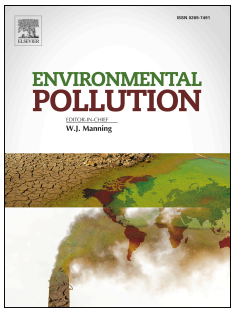
Journal Pre-proof



Evaluation of ketoprofen toxicity in two freshwater species: Effects on biochemical, physiological and population endpoints

G.D. Alkimin, A.M.V.M. Soares, C. Barata, B. Nunes

PII: S0269-7491(20)32174-6

DOI: https://doi.org/10.1016/j.envpol.2020.114993

Reference: ENPO 114993

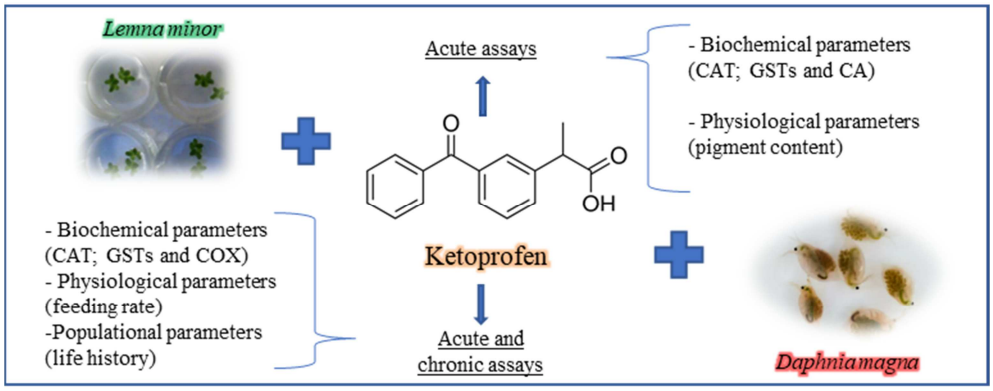
To appear in: *Environmental Pollution*

Received Date: 26 March 2020

Revised Date: 3 June 2020

Accepted Date: 6 June 2020

Please cite this article as: Alkimin, G.D., Soares, A.M.V.M., Barata, C., Nunes, B., Evaluation of ketoprofen toxicity in two freshwater species: Effects on biochemical, physiological and population endpoints, *Environmental Pollution* (2020), doi: https://doi.org/10.1016/j.envpol.2020.114993.



Evaluation of ketoprofen toxicity in two freshwater species: Effects on biochemical, physiological and population endpoints

Alkimin GD[1*], Soares AMVM[1], Barata C[2], Nunes B[1],

[1] Centre for Environmental and Marine Studies (CESAM), Campus de Santiago, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[2] Department of Environmental Chemistry, IDAEA-CSIC, Jordi Girona 18, 08034 Barcelona, Spain

*Corresponding author

Gilberto Dias de Alkimin

Departamento de Biologia, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

e-mail: alkimingd@ua.pt

Abstract

Among the most used non-steroidal anti-inflammatory drugs (NSAIDs), ketoprofen (KTF) assumes an important position. Nevertheless, its ecotoxicological effects are in non-target organisms poorly characterized, despite its use and frequency of occurrence in aquatic matrices. Thus, the aim of this study was to evaluate the possible toxicological effects of KTF contamination, in two freshwater species, *Lemna minor* and *Daphnia magna*, by measuring biochemical, physiological and population parameters. To attain this objective, both species were exposed to KTF at the same concentrations (0, 0.24, 1.2, 6 and 30 µg/L). *L. minor* plants were exposed during 4 d to these levels of KTF, and the enzymatic activity (catalase (CAT), glutathione S-transferases (GSTs) and carbonic anhydrase (CA)), and pigments content (chlorophyll a, b and total and carotenoids) were analyzed to evaluate the toxicity of this drug. *D. magna* was acutely and chronically exposed to KTF, and enzymatic activities (CAT, GSTs and cyclooxygenase (COX)), the feeding rates, and reproduction traits were assessed. In *L. minor*, KTF provoked alterations in all enzyme activities, however, it was not capable of causing any alteration in any pigment levels. On the other hand, KTF also provoked alterations in all enzymatic activities in *D. magna*, but did not affect feeding rates and life-history parameters. In conclusion, exposure to KTF, provoked biochemical alterations in both species. However, these alterations were not reflected into deleterious effects on physiological and populational traits of *L. minor* and *D. magna*.

Capsule: KTF demonstrates species-specific toxicity to different aquatic organisms affecting biochemical levels but not impairing physiological and population levels.

Keywords: Non-steroidal anti-inflammatory drugs; *Lemna minor*; *Daphnia magna*; Photosynthetic pigments; Biochemical markers; Reproduction.

Introduction

Thousands of tons of pharmaceutical substances are used yearly to prevent or treat illnesses (Kosjek et al., 2005; Lapworth et al., 2012). Drugs have been recognized as a large class of chemical contaminants which may originate from human and aquaculture usages, as direct results of excretions of metabolites and residues after metabolism (Eslami et al., 2015), among other sources. The presence and potential effects of these chemicals has been gaining attention due to the increase of their discharges, leading to an augmented frequency of their detection in the environment (Kosjek et al., 2005; Rzymski et al., 2017).

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most frequently prescribed drugs in modern medicine (Meek et al., 2010). It was estimated that the worldwide NSAIDs production is of several kilotons each year (Cleuvers 2004; Freches, 2017). These drugs are commonly used in the treatment of symptoms such as inflammation, pain, and fever (Simon, 2013; Al-Khateeb et al., 2017). This usage is due to their pharmacological activity, that occurs through cyclooxygenase (COX) inhibition, enzyme responsible for the production off prostaglandins, which are chemical mediators involved in inflammatory processes (Hernando et al. 2006). Beyond its use for the general purposes that NSAIDS are used for, KTF may also be used to treat rheumatoid arthritis and osteoarthritis, and to relieve muscle and joint pain (Prášková et al., 2011). Among NSAIDs that are nowadays in use for human therapeutics, it is possible to identify ketoprofen (KTF), which is the third most used NSAID; data from the literature show that, in Croatia, and between 2007 and 2013, the numbers of KTF consumption were just behind values for diclofenac and ibuprofen (Krnic et al., 2015). In 2015, in England, a total of about 25,400 prescriptions for KTF were accounted for (PACT 2016), while in 2017 this value reached 147,971, in the USA (ClinCalc, 2020).

KTF, in humans, is extensively and rapidly metabolized by the liver, mainly via conjugation with glucuronic acid (Gierse et al., 1999) and approximately 80% of the administered dose is excreted in the urine in a 24-hour period after the administration (Prášková et al., 2011); however, there is no information about these parameters for aquatic organisms, which is now critically required for the assessment of its putative toxicological environmental assessment.

NSAIDs are one of the most frequently detected pharmaceutical compounds in the aquatic environment, and their environmental distribution is widespread (Gentili 2007; aus der Beek et al., 2016, including KTF. These drugs, in general, are not efficiently eliminated in sewage treatment plants, being released to surface waters (Martinez-Sena et al., 2016). Thus, KTF is reported in aquatic environment up to 0.3 µg/L and up to 0.87 µg/L in Catalonia (Spain) rivers and effluents respectively (Farré et al., 2001), around 0.2 µg/L in German municipal sewage treatment plant (Ternes, 1998), 0.18 µg/L (Tixier et al, 2003) in Switzerland waste water treatment plant and 5.7 µg/L in effluents of Canadian sewage treatment plants (Metcalfe et al., 2003), besides that, KTF was recorded up to 2 µg/L in Finland influents (Lindqvist et al., 2005).

Considering the large number of NSAIDs, in general, their environmental fates and effects are still poorly understood, despite the already published data that evidence their persistent nature, and bioaccumulation potential in aquatic organisms (Geng et al., 2018). This is a pertinent gap in the knowledge of the environmental effects of drugs, since NSAIDs were designed to be biological active in a specific group of organisms (Kosjek et al., 2005; Ghlichloo and Gerriets, 2020). Mammalians, for instance, have both COX isoforms (COX-1 and COX-2, mainly), enzymes responsible for the regulation and onset of the inflammatory process (Heckmann et al., 2008a; Wongrakpanich et al., 2018). However, one COX isoform, at least, is generally present

in invertebrates and lower vertebrates (Rowley et al., 2005) and, consequently, these organisms may also be affected by NSAIDs too. Consequently, these pharmaceuticals may be responsible for a large number of toxicological effects in non-targets organisms, including macrophyte species (Alkimin et al., 2019a), mollusks (Almeida and Nunes, 2019; Piedade et al., 2020), polychaetes (Gomes et al., 2019) and fish (Nogueira et al., 2019). Toxicological effects of KTF have been mostly limited to a few studies, that measured its toxic effects on embryo-larval development and biochemical alterations, including the oxidative stress response, assessed in both larvae and adults of the fish species *Danio rerio* (Diniz et al., 2015; Rangasamy et al., 2018). Toxic effects of KTF were also determined in the freshwater fish *Cyprinus carpio* (Prášková et al., 2013), also focusing in embryo-larval development. In addition, KTF toxicity was analyzed in the microcrustacean *Daphnia magna*, and obtained data evidenced its capacity to alter the swimming behavior and physiological endpoints (heart rate, thoracic limb activity, and mandible movement) in this organism (Bownik et al., 2020)

Besides their pharmacological activity, which occurs through COX inhibition, some NSAIDs have the ability to interact with biota, thereby affecting other biological parameters. In humans, NSAIDs are capable of inhibiting the carbonic anhydrase (CA) activity (Knudsen et al., 2004), an enzyme responsible for carbon dioxide hydration (Lindskog, 1997). NSAIDs also induce the generation of reactive oxygen species (ROS) (Galati et al., 2002; Adachi et al., 2007; Ito et al., 2016) that can lead to oxidative stress. The effects of NSAIDs are not limited to the here-described toxicity, since they may also directly compromise the eicosanoids biosynthesis, an important molecule in reproductive traits of crustaceans (Heckmann et al., 2008a), and provoke physiological alterations on macrophyte species (Wrede, 2015). Thus, it is possible to assume that more studies about these effects associated to NSAIDs exposure, namely KTF, are

urgently required, in a comprehensive range of organisms. Besides the necessity to study the effects of KTF on a comprehensive range of distinct test organisms, it is equally necessary to determine its effects on a set of toxicological complimentary endpoints, from different levels of organization, including at the biochemical, physiological and population levels. This will allow creating a body of evidence about KTF toxicity to aquatic organisms, which is nowadays mostly inexistent. In addition, these studies were performed by testing much higher concentrations than those found in the aquatic environment, considering the already mentioned studies reporting the environmental presence and levels of this drug. To fully characterize the ecotoxicological potential of this drug, it is necessary to determine its toxicity at environmentally realistic concentrations, in aquatic organisms. Thus, the aim of this study was to evaluate the toxicological effects of KTF at realistic environmental concentrations and worst scenario case scenarios, in two freshwater species, namely a macrophyte species (*Lemna minor*) and a microcrustacean (*Daphnia magna*), by measuring biochemical, physiological and populational parameters.

Material and methods

Chemicals

Ketoprofen (CAS number: 22071-15-4) was purchased from Sigma-Aldrich (Belgium) with ≥ 98% of analytical purity; all other chemicals used in this study (buffers, protein determinations) had analytical purity and were purchased from Sigma Aldrich and Bio-rad® laboratories.

147 **Organisms culture and maintenance**

148 All organisms (*Lemna minor* and *Daphnia magna*) were cultured and maintained at the
149 Department of Biology at Aveiro University, as described by Alkimin et al. (2019b) for
150 *L. minor*, and by Daniel et al. (2019) for *D. magna*.

151

152 **Exposures of test organisms**

153 All experiments performed in this work involved exposing both species to the same
154 range of concentrations, based on already reported environmental levels (up to 5.7
155 µg/L) (Farré et al., 2001; Metcalfe et al., 2003; Lindqvist et al., 2005). The nominal
156 exposure concentrations were: 0, 0.24, 1.2, 6 and 30 µg/L; the control treatments
157 involved exposing organisms only to the culture medium of each species. The KTF
158 stock solutions were directly prepared in each specific culture medium by dissolving the
159 pure compound.

160

161 **Macrophytes – *Lemna minor***

162 Plants of the macrophyte species *L. minor* were exposed in a 6-well plate, filled with 10
163 mL of medium and/or stock solution per well. The test started with macrophytes
164 covering around 20% of the surface of each well, with 10 replicates per condition. The
165 test had 96 h of duration, a period that was chosen considering the assumptions
166 described by Alkimin et al. (2019b). This study established that 96h is a duration long
167 enough to cause measurable alterations in *Lemna* species after being exposed to
168 xenobiotics, including to some pharmaceutical drugs. Exposures were conducted
169 according to OECD (2006) under controlled conditions (temperature 23 ± 1 °C;

continuous light exposure; light intensity, ~84 µmol photons m^{-2} s^{-1}) in a climate chamber (Binder). After the exposure period, *Lemna* fronds from each treatment were collected, and this biomass was divided and placed in Eppendorfs microtubes, and stored at -80 ºC for the analyzes - quantification of pigments (chlorophyll a, b and total and carotenoids) and determination of biochemical endpoints (catalase, CAT; glutathione-S-transferases, GSTs; and carbonic anhydrase, CA, activities).

Microcrustacean – *Daphnia magna*

Three different experiments were performed with *D. magna*. In the first one, juveniles (5 d old) were exposed to KTF during 48 h in 300 mL glass flasks filled with 200 mL of KTF or medium solution. For each concentration, 5 replicates with 12 organisms each were adopted. This experiment was repeated thrice, each one for obtaining biological samples to quantify a specific enzyme (biochemical markers). At the end of the exposure periods, animals were collected with a plastic pipette to Eppendorf microtubes and stored at -80 ºC for ulterior analysis (determination of CAT, GSTs, and COX activities). Feeding rate experiments were conducted in test-chambers consisted of 50 mL glass flasks filled with test solution, where five 5 d old daphnids were positioned (five replicates per treatment). Briefly, this test was used to measure the filtration capacity on algal cells (*Raphidocelis subcapitata*) by test organisms, when exposed to the drug. The animals were exposed for 24 h, in the dark, to all experimental conditions, with $5x10^5$ cells/mL of the algae *R. subcapitata* in the test-chambers. Afterwards, animals were transferred to a new test-chamber (with clean media), also in the dark, for 4 h, with the same algae density (post-exposure period), to evaluate the recovery from exposure to toxicants. Blank controls (media with no daphnids) were included in the experimental design in both cases. At the end of each period, the algae cell densities

were spectrophotometrically measured (Thermo Scientific Multiskan (ScanIt Software 2.4.4)) at 440 nm. Finally, the feeding rate was calculated converting the obtained values to proportional algae consumption (%) relative to control. Finally, a chronic reproduction test was conducted, based on the OECD guideline 211 (OECD 2012) with the exposure period modified according to Ribeiro et al. (2011) and Alkimin et al. (2020). The test duration was approximately 16 days and/or the third brood. The experiment was performed in 50 mL glass flasks, and 10 replicates with < 24 h old neonates were used for each concentration. Medium test was totally renewed every other day, and the animals were maintained in the same culture conditions. The parameters day of first brood, number of neonates from first brood, and total number of neonates were the evaluated endpoints.

Quantification of physiological and biochemical parameters

Pigments analysis (chlorophylls and carotenoids)

Total, a, and b chlorophylls (TChl; Chl a; Chl b), and carotenoids (Car) amounts were determined spectrophotometrically, according to the method described by Hiscox and Israelstam (1979). Pigments were extracted from the previously exposed fronds of *L. minor* (about 10 mg per replicate - fresh weight, FW) in 1.8 mL of dimethyl sulphoxide (DMSO). The extract was placed in water at 65 ºC during 30 min and allowed to cool in the dark and at room temperature. The obtained supernatants were used to quantify the amounts of chlorophylls (a, b, and total), and carotenoids levels, by spectrophotometry, by measuring the absorbance of the extracts, at wavelengths of 470, 645, 646, and 663. The calculation of the pigments followed the equations proposed by Arnon (1949) and demonstrated by Hiscox and Israelstam (1979) to be suitable if the extraction was

undertaken with DMSO, to calculate the amounts of Chl (a, b, and total), and by Lichtenthaler (1987) to quantify the Car amount. In addition, two ratios were determined: [Chl a] / [Chl b]; and [TChl] / [Car].

Tissue homogenization – biochemical markers

For CAT and GSTs determinations, samples were macerated with mortar and pestle (*L. minor*) or sonicated (*D. magna* – Branson sonicator, model 250) in ice-cold phosphate buffer (50 mM, pH 7.0, with 0.1% Triton X-100). Each homogenate sample was composed by ~ eight fronds of *L. minor*, or 12 individuals of *D. magna*, and 1.2 mL phosphate buffer. Homogenized samples were centrifuged at 15,000 g at 4ºC for 10 min. For carbonic anhydrase activity (CA) determination, samples of *L. minor* were macerated in 1 mL ice-cold Tris-sulfate 25 mM (pH 7.5) with 25 mM of sodium sulfate. Homogenized samples were centrifuged at 10,000 g at 4 ºC for 40 min. Cyclooxygenase determination (*D. magna*) was performed using 0.8 mL of 0.1 M Tris-HCl buffer (pH 7.8) with 1 mM EDTA to sonicate animals, followed by a cycle of centrifugation at 10,000 g at 4 ºC for 15 min. After the centrifugation process, supernatants were divided into aliquots, which were used for the different enzymatic determinations, as described below.

Catalase activity determination

Catalase activity was determined in a 96-well microplate, by the procedure described by Aebi (1984), based on the degradation rate of the substrate H_2O_2, monitored at 240 nm for 5 min. The results were expressed by considering that one unit of CAT activity equals the number of moles of H_2O_2 degraded per minute, per milligram of protein.

Glutathione-S-transferases activity determination

The procedure to determine GSTs activity monitored the increment of absorbance at 340 nm of a thioether resulting from the catalysis of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione by GSTs, as described by Habig et al. (1974), adapted to a 96 well microplate. Results were expressed as millimoles of thioether produced per minute, per milligram of protein.

Carbonic anhydrase activity determination

To determine CA activity, the method by Verpoorte et al. (1967) was adapted to 96-well microplates. This methodology is based on the hydrolysis of the substrate p-nitrophenol acetate (pNPAc), catalyzed by CA. This reaction was then monitored for 5 min at a wavelength of 400 nm. One unit of CA is defined as the amount of enzyme needed to hydrolyze one µmol of pNPAc per minute, and the data were expressed in U/milligram of protein.

Cyclooxygenase activity determination

This method is based on the ability of cyclooxygenase (COX) to convert arachidonic acid into a hydroperoxy endoperoxide (Prostaglandin G2; PGG2). This is reduced in the presence of N, N, N', N'-tetramethyl-p-phenylenediamine (TMPD) to its alcohol (Prostaglandin H2; PGH2); then, the oxidation of TMPD was monitored during 5 min at 590 nm (Petrovic and Murray, 2010).

Protein determination

Protein quantification was performed at 595 nm using the Bradford method (Bradford 1976), adapted to microplate, with bovine γ-globulin as standard (1mg/ml), in order to express enzymatic activities per mg of protein of the analyzed samples. Finally, all spectrophotometric readings were performed in a microplate reader Thermo Scientific Multiskan (ScanIt Software 2.4.4).

Statistical analysis

Data for all parameters were tested for homogeneity and normality, and a one-way analysis of variance (ANOVA) was performed, followed by a post-hoc Dunnett's test (or the equivalent non-parametric test, according to the data). Statistical analysis was performed with SigmaPlot v.14.0. The adopted level of significance was 0.05.

Results

The lowest concentration (0.24 µg/L) of KTF provoked a decrease in GSTs activity in *L. minor*; on the contrary, this enzyme´s activity was increased in plants exposed to all other concentrations (1.2, 6, and 30 µg/L) ($F_{(4, 28)} = 57.177$, $p < 0.001$; Figure 1A). With the exception of the highest concentration (30 µg/L), KTF increased CAT activity ($F_{(4, 32)} = 23.157$, $p<0.001$; Figure 1B). KTF caused a decrease of CA activity in plants exposed to levels of 0.24 and 1.2 µg/L ($F_{(4, 46)} = 10.055$, $p<0.001$; Figure 1C) and no effect was observed in plants exposed to the two highest concentrations (6, and 30 µg/L).

Photosynthetic pigments (Chl a, b and TChl) measured in *L. minor* did not suffer any alteration after KTF exposure ($F_{(4, 47)}$ = 0.922, p = 0.460; $F_{(4, 47)}$ = 1.426, p = 0.242 and $F_{(4, 47)}$ = 1.003, p = 0.416, respectively). No alterations were also detected for Car content ($F_{(4, 47)}$ = 1.139, p = 0.351,) or for both determined ratios (Chl a/b – H_{4df} = 4.255, p = 0.373; TChl/Car – $F_{(4, 47)}$ = 2.022, p = 0.108) (Figure 2A – F).

In *D. magna*, KTF caused an increase in CAT activity ($F_{(4,20)}$=31.597; p<0.001) in a concentration related manner (Figure 3A); GSTs activity also presented an increase, but only statistically different in organisms exposed to concentrations of 1.2 and 6 µg/L ($F_{(4,22)}$=5.048; p=0.007)(Figure 3B). On the other hand, COX activity was reduced after KTF exposure ($F_{(4,18)}$=4.695; p=0.013) for organisms exposed to all tested concentrations, in a dose-response manner (Figure 3C).

Ketoprofen did not affect *D. magna* feeding rates during exposure ($F_{(4,24)}$= 3.515; p = 0.025) or during recovery period ($F_{(4,24)}$= 1.636 p=0.0204; Figure 4).

Ketoprofen did not affect reproductive endpoints in *D. magna*, which included day of first brood (H_{4df} =4.091; p=0.394; Figure 5A), number of neonates from first brood (H_{4df}=2.803; p=0.591; Figure 5B), and total number of neonates ($F_{(4, 44)}$=0.938; p=0.452; Figure 5C).

Discussion

Lemna minor

NSAIDs, including diclofenac and paracetamol, are capable to induce ROS production in *L. minor*, as already demonstrated by Kummerová et al. (2016). This is highly important in our context, considering that reactive oxygen species (ROS), and

311 particularly hydrogen peroxide (H_2O_2), are generated during the normal plant
312 metabolism and are involved in a diversity of signaling cascades and reactions
313 necessary for the physiological regulation of diverse aspects, such as plant growth
314 (Cheeseman, 2007). Exposure to anthropogenic xenobiotics, such as the referred drugs,
315 may enhance this production, leading to toxic effects in plants. A similar finding was
316 made clear in this study, since CAT activity was increased following KTF exposure.
317 Increased CAT activity must be understood as the activation of enzymatic detoxification
318 mechanisms that to minimize cellular levels of superoxide radicals (O_2^{-}) and H_2O_2. This
319 is the main role of antioxidant enzymes such as CAT (Sairam and Tyagi, 2004). It is
320 thus possible to hypothesize that low levels of KTF were causative of the triggering of
321 an antioxidant response. Conversely, the activity of CAT measured in plants exposed to
322 the highest KTF concentration was closely similar to the control values; this can be
323 assumed as a possible plant resilience mechanism, since macrophytes are known for
324 their ability to recover along time (Wang, 1990), when kept in a contaminated
325 environment by different contaminants, such as metals (Jaramillo et al., 2019) and
326 pharmaceutical drugs (Alkimin et al., 2019a). The normalization of CAT means that
327 plants were able to activate alternative detoxification and antioxidant mechanisms, such
328 as ascorbate peroxidase and superoxide dismutase, another two enzymes very important
329 for the antioxidant defense system of plants (Mittler, 2002). For example, the different
330 affinities of ascorbate peroxidase (APX; µm range) and CAT (mm range) for H_2O_2
331 indicated that they belong to different classes of ROS-scavenging enzymes, namely,
332 H_2O_2 (Mittler, 2002). This means that the fine modulation of ROS signaling might
333 occurs through APX, whereas CAT might be responsible for the removal of excess ROS
334 during stress (Mittler, 2002).

335 The alterations of CAT activity may be linked to the observed changes in GSTs activity.
336 Plants exposed to the lowest concentration of KTF had a decrease in GSTs activity. It is
337 possible to suggest that the overproduction of ROS by KTF exposure (signaled by the
338 enhancement of CAT activity) may have caused direct damages in cellular molecules.
339 This a common feature of ROS, and their damaging action may include deleterious
340 alterations of DNA, lipids, and proteins; this often causes a decrease in enzymatic
341 activity by denaturation (Schieber and Chandel, 2014), an effect that may be held
342 accountable for the here-observed decrease of GSTs activity. On the other hand, plants
343 exposed to the other tested concentrations were capable of increasing their GSTs
344 activity. This pattern is indicative of distinct effects, related to the increase of
345 biotransformation capacity of the organism to cope with the excess of this xenobiotic.
346 Since, this isoenzyme group is part of phase II metabolic mechanisms that is responsible
347 for the detoxification of exogenous compounds; conjugating glutathione (GSH) with
348 compounds containing an electrophilic center to modify the substrate into a more water
349 soluble, less toxic complex (Habig et al., 1974). In addition, GSTs had also a role in
350 resisting pro-oxidative effects, being useful in enhancing plant survival on toxic sites
351 (Cummins et al., 2011). This effect occurs since GSTs contribute for the efficacy of the
352 antioxidant defense system, which does not directly relate to their role in xenobiotic
353 detoxification (Moons, 2005).

354 Macrophytes (such as *L. minor*) are primary producers in the aquatic environment,
355 consequently being at the basis of the food web of freshwater systems; thus, adverse
356 effects that may occur in these plants can also deleteriously affect the entire aquatic
357 food web. So, if the *L. minor* antioxidant stress system was not able to efficiently reduce
358 the adverse effect of xenobiotics upon exposure, deleterious effects might occur in this
359 species, from cell death to other unpredictable consequences (Demidchik, 2015; Xie et

al., 2019). Despite the general absence of data for plant organisms, it is possible to consider the assumptions made by Monaghan et al. (2009), when referring that life history trade offs in animals are likely to be associated to oxidative stress and to the antioxidant response, which occur at the most fundamental metabolic activities of living organisms. Such traits are likely to impact not only the individual, but to escalate at higher levels of organization, with unforeseen outcomes. So, changes in plants of *L. minor* may indeed correspond to alterations at the ecosystem level.

Carbonic anhydrase is a zinc-containing enzyme that catalyzes the rapid conversion of CO_2 over water in a proton and bicarbonate ion (HCO_3^- - reversible hydration/dehydration reaction) (Coleman, 2000; Escudero-Almanza et al., 2012). In plants, CA is important in many physiological functions that involve carboxylation or decarboxylation reactions (Moroney et al., 2001) by increasing CO_2 concentrations in the chloroplast to increase the carboxylation rate of the ribulose 1,5-disphosphate carboxylase (RuBisCO) enzyme. This chemical reaction is responsible for the incorporation of CO_2 into carbohydrates during photosynthesis; however, it and can only use C coming from CO_2, instead of C from carbonic acid or bicarbonate (Escudero-Almanza et al., 2012). In addition, CA also actively participates in the inorganic carbon transportation into actively photosynthesizing cells or away from actively respiring cells (Henry 1996). Few studies reported the effects of pharmaceutical drugs in CA enzymatic activity of macrophytes. Previous data have shown that acetazolamide (diuretic drug) and salicylic acid (NSAID), had the capacity to decrease CA activity, also in a macrophyte species, namely *L. gibba* (unpublished data). However, the here obtained data showed a decrease of CA activity only in plants exposed to the lowest concentrations of KTF (0.24 and 1.2 µg/L), which may indicate hormesis. In general, a hormesis effect represents an adaptive response of organisms to

environmental or self-imposed challenges through which they improve its functionality and/or tolerance to more aggressive challenges (Calabrese and Mattson, 2017). Such challenging conditions, in this case, correspond to the higher concentrations of the tested pharmaceutical. The exposed plants showed to be resilient, by decreasing their CA activity. Hormesis effects were also observed in CAT activity, which only increased in plants exposed to the lowest concentrations.

Generally, higher plants have three groups of pigments: carotenoids, chlorophylls, and phycobilins; the most important pigments involved in photosynthesis are chlorophylls, which are prone to be targets for toxic alterations. Changes of the qualitative composition or of quantitative content of pigments, are physiologically important characteristics that indicate the general health condition of plants, informing also on the function of photosynthetic apparatus, including the adaptive responses after being subjected to stressful situations (Belous et al., 2018). Levels of the main photosynthetic pigments here analyzed (Chl a, b and total) did not suffer any alteration after KTF exposure; in addition, the ratio between levels of chlorophylls a and b were not altered. This ratio is a calculation that characterizes the photosynthetic apparatus operation, by indicating the potential photochemical activity of leaves. The absence of effects may lead us to conclude that KTF does not compromise the normal photochemical potential in *L. minor*, despite pervious indications that, in general, NDSAIDs may exert this effect. As far as we know, no data are available concerning this parameter, for macrophyte species after being exposed to KTF. However, as mentioned, NSAIDs are capable to cause alterations in chlorophyll content in plants. For example, diclofenac (100 µg/L; 314.3 nM) and paracetamol (100 µg/L; 661.5 nM) were capable to decrease the contents of photosynthetic pigments in *L. minor* after 7 days of exposure (Kummerová et al., 2016). Diclofenac (1 mg/L; 3.1 mM) was also responsible for the

decrease of Chl a and b content in chicory (*Cichorium intybus*), after 22 days of exposure (Podio et al., 2020). Thus, we can hypothesize that environmental concentrations of KTF are safe to *Lemna* species in relation to this specific endpoint, being KTF a less toxic pharmaceutical drug among the NSAID class to this genera. We may also suggest that the here studied period of exposure was not long enough to trigger alterations in this endpoint after KTF exposure. This is an important set of results, since, to the best of our knowledge, this is the first report about KTF toxicity evaluation in *Lemna* species.

Carotenoids are well known free-radical scavengers, also playing an important role in photoprotection of photosynthetic apparatus (Sytar et al., 2013). KTF-exposed plants did not show changes in Car, in terms of their presence, quantity, and proportion (in comparison with chlorophylls, assessed by the determination of TChl/Car ratio). The ratio of TChl to Car is more informative that the simple measurement of pigment levels, because it indicates the degree of adaptation of plants to light and to adverse conditions. In this case, after the evaluation of the obtained results, it is possible to suggest that the here tested KTF concentrations were not capable to cause alterations of this physiological parameter, showing an adaptive capacity of plants when challenged by this adverse condition. This assumption can be reinforced since, even after being exposed to other NSAIDs, plants were capable to adapt to this source of chemical stress, and were not prone to suffer alterations in the Car content, along the entire duration of the exposure (Alkimin et al., 2019a). This set of results concerning the levels of pigments demonstrates that *L. minor* was physiologically adapted to KTF, and no deleterious effects were caused by KTF in terms of the pigments levels of exposed organisms.

Finally, the observed biochemical effects were not evident enough to sustain the occurrence of physiological alterations. Despite the occurrence of biochemical changes caused by KTF exposure, these were not followed by deleterious changes in photosynthetic pigments (Chl a, b, total and Car). Thus, it is possible to assume that the antioxidant and biotransformation systems of *L. minor* were efficient enough to protect these plants from other injuries.

Daphnia magna

The genus *Daphnia* is considered as a dominant herbivorous in the zooplankton group, and animals from this genus play an important role in temperate freshwater ecosystems as primary consumers (Smirnov, 2013). Thus, their feeding activity is an important aspect in their ecology, being necessarily assessed in a stressful situation, including exposure to contamination by pharmaceutical drugs. In this work, it was possible to observe that KTF, in the tested concentrations, did not alter feeding; animals subjected to the recovery period evidenced a similar response, and no substantial effects were ascertained in these organisms. However, other NSAIDs, such as diclofenac, are capable of reducing *D. magna* feeding rate (Nkoom et al., 2019) in concentrations between 5 and 100 µg/L. However, it is necessary consider that Nkoom et al. (2019) experiment was conducted with neonates <24 h old, and our work involved only juveniles (5 d old), which can be more tolerant (Hoang and Kaine, 2007). Feeding rate can affect a large number of life-history traits that may have context-dependent effects on fitness (Garbutt and Little, 2014), being putatively associated with the reproduction. Indeed, KTF did not affect the studied life-history traits. Data for another crustacean species, namely *Ceriodaphnia dubia*, showed the absence of effects after exposure to KTF at concentrations up to 100 µg/L, with a decrease in the offspring per female, when

459 animals were exposed to 1 mg/L (Mennillo et al., 2017). This level is nevertheless much
460 higher than the here tested concentrations, and also well above the amounts detected in
461 the environment, as already cited. These data are coherent, since the *D. magna* 48h
462 EC_{50}, is higher than 100 mg/L of KTF (Boström and Berglund, 2015), a value
463 approximately 3300 times higher than the levels tested in this study.

464 Despite not causing alterations in the feeding behavior and in life-history traits, KTF
465 was able to trigger the *D. magna* antioxidant system, increasing the CAT activity of
466 exposed animals. This occurred most likely as an attempt to cope with the putatively
467 higher levels of generated ROS. Among NSAIDs, KTF has been shown to be more
468 toxic than other drugs from this class to the *D. magna* antioxidant system, generating
469 changes in the antioxidant mechanisms in concentration as low as 0.24 µg/L of KTF
470 (943.8 pM). Daniel et al. (2019) reported an increase of CAT activity in animals
471 exposed to 2.56 mg/L (16.9 µM) of paracetamol, and, even a concentration of 50 µg/L
472 (242.4 nM) of ibuprofen was not able to trigger this system (Wang et al., 2016).

473 KTF seems also to have activated the biotransformation system in *D. magna*, by
474 increasing the activity of GSTs, a response that occurred for animals exposed to the
475 intermediate concentrations (1.2 and 6 µg/L). Phase II enzymes are crucial in
476 eliminating NSAIDs, but the interaction between GSTs and KTF is poorly understood
477 for aquatic organisms. In other animals, such as mammals, KTF can be eliminated in its
478 free forms: conjugated via glucuronic acid, or as hydroxyl (OH) metabolite, with or
479 without conjugation (Alkatheeri et al., 1999). However, the metabolic pathway of
480 conjugation seems also to occur in aquatic organisms. In fact, KTF triggered the
481 biotransformation system, increasing the GSTs activity, in a fish PLHC-1 cell line
482 (Mennillo et al., 2017). Thus, it is possible to hypothesize that KTF can be excreted
483 following conjugation with glutathione (via GSTs) and/or with glucuronic acid, prior to

484 be delivered to phase III detoxification system. In addition, the lowest (0.24 µg/L) and
485 the highest (30 µg/L) concentrations did not cause any alteration of GST activity. Thus,
486 hermetic effect can be interpreted as follows: at low concentrations hydroxylation
487 processes were effective enough to detoxify the pharmaceutical, turning the phase II
488 biotransformation system of glutathione conjugation redundant (Bartha, 2012).
489 However, in animals exposed to higher concentrations, more complex metabolic
490 pathways should be activated by the drug (Bartha, 2012) leading to no alteration in the
491 GSTs activity. On the other hand, as already mentioned, ROS have the capacity to cause
492 molecular damage and this effect can be linked to a decrease in enzymatic activity by
493 denaturation (Schieber and Chandel, 2014) and consequently, may be responsible for
494 the decrease of GSTs activity in the higher concentration, attaining values near those of
495 the control treatment.

496 Eicosanoids are oxygenated metabolites of arachidonic acid (AA) with different
497 functions. Their biosynthesis may occur according to different pathways, such as the
498 cytochrome P450 epoxygenase pathway (epoxyeicosatrienoic acids), lipoxygenage
499 (LOX) pathway (leukotrienes and lipoxins), and cyclooxygenase (COX) pathway
500 (prostanoids: thromboxane and prostaglandins) (Stanley, 2006). As mentioned, COX or
501 PGH_2 synthase is an enzymatic form producing prostaglandins, and it is conserved in
502 crustaceans, such as daphnids (Kyoto Encyclopedia of Genes and Genomes, KEGG).
503 Prostaglandins are signaling molecules with crucial importance in crustacean
504 reproduction and immune system (Smirnov, 2017). NSAIDs can interfere/inhibit
505 prostaglandins biosynthesis, since NSAIDs are competitive inhibitors of some of the
506 involved enzymatic forms, by competing with AA, which is the physiological substrate
507 of COX; NSAIDs thereby affects eicosanoids biosynthesis and their physiological
508 functions (Charlier and Michaux, 2003). Heckmann and co-workers (2008a)

509 demonstrated the existence of a COX pathway in *D. magna*, which appears to be
510 simpler than the COX pathway observed in mammals. In addition, another study
511 indicated that ibuprofen, also a NSAID, is capable of affecting the sequential processes
512 of oogenesis and embryogenesis in daphnids, by interrupting the COX metabolic
513 pathway of eicosanoids (Heckmann et al., 2008b). By acting this way, this drug is
514 responsible for a dose-dependent decrease in *D. magna* reproduction (Han et al., 2010;
515 Heckmann et al., 2007) and also affects reproduction in another crustacean species,
516 namely *Moina macrocopa* (Han et al., 2010). The NSAID effect in COX was also
517 observed in this study, since KTF exposure clearly decreased COX activity in a dose-
518 response manner, as already mentioned before. This result was interpreted as a proxy
519 for the mode of action of these pharmaceutical drug; being a NSAIDs, its acute effects
520 resulted in the inhibition of COX, even at the low, albeit realistic here tested
521 concentrations, which were indisputably enough to trigger this mechanism in *D. magna*.
522 However, reproductive effects were not observed in *D. magna* after KTF exposure,
523 which means that NSAIDs affect crustacean reproduction at higher concentrations (in
524 the mg/L order) as observed by Han et al. (2010) and Heckmann et al. (2007). These
525 assumptions reinforce the notion that deleterious NSAIDs effects in the eicosanoids
526 biosynthesis pathway may be important for the reproduction of crustaceans. However,
527 the alterations that were observed in this study constitute a warning for the possible
528 long-term effects of KTF in a worst environmental case scenario.

529

530 **Conclusions**

531 In conclusion, both species responded to low, realistic levels of KTF. However, the
532 toxic effects of KTF were only limited to biochemical parameters, without extrapolating
533 to physiological and population modifications. However, changes of the biochemical

parameters were clearly different between both species, being *L. minor* more susceptible to low KTF concentrations, hypothesizing the resilience capacity and hormetic effects; while *D. magna*, in general, presented a dose response pattern. This comparison is highly interesting and allows us to assume that KTF toxicity is eminently species-specific. Finally, the results here obtained are a clear indication about the possibility of KTF in causing toxicological effects in the aquatic organisms, even when organisms were exposed to realistic conditions simulating the already described scenarios of contamination in the wild.

Acknowledgements

Bruno Nunes is hired by “ECO-R-pharmplast - Ecotoxicity of realistic combinations of pharmaceutical drugs and microplastics in marine ecosystems”, Fundação para a Ciência e a Tecnologia, FCT (reference POCI-01-0145-FEDER-029203). Gilberto Dias de Alkimin thanks FCT for the PhD grant (PD/BD/127922/2016). This research was financially supported by CESAM (UIDB/50017/2020+UIDP/50017/2020), by FCT/MCTES through national funds (PIDDAC), and by the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020.

References

Adachi, M., et al. (2007). Nonsteroidal anti-inflammatory drugs and oxidative stress in cancer cells. Histol Histopathol. 22: 437-442. DOI: 10.14670/HH-22.437

Aebi, H. (1984). Catalase in vitro. Methods in Enzymology, 6. 105-121

555 Alkatheeri, N. A., Wasfi, I. A., Lambert, M. (1999). Pharmacokinetics and
556 metabolism of ketoprofen after intravenous and intramuscular administration in camels.
557 J Vet Pharmacol Ther 22:127–135.

558 Al-Khateeb, L. A., Hakami, W., Salam, M. A. (2017). Removal of non-steroidal
559 anti-inflammatory drugs from water using high surface area nanographene: Kinetic and
560 thermodynamic studies. Journal of Molecular Liquids, 241, 733–741.
561 doi:10.1016/j.molliq.2017.06.068

562 Alkimin, G. D., Daniel, D., Dionísio, R., Soares, A. M. V. M., Barata, C.,
563 Nunes, B. (2019a). Effects of diclofenac and salicylic acid exposure on *Lemna minor*: Is
564 time a factor? Environmental Research, 177, 108609. doi:10.1016/j.envres.2019.108609

565 Alkimin, G. D., Daniel, D., Frankenbach, S., Serôdio, J., Soares, A. M. V. M.,
566 Barata, C., Nunes, B. (2019b). Evaluation of pharmaceutical toxic effects of non-
567 standard endpoints on the macrophyte species *Lemna minor* and *Lemna gibba*. Science
568 of The Total Environment, 657, 926–937. doi:10.1016/j.scitotenv.2018.12.002

569 Alkimin, G. D., Paisio, C., Agostini, E., Nunes, B. (2020). Phytoremediation
570 processes of domestic and textile effluents: evaluation of the efficacy and toxicological
571 effects in *Lemna minor* and *Daphnia magna*. Environ Sci Pollut Res 27, 4423–4441.
572 https://doi.org/10.1007/s11356-019-07098-3

573 Almeida, F., Nunes, B. (2019). Effects of acetaminophen in oxidative stress and
574 neurotoxicity biomarkers of the gastropod *Phorcus lineatus*. Environmental Science and
575 Pollution Research. doi:10.1007/s11356-019-04349-1

576 Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts, polyphenoxidase
577 in *Beta vulgaris*. Plant physiology, 24, 1-15.

578 Aus der Beek, T., Weber, F.-A., Bergmann, A., Hickmann, S., Ebert, I., Hein,
579 A., Küster, A. (2016) . Pharmaceuticals in the environment-Global occurrences and
580 perspectives. Environmental Toxicology and Chemistry, 35(4), 823–
581 835. doi:10.1002/etc.3339

582 Bartha, B. (2012). Uptake and Metabolism of Human Pharmaceuticals in Plants.
583 Identification of Metabolites and Specification of the Defense Enzyme Systems under
584 Pharmaceutical Exposure. (PhD thesis. Technische Universität München. Lehrstuhl für
585 Ökologischen Landbau und Pflanzenbausysteme).

586 Belous, O., Klemeshova, K., Malyarovskaya, V. (2018). Photosynthetic
587 Pigments of Subtropical Plants, Photosynthesis - From Its Evolution to Future
588 Improvements in Photosynthetic Efficiency Using Nanomaterials, IntechOpen, DOI:
589 10.5772/intechopen.75193.

590 Boström, M. L., Berglund, O. (2015). Influence of pH-dependent aquatic
591 toxicity of ionizable pharmaceuticals on risk assessments over environmental pH
592 ranges. Water Research, 72, 154–161. doi:10.1016/j.watres.2014.08.040

593 Bownik, A., Jasieczek, M., Kosztowny, E. (2020). Ketoprofen affects swimming
594 behavior and impairs physiological endpoints of Daphnia magna. Science of The Total
595 Environment, 138312. doi:10.1016/j.scitotenv.2020.138312

596 Bradford, M. (1976). A rapid and sensitive method for the quantification of
597 microgram quantities of protein utilizing the principle of protein dye binding. Anal.
598 Biochem. 72, 248-254.

599 Calabrese, E. J., Mattson, M. P. (2017). How does hormesis impact biology,
600 toxicology, and medicine? Npj Aging and Mechanisms of Disease, 3(1).
601 doi:10.1038/s41514-017-0013-z

602 Charlier, C., Michaux, C. (2003). Dual inhibition of cyclooxygenase-2 (COX-2)
603 and 5-lipoxygenase (5-LOX) as a new strategy to provide safer non-steroidal
604 antiinflammatory drugs. European Journal of Medicinal Chemistry 38: 645-659.

605 Cheeseman, J. M. (2007). Hydrogen peroxide and plant stress: a challenging
606 relationship Plant Stress, 1. 4-15

607 Cleuvers, M. (2004). Mixture toxicity of the anti-inflammatory drugs diclofenac,
608 ibuprofen, naproxen, and acetylsalicylic acid. Ecotoxicology and Environmental Safety,
609 59(3), 309–315.

610 ClinCalc. (2020). Ketoprofen - Drug Usage Statistics, United States, 2007 –
611 2017. Available: <https://clincalc.com/DrugStats/Drugs/Ketoprofen>.

612 Coleman, J. R. (2000). Carbonic Anhydrase and Its Role in Photosynthesis.
613 Advances in Photosynthesis and Respiration, 353–367. doi:10.1007/0-306-48137-5_15

614 Cummins, I., Dixon, D. P., Freitag-Pohl, S., Skipsey, M., Edwards, R. (2011).
615 Multiple roles for plant glutathione transferases in xenobiotic detoxification. Drug
616 Metabolism Reviews, 43(2), 266–280. doi:10.3109/03602532.2011.552910

617 Daniel, D., Dionísio, R., de Alkimin, G. D., Nunes, B. (2018). Acute and chronic
618 effects of paracetamol exposure on *Daphnia magna*: how oxidative effects may
619 modulate responses at distinct levels of organization in a model species. Environmental
620 Science and Pollution Research. doi:10.1007/s11356-018-3788-y

621 Demidchik, V. (2015). Mechanisms of oxidative stress in plants: From classical
622 chemistry to cell biology. Environmental and Experimental Botany, 109, 212–228.
623 doi:10.1016/j.envexpbot.2014.06.021

Diniz, M. S., Salgado, R., Pereira, V. J., Carvalho, G., Oehmen, A., Reis, M. A. M., Noronha, J. P. (2015). Ecotoxicity of ketoprofen, diclofenac, atenolol and their photolysis byproducts in zebrafish (*Danio rerio*). Science of The Total Environment, 505, 282–289. doi:10.1016/j.scitotenv.2014.09.103

Escudero-Almanza, D.J. et al. (2012). Carbonic Anhydrase and Zinc In Plant Physiology, Chilean Journal Of Agricultural Research 72(1)

Eslami, A., Amini, M. M., Yazdanbakhsh, A. R., Rastkari, N., Mohseni-Bandpei, A., Nasseri, S., … Asadi, A. (2015). Occurrence of non-steroidal anti-inflammatory drugs in Tehran source water, municipal and hospital wastewaters, and their ecotoxicological risk assessment. Environmental Monitoring and Assessment, 187(12). doi:10.1007/s10661-015-4952-1

Farré, M. et al. (2001). Determination of drugs in surface water and wastewater samples by liquid chromatography–mass spectrometry: methods and preliminary results including toxicity studies with *Vibrio fischeri*. Journal of Chromatography A, 938(1-2), 187–197. doi:10.1016/s0021-9673(01)01154-2

Freches, S. (2017). Medicamento que bate todos os recordes e até já foi à Lua faz 120 anos. Available <https://www.dn.pt/sociedade/medicamento-que-bate-todos-os-recordes-e-ate-ja-foi-a-lua-faz-120-anos-8830107.html>.

Galati, G., Tafazoli, S., Sabzevari, O., Chan, T. S., O'Brien, P. J. (2002). Idiosyncratic NSAID drug induced oxidative stress. Chemico-Biological Interactions, 142(1-2), 25–41. doi:10.1016/s0009-2797(02)00052-2

Gao, X., Geng, J., Du, Y. et al. (2018) Comparative study of the toxicity between three non-steroidal anti-inflammatory drugs and their UV/$Na_2S_2O_8$ degradation products on *Cyprinus carpio*. Sci Rep 8, 13512.

Garbutt, J. S., Little, T. J. (2014). Maternal food quantity affects offspring feeding rate in *Daphnia magna*. Biology Letters, 10(7), 20140356–20140356. doi:10.1098/rsbl.2014.0356

Gentili, A. (2007). Determination of non-steroidal anti-inflammatory drugs in environmental samples by chromatographic and electrophoretic techniques. Analytical and Bioanalytical Chemistry, 387(4), 1185–1202. doi: 10.1007/s00216-006-0821-7.

Ghlichloo, I., Gerriets, V. (2020). Nonsteroidal Anti-inflammatory Drugs (NSAIDs). In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing. Available: <https://www.ncbi.nlm.nih.gov/books/NBK547742/>.

Gierse, J. K., Koboldt, C. M., Walker, M. C, Seibert, K., Isakson, P. C. (1999). Kinetic basis for selective inhibition of cyclo-oxygenases. Biochem J 339: 607–614.6

Gomes A., Correia A.T., Nunes B. (2019). Worms on drugs: ecotoxicological effects of acetylsalicylic acid on the Polychaeta species *Hediste diversicolor* in terms of biochemical and histological alterations. Environmental Science and Pollution Research, 26, (13), 13619-13629.

Habig W. H., Pabst M. J., Jakoby W. B. (1974). Glutathione S-transferases—the first enzymatic step in mercapturic acid formation. J Biol Chem. 249, 7130–7139.

Han S, Choi K, Kim J, Ji K, Kim S, et al. (2010). Endocrine disruption and consequences of chronic exposure to ibuprofen in Japanese medaka *Oryzias latipes* and freshwater cladocerans *Daphnia magna* and *Moina macrocopa*. Aquatic Toxicology 98: 256-264.

669 Heckmann, L. H., Callaghan, A., Hooper, H. L., Connon, R., Hutchinson, T. H.,
670 et al. (2007). Chronic toxicity of ibuprofen to *Daphnia magna:* Effects on life history
671 traits and population dynamics. Toxicology Letters 172: 137-145.

672 Heckmann, L. H., Sibly, R. M., Connon, R., Hooper, H. L., Hutchinson, T. H., et
673 al. (2008a). Systems biology meets stress ecology: Linking molecular and organismal
674 stress responses in *Daphnia magna.* Genome Biology 9: R40 1-15

675 Heckmann, L.-H., Sibly, R. M., Timmermans, M. J., Callaghan, A. (2008b).
676 Outlining eicosanoid biosynthesis in the crustacean *Daphnia*. Frontiers in Zoology,
677 5(1), 11. doi:10.1186/1742-9994-5-11

678 Henry, R. P. (1996). Multiple roles of carbonic anhydrase in cellular transport
679 and metabolism. Annual Review of Physiology 58, 523–538.

680 Hernando, M. D., Heath, E., Petrovic, M., Barceló, D. (2006). Trace-level
681 determination of pharmaceutical residues by LC-MS/MS in natural and treated waters.
682 A pilot-survey study. Analytical and Bioanalytical Chemistry, 385(6), 985–991.

683 Hiscox, J. D., Israelstam, G. F. (1979). A method for the extraction of
684 chlorophyll from leaf tissue without maceration. Can J. Bot. 57, 1332-1334.

685 Hoang, T. C., Klaine, S. J. (2007). Influence of organism age on metal toxicity
686 to *Daphnia magna*. Environ Toxicol Chem. 26(6), 1198□1204. doi:10.1897/06-515r.1

687 Ito, H., Matsui, H., Hirayama, A., Indo, H. P., Majima, H. J., Hyodo, I. (2016).
688 Reactive oxygen species induced by non-steroidal anti-inflammatory drugs enhance the
689 effects of photodynamic therapy in gastric cancer cells. Journal of clinical biochemistry
690 and nutrition, 58(3), 180–185. https://doi.org/10.3164/jcbn.15-124

691 Jaramillo, E., Duarte, C., Labra, F.A. et al. (2019). Resilience of an aquatic
692 macrophyte to an anthropogenically induced environmental stressor in a Ramsar
693 wetland of southern Chile. Ambio 48, 304–312 https://doi.org/10.1007/s13280-018-
694 1071-6

695 KEGG – Kyoto Encycloedia of Genes and Genomes
696 <https://www.genome.jp/kegg/>.

697 Knudsen, J. F., Carlsson, U., Hammarström, P. et al. (2004). The
698 Cyclooxygenase-2 Inhibitor Celecoxib Is a Potent Inhibitor of Human Carbonic
699 Anhydrase II. Inflammation 28, 285–290. https://doi.org/10.1007/s10753-004-6052-1

700 Kosjek, T., Heath, E., Krbavcic, A. (2005). Determination of non-steroidal anti-
701 inflammatory drug (NSAIDs) residues in water samples. Environment International,
702 31(5), 679–685. doi:10.1016/j.envint.2004.12.001

703 Krnic D, Anic-Matic A, Dosenovic S, Draganic P, Zezelic S, Puljak L. (2015).
704 National consumption of opioid and nonopioid analgesics in Croatia: 2007–2013. Ther
705 Clin Risk Manag. 11:1305-1314.bhttps://doi.org/10.2147/TCRM.S86226

706 Kummerová, M., Zezulka, Š., Babula, P., & Tříska, J. (2016). Possible
707 ecological risk of two pharmaceuticals diclofenac and paracetamol demonstrated on a
708 model plant *Lemna minor*. Journal of Hazardous Materials, 302, 351–361.
709 doi:10.1016/j.jhazmat.2015.09.057

710 Lapworth, D. J., Baran, N., Stuart, M. E., Ward, R. S. (2012(. Emerging organic
711 contaminants in groundwater: A review of sources, fate and occurrence. Environmental
712 Pollution, 163, 287–303. doi:10.1016/j.envpol.2011.12.034

713 Lichtenthaler, H. K. (1987). Chlorophylls and Carotenoids Pigments of
714 Photosynthetic Biomembranes. Methods in Enzymology. 148, 350-382.

715 Lindqvist, N., Tuhkanen, T., Kronberg, L. (2005). Occurrence of acidic
716 pharmaceuticals in raw and treated sewages and in receiving waters. Water Research,
717 39(11), 2219–2228. doi:10.1016/j.watres.2005.04.003

718 Lindskog, S. (1997). Structure and mechanism of carbonic anhydrase.
719 Pharmacology & Therapeutics, 74(1), 1–20. doi:10.1016/s0163-7258(96)00198-2

720 Martinez-Sena, T., Armenta, S., Guardia, M., Esteve-Turrillas, F. A. (2016).
721 Determination of non-steroidal anti-inflammatory drugs in water and urine using
722 selective molecular imprinted polymer extraction and liquid chromatography. Journal of
723 Pharmaceutical and Biomedical Analysis, 131, 48–53. doi:10.1016/j.jpba.2016.08.006

724 Meek, I. L., Van de Laar, M. A. F. J., Vonkeman, E. H. (2010). Non-Steroidal
725 Anti-Inflammatory Drugs: An Overview of Cardiovascular Risks. Pharmaceuticals,
726 3(7), 2146–2162. doi:10.3390/ph3072146

727 Mennillo, E., Arukwe, A., Monni, G., Meucci, V., Intorre, L., Pretti, C. (2017).
728 Ecotoxicological properties of ketoprofen and the S(+)-enantiomer (dexketoprofen):
729 Bioassays in freshwater model species and biomarkers in fish PLHC-1 cell line.
730 Environmental Toxicology and Chemistry, 37(1), 201–212. doi:10.1002/etc.3943

731 Metcalfe, C. D., Koenig, B. G., Bennie, D. T., Servos, M., Ternes, T. A., &
732 Hirsch, R. (2003). Occurrence of Neutral and Acidic Drugs In The Effluents Of
733 Canadian Sewage Treatment Plants. Environmental Toxicology and Chemistry, 22(12),
734 2872. doi:10.1897/02-469 doi:10.1016/j.watres.2005.04.003

735 Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance Trends
736 Plant Sci., 7, 405-410 PMID: 12234732 52

737 Monaghan, P., Metcalfe, N. B. and Torres, R. (2009). Oxidative stress as a
738 mediator of life history trade□offs: mechanisms, measurements and interpretation.
739 Ecology Letters, 12: 75-92. doi:10.1111/j.1461-0248.2008.01258.x

740 Moons, A. (2005). Regulatory and Functional Interactions of Plant Growth
741 Regulators and Plant Glutathione S-Transferases (GSTs). Vitamins & Hormones, 155–
742 202. doi:10.1016/s0083-6729(05)72005-7

743 Moroney, J. V., Bartlett, S. G., Samuelsson, G. (2001). Carbonic anhydrases in
744 plants and algae. Plant, Cell and Environment, 24(2), 141–153. doi:10.1111/j.1365-
745 3040.2001.00669.x

746 Nkoom, M., Lu, G., Liu, J., Dong, H., Yang, H. (2019). Bioconcentration,
747 behavioral, and biochemical effects of the non-steroidal anti-inflammatory drug
748 diclofenac in *Daphnia magna*. Environmental Science and Pollution Research.
749 doi:10.1007/s11356-018-04072-3

750 Nogueira, A. F., Pinto, G., Correia, B., Nunes, B. (2019). Embryonic
751 development, locomotor behavior, biochemical, and epigenetic effects of the
752 pharmaceutical drugs paracetamol and ciprofloxacin in larvae and embryos of *Danio*
753 *rerio* when exposed to environmental realistic levels of both drugs. Environmental
754 Toxicology. doi:10.1002/tox.22819

755 OECD (2006) *Lemna sp*. growth inhibition test n. 221. OECD–Guideline for
756 Testing of Chemicals, Paris, 22 p.

OECD (2012) Test No. 211: *Daphnia magna* reproduction test, OECD guidelines for the testing of chemicals, section 2. OECD Publishing, Paris. https://doi.org/10.1787/9789264185203-en

PACT - Prescribing and Medicines Team Health and Social Care Information Centre. (2016). Prescription Cost Analysis, England 2015. Leeds (UK): Health and Social Care Information Centre. ISBN: 978□1□78386□680□9

Petrovic N., Murray M. (2010) Using N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) to Assay Cyclooxygenase Activity In Vitro. In: Armstrong D. (eds) Advanced Protocols in Oxidative Stress II. Methods in Molecular Biology (Methods and Protocols), vol 594. Humana Press, Totowa, NJ

Piedade, F., Bio, S., Nunes, B. (2019). Effects of common pharmaceutical drugs (paracetamol and acetylsalicylic acid) short term exposure on biomarkers of the mussel *Mytilus* spp. Environmental Toxicology and Pharmacology, 103276. doi:10.1016/j.etap.2019.103276

Podio, N. S., Bertrand, L., Wunderlin, D. A., Santiago, A. N. (2019). Assessment of phytotoxic effects, uptake and translocation of diclofenac in chicory (*Cichorium intybus*). Chemosphere, 125057. doi:10.1016/j.chemosphere.2019.125057

Praskova E, Voslarova E, Siroka Z, Macova S, Plhalova L, Bedanova I, Marsalek P, Pistekova V, Svobodova Z. (2011). Comparison of acute toxicity of ketoprofen to juvenile and embryonic stages of *Danio rerio*. Neuro Endocrinol Lett. 2011;32 Suppl 1:117-20.

Prášková, E., Štěpánová, S., Chromcová, L., Plhalová, L., Voslářová, E., Pištěková, V., … Svobodová, Z. (2013). The effects of subchronic exposure to

781 ketoprofen on early developmental stages of common carp. Acta Veterinaria Brno,
782 82(3), 343–347. doi:10.2754/avb201382030343

783 Rangasamy, B., Hemalatha, D., Shobana, C., Nataraj, B., Ramesh, M. (2018).
784 Developmental toxicity and biological responses of zebrafish (*Danio rerio*) exposed to
785 anti-inflammatory drug ketoprofen. Chemosphere.
786 doi:10.1016/j.chemosphere.2018.09.013

787 Ribeiro F., Ferreira, N. G. C., Ferreira, A., Soares, A. M. V. M., Loureiro, S.
788 (2011). Is ultraviolet radiation a synergistic stressor in combined exposures? The case
789 study of Daphnia magna exposure to UV and carbendazim. Aquat Toxicol 102(1–
790 2):114–122. https://doi.org/10.1016/j.aquatox.2011.01.007

791 Rowley, A. F., Voga, C. L., Taylor, G. W., Clare, A. S. (2005). Prostaglandins in
792 non-insectan invertebrates: recent insights and unsolved problems. Journal of
793 Experimental Biology, 208(1), 3–14. doi:10.1242/jeb.01275

794 Rzymski, P., Drewek, A., Klimaszyk, P. (2017(. Pharmaceutical pollution of
795 aquatic environment: an emerging and enormous challenge. Limnological Review,
796 17(2), 97–107. doi:10.1515/limre-2017-0010

797 Sairam R, Tyagi A. 2004. Physiology and molecular biology of salinity stress
798 tolerance in plants. Curr Sci-Bangalore. 86:407–421.

799 Schieber, M., Chandel, N. S. (2014). ROS Function in Redox Signaling and
800 Oxidative Stress. Current Biology, 24(10), R453–R462. doi:10.1016/j.cub.2014.03.034

801 Simon, L. S. (2013). Nonsteroidal anti-inflammatory drugs and their risk: a story
802 still in development. Arthritis Res Ther 15, https://doi.org/10.1186/ar4173

803 Smirnov, N. N. (2013) Physiology of the Cladocera. Academic Press, San
804 Diego, CA, USA.

805 Smirnov, N. N. (2017). Physiology of the Cladocera. 2nd Edition. Elsevier.
806 ISBN 978-0-12-805194-8

807 Stanley, D. (2006). Prostaglandins and Other Eicosanoids In Insects: Biological
808 Significance. Annual Review of Entomology, 51(1), 25–44.
809 doi:10.1146/annurev.ento.51.110104.151021

810 Sytar, O., Kumar, A., Latowski, D. et al. (2013). Heavy metal-induced oxidative
811 damage, defense reactions, and detoxification mechanisms in plants. Acta Physiol Plant
812 35, 985–999 https://doi.org/10.1007/s11738-012-1169-6

813 Ternes, T. A. (1998). Occurrence of drugs in German sewage treatment plants
814 and rivers. Water Research, 32(11), 3245–3260. doi:10.1016/s0043-1354(98)00099-2

815 Tixier, C., Singer, H. P., Oellers, S., Müller, S. R. (2003). Occurrence and Fate
816 of Carbamazepine, Clofibric Acid, Diclofenac, Ibuprofen, Ketoprofen, and Naproxen in
817 Surface Waters. Environmental Science & Technology, 37(6), 1061–1068.
818 doi:10.1021/es025834r

819 Verpoorte, J. A., Suchinta, M., Edsall, J. T. (1967). Esterase activities of human
820 carbonic anhydrase B and C. J. Biol. Chem. 242, 4221–4229.

821 Wang, L., Peng, Y., Nie, X., Pan, B., Ku, P., Bao, S. (2016). Gene response of
822 CYP360A, CYP314, and GST and whole-organism changes in *Daphnia magna* exposed
823 to ibuprofen. Comparative Biochemistry and Physiology Part C: Toxicology &
824 Pharmacology, 179, 49–56. doi:10.1016/j.cbpc.2015.08.010

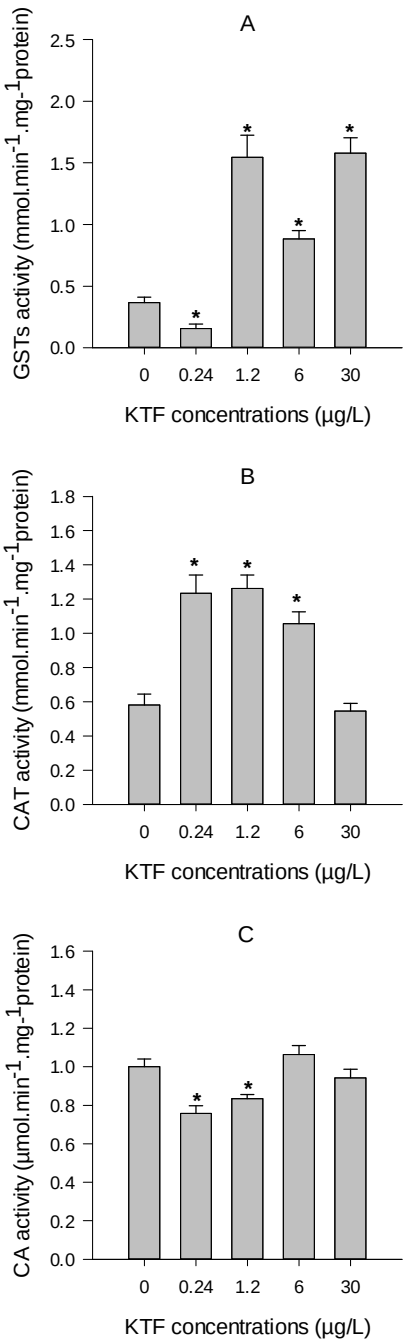
825 Wang, W. (1990). Literature review on duckweed toxicity testing.
826 Environmental Research, 52(1), 7–22. doi:10.1016/s0013-9351(05)80147-1

827 Wongrakpanich, S., Wongrakpanich, A., Melhado, K., Rangaswami, J. (2018).
828 A Comprehensive Review of Non-Steroidal Anti-Inflammatory Drug Use in The
829 Elderly. Aging and disease, 9(1), 143–150. https://doi.org/10.14336/AD.2017.0306

830 Wrede, J. D. (2015). Pharmaceutically active compounds might bioaccumulate
831 and cause effects on the free – floating macrophyte *Ceratophyllum demersum*. PhD
832 thesis. Lebenswissenschaftliche Fakultät der Humboldt-Universität zu Berlin. 103p.

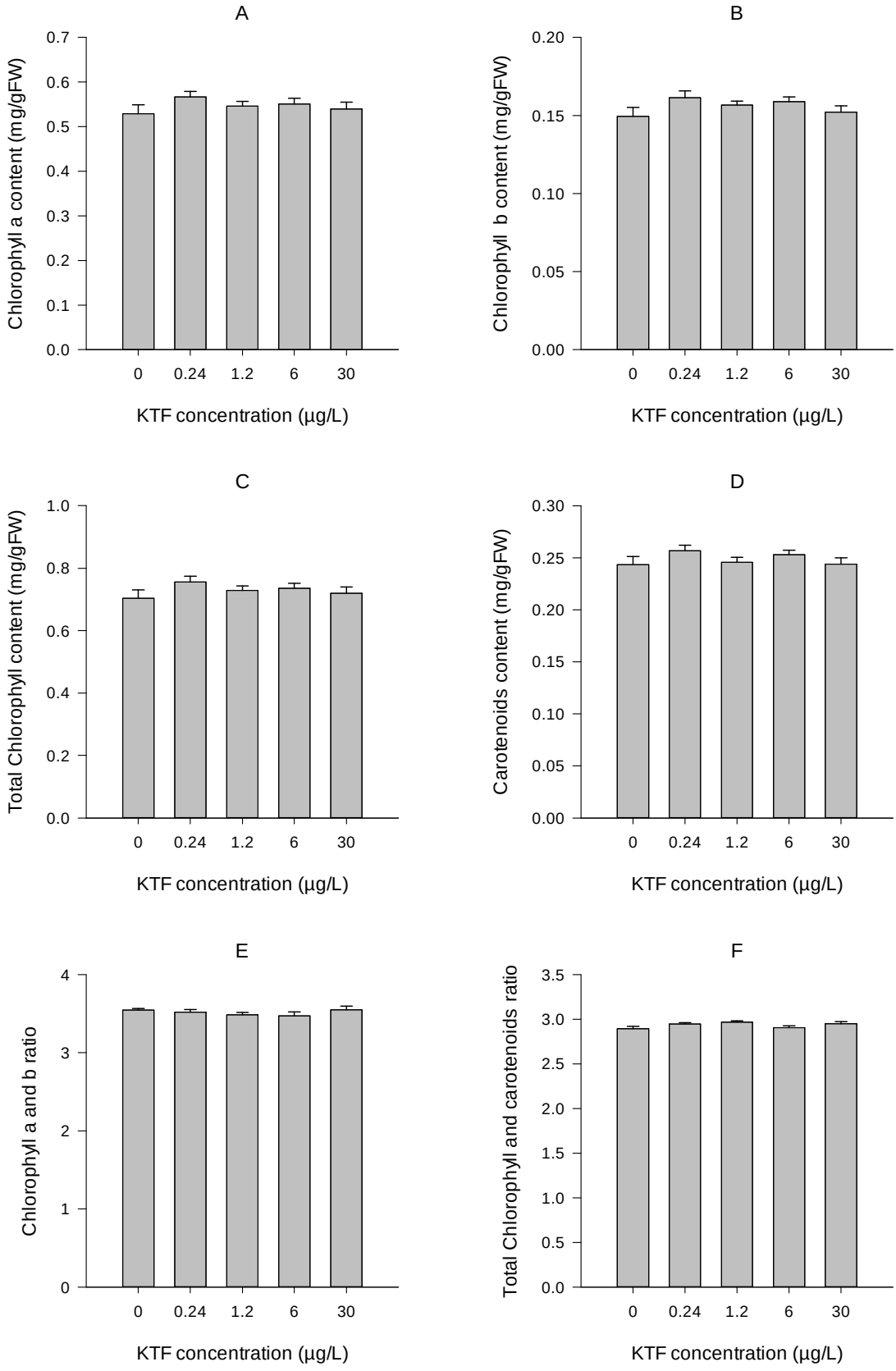
833 Xie, X., He, Z., Chen, N., Tang, Z., Wang, Q., Cai, Y. (2019). The Roles of
834 Environmental Factors in Regulation of Oxidative Stress in Plant. BioMed Research
835 International, 2019, 1–11. doi:10.1155/2019/9732325

836 **Figures**



837

838 Figure 1 – Effects of ketoprofen exposure in *L. minor* biomarkers upon exposure of 96 hours. A)
839 glutathione S-transferases activity; B) catalase activity; and C) carbonic anhydrase activity. Bars and
840 errors bars are Mean ± SE (N= 10). * stands for statistical differences in relation to control following
841 ANOVA and Dunnett's or the equivalent non-parametric test.



842

843 Figure 2 – Effects of ketoprofen exposure in *L. minor* during 96 hours. A) chlorophyll a content, B)
844 chlorophyll b content, C) total chlorophyll content, D) carotenoids content, E) chlorophyll a and b ratio
845 and F) total chlorophyll and carotenoids ratio. Bars and errors bars are Mean ± SE (N= 10).

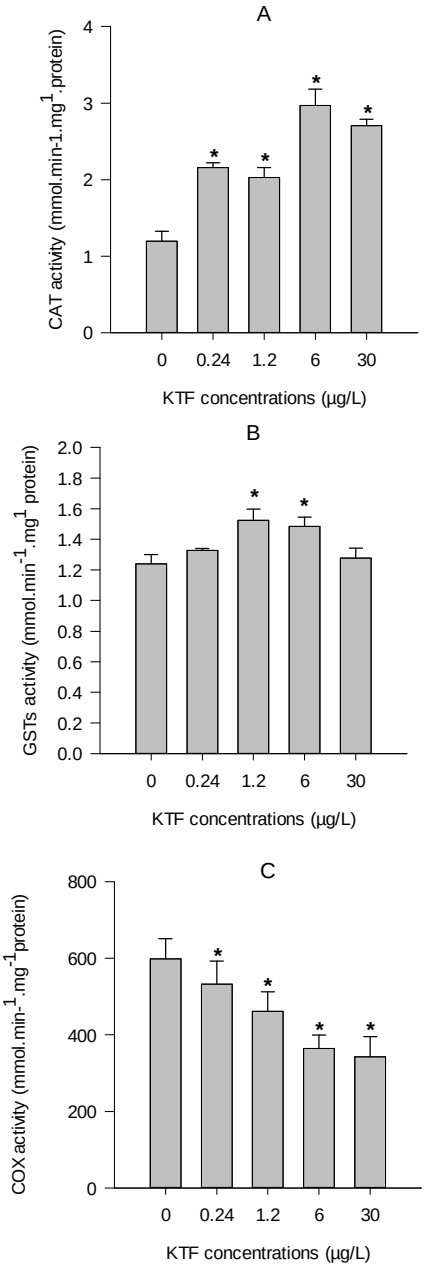
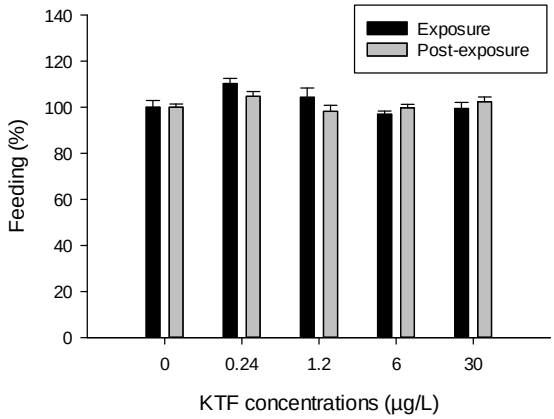


Figure 3 – Effects of ketoprofen in *D. magna* after acute exposure (48h). A) catalase activity B) glutathione S-transferases activity, and C) cyclooxygenase activity. Bars and errors bars are Mean ± SE (N= 5). * stands for statistical differences in relation to control following ANOVA and Dunnett's or the equivalent non-parametric test.

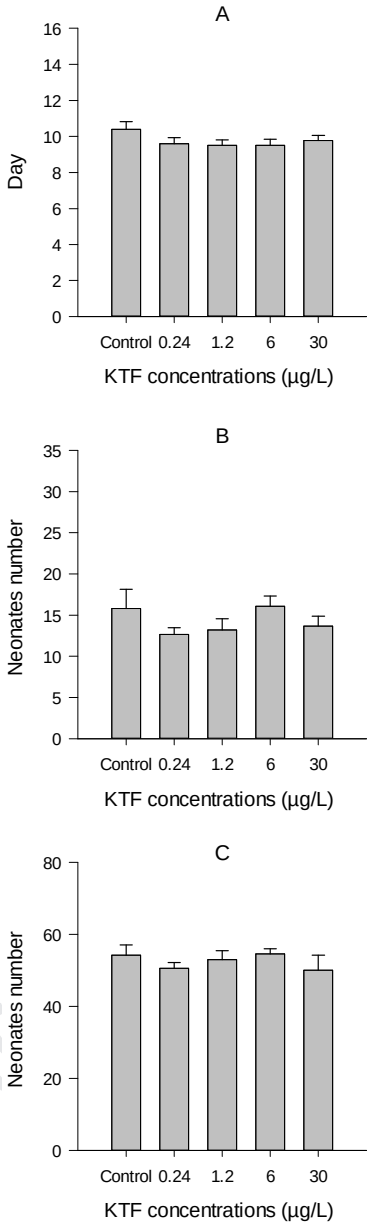


851

852 Figure 4 – Effects of ketoprofen in *D. magna* feeding behavior, after exposure and after recovery (post-
853 exposure). Bars and errors bars are Mean ± SE (N= 5).

854

855



856

857 Figure 5 – Ketoprofen effects in reproductive parameters in *Daphnia magna*. A) day of the first brood, B)
858 number of neonates from the first brood and C) total number of neonates (three broods). Bars and errors
859 bars are Mean ± SE (N= 10).

860

Highlights

- ✓ Ketoprofen (KTF) acts different on *Lemna minor* and on *Daphnia magna*;
- ✓ Effects of KTF were limited only to biochemical effects on both species;
- ✓ *L. minor* effects of low KTF concentrations suggest resilience and hormetic effects;
- ✓ *D. magna*, in general, presented a dose response pattern to KTF exposure.

Authors statement

GDA – Conceptualization; Methodology; Formal analysis; Investigation; Visualization; Writing - Original Draft; Writing - Review & Editing.

AMVMS Writing - Review & Editing; Supervision; Project administration; Funding acquisition

CB - Writing - Review & Editing; Supervision; Project administration; Funding acquisition

BN – Visualization; Writing - Review & Editing; Supervision; Project administration; Funding acquisition

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: