Insights into the ecotoxic impact of diclofenac using *Daphnia magna* as a model organism

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ABSTRACT

Residues of pharmaceutical compounds (PCs) are among the groups of contaminants of emerging concerns that have been reportedly detected in the aquatic environment. These compounds are widely distributed in diverse water bodies, thus, necessitating eco-toxicological assessment of PCs. However, data concerning the risk they pose to unintended non-target species in different ecosystems are still very scanty and scarce. This study investigated the ecotoxic effect of diclofenac, an analgesic, on freshwater aquatic ecosystem using the sensitive *Daphnia magna* (water flea) bioassay. The daphnid bioassay was carried out at concentrations 10 µg/L and 100 µg/L, which are the least and upper-end detection (LED & UED) values of the range of concentrations of diclofenac detected in the tested aquatic water columns, as well as reported levels in some international surveys. The 24 to 48-hr *Daphnia magna* test revealed a mortality rate of $\geq$ 75 % and 90 % for 100 µg/L diclofenac, respectively, in freshwater-spiked samples. These results suggest that the presence of PCs in aqueous ecosystems may pose a lethal impact on aquatic fauna at the detected levels in the environment.

Keywords: Bioassays, *Daphnia magna*, emerging contaminants, fauna, mortality rate

INTRODUCTION

Residues of pharmaceutical compounds (PCs) have often been reported to be present in river waters (Yang *et al.*, 2020, Boulard *et al.*, 2020, Madikizela *et al.*, 2020, Nannou *et al.*, 2020, Fernandes *et al.*, 2020, Su *et al.*, 2020, Li *et al.*, 2020, Kairigo *et al.*, 2020). Concerns over environmental contamination arising from residues of PCs have been a subject of interest. These compounds find their way into the environment through various routes and pathways, from post-consumption release to indiscriminate disposal of unused/expired drugs, with the significant entry route being effluent discharges from inefficient sewage treatment plants (Omotola and Olatunji, 2020). As a result, PCs along with other contaminants are discharged into receiving water bodies (Wilde *et al.*, 2017).

Some of these pharmaceutical compounds do not readily break down in the environment. In the worst situation where there is a continuous release of these bioactive compounds at a rate greater than their biodegradation, the PCs may accumulate and persist in polarity-compatible matrices for longer times after their release (Oluwole et al., 2020). This has a likelihood of causing detrimental ecological effects on microorganisms within the ecosystem, thereby disrupting the health and proper functioning of the interrelated biochemical processes.

Data and information on environmental monitoring and ecotoxicology of pharmaceutical contaminants are scanty and still emerging in Africa; hence, ecotoxicity studies based on reported concentrations of PC residues detected in the environment are rarely available. The few available data on ecotoxicity studies of some PCs using daphnids as test organisms were all conducted using therapeutic concentrations, such that effective concentration (EC50) up to 687,000 µg/L over a specific exposure duration have been reported (Minguez *et al.*, 2016, Havelkova *et al.*, 2016). Meanwhile, bearing in mind, the monotonic and non-monotonic responses of living organisms, the ecotoxicity of toxicants is not to be established based on logic, but on real terms since their environmental occurrence is at ultra-

low concentrations. Monotonic responses (MR) are somewhat linear dose responses elicited from organisms in response to toxic substances, and this increases proportionally with the level of exposure to the toxins (Lagarde *et al*., 2015), such that when a plot of chemical effect and the logarithm (log10) of the dose is plotted, it gives rise to a curvilinear or sigmoidal dose-response shape. Non-monotonic dose-response (NMDR), on the other hand, is a non-linear dose-response sometimes described as a biphasic dose-response (Andersson, 2015). Earlier studies were conducted at concentrations that may be too high for the most reality scenarios (Magdaleno *et al*., 2015, Havelkova *et al*., 2016, Drzymała and Kalka, 2020), hence this work investigated lower levels of PC that are representative of obtainable concentrations upon exposure. The present study focuses on the adverse effects of a commonly prescribed PC (diclofenac) at non-therapeutic levels.

This study, therefore, investigated the potential acute ecotoxic effect of diclofenac (Figure 1) at concentrations between 10 and 100 µg/L on aquatic fauna using *Daphnia magna* (freshwater flea) bioassay. Presently, diclofenac is approved only for human use but, it is illicitly used in veterinary medicine (Mahapatro and Arunkumar, 2014), thus, causing fatal consequences (Ågerstrand *et al*., 2015). Variable degrees of environmental contamination by diclofenac have since been detected in aquatic environments within African countries such as Nigeria (Olaitan *et al*., 2017, Ebele *et al*., 2020). *Daphnia magna* are filter-feeders because they can filter off tiny particles in the aquatic environment, which is part of the reason they are able to subsist in a freshwater environment. Oftentimes, the absence of *Daphnia magna* in a water body can signal water contamination. The choice of the PC was based on pre-informed knowledge of prevailing health needs, and detection in the Nigerian surface waters environment (Kirim *et al*., 2014, Ebele *et al*., 2020).

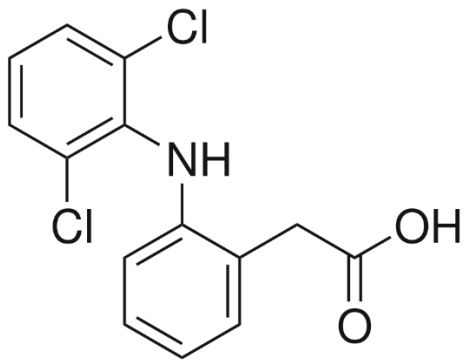

Figure 1: Chemical structure of diclofenac

MATERIALS AND METHODS

Materials

Neat standard of diclofenac was sourced from Sigma Aldrich, Germany. Daphtoxkit, with kit number DM576, ephippia (batch number-DM07119), and spirulina (batch number SP300118), were purchased from Tox Solutions Kits and Services (Stellenbosch, South Africa). A cool white light bulb for the illumination of ephippia was bought from South Africa. MilliQ water used in the LC analysis was prepared using RiOs^{TM}/Elix 5 Millipore Water Purifier (Model No PF05113). The Council for Scientific and Industrial Research (CSIR), Stellenbosch, South Africa, provided an aquarium air pump used in this study. All experiments were carried out at CSIR laboratory, Stellenbosch, South Africa.

Preparation of stock solution of analytes

About 0.01 g each of diclofenac was accurately weighed and dissolved in methanol in 10 mL volumetric flasks and made up to mark to achieve 1000 mg/L stock solution of the diclofenac. The diclofenac stock solution (10 mg/L) was prepared using pre-aerated freshwater (200 µL of 1000 mg/L made up in 20 mL standard flask). This solution was always freshly prepared for the bioassay. The PC

standard of different concentrations ranging between 10 and 100 µg/L, was prepared by serial dilution of an appropriate volume from the 10 mg/L stock solutions in aerated freshwater to achieve the concentration range needed for the bioassay.

Data interpretation

Descriptive statistics and other statistical analysis were carried out using Microsoft Excel 2016 package and the lethal dose 50 (LD50) curves obtained by the 'trend' function, from which respective values (LD50) were extrapolated. The curves were generated by plotting graphs of % response (mortality) of daphnids after 24 and 48 hr against the logarithmic concentration of test solutions. Simple fuzzy logic was employed in the classification of the response elicited from the *Daphnia magna*. These classifications include four categories: no lethal effect, mild lethal effect, severe lethal effect, and extremely severe lethal effects (Table 1).

Table 1: Classification of the lethal impact of targeted compounds using fuzzy logic principle

No lethal impact (W)	Mild lethal impact (X)	Severe lethal impact (Y)	Extremely severe lethal impact (Z)
$m < 25$	$m \geq 25 \leq 50$	$m > 50 \ \leq 75$	$m > 75$

Where $m\ is\ \%\ mortality\ of\ daphnids$

Daphnia 24-48 hr acute toxicity test

Acute immobilization test with *Daphnia magna* (freshwater fleas) was carried out according to the Organization for Economic Cooperation and Development (OECD) guidelines for the testing of chemicals, Tests No. 202 (O.E.C.D., 2004), which forms the basis of the kits manufacturer's protocol, with slight modification. The basic principle of this test is centered on the toxicity of the test solutions to the daphnids, as signified by motility and mortality rates. This is usually confirmed by tapping the assay plates for 15 seconds to observe drowning (dead daphnids are found at the base of the wells), and active/less movement according to the protocol (O.E.C.D., 2004).

RESULTS AND DISCUSSION

Daphnids' response to diclofenac dosages

The result obtained based on the % mortality of the daphnids after the acute exposure of the 10 µg/L concentration of the diclofenac solution ranged from 0 % to 25 % at 24 hr and 48 hr post-exposure, respectively. Using the 4-grid fuzzified indices, it could be said that the 10 µg/L diclofenac did not pose any lethal effect (W) to the daphnids. However, the observed % mortality of the daphnids after the acute exposure of the higher 100 µg/L concentration of diclofenac solution ranged from 75 % to 90 % at 24 hr and 48 hr post-exposure, respectively. This result showed that diclofenac exerted severe lethal impact (Z) both after 24 hr and 48 hr exposure to the daphnids, with 100 % survival on daphnids exposed to the control. The Figure below (Figure 2) summarizes the result obtained in this study.

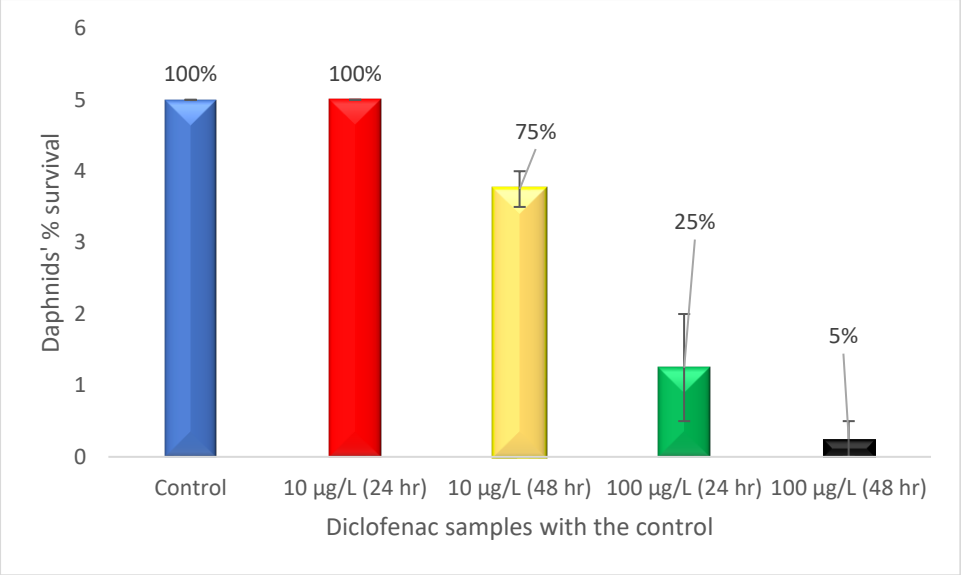

Figure 2: Daphnids' percent survival after 24-28 hr diclofenac exposure

The responses of daphnids to diclofenac at the different test concentrations (10 µg/L and 100 µg/L) were monotonic. This implied that the lower concentration of diclofenac had no effect on the daphnids, as extreme lethal impact was observed at higher concentration. This result could be linked to the fact that analgesics, specifically, acetaminophen is ascertained to induce pro-oxidant effects on biota leading to the activation of oxidative stress (Matić *et al.*, 2019). Oxidative stress induces the production of reactive oxygen species (ROS) in stressed organisms, which in turn may hunt the cell structures and possibly leading to the death of daphnids (Pizzino et al., 2017). Pro-oxidant effects on biota, however, depend on the favourability of thermodynamic parameters (Gonzalez-Rey and Bebianno, 2014, Bebianno *et al.*, 2017). Where a reduction of molecular oxygen to superoxide (or hydroxyl radicals) is favoured, ROS levels drop significantly and possibly to zero, and the rate of cell kill activities of ROS will be reduced, thereby allowing for the subsistence of aerobic life. Pro-oxidant activities such as this, facilitate an enhanced conducive environment for the daphnids.

Another parameter considered in this study to ascertain the ecotoxic nature of diclofenac on *D. magna* is the LD50 value. The LD50 values were obtained from a plot of daphnids' % response (mortality) after 24 and 48 hr against the logarithmic concentration of each test solution. The LD50 curve for diclofenac is presented in Figure 3.

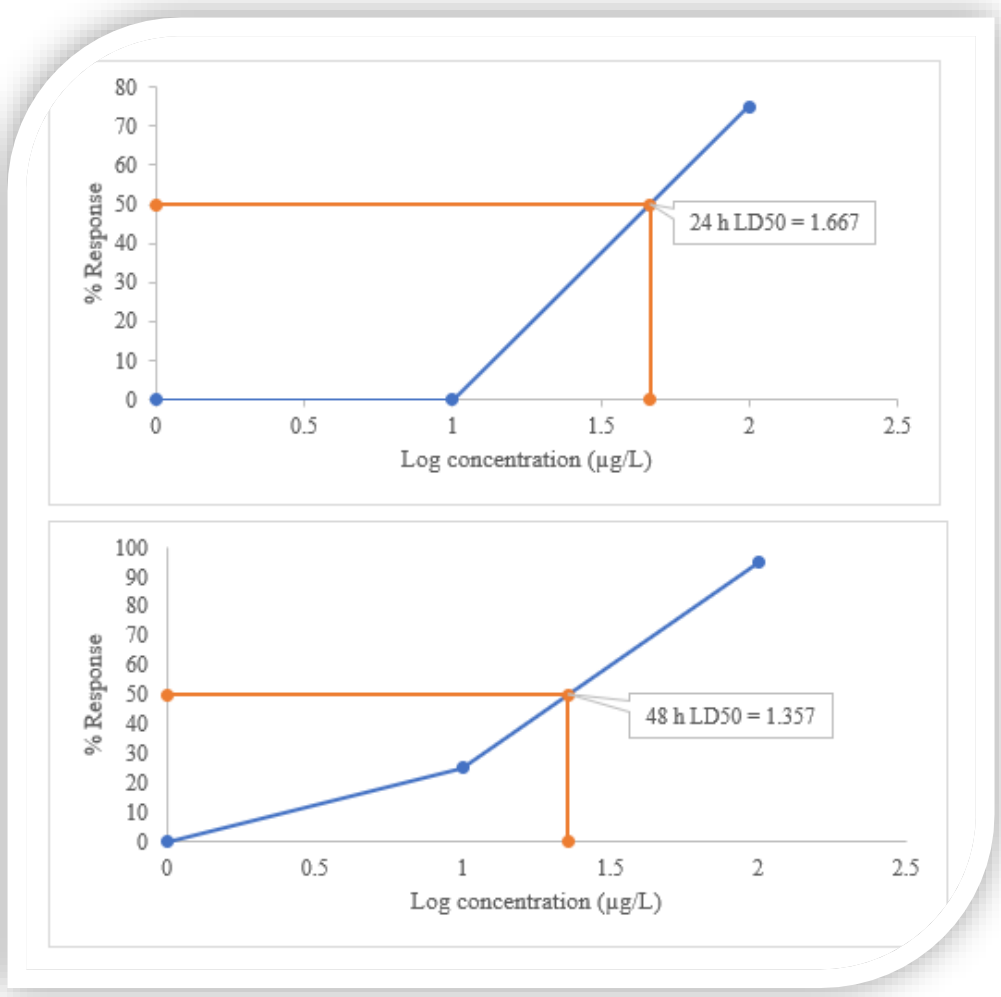

Figure 3: Plot of daphnia's % Response vs log concentration (ppb) to estimate the LD50 (24 and 48 hr) curves for diclofenac

The 24 hr and 48 hr LD50 values obtained for diclofenac were Log concentrations of 1.67 and 1.36, respectively, as shown in Figure 3. These results are equivalent to 46.8 µg/L and 22.9 µg/L, respectively. The implication of this result is that the presence of trace levels (in the ppb range) of diclofenac in the fresh aqueous environment (as revealed by the data of this study) has the potential to cause hazardous effects on aquatic biota, including fleas such as daphnia. In general, the hazardous effects of diclofenac on biota increased as a function of exposure time.

Very low toxicity effect (survival) on *D. magna* was earlier reported for diclofenac (Du *et al.*, 2016). This implies that lethal and sub-lethal toxicity endpoint concentrations of diclofenac in aqueous media may be in the low µg/L range. Therefore, diclofenac's environmental build-up is not desirable as non-target organisms might be adversely affected at ambient environmental concentration over a long exposure period. However, the mechanism of action of diclofenac with daphnids is not well-understood.

Comparison of results obtained in this study with other studies on aquatic organisms

As regards previous ecotoxicity studies on diclofenac, there are only a few studies reported. De Oliveira *et al.* (2016) reported the EC50 values of diclofenac as 123300 µg/L using *Daphnia magna* as bioindicator. Another team of authors that carried out similar work were Trombini *et al.*, (2016). The authors reported that diclofenac exposure to *Tisbe battagliai* had an EC50 value of 11300 µg/L. Finally,

Priyan *et al.* (2021) documented a lethal concentration 50 (LC50) value of 156990 µg/L using *Danio rerio*. It is noteworthy to mention that the values reported by these authors are not environmentally relevant concentrations. Despite the available scanty data and information, the mechanism of action of diclofenac that causes adverse reactions in non-target organisms is not easily explainable. Hence, ecotoxicity inferences were made based on their various actions and reactions, which can be monotonic or non-monotonic, on test organisms. Therefore, it is pretty difficult to conclude that, since 100 µg/L diclofenac led to fatality of almost all the test population, higher concentrations (e.g., 200 µg/L) of the same compound will result in kills of the whole test organisms, bearing in mind the monotonic and non-monotonic responses of living organisms.

CONCLUSIONS

The study investigated the ecotoxic actions of diclofenac using *Daphnia magna* as a model organism. An injury-related response in *Daphnia magna* survival was observed. Higher concentration of diclofenac (100 µg/L) was toxic, as evidenced by 95 % mortality of the daphnids after 48 hr exposure. On the contrary, a lower concentration of diclofenac (10 µg/L) exhibited no toxic effects on the organisms Thus, there may be no population effects on the ecosystem population and ecosystem functional balance with respect to this PC (10 µg/L diclofenac). However, chronic toxicities of this PC cannot be ruled out. Noteworthy is that previous studies on ecotoxicity bioassays of diclofenac were conducted using high concentrations, whereas these compounds are detected at trace and ultra-trace levels. This study, therefore, becomes one of the very few which considered the ecotoxic effects of environmentally relevant concentrations on aquatic fauna-*Daphnia magna*.

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