

Moving beyond standard toxicological metrics: The effect of diclofenac on planktonic host-parasite interactions

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ABSTRACT

Pharmaceuticals are increasingly released into surface waters and therefore ubiquitous in aquatic systems. While pharmaceuticals are known to influence species interactions, their effect on host-parasite interactions is still underexplored despite potential ecosystem-level consequences. Here, we ask whether diclofenac, a widely used non-steroid anti-inflammatory drug, affects the interaction between a phytoplankton host (*Staurastrum* sp.; green alga) and its obligate fungal parasite (*Staurastrumyces oculis*; chytrid fungus). We hypothesized that the effect of increasing diclofenac concentration on the host-parasite system depends on parasite exposure. We assessed acute and chronic effects of a wide range of diclofenac concentrations (0–150 mg/L) on host and parasite performance using a replicated long gradient design in batch cultures. Overall system response summarizing parameters related to all biotic components in an experimental unit i.e., number of bacteria and phytoplankton host cells along with photosynthetic yield (a measure of algal cell fitness), depended on diclofenac concentration and presence/absence of parasite. While host standing biomass decreased at diclofenac concentrations >10 mg/L in non-parasite-exposed treatments, it increased at ≥10 mg/L in parasite-exposed treatments since losses due to infection declined. During acute phase (0–48 h), diclofenac concentrations <0.1 mg/L had no effect on host net-production neither in parasite-exposed nor non-parasite-exposed treatments, but parasite infection ceased at 10 mg/L. During chronic phase (0–216 h), host net-production declined only at concentrations >10 mg/L in non-parasite-exposed cultures, while it was overall close to zero in parasite-exposed cultures. Our results suggest that chytrid parasites are more sensitive to diclofenac than their host, allowing a window of opportunity for growth of phytoplankton hosts, despite exposure to a parasite. Our work provides a first understanding about effects of a pharmaceutical on a host-parasite interaction beyond those defined by standard toxicological metrics.

1. Introduction

With an increasing and aging world population, the use and discharge of pharmaceuticals to the aquatic environment is projected to increase (Khetan and Collins, 2007). As pharmaceuticals are designed to be therapeutically active at low concentrations, even low environmental levels may already have far-reaching consequences for non-target organisms, their interactions and eventually also for ecosystem functioning (Morley, 2009; Van Donk et al., 2016). Conventional environmental risk assessment schemes of pharmaceuticals focus on the

direct effects of pharmaceuticals on individuals or populations. However, the influence of pharmaceuticals on interactions among species, in particular parasitic interactions is hardly explored.

Interactions between species are pivotal in maintaining biodiversity and ecosystem functions (Kirwan et al., 2009), therefore it is crucial to understand the severity of the effects of pharmaceuticals on species interactions. In aquatic systems, the effects of pharmaceuticals on higher trophic levels and their parasites have been explored (Morley, 2009). To exemplify, exposure of a fish species, *Squalius cephalus* to the central nervous system stimulant methamphetamine increased infection success

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rate by the obligate parasite larvae *glochidia* by nearly 20% (Douda et al., 2019). Combined effect of antiparasitic pharmaceuticals, oxamniquinone and praziquantel delayed the development of the trematode parasite, *Schistosoma mansoni* in the snail host, *Biomphalaria glabrata* (Mattos et al., 2007). Other studies have shown the potential of pharmaceuticals to disrupt aquatic host-parasite interaction. For example, an increase in virulence and synergistic reduction in host survival was observed in *Daphnia magna* (invertebrate host) – *Pasteuria ramosa* (obligate gram-positive bacteria) upon exposure to cyclosporine A (immunosuppressant) ranging between 0.01 mg/L to 0.1 mg/L (Schlüter-Vorberg and Coors, 2019). Diclofenac (0.5–2.0 µg/L) had a pre-oxidative toxic effect of on both parasite polychaeta, *Hediste diversicolor* and marine fish *Solea senegalensis* by increase in glutathione-S-transferases (Nunes et al., 2020). The effects of pharmaceuticals on the interactions at the basis of the aquatic food web remains yet underexplored.

Phytoplankton are commonly parasitized by chytrid fungi. Infection by chytrids can control phytoplankton biomass and species succession (Donk and Ringelberg, 1983) thereby changing aquatic food web structure as well as carbon and nutrient cycling (Sánchez Barranco et al., 2020). As parasites are completely dependent on their host for all their energetic and nutritional needs, they are also indirectly affected by the consequences that abiotic factors (including pharmaceuticals and pesticides) have on the condition and reproduction of their hosts (Pomati et al., 2017). Conversely, infection with parasites weakens or even kills the host, affecting host population dynamics and host condition. There is quite a large body of work on the temperature and nutrient context dependency in host-parasite interactions (Hatcher and Dunn, 2011; Wolinska and King, 2009), specifically in phytoplankton interactions (Gsell et al., 2013). However, effects of pharmaceuticals on non-target fungal parasites directly and on associated host-parasite dynamics is not well understood. Disregarding the effects of pharmaceuticals on parasites may therefore lead to an incomplete and/or inconclusive understanding of the effects of pharmaceuticals on ecosystems.

Diclofenac, a globally abundant non-steroidal anti-inflammatory, antipyretic drug is observed in the environment ranging from 45 ng/L–15 µg/L (Hanif et al., 2020; Walraven and Laane, 2008). In the environment, diclofenac can undergo several fates, with photodegradation as the prominent degradation pathway (Khetan and Collins, 2007; Packer et al., 2003). In acknowledgement of its ubiquitous occurrence and potential environmental impact (Ferrari et al., 2004; Verlicchi et al., 2012), diclofenac is included in the list of chemicals monitored in European Union surface waters (Domaradzka et al., 2016). Diclofenac impacts important primary producers such as phytoplankton, affecting physiological rates, mortality, and reproduction (Bonnefille et al., 2018; Ferrari et al., 2004). For example, direct phytotoxic effects of diclofenac have been shown to reduce fitness in algae (Majewska et al., 2021) which, in turn, may speed up or slow down the spread of infection, e.g. by lowering host resistance to infection (increasing infection), or host nutritional value (decreasing infection).

Here, we investigated how diclofenac concentrations affected the interaction between a phytoplankton host (*Staurastrum* sp.) and its obligate chytrid parasite (*Staurastrumyces oculus*) which depends completely on live and reproducing hosts. In this study we, for the first time, explore cues that are critical to formulate mechanistic hypotheses to understand the changes in the strength and outcome of host-parasite interactions upon exposure to a widely occurring pharmaceutical. We assessed fitness parameters in host cultures with and without exposure to the chytrid over a long gradient of diclofenac concentrations. This long gradient ranged from no diclofenac to measured environmental concentration ranges as well as high concentrations observed in hospital wastewater, accidental spills, and sewage overflows (Vieira et al., 2021). This study design allowed us to look at non-linear responses, e.g. the presence of hormetic effects where doses below toxicity threshold have a stimulatory effect (Calabrese et al., 1999). We hypothesized that the effect of increasing diclofenac concentration on the overall system (host and associated bacteria) depends on parasite exposure. We assessed

whether i) the overall system response to increasing diclofenac concentrations was modulated by the presence of a parasite; ii) the spread of infection decreased in response to increasing concentrations of diclofenac (reduction in prevalence); and iii) the combined impact of diclofenac and chytrid exposure decreased the host biomass accumulation in parasite-exposed treatments.

2. Material and methods

2.1. Experimental system

2.1.1. Host-parasite system

Our model system consisted of *Staurastrum* sp., a typical summer-blooming freshwater alga (Padisák et al., 2010), and its obligate fungal parasite *Staurastrumyces oculus*, both isolated from oligo-mesotrophic Lake Stechlin (E. Germany) (Van den Wyngaert et al., 2017). This chytrid parasite is characterized by a motile life stage (zoospores, diameter of 3–4 µm) that actively search for new hosts. Infection occurs by zoospores invading the host with a hyphen-like structure to absorb host nutrients while encysting in an external sporangium. Within the sporangium (diameter of 8–9 µm), the next generation of zoospores is formed either asexually (mitosis) or sexually, and eventually released by bursting of the sporangium. Sexual reproduction produces resting spores that can endure weeks to months before releasing the next generation of zoospores and are easily identified visually by their thicker sporangium walls. Within 2–3 days, infection by chytrids always leads to host death and precludes host reproduction before death (Van den Wyngaert et al., 2017).

2.1.2. Diclofenac

Diclofenac sodium, sodium 2-[(2, 6-dichlorophenyl) amino] phenylacetate (CAS No 15,307-79-6), is a derivative of phenyl acetic acid and is categorized as a NSAID. Diclofenac is used in human and veterinary medicine for its analgesic, antiarthritic and antirheumatic effects. In our experiment, diclofenac (sodium salt, brand name: Voltaren, Cayman Chemical Company, USA, ≥99% purity) was dissolved in demineralized water without addition of solvents, and subsequently filter-sterilized with a Sartorius Sartobran P300 filter (pore size 0.2 µm) to obtain stock solutions for the different concentrations. To determine the extent of photodegradation in the experimental matrix, we carried out a degradation experiment as outlined under 2.3.

2.2. Experimental design and methodology

We tested the effect of different diclofenac concentrations on host performance and host-parasite interaction using a replicated gradient design. As we were expecting a non-linear response to our broad range of exposures, we selected a gradient design rather than a classical ANOVA design (Kreyling et al., 2018). We exposed duplicated batch cultures of *Staurastrum* (“non-parasite-exposed”) and co-cultures of *Staurastrum* and *Staurastrumyces* (“parasite-exposed”) to 10 concentrations of diclofenac dissolved in water resulting in 20 experimental combinations and 40 experimental units. As *Staurastrumyces* is an obligate parasite, a parasite only treatment was not feasible. Our long diclofenac gradient included nominal concentrations reflecting environmental observations and effective concentration-50 (EC₅₀) values for green algal population growth (Ferrari et al., 2004; Verlicchi et al., 2012), ranging from 0 to 150 mg/L (0, 0.00001, 0.0001, 0.001, 0.01, 1, 10, 50, 100, 150 mg/L). The higher concentrations were chosen considering adverse events such as sewage overflows, and pollution accidents that lead to pulse additions of high concentrations of pollutants such as diclofenac. Hospital wastewaters are systems with diclofenac concentration in the range of 10 mg/L, for example, 19.82±0.054 mg/L concentrations were measured in hospital wastewater from Passo Fundo, Brazil (Vieira et al., 2021). Host and host-parasite co-cultures were acclimatized to the experimental light and temperature conditions for 14 days in batch

cultures. Host cultures were kept in an exponential growth phase by sequentially diluting them by 50% with WC medium every 4 days (Kilham et al., 1998). Host-parasite cultures were provided with uninfected host cultures to keep infection prevalence above 50% every second day. The experiment was started by setting up a total of 5000 host cells/mL in a final volume of 65 mL WC medium and verified by microscopic counting on day 0. Infection was achieved by adding additional 1600 infected host cells/mL from a 100% infected culture, resulting in a starting infection prevalence of ca 20%. Addition of infected cells did not contribute to the reproducing population as infected cells cease to divide and die due to infection within 2 days. Each experimental unit was inoculated with 5 mL of the respective diclofenac stock solution (or water in the case of the no diclofenac control).

All experimental units were created at the same time and placed in an incubator (SANYO Electric, Moriguchi, Japan) at 17 °C. The light was set to 40 $\mu\text{mol}/\text{m}^2/\text{s}$ intensity, provided by cool light fluorescent lamps (TL-D 30W/830) at a light cycle of 16: 8 h (light: dark). This light intensity is neither limiting nor inhibiting algal production (Dauta et al., 1990). 5 mL of subsamples were taken from all experimental units at 6 time points; 0, 24, 48, 72, 144 and 216 h. Based on OECD guidelines (OECD, 2002), the duration of 48 h is often used to assess acute toxicity and specifically growth of the algal population over a few generations. The final two time points were chosen to assess chronic toxicity and the potential effect of possible transformation products of diclofenac on the host and parasite performance.

Freshly harvested, live samples were stored in the dark for 10 min and then analyzed on a Multiwavelength-excitation PAM fluorometry PHYTO-PAM (Heinz Walz, Effeltrich, Germany) for two parameters: a) total chlorophyll a concentration ($\mu\text{g chl-a}/\text{L}$) indicating fluorescence intensity at the Measuring Light Frequency and b) effective quantum yield of photosynthetic energy conversion in photosystem II (yield). These two parameters were used to track relative changes in chl-a content and proportional changes in yield across treatments and time. Subsequently, all samples were fixed with glutaraldehyde (0.5% v/v) and stored at 4 °C for later enumeration. These preserved samples were used for microscopy enumeration of healthy and infection carrying host cells (see below). Additionally, 2 mL samples were acquired at 0, 48, 144 and 216 h, fixed with glutaraldehyde (0.5% v/v) and kept at -80 °C. They were used for bacterial population quantifications (see below). The time between sampling and fixation was at maximum 2.5 h.

2.2.1. Counting protocol

Staurastrum cells were counted under an inverted microscope at 400x magnification, according to the Utermöhl settling method (Utermöhl, 1931) on a 1 mL sample. The minimum range of counting was either 100 *Staurastrum* cells or, in low biomass samples, 40 fields of view. Each “Non-parasite-exposed” unit (*Staurastrum* only) sample was counted for the number of living cells/mL. Each “parasite-exposed” unit (*Staurastrum-Staurastrumyces*) sample was counted for: (i) number of living host cells/mL, and (ii) number of infected living host cells carrying one or more living chytrid infections/mL. As the sporangium wall does not disintegrate after rupture, it was possible to determine visually whether host death occurred due to infection or other causes. Dead *Staurastrum* cells carrying empty sporangia or resting spores of the parasite were excluded from the count as these did not contribute to the parasite population growth anymore.

The algal and chytrid cultures were not free of bacteria. To assess their population development, bacteria were counted by flow cytometry (Brussaard, 2004). In short, just before analysis, the frozen bacterial samples were thawed in a 37 °C water bath and diluted in a Tris-EDTA buffer (pH 8.2) to achieve an event rate between 100 and 1000 events/s. To distinguish bacteria from debris, the samples were stained with the DNA stain SYBR Green I nucleic acid gel stain (Molecular Probes, Invitrogen, Paisley, United Kingdom) for 15 min in the dark at room temperature (final concentration of 5×10^{-5} of commercial stock). These diluted and stained samples were then analysed on an Influx Cell Sorter

(BD Biosciences, Franklin Lakes, New Jersey, USA) equipped with a 200mW 488nm laser (Coherent Inc., Santa Clara, USA). The trigger was set to 530/40 (488nm) at a level of 0.7. Measurements were corrected with blanks, consisting of SYBR Green I stained Tris-EDTA buffer. The bacteria were gated in a plot of side scatter vs. 580/30-nm excitation and processed in the BD FACS software. The analyzed volume was calculated by weighing the sample before and after analysis.

2.3. Degradation experiment

As photodegradation is a major degradation pathway for diclofenac in the environment (Khetan and Collins, 2007; Packer et al., 2003), we determined its fate under our experimental conditions. Triplicates of 70 mL of WC medium with diclofenac concentrations 0, 1, 10, and 100 mg/L were incubated at light cycle and temperature similar to the experiment described above using a SNIJDERS Labs incubator. Light intensity was set to 70 $\mu\text{mol}/\text{m}^2/\text{s}$ which is non-limiting and non-inhibiting to algal production provided by cool light fluorescent lamps. From each experimental unit, 5 mL of samples were collected at 7 sampling timepoints: 0, 12, 24, 48, 72, and 216 h. These samples were used to measure optical density in the medium and to quantify diclofenac concentration. Optical density was measured at λ 750 nm determined against milliQ blank in using Unicam Helios Delta visible spectrophotometer (Thermo Spectronic, Cambridge, United Kingdom). Thereafter, 100 μL of the sample was stored in 70% acetonitrile (v/v in MilliQ) for further analyses. Diclofenac sodium in 100x diluted samples with 70% acetonitrile (v/v in MilliQ) were quantified in Agilent 1290 Infinity II liquid chromatography-triple quadrupole (LC-QQQ, Agilent) with Zorbax SB-C18 column (1.8 μm). The aliquots needed to establish a calibration curve were prepared in MilliQ water, as it was also the matrix for WC medium.

2.4. Statistical analysis

To address our research questions, the statistical analysis consisted of five parts. Our statistical analyses were focused on descriptive statistics, as this is an exploratory study. First, the overall system response to increasing diclofenac concentrations in presence or absence of parasitism was assessed by multivariate analysis of principal response curves (PRCs) using R package vegan (Van den Brink and Braak, 1999). A principal response curve analysis allows to quantify the temporal trends in treatment response of a multivariate set of biotic parameters relative to a reference situation (here the non-diclofenac treatments). This method is based on redundancy analysis (RDA), adjusted for the response over time as observed in a control (Van den Brink and Braak, 1999), allowing illustration of time-dependent treatment effects. We used standardized variables in our analyses, including number of bacteria cells, number of *Staurastrum* cells as well as the estimation of the photosynthetic yield, a measure of the fitness of an algal cell.

Second, the effect of a wide range of diclofenac concentrations on the spread of disease was evaluated on the time courses of infection prevalence (%) and total host density (cell/mL) in parasite-exposed cultures. For ease of comparison, we also plotted time courses of total host density in non-parasite-exposed cultures across all diclofenac concentrations. LOESS smoothing with 95% confidence interval was applied to all time series for easier visualization of trends.

Third, the combined impact of diclofenac and chytrid parasite on our phytoplankton host was analyzed by comparing the area under the curve (AUC), i.e., the accumulative host density (cells/mL over 9 days) of parasite-exposed and non-parasite-exposed cultures. LOESS smoothing with 95% confidence interval was applied to all plots to aid visual inspection. R package DescTools (DescTools Package - RDocumentation, n.d.) with spline interpolation was applied to calculate the AUC.

Fourth, to assess the impact of different diclofenac concentrations on biomass accrue in parasite-exposed vs non-parasite-exposed cultures, we compared net production based on the number of living host

cells/mL over a specific time interval. Net production was calculated as $(N_t - N_{t_0}) / (t - t_0)$ where, N_t is number of living host cells/mL over specific time interval (t) i.e., 48 h for the acute and 9 days for the chronic phase. N_{t_0} is the number of living host cells/mL at time 0 (t_0). Last, to determine the rate of diclofenac degradation under roughly the experimental light and temperature conditions, a Single First Order (SFO) kinetics model (mkin R package (Rankin et al., 2018)) was fitted to the observed concentrations in order to determine degradation of diclofenac over time in our experimental settings.

3. Results

3.1. Overall system response to diclofenac

We run the principal response curve ordination technique for both the non-parasite-exposed (Fig. 1a) as well as the parasite-exposed cultures (Fig. 1b). The principal response curve diagrams show that the systemic temporal response patterns to increasing diclofenac concentrations differed in absence (Fig. 1a) and presence (Fig. 1b) of the parasite. In absence of the parasite, the response of our cultures started to change markedly relative to the control situation at chronic exposure to 10, 50, 100 and 150 mg/L concentrations of diclofenac. As indicated by the parameter weights, which explain the extent to which a specific parameter changes explains the variation in ordination; at 10, 50, 100 and 150 mg/L the response was determined by a persistent increase in bacteria cells, and a simultaneous decrease in host cells and photosynthetic yield (a measure of photosystem II activity). In presence of the parasite, however, the systemic response started to change already at concentrations as low as 1 mg/L and > 3-day exposure relative to the control situation. Until day 6 as indicated by the parameter weights, pattern of response in the infected cultures relative to the control situation was determined by a decrease in bacterial cells and total number of cells of the host, and an increase in photosynthetic yield (indication of cell fitness i.e., active photosystem II fraction quantifying the photochemical use of absorbed light energy). After day 6, this pattern reversed with the number of host cells and bacteria decreasing and the fitness increasing. At exposure to higher concentrations, i.e., 50–150 mg/L, this effect was dampened, with no clear differences with the control situation. This implies that the absolute effect on individual parameters, such as spread of infection (see Section 3.2) may differ at high concentrations compared to the control, whereas this does not lead to a difference in

relative response. In both the control treatments and the treatments with highest diclofenac concentrations, the host cells -after an initial increase, decrease steadily over time, but the absolute concentrations of host cells at the end are much lower at the high concentrations compared to control treatment. Similarly, in both control treatments and the treatments with highest diclofenac concentrations, bacterial cells increase steadily over time, but the absolute concentrations of bacterial cells are 2-fold higher in the highest concentrations in comparison to the control situation.

3.2. Spread of infection in response to diclofenac exposure

Host density in non-parasite-exposed treatments increased near exponentially in all diclofenac concentrations up to ≤ 10 mg/L, above this concentration, host population growth slowed down strongly but remained positive at ≤ 100 mg/L (Fig. 2, gray lines). Overall, diclofenac treatment affected the spread of infection, indicating its effect on the interaction between our phytoplankton host and chytrid parasite. The effect of increasing diclofenac concentrations on the spread of infection was evaluated by comparing percentage of infection, i.e. prevalence (Fig. 2, blue lines) and total host cells/mL in parasite-exposed treatments over time (Fig. 2, black lines). While infection prevalence reached up to 100% in diclofenac concentrations < 10 mg/L, it stayed below 50% in diclofenac concentrations > 10 mg/L throughout 9 days, even disappearing completely under diclofenac concentrations above 50 mg/L. Hosts in parasite-exposed cultures was strongly infected and therefore total host density reduced to zero over time upon exposure to concentrations up to 1 mg/L, above which total host density did not reach zero and even increased between day 2 to 6 before reducing on day 9. Total biomass of parasite-exposed cultures was low in general, but the relative increase in the infected host cells is clearly visualized in the prevalence (Fig. 2, blue lines).

3.3. Combined effect of infection and diclofenac on host population

To test the combined effect of diclofenac exposure and infection on phytoplankton host net productivity, we calculated area under the curve (AUC; host cells /mL over 9 days) of both the parasite-exposed and the non-parasite-exposed cultures (Fig. 3). In the parasite-exposed cultures, however, we observed very low biomass accumulation up to exposure to diclofenac concentrations of > 10 mg/L. Also, the magnitude of this

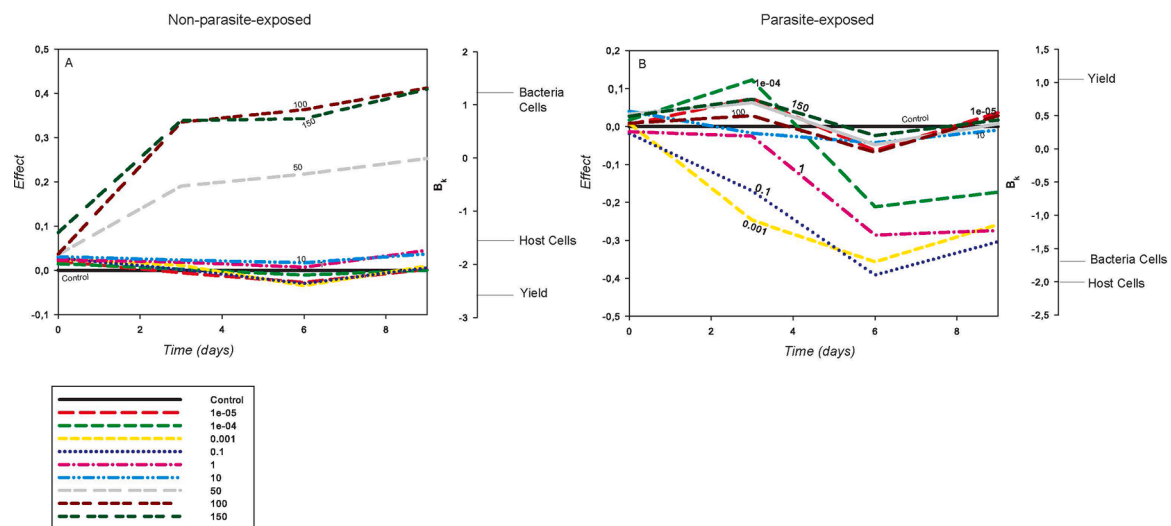


Fig. 1. Principal response curves (PRC) indicating the effect of 10 diclofenac treatments (concentrations were: 0.1×10^{-5} , 1×10^{-4} , 0.001, 0.01, 1, 10, 50, 100, and 150 mg/L) on *Staurastrum* host in the absence (1a) and presence (1b) of *Staurastrumyces oculus* parasite relative to control (no diclofenac, horizontal solid line). B_k values (right axis) are the parameter weights and explain the extent to which a specific parameter explains the variation in the ordination, with positive parameter weights on the right y-axis for a specific treatment having a positive relationship with the PRCs, and negative parameter weights having a negative relationship with the PRCs.

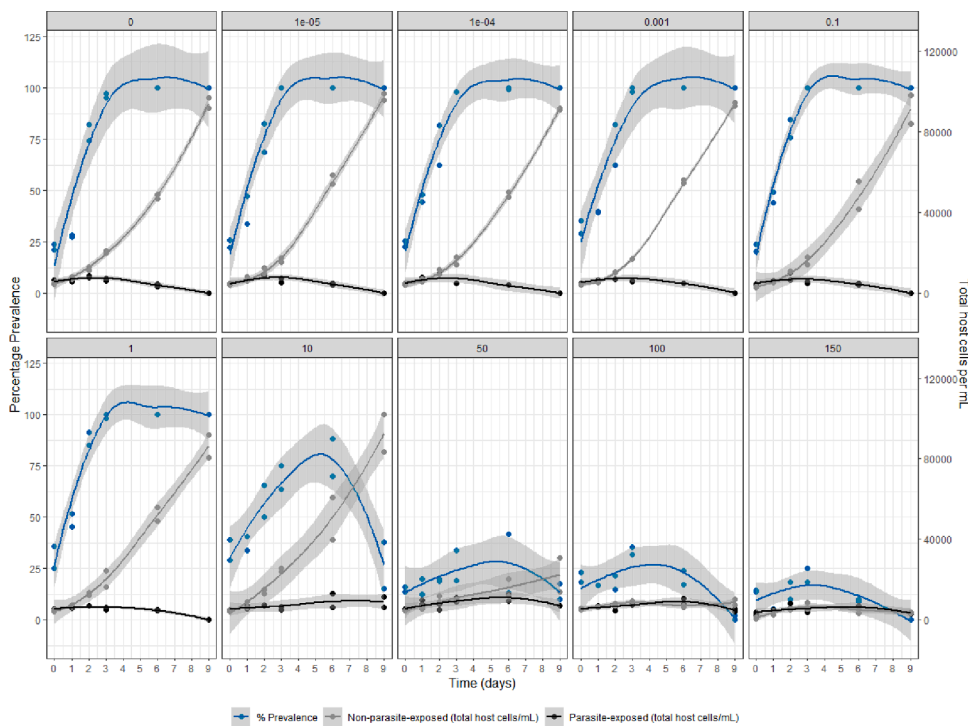


Fig. 2. Time series of prevalence (blue) and total host cells/mL in presence (black) and absence (gray) of *Staurastromyces oculis* parasite over 9 days indicating effect of 10 diclofenac concentrations (i.e. 0.1×10^{-5} , 1×10^{-4} , 0.001, 0.01, 1, 10, 50, 100, and 150 mg/L) on the spread of infection. Dots correspond to the scaled values for all three variables over 9 days. Lines are LOESS curve fits of prevalence, uninfected total host cells per mL and infected total host cells/mL with gray areas indicating the 95% confidence interval.

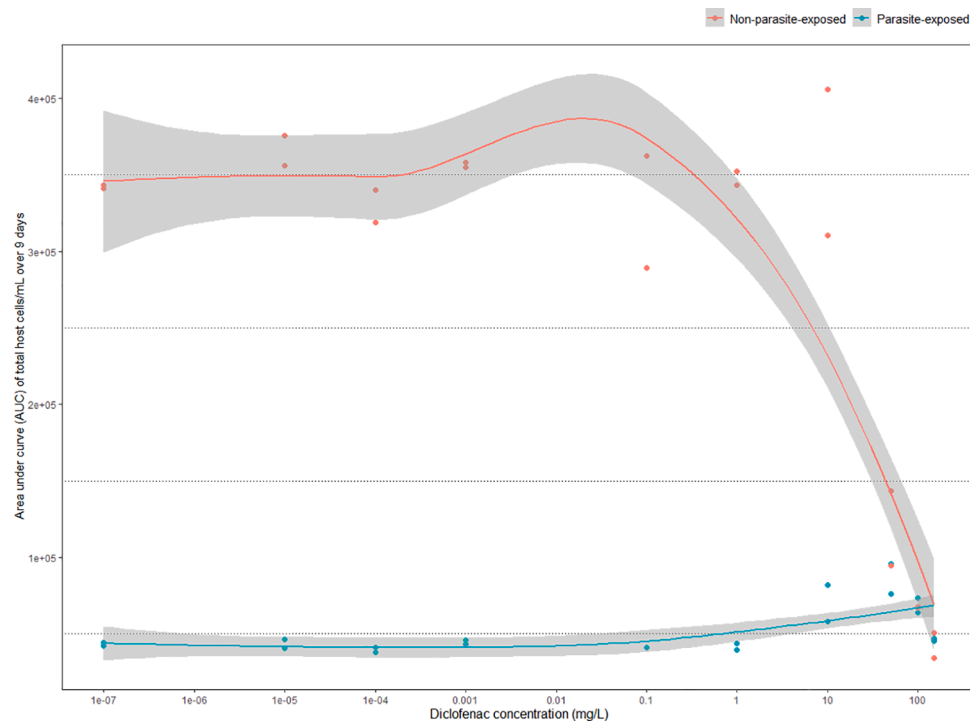


Fig. 3. Combined effect of *Staurastromyces oculis* parasite and diclofenac on *Staurastrum* host at 10 different diclofenac concentrations indicated by area under the curve of total host cells per mL over 9 days (AUC) in presence (blue dots) and absence (red dots) of *Staurastromyces oculis* parasite. Dashed lines indicate trends in AUC in presence (blue solid line) and absence (red solid line) of *Staurastromyces oculis* parasite with gray shaded areas indicating 95% confidence interval.

combined negative effect decreased in diclofenac treatments above 10 mg/L. At concentrations > 10 mg/L the non-parasite-exposed cultures showed a rapid decrease in biomass accumulation. The parasite-exposed cultures on the other hand, seemed to be released from infection (see also Fig. 2), resulting in a slightly higher biomass accumulation of parasite-exposed cultures exposed to diclofenac concentrations > 10

mg/L.

3.4. Response of host-parasite system to acute and chronic diclofenac exposure

To understand the difference between acute and chronic exposure to

diclofenac, the net production of non-parasite-exposed and parasite-exposed phytoplankton host across diclofenac concentrations were determined after 48 h (acute) and after 216 h (chronic) exposure (Fig. 4). During acute exposure (48 h, Fig. 4a), net production (host cells/mL in 48 h) in parasite-exposed treatments was generally lower than that in non-parasite-exposed ones. Relative to the control situation, non-parasite-exposed treatments showed little change up to diclofenac concentrations of 10 mg/L, above which their net production decreased strongly. parasite-exposed treatments showed a slight increase in net production up to diclofenac concentrations of 100 mg/L, above which their net production started to decline as well.

During chronic exposure (216 h, Fig. 4b), net production (host cells/mL in 216 h) in non-parasite-exposed treatments was high up to diclofenac concentrations of 10 mg/L, above which it decreased strongly. Conversely, net production in parasite-exposed treatments was close to zero up to diclofenac concentrations of 1 mg/L, above which their net production became positive again. At diclofenac concentrations of 100–150 mg/L, the net production of parasite-exposed treatments equaled or even surpassed that in non-parasite-exposed treatments.

3.5. Degradation of diclofenac during the experiment

We observed minimal degradation of diclofenac in our experimental matrix under roughly the same experimental light and temperature conditions across all tested concentrations (i.e. 1, 10, and 100 mg/L) over 72 h. Measured tested concentrations at the start were 0.93, 7.63, and 88.82 mg/L which reduced to 0.91, 7.2, and 83.72 mg/L at the end of 72 h. Degradation could be fitted with single first order (SFO) kinetics. Degradation constant derived by SFO was influenced by the initial concentration eventually altering the half-life or dissipation time 50 (DT₅₀). Lowest DT₅₀ was determined at an initial concentration of 10 mg/L i.e. 759 h which is still more than 3 folds higher than our experimental duration i.e. 216 h.

4. Discussion

In order to move beyond standard toxicological metrics, it is

important to know the impact of pharmaceuticals on non-target organisms and their interactions with other species. In this study, we investigated the effect of diclofenac on one of the least studied, but most abundant consumer strategies: host-parasite interactions. Direct effects of diclofenac on the performance and fitness of individual organisms have been studied extensively for fish, cladocerans and phytoplankton (Ferrari et al., 2004; Minguez et al., 2016), but little is known about the effects of diclofenac on fungal parasites, and their interactions with hosts. This gradient study is a first step towards understanding the impact of diclofenac on interaction between a phytoplankton host and its obligate chytrid parasite which is an important but less explored interaction. Our results show that the response of host population development and fitness as well as bacterial abundance of the overall system is modulated by the presence of infection. Also, we did not observe an additive effect of parasite and diclofenac on host biomass accumulation as there was no continued decrease in biomass accumulation upon > 10 mg/L diclofenac exposure. In contrast, host biomass in parasite-exposed treatments recovered in high diclofenac concentrations suggesting a parasite-free window of opportunity for the host at these conditions. Our degradation experiment showed that the half-life of diclofenac in our experiment is 3-fold higher than the duration of experiment i.e. 216 h indicating that all the results discussed further are responses to diclofenac and the role of daughter compounds are therefore not considered relevant.

4.1. Overall system response to diclofenac

We found that parasite presence modulates the overall system response to increasing diclofenac concentration. Upon exposure to concentrations > 10 mg/L, there was a decrease in photosynthetic yield of *Staurastrum*, suggesting reduced PSII activity (also a proxy for oxidative damage (Foyer, 2018)). Similarly, a 40% decrease in active PSII fraction upon a 10 h exposure of *Chlamydomonas reinhardtii* to 135 mg/L diclofenac was observed by Harshkova and co-authors (Harshkova et al., 2021). We also observed that the relationship between host and bacteria differed between parasite-exposed and non-parasite-exposed cultures, with hosts having a positive relationship

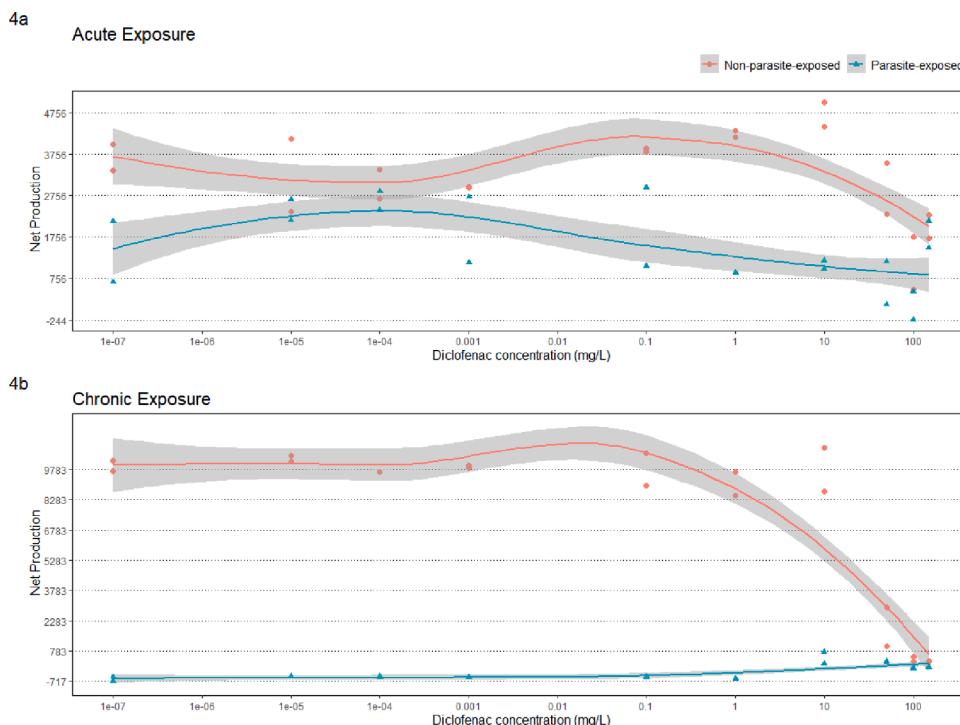


Fig. 4. Net production (host cells/mL over time) of 10 diclofenac concentrations (i.e. 0.1, 1, 10, 50, 100, 150 mg/L). (4a) Acute exposure: net production in non-parasite-exposed treatments (orange dots) and net production parasite-exposed treatments (blue triangles) over the first 48 h (4b) Chronic exposure: net production in non-parasite-exposed treatments (orange dots) and net production in parasite-exposed treatments (blue triangles) over 216 h (9 days). Dashed lines indicate trends in net production in presence (blue dashed line) and absence (red dashed line) of *Staurastrumyces oculus* parasite with gray shaded areas indicating 95% confidence interval.

with bacteria in parasite-exposed cultures, rather than a negative relationship as observed in non-parasite-exposed cultures. The negative co-occurrence of host and bacteria in the parasite-exposed treatments may be a result of infected and dying host releasing carbon compounds that serve as resources for bacterial growth (Klawonn et al., 2021; Senga et al., 2018). While the positive co-occurrence of host and bacteria in non-parasite-exposed treatments may reflect growth of both, host and bacteria populations over time.

4.2. Spread of infection in response to diclofenac exposure

We found a decrease in prevalence and increase in total host density indicating the difference in sensitivities of spread of infection to increasing diclofenac concentrations. Our observations on the diclofenac induced reduction in spread of infection could be a direct effect on the parasite due to a higher sensitivity of the chytrid parasite compared to the host, making it less successful in parasitizing the phytoplankton host. Sensitivity of parasites to a pharmaceutical usually should be quantified by traditional ecotoxicological parameters such as EC_{50} . One challenge posed by the parasitic lifestyle is that it is often difficult to measure the parasite response in isolation from the host response. Three possible parasite groups where such an EC_{50} measurement may be possible are: 1) protist or multicellular parasites in metazoans that survive the infection e.g., malaria clearance rates due to pharmaceutical treatment; 2) parasites with a measurable free-living life stage; 3) facultative parasites which can be grown on a non-living substrate.

The fact that our model species is an obligate parasite and is not able to grow in isolation of a host, prohibits us from calculating conventional ecotoxicological parameters such as effective concentration-50 (EC_{50}) for chytrid parasites. Understanding the sensitivity of chytrid parasite to diclofenac can only be feasible through changes in prevalence in our study. Ortiz-Cañavate et al. (2019) observed that fungicide, itraconazole at 100 and 1000 $\mu\text{g/L}$ concentrations suppressed the spread of infection by chytrid parasite, *Rhizophyidum megarrhizum* in cyanobacterium *Planktothrix* (Ortiz-Cañavate et al., 2019). A fungicide, thiophanate-methyl at 0.6 mg/L cleared *Batrachochytrium dendrobatidis* (chytrid fungus) infection in *Lithobates sphenoccephalus* tadpoles (Hanlon et al., 2012). Cuco et al. (2017) confirmed suppression of infection by *Metschnikowia bicuspidata* (parasite) in *Daphnia* spp. (host) upon exposure to fungicide- tebuconazole at concentrations 6.5 $\mu\text{g/L}$ and above (Cuco et al., 2017). Another fungicide-myclobutanil was also found to decrease parasite prevalence in the same *Metschnikowia bicuspidata* (parasite)- *Daphnia* spp.(host) system (Machado et al., 2022). Van Wyngaert et al. (2013) assessed the effect of herbicide diuron on the host-parasite relationship between the diatom *Asterionella formosa* and its obligate chytrid fungus *Zygorhizidium planctonicum* and showed that the spread of infection was initially reduced probably due to reduced chemotactic communication, but then actually enhanced with longer exposure time (Van den Wyngaert et al., 2013). Further studies on understanding the changes in chytrid extracellular enzymes like carbohydrate active enzymes in response to diclofenac would be needed to get a better understanding of the sensitivity of this obligate parasite (Lange et al., 2019).

The diclofenac induced reduced spread of infection could also be the consequence of a heightened immune response of the phytoplankton host. Although there is limited knowledge about the innate immune response in phytoplankton, presence of plant disease resistance gene-nucleotide binding site leucine-rich repeat (NBS-LRR) gene or R-gene in members of the green algal group Charophyta (*Coleochaete orbicularis* and *Nitella mirabilis*) has been explored (Gao et al., 2018). As our host species, *Staurostrum*, also belongs to the phylogenetic group of Charophyta thereby supporting speculation about the innate immune response of the phytoplankton host being evoked. Studies on chytrid enzymatic activity and NBS-LRR gene expression in phytoplankton hosts are needed to understand the effect of diclofenac on chytrid parasites and the role of this immune response in the phytoplankton host when

exposed to high doses of diclofenac.

4.3. Combined effect of infection and diclofenac on host population

We observed a marked decrease in the area under the curve (AUC) in non-parasite-exposed treatments > 10 mg/L which is comparable to the inhibition of *Scenedesmus vacuolatus* reproduction due to exposure to 23 mg/L diclofenac (Schmitt-Jansen et al., 2007). In the parasite exposed treatments, above 10 mg/L a slight but consistent increase in host biomass accumulation was observed due to an increasingly inhibited parasite infection spread and therefore less host mortality, while the diclofenac concentrations were still low enough to not reduce host population growth either. If there was a combined effect of diclofenac and chytrid exposure as we initially hypothesized, the decrease in biomass accumulation AUCs that we observed in < 10 mg/L diclofenac would have further decreased in higher concentrations. Simultaneous exposure to diclofenac and chytrid did not have an additive effect on phytoplankton hosts as we found the total host cells/mL at doses of diclofenac > 10 mg/L to increase (Fig. 3). The direct effect of diclofenac on chytrid parasites can also be an influencing factor for the combined effect of diclofenac and chytrid on phytoplankton host. Hence, even though diclofenac concentrations above 10 mg/L are already inhibiting host population growth, they can still be beneficial to hosts by reducing or even removing disease pressure and thereby providing hosts with a disease-free growth window.

4.4. Response of host-parasite system to acute and chronic diclofenac exposure

Net-production of phytoplankton host in both parasite-exposed and non-parasite-exposed treatments were found to be unaffected by the presence of diclofenac when exposed to concentrations < 0.1 mg/L. However, in parasite-exposed treatments upon exposure to diclofenac > 0.1 mg/L, we observed reduction in net production. This sharp decline is comparable to around 15% suppression of algal reproduction at 23 mg/L observed by Schmitt-Jansen et al. (2007). Generally, while both parasite-exposed and non-parasite-exposed treatments remained productive during the acute exposure, lower net production in infected treatments showed the impact of chytrid infections removing (large) parts of the host population. However, high diclofenac concentrations (> 10 mg/L) overall reduced net production, but at a stronger rate in non-parasite-exposed than parasite-exposed treatments.

No effect of diclofenac concentrations < 10 mg/L on net-production of host over chronic exposure in non-parasite-exposed cultures reflected the no observed effect concentration (NOEC) of 10 mg/L determined by (Ferrari et al., 2004) in *Desmodesmus subspicatus* over a 4-day exposure. In our study, parasite-exposed cultures showed nearly zero net production over chronic exposure throughout the gradient of diclofenac. Zero net production in parasite-exposed treatments at diclofenac concentrations < 10 mg/L resulted from prevalence, i.e. the percentage of infected cells in the host population, reaching 100% within the first 72 h (Fig. 2) effectively killing off the entire host population.

4.5. Degradation of diclofenac during the experiment

We found negligible degradation of diclofenac in our experimental matrix under roughly same experimental light and temperature conditions over 72 h indicating that effects and responses observed are likely neither influenced by a loss of active compound nor by the presence of photodegradation products. Half-life for photodegradation of diclofenac was reported to be between 0.2 to 1.7 h by (Poiger et al., 2001) and 5 days by (Andreozzi et al., 2003). However, we found the half-life of diclofenac in our experiments to be longer compared to earlier studies, which could be explained by at least 12 times lower light intensity in our experimental system compared to intensity of natural sunlight (i.e., 900–1500 $\mu\text{mol/m}^2/\text{s}$). Degradation experiments were conducted in a

filter sterilized axenic WC medium; therefore, the role of biodegradation products could not be assessed. In this study we focused on determining the half-life of photodegradation, considering that photodegradation is an important driver in the fate of diclofenac (Lonappan et al., 2016; Schulze et al., 2010). Poirier-Larabie et al. (2016) have reported 42% decrease in diclofenac upon bacterial degradation over 57 days with three transformation products (TPs): TP311 ($C_{14}H_{11}Cl_2NO_3$), TP265 ($C_{13}H_9Cl_2NO$) and m/z 295 ($C_{14}H_{10}Cl_2NO_2$) indicating the negligible role of bacterial degradation in our experiment carried out for 9 days (Poirier-Larabie et al., 2016). As we found an increase in bacterial cell counts over time (9 days) we cannot completely rule out that bacterial degradation may have played a role in breaking down the parent compound.

Conclusion

To our knowledge, we -for the first time- report the effects of simultaneous exposure to diclofenac and a parasite on a phytoplankton host, providing a baseline for future studies on these types of indirect effects. As host-parasite interactions are important in carbon and nutrient cycling, ignoring these types of interactions in environmental risk assessment schemes will likely underestimate the overall ecological impact of pharmaceuticals in surface waters. Our long gradient design allowed us to begin to understand how the interactions between an obligate parasite and its host can be affected by exposure to a ubiquitous pharmaceutical in surface waters. In support of our hypothesis, we observed that the effect of increasing diclofenac concentration on the overall system as reflected in the multivariate treatment response relative to the control, depends on whether the cultures were exposed to chytrid parasites or not. When we zoom in to treatment effects on individual experimental parameters, our results suggest that high diclofenac concentrations negatively impacted the spread of infection either because the host became less sensitive to the parasite, or the parasite became less virulent at high concentrations. This lower prevalence resulted in a resurgence of phytoplankton hosts at concentrations above 10 mg/L. This novel study provides us with cues to formulate mechanistic hypotheses to test in future studies exploring the mechanisms underlying the non-target effects of a pharmaceutical on an understudied interaction between key groups in the aquatic food web, primary producer hosts and their parasites, where the highest diclofenac concentrations studied reflect concentrations of hospital wastewater or accidental spills in the environment. However, to fully understand the mechanism, we need further physiological and molecular exploration of diclofenac toxicity on fungi and phytoplankton. Validation on the innate immune system in phytoplankton is crucial to confirm the combined effect of parasitism and diclofenac. Our study underlines the need for development of more holistic eco-toxicological measures, going beyond standard measures of EC_{50} for population growth or mortality to disentangle the effects of pharmaceuticals on key interactions among species.

CRediT authorship contribution statement

Nandini Vasantha Raman: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Visualization, Formal analysis. **Alena S. Gsell:** Conceptualization, Methodology, Investigation, Writing – original draft, Visualization, Writing – review & editing, Formal analysis, Supervision, Project administration. **Themistoklis Voulgarellis:** Methodology, Investigation, Writing – review & editing. **Nico W. van den Brink:** Writing – review & editing, Supervision, Formal analysis. **Lisette N. de Senerpont Domis:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Visualization, Formal analysis.

Declaration of Competing Interest

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.

Data Availability

Data will be made available on request.

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