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Effects of diclofenac on sentinel species and aquatic communities in semi-natural conditions

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ABSTRACT

Due to the potential hazard of diclofenac on aquatic organisms and the lack of higher-tier ecotoxicological studies, a long-term freshwater mesocosm experiment was set up to study the effects of this substance on primary producers and consumers at environmentally realistic nominal concentrations 0.1, 1 and 10 µg/L (average effective concentrations 0.041, 0.44 and 3.82 µg/L). During the six-month exposure period, the biovolume of two macrophyte species (*Nasturtium officinale* and *Callitriche platycarpa*) significantly decreased at the highest treatment level. Subsequently, a decrease in dissolved oxygen levels was observed. High mortality rates, effects on immunity, and high genotoxicity were found for encaged zebra mussels (*Dreissena polymorpha*) in all treatments. In the highest treatment level, one month after the beginning of the exposure, mortality of adult fish (*Gasterosteus aculeatus*) caused effects on the final population structure. Total abundance of fish and the percentage of juveniles decreased whereas the percentage of adults increased. This led to an overall shift in the length frequency distribution of the F₁ generation compared to the control. Consequently, indirect effects on the community structure of zooplankton and macroinvertebrates were observed in the highest treatment level. The No Observed Effect Concentration (NOEC) value at the individual level was < 0.1 µg/L and 1 µg/L at the population and community levels. Our study showed that in more natural conditions, diclofenac could cause more severe effects compared to those observed in laboratory conditions. The use of our results for regulatory matters is also discussed.

1. Introduction

Pharmaceutically active compounds (PhACs) are, extensively and increasingly used in human and veterinary medicine, and represent a class of emerging environmental contaminants (Académie Nationale de Pharmacie, 2019). Once consumed by humans or livestock, PhACs can enter the environment by different routes and ultimately end up in surface, drinking, groundwaters, and even soils (Lonappan et al., 2016). Various classes of PhACs such as beta – receptor blockers, antibiotics, psycho-active drugs, lipid regulators, analgesic non-steroidal anti-inflammatory drugs (NSAID) and hormone regulators are frequently detected in raw sewage, treated wastewater, drinking waters and rivers

throughout the world with concentrations ranging from a few ng/L to the µg/L (Barbosa et al., 2016; Heberer, 2002; Loos et al., 2009).

Among these compounds, diclofenac (DCF), a Non-Steroidal Anti-Inflammatory Drug (NSAID), is detected in almost all surveys probably due to its high consumption rate and polarity (Sathishkumar et al., 2020). Stumpf et al. (1996) were the first authors to report the presence of DCF in sewage and river waters in Germany. Since then, DCF is found in many countries in either wastewater influents and effluents and/or surface waters and/or drinking waters and/or ground waters at concentrations ranging from a few to thousands of ng/L. Worldwide median concentrations in river waters are 0.021 + /- 0.722 µg/L (Acuña et al., 2015). More specifically, in European river waters, values ranging from

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0.002 to 7.7 µg/L are reported by Lonappan et al. (2016) and Sathishkumar et al. (2020). Once released in the environment, the main degradation pathway of DCF in water is photodegradation with half-lives ranging from 1 to 3 h (Bartels and Von Tuempling, 2007; Packer et al., 2003; Tixier et al., 2003). DCF and the resulting photo-products can then be adsorbed on sediments (Kunkel and Radke, 2008) and/or can be metabolized by plants (Bartha et al., 2014; Huber et al., 2013; Matamoros et al., 2012), invertebrates (Parolini, 2020) and fish (Bickley et al., 2017; Brozinski et al., 2012; Cuklev et al., 2011; Kallio et al., 2010). Inhibition of the cyclooxygenase enzyme, which is responsible for the conversion of arachidonic acid to prostaglandin, is its main known mechanism of action (Gan, 2010).

DCF is responsible for the rapid decline of vulture populations in India, Pakistan, Nepal, and recently in Egypt (Cuthbert et al., 2006; Green et al., 2006; Oaks et al., 2004). Moreover, after short term (24–96 h) or rather long-term (28–30 days) laboratory exposures on mollusks (Parolini et al., 2013; Schmidt et al., 2001) and fish (Gröner et al., 2015; Hoeger et al., 2005; Mehinto et al., 2010; Saravanan et al., 2011; Triebkorn et al., 2004), concentrations as low as 0.5 µg/L affect many biochemical processes and/or physiological functions such as oxidative stress, proteins of biotransformation, metabolism, and immune parameters. In the latter fish studies, the lowest observed effect concentration (LOEC) for kidney, liver, and gill lesions is 1 µg/L. Furthermore, EC50 values on survival and/or, growth, and/or reproduction in chronic laboratory studies range from 16 to 350 mg/L for algae (Cleuvers, 2003; Ferrari et al., 2003), 7–47 mg/L for macrophytes (Cleuvers, 2003; Quinn et al., 2001), 28–140 mg/L for crustaceans (Garric et al., 2006; Lee et al., 2011; Quinn et al., 2001) and 5–70 mg/L for fish (Islas-Flores et al., 2013; Memmert et al., 2013; Praskova et al., 2014; Saravanan and Ramesh, 2013).

Considering the potential threat of diclofenac on terrestrial and aquatic organisms, the European legislation included diclofenac in the first Watch List of the Environmental Quality Standards Directive (2008/105/EC) (Commission, 2015). Using a traditional methodology to assess the reliability and relevancy of standard laboratory studies, an EQS of 0.05 µg/L for freshwater organisms was proposed (UBA, 2018). Results of non-standard long-term multi-species ecotoxicity tests such as mesocosms experiments are rarely considered to derive EQS values. Mesocosm experiments allow to concurrently study fate, direct and indirect effects of chemical substances in reconstructed model ecosystems (David et al., 2020). Assessment of the whole life cycle of organisms, with limited food resources and including ecological processes such as intra-specific competition and inter-specific predation is possible in these experiments. They also allow to bridge the gap between laboratory toxicity tests and natural ecosystems enabling to evaluate the cause and effects of chemicals substances in more environmentally realistic conditions (Caquet et al., 2000). This intermediate position may help to evaluate the relevance of the EQS value for DCF that is derived as mentioned above using only a collection of laboratory toxicity data.

Furthermore, according to the documented hazards and behavior of DCF in aquatic ecosystems, effects of diclofenac may be magnified in more natural experimental conditions. Indeed, photo-transformed DCF is reported to be five times more toxic to unicellular green algae (*Scenedesmus vacuolatus*) compared to the parent compound (Schulze et al., 2010). Moreover, unexpected fish mortality is found in more recent studies at concentrations as low as 100 µg/L for juvenile trout (Schwarz et al., 2017) and at 80 µg/L for juvenile three-spined sticklebacks (Näslund et al., 2017) which implies that direct effects can occur at relatively low concentrations. In this context, the primary objective of the present study is to evaluate both the direct and indirect effects of DCF at different levels of biological organization (cellular, individual, population, and community levels) on primary producers (macrophytes) and consumers (macroinvertebrates, zooplankton and more particularly on two sentinel species, *Dreissena polymorpha* and *Gasterosteus aculeatus*) in a lotic mesocosm experiment. We hypothesized that direct effects of DCF and its degradation products will occur at low concentrations at all

trophic levels especially on the two sentinel species. Indirect effects such as a decrease in food availability, shifts in water chemistry and inter-specific competition are also suspected due to the presence of a complex food web in our experimental conditions. To current knowledge, this is the first mesocosm study performed on DCF. Finally, the reliability and relevance of the No Observed Effect Concentrations (NOECs) derived in our study for all organisms and at all biological levels will *in fine* be evaluated and eventually used to assess the relevance of the DCF EQS value.

2. Material and methods

Our experimental design along with some of our sampling methods was standardized and published in Roussel et al. (2007b); de Kermoisan et al. (2013); David et al. (2020) (eg: mesocosm set up, water sampling for chemical analysis, physico-chemical methods, fish methods and sampling, macrophyte biovolume assessment, zooplankton and macro-invertebrate sampling). An appendix detailing these methods is thus provided when it is appropriate.

2.1. Mesocosm experimental set-up

2.1.1. Description of the mesocosm platform

The experiment was performed using 12 lotic mesocosms located in the North of France (INERIS, Verneuil-en-Halatte, France, 49.3°N – 2.52°E). See de Kermoisan et al. (2013) or Appendix A (Fig. S1) for a detailed description.

2.1.2. Biological composition of the mesocosms

From October 2012 to March 2013, each mesocosm was set up with macrophytes, zooplankton, benthic and pelagic invertebrates (See Appendix A, Mesocosm set up for a detailed description). Furthermore, two species, three-spined sticklebacks (*Gasterosteus aculeatus*) and zebra mussels (*Dreissena polymorpha*) were selected as they are sentinel species frequently used for freshwater biomonitoring including passive biomonitoring studies (Catteau et al., 2021; Palos Ladeiro, 2017).

The three-spined stickleback *Gasterosteus aculeatus*, a small-bodied teleost fish, is a major component of shallow-water food webs in the northern hemisphere (Wootton, 1976). The fish used in this study came from our breeding facilities (INERIS, Verneuil-en-Halatte, France). Fish under 25 mm long were selected in October 2012 (less than one year old at the beginning of the experiment) and reared in the same conditions until the beginning of the experiment (rearing in opaque plastic tanks, water renewed (1 L/h) and food (frozen *Chironomidae*) supplied *ad-libitum* every day).

On the 4th of March 2013, initial populations composed of 15 mature females and 10 mature males, were introduced in each mesocosm (called hereafter founder fish). Introduced fish were selected to have similar lengths and their gender was determined according to the method developed by de Kermoisan et al. (2012). Male lengths ranged from 35 to 46 mm (mean ± SD, 40.47 ± 2.67 mm, n = 120). Female lengths ranged from 40 to 45 mm (mean ± SD, 42.8 ± 1.38 mm, n = 180). Founder fish were individually marked with 1.2 × 2.7 mm alphanumeric tags [(VI Alpha Tags, Northwest Marine Technology, Shaw Island, WA, USA) which were implanted under their skin one week before their introduction (Lynch and Mensinger, 2011).

Zebra mussels (*Dreissena polymorpha*) used in our study were sampled at the end of March 2013 in the artificial lake “Der-Chantecoq” located in the northeast of France (Sainte Marie du Lac Nuisement 48°36'22.02", 4°46'34.0"E). Mussels were transported to the laboratory, cleaned up, and maintained for a minimum of two weeks in a temperature-controlled room (12 °C, photoperiodicity 16 h/8 h) in 20 L-aquariums filled with 7.5 L of the mineral water (brand: Cristalline Chantereine) and fed twice a week with a mixture of microalgae. Forty-eight hours before transplantation in the mesocosms, calibrated zebra mussels (2–2.5 cm shell length) were placed randomly into 2 mm-mesh

polyethylene experimental cages (2 cages of 120 individuals per mesocosm). In each mesocosm, cages were plunged to a depth of 0.5 m at 10 m from the water inlet.

2.2. Exposure monitoring

2.2.1. Diclofenac exposure concentrations

Three nominal concentrations of DCF were selected: 0.1, 1, and 10 µg/L. Each concentration had three replicates and three mesocosms served as controls. The first concentration is likely to occur frequently in the environment and is close to the EQS value. The second concentration is also commonly found in the environment and slight effects are suspected on the most sensitive species (eg: fish and invertebrates). The highest exposure concentration is likely to cause severe effects on different species, communities and ecosystem processes. Treatment began on the 16th of April 2013 (day 0) and ended on the 4th of October 2013 (day +171). Hereafter, time is expressed in days post beginning of the treatment and abbreviated “dpt”. Treatments will be considered in the rest of the paper as low (0.1 µg/L), medium (1 µg/L), and high (10 µg/L).

2.2.2. Contamination system

Two stock solutions composed of a technical grade of DCF (purity 99%, Sigma- Aldrich, France) in ethanol (purity 99%, Prolabo, France) at a concentration of 2 g.L⁻¹ were placed onto two separate 500 liters tanks supplied with tap water (Tank 1 and Tank 2). To obtain a nominal concentration of 0.5 mg/L of diclofenac in Tank 1, injection of 100 mL of the stock solution was performed. Peristaltic pumps connected to the Tank 1 and the different mesocosms then delivered DCF at the appropriate concentrations. Injection of the stock solutions, as well as water levels of the two tanks and peristaltic pumps, were controlled by an automatic system. When the water level of the Tank 1 was half empty, the automatic system commanded the injection in Tank 2. When Tank 1 was almost empty, the peristaltic pumps transferred to Tank 2. This operation was repeated every 8 h to ensure continuous contamination of the mesocosms. The stock solutions of diclofenac were changed every 12 days.

2.2.3. Diclofenac concentrations in water

DCF water concentrations in each of the 9 mesocosms were regularly monitored (every month) at different locations (0, 5, and 19 m from the water inlet) to measure the actual exposure concentrations. Samples were preserved at – 20 °C until analysis.

Concentrations were determined by liquid chromatography coupled to a mass spectrometric detector. The system was composed of a HP1200 Agilent Technologies liquid chromatograph coupled to a 3200 QTrap (ABSciex) triple quadrupole mass detector. The chromatographic separation was achieved on a Poroshell 120 EC-C18 (Agilent Technologies) column with a mobile phase composed of a gradient of methanol and water acidified with formic acid. The quantification was done by external calibration with at least six calibration levels of known amounts of DCF. The limits of detection and quantification were respectively 0.005 and 0.01 µg/L.

Average effective concentration (AEC) was calculated for each treatment using the mean values of the three replicates between 5 and 19 m following an integration method published by Van Wijngaarden et al. (1996) (See Appendix A Average effective concentration-Formula).

2.2.4. Diclofenac transformation products and metabolites in watercress

Many degradation products or metabolites were identified in the literature, in several matrices. Some of them are not stable or their identification is not complete (Boreen et al., 2003; Salgado et al., 2013). Considering the huge diversity of transformation products and metabolites, a focus was made on chemically stable and commercially available substances. Diclofenac (DCF), 4'-hydroxy-diclofenac (4'-OH-DCF), 5'-hydroxy-diclofenac (5'-OH-DCF), 2-[(2,6-Dichlorophenyl)amino]

benzoic acid (DCF-carboxylic acid), Diclofenac acyl-β-D-glucuronide (DCF-glucuronide), 2-[(2-chlorophenyl)amino] benzaldehyde (CPAB), ([2-[(2,6-dichlorophenyl)amino]phenyl]methanol) (DCF-alcohol), (1-(2,6-dichlorophenyl)1,3-dihydro-2 H-indol-2-one) (DCF-lactam), 2-Indolone and 2-[(2,6-Dichlorophenyl)amino]benzaldehyde (DCF-aldehyde) were the measured substances (See Appendix A, Table S1).

At the end of the experiment watercress (*Nasturtium officinale*) was sampled in each mesocosm. The samples were preserved at – 80 °C until analysis. The extraction of DCF along with its transformation products and metabolites was performed using a modified QuEChERS extraction. After optimization, the extraction was conducted with an acetate salt and a ratio acetonitrile/water of 10/5. The development, optimization, and validation of the analytical method are detailed in Danielle and Vuillet (2015). An H-Class UPLC system (Waters®, Saint-Quentin-en-Yvelines, France) was used for the liquid chromatographic separation of DCF and its transformation products and metabolites. The chromatographic system was coupled to a Xevo TQ-S triple-quadrupole mass spectrometer (Waters) equipped with a Step-Wave ion guide. The separation was achieved with a Poroshell 120 EC-C18 column (50 × 2.1 mm; 2.7 µm) from Agilent Technologies. The mobile phases were composed of (A) 0.01% formic acid in MilliQ water and (B) MeOH using a 0.6 mL/min flow rate.

2.3. Physico-chemical parameters

Temperature was measured every 10 min using water temperature sensors (HOBO 0257, Prosensor, Amanvilliers, France). In each mesocosm, the sensors were arranged at (i) 5 m from the water inlet in the middle of the mesocosm and (ii) 15 m from the water inlet at a water depth of 70 cm (on the bottom of the mesocosm).

Routine water parameters such as pH, conductivity, and dissolved oxygen were measured each week at 10 m from the water inlet in each mesocosm by using a WTW multi-parameter instrument equipped with digital sensors (MultiLine® IDS, Multi3430, WTW, Germany).

2.4. Macrophyte monitoring

The volume score of four macrophytes, i.e. watercress (*Nasturtium officinale*), various leaved water starwort (*Callitriche platycarpa*), eurasian water-milfoil (*Myriophyllum spicatum*) and filamentous green algae (*Spyrogyra* sp.) were estimated, in each mesocosm, every two weeks from the beginning of March to the beginning of October 2013 (-47 to +164 dpt) (Appendix A - Macrophyte monitoring for further details).

2.5. Sentinel species

2.5.1. Zebra mussel individual responses

For zebra mussels, after 2 (18/06/13, +63 dpt) and 5 (25/09/13, +162 dpt) months of exposure, 1 cage per mesocosm was sampled for biomarkers analysis. At each sampling date, the number of dead individuals was registered in each cage to calculate the percentage of survival.

For biochemical parameters (digestive enzyme activities, energy quantification, and Electron Transport System activity i.e. ETS) mussels were dissected and digestive glands (n = 9 for digestive enzyme activities) or the whole soft tissues (n = 9 for energy reserve and ETS) were weighted, preserved in liquid nitrogen and stored at – 80 °C until further analysis. The amylase activity was measured according to Palais et al. (2012). Concerning the energy reserves, lipids and glycogen were measured according to Van Handel and Plaistow et al., (1985, 2003); and total proteins were evaluated according to Bradford (1976). The activity of ETS was measured according to De Coen and Janssen (1997). The same individuals were used to define the condition index (CI) which was calculated as follows $CI = [\text{soft tissue wet weight (g)}] / \text{total body wet weight (g)}$.

For immune parameters, approximately 0.5 mL of haemolymph (5 pools of 5 mussels/mesocosm) was withdrawn from the adductor muscle sinus. The haemolymph was treated directly and kept in a physiological buffer to ensure haemocyte viability. Samples were then centrifuged at 400 g during 10 min (4 °C) and adjusted to 4×10^5 cells.m/L with Hank's Balanced Salt Solution (HBSS, Sigma). For each pool, morphological characteristics, haemocyte mortality (Propidium Iodide, 1.0 g/L, Molecular Probes; 10 min on ice), phagocytosis percentage (FluoSpheres carboxylate-modified microspheres, diameter 1.712 µm, Life Technologies, Carlsbad, CA, USA; 1 h at room temperature) and oxidative stress were analyzed.

Concerning oxidative activity, the method used 2',7'-DCFH-DA and flow cytometry (Beckman Coulter) as described by [Le Guernic et al. \(2015\)](#). During incubation with hemocytes, DCFH-DA diffuses into the cells. In the cytoplasm the acetate groups (-DA) are removed by esterase; DCFH is thereby trapped within the cells. The intracellular DCFH, a non-fluorescent fluorescein analog, is oxidized to highly fluorescent dichlorofluorescein (DCF) by hemocytes stimulated to produce ROI by PMA. Intracellular DCFH oxidation is quantitatively related to the oxidative metabolism of the hemocyte and mediated by H₂O₂. DCF production results in green fluorescence, measurable on the FL1 detector of a flow cytometer.

DNA strand breaks were measured by the comet assay in circulating hemocytes punctured for a minimum of four zebra mussels per mesocosm (12 mussels per condition). Briefly, cell membrane integrity (> 90%) was microscopically estimated with the trypan blue dye exclusion test ([Le Guernic et al., 2015](#)) before performing the alkaline comet assay to avoid a possible cytotoxic effect. DNA strand breaks were quantified according to the alkaline protocol described by [Singh et al. \(1988\)](#) with minor modifications (Appendix A, Zebra Mussels- Material and Methods for more details).

2.5.2. Fish individual responses

As for the sentinel fish species, at the end of the experiment, 30 fish each with a length superior to 35 mm were randomly selected in each mesocosm. Each fish was sacrificed by cervical dislocation, measured, and weighed. As described by [Janssen et al. \(1995\)](#), the condition factor (CF) was calculated as $CF = [100 \times \text{body weight (g)}] / \text{length}^3 \text{ (cm)}$. The liver somatic index (LSI) was calculated as follows: $LSI = [100 \times \text{liver wet weight (g)}] / \text{total body weight (g)}$ ([Slooff et al., 1983](#)). The gonadal somatic index (GSI) was calculated as $GSI = [100 \times \text{gonad weight (g)}] / \text{fish weight (g)}$ ([Lofts et al., 1966](#)). Then, the liver was sampled for oxidative stress analysis and energy allocation while the spleen was sampled for immunomarker analysis.

Concerning oxidative stress, the liver was homogenized and centrifuged. The resulting post-mitochondrial fraction was used for biomarker measurements. Protein concentration was determined using the method of [Bradford \(1976\)](#). Hepatic activities of lipidic lipoperoxidation (TBARS), superoxide dismutase (SOD), glutathion total (GST), glutathion peroxydase (GPx) were assessed according to the method developed by [Paglia and Valentine \(1967\)](#); [Ohkawa et al. \(1979\)](#); [Paoletti et al. \(1986\)](#) and [Vandeputte et al. \(1994\)](#) respectively (See Appendix A, Fish- Material and Methods for more details).

Concerning energy allocation, the lipids were extracted from the liver using [Lu et al. \(2008\)](#) protocols. The lipid analysis was performed using a protocol adapted previously from a microplate analysis ([Cheng et al., 2011](#)).

Concerning immunomarkers, splenic leucocyte isolation was made following protocols described previously by [Bado-Nilles et al. \(2013\)](#). Then, analyses were carried out on whole leucocytes, using a Cyan™-ADP flow cytometer (Beckman Coulter). For each leucocyte sample, 10,000 cells were counted. Leucocyte distribution, cellular mortality (apoptotic and necrotic leucocytes), leucocyte respiratory burst ([Bado-Nilles et al., 2014](#)), lysosomal membrane integrity (LMI) ([Bado-Nilles et al., 2013](#)) and phagocytosis activity ([Bado-Nilles et al., 2011](#)) were performed.

2.5.3. Fish population abundance and structure

2.5.3.1. Larvae drift. Each day, drifting larvae were recovered in each mesocosm, and the total number of alive and dead larvae were counted. Results were expressed as total number of larvae per week and per mesocosm (See [de Kermoisan et al., 2013](#) for more details).

2.5.3.2. Final population structure. On the 7th of October 2013, all the individuals of each population were fished and killed. Thereafter, length and weight of all fish from each population were measured according to a protocol described in [de Kermoisan et al. \(2013\)](#) (See also Appendix A, Fish Material and Methods).

During this project, all experiments were conducted in accordance with the Commission recommendation 2007/526/EC on revised guidelines for the accommodation and care of animals used for experimental and other scientific purposes.

2.6. Community monitoring

2.6.1. Zooplankton

Zooplankton was sampled every 4 weeks (−49 to +148 dpt) in the upper and lower sections of each mesocosm ([de Kermoisan et al., 2013](#) and Appendix A, Invertebrates - Material and Methods for more details).

2.6.2. Invertebrates

Invertebrates were sampled every 4 weeks (−46 to +150 dpt) using different types of artificial substrates, i.e. tubes and tiles ([de Kermoisan et al., 2013](#) and Appendix A, Invertebrates - Material and Methods for more details).

2.7. Data analysis

Statistical analyses were performed with R 2.15.1 software ([Team, 2018](#)). The level of significance for all statistical analyses was 5%.

Repeated measures ANOVAs were performed to identify possible significant effects on the measured endpoints (macrophyte species bio-volume, physico-chemical parameters, zooplankton major taxon abundances, macroinvertebrate functional feeding group abundances) and a Dunnett's post hoc test was then used to compare each treatment to the control for the entire experimental period and for each sampling date. Assumptions of normality (Shapiro-Wilk normality test) and homogeneity of variance (Fligner-Killeen test) were verified before analysis. No Observed Effect Concentrations (NOEC) were determined for each sampling date and for the entire experimental period.

Fish individual biomarker responses were analysed using a two-way analysis of variance (ANOVA) to assess the combined effect of gender and the treatment on the different biological parameters. The Newman-Keuls post hoc test was performed to determine differences between the treatment and the control. For fish population data obtained at the end of the experiment, one-way ANOVA or binomial GLMs were performed. A Dunnett's post hoc test was then used to compare each treatment. If normality was not verified a non-parametric analysis of variance using Kruskal Wallis's test was performed. Dose-responses for each fish populational endpoint were computed with the R package DRC ([Ritz et al., 2015](#)) using log-normal distributions. For zebra mussel, one-way analysis of variance (ANOVA) was performed to assess the possible effect of diclofenac concentration on the different biological parameters. If normality was not verified, a non-parametric analysis of variance using Kruskal Wallis's test was performed followed by the post-hoc Dunn's multiple pairwise comparison test with a Bonferroni factor for the comet assay. No Observed Effect Concentrations (NOEC) were determined for each endpoint.

Responses of zooplankton and macroinvertebrate communities to DCF treatment were analyzed using the Principal Response Curve (PRC) method developed by [Van den Brink and Ter Braak \(1999\)](#). PRC analysis

is usually performed on $\log(a \cdot X + 1)$ transformed abundance data, where X stands for abundance, and by quantifying the distance between samples with the Euclidean distance. The abundance data was analyzed with this method, with a chosen so that $aX = 2$ when X was chosen as the lowest value in the dataset. Based on the findings by Tebby et al. (2017) zooplankton and macroinvertebrate abundance data were analyzed with the Hellinger distance on raw data with $\alpha = 0.5$. Significance of the overall treatment effects and the first PRC axis was estimated with Monte Carlo permutations of the time-series. Monte Carlo permutations were also performed at each sampling date, to test for the significance of treatment effects. Dunnett's test on PCA co-ordinates was used to determine the NOEC at the community level ($\text{NOEC}_{\text{community}}$) for each sampling date and thereon for the entire experimental period.

Validity of NOECs at the population and community levels for the entire experimental period was examined based on the dose-response relationship and the number of sampling dates with the same NOEC.

3. Results

Considering the large data set, a focus is made on the most relevant and significant results. Details for certain endpoints are provided in the Appendix A and results of statistical analysis are provided in Appendix B.

3.1. Diclofenac concentrations in water

Average DCF concentrations measured in water were lower than nominal values for all treatments and locations along each mesocosm. Mean measured concentrations at the inlet of the mesocosms were respectively 0.06 ± 0.02 , 0.46 ± 0.13 , and 4.36 ± 1.29 $\mu\text{g/L}$ for the 0.1, 1, and 10 $\mu\text{g/L}$ treatments during the entire experiment. Concentrations remained constant between 0 and 5 m and then dropped between 5 and 19 m. The average loss of DCF between 5 and 19 m for each treatment are, respectively for the 0.1, 1, and 10 $\mu\text{g/L}$ treatments, 41%, 44%, and 44%. Average effective concentrations (AECs) were constant throughout the experiment (Appendix A, Average effective concentrations, Table S2). The following average effective concentrations are used hereafter: 0.041 ± 0.016 , 0.44 ± 0.05 , 3.82 ± 0.47 for the low, medium, and high treatments respectively.

3.2. Diclofenac, transformation products and metabolites in watercress

DCF along with three of its nine identified transformation products were found in watercress, in the high treatment. DCF, 4' OH-DCF, and DCF-lactam were found in macrophyte tissue in all replicates, with concentrations ranging between 9.43 and 31 ng/g, 6.3–12.8 ng/g, and 0.3–1.3 ng/g, respectively. The metabolite 5' OH-DCF was found at a concentration of 0.9 ng/g in only one replicate (Appendix A, Diclofenac and transformation products in watercress, Table S3). Results in the control and the low and medium treatments are inferior to the limit of detection.

3.3. Physico-chemical parameters

Temperature and pH were not affected by the treatment (ANOVA, $F(3,8) = 3.4$ between treatment $p = 0.075$ for pH, ANOVA, $F(3,8) = 3.3$ between treatment $p = 0.078$ for temperature, Appendix A, Fig. S2 a,c). Dissolved oxygen was significantly affected by the treatment (RM-ANOVA, $F(3,8) = 20.6$ between treatment, $p = 4.10^{-4}$; Appendix A, Fig. S3). Indeed, levels were lower in the high treatment compared to the control at 8, 22, 30, 38, 51, 59, 93, 121, and 157 dpt (Appendix B, Physico-chemical parameters). Conductivity was significantly affected by the treatment (RM-ANOVA, $F(3,8) = 7.0$ between treatment, $p = 0.01$; Appendix A, Fig. S2 b) at two non-consecutive dates. Lower values were found in the low treatment compared to the control at -33 dpt. At +127 dpt, higher levels were found in the medium and high treatments

(Appendix B, Physico-chemical parameters). A NOEC of 0.44 $\mu\text{g/L}$ can be set for dissolved oxygen and a NOEC >3.82 $\mu\text{g/L}$ can be set, for pH and temperature. As significant effects were only found on two separate dates a NOEC >3.82 $\mu\text{g/L}$ is also set for conductivity.

3.4. Macrophytes

Volume scores of watercress, water starwort (Fig. 1a,b) and Eurasian water milfoil (Appendix A, Fig. S4 b) were significantly affected by the treatment (RM-ANOVA, $F(3,8) = 30.8$ between treatment $p = 9.5 \times 10^{-5}$ for watercress; RM-ANOVA, $F(3,8) = 14.05$ between treatment $p = 0.00149$ for water starwort, RM-ANOVA, $F(3,8) = 7.11$ between treatment $p = 0.012$ for water milfoil). Volume scores of filamentous algae were not affected by the treatment (RM-ANOVA, $F(3,8) = 2.5$ between treatment $p = 0.13$ Appendix A, Fig. S4 a).

In the control, peak values of the volume score of watercress were found in July (+77–91 dpt). Blossoming occurred from May to July (+36–91 dpt) and then the volume score decreased from July to August (+106–134 dpt). The appearance of newly formed stems occurred in September (+147–163 dpt). In the high treatment, watercress volume scores were negatively affected compared to the control at +36, 50, 64, 77, 91, and +106 dpt (Fig. 1a, Appendix B, Macrophyte responses, Watercress).

In the control, the volume score of water starwort increased throughout the experiment. In the high treatment, the volume score of water starwort was significantly lower at +22, 51, 78, and +107 dpt (Fig. 1b, Appendix B, Macrophyte responses, Water starwort) compared to the control.

In the control, the volume score of water milfoil increased slowly and gradually during the experiment (Appendix A, Fig. S4 b). Higher bio-volume levels were found in the medium treatment compared to the control at +116 and +135 dpt and in the low treatment at +135 dpt (Appendix B, Macrophyte responses- water milfoil).

A NOEC 0.44 $\mu\text{g/L}$ can be set for watercress and water starwort. A NOEC >3.82 $\mu\text{g/L}$ can be set for Eurasian water milfoil and filamentous algae. NOEC values for each species throughout time are presented in Appendix A (Table S4).

3.5. Sentinel species

3.5.1. Zebra mussel individual responses

Concerning zebra mussels, mortality rates increased between 2 (+63 dpt) and 5 months (+162 dpt) of exposure and a significant effect was observed at +162 dpt (Table 1, Kruskal-Wallis, $K = 19.4$ $p = 0.007$, $df = 7$). No effect of diclofenac was observed on the other parameters (condition index, ETS, amylase and energy reserve, Kruskal-Wallis, $p < 0.05$, Appendix B, Zebra Mussels). Only time influenced the condition index and ETS activity as a significant decrease was observed between 2 and 5 months.

Concerning immunomarkers, modifications of hemocyte immune responses were only observed at +63 dpt (Table 2). Hemocyte distribution was modified in the medium and high treatments compared to the control and low treatment (Table 2, two-way ANOVA, $F(22,84) = 5.4$, $p < 0.0001$, Appendix B: Zebra Mussels, Hemocyte distribution). In fact, for all treatments, hemocyte distribution was mainly made up of hyalinocytes sub-populations. The proportion of hyalinocytes decreased with increasing concentrations of diclofenac whereas the proportion of granulocytes increased. In the same way, cellular mortality increased in all treatments but was only statistically significant for the medium and high treatments (0.44 and 3.82 $\mu\text{g/L}$) (Table 2, Kruskal-Wallis, $K = 28.8$, $p = 0.00015$, $df = 7$, Appendix B, Zebra Mussels, Cellular mortality). Also, even if ROS production and phagocytosis activity were not statistically modified by exposure (Table 2, Kruskal-Wallis, $p > 0.05$, Appendix B, Zebra Mussels ROS production and phagocytosis), these parameters tend to decline in a dose-response manner.

A boxplot representation of the level of DNA strand breaks

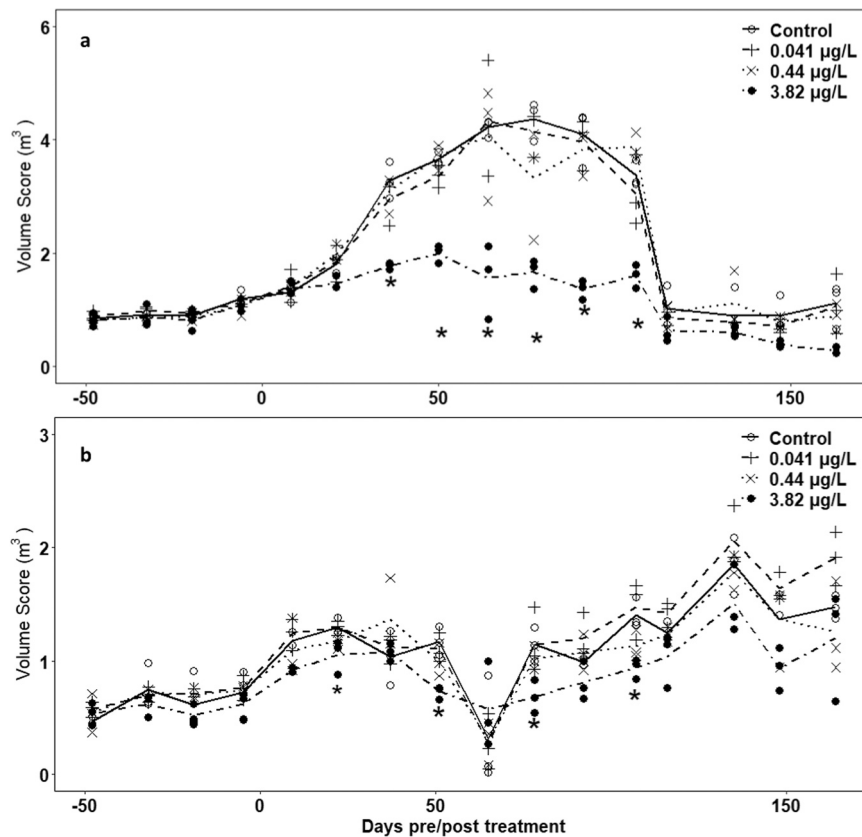


Fig. 1. Volume score (m^3) of watercress (a) and water-starwort (b) in the control (white dots) and the treatments [+ : 0.041, x: 0.44 and black dots: 3.82 $\mu g/L$] throughout time. Mean values are presented by a full-line, dashed-line, dotted-line and a dot-dashed-line respectively for the control, 0.041, 0.44, 3.82 $\mu g/L$ treatments. Significant differences between treatments and the control are marked by an asterisk.

Table 1

Individual parameters (mortality: mean \pm SE, N = 3 mesocosm per condition) and condition index (mean \pm SE, N = 9; except values with *, N = 4) and biochemical responses (mean \pm SE, N = 9; except values with *, N = 4) of zebra mussels after 2 and 5 months of exposure to different diclofenac concentrations. For each sampling date, different letters correspond to significant differences between treatments ($p \leq 0.05$).

		Cumulative Mortality (%)	Condition Index (AU)	ETS activity (mJ/mg ww/h)	Amylase activity (μg maltose/min/mg BSA)	Energy reserve (mJ/mg)
2 months(+63 dpt)	Control	7.8 \pm 8.4 ^a	0.19 \pm 0.02 ^a	8.9 \pm 3.8 ^a	98.4 \pm 44.8 ^a	2171 \pm 702 ^a
	0.041 $\mu g/L$	8.1 \pm 4.6 ^a	0.15 \pm 0.02 ^a	8.0 \pm 2.5 ^a	65.6 \pm 27.1 ^a	2509 \pm 746 ^a
	0.44 $\mu g/L$	11.4 \pm 3.1 ^a	0.16 \pm 0.04 ^a	7.4 \pm 3.2 ^a	67.9 \pm 31.3 ^a	2017 \pm 607 ^a
	3.82 $\mu g/L$	10.8 \pm 0.8 ^a	0.15 \pm 0.07 ^a	7.1 \pm 2.8 ^a	91.6 \pm 31.9 ^a	2682 \pm 757 ^a
5 months (+162 dpt)	Control	29.7 \pm 9.6 ^a	0.12 \pm 0.02 ^a	2.6 \pm 1.7 ^a	91.4 \pm 44.3 ^a	1971 \pm 479 ^a
	0.041 $\mu g/L$	37.5 \pm 2.2 ^b	0.12 \pm 0.02 ^a	2.6 \pm 1.9 ^a	92.9 \pm 45.7 ^a	2135 \pm 503 ^a
	0.44 $\mu g/L$	40.6 \pm 6.0 ^c	0.11 \pm 0.02 ^a	1.8 \pm 1.6 ^a	88.9 \pm 13.0 ^a	1709 \pm 418 ^a
	3.82 $\mu g/L$	57.2 \pm 8.4 ^d	0.10 ^a \pm 0.01 ^a	3.5 ^a \pm 0.5 ^a	ND	1862 ^a \pm 531 ^a

Table 2

Hemocyte immune activities (hemocyte distribution, hemocyte mortality, ROS production and phagocytosis activity) of zebra mussels (*Dreissena polymorpha*) were monitored in laboratory by flow cytometry after 2 and 5 months of exposure. Values correspond to means \pm standard error. Different letters correspond to significant differences between treatments ($p \leq 0.05$).

		Hemocyte distribution (%)		Hemocyte mortality (%)	ROS production (MFI)	Phagocytosis activity (%)
		Hyalinocytes	Granulocytes			
2 months (+63 dpt)	Control	90.3 \pm 3.5 ^a	9.7 \pm 0.9 ^a	4.6 \pm 0.2 ^a	26.7 \pm 1.5 ^a	21.7 \pm 2.5 ^a
	0.041 $\mu g/L$	85.8 \pm 0.9 ^a	14.2 \pm 0.9 ^a	7.4 \pm 0.4 ^{ab}	26.1 \pm 1.5 ^a	18.1 \pm 1.5 ^a
	0.44 $\mu g/L$	79.5 \pm 1.7 ^b	20.5 \pm 1.7 ^b	16.6 \pm 2.4 ^b	22.9 \pm 1.1 ^a	16.2 \pm 1.2 ^a
	3.82 $\mu g/L$	77.5 \pm 2.9 ^b	22.3 \pm 2.9 ^b	19.0 \pm 2.3 ^b	21.7 \pm 1.3 ^a	15.7 \pm 1.3 ^a
5 months (+162 dpt)	Control	71.1 \pm 1.4 ^a	28.9 \pm 1.4 ^a	17.6 \pm 2.1 ^a	38.9 \pm 2.5 ^a	37.6 \pm 1.9 ^a
	0.041 $\mu g/L$	78.9 \pm 1.6 ^a	21.1 \pm 1.6 ^a	14.5 \pm 2.2 ^a	52.2 \pm 4.3 ^a	27.6 \pm 1.6 ^a
	0.44 $\mu g/L$	77.9 \pm 1.7 ^a	22.1 \pm 1.7 ^a	20.7 \pm 4.5 ^a	34.4 \pm 3.7 ^a	27.9 \pm 1.0 ^a
	3.82 $\mu g/L$	79.1 \pm 0.7 ^a	20.9 \pm 0.7 ^a	12.0 \pm 1.8 ^a	37.3 \pm 2.2 ^a	21.7 \pm 1.0 ^a

(expressed in arbitrary units-AU) in hemocytes of zebra mussels for each treatment and sampling date is shown in Fig. 2. The average level of DNA strand breaks (AU \approx 20) was similar in control mussels throughout the experiment (from April to September), corresponding to the baseline level. This basal level linked to cellular metabolism was like the one observed in mussels originating from the same population and transplanted in “environmental conditions” (*Canal de l’Aisne à la Marne-Reims*) for one year (data not shown). An overall increase of DNA strand breaks was observed in hemocytes of zebra mussels exposed to DCF (between x2 to x3 compared to the control). After two months of exposure (+62 dpt), a significantly higher level of DNA strand breaks was observed in hemocytes of zebra mussels exposed to 0.041 $\mu\text{g/L}$ and especially to 3.82 $\mu\text{g/L}$ of DCF (Fig. 2). After 5 months of exposure (+162 dpt), a dose-effect relationship was observed, with significantly higher levels of DNA strand breaks in hemocytes of zebra mussels (Kruskal-Wallis, $H = 15.5$, $p < 0.0001$, $df = 8$).

Globally, for zebra mussels, 5 endpoints showed significant differences on the 10 explored individual endpoints. The NOEC value for mortality was 0.041 $\mu\text{g/L}$. Genotoxicity was high as a NOEC $< 0.041 \mu\text{g/L}$ was found for DNA strand breaks. Details on the different endpoints are provided in the Appendix A, Table S4 and Table S5.

3.5.2. Fish individual responses

After 6 months of exposure (+171 dpt), impacts were detected on some tested biomarkers in fish. Splenic immune leucocyte distributions of the three-spined stickleback were similar regardless of the condition and generation (Kruskal-Wallis, $K=0.6$, $p = 0.71$, $df = 2$ for F_0 and Kruskal-Wallis, $K=8.2$, $p = 0.41$ $df = 3$ for F_1 ; Table 3). An increase of leucocyte mortality, especially due to an apoptosis phenomenon, was shown only at the low treatment (0.041 $\mu\text{g/L}$) for both fish generations (Kruskal-Wallis, $K = 17.2$ $p = 0.001$, $df = 3$ for leucocyte mortality and Kruskal-Wallis, $K = 29.5$, $p < 0.0001$, $df = 3$ for apoptosis, Appendix B, Fish individual responses). Phagocytosis activity decreased in the high treatment for the F_1 generation only (Kruskal-Wallis, $K = 26.3$,

$p < 0.0001$, $df = 3$ Appendix B, Fish individual responses). Nevertheless, the most important immune destabilization concerned the ROS basal level which is representative of a leucocyte oxidative stress (Table 3, Kruskal-Wallis $K=15.6$, $p = 0.001$, $df = 3$, Appendix B, Fish individual responses).

The significant decrease of the ROS basal level could be correlated with the other biomarkers of oxidative stress. Indeed, TBARs measured in the liver have the same type of profile as the observed leucocyte oxidative stress (Fig. 3, two-way ANOVA, $F(7,111) = 8.4$, $p < 0.0001$ for the F_1 generation, two-way ANOVA, $F(5,81) = 8.6$, $p < 0.0001$ for the F_0 generation, Appendix B, Fish individual responses). Furthermore, GSH which protect cells from oxidative stress, were not significantly modified by the treatment (two-way ANOVA, $F(7,107) = 1.15$, $p = 0.33$). Only female GPx activities tend to increase in the low treatment (Fig. 3, Kruskal-Wallis, $K = 40.7$, $p < 0.0001$, $df = 7$ for the F_1 generation; Kruskal-Wallis, $K=53.2$, $p < 0.0001$, $df = 5$ for the F_0 generation, Appendix B, Fish individual responses) and SOD levels significantly decreased for the F_1 generation only in the high treatment for males (Fig. 3, two-way ANOVA, $F(7, 110) = 14.8$, $p < 0.0001$, Appendix B, Fish individual responses).

Concerning energy allocation, a significant increase of lipid storage was shown in the high treatment for females and males of the F_1 generation (Kruskal-Wallis, $K = 27.4$; $p = 4.8 \cdot 10^{-6}$, $df = 3$, Appendix A, Fig. S5, Appendix B, Fish individual responses).

3.5.3. Fish population responses

High mortality of founder fish (16 individuals in mesocosm 4, and 11 individuals in mesocosm 10) was observed in two out of three mesocosms exposed to the high treatment from the 22nd of May (+36 dpt) to the 1st of June (+46 dpt). Therefore, in the high treatment, larvae drift was negatively affected on week 23, 24, 26, and 28 (ANOVA, $F(3,8) = 18.1$, $p < 0.0006$, Appendix A, Fig. S6, Appendix B, Larvae drift).

Thus, at the end of the experiment, the total number of founder fish were lower in the high treatment compared to the control (ANOVA, F

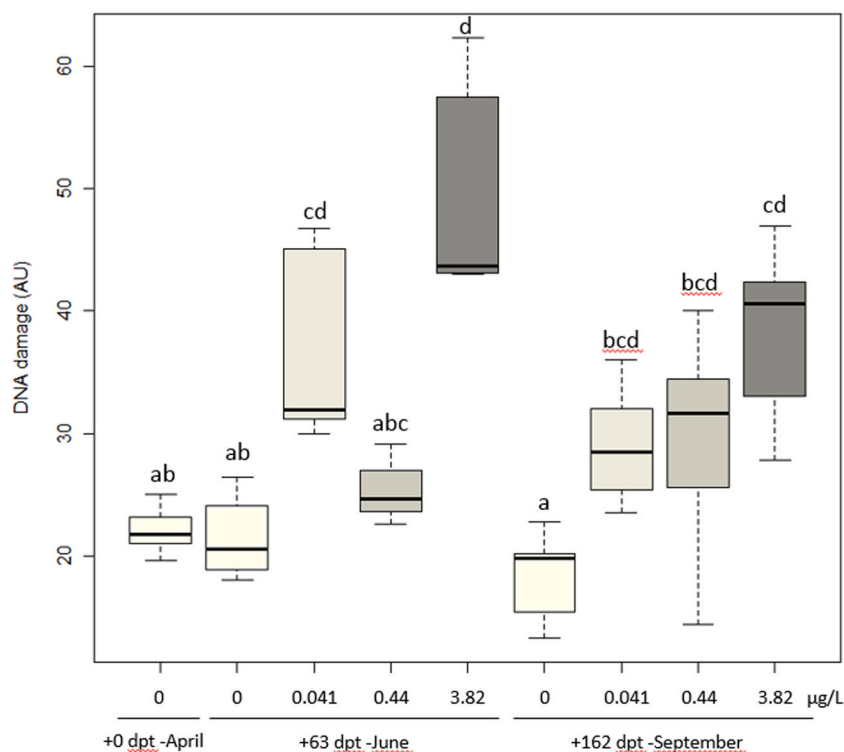


Fig. 2. Boxplot representation of the level of DNA strand breaks in hemocytes of zebra mussels before exposure (+ 0 dpt) and according to the time- (+ 63 or 162 dpt) and the treatment (control, 0.041, 0.44 and 3.82 $\mu\text{g/L}$). Boxplots with different letters are significantly different (with a p -level < 0.0014 according to the post-hoc Dunn’s multiple pairwise comparison test with a Bonferroni factor, after the non-parametric Kruskal-Wallis test).

Table 3
Splenic immune leucocyte activities (leucocyte distribution, cellular mortality, lysosomal membrane integrity (LMI), respiratory burst and phagocytosis) of the three-spined stickleback (*Gasterosteus aculeatus* L.) were monitored in laboratory by flow cytometry at the end of the experiment. Values correspond to means \pm standard error for founder fish and the first generation of fish. Boldface values corresponds to significantly different values from the control with $p \leq 0.05$.

		Leucocyte distribution (%)				Cellular mortality (%)				Respiratory burst values (MFI)				LMI (MFI)	Phagocytosis activity (%)
		Lymphocytes		Granulocytes-Macrophages		Total	Apoptosis	Necrosis	ROS basal level	ROS activated level	Stimulation index				
Founder fish	Control	63.7 \pm 2.1	36.3 \pm 2.1	8.2 \pm 2.8	2.1 \pm 0.2	6.1 \pm 2.8	107.7 \pm 10.1	200.3 \pm 35.2	2.1 \pm 0.3	11.4 \pm 0.3	34.9 \pm 1.2				
	0.041 μ g/L	65.4 \pm 2.0	34.6 \pm 2.0	10.0 \pm 0.4	3.7 \pm 0.4	6.3 \pm 0.3	81.5 \pm 14.0	131.3 \pm 17.9	1.9 \pm 0.2	11.3 \pm 0.2	31.9 \pm 1.2				
	0.44 μ g/L	63.6 \pm 1.5	36.4 \pm 1.5	9.9 \pm 1.0	3.6 \pm 0.6	6.3 \pm 0.6	147.4 \pm 27.9	325.4 \pm 69.5	2.1 \pm 0.2	10.3 \pm 0.2	30.1 \pm 1.8				
F ₁ generation	3.82 μ g/L	—	—	—	—	—	—	—	—	—	—				
	Control	68.7 \pm 1.4	31.3 \pm 1.4	5.2 \pm 0.4	2.1 \pm 0.2	3.1 \pm 0.4	109.7 \pm 27.0	133.7 \pm 17.4	1.6 \pm 0.2	12.0 \pm 0.2	36.9 \pm 2.5				
	0.041 μ g/L	68.3 \pm 1.3	31.7 \pm 1.3	7.3 \pm 0.5	4.5 \pm 0.6	2.8 \pm 0.3	53.7 \pm 6.6	84.4 \pm 8.7	1.7 \pm 0.1	12.1 \pm 0.1	28.2 \pm 1.2				
F ₁ generation	0.44 μ g/L	63.3 \pm 1.7	36.7 \pm 1.7	5.2 \pm 0.4	2.5 \pm 0.3	2.7 \pm 0.3	57.5 \pm 7.2	131.1 \pm 26.2	2.0 \pm 0.1	12.5 \pm 0.2	33.2 \pm 2.3				
	3.82 μ g/L	68.6 \pm 1.5	31.4 \pm 1.5	6.1 \pm 0.3	3.2 \pm 0.2	2.9 \pm 0.2	86.5 \pm 14.7	84.9 \pm 9.9	1.2 \pm 0.1	12.6 \pm 0.1	23.82 \pm 1.1				

(3,8) = 18.6, $p < 0.0005$ for F₀ females, ANOVA, F(3,8) = 5.4, $p = 0.024$ for F₀ males, Fig. 4c, d; Appendix B, Total number of founder fish). No effects were found for the other treatments. No effects were found on the mean standard length of male and female founder fish at the end of the experiment (ANOVA, F(3,6) = 0.9, $p = 0.51$ for F₀ females, ANOVA, F(3,6) = 25.5, $p = 0.154$ for F₀ males, Appendix A, Fig. S7, Appendix B, Mean standard length).

Total fish abundance (ANOVA, F(3,8) = 5.8, $p = 0.02$ for total abundance), and the percentage of juveniles (and thus adult percentage) along with sex-ratio were also negatively affected (GLM with complementary log-log link function (binomial family), $Z = 36.05$, $p < 2 \times 10^{-16}$

for the percentage of juveniles and GLM with complementary log-log link function (binomial family), $Z = 6.11$, $p = 9.9 \times 10^{-10}$ for sex ratio; Fig. 4a, b, f, Appendix B, Percentage of juveniles and sex ratio).

Finally, mean standard length of females and males of the F₁ generation was higher in the high treatment compared to the control (ANOVA, F(3,8) = 6.3, $p = 0.02$ for females; ANOVA, F(3,8) = 4.2, $p = 0.04$ for males, Fig. 4e and Appendix A, Fig. S8, Appendix B, Mean standard length F₁ generation). Juveniles were found in only one replicate thus no statistical analysis could be performed (Appendix A, Fig. S8).

Length frequency distributions of the different length classes for each group (female, males and juveniles) are in accordance with the latter observed effects (Appendix A, Fig. S9).

Globally, for sticklebacks, 6 endpoints showed significant differences on the 9 individual endpoints. At the population level, 9 endpoints showed significant differences for the 10 descriptive variables of the population. The lowest NOEC value at the individual and population levels are $< 0.041 \mu\text{g/L}$ and $0.44 \mu\text{g/L}$ respectively (see Appendix A, Table S6 for more details).

3.6. Community responses

3.6.1. Zooplankton

A total of 34 different taxonomic groups of zooplankton were sampled throughout the experiment including 23 taxa/species of rotifers (dominant taxa: *Lepadella sp.*, *Lecane sp.*, *Cephalodella sp.*), 10 species of cladocerans (dominant species: *Chydorus sphaericus*, *Alona rectangula*) and 1 order of copepod (Cyclopoida).

Using the Euclidean distance on Hellinger-transformed data, the community structure was significantly affected by the treatment (Monte Carlo Permutations on all axes; F(24,64) = 1.45; $p = 9 \times 10^{-4}$; Appendix A, Zooplankton results, Fig. S10). Time displayed explained 63.9% of the total variance. Treatment explained 12.7% of the total variance. Of this variance, 23.0% was displayed on the first PRC. Also, significance tests by axis showed that none of the PRC axes were significant (Monte Carlo Permutations on each axis; F(1,64) = 8.00 for the first axis; $p = 0.083$).

Two sampling dates, 38 and 92 dpt, displayed significant treatment effects (MC permutations on PCA at each sampling date, on the first PRC axis for 38 dpt (F(1,8) = 3.41, $p = 0.0018$, and on all PRC axes, F(3,8) = 2.23, $p = 0.0011$, F(3,8) = 1.39, $p = 0.033$ respectively, Appendix B, Table S7, PRC results for zooplankton).

The first principal response, which did not display significant effects of treatment, was characterized by higher abundances in the high treatment compared to the control except at + 92dpt. The principal response mainly reflected the abundance of *Lepadella*. Species with a negative weight such as *Mytilina* and *Testidumella* decreased in abundance, relative to the control, in the high treatment after +64dpt. *Notholca*, the second most contributing species to the first PRC, decreased in abundance at the first two sampling dates, before the treatment started (Appendix A, Zooplankton results, Fig. S10).

NOEC values for the zooplankton community and major taxon were respectively $> 3.82 \mu\text{g/L}$ and $0.44 \mu\text{g/L}$ (See Appendix A, Table S8 and Appendix B, Zooplankton taxon for more details).

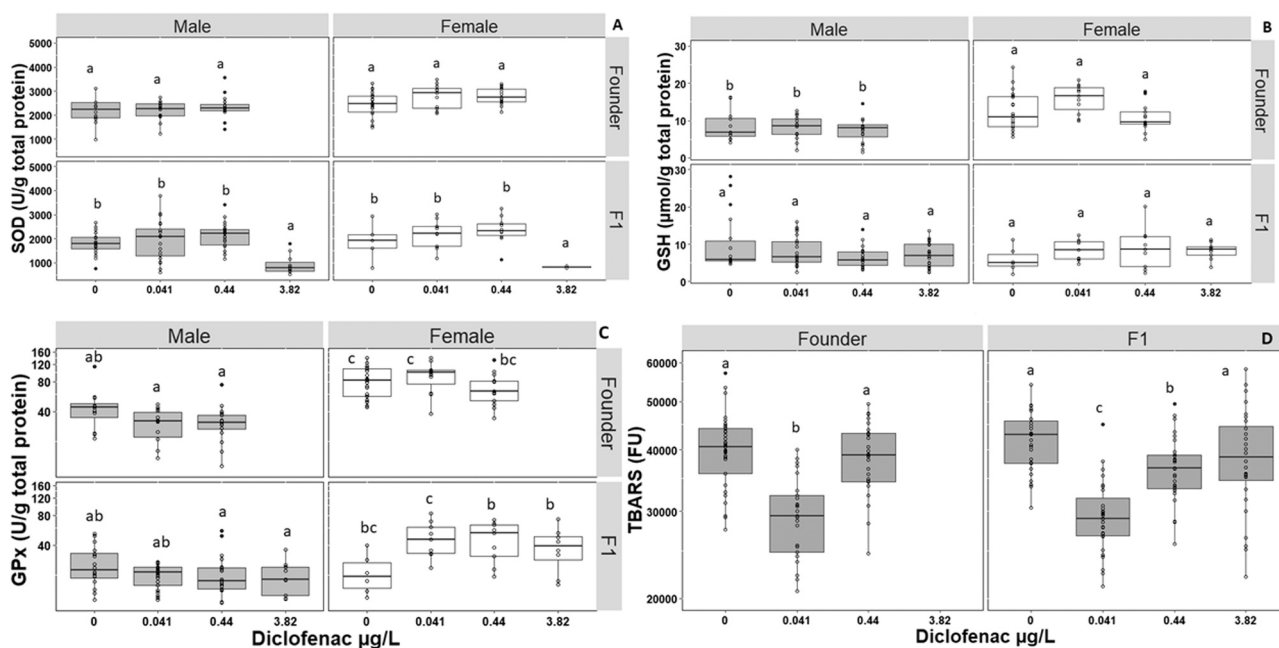


Fig. 3. Oxidative stress (A) SOD, (B) GSH, (C) GPx, (D) TBARS of the three spined stickleback (*Gasterosteus aculeatus* L.) following an exposure of six months in mesocosms exposed to diclofenac (from 0.041 to 3.82 µg/L). For GSH, GPx and SOD, male (M) and female (F) were treated separately due to significant difference between both gender in the analysis. Values correspond to means ± standard error. Different letters correspond to values significantly different with p ≤ 0.05.

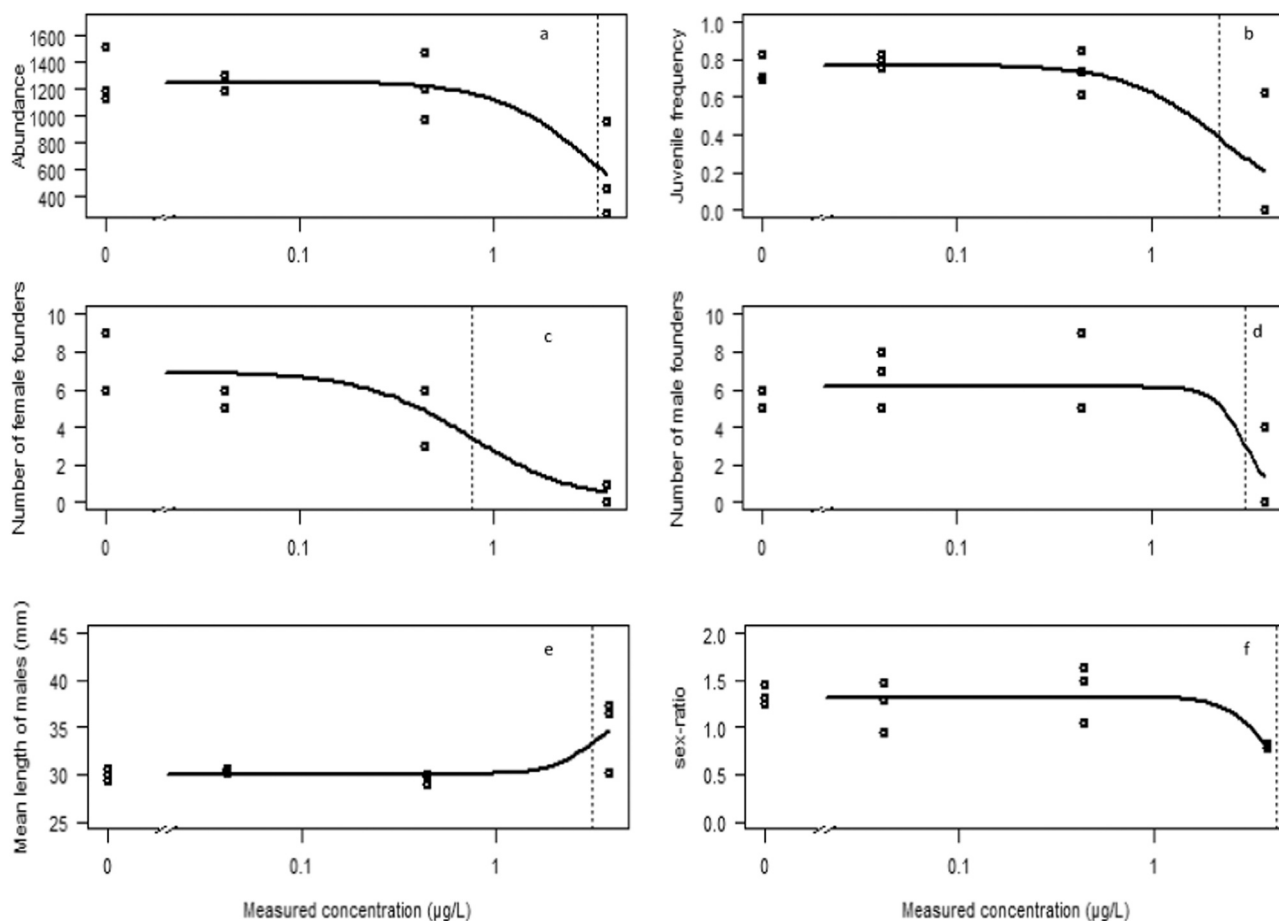


Fig. 4. (a) Total fish abundance, (b) juvenile frequency, (c) Number of female founders, (d) Number of male founders, (e) Mean length of males, (f) sex-ratio in the control and different treatments (0.041, 0.44 and 3.82 µg/L). The full black line represents the dose-responses fitted on the data points. Dashed lines represent the EC50 values for each endpoint.

3.6.2. Macroinvertebrates

A total of 22 different macroinvertebrate taxonomic groups were sampled throughout the experiment including 6 species of gastropods (*R. balthica*, *P. antipodorum*, *L. stagnalis*, *A. vortex*, *P. planorbis*, *P. acuta*), 2 of crustaceans (*G. pulex*, *A. aquaticus*), 2 taxa of oligochaeta (*S. lacustris*, *L. variegatus*), 1 of ephemeropterans (*C. dipterum*), 2 taxa of dipterans (Chironomidae, Tipulidae), 1 of tubellarians (*Dugesia*), 2 taxa of leeches (*Glossiphonia complanata* and *Erpobdellidae*), 1 taxa of heteropterans (Notonecta), 1 taxa of coleopterans (*Agabus*), 1 taxa of trichopterans (Limnephilidae) and 2 taxa of odonata (*Sympetrum* and *I. elegans*).

With the Euclidean distance on Hellinger-transformed data, the community structure was significantly affected by the treatment (9999 Monte-Carlo permutations on all axes; $F(24,64)=1.82$; $p=0.0001$; Appendix A, Invertebrate Results, Fig. S11) and the first PRC axis was significant (9999 Monte-Carlo permutations on the first axis; $F(1,64)=16.7$; $p=0.0002$). Time displayed explained 67.4% of the total variance. Treatment explained 12.9% of the total variance. Of this variance, 36.5% was displayed on the first PRC. With the Bray Curtis dissimilarity on log-transformed data, the results were very similar, with slightly less explained variance (65.6% by time and 11.8% by treatment).

The principal response was characterized by higher abundances in the high treatment compared to the control around +100dpt, and lower abundances compared to the control in the low and medium treatments in particular around +120 dpt. The principal response mainly reflected the abundance of *Radix balthica*, and to a lesser extent the population dynamics of *Asellus aquaticus* and *Erpobdella octoculata*, which followed the opposite dynamics. Species and/or taxa with species' weights between -0.25 and 0.25 were not represented on the graph (Appendix A, Invertebrate Results, Fig. S11).

Two sampling dates, 95 and 119 dpt, displayed significant treatment effects (MC permutations on PCA at each sampling date, on the first PRC axis ($F(1,8)=2.55$, $p=0.0125$, $F(1,8)=4.40$, $p=0.0086$ and on all PRC axes, $F(3,8)=1.39$, $p=0.0299$ and $F(3,8)=1.92$, $p=0.0077$ respectively, see Appendix B, PRC results for macroinvertebrates for details). Pairwise comparisons with Dunnett's test at these dates showed significant differences between the control and the high treatment.

NOEC values for the macroinvertebrate community (Appendix A, Table S10, NOEC values for macroinvertebrates) and major taxon were both $0.44 \mu\text{g/L}$ (Appendix B, Functional feeding groups for more details).

4. Discussion

The effects of DCF on primary producers and consumers were assessed in a simulated aquatic ecosystem. Although smaller and less complex than real ecosystems, mesocosm experiments allow to study both direct and indirect effects of chemical substances in more controlled conditions (Caquet et al., 2000). In our study, the direct action of DCF on different species will be discussed regarding the existing single -species literature data. As a consequence of these effects, species that are not directly impacted by DCF may be affected through indirect effects due to the presence of a complex food chain (Preston, 2002). Care will thus be taken to discuss the possible indirect effects that occurred during our study. The relevance of the EQS value for DCF will be discussed considering our results (mainly NOEC values).

4.1. Diclofenac exposure

Twenty-four hours after the beginning of the exposure, DCF concentrations in all treatments were approximately half of the nominal concentrations. The flow rates of each mesocosm along with the functioning of the contamination system were verified and adjustments were made when necessary. Despite this, DCF concentrations remained similar after one week of exposure. Degradation or adsorption in the mixing tanks supplying the mesocosms and/or in the silicon tubes delivering the substance occurred (data not shown). Nevertheless,

average effective concentrations reported hereafter were constant throughout time for each treatment.

In each mesocosm, a decrease of DCF concentrations was observed between 5 and 19 m from the water inlet. Partial photodegradation can explain this loss as it is the main degradation pathway for DCF (Yan and Song, 2014). Moreover, accumulation and biotransformation (metabolism phase I and phase II) of the parent compound by primary producers and consumers could also contribute to the decrease of the water concentrations (Kallio et al., 2010; Matamoros et al., 2012). Indeed, the metabolite 4'-OH DCF was found in fish (Daniele et al., 2016) and watercress in the high treatment which suggests that bioaccumulation and biotransformation are occurring in our study.

4.2. Macrophytes

In the high treatment, the biovolume of both watercress and water starwort were negatively affected compared to the control. Either direct and/or indirect effects could explain the observed responses.

Literature data on the effects of diclofenac on macrophytes is scarce and shows moderate toxicity of DCF. For example, inhibition of growth (EC_{50}) of *Lemna minor* was observed in three laboratory studies at relatively high concentrations compared to our tested concentrations (7.5, 47.6, and 0.1 mg/L for respectively Cleuvers, 2003; Kummerová et al., 2016; and Quinn et al., 2001) However, a decrease of photosynthetic pigments content and an increase in the amount of reactive nitrogen and oxygen species in roots were observed at concentrations as low as $0.1 \mu\text{g/L}$ (Kummerová et al., 2016). Moreover, when exposed to natural sunlight, phototoxicity of diclofenac was shown to increase for the unicellular chlorophyte, *Scenedesmus vacuolatus* (Schmitt-Jansen et al., 2007). Using effect-direct analysis, Schulze et al. (2010) identified 2-[(2-chlorophényl)amino] benzaldehyde (CPAB) as the main photo-transformation product responsible for the enhancement of the toxicity of DCF. Based on these results, we suspect that the effects on watercress and water starwort could be related to the presence of some photo-transformation products and/or metabolites. For this reason, DCF, CPAB and other metabolites of diclofenac were measured in watercress. Results obtained showed that DCF, 4'-OH DCF (4'-hydroxydiclofenac) and DCF-lactam (1-(2-6-dichlorophenyl)1,3-dihydro-2-H-indol-2-one) are present in watercress only in the high treatment (Daniele et al., 2016). As no literature data is available on the ecotoxicity of DCF-lactam to macrophytes, we can only suppose that this substance may be responsible for the observed effects.

Indirect effects such as an important development of the abundance of certain grazers such as snails could also explain the effects on macrophytes as they can feed on both periphyton (Beck et al., 2019; Hutchinson, 1975) and live macrophytes (Bakker et al., 2016). Nevertheless, Scheffer and Mormul et al., (1998, 2018) showed that freshwater gastropods graze preferably on epiphyton which indirectly favors macrophyte biomass and richness. Moreover, an increase of grazers in the high treatment is only observed after 2 months of exposure. Therefore, as effects on macrophytes and more especially on watercress was observed 15 days after the beginning of exposure, indirect effects on macrophytes due to a higher grazing pressure seem unlikely.

A decrease in macrophyte biomass will lead to a decrease in photosynthesis along with a decrease of the uptake of carbon dioxide and nutrients (Bakker et al., 2016; Scheffer, 1998). A decline in dissolved oxygen and pH levels along with an increase in conductivity is suspected. Indeed, one week after the beginning of the treatment and then during the experiment, dissolved oxygen was significantly lower in the high treatment compared to the control. The lower macrophyte biovolume in the high treatment probably significantly influenced dissolved oxygen levels. Similar results were found by Vervliet-Scheebaum et al. (2010) and by McGregor et al. (2008) who exposed macrophytes in semi-field conditions to respectively simazine and atrazine. These authors related the decrease in macrophyte biomass to the observed decrease in dissolved oxygen levels.

4.3. Sentinel species: from individual to population effects

4.3.1. Zebra mussels

High mortality was observed after 5 months of exposure in all treatments. Compared to literature data this result was unexpected as no mortality was recorded up to 1000 µg/L (Parolini, 2020; Quinn et al., 2001). A dose-effect relationship was observed on DNA integrity with lower DNA damage after 5 months of exposure. Surviving mussels may be more tolerant after 5 months of exposure compared to the ones sampled after 2 months of exposure. Moreover, the comet assay measures transitory DNA strand breaks, which considers both the genotoxic action of substance and reverse action performed by defense mechanisms such as DNA repair systems. After 2 months of exposure, DNA repair systems may be activated above a threshold level which may explain the observed results, as it has already been shown for invertebrate tissue (Bonnard et al., 2009). DNA repair systems could be activated and efficient at 0.44 µg/L but overwhelmed at 3.82 µg/L. Replacement of the most damaged cells in haemolymph in response to the genotoxic stress could also be occurring. Further investigations are necessary in order to validate these hypotheses.

These results were in accordance with previous studies underlying the genotoxicity of DCF alone (Binelli et al., 2001; Parolini, 2009) or in a mixture with ibuprofen and paracetamol (Parolini and Binelli, 2012). However, these previous studies were conducted *in vitro* (Parolini, 2009) or with high exposure concentrations which do not correspond to our experimental conditions (Binelli et al., 2001; Parolini and Binelli, 2012). Therefore, our results reveal that DNA integrity is also a sensitive endpoint in more natural conditions. Further studies are needed to specify the consequences of genotoxicity on the different functions of haemocytes and potentially on their death. Significant haemocyte mortality and haemocyte distribution were registered after 2 months of exposure. Only, the haemocyte mortality rate remained high after 5 months of exposure. This latter observation could be linked to seasonal (summer) and reproductive (gamete release) effects. In September during the post-spawning period, haemocytes participate to the elimination of residual gametes in gonads. This activity could explain the higher mortality rates. However, these latter effects were not associated with significant effects on the various measured haemocyte activities (phagocytosis, oxidative activity). In addition, no effect of DCF was observed regarding energy acquisition (digestive enzyme) reserves and mitochondrial biomarkers (ETS) which is surprising as Freitas et al. (2019) recorded a decrease of metabolic rate (decreased ETS) and an increase of endogenous reserves in marine mussel exposed to increasing temperature and DCF concentrations.

Thus, some of the biomarkers measured in this experiment may not be sensitive enough to highlight DCF effects on physiological functions (metabolism, immunity) of zebra mussels. New approaches such as metabolomics seem promising (Viant, 2007) as they allow to gain information without any a priori hypothesis about DCF effects. This strategy was successfully applied to marine mussels (Bonnefille et al., 2018). However, more knowledge on molecular mechanisms associated with certain functions is necessary in order to apply these methods to zebra mussels. Concerning immunity, Leprêtre et al. (2019) have recently provided new insight on zebra mussel immunity by describing molecular actors involved in the immune system in both hemocytes and plasma compartments which could represent interesting precocious biomarkers and alarm signals before hemocyte death.

Nevertheless, the relevance of using zebra mussels as a sentinel species in our study was shown as one biomarker (DNA integrity) was the most sensitive endpoint (NOEC < 0.041 µg/L).

4.3.2. Fish

Important mortality of founder fish was observed respectively two weeks and one month after the beginning of the exposure period in the high treatment in two out of three replicates. This mortality was shown to have consequences at the population level at the end of the

experiment (lower recruitment, absence of juveniles, effects on final population structure).

Cytological, and histological literature studies revealed effects of DCF down to water concentrations of 0.5 µg/L. These effects include a collapse of cellular compartmentation, glycogen depletion of hepatocytes in the liver, hyaline droplet degeneration in the kidneys, pillar cell necrosis in the gills, renal hematopoietic hyperplasia (Cuklev et al., 2011; Hong et al., 2007; Mehinto et al., 2010; Memmert et al., 2013). In several laboratory studies, median lethal concentrations (LC₅₀) for larvae and adults of different fish species such as *Cyprinus carpio*, *Oncorhynchus mykiss*, *Oryzias latipes*, *Danio rerio* were >1 mg/L for exposure durations ranging between 96 h and 3 months (Hallare et al., 2004; Islas-Flores et al., 2013; Linden and Lehtiniemi, 2005; Memmert et al., 2013; Praskova et al., 2014) which suggests that mortality of fish occurs at higher concentrations of DCF compared to the ones tested in our study. However, recent studies showed that direct mortality may occur also at lower concentrations such as 100 µg/L for juvenile trout (Schwarz et al., 2017) and 80 µg/L for juvenile three-spined sticklebacks (Näslund et al., 2017).

At the end of the experiment, an important immune destabilization due to leucocyte oxidative stress was observed in the F₁ generation in the high treatment. In addition, results obtained on biomarkers mainly TBARS and SOD showed an increase of antioxidant defenses and a loss of protective defenses which, like the results on the immune system, support the assumption of an increase in oxidative stress. These results are in line with other studies done on different fish species (Gröner et al., 2015; Islas-Flores et al., 2013; Saravanan et al., 2011).

Furthermore, p-benzoquinone imines and the hydroxylated metabolites mainly 4'- and 5'-hydroxydiclofenac are considered to be responsible for hepatotoxic adverse effects of DCF (Bort et al., 1998; Poon et al., 2001). In the present study, the metabolite 4'-hydroxydiclofenac was found in fish sampled at the end of the experiment only in the high treatment which supports that direct toxic effects may occur at the individual level (Daniele et al., 2016).

Furthermore, considering that mortality was episodic and in two out of three replicates of the high treatment, indirect effects are also suspected. Based on our experimental conditions which are similar to field conditions, the presence of diclofenac combined with other environmental stressors such as hypoxic conditions, temperature fluctuations, food scarcity, presence of parasites, microorganisms and/or toxic compounds formed by micro and macroalgae could lead to a reduction in individual fitness and eventually to death (Heugens et al., 2001; Schwaiger, 2001).

Bottom-up control of fish by the fluctuation of physico-chemical parameters may occur (Horppila et al., 1998). Indeed, low-oxygen (hypoxic) conditions can have serious impacts on fish populations, contributing to habitat loss and periodic mass mortalities (Breitburg, 2002). A decrease in dissolved oxygen levels observed prior to fish death in the high treatment may thus contribute to the observed founder fish and larvae mortality. Hypoxia of three-spined sticklebacks resulting in the eventual mortality of individuals was observed at 2 mg/L (Praskova et al., 2014; Wootton, 1976). As dissolved oxygen levels in the high treatment were never below 5 mg/L even during the night (data not shown), mortality due to hypoxia seems improbable.

Laboratory studies demonstrated that some chemicals (e.g. estradiol, cortisol, pesticides) cause immunomodulation in fish that is associated with a decrease of disease resistance to bacterial pathogens and parasites (Pickering and Christie, 1980; Shelley et al., 2012; Wenger et al., 2011) and an increase in the susceptibility of fish (Kreutz et al., 2010; Shelley et al., 2012). Moreover, some species of macrophytes and blue-green algae are known to release cyanotoxins (Christoffersen, 1996) and polyphenols (Linden and Lehtiniemi, 2005) which cause episodic mortality of invertebrates and fish species (Hallegraeff, 1993).

Based on our results and the literature data, we thus suppose that direct uptake of DCF by fish leads to an increase in oxidative stress and important immune destabilization. In turn, this indirectly caused a

greater susceptibility to endogenous bacterial pathogens and/or microalgae and/or macrophytes toxins and/or lower water quality. These degraded environmental conditions may be due to macrophyte biomass decomposition (lower oxygen and higher ammonium concentration) that were present in our experimental conditions. These direct and indirect effects are supposed to have led to the episodic mortality of adult fish and larvae. Further investigations are needed to conclusive determine the mechanisms responsible for this effect (e.g.: laboratory studies exposing fish to a combination of environmental stressors and DCF).

At the population level, impacts on the total abundance of fish (-), the percentage of juveniles (-) and adults (+), sex-ratio along with a shift in the length frequency distribution of the F₁ generation were observed in the high treatment compared to the control. The mean standard length of females, males, and juveniles were higher. Consequently, the length class with maximal abundance in populations exposed to the high treatment shifted to a bigger standard length. Juveniles were found in only one replicate. The mortality of founder fish and larvae at the beginning of the experiment is supposed to be responsible for the observed effects as reproduction occurred only once and recruitment success was lower than in the control. Therefore, density dependence competition was probably lower enabling the surviving juveniles to access to a greater food source (Rose et al., 2001). Furthermore, an increase in abundance of the zooplankton and macro-invertebrate populations that are consumed by fish (Wootton, 1976) was also observed in the high treatment (see below). As food availability and abundance were more important, growth of the remaining fish was greater. Moreover, an increase of lipid storage was observed in the liver of both females and males of the F₁ generation which suggests higher food consumption. Similar results were found for sticklebacks in previous studies (de Kermoisan et al., 2013; Roussel et al., 2007a).

Finally, in the high treatment sex-ratio was modified in the favor of males. This effect on sex-ratio is due to the shift in population structure involving a large number of young adults and no more reproductive active adults. Indeed, higher mortality was observed in reproductive active males (high energetic cost for parental care).

4.4. Community level effects

Macroinvertebrate and zooplankton community structures were impacted in the high treatment. Abundance of the following taxon increased: *Alona sp.*, *Cephalodella gibba* (zooplankton), scrapers (*Radix balthica*) and collector gatherers (*Stylaria lacustris*). Indirect effects are suspected. As shown in previous studies, three-spined sticklebacks juveniles feed mainly on cladocerans and copepods and thus have a great influence on macroinvertebrate community structure (de Kermoisan et al., 2013; Roussel et al., 2007a). As total fish abundance along with the percentage of juveniles and adults were impacted, in the high treatment, predation pressure was “released” enabling some species to considerably increase in abundance. Furthermore, as grazing by zooplankton and scrapers controls periphyton biomass and diversity, an increase in its pressure can thus also have an impact on primary production (Beck et al., 2019). This was not the case as no significant effects were observed on the overall periphyton biomass in the high treatment (data not shown).

4.5. Implication for water quality standards

The European Water Framework Directive (WFD) (2000/60/EC) highly recommends restoring all types of waters bodies to a ‘good’ chemical and biological status. One of the tools used to achieve this goal is environmental risk assessment for chemicals of concern. Risks are assessed by comparing chemical exposure to the identified hazard (Environmental Quality Standard, EQS) (Merrington et al., 2020). EQS values are derived using ecotoxicological laboratory studies that are selected according to methodologies recommended by Klimisch et al.

(1997); Moermond et al. (2016). Few guidelines exist on the use of higher-tier studies such as mesocosms for threshold evaluation and determination (de Jong et al., 2008). As these tools are intermediate between laboratory and field tests, they can be used to specifically address the uncertainty associated with a certain chemical depending on the area of concern identified earlier. For DCF, an EQS of 0.05 µg/L was proposed based on histological effects observed on *Oncorhynchus mykiss* (Mehinto et al., 2010). The ecological relevance of this individual-based effect is addressed; possible effects at higher levels such as the population are suspected using the weight of evidence approach (UBA, 2018). In our study, the lowest NOEC value at the individual level for fish was set to < 0.041 µg/L based on the immune responses and oxidative stress of founder fish and the F₁ generation. Mortality of founder fish also occurred at a higher concentration which in turn influenced the outcome at the population level (NOEC_{population} of 0.44 µg/L). Based on our results, the proposed EQS value seems relevant in terms of the protectiveness of potential higher-level effects. Nevertheless, other methods such as the tiered effects approach (EFSA, 2013) could be applied using conventional (standardized laboratory data) and unconventional endpoints (non-standardized laboratory data, mesocosm data) to derive a more environmentally realistic EQS value for DCF.

5. Conclusion

This first mesocosm experiment studying the effects of diclofenac in more natural environmental conditions revealed unexpected and novel results compared to the existing ecotoxicological data. Indeed, effects of diclofenac were observed at the individual (higher mortality rates, increase or decrease of biomarkers, responses of the immune system of both sentinel species), population (macrophytes, fish) and community (zooplankton and macroinvertebrate) levels. Furthermore, the duration of the study was a very interesting characteristic of this experiment, as some effects appeared only after one month of exposure, including mortality, increase in abundance of certain species and taxons, interspecific interactions. Moreover, enhanced ecotoxicity of some phototransformation products and metabolites of diclofenac is suspected. There remains a large need for investigating the environmental impact of photoproducts and metabolites of diclofenac that may be as toxic or even more toxic than the initial substance. In consideration of all the results, the no NOEC value is <0.041 µg/L at the individual level and 0.44 µg/L at the population and community levels. For regulatory matters, these latter NOECs may help evaluate how relevant and conservative the laboratory data based PNEC (predicted-no-effect concentration) or EQS (Environmental Quality Standard) values are.

CRedit authorship contribution statement

S. Joachim: Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **R. Beaudouin:** Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **G. Daniele:** Methodology, Writing - original draft, Writing - review & editing. **A. Geffard:** Methodology, Visualization, Formal analysis, Writing - original draft, Writing - review & editing. **A. Bado-Nilles:** Methodology, Visualization, Formal analysis, Writing - original draft, Writing - review & editing. **C. Tebby:** Formal analysis, Software, Writing - original draft, Writing - review & editing. **O. Palluel:** Methodology, Investigation, Resources, Formal analysis. **O. Dedourge-Geffard:** Methodology, Formal analysis. **M. Fieu:** Investigation, Resources, Data curation. **M. Bonnard:** Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **M. Palos-Ladeiro:** Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **C. Turies:** Investigation, Methodology, Software, Data curation. **E. Vulliet:** Validation, Project administration, Funding acquisition. **V. David:** Formal analysis, Data curation, Writing - original draft. **P. Baudoin:** Investigation, Resources, Data curation. **A. James:** Methodology, Writing -

original draft, Writing - review & editing. **S. Andres:** Supervision, Validation, Project administration, Funding acquisition. **J.M. Porcher:** Supervision, Validation, Project administration, Funding acquisition.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2020.111812](https://doi.org/10.1016/j.ecoenv.2020.111812).

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