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# Effects of diclofenac on the swimming behavior and antioxidant enzyme activities of the freshwater interstitial crustacean *Bryocamptus pygmaeus* (Crustacea, Harpacticoida)

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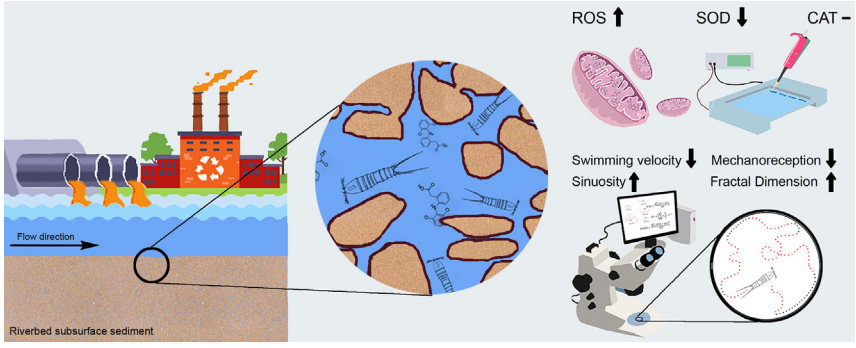
## HIGHLIGHTS

- The effect of 50 μg $L^{-1}$ diclofenac (DCF) was tested on *B. pygmaeus*.
- The environmentally relevant concentration affected the behavior of the species.
- Swimming, exploration ability and thigmotaxis were significantly affected.
- DCF also determined impairment in scavenging of reactive oxygen species (ROS).
- The mitochondrial SOD2 decreased in the individuals exposed to 50 μg $L^{-1}$ DCF.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Diclofenac (DCF) is one of the most widespread pharmaceutical compounds found in freshwaters as a pseudo-persistent pollutant due to its continuous release from point and diffuse sources, being its removal in Wastewater Treatment Plants incomplete. Moreover, DCF is particularly persistent in interstitial habitats and potentially toxic for the species that spend their whole life cycle among the same sediment grains. This study is aimed at offering a first contribution to the assessment of DCF effects on freshwater invertebrate species living in the interstitial habitats of springs, rivers, lakes and groundwaters. The Crustacea Copepoda are one of the main components of the freshwater interstitial communities, with the primacy taken by the worm-like and small-sized harpacticoids. A sub-lethal concentration of 50 μg $L^{-1}$ DCF significantly affected six out of the eight behavior parameters of the burrower/interstitial crustacean harpacticoid *Bryocamptus pygmaeus* recorded by video tracking analysis. DCF exposure reduced swimming speed, swimming activity, exploration ability and thigmotaxis, and increased swimming path tortuosity. The biochemical approach revealed a reduced level of the mitochondrial superoxide dismutase 2 in individuals exposed to DCF. It could be explained by a decline in mitochondrial performance or by a reduced number of functional mitochondria. Since mitochondrial dysfunction may determine ATP reduction, it comes that less energy is produced for maintaining the cell functions of the DCF-exposed individuals. In addition, the increasing energy demand for the detoxification process further contributes to decrease the total energetic budget allocated for other physiological activities. These observations can explain the changes we have observed in the swimming behavior of the copepod *B. pygmaeus*.



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## 1. Introduction

Emerging organic compounds (EOCs) are natural or synthetic substances not constantly monitored that can potentially induce negative effects on humans and ecosystems (Postigo and Barceló, 2015). This family of pollutants includes a wide variety of compounds mostly related to pharmaceuticals and medicinal products (Löffler and Ternes, 2003). Among pharmaceuticals that are commonly used, Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are worldwide gaining a growing concern (Swiacka et al., 2021). NSAIDs are used for medical and veterinary purposes and include analgesic, antipyretics, and anti-inflammatory drugs. Diclofenac (DCF) is one of the most used NSAIDs and its consumption is estimated to be around 1443 ± 58 t $y^{-1}$ on a global scale (Acuña et al., 2015). As most EOCs, DCF can reach the aquatic environment from point and diffuse sources (municipal effluents, aquaculture, irrigation, and surface runoff) and its removal in Wastewater Treatment Plants (WWTPs) is often partial (Zhang et al., 2008; Strenn et al., 2004; Ribeiro et al., 2015; Nunes et al., 2020). Due to its continuous release in freshwaters, DCF is considered a pseudo-persistent pollutant according to Ort et al. (2009) and one of the most widespread pharmaceutical compounds found in freshwaters (Hernando et al., 2006).

DCF was included in the European legislative framework in 2012 when it was introduced in the drinking water list of hazardous substances and, immediately after, listed in the Directive 2013/39/EU (European Commission, 2013). Later, DCF was included in the Surface Water Watch List (SWWL) with the Decision 2015/495/EU (European Commission, 2015). DCF was also among the 20 pharmaceuticals occurring with the highest concentrations in the Wastewater Treatment Plant (WWTP) influents and effluents, showing very low levels of removal in conventional sewage treatment plants (Lonappan et al., 2016). Recently, with the implementing Decision 2018/840/EU (European Commission, 2018), DCF has been proposed to be removed from the Surface Water Watch List as sufficiently high-quality monitoring data are now available, to be added to the candidate list of priority substances. DCF poses an environmental risk to European freshwater ecosystems at concentrations >50 ng $L^{-1}$ (Acuña et al., 2015; Comber et al., 2018) and to European groundwater ecosystems at concentration > 5 ng $L^{-1}$ (Di Lorenzo et al., 2021) and it is expected to also pose a similar environmental risk outside Europe, especially in developing countries worldwide (Rehman et al., 2015; Fekadu et al., 2019).

Despite the Measured Environmental Concentration (MEC) of DCF in European surface waters are within the range of ng $L^{-1}$ to μg $L^{-1}$ (Hernando et al., 2006), up to date, the available literature revolving around DCF concentrations in the European surface waters offers a partial vision of the future trend of concentration increase (Sathishkumar et al., 2020). Scheurell et al. (2009) reported the highest concentration of DCF (4.9 μg $L^{-1}$) in surface water bodies of Pakistan, with peaks up to 216 μg $L^{-1}$ detected in the Rawat industrial area (Hanif et al., 2020). In the WWTP effluents of Canada, 16 μg $L^{-1}$ DCF was reported by Lonappan et al. (2016). An increase of the environmental concentration of pollutants in surface waters is also predicted as an indirect effect of climate change which is expected to alter the hydrological regime of surface water bodies (European Commission, 2021). Furthermore, it should be noted that transient peaks of DCF concentrations exceeding 50 μg $L^{-1}$ may occur in surface waters located in the vicinity of pharmaceutical industries (Olaitan et al., 2014).

Toxic effects of DCF were detected for the first time by Oaks et al. (2004) on several populations of different vulture species in the Indian subcontinent. Then, DCF entered in the spotlight and its toxicity was studied in different organisms. With a few exceptions (Elazem and Abo-Kora, 2015; Abdel-Daim et al., 2018; Yurt et al., 2018; Adedara et al., 2020), most studies have been focused on aquatic organisms (Sathishkumar et al., 2020, and references therein). DCF toxicity experiments on aquatic standard test organisms (mostly algae, fish, crustacean daphnids) revealed different effects, such as DNA damage (Gómez-Oliván et al., 2014; Islas-Flores et al., 2017; Pandey et al., 2017), oxidative stress (Nieto et al., 2013; Stepanova et al., 2013; Oliveira et al., 2015; Guiloski et al., 2017), endocrine disruption (Gröner et al., 2015), morphological alterations (Lee et al., 2011; Chen et al., 2014; Ribeiro et al., 2015), histopathological damage (Schwarz et al., 2017; Derakhsh et al., 2020), behavioral modification (Ajima et al., 2015; Xia et al., 2017; Nkoom et al., 2019; Zhou et al., 2019), fertility reduction (Yokota et al., 2017), and adverse cardiovascular effects in mammalian and fish (Ghosh et al., 2016; Zhang et al., 2020).

DCF toxicity on freshwater invertebrate species living in the interstitial habitats (i.e., in the voids filled by water among sediment grains) of springs, rivers, lakes and groundwaters is still largely unexplored (Hernando et al., 2006; Nieto et al., 2017; Fu et al., 2020; Di Lorenzo et al., 2021), despite DCF persistence in interstitial habitats has been noted and attributed to low sorption properties under the conditions of slow sand filtration (Gröning et al., 2007). Moreover, microbial activity seemed not to be correlated with DCF depletion rate (Gröning et al., 2007), and this makes DCF particularly persistent in interstitial habitats and potentially toxic for the species with a holobenthic lifestyle (i.e., species that spend the whole life cycle among the same sediment particles). Copepoda (Crustacea) are one of the main components of the freshwater interstitial communities, with the primacy taken by the worm-like and small-sized order Harpacticoida (Galassi et al., 2009; Giere, 2009; Iannella et al., 2020). Freshwater harpacticoids also play an important role in providing ecosystem services like sediment bioturbation and carbon recycling (Schratzberger and Ingels, 2018), and several studies proved the suitability of the harpacticoid copepods for laboratory culture and tests (Bengtsson, 1978; Brown et al., 2005; Ward et al., 2011; Trombini et al., 2016; Guyon et al., 2018; Di Lorenzo et al., 2021). However, up to date, only a few studies have explored copepod response to DCF exposure, mainly concerning DNA methylation (Guyon et al., 2018), acute (Trombini et al., 2016; Castaño-Sánchez et al., 2021), and chronic toxicity (Di Lorenzo et al., 2021). Trombini et al. (2016) examined DCF toxicity in newly released nauplii (< 24-h old) of the marine harpacticoid copepod *Tisbe battagliai* and measured an LC50 value of 9.5 mg·$L^{-1}$ at 48 h. Castaño-Sánchez et al. (2021) assessed DCF toxicity in both copepodids and adults of the freshwater cyclopoid *Diacyclops crassicaudis crassicaudis* measuring LC50s of 62 and 103 mg $L^{-1}$ at 48 h, respectively. Finally, Di Lorenzo et al. (2021) measured an LC50 of 12 mg $L^{-}$1 at 96 h for the harpacticoid species *Nitocrella achaiae* and a NOEC (no-observed effect concentration) of 42 μg $L^{-1}$. Except for the study by Di Lorenzo et al. (2021), all the previous investigations concerned copepod survival at DCF exposures that were much higher than those found in the environment. The effects of sub-lethal environmentally relevant concentrations, which are hypothesized to affect species functional traits, such as swimming behavior, exploration aptitude and the ability to cope with impairment in scavenging of reactive oxygen species (ROS), had never been analyzed.

In this study, we investigated the short-term effect of a sub-lethal concentration of DCF on the harpacticoid copepod *B. pygmaeus* (Sars, 1863), an interstitial non-target species that is widely distributed in Europe (Dussart and Defaye, 1990). This harpacticoid species is ubiquitous in the interstitial environments of freshwater bodies (Fattorini et al., 2020), in springbed sediments, lakes, groundwater habitats, in the saturated and saturated alluvial and karst aquifers, and the hyporheic zone of streams and rivers (Di Lorenzo et al., 2013), with a typical holobenthic lifestyle like most harpacticoid copepods found in freshwaters. This species is a burrower, and lives preferentially in sandy-gravel sediments. It is a detritus feeder, eating on fine particulate organic matter and the microbial biofilm covering the sediments, and occasionally on algae. All these attributes make *B. pygmaeus* a good candidate for this type of study in the interstitial environment.

We aimed at analyzing changes in the species behavior by swimming video tracking and the potential biochemical mechanisms induced by DCF on the oxidative enzyme system of the species by using two common oxidative stress biomarkers: catalase (CAT) and the

mitochondrial superoxide dismutase 2 (SOD2). This integrated approach will give new insights into the effects of EOCs by analyzing for the first time freshwater invertebrate fauna living in sediments.

## 2. Material and methods

### 2.1. Experimental design and exposure

Diclofenac sodium salt ($C_{14}H_{10}Cl_2NNaO_2$; CAS 15307-79-6) was purchased from Sigma-Aldrich (Steinheim, Germany). Remineralized MILLIPORE® MILLI-Q® deionized water was used as dilution water with the following physico-chemical composition (pH = 7.6, electrical conductivity = 234 μS $cm^{-1}$, total hardness = 90.71 mg $L^{-1}$, $CaSO_4$ $2H_2O$ = 60 mg $L^{-1}$, $MgSO_4$ = 60 mg $L^{-1}$, $NaHCO_3$ = 96 mg $L^{-1}$, KCl = 4 mg $L^{-1}$; Di Lorenzo et al., 2021). A stock solution of DCF equal to 50 mg $L^{-1}$ was prepared by dissolving 50 mg of salt in 1 L of reconstituted water; scalar dilutions 100 ×, 10 ×, and 1 × were prepared to obtain the nominal concentration of 50 μg $L^{-1}$ DFC, 10 min prior to the sub-lethal exposure test. The concentration of 50 μg $L^{-1}$ is in the range of the DCF environmental measured concentrations in freshwaters worldwide and was chosen also based on the NOEC (no-observed effect concentration) of DFC for the groundwater interstitial harpacticoid species *Nitocrella achaiae* (NOEC = 42 μg $L^{-1}$; Di Lorenzo et al., 2021).

Two hundred adult individuals of *B. pygmaeus* were collected at the Vera Spring (coordinates: 42°22′21.42″N, 13°27′30.51″E; altitude 664 m asl) that is one of the main basal springs of the Gran Sasso karst aquifer (Central Italy). The site is known to be pristine and characterized by stable low temperature (~8 °C), stable discharge (~0.3 $m^3$ $s^{-1}$) and low concentration of nutrients throughout the hydrological year (Di Sabatino et al., 2021). To avoid impacting the local abundances of *B. pygmaeus*, the batch of organisms used in the behavioral tests and in biochemical analyses was obtained through monthly samplings carried out from September 2020 to November 2020. Specimens were collected with a hand net (mesh size: 60 μm) by gently moving the first 20 cm sediments of the springbed, and transported to the laboratory (University of L'Aquila, Central Italy) in a 1 L-plastic beaker filled with the spring water. In the laboratory, the individuals of *B. pygmaeus* were rapidly sorted under a stereomicroscope (Leica M205) at 12× magnification. Two hundred adult individuals of *B. pygmaeus* were distributed in 10 Petri dishes, each containing 20 individuals in 20 mL filtered spring water (culture medium) and fine sterile quartz sediment (0.1 g, φ = 0.25 mm) (Mapei S.p.A®, Milan, Italy) which is chemically inert in contact with most substances. The use of quartz sediments was considered appropriate for rearing *B. pygmaeus* because it is mainly an interstitial/burrower species. The ten vials were kept in a thermostatic cabinet (Velp Scientifica™ FOC 120E Cooled Incubator) at 8 ± 0.2 °C, in permanent darkness until December 2020. The dark condition was required because, during sorting under the stereomicroscope, *B. pygmaeus* showed an escape behavior (negative phototaxis, as also observed in other harpacticoid species; Ma and Johnson, 2017), finding rapidly refuge under the sediment grains. Culture medium was renewed once a week, and the individuals were fed on the organic matter present in the water collected from the sampling site (46.75 g $L^{-1}$ ± 15.12 g $L^{-1}$); before testing, a gradual transition to the dilution water was accomplished in 48 h following the procedure adopted by Di Lorenzo et al. (2019a, 2019b). Particulate organic matter (POM) was estimated as loss on ignition of six water samples taken from the sampling site. After removal of the fauna, samples were dehydrated at 105 °C (24 h) and weighted. The dry-weighted samples were burnt at 540 °C (4 h) in a muffle furnace and re-weighted to determine POM amount as the difference between dry and ash mass (Fischer et al., 2002).

A total of 80 adult individuals of *B. pygmaeus* (female length = 0.62 mm ± 0.07 mm; female width = 0.14 mm ± 0.02 mm; male length = 0.55 mm ± 0.07 mm; male width = 0.12 mm ± 0.02 mm), randomly chosen, were used for the tests. To avoid unbalanced exposure times between the first and the last recorded individuals, we performed the experiments in four independent runs within 21 days. The difference in age (of max 21 days) among the adult individuals of the four runs was considered negligible as the adult individuals of *B. pygmaeus* live for over 4 months in captivity (personal observation). In each run, 10 individuals were withdrawn and randomly assigned to the control group while other 10 were randomly assigned to the treatment group. We made sure that the sex ratio was close to 1 within each group. The individuals of the treatment group were exposed to a nominal concentration of 50 μg $L^{-1}$ DCF for 72 h in a 5 cm-diameter Petri dish containing 0.1 g of fine sterile quartz and 10 mL of test solution. The individuals of the control group were kept for 72 h in a 5 cm diameter Petri dish containing 0.1 g of fine sterile quartz and 10 mL of dilution water. Both the treatment and the control individuals were kept in permanent darkness at 8.0 ± 0.2 °C. At the end of the exposure, the individuals were individually transferred into transparent circular arenas (diameter = 1.5 cm, height = 0.1 cm), each filled with 150 μL of either test solution or dilution water and without sediments. After loading, the arenas were placed in the thermostatic cabinet for 60 min to rebalance the temperature. The temperature rebalancing time has been estimated in previous experiments (Di Lorenzo and Galassi, 2017; Di Lorenzo et al., 2019a).

### 2.2. Swimming behavior

The animals were individually recorded in two dimensions for 5 min with a 30-fps (frame per second) infrared sensitive digital camera (IDS camera, Loligo Systems®) mounted on the Leica M205 stereomicroscope. The animal trajectories were recorded in infrared (IR) light to avoid DCF photodegradation (Agüera et al., 2005) and the interference of visible light on *B. pygmaeus* individuals. IR illumination (wavelength = 850 nm) was supplied by a 470 mm × 210 mm × 20 mm panel (Loligo Systems®, Viborg, Denmark) placed beneath the arenas. In the process, water temperature was kept stable with a lab-made coil cooling system placed beside the arena. Temperature was monitored by a thermometer and maintained close to that measured in the sampling site. Trajectory videos were live recorded at 8 × magnification, using the uEye Cockpit software (IDS Imaging Development Systems GmbH®, Obersulm, Germany) and saved on a Solid-State Disc (SSD).

Digital videos were analyzed in LoliTrack 5 (Loligo Systems®, Viborg, Denmark) and frame-by-frame animals' centroid information (x, y, t) was exported as .xlsx files. Overall, 9000 frames were recorded for each individual. The .xlsx outputs were converted to .csv and imported in R Studio ver. 4.0.3 (R Development Core Team, 2003) to calculate trajectory parameters using the "trajr" package (McLean and Skowron Volponi, 2018). The trajectory digitalization phase tends to produce trajectories that suffer from noise. LoliTrack 5 perceives the animal as a cluster of points for which the centroid position is calculated frame-by-frame. Nevertheless, the cluster shape tends to slightly change due to the animal bending (e.g., slightly rotation along body axes) which generates changes in the centroid position that are basically false positives. For this reason, each trajectory was smoothed using a cubic spline interpolation before calculation of the trajectory parameters.

The trajectory parameters in this study were:

i) Average Speed (AS) calculated as in Eq. (1):

$$AS = \left(\sum_{i=1}^{n} sp_i\right) * n^{-1} \quad (1)$$

where $sp_i$ is the instantaneous speed expressed in mm $s^{-1}$ of the $i$-th pair of frames; $n$ is the total number of pairs. For each frame $sp_i$ is calculated as:

$$sp_i = \frac{\sqrt{(x_i - x_{i-1})^2 + (y_i - y_{i-1})^2}}{t}$$

where $x_i$ and $y_i$ are the spatial coordinates of the animal centroid at the frame $i$; $x_{i-1}$ and $y_{i-1}$ are the spatial coordinates of the animal centroid at the frame $i$-1; $t$ is the elapsed time between two frames (1/30 s in this study).

ii) Average Turning Angles (ATA) calculated as in Eq. (2):

$$ATA = \left(\sum_{i=1}^{n} a_i\right) * n^{-1} \quad (2)$$

where $a_i$ is the instantaneous turning angle associated with the step length of the $i$-th frame calculated in radians; $n$ is the total number of frames.

iii) Percentage of Activity (PA) calculated as in Eq. (3):

$$PA = \frac{af}{n} \quad (3)$$

where $af$ is the sum of the frames of animal activity. The animal was considered active when its instantaneous speed in the $i$-th frame was ≥0.15 mm $s^{-1}$ after a preliminary assessment of the minimum speed below which the sole movement of the cephalic or swimming appendages of an inactive animal is read by the software as active movement.

iv) Trajectory Convex Hull (CH) defined as the minimum area of the convex polygon containing all the coordinates of the trajectory expressed in $mm^2$. This measure was used as a proxy to infer *B. pygmaeus* habitat exploitation ability.

v) Inner Swimming Time (IST) expressed as the total time (in seconds) during which the animal movement was not influenced by the arena boundaries.

The swimming behavior was considered boundary-unconstrained when the animal was at a distance equal to 1.5 its body length from the arena boundary for more than 2 s. The spatial threshold was set following Schnörr et al. (2012) who studied the thigmotactic alteration in zebrafish embryos. The temporal limit was chosen in order to avoid erroneous conclusion related to the insufficient locomotion activity of the animal (Bouwknecht and Paylor, 2008). Following this criterion, for each individual trajectory, different numbers of unconstrained trajectory portions were exported (hereafter referred as Inner Swimming Clips, ISC) and counted; the IST accounted for the sum of the duration (in seconds) of each ISC. ISC durations lower than two seconds were discarded since the trajectory coordinates were insufficient to compute tortuosity indexes (see Eqs. (4) and (5)).

vi) Weighted Average Sinuosity (WAS) expressed as rad $mm^{-1/2}$ and calculated as in Eq. (4):

$$WAS = \frac{\sum_{i=1}^{N}(S_i * \Delta t_i)}{IST} \quad (4)$$

where $S_i$ is the sinuosity index defined by Bovet and Benhamou (1988) as a measure of the tortuosity of a random path, calculated for the $i$-th ISC expressed as rad $mm^{-1/2}$; $\Delta t_i$ is the time length of the $i$-th ISC expressed in seconds; $N$ is the total number of ISCs; IST is the sum of the duration of each ISC time. $S_i$ is calculated by multiplying 1.18 times the standard deviation of the turning angles divided by the square root of the mean step length in mm of the trajectory in the $i$-th ISC. Before calculation we checked the non-violation of the boundary conditions necessary for the $S_i$ computation (Benhamou, 2004).

vii) Weighted Average Fractal Dimension (WAFD) calculated as in Eq. (5):

$$WAFD = \frac{\sum_{i=1}^{N}(FD_i * \Delta t_i)}{IST} \quad (5)$$

where $FD_i$ is the Fractal Dimension value (Schmitt and Seuront, 2001; Nams, 2006; Uttieri et al., 2008a, 2008b, 2021; Seuront, 2009), which is a measure of tortuosity calculated for the $i$-th ISC; $\Delta t_i$ is the time length of the $i$-th ISC expressed in seconds; $N$ is the total number of the ISC; IST is sum of each ISC time. $FD_i$ is determined by rediscretizing the trajectory for each of several step lengths and plotting in a log - log plot the resulting path length as function of the step lengths used for each rediscretization (Dicke and Burrough, 1988). Since the FD and the S are different approaches to measure path tortuosity, we considered appropriate to exclude the trajectory portions in which the path shape was "forced" by the presence of the boundary (Uttieri et al., 2008a, 2008b, 2021).

As both FD and S refer to similar aspects of the motion, we compute both to assess path tortuosity of *B. pygmaeus*. This choice was made because some authors highly encourage the use of the FD (e.g., Seuront, 2009; Uttieri et al., 2008a, 2008b, 2021) while others criticize the use of the FD because of the non-fractal nature of the animals' trajectory (Turchin, 1996; Benhamou, 2004; Halley et al., 2004). Since, up to date, we do not know if an unequivocal tortuosity index conforms the motion of *B. pygmaeus*, we preferred to use both indices and discuss their outcomes separately.

The rationale of the ISC selection and IST, WAS and WAFD calculations is presented in Fig. 1.

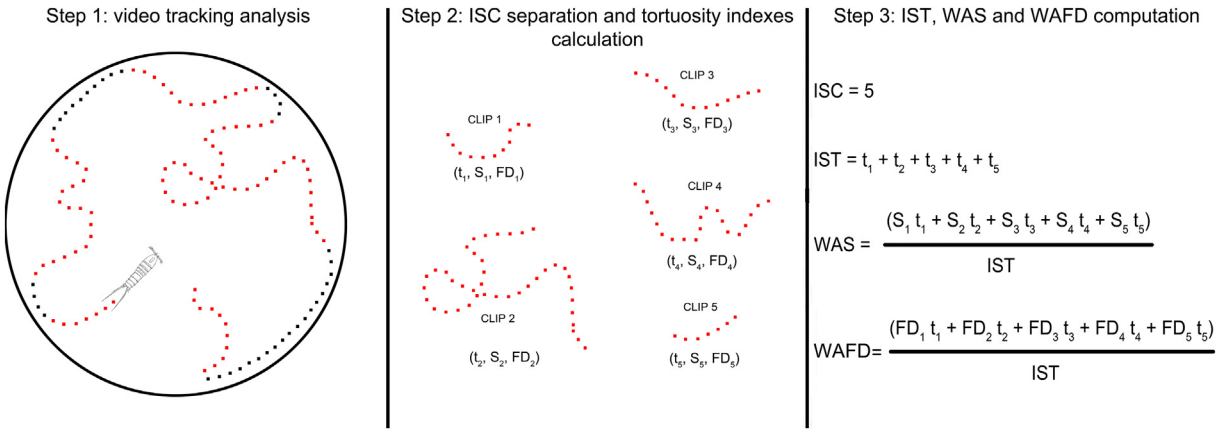


**Fig. 1.** Rationale of the steps required for calculating ISC (= Inner Swimming Clips), IST (= Inner Swimming Time), WAS (= Weighted Average Sinuosity), and WAFD (= Weighted Average Fractal Dimension). Red dotted lines indicate the portions of the animal trajectory not influenced by the arena boundary (unconstrained trajectory portions). For each portion, time span (t), sinuosity (S) and fractal dimension (FD) were calculated. Animal and arena dimensions are not in scale. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 2.3. Chemical analysis

At the end of each run, the DCF concentration from the test vials was analyzed by High Performance Liquid Chromatography (HPLC). HPLC analysis was carried out using a chromatographic system (Waters Corporation, Milford, MA) consisting of a Waters 600 pump with controller module, a 717 Plus auto-sampler, and a 996-photodiode array detector. The HPLC column was a Synergy Fusion (Phenomenex, Torrance, CA, USA) 250 × 4.6 mm, 4 μm particle size. The mobile phase consisted of a mixture 50:50 (V:V) of acetonitrile and phosphoric acid 0.1% in MILLIPORE® MILLI-Q® deionized water. The samples were mixed on a vortex agitator filtered using HPLC filters Whatman Spartan13/02 RC with pore size of 0.22 μm, and 20 μL were injected into the HPLC system. Photometric detection was performed in the range 210–400 nm, the chromatographic peak was monitored at 211 nm. The calibration curve of DCF was obtained by five replicates of five standard samples in the concentration range 0.03–1.0 μg $mL^{-1}$ by appropriate dilution of the working standard solutions with acetonitrile. The determination coefficient ($r^2$) of the calibration curve was 0.999. Limit of detection (LOD) and limit of quantitation (LOQ) were identified as the lowest analyte concentrations giving peaks whose signal to noise ratio is 3 and 10, respectively. In this study, the LOD and LOQ were obtained from the calibration curves according to the relationships LOD = 3.3 × $S_{x/y}$/b and LOQ = 10 × $S_{x/y}$/b where $S_{x/y}$ is the standard deviation of the response and b is the slope. LOD was 0.010 μg $mL^{-1}$ and LOQ 0.035 μg $mL^{-1}$.

### 2.4. Biomarker response

For Western blot analysis, we performed three independent experimental replicates per controls and treatments on 120 adult individuals of *B. pygmaeus* reared at the same time and under the same conditions set for the individuals used for the video tracking. Control and treatment individuals at the end of 72 h exposure were transferred in 1.5 mL Eppendorf tubes, and stored immediately at -80 °C before the biochemical analyses. Forty individuals for each of the three independent controls and three independent treatments, respectively were pooled for each experimental replicate in 50 mL of lysis buffer consisting of 50 mM Tris (Chem Cruz Santa Cruz Biotechnology, Inc., Dallas, TX, USA), 150 mM NaCl (sc-203274A ChemCruz), 1% Triton (Sigma-Aldrich Milano, Italy), 2% SDS (sc-264510B Chem Cruz Santa Cruz), 1 mM PMSF (Sigma-Aldrich). Then they were sonicated in an ultrasonic bath at 40KHz for three cycles (10 s on-10 s off) and centrifuged at +4 °C at 15,000 rpm for 30 min. The supernatant was recovered, and the protein assay was performed with the Qubit™ Protein Assay Kit (Thermo Fisher Scientific, DE, USA). SDS-PAGE was performed running samples on 10% polyacrylamide denaturing gels. Proteins were transferred on polyvinylidene fluoride (PVDF) sheets (Sigma-Aldrich) by wet electrophoretic transfer, and 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 (Sigma-Aldrich) (TBS-T) was used to block non-specific binding sites for 1 h at room temperature (RT). Membranes were incubated overnight at 4 °C with primary antibodies anti-Mn Sod (ADI-SOD-110 Stressgen bioreagent Victoria, British Columbia, Canada) 1:250 and anti-Catalase (100-4151 Rockland antibodies & assays Limerick, PA, USA) 1:250. After this step, membranes were incubated with an anti-rabbit secondary antibody diluted in blocking solution for 1 h at room temperature. Protein bands were visualized and acquired with Alliance 7 instrument (Uvitec Limited, Cambridge, UK). Actin (MAB-24008 Immunological Science Rome Italy) was used as housekeeping protein for densitometry using ImageJ software, and values were refereed as relative units (R.U.).

### 2.5. Statistical analysis

Data from the four runs were cumulated since the differences in the DFC concentrations were considered negligible (see paragraph 3.1); before testing the differences between Factor 1 (Sex) and Factor 2 (Condition), normality and homoscedasticity of each variable were tested by Shapiro-Wilk test (α = 0.05) and Bartlett test (α = 0.05). When at least one of the two null hypotheses was rejected, we attempted to achieve parametric conditions through data transformation. Data were transformed applying a hierarchical criterion from the less to the most powerful transformation in the following order: $x^{1/2} > x^{1/4} > \text{Log}(x + 1)$. For the PA data (percentages) the arccosine transformation was applied. We tested differences in behavioral parameters using a two-way permutational analysis of variance (two-way PERMANOVA; Anderson, 2001; Anderson et al., 2008) with the following setting: α = 0.05, permutations = 9999, distance measure = Euclidean, Factor 1 = Sex, Factor 2 = Condition, Type III SS and permutation of residuals under a reduced model.

The effects of DCF exposure on SOD2 and CAT levels between control and treatment individuals were tested with an unpaired two-tailed *t*-test (α = 0.05).

Statistical analysis and graphic outputs were made in R ver. 4.0.3 (R Development Core Team, 2003).

## 3. Results

### 3.1. Swimming behavior

The difference among the DCF concentrations of each run was low (max ±2 μg $L^{-1}$; S_Table 1). The results of the behavioral tests are presented in Table 1. Significant differences emerged only for the Factor 2 (Condition) while no significant differences were highlighted for the Factor 1 (Sex) or the interaction of the two factors (Supplemental File_STable_PERMANOVA), except for PA for which significant differences were obtained for both Factor 1 and Factor 2. DCF significantly affected six out of eight behavior parameters analyzed in this study in both males and females (AS, PA, CH, ISC, WAS, WAFD). No statistical significance was observed for ATA, the values of which were close to 0 rad for both the control and treatment individuals, indicating that no preferential direction was chosen by the individuals during the tests. DCF-exposed individuals swam slower and were less active than the control individuals. In particular, males and females swam respectively

**Table 1**
Summary (mean ± SD) of trajectory parameters (TP) in the control males (CM), control females (CF), treatment males (TM) and treatment females (TF) and *p*-values of the PERMANOVA tests. ISC: Inner Swimming Clips (number of); AS = average speed (mm $s^{-1}$); ATA = Average Turning Angle (rad); PA = Percentage of Activity (%); CH = Trajectory Convex Hull ($mm^2$); IST = Inner Swimming Time (*sec*); ISC = Inner Swimming Clips (number of); WAS = Weighted Average Sinuosity (rad $mm^{-1/2}$); WAFD = Weighted Average Fractal Dimension. Transformations are superscripts on the variables: S = square root; A = arccosine. p(F1) = p-values for the Factor 1 (Sex); p(F2) = p-values for the Factor 2 (Condition); p(F1 x F2) = p-values for the interaction of the two factors. Statistically significant results are highlighted in bold.

| TP | CM | CF | TM | TF | P(F1) | P(F2) | P(F1xF2) |
|---|---|---|---|---|---|---|---|
| $AS^S$ | 0.73 ± 0.23 | 0.65 ± 0.32 | 0.52 ± 0.28 | 0.43 ± 0.19 | 0.082 | **0.003** | 0.869 |
| ATA | 4e-04 ± 5e-03 | 9e-05 ± 5e-03 | 3e-04 ± 5e-03 | 1e-03 ± 6e-03 | 0.452 | 0.602 | 0.681 |
| $PA^A$ | 80.93 ± 14.16 | 70.26 ± 19.89 | 65.41 ± 20.93 | 62.39 ± 14.65 | **0.032** | **0.002** | 0.385 |
| CH | 91.71 ± 37.17 | 99.72 ± 24.34 | 84.81 ± 28.98 | 77.37 ± 35.26 | 0.919 | **0.04** | 0.318 |
| IST | 102.25 ± 42.19 | 82.49 ± 44.75 | 90.04 ± 32.07 | 107.48 ± 52.02 | 0.89 | 0.559 | 0.118 |
| ISC | 10.05 ± 3.87 | 9.25 ± 5.18 | 7.06 ± 2.74 | 6.95 ± 3.72 | 0.418 | **0.004** | 0.715 |
| $WAS^S$ | 3.80 ± 0.89 | 3.95 ± 1.28 | 4.98 ± 1.78 | 4.97 ± 0.98 | 0.194 | **0.001** | 0.589 |
| WAFD | 1.03 ± 0.03 | 1.01 ± 0.01 | 1.055 ± 0.05 | 1.05 ± 0.03 | 0.709 | **0.019** | 0.625 |

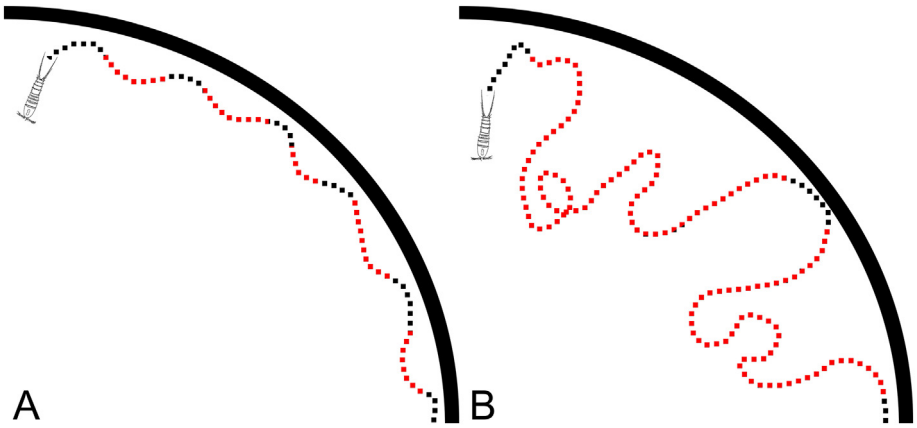


**Fig. 2.** Graphical representation of DCF effect on the harpacticoid copepod *B. pygmaeus*. (A) Control individuals swim closer to the arena boundary and ISCs (red dotted lines) display straighter trajectory; (B) DCF-exposed individuals reorient their bodies towards the boundary less efficiently, increasing the path tortuosity of the ISCs. Note that the total number of ISCs is higher in the control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

0.21 mm $s^{-1}$ and 0.22 mm $s^{-1}$ slower than the control individuals and were 15.52% and 7.87% less active. DCF exposure also affected the exploratory behavior of the individuals since the CH values were on average 6.9 $mm^2$ lower for the males and 22.35 $mm^2$ lower for the females. The statistical tests highlighted significant differences for the ISC values. For the control males it was possible to isolate on average 2.99 more ISC, while for the control females 2.30 more ISC were isolated. Nevertheless, control and treatment individuals spent an equal amount of swimming time far from the arena boundary since no significant differences emerged for the IST (Table 1). The test outcomes of ISC and IST highlighted that DCF-exposed individuals tended to reorient their swimming towards the arena boundary less frequently than the controls independently from the sex. The results obtained for the tortuosity indexes WAS and WAFD were consistent. Both parameters indicated that DCF significantly increased the trajectory tortuosity when the animals swam towards the arena inner zone. DCF exposure increased by 1.18 rad $mm^{1/2}$ the WAS values in the males and by 1.02 rad $mm^{1/2}$ in the females. With regards to the WAFD, the males exhibited on average values greater by 0.025 units while the females had values in average higher by 0.04 units with respect to the controls (Fig. 2) (Supplemental File_STable Controls, _STable Treatments, Supplemental File_Trajplots).

### 3.2. Biomarker analysis

Western blot analysis of SOD2 (MnSOD) and CAT are shown in Fig. 3. SOD2 showed a value of 1.43 ± 0.55 R.U. ($n = 3$) in the control individuals of *B. pygmaeus*, while a significant decrease of the protein levels was observed in the treated individuals with a mean value of 0.55 ± 0.35 R.U. ($n = 3$) ($p = 0.0439$). For CAT, a different trend was observed, because no significant differences were observed in control and treatment individuals ($p = 0.7101$), as the measured values were almost comparable between control and treatment individuals, with mean values of 1.05 ± 0.25 R.U. ($n = 3$) and 0.97 ± 0.25 R.U. ($n = 3$) for control and treatment individuals, respectively.

## 4. Discussion

### 4.1. Swimming behavior

Studies on the alteration of the swimming behavior in freshwater invertebrates are scanty, if we exclude those performed on the planktonic model species belonging to the genus *Daphnia* (Bownik, 2017, and references therein). For interstitial small-sized freshwater invertebrates, a few contributions are available from literature; however, changes in swimming behavior were observed by visual analyses (Boyd et al., 2002; Pallarés et al., 2020), not always tracking the individuals. Recently, one study tracked the swimming behavior of a freshwater obligate groundwater-dweller cyclopoid copepod *Diacyclops belgicus* under propranolol exposure (Di Lorenzo et al., 2019a), however this species is not strictly interstitial, being able to live either in free open water, close to the sediments (hyperbenthic), or in the interstitial environment.

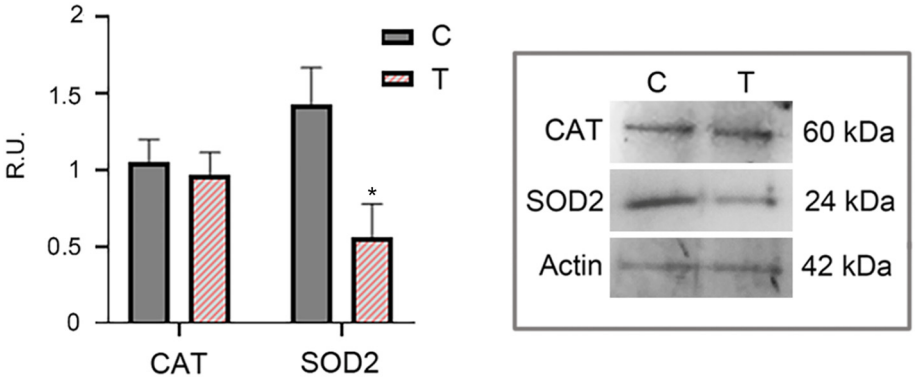


**Fig. 3.** Western blot analysis of SOD2 and CAT proteins after 72 h treatment with 50 μg $L^{-1}$ DCF. A significant down-regulation of SOD2 ($p$-value = 0.0439; * indicates significance) was observed while no significant differences in regulation of CAT protein levels were observed (p-value = 0.7101). Data are given as mean ± SEM of three independent replicates (40 individuals for each condition in three independent replicates).

To the best of our knowledge, our results are the first evidence of behavioral alterations detected by swimming tracking of a freshwater interstitial copepod species. The short-term exposure to the sub-lethal DCF concentration of 50 μg $L^{-1}$ negatively affected the swimming behavior of *B. pygmaeus* in various respects. Interestingly, while the average speed (AS) revealed no significant differences between males and females, the percentage activity (PA) was significantly higher in males than in females. This observation would suggest that females make a greater number of stops in their path, but that in the movement phase they have greater propulsion and move faster in both controls and treatments.

Our results showed that *B. pygmaeus* swims slower after being exposed to DCF, also reducing its activity and exploration ability. Swimming speed decline in relation to DCF exposure has been already studied in different aquatic species and our results are in line with studies performed on amphibian larval stages. Peltzer et al. (2019) observed significant decreasing swimming speed and lower global activity of the species *Trachycephalus typhonius* and *Physalaemus albonatus* when exposed, for 96 h, to 125, 250, and 500 μg $L^{-1}$ DCF, while higher swimming speed and global activity were observed by the same authors for concentrations >1000 μg $L^{-1}$. A reduced swimming speed was also observed by Melvin (2016) for *Limnodynastes peronii* tadpoles after being exposed, for 30 days, to DCF nominal concentrations of 0.1, 1.0, 10, 100 and 1000 μg $L^{-1}$. Swimming speed is a crucial trait for aquatic animals, crustacean copepods included, since their fitness in terms of survival probability, reproductive success, feeding, predators' avoidance and habitat exploitation strictly depend on the ability to swim. In general, feeding success depends on four chronological steps: i) encounter with prey or food particles; ii) prey or food particle recognition; iii) prey or food particle capture; iv) prey or food particle ingestion. The same chronological step model is applicable when considering encounter probability with conspecifics for mating. While the second, third and fourth step listed above depend on the animal mechanoreception and chemoreception, the first step is strictly related to the copepod swimming speed. In an encounter probability model proposed by Gerritsen and Strickler (1977), the authors demonstrated that the encounter rate with a prey, or a conspecific, is remarkably linked to the average swimming speed of generic zooplankters, copepods included, moving in a random way. They highlighted that even a slightly decrease in swimming speed can highly reduce the encounter rate with food resources or potential mates.

The success of a productive encounter (e.g., feed ingestion or mating) and the avoidance of a detrimental one (e.g., predator or unfavorable environmental conditions) do not only rely on swimming speed. A fundamental role is also played by chemoreception and mechanoreception. Chemoreception and mechanoreception together shape the so called "encounter radius" of the copepods (Strickler and Twombly, 1975; Gerritsen and Strickler, 1977; Buskey, 1998; Uttieri et al., 2008a, 2008b; Sabia et al., 2014). The encounter radius determines copepod surrounding volume in which the individual can perceive the mechanical and chemical stimuli that come from the surrounding environment. Regarding mechanoreception, our findings highlighted that DCF affected both thigmotactic and hydrodynamic responses of *B. pygmaeus*. Control individuals tended to stay closer to the arena boundary most of the time and when they detached from it, they reoriented their swimming direction quickly towards the arena walls. This behavior is the reflection of the strong thigmotaxis displayed by *B. pygmaeus* and most crustacean harpacticoids because conferring success in adhering to sediment grains and pushing against them for enhancing locomotion in the darkness, and it is not related to anxiolytic-like behavior as inferred for other invertebrates (Kohler et al., 2018). DCF-exposed individuals also showed the same tendency of the controls and tried to swim closer to the arena boundary; however, when they moved far from the boundary, they reoriented towards the borders less efficiently, drawing more complex and winding paths. The increased path tortuosity exhibited by the treated copepods when they were far from the arena borders could be explained by supposing an alteration of their mechanoreception. A good mechanical response is crucial for holobenthic species, like *B. pygmaeus*, because their habitats are characterized by the presence of sediments of different types, size, and shape, and the intensity of light is reduced or absent. It is, therefore, obvious that relying only on chemoreception and photoreception could not be sufficient to assure copepod survival and fitness in benthic interstitial environments. Copepods encode mechanical stimuli through sensory setae on their antennules. When these setae are bended and deflected by the fluid pressure or a touch stimulus, a neural cell reaction is triggered along their cephalic appendages. Thus, the rate and the angle of the deflection provide the animal with information about the speed and the volume of the fluid movement (Fields and Yen, 2002, and references therein). An adequate copepod response to a mechanical stimulus could be achieved only if three fundamental conditions are met (Fields and Weissburg, 2004): i) the electric impulse of the neural cells must travel from the receptor to the target region, ii) the neural impulse must contain sufficient information in order to recognize different kinds of stimuli, iii) the coming information has to provide the spatial location of the stimulus. It could be possible that sub-lethal DCF exposure interferes with one or more of these three steps.

### 4.2. Biomarker response

Oxidative stress can arise in several ways: (i) increased ROS production, (ii) decreased ROS elimination, and (iii) a mismatched combination of production versus elimination. For SOD2, a decrease in protein levels was observed in DCF-exposed individuals of *B. pygmaeus*, indicating an impairment of mitochondrial oxidative defense, likely via a mismatch of ROS production and elimination, as observed in other invertebrate and vertebrate species living in different habitats. The SODs are involved in mitochondrial superoxide anion detoxification, a major mitochondrial ROS. Moreover, we did not observe changes in CAT protein levels, likely because lower $H_2O_2$ amount is produced by mitochondrial SOD2 and because CAT is mainly a cytosolic enzyme, not involved in mitochondrial scavenger activity. DCF increases the production of reactive oxygen species (ROS) in the crustacean cladoceran *Daphnia magna* along with a decrease in superoxide dismutase forms (SODs) when the species is exposed to concentration ranging from 5 to 100 μg $L^{-1}$ (Nkoom et al., 2019). Similar results were obtained for fish, such as *C. carpio* and *Danio rerio* (Islas-Flores et al., 2017; Bio and Nunes, 2020). Superoxide dismutase 2 (SOD2) is an antioxidant mitochondrial enzyme which plays a crucial role in maintaining mitochondrial ROS balance. This enzyme catalyzes the dismutation of the superoxide anion ($O_2^-$) in hydrogen peroxide ($H_2O_2$), a less reactive form of oxygen. The decrease of SOD activity is responsible for increased $O_2^-$ levels and further oxidative stress able to damage mitochondria and cellular structures and molecules. The hydrogen peroxide is detoxified by the catalase to $H_2O$ and $O_2$. The optimal cell protection is achieved only when an appropriate balance among these enzymes is maintained. The reduced activity of SOD2 in individuals exposed to DCF could be explained by a decline in mitochondrial function or in a reduced number of functional mitochondria. These findings can explain the changes we have observed in the swimming behavior of the copepod *B. pygmaeus* (see paragraph 4.1). Since mitochondrial dysfunction determines ATP reduction, it comes that less energy is produced for maintaining the cell functions of the DCF-exposed individuals. In addition, the increasing energy demand for the detoxification process further contributes to decrease the total energetic budget allocated for other physiological activities. In our study, the observed decrease in speed and activity could be explained by the fact that less energy is stored for the swimming of *B. pygmaeus*. With regards to the altered mechanoreception, it could be possible that DCF also has neurotoxic effects on *B. pygmaeus*, which means that DCF could inhibit the neurotransmission of the mechanical stimuli coming from the surrounding environment to the animal. In this study we did not explore the neurotoxicity of DCF at the cellular

level, nonetheless Oliveira et al. (2015) observed a decreased level of the acetylcholinesterase enzyme (AChE) in *Daphnia magna* when the species undergoes long-term exposure to environmental relevant DCF concentrations. Further, Gonzalez-Rey and Bebianno (2014) in a study on the effect of DCF in the mussel *Mytilus galloprovincialis* advanced the hypothesis that the inhibition of the AChE activity could be modulated by the decreased SOD activity. Indeed, previous studies have found a positive correlation between AChE activity and locomotion behavior parameters in invertebrates (Engenheiro et al., 2005; Xuereb et al., 2009).

## 5. Conclusion

The coupling between behavioral and biochemical effects of pharmaceutical compounds in aquatic animals is still poorly investigated and this study represents the first evidence in a species of interstitial copepods. The results obtained from biomarker analysis seem to explain the behavioral alterations observed in *B. pygmaeus*, but further studies are needed to obtain a clear understanding of the neurophysiology of this species in response to DCF exposure. The results of this study reinforce the evidence that environmentally relevant concentrations ($\mu g\ L^{-1}$) of pharmaceutical compounds, if not leading aquatic organisms to death, can cause an alteration of biochemical processes that, in turn, trigger an energetic imbalance to the detriment of the behavior of aquatic species. This phenomenon can generate effects not only on individual populations, due to the reduction of fitness in the food search and mating, but also at the community level (through alterations of the interactions associated with predation or competition) that may further cascade to disrupt ecosystem function and services. The video tracking method used in this study can analyze multiple individuals at a time and therefore, future studies should be aimed at examining the effect of sub-lethal concentrations of pharmaceutical compounds at the population and community levels.

## CRediT authorship contribution statement

Mattia Di Cicco: Methodology, Experiments, Conceptualization, Writing-Review & Editing.

Tiziana Di Lorenzo: Methodology, Conceptualization, Writing-Review.

Barbara Fiasca: Experiments, Methodology.

Fabrizio Ruggieri: Chemical Methodology, Experiments, Writing-Review of the related section.

Annamaria Cimini: Biochemical methodology, Review of the related section, *Co*-funding acquisition.

Gloria Panella: Biochemical methodology, Experiments.

Elisabetta Benedetti: Biochemical methodology, Experiments, Writing-Review of the related section.

Diana M. P. Galassi: Conceptualization, Writing-Review & Editing, Project administration, Funding acquisition.

## Data availability statement

All the data supporting this article are made available in the main text and supplementary material.

## 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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.149461.

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