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Effects of diclofenac on the gametes and embryonic development of *Arbacia lixula*

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Abstract

Diclofenac (DCF), a non-steroidal anti-inflammatory drug (NSAID), is among the main pollutants of aquatic environments. Nevertheless, even if several authors evaluated its effects on marine organisms, no work has ever analysed its impact on the sea urchin *Arbacia lixula*. The purpose of this study was to analyse, for the first time, the impact of DCF at different concentrations (50 mg/l, 5 mg/l, 0.5 mg/l, 0.05 mg/l) on *A. lixula* embryos and gametes to indicate possible safe minimum levels of release of this drug in marine environments. Our results showed significant concentration-dependent effects on embryonic development (decrease in the number of developed embryos and increase in the quantity of degenerated eggs) and significant levels of bioaccumulation at DCF concentrations of 50 mg/l in both gametes and embryos. DCF showed a significant impact on the fertilisation of *A. lixula* due to its effects on gametes, confirming the possibility of environmental risk and highlighting the need to improve wastewater treatment and drug disposal processes.

Keywords: Anti-inflammatory, developed embryos, drugs, marine pollution, sea urchin

Introduction

To date, pharmaceuticals compounds (PhACs) released in marine environment (e.g. antibiotic, anti-inflammatory, antidepressants) are a threat for marine ecosystems (Branchet et al. 2021; Mezzelani & Regoli 2022). Among 70%–80% of this pollution is caused by humans, who expel PhACs as active metabolites via urine or faeces after ingestion (Zuccato et al. 2000). The remaining 20%–30% is caused by illegal industrial disposal (Heberer 2002; Bonnefille et al. 2018). Prior to its release into the aquatic environment, the waste passes through purification plants which are not yet efficient (Ojemaye & Petrik 2019; Angeles et al. 2020) at removing drug traces in surface water, ground water, and sediments (Zuccato et al. 2000; Farré et al. 2001; Bonnefille et al. 2018). These substances released in aquatic environments are foreign to the diets and metabolisms of marine organisms and can significantly affect marine

communities and ecosystems (Desbiolles et al. 2018; Sehonova et al. 2018; Patel et al. 2019). One of the world's most popular drugs is diclofenac (DCF; 2-[(2,6-dichlorophenyl) amino] phenylacetic acid) — the active ingredient in a number of non-steroidal anti-inflammatory medications (NSAIDs; Acuña et al. 2015; Fabbri & Franzellitti 2016). Its presence in seawater has been identified at concentrations ranging from 0.6 to 843.0 ng/L (Fent et al. 2006; Ankley et al. 2007; Gaw et al. 2014), and a number of scientific studies have also found it in drinking water, surface water, sewage, and aquatic organisms (Heberer & Feldmann 2005; Liu et al. 2015; Xie et al. 2015, 2017; Tran et al. 2018; Praveena et al. 2019). In detail, concentrations of DCF in water bodies vary from a few ng/l to µg/l (Zhang et al. 2008; Wang et al. 2010; Gonzalez-Rey et al. 2015; Xie et al. 2017; Nantaba et al. 2020), while concentrations in surface and wastewater range

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from a number of ng/L to a few mg/L (Vieno & Sillanpää 2014; Lonappan et al. 2016; Sousa et al. 2019; Sathishkumar et al. 2020). DCF is on the European Commission's "Checklist" concerning the control of water bodies (European Union, EU 2015/495) and was added to the "EU Water Framework Directive" watch list in 2013 (EU, 2013). Due to its biologically and pharmacokinetically high activity, it can damage aquatic biota (Jobling et al. 2003); in fact, several authors showed delay in the development of fish *Danio rerio* (Van den Brandhof & Montforts 2010; Ribeiro et al. 2015; Zhang et al. 2020), damages in liver, kidneys, and gills of fish *Oncorhynchus mykiss* (Triebkorn et al. 2004; Groner et al. 2017), teratogenesis, and embryotoxicity in amphibians *Xenopus laevis* and *Lithobates catesbeianus* (Cardoso-Vera et al. 2017). Although most scientific studies involved freshwater species (Aguirre-Martínez et al. 2013; Hughes et al. 2013), marine organisms, key sources of bioactive molecules (Lazzara et al. 2019; Inguglia et al. 2020; Luparello et al. 2020a, b, Luparello 2021; Mauro et al. 2020a) and important environmental bioindicators (Cammilleri et al. 2019; Chiamonte et al. 2020; Vazzana et al. 2020a, b; Mauro et al. 2020b, 2021) can be adversely affected by drug pollution, and marine invertebrates in particular are among the organisms most threatened by drug pollution (Gagné et al. 2006; Lonappan et al. 2016). DCF is classified in European Union Directive 93/67/EEC as a very toxic substance to gametes of starfish *Asterias rubens*, sea urchin *Psammechinus miliaris*, and bloodworm *Arenicola marina* (EC50 = 100 and 1000 µg/l). In literature, several scientific studies highlighted that DCF can affect marine invertebrates at biochemical and physiological levels as in the case of mollusca *Ruditapes philippinarum* (Matozzo et al. 2012; Munari et al. 2018), crab *Carcinus maenas* (Eades & Waring 2010), mollusca *Mytilus galloprovincialis* and *Mytilus edulis* (Schmidt et al. 2011; Cunha et al. 2017; Mezzelani et al. 2018; Munari et al. 2018), *Perna perna*, *Dreissena polymorpha*, and planktonic crustacean *Daphnia magna* (Quinn et al. 2011; Cortez et al. 2012; Riva et al. 2012; Liu et al. 2017). Given the risk that DCF pollution can cause, it is now urgent to acquire further data regarding its effects on marine species to fully understand the toxicity and environmental risks. The aim of this study was to evaluate, for the first time, the effects of DCF at different concentrations on embryonic development and accumulation in embryos and gametes (eggs and spermatozoa) using *A. lixula* sea urchin as a model organism. It was decided to analyse the first phases of the life cycle because as well as being important for the survival of the species, these phases

are particularly sensitive to environmental conditions and are used in the ecotoxicological approach to assess the quality of the environment (De Campos et al. 2022; Thompson et al. 2022; Venâncio et al. 2022). The objective of the study is to provide a better understanding of the effects of diclofenac in seawater to establish the potential toxicity and understand the relationship between concentrations and the effects on non-target organisms. The results obtained could help to fill the lack of information regarding the real effects (likely species-dependent) of DCF on marine species and to provide additional indications to establish limit values of the release of DCF into marine environments for the protection of biodiversity.

Materials and methods

Experimental animals

Sixty specimens of *A. lixula* were collected in spring from the coast of Terrasini (Palermo, Italy) and were maintained (while respecting the photo period) in three tanks at the same conditions (20 individuals per tank) containing aerated, filtered, and refrigerated seawater ($16 \pm 1^\circ\text{C}$) at the University of Palermo's aquarium (Dept. STEBICEF). The seawater used was that of the sampling site, and one water change (one third of the volume) was made during the acclimatization period (lasted for a week). The animals were fed *ad libitum* with pelleted invertebrate food (Azoo, Taikong Corp. Taiwan) until 24 h before the start of the experiments.

Experimental samples

From 50 total individuals maintained in tanks, 9 females and 9 males were selected randomly and identified using gamete emissions to perform the experimental plan. Emissions (eggs and sperm) were induced from each individual using an intracoelomatic injection of 0.5 ml of KCl 0.5M into the peristomial membrane (His et al. 1999; Marin et al. 2000; Au et al. 2001; Volpi Ghirardini et al. 2005). The gametes' sperm and eggs were then sampled separately after a few minutes and washed twice with a marine solution (MS) (CaCl₂ 12 mM, KCl 11 mM, MgCl₂ 26 mM, NaCl 0.45 M, Trizma Base 45 mM a pH 7.4). The quality of the gametes was evaluated using a 40X optical microscope, and only completely spherical/normal eggs and sperm with good/excellent motility were employed in the experiments (Fernandez & Beiras 2001; Bellas 2007).

The gametes were counted using a Neubauer chamber and the formula:

$$N^{\circ} \text{ cells}/0.02 * 1000 * \text{FD (dilution factor)}$$

Diclofenac treatment

The DCF solutions used in the experiment were prepared by heat-dissolving the drug (99% purity, Sigma-Aldrich) in a MS. We first assessed the effects of different DCF concentrations on the embryonic development and, subsequently, the eggs and sperm of *A. lixula* to identify whether there was an impact on fertilisation.

Embryotoxicity were evaluated using eggs and sperm at a 1:8 ratio ($1 \times 10^6/\text{ml}$ eggs and $8 \times 10^6/\text{ml}$ sperm). These were simultaneously combined on multiwells plates with different DCF solutions at final concentrations of 50 mg/L, 5 mg/L, 0.5 mg/L and 0.05 mg/L (10 mL for each well). The control sample was obtained by combining gametes in a MS. Two type of assessments were carried out to identify the impact of the different concentrations of DCF on early development. The former were

performed using an inverted microscope to determine the number of normal embryos, degenerated eggs and unfertilised eggs 3 h after the start of their incubation with the drug. For all experimental concentrations and controls a total of 100 embryos/unfertilized eggs/degenerated eggs were counted and data were expressed as a percentage of each single category (embryos, unfertilized eggs and degenerated eggs). The second type of analyses were conducted to evaluate the levels of DCF accumulated by the embryos. In more detail, the samples were centrifuged at 2200 rpm for 10 min at 4°C and the pellets obtained were washed with a MS and stored at -80°C for the subsequent high-performance liquid chromatography (HPLC) analysis. The evaluations of the DCF-accumulation levels were conducted while respecting the equal weights of all the samples (240 mg). The assessments described were repeated nine times using nine different pairs of animals (nine female and nine male in total as reported above) (Figure 1).

To evaluate the gametes, eggs and sperm were incubated for 3 h separately with each DCF solution using polystyrene well plates (as reported above).

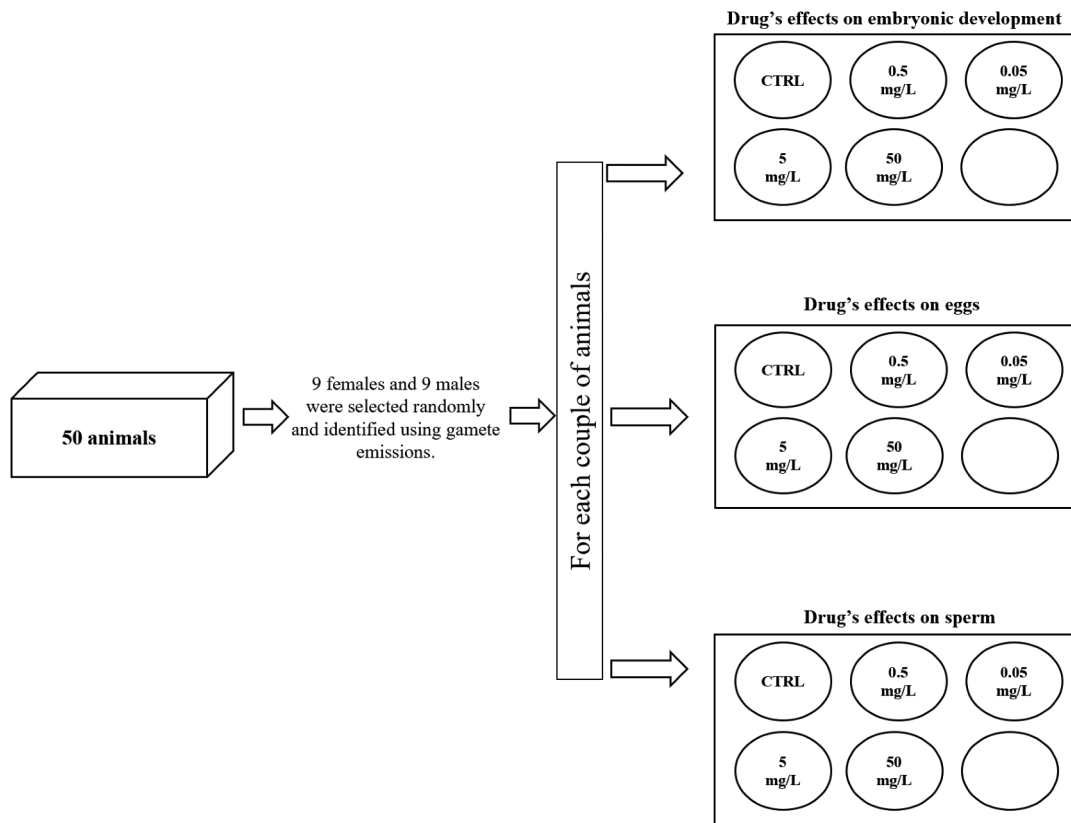


Figure 1. Scheme of experimental design.

Egg/sperm samples of 1 ml were incubated with 10 ml of each DCF solution; 10 ml of a MS was used for the control. The quantitative analysis was performed using HPLC analysis as described above. The same final pellet weight (240 mg) was used for all the samples in this analysis (corresponding to 3×10^6 cells/ml for the eggs and 18×10^9 cells/ml for the sperm). To evaluate the gametes' accumulation of DCF, this experiment was repeated nine times for the eggs and sperm separately using the same individuals used previously in fertilization analysis (18 individuals in total, nine for eggs and nine for spermatozoa) (Figure 1).

HPLC analysis

Reagents and standards. Analytical grade (>99% purity) phosphoric acid, perchloric acid and the DCF standard were purchased from Sigma Aldrich (Amsterdam, Holland). All the samples were weighed and mixed with 20 μ l of 85% phosphoric acid and 150 μ l of 60% perchloric acid, and the solutions were centrifuged at 15,000 rpm for 10 min (room temperature). About 100 μ l of supernatant were collected and placed on 150 μ l vials for the HPLC analysis. This was conducted on an Agilent 1200 HPLC with an ultraviolet diode array detector (UV/DAD) using a Synergi 4 μ m MAX-RP 80A HPLC column (250 x 4.6 mm; Phenomenex, Torrance, California, USA). The instrument conditions are set out in Table I.

The method used employed an elution obtained after the HPLC column was conditioned in the mobile phase for 1 h. Acetonitrile acid (85:15, v/v) and phosphoric acid (0.01 M) were used as eluents, as per the gradients listed in Table II. The method was validated for its linearity, detection capability (CC β), trueness of recovery and repeatability according to the European Commission Decision 2002/657/EC. The linearity was calculated in a solution taking into account six concentration points (50–100–200–500–1000 μ g/L), with $r^2 > 0.99$. The recovery and repeatability were determined with blank samples spiked at 500 μ g/Kg.

Table I. HPLC-UV/DAD instrument conditions.

Parameter	Value
Loop	50 μ l
Flux	1.2 ml/min
Column temperature	40°C
Autosampler temperature	15°C
Stop time	20 min
Detector	UV 240–254–278–290 nm

Table II. The gradients of the eluents after the HPLC column was conditioned in the mobile phase for 1 h.

Time (min)	Acetonitrile (%)	Phosphoric acid (%)
0	35	65
2	50	50
4	75	25
15	75	25
16	50	50
20	35	65

Statistical analysis

According to Helsel (2005), the samples in which no DCF was detected by the method described above were treated as having half the detection capability (0.025 mg/L) for the purposes of the statistical analysis. The normal distribution of the dataset was tested using the Shapiro-Wilk test ($p > 0.05$). Wilcoxon rank-sum and t-tests were performed to evaluate differences in the levels of DCF present in the male and female gametic cells (including the controls). The Kruskal-Wallis test was employed to examine differences in the DCF accumulation level of control and treated sperm and egg samples and was also used to evaluate embryonic development (in terms of embryos and degenerated and unfertilised eggs). In particular, the test was employed to verify differences in DCF levels in the controls and the treated samples, as well as during the early stages of their development. An ANOVA test was carried out to identify differences in the amounts of DCF present in the controls and the treated embryos. All the statistical analyses were conducted using the R®3.0.3 software package.

Results

The results in relation to embryotoxicity test (Figure 2) revealed a reduction in successful fertilisation that depended on the concentration of DCF introduced during the incubation period: as the DCF level increased, there was a significant decrease in the number of correctly developed embryos (Kruskal-Wallis chi-squared = 17.318, $df = 4$, $p = 0.002$; Figure 2A) and a significant increase in the number of degenerated eggs (Kruskal-Wallis chi-squared = 22.664, $df = 4$, $p = 0.000$; Figure 2C). However, in comparison to control samples, only DCF concentrations of 50 mg/l led to significant reductions in the number of normal embryos. The number of unfertilised eggs rose as the DCF concentration increased, although these changes were not statistically significant (Kruskal-

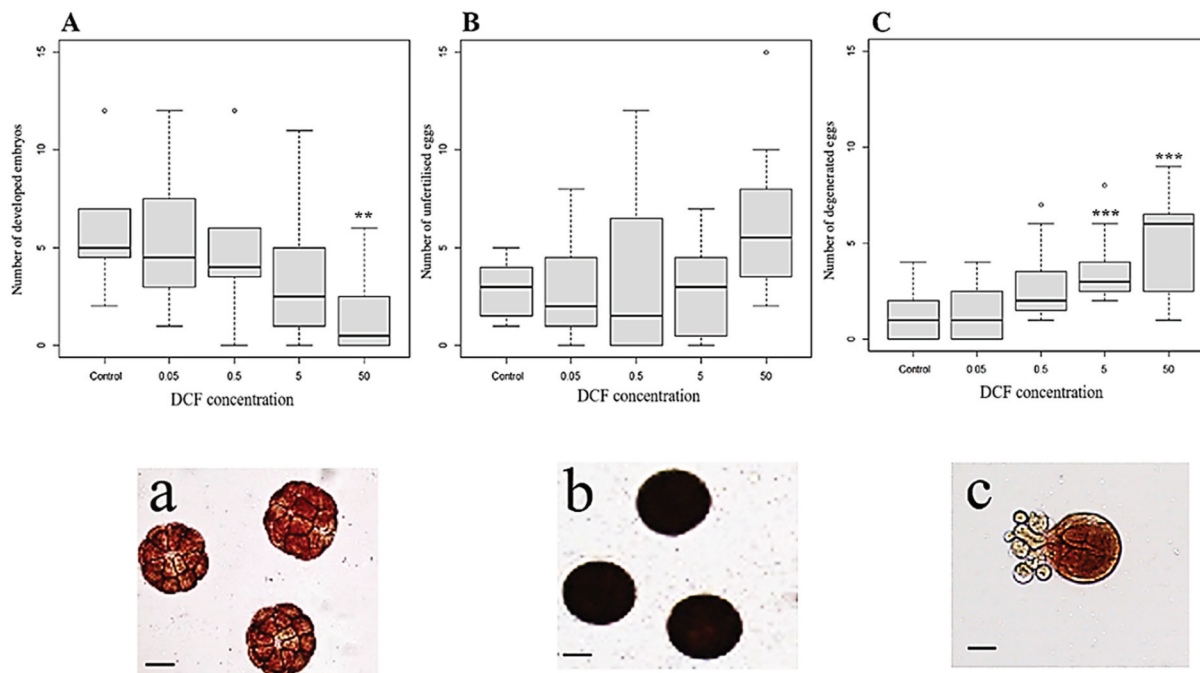


Figure 2. **A**, **B** and **C**: Box-plots obtained using the Kruskal-Wallis test on the developed embryos and the unfertilised and degenerated eggs (from left to right). The circles represent the outlier; **a**, **b**, **c**: microscopic photographs of the embryos and the unfertilised and degenerated eggs. Scale bar 50 μ m. The asterisks represent statistical differences (** $p < 0.01$, *** $p < 0.001$).

Wallis chi-squared = 9.126, $df = 4$, $p = 0.058$). In relation to the degenerated eggs, there was a significant increase in their number at DCF concentrations of 5 mg/L and 50 mg/L compared to the controls. This was also the case when the samples containing DCF concentrations of 0.05 mg/L and 5 mg/L were compared to those with DCF levels of 50 mg/L.

The levels of DCF present in the embryos were evaluated using an HPLC analysis (Figure 3) to verify the actual degree of accumulation. The ANOVA and Tukey post hoc tests confirmed that there was greater and significant accumulation of the drug respect to the control when the highest concentrations were introduced during the incubation period (F value = 7.357; $df = 4$; $p = 0.001$). The DCF accumulation was dose-dependent and directly correlated to the exposure concentration. This was significant when the different treatments were compared, in particular when comparing DCF levels of 0.05 ($p < 0.01$), 0.5 ($p < 0.01$) and 5 mg/L ($p < 0.05$) to those of 50 mg/L.

The HPLC analyses of the eggs (Figure 4A) and sperm (Figure 4B) incubated with different DCF concentrations revealed significant increases ($p < 0.05$) in accumulation compared to the

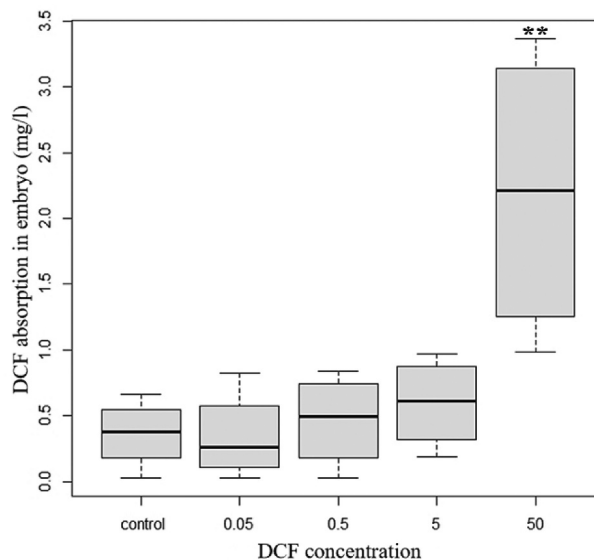


Figure 3. DCF accumulation in embryos. Values are expressed as the mean \pm SD. The asterisks represent statistical differences (** $p < 0.01$).

controls (for eggs: Kruskal-Wallis chi-squared = 29.79, $df = 4$, p -value = 0.000; for sperm: Kruskal-Wallis chi-squared = 22.094, $df = 4$, $p = 0.000$): the accumulation were statistically

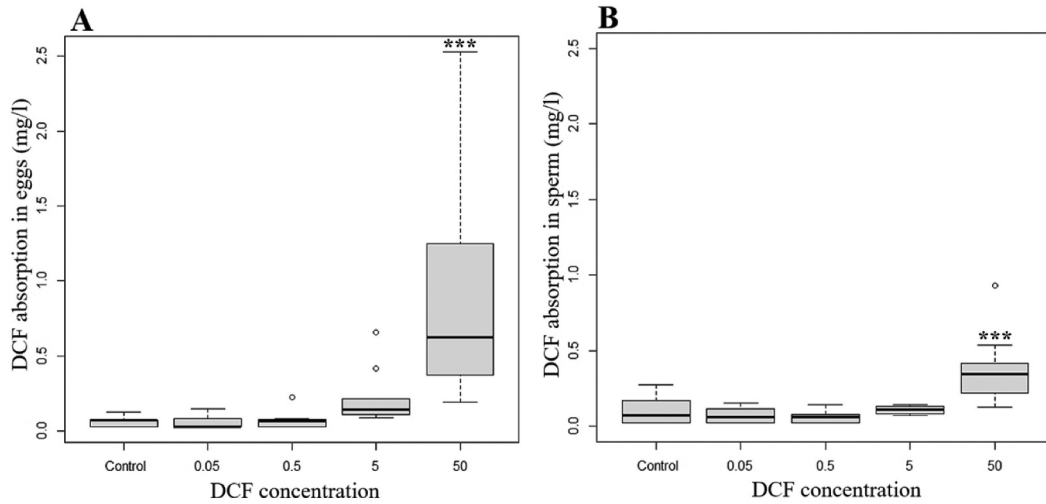


Figure 4. DCF accumulation in *A. lixula* eggs (A) and sperm (B) measure by HPLC analysis. The circles represent the outlier. Significant accumulation were observed at concentrations over 50 mg/L. The asterisks represent statistical differences (***) ($p < 0.001$).

significant when the eggs were incubated with 50 mg/L of DCF. Moreover, the increase in accumulation was dependent on the concentration of DCF introduced and was significant when levels of 0.05 and 0.5 mg/L were compared to one of 50 mg/L. Table III sets out the mean values of DCF accumulation in egg and sperm samples.

Discussion

To date, among PhACs, Diclofenac (DCF) is one of the most commonly found NSAIDs worldwide (Bonnefille et al. 2018; Ajibola et al. 2021; Pap et al. 2021). In literature, it is known that DCF has negative effects on marine vertebrate and invertebrate organisms (Bonnefille et al. 2018). For this reason, it is important to fully understand its toxicity, environmental risks, and the relationship between its concentrations and effects. Echinoderms are important test species in marine ecotoxicology (Sugni et al. 2007) and in light of this, we studied the effects of DCF on the sea urchin *A. lixula*, focusing on the first phases of the life cycle and gametes, essential for the survival of the species and sensitive to environmental conditions (De Campos et al. 2022; Thompson et al. 2022; Venâncio et al. 2022). We also analysed their

bioaccumulation capacity which can influence the biomagnification effect along the trophic chain. All these aspects are important to enlarge the diversity of animal model used for testing the toxicity of DCF. Although DCF concentrations found in seawater are generally below 1 µg/L, the European Union has included DCF in its first checklist of the Water Framework Directive (EU 2015/495, European Commission). This is because DCF often enters aquatic environments via Waste Water – treatment Plants (WWTP) inputs, and the extent of its degradation depends on the wastewater and the treatment technology used (Jiang et al. 2021; Kiejza et al. 2021). Furthermore, WWTP treatment of DCF is also dependent on sunlight, which promotes photolysis (Mestre & Carvalho 2019). For all these reasons, the distribution and real concentration of this drug in the aquatic environment is not yet clear. Four concentrations of DCF were used in this study, some even higher than the environmental concentrations.

These were chosen in agreement with other authors who examined the effects at concentrations ranging from a few ng/L to several mg/L, showing that marine organisms have different sensitivities, depending on the species, of the development stage and measured endpoints (reviewed by Bonnefille et al. 2018). Moreover, to date, the behaviour and

Table III. Concentrations of DCF absorbed in the sperm and egg samples, expressed as the mean \pm standard deviation.

DCF treatments	0 mg/L	0.05 mg/L	0.5 mg/L	5 mg/L	50 mg/L
Egg	0.06 \pm 0.04	0.05 \pm 0.05	0.06 \pm 0.06	0.21 \pm 0.20	0.91 \pm 0.80
Sperm	0.1 \pm 0.09	0.07 \pm 0.05	0.06 \pm 0.04	0.10 \pm 0.03	0.40 \pm 0.24

distribution of this drug in aquatic environments are not yet clear, and the evaluation of maximum drug concentrations in an *in vitro* study could be useful to understand up to which concentration the negative effects are visible and important.

In this study, we demonstrated negative effects on embryos and gametes dependent on DCF concentration, causing a decrease in number of developed embryos and an increase in number of degenerated eggs as shown by other authors in mussels, crustacean, and echinoderms (Riberio et al. 2015; Liu et al. 2017; Balbi et al. 2018). Our results are in agreement with other authors which showed

important effects depending on pollutants (e.g. alkylphenols, triorganotin compounds, bisphenol A, polystyrene microbeads, nanoparticles) concentration on *A. lixula*, *P. lividus* and *Strongylocentrotus intermedius* embryos and sperm highlighting closely concentration-dependent damages causing malformations and growth inhibition of embryos in the early life stages (Novelli et al. 2002, 2003; Arslan & Parlak 2007; Özlem & Hatice 2008; Messinetti et al. 2018; Pikula et al. 2020).

Moreover, also in oyster, genotoxic effects of herbicides depending on differences in pollutant sensitivity and affecting the sperm function and the fertilization were observed on embryos and spermatozoa (Akcha et al. 2012). The negative effects observed in this study on embryo development are likely due to drug bioaccumulation, which can cause changes in the expression of important genes (e.g., expression of vitellogenin the precursor of the common egg yolk protein vitellin) involved in the detoxification of the metabolism, growth, development, and reproduction (Dennerly 2007; Liu et al. 2017). The observed effects on embryonic development could be due to a bioaccumulation of the drug in the gametes before fertilization. In fact, we showed that the bioaccumulation in gametes (eggs and spermatozoa) was significantly greater at the highest tested concentration. These data are very important because very few studies have analysed the bioaccumulation and have mainly been conducted on bivalves (Ericson et al. 2010; Bonnefille et al. 2017, 2018; Fu et al. 2020). Radenac et al. (2001) evaluated the bioaccumulation and the frequencies of abnormalities of four metals in larvae of *Paracentrotus lividus* highlighting in agreement with ours results that the concentrations bioaccumulated were directly dependent to the concentration exposure. In our study, the levels of DCF bioaccumulation at high exposure concentrations could have caused both the increase in the number of degenerate eggs and the decrease in the number of developed embryos as shown by Lewis and Galloway (2009).

The observed increase, even if not significant, in the number of unfertilized eggs could also be due to the accumulation of the drug in the spermatozoa, which is capable of compromising their movement and the acrosome reaction as shown by several authors (Caldwell et al. 2004; Du et al. 2016; Zanuri et al. 2017; Fontes et al. 2018). In fact, as observed in the spermatozoa of marine invertebrate species, it is possible that the drug may cause an alteration in the functioning of the ion channels (Espinal-Enríquez et al. 2014), the stability of the lysosomal membrane (Fontes et al. 2018), the oxidative stress and polyspermia (Gonzalez-Rey & Bebianno 2012).

In conclusion, although the toxicity of DCF is well known (Lonappan et al. 2016) and the drug is on the EU's Water Framework Directive's Watch List of Priority Substances (European Commission 2013, 2015; Barbosa et al. 2016; Tiedeken et al. 2017), there are no restrictions on its emission into the sea. To date, the sea urchin embryotoxicity test (ASTM 2021: ASTM E1563 – 21 Standard Guide for Conducting Short-Term Chronic Toxicity Tests with Echinoid Embryos, ASTM International, West Conshohocken, PA, 2021) provides a good indication about the acceptability of pollutants dangerous concentrations. Our results represent the first indication of the negative effects of the acute exposure of DCF on sea urchin *A. lixula*, a marine invertebrate of ecological importance highlighting that analysing the early life stages is a promising approach for assessing the toxicity levels of pharmaceuticals, confirming that the presence of anthropogenic stressors, such as DCF, could compromise their developmental processes and therefore their survival (Hamdoun & Epel 2007). Understanding the effects on developing embryos and on the accumulation capacity of the drug by gametes can therefore provide predictive tools to identify potentially harmful compounds and provide indications on safe levels of release of this drug into the sea. All of this confirms the environmental risk of DCF and highlights the need to improve wastewater treatment and drug disposal processes.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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