Assessment of ecotoxicological effects of ciprofloxacin in *daphnia magna*: life history traits, biochemical and genotoxic effects

B. Nunes, C. Leal, S. Rodrigues and S. C. Antunes

ABSTRACT

Antibiotics (e.g. ciprofloxacin) have been detected in surface water and groundwater for several decades. In order to understand the potential impact of the continuous exposure of aquatic organisms to ciprofloxacin, a chronic assay was carried out with *Daphnia magna*. This approach allowed evaluating the effects of ciprofloxacin in life-history and sub-individual parameters (antioxidant status and metabolic response: activities of catalase and glutathione S-transferases – GSTs; peroxidative damage, thiobarbituric acid reactive substances and genotoxic effects (Genotoxic Damage Index, measured by the comet assay). Life-history parameters of *D. magna* showed no significant effects after ciprofloxacin exposure. Concerning oxidative stress and metabolism parameters, no significant alterations were reported for catalase and GSTs activities. However, a dual response was observed, with a significant decrease in lipid peroxidation levels at low ciprofloxacin concentrations (<0.013 mg/L), while a significant increase was verified at high ciprofloxacin concentrations (0.078 mg/L). The genotoxicity assay detected a significant increase in genetic damage index up to0.013 mg/L of ciprofloxacin. The here-tested ciprofloxacin concentrations, which are ecological relevant, did not cause significant impacts concerning the life history parameters of *D. magna*, however, at the same levels of ciprofloxacin an oxidative stress and genotoxic damage scenario were recorded.

Key words | antibiotics, chronic assays, comet assay, fluoroquinolones, oxidative stress biomarkers, reproductive parameters

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INTRODUCTION

Pharmaceutical drugs play a decisive role in modern human medicine by treating, preventing and diagnosing diseases. The use of pharmaceuticals has been increasing along time, and nowadays more than 3,000 different substances are in use for human therapeutics alone in the European Union market (Kronimus *et al.* 2004). However, only during the 90 s, it was possible to know in detail their widespread presence in ecosystems, especially in the aquatic compartment, encompassing the advances in analytical methodologies and techniques that allowed significantly lower limits of detection and a vast number of substances detected (Schulman *et al.* 2002; Santos *et al.* 2010). These compounds have been found in tissues of multiple living organisms from different compartments of the ecosystem, evidencing their worldwide dispersion (Daughton & Ternes 1999; Halling-Sørensen *et al.* 2000; Kronimus *et al.* 2004). In addition, the use of some of these chemicals for veterinary purposes and food productions (including aquaculture practices) increased their direct dispersal and consequent presence in the aquatic compartment (Bottoni *et al.* 2010). On the other hand, the screening of influents and effluents of sewage treatment plants has shown that pharmaceutical drugs, in general, are not effectively removed by traditional treatment processes and systems (Han *et al.* 2006; Bartelt-Hunt *et al.* 2009).

Pharmaceuticals are distinct from other contaminants that may coexist in the same environmental matrix, since they necessarily present biological activity, from which their therapeutic activity derives. They are usually bioavailable and pharmacologically potent chemicals, and these

doi: 10.2166/wst.2018.255

activities remain even when they are in the wild (Daughton & Ternes 1999; Halling-Sørensen *et al.* 2000). Pharmaceutical drugs are also moderately lipophilic (readily permeating biological membranes), and are resistant to biotransformation, to act for longer periods before being excreted; This set of characteristics allows concluding that pharmaceutical drugs are likely to be persistent being responsible for several potential environmental risks (Daughton & Ternes 1999; Halling-Sørensen *et al.* 2000). Among drugs found in the wild, antibiotics are particularly troublesome, since these substances are also one of the most used group of pharmaceutical drugs (Wise 2002). Despite the absence of legislation in specific countries to prevent their excessive use, namely in animal husbandry (Marshall & Levy 2011), the global consumption of these substances is still increasing by circa 4% for year (Hamad 2010). The most common subclasses of antibiotics include tetracyclines, penicilines, macrolids, sulphonamides, and fluoroquinolones, among others (Kümmerer 2009). Fluoroquinolones (FQs) are a group of antibiotics widely used both in human (e.g. treatment of urinary infections) (EMA 2009) and veterinary (e.g. treatment of prostatitis and severe gastroenteritis). The mechanism of action of FQs on target microorganisms relies on the inhibition of DNA gyrase and of topoisomerase IV, thereby preventing DNA replication of bacterial cells, and thus compromising their cellular proliferation (Pommier *et al.* 2010).

One of the most employed FQs is ciprofloxacin (CIP), which was first marketed in 1987 and was included in the list of the World Health Organization as one of the most important drugs to be used in the basic medical healthcare system (Couper 1997). CIP is a recent large spectra FQ whose activity is not shared with other FQs. The presence of CIP in the environment has been demonstrated, in levels reaching up to 2.45×10^{-4} mg/L (Rodrigues-Silva *et al.* 2014) and 6.3×10^{-4} mg/L in surface waters (Halling-Sørensen *et al.* 2000), and concentrations varying from 0.0007 to 0.1245 mg/L in hospital effluents (Hartmann 1999). Given its high environmental levels and potential ecotoxicity, de Voogt *et al.* (2009) classified CIP as a top priority drug, whose effects in the wild must be mandatorily assessed. In addition, and considering its wide activity against bacteria, several studies have been conducted to determine the putative ecotoxicological effects of CIP towards organisms from distinct ecosystems (Zuccato *et al.* 2010; Bona *et al.* 2014). Despite not having the same mechanism of action towards nonbacterial cells, ciprofloxacin (and other FQs) have been identified as putative genotoxicants (Gorla *et al.* 1999; Herbold *et al.* 2001; Gurbay *et al.* 2006), being also capable of exerting pro-oxidative effects in distinct organisms (Tu *et al.* 2008; Lowes *et al.* 2009; Afolabi & Oyewo 2011; Talla & Veerareddy 2011; Qin & Liu 2013; Gomes *et al.* 2017; Michalak *et al.* 2017). These indications reinforced the ecotoxicological significance of FQs (especially ciprofloxacin), if one considers their persistence and the toxicity of their metabolites. In addition, metabolites and photodegradation products of FQs are biological active and can, in some cases, be more toxic than parental compounds (Li *et al.* 2011).

Considering the above-mentioned features of CIP, it is important to obtain more information about its final environmental fate and putative toxic effects, prioritizing studies where environmentally relevant concentrations are tested. Furthermore, the need for additional ecotoxicological data is augmented if one considers that CIP was recently included in the list of high priority pharmaceuticals to be monitored in the wild (de Voogt *et al.* 2009).

The main goals of this study were to assess the chronic ecotoxicological effects of *Daphnia magna* after exposure to a range of ecologically relevant concentrations of CIP, i.e. close to realistic scenarios of contamination, at several levels of organization. The adopted ecotoxicological endpoints were measured in terms of the individual (life-history parameters: age at first reproduction, reproductive output, somatic growth rate, rate of population increase), and sub-individual level (oxidative stress biomarkers: catalase – CAT and glutathione S-transferases (GSTs) activities; lipid peroxidation; genotoxicity, with the determination of the genetic damage index (GDI) – measured by the comet assay).

MATERIALS AND METHODS

Daphnia magna cultures

Daphnia magna was the model species chosen for this study. *D. magna* is an aquatic microcrustacean considered a good indicator due to its high sensitivity to toxicants, being widely used as an ecotoxicological model (OECD 2012). For the purpose of this study, clone A was used (as in Antunes *et al.* 2004).

Group cultures, constituted by 25–30 asexual females with the same age, were maintained in reconstituted hard water, supplemented with a standard organic extract (Antunes *et al.* 2004; OECD 2012). Cultures were kept in a growth chamber with controlled conditions of light intensity (10–20 μmol/m^{-}/s), photoperiod (16 h^{L}:8 h^{D}), and

temperature (20 ± 2 °C). Animals were fed with a microalgae (*Raphidocelis subcapitata*) suspension of 3.0×10^5 cells/mL every other day. For further details on rearing procedures of *D. magna* and *R. subcapitata* cultures, see Antunes *et al.* (2004). Experiments were carried out with individuals less than 24 h old (neonates), born between the 3rd and 5th brood (for homogenization and standardization purposes).

Chemicals

Ciprofloxacin (CAS - 85721-33-1 at 98% of purity) was acquired at Sigma-Aldrich.

Chronic assay

Chronic assay with *D. magna* was conducted following general recommendations (OECD 2012, guideline 211 reproduction assay), and lasted for 21 days. Experiments were initiated with neonates, obtained from a healthy stock, and test vessels (25 mL glass beakers) were kept under the controlled conditions described above. The experiment consisted of 10 organisms, held individually in separate vessels, exposed to each CIP concentration. Test concentrations (geometric series) were defined according to data from the literature: 0.000 mg/L (negative control); 0.005 mg/L; 0.013 mg/L; 0.031 mg/L; 0.078 mg/L (concentrations found in the aquatic ecosystem) and 0.195 mg/L (levels detected in hospital effluents). These different concentrations of CIP were prepared by diluting a concentrate stock solution of the antibiotic in distilled water, immediately before the start of the assay. All the presented concentrations are nominal and, since the assay was conducted under semi-static conditions, we assume that all CIP levels were kept stable during the assay. Daphnids were transferred to newly-prepared CIP dilutions every other day and daily fed with their respective *R. subcapitata* ration (3.0×10^5 cells/mL). During the assay period, daphnids were monitored daily for mortality and reproductive state, and the following parameters were determined: age at first reproduction, reproductive output (cumulative number of offspring produced until day 21), somatic growth rate, and the *per capita* intrinsic rate of population increase (a measure of population growth potential). Reproductive output considers the contribution of all test organisms, whether they survived or not until the end of the assay; as such, it is a more relevant endpoint than fecundity (number of offspring per surviving female), since it considers the combined effects of stressors on both survivorship and fecundity (OECD 2012). The somatic growth rate was estimated from the initial and final body size of the daphnids, according to the following expression:

$$\text{Somatic growth rate (day}^{-1}) = \frac{\ln(lf) - \ln(li)}{\Delta t}$$

where *lf* is the body size (in mm) of the test organism at the end of the test, *li* is the average body size (in mm) of a subsample ($n = 20$) of neonates coming from the same batch of neonates that initiated the test, and Δt is the time interval (in days) (Antunes *et al.* 2004).

The *per capita* rate of population increase was iterated from the Euler–Lotka equation, using the survival and fecundity estimates:

$$1 = \sum_{x=0}^{n} e^{-rx} l_x m_x$$

where r is the intrinsic rate of increase (in day^{-1}), x is the age class in days ($0 \ldots n$), l_x is the probability of surviving to age x, and m_x, is the fecundity at age x. Because data from all individuals of each experimental treatment are needed for the calculation, individual pseudo-values for r were generated by jack-knife re-sampling (Meyer *et al.* 1986).

At the end of the measures of survival individuals, two pools of organisms of each treatment were preserved for posterior determination (oxidative stress and genotoxicity biomarkers). For oxidative stress biomarkers quantifications, a pool of 7 organisms from each treatment were collected and stored in Eppendorf microtubes at −20 °C until analyses. For the assessment of genotoxic effects, a pool of 3 organisms per treatment was collected and immediately processed (*see* Genotoxicity section).

Biomarker determinations

Specific oxidative stress biomarkers were determined, including catalase and GSTs activities, and levels of lipid peroxidation (concentration of thiobarbituric acid reactive substances (TBARS)). Samples were homogenized in ice-cold phosphate buffer (50 mM, pH = 7.0 with 0.1% of Triton X-100) using a rotary tissue homogenizer at 14.000 rpm. Homogenates were subsequently centrifuged at 14,000 *g* for 10 min at 4 °C. All the quantifications were adapted to microplate and the absorbance values were measured in a Thermo Scientific Multiskan EX spectrophotometer (Ascent Software 2.6).

Catalase (CAT) activity was determined spectrophotometrically by measuring the decrease in absorbance at

240 nm (ε 240 = 0.00394 ± 0.0002 mM^{-1} mm^{-1}) as described by Aebi (1984), due to the enzymatic decomposition of H_2O_2 to H_2O and O_2. Changes in absorbance were spectrophotometrically monitored at 240 nm for 30 s, and activities were expressed as nmoles H_2O_2 consumed per minute, per milligram protein.

GSTs activity was determined spectrophotometrically according to the method by Habig *et al.* (1974). GSTs catalyze the conjugation of glutathione with the substrate 1-chloro-2,4-dinitrobenzene (CDNB), forming a thioether whose formation can be followed by the increase of absorbance at a wavelength of 340 nm. Enzymatic activities were expressed as nmol of thioether produced per minute, per milligram of protein.

Lipid peroxidation was measured through the quantification of the levels of TBARS, according to Buege & Aust (1978). Malondialdehyde (MDA) and MDA-like compounds are the main by-products of the oxidative damage to lipid membranes caused by reactive oxygen species – ROS. This methodology is based on the reaction of compounds such as malondialdehyde (MDA) formed by degradation of initial products of free radical attack, with 2-thiobarbituric acid (TBA). Absorbance readings of each sample were measured at a wavelength of 535 nm. TBARS concentrations were expressed as MDA equivalents (in nmoles), per mg of protein.

Protein concentration of the samples was determined according to the spectrophotometric (wavelength 595 nm) method of Bradford (1976), adapted to microplate using γ-globulin as a standard, in order to express enzymatic activities as function of the protein content.

Genotoxicity

The alkaline version of the comet assay was performed according to the methodology by Azqueta & Collins (2011) with slight modifications. Three organisms per concentration were carefully cut with a scalpel in a 0.5 mL of saline phosphate buffer (10% DMSO, 20 mM EDTA, PBS - 1.5 mM KH_2PO_4, 2 mM $Na_2HPO_4{\cdot}12H_2O$, 2.7 mM KCl, 0.14 M NaCl, pH = 7.4). A set of two successive centrifugations were conducted; in the first (20 s, 200 *g*) the supernatant was collected, and in the second centrifugation (10 min, 200 *g*) the pellet was retained. The pellet was then mixed with 200 μL of the low melting point agarose solution (1%) in a 1.5 mL microcentrifuge tube. One drop of 130 μL of the previous mixture was placed on one glass microscope slide (two slides per treatment were prepared, representing two replicates), precoated with 1% normal melting point agarose. Then, gels were immersed for a 1–24 h in a lysis solution (0.2 M NaOH, 100 mM $Na_2EDTA2H_2O$, 10 mM TRIS, 2.5 M NaCl, 1% Triton x-100, 10% DMSO, pH = 10, 4 °C). After this period, slides were placed in the electrophoresis solution (0.3 M NaOH, 1 mM Na_2EDTA, pH > 13) and DNA migration was allowed at a fixed voltage of 0.8 V/cm and 300 mA for 30 min. A period of 15 min of neutralization (PBS) and 15 min of washing were allowed. After washing the slides were placed in 70% ethanol for 10 min and then in absolute ethanol for 10 min. Finally, the coloration of slides was done with an ethidium bromide solution (0.01 mg/mL) for 20 min. At the end of this procedure, slides were stored in boxes, protected from light, until observation. The subsequent procedure required using a fluorescence microscope Nikon Eclipse G-2A (Nikon, Tokyo, Japan) equipped with an ethidium bromide compatible filter (excitation filter: 510–560 nm; dichroic mirror: 565; absorption filter: 590 nm), with which 50 nucleoids per gel were scored and classified, randomly. This score/classification, dividing each nucleoid into one of five categories, was attributed according to the tail and head intensity. The DNA damage was quantified by visual classification of nucleoids according to the tail intensity and length, from 0 (no tail) to 4 (almost all DNA in tail) Azqueta & Collins (2011). The GDI was calculated multiplying the mean percentage of nucleoids in each class by the corresponding factor Azqueta & Collins (2011), according to the equation:

$$\begin{aligned}\textbf{GDI} = {} & (\%\ \textit{of nucleoids in class}\ 0 \times 0) \\ & + (\%\ \textit{of nucleoids in class}\ 1 \times 1) \\ & + (\%\ \textit{of nucleoids in class}\ 2 \times 2) \\ & + (\%\ \textit{of nucleoids of class}\ 3 \times 3) \\ & + (\%\ \textit{of nucleoids of class}\ 4 \times 4)\end{aligned}$$

GDI results were expressed as arbitrary units, on a scale of 0–400 per 100 scored nucleoids (as average value for the two gels observed per fish). As positive controls, blood cells of control animals were treated with 50 μM of H_2O_2 for 5 min.

Statistical analyses

All tested variables (life-history parameters, biomarkers, GDI and damages classes) were checked for normality and homogeneity of variances prior to statistical analysis. One-way analysis of variance (ANOVA) was used to test for differences among CIP concentrations, followed by

Dunnett's test (when applicable) to discriminate significant differences relatively to the control. All statistical analyses were performed using SPSS Statistics v23 and MS Excel, using a significance level of 0.05.

RESULTS AND DISCUSSION

Figure 1 depicts life history parameters of *D. magna* after chronic exposure to CIP. No statistical differences were recorded for all measured parameters along the range of tested CIP concentrations (Figure 1(a)–1(d) and Table 1). However, a non-significant decrease was observed for the rate of population increase parameter along the tested concentrations. From previously published data, CIP effects on these parameters may in fact occur, but only for high levels of exposure, that are far from being realistic in ecological terms (Martins *et al.* 2012; Bona *et al.* 2014, 2015). According to the study by Martins *et al.* (2012), exposure of *D. magna* to CIP caused a significant delay in population traits, including in the age of first brood, but for levels above 25 mg/L; effects were also reported concerning the rates of somatic growth and population increase, which were only compromised for levels above 15 mg/L. The same study also showed fecundity impairment for organisms exposed to concentrations above 8 mg/L, while the size of neonates was affected by levels of CIP in excess of 2 mg/L. Similarly, data published by Bona *et al.* (2015) identified a significant decrease in the rate of somatic growth in individuals of *D. magna* exposed to concentrations above 30 mg/L. Moreover, Bona *et al.* (2014) showed that the age at first brood of *D. magna* was significantly anticipated after being chronically exposed to a concentration of 12.5 mg/L of CIP, which are well above the here-adopted range of concentrations. Similar effects concerning *D. magna* population performance have also been reported for other FQs; enrofloxacin for instance was shown to reduce the reproduction rate and the population growth rate at levels, but at levels of 15 mg/L (Park & Choi 2008). This entire set of data evidences that the ecotoxicity of FQs, and specifically of CIP, only occurs following exposure to extremely high levels of contamination, that exceed those

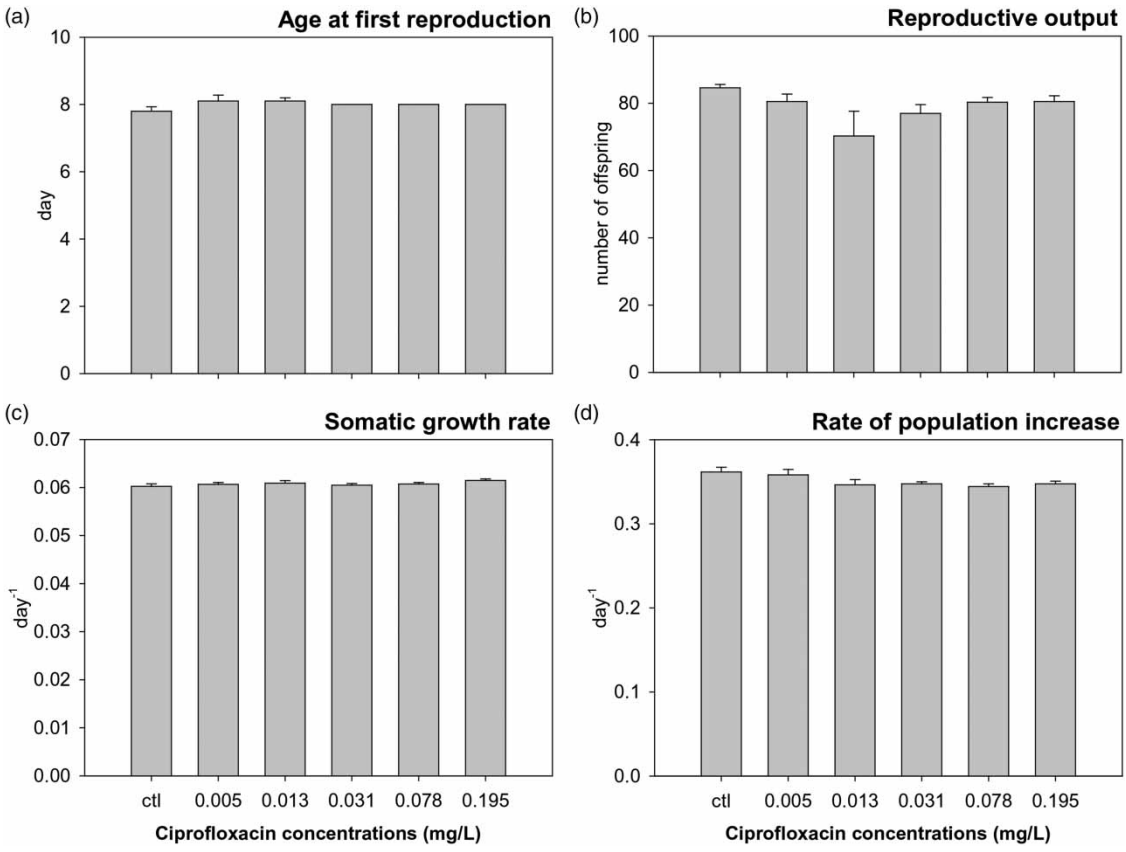

Figure 1 | Life-history results of *Daphnia magna* after chronic exposure of to a range of ciprofloxacin concentrations. Data are expressed as mean ± standard error (SE).

Table 1 | Summary of one-way ANOVA for all ecotoxicological parameters measured

Parameters	g. l.	F	P
Age at first reproduction	5, 54	1.200	0.322
Reproductive output	5, 54	1.965	0.099
Somatic growth rate	5, 52	1.072	0.387
Rate of population increase	5, 54	2.180	0.072
CAT	5, 12	1.769	0.194
GSTs	5, 16	11.40	<0.001
TBARS	5, 12	16.71	<0.001
Genetic Damage Index	5, 6	578.7	<0.001
Damage class 0	5, 6	58.88	<0.001
Damage class 1	5, 6	129.5	<0.001
Damage class 2	5, 6	32.81	<0.001
Damage class 3	5, 6	856.7	<0.001
Damage class 4	5, 6	299.5	<0.001

adopted in this study, and that are not likely to occur in the wild.

Concerning the oxidative stress biomarkers measured, no significant alterations were recorded in terms of CAT activity (Figure 2(a) and Table 1). However, a significant decrease of GSTs activity was observed for organisms exposed to the concentration of 0.013 mg/L of CIP (Figure 2(b) and Table 1). TBARS content was also significantly decreased, namely in organisms exposed to the lowest concentrations tested, 0.005 and 0.013 mg/L of CIP (Figure 2(c) and Table 1). Contrarily, a significant increase of TBARS content was observed for those organisms exposed to 0.078 mg/L of CIP (Figure 2(c) and Table 1). Despite the occurrence of significant effects that may be related to oxidative stress and damage (namely increased TBARS levels), it is not possible to state that oxidative stress was a clear outcome following CIP exposure. In fact, these results are corroborated by literature data. Plhalova *et al.* (2014) showed that *Danio rerio* was not significantly affected after exposed to up to 3 mg/L of CIP) in terms of the antioxidant enzyme catalase. However, compounds from the same antibiotic group (FQs), can indeed exert pro-oxidative effects; norfloxacin was capable of inducing CAT activity also in *D. rerio* in levels above 0.1 μg/L (Bartoskova *et al.* 2013). It was made clear by the earlier mentioned studies that an adaptive antioxidant response elicited by both FQ compounds was triggered, which was not observed in our study. However, it must be again stressed that pro-oxidative effects reported in the studies from the literature were only attained at levels that considerably exceeded those

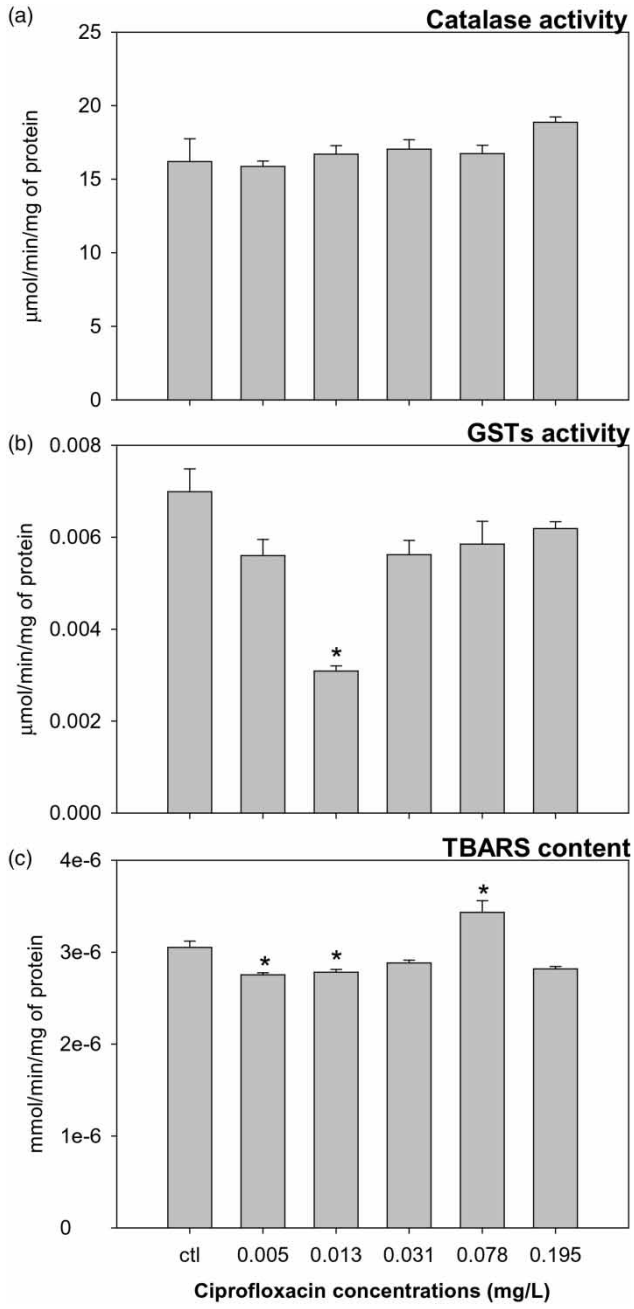

Figure 2 | Biomarker results ((a) – Catalase, (b) – GSTs; and (c) – TBARS) of *Daphnia magna* after chronic exposure to a range of ciprofloxacin concentrations. Data are expressed as mean ± standard error (SE), * stands for significant differences when compared to control ($p < 0.05$).

here-adopted. Similarly, a significant increase for the phase II metabolism biomarker, glutathione-S-transferases, was described by Plhalova *et al.* (2014) after exposing *D. rerio* to 0.7 μg/L and 100 μg/L of CIP. Bartoskova *et al.* (2013) has also shown that *D. rerio* was responsive to the FQ norfloxacin; however, this response was obtained only for low

levels of this antibiotic (0.1 μg/L), evidencing that the metabolism of this specific FQ requires the activation of the GSH conjugation pathway. According to both studies, these results indicate that low dosages of FQs are causative of GSTs induction, to increase the conjugation capability of exposed organisms; on the contrary, exposure to higher amounts of FQs can cause failure of the antioxidant defense system of this fish species. This pattern was not observed in our study, since modifications of GSTs activity (namely, a decrease) were only noticeable for the concentration of 0.013 mg/L. This shows that the interspecific difference in sensitivity towards specific xenobiotics must always be accounted for. FQs (and ciprofloxacin in particular) are long known to be causative of oxidative stress, with multilevel effects that ultimately result in cellular dysfunction in experimental organisms, as summarized by Michalak *et al.* (2017). The overproduction of ROS that is stimulated by CIP may end up opening the permeability transition pore of the mitochondria of affected cells, leading to the ultimate loss of their metabolic function. Loss of metabolic capability may in turn compromise specific cellular functions that require energy, such as the biosynthesis of scavengers to be used against oxidative insult, such as glutathione. In fact, data from the literature support this possibility. CIP exposure causes the progressive depletion of glutathione, in its reduced form GSH, as shown by Talla & Veerareddy (2011), favoring a progressive establishment of an oxidative stress deleterious scenario. In this scenario of oxidative stress, it is not possible to exclude that GSTs may themselves be the target of ROS. In fact, the activity of GSTs may be compromised by pro-oxidative compounds, as demonstrated by Birben *et al.* (2012), due to the potential direct binding of toxicants (and/or of the ROS produced during their metabolism) to their sulfhydryl (–SH) groups of these specific isoenzymes. Considering the clear pro-oxidative features of CIP, the effect of multiple mechanisms of toxicity should not be excluded, with distinct outcomes and involvement of diverse pathways and responses. The here-obtained data for lipid peroxidation showed a significant decrease for animals exposed to the lowest concentrations tested, 0.005 and 0.013 mg/L of CIP. This trend evidences the absence of a clear scenario of oxidative damage. Even if ROS were produced as a consequence of CIP metabolism (which is a common outcome, *see* previous paragraph), the antioxidant defense system of exposed *D. magna* individuals was effective enough to prevent lipid membrane damage. Similarly, data obtained by Plhalova *et al.* (2014) showed a decrease in TBARS levels for *D. rerio* exposed to a concentration of 100 μg/L of CIP, which was in the same order of magnitude as the lowest concentrations tested in this study. A somewhat similar trend (low CIP levels induce a decrease in TBARS levels; high CIP levels caused an increase in lipid peroxidation) was again observed by Bartoskova *et al.* (2013), after exposing *D. rerio* to levels up to 30 mg/L of enrofloxacin. Despite the phylogenetic difference between *D. rerio* and *D. magna*, and the putative detoxification and antioxidant defense mechanisms involved in CIP metabolism, it is possible to hypothesize that this pattern is the result of the successful activation of the antioxidant defense at low dosages, and its failure at higher levels of exposure, with the occurrence of oxidative damage by ROS produced by FQ metabolism.

Daphnia magna genotoxicity data is presented in Figure 3, and in Tables 1 and 2. GDI showed a significant increase from the second CIP concentration tested onwards, 0.013 mg/L (Figure 3 and Table 1). When looking to the % of damage classes, a significant increase of % of the higher damage was observed, following the increase of CIP concentrations (Tables 1 and 2), with a decrease of the % of the lowest classes of damage. Indeed, for the lowest class of damage (0) a significant decrease in its percentage was observed along with the increase of CIP concentrations tested (Table 2). As already described in the introduction section, ciprofloxacin is a quinolone widely used in antimicrobial treatments, acting by binding to the bacterial DNA gyrase, compromising its function, and consequently, preventing cell replication and bacterial proliferation. In addition, FQs may also bind to topoisomerase II

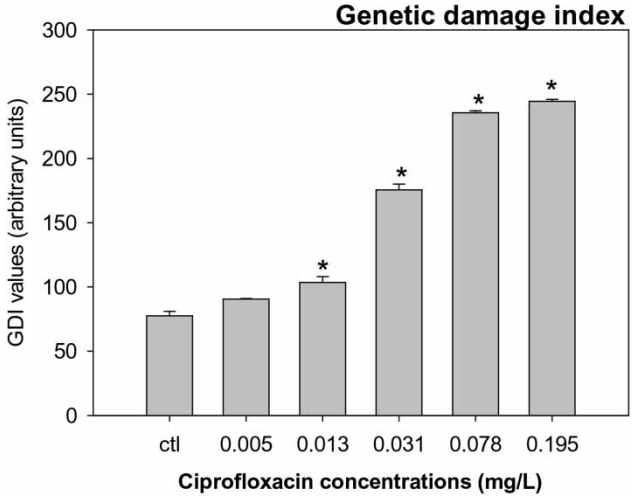

Figure 3 | Mean Genetic damage index (GDI) measured by the comet assay in *Daphnia magna* after chronic exposure to a range of ciprofloxacin concentrations. Data are expressed as mean ± standard error (SE),* stands for significant differences when compared to control ($p < 0.05$).

Table 2 | Mean frequency (%) of each DNA damage class (± standard error). Measured by the comet assay, in *Daphnia magna* after chronic exposure to ciprofloxacin

	Damage Classes				
Ciprofloxacin (mg/L)	**D0**	**D1**	**D2**	**D3**	**D4**
Ctl	42.0 ± 2.0	41.0 ± 1.0	14.5 ± 0.5	2.50 ± 0.5	0
0.005	37.5 ± 2.5	44.0 ± 3.0	12.0 ± 1.0	3.50 ± 0.5	3.0 ± 1.0*
0.013	34.5 ± 1.5	39.5 ± 0.5	15.5 ± 1.5	9.00 ± 0*	1.5 ± 0.5
0.031	20.5 ± 1.5*	21.5 ± 0.5*	28.5 ± 1.5*	21.0 ± 0*	8.5 ± 0.5*
0.078	14.0 ± 1.0*	11.0 ± 1.0*	18.5 ± 0.5	38.5 ± 0.5*	18.0 ± 0*
0.195	13.5 ± 0.5*	8.50 ± 0.5*	20.5 ± 0.5*	35.0 ± 1.0*	22.5 ± 0.5*

(homologous enzyme of DNA gyrase) present in eukaryotic cells. Thus, FQs such as ciprofloxacin, have been shown to exert genotoxicity in both prokaryotic and eukaryotic cells; this effect is nevertheless more evident in prokaryotic cells, since the affinity of ciprofloxacin for the bacterial DNA gyrase is higher than for the eukaryotic topoisomerase II (Herbold *et al.* 2001). Indeed, several studies have shown that CIP induces genotoxicity in human or rodent cells (Gorla *et al.* 1999; Herbold *et al.* 2001; Gurbay *et al.* 2006). Gurbay *et al.* (2006) demonstrated that ciprofloxacin causes a significant increase in DNA damage in rodent astrocytes at concentrations of 150 and 300 mg/L. Ciprofloxacin in concentrations of 0 to 50 μg/mL for 72 h was also capable of causing genotoxicity in human lymphocytes (Gorla *et al.* 1999).

Some studies indicate that, when DNA damage is of low intensity (D1 or D2 classes), organisms are able to trigger defensive mechanisms to revert the stressful conditions; however, this damage may be irreversible and can cause cellular apoptosis (e.g. Ahamed *et al.* 2008). The here-obtained results showed that, with increasing concentrations of ciprofloxacin, the incidence of genomic damages of lowest intensity (those that can be reverted, D1 and D2) decreases, being replaced by irreversible damages that can lead to cell death (D3 and D4). These results corroborate previous studies with the same compound (Gorla *et al.* 1999; Gurbay *et al.* 2006). The study by Gurbay *et al.* (2006), performed with rat astrocytes exposed to concentrations of 0, 5, 150 and 300 mg/L of CIP, demonstrated a clear dose-dependent increase in tail. Another study with *Escherichia coli* exposed to ciprofloxacin (0.1, 1 and 10 μg/L) for 40 min, concluded that comets were almost all of the D4 class in cells exposed to the highest concentration, whose repair efficacy was quite limited, and only observed after 4 h of incubation in the absence ciprofloxacin (Tamayo *et al.* 2009).

The failure of the antioxidant defense mechanisms may result in oxidative stress, which culminates in molecular damage, especially to highly important cellular macromolecules, including DNA, proteins, and cellular lipids (Bartoskova *et al.* 2013). So, genotoxicity can be a common outcome of oxidative stress; Conger & Fairchild (1952) have shown that an increase in intracellular oxygen levels is connected to the accumulation of chromosomal aberrations. Despite this association, genetic damage cannot be explained solely by the potential occurrence of oxidative stress. Other sources of damaging conditions may exist, including radiation and other chemicals that may be ultimately responsible for genotoxicity (Natarajan 2002; Kumaravel & Jha 2006). In our study, and considering the absence of unequivocal data showing pro-oxidant effects after CIP exposure, it is not possible to sustain that oxidative stress is the main driving factor that explains genotoxic effects. However, despite the absence of clear pro-oxidative effects, several evidences indicate the activation of antioxidant mechanisms. In addition, genotoxic effects were clearly observed following CIP exposure, indicating that, despite the underlying toxic mechanisms of action, this drug caused adverse effects at the sub individual level in exposed organisms. On the contrary, no population traits were compromised following CIP exposure, showing that such endpoints are not adequate to diagnose exposure to this type of xenobiotics. In fact, significant alterations at lower levels of organization were not reflected by changes at the individual level. According to this observation, the inadequacy of standard ecotoxicological tools and testing guidelines became clear, when compared to biochemical markers of toxicity (that pinpoint subtle toxic effects), to address the issue of potential aquatic contamination by pharmaceutical drugs, under realistic conditions similar to those that were already documented to occur in the wild. Despite the lack of a full scenario of oxidative stress, tour

data sustain the occurrence of deleterious modifications of the genome of exposed *D. magna*, which were not signaled by any population trait.

In merely scientific and ecological terms, this study shows the responsiveness of a model species of a crustacean, exposed to low and ecological relevant levels of an antibiotic, following standard recommendations under controlled abiotic conditions; however, the interpretation of the here-obtained data require an alternative approach to the analyzed parameters. No regulation encompasses the amount of pharmaceutical drugs in the wild; however, toxicological data for CIP establish that residual doses, below the determined levels in the wild, may exert both short term (defensive, transient adaptive responses, in oxidative stress and metabolic pathways) and long term, potentially irreversible effects (including genome alterations). Moreover, we cannot exclude that full life cycle or transgenerational experiments may result in deleterious population alterations that cannot be properly assessed with the here-adopted experimental design and testing guidelines.

CONCLUSIONS

The here-obtained results showed that CIP, did not cause significant effects in the life history parameters of *D. magna*, after exposure to CIP ecological relevant levels. These results allow inferring that, for the CIP concentrations tested no significant effects were observed for daphnids population. Despite the occurrence of mild, but significant biochemical effects, suggesting the establishment of pro-oxidative conditions, a full scenario of oxidative stress was not indeed established. On the other hand, genotoxic damages were observed after CIP exposure, even at low dosages here-adopted. Nevertheless, according to our results it was not possible to establish a causal relationship between genotoxicity and oxidative stress, suggesting that alternative mechanisms may underlie the genotoxicity shown to occur after CIP exposure.

ACKNOWLEDGEMENTS

This research was partially supported by the Strategic Funding CIIMAR – UID/Multi/04423/2013 and CESAM – UID/AMB/50017 through national funds provided by FCT – Foundation for Science and Technology and European Regional Development Fund (ERDF), in the framework of the programme PT2020. Sara Antunes and Sara Rodrigues are recipient of individual grant (SFRH/BPD/109951/2015 and SFRH/BD/84061/2012, respectively) by the Portuguese Foundation for Science and Technology (FCT). Bruno Nunes was supported by FCT (researcher contract IF/01744/2013).

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First received 29 November 2017; accepted in revised form 24 May 2018. Available online 7 June 2018