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Increasing toxicity of enrofloxacin over four generations of *Daphnia magna*

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ABSTRACT

The effects of both continuous and alternate exposure to 2 mg L^{-1} of enrofloxacin (EFX) on survival, growth and reproduction were evaluated over four generations of *Daphnia magna*. Mortality increased, reaching 100% in most groups by the end of the third generation. Growth inhibition was detected in only one group of the fourth generation. Reproduction inhibition was > 50% in all groups and, in second and third generations, groups transferred to pure medium showed a greater inhibition of reproduction than those exposed to EFX. To verify whether the effects observed in these groups could be explained by the perinatal exposure to the antibacterial, a reproduction test with daphnids obtained from *in vitro* exposed *D. magna* embryos was also carried out. Perinatal exposure to EFX seemed to act as an 'all-or-nothing' toxicity effect as 31.4% of embryos died, but the surviving daphnids did not show any inhibition of reproduction activity. However, the embryonic mortality may at least partially justify the inhibition of reproduction observed in exposed groups along the multigenerational test. Concluding, the multigenerational test with *D. magna* did show disruption to a population that cannot be evidenced by the official tests. The increasing deterioration across generations might be inferred as the consequence of heritable alterations. Whilst the concentration tested was higher than those usually detected in the natural environment, the increasing toxicity of EFX across generations and the possible additive toxicity of fluoroquinolone mixtures, prevent harm to crustacean populations by effects in the real context from being completely ruled out.

1. Introduction

Enrofloxacin (EFX) is a fluoroquinolone antibacterial agent largely used in veterinary medicine (Li et al., 2015). Both EFX and ciprofloxacin (CPX), its active metabolite/degradation product (Sukul and Spiteller, 2007), commonly used in human medicine, are water contaminants (CWF, 2011) and they have been repeatedly detected in the natural aquatic environment. Concentrations were usually below 1 μg L^{-1} (He et al., 2014; Santos et al., 2010; Tong et al., 2011) but occasionally higher (Guedes-Alonso et al., 2013) and even in the mg L^{-1} range (Fick et al., 2009; Larsson et al., 2007). In calves, goats, sheep, swine, poultry and rabbits, EFX is indicated for the treatment of various infections such as colibacillosis, salmonellosis, mycoplasmosis and pasteurellosis, and is available on the market in the EU as a solution intended for oral administration in drinking water or milk supplements. Using this formulation, EFX can be administered to a large collection of animals when disease has been diagnosed only in few of them (metaphylactic treatment) or indeed when no disease has been diagnosed (prophylactic treatment). In practice, to prevent respiratory and intestinal diseases when animals are subjected to stressful conditions as transportation or overcrowding, mass administration of antibacterials is routinely used in intensive farming (CWF, 2011). These routine mass treatments are criticized for fear of boosting the emergence and spread of antibacterial resistant microorganisms (Laxminarayan et al., 2013), and also because they can result in a considerable environmental burden of drugs and/or metabolites when the spent litter is used for the fertilization of agricultural fields (Boxall et al., 2003). In soil, the easy sorption of EFX to the matrix may lead to its accumulation and subsequent slow desorption and contamination of the aquatic environment (Picó and Andreu, 2007). Furthermore, EFX may directly contaminate surface water in those countries where its use extends to aquaculture (Quesada et al., 2013; Rico et al., 2013).

For many years, *D. magna* has been recognised as a keystone species in the food webs of many continental water bodies, and

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has served as an important model for ecotoxicological research (Seda and Petrusek, 2011). The classic reproduction test with *D. magna* (OECD, 2012) lasts 21 days and involves only one generation of the microcrustacean. However, to better identify effects of prolonged exposure over generations at population level, various authors have performed multigenerational tests on daphnids (Arndt et al., 2014; Borgatta et al., 2015; Brennan et al., 2006; Dietrich et al., 2010; Vandegehuchte et al., 2010). Impacts on daphnid populations may reverberate across the whole aquatic ecosystem as they are principal grazers of algae and primary forage for fish in lentic in-land ecosystems (Colbourne et al., 2011). In a prior two-generation study on *Daphnia magna* (Dalla Bona et al., 2015) we observed a significant increase in EFX toxicity in F1 generation with respect to F0, even when the F1 generation had been returned to pure medium. In the current study a four-generation test was performed, in order to verify whether the trend persists into subsequent generations, thus leading to a progressive population level damage. To reproduce different possible scenarios across generations, both a continuous and an alternate exposure were explored. In multigenerational tests with alternate exposure scheme a direct exposure of embryos in the brood chamber to the tested substance is possible because daphnids actively exchange the fluid in the brood chamber for environmental water, in order to support embryonic oxygen demand (Abe et al., 2001). Furthermore, a brief exposition of neonates between their delivery by the mother and their collection and transfer to the clean solution should also be taken into account. For these reasons a perinatal exposure is unavoidable. Then, in order to verify whether the effects observed in those groups returned to pure medium could be explained by the perinatal antibacterial exposure, a reproduction test in pure medium with daphnids obtained from D. magna embryos exposed *in vitro* to EFX was also carried out.

2. Materials and methods

2.1. Test chemical

EFX was purchased from Sigma-Aldrich (Milano, Italy) (CAS number 93,106-60-6). Purity was $\geq$98%. Solubilisation in Aachener Daphnien Medium (ADaM: hardness 193 $mgCaCO_3$ L^{-1}) (Klüttgen et al., 1994a, 1994b) was achieved by returning the pH of the medium to its original value (8.0) using 1 M NaOH. The pH was measured using a BASIC 20 pH-meter (CRISON, Carpi, Italy). The concentration of EFX in stock solutions was checked using an already reported HPLC method (Dalla Bona et al., 2015) and was always in the range $\pm$7% of the nominal values. The stability of the compound in the conditions (Light, Temperature and Renewal Time) employed for the tests was previously verified (Dalla Bona et al., 2015). Therefore, test results were based on the nominal concentration (2 mg L^{-1}).

2.2. Culture conditions

Resting eggs of *Daphnia magna* were originally provided by ECOTOX (Milano, Italy). A single clone culture was selected based on its adequate sensitivity to potassium dichromate (ISO, 1996). The sensitivity was then checked periodically (every 4 months). The organisms were maintained in ADaM at 20 $\pm$ 1 °C, with a 16-h light (2.6 mE m^{-2} s^{-1}): 8-h dark photoperiod. They were fed three times per week with *Scenedesmus dimorphus* (8×10^5 cells mL^{-1}). Further details about the culturing method have already been reported (De Liguoro et al., 2012).

2.3. Four-generations reproduction test

A four generations reproduction test was conducted, adapted from the OECD Test Guideline 211 (OECD, 2012), with only a single concentration of EFX assayed, due to the complexity of the experimental design (see below). The chosen concentration (2 mg L^{-1}) was based on previous results (Dalla Bona et al., 2015) which indicated an EC_{20} of 2.7 mg L^{-1} for reproduction inhibition in F0.

Each test group was composed of 10 third-brood neonates $<$24 h old (OECD, 2012) allocated individually in a 150 mL beaker containing 50 mL either of the test solution (EFX 2 mg L^{-1}) or of pure ADaM, and incubated for 21 days at the same conditions (light, temperature) used for culturing. After exposing the group (E0) of the first generation, 20 specimens from the collected offspring (third-brood) were randomly assigned to two groups (n=10). The first group (EE1) was exposed again to EFX while the second one (EN1) was returned to clean medium. This continuous and alternate exposure scheme was to be followed across the four generations, therefore a total of 15 (1+2+4+8) test groups plus 4 control groups were originally scheduled (Fig. 1). However, where too few offspring were produced, due to excessive mortality and/or excessive inhibition of reproduction, the consequent tests on the subsequent generations were not carried out.

During each test, solutions were renewed every other day, the neonates removed and counted, and the feed (*S. dimorphus*, 8×10^5 cells mL^{-1}) supplied. Old (48-h) solutions were then pooled and monitored for pH, conductivity and dissolved oxygen using YSI 85 Multiparameter Instrument (YSI Incorporated, Yellow Springs, OH, USA).

2.4. Test on D. magna embryos

Gravid daphnids were collected from cultures and examined microscopically for the level of development of embryos in the brood chamber. Twenty specimens, carrying embryos in early development (stage 1) (LeBlanc et al., 2000), were selected for the experiment. The embryos were extracted by immobilizing the head of the adult with a dissecting probe while a second probe was used to gently free the embryos by separating the carapace (LeBlanc et al., 2000). Forty-eight of the collected embryos were randomly taken and individually transferred to each well of a 24-well Suspension Culture Plate (CELLSTAR, Greiner bio-one) containing either 2 mL of test solution (n=24) or 2 mL of ADaM (n=24). They were incubated for 3 days at the conditions (light and temperature) normally used for *D. magna* culture (see above). After incubation, the number of hatched embryos was recorded and 10 neonates obtained from exposed embryos (EFX 2 mg L^{-1}) and 10 obtained from controls were randomly taken and assigned to group 1 (perinatal exposure) and group 2 (controls) respectively. Daphnids from both groups were allocated to individual 150 mL beaker containing 50 mL of pure ADaM and incubated for 21 days in the chosen culturing conditions with medium renewed every other day. New neonates produced were removed and counted, and feed (*S. dimorphus*, 8×10^5 cells mL^{-1}) supplied. At the end of the test the growth rate, reproduction activity and mortality rate of the two groups were measured and compared.

2.5. Endpoints in multigenerational test

Evaluated endpoints were mortality, reproduction inhibition and growth inhibition.

Mortality among the parent animals was recorded every other day, at the same times as offspring were counted. An animal was recorded as dead when it was immobile, *i.e.* when was not able to swim, or if there was no observed movement of appendages or

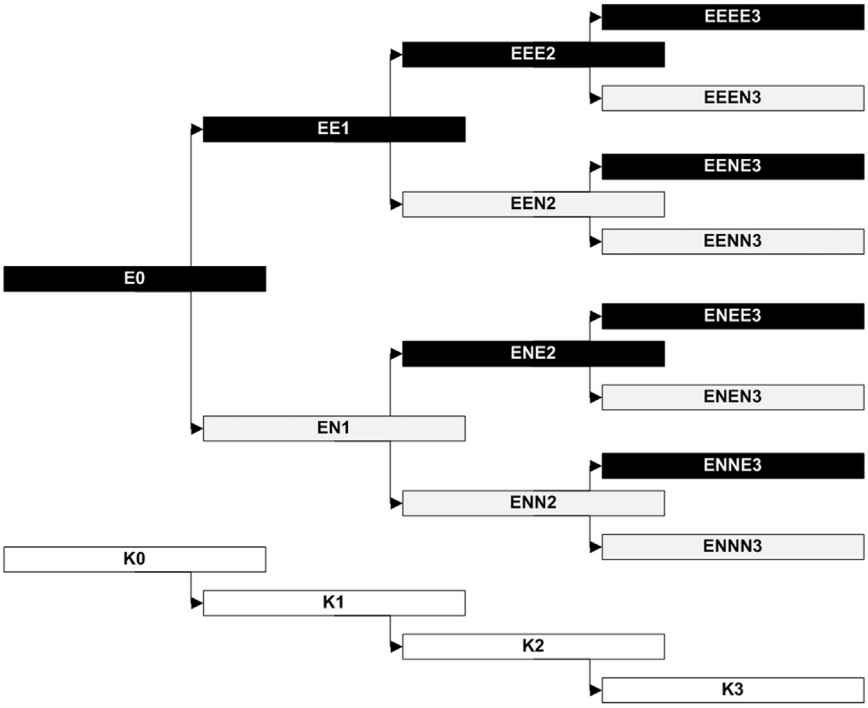

Fig. 1. Flow chart of the planned multigenerational test. Each bar represents a 21 d reproduction inhibition test (OECD, 2012). Black bars: groups ($n=10$) exposed to 2 mg L^{-1} EFX.Grey bars: groups ($n=10$) exposed to pure medium.White bars: controls.

postabdomen, within 15 s of gentle agitation of the beaker. Reproduction performance was measured as the average number of living offspring produced per parent animal alive at the end of the test. Before the beginning of each experiment, in order to measure growth inhibition, 30 offspring not intended for the test were isolated, fixed in 70% v/v ethanol and their length measured under a microscope. Length was defined as the distance from the upper edge of the compound eye to the base of the tail spine. At the end of each experiment adult daphnids were collected and measured as above, and their daily growth rates calculated.

Reproduction and growth were normalized to the control mean and inhibition rates were reported as percentages.

2.6. Statistical analysis

Data were analyzed using GraphPad Prism 5.02 (La Jolla, California, USA). The assumptions of normality and homogeneity of variances were tested by using the Shapiro-Wilk test and the Bartlett test respectively. Since data were normally distributed and variances were homogeneous, differences of reproduction and growth between groups in multigenerational test were assessed using the analysis of variance (ANOVA) followed by the Bonferroni post-hoc test. To infer differences in growth and reproduction between the control and the exposed group in the embryo test, the Student *T*-test was used. *P* values <0.01 were considered significant. Mortality data were reported as raw percentages.

3. Results and discussion

In all tests, the recorded values of water quality parameter were always within the following ranges: pH 7.8–8.2, dissolved oxygen 6.75–8.40 mg L^{-1}, and conductivity 1042–1184 μS cm^{-1}.

Mortality rate was already 50% in E0 and increased over subsequent generations, reaching 100% in three groups of the F3 generation groups, whilst remaining $\leq 20\%$ in the control groups (Table 1). In the perinatal exposed group of *in vitro* cultured *D. magna* embryos, mortality was 31.4% with no mortality observed during the subsequent reproduction test in pure ADaM (Table 2).

Reproduction inhibition in tested groups remained above 50%. F1 and F2, groups returned to pure medium showed a greater inhibition than the re-exposed ones (Table 1 and Fig. 2). In adult daphnids coming from the perinatal exposed group of *in vitro* cultured *D. magna* embryos, no significant reproduction inhibition was observed (Table 2).

No significant growth inhibition was observed in F0, F1 and F2 generations. In F3, due to the high mortality rate, the end point was evaluated only for the EENN3 group, which showed significant inhibition of growth (51%). In the perinatal exposed group of *in vitro* cultured *D. magna* embryos, no significant growth inhibition was observed (Table 2).

The current study indicates that prolonged exposure (continuous or alternating) to 2 mg L^{-1} of EFX may have a serious impact on *D. magna* population, while the classic (single generation) reproduction test had previously indicated a NOEC of 5 mg L^{-1} EFX for reproduction inhibition and adult survival of *D. magna* (Park and Choi, 2008). This underlines the importance of testing xenobiotic toxicity on multiple generations of daphnids. The increasing toxicity of EFX over four generations of *D. magna* was demonstrated not only by the progressive impairment of reproduction performances but also by the mortality rate that reached 100% in three out of four tested groups in the fourth generation. Reproduction performances of daphnids born to EFX-exposed mothers were invariably compromised, regardless of their subsequent incubation either in EFX-containing or pure medium. Apparently, the effect was even worse in the non-re-exposed groups (see Table 1). In particular, the high reproduction inhibition of the group EN1 made it impossible to continue the test along that specific arm of the experimental flow chart (Fig. 2) as the number of neonates harvested from the third brood was insufficient for this purpose.

Table 1
Multigenerational test with enrofloxacin (2 mg L^{-1}): mortality rate, reproduction performances and daily growth of *D. magna*.

Generation	Group	Mortality rate (%) $n=10$	Time to production of first brood (days)	Neonates per female (mean ± sd)	Reproduction Inhibition (%)	Daily growth, μm (mean ± sd)	Daily growth inhibition %
F0	*K0*	*20*	*9*	*96 ± 55*		*140 ± 9*	
	E0	50	9	34 ± 32	64.6**	127 ± 9	9.2
F1	*K1*	*0*	*10*	*109 ± 42*		*147 ± 12*	
	EE1	50	12	9 ± 20	91.7***	123 ± 23	16.3
	EN1	30	12	4 ± 9	96.3***	135 ± 16	8.1
F2	*K2*	*0*	*10*	*82 ± 31*		*138 ± 9*	
	EEE2	70	14	8 ± 15	90.2***	132 ± 7	4.3
	EEN2	70	14	4 ± 8	95.1***	114 ± 18	17.3
	ENE2	*NP*					
	ENN2	*NP*					
F3	*K3*	*20*	*9*	*113 ± 10*		*137 ± 10*	
	EEEE3	100	–	0	100***	–	–
	EEEN3	100	–	0	100***	–	–
	EENE3	100	–	0	100***	–	–
	EENN3	50	14	1 ± 2	99.1***	67 ± 2	51.1***
	ENEE3	*NP*					
	ENEN3	*NP*					
	ENNE3	*NP*					
	ENNN3	*NP*					

** $p < 0.01$;
*** $p < 0.001$; *NP* Not Performed.

Table 2
Mortality, reproduction performances and daily growth of *D. magna* after being exposed for three days to 2 mg L^{-1} of enrofloxacin (EFX) during embryonic development.

Group	Embryonic Mortality ($n=35$) (%)	Adult mortality ($n=10$) (%)	Neonates per female ($n=10$) (mean ± sd)	Daily growth ($n=10$) (mean ± sd)
EFX	31.4	0	118.70 ± 45.57	177 ± 0.3 μm
Control	0.03	0	121.70 ± 27.03	183 ± 0.3 μm

As already indicated, in multigenerational tests a perinatal exposure of daphnids born to exposed mothers is unavoidable. Therefore, it is important to clarify whether the toxic effects observed in 'non-exposed' groups are simply the consequence of perinatal exposure. On this regard, it is worth noting that strong inhibitory effects on reproduction were observed also in group EENN3 which was generated by 'non-exposed' mothers (group EEN2). Hence, the effects were not due to a perinatal exposure of the daphnids; a fact that was confirmed by the results of the targeted test on *D. magna* embryos where daphnids exposed to 2 mg L^{-1} EFX only during embryo development showed a reproduction performance equal to that of the control group. More specifically, perinatal exposure to EFX seemed to act as an 'all-or-nothing' toxicity effect as 31.4% of embryos died, but the surviving daphnids did not show any inhibition of reproduction activity. On the other hand, embryo mortality may at least partially justify the reproduction inhibition observed in exposed groups in the multigenerational test.

Altogether, there must be damage to adult daphnids exposed to 2 mg L^{-1} EFX that is transmitted to subsequent generations. Considering that EFX may exert genotoxic (Thomé et al., 2012) or epigenetic (Csoka and Szyf, 2009) effects in eukaryotes, transmission of genetic alterations or regulations to the *Daphnia* offspring can be hypothesized. In the real context, even if the pharmaceutical entity disappears from the aquatic environment, due to dilution and/or degradation, the *D. magna* population may pay the consequences of its historical presence, hence what might be intermittent exposure does not necessarily imply a reduction in risk for this crustacean.

Generally speaking, a fluoroquinolone (FQ) concentration such as the one evaluated in this experiment (2 mg L^{-1}) should be considered environmentally unrealistic, save in case of exceptional contaminations that may occur in relation to poorly controlled wastewaters from pharmaceutical factories (Gunnarsson et al., 2009) or excessive use of prophylactic treatments in aquaculture ponds (Le and Munekage, 2004). Nevertheless, given the toxicity trend observed over the generations in this study, the long-term survival of *D. magna* populations cannot be assured even at commonly detected concentrations. A number of different FQs, coming not only from veterinary metaphylactic treatments but also from human therapy may reach the aquatic environment (Oldenkamp et al., 2013; Seifrtová et al., 2009) and exert their effects additively (Dalla Bona et al., 2014; Van Doorslaer et al., 2014). The presence of complex mixtures of different FQ residues in water has been frequently reported (Speltini et al., 2010). Furthermore, the effects of FQs on organisms from different trophic levels must be considered. For example, EFX and its metabolite ciprofloxacin (CPX) are highly toxic to some photoautotrophic aquatic species, with EC_{50} for the cyanobacterium *Anabaena flos-aquae* and the aquatic macrophyte *Lemna minor* in the range of 10–173 μg L^{-1} (Ebert et al., 2011). Due to their tendency to accumulate in sediments (Andrieu et al., 2015), FQs may have an impact not only on bacterial populations but also on sediment dwelling organisms. Hence, the effects of FQs on crustaceans may be compounded by the perturbation these compounds can cause to the whole aquatic ecosystem.

4. Conclusions

The multigenerational test with daphnids was able to evidence population disturbances that official acute and chronic tests cannot. The increasing deterioration observed from one generation to the next might be the consequence of genetic or epigenetic alterations. Daphnids returned to pure medium failed to recover

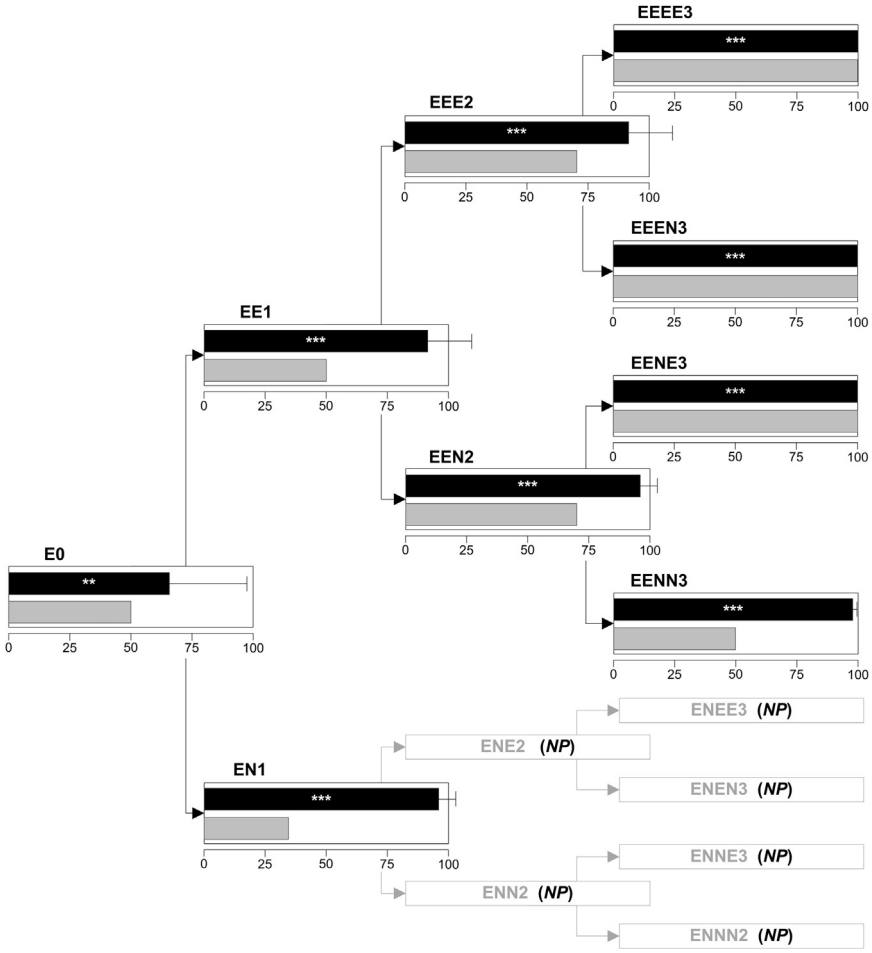

Fig. 2. Increasing mortality (grey bars, raw percentage) and reproduction inhibition (black bars, percentage of control mean, with SD) across four generations of *D. magna* exposed, continuously or alternately, to 2 mg L^{-1} EFX. ** $p < 0.01$;*** $p < 0.001$; *NP* Not Performed.

from reproduction inhibition, indicating that with EFX, intermittent exposure scenarios are not necessarily less harmful than continuous ones. Whilst the concentration tested (2 mg L^{-1}) is higher than those usually detected in the aquatic environment, the increasing toxicity over generations and the possible additive toxicity of FQ mixtures, prevent the exclusion of probable harm to crustacean populations in the real context. Further investigations are needed to clarify the mechanism of EFX transgenerational toxicity and to verify the additive or synergistic effects of FQ mixtures, particularly within a multigenerational frame of reference.

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