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Effects of enrofloxacin, ciprofloxacin, and trimethoprim on two generations of *Daphnia magna*

Mirco Dalla Bona [a,*], Radka Zounková [b], Roberta Merlanti [a], Ludek Blaha [b], Marco De Liguoro [a]

[a] Department of Comparative Biomedicine and Food Science, University of Padua, Italy
[b] Masaryk University, Faculty of Science, RECETOX, Kamenice 5, 62500 Brno, Czech Republic

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ABSTRACT

Multigenerational tests on *Daphnia magna* were performed exposing two subsequent generation to enrofloxacin (EFX) and its metabolite ciprofloxacin (CPX), and to trimethoprim (TMP). Mortality rate of 100% and 50% was detected in F0 at concentrations of ≥ 13 mg L^{-1} (EFX) and 50 mg L^{-1} (TMP), respectively. In F1 with respect to F0, both for growth and reproduction, a worsening trend of the response with EFX, a similar response with CPX and an attenuating trend with TMP was observed. Furthermore, the lowest EC_{20} for reproduction inhibition (1.3 mg L^{-1}) was calculated for F1 exposed to EFX. However, other experimentations, longer and more complex, are necessary in order to confirm that EFX is more hazardous to daphnids than CPX and TMP. EC_{50} measured for the three assayed antibacterials were in the 6.5–37 mg L^{-1} range therefore environmental unrealistic, except in case of exceptional contaminations that may occur in relation to poorly controlled wastewaters from pharmaceutical factories or excessive use of prophylactic treatments in aquaculture.

1. Introduction

Veterinary pharmaceuticals, in particular antibacterials, are pollutants of relatively new concern. Most of them can reach the aquatic environment via different pathways, principally as a consequence of the use in aquaculture or in livestock treatments. Emissions during manufacturing and improper disposals also play a role in the environmental burden of these compounds (Boxall et al., 2004; Halling-Sørensen et al., 1998). Notwithstanding the enforcement, in some developed countries, of legislations aimed to avoid the overuse of antibacterials in food animals (EC, 2005; Marshall and Levy, 2011), the global consumption of these compounds is constantly increasing, with an estimated annual rate of 4% (Hamad, 2010).

In EU fluoroquinolones (FQs) and trimethoprim (TMP), account for 3.2% of antibacterial agents sold for food producing animals (EMA, 2011). Residues of FQs and TMP have been found worldwide in surface waters, the concentrations usually ranging from 10 ng L^{-1} to 10 μg L^{-1} (Santos et al., 2010) with exceptional peaks (mg L^{-1}) detected in effluents from drug manufactures (Larsson et al., 2007) and aquaculture facilities (Le and Munekage, 2004).

FQs mechanism of action is directed toward bacterial DNA-gyrases and topoisomerase IV (Martinez et al., 2006). However, as a consequence of their weak affinity also with eukaryotic topoisomerase, FQs can cause DNA damage both to vertebrate and invertebrate organisms (Albertini et al., 1995; Pommier et al., 2010; Thomé et al., 2012). Trimethoprim (TMP) disrupts the synthesis of DNA by competitively inhibiting dihydrofolate reductase, which catalyses the conversion of dihydrofolate to tetrahydrofolate (Abou-Eisha et al., 1999). It is largely employed both in veterinary and human medicine, alone or in association with sulphonamides. Genotoxic effects of TMP have been reported in aquatic non-target organisms and justified as an indirect consequence of the disruption of DNA synthesis (Abou-Eisha et al., 1999; Binelli et al., 2009). Other crucial aspects of FQs regard their persistence and the toxicity of metabolites. In fact, even though FQs are not particularly stable when exposed to light (Knapp et al., 2005; Sturini et al., 2010), their ready sorption to soil particles may lead to the accumulation and to the subsequent slow desorption and contamination of the aquatic environment (Picó and Andreu, 2007). Furthermore, FQs metabolic and photolysis products may be active and, in some cases, even more toxic than the parent compounds (Li et al., 2011).

Due to its high efficacy and wide spectrum, the veterinary FQ enrofloxacin (EFX) is used worldwide for livestock diseases and, in some countries, also for aquaculture treatments (Quesada et al., 2013; Rico et al., 2013). In shallow waters, EFX photodegrades to

* Corresponding author. Fax: +39 049 827 2973.
E-mail address: mircodb@gmail.com (M. Dalla Bona).

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Table 1
Some physico-chemical properties of enrofloxacin (EFX), ciprofloxacin (CPX), and trimethoprim (TMP).

Compound	CAS number	Log Kow	Water solubility (g L^{-1})	pKa
EFX	93106-60-6	1.88[a]	130[b]	6.38[b]
CPX	85721-33-1	0.65[a]	30[b]	6.27[b]
TMP	738-70-5	0.91[c]	0.5[c]	6.6[c]

[a] Predicted ACD/Lab (Chemspider, 2014).
[b] Nowara et al. (1997).
[c] Pérez et al. (2005)

its main metabolite Ciprofloxacin (CPX) (Knapp et al., 2005; Li et al., 2011), a pharmaceutical mainly used in human medicine.

Based on their occurrence in the environment and the available toxicity data, various authors have suggested that CPX (Kim et al., 2008), EFX (Boxall et al., 2003) and TMP (Capleton et al., 2006) are likely to be hazardous for the environment and/or for human health, thus representing a priority as drugs for in depth environmental risk assessment. Their physico-chemical properties are reported in Table 1.

Multigenerational tests are time consuming and laborious, and only few research studies have thus far applied this kind of tests in aquatic ecotoxicology (Chen et al., 2014; Dietrich et al., 2010; Kim et al., 2012; Lamichhane et al., 2013; Tsui and Wang, 2005; Vandegehuchte et al., 2010). Nevertheless, when compared to the acute toxicity test or to the one-generation chronic test, they allow to obtain a more representative picture of the population effects of tested compounds. This may be of particular value with antibacterials, the real pattern of environmental exposure to them being low throughout the entire life cycles over numerous generations (Kim et al., 2012). Effects on *Daphnia* population may reverberate on the whole aquatic ecosystem as waterfleas are principal grazers of algae and primary forage for fish in lentic inland ecosystems (Colbourne et al., 2011).

In this work, lethal and sub-lethal (inhibition of reproduction and growth) effects of EFX, CPX, and TMP were evaluated throughout two generations of *Daphnia magna.*

2. Materials and methods

2.1. Test chemicals

EFX (CAS number 93106-60-6), CPX (CAS number 85721-33-1), and TMP (CAS number 738-70-5) were purchased from Sigma-Aldrich (Milano, Italy); their purity ranged between 95% and 99%. Their solubilisation in Aachener Daphnien Medium (ADaM: hardness 193 mg $CaCO_3$ L^{-1}) (Klüttgen et al., 1994a, 1994b) was achieved by bringing back the pH of the medium to the original value (8.0) using 1 M NaOH or HCl. The pH was measured using a BASIC 20 pH-meter (CRISON, Carpi, Italy).

2.2. Analytical confirmation

To check for the stability of the compounds, 3 samples of freshly prepared and 3 samples of old test solutions were collected during each test, at renewal time, from the highest and lowest concentration tested. As the ADaM medium did not interfere with the analysis, extraction and purification were unnecessary. Samples were simply filtered through Phenex-PTFE 0.45 µm (Phenomenex, Castel Maggiore, Italy) and then analysed by means of HPLC. The HPLC system (Jasco, Tokyo, Japan) consisted of a PU-980 HPLC pump equipped with an LG-980-02 ternary gradient unit, an UV-975 detector and an AS-950 autosampler. The applied analytical columns were a Zorbax XDB C18 (Agilent Technologies, USA) for TMP and a Sinergy Fusion RP (Phenomenex, Castel Maggiore, Italy) for FQs. Analytical conditions for TMP were the following: injection volume 10 µL, mobile phase in isocratic elution composed by 85% of a 25 mM solution of orthophosphoric acid (pH 3) and 15% acetonitrile, with a 0.5 mL min^{-1} flux with the detector UV-975 (Jasco, Tokyo, Japan) set at 268 nm. Analytical conditions for FQs were the following: injection volume 10 µL, mobile phase consisting of (A) 25 mM solution of orthophosphoric acid (pH 3) and (B) acetonitrile in gradient elution, fluorescence detector FP-920 (Jasco, Tokyo, Japan) set at 280 nm excitation and 450 nm emission. Mobile phase composition (A/B, v/v) was 80:20 from 0 to 4 min, 20:80 from 8 min to 10 min, 80:20 from 12 to 14 min. For each compound, the linearity of response was verified in the 0.050–6.3 mg L^{-1} range by analysing, in triplicate, control solution spiked with 5 concentration of the drug. In order to match this concentration range, samples were accordingly diluted in HPLC mobile phase before injection.

2.3. 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 ± 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).

2.4. Chronic test

The OECD 211 test guideline (OECD, 2012) was applied both to the first and to the second generation of daphnids. The first generation (F0) was composed of neonates produced by the monoclonal culture between second and fifth broods, while the second generation (F1) was composed of neonates produced by F0 during the last day (21st) of exposition to each pharmaceutical.

An adequate volume of stock solution of each compound was prepared at the beginning of each test by dissolving the drug in ADaM medium at a concentration higher than the maximum concentration to be tested. Each stock solution was stored at 4 °C in the dark and, prior to use, was diluted accordingly in ADaM to obtain the various concentrations required for the test. For each exposure level (control included) 10 daphnids were used, each allocated in a 150 mL beaker containing 50 mL of test solution. During the tests, solutions were renewed every other day, the neonates removed and counted, and feed supplied. F0 daphnids were exposed for 21 days to 5 scaled (with a factor of 2) concentrations of the three drugs in the following ranges: 1.6–25 mg L^{-1} (EFX), 1.9–30 mg L^{-1} (CPX) and 3.1–50 mg L^{-1}. During the following 21 days, F1 daphnids were either exposed to the same concentration as their parents (F1e) or returned to a clean medium (F1n) (Fig. 1). This design has been used in order to assess both the cumulative effects of the drugs after two generations exposition and the capacity of the crustaceans to recover from the parental and perinatal exposure. However, where few offspring were produced, due to excessive mortality and/or excessive reproduction inhibition in F0, the test on F1 was not carried out.

2.5. End points measurement

The evaluated end points were mortality, growth inhibition and reproduction inhibition. Before the beginning of each experiment, in order to measure growth inhibition, 30 offspring not intended

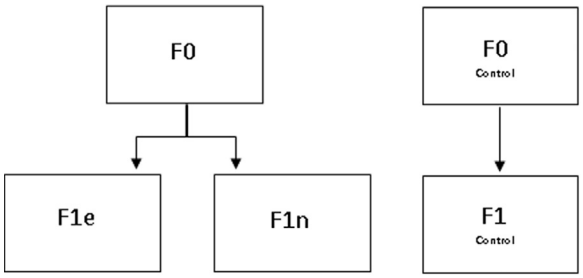

Fig. 1. Experimental design of two generation test on *D. magna*. F0, exposure group; F1e offspring re-exposed at the parental conditions; F1n offspring returned to clean medium; $F0_{control}$ controls of first generation, and $F1_{control}$ controls of second generation.

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.

2.6. Statistical analysis

Data were analysed using GraphPad Prism® 5.02 (La Jolla, California, USA). EC_{20} and EC_{50} for reproduction inhibition and average daily growth were obtained from a four-parameter logistic dose response model ($Y = Bottom + (Top - Bottom)/(1 + 10^{((Log\ EC_{50-X}) \times Hill\ Slope)})$) with the top and the bottom asymptote fixed to 100% and 0% effect. Since data were normally distributed (Shapiro–Wilk test) and variances homogeneous (Bartlett test), LOEC and NOEC were determined using the analysis of variance (ANOVA) followed by the Bonferroni post-hoc test.

2.7. Water quality parameters

Dissolved oxygen, conductivity and pH were checked at the time of each renewal by using a YSI 85 Multiparameter Instrument (YSI Incorporated, Yellow Springs, OH, USA) and a BASIC 20 pH meter (CRISON, Carpi, Italy).

3. Results

3.1. Analytical confirmation

Calibration curves were linear over the entire concentration range (0.050–6.3 mg L^{-1}) with a correlation coefficient that always exceeded 0.996. Concentration measured in fresh and spent solutions are reported in Table 2. As the concentration of the three tested substances was maintained within ± 20% of the initial concentration, test results were based on nominal values (OECD, 2012).

3.2. Water quality parameters

In all tests the recorded values were always within the following ranges: pH 7.6–8.4, dissolved oxygen 6.54–8.40 mg L^{-1}, and conductivity 1005–1120 µS cm^{-1}.

3.3. Mortality

Survival in the control groups was always ≥ 80%. The F0 generation exposed to 13 mg L^{-1} and 25 mg L^{-1} of EFX showed 100% mortality while 50% mortality was observed in F0 exposed to 50 mg L^{-1} of TMP and 30% mortality in F0 exposed to 30 mg L^{-1} of CPX. Some mortality was detected also in F1e and F1n born to the EFX exposed F0, with an apparent negative correlation between mortality and parental exposure in F1n (Table 3).

3.4. Inhibition of reproduction

In F0 a significant inhibition of reproduction was observed with 6.3 mg L^{-1} of EFX, 30 mg L^{-1} of CPX and ≥ 13 mg L^{-1} of TMP (Table 3). In F1e significant inhibitory effects were observed at concentrations of ≥ 3.1 mg L^{-1} EFX and 30 mg L^{-1} CPX (Table 3). In F1n, only the group exposed to 6.3 mg L^{-1} of EFX showed a significant decrease of reproduction. The EC_{20} and EC_{50} values for reproduction inhibition are reported in Table 4 while Fig. 2 shows the reproduction inhibition curves for EFX, CPX and TMP.

3.5. Average daily growth

Significant reduction in daily growth was observed in F0 when exposed to 3.1 mg L^{-1} and 6.3 mg L^{-1} of EFX, 30 mg L^{-1} of CPX and ≥ 13 mg L^{-1} of TMP (Table 3). The F1e daphnids were inhibited when exposed to 6.3 mg L^{-1} of EFX or to 30 mg L^{-1} of CPX. Only F1n generated by mothers exposed to 6.3 mg L^{-1} of EFX showed a significant decrease in average daily growth (Table 3). EC_{50} and EC_{20} for growth inhibition were calculated only among the F0 groups exposed to TMP, as with the other groups the inhibition was generally weak (Table 3).

4. Discussion

For obvious reasons, both high mortality and strong reproduction inhibition represent an obstacle to the performing of multigenerational tests, particularly when they already occur in F0. In the present study, only with CPX it was possible to carry on the test on F1 at all five exposure levels, while only three concentrations were assayed with EFX and TMP due to the excessive mortality or reproduction inhibition observed in F0. Consequently, EC_{50} measured in F1 for EFX and TMP were obtained from curves fitted on only three points and were rather approximate.

Table 2
Analytical results for enrofloxacin (EFX), ciprofloxacin (CPX) and trimethoprim (TMP): average recovery, correlation coefficient of the calibration curves and concentrations measured in fresh and spent solutions.

Test chemical	Recovery from spiked blank solutions (%)	Calibration curve (R^2)	Nominal concentration (mg/L^{-1})	Concentration measured in fresh solution ($n=3$)	Concentration measured in spent solution ($n=3$)
EFX	85 ± 4	0.996	1.6	1.6 ± 0.18	1.6 ± 0.18
			25	24 ± 1.0	24 ± 0.87
CPX	88 ± 3	0.997	1.9	1.8 ± 0.13	1.8 ± 0.11
			30	32 ± 0.04	31 ± 1.0
TMP	92 ± 7	0.997	3.1	3.1 ± 0.16	3.1 ± 0.14
			50	49 ± 0.25	49 ± 0.33

Table 3
Survival rate, offspring production and average daily growth of *Daphnia magna* ($n=10$) exposed to enrofloxacin (EFX), ciprofloxacin (CPX) and trimethoprim (TMP) during two generations.

Copmpound	Generation	Level of exposure (mg L^{-1})	Survival (%)	Total number of neonates	Neonates per female (mean ± SE)	Average daily growth (length, μm ± SE)
EFX	F0	Control	90	1038	115 ± 8.7	154 ± 2.5
		C5 (1.6)	100	962	96 ± 7.0	149 ± 2.1
		C4 (3.1)	100	957	96 ± 7.9	143 ± 1.4**
		C3 (6.3)	80	447	56 ± 6.6***	131 ± 2.8***
		C2 (13)	0	–	–	–
		C1 (25)	0	–	–	–
	F1e	Control	80	770	97 ± 13	149 ± 5.8
		C5 (1.6)	90	620	69 ± 6.6	142 ± 2.6
		C4 (3.1)	100	596	60 ± 8.0*	143 ± 2.3
		C3 (6.3)	50	143	29 ± 13***	100 ± 12***
	F1n	C5 (0)	40	320	80 ± 19	147 ± 8.0
		C4 (0)	60	394	66 ± 13	152 ± 3.1
		C3 (0)	70	107	15 ± 5.3***	97 ± 8.4***
CPX	F0	Control	100	1183	118 ± 7.5	154 ± 1.8
		C5 (1.9)	100	1261	126 ± 14	156 ± 2.2
		C4 (3.8)	100	1362	136 ± 11	156 ± 2.2
		C3 (7.5)	100	817	82 ± 13	146 ± 8.0
		C2 (15)	90	866	96 ± 7.0	128 ± 1.8
		C1 (30)	70	314	45 ± 6.4***	127 ± 2.0***
	F1e	Control	100	858	113 ± 13	152 ± 2.1
		C5 (1.9)	100	1016	102 ± 10	161 ± 1.8
		C4 (3.8)	100	1396	140 ± 14	160 ± 3.2
		C3 (7.5)	80	989	124 ± 8.6	161 ± 2.1
		C2 (15)	80	879	110 ± 9.3	156 ± 2.5
		C1 (30)	100	775	78 ± 5.6***	143 ± 2.2*
	F1n	C5 (0)	100	1138	114 ± 3.1	155 ± 1.3
		C4 (0)	100	1267	127 ± 5.9	158 ± 1.6
		C3 (0)	100	1121	112 ± 14	156 ± 2.2
		C2 (0)	100	1173	117 ± 6.5	156 ± 1.3
		C1 (0)	100	1050	105 ± 7.9	154 ± 1.7
TMP	F0	Control	100	1298	130 ± 5.2	168 ± 2.4
		C5 (3.1)	80	962	120 ± 12	165 ± 4.8
		C4 (6.3)	90	1119	124 ± 4.1	159 ± 1.5
		C3 (13)	100	1040	104 ± 3.2*	156 ± 2.2*
		C2 (25)	80	34	4.3 ± 2.6***	120 ± 1.8***
		C1 (50)	50	0	0***	60 ± 4.2***
	F1e	Control	100	1506	151 ± 10	166 ± 2.7
		C5 (3.1)	100	1411	141 ± 5.4	159 ± 1.8
		C4 (6.3)	90	1198	133 ± 2.7	160 ± 1.9
		C3 (13)	100	1334	133 ± 10	162 ± 2.8
	F1n	C5 (0)	100	1308	131 ± 2.6	161 ± 2.1
		C4 (0)	100	1351	135 ± 3.6	165 ± 1.5
		C3 (0)	90	966	129 ± 13	158 ± 2.2

* Significantly different from the controls $p < 0.05$.
** Significantly different from the controls $p < 0.01$.
*** Significantly different from the controls $p < 0.001$.

4.1. Mortality

In order to avoid lethal effects, tested concentrations of the three compounds were chosen taking into account available data on their acute toxicity in *D. magna*. However a 100% mortality rate was recorded when exposing F0 daphnids to 13 or 25 mg L^{-1} of EFX. During the chronic test, these lethal effects were already evident after 48 h of exposition; this does not correspond to findings of Robinson et al. (2005) claiming absence of lethal effects in *D. magna* exposed for 48 h to 10 mg L^{-1} of EFX. More recent data (Dalla Bona et al., 2014) indicate for EFX an acute EC_{50} of 16 mg L^{-1} in *D. magna* which may explain the 100% mortality observed in the chronic test and allows the calculation of an ACR (EC_{50} acute/EC_{50} chronic ratio) of 2.5. A similar ACR (3.5) can be calculated for CPX (EC_{50} 87 mg L^{-1}) (Dalla Bona et al., 2014) and an ACR of 5.6 was reported for ofloxacin in *Ceriodaphnia dubia* (Isidori et al., 2005). These values are quite low when compared with those obtained by various authors testing other classes of antibacterials on *D. magna* (De Liguoro et al., 2009; Wollenberger et al., 2000; Zounková et al., 2011) and underline the difficulty of obtaining complete data from chronic tests on daphnids with FQs. As a consequence, while the chronic test with EFX showed limitations due to the excessive mortality, the one with CPX was able to show only slight effects on reproduction.

4.2. Reproduction and average daily growth

Previous studies have already considered the chronic effects of TMP and EFX on *D. magna* (De Liguoro et al., 2012; Park and Choi, 2008) outlining toxicity effects at concentrations similar to those obtained in the present study with F0. In particular, Park and Choi (2008) reported no effects on reproduction in daphnids exposed to 5 mg L^{-1} of EFX and to 6 mg L^{-1} of TMP, while De Liguoro et al. (2012) indicated an EC_{50} for TMP reproduction inhibition

Table 4
Two generations chronic test: EC_X with 95% confidence interval (mg L^{-1}) and slope of dose/response curves for the assayed antibacterials.

		Reproduction			Growth		
		EC_{20} (CI 95%) mg L^{-1}	EC_{50} (CI 95%) mg L^{-1}	Slope	EC_{20} (CI 95%) mg L^{-1}	EC_{50} (CI 95%) mg L^{-1}	Slope
EFX	F0	2.7 (1.8–4.0)	6.5 (4.5–9.4)	1.6	–	–	
	F1e	1.3 (0.57–2.8)	3.9 (2.5–5.9)	1.3	–	–	
	F1n	2.5 (1.6–3.8)	3.8 (2.9–4.9)	3.3	–	–	
CPX	F0	11 (6.4–19)	24 (16–38)	1.7	–	–	
	F1e	24 (15–36)	36 (23–58)	3.2	–	–	
	F1n	–	–		–	–	
TMP	F0	13 (11–14)	15 (13–18)	7.0	20 (19–22)	38 (36–41)	2.2
	F1e	–	–		–	–	
	F1n	–	–		–	–	

(8.2 mg L^{-1}) which is slightly lower than the 15 mg L^{-1} obtained in this study (Table 4). However, those studies did not consider the exposure of subsequent generations, which better represents the real environmental conditions. Instead, no data were available regarding chronic effects of CPX on *D. magna* and the EC_{50} calculated in F0 (Table 4) shows that this compound is less toxic than EFX. Considering that the two compounds have an identical mechanism of action, a possible explanation may be that CPX being more polar and hydrophilic than EFX is less absorbed by daphnids. Thus, the EFX metabolization and/or degradation to CPX may represent a favourable event for daphnids. However, the effective concentrations determined in the current study are not environmentally realistic (Santos et al., 2010), except in very rare cases (Larsson et al., 2007; Le and Munekage, 2004).

Interestingly, effect levels for reproduction inhibition in F1, when compared to those obtained in F0, showed a different trend for each of the three compounds. With EFX there was correspondence to F0 both in F1e and F1n. With CPX, only F1e showed corresponding results to F0. With TMP there was no correspondence between the two generations, as no effects were observed in F1. Analogous considerations can be made with the three compounds also for growth inhibition effects. The latter are frequently observed after exposition of daphnids to toxicants and may be explained by the 'principle of allocation' (Kim et al., 2012).

The effects observed with EFX in F1n, notwithstanding the return of the offspring to clean medium, have already been observed with other contaminants such as uranium and microcystin (Massarin et al., 2010; Ortiz-Rodríguez et al., 2012) and may be the consequence of the perinatal exposition of daphnids to the antibacterial. As suggested by Abe et al. (2001), a direct exposure of embryos in the brood chamber to environmental pollutants is possible because daphnids actively exchange the fluid in the brood chamber for environmental water, in order to support embryonic oxygen demand. Furthermore, in multigenerational tests, 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. Another hypothesis could be that the exposed mothers, due to the stress caused by the toxicant, produced an offspring weaker than that produced by the controls. However, at least the body length of F1 newborns was not significantly different from that of controls. Finally, considering that EFX may

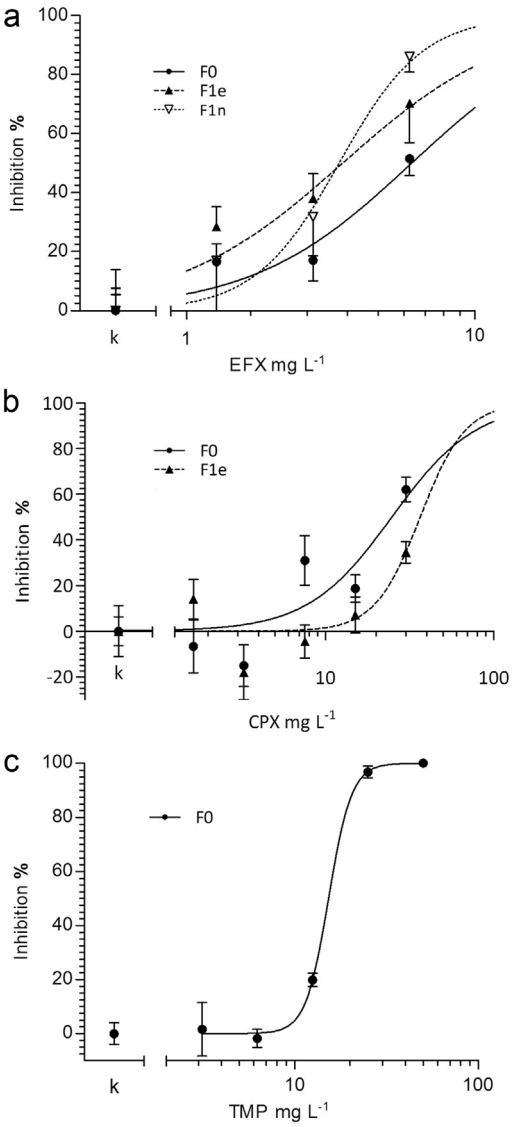

Fig. 2. Dose response curves for inhibition of reproduction. Error bars represent standard error of the means. (a) Enrofloxacin; (b) ciprofloxacin; and (c) trimethoprim.

exert genotoxic (Thomé et al., 2012) or epigenetic (Csoka and Szyf, 2009) effects in eukaryotes, the possible transmission of genetic alterations to the offspring cannot be excluded.

The absence of effects observed in F1e with TMP points to a certain degree of adaptation throughout generations or, more probably, to acclimatisation in F1e. However, as the resistance to TMP was observed in F1e but not in F0, a possible explanation for this difference can be that a prenatal exposure to drugs is necessary for daphnids to acclimatise to these toxic substances (Dietrich et al., 2010). The phenomenon has already been observed in *D. magna* with various compounds including for example diclofenac, ethinylestradiol and mercury chloride (Dietrich et al., 2010; Tsui and Wang, 2005). More specifically, Dietrich et al. (2010) studied effects on reproduction of different pharmaceuticals along 6 generations of *D. magna* and showed that, with some compounds (carbamazepine, metoprolol, diclofenac, ethinylestradiol) after an initial acclimatisation in F1 and F2, the effects can reappear in the subsequent generations. This event, which the authors explained with the difficulty of the organisms to sustain the resistance energetic cost, might also be possible with TMP. Thus, the absence of effects in the F1 generation does not necessarily

imply the possibility of the population of tolerating the assayed levels of TMP after prolonged exposures.

Less interesting were the results obtained with CPX where, as it could be expected, the F1e presented a sensitivity comparable to that of F0 while no effects were observed in F1n.

5. Conclusions

EC_{50} measured for the three studied antibacterials were in the 6.5–37 mg L^{-1} range and therefore environmental unrealistic, save in case of exceptional contaminations that may occur in relation to poorly controlled wastewaters from pharmaceutical factories or excessive use of prophylactic treatments in aquaculture.

Taking the uncertainty in long-term toxicity tests into consideration and the uncertainty in estimating EC values based on only three concentrations, the two generation study provided indication of trends that may be critical for the toxicity of the three compounds at population level. In F1 with respect to F0, both for growth and reproduction, the observed responses differed by test substance; a worsening response to EFX, an equivalent response to CPX and an attenuating response to TMP. This could lead to the conclusion that, at least for *D. magna* population, EFX is more hazardous than the other two compounds. However, other experimentations, longer and more complex, are necessary in order to confirm this conclusion because previous works from other authors, performed on several generations of the crustacean, have shown that the trend exhibited by F1 is not necessarily maintained throughout the subsequent generations.

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