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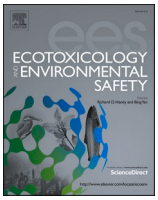

# Delayed toxicity of three fluoroquinolones and their mixtures after neonatal or embryonic exposure, in *Daphnia magna*

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A R T I C L E I N F O

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A B S T R A C T

Fluoroquinolones (FQs) are antibacterial drugs, used both in human and veterinary medicine, that are currently considered as emerging micropollutants. This study investigated the delayed toxic effects of enrofloxacin (ENR), flumequine (FLU), levofloxacin (LEV) and their binary mixtures in *D. magna*. For this purpose, a 10-day follow-up in pure medium was added to the standard *D. magna* immobilization test. During this follow-up, phenotypic alterations were evidenced, which were related to scarce or zeroed egg production and early mortality. Consequently, the $EC_{50\ s}$ recalculated at the end of the follow-up were always remarkably lower than those obtained after the 48 h immobilization test: ENR 3.13 vs. 16.72 mg $L^{-1}$; FLU 7.18 vs. 25.35 mg $L^{-1}$; LEV 15.11 vs. > 40 mg $L^{-1}$. To analyse the possible interactions within the binary mixtures, the method of nonlinear additive isoboles was applied. The three compounds showed invariably to follow the principle of concentration addition. Furthermore, as previous experiments showed toxicity of FLU and ENR after embryonic exposure of *D. magna* at a concentration of 2 mg $L^{-1}$, an additional two embryonic tests were conducted with identical design: one with 2 mg $L^{-1}$ LEV and the other with a ternary mixture containing 0.66 mg $L^{-1}$ of each of the three FQs. The embryos were exposed for three days in vitro to the drug solutions and were then reconducted to pure medium for 21 days observation. Both the tests ended-up with only non-significant effects on growth and reproduction, confirming the lower toxicity of LEV, when compared to ENR and FLU, and the absence of any evident synergistic interaction among the three FQs. Overall, these studies have shown two relevant features related to the toxicity of the three FQs: (1) they give rise to delayed toxic effects in *D. magna* that are undetectable by the standard immobilization test; (2) their interaction in mixtures follow the principle of Concentration Addition. Both these indications concern the Environmental Risk Assessment of FQs and may be of interest to regulatory authorities.

## 1. Introduction

Antibacterial drugs play a crucial role in the treatment and prevention of diseases in human and veterinary medicine. In recent decades, global consumption of these compounds has increased drastically (Klein et al., 2018) and there is growing concern about the potential impact on biota given their continuous release into the environment (Kümmerer, 2009; Tijani et al., 2013).

The environmental prevalence of these agents has been increasingly evident thanks to the progress of analytical chemistry, and their possible harmful effects on ecosystems made clear by the development of refined ecotoxicity tests. It is now straightforward for toxicologists to evidence the sublethal effects that may be critical for the dynamics of wild populations (Beiras, 2018).

In intensive animal farming, antibacterials are used mainly for mass prophylactic/metaphylactic treatment. Their use as growth promoters was banned in the EU in 2006 (Sarmah et al., 2006), but has continued until recently in many countries outside the EU, China and India included (Laxminarayan et al., 2015). Use of any of these agents generates a non-negligible environmental load of either parent compounds or active metabolites because slurry and manure are used for soil fertilization (Boxall et al., 2003a). Antibacterials can be transferred from contaminated soils to surface water through ground seepage and/or runoff (Tarazona et al., 2010). Another significant, more direct impact of antibacterials on surface water, relates to fish farming.

In addition, the use of antibacterials in humans results in the contamination of municipal sewage, from both households and hospitals. This can be transferred to surface water through deliberate or accidental sewer overflow during periods of high rainfall (Laville, 2021) or inefficient wastewater treatment (Vieno et al., 2007). Another

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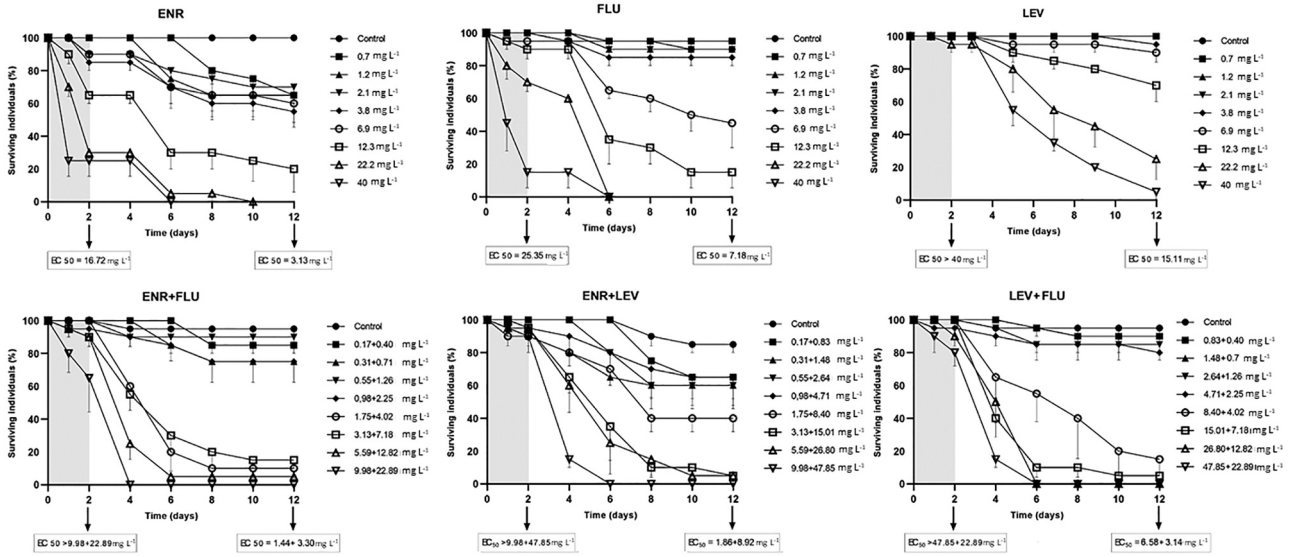


**Fig. 1.** Survival curves of *D. magna* over 48 h exposure to fluoroquinolones, individually or in binary combinations, with 10 days follow-up in pure medium. Error bars show standard error (ENR, enrofloxacin; FLU, flumequine; LEV, levofloxacin).

possible source of surface water contamination is discharge from pharmaceutical manufacturing facilities, particularly in countries with less stringent controls (Scott et al., 2018).

Fluoroquinolones (FQs) have been widely used both in human (Hamad, 2010), and veterinary medicine (EMA, 2011). They act through the inhibition of bacterial DNA-gyrase and topoisomerase IV, with consequent bactericidal action (Martinez et al., 2006). In human medicine, they are mainly used in the treatment of urinary and respiratory infections, whilst their primary indication in farm animals is the control of diseases such as colibacillosis, salmonellosis, mycoplasmosis and pasteurellosis.

Significant factors that contribute to the potential environmental impact of FQs are the persistence of the parent compounds and the toxicity of their metabolites. Indeed, both can persist and accumulate in soil and sediments (Sukul and Spiteller, 2007; Gao et al., 2012). Furthermore, when FQs are degraded by sunlight, their photolysis products may also be active, and can be even more toxic than the parent compounds in some cases (Li et al., 2011).

Given their environmental prevalence, FQs have been detected repeatedly in surface water (Gunnarsson et al., 2009; Santos et al., 2010) and the most troubling agents have been given high priority for environmental risk assessment (Boxall et al., 2003b; Kim et al., 2008). Whilst actual concentrations in watercourses and standing water are usually very low (ng to μg per liter), there are notable exceptions where remarkably high levels of contamination occur. Concentrations as high as mg $L^{-1}$ have been evidenced in relation to manufacturing facilities (Larsson et al., 2007; Gothwal and Thatikonda, 2017) or fish farming (Le and Munekage, 2004). Moreover, given their tendency to adsorb to solid matrices, higher concentrations of FQs are generally detected in freshwater sediments, from 210 ng $kg^{-1}$ up to 20 mg $kg^{-1}$, with an average of 760 μg $kg^{-1}$ (Van Doorslaer et al., 2014).

The potential environmental impact of three different FQs, Flumequine (FLU), Enrofloxacin (ENR) and Levofloxacin (LEV) was considered in this study. FLU and ENR are largely used in farm animals, whilst LEV, together with Ciprofloxacin (a metabolite of ENR), are amongst the most widely-used FQs in human medicine. The cladoceran crustacean *Daphnia magna* was the model organism selected for testing the ecotoxicity of the three FQs, given its well documented sensitivity to this class of pharmaceuticals (Isidori et al., 2005; Yang et al., 2013; Dalla Bona et al., 2016; De Liguoro et al., 2019).

Preliminary, acute immobilisation tests (OECD, 2004) run in our laboratory showed lethargic behavior of *D. magna* after 48 h exposure to FLU or ENR. The same observation was made by Robinson and coll (2005) for both FLU and clinafloxacin. In order to investigate any possible delayed toxic effects of ENR, FLU and LEV, in this study our experimental design was based on a standard 48 h exposure with a follow-up of ten days in pure medium. Moreover, this experimental design involved exposure of the crustacean to binary mixtures of the three FQs and thus, any possible interactions between these compounds was assessed. This type of evaluation is worthwhile because various FQs can be present simultaneously in contaminated waters (Gao et al., 2012; Rutgersson et al., 2014). Whether any such interactions are less than additive, additive or synergistic is of considerable interest in environmental risk assessment (Cedergreen, 2014).

Finally, as previous experiments have shown toxicity of FLU and ENR after embryonic exposure of *D. magna*, an additional two embryonic experiments were conducted with identical design; one with LEV and the other with a ternary mixture of the three FQs.

The standard *D. magna* immobilization test has a pivotal role in Environmental Risk Assessment, however, neither does it consider delayed toxicity, nor is there a single agreed protocol to evaluate mixtures of environmental micropollutants (Cedergreen, 2014).

Given the significance of this emerging class of environmental micropollutants (Van Doorslaer et al., 2014), it is hoped that data generated in this study may contribute to the further evolution of the methodologies used in Environmental Risk Assessment.

## 2. Materials and methods

### 2.1. Chemicals

Enrofloxacin (ENR), flumequine (FLU) and levofloxacin (LEV) were supplied by Sigma-Aldrich (Milan, Italy). Their purity was ≥ 98%.

For each compound, a 100 mg $L^{-1}$ stock solution in Rocchetta© still mineral water (pH 7.6, dry residue 181.6 mg $L^{-1}$) was prepared before each test and stored in the dark at 4 °C. Solubilization of the test compounds was obtained by gentle stirring overnight at 37 °C, and pH was measured using a BASIC20 pH-meter (CRISON, Carpi, Italy).

### 2.2. Test organism and culture conditions

*Ephippia* of *D. magna* were originally provided by ECOTOX (Milan, Italy) and a single cloned population was cultured in our lab. During culturing, the medium was Rocchetta© water. A temperature of 20 °C

+/- 1 °C temperature was maintained by a thermo-refrigerated incubator. A photoperiod of 16 h light (100 lx): 8 h dark was selected. The sensitivity of the *D. magna* clone was checked every 4 months by exposure to potassium dichromate (ISO, 1996). The high quality health status of the culture was evidenced over time by the low mortality rate ($\leq$ 2% per week), the high reproduction rate (about 10 neonates per day per individual), and the absence of *ephippia* and/or males. Daphnids were fed three times per week with *Scenedesmus dimorphus* ($8 \times 10^5$ cells $mL^{-1}$). Details of the algal culturing method have previously been reported (De Liguoro et al., 2012).

### 2.3. Acute immobilization test with 10-day follow-up

The acute immobilization test itself was performed in accordance with the Guideline 202 "*Daphnia* sp., Acute immobilization Test" (OECD, 2004). However, in order to evidence any delayed toxicity, a follow-up of 10 days in pure medium was added to the standard incubation time (48 h).

The three FQs were assayed individually (ENR; FLU; LEV) or in binary combinations (ENR+FLU; ENR+LEV; FLU+LEV). In each test, we used a negative control (pure medium) and eight sequential concentrations in a geometric series, with a separation factor of 1.8. The individual tests were run at the following concentrations: 0.7, 1.2, 2.1, 3.8, 6.9, 12.3, 22.2, and 40 mg $L^{-1}$. The concentrations of compounds within the binary mixtures followed an equi-toxicity ratio design that was based on the $EC_{50}$ calculated at the end of the three individual tests (Fig. 1).

Before the beginning of each test, in order to evaluate growth inhibition, 30 offspring not intended for the tests were isolated, fixed in 70% ethanol and photographed. Their length was measured, using Photoshop© software, as the distance between the eye and the base of the tail spine. The same procedure was repeated with all surviving individuals at the end of the assay, and hence their daily growth calculated.

For the test, only daphnids younger than 24 h and obtained from the third to fifth brood were used. They were fed 1 h before the beginning of the experiment with spirulina powder (15 mg in 100 mL of Rocchetta© water) and then incubated in 4 groups of 5, in 10 mL pure medium (control) or drug solutions, under the same light and temperature conditions used for culturing. Pre-feeding of the organisms is not deemed necessary by the test guideline, however in our experience is beneficial and therefore advisable (as it helps to sustain 100% survival in the control groups). After 48 h of incubation, the number of immobilized daphnids was recorded and the $EC_{50}$ calculated. Then, the follow-up in pure medium was carried out: surviving individuals were transferred to 36 individual beakers (preserving the group/exposure identity). Each beaker contained 50 mL of Rocchetta© water and the daphnids fed in every other day by adding *S. dimorphus* to a concentration of $8 \times 10^5$ cells $mL^{-1}$. Follow-up lasted 10 days during which time *D. magna* ability to survive, grow and produce eggs was evaluated. At day 12, when many daphnids had already produced their first clutch, all surviving adults were collected, fixed and measured. The endpoints considered were mortality and growth inhibition.

### 2.4. Embryonic test with 21-day follow-up

As previous experiment had shown toxicity of FLU and ENR after embryonic exposure of *D. magna* at a concentration of 2 mg $L^{-1}$ (Dalla Bona et al., 2016; De Liguoro et al., 2019), an analogous test with 2 mg $L^{-1}$ LEV was performed. Then, in order to understand whether one of the three different toxicity patterns displayed by the individual compounds would prevail on exposure to their mixture, another test was run with a combination containing 0.66 mg $L^{-1}$ of each one. In both tests the following procedure was used. Gravid daphnids were collected from cultures and examined microscopically for the level of embryo development in the brood chamber. To obtain sufficient embryos, approximately thirty specimens, carrying embryos in early development (stage1) (LeBlanc et al., 2000), were selected. Embryos were extracted by immobilizing the head of the adult with a dissecting probe whilst a second probe was used to free the embryos gently by separating the carapace (LeBlanc et al., 2000). The collected embryos were taken at random and transferred individually to each well of 24-well Suspension Culture Plate (CELLSTAR, Greiner bio-one) containing either 1 mL of the drug solution ($n = 72$) or 1 mL of Rocchetta© ($n = 72$). They were incubated for 3 days under the conditions (light and temperature) normally used for *D. magna* culture (see above). After incubation, the number of embryos hatched was recorded and 10 apparently-healthy neonates from the population of exposed embryos were randomly selected and assigned to group 1 (prenatal exposure), whilst 10 neonates randomly selected from the control group were assigned to group 2 (controls). Daphnids from each group were individually allocated to 100 mL beakers containing 50 mL of pure Rocchetta© water and incubated for 21 days under the culture conditions described earlier. Every other day the medium was replaced, the neonates removed and counted, and feed (*S. dimorphus*, $8 \times 10^5$ cells $mL^{-1}$) supplied. At the end of the test, the growth rate, reproductive activity and mortality rate of the two groups were measured and compared (OECD, 2012).

### 2.5. Data analysis

The $EC_x$ values with confidence limits were calculated using "Probit Analysis" software (USEPA, 2012). The applied Concentration Addition (CA) model for binary mixture toxicity prediction was calculated as:

$$\sum_{i=1}^{N} \frac{dA_i}{DA_i} = 1$$

where dAi is the dose/concentration of Ai in a mixture that produces a specified effect, and DAi is the dose/concentration of the single agent which on its own elicits the same effect as the mixture (Kortenkamp and Altenburger, 1998). To strictly evaluate any possible deviation from the CA model, a curvilinear isobologram analysis at $EC_{50}$ was performed, with confidence limits based on Hill coefficient variability (De Liguoro et al., 2018). According to Tallarida (2006), for compounds with a variable potency ratio, synergy (or antagonism) is detected only if the $EC_{50}$ of the mixture lies below (or above) the region of the plane bounded by the two curves of additivity for a 50% effect.

To infer differences in growth and reproduction between the control and the exposed group in the immobilization and embryo tests, the Student T-test was used. P values $< 0.05$ were considered significant.

## 3. Results

Considering the exposure conditions adopted in the various tests (incubation 48–72 h, temperature 20 $\pm$ 1 °C, 100 lux), any degradation of the three FQs under study can be considered negligible. Indeed, previous studies have already indicated that ENR (Dalla Bona et al., 2015) and FLU (De Liguoro et al., 2019) are largely stable under these conditions, while LEV was found to be stable in three different saline solutions, even after a 4-week exposure to daylight (Czyrski et al., 2019). Based on the Criteria for Reporting and Evaluation of ecotoxicity Data (CRED; Moermond et al., 2016), in acute toxicity tests with stable substances, nominal concentrations without further measurements are acceptable. Accordingly, in the present study the use of HPLC analysis was deemed redundant and consequent undesirable excess use of solvents was avoided, with test results being based on nominal concentrations. In the immobilization tests, validity criteria (OECD, 2004) were fulfilled as control survival (mobility) was $\geq$ 90%, and the recorded values of water quality parameters were always within the following ranges: pH 7.5–7.7, dissolved oxygen 7.9–8.4 mg $L^{-1}$.

Data from acute immobilization tests with follow-up are shown in Fig. 1. Invariably, a decline in survival was recorded during the 10-day follow-up. As a result, the calculated 48 h $EC_{50\,s}$ were considerably

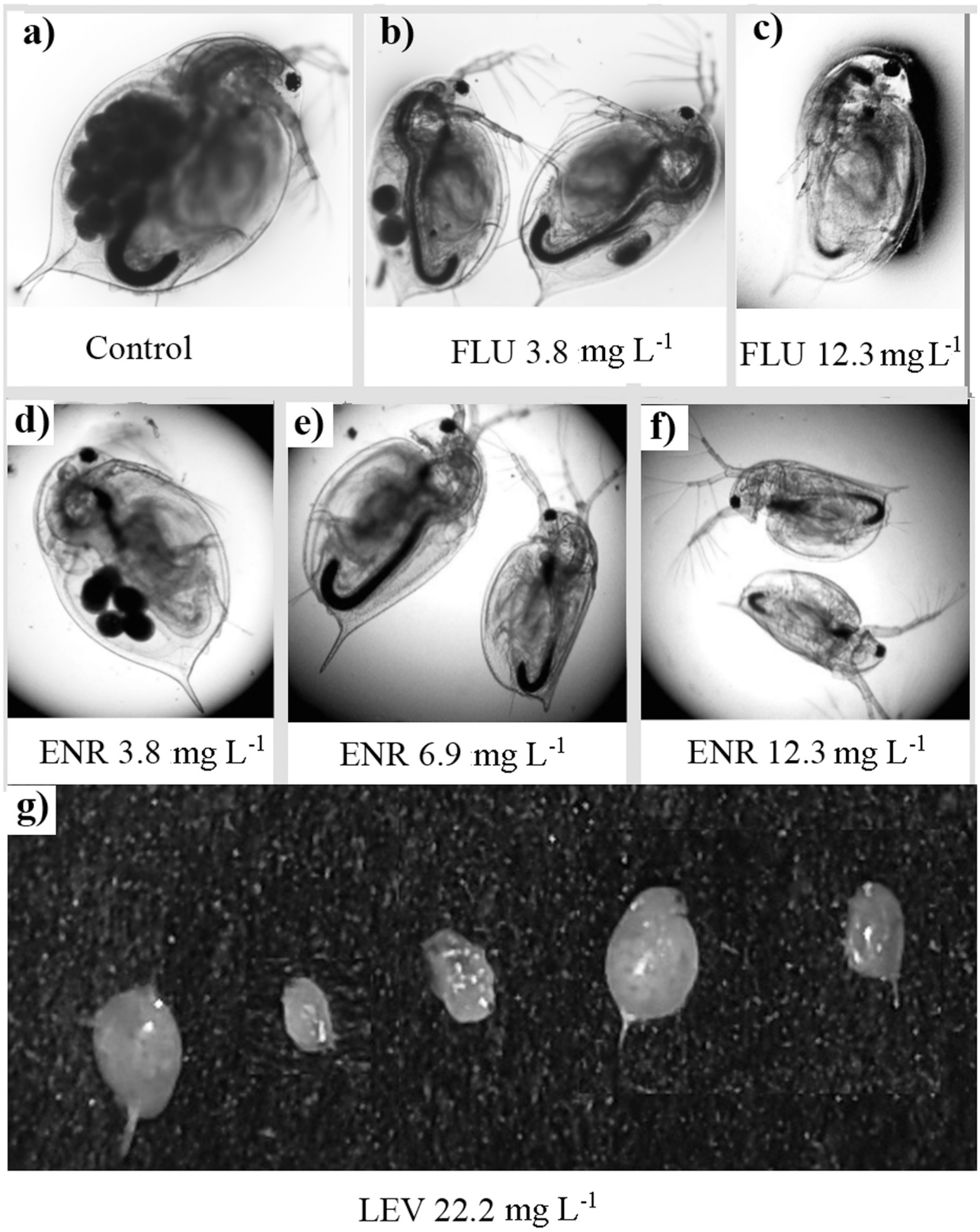


**Fig. 2.** Phenotypic and functional alterations in *D. magna* after 48 h exposure to fluoroquinolones, individually or in binary combinations, with 10 days follow-up in pure medium. a) Control; b-f) individuals exposed to different concentrations of FLU and ENR; g) surviving individuals from a single group, exposed to LEV (ENR, enrofloxacin; FLU, flumequine; LEV, levofloxacin).

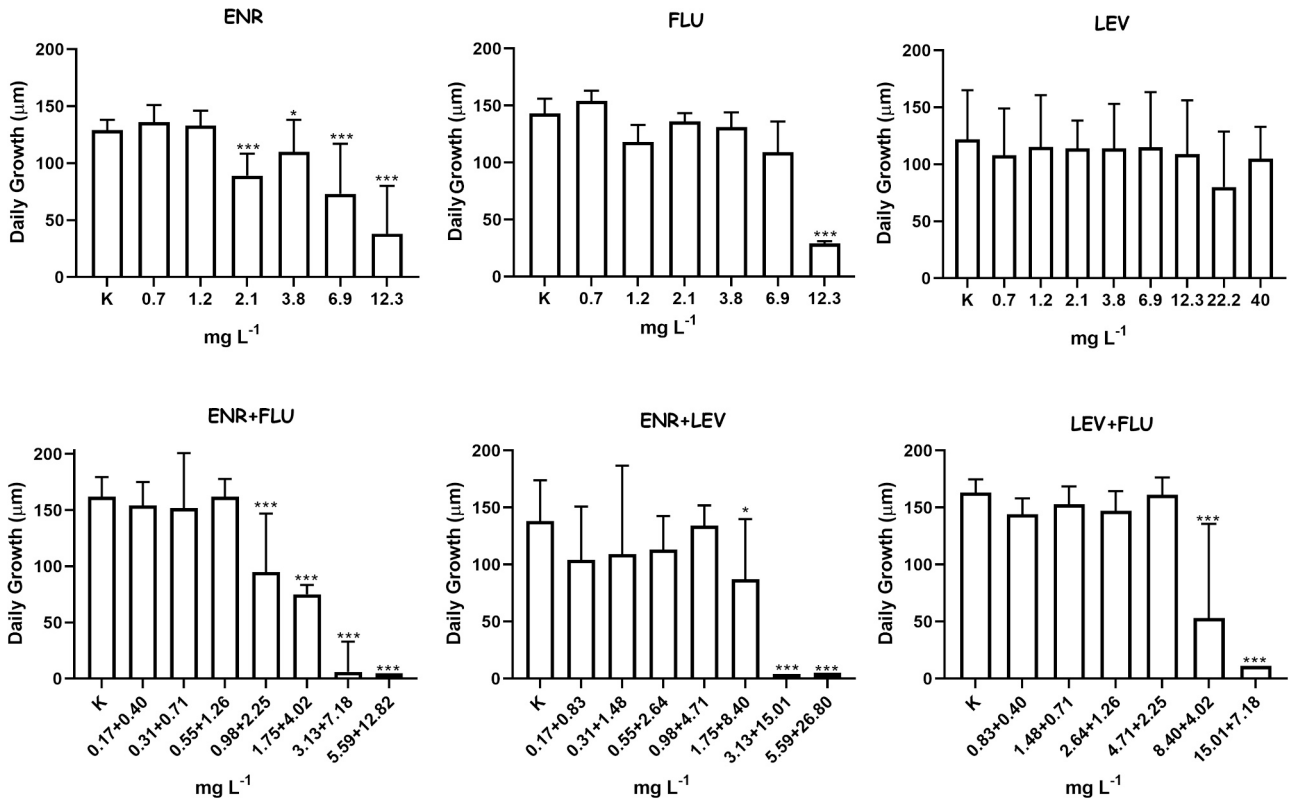


**Fig. 3.** Daily growth of *D. magna* after 48 h exposure to fluoroquinolones, individually or in binary combinations, with 10 days follow-up in pure medium. Error bars show standard deviation (ENR, enrofloxacin; FLU, flumequine; LEV, levofloxacin).

higher than those calculated at the end of the test (12 days). In particular, for the individual tests: ENR 16.72 vs. 3.13 mg $L^{-1}$; FLU 25.35 vs. 7.18 mg $L^{-1}$; LEV > 40 vs. 15.11 mg $L^{-1}$. And for the binary tests: ENR+FLU 9.98 + 22.89 vs 1.44 + 3.30 mg $L^{-1}$; ENR+LEV 9.98 + 47.85 vs 1.86 + 8.92 mg $L^{-1}$; LEV+FLU > 47.85 + 22.89 vs 6.58 + 3.14 mg $L^{-1}$. Thanks to the follow-up, effects on development were evidenced in some individuals. These were testified by the scarcity or absence of eggs/embryos in the brood chamber. In Fig. 2, a control individual (a) is compared to exposed daphnids displaying proportionate dwarfism, which apparently was dose-correlated (b-f), and to surviving individuals from a single group exposed to 22.2 mg $L^{-1}$ LEV (g), ranging from perfectly healthy to seriously altered. Reproductive potential was inevitably compromised by these phenotypic alterations, which were generally also the precursor to premature death. Significant effects on daily growth were detected mainly at higher concentrations, in some cases with large within-group variability. These effects were more evident with ENR in the individual tests and with ENR+FLU in the binary tests (Fig. 3).

In Fig. 4, the predicted dose-response curves based on the CA principle were matched to the curves obtained by testing the binary mixtures. In all cases the two curves were closely similar to each other, thereby indicating no relevant deviation from the CA principle. This tendency was confirmed by the curvilinear isobolograms (Tallarida, 2006), where the $EC_{50\,s}$ of the three mixtures invariably lay within the plane bounded by the two curves of additivity and their confidence limits (Fig. 5).

The exposure of *D. magna* embryos to 2 mg $L^{-1}$ LEV did not affect their survival (Table 1); however, two newborns (out of 70) showed an incorrect arrangement of their antennae (Fig. 6.a,b) and were unable to swim properly. Healthy newborns transferred to pure medium and followed-up for 21 days showed no phenotypic alterations, and their reproduction and growth rates were not significantly lower than those of the control group (Table 1). Similarly, the exposure of *D. magna* embryos to the mixture composed by 0.66 mg $L^{-1}$ of each compound caused only a non-significant reproduction inhibition (Table 1). 10% mortality during the 21-day follow-up should be considered casual from a statistical point of view; however, the only dead individual (out of ten) showed (Fig. 6.c) the typical phenotypic alteration (discolouration, poor growth) known to be brought about by FQs (De Liguoro et al., 2019).

## 4. Discussion

As already observed in a previous, multigenerational experiment (De Liguoro et al., 2019), the toxic effects of FQs on the cladoceran crustacean *D. magna* occur stochastically in a certain percentage of individuals. They are characterized by phenotypic alterations of varying severity, accompanied by functional effects, ranging from limitation of reproduction rate to early mortality. The exposure to eight concentrations in a geometric series permitted the observation that both the percentage of harmed individuals (Fig. 1) and the severity of damage (Fig. 2.b-f) increase with increasing concentrations of FQs. Based on the measured $EC_{50\,s}$, the following toxicity ranking was inferred: ENR > ~ FLU > LEV. This ranking was apparent also when taking into account the growth inhibition endpoint (Fig. 3). Considering that, at neutral pH, ENR has a lower aqueous solubility and a higher membrane permeability than LEV (Blokhina et al., 2016), a possible explanation for its higher toxicity could be the easier absorption by the cells of *D. magna*. However, the toxic effects of the three drugs appeared to be qualitatively alike. For example, the alterations observed after acute exposure to LEV (Fig. 2.g) are strikingly similar to those previously found after chronic exposure to FLU (De Liguoro et al., 2019).

In general, inhibition of daily growth was poorly correlated to the exposure level since the measurement of this endpoint was limited to individuals alive at the end of the test. Indeed, randomly harmed individuals within groups either survived till the end of the test or died prematurely, thereby affecting in one sense or another the average daily

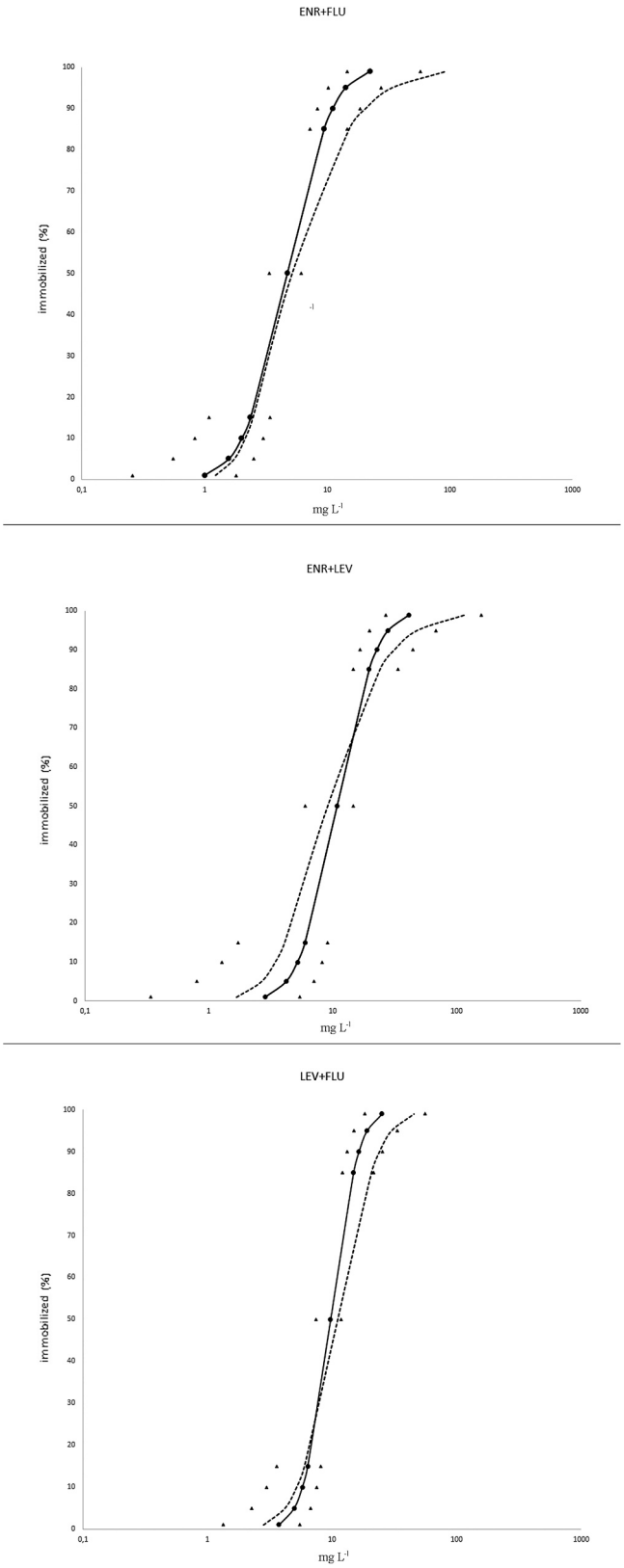


**Fig. 4.** Concentration-effect curves of fluoroquinolone binary mixtures assayed on *D. magna*, obtained by connecting the $EC_x$ values generated by Probit analysis of the experimental data. Triangles indicate the 95% confidence limits. Dashed lines are the predicted concentration-effect curves according to the Concentration Addition principle (ENR, enrofloxacin; FLU, flumequine; LEV, levofloxacin).

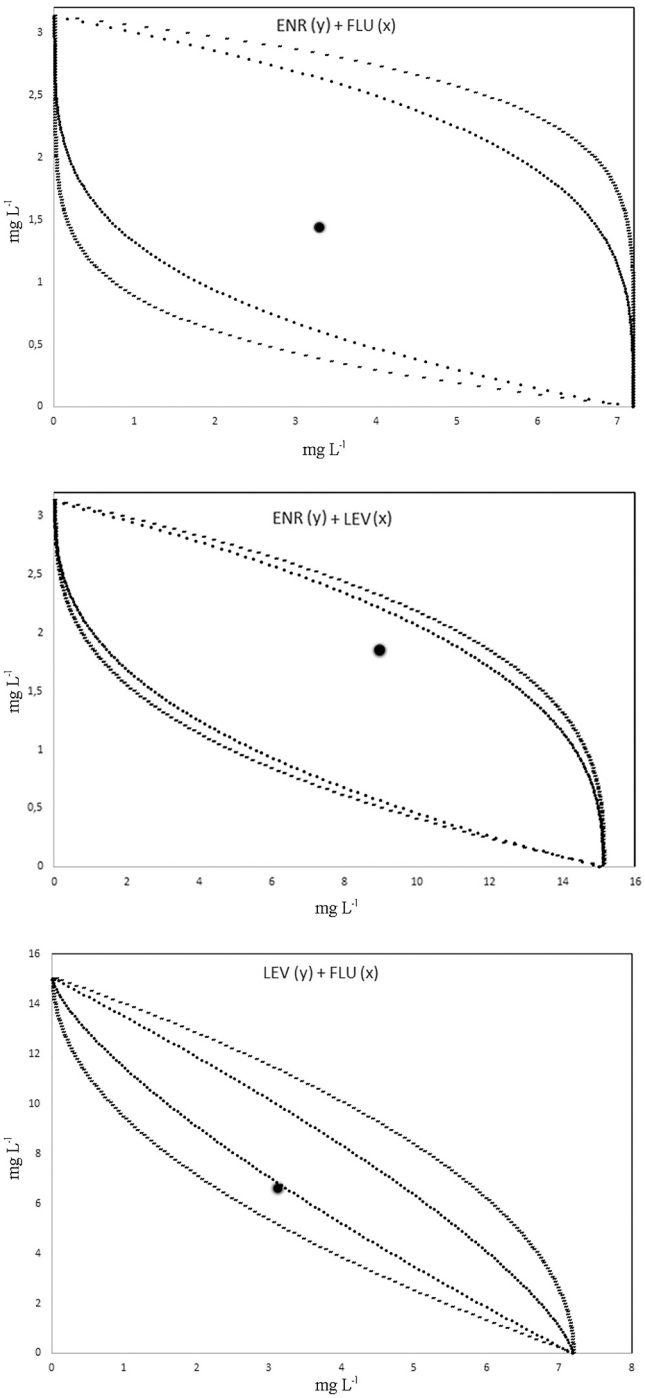


**Fig. 5.** Isobolograms of fluoroquinolone binary mixtures assayed on *D. magna*. Dotted lines represent curves of additivity. Dashed lines are their confidence limits based on Hill coefficient variability (ENR, enrofloxacin; FLU, flumequine; LEV, levofloxacin).

growth of their group. Moreover, the occasional presence of healthy and harmed individuals in the same group, explains the large standard deviation of the dataset seen in some cases (Fig. 3).

The follow-up to the acute immobilization test evidenced, for all the compounds tested and their binary mixtures, delayed toxic effects that may have crucial consequences for *D. magna* populations. The $EC_{50}$ measured at 48 h is clearly overstated when compared to that recalculated after 10 days of maintenance in pure medium. Considering that the $EC_{50}$ of *Daphnia* immobilization test is, within the Environmental Risk Assessment of pharmaceuticals (EMEA, 2005), one of the primary

**Table 1**
Embryonic test with 21-day follow-up.

| Group | Healthy newborns | Early developmental arrest | Late developmental arrest | Stillbirth | Birth defects (antennae or tail spine) | References |
|---|---|---|---|---|---|---|
| **a)** | | | | | | |
| Control (n:35) | 97% | 1 | 0 | 0 | 0 | Dalla Bona et al. (2016) |
| ENR | 69% | 9 | 2 | 0 | 0 | |
| Control (n:72) | 93% | 3 | 1 | 1 | 0 | De Liguoro et al. (2019) |
| FLU | 78% | 6 | 1 | 3 | 6 | |
| Control (n:72) | 92% | 4 | 1 | 1 | 0 | This work |
| LEV | 93% | 1 | 1 | 1 | 2 | |
| Control (n:72) | 92% | 6 | 0 | 0 | 0 | This work |
| ENR+FLU+LEV | 94% | 4 | 0 | 0 | 0 | |

| **b)** Group | Mortality rate (%) ($n = 10$) | Time to production of first brood (days) | Neonates per parent animals at the start of the test | Neonates per surviving parent animals | Average daily growth (length, μm) | Daily growth inhibition (%) | References |
|---|---|---|---|---|---|---|---|
| Control | 0 | n.d. | 122 ± 27 | 122 ± 27 | 183 ± 30 | | Dalla Bona et al. (2016) |
| ENR | 0 | n.d. | 119 ± 46 | 119 ± 46 | 177 ± 30 | 3 | |
| Control | 10 | 10 | 73 ± 30 | 79 ± 26 | 152 ± 31 | | De Liguoro et al. (2019) |
| FLU | 70 | 10 | 17 ± 30*** | 59 ± 27 | 152 ± 24 | 0 | |
| Control | 20 | 10 | 74 ± 9 | 73 ± 8 | 160 ± 30 | | This work |
| LEV | 20 | 10 | 60 ± 26 | 64 ± 15 | 148 ± 35 | 8 | |
| Control | 0 | 10 | 107 ± 18 | 107 ± 18 | 141 | | This work |
| ENR+FLU+LEV | 10 -2 | 10 | 84 ± 33 | 93 ± 15 | 144 | | |

a) Results of the 3-day in vitro test on D. magna embryos exposed to 2 mg $L^{-1}$ of ENR, FLU or LEV, or to a ternary mixture (ENR+FLU+LEV) containing 0.66 mg $L^{-1}$ of each compound (ENR, enrofloxacin; FLU, flumequine; LEV, levofloxacin). b) Mortality, reproduction performance and daily growth in the 21-day follow-up of apparently healthy individuals hatched after 3-day embryo exposure to ENR, FLU or LEV, or to a ternary mixture (ENR+FLU+LEV) (ENR, enrofloxacin; FLU, flumequine; LEV, levofloxacin).
*** $p < 0.001$

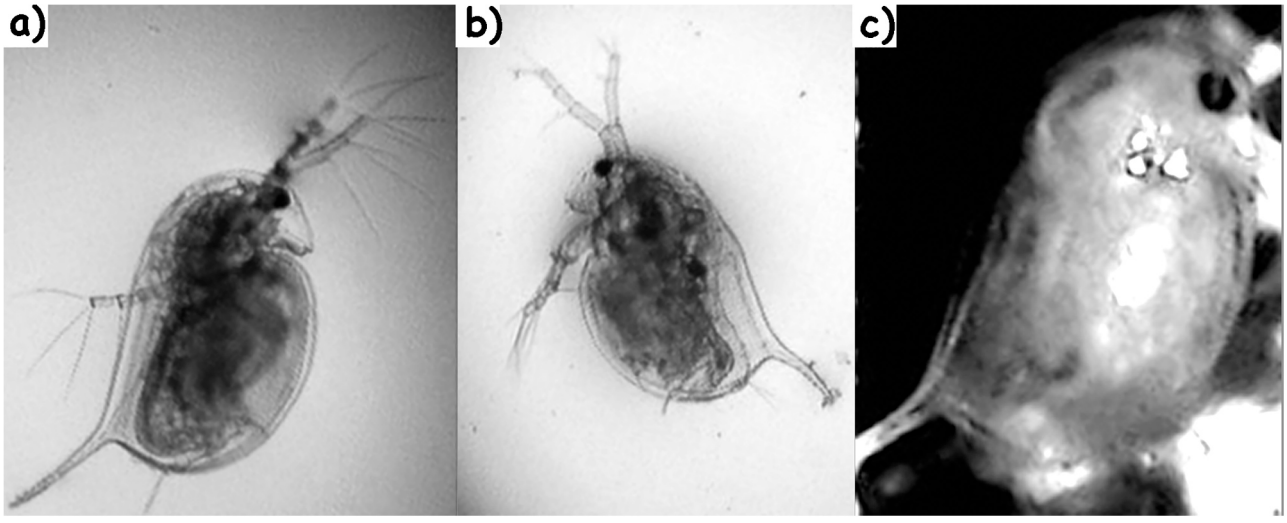


**Fig. 6.** Phenotypic and functional alterations in *D. magna* after 3 days embryo exposure to LEV individually or to ternary mixture, with 21 days follow-up in pure medium. a-b) Individuals exposed to 2 mg $L^{-1}$ levofloxacin (LEV); c) individual exposed to the ternary mixture of fluoroquinolones.

parameters used for the calculation of the PNEC (Predicted No Effect Concentration), its overstatement implies an underestimation of the Risk Quotient (PEC/PNEC) and potentially inadequate protection of the environment. Furthermore, the same test is required under EU regulations concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) (EC, 2006). For these reasons, we believe that a follow-up period post acute immobilisation test should always be considered in those cases where, after 48 h exposure, the surviving daphnids display signs of lethargy, discolouration, or any other evident harm.

The aim of testing binary mixtures was to verify if the three FQs could interact synergistically, and thereby jointly exert a larger effect than predicted by the CA principle. This information is crucial because, for those regulations where mixtures are considered, CA is proposed as the default model (Backhaus et al., 2010). Data generated thus far on the toxicity of chemical mixtures, justify this proposition, as in 95% of cases no interaction (simple additivity) or antagonism (less than additivity) have been detected (Cedergreen, 2014). The compounds investigated in this experiment were no exception to the rule; invariably, they acted in combination by following the principle of CA. Therefore, at least for

these three FQs, the simple additivity concept seems adequate for evaluating the ecotoxicity of their mixtures using *D. magna* populations. However, the assayed binary mixtures were all composed of equi-toxic concentrations of the three pharmaceuticals and synergic or antagonistic interactions at other toxic ratios cannot be excluded. Indeed, the mixture-ratio dependence of drug interactions is not an unusual phenomenon and has been observed by various authors (Berenbaum, 1989; Kortenkamp and Altenburger, 1998; De Liguoro et al., 2009). Moreover, any generalization of the obtained results to the entire class of FQs should be avoided: the fact that FQs share the same mechanism of action does not necessarily imply that any or all of their possible combinations should follow the CA principle. Indeed, in previous tests on *D. magna* with sulphonamides, another class of antibacterials, after testing 15 different binary combinations, a tendency towards a range of possible interactions (antagonistic, additive, synergistic) was observed (De Liguoro et al., 2018).

The embryonic test with 2 mg $L^{-1}$ LEV confirmed its lower toxicity to *D. magna* when compared to the other two FQs. Taking into account previously published data from analogous tests conducted with ENR and FLU (Dalla Bona et al., 2016; De Liguoro et al., 2019), the three FQs showed different impact patterns after prenatal exposure of the crustacean (Table 1): ENR featured an 'all or nothing' toxicity pattern, causing the death of a substantial percentage of embryos without any delayed toxicity over postnatal development and reproduction; FLU caused a slight mortality of embryos and some birth defects, but had also delayed, relevant effects on survival and reproduction; LEV had no apparent effect, with the exception of two cases of phenotypic alterations in *D magna* newborns. The two individuals (Fig. 6.a,b) bore an altered conformation of the antennae and, consequently, they were unable to swim properly, which would have destined them to a very short life in a natural environment, given the crucial role of swimming in finding the optimum position relative to food availability and predator pressure (Christensen et al., 2005).

The results of the test with the ternary mixture (ENR+FLU+LEV) indicate a pattern very similar to the one elicited by LEV alone. Considering that LEV was the least toxic of the three compounds composing the mixture, any synergistic interaction can be excluded for the assayed ternary combination.

## 5. Conclusions

A 10-day follow-up in pure medium added to the standard (48 h) immobilization test on *D. magna*, led to lower values of acute $EC_{50}$ with each of three FQs (ENR, FLU and LEV). At the end of the standard immobilization test, in the case of some survived daphnids displaying signs of illness, this addition of a 10-day follow-up in pure medium can be recommended for all the survived individuals. Indeed, in that it can evidence delayed mortality, it permits a more accurate environmental risk-assessment.

Tests using mixtures of the three compounds have indicated that their toxicity to the crustacean tends to obey the reference model of Concentration Addition. Whilst this limited observation cannot be generalized to the entire class of FQs, it strengthens the concept that for drugs sharing the same mechanism of action, such as the FQs, the Concentration Addition model (simple additivity) is probably appropriate.

The exposure of *D.magna* embryos to 2 mg $L^{-1}$ LEV did not cause any mortality and had only non-significant effects on subsequent daily growth and reproduction activity. This confirmed the lower toxicity of LEV when compared to ENR and FLU, already observed with the extended immobilization test. Overall, the three compounds can harm this crustacean at concentrations of few mg $L^{-1}$. Whilst levels of FQs in the freshwater environment are generally very low (ng to μg $L^{-1}$), far higher concentrations (mg $L^{-1}$) have been occasionally reported either in the water column or sediment. Consequently, possible harm to crustacean populations in heavily contaminated watersheds cannot be ruled out.

Given the mechanism of action of FQs, any future research aimed at investigating possible alteration or regulation of *D. magna* genetic material would contribute to a better understanding of the delayed toxicity evidenced in this study and the previously reported transgenerational toxicity.

## CRediT authorship contribution statement

Conceptualization: Marco De Liguoro and Roberta Tolosi, Laboratory Activities/Investigation: Roberta Tolosi, Formal analysis: Marco De Liguoro, Writing: Marco De Liguoro and Roberta Tolosi, Project administration: Marco De Liguoro, Funding acquisition: Marco De Liguoro.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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