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*Title Page

Enantiomer stability and combined toxicity of duloxetine and econazole on *Daphnia Magna* using real concentrations determined by capillary electrophoresis

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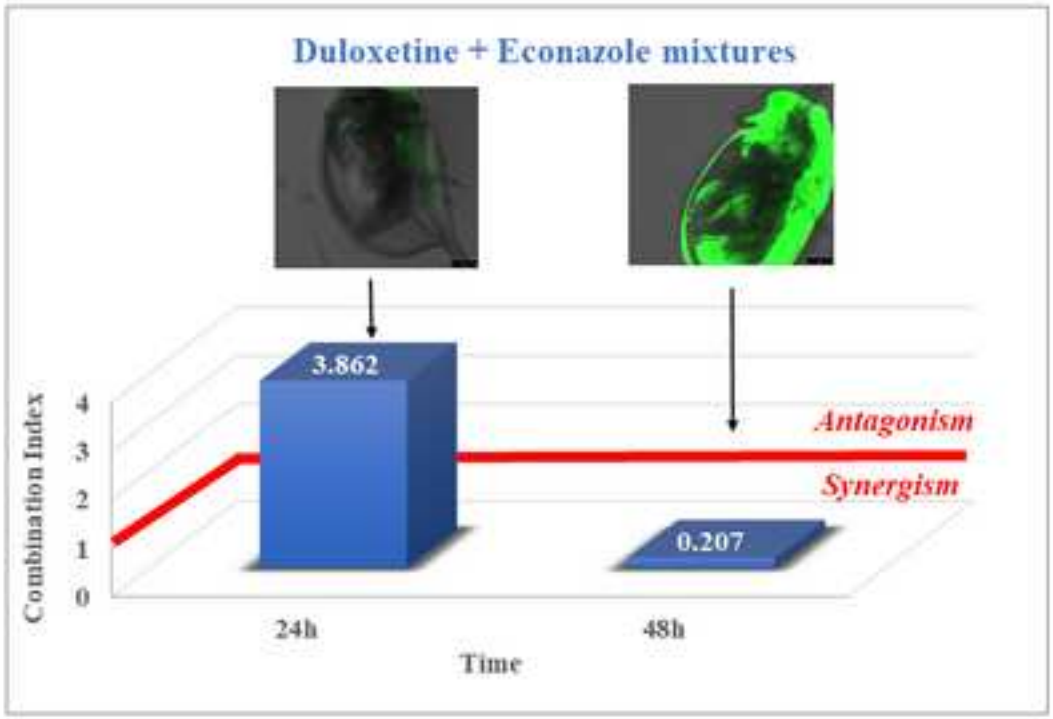
*Graphical Abstract



Highlights

- Stability of each drug or mixture was evaluated under abiotic and biotic conditions
- Toxicity of both drugs and their mixtures for *Daphnia magna* was established
- Real concentrations of drug enantiomers were determined by chiral CE
- Strong synergism observed for drug mixture at 48 h exposure and any effect level

1. Introduction

The wide use of pharmaceuticals for human and animal disease treatment has originated their presence in the environment and their consideration as emerging pollutants. A lot of work has been carried out to evaluate the presence of these pollutants in water or soil samples. However, although the increasing pollution has mainly impact on ground and surface waters or soils, biota is also affected without having paid considerable attention to the risk that these emerging pollutants could suppose for the metabolism and hormonal balance of non-target organisms (**Weber et al., 2016; Sanganyado et al., 2017**) even when they are at as low concentrations as µg/L and ng/L (**Sanderson et al., 2003**).

An example of a non-target organism is *Daphnia magna*, a freshwater organism belonging to the microcrustacean family (**Minguez et al., 2016**) that can be found in lakes, rivers and rocky pools. Its lifespan depends on the environmental conditions, such as temperature, food availability (since this organism is fed with algae, bacteria and detritus), and the presence of pollutants (**Animal Diversity Web, 2014**). *Daphnia magna* is an indicator organism for water quality and it is employed in test of water toxicity. It is noteworthy to stand out that this organism can degrade chiral compounds by means of estereospecific enzymatic processes with a variation in their enantiomeric ratio (**Stanley et al., 2006**). In addition, *Daphnia magna* possesses transparent physiology (**Paul et al., 1997**) that allows to carry out non-invasive assays in order to investigate changes in the organism during the toxic process (**Colmorgen and Paul, 1995**).

Duloxetine (N-methyl-3-(naphthalen-1-yloxy)-3-(thiophen-2-yl)propan-1-amine) belongs to the family of selective serotonin and norepinephrine reuptake inhibitors that affect neurotransmitters in order to restore the balance in brain and treat

depression and anxiety disorder (**MedicineNet, 1996**). It is a chiral compound that possesses two active enantiomers although with different activity, being higher the activity of the S-enantiomer (**Wong et al., 1993**), reason that has originated its commercialization as pure enantiomer. Econazole (1-[2-[(4-chlorophenyl)methoxy]-2-(2,4-dichlorophenyl) ethyl] imidazole) is a chiral drug belonging to imidazole family, with antimicrobial activity, that is widely employed as antifungal in the treatment of mycosis infections (**Heel et al., 1978**). It is also used in different kinds of ringworm (**Instituto Químico Biológico, 2004**), being commercialized as racemate although the antifungal activity has been reported only for the R-enantiomer (**Furuta and Doi, 1994**). Stability studies were carried out for pharmaceutical production for duloxetine and econazole. Duloxetine is degraded under acid, alkaline and neutral hydrolysis (**Chadha et al., 2016**) and under UV photodegradation (**Datar and Waghmare, 2014**) and it remains stable under thermal and oxidative stress conditions (**Chadha et al., 2016**). Econazole demonstrated full stability under several stress conditions when neutral, acidic and alkaline hydrolysis were employed at high temperatures (90ºC) as well as with thermal degradation, showing instability only under oxidation conditions (**Baker et al., 2016**). Moreover, in the context of environmental studies, it has been shown that econazole bioaccumulates and has a low biodegradability (**Lindberg et al., 2010; Jean et al., 2012**).

Enantiomers of chiral compounds possess identical physical and chemical properties in a symmetrical environment. However, when they are present in a chiral biological environment, they can exhibit different enantiospecific biological activity with differences between the enantiomers that can reach 500-fold in some cases such as β-blockers (**Ma et al., 2014**). The different pharmacokinetics, pharmacodynamics, toxicity and degradation rates (**Sanganyado et al., 2017**), confer a high interest to the

investigation of the stability and toxicity for the individual enantiomers of chiral drugs under abiotic and biotic conditions. However, this study requires to have analytical tools able to determine the individual concentrations of these enantiomers. In this context, Capillary Electrophoresis (CE) is considered one of the most powerful techniques to achieve the separation of enantiomers. With this aim, a chiral selector has to be added to the separation media in the most frequently employed chiral separation mode, which is Electrokinetic Chromatography.

Although Minguez et al. (**Minguez et al., 2016**) studied the ecotoxicity of duloxetine and econazole on *Daphnia magna*, this study was performed using nominal and not real concentrations of each drug for the determination of the EC50 values in solutions containing only one of both drugs and not their mixtures.

In this work, real concentrations of duloxetine and econazole were determined by CE in culture medium for *Daphnia magna* under abiotic and biotic conditions in order to make possible the individual evaluation of the toxicity of these drugs in single drug solutions and their mixtures. In addition, the individual estimation of the stability of drug enantiomers was also carried out in single drug solutions and their mixtures and under abiotic and biotic conditions.

2. Materials and methods

2.1. Chemicals

High purity standards (>99%) of (R,S)-duloxetine HCl and of (R,S)-econazole nitrate were from IS Chemical Technology (Shanghai, China) and from Sigma-Aldrich, respectively. Sulfated-β-CD (S-β-CD), sodium hydroxide (NaOH), and ortophosphoric acid 85% were from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid (HCl)

37% and methanol (MeOH) were from Scharlau Chemie (Barcelona, Spain). 2′,7′-dichlorofluorescein diacetate (H_2DCFDA, ≥97%) was acquired from Sigma Aldrich. Water used to prepare solutions was purified through a Milli-Q System from Millipore (Bedford, MA, USA).

2.2. Enantiomeric determination of duloxetine and econazole by CE

A HP^{3D}CE instrument from Agilent Technologies (Palo Alto, CA, USA) with a diode array detector (DAD) was employed. The electrophoretic system was controlled by HP^{3D}CE ChemStation software and included the data collection and analysis. Separations were performed in an uncoated fused-silica capillaries of 50 µm I.D. (375 µm O.D.) with a total length of 58.5 cm (50 cm effective length) purchased from Polymicro Technologies (Phoenix, AZ, USA). The detection wavelengths were 200 nm for econazole and 220 nm for duloxetine with a bandwidth of 5 nm, and response time of 1.0 s when the quantitation of these compounds was achieved. An intermediate wavelength of 210 nm was also employed to record electropherograms for binary mixtures of both drugs.

The preparation of stock standard solutions of duloxetine and econazole was carried out by dissolving each compound in MeOH at a 1000 mg/L concentration and diluting with Milli-Q water to obtained the desired concentration. All solutions were stored at 4 ºC until use, and filtered (through a 0.45 µm pore size nylon filter from Scharlau Chemie) and degassed (in an ultrasonic bath from Penta Manufacturing Company (Livingston, NJ, USA)), before use.

In order to prepare buffer solutions, the appropriate volume of phosphoric acid was dissolved in Milli-Q water and the pH was adjusted at pH 3.0 with 1M NaOH. Milli-Q water was added to complete the volume necessary to reach the desired buffer

concentration. The appropriate amount of S-β-CD was dissolved in the buffer solution to obtain the background electrolyte (BGE).

When new capillaries were employed, a special procedure was followed which involved rinsing with MeOH for 5 min, 1 M NaOH for 25 min, Milli-Q water for 5 min followed by 5 min with 1M HCl (at 1 bar pressure). At the beginning of each working day, the capillary was conditioned with buffer solution for 20 min and 10 min with the BGE. At the end of the day, it was flushed with NaOH 0.1 M and Milli-Q water, both of them for 5 min. Between injections, the capillary was flushed with 0.1 M HCl for 2 min, Milli-Q water for 1 min and BGE for 5 min.

The separation and determination of duloxetine and econazole enantiomers were achieved using a 25 mM phosphate buffer (pH 3.0) containing 1.5% S-β-CD at a temperature of 30ºC and reverse polarity at -20 kV. Hydrodynamic injection of standard solutions and samples was carried out at 50 mbar for 10 s. Seven standard solutions for duloxetine racemate (2, 12, 20, 30, 40, 50, 60 mg/L) and six for econazole racemate (6, 8, 12, 15, 18, 20 mg/L) were employed for quantitation of both compounds using the external calibration method. Calibration by the standard addition method was achieved by adding similar concentrations employed for external calibration to culture medium samples for *Daphnia magna*. Comparison of slope values for both calibration methods was performed using Statgraphics Centurion XVI (**Statgraphics Centurion, 2013**) which was also employed for other statistical data analysis.

2.3. Biological material and pre-culture conditions

Daphnia magna eggs and concentrated solution for nutritive medium were obtained from the MicroBio Tests Inc. (Belgium).

Eggs were incubated in the nutritive medium at a temperature of 20±1 ºC with a continuous illumination at 6000 lux inside a growth chamber acquired from IBERCEX, S.L. (Spain), with the aim of achieving the hatching.

The stability of duloxetine and econazole was evaluated under abiotic and biotic conditions using toxicant concentrations ranging from 3 to 30 mg/L. The abiotic runs were carried out in the absence of daphnids, in order to evaluate the possible hydrolysis of compounds into aqueous reaction media and the effect of the variation of any physico-chemical parameter. Concentration of each contaminant in the liquid fraction was determined at the beginning (0 h) and along the exposure time (24, 48 and 72 h). Each assay condition was replicated three times.

2.4. Toxicity tests

Once the eggs hatching was carried out, toxicity bioassays with neonates of 24 h of lifetime were developed in accordance with the international standard OECD 202 Guideline (**OECD, 2004**). The experiments were conducted in plates containing 5 neonates and 10 mL of culture media supplemented with either no added toxicants (Control) and predefined concentration of toxicants, per quadruplicate, being all sets of experiments conducted under darkness. Both pollutants were first assayed in the range 0.1-20 mg/L and mixtures were tested based on the EC50 values of the individual components at 24 h of exposure. Acute toxicity was expressed by the EC50 value, being this the concentration provoking the immobilization of 50% of organisms during the exposure time (24, 48 and 72 h), using the control experiment as the reference with 0% of inhibition.

2.5. Evaluation of oxidative stress

The toxicity of the target compounds was also evaluated in terms of Reactive Oxygen Species (ROS) amount produced in *D. magna* by H_2DCFDA assay (**Galdiero et al., 2017**). In brief, after exposure to toxicants at the EC50 concentration, as previously described in section 2.4, 10 daphnids were taken and incubated with 1 mL of 10 mM H_2DCFDA for 2 h at 20°C in the dark. ROS level was monitored by fluorescence (excitation wavelength of 350 nm and emission wavelength of 600 nm) using a Confocal Microscope Leica TCS SP5 (Germany).

2.6. Equations for the evaluation of toxicity parameters

The median-effect/combination index (CI)-isobologram equation, was used the calculation of toxicity parameters. This equation proposed by Chou and Talalay (**Chou and Talalay, 1984**) is derived from mass-action law:

$$\frac{f_a}{1-f_a} = \left(\frac{D}{D_m}\right)^m$$

D is a concentration of drug that provokes damage on a population fraction f_a. D_m is the median effective concentration (EC50) and the parameter m accounts for the shape of the same dose-effect curve.

EC50 and m values are used for calculating the CI values; CI <1, =1, and >1 indicate synergism (S), additive effect (Add), and antagonism (A), respectively. EC10, EC50 and EC95, are the doses required to inhibit 10, 50 and 95%, respectively. Computer software CompuSyn (**Chou and Martin, 2005**) was used for automated calculation and simulation.

To the evaluation of the combined effect of duloxetine and econazole, it was tested mixtures of pollutants in 1:1 fixed constant ratio based on the EC50 values of

single drugs for 24h of exposition time, over a wide range of effect levels. Calculation of the CI values was done according to the combination index equation (**Chou, 2006**):

$$(CI)_x^n = \sum_j^n \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^n \frac{(D_x)_{1-n}\left\{\frac{D_j}{\sum_1^n [D]}\right\}}{(D_m)_j\left\{\frac{(f_{ax})_j}{[1-(f_{ax})_j]}\right\}^{1/mj}}$$

Where $(CI)_x^n$ is the combination index for n chemicals at a certain x% inhibition (e.g., mobility of daphnids), $(D_x)_{1-n}$ is the sum of the concentrations of n toxicants exerting x% of inhibition in combination $\{[D]_j / \sum_1^n [D]\}$ is the ratio of a given (j) drug inducing a x% inhibition in combination and $(D_m)_j\{(f_{ax})_j/[1-(f_{ax})_j]\}^{1/mj}$ is the dose of each compound alone producing the same effect. CI indicates additivity (CI = 1), synergism (CI < 1) or antagonism (CI > 1). The calculations were performed using CompuSyn software (**Chou and Martin, 2005**).

3. Results and discussion

3.1 Analytical characteristics of the CE method employed for the simultaneous determination of duloxetine and econazole enantiomers

In a previous work of our research team (**Valimaña-Traverso et al., 2019**) a CE method was optimized enabling the simultaneous enantiomeric separation of duloxetine and econazole in 7.5 min with enantiomeric resolutions of 7.9 and 6.5, respectively. The experimental conditions employed are detailed in Materials and Methods (see section 2.2). In order to assure the adequate performance of this method to analyse duloxetine and econazole enantiomers in the culture medium samples employed in this work under abiotic and biotic conditions, the analytical characteristics of this method were evaluated. As linearity was assessed in our previous work using standard solutions

(linear range from 1.8 to 60 mg/L for duloxetine and from 4.8 to 20 mg/L for econazole), in this work, precision, accuracy, LOD and LOQ, and the existence of matrix interferences were evaluated using the culture medium of *Daphnia magna* under abiotic and biotic conditions. To assess method variability related to the incubation process with duloxetine and econazole mixtures in the culture medium of *Daphnia magna*, precision was expressed as repeatability and intermediate precision at two concentration levels. As shown in Table 1, repeatability (expressed as RSD values) was better than 1.3 % for migration times and lower than 2.5 % for corrected peak areas, both for duloxetine and econazole enantiomers. RSD values obtained for intermediate precision were lower than 1.6 % for migration times and 2.8 % for corrected peak areas for drug enantiomers. The study of matrix interferences was carried out by comparison of the slopes obtained by the external and the standard addition calibration methods. No statistically significant differences existed (p *value*>0.05) between these slope values showing the absence of matrix interferences and the possibility of using the external calibration method for the quantitation of duloxetine and econazole enantiomers (see Table 1). Finally, average recovery values at two concentrations levels ranged from 98 to 102 % for duloxetine and from 98 to 104 % for econazole assuring method accuracy, and LOD and LOQ values were close to 0.4 and 1.3 mg/L for duloxetine enantiomers and close to 1.1 and 3.6 mg/L for econazole enantiomers, respectively.

3.2 Stability of duloxetine and econazole enantiomers and their mixtures

The stability of duloxetine and econazole enantiomers was evaluated in individual solutions of each drug under abiotic and biotic conditions. It was observed that the variations of duloxetine concentrations were negligible in any case while econazole was not stable disappearing after incubation with the culture medium and in the presence of daphnis (results not shown). Similar results were obtained for econazole when mixtures

of duloxetine and econazole enantiomers were incubated under abiotic and biotic conditions (Figures 1C and 1D). However, stability profiles for racemic duloxetine and each of its enantiomers were different in mixtures of both drugs after 72 h incubation under abiotic (Figure 2A) and biotic conditions (Figure 2B) and different racemate initial nominal concentrations. It was observed that in absence of *Daphnia magna*, the concentration of each duloxetine enantiomer is stable with variations lower than 0.4% of the initial nominal concentration (see Figure 2A). Nevertheless, in the presence of *Daphnia magna*, a concentration decay was observed for racemic duloxetine as well as for the individual enantiomers (Figure 2B). Decay percentages for duloxetine enantiomers ranged from 46 to 77 % depending on the initial nominal concentrations assayed. In fact, for the lowest initial nominal concentrations (3 and 7 mg/L racemate), duloxetine concentrations after incubation could not be determined because they were lower than the LOQ of the analytical method.

3.3. Toxicity of duloxetine and econazole mixtures.

The toxicity of mixtures of duloxetine and econazole on *Daphnia magna* was determined for the first time in this work, and compared with the toxicity evaluated for single drug solutions. Table 2 groups the values of the toxicity parameters for the mixtures and the single solution of each drug, in both cases for 24, 48 and 72 h of exposure time. Real concentrations determined by CE were employed for calculations for both drugs although in the case of econazole only initial concentrations could be used since this drug disappeared after incubation. It can be observed that EC50 values measured for single drug solutions decreased with the incubation time for both compounds. In fact, EC50 values were a 73% lower for duloxetine and a 36 % for econazole at 48 h incubation time referred to the typical reference time for *Daphnia magna* mobility test (24 h incubation time). Decreases of 82 % and 85 % were observed

for duloxetine and econazole, respectively, when an incubation time of 72 h is considered. These results show the high toxicity of duloxetine and econazole according to the European Regulation EC 1272/2008 (**EC, 2008**), although duloxetine toxicity was higher than that of econazole. EC50 values obtained in this work could be compared with those determined previously by other authors but only for 48 h incubation time (**Minguez et al., 2016**). In the case of duloxetine, an EC50 value of 3.35 mg/L was previously reported (**Minguez et al., 2016**) which was considerably higher than that obtained in this work (0.12 mg/L). However, differences for EC50 values were lower in the case of econazole (0.4 and 0.24 mg/L in (**Minguez et al., 2016**) and in this work, respectively). The highest differences in the EC50 values for duloxetine with respect to the previous work (**Minguez et al., 2016**) could be explained by the use of real concentrations determined in this work by CE while nominal concentrations were used for calculations in the previous work. In fact, these differences in EC50 values decreased for econazole for which initial concentrations were used in this work as this drug disappeared as explained above.

Regarding the mixtures of duloxetine and econazole, EC50 values decreased when increasing the incubation time, as previously observed for the single solution of each drug, with EC50 values up to 98 % lower than that obtained for 24 h (see Table 2). Table 2 also shows the higher toxicity of the mixtures at 48 h incubation time compared with the single drug solutions. Regarding the combination index, Figure 3 shows that this parameter is lower than 1 at any effect level for 48 h incubation time (Fig. 3B) contrary to that observed for 24 (Fig. 3A) and 72 h (Fig. 3C) showing the synergism existing between both drugs at 48 h incubation time. At short exposure times (Fig. 3A), the interaction profile is different from that obtained at 48 h with synergism effect only until 0.3 effect level. Similarly, Fig. 3C shows that synergism occurs up to 0.75 effect

level with antagonism appearing when increasing this variable. These results showing the different interaction profiles for the drug studied in this work with *Daphnia magna* agree with those previously obtained by our research team for the same compounds and the aquatic plant *Spirodela polyrhiza* (**Valimaña-Traverso et al., 2019**) and also for other organisms (green alga, cyanobacteria (**González-Pleiter et al., 2013**), and biological activated sludge (**Amariei et al., 2017**), and other drug mixtures such as a combination of five antibiotics (amoxicillin, erytromycin, levofloxacin, norfloxacin and tetracycline) (**González-Pleiter et al., 2013**), and a binary mixture containing an antimicrobial (triclosan) and a non-steroidal antiinflamatory drug (ibuprofen)) (**Amariei et al., 2017**) using in all these cases the combination index model. Moreover, using a different model (Concentration Addition (CA)), deviations observed from predictions, suggested a synergistic effect for duloxetine in mixtures with other eight antidepressants when two different green algae were studied (**Minguez et al., 2018**). Traditional CA and Independent Action (IA) models, predict the toxicity of a mixture based on the effect of individual components, and consider deviations from additivity (antagonism and synergy) toxicologically irrelevant and corresponding to unusual situations (Backhaus, et al., 2003; European Commission, 2011). However, the results obtained in this work, together with those reported in our previous works (**González-Pleiter et al., 2013**, **Amariei et al., 2017**) show that deviations from additivity exist and can be strong in many systems.

Toxicity of duloxetine, econazole and their mixtures was also investigated by evaluating the oxidative stress on *Dapnia magna* using a biochemical marker and fluorescence images as described in section 2.5. The results obtained are shown in Figure 4. A light stress (background fluorescence) was observed in control assays at 24 and 48 h of incubation without toxicants probably due to changes in aqueous media when

H_2DCFDA was added and to the handling of the crustaceans for confocal microscopy observation. No increase on the fluorescence was detected at 24 h incubation time for the mixture supporting the above-mentioned comments on the antagonistic effect of this mixture at EC50 concentration value contrary to what it was observed at 48 h exposure time (see Table 2). However, as shown in Fig. 4, a considerable increase of fluorescence was observed with the exposure time also corroborating the above-mentioned results on the toxicity of these compounds as illustrated in Table 2. In fact, the highest increase in fluorescence took place for duloxetine at 48 h exposure time and for the mixture of both drugs at the same exposure time. Moreover, a widespread distribution of fluorescence occurred within the daphnids body, regardless of the compounds tested and the exposure time, contrasting this distribution with that reported when *Daphnia magna* was exposed to metallic nanoparticles (**Galdiero et al., 2017**). In this case, the increase in fluorescence was limited to the gastrointestinal tract and broad egg chamber of daphnis. Other authors reported previously oxidative stress for *Daphnia magna* induced by pharmaceuticals (**Gómez-Oliván et al., 2014a; Gómez-Oliván et al., 2014b**) although duloxetine and econazole were not investigated in any case.

The results obtained in this work are the first described for mixtures of duloxetine and econazole using the microcrustacean *Daphnia magna*. No previous results were reported for comparison.

4. Conclusions

The stability of duloxetine and econazole enantiomers in individual solutions and their mixtures under the ecotoxicity test conditions for *Daphnia magna* was evaluated for the first time in this work. Results showed that the variations of duloxetine concentrations

were negligible in any case in single solutions while the stability profiles for racemic duloxetine and each of its enantiomers were different in mixtures of both drugs after 72 h incubation. Decay percentages for duloxetine enantiomers ranged from 46 to 77 % depending on the initial nominal concentrations. However, econazole was not stable disappearing after incubation and this was true for single solutions and mixtures of both drugs. The toxicity of the mixtures of duloxetine and econazole was determined on *Dapnia magna* for the first time in this work and compared with the toxicity of single solutions. Mixtures at 48 h incubation time showed a high toxicity and synergism at any effect level which should be taken into account when evaluating environmental risk in aquatic ecosystems. A good correlation was observed between toxicity parameters calculated by the ecotoxicity test and the interaction profiles, and the fluorescence images obtained for *Daphnia magna* using a reactive oxygen species biochemical marker. Real drug concentrations were determined by CE in this work which could justify the big differences observed for EC50 values obtained for duloxetine with respect to the only EC50 value reported for this drug in the bibliography in a single solution.

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443 **Table Legends.**

444 **Table 1.** Precision, study on the existence of matrix interferences, LOD, LOQ, and
445 accuracy of the CE method, evaluated using the culture medium of *Daphnia magna*
446 under abiotic and biotic conditions.

447 **Table 2.** Dose-effect relationship parameters and mean combination index (CI) values
448 of duloxetine (D), econazole (E) and their mixtures for toxicity tests.

Figure Captions

Figure 1. Electropherograms corresponding to the analysis of a mixture of duloxetine and econazole racemates at racemic concentrations of 20 mg/L of each drug in: A) an aqueous standard solution; B) culture medium at zero time; C) culture medium at 72 h incubation (abiotic conditions); D) culture medium in presence of *Daphnia magna* at 72 h incubation. Experimental conditions: 25 mM phosphate buffer (pH 3.0) with a 1.5% S-β-CD at a temperature of 30ºC and a separation voltage of -20 kV; detection at λ= 210 nm; effective capillary length 50 cm, internal diameter 50 µm, hydrodynamic injection 50 mbar x 10 s.

Figure 2. Stability profiles for racemic duloxetine and its enantiomers after 72 h incubation under abiotic (A) and biotic conditions (B) in mixtures of duloxetine and econazole at different initial nominal concentrations of both racemic drugs. Each percentage is the average of three results. Error bars correspond to a 95% confidence interval.

Figure 3. Combination index calculated for different effect levels in binary mixtures of duloxetine and econazole at different exposition times: A) 24 h., B) 48 h., C) 72 h. Error bars correspond to a 95% confidence interval.

Figure 4. Fluorescence images of *Daphnia magna* after 24 h and 48 h of exposure to duloxetine, econazole and their mixtures at their specific EC50 values.

Table 1
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Table 1. Precision, study on the existence of matrix interferences, LOD, LOQ, and accuracy of the CE method, evaluated using the culture medium of *Daphnia magna* under abiotic and biotic conditions.

	Duloxetine				**Econazole**			
	Enantiomer 1		**Enantiomer 2**		**Enantiomer 1**		**Enantiomer 2**	
Precision[1]								
Concentration (mg/L)	**2**	**30**	**2**	**30**	**4**	**10**	**4**	**10**
Repeatability (n=6)[2]								
t_m, RSD (%)	0.3	1.0	0.8	1.3	1.0	1.1	1.1	1.3
A_c, RSD (%)	1.0	2.5	1.2	2.5	2.0	2.3	2.4	2.2
Intermediate precision (n=9)[3]								
t_m, RSD (%)	0.4	1.2	0.9	1.5	1.3	1.4	1.3	1.6
A_c, RSD (%)	1.1	2.8	1.4	2.7	2.1	2.6	2.8	2.4
Standard additions calibration method (n=6)								
Range (mg/L)	**1-30**				**3-10**			
Equation (bx ± a)	0.361x - 0.202		0.370x – 0.270		0.396x – 0.878		0.372x – 0.784	
Standard errors	S_a=0.154, S_b=0.012		S_a=0.194, S_b=0.016		S_a=0.048, S_b=0.007		S_a=0.044, S_b=0.006	
Correlation coefficient (r)	0.9982		0.9972		0.9976		0.9977	
a ± t x S_a	-0.202±0.490		-0.270±0.617		-0.878 ± 0.133		-0.784 ± 0.122	
b ± t x S_b	0.361±0.038		0.370±0.051		0.396 ± 0.019		0.372 ± 0.017	
LOD (mg/L)	0.3		0.4		1.0		1.1	
LOQ (mg/L)	1.0		1.3		3.3		3.6	
Accuracy								
Study of matrix interferences p-value of t test	0.5528		0.8285		0.8765		0.5925	
Concentration (mg/L)	**2**	**30**	**2**	**30**	**4**	**10**	**4**	**10**
Recovery (%)	98 ± 3	101 ± 4	100 ± 1	102 ± 5	102 ± 5	98 ± 4	101 ± 3	104 ± 6

[1] Precision: This variability included the incubation process with duloxetine and econazole mixtures in culture medium of *Daphnia magna* organisms. [2] Six consecutive injections of the culture medium of *Daphnia magna* with a mixture of duloxetine and econazole racemates. [3] Three replicates of the culture medium of *Daphnia magna* with a mixture of duloxetine and econazole racemates were injected in triplicate.
a: intercept; b: slope; S_a: intercept standard deviation; S_b: slope standard deviation; Confidence interval at 95% as confidence level (n = 9); Enantiomer1: first-migrating enantiomer; Enantiomer2: second-migrating enantiomer; Ac: corrected peak area; tm: migration time; LOD: limit of detection; LOQ: limit of quantification.

Table 2. Dose-effect relationship parameters and mean combination index (CI) values of duloxetine (D), econazole (E) and their mixtures for toxicity tests.

SINGLE TOXICANT						
Exposure time (h)	**Duloxetine** **Dose-effect parameters**			**Econazole** **Dose-effect parameters**		
	EC_{50} (mg/L)	**m**	**r**	**EC_{50} (mg/L)**	**m**	**r**
24	0.45±0.01	1.0±0.1	0.98	0.73±0.02	3±1	0.90
48	0.12±0.01	0.8±0.2	0.89	0.24±0.01	1.6±0.3	0.93
72	0.08±0.01	0.9±0.2	0.96	0.11±0.01	1.7±0.4	0.96
BINARY COMBINATION DULOXETINE AND ECONAZOLE						
Exposure time (h)	**Dose-effect parameters**			**IC values**		
	EC_{50} (mg/L)	**m**	**r**	**EC_{10}**	**EC_{50}**	**EC_{95}**
24	2.52±0.02	0.43±0.02	0.98	**0.09**±0.01	3.86±0.05	977±1
48	0.04±0.01	1.6±0.8	0.90	**0.39**±0.02	**0.21**±0.01	**0.15**±0.02
72	0.07±0.01	1.8±0.6	0.91	1.07±0.01	**0.67**±0.02	**0.50**±0.03

Figure 1
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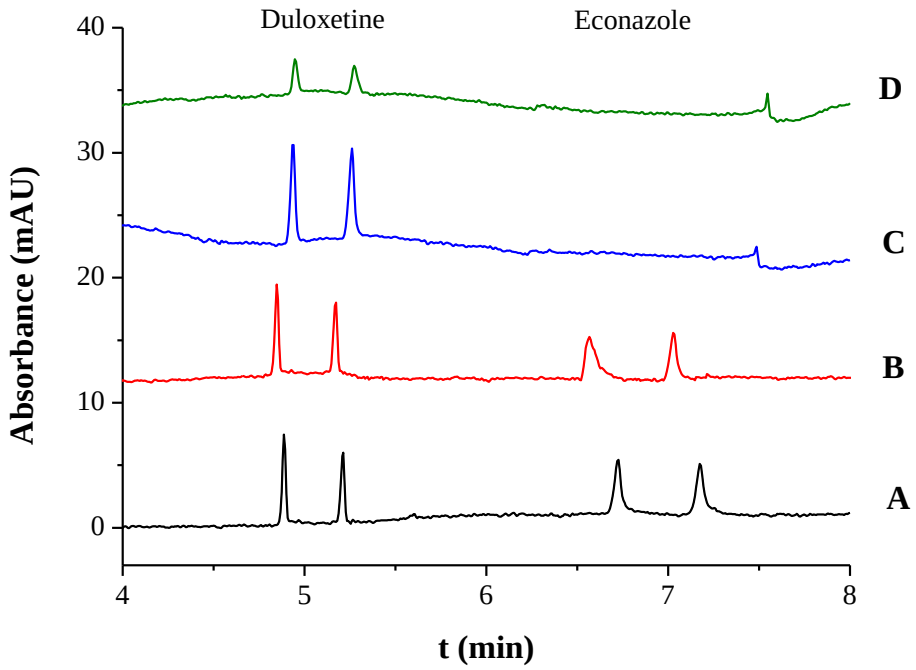

Figure 1.

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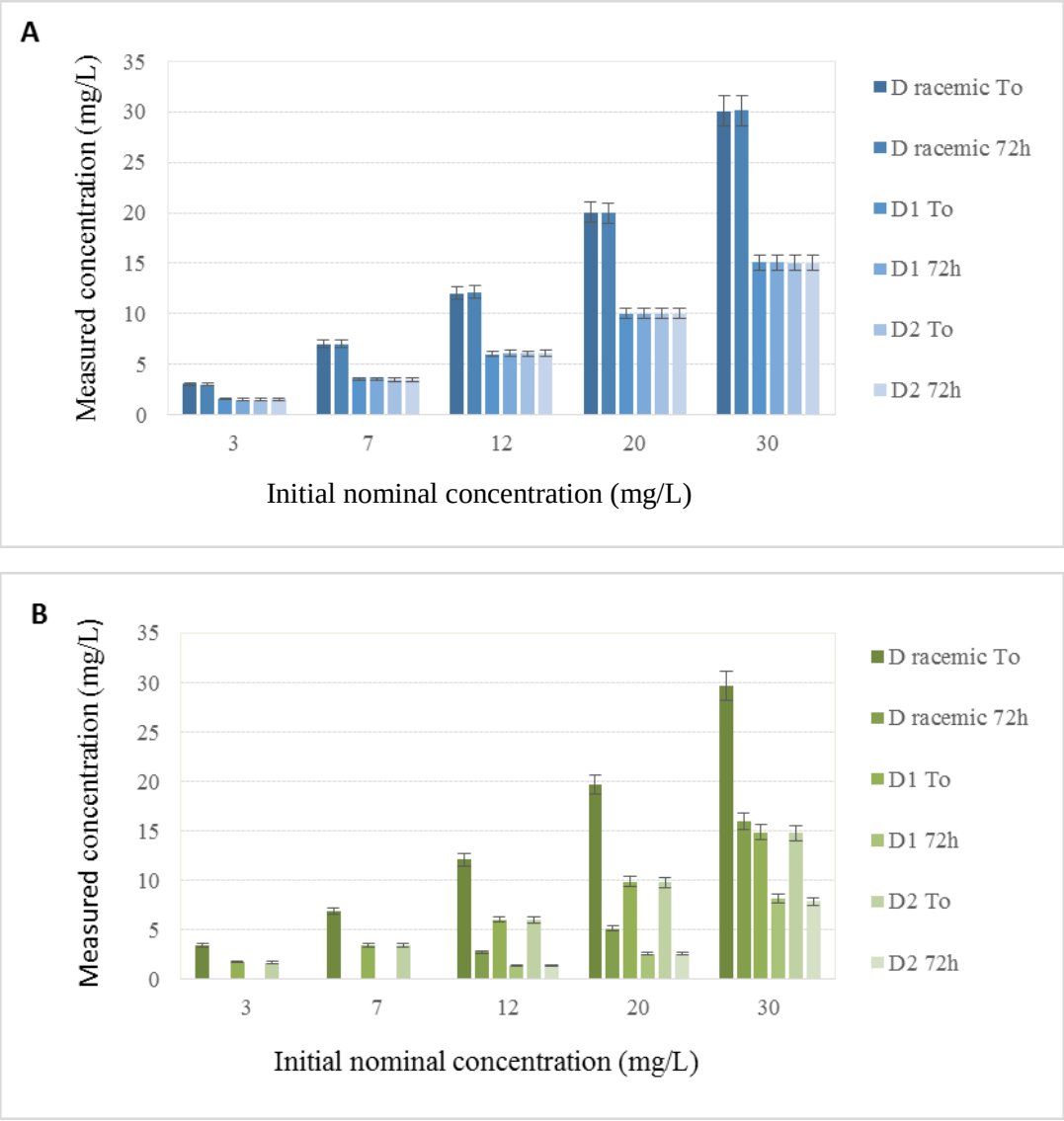

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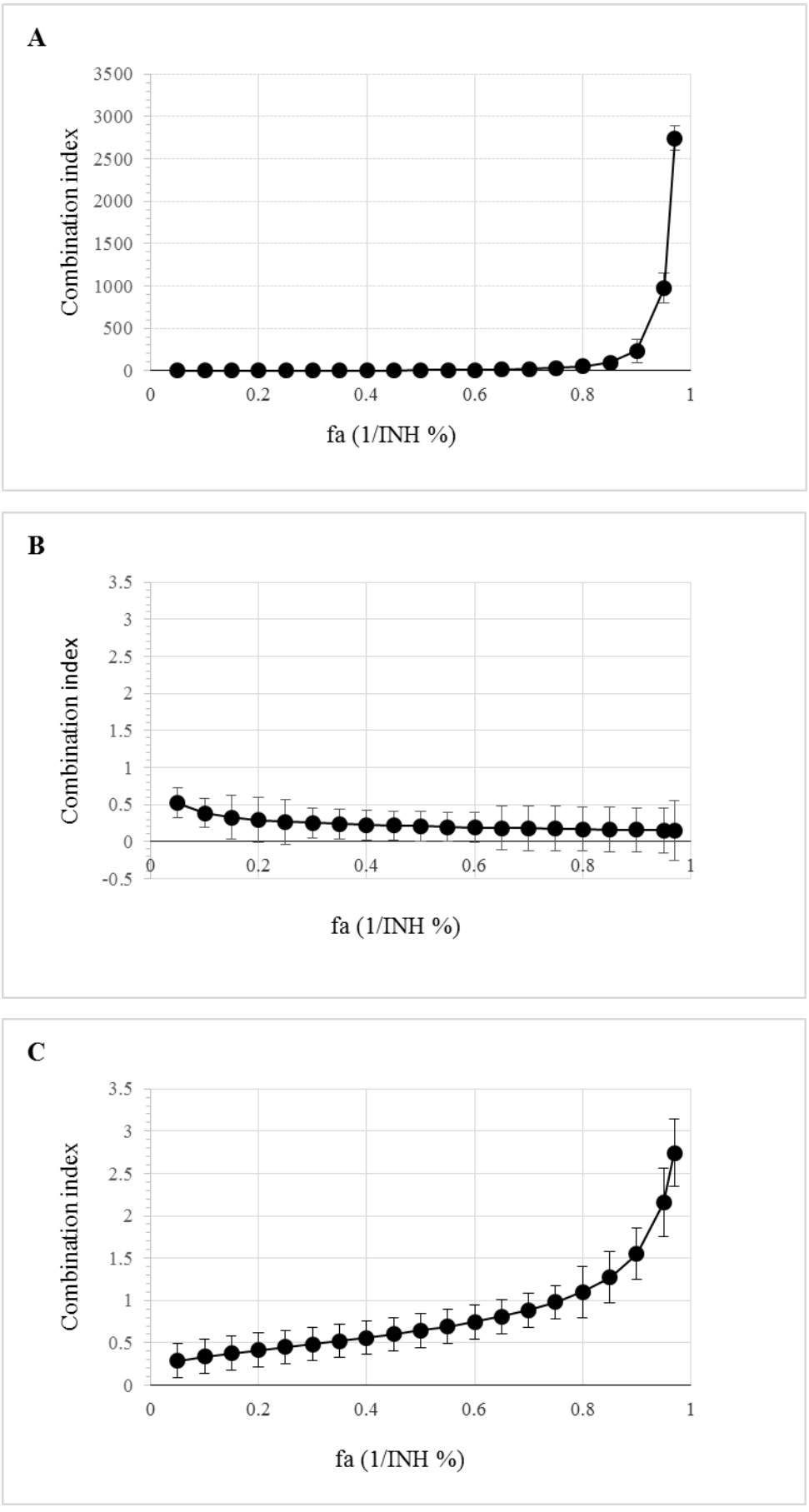

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Figure 4

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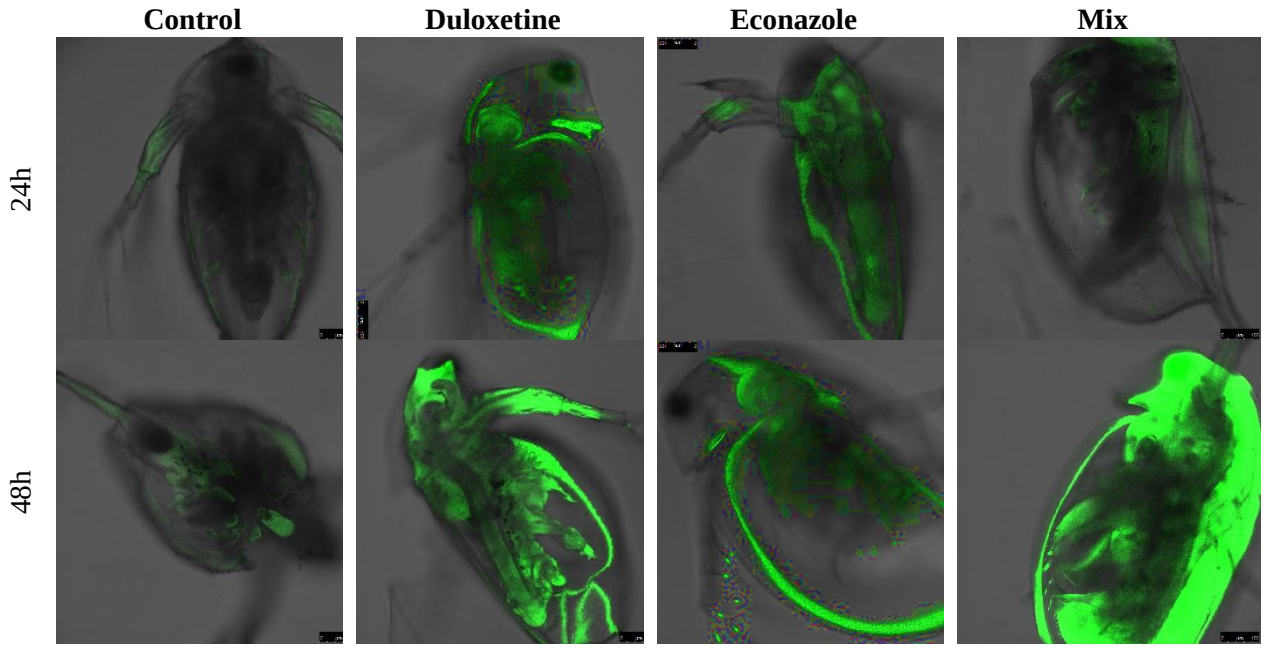

Figure 4.