

Ecotoxicity and Biodegradability of Oxytetracycline and Ciprofloxacin on Terrestrial and Aquatic Media

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Abstract:

Antibiotics are widely found in the environment. For this work, two antibiotics were chosen: oxytetracycline (OTC), used for human and veterinary purposes, and ciprofloxacin (CIP), prescribed for human medicine. The aim of this study was to assess ecotoxicological effects under standardized methods and the biodegradability in terrestrial and aquatic environments for both molecules. In soil, were tested soil respiration, nitrification, and the growth of *Allium cepa* L. (onion), *Lolium perenne* L. (ryegrass) and *Raphanus sativus* L. (radish). *Vibrio fischeri* bioluminescence test and BOD (5 and 28 days) were performed in water. Assays were done in concentrations ranging 0.1, 1, 10, 100, 1,000, 5,000 and 10,000 mg.kg⁻¹/ mg.L⁻¹. Biodegradability was determined by HPLC/UV in the soil and water. OTC was more persistent in water while CIP better persisted in soil. Both antibiotics elicited negative effects on nitrification at highest doses, whereas CIP produced slight inhibition on soil respiration. Lowest values for EC50 plants growth were: 10 mg.kg⁻¹ (CIP) for *A. cepa* (root) and 40 mg.kg⁻¹ (OTC) for *R. sativus* (stem). CIP stimulated *R. sativus* root growth with 1 mg.kg⁻¹ and was harmless to *L. perenne*. This work contributes to increasing knowledge about the toxicity and biodegradability of OTC and CIP for some trophic levels in terrestrial and aquatic media.

Keywords: antibiotics; environment assessment; fluoroquinolone; soil; tetracycline

1. Introduction

Antibiotics act effectively in low doses; they are resistant to degradation and often can reach different environmental matrices [1]. The release of antibiotic compounds in the environment can be harmful to non-target organisms, affecting them directly or through alterations between ecological relations [1, 2]. Concerns about its ecological impacts, as the inhibition of processes mediated by microorganisms - C and N mineralization and contaminant degradation, are demanding more research [3]. Besides that, the potential bioaccumulation and the possible effects on environmental quality and human health should be considered [4, 5]. In addition, the investigation of their influence on potential resistance

mechanisms on microbial population of urban [6, 7] and agricultural environments [8, 9] are crucial. The continuous application of animal manure with antibiotic residues can contribute to the expansion of a reservoir of antibiotic resistance genes (ARGs) on the environment [10, 11]. Among the different groups of antibiotics, tetracyclines (TCs) and fluoroquinolones (FQs) exhibit a broad spectrum of antimicrobial activity, which explains their wide use in human and veterinary medicine [12, 13]. TCs act by inhibiting synthesis of proteins, behaving as bacteriostatic, and as bactericidal in sensitive organisms, while FQs inhibit DNA replication and transcription, acting on DNA gyrase (Gram-negative bacteria) or topoisomerase IV (Gram-positive) [14]. Oxytetracycline (OTC), as all tetracyclines, is

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slightly metabolized in the digestive tract of animals, with 50 - 80% excreted as parental compounds [15]. Among the FQs, the most prescribed drug is ciprofloxacin (CIP) which is the major metabolite of enrofloxacin, a widely used veterinary antibiotic [3, 16]. OTC and CIP are amphoteric compounds with high sorption potential in different soils [15, 17, 18], which suggests this matrix can storage these compounds. Due to their bio-resistance and chemical stability, none of the antibiotics can be totally eliminated by conventional water treatment methods [19, 20]. They may reach surface waters, groundwater, sediments [21], and even marine environments [22]. Previous studies have demonstrated negative effects of antibiotics on aquatic (eg. bacteria, microalgae) and terrestrial environments (eg. soil microbiota, plants) [1, 2, 5, 12]. In general, through acute and chronic tests, studies assess effects on ecosystem functions, choosing some indicators such as basal respiration and O₂ consumption, nitrification and microbial biomass, while in species, are assessed lethal and sub-lethal effects. This study aims to assess ecotoxicological effects of OTC and CIP on terrestrial and aquatic media, as well as to evaluate its biodegradability in both micro-ecosystems. The species (*Vibrio fischeri* marine bacteria and edible plants) or micro-ecosystems used are included in standardized methodologies as bio-indicators of environmental quality, besides being of economic and public health interest.

2. Results and Discussion

2.1 Soil respiration and nitrification

In soil tests (Fig. 1), OTC did not affect the soil respiratory function at all doses. Boleas et al. [23], observed in a poor organic matter soil low microbiota sensibility to OTC, with soil respiration decreases of 16-25% and 28-38% at concentrations of 100 and 1,000 mg.kg⁻¹, showing significant effects only at high doses. Similar results were observed by Zhang et al. [24], that reported respiration inhibitory effects in sediments between 25% (100 mg.kg⁻¹) and 38% (1,000 mg.kg⁻¹). Thiele-Bruhn and Beck [25], defend that the absence of OTC effects on basal soil respiration (1,000 mg.kg⁻¹) is due to its role only as bacteriostatic antibiotic, not biocide. In this case, OTC would not affect dormant microorganisms and, metabolic oxidizing internal

energy sources could be affecting results. Zielezny et al. [26], observed growth inhibition on 12 bacteria isolated from soil caused by chlortetracycline.

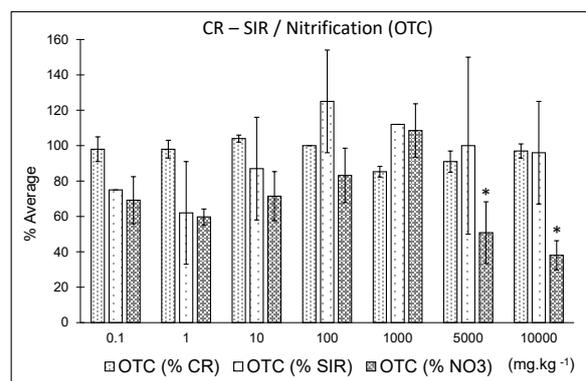


Figure 1. Percentage average relative to control of cumulative respiration (CR), substrate-induced respiration (SIR) and nitrification with oxytetracycline addition in different doses (mg.kg⁻¹). (*) Significant inhibition compared to control ($p < 0.05$).

At SIR test, changes can be observed with 25% increase related control at 100 mg.kg⁻¹ (OTC). SIR indicates the potential activity of the microbiota remaining after toxic stress. Still about SIR tests, there was a great variability, verified by the wide ranges of standard deviation. This variation evidences different soil microbiota response pattern among the treatments. Regarding nitrification tests, the soil microbiota maintained the edaphic function even at 1,000 mg.kg⁻¹, avoiding NO₂⁻ accumulation, a particularly toxic form [27]. Among all tests with OTC, a significant inhibition ($p < 0.05$) was only observed for nitrification at high doses of 5,000 and 10,000 mg.kg⁻¹. Compared with the soil respiration function, this function is more sensitive, being fulfilled by a more specific microbiota [27]. In CIP tests (Fig. 2), adverse effects were observed in both functions, even at low doses (1 mg.kg⁻¹). Again, with CIP (SIR and nitrification), there was a wide variation demonstrated in standard deviation intervals.

A previous study found strong evidence of CIP high persistence and demonstrated negative effects of FQs on soil, contradicting earlier assessments that claimed a low persistence and ecological risk [3]. Some factors may have influenced the absence of effects in

environmentally relevant concentrations on the two functions investigated. Antibiotics as TCs and FQs, due to their physical and chemical characteristics, have high potential of sorption in soils [17, 12]. For ionizable compounds, electrostatic interactions appear to have greater influence on adsorption processes than parameters as Log K_{ow} [12].

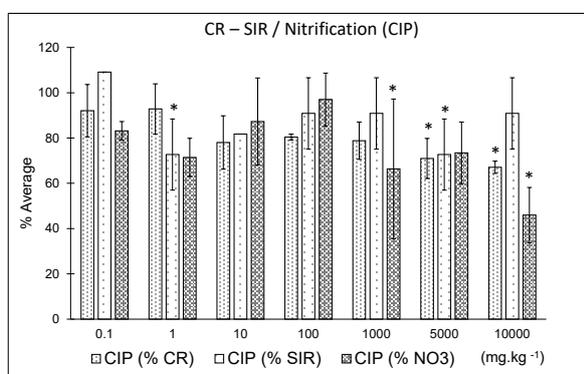


Figure 2. Percentage average relative to control of cumulative respiration (CR), substrate-induced respiration (SIR) and nitrification with ciprofloxacin addition in different doses (mg.kg^{-1}). (*) Significant inhibition compared to control ($p < 0.05$).

Furthermore, different grain sizes can influence their bioavailability and, consequently, their contact with microorganisms [13], minimizing or slowing potential effects. Antibiotics on soil are responsible for structure changes on microbiota communities [28, 5], due to their particular effect on some species, resulting in the development of some more competitive ones [6], such as archaea, fungi, and microorganisms which are tolerant to antibiotics, as well as some pseudomonades [3]. In this case, both functions

(C and N mineralization) could be performed by microorganisms not affected by OTC and CIP, helping to maintain them in the soil.

2.2 OTC and CIP (bio)degradability in soil

Recoveries of OTC (Table 1) ranging between 58 and 81% at the beginning of the experiment (0 day) can be seen in soils submitted to the respirometry tests. This might be due to sorption processes. At the end of the respirometric process, the recoveries are low (2 - 27%), especially in the lowest application dose. The respiration process could favor the biotic degradation of the substance, whereas many other sorption processes can be operating due to the time of contact elapsed. Depending on soil characteristics, OTC molecules can be presented as cations, anions or zwitterions, making it difficult to predict their characteristics in terms of adsorption, bioavailability and potential toxicity [15]. According [29], in soil tests spiked with 1 mg.kg^{-1} OTC, observed half-life - $DT_{50} < 103$ days - and dissipation time - $DT_{90} > 152$ days. Each portion of the molecule has its own balance. In addition, chemical and biological reactivity on soil causes recoveries of these ionic compounds complex and variable [30]. Low recoveries of amphoteric molecules are also due to physicochemical factors, besides the biological processes. At the pH of the soil used (5.9), OTC remains in the neutral molecular form, so the processes taking place could be organic matter interactions and eventual biotic and abiotic degradation processes. Notably, high recovery degree was observed with CIP (Table 1), indicating low dissipation (degradability) during soil respirometry tests.

Table 1. Recovery and degradation rates quantified in soil samples incubated 28 days in respirometric test. Concentration at the beginning (0 day) and at the end of the incubation (28th day) in % related to the initial one.

Dose (mg.kg^{-1})	Oxytetracycline (OTC)					Ciprofloxacin (CIP)				
	0day (%)	CV ^a	28day (%)	CV ^a	Degradation (%)	0day (%)	CV ^a	28day (%)	CV ^a	Degradation (%)
1	79.0	10.1	2.1	3.8	97.4	99.6	32.7	N.d ^b	N.q. ^c	N.q. ^c
10	63.6	17.6	27.7	5.2	56.4	N.q. ^c	N.q. ^c	N.q. ^c	N.q. ^c	N.q. ^c
100	58.4	4.3	12.6	1.2	78.4	77.9	4.8	33.8	13.0	56.6
1,000	69.3	3.2	17.9	14.1	74.1	64.9	4.6	46.9	8.5	27.8
5,000	81.1	3.8	21.2	11.8	73.9	58.8	3.8	43.5	4.3	26.1
10,000	60.1	10.8	18.9	7.5	68.6	46.7	2.8	35.2	9.6	24.6

a: Coefficient of variation; b: Not detected; c: Not quantified

Girardi et al. [3], observed low CIP degradation (0.9%) using soil spiked with 20 mg.kg⁻¹. FQs are chemically stable, resistant to hydrolysis and not easily biodegradable [27]. They also present low mobility and long half-life in soil. A previous study [29], observed high half-life time (DT50 > 152 days) in soil spiked with 1 mg.kg⁻¹ enrofloxacin. This half-life is classified according to soil degradability pesticides parameters as "slightly degradable" [31]. Therefore, this matrix can act as a reservoir of this antibiotic [32,33,12]. At the present soil pH, the molecule takes an anionic-neutral (50%) form.

2.3 Toxicity to terrestrial plants

In tests with *A. cepa* (Fig. 3), OTC inhibited plant growth at low doses and significantly ($p < 0.05$) at high doses. In addition, there was a growth increase of 130% (leaves) and 124% (roots) at 100 mg.kg⁻¹. On the other hand, [23] observed growth increase in tests with OTC and *Triticum aestivum* L. (wheat) at low doses (0.01 mg.kg⁻¹). Both species are monocotyledonous, however different effects can be derived from soil type, which directly affects compounds bioavailability, or even by the difference in OTC uptake between species. No adverse effects were observed to *L. perenne* (Fig. 4) by CIP, even at the highest doses, while OTC affected significantly the plants growth from 1,000 mg.kg⁻¹.

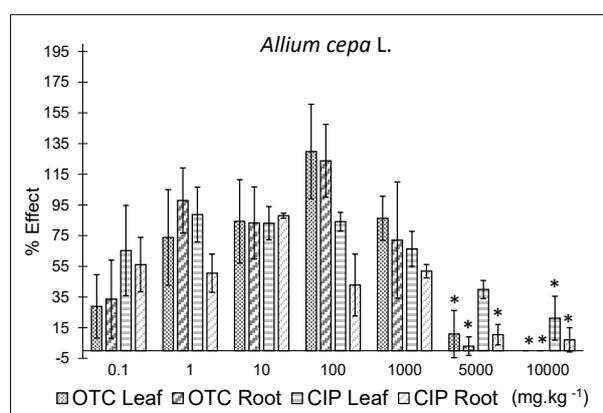


Figure 3. Average growth respect to control of *Allium cepa* L. (leaf) and root grown in soil with OTC and CIP addition in different doses (mg.kg⁻¹). (*) Significant inhibition compared to control ($p < 0.05$).

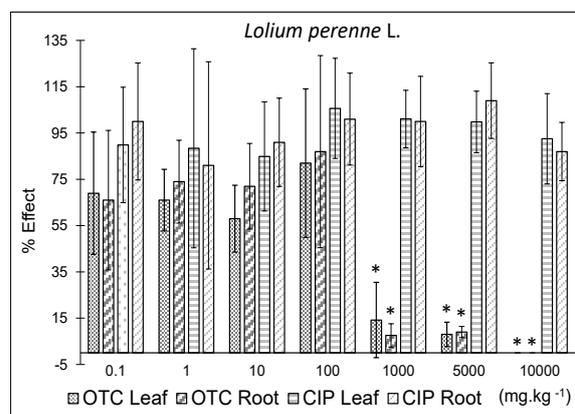


Figure 4. Average growth respect to control of *Lolium perenne* L. (leaf) and root grown in soil with OTC and CIP addition in different doses (mg.kg⁻¹). (*) Significant inhibition compared to control ($p < 0.05$).

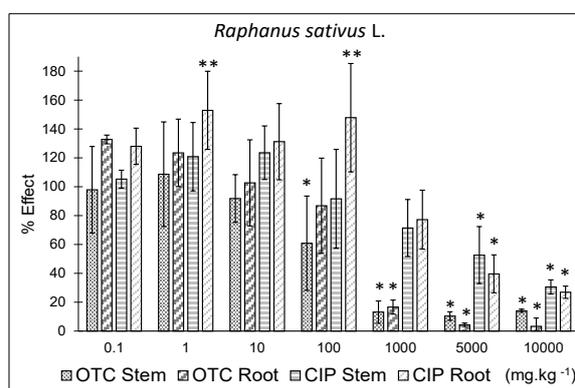


Figure 5. Average growth respect to control of *Raphanus sativus* L. (stem) and root grown in soil with OTC and CIP addition in different doses (mg.kg⁻¹). (*) Significant inhibition and (**) stimulation compared to control ($p < 0.05$).

According a previous study, with two wheat varieties (OTC tolerant and OTC sensitive), were observed negative effects on growth in both of them [34]. Another study with the same species, mentions OTC negative effects on growing roots with EC50 7.1 mol.L⁻¹ [35]. This value would be equivalent, based on the methodology of the current work, to a dose ranging between 0.88 and 5.28 mg.kg⁻¹ in soil. These low values comparable to the present study in soil suggest that plants are more exposed to contaminants in aqueous medium. In soils, these molecules may become less bioavailable depending on the physicochemical characteristics of the substrate. In CIP tests with *R. sativus* (Fig. 5), it was observed a root increase at 1 and 100 mg.kg⁻¹

doses, followed by a negative effect on the highest doses (5,000 and 10,000 mg.kg⁻¹), characterizing hormesis phenomenon [36]. Migliori et al. [37], observed the same phenomenon at low concentrations (50 and 100 mg.L⁻¹) and high concentration toxicities (5,000 mg.L⁻¹) in *R. sativus* root and aerial parts. The same authors reported that plants were still able to metabolize enrofloxacin to CIP as animals do. OTC significantly affected *R. sativus* aerial part growth from 100 mg.kg⁻¹. *R. sativus* had a greater root growth among the tested species. Its axial root, characteristic of dicotyledon, has greater root extension on the roots of *A. cepa* and *L. perenne*, both monocotyledons. In experiments with OTC and alfalfa (*Medicago sativa* L.), a dicotyledon, a previous study reported more sensitivity regarding roots than leaf, with 85% and 61% decreased fresh weight [38]. In addition to possible effects on plant growth, there is a concern about antibiotics uptake by edible plants [29, 13]. Kumar et al., mentioned chlortetracycline absorption by *A. cepa* through soil fertilized with manure [39]. Previous studies reported OTC transfer from pig manure to edible aquatic plants [40]. As in the previous experiment, soil characteristics could be influencing bioavailability and effects on plants,

with OTC and CIP.

2.4 *Vibrio fischeri* bioluminescence test

The results of the microtox® test give for OTC an EC₅₀ (15 min) of 49.4 mg.L⁻¹ (confidence interval - CI: 42.9 - 55.8 mg.L⁻¹). In tests with tetracycline, previous studies observed EC₅₀ (24h) 0.0251 mg.L⁻¹ [41] and EC₅₀ (30 min) 64.5 mg.L⁻¹ [42]. Current results are comparable to those for similar contact time. In the case of CIP test, the EC₅₀ (15 min) was 41 mg.L⁻¹ (CI: 35.1 - 47 mg.L⁻¹). According Hernando et al. [43], EC₅₀ values point out that OTC could have harmful or very toxic to representative organisms, as the FQ oxofloxacin for bacteria. Indeed, authors mentioned high sensitivity of cyanobacterium *Anabaena flosaquae* with low value for CIP (EC₅₀ 0.005 mg.L⁻¹) [33].

2.5 Toxicity and (bio)degradability in water

In BOD test it was used OTC and CIP as sole carbon sources (Table 2). High O₂ consumption for both antibiotics can be observed with concentration of 5 mg.L⁻¹.

Table 2. BOD (%) respect to control, (%) recovery average and standard deviation to OTC and CIP from BOD incubation at 5th and 28th days to 5 and 100 mg.L⁻¹.

Days/ Dose	Oxytetracycline (OTC)			Ciprofloxacin (CIP)		
	% BOD ^a	% Recovery	SD ^b	% BOD ^a	% Recovery	SD ^b
BOD ₅ 5 mg.L ⁻¹	77.6	104	8.27	82.6	N.d. ^c	N.d. ^c
BOD ₂₈ 5 mg.L ⁻¹	181	83.7	5.10	125	103	12.2
BOD ₅ 100 mg.L ⁻¹	89.1	87.4	8.63	78.3	85.9	23.7
BOD ₂₈ 100 mg.L ⁻¹	65.7	64.7	9.61	149	50.8	10.1

a: Biological oxygen demand; b: Standard deviation; c: Not determined

For the test with 5 mg.L⁻¹ OTC, it was verified very apparent presence of fungi at the end of incubation time, which could have contributed to the O₂ consumption detected. In fact, this functional redundancy is inherent in natural environment [5]. It was also observed at the end of experiment with 5 mg.L⁻¹, high recoveries, indicating low degradation ability for both drugs. OTC at 100 mg.L⁻¹ affected the function with a decrease of O₂ metabolic consumption, indicating toxicity to the microbiota or scarce available population to perform this metabolic function. For CIP (100 mg.L⁻¹), it was registered a large

increase in respiration rate related to control. These values indicate good biodegradability due to population adaptive capacity, or lower compound toxicity, since the compounds are available as the only carbon source for the inoculum. Girardi et al. [3], observed no CIP biodegradation in water related to biostatic activity. The difference in results between experiments may be due to the nature of the bacterial inoculum used for each test. The degradation of these molecules may be dependent on biotic possibilities, but may also be due to abiotic factors. In surface water, the main

processes of drug abiotic degradation are hydrolysis and photolysis [44]. Previous studies demonstrate instability of TCs to hydrolysis, while FQs were resistant to this process [3, 27, 44]. According to Babić et al. [44], solar radiation contributes significantly to CIP degradation but, as the incubations were performed in the dark, it is likely that the degradation observed in CIP was due to biotic factors.

2.6 Ecotoxicity summary

From the ecotoxicity tests, half maximal effective concentration (EC) values were estimated as a reference for risk assessment of

chemicals in the environment (Tables 3 and 4). According to the performed tests, soil functions were slightly affected. LOAEL (OTC soil respirometry) could not be estimated because no significant adverse effects were observed. Even though, continuous exposure to antibiotics can change or inhibit microorganisms growth, affecting soil enzyme activity [39] and thus, environmental functioning. Risk quotient (RQ) calculations to OTC and CIP indicate medium to high adverse effects to bacteria and other soil organisms [45, 46]. According to EC50 values for *V. fischeri*, OTC and CIP can be considered "Harmful to aquatic organisms, categorie III acute" [47].

Tables 3. OTC: LOAEL, EC values and confidence interval (CI) from ecotoxicity tests. ECx: mg.kg⁻¹ terrestrial/mg.L⁻¹ aquatic.

OTC tests	LOAEL ^a		EC20 ^b / CI ^c		EC50 ^d / CI ^c	
Soil respiration	N.q. ^e		N.q. ^e		N.q. ^e	
Nitrification	5,000		354 (87 - 1,447)		774 (415 - 1,443)	
<i>Vibrio fischeri</i>	N.q. ^e		N.q. ^e		49*	
Terrestrial plants	Leaf/Stem	Root	Leaf/Stem	Root	Leaf/Stem	Root
<i>A. cepa</i>	5,000	5,000	69 (31-153)	3 (1-13)	130 (84-200)	112 (58-216)
<i>L. perenne</i>	1,000	1,000	49 (24-102)	105 (58-192)	113 (79-161)	187 (129-271)
<i>R. sativus</i>	100	1,000	15 (5-44)	68 (17-274)	40** (5-44)	123 (60-253)

a: Lowest observed adverse effect level; b: Effective concentration 20% population; c: Confidence interval; d: Effective concentration 50% population; e: Not quantified; (*) Harmful to aquatic life; (**) Hazardous to terrestrial organisms: Reg. EC N° 1272/2008

Table 4. CIP: LOAEL, EC values and confidence interval (CI) from ecotoxicity tests. ECx: mg.kg⁻¹ terrestrial/mg.L⁻¹ aquatic.

CIP tests	LOAEL ^a		EC20 ^b / CI ^c		EC50 ^d / CI ^c	
Soil respiration	5,000		N.q. ^e		N.q. ^e	
Nitrification	1,000		255 (35 - 1,834)		1,700 (266 - 10,874)	
<i>Vibrio fischeri</i>	N.q. ^e		N.q. ^e		41*	
Terrestrial plants	Leaf/Stem	Root	Leaf/Stem	Root	Leaf/Stem	Root
<i>A. cepa</i>	5,000	100	125 (17-922)	1 (0-6)	288 (152-543)	10** (3-30)
<i>L. perenne</i>	N.q. ^e	N.q. ^e	N.q. ^e	N.q. ^e	N.q. ^e	N.q. ^e
<i>R. sativus</i>	5,000	5,000	35 (9-136)	38 (11-128)	219 (105-453)	162 (83-314)

a: Lowest observed adverse effect level; b: Effective concentration 20% population; c: Confidence interval; d: Effective concentration 50% population; e: Not quantified(*) Harmful to aquatic life, (**) Hazardous to terrestrial organisms: Reg. EC N° 1272/2008.

Although this regulation is not specific to these compounds, it may serve as a benchmark for the protection of human health and the environment. Previous studies cited good relations between EC50 to *V. fischeri* and LC50 to other aquatic species (e.g. water fleas - *Daphnia* spp., the ciliate

- *Tetrahymena pyriformis*, algae species and fish - catfish, goldfish and zebrafish) [48]. Ashfaq et al. [49], mentioned high risk quotient (3,300) for ofloxacin, a FQ, in tests with the same species. For CIP, same authors calculated RQ 3,500 to cyanobacteria *M. aeruginosa*. Values RQ > 1 are

considered high risk [50]. According Chen et al. [51], in a risk assessment in coastal environments with OTC, also show high RQ values for marine organisms. Adverse effects on different organisms can be quite diverse, tests with unique species can be more sensitive than those involving communities or populations. However, side effects are even more difficult to assess due to changes in the natural balance. Also, in the same test, toxicity synergistic effects of both antibiotics were not assessed. Due to EC50 values for *A. cepa* (root) and *R. sativus* (leaves), OTC and CIP can be considered as "Hazardous to soil organisms categories chronic IV and acute I" [47].

3. Material and Methods

3.1 Reagents and material

The antibiotics oxytetracycline hydrochloride purity $\geq 95\%$ and ciprofloxacin purity $\geq 98\%$ were purchased from Sigma-Aldrich (Saint Louis, USA). All solvents used were of HPLC grade. Soil samples were taken from an A horizon of a Haplic Arenosol [52], which is a granitic, sandy textured soil, poor in organic matter (1.3% oxidizable carbon and 21.5 C/N ratio), with low water holding capacity and cation exchange and pH 5.9. Samples were air dried and sieved (2 mm) previously. This soil meets OECD recommendation for ecotoxicological tests [53, 54].

3.2 Soil respiration and nitrification

Two different tests were performed to assess OTC and CIP toxicity on soil respiration and nitrification processes. Soil respiration was tested according to standard [54] with Respirometry Oxitop® system (WTW) to allow the manometric measure of O₂ consumption. It was done in glass bottles (0.5 L) with 50 g soil and 10 mL of solutions patterns for concentrations between 0.1; 1; 10; 100; 1,000; 5,000 and 10,000 mg.kg⁻¹ plus a control with distilled water, and three replicates per treatment. Thus, sample humidity was adjusted to 60% of the water holding capacity. Samples were incubated in the dark at 25 °C for 28 days, with automatic registration every 0.2 hours to determine cumulative respiration (CR). Once the first test was finished, the substrate

induced respiration (SIR) was determined by the addition of 4,000 mg.kg⁻¹ glucose solution to the same samples, measuring O₂ consumption (12 hour). Nitrification was performed according to the standardized method [53]. It was prepared in 50 mL plastic recipients with 5 g soil, 0.05 g dehydrated alfalfa, moisture and concentrations were the same used in the first test. Five replicates per treatment were done, including incubation aerobically over 28 days in the dark at 30 °C. Nitrate was extracted with KCl 0.1 M 1:5 (w/v), 1h with an axial stirrer, then filtered (Whatman® N° 42), and nitrate concentrations determined by the Brucine colorimetric method [55].

3.3 Toxicity to terrestrial plants

The assays were performed following [56] with *Allium cepa* (onion), *Lolium perenne* (ryegrass) and *Raphanus sativus* (radish). The seedling was done in plastic seedbeds with 15 g soil, spiked with the same concentrations described in the topic 3.2 and humidity equivalent to 50% of the water holding capacity. There were four replicates with five seeds planted, placed in natural lighting, in temperature around 25 °C and keeping soil moisture every two days. Finally, leaf (monocotyledon), stem (dicotyledon), and root lengths were measured after 9 to 16 days growth.

3.4 *Vibrio fischeri* bioluminescence test

Acute toxicity on *Vibrio fischeri* bacteria strains (NRRL B-11177), purchased from SDI Europe, Hampshire, UK, was assessed by Microtox® test [57]. Isotonic solutions were prepared using 2% NaCl. First concentrations were 0.1% and 0.01% (1:10 ratio); then, four serial dilutions were prepared in 1:2 ratio. Luminescence decreasing was measured after 15 minutes in contact with antibiotics at 15 °C, by means of a Microtox® M500 Analyzer. With the test results, using a log-linear model, a dose response curve was interpolated with 50% inhibitory concentration (IC50) with 95% confidence interval.

3.5 Biological oxygen demand (BOD)

The BOD of the samples was measured manometrically, according [58] in Respirometry

Velp® System devices. The test was done in concentrations of 5 and 100 mg.L⁻¹, with four replicates for each treatment, using an inoculum from sewage treatment plant and only distilled water for control. Incubation was done in the dark for 28 days at 22 °C. BOD was registered at the 5th and the 28th days.

3.6 OTC/CIP extraction and quantification

The extraction of OTC from soil samples (Soil respiration test) was adapted from [59]. Soil samples (5 g) were extracted with 30 mL solution made of methanol, 0.1M EDTA and Mc Ilvaine's buffer at pH 7 (2:1:1), in 12 hours axial stirrer. CIP extraction method was adapted from [60]. Soil samples (5 g) were extracted with 30 mL of solution with MgNO₃ 1M, NH₃ (pH 9), in 12 hours axial stirrer and filtered with slow filter paper Whatman® n° 42, 2.5 µm (Kent, England). Spiked soil samples with OTC and CIP concentrations were quantified before incubation. Quantification in samples from BOD test and from Soil respiration test were done by high pressure liquid chromatography (HPLC) with UV-visible detector, Kromasil® 100-5C18 column with 15 cm / 4.6 mm (5 µm), Teknokroma® TR-C-160-1 (ODS) precolumn and 1 mL/min flow mobile phase. OTC mobile phase consisted of 1% formic acid and acetonitrile (ACN) in starting proportions of 85% and 15%, increasing ACN concentration to 75% between 6 and 12 minutes and restoring start conditions in 15 minutes. The wavelength was set at 360 nm. CIP mobile phase consisted of 0.025 M phosphoric acid, and ACN with triethylamine (pH 3) as organic phase, with starting proportions 88.5% aqueous phase, decreasing to 47% at 17 min. and restoring initial conditions at 20 min. The wavelength was set at 278 nm.

3.7 Statistical analysis

Statistical analysis was done using IBM SPSS® software. A simple analysis of variance (ANOVA) and Duncan test comparing groups of means, both with a p<0.05 significance level, were done to assess significant differences between treatments, which allowed to deduce experimental values of lower observed adverse effect level (LOAEL). Dose-response curves were generated using Statistica® 6.0, adjusting the results to three sigmoid nonlinear regression

models: Gompertz, Hormesis and Logistic [36]. For each case, the best fit model was selected [61] to estimate effective concentrations EC50 and EC20, using R correlation coefficient as criteria. The EC50 value is widely used in EU legislation and EC20 is an index of least significant alteration, comparable to LOAEL value considering that this research was conducted in experimentally controlled laboratory conditions. The charts were performed using Microsoft Office Excel® (2016).

4. Conclusions

In soil toxicity tests, the soil function maintenance, even in the case of nitrification, pointed to the potential soil capacity on antibiotics immobilization, as well as the soil microbiota ability to adapt and fulfill the functions investigated. The low OTC recovery (high % degradation) in soil tests compared to the one observed for CIP, suggests a higher degradability of this antibiotic under the study conditions. High values in BOD tests (% respect to control) with CIP indicate good microbiota adaptability or lower toxicity, whereas OTC has been shown to be more unfavorable to the performance of the evaluated function. Considering our data on *V. fischeri*, acute and chronic toxicity tests should be performed on other planktonic organisms with extended dosing intervals to establish toxicity values. In plant growth experiments, OTC affected negatively species in the following order: *R. sativus* (leaf/stem) > *R. sativus* (root), *L. perenne* (both) > *A. cepa* (both). CIP produced negative effects in this sequence: *A. cepa* (root) > *A. cepa* (leaf) / *R. sativus* (both). The estimated EC50 for *A. cepa* (root) exposed to CIP in an environmentally relevant concentration (10 mg.kg⁻¹) point out that these antibiotic under the tested conditions can potentially affect edible plants growth. Chemically these molecules perform very complex and require a follow-up study with extended dosing intervals to establish more precise toxicity values and considering more trophic levels and endpoints.

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