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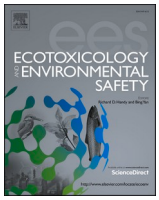

Ecotoxicity of five veterinary antibiotics on indicator organisms and water and soil communities

María Rosa Pino-Otín [*,1], Antonio Valenzuela, Cristina Gan, Guillermo Lorca, Natalia Ferrando, Elisa Langa, Diego Ballestero

Universidad San Jorge, Villanueva de Gállego, Zaragoza 50830, Spain

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ABSTRACT

This study explores the environmental effects of five common veterinary antibiotics widely detected in the environment, (chlortetracycline,CTC; oxytetracycline,OTC; florfenicol,FF; neomycin, NMC; and sulfadiazine, SDZ) on four bioindicators: *Daphnia magna*, *Vibrio fischeri*, *Eisenia fetida*, and *Allium cepa*, representing aquatic and soil environments. Additionally, microbial communities characterized through 16 S rRNA gene sequencing from a river and natural soil were exposed to the antibiotics to assess changes in population growth and metabolic profiles using Biolog EcoPlates™. Tetracyclines are harmful to *Vibrio fisheri* (LC_{50} ranges of 15–25 μg/mL), and the other three antibiotics seem to only affect *D. magna*, especially, SDZ. None of the antibiotics produced mortality in *E. fetida* at concentrations below 1000 mg/kg. NMC and CTC had the highest phytotoxicities in *A. cepa* (LC_{50} = 97–174 μg/mL, respectively). Antibiotics significantly reduced bacterial metabolism at 0.1–10 μg/mL. From the highest to the lowest toxicity on aquatic communities: OTC > FF > SDZ ≈ CTC > NMC and on edaphic communities: CTC ≈ OTC > FF > SDZ > NMC. In river communities, OTC and FF caused substantial decreases in bacterial metabolism at low concentrations (0.1 μg/mL), impacting carbohydrates, amino acids (OTC), and polymers (FF). At 10 μg/mL and above, OTC, CTC, and FF significantly decreased metabolizing all tested metabolites. In soil communities, a more pronounced decrease in metabolizing ability, detectable at 0.1 μg/mL, particularly affected amines/amides and carboxylic and ketonic acids ($p < 0.05$). These new ecotoxicity findings underscore that the concentrations of these antibiotics in the environment can significantly impact both aquatic and terrestrial ecosystems.

1. Introduction

Antibiotics (ABXs) have been used on a large scale not only to treat human infections, but also in livestock where they have also been used preventively or for promoting growth (Hao et al., 2014).

Despite the discrepancies in the data collection methods (Oliver et al., 2020), an average annual antimicrobial consumption per kilogram of animal produced has been estimated at 172 mg/kg for pigs, 148 mg/kg for chicken and 45 mg/kg for cattle. Moreover, between 2010 and 2030, the global consumption of veterinary antimicrobials is expected to increase from 63151 ± 1560 tons to 105596 ± 3605 tons, a 67% increase (Van Boeckel et al., 2015). Other estimates predict increases of up to 200000 tons in 2030 (Klein et al., 2018).

The most commonly sold antibiotic classes in 31 European countries were (after penicillins, 31.2%) tetracyclines (chlortetracycline and oxytetracycline, 25.8%), sulfonamides such as sulfodiazine (9.9%) and aminoglycosides such as neomycin (5.9%). Amphenicols (Florfenicol) already account for 2.8% (EMA, 2022 report).

This massive consumption has generated a series of problems encompassing human, animal and environmental health (Berkner et al., 2014; Bielen et al., 2017; Carvalho and Santos, 2016; Pino-Otin et al., 2022), which are interrelated areas in the holistic concept of One Health (Robinson et al., 2016).

Animals, as well as humans, are not able to metabolize antibiotics efficiently, so a high percentage of the whole or metabolized product is excreted via urine or feces. For example, up to 90% of the active

* Correspondence to: Universidad San Jorge, Campus Universitario Villanueva de Gállego, Autovía A- 23 Zaragoza-Huesca, km. 510, Villanueva de Gállego, Zaragoza 50830, Spain.

E-mail addresses: rpino@usj.es (M.R. Pino-Otín), avalenzuela@usj.es (A. Valenzuela), cgan@usj.es (C. Gan), glorca@usj.es (G. Lorca), nferrando@usj.es (N. Ferrando), elanga@usj.es (E. Langa), dballestero@usj.es (D. Ballestero).

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ingredient of sulfonamides or up to 65% of chlortetracycline is eliminated in this way (Quaik et al., 2020). These wastes can be channeled to wastewater treatment plants (WWTPs) that do not completely mineralize these substances, so antibiotics or their degradation products can reach waterbodies when wastewater or sewage is discharged into the environment, including rivers and lakes, as well as the marine environment (Haenelt et al., 2023; Korkmaz et al., 2022; Moldovan, 2006; Omuferen et al., 2022; Wang et al., 2021b). It is estimated that thousands of tons of antimicrobials and their transformation products enter the environment annually (Harnisz et al., 2015; Ji et al., 2012b).

The average concentrations of antibiotics in different aquatic compartments range between few ng/L to few μg/L (Barbosa et al., 2016; Bhagat et al., 2020; Loos et al., 2013; Michael et al., 2013). CTC was found in water samples of US streams with maximum concentrations of 0.69 μg/L (Kolpin et al., 2002). Detected SDZ values were as high as 840 ng/L in surface water in Nairobi (Ngigi et al., 2020) or 1.181 ng/L in the Chaobai River in China (Su et al., 2020). In river basins of China OTC was found to be the most frequent antibiotic in the aquatic environment (Guo et al., 2022), and the most abundant in river basins with concentrations of up to 361.1 μg/L and 56.1 μg/L have been detected in northern China and Colorado, USA, respectively (Jiang et al., 2014; Karthikeyan and Meyer, 2006). In literature reviews, the highest maximum concentrations, i.e., 560 μg/L, were observed for OTC in surface waters in Asia (Kovalakova et al., 2020). Fish farms can also be a source of antibiotic discharges into watercourses, especially OTC, florfenicol (FF) and sulfadiazine (SDZ) (Jara et al., 2021; Lulijwa et al., 2020).

ABXs can also reach the soil when sewage or sewage sludge is used for irrigation and can accumulate after repeated fertilizations, with tetracyclines being the most frequently detected group of antibiotics in particular CTC. For example, up to 2668.9 mg/kg of CTC were detected in soils around pig feedlots in China (Ji et al., 2012a); 9.5 μg/kg of CTC was detected in the top 10 cm of soil from eight fields fertilized with animal manure (Kolpin et al., 2002). The highest concentration (143.97 mg/Kg) of an ABX found in manure in a study in Shenyang (China) was also CTC (An et al., 2015). ABXs can leach into groundwater or even be taken up by crops (Aydin et al., 2022; Aznar et al., 2014; Bastos et al., 2020). For example, chlortetracycline (CTC) has been detected at concentrations of 0.139 mg/kg in Leek, celery, pakchoi cabbage and radish (Wang and Han, 2008) and 0.017 mg/kg in *Allium cepa* (Kumar et al., 2005); OTC ranged from 0.041 to 0.174 mg/kg in a study with more than nine crops like lettuce, carrot and potato (Yao et al., 2010), and FF was detected in lettuce and carrots (15–38 μg/Kg) (Boxall et al., 2006). Therefore, antibiotics may contaminate the human food chain (Kim et al., 2012).

While the emergence of resistance mechanisms in bacteria remains a significant environmental concern, the potential harm of antibiotics to non-target organisms is also concerning (Gonzalez-Pleiter et al., 2013; Patil et al., 2020) due their persistent discharge, bioactive characteristics and their continual presence, even at comparatively low levels ranging from ng/L to μg/L in surface waters and from ng/g to μg/g in sediments (Hernando et al., 2006; Kummerer, 2010).

Perhaps one of the best bioindicators that can estimate the impact of these antibiotics on aquatic ecosystems are the microbial communities that provide basic ecosystem services. There are not many ecotoxicity studies on riverine microbial communities, but they do show that exposure to antibiotics such as OTC and FF can produce a decrease in the microbial diversity and changes in the structure of the community (Gao et al., 2018; Harrabi et al., 2019a). Among other aquatic organisms, a recent review (Duan et al., 2022) suggested that some antibiotics, such as sulfadiazine (SDZ), pose a great risk to the aquatic system as well as algae, crustaceans and fish (Chen et al., 2020; De Orte et al., 2013; Lin et al., 2014; Wang et al., 2017), insects (Xie et al., 2019). OTCs are ranked as "toxic" to algae and aquatic plants, and "very toxic" to cyanobacteria (Kovalakova et al., 2020). OTCs an CTCs present effects (Guo and Chen, 2012; Zounkova et al., 2011) on freshwater phytoplankton alone or combined (Carusso et al., 2018) as microalgae (Bialk-Bielinska et al., 2013; Kolar et al., 2014; Siedlewicz et al., 2020; Zounkova et al., 2011), diatoms (Phaeodactylum tricornutum) and cyanobacteria (van der Grinten et al., 2010; Zhou et al., 2020). Tetracyclines also affect fish (Park and Choi, 2008), crustaceans (Mayor et al., 2008) and amphibians with an LC_{50} of 64.04 mg/L and alterations in the oxidative stress biomarkers of larvae (Lourido et al., 2022). In the same way, it was found that OTC and FF have stronger adverse effects on aquatic plants such as *Lemmna minor* (LC_{50} ranges from 0.68 to 3.26 mg/L; (Bialk-Bielinska et al., 2013; Kolodziejska et al., 2013; Machado et al., 2016; Zounkova et al., 2011). FF also appears to be toxic to algae (Goncalves Ferreira et al., 2007; Lai et al., 2009) and affects fish such as *Nile tilapia* larvae (Mattioli et al., 2020) and snails (Florencio et al., 2014). Microalgae can also be affected by neomycin (NMC) (Lee et al., 2021) with LC_{50} = 4.60 mg/L values. The aquatic invertebrate *Daphnia magna* and the bacterium *Vibrio fisheri* can serve as robust individual bioindicators for assessing the impact of antibiotics in aquatic ecosystems. *D. magna* susceptible to contaminants through both surface contact and ingestion as a filter feeder, exhibits heightened sensitivity to environmental changes (Martins et al., 2007). *V. fisheri*, with its high sensitivity, ease of use, and reliability, also proves to be an effective bioindicator (Abbas et al., 2018).

In terrestrial ecosystems, ABXs such as CTC could affect enzyme activities (Liu et al., 2015) and change microbial-mediated nitrogen behavior in soils (Stone et al., 2011), and CTC, OTC and SDZ affect microbial community structure (Hammesfahr et al., 2008; Yin et al., 2018; Zhou et al., 2020). SDZ reduces rates of nitrification and N mineralization in soils (Hammesfahr et al., 2011) and have the potential to disturb soil organic matter cycling (Qiu et al., 2021). OTC also showed toxic effects in the reproduction of terrestrial invertebrates (Giordano et al., 2010), and CTC causes DNA damage and biochemical toxicity in the earthworm *E. fetida* (Dong et al., 2012; Lin et al., 2012). These ABXs can also affect terrestrial plants. There is evidence that OTC, CTC and FF exhibit inhibitory and genotoxicity effects on root and shoot elongation of different crops (Jin et al., 2009; Kong et al., 2007; Xie et al., 2010) and OTC inhibits sorghum seed germination (Wieczerzak et al., 2018). Hence, the terrestrial invertebrate *Eisenia fetida* and the plant *Allium cepa* are commonly employed as suitable indicators for assessing soil ecotoxicity. *E. fetida*, with pivotal roles as decomposers, soil engineers, and contributors to nutrient cycling, demonstrates high sensitivity to contaminants, establishing it as an excellent indicator of soil quality (Pino-Otín et al., 2015; Wang et al., 2012). Moreover, the *A. cepa* root growth assay has seen frequent utilization as a phytotoxicity test (Caetano et al., 2018; Pino-Otín et al., 2019), attributed to its sensitivity, rapid response, ease of handling, and ability to represent effects on higher plants. The capacity of both indicators to undergo long-term assays further amplifies their utility in ecological studies.

The objective of this research is to conduct a comprehensive ecotoxicity study of five veterinary antibiotics -widely detected in the environment with evidence of damaging aquatic and terrestrial ecosystems- both, in individual non-target organisms and in communities, at different trophic levels.

To this end, the aim is to: (1) Quantify the ecotoxicity of the five ABXs on four bioindicators, two from aquatic environments (the invertebrate *Daphnia magna* and the marine bacterium *Vibrio fischeri*) and two from soils (the earthworm *Eisenia fetida* and the plant *Allium cepa*); (2) to evaluate the impact of these five ABXs on microbial communities characterized through 16 S rRNA gene sequencing and obtained from a river and natural soil on population growth, (3) Analyze sublethal effects on microbial communities, measured as changes in the metabolic profile of the microorganisms.

2. Material and methods

2.1. Antibiotics

Five veterinary antibiotics were used belonging to four families: the tetracyclines chlortetracycline (CTC) and oxytetracycline (OTC), the amphenicol florfenicol (FF), the aminoglycoside neomycin (NMC) and the sulfonamide, sulfadiazine (SDZ). CAS number, supplier, purity and other details are shown in Table 1.

2.2. Daphnia magna assay

Daphnia magna (water flea) assays were conducted in accordance with OECD 202 (2004) guidelines following the standard operational procedures outlined in the Daphtoxkit FTM magna (1996) from Vidrafoc (Spain) (ref. DM121219).

The kit was stored at 5 °C until use. The Daphnia eggs were incubated for 72 h at 20–22 °C under 6000-lux light conditions in a TOXKIT model CH-0120D-AC/DC incubator (provided by ECOTEST, Spain). The neonates were pre-fed with one vial of spirulina microalgae 2 h prior to exposure to ABXs. Various solutions of ABXs were prepared in synthetic freshwater (ISO 6341 2012) at the following test concentrations: CTC (50, 100, 200, 300 and 400 μg/mL), OTC (200, 300, 400, 500 and 600 μg/mL), FF (62.5, 125, 250, 500 and 1000 μg/mL), NMC (62.5, 125, 250, 500 and 1000 μg/mL) and SDZ (62.5, 125, 250, 500 and 1000 μg/mL).

Synthetic freshwater served as the negative control, and the pH of the solutions was adjusted to 7–7.5 using 0.1 M NaOH or HCl if needed. Each concentration of ABXs was tested in five replicates per plate, with five organisms per well. Daphnids were incubated in complete darkness for 24 h at 20–22 °C. Following the 24 h exposure period, daphnids that failed to swim for 15 seconds after gentle agitation of the test vial were deemed immobile. The results were calculated as the LC_{50} (the concentration of the compound resulting in 50% of lethality).

2.3. Vibrio fischeri assay

The bioluminescence inhibition assays were conducted following the established methodology for the *V. fischeri* acute toxicity test (UNE-EN-ISO 11348–3 2009). The *V. fischeri* strain NRRL-B-11,177, obtained from Macherey-Nagel (ref. 945 006), was used in this assay. The lyophilized *V. fischeri* were rehydrated using the provided reactivation solution and stored at 4 ºC for 5 min. Antibiotic stock solutions were prepared using a 2% NaCl stock solution (v/v) at different concentrations according to the solubility of each compound: CTC (6.25, 12.5, 25, 50 and 100 μg/mL), OTC (7.8, 15.6, 31.2, 62.5 and 125 μg/mL), FF (62.5, 125, 250, 500 and 1000 μg/mL), NMC (250, 500, 1000, 2000 and 2500 μg/mL) and SDZ (12.5, 25, 50, 100 and 200 μg/mL). NaOH and HCl 0.1 M solutions were used to adjust the pH of the test solutions to a range between 6 and 8. The assay was performed in quadruplicate, with four tubes containing bacteria but no ABX solutions serving as negative controls.

To initiate the assay, the luminescence baseline was measured. Subsequently, 0.5 mL of each ABX dilution to be tested was added to the respective tubes, and after 30 minutes, the second measurement of luminescence inhibition was performed. The measurements were obtained using a Biofix® Lumi-10 luminometer (Macherey-Nagel). The endpoint of the test was determined by the reduction in bacterial light production. The EC_{50} (the concentration of product that generates 50% of the measured effect), was expressed as a percentage of luminescence inhibition and calculated for each concentration compared to the control.

2.4. Eisenia fetida assays

Adult *Eisenia fetida* individuals were obtained from composters at Todo Verde (Spain). Prior to testing, the earthworms were acclimated for 15 days in sphagnum peat-conditioned substrate provided by the Spanish Flowers Company (Spain) and maintained under stable conditions: 18–25 ∘C, pH 7.5–8 and 80–85% humidity.

For the ecotoxicity assessment, adult earthworms above 60 days old, exhibiting a clitellum and weighing 300–600 mg were selected. The toxicity tests were conducted following the guidelines of the OECD 207 (1984) methodology, as previously described(Pino et al., 2015) in standardized soil substrate: quarzitic sand (Imerys Ceramics España, S. A., Spain), kaolinic clay (Imerys Ceramics España, S.A., Spain) and sphagnum peat (Verdecora vivarium, Spain) in a 7:2:1 ratio.

Polypropylene containers with a capacity of 1 L and perforated lids for ventilation and to minimize moisture loss were filled with 600 mg of this artificial soil. Each box contained ten earthworms and ABX solutions with final concentrations of 0.1, 1.0, 10, 100 and 1000 mg/Kg and sufficient deionized water to adjust the humidity to a level of 35–45% of the dry soil weight. Negative controls were prepared following the same procedure but without ABX. Each concentration was tested in triplicate. The containers were maintained under controlled environmental conditions at 20 ± 2 ∘C, 80–85% relative humidity and 400–800 lx light intensity. Earthworm mortality was assessed after 14 days of treatment and LC_{50} calculated.

2.5. Allium cepa assay

Bulbs of *A. cepa* (var. Stuttgarter Riesen, 14/21 gauge) were obtained from the Fitoagrícola Company (Spain). Prior to the test, young bulbs

Table 1
Properties of the antibiotics studied.

Antibiotic	Abbreviation	Family	CAS number	Supplier	Purity	Molecular weight (g/mol)	Water solubility (mg/mL)	pKa1/ pKa2	Log Ko/ w
Chlortetracycline	CTC	Tetracyclines	57–62–5	Sigma-Aldrich	>99%	478.88	0742[a]	3,3[e]	0,72[a]
Oxytetracycline	OTC		2058–46–0	Sigma-Aldrich	>99%	496.89	100[b]	3,18[f]	-2,9[a]
Florfenicol	FF	Amphenicols	73231–34–2	Laboratorios Karizoo S.A	-	358.21	9,94[a]	10,73[e]	-0,04[a]
Neomycin	NMC	Aminoglycosides	1404–04–2	Laboratorios Karizoo S.A	-	614.64	>250[c]	12,9[g]	-3,7[g]
Sulfadiazine	SDZ	Sulfonamides	68–35–9	Acofarma	>99%	250.28	0077[d]	6,36[h]	-0,09[i]

[a] (Rathborey Chan et al., 2020)
[b] (National Center for Biotechnology Information, 2023a)
[c] (National Center for Biotechnology Information, 2023b)
[d] (Rose-Marine Dannenfelser et al., 1991)
[e] (Settimo L et al., 2014)
[f] (Drugbank, 2023)
[g] (Sarmah AK et al., 2006)
[h] (Ritter S et al. 1995)
[i] (Corwin Hansch et al., 1995)

were carefully peeled, taking care to avoid damage to the root ring. Acute toxicity experiments with *A. cepa* were conducted following the method outlined by Fiskesjo (1993). The bulbs were placed in 15 mL tubes and mineral water (VERI, Aguas de San Martín de Veri S.A., Spain) was used as the growth medium due to its appropriate calcium and magnesium content (https://www.veri.es/es/el-producto). Ecotoxicological tests were performed with 12 replicates for each concentration: 0.03, 0.3, 3.0, 30 and 300 mg/L. The negative control consisted of water. The bulbs were cultivated in an incubator under light conditions at 25 °C for 72 hours, with the test solutions being refreshed every 24 hours. Root growth inhibition was measured as end point and EC50 calculated.

2.6. River and soil microorganism assays

2.6.1. River samples

Water samples were collected on October 2022 from the Gallego River (Zaragoza, Spain) and transported to the laboratory according to standard procedures (ISO 19458:2006, AENOR ISO 19458:2006, AENOR). This sample was used for genetic analysis, chemical analysis and Biolog EcoPlates™ assays (Tiselab S.L., Spain). In situ parameters were also measured: water temperature 17 ºC (Nahita thermometer); pH = 7.5 (PanReac AppliChem, A011435) and conductivity of 2.8 mS (conductivity meter Hanna HI8733).

For genetic analysis, microorganisms were obtained from 5 L of river water. The water was filtered through a 0.22 μm cellulose nitrate filter (Sartorius) using a vacuum flask. The filtered microorganisms were then resuspended in a sterile Falcon tube containing 50 mL of phosphate-buffered saline (PBS) and centrifuged at 5000 g for 10 min. The supernatant was discarded, and the resulting pellet was stored at −80 °C for subsequent sequencing.

For the ecotoxicity assays, 1 L of river water was filtered through a 70 μm nylon sieve (BD Falcon) to remove debris. The filtered water was then stored at 4 °C in the dark until being used in Biolog EcoPlates™. Two liters were taken the same day of sampling to the Laboratorios Valero Analítica (Zaragoza, Spain) for physicochemical analysis.

2.6.2. Soil samples

Soil samples were collected on November 2022 from a pesticide-free and contaminant-free crop field located at Agri-food research and technology center of Aragon (CITA, www.cita-aragon.es) in Zaragoza, NE Spain. The soil composition was analyzed by the CITA Soil and Irrigation Unit (Supporting Information 1).

For the genetic analysis, 20 g of soil were mixed with 100 mL of sterile water. The mixture was stirred for 30 min under sterile conditions and allowed to settle for 1 h. Next, 10 mL of the sample was transferred to Falcon tubes and subjected to sonication for 1 min, followed by centrifugation at 1000 g for 10 min. The supernatant was collected under sterile conditions, and the soil microorganisms were obtained by filtering the supernatant through a 0.22 μm cellulose nitrate filter (Sartorius) using a vacuum flask. The content of the filter was carefully washed with sterilized PBS, then centrifuged at 5000 g for 10 min. The resulting pellets were collected with an eyedropper and stored at −80 °C until sequencing.

Before conducting the ecotoxicity assays, 10 g of soil were passed through a 2 mm sieve (Becton Dickinson, Spain). To this pre-sieved soil, 95 mL of sterile water was added, and the sample was stirred in an Erlenmeyer flask for 30 min and allowed to settle for 1 h. Then, 10 mL of the upper portion of the flask was transferred to Falcon tubes and centrifuged at 1000 g for 10 min, with the supernatant collected under sterile conditions. This process was repeated five times. The total obtained supernatant was filtered through a 70 μm nylon sieve (Becton Dickinson, Spain) to remove suspended soil debris, resulting in a sufficient sample volume for inoculation in Biolog plates.

2.6.3. Community-level physiological profiling (CLPP) of river and soil microorganisms

To evaluate the impact of ABXs on the metabolic activity of microbial communities in water and soil, we employed the Biolog EcoPlate test (Tiselab S.L., Spain). This test allowed us to monitor changes in the utilization of 31 different carbon sources, as previously described (Pino-Otin et al., 2017; Rosa Pino-Otin et al., 2019). For the ecotoxicity assessment, we prepared various concentrations of ABXs (0.1, 10 and 100 μg/mL) in a final volume of 150 μL within the wells of a Biolog plate. We used prefiltered river water (refer to Section 2.6.1) or the supernatant obtained from the soil sample (refer to Section 2.6.2) to evaluate the impact of ABXs on microbial communities in water and soil, respectively. The pH of the solutions ranged between 6 and 7. Each concentration was tested in triplicate, and all procedures were conducted under sterile conditions within a flow chamber. The plates were then placed in the dark at 25 °C for 7 days, maintaining sterile conditions throughout. Optical density (OD) measurements were taken at a wavelength of 590 nm immediately after inoculation and once daily using a Synergy H1 Microplate reader (BIO-TEK, USA) and Gen5™ data analysis software. The carbon utilization rate was determined by assessing the reduction of tetrazolium violet redox, as described by Pohland and Owen (2009).

2.6.4. Genetic identification of microbial populations

The prefiltered solution obtained at the end of Section 2.6.1 was subjected to an additional filtration step using Sartori 0.2 μm cellulose nitrate filters that had been thoroughly washed in PBS (Phosphate Buffered Saline) solution with a pH of 7.5. The PBS solution was collected in Falcon tubes and centrifuged at 5000 g for 10 min. After careful removal of the supernatants, the resulting pellets were frozen at −80 °C for genetic analysis.

DNA was extracted using the kit AllPrep® PowerViral® DNA/RNA Kit (QiaGen), following the manufacturer's instructions. After extraction, purified DNA samples were fluorimetrically quantified using Picogreen® and 1.5 ng of input DNA from each sample was used to amplify the V3–V4 region of 16 S rRNA gene. PCR primers used were 5'-ACACTGACGACATGGTTCTACACCTACGGGNGGCWGCAG-3' and 5'-TACGGTAGCAGAGACTTGGTCTGACTACHVGGGTATCTAATCC-3, which include a common extension to allow for further library preparation. The V3–V4 specific PCR consisted of 21 cycles and was made using the Q5® Hot Start High-Fidelity DNA Polymerase (New England Biolabs) and 100 nM primers. After amplification, positive 16 S derived bands were assessed by agarose gel electrophoresis, DNA products were diluted and a second PCR of 13 cycles was performed in the presence of 400 nM primers: 5′-AATGATACGGCGACCACCGAGATCTACACTGACGACATGGTTCTACA-3′ and 5′-CAAGCAGAAGACGGCATACGAGAT-[10 nucleotides barcode]-TACGGTAGCAGAGACTTGGTCT-3′, which belong to the collection Access Array Barcode Library for Illumina Sequencers (Fluidigm). This second PCR completes the Illumina library construction and labels each sample with a unique barcode. After individual library preparation, samples were checked for size and concentration in a Tape Station (Agilent) and an equimolar pool was made, purified using AMPure beads and titrated by quantitative PCR using the "Kapa-SYBR FAST qPCR kit forLightCycler480" and a reference standard for quantification.

The pool of amplicons was denatured prior to be seeded on a flowcell at a density of 10pM, where clusters were formed and sequenced using a "MiSeq Reagent Kit v3", in a 2×300 pair-end sequencing run on a MiSeq sequencer.

The fastq files were constructed using the bcl2fastq integrated in the Illumina sequence workflow. Phylogenetic analysis was made using the 16 S Metagenomics app of Base Space v1.1.0 (Illumina). The Greengenes (13_5) database was used for taxon assignment.

2.7. Statistical analysis and visualization

To determine the dose-response curves for *D. magna* mobility, *E. fetida* survival, *A. cepa* root elongation and *V. fischeri* luminescence, we employed a logit logistic regression using the XLSTAT software (version 2014.5.03). This allowed us to calculate the corresponding LC_{50} and EC_{50} values and the confidence limits.

The microbial activity of each Biolog EcoPlate was quantified as the the Average Well Color Development (AWCD), following the method described by Garland and Mills (1991), as previously reported in other studies (Pino-Otin et al., 2021).

Graphical representations of the results were generated using appropriate visualization techniques.

$$AWCD = \sum_{i=0}^{i=12} (OD_{t=xi} - OD_{t=x0}) \quad (1)$$

where ODi is the optical density value from each well at any given time after subtracting ODt = X0 from ODt = Xi of that well.

The significance of Average Well Color Development (AWCD) differences compared to the control was assessed using a Kruskal-Wallis one-way ANOVA for non-parametric data, conducted with SPSS software (version 28.0.1.0, 142), and Student's t-test for two independent samples, utilizing XLSTAT software (version 2014.5.03) in the case of absorbances of metabolites that exhibited a normal distribution.

Finally, AWCD curves were fitted to a logistic model (Eq. 2) for sigmoid microbial growth (Peleg et al., 2007) with the Excel Solver (Microsoft 365) complement:

$$AWCD = \frac{C\max}{1 + e^{b-rt}} \quad (2)$$

where C_{max} is the carrying capacity, that is, the maximum reachable population density, r (intrinsic rate of population increase), and b is a fitting parameter. C_{max} and r were obtained for each ABX.

3. Results

3.1. Ecotoxicology of the antibiotics in Daphnia magna

Fig. 1a shows the dose-response curves of *D. magna* exposed for 24 h to the five antibiotics. There was a clear dose effect for the four ABXs tested, although with very similar LC_{50} ranges. NMC is not represented because it produced no effect at doses lower than 1000 μg/mL. LC_{50} values are shown in Table 2. Toxicity from highest to lowest was (LC_{50} values in μg/mL): NMC > CTC > FF > OTC > SDZ. All toxicity values analyzed with the chi-squared test were highly significant ($p < 0.0001$).

3.2. Ecotoxicology of the antibiotics in Vibrio fischeri

The dose-response curves of the bacteria *V. fischeri* exposed for 30 min to both tetracyclines are plotted in Fig. 1b. The other antibiotics presented LC_{50} values > 200 μg/mL. The EC_{50} values of the tetracyclines are shown in Table 2. The other three ABXs (FF, NMC and SDZ) had LC_{50} values higher than the concentrations tested. The chi-squared tests were highly significant ($p < 0.0001$) in both cases.

3.3. Ecotoxicology of the antibiotics in Allium cepa

In Fig. 1c, the dose-response curves of *A. cepa* exposed for 72 hours to CTC and NMC are shown. The other antibiotics presented EC_{50} values > 300 μg/mL. All values can be seen in Table 2. The chi-squared tests were highly significant ($p < 0.0001$) in both cases.

3.4. Ecotoxicology of the antibiotics in E. fetida

None of the five antibiotics tested on *E. fetida* showed toxicity. The

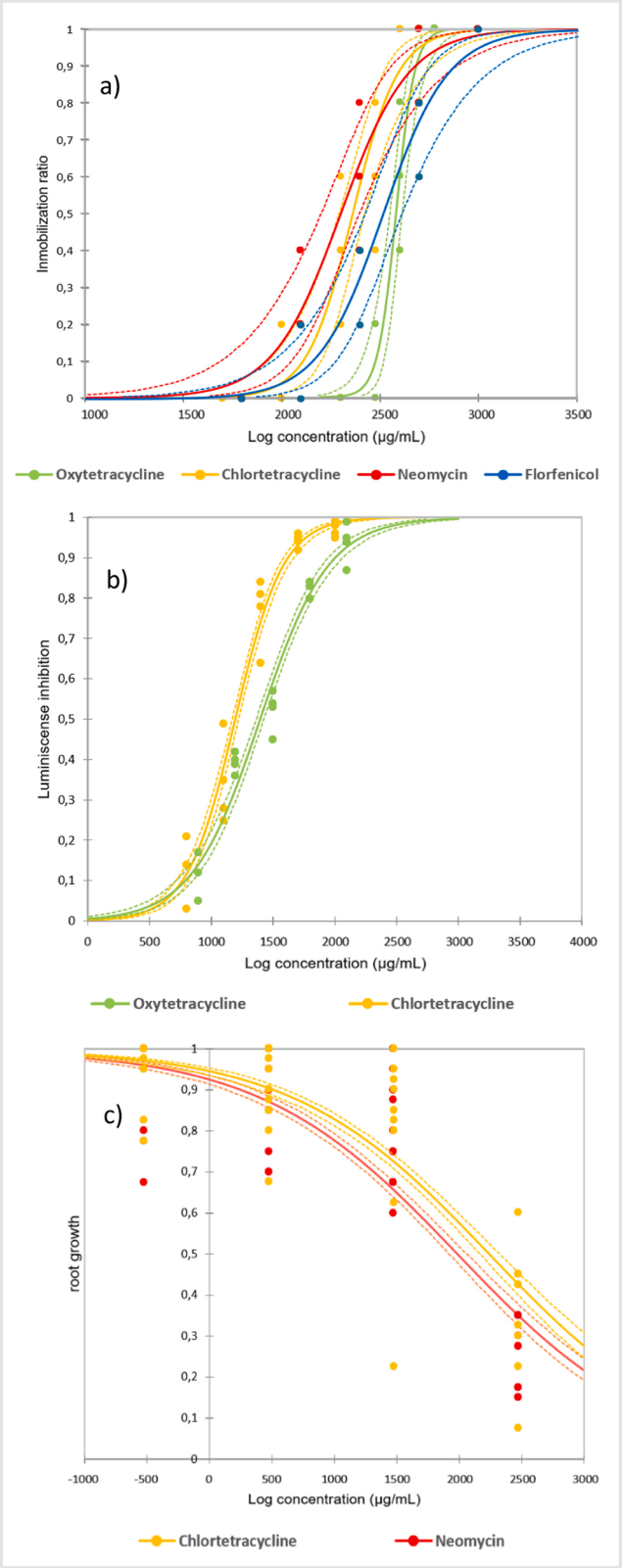

Fig. 1. Dose-response curve for (a) *Daphnia magna* after 24 h of exposure to oxytetracycline (OTC), chlortetracycline (CTC), neomycin (NMC) and florfenicol (FF), (b) *Vibrio fischeri* after 30 min of exposure to OTC and CTC and (c) *Allium cepa* after 72 h of exposure to CTC and NMC. Dashed lines indicate the confidence limits (95%). Antibiotics with LC_{50} or EC_{50} outside the studied range have not been represented.

Table 2
Ecotoxicology of antibiotics on bioindicators and river and soil microorganism communities. The last row contains values from the literature for tests under the same conditions.

Antibiotic	Daphnia magna (μg/mL)		Vibrio fischeri (μg/mL)		Eisenia fetida (mg/Kg)		Allium cepa (μg/mL)		River Microrganisms (μg/mL)		Soil Microorganisms (μg/mL)	
	$LC_{50\ (24\ h)}$	$LC_{10\ (24\ h)}$	$EC_{50(30')}$	$EC_{10(30')}$	LC_{50}	LC_{10}	EC_{50}	EC_{10}	$LC_{50\ (120\ h)}$	$LC_{10(120\ h)}$	$LC_{50(120\ h)}$	$LC_{10(120\ h)}$
Chlortetracycline	**229.6**[(1)] (195.3–264.2)	**128.0** (84.5–158.2)	**15.6** (14.7–16.6)	**5.8** (5.17–6.5)	**>1000**	**94.4** (8.7–290.1)	**174** (146.3–210.5)	**3.2** (2.5–4.1)	**11.7** (10.3–32.9)	**5.9** (0.2–7.8)	**4.6** (1.6–7.3)	**0.7** (0.04–1.8)
Oxytetracycline	**383.1**[(2)] (354.8–409.6)	**302.1** (254.5–331.2)	**24.9**[(4)] (23.1–26.7	**6.2** (5.2–7.1)	**>1000**	**>10**	**>300**	-	**<10**	**<0.1**	**2.4** (0.03–6.9)	**0.02** (0.0–0.3)
Florfenicol	**326.4** (263.5–406.3)	**141.6** (87.8–186.1)	**>1000**[(5)]	-	**>1000**	**>1000**	**>300**	-	**<10**	**<0.1**	**14.9** (9.7–21.0)	**1.4** (0.5–2.7)
Neomycin	**200.6**[(3)] (159.4–249.5)	**83.8** (49.3–112.7)	**2587** (2533–2660)	**2073** (1995–2131)	**>1000**	**>10**	**97.4** (83.2–115.2)	**1.2** (1.4–2.3)	**36.7** (26.5–50.0)	**4.2** (2.0–7.0)	**45.9** (25.5–75.7)	**0.6** (0.08–1.9)
Sulfadiazine	**>1000**	**563.3** (251.0–760.3)	**>200**	-	**>1000**	**>1000**	**>300**	-	**<10**	**<0.1**	**0.2** (0.0–4.3)	**0.0** (0.0–0.0)

Daphnia magna
[(1)] $LC_{50\ (24\ h)}$= 380,1(Park and Choi, 2008)
[(2)] $LC_{50\ (24\ h)}$= >100 (Wollenberger et al., 2000)
>400 (Zounkova et al., 2011).
22,64 (Isidori et al., 2005)
>800 (Park and Choi, 2008)
[(3)] $LC_{50\ (24\ h)}$= 116,6 (Park and Choi, 2008)
Vibrio fischeri
[(4)] $LC_{50\ (30')}$= 64,50 (Isidori et al., 2005)
108 (Kolodziejska et al., 2013)
121,01 (Lalumera et al., 2004)
[(5)] $LC_{50\ (30')}$= 29,4 (Kolodziejska et al., 2013).

LC_{50} values obtained were > 1000 mg/kg. In some cases, such as CTC, the LC_{10} could be estimated (Table 2). Only in the cases of CTC, OTC and NMC, one or two worm deaths were observed at 100 mg/kg.

3.5. Ecotoxicology of the antibiotics in river microorganism communities

3.5.1. Genetic 16 s sequencing of river microbial communities

The taxonomic analysis of bacteria in the water samples collected from the Gállego River is presented in Fig. 2a. The figure highlights the most prevalent taxa (> 1% of the phylum, class and order and > 2% of the family, genus and species due to greater variability). Total reads were 103496. More than 95% of the microorganisms at the taxonomic level of kingdom, phylum, class and order were identified. In the remaining levels, it was lower: 61.88% at the family level, 58.76% at the genus level and 21.27% at the species level.

3.5.2. Impact on global microbial activity

The first graphs in Figs. 3–7 (a1) show the effect of the five ABXs on the river microorganisms throughout the 6 days of the assay measured as AWCD compared to the control (black line). A clear dose effect can be seen in all cases.

At lower concentrations (0.1 μg/mL) all ABXs except NMC produced an appreciable reduction in bacterial growth, although no one showed significant differences with respect to the control ($p>0.05$). However, at 10 μg/mL OTC and FF had significant differences ($p<0.05$) from 72 h to the end of the measures. At 100 μg/mL all ABXs showed strong growth inhibition compared to the control ($p < 0.01$) except OTC and CTC which showed $p<0.05$ starting at 72 h and 96 h respectively.

The LC_{50} values were calculated for 168 hours when the growth kinetics had already reached the stationary phase (Table 2); although in some cases only estimates could be made due to the high inhibition produced, almost all values were zero.

The values defining the kinetics of the AWCD curves at all concentrations (Cmax and r) can be seen in Supporting Information 2. According to the Cmax values, we can order the five ABXs from highest to lowest toxicity on aquatic communities as follows: OTC > FF > SDZ ≈ CTC > NMC. ABXs, however, hardly affect the growth rate (r) with respect to the control.

3.5.3. Community-level physiological profiling (CLPP) of river microorganisms

Figs. 3–7 (a2–a4) show the impact of the five ABXs on the ability of river microorganisms to metabolize the five substrates of the Biolog EcoPlates™ as the difference from the control (which is 0 on the x-axis). As can be seen, the decrease in the ability to metabolize the different metabolites is dose-dependent, increasing as the concentration of the antibiotic increases.

OTC and FF caused the greatest decreases in bacterial metabolism, which was noticeable as early as 0.1 μg/mL and was significant ($p < 0.05$) for carbohydrates and amino acids in the case of exposure to OTC and polymers in the case of FF (see asterisks in Fig. 3 [a2–a4]).

At 10 μg/mL, OTC and FF produced the greatest decreases, OTC in all metabolites ($p < 0.0001$) and FF in carbohydrates ($p < 0.0001$), carboxy and ketonic acids ($p = 0.001$) and polymers ($p < 0.05$). OTC also produced significant decreases ($p < 0.001$) in polymers and amines and amides and carbohydrates ($p = 0.001$).

At 100 μg/mL, ABXs, including NMC and SDZ, produced a generalized and highly significant decrease ($p < 0.0001$) for almost all metabolites.

3.6. Ecotoxicology of the antibiotics in soil microorganism communities

3.6.1. Genetic 16 s sequencing of soil microbial communities

The sequencing of soil microorganisms shown in Fig. 2b reflects a great diversity of taxa highlighting the most prevalent ones as in the river sequencing. In this case, of the 65193 reads it was possible to

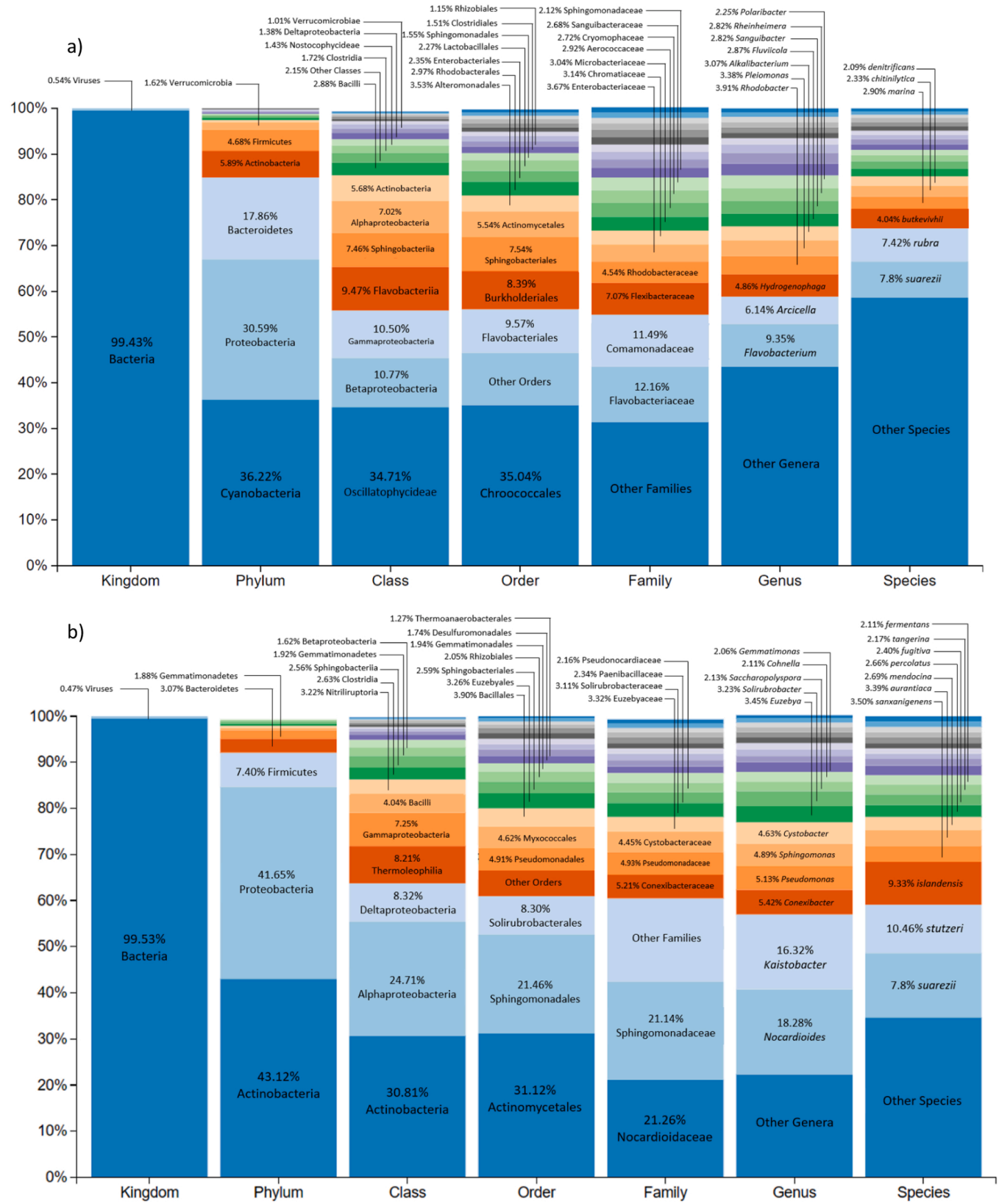

Fig. 2. Relative abundance of genetically sequenced microorganisms from river (a) and soil (b) within their taxonomic classifications at each level.

identify more than 90% of taxa at the taxonomic levels of kingdom, phylum, class and order, 88.25% to the genus and only 25.88% of species.

3.6.2. Impact on global microbial activity

Figs. 3–7 (b1) show the effect of the five ABXs on the soil microbial activity throughout the 7 days of the assay measured as AWCD. As in the case of the river microorganisms, a clear dose effect was observed, and

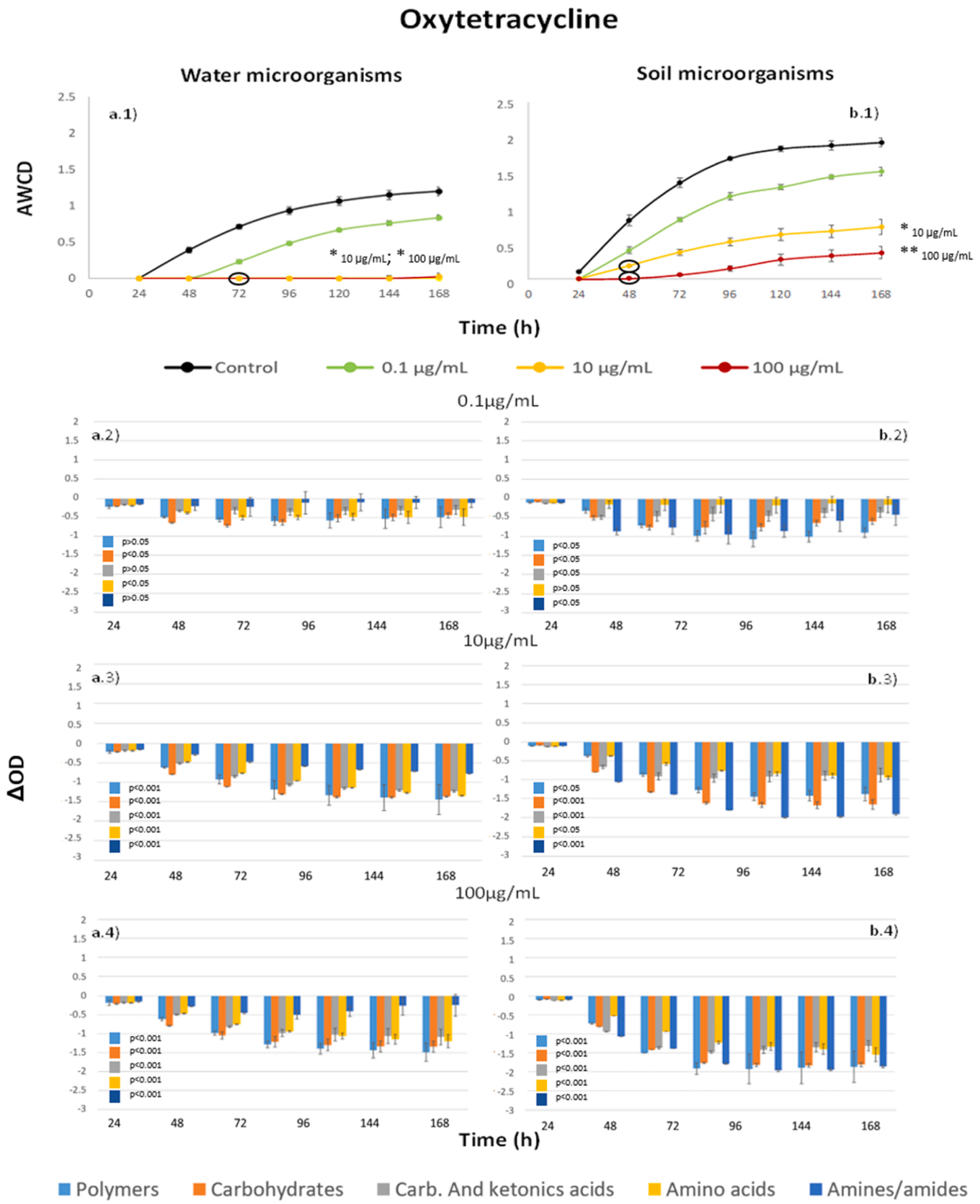

Fig. 3. Microbial growth quantified as the Average Well Color Development (AWCD) in Biolog EcoPlates based on 168 h incubation of (a.1) river and (b.1) soil microorganisms exposed to oxytetracycline. The circle surrounding the point on the AWCD curves indicates the time at which differences from the control become significant. The asterisks denote the degree of significance, *$p < 0.05$ and ** $p < 0.01$. Bars represent metabolic effect differentiation by carbon sources of the (a.2 to a.4.) river and (b2 to b4) soil microorganisms exposed in different concentrations to oxytetracycline respect to the control (O axis), measured as optical density (ΔOD). Each point is the average value of three replicates.

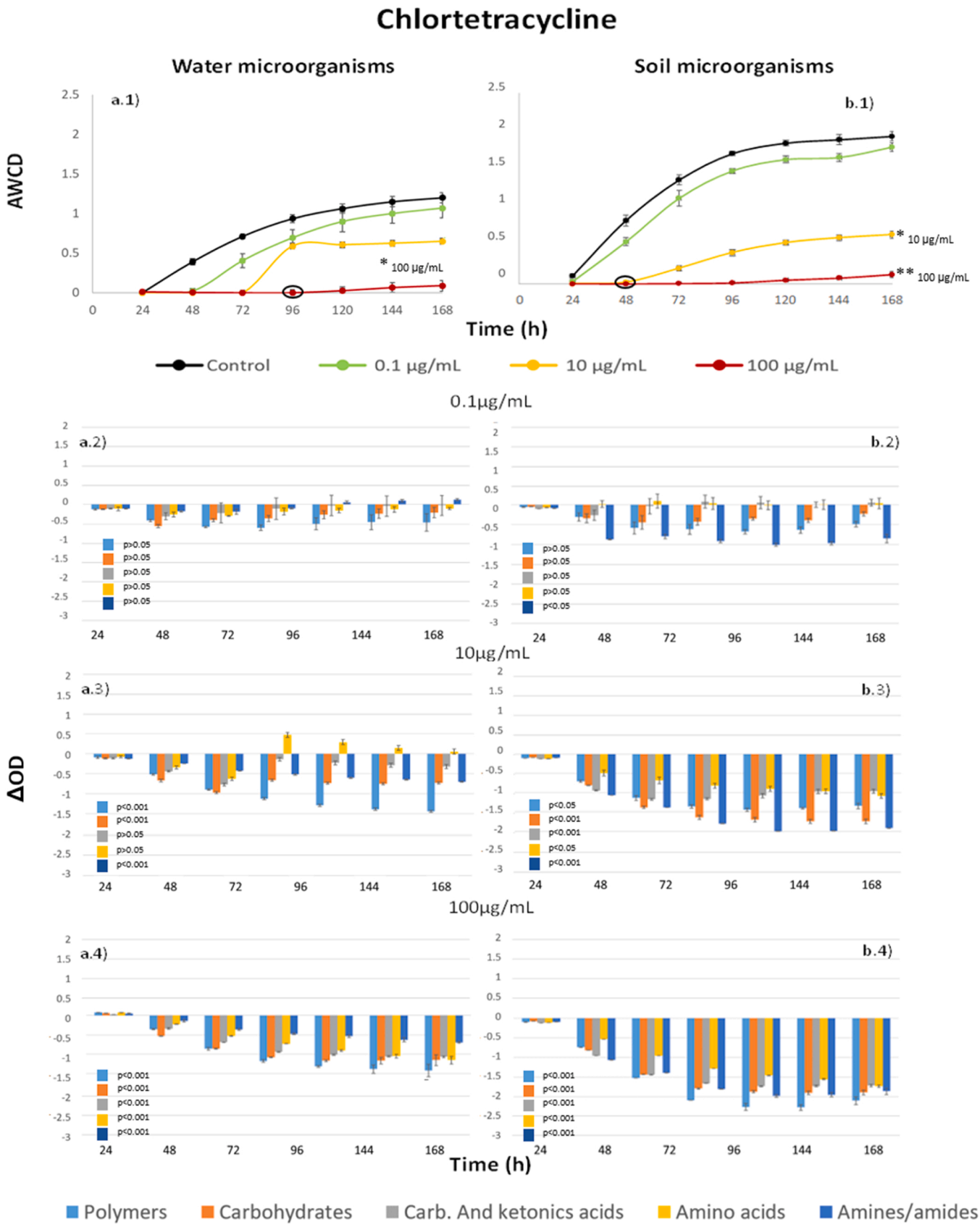

Fig. 4. Microbial growth quantified as the Average Well Color Development (AWCD) in Biolog EcoPlates based on 168 h incubation of (a.1) river and (b.1) soil microorganisms exposed to chlortetracycline. The circle surrounding the point on the AWCD curves indicates the time at which differences from the control become significant. The asterisks denote the degree of significance, *$p < 0.05$ and ** $p < 0.01$. Bars represent metabolic effect differentiation by carbon sources of the (a.2 to a.4.) river and (b2 to b4) soil microorganisms exposed in different concentrations to chlortetracycline respect to the control (O axis), measured as optical density (ΔOD). Each point is the average value of three replicates.

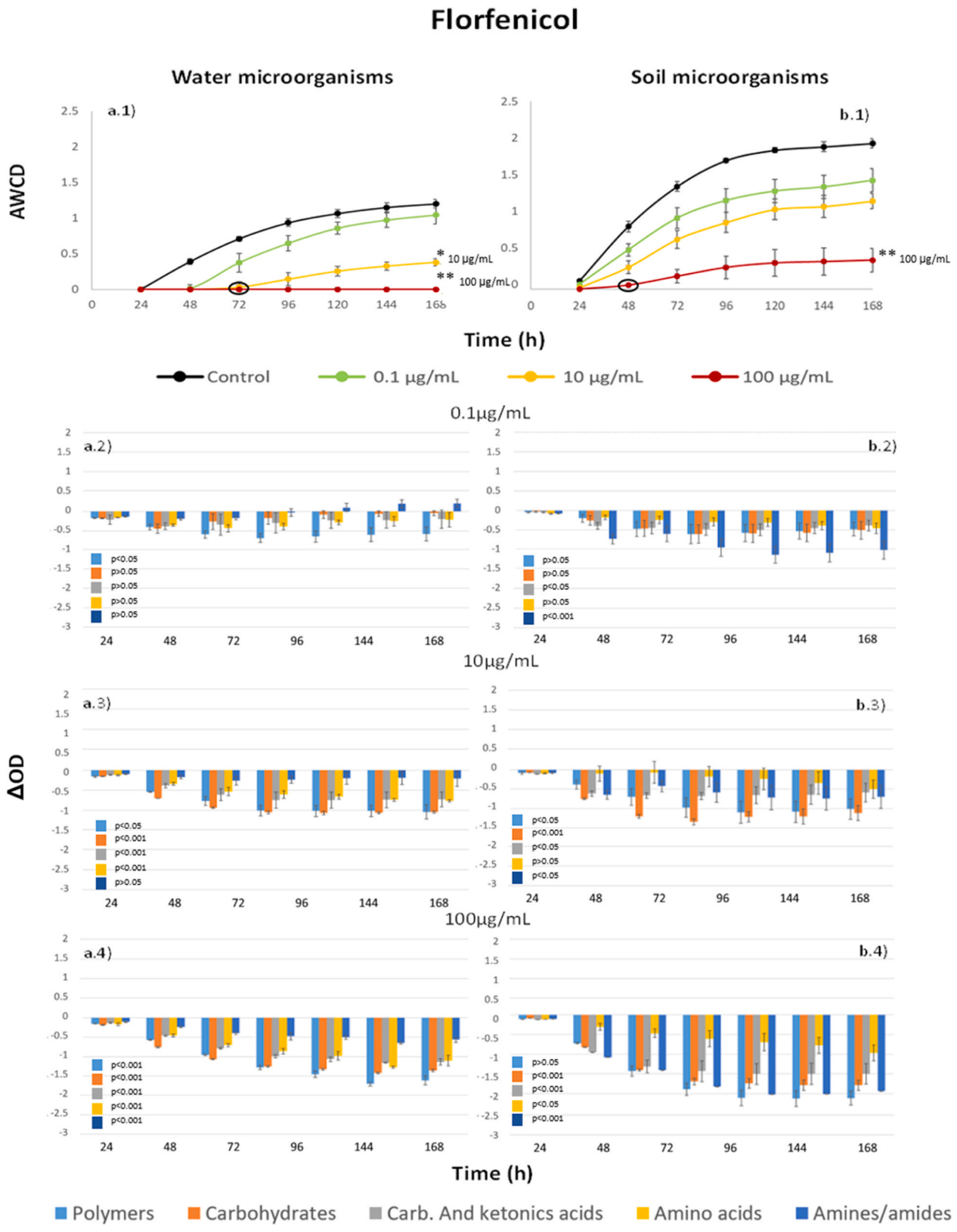

Fig. 5. Microbial growth quantified as the Average Well Color Development (AWCD) in Biolog EcoPlates based on 168 h incubation of (a.1) river and (b.1) soil microorganisms exposed to florfenicol. The circle surrounding the point on the AWCD curves indicates the time at which differences from the control become significant. The asterisks denote the degree of significance, *$p < 0.05$ and ** $p < 0.01$. Bars represent metabolic effect differentiation by carbon sources of the (a.2 to a.4.) river and (b2 to b4) soil microorganisms exposed in different concentrations to florfenicol respect to the control (O axis), measured as optical density (ΔOD). Each point is the average value of three replicates.

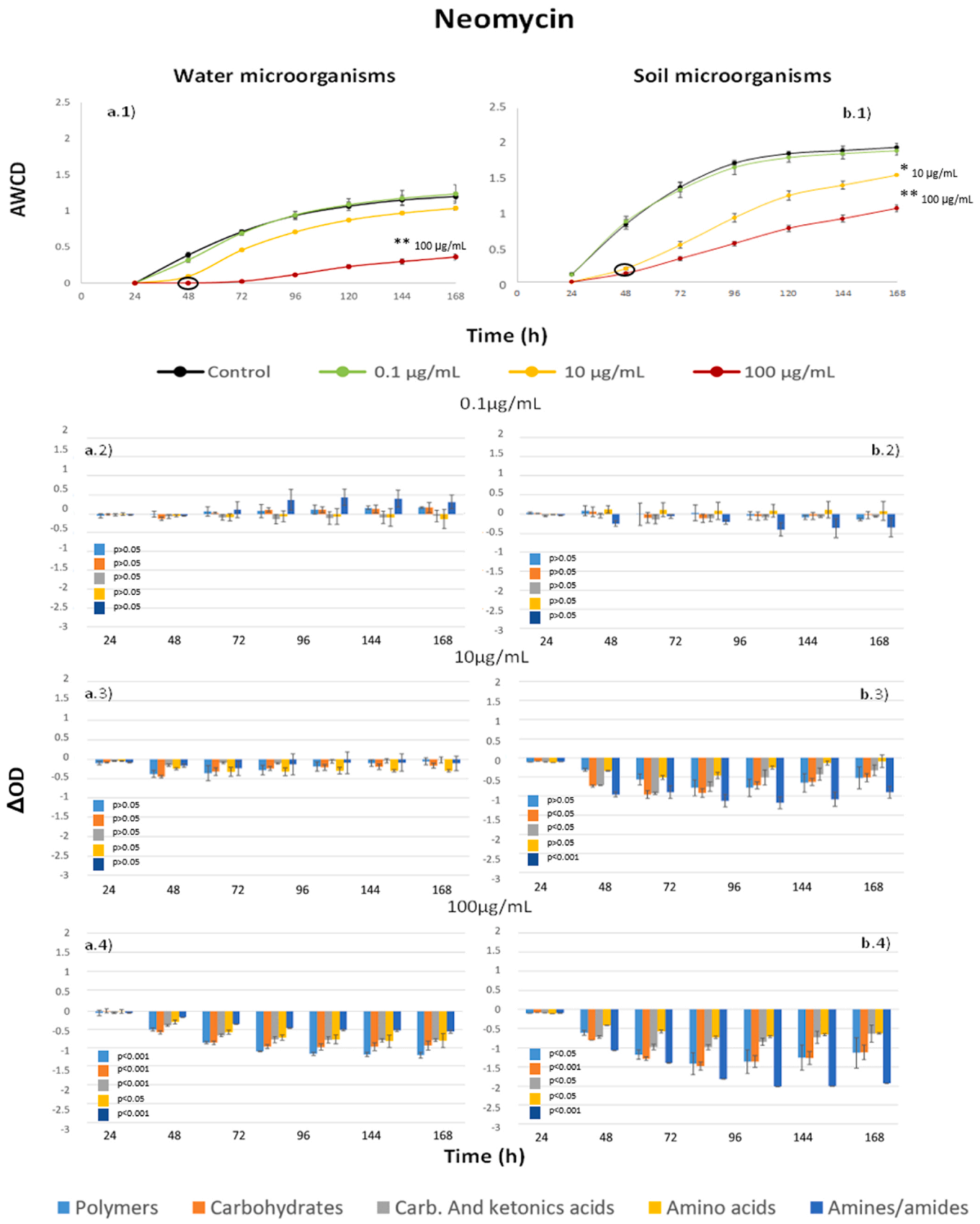

Fig. 6. Microbial growth quantified as the Average Well Color Development (AWCD) in Biolog EcoPlates based on 168 h incubation of (a.1) river and (b.1) soil microorganisms exposed to neomycin. The circle surrounding the point on the AWCD curves indicates the time at which differences from the control become significant. The asterisks denote the degree of significance, *p < 0.05 and ** p < 0.01. Bars represent metabolic effect differentiation by carbon sources of the (a.2 to a.4.) river and (b2 to b4) soil microorganisms exposed in different concentrations to neomycin respect to the control (O axis), measured as optical density (ΔOD). Each point is the average value of three replicates.

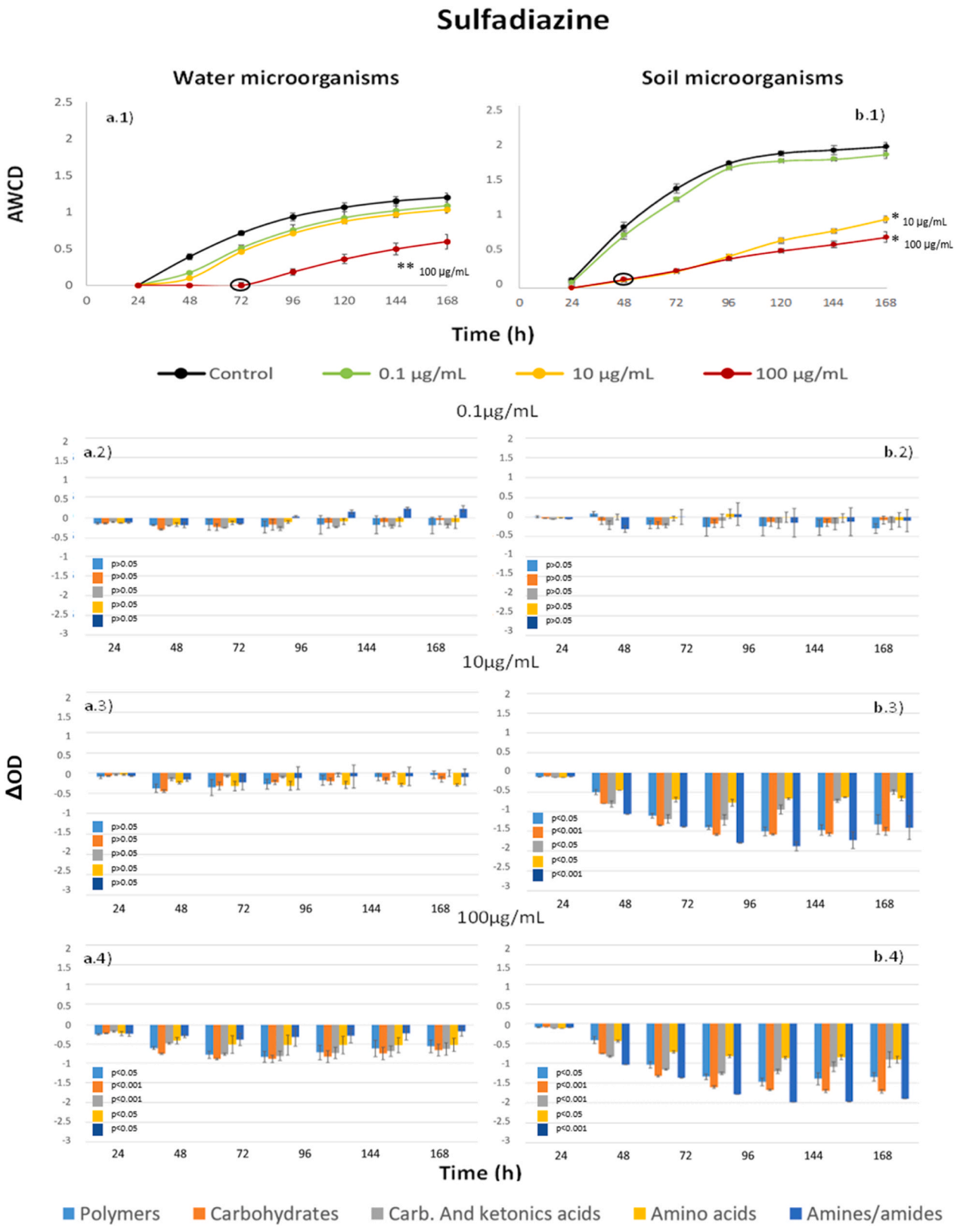

Fig. 7. Microbial growth quantified as the Average Well Color Development (AWCD) in Biolog EcoPlates based on 168 h incubation of (a.1) river and (b.1) soil microorganisms exposed to sulfadiazine. The circle surrounding the point on the AWCD curves indicates the time at which differences from the control become significant. The asterisks denote the degree of significance, *$p < 0.05$ and ** $p < 0.01$. Bars represent metabolic effect differentiation by carbon sources of the (a.2 to a.4.) river and (b2 to b4) soil microorganisms exposed in different concentrations to sulfadiazine respect to the control (O axis), measured as optical density (ΔOD). Each point is the average value of three replicates.

all ABXs produced significant impacts on the growth kinetics of microbial populations. ABXs did not show significant differences with respect to the control at 0.1 μg/mL ($p > 0.05$), however, from a concentration of 10 μg/mL all ABXs except FF showed significant differences in their growth ($p<0.05$) starting in all cases at 48 h and remaining until the end of the test. Finally, at 100 μg/mL, all ABXs showed strong growth inhibition compared to the control ($p<0.01$) from 48 h except SDZ which significance level started at the same time but was less determining ($p<0.05$).

LC_{50} values were also calculated for 168 hours (Table 2). All ABXs had a $LC_{50} < 10$ μg/mL except FF and NMC.

The Cmax and r values can be seen in Supporting Information 2. According to the Cmax values, we can order the five ABXs from highest to lowest toxicity on edaphic communities as follows: CTC ≈ OTC > FF > SDZ > NMC.

In this case, the microbial growth rate (r) was also affected, especially OTC, which decreased the r value by 16% on average, followed by NMC and SDZ (14% and 13%, respectively). CTC had a very small effect (10%) and FF did not affect this parameter at all.

3.6.3. Community-level physiological profiling (CLPP) of soil microorganisms

According Figs. 3–7 (b2–b4) all five antibiotics produced a dose-dependent decrease in the ability to metabolize all metabolites in all cases and that this decrease was even more pronounced than in the case of aquatic microorganisms and was already detectable at 0.1 μg/mL. At this concentration, OTC had the greatest effect on the ability of soil microorganisms to metabolize metabolites (all but aminoacids), unlike river microorganisms. OTC and FF affected some groups (amines/amides and carboxylic and ketonic acids, $p < 0.05$), but these groups were different from those affecting river microorganisms. At 10 μg/mL there was a significant decrease ($p < 0.05$ and $p < 0.001$) of almost all metabolites for all ABXs, slightly less for NMC. At 100 μg/mL all ABXs produced large decreases in the ability of soil microorganisms to metabolize all metabolites ($p < 0.001$ in virtually all cases).

4. Discussion

According to our results, at environmental concentrations a direct acute toxicity effect of ABXs, on aquatic invertebrates such as *D. magna* may be unlikely. However, concentrations of up to 0.5 μg/mL could be detected in different effluents, especially OTC (Kovalakova et al., 2020). Results of river microbial communities show substantial changes in both growth and metabolic profile from 0.1 to 10 μg/mL, especially when exposed to OTC (see Fig. 3). This indicates that environmental concentrations can cause changes in the microbial community which, being key organisms in the river ecosystem and the basis of food chains, can alter the balance of the aquatic ecological environment and cause indirect environmental risks to other organisms (Zhou et al., 2020). It should not be forgotten that, although the point concentrations of ABXs to which they are exposed are low, they are persistent over time and multiple ABXs can interact with each other (Brosche and Backhaus, 2010) or with other stressors (Kong et al., 2006), so cumulative effects cannot be ruled out. In addition, some of the ABXs may bioaccumulate, as in the case of SDZ, which showed a high potential for bioaccumulation in benthic organisms (Wu et al., 2021).

Considering also the existing data on the presence of these antibiotics in the soil, even more than in the river environment, the repeated application of contaminated manure on fields or its use over prolonged periods of time can lead to the accumulation of very high concentrations of antibiotics, reaching levels as high as 1067.1 mg/kg for CTC (Quaik et al., 2020).

4.1. Ecotoxicology in aquatic indicators: Daphnia magna and Vibrio fischeri

Tetracyclines are especially harmful to marine bacteria, although they also exhibit toxicity for *D. magna*. However, the other three antibiotics seem to affect only *D. magna*, especially SDZ.

OTC and CTC are broad-spectrum antibiotics that inhibit protein synthesis by binding to the 30 S subunit of the bacterial ribosome, preventing the elongation of the polypeptide chain. The presence of the cellular target is undoubtedly the main factor in sensitivity of *V. fischeri* to tetracyclines. However, they are also small molecules of lipophilic nature (Table 1) that can cross cell membranes (both the outer membrane of the gram negative bacteria and the cell membrane) by passive diffusion and do so more efficiently in prokaryotic cells because they have a higher proportion of saturated fatty acids in their membrane or the presence of porins in the outer membrane (Thanassi et al., 1995). In addition, CTC is more lipophilic than OTC due to the presence of a chlorine group in its structure which increases its polarizability and the ability of this ABX to penetrate lipid membranes and may contribute to the higher toxicity in both *V. fischeri* and *D. magna* that we observed.

The antimicrobial spectrums of FF and SDZ exhibit broad ranges of Gram-positive and Gram-negative bacteria. NMC antimicrobial activity is primarily restricted to Gram-negative species. Both FF and NMC act by binding to the 50 S and 30 S subunits of the bacterial ribosome, respectively, inhibiting protein synthesis. The mechanism of action of SDZ is somewhat different: it acts by interfering with synthesis of folic acid because it is a structural analog of para-aminobenzoic acid (PABA), a precursor in the synthesis of folic acid, which is essential for the growth and replication of microorganisms.

According to our results, these three antibiotics are not harmful to *V. fischeri*, despite their specific mechanism of action against Gram-negative bacteria. It is possible that their physicochemical properties influence their ability to pass through the bacterial envelope and reach its target site in the bacterial cell. For example, NMC is a relatively large molecule, which might hinder its ability to pass through the pores of the outer membrane of Gram-negative bacteria, and its high hydrophilicity may make it difficult to cross the hydrophobic lipid bilayer of the bacterial outer membrane. In addition, at physiological pH, its amino group are protonated, which confers it a net positive charge, so it can be repelled by the negatively charged lipopolysaccharides (LPS) on the surface of Gram-negative bacteria. These factors would not occur in the case of FF or SDZ because they are neutral molecules at physiological pH, preventing electrostatic repulsions between these ABXs and the surface of Gram-negative bacteria. They have small sizes and lipophilic natures, making them more likely to pass through the porins of the bacterial outer membrane. Resistance mechanisms developed against these ABXs could perhaps explain their negligible effect on Gram-negative bacteria. For FF, studies detected increased expression of resistance genes in the genus *Vibrio*, among others (Kadlec et al., 2007; Li et al., 2020).

Tetracyclines can also pass through eukaryotic cells by passive diffusion, affecting *D. magna*. Likewise, eukaryotic cells possess various transporter proteins that facilitate the entry and efflux of molecules, including tetracyclines. For instance, organic anion transporters (OATs) have been implicated in the uptake of tetracyclines in certain cell types (Kobayashi et al., 2005).

The moderate lipophilicity of FF, on the other hand, allows it to dissolve in both lipid-based and aqueous environments, which, along with its relatively small molecular weight (see Table 1), facilitates it passing through the lipid bilayer of cell membranes via passive diffusion, which would explain its effect on *D. magna* being similar to tetracyclines.

It should also be noted that *D. magna* is a filter-feeding organism, so exposure to ABXs also occurs via the digestive tract. Some authors have suggested that damage after OTC exposure is produced due to the disruption of the intestinal biota of *D. magna* that could have

implications on long-term survival, energy and expenditure (Lovern and Van Hart, 2022). It is evident in any case that concentrations lower than those that produce lethality are capable of producing sublethal effects such as reproductive capacity, where lower LC_{50} values such as 86 μg/mL (Zounkova et al., 2011) or 46.2 μg/mL (Wollenberger et al., 2000) have been found. In the case of NMC, where its large size and hydrophilic nature make it difficult to cross cell membranes, its effect on *D. magna* is probably via digestive penetration. The mechanism of action of SDZ is too specific to bacterial metabolic pathways, which could explain its limited effect on *D. magna* despite being ingested.

Few studies on these antibiotics can be found in the literature, mainly focused on tetracyclines and comprising very diverse values, although in the case of *D. magna* in ranges similar to those we have found (See Table 2).

4.2. Ecotoxicology in edaphic indicators: Allium cepa and Eisenia fetida

None of the antibiotics caused mortality in *E. fetida* at concentrations below 1000 mg/Kg, however, the worms showed morphological changes such as decreases in body size and thickness in all cases. This is consistent with the observations of Cao (Cao et al., 2015) that 100 mg/kg OTC-contaminated soil (prepared with mycorrhizae and maize) did not produce *E. fetida* mortality. However, sublethal effects have been detected for both tetracyclines. For example, some morphological changes in the worms after 15 days of exposure were observed for OTC (Zhao et al., 2019). CTC induced DNA damage and biochemical stress was detected by analysis of superoxide dismutase and catalase enzyme activities in this earthworm (Dong et al., 2012; Lin et al., 2012). Damage to the integrity of lysosomal membranes and increased apoptosis of coelomocytes after exposure to OTC were also observed in *E. fetida* (Gao et al., 2015). In addition, proteomic analysis found changes in actin protein expression after OTC exposure, affecting the cell cytoskeleton, which could be a target of oxidative stress. It is noteworthy that the presence of OTC in soil at concentrations $>$ 1000 mg/kg results in a significant avoidance response (Gao et al., 2016).

As far as we can tell, there is little information on the other three ABXs and this is the first time that ecotoxicity data have been provided for *E. fetida*.

E. fetida is widely used in terrestrial ecotoxicology because it is a sensitive indicator of soil quality as it is exposed by two routes to contaminants present in the soil: the percutaneous route as they have a thin epidermal cuticle and a glandular orifice that communicates with the environment as well as a digestive route when stirring the soil. Uptake of 14 C-sulfadiazine in the earthworms, for example, was clearly detected (Norr and Riepert, 2007). Perhaps these sublethal effects lie in an impact on the gut microbiota of *E. fetida* that may determine their responses to antibiotic stress but is not aggressive enough to cause mortality. This has been suggested in the case of OTC (Saha et al., 2021) and SDZ exposure (Kotzerke et al., 2010). In fact, *E. fetida* is able to accelerate the degradation of OTC in soil, which may aid bioremediation, and would explain its resistance to ABX (Liu et al., 2020; Yang et al., 2023). For other terrestrial Oligochaeta exposed to OTC, the literature reports EC_{50} values found in ranges $>$ 3000 mg/kg, although reproduction was generally a more sensitive endpoint than survival (Baguer et al., 2000).

Although studies have shown that some of the antibiotics we studied show phytotoxicity, to our knowledge this is the first time that the ecotoxicity (EC_{50}) of these antibiotics in *Allium cepa* has been quantified.

According to our results, NMC and CTC are the antibiotics with the highest phytotoxicity on *A. cepa.* There was evidence of root length inhibition of CTC in other plants such as wheat, tobacco, lettuce and alfalfa (Kim et al., 2018; Kong et al., 2007; Wang et al., 2022; Xie et al., 2010), but little has been elucidated so far about *A. cepa* except that it did not produce effects of genotoxicity in seed germination (Magdaleno et al., 2017). There is evidence, however, that crops of *A. cepa* are able to absorb CTC (Kumar et al., 2005), which could explain the root exposure to this ABX. It should also be considered that the physicochemical properties of CTC, such as lipophilicity, size and hydrogen bonding may facilitate its entry into the plant cell and passage through the vegetal cell envelope. It has similar properties to NMC, which enables it to pass through plant cell coatings. Moreover, it is a polycationic molecule due to the presence of amino groups. Plant cell walls are negatively charged due to the presence of pectin and other anionic compounds, so electrostatic interactions between the positively charged NMC and the negatively charged cell wall components could potentially facilitate its passage through the cell wall.

Once inside the plant cell, there are indications that NMC acts as an antagonist of Phospholipase-C (Reggiani and Laoreti, 2000), which in the plant root is involved in signal transduction pathways, calcium signaling, root hair development and abiotic stress responses, which, taken together, could explain why it has the strongest antibiotic effect on A. cepa. Accordingly, Wilson (Wilson, 1950) found morphological changes in the root of *A. cepa* exposed to NMC from 3 μg/mL. In other plants such as *Arabidopsis* exposed to NMC, root elongation was also affected (Andreeva et al., 2010).

We found that CTC, FF and SDZ are low phytotoxic to *A. cepa* and little information is available on the ecotoxicity of these ABXs on plants. There is evidence that CTCs affect the seed germination of *A. cepa* (Taveira Parente et al., 2018), although they do not appear to be genotoxic to this plant (Magdaleno et al., 2017). Bartikova (Bartikova et al., 2015) demonstrated that FF can be taken up by carrot roots and lettuce leaves under laboratory conditions with enriched soil, but very little or no SDZ.

4.3. Effects on river and soil microorganism communities' metabolisms

The five antibiotics have detectable impacts on the growth and metabolism of the studied microbial communities, both fluvial and edaphic, at the concentrations tested.

The broad antibacterial spectrum of these ABXs leads to high antimicrobial activity (Luis Martinez, 2009) and the presence of their targets in the bacterial communities would explain this strong impact on microorganisms. The slight variations in the sensitivity of these communities to the different ABXs may be due to the great diversity of taxa in soil and river samples (see Fig. 2), the different physicochemical properties of the ABXs as well as differences in bacterial cell wall architecture, which will determine their ability to access the prokaryotic cell. This will cause a decrease in the most sensitive bacteria, and niches that are occupied by the more tolerant ones will predominate. This may decrease diversity even though biomass may be maintained (Suga et al., 2013) and may explain the differences in the changes we have detected in the metabolic profile of the whole community. Different resistance strategies to these ABXs cannot be ignored either (Grossman, 2016).

OTC has been reported to cause changes in the composition of prokaryotic microbial communities (Zhou et al., 2020), sometimes decreasing aquatic microbial diversity (Harrabi et al., 2019b; Hu et al., 2021). OTC seems to increase Proteobacteria (which represent 30.4% of our samples) but decreases Bacteroidetes (17% of our samples), especially Flavobacterium (Zhou et al., 2020). In the case of SDZ, the affect that we detected on the growth and metabolic capacity of river bacteria, although less than in the other antibiotics, could be due to a decrease of the bacteria that are more sensitive to this antibiotic such as Proteobacteria (30.4% of our samples) or Commamonadaceae (7.11%) (Bai et al., 2019).

As far as we know, this is the first time that the effects of these antibiotics on the metabolic profile of river bacteria has been studied, although there is previous evidence of the impact of some of these antibiotics on the compositions of bacterial communities. For example, FF seems to cause a decrease in bacteria involved in the nitrification process (Gao et al., 2018). However, Zhang (Zhang et al., 2022) detected little impact on the bacterial community structure at 0.1 μg/mL but found a dramatic effect at 100 μg/mL, which would explain the potent effect of decreased metabolizing capacity of river microorganisms exposed to FF

that we detected at this concentration for all metabolites ($p < 0.0001$).

Other literature on exposure of soil microbial communities to these ABXs exists, but it is mainly focused on analyzing changes in community structure and more partial aspects of metabolism that Biolog EcoPlates can assess. For example, in the case of SDZ, changes in soil respiration activity (Fang et al., 2014a) or in soil potential nitrification rate (PNR) and diversity of ammonium oxidizing microbes. They all detected a decrease in PNR with increasing SDZ concentrations and a reduction in the diversity of related ammonia-oxidizing microbes (Hammesfahr et al., 2011; Hou et al., 2023; Li et al., 2022; Liao et al., 2019; Radl et al., 2015). In addition, most studies are conducted by sprinkling ABX in soil samples or applying contaminated manure, which makes comparisons difficult due to differential soil composition or different doses of ABX in manure samples. For instance, in the case of SDZ, the literature indicates that it reduces the activity of soil microorganisms and causes changes in the growth and structure of the community and in its metabolism, but these studies were performed by applying ABX to soil samples (Hammesfahr et al., 2008; Liao et al., 2019; Qiu et al., 2021) or they used sulfadiazine-contaminated manure (Ding et al., 2014; Li et al., 2022; Radl et al., 2015; Xu et al., 2016). To our knowledge, only one study used soil bacterial isolates as we did (Zielezny et al., 2006), but the tests of soil respiration as well as the bacterial community structure were performed with agar diffusion tests and therefore were hardly comparable to ours. Other studies showed that high concentrations of SDZ (50 mg/kg) applied to soil induced a larger effect on the structure of the soil bacterial community as well as bacterial growth as a whole (Santas-Miguel et al., 2020; Xu et al., 2016).

In some cases, the sequenced communities were very similar to ours, with predominance of Actinobacteria and Proteobacteria (Qiu et al., 2021). Interestingly, Proteobacteria, Firmicutes and Clostridium seem to increase after exposure, while Actinobacteria and Pseudomonas seem to decrease (Ding et al., 2014; Lin et al., 2016; Qiu et al., 2021). This change in community structure may explain the disturbance in the microbial metabolic pattern that we detected after exposure to this antibiotic, especially in the case of carbohydrates. Other authors found that SDZ exposure in soil seems to promote carbohydrate metabolism, although amino acid metabolism was inhibited as in our case (Qiu et al., 2021).

The few existing studies on the effect of FF on aquatic microorganisms focused on changes in microbial community structure (Uddin et al., 2019) and point to a decrease in Actinobacteria (40% of bacteria in our samples) and Firmicutes (7%), which could explain the large decrease in bacterial metabolism we detected for this ABX as Proteobacteria appear to be more resistant (Wang et al., 2021a).There is little information on the impact on the bacterial metabolic profile produced by FF and studies focused on the effect on microorganisms associated with the nitrogen cycle (Wang et al., 2021a; Zhou et al., 2021).

The changes detected in the activity and metabolic profile of the soil microorganisms produced by NMC are consistent with the study of (Benassi-Borba et al., 2021) who found that NMC caused a significant reduction in soil enzyme activity which may be due to the loss of key microbial community members. For example, Zhang (Zhang et al., 2017) found that Pseudomonas disappear in soils treated with NMC while gammaproteobacteria were able to survive.

The effects of tetracyclines on soil microorganisms have been studied in more depth. Kong(Kong et al., 2006) found that OTC applied to soil-extracted bacteria produce a significant decrease of the AWCD and substrate utilization, but in other studies the application of the antibiotic (OTC or CTC) was done on soil, so that, again, the results are not comparable given the different characteristics of the soils (Liu et al., 2015, 2012; Ma et al., 2016; Solis et al., 2011). In general, the AWCD results showed a lower effect than direct application on bacteria, which is to be expected since in a soil substrate the exposure is necessarily lower. The effect is also less when manure contaminated with these antibiotics is applied (Chronakova et al., 2021; Fang et al., 2014b). Fang also found that Proteobacteria and Actinobacteria appear to resist CTC exposure well, although Firmicutes appear to be more sensitive. Other authors note that in fertilized soils exposed to CTC, the relative abundance of Actinobacteria and Firmicutes on antibiotic treatment increased significantly, but that of Proteobacteria decreased (Han et al., 2019). The strong impact we detected for this antibiotic on the edaphic communities studied may therefore be due to this change in the proportions of these phyla, which were predominant in our samples.

Most studies on the impacts of these ABXs on soil microbial communities also focused on more partial aspects of soil metabolism, such as the activity of soil ammonia oxidizing bacteria (Liu et al., 2020), the analysis of phospholipid fatty acids (Chen et al., 2023) and soil enzyme activities after OTC exposure. In general, high inputs of OTC and CTC decreased soil enzyme activities (about 200 mg/kg) and low and moderate inputs of OTC and CTC (e.g., 0.1 and 1 mg/Kg) had no (or very little) adverse impact on soil enzyme activities.

5. Conclusions

Our results demonstrate that five widely used veterinary antibiotics present in the environment have moderate toxicity towards non-target aquatic and terrestrial organisms, including *D. magna*, *V. fischeri*, and *A. cepa*, and very low toxicity in the case of the earthworm *E. fetida*. However, they have a deep impact on aquatic and terrestrial microorganism communities from river and soil ecosystems.

Consistent with other studies, we have observed that tetracyclines exhibit toxicity to the two aquatic indicators tested, while NMC, FF, and SDZ affect mainly *D. magna*. We have quantified, for the first time, the ecotoxicity of these ABXs on the terrestrial indicators *E. fetida* and *A. cepa*, finding that *E. fetida* survives well under exposure to concentrations >1000 μg/mL, and that NMC and CTC are the antibiotics with the highest phytotoxicity on *A. cepa* in the range of approximately 100 μg/mL.

There was evidence of the impact of some of these antibiotics on the compositions of bacterial communities, but now we have found that all five antibiotics affect the growth and metabolism of microbial communities, both aquatic and soil, within the concentration range of 0.1–10 μg/mL in a dose-dependent manner. The impact is more pronounced on soil microorganisms, and there are differences in the substrates on which the metabolic capacity of aquatic communities is lost compared to edaphic ones.

The detected environmental concentrations of these antibiotics suggest that acute ecotoxicity effects on non-target indicators belonging to different trophic levels studied are unlikely. However, at the concentrations of these ABX detected in the environment, the impact on the composition and functionality of aquatic and soil microorganism communities, which are the basis of the trophic chains, can undoubtedly cause environmental changes that should be studied in the long term.

CRediT authorship contribution statement

Cristina Gan: Data curation, Investigation. **Antonio Valenzuela:** Data curation, Formal analysis, Investigation, Writing – original draft. **Natalia Ferrando:** Investigation. **Guillermo Lorca:** Investigation. **Mª Rosa Pino-Otín:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Diego Ballestero:** Funding acquisition, Supervision, Validation. **Elisa Langa:** Supervision, Validation.

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.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.116185.

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