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Ecotoxicity and risk assessment of eight common antibiotics using soil and aquatic bioindicators

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Abstract

Background The extensive use of antibiotics in both human and veterinary medicine has resulted in their release into terrestrial and aquatic environments, raising concerns about ecological impacts and antimicrobial resistance. This study assesses the ecotoxicity and environmental risk of eight widely used antibiotics: amoxicillin, ampicillin, penicillin, erythromycin, chloramphenicol, gentamicin, streptomycin, and tetracycline. Acute toxicity tests were conducted using five bioindicators spanning different trophic levels and environmental compartments: *Aliivibrio fischeri*, *Daphnia magna*, *Desmodesmus subspicatus*, *Eisenia fetida*, and *Allium cepa*. EC₅₀ values were experimentally determined or sourced from literature to calculate predicted no-effect concentrations (PNECs) and hazard quotients (HQs) for water and soil.

Results Toxicity varied considerably among species. *A. fischeri* was most sensitive to penicillin, while *D. magna* showed highest sensitivity to chloramphenicol. In *A. cepa*, amoxicillin and gentamicin elicited the strongest phytotoxic responses. *E. fetida* exhibited no mortality at concentrations below 1000 mg/kg. HQ-based risk assessment identified erythromycin, tetracycline, and amoxicillin as posing the greatest environmental risks, with HQ > 1 in both acute and chronic scenarios. Ampicillin consistently showed the lowest risk.

Redundancy analysis indicated that the number of rotatable bonds was the most significant physicochemical predictor of EC₅₀ values across species. This suggests that molecular flexibility, associated with structural promiscuity, may increase the potential for off-target interactions.

Conclusions Overall, the study underscores the environmental risks posed by common antibiotics and highlights the relevance of molecular structure in predicting ecotoxicity. These insights support the development of targeted environmental management and regulatory frameworks.

The apparent dissociation between the relatively low toxicity observed in individual non-target organisms and the high environmental risk estimated for these compounds reveals that the true threat of antibiotics lies in their silent yet persistent erosion of ecosystem resilience, biodiversity, and ecological functions. These findings emphasize the need to consider sublethal and community-level effects in environmental risk assessments.

Keywords Antibiotic ecotoxicity, *Eisenia fetida*, *Allium cepa*, *Daphnia magna*, *Desmodesmus subspicatus*, *Aliivibrio fischeri*

Background

Antibiotics (ABXs) have transformed the treatment of bacterial infections in human medicine and animal production systems [1, 2]. However, their extensive use has led to the unintended release of these compounds

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into various environmental compartments, raising concerns about their behaviour and potential ecological and human health risks [2]. Their presence in the environment originates from various exposure pathways, including discharges from pharmaceutical manufacturing, agricultural and livestock activities, and the excretion of unmetabolized residues following ABX consumption [3, 4].

The presence of these residues in the environment is particularly concerning due to their potential to exert toxic effects on non-target organisms, triggering cascading effects throughout food webs and disrupting ecological interactions [5]. One key issue associated with ABXs in the environment is their accumulation in soil and aquatic compartments, where they can persist and exhibit bioactive properties even at low concentrations [6]. This environmental persistence is exacerbated by the variable removal efficiency of ABXs during wastewater treatment. Once municipal wastewater reaches sewage treatment plants, the removal rates of ABX compounds can vary significantly [7]. Residual contaminants may persist in liquid effluents, sewage sludge, or biosolids [8, 9], which are often reused for irrigation or as fertilizers in agricultural soils [10, 11]. ABX concentrations in these matrices range from ng/kg to µg/kg [12, 13]. ABXs such as tetracycline (TC) and chloramphenicol (CHL) have been detected in water bodies at concentrations as high as 400 and 75 ng/L [14, 15], respectively. And in soils surrounding agricultural sites at levels up to 1010 µg/kg [16]. Repeated or prolonged application of such contaminated amendments contributes to sustained and widespread accumulation of ABXs in soils [17]. The environmental fate of each ABX class is modulated by its physicochemical properties, interactions with soil matrices, and local climatic conditions [18].

The potential consequences of ABX residues extend beyond immediate toxic effects. The occurrence of ABX-resistant bacteria and genes in the environment is another significant concern associated with the use of these antimicrobials because the environment plays a significant role in their dissemination [19, 20]. The environment serves as a reservoir for ABX resistance genes (ARGs), which can be transferred to human pathogens, leading to the emergence of multidrug-resistant strains [20]. The World Health Organization (WHO) has declared that the emergence of multidrug-resistant (MDR) pathogens is one of the greatest threats to global health, food security, and development [21]. This dual threat underscores the urgency of evaluating the environmental risks of these substances to inform regulatory frameworks and mitigation strategies.

Recent ecotoxicity research has increasingly emphasized that antibiotics, regardless of their pharmacological

class, can exert a wide spectrum of effects on non-target organisms and environmental functions [22, 23]. Current studies highlight the need to complement traditional acute-toxicity endpoints with the assessment of sublethal, chronic and community-level impacts, including alterations in microbial diversity, metabolic activity and primary production [23, 24]. A growing body of work also shows that antibiotics can induce hormetic responses, oxidative stress and functional impairment in algae, bacteria and soil microbiota, even at concentrations far below those causing overt lethality [25, 26]. Moreover, environmental monitoring increasingly reveals the persistence and widespread occurrence of multiple antibiotic classes in water and soils, raising concerns about long-term ecosystem disruption and antibiotic-resistance dynamics [27]. Overall, these trends underscore the emerging consensus that antibiotic ecotoxicity is driven not only by acute toxicity but also by low-dose, multispecies and functional effects, which are now central to contemporary environmental risk-assessment frameworks.

This paper aims to provide updated scientific information on ecotoxicity and risk assessment of eight widely used human ABXs. The findings of this paper can support future regulatory decisions and contribute to conclusions about the reduction of environmental ABX contamination.

To this end, the present study aims to: (1) assess the ecotoxicity of eight ABXs on five bioindicators, two from soil environments (the earthworm *Eisenia fetida* and the plant *Allium cepa*) and three from aquatic (the invertebrate *Daphnia magna*, the marine bacterium *Aliivibrio fischeri* and the green algae *Desmodesmus subspicatus*); (2) to review existing literature and conduct a hazard assessment by calculating predicted no-effect concentrations (PNECs) and hazard quotients (HQ); (3) analyse the influence of key physico-chemical properties of ABXs—such as molecular weight, hydrogen bonding capacity, polarity, lipophilicity, number of rotatable bonds and molecular charge—on their ecotoxicity profiles using redundancy analysis (RDA), in order to identify structural features driving differential toxicity across species.

Methods

Antibiotics

Eight widely used ABXs were tested, that is, gentamicin (GTM), streptomycin (STM), erythromycin (ERY), tetracycline (TC) and chloramphenicol (CHL) were purchased from Acofarma. Penicillin G (PEN), ampicillin (AMP) and amoxicillin (AMO) were purchased from Sigma-Aldrich. Detailed information for each compound is summarized in Table S1. Eight ABXs were selected for this study based on their pharmacological diversity, clinical relevance, and environmental occurrence.

The compounds represent different antibiotic families, encompassing a range of mechanisms of action and physicochemical properties that may influence their environmental fate and biological effects.

***Daphnia magna* assay**

The acute immobilization test of *Daphnia magna* was conducted in accordance with OECD guideline 202 and following the protocol outlined in Daphtoxkit FTM magna provided by Vidrafoc (Spain), ref. DM121219. *D. magna* ehippia, synthetic freshwater medium (ISO 6341, 2012), and Spirulina microalgae (a nutritional source for *D. magna*) were obtained from Vidrafoc (Daphtoxkit). Ehippia were incubated for 72 h at 20–22 °C in an ERI 180 DBO, Equitec® refrigerated incubator under continuous light conditions of 6000 lx. Upon reaching an adequate number of neonates (young daphnids aged less than 24 h), they were pre-fed with Spirulina microalgae for 2 h.

Pre-fed *D. magna* neonates were subsequently exposed to varying concentrations (from 200 to 10,000 mg/L, depending on the solubility and range-finding concentration test) of each of the 8 ABXs, PEN and AMO (500, 1000, 1500, 2000 and 2500 mg/L), AMP (625, 1250, 2500, 5000 and 10,000 mg/L), GTM (600, 800, 1000, 1200 and 1400 mg/L), STM (312.5, 625, 1250, 2500 and 5000 mg/L), ERY (125, 250, 500, 1000 and 1500 mg/L), TC (200, 300, 400, 500 and 600 mg/L) and CHL (200, 400, 600, 800 and 1000 mg/L). All dilutions were prepared in *Daphnia* culture medium. Each test concentration used 25 organisms, with five replicates of five neonates per well in a volume of 10 mL per well. The daphnids were then subjected to incubation in complete darkness for 24 h at 20–22 °C.

Following the incubation period, immobilization was assessed and compared to control values. This was determined by observing the number of organisms that could not swim for 15 s after mild agitation. In addition, abnormal behaviour or appearance was documented. The obtained results were analysed to calculate the EC₅₀, defined as the concentration of the chemical resulting in 50% effect relative to the control. In the case of *D. magna*, this effect corresponds to 50% reduction in the number of active neonates (i.e., mobile individuals).

***Aliivibrio fischeri* assay**

The assay was conducted following the prescribed test conditions outlined in the *Aliivibrio fischeri* acute toxicity test (UNE-EN-ISO 11348–3 2009). Lyophilized *A. fischeri* (strain NRRL-B-11,177) utilized for bioluminescence inhibition assays was procured from Vidrafoc in the Bio-Fix® Kit format.

Five solutions with varying concentrations (from 1 to 10,000 mg/L, depending on the solubility and range-finding concentration test), PEN (1, 12.5, 125, 250 and 500 mg/L) AMO (500, 750, 1000, 1250 and 1500 mg/L), AMP (25, 250, 500, 1000 and 2000 mg/L), GTM (25, 100, 1000, 2500 and 5000 mg/L), STM (625, 1250, 2500, 5000 and 10,000 mg/L), ERY (12.5, 125, 250, 500 and 1000 mg/L), TC (1, 5, 10, 50 and 100 mg/L) and CHL (62.5, 125, 250, 500 and 1000 mg/L). Each of the 8 ABXs were prepared in 2% NaCl solution (v/v). The pH of all solutions was meticulously monitored and maintained within the range of 6–8, with adjustments using 0.1 M NaOH if necessary.

Prior to the commencement of the assay, freeze-dried bacteria were rehydrated using the reactivation solution provided by the manufacturer. The rehydrated bacteria were allowed to equilibrate for 5 min at 4 °C. Subsequently, 0.5 mL of the reactivated bacteria were transferred to cuvettes positioned in a bath set at 15 °C for a 10 min-equilibration period. Upon equilibration, basal luminescence measurements were obtained using a BioFix® Lumi-10 luminometer (Macharey-Nagel) covering the 380–660 nm spectral range. Following this, 0.5 mL of the ABX dilutions under investigation were added to each cuvette. Luminescence measurements were repeated after a 30 min-incubation period as the UNE EN ISO 11348–3 (2009) protocol establishes. Each test was carried out in quadruplicate.

Toxic effects were assessed through the percentage of luminescence inhibition, calculated as the reduction in the ratio of bacterial light production to the remaining light post-testing, for each concentration relative to the control. The obtained results were subjected to analysis to determine the EC₅₀, representing the chemical concentration resulting in a 50% reduction in bioluminescence.

***Desmodesmus subspicatus* assay**

The *Desmodesmus subspicatus* assay aimed at assessing algal growth inhibition. It was conducted following OECD guideline 201 and ISO standard 8692 protocols. The microalga *D. subspicatus* BEA 0141B, sourced from the publicly available and officially recognized culture collection of the Spanish Bank of Algae, was utilized. Algal cultures were routinely maintained and precultured in accordance with OECD 201 guidelines.

The assay was carried out in 24-well sterile plates (Falcon 24 Well Clear Flat Bottom TC-Treated Polystyrene Multiwell Cell Culture Plate). Stock solutions of the ABXs were prepared in BG-11 culture medium obtained from the Spanish Bank of Algae. The initial cell density was adjusted to 2–5·10³ cells/mL, corresponding to the exponential growth phase preculture, utilizing a calibration

curve of chlorophyll fluorescence (Ex: 450 nm, Em: 680 nm) versus cell number ($R^2=0.9948$).

In the initial range-finding test, algae were exposed to five concentrations of each of the eight ABXs (from 0.1 to 1000 mg/L), with six control replicates and three treatment replicates used for each experiment, with a final volume of 2 mL per well. For the ABXs GTM, CHL, ERY, and TC, a second test was conducted under identical assay conditions, with concentrations of 125, 250, 500, 750 and 1000 mg/mL to adjust the dose–effect curves.

The exposed algae were then incubated at 23 ± 2 °C under continuous white light and agitation to ensure algal suspension and proper oxygen exchange. Algal growth was quantified at 0, 24, 48 and 72 h using a fluorometric method with a BioTek™ Synergy H1 hybrid multimode microplate reader (Ex: 450 nm, Em: 680 nm). Prior to measurement, samples were vigorously shaken for 10 s to ensure cell suspension. pH measurements were conducted before and after each experiment to ensure proper oxygenation of the sample. The pH was maintained in the range of 6–7.5 throughout the experiment.

The results were quantified in terms of average growth rates calculated from cell numbers based on chlorophyll fluorescence measurements, Eq. 1.

$$\% \text{ growth inhibition} = [(\mu_e - \mu_c) / \mu_c] \times 100 \quad (1)$$

where μ_c is the control luminescence, μ_e the luminescence of solution with ABX.

The results were subjected to analysis to determine the EC_{50} , representing the chemical concentration resulting in a 50% of growth inhibition.

Eisenia fetida assay

The *Eisenia fetida* test was conducted in accordance with OECD guideline 207. Adult *E. fetida* worms were sourced from Flower (Spain). These organisms were carefully maintained under controlled environmental conditions, ensuring temperature and humidity levels between 18–25 °C and 80–85%, respectively, within a sphagnum peat substrate (Flower, Spain) for a minimum acclimatization period of 15 days. Worms selected for experimentation were a minimum of 2 months old and exhibited a well-developed clitellum. Experiments were conducted in ½ L polyethylene terephthalate containers with perforated lids, designed to minimize water evaporation and prevent animal escape while facilitating soil aeration. A standardized OECD soil substrate, comprising industrial fine sand, kaolin clay, and sphagnum peat mixed in a ratio of 7:2:1, respectively, was prepared. The water content of this mixture was adjusted to 35% of the dry weight by adding deionized water (SIEMENS Ultra Clear™) and vigorously mixing.

Concentrations ranging from 0.1 to 1000 mg/kg of the eight ABXs under investigation were tested. Subsequently, ten worms were introduced into each container. Three independent replicates were conducted for each concentration. The experimental setup was maintained at a temperature of 20 ± 2 °C, with relative humidity levels maintained between 80–85% under constant illumination for a duration of 14 days. Following the incubation period, mortality rates were assessed, and the LC_{50} value, representing the chemical concentration resulting in 50% mortality of the *E. fetida* population, was calculated.

Allium cepa assay

The acute toxicity test for root growth inhibition using *Allium cepa* was conducted following the methodology outlined by Fiskesjö [28].

A. cepa bulbs of the Stuttgarter Riesen variety (diameter 14/21) were procured from Fitoagrícola Company (Spain) and stored under controlled conditions at 10–20 °C in complete darkness and low-humidity environment until required. For the ecotoxicological assessment, various dilutions of the eight ABXs (0.03, 0.3, 3, 30 and 300 mg/L) were prepared in a growth medium. The growth medium comprises water enriched with the following mineral ion content (mg/L): calcium, 32.1; sodium, <0.5; magnesium, 9.2; bicarbonates, 140; sulphates, 3.3; chlorides, 0.6.

The *A. cepa* bulbs underwent meticulous cleaning and peeling, ensuring the integrity of the root ring. Subsequently, bulbs were carefully placed in test tubes containing growth medium as the control and varying concentrations of ABXs. Throughout the test duration, the root zone remained consistently immersed in the liquid medium. Each concentration was replicated twelve times to ensure statistical robustness. Incubation of the bulbs was carried out in a controlled chamber under indirect lighting conditions at 20 °C for a duration of 72 h. At the conclusion of the incubation period, average root length was recorded.

Phytotoxic effects were assessed by calculating the percentage inhibition of root growth relative to the control. The obtained data were subjected to analysis to determine the EC_{50} , representing the chemical concentration at which a 50% reduction in root length occurred.

Calculation of effect concentrations

Dose–response curves for the five indicators were calculated with logistic logit regression using XLSTAT software (2014.5.03) to obtain the corresponding EC_{50} or LC_{50} and EC_{10} or LC_{10} values with 95% confidence intervals. The dose–response models were statistically tested using the chi-square test.

Risk assessment

Hazard Quotient (HQ) was used to assess the environmental risk of chemicals and their potential to cause adverse effects in the environment and was calculated as follows, Eq. 2:

$$HQ = \frac{MEC}{PNEC} \quad (2)$$

where PNEC is the Predicted No-Effect Concentration and MEC is the Measured Environmental Concentration. MEC data were obtained from literature. The risk levels were classified into no risk ($HQ < 0.01$), low risk ($0.01 \leq HQ < 0.1$), medium risk ($0.1 \leq HQ < 1$), and high risk ($HQ \geq 1$) [29].

The PNEC is the concentration of the substance below which adverse effects in the environmental sphere of concern are not expected to occur, and was obtained as follows, Eq. 3:

$$PNEC = \frac{\text{Lowest } EC_{50} \text{ value}}{AF} \quad (3)$$

where, according to Park and Choi [30], the lowest EC_{50} can be obtained from the current study or the literature, while the Assessment Factor (AF) equals 1000 if only acute toxicity tests are available and 100 if chronic data exist [31].

When no acute or chronic EC_{50} values were found for soil, their soil PNECs were obtained from their respective water PNECs and the water-organic matter partition coefficient (Koc) through the application of the following equation [1]:

$$PNEC_{\text{soil}} = PNEC_{\text{water}} \cdot Koc \quad (4)$$

Redundancy analysis

The effect of the physico-chemical properties of the ABX on EC_{50} or LC_{50} value of each organism was analyzed by redundancy analysis (RDA). RDA is an extension of multiple regression that generates a model to determine the effect of the explanatory variables, that is, molecular weight (MW), number of rotatable bonds, number of H-donors, number of H-acceptors, topological Polar Surface Area (PSA), logarithm of the distribution coefficient (LogD) and charge of the molecule, both at pH=7, on a response variable matrix consisting of the EC_{50} values of the organisms exposed to the eight different ABXs. Table S2 offers the specific values of each property for the 8 ABXs tested.

MW, number of rotatable bonds, number of H-donors and acceptors, PSA, number of heavy atoms and water

solubility data were directly obtained from PubChem website using the CAS number of the ABXs. LogD was calculated using the ChemAxon calculator (<https://playground.calculators.cxn.io/>). This free calculator also provides the different molecular structures for a compound together with the charge for each of them and the proportion at which they are found in water solution depending on their ionizable capability. The average charge was obtained as the weighted average of the charge of all possible ionizable structures of a given ABX at pH=7, the pH at which most of the ecotoxicity tests were performed.

All analysis were performed using R software version 4.1.2. Briefly, the explanatory variables measured in different units were scaled using scale function. This function normalizes the values by subtracting the mean and dividing by the standard deviation of each variable. Response variables were transformed in abundance profiles by Hellinger transformation using *decostand* function to reduce the influence of dominant species. This transformation calculates the square root on the division of the original EC_{50} produced by a given ABX in each organism by the summatory of the EC_{50} for all ABXs in the same organism. Subsequently, *rda* function of *vegan* R package [32] was implemented to obtain the model that allows the simultaneous analysis between response and explanatory variables using the option *scaling*=2, where the angles between the variables reflect their correlation.

Significance contribution of physico-chemical properties that conform the RDA model was tested using the function *anova.cca* with the option *by*="term". Statistic F was obtained and *p*-value calculated using 999 permutations, a value below 0.05 were considered as statistically significant.

Results and discussion

Effects of antibiotics on *Aliivibrio fischeri* bioluminescence

Figure 1 shows the dose–response curve for *A. fischeri* bioluminescence inhibition when these bacteria were exposed to the ABXs. The EC_{50} values for each ABX can be found in Table 1. In Fig. 1, only ABXs with EC_{50} values placed in the range studied were shown. According to this plot, the behaviour of ERY is the only one that differs from that of AMP, PEN and CHL. ERY exhibited a markedly steeper slope, indicating a narrower margin between sublethal and inhibitory concentrations. According to EC_{50} values, the least harmful compounds were the aminoglycosides and AMO, which showed no toxicity at the concentrations tested. On the contrary, the most toxic compound was PEN, followed by CHL, ERY and AMP with EC_{50} ranging from 84.29 to 363.52 mg/L. TC could not be tested because the coloured solution interfered with the readout measurements. From the

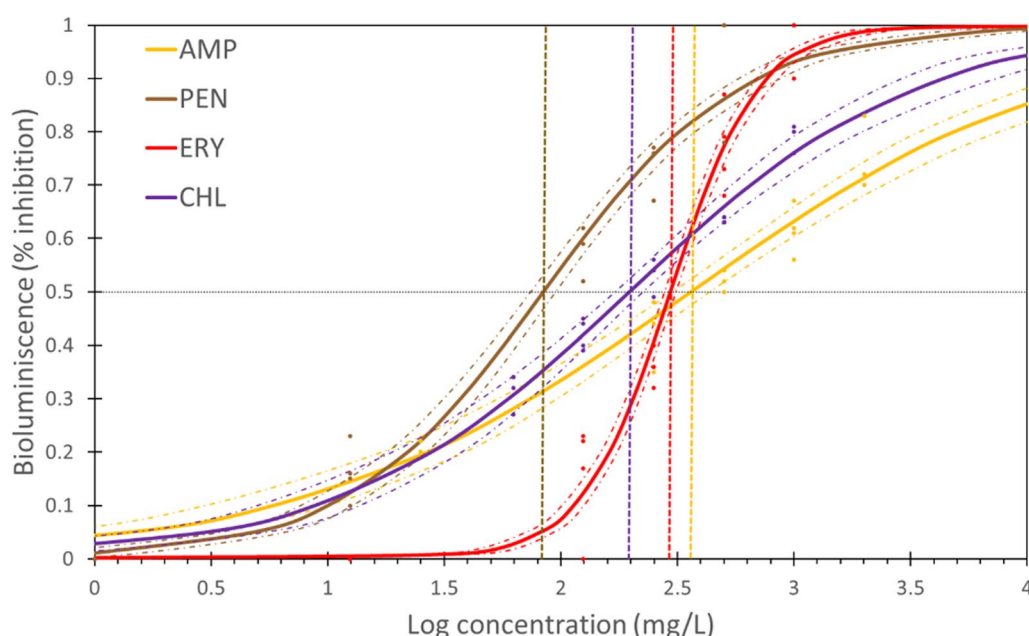


Fig. 1 Dose–response curve for *Aliivibrio fischeri* after 30 min-exposure to ampicillin (AMP), penicillin (PEN), erythromycin (ERY) and chloramphenicol (CHL). Solid line is the model, dashed lines are the superior and inferior confidence limits (95%) and dots are the experimental data. Vertical lines denote the EC_{50} values calculated for each antibiotic. Antibiotics with EC_{50} outside the studied range have not been enclosed

highest to the lowest toxicity according to their EC_{50} values (Table 1), the following order was established: $PEN > CHL > ERY > AMP > AMO$ -GTM.

In line with the toxicity classification designed by European Chemicals Agency [33], compounds are categorized as very toxic ($LC_{50} < 1$ mg/L), toxic ($1 \text{ mg/L} \leq LC_{50} < 10$ mg/L), little toxic ($10 \text{ mg/L} \leq LC_{50} < 100$ mg/L) and non-toxic ($100 \text{ mg/L} \leq LC_{50}$). From the point of view of *A. vibrio* assay, these compounds can be classified as non-toxic, with the only exception of PEN, which can be classified as little toxic.

The low toxicity of most ABXs to *A. fischeri* can be explained by a combination of factors related to their mechanisms of action, bacterial physiology, and, most importantly, the design of the test.

β -lactam ABXs (PEN, AMP, AMO) inhibit peptidoglycan synthesis and are primarily effective against Gram-positive bacteria. In the 30-min assay, the β -lactams tested produced almost no inhibition of *A. fischeri* luminescence. For instance, Havelkova et al. [22] investigated the effects of PEN on several aquatic and soil organisms, but were unable to determine an EC_{50} for *A. fischeri* within the tested concentration range. AMP and AMO also did not elicit toxic responses (see Table 1). Other authors conducting similar tests (albeit with shorter exposure durations from 5 to 15 min) reported EC_{50} values between 1000 and 4000 mg/L ([30, 34, 35]. Even

under extended exposure (24 h), Ioele et al. [36] found EC_{50} values above 150 mg/L—a concentration unlikely to be encountered in the environment. The absence of toxicity may be attributed to a combination of the ABXs' mode of action and the experimental setup. β -lactams act by covalently binding to penicillin-binding proteins and inhibiting peptidoglycan crosslinking, a process mostly effective in actively dividing bacteria [37]. Additionally, many marine bacteria, including *A. fischeri*, possess β -lactamase genes such as *ampC*, which hydrolyse the β -lactam ring and likely inactivate the ABXs before they reach their target [38].

Similarly, the aminoglycosides GTM and STM, which irreversibly bind to the 16S rRNA of the 30S ribosomal subunit to induce mistranslation [39], showed no detectable toxicity. GTM displayed high EC_{50} values, consistent with data from Lomba et al. [40], confirming that this aminoglycoside poses minimal acute toxicity on *A. fischeri*. Our results for STM align with those of Grinten et al. [26], who reported EC_{50} values higher than 0.4 mg/L and with Backhaus and Grimme [41], who obtained a lower EC_{50} at higher exposure time, Table 1. These findings are likely due to impaired ABX uptake: aminoglycoside uptake occurs in two energy-dependent phases, both of which rely on the proton motive force (PMF) [42, 43], both of which can be reduced under test conditions, high salinity [43, 44] and low temperature [45, 46]. As a result, intracellular accumulation of the ABX may be insufficient

Table 1 Experimental and literature ecotoxicity values (EC_{50} or LC_{50}) for antibiotics on *D. magna*, *A. fischeri*, *D. subspicatus*, *E. fetida*, *A. cepa* and other non-target organisms. Concentration is given in mg/L and mg/kg (for soil organisms)

Water					
ABX	Type of organism	Name	Test time	EC_{50} or LC_{50} (mg/L or mg/kg)	References
AMO	Bacteria	<i>Aliivibrio fischeri</i>	5 min	1320.0 (973–179)	[30]
			15 min	3597.0 (519.5–24,760)	[30]
				3597	[57]
				4000 (3600–5000)	[35]
			30 min	> 50	[36]
		<i>Microcystis aeruginosa</i>		> 1500	This study
			24 h	150.02	[36]
			7 d	0.0037	[58]
				0.008	[59]
			3 d	56.3	[24]
	Crustacean	<i>Synechococcus sp.</i>	4 d	0.0022	[60]
		<i>Daphnia magna</i>	24 h	> 1000	[30]
				> 2500	This study
			48 h	> 1000	[30]
			96 h	6950	[61]
		<i>Moina macrocopa</i>	24 h	> 1000	[30]
			48 h	> 1000	[30]
	Algae	<i>Desmodesmus subspicatus</i>	3 d	> 1000	This study
			3 d	> 1500	[24]
				> 2000	[62]
				630	[59]
				> 1000	[63]
		<i>Rhodomonas salina</i>	7 d	> 250	[58]
			7 d	3018	[58]
			3 d	> 1000	[63]
	Aquatic plants	<i>Lemna gibba</i>		> 1	[64]
	Fish	<i>Oryzias latipes</i>	48 h	> 1000	[30]
			96 h	> 1000	[30]

Table 1 (continued)

Water					
ABX	Type of organism	Name	Test time	EC ₅₀ or LC ₅₀ (mg/L or mg/kg)	References
AMP	Bacteria	<i>Aliivibrio fischeri</i>	5 min	1056.0 (562–199)	[30]
			15 min	2627.0 (384.8–17,930)	[30]
			30 min	> 50	[36]
				363.52 (302.61–435.65)	This study
	Crustacean	<i>Daphnia magna</i>	24 h	163	[36]
			24 h	> 1000	[30]
				4324.13 (3549.97–5312.77)	This study
			48 h	> 1000	[30]
		<i>Moina macrocopa</i>	24 h	> 1000	[30]
			48 h	> 1000	[30]
	Algae	<i>Desmodesmus subspicatus</i>	3 d	> 1000	This study
	Aquatic plants	<i>Lemna gibba</i>	7 d	> 1	[64]
	Fish	<i>Oryzias latipes</i>	48h	> 1000	[30]
			96h	> 1000	[30]
CHL	Bacteria	<i>Aliivibrio fischeri</i>	5 min	589.0 (423.4–819.4)	[65]
			15 min	537.5 (222.0–591.0)	[65]
			30 min	197.25 (171.57–225.51)	This study
			24 h	0.064	[41]
	Crustacean	<i>Daphnia magna</i>	24 h	1086	[66]
				542.86	[67]
				285.63 (235.12–333.92)	This study
			48 h	327.4 (300.2–444.5)	[65]
				363 (304–460)	[68]
			72 h	122 (81–154)	[68]
			72 h, 20 °C	283.86	[69]
			72 h, 25 °C	85.18	[69]
			96 h	81.2 (54.5–107.8)	[65]
		<i>Moina macrocopa</i>	48 h	511.5	[65]
			96 h	273.3	[65]
				305	[66]
		<i>Streptocephalus proboscideus</i>	24 h	305	[66]
		<i>Artemia salina</i>	24 h	2042	[66]
	Rotifer	<i>Brachionus calyciflorus</i>	24 h	2074	[66]
	Algae	<i>Desmodesmus subspicatus</i>	3 d	669.66 (644.27–695.78)	This study
	Fish	<i>Oryzias latipes</i>	48 h	> 1000	[65]
			96 h	> 1000	[65]

Table 1 (continued)

Water					
ABX	Type of organism	Name	Test time	EC ₅₀ or LC ₅₀ (mg/L or mg/kg)	References
ERY	Bacteria	<i>Aliivibrio fischeri</i>	30 min	Not determined because of its low water solubility	[36]
				> 100	[25]
				560 Extrapolated	[70]
				294.84 (278.52–312.05)	This study
		<i>Anabaena</i> spp. CPB4337	3 d	0.022	[24]
		<i>Anabaena cylindrica</i>	6 d	0.035	[71]
		<i>Anabaena flos-aquae</i>	6 d	0.27	[71]
		<i>Anabaena variabilis</i>	6 d	0.43	[71]
		<i>Synechococcus</i> sp.	6 d	0.23	[72]
		<i>Synechococcus leopoldensis</i>	6 d	0.16	[71]
		<i>Microcystis wessenbergii</i>	6 d	0.023	[71]
		<i>Nostos</i> sp.	6 d	0.2	[71]
	Crustacean	<i>Daphnia magna</i>	24 h	387.70 (282.5–532.0)	[73]
				22.45 (16.98–29.85)	[25]
				498.72 (405.55–608.40)	This study
		<i>Moina macrocopa</i>	48 h	135.5	[74]
		<i>Thamnocephalus platyurus</i>	24 h	> 100	[75]
				17.68	[25]
		<i>Clubiona dubia</i>	24 h	10.23	[25]
			7 d	0.22	[76]
	Rotifer	<i>Brachionus calyciflorus</i>	24 h	27.53	[25]
			48 h	0.94	[25]
	Algae	<i>Desmodesmus subspicatus</i>	3 d	2818.47 (1689.40–5478.96)	This study
		<i>Pseudokirchneriella subcapitata</i> or <i>Selenastrum capricornutum</i> or <i>Raphidocelis subcapitata</i>	3 d	0.02	[25]
				0.0366	[63]
				0.35	[24]
				0.13—0.24	[70]
				0.038	[77]
			4 d	0.0246	[78]
				0.044	[79]
				0.056	[80]
			6 d	0.0366	[71]
	Aquatic plants	<i>Chlorella vulgaris</i>	3 d	33.8	[63]
			4 d	0.36	[81]
		<i>Lemna gibba</i>		> 1	[64]
		<i>Lemna minor</i>	7 d	5.62	[63]
	Fish	<i>Oryzias latipes</i>	96 h	> 100	[75]
GTM	Bacteria	<i>Aliivibrio fischeri</i>	30 min	> 10,000	[40]
				> 5000	This study
	Crustacean	<i>Daphnia magna</i>	24 h	875.5 ± 180	[40]
				840.75 (742.70–927.24)	This study
	Algae	<i>Desmodesmus subspicatus</i>	3 d	820.41 (765.12–888.14)	This study
		<i>Pseudokirchneriella subcapitata</i> or <i>Selenastrum capricornutum</i> or <i>Raphidocelis subcapitata</i>	3 d	19.2	[62]

Table 1 (continued)

Water					
ABX	Type of organism	Name	Test time	EC ₅₀ or LC ₅₀ (mg/L or mg/kg)	References
PEN	Bacteria	<i>Aliivibrio fischeri</i>	15 min	> 100	[22]
			30 min	84.29 (74.29–95.09)	This study
	Crustacean	<i>Microcystis aeruginosa</i>	7 d	0.006	[82]
		<i>Daphnia magna</i>	24 h	> 2500	This study
			48 h	1496.9 (1422.7–1574.9)	[22]
	Algae	<i>Desmodesmus subspicatus</i>	3 d	> 1000	This study
		<i>Pseudokirchneriella subcapitata</i> or <i>Selenastrum capricornutum</i> or <i>Raphidocelis subcapitata</i>	3 d	7114.3	[22]
STM	Bacteria	<i>Aliivibrio fischeri</i>	30 min	> 0.4	[26]
				> 5000	This study
			24 h	8.21	[41]
		<i>Microcystis aeruginosa</i>	24 h	0.034	[26]
			6 d	0.023	[71]
	Crustacean		7 d	0.007	[82]
		<i>Daphnia magna</i>	24 h	772.37 (499.27–1087.04)	This study
	Algae	<i>Desmodesmus subspicatus</i>	3 d	> 1000	This study
		<i>Pseudokirchneriella subcapitata</i> or <i>Selenastrum capricornutum</i> or <i>Raphidocelis subcapitata</i>	24 h	1.5	[26]
	Crustacean		3 d	0.133	[82]
		<i>Daphnia magna</i>	24 h	947 (778 ± 1181)	[83]
			48 h	487 (346 ± 721)	[83]
TC	Bacteria	<i>Aliivibrio fischeri</i>	21 d	> 64	[83]
			15 min	17.58	[22]
				560 (430–690)	[70]
			30 min	6.7	[22]
			60 min	173.8	[84]
			24 h	0.0251	[41]
		<i>Microcystis aeruginosa</i>	3 d	0.09	[58]
		<i>Anabaena</i> spp. CPB4337	3 d	6.2	[24]
	Crustacean	<i>Daphnia magna</i>	24 h	> 340	[83]
				391.07 (352.71–430.33)	This study
			48 h	8.16 (6.61–10.10)	[22]
	Algae			36.52 (± 5.71)	[85]
				> 340	[83]
			21 d	44.8 (40.3–49.8)	[83]
		<i>Desmodesmus subspicatus</i>	3 d	200.7 (166.96–232.30)	This study
		<i>Pseudokirchneriella subcapitata</i> or <i>Selenastrum capricornutum</i> or <i>Raphidocelis subcapitata</i>	3 d	2.2	[82]
				3.3	[24]
				0.31	[59]
Aquatic plants	Fish			1.82	[22]
		<i>Lemna gibba</i>		0.723	[86]
	Fish	<i>Lemna minor</i>	7d	1.06	[63]
		<i>Salvelinus namaycush</i>	24 h	220	[57]
	Fish	<i>Morone saxatilis</i>	24, 48, 96 h	> 182	[57]

Table 1 (continued)

Soil					
ABX	Type of organism	Name	Test time	EC50 or LC50 (mg/kg)	References
AMO	Worm	<i>Eisenia fetida</i>	15 d	> 1000	This study
	Terrestrial plants	<i>Allium cepa</i>	3 d	45.19 (35.11–59.37)	This study
AMP	Worm	<i>Eisenia fetida</i>	15 d	> 1000	This study
	Terrestrial plants	<i>Allium cepa</i>	3 d	> 300	This study
CHL	Worm	<i>Eisenia fetida</i>	15 d	> 1000	This study
	Terrestrial plants	<i>Allium cepa</i>	3 d	102.34 (84.09–126.42)	This study
ERY	Worm	<i>Eisenia fetida</i>	15 d	> 1000	This study
	Terrestrial plants	<i>Allium cepa</i>	3 d	> 300	This study
GTM	Worm	<i>Eisenia fetida</i>	15 d	> 1000	This study
	Terrestrial plants	<i>Allium cepa</i>	3 d	42.57 (30.74–61.07)	This study
PEN	Worm	<i>Eisenia fetida</i>	15 d	> 1000	This study
			56 d	348 (208–487)	[22]
	Springtail	<i>Folsomia candida</i>	28 d	328	[22]
	Terrestrial plants	<i>Allium cepa</i>	They found very few mitotic aberrations, although up to 200 mg/L, there seems to be some additional mitotic activity		[87]
			3 d	> 300	This study
STM	Worm	<i>Eisenia fetida</i>	15 d	> 1000	This study
	Terrestrial plants	<i>Allium cepa</i>	3 d	> 300	This study
TC	Worm	<i>Eisenia fetida</i>	15 d	> 2000	[88]
				> 1000	This study
	Springtail	<i>Folsomia candida</i>	28 d	2560	[22]
	Terrestrial plants	<i>Allium cepa</i>	Plant meiosis was affected by TC and that genetic damage can be potentially transmitted to the descendants via male gametes, leading to congenital abnormalities		[89]
			3 d	106.58 (82.33–141.47)	This study

Data provided by this study are highlighted in bold

within 30 min, thus, little ribosomal binding occurs during the assay.

ERY, a macrolide, binds to the 50S ribosomal subunit and blocks peptide elongation [47]. Although it is considered a broad-spectrum ABX, Gram-negative bacteria are intrinsically less susceptible due to permeability barriers and the action of efflux pumps [48]. In our *A. fischeri* test, ERY caused minimal luminescence inhibition, which can be explained by the same cause of previously mentioned ABXs: the ABX never reached its ribosomal target in high enough concentrations during the 30-min exposure enough to suppress protein synthesis in a way that would affect luminescence. This ABX was reported by Ioele et al. [36] as a substance that displays hormesis, a behaviour formerly registered for some drugs by other investigators using *A. fischeri* [49, 50]. This phenomenon consists of a “dose–response phenomenon characterised by a low dose stimulation and a high dose inhibition” as an adaptive

response of the organisms to the effects of toxic chemicals exposure [51]. We did not observe this effect on *A. fischeri* since the minimum concentration tested by us (125 mg/L) doubled by far the maximum concentration in the study performed by Ioele et al. (50 mg/L).

CHL, a bacteriostatic agent that inhibits peptidyl transferase activity by binding to the 50S ribosomal subunit [52], also exhibited low toxicity to *A. fischeri* in the 30-min bioluminescence inhibition test. This aligns with its mode of action: CHL inhibits bacterial growth rather than directly causing cell death.

No results for TC on *A. fischeri* could be obtained since this chemical interferes with the luminescence test itself at the concentrations studied. Reported data about TC toxicity on *A. fischeri* are, then, contradictory. On the one hand, Havelkova et al. [22] and Brandt et al. [23] suggested that *A. fischeri* is insensitive to ABX because the activity inhibition of luciferase (bioluminescence) after short-term exposure is not related to the mechanism of

toxic action of most of this type of drugs. On the other hand, Backhaus and Grimme [41] stated that TC is, according to the 93/21/ECC directive, a very toxic compound to aquatic organisms because its EC_{50} (0.0251 mg/L) is below 1 mg/L after 24 h. As far as we know, this is the first reported value of EC_{50} for PEN and CHL when using a 30 min exposure test to *A. fischeri*.

The standard 30-min bioluminescence assay may underestimate the toxicity of ABXs. Most bactericidal agents act during cell replication, and many bacteriostatic agents require extended contact times to suppress protein synthesis and impact cell metabolism. For example, in the case of CHL, requires longer exposures (6–9 h), especially in non-dividing populations [53, 54]. During the 30-min assay, *A. fischeri* cells are not undergoing exponential growth. As a result, the assay predominantly captures immediate metabolic disruptions, measured via luminescence inhibition, rather than longer-term impacts on growth or viability [55, 56]. It is therefore very important to have a battery of bioindicators, and not just one, in order to realistically assess the toxicity of these antibiotics in the environment.

Effects of antibiotics on *Daphnia magna* mobility

The second set of bioassays focused on the effects of ABX exposure on *D. magna*, a standard model organism for evaluating freshwater invertebrate sensitivity to contaminants. Figure 2 shows the dose–response curves for

the effect of AMP, ERY, CHL, GTM, TC and STM on *D. magna*. PEN and AMO are not included because their EC_{50} were not reached in the range of concentrations tested. Besides, specific EC_{50} values and confidence intervals are given in Table 1. Ours is the first study to evaluate the toxicity of PEN and STM using a 24 h *D. magna* test.

Among the compounds tested, β -lactams (PEN, AMP, and AMO) showed the lowest toxicity, with EC_{50} values all above 2500 mg/L, indicating low ecotoxicity related to *D. magna*. CHL displayed the highest inhibitory effect on this organism, with an EC_{50} of 285.63 mg/L. The remaining ABX families showed EC_{50} values ranging from 391.07 to 840.75 mg/L.

From highest to lowest toxicity based on their EC_{50} values: CHL > TC > ERY > STM > GTM > AMP. However, as shown, all compounds exhibit insignificant toxicity (EC_{50} > 100 mg/L) towards *D. magna*, according to the ECHA classification [33].

The dose–response curves (Fig. 2) for most of the ABXs followed the same trend regardless the compound, which consists of a pronounced slope, except for STM, which showed a more gradual one. This suggests that although STM is not the most toxic compound, as mentioned above, its effects are observed at lower concentrations when compared to the other antimicrobials.

Our results for PEN agree with those of Havelkova et al. [22]. The small differences (see Table 1) may be

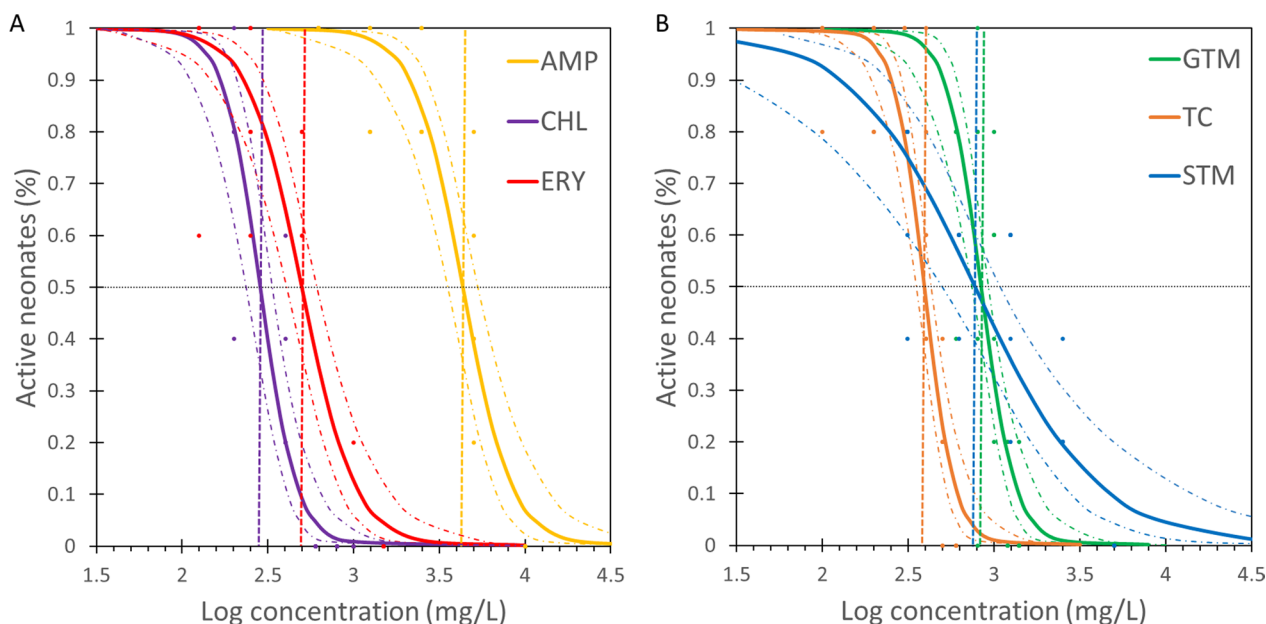


Fig. 2 Dose–response curve for *Daphnia magna* after 24 h-exposure to (A) ampicillin (AMP), erythromycin (ERY) and chloramphenicol (CHL) and (B) gentamicin (GTM), tetracycline (TC) and streptomycin (STM). Solid line is the model, dashed lines are the superior and inferior confidence limits (95%) and dots are the experimental data. Vertical lines denote the EC_{50} values calculated for each antibiotic. Antibiotics with EC_{50} outside the studied range have not been enclosed

partly explained by the shorter test duration in our study (24 h vs. 48 h), as longer exposures often reveal greater toxicity [34].

AMP and AMO similarly showed low toxicity to *D. magna*, confirming previous findings from other authors [30, 61].

Although *D. magna* seems to be slightly more sensitive to GTM, ERY and TC, the specific molecular targets of these ABXs in *D. magna* are not well-defined. On *D. magna*, STM caused nor reproductive (up to 64 mg/L), nor acute effects according to Wollenberger et al. [83]. However, these same authors observed reproductive effects on the crustacean when TC was tested in the range of 5 to 50 mg/L and mortality in the parent generation during long-term exposure (3 weeks). This is in agreement with the fact that our EC_{50} (Table 1) is higher than this last concentration. Wollenberger et al. (2000) also highlighted that indirect toxicity may occur if ABXs affect *D. magna*'s food source, such as algae or bacteria in test systems and not directly on the *D. magna* organisms [83].

Regarding CHL, its toxicity appears linked to oxidative stress mechanisms. Barata et al. [90] reported an antioxidant response in *D. magna* when tested against CHL due to tissue oxidation (lipid peroxidation). In this response, the enzymes CHL acetyltransferase, superoxide dismutase, and glutathione-S-transferase were involved. In 2021, Zhang et al. [68] monitored the

levels of MDA (a product of lipid peroxidation that is related to damage in cell membranes), GSH and catalase (CAT) activity, together with the growth and population levels of daphnids. CHL concentrations higher than 100 mg/L significantly increased the levels of MDA and CAT with respect to the control, while GSH significantly decreased when compared to that of the control, as expected when *D. magna* reacts against oxidative stress, previously reported by Barata et al. [90]. These results are coherent with our EC_{50} value, which is higher than 100 mg/L (Table 1).

Taken together, the results support the notion that ABX toxicity in *D. magna* is compound-specific and may involve oxidative stress, metabolic disruption, or indirect trophic effects.

Effects of antibiotics on *Desmodesmus subspicatus* growth

The third set of assays evaluated the growth inhibition of the green microalga *D. subspicatus*, a sensitive freshwater species frequently used in ecotoxicological risk assessment of chemicals, exposed to the eight ABXs. As shown in Table 1 and Fig. 3, most of the tested compounds exhibited low toxicity toward this species, with EC_{50} values exceeding 1000 mg/L. Notable exceptions included TC (EC_{50} =200.70 mg/L), CHL (EC_{50} =669.66 mg/L), and, to a lesser extent, GTM (EC_{50} =820.41 mg/L). In Fig. 3, GTM and CHL exhibited steeper dose-response curves, suggesting more abrupt growth inhibition above

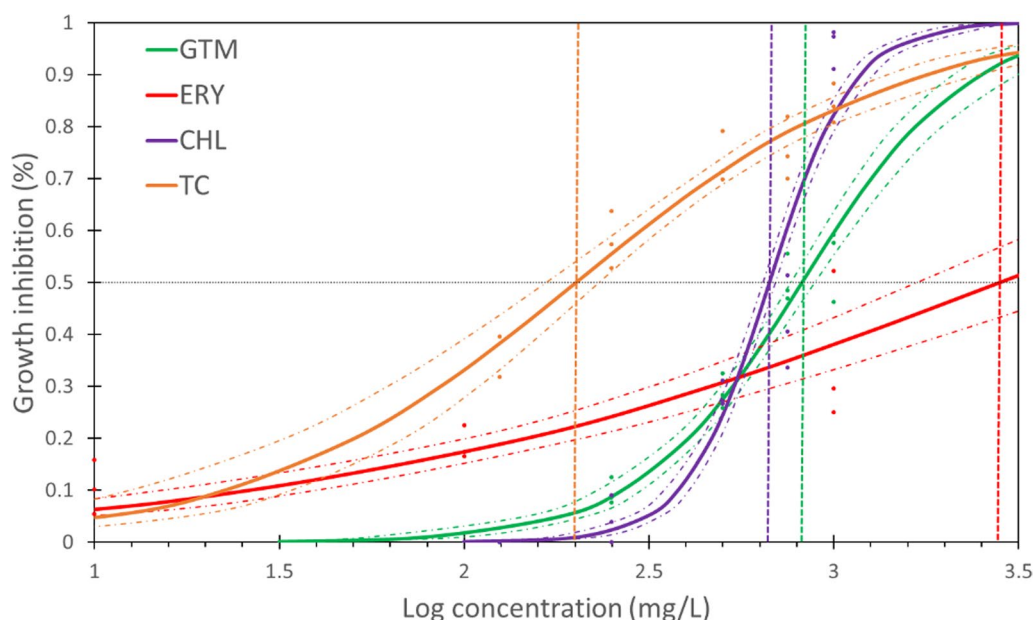


Fig. 3 Dose-response curve for *Desmodesmus subspicatus* growth inhibition after 72 h exposure to gentamicin (GTM), erythromycin (ERY), chloramphenicol (CHL) and tetracycline (TC). Solid line is the model, dashed lines are the superior and inferior confidence limits (95%) and dots are the experimental data. Vertical lines denote the EC_{50} values calculated for each antibiotic. Antibiotics with EC_{50} outside the studied range have not been enclosed

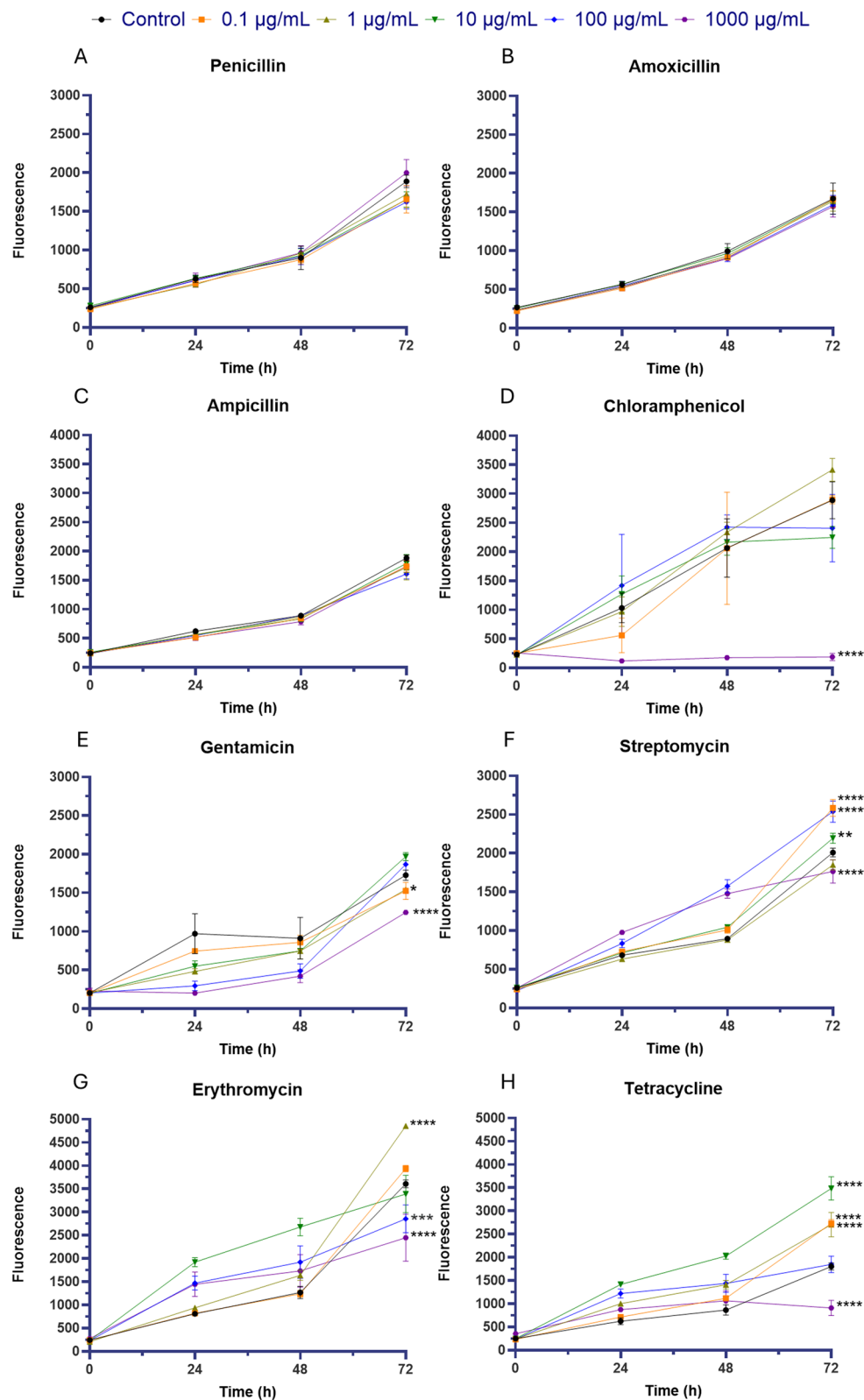


Fig. 4 Kinetic growth curve of *Desmodesmus subspicatus* exposed to 5 different concentrations of the eight tested antibiotics for 3 days, **A**, penicillin; **B**, amoxicillin; **C**, ampicillin; **D**, chloramphenicol; **E**, gentamicin; **F**, streptomycin; **G**, erythromycin; **H**, tetracycline. Statistical significance at 72 h was determined using two-way ANOVA (****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05). Absence of notation indicates no statistically significant differences

threshold concentrations. In contrast, TC and ERY showed more gradual declines in algal growth, indicative of broader response variability among individual cells. From highest to lowest toxicity according to their EC_{50} values: TC > CHL > GTM > ERY. As with *D. magna*, all compounds show insignificant toxicity ($EC_{50} > 100$ mg/L) towards *D. subspicatus*, according to the ECHA classification [33].

In Fig. 4, a kinetic overview of the tested ABXs on *D. subspicatus* over a 72-h period is offered. For AMO, AMP and PEN no significant differences are found at any time among the various doses tested, reinforcing the minimal impact of β -lactam ABXs on this algal species.

To our knowledge, no prior studies have assessed the toxicity of these ABXs on *D. subspicatus*. However, the minimal toxicity of β -lactam ABXs is well documented in related microalgae. This has been attributed to their mode of action, which specifically targets bacterial cell wall biosynthesis, a missing process in algae [63, 82]. Similar results were found by Andreozzi et al. [91] when they analysed the effect of AMO on a comparable algae, *Raphidocelis subcapitata*. In general terms, it is said that the mode of action of ABXs on microalgae mainly include three aspects: photosynthesis, oxidative stress and macromolecular alterations [92].

GTM produced a clear dose-dependent inhibition of algal fluorescence (Fig. 4E), whereas ERY, TC, and STM (Fig. 4G, H and F, respectively) unexpectedly increased fluorescence at nearly all concentrations tested. This response is characteristic of hormesis, a phenomenon previously reported for several ABXs in microalgae [93–95], including TC in *Coelastrella* sp. and *Synechocystis* sp. [96]. Given that ABX concentrations in natural environments are typically below EC_{50} thresholds, hormetic stimulation could occur frequently in aquatic ecosystems, potentially disrupting primary production and trophic balance. Some other authors have attributed the toxicity of TCs on microalgae, specifically chlortetracycline, to the modification of chloroplast metabolism [62]. When analysing the effect of ERY on *R. subcapitata*, an adaptive mechanisms was hypothesized, which would be consistent to hormesis phenomenon and our results [79].

CHL displayed a mixed behaviour (Fig. 4D). At the highest dose, it inhibits fluorescence, but at medium and low concentrations, it promotes it. This pattern is consistent with previous finding by Zhang et al. [97] when studying the effect of CHL on *Chlorella pyrenoidosa*, and may be linked to inhibition of protein or protochlorophyll biosynthesis, as observed in *Scenedesmus obliquus* [97].

Effects of antibiotics on *Eisenia fetida* mortality

The potential toxicity of the tested ABXs on the soil invertebrate *E. fetida* was assessed through a standardized 14-day toxicity test. *E. fetida* is considered a non-target organism with a dual exposure pathways: dermal absorption through contact with contaminated soils [98] and digestive uptake through ingestion of soil particles, as these organisms typically process large volumes of substrate in search of organic matter, inadvertently ingesting soil particles adhered to organic residues [99].

Despite the multiple exposure routes, LC_{50} values for all tested ABXs exceeded 1000 mg/kg (Table 1), indicating an absence of acute toxicity under the conditions of the assay. Among the tested compounds, only CHL and TC induced observable mortality at the highest tested concentration (1000 mg/kg), causing 40% and 27% mortality, respectively.

To the best of our knowledge, this study constitutes the first assessment of the effects of AMP, AMO, GTM, STM, ERY and CHL on *E. fetida*. Although literature on this topic remains scarce, some previous studies have evaluated the effects of selected ABXs on this key soil bioindicator. For instance, although PEN did not induce significant toxicity in *E. fetida* in our 14-day assay, similar findings were reported by Havelková et al. [22], who determined an LC_{50} value of 348 mg/kg for PEN, but only after a prolonged exposure of 56 days. This is coherent with our results and supports the notion that longer exposure durations may reveal sub-lethal or chronic toxic effects not evident under shorter testing periods. Regarding TC, our results are in agreement with previous studies, which also reported low toxicity in *E. fetida*. For instance, Pino et al. [88] found an $LC_{50} > 2000$ mg/kg, and Havelková et al. [22], determined a similar value for chlortetracycline (2735 mg/kg), a structurally related analogue.

E. fetida is considered a non-target organism for ABXs because these drugs are designed to act on bacteria rather than multicellular soil invertebrates. Nonetheless, exposure to ABX-contaminated soil can adversely affect earthworms and other terrestrial organisms through indirect pathways [100]. Studies report that increasing ABX levels in soil (e.g. from manure amendments) lead to reduced earthworm growth and survival, with worms often exhibiting avoidance behaviour (fleeing the contaminated soil) and other stress responses [100]. Some of these effects are attributed to off-target mechanisms: ABXs can disrupt the earthworm's gut microbiota and alter the soil microflora [101]. In particular, Rutkoski et al. (2024) found that exposure to TC did not affect the survival of *E. fetida*, a result consistent with our own findings, however, despite the absence of mortality, multiple types of toxicity were observed at different biological levels, including

cellular and histological effects. For instance, exposure to the highest TC concentration led to a reduction in coelomocyte viability, decreased acetylcholinesterase activity (indicating neurotoxicity), and a reduction of mucopolysaccharide content in the epidermis, suggesting impaired mucus production [101]. Furthermore, exposure to TC has been found to significantly shift soil microbial communities, and these alterations in functional diversity were correlated with oxidative stress markers, such as increased malondialdehyde levels, in *E. fetida* [102]. Such findings suggest that the toxicity observed in *E. fetida* may also arise not from direct action on earthworm tissues but rather through microbiome-mediated indirect effects. In addition, a study evaluating the impact of eight commonly used ABXs on soil bacterial communities found that CHL had the most pronounced disruptive effect [103], which aligns with our findings, as CHL was also the ABX that induced the highest mortality in *E. fetida* during our study.

Effects of antibiotics on *Allium cepa* roots inhibition

Root growth inhibition in *A. cepa* is a widely used plant-based bioassay for evaluating the cytotoxic and genotoxic potential of environmental contaminants. According to the EC_{50} values of *A. cepa* root inhibition effect (Table 1 and Fig. 5), ABXs in this study can be classified in 3 groups [33]: (i) AMO and GTM, exhibited moderate toxicity, with EC_{50} values around 45 mg/L, falling within the slightly toxicity category ($10 < EC_{50} \leq 100$ mg/L); (ii) CHL

and TC showed EC_{50} values just above 100 mg/L, however, the lower bounds of their 95% confidence intervals fall within the 10–100 mg/L range, suggesting that they are close to the threshold between low toxicity and no toxicity, and (iii) STM, ERY, PEN and AMP exhibited no observable phytotoxicity with EC_{50} values higher than 300 mg/L.

Historical studies support these trends, Wilson [87] probed different concentrations of PEN (from 100 to 800 ppm) on *A. cepa* for 12 h and up to 7 days. Wilson found very few mitotic aberrations, although up to 200 ppm (200 mg/L), there seems to be some additional mitotic activity than with respect to the control and prophase reductional groupings were relatively frequent. Drury and Khan [104] reported deformities in the ultrastructure of cellular organelles of *A. cepa* after having been treated with STM sulphate, which could be attributed to the inhibition of nucleic acid synthesis. This work was consistent with the one by Wilson [87], where obvious cytological effects were found for concentrations higher than 100 ppm. Besides, the chromosomes became shorter at full metaphase after treating *A. cepa* with all STM concentrations.

Mann et al. [89] observed how *A. cepa* plant meiosis was affected by TC and that genetic damage can be potentially transmitted to the descendants via male gametes, leading to congenital abnormalities. All this agrees with the fact that TC showed toxic effects on onion plants after 72 h at 108.58 mg/L (Table 1).

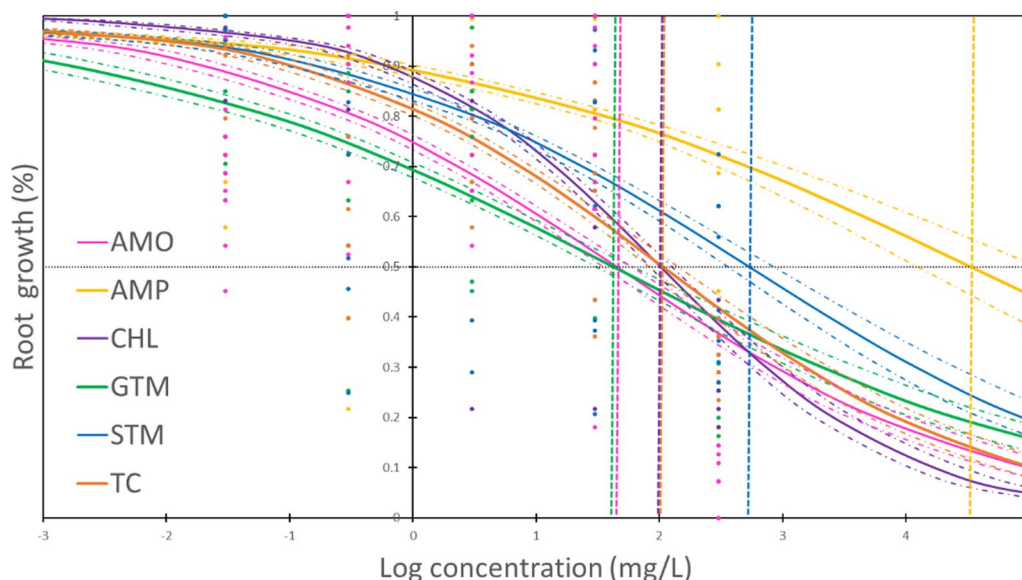


Fig. 5 Dose–response curve for *Allium cepa* after 72 h-exposure to amoxicillin (AMO), ampicillin (AMP), chloramphenicol (CHL), gentamicin (GTM), streptomycin (STM) and tetracycline (TC). Solid line is the model, dashed lines are the superior and inferior confidence limits (95%) and dots are the experimental data. Vertical lines denote the EC_{50} values calculated for each antibiotic. Antibiotics with EC_{50} outside the studied range have not been enclosed

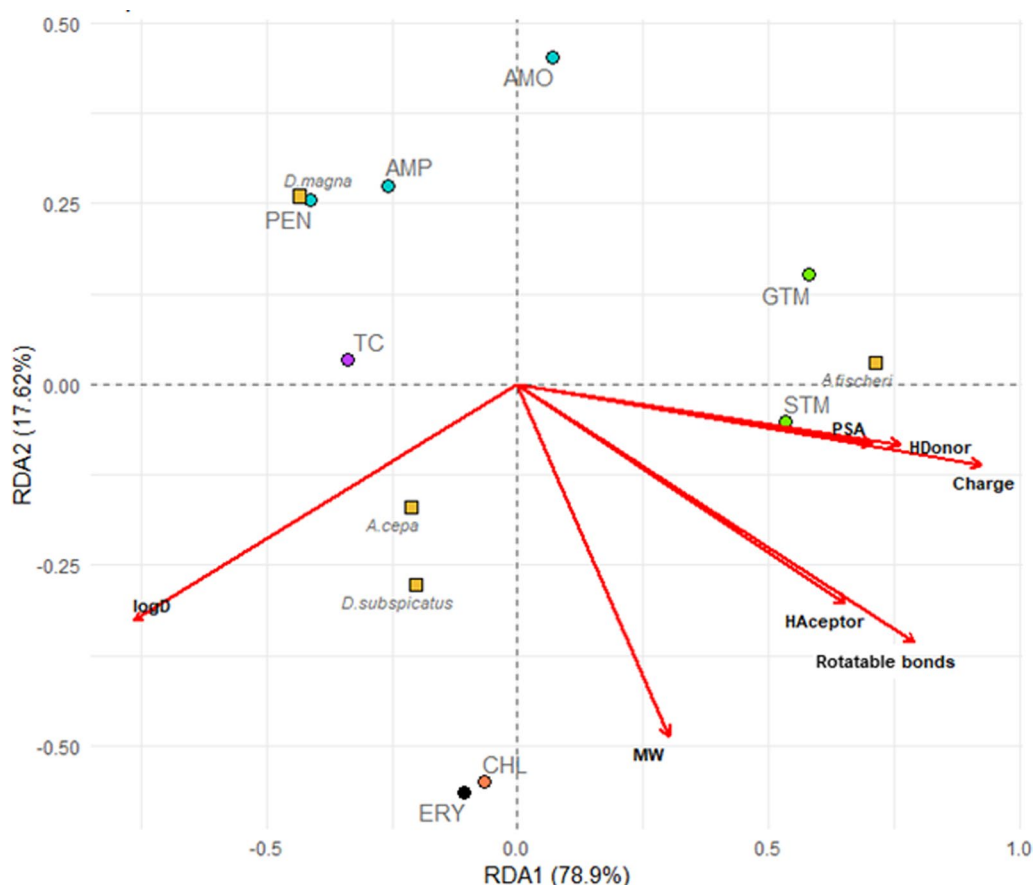


Fig. 6 Triplot of the RDA, dots represent the antibiotics (same colour means the antibiotics belong to the same family), yellow squares the organisms and red arrows the explanatory variables, that is, the physico-chemical properties

Redundancy analysis

To further investigate the differences in ecotoxicity observed among the tested ABXs, a RDA was performed to explore how selected physico-chemical properties of ABXs influence their toxicity profiles across test organisms, as determined by EC_{50} values. Figure 6 shows the triplot of the RDA, blue dots represent the ABXs, yellow squares the organisms and red arrows the explanatory variables, that is, the physico-chemical properties. As can be seen in Fig. 6, the 96.52% of the variance of the model was explained by the two first components (78.9% and 17.62%, respectively) reflecting that the selected physico-chemical properties effectively describe the behaviour of the observed EC_{50} values.

On the one hand, the relative position of organisms and ABXs reflect the influence of the ABX on the EC_{50} of the organism. As EC_{50} is inversely related to toxicity, a shorter distance between an ABX and an organism indicates a higher EC_{50} value and thus a lower toxic effect. Accordingly, *A. fischeri* exhibited higher EC_{50} values (i.e., lower sensitivity) in response to ABXs GTM and STM.

Similarly, *D. magna* was less affected by PEN and AMP, suggesting reduced susceptibility to these compounds. The remaining organisms, *A. cepa* and *D. subspicatus*, showed a more homogeneous response to all ABXs.

On the other hand, the effect of physico-chemical characteristics in the ability of the ABXs to produce the observed EC_{50} in the organisms could be clustered into four different groups (as many as the directions where the red arrows are placed). The correlation between the property and the ABX was determined by the angle of the arrow, meanwhile the length of the arrow reflects the strength of the effect. PSA, H-donors, and molecular charge exhibited strong positive correlations with GTM and STM, suggesting that these properties contribute to reduced toxicity (higher EC_{50} values). In contrast, PEN, AMP, and TC showed an opposite relationship, with these properties potentially enhancing their toxic effect. ABXs such as AMO, ERY, and CHL displayed little to no correlation with these variables, indicating that other factors may drive their bioactivity. Furthermore, H-acceptors and rotatable bonds showed variable influence: a

slight positive correlation was observed with GTM, STM, ERY, and CHL, whereas a negative correlation appeared with PEN, AMP, and TC. MW seemed to show a high influence on ERY and CHL ABXs in comparison to the rest of ABXs, because no correlation was found, as it was located nearly perpendicular to their positions. Lastly, the direction and length of the logD vector suggest that the hydrophilic nature of GTM and STM correlates with lower EC_{50} values.

To study the explanatory variables that could be more important in the described model and ANOVA analysis was performed. For that, based on the arrow length and distribution of the triplot, the model was simplified to be able to implement the *anova.cca* function. Therefore, a new model considering the response variables and only the physico-chemical properties: charge, rotatable bonds (these two were the longest arrows of their groups), logD and MW, as explanatory variables, was obtained. Results returned rotatable bonds as the most important physico-chemical characteristics on the performance of ABXs as it was found statistically significant for the model ($p=0.012$).

In summary, the redundancy analysis revealed that specific physicochemical properties are associated with the differential toxicity of ABXs across test species. Among these, the number of rotatable bonds emerged as the most statistically significant factor ($p=0.012$), likely due to its influence on molecular flexibility and, consequently, on the ability of ABXs to interact with cellular targets. Higher flexibility allows molecules to adopt conformations that facilitate membrane permeation and binding to intracellular structure.

Risk assessment evaluation

The HQ for each of the eight ABXs evaluated in this study is presented in Table 2. MEC data were acquired, for soil and water environments, from a wide variety of types of water and soil and locations all over the world from literature. From every location, we chose the highest value when different measurements were available. Soil and water PNEC values for the 8 ABXs were calculated according to Eq. (3). PNECs were obtained from EC_{50} (or LC_{50}) data for both acute and chronic toxicity tests. After obtaining the PNEC values, HQ was calculated applying Eq. (2). For AMP and STM no soil HQ could be calculated and for STM, the water HQ could not be calculated either because no MEC values were found in either environment.

Although most HQs for the ABXs assessed in this study were obtained using literature-derived data for both acute and chronic toxicity scenarios, it is important to highlight that experimentally determined EC_{50} or LC_{50} values from our study were also employed. Specifically,

EC_{50} values obtained from *A. fischeri* assays were used to determine acute HQs in aquatic environments for PEN, AMP and CHL. Likewise, EC_{50} values from *A. cepa* assays were used to calculate acute HQs in soil environments for AMO, GTM and TC, Table 2.

According to the reviewed literature, the ABXs with the highest number of acute and chronic ecotoxicity records in aquatic environments are AMO, ERY and TC, with 17, 24, and 13 records respectively. In soil environments, ERY and TC are the most frequently studied, with 11 and 13 records, respectively (see Table 2).

Given the variability in HQ values, Fig. 7 provides a visual synthesis of the proportion of risk categories (no, low, medium and high) reported for each antibiotic in both aquatic and soil compartments. These proportions were derived from literature-based MECs and PNECs, supplemented in some cases with experimentally determined EC_{50} values from this study (see Table 2). In aquatic environments (Fig. 7A and B), AMP consistently posed negligible risk, while AMO, although low risk in acute scenarios, was predominantly associated with high chronic risk. ERY and TC were among the compounds most frequently associated with high risk across both acute and chronic exposures. GTM showed moderate acute risk, and data gaps limited conclusions for STM.

In soil environments (Fig. 7C and D), risk profiles varied widely and were often limited by data gaps. AMO, ERY, and GTM consistently exhibited high risk based on available records. TC displayed mixed patterns: predominantly high risk in acute scenarios, but reduced risk under chronic exposure. CHL showed low to moderate risk, while PEN indicated high risk. No HQs could be calculated for AMP and STM due to insufficient ecotoxicity or exposure data. Importantly, soil PNEC values for AMO, ERY, TC, and CHL were not precedent from chronic toxicity EC_{50} data obtained directly from soil tests. Instead, these values were estimated using Eq. 4 [1], based on chronic aquatic PNEC values for the same ABXs and literature-derived soil organic carbon–water partition coefficient, as detailed in Table 2.

Comparing HQ values across different ABXs is a complex task, as this parameter is highly dependent on other variables such as the MEC, which exhibits substantial variability depending on the type of water or soil considered, and the EC_{50} or LD_{50} , which varies depending on the reference organism used for each ABX. Nonetheless, the highest HQ values were observed in wastewater samples [106, 108, 122–125, 130], with the exception of AMO, GTM, and TC, for which the highest HQ values were derived from river water samples collected in countries such as Iraq [119] and Brazil [114]. Regarding HQ values for ABXs in soil samples, the results were also consistent with expectations, as the highest values were

Table 2 Hazard quotient (HQ), measured environmental concentrations (MEC) and predicted no effect concentrations (PNE) for soil and water environments and for both acute and chronic toxicity tests for the 8 antibiotics

Water														
ABX	Type of water	MEC (µg/L)	Lowest EC ₅₀ acute tests (µg/L)	PNEC acute (µg/L)	HQ acute	Risk level if acute toxicity	Lowest EC ₅₀ chronic (µg/L)	PNEC chronic (µg/L)	HQ chronic	Risk level if chronic	Country	References MEC	References EC ₅₀ acute	References EC ₅₀ chronic
PEN	SW	0.003	84,290	84.29	3.56·10 ⁻⁵	No risk	6	0.06	0.0500	Low risk	Germany	[105]	A. fischeri 30 min, this study	M. aerugi- nosa 7d, [82]
	STPE+WWTP+SW	0.5033			0.0060	No risk			8.388	High risk	South- Africa	[106]		
	Drinking water	0.001			1.19·10 ⁻⁵	No risk			0.0167	Low risk	China	[107]		
	STPE	0.2			0.0024	No risk			3.333	High risk	Germany	[105]		
AMP	Drinking water	0.0001	363,520	363.52	2.75·10 ⁻⁷	No risk	163,000	1630	0.0000	No risk	China	[107]	A. fischeri 30 min, this study	A. fischeri 24 h, [36]
	WWTPI	1.805			0.0050	No risk			0.0011	No risk	Greece	[108]		
	WWTPE	0.0994			0.0003	No risk			0.0001	No risk	Several European countries	[109]		
AMO	SW	1.654	630,000	630	2.6·10 ⁻³	No risk	2.2	0.022	75.18	High risk	Turkey	[110]	P. subcapi- tata or S. capricornu- tum or R. subcapitata 3d [59]	Synechococ- cus sp 4d [60]
	River	0.9			1.4·10 ⁻³	No risk			40.91	High risk	Kenya	[111]		
	WWTPI	4.6			7.3·10 ⁻³	No risk			20.91	High risk				
	WWTPE	1.6			2.5·10 ⁻³	No risk			72.73	High risk				
	River	0.00991			1.5·10 ⁻⁵	No risk			45.04	High risk	Italy	[112]		
	WWTPI	0.018			2.9·10 ⁻⁵	No risk			81.82	High risk				
	River	0.01555			2.5·10 ⁻⁵	No risk			70.68	High risk	Iran	[113]		
	WWTPI	0.5863			9.3·10 ⁻⁴	No risk			26.65	High risk				
	WWTPE	0.09457			1.5·10 ⁻⁴	No risk			4.299	High risk				
	River	4.63			7.3·10 ⁻³	No risk			210.4	High risk	Brazil	[114]		
	River	0.021			3.3·10 ⁻⁵	No risk			0.9545	Medium risk	France	[115]		
	WWTPE	0.02			3.3·10 ⁻⁵	No risk			0.9091	Medium risk				
	WWTPI	1.27			2.0·10 ⁻³	No risk			57.72	High risk	Germany	[116]		
	WWTPE	0.187			3.0·10 ⁻⁴	No risk			8.500	High risk				
	WWTPI	0.087			1.4·10 ⁻⁴	No risk			3.954	High risk	UK	[117]		
	WWTPE	0.031			4.9·10 ⁻⁵	No risk			1.409	High risk	UK	[117]		
	SW		0.622			9.8·10 ⁻⁴	No risk			28.27	High risk	UK	[118]	

Table 2 (continued)

Water														
ABX	Type of water	MEC (µg/L)	Lowest EC ₅₀ acute tests (µg/L)	PNEC acute (µg/L)	HQ acute	Risk level if acute toxicity	Lowest EC ₅₀ chronic (µg/L)	PNEC chronic (µg/L)	HQ chronic	Risk level if chronic	Country	References MEC	References EC ₅₀ acute	References EC ₅₀ chronic
GTM	SW	12.5	19,200	19.2	0.6510	Medium risk	No chronic data available	No chronic data available	–	–	Iraq	[119]	<i>P. subcapitata</i> or <i>S. capricornutum</i> or <i>R. subcapitata</i> 3d, [62]	No chronic data available
STM	–	Not MEC data found	–	–	–	–	–	–	–	–	–	Not MEC data found	–	–
ERY	SW	1.7	20	0.02	85.00	High risk	22	0.22	7.727	High risk	Germany	[120]	<i>P. subcapitata</i> or <i>S. capricornutum</i> or <i>R. subcapitata</i> 3d, [24]	<i>Anabaena</i> spp. CPB4337 3d, [24]
	SW	0.131			6.550	High risk			0.5955	Medium risk	Turkey	[110]		
	SW	0.0295			1.475	High risk			0.1341	Medium risk	Germany	[120]		
	SW	0.00816			0.4080	Medium risk			0.0371	Low risk	USA	[121]		
STPE +WWTP + SW		0.14263			7.131	High risk			0.6483	Medium risk	South-Africa	[106]		
Drinking water		0.009			0.4500	Medium risk			0.0409	Low risk	China	[107]		
WWTPI		2.53			126.5	High risk			11.50	High risk	UK	[122–125]		
WWTPE		1.385			69.25	High risk			6.295	High risk				
SW		1.022			51.10	High risk			4.645	High risk				
River		0.00812			0.4060	Medium risk			0.0369	Low risk	Italy	[112]		
WWTPI		0.012			0.6000	Medium risk			0.0545	Low risk				
WWTPE		0.052			2.600	High risk			0.2364	Medium risk				
River		0.0069			0.3450	Medium risk			0.0314	Low risk	China	[126]		

Table 2 (continued)

Water														
ABX	Type of water	MEC (µg/L)	Lowest EC ₅₀ acute tests (µg/L)	PNEC acute (µg/L)	HQ acute	Risk level if acute toxicity	Lowest EC ₅₀ chronic (µg/L)	PNEC chronic (µg/L)	HQ chronic	Risk level if chronic	Country	References MEC	References EC ₅₀ acute	References EC ₅₀ chronic
TC	WWTP1	0.24864			12.43	High risk			1.130	High risk	Czech Republic	[127]		
	WWTPE	0.2042			10.21	High risk			0.9282	Medium risk				
	River	0.02065			1.032	High risk			0.0939	Low risk	China	[128]		
	WWTP1	0.25864			12.93	High risk			1.176	High risk				
	WWTPE	0.14821			7.410	High risk			0.6737	Medium risk				
	River	0.913			45.65	High risk			4.150	High risk	France	[115]		
	WWTPE	1.492			74.60	High risk			6.782	High risk				
	WWTP1	0.32			16.00	High risk			1.454	High risk	Greece	[108]		
	WWTPE	0.287			14.35	High risk			1.304	High risk	Germany	[129]		
	STPE	0.4			20.00	High risk			1.818	High risk	Germany	[130]		
	GW	0.01766			0.8830	Medium risk			0.0803	Low risk	USA	[121]		
	SW	43.57	310	0.31	140.5	High risk	25.1	0.251	173.6	High risk	Iraq	[119]	<i>P. subcapitata</i> or <i>S. capricornutum</i> or <i>R. subcapitata</i> 3d, [59]	<i>A. fischeri</i> 24 h, [41]
	SW	0.0225			0.0726	Low risk			0.0896	Low risk	China	[131]		
	Drinking water	0.0079			0.0255	Low risk			0.0315	Low risk	China	[107]		
	River	0.0783			0.2526	Medium risk			0.3120	Medium risk	China	[132]		
	River	0.00186			0.0060	No risk			0.0074	No risk	China	[126]		
	River	0.036			0.1161	Medium risk			0.1434	Medium risk	China	[128]		
	WWTP1	0.47864			1.544	High risk			1.907	High risk				
	WWTPE	0.02052			0.0662	Low risk			0.0818	Low risk				
River	0.009			0.0290	Low risk			0.0359	Low risk	France	[115]			
WWTPE	0.238			0.7677	Medium risk			0.9482	Medium risk					
STPE + WWTP + SW	1.75651			5.666	High risk			6.998	High risk	South-Africa	[106]			
WWTPE	0.2312			0.7458	Medium risk			0.9211	Medium risk	Several European countries	[109]			
GW	0.4			1.290	High risk			1.594	High risk	USA	[133]			

Table 2 (continued)

Water														
ABX	Type of water	MEC	Lowest EC ₅₀ acute tests	PNEC acute	HQ acute	Risk level if acute toxicity	Lowest EC ₅₀ chronic	PNEC chronic	HQ chronic	Risk level if chronic	Country	References MEC	References EC ₅₀ acute	References EC ₅₀ chronic
		(µg/L)	(µg/L)	(µg/L)			(µg/L)	(µg/L)						
CHL	SW	0.06	197,250	197.25	0.0003	No risk	64	0.64	0.0938	Low risk	Germany	[120]	<i>A. fischeri</i> 30 min, this study	<i>A. fischeri</i> 24 h, [41]
	Drinking water	0.0008			4.06·10 ⁻⁶	No risk			0.0013	No risk	China	[107]		
	WWTP1	0.248			0.0013	No risk			0.3875	Medium risk	UK	[117, 118, 123]		
	WWTPE	0.021			0.0001	No risk			0.0328	Low risk				
	SW	0.04			0.0002	No risk			0.0625	Low risk				
	STPE	190			0.9632	Medium risk			296.9	High risk	Germany	[130]		
	STPE	0.56			0.0028	No risk			0.8750	Medium risk	Germany	[120]		
Soil														
ABX	Type of soil	MEC	Lowest EC ₅₀ acute	PNEC acute	HQ Acute	Risk level if acute toxicity	Lowest EC ₅₀ chronic	PNEC chronic	HQ chronic	Risk level if chronic	Country	References MEC	References EC ₅₀ acute	References EC ₅₀ chronic
		(mg/kg)	(mg/kg)	(mg/kg)			(mg/kg)	(mg/kg)						
PEN	Soil (abandoned and active landfill)	120.52	> 300	0.2259	533.5	High risk	328	3.28	36.74	High risk	Ghana	[27]	No acute data found, PNEC data obtained as PNECacute in water-Koc. Koc obtained from [1]	<i>Folsomia candida</i> 28 d, data obtained [22]
AMP	–	Not MEC or Koc data found	No acute data available	No acute data available	–	–	No chronic data available	No chronic data available	–	–	–	Not MEC or Koc data found	No acute data available	No chronic data available
AMO	Pig sediment	74.00	45.19	0.04519	1637	High risk	No chronic data found for soil	0.0190	3894.7	High risk	Thailand	[80]	<i>A. cepa</i> 3d, this study	No chronic data found, PNEC data obtained as PNEC-chronic in water-Koc. Koc obtained from [1]
	Pig manure	3300.00			73,025	High risk			173,684.2	High risk				
	Soil (abandoned and active landfill)	76.62			1695	High risk			4032.6	High risk	Ghana	[27]		
	Vineyard crop soil	0.06			1.261	High risk			3.158	High risk	Spain	[134]		

Table 2 (continued)

Soil														
ABX	Type of soil	MEC (mg/kg)	Lowest EC ₅₀ acute (mg/kg)	PNEC acute (mg/kg)	HQ Acute	Risk level if acute toxicity	Lowest EC ₅₀ chronic (mg/kg)	PNEC chronic (mg/kg)	HQ chronic	Risk level if chronic	Country	References MEC	References EC ₅₀ acute	References EC ₅₀ chronic
	Sediment	0.0035			0.0328	Low risk			0.0001	No risk	China	[126]		
	Field fertilized with liquid manure	0.3074			2.884	High risk			0.0131	Low risk	Germany	[146]		
	Manure	0.117			1.098	High risk			0.0050	No risk	China	[141]		
	Manure	0.4			3.753	High risk			0.0171	Low risk	China	[14]		
	Pig farm sediment	300			2815	High risk			12.81	High risk	Thailand	[80]		
CHL	Soil with crops around	0.0223	102.34	0.10234	0.2179	Medium risk	No chronic data found	0.7392	0.0302	Low risk	China	[14]	A. cepa 3d, this study	No chronic data found, PNEC data obtained as PNEC-chronic in water;Koc. Koc obtained from [147]

PNEC data for PEN and ERY were obtained as follows: PNEC soil = PNEC water-Koc, where Koc is the soil organic carbon–water partition coefficient extracted from [1] and [147]
STPE Sewage treatment plant effluent, WWTP Wastewater treatment plant, SW surface water, GW ground water

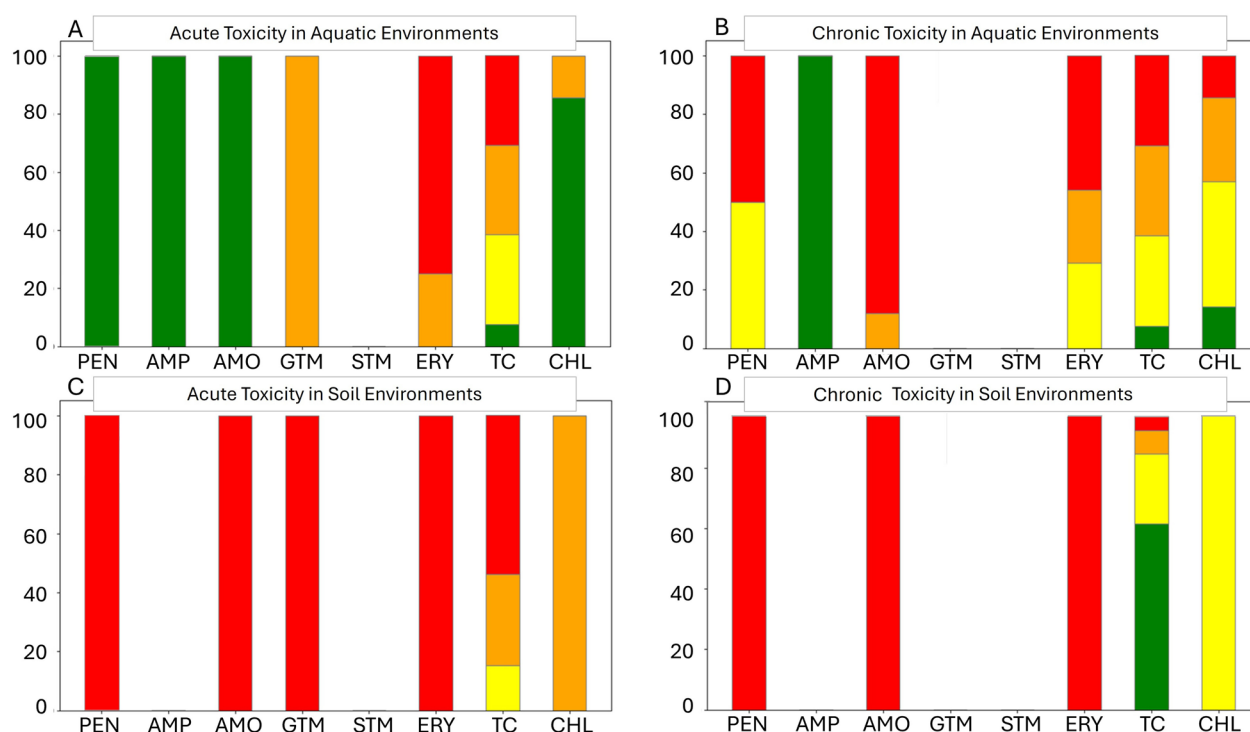


Fig. 7 Proportion of records (%) of HQ values calculated for soil and water, based on both acute and chronic toxicity data, classified as no risk (green), low risk (yellow), medium risk (orange), and high risk (red)

observed in soils collected from pig farms in countries such as Thailand [80].

In terms of environmental persistence and mobility, the degradation behaviour and adsorption properties of ABXs play a critical role in determining their risk potential. Many pharmaceuticals and personal care products show relatively long half-lives in water; for example, CHL requires approximately 60 days for 50% degradation [65]. However, penicillins are rarely detected in aquatic environments due to their high susceptibility to hydrolysis, driven by the instability of the β -lactam ring, which is rapidly cleaved by β -lactamases or via chemical degradation [148]. PEN is among the most unstable ABXs studied, as its degradation was generally around 50% at 4 °C and complete at 25 °C after a week. The degradation depends also on the pH [149], but it is reduced to a minimum when pH of the aqueous solution is set between 5 and 7 [22]. Thus, intact penicillins are usually not found in the environment [150]. This might be the reason why there are very few studies reporting ecotoxicity values for this ABX. AMP and AMO exhibit a quite similar decomposition pattern. After a week, around 80% of the total amount of both is unaltered at 25 °C. This proportion decreased to 60% for AMP and 70% for AMO after 2 weeks at the same temperature [149]. Other researchers analysed how pH influences this degradation proportion

of AMO. They found that after 16 days, only 30.9% of AMO was unmodified at buffer phosphate pH7 [151]. In contrast, aminoglycosides (GTM and STM) and TC are more persistent in water. GTM is thermally stable and maintains its activity even post-autoclaving [152]. STM displays a half-life of nearly 60 days at 20 °C and pH=7.04 [153]. ERY degrades in aqueous solution following first-order kinetics dependent on pH. It is particularly unstable at pH<5.5 or >10.0, regardless of the nature of its salt [154]. The amount of unaltered TC in water was studied by Honnorat-Benabbou et al. They found that after 3 days at 37 °C, 88% of the total amount of TC remained unaltered, while this value is reduced to 70% after 10 days [155].

The bioavailability of ABXs in soil, defined as the fraction accessible for uptake by organisms, is a major determinant of their ecotoxicological impact and is governed by their adsorption behaviour. This bioavailable fraction depends not only on total concentration, but on how ABXs interact with soil matrices, governed by their physicochemical properties (e.g., water solubility, pKa, LogD) and key soil factors such as pH, organic matter content, and cation exchange capacity [1, 156]. In broad terms, the more hydrophilic an ABX (lower LogD), the more it tends to remain dissolved in soil pore water and move with water flow, unless it carries positive charges that enable

strong electrostatic adsorption to negatively charged clay and organic matter [1, 156]. More lipophilic ABXs (higher LogD) are typically retained in the solid phase via hydrophobic interactions, especially with soil organic matter. However, ionization status, shaped by compound pKa values and soil pH, can override simple hydrophobicity. Conversely, moderately hydrophobic ABXs that are anionic at neutral pH may be mobile due to charge repulsion. Soil organic matter enhances sorption of neutral and hydrophobic ABXs and provides pH-dependent charge sites that bind cationic drugs [157, 158].

ABXs studied in this work differ widely in their structure, ionization state, and polarity, which strongly affect their adsorption to soil and resulting mobility. Aminoglycosides (GTM and STM) are highly hydrophilic and strongly cationic at environmental pH, these ABXs exhibit strong adsorption to negatively charged clay and organic matter via cation exchange. Their mobility and bioavailability are low, and they degrade relatively quickly in soil [159, 160]. ERY is moderately lipophilic (LogD ~ 0.6, Table S2) and cationic at neutral pH (Table S2). It shows moderate sorption through a combination of electrostatic and hydrophobic interactions. It is less mobile than aminoglycosides but more bioavailable [157]. TC is amphoteric with multiple ionizable groups and strong chelating ability and it adsorbs very strongly in soil via complex mechanisms including metal bridging and cation exchange. Despite its polarity, its mobility is extremely low and its persistence high, leading to limited bioavailability but long-term presence in soils [161]. CHL is a neutral and moderately polar (LogD ~ 0.9, Table S2) molecule at soil pH. CHL adsorbs weakly and remains largely in soil pore water, this likely contributes to the higher toxicity observed in *E. fetida* in our study [157]. β -lactams (AMO, AMP and PEN) are all anionic compounds at pH7 with low LogD values (-2.5 to -2.2, Table S2), resulting in minimal sorption and high mobility in soil. However, they are highly unstable and degrade rapidly via hydrolysis and microbial breakdown, limiting their persistence and long-term exposure risks [156].

In summary, strongly adsorbed ABXs such as TC and aminoglycosides show low bioavailability and mobility but can persist longer in soil, whereas mobile, weakly adsorbed ABXs like β -lactams and CHL are more bioavailable in the short term but may be quickly degraded or leached. These dynamics directly influence the likelihood and severity of toxic effects in soil organisms, like *E. fetida*, and must be considered when assessing ABX in terrestrial ecosystems.

When considered together, the ecotoxicological data, RDA output, and environmental risk profiles enable a compound-specific diagnosis of the environmental

relevance of the antibiotics studied. Starting with the β -lactam antibiotics (PEN, AMP, and AMO), these compounds consistently exhibited low toxicity across aquatic and terrestrial test organisms ($EC_{50} > 100$ mg/L in most assays) and were generally associated with no risk in acute water toxicity scenarios and low to medium risk in chronic aquatic scenarios. However, in soil, the limited data available suggest high risk, likely due to their high mobility and bioavailability in this matrix. Despite their potential for environmental exposure, their rapid degradation via hydrolysis and microbial activity, driven by the instability of the β -lactam ring, reduces the likelihood of sustained toxicity under realistic conditions. The RDA further supports that, based on their physicochemical profiles, these compounds are not expected to exhibit marked toxicity against any of the tested bioindicators, for instance, it is reflected by their position in the RDA plot showing minimal toxicity to *D. magna*. This relative “innocuity” appears to be negatively correlated with the number of hydrogen bond acceptors and rotatable bonds, parameters for which the β -lactam antibiotics exhibited some of the lowest values among all compounds analyzed in this study.

Aminoglycosides (GTM and STM) present a more complex profile. While both displayed low to moderate toxicity overall – being the least toxic to *A. fischeri* – the absence of environmental concentration data for STM and chronic data for GTM limits definitive risk assessment. GTM, however, showed medium to high HQ values under acute scenarios, contradicting its observed low ecotoxicity. The RDA also indicates that, based on their physicochemical characteristics, these compounds are expected to exhibit negligible toxicity toward *A. fischeri* and only mild toxicity to *A. cepa* and *D. subspicatus*. Their toxicological profiles appear to be primarily correlated to LogD, and inversely associated with PSA, hydrogen bond donors, and molecular charge. These features typically limit diffusion across biological membranes and reduce cellular uptake, thereby correlating with higher EC_{50} values (i.e., lower toxicity) for these two antibiotics. Despite their high hydrophilicity, which could promote bioactivity, their strong ionic character likely limits effective cellular uptake, thereby reducing overall toxicity.

For the macrolide ERY, EC_{50} values were among the lowest across the test species, compared with the other ABXs in this study. Consistently, its HQ values were elevated in both soil and aquatic compartments, under acute and chronic exposure scenarios. ERY's moderate lipophilicity (LogD $\approx$ 0.6) and cationic nature at neutral pH suggest enhanced bioavailability, particularly in aqueous media. In the RDA triplot, ERY was positioned close to vectors representing MW and rotatable bonds, while showing no significant correlation with charge, hydrogen

bond donors, or PSA. This particular combination of physicochemical attributes, moderate mobility, reduced environmental dispersion, and relatively high bioavailability, may account for both its persistent environmental presence and the consistently elevated risk values observed. CHL showed a profile closely resembling ERY, both in its RDA positioning and ecotoxicological behaviour. CHL was among the most toxic antibiotics tested and appeared distant from bioindicator vectors in the RDA, reinforcing its generalized ecotoxicity. It exhibited the full range of risk levels across the four evaluated scenarios, likely due to variability in reported MEC values. As a neutral and moderately polar compound ($\log D \sim 0.9$), CHL remains bioavailable and persistent, especially in pore waters, which likely contributes to its pronounced toxicity in soil organisms like *E. fetida* and *A. cepa*.

TC showed intermediate toxicity, with EC_{50} values between 100 and 400 mg/L. Its HQs were highly variable, reflecting widespread environmental occurrence and a broad range of MEC values across geographic regions. TC is a molecule known to exhibit strong sorption to soil matrices, a characteristic that typically reduces its bioavailability in terrestrial environments. This is corroborated by the RDA, where its reduced toxicity is positively correlated only with the physicochemical descriptor LogD. In contrast, other properties, namely molecular charge, number of hydrogen bond donors and acceptors, PSA, and rotatable bonds, were all negatively associated with EC_{50} values, suggesting their potential contribution to TC's residual toxicity. These results highlight that even antibiotics with limited mobility due to strong sorption can exert chronic ecotoxicological effects, reinforcing the classification of TC as a compound of environmental concern.

Taken together, these findings demonstrate that the interplay between physicochemical properties, environmental exposure, and biological sensitivity must be considered to fully understand the ecological impact of antibiotics. While some compounds (e.g., ERY, CHL, TC) align across toxicity, risk, and bioavailability variables, others (e.g., GTM, STM, AMO) diverge, highlighting the need for compound-specific and matrix-specific risk frameworks.

Effects of antibiotics on ecosystems and biodiversity

Our ecotoxicity assays using non-target organisms were designed to cover different trophic levels and environmental compartments, allowing a broader understanding of the potential ecosystem-scale impacts of the tested antibiotics.

Aquatic ecosystems

In aquatic primary producers such as microalgae, which form the base of freshwater food webs, all antibiotics displayed low acute toxicity according to ECHA classification ($EC_{50} > 100$ mg/L) [33]. β -lactams (PEN, AMO, AMP) produced no detectable acute effects, whereas some compounds induced sublethal responses, including growth stimulation (hormesis) for TC, ERY and STM, and clear inhibition in the case of GTM. Studies on natural riverine microbial communities report similar patterns: overall low acute toxicity but pronounced sublethal alterations in population growth and metabolic activity at concentrations around 100 μ g/mL, particularly for antibiotics targeting prokaryotic 70S ribosomes (e.g., CHL, ERY, GTM). These responses often include reduced utilization of polymers, carbohydrates and organic acids, and mild hormetic stimulation at very low concentrations [162].

Although these effects are not apparent in single-species assays such as *A. fischeri*, they become ecologically relevant in complex communities. Sublethal antibiotic exposure can alter species succession, diversity and competitive balance, favouring tolerant taxa over sensitive ones [163]. This may lead to shifts in phytoplankton composition, including reductions in diatoms and cyanobacteria and relative increases in green algae [162, 164]. Functional consequences include impaired nitrogen fixation by diazotrophic cyanobacteria, reduced nitrification and denitrification, and modified heterotrophic decomposition, ultimately disrupting key biogeochemical processes [165]. Such structural and functional changes propagate through food webs: zooplankton such as *D. magna* may be indirectly affected by altered prey availability, and antibiotic-driven disruption of bacteria–phytoplankton symbioses can promote the dominance of tolerant or harmful taxa such as *Microcystis spp.*, increasing the risk of harmful algal blooms [166].

Integration of our results with literature-derived MEC–PNEC data showed that ERY and TC pose the highest acute aquatic risk, CHL and GTM moderate risk, while other compounds present negligible acute hazard. However, the scenario changes under chronic exposure: except for AMP (and GTM/STM where data were scarce), all antibiotics exhibited high chronic risk, highlighting that sublethal effects arise at concentrations far below those causing mortality [167]. Chronic exposure to sub-inhibitory levels leads to progressive, cumulative and often irreversible ecological alterations, driven by continuous inputs from WWTP effluents, hospital wastewater and agricultural runoff. These exposures erode functional redundancy, eliminate sensitive taxa, facilitate antibiotic-resistance development, and reduce ecosystem resilience [168].

Chronic antibiotic effects persist across generations: even without overt lethality, fecundity, gamete viability and larval survival decrease over time, and epigenetic modifications may alter immune responses and stress tolerance [169, 170]. Community restructuring can become permanent, as chronic exposure causes the loss of functionally important taxa, reduction of diversity, and homogenization of microbial assemblages. Diminished functional redundancy increases vulnerability to secondary disturbances. Moreover, chronic exposure disproportionately affects keystone species and destabilizes bacteria–primary producer mutualisms, enabling alternative stable states dominated by resistant cyanobacteria [171]. Prolonged exposures also enhance synergistic effects with co-occurring pollutants and strongly promote the selection and horizontal transfer of antibiotic-resistance genes, even at concentrations as low as 2–6% of the MIC [172].

Terrestrial ecosystems

In soil bioindicators, we observed minimal acute toxicity: *E. fetida* showed no mortality, and *A. cepa* displayed only mild inhibition for AMO and GTM, with negligible effects for CHL and TC. However, studies on soil microbial communities using the same antibiotics reveal substantial sublethal impacts at ~100 µg/mL, including reduced microbial growth and altered metabolic profiles, particularly affecting the degradation of amino acids, carboxylic acids and polymers [103]. Because soil microbiomes possess lower functional redundancy than aquatic communities, these alterations can be more pronounced and ecologically consequential [173].

This contrast, absence of acute toxicity in macro-organisms versus strong sublethal impacts on microbial communities, has major implications for terrestrial ecosystems. Although earthworms survive antibiotic exposure at environmentally relevant concentrations, numerous studies report oxidative stress, enzyme disruption, cytoskeletal damage and shifts in gut microbiota, which may impair essential functions such as bioturbation, organic-matter decomposition and nutrient cycling [88, 174, 175]. Similarly, *A. cepa* may show limited growth inhibition while still exhibiting chromosomal aberrations, mitotic disturbances and oxidative DNA damage [176].

Soil microbial communities show EC₅₀ values 10–30 times lower than those for *E. fetida*, indicating far higher sensitivity and a strong likelihood of ecosystem-level effects [177]. Antibiotics tend to exert more persistent effects in soils than in aquatic systems due to slower degradation (DT₅₀ of weeks to months), strong

sorption to organic matter and continuous release from manure and biosolids applications. These factors result in chronic, steady-state exposures that prevent microbial recovery and lead to progressive erosion of microbial functional diversity, reductions in nutrient recycling (N, C, P), impaired soil formation and decreased plant growth support.

Consequently, the absence of acute lethality in *E. fetida* and *A. cepa* does not imply ecological safety. The high chronic risk observed for all antibiotics with available data indicates that terrestrial antibiotic ecotoxicity operates primarily through sublethal, community-level and functional pathways, not through direct mortality. These mechanisms represent a persistent and often irreversible threat to soil ecosystem functioning and resilience.

Conclusions

In conclusion, the present study assessed the ecotoxicity and environmental risks associated with eight commonly used ABXs (AMP, PEN, AMO, CHL, ERY, GTM, STM and TC) across five representative bioindicators from water and soil. The results reveal significant variability in toxicity profiles influenced by ABX class and environmental matrix. ABXs with higher persistence and bioavailability, such as ERY, CHL, and TC, presented greater ecological risks. In contrast, antibiotics like β-lactams and aminoglycosides showed lower acute toxicity and environmental risk. Redundancy analysis revealed that molecular flexibility, indicated by the number of rotatable bonds, significantly influenced antibiotic toxicity across species. Hazard quotient analyses highlighted ERY, TC, and AMO as posing the greatest environmental risks under various scenarios. Collectively, these findings emphasize the importance of considering physicochemical properties, environmental fate, and organism-specific sensitivities in ABX risk assessments to support targeted environmental management and regulatory strategies.

Moreover, the apparent disconnect between the low toxicity detected in individual non-target organisms and the high environmental risk estimated for these antibiotics highlights that their true ecological threat stems from the progressive and potentially irreversible disruption of entire ecosystems through sublethal impacts on microbial communities and their functional networks, rather than from organism-level lethality. Such degradation of ecosystem resilience, biodiversity and functional services unfolds subtly and cumulatively under chronic exposure, ultimately undermining the capacity of ecosystems to sustain their structure, function and ability to recover from disturbance.

Abbreviations

ABX Antibiotics

AF	Assessment factor
AMO	Amoxicillin
AMP	Ampicillin
ARG	Antibiotic resistance genes
CHL	Chloramphenicol
D	Distribution coefficient
EC	Effective dose
ERY	Erythromycin
GTM	Gentamicin
HQ	Hazard quotient
Koc	Water-organic matter partition coefficient
LD	Lethal dose
MDR	Multidrug-resistant
MEC	Measured environmental concentration
MW	Molecular weight
OECD	Organization for the economic cooperation and development
PEN	Penicillin G
PNEC	Predicted no-effect concentration
PSA	Polar surface area
RDA	Redundancy analysis
STM	Streptomycin
TC	Tetracycline
WHO	World health organization

Supplementary Information

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Supplementary material 1

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Author contributions

****CG:**** Investigation, Validation, Data curation, Writing original draft, Writing—Review & Editing, Methodology, Formal analysis, Visualization. ****MRP-O:**** Conceptualization, Writing—Review Editing, Methodology, Supervision, Validation. ****BR:**** Investigation, Data curation, Methodology. ****DB:**** Project administration, Funding acquisition, Methodology, Validation, Resources. ****EL:**** Writing original draft, Writing—Review and Editing, Supervision, Methodology, Conceptualization, Visualization.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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