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Current Research on Soil Science and Related Aspects of Environmental Sciences in Galicia

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Current Research on Soil Science and Related Aspects of Environmental Sciences in Galicia

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With this Special Issue, the Spanish Journal of Soil Science starts a series of regionally- and/or country-focused Special Issues. In this Special Issue, Galician researchers, and/or researchers from any country working on Soil Science (and/or related aspects of Environmental Sciences) dealing with Galicia have published manuscripts.

This geographic area shares borders with Portugal, the Spanish Autonomous Communities of Castilla-León, and Asturias, and the Atlantic Ocean, and has a marked diversity in geology, climate, geomorphology, landscapes, soils, forests, crops, fauna, and the whole environment, as well as specific social, cultural, industrial and economy aspects.

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EDITORIAL
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Editorial: Current Research on Soil Science and Related Aspects of Environmental Sciences in Galicia

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Keywords: editorial, current research, soil science, environmental sciences, Galicia

Editorial on the Special Issue

Current Research on Soil Science and Related Aspects of Environmental Sciences in Galicia

As recently as under 200 years ago, soil was almost unknown as subject of scientific study, and its status was far from being part of the disciplines taught at universities (Brevik, 2005; Brevik and Hartemink, 2010; Díaz-Raviña and Caruncho, 2022; Reyes-Sánchez and Irazoque, 2022). Nowadays, however, Soil Science is a consolidated field of knowledge, with clear recognition at academic, scientific, and technical levels. In Spain, the Spanish Society of Soil Science (*Sociedad Española de la Ciencia del Suelo*, SECS) has reached 75 years of history, while in Galicia, Soil Science is commemorating 70 years. Throughout all this time, many professors and researchers have contributed their dedication and efforts to promote Soil Science in Spain and Galicia. Among them, Dr. E. Huguet del Villar, Dr. J. M. Alvareda, Dr. F. Guitián Ojea, and many others who followed them and who unfortunately are no longer with us. Those of us who continue to work in this field, as heirs to their legacy, will try to maintain and transmit their in-depth knowledge and impression to the next generations.

The first studies on the soils of Galicia date back to the 1930s, carried out by Huguet del Villar, with his publications on the types of soils in Galicia (Huguet del Villar, 1935) and the Soil Map of the Lusitano-Iberian Peninsula (Huguet del Villar, 1938). After various other studies that followed those original ones, more recently several books were also published on the types of soils in Galicia, such as the one focusing on Soils in A Coruña province (Macías and Calvo, 1992), a chapter on Galician soils (Macías and Calvo, 2001), or the review on the soils of a wider area (the temperate-humid zone of Spain) carried out by Carballas et al. (2016).

Soil has played a crucial role throughout human history. Regarding Soil Science, the knowledge generated by soil scientists has contributed to alleviating hunger in many parts of the world, has favored the restoration of degraded ecosystems, and currently helps us to face new challenges, such as the fight against global warming or the eutrophication of surface waters. However, we must also bear in mind that many soil scientists have the perception that soil and Soil Science do not receive the same social recognition as other related areas. For this reason, while advancing in scientific knowledge, we should also move ahead in the dissemination of our science. In this sense, the present SI of the SJSS aims to collect the results of research lines that address relevant and/or novel aspects related to Galician soils and other environmental compartments, ranging from the incidence of antibiotics in agricultural soils to the quantification of Blue C in *Zostera* soils in the Ría of Ferrol, among others. In fact, we take into account that, as artistic shows do, Science must go on and must be continuously updated and fed with the latest findings. The Editors of this Special Issue hope that all the papers included in it will contribute to showing what could be seen as a kind of interesting and stimulating performance focused on hot topics in Soil and Environmental Sciences, which take place in this geographic area, but are also relevant at a global level.

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Specifically, the paper by Barreiro et al. focused on quantifying a variety of antibiotics present in sewage sludge, soils, corn, and grape samples after the spreading of sludge, whereas the one by de la Cerda-Marin et al. dealt with the study of blue carbon stock in *Zostera marina* meadows in the Ría of Ferrol. In turn, the study by Chaves and Monterroso reported details on the agricultural use of pesticides in Galicia during the 20th century, providing an inventory of active substances and their geographic distribution, while the research by Gómez-Armesto et al. was directed towards determining mercury content and pools in complex polycyclic soils from a mountainous area in Galicia. López-Mateo et al. investigated toxic and trace metals in grassland soils of Galicia, while the work by Rodríguez-Blanco et al. was about "*Nitrate and Phosphorus Transport in a Galician River (NW Iberian Peninsula): Insights From Fourteen Years of Monitoring*." Rodríguez-González et al. carried out a study on microbial communities affected by clarithromycin addition in four acid soils, whereas Rodríguez-López et al. investigated the "*Influence of pH, Humic Acids, and Salts on the Dissipation of Amoxicillin and Azithromycin under Simulated Sunlight*." Finally, the paper by Santás-Miguel et al. studied "*Soil Bacterial Community Tolerance to Three Tetracycline Antibiotics Induced by Ni and Zn*".

We really hope that this SI will contribute to stimulating further research on Soil and Environmental Sciences in Galicia, in Spain, and globally, thus continuing the work of our predecessors. We are confident that present and future researchers will be able to reap what these key soil scientists have sowed. To end this editorial piece, just a wish for all of us: May Science be with you!

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

Brevik, E. C., and Hartemink, A. E. (2010). "History, Philosophy, and Sociology of Soil Science," in *Soils, Plant Growth and Crop Production. Encyclopedia of Life Support Systems (EOLSS), Developed under the Auspices of the UNESCO.* Editor W. Verheye (Oxford, UK: EOLSS Publishers). Available at: http://www.eolss.netUH.

Brevik, E. C. (2005). "A Brief History of Soil Science," in *Global Sustainable Development, Theme 1.05: Land Use and Cover*. Editor W. Verheye (Oxford, UK: EOLSS Publishers). Available at: http://www.eolss.net.

Carballas, T., Rodríguez-Rastrero, M., Artieda, O., Gumuzzio, J., Díaz-Raviña, M., and Martín, A. (2016). "Soils of the Temperate Humid Zone," in *The Soils of Spain. World Soils Book Series*. Editor J. F. Gallardo (Berlin, Germany: Springer), 49–144. doi:10.1007/978-3-319-20541-0_3

Díaz-Raviña, M., and Caruncho, C. (2022). A Brief Analysis of the Contribution of Women to Soil Science. *Span. J. Soil Sci.* 12. doi:10.3389/sjss.2022.10658

Huguet del Villar, E. (1935). *Los tipos de suelo de Galicia. Las Ciencias, año II, no 4*, 970–1000.

Huguet del Villar, E. (1938). *Soils of the Lusitano-Iberian Peninsula (Spain & Portugal)*. London: Thomas Murby & Co.

Macías, F., and Calvo, R. (1992). *Suelos de la provincia de La Coruña*. A Coruña: Diputación Provincial de A Coruña, 85.

Macías, F., and Calvo, R. (2001). "Atlas de Galicia. Los Suelos. Tomo I, 173-217," in *Atlas de Galicia. Tomo I*. Editors A. Precedo and J. Sancho (Sociedade para o Desenvolvemento Comarcal de Galicia), 50.

Reyes-Sánchez, L. B., and Irazoque, A. (2022). Reevaluating Diversity and the History of Women in Soil Science: A Necessary Step for a Real Change. *Span. J. Soil Sci.* 12. doi:10.3389/sjss.2022.10401

ORIGINAL RESEARCH
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Influence of pH, Humic Acids, and Salts on the Dissipation of Amoxicillin and Azithromycin Under Simulated Sunlight

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This work is focused on the study of the dissipation of the antibiotics amoxicillin (AMX) (an amino penicillin) and azithromycin (AZT) (belonging to the macrolide group), performed at a laboratory scale, under simulated sunlight and in the dark, at pH values 4.0, 5.5, and 7.2, and in the presence of humic acids and different inorganic salts. The results indicate that AMX is more affected than AZT by simulated sunlight, with half-lives ranging 7.7–9.9 h for AMX and 250–456 h for AZT. The lowest half-life values were obtained at pH 7.2 for AMX (7.7 h) and at pH 4.0 for AZT (250 h). Regarding the effect of various salts, the presence of $NaNO_3$ causes that C/C_0 decreases from 0.6 to 0 under simulated sunlight, having no effect on the dissipation of AMX in the dark. However, in the presence of $FeCl_3$ at concentrations of 500 mg L^{-1}, AMX suffered total dissipation, both under simulated sunlight and in the dark. For AZT the influence was lower, and the salts that caused a higher increase in its dissipation were NaCl (with C/C_0 decreasing from 0.5 to 0.2) and $CaCl_2$ (C/C_0 decreasing from 0.5 to 0.3). The presence of humic acids caused a slight increase in the dissipation of AMX, both under simulated sunlight and in the dark, a fact that was attributed to the adsorption of the antibiotic onto these organic substances, which, however, caused a more marked increase in the dissipation of AZT (reaching 68%) under simulated sunlight. The overall results of this research can be considered clearly relevant, mainly to determine the fate of AMX and AZT when these antibiotics reach the environment as pollutants, either as regards their exposure to natural sunlight, or in relation to the use of inactivation/photo-degradation systems in decontamination procedures focused on environmental compartments.

Keywords: amoxicillin, azithromycin, humic acids, inorganic salts, photo-degradation

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INTRODUCTION

Antibiotics reaching environmental compartments are considered emerging pollutants, and its presence in soils can cause a variety of public health and environmental issues (Tello et al., 2012; Manyi-Loh et al., 2018; Iwu et al., 2020; Serwecińska, 2020). The passage of antibiotics from soils to surface-, subsurface- and ground-water (and its direct arrival to riverbeds through wastewater), as well as their entry into the food chain through crops or aquatic organisms, is considered a particularly relevant risk (Zhao et al., 2019; Kumar et al., 2020; Muhammad et al., 2020; Hassan et al., 2021). But a particular aspect, specific to the soil, is the potential harmful effects of antibiotics on edaphic microorganisms (Brandt et al., 2015; Cycoń et al., 2019; Santás-Miguel et al., 2020), both due to the direct toxicity on these microorganisms, and to the development of pathogenic antibiotic resistant bacteria (Forsberg et al., 2012; Cycoń et al., 2019). In this regard, as indicated in Salam and Obayori (2019), antibiotic resistance is common in the environment, and the meta-genomic approach has recently shown its increasing relevance, mainly in cultivated soils and environmental compartments affected by anthropogenic activities.

One of the main routes for antibiotics to reach soils and the overall environment is through wastewater and sewage sludge spreading, and since many treatment plants (WWTPs) have low efficiency in removing these compounds (Wang and Wang., 2016; Yang et al., 2016), different additional methods have been tested to inactivate/remove them, among which oxidation processes, adsorption or biological treatments stand out (Han et al., 2019; Han et al., 2021; Nippes et al., 2021). This low purification efficiency reached in WWTPs, is one of the reasons why relevant concentrations of antibiotics appear in both surface and groundwater, and even in drinking water (Kemper, 2008).

Once these antibiotics reach watercourses, their degrees of susceptibility to photo-degradation are of main importance to establishing their persistence, as occurs in soils and other environmental compartments (Cycoń et al., 2019; Nnadozie and Odume, 2019; Arun et al., 2020). This susceptibility to photo-degradation can be studied in the laboratory under simulated sunlight, allowing to determine the persistence of the antibiotics in the environment (particularly in aqueous media), which affects their probability of generating environmental and public health issues (Bilal et al., 2020; Chaturvedi et al., 2021).

Given the complexity and importance of this problem, it would be clearly interesting to obtain new experimental data regarding the degradation of antibiotics that takes place in aqueous media (which could correspond to watercourses, but could also be representative of the soil solution). In a latter step, this would help in modeling the behavior of these compounds in a variety of environmental situations, allowing to predict and fight related pollution and public health issues (Rico et al., 2019; Su et al., 2019; Tang et al., 2019; Khan et al., 2020; Bavumiragira et al., 2022).

In addition, this kind of information on photo-degradation, obtained through laboratory experiments, could be useful to find the most favorable conditions promoting the removal/inactivation of antibiotics present in wastewater, which could be studied in later phases, at pilot scale, before entering the real world scale if justified (Liu et al., 2018; Almansba et al., 2021; Silva et al., 2021).

With the above background in mind, this work was conceived to study the laboratory photo-degradation of two antibiotics: amoxicillin (AMX), a semi-synthetic derivative of penicillin that is among the most widely used antibiotic substances, and has been detected in waters of different countries (Tran et al., 2019; Sodhi et al., 2021; Yang et al., 2021), and azithromycin (AZT), a macrolide which was also detected in different water samples (Yi et al., 2019; Yang et al., 2021). This is the first study on the photo-degradation of both antibiotics in aqueous media in which the pH is modified, as well as the concentrations of different salts and humic acids. The results of this research could shed light on these processes, which are highly relevant at the level of different environmental compartments, public health and the overall environment.

MATERIALS AND METHODS

Chemicals and Reagents

The antibiotics used were Amoxicillin tri-hydrate (AMX) with 95% purity, and Azithromycin (AZT), with 98% purity, both from by Sigma Aldrich (Barcelona, Spain). MilliQ water was used to prepare the AMX solution (at 50 μM), while AZT was prepared at 5 mM in 96% ethanol, and from this a 50 μM concentration was obtained by dilution with MilliQ water. All the reagents used for HPLC analyses (acetonitrile, phosphoric acid and monopotassium phosphate), and the different inorganic salts used, were of high purity analytical grade, supplied by Sigma-Aldrich (Barcelona, Spain). The most relevant physicochemical characteristics of both antibiotics are presented in **Table 1**.

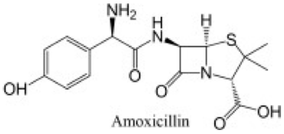
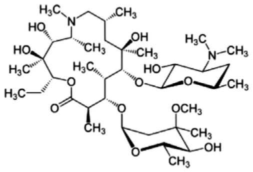
Antibiotics Quantification

The equipment used for the quantification of the antibiotics was an UltiMate 3000 HPLC (Thermo Fisher Scientific, United States), with a quaternary pump, an auto-sampler, a thermosstatted column compartment and an UltiMate 3000 series ultraviolet-visible detector. This equipment contains a computer with version 7 of the Chromeleon software (Thermo Fisher Scientific, United States). Chromatographic separations were performed on a Luna C18 analytical column (150 mm long; 4.6 mm internal diameter; 5 μm particle size) from Phenomenex (Madrid, Spain), and a security column (4 mm long; 3 mm internal diameter; 5 μm particle size), packed with the same material as the column.

As the quantification limits were 0.01 μM for AMX and 0.5 μM for AZT, the injection volumes were lower for the former (50 μL), while it was 200 μL for AZT. The flow rates were 1.5 ml min^{-1} for AMX and 1 ml min^{-1} for AZT. The temperature was kept constant at 25 ± 1°C throughout the analysis. Finally, it should be noted that between each measurement, the syringe was washed with a methanol:water solution (ratio 50:50).

The conditions used to achieve the separation of the antibiotic AZT were the following: the mobile phase was formed by acetonitrile (phase A), and by monopotassium phosphate 0.025 M (phase B). The linear gradient elution program was executed from 5 to 70% for phase A and from 95 to 30% for phase B with a time of 18 min. The initial conditions were restored in 2 min and held for 3 min. The total time for analysis was 25 min, with a retention time of 9.7 min, and the wavelength used for detection was 210 nm.

TABLE 1 | Properties of the antibiotics studied.

Common name	Chemical structure	Chemical formula	Molecular weight ($g\ mol^{-1}$)	Log K_{OW}	pK_a	Water solubility ($mg\ L^{-1}$)
Amoxicillin[a]		$C_{16}H_{19}N_3O_5S$	365.4	0.87	3.23–7.22	3430
Azithromycin[b]		$C_{38}H_{72}N_2O_{12}$	749.0	4.02	12.53–9.57	2.37

[a]Solliec et al. (2016), Ghirardini et al. (2020).
[b]Ghirardini et al. (2020), Talaiekhozani et al. (2020).

AMX was separated under the following conditions: the mobile phase was acetonitrile (phase A) as for AZT, but with phase B being 0.01 M phosphoric acid (at pH = 2). The linear gradient elution program was executed from 5 to 15% for phase A and from 95 to 85% for phase B, within 4 min. The initial conditions were restored in 2 min. The total analysis time was 10 min, with a retention time of 3.3 min, and the wavelength used was 230 nm.

Additionally, in the aqueous solutions in which photo-degradation was studied, the total organic carbon (TOC) was determined at times of 0, 2, 16, and 72 h of exposure to light, to determine the degree of antibiotic mineralization. However, it was performed just for AMX, as AZT had been prepared in ethanol, as indicated above. Specifically, the quantification of TOC was carried out by means of a Multi N/C2100 equipment (Analytikjena, Jena, Germany).

Photo-Degradation Experiments

Influence of pH on AMX and AZT Photo-Degradation

The effect of pH on degradation was studied, both under simulated sunlight and in the dark, adjusting the solutions to three pH values (4.0, 5.5, and 7.0) with 0.5 M NaOH and 0.5 M HCl. The following conditions were used to carry out the study in the dark: Six (6) ml of the solution of each antibiotic (at concentrations of 50 μM) were placed in opaque glass EPA vials that were kept at a constant temperature (22 ± 2°C); the concentration of each antibiotic was measured at different times (between 0.1 and 72 h for AMX, and between 0.1 and 192 h for AZT) using the HPLC equipment. In the simulated sunlight experiments, the samples were placed in a Suntest CPS + Atlas cabinet (Germany) equipped with a 550 Wm^{-2} xenon lamp and quartz filters with a wavelength cut-off at 285 nm. The contact times were the same as those used in the experiments in the dark.

Effects of Inorganic Salts and Humic Acids on AMX and AZT Photo-Degradation

For these experiments, specific samples were prepared with a 50 μM concentration of each antibiotic and with different concentrations of each salt (0, 5, 10, 50, and 500 $mg\ L^{-1}$) and humic acids (0, 0.05, 0.1, 0.2, 0.5, 1, and 10 $mg\ L^{-1}$). The salts used were NaCl, $CaCl_2$, $NaNO_3$, Na_2HPO_4, and $FeCl_3$. The solutions were adjusted to pH 4.0 with 0.5 M NaOH and 0.5 M HCl, except for the samples with humic acids, which were adjusted to pH 5.5, since these substances precipitate at more acidic pH values.

The exposure to light or dark conditions took place during a time dependent on the half-life of each antibiotic, calculated from the experimental data of degradation in water at pH 4.0, which were described with an exponential decay model presented below, being 8 h for AMX and 216 h for AZT. All experiments were performed in triplicate.

Decay Model

The experimental data were fitted to an exponential decay model (**Eq. 1**)

$$C/C_0 = e^{-kt} \tag{1}$$

where C/C_0 is the fraction of the initial concentration C_0 that remains in the suspension after a given time t (expressed in min), and k (min^{-1}) is the kinetic dissipation constant. The half-life ($t_{1/2}$) of each compound was calculated as per **Eq. 2**:

$$t_{\frac{1}{2}} = \frac{\ln 2}{k} \tag{2}$$

RESULTS

Effect of pH on AMX and AZT Photo-Degradation in Absence of Inorganic Salts and Humic Acids

Figure 1 shows the dissipation results for AMX and AZT at different pH values, both under simulated sunlight and in the

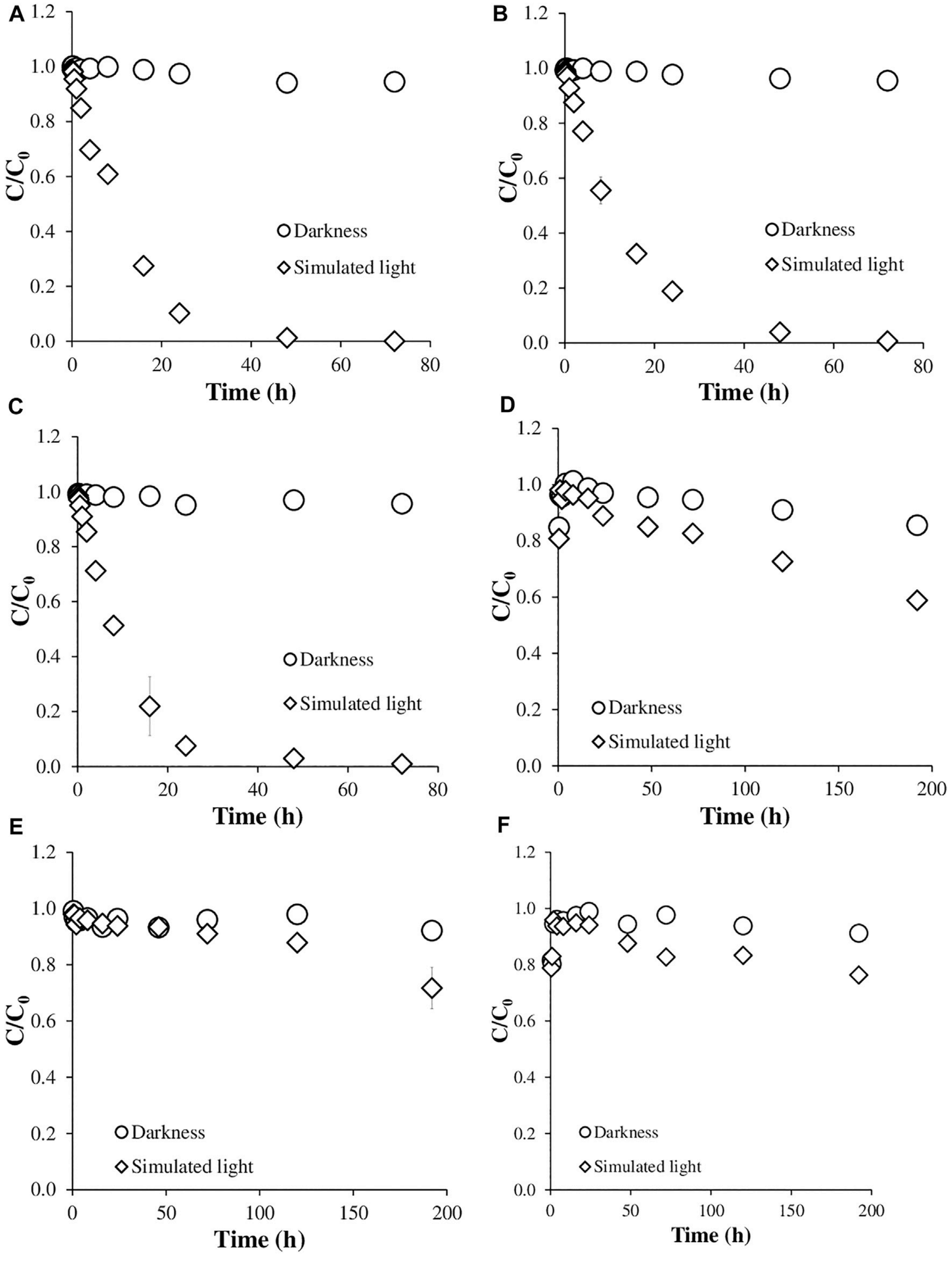


FIGURE 1 | Degradation kinetics of the antibiotics studied. **(A)** Amoxicillin at pH 4.0; **(B)** Amoxicillin at pH 5.5; **(C)** Amoxicillin at pH 7.0; **(D)** Azithromycin at pH 4.0; **(E)** Azithromycin at pH 5.5; **(F)** Azithromycin at pH 7.0.

dark. For AMX, there was no dissipation in the dark at the three pHs studied, keeping the value of the ratio C/C_0 close to 1 (**Figures 1A–C**), while significant degradation occurred under simulated sunlight, although without showing a clear trend as a function of pH (**Figures 1A–C**), reaching C/C_0 values close to 0 after 40 h of contact/exposure.

TABLE 2 | Data corresponding to the exponential decay model showing the behavior of the antibiotics in filtered milliQ water at different pH values; *k*: rate constant (h^{-1}); $t_{1/2}$: half-life (h).

Amoxicillin	*k* (h^{-1})	$t_{1/2}$ (h)	R^2
pH 4.0	0.0791 ± 0.0037	8.8	0.995
pH 5.5	0.0701 ± 0.0008	9.8	0.999
pH 7.2	0.0900 ± 0.0026	7.7	0.993
Azithromycin			
pH 4.0	0.0028 ± 0.0002	250.2	0.983
pH 5.5	0.0015 ± 0.0001	455.9	0.913
pH 7.2	0.0016 ± 0.0002	440.7	0.928

The behavior of AZT in dark conditions was similar to that of AMX, keeping the C/C_0 values close to 1 for all the contact times considered (**Figures 1D–F**). However, the degradation under simulated sunlight was much lower than that of AMX, in spite of using longer times of exposure to light (**Figures 1D–F**), with C/C_0 values always above 0.5. In this case, the degradation was higher at pH 4.0 than at pH 5.5 and 7.2 (**Figures 1D–F**).

The exponential decay model used to calculate half-lives (**Eq. 1**) proved to be useful for this type of estimation, with R^2 scoring between 0.913 and 0.999 (**Table 2**). For AMX, the kinetic constants (k) did not show clear variation when pH went from 4.0 to 7.2, with just a slight increase at pH 7.2, with the half-life following the same trend, going from 8.8 h (at pH 4.0) to 7.7 h (at pH 7.2), and reaching a value of 9.9 h at the intermediate pH of 5.5 (**Table 2**).

Furthermore, changing the pH between 4 and 7.2 does not produce significant modifications in the AMX adsorption spectra (**Figure 2**), an aspect that could also influence its degradation, but it does not seem to be relevant in this case.

Figure 3 presents TOC data corresponding to the experiments with AMX under simulated sunlight at different pHs and at different times of exposition to light. With 2 and 16 h of exposure, TOC does not decrease significantly, despite the fact that the concentration of AMX does reduce by more than 50% at the three pH

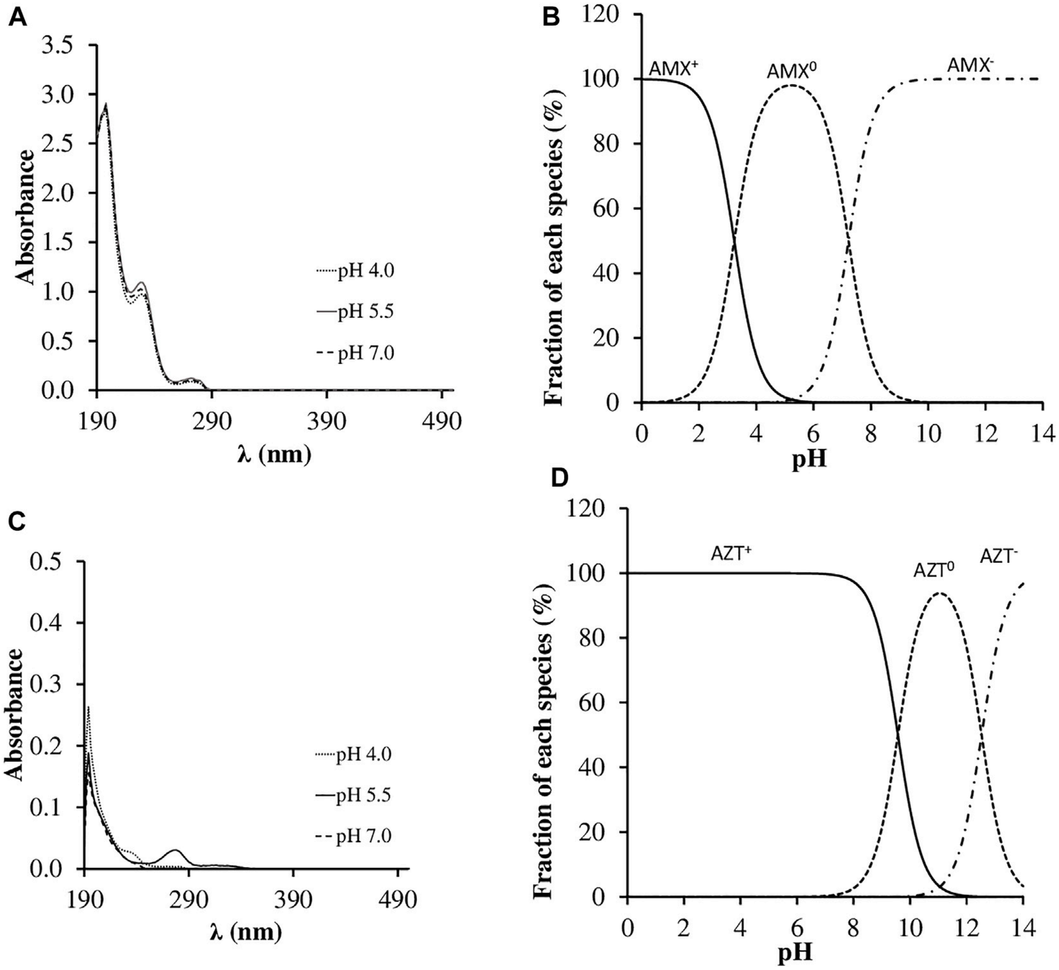


FIGURE 2 | Spectra of antibiotics adsorption and speciation at different pH values (4.0, 5.5 and 7.2). **(A)** Spectrum of Amoxicillin (AMX); **(B)** Speciation of AMX; **(C)** Spectrum of Azithromycin (AZT); **(D)** Speciation of AZT.

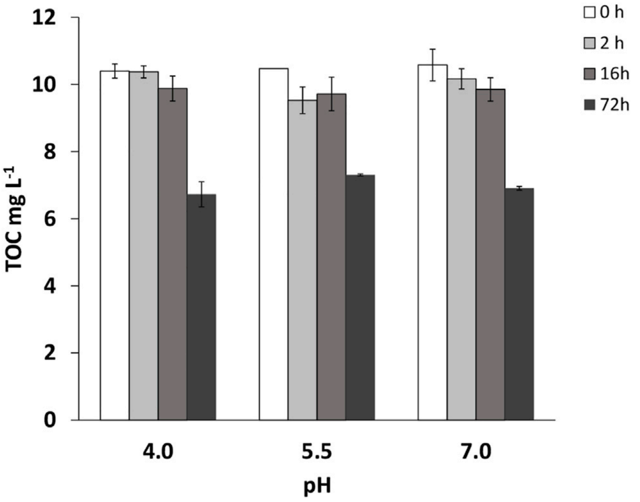


FIGURE 3 | Total organic carbon (TOC) values (mg L^{-1}) for Amoxicillin (AMX), at times: 0, 2, 16, and 72 h, in the experiments under simulated sunlight. Average values for three replicates (n = 3), with vertical bars corresponding to standard deviations.

values studied (**Figures 1A–C**). After 72 h of simulated sunlight, AMX was below the quantification limit, but the TOC measurements only decreased between 30 and 35%, without significant differences among the three pH values studied (**Figure 3**).

The degradation of AZT was different from that of AMX, with much lower kinetic constants (ranging between 0.0016 and 0.0028 h^{-1}) and much longer half-lives (between 250 and 456 h), the lowest values corresponding to pH 4.0. This indicates that AZT suffers higher degradation at acidic pH (4.0) (**Table 2**), with a minimum degradation at pH 5.5, which corresponds to a longer half-life (**Table 2**).

Effects of Inorganic Salts and Humic Acids on AMX and AZT Photo-Degradation

The effects of the presence of the inorganic salts NaCl, $CaCl_2$, $NaNO_3$, Na_2HPO_4, and $FeCl_3$, and of soluble organic matter (humic acids) on the degradation of the two antibiotics studied, both in the dark and under simulated sunlight, are presented in **Figures 4**, **5**.

Figure 4 shows the data corresponding to AMX. The results indicate that the degradation of AMX is dependent on exposition to light, as well as on the matrix, and on the characteristics of the antibiotic. In the dark, no effect on the degradation of AMX was found for the different inorganic salts studied, except for $FeCl_3$ at its highest concentration (500 mg L^{-1}), which causes a 100% dissipation of AMX; however, under simulated sunlight, AMX degradation was greatly increased in the presence of NaCl and $NaNO_3$ (**Figures 1A,C**). The inorganic salt NaCl had an effect favoring the dissipation of AMX, with C/C_0 passing from a value of 0.61 in the sample without NaCl to 0.34 in the presence of 500 mg L^{-1} of NaCl, which means a decrease of 44% for this antibiotic (**Figure 4A**). The presence of $NaNO_3$ caused the dissipation of practically 100% of AMX for the highest concentration of the salt used (500 mg L^{-1}) (**Figure 4C**).

The effects of $CaCl_2$ and Na_2HPO_4 under simulated sunlight were slightly lower, with C/C_0 values changing from 0.60 to 0.48, and from 0.65 to 0.57, respectively, which represents 21% and 12% dissipation, respectively (**Figures 4B,D**). As was the case in the dark, the presence of $FeCl_3$ made AMX degradation very high (**Figure 1E**).

The effect of the presence of inorganic salts on the dissipation of AZT is shown in **Figure 5**. In the dark, no significant effect was observed, while under simulated sunlight the salts that had the greatest effect on increasing the degradation of AZT were NaCl, (with C/C_0 changing from 0.49 to 0.24, which means a dissipation of 51% for the antibiotic) and $CaCl_2$ (with C/C_0 changing from 0.52 to 0.27, which means 47% of dissipation) (**Figures 5A,B**); $NaNO_3$, Na_2HPO_4 and $FeCl_3$ did not cause increases in AZT degradation (**Figures 5C–E**). The fundamental difference of AZT with respect to AMX is the lack of effect of $FeCl_3$, and the effect of NaCl and $NaNO_3$ favoring the degradation for AZT, with respect to AMX (**Figures 4**, **5**).

Finally, the presence of dissolved organic matter (humic acids) had very scarce effect on AMX degradation, both in the dark and under simulated sunlight (**Figure 4F**). There is a slight dissipation taking place both in the dark and under simulated sunlight (4%–19% of increase in dissipation) that can be related to the adsorption of AMX onto humic acids. However, for AZT the results are similar to those of AMX in the dark (6% of increase in dissipation), whereas under simulated sunlight the dissipation is very intense, reaching 68% (**Figure 5F**).

DISCUSSION

Effect of pH on AMX and AZT Photo-Degradation

Regarding previous publications, there are few data on the photo-degradation of AMX in aqueous solutions without the intervention of a photo-catalyst, but the trend is that the degradation increases as the pH rises, as has been pointed out in studies using TiO_2 nanoparticles (Moosavi and Tavakoli, 2016; Li et al., 2019), or ZnO (Elmolla and Chaudhuri, 2010). However, there are also other studies indicating that changing pH does not affect AMX degradation, such as that of Dimitrakopoulou et al. (2012), who reported that the change in pH from 5 to 7.5 had not effect on AMX degradation, but it did slow down the mineralization of the antibiotic. This increase in degradation as the pH rises is usually related to the different sensitivity of the various species present, which is dependent on pH. Specifically, the zwitterion and negatively charged forms grow as the pH increases (**Figure 2**), which is determined by their pK_a values. The effect of increasing photo-degradation with rising pH has also been highlighted for other antibiotics, such as tetracyclines (Conde-Cid et al., 2018a), sulfonamides

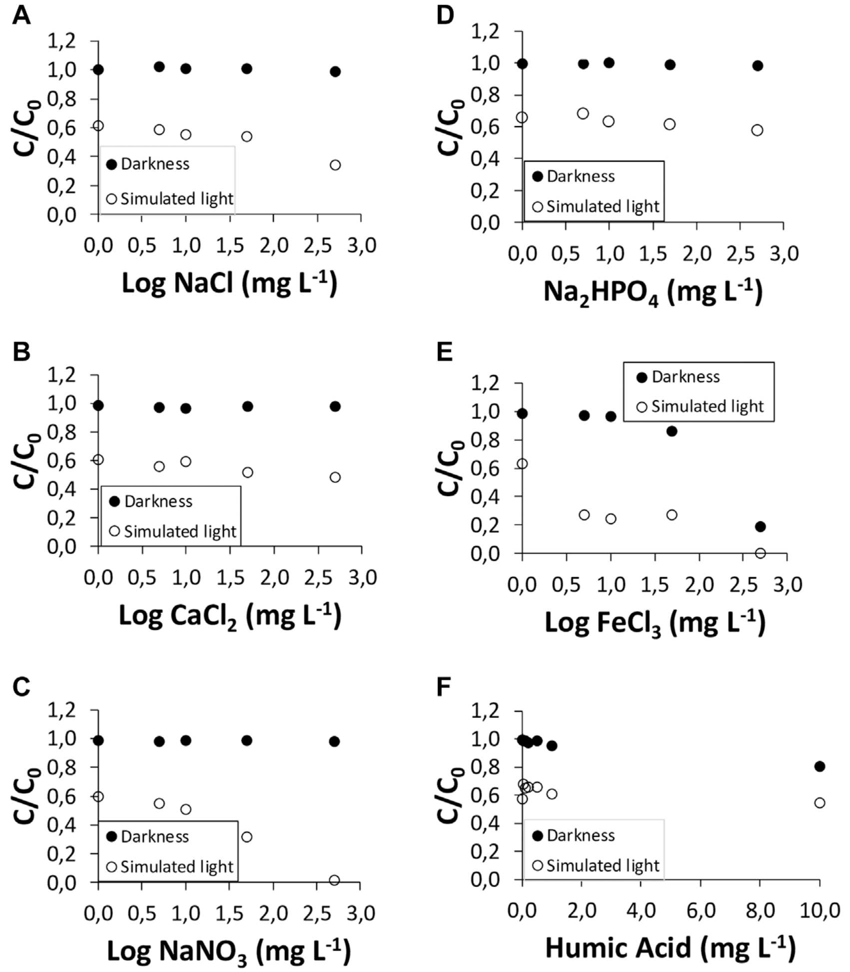


FIGURE 4 | Amoxicillin (AMX) degradation under simulated sunlight and in the dark, in the presence of the inorganic salts: **(A)** NaCl; **(B)** $CaCl_2$; **(C)** $NaNO_3$; **(D)** Na_2HPO_4; **(E)** $FeCl_3$; and in the presence of **(F)** humic acids.

(Canonica et al., 2008; Conde-Cid et al., 2018b) and other families of antibiotics (Canonica et al., 2008; Rodríguez-López et al., 2021; Álvarez-Esmorís et al., 2022).

Regarding TOC analyses, the results obtained indicate that the mineralization of AMX is not complete and that intermediate molecules are formed through different degradation pathways, such as hydroxylation, hydrolysis and decarboxylation (Li et al., 2019; Zhang et al., 2019).

The behavior of presenting a maximum stability at pH between 5.0 and 6.5, has already been described for AZT by other authors (Farghaly and Mohamed, 2004; Zhang et al., 2009). In addition, Čizmič et al. (2019) described a lack of effect on the degradation of AZT due to varying pH between 3.0 and 7.0, but they did observe an increase in the degradation of AZT when pH reached 10. The greater or minor degree of photo-degradation in this case is not related to different ionic forms of the molecule, since AZT has a predominantly positive charge in the pH range studied in the current work, due to its very high pK_a values (**Table 1**; **Figure 2D**). Although it was not possible to verify this fact with TOC measurements, numerous intermediate degradation products have also been detected for AZT, generating different fragments due to modifications in the macrolide ring (Tong et al., 2011).

Effects of Salts and Humic Acids on Photo-Degradation

The effect of Fe favoring the degradation of AMX has already been described by several authors, both through the Fenton reagent (Ay and Kargi, 2010) and through Fe particles (Ghauch et al., 2009), the fundamental mechanism being the formation of OH radicals that favor the degradation of the antibiotic (Buitrago et al., 2020).

Comparing with previous publications, the results regarding the effects of different water matrices on the degradation of the antibiotics are contradictory, which can be attributed to the different experimental conditions and the difference in sensitivity of the antibiotics. In this sense, Canonica et al. (2008) indicate that the effects of photosensitizers on the degradation of antibiotics depended on the type of antibiotic and the light source. Different results are also described even within the same family of antibiotics,

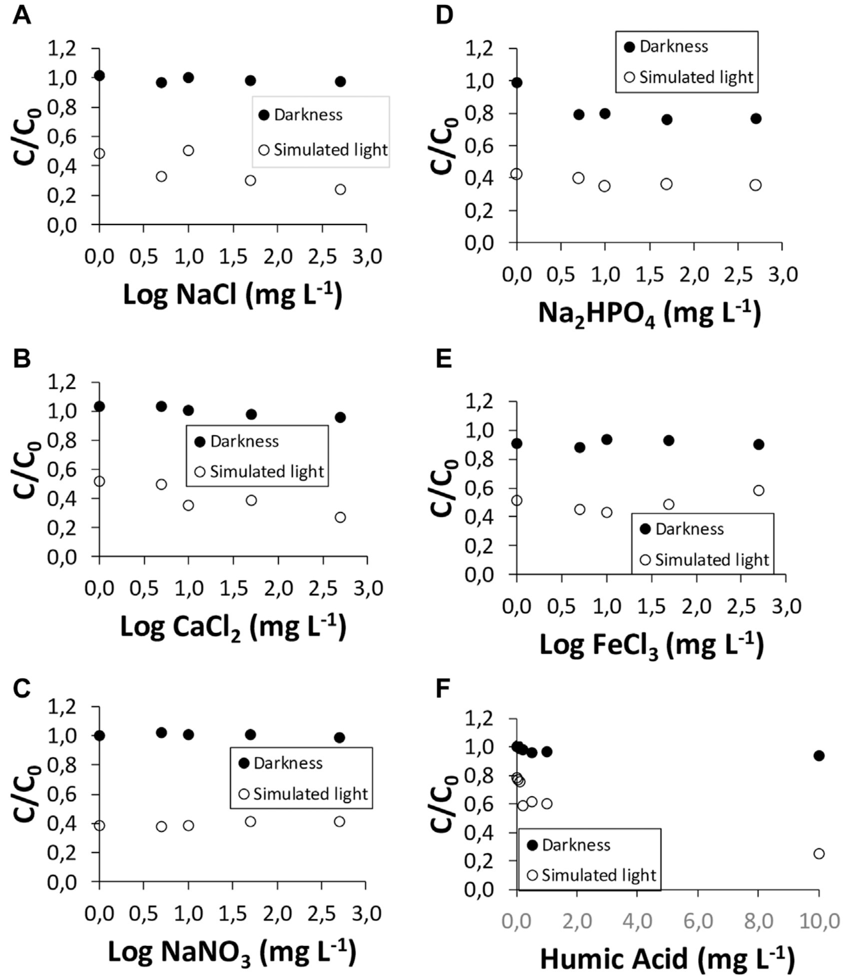


FIGURE 5 | Azithromycin (AZT) degradation under simulated sunlight and in the dark, in the presence of the inorganic salts: **(A)** NaCl; **(B)** $CaCl_2$; **(C)** $NaNO_3$; **(D)** Na_2HPO_4; **(E)** $FeCl_3$; and in the presence of **(F)** humic acids.

as is the case of the various responses due to the presence of dissolved organic matter and nitrates on the degradation of different cephalosporin molecules (Jiang et al., 2010). In general, the presence of nitrates in water has been related to an increase in the photolysis of AZT (Tang et al., 2019). In addition, an increase in ionic strength was related to a higher rate of degradation of AZT (Zhang et al., 2009). Other authors found an inhibitory effect due to the presence of anions such as Cl^-, HCO_3^-, NO_3^-, and SO_4^{2-}, which has been observed for AMX (Zhang et al., 2019), while some other authors did not find neither inhibition nor promotion of degradation (Mavronikola et al., 2009).

Regarding the effect of humic acids on the dissipation of antibiotics, it probably depends on the content of these organic substances, since at low concentrations they can work as promoters of the photosensitivity of antibiotics, while at high concentrations they can act as a preventer of photo-degradation by increasing the turbidity of the liquid medium (Thiele-Bruhn, 2003; Xuan et al., 2010). This is one of the reasons why contradictory results are found regarding its effect on the degradation of antibiotic molecules. As example, an effect of increasing the dissipation of sulfonamides has been previously reported (Conde-Cid et al., 2018b), but their presence caused no effect on the dissipation of tetracycline (Chen et al., 2008).

Conclusion

Among the main facts to remark from the current study, it should be noted that the antibiotics AMX and AZT did not show significant dissipation in the dark. AMX presented a half-life under simulated sunlight that ranged between 7.7 and 8.7 h, with the lowest value associated to the highest pH in the range investigated (pH 7.2). On the other hand, AZT presented a higher half-life, with values that ranged between 250 and 456 h, showing the highest value for an intermediate pH (5.5). AMX completely dissipated after 50 h of simulated sunlight, but the evaluation of the TOC values indicates that the mineralization ranged between 30% and 35%, which corresponds to the formation of intermediate compounds. However, AZT did not completely dissipate even for the longest contact time used (216 h) for the three pH values tested (4.0, 5.5, and 7.2). The presence of NaCl had a favorable effect on the dissipation of both antibiotics, with an increase in degradation of 44% and 51% for

AMX and AZT, respectively. The presence of $CaCl_2$ increased the dissipation of AMX by 21%, and 47% for AZT. $NaNO_3$ caused the total dissipation of AMX when this salt was present at high concentrations, while it had no effect on AZT. The presence of Na_2HPO_4 had no effect on the dissipation of the two antibiotics studied, while $FeCl_3$ drastically increased AMX dissipation both in the dark and under simulated sunlight, and had no effect on AZT. The presence of humic acids only slightly increased the dissipation of AMX, both in the dark (17%) and under simulated sunlight (4%), having a similar effect for AZT in the dark (6% increase in dissipation), and being more pronounced under simulated sunlight, with an increase in dissipation of 68% for the highest concentration of these substances (10 mg L^{-1}). These results can be considered relevant to understand and predict the fate of both antibiotics when they reach environmental compartments, and could help to define appropriate conditions to achieve the inactivation/degradation/removal of these emerging pollutants.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

Almansba, A., Kane, A., Nasrallah, N., Wilson, J. M., Maachi, R., Lamaa, L., et al. (2021). An Engineering Approach Towards the Design of an Innovative Compact Photo-Reactor for Antibiotic Removal in the Frame of Laboratory and Pilot-Plant Scale. *J. Photochem. Photobiol. A Chem.* 418, 113445. doi:10.1016/j.jphotochem.2021.113445

Álvarez-Esmorís, C., Rodríguez-López, L., Fernández-Calviño, D., Núñez-Delgado, A., Álvarez-Rodríguez, E., and Arias-Estévez, M. (2022). Degradation of Doxycycline, Enrofloxacin, and Sulfamethoxypyridazine Under Simulated Sunlight at Different pH Values and Chemical Environments. *Agronomy* 12, 260. doi:10.3390/agronomy12020260

Arun, S., Kumar, R. M., Ruppa, J., Mukhopadhyay, M., Ilango, K., and Chakraborty, P. (2020). Occurrence, Sources and Risk Assessment of Fluoroquinolones in Dumpsite Soil and Sewage Sludge from Chennai, India. *Environ. Toxicol. Pharmacol.* 79, 103410. doi:10.1016/j.etap.2020.103410

Ay, F., and Kargi, F. (2010). Advanced Oxidation of Amoxicillin by Fenton's Reagent Treatment. *J. Hazard. Mater.* 179, 622–627. doi:10.1016/j.jhazmat.2010.03.048

Bavumiragira, J. P., Ge, J. n., and Yin, H. (2022). Fate and Transport of Pharmaceuticals in Water Systems: A Processes Review. *Sci. Total Environ.* 823, 153635. doi:10.1016/j.scitotenv.2022.153635

Bilal, M., Mehmood, S., Rasheed, T., and Iqbal, H. M. N. (2020). Antibiotics Traces in the Aquatic Environment: Persistence and Adverse Environmental Impact. *Curr. Opin. Environ. Sci. Health* 13, 68–74. doi:10.1016/j.coesh.2019.11.005

Brandt, K. K., Amézquita, A., Backhaus, T., Boxall, A., Coors, A., Heberer, T., et al. (2015). Ecotoxicological Assessment of Antibiotics: A Call for Improved Consideration of Microorganisms. *Environ. Int.* 85, 189–205. doi:10.1016/j.envint.2015.09.013

Buitrago, J. L., Sanabria, J., Gútierrez-Zapata, H. M., Urbano-Ceron, F. J., García-Barco, A., Osorio-Vargas, P., et al. (2020). Photo-Fenton Process at Natural Conditions of pH, Iron, Ions, and Humic Acids for Degradation of Diuron and Amoxicillin. *Environ. Sci. Pollut. Res.* 27, 1608–1624. doi:10.1007/s11356-019-06700-y

Canonica, S., Meunier, L., and von Gunten, U. (2008). Phototransformation of Selected Pharmaceuticals During UV Treatment of Drinking Water. *Water Res.* 42, 121–128. doi:10.1016/j.watres.2007.07.026

Chaturvedi, P., Giri, B. S., Shukla, P., and Gupta, P. (2021). Recent Advancement in Remediation of Synthetic Organic Antibiotics from Environmental Matrices: Challenges and Perspective. *Bioresour. Technol.* 319, 124161. doi:10.1016/j.biortech.2020.124161

Chen, Y., Hu, C., Qu, J., and Yang, M. (2008). Photodegradation of Tetracycline and Formation of Reactive Oxygen Species in Aqueous Tetracycline Solution under Simulated Sunlight Irradiation. *J. Photochem. Photobiol. A Chem.* 197, 81–87. doi:10.1016/j.jphotochem.2007.12.007

Čizmić, M., Ljubas, D., Rožman, M., Ašperger, D., Ćurković, L., and Babić, S. (2019). Photocatalytic Degradation of Azithromycin by Nanostructured TiO_2 Film: Kinetics, Degradation Products, and Toxicity. *Materials* 12, 873. doi:10.3390/ma12060873

Conde-Cid, M., Fernández-Calviño, D., Nóvoa-Muñoz, J. C., Arias-Estévez, M., Díaz-Raviña, M., Fernández-Sanjurjo, M. J., et al. (2018a). Biotic and Abiotic Dissipation of Tetracyclines Using Simulated Sunlight and in the Dark. *Sci. Total Environ.* 635, 1520–1529. doi:10.1016/j.scitotenv.2018.04.233

Conde-Cid, M., Fernández-Calviño, D., Nóvoa-Muñoz, J. C., Arias-Estévez, M., Díaz-Raviña, M., Núñez-Delgado, A., et al. (2018b). Degradation of Sulfadiazine, Sulfachloropyridazine and Sulfamethazine in Aqueous Media. *J. Environ. Manag.* 228, 239–248. doi:10.1016/j.jenvman.2018.09.025

Cycoń, M., Mrozik, A., and Piotrowska-Seget, Z. (2019). Antibiotics in the Soil Environment-Degradation and Their Impact on Microbial Activity and Diversity. *Front. Microbiol.* 10, 338. doi:10.3389/fmicb.2019.00338

Dimitrakopoulou, D., Rethemiotaki, I., Frontistis, Z., Xekoukoulotakis, N. P., Venieri, D., and Mantzavinos, D. (2012). Degradation, Mineralization and Antibiotic Inactivation of Amoxicillin by UV-A/TiO_2 Photocatalysis. *J. Environ. Manag.* 98, 168–174. doi:10.1016/j.jenvman.2012.01.010

Elmolla, E. S., and Chaudhuri, M. (2010). Degradation of Amoxicillin, Ampicillin and Cloxacillin Antibiotics in Aqueous Solution by the UV/ZnO Photocatalytic Process. *J. Hazard. Mater.* 173, 445–449. doi:10.1016/j.jhazmat.2009.08.104

Farghaly, O. A. E.-M., and Mohamed, N. A. L. (2004). Voltammetric Determination of Azithromycin at the Carbon Paste Electrode. *Talanta* 62, 531–538. doi:10.1016/j.talanta.2003.08.026

Forsberg, K. J., Reyes, A., Wang, B., Selleck, E. M., Sommer, M. O. A., and Dantas, G. (2012). The Shared Antibiotic Resistome of Soil Bacteria and Human Pathogens. *Science* 337, 1107–1111. doi:10.1126/science.1220761

Ghauch, A., Tuqan, A., and Assi, H. A. (2009). Antibiotic Removal from Water: Elimination of Amoxicillin and Ampicillin by Microscale and Nanoscale Iron Particles. *Environ. Pollut.* 157, 1626–1635. doi:10.1016/j.envpol.2008.12.024

Ghirardini, A., Grillini, V., and Verlicchi, P. (2020). A Review of the Occurrence of Selected Micropollutants and Microorganisms in Different Raw and Treated Manure - Environmental Risk Due to Antibiotics after Application to Soil. *Sci. Total Environ.* 707, 136118. doi:10.1016/j.scitotenv.2019.136118

Han, N., Yao, Z., Ye, H., Zhang, C., Liang, P., Sun, H., et al. (2019). Efficient Removal of Organic Pollutants by Ceramic Hollow Fibre Supported Composite Catalyst. *Sustain. Mater. Technol.* 20, e00108. doi:10.1016/j.susmat.2019.e00108

Han, N., Race, M., Zhang, W., Marotta, R., ZhangBokhari, C. A., Bokhari, A., et al. (2021). Perovskite and Related Oxide Based Electrodes for Water Splitting. *J. Clean. Prod.* 318, 128544. doi:10.1016/j.jclepro.2021.128544

Hassan, M. M., El Zowalaty, M. E., Lundkvist, Å., Järhult, J. D., Khan Nayem, M. R., Tanzin, A. Z., et al. (2021). Residual Antimicrobial Agents in Food Originating from Animals. *Trends Food Sci. Technol.* 111, 141–150. doi:10.1016/j.tifs.2021.01.075

Iwu, C. D., Korsten, L., and Okoh, A. I. (2020). The Incidence of Antibiotic Resistance within and beyond the Agricultural Ecosystem: A Concern for Public Health. *MicrobiologyOpen* 9, e1035. doi:10.1002/mbo3.1035

Jiang, M., Wang, L., and Ji, R. (2010). Biotic and Abiotic Degradation of Four Cephalosporin Antibiotics in a Lake Surface Water and Sediment. *Chemosphere* 80 (11), 1399–1405. doi:10.1016/j.chemosphere.2010.05.048

Kemper, N. (2008). Veterinary Antibiotics in the Aquatic and Terrestrial Environment. *Ecol. Indic.* 8, 1–13. doi:10.1016/j.ecolind.2007.06.002

Khan, H. K., Rehman, M. Y. A., and Malik, R. N. (2020). Fate and Toxicity of Pharmaceuticals in Water Environment: An Insight on Their Occurrence in South Asia. *J. Environ. Manag.* 271, 111030. doi:10.1016/j.jenvman.2020.111030

Kumar, S. B., Arnipalli, S. R., and Ziouzenkova, O. (2020). Antibiotics in Food Chain: The Consequences for Antibiotic Resistance. *Antibiotics* 9, 688. doi:10.3390/antibiotics9100688

Li, Q., Jia, R., Shao, J., and He, Y. (2019). Photocatalytic Degradation of Amoxicillin via TiO_2 Nanoparticle Coupling with a Novel Submerged Porous Ceramic Membrane Reactor. *J. Clean. Prod.* 209, 755–761. doi:10.1016/j.jclepro.2018.10.183

Liu, X., Zhou, Y., Zhang, J., Luo, L., Yang, Y., Huang, H., et al. (2018). Insight into Electro-Fenton and Photo-Fenton for the Degradation of Antibiotics: Mechanism Study and Research Gaps. *Chem. Eng. J.* 347, 379–397. doi:10.1016/j.cej.2018.04.142

Manyi-Loh, C., Mamphweli, S., Meyer, E., and Okoh, A. (2018). Antibiotic Use in Agriculture and its Consequential Resistance in Environmental Sources: Potential Public Health Implications. *Molecules* 23, 795. doi:10.3390/molecules23040795

Mavronikola, C., Demetriou, M., Hapeshi, E., Partassides, D., Michael, C., Mantzavinos, D., et al. (2009). Mineralisation of the Antibiotic Amoxicillin in Pure and Surface Waters by Artificial UVA- and Sunlight-Induced Fenton Oxidation. *J. Chem. Technol. Biotechnol.* 84, 1211–1217. doi:10.1002/jctb.2159

Moosavi, F. S., and Tavakoli, T. (2016). Amoxicillin Degradation from Contaminated Water by Solar Photocatalysis Using Response Surface Methodology (RSM). *Environ. Sci. Pollut. Res.* 23, 23262–23270. doi:10.1007/s11356-016-7349-y

Muhammad, J., Khan, S., Su, J. Q., Hesham, A. E.-L., Ditta, A., Nawab, J., et al. (2020). Antibiotics in Poultry Manure and Their Associated Health Issues: A Systematic Review. *J. Soils Sediments* 20, 486–497. doi:10.1007/s11368-019-02360-0

Nippes, R. P., Macruz, P. D., da Silva, G. N., and Neves Olsen Scaliante, M. H. (2021). A Critical Review on Environmental Presence of Pharmaceutical Drugs Tested for the Covid-19 Treatment. *Process Saf. Environ. Prot.* 152, 568–582. doi:10.1016/j.psep.2021.06.040

Nnadozie, C. F., and Odume, O. N. (2019). Freshwater Environments as Reservoirs of Antibiotic Resistant Bacteria and Their Role in the Dissemination of Antibiotic Resistance Genes. *Environ. Pollut.* 254 (B), 113067. doi:10.1016/j.envpol.2019.113067

Rico, A., Vighi, M., Van den Brink, P. J., Horst, M., Macken, A., Lillicrap, A., et al. (2019). Use of Models for the Environmental Risk Assessment of Veterinary Medicines in European Aquaculture: Current Situation and Future Perspectives. *Rev. Aquacult.* 11, 969–988. doi:10.1111/raq.12274

Rodríguez-López, L., Cela-Dablanca, R., Núñez-Delgado, A., Álvarez-Rodríguez, E., Fernández-Calviño, D., and Arias-Estévez, M. (2021). Photodegradation of Ciprofloxacin, Clarithromycin and Trimethoprin: Influence of pH and Humic Acids. *Molecules* 26, 3080. doi:10.3390/molecules.26113080

Salam, L. B., and Obayori, O. S. (2019). Structural and Functional Metagenomic Analyses of a Tropical Agricultural Soil. *Span. J. Soil Sci.* 9 (1), 1–23. doi:10.3232/SJSS.2019.V9.N1.01

Santás-Miguel, V., Díaz-Raviña, M., Martín, A., García-Campos, E., Barreiro, A., Núñez-Delgado, A., et al. (2020). Medium-Term Influence of Tetracyclines on Total and Specific Microbial Biomass in Cultivated Soils of Galicia (NW Spain). *Span. J. Soil Sci.* 10, 217–232. doi:10.3232/SJSS.2020.V10.N3.05

Serwecińska, L. (2020). Antimicrobials and Antibiotic-Resistant Bacteria: A Risk to the Environment and to Public Health. *Water* 12, 3313. doi:10.3390/w12123313

Silva, C., Louros, V., Silva, V., Otero, M., and Lima, D. (2021). Antibiotics in Aquaculture Wastewater: Is it Feasible to Use a Photodegradation-Based Treatment for Their Removal? *Toxics* 9, 194. doi:10.3390/toxics9080194

Sodhi, K. K., Kumar, M., and Singh, D. K. (2021). Insight into the Amoxicillin Resistance, Ecotoxicity, and Remediation Strategies. *J. Water Process Eng.* 39, 101858. doi:10.1016/j.jwpe.2020.101858

Solliec, M., Roy-Lachapelle, A., Gasser, M.-O., Coté, C., Généreux, M., and Sauvé, S. (2016). Fractionation and Analysis of Veterinary Antibiotics and Their Related Degradation Products in Agricultural Soils and Drainage Waters Following Swine Manure Amendment. *Sci. Total Environ.* 543, 524–535. doi:10.1016/j.scitotenv.2015.11.061

Su, C., Zhang, H., Cridge, C., and Liang, R. (2019). A Review of Multimedia Transport and Fate Models for Chemicals: Principles, Features and Applicability. *Sci. Total Environ.* 668, 881–892. doi:10.1016/j.scitotenv.2019.02.456

Talaiekhozani, A., Joudaki, S., Banisharif, F., Eskandari, Z., Cho, J., Moghadam, G., et al. (2020). Comparison of Azithromycin Removal from Water Using UV Radiation, Fe (VI) Oxidation Process and ZnO Nanoparticles. *Int. J. Environ. Res. Pub. Heal.* 17, 1758. doi:10.3390/ijerph17051758

Tang, J., Wang, S., Fan, J., Long, S., Wang, L., Tang, C., et al. (2019). Predicting Distribution Coefficients for Antibiotics in a River Water-Sediment Using Quantitative Models Based on Their Spatiotemporal Variations. *Sci. Total Environ.* 655, 1301–1310. doi:10.1016/j.scitotenv.2018.11.163

Tello, A., Austin, B., and Telfer, T. C. (2012). Selective Pressure of Antibiotic Pollution on Bacteria of Importance to Public Health. *Environ. Health Perspect.* 120, 1100–1106. doi:10.1289/ehp.1104650

Thiele-Bruhn, S. (2003). Pharmaceutical Antibiotic Compounds in Soils - A Review. *J. Plant Nutr. Soil Sci.* 166, 145–167. doi:10.1002/jpln.200390023

Tong, L., Eichhorn, P., Pérez, S., Wang, Y., and Barceló, D. (2011). Photodegradation of Azithromycin in Various Aqueous Systems under Simulated and Natural Solar Radiation: Kinetics and Identification of Photoproducts. *Chemosphere* 83, 340–348. doi:10.1016/j.chemosphere.2010.12.025

Tran, N. H., Hoang, L., Nghiem, L. D., Nguyen, N. M. H., Ngo, H. H., Guo, W., et al. (2019). Occurrence and Risk Assessment of Multiple Classes of Antibiotics in Urban Canals and Lakes in Hanoi, Vietnam. *Sci. Total Environ.* 692, 157–174. doi:10.1016/j.scitotenv.2019.07.092

Wang, J., and Wang, S. (2016). Removal of Pharmaceuticals and Personal Care Products (PPCPs) from Wastewater: A Review. *J. Environ. Manag.* 182, 620–640. doi:10.1016/j.jenvman.2016.07.049

Xuan, R., Arisi, L., Wang, Q., Yates, S. R., and Biswas, K. C. (2010). Hydrolysis and Photolysis of Oxytetracycline in Aqueous Solution. *J. Environ. Sci. Health, Part B* 45, 73–81. doi:10.1080/03601230903404556

Yang, C.-W., Hsiao, W.-C., and Chang, B.-V. (2016). Biodegradation of Sulfonamide Antibiotics in Sludge. *Chemosphere* 150, 559–565. doi:10.1016/j.chemosphere.2016.02.064

Yang, Q., Gao, Y., Ke, J., Show, P. L., Ge, Y., Liu, Y., et al. (2021). Antibiotics: An Overview on the Environmental Occurrence, Toxicity, Degradation, and Removal Methods. *Bioengineered* 12, 7376–7416. doi:10.1080/21655979.2021.1974657

Yi, X., Lin, C., Ong, E. J. L., Wang, M., and Zhou, Z. (2019). Occurrence and Distribution of Trace Levels of Antibiotics in Surface Waters and Soils Driven by Non-point Source Pollution and Anthropogenic Pressure. *Chemosphere* 216, 213–223. doi:10.1016/j.chemosphere.2018.10.087

Zhang, Y., Liu, X., Cui, Y., Huang, H., Chi, N., and Tang, X. (2009). Aspects of Degradation Kinetics of Azithromycin in Aqueous Solution. *Chroma* 70, 67–73. doi:10.1365/s10337-009-1116-x

Zhang, Y., Xiao, Y., Zhong, Y., and Lim, T.-T. (2019). Comparison of Amoxicillin Photodegradation in the UV/H_2O_2 and UV/Persulfate Systems: Reaction Kinetics, Degradation Pathways, and Antibacterial Activity. *Chem. Eng. J.* 372, 420–428. doi:10.1016/j.cej.2019.04.160

Zhao, F., Yang, L., Chen, L., Li, S., and Sun, L. (2019). Bioaccumulation of Antibiotics in Crops under Long-Term Manure Application: Occurrence, Biomass Response and Human Exposure. *Chemosphere* 219, 882–895. doi:10.1016/j.chemosphere.2018.12.076

ORIGINAL RESEARCH
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Occurrence of Nine Antibiotics in Different Kinds of Sewage Sludge, Soils, Corn and Grapes After Sludge Spreading

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The huge worldwide use of antibiotics triggers the accumulation of these substances in sludge from wastewater treatment plants (WWTP) and the possible contamination of soils amended with it, as well as of crops growing in these soils. In this work we analyzed the presence of the antibiotics amoxicillin (AMO), cefuroxime (CEF), ciprofloxacin (CIP), clarithromycin (CLA), levofloxacin (LEV), lincomycin (LIN), norfloxacin (NOR), sulfadiazine (SUL), and trimethoprim (TRI), in sludge from different WWTPs in Galicia (NW Spain), as well as in sludge technically treated by waste-managers, in soils where treated sludge was applied, and in crops (corn and vineyard) growing in the amended areas. The antibiotics were quantified by means of high resolution HPLC-mass-chromatography. The results indicate that almost all the sludge samples contained antibiotics, being ciprofloxacin and levofloxacin the most abundant reaching maximum values of 623 and 893 ng/g, respectively. The sludge treatment significantly reduced the number and the concentrations of antibiotics. In 12% of the soil samples where sludge was applied, some antibiotics were detected, but always in small concentrations. Regarding the crops, no antibiotic was detected in the roots, stalk, leaves and grain of corn, neither in grapes sampled in vineyards. It can be concluded that the treatments currently applied in the WWTPs under study are not totally effective in removing antibiotics from the sludge, although the findings of this research suggest that the additional specific treatment of the sludge derived from these WWTPs is effective in reducing the risk of environmental pollution due to a variety of antibiotics, and specifically in the case of soils amended with these organic materials and crops growing on it.

Keywords: antibiotics, composting, crop soils, sludge treatment, wastewater treatment plants

INTRODUCTION

Antibiotics have been widely used in human medicine since the discovering of penicillin in 1928, mainly to treat bacterial infections. Their consumption reaches globally up to 15.7 daily doses (DDDs) per 1,000 people (Sriram et al., 2021) with a slightly higher average for Europe, with 16.4 DDD per 1,000 inhabitants (ECDC, 2021). These pharmaceuticals are also provided as food supplement to animals for growth promotion, although this it is banned in Europe (Spielmeyer, 2018), and for animal health control which can lead to the soil persistence of these compounds after repeated application of manure and slurry (Gros et al., 2019). Globally, between the years 2000–2015, 42.3 billion DDD were consumed by humans and the antibiotics consumption rate increased 39%, being higher in high-income countries (Klein et al., 2018), with amoxicillin and amoxicillin/clavulanic acid being the most frequently consumed antibiotics in most countries (Aldeyab et al., 2020). Within the European Union, the antibiotics more consumed are the ones from the group of β-Lactam antibacterial molecules and penicillins (42.3%) and the general consumption pattern has not significantly change in the period 1997–2017 (Bruyndonckx et al., 2021). The country raking of antibiotic consumption in Europe is led by Greece, with Cyprus and Spain in second and third place, respectively (Bruyndonckx et al., 2021).

After its administration, these compounds are partially metabolized and then excreted, reaching the sewage systems and wastewater treatment plants (WWTP). The sludge generated in the WWTP has a great capacity for adsorbing antibiotics, favoring their accumulation, which has been previously described. Specifically, in a study carried out in China, 21 different antibiotics were detected with concentrations up to 5,800 ng g^{-1} in two WWTPs (Zhou et al., 2013), and in Sweden 9 different antibiotics were detected in 11 WWTPs (Östman et al., 2017). Some of the most relevant antibiotics present in WWTPs are: ciprofloxacin, appearing in 90% of the sampled WWTP effluents in a European survey (Loos et al., 2013); lincomycin, which is among the three main individual pollutants usually found in concentrations over 10 μg L^{-1} in the sewage influents in Korea (Behera et al., 2011); and clarithromycin, detected in 73% of sludge samples analyzed in Sweden (Östman et al., 2017) and in effluents from WWTPs in Switzerland (Mcardell et al., 2003). Other antibiotics like levofloxacin (Okuda et al., 2009), norfloxacin (Gao et al., 2012) and trimethoprim (Östman et al., 2017) have been detected as well in the sludge of different WWTPs. Amoxicillin has been detected both in wastewater (Henninger et al., 2001) and in WWTP sludge (Kimosop et al., 2016). Namely, the conventional treatments implemented in WWTPs are not efficient in removing antibiotics from both water and sludge, and additional treatments are required (Sabri et al., 2020). These compounds are considered among the most critical pharmaceuticals and personal care products (PPCPs) regarding environmental risk (Verlicchi and Zambello 2015).

According to the Spanish National Sludge Register, 1.2 million tons of sludge (dry matter) are produced annually in Spain and are considered residues, therefore specific residues regulations are applied related to their disposal and agricultural use (MAMRM, 2009). The sludge should be treated before field application, that in Spain represented around 80% of the sludge use (MAMRM, 2009). These treatments can be applied in the WWTP or after transfer to authorized residue managers. The current normative regulates the maximum concentration of heavy metals allowed in the sludge, meanwhile presence of antibiotics is not controlled. Despite the regulations regarding sludge management, antibiotics might end up in agricultural soils amended with the sludge, and therefore available to the crops growing in these soils (Verlicchi and Zambello 2015). The plants would tend to accumulate antibiotics in the roots, after uptake from the soil solution (Azanu et al., 2016). The presence of these pharmaceuticals has been identified as a new threat due to the application of sewage sludge in agriculture (Ivanová et al., 2018), generating risks for the consumers (Buta et al., 2021). Several studies have detected antibiotic uptake in crops like carrot and barley (Eggen et al., 2011), corn, green onion and cabbage (Kumar et al., 2005), lettuce and radish (Bassil et al., 2013) and grass, corn and wheat (Conde-Cid et al., 2018). It has been shown that different antibiotics may also impact on the soil microbial biomass (Santás-Miguel et al., 2020), and that their degradation in the soil environment is affected by variables such as soil pH, humic acids and salts (Rodríguez-López et al., 2022), which could substantially change after sludge spreading.

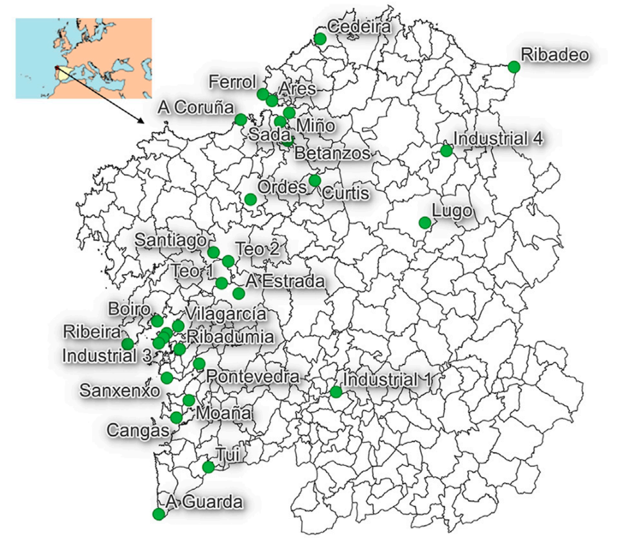

FIGURE 1 | Location of the wastewater treatment plants included in the study, all of them in Galicia (NW Spain).

The aim of the current research is to screen the presence of antibiotics in sludge from different WWTPs in Galicia (NW Spain), as well as in sludge treated by residue managers, in soils where the treated sludge mixtures were applied, and in crops (corn and vine plants) growing on these soils. The study will focus on assessing the eventual success as regards the following aspects: 1) the eventual decrease in the amount of antibiotics present in sewage sludge subjected to technical treatment applied to these residues, and 2) after the spreading of the treated sludge, the

TABLE 1 | Treatments applied by the residue managers (RM) to the wastewater treatment plants (WWTP) sludge, by adding and mixing different by-products during certain time. Sp: spring sampling; Au: autumn sampling.

		WWTP	Additions	Time
RM 1	Mixture 1 (Sp)	Betanzos, Ribeira, Tui	Chicken manure, wood chips and lime	3 days
	Mixture 2 (Sp)	Ares, Betanzos, Cedeira	Sludge from meat industry, mussel shell and lime	
	Mixture 1 (Au)	A Guarda	Sludge from industrial WWTP, wood chips, chicken manure, crop substrate, compost, lime	
	Mixture 2 (Au)	Cedeira, Lugo		
	Mixture 3 (Au)	Sada, Moaña, Cangas		
RM 2	Mixture 1	Miño, Ordes, Ribadeo	Sludge from industrial WWTP, animal rest (blood, stomach intestine, meat), mussel shell, wood chips, milk	8 weeks (Register fertilizer)
RM 3	Mixture 1	Ferrol	Sludge from cannery industry, dairy industry, food leftovers, wood chips	8 weeks
RM 4	Mixture 1	A Estrada, Teo 1-2, Santiago	Wood chips	2 weeks
	Mixture 2	Sanxenxo, Vilagarcía, Industrial 1		
	Mixture 3	Boiro, Vilagarcía, Industrial 1		

concentrations of antibiotics present in soils and crops would remain low. In addition, the research could shed further light on processes taking place and affecting the fate of these emerging pollutants in the environment, which is a matter of real concern as regards environmental and human health.

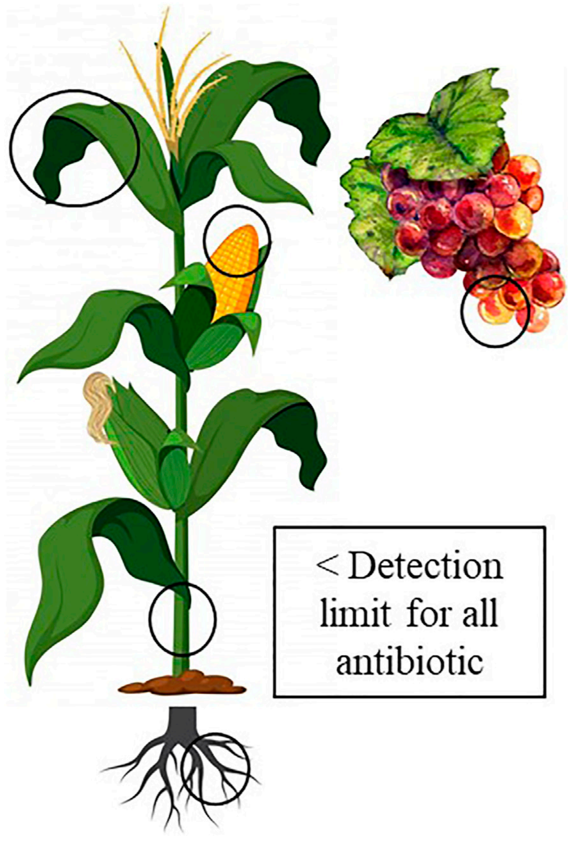

FIGURE 2 | Scheme of plant material sampling. It is shown that all the antibiotics studied in plant samples were below their detection limits using high performance HPLC-mass-chromatography.

MATERIALS AND METHODS

Experimental Design

Sludge samples were collected from 30 different WWTPs in Galicia (NW Spain) (**Figure 1**) during two sampling campaigns, in spring and autumn of 2019. In the spring campaign, 21 urban WWTP, plus 1 industrial WWTP, were sampled. The urban WWTPs were placed in cities with 65,000–248,000 habitants (A Coruña, Ferrol, Pontevedra, Santiago de Compostela), as well as in villages with above 17,000 habitants (A Estrada, Boiro, Ribeira, Teo, Tui, Sanxenxo, Vilagarcía), and in smaller villages (A Guarda, Ares, Betanzos, Cedeira, Curtis, Miño, Ordes, Ribadeo, Ribadumia). In the second sampling campaign (autumn), 28 WWTP sludge samples were taken (the same as for the first sampling, except Cedeira, Ferrol and Teo 2), plus additional WWTPs from Cambados, Cangas, Moaña, Sada, Lugo, and 3 more industrial WWTPs. The industrial ones belong to the agri-food industry, poultry and canned fish production. Regarding configurations adopted in the treatment lines, all urban WWTPs use both primary (gritting, sieving, sedimentation) and secondary treatments (organic matter removal), while tertiary treatments are applied differently in each WWTP (only UV disinfection is applied is most of them, **Supplementary Table S1**).

Within Spain, the specific Galician legislation requires the sludge to be treated before being suitable to be used as an amendment in agricultural soils. The sludge generated from different WWTPs is processed by residue managers, generating compost or not composted mixtures. These processed sludge-derived materials were sampled in spring and autumn, from four different residue managers (RM) who produced different mixtures of WWTP sludges with several animal and vegetal by-products (**Table 1**). These treated mixtures were applied as fertilizers to several agricultural fields, where corn and vineyard were cultivated. Also soils and crops were sampled, as indicated below.

Sludge, Compost, Soil, and Plant Sampling

The WWTP sludge and treated mixtures were sampled at the WWTPs and residue managers facilities, freeze-dried and stored at −4°C until analysis. Soils from amended fields, which had received that kind of fertilization during at least the last 3 years, specifically with the treated mixtures from residue managers (RM) RM1, RM2 and RM4 were sampled at harvest time. A total of 59 amended soils were sampled, 27 under corn (10 amended by RM1, 5 by RM2, and 12 by RM4) and 32 under vineyard (all amended by RM4). The fields were located in different parts of Galicia with (according to the Köppen-Geigen classification) Csb climate (warm temperature with dry and warm summers) and characterized by high precipitations. In each field 10 subsamples were collected in a zigzag transect in the soil surface (depth 0–20 cm) and mixed as a composite sample per field. Soil was dry (at 40°C) until stable weight, sieved (by 2 mm mesh) and stored until analysis. At those same fields vegetal material was sampled at harvest time. In each corn field, three corn plants were randomly selected, and leaves, stem, grains, and roots were taken, making a composite sample per site (**Figure 2**). In the vineyard fields, three plants were also randomly selected, and several bunches of grapes were sampled from each plant making a composite sample per site. In the laboratory, the roots were washed with distilled water, and in stems and leaves the parts closer to the cob were selected for analysis, discarding the central nerve and the sides in the leaves.

Physicochemical Analyses of Soil and Sludge Samples

Standard procedures were used to carry out the analyses (Guitián Ojea and Carballas, 1976; Tan, 1996). Soil texture was determined using the international method of Robison Pipette. For both soil and sludge samples, pH values were obtained in water and KCl (1: 2.5), using a pH-meter CRISON, model 2001. Total C and N were determined by elemental analysis using a LECO equipment, model TRUSPEC CHNS. Extractable P was measured in soil samples using the Olsen method (Olsen and Sommers, 1982). Soil exchange cations (Ca, Mg, Na, K, Al) were extracted using NH_4Cl 1M (Peech et al., 1947) in 1:10 solution. Ca, Mg and Al were measured by atomic absorption spectrophotometry; Na and K were quantified using emission spectrophotometry. The sum of all this exchange cations was considered the effective cation exchange capacity (eCEC) (Kamprath., 1970). Non-crystalline Al and Fe (Blackmore, 1978) and organic Al and Fe (Bascomb, 1968) were measured by atomic absorption after specific extractions. In sludge samples, total element analysis was performed according to the EPA 3051 method, using ICP-MS for measuring Na, Mg, Al, K, P, Ca, Cr, Mn, Fe, Ni, Cu, Zn, As, Cd, Hg and Pb concentrations.

Chemicals, Reagents, and Stock Solutions for the Quantification of the Antibiotics

Amoxicillin, cefuroxime, ciprofloxacin, clarithromycin, levofloxacin, lincomycin, norfloxacin, sulfadiazine, and trimethoprim (purity >98%) and formic acid (purity >99% for analysis) were bought from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol HPLC grade were obtained from Merk (Darmstadt, Germany) and Milli-Q water was obtained from a Millipore system (Bedford, MA, USA) installed in the laboratory.

For each antibiotic, an amount of 20 mg was accurately weight (±0.1 mg) and transferred to a 25 ml volumetric flask containing approximately 10 ml of solvent, the volume was finally made up to the graduation mark and the concentration was calculated taking in consideration the purity of the compound. Specifically, ciprofloxacin was dissolved in methanol:water:formic acid (50: 49.95:0.05), amoxicillin in water acidified with formic acid, and the rest of antibiotics in methanol. Each day of the analysis a new standard solution of amoxicillin was prepared as this pharmaceutical is very unstable and it degrades easily. The other standard solutions were stored in the dark at −18°C. These stock solutions were mixed and diluted several times with a mixture of methanol and water (50:50) to obtain a working standard solution mixture of antibiotics at 1 and 10 $\mu g\ ml^{-1}$.

Antibiotics Extraction Protocol

The extraction solvent was not the same for all matrices, however, independently of the matrix type, 2 g of sample was weight in a 15 ml Falcon tube. From roots, leaves and soils, the antibiotics were extracted with a mixture of acetonitrile:water: formic acid (50:49.95:0.05), whereas from grapes and stalk were extracted with acetonitrile acidified with 0.2% of formic acid, and from sewage sludge with acetonitrile. After the addition of the extraction solvent, the falcon tubes were shaken on a rotary shaker for 30 min and centrifuged at 4,500 rpm for 15 min. A portion of 1 ml of the supernatant was filtered through an Acrodisk Syringe Filter (Waters, USA) and transferred to a HPLC amber vial for analysis. For each batch of samples and type of matrix (soil, sludge, stalk, roots, leaves and grapes) a matrix-matched calibration curved with analyte-free sample was prepared. The concentration of each antibiotic in the calibration curved varied with the matrix but in average ranged from 10 to 1,000 $ng\ g^{-1}$. These calibration curves were used to identify and correctly quantify each of the antibiotics.

Analyses of the Antibiotics by HPLC-MS/MS

The samples were analyzed in an LC-MS/MS system from Bruker (Bremen, Germany) consisting of a UPLC® Elute with degasser, pump, oven, column and autosampler connected to an Evoq Elite triple quadrupole detector. Analytes were separated on an HPLC column Kinetex biphenyl (2.6 µm, 2.1 × 50 mm) from Phenomenex (Torrance, CA, USA). The temperature of the column was set up at 43°C, and the separation of the analytes was achieved on a gradient mode mixture of phase A (0.1% acidified water with formic acid) and phase B (acetonitrile 0.1% acidified with formic acid). The flow was maintained at 375 $\mu L\ min^{-1}$, the column temperature at 43°C, and the samples at 8°C. The mass spectrometer was calibrated as follows: the capillary voltage at 4,800 V, the cone pressure at 20 psi, the cone temperature at 300°C, the source pressure at 30 psi, the source temperature at 500°C, and the misting gas pressure at 60 psi. The injection volume was 15 µL. The MRM transitions monitored for each compound as well as the collision energy employed are compiled in **Table 2**.

TABLE 2 | Ionization mode, precursor and product ion and collision energy employed for pharmaceuticals quantification.

	Ionization mode	Precursor Ion	Product Ions	Collision energy
Amoxicillin	Positive	366.101	114.000	29
			208.100	15
Cefuroxime	Negative	422.9	207.0	15
			318.0	15
Ciprofloxacin	Positive	331.95	231.100	41
			288.100	31
Clarithromycin	Positive	749.846	158.200	33
			83.100	77
Levofloxacin	Positive	362.362	261.000	31
			318.200	21
Lincomycin	Positive	407.3	126.2	22
			359.2	12
Norfloxacin	Positive	320.0	302.0	15
			276.0	15
Sulfadiazine	Positive	252.1	156.0	12
			108.0	19
Trimethoprim	Positive	291.272	261.100	21
			81.000	59

TABLE 3 | Concentration (ng g^{-1}) of antibiotic in the sludge from the different WWTP at the spring (Sp) and autumn (Au) sampling time. Antibiotic detected: ciprofloxacin (CIP), clarithromycin (CLA), levofloxacin (LEV), lincomycin (LIN), norfloxacin (NOR), sulfadiazine (SUL), trimethoprim (TRI).

WWTP	CIP		CLA		LEV		LIN		NOR		SUL		TRI	
	Sp	Au	Sp	Au	Sp	Au	Sp	Au	Sp	Au	Sp	Au	Sp	Au
A Coruña	<	<	<	<	<	<	<	<	<	<	<	<	9	<
A Estrada	<	<	<	<	<	<	<	<	<	<	<	<	<	<
A Guarda	599	<	<	<	<	<	<	<	<	<	<	<	<	<
Ares	105	<	63	<	<	<	<	<	<	<	<	<	21	<
Betanzos	<	<	<	<	<	<	<	<	<	<	<	<	24	<
Boiro	274	<	85	<	525	<	<	<	<	<	<	<	37	<
Cambados	-	<	-	<	-	<	-	<	-	<	-	<	-	<
Cangas	-	<	-	<	-	31	-	<	-	<	-	<	-	<
Cedeira	<	-	<	-	<	-	<	-	<	-	<	-	7	-
Curtis	<	49	<	<	<	18	<	<	<	<	<	<	<	<
Ferrol	<	-	<	-	<	-	<	-	<	-	<	-	<	-
Industrial 1	623	<	<	<	<	<	<	<	144	<	<	<	<	<
Industrial 2	-	<	-	<	-	<	-	<	-	<	-	<	-	<
Industrial 3	-	<	-	<	-	<	-	<	-	<	-	<	-	<
Industrial 4	-	<	-	<	-	<	-	<	-	<	-	<	-	<
Lugo	-	<	-	<	-	<	-	<	-	<	-	<	-	<
Miño	<	<	<	<	<	98	<	<	<	32	<	<	<	<
Moaña	-	1,573	-	<	-	92	-	<	-	<	-	<	-	<
Pontevedra	390	<	<	<	<	<	<	<	<	<	<	<	<	<
Ordes	<	<	256	<	118	<	<	<	<	<	<	<	<	<
Ribadeo	76	<	<	<	<	<	<	<	<	<	<	<	<	<
Ribadumia	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Ribeira	150	<	<	<	137	56	<	<	<	<	<	<	114	20
Sada	-	<	-	<	-	<	-	<	-	<	-	15	-	27
Santiago	351	111	<	<	649	<	<	<	65	<	<	<	23	<
Sanxenxo	319	297	<	<	892	<	<	<	<	<	<	<	43	<
Teo 1	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Teo 2	<	-	95	-	<	-	<	-	<	-	<	-	<	-
Tui	<	<	<	<	<	<	<	31	<	<	<	<	<	<
Vilagarcía	293	<	<	<	37	<	<	<	<	<	400	<	57	<

< indicates the antibiotic was below detection level and – indicates no data.

Statistical Treatment

Mean ± SD values of the general soil properties were used to compare the soils amended with different sludges and under different crops. These data were analyzed by performing a standard analysis of variance (ANOVA 1), and in the cases of significant F statistics, the Tukey's minimum significant different test was used to separate the means, using the statistical package SPSS 27.0 (IBM, USA). For the sludge and mixtures, the study of Pearson correlations ($p < 0.01$) among concentrations of antibiotics and total elements and physicochemical properties was performed using the R software package (R studio, version 4.1.0).

RESULTS AND DISCUSSION

Presence of Antibiotics in Sludge From WWTPs

Altogether in both sampling campaigns (spring and autumn of 2019) seven different antibiotics were detected in the WWTP sludge samples analyzed, with concentrations generally bigger in the first than in the second sampling date. In the first sampling 15 out of 22 of the sludge samples (68%) had at least one antibiotic detected, while 7 of the sludge samples had concentrations below the detection level for all the antibiotics analyzed (**Table 3**).

In the second sampling, which was performed in autumn, just 32% of the samples (9 out of 28) presented some antibiotic at detectable concentrations, even though one the samples showed the highest antibiotic concentration detected in the whole study, which reached 1,573 ng g^{-1} for ciprofloxacin (**Table 3**). Previous studies have shown that the presence of antibiotics in WWTP sludge has a seasonal pattern. In this regard, Golovko et al. (2014) found in water from WWTPs higher concentrations of different antibiotics in winter than in the rest of the year. In the northern hemisphere, antibiotics consumption peaks between January and March (Van Boeckel et al., 2014), which can lead to a total daily pharmaceutical load in the sludge as high as 1 kg day^{-1} in that season (Aydın et al., 2022). In the current study, the higher presence of antibiotics in spring than in autumn might be due to this seasonal consumption pattern.

The number of antibiotics detected in the present study was generally lower than values reported by other authors. Aydın et al. (2022) found ten different antibiotics in WWTP sludge in Turkey, with maximum concentrations detected in the same range that in the current study. In different works carried out in China, the maximum concentration of antibiotic detected in sewage sludge was higher than in our study. Zhou et al. (2013) detected 21 different antibiotics with maximum values up to 5,800 ng g^{-1}, 4-fold higher than our maximum; while Huang et al. (2020) described the presence of 24 different antibiotics in WWTP sludge with maximum concentration of the antibiotics reaching up to 11,000 ng g^{-1}, 7-fold higher than our maximum values. Ivanová et al. (2018) reported the presence of 15 different antibiotics in five WWTP in Slovakia, but the maximum concentration was relatively low, reaching 336 ng g^{-1} for azithromycin, antibiotic which was not detected in our study. On the other hand, Östman et al. (2017) detected only four different antibiotics in sludge samples in Sweden, but with the maximum concentration being 7-fold higher than for our samples. In the same geographic area of our study, the NW of Spain, Conde-Cid et al. (2018) found 8 different antibiotics in 42% of manure samples analyzed, but with maximum concentration values being more than 60-fold higher than those corresponding to our results for sludge.

The WWTP´s with greater number of antibiotics detected in our first sampling were Boiro, Santiago de Compostela and Vilagarcía (**Table 3**). Ciprofloxacin was the more frequently detected antibiotic in this sampling, present in 45% of the sampled sludge, with a maximum value of 623 ng g^{-1} detected in the industrial WWTP (**Table 3**). This same antibiotic showed the maximum concentration detected in our study, found in the second sampling, with a concentration as elevated as 1,573 ng g^{-1} in Moaña´s WWTP (**Table 3**). Several authors have described this antibiotic (ciprofloxacin) as one of the dominant emerging contaminants in all environmental compartments, detecting maximum values of 1,040 ng g^{-1} in sludge (Huang et al., 2020), or founding it in most of the WWTP effluents, with maximum concentration from 264 ng L^{-1} (Loos et al., 2013) to 2,733 96 ng L^{-1} (Nas et al., 2021). In the current study, the second most frequent antibiotic detected in the spring sampling was trimethoprim, which was present in 41% of the samples, with maximum values up to 114 ng g^{-1} in Ribeira´s WWTP sludge (**Table 3**). In a previous investigation this antibiotic was detected in 93% of the WWTP effluents analyzed in Europe (Loos et al., 2013). In the present work, the high frequency of trimethoprim detected could be related to the lack of a specific tertiary treatment (a sand filtration system) which has been described to reduce 60% of the amount of this antibiotic in activated sludge (Gobel et al., 2005). As shown in **Supplementary Table S1**, this filtration system was not present in any of the WWTPs of our study.

Among the less frequently detected antibiotics in the spring sampling, levofloxacin appeared just in 6 of the WWTPs (27%) but showed the highest antibiotic concentration detected at this sampling campaign, 892 ng g^{-1} in Sanxenxo´s WWTP. Other authors have included this antibiotic among the emerging compounds with highest concentration in sludge in WWTPs (Okuda et al., 2009), with values ranging from 204 ng g^{-1}–8,680 ng g^{-1}. Also, the difficulty to remove it by means of the treatments usually applied in WWTPs has been previously described (Martínez-Orgániz et al., 2021). The other antibiotics detected, clarithromycin, norfloxacin and sulfadiazine, were present in 4, 2 and 1 of the samples, respectively, with the maximum value found for the later (reaching 400 ng g^{-1}) in Vilagarcía´s WWTP. Contrarily to our results, other authors detected clarithromycin in 67% of the sludge samples (Aydın et al., 2022), and norfloxacin in 100% of the samples (Gao et al., 2012), with maximum values of 1,496 and 3,200 ng g^{-1} respectively. Loos et al. (2013), in a study of the WWTP effluents in Europe detected sulfadiazine only in 3% of the samples.

In the second sampling campaign of the current study (autumn) the most frequent antibiotic was levofloxacin, which was present in 5 samples (one of them present in the first sampling as well) with maximum values of 98 ng g^{-1} in Miño´s WWTP. Ciprofloxacin was present in 4 samples, showing the highest concentration found in this research, and was present as well in the first sampling in 2 of these WWTPs (**Table 3**). Norfloxacin and trimethoprim appeared in 2 samples, with maximum values of 42 and 27 ng g^{-1}, respectively, while

TABLE 4 | Concentration (ng g^{-1}) of antibiotic in the sludge mixtures from the different residue managers (RM) at the spring and autumn sampling time. Antibiotic detected: ciprofloxacin (CIP), levofloxacin (LEV), norfloxacin (NOR), lincomycin (LIN).

Mixtures Spring	CIP	LEV	NOR	LIN
RM1 – Mixture 1	<	<	551	<
RM1 – Mixture 2	<	<	<	<
RM3 – Mixture 1	<	<	587	<
RM4 – Mixture 1	286	369	<	<
RM4 – Mixture 2	391	289	<	<
RM4 – Mixture 3	394	393	<	<
Mixtures Autumn				
RM1 – Mixture 1	<	<	<	40
RM1 – Mixture 2	<	<	<	40
RM1 – Mixture 3	<	<	<	26
RM2 – Mixture 1	<	<	<	<
RM4 – Mixture 1	<	<	<	<
RM4 – Mixture 2	<	<	<	<
RM4 – Mixture 3	<	169	<	<

< indicates the antibiotic was below detection level.

sulfadiazine and lincomycin were present in just one sample (at 15 and 31 ng g^{-1} respectively). This last one antibiotic only appeared in this sampling, but was found in 37% of the WWTP effluents in an European survey (Loos et a., 2013).

Several previous studies have indicated that the primary and secondary treatments usually applied in WWTPs are not enough for removing many emerging pollutants, such as most antibiotics from both water effluents and sludge (Jelic et al., 2011; Verlicchi et al., 2012; Verlicchi and Zambello 2015; Nguyen et al., 2021), even though some tertiary treatments, like ozone disinfection, can provoke the degradation of macrolide antibiotics such as clarithromycin (Lange et al., 2006). In our study, only one of the WWTPs that presented this antibiotic in the sludge had a disinfection treatment, but was with UV and not ozone, which was not used in any of the WWTPs covered. In our samples the less frequent antibiotics were lincomycin and sulfadiazine. The latest belongs to the sulfonamides group, which have been reported to be easily biodegradable (García-Galán et al., 2011) and transformed during anaerobic sludge digestion (Gobel et al., 2005), a secondary treatment present in most WWTPs.

Presence of Antibiotics in the Sludge Treated by Residue Managers

The sludge treated by the different residue managers (RM) showed a reduced number of antibiotics, compared with the WWTP sludge not subjected to treatment. Specifically, the mixtures from RM1 presented norfloxacin (551 ng g^{-1}) in one of the mixtures from the spring sampling, and lincomycin in the three mixtures from the autumn sampling (maximum value 40 ng g^{-1}) (**Table 4**). The mixture from RM3 had, similarly to RM1, a high concentration of norfloxacin (587 ng g^{-1}), while no antibiotic was detected in the mixture from RM2. The three mixtures from RM4 sampled in spring showed two antibiotics, ciprofloxacin and levofloxacin, with maximum values of 394 ng g^{-1} and 393 ng g^{-1}, respectively, while in the autumn sampling only the mixture 3 presented levofloxacin (at 169 ng g^{-1}, **Table 4**).

As indicated by Nguyen et al. (2021) and Jelic et al. (2011), most of the treatments currently applied in WWTPs are not effective for removing antibiotics, some of which are accumulated in sludge, triggering the need of further sludge treatment to be carried out by residue managers. In the current work, this management consisted in the addition of other organic wastes (animal or vegetal) obtaining a final mixture, in some cases subjected to composting. The comparison between the concentrations of antibiotics detected in the sludge used by each RM and the resulting mixture (**Figure 3**), indicates the effectiveness of the residue management in the antibiotic removal. Specifically, RM1 successfully removed ciprofloxacin, clarithromycin, levofloxacin, sulfadiazine and trimethoprim from the sludge, even though two other antibiotics that were not detected in the sludge (lincomycin and norfloxacin) appeared later in the treated mixtures (**Figure 3**), the latter in high concentrations, which could be originated in the materials added by this RM. In this regard, it should be noted that the mixture 1, that showed high norfloxacin concentrations, had chicken manure included and poultry production industry uses norfloxacin for the prevention and therapy of infectious diseases in some countries (Al-Mustafa and Al-Ghamdi, 2000). However, in the European Union norfloxacin is used in human medicine, but it is not allowed as a veterinary medicine (Pereira et al., 2018), remaining unclear the origin of the norfloxacin in this mixture in the present work. Also to note that the same RM generates three other mixtures that presented small concentrations of lincomycin, without being this antibiotic present in the urban sludge used for the mixing. Sludge from an additional industrial WWTP was added to these mixtures, suggesting a possible industrial origin of the lincomycin, since this antibiotic has been detected in all the WWTPs analyzed in a study in an industrial city in Korea (Behera et al., 2011). However, in the current study we found it only in one of the urban samples and in none on the industrial ones analyzed, thus its origin also being unclear.

RM2 successfully removed levofloxacin and norfloxacin from the sludge. The sludge used by RM3 had no antibiotic detected, but the residue management introduced norfloxacin in the treated mixture. This residue manager, like RM1, added different sludge from industrial WWTPs that may introduce antibiotics that were no present in the urban sludge. On the other hand, RM4 managed to remove most of the antibiotics detected in the sludge, and decreases the amount of ciprofloxacin and levofloxacin, which showed high concentrations in the WWTP sludge (**Figure 3**). This RM carried out a composting process with wood chips. Previous studies reported the removal of levofloxacin from aqueous solutions with a biochar made of wood chips (Yi et al., 2016) and the persistence of ciprofloxacin in composted sludges (Khadra et al., 2019). The compost process is a well-known technology effective in the reduction of antibiotics from sludge, with ranges of removal 17%–100% (Verlicchi and Zambello 2015; Ezzariai et al., 2018). In general, in the current study the management executed by the different residue

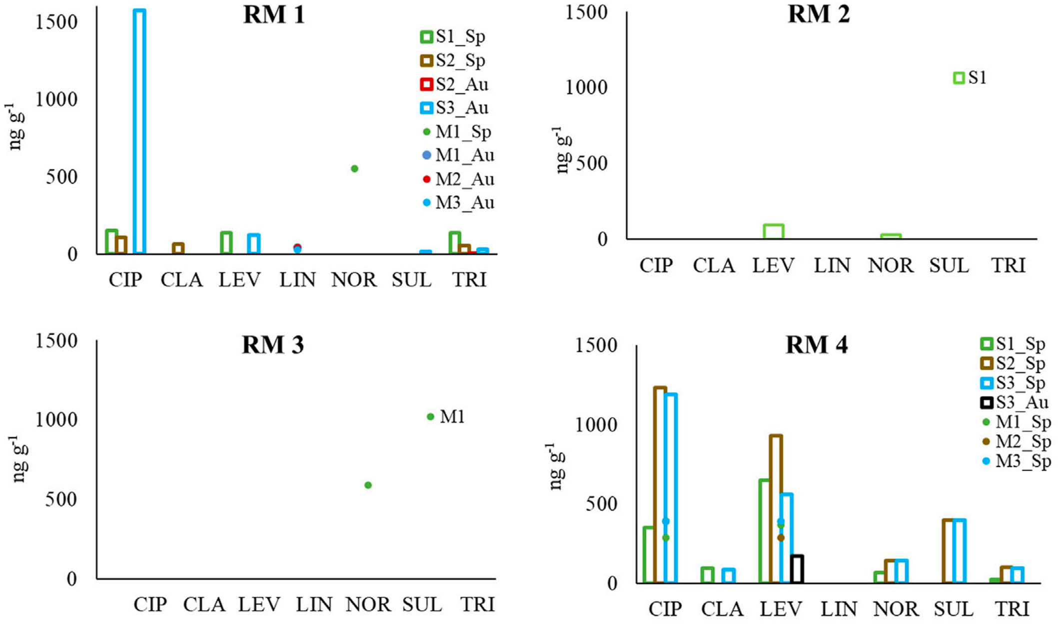

FIGURE 3 | Concentration (ng g^{-1}) of antibiotics present in the sludge (S) from wastewater treatment plants (sum of all antibiotics present in the sludge used for each mixture) and in the mixtures (M) generated by different residue managers (RM). Antibiotics detected: ciprofloxacin (CIP), clarithromycin (CLA), levofloxacin (LEV), lincomycin (LIN), norfloxacin (NOR), sulfadiazine (SUL), trimethoprim (TRI).

managers was successful in the removal or reduction of antibiotics concentrations from the WWTPs sludge.

Physicochemical Characteristics of Sludge and Mixtures

The physicochemical properties of the various sludges and mixtures differed widely, with ranges 5-12 for pH, 2.25%–57.47% for C, and 0.75%–60.85% for N (**Supplementary Table S2**). Similarly, the total elements contents showed great variation among the different sludge and mixture samples, with values (in mg kg^{-1}) between 107-9078 (for Na), 549-6395 (Mg), 143-26106 (Al), 0.6-25.1 (P), 343-8161 (K), 3-230 (Ca), 0-797 (Cr), 25-3595 (Mn), 133-48800 (Fe), 0.9-426 (Ni), 7-1229 (Cu), 0-1777 (Zn), 0.5-120 (As), 0-29 (Cd), 0-447 (Hg), and 0.4-110 (Pb). The WWTPs that presented the highest concentrations of total elements were Lugo (Na and P), Pontevedra (Cr, Fe, and Ni), Curtis (Cu and As), and Ferrol (Hg and Pb) (**Supplementary Table S2**). Regarding the sludge mixtures, the highest values for most of the elements were detected in mixtures from RM1 (Na, P, K, Ca, Cr, Mn, Ni, Cu, Zn, Cd, Hg and Pb), meanwhile a mixture from RM2 had higher concentration of Fe, and mixtures from RM4 showed the highest scores for Mg, Al, Cr and As (**Supplementary Table S2**).

Comparing with other studies, the values of pH were in the same range (Onchoke et al., 2022), while another study carried out in the USA with a lower number of WWTPs (Onchoke et al., 2018) showed narrower ranges for C (30–32%) and N (8–10%), and higher values of P (935–1705 mg kg^{-1}), and Ca (1,521–3,869 mg kg^{-1}), but the maximum values of Mg (709 mg kg^{-1}), K (679 mg kg^{-1}), Na (2,303 mg kg^{-1}), Al (12,857 mg kg^{-1}), Cr (20 mg kg^{-1}), Mn (1,103 mg kg^{-1}), Fe (22,688 mg kg^{-1}), Ni (19.8 mg kg^{-1}), Cu (550 mg kg^{-1}), Zn (772 mg kg^{-1}), As (13 mg kg^{-1}), Cd (0.5 mg kg^{-1}), Hg (0.6 mg kg^{-1}), and Pb (34 mg kg^{-1}) were significantly smaller than the maximum concentrations detected in our samples. Similarly, in a study in Poland Latosinska et al. (2021) reported that the maximum concentrations of Cu (196 mg kg^{-1}), Cr (2,760 mg kg^{-1}), Cd (12 mg kg^{-1}), and Ni (44 mg kg^{-1}) in sludge were smaller than in our samples, but Pb (427 mg kg^{-1}) and Zn (5,351 mg kg^{-1}) showed higher concentrations.

According to the Spanish regulation regarding the maximum concentration of heavy metals allowed in sludge to be suitable for field application (Royal Decree 1,310/1990), practically all the sludge samples from the spring sampling had values above the threshold allowed for Hg (16–25 mg kg^{-1}). The values detected in our sludge samples, with maximum of 447 mg kg^{-1}, far outweigh the results from other authors, which were in the range 0.6–6.1 mg kg^{-1} of total Hg (Mao et al., 2016; Onchoke et al., 2018). The management of the sludge did not manage to remove Hg in three of the spring mixtures (mixtures 1 and 2 from RM1, and RM3 mixture), as they presented Hg concentrations in the range 171–337 mg kg^{-1}. The mixture from RM3 was no applied to the field, but the mixtures from RM4 were spread, which supposes a clear pollution risk.

The fact that the sludge from the industrial WWTPs had Hg values conforming to the regulations, contrary to the urban ones, and that the samples with the highest concentrations belong to the WWTPs of the bigger cities, suggests a domestic origin of these elevated concentration of Hg, such as amalgams, food, laundry detergent, or other, like dentist business of pipe sediments

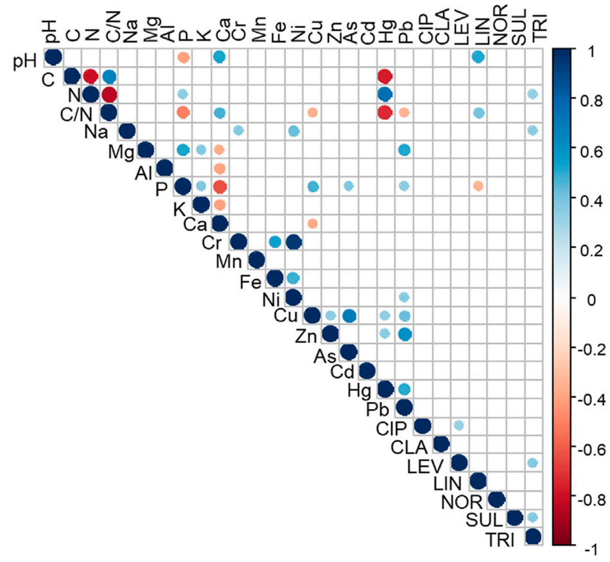

FIGURE 4 | Correlations among the sludge and sludge mixtures physicochemical properties, total element concentration and the sludge and sludge mixtures antibiotics concentrations. Blue and red dots show positive or negative (respectively) significant correlation Pearson coefficients ($p < 0.01$). Ciprofloxacin (CIP), clarithromycin (CLA), levofloxacin (LEV), lincomycin (LIN), norfloxacin (NOR), sulfadiazine (SUL), trimethoprim (TRI).

(Sörme et al., 2003). Previous studies correlated the high concentration of Hg detected in cattle in Galicia with the coal-fired power plants present in the area, that caused high Hg dispersion and deposition (López-Alonso et al., 2003), however nowadays these stations have been closed. The concentrations of the other heavy metals across the different WWTPs were among the Spanish regulation limits, with punctual exceptions.

The analysis of the correlations among antibiotics concentrations and chemical properties in sludge and sludge mixtures showed that lincomycin was correlated with pH (R = 0.52, $p < 0.001$), C/N ratio (R = 0.4, $p < 0.05$) and P content (R = -0.34, $p < 0.05$); while trimethoprim was correlated with N (R = -0.32, $p = 0.01$); and Na (R = 0.34, $p < 0.05$) (**Figure 4**). To note that parameters related to organic matter appear in the correlations, evidencing its marked influence on the retention of antibiotics the soil environment. Physicochemical properties are determinant in the adsorption of antibiotics in soils or bio-adsorbents (Cela-Dablanca et al., 2021, 2022), and similar adsorption mechanisms might take place in sludge. The concentrations of some of the antibiotics were positively correlated with each other: trimethoprim with levofloxacin (R = 0.37, $p < 0.01$) and with sulfadiazine (R = 0.37, $p < 0.05$), and ciprofloxacin with levofloxacin (R = 0.33, $p < 0.01$). In this regard, it should be noted that trimethoprim is used against different infections, from urinary to respiratory ones, and the antibiotic levofloxacin has emerged as an alternative for respiratory infections (Sarzynski et al., 2022). On the other hand, trimethoprim is sometimes administrated simultaneously with sulfadiazine to combat bacterial infections in animals, and ciprofloxacin and levofloxacin (both quinolone antibiotics) are used in a wide range of bacterial infections.

Impact on Agricultural Soils and Crops due to the Spreading of Treated Sludge

The physicochemical properties of the soils amended with the mixtures treated by the different residue managers are shown in **Table 5**. Regarding grain sizes and soil texture, the sand fraction clearly predominates, with a percentage above 45% in all cases. Soils amended with mixtures from RM1 (corn) and RM4 (vineyard) show high pH values (7.90 and 7.01, respectively), meanwhile the corn soils amended with mixtures from RM2 and RM4 had an acidic pH (5.32 and 5.53, respectively). The dominant exchange cation was Ca in all cases, and it is especially clear in the RM1-amended soils. These soils also presented the highest values of the other exchange cations, as well as the lowest values of exchangeable Al, coinciding with the highest pH. Also noteworthy are the high values of organic matter in the soils with the mixtures from RM1 (13.04%), while in the other soils the values were lower (between 6 and 6.7%) (**Table 5**). Non-crystalline Fe compounds (Fe_{ox} and Fe_{pir}) were more abundant in soils amended with RM2 and RM4 (corn). Non-crystalline Al compounds, however, showed higher levels in soils amended with RM1 mixtures.

Out of the 59 soils sampled, only seven soils showed detectable levels of antibiotics: two soils amended by RM2, and the other five by RM4 (**Table 6**). The two soils amended by RM2 had corn as crop, and one of them showed a concentration of 57 ng g^{-1} of amoxicillin, while the other one had 41 ng g^{-1} of clarithromycin. Regarding the soils amended by RM4, the three soil samples under corn contained the antibiotic cefuroxime, with maximum values of 276 ng g^{-1}, while the other two soils which were placed under vineyard showed small amounts of amoxicillin (**Table 6**). The mixture from RM2 did not show clarithromycin and the other two antibiotics were not detected in any of the sludge samples, nor for those coming directly from WWTPs, neither after the processing in the residue manager facilities (**Tables 3**, **4**). The presence of antibiotics in these soil samples might be due to their dispersion through contaminated WWTPs effluents (Zuccato et al., 2010) or to the persistence of antibiotics in the soil after several years of sludge application, even though long-term application of sludge in arable lands has been shown to not being a probable cause of antibiotics accumulation in soils (Rutgersson et al., 2020). On the other hand, the continues application of slurry and manure, especially swine slurry, has been demonstrated that contributes building up persistent antibiotic residues in the uppermost layers of the soil (Gros et al., 2019). No antibiotic was detected in any of the leaves, stem, grain, roots or grape samples (**Figure 2**). The post-WWTP management of the sludge is crucial to avoid soil contamination with antibiotics. Our results, with just 12% of the sampled soils presenting one antibiotic per sample, indicate the efficiency of the different management methods. Conde-Cid et al. (2018) studied the presence of veterinary antibiotics in soils amended with manure, detecting these compounds in 17% of the soil samples, with maximum values of 600 ng g^{-1}, doubling the maximum detected in the soils amended with the managed sludge in the current research. Composting have been proved to reduce antibiotics in both manure and sludge samples (Dolliver

TABLE 5 | Physicochemical properties (average ± DE) of soil amended with mixtures from different residue managers (RM) and under corn and vineyard crops. OM (organic matter); eCEC (effective cation exchange capacity); Al_e (extractable Al); Fe ox (non-crystalline Fe); Fe pir (organic Fe); Al ox (non-crystalline Al); Al pir (organic Al). Different letters mean significant differences $p < 0.05$.

	Soil under corn		Soil under vineyard	
	RM1	**RM2**	**RM4**	**RM4**
pH (H_2O)	7.90 ± 0.10c	5.32 ± 0.28a	5.53 ± 0.42a	7.01 ± 0.13b
pH (KCl)	7.67 ± 0.07d	4.71 ± 0.18a	5.29 ± 0.50b	6.45 ± 0.19c
Sand (%)	53.95 ± 3.90b	46.24 ± 5.41a	49.16 ± 6.64ab	67.73 ± 2.34c
Silt (%)	28.53 ± 3.89b	32.99 ± 6.06c	28.36 ± 3.34b	15.63 ± 2.41a
Clay (%)	17.52 ± 3.29a	20.77 ± 1.69ab	22.48 ± 4.45b	16.64 ± 2.90a
Ca $cmol_{(+)}kg^{-1}$	47.28 ± 5.80c	5.30 ± 0.84a	8.85 ± 5.47ab	11.01 ± 2.77b
Mg $cmol_{(+)}kg^{-1}$	3.05 ± 0.86b	1.04 ± 0.09a	0.88 ± 0.35a	0.88 ± 0.31a
Na $cmol_{(+)}kg^{-1}$	0.70 ± 0.30b	0.20 ± 0.05a	0.05 ± 0.05a	0.15 ± 0.11a
K $cmol_{(+)}kg^{-1}$	1.38 ± 0.67b	0.19 ± 0.03a	0.42 ± 0.20a	0.47 ± 0.16a
Al $cmol_{(+)}kg^{-1}$	0.02 ± 0.01a	0.51 ± 0.35c	0.26 ± 0.24b	0.03 ± 0.01a
eCEC $cmol_{(+)}kg^{-1}$	52.43 ± 6.99b	7.23 ± 0.75a	10.46 ± 5.83a	12.54 ± 3.16a
Al_e (%)	0.05 ± 0.02a	7.23 ± 4.92c	3.50 ± 3.48b	0.27 ± 0.27a
P mg kg^{-1}	181.89 ± 27.47c	16.09 ± 1.85a	52.09 ± 12.27b	25.33 ± 9.99a
C (%)	7.56 ± 1.54b	3.53 ± 0.21a	3.56 ± 1.55a	3.90 ± 0.83a
OM (%)	13.04 ± 2.66b	6.08 ± 0.36a	6.13 ± 2.67a	6.72 ± 1.44a
N (%)	0.55 ± 0.15c	0.36 ± 0.03b	0.34 ± 0.12ab	0.24 ± 0.06a
C/N	14.07 ± 1.39b	9.85 ± 0.51a	10.26 ± 0.70a	16.66 ± 1.48c
Fe ox (mg kg^{-1})	4,929 ± 1339b	8,161 ± 2030d	6010 ± 646c	2,450 ± 488a
Fe pir (mg kg^{-1})	2,552 ± 585b	3,362 ± 319c	3,281 ± 224c	1,396 ± 267a
Al ox (mg kg^{-1})	7235 ± 2552a	1,632 ± 1170a	4,561 ± 2342b	4,126 ± 948b
Al pir (mg kg^{-1})	3,392 ± 1012b	1,409 ± 140a	3,189 ± 1135b	2,548 ± 633b

TABLE 6 | Concentration (ng g^{-1}) of antibiotic in soils under corn and vineyard crops and amended with compost/mixtures from the different residue managers (RM). Antibiotic detected: amoxicillin (AMO), cefuroxime (CEF), clarithromycin (CLA).

	RM	AMO	CEF	CLA
Soil under corn	RM2	57	<	<
		<	<	41
	RM4	<	150	<
		<	213	<
		<	276	<
Soil under vineyard	RM4	*	<	<
		*	<	<

*< indicates the antibiotic was below detection level and * indicated below quantification level.*

et al., 2008; Ezzariai et al., 2018). In the present study, the presence of antibiotics in the mixtures that were lacking in the initial sludge highlights the need of carefully selecting the materials to be combined with the sludge for the composting process. Indeed, the co-composting of contaminated sludges with agricultural wastes has been suggested as a good strategy for the sanitation of compost (Alvarenga et al., 2015). These latter authors mentioned, however, that the risk of heavy metals accumulation driven by the composting process should be considered.

In previous investigations, different crops growing in manure-applied soils have shown antibiotic uptake (Bassil et al., 2013), but in some cases the values were below the quantification limit (Kang et al., 2013). In Galicia, previous studies showed that 44% of the crops amended with manure had detectable concentrations of veterinary antibiotics (Conde-Cid et al., 2018), these crops including grass, corn and wheat samples, with maximum values detected in corn, reaching up to 600 ng g^{-1} for some veterinary antibiotics. The absolute absence of any antibiotic detected in plant material in the current research highlights the importance of the residue management before application to agricultural fields. In this sense, the spreading of manure, contrarily to what happens with sludge, is not regulated, and no management its required before field application in Spain, with less than 10% of the total animal manure being processed at the European level (Foged et al., 2011). It is possible that the situation in other regions and countries differ from this, although it means that we need to do further efforts regarding emerging pollutants monitoring. The impact of antibiotic contamination includes the presence of ARGs (antibiotic resistance genes) that has been reported in crops growing in soils where un-treated WWTP sludge was applied (Rahube et al., 2016), which is a real issue of concern as regards environmental and human health.

CONCLUSION

The treatments applied in the wastewater treatment plants facilities investigated in the current study were not effective in removing the variety of antibiotics used in human medicine that were determined, which were specifically accumulated in sludge. In fact, most of the sludge studied contained some antibiotics, especially in the spring sampling, which is related to the greater consumption of these drugs in certain months. The antibiotics most frequently detected and in the greatest quantity in these sludge materials were ciprofloxacin and levofloxacin. The lack of specific regulation regarding these emergent pollutants in sludge and soils makes this an environmental problem needing further efforts to be correctly addressed. Our findings suggest that additional treatments of the sludge derived from these WWTP, carried out by waste managers, are effective in reducing the risk of environmental pollution due to antibiotics, specifically in the case of soils amended with these organic materials, preventing their entry in the food chain through the crops that grow in those agricultural soils.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/sjss.2022.10741/full#supplementary-material

REFERENCES

Al-Mustafa, Z. H., and Al-Ghamdi, M. S. (2000). Use of Norfloxacin in Poultry Production in the Eastern Province of Saudi Arabia and its Possible Impact on Public Health. *Int. J. Environ. Health Res.* 10, 291–299. doi:10.1080/0960312002001483

Aldeyab, M., López-Lozano, J. M., and Gould, I. M. (2020). "Global Antibiotics Use and Resistance," in *Global Pharmaceutical Policy*. Editor Z. Babar (Singapore: Palgrave Macmillan), 331.

Alvarenga, P., Mourinha, C., Farto, M., Santos, T., Palma, P., Sengo, J., et al. (2015). Sewage Sludge, Compost and Other Representative Organic Wastes as Agricultural Soil Amendments: Benefits versus Limiting Factors. *Waste Manag.* 40, 44–52. doi:10.1016/j.wasman.2015.01.027

Aydın, S., Ulvi, A., Bedük, F., and Aydın, M. E. (2022). Pharmaceutical Residues in Digested Sewage Sludge: Occurrence, Seasonal Variation and Risk Assessment for Soil. *Sci. Total Environ.* 817, 152864. doi:10.1016/j.scitotenv.2021.152864

Azanu, D., Mortey, C., Darko, G., Juhl Weisser, J., Styrishave, B., and Abaidoo, R. C. (2016). Uptake of Antibiotics from Irrigation Water by Plants. *Chemosphere* 157, 107–114. doi:10.1016/j.chemosphere.2016.05.035

Bascomb, C. (1968). Distribution of Pyrophosphate-Extractable Iron and Organic Carbon in Soils of Various Groups. *J. Soil Sci.* 19, 251–268. doi:10.1111/j.1365-2389.1968.tb01538.x

Bassil, R. J., BashourII, Sleiman, F. T., and Abou-Jawdeh, Y. A. (2013). Antibiotic Uptake by Plants from Manure-Amended Soils. *J. Environ. Sci. Health Part B* 48, 570–574. doi:10.1080/03601234.2013.774898

Behera, S. K., Kim, H. W., Oh, J. E., and Park, H. S. (2011). Occurrence and Removal of Antibiotics, Hormones and Several Other Pharmaceuticals in Wastewater Treatment Plants of the Largest Industrial City of Korea. *Sci. Total Environ.* 409, 4351–4360. doi:10.1016/j.scitotenv.2011.07.015

Blackmore, L. C. (1978). "Exchange Complex Dominated by Amorphous Material (ECDAM)," in *The Andisol Proposal*. Editor G. D. Smith (Lower Hutt, New Zealand: New Zealand Soil Bureau), 21.

Bruyndonckx, R., Adriaenssens, N., Versporten, A., Hens, N., Monnet, D. L., Molenberghs, G., et al. (2021). Consumption of Antibiotics in the Community, European Union/European Economic Area. *J. Antim Chemo*, 76. Supplement_2 ii7-ii13. doi:10.1093/jac/dkab172

Buta, M., Hubeny, J., Zieliński, W., Harnisz, M., and Korzeniewska, E. (2021). Sewage Sludge in Agriculture – the Effects of Selected Chemical Pollutants and Emerging Genetic Resistance Determinants on the Quality of Soil and Crops – a Review. *Ecotoxicol. Environ. Saf.* 214, 112070. doi:10.1016/j.ecoenv.2021.112070

Cela-Dablanca, R., Barreiro, A., López, L. R., Santás-Miguel, V., Arias-Estévez, M., Núñez-Delgado, A., et al. (2022). Relevance of Sorption in Bio-Reduction of Amoxicillin Taking Place in Forest and Crop Soils. *Environ. Res.* 208, 112753. doi:10.1016/j.envres.2022.112753

Cela-Dablanca, R., Nebot, C., López, L. R., Fernández-Calviño, D., Arias-Estévez, M., Núñez-Delgado, A., et al. (2021). Efficacy of Different Waste and By-Products from Forest and Food Industries in the Removal/retention of the Antibiotic Cefuroxime. *Processes* 9, 1151. doi:10.3390/pr9071151

Conde-Cid, M., Álvarez-Esmorís, C., Paradelo-Núñez, R., Nóvoa-Muñoz, J. C., Arias-Estévez, M., Álvarez-Rodríguez, E., et al. (2018). Ocurrence of Tetracyclines and Sulfonamides in Manures, Agricultural Soils and Crops from Different Areas in Galicia (NW Spain). *J. Clean. Prod.* 197, 491–500. doi:10.1016/j.jclepro.2018.06.217.2

Dolliver, H., Gupta, S., and Noll, S. (2008). Antibiotic Degradation during Manure Composting. *J. Environ. Qual.* 37, 1245–1253. doi:10.2134/jeq2007.0399

ECDC (2021). *European Centre for Disease Prevention and Control. Antimicrobial Consumption in the EU/EEA (ESAC-Net)*. Stockholm: Annual Epidemiological Report 2020.

Eggen, T., Asp, T. N., Grave, K., and Hormazabal, V. (2011). Uptake and Translocation of Metformin, Ciprofloxacin and Narasin in Forage- and Crop Plants. *Chemosphere* 85, 26–33. doi:10.1016/j.chemosphere.2011.06.041

Ezzariai, A., Hafidi, M., Khadra, A., Aemig, Q., El Fels, L., Barret, M., et al. (2018). Human and Veterinary Antibiotics during Composting of Sludge or Manure: Global Perspectives on Persistence, Degradation, and Resistance Genes. *J. Hazard. Mat.* 359, 465–481. doi:10.1016/j.jhazmat.2018.07.092

Foged, H. L., Flotats, X., Blasi, A. B., Palatsi, J., Magri, A., and Schelde, K. M. (2011). "Inventory of Manure Processing Activities in Europe,". Technical Report No. I in *Concerning 'Manure Processing Activities in Europe' to the European Commission* (Directorate General Environment), 138

Gao, L., Shi, Y., Li, W., Niu, H., Liu, J., and Cai, Y. (2012). Occurrence of Antibiotics in Eight Sewage Treatment Plants in Beijing, China. *Chemosphere* 86, 665–671. doi:10.1016/j.chemosphere.2011.11.019

García-Galán, M. J., Diaz-Cruz, M. S., and Barceló, D. (2011). Occurrence of Sulfonamide Residues along the Ebro River Basin Removal in Wastewater Treatment Plants and Environmental Impact Assessment. *Environ. Int.* 37, 462–473. doi:10.1016/j.envint.2010.11.011

Gobel, A., Thomsen, A., McArdell, C. S., Joss, A., and Giger, W. (2005). Occurrence and Sorption Behavior of Sulfonamides, Macrolides, and Trimethoprim in

Activated Sludge Treatment. *Environ. Sci. Technol.* 39, 3981–3989. doi:10.1021/es048550a

Golovko, O., Kumar, V., Fedorova, G., Randak, T., and Grabic, R. (2014). Seasonal Changes in Antibiotics, Antidepressants/psychiatric Drugs, Antihistamines and Lipid Regulators in a Wastewater Treatment Plant. *Chemosphere* 111, 418–426. doi:10.1016/j.chemosphere.2014.03.132

Gros, M., Mas-Pla, J., Boy-Roura, M., Geli, I., Domingo, F., and Petrović, M. (2019). Veterinary Pharmaceuticals and Antibiotics in Manure and Slurry and Their Fate in Amended Agricultural Soils: Findings from an Experimental Field Site (Baix Empordà, NE Catalonia). *Sci. Total Environ.* 654, 1337–1349. doi:10.1016/j.scitotenv.2018.11.061

Guitián Ojea, F., and Carballas, T. (1976). *Técnicas de análisis de suelos*. Spanish: Pico Sacro.

Henninger, E., Herrel, M., Strehl, E., and Kummer, K. (2001). "Emission of Pharmaceuticals, Contrast Media, Disinfectants, and AOX from Hospitals," in *Pharmaceuticals in the Environment Sources, Fate, Effects, and Risks* (Berlin: Springer), 29–41.

Huang, F., An, Z., Moran, M. J., and Liu, F. (2020). Recognition of Typical Antibiotic Residues in Environmental Media Related to Groundwater in China (2009-2019). *J. Hazard. Mat.* 399, 122813. doi:10.1016/j.jhazmat.2020.122813

Ivanová, L., Mackuľak, T., Grabic, R., Golovko, O., Koba, O., Staňová, A. V., et al. (2018). Pharmaceuticals and Illicit Drugs - A New Threat to the Application of Sewage Sludge in Agriculture. *Sci. Total Environ.* 634, 606–615. doi:10.1016/j.scitotenv.2018.04.001

Jelic, A., Gros, M., Ginebreda, A., Cespedes-Sánchez, R., Ventura, F., Petrovic, M., et al. (2011). Occurrence, Partition and Removal of Pharmaceuticals in Sewage Water and Sludge during Wastewater Treatment. *Water Res.* 45, 1165–1176. doi:10.1016/j.watres.2010.11.010

Kamprath, E. (1970). Exchangeable Aluminum as a Criterion for Liming Leached Mineral Soils. *Soil Sci. Soc. Am. J.* 34, 252–254. doi:10.2136/sssaj1970.03615995003400020022x

Kang, D. H., Gupta, S., Rosen, C., Fritz, V., Singh, A., Chander, Y., et al. (2013). Antibiotic Uptake by Vegetable Crops from Manure-Applied Soils. *J. Agric. Food Chem.* 61, 9992–10001. doi:10.1021/jf404045m

Khadra, A., Ezzariai, A., Merlina, G., Capdeville, M. J., Budzinski, H., Hamdi, H., et al. (2019). Fate of Antibiotics Present in a Primary Sludge of WWTP during Their Co-composting with Palm Wastes. *Waste Manag.* 84, 13–19. doi:10.1016/j.wasman.2018.11.009

Kimosop, S. J., Getenga, Z. M., Orata, F., Okello, V. A., and Cheruiyot, J. K. (2016). Residue Levels and Discharge Loads of Antibiotics in Wastewater Treatment Plants (WWTPs), Hospital Lagoons, and Rivers within Lake Victoria Basin, Kenya. *Environ. Monit. Assess.* 188, 532. doi:10.1007/s10661-016-5534-6

Klein, E. Y., Van Boeckel, T., Martinez, E. M., Pant, S., Gandra, S., Levin, S. A., et al. (2018). Global Increase and Geographic Convergence in Antibiotic Consumption between 2000 and 2015. *Proc. Natl. Acad. Sci. U. S. A.* 115, E3463-E3470–E3470. doi:10.1073/pnas.1717295115

Kumar, K., Gupta, S. C., Baidoo, S. K., Chander, Y., and Rosen, C. J. (2005). Antibiotic Uptake by Plants from Soil Fertilized with Animal Manure. *J. Environ. Qual.* 34, 2082–2085. doi:10.2134/jeq2005.0026

Lange, F., Cornelissen, S., Kubac, D., Sein, M. M., von Sonntag, J., Hannich, C. B., et al. (2006). Degradation of Macrolide Antibiotics by Ozone: A Mechanistic Case Study with Clarithromycin. *Chemosphere* 65, 17–23. doi:10.1016/j.chemosphere.2006.03.014

Latosinska, J., Kowalik, R., and Gawdzik, J. (2021). Risk Assessment of Soil Contamination with Heavy Metals from Municipal Sewage Sludge. *Appl. Sci. (Basel).* 11, 548. doi:10.3390/app11020548

Loos, R., Carvalho, R., António, D. C., Comero, S., Locoro, G., Tavazzi, S., et al. (2013). EU-Wide Monitoring Survey on Emerging Polar Organic Contaminants in Wastewater Treatment Plant Effluents. *Water Res.* 47, 6475–6487. doi:10.1016/j.watres.2013.08.024

López Alonso, M., Benedito, J. L., Miranda, M., Fernández, J. A., Castillo, C., Hernández, J., et al. (2003). Large-scale Spatial Variation in Mercury Concentrations in Cattle in NW Spain. *Environ. Pollut.* 125, 173–181. doi:10.1016/S0269-7491(03)00073-3

MAMRM (2009). Characterization of Wastewater Treatment Plants Sludge Genetared in Spain (In Spanish)." in *Ministerio Medio Ambiente Y Medio Rural Y Marino*.

Mao, Y., Cheng, L., Ma, B., and Cai, Y. (2016). The Fate of Mercury in Municipal Wastewater Treatment Plants in China: Significance and Implications for Environmental Cycling. *J. Hazard. Mat.* 306, 1–7. doi:10.1016/j.jhazmat.2015.11.058

Martínez-Orgániz, A., Becerril Bravo, J. E., Llompart, M., Dagnac, T., Lamas, J. P., Vázquez, L., et al. (2021). Emerging Pollutants and Antibiotics Removed by Conventional Activated Sludge Followed by Ultraviolet Radiation in a Municipal Wastewater Treatment Plant in Mexico. *Wat Q. Res. J.* 56, 167–179. doi:10.2166/wqrj.2021.013

Mcardell, C. S., Molnar, E., Suter, M. J. F., and Giger, W. (2003). Occurrence and Fate of Macrolide Antibiotics in Wastewater Treatment Plants and in the Glatt Valley Watershed, Switzerland. *Environ. Sci. Technol.* 37, 5479–5486. doi:10.1021/es034368i

Nas, B., Dolu, T., and Koyuncu, S. (2021). Behavior and Removal of Ciprofloxacin and Sulfamethoxazole Antibiotics in Three Different Types of Full-Scale Wastewater Treatment Plants: A Comparative Study. *Water Air Soil Pollut.* 232, 127. doi:10.1007/s11270-021-05067-6

Nguyen, A. Q., Vu, H. P., Nguyen, L. N., Wang, Q., Djordjevic, S. P., Donner, E., et al. (2021). Monitoring Antibiotic Resistance Genes in Wastewater Treatment: Current Strategies and Future Challenges. *Sci. Total Environ.* 783, 146964. doi:10.1016/j.scitotenv.2021.146964

Okuda, T., Yamashita, N., Tanaka, H., Matsukawa, H., and Tanabe, K. (2009). Development of Extraction Method of Pharmaceuticals and Their Occurrences Found in Japanese Wastewater Treatment Plants. *Environ. Int.* 35, 815–820. doi:10.1016/j.envint.2009.01.006

Olsen, S. R., and Sommers, L. E. (1982). "Phosphorus," in *Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties*. Editor A. L. Page (Madison, WI: Agronomy Monographs), 9.

Onchoke, K. K., Franclemont, C. M., and Weatherford, P. W. (2018). Structural Characterization and Evaluation of Municipal Wastewater Sludge (Biosolids) from Two Rural Wastewater Treatment Plants in East Texas, USA. *Spectrochimica Acta Part A Mol. Biomol. Spectrosc.* 204, 514–524. doi:10.1016/j.saa.2018.06.096

Onchoke, K. K., Oluwadamilola, O. F., Friedfeld, R. B., and Weatherford, P. W. (2022). Evaluation and Analysis of Perlite and Municipal Wastewater Sludge (Biosolids) from Three Wastewater Treatment Plants in East Texas, USA. *Environ. Monit. Assess.* 194, 121. doi:10.1007/s10661-022-09794-z

Östman, M., Lindberg, R. H., Fick, J., Björn, E., and Tysklind, M. (2017). Screening of Biocides, Metals and Antibiotics in Swedish Sewage Sludge and Wastewater. *Water Res.* 115, 318–328. doi:10.1016/j.watres.2017.03.011

Peech, L., Alexander, L. T., and Dean, L. A. (1947). *Methods of Soil Analysis for Soil-Fertility Investigations*. Washington D.C. USA: USDA Cir. N° 757.

Pereira, A. M. P. T., Silva, L. J. G., Rodrigues, J., Lino, C., and Pena, A. (2018). Risk Assessment of Fluoroquinolones from Poultry Muscle Consumption: Comparing Healthy Adult and Pre-school Populations. *Food Chem. Toxicol.* 118, 340–347. doi:10.1016/j.fct.2018.05.035

Rahube, T. O., Marti, R., Scott, A., Tien, Y. C., Murray, R., Sabourin, L., et al. (2016). Persistence of Antibiotic Resistance and Plasmid-Associated Genes in Soil Following Application of Sewage Sludge and Abundance on Vegetables at Harvest. *Can. J. Microbiol.* 62, 600–607. doi:10.1139/cjm-2016-0034

Rodríguez-López, L., Santás-Miguel, V., Núñez-Delgado, A., Álvarez-Rodríguez, E., Pérez-Rodríguez, P., and Arias-Estévez, M. (2022). Influence of pH, Humic Acids, and Salts on the Dissipation of Amoxicillin and Azithromycin under Simulated Sunlight. *Span. J. Soil Sci.* 12, 10438. doi:10.3389/sjss.2022.10438

Rutgersson, C., Ebmeyer, S., Lassen, S. B., Karkmana, A., Fick, J., Kristiansson, E., et al. (2020). Long-term Application of Swedish Sewage Sludge on Farmland Does Not Cause Clear Changes in the Soil Bacterial Resistome. *Environ. Int.* 137, 105339. doi:10.1016/j.envint.2019.105339

Sabri, N. A., van Holst, S., Schmitt, H., van der Zaan, B. M., Gerritsen, H. W., Rijnaarts, H. H. M., et al. (2020). Fate of Antibiotics and Antibiotic Resistance Genes during Conventional and Additional Treatment Technologies in Wastewater Treatment Plants. *Sci. Total Environ.* 741, 140199. doi:10.1016/j.scitotenv.2020.140199

Santás-Miguel, V., Díaz-Raviña, M., Martín, A., García-Campos, E., Barreiro, A., Núñez-Delgado, A., et al. (2020). Medium-term Influence of Tetracyclines on Total and Specific Microbial Biomass in Cultivated Soils of Galicia (NW Spain). *Span. J. Soil Sci.* 10, 217–232. doi:10.3232/SJSS.2020.V10.N3.05

Sarzynski, S. H., Warner, S., Sun, J., Matsouaka, R., Dekker, J. P., Babiker, A., et al. (2022). Trimethoprim-sulfamethoxazole versus Levofloxacin for S*tenotrophomonas Maltophilia* Infections: A Retrospective Comparative

Effectiveness Study of Electronic Health Records from 154 US Hospitals. *Open Forum Infect. Dis.* 9, ofab644. doi:10.1093/ofid/ofab644

Sörme, L., Lindqvist, A., and Söderberg, H. (2003). Capacity to Influence Sources of Heavy Metals to Wastewater Treatment Sludge. *Environ. Manage.* 31, 421–428. doi:10.1007/s00267-002-2810-8

Spielmeyer, A. (2018). Occurrence and Fate of Antibiotics in Manure during Manure Treatments: A Short Review. *Sustain. Chem. Pharm.* 9, 76–86. doi:10.1016/j.scp.2018.06.004

Sriram, A., Kalanxhi, E., Kapoor, G., Craig, J., Balasubramanian, R., Brar, S., et al. (2021). *State of the World'santibiotics 2021: A Global Analysis of Antimicrobial Resistance and its Drivers*. Washington DC: Center for Disease Dynamics, Economics & Policy.

Tan, K. H. (1996). *Soil Sampling, Preparation, and Analysis*. New York, USA: Marcel Dekker.

Van Boeckel, T. P., Gandra, S., Ashok, A., Caudron, Q., Grenfell, B. T., Levin, S. A., et al. (2014). Global Antibiotic Consumption 2000 to 2010: an Analysis of National Pharmaceutical Sales Data. *Lancet Infect. Dis.* 14, 742–750. doi:10.1016/S1473-3099(14)70780-7

Verlicchi, P., Al Aukidy, M., and Zambello, E. (2012). Occurrence of Pharmaceutical Compounds in Urban Wastewater: Removal, Mass Load and Environmental Risk after a Secondary Treatment-A Review. *Sci. Total Environ.* 429, 123–155. doi:10.1016/j.scitotenv.2012.04.028

Verlicchi, P., and Zambello, E. (2015). Pharmaceuticals and Personal Care Products in Untreated and Treated Sewage Sludge: Occurrence and Environmental Risk in the Case of Application on Soil - A Critical Review. *Sci. Total Environ.* 538, 750–767. doi:10.1016/j.scitotenv.2015.08.108

Yi, S., Gao, B., Sun, Y., Wu, J., Shi, X., Wu, B., et al. (2016). Removal of Levofloxacin from Aqueous Solution Using Rice-Husk and Wood-Chip Biochars. *Chemosphere* 150, 694–701. doi:10.1016/j.chemosphere.2015.12.112

Zhou, L. J., Ying, G. G., Liu, S., Zhao, J. L., Yang, B., Chen, Z. F., et al. (2013). Occurrence and Fate of Eleven Classes of Antibiotics in Two Typical Wastewater Treatment Plants in South China. *Sci. Total Environ.* 452-453, 365–376. doi:10.1016/j.scitotenv.2013.03.010

Zuccato, E., Castiglioni, S., Bagnati, R., Melis, M., and Fanelli, R. (2010). Source, Occurrence and Fate of Antibiotics in the Italian Aquatic Environment. *J. Hazard. Mat.* 179, 1042–1048. doi:10.1016/j.jhazmat.2010.03.110

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Soil Bacterial Community Tolerance to Three Tetracycline Antibiotics Induced by Ni and Zn

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A laboratory work has been carried out to determine the tolerance of soil bacterial communities to Ni and Zn and co-tolerance to tetracycline antibiotics (chlortetracycline (CTC), oxytetracycline (OTC) and tetracycline (TC)) in soils individually spiked with five different concentrations of Ni or Zn (1,000, 750, 500, 250, and 125 mg kg^{-1}), and an uncontaminated (0 mg kg^{-1}) control soil. The PICT parameter (pollution-induced community tolerance) was estimated for the bacterial community using the tritium (^{3}H)-labeled leucine incorporation technique, and the values corresponding to log IC$_{50}$ were used as toxicity index. The mean log IC$_{50}$ values observed in the uncontaminated soil samples indicate that Zn (with log IC$_{50}$ = −2.83) was more toxic than Ni (log IC$_{50}$ = −2.73). In addition, for the soil with the lowest carbon content (C = 1.9%), Ni-contaminated samples showed increased tolerance when the Ni concentrations added were ≥500 mg kg^{-1}, while for the soils with higher carbon content (between 5.3% and 10.9%) tolerance increased when Ni concentrations added were ≥1,000 mg kg^{-1}. Regarding the soils contaminated with Zn, tolerance increased in all the soils studied when the Zn concentrations added were ≥125 mg kg^{-1}, regardless of the soil carbon content. The co-tolerance increases obtained after exposure of the bacterial suspension to TC, OTC and CTC showed an identical behavior within these tetracycline antibiotics. However, it was dependent on the heavy metal tested (Ni or Zn). In the case of soils 1 (C = 1.1%) and 2 (C = 5.3%), the soil bacterial communities showed increases in co-tolerance to TC, OTC and CTC for Ni concentrations added of ≥125 mg kg^{-1}, while for soil 3 (with C = 10.9%) co-tolerance took place when Ni was added at ≥1,000 mg kg^{-1}. However, in soils contaminated with Zn, increases in co-tolerance to CTC, OTC and TC occurred at Zn concentrations added of ≥125 mg kg^{-1} for the 3 soils tested. These results can be considered relevant when anticipating possible environmental repercussions related to the simultaneous presence of various types of pollutants, specifically certain heavy metals and antibiotics.

Keywords: antibiotics, bacterial growth, heavy metals, leucine incorporation method, PICT

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INTRODUCTION

Soil microorganisms are key components in ecosystem, by their relevance in process such as organic matter recycling or cycling of nutrients. The correct maintenance of biological activity in soils is considered a key characteristic to guarantee the functions of an ecosystem and its sustainable productivity (Swift, 1994). The presence of contaminants in the soil can endanger the proper functioning of its biological activity since soil microorganisms are more sensitive than other organisms to some contaminants such as heavy metals (Brookes, 1995; Giller et al., 1999). Although, heavy metals are of moderate to high physiological importance as they are essential micronutrients required for various cellular components and functions, such as Zn for DNA and RNA polymerase, and Ni for urease (Seiler and Berendonk, 2012), the presence of these compounds in high concentrations can bring negative effects. Heavy metals can be naturally present in soils, although there are also various anthropic activities, such as agricultural, livestock, and industrial production, as well as mining, which can promote a high entry of these substances into terrestrial ecosystems (Kabata-Pendias, 2000; Abdu et al., 2017). Agricultural soils can suffer from high concentrations of heavy metals due to the repeated application of various amendments, such as sewage sludge, phosphate fertilizers, nitrate fertilizers, manure, lime, and composted residues. As indicated in various publications (European Union, 2006; Wu et al., 2013) heavy metals are frequently used in livestock production, with many commercial feedings being added with Zn, Cu and other essential elements, mainly used with the aim of preventing different diseases.

In addition to the use of heavy metals in livestock production, the use of veterinary antibiotics to treat infectious diseases in animals or as growth promoters has been implemented in the last century (Sapkota et al., 2008). In addition, the use of veterinary antibiotics to treat infectious diseases is common in livestock production. The most widely used antibiotics in Europe are tetracyclines, among which chlortetracycline (CTC), oxytetracycline (OTC), and tetracycline (TC) stand out (European Medicines Agency, 2016). Once administered to cattle, antibiotics and heavy metals are poorly absorbed by the animal intestine and a high percentage of these compounds are excreted and accumulated in feces and urine (Sarmah et al., 2006; Zhang et al., 2012; Hejna et al., 2018). Tetracycline antibiotics and heavy metals tend to accumulate in agricultural soils due to frequent spreading of manures and slurries as organic amendments (Hamscher et al., 2002; He et al., 2009), being a widespread problem throughout the world.

The toxicity of antibiotics and heavy metals on soil microorganisms depends on their concentration and their chemical form (Nies, 1999). In this regard, there are several studies that have evaluated the toxicity of heavy metals in soil microbial communities (Hattori, 1992; Giller et al., 1998; Giller et al., 2009; Abdu et al., 2017). On the other hand, there are also previous studies focused on studying the effects of antibiotics on soil microorganisms (Thiele-Bruhn, 2005; Toth et al., 2011; Caban et al., 2018; Urra et al., 2019), however, there are few studies that focus on the study of tetracyclines in soil bacterial communities, such as Santás-Miguel et al. (2020a); Santás-Miguel et al., 2020c; Santás-Miguel et al., 2020d), or on the temporal evolution of various antibiotics affected by environmental conditions, such as Rodríguez-López et al. (2022). On the other hand, several works evaluated the co-tolerance to antibiotics of soil bacterial communities, at the functional and genetic level, in soils contaminated with heavy metals (Bååth, 1989; Berg et al., 2010; Oyetibo et al., 2010; Ji et al., 2012; Dickinson et al., 2019; Santás-Miguel et al., 2020b; Santás-Miguel et al., 2022). However, few studies focused on the concentration levels present in soils that would be the starting point to cause increases in co-tolerance of soil bacterial communities to antibiotics when simultaneously contaminated with heavy metals (Fernández-Calviño and Bååth, 2013; Song et al., 2017; Santás-Miguel et al., 2020b). Knowledge of these concentrations would shed light on the vulnerability of soils contaminated with heavy metals to the presence of bacteria resistant to antibiotics, without previously having been in contact with them. These antibiotic-resistant bacteria, through horizontal gene transfer, can confer resistance to human pathogens, becoming a threat to public and environmental health (Davies, 1994; Knapp et al., 2010; Serwecińska, 2020).

Therefore, the main objective of this study was to determinate the concentrations from which increases in tolerance to Ni and Zn and co-tolerance to tetracycline antibiotics (CTC, OTC, and TC) occur in 3 agricultural soils contaminated separately with 5 different concentrations of Ni and Zn (1,000, 750, 500, 250, and 125 mg kg^{-1}) compared with a control (0 mg kg^{-1}). This study could shed light on the vulnerability of soils contaminated with heavy metals to achieve increased tolerance to antibiotics. This information could facilitate an ulterior design and implementation of appropriate management practices to mitigate these undesirable effects.

MATERIAL AND METHODS

Chemicals

The metals in this research were Zn (as $Zn(NO_3)_2\ 6H_2O$, CAS; 10196–18-6) and Ni (as $Ni(NO_3)_2\ 6H_2O$, CAS; 13478-00-7). They were supplied by Panreac (Barcelona, Spain).

The antibiotics used in the tests were chlortetracycline hydrochloride ($C_{22}H_{23}ClN_2O_8$) (≥97% in purity; CAS 64-72-2), oxytetracycline hydrochloride ($C_{22}H_{24}N_2O_9$) (≥95% in purity; CAS 2058-46-0), and tetracycline hydrochloride ($C_{22}H_{24}N_2O_8$) (≥95% in purity; CAS 64-75-5), from Sigma–Aldrich (Steinheim, Germany).

Soil Samples

Three agricultural soil samples from A Limia area in Galicia (NW of Spain), were selected form a set previously characterized (Conde-Cid et al., 2018). These soil samples were previously used by Santás-Miguel et al. (2020c) and Santás-Miguel et al. (2020d) for measuring the bacterial growth in soils polluted with tetracycline antibiotics. The methods used to analyze these soils are included in **Supplementary Material S1**.

The main characteristics of the soils studied are shown in **Supplementary Table S1**. Briefly, the 3 soils present similar values of pH measured in water, ranging 4.5–4.8. However, they show very different organic carbon contents, with values between 1.1% and 10.9%, while DOC varies between 210.5 and 572 mg kg^{-1}. The sand, silt and clay contents varied between 40.6 and 70.4, between 11.9 and 25.8, and between 17.7 and 33.6 respectively. Therefore, the texture of soil samples was sandy loam for soil 1, clay loam for soil 2 and sandy clay loam for soil 3. The cation exchange capacity ranged between 4.1 and 11.7 cmol$_c$ kg^{-1}. The Al$_o$ and Fe$_o$ values ranged 855–3,535 mg kg^{-1} and 1,150–2080 mg kg^{-1}, respectively. Total Ni and Zn contents ranged 14.0–19.7 and 64–141 mg kg^{-1}, respectively. These values are similar to those found by Macías and Calvo (2008) in uncontaminated soils in the area. The concentration of bioavailable heavy metals to most heavy metal concentration added (1,000 mg kg^{-1}) at days 0 and 42 is shown in **Supplementary Table S2**. The concentrations of bioavailable heavy metals at day 0 in soil 1, 2 and 3, determined in polluted soil samples extracted with $CaCl_2$ were 413.6, 365.9 and 356.6 mg kg^{-1}, respectively, for Ni and 558.5, 506.6 and 585.4 mg kg^{-1} respectively, for Zn. On the other hand, the soil samples extracted with EDTA in soil 1, 2 and 3 at day 0, they were 900.8, 560.6 and 503.1 mg kg^{-1} for Ni, respectively, and 878.6, 692.2 and 615.2 mg kg^{-1} for Zn (**Supplementary Table S2**). The bioavailable concentrations determined in polluted soil samples at day 42 of incubation in soil 1, 2 and 3 were 288.2, 271.5 and 257.7 mg kg^{-1} for Ni and were 527.8, 490.9 and 505.5 mg kg^{-1} for Zn, when extracted with $CaCl_2$, while they were 731.2, 367.8 and 333.5 mg kg^{-1} corresponding to Ni and 788.3, 528.7 and 517.9 mg kg^{-1} corresponding to Zn, when extracted with EDTA (**Supplementary Table S2**).

Experimental Design

Each of the 3 soil samples were introduced into polyethylene bottles of 500 mL (84 g per bottle) and moistened up to 70% of their water holding capacity, being incubated for 1 week (at 22°C, in the dark), as indicated by Meisner et al. (2013) to facilitate the correct growth of the soil bacterial communities after adjustment of moisture. After this time, the soil samples were distributed in 6 polypropylene tubes of 50 mL (14 g wet weight) and separately spiked with 5 solution with different concentrations of Ni or Zn to reach a final concentration of 1,000, 750, 500, 250, and 125 mg kg^{-1}, also preparing an uncontaminated control soil with water (0 mg kg^{-1}). Then, for each metal (Ni or Zn), the 6 microcosms were distributed in 18 polypropylene tubes of 50 mL (6 microcosms x 3 replication), with 4.6 g (expressed in dry weight) included in each of the tubes, and then incubated (in dark conditions) during 42 days, being a total of 108 microcosms for 3 soil samples and each heavy metal (Ni or Zn). A schematic description of the experimental design is shown in **Supplementary Figure S1**. The procedure detailed in Díaz-Raviña et al. (1994) and in Díaz-Raviña and Baath. (1996) was followed to determine the bacterial community tolerance (although with some modifications), complemented by the details published by Díaz-Raviña and Bååth. (2001) and Díaz-Raviña et al. (1994) as regards the leucine incorporation method. For this, soil samples corresponding to each microcosm were added with distilled water (at 1:20 (w/v)), shaking with a multivortex (at maximum intensity) for 3 min. Then, the soil + water mixture was centrifuged during 10 min at 1,000 *x g*, obtaining the soil bacterial suspension (supernatant). Volumes of 1.50 mL of the supernatant were transferred to Eppendorf vials of 2 mL, in which 150 μL of pollutant (heavy metal or antibiotic) were subsequently added. The different concentrations of heavy metals (Ni and Zn) and antibiotics (CTC, OTC, and TC) added to the bacterial suspension plus a control sample (with only water added) made a total of 8 concentrations of pollutants used for each compound and soil sample. The final heavy metal concentrations added in liquid to bacterial suspension ranged between 10^{-2} and 10^{-5} mol L^{-1}, while for the antibiotics ranged between 400 and 9.7×10^{-2} mg L^{-1}. The bacterial community growth was estimated after a pre-incubation step of 24 h of bacterial suspension containing the different antibiotics concentrations added before performing the leucine incorporation assay (Berg et al., 2010; Fernández-Calviño and Bååth, 2013). On each micro-centrifugation tube, [^{3}H] leucine incorporation was then measured as follows: 0.2 μL [^{3}H] Leu (3.7 MBq mL^{-1} and 0.574 TBq mmol^{-1}; Amersham) incorporated with non-labeled Leu into each of the tubes, giving 275 nM Leu in the bacterial suspensions. To stop bacterial growth 75 μL of trichloroacetic acid (at 100%) were added after 3 h of incubation. The indications from Díaz-Raviña and Bååth. (2001) for washing, whereas a liquid scintillation counter (Tri-Carb 2810 TR, PerkinElmer, USA) was used for the radioactivity measurement. For determining the co-tolerance, the results of bacterial growth that were obtained after the exposure of the bacterial suspension to 8 concentrations (from o to 400 mg L^{-1}) of the tetracycline antibiotics (CTC, OTC, and TC, separately), were normalized with respect to the control, obtaining dose-response curves for the different soil samples contaminated with the various Ni or Zn concentrations added (1,000, 750, 500, 250, and 125 mg kg^{-1}, and the unpolluted control sample).

Data Analyses

To estimate the tolerance of the bacterial community to Ni and Zn, and to CTC, OTC and TC, the logarithm of the concentration that resulted in 50% inhibition of bacterial community growth (log IC$_{50}$) was used. Log IC$_{50}$ was calculated using a logistic model, detailed below (Sebaugh, 2011):

$$Y = c\Big/\left[1 + e^{b(x-a)}\right] \qquad (1)$$

Where Y is the level of Leu incorporation (measured), X is the logarithm of the concentration of the substance (Ni and Zn, or CTC, OTC and TC) added to the bacterial suspension, a is the log IC$_{50}$, c the bacterial growth in absence of the toxics, and b is a slope parameter indicative of the inhibition rate. High log IC$_{50}$ values indicate higher community tolerance, whereas lower values indicate higher toxicity to the bacterial community.

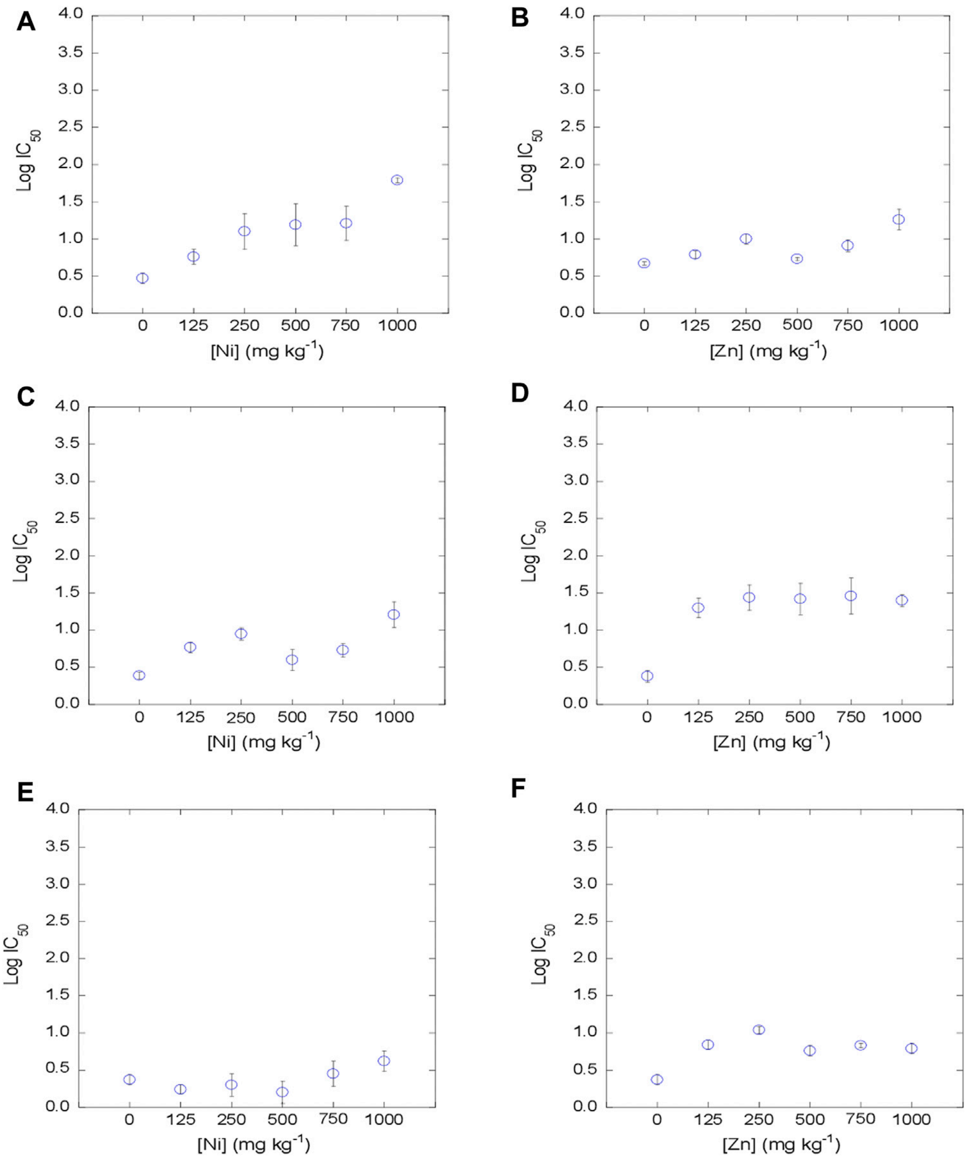

FIGURE 1 | Evolution of bacterial community tolerance to Ni and Zn (as log IC_{50}), for increasing Ni and Zn concentrations in the soil (0, 125, 250, 500, 750 and 1,000 mg kg^{-1}). **(A, B)**: soil 1; **(C, D)**: soil 2, **(E, F)**: soil 3.

RESULTS AND DISCUSSION

Tolerance of Soil Bacterial Communities to Ni and Zn

The results of bacterial growth measured for each dose of Ni and Zn [1,000, 750, 500, 250, 125, and 0 mg kg^{-1})] were obtained after exposing the bacterial suspensions to the various concentrations of heavy metals (which expressed in molar concentrations went from 0 to 10^{-2} mol L^{-1}). After being normalized compared to the control, dose-response curves were obtained for the 3 tested soils (**Supplementary Figure S2**). These dose-response curves were of sigmoidal type, that is, low concentrations of added heavy metal do not cause inhibition of bacterial growth, however, as the heavy metal concentration added is increased, bacterial growth is progressively inhibited. The dose-response curves show a shift to the right compared to the control, which suggests increases in the tolerance of the bacterial communities to the both heavy metals tested.

The dose-response curves presented good fits (**Supplementary Tables S3, S4**) to the logistic model (Eq. **1**) with R^2 from 0.873 to 0.996 (Mean = 0.960) in soils contaminated with Ni, and R2 from 0.824 to 0.999 (Mean = 0.975) for soils contaminated with Zn. From the dose-response curves obtained for Ni and Zn in the 3 soils studied, the log IC_{50} values were estimated for the 6 concentrations tested, as represented in **Figure 1**. The log IC_{50} values obtained for the control samples (0 mg kg^{-1}) for each soil show scores between −3.05 and −2.36 (mean = −2.73) for Ni, and between −2.99 and −2.69 (mean = −2.83) for Zn. Observing the mean log IC_{50} values, the concentration that inhibits 50% of the bacterial population is slightly lower for Zn than that observed for Ni, indicating that Zn seems has higher toxicity than Ni on bacterial communities in the soil samples studied. The log IC_{50} values obtained in the present study for the control sample are similar to those found in previous works by Santás-Miguel et al. (2022), with log IC_{50} values of −2.56 for Ni and −2.89 for Zn.

The increases in tolerance to heavy metals were different depending on the soil and the heavy metal tested. Soil bacterial communities show increased tolerance to Ni at concentrations ≥500 mg kg^{-1} for soil 1 (C = 1.1%) with respect to the control. However, increases in tolerance to Ni in soils with higher carbon contents (5.3% for soil 2, and 10.9% for soil 3) occur at high metal concentrations added (≥1,000 mg kg^{-1}). On the other hand, regarding Zn, the increase in tolerance with respect to this metal is not dependent on the carbon content of the soil, since there is an increase in tolerance to Zn with respect to the control in all soils at concentrations ≥125 mg kg^{-1}.

The results of the current study are similar to those shown by various authors who also observed increased tolerance to Ni and Zn in soils polluted with different concentrations of these metals (Duxbury and Bicknell, 1983; Díaz-Raviña et al., 1994; Díaz-Raviña and Bååth, 2001; Almås et al., 2004; Davis et al., 2004; Stefanowicz et al., 2009; Zhong et al., 2021; Santás-Miguel et al., 2022). For example, Díaz-Raviña et al. (1994) after 9 months of incubation observed a rise in tolerance with respect to the control for all the concentrations of Zn and Ni tested in agricultural soils from southern Sweden, which were polluted with 1878 mg kg^{-1} of Ni and between 1,046 and 2093 mg kg^{-1} of Zn. Stefanowicz et al. (2009) measured the tolerance of bacterial communities in soils near galvanized electricity pylons contaminated with Zn using the Biolog plate technique and observed an increased tolerance to Zn at concentrations ≥300 mg kg^{-1}.

Observing the results obtained in the present research, it is shown that Ni toxicity on soil bacterial communities has a slight dependence on soil carbon content, while it does not happen for Zn. In this regard, it should be bear in mind that the affinity shown by heavy metals for organic matter increases with pH, therefore, at acidic pH, the affinity will be lower than at basic pH (Spark et al., 1997). Taking into account that the pH of the soils here studied is between 4.5 and 4.8, the results obtained may be due to the affinity that these metals have for the organic matter of the soil. In fact, it is classified, according to (Gluskoter, 1977), as a low affinity for Zn and medium affinity for Ni. This difference in heavy metals affinity for soil organic matter has been demonstrated by a large number of authors (Khan and Schnitzer, 1978; McBride, 1994; Rule, 1999; Sauvé et al., 2003; Refaey et al., 2014). In this sense, Piccolo (1989) measured the effect of humic acids on the immobilization of different heavy metals in soils and observed the following sequence: Pb > Cu > Cd > Ni > Zn, with Zn being the metal with the highest solubility followed by Ni and, therefore, having greater bioavailability in soils than other heavy metals. However, considering that other studies such Milne et al. (2003) showed a slightly lower Ni affinity for humic acids than Zn, other causes different than affinities for organic matter cannot be discarded. Thus, log IC_{50} values found in non-amended soil samples showed a slightly higher toxicity of Zn than Ni on bacterial communities (**Supplementary Tables S3, S4**).

The high bioavailability of these metals in the soil causes the bacterial communities in the soil to be exposed to this contaminant and therefore the selection pressure exerted on the communities is higher (Blanck, 2002). Zn, therefore, exerts higher selection pressure than Ni, which caused that metal tolerance increases take place from lower Zn concentrations than those needed for Ni.

Co-Tolerance of Soil Bacterial Communities to Tetracycline Antibiotics in Ni and Zn Polluted Soils

The results of bacterial growth obtained after exposure of the bacterial suspension to 8 concentrations of the three tetracycline antibiotics (400–0 mg L^{-1}), added separately, were normalized with respect to the control, giving sigmoidal-type dose-response curves for the different soil samples contaminated with the various concentrations of Ni and Zn (1,000, 750, 500, 250, 125, and 0 mg kg^{-1}) (**Supplementary Figures S3–S5**). The dose-response curves show a clear shift to the right for most heavy metals concentrations, indicating that there is a clear increase in co-tolerance to CTC, OTC and TC in most of the soils contaminated with the different Ni and Zn concentrations.

The dose-response curves were satisfactorily fitted to the logistic model, with R^2 values between 0.890 and 0.999 (mean = 0.983) for the 3 tetracyclines in Ni-contaminated soils, and with R^2 values between 0.874 and 0.999 (mean = 0.982) in Zn-contaminated soils. Log IC_{50} values were estimated from the dose-response curves obtained for each concentration of metal, soil and antibiotic added (**Figures 2–4**). The mean log IC_{50} values corresponding to the uncontaminated control (0 mg kg^{-1} of Ni and Zn) after the addition of the tetracycline antibiotics to the bacterial suspension were 0.76 for TC, 0.78 for OTC and 0.41 for CTC, indicating that CTC is the most toxic among them, while TC and OTC show similar values. This sequence of toxicities is similar to that found in previous works, where CTC >> OTC ≥ TC (Santás-Miguel et al., 2020c; Santás-Miguel et al., 2020d).

The co-tolerance increases obtained after exposure of the bacterial suspension to TC, OTC and CTC showed an identical behavior within these tetracycline antibiotics. However, it was dependent on the heavy metal tested (Ni or Zn). On the one hand, the tolerance increases of TC, OTC and CTC in soils contaminated with Ni were evidenced at concentrations ≥125 mg kg^{-1} in soil 1 (C = 1.1%) and 2 (C = 5.3%), whereas in soil 3, with a very high carbon content (C = 10.9%), increases in co-tolerance to tetracycline antibiotics took place at very high concentrations (≥1,000 mg kg^{-1}). Regarding soils contaminated with different concentrations of Zn, co-tolerance increases were observed for TC, OTC and CTC at the lowest concentration tested of ≥125 mg kg^{-1} for the 3 soils. This behavior shown for tetracycline antibiotics is similar to that observed for heavy metals in the previous section, where Ni tolerance increases showed a slight dependence on soil carbon content, while Zn tolerance increases were independent of the soil carbon content. Therefore, the evidence from studies

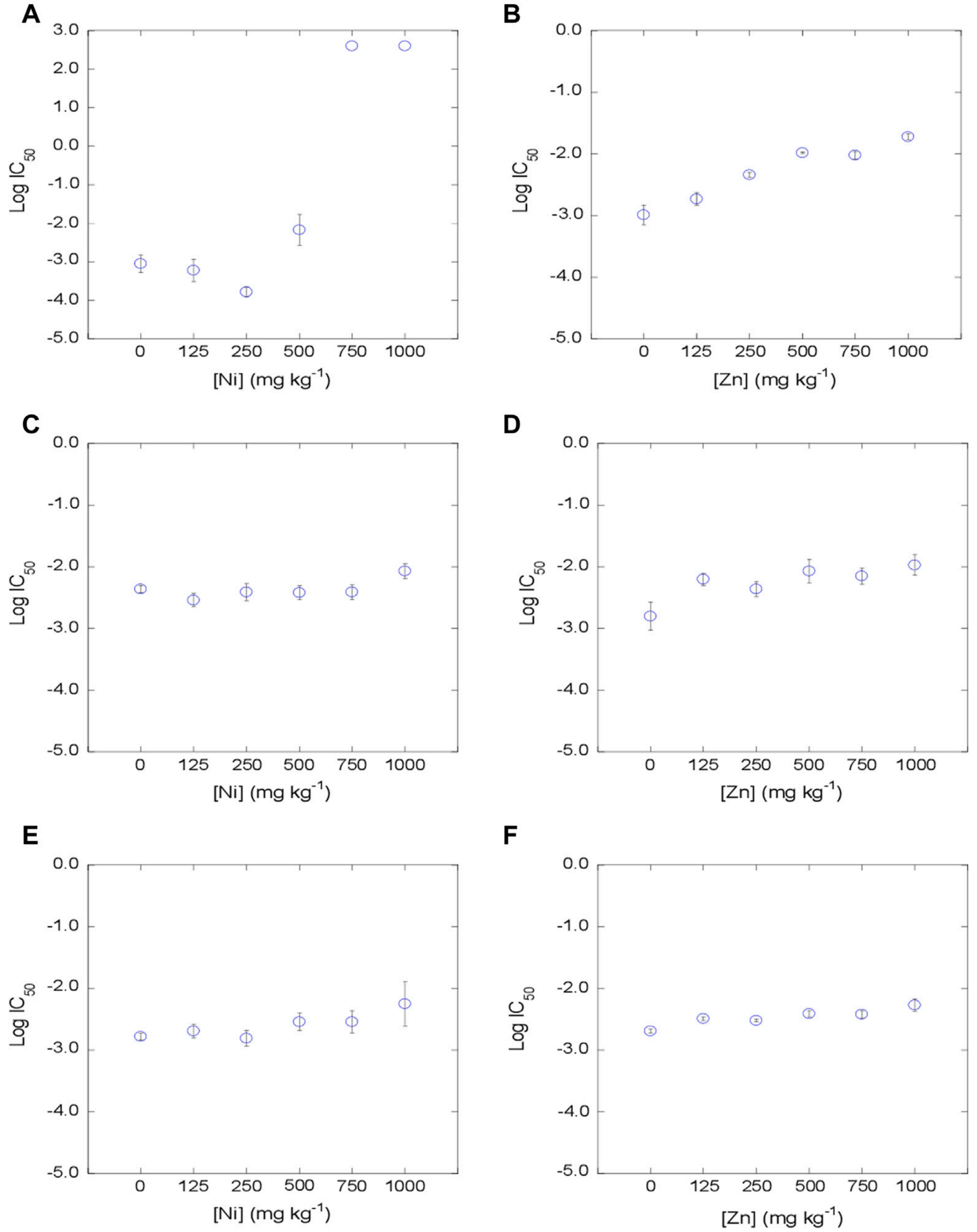

FIGURE 2 | Evolution of bacterial community tolerance to TC (as log IC_{50}), for increasing Ni and Zn concentration in the soil (0, 125, 250, 500, 750 and 1,000 mg kg^{-1}). **(A, B)**: soil 1; **(C, D)**: soil 2, **(E, F)**: soil 3.

suggests that bacterial communities that have been previously exposed to heavy metals may also develop co-tolerance to antibiotics. There are several studies that show that environmental contamination with heavy metals shows a high correlation with antibiotic resistance genes (ARGs) (Ji et al., 2012; Pal et al., 2015; Chen et al., 2019; Lu et al., 2020). Lu et al. (2020) studied the abundance of heavy metals, antibiotics and ARGs in sediments from China with organic matter values between 89 and 120 g kg-1. These authors found that there is a positive correlation between most of the tetracycline ARGs analyzed (*tetC, tetG, tetO, tetE*) and the presence of Zn in the sediments. However, in the case of Ni, no correlations were found with most of the tetracycline ARGs studied (only with *tetA* and *tetQ*). However, there are various mechanisms that can participate in the acquisition of resistance to heavy metals and antibiotics, such as co-resistance, cross-resistance and co-regulated resitant.

As previously commented, many studies show that there are increases in co-tolerance to antibiotics in the presence of different heavy metals from a functional and genetic point of view (Berg et al., 2010; Knapp et al., 2011; Hu et al., 2017; Knapp et al., 2017; Song

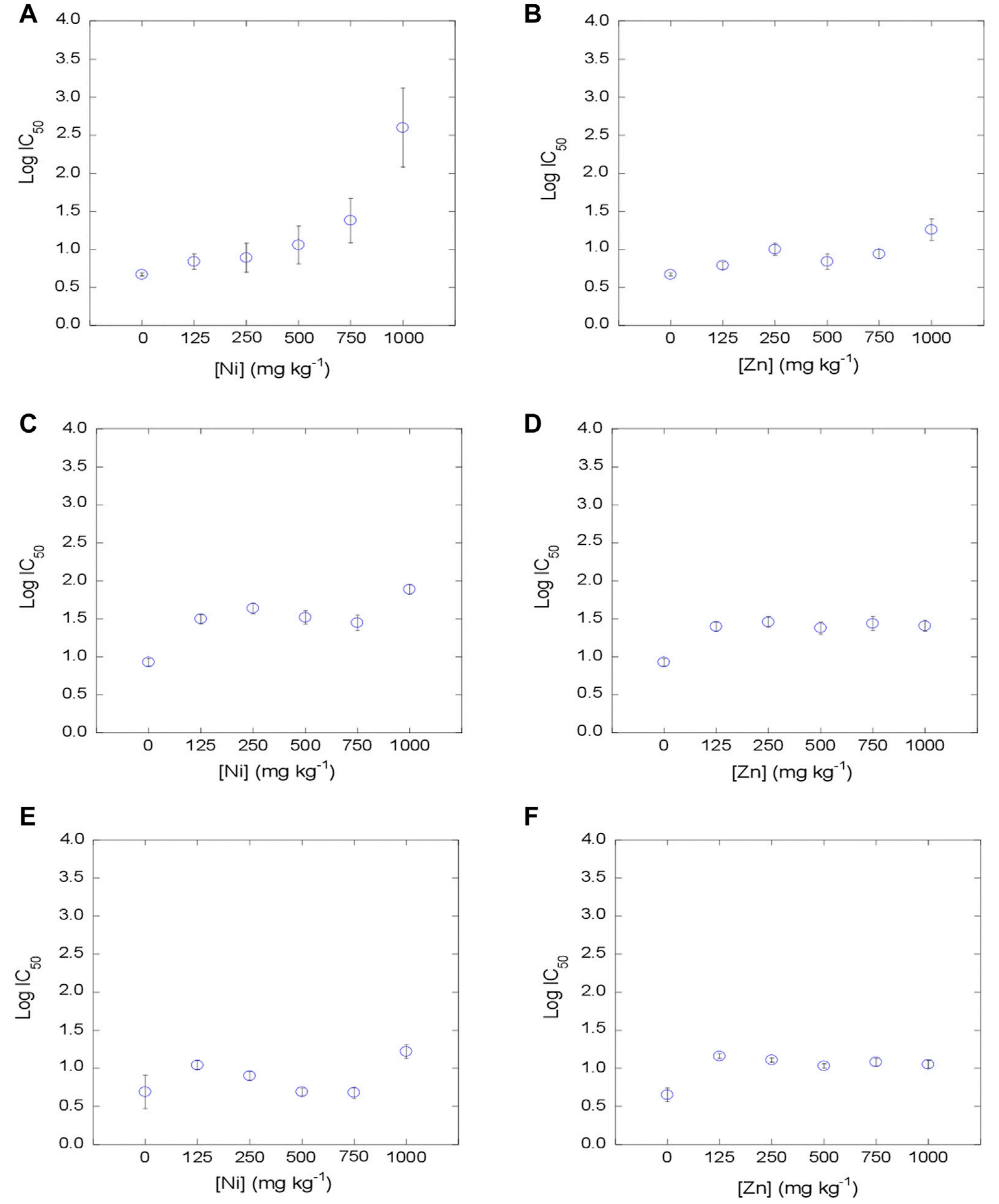

FIGURE 3 | Evolution of bacterial community tolerance to OTC (as log IC_{50}), for increasing Ni and Zn concentration in the soil (0, 125, 250, 500, 750 and 1,000 mg kg^{-1}). **(A, B)**: soil 1; **(C, D)**: soil 2, **(E, F)**: soil 3.

et al., 2017; Xu et al., 2017; Nguyen et al., 2019; Santás-Miguel et al., 2020b; Zhong et al., 2021; Santás-Miguel et al., 2022). But rather few studies have focused on the concentrations of heavy metals present in soils triggering the co-tolerance of soil bacterial communities to antibiotics and, more specifically, to tetracycline antibiotics. In this regard, Santás-Miguel et al. (2022) observed increases in co-tolerance to tetracycline antibiotics at 1,000 mg kg^{-1} of 7 heavy metals separately added to the soil, including Ni and Zn among them. Song et al. (2017) observed increases in co-tolerance to tetracycline in a soil sample (%C = 1.4) contaminated with Zn at concentrations ≥264 mg kg^{-1}. However, Zhong et al. (2021) did not observe an increase in co-tolerance to TC in soils contaminated with different concentrations of Zn (33–3,811 mg kg^{-1}). As for Ni, there are very few studies dealing with increases in co-tolerance to tetracycline antibiotics. Hu et al. (2017) observed an increase in antibiotic resistance genes (ARGs) at Ni concentrations in soil ≥100 mg kg^{-1}, concentrations similar to those observed in the present study for soils with a carbon content ≤5.3% (soil 1 and 2). Therefore, although the effect of heavy metals on soil bacterial communities is a widely studied topic, additional research should be carried out in the future, to be added to the current investigation, to shed further light on what are the concentrations

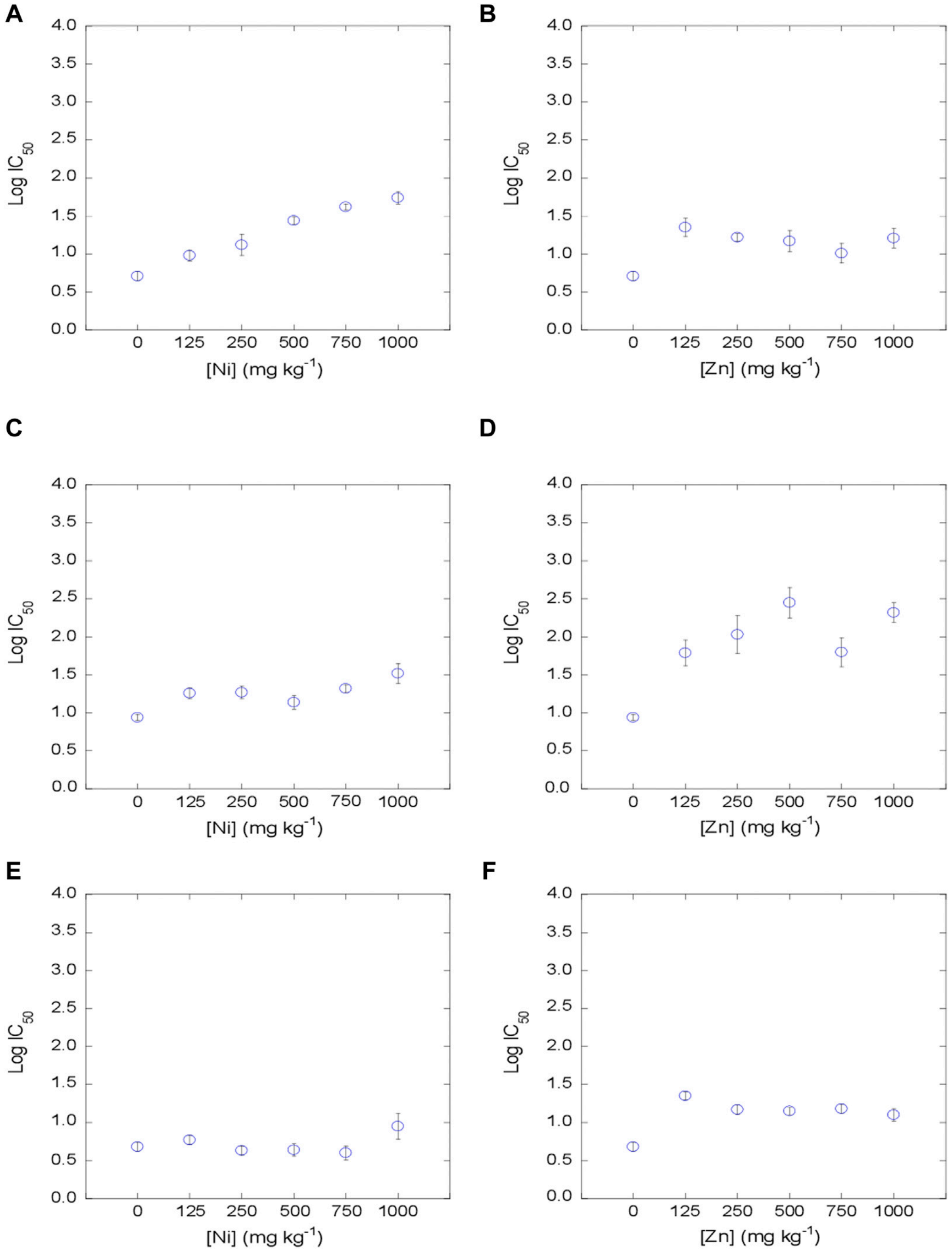

FIGURE 4 | Evolution of bacterial community tolerance to CTC (as log IC_{50}), for increasing Ni and Zn concentration in the soil (0, 125, 250, 500, 750 and 1,000 mg kg^{-1}). **(A, B)**: soil 1; **(C, D)**: soil 2, **(E, F)**: soil 3.

of different heavy metals from which there is a risk of increased co-tolerance to a vast variety of antibiotics. This environmental problem must be treated as a priority to recognize which are the soils with potential risk of presenting bacterial communities resistant to antibiotics and thus avoid the dissemination of antibiotic resistance genes in the environment.

CONCLUSION

In the present study it was observed that, for the 3 agricultural soils studied, Zn presented higher toxicity than Ni on soil bacterial communities. In addition, the soils contaminated with different concentrations of Ni and Zn showed increased tolerance to both metals, with a slight dependence on the soil carbon content in the case of Ni. However, for Zn these increments in tolerance were independent of the soil carbon content. In addition, the samples contaminated with Ni showed increases in co-tolerance from low concentrations of these heavy metals (≥125 mg kg^{-1}) for tetracycline antibiotics (TC, OTC and CTC) for soils with carbon content ≤5.3%, whereas, for soil with a high carbon content (C = 10.9%), co-tolerance increases occur at concentrations ≥1,000 mg kg^{-1}. On the other hand, the samples contaminated with Zn showed increases in co-tolerance from low concentrations of this metal (≥125 mg kg^{-1}) for the 3 tetracycline antibiotics. As a suggestion for future research, the study of the interactions between different contaminants present in agricultural soils and the selection pressure they exert on soil bacterial communities should be addressed in more depth. The acquisition of this knowledge will allow the implementation of more appropriate agricultural soil management practices, such as the improvement of high-carbon amendments to reduce the bioavailability of these elements and thus reduce the risk to human and environmental health that contaminated soils show.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

Conceptualization; MA-E, EÁ-R, DF-C, AN-D, MD-R; Methodology: MA-E, EÁ-R, DF-C, AN-D, MD-R; Software: LR-G, VS-M; Data curation: MA-E, EÁ-R, DF-C, MD-R; Writing—Original draft preparation: VS-M, MA-E, DF-C, MD-R; Visualization: MA-E, EÁ-R, DF-C, AN-D, MD-R, LR-G, VS-M; Investigation: LR-G, VS-M; Supervision: MA-E, EÁ-R, DF-C, AN-D, MD-R; Validation: MA-E, EÁ-R, DF-C, AN-D, MD-R; Writing—Reviewing and Editing: MD-R, AN-D.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/sjss.2023.10799/full#supplementary-material

REFERENCES

Abdu, N., Abdullahi, A. A., and Abdulkadir, A. (2017). Heavy Metals and Soil Microbes. *Environ. Chem. Lett.* 15 (1), 65–84. doi:10.1007/s10311-016-0587-x

Almås, Å. R., Bakken, L. R., and Mulder, J. (2004). Changes in Tolerance of Soil Microbial Communities in Zn and Cd Contaminated Soils. *Soil Biol. biochem.* 36 (5), 805–813. doi:10.1016/j.soilbio.2004.01.010

Bååth, E. (1989). Effects of Heavy Metals in Soil on Microbial Processes and Populations (A Review). *Water Air Soil Pollut.* 47 (3), 335–379. doi:10.1007/BF00279331

Berg, J., Thorsen, M. K., Holm, P. E., Jensen, J., Nybroe, O., and Brandt, K. K. (2010). Cu Exposure under Field Conditions Coselects for Antibiotic Resistance as Determined by a Novel Cultivationindependent Bacterial Community Tolerance Assay. *Environ. Sci. Technol.* 44, 8724–8728. doi:10.1021/es101798r

Blanck, H. (2002). A Critical Review of Procedures and Approaches Used for Assessing Pollution-Induced Community Tolerance (PICT) in Biotic Communities. *Hum. Ecol. Risk Assess.* 8 (5), 1003–1034. doi:10.1080/1080-700291905792

Brookes, P. C. (1995). The Use of Microbial Parameters in Monitoring Soil Pollution by Heavy Metals. *Biol. Fertil. Soils.* 19 (4), 269–279. doi:10.1007/BF00336094

Caban, J. R., Kuppusamy, S., Kim, J. H., Yoon, Y. E., Kim, S. Y., and Lee, Y. B. (2018). Green Manure Amendment Enhances Microbial Activity and Diversity in Antibiotic Contaminated Soil. *Appl. Soil Ecol.* 129, 72–76. doi:10.1016/j.apsoil.2018.04.013

Chen, J., Li, J., Zhang, H., Shi, W., and Liu, Y. (2019). Bacterial Heavy-Metal and Antibiotic Resistance Genes in a Copper Tailing Dam Area in Northern China. *Front. Microbiol.* 10, 1916. doi:10.3389/fmicb.2019.01916

Conde-Cid, M., Álvarez-Esmorís, C., Paradelo-Núñez, R., Nóvoa-Muñoz, J. C., Arias- Estévez, M., Álvarez-Rodríguez, E., et al. (2018). Occurrence of Tetracyclines and Sulfonamides in Manures, Agricultural Soils and Crops from Different Areas in Galicia (NW Spain). *J. Clean. Prod.* 197, 491–500. doi:10.1016/j.jclepro.2018.06.217

Davies, J. (1994). Inactivation of Antibiotics and the Dissemination of Resistance Genes. *Science* 264 (5157), 375–382. doi:10.1126/science.8153624

Davis, M. R., Zhao, F. J., and McGrath, S. P. (2004). Pollution-induced Community Tolerance of Soil Microbes in Response to a Zinc Gradient. *Environ. Toxicol. Chem.* 23 (11), 2665–2672. doi:10.1897/03-645

Diaz-Ravina, M., and Baath, E. (1996). Development of Metal Tolerance in Soil Bacterial Communities Exposed to Experimentally Increased Metal Levels. *Appl. Environ. Microbiol.* 62 (8), 2970–2977. doi:10.1128/aem.62.8.2970-2977.1996

Díaz-Raviña, M., Bååth, E., and Frostegård, Å. (1994). Multiple Heavy Metal Tolerance of Soil Bacterial Communities and its Measurement by a Thymidine Incorporation Technique. *Appl. Environ. Microbiol.* 60 (7), 2238–2247. doi:10.1128/aem.60.7.2238-2247.1994

Dıaz-Ravina, M., and Bååth, E. (2001). Response of Soil Bacterial Communities Pre-exposed to Different Metals and Reinoculated in an Unpolluted Soil. *Soil Biol. biochem.* 33 (2), 241–248. doi:10.1016/S0038-0717(00)00136-X

Dickinson, A. W., Power, A., Hansen, M. G., Brandt, K. K., Piliposian, G., Appleby, P., et al. (2019). Heavy Metal Pollution and Co-selection for Antibiotic Resistance: A Microbial Palaeontology Approach. *Environ. Int.* 132, 105117. doi:10.1016/j.envint.2019.105117

Duxbury, T., and Bicknell, B. (1983). Metal-tolerant Bacterial Populations from Natural and Metal-Polluted Soils. *Soil Biol. biochem.* 15 (3), 243–250. doi:10.1016/0038-0717(83)90066-4

EC (2006). "European Commission Regulation EC 1881/2006. Setting Maximum Levels for Certain Contaminants in Foodstuffs"," in *Official Journal of the European Union EC 1881/2006*. (20.12.2006), L364/365-L364/324.

European Medicines Agency (2016). "European Surveillance of Veterinary Antimicrobial Consumption," in *Sales of Veterinary Antimicrobial Agents in 29 European Countries in 2014*. EMA/61769/2016.

Fernández-Calviño, D., and Bååth, E. (2013). Co-selection for Antibiotic Tolerance in Cu-Polluted Soil Is Detected at Higher Cu-Concentrations Than Increased Cu-Tolerance. *Soil Biol. biochem.* 57, 953–956. doi:10.1016/j.soilbio.2012.08.017

Giller, K. E., Witter, E., and McGrath, S. P. (1999). Assessing Risks of Heavy Metal Toxicity in Agricultural Soils: Do Microbes Matter? *Hum. Ecol. Risk Assess.* 5 (4), 683–689. doi:10.1080/10807039.1999.9657732

Giller, K. E., Witter, E., and McGrath, S. P. (2009). Heavy Metals and Soil Microbes. *Soil Biol. biochem.* 41 (10), 2031–2037. doi:10.1016/j.soilbio.2009.04.026

Giller, K. E., Witter, E., and Mcgrath, S. P. (1998). Toxicity of Heavy Metals to Microorganisms and Microbial Processes in Agricultural Soils: a Review. *Soil Biol. biochem.* 30 (10-11), 1389–1414. doi:10.1016/S0038-0717(97)00270-8

Gluskoter, H. J. (1977). *Trace Elements in Coal: Occurrence and Distribution*. Urbana, IL. Circular no. 499.

Hamscher, G., Sczesny, S., Höper, H., and Nau, H. (2002). Determination of Persistent Tetracycline Residues in Soil Fertilized with Liquid Manure by High-Performance Liquid Chromatography with Electrospray Ionization Tandem Mass Spectrometry. *Anal. Chem.* 74 (7), 1509–1518. doi:10.1021/ac015588m

Hattori, H. (1992). Influence of Heavy Metals on Soil Microbial Activities. *Soil Sci. Plant Nutr.* 38 (1), 93–100. doi:10.1080/00380768.1992.10416956

He, Z., Endale, D. M., Schomberg, H. H., and Jenkins, M. B. (2009). Total Phosphorus, Zinc, Copper, and Manganese Concentrations in Cecil Soil through 10 Years of Poultry Litter Application. *Soil Sci.* 174 (12), 687–695. doi:10.1097/SS.0b013e3181c30821

Hejna, M., Gottardo, D., Baldi, A., Dell'Orto, V., Cheli, F., Zaninelli, M., et al. (2018). Review: Nutritional Ecology of Heavy Metals. *Animal* 12 (10), 2156–2170. doi:10.1017/S175173111700355X

Hu, H. W., Wang, J. T., Li, J., Shi, X. Z., Ma, Y. B., Chen, D., et al. (2017). Long-term Nickel Contamination Increases the Occurrence of Antibiotic Resistance Genes in Agricultural Soils. *Environ. Sci. Technol.* 51 (2), 790–800. doi:10.1021/acs.est.6b03383

Ji, X., Shen, Q., Liu, F., Ma, J., Xu, G., Wang, Y., et al. (2012). Antibiotic Resistance Gene Abundances Associated with Antibiotics and Heavy Metals in Animal Manures and Agricultural Soils Adjacent to Feedlots in Shanghai; China. *J. Hazard. Mat.* 235, 178–185. doi:10.1016/j.jhazmat.2012.07.040

Kabata-Pendias, A. (2000). *Trace Elements in Soils and Plants*. Boca Raton, FL: CRC Press.

Khan, S. U., and Schnitzer, M. (1978). *Soil Organic Matter (No. 631.42 SOI)*.

Knapp, C. W., Callan, A. C., Aitken, B., Shearn, R., Koenders, A., and Hinwood, A. (2017). Relationship between Antibiotic Resistance Genes and Metals in Residential Soil Samples from Western Australia. *Environ. Sci. Pollut. Res.* 24 (3), 2484–2494. doi:10.1007/s11356-016-7997-y

Knapp, C. W., Dolfing, J., Ehlert, P. A., and Graham, D. W. (2010). Evidence of Increasing Antibiotic Resistance Gene Abundances in Archived Soils since 1940. *Environ. Sci. Technol.* 44 (2), 580–587. doi:10.1021/es901221x

Knapp, C. W., McCluskey, S. M., Singh, B. K., Campbell, C. D., Hudson, G., and Graham, D. W. (2011). Antibiotic Resistance Gene Abundances Correlate with Metal and Geochemical Conditions in Archived Scottish Soils. *PloS One* 6 (11), e27300. doi:10.1371/journal.pone.0027300

Lu, L., Liu, J., Li, Z., Zou, X., Guo, J., Liu, Z., et al. (2020). Antibiotic Resistance Gene Abundances Associated with Heavy Metals and Antibiotics in the Sediments of Changshou Lake in the Three Gorges Reservoir Area, China. *Ecol. Indic.* 113, 106275. doi:10.1016/j.ecolind.2020.106275

Macías, F., and Calvo, R. (2008). "Niveles genéricos de referencia de metales pesados y otroselementos traza en suelos de Galicia (Reference values for heavy metals and other traceelements in soils in Galicia)," in *Consellería de Medio Ambiente e Desenvolvemento Sostible* (Santiago de Compostela, Spain: Xunta de Galicia), 229.

McBride, M. B. (1994). *Environmental Chemistry of Soils*. New York: Oxford University Press.

Meisner, A., Bååth, E., and Rousk, J. (2013). Microbial Growth Responses Upon Rewetting Soil Dried for Four Days or One Year. *Soil Biol. Biochem.* 66, 188–192. doi:10.1016/j.soilbio.2013.07.014

Milne, C. J., Kinniburgh, D. G., Van Riemsdijk, W. H., and Tipping, E. (2003). Generic NICA—Donnan Model Parameters for Metal-Ion Binding by Humic Substances. *Environ. Sci. Technol.* 37 (5), 958–971. doi:10.1021/es0258879

Nguyen, C. C., Hugie, C. N., Kile, M. L., and Navab-Daneshmand, T. (2019). Association between Heavy Metals and Antibiotic-Resistant Human Pathogens in Environmental Reservoirs: a Review. *Front. Environ. Sci. Eng.* 13 (3), 46–17. doi:10.1007/s11783-019-1129-0

Nies, D. H. (1999). Microbial Heavy-Metal Resistance. *Appl. Microbiol. Biotechnol.* 51 (6), 730–750. doi:10.1007/s002530051457

Oyetibo, G. O., Ilori, M. O., Adebusoye, S. A., Obayori, O. S., and Amund, O. O. (2010). Bacteria with Dual Resistance to Elevated Concentrations of Heavy Metals and Antibiotics in Nigerian Contaminated Systems. *Environ. Monit. Assess.* 168 (1), 305–314. doi:10.1007/s10661-009-1114-3

Pal, C., Bengtsson-Palme, J., Kristiansson, E., and Larsson, D. G. (2015). Co-occurrence of Resistance Genes to Antibiotics, Biocides and Metals Reveals Novel Insights into Their Co-selection Potential. *BMC genomics* 16 (1), 964–1014. doi:10.1186/s12864-015-2153-5

Piccolo, A. (1989). Reactivity of Added Humic Substances towards Plant Available Heavy Metals in Soils. *Sci. Total Environ.* 81, 607–614. doi:10.1016/0048-9697(89)90169-1

Refaey, Y., Jansen, B., El-Shater, A. H., El-Haddad, A. A., and Kalbitz, K. (2014). The Role of Dissolved Organic Matter in Adsorbing Heavy Metals in Clay-Rich Soils. *Vadose Zone J.* 13 (7), vzj2014010009. doi:10.2136/vzj2014.01.0009

Rodríguez-López, L., Santás-Miguel, V., Núñez-Delgado, A., Álvarez-Rodríguez, E., Pérez-Rodríguez, P., and Arias-Estévez, M. (2022). Influence of pH, Humic Acids, and Salts on the Dissipation of Amoxicillin and Azithromycin under Simulated Sunlight. *Span. J. Soil Sci.* 12. doi:10.3389/sjss.2022.10438

Rule, J. H. (1999). Trace Metal Cation Adsorption in Soils: Selective Chemical Extractions and Biological Availability. *Stud. Surf. Sci. Catal.* 120, 319–349. doi:10.1016/S0167-2991(99)80364-4

Santás-Miguel, V., Arias-Estévez, M., Díaz-Raviña, M., Fernández-Sanjurjo, M. J., Álvarez-Rodríguez, E., Núñez-Delgado, A., et al. (2020b). Bacterial Community Tolerance to Tetracycline Antibiotics in Cu Polluted Soils. *Agronomy* 10, 1220. doi:10.3390/agronomy10091220

Santás-Miguel, V., Arias-Estévez, M., Díaz-Raviña, M., Fernández-Sanjurjo, M. J., Álvarez-Rodríguez, E., Núñez-Delgado, A., et al. (2020d). Effect of Oxytetracycline and Chlortetracycline on Bacterial Community Growth in Agricultural Soils. *Agronomy* 10 (7), 1011. doi:10.3390/agronomy10071011

Santás-Miguel, V., Arias-Estévez, M., Díaz-Raviña, M., Fernández-Sanjurjo, M. J., Álvarez-Rodríguez, E., Núñez-Delgado, A., et al. (2020c). Interactions between Soil Properties and Tetracycline Toxicity Affecting to Bacterial Community Growth in Agricultural Soil. *Appl. Soil Ecol.* 147, 103437. doi:10.1016/j.apsoil.2019.103437

Santás-Miguel, V., Díaz-Raviña, M., Martín, A., García-Campos, E., Barreiro, A., Núñez-Delgado, A., et al. (2020a). Medium-term Influence of Tetracyclines on Total and Specific Microbial Biomass in Cultivated Soils of Galicia (NW Spain). *Span. J. Soil Sci.* 10, 217–232. doi:10.3232/SJSS.2020.V10.N3.05

Santás-Miguel, V., Núñez-Delgado, A., Álvarez-Rodríguez, E., Díaz-Raviña, M., Arias-Estévez, M., and Fernández-Calviño, D. (2022). Tolerance of Soil Bacterial Community to Tetracycline Antibiotics Induced by as, Cd, Zn, Cu, Ni, Cr, and Pb Pollution. *SOIL* 8 (1), 437–449. doi:10.5194/soil-8-437-2022

Sapkota, A., Sapkota, A. R., Kucharski, M., Burke, J., McKenzie, S., Walker, P., et al. (2008). Aquaculture Practices and Potential Human Health Risks: Current Knowledge and Future Priorities. *Environ. Int.* 34 (8), 1215–1226. doi:10.1016/j.envint.2008.04.009

Sarmah, A. K., Meyer, M. T., and Boxall, A. B. (2006). A Global Perspective on the Use, Sales, Exposure Pathways, Occurrence, Fate and Effects of Veterinary Antibiotics (VAs) in the Environment. *Chemosphere* 65 (5), 725–759. doi:10.1016/j.chemosphere.2006.03.026

Sauvé, S., Manna, S., Turmel, M. C., Roy, A. G., and Courchesne, F. (2003). Solid–Solution Partitioning of Cd, Cu, Ni, Pb, and Zn in the Organic Horizons of a Forest Soil. *Environ. Sci. Technol.* 37 (22), 5191–5196. doi:10.1021/es030059g

Sebaugh, J. L. (2011). Guidelines for Accurate EC50/IC50 Estimation. *Pharm. Stat.* 10 (2), 128–134. doi:10.1002/pst.426

Seiler, C., and Berendonk, T. U. (2012). Heavy Metal Driven Co-selection of Antibiotic Resistance in Soil and Water Bodies Impacted by Agriculture and Aquaculture. *Front. Microbiol.* 3, 399. doi:10.3389/fmicb.2012.00399

Serwecińska, L. (2020). Antimicrobials and Antibiotic-Resistant Bacteria: a Risk to the Environment and to Public Health. *Water* 12 (12), 3313. doi:10.3390/w12123313

Song, J., Rensing, C., Holm, P. E., Virta, M., and Brandt, K. K. (2017). Comparison of Metals and Tetracycline as Selective Agents for Development of Tetracycline Resistant Bacterial Communities in Agricultural Soil. *Environ. Sci. Technol.* 51 (5), 3040–3047. doi:10.1021/acs.est.6b05342

Spark, K. M., Wells, J. D., and Johnson, B. B. (1997). The Interaction of a Humic Acid with Heavy Metals. *Soil Res.* 35 (1), 89–102. doi:10.1071/S96008

Stefanowicz, A. M., Niklińska, M., and Laskowski, R. (2009). Pollution-induced Tolerance of Soil Bacterial Communities in Meadow and Forest Ecosystems Polluted with Heavy Metals. *Eur. J. Soil Biol.* 45 (4), 363–369. doi:10.1016/j.ejsobi.2009.05.005

Swift, M. J. (1994). "Maintaining the Biological Status of Soil: a Key to Sustainable Land Management," in *Soil Resilience and Sustainable Land Use*. Editors D. J. Greenland, and I. Szabolcs (Wallingford: CAB), 33–39.

Thiele-Bruhn, S. (2005). Microbial Inhibition by Pharmaceutical Antibiotics in Different Soils—Dose-response Relations Determined with the Iron (III) Reduction Test. *Environ. Toxicol. Chem.* 24 (4), 869–876. doi:10.1897/04-166R.1

Toth, J. D., Feng, Y., and Dou, Z. (2011). Veterinary Antibiotics at Environmentally Relevant Concentrations Inhibit Soil Iron Reduction and Nitrification. *Soil Biol. biochem.* 43 (12), 2470–2472. doi:10.1016/j.soilbio.2011.09.004

Urra, J., Alkorta, I., Lanzén, A., Mijangros, I., and Garbisu, C. (2019). The Application of Fresh and Composted Horse and Chicken Manure Affects Soil Quality, Microbial Composition and Antibiotic Resistance. *Appl. Soil Ecol.* 135, 73–84. doi:10.1016/j.apsoil.2018.11.005

Wu, L., Pan, X., Chen, L., Huang, Y., Teng, Y., Luo, Y., et al. (2013). Occurrence and Distribution of Heavy Metals and Tetracyclines in Agricultural Soils after Typical Land Use Change in East China. *Environ. Sci. Pollut. Res.* 20, 8342–8354. doi:10.1007/s11356-013-1532-1

Xu, Y., Xu, J., Mao, D., and Luo, Y. (2017). Effect of the Selective Pressure of Sub-lethal Level of Heavy Metals on the Fate and Distribution of ARGs in the Catchment Scale. *Environ. Pollut.* 220, 900–908. doi:10.1016/j.envpol.2016.10.074

Zhang, F., Li, Y., Yang, M., and Li, W. (2012). Content of Heavy Metals in Animal Feeds and Manures from Farms of Different Scales in Northeast China. *Int. J. Environ. Res. Public Health.* 9 (8), 2658–2668. doi:10.3390/ijerph9082658

Zhong, Q., Cruz-Paredes, C., Zhang, S., and Rousk, J. (2021). Can Heavy Metal Pollution Induce Bacterial Resistance to Heavy Metals and Antibiotics in Soils from an Ancient Land-Mine? *J. Hazard. Mat.* 411, 124962. doi:10.1016/j.jhazmat.2020.124962

ORIGINAL RESEARCH
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Nitrate and Phosphorus Transport in a Galician River (NW Iberian Peninsula): Insights From Fourteen Years of Monitoring

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The long-term development of water quality metrics is critical to estimate the status of water resources and successful catchment management. This study looks at the temporal evolution of nitrate and phosphorus loads and concentrations in a stream draining rural catchment in Galicia (NW Iberian Peninsula) during 14 hydrological years. The concentrations of both nutrients are relatively low because of the rural nature of the research catchment and low-intensity agriculture. However, catchment nutrient reference levels were widely exceeded. The hydrology controls nutrient fluxes as flow is the main driver of nutrient transfer. For both nutrients there were no consistent trends in nutrient fluxes. The analysed nutrients, however, have been seen to behave differently. As a result, N seems to be decreasing, mainly due to decreased transit in the autumn and spring. Phosphorus, which is primarily transported as particulate matter, exhibits an increase in winter linked to a greater flow and sediment transfer.

Keywords: long-term, nutrients, Galicia, rural catchment, land use

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INTRODUCTION

The natural quality of inland waters is related to the geomorphology, climate, and land use in the catchment. Thus, size and slope of the catchment, rainfall, temperatures, vegetation, erosion and soil structure play an important role in water quality. However, due to human activities, the high loads of sediments and nutrients transported by rivers have become one of the greatest problems causing the deterioration of the quality of surface waters on a global scale (Bennet et al., 2001; Worrall et al., 2016; Grizzetti et al., 2021; Kelly et al., 2022), and this explains why in recent years, determined efforts have been made both in the field of research and management and restoration.

Although water management and use has been one of the main concerns of the European Water Policy since the 1960s, it was only in the year 2000 that progress was made in the protection of aquatic resources with the publication of the Water Framework Directive (WFD; Directive, 2000) by the European Parliament and the Council of the European Union, in which a community action framework was established in the field of water policy. The WFD focuses on the protection of all water bodies present in the territory of the European Community. Its objectives are to prevent the deterioration of water resources, protect and improve their condition, promote the sustainable use of the resource, guarantee a reduction in pollution, prevent the effects of floods and droughts, and achieve at least good ecological status by the year 2015. Despite the progress made with the scope of improving the environmental quality of the various water sources in Europe, the last report by the

European Environment Agency (European Environment Agency, 2018) indicated that 47% of European surface waters continue to fail to meet the minimum objective of good ecological status, with agricultural activities and the urbanisation of rural landscapes being the main threats to fluvial courses (European Environment Agency, 2018). Therefore, the frequent use of agrochemicals to meet production demands means that agricultural systems play an important role as a source of contamination (non-point source) of water bodies (Oenema et al., 2005; Sharpley et al., 2008; Lassaletta et al., 2009). Moreover, over the last few decades, the progressive implementation of modern agriculture and livestock methods, with the introduction of increasingly intensive tillage techniques, an increase in the size of plots, the establishment of monocultures, etc. has promoted runoff and soil erosion (Panagos et al., 2015). In general, chemicals from non-point sources are transported to nearby water bodies either in dissolved form or along with sediment during heavy rainfall and erosion events, even in gently sloping areas (Sharpley et al., 2008; Dupas et al., 2017).

The latest reports from the European Environment Agency noted an improvement in 20% of the stations, directly attributed to the implementation of measures to control diffuse (non-point) contamination and increased efficiency in wastewater treatment (Carvahlo et al., 2019). Thus, based on reported long-term data for nitrates in European waters, there was a falling trend for nitrate concentration in rivers, although this has levelled off since 2009. The reason for nitrate persistence is due to overuse of nitrogen, which can remain in the subsurface flow for decades in catchments with long time response and add to increased nitrate loads long after application practices have changed, or mitigation strategies have been implemented (Fovet et al., 2015; Ehrbardt et al., 2021). These and other provenances of nitrate create a heterogeneous patchwork of source areas across the landscape that can be activated and deactivated in response to changing hydrological conditions (Dupas et al., 2019). For phosphorus, a marked decline was reported due to improved wastewater treatment, reduction of P in detergents, as well as reduction in soil fertilization rates and implementation of measures to reduce soil erosion and connectivity to the receiving waters (Oenema eta l., 2005; Minaudo et al., 2015; Moatar et al., 2017). However, phosphorus may persist through soil enrichment, and therefore it may take a long time before any major water quality improvements can be detected in the recipient water bodies after decreasing fertilisation of agricultural fields (Withers and Lord, 2002; Ekholm et al., 2015).

Correct assessment of the quality of water resources in the catchment is essential to estimate the status of the resources and start management and planning, if required. In fact, the WFD urges members to carry out studies on trends of different contaminants (with trend understood to be the monotonic variation of the concentration in relation to time) and identify those water bodies exceeding established reference nutrient levels. This requires sufficiently frequent monitoring of the water quality over long periods of time, which is an arduous, costly, and time-consuming task. At present, several countries, including Spain, must improve their control programs to guarantee broad and consistent monitoring of water bodies, with appropriate coverage of all relevant quality indicators, since there are still significant deficiencies. However, the number of checkpoints has decreased in recent years as compared to the first planning cycles of the WFD. This includes the Galician region, popularly known as "the country of a thousand rivers." Despite this, the scientific community did not pay much attention to the health of Galician rivers. To date, they have not undergone extensive monitoring because hydrological and hydrochemical studies based on catchment monitoring are relatively recent in the community. This underlines the need to continue long-term monitoring efforts for water quality assessments and to identify trends.

The objective of this paper was to explore the temporal evolution of nitrate and phosphorus loads and concentrations in a stream draining a typical rural catchment in Galicia (NW Iberian Peninsula), i.e., small population centres and low-intensity agriculture. The study is based on information collected over a period of 14 years in which regular manual sampling was combined with automatic sampling during runoff episodes.

MATERIAL AND METHODS

Study Area

The study site is the Corbeira stream headwater catchment located in NW Spain (**Figure 1**, gauge at 43° 12′58″N, 8° 13′48″W). The Corbeira is a right-bank tributary of the Mero River, the main river contributing to the Cecebre reservoir (water supply for the city of A Coruña and surroundings (about 450,000 inhabitants) discharging into the Ria do Burgo, in the vicinity of the city of A Coruña (**Figure 1**). The total length of the stream is 10 km, and the whole catchment area covers about 16 km^2, with altitudes ranging from 65 m a.s.l. at the confluence with the Mero River to 470 m a.s.l. at the highest part. The mean slope is 19%, although approximately 27% of the catchment is steep, i.e., slopes higher than 25%. From a geological point of view, it is located within the Ordenes Complex and is dominated by basic schists (IGME, 1981), with phyllites and biotitic schists the most common petrological types in the area, which confers a certain uniformity to the lithological substrate. Schists are made up of easily alterable minerals, such as biotite (sometimes chlorite), plagioclase, and amphiboles. The soils are Umbrisols and Cambisol (IUSS Working Group WRB, 2014) with a silt and silty loam texture developed. The organic matter content in the upper horizon varies from less than 5.3% in cultivated lands, to 10.5% in forest soils, highlighting the importance of the latter in carbon sequestration. Total N soil concentrations ranged between 0.26% on cultivated land and 0.47% on grassland, whereas forest soils showed a mean value of 0.39% (Rodríguez-Blanco et al., 2015). The P Olsen content varied between 14.0 mg kg^{-1} in the forest soils and 69.0 mg kg^{-1} in cultivated fields, with the mean content of grassland 19.5 mg kg^{-1} (Rodríguez-Blanco et al., 2013).

The main land use is forest, which covers 65% of the catchment area and is mostly used for commercial plantations of eucalyptus and pine. In addition, there is, as in most Galician River catchments, a dense riparian vegetation basically consisting

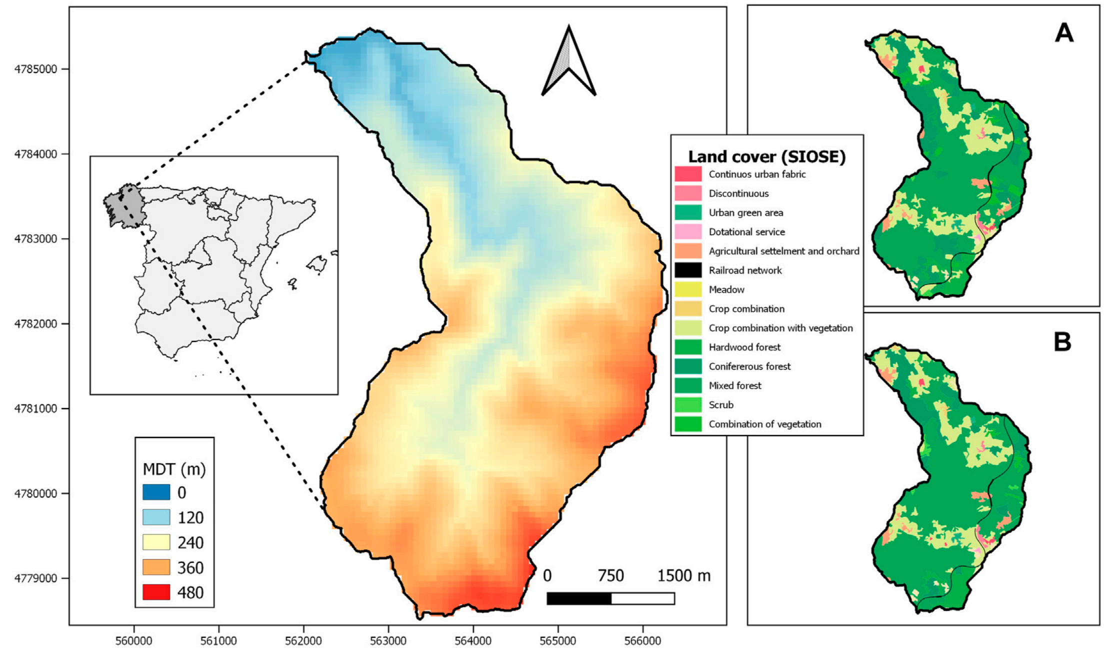

FIGURE 1 | Location of the study area. Changes in land use observed in the study area: **(A)** 2000 and **(B)** 2018 based on SIOSE.

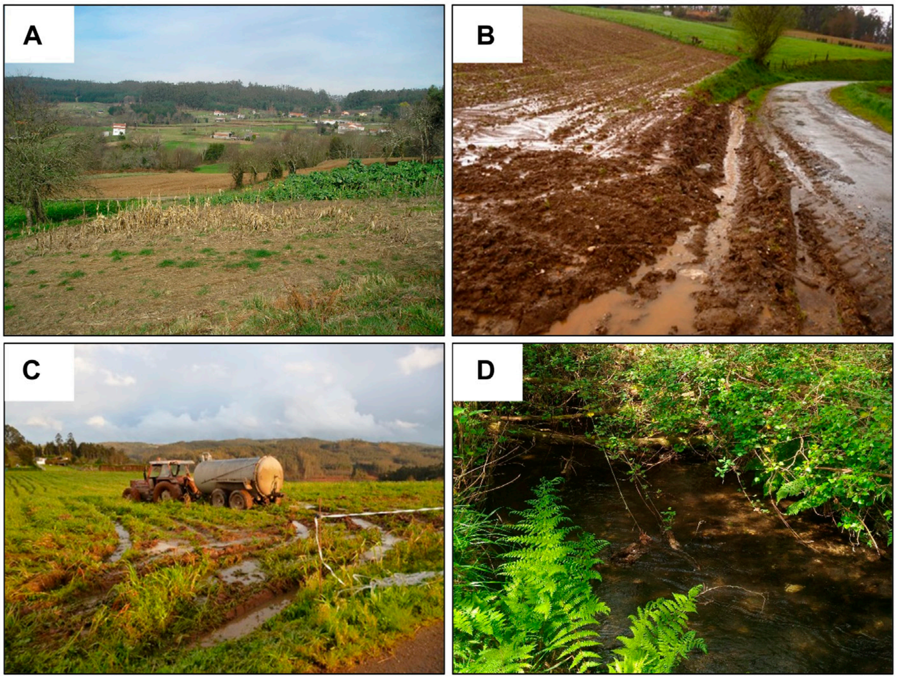

FIGURE 2 | Illustrative photographs of the study area. **(A)** Landscape mosaic in which meadows, cultivated land and forest area; **(B)** runoff generated on cultivated land devoid of vegetation and channeled through the ditch; **(C)** slurry application on the ground at field capacity, and **(D)** riverside vegetation.

of *Alnus glutinosa*, *Betula alba* and *Salix spp*. The agricultural surface represents 30% of the catchment and is dominated by pasture and meadows (26%), the latter being humid areas mainly in flood zones. They are used to produce green, dry, or ensiled forage for consumption. Cropland comprises approximately 4% of the catchment area and is essentially used for forage crops, such

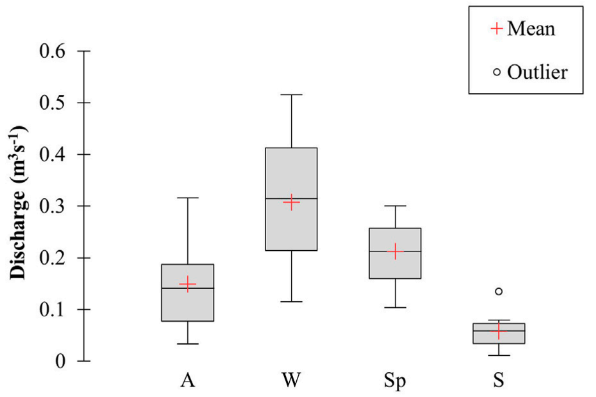

FIGURE 3 | Box plot of seasonal distribution of discharge in the Corbeira catchment. The length of the box plot shows the interquartile range, comprising 50% of the values, and the straight horizontal line inside the box represents the median. (Data from Rodríguez-Blanco et al., 2020). A: autumn, W: winter, Sp: spring, S: summer.

as maize and winter cereals. Potatoes, vegetables, and fruit trees are cultivated in small kitchen gardens and orchards. The remaining 5% of the catchment's surface is occupied by impervious areas, mostly road infrastructures (roads and forest paths) distributed throughout the catchment, crossing the main channel and its tributaries at numerous points. This information is based on land use maps from the Spanish Land Cover/Land Use Information System (SIOSE), used to calculate the cover (%) of land use for the years 2004 and 2018. A slight decrease was observed in the agricultural area during this period, whereas forests increased due to the replacement of cultivated land by plantations of fast-growing tree species, mainly eucalyptus. There is also a decrease in stocking density (dominated by cattle) during the study period, going from 0.96 livestock units (LU) per ha of utilised agriculture area (UAA) in 2004 to 0.73 LU per ha of UAA in 2018.

The distribution of land uses in the catchment is characterized by the mosaic presented in agricultural areas (**Figure 2**), which is generally bordered by meadows and, in some cases, by small stone walls, characteristic of smallholdings in Galicia. This distribution could be classified as a low-intensity agricultural mosaic (cropland and grassland), with a light input of inorganic fertilizer, small field sizes and low livestock density (Dou et al., 2021).

The study area has a marked rural nature (<150 inhab. km^{-2}; Organization for Economic Co-operation and Development-OECD, 2007), characterized by a low population density, which has gone from 35 inhab. km^{-2} at the beginning of the study (2004) to 32 inhab. km-^{2} in 2018 (rates calculated from data of Spanish Statistical Office-INE, 2019)- a 15% decrease in just 15 years. In fact, the Ministry of Agriculture statistics place Galicia at the head of the most isolated communities in rural areas. Between the years 2010–2018, the population in Galician villages fell by 17.9% (INE, 2019). Most of the population is distributed in scattered dwellings that lack a sewage system, so that domestic wastewater is stored in cesspools constituting small sources of contamination.

The climate is temperate oceanic, with an annual average temperature of 13.5°C ranging from 8.9°C in January and February (winter) to 18.8°C in July and August (summer). The rainfall is abundant (1,074 mm- period 1983/1984–2017/2018) and occurs throughout the year, although it is concentrated in the autumn and winter, with October, November and December being the wettest months and July and August the driest. The hydrological regime is pluvial oceanic; which implies two alternate periods: a high-discharge period during the wet period (i.e., late autumn and winter) and a low-discharge period during the dry season (i.e., late spring and summer) (**Figure 3**; Rodríguez-Blanco et al., 2020). Mean stream discharge is 0.181 m^{3}s^{-1}, ranging from 0.08 to 0.273 m^{3}s^{-1} (Rodríguez-Blanco et al., 2020). Baseflow contribution to the total streamflow (i.e., baseflow index), calculated according to Gustard et al. (1992), varies between 0.61 and 0.78 with an average of 0.71 (unpublished data), indicating that 71% of long-term streamflow in the study area is originated from groundwater.

Field Instrumentation and Water Sampling

The data used in this study covers 14 hydrological years from October 2004 to September 2018. Monitoring within the catchment included rainfall and discharge measurements and automatic and manual sampling of stream water at the catchment outlet. Rainfall was monitored at three locations across the catchment at 10-min intervals using tipping bucket rain gauges (0.2 mm resolution), followed by the Thiessen Polygon method (Linsely et al., 1949) to calculate the mean rainfall in the area. Stream water level was measured at the catchment outlet using a pressure transducer (ISCO 720), and discharge was calculated from the rating curve. Measured water levels at 1 min intervals were aggregated to 10-min values. Water sampling was carried out under baseflow conditions (manual sampling every 2 weeks) and during runoff events (automatic sampling, ISCO 6712-FS). Full details of the design of the monitoring and water sampling can be found in Rodríguez-Blanco et al. (2013, 2015).

Laboratory Analyses

Water samples were immediately brought back to the laboratory and refrigerated (4°C) until analysed for nitrate and total and dissolved phosphorus concentrations. After sample filtration (0.45 µm), nitrate concentrations (expressed as mg NO_3^- L^{-1}) were determined by capillary electrophoresis. Total phosphorus (TP) (µg P L^{-1}) was determined in unfiltered water samples by acid digestion with ammonium persulfate (APHA, 1998) followed by colorimetric determination using the molybdate-ascorbic acid method developed by (Murphy and Riley, 1962). Following water sample filtration, dissolved phosphorus (DP) was determined by ICP-MS. Particulate phosphorus (PP) was calculated as the difference between TP and DP.

Data and Statistical Analysis

Since the main objective of this work was to detect trends of nitrate and phosphorus in long time series, the load (of these nutrients) for different periods of time (monthly, seasonal, or annual) was calculated by summing up the products from the mean concentration between two consecutive samples and cumulative discharge for each time interval between two water samples. The discharge-weighted mean concentration of each nutrient for each specific period (monthly, seasonal, or annual) was calculated as the nutrient load of each period divided by the total discharge of the period. For the seasonal trend, the year was divided into four seasons: autumn (October, November, and December), winter (January, February, and March), spring (April, May, and June) and summer (July, August, and September).

Once the nutrient concentrations were estimated, two different methodologies were used to calculate the trend of nitrate concentrations. The first refers to the approach used by the member states in the context of the Nitrates Directive, which is based on calculating the differences between the average concentration of nitrates taken from two consecutive periods of 4 years. Differences in the range between −1 and 1 mg L^{-1} indicates the absence of trend, whereas those higher than 1 and 5 mg L^{-1} are rated as a positive and strongly upward trend. In the second approach, trend analysis was performed using the non-parametric Mann-Kendall test and the Sen method in the Maksesen program (developed by the Finnish Meteorological Office). Both tests are widely used for trend detection in hydro-meteorological records and water quality (Bouraoui and Malagó, 2020) and have also been recommended by the World Meteorological Organisation for analysing the trends in hydrometeorological time series (Kumar et al., 2009). The presence of a monotonic upward or downward trend was tested with the Mann-Kendall and the magnitude (i.e., slope of a linear trend) was estimated with the Sen method.

RESULTS

Exploratory Analysis: Annual and Seasonal Nitrate and Phosphorus Loads and Concentrations

The average nitrate and phosphorus export during the study period was 19.04 kg ha^{-1} $year^{-1}$ (7.36 and 30.07 kg ha^{-1} $year^{-1}$) and 0.13 kg ha^{-1} $year^{-1}$ (0.08 and 0.22 kg ha^{-1} $year^{-1}$) for nitrate and phosphorus respectively (**Figure 4**). In both cases, the highest values were registered in the hydrological year 2009/2010 and the lowest were measured in the hydrological year 2016/17 (**Figure 4**), similar to discharge variations in the study period. This highlights the importance of the hydrological response in the nutrient export in the study catchment, because small streams can be very sensitive to changes in the precipitation regime and runoff episodes compared to large basins (Bol et al., 2018). The results showed that PP is the main fraction of P mobilized from the catchment, representing approximately 72% of the phosphorus exported, although the proportions vary between 39% in the hydrological year 2013/14 and 86% in 2017/18. This variability is probably related to the characteristics of the rainfall-runoff events, management activities within the catchment in relation to meteorological conditions, and the source of sediments delivered to the river channel. The P losses occur primarily through erosion and surface runoff as P is mobilized and transported during runoff events (Rodríguez-Blanco et al., 2015).

Mean annual concentrations ranged from 4.1 to 5.2 mg L^{-1} for NO_3^-, and 17.2 and 35.1 μg L^{-1} for TP (**Figure 4**), reaching mean values of 4.8 mg L^{-1} and 26.3 μg L^{-1}, respectively, which signifies a greater variability of phosphorus (CV = 20%) concentrations than nitrate (CV = 6%). The highest mean concentration of nitrate was recorded in the hydrological year 2006/07, while the lowest values were obtained in 2004/05. For total phosphorus, the highest mean concentrations were recorded in hydrological year 2005/06 while the lowest values, also for dissolved phosphorus, were recorded in 2007/08. Therefore, the lowest concentrations are usually recorded in the driest years and with lower flow rates (**Figure 4**).

Transport throughout the year (**Figure 5**) showed that the highest nitrate loads were obtained in winter (8.45 kg ha^{-1} $year^{-1}$), followed by spring (4.78 kg ha^{-1}) and autumn (4.54 kg ha^{-1}), with the lowest values recorded in summer (1.25 kg ha^{-1}). The pattern for phosphorus was similar, which also returned the highest records in winter (0.050 kg ha^{-1}), followed by autumn (0.030 kg ha^{-1}) and spring (0.031 kg ha^{-1}), with the lowest values observed in summer (0.006 kg ha^{-1}) (**Figure 5**). The seasonal distribution of the concentrations (**Figure 6**) followed the same pattern as the loads, meaning that nitrate showed the following decreasing order: winter (5.5 mg L^{-1}) > autumn (5.3 mg L^{-1}) > spring (4.5 mg L^{-1}) > summer (4.1 mg L^{-1}), while phosphorus displayed the sequence: autumn (TP: 32.75 μg L^{-1}; PP: 22.91 μg L^{-1}; DP: 10 μg L^{-1}) > winter (TP: 33 μg L^{-1}; PP: 23 μg L^{-1}; DP: 10 μg L^{-1}) > spring (TP: 24 μg L^{-1}; PP: 15 μg L^{-1}; DP: 8 μg L^{-1}) > summer (TP: 16 μg L^{-1}; PP: 9 μg L^{-1}; DP: 7 μg L^{-1}). Phosphorus concentrations showed large differences between autumn and winter against summer.

Trends in Nitrate and Phosphorus Loads and Concentrations

Tables 1–3 present the results obtained with the Mann-Kendall test. Statistically relevant trends in annual values were not observed for both nutrients (**Table 1**). Nitrate seems to show a limited downward prospect. The same results were obtained using the Nitrate Directive approach, because the difference between the NO_3^- flow-weighted concentrations of two consecutive reporting periods ranged between −1 and 1 mg L^{-1}, indicating the absence of a trend. On a seasonal scale, a meaningful decrease is only observed in autumn for the nitrate load (**Table 2**), mainly linked to the decrease in October, as well as an increase in TP concentrations in winter (**Table 3**). A negative trend is also seen in the load of NO_3^- and P in the month of September, while the concentrations show a positive pattern, which could be related to the lower discharge in this month and the enrichment of the flow due to less dilution.

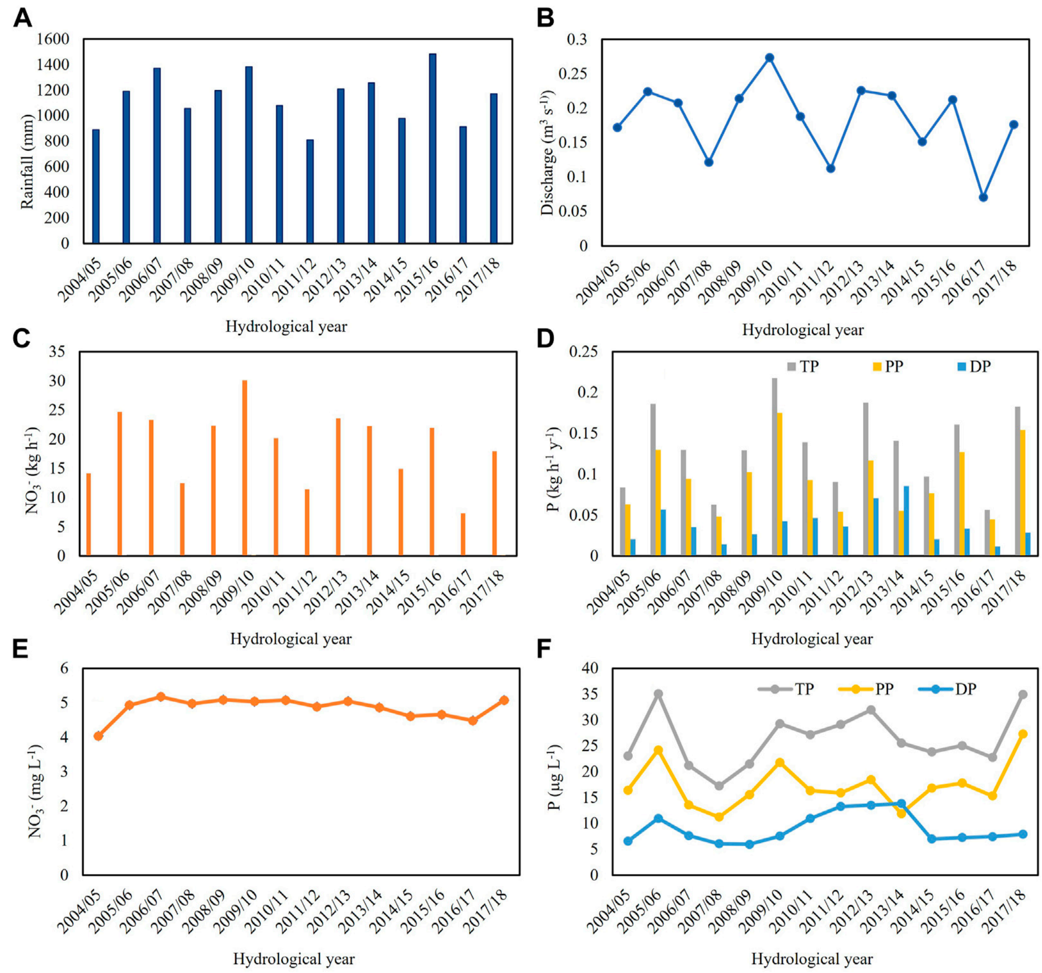

FIGURE 4 | Temporal evolution of annual **(A)** rainfall, **(B)** discharge, **(C)** NO_3^- load, P load **(D)**, mean **(E)** NO_3^- concentration and **(F)** P mean concentrations during the study period. TP: total phosphorus, PP: particulate phosphorus, DP: dissolved phosphorus.

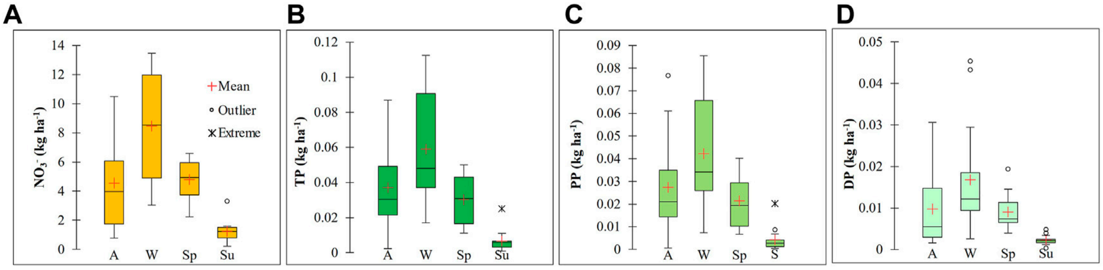

FIGURE 5 | Box plot with the load of **(A)** nitrate (NO_3^-), **(B)** total phosphorus (TP), **(C)** particulate phosphorus (PP) and **(D)** dissolved phosphorus (DP). The length of the box plot shows the interquartile range, comprising 50% of the values, and the straight horizontal line inside the box represents the median. A: autumn, W: winter, Sp: spring, S: summer.

DISCUSSION

NO_3^- and P loads were similar or lower compared with other studies carried out in Galicia (Bernárdez et al., 2013; Serrano et al., 2015) and suggest that agricultural activities and forest plantation management in the study area have only a slight impact on surface waters of the Corbeira catchment relative to other mixed land use catchments in Europe (Fovet et al., 2015; Dupas et al.,

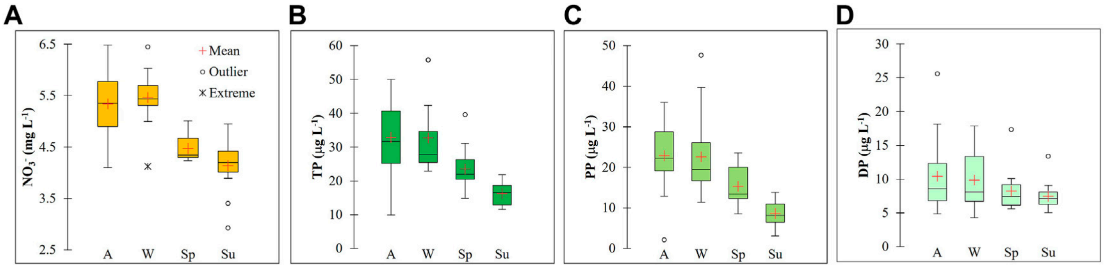

FIGURE 6 | Box plot with the concentration of **(A)** nitrate (NO_3^-), **(B)** total phosphorus (TP), **(C)** particulate phosphorus (PP) and **(D)** dissolved phosphorus (DP). The length of the box plot shows the interquartile range, comprising 50% of the values and the straight horizontal line inside the box represents the median. A: autumn, W: winter, Sp: spring, S: summer.

TABLE 1 | Mann-Kendall trend test results for nutrient yield and concentrations.

Variable	Mann-Kendall test	Sen's slope
NO_3^- (kg ha^{-1} year^{-1})	−1.20	−0.485
TP (kg ha^{-1} year^{-1})	0.33	0.001
PP (kg ha^{-1} year^{-1})	0.00	0.001
DP (kg ha^{-1} year^{-1})	−0.11	0.000
NO_3^- (mg L^{-1})	−0.99	−0.018
TP (µg L^{-1})	0.55	0.277
PP (µg L^{-1})	0.55	0.176
DP (µg L^{-1})	0.88	0.154

A positive Sen´s slope reveals increasing values, whereas a negative slope indicates decreasing values.

2017; Bouraoui and Malago, 2020; Ehrhardt et al., 2021). In fact, annual mean concentrations of both elements were well below the recommendations of WFD, the threshold proposed by Camargo and Alonso (2006) for protecting the most sensitive freshwater species and the threshold stablished by Carleton et al. (2009) to prevent nuisance level of periphyton and cyanobacteria (**Table 4**). When the mean nitrate and phosphorus concentrations obtained in this study are compared to the threshold values reported by Poikane et al. (2021), based on their relationship with macrophytes and phytobenthos, it is observed that mean nutrient concentrations of this study are within the range of predicted nitrogen good-moderate class boundaries for low alkalinity lowland and upland rivers (**Table 4**). They also are in the boundary values proposed by de Vries et al. (2013) to protect aquatic ecosystems from eutrophication. However, they exceed the threshold reported by Stevenson et al. (2008). In this case, the values reported by this last author coincide with concentrations in the catchment reference site (TP: 15 ± 7 µg L^{-1}; mean and standard deviation from 120 water sampled collected from 2004 to 2016, unpublished data); i.e., in the headwaters of the studied stream, which drains a forest area and is assumed to be free of nutrient inputs from human activities. So, protection of stream with this threshold was deemed impractical. This highlights the need to adapt the thresholds to individual water bodies to protect aquatic ecosystems from eutrophication.

Nitrate concentration exceeds the concentration collected by Macías et al. (1991) as reference values in slightly polluted rivers

TABLE 2 | Mann-Kendall trend test results for seasonal and monthly nutrient loads.

	$NO3^-$		*TP*		PP		DP	
Variable	**Sen´s slope**		**Sen´s slope**		**Sen´s slope**		**Sen´s slope**	
Autumn	−1.86$^+$	−0.400	−1.31	0.003	−1.42	−0.002	−1.09	0.000
Winter	0.77	0.194	0.99	0.003	1.53	0.002	0.33	0.000
Spring	−0.33	−0.040	0.00	0.000	0.33	0.000	0.33	0.000
Summer	−1.09	−0.052	0.00	0.000	0.00	0.000	−1.64	0.000
October	−2.19*	−114.638	−1.42	−0.582	−1.42	−0.540	−1.86	−0.136
November	−1.53	−199.571	−1.64	−1.773	−1.86$^+$	−1.376	−1.31	−0.381
December	−1.64	−372.552	−1.09	−2.275	−1.09	−0.628	−0.77	−0.387
January	0.55	72.032	1.31	1.558	0.99	1.253	0.33	0.078
February	0.88	104.815	1.31	1.963	1.53	2.083	0.00	0.032
March	0.44	80.935	0.44	0.486	0.33	0.224	0.77	0.240
April	−0.22	−28.660	0.99	1.038	0.55	0.243	0.99	0.206
May	−1.42	−63.505	−0.77	−0.349	−1.09	−0.289	0.11	0.007
June	−0.88	−47.283	−0.44	−0.092	−0.88	−0.172	−0.44	−0.029
July	−0.66	−32.810	−0.66	−0.156	−0.66	−0.072	−1.09	−0.072
August	−1.09	−16.652	−0.99	−0.074	−0.33	−0.022	−1.97*	−0.059
September	−1.86$^+$	−34.130	−1.86$^+$	−0.114	−1.53	−0.044	−2.08*	−0.056

*A positive Sen´s slope reveals increasing values, whereas a negative slope indicates decreasing values. Significance level: *: 0.05; $^+$: 0.1.*

TABLE 3 | Mann-Kendall trend test results for seasonal and monthly nutrient concentrations.

	NO3-		***TP***		**PP**		**DP**	
Variable	**Sen´s slope**		**Sen´s slope**		**Sen´s slope**		**Sen´s slope**	
Autumn	−1.31	−0.080	−0.33	−0.135	−0.55	−0.342	0.22	0.093
Winter	0.11	0.007	2.30*	1.006	1.53	0.832	0.77	0.168
Spring	−0.22	−0.003	0.44	0.228	−0.11	−0.023	1.53	0.150
Summer	0.00	0.006	1.42	0.393	1.31	0.292	−0.22	−0.056
October	−1.53	−0.088	−0.77	−0.855	−1.20	−1.135	−0.22	−0.137
November	−1.75^{+}	−0.124	−0.66	−0.873	−1.09	−1.239	0.00	−0.024
December	−0.66	−0.084	0.77	0.458	0.11	0.173	1.31	0.285
January	0.33	0.011	1.31	1.659	0.77	0.872	0.00	−0.012
February	0.00	−0.011	1.20	0.939	0.99	0.696	0.66	0.165
March	0.00	0.002	0.00	−0.076	−0.33	−0.269	1.42	0.244
April	−1.20	−0.031	0.11	0.144	0.11	0.128	1.31	0.165
May	−0.22	−0.007	0.33	0.129	−0.33	−0.320	1.42	0.251
June	−0.22	−0.013	0.22	0.209	0.00	−0.005	1.75^{+}	0.136
July	0.55	0.014	0.22	0.215	0.00	0.016	−0.22	−0.031
August	0.44	0.033	0.66	0.208	1.42	0.284	−0.33	−0.064
September	0.44	0.017	1.31	0.196	0.88	0.133	0.77	0.220

*A positive Sen´s slope reveals increasing values, whereas a negative slope indicates decreasing values. Significance level: *: 0.05; $^{+}$: 0.1.*

TABLE 4 | Nutrient thresholds establish by different ecology-based strategies.

Reference	Nutrient criteria	Approach for establishing criteria
Camargo and Alonso (2006)	0.5–1.0 mg TN L^{-1}	Protect aquatic ecosystems from eutrophication and acidification
Carleton et al. (2009)	100 µg TP L^{-1}; 2.7 mg TN L^{-1}	Prevent nuisance level of periphyton and cyanobacteria
Poikane et al. (2021)	1.63 mg TN L^{-1} (.71–4.19), 32 µg SRP L^{-1} (18–58)	Good moderate class boundary low alkalinity lowland rivers
	1.79 mg N L^{-1} (1.02–4.04), 40 µg L^{-1} (19–78)	Good moderate class boundary low alkalinity upland rivers
Stevenson et al. (2008)	10–12 µg TP L^{-1}	Protect high-quality biological conditions in streams (Mid-Atlantic Highlands)
de Vries et al. (2013)	1.0–2.5 mg N L^{-1}	Protect aquatic ecosystems from eutrophication and acidification

TP, Total phosphorus; SRP, soluble phosphorus; TN, total nitrogen.

(1.19 mg L^{-1}), the background concentration proposed by Meybeck (1982) for the major unpolluted rivers (0.44 mg L^{-1}), as well as the levels suggested for uncontaminated natural rivers in Galicia (1–4 mg L^{-1}, Antelo and Arce, 1996) and the mean concentration in the catchment reference site (1.99 ± 0.54 mg L^{-1}; mean and standard deviation from 120 water sampled collected from 2004 to 2016, unpublished data). Naves et al. (2019) observed nitrate contamination in the groundwaters (concentrations between 2.54 and 47.1 mg L^{-1}) of the study area linked to an inadequate management of the manure in the field and even to an occasional discharge of slurry from local farms. However, the results of this study are far from what was observed by these authors, since nitrate concentrations in summer (mean = 4.13 mg L^{-1}) -when discharge is dominated by groundwater-are well below the values reported by Naves et al. (2019) as a reference in the study area (1.2–9.9 mg L^{-1}).

Loads and the concentrations of both nutrients showed a marked seasonal pattern related to the hydrological behaviour of the catchment, that is, the highest values were recorded in winter and autumn, coinciding with the highest discharge and runoff events (Rodríguez-Blanco et al., 2019), while the lowest were obtained in summer, coinciding with the period of low waters and the greatest influence from the baseflow. This behaviour suggests a greater nutrient mobilization during the wet period (October-March), which is consistent with a predominant diffuse source of nitrate and phosphorus (Abbott et al., 2018; Dupas et al., 2019; Garzon-Vidueira et al., 2020). Thus, wet periods increase the connectivity between nutrient sources and the stream network (Rodríguez-Blanco et al., 2013; 2015), promoting the transfer of nutrients. Corbeira is a transport-limited catchment, where the nutrient fluxes are controlled by the hydrology, as the flow is the main driver of nutrient transport, since it controls the connectivity between the source (e.g., soils) and the stream. Nitrate is mainly transported in subsurface flow (Rodríguez-Blanco et al., 2015) while P (mainly associated with sediment) in surface runoff (Rodríguez-Blanco et al., 2013).

The results showed the lack of consistent trends of N and P over time, although a decreasing trend appears for N loads, mainly linked to the reduction of nitrate transport during autumn and spring (**Table 2**), although it might also be linked to a decline in agricultural activities in the catchment, which leads to a decrease in the application of manure and fertilisers—ideally in the autumn and spring. Forest plantation cutting cycles should also be considered since forest cut decrease nutrient uptake, making more nutrients available for leaching into water courses (Thompson et al., 2009). Forest roads, especially when intercepting the stream can be an important source of nutrients associated (mainly P) with sediments in plantation areas. Phosphorus points to an upward trend, mainly in winter, which could be related to the positive flow trend in the study area (Rodríguez-Blanco et al., 2020). An increase in discharge levels translates into a greater flow velocity and movement of sediments, which is essential for the transport of P in the catchment since it is intrinsically related to the particulate fraction. A similar pattern was observed in another mixed land use catchment (Minaudo et al., 2015; Bol et al., 2018), demonstrating the significance of suspended sediment in the dynamics of P. The results also show an increase in the nutrient concentrations, despite the fact that discharge and nutrient load decrease in the summer, meaning that the flow is being enriched owing to lesser dilution. As has been reported in several other catchments with forestry and agricultural uses (e.g., Abbott et al., 2018), this could be indicative of surplus nutrients applied to the soil being available for export to water bodies by drainage or runoff.

Despite the decline in population and reduced agricultural activities in the catchment, only a limited improvement in nitrate concentrations was observed. This may be due to the potentially long transit time in the catchment, so reducing non-point source nutrients may take decades to affect the water chemistry of rivers (Fovet et al., 2015; Dupas et al., 2017). The increase in winter flow could also counteract the effect of the reduction in the number of people, so that no trend at annual scale will be detected.

CONCLUDING REMARKS

The concentrations of N and P measured in the catchment are relatively low, which shows that agricultural practices and the forest plantation management in the catchment do not jeopardise (for the moment) the water quality, although the catchment nutrient reference levels were widely exceeded. The variability of export fluxes is high, despite their "natural nature," due to the fact that connectivity between the nutrient source area and the stream is dependent on flow conditions. A lack of consistent trends of N and P over time was observed, but the nutrients analysed behaved differently. Thus, N appeared to show a downward trend, mainly associated with declining transport in autumn and spring. Phosphorus showed an upward trend in winter linked to a greater flow and sediment transfer. As a result, any increase in erosion and runoff will almost certainly result in an increase phosphorus load in the study area.

Despite the decrease in population and abandonment of agricultural land in the catchment, the concentrations of nitrate do not show a sizable reduction, which could be indicative of surplus nutrients provided to the soil being available for export to water bodies *via* drainage or runoff. As a result, a decrease in inputs from various sources does not result in rapid improvement in riverine water quality.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the data are part of a large database. Requests to access the datasets should be directed to maria.luz.rodriguez.blanco@uvigo.es.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

Abbott, B. W., Moatar, F., Gauthier, O., Fovet, O., Antoine, V., and Ragueneau, O. (2018). Trends and Seasonality of River Nutrients in Agricultural Catchments: 18 Years of Weekly Citizen Science in France. *Sci. Total Environ.* 624, 845–858. doi:10.1016/j.scitotenv.2017.12.176

Antelo, J. M., and Arce, F. (1996). "Características fisicoquímicas das augas superficiais," in *As Aguas de Galicia*. Editor F. Díaz-Fierros (Santiago de Compostela, Spain: Consello da Cultura Galega), 353–446.

APHA (1998). *Standard Methods for the Examination of Water and Wastewater 20th Edition*. Washington, DC: American Public Health Association, American Water Work Association, Water Environment Federation.

Bennett, E. M., Carpenter, S. R., and Caraco, N. F. (2001). Human Impact on Erodable Phosphorus and Eutrophication: A Global Perspective. *Bioscience* 51 (3), 227–234. doi:10.1641/0006-3568(2001)051[0227:hioepa]2.0.co;2

Bernárdez, P., Ospina-Álvarez, N., Caetano, M., and Prego, R. (2013). Fluvial Contributions of Nutrient Salts, Dissolved Trace Elements and Organic Carbon to the Sea by Pristine Temperate Rivers (SW Europe). *Environ. Chem.* 10 (1), 42–53. doi:10.1071/en12123

Bol, R., Gruau, G., Mellander, P., Dupas, R., Bechmann, M., Skarbøvik, E., et al. (2018). Challenges of Reducing Phosphorus Based Water Eutrophication in the Agricultural Landscapes of Northwest Europe. *Front. Mar. Sci.* 5, 276. doi:10.3389/fmars.2018.00276

Bouraoui, F., and Malagó, A. (2020). Trend Analysis of Nitrate Concentration in Rivers in Southern France. *Water* 12 (12), 3374. doi:10.3390/w12123374

Camargo, J. A., and Alonso, Á. (2006). Ecological and Toxicological Effects of Inorganic Nitrogen Pollution in Aquatic Ecosystems: a Global Assessment. *Environ. Int.* 32 (6), 831–849. doi:10.1016/j.envint.2006.05.002

Carleton, J. N., Park, R. A., and Clough, J. S. (2009). Ecosystem Modeling Applied to Nutrient Criteria Development in Rivers. *Environ. Manage* 44 (3), 485–492. doi:10.1007/s00267-009-9344-2

Carvalho, L., Mackay, E. B., Cardoso, A. C., Baattrup-Pedersen, A., Birk, S., Blackstock, K. L., et al. (2019). Protecting and Restoring Europe's Waters: An Analysis of the Future Development Needs of the Water Framework Directive. *Sci. Total Environ.* 658, 1228–1238. doi:10.1016/j.scitotenv.2018.12.255

de Vries, W., Kros, J., Kroeze, C., and Seitzinger, S. P. (2013). Assessing Planetary and Regional Nitrogen Boundaries Related to Food Security and Adverse Environmental Impacts. *Curr. Opin. Environ. Sustain.* 5 (3-4), 392–402. doi:10.1016/j.cosust.2013.07.004

Directive, W. F. (2000). Water Framework Directive. *J. Ref. OJL* 327, 1–73.

Dou, Y., Cosentino, F., Malek, Z., Maiorano, L., Thuiller, W., and Verburg, P. H. (2021). A New European Land Systems Representation Accounting for Landscape Characteristics. *Landsc. Ecol.* 36 (8), 2215–2234. doi:10.1007/s10980-021-01227-5

Dupas, R., Abbott, B. W., Minaudo, C., and Fovet, O. (2019). Distribution of Landscape Units within Catchments Influences Nutrient Export Dynamics. *Front. Environ. Sci.* 7, 43. doi:10.3389/fenvs.2019.00043

Dupas, R., Mellander, P., Gascuel-Odoux, C., Fovet, O., McAleer, E. B., McDonald, N. T., et al. (2017). The Role of Mobilisation and Delivery Processes on Contrasting Dissolved Nitrogen and Phosphorus Exports in Groundwater Fed Catchments. *Sci. Total Environ.* 599, 1275–1287. doi:10.1016/j.scitotenv.2017.05.091

Ehrhardt, S., Ebeling, P., Dupas, R., Kumar, R., Fleckenstein, J. H., and Musolff, A. (2021). Nitrate Transport and Retention in Western European Catchments Are Shaped by Hydroclimate and Subsurface Properties. *Water Resour. Res.* 57 (10), e2020WR029469. doi:10.1029/2020wr029469

Ekholm, P., Rankinen, K., Rita, H., Räike, A., Sjöblom, H., Raateland, A., et al. (2015). Phosphorus and Nitrogen Fluxes Carried by 21 Finnish Agricultural Rivers in 1985–2006. *Environ. Monit. Assess.* 187 (4), 216–217. doi:10.1007/s10661-015-4417-6

European Environment Agency (2018). *European Waters Assessment. EEA Report N° 7/2018*. Copenhage, Denmark: EEA.

Fovet, O., Ruiz, L., Faucheux, M., Molénat, J., Sekhar, M., Vertès, F., et al. (2015). Using Long Time Series of Agricultural-Derived Nitrates for Estimating Catchment Transit Times. *J. Hydrology* 522, 603–617. doi:10.1016/j.jhydrol.2015.01.030

Garzon-Vidueira, R., Rial-Otero, R., Garcia-Nocelo, M. L., Rivas-Gonzalez, E., Moure-Gonzalez, D., Fompedriña-Roca, D., et al. (2020). Identification of Nitrates Origin in Limia River Basin and Pollution-Determinant Factors. Agric. *Ecosyst. Environ.* 290, 106775.

Grizzetti, B., Vigiak, O., Udias, A., Aloe, A., Zanni, M., Bouraoui, F., et al. (2021). How EU Policies Could Reduce Nutrient Pollution in European Inland and Coastal Waters. *Glob. Environ. Change* 69, 102281. doi:10.1016/j.gloenvcha.2021.102281

Gustard, A., Bullock, A., and Dixon, J. M. (1992). *Low Flow Estimation in the United Kingdom*. England: Institute of Hydrology, 19.

IGME (Instituto Tecnológico Geominero de España) (1981). *Mapa Geológico de España, 1:50,000*. Betanzos Spain. Hoja 45.

INE (2019). *Nomenclator: Población del padrón continuo por unidad poblacional*. Madrid: Instituto Estadístico de España.

Iuss Working Group Wrb (2014). in *International Soil Clasification System for Naming Soils and Creating Legends for Soil Maps* (Rome: World Soil Resources Reports. N° 106FAO).*World Reference Base for Soil Resources.*

Kelly, M. G., Phillips, G., Teixeira, H., Várbíró, G., Herrero, F. S., Willby, N. J., et al. (2022). Establishing Ecologically-Relevant Nutrient Thresholds: A Tool-Kit with Guidance on its Use. *Sci. Total Environ.* 807, 150977. doi:10.1016/j.scitotenv.2021.150977

Kumar, S., Merwade, V., Kam, J., and Thurner, K. (2009). Streamflow Trends in Indiana: Effects of Long Term Persistence, Precipitation and Subsurface Drains. *J. Hydroloy* 374 (1-2), 171–183. doi:10.1016/j.jhydrol.2009.06.012

Lassaletta, L., García-Gómez, H., Gimeno, B. S., and Rovira, J. V. (2009). Agriculture-induced Increase in Nitrate Concentrations in Stream Waters of a Large Mediterranean Catchment over 25 Years (1981–2005). *Sci. Total Environ.* 407 (23), 6034–6043. doi:10.1016/j.scitotenv.2009.08.002

Linsely, R. K., Kohler, M. A., and Paulhus, J. C. (1949). *Applied Hydrology*. New York: McGraw-Hill Book Co.

Macías, F., Álvarez Rodríguez, E., and Calvo de Anta, R. (1991). Impactos de origen agrario y urbano en la cuenca del río Ulla. *Ecología* 5, 73–86.

Meybeck, M. (1982). Carbon, Nitrogen, and Phosphorus Transport by World Rivers. *Am. J. Sci.* 282 (4), 401–450. doi:10.2475/ajs.282.4.401

Minaudo, C., Meybeck, M., Moatar, F., Gassama, N., and Curie, F. (2015). Eutrophication Mitigation in Rivers: 30 Years of Trends in Spatial and Seasonal Patterns of Biogeochemistry of the Loire River (1980–2012). *Biogeosciences* 12 (8), 2549–2563. doi:10.5194/bg-12-2549-2015

Moatar, F., Abbott, B. W., Minaudo, C., Curie, F., and Pinay, G. (2017). Elemental Properties, Hydrology, and Biology Interact to Shape Concentration-discharge Curves for Carbon, Nutrients, Sediment, and Major Ions. *Water Resour. Res.* 53 (2), 1270–1287. doi:10.1002/2016wr019635

Murphy, J., and Riley, J. P. (1962). A Modified Single Solution Method for the Determination of Phosphate in Natural Waters. *Anal. Chim. Acta* 27, 31–36. doi:10.1016/s0003-2670(00)88444-5

Naves, A., Samper, J., Mon, A., Pisani, B., Montenegro, L., and Carvalho, J. M. (2019). Demonstrative Actions of Spring Restoration and Groundwater Protection in Rural Areas of Abegondo (Galicia, Spain). *Sustain. Water Resour. Manag.* 5 (1), 175–186. doi:10.1007/s40899-017-0169-5

OECD (2007). *OECD Regions at a Glance*. Paris: OECD Publishing, 252.

Oenema, O., van Liere, L., and Schoumans, O. (2005). Effects of Lowering Nitrogen and Phosphorus Surpluses in Agriculture on the Quality of Groundwater and Surface Water in the Netherlands. *J. Hydrology* 304 (1-4), 289–301. doi:10.1016/j.jhydrol.2004.07.044

Panagos, P., Borrelli, P., Poesen, J., Ballabio, C., Lugato, E., Meusburger, K., et al. (2015). The New Assessment of Soil Loss by Water Erosion in Europe. *Environ Sci Policy* 54, 438–447. doi:10.1016/j.envsci.2015.08.012

Poikane, S., Várbíró, G., Kelly, M. G., Birk, S., and Phillips, G. (2021). Estimating River Nutrient Concentrations Consistent with Good Ecological Condition: More Stringent Nutrient Thresholds Needed. *Ecol. Ind.* 121, 107017. doi:10.1016/j.ecolind.2020.107017

Rodríguez-Blanco, M. L., Taboada-Castro, M. M., and Taboada-Castro, M. T. (2020). An Assessment of the Recent Evolution of the Streamflow in a Near-Natural System: A Case Study in the Headwaters of the Mero Basin (Galicia, Spain). *Hydrology* 7 (4), 97. doi:10.3390/hydrology7040097

Rodríguez-Blanco, M. L., Taboada-Castro, M. M., and Taboada-Castro, M. T. (2019). An Overview of Patterns and Dynamics of Suspended Sediment Transport in an Agroforest Headwater System in Humid Climate: Results from a Long-Term Monitoring. *Sci. Total Environ.* 648, 33–43. doi:10.1016/j.scitotenv.2018.08.118

Rodríguez-Blanco, M. L., Taboada-Castro, M. M., and Taboada-Castro, M. T. (2013). Phosphorus Transport into a Stream Draining from a Mixed Land Use Catchment in Galicia (NW Spain): Significance of Runoff Events. *J. Hydrology* 481, 12–21. doi:10.1016/j.jhydrol.2012.11.046

Rodríguez-Blanco, M. L., Taboada-Castro, M. M., Taboada-Castro, M. T., and Oropeza-Mota, J. L. (2015). Relating Nitrogen Export Patterns from a Mixed Land Use Catchment in NW Spain with Rainfall and Streamflow. *Hydrol. Process* 29 (12), 2720–2730. doi:10.1002/hyp.10388

Serrano, L., Palleiro, L., Rodríguez-Blanco, M. L., Taboada-Castro, M. M., and Taboada-Castro, M. T. (2015). Preliminary Assessment of the Riverine Nitrogen Concentration and Load in an Agroforestry Basin in NW Spain. *Commun. Soil Sci. Plant Anal.* 46, 326–331. doi:10.1080/00103624.2014.989071

Sharpley, A. N., Kleinman, P. J., Heathwaite, A. L., Gburek, W. J., Folmar, G. J., and Schmidt, J. P. (2008). Phosphorus Loss from an Agricultural Watershed as a Function of Storm Size. *J. Environ. Qual.* 37 (2), 362–368. doi:10.2134/jeq2007.0366

Withers, P. J., and Lord, E. I. (2002). Agricultural Nutrient Inputs to Rivers and Groundwaters in the UK: Policy, Environmental Management and Research Needs. *Sci. Total Environ.* 282, 9–24. doi:10.1016/s0048-9697(01)00935-4

Worrall, F., Jarvie, H. P., Howden, N. J., and Burt, T. P. (2016). The Fluvial Flux of Total Reactive and Total Phosphorus from the UK in the Context of a National Phosphorus Budget: Comparing UK River Fluxes with Phosphorus Trade Imports and Exports. *Biogeochemistry* whit130 (1), 31–51. doi:10.1007/s10533-016-0238-0

ORIGINAL RESEARCH
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Mercury Content and Pools in Complex Polycyclic Soils From a Mountainous Area in Galicia (NW Iberian Peninsula)

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Atmospheric mercury (Hg) usually tends to accumulate in the upper horizons of soils. However, the physico-chemical characteristics of some soils, as well as pedogenetic processes, past climate changes, or soil degradation processes, can lead to a redistribution of mercury through the soil profile. In this work, the presence and accumulation of mercury was studied in three deep polycyclic soils from a mountainous area in NW Iberia Peninsula. The highest total Hg values (Hg_T) were found in the organic matter-rich O and A horizons of FL and MF profiles (169 and 139 μg kg^{-1}, respectively) and in the illuvial horizon of RV (129.2 μg kg^{-1}), with the latter two samples showing the maximum Hg reservoirs (29.3 and 29.0 mg m^{-2}, respectively). Despite finding the highest Hg content in the surface horizons, considerable Hg reservoirs were also observed in depths higher than 40–50 cm, indicating the importance of taking into account these soil layers when Hg pools are evaluated at a global scale. Based on the mass transfer coefficients, we can rule out the contribution of parent material to the Hg accumulation in most of the horizons, thus indicating that pedogenetic processes are responsible for the Hg redistribution observed along the soil profiles. Finally, by means of principal component analysis (PCA) and stepwise linear regression we could assess the main soil components involved in the Hg accumulation in each soil horizon. Therefore, PC1 (organic matter and low stability Al-hummus complexes) showed a higher influence on the surface horizons, whereas PC2 (reactive Al-Fe complexes and medium-high Al-hummus complexes) and PC4 (crystalline Fe compounds and pH_w) were more relevant in the Hg distribution observed in the deepest soil layers.

Keywords: soil organic matter, Hg storage, Hg vertical patterns, pedogenetic processes, Al and Fe compounds

INTRODUCTION

Hg is a global pollutant of environmental concern, which can cause serious damage to the health of both humans and wildlife, especially in the methylated form (Clarkson and Magos, 2006; Driscoll et al., 2013). Hg is mainly emitted to the atmosphere by both natural and anthropogenic sources and transported far away from its emission point. Therefore, one of the biggest Hg reservoirs in the biosphere is the atmosphere (Obrist et al., 2018). After a certain period of permanence in the atmosphere (up to 2 years, Schroeder and Munthe, 1998), Hg is deposited over terrestrial ecosystems through direct wet and dry precipitation and *via* litterfall. Because of its predominant atmospheric origin and its high affinity for the reduced sulphur groups of the organic matter, Hg tends to accumulate in the organic-matter rich superficial soil layers (i.e., 30–50 cm) (Skyllberg et al., 2006; Smith-Downey et al., 2010; Obrist et al., 2014; Obrist et al., 2017). For this reason, most of the Hg studies in terrestrial ecosystems are usually focused on the top few centimetres of soils (Xin and Gustin, 2007). However, on a minor scale, Hg can also occur in soils as a result of the weathering of soil parent material (Roulet et al., 1998; Guédron et al., 2006; Peña-Rodríguez et al., 2014; Kroonenberg et al., 2022). As a consequence, Hg derived from lithological sources could be also contributing significantly to the total Hg soil pool, especially in the deepest soil horizons (Fiorentino et al., 2011; Richardson et al., 2018; Kroonenberg et al., 2022). The participation of the deep soil layers in Hg storage would strengthen the role of soils as a main reservoir of atmospheric Hg in terrestrial ecosystems, as well as making it less probable that Hg would reach aquatic ecosystems (Amos et al., 2013). Unfortunately, few recent studies have explored the relevance of soil deep horizons in the biogeochemical fate of Hg in soil, as most of them were restricted to the surface soil horizons (Richardson et al., 2018; Schuster et al., 2018; Kroonenberg et al., 2022; Richardson, 2022; Richardson, 2022). In the deepest soils layers, soil components other than C, such as Al and Fe oxyhydroxides or organic Al and Fe compounds, have been recognized to influence the vertical pattern of Hg in soils (Do Valle et al., 2005; Guédron et al., 2006; Navrátil et al., 2014; Richardson et al., 2018; Gómez-Armesto et al., 2020).

Podzols and soils with podzolic characteristics can serve as examples of soils with a recognized ability to accumulate Hg in the deeper soil horizons. In Podzols, Hg can be mobilized through the soil profile together with organic matter and Al and Fe compounds and afterwards immobilized due to its adsorption to metal (Al, Fe)-humus complexes and Al and Fe oxyhydroxides, leading to the characteristic soil vertical pattern of Hg reported in different works (Guédron et al., 2009; Navrátil et al., 2014; Richardson et al., 2018; Gómez-Armesto et al., 2020; Gómez-Armesto et al., 2021a; Gómez-Armesto et al., 2021b).

Besides the particular chemical properties that determine Hg occurrence in soils such as Podzols, the distribution of Hg in other soils can also be defined by pedogenetic processes, past climate changes, or soil degradation processes (forest fires, deforestation, erosion) as reported by Obrist et al. (2018). In some cases, the soil can be removed whole or in part by climatic and/or erosive causes and deposited far away from its original area, whereas in other cases, surface soil layers are buried with soil mobilized through erosion. When this happens, it is quite probable that the appearance of polycyclic soils is a consequence of the action of several pedogenetic processes, as these soils show a complex mixture of chemical properties, soil compounds, and morphological features (Taboada-Castro et al., 1995; Peiteado Varela et al., 2002). In fact, a polycyclic soil is a soil developed under different climatic scenarios that led to different and, sometimes, contrasting pedogenetic processes, resulting in soil horizons that are not genetically related (Canarache et al., 2006). Thus, the multilingual dictionary of soil science supported by the Spanish Society of Soil Science (SECS-SLCS-IEC, 2023) indicates that the deeper part of a polycyclic soil derived from old pedogenetic processes may serve as parent material of the more recent and superficial part of the soil. The involvement of polycyclism processes, through the vertical arrangement of organic matter and Al and Fe oxyhydroxides along the soil profile, was reported to be responsible for vertical patterns of Hg in mountain forest soils from Tierra del Fuego, Argentina (Peña-Rodríguez et al., 2014). In summary, the substantial role of the pedogenetic processes in the soil Hg sequestration should be deciphered to assess the transcendence of soils in the biogeochemical cycle of Hg in terrestrial ecosystems.

The present study assess the occurrence, accumulation, and vertical distribution of Hg in three deep polycyclic soils from a mountain area in NW Iberia Peninsula. This study aims to evidence the relevance of deeper soil layers in terms of soil Hg pools, and attempts to further understand the main soil chemical characteristics and soil processes involved in the observed Hg patterns with soil depth.

MATERIAL AND METHODS

Study Area and Sampling Procedure

This study was carried out in the Xistral Mountains, a medium altitude mountain range (maximum 1,060 m a.s.l.) located in the north of the province of Lugo (Galicia). This area is characterized by a mean annual temperature between 7°C and 10°C, and a moderate total annual rainfall (1,400–1800 mm) with a scarce rainfall seasonality and abundant fogs throughout the year above 600 m a.s.l. (Pontevedra-Pombal et al., 2012). In the Xistral Mountains, three soil profiles developed from granitic materials and with a complex morphology were selected for the present study. These soils were denoted as FL (43° 31′42.8″ N, 7° 31′3.9″ W) MF (43° 28′6.7″ N, 7° 31′33.5″ W), and RV (43° 31′22.6″ N, 7° 29′54.9″ W), with a depth of 185, 190, and 155 cm, respectively. The vegetation in the three sampling sites was mostly dominated by different species of heather (*Erica* sp.), some individuals of shrubs species from genus *Ulex* and *Cytisus*, small patches of mosses (*Sphagnum* sp.), and several herbaceous species such as *Molinia caerulea* and *Agrostis curtisii*.

At each soil sampling site, samples were collected on a surface cut of a forest track, removing the first 30 cm-thickness of the profile in order to obtain a fresh soil surface and avoid potential anthropogenic disturbances. From each individual horizon identified in the field, soil samples were collected at an interval of 10–20 cm with a plastic garden trowel, which was rinsed twice between samples with a diluted HNO_3 solution and then dried. The total number of soil samples collected was 45. In addition, a fresh rock sample representing the parent material for each sampled soil was collected. Soil and rock samples were stored in plastic bags and transported to the laboratory in a portable fridge at 4°C. Once in the laboratory, after plant debris and stone removal, soil samples were air-dried and sieved (2-mm mesh), whereas rock samples were washed with distilled water and any soil residue removed. Approximately 0.5 kg of sieved soil was quartered with a stainless steel riffle-splitter to obtain samples with enough representativeness and homogeneity for subsequent general chemical characterization and Hg analyses.

Analytical Methods

The physico-chemical characterization of soil samples was carried out in the fine earth fraction (<2 mm). Soil pH was determined in soil suspensions obtained after addition of distilled water (pH_w) or saline solution (0.1 M KCl, pH_K) to soil samples, maintaining a 1:2.5 soil:solution ratio. The total content of biophilic elements (i.e., C and N) were determined in an autoanalyzer after combustion of milled soil samples, with total C values assumed to be organic C due to the absence of inorganic carbonates in the studied soils. Neutral saline solutions, 1 M NH_4Cl (Peech et al., 1947) and 1 M KCl (Al_K; Lin and Coleman, 1960) were used to displace exchangeable base cations (K_e, Na_e, Ca_e, Mg_e) and exchangeable Al. The sum of all displaced exchangeable cations was considered an estimate of the effective cation exchange capacity (eCEC). Soil texture was obtained on the basis of the particle-size distribution, which was determined after wet sieving (sand fractions) and the pipette method based in the sedimentation rate of soil particles for silt and clay fractions (Gee and Bauder, 1986).

The fractionation of Al and Fe in soil samples was carried out following the procedures used by García-Rodeja et al. (2004). Aluminium and Fe complexed by soil organic matter were obtained after extraction with 0.1 M Na-pyrophosphate solution (Al_p, Fe_p). A 0.2 M ammonium oxalate-oxalic acid solution buffered at pH 3 was used to dissolve all non-crystalline Al and Fe compounds (Al_o, Fe_o) including poorly-ordered inorganic Al and Fe oxyhydroxides and metal (Al, Fe)-humus complexes. To estimate the total free pools, a 0.5 M NaOH solution was used in the case of Al (Al_n) while an Na-dithionite-citrate solution was applied for Fe (Fe_d). After these extractions, the following fractions of Al and Fe were operatively defined: a) metal (Al, Fe)-humus complexes which are equal to Al_p and Fe_p; b) inorganic non-crystalline Al and Fe compounds (Al_{ia}, Fe_{ia}) as the subtraction among the amounts of Al and Fe extracted with oxalate-oxalic acid and Na-pyrophosphate; c) crystalline Al and Fe compounds (Al_c, Fe_c) from the result of total free Al and Fe minus oxalate-oxalic acid extractable Al and Fe. To extend the characterization of the organically complexed Al pool, two additional extractions were carried out using two chloride salt solutions, namely, 0.5 M $CuCl_2$ (Juo and Kamprath, 1979) and 0.33 M $LaCl_3$ (Bloom et al., 1979), denoted as Al_{Cu} and Al_{La}, respectively. Following previous studies (Urrutia et al., 1995; García-Rodeja et al., 2004), chloride salt solutions provide the operative differentiation of Al-humus complexes in the following types: high stability Al-humus complexes (Al_{oh}, as Al_p–Al_{Cu}), moderate stability (Al_{om}, as Al_{Cu}–Al_{La}) and low stability (Al_{ol}, Al_{La}–Al_K). The concentration of Al and Fe in the different extractions was determined by flame-AAS spectrometry. Carbon solubilized during the Na-pyrophosphate extract (C_p) was determined by titration with 0.1 N Mohr salt and considered an estimation of well humified soil organic matter.

For the Hg measurement, samples of soil and rock were milled in a mechanical agate mortar (Retsch RM100, Retsch RM200). About 100 mg of each sample was analyzed twice using a DMA-80 Hg analyzer (Milestone), which is based on thermal decomposition and atomic absorption spectroscopy. Measurements were repeated when the coefficient of variation was higher than 10%. In order to test the accuracy of the method (for quality assurance and quality control purposes), different standard reference materials were analyzed at the start of each sample run and every fifteen samples, obtaining recovery percentages of 91% for GBW 07402 (average 13.6 ± 0.7 $\mu g\ kg^{-1}$; n = 12) and 91% for GBW 07427 (average 47.4 ± 9.8 $\mu g\ kg^{-1}$; n = 9). Finally, the detection limit of the method was 0.043 $\mu g\ kg^{-1}$.

Mercury Reservoir Evaluation

For each sampled depth, the pool of Hg (Hg_{Res_d}) was calculated taking into account the corresponding thickness (T_d), bulk density (B_d), coarse fragment proportion (C) (%), and total Hg content (HgT) as in Wang et al. (2017).

$$Hg_{Res\_d} = Hg_T\ x\ B_d\ x\ T_d\ x\ (1 - C)$$

The Hg reservoir in each soil horizon identified (Hg_{Tres}) was calculated as the sum of all Hg_{Res_d} included in the same horizon.

Relative Hg Losses and Gains During Weathering

Mass transfer coefficients of Hg (τ_{Hg}) were calculated following the open-system mass transport function (Guédron et al., 2006; Fiorentino et al., 2011; Guédron et al., 2013; Richardson, 2022; Spinola et al., 2022) for each sample depth, in order to evaluate Hg depletions or enrichments and to discriminate among possible sources of Hg (atmospheric deposition or lithogenic origin) in the soil profiles. The chosen immobile element for calculations was zirconium and its concentrations in soil (*subscript s*) and parent material (*subscript r*) of both elements (Hg as mobile element and Zr as immobile element) were used as follows:

$$\tau_{Hg} = \frac{Hg_{T,s}}{Zr_{T,s}} * \frac{Zr_{T,r}}{Hg_{T,r}} - 1$$

The τ_{Hg} for each soil horizon identified was calculated as the average of all Tau values included in the corresponding depths analysed. As Spinola et al. (2022) indicated, positive τ values mean Hg enrichment of soil horizons regarding soil

TABLE 1 | Mean values per soil horizon of some chemical characteristics of FL, MF, and RV soils.

Profile	Horizon	Depth	[a]n	[b]pH$_w$	[b]pH$_K$	C	[c]C$_p$	N	S	[d]BS	[d]Al$_K$
		cm				----------- % -----------				cmol$_c$ kg^{-1}	
FL	O	0–10	1	4.7	4.3	20.8	10.3	1.5	0.05	5.9	1.3
	A	10–30	2	4.8	3.9	11.3	7.1	0.7	0.05	1.3	6.5
	AC	30–50	2	5.1	4.1	5.0	4.3	0.3	0.02	0.3	4.3
	2A	50–60	1	4.8	4.2	5.7	5.1	0.3	0.01	0.3	3.7
	2AC	60–74	1	4.7	4.3	4.5	2.2	0.2	0.01	0.2	4.0
	2BC	74–90	1	4.7	4.4	3.2	2.9	0.1	0.01	0.2	1.9
	3BC	90–150	3	4.6	4.5	2.4	1.5	0.1	0.01	0.2	1.8
	3C	150–185	3	4.9	4.4	0.6	0.0	0.0	0.00	0.2	0.7
MF	A	0–38	3	4.8	3.6	10.9	5.9	0.6	0.03	1.6	5.2
	Bhs	38–48	1	5.0	4.3	3.0	1.7	0.1	0.01	0.2	2.1
	Bs	48–68	1	5.0	4.4	3.9	3.2	0.2	0.01	0.3	2.1
	2CB	68–100	2	5.1	4.5	1.6	1.0	0.1	0.00	0.2	1.3
	3C	100–190	6	5.2	4.3	0.6	0.1	0.0	0.00	0.4	1.2
RV	A	0–43	4	4.8	4.2	7.7	5.7	0.5	0.03	0.6	4.2
	B	43–75	6	4.8	4.5	4.5	3.9	0.3	0.02	0.4	1.8
	2A	75–95	3	4.7	4.4	3.1	2.2	0.2	0.01	0.4	1.7
	2AB	95–110	2	4.7	4.5	1.2	0.9	0.1	0.01	0.4	1.1
	2B	110–140	2	4.8	4.3	0.3	0.1	0.0	0.01	0.4	1.5
	2C	140–155	1	4.8	4.0	0.1	0.0	0.0	0.01	0.3	1.8

[a] *n is the number of samples for each soil horizon.*

[b] *pH$_w$ and pH$_K$ are soil pH in water and in saline solution.*

[c] *C$_p$ is the pyrophosphate-extracted C.*

[d] *BS is the sum of base cations (Ca, Mg, Na, K) and Al$_K$ is the exchangeable Al.*

parent material, negative τ values signal Hg losses, and if zero values are obtained Hg is stable in the horizon in relation to the parent material or there is net gain/loss of Hg in that horizon.

Statistical Treatment of Data and Calculations

All the statistical analyses described in this section were done using SPSS version 25.0 software for Windows.

In order to reduce the number of soil variables studied into a few components, a principal component analysis (PCA) was conducted, applying varimax rotation that maximizes the sum of the variances of the square loadings. Each principal component consisted of variables with loadings higher than 0.50 (Abdi and Williams, 2010).

A principal component regression (PCR) analysis was carried out with the "new" variables obtained in the PCA as independent variables, and the principal components which are not correlated between them (orthogonal) and Hg as dependent variables. Using this method, we could predict Hg concentration and distinguish which soil properties are most involved in the Hg depth distribution observed. The weight of each component (wPC) was used to calculate its participation in the Hg prediction and it was estimated by multiplying the score of each component by the corresponding standardized regression coefficient (Liu et al., 2003). The accuracy of the model was checked through root mean squared error (RMSE) calculation and through the representation of Hg observed vs. predicted.

RESULTS AND DISCUSSION

Main Characteristics of Soils

The general chemical properties of the three soil profiles are shown in **Table 1** as an average per soil horizon type. The pH in distilled water (pH$_w$) ranged from 4.6 to 5.2, and in saline solution (pH$_K$) was about 0.2–1.1 units lower than pH$_w$. The range of pHw is quite similar to that reported in previous studies on forest soils derived from granitic material, varying from 4.5 to 5.3 (Álvarez et al., 2002; Eimil-Fraga et al., 2015). The strong acidity showed by the studied soils, which was more evident in the A horizons, is expected considering the low content of weatherable minerals in the soil parent material as well as the climatic conditions that favour the loss of base cations through leaching (Macías et al., 1982). In addition, the contribution of the exchangeable acidity associated with proton release from organic matter functional groups and Al-humus complexes can also contribute to the observed acidity, as was evidenced in highly complex colluvial soils (Kaal et al., 2008) and in podzolic soils (Ferro-Vázquez et al., 2014). Since there are no carbonates in these soils, the total carbon is assumed to be equal to organic C and it ranged from 20.8% in organic horizons to 0.1% in the deepest C soil horizons. The highest Na-pyrophosphate extracted C (C$_p$)/C ratios occurred in the buried A horizons as well as in the illuvial horizons of MF, indicating the presence of well-humified and reactive organic matter in them which may be mobilized as metal-humus complexes (Ferro-Vázquez et al., 2014). As expected, due to the strong acidity of the studied soils, the sum of the base cations was very low in all horizons (0.2–5.9 cmol$_c$ kg^{-1}), while the exchangeable Al (Al$_K$) was slightly high

TABLE 2 | Mean contents per soil horizon of Al and Fe compounds and Hg (Hg_T), total reservoir of Hg ($\sum Hg_{Tres}$) and Tau.

Profile	Horizon	Depth	[a]n	[b]Al_p	[b]Al_o	[b]Al_n	[b]Fe_p	[b]Fe_o	[b]Fe_d	[c]Hg_T	[c]$\sum Hg_{Tres}$	[d]τ_{Hg}
		cm		g kg^{-1}						µg kg^{-1}	mg m^{-2}	
FL	O	0–10	1	4.7	4.8	5.2	4.9	5.8	7.1	169	8.6	460.4
	A	10–30	2	4.4	4.7	5.8	4.7	6.5	6.9	117	20.2	199.0
	AC	30–50	2	4.6	5.5	5.7	4.9	4.9	6.3	44	7.0	72.5
	2A	50–60	1	8.6	10.0	10.7	7.1	9.0	9.9	79	6.6	135.3
	2AC	60–74	1	8.9	9.8	12.8	5.3	6.4	7.3	91	10.0	137.3
	2BC	74–90	1	6.8	9.9	10.6	2.4	2.5	3.8	47	7.0	83.4
	3BC	90–150	3	6.3	9.3	16.2	1.2	1.7	1.7	36	22.6	58.2
	3C	150–185	3	2.2	3.0	17.5	0.6	0.8	2.7	15	5.7	20.4
MF	A	0–38	3	4.4	5.2	5.9	4.8	7.1	8.8	139	29.3	59.4
	Bhs	38–48	1	6.7	7.7	10.2	4.6	8.1	9.4	52	3.5	44.6
	Bs	48–68	1	11.3	13.3	18.9	2.8	3.7	6.8	93	11.3	23.3
	2CB	68–100	2	4.7	9.0	9.3	1.6	2.2	4.8	40	9.9	13.7
	3C	100–190	6	2.1	4.1	18.3	1.5	1.9	6.0	22	12.5	9.4
RV	A	0–43	4	9.4	10.0	11.9	7.3	7.5	12.5	113	29.0	3.2
	B	43–75	6	9.0	15.0	16.2	7.2	8.6	14.2	129	32.5	5.0
	2A	75–95	3	5.7	10.0	14.8	3.4	3.9	7.5	88	13.3	2.3
	2AB	95–110	2	2.7	5.7	13.7	1.2	1.6	4.0	28	3.0	0.4
	2B	110–140	2	1.3	2.8	16.7	0.8	1.2	3.1	15	4.1	–0.5
	2C	140–155	1	0.7	1.4	17.5	0.4	0.6	2.7	9	1.5	–0.6

[a] *n is the number of samples for each soil horizon.*

[b] *Al_p (Fe_p), Al_o (Fe_o), Al_n and Fe_d are Al (Fe) extracted with Na-pyrophosphate (p), ammonium oxalate-oxalic acid (o), Al extracted with Na hydroxide (n) and Fe extracted with Na-dithionite-citrate (d), respectively.*

[c] *Hg_T and Hg_{Tres} are total mercury content and the mass of total Hg in areal basis for each whole horizon.*

[d] *τ_{Hg} are the mass transfer coefficients of Hg for each whole horizon.*

(0.7–6.5 $cmol_c$ kg^{-1}), especially in the surface and buried A horizons, where it dominated the cation exchange capacity of the soils. Both the exchangeable base cations and exchangeable Al are in the range of values reported for acid forest soils developed from parent materials poor in weatherable minerals such as granite, micaceous schist, slates, or quartzites (Álvarez et al., 2002; Eimil-Fraga et al., 2015; Cutillas-Barreiro et al., 2016). Unless in the case of the O layer of FL soil, Al dominated the cation exchange complex in the studied soils (range 0.7–6.5 $cmol_c$ kg^{-1}) which is characteristic in acid forest soils.

The distribution of the Al and Fe compounds in the soil solid phase of the different horizons studied is summarized in **Table 2**. The Al distribution is dominated by the non-crystalline compounds (Al_o) in the O, A, and B horizons ranging from 4.7 to 15.0 g kg^{-1}, although in most of the cases Al-hummus complexes (Al_p) represented more than 60% of total non-crystalline Al compounds. The predominance of non-crystalline Al compounds is often reported in mountain acid forest soils from NW Iberian Peninsula (García-Rodeja and Macías, 1986; Álvarez et al., 2002). On the other hand, Al_c (secondary crystalline Al compounds, estimated as the difference of Al_n minus Al_o) showed a notable predominance in the deepest soil layers (mostly C or transitional B/C horizons) representing 42%–92% of the total free Al pool. A greater abundance of crystalline compounds, compared to other non-ordered Al compounds, was shown by García-Rodeja and Macías (1986) in low organic C content horizons of soils derived from weathered granitic materials located in mountain areas. With regard to organically-complexed Al compounds, its distribution is dominated by Al-humus complexes of moderate (Al_{om}) and high stability (Al_{oh}) below 20–30 cm depth in the three soil profiles, showing values in ranges of 4–37 and 4–91 $cmol_c$ kg^{-1}, respectively. These values are in the same order as those reported in previous studies where Al fractionation in acid forests soils was assessed (Álvarez et al., 2002; Kaal et al., 2008; Ferro-Vázquez et al., 2014), and combined, Al_{om} and Al_{oh} accounted for 51%–99% of the total Al-humus complexes (**Figure 1**). Consistently with Eimil-Fraga et al. (2015), the degree of organic matter humification in the FL, MF, and RV soils, indirectly assessed through the C/N ratio, would favour the organic matter-Al interactions and the consequent formation of high stability Al-humus complexes.

In the case of the Fe distribution, organically-complexed Fe (Fe_p) dominate in the A horizons (4.7–7.3 g kg^{-1}), whereas in the B and C horizons there is an equal partition between Fe-hummus complexes (Fe_p) and crystalline compounds (Fe_c) (**Table 2**). The range of Fe values extracted with different solutions (0.4–14.2 g kg^{-1}) is quite similar to that reported by Kaal et al. (2008) for soils with a noticeable polycyclic pedogenetic character. The trend observed in the fractionation of Fe in FL, MF, and RV coincides with that reported for acid soils from mountain areas (Ferro-Vázquez et al., 2014; Gómez-Armesto et al., 2021a).

Considering the morphological features observed during field sampling such as the horizon diversity and discontinuities recognisable by the occurrence of stone lines and charcoal and the previous discussion of main soil physicochemical characteristics, the three soils studied are representative of complex polycyclic soils typically found in the mountain landscapes from NW Iberian Peninsula. According to this and

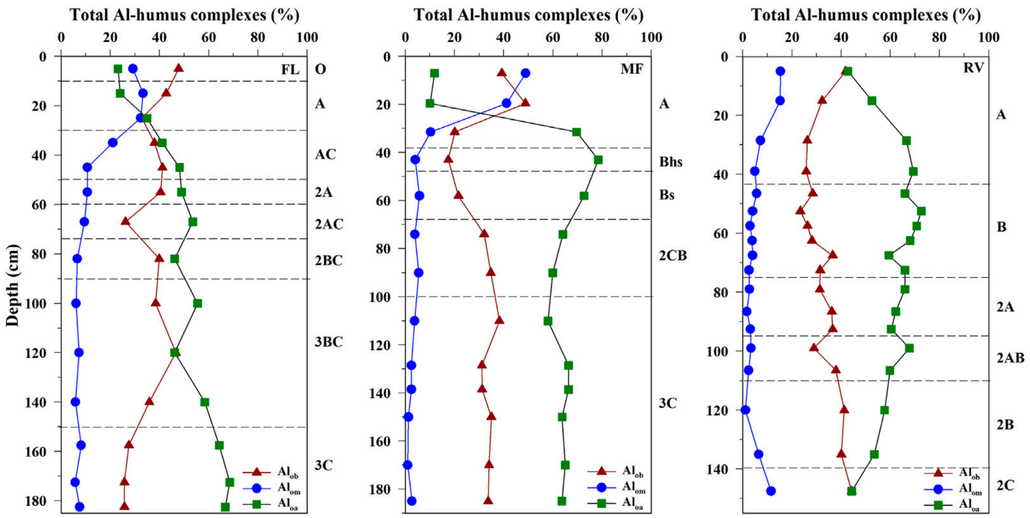

FIGURE 1 | Vertical patterns of Al-humus complexes of low (Al_{ob}), moderate (Al_{om}) and high stability (Al_{oa}) in the soils FL, MF, and RV.

following the IUSS-WRB (2014), although some classification requirements could be not wholly satisfied such as colour in the illuvial horizons, a tentative classification of the studied soil was Folic Umbrisol for soils FL and MF and Cambic Umbrisol in the case of RV soil.

Total Mercury Content and Pools

The total mercury content (Hg_T) is shown in **Table 2** as an average per type of horizon. In general, the highest Hg concentrations are found in the organic matter rich horizons of the three soils (O and A) as well as in illuvial horizons, whereas the minimum Hg_T is observed in the C horizons of the studied soil profiles. Values of Hg_T in the uppermost soils layers of the three soils were higher than the range 43–92 μg kg^{-1} estimated through deep neural network-regression kriging in topsoil samples (0–20 cm) from the NW Iberian Peninsula in a large-scale study carried out in the European Union (Ballabio et al., 2021). Greater values of Hg_T than expected in A and O horizons could be justified by, besides other factors, the altitude at which the studied soils were located, as according to previous research greater atmospheric Hg deposition is favoured in these areas (Blackwell and Driscoll, 2015; Wang et al., 2017). Indeed, in the abovementioned study of Ballabio et al. (2021), moderate Hg levels were found for the Cantabrian Mountains in whose western foothills are located the Xistral Mountains. Detailed revision of values of Hg_T in the studied soils reveals that maximum Hg_T in the FL profile was in the O horizon (169 μg kg^{-1}), diminishing steadily with the soil depth until the first buried soil appeared (horizons 2A and 2AC) where Hg ranges between 79 and 91 μg kg^{-1} (**Figure 2**; blue line). In the MF soil, the highest Hg content was observed in the surface A horizon (139 μg kg^{-1}) then decreasing in the Bhs horizons to increase again in the spodic Bs horizon (93 μg kg^{-1}). Contrary to the FL profile, the remains of older soil cycles in the MF site do not lead to subsurface peaks of Hg_T and this diminishes, progressively reaching the lowest values in the deepest horizon analysed (3C). As it can be seen in **Figure 2**, the maximum Hg_T in the RV soil does not occur in the uppermost soil layer (A horizon) but in the B horizon (129.2 μg kg^{-1}), although both horizons showed a relatively similar mean value. The buried soil in RV shows a noticeable Hg_T level in the 2A horizon (88 μg kg^{-1}), although somewhat lower than in the present soil cycle. Below the horizon 2A, Hg_T decreases with depth in the soil RV and the lowest value is observed in the deepest horizon with 9 μg kg^{-1}.

Although the Hg_T in the samples of the three soils studied were considerably higher than the values of Hg_T found in their parent material (0.7, 1.3, and 4.0 μg kg^{-1} for soils FL, MF and RV, respectively), most of the samples were below 130 μg kg^{-1}, the critical load of Hg in soils (Tipping et al., 2010). Previous studies have shown that in soils with background Hg levels, such as those from the present study, the proportion of available Hg scarcely reaches a percentage of 0.31% of Hg_T in acid soils (Frey and Rieder, 2013). Therefore, considering both the levels of Hg_T and the equivalent fraction of available Hg, the bacterial and fungal communities of the studied soils are not likely to be affected, as detrimental effects on these are only likely with values of Hg_T above 320 μg kg^{-1} (Frossard et al., 2017), almost twice that of the measured values. Moreover, only the surface horizons (O and A) and the B horizon of the soil RV have Hg concentrations somewhat higher than the natural background considered for non-polluted soils (<100 μg kg^{-1}; Xin and Gustin, 2007). This value of Hg_T (100 μg kg^{-1}) was also considered by Harris-Hellal et al. (2009) as a threshold below which no modifications were observed in the bacterial community structure. For plants and terrestrial invertebrates, toxicity effects were observed for Hg_T levels several orders of magnitude higher than those found in FL, MF, and RV soils (Mahbub et al., 2017).

In general, the values of Hg_T obtained in the present study are in the same order as those reported for acid forest soils not directly affected by Hg emission point sources worldwide (Du et al., 2019; Gruba et al., 2019; Nave et al., 2019; Gómez-Armesto et al., 2021a, 2021b; Kroonenberg et al., 2022). Beyond the values

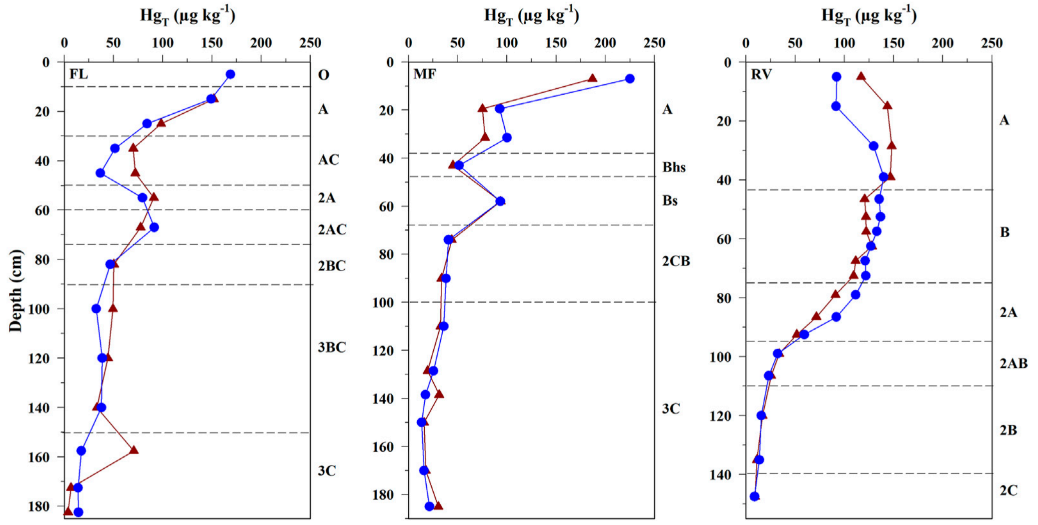

FIGURE 2 | Variation of observed (blue circles) vs. predicted Hg (red triangles) with soil depth in the soils FL, MF, and RV.

of Hg$_T$, the variation of Hg concentrations with soil depth should also be considered. The progressive diminution of Hg$_T$ values with soil depth observed in the three studied soils, besides the very low values of Hg in the parent material, indicates a predominance of atmospheric against lithological Hg source, which reinforces atmospheric deposition as the main mechanism responsible for the occurrence of Hg in the soil surface. Previous studies that focused on the variation of Hg with soil depth also showed a noticeable diminution of Hg from the surface to bottom horizons (Fiorentino et al., 2011; Richardson et al., 2018; Gómez-Armesto et al., 2020; Kroonenberg et al., 2022; Richardson, 2022). However, as it can be seen from **Figure 2**, some parts of the soil profiles can depart from this general trend due to the effects of pedogenetic processes.

The Hg reservoir (Hg$_{Tres}$), shown in **Table 2**, was calculated as the sum of the absolute amount of Hg in each soil sample that compounds the horizon described for the studied soil profiles. The total Hg accumulated in the whole soil profile was 87.7, 66.5, and 83.4 mg m^{-2} for the FL, MF, and RV soils, respectively. On average, the studied soil profiles accumulated 79.2 mg m^{-2}, higher than the total Hg pool of the upper 40 cm of forest soils reported by Obrist et al. (2009) (from 4 to 7 mg m^{-2}), Zhou et al. (2017) (from 7.2 to 10.7 mg m^{-2}), and Obrist (2012) (from 3 to 40 mg m^{-2}). When evaluating the upper 60 cm of forest soils, Méndez López et al. (2023) assessed between 32 and 42 mg m^{-2} in the complete mineral soil profiles studied. Hg pools reported by Demers et al. (2007) (22 mg m^{-2} and 9.6 mg m^{-2} for deciduous and coniferous stands, respectively) are also lower than those reported in the almost 2-m-depth soil profiles of the current study. When comparing Hg pools from different studies, in addition to the uncertainties arising from their calculation for specific soil horizons and/or the complete soil profile (Zhou et al., 2020), different soil profiles depths should also be of concern. In fact, Hg$_{Tres}$ reported for the upper meter of six different 10-m depth soil profiles by Richardson et al. (2018) were 26, 33, 48, 69, 98, and 246 mg Hg m^{-2}.

The Hg reservoir (8.6 mg m^{-2}) of the soil horizon with the highest Hg$_T$ content (O horizon - FL soil profile) was higher than the range (0.02–7 mg m^{-2}) found for this type of horizon worldwide (Evans et al., 2005; Demers et al., 2007; Friedli et al., 2007; Obrist, 2012; Richardson et al., 2013 or Méndez López et al., 2023). The O layers are usually considered as the soil horizons more influenced by atmospheric Hg deposition, but despite this, higher Hg stocks were detected in singular horizons from these polycyclic soils like 2AC (FL soil), Bs and 3C (MF soil) or 2A (RV soil).

The horizons with the highest Hg$_{Tres}$ were the B-horizon from RV soil (32.5 mg m^{-2}), located at 43–75 cm depth followed by both the surface A horizons from RV and MF soil profiles (29.0 and 29.3 mg m^{-2}, respectively). Similar thickness (32–41 cm depth), bulk density (0.81–0.99 g cm^{-3}) and high total Hg contents (113–139 μg kg^{-1}, **Table 2**) were responsible for similar Hg pools of these horizons. As an average, the Hg stored in the surface mineral horizons (A horizons upper 30 cm, ~26.2 mg m^{-2}) is comparable to that found by Richardson et al. (2018) in the upper 30 cm of several soils with values between 26 and 38 mg m^{-2}. At greater soil depths; Richardson et al. (2018) estimated higher Hg inventories as we observed in subsurface mineral horizons such as B from RV soil and the transition layer 3BC from FL soil. The Hg$_{Tres}$ estimated for the 3BC layer (22.6 mg Hg m^{-2}) is mainly a consequence of its thickness (60 cm) and, to a lesser extent, to Hg$_T$ content (36 μg kg^{-1}).

The lowest Hg$_{Tres}$ occurred in the deepest sampled horizons from FL and RV soil profiles (3C: 5.7 mg Hg m^{-2} and 2C: 1.5 mg Hg m^{-2}, respectively) manly due to their low Hg$_T$ contents and the Bhs horizon from MF soil (3.5 mg Hg m^{-2}) because of its low thickness (10 cm) and high coarse fragment proportion (54%). Therefore, the commonly observed declining pattern of Hg concentrations with depth (Zhou et al., 2020) is broken when areal mass is taken into account because in addition to Hg concentrations, soil horizon properties related to soil genesis (density, coarse fraction content, horizon thickness, organic matter content, etc.) will influence the pattern distribution of $\mathrm{Hg_{Tres}}$. As a result, deeper and subsurface soil horizons that are not commonly evaluated (>40–50 cm) because they are not suspicious for accumulating atmospheric Hg can evolve as true reservoirs with important environmental and health implications. The highest Hg pools observed in the deepest horizons (>40–50 cm) represented about 50%–65% of all the Hg stored in those soil profiles. This fact suggests that, if deep layers of soils are not evaluated, a big proportion of the Hg stored at a global level in soils will be systematically underestimated. Fluctuations in the pool of Hg observed with soil depth are even more prominent when looking at polycyclic soils due to the complex soil genesis involved. This has a great transcendence in environmental terms since Hg accumulated in deep soil horizons is associated with organic matter and Al and Fe compounds which are expected to contribute to the most recalcitrant soil Hg pools (Smith-Downey et al., 2010). In absence of anthropogenic perturbations such as forest fire or land-use changes, the biogeochemical stability of this soil Hg pool prevents its mobility to other components of terrestrial ecosystems, leaving aside toxicity risks to wildlife and human health as well as a decline in the quality of groundwater and surface waters.

The complexity of polycyclic soils is also conspicuous when evaluating possible gains and losses of Hg regarding parent material. The highest τ_{Hg} values in each soil profile (**Table 2**) were detected in current or older surface horizons (O, A and even AC horizons) showing that atmospheric deposition of Hg was, and still is, the main source of Hg in the soils evaluated. Moreover, regardless ofthose buried surface horizons, τ_{Hg} values decrease as soil profile depth increases and the lowest values were always calculated for the deepest horizons of each soil profile showing almost no influence of Hg from lithogenic sources. Close to zero τ_{Hg} negative values (−0.5 and −0.6 from 2B to 2C horizons from RV soil, respectively) were also interpreted as indicative of almost no atmospheric deposition. Compared to FL and MF soil horizons, τ_{Hg} values from RV soil are very low. They are comparable with those reported by different authors for a nearby active quarry soil profile in Brasil (Fiorentino et al., 2011), for different sites in a New York-Tennessee transect (Richardson, 2022), and for different forest soils in a nearby study area (Gómez Armesto et al., 2021). In all cases, τ_{Hg} values are mainly positive but less than 10. When focussing in FL and MF soil profiles, similar values of τ_{Hg} were only found in the deepest soil horizons. The soil FL is that with the highest τ_{Hg} values which are comparable to those found in soils nearby to gold mines (Guédron et al., 2006).

The mass transfer coefficients of Hg calculated for the three soil profiles were of different orders of magnitude but all indicated a net gain of Hg in the soil horizons regarding their parent material. In fact, the contribution from parent material to soil Hg accumulation is very low in almost all of the horizons evaluated. Taking into account that the studied polycyclic soils are located relatively close to one another and therefore climate conditions are assumed to be the same, in addition to exogenic inputs associated to atmospheric Hg deposition, pedogenetic processes should be also involved in Hg distribution accounting for its gains and losses throughout the soil profiles as it was indicated by Guédron et al. (2006).

Relationship Between Soil Properties and Hg Distribution

In order to understand the involvement of soil processes in Hg distribution through soil profiles, in addition to common soil properties derived from the general characterization of soil samples, the different forms of Fe and Al associated to the soil solid phase together with Hg concentrations were included in the PCA analysis. The PCA extracted four components (**Table 3**) that accounted for 87% of the variance. The soil properties included in each PC and their corresponding loadings are shown in **Table 3**. The highest variation of the data was explained by PC1 and PC2, which accounted for 36% and 30%, respectively. PC1 is mainly associated with soil organic matter, including variables such as C, N, or S, but

TABLE 3 | Loadings of the soil properties used in the principal component analysis.

	PC1	PC2	PC3	PC4	Com
N	**0.97**	0.18	0.09	0.01	0.98
C	**0.96**	0.21	0.14	−0.01	0.98
eCEC	**0.92**	0.01	0.33	−0.07	0.96
S	**0.91**	0.21	0.01	0.08	0.89
BS	**0.90**	−0.18	−0.15	−0.01	0.86
$\mathrm{Al_K}$	**0.84**	0.07	0.45	−0.09	0.93
$\mathrm{C_p}$	**0.83**	0.43	0.26	0.08	0.96
$\mathrm{Al_{La}}$	**0.78**	0.22	0.53	−0.12	0.96
$\mathrm{Al_{ol}}$	**0.74**	0.26	0.54	−0.13	0.93
$\mathrm{pH_K}$	**−0.69**	0.33	−0.53	0.02	0.87
$\mathrm{Al_o}$	−0.10	**0.93**	−0.19	0.11	0.93
$\mathrm{Al_p}$	0.12	**0.93**	0.12	0.15	0.92
$\mathrm{Al_{om}}$	0.15	**0.91**	0.11	−0.06	0.86
$\mathrm{Al_{oh}}$	−0.09	**0.89**	−0.01	0.26	0.86
$\mathrm{Al_{Cu}}$	0.47	**0.76**	0.32	−0.09	0.90
$\mathrm{Al_c}$	−0.39	**−0.76**	−0.31	0.15	0.84
$\mathrm{Fe_p}$	0.30	**0.72**	0.30	0.49	0.94
$\mathrm{Fe_o}$	0.29	**0.70**	0.46	0.42	0.95
$\mathrm{Fe_d}$	0.16	**0.63**	0.17	0.72	0.97
Alia	−0.35	**0.51**	−0.48	0.01	0.62
Feia	0.12	0.31	**0.70**	0.04	0.60
$\mathrm{Al_n}$	−0.56	−0.07	**−0.57**	0.31	0.75
$\mathrm{Fe_c}$	−0.10	0.29	−0.34	**0.84**	0.91
$\mathrm{pH_w}$	−0.28	−0.45	0.08	**0.49**	0.53
Eigv	8.6	7.1	3.0	2.2	
Var	35.6	29.6	12.7	9.1	

PC1—PC4: components.

Com: communality, proportion of the variance of each parameter explained by the extracted components.

Eigv: eigenvalue.

Var: percentage of variance explained by each component.

Soil properties determining each principal component (PC) appear in bold.

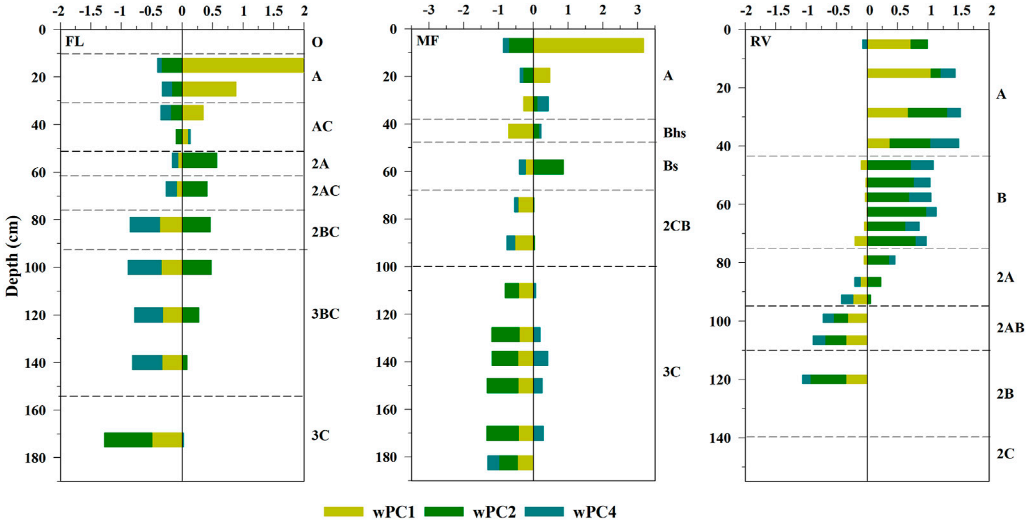

FIGURE 3 | Contribution of each principal component (wPC) to the predicted Hg_T of each sample (weight: regression standardized coefficient for a given component multiplied by the score).

also exchangeable cations and Al-hummus complexes of low stability. PC2 comprised reactive Al and Fe complexes, particularly those Al-hummus complexes of medium to high stability. PC3 explained 13% of the variance of the data and it was related to inorganic non-crystalline Fe compounds and total reactive Al with opposite sign. Finally, PC4 included crystalline Fe compounds and pH_w, accounting for 9% of the data variability.

In order to elucidate the influence of the soil properties in the Hg distribution observed in the three soil profiles studied, we performed a stepwise regression analysis using the scores of the four PCs extracted by PCA, considering them as new soil variables. The best model obtained included PC1, PC2, and PC4 with and adjusted R^2 of 0.893. The predicted Hg (Hg_{pred}) for each soil sample was estimated through the following Eq. **1**:

$$Hg_{pred} = 70.5 + 36.1*PC1 + 28.9*PC2 + 13.1*PC4 \quad (1)$$

The model is rather accurate with a root mean-square error (RMSE) of 18 μg kg^{-1} for the whole group of samples as it can be seen in **Figure 2** in which we represented observed Hg_T values (blue circles) against predicted Hg (Hg_{pred}, red triangles). When focussing on how the principal soil components: PC1 (organic matter and low stability Al-hummus complexes), PC2 (reactive Al-Fe complexes and medium-high Al-hummus complexes), and PC4 (crystalline Fe compounds and pH_w) influence the depth Hg distribution of the studied soils (**Figure 3**), differences among the polycyclic soil horizons were observed. The main soil characteristics implied in the Hg_T distribution observed in A horizons are soil organic matter and low-stability Al-hummus complexes (PC1). In this sense, mercury is widely recognized to show a high affinity for the reduced sulphur groups of the organic matter (Khwaja et al., 2006; Skyllberg et al., 2006).

In the case of the FL soil, there is a clear effect of the PC2 (Al and Fe compounds), from the 2A horizon (50–60 cm) up to the 3C horizon in which, due to the low Hg values, no component showed influence. In the MF profile, of podzolic features, Al and Fe compounds, especially Al (Fe)-hummus complexes, showed the highest weights in Hg prediction. However, in the 3C horizon the crystalline Fe forms were the most important components in the estimation of the Hg content.

In the RV soil, the Hg content in the B and 2A horizons is mostly influenced by Al and Fe complexes (PC2) but also crystalline Fe (PC4) to a lesser extent. In this sense, secondary Fe minerals are recognized to promote Hg accumulation instead of organic compounds in those soil layers deeper than 1 m, as it was suggested by Richardson et al. (2018).

CONCLUSION

The results of this work indicated a noticeable contribution of the Hg deposition from the atmosphere to the Hg concentrations found in the soils studied instead of the lithological source. This fact is supported by the highest Hg values in surface horizons that diminished with depth and peaks in illuvial horizons which were, in all cases, considerably higher than the Hg concentrations of the parent material samples. The Hg depth distribution of the three soils studied was determined by the presence of soil components such as organic matter and Al and Fe complexes. Despite the fact that the maximum Hg contents were observed in O and A horizons, the highest Hg pools appeared in the deepest soil layers (>40–50 cm), representing about 50%–65% of all the Hg stored in those soil profiles. For this reason, subsurface soil horizons should be systematically evaluated in works about Hg distribution in soils, since they could contribute substantially to the total Hg accumulated in soils worldwide. In addition, the biogeochemical stability of Hg accumulated in deep soil layers prevents its mobility to other components of terrestrial ecosystems, leaving aside toxicity risks to wildlife and human health as well as a decline in the quality of groundwater and surface waters.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

Abdi, H., and Williams, L. J. (2010). Principal Component Analysis. *Wiley Interdiscip. Rev. Comput. Stat.* 2, 433–459. doi:10.1002/wics.101

Álvarez, E., Monterroso, C., and Fernández Marcos, M. L. (2002). Aluminium Fractionation in Galician (NW Spain) Forest Soils as Related to Vegetation and Parent Material. *For. Ecol. Manag.* 166, 193–206. doi:10.1016/S0378-1127(01)00658-2

Amos, H. M., Jacob, D. J., Streets, D. G., and Sunderland, E. M. (2013). Legacy Impacts of All-Time Anthropogenic Emissions on the Global Mercury Cycle. *Glob. Biogeochem. Cycle* 27, 410–421. doi:10.1002/gbc.20040

Ballabio, C., Jiskra, M., Osterwalder, S., Borrelli, P., Montanarella, L., and Panagos, P. (2021). A Spatial Assessment of Mercury Content in the European Union Topsoil. *Sci. Total Environ.* 769, 144755. doi:10.1016/j.scitotenv.2020.144755

Blackwell, B. D., and Driscoll, C. T. (2015). Deposition of Mercury in Forests along a Montane Elevation Gradient. *Environ. Sci. Technol.* 49, 5363–5370. doi:10.1021/es505928w

Bloom, P. R., McBride, M. B., and Weaver, R. M. (1979). Aluminum Organic Matter in Acid Soils: Salt-Extractable Aluminum. *Soil Sci. Soc. Am. J.* 43, 813–815. doi:10.2136/sssaj1979.03615995004300040041x

Canarache, A., Vintila, I., and Munteanu, I. (2006). *Elsevier's Dictionary of Soil Science: Definitions in English with French, German, and Spanish Word Translations*. 1st. Amsterdam: Elsevier Science, 1354.

Clarkson, T. W., and Magos, L. (2006). The Toxicology of Mercury and its Chemical Compounds. *Crit. Rev. Toxicol.* 36 (8), 609–662. doi:10.1080/10408440600845619

Cutillas-Barreiro, L., Pérez-Rodríguez, P., Gómez-Armesto, A., Fernández-Sanjurjo, M. J., Álvarez-Rodríguez, E., Núñez-Delgado, A., et al. (2016). Lithological and Land-Use Based Assessment of Heavy Metal Pollution in Soils Surrounding a Cement Plant in SW Europe. *Sci. Total Environ.* 562, 179–190. doi:10.1016/j.scitotenv.2016.03.198

Demers, J. D., Driscoll, C. T., Fahey, T. J., and Yavitt, J. B. (2007). Mercury Cycling in Litter and Soil in Different Forest Types in the Adirondack Region, New York, USA, *Ecol. Appl.* 17-5, 1341–1351. doi:10.1890/06-1697.1

Do Valle, C. M., Santana, G. P., Augusti, R., Egreja-Filho, F. B., and Windmöller, C. C. (2005). Speciation and Quantification of Mercury in Oxisol, Ultisol, and Spodosol from Amazon (Manaus, Brazil). *Chemosphere* 58, 779–792. doi:10.1016/j.chemosphere.2004.09.005

Driscoll, C. T., Mason, R. P., Chan, H. M., Jacob, D. J., and Pirrone, N. (2013). Mercury as a Global Pollutant: Sources, Pathways, and Effects. *Environ. Sci. Technol.* 47, 4967–4983. doi:10.1021/es305071v

Du, B., Zhou, J., Zhou, L., Fan, X., and Zhou, J. (2019). Mercury Distribution in the Foliage and Soil Profiles of a Subtropical Forest: Process for Mercury Retention in Soils. *J. Geochem. Explor.* 205, 106337. doi:10.1016/j.gexplo.2019.106337

Eimil-Fraga, C., Álvarez-Rodríguez, E., Rodríguez-Soalleiro, R., and Fernández-Sanjurjo, M. J. (2015). Influence of Parent Material on the Aluminium Fractions in Acidic Soils under *Pinus pinaster* in Galicia (NW Spain). *Geoderma* 255-256, 50–57. doi:10.1016/j.geoderma.2015.04.026

Evans, G., Norton, S. A., Fernandez, I. J., Kahl, J. S., and Hanson, D. (2005). Changes in Concentrations of Major Elements and Trace Metals in Northeastern U.S.-Canadian Sub-alpine Forest Floors. *Water Air Soil Pollut.* 163, 245–267. doi:10.1007/s11270-005-0435-2

Ferro-Vázquez, C., Nóvoa-Muñoz, J. C., Costa-Casais, M., Klaminder, J., and Martínez-Cortizas, A. (2014). Metal and Organic Matter Immobilization in Temperate Podzols: A High Resolution Study. *Geoderma* 217-218, 225–234. doi:10.1016/j.geoderma.2013.10.006

Fiorentino, J. C., Enzweiler, J., and Angélica, R. S. (2011). Geochemistry of Mercury along a Soil Profile Compared to Other Elements and to the Parental Rock: Evidence of External Input. *Water Air Soil Pollut.* 221, 63–75. doi:10.1007/s11270-011-0769-x

Frey, B., and Rieder, S. R. (2013). Response of Forest Soil Bacterial Communities to Mercury Chloride Application. *Soil Biol. Biochem.* 65, 329–337. doi:10.1016/j.soilbio.2013.06.001

Friedli, H. R., Radke, L. F., Payne, N. J., McRae, D. J., Lynham, T. J., and Blake, T. W. (2007). Mercury in Vegetation and Organic Soil at an Upland Boreal Forest Site in Prince Albert National Park, Saskatchewan, Canada. *J. Geophys. Res.* 112, G01004. doi:10.1029/2005JG000061

Frossard, A., Hartmann, M., and Frey, B. (2017). Tolerance of the Forest Soil Microbiome to Increasing Mercury Concentrations. *Soil Biol. Biochem.* 105, 162–176. doi:10.1016/j.soilbio.2016.11.016

García-Rodeja, E., Nóvoa, J. C., Pontevedra, X., Martínez-Cortizas, A., and Buurman, P. (2004). Aluminium Fractionation of European Volcanic Soils by Selective Dissolution Techniques. *Catena* 56, 155–183. doi:10.1016/j.catena.2003.10.009

García-Rodeja, E., and Macías, F. (1986). Aplicación de técnicas de disolución selectiva al estudio de componentes no cristalinos de una secuencia de suelos sobre granito en la sierra de Ancares (Lugo, Galicia). *An. Edaf. Agrob.* 45, 347–366.

Gee, G. W., and Bauder, J. W. (1986). "Particle Size Analysis," in *Methods of Soil Analysis. Part A.* Editor A. Klute (Madison: American Society of Agronomy), 383–411.

Gómez-Armesto, A., Martínez-Cortizas, A., Ferro-Vázquez, C., Méndez-López, M., Arias-Estévez, M., and Nóvoa-Muñoz, J. C. (2020). Modelling Hg Mobility in Podzols: Role of Soil Components and Environmental Implications. *Environ. Pollut.* 260, 114040. doi:10.1016/j.envpol.2020.114040

Gómez-Armesto, A., Méndez-López, M., Pontevedra-Pombal, X., García-Rodeja, X., Alonso-Vega, F., Arias-Estévez, M., et al. (2021a). Soil Properties Influencing Hg Vertical Pattern in Temperate Forest Podzols. *Environ. Res.* 193, 110552. doi:10.1016/j.envres.2020.110552

Gómez-Armesto, A., Méndez-López, M., Marques, P., Pontevedra-Pombal, X., Monteiro, F., Madeira, M., et al. (2021b). Patterning Total Mercury Distribution in Coastal Podzolic Soils from an Atlantic Area: Influence of Pedogenetic Processes and Soil Components. *Catena* 206, 105540. doi:10.1016/j.catena.2021.105540

Gruba, P., Socha, J., Pietrzykowski, M., and Pasichnyk, D. (2019). Tree Species Affects the Concentration of Total Mercury (Hg) in Forest Soils: Evidence from a Forest Soil Inventory in Poland. *Sci. Total. Environ.* 647, 141–148. doi:10.1016/j.scitotenv.2018.07.452

Guédron, S., Grangeon, S., Jouravel, G., Charlet, L., and Sarret, G. (2013). Atmospheric Mercury Incorporation in Soils of an Area Impacted by a Chlor-Alkali Plant (Grenoble, France): Contribution of Canopy Uptake. *Sci. Total Environ.* 445-446, 356–364. doi:10.1016/j.scitotenv.2012.12.084

Guédron, S., Grangeon, S., Lanson, B., and Grimaldi, M. (2009). Mercury Speciation in a Tropical Soil Association; Consequence of Gold Mining on Hg Distribution in French Guiana. *Geoderma* 153, 331–346. doi:10.1016/j.geoderma.2009.08.017

Guédron, S., Grimaldi, C., Chauvel, C., Spadini, L., and Grimaldi, M. (2006). Weathering versus Atmospheric Contributions to Mercury Concentrations in French Guiana Soils. *Appl. Geochem.* 21, 2010–2022. doi:10.1016/j.apgeochem.2006.08.011

Harris-Hellal, J., Vallaeys, T., Garnier-Zarli, E., and Bousserrhine, N. (2009). Effects of Mercury on Soil Microbial Communities in Tropical Soils of French Guyana. *Appl. Soil Ecol.* 41, 59–68. doi:10.1016/j.apsoil.2008.08.009

IUSS Working Group WRB (2014). International Soil Classification System for Naming Soils and Creating Legends for Soil Maps. World Reference Base for Soil Resources 2014. World Soil Resources Reports No. 106. Rome: FAO, 181.

Juo, A. S. R., and Kamprath, E. J. (1979). Copper Chloride as an Extractant for Estimating the Potentially Reactive Aluminum Pool in Acid Soils. *Soil Sci. Soc. Am. J.* 43, 35–38. doi:10.2136/sssaj1979.03615995004300010006x

Kaal, J., Costa-Casais, M., Ferro-Vázquez, C., Pontevedra-Pombal, X., and Martínez-Cortizas, A. (2008). Soil Formation of "Atlantic Rankers" from NW Spain-A High Resolution Aluminium and Iron Fractionation Study. *Pedosphere* 18, 441–453. doi:10.1016/S1002-0160(08)60035-1

Khwaja, A. R., Bloom, P. R., and Brezonik, P. L. (2006). Binding Constants of Divalent Mercury (Hg^{2+}) in Soil Humic Acids and Soil Organic Matter. *Environ. Sci. Technol.* 40, 844–849. doi:10.1021/es051085c

Kroonenberg, S., Wong, T., Bijnaar, G., Finkie, R., Goenopawiro, K., Asneel, S., et al. (2022). Mercury Background Values in Soils and Saprolites in the Gold-Rich Greenstone Belt of Suriname, Guiana Shield: The Role of Parent Rock and Residual Enrichment. *Sci. Total Environ.* 848, 157631. doi:10.1016/j.scitotenv.2022.157631

Lin, C., and Coleman, N. T. (1960). The Measurement of Exchangeable Aluminum in Soils and Clays. *Soil Sci. Soc. Am. J.* 24, 444–446. doi:10.2136/sssaj1960.03615995002400060009x

Liu, R. X., Kuang, J., Gong, Q., and Hou, X. L. (2003). Principal Component Regression Analysis with SPSS. *Comput. Methods Progr. Biomed.* 71, 141–147. doi:10.1016/S0169-2607(02)00058-5

Macías, F., Calvo, R., García-Rodeja, E., García, C., and Silva, B. (1982). El material original: su formación e influencia en las propiedades de los suelos de Galicia. *An. Edafol. Agrobiol.* 47: 1747–1768.

Mahbub, K. R., Krishnan, K., Naidu, R., Andrews, S., and Megharaj, M. (2017). Mercury Toxicity to Terrestrial Biota. *Ecol. Indic.* 74, 451–462. doi:10.1016/j.ecolind.2016.12.004

Méndez-López, M., Parente-Sendín, A., Calvo-Portela, N., Gómez-Armesto, A., Eimil-Fraga, C., Alonso-Vega, F., et al. (2023). Mercury in a Birch Forest in SW Europe: Deposition Flux by Litterfall and Pools in Aboveground Tree Biomass and Soils. *Sci. Total Environ.* 856, 158937. doi:10.1016/j.scitotenv.2022.158937

Nave, L. E., Covarrubias Ornelas, A., Drevnick, P. E., Gallo, A., Hatten, J. A., Heckman, K. A., et al. (2019). Carbon–mercury Interactions in Spodosols Assessed through Density Fractionation, Radiocarbon Analysis, and Soil Survey Information. *Soil Sci. Soc. Am. J.* 83, 190–202. doi:10.2136/sssaj2018.06.0227

Navrátil, T., Shanley, J., Rohovec, J., Hojdová, M., Penížek, V., and Buchtová, J. (2014). Distribution and Pools of Mercury in Czech Forest Soils. *Water Air Soil Pollut.* 225, 1829. doi:10.1007/s11270-013-1829-1

Obrist, D., Agnan, Y., Jiskra, M., Olson, C. L., Colegrove, D. P., Hueber, J., et al. (2017). Tundra Uptake of Atmospheric Elemental Mercury Drives Arctic Mercury Pollution. *Nature* 547, 201–204. doi:10.1038/nature22997

Obrist, D., Johnson, D. W., and Lindberg, S. E. (2009). Mercury Concentrations and Pools in Four Sierra Nevada Forest Sites, and Relationships to Organic Carbon and Nitrogen. *Biogeosciences* 6, 765–777. doi:10.5194/bg-6-765-2009

Obrist, D., Kirk, J. L., Zhang, L., Sunderland, E. M., Jiskra, M., and Selin, N. E. (2018). A Review of Global Environmental Mercury Processes in Response to Human and Natural Perturbations: Changes of Emissions, Climate, and Land Use. *Ambio* 47, 116–140. doi:10.1007/s13280-017-1004-9

Obrist, D. (2012). Mercury Distribution across 14 U.S. Forests. Part II: Patterns of Methyl Mercury Concentrations and Areal Mass of Total and Methyl Mercury. *Environ. Sci. Technol.* 46, 5921–5930. doi:10.1021/es2045579

Obrist, D., Pokharel, A. K., and Moore, C. (2014). Vertical Profile Measurements of Soil Air Suggest Immobilization of Gaseous Elemental Mercury in Mineral Soil. *Environ. Sci. Technol.* 48, 2242–2252. doi:10.1021/es4048297

Peech, M., Alexander, L. T., Dean, L. A., and Fielding Reed, J. (1947). *Methods of Soil Analysis for Soil-Fertility Investigations*. U.S. Dept. of Agriculture. Washington DC, 757.

Peiteado Varela, E., Piñeiro Rebolo, R., and Martínez Cortizas, A. (2002). Distribución de algunos elementos mayores (K, Ca, Ti,Fe) y traza (Ga, Rb, Sr, Y, Zr, Br) en dos suelos policíclicos podsólicos. *Edafología* 9, 61–84.

Peña-Rodríguez, S., Pontevedra-Pombal, X., Gayoso, E. G. R., Moretto, A., Mansilla, R., Cutillas-Barreiro, L., et al. (2014). Mercury distribution in a toposequence of sub-Antarctic forest soils of Tierra del Fuego (Argentina) as consequence of the prevailing soil processes. *Geoderma* 232-234, 130–140. doi:10.1016/j.geoderma.2014.04.040

Pontevedra-Pombal, X., Rey-Salgueiro, L., García-Falcón, M. S., Martínez-Carballo, E., Simal-Gándara, S., and Martíez-Cortizas, A. (2012). Pre-industrial Accumulation of Anthropogenic Polycyclic Aromatic Hydrocarbons Found in a Blanket Bog of the Iberian Peninsula. *Environ. Res.* 116, 36–43. doi:10.1016/j.envres.2012.04.015

Richardson, J. B., Aguirre, A. A., Buss, H. L., Toby O'Geen, A., Gu, X., Rempe, D. M., et al. (2018). Mercury Sourcing and Sequestration in Weathering Profiles at Six Critical Zone Observatories. *Glob. Biogeochem.* 32, 1542–1555. Cycles. doi:10.1029/2018GB005974

Richardson, J. B., Friedland, A. J., Engerbretson, T. R., Kaste, J. M., and Jackson, B. P. (2013). Spatial and Vertical Distribution of Mercury in Upland Forest Soils across the Northeastern United States. *Environ. Pollut.* 182, 127–134. doi:10.1016/j.envpol.2013.07.011

Richardson, J. B. (2022). Shale Weathering Profiles Show Hg Sequestration along a New York–Tennessee Transect. *Environ. Geochem. Health* 44, 3515–3526. doi:10.1007/s10653-021-01110-x

Roulet, M., Lucotte, M., Saint-Aubin, A., Tran, S., Rhéault, I., Farella, N., et al. (1998). The Geochemistry of Mercury in Central Amazonian Soils Developed on the Alter-Do-Chão Formation of the Lower Tapajós River Valley, Pará State, Brazil1The Present Investigation Is Part of an Ongoing Study, the CARUSO Project (IDRC-UFPa-UQAM), Initiated to Determine the Sources, Fate, and Health Effects of MeHg in the Lower Tapajós area.1. *Sci. Total Environ.* 223, 1–24. doi:10.1016/s0048-9697(98)00265-4

Schroeder, W. H., and Munthe, J. (1998). Atmospheric Mercury - an Overview. *Atmos. Environ.* 32, 809–822. doi:10.1016/S1352-2310(97)00293-8

Schuster, P. F., Schaefer, K. M., Aiken, G. R., Antweiler, R. C., Dewild, J. F., Gryziec, J. D., et al. (2018). Permafrost Stores a Globally Significant Amount of Mercury. *Geophys. Res. Lett.* 45, 1463–1471. doi:10.1002/2017GL075571

SECS-SLCS-IEC (2023). Diccionario Multilingüe de la Ciencia del Suelo, español, catalán, gallego y portugués, con equivalencias en francés e inglés. Available from: https://cit.iec.cat/DiccMCS/inici.html (Accessed February 27, 2023).

Skyllberg, U., Bloom, P. R., Qian, J., Lin, C., and Bleam, W. F. (2006). Complexation of Mercury(II) in Soil Organic Matter: EXAFS Evidence for Linear Two-Coordination with Reduced Sulfur Groups. *Environ. Sci. Technol.* 40, 4174–4180. doi:10.1021/es0600577

Smith-Downey, N. V., Sunderland, E. M., and Jacob, D. J. (2010). Anthropogenic Impacts on Global Storage and Emissions of Mercury from Terrestrial Soils:

Insights from a New Global Model. *J. Geophys. Res. G. Biogeosci.* 115, G03008. doi:10.1029/2009JG001124

Spinola, D., Portes, R., Fedenko, J., Lybrand, R., Dere, A., Biles, F., et al. (2022). Lithological Controls on Soil Geochemistry and Clay Mineralogy across Spodosols in the Coastal Temperate Rainforest of Southeast Alaska. *Geoderma* 428, 116211. doi:10.1016/j.geoderma.2022.116211

Taboada Castro, M. T., Ramil Rego, P., and Díaz-Fierros, F. (1995). *Formación de suelos policíclicos durante el Cuaternario reciente en el Monte Borrelho (N. de Portugal)*, 20. Cuadernos del Laboratorio Xeolóxico de Laxe, 27–36.

Tipping, E., Lofts, S., Hooper, H., Frey, B., Spurgeon, D., and Svendsen, C. (2010). Critical Limits for Hg(II) in Soils, Derived from Chronic Toxicity Data. *Environ. Pollut.* 158, 2465–2471. doi:10.1016/j.envpol.2010.03.027

Urrutia, M., Macías, M., and García-Rodeja, E. (1995). Evaluación del $CuCl_2$ y del $LaCl_3$ como extractantes de aluminio en suelos ácidos de Galicia. *Nova Acta Cient. Compostelana* 5, 173–182.

Wang, X., Luo, J., Yin, R., Yuan, W., Lin, C. J., Sommar, J., et al. (2017). Using Mercury Isotopes to Understand Mercury Accumulation in the Montane Forest Floor of the Eastern Tibetan Plateau. *Environ. Sci. Technol.* 51, 801–809. doi:10.1021/acs.est.6b03806

Xin, M., and Gustin, M. S. (2007). Gaseous Elemental Mercury Exchange with Low Mercury Containing Soils: Investigation of Controlling Factors. *Appl. Geochem.* 22, 1451–1466. doi:10.1016/j.apgeochem.2007.02.006

Zhou, J., Du, B., Shang, L., Wang, Z., Cui, H., Fan, X., et al. (2020). Mercury Fluxes, Budgets, and Pools in Forest Ecosystems of China: A Review. *Crit. Rev. Environ. Sci. Technol.* 50, 1411–1450. doi:10.1080/10643389.2019.1661176

Zhou, J., Wang, Z., Zhang, X., and Gao, Y. (2017). Mercury Concentrations and Pools in Four Adjacent Coniferous and Deciduous Upland Forests in Beijing, China. *J. Geophys. Res. Biogeosci.* 112, 1260–1274. doi:10.1002/2017JG003776

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Agricultural Use of Pesticides in Galicia (NW Spain) During the 20th Century: Inventory of Active Substances and Their Geographic Distribution

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Production of agricultural pesticides underwent an exponential increase during the 20th century. Many of these pesticides have since been prohibited because they are toxic and because they are bioaccumulated and persist in the environment. Detailed knowledge about any substance that is applied to land is essential to enable evaluation of potential contamination effects and the risk of latent exposure to these types of substances. However, accurate determination of the type and amounts of pesticides used throughout the history of agriculture in Spain is greatly complicated by the absence of historical records, scarce control of use and the lack of effective legislation until well into the 21st century. The aims of this study were: 1) to elaborate an inventory of the active substances of the agricultural pesticides (phytosanitary products) used in Galicia throughout the 20th century, 2) to develop a model of their geographic distribution by using a geographic information system (GIS), and 3) to contrast the geographical model obtained for selected persistent pesticides with the presence of these compounds in raw water. We consulted a large variety of sources of information and carried out a geographic analysis for the most commonly used pesticides in different crops. We inventoried a total of 176 active substances belonging to more than 50 different classes of chemicals and observed a clear geographic zoning according to the chemical class and mode of action of the substances. The presence of pesticide residues at drinking water extraction points is consistent with the geographic delimitation established in the study.

Keywords: pesticides, phytosanitary products, organochlorines, triazine, GIS

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INTRODUCTION

The 20th century was a period of intense development for many industries, including the agrochemical sector. Major growth of this industry occurred after the Second World War, particularly during the Green Revolution in the 1960s. New organic synthetic pesticides were produced during this period by one of the most rapidly adopted technologies in the history of agriculture worldwide (March et al., 2010). The consumption of pesticides increased exponentially between the 1970s and 1990s, when it reached maximal levels (Carvalho, 2006; Zhang W. et al., 2011). At the end of the 20th century, some 700 active pesticide substances were authorized for use in

Europe and some 6000 different pesticides are authorized for use throughout the world (Juraske, 2007).

The benefits provided by pesticides in relation to the control of pests and diseases, agricultural development and food quality are widely recognized (Navarro et al., 2007). Currently, almost 3 million tons of pesticides per year are used worldwide and more than 400,000 tons are applied in Europe alone (FAOSTAT, 2021). However, only a very small portion of the amounts applied to crops, generally less than 0.1 percent, reaches the target pest (Pimentel, 1995; Torres and Capote, 2004). Pesticides are mobile in the atmosphere and water and are accumulated and transformed mainly in the soil, where they can be toxic to non-target species, persistent, bioaccumulative and/or biomagnified. Then, although pesticides are designed to provide a highly specific mode of action, their intensive and prolonged use, inappropriate application, and the generation of residues contribute to environmental pollution and represent a risk to human and animal health. The environmental persistence is perhaps best exemplified by the organochlorine compounds, the residues of which have been identified in diverse environmental matrices worldwide (Skrbic and Durisic-Mladenovic, 2007).

The first specific European Directives relating to pesticides concerned the maximum allowed contents of pesticides in fruits and vegetables (Council Directive 76/895/EEC) and the prohibition of active substances due to their toxic effects on human and animal health and on the environment (Council Directive 79/118/EEC). Council Directives 91/414/EEC and 98/8/CE relating respectively to the commercialization of phytosanitary products (agricultural pesticides) and the commercialization of biocides (no agricultural pesticides) appeared in the 1990s. These Directives controlled the commercialization of pesticides in the European Union until well after the beginning of the 21st century.

Despite this regulatory framework, the European Union recognized that the prevailing legislation was not completely effective in protecting against the risks to health and the environment derived from the use of pesticides (European Commission, 2006). To correct this deficiency and to create a consistent global framework (regarding pesticide use), the *Thematic Strategy for the Sustainable Use of Pesticides* was developed within the VI Environmental Program established by the European Council and Parliament for the period 2002–2012 and was adopted by the European Commission on 12 July 2006. Recently, the *Farm to Fork Strategy* (F2FS) for a fair, healthy and environmentally friendly food system was adopted as a key element of the *Green Deal.* The F2FS aims, among other things, to reduce by 50% the overall use and risk of chemical pesticides and the use of more hazardous pesticides by 50% by 2030 (European Commission, 2020).

Spain incorporated the main rules derived from the directive and the aforementioned strategy (Directive 2009/128/EC and Regulation 1107/2009/EC) *via* Royal Decree 1311/2012, 14 September, which established the framework of action for the sustainable use of phytosanitary products. The National Action Plan for the sustainable use of phytosanitary products (PAN, Spanish acronym for *Plan de Acción Nacional*) came into force on 10 December 2012 (PAN 2013–2017), which was later reviewed and updated (PAN 2018–2022, PAN 2023–2024) (MAPA, 2022a).

FIGURE 1 | Location of the autonomous community of Galicia within Europe.

The first strict rule that appeared in Spain regarding information about the amounts of phytosanitary products consumed was Order APA/326/2007, 9 February, which was established because of the transposition of Regulation 852/2004/CE, 29 April, regarding food product hygiene. However, it was not until 2011, *via* another regulation derived from "The Strategy" (Regulation 1185/2009/EC), that a questionnaire on the *Commercial Use of Phytosanitary Products* began to be administered to all producers/sellers of these products. In 2014, as part of the National Action Plan, statistics on the use of phytosanitary products were compiled from the results of questionnaires administered to the owners/managers of agricultural exploitations. Both instruments provide accurate statistical data on the amounts of active substances produced, consumed, and applied to the main crops grown in the Spanish agricultural sector.

It can be concluded that during the 20th century development of pesticide-related legislation did not occur at the same rate as either the pesticide industry or the agricultural sector. It was not until after the beginning of the 21st century that strict rules began to be applied in relation to controlling pesticide use in agriculture by compulsory recording of the exact amounts of pesticides used. Consequently, information about the types and amounts of phytosanitary products applied and about the crops to which they were applied is scarce, disperse and difficult to access.

The objectives of this study were: 1) to compile an inventory of the active substances of the agricultural pesticides (phytosanitary products) used in Galicia throughout the 20th century, 2) to develop a model of their geographic distribution by means of a geographic information system (GIS), and 3) to contrast the geographical model obtained for selected persistent pesticides with the presence of these compounds in raw water. For this purpose, we used a variety of sources of information and carried out a combined geographic analysis of the most commonly used pesticides and of the agricultural land destined for production of the main crops grown in Galicia.

MATERIALS AND METHODS

Study Area

This study focuses on the autonomous community of Galicia, located in north-west Spain and bordering with northern Portugal (**Figure 1**). Occupying a surface area of 29.547 km^2, Galicia is the seventh largest autonomous community in Spain and is slightly larger than the region of Valencia and of similar size to Belgium. It covers four provinces (Coruña, Lugo, Ourense and Pontevedra) and three hundred and thirteen municipalities wich are the smallest administrative subdivisions in Spain and represent the minimum mapping unit of most Spanish statistical surveys (Serra et al., 2014).

The climate in the region varies from oceanic to continental Mediterranean. Mean precipitation levels are high, although variable, with some zones receiving less than 700 mm yr^{-1} and others more 2000 mm yr^{-1} (**Supplementary Figure S1**). The mean temperature ranges between 8°C and 14°C (Sánchez et al., 2014). The frost risk period lasts between less than 3 months in coastal areas, to more than 9 months in the highest mountain areas in the provinces of Ourense and Lugo (Corbelle et al., 2014). The lithology is varied, there being a predominance of acid rocks (granite, quartz schists and shales) and a lower proportion of basic (gabbros and amphibolites) and ultramafic rocks (serpentinites). According to World Reference Base (IUSS Working Group WRB, 2015), the soils are mainly classified as Haplic/Andic Umbrisols on acid rocks, Umbric Andosols on basic rocks and Haplic Phaeozems on serpentinites (Calvo de Anta et al., 2015).

Galician agriculture, which is characterized by smallholdings, polyculture and intensive labor, has been classified as subsistence agriculture since the second half of the 20th century. Agricultural land use in Galicia has traditionally been closely linked to the geochemical and morphological characteristics of the land. The relief is hilly in a large part of the region (more than 76% of the land in Galicia is located at altitudes of between 201 and 1000 m) with a predominance of shallow, acid soils typically with low levels of natural fertility and other limiting edaphic features (leading them to be described as, e.g., stony, hydromorphic and of heavy texture) (Macías, 1992). Until the first half of the 20th century, the main crops were grown in the area were as follows: corn, first grown for grain, with later incorporation of forage varieties; wheat, rye, and barley, rotated annually with corn; potato, initially grown as a fodder crop and later for human consumption; grapes, produced in certain regions; and fruit trees, distributed widely throughout the region. This system went into crisis in the early 1960s, and the subsequent adaptation to market conditions and to social changes greatly modified the agricultural landscape in Galicia (Macías, 1992; Torres Luna, 1992). Greenhouse cultivation of vegetables and flowers began to be introduced in this period.

The incorporation of Galicia (as part of Spain) in the European Union (1986), considered unsatisfactory by some authors (Colino et al., 1999; Soto, 2006; Sineiro, 2008), was definitive in the transformation of the agricultural landscape. Between 1985 and 2005 volume of cereals and potatoes produced decreased, while the value of vegetable production did not increase, and although wine production increased in value, there was no increase in the amount produced (Sineiro, 2008). The most notable change in the farming sector during this period was the increase in milk production, closely related to the cultivation of mixed artificial grassland and forage for dairy livestock. At the beginning of the 21st century, the intense specialization of farming in Galicia determined the land use distribution, with production of vegetable fodder crops predominating (López, 2000; Xunta de Galicia, 2002; Balboa et al., 2004).

Period of Study and Sources of Information

The use of agricultural pesticides in Galicia during the period 1900 to 2000 was analyzed in the present study. For this, we consulted numerous and diverse sources of information throughout the study.

First, we obtained historical information about pesticide consumption, in terms of economic value, from statistical records and studies of agrarian macroeconomic magnitudes in the primary sector (Soto, 2002; MAPA, 2021). We also consulted published data on the amounts of phytosanitary products produced at the end of the 1980s, made available by the *Asociación Empresarial para la Protección de las Plantas* (AEPLA) (Business Association for Plant Protection) to different public organizations. Some of this information has recently been analyzed in relation to the mode of pesticide action (fungicides, insecticides, acaricides, herbicides, etc.) (IAEST, 2015).

On the other hand, although the information obtained from bibliographic references and sources is disperse and incomplete, we have consulted the summaries and abstracts published in historical archives: "*Boletín de la Granja-Escuela Experimental de La Coruña*" (Ministerio de Agricultura, 1899), now the Centro de Investigaciones Agrarias de Mabegondo; "*Cuadernos da Área de Ciencias Agrarias*" (Muñiz de las Cuevas, 1988); the publication "*100 años de Investigación Agraria. 1888–1988*" (Pérez Vidal, 1992), and in other monographs (Xunta de Galicia, 1991). The most recent data were reproduced from Carballeira and Aboal (2000), who detailed the amounts of pesticides (grouped by mode of action) applied to the main crops grown in Galicia in the 1980s and 1990s.

The recent introduction of ICTs (Information and Communication Technologies) has enabled comparison of larger amounts of data *via* the phytosanitary notices published, since 1981, by *La Estación Fitopatolóxica do Areeiro* (the Areeiro Phytopathological Station) (Estación Fitopatolóxica do Areerio, 1981), and to find the most up-to-date information on different web portals, such as those of the *Asociación Empresarial para la Protección de las Plantas* (Business Association for Plant Protection) (AEPLA, 2016) (reports and statistics), the *Ministerio de Agricultura, Pesca y Alimentación de España* (Spanish Ministry of Agriculture, Fisheries and Food (MAPA, 2022b) (Phytosanitary records), and of various multinational pesticide producers (Bayer, Syngenta, Dupont, etc.) as well as other web portals such as InfoAgro and the associated "Agrovademecum."

Finally, we obtained further information *via* semi-structured interviews and administration of questionnaires to a selected group of interest. After making initial contacts *via* various public and private organizations (public authorities, universities, *CSIC*, agricultural syndicates, commercial industries), we selected a group for interview that comprised people of recognized importance in the field of plant health in Galicia, belonging to leading scientific, professional and business institutions (i.e., Areeiro Phytopathology monitoring Station-*CSIC,* Galician agricultural syndicate *Unións Agrarias,* Bayer CropScience Spain for Galicia, and editorial of the *Almanaque Agrícola, ZZ—Syngenta España*).

Database of Active Substances

The information obtained was prepared in three stages, with the aim of compiling a comprehensive, standardized database: 1) comparison and combination of the bibliographic sources and personal inputs; 2) elimination of inconsistent data; 3) chronological ordering of the information.

Once the information was organized and revised, the *Clasificación Armonizada de Sustancias* (Standardized Classification of Substances) established in the annex of Commission Regulation (EU) 656/2011, 7 July 2011, which replaced annex III of Regulation 1185/2009, was applied in order to group the substances in relation to their mode and action and standardized chemical classification. In addition, the most recent update of the "*Lista Comunitaria de Sustancias Activas Aprobadas, Excluidas y en Evaluación Comunitaria, Sustancias de Bajo Riesgos y Lista de Sustancias Básicas*" (Community List of Active Substances Approved, Excluded and Under Community Consideration, Low Risk Substances, and List of Basic Substances) (MAPA, 2022c), dated 1 July 2022, was consulted in order to establish the prevailing regulations concerning each of the active substances inventoried. The following database categories were thus established: active substance, chemical formula, year of introduction, commercial products, target crop, chemical class, mode of action and legal status. The following modes of action were considered: insecticide, herbicide, fungicide, soil sterilizer and bird repellent.

Geographic Model of Use of Active Substances

For the geographical analysis, the variable *accumulated use* of active substances was calculated in each of the municipalities of Galicia. This variable considered: a) the area of agricultural land destined to a specific crop in each municipality, and b) the total number of active substances applied to each of these crops in the period 1900–2000. To simplify the analysis, the distribution of agricultural land (main crops) in Galicia in 1997 (Xunta de Galicia, 1999) was considered representative of the agricultural system at the end of the 20th century. The *accumulated use* of active substances (AU) was calculated (**Eq. 1**) according to: 1) their mode of action (MA; i.e., insecticides, acaricides, fungicides, herbicides, soil sterilizers and bird repellents), or 2) standardized chemical classification (CC; i.e., Organophosphate, carbamate, inorganic, conazole,....). In relation to the chemical classification, *accumulated use* was calculated for the 14 chemical classes of active substances most commonly applied (**Table 1**). Data are available as **Supplementary Material**.

$$\mathrm{AU}_{(\mathrm{MA\,or\,CC})\,j} = \sum_{i=1}^{n} \left(Scul_i \times \mathrm{T}_{(\mathrm{MA\,or\,CC})}\mathrm{cul}_i \right) \tag{1}$$

where,

$\mathrm{AU}_{(\mathrm{MA\,or\,CC})\,j}$: Accumulated use of active substances, according to mode of action (MA) or chemical class (CC), for municipality *j*.

$Scul_i$: % area of land destined for crop *i*.

$\mathrm{T}_{(\mathrm{MA\,or\,CC})}\mathrm{cul}_i$: Total number of Active Substances applied to crop *i* in the period 1900–2000, according to mode of action (MA) or chemical class (CC).

The geographic analysis was carried out with *gvGIS* vs. *2.1.* software, by classifying the variable *accumulated use* into 5 intervals. The intervals were calculated according to the method described by Jenk[1] for optimizing the natural localization of intervals, considering five levels (*very low, low, intermediate, high, and very high*) of *accumulated use* of active substances, for each mode of action and chemical class.

Persistent Pesticides in Water

In order to contrast the geographical model obtained for selected persistent pesticides we consulted the records of the analysis of phytosanitary products in raw waters by different river basin development authorities (*Demarcación Hidrográfica de Galicia Costa y la Confederación Hidrográfica Miño-Sil*) (SERGAS, 2016).

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[1] The categories were constructed using an algorithm that groups similar values and maximizes the differences between classes. The class boundaries are located where there are large increases in the values of the data. This is the method most commonly used in GIS applications.

TABLE 1 | Main standard chemical classes and main active substances introduced in Galicia between 1900 and 2000, classified according to the current legal status (Regulation 1107/2009, 1 July 2022).

Chemical classification	Active substances (mode of action)	
	Authorized	Not authorized
Organophosphate insecticides, fungicides and herbicides	Dimethoate (I), Fenamiphos (I), Chlorpyrifos (I), Malathion (I), Methyl chlorpyrifos (I), Ethoprophos (I), Pyrimiphos-methyl (I), Glyphosate (H), Sulfosate (H), Fosetyl (F)	Parathion (I), Methyl parathion (I), Trichlorfon(I), Demethon (O + S) (I) Fenthion (I), Demethon-S-methyl (I), Phosalone (I), Methidathion (I), Aziniphos-methyl (I), Foxime (I), Vamidothion (I), Diazinone (I), Acephate (I), Heptenophos (I), Ethion (I), Fenitrothion (I), Methamidophos (I), Phosmet (I), Glufosinate (H)
Carbamate insecticides, fungicides and herbicides	Pirimicarb (I), Iprovalicarb (F)	Carbaryl (I), Propoxur (I), Ethiofencarb (I) Carbofuran (I), Aldicarb (I), Asulam (H), Methiocarb (I), Fenoxycarb (I)
Inorganic fungicides and insecticides	Inorganic sulphur (F), Calcium oxide powder (F), Bordeaux mixture (F), Copper oxychloride (F)	Lead arsenate (I), Barium polysulphate (F), Sodium arsenite (F), Carbon disulphide (I), Potassium sulphide (F), Carbon powder (F)
Conazole fungicides	Tebuconazole (F), Difenoconazole (F), Penconazole (F)	Triadimefon (F), Bitertanol (F), Hexaconazole (F), Triadimenol (F), Cyproconazole (F)
Pyrethroid insecticides	Deltamethrin (I), Teflutrin (I), Lambda cyhalothrin (I), Cypermethrin (I)	Cyfluthrin (I), Fenvalerate (I), Permethrin (I)
Dithiocarbamate fungicides	Metiram (F), Ziram (F)	Zineb (F), Mancozeb (F), Thiram (F), Propineb (F), Maneb (F)
Organochlorine insecticides and acaricides		Hexachlorobenzene (I), HCH (I), DDT (I), Lindane (gamma-HCH) (I), Heptachlor (I), Endosulfan (I), Tetradifon (A)
Sulfonylurea herbicides	Iodosulfuron (H), Iodosulfuron-methyl-sodium (H), Mesosulfuron-methyl (H), Nicosulfuron (H)	Triasulfuron (H)
Amide fungicides and herbicides	Metalaxyl (F), Metalaxyl-M (F), Zoxamida (F)	Dimenthenamide (H), Benalaxyl (F)
Triazine herbicides	Terbuthylazine (H)	Anilazine (H), Simazine (H), Atrazine (H), Terbutryn (H)
Benzoylurea insecticides		Flufenoxuron (I), Diflubenzuron (I), Triflumuron (I), Lufenuron (I)
Urea herbicides	Diuron (H), Linuron (H)	Metabenzthiazuron (H), Isoproturon (H)
Chloroacetanilide herbicides	S-Metolachlor (H)	Alachlor (H), Metolachlor (H), Acetochlor (H)
Soil sterilizers	Metam-sodium (S), Metam-potassium (S)	1,3-Dichloropropene (DD) (S), Methyl bromide (S)

Mode of action: I, insecticide; A, acaricide; H, herbicide; F, fungicide; S, soil sterilizer.

RESULTS AND DISCUSSION

Database of Active Substances

The research and literature review carried out in the present study revealed that a total of 176 active substances were introduced in Galicia during the period 1900–2000. In relation to mode of action, 41% of these were insecticides/acaricides, followed in order of abundance by fungicides (30%), herbicides (27%), soil sterilizers (2%) and bird repellents (1%) (**Figure 2A**). The greatest increase in the number of active substances introduced occurred between 1950 and 1980, coinciding with the period of highest consumption of pesticides in Galicia (Soto, 2002; MAGRAMA, 2016a). After 1980, the number of new substances introduced leveled off, although the number of herbicides introduced increased in line with the development of artificial grassland and forage corn in Galicia and the need for weed control. Of all of the active substances inventoried, 106 are currently prohibited and major of these (~70%) were introduced between the 1950s and 1980s (**Figure 2B**).

The active substances inventoried belong to more than 50 different chemical classes (**Table 1**; **Figure 3**). Fourteen of these chemical classes include more than 61% of all of the active substances inventoried and 66% of those substances currently prohibited under Regulation 1107/2009 (**Table 1**). Up until the mid 20th century, the most commonly used pesticides were the inorganic fungicides and insecticides, the biological insecticides, organochlorine insecticides, phenoxy herbicides and some mercurial fungicides and dinitrophenol derivatives. From the 1950s onwards, there was a great increase in the diversity of chemical classes available, and, e.g., the organophosphate insecticides, the chemical class that includes the largest number of substances, appeared during this period. Other important groups include the carbamate and pyrethroid insecticides, the dithiocarbamate fungicides, the chloroacetanilide, sulphonylurea and triazine herbicides, amongst many others.

Geographic Model of Use of Active Substances

The results of the geographic analysis of the *accumulated use* of active substances during the 20th century, in terms of mode of action, shows a clear zoning within Galicia (**Figure 4**). The resulting *Fungicides* map (**Figure 4A**) reveals use of a greater number of substances in the Atlantic zone and the south of the province of Ourense, with a predominance of vine and potato crops. The *Herbicides* map (**Figure 4B**) shows the highest levels of use in areas specialized in growing fodder crops for livestock, as in the regions of Santiago and Xallas (A Coruña), Deza (Pontevedra) and Terra Cha (Lugo). The *Insecticides* map (**Figure 4C**) shows that the use of these compounds is extended throughout Galicia,

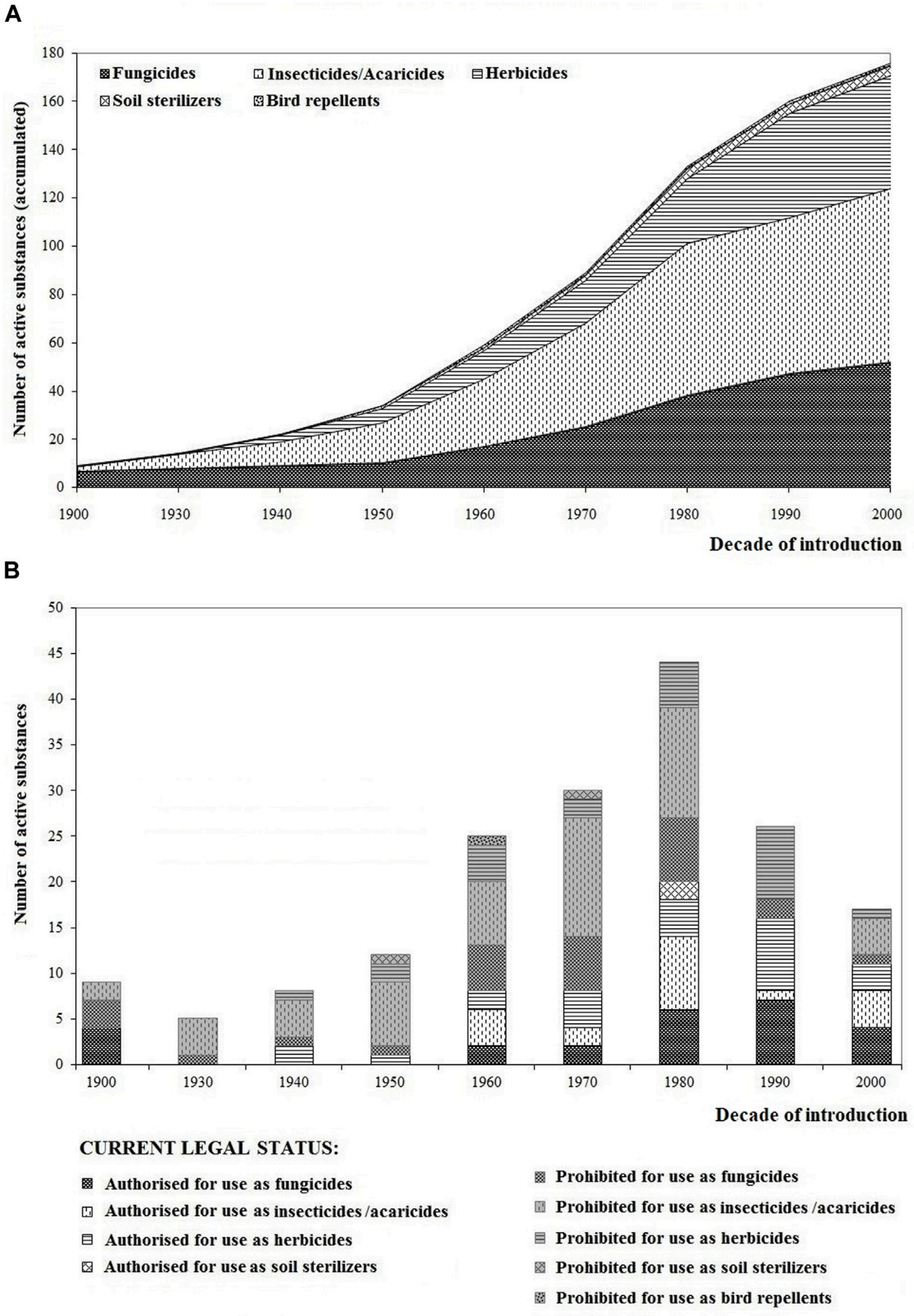

FIGURE 2 | Accumulated number of active substances introduced in Galicia during the period 1900–2000, by **(A)** mode of action and **(B)** mode of action/legal status according to Regulation 1107/2009, 1 July 2022.

with the intensity of use depending on the predominant crops and the climate, with consumption being particularly high in the above-mentioned areas in the province of Pontevedra. The map of *Other substances* (soil sterilizers and bird repellents; **Figure 4D**) showed a similar pattern to that of the insecticides. The zoning of the pesticide use obtained in this

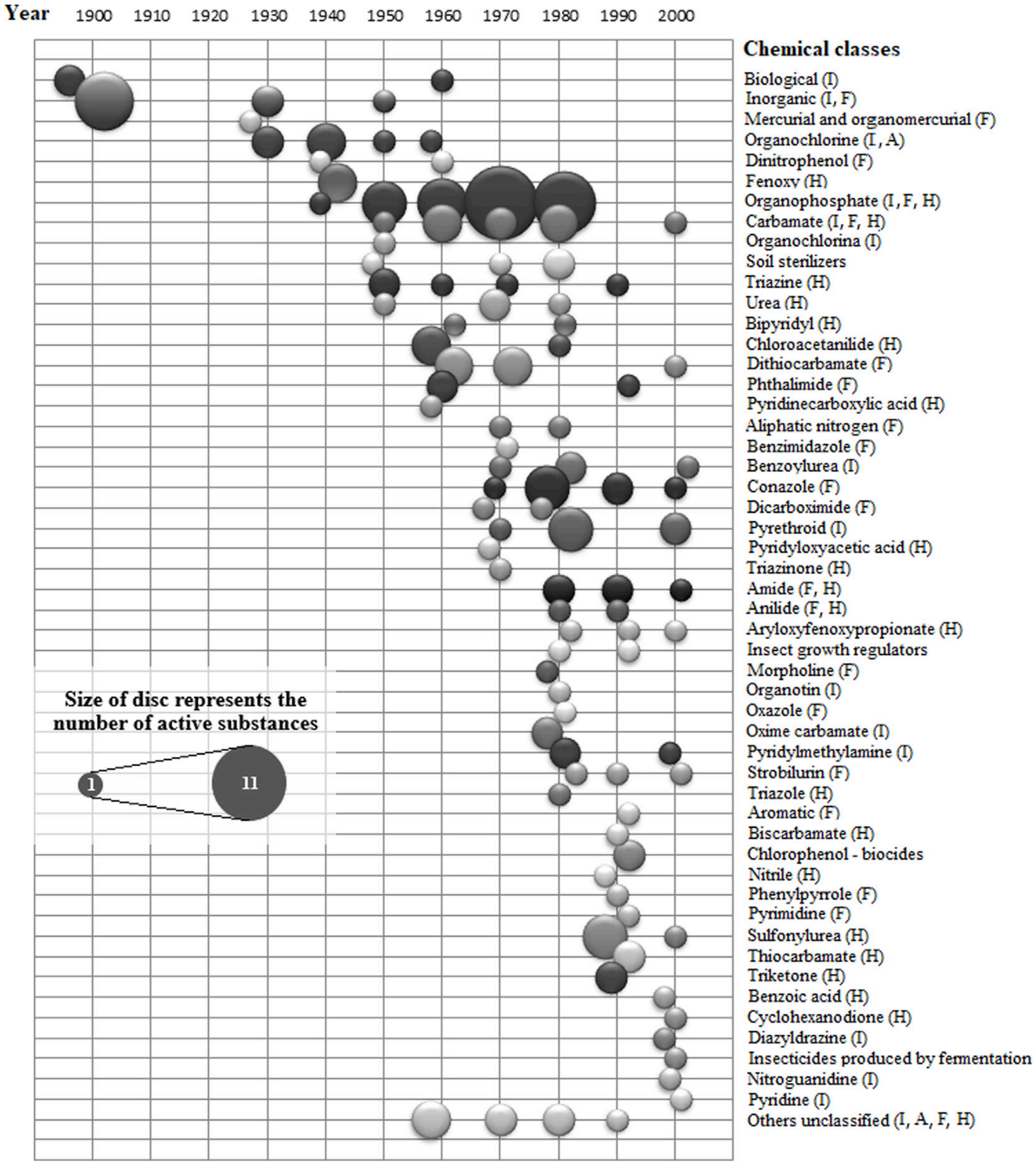

FIGURE 3 | Abundance of active substances introduced in Galicia per year, during the period 1900–2000, according to standard chemical class. The modes of action in each chemical class are indicated in parentheses (I, insecticide; A, acaricide; H, herbicide; F, fungicide).

work showed a close relationship with the climatic variables (temperature, humidity, and duration of the frost period; **Supplementary Figure S1**), which, together with the type of crop, determine the incidence and proliferation of pests. Additionally, this zoning is consistent with that derived from the analysis of the amounts of pesticides (grouped by mode of action) used in the different provinces between 1990 and 2000 (Carballeira and Aboal, 2000). According to the aforementioned authors, the amounts of pesticides consumed, in relation to mode of action, depends on the degree of agricultural intensification in each province and on the type of substance and formulation of each pesticide. For example, in Pontevedra and Ourense, greater amounts of fungicides than of other types of pesticides were consumed, reflecting the large amounts of copper oxychloride and copper sulfate applied to potato crops and vines (Carballeira and Aboal, 2000). These results indicate, in general, a direct relationship between the number and amount of active substances used.

On the other hand, the results of the geographic analysis of the *accumulated use* of active substances according to chemical class (**Table 1**) also reveals a marked zoning in Galicia in relation to the class of pesticide considered (**Figure 5**, and **Supplementary Figure S2**). For example, the greatest numbers (highest *accumulated use*) of inorganic pesticides (mainly fungicides; **Figure 5A**), and pyrethroids (mainly insecticides; **Figure 5B**), were used in the following areas: the Atlantic zone and the south

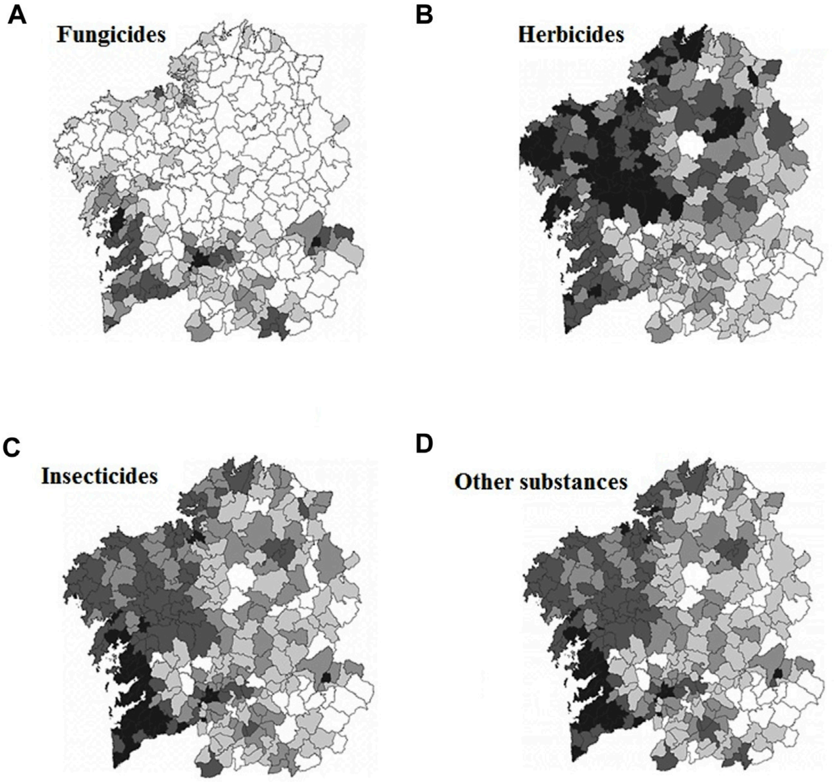

FIGURE 4 | Geographic distribution of the accumulated use of pesticides for the period 1900–2000 according to the mode of action: **(A)** Fungicide, **(B)** Herbicide, **(C)** Insecticide/acaricide, and **(D)** Other substances (soil sterilizers and bird repellents).

of the province of Pontevedra, where there is an important tradition of growing vines and vegetable crops; the vine-growing regions of O Ribeiro, Monterrei and Valdeorras, and the region of A Limia (province of Ourense), known for producing potatoes; and to a lesser extent the regions of Coruña, Betanzos and Eume, in the province of A Coruña, where potato and vegetable growing are important (Xunta de Galicia, 1999). The climate in these areas, i.e., mild mean annual temperature (>13°C) and a short frost risk period (~3 months), is also favorable both for agriculture and for proliferation of most pests.

The distribution map for dithiocarbamate use (**Figure 5C**) is similar to that established for the inorganic pesticides as these compounds are also fungicides and are mainly applied to the same crops in the same areas as already mentioned. Use of the conazole fungicides was clearly delimited in the vine and fruit growing areas of Galicia at the end of the 20th century (**Figure 5D**). Use of the sulfonylureas (herbicides) was mainly concentrated in the northern half of Galicia, in the provinces of A Coruña and Lugo, where cereals (wheat) and fodder crops are traditionally grown (**Figure 5E**). The use of triazines, herbicides applied massively to corn and vines, was concentrated in the western half of Galicia (**Figure 5F**). The organophosphates, wide spectrum insecticides applied to many types of crops, were more widely distributed, although use in the Atlantic zone of Pontevedra is again highlighted, revealing this as one of the top pesticide-consuming areas in Galicia (**Figure 5G**).

Spatial distribution of the organochlorines appears mostly concentrated in Atlantic fringe of Galicia, where could had been applied mainly as insecticide to corn (**Figure 5H**). Another group of chlorinated pesticides, the chloroacetanilides, are herbicides that has been commonly used in a large number of crops (Liu et al., 2000), and its spatial distribution coincides with fodder and corn for grain cultivation areas in Galicia (**Figure 5I**).

Persistent Pesticides in Water

The long-term behavior and persistence of pesticides in soil depends on a number of factors involving the characteristics of the substances (volatility, solubility), the environmental factors (temperature, precipitation), and the soil properties (structure, organic matter content, clay content, microbial activity), (e.g., Edwards, 1975).

The pesticides most persistence in soils in this inventory are the chlorinated compounds (Zhang N. et al., 2011; Tzanetou and Karasali, 2022). The active substances of this group are now totally prohibited: DDT, lindane, endosulphan, heptachlor, etc. (organochlorines) and alachlor, metolachlor, acetochlor (chloroacetanilides). However, as a result of the

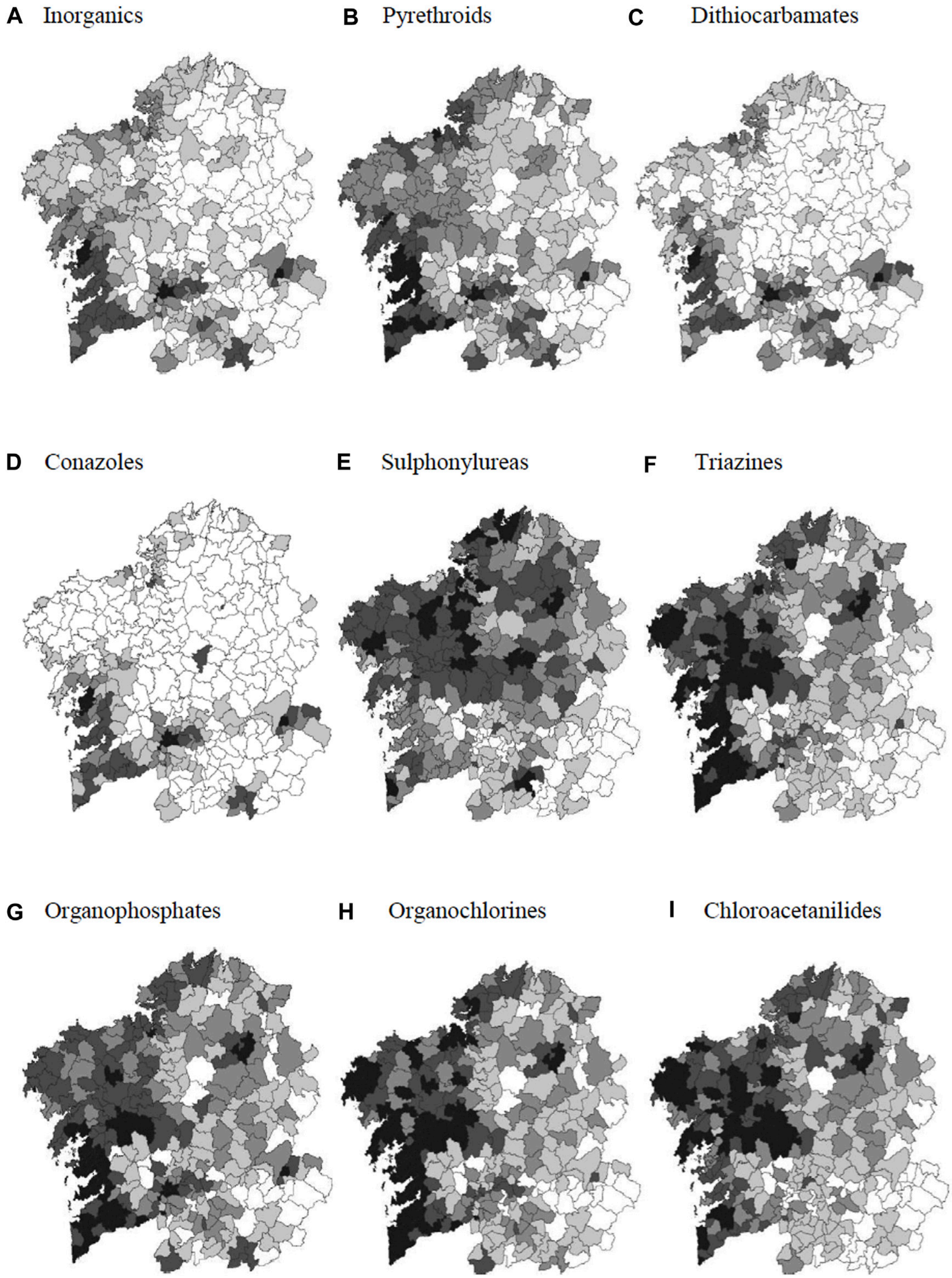

FIGURE 5 | Geographic distribution of the accumulated use of pesticides for the period 1900–2000 according to standard chemical class: **(A)** Inorganic, **(B)** pyrethroids, **(C)** dithiocarbamates, **(D)** Conazoles. **(E)** Sulphonylureas, **(F)** triazines, **(G)** organophosphates, **(H)** organochlorines, **(I)** chloroacetanilides.

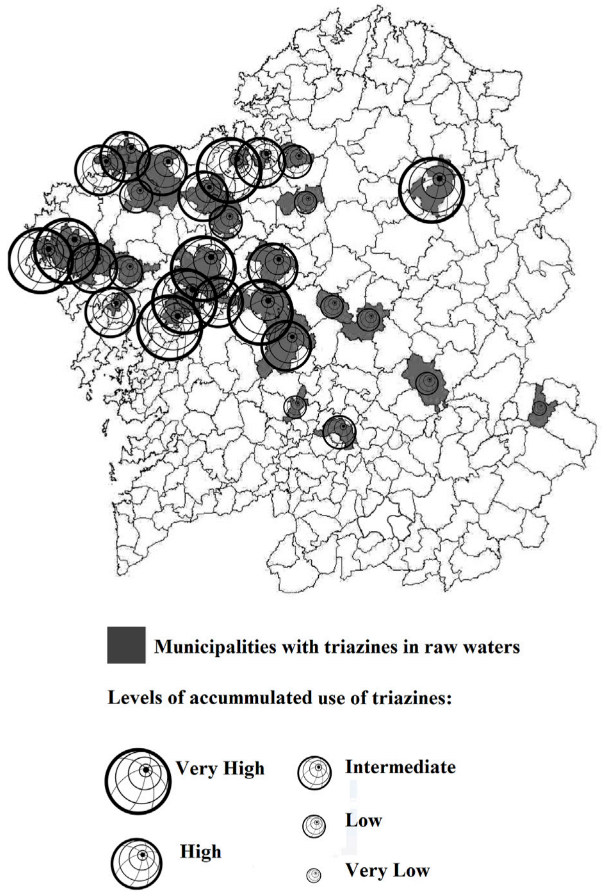

FIGURE 6 | Municipalities of Galicia in which triazines have been detected in raw water during 2010–2014 (SERGAS, 2015) and level of *accumulated use* of triazines obtained in those municipalities.

environmental persistence of organochlorines, residues of these compounds are still being detected in different environmental matrices worldwide (Skrbic and Durisic-Mladenovic, 2007). Of the chemical class of chloroacetanilides, alachlor degrades relatively rapidly in the soil, although it can persist in surface waters for up to 500 days (FAO-UNEP, 2011). Lindane and metolachlor have recently been detected in surface waters in Galicia, during surveys carried out between 2010 and 2014 by local river basin authorities ("*Demarcación Hidrográfica Galicia Costa*" and the "*Confederación Hidrográfica Miño-Sil*" (SERGAS, 2016).

Substances belonging to the triazine class, specifically terbutylazine and atrazine, the latter of which is no longer authorized for agricultural use, were also detected in the same surveys. Comparison of the distribution of pesticides detected in raw waters in the surveys carried out between 2010 and 2014 (SERGAS, 2016) and the regional distribution map of the use of triazines elaborated in the present study showed that 79% of the regions where the substances in this groups were detected by the river basin authorities correspond to intermediate to very high levels of accumulated use of triazines established in the present study (**Figure 6**). The main discrepancy observed in this comparison is in the province of Pontevedra, where these substances were not detected in surface waters, despite the high level of use (**Figure 5F**). Triazines were mainly used in this area to control weeds in vineyards, but this practice was discontinued in the 1990s when it was discovered that the vegetation growing in vineyard soils helped to protect the soil and enhance its fertility. Additionally, vineyard soils usually present sandy textures and low organic matter content, compared to cereal/orchard soils in other areas (Calvo de Anta et al., 2015), which could have contributed to a high bioavailability of these pesticides, and further biodegradation.

CONCLUSION

Compilation of the inventory of the active substances of agricultural pesticides used in Galicia between 1900 and 2000 was complex, mainly due to the absence of historical records, as well as the particular characteristics of Galician agriculture and the mode of introduction of the treatments (*via* commercial businesses, agricultural extension agencies and agricultural cooperatives). The inventory includes 176 active substances applied to the main crops grown in Galicia during the 20th century, of which 106 are currently prohibited. The substances inventoried belong to 50 different chemical classes.

The greatest numbers of active substances were introduced for use in Galician in the period between the 1950s and the 1980s, coinciding with the Green Revolution worldwide. Thereafter, the appearance of new substances decreased and was influenced by reformation of the agricultural economics in Galicia, brought about by the entry of Spain in the European Union. A further substantial reduction is expected in the coming years thanks to the recently adopted European farm-to-fork strategy.

The geographic analysis of the accumulated use of active substances throughout the 20th century revealed a clear zoning of the region according to the mode of action and chemical classification. The spatial distribution was consistent with the pattern of agricultural production in Galicia, with the particular climate conditions in each area and with the data obtained regarding the amounts of pesticides (tonnes) used during the 1990s.

Many of the substances previously used in Galicia, and now prohibited, continue to appear in pesticide detection surveys, in, e.g., raw waters. The points where triazine pesticides were detected in this matrix and in recent surveys carried out by the *Organismos Oficiales de Cuenca* (River Basin Authorities) are consistent with intermediate to high levels of accumulated use in the geographic distribution map elaborated in the present study.

The maps produced in this work can serve as a basis for exploratory sampling designs aimed at detecting inventoried pesticides in different media (soils, water, biota) and contribute to environmental and biological monitoring programs to quantify exposure and assess pesticide risks.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

RC is responsible for design, data mining, modeling, and writing; CM is responsible for the design, discussion, writing, and revision of the manuscript.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/sjss.2023.11197/full#supplementary-material

REFERENCES

AEPLA (2016). *Memoria de actividad de la Asociación Empresarial para la Protección de las Plantas 2015*. Available from: http://www.aepla.es/tmp/images/publicaciones/P0022_Memoria_AEPLA_2015.pdf (Access September 20, 2016).

Balboa, X., Fernández, L., Soto, D., Besteiro, B., Fernández, X., Jordán, M., et al. (2004). La devolución de la propiedad vecinal en Galicia (1960-1985). Modos de uso y conflicto de propiedad. *Hist. Agrar.* 33, 105–130.

Calvo de Anta, R., Luis Calvo, E., Casás Sabarís, F., Galiñanes Costa, J. M., Matilla Mosquera, N., Macías Vázquez, F., et al. (2015). Soil Organic Carbon in Northern Spain (Galicia, Asturias, Cantabria and País Vasco). *Span. J. Soil Sci.*, 5(1), 41–53. doi:10.3232/sjss.2015.v5.n1.04

Carballeira, A., and Aboal, J. (2000). *Banco de especímenes ambientales: Una propuesta para Galicia*. Ed. 105. Santiago de Compostela: Universidade de Santiago de Compostela, Servicio de Publicacións e Intercambio Científico, 110.

Carvalho, F. (2006). Agriculture, Pesticides, Food Security and Food Safety. *Environ. Sci. Policy* 9, 685–692. doi:10.1016/j.envsci.2006.08.002

Colino, J., Noguera, P., and Aboal, M. (1999). *La agricultura gallega en la Unión Europea: balance del primer decenio*. Galicia: Fundación La Caixa Galicia, Santiago de Compostela. ISBN 84-89231-94-X.

Corbelle, E., Vila, D., Crecente, R., and Díaz-Fierros, F. (2014). Dixitalización Do mapa de capacidade produtiva dos solos de Galicia. *Recur. Rurais, núm* 10, 35–43. doi:10.15304/rr.id3321

de Galicia, X. (2002). *Anuario de Estadística Agraria 2000. Consellería de Medio Rural*. ISBN 978-84-453-3323-5.

de Galicia, X. (1999). Anuario de estatística agraria 1997. *Cons. Medio Rural*, 369p. ISBN 978-84-453-2366-3.

Edwards, C. A. (1975).Factors that Affect the Persistence of Pesticides in Plants and Soils. In *Pesticide Chemistry*, 3. Butterworth-Heinemann, 39–56.

Estación Fitopatolóxica do Areerio (1981). Avisos Fitosanitarios. *Diputación de Pontevedra*. Available from: http://areeiro.es/es/Servicios/Info_Fitosanitaria/Avisos/Anteriores/Aviso1_Pag1.HTM (Access July 12, 2015).

European Commission (2006). *La política comunitaria para un uso sostenible de plaguicidas. Origen de la estrategia*. Luxembourg: Oficina de Publicaciones Oficiales de las Comunidades Europeas. ISBN 92-79-03221-6.

European Commission (2020). *Farm to Fork Strategy: For a Fair, Healthy and Environmentally-Friendly Food system.Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions*. Available from: https://ec.europa. eu/food/farm2fork_en (Accessed December 1, 2022).

FAO-UNEP (2011). *Alaclor. Documento de orientación para la adopción de decisiones. Secretaría del Convenio de Rotterdam sobre el procedimiento de consentimiento fundamentado previo aplicable a ciertos plaguicidas y productos químicos peligrosos objeto de comercio internacional*. Geneva.

FAOSTAT (2021). *Pesticides Use*. Available from: https://www.fao.org/faostat/en/#data/RP/visualize (Accessed July 15, 2021).

Instituto Aragonés de Estadística, (2015). *Consumo agrícola de plaguicidas/pesticidas por tipos de producto, por CC.AA. España. Serie 1999-2014*. Huesca: Departments and Public Organizations. Government of Aragon. Available from: http://www.aragon.es/DepartamentosOrganismosPublicos/Institutos/InstitutoAragonesEstadistica/AreasTematicas/14_Medio_Ambiente_Y_Energia/01_SectoresProductivos/ci.01_Agricultura_medio_ambiente.detalleDepartamento (Access 15 June 2016).

IUSS Working Group WRB (2015). “World Reference Base for Soil Resources 2014 (Updated in 2015),” in *International Soil Classification System for Naming Soils and Creating Legends for Soil Maps*. World Soil Resources Reports No. 106 (Rome: FAO).

Juraske, R. (2007). *Advances in Life Cycle Impact Assessment of Pesticides: Methodological Improvements and Experimental Studies*. Tarragona, Spain: Doctoral thesis. Universitat Rovira i Virgili.

Liu, W., Gan, J., Papiernik, S-K., and Yates, S-R. (2000). Structural Influences in Relative Sorptivity of Chloroacetanilide Herbicides on Soil. *J. Agric. Food Chem.* 48 (9), 4320–4325. doi:10.1021/jf990970+

López, E. (2000). El sector agrario gallego a las puertas del siglo XXI: balance de sus transformaciones recientes. *Rev. Galega Econ.* 9 (1), 1–30.

Macías, F. (1992). El uso del Suelo en Galicia. *Guia Nat. Galicia* 19, 361–380.

MAPA (2022a). Plan de Acción Nacional para el uso sostenible de productos fitosanitarios. *Secretaría General de Agricultura y Alimentación, Dirección General de Sanidad de la Producción Agraria. Miniterio de Industria, Pesca y Alimentación*. Madrid, España. Available from: https://www.mapa.gob.es/es/agricultura/temas/sanidad-vegetal/plandeaccionnacional2023-2024_tcm30-639977.pdf (Access December 20, 2022).

MAPA (2022b). *Registro de Productos Fitosanitarios*. Pesca y Alimentación: Ministerio de Agricultura. Available from: https://www.mapa.gob.es/es/agricultura/temas/sanidad-vegetal/productos-fitosanitarios/registro-productos/ (Access July 5, 2022).

MAPA (2022c). *Lista Comunitaria de Sustancias Activas Aprobadas, Excluidas y en Evaluación Comunitaria, Sustancias de Bajo Riesgos y Lista de Sustancias Básicas*. Madrid, España: Dirección General de Sanidad de la Producción Agraria, Ministerio de Agricultura, Pesca y Alimentación. Available from: https://www.mapa.gob.es/es/agricultura/temas/sanidad-vegetal/listasustanciasactivasaceptadasexcluidas_tcm30-618972.pdf (Access Jully 5, 2022).

MAPA (2021). *Resultados Renta Agraria Regional (serie histórica 1990 - 2000).* Galicia: Evolución macromagnitudes agrarias. *Miniterio de Industria, Pesca y*

Alimentación. Madrid, España. Available from: https://www.mapa.gob.es/es/estadistica/temas/estadisticas-agrarias/galicia_tcm30-122209.pdf (Access September 20, 2013).

March, G., Oddino, C., and Marinelli, A. (2010). *Manejo de Enfermedades de los Cultivos según Parámetros Epidemiológicos*, 193. Argentina: Biglia Impresores. ISBN 978-987-05-9063-7.

Ministerio de Agricultura (1899). Boletín de la Granja Experimental de La Coruña, 1899-1901. *Números del 1 al 30*. Coruña: Centro de Investigacións Agrarias de Mabegondo (A Coruña). Available from: http://biblioteca.galiciana.gal/es/consulta/registro.do?control=BDG20080023577 (Access March 1, 2014).

Muñiz de las Cuevas, R. (1988). Alternativas estructurais e producción agrícola-gandeira. Cuadernos da área de ciencias agrarias (Coruña: Edicións do Castro), 8, 260. ISBN 978-84-7492-393-3.

Navarro, S., Vela, N., and Navarro, G. (2007). *Review*. An Overview on the Environmental Behaviour of Pesticide Residues in Soils. *Span. J. Agric. Res.* 5 (3), 357–375. doi:10.5424/sjar/2007053-5344

Pérez Vidal, T. (1992). *100 años de investigación agraria: 1888-1988. Tomos I y II. Servicio de Estudios e Publicacións. Consellería de Agricultura Gandería e Montes*. Santiago de Compostela: Xunta de Galicia.

Pimentel, D. (1995). Amounts of Pesticides Reaching Target Pests: Environmental Impacts and Ethics. *J. Agric. Environ. Ethics*. 8, 17–29. doi:10.1007/bf02286399

Sánchez, R., Gómez, P., and Orro, M. (2014). Centenario del Observatorio de A Coruña. Un siglo de registros climáticos en Galicia. *Agencia Estatal de Meteorología*. Madrid: Ministerio de Agricultura, Alimentación y Medio Ambiente, 229. ISBN 978-84-78378-091-7.

SERGAS (2015). Fitosanitarios. *Servicio Galego de Saúde, Consellería de Sanidade, Xunta de Galicia*. Available from: https://www.sergas.es/Saude-publica/Documents/2891/FITOSANITARIOS_23042015.pdf (Access September 1, 2016).

Serra, P., Vera, A., Tulla, A. F., and Salvati, L. (2014). Beyond Urban–Rural Dichotomy: Exploring Socioeconomic and Land-Use Processes of Change in Spain (1991–2011). *Appl. Geogr.* 55, 71–81. doi:10.1016/j.apgeog.2014.09.005

Sineiro, F. (2008). La integración de la agricultura gallega en la unión europea. *Rev. gallega Econ.* 17, 1–14.

Skrbic, B., and Durisic-Mladenovic, N. (2007). Principal Component Analysis for Soil Contamination with Organochlorine Compounds. *Chemosphere* 68, 2144–2152. doi:10.1016/j.chemosphere.2007.01.083

Soto, D. (2006). "Historia Dunha Agricultura Sustentábel: Transformacións Produtivas Na Agricultura Galega Contemporánea," in *Xunta de Galicia. Consellería do Medio Rural* (Santiago de Compostela). ISBN: 978-84-453-4308.4.

Soto, D. (2002). *Transformacións productivas na agricultura galega contemporánea. Da Agricultura Orgánica á Revolución Verde (1752-1986).Unha aproximación a partir das macromagnitudes*. Doctoral thesis. Santiago de Compostela: Universidad de Santiago de Compostela.

Torres, D., and Capote, T. (2004). Agroquímicos un problema ambiental global: uso del análisis químico como herramienta para el monitoreo ambiental. *Ecosistemas* 13 (3), 2–6.

Torres Luna, M. P. (1992). La Agricultura Gallega. Una Visión Geográfica. Actas do I Congreso Internacional da Cultura Galega (Santiago de Compostela: Xunta de Galicia), 221–233. ISBN 84-453-0595-6.

Tzanetou, E-N., and Karasali, H. (2022). A Comprehensive Review of Organochlorine Pesticide Monitoring in Agricultural Soils: The Silent Threat of a Conventional Agricultural Past. *Agriculture* 12 (5), 728. doi:10.3390/agriculture12050728

Xunta de Galicia, X. (1991). *A Estatística Na Agricultura. Estudios Monográficos*. Santiago de Compostela: Instituto Galego de Estadística, 218. 978-84-453-0368-9.

Zhang, N., Yang, Y., Tao, S., Liu, Y., and Shi, K-L. (2011b). Sequestration of Organochlorine Pesticides in Soils of Distinct Organic Carbon Content. *Environ. Pollut.* 159, 700–705. doi:10.1016/j.envpol.2010.12.011

Zhang, W., Jian, F., and Ou, J. (2011a). Global Pesticide Consumption and Pollution: with China as a Focus. *Proc. Int. Acad. Ecol. Environ. Sci.* 1, 125–144.

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Forms of Toxic and Trace Metals in Grassland Soils of Galicia, Spain

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The application of cattle slurry to agricultural soils contributes to the circular economy, while enriching the soil in macro and micronutrients and organic matter. However, this practice can have deleterious environmental effects, by adding toxic metals and other contaminants. The pseudo-total concentrations of nine potentially toxic and trace metals (Fe, Mn, Zn, Cu, Ni, Co, Cr, Cd and Pb) as well as metals extracted by DTPA, Mehlich 3 and 0.01 M $CaCl_2$ were determined in Galician (NW Spain) grassland soils regularly receiving cattle slurry. Four soil depths (0–5, 5–10, 10–20 and 20–40 cm) were sampled and analysed. The pollution condition was assessed by comparing the pseudo-total concentrations with generic reference levels for Galician soils and by using pollution indices. The results indicated the absence of soil pollution by Fe, Ni, Co, Cr and Pb and a situation of no pollution to moderate pollution by Mn, Zn and Cu. Cd was the element most frequently enriched in the studied soils according to the pseudo-total, DTPA and Mehlich-3 concentrations, while the extraction by $CaCl_2$ pointed to no environmental risk. The study supports the lithogenic character of Fe, Ni, Co and Cr, the mixed lithogenic and anthropogenic nature of Mn, Zn and Cu and the anthropogenic origin of Cd in these soils. The latter element can be added by both the application of cattle slurry and inorganic phosphate fertilisers.

Keywords: heavy metals, grassland soils, Mehlich-3 extraction, $CaCl_2$ extraction, DTPA extraction, pastureland, microwave digestion, availability

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INTRODUCTION

The term “heavy metals” has been used to refer to metals (sometimes metalloids are included) that in the elemental state have a density greater than 5 g cm^{-3} and can be toxic to organisms or ecosystems. Many of these elements are present in soils and the lithosphere in tiny concentrations (<0.1%) and are known as “trace elements.” Some trace elements are micronutrients for plants or animals, while others do not have any known biological function. The use of the term “heavy metals” has been questioned, especially in recent years (Nieboer and Richardson, 1980; Duffus, 2002; Hodson, 2004) and the terms “trace elements,” “toxic elements,” “potentially toxic elements,” “soft acid metal ions” or “class B metal ions” are preferred by some authors (Nieboer and Richardson, 1980; Madrid, 2010; Pourret and Hursthouse, 2019; Zhang et al., 2022). However, the term “heavy metals” is often found in current publications and other authors support continuing to use it, as a well-established expression utilised for decades in the environmental field (Batley, 2012; Gustin et al., 2021).

Potentially toxic metals are present in natural soils, usually at trace levels, originating from the parent material. Anthropogenic activities can contribute potentially toxic metals to the soil, promoting the build-up of these elements. Among the anthropogenic sources of potentially toxic metals in soils, mining, metal smelting, burning of fossil fuels, waste disposal and incineration, and traffic of vehicles are worth mentioning. Agricultural activities can also add potentially toxic metals to the soil.

Potentially toxic metals can be found in soil in various chemical forms, those that are soluble or mobile being the most worrying from an environmental point of view in the short/medium term. The soil is often capable of effectively retaining potentially toxic metals and other pollutants, preventing their passage into water bodies and vegetation (Nunez-Delgado et al., 2002). A polluted soil no longer plays a protective role of other environmental compartments, becoming instead a source of pollutants (Richter et al., 1997).

Human being exerts a series of disturbing actions on agricultural soils, among them the addition of fertilisers and pesticides. Various agricultural practices contribute potentially toxic metals to the soil. Many traditionally used fungicides and insecticides supply Cu, Hg, Mn, Pb and Zn (Nicholson et al., 2003). Other agricultural practices contribute potentially toxic metals to the soil from fuels, lubricants, tires and sewage sludge (Nicholson et al., 2003; Antoniadis et al., 2008; Proshad et al., 2021; Li et al., 2022). Manures and slurries from livestock activities may contain significant amounts of Zn, Cu and other heavy metals, which come from additives in animal feed (Mantovi et al., 2003; Jakubus et al., 2013). Zn and Cu are essential elements to plants, but they are toxic at high concentrations. Also chemical fertilisers, in particular phosphates, can contain toxic metals as impurities (Loganathan et al., 2008; Cakmak et al., 2010). Heavy metals can be toxic to soil microorganisms, plants and animals as well as be exported to water bodies and to the food chain (Liang and Tabatabai, 1977; Fernandez-Calvino et al., 2010; Nagajyoti et al., 2010; Antunes et al., 2011; Kabata-Pendias, 2011).

The use of livestock manures and slurries as fertilisers is a sound way of disposal of these animal wastes and allows the use of synthetic fertilisers to be reduced, improving soil properties and crop yields while contributing to a circular economy (Poulsen et al., 2013; Xia et al., 2017). Despite their overall benefit for agriculture, their contribution of potentially toxic metals as well as pathogens and antibiotics residues requires careful management of the application of manure and slurry to agricultural soils. The application of slurry to soil has been reported to be a cause of toxic metal contamination (Del Castilho et al., 1993; Baize, 1997; L'Herroux et al., 1997; Smith, 1997; Romkens and Salomons, 1998; De la Torre et al., 2000; Aldrich et al., 2002; Moreno-Caselles et al., 2002; Wilcke et al., 2002; De Temmerman et al., 2003). The metal toxicity in soils depends not only on their total concentration but, especially, on the concentration of soluble or easily mobilisable forms (McBride, 1989; Alloway and Jackson, 1991; Gupta et al., 1996). In addition, soil contamination can lead to contamination of aquifers as well as contamination of the food chain through vegetation. In relation to the possible contamination of other media, the soil can play a filter role, retaining heavy metals and preventing their passage to groundwater or vegetation (L'Herroux et al., 1997; Nunez-Delgado et al., 2002).

In Galicia (NW Spain), the application of cattle slurry to pastureland is a common practice (Franco-Uria et al., 2009), which provides the soil with macro and micronutrients as well as organic matter. Few published data exist relative to the presence of toxic and trace metals in Galician soils treated with cattle slurry (Paz-González et al., 2000; Caridad-Cancela et al., 2002; Lopez-Mosquera et al., 2005; Franco-Uria et al., 2009; Seco-Reigosa et al., 2015).

Franco-Uría et al. (2009) studied the total concentrations of heavy metals in the surface horizons of grassland soils in the river Magdalena basin (Lugo, Galicia, Spain), which usually receive dairy cattle slurry, with the main aim of identifying the sources of the different heavy metals. The present study addresses the occurrence and quantification of different forms of heavy metals at various soil depths in the same basin, to assess the soil pollution degree, the vertical distribution of metals and the risk of toxic metals mobilisation. Among the studied elements, iron (Fe) is not a trace element in geochemistry (its concentration in the Earth's crust is 5%), but is a trace element in biochemistry (IUPAC, 2022) and a plant micronutrient.

MATERIALS AND METHODS

Study Area

The study was carried out in the basin of the river Magdalena, in A Pastoriza, province of Lugo, Spain (43° 16′44″N, 7° 20′50″W). The basin (**Figure 1**) covers an area of 2,250 ha and is mainly occupied by grasslands (84% of the surface). The principal economic activity in the area is dairy farming. The basin can be considered representative of the zones of intensive cattle breeding in Galicia (NW Spain). The climate is humid temperate, with an annual average temperature of 12°C and average annual precipitation of 1,350 mm (MeteoGalicia, 2022). The soils, developed from tertiary and quaternary sediments and quartz-rich slates, are classified as Umbrisols (IUSS Working Group WRB, 2022). The grassland soils, acid and rich in organic matter (Franco-Uria et al., 2009), are regularly fertilised with cattle slurry, coming from the farms, and inorganic fertilisers. The average contribution of slurry to the soils of the basin is 90 m^3 ha^{-1} $year^{-1}$. The soil textures range from sandy loam to silt loam and clay loam.

Soil Sampling and Analysis

Twenty farms were selected in the river Magdalena basin, where representative soils were sampled at four depths (0–5, 5–10, 10–20 and 20–40 cm). Each soil sample was made up of 10–15 sub-samples, taken at random from the whole area of each plot with a split-tube sampler. Samples were air-dried and sieved (<2 mm) before analysis. The soil pH in water was 5.42 ± 0.34 at 0–5 cm and 5.28 ± 0.29 at 20–40 cm. The concentration of organic carbon was 7.76% ± 2.01% at 0–5 cm and 3.64% ± 1.20% at 20–40 cm.

Various forms (pseudo-total, DTPA-extractable, Mehlich 3-extractable and 0.01 M $CaCl_2$-extractable) of iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), chromium (Cr),

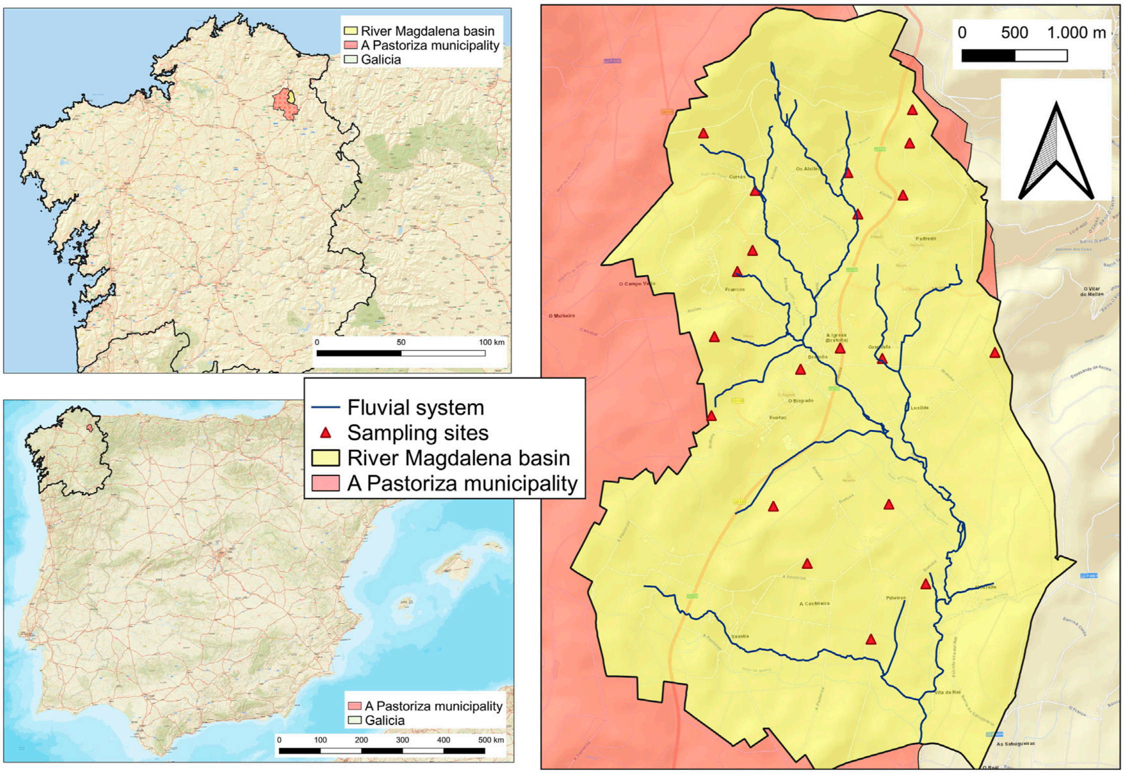

FIGURE 1 | Location of the studied soils in Spain, Galicia and A Pastoriza.

TABLE 1 | Limits of detection (LOD) for the analysed metals.

	Pseudo-total concentration, mg kg^{-1}	DTPA extractable concentration, mg kg^{-1}	Mehlich-3 extractable concentration, mg kg^{-1}	0.01 M $CaCl_2$ extractable concentration, µg kg^{-1}
Fe	8.3	0.077	0.345	2.45
Mn	1.0	0.0085	0.044	0.36
Zn	6.1	0.054	0.216	2.01
Cu	1.1	0.0098	0.044	0.45
Cr	2.3	0.011	0.0061	0.47
Co	0.03	0.0004	0.0013	0.0080
Ni	0.32	0.0036	0.014	0.0096
Cd	0.006	0.0001	0.0003	0.0016
Pb	1.0	0.0097	0.034	0.29

cobalt (Co), nickel (Ni), cadmium (Cd) and lead (Pb) were analysed. All of them are class B or mostly borderline metal ions (Nieboer and Richardson, 1980; Duffus, 2002). Fe, Mn, Zn, Cu and Ni are plant micronutrients. They can all be toxic above a concentration threshold.

Pseudo-total metals were determined by microwave-assisted digestion, with concentrated HNO_3 and HCl (HCl:HNO_3 ratio 1: 3), according to the EPA 3051A method (USEPA, 2007) (**Supplementary Data Sheet S2**). Available metals were extracted by DTPA (Lindsay and Norvell, 1978) (**Supplementary Data Sheet S2**). The soil samples were also extracted by Mehlich 3 reagent (Mehlich, 1984) (**Supplementary Data Sheet S2**) to compare the results obtained with those from DTPA extraction. DTPA and Mehlich 3 reagent are commonly used to determine available trace elements in soils. The Mehlich 3 extraction is less time-consuming than the DTPA extraction and allows the determination of available macronutrients, such as phosphorus, in the same extract. Soluble (mobile) metals were extracted by 0.01M $CaCl_2$ at a soil:solution ratio of 1:10 (Houba et al., 1990). The digestion and extraction methods are described in **Supplementary Data Sheet S2.** In all cases, the metals were determined in the digestate or extract by ICP-MS. The limits of

TABLE 2 | Generic reference levels (GRL) for various metals in Galician soils (Macías and Calvo de Anta, 2009), including the specific values for soils on slates and the average concentration of iron in the Earth's crust.

	Lithological GRL (edaphogeochemical background)	Lithological GRL (edaphogeochemical background) for slate soils	Risk-based GRL	Risk-based GRL for slate soils	Average concentration in the earth crust
Fe, mg kg^{-1}					50,000
Mn, mg kg^{-1}	850		850		
Zn, mg kg^{-1}	100		300		
Cu, mg kg^{-1}	45		50		
Cr, mg kg^{-1}	80	85	80	85	
Co, mg kg^{-1}	40		40		
Ni, mg kg^{-1}	65	80	75	80	
Cd, mg kg^{-1}	0.25		2		
Pb mg kg^{-1}	55		100		

detection were calculated as 3 times the standard deviation of blank values and are presented in **Table 1**.

Pollution Assessment

The pseudo-total metal concentrations were compared with the generic reference levels (GRL) for trace elements in Galician soils (**Table 2**) (Macías-Vázquez and Calvo de Anta, 2009). These levels represent the "concentration of a contaminating substance in the soil that does not carry a risk greater than the maximum acceptable for human or ecosystem health."

We used two indices to assess pollution in the studied soils. The first one is the enrichment factor (EF) developed by Buat-Menard and Chesselet (1979), which allows to compare a metal concentration in soil with the background concentration, both standardised against a reference element (Al, Fe, or Ti). In the present study Fe was chosen as the reference element, following the recommendations of Daskalakis and Oconnor (1995), Acevedo-Figueroa et al. (2006) and Rodriguez-Seijo et al. (2017), so that EF was calculated as follows:

$$EF_X = (C_X/C_{Fe})_{soil} / (C_X/C_{Fe})_{background},$$

where X is the metal of interest; EF_X, the enrichment factor of the element X; C_X, the total concentration of X; C_{Fe}, the total concentration of iron (a lithogenic reference element); "soil" refers to the assessed soil, and "background" to the situation of natural unpolluted soils in the study area, taken as a reference. In the present study, the edaphogeochemical background (lithological GRL) values for Galician soils (**Table 2**) were used as references. EF values below 2 indicate minimum enrichment; values between 2 and 5 represent moderate enrichment; values between 5 and 20 denote significant enrichment; EF values higher than 20 show very high enrichment; values above 40 reveal extremely high enrichment (Rodriguez-Seijo et al., 2017).

The second index used is the geoaccumulation index (I_{geo}) (Müller, 1979), calculated as:

$$I_{geo} = \log_2 (C_X/1.5\, B_x),$$

where C_X is the total concentration of the element X and B_X is the background concentration of the element X. The factor 1.5 is used to minimise the effect of potential variations in the background value because of lithological variations. We used as background the edaphogeochemical backgrounds for Galician soils (**Table 2**) and the average concentration in the Earth's crust for iron. The I_{geo} values allow to classify the soils into seven categories: <0 = practically unpolluted, 0–1 = unpolluted to moderately polluted, 1–2 = moderately polluted, 2–3 = moderately to strongly polluted, 3–4 = strongly polluted, 4–5 = strongly to extremely polluted, and >5 = extremely polluted (Rodriguez-Seijo et al., 2017).

Statistical Analysis

The analysis of variance was used to assess the influence of depth on the concentrations of the various forms of metals. Correlation analysis was performed between the different forms of each metal. The statistical analysis was performed using the IBM SPSS Statistics 28.0.

RESULTS

Pseudo-Total Metal Concentrations

The concentrations of pseudo-total metals at various depths (means and standard deviations) are presented in **Figure 2**. **Table 2** shows the generic reference levels (GRL) for the studied metals in Galician (NW Spain) soils (Macías-Vázquez and Calvo de Anta, 2009). The specific (exceptional) values for soils on slates are presented when they exist. For iron the average concentration in the Earth's crust is presented (there is no generic reference level for Fe).

The Fe concentrations (**Figure 2**) ranged between 17.63 and 58.70 g kg^{-1}, being below or very close to its average concentration in the Earth's crust. The Mn concentrations, ranging from 184 to 2,276 mg kg^{-1}, were above the GRL of 850 mg kg^{-1} at all depths in seven soils and in the surface (0–5 or 0–10 cm) of four other soils. The Zn concentrations, ranging from 20 to 146 mg kg^{-1}, were above the edaphogeochemical background (100 mg kg^{-1}) with some frequency, but never reached the risk-based GRL (300 mg kg^{-1}). The Cu concentrations, ranging from 2 to 72 mg kg^{-1}, were below the edaphogeochemical background (45 mg kg^{-1}) and the risk-based GRL (50 mg kg^{-1}), excepting two soils. The Cr concentrations ranging from 26 to

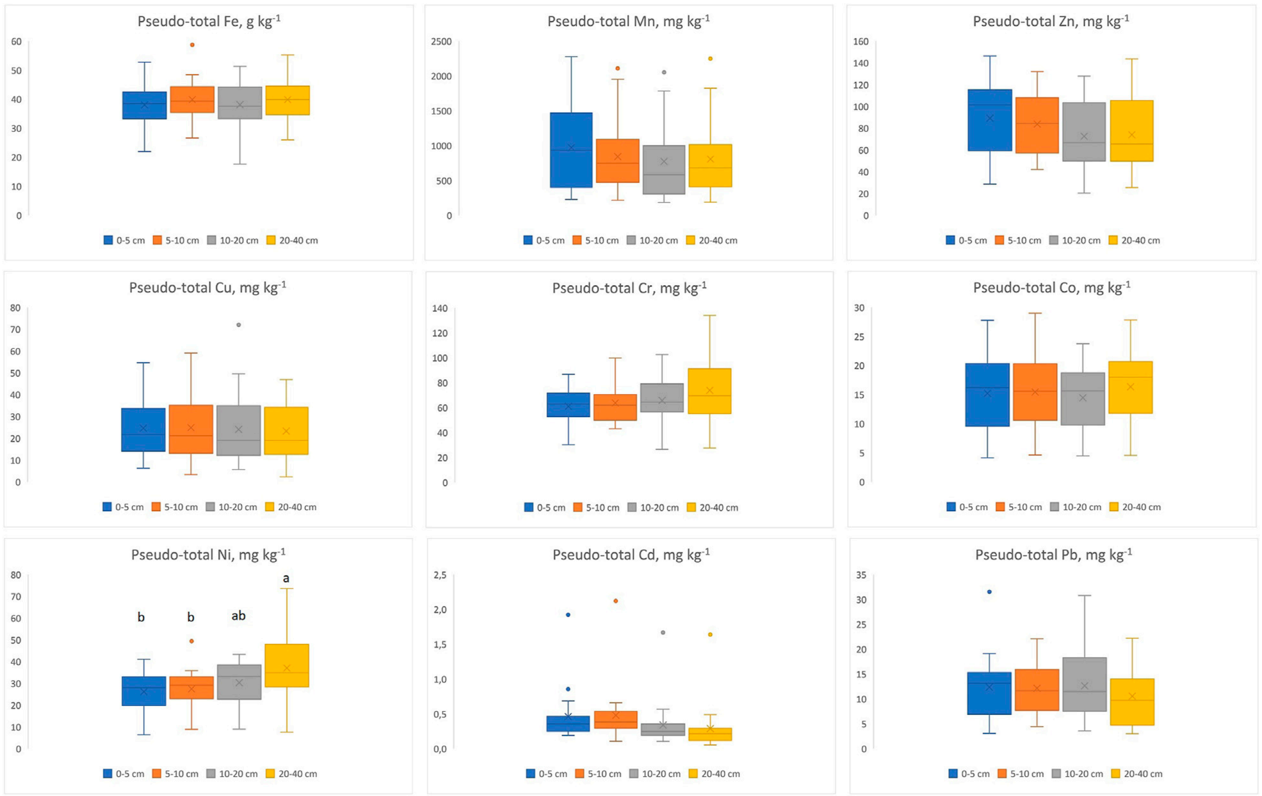

FIGURE 2 | Concentrations of pseudo-total metals for different soils as a function of depth. Different letters denote significant differences ($p < 0.05$) among depths.

134 mg kg^{-1}, exceeded the reference value for slate soils (85 mg kg^{-1}) in various cases, particularly in samples at greater depth. The concentrations of Co (4–29 mg kg^{-1}) and Ni (6–74 mg kg^{-1}) were always below the reference values (40 and 80 mg kg^{-1}, respectively). The Cd concentrations, ranging from 0.05 to 2.12 mg kg^{-1}, were often above the edaphogeochemical background of 0.25 mg kg^{-1}; only one outlier (at 5–10 cm depth) exceeded the risk-based GRL of 2 mg kg^{-1}. The Pb concentrations, ranging from 3 to 32 mg kg^{-1}, were always below the edaphogeochemical background (55 mg kg^{-1}) and the risk-based GRL (100 mg kg^{-1}).

Only for Ni the pseudo-total concentration varied significantly with depth ($p < 0.05$), being highest in the deepest sampled layer (20–40 cm) and lowest at 0–10 cm (**Figure 2**). For most metals there was a trend to decrease with depth, although the differences were not significant, or to remain stable (**Figure 2**). For Ni, Co and Cr there was a trend to increase with depth, the differences being significant only for Ni (**Figure 2**).

Pollution Indices

The enrichment factors (EF, **Table 3**) of Co (0.20–1.35), Ni (0.17–1.22) and Pb (0.08–0.88) were always lower than 2, indicating minimum enrichment with respect to the edaphogeochemical background (lithological GRL). The Mn EF values ranged between 0.28 and 5.50. Various soils had Mn EF between 2 and 5, indicating moderate enrichment with respect to the edaphogeochemical background; one sample had EF = 5.50, denoting significant enrichment. The EF values varied in the ranges 0.32–2.46, 0.09–2.37 and 0.37–2.03 for Zn, Cu and Cr, respectively. One to three samples from 10–20 or 20–40 cm depth had Zn, Cu and Cr EF slightly higher than 2, indicating moderate enrichment with respect to the edaphogeochemical background. The highest enrichment factors were found for Cd (0.20–8.70), often showing moderate enrichment (EF from 2 to 5) and significant enrichment (EF from 5 to 20) at all depths in one soil. The enrichment factor (**Table 3**) showed a trend to decrease with depth for some metals, while for other no visible trend was observed. However, the differences among depths were significant ($p <$

TABLE 3 | Enrichment factors (EF) of metals (mean ± standard deviation) at various depths.

	0–5 cm	5–10 cm	10–20 cm	20–40 cm
Mn	1.57 ± 1.10	1.34 ± 0.88	1.29 ± 1.20	1.31 ± 0.97
Zn	1.16 ± 0.41	1.10 ± 0.32	0.98 ± 0.52	1.01 ± 0.53
Cu	0.70 ± 0.35	0.72 ± 0.37	0.73 ± 0.58	0.71 ± 0.50
Cr	0.95 ± 0.16	1.00 ± 0.18	1.03 ± 0.27	1.16 ± 0.35
Co	0.49 ± 0.18	0.50 ± 0.16	0.49 ± 0.24	0.55 ± 0.22
Ni	0.42 ± 0.12^{b}	0.45 ± 0.11^{b}	0.51 ± 0.22^{ab}	0.62 ± 0.26^{a}
Cd	2.37 ± 1.58	2.46 ± 1.74	1.73 ± 1.32	1.52 ± 1.47
Pb	0.30 ± 0.16	0.30 ± 0.17	0.31 ± 0.17	0.26 ± 0.17

Different letters denote significant differences (p *< 0.05*) *among depths.*

TABLE 4 | Geoaccumulation indices of metals (mean ± standard deviation) at various depths.

	0–5 cm	5–10 cm	10–20 cm	20–40 cm
Fe	−1.01 ± 0.29	−0.94 ± 0.26	−1.01 ± 0.35	−0.93 ± 0.26
Mn	−0.71 ± 1.03	−0.88 ± 0.94	−1.06 ± 1.02	−0.94 ± 0.94
Zn	−0.88 ± 0.68	−0.92 ± 0.52	−1.21 ± 0.77	−1.16 ± 0.67
Cu	−1.70 ± 0.89	−1.70 ± 0.96	−1.82 ± 1.01	−1.83 ± 1.06
Cr	−1.02 ± 0.39	−0.95 ± 0.34	−0.94 ± 0.49	−078 ± 0.51
Co	−2.12 ± 0.70	−2.07 ± 0.63	−2.16 ± 0.60	−1.99 ± 0.63
Ni	−2.01 ± 0.65	−1.92 ± 0.56	−1.79 ± 0.63	−1.53 ± 0.70
Cd	0.03 ± 0.81^{a}	0.05 ± 0.90^{a}	−0.44 ± 0.82^{ab}	−0.89 ± 1.19^{b}
Pb	−2.92 ± 0.78	−2.91 ± 0.68	−2.92 ± 0.82	−3.20 ± 0.87

Different letters denote significant differences (p < 0.05) among depths.

0.05) only for Ni, whose EF increased with depth, in accordance to the variation of its pseudo-total concentration.

The indices of geoaccumulation (I_{geo}, **Table 4**) were always negative (practically unpolluted) for Fe (−2.09 to −0.35), Zn (−2.88 to −0.04), Co (−3.85 to −1.05), Ni (−3.92 to −0.41) and Pb (−4.78 to −1.39). This index was between 0 and 1 (unpolluted to moderately polluted) for Cu (range −4.84 to 0.09) and Cr (range −2.18 to 0.16) in one sample (in the case of Cr coinciding with the sample identified by EF as having moderate enrichment). The Mn I_{geo} (−2.79 to 0.84) was often between 0 and 1 (unpolluted to moderately polluted), in most cases coinciding with values of EF between 2 and 5 (moderate enrichment). The same as for EF, the highest values of I_{geo} were found for Cd (−2.82 to 2.50), with frequent values between 0 and 1 (unpolluted to moderately polluted); in one case the value was between 1 and 2 (moderately polluted); I_{geo} values between 2 and 3 (moderately to strongly polluted) were determined for the soil showing significant enrichment (EF from 5 to 20). The trends of variation of I_{geo} with depth were similar to those of pseudo-total concentrations and EF. The differences in I_{geo} values among depths were significant only for Cd, showing the highest values at 0–10 cm (**Table 4**).

Available and Soluble Metals

Available metal concentrations (DTPA and Mehlich 3) are shown in **Figure 3**. Metal concentrations determined by DTPA and Mehlich 3 extractions correlated significantly ($p < 0.001$) for all metals except Cr (**Table 5**).

DTPA concentrations (mg kg^{-1}) varied widely in the ranges: 36–455 for Fe, 0.63–119 for Mn, 0.14–11.36 for Zn, 0.11–5.12 for Cu, 0.000–0.054 for Cr, 0.021–0.767 for Co, 0.169–1.413 for Ni, 0.008–0.180 for Cd, and 0.176–2.663 for Pb. Mehlich-3 concentrations (mg kg^{-1}) varied widely in the ranges: 13–388 for Fe, 0.74–118 for Mn, 0.19–15.21 for Zn, 0.04–7.06 for Cu, 0.000–0.185 for Cr, 0.010–1.260 for Co, 0.106–0.949 for Ni, 0.000–0.186 for Cd, and 0.083–3.215 for Pb. As a general trend, the available metal concentrations decreased with depth, although the differences were not always

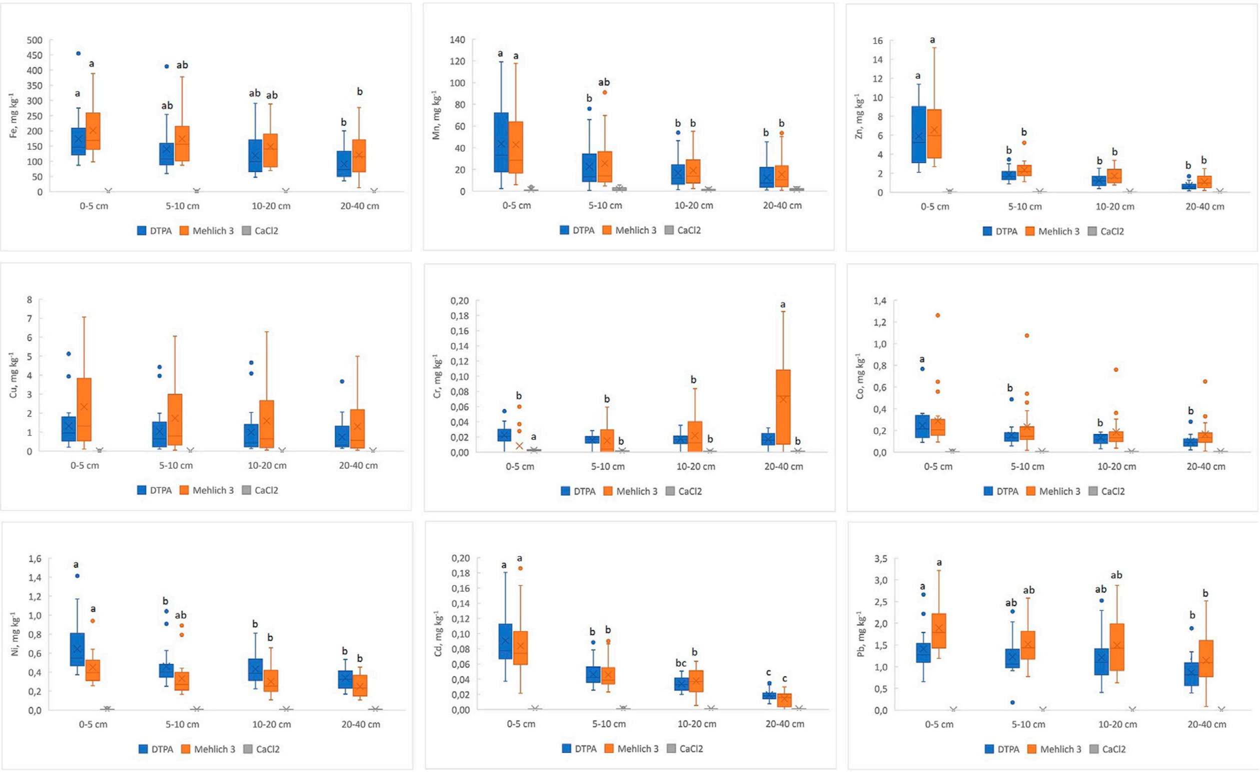

FIGURE 3 | Concentrations of available (extractable by DTPA and Mehlich 3) and soluble (extractable by 0.01M $CaCl_2$) metals for different soils as a function of depth. Different letters denote significant differences (p < 0.05) among depths.

TABLE 5 | Regression equations between Mehlich 3 and DTPA extractions. The concentration determined by DTPA extraction is the dependent variable (y) and that determined by Mehlich 3 extraction is the independent variable (x).

Metal, units	Equation	r^2	Significance
Fe, mg kg^{-1}	y = −7.529 + 0.859 x	0.789	<0.001
Mn, mg kg^{-1}	y = 0.418 + 0.911 x	0.933	<0.001
Zn, mg kg^{-1}	y = −0.283 + 0.925 x	0.950	<0.001
Cu, mg kg^{-1}	y = 0.223 + 0.463 x	0.590	<0.001
Cr, µg kg^{-1}			n.s.
Co, mg kg^{-1}	y = 0.052 + 0.484 x	0.754	<0.001
Ni, mg kg^{-1}	y = 0.078 + 1.170 x	0.881	<0.001
Cd, µg kg^{-1}	y = 5.028 + 0.938 x	0.878	<0.001
Pb, mg kg^{-1}	y = 0.224 + 0.633 x	0.593	<0.001

n.s. = not significant.

significant (**Figure 3**). An exception is chromium extracted by Mehlich 3 reagent, which increased significantly with depth.

The fraction of the available metal in relation to the pseudo-total metal varied widely among the different metals, from negligible values for chromium to very high values for cadmium or lead (**Supplementary Tables S4, S5**). The ranges of variation were: 0.10%–1.07% for DTPA Fe, 0.04%–1.54% for Mehlich-3 Fe, 0.19%–12.65% for DTPA Mn, 0.19%–13.39% for Mehlich-3 Mn, 0.24%–39.60% for DTPA Zn, 0.40%–53.11% for Mehlich-3 Zn, 0.48%–49.19% for DTPA Cu, 0.21%–73.03% for Mehlich-3 Cu, 0.00%–0.13% for DTPA Cr, 0.00%–0.29% for Mehlich-3 Cr, 0.14%–18.48% for DTPA Co, 0.06%–30.36% for Mehlich-3 Co, 0.43%–18.19% for DTPA Ni, 0.34%–14.61% for Mehlich-3 Ni, 1.08%–61.45% for DTPA Cd, 0.00%–59.24% for Mehlich-3 Cd, 1.98%–43.49% for DTPA Pb, and 1.80%–58.07% for Mehlich-3 Pb. The values showed for all metals a trend to decrease with depth, that is, the fraction of available metal is highest at soil surface, except for Mehlich-3 Cr, which increased with depth. Significant differences among depths were found for the fractions of DTPA Fe, Mn, Zn, Ni and Cd and for the fractions of Mehlich-3 Fe, Mn, Zn, Cr, Ni and Cd.

The concentrations of metals soluble in 0.01M $CaCl_2$ (**Figure 3**) were a tiny fraction of pseudo-total or available metals (**Supplementary Table S6**), ranging from 0 µg Pb kg^{-1}, 0–2 µg Cd kg^{-1} or 0–4 µg Cr kg^{-1} to 0–339 µg Zn kg^{-1}, 0–352 µg Fe kg^{-1} or 0–6,902 µg Mn kg^{-1}. Iron and manganese were the most soluble metals, the soluble fraction being on average 0.16% and 0.17% of pseudo-total metal, respectively. The soluble fraction of cadmium was on average 0.35% of pseudo-total metal. Lead concentrations in the $CaCl_2$ extracts were always below the limit of detection. The concentrations of metals soluble in 0.01M $CaCl_2$ often correlated significantly with DTPA and/or Mehlich 3 extractions. Correlations with DTPA extractions are shown in **Table 6**. Only in the case of Cr significant ($p < 0.05$) differences in soluble metal were observed among soil depths (**Figure 3**).

DISCUSSION

Pseudo-Total Metal Concentrations and Pollution Indices

The pseudo-total iron concentrations were below or very close to the average iron concentration in the Earth's crust, and the iron geoaccumulation indices (I_{geo}) were always negative. This indicates the absence of iron pollution. Thus, iron will be considered mainly in its micronutrient condition. The concentrations were higher than the total concentrations, determined by X-ray fluorescence, reported by Seco-Reigosa et al. (2015) for four pasture soils of the same area. In the case of manganese, 39% of the samples analysed, mainly in the surface soil, had a pseudo-total concentration higher than the GRL. The values were higher than those reported by Seco-Reigosa et al. (2015) for pasture and forest soils of the same area and similar to those reported by Caridad-Cancela et al. (2002) for natural soils in Galicia. Moreover, the maximum allowable concentration range for Mn (1,500–3,000 mg kg^{-1}, according to Kabata-Pendias and Szteke (2015)) was never exceeded. The EF values of 14% of the samples indicated moderate enrichment and that of one sample (out of 80) indicated significant enrichment. The I_{geo} values indicated an unpolluted to moderately polluted situation in 18% of the samples. Although Mn is a relatively abundant element in the Earth's crust, the data indicate in some cases a moderate anthropogenic enrichment, which can be related to the application of slurry or inorganic fertilisers. Slurry from the river Magdalena basin has a pseudo-total Mn concentration of 574 ± 138 mg kg^{-1} dry matter (Calvo-Rodríguez, 2003). Given that manganese is not a highly toxic element, the situation relative to this element in the river Magdalena basin does not appear to be of concern.

Thirty two percent of the samples analysed presented pseudo-total zinc concentrations higher than the edaphogeochemical background for Galician soils (100 mg kg^{-1}), but the values were always much lower than the risk level (300 mg kg^{-1}) and lower than the maximum allowable concentration (MAC) range for agricultural soils (100–300 mg kg^{-1}) according to Kabata-Pendias (2011). Twelve percent of the samples analysed exceeded the edaphogeochemical background for copper in Galician soils (45 mg kg^{-1}) and three samples (4%) exceeded the risk level (50 mg kg^{-1}) but did not exceed the MAC range for agricultural soils (60–150 mg kg^{-1}) according to Kabata-Pendias (2011). Most samples presented zinc and copper EFs lower than 2 and negative I_{geo} values. Three samples presented zinc EF slightly higher than 2, while two samples presented copper EF slightly higher than 2 (moderate enrichment). One sample presented a slightly positive copper I_{geo} (0.09). These

TABLE 6 | Regression equations between $CaCl_2$ and DTPA extractions. The concentration determined by DTPA extraction is the dependent variable (y) and that determined by $CaCl_2$ extraction is the independent variable (x).

Metal	Equation	r^2	Significance
Fe	y = 110.205 + 0.333 x	0.094	<0.01
Mn	y = 10.871 + 0.009 x	0.371	<0.001
Zn			n.s.
Cu	y = 0.599 + 0.210 x	0.663	<0.001
Cr	y = 14.831 + 3.650 x	0.121	<0.01
Co	y = 0.106 + 0.011 x	0.165	<0.001
Ni	y = 0.368 + 0.016 x	0.078	<0.05
Cd			n.s.

The units are mg kg^{-1} for DTPA extraction and µg kg^{-1} for $CaCl_2$ extraction, excepting Cr, where units are µg kg^{-1} for both extractions. n.s. = not significant.

results support the mixed lithogenic and anthropogenic nature of Zn and Cu in the soils of the river Magdalena basin (Franco-Uria et al., 2009). The strong correlation between the pseudo-total Zn and Cu concentrations (**Supplementary Table S1**) is in accordance with a common origin of these elements. In a similar way, Sterckeman et al. (2006) reported the association of Zn and Cu in soils developed from sedimentary materials in Northern France. The concentrations of zinc and copper are moderate in the cattle slurry from farms in the river Magdalena basin (260 ± 117 mg Zn kg^{-1} dry matter and 43 ± 18 mg Cu kg^{-1} dry matter (Calvo-Rodríguez, 2003)), although low compared to published values (300–410 mg Zn kg^{-1} and 74–100 mg Cu kg^{-1}) for Dutch farms (Jakubus et al., 2013). These values are well below the maximum concentrations of Zn and Cu (2,500 mg Zn kg^{-1} and 1,000 mg Cu kg^{-1}) in materials allowed to be applied to agricultural soils according to the Spanish legislation, even though manure is excluded from this limitation (MINISTERIO DE LA PRESIDENCIA, 2022). Despite the Zn and Cu contribution from slurry, the soils of the basin did not show significant enrichment or contamination by these metals. Unlike our results, Seco-Reigosa et al. (2015) reported values of the Zn and Cu total concentrations below the GRL. The pseudo-total concentrations of Zn and Cu in the present study were comparable to those reported by Paz-González et al. (2000), Caridad-Cancela et al. (2002) and Lopez-Mosquera et al. (2005) for Galician soils.

Twelve percent of the samples exceeded the GRL for Cr (85 mg kg^{-1}). Seco-Reigosa et al. (2015) reported Cr concentrations above the GRL for pasture and forest soils of the same area. The GRL for Co and Ni (40 and 80 mg kg^{-1}, respectively) were never exceeded, consistently with Seco et al. (Seco-Reigosa et al., 2015). The MAC ranges for Cr and Co in agricultural soils according to Kabata-Pendias (2011) (50–200 and 20–50 mg kg^{-1}, respectively) were not exceeded either, while the MAC range for Ni in agricultural soils (20–60 mg kg^{-1}) was exceeded by one sample (20–40 cm depth). In soils with a pH below 7, Cr is found predominantly in the form of Cr^{3+} (Bartlett and Kimble, 1976), a much less mobile and less toxic form than CrO_4^{2-} (Fendorf, 1995). The EF values for these three elements indicated minimum enrichment in all cases, consistently with the situation reported by Sterckeman et al. (2006) for soils derived from sediments in France, while the I_{geo} values indicated a situation of no pollution. These results are in accordance with the identification of a lithogenic origin for these elements in the river Magdalena basin (Franco-Uria et al., 2009). The significant increases of Ni pseudo-total concentration and EF with depth (**Figure 2**; **Table 3**) also indicate a lithological origin. Similarly, the (not significantly) higher values at depth of Cr and Co concentrations, EF and I_{geo} and Ni I_{geo} are in accordance with the lithological origin of these metals. Moreover, this origin is supported by the strong bivariate correlations of the pseudo-total concentrations of Ni, Cr and Co with those of Fe, a lithogenic element (**Supplementary Table S1**). Consistent with our results, Cr, Co, and Ni were associated with Fe and Al in soils developed from sedimentary materials in northern France (Sterckeman et al., 2006). The pseudo-total concentrations of Cr, Co and Ni were comparable to those reported by Paz-González et al. (2000), Lopez-Mosquera et al. (2005) and Seco-Reigosa et al. (2015) for Galician soils.

Cadmium was the element whose pseudo-total concentrations most frequently exceeded the edaphogeochemical background for Galician soils (0.25 mg kg^{-1}; 56% of the analysed samples), only one sample exceeded the risk level (2 mg kg^{-1}), and the MAC range for agricultural soils (1–5 mg kg^{-1}) according to Kabata-Pendias (2011) was never exceeded. The values were in accordance with those reported by Lopez-Mosquera et al. (2005) for pasture soils of Galicia. Sterckeman et al. (2006) reported Cd total concentrations in the range 0.01–8.98 mg kg^{-1} for soils derived from sediments in France. EF indicating moderate enrichment were found in 27% of samples, while 5% of samples (all depths from one soil) showed significant enrichment. The I_{geo} indicated unpolluted to moderately polluted soils in 27% of samples, moderately polluted soil in one sample and moderately to strongly polluted soil at all depths of the soil presenting significant enrichment. Cd is an element very toxic to plants, animals and humans (Alvarez-Ayuso, 2008) and has an anthropogenic origin in the river Magdalena basin (Franco-Uria et al., 2009). It is rather mobile and available in soils, having a high potential of transfer from soil to plants (McLaughlin et al., 1996; Pereira et al., 2011). The cadmium concentrations in the slurry from the basin were 23.4 ± 4.7 mg kg^{-1} dry matter (Calvo-Rodríguez, 2003), what is higher than the maximum concentration (10 mg Cd kg^{-1} dry matter) in materials allowed to be applied to agricultural soils according to the Spanish legislation (MINISTERIO DE LA PRESIDENCIA, 2022), although manure is excluded from this limitation. Furthermore, Cd is contributed to soil with inorganic phosphate fertilisers (Williams and David, 1976; McLaughlin et al., 1996; Nziguheba and Smolders, 2008; Cakmak et al., 2010). The pseudo-total Cd concentrations in the present study were slightly higher than those reported by Andrews et al. (1996) and Loganathan and Hedley (1997) for New Zealand pasture soils with a phosphate fertilisation history. According to McLaughlin et al. (1996) and Loganathan et al. (2008), Cd is the element of most concern among the contaminants present as impurities in inorganic fertilisers. As stated by Sterckeman et al. (2006), the high Cd concentrations can also be related to Cd enrichment of sedimentary parent materials relative to the average Earth's crust. The significantly higher Cd I_{geo} in surface soil (**Table 4**), as well as the (non-significant) trend to decrease at depth of Cd concentration and EF (**Figure 2**; **Table 3**), are in accordance with the anthropogenic origin of this metal. The Cd enrichment in surface soils is consistent with the results for soils developed from sediments in northern France (Sterckeman et al., 2006), including forest, agriculture and grassland soils. That study reported higher enrichment in permanent grassland than in cultivation or forest soils. Contrary to Loganathan and Hedley (1997) and Loganathan et al. (2008), that reported limited Cd downward movement in pastoral soils, in the present study enrichment factors above 2 and positive geoaccumulation indices were found in a few soils at a depth of 20–40 cm. In contrast to Loganathan and Hedley (1997) and Loganathan et al. (2008) results, Sterckeman et al. (2000) found traces of Cd and Zn contamination at a depth of 2 m in the vicinity of lead and zinc smelters and reported that Cd is more mobile than Pb and Zn. The Cd mobility is attributed to the rather weak adsorption to silicate clays, oxides and organic matter in acid soils (Sterckeman et al., 2006; Alvarez-Ayuso, 2008; Pereira et al., 2011). Only one soil in the present study showed high pseudo-total Cd concentrations at all depths (1.64–2.12 mg kg^{-1}, displayed as extreme values in **Figure 2**), close to the risk level, with enrichment factors between 6.72 and 8.70 (significant enrichment) and I_{geo} values between 2.13 and 2.50 (moderately to strongly polluted). Excepting this soil, the Cd pseudo-total concentrations were below 1 mg kg^{-1}. There is no currently generalised risk associated to Cd pollution in the basin. However, if the application of slurry and superphosphate continues, the cadmium build-up may grow. Moreover, there is a risk of downward movement of Cd. It is necessary to monitor the concentration of cadmium in the soil and to determine cadmium in the fertilisers applied, limiting the application of slurry and avoiding the application of cadmium-rich phosphates.

The pseudo-total lead concentrations were always below the edaphogeochemical background for Galician soils (55 mg kg^{-1}) and the MAC for agricultural soils (20–300 mg kg^{-1}; Kabata-Pendias, 2011). The EF always indicated minimum enrichment, while the I_{geo} indicated that the soils were practically unpolluted. Although Pb in soils is mainly anthropogenic (Franco-Uria et al., 2009; Kabata-Pendias, 2011), the rural nature of the river Magdalena basin explains the absence of Pb pollution. The concentrations were similar to those reported by Lopez-Mosquera et al. (2005) and Seco-Reigosa et al. (2015) and lower than some of the values presented by Paz-González et al. (2000) for agricultural topsoils in Galicia, Spain.

The pseudo-total Zn, Cu and Pb concentrations at 0–5 cm depth were lower than those reported by Guo et al. (2018) for the same depth in Chinese crop soils treated with pig manure. Ni concentrations were slightly lower, while Cr and Cd concentrations were similar to those reported by Guo et al. (2018).

Available and Soluble Metal Concentrations

The total element concentrations in soils do not allow predicting their mobility and plant availability, which depend on soil properties such as pH, clay content and type, contents of Al, Fe and Mn oxyhydroxides or organic matter. In the short and medium term, metals extracted by appropriate reagents are a better estimation of their bioavailability. For trace metals, EDTA (Trierweiler and Lindsay, 1969), DTPA (Lindsay and Norvell, 1978) and Mehlich 3 (Mehlich, 1984) are reagents usually employed to extract bioavailable forms. $CaCl_2$ solutions can be used to estimate soluble forms (Lebourg et al., 1996). These reagents are useful to assess the availability of micronutrients to vegetation and the risk associated to the presence of toxic elements in soils. Some studies reveal medium to good correlations between the concentrations of trace metals determined with DTPA and Mehlich 3 reagents. Their use in the present study aims to compare both extractions and check if there is a good correlation between the concentrations of trace metals determined with both reagents in acid soils of NW Spain.

The concentrations of DTPA Fe (**Figure 3**) were always well above the threshold value of 4.5 mg kg^{-1} reported by Lindsay and Norvell (1978) for Fe deficiency. Similarly, the concentrations of DTPA Mn were well above the threshold value of 0.2 mg kg^{-1} reported by Sims and Johnson (1991). The Zn concentrations at 0–10 cm depth were above the threshold value of 0.8 mg kg^{-1} (Lindsay and Norvell, 1978), but were often below this value at 10–20 and especially at 20–40 cm depth. The Cu concentrations at 0–5 cm depth were above the threshold value of 0.2 mg kg^{-1} (Lindsay and Norvell, 1978); at 5–10, 10–20 and 20–40 cm depth, the Cu concentrations were below the threshold value in two to four soils.

The Mehlich-3 Mn concentrations (**Figure 3**) were always above the deficiency threshold of 3.9 mg kg^{-1} (Sims and Johnson, 1991) in 0–5 and 5–10 cm samples and in most 10–20 and 20–40 cm samples. The Mehlich-3 Zn concentrations were always above the critical deficiency range of 1.15–1.62 mg kg^{-1} (Sims and Johnson, 1991) in 0–5 and 5–10 cm samples and often in 10–20 cm samples, while the samples at 20–40 cm depth were often below this threshold. For Mehlich-3 Cu, Sims and Johnson (1991) reported the critical deficiency range of 1–10 mg kg^{-1}. The value of 1 mg kg^{-1} was exceeded by most of the 0–5 cm samples, while at higher depths most samples were below this value.

In short, the micronutrients Fe and Mn were available at sufficiency levels in most of the studied soils at all depths (0–40 cm), while Zn and Cu were at sufficiency levels at 0–5 cm and below these levels with increasing frequency at higher depths.

The critical level of 200 mg kg^{-1} extracted by DTPA reported by Monterroso et al. (1999) for Fe toxicity was exceeded by six samples at 0–5 cm depth and with decreasing frequency at 5–10 and 10–20 cm depth (**Figure 3**). The critical level of 60 mg kg^{-1} for Mn toxicity (Monterroso et al., 1999) was exceeded by six samples at 0–5 cm depth and by two samples at 5–10 cm depth.

The critical level of toxicity for Zn (2 mg kg^{-1}, Sims and Johnson, 1991) was exceeded by all the soils analysed at a depth of 0–5 cm and with decreasing frequency at greater depths. For Cu, the toxicity threshold of 2 mg kg^{-1} (Sims and Johnson, 1991) was exceeded by two soils at all depths (**Figure 3**).

The available Ni and Pb concentrations were within the ranges of critical levels of toxicity (0.03–5.34 mg kg^{-1} for DTPA Ni; 0.04–5.93 mg kg^{-1} for Mehlich-3 Ni; 0.44–39 mg kg^{-1} for DTPA Pb; 0.66–29 mg kg^{-1} for Mehlich-3 Pb) reported by Silva et al. (2020), despite the lithogenic origin of these elements and the absence of pollution indicated by the total concentrations and the pollution indices. However, according to Silva et al. (2020), the critical levels for these elements vary widely among soils and might not apply to the soils in the present study. Therefore, the absence of Ni and Pb pollution appears most plausible.

The available Cd concentrations (extracted either by DTPA or Mehlich 3) were above or close to the critical levels of toxicity (0.01–0.04 mg kg^{-1}) reported by Silva et al. (2020). However, contrary to pseudo-total Cd (**Figure 2**), the concentrations of DTPA and Mehlich-3 Cd (**Figure 3**) decreased significantly with depth, indicating a decreased risk by Cd pollution.

In summary, the toxicity thresholds for Fe and Mn were exceeded by a few samples, mainly at low depths. Given that these metals are not highly toxic, the situation regarding these elements does not seem worrisome, as already indicated by the data on pseudo-total concentrations and the values of the pollution indices. The toxicity threshold for Zn was often exceeded, mainly at low depths. It is worth highlighting the short distance between published Zn deficiency and toxicity thresholds. Taking into account the rapid decrease with depth (**Figure 3**), the situation does not seem worrisome for Zn either. As for Cu, only two soils could be at risk of toxicity (**Figure 3**). No risk of Ni or Pb toxicity appears either. As for Cd, the results show decreased toxicity with depth.

The significant decrease with depth in the concentrations of most metals in available form (**Figure 3**; **Supplementary Tables S4, S5**) points to a greater availability of metals of anthropogenic origin (abundant in surface soils), while metals of lithological origin (abundant in deep soils) would be largely in non-available form. An exception is Mehlich-3 Cr, which increases with depth, in a way similar to pseudo-total Cr (**Figure 2**), although it is a very small fraction of the pseudo-total Cr. In fact, the Cr presented a very low availability in all samples. The opposite variations with depth of DTPA Cr and Mehlich-3 Cr indicate that these reagents extract different pools of Cr.

The Mehlich 3 reagent extracted more Fe, Zn, Cu, Co and Pb, less Ni and similar quantities of Mn and Cd compared to DTPA. According to Monterroso et al. (1999), the acidity of Mehlich 3 reagent (pH 3) is responsible for its higher extracting power. The regression equations in **Table 5** allow to estimate the DTPA concentrations from the Mehlich-3 concentrations (or vice-versa), the Mehlich 3 extraction being less time-consuming and allowing the determination of available macronutrients in the same extract. The lack of significant correlation between DTPA Cr and Mehlich-3 Cr is in accordance with the fact that the two reagents extract different pools of chromium.

Significant bivariate correlations, in most cases positive, were found among different metals extracted either by DTPA or Mehlich 3 reagent (**Supplementary Tables S2, S3**), suggesting associations between the metals presenting the strongest correlations. It is worth mentioning that DTPA Cr correlated significant and positively with most other metals (excepting Fe and Pb), while Mehlich-3 Cr correlated negatively with most other metals. This is consistent with the fact that these reagents extract different pools of Cr and suggests that DTPA is more suitable than Mehlich 3 to estimate available Cr.

The available metal concentrations (determined either by DTPA or Mehlich 3) as percent of pseudo-total concentrations (**Supplementary Tables S4, S5**) indicate that most chromium is in a non-available form, while a considerable proportion of Cd and Pb are in available form.

The available Zn, Cu, Co, Ni and Pb were comparable to the values reported by Paz-González et al. (2000) for the same metals extracted by EDTA from agricultural topsoils in Galicia, Spain.

The DTPA and Mehlich-3 extracted Fe, Mn, Zn and Cu were similar or slightly higher than those reported by Caridad-Cancela et al. (2002) for the same elements extracted by the same reagents from natural soils of Galicia, Spain. This is consistent with the fact that the soils of the river Magdalena basin are representative of Galician pasture soils.

The DTPA Zn, Cu and Pb concentrations at 0–5 cm depth were much lower, those of Cd and Ni were lower and those of Cr were similar to those reported by Guo et al. (2018) for the same depth in Chinese crop soils treated with pig manure, in accordance with the much lower Cu and Zn concentrations in cattle manures compared with pig manures.

The analysis of soluble metals (extractable with 1 M $CaCl_2$) will provide complementary information on the downward movement of heavy metals. As expected, 0.01 M $CaCl_2$ extracted much less metals than DTPA and Mehlich 3 reagents (**Figure 3**). Fe, Mn and Cd were the elements extracted in highest proportion (**Supplementary Table S6**). The variation with depth is significant only in the case of chromium.

$CaCl_2$ has been suggested as the best predictor of phytoavailable metals, especially Cd, in soils (Whitten and Ritchie, 1991; Andrews et al., 1996; Lebourg et al., 1996; McLaughlin et al., 2000; Adamo et al., 2018). The $CaCl_2$-extractable Cd concentrations were well below the critical value of 80 $\mu g\,kg^{-1}$ proposed by Podlešáková et al. (2002). Despite the slightly higher concentrations of pseudo-total Cd, the soils in the present study had much lower concentrations of Cd extracted by 0.01 M $CaCl_2$ than those reported by Andrews et al. (1996) and Loganathan and Hedley (1997) for New Zealand pasture soils with a phosphate fertilisation history and similar pH and organic carbon (0.009–0.034 mg Cd kg^{-1}). This suggests that Cd in the soils of the river Magdalena basin is much less available than in the New Zealand soils studied by Andrews et al. (1996) and Loganathan and Hedley (1997). It is worth noticing that in those New Zealand soils the reported values of Cd extracted by EDTA are very close to total concentrations. Cadmium contributed by cattle slurry seems to be less mobile than that supplied by soluble phosphate fertilisers. Contrary to other forms of Cd, the concentrations of Cd extracted by 0.01 M $CaCl_2$ indicate that there is no risk of cadmium transfer from soil to plants or to water bodies. Chelating agents, such as DTPA or Mehlich-3 reagent, can extract organically bound metals and metals specifically adsorbed by the clay fraction, so overestimating the bioavailable fraction of some elements (Adamo et al., 2018).

In a similar way to Cd, the $CaCl_2$-extracted Mn, Zn, Cu, Co, Cr, Ni and Pb were well below the critical values proposed by Podlešáková et al. (2002) (30, 2, 0.2, 0.5, 0.05, 100 and 7.5 $mg\,kg^{-1}$, respectively). This confirms the general non-problematic situation regarding these metals indicated by the results of total and available concentrations.

The values of pseudo-total Cd as well as the cadmium extracted by chelating agents make it advisable to limit the contribution of phosphate fertilisers in coarser textured soils and to apply limestone as an amendment to reduce the cadmium mobility in those soils with higher concentrations (Andrews et al., 1996; Loganathan and Hedley, 1997; Gray et al., 1999; Alvarez-Ayuso, 2008).

CONCLUSION

The study reveals the absence of pollution by the lithogenic Fe, Ni, Co and Cr. Most samples presented minimum enrichment in the mixed-origin Mn, Zn and Cu, while a few samples showed moderate enrichment, resulting in unpolluted to moderately polluted soils. The soils of the river Magdalena basin were unpolluted by Pb.

Cadmium was the element that most frequently presented situations of pollution (moderate in most cases), revealed both by the values of pseudo-total and available (extracted by DTPA or Mehlich 3) concentrations. Furthermore, it appeared to be moderately mobile in these acid soils, even though the concentrations in available form decreased more significantly with depth than the pseudo-total concentrations. Despite the high values of pseudo-total Cd and Cd extracted by chelating agents, the extraction by 0.01 M $CaCl_2$ pointed to very low environmental risk related to Cd pollution.

The micronutrients Fe and Mn were at sufficient levels for plant growth, while Zn and Cu were above the sufficiency level in the surface soil (0–5 or 0–10 cm) but deficiency situations might appear in some cases below 10 cm depth.

It is shown that the application of cattle slurry to grassland soils in Galicia, NW Spain, does not entail an environmental risk associated to the contribution of toxic metals, excepting Cd, for which precautions are advised to avoid possible future risk situations in the event that cadmium continues to be added to the soil.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

Conceptualization: MF-M and FD-R. Methodology: MF-M and FD-R. Soil sampling: CL-M, MF-M, and FD-R. Samples processing and laboratory analysis: CL-M and RM-R. Data analysis: CL-M, MF-M, and FD-R. Writing: MF-M. Funding acquisition: MF-M and FD-R.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/sjss.2023.11201/full#supplementary-material

Supplementary Data Sheet 1 | Supplementary Tables.

Supplementary Data Sheet 2 | Analytical methods.

REFERENCES

Acevedo-Figueroa, D., Jimenez, B. D., and Rodriguez-Sierra, C. J. (2006). Trace Metals in Sediments of Two Estuarine Lagoons from Puerto Rico. *Environ. Pollut.* 141, 336–342. doi:10.1016/j.envpol.2005.08.037

Adamo, P., Agrelli, D., and Zampella, M. (2018). Chemical Speciation to Assess Bioavailability, Bioaccessibility and Geochemical Forms of Potentially Toxic Metals (PTMs) in Polluted Soils. *Environ. Geochem. (Second Ed.)* 2018, 153. doi:10.1016/B978-0-444-63763-5.00010-0

Aldrich, A. P., Kistler, D., and Sigg, L. (2002). Speciation of Cu and Zn in Drainage Water from Agricultural Soils. *Environ. Sci. Technol.* 36, 4824–4830. doi:10.1021/es025813x

Alloway, B. J., and Jackson, A. P. (1991). The Behaviour of Heavy Metals in Sewage Sludge-Amended Soils. *Sci. Total Environ.* 100, 151–176. doi:10.1016/0048-9697(91)90377-q

Alvarez-Ayuso, E. (2008). Cadmium in Soil-Plant Systems: an Overview. *Int. J. Environ. Pollut.* 33, 275–291. doi:10.1504/ijep.2008.019399

Andrews, P., Town, R. M., Hedley, M. J., and Loganathan, P. (1996). Measurement of Plant-Available Cadmium in New Zealand Soils. *Aust. J. Soil Res.* 34, 441–452. doi:10.1071/sr9960441

Antoniadis, V., Robinson, J. S., and Alloway, B. J. (2008). Effects of Short-Term pH Fluctuations on Cadmium, Nickel, Lead, and Zinc Availability to Ryegrass in a Sewage Sludge-Amended Field. *Chemosphere* 71, 759–764. doi:10.1016/j.chemosphere.2007.10.015

Antunes, S. C., Pereira, R., Marques, S. M., Castro, B. B., and Goncalves, F. (2011). Impaired Microbial Activity Caused by Metal Pollution: A Field Study in a Deactivated Uranium Mining Area. *Sci. Total Environ.* 410, 87–95. doi:10.1016/j.scitotenv.2011.09.003

Baize, D. (1997). Detection of Moderate Contamination by Trace Metals in Agricultural Soils. *Analusis* 25, M29–M35.

Bartlett, R. J., and Kimble, J. M. (1976). Behavior of Chromium in Soils: I. Trivalent Forms. *J. Environ. Qual.* 5, 379–383. doi:10.2134/jeq1976.00472425000500040009x

Batley, G. E. (2012). Heavy Metal"-Aa Useful Term. *Integr. Environ. Assess. Manag.* 8, 215. doi:10.1002/ieam.1290

Buat-Menard, P., and Chesselet, R. (1979). Variable Influence of the Atmospheric Flux on the Trace-Metal Chemistry of Oceanic Suspended Matter. *Earth Planet. Sci. Lett.* 42, 399–411. doi:10.1016/0012-821x(79)90049-9

Cakmak, D., Saljnikov, E., Mrvic, V., Jakovljevic, M., Marjanovic, Z., Sikiric, B., et al. (2010). Soil Properties and Trace Elements Contents Following 40 Years of Phosphate Fertilization. *J. Environ. Qual.* 39, 541–547. doi:10.2134/jeq2009.0216

Calvo-Rodríguez, F. (2003). *Caracterización dos puríns de vacuno das explotacións da conca do río Magdalena (A Pastoriza).* Master Thesis Dissertation (Santiago, Spain: University of Santiago de Compostela).

Caridad-Cancela, R., de Abreu, C. A., and Paz-Gonzalez, A. (2002). DTPA and Mehlich-3 Micronutrient Extractability in Natural Soils. *Commun. Soil Sci. Plant Analysis* 33, 2879–2893. doi:10.1081/css-120014488

Daskalakis, K. D., and Oconnor, T. P. (1995). Normalization and Elemental Sediment Contamination in the Coastal United States. *Environ. Sci. Technol.* 29, 470–477. doi:10.1021/es00002a024

de la Torre, A. I., Jimenez, J. A., Carballo, M., Fernandez, C., Roset, J., and Munoz, M. J. (2000). Ecotoxicological Evaluation of Pig Slurry. *Chemosphere* 41, 1629–1635. doi:10.1016/s0045-6535(00)00038-2

de Temmerman, L., Vanongeval, L., Boon, W., Hoenig, M., and Geypens, M. (2003). Heavymetal Content of Arable Soils in Northern Belgium. *Water Air Soil Pollut.* 148, 61–76. doi:10.1023/a:1025498629671

Del Castilho, P., Chardon, W. J., and Salomons, W. (1993). Influence of Cattle-Manure Slurry Application on the Solubility of Cadmium, Copper, and Zinc in a Manured Acidic, Loamy-Sand Soil. *J. Environ. Qual.* 22, 689–697. doi:10.2134/jeq1993.00472425002200040009x

Duffus, J. H. (2002). "Heavy Metals" - A Meaningless Term? (IUPAC Technical Report). *Pure Appl. Chem.* 74, 793–807. doi:10.1351/pac200274050793

Fendorf, S. E. (1995). Surface-reactions of Chromium in Soils and Waters. *Geoderma* 67, 55–71. doi:10.1016/0016-7061(94)00062-f

Fernandez-Calvino, D., Soler-Rovira, P., Polo, A., Diaz-Ravina, M., Arias-Estevez, M., and Plaza, C. (2010). Enzyme Activities in Vineyard Soils Long-Term Treated with Copper-Based Fungicides. *Soil Biol. Biochem.* 42, 2119–2127. doi:10.1016/j.soilbio.2010.08.007

Franco-Uria, A., Lopez-Mateo, C., Roca, E., and Fernandez-Marcos, M. L. (2009). Source Identification of Heavy Metals in Pastureland by Multivariate Analysis in NW Spain. *J. Hazard. Mater.* 165, 1008–1015. doi:10.1016/j.jhazmat.2008.10.118

Gray, C. W., Mclaren, R. G., Roberts, A. H. C., and Condron, L. M. (1999). Effect of Soil pH on Cadmium Phytoavailability in Some New Zealand Soils. *N. Z. J. Crop Hortic. Sci.* 27, 169–179. doi:10.1080/01140671.1999.9514093

Guo, T., Lou, C. L., Zhai, W. W., Tang, X. J., Hashmi, M. Z., Murtaza, R., et al. (2018). Increased Occurrence of Heavy Metals, Antibiotics and Resistance Genes in Surface Soil after Long-Term Application of Manure. *Sci. Total Environ.* 635, 995–1003. doi:10.1016/j.scitotenv.2018.04.194

Gupta, S. K., Vollmer, M. K., and Krebs, R. (1996). The Importance of Mobile, Mobilisable and Pseudo Total Heavy Metal Fractions in Soil for Three-Level Risk Assessment and Risk Management. *Sci. Total Environ.* 178, 11–20. doi:10.1016/0048-9697(95)04792-1

Gustin, M. S., Hou, D., and Tack, F. M. G. (2021). The Term "heavy Metal(s)": History, Current Debate, and Future Use. *Sci. Total Environ.* 789, 147951. doi:10.1016/j.scitotenv.2021.147951

Hodson, M. E. (2004). Heavy Metals - Geochemical Bogey Men? *Environ. Pollut.* 129, 341–343. doi:10.1016/j.envpol.2003.11.003

Houba, V. J. G., Novozamsky, I., Lexmond, T. M., and Vanderlee, J. J. (1990). Applicability of 0.01 M $CaCl_2$ as a Single Extraction Solution for the Assessment of the Nutrient Status of Soils and Other Diagnostic Purposes. *Commun. Soil Sci. Plant Analysis* 21, 2281–2290. doi:10.1080/00103629009368380

IUPAC (2022). *Trace element [Online]*. Available at: https://goldbook.iupac.org/terms/view/T06421 (Accessed Sepember, 2022).

IUSS Working Group WRB (2022). *World Reference Base for Soil Resources. International Soil Classification System for Naming Soils and Creating Legends for Soil Maps*. 4th ed. Vienna, Austria: International Union of Soil Sciences (IUSS).

Jakubus, M., Dach, J., and Starmans, D. (2013). Bioavailability of Copper and Zinc in Pig and Cattle Slurries. *Fresenius Environ. Bull.* 22, 995–1002.

Kabata-Pendias, A., and Szteke, B. (2015). *Trace Elements in Abiotic and Biotic Environments*. Abingdon-on-Thames, Oxfordshire, UK: Taylor & Francis.

Kabata-Pendias, A. (2011). *Trace Elements in Soils and Plants*. Boca Raton, FL, USA: CRC.

L'Herroux, L., Leroux, S., Appriou, P., and Martinez, J. (1997). Behaviour of Metals Following Intensive Pig Slurry Applications to a Natural Field Treatment Process in Brittany (France). *Environ. Pollut.* 97, 119–130. doi:10.1016/s0269-7491(97)00072-9

Lebourg, A., Sterckeman, T., Ciesielski, H., and Proix, N. (1996). Intérêt de différents réactifs d'extraction chimique pour l'évaluation de la biodisponibilité des métaux en traces du sol. *Agronomie* 16, 201–215. doi:10.1051/agro:19960401

Li, Y., Xu, Z. Q., Ren, H. H., Wang, D., Wang, J., Wu, Z., et al. (2022). Spatial Distribution and Source Apportionment of Heavy Metals in the Topsoil of Weifang City, East China. *Front. Environ. Sci.* 10. doi:10.3389/fenvs.2022.893938

Liang, C. N., and Tabatabai, M. A. (1977). Effects of Trace Elements on Nitrogen Mineralisation in Soils. *Environ. Pollut.* 12, 141–147. doi:10.1016/0013-9327(77)90017-9

Lindsay, W. L., and Norvell, W. A. (1978). Development of a DTPA Soil Test for Zinc, Iron, Manganese, and Copper. *Soil Sci. Soc. Am. J.* 42, 421–428. doi:10.2136/sssaj1978.03615995004200030009x

Loganathan, P., and Hedley, M. J. (1997). Downward Movement of Cadmium and Phosphorus from Phosphatic Fertilisers in a Pasture Soil in New Zealand. *Environ. Pollut.* 95, 319–324. doi:10.1016/s0269-7491(96)00142-x

Loganathan, P., Hedley, M. J., and Grace, N. D. (2008). Pasture Soils Contaminated with Fertilizer-Derived Cadmium and Fluorine: Livestock Effects. *Rev. Environ. Contam. Toxicol.* 192, 29. doi:10.1007/978-0-387-71724-1_2

Lopez-Mosquera, M. E., Barros, R., Sainz, M. J., Carral, E., and Seoane, S. (2005). Metal Concentrations in Agricultural and Forestry Soils in Northwest Spain: Implications for Disposal of Organic Wastes on Acid Soils. *Soil Use Manag.* 21, 298–305. doi:10.1079/sum2005324

Macías-Vázquez, F., and Calvo de Anta, R. (2009). *Niveles genéricos de referencia de metales pesados y otros elementos traza en suelos de Galicia*. Santiago de Compostela, Xunta de Galicia: Consellería de Medio Ambiente e Desenvolvemento Sostible.,

Madrid, L. (2010). Heavy Metals": Reminding a Long-Standing and Sometimes Forgotten Controversy. *Geoderma* 155, 128–129. doi:10.1016/j.geoderma.2009.11.031

Mantovi, P., Bonazzi, G., Maestri, E., and Marmiroli, N. (2003). Accumulation of Copper and Zinc from Liquid Manure in Agricultural Soils and Crop Plants. *Plant Soil* 250, 249–257. doi:10.1023/a:1022848131043

Mcbride, M. B. (1989). "Reactions Controlling Heavy Metal Solubility in Soils," in *Advances in Soil Science*. Editor B. A. Stewart (New York, NY: Springer New York), Vol. 10.

Mclaughlin, M. J., Tiller, K. G., Naidu, R., and Stevens, D. P. (1996). Review: The Behaviour and Environmental Impact of Contaminants in Fertilizers. *Aust. J. Soil Res.* 34, 1–54. doi:10.1071/sr9960001

Mclaughlin, M. J., Zarcinas, B. A., Stevens, D. P., and Cook, N. (2000). Soil Testing for Heavy Metals. *Commun. Soil Sci. Plant Analysis* 31, 1661–1700. doi:10.1080/00103620009370531

Mehlich, A. (1984). Mehlich 3 Soil Test Extractant: A Modification of Mehlich 2 Extractant. *Commun. Soil Sci. Plant Analysis* 15, 1409–1416. doi:10.1080/00103628409367568

Meteogalicia, C. D. M. A. (2022). Xunta de Galicia, Spain. Available at: http://www.meteogalicia.gal (Accessed September, 2022).

Ministerio de la Presidencia, R. C. L. C. Y. M. D. (2022). *Real Decreto 1051/2022, de 27 de diciembre, por el que se establecen normas para la nutrición sostenible en los suelos agrarios*. Madrid, Spain: Boletín Oficial Del Estado.

Monterroso, C., Alvarez, E., and Marcos, M. L. F. (1999). Evaluation of Mehlich 3 Reagent as a Multielement Extractant in Mine Soils. *Land Degrad. Dev.* 10, 35–47. doi:10.1002/(sici)1099-145x(199901/02)10:1<35::aid-ldr319>3.0.co;2-6

Moreno-Caselles, J., Moral, R., Perez-Murcia, M., Perez-Espinosa, A., and Rufete, B. (2002). Nutrient Value of Animal Manures in Front of Environmental Hazards. *Commun. Soil Sci. Plant Analysis* 33, 3023–3032. doi:10.1081/css-120014499

Müller, G. (1979). Heavy Metals in the Sediments of the Rhine - Changes since 1971. *Umschau Wissenschaft Und Tech.* 79, 778–783.

Nagajyoti, P. C., Lee, K. D., and Sreekanth, T. V. M. (2010). Heavy Metals, Occurrence and Toxicity for Plants: a Review. *Environ. Chem. Lett.* 8, 199–216. doi:10.1007/s10311-010-0297-8

Nicholson, F. A., Smith, S. R., Alloway, B. J., Carlton-Smith, C., and Chambers, B. J. (2003). An Inventory of Heavy Metals Inputs to Agricultural Soils in England and Wales. *Sci. Total Environ.* 311, 205–219. doi:10.1016/s0048-9697(03)00139-6

Nieboer, E., and Richardson, D. H. S. (1980). The Replacement of the Non-descript Term Heavy-Metals by a Biologically and Chemically Significant Classification of Metal-Ions. *Environ. Pollut. Ser. B-Chemical Phys.* 1, 3–26. doi:10.1016/0143-148x(80)90017-8

Nunez-Delgado, A., Lopez-Periago, E., and Diaz-Fierros-Viqueria, F. (2002). Pollution Attenuation by Soils Receiving Cattle Slurry after Passage of a

Slurry-like Feed Solution. Column Experiments. *Bioresour. Technol.* 84, 229–236. doi:10.1016/s0960-8524(02)00050-0

Nziguheba, G., and Smolders, E. (2008). Inputs of Trace Elements in Agricultural Soils via Phosphate Fertilizers in European Countries. *Sci. Total Environ.* 390, 53–57. doi:10.1016/j.scitotenv.2007.09.031

Paz-González, A., Taboada-Castro, T., and Taboada-Castro, M. (2000). Levels of Heavy Metals (Co, Cu, Cr, Ni, Pb, and Zn) in Agricultural Soils of Northwest Spain. *Commun. Soil Sci. Plant Analysis* 31, 1773–1783. doi:10.1080/00103620009370536

Pereira, B. F. F., Rozane, D. E., Araujo, S. R., Barth, G., Queiroz, R. J. B., Nogueira, T. A. R., et al. (2011). Cadmium Availability and Accumulation by Lettuce and Rice. *Rev. Bras. De. Cienc. Do Solo* 35, 645–654. doi:10.1590/s0100-06832011000200033

Podlešáková, E., Němeček, J., and Vácha, R. (2002). Critical Values of Trace Elements in Soils from the Viewpoint of the Transfer Pathway Soil - Plant. *Plant, Soil Environ.* 48, 193–202. doi:10.17221/4224-pse

Poulsen, P. H. B., Magid, J., Luxhoi, J., and de Neergaard, A. (2013). Effects of Fertilization with Urban and Agricultural Organic Wastes in a Field Trial - Waste Imprint on Soil Microbial Activity. *Soil Biol. Biochem.* 57, 794–802. doi:10.1016/j.soilbio.2012.02.031

Pourret, O., and Hursthouse, A. (2019). It's Time to Replace the Term "Heavy Metals" with "Potentially Toxic Elements" when Reporting Environmental Research. *Int. J. Environ. Res. Public Health* 16, 4446. doi:10.3390/ijerph16224446

Proshad, R., Islam, M. S., Kormoker, T., Sayeed, A., Khadka, S., and Idris, A. M. (2021). Potential Toxic Metals (PTMs) Contamination in Agricultural Soils and Foodstuffs with Associated Source Identification and Model Uncertainty. *Sci. Total Environ.* 789, 147962. doi:10.1016/j.scitotenv.2021.147962

Richter, R., Rimovsky, K., and Hlusek, J. (1997). The Productivity of a Crop Rotation under Conventional and Organic Method of Management in Conditions of Increased Content of Heavy Metals in the Soil. *Acta Univ. Agric. Silvic. Mendelianae Brunensis* 45, 83–90.

Rodriguez-Seijo, A., Andrade, M. L., and Vega, F. A. (2017). Origin and Spatial Distribution of Metals in Urban Soils. *J. Soils Sediments* 17, 1514–1526. doi:10.1007/s11368-015-1304-2

Romkens, P., and Salomons, W. (1998). Cd, Cu and Zn Solubility in Arable and Forest Soils: Consequences of Land Use Changes for Metal Mobility and Risk Assessment. *Soil Sci.* 163, 859–871. doi:10.1097/00010694-199811000-00003

Seco-Reigosa, N., Fernandez-Sanjurjo, M. J., Nunez-Delgado, A., Cutillas-Barreiro, L., Gomez-Armesto, A., Novoa-Munoz, J. C., et al. (2015). Heavy Metals in Pastureland Soils Situated in A Pastoriza (NW Spain) Treated with Cattle Slurry and NPK Fertilizers. *Span. J. Soil Sci.* 5, 154–164. doi:10.3232/sjss.2015.v5.n2.05

Silva, E. B., Alves, I. S., Alleoni, L. R. F., Grazziotti, P. H., Farnezi, M. M. M., Santos, L. L., et al. (2020). Availability and Toxic Level of Cadmium, Lead and Nickel in Contaminated Soils. *Commun. Soil Sci. Plant Analysis* 51, 1341–1356. doi:10.1080/00103624.2020.1763396

Sims, J. T., and Johnson, G. V. (1991). Micronutrient Soil Tests. *Micronutr. Agric.* 4. doi:10.2136/sssabookser4.2ed.c12

Smith, S. R. (1997). Rhizobium in Soils Contaminated with Copper and Zinc Following the Long-Term Application of Sewage Sludge and Other Organic Wastes. *Soil Biol. Biochem.* 29, 1475–1489. doi:10.1016/s0038-0717(97)00036-9

Sterckeman, T., Douay, F., Baize, D., Fourrier, H., Proix, N., and Schvartz, C. (2006). Trace Elements in Soils Developed in Sedimentary Materials from Northern France. *Geoderma* 136, 912–929. doi:10.1016/j.geoderma.2006.06.010

Sterckeman, T., Douay, F., Proix, N., and Fourrier, H. (2000). Vertical Distribution of Cd, Pb and Zn in Soils Near Smelters in the North of France. *Environ. Pollut.* 107, 377–389. doi:10.1016/s0269-7491(99)00165-7

Trierweiler, J. F., and Lindsay, W. L. (1969). EDTA-ammonium Carbonate Soil Test for Zinc. *Soil Sci. Soc. Am. Proc.* 33, 49–54. doi:10.2136/sssaj1969.03615995003300010017x

USEPA (2007). *Method 3051A. Microwave Assisted Acid Digestion of Sediments, Sludges, Soils, and Oils [Online]*. Washington, DC, USA: USEPA. Available at: https://www.epa.gov/hw-sw846/sw-846-test-method-3051a-microwave-assisted-acid-digestion-sediments-sludges-soils-and-oils (Accessed September, 2022).

Whitten, M. G., and Ritchie, G. S. P. (1991). Calcium-chloride Extractable Cadmium as an Estimate of Cadmium Uptake by Subterranean Clover. *Aust. J. Soil Res.* 29, 215–221. doi:10.1071/sr9910215

Wilcke, W., Bol, R., and Amelung, W. (2002). Fate of Dung-Applied Copper in a British Grassland Soil. *Geoderma* 106, 273–288. doi:10.1016/s0016-7061(01)00128-8

Williams, C. H., and David, D. J. (1976). Accumulation in Soil of Cadmium Residues from Phosphate Fertilizers and Their Effect on Cadmium Content of Plants. *Soil Sci.* 121, 86–93. doi:10.1097/00010694-197602000-00004

Xia, L., Lam, S. K., Yan, X., and Chen, D. (2017). How Does Recycling of Livestock Manure in Agroecosystems Affect Crop Productivity, Reactive Nitrogen Losses, and Soil Carbon Balance? *Environ. Sci. Technol.* 51, 7450–7457. doi:10.1021/acs.est.6b06470

Zhang, X., Barcelo, D., Clougherty, R. J., Gao, B., Harms, H., Tefsen, B., et al. (2022). Potentially Toxic Element"-Something that Means Everything Means Nothing. *Environ. Sci. Technol.* 56, 11922–11925. doi:10.1021/acs.est.2c03056

ORIGINAL RESEARCH
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Microbial Communities as Affected by Clarithromycin Addition in Four Acid Soils (NW Iberian Peninsula)

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A laboratory experiment was carried out to investigate the response of the microbial communities in acid agricultural soils located in the NW Iberian Peninsula to the presence of clarithromycin. Four soils, with different organic C content and similar pH, and seven different concentrations of clarithromycin (0.49, 1.95, 7.81, 31.25, 125, 500 and 2,000 mg kg^{-1} of soil) were used, and microbial estimates were made after 8 and 42 incubation days. The phospholipid fatty acids (PLFA) technique was used to estimate the total microbial biomass and biomass of specific microbial groups as well as the microbial community structure (PLFA pattern). The microbial biomass (total and specific groups) was different in the four studied soils, the lowest values being exhibited by soils with the lowest organic C. The antibiotic addition showed a positive effect on microbial biomass (total and specific groups), especially at the highest dose; the effect being similar or even more accentuated with time passed after the addition (42 days ≥8 days). Principal component analysis (PCA) of the PLFA data carried out with the whole data set showed that the main determining factors of the microbial structure followed the order: soil > time incubation ≥ antibiotic dose. When the PCA was performed individually for each incubation time, the results indicated that microbial communities of the four soils were different. Likewise, for each soil, different microbial communities were observed depending on antibiotic concentration. The microbial biomass and PLFA pattern data were coincidentally showing that the clarithromycin addition favored fungi and G^- bacteria more that bacteria and G^+ bacteria; the effect being dose-dependent. Our data (microbial biomass, PLFA pattern) also demonstrated that the effect of clarithromycin addition on microbial communities in these four acid agricultural soils persisted even after 42 incubation days.

Keywords: polluted soil, phospholipid fatty acids, persistent effects, antibiotics, microbial biomass

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INTRODUCTION

Antibiotics, used worldwide for human health, are excreted in feces and urine as the origin compound and/or as secondary metabolites, reaching the environment and causing serious damage to the soil and aquatic ecosystems (Thiele-Bruhn, 2003; Kümmerer, 2009). These emergent contaminants of human origin, which are present in wastewater destined for wastewater treatment plants (WWTPs), can enter into soil following both agricultural irrigation with liquid effluents and the application of solid effluents (sewage sludge) as organic amendments to the soil (Fijalkowski et al., 2017; Aydin et al., 2022). Thus, residues of both parent veterinary and pharmaceutical compounds and their degradation products have been detected in soils (Biel-Maeso et al., 2018; Conde-Cid et al., 2018; Nuñez-Delgado et al., 2019; Mejías et al., 2021). These findings are being taken into account by the regulatory bodies of the European Union, leading in the last two decades to increased investigations concerning the presence of these emergent contaminants such as antimicrobial compounds on soil-plant ecosystems (Caracciolo et al., 2015; Tasho and Cho, 2016; Pan and Chu, 2017; Madikizela et al., 2018; Gworek et al., 2021; Bolesta et al., 2022; Yin et al., 2023).

Soil microorganisms play a major role in soil quality and long-term sustainability of agricultural terrestrial ecosystems since they control the breakdown of organic matter and hence the net fluxes of carbon and nutrients through the decomposition, mineralization, and immobilization processes (Pankhurst et al., 1996; Nannipieri et al., 2003). However, most studies concerning the impact of emergent contaminants such as antibiotics on terrestrial ecosystems are focused on their presence, behaviour, and risk assessment on human health and aquatic systems (Li, 2014; Bastos et al., 2020; Aydin et al., 2022). It is well known that antimicrobials for veterinary and human medicine can drastically modify non-target organisms living in the agricultural soils and hence alter biodiversity and ecosystem functions. Surprisingly, despite interest in the topic, the investigations addressing the impact of antibiotics on autochthonous microorganisms in the terrestrial ecosystems are scarce. The investigations concerning the impact of different groups of pharmaceutical compounds on microbial communities of agricultural soils located through the world were collected in a recent review by Cycón et al. (2019). The review clearly evidences that the response of microorganisms to the presence of these compounds measured by means of different parameters related to their mass, activity, and diversity (microbial C, total PLFA biomass and specific microbial groups PLFA biomass, soil respiration, bacterial growth, soil enzymes, microbial community structure), were variable; thus, although a negative temporal effect was often observed on microorganisms living in the soil, non effect, or even a positive effect, could also be detected. This is in agreement with recent investigations conducted by our research group performed with acid agricultural soils located in Galicia (NW Iberian Peninsula) added with antibiotics of different groups (tetracyclines, sulfonamides, fluorquinones, penicillins, cephalosphorine, diaminopyridine, β-lactams) (Santás-Miguel et al., 2020a; Santás-Miguel et al., 2020b; Santás-Miguel et al., 2020c; Rodríguez-González et al., 2021; Santás-Miguel et al., 2021; Rodríguez-González et al., 2022; Santás-Miguel et al., 2022; Rodríguez-González et al., 2023).

The World Health Organization (2017) considers the macrolides to be critical antibimicrobials of the highest priority, besides being the second most commonly used group of antibiotics in Europe (European Centre for Disease Prevention and Control, 2018). Clarithromycin is one of the most frequently prescribed macrolides in human medicine, therefore environmental risk investigations about the presence of this compound in soils are necessary (McLaughlin and Belknap, 2008; Baumann et al., 2015; Senta et al., 2019; Aydin et al., 2022). Recently, we examined the bacterial growth of 12 agricultural soils, both with no additions and with the addition of increasing concentrations of clarithromycin over time (1, 8, and 42 incubation days) (Rodríguez-González et al., 2021). In general, the data showed an initial inhibitory (toxic) effect which disappeared over time; however, in some cases, surprisingly, bacterial growth rates reached values higher than those in the corresponding unpolluted soil after 42 days of incubation. The aim of this laboratory experiment is to determine whether these bacterial growth changes were accompanied by changes in biomass and microbial community structure.

MATERIAL AND METHODS

Soils

The study was conducted with four agricultural soils located in the temperate humid zone (Galicia, NW Iberian Peninsula) which have not been previously treated with antibiotics. For each soil, 15–20 subsamples, collected randomly from the top 20 cm of the A horizon, were mixed, sieved (<2 mm), thoroughly homogenized, and air-dried. The main soil properties, which have been previously determined by Rodríguez-González et al. (2022), are shown in **Table 1**. They showed different texture, similar pH (ranges of pH in water 5.1–5.6), and different organic matter content (1.61%–6.8% C).

Experimental Set Up

Of each of the four air-dried soil samples, 100 g was moistened to up to 70% of the water holding capacity and incubated in the dark at 22°C for 15 days to recover and achieve the stabilization of bacterial growth (Meisner et al., 2013). After that, they were

TABLE 1 | Main properties of the four acid soils studied (adapted from Rodríguez-González et al., 2022).

Soil	3	5	10	12
pH_W	5.6	5.7	5.2	5.1
pH_{KCl}	4.8	4.9	4.7	4.5
C (%)	1.1	4.8	3.2	6.8
N (%)	0.18	0.40	0.41	0.59
C/N	6.1	12.0	7.8	11.5
eCEC (cmol$_c$ kg^{-1})	6.0	17.4	4.1	5.5
OC (mg kg^{-1})	243.1	332.9	327.1	318.4
Sand (%)	44	58	55	34
Silt (%)	33	20	28	38
Clay (%)	23	22	17	28
Texture	loam	Sandy clay loam	Sandy loam	Clay loam
Fe$_o$ (g kg^{-1})	2.2	2.8	8.9	8.9
Al$_o$ (g kg^{-1})	1.0	5.3	3.1	8.7

pH_W is pH measured in water; pH_{KCl} is pH measured in 0.1 M KCl; C is total carbon; eCEC is Cationic Exchange Capacity; OC is organic carbon; Al_o, Fe_o: Al and Fe extracted with ammonium oxalate.

separated into 8 centrifuge tubes (12 g in each tube) to be spiked with 7 different concentrations of clarithromycin (0.49, 1.95, 7.81, 31.25, 125, 500, and 2,000 mg kg^{-1} of soil), using talc as carrier to facilitate the mixture of the antibiotic with the soil and a blank to which the same amount of talc (48 mg) was added, but without antibiotic. These concentrations allowed the study of the effects of the antibiotic in a range that encompasses the toxicity of the antibiotic on soil bacteria from blank (no toxicity) to levels that suppress bacterial growth at high levels (Rodriguez-Gonzalez et al., 2021). The different mixtures of soil and antibiotic were incubated under controlled conditions (22°C, 70% of water holding capacity, darkness). After 8 days of incubation, 2 g samples were extracted in triplicate from each mixture and PLFA analysis was made. The same process was repeated after 42 days. Sampling was destructive for each treatment-time combination, resulting in a total of 192 microcosms (4 soils × 8 concentrations × 3 replicates × 2 times). The PLFA estimates were made using a representative composite soil sample of each antibiotic treatment obtained by mixing the three incubation replicates.

Phospholipid Fatty Acids (PLFA) Analysis

The total biomass (TotalPLFA) and the biomass of the specific microbial groups as well as the microbial community structure were estimated using the procedure and the nomenclature described by Frostegård et al. (1993). A detailed description of the method is given by Rodríguez-González et al. (2022). TotalPLFA was estimated as the sum of all the extracted PLFAs. The following PLFAs fatty acids were used as indicators of biomass of specific microbial groups: bacterial biomass (BactPLFA), the sum of i15:0, a15:0, 15:0, i16:0, 16:1ω9, 16:1ω7t, i17:1ω8, i17:0, a17:0, 17:0, cy17:0, 18:1ω7, and cy19:0 PLFAs; fungal biomass (FungPLFA), 18:2ω6 PLFA; actinobacteria biomass (ActPLFA), the sum of 10Me16:0, 10Me17:0, and 10Me18:0 PLFAs; Gram-positive bacteria biomass (G^+PLFA), the sum of i14:0, i15:0, i16:0, a15:0, i17:0, and a17:0 PLFAs; and Gram-negative bacteria biomas (G^-PLFA), the sum of cy17:0, cy19:0, 16:1ω7c, and 18:1ω7 PLFAs (Santás-Miguel et al., 2020b). In order to detect environmental effects (soil, incubation, antibiotic addition) on the soil microbial community structure, the concentrations of all the individual PLFAs, expressed in mole percent and logarithmically transformed, were subjected to principal component analysis (PCA). All statistical analyses were made using the SPSS 15.0 statistical package.

RESULTS AND DISCUSSION

The values of total (TotalPLFA) and specific microbial biomass (FungPLFA, BactPLFA, G^-PLFA, G^+PLFA, and ActPLFA) obtained for soil groups with nothing added and with different doses of the antibiotic clarithromycin added after 8 and 42 days of its application are shown in **Figures 1**, **2**, respectively. In general, the values observed for the two sampling times were quite similar. Therefore, for each soil type, the data obtained for un-treated and treated samples after 8 and 42 days of incubation were grouped together. Furthermore, in order to facilitate the interpretation of the results, the mean values of soil samples added with the 7 doses of clarithromycin were compared with the corresponding soil without antibiotic addition. In control S3 soil, total biomass showed values (mean values ±SE) of 35 ± 6 nmol g^{-1} and FungPLFA, BactPLFA, G^-PLFA, G^+PLFA, and ActPLFA values of 0.47 ± 0.16, 17.8 ± 3.1, 6.6 ± 0.8, 9.9 ± 2.1, and 2.7 ± 0.5 nmol g^{-1}, respectively. Similar values were found in the samples treated with clarithromycin, in which TotalPLFA, FungPLFA, BactPLFA, G^-PLFA, G^+PLFA, and ActPLFA values were 37 ± 3, 0.52 ± 0.07, 18.4 ± 1.1, 7 ± 0.5, 10.2 ± 0.5, and 2.9 ± 0.6 nmol g^{-1}, respectively. In S5, S10, and S12 soils, the magnitude levels of the total and specific biomass values were quite similar and much higher than those observed for S3 soil (**Figures 1**, **2**). These results can be explained by the organic C content of the soils, which is found to be positively correlated with the biomass estimates (Díaz-Raviña et al., 1988; Díaz-Raviña et al., 1993; Leirós et al., 2000). In general, as a consequence of antibiotic addition, the values of total and specific biomass of the S5, S10, and S12 soils increased. Thus, TotalPLFA biomass in these soils ranged from 117 ± 20 and 168 ± 33 nmol g^{-1} in control samples and from 162 ± 30 and 224 ± 56 nmol g^{-1} in antibiotic treated samples. FungPLFA biomass showed the lowest values of the specific microbial groups, ranging from 1.31 ± 0.32 and 1.88 ± 0.56 nmol g^{-1} in control soils and from 1.49 ± 0.47 and 4.16 ± 3.5 nmol g^{-1} in the soils added with clarithromycin. In contrast, BactPLFA biomass showed the highest values of the specific groups ranging from 49.3 ± 8.6 and 80.8 ± 18.5 nmol g^{-1} in un-treated control soils, and from 70.3 ± 26.4 and 104.2 ± 24.8 nmol g^{-1} in antibiotic polluted soils. G^-PLFA biomass values varied from 16 ± 2.3 and 31.2 ± 10.2 nmol g^{-1} in control soils and from 26.7 ± 14 and 35 ± 7.9 nmol g^{-1} in treated soils, while G^+PLFA biomass ranged from 29.3 ± 5.8 and 40.3 ± 9.7 nmol g^{-1} in control soils and from 41.2 ± 11.5 and 64.15 ± 15.33 nmol g^{-1} in antibiotic polluted soils. Finally, ActPLFA biomass ranged from 10.9 ± 1.9 and 14.9 ± 3.2 nmol g^{-1} in un-treated control soils and from 14 ± 4.6 and 20 ± 5.1 nmol g^{-1} in antibiotic polluted soils.

The total and specific biomass PLFA values in both the control studied soils and the corresponding soils added with clarithromycin, were within the reported range given for other soils of the same area added with other organic compounds such as atrazine (Mahía et al., 2011), tetracyclines, cyproflaxin, trimethoprim, and amoxicillin (Santás-Miguel et al., 2021; Rodríguez-González et al., 2022). Likewise, the relative importance of different specific groups (FungPLFA, BactPLFA, G^-PLFA, G^+PLFA, and ActPLFA) with respect to the TotalPLFA, as well as the ratios of Fung/Bact PLFA and Gr^-/G^+ PLFA, were similar to those reported in the mentioned studies. In agreement with a previous study, the lowest total and specific biomass values were exhibited by the soil with the lowest organic matter content (Rodríguez-González et al., 2022).

In four studied soils, after the incubation of the soils added with clarithromycin, the values of the total and specific biomass were generally similar (S3 soil) or higher (soils S5, S10, and S12 soils) than those in the equivalent control soils. Consequently, a positive effect of antibiotic addition, with

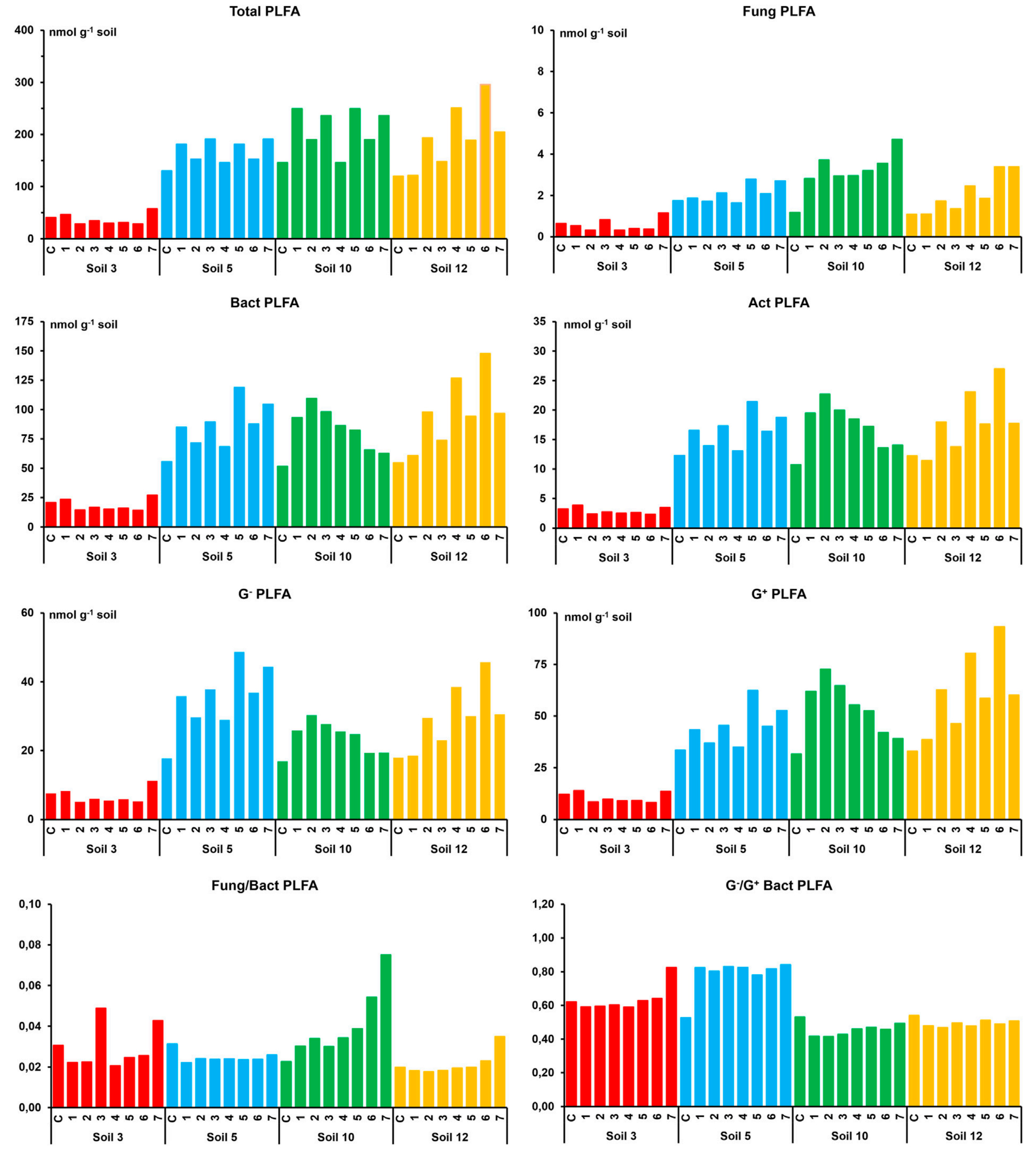

FIGURE 1 | Total and specific microbial biomass values (fungal, bacterial, actinobacteria, Gram-negative bacteria and Gram-positive bacterial) in four studied soils with nothing added (C) and with the different doses of clarithromycin after 8 days of incubation. Doses: 1 (0.49), 2 (1.95), 3 (7.81), 4 (31.25), (5) 125, 6 (500), and 7 (2000) mg kg^{-1} of soil.

increases up to 2 times the control value, was detected at medium- (8 incubation days) and long-term (42 days). Comparison of four soil trends at two sampling times showed slight differences among them, with increases slightly higher at 42 days for S3 and S5 soils while the opposite was observed for S10 and S12 soils. The higher biomass values, which were dose dependent, showed that fungal and Gram^{-} bacterial groups are more favoured by the antibiotic addition than the bacterial and Gram^{+} bacterial groups. This trend was evidenced by the values of the Fung/Bact PLFA and Gram^{-}/G^{+} PLFA, which is

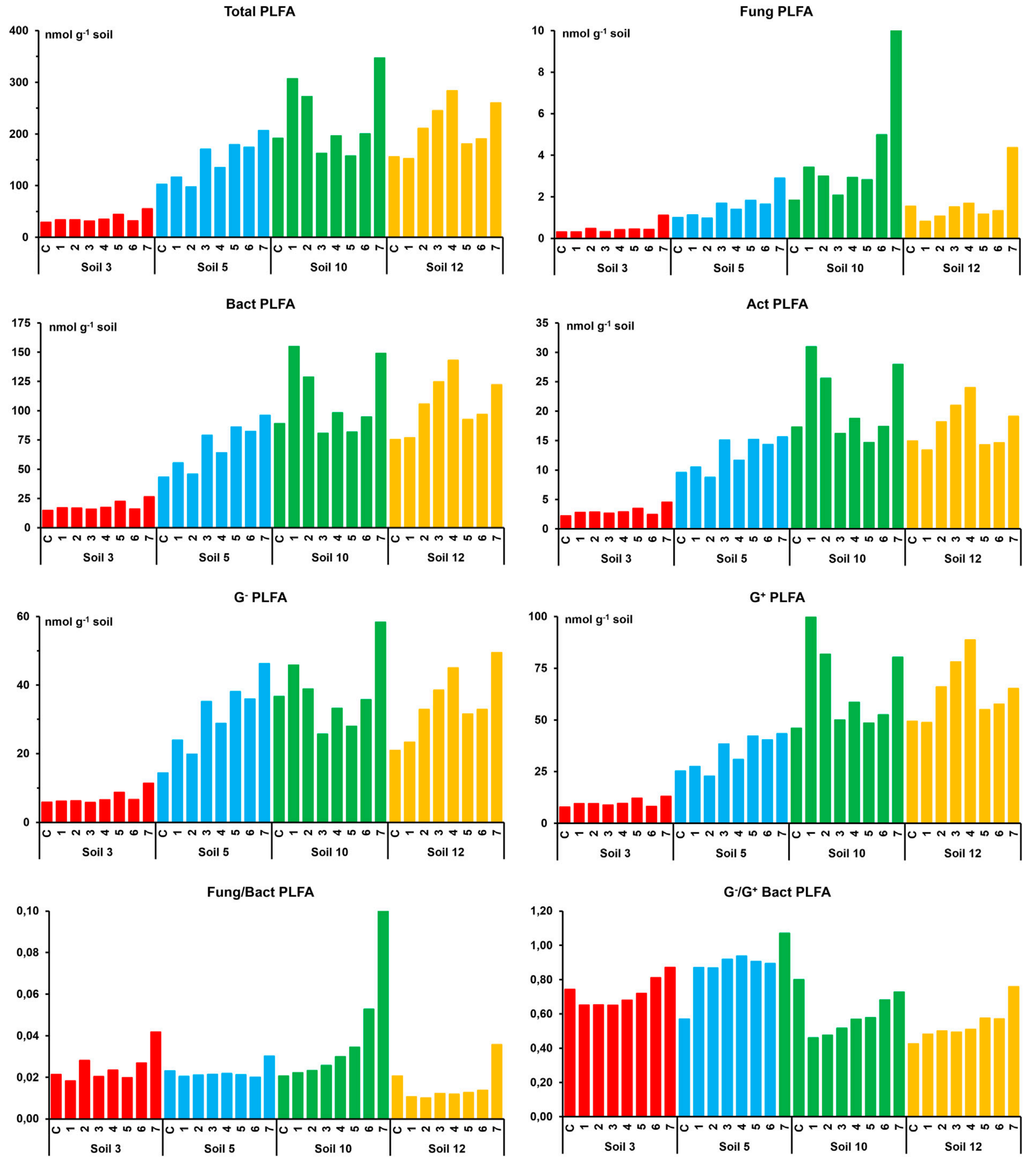

FIGURE 2 | Total and specific microbial biomass values (fungal, bacterial, actinobacteria, Gram-negative bacteria, and Gram-positive bacterial) in four studied soils with nothing added (C) and with the different doses of clarithromycin after 42 days of incubation. Doses: 1 (0.49), 2 (1.95), 3 (7.81), 4 (31.25), (5) 125, 6 (500), and 7 (2000) mg kg^{-1} of soil.

coincident with findings of other studies on this topic (Hammesfahr et al., 2008; Unger et al., 2012; Chen et al., 2013; Rodríguez-González et al., 2022). Inconsistent results were reported in the literature concerning the impact of veterinary and human antibiotics on biomass of soil microbial communities estimated by the PLFA method. According to the results of the present investigation, some authors showed that microbial biomass increased (Cordova-Kreylos and Scow, 2007) while other researchers observed that PLFA biomass values were unaffected or decreased as a consequence of the antibiotic presence (Thiele-Bruhn and Beck, 2005; Cui et al., 2014). The studies also showed that this variable response of microbial

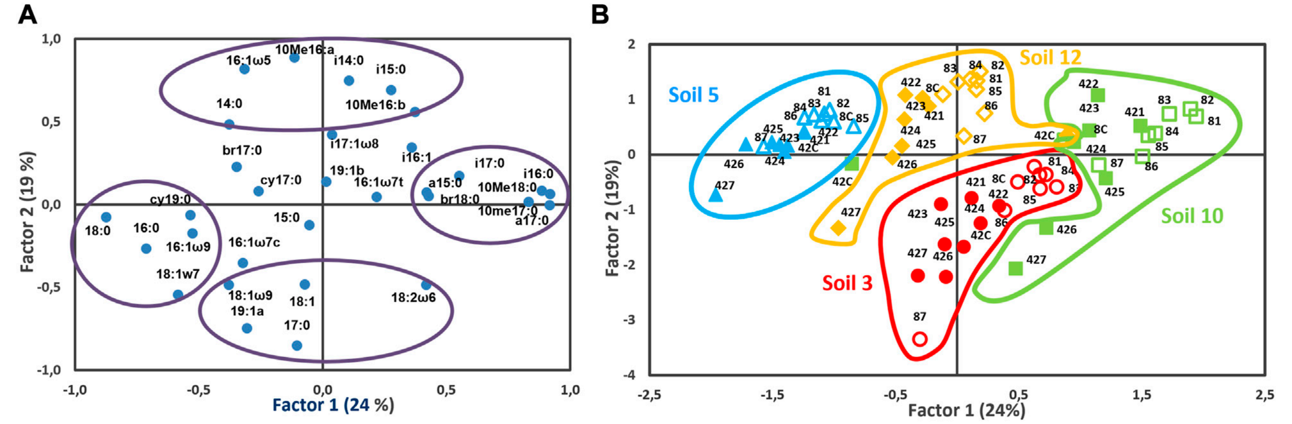

FIGURE 3 | Principal component analysis (**(A)**, variables; **(B)**, samples) performed with the PFLA data set for the four studied soils with nothing added (C) and with the different doses of clarithromycin after 8 (open symbols) and 42 (filled symbols) days of incubation. Doses: 1 (0.49), 2 (1.95), 3 (7.81), 4 (31.25), (5) 125, 6 (500), and 7 (2000) mg kg^{-1} of soil.

communities to the antibiotic addition depended on soil characteristics, type, and dose of antibiotic as well as time passed after its application (Cycón et al., 2019; Santás et al., 2020b; Santás et al., 2021; Rodríguez-González et al., 2022).

Figure 3 shows the results of the principal component analysis performed with the whole PLFA data set obtained for the four studied soils (S3, S5, S10, S12) with nothing added (control soil) and with different concentrations of clarithromycin added (0.49, 1.95, 7.81, 31.25, 125, 500 and 2,000 mg kg^{-1} of soil) after 8 and 42 days of incubation. The plane defined by the first and second component, accounting for the 43% of the variation, separated the soils independently of antibiotic addition and the time passed after its application. Samples of the same soil at two sampling times (8 and 42 days) and the antibiotic addition were grouped together and separated from the rest of the soils. S10 samples, having higher concentrations of i16:0, a17:0, 10Me18:0 and 10Me17:0 PLFAs, were located in the positive part of axis 1, S12 and S3 samples in the middle part and, finally, S5 samples, having higher concentrations of PLFAs 18:0, 16:0, cy19:0, 16:1ω9, and 18:1ω7, in the negative part. The axis 2 clearly separated S3 samples, having higher concentrations of PLFAs, at 17:0, 19:1a, 18:2ω6, 18:1, and 18:1ω9, from the rest of the soils (S5, S10, and S12), with higher values of 10Me16:0, 16:1ω5, i14:0, and i15:0. This axis also differentiated samples showing higher antibiotic concentrations, located in its negative part, which exhibited higher concentrations of PLFAs 18:2ω6 and 18:1ω9, indicative of fungi, and PLFA 19:1a, characteristic of G^- bacteria. Likewise, for each soil, a slight separation of samples incubated for 8 and 42 days was observed along axis 1 as well as a more defined separation of samples according the antibiotic addition, particularly after 42 days of incubation at higher doses.

To properly analyze the influence of the antibiotic addition on microbial community structure without the masking effect of time passed after its application, principal component analysis was made for each time separately (8 and 42 days after incubation) (**Figure 4**). Similar results were observed independently of sampling time (8 and 42 days of incubation). Again, the plane was defined by axis 1 and 2, accounting for 46% of the variation, samples of same soil were grouped together and separated from the other soils. Along axis 1, accounting for 23% of the variation, samples of S3 and S10 were located in the positive part of axis 1, while samples of S5 and S12 were situated in the negative part. The axis 2, accounting for 23% of the variation, clearly distinguished between samples with nothing added and those with an increasing dose of clarithromycin. Thus, in general, for all soils, independently of soil and sampling time, the highest effect of clarithromycin was observed at higher doses. It should be noted that, for all soils, the samples with higher doses of clarithromycin antibiotic had a relatively higher abundance of fungi, as indicated by PLFAs 18:2ω6 and 18:1ω9, and G^- bacteria, as indicated by PLFAs 18:1ω7, 19:1a, 16:1ω7c, and cy19:0.

Therefore, our data clearly demostrated that the main factor determining the source of variation of PLFA profiles among samples are soil characteristics followed by the incubation time and, to a lesser extent, the antibiotic dose. This is consistent with numerous field and laboratory studies concerning phospholipid fatty acid profiles performed with different soils following the impact of numerous stress agents (e.g., wildfire, prescribed fire, soil heating, fire retardants, post-fire rehabilitation treatments, vegetation type, soil management, climatic conditions, herbicide application, heavy metal pollution) which found that microbial communities of the same soil with different disturbances were grouped together and separated from microbial communities of other soils (Bossio et al., 1998; Díaz-Raviña et al., 2006, 2018; Fernández-Calviño et al., 2010; Mahía et al., 2011; Barreiro et al., 2015; Lombao et al., 2015). Likewise, a similar medium-term response of microbial communities was observed following the addition of antibiotics of both veterinary use such as tetracyclines (Santás-Miguel et al., 2021) and human use such as ciprofloxacin, trimethoprim, and amoxicillin (Rodríguez-González et al., 2022) to some soils of the same temperate humid zone.

The results of total and specific microbial biomass and microbial community structure determined by means of PLFA

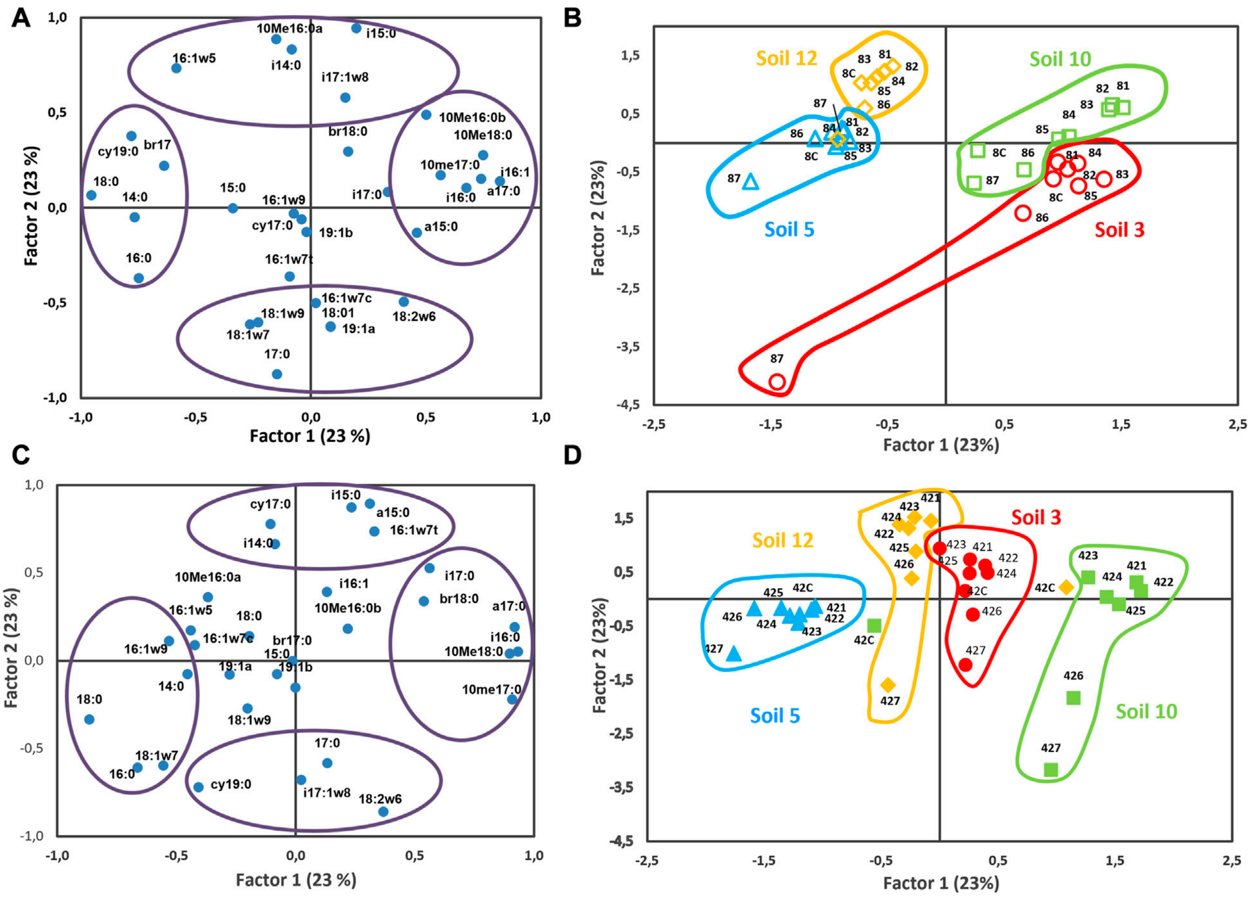

FIGURE 4 | Principal component analysis performed with the PFLA data set for the four studied soils with nothing added (C) and with the different doses of clarithromycin after 8 **(A,B)** and 42 **(C,D)** days of incubation. Doses: 1 (0.49), 2 (1.95), 3 (7.81), 4 (31.25), (5) 125, 6 (500), and 7 (2000) mg kg^{-1} of soil.

analysis were somehow coincident, showing that: a) the clarithromycin addition favoured fungi and G$^-$ bacteria more than bacteria and G$^+$ bacterial and b) the effects observed short-term (8 days of incubation) changed very little at medium-term (42 days of indication). The PLFA pattern distinguished different microbial communities following the application of increasing concentrations of clarithromycin; however, this analysis does not allow us to determine if its impact is positive or negative. This information is derived from total and specific microbial biomass data which showed a positive influence on soil microbial communities. The results of a study of Díaz-Raviña et al. (1988) concerning the soil respiration and mineralization of soil organic matter (SOM) in soils with nothing added and with glucose added, located in the temperate humid zone, demonstrated that the C availability is the limiting factor of microbial community activity. Thus, the positive effect of clarithromycin on soil microorganisms can be explained by the use of the antibiotic as a source of C (Liao et al., 2016; Schofield, 2018) and/or the increase of C and nutrient availability derived from the death of non-target bacteria (bactericide effect) (Díaz-Raviña and Bååth, 1996; Rajapaksha et al., 2004). Likewise, an increased mineralization of the native SOM (priming effect) caused by trace concentrations of "tigger solutions" of original chlaritromycin and its secondary metabolites can not be discarded. The magnitude and the duration (8 and 42 days) of the increases of the microbial biomass seem to support the latter hypothesis. Our results are also in agreement with findings of other authors concerning the respiration or bacterial growth rate values following the addition of glucose, amino acids, root exudates (De Nobili et al., 2001), herbicides (Mahía et al., 2008), and antibiotics of veterinary or human origin (Rodríguez-González et al., 2022) to different soils. It should be noted that an increased biomass and bacterial growth rates cannot be necessarily related to an increase in the soil quality status, particularly in these acid soils with low C and nutrient availability where soil quality is closely related to quantity and quality of soil organic matter (SOM) (Carballas et al., 2015). Thus, for example, in soil S3 exhibiting the lowest SOM content and C/N ratio, an increased SOM over a prolonged time (more than 42 days) can provoke a decrease in SOM content and hence also in soil quality.

The values reported in the literature for half-lifes of clarithromycin in soils, estimated in laboratory experiments using labelled compounds, ranged from 8 days to almost no dissipation (Kodešová et al., 2016; Topp et al., 2016). This behavior can be explained by the characteristics and concentration of the clarithromycin, the soil properties, as well as the time passed after application. In general, the higher half-life values or long-term persistence of clarithromycin were observed in more polluted soils with high sorption capacity (Cycón et al., 2019). This is associated not only with clarithromycin affinity by soil and chemical properties such as pH and properties related to the effective cation exchange properties (Rodríguez-González et al., 2022), but also with the soil microbial properties. The latter hypothesis is confirmed by Kodešová et al. (2016) who found higher dissipation half-lives in soils of better quality and, hence, with better microbial conditions for biodegradation processes than in lower quality soils. The history of field exposure to clarithromycin also showed a great influence on the response of microbial communities to the recent application of this antibiotic to the same soil; thus, repeated annual exposure to the antibiotic provided selective pressure to provoke changes in microbial composition (increase of tolerant microorganisms and decrease of sensitive ones) and, hence, a faster dissipation of clarithromycin (Topp et al., 2016; Lau et al., 2020). Evidence for long-term exposure leading to accelerated biodegradation of organic compounds in soils was previously detected for herbicides (Barriuso and Houot, 1996; Mahía et al., 2011).

In a previous investigation, we examined the bacterial growth rates of soils S3, S5, S10, and S12 not added and added with the same increasing concentration of clarithromycin over time (1, 8, and 42 days) (Rodríguez-González et al., 2022); therefore, data can be properly compared with those obtained in this investigation. For all soils, an inhibitory effect of clarithromycin on bacterial growth was detected; however, this toxicity disappeared gradually with time (8 days) and was no longer found after 42 days. In fact, for soil S3 after 8 days and for all soils after 42 days of incubation, bacterial growth rates increased exponentially between 8 and 42 days at higher doses of the antibiotic. In the present work, a positive effect of antibiotic was shown on total and specific microbial biomass values after both 8 and 42 days, the amended soils reaching values 1.5–2 times higher than those in the corresponding unpolluted soil. Therefore, the data supported our initial hypothesis about the fact that changes in bacterial growth observed as a consequence of clarithromycin addition were accompanied by changes in both microbial community composition and total and specific microbial biomass.

CONCLUSION

In summary, the results of the present study clearly demonstrated residual effects on total and specific microbial biomass (shifts in the positive direction) in these agricultural acid soils as a consequence of the clarithromicyn addition after 42 days of application. Since these microbial PLFAbiomass changes are associated with shifts in the microbial composition (PLFA pattern), it is quite probable that clarithromycin can have also an impact on functional microbial diversity. Further investigations including the measurements of microbial parameters related to mass, activity, microbial community structure, and functional microbial diversity should be conducted in short-, medium- and long-term studies in order to gain a deeper understanding of the persistence period of clarythromycine in terrestrial ecosystems as well as its risk assessment in human health, which is associated with its potential negative impact on soil functioning.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

Conceptualization; MA-E, DF-C, MD-R; Methodology: MA-E, DF-C, MD-R; Software: LR-G, VS-M, ÁM; Data curation: EG-C, ÁM, LR-G, MD-R; Writing-Original draft preparation: LR-G, VS-M, MD-R, ÁM; Visualization: MA-E, DF-C, MD-R, LR-G, VS-M; Investigation: MD-R, LR-G, VS-M, EG-C; Supervision: MA-E, DF-C, MD-R; Validation: MD-R, MA-E, DF-C; Writing-Reviewing and Editing: LR-G, MD-R, VS-M.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

Aydin, S., Ulvi, A., Bedük, F., and Aydin, M. E. (2022). Pharmaceutical Residues in Digested Sewage Sludge: Occurrence, Seasonal Variation and Risk Assessment for Soil. *Sci. Total Environ.* 817, 152864. doi:10.1016/j.scitotenv.2021.152864

Barreiro, A., Fontúrbel, M. T., Lombao, A., Martín, A., Vega, J. A., Carballas, T., et al. (2015). Using Phospholipid Fatty Acid and Community Level Physiological Profiling Techniques to Characterize Soil Microbial Communities Following an Experimental Fire and Different Stabilization Treatments. *Catena* 135, 419–429. doi:10.1016/j.catena.2014.07.011

Barriuso, E., and Houot, S. (1996). Rapid Meneralization of the S-Triazine Ring of Atrazine in Soils in Relation to Soil Management. *Soil Biol. Biochem.* 28, 1341–1348. doi:10.1016/S0038-0717(96)00144-7

Bastos, M. C., Soubrand, M., Guet, T. L., Floch, É. L., Baudu, M., Casellas, M., et al. (2020). Occurrence, Fate and Environmental Risk Assessment of

Pharmaceutical Compounds in Soils Amended with Organic Wastes. *Geoderma* 375, 114498. doi:10.1016/j.geoderma.2020.114498

Baumann, M., Weiss, K., Maletzki, D., Schüssler, W., Schudoma, D., Kopf, W., et al. (2015). Aquatic Toxicity of the Macrolide Antibiotic Clarithromycin and its Metabolites. *Chemosphere* 120, 192–198. doi:10.1016/j.chemosphere.2014.05.089

Biel-Maeso, M., Corada-Fernández, C., and Lara-Martín, P. A. (2018). Monitoring the Occurrence of Pharmaceuticals in Soils Irrigated with Reclaimed Wastewater. *Environ. Pollut.* 235, 312–321. doi:10.1016/j.envpol.2017.12.085

Bolesta, W., Głodniok, M., and Styszko, K. (2022). From Sewage Sludge to the Soil—Transfer of Pharmaceuticals: a Review. *Int. J. Environ. Res. Public Health.* 19, 10246. doi:10.3390/ijerph191610246

Bossio, D. A., Scow, K. M., Gunapala, N., and Graham, K. J. (1998). Determinants of Soil Microbial Communities: Effects of Agricultural Management, Season, and Soil Type on Phospholipid Fatty Acid Profiles. *Microb. Ecol.* 36, 1–12. doi:10.1007/s002489900087

Caracciolo, A. B., Topp, E., and Grenni, P. (2015). Pharmaceuticals in the Environment: Biodegradation and Effects on Natural Microbial Communities. *A Rev. Pharm. Biomed. Anal.* 106, 25–36. doi:10.1016/j.jpba.2014.11.040

Carballas, T., Rodríguez-Rastrero, M., Artieta, O., Gumuzzio, J., Díaz-Raviña, M., Martín, A., et al. (2015). "Soils of Temperate Humid Zone," Editorial in *The Soils of Spain* (Switzerland: Springer), 49–144. Lancho, Coord.

Chen, W., Liu, W., Pan, N., Jiao, W., and Wang, M. (2013). Oxytetracycline on Functions and Structure of Soil Microbial Community. *J. Soil. Sc. Plant Nutr.* 13, 0–975. doi:10.4067/S0718-95162013005000076

Conde-Cid, M., Álvarez-Esmorís, C., Paradelo-Nuñez, R., Nóvoa-Muñoz, J. C., Arias-Estévez, M., Álvarez-Rodríguez, E., et al. (2018). Occurrence of Tetracyclines and Sulfonamides in Manures, Agricultural Soils and Crops from Different Areas in Galicia (NW Spain). *J. Clean. Prod.* 197, 491–500. doi:10.1016/j.jclepro.2018.06.217

Cordova-Kreylos, A. L., and Scow, K. M. (2007). Effects of Ciprofloxacin on Salt Marsh Sediment Microbial Communities. *ISME J* 1 (7), 585–595. doi:10.1038/ismej.2007.71

Cui, H., Wang, S. P., Fu, J., Zhou, Z. Q., Zhang, N., and Guo, L. (2014). Influence of Ciprofloxacin on Microbial Community Structure and Function in Soils. *Biol. Fertil. Soils.* 50 (6), 939–947. doi:10.1007/s00374-014-0914-y

Cycón, M., Mrozik, A., and Piotrowska-Seget, Z. (2019). Antibiotics in the Soil Environment—Degradation and Their Impact on Microbial Activity and Diversity. *Front. Microbiol.* 10, 338. doi:10.3389/fmicb.2019.00338

De Nobili, M., Contin, M., Mondini, C., and Brookes, P. C. (2001). Soil Microbial Biomass Is Triggered into Activity by Trace Amounts of Substrate. *Soil Biol. biochem.* 32, 1163–1170. doi:10.1016/S0038-0717(01)00020-7

Díaz-Raviña, M., and Bååth, E. (1996). Thymidine and Leucine Incorporation into Bacteria from Soils Experimentally Contaminated with Heavy Metals. *Appl. Soil Ecol.* 3, 225–234. doi:10.1016/0929-1393(95)00086-0

Díaz-Raviña, M., Carballas, T., and Acea, M. J. (1988). Microbial Biomass and Metabolic Activity in Four Acid Soils. *Soil Biol. Biochem.* 20, 817–823. doi:10.1016/0038-0717(88)90087-9

Díaz-Raviña, M., Acea, M. J., and Carballas, T. (1993). Microbial Biomass and its Contribution to Nutrient Concentrations in Forest Soils. *Soil Biol. biochem.* 25, 25–31. doi:10.1016/0038-0717(93)90237-6

Díaz-Raviña, M., Bååth, E., Martín, A., and Carballas, T. (2006). Microbial Community Structure in Forest Soils Treated with a Fire Retardant. *Biol. Fertil. Soils* 42 (6), 465–471. doi:10.1007/s00374-005-0036-7

Díaz-Raviña, M., Lombao, A., Barreiro, A., Martín, A., and Carballas, T. (2018). Medium-term Impact of Post-fire Emergency Rehabilitation Techniques on a Shrubland Ecosystems in Galicia (NW Spain). Span. *J. Soil Sci.* 8, 322–346.

European Centre for Disease Prevention and Control (ECDC) (2018). "Surveillance Report," in *Annual Epidemiological Report for 2016; Antimicrobial Consumption* (Sweden: ECDC: Solna Municipality).

Fernández-Calviño, D., Martín, A., Arias-Estévez, M., Bååth, E., and Díaz-Raviña, M. (2010). Microbial Community Structure of Vineyard Soils with Different pH and Copper Content. *Appl. Soil Ecol.* 46, 276–282. doi:10.1016/j.apsoil.2010.08.001

Fijalkowski, K., Rorat, A., Grobelak, A., and Kacprzak, M. J. (2017). The Presence of Contaminations in Sewage Sludge. The Current Situation. *J. Environ. Manag.* 203, 1126–1136. doi:10.1016/j.jenvman.2017.05.068

Frostegård, A., Bååth, E., and Tunlip, A. (1993). Shifts in the Structure of Soil Microbial Communities in Limed Forests as Revealed by Phospholipid Fatty Acid Analysis. *Soil Biol. Biochem.* 25, 723–730. doi:10.1016/0038-0717(93)90113-P

Gworek, B., Kijeéwska, M., Wrzosek, J., and Graniewska, M. (2021). Pharmaceuticals in the Soil and Plant Environment: a Review. *Water Air Soil Pollut.* 232, 145. doi:10.1007/s11270-020-04954-8

Hammesfahr, U., Heuer, H., Manzke, B., Smalla, K., and Thiele-Bruhn, S. (2008). Impact of the Antibiotic Sulfadiazine and Pig Manure on the Microbial Community Structure in Agricultural Soils. *Soil Biol. Biochem.* 40 (7), 1583–1591. doi:10.1016/j.soilbio.2008.01.010

Kodešová, R., Kočárek, M., Klement, A., Golovko, O., Koba, O., Fér, M., et al. (2016). An Analysis of the Dissipation of Pharmaceuticals under Thirteen Different Soil Conditions. *Sci. Total Environ.* 544, 369–381. doi:10.1016/j.scitotenv.2015.11.085

Kümmerer, K. (2009). Antibiotics in the Aquatic Environment—A Review—Part I. *Chemosphere* 75, 417–434. doi:10.1016/j.chemosphere.2008.11.086

Lau, C. H. L., Tien, Y. C., Stedtfeld, R. D., and Topp, E. (2020). Impacts of Multi-Year Field Exposure of Agricultural Soil to Macrolide Antibiotics on the Abundance of Antibiotic Resistance Genes and Selected Mobile Genetic Elements. *Sci. Total Environ.* 727, 138520. doi:10.1016/j.scitotenv.2020.138520

Leirós, M. C., Trasar-Cepeda, C., Seoane, S., and Gil-Sotres, F. (2000). Biochemical Properties of Acid Soils under Climax Vegetation (Atlantic Oakwood) in an Area of the European Temperate–Humid Zone (Galicia, NW Spain): General Parameters. *Soil Biol. Biochem.* 32, 733–745. doi:10.1016/S0038-0717(99)00195-9

Li, W. C. (2014). Occurrence, Sources, and Fate of Pharmaceuticals in Aquatic Environment and Soil. *Environ. Pollut.* 187, 193–201. doi:10.1016/j.envpol.2014.01.015

Liao, X., Li, B., Zou, R., Dai, Y., Xie, S., and Yuan, B. (2016). Biodegradation of Antibiotic Ciprofloxacin: Pathways, Influential Factors, and Bacterial Community Structure. *Environ. Sci. Pollut. Res.* 23 (8), 7911–7918. doi:10.1007/s11356-016-6054-1

Lombao, A., Díaz-Raviña, M., Martín, M., Fontúrbel, M. T., Vega, J. A., Fernández, C., et al. (2015). Influence of Straw Mulch Application on the Properties of a Soil Affected by a Forest Wildfire. *J. Soil Sci.* 5, 26–40. doi:10.3232/SJSS.2015.V5.N1.03

Madikizela, L. M., Ncube, S. B., and Chimuka, L. (2018). Uptake of Pharmaceuticals by Plants Grown under Hydroponic Conditions and Natural Occurring Plant Species: A Review. *Sci. Total Environ.* 636, 477–486. doi:10.1016/j.scitotenv.2018.04.297

Mahía, J., Cabaneiro, A., Carballas, T., and Díaz-Raviña, M. (2008). Microbial Biomass and C Mineralization in Agricultural Soils as Affected by Atrazine Addition. *Biol. Fertil. Soils* 45, 99–105. doi:10.1007/s00374-008-0318-y

Mahía, J., González-Prieto, S. J., Martín, A., Bååth, E., and Díaz-Raviña, M. (2011). Biochemical Properties and Microbial Community Structure of Five Different Soils after Atrazine Addition. *Biol. Fertil. Soils* 47 (5), 577–589. doi:10.1007/s00374-011-0569-x

McLaughlin, A., and Belknap, A. (2008). *Annual Kg Quantity of Medicinal Ingredients Distributed and Dispensedin Canada: Analysis if Intercontinental Medical Stadistics (IMS) Data for 2007*. Excel Formal data. Environmental Impact Initiative, HPFB, Health Canada.

Meisner, A., Bååth, E., and Rousk, J., (2013). Microbial Growth Responses upon Rewetting Soil Dried for Four Days or One Year. *Soil Biol. biochem.* 66, 188–192. doi:10.1016/j.soilbio.2013.07.014

Mejías, C., Martín, J., Santos, J. L., Aparicio, I., and Alonso, E. (2021). Ocurrence of Pharmaceuthicals and Their Metabolites in Sewagw Sludge and Soil: a Review on Their Distribution and Environmental Risk Assessment. *Trends Environ. Anal. Chem.* 30, e00125. doi:10.1016/j.teac.2021.e00125

Nannipieri, P., Ascher, J., Ceccherini, M. T., Landi, L., Pietramellara, G., and Renella, G. (2003). Microbial Diversity and Soil Functions. *Eur. J. Soil Sci.* 54, 655–670. doi:10.1046/j.1351-0754.2003.0556.x

Nuñez-Delgado, A., Zhou, A., Necibi, C., Xu, Y., and Fernández-Calviño, D. (2019). Editorial of the VSI "Antibiotics and Heavy Metals in the Environment: Facing the Challenge". *Sci. Total Environ.* 678, 30–32. doi:10.1016/j.scitotenv.2019.04.421

Pan, M., and Chu, L. M. (2017). Fate of Antibiotics in Soil and Their Uptake by Edible Crops. *Sci. Total Environ.* 599, 500–512. doi:10.1016/j.scitotenv.2017.04.214

Pankhurst, C. E., Ophel-Keller, K., Doube, B. M., and Gupta, V. V. S. R. (1996). Biodiversity of Soil Microbial Communities in Agricultural Systems. *Biodivers. Conserv.* 5, 197–209. doi:10.1007/BF00055830

Rajapaksha, R. M. C. P., Tobor-Kaplon, M. A., and Bååth, E. (2004). Metal Toxicity Affects Fungal and Bacterial Activities in Soil Differently. *Appl. Environ. Microbiol.* 70, 2966–2973. doi:10.1128/AEM.70.5.2966-2973.2004

Rodríguez-González, L., Santás-Miguel, V., Campillo-Cora, C., Arias-Estévez, M., and Fernández-Calviño, D. (2021). The Effect of Clarithromycin Toxicity on the Growth of Bacterial Communities in Agricultural Soils. *Processes* 9 (8), 1303. doi:10.3390/pr9081303

Rodríguez-González, L., Nuñez-Delgado, A., Álvarez-Rodríguez, E., García-Campos, E., Martín, A., Díaz-Raviña, M., et al. (2022). Effects of Ciprofloxacin, Trimethoprim, and Amoxicillin on Microbial Structure and Growth as Emerging Pollutants Reaching Crop Soils. *Environ. Res.* 214, 113916. doi:10.1016/j.envres.2022.113916

Rodríguez-González, L., Nuñez-Delgado, A., Álvarez-Rodríguez, E., Díaz-Raviña, M., Arias-Estévez, M., Fernández-Calviño, D., et al. (2023). Direct Toxicity of Six Antibiotics on Soil Bacterial Communities Affected by the Addition of Bio-Adsorbents. *Environ. Pollut.* 322, 121161. doi:10.1016/j.envpol.2023.121161

Santás-Miguel, V., Arias-Estévez, M., Díaz-Raviña, M., Fernández-Sanjurjo, M. J., Álvarez-Rodríguez, E., Núñez-Delgado, A., et al. (2020a). Effect of Oxytetracycline and Chlortetracycline on Bacterial Community Growth in Agricultural Soils. *Agronomy* 10 (7), 1011. doi:10.3390/agronomy10071011

Santás-Miguel, V., Díaz-Raviña, M., Martín, A., García-Campos, E., Barreiro, A., Núñez-Delgado, A., et al. (2020b). Medium-term Influence of Tetracyclines on Total and Specific Microbial Biomass in Cultivated Soils of Galicia (NW Spain). *Span. J. Soil Sci.* 10 (3), 217–232. doi:10.3232/SJSS.2020.V10.N3.05

Santás-Miguel, V., Rodríguez-González, L., Núñez-Delgado, A., Díaz-Raviña, M., Arias-Estévez, M., and Fernández-Calviño, D. (2020c). The Toxicity Exerted by the Antibiotic Sulfadiazine on the Growth of Soil Bacterial Communities May Increase over Time. *Int. J. Environ. Res. Public Health* 17 (23), 8773. doi:10.3390/ijerph17238773

Santás-Miguel, V., Díaz-Raviña, M., Martín, A., García-Campos, E., Barreiro, A., Núñez-Delgado, A., et al. (2021). Soil Enzymatic Activities and Microbial Community Structure in Soils Polluted with Tetracycline Antibiotics. *Agronomy* 11, 906. doi:10.3390/agronomy11050906

Santás-Miguel, V., Rodríguez-González, L., Núñez-Delgado, A., Álvarez-Rodríguez, E., Díaz-Raviña, M., Arias-Estévez, M., et al. (2022). Time-course Evolution of Bacterial Community Tolerance to Tetracycline Antibiotics in Agricultural Soils: A Laboratory Experiment. *Chemosphere* 291, 132758. doi:10.1016/j.chemosphere.2021.132758

Schofield, C. J. (2018). Antibiotics as Food for Bacteria. *Nat. Microbiol.* 3 (7), 752–753. doi:10.1038/s41564-018-0181-z

Senta, I., Kostanjevecki, P., Kritzman-Matasic, I., Terzic, S., and Ahel, M. (2019). Occurrence and Behavior of Macrolide Antibiotics in Municipal Wastewater Treatment: Possible Importance of Metabolites, Synthesis Byproducts, and Transformation Products. *Environ. Sci. Technol.* 53, 7463–7472. doi:10.1021/acs.est.9b01420

Tasho, R. P., and Cho, J. Y. (2016). Veterinary Antibiotics in Animal Waste, its Distribution in Soil and Uptake by Plants: A Review. *Sci. Total Environ.* 563, 366–376. doi:10.1016/j.scitotenv.2016.04.140

Thiele-Bruhn, S., and Beck, I. (2005). Effects of Sulfonamide and Tetracycline Antibiotics on Soil Microbial Activity and Microbial Biomass. *Chemosphere* 59, 457–465. doi:10.1016/j.chemosphere.2005.01.023

Thiele-Bruhn, S. (2003). Pharmaceutical Antibiotic Compounds in Soils – a Review. *J. Plant Nutr. Soil Sci.* 166, 145–167. doi:10.1002/jpln.200390023

Topp, E., Renaud, J., Surumah, M., and Sabourin, L. (2016). Reduced Persistence of the Macrolide Antibiotics Erythromycin, Clarithromycin and Azithromycin in Agricultural Soil Following Several Years of Exposure in the Field. *Sci. Total Environ.* 562, 136–144. doi:10.1016/j.scitotenv.2016.03.210

Unger, I. M., Goyne, K. W., Kennedy, A. C., Kremer, R. J., McLain, J. E. T., and Williams, C. F. (2012). Antibiotic Effects on Microbial Community Characteristics in Soils under Conservation Management Practices. *Soil Sci. Soc. Am. J.* 77, 100–112. doi:10.2136/sssaj2012.0099

World Health Organization (2017). "Critically Important Antimicrobials for Human Medicine 5th Revision 2016," in *Ranking of Medically Important Antimicrobials for Risk Management of Antimicrobial Tesistance Due to Non-human Use Uull* (Geneva, Switzerland: World Health Organization).

Yin, L., Wang, X., Li, Y., Liu, Z., Mei, Q., and Chen, Z. (2023). Uptake of the Plant Agriculture-Used Antibiotics Oxytetracycline and Streptomycin by Cherry Radish─ Effect on Plant Microbiome and the Potential Health Risk. *J. Agric. Food Chem.* 71, 4561–4570. doi:10.1021/acs.jafc.3c01052

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Blue Carbon Stock in *Zostera marina* Meadows in the Ría de Ferrol (NW Iberian Peninsula)

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The increase in greenhouse gases (GHG) has been constant since the Industrial Revolution. For this reason, different international organizations have devoted special attention to GHG sinks such as terrestrial soils and ecosystems. However, the initially estimated balances did not include the carbon stock associated with ocean waters, coastal soils and sediments, known as *blue carbon*. Currently, blue carbon sinks are the subject of numerous studies due to the limited information available, especially on the Atlantic coast of the Iberian Peninsula. We studied the organic C stock present in soils and in *Zostera marina* biomass in the two main meadows in the Ría de Ferrol (O Baño and Castelo de San Felipe). The carbon stock associated with biomass was 0.37 Mg C ha^{-1}, with 0.18 Mg C ha^{-1} corresponding to the epigeal portion and 0.19 Mg C ha^{-1} to the hypogeal portion. Soil carbon stock was much higher: 4.11 Mg C ha^{-1} at a depth of 5 cm and 82.14 Mg C ha^{-1} at a depth of 1 m. Together with carbon values in biomass, a stock of 82.5 Mg C ha^{-1} was obtained for the whole Ría de Ferrol. *Z. marina* accounted for 8.25% of total C in the Ría de Ferrol. These values were higher than those found in other regions. Isotope ratios (δ13C, δ15N) and C/N ratios indicated that the organic C stock in the O Baño soil may receive important organic matter inputs of terrestrial origin, while in San Felipe, it seems to have a marine origin.

Keywords: seagrass, biometric parameters, isotope ratios, carbon, nitrogen

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HIGHLIGHTS

- Carbon stock in biomass was related to leaf length and shoot density of *Z. marina*.
- Carbon stock in soil was much higher than in biomass.
- Isotope and elemental ratios indicated the influence of allochthonous sources of soil carbon stock.

INTRODUCTION

Human-induced deforestation, whose beginnings date back from preindustrial times, along with fossil fuel burning and changes in land use, are leading to an exponential increase in carbon dioxide (CO$_2$) and

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Abbreviations: SBD, soil bulk density; SCD, soil carbon density; SCS, soil carbon stock.

other greenhouse effect gases (Mcleod et al., 2011; IPCC, 2021). Thus, the preindustrial baseline atmospheric CO_2 concentrations have multiplied by 1.5, going from 280 to 416.58 ppmv, at an annual increase rate of 2.31 ppmv (NOAA, 2021).

Ocean waters are the main carbon reservoir on the Earth's surface, and coastal ecosystems with vascular plants play a key role as atmospheric CO_2 sinks. Blue carbon is the proposed term to designate carbon stored by coastal marine ecosystems, particularly saltmarshes, mangroves, and seagrass meadows (Pendleton et al., 2012; Röhr et al., 2018). These ecosystems occupy only 0.2% of the ocean surface but store more than half the carbon present in terrestrial systems (green C) and 33% of the total carbon stored in oceans (Fourqurean et al., 2012). However, unlike green C, a highly relevant aspect for the study of carbon stocks is the long residence time of blue carbon. Green carbon can be retained for decades, while blue carbon can be stored for millennia due to the anaerobic conditions that are predominant in coastal sedimentary environments, along with other factors (e.g., refractance of organic compounds, toxicity associated with reduced Fe and S forms, etc.) that inhibit decomposition of organic matter (Fourqurean et al., 2012; Pendleton et al., 2012; Herr and Landis, 2016).

Seagrass meadows are formed by 60 phanerogam species included in the order Alismatales (Kuo and Den Hartog, 2001). They are among the most productive ecosystems worldwide, accounting for 15% of the ocean's net primary production (Duarte and Chiscano, 1999). For this reason, they are considered an essential CO_2 sink for climate change mitigation (Duarte et al., 2013). They currently occupy 30 million hectares (ha), but they are experiencing a sharp decline due to anthropogenic action and to the consequences of global change (Duarte et al., 2013). Estimates suggest that 29% of their area has already been lost and, if loss continues to occur at the current rates, 40% will have been lost 100 years from today (Pendleton et al., 2012; Claes, et al., 2022). Their disappearance entails the loss of an effective CO_2 sink and the simultaneous release of CO_2 and/or methane (CH_4) into the atmosphere; the greenhouse effect of the latter gas is 23–25 times higher than that of CO_2 (Short and Neckles, 1999; Pendleton et al., 2012; IPCC, 2021).

Despite the evidence supporting their importance in CO_2 removal and retention, isotopy and elemental composition both of their biomass and soils are worth taking into account. The proportions of stable isotopes δ15N and δ13C, as well as C/N ratios, are a result of the origins of stored carbon (Prentice et al., 2020). The continental area can act as a carbon source; this has been observed in *Zostera marina* meadows in temperate areas (Prentice et al., 2020). Seagrass meadows are considered to be hotspots for allochthonous carbon sequestration, since isotopy has shown that only 50% of the carbon stock associated with these ecosystems is produced by phanerogam tissues. However, through the formation of meadows, phanerogams are able to remove particles from the water column and transfer them to the bottom (Kennedy et al., 2010).

Zostera marina is the most widely distributed species among marine phanerogams; in Galicia, it forms vast meadows in intertidal and shallow subtidal areas (Míguez, 2003; Cacabelos et al., 2015; García-Redondo et al., 2019). The Galician coastline features rias and inlets that provide optimal conditions for the growth of *Zostera marina* (García-Redondo et al., 2019).

The main objectives of this research were to quantify the total C stock (both associated with biomass and present in soils) in the two main *Zostera marina* meadows in the Ría de Ferrol; to study the variability in total C between the two main meadows in the Ría de Ferrol: O Baño and San Felipe; and to analyze whether said variability is related to plant biometry in meadows and/or to nutrient origin. To this end, the most relevant biometric parameters of *Z. marina* (i.e., epigeal and hypogeal biomass, leaf length and width), soil composition and properties, organic C content, and isotopic ratios ($\delta^{13}C$, $\delta^{15}N$) and organic C stock (Mg C ha^{-1}) in *Z. marina* biomass and in soil were determined.

MATERIAL AND METHODS

Study Area

The study area covers the Ría de Ferrol (NW Iberian Peninsula, A Coruña). Its area is 21 km^2, and its largest tributary is the Xubia River (**Figure 1**). *Zostera marina* occupies a total area of 11.42 ha in the Ría de Ferrol, divided among four meadows: O Baño (9.11 ha), Castelo de San Felipe (1.48 ha), O Segaño (0.66 ha), and San Felipe Núcleo (0.17 ha). The two main meadows have been sampled for this study: O Baño (43° 27′ 26.5″ N, 8° 16′ 0.6″ W) and Castelo de San Felipe (43° 27′ 51.1″ N, 8° 17′ 0.3″ W).

The O Baño meadow is located in the intertidal area within an inlet that is relatively sheltered from marine currents; the granulometry of its substrate is silt. The *Z. marina* meadow in Castelo de San Felipe is located in the subtidal area, at a depth of 2 m at low tide. Exposure to marine currents is greater in Castelo de San Felipe, and substrate is mostly sandy, with abundant gravel.

Sample Collection

Zostera marina Sampling

Sampling was performed in September 2021 following the instructions in the *Blue Carbon Manual* (Howard et al., 2014) in order to use standardized methods, thus allowing for result comparison among studies. Ten sampling points were defined in the O Baño meadow, distributed across the meadow following a zigzag pattern. Samples were collected using metal squares with dimensions 30 cm × 30 cm and a depth of 10 cm to collect the epigeal and hypogeal portions, i.e., leaves and rhizomes, respectively. Samples were frozen at 3°C. In the Castelo de San Felipe meadow, six squares with the above described measurements were collected.

Soil Sampling

The same number of soil samples were collected in both meadows. Eighteen soil samples were collected, 10 of which were taken from within the meadows, next to each of the plots from which plant samples were taken; these soil samples were collected using 8 cm wide, 15 cm deep PVC cores. The remaining 8 samples were collected using 5 cm deep PVC cores; 4 of them were collected within the meadow, following the zig-zag method, while the remaining 4 were collected in adjacent areas, at 4 sites around the perimeter of the meadow and outside the meadow, to serve as control samples. pH, electrical conductivity, and redox potential (Eh) were measured *in situ* using a Hanna multiparameter probe. Samples were then kept at −20°C until analysis in the laboratory.

FIGURE 1 | Seagrass meadows in the Ría de Ferrol.

Sample Analysis

Flood Water Analysis

A Hanna multiparameter probe was used to measure pH, electrical conductivity, redox potential (Eh), and salinity *in situ* in flood water in the two main meadows.

Plant Analysis

Prior to analysis, *Zostera marina* samples were washed in distilled water to remove all adhered materials. For each square, the number of bundles was counted to determine density. Subsequently, 15 bundles were randomly selected within each plot and divided into their hypogeal and epigeal portions. The hypogeal portion of each bundle was measured, the number of leaves was counted, and leaves were divided by maturity status from the outside towards the inside of the bundle, where new leaves sprout from the central portion, sheathed in one another. The following measurements were taken for each leaf within the bundle:

1. Category: 1: whole mature leaf, 2: large (>20 cm) immature leaf, 3: small (<20 cm) immature leaf, 4: broken leaf.
2. Leaf length,
3. Length of the petiole or base of the leaf (present only in mature leaves)
4. Leaf width (mid-leaf).

Moreover, biomass was measured as dry weight (at 105°C) both for the epigeal and the hypogeal portion, as well as total biomass. To determine organic C content and to perform isotope and elemental analyses, they were divided in 5-bundle groups (pooled sample), separating the epigeal and hypogeal sections, to reach sufficient weight for the required analyses. Each pool was ground using a Retsch MM400 electrical mill. From each ground hypogeal portion sample, 0.5 g were used to measure carbonate content using a BD brand, LOGL model digital calcimeter. A 6M HCl dissolution was used to dissolve carbonates. Total carbon content in plants was measured by combustion using a LECO CN828 autoanalyzer. Organic C content in the hypogeal portion was determined as the difference between total C content and carbonate content, while for the epigeal portion, total C was considered as organic C. Carbonates were absent from the epigeal portion, contrasting with the presence of carbonates found in the hypogeal portion.

The C stock associated with *Zostera marina* was calculated by multiplying the three carbon reservoirs (epigeal, hypogeal, and total) times the % of organic carbon and times the area of the plot (900 m^2). In order to obtain comparable results, they were expressed in Mg (Mg = metric ton) C ha^{-1}. Total biomass and total organic C were calculated by averaging the values from the hypogeal and epigeal portions.

Isotope ratios for $\delta^{13}C$ (‰ vs. VPDB) and $\delta^{15}N$ (‰ vs. Air) were analyzed at the University of A Coruña's Research Support Services (Servicios de apoyo a la investigación, SAI) using a FlashEA1112 autoanalyzer (Thermo Finnigan) coupled with a Deltaplus isotope ratio mass spectrometer (ThermoFinnigan). Nitrogen percentages were also analyzed at the SAI.

Soil Analysis

Out of the 18 soil samples collected at each site, 10 were selected for the following depths: 0–2, 2–4, 4–6, 6–10, and 10–15 cm, and subsequently dried. From each section, 10 g were weighed and sieved to determine the proportion of gravel (>2 mm), coarse sand (2–0.20 mm), fine sand (0.20–0.05 mm), and clay and silt (<0.05 mm). Carbonate content, total C content, organic C content, and $\delta^{13}C$ and $\delta^{15}N$ isotope ratios, as well as elemental ratios, were determined using the same method as for plants. The remaining 8 samples from each site, 4 from control areas and 4 from meadows, were used to analyze soil bulk density [SBD (g cm^{-3}) = Mass of dry soil (g)/volume (cm^3)]. To this end, their height and width were measured, and they were weighed after drying at 105°C. In the 4 control samples, the percentages of carbonates, total C, and organic C present were also measured, along with isotopy and elemental ratios.

To obtain C stock in soil (SCS; Eq. **2**), soil C density was first calculated (SCD; Eq. **1**) (Howard et al., 2014).

$$SCD = SDB\ (g\,cm^{-3})\,^{*}\ (\%C.O./100) \tag{1}$$

$$SCS\ (Mg\,C\,ha^{-1}) = SCD\,^{*}\ depth\ (5\,cm)^{*}\ 100 \tag{2}$$

The mean %CO value for the first three sections of the 15 cm deep cores and the apparent density value of 5 cm deep cores were used to calculate the SCS value for the upper 5 cm.

To calculate total SCS in the Ría de Ferrol, SCS values from the *Z. marina* meadows in the O Segaño and San Felipe Núcleo sites were also included. The values for these two meadows were estimated based on the data obtained for Castelo de San Felipe, which is the closest meadow and shows similar environmental parameters.

Statistical Analysis

A t-Student test was used for biometric parameters of *Z. marina*, except for those that did not follow a normal distribution, for which a Mann-Whitney test was used. In the case of soil, granulometric data were analyzed using a t-Student test, while C stock data were analyzed using a one-way Anova.

RESULTS

Flood Water Parameters

Flood water from both sites was characterized by similar pH values between sites, as well as similar to seawater (pH ~8.2; **Table 1**), and a high ion concentration resulting in a high electrical conductivity (54–55 mS cm^{-1}) and salinity (**Table 1**), similar to seawater, which suggests a low influence of continental freshwater. Redox potential (Eh) showed mean values over 500 mV, which is typical of oxic environments.

TABLE 1 | Physicochemical properties of flood water.

Location	Sample size	pH	Eh	EC	Salinity
			mV	mS cm^{-1}	
O Baño	3	8.3 ± 0.07	611 ± 54	55 ± 0.1	36.4
S. Felipe	10	8.2 ± 0.03	590 ± 22	54 ± 0.2	na

na, not analyzed.

Characterization of Soils of *Z. marina* Meadows

Soils from the O Baño meadow were characterized by mean pH values close to neutral (pH: 7.2 ± 0.1; **Table 2**), redox potential values typical of suboxic sedimentary environments (Eh 247 ± 70 mV), and electrical conductivity values notably lower than those of flood water (16 ± 4.3 mS cm^{-1}). Granulometric composition was dominated by the sandy fraction, with an average proportion of 79.4%, which showed high contents of biogenic carbonates (21.0 ± 0.2%; mostly shells), and organic C content was 0.73 ± 0.24%.

Soils from the *Zostera marina* meadow of Castelo San Felipe showed differences with respect to those from the O Baño population. pH (7.9 ± 0.2) and redox potential (Eh: 439 ± 18 mV) were higher, defining an environment with oxic conditions, consistently with their granulometric composition with a greater proportion of sand and lower organic C contents. Another substantial difference was the lower concentration of salts in soils from Castelo San Felipe than in those from O Baño.

Variability in Biometric Parameters of *Zostera marina*

Significant differences were observed in terms of shoot density (O Baño 351.1 ± 131 shoots m^{-2}; Castelo San Felipe 139 ± 65.1 shoots m^{-2}), leaf width (cm) (O Baño 0.51 ± 0.06 cm; Castelo San Felipe 0.60 ± 0.15 cm), petiole length (cm) (O Baño 9.13 ± 2.12; Castelo San Felipe 14.3 ± 4.77 cm), and leaf category, with category 3 as the predominant one in O Baño and category 4 in Castelo San Felipe. Conversely, no significant differences were found either in the number of leaves (O Baño 5.05 ± 0.44 shoots; Castelo San Felipe 5.16 ± 0.50 shoots), in their length (cm) (O Baño 22.2 ± 6.29; Castelo San Felipe 26.9 ± 8.9 cm; **Table 3**), or in rhizome length (cm) (O Baño 8.27 ± 1.57; Castelo San Felipe 7.35 ± 0.24 cm). Similarly, no differences were found for epigeal biomass (O Baño 49.4 ± 27.0; Castelo San Felipe 48.1 ± 33.4 g m^{-2}), hypogeal biomass (O Baño 60.2 ± 33.9; Castelo San Felipe 41.5 ± 16.2 g m^{-2}; **Table 3**), or total biomass (O Baño 109.6 ± 59.1; Castelo San Felipe 89.5 ± 46.3 g m^{-2}; **Table 3**).

Carbon Stock in Plant Biomass and in Soils

The C stock associated with *Z. marina* biomass did not show significant differences either for epigeal (O Baño 0.18 ± 0.12; San Felipe 0.17 ± 0.13 Mg C ha^{-1}), hypogeal (O Baño 0.21 ± 0.12; Castelo San Felipe 0.12 ± 0.05 Mg C ha^{-1}), or total carbon (O Baño 0.39 ± 0.23; Castelo San Felipe 0.30 ± 0.17 Mg C ha^{-1}) (**Figure 2**).

TABLE 2 | Geochemical characterization and composition of the soils on which *Zostera marina* grows in the ría de Ferrol.

Site	Sample size	pH	Eh	EC	Coarse sand	Fine sand	Silt + Clay	Carbonates	Organic C
			(mV)	(mS cm^{-1})	(%)				
O Baño	10	7.2 ± 0.1	247 ± 70	16 ± 4.3	13.9 ± 5.2	65.5 ± 5.5	20.1 ± 0.2	21.0 ± 0.2	0.73 ± 0.24
Castelo S. Felipe	6	7.9 ± 0.1	439 ± 18	4 ± 3.3	62.9 ± 3.8	29.8 ± 3.5	7.3 ± 1.7	0.68 ± 0.2	0.35 ± 0.32

TABLE 3 | Biometric characterization of *Zostera marina* in the two main meadows of the ría de Ferrol. Different letters indicate significant differences between meadows.

Parameter	O Baño	Castelo san Felipe
Density (Shoot m^{-2})	351.1 ± 131.3 a	138.9 ± 65.1 b
Number leaves	5.05 ± 0.44 a	5.16 ± 0.50 a
Leaves length (cm)	22.2 ± 6.29 a	26.9 ± 8.9 a
Leaves width (cm)	0.51 ± 0.06 a	0.60 ± 0.15 b
Petiole length (cm)	9.13 ± 2.12 a	14.3 ± 4.77 b
Rhizome length (cm)	8.27 ± 1.57 a	7.35 ± 1.24 a
Leaf biomass (g m^{-2})	49.4 ± 27.0 a	48.1 ± 33.4 a
Rhizome biomass (g m^{-2})	60.2 ± 33.9 a	41.5 ± 16.2 a
Total biomass (g m^{-2})	109.6 ± 59.1 a	89.5 ± 46.3 a

Carbon stock in soil (**Figure 3**) was significantly higher in the O Baño *Z. marina* meadow (4.58 ± 1.46 Mg C ha^{-1}) than in the Castelo San Felipe one (2.27 ± 1.69 Mg C ha^{-1}; t = 3.76; p-value = 0.005). Significant differences were also observed between O Baño and the Castelo San Felipe control site (0.30 ± 0.1 Mg C ha^{-1}; t = 5.26; p-value <0.001). However, no significant differences were observed between the O Baño control site (2.51 ± 0.40 Mg C ha^{-1}) and the Castelo San Felipe meadow (t = 0.29; p-value = 0.78). No significant differences were observed between the control sites (t = 2.27; p-value = 0.06) or between control sites and their corresponding meadows (O Baño t = 2.55; p-value = 0.07; Castelo San Felipe t = 2.42; p-value = 0.07) (**Figure 3**).

Isotope Ratio (δ^{13}C, δ^{15}N) in Plants and in Soils

No significant differences in terms of δ^{13}C content were observed in any of the sites, either for the epigeal (δ^{13}C**:** O Baño −8.67 ± 0.51‰; Castelo San Felipe −8.87 ± 1.32‰) or the hypogeal portion of plants (δ^{13}C: O Baño −10.00 ± 0.82‰; Castelo San Felipe −9.83 ± 0.95‰). Likewise, no significant differences in terms of δ^{15}N content were observed for the epigeal (δ^{5}N: O Baño 5.83 ± 0.80‰; San Felipe 6.43 ± 0.49‰) or the hypogeal portion

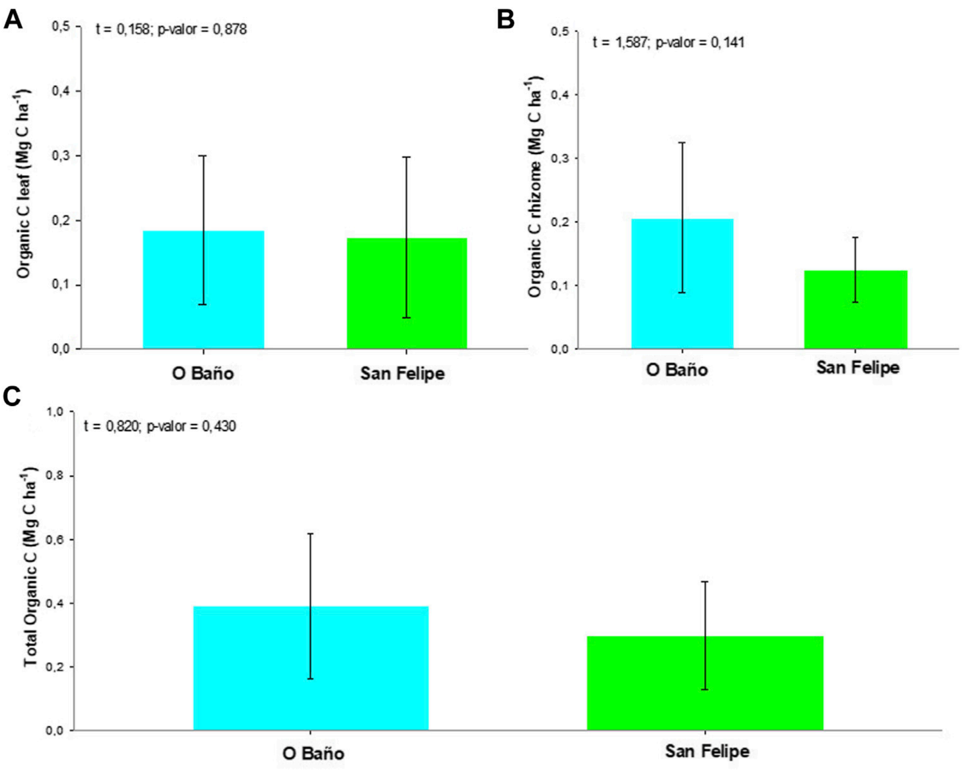

FIGURE 2 | Mean organic C ± SD (Mg C ha^{-1}) in each location. **(A)** Organic C in leaves, **(B)** in rhizomes, and **(C)** total.

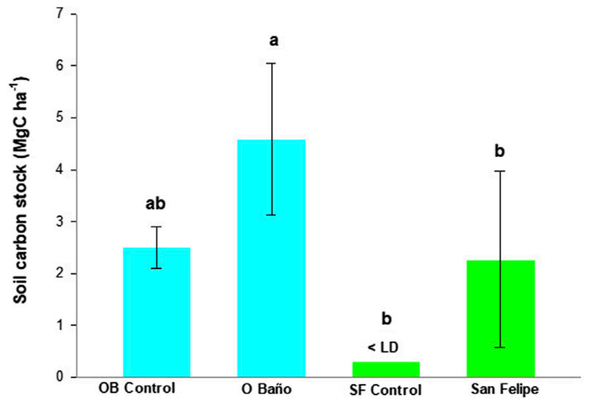

FIGURE 3 | Mean organic C ± SD (Mg C ha^{-1}) in each location.

of plants (δ^{5}N: O Baño 5.57 ± 0.31‰; Castelo San Felipe 6.43 ± 0.50‰).

As for soils, δ^{13}C did not show any significant differences between meadows and their matched control sites (δ^{13}C: O Baño −22.25 ± 1.71‰ and O Baño control site −22.17 ± 0.47‰; Castelo San Felipe −5.40 ± 5.41‰ and Castelo San Felipe control site −6.60 ± 2.66‰). However, there were differences between both meadows (δ^{13}C: O Baño −22.25 ± 1.71‰; Castelo San Felipe −5.40 ± 5.41‰). A similar situation was found for δ^{15}N: there were no differences between soils from each meadow and their matched control site, but differences were observed between the two meadows (δ^{15}N: O Baño 5.32 ± 0.075‰ and O Baño control site 5.10 ± 0.27‰; Castelo San Felipe 5.73 ± 0.39‰ and Castelo San Felipe control site 5.57 ± 0.15‰).

DISCUSSION

C Stock in *Zostera marina* Biomass

Considering the area occupied by each one of the four meadows present in the Ría de Ferrol, blue carbon associated with *Z. marina* in the Ría de Ferrol was 4.25 Mg C, with the highest values found in the O Baño meadow, with 3.56 Mg C, followed by Castelo de San Felipe (0.44 Mg C), O Segaño (0.20 Mg C), and San Felipe Núcleo (0.05 Mg C). Carbon stocks in the O Segaño and San Felipe Núcleo meadows were estimated from the values obtained for the closest meadow: Castelo de San Felipe. The value obtained for biomass-associated carbon, 0.37 Mg C ha^{-1}, was lower than the value observed in the Eastern Atlantic by Röhr et al., 2018 (1.29 Mg C ha^{-1}; **Table 4**), who recorded C stock data for a wide range of *Z. marina* meadows across its entire distribution area. Mean density in the Ría de Ferrol was found to be much lower than the expected values for Western Atlantic *Zostera marina* meadows (774 ± 275 shoots m^{-2}). These values were closer to those established for the Mediterranean Sea, Western Pacific, and the Korean coast (Kim et al., 2022; **Table 4**), the areas with the lowest density values. Epigeal biomass is much lower than any values described by said authors (**Table 4**). However, hypogeal biomass values were similar to previously detected, although they were at the lowest end of the range.

As for leaf category, petiole length, and rhizome length, no detailed studies have been found. Our results allowed inferring that Castelo San Felipe showed more mature leaves (petiole length: 14.3 cm; category 4) than O Baño (petiole length: 9.13 cm; category 3). Moreover, our results showed a high biometric variability between both meadows, consistently with previous studies (Samper-Villarreal et al., 2016; García-Redondo et al., 2019). Said variability is common in the studied species due to its phenotypic plasticity, enhanced by spatial and seasonal variations (Samper-Villarreal et al., 2016; Bertelli et al., 2021), which makes data extrapolation difficult.

In our case, biometric variables have been observed to relate to plant biomass and to its C stock. Leaf length, which did not show any significant differences between the two meadows, appears to be a good predictor for epigeal biomass, as well as for epigeal and total carbon stock (**Figure 4**). Shoot density was highly correlated with hypogeal and total carbon stock (**Figure 4**). Therefore, correlations were obtained between biometric variables and carbon content in plants, as observed in other studies (Kennedy et al., 2010; Samper-Villarreal et al., 2016).

TABLE 4 | Biometric parameters and organic C stock in soils from different geographic sites with *Zostera marina* in relation with meadows of the Ría de Ferrol.

Sites	Density	Leaf biomass	Rhizome biomass	Organic C leaf & rhizome	Soil O.C stock	References
	Shoot m^{-2}	g m^{-2}	g m^{-2}	Mg C ha^{-1}	Mg C ha^{-1}	
Baltic sea	397 ± 63	118 ± 17	102 ± 19	0.79	23.1	Röhr et al. (2018)
Black sea	736 ± 459	120 ± 56	72 ± 32	0.63	29	
Eastern Atlantic	774 ± 275	78 ± 10	264 ± 134	1.29	55.4	
Western Atlantic	381 ± 202	183 ± 46	180 ± 41	1.00	54	
Eastern Pacific	549 ± 316	232 ± 61	111 ± 47	1.07	69.4	
Western Pacific	287 ± 80	194 ± 53	56 ± 16	0.86	93.7	
Kattegat-Skagerrak	319 ± 35	129 ± 15	125 ± 26	0.8	194.5	
Mediterranean Sea	223 ± 55	73 ± 14	144 ± 40	0.62	351.7	
South Korean Coast	154 ± 22.0	303 ± 43.5	71.1 ± 8.1	—	49.1	Kim et al. (2022)
East Korean Coast	219 ± 30.2	371.8 ± 85.7	67.2 ± 12.4	—	58.5	
West Korean Coast	265 ± 16.1	331.4 ± 21.1	45.15 ± 6.7	—	71.3	
Ría Ferrol	245 ± 98.2	48.8 ± 30.2	50.9 ± 25.0	0.37	82.14	

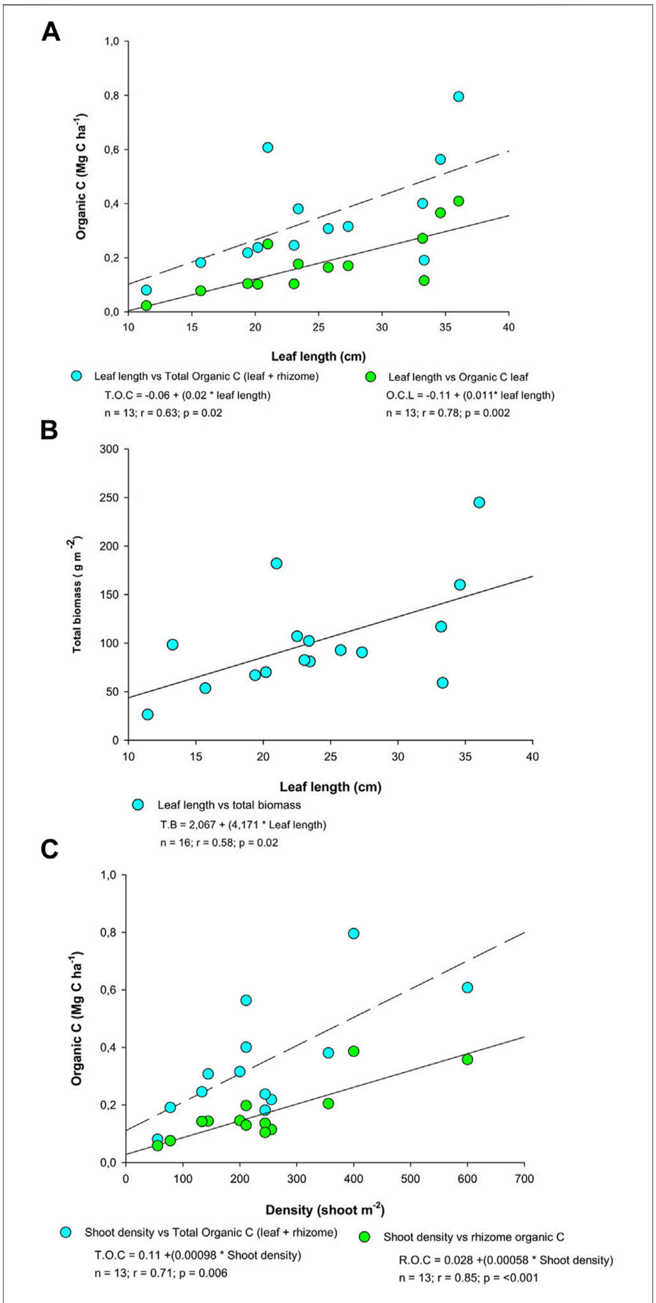

FIGURE 4 | Individual relationships between **(A)** total and leaf carbon content (Mg C ha^{-1}) and leaf length (cm); **(B)** total biomass (g m^{-2}) and leaf length; and, **(C)** total and rhizome carbon content and shoot density (m^{-2}).

Conversely, no correlation has been observed between biometric parameters and soil C stock. Kennedy et al. (2010) observed that carbon inputs from seagrass meadows into soils accounts for around 50% of total C. Nevertheless, other authors obtained lower estimates. Samper-Villarreal et al. (2016) observed that the meadow's carbon contribution to the substrate depended on the environment's physical characteristics (e.g., water turbidity and depth), ranging from 11.4% to 44.5%, while Röhr et al. (2016) observed that inputs from *Z. marina* explained 10.9% of variations in the substrate's carbon stock.

Blue Carbon in Seagrass Meadow Soils

Carbon stock values in soils of *Z. marina* meadows were 41.7 Mg C in O Baño, 3.36 Mg C in Castelo San Felipe, 1.45 Mg C in Segaño, and 0.39 Mg C in San Felipe Núcleo, which adds up to a total 46.9 Mg C for the whole ria, or 4.11 Mg C ha^{-1} when expressed per unit of area, taking into account the upper 5 cm of the soil layer. Extrapolating to a soil depth of 100 cm and based on the assumption of homogeneous carbon, thus allowing for result standardization (Lavery et al., 2013; Howard et al., 2014; Röhr et al., 2018; Prentice, et al., 2020; Kim et al., 2022), carbon stock was estimated at 82.14 Mg C ha^{-1}.

Soil carbon stock was higher than expected for Eastern Atlantic meadows, as well as for meadows in the Western Atlantic and in most areas of the world (**Table 4**). The sum of carbon contained in *Z. marina* biomass and the carbon stock in soil was 4.48 Mg C ha^{-1} for the upper 5 cm and 82.5 Mg C ha^{-1} for the upper 100 cm, which suggests that the plant portion accounted for 8.25% of total C, a value within the ranges described by other authors (Kennedy et al., 2010; Röhr et al., 2016; Samper-Villarreal et al., 2016).

Our results show great differences between carbon stock in soil and in meadows, suggesting that organic carbon in the substrate can have other origins besides the meadows themselves. The origin of organic carbon in soil can be inferred based on different proxies, such as isotope ratios (δ13C and δ15N) or the C/N ratio; based on these ratios, the marine or continental origin of organic matter in soils and sediments can be inferred (for additional details, see García Moya, 2014; Lamb et al., 2006; Kennedy et al., 2010; Röhr et al., 2018; and Samper-Villarreal et al., 2016; **Figure 5**). According to these ratios, organic carbon in the O Baño meadow could receive continental inputs of organic matter, while in Castelo San Felipe, organic matter seems to have an oceanic origin.

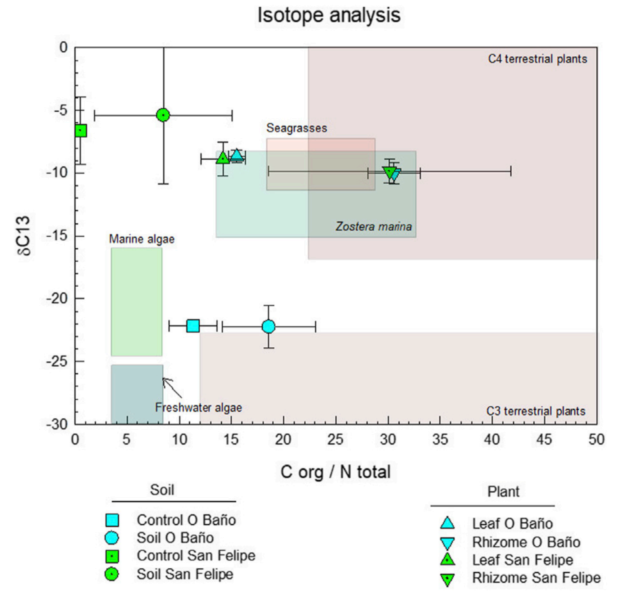

FIGURE 5 | Typical δ13C and C/N ranges for organic matter inputs to coastal environments, together with data obtained from leaves, rhizomes, and soil in both meadows and in control sites. Data compiled from: Lamb et al. (2006), García Moya (2014), and Fourqurean, et al. (1997).

In the O Baño site, the low value obtained for δ13C (−22.25‰) and the high C/N ratio (18.58) were similar to those observed for C3 terrestrial plants, while in Castelo San Felipe, the higher values for δ13C (−5.40‰) and the lower C/N ratio (8.49) seem to suggest a marine source of organic matter (Kennedy et al., 2010) (**Figure 5**). Previous studies have observed that, due to the scarcity of nitrogen and to the fact that the light isotope does not tend to remain, the values of δ^{15}N tend to be higher in sheltered meadows than in exposed ones (Fourqurean et al., 1997; Kennedy et al., 2010; Samper-Villarreal et al., 2016). In our case, the variability observed was minimal, and the correlation was not found, given that values in O Baño (5.3‰) were lower than in Castelo San Felipe (5.7‰).

Finally, it is worth noting that the difference in C/N ratios between O Baño and Castelo San Felipe may also be influenced by their location (Fourqurean et al., 1997). Since O Baño is located towards the innermost area, terrestrial matter tends to be deposited, and its low nitrogen contents (due to the presence of lignin and cellulose) result in high C/N ratios (Fourqurean et al., 1997). The low exposure levels lead to a bottom with a silt-clay granulometry, which promotes a higher degree of recalcitrance, in addition to favoring organic matter stabilization and retention, which could lead to a greater carbon stock. In Castelo San Felipe, the values obtained for the C/N ratio could be influenced by horizontal export processes due to exposure (Lamb et al., 2006; Röhr et al., 2016).

CONCLUSION

Blue carbon associated with *Zostera marina* biomass in the Ría de Ferrol (0.37 Mg C ha^{-1}) was much lower than soil carbon stock (4.11 Mg C ha^{-1}) and clearly lower than values observed in meadows of this species. However, soil carbon stock in the Ría de Ferrol for the upper 100 cm (82.1 Mg C ha^{-1}) was higher than the data described by other authors for most meadows worldwide, likely as a consequence of inputs of organic matter of terrestrial origin received at some sites, as isotope ratios and C/N ratios seem to suggest. However, a more detailed characterization of the composition of organic matter (e.g., by NMR or pyrolysis) could help more accurately confirming its origin.

A high spatial variability was observed between both sites in terms of carbon stock in the substrate, which is related to the characteristics of the environment in which the meadows grow (e.g., degree of exposure to marine currents or content of silt).

Finally, the high correlation observed between certain biometric parameters of the plants, such as leaf length, and biomass carbon stock in meadows can constitute a good predictor to estimate the blue carbon globally associated with *Z. marina* meadows. Further studies in different geographical areas and during different seasons could provide additional data that are essential to develop models for a more accurate estimation of global carbon content in seagrass meadows.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

Conceptualization; MdC, XO, and GD-A; methodology: MdC, XO, GD-A, MCR, RRT, AdSE, and MPS; software: MdC and XO; data curation: MdC, XO, GD-A, MCR, RRT, AdSE, and MPS; writing—original draft preparation: MdC, XO, GD-A, MCR, RRT, AdSE, and MPS; supervision: MdC, XO, and GD-A.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REFERENCES

Bertelli, C. M., Bull, J. C., Cullen-Unsworth, L. C., and Unsworth, R. K. (2021). Unravelling the Spatial and Temporal Plasticity of Eelgrass Meadows. *Front. Plant Sci.* 12, 664523. doi:10.3389/fpls.2021.664523

Cacabelos, E., Quintas, P., Troncoso, J., Bárbara, I., García-Redondo, V., Cremades, J., et al. (2015). "Las praderas marinas de España: una visión general. Cuadro temático 1. La biodiversidad de las praderas españolas, Atlántico norte," in *Atlas de las praderas marinas de España* (IEO/IEL/UICN, Murcia-Alicante-Málaga), 87–91.

Claes, J., Hopman, D., Jaeger, G., and Rogers, M. (2022). *Blue Carbon: The Potential of Coastal and Oceanic Climate Action*. Hong Kong, China: McKinsey & Company.

Duarte, C. M., and Chiscano, C. L. (1999). Seagrass Biomass and Production: a Reassessment. *Aquat. Bot.* 65, 159–174. doi:10.1016/S0304-3770(99)00038-8

Duarte, C. M., Sintes, T., and Marbà, N. (2013). Assessing the CO 2 Capture Potential of Seagrass Restoration Projects. *J. Appl. Ecol.* 50 (6), 1341–1349. doi:10.1111/1365-2664.12155

Fourqurean, J. W., Duarte, C. M., Kennedy, H., Marbà, N., Holmer, M., Mateo, M. A., et al. (2012). Seagrass Ecosystems as a Globally Significant Carbon Stock. *Nat. Geosci.* 5 (7), 505–509. doi:10.1038/ngeo1477

Fourqurean, J. W., Moore, T. O., Fry, B., and Hollibaugh, J. T. (1997). Spatial and Temporal Variation in C: N: P Ratios, δ15N, and δ13C of Eelgrass *Zostera Marina* as Indicators of Ecosystem Processes, Tomales Bay, California, USA. *Mar. Ecol. Prog. Ser.* 157, 147–157. doi:10.3354/meps157147

García Moya, A. (2014). *Estudio de origen y variación temporal de la materia óraganica en los sedimentos de Refugio de Fauna" Laguna Guanaroca", Cienfuegos, Cuba*. Doctoral dissertation (Las Villas: Universidad Central" Marta Abreu").

García-Redondo, V., Bárbara, I., and Díaz-Tapia, P. (2019). Zostera Marina Meadows in the Northwestern Spain: Distribution, Characteristics and Anthropogenic Pressures. *Biodivers. conservation* 28 (7), 1743–1757. doi:10.1007/s10531-019-01753-4

Herr, D., and Landis, E. (2016). "Coastal Blue Carbon Ecosystems," in *Opportunities for Nationally Determined Contributions. Policy Brief* (Gland, Switzerland: IUCN).

Howard, J., Hoyt, S., Isensee, K., Pidgeon, E., and Telszewski, M. (2014). "Coastal Blue Carbon Methods for Assessing Carbon Stocks and Emissions Factors in Mangroves, Tidal Salt Marshes, and Seagrasses Meadows," in *Conservation International, Intergovernmental Oceanographic Commission of UNESCO, International Union for Conservation of Nature* (Arlington, Virginia, USA: UNESCO).

IPCC (2021). *The Physical Science Basis. Contribution of Working. Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate. Change*. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press.

Kennedy, H., Beggins, J., Duarte, C. M., Fourqurean, J. W., Holmer, M., Marbà, N., et al. (2010). Seagrass Sediments as a Global Carbon Sink: Isotopic Constraints. *Glob. Biogeochem. cycles* 24 (4). doi:10.1029/2010gb003848

Kim, S. H., Suonan, Z., Qin, L. Z., Kim, H., Park, J. I., Kim, Y. K., et al. (2022). Variability in Blue Carbon Storage Related to Biogeochemical Factors in Seagrass Meadows off the Coast of the Korean Peninsula. *Sci. Total Environ.* 813, 152680. doi:10.1016/j.scitotenv.2021.152680

Kuo, J., and Den Hartog, C. (2001). "Seagrass Taxonomy and Identification Key," in *Global Seagrass Research Methods*. Editors F. T. Short and R. G. Coles (Amsterdam: Elsevier Science BV), 33, 31–58. doi:10.1016/B978-044450891-1/50003-7

Lamb, A. L., Wilson, G. P., and Leng, M. J. (2006). A Review of Coastal Palaeoclimate and Relative Sea-Level Reconstructions Using δ13C and C/N Ratios in Organic Material. *Earth-Science Rev.* 75 (1-4), 29–57. doi:10.1016/j.earscirev.2005.10.003

Lavery, P. S., Mateo, M. Á., Serrano, O., and Rozaimi, M. (2013). Variability in the Carbon Storage of Seagrass Habitats and its Implications for Global Estimates of Blue Carbon Ecosystem Service. *PloS one* 8 (9), e73748. doi:10.1371/journal.pone.0073748

Mcleod, E., Chmura, G. L., Bouillon, S., Salm, R., Björk, M., Duarte, C. M., et al. (2011). A Blueprint for Blue Carbon: toward an Improved Understanding of the Role of Vegetated Coastal Habitats in Sequestering CO2. *Front. Ecol. Environ.* 9 (10), 552–560. doi:10.1890/110004

Míguez, B. M. (2003). *Descripción dinámica de la circulación en dos Rías Baixas: Vigo y Pontevedra*. Doctoral dissertation, PhD Thesis (Vigo: University of Vigo).

NOAA (2021). EarthDay: Invest in Our Changing Planet Now to Secure a More Livable Future. Available from: https://gml.noaa.gov/ccgg/trends/weekly.html.

Pendleton, L., Donato, D. C., Murray, B. C., Crooks, S., Jenkins, W. A., Sifleet, S., et al. (2012). Estimating Global "Blue Carbon" Emissions from Conversion and Degradation of Vegetated Coastal Ecosystems. *PLoS One* 7 (9), e43542. doi:10.1371/journal.pone.0043542

Prentice, C., Poppe, K. L., Lutz, M., Murray, E., Stephens, T. A., Spooner, A., et al. (2020). A Synthesis of Blue Carbon Stocks, Sources, and Accumulation Rates in Eelgrass (Zostera Marina) Meadows in the Northeast Pacific. *Glob. Biogeochem. Cycles* 34 (2), e2019GB006345. doi:10.1029/2019gb006345

Röhr, M. E., Boström, C., Canal-Vergés, P., and Holmer, M. (2016). Blue Carbon Stocks in Baltic Sea Eelgrass (*Zostera marina*) Meadows. *Biogeosciences* 13 (22), 6139–6153. doi:10.5194/bg-13-6139-2016

Röhr, M. E., Holmer, M., Baum, J. K., Björk, M., Boyer, K., Chin, D., et al. (2018). Blue Carbon Storage Capacity of Temperate Eelgrass (*Zostera Marina*) Meadows. *Glob. Biogeochem. Cycles* 32 (10), 1457–1475. doi:10.1029/2018gb005941

Samper-Villarreal, J., Lovelock, C. E., Saunders, M. I., Roelfsema, C., and Mumby, P. J. (2016). Organic Carbon in Seagrass Sediments Is Influenced by Seagrass Canopy Complexity, Turbidity, Wave Height, and Water Depth. *Limnol. Oceanogr.* 61 (3), 938–952. doi:10.1002/lno.10262

Short, F. T., and Neckles, H. A. (1999). The Effects of Global Climate Change on Seagrasses. *Aquat. Bot.* 63 (3-4), 169–196. doi:10.1016/s0304-3770(98)00117-x

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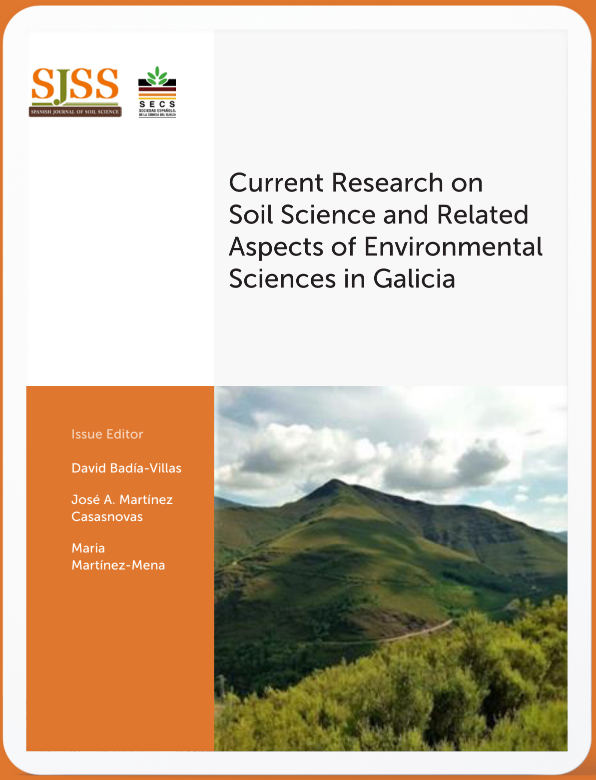

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