



## Article

# The Effect of Clarithromycin Toxicity on the Growth of Bacterial Communities in Agricultural Soils

Laura Rodríguez-González, Vanesa Santás-Miguel \*, Claudia Campillo-Cora, Manuel Arias-Estévez   
and David Fernández-Calviño 

Área de Edafología e Química Agrícola, Departamento de Biología Vexetal e Ciencia do Solo, Facultade de Ciencias, Universidade de Vigo, Campus As Lagoas, s/n, 32004 Ourense, Spain; laura.rodriguez.gonzalez@uvigo.es (L.R.-G.); ccampillo@uvigo.es (C.C.-C.); mastevez@uvigo.es (M.A.-E.); davidfc@uvigo.es (D.F.-C.)

\* Correspondence: vsantas@uvigo.es

**Abstract:** The presence of antibiotics in different environmental matrices is a growing concern. The introduction of antibiotics into the soil is mainly due to sewage treatment plants. Once in the soil, antibiotics may become toxic to microbial communities and, as a consequence, can pose a risk to the environment and human health. This study evaluates the potential toxicity of the antibiotic clarithromycin (CLA) in relation to the bacterial community of 12 soils with different characteristics. Bacterial community growth was evaluated in soils spiked in the laboratory with different concentrations of CLA after 1, 8, and 42 incubation days. The results indicated that the addition of clarithromycin to the soil may cause toxicity in the bacterial communities of the soil. In addition, it was observed that toxicity decreases between 1 and 8 incubation days, while the bacterial community recovers completely in most soils after 42 incubation days. The results also show that soil pH and effective cation exchange capacity may influence CLA toxicity.

**Keywords:** human use antibiotic; macrolide; leucine incorporation; soil interaction; inhibition



**Citation:** Rodríguez-González, L.; Santás-Miguel, V.; Campillo-Cora, C.; Arias-Estévez, M.; Fernández-Calviño, D. The Effect of Clarithromycin Toxicity on the Growth of Bacterial Communities in Agricultural Soils. *Processes* **2021**, *9*, 1303. <https://doi.org/10.3390/pr9081303>

Academic Editors: Marco Race and Juan Francisco García Martín

Received: 31 May 2021  
Accepted: 23 July 2021  
Published: 28 July 2021

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## 1. Introduction

The consumption of antibiotics to treat human diseases has increased considerably worldwide [1], reaching between 100,000 and 200,000 tons per year [2]. Antibiotics are poorly metabolized in the human digestive tract; therefore, a high percentage of the antibiotics consumed (30–90%) are excreted in urine and/or feces [3] either as the original compound and/or as secondary metabolites [4]. The intensive use, both domestic and in hospitals, of these substances causes a high presence of antibiotics in wastewater destined for wastewater treatment plants (WWTPs), where they are only partially eliminated [5–8]. In addition, pharmaceutical products that enter the wastewater plants tend to accumulate in sewage sludge [9]. Therefore, WWTPs become the main sources of human antibiotics released into the environment [10–12]. The antibiotics present in the WWTPs may be released into the soil through two routes: liquid effluents used for agricultural irrigation [13] and solid effluents (sewage sludge) used as fertilizers. The amendment of agricultural soils with sewage sludge is a widespread and approved practice in many jurisdictions in Europe [14] because it improves soil fertility [15]. However, there is a potential risk in using sewage sludge as an organic amendment for agricultural soils, as it contains various inorganic and organic contaminants, including antibiotics for human use [16–19].

Once in the soil, antibiotics can affect non-target organisms such as bacterial communities [20]. Moreover, risks arising from the arrival of antibiotics in the soil lie in their inhibitory effect on the growth of natural bacterial communities [21]. The effect of antibiotics on soil bacterial communities may also affect their structure and diversity [22–24] and also their environmental functions [22,25–32]. Moreover, the toxicity exerted by organic compounds on soil microbial communities may be dependent of their availability,

which may be highly affected by the physico-chemical characteristics of soils such as pH or organic matter content [33].

Macrolides are a widely used group of antibiotics (the second highest in Europe) [34], and the World Health Organization has classified them as critical antimicrobials of the highest priority [35]. Within the macrolides group, clarithromycin (6-O-methylerythromycin) is one of the most prescribed in human medicines [36]. Clarithromycin is a stable semisynthetic acid antibiotic with a broad spectrum of activity [37]. Previous studies on the effect of clarithromycin on bacterial communities focused mainly on sewage sludge treatment processes [38–40]. However, no studies have been found on clarithromycin's effects on the structure and/or function of soil bacterial communities.

In view of this background, this study has hypothesized that clarithromycin may have toxic effects on soil bacterial communities and the toxicity may be dependent on the characteristics of soils. Therefore, the main objective of this study is to evaluate the effect of soil pollution with clarithromycin on the growth of bacterial communities in soils with different general characteristics. For this purpose, 12 agricultural soils with different characteristics in terms of organic carbon and pH were selected and spiked with eight different concentrations of clarithromycin. The results of this study may provide relevant information on the potential effects of clarithromycin pollution on soil bacterial communities' growth and tolerance.

## 2. Materials and Methods

### 2.1. Chemicals and Soil Samples Used

Clarithromycin (CAS: 81103-11-9, 95% purity; CLA) and talc, (CAS 14807-96-6) were supplied by Sigma-Aldrich (Steinheim, Germany). Twelve soil samples, collected from different areas of Galicia (NW Iberian Peninsula), were selected from a larger pool of soils in order to select soil samples that show high variability in their properties such as pH and organic carbon. The selected soils have not been previously treated with antibiotics. On each sampling site 10–20 sub-samples were taken in the soil surface horizon (0–20 cm) using an Edelman probe, and were subsequently mixed in one composite sample. Then, the soil samples were air-dried, sieved through a 2 mm mesh, and stored in polyethylene bottles until analysis. Soil characteristics were determined following standard methods [41]. Soil pH was determined in water ( $\text{pH}_W$ ) and in 0.1 M KCl ( $\text{pH}_{\text{KCl}}$ ) (soil ratio: 1:2.5), using a combined glass electrode. Organic carbon and total nitrogen were determined by elemental analysis in a LECO CHN-1000 (LECO Corporation, St. Joseph, MI, USA). In this study, dissolved organic carbon (DOC) was analyzed using distilled water as the extraction solution (soil/water ratio, 1:10) and measuring it in a total organic carbon (TOC) analyzer. Particle size distribution (texture) was analyzed with wet sieving followed by the pipette method. Exchangeable basic cations (Ca, Mg, Na, and K) were extracted with 0.2 M  $\text{NH}_4\text{Cl}$  [42], while exchangeable Al was extracted with 1 M KCl [43], and then determined by flame atomic absorption (Ca, Mg, and Al) or emission spectroscopy (Na and K). The effective cation exchange capacity (eCEC) was estimated as the sum of the exchangeable basic cations and Al. Non-crystalline Fe and Al oxides ( $\text{Fe}_o$  and  $\text{Al}_o$ ) were extracted with 0.2 M ammonium oxalate-oxalic acid [44]. All samples were analyzed by triplicate.

### 2.2. Experimental Design

The 12 soil samples were moistened up to 60–80% of water holding capacity and incubated at 22 °C in darkness for 15 days, enough time to recover the microbial activity and soil bacterial community growth stabilization [45]. After the incubation period, the 12 soils were spiked with clarithromycin (by triplicate) using different doses in order to achieve the following eight concentrations: 0, 0.49, 1.95, 7.81, 31.25, 125, 500, and 2000  $\text{mg kg}^{-1}$  of soil. The concentrations were selected in order to obtain adequate short-term dose-response curves, allowing the estimation of toxicity indices in a reliable way [46]. These concentrations were also satisfactorily used in previous studies testing the effects of different tetracycline antibiotics on soil bacterial communities [31,32]. Clarithromycin was

added to soil using talc as a carrier for equalizing the amount of dry material added to each microcosm and facilitating the mixture with the soil [47]. The mixtures of antibiotic and soil obtained resulted in a total of 288 microcosms (12 soils  $\times$  8 concentrations  $\times$  3 replications). Once the soils became spiked with clarithromycin, the soil microcosms were incubated at 22 °C in darkness and the bacterial community growth was determined after 1, 8, and 42 days. These incubation times have been selected to estimate short-term (immediate toxicity, day 1), medium-term (day 8) and long-term (day 42) toxicity. In general, 42 days of incubation is enough time for the stabilization of the soil bacterial communities after an impact [48].

### 2.3. Estimation of Bacterial Community Growth

The bacterial community growth was estimated using the leucine incorporation technique [49,50]. Briefly, 1 g of soil (fresh weight) was mixed with 10 mL distilled water using a multivortex shaker at maximum intensity for 3 min, followed by low-speed centrifugation at  $1000 \times g$  for 10 min to create a bacterial suspension in the supernatant. An aliquot (1 mL) of this suspension was transferred to 2 mL microcentrifugation tubes. Then, 2  $\mu$ L [ $^3$ H]Leu (3.7 MBq ml $^{-1}$  and 0.574 TBq mmol $^{-1}$ ; Perkin Elmer, Waltham, MA, USA) was added with non-labeled Leu to each tube, resulting in 300 nM Leu in the bacterial suspensions. After incubation for 2 h at 22 °C, growth was stopped with 75  $\mu$ L 100% trichloroacetic acid. Finally, the bacteria in the microcentrifugation tubes were washed as described by Bååth et al. [50] and radioactivity determined using scintillation liquid counting (Tri-Carb 2810 TR, Perkin Elmer). This methodology was previously used for other antibiotics such as streptomycin [51], sulfadiazine [52] and tetracycline antibiotics [31,32,51].

### 2.4. Data Analysis

The resulting bacterial community growth data were normalized dividing each value by mean control values (without clarithromycin) for each soil sample. The logarithm of added clarithromycin concentration that inhibited 50% of bacterial community growth (Log IC $_{50}$ ) was estimated for each soil using the following logistic model [53–55] (1):

$$Y = c/[1 + e^{b(a-x)}], \quad (1)$$

where  $Y$  is the Leu incorporation (bacterial community growth) determined for each added clarithromycin concentration,  $x$  is the logarithmic value of the added clarithromycin concentration,  $a$  is the value of Log IC $_{50}$ ,  $b$  is a parameter related to the slope of the inhibition curve, and  $c$  is the bacterial growth rate observed in the control sample (antibiotic-free). High Log IC $_{50}$  values indicate low clarithromycin toxicity on bacterial growth, whereas low Log IC $_{50}$  values indicate high clarithromycin toxicity. Moreover, Log IC $_{10}$  values (the logarithm of added clarithromycin concentration that inhibited 10% of bacterial community growth) were calculated using Equation (2), from estimated data using Equation (1):

$$\text{Log IC}_{10} = a - (\ln((c/0.9) - 1))/b. \quad (2)$$

### 2.5. Statistics

The differences between Log IC $_{50}$  or Log IC $_{10}$  values with incubation time (1, 8 and 42 days) were checked using a paired t-test, while the relationships between soil properties and clarithromycin toxicity were studied using the Pearson correlation. SPSS Statistics 21.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. Figures were drawn using the KaleidaGraph software (Synergy Software, Reading, PA, USA).

## 3. Results

### 3.1. General Characteristics of Soils

The general properties of soil samples are shown in Table 1. Briefly, these soil samples present pH values measured in water (pH $_W$ ) between  $4.1 \pm 0.1$  and  $6.1 \pm 0.2$ , and pH values measured in 1 M KCl (pH $_{KCl}$ ) between  $3.7 \pm 0.2$  and  $5.3 \pm 0.2$ . The organic carbon

contents and nitrogen contents vary between  $0.6 \pm 0.2\%$  and  $6.80 \pm 0.3\%$  and  $0.1 \pm 0.0$  and  $0.6 \pm 0.3\%$ , respectively. The DOC measured in soils samples ranged between  $121 \pm 10$  and  $634 \pm 47 \text{ mg kg}^{-1}$ . The studied soils present different textures and show sand content between  $34 \pm 2\%$  and  $81 \pm 7\%$ , silt content between  $10 \pm 0\%$  and  $38 \pm 3\%$ , and clay content between  $9 \pm 0\%$  and  $28 \pm 2\%$ . The effective cation exchange capacity (eCEC) values varied between  $3.2 \pm 0.1$  and  $37.2 \pm 1.3 \text{ cmol}_c \text{ kg}^{-1}$ . Lastly,  $\text{Al}_o$  and  $\text{Fe}_o$  values ranged between  $0.7 \pm 0.0$  and  $8.7 \pm 1.3 \text{ g kg}^{-1}$ , and between  $1.3 \pm 0.1$  and  $8.9 \pm 0.3 \text{ g kg}^{-1}$ , respectively.

**Table 1.** General characteristics of studied soils mean value  $\pm$  standard error ( $n = 3$ ).

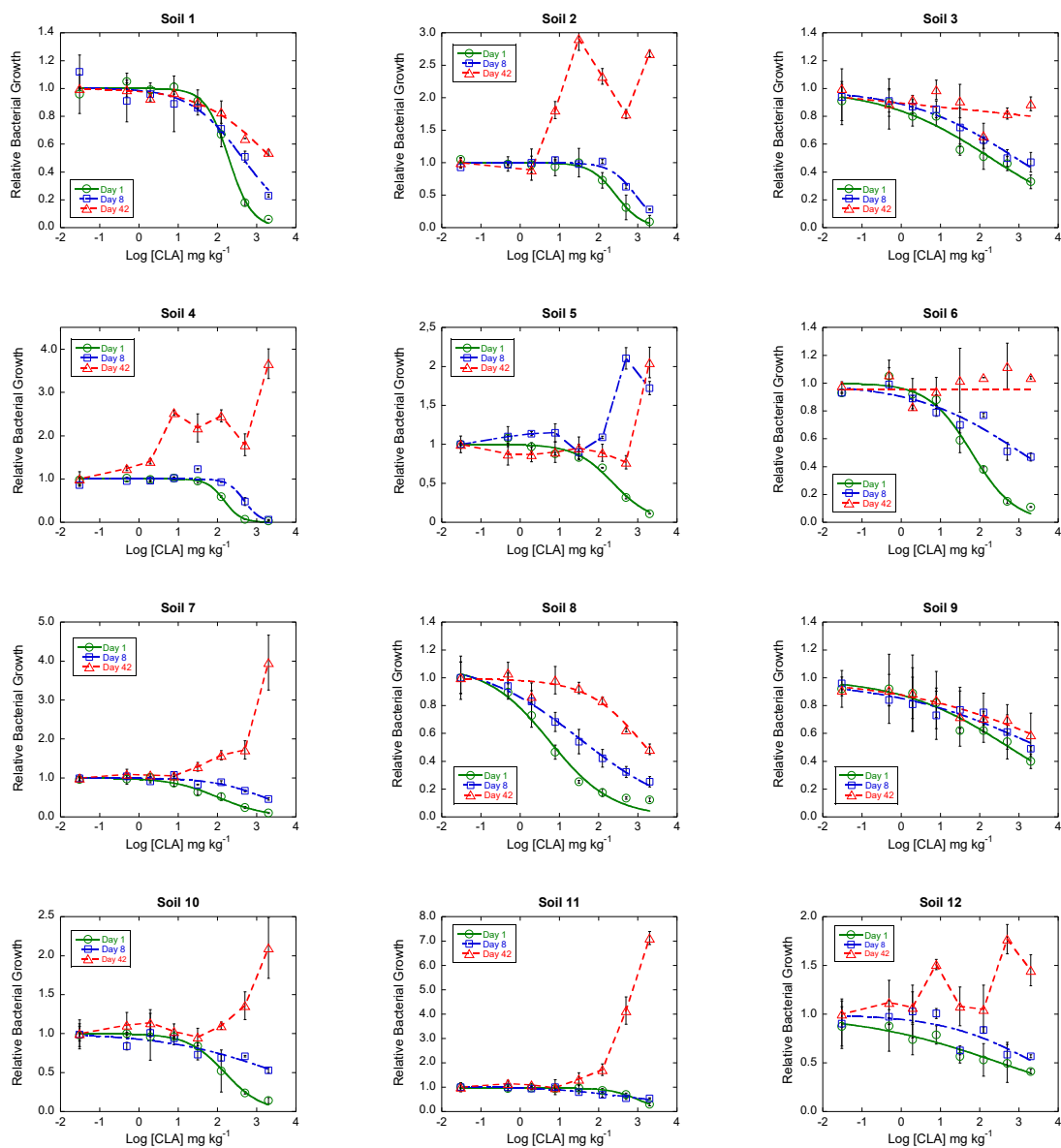
Soil	pH <sub>W</sub>	pH <sub>KCl</sub>	C (%)	N (%)	eCEC (cmol <sub>c</sub> kg <sup>-1</sup> )	DOC (mg kg <sup>-1</sup> )	Sand (%)	Silt (%)	Clay (%)	Fe <sub>o</sub> (g kg <sup>-1</sup> )	Al <sub>o</sub> (g kg <sup>-1</sup> )
1	5.6 $\pm$ 0.0	4.2 $\pm$ 0.1	0.6 $\pm$ 0.2	0.1 $\pm$ 0.0	6.0 $\pm$ 0.2	120.9 $\pm$ 10	61 $\pm$ 5	25 $\pm$ 1	13 $\pm$ 0	1.3 $\pm$ 0.1	0.7 $\pm$ 0.0
2	5.6 $\pm$ 0.1	4.9 $\pm$ 0.1	3.7 $\pm$ 0.0	0.3 $\pm$ 0.2	19.5 $\pm$ 0.4	263.3 $\pm$ 15	69 $\pm$ 4	17 $\pm$ 1	14 $\pm$ 1	3.5 $\pm$ 0.1	5.8 $\pm$ 0.2
3	5.6 $\pm$ 0.2	4.8 $\pm$ 0.1	1.6 $\pm$ 0.1	0.2 $\pm$ 0.1	6.0 $\pm$ 0.0	243.1 $\pm$ 13	44 $\pm$ 3	34 $\pm$ 2	23 $\pm$ 1	2.2 $\pm$ 0.0	1.0 $\pm$ 0.0
4	6.1 $\pm$ 0.2	5.3 $\pm$ 0.2	2.8 $\pm$ 0.1	0.2 $\pm$ 0.1	37.2 $\pm$ 1.3	265.0 $\pm$ 19	61 $\pm$ 5	21 $\pm$ 1	18 $\pm$ 1	3.6 $\pm$ 0.2	4.4 $\pm$ 0.1
5	5.7 $\pm$ 0.0	4.9 $\pm$ 0.0	4.8 $\pm$ 0.2	0.4 $\pm$ 0.1	17.4 $\pm$ 0.9	332.9 $\pm$ 24	58 $\pm$ 4	20 $\pm$ 1	21 $\pm$ 2	2.8 $\pm$ 0.2	5.3 $\pm$ 0.3
6	5.5 $\pm$ 0.2	4.2 $\pm$ 0.2	6.6 $\pm$ 0.2	0.5 $\pm$ 0.3	10.5 $\pm$ 0.4	350.6 $\pm$ 16	66 $\pm$ 4	13 $\pm$ 1	21 $\pm$ 1	5.5 $\pm$ 0.1	3.1 $\pm$ 0.1
7	4.8 $\pm$ 0.1	4.4 $\pm$ 0.1	3.1 $\pm$ 0.0	0.4 $\pm$ 0.3	5.2 $\pm$ 0.3	633.9 $\pm$ 47	70 $\pm$ 5	18 $\pm$ 1	12 $\pm$ 0	1.8 $\pm$ 0.0	2.1 $\pm$ 0.1
8	4.6 $\pm$ 0.1	4.2 $\pm$ 0.3	2.4 $\pm$ 0.1	0.3 $\pm$ 0.1	3.2 $\pm$ 0.1	336.2 $\pm$ 28	77 $\pm$ 4	14 $\pm$ 1	9 $\pm$ 0	1.6 $\pm$ 0.0	2.2 $\pm$ 0.2
9	4.1 $\pm$ 0.1	3.7 $\pm$ 0.2	3.3 $\pm$ 0.1	0.3 $\pm$ 0.0	4.3 $\pm$ 0.2	599.8 $\pm$ 39	81 $\pm$ 7	10 $\pm$ 0	9 $\pm$ 0	2.3 $\pm$ 0.1	1.8 $\pm$ 0.0
10	5.2 $\pm$ 0.2	4.7 $\pm$ 0.1	3.2 $\pm$ 0.2	0.4 $\pm$ 0.2	4.1 $\pm$ 0.2	327.1 $\pm$ 25	55 $\pm$ 2	28 $\pm$ 2	17 $\pm$ 1	8.9 $\pm$ 0.5	3.1 $\pm$ 0.1
11	5.6 $\pm$ 0.3	5.1 $\pm$ 0.2	3.4 $\pm$ 0.1	0.4 $\pm$ 0.1	11.2 $\pm$ 0.8	356.4 $\pm$ 23	45 $\pm$ 2	33 $\pm$ 2	22 $\pm$ 1	8.1 $\pm$ 0.2	3.8 $\pm$ 0.1
12	5.1 $\pm$ 0.0	4.5 $\pm$ 0.0	6.8 $\pm$ 0.3	0.6 $\pm$ 0.3	5.5 $\pm$ 0.3	318.4 $\pm$ 21	34 $\pm$ 2	38 $\pm$ 3	28 $\pm$ 2	8.9 $\pm$ 0.3	8.7 $\pm$ 0.4

pH<sub>W</sub> is pH measured in water; pH<sub>KCl</sub> is pH measured in 0.1 M KCl; C is total carbon; eCEC is Cationic Exchange Capacity; DOC is dissolved organic carbon; Al<sub>o</sub>, Fe<sub>o</sub>: extracted with ammonium oxalate (mg kg<sup>-1</sup>).

### 3.2. Toxicity of Clarithromycin on the Growth of Soil Bacterial Communities

The inhibition curves obtained with the addition of clarithromycin to the 12 studied soils after 1, 8, and 42 incubation days are shown in Figure 1. As a general trend, the soil samples showed sigmoid dose–response curves after 1 and 8 incubation days, i.e., low clarithromycin antibiotics doses did not inhibition bacterial growth, but at high doses, the extent of inhibition increased with the dose. The dose–response curves obtained for the 12 soils for 1 and 8 incubation days showed a shift to the right with time, i.e., the toxicity exerted by clarithromycin on soil bacterial communities decreased with time. In addition, there is a total recovery and even an increase in bacterial growth in many of the soils studied to day 42 of incubation.

The dose–response curves to all soils for 1 and 8 incubation days were generally well described by the logistic model (Equation (1)), with  $R^2$  values ranged between 0.925 and 0.998 (mean  $R^2 = 0.978$ ) for 1 day and from 0.682 to 0.993 (mean  $R^2 = 0.900$ ) for day 8. Only one curve (soil 5, day 8) was not well described (Table 2). However, for day 42, only three soils were well described by the logistic model (Table 2), with  $R^2$  values between 0.932 and 0.976 (mean  $R^2 = 0.947$ ). Table 2 also shows estimated Log IC<sub>50</sub> and Log IC<sub>10</sub> values for each soil and incubation time tested from inhibition curves (Table 2). The Log IC<sub>50</sub> values estimated after 1 day of incubation of clarithromycin ranged between  $0.75 \pm 0.18$  and  $3.02 \pm 0.04$  (mean = 2.22); after 8 days of incubation between  $1.52 \pm 0.17$  and  $3.57 \pm 0.51$  (mean = 2.96); and after 42 days of incubation ranged between  $3.22 \pm 0.15$  and  $4.12 \pm 0.34$  (mean = 3.57). Similar trends were found for Log IC<sub>10</sub> values. Thus, after 1 day of incubation the Log IC<sub>10</sub> ranged between  $-1.48$  and  $2.07$  (mean = 0.64); after 8 days between  $-0.89$  and  $2.23$  (mean = 0.75) and after 42 days of incubation the Log IC<sub>10</sub> values ranged between  $-0.45$  and  $1.60$  (mean = 0.86). These results, together with the graphical analysis, show that the toxicity of clarithromycin decreases with incubation time, i.e., the toxicity of clarithromycin on soil bacterial communities is greater at day 1 of incubation (low Log IC<sub>50</sub> values) than at day 42 (high Log IC<sub>50</sub> values). This is evident for 42 days, when most of the soils totally recover the bacterial growth (Figure 1). The Log IC<sub>50</sub> values were used to observe whether there are significant differences between 1 and 8 incubation days by performing the paired t-tests, showing that Log IC<sub>50</sub> values are significantly different ( $p < 0.05$ ;  $t = -6.338$ ). This result confirms that the toxicity exerted by clarithromycin on the growth of bacterial communities decrease with time.



**Figure 1.** Relative bacterial community growth as a function of clarithromycin (CLA) concentration in the 12 soils studied, after 1, 8, and 42 days of incubation.

**Table 2.** Toxicity exerted by clarithromycin on the growth of soil bacterial communities estimated as Log IC<sub>10</sub> and Log IC<sub>50</sub> values (mean values with the standard error range in brackets) after 1, 8, and 42 days of incubation. R<sup>2</sup> values represent the coefficients of determination for model fits used for Log IC<sub>50</sub> determination.

Soil	Day 1			Day 8			Day 42		
	Log IC <sub>50</sub> ± Error	Log IC <sub>10</sub>	R <sup>2</sup>	Log IC <sub>50</sub> ± Error	Log IC <sub>10</sub>	R <sup>2</sup>	Log IC <sub>50</sub> ± Error	Log IC <sub>10</sub>	R <sup>2</sup>
1	2.29 ± 0.04	1.63	0.994	2.64 ± 0.14	1.20	0.954	3.37 ± 0.10	1.43	0.976
2	2.45 ± 0.05	1.72	0.989	2.94 ± 0.07	2.23	0.962			
3	2.28 ± 0.31	−0.77	0.960	2.92 ± 0.17	−0.17	0.976			
4	2.17 ± 0.02	1.70	0.998	2.68 ± 0.10	2.23	0.917			
5	2.37 ± 0.09	1.28	0.979						
6	1.80 ± 0.10	0.58	0.984	3.07 ± 0.33	0.03	0.900			
7	2.02 ± 0.10	0.70	0.985	3.19 ± 0.17	1.76	0.897			
8	0.75 ± 0.18	−0.50	0.983	1.52 ± 0.17	−0.28	0.993	3.22 ± 0.15	1.60	0.932
9	2.74 ± 0.27	−0.35	0.953	3.57 ± 0.51	−0.89	0.894	4.12 ± 0.34	−0.45	0.934
10	2.19 ± 0.05	1.14	0.972	3.53 ± 0.45	0.47	0.804			
11	3.02 ± 0.04	2.07	0.989	3.09 ± 0.26	0.86	0.924			
12	2.52 ± 0.61	−1.48	0.925	3.42 ± 0.55	0.79	0.682			



### 3.3. Effect of Soil Characteristics on Clarithromycin Toxicity

A Pearson correlation analysis was carried out to check the relationships between the toxicity exerted by clarithromycin on soil bacterial communities (Log IC<sub>50</sub> and Log IC<sub>10</sub> values) and the properties of the studied soils (Table 3). No significant correlation was found between the soil properties and Log IC<sub>50</sub> values at any incubation time. However, Log IC<sub>10</sub> values were significantly ( $p < 0.05$ ) and positively correlated with pH measured in water (pH<sub>W</sub>) and in KCl (pH<sub>KCl</sub>), and also with the effective cation exchange capacity (eCEC) after 8 incubation days (Table 3), i.e., the clarithromycin toxicity decreases when the pH<sub>W</sub>, pH<sub>KCl</sub>, and eCEC increase. After 1 day of incubation the correlation coefficients showed the same sign for those variables as after 8 days, but with a different  $p$ -value ( $p < 0.1$ ). The same trend was observed after 42 days, despite having only data from three soils.

**Table 3.** Pearson correlation coefficients between soil characteristics and Log IC<sub>50</sub>/IC<sub>10</sub> values estimated after 1, 8, and 42 days of incubation.

	Sand	Silt	Clay	C	N	pH <sub>W</sub>	pH <sub>KCl</sub>	TOC	CIC <sub>e</sub>
Log IC <sub>50</sub> Day 1 (n = 12)	−0.438	0.452	0.336	0.070	0.206	0.172	0.285	0.044	0.055
Log IC <sub>50</sub> Day 8 (n = 11)	−0.308	0.250	0.356	0.401	0.474	−0.081	−0.026	−0.111	0.368
Log IC <sub>50</sub> Day 42 (n = 3)	−0.672	0.600	0.832	0.059	0.045	0.286	0.564	0.524	0.813
Log IC <sub>10</sub> Day 1 (n = 12)	0.137	−0.136	−0.154	−0.256	−0.275	0.569 *	0.501 *	−0.232	0.532 *
Log IC <sub>10</sub> Day 8 (n = 11)	−0.115	0.122	0.065	−0.064	−0.176	0.604 **	0.640 **	−0.233	0.656 **
Log IC <sub>10</sub> Day 42 (n = 3)	−0.596	0.651	0.434	−0.708	−0.608	0.705	0.997 **	−0.858	0.042

\*  $p < 0.1$ , \*\*  $p < 0.05$ .

## 4. Discussion

The addition of clarithromycin to the studied soil samples inhibited the growth of bacterial communities in the soil after one incubation day, showing clear dose–response curves similar to those found by other authors with other antibiotics such as tetracyclines and streptomycin [31,32,51]. The toxicity exerted by clarithromycin on the growth of bacterial communities is time dependent. Thus, after eight incubation days the toxicity is still present for most of the soils, but with a lower magnitude than after one incubation day. After 42 days, the bacterial community growth was totally recovered in most of the studied soils. This type of recovery has been previously observed in soils contaminated with other pollutants such as Cu [48,56] and Zn. [48]. Therefore, the clarithromycin toxicity effect on the growth of soil bacterial communities has a low persistence in the soil, contrary to the behavior found for other antibiotics such as tetracyclines [31,32] or sulfadiazine [52], and other organic substances such as propiconazole [57] or terbutryn [58].

The decrease in clarithromycin toxicity over time may be due to different reasons. First, clarithromycin can be adsorbed by soil components [59] and thus be less bioavailable for bacterial communities. Sorption processes may be increased with time in soils, causing an aging effect [60], and therefore reduce the toxicity exerted by clarithromycin on soil bacteria. A second possibility is clarithromycin degradation in soil, a time-dependent process. The half-lives of antibiotics in soils depend on the initial concentration of the antibiotics and the physical–chemical and biological properties of soils [24,61–63]. It should also be noted that the degradation of clarithromycin in the soil may also be caused by soil microorganisms, since the biodegradation of clarithromycin has been demonstrated in several studies [64–67]. Different authors have found different half-lives of clarithromycin in soils. Thus, Kodešová et al. [63] observed that the lowest value of clarithromycin half-life obtained for the different soils was 88.9 days, whereas Topp et al. [67] obtained half-life values of 37 days in soils where there was no previous application of clarithromycin, although the half-life times decreased to 16 and 10 days in soils with a history of application of antibiotics of 0.1 and 10 mg kg<sup>−1</sup>. In general, clarithromycin may show a high persistence in the soil for 42 days, especially for high antibiotic concentrations. The third option is

the development of tolerance to clarithromycin by soil bacteria, since the presence of any contaminant in the soil can induce bacterial community tolerance to that contaminant [68]. The induction of bacterial community tolerance to antibiotics depends on factors such as the amount of antibiotic applied and the frequency of application [69]. Few works have studied the effect of clarithromycin on soil microorganisms. Thus, Lau et al. [70] studied the presence of resistance genes derived from the addition of clarithromycin in soils, showing that low concentrations ( $0.1 \text{ mg kg}^{-1}$ ) didn't increase the abundance of clarithromycin-associated resistance genes. However, adding  $10 \text{ mg kg}^{-1}$ , the abundance of resistance genes in the soil increased.

In some of the soils, further to a full recovery of bacterial growth after 42 incubation days, exponential increases of bacterial community growth were observed. These exponential increases may be due to the fact that antibiotics can be used by soil bacteria as sources of carbon and nitrogen [71–73], since these nutrients may be limiting factors for soil bacterial growth [74]. In addition, the introduction of antibiotics into soil microcosm may cause the death of a large number of bacteria, dead bacteria being a possible future source of energy for bacterial groups that resist antibiotic concentrations in the soil [48]. Reischke et al. [75] studied the effect of the addition of glucose (as a labelled carbon source) on the microbial activity of the soil and observed an increase in bacterial growth in the soil. Since clarithromycin may be bacteriostatic or bactericidal depending on the organism and drug concentration, this effect may be present, especially for higher clarithromycin concentrations.

Soil properties did not show a clear effect of the toxicity exerted by clarithromycin on the growth of soil bacterial communities based on  $\text{Log IC}_{50}$  values. However, based on  $\text{Log IC}_{10}$  values, there is a relationship between clarithromycin toxicity and soil pH and eCEC. Clarithromycin speciation is strongly pH dependent, occurring predominantly in cationic form at soil pH values (4.1 to 6.1) [76]. Since soil charge is pH dependent and the negative charge increased with pH [77,78], clarithromycin adsorption in the soil may increase with pH, as described previously for humic substances [79]. This behavior is consistent with the results obtained in this study, showing that the increase in pH values may reduce the toxicity that clarithromycin exerts on the growth of soil bacterial communities. Furthermore, there is also a positive correlation between  $\text{Log IC}_{10}$  values and eCEC, i.e., high eCEC values caused lower toxicity of clarithromycin on the growth bacterial communities than low eCEC values. High eCEC values allow high antibiotic adsorption on soils, especially given that antibiotic–soil interactions occur mainly through cation exchange. This type of interaction has been observed by Sibley and Pedersen [76] in a study of the sorption of clarithromycin by humic acids. Moreover, similar results were observed by Kodešová, et al. [59], who found a positive correlation of clarithromycin adsorption with pH and with variables related to the exchange complex.

After one incubation day,  $\text{Log IC}_{50}$  values ranged between 0.75 and 3.02 (Table 2), i.e.,  $\text{IC}_{50}$  ranged between 5.6 and  $1050 \text{ mg kg}^{-1}$  of clarithromycin in soils. Considering that the concentrations of clarithromycin found in soils are between 1 and  $100 \text{ } \mu\text{g kg}^{-1}$  [80], much lower than  $\text{IC}_{50}$  values, these soils present a low risk of high clarithromycin toxicity in relation to soil bacterial growth. However,  $\text{Log IC}_{10}$  values ranged between  $-1.48$  and 2.07 (Table 2), i.e.,  $\text{IC}_{10}$  values ranged between 0.03 and  $120 \text{ mg kg}^{-1}$  of clarithromycin in soils, the lower value being close to those found in soils. Therefore, in some clarithromycin-polluted soils, low toxicity may be present in soil bacterial growth.

## 5. Conclusions

The addition of clarithromycin (CLA) to soils has a dose-dependent inhibitory effect on bacterial communities after one incubation day. This effect remained after eight incubation days, but at a low level (i.e., the toxicity decreased), while after 42 incubation days the bacterial community growth was totally recovered in most of the studied soils. In fact, in some of them the growth was higher in clarithromycin-amended soils than in the control after 42 incubation days. No relationships were observed between different soil

characteristics and clarithromycin toxicity at the doses needed to decrease the growth of bacterial communities by 50% (IC<sub>50</sub>) However, Log IC<sub>10</sub> was related to soil pH and effective cation exchange capacity. The higher these parameters were, the lower the clarithromycin toxicity according to Log IC<sub>10</sub> values.

**Author Contributions:** Data curation, L.R.-G. and C.C.-C.; formal analysis, L.R.-G. and C.C.-C.; investigation, L.R.-G., C.C.-C., V.S.-M., M.A.-E. and D.F.-C.; methodology, V.S.-M. and D.F.-C.; project administration, M.A.-E.; supervision, M.A.-E.; writing- original draft, L.R.-G., V.S.-M. and M.A.-E.; writing – review & editing, V.S.-M. and D.F.-C. All authors have read and agreed to the published version of the manuscript.

**Funding:** This study has been funded by the Spanish Ministry of Science, Innovation and Universities through the projects RTI2018-099574-B-C21 and RTI2018-099574-B-C22 (FEDER Funds). David Fernández Calviño holds a Ramón y Cajal contract (RYC-2016-20411) financed by the Spanish Ministry of Economy Industry and Competitiveness. Vanesa Santás Miguel, Claudia Campillo Cora and Laura Rodríguez González hold a pre-doctoral fellowship (ED481A-2020/089, ED401A-2020/084 and ED481A 2021/309, respectively) financed by Xunta de Galicia.

**Conflicts of Interest:** The authors declare no conflict of interest.

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