ISTA 14 - Impact of antibiotics from pig slurry on soil microbial communities, including the basidiomycete *Trametes versicolor*

Running head: Impact of antibiotics from pig slurry on micro-organisms

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Abstract

Livestock slurry containing antibiotics is a source of contamination of agricultural soils, with possible effects on soil function and micro-organisms. Extracellular oxido-reductases and hydrolases from the fungus *T. versicolor* and fungal growth were monitored in liquid cultures in the presence of tetracycline, lincomycine, sulfadiazine and ciprofloxacin for 10 days, in order to assess the suitability of these enzymes as biomarkers. Among the conditions of treatment, statistical analysis demonstrated an increase in manganese-dependent peroxidase after exposure to sulfadiazine at 1 mg/L when compared to the control. Acid phosphatase activity was decreased by lincomycine at 1 or 10 mg/L. Conversely, β-glucosidase activity increased in the presence of this antibiotic at 10 mg/L. In Terrestrial Model Ecosystems spiked with contaminated pig slurry, lincomycine at the concentration of 8 or 80 µg/kg dry soil, and ciprofloxacin at 250 ng/kg dry soil decreased the activity of soil dehydrogenase, when compared to a green slurry treatment, over 28-day incubations. Laccase activity was similarly decreased in the presence of the highest concentration of antibiotics. We determined bacterial and fungal biomasses using Q-PCR. Bacterial biomass was increased in the presence of lincomycine at 80 µg/kg whatever the time of exposure, and to a lesser extent in the presence of ciprofloxacin at 250 ng/kg, but only at day 28. In contrast, both antibiotics, whatever their concentrations, did not modify fungal biomass in soil. In conclusion, we were unable to demonstrate important effects of antibiotics at concentrations found in the agricultural environment.

Keywords: veterinary medicine, pig slurry, enzymes, biomarkers, soil micro-organisms

INTRODUCTION

Antibiotics, which are used worldwide for the treatment of microbial infections, and thus as growth promoters, are administered in large amounts to livestock. Most of these veterinary medecines are excreted from in faeces, which are often used for the fertilization of agricultural soils (see the reviews by Kemper, 2008; Sarmah *et al.*, 2006; Thiele-Bruhn, 2003). Tetracycline, lincomycine, sulfadiazine and enrofloxacin (the later converted to its metabolite ciprofloxacin, Zhou *et al.*, 2008) are compounds frequently used in pig rearing. As a consequence, residues of these chemicals have been discovered in slurry-amended or manured soils in concentrations up to 900,000 ng/kg for tetracycline, 8,500 ng/kg for lincomycine, 1,000 ng/kg for sulfadiazine and 52,000 ng/kg for ciprofloxacin (Kemper, 2008). Their persistence in soils often varies mainly from several days to several months. Although natural antibiotics are released by micro-organisms in soils, unwanted ecotoxicological effects due to exogenous chemicals, such as alteration of microbial turnover processes in soils and spreading of antimicrobial resistance genes among environmental bacteria, can be expected (Kemper, 2008). It is thus important to understand the effects of pharmaceutical antibiotics on soil micro-organisms, particularly concerning lincomycine and ciprofloxacin, whose fates and effects in soils have been poorly studied to date.

One reason for our lack of knowledge is the death of efficient tools to assess the impacts of chemicals on the functional diversity of micro-organisms in soils. It is thus necessary to understand the molecular basis for the enzymatic secretion and to to develop selective and sensitive biomarkers for the ecotoxicity assessment of soils. Fungal liquid cultures allowing high contaminant bioavailability appear suitable for that purpose. The white-rot basidiomycete *Trametes versicolor* has been studied for many years for its suitability to produce biomarkers for soil ecotoxicity assessment (Mougin *et al.*, 2003). For example, its laccases have been shown to be induced after exposure to environmental contaminants such as pesticides and polycyclic aromatic compounds (Mougin *et al.*, 2002). Lebrun *et al.* in 2010 demonstrated that the activities of several enzymatic systems of this fungus were affected after exposure to copper.

Secondly, the standard tests for toxicity assessment of antibiotics are often conducted under unrealistic conditions. As integrated systems, Terrestrial Model Ecosystems, where natural parameters (moisture, temperature…) can be controlled in order to reduce the variability during the assays, offer more realistic manure application conditions to assess both the environmental fate and effects of chemical contaminants on different soil communities (Ghanem *et al.*, 1996; Fernandez *et al.*, 2004, Boleas *et al.*, 2005).

The goals of our programme are to investigate the impact of manure treatment and storage on its antibiotic content, the fate of antibiotics in slurry-amended and manured soils, and the resulting effects of these medicines on the organisms in the topsoil.

In the present study, our specific objectives are 1) to assess the suitability of fungal biomarkers to monitor the effects of antibiotics in liquid cultures and 2) to assess the ecotoxicity of selected antibiotics on the indigenous microflora in soils amended with pig slurry through functional and structural parameters determined in Terrestrial Model Ecosystems. We provide new information concerning the impact of antibiotics on soil micro-organisms in simulated agricultural situations.

MATERIALS AND METHODS

Chemicals and Reagents

Antibiotics and all other high purity chemicals (>99%): e.g. tetracycline (TET), lincomycine (LIN), sulfadiazine (SDZ) and ciprofloxacin (CIP), were purchased from Sigma-Aldrich-Fluka (France). Analytical grade solvents were obtained from Carlo-Erba (France).

Soil characteristics

The silt loam was collected in the 10–20 cm layer of an experimental site (La Cage, INRA, Versailles, France). It comprises 21.6% sand, 61.1% silt, and 17.3% clay. Its content in organic carbon is 0.98%, and its pH_{wat} was 7.1. Its water holding capacity (WHC) was 15.64 ± 0.21% at pF=2.5 and 6.79 ± 0.21% at pF=4.2. The soil was roughly homogenized, sieved at 2 mm, and used without storage for the preparation of the Terrestrial Model Ecosystems (TMEs).

Pig slurry sampling and characterisation

The pig slurry, referred to here as green slurry, was from the quarantine livestock building of the IFIP (French Pig Institute) at Romillé (Brittany, France). The animals were cross-bred from Large White and Landrace and weighed 40-100 kg (fattening pigs). They were fed an unchanging cereal formula consisting of wheat (44%), barley (30%), soybeans (18%), molasses (2%) and amino acids and minerals (6%). They did not receive antibiotics.

The pig slurry resulting from about three months of manure accumulation from 20 pigs was homogenized in the pit (about 25 m^3) using a submersible pump for about 20 minutes. Then 20 litres of slurry were collected and stored at 4°C for one day until use.

The slurry pH (7.5), dry matter content (26.2 +/- 0.1 g/kg), Chemical Oxygen Demand (36 +/- 2 gO_2/L), Total Kjeldahl Nitrogen (3.05 +/- 0.03 g/kg) and Total Ammonia-N (2.3 +/- 0.1 g/kg) were determined according to standard methods (APHA, 1992). All parameters were analysed in

triplicate. These data are in agreement with previous reports on pig slurry characterisation in French husbandry (Burton and Turner, 2003).

Micro-organism and culture conditions

Trametes versicolor ATCC 32745 was grown on the culture medium described by Abadulla *et al.* (2000), containing glucose (10 g/L) and NH_4CL (2.5 g/L) as carbon and nitrogen sources. The medium also contained 0.5 g/L $MgSO_4$ $7H_2O$, 5 g/L yeast extract, 0.1 g/L $CaCl_2$ and 0.5 g/L KCl. $FeSO_4$, $ZnSO_4$, $MnSO_4$ and $CuSO_4$ were added to achieve the final concentration of 0.01 g/L. A mycelial mat on agar plug (10 mm diam.) was inoculated into 10 mL of the culture medium in each 150 mL Erlenmeyer flask. Cultivation was carried out statically in the dark at 25°C for 10 days. Solutions of antibiotics in water (TET, LIN), diméthylformamide (SDZ) and dimethylsulfoxide (CIP) were added to the cultures to give 0.1, 1 and 10 mg/L (100 µL solution per Erlenmeyer flask). Biomass and enzymatic determinations were achieved at the beginning of the incubations, then after 3, 5, 7 and 10 days. Each experiment was performed in triplicate.

Set up of Terrestrial Model Ecosystems

Three days before the beginning of the experiments, each Terrestrial Model Ecosystem (TME) was filled with 1 kg dry soil moistened to 50% of its WHC. The soil was then amended with 25 mL pig slurry to simulate agronomic conditions (equivalent to 30 T dry manure/ha). Slurry was spiked or not with antibiotics. CIP was added to the final concentrations of 1 and 10 µg/L in the slurry, 25 and 250 ng/kg in dry soil. LIN was applied in final concentrations of 320 and 3200 µg/L in slurry, equivalent to 8 and 80 µg/kg in dry soil. After mixing, the amended soil was set in the TMEs, a litter-bag (7 x 7 cm, 2 g dry wheat straw) was inserted at mid-height of the soil, and water was added to achieve 100% WHC. TMEs were incubated in the dark at 18 ± 1°C. Biomass and enzyme activities were measured at the beginning of incubation, then after 7, 14 and 28 days. After 28 days, the bags were removed, washed, dried and the straw was weighted. Each experiment was performed in triplicate.

Enzyme activity measurements

Laccase (LAC; E.C. 1.10.3.2) production was assessed in liquid cultures of *T. versicolor* by measurement of enzymatic oxidation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)(ABTS) at 420 nm (ε = 3.6 x 10^4 cm^{-1} M^{-1}) according to Wolfenden & Wilson (1982). The reaction mixture contained 20 µL extracellular fluid and 980 µL 1 mM ABTS in 0.1 M KH_2PO_4/citric acid buffer (pH 3.0) at 30°C. The buffer solution was saturated with air by bubbling prior to the experiment. Manganese-dependent peroxidase (MNP; E.C. 1.11.1.14) activity was

monitored by the oxidation of 20 µL 5 mM 4-(4-hydroxy-3-methoxyphenyl)-3-buten-2-one at 334 nm, 200 µL extracellular fluid, 20 µL 5 mM $MnSO_4$, 20 µL 16mM hydrogen peroxide in 740 µL 0.1 M KH_2PO_4/citric acid buffer (pH 5.0), as described by Paszczynski *et al.* (1986). One unit of enzyme activity is defined as the amount of enzyme that oxidises one µmol ABTS per min.

In TMEs, we used a protocol adapted from Floch *et al.* (2007) to determine laccase activity. The reaction mixture contained 1mL soil solution (obtained from 1 g soil in 6 mL KH_2PO_4/citric acid buffer pH 3.0), and 600 µl of a 50 mM guaiacol solution. After incubation at 37 °C for 20 min, the mixture was centrifuged at 12000 rpm at 4 °C for 2 min. The formation of tetrameric guaiacol in the supernatant was measured at 470 nm.

Measurement of dehydrogenase activity (DHA; E.C. 1.1.1.1), indicating the level of global metabolic process in the soil, was adapted from Schaefer (1963). 1 mL distilled water and 250 µL aqueous solution of 1,3,5-triphenyltetrazolium chloride (TTC) at 40 g/L were mixed with 1 g fresh soil in 30-mL centrifuge tubes. Controls were prepared without TTC. Incubation was performed at 37°C for 16 hours. After that period, 5 mL ethanol was added to the mixture which was shaken for 5 min in the dark. The medium was incubated at 37°C for 2 hours, and shaked after one hour. Finally, TTC was added to the control and all the mixtures were centrifuged 5 min at 6000 g. The absorbance of the supernatant (red colour in case of formazan formed) was measured at 485 nm. Results were expressed in mU per g dry soil.

Acid phosphatase (ACP; E.C. 3.1.3.2, β-glucosidase (GLU; E.C. 3.2.1.21) activities were measured according to a method adapted from Dick (1997). 16 g soil were shaken in 100 mL distilled water. Then, 125 µL of soil suspension were incubated with 25 µL 0.05 M substrate (4-nitrophenyl phosphate for the phosphatase or 4-nitrophenyl ß-D-galactopyranoside for the glucosidase) at 37°C for one hour in multiwell plates. For each soil sample, a control without substrate was performed. At the end of the incubation, 25 µL of $CaCl_2$ (0.5 M) were added in order to stabilize humic acid, and 100 µL Tris 0,1 M pH12 were used to stop the reaction. The amount of p-nitrophenol (PNP) formed was obtained by the measurement of the supernatant absorbance at 405 nm. Results were expressed in mU per g dry soil.

Biomass determination

Biomass was determined from soil genomic DNA. Total genomic DNA was extracted from 0.5g of frozen soil using the Fast DNA kit for soil (MP Biomedical) and suspended in 100 µL of sterile deionised water. rDNA was then amplified in 20 µL final qPCR mixture containing the following total amounts: 10 ng of total soil DNA, 25 pmoles of each primer, 10 µL of iq-SYBR®Green super Mix (Biorad). For bacterial 16S-rDNA, the forward primer used was S-BCT 5'-CAG GCC TAA CAC ATG CAA GTC-3' (primer 63f from Marchesi *et al.*, 1998) and the reversed primer was AS-

BCT1 5'-CTG CTG CCT CCC GTA GG-3' (primer 341F from Muyzer *et al.*, 1993). For fungal 18S-rDNA, the forward primer was S-FUN1 5'-GGA AAC TCA CCA GGT CCA GA-3' (primer nu-SSU-1196 from Borneman and Hartin, 2000) and the reversed primer was AS-FUN1 5'-ATT GCA ATG CYC TAT CCC CA-3' (primer nu-SSU-1536 from Borneman and Hartin, 2000). All cycles were programmed on Chom4 (Biorad) to perform 35 cycles consisting of: 95°C for 40s, 64°C for 45s and 72°C for 30s with bacterial rDNA and 95°C for 20s, 62°C for 30s and 72°C for 30s with fungal rDNA. Each amplification was followed by a final extension step of 10 minutes at 72°C.

Data analysis

Prior to statistical analyses, Shapiro and Bartlett tests were used to verify the normality and the homoscedasticity of the data, respectively. If both hypotheses were not refuted, ANOVAs and Tukey significant differences test were performed. In all other cases, equivalent non-parametric tests were preferred (Kruskall-Wallis and Kolmogorov-Smirnov tests, respectively).

For liquid culture results, two-way ANOVA was used to test the effect of "exposure time" and "antibiotic concentration" factors on fungi biomass and enzyme activities. Exposure times were 0, 3, 5, 7 and 10 days. The concentration levels were 0 (*i.e.* the corresponding control), 0.1, 1 and 10 mg/L for all antibiotics. When ANOVA was significant, Tukey HSD tests for mean comparison were realized. Similar analyses were made for TMEs with "exposure time" (0, 7, 14 and 28 days) and the "antibiotic concentration" as the independent variables. The spread concentrations were 0 (control), 8 and 80 µg/kg dry soil for lincomycine and 0 (control), 25 and 250 ng/kg dry soil for ciprofloxacine. All tests were realized with R software (R core development team, 2009) at the rejection level $\alpha = 0.05$.

RESULTS AND DISCUSSION

Impact of antibiotics on *T. versicolor* cultured in liquid conditions

The effects of incubation time and antibiotic concentration on *T. versicolor* cultured in liquid conditions have been assessed on fungal growth and activities of extracellular oxido-reductases (laccase, LAC; Mn-dependant peroxidase, MNP) and hydrolases (β-glucosidase, GLU; acid phosphatase, ACP). Levels of medium spiking (0.1, 1 and 10 mg/L) were chosen to cover a range of values previously reported to modify fungal protein activity. Considering overall effects, most of the variance in data was explained by exposure time (Table 1). Bell-shape curves were observed, with maxima noticed after 5 days for fungal biomass, and after 7 days for extracellular hydrolases. In contrast, LAC activity presented two peaks, after 3 and 10 days of exposure, whereas MNP

activity increased during the course of incubation with maxima noticed after 10 days. These results are consistent with the previous study of Lebrun *et al.* (2010).

On the other hand, few statistical differences appeared when regarding the effect of antibiotic concentration on the enzymatic activities used as biomarkers. TET and CIP from 0.1 to 10 mg/L did not exhibit any impact on the selected activities in our experimental conditions. It remains noteworthy that the well known inducer xylidine (Kollmann *et al.*, 2005), used as a positive control in the present experiments, led to a 13-fold increase of LAC after 3 days of fungal exposure. SDZ presented an effect only on MNP, with higher activities in 1 mg/L concentration treatment than in other cases (Figure 1). LIN significantly influenced both ACP and GLU. The activity of ACP was decreased when the antibiotic was brought at 1 or 10 mg/L (*e.g.* -34% at the peak of activity, $p < 0.05$)(Figure 2A). Conversely, GLU activity increased in the presence of LIN at 10 mg/L when compared to the control (+10%, $p < 0.05$)(Figure 2B).

Our knowledge of the possible effects of antibiotics on filamentous environmental fungi is scarce. Because of their molecular mode of action, it was unlikely that the antibiotics in the present study exert direct effects on *T. versicolor*. Nevertheless, Dhawan *et al.*, (2005) demonstrated that TET at 200 mg/L inhibited fungal growth and increased laccase production from *Cyathus bulleri* and *Pycnoporus cinnabarinus*. Because environmental contaminants (PAHs, pesticides, Mougin *et al.*, 2003; xylidine, Kollmann *et al.*, 2005) modify the secretion of enzymes from *T. versicolor*, they were intended to be used as potential biomarkers for ecotoxicity assessment. Our results show that fungal oxido-reductases and hydrolases do not behave as relevant biomarkers of exposure to antibiotics. That poor sensitivity could be due to the ability of white rot basidiomycetes to secrete extracellular lignin-degrading enzymes efficiently degrading antibiotics, as reported for TET (Wen *et al.*, 2009).

Impact of antibiotics on soil functional diversity in Terrestrial Model Ecosystems

LIN and CIP are two antibiotics widely quantified in French slurry or manure samples (our programme, but also data reported by Kuchta and Cessna, 2009; Zhou *et al.*, 2008). Our knowledge of the effects of these chemicals on soils organisms is scarce. We observed a slight effect of LIN on the ACP and GLU activities of *T. versicolor*. CIP was recently described to affect bacterial communities in soils (Zhou *et al.*, 2008). Here we studied their effects in TMEs filled with a silt loamy soil supplemented by green or spiked slurry samples, each applied at 30T/ha. Final concentrations used in our study, of agricultural relevance, were 8 and 80 µg/kg dry soil for LIN, 25 and 250 ng/kg dry soil for CIP. Modification of soil functional biodiversity was assessed by enzyme activity measurements (LAC, DHA, GLU, ACP).

Among enzymatic activities, two patterns of response appeared, according to the type of enzyme, which were shared by the two antibiotics. Hydrolase enzymes merely responded to the time of incubation, as shown by the evolution of activities of ACP and GLU in our TMEs. For instance, in the presence of LIN, GLU activity was influenced by the time exposure ($F_{3,32}$ = 25.7, $p<0.001$ and $F_{3,32}$ = 19.1, $p<0.001$) but not by antibiotic concentration ($F_{3,32} = 2.8^{ns}$ and $F_{3,32} = 0.8^{ns}$) (Figure 3).

In contrast, oxydo-reductases were influenced by antibiotic concentration rather than time of incubation. The activity of DHA was constant during the course of the experiment with green slurry. It was affected by the presence of each of the antibiotics. Spreading LIN-containing slurry decreased the activity of DHA, either at 8 or 80 µg/kg, in comparison with green slurry alone (Figure 4A). For instance, after 7 days of incubation, DHA activity was half lower (-54%) in the LIN-80 µg/kg treatment, than in green slurry one. Regarding CIP effects, only treatment spread at the highest concentrations induced a significant decrease in DHA activity (Figure 4B). For instance, after 7 days, DHA activity in CIP-250 ng/kg treatment was decreased by 45% when compared to green slurry treatment.

LAC activity was similarly decreased in the presence of the highest concentration of antibiotics (data not shown). For example, in the LIN-80 µg/kg treatment, activities after 7 and 14 days were 34% and 23% lower, respectively, than in the control. In the CIP-250 ng/kg treatment, decreases were comparable for the same exposure times (-25% and -40% respectively). Nevertheless, after 28 days no differences were observed between treatments.

Whatever the incubation conditions, 18-24% of the initial amounts of straw were degraded in the litter-bag assays, without a significant effect of the antibiotics (data not schown).

Data concerning the effects of LIN and CIP on soil enzyme activities are lacking. Our results contrast with the study of Zhou et al. (2008), who reported no impact of enrofloxacin and its metabolite CIP on populations of soil micro-organisms at concentrations below 0.2 mg/kg. It should be noted that the effects of antibiotics in soils are influenced by their bioavailability for micro-organisms.

Impact of antibiotics on soil microbial biomass in Terrestrial Model Ecosystems

In our experiments, bacterial and fungal biomasses were specifically determined using Q-PCR on DNA from soil samples. Bacterial biomass was affected by spreading with antibiotic spiked-slurry. It was increased in the presence of LIN at 80 µg/kg whatever the time of exposure (Figure 5A), and to a lesser extent in the presence of CIP at 250 ng/kg, but only at day 28 (Figure 5B). In contrast, LIN and CIP, whatever their concentrations, did not modify fungal biomass in soil TMEs. No data are available to date concerning the effects of LIN on microbial communities in agricultural soils. However, recent laboratory experiments demonstrated contrasted shifts in bacterial diversity

according to pH in forest soils (Cermak et al., 2008). Only one study (Zhou *et al.*, 2008) reported an impact of enrofloxacin and its metabolite CIP on the edaphon community, leading to the promotion of bacterial growth utilizing the fluoroquinolones as a nutrient source. Antibiotics (*e.g.* sulfonamide, SDZ and tetracycline compounds) increased the fungal:bacterial ratio, although in higher concentrations within the soil (Hammesfahr et al., 2008; Thiele-Bruhn and Beck, 2005).

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Table 1. Anova results (F values) on the effect of the factors “antibiotic concentration” (0, 0.1, 1 and 10 mg L^{-1}), “exposure time” (0, 3, 5, 7 and 10 days) and their interaction on the response of 5 extracellular enzymatic activities of *T. versicolor* in liquid conditions.

TET: tetracycline, LIN: lincomycine, SDZ: sulfadiazine, CIP: ciprofloxacin, ACP: acid phosphatase, GLU: β-glucosidase, LIP: lignin peroxydase, MNP: manganese peroxydase, LAC: laccase. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Antibiotic	Factor	ACP	GLU	LIP	MNP	LAC
TTC	Antibiotic concentration	0.18	1.53	0.08	0.04	0.16
	Exposure time	**89.00*** **	**42.84*** **	**5.87***	2.13	**12.91*** **
	Interaction of factor	1.73	2.16	0.83	0.32	2.82
LYN	Antibiotic concentration	0.10	1.95	0.67	0.76	0.04
	Exposure time	**62.70*** **	**48.32*** **	1.56	**17.12*** **	**31.33*** **
	Interaction of factor	0.02	1.90	0.67	0.12	0.06
SDZ	Antibiotic concentration	0.24	0.71	1.05	0.38	0.58
	Exposure time	**38.59*** **	**30.60*** **	3.39	**4.99*** **	**12.41*** **
	Interaction of factor	0.48	0.60	0.62	<0.01	<0.01
CIP	Antibiotic concentration	0.08	0.16	2.00	<0.01	0.04
	Exposure time	**106.90*** **	**63.26*** **	**6.44***	**6.56***	**2.33**
	Interaction of factor	0.13	0.28	3.27	0.11	0.04

Fig 1. Effect of sulfadiazine (SDZ) at various concentrations (mg/L) on Mn-dependant peroxidase (MNP) in liquid cultures of *T. versicolor*. Results are expressed as means ± standard errors.

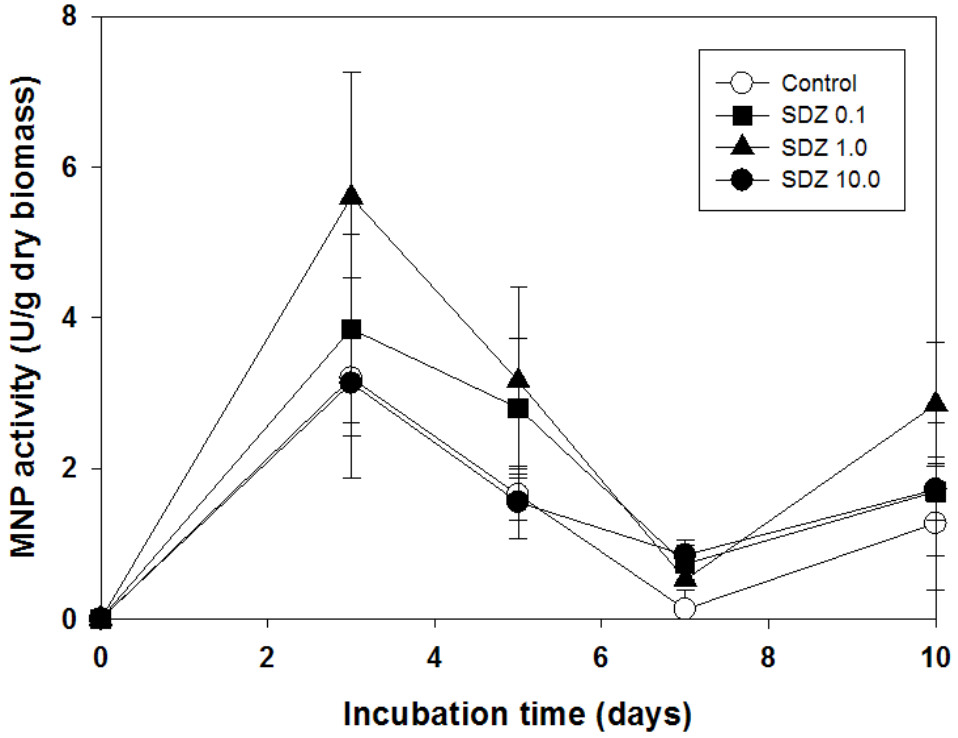

Fig 2. Effect of lincomycine (LIN) at various concentrations (mg/L) on (A) acid phosphatase (ACP) and on (B) β-glucosidase (GLU) in liquid cultures of *T. versicolor*. Results are expressed as means ± standard errors.

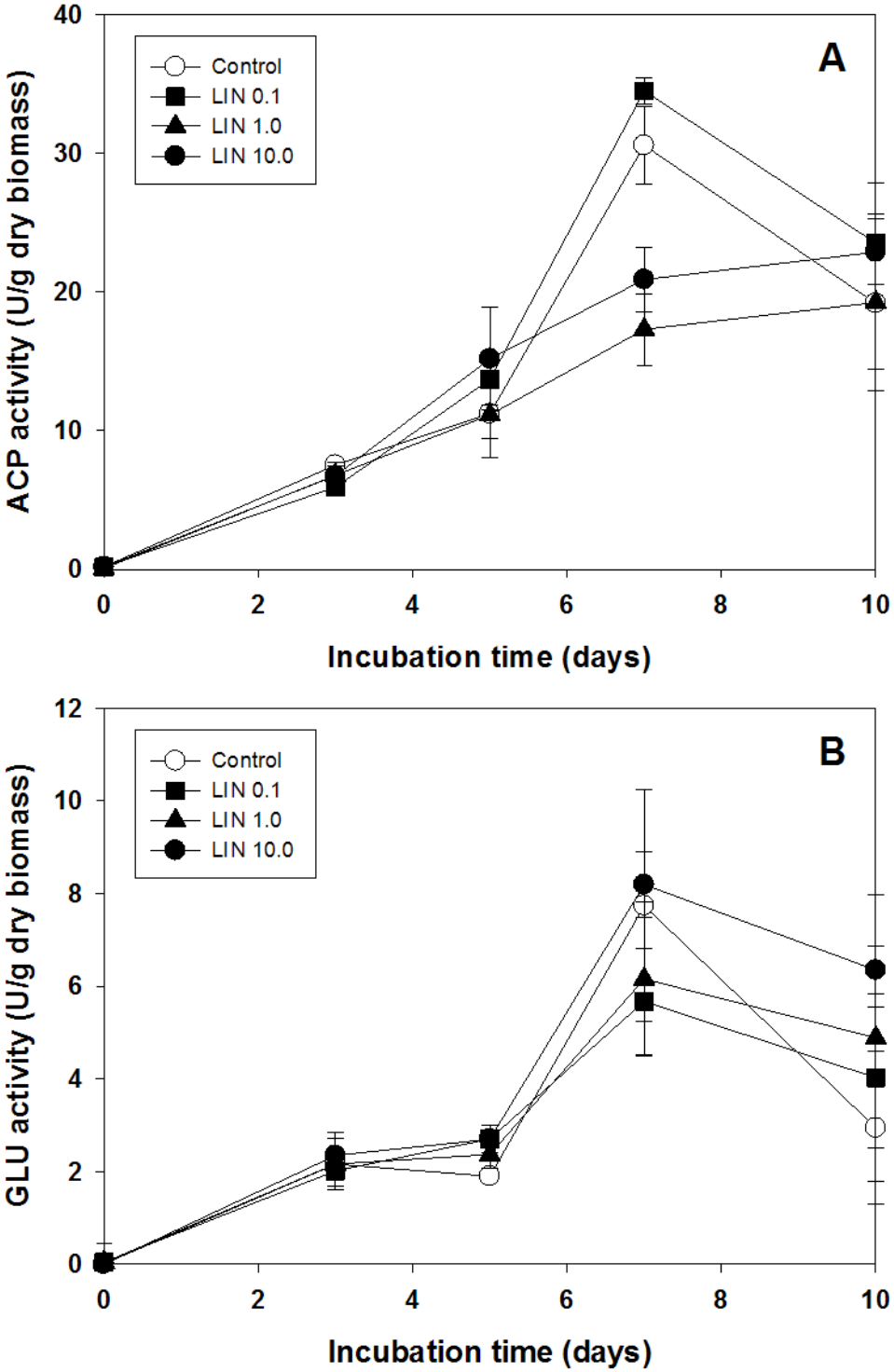

Fig 3. Evolution of β-glucosidase activity (GLU) in TMEs filled with green or slurry spiked with lincomycine (LIN) at two concentrations (µg/kg). Results are expressed as means ± standard errors.

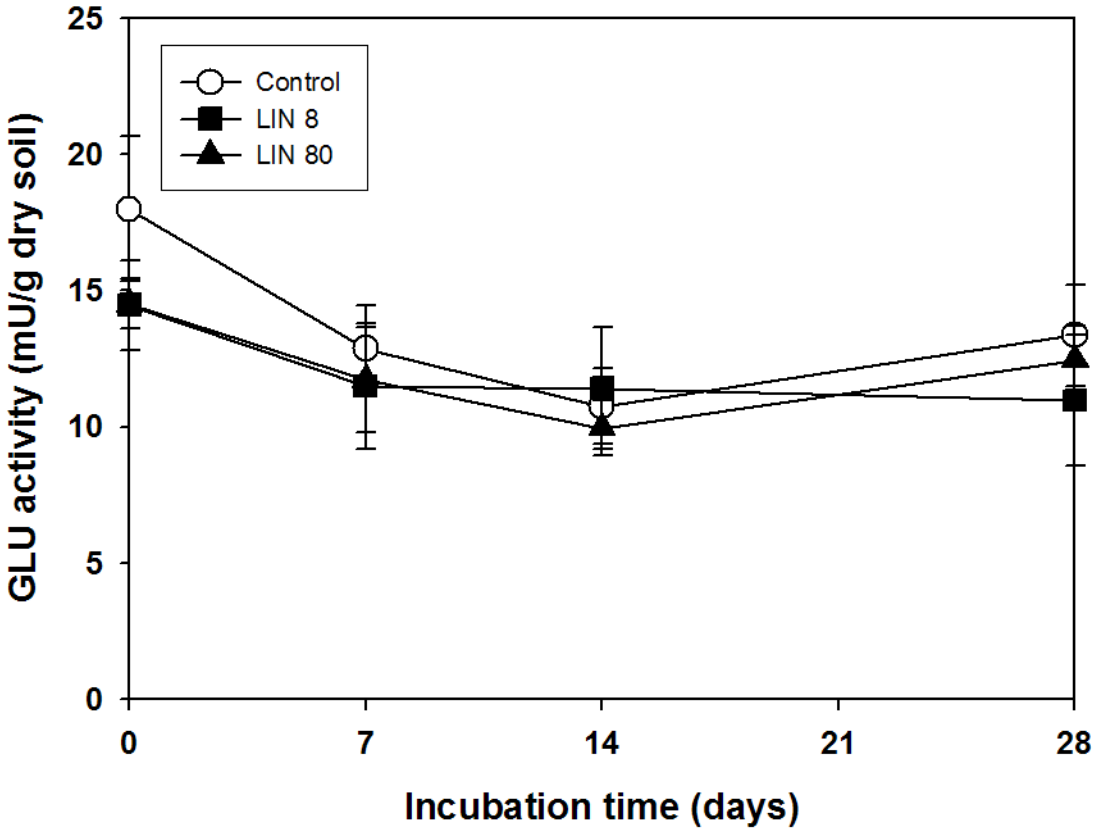

Figure 4: Evolution of dehydrogenase activity (DHA) in TMEs filled with green or slurry spiked with (A) lincomycine (LIN) at two concentrations (µg/kg) or (B) ciprofloxacin (CIP) at two concentrations (ng/kg). Results are expressed as means ± standard errors.

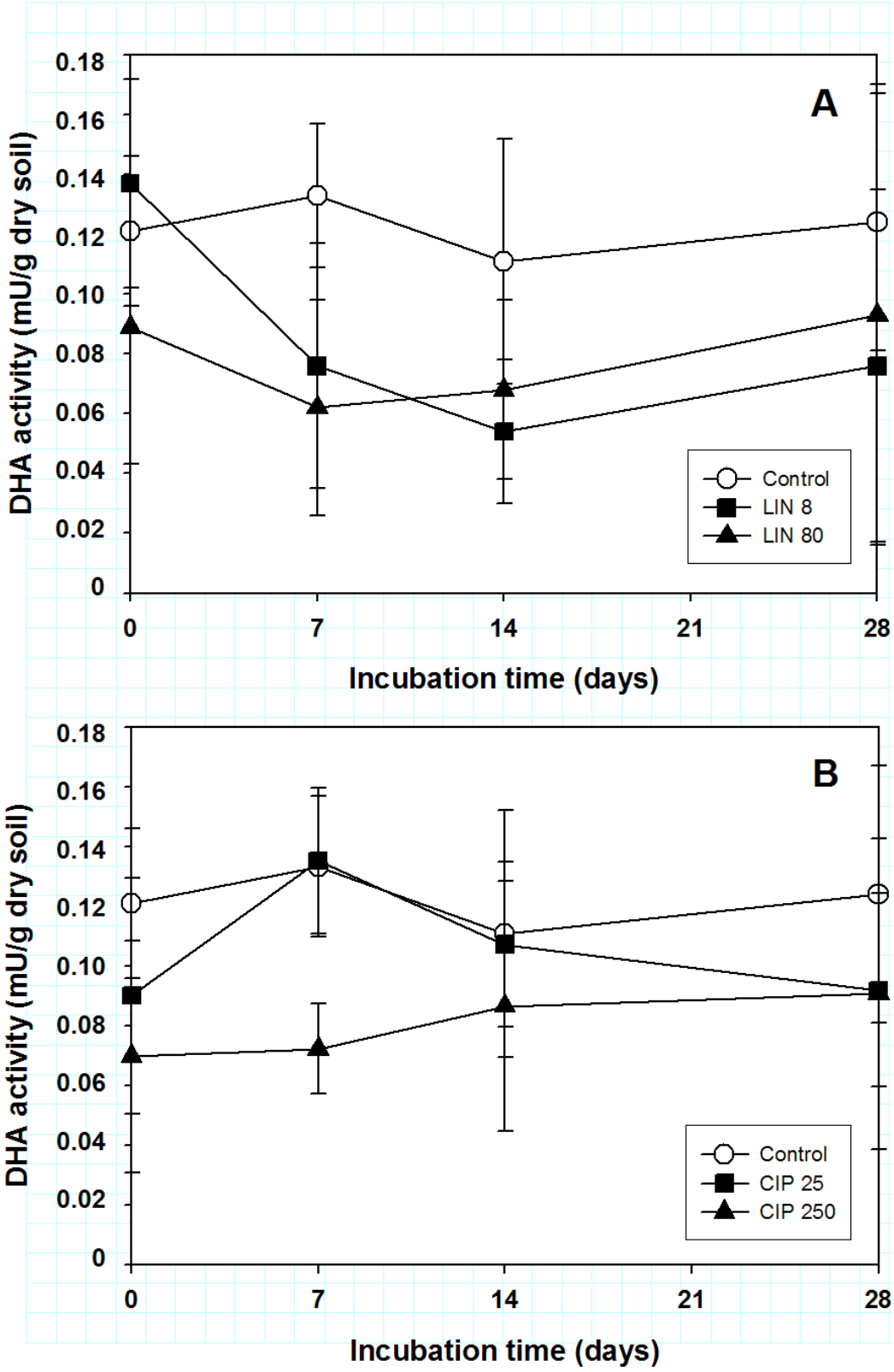

Figure 5: Evolution of bacterial biomass in TMEs filled with green or slurry spiked with (A) lincomycine (LIN) at two concentrations (µg/kg) or (B) ciprofloxacin (CIP) at two concentrations (ng/kg). Results are expressed as means ± standard errors.