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Pesticide dissipation capacity of an organic biomixture used in the agriculture exposed to copper oxychloride

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1. Introduction

With the increased global demand of food, and industrialization of the agricultural sector, the use of pesticides has increased considerably worldwide. However, together with large quantities of applied pesticides, a large amount of harmful environmental and health effects has also appeared (Pimentel and Burgess, 2014; Migheli, 2017; Peña et al., 2019; Persson et al., 2018). In this regard, it has also been necessary to invest in technologies, resources and energy for the recovery of contaminated sites and improve human health (Nicolopoulou-Stamati et al., 2016; Morillo and Villaverde, 2017). Therefore, patterns of pesticide use, and application of biotechnological innovations aimed to achieve a more environmentally friendly activity is the main concern to allow a cleaner production in agriculture (Abadi, 2018).

The presence of pesticides in water (groundwater or surface) has been systematically reported in different latitudes (Cruzeiro et al., 2016; Migheli, 2017; Glinski et al., 2018). In this regard, point source has been considered as a major cause of water pollution by pesticides (Helweg, 1994; Spliid et al., 1999; Babut et al., 2013; Persson et al., 2018). On-farm biopurification systems (OBS) comprise a cheap biotechnological tool developed for to avoid point source contamination, by means of biological removal that reduces the cost and energy necessary to remediate contaminated environment and human health (Saint-Ges and Bélis-Bergouignan, 2009). On-farm biopurification systems are widely employed in farm activities in several countries worldwide, reducing effectively the concentration of active ingredients in wastewaters of agricultural origin (Castillo et al., 2008; Góngora-Echeverría et al., 2017). In OBS, the organic biomixture is the main component and responsible for the high biological activity and consequently for an efficient pesticide dissipation; the biomixture is composed by agricultural soil, peat and wheat straw (25:25:50% by volume) (Castillo et al., 2008). Several works have demonstrated that the biomixture is able to dissipate several types of pesticide (alone or mixtures) and at high concentrations (Tortella et al., 2012; Tortella et al., 2013a, b; Marinozzi et al., 2013; Tortella et al., 2014; Chin-Pampillo et al., 2015; Holmsgaard et al., 2017; Diez et al., 2017; Diez et al., 2018).

On the other hand, in order to achieve the long-term sustainability of the biomixture, microbiological studies have also been performed (Coppola et al., 2011; Tortella et al., 2013a, 2013b; Marinozzi et al., 2013; Murillo-Zamora et al., 2017; Castro-Gutierrez et al., 2017; Góngora-Echeverría et al., 2018). The biomixture has shown a notoriously stable microbiological activity, allowing quick pesticide dissipation in many cases. In fact, several reports showed that biological activities are only temporarily affected after pesticide application, as they suffer a quick recovery afterwards (Tortella et al., 2013a, 2013b).

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Likewise, Tortella et al. (2013a, b; 2014) found that high pesticide concentrations in the biomixture, do not usually cause an adverse response on the microbial community structure. Contrastingly, Marinozzi et al. (2013) reported different alterations in the microbial communities after pesticide application. However, the negative effects were transitory, and the biological activities were recovered after 60 days.

During the pest control activities, the pesticides are often co-applied with others compounds such as antibiotics or copper-based pesticides such as copper oxychloride. In this regard, recent studies have demonstrated that the presence of antibiotics in the biomixture does not cause alterations in both, pesticide dissipation and microbial communities (Castillo-González et al., 2017; Jiménez-Gamboa et al., 2018; Huete-Soto et al., 2017a, b). On the other hand, copper-based pesticides are used in agricultural practices worldwide (Flores-Vélez et al., 1996; Bünemann et al., 2006). Among them, copper oxychloride (Ox) is annually applied to control several plant diseases (Nel et al., 1999). Nevertheless, copper tends to accumulate in surface layers of soil (Rusjan et al., 2007; McBride et al., 1981), which may eventually exert adverse eco-toxicological effects including interference on production and enzyme functionality of soil microorganisms (Gadd et al., 2001; Wang et al., 2009; Wightwick et al., 2010) and the decrease in pesticide degradation ability of soils at high copper levels (Jindal et al., 2000; Gunasekara et al., 2005 Gaw et al., 2006; Liu et al., 2007; Kim et al., 2011). In this context, Kunito et al. (1999) and Viti et al. (2008) reported that a copper concentration of 100 mg kg^{-1} produced alterations in microbial communities. However, current literature lacks research about the potential impact of wastewaters containing copper-based pesticides on the biomixture microbial communities and therefore, on pesticide dissipation. This effect is still unknown, but relevant for the optimized use of biopurification systems.

Considering that the application of copper could lead to similar effects in the biomixture of on-farm biopurification systems than those reported in soil, the main objective of this study was to evaluate the pesticide dissipation and the biological performance of a OBS at laboratory-scale exposed to a simultaneous addition of copper (as copper oxychloride) and three pesticides commonly used in farming activities. Data will provide useful information to unveil the risks associated to the practice of pesticide and copper co-disposal for the efficient use of biopurification systems.

2. Materials and methods

2.1. Chemicals

Analytical standards (99% purity) of diazinon (DZN), atrazine (ATZ) and carbendazim (CARB) were purchased from Chem Service (West Chester, USA). Copper oxychloride (36%) was supplied by Anasac Chile S.A. MBTH and DMAB from Sigma Aldrich (Chile). Other chemicals reagents were of analytical reagent grade and purchased from Merck S.A. (Chile). ATZ, DZN and CARB were selected based on their normal use in agriculture, contrasting solubility and because they have been effectively dissipated in the biomixture (Tortella et al., 2013a, b; Tortella et al., 2014).

2.2. Organic biomixture

The organic biomixture was prepared by homogeneously mixing agricultural top soil, commercial peat (Anasac-Chile 36.6% OC) and wheat straw (34% OC) in a volumetric proportion of 25:25:50. As the soil is a source of degrading microorganisms as well as adsorption site it is important that the clay content should be low (Castillo et al., 2008). On the other hand, if agricultural soil used was previously exposed to pesticide could be a source of microorganisms with a greater capacity to pesticide degradation (Castillo et al., 2008). In our study, the soil (without pesticide application history) was collected from the upper layer (0–20 cm) at the experimental station Maquehue (Freire series; 38500S, 72,410 W). According to the World Reference Base for Soil Resources (WRB; IUSS Working Group 2014), the soil used is classified as an Andosol and developed in glass-rich volcanic ejecta, with high Al and Fe content and high P fixation (WRB; IUSS Working Group 2014). The main physicochemical characteristics of the soil used were: pH (6.1 ± 0.1), organic matter content (18 ± 0.4%); copper (1.3 ± 0.2 mg kg^{-1}); and its granulometric composition is sand, silt, clay content at 28.8 ± 1.2, 43.8 ± 0.9 and 27.4 ± 0.7%, respectively. All the employed substrates were processed as previously reported in Tortella et al. (2013a). The main chemical characteristics of the biomixtures prepared were: Organic carbon (21.2%), pH (5.1), organic matter (38.1%), CICE (30.6 cmol + kg^{-1}) and C/N relation (22.8).

2.3. Pesticides dissipation

For the establishment of the laboratory-scale biopurification systems (LSBS), a portion of biomixture (3.0 kg dry weight) was weighed into glass boxes (20 × 25 × 15 cm) and artificially contaminated (by spraying) with a mixture of three active ingredients (DZN, ATZ and CARB, 10 mg a.i kg^{-1} each) and three different Ox doses (10, 100 or 1000 mg kg^{-1}). Tested concentrations used of both contaminants (copper and pesticides) were selected because the biomixture in BPS was designed to dissipate high concentration of active ingredients in situations such as accidental spills and equipment washing, among others. Therefore, these not represent environmental relevant concentrations. Biomixture without pesticide or Ox were used as controls. Moreover, biomixtures containing only the active ingredient mixture, or Ox were also carried out to evaluate their individual effects. All assays were established in triplicate. Controls with single pesticides were not run, because their dissipation and individual effects on the microbiological properties of the biomixture have been previously reported (Tortella et al., 2013a, b; Tortella et al., 2014). Additionally, a control with sterilized biomixture was carried out to evaluate the abiotic dissipation of pesticides. LSBS were incubated in the dark at 25 ± 2 °C for 30 days. The moisture (50%) was maintained by regular addition of sterilized distilled water. The LSBS were regularly sampled to evaluate the pesticide dissipation and to perform microbiological analyses. Each biomixture sample from LSBS was a composite of six cores (5 cm diameter). After pesticide spiking, the first samples were taken at 12 h (time 0) to evaluate the initial microbiological effects. For the determination of residual pesticides, DZN and CARB were extracted from the biomixture (10 g) by agitation (350 rpm, 2 h) and sonication (0.5 h) in 30 mL of acetonitrile (HPLC grade) and the supernatant (obtained by centrifugation in Teflon tubes at 10,000 rpm) was filtered (0.2 μm-PTFE) and quantified by High Performance Liquid Chromatography analysis (HPLC) using Merck Hitachi L-2130 pump, a Rheodyne 7725 injector with a 20 μL loop and a Merck Hitachi L-2455 diode array detector. A column Purosphere Star C18, 4.6 μm × 100 mm was used for the chromatographic separation. Mobile Phase acetonitrile/1% phosphoric acid (75/25%), methanol/0.1% phosphoric acid (70/30%) and methanol/water/ammonium acetate (30/65/5%) were used for DZN, ATZ and CARB respectively. ATZ was extracted in the same way but using methanol (HPLC grade) as the solvent. The extraction of pesticides in the samples was performed three consecutive times before the analysis. The recovery of the pesticides was 92 ± 3%, 94 ± 4% and 89% ± 2% for ATZ, CARB and DZN respectively. Detection limit were 0.08, 0.187 and 0.006 mg L^{-1} for ATZ, DZN and CARB respectively. Pesticide dissipation was described with the first-order kinetic model, $C = C_0\, e^{-kt}$, and half-lives ($t_{½}$) were determined using the equation $t_{½} = Ln\ (2)/k$.

2.4. Enzyme activities

Given that in the same way that in soil, all biochemical reactions in the biomixture are catalyzed by a widely group of enzymes, these are

suitable as biological activity indicators (Alkorta et al., 2003). Moreover, determination of enzyme activities is easy, rapid, and low-cost method to monitor biomixture health. Acid phosphatase, β-glucosidase and fluorescein diacetate hydrolysis were selected because are frequently used as indicators of contamination in soil and biomixture (Alkorta et al., 2003; Trasar-Cepeda et al., 2016; Tortella et al., 2013a, 2013b). Phenoloxidase activity was selected because is the main enzyme activity associated to pesticide degradation in the biomixture of biobeds system (Castillo et al., 2008). Acid phosphatase was determined using *p*-nitrophenylphosphate (0.05 M) as a substrate (Tabatabai and Bremner, 1969). Phenoloxidase activity was determined by MBTH/DMAB method (Castillo et al., 1994). Fluorescein diacetate hydrolysis (FDA) was evaluated according to Schnürer (1982). β-glucosidase was determined by the methodology proposed by Verchot and Borelli (2005) using *p*-nitrophenyl-β-D-glucopyranoside as a substrate.

2.5. Analysis of soil microbial communities

Total DNA was extracted using NucleoSpin Soil DNA Isolation Kit (Macherey Nagel. GmbH & Co., Germany) according to manufacturer's instructions. ITS and 16S of fungal or bacterial rRNA genes were amplified to evaluate fungal and bacterial communities respectively. The primers used were F341 and R534 for bacteria (Muyzer et al., 1993) and ITS4 and ITS3 for fungi (White et al., 1990). *Anthracophyllum discolor* and *Pseudomonas* spp, were run as positives controls for fungi and bacteria, respectively. All PCR conditions are described in previous work (Tortella et al., 2013a). Amplicons from PCR were used in DGGE analysis in gels containing urea-formamide denaturing gradient (40–70% for bacteria and 30–60% for fungi). The amplicons obtained were analyzed by gel electrophoresis (17 h, 60 °C and 80 V) and stained with silver nitrate (Sanguinetti et al., 1994). Phoretix 1D analysis software (Nonlinear Dynamics, Durham, USA) was used to evaluate the cluster analysis of DGGE banding profiles.

The abundance of fungal and bacterial ribosomal gene copy numbers was evaluated using StepOnePlus Real-Time PCRT System (Applied Biosystems) and HOT FIRE Pol® EvaGreen® qPCR Mix Plus (Soils Biodyne). Full details of the qPCR conditions used are described in Tortella et al. (2014). Bacterial 16S rRNA was estimated using the primer sets Eub338 and Eub518 (Muyzer et al., 1993) and fungal 18S rRNA was determined using the primers set FF390 and FR1 (Vainio and Hantula, 2000). DNA standards curves were obtained using serial dilutions of the target genes synthesized by GeneArt (Life Technologies). The PCR efficiencies were higher that 85% with $R^2 > 0.9$.

2.6. Statistical analysis of data

Data normality was analyzed according to Kolmogorov's test. The results obtained in enzyme assays and qPCR were analyzed by a one-way analysis of variance (ANOVA) and compared by Tukey test, using SPSS software (SPSS, Inc.) ($n = 3$).

For the DGGE, the similarity in the band profiles were calculated based on the Pearson correlation coefficient with the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering algorithm. Based on the matrix obtained from Phoretix 1D analysis, non-metric multidimensional scaling (NMDS) using PRIMER 7 software (Primer-E, Plymouth, UK) with Bray–Curtis similarity index was used to analyze changes in the band profiles.

3. Results and discussion

3.1. Pesticide dissipation

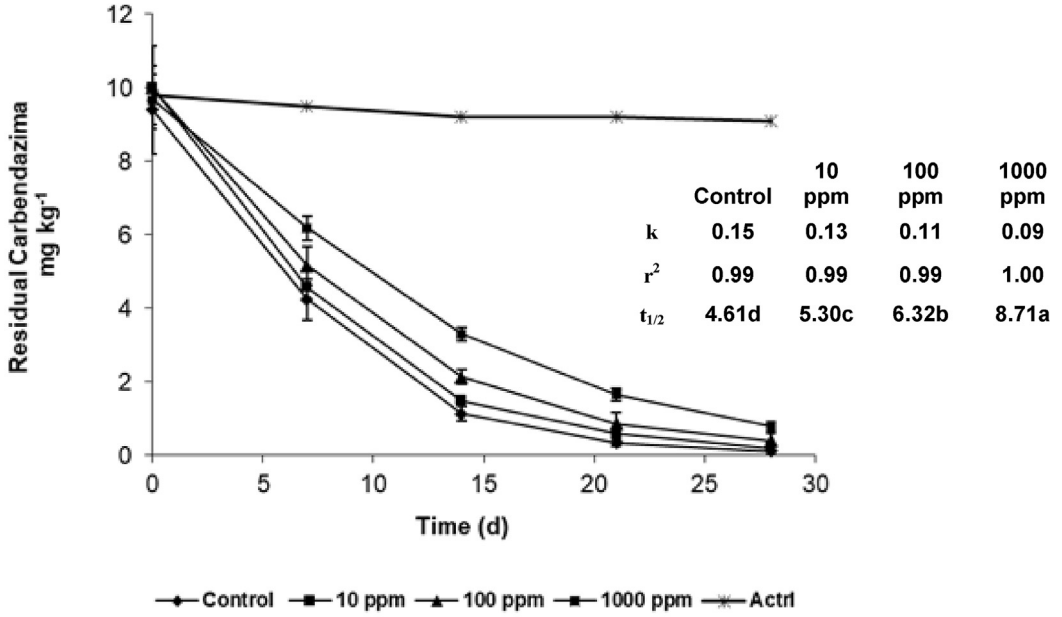
As shown in Fig. 1, a fast dissipation of ATZ, CARB and DZN was achieved in all treatments and control after 30 d of incubation period. The residual pesticides in the biomixture were less than 20% of the initial concentration applied. Abiotic control revealed that most of pesticide dissipation was mainly due to microbial activity. The kinetic data derived from the applied first-order model showed that dissipation rate constants for the three pesticides ranged from 0.13 to 0.14 d^{-1}, 0.10–0.13 d^{-1} and 0.09 d^{-1} in the treatment with 10, 100 or 1000 mg kg^{-1} of Ox, respectively, and 0.14–0.16 d^{-1} in the controls, evidencing a delay in the dissipation rate due to increase in Ox concentrations. On the other hand, although the dissipation at the end of the experiment was higher than 80%, significant differences ($p < 0.05$) were found in the half-lives ($t_{1/2}$) of the three pesticides with the three copper concentrations evaluated. As shown in Fig. 1, the presence of Ox in the biomixture at low concentration (10 mg kg^{-1}) caused significant differences in $t_{1/2}$ only for DZN, probably due to that this pesticide cause additive negative effects together with Ox at low concentrations. In this regard, Banks et al. (2003) reported that joint toxicity of DZN and copper at low concentrations caused severe toxicity on *Ceriodaphnia dubia*. At highest dose (100 and 1000 mg kg^{-1}) $t_{1/2}$ increased the for all pesticides evaluated and significant differences ($p < 0.05$) were found.

Previous works have demonstrated that after 30 days of incubation the dissipation of ATZ, CARB and DZN at 30 mg kg^{-1} in the biomixture was efficient (Tortella et al., 2013a, b; Tortella et al., 2014), even in presence of other active ingredients such as chlorpyrifos and isoproturon at higher concentrations (100 and 200 mg a.i kg^{-1}) (Diez et al., 2013). Other works have reported an effective dissipation of other kinds of pesticides, alone or in mixtures (Coppola et al., 2011; Marinozzi et al., 2013; Holmsgaard et al., 2017; Diez et al., 2017). However, it was until now unknown the implications of simultaneous exposures of copper and pesticides and the potential deleterious effects on the dissipation efficiency of the biomixture. The above-mentioned aspect is a key parameter, because it has been shown that copper can cause a negative impact on pesticide dissipation in soil (Gunasekara et al., 2005; Gaw et al., 2006; Liu et al., 2007; Kim et al., 2011; Dewey et al., 2012). In this regard, Dewey et al. (2012) reported that ATZ degradation in a sandy loam agricultural soil was adversely affected by copper sulfate at 100 or 1000 mg kg^{-1}. The authors indicate that was probably due to a negative effect on the pesticide degrading microorganisms. Although this is a first report that reveals the effects of copper in the biomixture, the results obtained agree previous reports that describe the co-application of pesticides with others chemicals of agricultural use, such as antibiotics, where the dissipation of the most pesticides evaluated was not inhibited significantly (Castillo-González et al., 2017; Huete-Soto et al., 2017a, b; Jiménez-Gamboa et al., 2018).

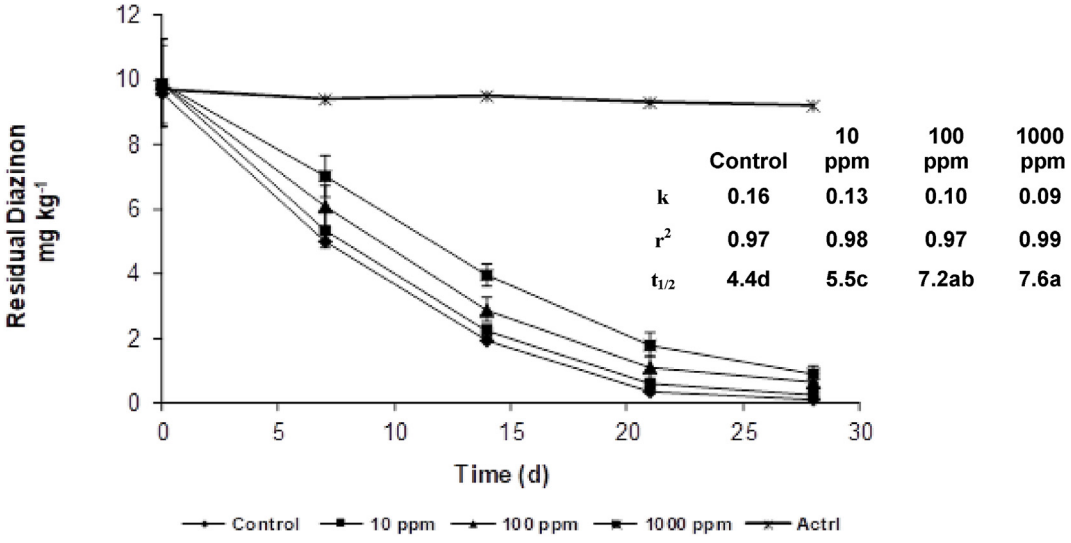
Our results demonstrated that in the same way that soil, copper caused some deleterious effects on pesticide dissipation in the biomixture. However, this was capable to sustain a favorable pesticide dissipation, although it should be taken into account that it was only one application and that the evaluation of successive applications is necessary in futures works.

3.2. Enzyme activities in the biomixture

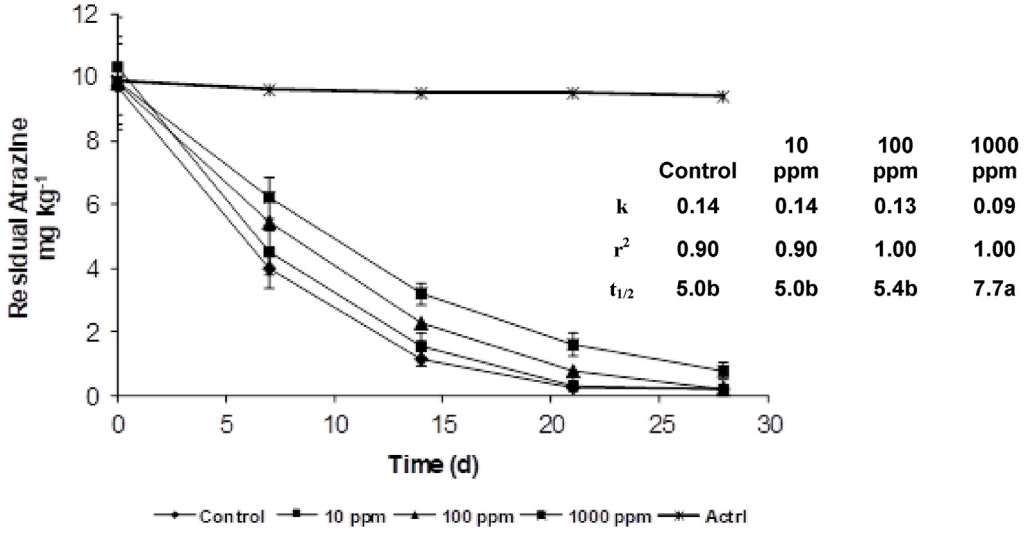
Contamination with trace elements such as metals may adversely affect the microorganisms causing alterations in soil microbial community structure, as well as, in enzyme activities (Gawet al., 2006; Wang et al., 2009; Dewey et al., 2012). As shown in Fig. 2A, immediately after pesticide application (0 d) no significant differences ($p < 0.05$) between the treatments and control were observed in FDA activity reaching values of 170 μg g^{-1} h^{-1} approximately. After 7- and 14-days post application were evident the negative effects caused due to the application of pesticide mixture (denoted in Fig. 1 as Pm), Ox alone, and the combination of these. As shown in Fig. 2A, after 7 days post application, pesticide mixture caused a 10% approximately of decrease in FDA activity and up to 21% of decrease respect to control in the biomixtures exposed to Ox alone. However, a greater reduction in FDA activity respect to control ($p < 0.05$) was observed in biomixtures treated with Ox combined with pesticide mixture. A reduction of ~50%



	Control	10 ppm	100 ppm	1000 ppm
k	0.15	0.13	0.11	0.09
r^2	0.99	0.99	0.99	1.00
$t_{1/2}$	4.61d	5.30c	6.32b	8.71a



	Control	10 ppm	100 ppm	1000 ppm
k	0.16	0.13	0.10	0.09
r^2	0.97	0.98	0.97	0.99
$t_{1/2}$	4.4d	5.5c	7.2ab	7.6a



	Control	10 ppm	100 ppm	1000 ppm
k	0.14	0.14	0.13	0.09
r^2	0.90	0.90	1.00	1.00
$t_{1/2}$	5.0b	5.0b	5.4b	7.7a

(caption on next page)

Fig. 1. Residual pesticide, half-life ($t_{1/2}$) and rate constant (k) in the biomixture after copper addition at 10, 100 and 1000 mg kg^{-1} and incubated at 25 ± 2 °C during 30 d. Control represent the residual pesticide in the biomixture without copper application. (Actrl) represent residual pesticide in abiotic control. Error bars represent the standard error of the mean of three replicates (n = 3). Different letters in ($t_{1/2}$) represent significant differences ($p < 0.05$).

was observed at 10 or 100 mg kg^{-1} of Ox (Ox10 + Pm or Ox100 + Pm) and ~61% at 1000 mg kg^{-1} (Ox1000 + Pm) as shown in Fig. 2A. After 14 days post-application a slight increase in FDA was observed in all treatments, however the tendency was similar that at 7 days. Interestingly, after 30 days post-application a notorious recovery in the enzyme activity was observed in all treatments. Although, significant differences ($p < 0.05$) respect to the control was observed in the biomixture exposed to Ox at 1000 mg kg^{-1} and pesticide mixture (Ox1000 + Pm). These results clearly demonstrated that pesticide mixture (ATZ, DZN and CARB) at 10 mg kg^{-1} each cause a slight negative effect in FDA activity, as well as, Ox at 10, 100 or 1000 mg kg^{-1} alone. However, notorious synergistic negative effects between pesticides and Ox were observed, even at low Ox concentrations (10 mg kg^{-1}).

As shown in Fig. 2B, similar results were found for acid phosphatase activity as shown in Fig. 2B. However, contrary to FDA activity a significant decrease ($p < 0.05$) was noted after few hours post application of the pesticides in combination with Ox (day 0). Pesticide mixture alone or Ox alone (in all concentrations) did not cause a significant reduction in phosphatase activity at 0 days, compared with a simultaneous presence of pesticides and Ox. After 7 and 14 days of incubation, the residual toxic effects of pesticides combined with Ox were clear, and a significant reduction ($p < 0.05$) in the phosphatase activity was observed; this effect was also observed in the biomixture containing the highest dose of Ox alone (1000 mg kg^{-1}). Although the effects of simultaneous presence of metals and pesticides has not been evaluated in biopurification systems, has been reported that phosphatase activity (acid or alkaline) in soil is highly sensitive to the presence of heavy metals (Zheng-Chung Rong et al., 1999), which could be related to the earliest negative effects in phosphatase activity in the biomixture. After 30 days post application, phosphatase activity was recovered, but slowly in the biomixture contaminated with pesticides combined with 1000 mg kg^{-1} of Ox (Fig. 2B).

Phenoloxidase activity patterns showed similar results to those obtained with acid phosphatase (Fig. 3A). Initially (day 0) phenoloxidase activity was negatively affected in the biomixtures treated with pesticides and combined with 100 and 1000 mg kg^{-1} of Ox (Pm + Ox100 and Pm + Ox1000 respectively). After 7 days post application, a notorious decreasing was observed in all treatments with pesticide mixture combined with all Ox concentrations (10,100 and 1000 mg kg^{-1}). After 30 days of incubation the activity was completely recovered, and no significant differences ($p < 0.05$) were found. Phenoloxidase activity (laccase, manganese peroxidase and lignin peroxidase) is well known as one of the most important biological activity in the biomixture, and related with pesticide degradation (Castillo et al., 2008). In this regard, an initial perturbation of phenoloxidase activity (between 0 and 15 days), as consequence of the simultaneous presence of Ox and pesticides (Fig. 3A) could be related to the slight delay of pesticide degradation (Fig. 1). In general, the decrease of phenoloxidase activity has been observed in biomixtures right after pesticide application (at high levels), with a subsequent increase that restores the activity levels (Tortella et al., 2013a, 2013b; Diez et al., 2017). However, other authors showed no positive correlation between pesticide dissipation and phenoloxidase activity (Karanasios et al., 2010). These observations could be associated to the biomixture composition, given that the original constituents (straw, peat and soil) have been modified (type and proportions) in several works (Henriksen et al., 2003; Vischetti et al., 2004; Coppola et al., 2007; Holmsgaard et al., 2017), to be adapted to local conditions as well as the availability of lignocellulosic substrates. In this regard, different biomixture composition could favor or disfavor phenoloxidase activity. In this sense, Karanasios et al. (2010) reported the hardness to associate a specific enzyme activity to the dissipation of pesticides, given that the dissipation is the results of a combination of biological activities regulated by fungal, bacterial o actinobacterial communities.

Finally, as shown in Fig. 3B, β-glucosidase activity was the least sensitive enzyme activity. β-glucosidase was only initially affected (day 0) due to the co-application of Ox at 1000 mg kg^{-1} and pesticides (denoted as Pm + Ox1000 in Fig. 3B). Afterwards, the enzyme activity was recovered, and an increase in the activity was observed at 14 days post application of contaminants. It has been reported that β-glucosidase activity in soil polluted with heavy metals is negligible (Adetunji et al., 2017). However, given that β-glucosidase activity is closely associated to organic matter in soil, and addition of organic substrates increase this biological activity (Adetunji et al., 2017), it is possible hypothesize that due to the composition of the biomixture (straw, peat and soil) and its the high organic matter content (~18%) would serve as buffer to the toxic effects of pesticides and Ox, and therefore to support bacterial growth in the biomixture, which would be related to the values obtained in qPCR assays (see below).

As mentioned above, co-application of pesticides and other agrochemical products in the biomixture has been evaluated only with antibiotics (Castillo-González et al., 2017; Huete-Soto et al., 2017a, b; Jiménez-Gamboa et al., 2018). The main findings from these works are that microbial activity is initially affected with a quick recovery that produces efficient pesticide dissipation. Moreover, Similar results have been reported in biomixtures that treated with acute or chronic addition of pesticides (alone o mixture) (Karanasios et al., 2010; Coppola et al., 2011; Marinozzi et al., 2013; Chin-Pampillo et al., 2015; Holmsgaard et al., 2017; Diez et al., 2018).

The results obtained in this work demonstrated that enzyme activities in the biomixture can be affected by co-application of copper (as oxychloride) and pesticides. Specifically, it is clear that additive negative effects were produced by the simultaneous presence of both contaminants, and mainly when Ox was added at 1000 mg kg^{-1}, which could be related to the slight pesticide dissipation achieved in this treatment (Fig. 1). Given that transitory alterations in biological activities due to the presence of copper in the biomixture, revealed a slight delay in pesticide dissipation, more detailed studies are necessary to evaluate specifically if these or other enzyme activities associated with specific microbiological groups produce this alteration in pesticide degradation in the biomixture. Similar results were reported by Demanou et al. (2006), who described synergistic negative effects on functional diversity of soil microorganisms when mefenoxam and copper (CuO) were co-applied, but the same effects were not observed when these compounds were applied separately.

3.3. Analysis of the microbial community structure

Microbial community structure evaluated by DGGE and qPCR analyses were carried out to evaluate alterations in bacteria and/or fungi in the biomixture, as a consequence of co-application of pesticides and Ox. As shown in Fig. 4A, DGGE profiles corresponding to bacterial populations showed a high similarity. In general, NMDS analysis revealed three main groups, which were chronologically grouped (0, 15 or 30 d) with a similarity close to 80% among them. Each of these group arranged chronologically showed a high similarity (90%), suggesting that minimal changes occurred in bacterial communities due to the presence of pesticides and Ox compared to the control. However, after 15 days of incubation the treatment containing Ox at 1000 mg kg^{-1} combined with pesticides (denoted as Oxi 1000 + P) was not included in the groups as shown in Fig. 4A, indicating that at 15 days a high

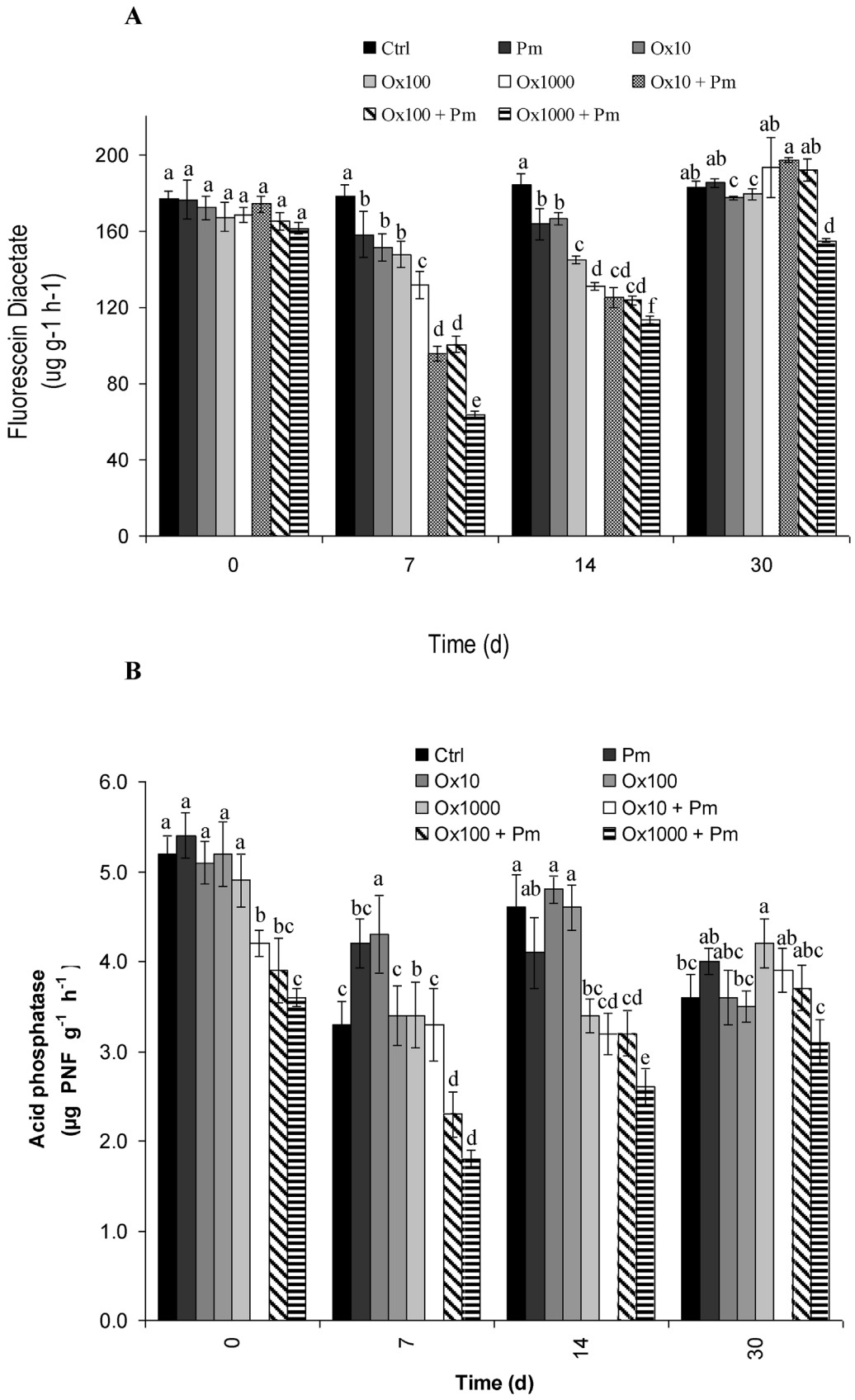


Fig. 2. Fluorescein diacetate hydrolysis (FDA) (A) and phosphatase activity (B) in the biomixture after copper addition at 10, 100 and 1000 mg kg^{-1} after 30 d of incubation at 25 ± 2 °C. Treatments are denoted as Ctrl (control), Pm (active ingredient mixture alone), Ox10, Ox100 or Ox1000 (copper alone at different concentrations 0, 10 or 1000 mg kg-1) and Ox + Pm (copper combined with active ingredient mixture). Error bars represent the standard error of the mean of three replicates (n = 3). Different letters represent significant differences between treatments ($p < 0.05$).

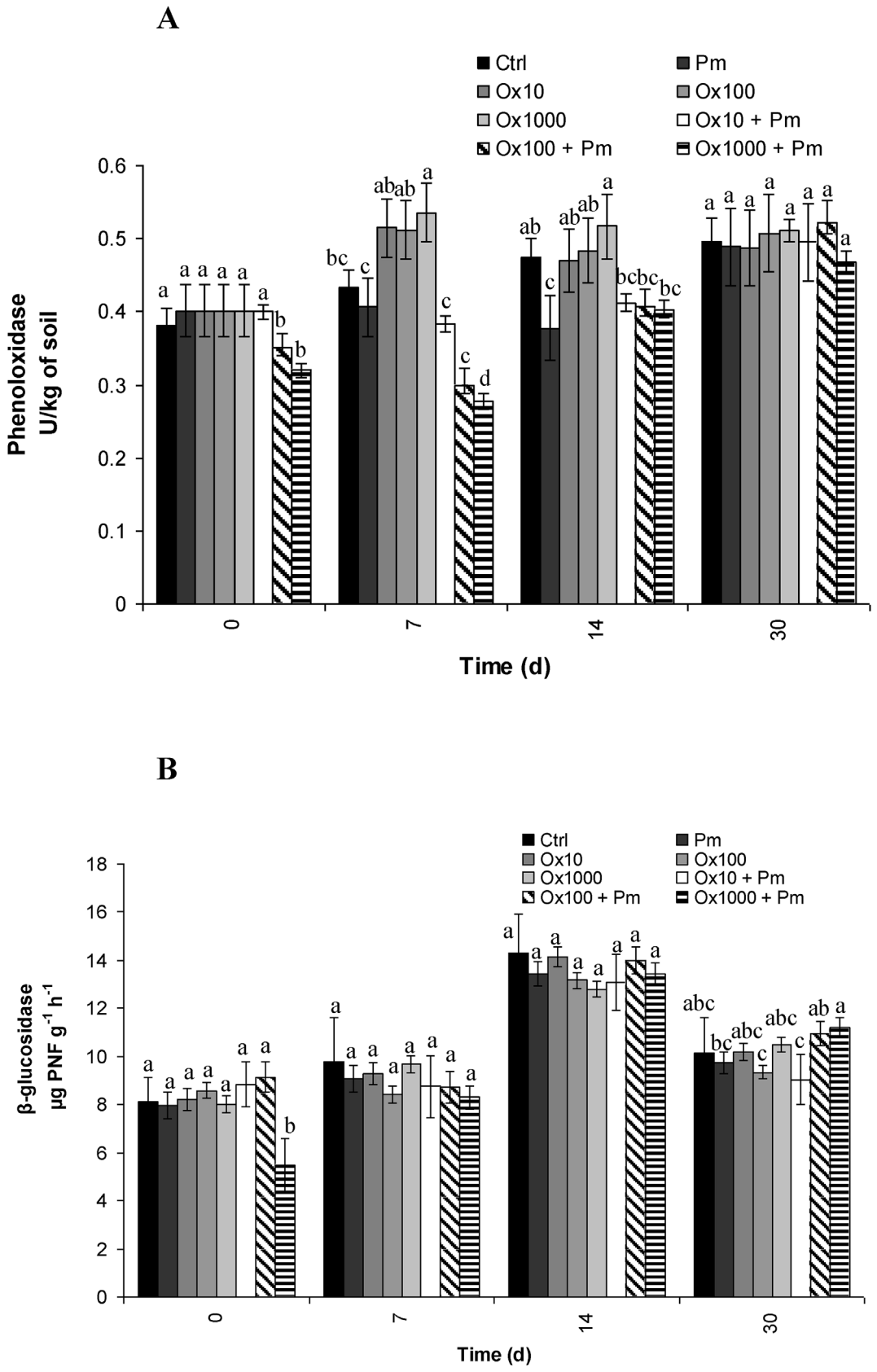


Fig. 3. Effect of copper at 10, 100 and 1000 mg kg^{-1} on phenoloxidase activity (A) and β-glucosidase activity (B) in the biomixture after 30 d of incubation at 25 ± 2 °C. Treatments are denoted as Ctrl (control), Pm (active ingredient mixture alone), Ox10, Ox100 or Ox1000 (copper alone at different concentrations 0, 10 or 1000 mg kg^{-1}) and Ox + Pm (copper combined with active ingredient mixture). Error bars represent the standard error of mean of three replicates (n = 3). Different letters represent significant differences between treatments ($p < 0.05$).

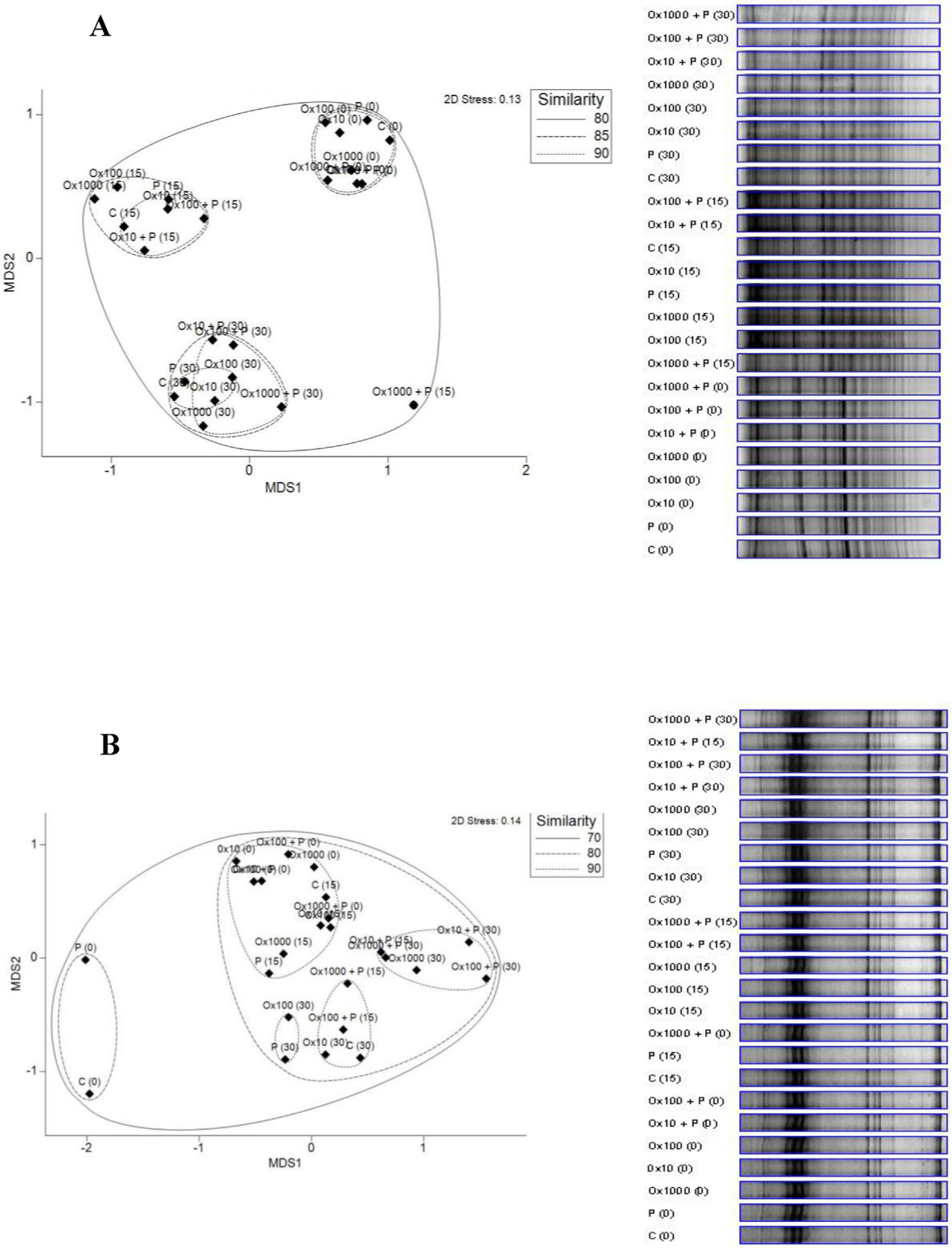


Fig. 4. Non-metric multidimensional scaling (MDS) analysis of DGGE profiles of 16S rRNA of bacterial amplicons (A) and 18S rRNA of fungal amplicons (B). The DGGE lanes from the contaminated biomixture and the control were denoted as C (control), P (active ingredient mixture without copper), Ox10, Ox100 or Ox1000 (copper alone) and Ox + P (copper combined with active ingredient mixture). Number in parenthesis represent the sample days (0, 15 and 30 days).

concentration of Ox (1000 mg kg^{-1}) co-applied with the pesticide mixture caused a greater alteration in bacterial communities. However, after 30 days the similarity was increased, again demonstrating the high resilience of bacterial communities in the biomixture.

For fungal communities, the application of Ox caused a higher variability in DGGE profiles, where five groups of high similarity (90 or 95%) were formed in the clustering (Fig. 4B). Interestingly, NMDS analysis revealed the formation of one group that includes the control and the biomixture treated with the pesticide mixture alone at the beginning of the assays (day 0) (denoted as C(0) and P(0) in Fig. 4B),

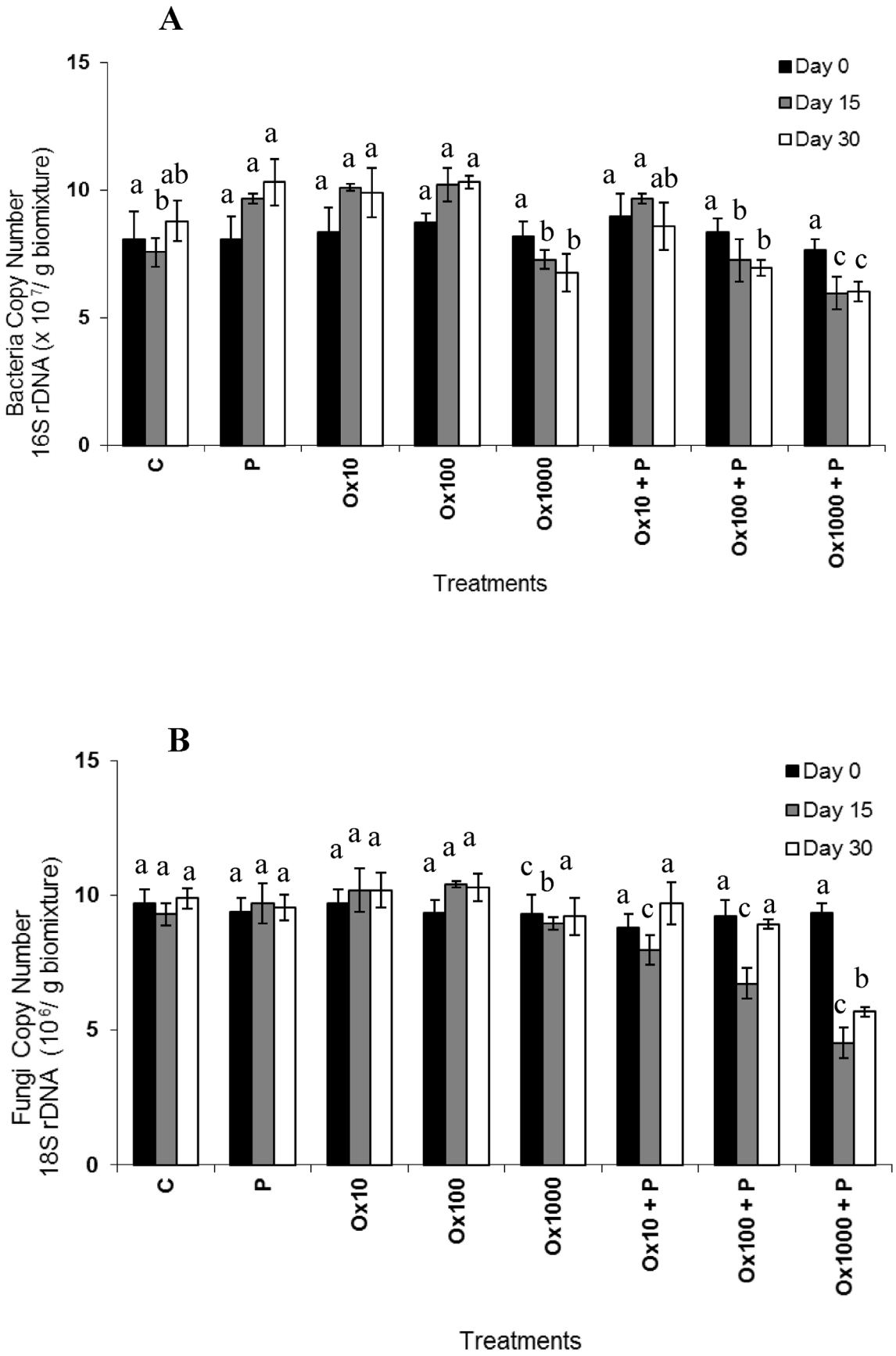


Fig. 5. Effect of copper addition at 10, 100 and 1000 mg kg^{-1} on total abundance of 16S rDNA gene of bacteria (A) and fungi (B) in the biomixture. Treatment were denoted as C (control), P (active ingredient mixture without copper), Ox10, Ox100 or Ox1000 (copper alone) and Ox + P (copper combined with active ingredient mixture). Error bars represent the standard error of mean of three replicates (n = 3). Different letters represent significant differences between treatments ($p < 0.05$).

which showed a lower similarity (70%) compared with the other treatments at different sampling times, suggesting an initial perturbation caused by the presence of Ox in the biomixture. This finding could be linked to the decrease in the phenoloxidase activity (Fig. 3A) and the decrease in the pesticide degradation rates. However, given that the pesticide dissipation it not only dependent of fungal communities in the biomixture, this hypothesis needs to be addressed in more detail in futures studies.

Considering that DGGE revealed only qualitative changes in microbial communities, qPCR analysis was carried out to determine quantitatively the changes observed in the biomixtures. As shown in Fig. 5A and B, a clear decrease in 16S and 18S rRNA copy numbers of bacteria and fungi, respectively, was observed in the biomixtures exposed to pesticides combined with Ox at the highest doses (100 and 1000 mg kg^{-1}), compared to control and the other treatments ($p < 0.05$). In general, the pesticide mixture alone (at 10 mg kg^{-1} each) or Ox alone (at 10 or 100 mg kg^{-1}) did not decrease the 16S or 18S rRNA copy numbers; on the contrary, these treatments increased slightly these parameters. It was remarkable that, although an initial decrease in fungal and bacterial rRNA copy numbers was observed with high doses of Ox combined with pesticides, a clear recovery occurred in time. These results are in agree with the delay in pesticide removal observed in these treatments. Nonetheless, the recovery in the biological parameters highlights the use of biomixtures as a robust tool for the treatment of pesticides, even in presence of antimicrobial agents.

Previous reports have demonstrated that heavy metals cause alterations in soil microbial populations and enzyme activities (Bhattacharyya et al., 2008; Jin et al., 2014). In this study, we evaluated the potential adverse effects of Ox addition on pesticide dissipation in a biomixture traditionally used in on-farm biopurification systems, since copper is usually applied and combined with pesticides for pest control in farm activities. The obtained results demonstrated that Ox did not cause permanent significative negative effects on microbiological parameters and pesticide dissipation. Even when Ox was added alone at 100 mg kg^{-1}, no significant effects compared to an unspiked control were found. Moreover, a slight negative effect was observed when Ox was added at 1000 mg kg^{-1}, alone or co-applied with pesticides. The initial detrimental effects on enzyme activities and microbial populations could partially explain the delay in pesticide dissipation in these cases. Also, a clear recovery of biological activities was observed over time, evidencing the microbiological robustness of the biomixture. Therefore, according to our findings, we conclude that Ox at concentrations of $\leq$100 mg kg^{-1} could be added in combination with pesticides to the biomixture, without resulting in significant perturbations in microbial communities, enzyme activities and pesticide dissipation capacity. However, it is necessary to consider that Ox was added as a single application and that trace concentrations in the biomixture not caused an irreversible damage in microbial communities or biological activities. Therefore, complementary studies are necessary to evaluate, the potential effects produced by a chronic exposure, as well as the evaluation of other biomixture types, which could be built with more fragile soils as sandy soils. Moreover, a more specific study focused in the interaction of copper with the components of the biomixture, is recommended to elucidate its bioavailability in the matrix. Nevertheless, the data obtained constitutes an important progress to describe the microbiological response and pesticide dissipation performance when the biomixture is exposed to the co-application of pesticides and other chemical compounds usually applied in farm activities.

Author contributions

Tortella, G: Conceptualization, Methodology, Resourses, Writing - Original Draft, Funding acquisition. Cuozzo, S: Methodology, Writing - Review & Editing Diez, M.C: Validation, Writing - Review & Editing, Rodríguez-Rodríguez, C: Methodology, Formal analysis, Writing - Review & Editing. Durán, P: Investigation, Data Curation. Masís-Mora, M: Validation. Parada, J: Investigation, Writing - Review & Editing. Rubilar, O: Resources, Writing - Review & Editing.

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