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Title: **Pesticides dissipation and enzyme activities in ungrassed and grassed biomixtures, composed of winery wastes, used in biobed bioremediation systems.**

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Abstract

The biomixture composition and the presence of a grass layer in a biobed bioremediation system can improve the performance of these systems to minimize pesticide point-source contamination. In this study, a novel biomixture composed with organic wastes from vineyards and wine industries (vermicompost of winery wastes and vine shoots) and top soil (W) was elaborated. The impact of three pesticides, commonly used in vineyards, on its microbial activity and on the development of turfgrass was determined in a short-term experiment. Moreover, the dissipation of the assayed pesticides was evaluated to stablish their distribution patterns between the turfgrass and the biomixture. For comparison, the original biomixture composed with top soil, peat and straw (P) was also studied. After 15 days of pesticide application, the development of the turfgrass in both biomixtures was similar. However, the oxidoreductases (dehydrogenase and orthodiphenol oxidase) and the hydrolytic (FDA and β-glucosidase) enzymes activities were greater in W-biomixture than in P-biomixture. The dissipation of metalaxyl and imidacloprid recorded in the W-biomixtures was significantly greater than in the P-biomixtures. The pesticide dissipation in W-biomixtures followed the same order of their octanol water partition coefficients. Except for tebuconazole, the lower biological activity in the P-biomixture would explain the limited pesticide dissipation. In the grassed biomixtures, most (>83%) of the non-dissipated imidacloprid and tebuconazole remained in the biomixtures, while metalaxyl was rapidly translocated to the aerial part of the turfgrass. Our results show the potential capability of the novel biomixture as an alternative to the original one in a biobed

.**Keywords**: Turfgrass; oxidoreductase enzymes; hydrolytic enzymes; insecticide; fungicides; mass balance

1. Introduction

Pesticide pollution of surface and ground water constitutes a considerable hazard for the aquatic environment in developed and developing countries. In many cases, the contamination of water resources is more due to the inappropriate use of pesticides (point source contamination) than to the widespread (diffuse contamination) after their application. Point-source (high concentration of pesticides in a small area) contamination is caused by the spills from the devices used for pesticides application, the equipment- washing water and the uncontrolled disposal of pesticides residues, among others (Fait et al. 2007).

In the last two decades, different low-cost systems, such as biobed, have been developed as biotechonological tools to minimize pesticide point source contamination. These bioremediation systems include a biomixture of different organic materials and soil, which retains and degrades pesticides. The most commonly used biomixture includes soil, peat and straw (25:25:50 v:v:v) -original swedish biobed- (Torstensson and Castillo 1997, Castillo et al. 2008), but also other local organic materials from agricultural, agroindustrial and energy production have been efficiently used (Delgado-Moreno et al. 2017a, Ruiz-Hidalgo et al. 2015, Vischetti et al. 2007), because peat is not feasible due to its higher cost and limited availability. In some cases, the surface of the biomixture is covered by a grass layer in order to regulate the moisture in the biomixture, to maintain optimal level of temperature for microbial activity and to serve as an indicator of pesticide spillage (Castillo et al. 2008). However, scarce information is available on the effect of the grass layer on the dissipation of pesticides in the biomixture (Delgado-Moreno et al. 2017a, Diez et al. 2015).

Enzymes activity are useful indicators of biological activity in different natural and anthropogenic ecosystems. In addition, these parameters have been used as indicators of the progress of different biological processes of degradation of organic wastes such as composting and vermicomposting, among others (Castillo-Diaz et al. 2013, Vargas-Garcia et al. 2010). In biobed biomixtures, ligninolytic enzymes (peroxidases and laccases), have been commonly used because they are responsible for the degradation of a wide variety of pesticides (Castillo et al. 2008). However, the use of other enzyme activities to evaluate the impact of pesticides on the microbial activity or on the hydrolytic potential of the biobed biomixtures has been scarcely studied (Delgado-Moreno et al. 2017a; Vischetti et al. 2006).

Viticulture and the subsequent winery agro-industry produce great amounts of wastes, whose disposal represents a serious environmental issue in the main grape-growing regions. The main waste of viticulture is the

vine shoot, a lignocellulosic material that turns into waste during the pruning of the grapevines. The primary waste of the winery industry is the grape marc, containing grape stalks, seeds, and skins left over after the crushing, draining, and pressing stages of wine production. In addition, wine making has other by-products, such as the wine lees, the dregs that accumulate in the bottom of grape-juice or wine-fermentation tanks. Finally, the distillation of the alcohol from low-quality wine, wine lees and grape marc produces large volumes of a polluted and acidic wastewater known as vinasse. Vinasse is commonly treated to yield a purified liquid effluent and a solid residue named biosolid vinasse. Winery wastes have been successful recycled through composting and vermicomposting processes to obtain mature organic amendments for agricultural purposes (Bustamante et al. 2009; Castillo-Diaz et al. 2013). In addition, winery wastes can be converted, through combustion, gasification, pyrolysis and anaerobic digestion processes, into renewable energy and other end products, some of them with good agronomic characteristics (Da Ros et al. 2017, Zhang et al. 2017). However, little information is available on the use of raw or transformed winery wastes as low-cost and novel materials for biobed biomixtures (Castillo-Diaz et al. 2016).

The objective of this study was to evaluate the initial impact of a high dosage of pesticides, commonly used in vineyard crops, on a biomixture containing winery wastes in terms of microbial activity and development of grass layer. For that purpose, different enzyme activities (dehydrogenase activity, ortho-diphenol oxidase, FDA hydrolytic activity, β-glucosidase, acid phosphatase, and urease activity) were analyzed and the biomass of the shoot and root of the turfgrass weighted. Results were compared with those obtained for the original biomixture, containing peat and straw, in order to stablish the potential capability of the alternative biomixture as a substitute of the original one. In addition, the dissipation of the assayed pesticides was determined to stablish their distribution patterns between the plant and the biomixture in a biobed system with grass layer.

2. Material and Methods

2.1 Chemicals

The registered products of one insecticide Imidacloprid (I) 20% w/v (Confidor ® 20 LS Bayer, Leverkusen, Germany), and two fungicides Metalaxyl (M) 25%w/w (Artemil ® 25WP, IQV Agro) and Tebuconazole (T) 25% w/v (Genius ® WG Saravia S.A) were selected as representative pesticides used in vineyards. The solubility of I, M and T in water was 0.51, 8.4 and 0.032 g L^{-1}, respectively and their octanol/water partition

coefficients (log Kow) were 0.57, 1.71 and 3.7, respectively. All other solvents and chemicals used were of HPLC grade.

2.2 Soil, organic materials and preparation of biomixtures

A calcareous, silty clay loam soil containing 23 g kg^{-1} organic carbon (OC), 2.1 g kg^{-1} nitrogen and pH 8.2, was collected from the upper soil layer (0-20 cm) of an agricultural area in Deifontes, Granada, Southern Spain. It was sieved through a 5 mm. The soil had never been treated with any of the assayed pesticides. The peat was collected from Turbera del Agia (Padul, Granada, Southern Spain) and it had pH 4.5, 301 g kg^{-1} OC and 8 g kg^{-1} of nitrogen. The mature vermicompost was developed on a pilot scale, as described by Castillo-Diaz et al. (2013) from a mixture of wine shoot: biosolid vinasse (4:1, dry weight). After vermicomposting, the vermicompost was allowed to mature and dry for two more months. This mature vermicompost had pH 7.6, 317 g kg^{-1} OC and 28 g kg^{-1} of nitrogen. Dry barley straw and dry wine shoots had, respectively, 571 and 445 g kg^{-1} and 1.8 and 7.2 g kg^{-1} of nitrogen. All organic materials assayed were passed through a 4 mm sieve.

Two biomixtures were prepared. The first was composed of soil, straw and peat (P) and the second consisted of soil, vine shoots and vermicompost (W), both prepared at volumetric proportions of 1:1:2. The constituents were mixing vigorously to obtain a homogeneous biomixture. The main chemical properties of both biomixtures are presented in Table 1.

2.3 Experimental layout

A bulk sample from each biomixture was separated into 9 sub-samples (160 g dw each) and placed in plastic pots (0.67 L). All the biomixtures were watered with distilled water to 80% of its water holding capacity (WHC) and placed in a camera in darkness (Initial time, I). Two grams of a mixture of turfgrass seeds (*Bromus perenne* cv halcón (25%), *Lolium perenne* cv NUT (25%) and *Lolium multiflorum* cv Serenade (50%) were directly sown on six pots of each biomixture. After the germination of the seeds (2 days), the pots were placed in a greenhouse under a day/night cycle of 16:8 h, 21–15°C and 50% relative humidity. Samples were irrigated every two days with distilled water to maintain 80% of the biomixture WHC. After 21 days, the turfgrass had reached 10 cm length and an aqueous solution containing a mixture of the pesticides imidalcloprid, tebuconazole and metalaxyl, at a concentration of 50 µg g^{-1} biomixture each one (Co), was sprayed to three pots containing turfgrass

(+G,+ITM) and to three pots without turfgrass (-G, +ITM). The other three pots containing turfgrass were not sprayed with pesticides and used as a control (+G). To prevent pesticides loss from the pots, glass trays were placed under each pot and the possible leachates collected were put back into the respective pots. However, no leachates were detected from the pots during the experimental period. All the nine pots were kept at the same greenhouse conditions as described before for 15 days. After 15 days of pesticide application (end of the experimental period), all the nine pots were collected. The shoot of the turfgrass was harvested and the roots were separated from the final biomixtures. The fresh turfgrass roots and shoot and the biomixtures were weighed and stored in plastic bags at -20°C until analysis.

2.4. Analytic procedure

2.4.1 Extraction and pesticide analysis in biomixtures, roots and shoots.

For the extraction of pesticides from the final biomixtures, an aliquot of 3 g of the homogenized sample of each pot was weighed into a 50 mL centrifuge tube and 5 mL of acetonitrile were added. The mixture was vortexed for 1 minute and after that added with 1 g of QuEChERS EN Puch (Agilent Technologies, Santa Clara, CA). Then, samples were vortexed for 1 min and centrifuged at 3000 rpm at 10ºC temperature for 5 min. To eliminate interfering substances a clean-up procedure was carried out. Briefly, an aliquot (1 ml) of the supernatant was transferred to a vial containing 1.5 g of dispersive SPE, Fruit and Veg EN (Agilent Technologies, Santa Clara, CA). Subsequently, 3 mL of acetonitrile were added to the vial. Samples were manually agitated for 1 min, and then centrifuged at 3000 rpm at 10ºC temperature for 5 min. An aliquot (0.8 mL) of the supernatant was diluted with deionized water to a final volume of 2 mL.

For the extraction of pesticides from shoots and roots, an aliquot of 1g of crushed sample of each pot was vortexed for 1 min with 2 and 3 mL of acetonitrile, respectively. Then, 0.3 g and and 0.6 g of QuEChERS were added to shoot and root sample, respectively. Samples were vortexed and centrifuged as described above and an aliquot of the supernatants was diluted by a factor of 2 with deionized water.

All the samples were passed through a 0.2 µm PTFE filter (Thermo Fisher Scientific Inc. Waltham, MA) prior to pesticide analysis. Pesticides analysis was performed in an 1100 high pressure liquid chromatograph equipped with a diode-array detector (Agilent Technologies, Santa Clara, CA). A Zorbax RX-C8 (150×2.1-mm i.d.) analytical column packed with diisopropyl n-octyl (5 µm) and a guard cartridge Eclipse XDB-C8 (12.5×2.1-

mm i.d.), packed with the same material (both from Agilent Technologies, Santa Clara, CA) were used. The mobile phase was acetonitrile and water adjusted to pH 3 with sulfuric acid. In order to get good separation of each analyte a solvent gradient was used from 50% to 70% of acetonitrile. Detection and quantification of imidacloprid, metalaxyl and tebuconazole were performed at 254, 210 and 195 nm respectively, with retention times of 4.6, 8.9 and 15.89 min respectively.

Analysis of fortified samples was conducted to verify the extraction efficiency of the methods described above. Recovery tests were performed in triplicate, in the biomixture (at concentration of 100 µg g^{-1} of each pesticide) and in the shoot and roots (at concentration of 20 µg g^{-1} of each pesticide). The mean percentages of recovery for imidacloprid in the biomixtures, shoot and root samples were 82.7, 85.4 and 91.4 % respectively (CV < 4, 0.9 and 2.1 %, respectively), while the recoveries of metalaxyl were 84.9, 84.0 and 97.6 % respectively (CV < 3.3, 6.3 and 0.7 %, respectively). Finally, the recovery percentages for tebuconazole were 89.6, 105.6 and 87.8 %, respectively (CV <6.1, 9.9 and 4.6 %, respectively). The detection limit was 0.2 mg L^{-1} for every pesticide tested.

2.4.2 Chemical and enzyme activities in the soil, organic materials and biomixtures

Soil texture was determined by the pipette method (MAPA 1986). The pH and electrical conductivity (EC) were measured in a 1:10 sample:water (w/v) ratio. Total OC (TOC) and total Kjeldhal nitrogen (TKN) were determined using the dichromate oxidation and Kjeldahl methods, respectively (MAPA 1986). Water soluble carbon (WSC) was extracted at 60 ºC for 1 h with distilled water (1:10 sample:water w/v) and determined using the dichromate oxidation method. Hemicellulose, cellulose, lignin, and total phenolic compounds were determined according to validated methods described by Romero et al. (2006).

Total enzyme activities in the initial and final biomixtures, were determined in triplicate, which each reaction tube containing 0.2 g of sample. Dehydrogenase activity (DHA) was measured after the extraction of iodonitrotetrazolium formazan (INTF), produced by the reduction of 2-*p*-iodophenyl-3*p*-nitrophenyl-5 tetrazolium chloride, with a mixture of 1.5:1 acetone:tetrachloroethylene. The INTF was measured in a spectrophotometer at 490 nm (García et al., 1997). The enzyme activities β-glucosidase and acid phosphatase were quantified by estimating the amount of *p*-nitrophenol (PNP) produced from 4-nitrophenyl-β- D glucanopyranoside (PNG) and 4-nitrophenyl phosphate (PNPP) substrates (Fernandez-Gomez et al. 2012). Urease activity was determined using urea as substrate (Nannipieri et al. 1980). The NH_4 produced was

measured after extraction with 2 M KCl using a modified salicylate–nitroprusside colorimetric method (Kandeler and Gerber 1988). Ortho-diphenol oxidase (*o*-DPO) activity was determined by using catechol as the substrate (Perucci et al. 2000a). Total hydrolytic enzyme activity was estimated by using the fluorescein diacetate (FDA) method as reported Perucci et al. (2000b)

2.5. Statistical analysis

All results are the means of three replicates. The data were subjected to an one-way ANOVA analysis using the SPSS 21 Statistical software (IBM Corp, Armonk, NY, USA) and Duncan's Multiple Range Test was used to discriminate the means with an overall significance level of 0.05. Pearson's correlations were established between the enzyme activities of the biomixtures.

3. Results and discussion

The shoot biomass of the turfgrass was significantly higher (1.5 fold) in the W-biomixture compared to P-biomixture (Figure 1). In contrast, the root biomass was similar in both biomixtures. The positive effect observed in the shoot biomass by W-biomixture might be related to the presence of mature vermicompost, which could have better balanced the composition of nutrients than peat. Previous studies have also shown an improvement in plant growth and fruit quality when peat was substituted for compost and vermicomposts in both potting media substrates used for horticultural crops (Zaller 2007) and soil organic amendments used for agricultural and forage crops, including turfgrass (Gardner 2004). The application of imidacloprid, metalaxyl and tebucolazone (+IMT) did not adversely affect the grass layer in both biomixtures, which might indicate that the pesticide uptake by the plant did not have a detrimental effect on its development.

3.2 Changes in enzyme activities of the biomixtures

The DHA enzyme has been considered as an indicator of overall microbial activity in soils, organic amendments and sediments because it occurs intracellularly in all living microbial cells (Nannipieri et al. 2002). This enzyme activity was significantly higher in W-biomixtures (between 2.1 and 2.4 fold) than in P-biomixtures (Figure 2a). The W-biomixture has in their composition vermicompost from wine shoot and biosolid vinasse, and is well

known that vermicomposts and composts, unlike peat, host a wide variety and quantity of microorganisms (Fernandez Gomez et al. 2013). In our study, the value of DHA activity in the winery vermicompost was 145±11 µg INTF $g^{-1}h^{-1}$, while that was negligible in the peat (0.38±0.07 µg INTF g^{-1} h^{-1}). In addition, the initial W-biomixture has higher content of TOC, easily available carbonaceous compounds, measured as water soluble carbon (WSC), and nitrogen than the P-biomixture (Table 1), which would promote a better development and activity of the microorganisms. As compared to the initial biomixtures, DHA activity increased significantly in both biomixtures at the end of the experimental period. Rewetting of the initial biomixtures and maintaining an optimal moisture level (80% WHC) in the biomixtures and an optimal greenhouse temperature during the experimental period might have favoured the decomposition of organic material increasing the number and diversity of microorganisms, which enhance DHA activity (Wolińska and Stępniewska 2012). However, after 15 days of the pesticide application, no significant differences were observed between DHA activity found in the grassed non-pesticide treated biomixtures (+G) and in the grassed or ungrassed IMT-treated biomixtures (+G, IMT; -G, IMT). Contrary to these results, other authors (Bending et al. 2007; Cycoń and Piotrowska-Seget 2015; Monkiedje and Spiteller, 2002) have observed that application of imidacloprid, metalaxyl and tebucanozole inhibited soil DHA activity. In general, this negative effect was more apparent when these pesticides were applied at doses higher than those used in our study and after a longer period of time after its application. However, in biobed systems, Delgado et al. (2017b) also detected an increase of DHA activity few days after the application of high doses of imidacloprid, diuron, dimethoate, tebuconazole and oxyfluorfern to different types of biomixtures, which was attributed to the attenuation of the pesticide impact by the high retention capability of the biomixtures.

The *o*-DPO is a ligninolytic enzyme that catalyses the oxidation of phenolic compounds to quinones and indicates the capacity of the microorganisms to degrade recalcitrant organic substances (Perucci et al. 2000a). Although no *o*-DPO activity was detected in the peat, unlike the winery vermicompost (4.6±0.9 µmol oxidized catechol g^{-1} 1 min^{-1}), initial *o*-DPO values for both biomixtures had not marked differences (Figure 2b). It would be because both mixtures contain 25% of vine shoots or straw, lignin-rich materials which would stimulate the growth of ligninolytic microorganisms and the production of *o*-DPO, among other phenoloxidases enzymes (Castillo et al. 2008). At the end of the experimental period, the *o*-DPO activities recorded in the W-mixtures were significantly higher (between 1.3 and 1.9 fold) than at initial time and no significant differences were observed between treatments. In the P-biomixture, higher *o*-DPO activities were determined in the grassed biomixtures (+G and +G,+IMT), which are in agreement with Urrutia et al. (2015) and Delgado et al. (2017a)

who observed that the presence of plants root increased the phenoloxidase activity and o-DPO activity, respectively, compared with ungrassed biomixtures.

FDA hydrolase activity provides a measurement of the amount and activity of microbial biomass in a sample by determining a spectrum of microbial enzyme activities (proteases, lipases, esterases). In this study, FDA activity was significantly higher in W-biomixture (between 1.5 and 3.3 fold) than in P-biomixture (Figure 2c) which indicates that W-biomixture was biologically more active or contain a higher microbial biomass. The FDA activities recorded in both mixtures at the end of the experimental period significantly correlated to DHA activity (0.796, $p<0.05$). In comparison with DHA, few studies have examined the response of FDA hydrolase activity to the presence of pesticides in soil, and no there is no clear trend in fluorescein diacetate hydrolase after the addition of pesticides (Riah et al. 2014). According to Karanasios et al. (2010), temporal changes in FDA activity depend on the biomixtures composition, the pesticide properties and dose applied, so that of FDA activity can be stimulated or inhibited over time after the application of pesticides.

β-glucosidases, which catalyze the hydrolysis of cellobiose and other disaccharides; ureases, which catalyse the hydrolysis of urea into carbon dioxide and ammonia; and acid phosphatases, which catalyse the hydrolysis of organic phosphomonoester to an inorganic phosphate form, play a major role in the decomposition of organic C, N and P compounds, respectively (Romero et al 2005). Similar to DHA and FDA activities, the initial β-glucosidase activity was significantly higher (between 3.1 and 4.1 fold) in treatments containing the W-biomixture respect to the P-biomixture (Figure 2 d). It can be explained by the higher content of WSC in this biomixture (Table 1). In contrast, the initial urease activities were similar between both biomixtures (figure 2e). At the end of the experimental period, the presence of turfgrass in both mixtures enhanced significantly both extracellular enzyme activities. The release of plant root exudates, which contain C-rich compounds and easily degradable N-compounds, and the possible direct release of enzymes by roots might have stimulated both hydrolytic enzyme activities (Gianfreda 2015). The joint application of the three pesticides to the biomixtures did not have a detrimental effect on β-glucosidase and urease activities, in presence or absence of turfgrass. It is well known that extracellular enzymes in the soil can be stabilized and protected against degradation due to the formation of complexes with humified organic matter and/or because they can get trapped within soil clays (Nannipieri et al. 1996). These interactions would avoid their inhibition in the biomixtures due to pesticides. Unlike β-glucosidase and urease activities, similar values of acid phosphatase activities were recorded in all treatments in W- and P-biomixtures (Figure 2f). This fact implies that both biomixtures contained sufficient available organic phosphorus to maintain this activity or this enzyme was partly immobilized in the microbial

cells of the biomixtures, which would mask the possible effects of the grass layer and/or the pesticide application.

3.3 Pesticide dissipation in biomixtures

Although 15 days is a short time for the dissipation of the total amount of pesticides added to both biomixtures, some significant differences were observed (Figure 3). Metalaxyl in the W-biomixture was the pesticide that showed the greater dissipation (> 40 %) followed by imidacloprid (> 21 %) and tebuconazole (>13 %). The turfgrass in the W-biomixtures (+G) showed different effect depending on the pesticide type. Thus, increased for metalaxyl dissipation but decreased for imidacloprid and tebuconazole. In P-biomixtures, the dissipation of metalaxyl and imidaloprid was lower than in W-biomixture but the percentage of dissipated tebuconazole was similar between biomixtures. The presence of the turfgrass (+G) in P-biomixture did not affect imidacloprid and tebuconazole dissipation but increased the dissipation of metalaxyl.

The pesticide dissipation order observed in the W-biomixtures was: metalaxyl> imidacloprid > tebuconazole agree with their water solubility's, but not in P-biomixture, suggesting that the pesticide removal must be linked to other factor. As indicated above, the W-biomixture had higher DHA, FDA and glucosidase activities than the P-biomixture and, therefore, it would be biologically more active, enhancing the dissipation of the added pesticides. In addition, the W-biomixture containing winery vermicompost would have higher variety and quantity of microorganisms than the P-biomixture containing peat. Castillo et al. (2013) indicated that the total abundance of bacteria and fungi, quantified by real-time PCR in a similar winery vermicompost, was 7.2 x 10^9±0.2 x 10^9 copies g^{-1} and 148 x 10^6±18 x 10^6 copies g^{-1}, respectively. Some of those microorganisms would have pesticide-degrading activities, as has been observed in other studies with vermicomposts (Anastasi et al. 2005; Fernandez-Gomez et al. 2011). The W-biomixture contained also more lignin, cellulose and hemicellulose than the P-biomixture (Table 1). This fact could favour the development of different fungi communities affecting the level of lignin-degrading enzymes which might enhance the capability of the W-biomixture to degrade pesticides (Coppola et al. 2010). However, the values of the ligninolytic enzyme activity (*o*-DPO) determined in this study were similar, with some exception, in both mixtures (Figure 2b).

The low pesticide dissipation recorded in the P-biomixture would be associated with its lower biological activity, as mentioned above, and with higher pesticide sorption capacity which might have limited the pesticide bioavailability and thus, their biodegradation. Finally, the scarce influence of turfgrass indicates that no

rhizosphere degradation took place in this study. Probably it was due to the short time elapsed since the pesticide application. The observed pesticide dissipation was more dependent on the composition of each biomixture. Delgado-Moreno et al. (2017a) observed, in a long time (300 days), pilot-scale biobed experiment, that grass layer modified the dissipation of tebuconazole and metalaxyl depending on the composition of biomixture.

3.4. Mass balance of pesticide residues in grassed biomixtures

The percentages of the pesticides residues extracted from the biomixtures, roots and shoot of the turfgrass, two weeks after pesticide application, revealed that more than 83% of the non-dissipated imidacloprid and 88% of the non-dissipated tebuconazole remained in the W-biomixture (Figure 4). In contrast, the percentage of the non-dissipated metalaxyl was appreciably lower (65%). In the P-biomixtures percentages of non-dissipated pesticides were higher, ranging from 80% for metalaxyl to 93% for tebuconazole, confirming the greater adsorption potential of the pesticides in this biomixture. Pesticides uptake into roots of grass layer was low (between 2-8%), being greater in the W-biomixture. In the aerial part of the turfgrass developed on the W-biomixture, a significant proportion of metalaxyl was detected (>30%), which was between 3 and 15 times higher than the percentages of imidacloprid and tebuconazole, respectively. In the P-biomixture, metalaxyl, imidacloprid and tebuconazole uptake by the aerial part of the turfgrass were almost half compared to the W-mixture.

The highest solubility and the low lipophilicity (log Kow=1.7) of the metalaxyl favored its root uptake and fast acropetal translocation in the turfgrass. In other studies, rapid root absorption and upward translocation to aerial part of crops, mainly due to movement with the transpiration stream, has been observed when metalaxyl was applied as a soil drench (Carris and Bristow 1987; Maruchinni et al. 1983). Although imidacloprid also has low lipophilicity (Log Kow= 0.57), its solubility in water was significantly lower than metalaxyl, which would reduce or/ and retard its translocation from roots to the turfgass shoot. Sun et al. (2017) observed, in a maize crop grown in aqueous solutions, than imidacloprid was strongly absorbed by roots, and its accumulation in the leaves was time dependent. Tebuconazole with lower solubility in water and with medium/high lipophilicity (log Kow=3.7) would be less absorbed and translocated throughout turfgrass than the other assayed pesticides.

4. Conclusions

The alternative biomixture containing vine shoot and vermicompost from a mixture of wine shoot: biosolid vinasse (4:1dry weight) was more effective for the growth of a turfgrass and for enhancing the dissipation of metalaxyl and imidacloprid than the referential biomixture containing straw and peat. Higher dehydrogenase, FDA and glucosidase activities, and therefore, higher biological activity in this novel biomixture explain in part its relative higher efficiency for pesticide removal. The presence of a grass layer in both biomixtures had no effect, as compared with its absence, on the pesticide dissipation probably due to the short time elapsed since the pesticide application. At the end of the experimental period and in the grassed biomixtures, most of the residual amount of imidacloprid and tebuconazole remained in the biomixtures (>83%), while metalaxyl was rapidly translocated to the aerial part of the turfgrass. The results obtained in this short term study point out the effectiveness of the winery wastes as biobed biomixtures. In addition, the study shows the scarce influence of the turfgrass in the pesticide removal in these bioremediation systems. However, this should be confirmed in future studies, at long-term scale, in order to implement these biomixtures and grass layer in wine regions.

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Figure legends

Fig. 1. Fresh matter yield of root and shoot of turfgrass in W and P biomixtures without pesticide application (-IMT) or sprayed with the pesticide mixture (+IMT). Error bars represent the mean standard error. For each parameter, means followed by the same letter do not significantly differ ($p < 0.05$).

Fig. 2. Enzyme activities in the W and P biomixtures at initial time (I) and at the end of the experimental period (after 15 days of pesticide application) in grassed biomixtures without pesticide application (+G) and in grassed and ungrassed biomixtures sprayed with the pesticide mixture (+G,+IMT; -G+IMT). For each enzyme activity, different capital or lowercase letters indicate significant differences ($p < 0.05$) among W-biomixtures or P-biomixtures, respectively. The asterisk (*) indicates a significant difference between the W-biomixtures and P-biomixtures fron each treatment.

Fig. 3. Relative concentration (C/CO x100) (grey) and degraded percentage (white) of imidacloprid, metalaxyl and tebuconazole in the W and P biomixtures, in absence (-G) or presence (+G) of the turfgrass after 15 days of the pesticide application. Error bars represent the mean standard error. For each pesticide, means followed by the same letter do not significantly differ ($p < 0.05$).

Fig. 4. Percentage (means ±SD) of pesticide residues remained in the shoot and root of the turfgrass and in the biomixtures after 15 days of the pesticide application

Table 1 Chemical properties (mean ± SD, n=3) of the two assayed biomixtures

	W	P
pH	8.7 ± 0.1	7.1 ± 0.09
EC dS m^{-1}	1.2 ± 0.06	2.96 ± 0.14
TOC g kg^{-1}	259 ± 5	171 ± 9
TKN g kg^{-1}	18 ± 1	6 ± 0.3
C/N	15 ± 1	29 ± 2
WSC g kg^{-1}	6.7 ± 0.3	2.3 ± 0.2
Hemicellulose g kg^{-1}	111±3	38± 27
Cellulose g kg^{-1}	104±16	35± 34
Lignin g kg^{-1}	124±7	45± 44

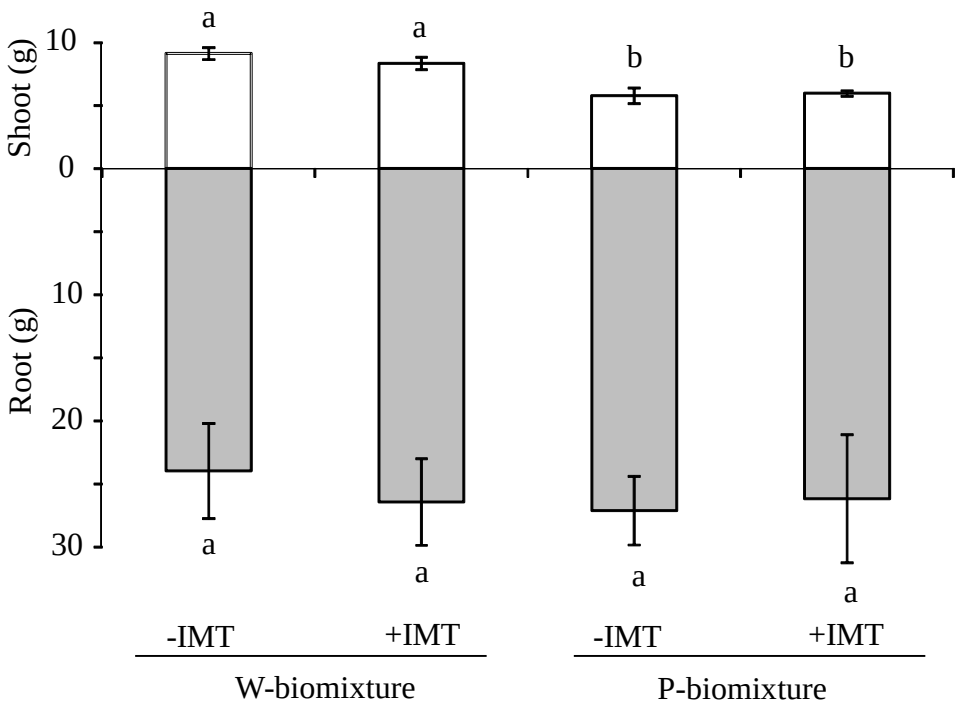

Figure 1

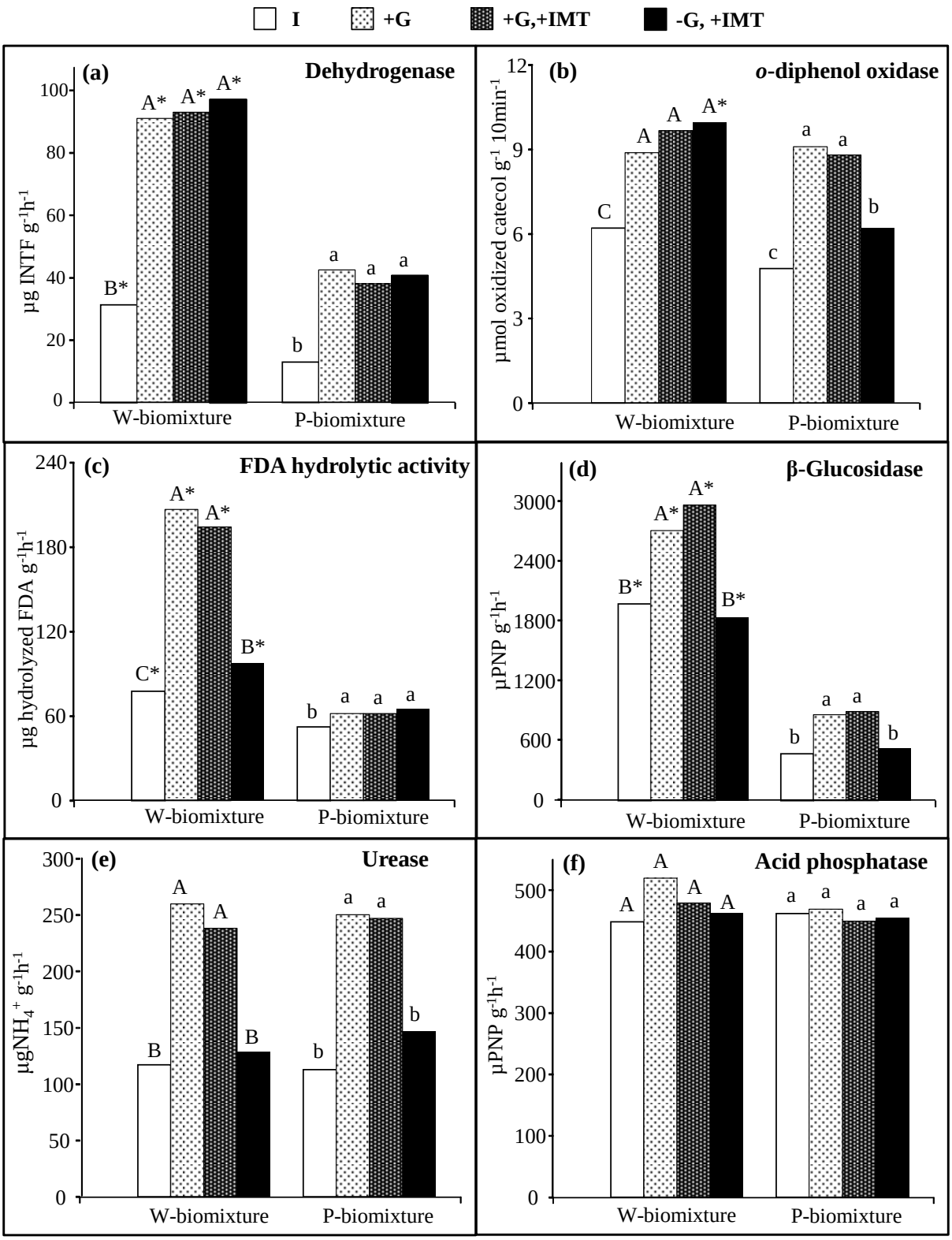

Figure 2

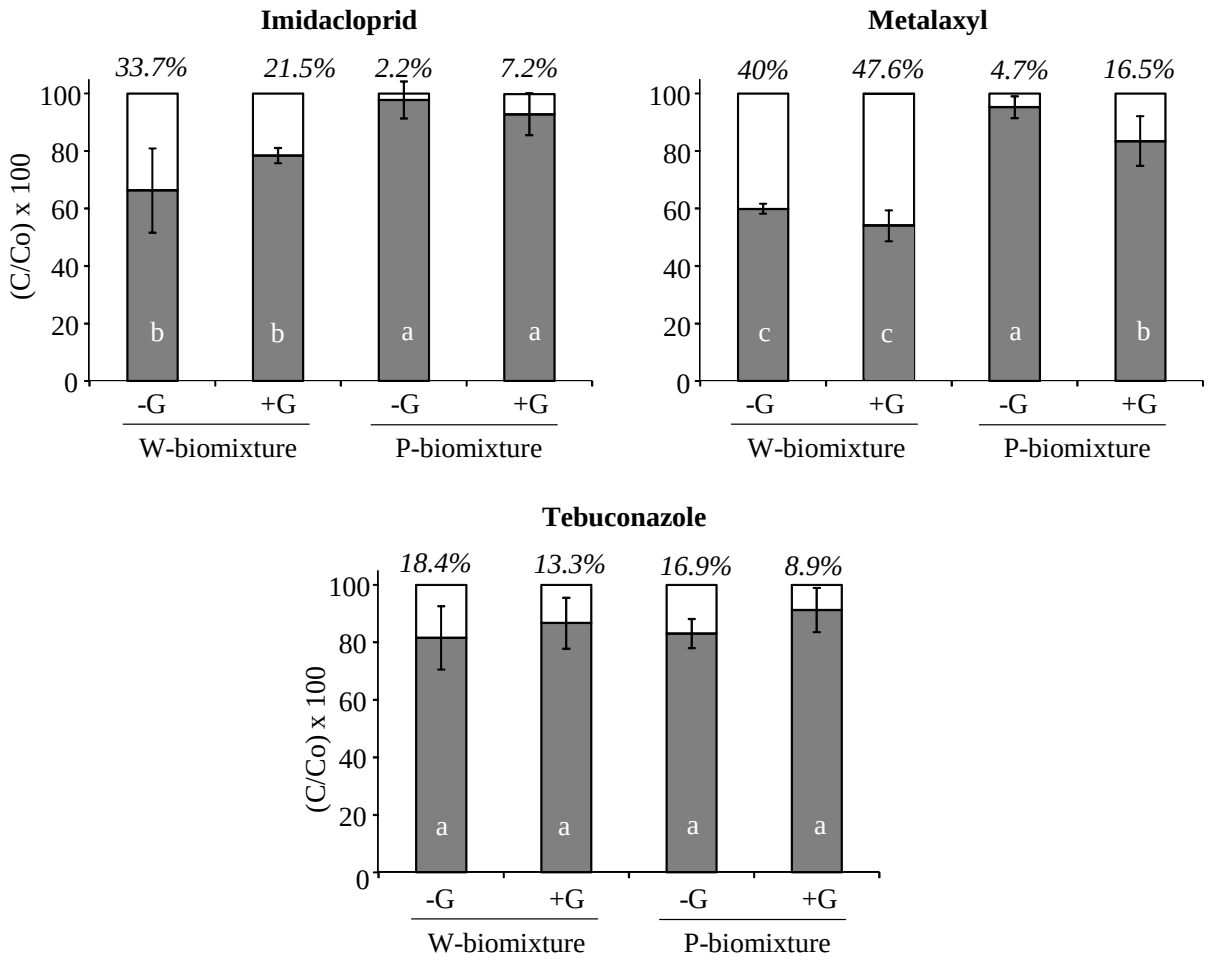

Figure 3

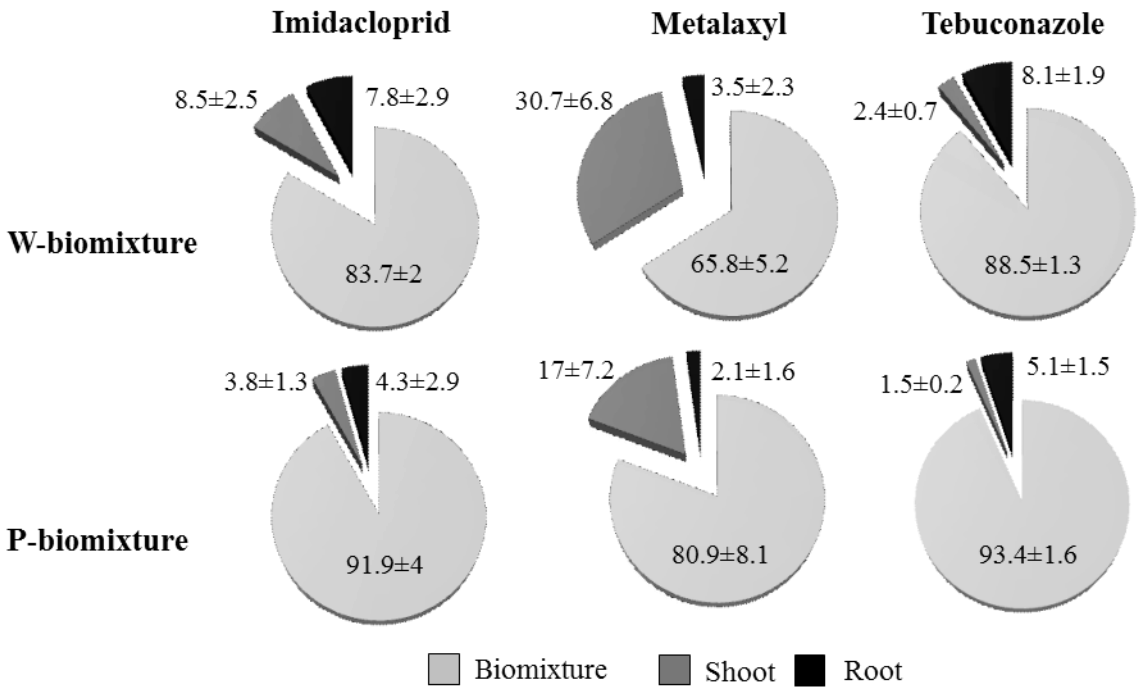

Figure 4