Effect of different rates of spent mushroom substrate on the dissipation and bioavailability of cymoxanil and tebuconazole in an agricultural soil

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ABSTRACT

Physicochemical methods to immobilize pesticides in vulnerable soils are currently being developed to prevent water contamination. Some of these methods include the use of different organic residues to modify soils because they could limit the transport of pesticides and/or facilitate their dissipation. Spent mushroom substrate (SMS) may be used for these purposes. Accordingly a study was conducted under laboratory conditions to know the dissipation and bioavailability of the fungicides cymoxanil and tebuconazole over time in a vineyard soil amended with two rates of spent mushroom substrate (SMS) (5% and 50% (w/w)), selected to prevent the diffuse or point pollution of soil. The dissipation of cymoxanil was more rapid than that of tebuconazole in the different soils studied. The dissipation rate was higher in the amended soil than in the unamended one for both compounds, while no significant differences were observed between the amended soils in either case. An apparent dissipation occurred in the amended soil due to the formation of non-extractable residues. Bound residues increased with incubation time for tebuconazole, although a proportion of this fungicide was bioavailable after 303 days. The major proportion of cymoxanil was tightly bound to the amended soil from the start, although an increasing fraction of bound fungicide was bioavailable for mineralization. Soil dehydrogenase activity was significantly affected by SMS application and incubation time; however, it was not significantly modified by fungicide application. The significance of this research suggests that SMS applied at a low or high rate to agricultural soil can be used to prevent both the diffuse or point pollution of soil through the formation of non-extractable residues, although more research is needed to discover the time that fungicides remain adsorbed into the soil decreasing either bioavailability (tebuconazole) or mineralization (cymoxanil) in SMS-amended soils.

Keywords: dissipation, bioavailability, fungicide, soil, amendment rate, pollution

1. Introduction

Pesticide residues are now being detected in waters and soils in different areas around the world due to the intensive application of these compounds in agriculture (Rabiet et al., 2010; Herrero-Hernández et al., 2013; Pose-Juan et al., 2015a). Consequently, soil and water contamination is a growing concern, as these compounds could be toxic and cause health and environmental problems. European and Spanish legislation (Directive 2009/128/EC and Royal Decree 1311/2012, respectively) on this matter advises introducing specific measures to prevent soil contamination and limit the transport of contaminants through water resources, especially groundwater, to reduce the risks and impacts of pesticide use (EC, 2009; MPR, 2012).

Pesticide application and management in agriculture may cause the diffuse and point pollution of soil and water bodies. Diffuse sources of soil pollution include spray drift, run-off, leaching, etc., whereas point sources include farmyard activities, direct contamination, and over-spray, among others (Carter, 2000; Balderacchi et al., 2013). Physicochemical methods to immobilize pesticides in vulnerable soils are currently being developed to prevent water contamination. Some of these methods include the use of different organic residues to modify soils, as their high organic matter (OM) content could limit the transport of pesticides from soil to groundwater and/or facilitate their dissipation, avoiding the diffuse or point pollution of waters due to the intensive use of these compounds (Rodríguez-Cruz et al., 2012; Álvarez-Martín et al., 2015).

Spent mushroom substrate (SMS) is an organic residue from mushroom production. In 2012, Spain was the fourth largest producer of mushrooms in Europe,

with an output of 146000 t (FAOSTAT, 2015). Mushroom production generates 170000 t of SMS per year (MAGRAMA, 2015). SMS has a high content of OM and nutrients, and can be used as an amendment to improve soil properties and quality (Brunetti et al., 2009; Peregrina et al., 2012). Furthermore, due to its high OM content, SMS could be a useful tool to control the behaviour of pesticides in soils modified with this residue. Previous works have studied the use of SMS to immobilize pesticides in amended soils (Marín-Benito et al. 2012a) or in biobeds (Karanasios et al., 2010; Gao et al., 2015), as well as its effect for controlling the leaching or biodegradation of pesticides (Kadian et al., 2012) and PAHs (García-Delgado et al. 2013).

Cymoxanil and tebuconazole are two fungicides with very different properties that are widely used on vineyards. Previous studies have reported the dissipation of some fungicides in a vineyard soil amended with fresh and re-composted SMS (Marín-Benito et al., 2012b). Furthermore, Herrero-Hernández et al. (2011) have studied the dissipation of tebuconazole in a vineyard soil amended with SMS under field conditions. Both studies highlight the effect of SMS characteristics on the dissipation of fungicides. However, the effect of SMS on the bioavailability and dissipation mechanism of fungicides in amended soil when SMS is applied at two contrasting rates in order to predict their persistence in the soil has not been reported. SMS contrasting rates could be used to prevent diffuse or point soil pollution and results could provide relevant knowledge about these processes. The dissipation of pesticides in soils amended with different rates of other organic residues has hardly been studied (Karanasios et al., 2010), and, in general, a very low range of amendment rates was applied to the soil in these experiments (López-Piñeiro et al., 2013; Sopena and Bending, 2013).

Cymoxanil [1-(2-cyano-2-methoxyiminoacetyl)-3-ethylurea] is an aliphatic nitrogen fungicide that is effective against grape downy mildew. The dissipation of this fungicide has scarcely been studied (Liu et al., 2014). It is considered a non-persistent compound that degrades rapidly, with a time to 50% degradation (DT_{50}) value of 1.2 days in soils under laboratory aerobic conditions (PPDB, 2015). Tebuconazole [(*RS*)-1-*p*-chlorophenyl-4,4-dimethyl-3-(1*H*-1,2,4-triazol-1-ylmethyl)pentan-3-ol] belongs to the fungicide group of triazoles, which is used to control smut and bunt diseases of cereals and other field crops and powdery mildew in grapevines. Tebuconazole is a hydrophobic fungicide with low solubility in water, and it degrades slowly in soil ($DT_{50} > 365$ days; very persistent), being slightly mobile ($K_{foc}=769$) (PPDB, 2015). Tebuconazole dissipation has been studied mainly in unamended soils (Strickland et al., 2004; Potter et al., 2005; EFSA, 2008b, Fenoll et al., 2011), but there is little information on its dissipation in amended soils (Herrero-Hernández et al., 2011). Tebuconazole and cymoxanil have been detected at concentrations of up to 3.2 $\mu\text{g/L}$ and 0.9 $\mu\text{g/L}$ in surface and ground waters, respectively, from La Rioja region (Spain), exceeding the EU's 0.1 $\mu\text{g L}^{-1}$ limit (Herrero-Hernández et al 2013).

The bioavailability of pesticides in soils and the dissipation mechanism from a mass balance including aqueous and organic extractable fractions and mineralized and non-extractable fractions have been studied in recent years under different environmental conditions and agricultural practices (Mamy et al., 2005, Alonso et al., 2015). However, as far as we know, there are no studies on amended soils under laboratory conditions for cymoxanil and tebuconazole.

The aim of this paper was to study the impact that the SMS applied to soil at two different rates (5% and 50% on a dry weight basis) had on the bioavailability and dissipation mechanism of two fungicides with very different characteristics, namely,

tebuconazole and cymoxanil. Studies were therefore carried out on dissipation kinetics (i) and mass balance (ii). Furthermore, soil dehydrogenase activity was assessed (iii) as a soil biochemical parameter to evaluate the impact of organic amendment and fungicides on soil microbial communities, and its possible effect on their dissipation (Muñoz-Leoz et al., 2012; Pose-Juan et al., 2015b). Our findings support the development of strategies for optimizing the dissipation of these fungicides in amended soils in order to restrict the contamination of water by these compounds.

2. Materials and methods

2.1. Pesticides and reagents

Unlabeled analytical standards of tebuconazole PESTANAL[®] and cymoxanil PESTANAL[®] (>99% purity) were supplied by Sigma-Aldrich Química S.A. (Madrid, Spain). Labelled [triazole-U-¹⁴C]-tebuconazole and [acetyl 2-¹⁴C]-cymoxanil (specific activities of 4.72 and 10.08 MBq mg⁻¹ and 98.06% and 97.79% purities, respectively) were supplied by IZOTOP Co. Ltd. (Budapest, Hungary). The characteristics of the fungicides are included in Table 1 (PPDB, 2015).

HPLC grade acetonitrile and chloroform anhydrous (>99% purity) were supplied by VWR International Eurolab (Spain). 2,3,5-Triphenyltetrazolium chloride (TTC) and 2,3,5-triphenylformazan (TPF) were supplied by Sigma-Aldrich Química S.L. (Madrid, Spain).

2.2. Organic amendment

Spent mushroom substrate (SMS) from *Agaricus bisporus* cultivation is a pasteurized mixture of wheat straw, poultry manure, urea, and gypsum. This residue was supplied by Sustratos de La Rioja S.L. (Pradejón, Spain). The characteristics of

SMS were determined in air-dried samples. The pH was determined in a residue/water suspension (1/2.5 w/v ratio). Organic carbon (OC) content was determined by oxidation (Walkley–Black method). Dissolved organic carbon (DOC) was determined in a suspension of residue in Milli-Q ultrapure water (1/100 w/v ratio), as described by Marín-Benito et al. (2012b). Total N content was determined according to the Kjeldahl method. The organic amendment applied had the following characteristics (on a percentage dry weight basis): pH 6.97, ash content 33.6%, OC content 24.5%, DOC content 1.91%, N content 1.75% and C/N 13.9. Moisture content was 64.5%.

2.3. Unamended and amended soil characterization

A soil sample (S) was collected from the surface horizon (0-30 cm) of a vineyard located in Sajazarra (La Rioja, Spain). The soil was sieved (<2 mm), and the characteristics were determined by standard analytical methods (MAPA, 1986), and they are included in Table 2. The pH was determined in a soil/water suspension (1/2 w/v ratio), and particle size distribution was determined using the pipette method. The OC and DOC contents in soil extracts (1/2 w/v ratio) in Milli-Q ultrapure water were determined as previously indicated for SMS. Its texture was classified as sandy clay loam.

Amended soils were prepared by uniformly mixing soil with SMS at rates of 5% (S+SMS5) and 50% (S+SMS50) w/w, on a dry weight basis. The soils were mixed without sieving (undisturbed), and SMS was also used as received. The initial moisture content of the soils was adjusted to 40% of their maximum water-holding capacity. The unamended and amended soils (10 kg) were incubated in containers outdoors. Soil subsamples were taken one month after the soil had been amended. These samples were air-dried and sieved (<2 mm) prior to their use in the dissipation experiment.

Subsamples were analyzed to assess both OC and DOC contents and pH, as previously indicated (Table 2).

2.4. Dissipation studies

The fungicide dissipation experiments (Lynch et al., 1995) were conducted in duplicate. Unlabeled tebuconazole and cymoxanil were added to portions of 500 g of unamended (S) and amended (S+SMS5 and S+SMS50) soils to give a concentration of 2 mg kg⁻¹ dry soil. The soil samples were incubated at 20 °C in the dark. The moisture content of the soil samples was adjusted to 40% of the maximum soil water-holding capacity, and it was maintained by adding sterile Milli-Q ultrapure water when necessary. A sterilized soil sample was also prepared by autoclaving soil at 120 °C for 1 h on three consecutive days. The sterilized unamended soil was treated with each fungicide and incubated as indicated above, and these samples were used as controls to check the chemical degradation of fungicides. Finally, soils for microbiological control were prepared by adding only sterile Milli-Q ultrapure water. All the soils were thoroughly stirred with a sterilized spatula, and all the steps were performed in a sterile cabinet. Sampling was performed at different times, and fungicide was extracted from the soil at different time intervals (up to 303 days), depending on each compound's degradation rate.

Simultaneous incubations were carried out with ¹⁴C-labelled fungicides to study the dissipation mechanism and bioavailability of pesticides. Aqueous solutions of an appropriate concentration of an unlabelled fungicide were labelled with ¹⁴C-fungicides, and a volume of 10 mL of these solutions was added to 500 g fresh weight of unamended (S) or amended (S+SMS5 and S+SMS50) soils to give a concentration of 2 mg kg⁻¹ dry soil, and an activity of approximately 100 Bq g⁻¹. In these soil samples, a

$^{14}\text{CO}_2$ trap, consisting of a scintillation vial containing 1 M NaOH (1 mL), was attached to the lid via a stainless steel clip (Reid et al., 2002).

2.5. Extraction and determination of cymoxanil and tebuconazole

At each sampling time, 2×5 g of each duplicate treatment were shaken in glass test tubes at 20 °C for 24 h, with methanol (10 mL) for tebuconazole and methanol/water 50:50 (10 mL) for cymoxanil. The samples were then centrifuged at 5045 g for 15 min, and the fungicide extracts were filtered in a Minisart NY 25 filter (Sartorius Stedim Biotech, Germany) to remove particles $> 0.45 \mu\text{m}$. For the determination of the fungicides, a volume of the extract (5 mL) was transferred to a clean glass test tube and evaporated until dry at 25 °C under a nitrogen stream using an EVA-EC2-L evaporator (VLM GmbH, Bielefeld, Germany). The residue was dissolved in 0.5 mL of acetonitrile and then transferred to a glass vial for analysis. The recoveries of the extraction method were determined by spiking three unamended and amended soil samples with analytical grade fungicide to a final concentration of 2 mg kg^{-1} , performing the extraction procedure as described above. The mean recovery values varied between 70% and 111% for both the fungicides studied.

Tebuconazole and cymoxanil were determined by HPLC-MS using a Waters chromatograph (Waters Assoc., Milford, MA, USA) with a Phenomenex Luna (3μ C18, 150 x 4.60 mm) column. The mobile phase was 70:30 acetonitrile:water for cymoxanil, and 90:10 acetonitrile:water+0.1% formic acid for tebuconazole. The flow rate was 0.3 mL min^{-1} for cymoxanil and 0.4 mL min^{-1} for tebuconazole, and the sample injection volume was 10 μL for cymoxanil and 20 μL for tebuconazole. The MS parameters were as follows: capillary voltage, 3.1 kV; source temperature, 120°C; desolvation temperatures and desolvation gas flow set at 300°C and 400 L h^{-1} , respectively, and

cone gas flow set at 60 L h⁻¹. The positive molecular ion (m/z) was 199.19 for cymoxanil and 308.80 for tebuconazole. Retention times for cymoxanil and tebuconazole were 7.9 and 6.2 min, respectively. Monitoring also involved positive molecular ions (m/z) 128.09 and 216.2 for cymoxanil metabolites (cyano(methoxyimino)acetic acid and 3-ethyl-4-(methoxyamino)-2,5-dioxoimidazolidine-4-carboxamide, respectively), and 168, 240, 224 and 322 for tebuconazole metabolites (3,3-dimethyl-1-([1,2,4]triazol-1-yl)-butan-2-one, 4-hydroxy-5,5-dimethyl-4-(1*H*-1,2,4-triazol-1-ylmethyl)hexanoic acid, 5-*tert*-buthyl-5-(1*H*-1,2,4-triazol-1-ylmethyl)hexanoic acid and 1-(4-chlorophenyl)-3-hydroxy-4,4-dimethyl-3-([1,2,4]triazol-1-ylmethyl)-pentan-1-one). The formation of metabolites during the dissipation experiment was only qualitatively monitored because metabolite standards were not available. The method validation was described previously (Pose-Juan et al., 2014). The limit of detection (LOD) and quantification (LOQ) values obtained were 0.022 and 0.069 µg L⁻¹ for cymoxanil, and 0.012 and 0.035 µg L⁻¹ for tebuconazole.

2.6. Extraction and determination of ¹⁴C-cymoxanil and ¹⁴C-tebuconazole

The extraction of the ¹⁴C-pesticides from the soil was carried out in two sequential steps: initially, 2 × 5 g of each duplicate treatment were extracted with 10 mL of a 0.01 M CaCl₂ Milli-Q ultrapure water solution for 24 h, and then a second extraction with 10 mL of the organic solvent methanol was carried out for 24 h.

The quantitative determination of ¹⁴C-tebuconazole and ¹⁴C-cymoxanil after extraction was performed by liquid scintillation using a Beckman LS 6500 liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA). The radioactivity of the solution was measured in disintegrations per minute (dpm), being determined in

duplicate in 1 mL of aqueous methanol extract to which 4 mL of scintillation cocktail was added (Ecoscint TMA, National Diagnostics, Atlanta, GA).

The residues of ^{14}C -fungicides remaining in the soil after extraction were determined by the combustion of triplicate 1 g dried soil samples, using a Biological Oxidizer (RJ. Harvey OX-500 Instrument Corporation, NJ) under O_2 excess at 900°C . The $^{14}\text{CO}_2$ generated was trapped in a mixture of ethanolamine (1 mL) and scintillation cocktail (Oxysolve C-400, Zinsser Analytic, Berkshire, UK, 15 mL), and determined as indicated above. $^{14}\text{CO}_2$ from mineralized ^{14}C -fungicides in the scintillation vial containing 1 M NaOH (1 ml) was determined at the different sampling times by mixing with 4 mL of scintillation cocktail, and determined as previously indicated.

2.7. Soil dehydrogenase activity

Soil dehydrogenase activity (DHA) was determined following the Tabatabai method (Tabatabai, 1994) at different times after fungicide application.

2.8. Data analysis

The degradation kinetics for the fungicide was fitted to a single first-order (SFO) kinetic model ($C = C_0 e^{-kt}$) or first order multi-compartment (FOMC) model ($C = C_0 / ((t / \beta) + 1)^\alpha$), known also as the Gustafson and Holden model. C is the fungicide concentration at time t , C_0 is the initial fungicide concentration, k (day^{-1}) is the degradation rate, α is a shape parameter determined by the coefficient of variation of k values, and β is a location parameter. For the selection of the kinetic model that best describes the degradation results, FOCUS work group guidelines were followed (FOCUS, 2006). The coefficient of determination (r^2) and the chi-square (χ^2) test were calculated as indicators of the goodness of fit. The time to 50% degradation, or DT_{50}

value, was used to characterise the decay curves and compare variations in degradation rates. The parameters of the kinetic models were estimated using the Excel Solver add-in Package (FOCUS, 2006).

Analysis of variance (ANOVA) was used to evaluate the effects of the different treatments on the dissipation of fungicides. Standard deviation (SD) was used to indicate variability among replicates, and the least significant difference (LSD), at a confidence level of 95%, was determined to evaluate the effects of different soil treatments on DT₅₀ values and dehydrogenase activity. Statgraphics Plus version 5.1 statistical software (Statgraphics Plus Corp., Princeton, NJ) was used.

3. Results and discussion

3.1. Dissipation of tebuconazole and cymoxanil in unamended and amended soils

The dissipation kinetics of tebuconazole and cymoxanil (expressed as a percentage of the amount of fungicide initially applied versus the incubation time) in an unamended soil and a soil amended with SMS at rates of 5% and 50% are included in Figure 1. At the end of the incubation time (303 days for tebuconazole and 15 days for cymoxanil), the dissipation of tebuconazole ranged between 34.8% and 76.7%, and cymoxanil was fully dissipated in the different soil samples.

The dissipation pattern of both fungicides was fitted to SFO or FOMC models, and kinetic parameters were calculated for each fungicide and soil treatment (Table 3). The dissipation kinetics of tebuconazole in both the unamended and S+SMS5 soils fitted the FOMC model better, whereas in the S+SMS50 it fitted the SFO model better. In turn, the dissipation kinetics of cymoxanil in all the soils studied fitted the SFO model better. Previous works have reported the dissipation curves of tebuconazole in unamended and amended soils fitted to a SFO or a biphasic model (Strickland et al.,

2004; Potter et al., 2005; Herrero-Hernández et al., 2011). The dissipation kinetics of cymoxanil in unamended soils was fitted to SFO or FOMC models in previous studies (EFSA, 2008a).

Cymoxanil dissipation was very rapid (< 1 day) in both the unamended and amended soils. The DT_{50} values between 0.4 and 0.8 days were consistent with the range of values reported for this fungicide in soils under similar conditions as in this study, which varied between 0.2-7.3 days (PPDB, 2015), 0.1-4.3 days (EFSA, 2008a) or < 1 day (Liu et al., 2014). The dissipation rate of cymoxanil increased when SMS was applied to the soil, although cymoxanil adsorption of fungicide by the amended soils increased. The adsorption constant of cymoxanil by the unamended soil ($K_f=0.46$) was lower than those by amended soils ($K_f=0.81$ and 6.79 , respectively by S+SMS5 and S+SMS50) (Alvarez-Martin et al., 2015). The influence of adsorption on decreasing the dissipation rate of pesticides in soils due to a decrease in the bioavailability and biodegradation of organic compounds adsorbed by the soil has not been observed for cymoxanil, although it has been reported for other pesticides, such as diazinon and myclobutanil (Marín-Benito et al., 2014), or fungicides, such as penconazole, metalaxyl and iprovalicarb (Marín-Benito et al., 2012a) in SMS-amended soil, but changes in cymoxanil dissipation rates were not very relevant between the soils amended at 5% and 50% of SMS.

The DT_{50} values for the dissipation of tebuconazole were higher than those for cymoxanil in all the soils studied (Table 3). DT_{50} in the unamended soil (> 1000 days) was higher than that reported in previous works (from 43 to 693 days) for agricultural soils under laboratory conditions (Strickland et al., 2004; Potter et al., 2005; EFSA, 2008b, Fenoll et al., 2011; PPDB 2015). However, the tebuconazole dissipation rate increased significantly in the SMS-amended soils. DT_{50} values were 126 and 168 days

for 5% and 50% SMS-amended soils, respectively). They decreased up to 293 times compared to the unamended soil, but the SMS rate had no significant effect on fungicide dissipation. The lower adsorption coefficients of tebuconazole by the unamended soil ($K_f=3.06$) than by the SMS-amended soils ($K_f = 6.24$ and 36.6 by S+SMS5 and S+SMS50, respectively) (Álvarez-Martín et al., 2015) could not explain the higher residual amount of fungicide found at the end of incubation, and the higher persistence of fungicide in the unamended soil than in the amended one. These results could be supported by considering that (i) the application of organic residues such as SMS to soil can increase the soil microbial biomass, its microbial activity, and its capacity to biodegrade the pesticide (Karanasios et al., 2010), leading to higher dissipation, or (ii) the increase in soil OM due to the application of the amendment could help to enhance pesticide adsorption and the formation of non-extractable residues over time (Kim et al., 2003; Marín-Benito et al., 2012b), which could explain the more rapid apparent dissipation of tebuconazole in amended soils. Similar results have been reported for other hydrophobic pesticides such as linuron in soils amended with different organic residues (sewage sludge, grape marc, or SMS) (Marín-Benito et al., 2014). Some authors have reported that the persistence of pesticides depends on the dissipation mechanism of these compounds in amended soils (Kim et al., 2003).

The degradation of fungicides in sterilized soil was slower than in non-sterilized soil (data not shown), which indicates that the soil microbial community played an important role in fungicide degradation. In sterilized soil, the percentage of fungicide degraded at the end of the incubation period was 18.7% for tebuconazole and 74.6% for cymoxanil. Previous papers have reported that the degradation of tebuconazole is mediated mainly by microorganisms (Bending et al., 2007; Muñoz-Leoz et al., 2011; Li

et al., 2015). No information about the biodegradation of cymoxanil is available in the literature.

During the incubation experiments, some metabolites of tebuconazole and cymoxanil were qualitatively monitored. The monitoring of the formation of tebuconazole metabolites (peak area) over time in the soil extracts from unamended and amended soils is presented in Figure 2. Two degradation products from tebuconazole, 3,3-dimethyl-1-([1,2,4]triazol-1-yl)-butan-2-one and 1-(4-chlorophenyl)-3-hydroxy-4,4-dimethyl-3-([1,2,4]triazol-1-ylmethyl)-pentan-1-one (molecular weights 167 and 321, respectively) were detected in the soil extracts up to 303 days. These metabolites were previously detected in other degradation studies on tebuconazole under laboratory or field conditions (Herrero-Hernández et al., 2011; Potter et al., 2005; Strickland et al., 2004). Both metabolites steadily increased with time between 90 and 160 days for the unamended or SMS-amended soils, and then their formation decreased at the end of the experiment. Metabolite formation was higher in the soil treated with a low rate of SMS than in the one treated with a high rate. The highest rate of amendment increased tebuconazole retention, initially protecting the fungicide from degradation.

Traces of the cymoxanil metabolite, cyano(methoxyimino)acetic acid (molecular weights 127) were also detected in the soil extracts up to seven days, although no differences in its detection between soils were observed. It has been reported that cymoxanil metabolites degrade very quickly in soil (EFSA, 2008a).

3.2. Mass balance of ^{14}C -tebuconazole and ^{14}C -cymoxanil in unamended and amended soils and dissipation mechanism

The total ^{14}C balance for ^{14}C -tebuconazole and ^{14}C -cymoxanil corresponding to mineralized, CaCl_2 -extracted, methanol-extracted, and non-extractable (bound residues)

fractions of ^{14}C -tebuconazole and ^{14}C -cymoxanil in unamended and SMS-amended soils over time is shown in Figure 3. The total mass balance (expressed as a percentage of the ^{14}C initially applied) was as follows: $94 \pm 3\%$, $100 \pm 5\%$, $112 \pm 5\%$ for ^{14}C -tebuconazole, and $90 \pm 1\%$, $86 \pm 1\%$, and $92 \pm 2\%$ for ^{14}C -cymoxanil in the unamended and S+SMS5 and S+SMS50 soils, respectively.

The mineralization of cymoxanil was fast after six days, and there was a steady increase in $^{14}\text{CO}_2$ evolution during the incubation time studied (up to 102 days). Cymoxanil was ^{14}C -labelled in the 2-acetyl group, with the rapid $^{14}\text{CO}_2$ evolution indicating that this group degraded very quickly. The mineralization of ^{14}C -cymoxanil was always much higher than that of ^{14}C -tebuconazole. Tebuconazole was ^{14}C -labelled in the triazole ring, and mineralization increased slowly up to 303 days, indicating that this triazole group is less accessible for microorganisms to be mineralized. These results are consistent with the dissipation rates of both fungicides.

The mineralization of ^{14}C -cymoxanil and ^{14}C -tebuconazole was significantly higher in the unamended soil compared to the SMS-amended soils. The amounts of ^{14}C -cymoxanil mineralized after 102 days were 59.3%, 57.8% and 38.5% in the unamended, S+SMS5 and S+SMS50 soils, respectively. For ^{14}C -tebuconazole, the amounts mineralized were 3.37%, 1.13% and 0.19% in the unamended, S+SMS5 and S+SMS50 soils, respectively, after 303 days of incubation. The higher adsorption of the fungicides by the amended soils, as previously indicated, could explain these results, especially in the soil amended with the highest SMS rate. The influence of adsorption on decreasing mineralization has been reported for other pesticides in unamended and amended soils (Houot et al., 1998; Kim et al., 2003; Marín-Benito et al. 2012b, 2014).

The high mineralization of cymoxanil (60.4% in unamended soil) has been reported (EFSA, 2008a). The low mineralization of tebuconazole was similar to that for

other triazole fungicides, such as propiconazole (Kim et al., 2003; Barriuso et al., 2008) or penconazole (Marín-Benito et al., 2012b), being explained by the difficulty microorganisms have to degrade the triazole ring, where ^{14}C is labelled. In SMS-amended soils, the amount of $^{14}\text{CO}_2$ evolved was below the impurity level, so the true mineralization of ^{14}C -tebuconazole may be lower than indicated. A very low mineralization (<0.1%) of ^{14}C -tebuconazole has been observed in a previous report (EFSA, 2008b).

The extracted amounts of both ^{14}C -fungicides in 0.01M CaCl_2 and methanol decreased with incubation time in the unamended and SMS-amended soils. According to Alonso et al. (2015), fungicide degradation and the formation of bound residues could explain the decrease in extractability with incubation time.

For tebuconazole, the ^{14}C amounts extracted with a water solution were initially higher in the unamended soil (40.2%) than in the amended ones (11.5%-23.6%), and they were lower in S+SMS50 than in S+SMS5, indicating the effect of the SMS rate in the water extractable fraction. These amounts decreased over time, but after 303 days they were still higher in the unamended soil. These results are related with the adsorption coefficient of tebuconazole, which is higher for the SMS-amended soils, indicating a higher bioavailability of the fungicide in the unamended soil than in the amended ones. However, the amounts of ^{14}C -tebuconazole extractable with methanol followed the order: S+SMS50 (67.1%) > S+SMS5 (62.0%) > S (45.5%), but the decrease over time was higher in the SMS-amended soils compared to the unamended soil according to the highest dissipation rate of fungicide in the amended soils (Figure 1, Table 3). At the end of the incubation period, the extracted ^{14}C amounts were higher in the unamended soil (36.3%) compared to the SMS-amended soils (25.2%-28.6%).

The water extractable ^{14}C residues for cymoxanil were initially higher than for tebuconazole, in agreement with its potential bioavailability, and they decreased over time. These amounts were higher in the unamended soil (48.2%), and decreased when the SMS rate applied to the soil increased (43.7%-19.1%). At the end of the incubation period, the water extractable ^{14}C residues decreased in all the soils, ranging between 1.19% and 2.25%. The methanol extracts for ^{14}C -cymoxanil were initially higher (6.70%-7.77%) in the SMS-amended soils than in the unamended one (3.96%), and decreased quickly in all the soils, with the extracted amounts being $< 1\%$ after nine days. The water and methanol extractable ^{14}C amounts correspond to cymoxanil and its metabolites formed during degradation, and these results were consistent with the higher extraction of labelled fungicide compared to non-labelled cymoxanil.

The amounts of non-extractable residues in the soils were initially lower for tebuconazole than for cymoxanil. These amounts increased with incubation time for tebuconazole, whereas a decrease was observed for cymoxanil. The formation of non-extractable ^{14}C -cymoxanil residues decreased with incubation time due to an increase in the $^{14}\text{CO}_2$ evolved. Non-extractable residues could be available for degradation or mineralization due to re-equilibration between the sorbed and soluble phases (Alonso et al., 2015). However, the formation of bound residues for tebuconazole continued throughout the incubation period, indicating a continuous incorporation of new residues in the non-extractable residues pool, and greater physicochemical interactions between the fungicide and the soil, as reported for other pesticides in previous works (Barriuso et al., 2008; Fenlon et al., 2011; Marín-Benito et al., 2014; Alonso et al., 2015).

The percentages of bound residues of cymoxanil after 102 days were similar in both the unamended and S+SMS5 soils (28.2% and 27.8%, respectively) and increased in S+SMS50 (50.5%). For tebuconazole, these percentages increased in the SMS

amended soils (53.2% and 63.7% in S+SMS5 and S+SMS50, respectively) compared to the unamended soil (35.2%) after 303 days of incubation. This is consistent with the higher dissipation rate of tebuconazole in the amended soils compared to the unamended one (Table 3), with the formation of bound residues decreasing its bioavailability. The non-extractable residues could come from the parent compounds or metabolites of fungicides, as shown for other pesticides (Marín-Benito et al., 2014), and it seems to be controlled by the soil OC content (Mamy et al., 2005).

3.3. Effect of SMS and fungicides on soil dehydrogenase activity

Dehydrogenase activity (DHA) was analyzed for the unamended and amended soils, either untreated or treated with fungicides during the dissipation period, as a useful indicator of overall microbial activity in soil to evaluate the impact of disturbances on soil quality (García-Izquierdo et al., 2003).

The DHA values obtained were dependent upon the amendment amount and incubation time for both fungicides studied (Figure 4). The addition of SMS at different rates to the soil significantly increased the DHA values ($p < 0.001$), showing the stimulatory effect of the amendment on soil microbial activity, as previously reported (Kadian et al., 2012; Marín-Benito et al., 2014). Soil DHA follows the order $S < S+SMS5 < S+SMS50$ for the untreated soils or those treated with both fungicides. Gao et al. (2015) have reported higher activities in SMS-biomixtures due to a more readily available carbon. However, DHA decreased significantly with incubation time in all the soils and treatments studied ($p < 0.001$ and $p < 0.05$ for soils treated with tebuconazole or cymoxanil, respectively). This decrease in DHA recorded in the soils treated with both fungicides over time is because the fungicides were dissipated with incubation time, and they were less available for microorganisms.

The effect of fungicide on soil DHA was not significant ($p > 0.1$) for either fungicide treatment when considering all the soils together. At the beginning of incubation, the DHA values in the SMS amended-soils treated with tebuconazole were higher than the control soils (without fungicide), indicating that soil microbial activity was stimulated by an extra carbon source provided by the addition of pesticides and amendment to the soil (Herrero-Hernández et al., 2011; Muñoz-Leoz et al., 2013). This effect was also observed for cymoxanil, but only in the soil amended with the highest rate of SMS. Previous works, nevertheless, have reported that tebuconazole leads to a reduction in microbial activity, probably due to its negative effect on soil fungal communities (Muñoz-Leoz et al., 2011; Cycon et al., 2006).

4. Conclusions

The results obtained in this work reveal changes in the dissipation and bioavailability of tebuconazole and cymoxanil in soil amended with SMS. The dissipation rate of both fungicides was lower in the unamended soil than in both the SMS-amended soils. The apparent increase in the dissipation rate of both compounds in amended soils was due to an increase in the adsorption by these soils in an unavailable form. Bound residues of tebuconazole increased in all the soils over incubation time, although its bioavailability was lower in the SMS-amended soils. Bound residues of cymoxanil were higher in the SMS-soil, and they decreased over incubation time in all the soils because cymoxanil was mineralized from the start of the incubation period. The mineralization of ^{14}C -tebuconazole and ^{14}C -cymoxanil was higher in the unamended soil compared to the SMS-amended soils due to the higher fungicide retention by the amended soils. Therefore, SMS applied at different rates to soil revealed a potential for designing strategies to prevent the diffuse or point

contamination of soils because it could reduce the extractable fraction of fungicides through the formation of non-extractable residues. However, soil particles containing these less-extractable residues of fungicides may be mobilized and transported into aquatic ecosystem where they can be released and other processes such as fungicide leaching or runoff should be taken into account to have a further view of the effect of the proposed soil amendment strategy..

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Figure legends

Figure 1. Degradation kinetics of tebuconazole and cymoxanil in unamended soil and soil amended with SMS at rates of 5% and 50%. Bars indicate the standard deviation of the mean value (n=4).

Figure 2. Peak areas of tebuconazole metabolites (m/z=322 and 168) over time in unamended soil and soil amended with SMS at rates of 5% and 50%. Error bars indicate standard deviation of the mean value (n=4).

Figure 3. Mineralized, CaCl₂-extractable, methanol-extractable and non-extractable fractions of ¹⁴C-tebuconazole and ¹⁴C-cymoxanil in unamended and SMS-amended soils.

Figure 4. Soil dehydrogenase activity for unamended (S) and SMS-amended soils (S+SMS5 and S+SMS50), untreated (control) and treated with tebuconazole or cymoxanil (2 mg kg⁻¹) at different sampling times. Bars indicate the standard deviation of the mean (n=4). Different letters indicate significant differences (p < 0.05), considering effects of amendment, fungicide and incubation time.

Table 1. Chemical structure and physicochemical properties of fungicides.

Common name	Chemical structure	WS ^a	log Kow ^b	Koc ^c	DT ₅₀ ^d	GUS ^e
IUPAC name		(mg L ⁻¹)		(mL g ⁻¹)	(days ⁻¹)	index
Tebuconazole		36	3.7	769	63-365	2.0
Cymoxanil		780	0.0, -0.79	43.6	0.7-3.5	-0.37

^a Solubility in water at 20 °C, ^b Octanol/water partition coefficient at pH 7-8 and 20 °C, ^c Sorption coefficient normalized to organic carbon content. ^d Time to degradation 50% of compound, ^e Gustafson mobility index of fungicides (Data taken from PPDB 2015).

Table 2. Characteristics of unamended and amended soils.

	pH	OC	DOC	C/N	Sand	Silt	Clay
		(%)	(%)		(%)	(%)	(%)
Soil	7.52	0.67	<0.01	11.2	67.0	11.9	21.1
S+SMS5	7.26	1.73	0.06				
S+SMS50	7.19	16.3	0.44				

Table 3. Kinetics parameters for the dissipation of tebuconazole and cymoxanil in unamended and SMS amended soils.

	SFO model				FOMC model				
	k (day ⁻¹)	DT ₅₀ (days)	r ²	χ ²	α	β	DT ₅₀ (days)	r ²	χ ²
Tebuconazole									
Soil	0.001	592±119	0.78	6.0	0.06	0.60	> 1000	0.91	3.6
S+SMS5	0.005	141±9.26	0.93	5.9	1.25	1.70 x 10 ²	126±29.8	0.93	5.5
S+SMS50	0.004	168±18.2	0.90	10.6	1.03 x 10 ³	2.54 x 10 ⁵	168±21.1	0.90	10.9
Cymoxanil									
Soil	0.90	0.8±0.0	0.99	6.4	6.74 x 10 ⁴	7.50 x 10 ⁴	0.8±0.0	0.99	6.8
S+SMS5	1.90	0.4±0.0	0.99	5.4	1.24	0.30	0.2±0.0	0.98	5.9
S+SMS50	1.62	0.4±0.1	0.99	1.0	6.64 x 10 ²	4.10 x 10 ²	0.4±0.1	0.98	1.7

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