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DISSIPATION OF HERBICIDES AFTER REPEATED APPLICATION IN SOILS AMENDED WITH GREEN COMPOST AND SEWAGE SLUDGE

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ABSTRACT

Certain agricultural practices, such as the repeated application of herbicides or organic amendments to soil, can influence herbicide dissipation. This research has studied the effects of two repeated applications of mesotrione, pethoxamid, and triasulfuron on their dissipation rates in unamended soil (S) and soil amended with green compost (S+GC) or sewage sludge (S+SS). The dissipation experiment has also included an evaluation of the adsorption of the three herbicides by soils and of changes in soil dehydrogenase activity (DHA). The adsorption of the three herbicides by amended soils (Kf range 0.83-2.98) was higher than by unamended soil (Kf range 0.20-0.64). The adsorption coefficients (Kd) of mesotrione and triasulfuron were higher for S+SS, while that of pethoxamid was higher for S+GC, but no relationship between values for the time to 50% degradation (DT_{50}) and adsorption coefficients could be determined. The repeated application of mesotrione decreased its dissipation rate in unamended soil (DT_{50} increased from 4.75 to 8.15 days) and amended soils (DT_{50} increased from 11.7 to 28.2 days in S+GC and from 17.7 to 37.9 in S+SS), whereas the repeated application of pethoxamid increased its dissipation rate in all the treatments, and the rate for triasulfuron increased only in amended soils. The highest DT_{50} values for pethoxamid (12.3 days) and triasulfuron (57.1 days) were in S+GC, and the lowest in S+SS (8.35 and 24.7 days). Soil DHA was stimulated by the presence of GC in the soil and by the first application of mesotrione. The second application of mesotrione and pethoxamid positively affected soil DHA, but this did not occur for triasulfuron. The repeated applications of herbicides and soil organic amendments have different effects on herbicide dissipation, adsorption, and soil DHA, and they should be taken into account when assessing soil quality and other potential environmental implications of pesticide use.

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Keywords: herbicide; repeated application; soil organic amendment; dissipation; adsorption; dehydrogenase activity

1. Introduction

Soils in the Spanish region of "Castilla y Leon" belong to the Mediterranean area, where the organic matter (OM) content is low (< 1%). The use of organic amendments in soils to increase the organic carbon (OC) content and improve their fertility and stability is becoming more frequent (Diacono and Montemurro, 2010). Furthermore, this practice could mean that pesticides are not lost through runoff or leaching because soil OC affects pesticide fate involving adsorption, transport and degradation processes with various chemical interactions (Rodríguez-Cruz et al., 2012). The soluble organic carbon (DOC) provided by organic amendments can also modify the adsorption and leaching of pesticides in soils (Marín-Benito et al., 2013; Álvarez-Martín et al., 2017). The influence of OC and DOC contents on the adsorption of pesticides with different characteristics has already been addressed (Marín-Benito et al., 2012; Gamiz et al., 2018; Paradelo et al., 2018)

Some authors have reported a lower adsorption of hydrophobic compounds by soils with DOC (Wanner et al., 2005). On the other hand, some studies have indicated that DOC could be adsorbed by the soil, increasing pesticide adsorption (Singh et al., 2003; Said-Pullicino et al., 2004; Rodríguez-Liebana and Peña, 2018).

Studies on the dissipation of pesticides in unamended and amended soils are usually carried out with the single application of one compound. However, the repeated application of individual pesticides to the same crop is a common agricultural practice, particularly in areas of intensive agriculture (horticultural crops, vine and fruit crops, etc.) (Singh et al., 2002; Fang et al., 2018). The repeated application of pesticides over a

long period can influence pesticide dissipation by accelerating biodegradation by adapted soil microorganisms, which may reduce or eliminate the pesticide's efficacy for controlling the target pest, disease or weed (Fang et al., 2015; Wang et al., 2015). On the other hand, pesticide residues could remain in the soil after repeated applications, having an effect on soil microbial function and structure (Singh et al., 2002; Xu et al., 2014; Wang et al., 2015). In some cases, repeated application can have a detrimental effect on the microbial community degrading the pesticide, significantly compromising its performance (Singh et al., 2002; Wang et al., 2015).

Three herbicides (mesotrione, pethoxamid, and triasulfuron) were used in this study. These herbicides have different characteristics and a broad spectrum of activity against weeds. They are extensively used worldwide, although there are only a few reports on the fate of these compounds in amended soils, and especially on their dissipation and effect on soil microbial communities (Pose Juan et al., 2015a, 2017).

Mesotrione (2-(4-mesyl-2-nitrobenzoyl) cyclohexane-1,3-dione) is a triketone herbicide used for the pre- and post-emergence control of broad-leaved weeds and annual grass weeds in maize (PPDB, 2017). It is weakly acid, non-volatile, and highly soluble in water (Dumas et al., 2017). Its anionic character means its adsorption to soil is low, indicating leaching potential (Dyson et al., 2002; Shaner et al., 2012; Mendes et al., 2016). Mesotrione is mostly degraded by microorganisms (Su et al., 2017). Most of the studies on the adsorption and degradation of mesotrione have been carried out in unamended soils (Dyson et al., 2002; Chen et al., 2012; Mendes et al., 2016). The influence of application dose, pH, temperature, soil moisture, soil OC content, soil texture, soil microorganisms and sunlight on its dissipation and soil microbial function and diversity has been mainly studied in unamended soils (Crouzet et al., 2010; Barchanska et al., 2016; Mendes et al., 2017; Su et al., 2017).

Pethoxamid (2-chloro-N-(2-ethoxyethyl)-N-(2-methyl-1-phenylprop-1-enyl) acetamide) is a chloroacetamide herbicide used for both grass and broadleaf weeds control in maize and soybean in pre-emergence and post-emergence. It is moderately soluble in water, and records low volatility and leachability (PPDB, 2017). This substance has only recently (2001) been included in Annex I of Directive 91/414/EEC. Hence there are few studies on its behaviour in soils, most of which are related to its toxicity or to its efficacy and selectivity (Dhareesank et al., 2005; Jursík et al., 2013). It records low adsorption, a medium level of leaching in soil, being considered a non-persistent herbicide in soil (Jursík et al., 2013; PPDB, 2017).

Triasulfuron (1-[2-(2-chloroethoxy) phenylsulfonyl]-3-(4-methoxy-6-methyl-1, 3, 5-triazin-2-yl) urea is a sulfonylurea herbicide used in pre- and post-emergence for weed control in crops such as rice, wheat, soybean, sugar beet, and maize. It is a weak acid with low volatility, and high solubility in water and leachability (PPDB, 2017). To our knowledge, most of the studies developed so far have been on adsorption in unamended and amended soils (Said-Pullicino et al., 2004; Gennari et al., 2008; Pinna et al., 2009). Triasulfuron is mostly degraded by microorganisms and by chemical hydrolysis, and this degradation is influenced by soil OM and clay content, pH, fertilizers and amendments, and climatic conditions (Pose-Juan et al., 2017).

As part of the same research project, the authors have already conducted studies on the dissipation of mesotrione, pethoxamid, and triasulfuron applied in increasing doses to unamended and amended soils, and their effect on soil microbial communities (Pose-Juan et al., 2015a, 2015b, 2017). However, to our knowledge, no research is available on the dissipation of these herbicides in unamended and amended soils when repeated applications are carried out.

The aim of this work was therefore to evaluate the influence of two repeated applications (dose = 2 mg kg^{-1} / application) of the herbicides mesotrione, pethoxamid, and triasulfuron on their dissipation in an unamended soil and this one amended with two organic amendments: green compost (GC) and sewage sludge (SS). To support this research, studies were simultaneously carried out to determine the following: (i) the adsorption of the three herbicides by unamended and amended soils, and (ii) soil dehydrogenase activity (DHA) as an indicator of soil microbial activity.

2. Materials and methods

2.1. Chemicals

Analytical standards of mesotrione (2-(4-mesyl-2-nitrobenzoyl) cyclohexane-1,3-dione; 99.9% a.i.), pethoxamid (2-chloro-*N*-(2-ethoxyethyl)-*N*-(2-methyl-1-phenylprop-1-enyl) acetamide; 94.8% a.i.), and triasulfuron (1-[2-(2-chloroethoxy) phenylsulfonyl]-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl) urea; 97.1% a.i.) from PESTANAL® were supplied by Sigma-Aldrich Química S.L. (Madrid, Spain). The characteristics of these compounds are presented in **Table 1** (PPDB, 2017).

HPLC grade acetonitrile, acetone, methanol and water were supplied by VWR International Eurolab (Spain). Calcium carbonate, 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 residues and soil

Green compost (GC), which is comprised of pruning wastes from gardens and parks, was supplied by the Salamanca Local Authority (Salamanca, Spain). Sewage sludge (SS) from a domestic waste treatment plant and stabilized by anaerobic digestion

125 was supplied by Aqualia SA (Salamanca, Spain). Some characteristics of these organic
126 residues were determined in samples previously air dried, homogenized and sieved (< 2
127 mm) (Table 2). The pH was determined in a residue/water suspension (1/2.5 w/v ratio).
128 Organic carbon (OC) content was determined by oxidation (Walkley-Black method).
129 Dissolved organic carbon (DOC) was determined in a suspension of residue (1/100 w/v
130 ratio) in Milli-Q ultrapure water after residue shaking (24 h at 20ºC), centrifugation (20
131 min at 10000 rpm), and filtering (Minisart NY 25 filter 0.45µm, Sartorius Stedim
132 Biotech, Germany) using a Shimadzu 5050 (Shimadzu, Columbia, MD, USA) organic
133 carbon analyzer. Total N was determined by the Kjeldahl method.

134 The soil used in all the experiments was taken from the surface horizon (0-30
135 cm) of an agricultural farm in Zamora (NW-Spain). It is a Typic Xerorthent (Soil
136 Survey Staff, 2006), and its texture was classified as sandy loam (83.4% sand, 5.9% silt,
137 and 10.7% clay). The amended soils were prepared in October 2012 by uniformly
138 mixing soil with GC or SS *in situ* using an agricultural rotovator at a rate of 50 t ha^{-1}
139 (considering a soil depth of ~10 cm and a soil density of 1.3 g cm^{-3}). As noted earlier,
140 unamended soil is referred to as S, whilst soil amended with GC or SS is referred to as
141 S+GC and S+SS, respectively. Undisturbed soil samples of all the treatments (~30 kg)
142 were kept under environmental conditions in 60 x 40 x 25 cm trays on site at IRNASA
143 (Salamanca, Spain) allowing drainage. After three months of incubation, fresh samples
144 of unamended and amended soils were sieved (<2 mm) prior to their use in the
145 adsorption and dissipation experiments. Characteristics of unamended and amended
146 soils were determined using standard analytical methods (Sparks, 1996) and are
147 included in Table 2. The DOC was determined in soil extracts (1/2 w/v ratio) as
148 previously indicated for organic residues. The particle size distribution was determined
149 using the pipette method. Clay minerals (smectite, illite and kaolinite) were qualitatively

identified in the soil clay fraction by X-ray diffraction using CuKα radiation on a Philips PW1710 diffractometer (Eindhoven, The Netherlands).

2.3. Dissipation studies

Initially, the herbicides were individually dissolved in acetonitrile to give a concentration of 1000 mg L^{-1}. Solutions of the herbicides were then prepared in Milli-Q ultrapure water, and a volume of 10 mL of appropriate concentration was spiked to 800 g of fresh weight of unamended or amended soils to give a concentration of 2 mg kg^{-1} dry soil. Soil samples were incubated at 20 ^{o}C in the dark. The moisture content of the soil samples was previously adjusted to 40% of the maximum soil water-holding capacity, and it was maintained by adding sterile Milli-Q ultrapure water when necessary. Each soil treatment was prepared in duplicate. All the soils were thoroughly stirred with a sterilized spatula, and all the steps were performed in a sterile cabinet. Samples of the soils individually treated with mesotrione, pethoxamid, and triasulfuron were taken for pesticide analysis at different time intervals for 99, 43 and 144 days, respectively (according to the dissipation rates of each pesticide). A second treatment of the soils with the herbicides was applied at the same dose of 2 mg kg^{-1} when the concentrations of each specific herbicide were between 30% and 70% of the amount initially added. This second treatment was applied to the soils previously treated with mesotrione, pethoxamid, and triasulfuron at 14, 9 and 35 days, respectively.

2.4. Extraction and determination of herbicides

Duplicate 5 g samples of each treatment (800 g of unamended or amended soil) were taken at each sampling time and shaken in glass tubes at 20 ^{o}C for 2 h (mesotrione and triasulfuron) or 24 h (pethoxamid) with 10 mL of methanol: water (1:1),

175 acetone:acetonitrile (1:1) or methanol: acetone (1:1) for mesotrione, pethoxamid, or
176 triasulfuron extraction, respectively. The soil samples treated with mesotrione or
177 triasulfuron were then sonicated for 1 h. In all cases, the samples were centrifuged at
178 5045 g for 15 min, and the herbicide extracts were passed through a Minisart NY 25
179 filter (Sartorius Stedim Biotech, Germany) to remove particles > 0.45 μm. For the
180 determination of the herbicides, a volume of the extract was transferred to a glass vial
181 for analysis. The recoveries of the extraction method were determined by spiking three
182 unamended and amended soil samples with an analytical grade of each herbicide to a
183 final concentration of 2 mg kg^{-1}, and then performing the extraction procedure as
184 described above. The mean recovery values were > 70% for the three herbicides studied
185 (Pose-Juan et al. 2015b, Pose-Juan et al., 2017).

186 Herbicides were quantified by HPLC with a diode array detector (DAD) and a
187 mass spectrometer (MS) (Waters Associates, Milford, MA), using Empower software as
188 the data acquisition and processing system. The analytical column was a Luna PFP (2)
189 (150 × 4.6 mm i.d., 3.0 μm) (Phenomenex, Torrance, CA, USA). The mobile phase was
190 90:10 (v/v) acetonitrile/water (1% formic acid for mesotrione and triasulfuron, and
191 0.1% formic acid for pethoxamid). The flow rate of the mobile phase was 0.4 mL min^{-1}
192 and the sample injection volume was 10 μL. Retention times were 5.6, 6.1, and 5.1 min,
193 for mesotrione, pethoxamid, and triasulfuron, respectively.

194 Quantitative analysis was performed using the peak area of the herbicides
195 obtained from the total ion chromatogram (TIC) in SIM mode. The molecular ions
196 (m/z) corresponding to mesotrione, pethoxamid, and triasulfuron in the positive
197 ionization mode $[M + H]^+$ were 340.0, 296.8, and 402.8, respectively. Calibration was
198 performed from 0.5 to 25 μg mL^{-1} ($r \geq 0.99$), and the limit of detection (LOD) and limit
199 of quantification (LOQ) were 0.0018 and 0.020 μg mL^{-1}, 0.0031 and 0.010 μg mL^{-1}, and

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200 0.012 and 0.113 μg mL^{-1} for mesotrione, pethoxamid, and triasulfuron, respectively.

201

202 *2.5. Adsorption studies*

203 The adsorption curves of pesticides for unamended and amended soils were
204 obtained using the batch equilibrium technique. Duplicate soil samples (2.5 g for
205 mesotrione and triasulfuron, and 5 g for pethoxamid) were equilibrated with 10 mL of a
206 0.01 M $CaCl_2$ Milli-Q ultrapure water solution of each herbicide at concentrations of 1,
207 5, 10, 15, 20 and 25 μg mL^{-1}. The soil/solution ratios were previously optimized. The
208 suspensions were shaken at 20 ºC for 24 h in a thermostated chamber, with intermittent
209 shaking for 2 h at three-hour intervals. Preliminary experiments revealed that contact for
210 24 h was long enough for attaining equilibrium. The suspensions were subsequently
211 centrifuged at 5045 g for 15–30 min, filtered, and the equilibrium concentration of each
212 herbicide was determined by HPLC-MS, as indicated above. The amount of herbicide
213 adsorbed was calculated as the difference between that initially present in the solution
214 and that remaining after equilibration with the soil.

215

216 *2.6. Soil dehydrogenase activity (DHA)*

217 Soil dehydrogenase activity (DHA) was determined following the Tabatabai
218 method (Tabatabai, 1994), which is based on the extraction and colorimetric
219 determination of the intensely coloured 2,3,5-triphenylformazan (TPF) produced by
220 reducing colourless triphenyltetrazolium chloride (TTC) in soils. Soil DHA was
221 determined at the beginning of the dissipation experiments (0 days), and after 14, 36
222 and 99 days for the mesotrione experiment, 9 and 43 days for the pethoxamid
223 experiment, and 35, 98 and 144 days for the triasulfuron experiment. These sampling
224 times were set after the first and second herbicide application, at an intermediate time in

the second dissipation kinetics of each herbicide, and when the herbicides were almost completely dissipated or at the end of the incubation of soils with herbicides.

2.7. Data analysis

Herbicide adsorption data were fitted to the Freundlich equation, and adsorption and non-linearity coefficients, K_f (μg^{1-nf} g^{-1} mL^{nf}) and n_f, respectively, were obtained. Distribution coefficients, K_d (mL g^{-1}), were determined for equilibrium concentrations (Ce) of 5 and 15 μg mL^{-1} (K_d5 and K_d15) of each herbicide.

The dissipation kinetics for herbicides was fitted to a single first-order (SFO) kinetic model or first order multicompartment (FOMC or Gustafson and Holden) model. FOCUS work group guidance recommendations were followed for selecting the kinetic model that best describes the dissipation results (FOCUS, 2006). The coefficient of determination (r^2) and the chi-square (χ^2) test were calculated as indicators of goodness of fit. Values for the time to 50% dissipation (DT_{50}) were used to characterize the decay curves and compare variations in dissipation rates. The parameters of the kinetic models were estimated using the Excel Solver add-in package (FOCUS, 2006).

Standard deviation (SD) was used to indicate variability among replicates. Data were analysed by one-way or two-way ANOVA. Fisher´s least significant difference (LSD) method was used, at a confidence level of 95%, to determine significant differences between means and evaluate the effects of the different treatments on DT_{50}, Kf and Kd and soil DHA values. SPSS Statistics v22.0 software for Windows (IBM Inc., Chicago, ILL, USA) was used.

3. Results and discussion

3.1. Adsorption studies

Fig. 1 includes the adsorption curves of mesotrione, triasulfuron, and pethoxamid for unamended (S) and amended soils (S+GC, and S+SS). The adsorption curves fitted the Freundlich equation with r^2 values in the range 0.80-0.98 ($p < 0.05$-0.01), and the K_f, and n_f parameters determined from the linear form of this equation are included in **Table 3**.

The adsorption curves of the weak acid herbicides mesotrione and triasulfuron were, in general, non-linear, L-type. These herbicides were in an anionic form because the pH of soils is higher than the pKa of compounds (Table 2), and due to the presence of two plateaus the curves obtained were classified as sub-group 4 (Giles et al. (1960). Similar adsorption curves were obtained for other anionic compounds with different sorbents (Atay et al., 2002; Sánchez-Martín et al., 2008), with the second rise in the curves being attributed to the development of a fresh surface on which adsorption can occur, with the second plateau representing the complete saturation of this new surface (**Fig. 1**). The n_f values were in the range 0.68-0.90 and 0.70-1.27 for the adsorption of mesotrione and triasulfuron by unamended and amended soils, respectively. These values for mesotrione indicate a relatively higher affinity for all the soils at a low concentration, and a reduction in adsorption at a higher concentration (Pinna et al., 2014). The L-type curves of mesotrione could be due to herbicide adsorption on soil organic matter (Alekseeva et al., 2014). This behaviour was also observed for the adsorption of triasulfuron by amended soils, while it did not occur for the unamended soil (Table 3). The high water solubility of triasulfuron could prevent the herbicide's adsorption by the soil at low concentrations when herbicide compete with the water by soil surfaces as reported for some polar compounds (Stangroom et al., 2000).

The adsorption curve of pethoxamid for unamended soil was of S-type ($n_f > 1$), while its adsorption by S+SS and S+GC was close to linearity (C-type) (n_f =1.16 and

0.96). These different types of curves are to be expected considering that pethoxamid is a compound with a slightly hydrophobic character, but with high water solubility (Table 1). Non-linear curves are generally obtained when specific interactions or different interaction mechanisms are involved in the adsorption of a compound by an adsorbent. This could occur here, considering the herbicides' characteristics and the changes in soil surface caused by the different amendments (Marín-Benito et al., 2009; Said-Pullicino et al., 2004).

The K_f values for the adsorption of the anionic herbicides mesotrione and triasulfuron by unamended and amended soils fell within the ranges 0.64-1.12 and 0.61-2.66, respectively, while it was 0.20-2.98 for the adsorption of the non-ionic herbicide pethoxamid. The adsorption of the more hydrophobic herbicide pethoxamid by the unamended soil was lower than that of the anionic herbicides. However the adsorption by the amended soils increased for the three herbicides due to their higher OC content. In general, this effect was also recorded for the distribution coefficients (K_d) determined at equilibrium concentrations of 5 and 15 μg mL^{-1} to compare the adsorption capacity of herbicides by soils, because the n_f values of the adsorption curves were different to 1. The K_d5 and K_d15 values for the herbicide mesotrione and K_d5 for the herbicide triasulfuron were higher in S+SS than in S+GC, although these values were higher for pethoxamid in S+GC (**Table 3**). The lower pH of S+SS could increase the adsorption of anionic herbicides observed in some cases by this soil together with the higher OC and DOC contents, which could give rise to the formation of new hydrophobic surfaces, as observed for other compounds (Álvarez-Martín et al., 2016). However, the K_d15 values were in general lower than the K_d5 values for the most hydrophilic compounds (mesotrione and triasulfuron), and higher for pethoxamid (more hydrophobic) in agreement with the adsorption curves obtained. These curves showed a plateau for

hydrophilic herbicides representing the complete saturation of adsorbent surface with the increase of herbicide concentration. This behaviour could be explained taking into account that different adsorption mechanism could be involved for the adsorption of the anionic herbicides and the non-ionic herbicide pethoxamide as reported previously (Rodríguez-Cruz et al., 2009).

A correlation analysis between the adsorption parameters and the soil characteristics was carried out to extend the information on the influence of soil parameters on the adsorption of these herbicides. OC and DOC contents and pH were the parameters considered in the analysis because they are the parameters influencing the adsorption of organic compounds with different characteristics (Stangroom et al., 2000; Rodríguez-Cruz et al., 2012). The CEC of soils was also reported as a factor influencing the adsorption of ionic compounds. However in this study CEC were not considered in the analysis because a highly significant relationship between CEC and OC contents of soils was found (r=0.993).

For mesotrione, the results reveal no significant correlation of K_f values with any soil parameters. However, there was a positive relationship (not significant because of the low number of samples examined) between the distribution coefficients (K_d5 and K_d15) and soil OC content. This indicates the influence of soil OC content in the adsorption when the concentration of mesotrione in solution increased ($Ci \geq 10$ µg mL^{-1}). A positive, significant relationship was observed between K_d15 values and soil DOC contents ($r = 0.99$, $p < 0.1$), while no significant correlation was observed between any adsorption coefficients and soil pH. Mesotrione is an ionic compound, and relationships between adsorption and soil pH have been reported (Dyson et al., 2002). The influence of OC and clay mineral contents and CEC have also been reported (Dyson et al., 2002; Shaner et al., 2012; Alekseeva et al., 2014; Pinna et al., 2014; Mendes et al., 2016).

The results for triasulfuron also indicate the influence of OC and DOC contents on its adsorption by soils, although positive relationships were only observed at low concentrations of herbicide in solution (Ci = 1-5 μg mL^{-1}), that is, between K_f or K_d5 and soil OC and DOC contents, significant in the case of K_f values and OC contents ($r = 0.99$, $p < 0.1$). A negative but non-significant correlation between K_d15 and soil pH was also obtained. Data on the influence of soil pH and soil OC, DOC and humic acid contents on the adsorption of triasulfuron by unamended and amended soils have been also reported (Said-Pullicino et al., 2004; Pinna et al., 2009). There is a higher adsorption of this herbicide by unamended soil than by the amended soils at higher concentrations of triasulfuron in solution (Ci≥20 μg mL^{-1}), which could indicate that the clay fraction and the smectite mineral present in this soil is also important for adsorption, as observed for other ionic and water-soluble compounds (Azejjel et al., 2008).

For pethoxamid, increased adsorption was obtained by the amended soils independently of the initial herbicide concentration in solution. Adsorption was higher by S+GC, with lower OC content, than by S+SS, and a non-significant correlation was observed between the adsorption coefficients and soil parameters. The higher DOC content in S+SS than in S+GC could explain this behaviour by pethoxamid, considering the contrary effect of DOC on the adsorption reported for some hydrophobic compounds (Marín-Benito et al., 2009, 2012).

3.2 Dissipation studies

Fig. 2 shows the dissipation curves of mesotrione, triasulfuron and pethoxamid after the first and second repeated applications of 2 mg kg^{-1} in unamended and amended soils**.** The data for the residual concentrations of herbicides as a function of time were

fitted to the SFO kinetic model ($r^2 \geq 0.95$ for mesotrione, $r^2 \geq 0.98$ for pethoxamid, and $r^2 \geq 0.96$ for triasulfuron) to calculate the dissipation rate constants (k) and DT_{50} values. These kinetic parameters for the first application of the herbicides were calculated from dissipation curves between 0 and 14, 35 or 9 days of mesotrione, triasulfuron, and pethoxamid application, respectively. The percentages of the residual amounts of herbicides at the end of the first application were between 30% and 70%. The second herbicide application was then carried out, and the kinetic parameters were calculated from dissipation curves between 14 and 99 days, 35 and 144 days and 9 and 43 days for mesotrione, triasulfuron, and pethoxamid, respectively. The percentages of residual amounts of herbicides at the end of the second application were < 25% **(Fig. 2)**.

The DT_{50} values of mesotrione after the first application were 4.75, 11.7, and 17.7 days in S, S+GC, and S+SS, respectively, and they increased to 8.15 (S), 28.2 (S+GC), and 37.9 (S+SS) days after the second application of the herbicide (Table 4). These DT_{50} values agree with the wide range of DT_{50} values (4.5-34 days) reported in the literature for mesotrione dissipation in different unamended soils (Dumas et al., 2017; PPDB, 2017). These results indicate a significant increase in DT_{50} values (up to ≈ 2.5 times) in all the treatments after the second application (LSD = 4.80, $p < 0.05$), and a reduction in the dissipation rates of mesotrione, or inhibited degradation when it was repeatedly applied. This is contrary to accelerated degradation expected due to microbial adaptation as usually reported by repeated application of herbicides (Celis et al., 2015), although a similar effect was also obtained when higher doses of mesotrione were applied in the unamended and amended soils. The effect on the decrease in dissipation by the repeated application of the herbicide was less significant than the application of the highest single dose of herbicide (2-50 mg kg^{-1}) in similar soils, as in that case DT_{50} increased up to five times (Pose-Juan et al., 2015b).

375 The dissipation of mesotrione was faster in S than in S+GC and in S+SS before
376 and after the repeated application of the herbicide consistent with the highest K_d
377 adsorption coefficients by the amended soils, as indicated above (Table 3). Su et al.
378 (2017) have reported that the use of farmyard manure as soil amendment decreases the
379 mesotrione degradation rate due to higher herbicide adsorption by soils. Furthermore,
380 the adsorption of mesotrione by these soils after repeated application could increase
381 with time, with slower dissipation rates if the mass transfer from the sorbed phase to the
382 solution is limited, as previously reported (Shaner et al., 2012). Similarly, the slower
383 dissipation rates of other pesticides after repeated application in soils were also
384 attributed to the toxicity of the increased amount of pesticide or their degradation
385 products for soil microorganisms or the lack of easily degradable OM in the soil (Singh
386 et al., 2002; Celis et al., 2015).

387 The DT_{50} values of triasulfuron after the first application were 20.4 (S), 57.1
388 (S+GC), and 24.7 (S+SS) days, and these values were 22.9 (S), 33.2 (S+GC), and 21.6
389 (S+SS) days after the second application. The DT_{50} values obtained are consistent with
390 other values reported in unamended soils (15-48 days) (EFSA, 2015). DT_{50} values were
391 similar in S and S+SS, and increased significantly in S+GC after the first and second
392 applications (LSD = 6.58, $p < 0.05$) (**Table 4**). The repeated application of herbicide in
393 the S+GC soil enhanced the dissipation rate of triasulfuron, while the rate in S or S+SS
394 was less affected. Consequently, the dissipation rates were unrelated to the adsorption of
395 this herbicide by the soils, which was higher in S+SS (Table 3). The dissipation of
396 triasulfuron did not follow a similar trend when the effect of higher doses of herbicide
397 was evaluated (Pose-Juan et al., 2017). DT_{50} values of triasulfuron increased by around
398 twice for doses applied up to 50 mg kg^{-1} in both S+GC and S+SS. The behaviour of
399 triasulfuron was different to the other anionic herbicide mesotrione because its

dissipation was slower than that of mesotrione after the first and second repeated applications. The higher adsorption of triasulfuron by unamended and amended soil than that of mesotrione could explain its lower bioavailability to soil microorganisms in spite of its characteristics (low hydrophobic character and high water solubility).

The DT_{50} values for pethoxamid after the first application were 11.9, 12.3, and 8.35 days in S, S+GC, and S+SS, respectively, and they decreased up to 8.05 days (S), 10.0 days (S+GC), and 6.75 days (S+SS) after the second application ($p < 0.05$) (**Table 4**). The DT_{50} values for the dissipation of pethoxamid applied at both repeated doses in unamended soil fell within the range of the DT_{50} values reported (6-14.8 days) (PPDB, 2017). The DT_{50} values indicated an increase in the dissipation rate by the repeated application of the herbicide. These results are consistent with previous findings for other pesticides after repeated applications, which were explained by accelerated degradation due to microbial adaptation leading to faster metabolism (Celis et al., 2015). The dissipation rate of pethoxamid in S+GC was the lowest after the first and second applications, while the highest dissipation rate was in S+SS. The DT_{50} values obtained could be explained by the higher adsorption constants of pethoxamid for S+GC than those for S or S+SS (Table 3). The low DT_{50} values in S+SS could be explained by its high content in DOC. The herbicide could be adsorbed by DOC, and the bioavailability for degradation could be enhanced if it remains adsorbed in solution, as indicated for other pesticides (Marín-Benito et al., 2012). The dissipation of pethoxamid was faster than that of the anionic herbicides mesotrione and triasulfuron, indicating the different bioavailability of the herbicides in the soil to be degraded by microorganisms. The apparent rapid dissipation of pethoxamid in amended soils could be explained by its adsorption in a more irreversible form over time after the application due to its hydrophobic character (Dhareesank et al., 2016).

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No significant correlations between DT_{50} values and soil pH or OC content were observed for any one the three herbicides, possibly due to the low number of soil samples considered in this study.

3.3. Soil dehydrogenase activity (DHA)

Soil DHA was determined in soils with different treatments, and **Fig. 3** shows the effects of the amendments (GC or SS) and herbicides (mesotrione, pethoxamid, and triasulfuron) at different incubation times after the repeated application of herbicides.

In soil without herbicides (control soils) the application of amendments to soil stimulated DHA in the case of GC, but the application of SS did not increase the mean values of DHA with respect to S (**Fig. 3**, different upper-case letters denote significant differences within treatments for unamended and amended soils untreated and treated with herbicides). The soil DHA enhancement in S+GC was expected due to the increase in soil OC and the addition of a new microbial population with this amendment, as reported previously for soils amended with different organic residues (Marín-Benito et al., 2012; Pose-Juan et al., 2015b, 2017). However, the DHA values in S+SS either decreased or were similar to those of S, although OC content in this soil also increased. Similar results have been reported in previous works, being explained by a possible toxic effect of SS for soil microorganisms (Mingorance et al., 2014; Pose-Juan et al., 2015a)

The effects of herbicides on soil DHA were variable. Mesotrione stimulated soil DHA in unamended and amended soils after the application of the first dose, but this effect did not occur for the herbicides triasulfuron (in any soil) or pethoxamid (in S and S+GC) (**Fig. 3**, different lower-case letters denote significant differences between sampling times for untreated or soil treated with herbicides within the same unamended

or amended soil). Soil DHA increased initially with the application of the second dose of mesotrione after 14 days and of pethoxamid after 9 days in the unamended and amended soils, but DHA did not increase (or decrease) for triasulfuron after 35 days (second sampling time in **Fig. 3**), indicating a positive or negative effect of the herbicides on soil microbial activity. At the end of the soil-amendment-herbicide incubation period, DHA decreased in both amended soils treated with mesotrione (99 days), in S+SS treated with pethoxamid (43 days), and in S+GC treated with triasulfuron (144 days) (**Fig. 3**).

These findings are consistent with previous results in which soil DHA was stimulated following the application of mesotrione at the recommended dose (Kacynski et al., 2016; Pose-Juan et al., 2015b). However, Borowik et al. (2017) and Kacynski et al. (2016) have reported that mesotrione applied in herbicide mixtures of mesotrione + terbuthylazine + S-metolachlor and mesotrione + S-metolachlor inhibit DHA when the herbicides are applied at doses higher than the recommended field rate. It has been reported accordingly that the application of herbicides mixtures to soil can inhibit DHA (Kacynski et al., 2016; Wyszkowska et al., 2016).

The influence of herbicides on DHA was corroborated by the relationship between the amounts of herbicides present in the different soils and the DHA values measured at the different sampling times. A positive significant relationship was obtained for mesotrione in S+GC ($r=0.716$, $p < 0.05$) and S+SS ($r=0.909$, $p < 0.01$) or when all the soils were considered ($r=0.615$, $p < 0.01$). For pethoxamid, there was a positive significant relationship between herbicide amounts and DHA values in S+SS ($r=0.902$, $p < 0.02$), and for triasulfuron this positive relationship was only significant in S ($r=0.747$, $p < 0.05$), and hardly significant in S+GC ($r=0.618$, $p \leq 0.1$). The results corroborate the stimulatory effect of the repeated application of mesotrione on DHA,

475 especially in amended soils, where mesotrione was dissipated at a slower rate after the
476 second application. However, the positive effect of the herbicides pethoxamid and
477 triasulfuron on DHA was only significant for some individual soil samples because
478 dissipation was faster after the second application, or because of a possible toxic effect
479 of the herbicide on soil microorganisms (Sofo et al., 2012).
480

481 **4 CONCLUSION**

482 The repeated application of herbicides in soils leads to dissipation effects that differ to
483 those observed with just a single application. For mesotrione, this repeated application
484 had an inhibitory effect on its dissipation rate in the unamended and amended soils
485 studied, and the herbicide could become more persistent or increase its accumulation in
486 amended soils. However, repeated applications of pethoxamid and triasulfuron
487 accelerated the dissipation of these compounds in amended soils through the possible
488 formation of bound residues over time, decreasing the bioavailability of the compound
489 to be degraded. In this case, faster dissipation could lead to a loss of efficacy. The
490 dissipation mechanism of herbicides could be explained by the adsorption constants
491 determined, which indicate the highest adsorption by amended soils. On the other hand,
492 soil DHA was stimulated by the application of GC, but not by SS. The first and second
493 application of mesotrione stimulated soil DHA, while only the second application of
494 pethoxamid increased DHA values, and DHA was not stimulated for any application of
495 triasulfuron. Therefore, the repeated application of herbicides in soils amended with
496 organic residues can modify their dissipation and affect soil microbial activity
497 depending on the type of herbicide and the characteristics of the amended soil. These
498 results should be taken into account when assessing soil quality and other potential
499 environmental implications of pesticide use.

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Figure captions

Fig. 1. Adsorption curves of mesotrione, triasulfuron and pethoxamid by unamended soil and soil amended with green compost (GC) or sewage sludge (SS) fitted to the Freundlich equation. Cs (μg g^{-1}) is the amount of adsorbed herbicide; Ce (μg mL^{-1}) is the herbicide's equilibrium concentration in the solution. Error bars represent the standard deviation of the Cs mean value (n=2).

Fig. 2. Dissipation kinetics of mesotrione, triasulfuron and pethoxamid in unamended soil and soil amended with green compost (GC) or sewage sludge (SS). The initial time of each kinetics is indicated as T_0-1st and T_0-2nd for the first and second herbicide application, respectively. Error bars represent the standard deviation of the mean value (n=2).

Fig 3. Soil dehydrogenase activity (DHA) in unamended and amended soils, untreated (control) and treated with mesotrione, triasulfuron and pethoxamid at different incubation times after the repeated application of herbicides. The second herbicide application is indicated with an arrow. Error bars represent the standard deviation of the mean (n=2). Different upper case letters denote significant differences between treatments for unamended and amended soils untreated and treated with herbicides and different lower case letters denote significant differences between sampling times for untreated or treated soil with herbicides within the same unamended or amended soil).

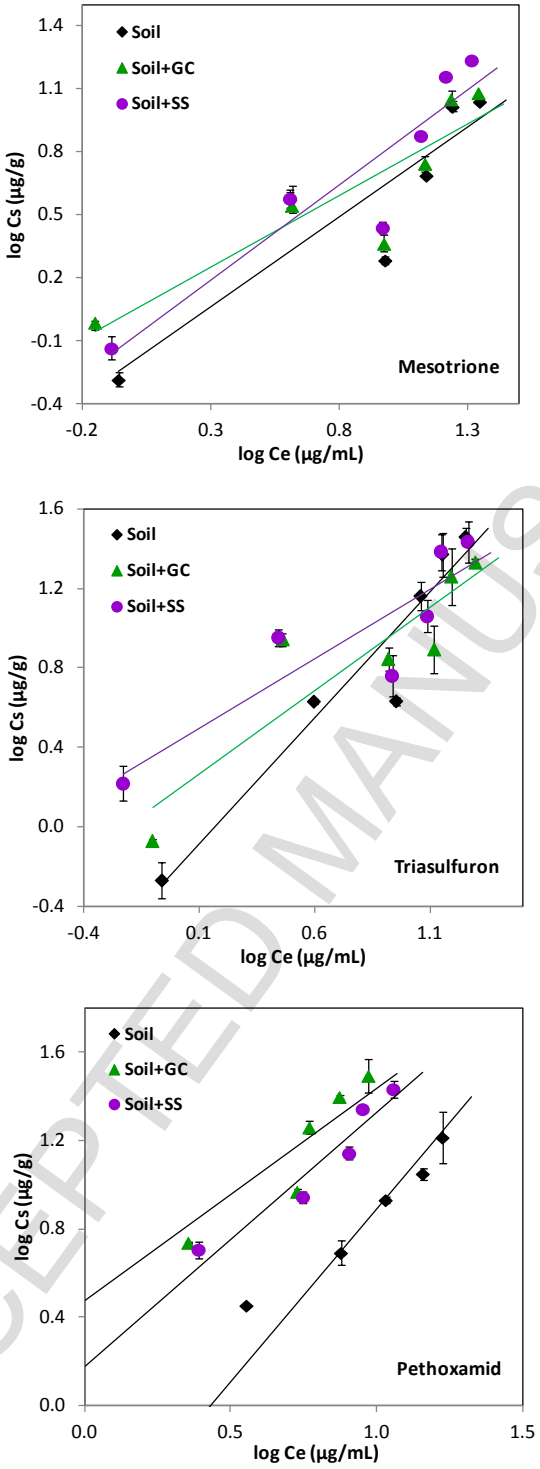


Fig. 1.

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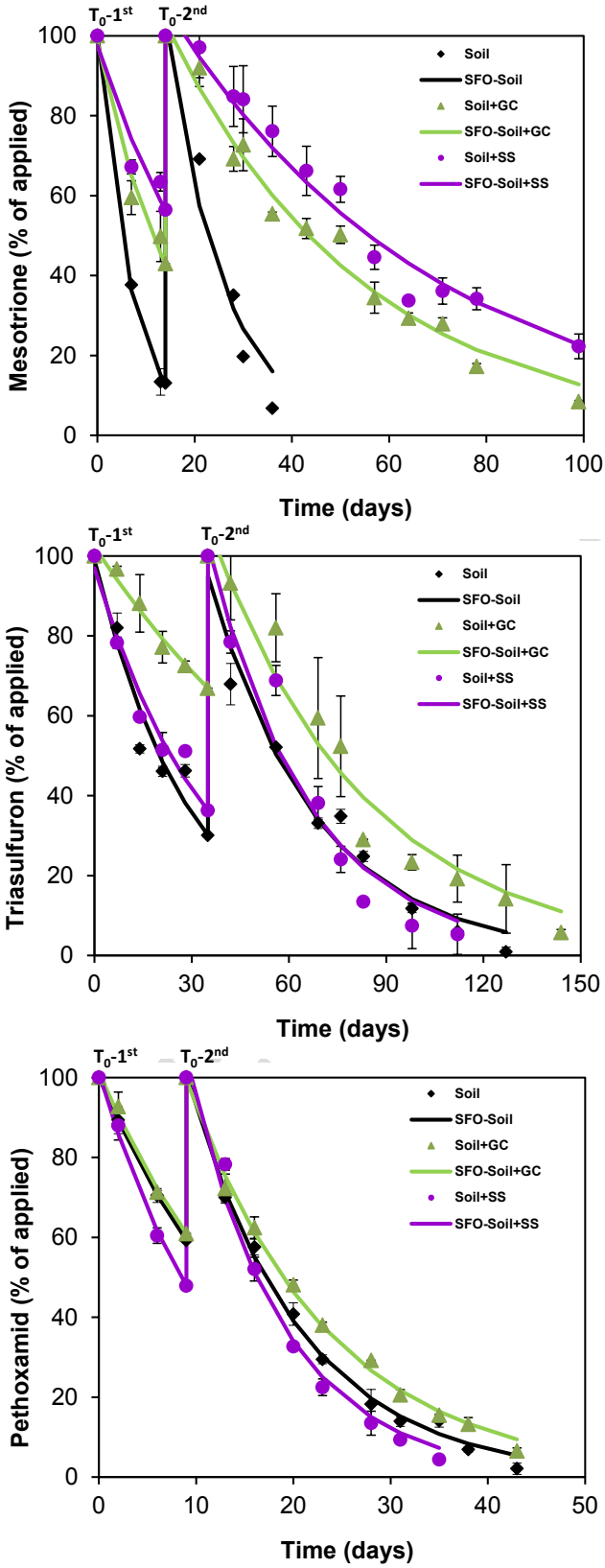

T0-1st
T0-2nd
Soil
SFO-Soil
Soil+GC
SFO-Soil+GC
Soil+SS
SFO-Soil+SS
Mesotrione (% of applied)
Time (days)
Triasulfuron (% of applied)
Pethoxamid (% of applied)

Fig. 2.

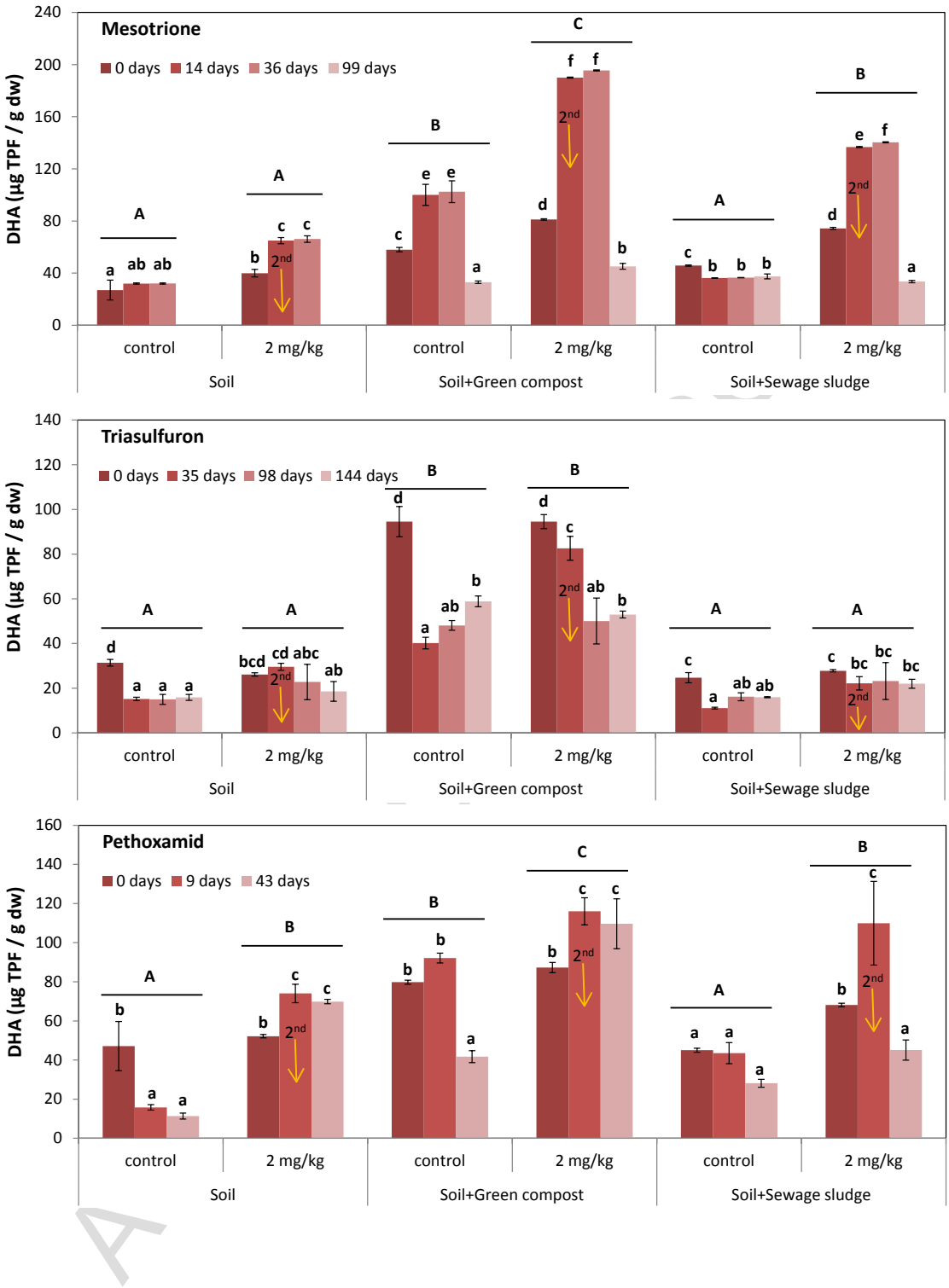

Mesotrione
0 days 14 days 36 days 99 days
DHA (µg TPF / g dw)
control 2 mg/kg control 2 mg/kg control 2 mg/kg
Soil Soil+Green compost Soil+Sewage sludge
Triasulfuron
0 days 35 days 98 days 144 days
DHA (µg TPF / g dw)
control 2 mg/kg control 2 mg/kg control 2 mg/kg
Soil Soil+Green compost Soil+Sewage sludge
Pethoxamid
0 days 9 days 43 days
DHA (µg TPF / g dw)
control 2 mg/kg control 2 mg/kg control 2 mg/kg
Soil Soil+Green compost Soil+Sewage sludge

Fig. 3.

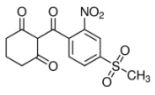
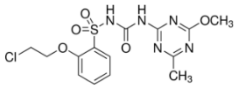
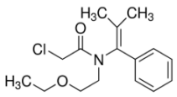
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Highlights

- Dissipation of herbicides after repeated application was evaluated in amended soils.
- Green compost and sewage sludge increased the herbicide adsorption by amended soils.
- Repeated mesotrione application decreased its dissipation in unamended and amended soils.
- Dissipation of repeated triasulfuron or pethoxamid application increased in amended soils.
- Soil dehydrogenase activity was stimulated by green compost and repeated mesotrione application.

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Table 1. Chemical structure and physicochemical properties of herbicides.

Common name IUPAC name	Chemical structure	WS[a] (mg L^{-1})	Log K_{ow}[b]	pKa	Koc[c] (mL g^{-1})	DT_{50}[d] (days)	GUS[e] index
Mesotrione (2-(4-mesyl-2-nitrobenzoyl)cyclohexane-1,3-dione)		160-1500	0.11	3.12	122	5-19.6	2.69
Triasulfuron (1-[2-(2-chloroethoxy)phenylsulfonyl]-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)urea)		32-815	-0.59	4.64	60	38-59	5.12
Pethoxamid (2-chloro-*N*-(2-ethoxyethyl)-*N*-(2-methyl-1-phenylprop-1-enyl)acetamide)		400	2.96	-	211	6-14.8	1.34

[a]Solublity in water at 20 ºC and pH 5-7, [b]Octanol/water partition coefficient at 20 ºC and pH 7, [c]Adsorption coefficient normalized to organic carbon content. [d]Time to degradation 50% of compound, [e]Gustafson mobility index (Data taken from PPDB, 2017).

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Table 2. Characteristics of organic residues and unamended and amended soils.

	Green compost (GC)	Sewage sludge (SS)	Soil (S)	S+GC	S+SS
pH	7.08	6.73	6.29	7.05	6.16
OC (%)	8.06	27.0	0.49	1.50	2.20
DOC[a] (mg g^{-1})	1.02	21.7	0.05	0.07	0.68
N (%)	0.79	4.76	0.04	0.18	0.35
C/N	10.2	5.67	12.2	8.33	6.28
CEC[b] ($cmol_c$ kg^{-1})	18.7	64.5	4.38	6.87	7.95
Na ($cmol_c$ kg^{-1})	0.27	1.22	0.03	0.03	0.02
K ($cmol_c$ kg^{-1})	56.4	4.99	0.66	2.78	0.78
Ca ($cmol_c$ kg^{-1})	28.9	24.5	6.96	17.2	10.8
Mg ($cmol_c$ kg^{-1})	3.83	14.2	2.40	3.16	4.33

[a] Dissolved organic carbon. [b] Cationic exchange capacity.

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Table 3. Freundlich adsorption constants (K_f and n_f) and distribution coefficients (K_d) of mesotrione, triasulfuron and pethoxamid by unamended soil (S) and soil amended with green compost (S+GC) or sewage sludge (S+SS).

Soil	K_f±SD[a]	n_f±SD	r^2	K_d5[b]±SD	K_d15[c]±SD
			Mesotrione		
S	0.64±0.02a	0.85±0.01	0.81	0.51±0.01a	0.43±0.01a
S+GC	1.12±0.10a	0.68±0.04	0.81	0.67±0.01ab	0.47±0.02a
S+SS	0.83±0.11a	0.90±0.04	0.87	0.71±0.07b	0.64±0.01b
LSD ($p<0.05$)	0.51			0.19	0.06
			Triasulfuron		
S	0.61±0.08a	1.27±0.09	0.92	0.94±0.08a	1.26±0.20a
S+GC	1.53±0.06b	0.84±0.12	0.81	1.18±0.20ab	0.99±0.28a
S+SS	2.66±0.11c	0.70±0.02	0.80	1.65±0.10b	1.19±0.19a
LSD ($p<0.05$)	0.39			0.62	1.02
			Pethoxamid		
S	0.20±0.06a	1.68±0.44	0.94	0.59±0.03a	1.26±0.11a
S+GC	2.98±0.77b	0.96±0.10	0.94	2.79±0.19c	2.67±0.14b
S+SS	1.49±0.05ab	1.16±0.05	0.98	1.92±0.11b	2.28±0.27b
LSD ($p<0.05$)	2.01			0.58	0.84

[a]SD, standard deviation (n=2). [b]Distribution coefficient for an equilibrium concentration of 5 μg mL^{-1}. [c]Distribution coefficient for an equilibrium concentration of 15 μg mL^{-1}. For each herbicide, K_f, K_d5 or K_d15 values followed by different letter are significantly different ($p < 0.05$).

Table 4. Kinetics parameters for the degradation of mesotrione, triasulfuron and pethoxamid after two repeated applications (1 and 2) in unamended and amended soils obtained from fitting kinetics to a single first order (SFO) model ($C=C_0\ e^{-kt}$).

Soil	Application	k ($days^{-1}$)	DT_{50} (days)	r^2	χ^2
			Mesotrione		
S	1	0.145	4.75 ± 0.2a	0.99	2.3
	2	0.085	8.15 ± 0.8ab	0.97	13.5
S+GC	1	0.059	11.7 ± 2.2b	0.97	5.2
	2	0.025	28.2 ± 0.9d	0.99	6.3
S+SS	1	0.039	17.7 ± 1.5c	0.95	5.9
	2	0.018	37.9 ± 0.5e	0.98	6.0
LSD ($p<0.05$)			4.80		
			Triasulfuron		
S	1	0.034	20.4 ± 0.7a	0.97	7.4
	2	0.030	22.9 ± 0.1a	0.99	10.5
S+GC	1	0.012	57.1 ± 3.0c	0.97	1.8
	2	0.021	33.2 ± 2.3b	0.96	11.6
S+SS	1	0.028	24.7 ± 1.3a	0.98	5.1
	2	0.032	21.6 ± 0.2a	0.97	14.1
LSD ($p<0.05$)			6.58		
			Pethoxamid		
S	1	0.058	11.9 ± 0.8cd	0.99	0.2
	2	0.086	8.05 ± 0.4a	0.99	4.5
S+GC	1	0.057	12.3 ± 0.2d	0.98	1.6
	2	0.069	10.0 ± 0.2bc	0.99	3.5
S+SS	1	0.083	8.35 ± 0.6ab	0.99	1.7
	2	0.102	6.75 ± 0.1a	0.99	7.9
LSD ($p<0.05$)			1.88		

For each herbicide, DT_{50} values followed by different letter are significantly different ($p < 0.05$).