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Changes in activity and structure of the soil microbial community after application of azoxystrobin or pirimicarb and an organic amendment to an agricultural soil

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ABSTRACT

Pesticides and organic amendments are widely used in agricultural soils, the former for pest control and to improve crop yield, and the latter to maintain soil quality and to mitigate pesticide leaching. Both amendments and pesticides can affect soil microbial communities, which may impact crop fitness. Therefore, the objective of the current study was to investigate the changes that occur to soil microbial communities following use of the low-cost organic residue spent mushroom substrate (SMS) (2% and 5% on a dry weight basis) as an amendment to an agricultural soil in combination with the widely used pesticides azoxystrobin or pirimicarb (at 2 and 25 mg kg⁻¹ rates). Dissipation experiments showed that azoxystrobin was more persistent than pirimicarb over the incubation time (90 days). The half-life (DT₅₀) values of azoxystrobin (2 mg kg⁻¹) and pirimicarb (2 and 25 mg kg⁻¹) decreased, while DT₅₀ values of azoxystrobin (25 mg kg⁻¹) increased when SMS was applied to soil. Broad-scale methods such as soil dehydrogenase activity (DHA) and soil respiration showed that SMS rate, pesticide dose and incubation time all affected microbial community metabolism. DHA increased in SMS-amended soils and increased (azoxystrobin) or decreased (pirimicarb) over incubation time. Respiration increased in the soils when the concentration of the pesticide increased and decreased with increasing incubation time. Illumina MiSeq analysis of 18S rRNA genes demonstrated a strong effect of the SMS and incubation time, but not the pesticides, on micro-eukaryote community composition.

Keywords: Pesticides, spent mushroom substrate, dissipation, soil microbial community, next generation sequencing, microbial parameters

1. Introduction

Soil microbial communities are key contributors to soil biological quality and can affect nutrient availability, plant development and environmental quality. They play key roles in soil ecosystem functions, including pollutant biodegradation, biogeochemical cycling and soil formation (Gianfreda and Rao, 2008; Singh et al., 2014). These communities could be potentially impacted by pesticide application to soil (Bending et al., 2007) or by the use of organic residues (Poulsen et al., 2013) either as soil amendments (Herrero-Hernández et al., 2011;) or as a tool to protect water contamination by these compounds (Álvarez-Martín et al., 2016). Therefore it is important to understand how these different agricultural practices affect microbial community structure and functioning.

Pesticides are used intensively in agriculture for pest control and to improve crop yield (Arias-Estévez et al., 2008). As a consequence, pesticide residues may reach surface and ground waters through non-point and point pollution sources by direct run-off and leaching, or following washing and disposal of empty containers post application (Carter, 2000, Herrero-Hernández et al., 2013). The pollution of surface and ground waters by pesticides is governed by the physicochemical characteristics of the compounds, and soil and environmental variables, which together govern their behaviour (e.g. sorption and degradation) in the environment (Arias-Estévez et al., 2008).

Recently, the use of organic amendments in agricultural soils has been demonstrated to be a feasible way to prevent soil and water pollution by pesticides, because the organic matter (OM) incorporated into the soil with the amendment can immobilize and/or enhance degradation of the pollutant and its metabolites and therefore avoid their dispersion to the environment (Rodríguez-Liebana et al., 2014). At the same time such additions of residue to soil can improve soil quality and plant

productivity (Cotxarrera et al., 2012). Furthermore, use of organic substrates such as soil amendments helps to solve environmental problems associated with the disposal of organic materials into landfill (Fait et al., 2007).

Common amendments with potential value for application to agricultural soils include biochar, sewage sludge, vinasse and spent mushroom substrate (SMS) (Pérez-Piqueres et al., 2006; Castillo et al., 2008). SMS is an organic amendment applied to some Spanish agricultural soils in regions where mushrooms are grown in large amounts (www.magrama.gob.es). SMS shows potential to sorb hydrophobic pesticides due to its high OM content. Recently, the effect of SMS as a soil amendment to promote sorption or immobilization and degradation of pesticides, and to reduce their mobility in the environment, has been studied in detail (Marín-Benito et al., 2012a, 2012b, 2012c). Results of these studies have identified that organic carbon (OC) and dissolved organic carbon (DOC) content of SMS determine its capacity as a sorbent of pesticides and thereby their bioavailability to soil microorganisms and their degradation rates and retention in soil.

However the addition of both pesticides and organic amendments to agricultural soils can affect soil microbial communities. In general the biodegradation of pesticides applied to crops can be altered by addition of organic amendments to the soil via i) increasing sorption and reducing bioavailability to microorganisms, ii) introducing exogenous microorganisms with biodegradation abilities or iii) changing soil properties (eg, pH and available nutrients), thereby altering the composition and activity of indigenous microflora, including degrader communities (Arias-Estévez et al., 2008; Cabrera et al., 2014).

In the case of SMS, previous reports have shown that the addition of this amendment to soil stimulates the soil microbial biomass and the activities of the soil

microbial community (Pérez-Piqueres et al., 2006; García-Delgado et al., 2015) and can inhibit or stimulate degradation of pesticides depending on the nature and stabilization of SMS and pesticide properties (Herrero-Hernández et al., 2011; Marín-Benito et al. 2012b; Marín-Benito et al., 2014).

Soil microbial diversity is important in maintaining the wide functional potential of the soil ecosystem (Feld et al., 2015). However, the relative impacts and interactions of SMS and pesticides on soil microbial communities, and their contribution to soil ecosystem functions such as OM breakdown, nutrient cycling, plant growth or biodegradation of pollutants, are unclear. These impacts on soil microbial communities are usually assessed measuring overall activities (respiration, enzyme activities). However, application of next-generation sequencing techniques to evaluate the effects of pesticide and/or organic residues on the soil microbial diversity are scarce (Jacobsen and Hjelmsø, 2014; Feld et al., 2015).

The objective of the current work was to study the changes that occur in soil microbial communities following the combined use of the pesticides azoxystrobin or pirimicarb and SMS as an organic amendment to a vineyard soil from La Rioja region (Spain). Both compounds are used in a broad range of crops for the control of fungal and aphid crop pathogens. These two pesticides were applied to an unamended and SMS-amended soil at two rates for characterisation of: 1) Dissipation kinetics of pesticides following extraction with an organic solvent to measure total pesticide concentrations; 2) Soil dehydrogenase activity (DHA) and soil respiration to give an indication of broad-scale impacts of the pesticides and SMS amendment on the soil microbial community, and 3) Structure and diversity of micro-eukaryote communities.

2. Material and methods

2.1. Study site and soil characterization

The soil was a Calcisol sandy loam (Typic Calcixerepts) (Soil Survey Staff, 2010). The soil was taken from the surface horizon (0–30 cm) of a vineyard farm in Alcanadre (42°24'20"N, 2°7'6"W, La Rioja, NE-Spain). The soil was sieved (<2 mm) prior to use and its characteristics were determined using standard analytical methods (Sparks, 1996). The soil pH was 7.49. The OC content was 0.89%. The particle size distribution determined using the pipette method was 79.6%, 9.0%, 11.4% sand, silt and clay respectively. Inorganic carbon content, determined as CaCO₃ with a Bernard calcimeter, was 24.1%.

2.2. Chemicals

Pirimicarb [2-dimethylamino-5,6-dimethylpyrimidin-4-yl dimethylcarbamate] is a carbamate insecticide. Pirimicarb (water solubility 3100 mg L⁻¹, log K_{ow} 1.7) acts by disabling the nervous system by the inhibition of acetylcholinesterase. Azoxystrobin [methyl (E)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate] is a strobilurin fungicide. Azoxystrobin (water solubility 6.7 mg L⁻¹, log K_{ow} 2.5) is the heme-containing component of the cytochrome bc₁ complex, which accepts electrons from Rieske protein and transfer electrons to cytochrome c in the mitochondrial respiratory chain (Berman et al., 2000). Both pesticides were supplied by Sigma-Aldrich Química SA (Madrid, Spain).

All organic solvents and other reagents used were of analytical grade and were supplied by Fisher Scientific Ltd. (Loughborough, UK) and Sigma-Aldrich Química SA (Madrid, Spain).

2.3. Amendment

Spent mushroom substrate (SMS) from *Agaricus bisporus* cultivation is a pasteurized mixture of cereal straw and poultry litter, urea, and gypsum. This residue was supplied by Sustratos de La Rioja SL (Pradejón, Spain). SMS was sieved <2 mm prior to use.

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) (Jackson, 1964). 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 (Bremner, 1996). The organic amendment applied showed the following characteristics (on a percentage dry weight basis): pH 6.97, ash content 33.6%, OC content 24.5%, DOC 1.91%, N content 1.75% and C/N 14.0. Moisture content was 64.5%.

2.4. Experimental design and incubation

The experiment had a completely randomized block design including a total of 162 samples with three replications of the following treatments: 3 soils (unamended soil, and soil mixed with 2 and 5 % SMS), 3 pesticide application doses (0, 2 and 25 mg kg⁻¹), 2 pesticides (azoxystrobin and pirimicarb), and 3 harvest intervals (0, 30 and 90 days).

The amended soils were prepared by uniformly mixing soil with SMS at a rate of 2% and 5% on a dry weight basis. For soil amended with 2% SMS, the pH was 7.39 and the OC was 1.22, while for soil with 5% SMS, pH and OC were 7.26 and 2.22.

Prior to the addition of pesticides, the soils were incubated in the dark at 20°C

for 48 h to equilibrate. The pesticides were dissolved in acetone and added to soil gradually during a 10 min mixing period to allow the acetone to volatilise. An equal volume of acetone was added to the control samples (i.e. soil and SMS treatments without pesticide). The pesticide concentrations used represented the maximum agronomic dose and 12.5 times the agronomic dose (2 and 25 mg kg⁻¹) considering a soil depth of 5 cm and a bulk density of 1.5 g cc⁻¹. The highest dose of azoxystrobin was similar to that used in previous work, which was shown to alter soil fungal community structure (Bending et al., 2007; Sopeña and Bending, 2013). The same high dose of pirimicarb was used for comparison. Pesticides were studied separately to avoid synergistic effects. Soil moisture content was adjusted to 40% of the maximum soil water-holding capacity. After mixing thoroughly using sterilized spatulas, 50 g was placed in sterilized bottles and incubated in darkness at 20°C for 24 h (time 0), 30 and 90 days. Soil moisture content was maintained by the addition of sterile distilled water as necessary.

2.5. Pesticide dissipation

To determine how pesticide persistence in soil was affected by SMS, methanol extraction was carried out. Samples of incubated soil (5 g) were shaken with 10 mL of methanol for 24 h and the extracted pesticide analysed by HPLC/MS using a Waters (Milford, MA, USA) system. Half-life (DT₅₀) was calculated according to FOCUS guidelines (FOCUS, 2006).

HPLC/MS conditions were set up with a mobile phase of acetonitrile:water + 0.1% formic acid, 90:10 and 80:20 (v/v) for pirimicarb and azoxystrobin, respectively. Flow rate was 0.4 ml min⁻¹ and retention time was 6.3 min for both pesticides. The studied ion (m/z) [M]⁺ was 404.0 for azoxystrobin and 239.4 for pirimicarb.

2.6. Dehydrogenase activity and respiration in soils

Soil dehydrogenase activity (DHA) and respiration analysis were performed to give an indication of broad-scale impacts of the SMS and pesticides. DHA was determined following the Tabatabai method (Tabatabai, 1994). The method is based on the extraction and colorimetric determination of the intensely coloured 2,3,5-triphenyl formazan (TPF) produced by the reduction of colourless 2,3,5- triphenyltetrazolium chloride (TTC) in soils. Basal respiration was determined by measuring O₂ absorption by microorganisms in 50 g portions of soil incubated at 25°C for 60 h using Oxi-Top Control equipment (WTW, Weilheim, Germany) according to DIN ISO 16072.

2.7. Microbial community structure analysis

DNA was extracted from all unamended and amended soil samples (0.5 g) using the Fast DNA® Spin Kit for Soil (MP Biomedicals, LLC, UK) according to the manufacturer's instructions. Quality and quantity of the extracted DNA was elucidated by Qubit ® BR DNA kit (Life Technologies Corporation). The Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs ® inc.) was used to amplify 18S rRNA genes using the primers 18S Euk_1391f/ 18S EukBr (Amaral-Zettler et al., 2009). PCR products were purified using AMPure XP beads (Beckman Coulter, USA) using the manufacturer's handbook. Index PCR was carried out with the commercial i5 and i7 indexed primers provided by the Illumina Nextera® Index Kit. These PCR products were also purified as mentioned above and DNA concentration was checked using Qubit. The DNA concentration was normalized to 10 nM and the samples were sent to TGAC (The Genome Analysis Centre, Norwich, UK) for Miseq v2 2 x 250 bp sequencing.

2.8. Processing of Illumina MiSeq Sequence Data

Paired-ends reads were assembled by aligning the forward and reverse reads, trimming primers and quality filtering (-fastq_maxee 0.5) using USEARCH and UPARSE software (Edgar, 2010; Edgar *et al.*, 2011) (version 8.1.1861). Unique sequences were sorted by abundance, and singletons in the data set were discarded. Sequences were clustered, followed by chimera filtering using the SILVA database as reference (Pruesse *et al.*, 2007). To obtain the number of reads of each OTU, reads were mapped back to OTUs with a minimum identity of 97%. Taxonomy was assigned using Quantitative Insights into Microbial Ecology (QIIME 1.8) (Caporaso *et al.*, 2010) and the SILVA database (Pruesse *et al.*, 2007).

2.9. Statistical analyses

One-way and two-way analysis of variance (ANOVA) were used to evaluate the effects of the different treatments (pesticide concentration, type of soil sample and incubation time) as independent factors on DHA and respiration, and to investigate any interaction between them. Standard deviation (SD) was used to indicate variability among replicates and Fisher's post-hoc test at a confidence level of 95 % was used to establish significant differences between values. SPSS Statistics 22.0 software for windows (SPSS Inc. Chicago, USA) was used.

Rarefaction Curves were used to determine sequencing sampling depth (Chao, 1984). Significant differences in micro-eukaryote community structure between treatments over the course of the experiment were analysed using Bray-Curtis resemblance matrices using non-metric multidimensional scaling (NMDS) in the program PRIMER Version 6.1.9 (PRIMER-E Ltd., Plymouth, UK). ANOSIM reports

the level of dissimilarity between sample groups (global R) and the associated level of significance (P). R is scaled to be within the range +1 to -1. Positive R values indicate that samples are more dissimilar between groups than within groups. R values close to zero occur if the high and low similarities are perfectly mixed and bear no relationship to the group. Negative R values indicate that dissimilarities within groups are greater than dissimilarities between groups (Clarke, 1993). Additionally, similarity percentage (SIMPER) analysis was used to elucidate which specific OTUs contributed to differences in the similarity matrix structure between treatments (Clarke and Warwick, 2001).

3. Results

3.1. Dissipation of azoxystrobin and pirimicarb in unamended and amended soil

Pesticide dissipation followed single first order kinetics with $r^2 > 0.91$ for azoxystrobin and $r^2 > 0.98$ for pirimicarb. The DT_{50} values ranged between 122.5 and 268.2 days for azoxystrobin and 20.0 and 42.0 days for pirimicarb depending on the soil treatment and pesticide dose (Table 1).

DT_{50} values of azoxystrobin decreased (low dose) or increased (high dose) when applied to SMS amended soil ($p < 0.05$), with no significant effect of SMS amendment rate. In all cases, degradation rate of azoxystrobin was slower (4-12 times) than that of pirimicarb. SMS application at 5 %, but not 2 %, reduced DT_{50} values of pirimicarb ($p < 0.05$).

3.2. Dehydrogenase activity in soil

Soil dehydrogenase activity (DHA) was measured to give an indication of broad-scale impacts of the pesticides and SMS on the microbial community and results are

included in Fig. 1. In both azoxystrobin and pirimicarb treated soils, DHA activity increased significantly ($p < 0.001$) following SMS application, with the increase greater following 5 % relative to 2 % addition. Furthermore, DHA increased with incubation time ($p < 0.001$) in all treatments (Fig. 1). However, DHA activity was not significantly affected by the concentration of fungicide applied (0, 2 and 25 mg kg⁻¹) in unamended or amended soils. Two-way analysis of variance indicated that DHA was significantly affected by the interaction of time and soil sample ($p < 0.01$), but there were no other significant interactions.

In the case of pirimicarb, a significant decrease in DHA was seen as the concentration of the insecticide increased ($p < 0.05$) and with increasing incubation time ($p < 0.001$). There was a significant interaction ($p < 0.05$) between soil sample and insecticide concentration, but there were no other significant interactions.

3.3. Soil respiration

In the case of azoxystrobin, SMS application had no significant effect on respiration (Fig. 2). At time 0, respiration was significantly ($p < 0.05$) affected by the concentration of fungicide applied, with a dose dependent increase seen in all treatments. Respiration significantly decreased with incubation time ($p < 0.005$) in all samples (Fig. 2). Two-way analysis of variance indicated that time and fungicide concentration interacted to affect respiration ($p < 0.05$), but there were no other significant interactions.

For pirimicarb treated soils, SMS application had no significant effect on respiration (Fig. 2). At time 0 there was a significant increase in respiration across all treatments when the concentration of the insecticide increased ($p < 0.05$), although respiration subsequently significantly decreased with increasing incubation time

($p < 0.05$) (Fig. 2). Furthermore a significant interaction ($p < 0.05$) between time and insecticide concentration was observed but there were no other significant interactions.

3.4. Soil micro-eukaryote community analysis

Illumina sequencing of micro-eukaryote communities in pirimicarb treated soils (8549617 processed reads) revealed 6293 OTUs with average length of 112 bp, and an average 105551 reads per sample. Among these, 1185 OTUs were assigned to fungi.

In the case of azoxystrobin treated soils (8438383 processed reads) there were 5119 micro-eukaryote OTUs with average length of 109 bp, and an average of 104177 sequences per sample. Among these, 943 OTUs were assigned to fungi.

Chao1 analysis of the soils treated with pirimicarb (Fig. 3a), showed that there were significant differences (both $p \leq 0.003$) between the unamended soil and SMS amended soils, with amended soils showing 19% higher richness. SMS amendment rate, time and pesticide concentration had no effect on OTU richness (data not shown). Similar results were found for azoxystrobin (Fig. 3b), with significant differences in OTU richness between unamended soil and soil receiving SMS at 2 % and 5% rates, which both showed an increase of 12% richness ($p \leq 0.003$ and $p \leq 0.015$ respectively).

NMDS and ANOSIM analyses showed that micro-eukaryote communities in each soil treatment were significantly different ($p < 0.001$) within both azoxystrobin and pirimicarb treated soils (Fig. 4, Table 2). Additionally, within each type of soil (0% SMS, 2% and 5% of SMS), there were significant differences across harvest times (0, 30 and 90 days) (Table S1). However pesticide application had no significant effect on community structure. Furthermore, specific analysis of sequences assigned to fungi, using NMDS and ANOSIM, showed similar patterns of variation to the micro-eukaryote analysis (Fig. S1, Tables S2 and S3), with soil treatment and time, but not

pesticide application, significantly affecting community structure. SMS addition was associated with increases in the relative abundance of sequences assigned to the Stramenopiles (7-39%), Rhizaria (49-119%) and Heterolobosea (529-1171%), and decreased abundance of sequences assigned to Viridiplantae (27-51%) and Fungi (23-27%) (Fig. 5).

SIMPER analysis was used to identify taxa responsible for differentiating micro-eukaryote communities between soil treatments. For both azoxystrobin and pirimicarb treated soils, the main taxa responsible for the differences in the micro-eukaryote community across treatments were OTU assigned to the arthropod genera *Symphypleona*, protists related to *Hartmannellidae* sp. and *Stachyamoeba*, and plant sequences related to *Moniliformopses* (Table 3, Table 4). In all cases except for *Stachyamoeba*, relative abundance was decreased in the presence of the amendment.

Different fungal taxa were responsible for differentiating communities across soil treatments in the azoxystrobin and pirimicarb amended soils. In the case of azoxystrobin, the major changes were associated with *Agaricomycetes*, *Eurotiomycetes* and *Saccharomycetes* (Table S6), while pirimicarb, they were associated with *Eurotiomycetes*, *Saccharomycetes* and *Chyridiomyce* (Fig S7). Differences in micro-eukaryote community composition across harvest times were associated with *Symphypleona*, *Moniliformopses* and *Hartmannellidae* for both azoxystrobin and pirimicarb (Tables S4 and S5). When fungal communities were considered, we found the taxa most responsible differentiating communities across time were *Agaricomycetes*, *Eurotiomycetes*, *Dothideomycetes* and *Saccharomycetes* (Tables S8 and S9).

4. Discussion

4.1. Dissipation of azoxystrobin and pirimicarb in unamended and amended soil

We found that the addition of SMS to soil increased soil microbial activity, and that this was associated with enhanced rates of pirimicarb degradation at both high and low application rates, and of azoxystrobin when applied at the low dose. In the case of azoxystrobin applied at 25 mg kg⁻¹, higher immobilization of the pesticide in SMS amended soils resulted in reduced rates of degradation relative to the unamended soil.

Calculated DT₅₀ values of both pesticides are in accordance with those reported in previous studies (Bending et al., 2006, 2007; Kah et al., 2007; Howell et al. 2014; PPDB, 2015), and indicate that these pesticides are moderately persistent.

Previous research has correlated the degradation of pesticides in soil with pH and OC. In particular, azoxystrobin dissipation has been inversely correlated with pH (Bending et al., 2006; Singh et al., 2010). SMS induced a decrease in soil pH and increased the OC content, which could have affected the interaction between SMS and the pesticide, thereby enhancing pesticide sorption and reducing bioavailability.

For both pesticides, bioavailability may be higher when applied at a low dose (2 mg kg⁻¹) relative to a higher dose (25 mg kg⁻¹), as previously described (Sopeña and Bending 2013; Howell et al. 2014), and this could explain the lower DT₅₀ values found for both pesticides applied at a lower dose.

4.2. Soil dehydrogenase activity and respiration

In our study we found that DHA activity was stimulated by the SMS. These changes could reflect the amendments providing a source of labile carbon for the soil microbial community, or result from changes in microbial activity induced by modification to soil physicochemical properties, which may impact the pore structure and thereby aeration and water availability (Gao et al., 2015).

In addition, DHA was affected by the concentration of pirimicarb, decreasing after 90 days in the 25 mg kg⁻¹ treatment in SMS treated soils, suggesting that the microbial community had a low resistance to pirimicarb application (Smith et al., 2000). Several studies (Pandey and Singh, 2006; Muñoz-Leoz et al., 2013) have also reported sensitivity of soil DHA to other insecticides suggesting death or metabolic inhibition of a microbial fraction sensitive to these chemicals at high doses. However azoxystrobin did not have a significant effect on DHA activity at any time point. Moreover the dissipation studies show the persistence of azoxystrobin in the soil, indicating that there was not a direct link between azoxystrobin and DHA. Although decreased DHA has been shown previously in soils treated with this fungicide (Bending et al., 2007; Sopena and Bending, 2013), this discrepancy could be due to adaptation towards more tolerant microbial communities, which are unaffected by pesticide exposure, in agreement with our sequencing results (see below).

Respiration increased in pesticide treated relative to untreated soils. Respiration has been extensively used as an ecophysiological indicator that can be used as a factor to evaluate environmental stress in microbial communities. Therefore, when the same biomass shifts to a higher respiratory activity it can reflect stress conditions, inducing more active metabolism and lower incorporation of OC into the microbial biomass (Anderson and Domsch, 1985). In the current study respiration increased in a similar way following application of both pesticides, which did not correlate with pesticide persistence. This could reflect either increased stress, or microbial growth on biomass killed by pesticide application, but probably not the use of the pesticide as carbon source (Marinozzi et al., 2013).

4.3. Soil micro-eukaryote community analysis

Sequence analysis of the micro-eukaryote community revealed that the concentration of amendment influenced community composition. Also, the diversity, as measured by taxa richness, was lower in the presence of SMS. These results are consistent with low resolution t-RFLP analysis of bacterial and fungal communities, carried out by Pérez-Piqueres et al. (2006).

Furthermore, within each soil treatment, there were significant changes in community composition over time. This supports evidence from the DHA and respiration experiments, which indicated that microbial activity showed variation across time points.

The predominant factor influencing micro-eukaryote community structure was SMS addition. Key taxa which responded to SMS application included microarthropods such *Collembola*, which increased in the presence SMS, as found for other soils amended with organic materials (Jørgensen and Hedlund, 2013) and protists, which also increased, probably due to the higher microbial turnover in the treatments (Forge et al., 2003).

In contrast there was a decline in abundance of plant sequences. This suggests reduced relative abundance of plant sequences in the 'seed bank' (Davies et al., 2013), which could reflect either a dilution by the amendment or enhanced decomposition of plant debris following stimulated microbial activity after incorporation of the amendment. Similarly, one of the fungal taxa more responsible differentiating communities across treatments was, as expected, *Agaricomycetes*, which increased with SMS amendment, indicating the presence of mushroom residues.

Interestingly, Howell et al. (2014) found a significant influence of azoxystrobin on microbial communities when profiling communities using RNA but not DNA, suggesting a change in the active population rather than the total population.

Additionally, it is important to note that in the study of Howell et al. (2014), non-agricultural soils were used. Therefore in our agricultural soil, where pesticides have been used for decades, the endogenous microbial structures have probably been adapted to the stress caused by pesticide application, so that they have greater resistance and resilience to the effects of pesticides (Widenfalk et al., 2008; Howell et al., 2014).

In the current study micro-eukaryote but not prokaryote communities were profiled. The data shows the power of micro-eukaryote sequencing to resolve taxa responding to agricultural management practices, thereby identifying indicators at a range of trophic levels, including fungi, protists and invertebrates. While the fungicide and insecticide used here could potentially directly affect micro-eukaryote communities, direct effects on bacterial communities could occur, associated with proliferation of degraders, and this could result in indirect effects on micro-eukaryote communities through processes such as competition and predation (Adetutu et al., 2008; Arabet et al., 2014). However, our results clearly demonstrate that pesticides had little influence on the structure of micro-eukaryote communities.

5. Conclusions

In this work we have studied the effect of the addition of azoxystrobin, pirimicarb and/or SMS to an agricultural soil on microbial communities using both broad and fine scale analyses. Dissipation studies revealed that both pesticides were bioavailable in both amended and unamended soils. We found that SMS facilitated the degradation of pirimicarb at both studied concentrations and of azoxystrobin at the lower concentration. Analysis of DHA and respiration suggested that while SMS and time affected communities, pesticides had little effect. Both DHA and respiration decreased with incubation time. Next generation sequencing revealed significant effects

of both SMS amount and incubation time, but no impact of pesticides, on micro-eukaryote communities. Therefore, the use of SMS as an organic amendment in agricultural soils could not only affect pesticide dissipation but also microbial community composition.

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References

- Adetutu, E.M., Ball, A.S., Osborn, A.M., 2008. Azoxystrobin and soil interactions: degradation and impact on soil bacterial and fungal communities. *J. Appl. Microbiol.* 105, 1777-1790.
- Álvarez-Martín, A., Sánchez-Martín, M.J., Pose-Juan, E., Rodríguez-Cruz, M.S., 2016. Effect of different rates of spent mushroom substrate on the dissipation and bioavailability of cymoxanil and tebuconazole in an agricultural soil. *Sci. Total Environ.* 550, 495-503.
- Amaral-Zettler, L., McCliment, E., Huse, S., 2009. A method for studying protistan diversity using massively parallel sequencing of V9 hypervariable regions of small-subunit ribosomal RNA genes. *PLoS ONE* 4, e6372.
- Anderson, T.H., Domsch, K.H., 1985. Determination of ecophysiological maintenance carbon requirements of soil microorganisms in a dormant state. *Biol. Fert. Soils* 1, 81-89.

- Arabet, D., Tempel, S., Fons, M., Denis, Y., Jourlin-Castelli, C., Armitano, J., Redelberg, D., Iobbi-Nivol, C., Boulahrouf, A., Méjean, V., 2014. Effects of a sulfonylurea herbicide on the soil bacteria community. *Environ. Sci. Pollut. Res.* 21, 5619-5627.
- Arias-Estévez, M., López-Periago, E., Martínez-Carballo, E., Simal-Gándara, J., Mejuto, J.C., García-Río, L., 2008. The mobility and degradation of pesticides in soils and the pollution of groundwater resources. *Agr. Ecosyst. Environ.* 123, 247–260.
- Bending, G.D., Lincoln, S.D., Edmondson, R.N., 2006. Spatial variation in the degradation rate of the pesticides isoproturon, azoxystrobin and diflufenican in soil and its relationship with chemical and microbial properties. *Environ. Pollut.* 132, 279-287.
- Bending, G.D., Rodríguez-Cruz, M.S., 2007. Microbial aspects of the interaction between soil depth and biodegradation of the herbicide isoproturon. *Chemosphere* 66, 664-671.
- Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., Shindyalov, I.N., Bourne, P.E., 2000. The protein data bank. *Nucleic Acids Res.* 28, 235-242.
- Bremner, J.M., 1996. Nitrogen total. In: Sparks, D.L. (Ed.), *Methods of Soil Analysis. Part 3, Chemical Methods*. Soil Science Society of America, Inc, Madison, Wisconsin, USA.
- Cabrera, A., Cox, L., Spokas, K., Hermosín, M.C., Cornejo, J., Koskinen, W.C., 2014. Influence of biochar amendments on the sorption–desorption of aminocyclopyrachlor, bentazone and pyraclostrobin pesticides to an agricultural soil. *Sci. Total Environ.* 470, 438-443.

- Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., et al., 2010. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335-336.
- Carter A., 2000. How pesticides get into water – and proposed reduction measures. *Pesticide Outlook* 11, 149-152.
- Castillo M.P., Torstensson L, Stenstrom J., 2008. Biobeds for environmental protection from pesticide use. A review. *J. Agric. Food Chem.* 56, 6206-6219.
- Chao, A., 1984. Nonparametric estimation of the number of classes in a population. *Scand. J. Stat.* 11, 265-270.
- Clarke, K.R., 1993. Non-parametric multivariate analyses of changes in community structure. *Aust. J. Ecol.* 18, 117-143.
- Clarke, K.R., Warwick, R.M., 2001. Change in Marine Communities. An Approach to Statistical Analysis and Interpretation. 2nd edn, PRIMER-E Ltd.
- Cotxarrera, L., Trillas-Gay, M.I., Steinberg, C., Alabouvette, C., 2002. Use of sewage sludge compost and *Trichoderma asperellum* isolates to suppress *Fusarium* wilt of tomato. *Soil Biol. Biochem.* 34, 467-476.
- Davies, O.L., Schäfer, H., Marshall, S., Bramke, I., Oliver, R.G., Bending, G.D., 2013. Light structures phototroph, bacterial and fungal communities at the soil surface. *PLOS One* 8, e69048.
- Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460-2461.
- Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194-2200.

- Fait, G., Nicelli, M., Fragoulis, G., Trevisan, M., Capri, E., 2007. Reduction of point contamination sources of pesticide from a vineyard farm. *Environ. Sci. Technol.* 41, 3302-3308.
- Feld, L., Hjelmsø, M.H., Nielsen, M.S., Jacobsen, A.D., Rønn, R., Ekelund, F., Krogh, P.H., Strobel, B.W., Jacobsen, C.S., 2015. Pesticide side effects in an agricultural soil ecosystem as measured by *amoA* expression quantification and bacterial diversity changes. *PLoS ONE* 10(5): e0126080.
- FOCUS, 2006. Report of the FOCUS Work Group on Degradation Kinetics EC Document Reference Sanco/10058/2005 version 2.0.
- Forge, T.A., Hogue, E., Neilsen, G., Neilsen, D., 2003. Effects of organic mulches on soil microfauna in the root zone of apple: implications for nutrient fluxes and functional diversity of the soil food web. *Appl. Soil Ecol.* 22, 39-54.
- Gao, W., Liang, J., Pizzul, L., Feng, X.M., Zhang, K., Castillo, M.P., 2015. Evaluation of spent mushroom substrate as substitute of peat in Chinese biobeds. *Int. Biodeter. Biodegr.* 98, 107-112.
- García-Delgado, C., D'Annibale, A., Pesciaroli, L., Yunta, F., Crognale, S., Petruccioli, M., Eymar, E., 2015. Implications of polluted soil biostimulation and bioaugmentation with spent mushroom substrate (*Agaricus bisporus*) on the microbial community and polycyclic aromatic hydrocarbons biodegradation. *Sci. Total Environ.* 508, 20-28.
- Gianfreda, L., Rao, M.A., 2008. Interactions between xenobiotics and microbial and enzymatic soil activity. *Crit. Rev. Env. Sci. Tec.* 38, 269-310.
- Herrero-Hernández, E., Andrades, M.S., Álvarez-Martín, A., Pose-Juan, E., Rodríguez-Cruz, M.S., Sánchez-Martín, M.J., 2013. Occurrence of pesticides and some of their degradation products in waters in a Spanish wine region. *J. Hydrol.* 486, 234-245.

- Herrero-Hernández, E., Andrades, M.S., Marín-Benito, J.M., Sánchez-Martín, M.J., Rodríguez-Cruz, M.S., 2011. Field-scale dissipation of tebuconazole in a vineyard soil amended with spent mushroom substrate and its potential environmental impact. *Ecotoxicol. Environ. Saf.* 74, 1480-1488.
- Howell, C.C., Semple, K.T., Bending, G.D., 2014. Isolation and characterisation of azoxystrobin degrading bacteria from soil. *Chemosphere* 159, 776-781.
- Jackson, M.L. 1964. *Soil Chemical Analysis*. Prentice-Hall, Inc. Englewood Cliffs, N.J., USA.
- Jacobsen, C.S., HjelmsØ, M.H., 2014. Agricultural soils, pesticides and microbial diversity. *Curr. Opin. Biotech.* 27, 15-20.
- Jørgensen, H.B., Hedlund, K., 2013. Organic amendment and fungal species in combination can alter collembolan fitness. *Soil Biol. Biochem.* 65, 316-321.
- Kah, M., Beulke, S., Brown, C.D., 2007. Factors influencing degradation of pesticides in soil. *J. Agric. Food Chem.* 55, 4487-4492.
- Marín-Benito, J.M., Andrades, M.S., Rodríguez-Cruz, M.S., Sánchez-Martín, M.J., 2012a. Changes in the sorption-desorption of fungicides over time in an amended sandy clay loam soil under laboratory conditions. *J. Soils Sediments* 12, 1111-1123.
- Marín-Benito, J.M., Andrades, M.S., Sánchez-Martín, M.J., Rodríguez-Cruz, M.S., 2012b. Dissipation of fungicides in a vineyard soil amended with different spent mushroom substrates. *J. Agric. and Food Chem.* 60, 6936-6945.
- Marín-Benito, J.M., Herrero-Hernández, E., Andrades, M.S., Sánchez-Martín, M.J., Rodríguez-Cruz, M.S., 2014. Effect of different organic amendments on the dissipation of linuron, diazinon and myclobutanil in an agricultural soil incubated for different time periods. *Sci. Total Environ.* 476–477, 611–621.

- Marín-Benito, J.M., Rodríguez-Cruz, M.S., Andrades, M.S., Sánchez-Martín, M.J., 2012c. Assessment of spent mushroom substrate as sorbent of fungicides: Influence of sorbent and sorbate properties. *J. Environ. Qual.* 41, 814-822.
- Marinozzi, M., Coppola, L., Monaci, E., Karpouzas, D.G., Papadopoulou, E., Menkissoglu-Spiroudi, U., Vischetti, C., 2013. The dissipation of three fungicides in a biobed organic substrate and their impact on the structure and activity of the microbial community. *Environ. Sci Pollut. Res.* 20, 2546-2555.
- Muñoz-Leoz, B., Garbisu, C., Charcosset, J.Y., Sánchez-Pérez, J.M., Antigüedad, I., Ruiz-Romera, E., 2013. Non-target effects of three formulated pesticides on microbially-mediated processes in a clay-loam soil. *Sci. Total Environ.* 449, 345-354.
- Pandey, S., Singh, D.K., 2006. Soil dehydrogenase, phosphomonoesterase and arginine deaminase activities in an insecticide treated groundnut (*Arachis hypogaea L.*) field. *Chemosphere* 63, 869-880.
- Pérez-Piqueres, A., Edel-Hermann, V., Alabouvette, C., Steinberg, C., 2006. Response of soil microbiota communities to compost amendments. *Soil Biol. Biochem.* 38, 460-470.
- Poulsen, P.H.B., Al-Soud, W.A., Bergmark, L., Magid, J., Hansen, L.H., Sørensen, S.J., 2013. Effects of fertilization with urban and agricultural organic wastes in a field trial – Prokaryotic diversity investigated by pyrosequencing. *Soil Biol. Biochem.* 57, 784-793.
- PPDB, Pesticide Properties Data Base, 2015. University of Hertfordshire. [<http://sitem.herts.ac.uk/aeru/ppdb/en/index.htm>]
- Singh, B.K., Quince, C., Macdonald, C.A., Khachane, A., Thomas, N., Al-Soud, W.A., Sørensen, S.J., He, Z., White, D., Sinclair, A., Crooks, B., Zhou, J., Campbell,

- C.D., 2014. Loss of microbial diversity in soils is coincident with reductions in some specialized functions. *Environ. Microbiol.* 16, 2408-2420.
- Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J., Glöckner, F.O., 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* 35, 7188-7196.
- Singh, N., Singh, S., Mukerjee, I., Gupta, S., Gajbhiye, V., Sharma, P., Goel, M., Dureja, M., 2010. Metabolism of ¹⁴C-azoxystrobin in water at different pH. *J. Environ. Sci. Heal. B* 45, 123-127.
- Smith, M.D., Hartnet, D.C., Rice, C.W., 2000. Effects of long-term fungicide applications on microbial properties in tall grass prairie soil. *Soil Biol. Biochem.* 32, 935-946.
- Soil Survey Staff, 2010. Keys to Soil Taxonomy, 11th ed. USDA-Natural Resources Conservation Service, Washington, DC.
- Sopeña, F., Bending, G.D., 2013. Impacts of biochar on bioavailability of the fungicide azoxystrobin: A comparison of the effect on biodegradation rate and toxicity to the fungal community. *Chemosphere* 91, 1525-1533.
- Sparks, D.L., 1996. Methods of Soil Analysis. Part 3-Chemical Methods. Soil Science Society of America, Inc., Madison, WI.
- Rodríguez-Liebana, J.A., Mingorance, M.D., Peña, A., 2014. Pesticide mobility and leachate toxicity in two abandoned mine soils. Effect of organic amendments. *Sci. Total Environ.* 497, 561-569.
- Tabatabai, M.A., 1994. Soil enzymes. In: Weaver, R.W. (Ed.), Methods of Soil Analysis: Part 2 Microbiological and Biochemical Properties. Soil Science society of America, Madison, WI, pp. 903-947.

Widenfalk, A., Bertilsson, S., Sundh, I., Goedkoop, W., 2008. Effects of pesticides on community composition and activity of sediment microbes – response at various levels of microbial community organization. *Environ. Pollut.* 152, 576-584.

Figure captions

Fig. 1. Soil dehydrogenase activity for unamended and SMS-amended soils, untreated and treated with 2 and 25 mg kg⁻¹ of azoxystrobin or pirimicarb after 0, 30 and 90 days of incubation. Bars indicate the standard deviation of the mean (n=3).

Fig. 2. Soil basal respiration for unamended and SMS-amended soils, untreated and treated with 2 and 25 mg kg⁻¹ of azoxystrobin or pirimicarb after 0, 30 and 90 days of incubation. Bars indicate the standard deviation of the mean (n=3).

Fig. 3. Chao1 estimate of micro-eukaryote diversity in the three different soils treated with pirimicarb (a) and azoxystrobin (b). Error bars are ± 0.1 S.E.

Fig. 4. Non-Metric Multi-Dimensional Scaling of micro-eukaryote community structure based on the azoxystrobin (a) and pirimicarb (b) samples from the three different soils.

Fig. 5. Micro-eukaryote taxa richness and relative abundance across treatments (amendment doses, harvest time (0, 30 and 90 days) and pesticide concentrations (0, 2 and 25 mg kg⁻¹)) for azoxystrobin (a) and pirimicarb (b).

Table 1. Dissipation rate (k) and half-life (DT₅₀) values for azoxystrobin and pirimicarb applied at two doses (2 and 25 mg kg⁻¹) in unamended and SMS-amended soils.

	Azoxystrobin			Pirimicarb		
	k (days ⁻¹)	DT ₅₀ ±SD [†] (days)	r ²	k (days ⁻¹)	DT ₅₀ ±SD [†] (days)	r ²
(2 mg kg ⁻¹)						
S	0.004	178.8±15.6	0.98	0.018	39.0±7.5	0.99
S+2%SMS	0.006	122.5±10.2	0.99	0.022	31.1±2.4	0.99
S+5%SMS	0.005	139.4±15.0	0.99	0.026	26.2±1.7	0.99
LSD (95%)	0.001	27.59		0.005	9.266	
(25 mg kg ⁻¹)						
S	0.004	190.1±40.4	0.94	0.016	42.6±7.5	0.99
S+2%SMS	0.003	240.6±29.9	0.91	0.019	36.0±3.0	0.99
S+5%SMS	0.003	268.2±27.6	0.97	0.035	20.0±0.6	0.98
LSD (95%)	0.001	66.22		0.004	9.394	

[†]SD, standard deviation (n=3).

Table 2. Analyses of similarity (ANOSIM) evaluating the variation of micro-eukaryote communities. (Comparison of percentage of amendment)

Soils comparisons	Azoxystrobin		Pirimicarb	
	R	p	R	p
S, S+2%SMS	0.315	0.001	0.493	0.001
S, S+5%SMS	0.790	0.001	0.934	0.001
S+2%SMS, S+5%SMS	0.354	0.001	0.577	0.001

Table 3. Analysis of similarity percentage (SIMPER) for azoxystrobin micro-eukaryote communities. (Comparison between percentages of amendment)

Soil comparisons A vs. B	Overall Average Dissimilarity	Five most influential taxa	Percent contribution to difference	Average percent abundance in class A±SEM	Average percent abundance in class B±SEM
S vs. S+2%SMS	45.6	Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona	16.2	12.7 ± 2.97	10.6 ± 2.46
		Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Moniliformopses	13.8	18.7 ± 2.29	9.49 ± 1.43
		Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae	8.91	8.75 ± 1.35	10.1 ± 1.44
		environmental samples;uncultured eukaryote;Other;Other;Other	8.71	11.1 ± 1.18	13.1 ± 2.15
		Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stachyamoe ba sp. ATCC 50324	4.98	0.53 ± 0.20	5.04 ± 0.38
S vs. S+5%SMS	56.3	Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Moniliformopses	13.3	18.7 ± 2.29	4.23 ± 0.73
		Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona	12.3	12.7 ± 2.97	8.25 ± 1.99
		Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stachyamoe ba sp. ATCC 50324	8.25	0.53 ± 0.20	9.83 ± 0.58
		Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae	6.48	8.75 ± 1.35	8.50 ± 1.12
		Viridiplantae;Chlorophyta;Chlorophyceae;Pseudomuriella;Pseudomuriell a sp. Itas 9/21 14-1d	5.77	6.66 ± 0.45	0.15 ± 0.02
S+2%SMS vs. S+5%SMS	43.9	Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona	13.9	10.6 ± 2.46	8.25 ± 1.99
		Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae	8.70	10.1 ± 1.44	8.50 ± 1.12
		environmental samples;uncultured eukaryote;Other;Other;Other	8.65	13.1 ± 2.15	11.0 ± 0.92
		Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Moniliformopses	8.05	9.49 ± 1.43	4.23 ± 0.73
		Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stachyamoe ba sp. ATCC 50324	5.85	5.04 ± 0.39	9.83 ± 0.58

Table 4. Analysis of similarity percentage (SIMPER) for pirimicarb micro-eukaryote communities. (Comparison between percentages of amendment)

Soil comparisons A vs. B	Overall Average Dissimilarity	Five most influential taxa	Percent contribution to difference	Average percent abundance in class A $\pm$ SEM	Average percent abundance in class B $\pm$ SEM
S vs. S+2%SMS	39.2	Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona	14.4	11.4 $\pm$ 2.17	8.02 $\pm$ 1.73
		Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Moniliformopses	9.71	12.3 $\pm$ 1.09	9.28 $\pm$ 1.36
		Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae	7.74	7.59 $\pm$ 1.06	8.25 $\pm$ 1.07
		environmental samples;uncultured eukaryote;Other;Other;Other	6.25	12.4 $\pm$ 0.69	13.4 $\pm$ 1.03
		Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stachyamoe ba sp. ATCC 50324	4.92	0.24 $\pm$ 0.08	4.10 $\pm$ 0.55
S vs. S+5%SMS	51.7	Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona	10.9	11.4 $\pm$ 2.17	10.2 $\pm$ 1.75
		Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Moniliformopses	8.87	12.3 $\pm$ 1.09	3.91 $\pm$ 0.74
		Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stachyamoe ba sp. ATCC 50324	7.96	0.24 $\pm$ 0.08	8.48 $\pm$ 0.44
		Viridiplantae;Chlorophyta;Chlorophyceae;Pseudomuriella;Pseudomuriell a sp. Itas 9/21 14-1d	6.08	6.39 $\pm$ 0.42	0.09 $\pm$ 0.01
		Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae	5.89	7.59 $\pm$ 1.06	7.11 $\pm$ 1.76
S+2%SMS vs. S+5%SMS	38.4	Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona	12.9	8.02 $\pm$ 1.73	10.2 $\pm$ 1.75
		Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Moniliformopses	8.94	9.28 $\pm$ 1.36	3.91 $\pm$ 0.74
		Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae	7.71	8.25 $\pm$ 1.07	7.11 $\pm$ 1.76
		Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stachyamoe ba sp. ATCC 50324	6.62	4.10 $\pm$ 0.55	8.48 $\pm$ 0.44
		environmental samples;uncultured eukaryote;Other;Other;Other	6.57	13.4 $\pm$ 1.03	11.4 $\pm$ 0.68

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Changes in activity and structure of the soil microbial community after application of azoxystrobin or pirimicarb and an organic amendment to an agricultural soil

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Fig. S1. Non-metric Multi-Dimensional of fungal community structure based on the azoxystrobin (a) and pirimicarb (b) fungal samples from the three different soils.

Table S1. Analyses of similarity (ANOSIM) evaluating the variation of micro-eukaryote communities (Comparison of harvest time in each type of soil)

		Azoxystrobin		Pirimicarb	
		R	p	R	p
S	0 days, 30 days	0.124	0.002	0.631	0.001
	0 days, 90 days	0.683	0.001	0.695	0.001
	30 days, 90 days	0.206	0.026	0.362	0.006
S + 2%SMS	0 days, 30 days	0.613	0.001	0.615	0.001
	0 days, 90 days	0.905	0.001	0.929	0.001
	30 days, 90 days	0.366	0.006	0.381	0.001
S + 5%SMS	0 days, 30 days	0.572	0.001	0.483	0.003
	0 days, 90 days	0.848	0.001	0.750	0.001
	30 days, 90 days	0.424	0.001	0.458	0.001

Table S2. Analyses of similarity (ANOSIM) evaluating the variation of fungal communities. (Comparison of percentage of amendment)

Soils comparisons	Azoxystrobin		Pirimicarb	
	R	p	R	p
S, S+2%SMS	0.306	0.001	0.510	0.001
S, S+5%SMS	0.770	0.001	0.966	0.001
S+2%SMS, S+5%SMS	0.432	0.001	0.649	0.001

Table S3. Analyses of similarity (ANOSIM) evaluating the variation of fungal communities. (Comparison of harvest time in each type of soil)

		Azoxystrobin		Pirimicarb	
		R	p	R	p
S	0 days, 30 days	0.135	0.034	0.477	0.002
	0 days, 90 days	0.322	0.001	0.439	0.001
	30 days, 90 days	0.163	0.025	0.219	0.004
S + 2%SMS	0 days, 30 days	0.352	0.001	0.239	0.001
	0 days, 90 days	0.536	0.001	0.430	0.001
	30 days, 90 days	0.180	0.004	0.217	0.007
S + 5%SMS	0 days, 30 days	0.039	0.169	0.319	0.001
	0 days, 90 days	0.728	0.001	0.413	0.001
	30 days, 90 days	0.496	0.001	0.368	0.001

Table S4. Analysis of similarity percentage (SIMPER) for azoxystrobin micro-eukaryote communities. (Comparison between harvest time)

Soil comparisons A vs. B	Overall Average Dissimilarity	Five most influential taxa	Percent contribution to difference	Average percent abundance in class A $\pm$ SEM	Average percent abundance in class B $\pm$ SEM
0 days vs. 30 days	46.1	Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona	14.8	11.3 $\pm$ 2.28	11.0 $\pm$ 2.71
		Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Monilifor mopses	14.4	18.9 $\pm$ 2.36	7.82 $\pm$ 1.23
		Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae	11.1	1.28 $\pm$ 0.18	11.5 $\pm$ 0.95
		Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stach yamoeba sp. ATCC 50324	5.77	5.85 $\pm$ 1.01	5.32 $\pm$ 0.80
		Viridiplantae;Chlorophyta;Chlorophyceae;Pseudomuriella;Pseudo muriella sp. Itas 9/21 14-1d	4.62	3.74 $\pm$ 0.61	4.52 $\pm$ 0.93
0 days vs. 90 days	52.7	Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Monilifor mopses	13.7	18.9 $\pm$ 2.36	5.72 $\pm$ 1.02
		Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona	12.7	11.3 $\pm$ 2.28	9.20 $\pm$ 2.55
		Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae	12.5	1.28 $\pm$ 0.18	14.5 $\pm$ 0.76
		environmental samples;uncultured eukaryote;Other;Other;Other	9.91	8.57 $\pm$ 0.34	18.2 $\pm$ 2.07
		Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stach yamoeba sp. ATCC 50324	4.87	5.85 $\pm$ 1.01	4.23 $\pm$ 0.67
30 days vs. 90 days	43.5	Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona	15.7	11.0 $\pm$ 2.71	9.20 $\pm$ 2.55
		environmental samples;uncultured eukaryote;Other;Other;Other	12.1	8.44 $\pm$ 0.37	18.2 $\pm$ 2.07
		Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Monilifor mopses	7.20	7.82 $\pm$ 1.23	5.72 $\pm$ 1.02
		Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae	6.30	11.5 $\pm$ 0.95	14.5 $\pm$ 0.76
		Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae	5.03	10.9 $\pm$ 0.72	10.0 $\pm$ 0.52

Table S5. Analysis of similarity percentage (SIMPER) for pirimicarb micro-eukaryote communities. (Comparison between harvest time)

Soil comparisons A vs. B	Overall Average Dissimilarity	Five most influential taxa	Percent contribution to difference	Average percent abundance in class A $\pm$ SEM	Average percent abundance in class B $\pm$ SEM
0 days vs. 30 days	41.7	Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona	14.8	13.5 $\pm$ 2.03	6.81 $\pm$ 1.88
		Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae	11.6	1.97 $\pm$ 0.72	10.9 $\pm$ 0.72
		Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Monilifor mopses	10.4	13.5 $\pm$ 1.46	7.24 $\pm$ 0.91
		Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stach yamoeba sp. ATCC 50324	5.37	4.24 $\pm$ 0.73	4.61 $\pm$ 0.85
		Viridiplantae;Chlorophyta;Chlorophyceae;Pseudomuriella;Pseudo muriella sp. Itas 9/21 14-1d	4.26	3.49 $\pm$ 0.60	3.70 $\pm$ 0.64
0 days vs. 90 days	44.7	Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona	12.2	13.5 $\pm$ 2.03	9.34 $\pm$ 1.57
		Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Monilifor mopses	10.9	13.5 $\pm$ 1.46	4.76 $\pm$ 0.68
		Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae	9.85	1.97 $\pm$ 0.72	10.0 $\pm$ 0.52
		environmental samples;uncultured eukaryote;Other;Other;Other	7.61	9.83 $\pm$ 0.40	16.4 $\pm$ 0.80
		Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stach yamoeba sp. ATCC 50324	4.65	4.24 $\pm$ 0.73	3.98 $\pm$ 0.73
30 days vs. 90 days	36.2	Metazoa;Arthropoda;Hexapoda;Collembola;Symphypleona	13.5	6.81 $\pm$ 1.88	9.34 $\pm$ 1.57
		environmental samples;uncultured eukaryote;Other;Other;Other	8.48	11.0 $\pm$ 0.57	16.4 $\pm$ 0.80
		Viridiplantae;Streptophyta;Embryophyta;Tracheophyta;Monilifor mopses	6.62	7.24 $\pm$ 0.91	4.76 $\pm$ 0.68
		Heterolobosea;Schizopyrenida;Gruberellidae;Stachyamoeba;Stach yamoeba sp. ATCC 50324	6.01	4.61 $\pm$ 0.85	3.98 $\pm$ 0.73
		Amoebozoa;Tubulinea;Euamoebida;Tubulinida;Hartmannellidae	5.03	10.9 $\pm$ 0.72	10.0 $\pm$ 0.52

Table S6. Analysis of similarity percentage (SIMPER) for azoxystrobin fungal communities. (Comparison between percentages of amendment)

Soil comparisons A vs. B	Overall Average Dissimilarity	Five most influential taxa	Percent contribution to difference	Average percent abundance in class A $\pm$ SEM	Average percent abundance in class B $\pm$ SEM
S vs. S+2%SMS	40.4	Fungi;Dikarya;Basidiomycota;Agaricomycotina;Agaricomycetes	15.2	1.58 $\pm$ 0.62	0.45 $\pm$ 0.14
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes	9.52	1.91 $\pm$ 0.14	1.38 $\pm$ 0.08
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Dothideomycetes	8.41	1.01 $\pm$ 0.12	0.56 $\pm$ 0.07
		Fungi;Fungi incertae sedis;Basal fungal lineages;unclassified zygomycetes;zygomycete sp. AM-2008a	5.91	0.82 $\pm$ 0.13	0.39 $\pm$ 0.03
		Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes	4.88	0.14 $\pm$ 0.01	0.49 $\pm$ 0.06
S vs. S+5%SMS	53.7	Fungi;Dikarya;Basidiomycota;Agaricomycotina;Agaricomycetes	11.8	1.58 $\pm$ 0.62	0.57 $\pm$ 0.17
		Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes	10.4	0.14 $\pm$ 0.01	1.25 $\pm$ 0.26
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes	9.90	1.91 $\pm$ 0.14	0.89 $\pm$ 0.06
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Dothideomycetes	7.64	1.01 $\pm$ 0.12	0.21 $\pm$ 0.2
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Lecanoromycetes	7.51	0.10 $\pm$ 0.01	0.84 $\pm$ 0.05
S+2%SMS vs. S+5%SMS	41.1	Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes	12.1	0.49 $\pm$ 0.06	1.25 $\pm$ 0.26
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes	8.86	1.38 $\pm$ 0.08	0.89 $\pm$ 0.06
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Lecanoromycetes	6.75	0.43 $\pm$ 0.3	0.84 $\pm$ 0.05
		Fungi;Fungi incertae sedis;Basal fungal lineages;Mucoromycotina;Mucorales	6.74	0.37 $\pm$ 0.04	0.77 $\pm$ 0.05
		Fungi;Dikarya;Basidiomycota;Agaricomycotina;Agaricomycetes	6.41	0.45 $\pm$ 0.14	0.57 $\pm$ 0.17

Table S7. Analysis of similarity percentage (SIMPER) for pirimicarb fungal communities. (Comparison between percentages of amendment)

Soil comparisons A vs. B	Overall Average Dissimilarity	Five most influential taxa	Percent contribution to difference	Average percent abundance in class A $\pm$ SEM	Average percent abundance in class B $\pm$ SEM
S vs. S+2%SMS	37.2	Fungi;Dikarya;Ascomycota;Pezizomycotina;Dothideomycetes	12. 1	1. 64 $\pm$ 0. 21	0. 62 $\pm$ 0. 05
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes	11. 9	2. 45 $\pm$ 0. 26	1. 54 $\pm$ 0. 07
		Fungi;Chytridiomycota;environmental samples;uncultured	6. 96	1. 12 $\pm$ 0. 09	0. 55 $\pm$ 0. 04
		Chytridiomycota;Other			
		Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes	5. 65	0. 13 $\pm$ 0. 01	0. 63 $\pm$ 0. 07
		Fungi;Fungi incertae sedis;Basal fungal lineages;unclassified zygomycetes;zygomycete sp. AM-2008a	5. 55	1. 01 $\pm$ 0. 07	0. 57 $\pm$ 0. 03
S vs. S+5%SMS	54.4	Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes	12. 5	2. 45 $\pm$ 0. 26	0. 94 $\pm$ 0. 07
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Dothideomycetes	11. 8	1. 64 $\pm$ 0. 21	0. 19 $\pm$ 0. 01
		Fungi;Chytridiomycota;environmental samples;uncultured	9. 44	0. 13 $\pm$ 0. 01	1. 24 $\pm$ 0. 14
		Chytridiomycota;Other			
		Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes	7. 1	1. 12 $\pm$ 0. 09	0. 27 $\pm$ 0. 02
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Lecanoromycetes	6. 97	0. 07 $\pm$ 0. 08	0. 89 $\pm$ 0. 05
S+2%SMS vs. S+5%SMS	37.5	Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes	10. 5	0. 63 $\pm$ 0. 07	0. 63 $\pm$ 0. 07
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes	9. 44	1. 54 $\pm$ 0. 07	1. 54 $\pm$ 0. 07
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Dothideomycetes	5. 81	0. 62 $\pm$ 0. 05	0. 62 $\pm$ 0. 05
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Lecanoromycetes	7. 72	0. 51 $\pm$ 0. 03	0. 51 $\pm$ 0. 03
		Fungi;Dikarya;Basidiomycota;Agaricomycotina;Agaricomycetes	5. 47	0. 73 $\pm$ 0. 16	0. 58 $\pm$ 0. 03

Table S8. Analysis of similarity percentage (SIMPER) for azoxystrobin fungal communities. (Comparison between harvest time)

Soil comparisons A vs. B	Overall Average Dissimilarity	Five most influential taxa	Percent contribution to difference	Average percent abundance in class A $\pm$ SEM	Average percent abundance in class B $\pm$ SEM
0 days vs. 30 days	39.9	Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes	10.97	0.81 $\pm$ 0.15	0.75 $\pm$ 0.26
		Fungi;Dikarya;Basidiomycota;Agaricomycotina;Agaricomycetes	10.05	0.64 $\pm$ 0.19	1.00 $\pm$ 0.51
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes	9.04	1.53 $\pm$ 0.10	1.52 $\pm$ 0.16
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Dothideomycetes	8.54	0.97 $\pm$ 0.14	0.48 $\pm$ 0.06
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Lecanoromycetes	5.47	0.43 $\pm$ 0.06	0.54 $\pm$ 0.07
0 days vs. 90 days	44.6	Fungi;Dikarya;Basidiomycota;Agaricomycotina;Agaricomycetes	10.9	0.64 $\pm$ 0.19	0.96 $\pm$ 0.04
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Dothideomycetes	8.93	0.97 $\pm$ 0.14	0.30 $\pm$ 0.04
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes	8.76	1.53 $\pm$ 0.10	1.13 $\pm$ 0.09
		Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes	8.38	0.81 $\pm$ 0.15	0.31 $\pm$ 0.04
		Fungi;Chytridiomycota;environmental samples;uncultured Chytridiomycota;Other	4.98	0.59 $\pm$ 0.05	0.23 $\pm$ 0.02
30 days vs. 90 days	43.8	Fungi;Dikarya;Basidiomycota;Agaricomycotina;Agaricomycetes	13.2	1.00 $\pm$ 0.51	0.96 $\pm$ 0.04
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes	9.6	1.52 $\pm$ 0.16	1.13 $\pm$ 0.09
		Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes	8.03	0.75 $\pm$ 0.26	0.31 $\pm$ 0.04
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Lecanoromycetes	5.99	0.54 $\pm$ 0.07	0.48 $\pm$ 0.06
		Fungi;Fungi incertae sedis;Basal fungal lineages;Mucoromycotina;Mucorales	5.12	0.59 $\pm$ 0.06	0.37 $\pm$ 0.04

Table S9. Analysis of similarity percentage (SIMPER) for pirimicarb fungal communities. (Comparison between harvest time)

Soil comparisons A vs. B	Overall Average Dissimilarity	Five most influential taxa	Percent contribution to difference	Average percent abundance in class A $\pm$ SEM	Average percent abundance in class B $\pm$ SEM
0 days vs. 30 days	37.1	Fungi;Dikarya;Ascomycota;Pezizomycotina;Dothideomycetes	12.17	1.31 $\pm$ 0.25	0.58 $\pm$ 0.09
		Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes	10.8	0.94 $\pm$ 0.17	0.64 $\pm$ 0.09
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes	9.97	1.40 $\pm$ 0.11	1.79 $\pm$ 0.15
		Fungi;Chytridiomycota;environmental samples;uncultured Chytridiomycota;Other	6.88	0.70 $\pm$ 0.09	0.77 $\pm$ 0.11
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Lecanoromycetes	5.40	0.44 $\pm$ 0.06	0.57 $\pm$ 0.08
0 days vs. 90 days	41.9	Fungi;Dikarya;Ascomycota;Pezizomycotina;Dothideomycetes	10.9	1.31 $\pm$ 0.25	0.56 $\pm$ 0.08
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes	10.0	1.40 $\pm$ 0.11	1.73 $\pm$ 0.09
		Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes	9.28	0.94 $\pm$ 0.17	0.41 $\pm$ 0.06
		Fungi;Dikarya;Basidiomycota;Agaricomycotina;Agaricomycetes	5.53	0.73 $\pm$ 0.11	0.62 $\pm$ 0.13
		Fungi;Chytridiomycota;environmental samples;uncultured Chytridiomycota;Other	5.02	0.70 $\pm$ 0.09	0.46 $\pm$ 0.05
30 days vs. 90 days	37.6	Fungi;Dikarya;Ascomycota;Pezizomycotina;Eurotiomycetes	13.1	1.79 $\pm$ 0.15	1.73 $\pm$ 0.09
		Fungi;Dikarya;Ascomycota;Saccharomycotina;Saccharomycetes	6.31	0.64 $\pm$ 0.09	0.41 $\pm$ 0.06
		Fungi;Dikarya;Ascomycota;Pezizomycotina;Lecanoromycetes	6.07	0.57 $\pm$ 0.08	0.47 $\pm$ 0.07
		Fungi;Chytridiomycota;environmental samples;uncultured Chytridiomycota;Other	6.04	0.77 $\pm$ 0.11	0.46 $\pm$ 0.05
		Fungi;Fungi incertae sedis;Basal fungal lineages;unclassified zygomycetes;zygomycete sp. AM-2008a	5.86	0.67 $\pm$ 0.06	0.70 $\pm$ 0.10