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# **Fate of deoxynivalenol (DON) and impact on the soil microflora and soil fauna**

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INRAE: National Research Institute for Agriculture, Food and Environment

1 **Abstract:**

2 *Fusarium graminearum* is a plant-pathogenic fungus that causes the devastating disease  
3 “Fusarium head blight” (FHB) in cereal crops such as wheat (*Triticum aestivum*). It also  
4 contaminates grains with mycotoxins, including deoxynivalenol (DON), which turn toxic to  
5 humans and animals. This fungus overwinters in crop residues left in the field. The fate of  
6 mycotoxins in these crop residues in the soil and their ecological role are still unexplored.  
7 Therefore, our objective was to assess whether mycotoxins are maintained in the soil, impact  
8 the soil biome and benefit the survival of *F. graminearum*. A six-month study in microcosms  
9 was performed to examine the fate of DON in *F. graminearum*-contaminated wheat straw and  
10 soil, and its impact on soil communities. DON was extracted from straw and soil mixtures,  
11 and quantified by high-performance liquid chromatography (HPLC). *Fusarium graminearum*  
12 and total fungal and bacterial molecular biomasses were quantified using real-time  
13 polymerase chain reaction (Q-PCR). Nematode and earthworm densities were quantified  
14 through binocular observations. Changes in the genetic structure of fungi, bacteria, protozoa,  
15 and nematodes communities were determined by terminal restriction fragment length  
16 polymorphism (T-RFLP) analyses. Results revealed that DON disappeared from the straw and  
17 the soil over time. The rate of disappearance was accelerated when straw was incorporated  
18 into the soil and when microcosms contained earthworms. Fungal and bacterial biomass, first  
19 stimulated during the incorporation of straw, decreased after 2 weeks and until the end of the  
20 experiment (24 weeks). It decreased more strongly in the presence of DON. This negative  
21 impact of DON was temporary and at the end of the experiment, the bacterial and fungal  
22 biomass was higher in the treatments that received DON than in the other treatments while the  
23 population of *F. graminearum* was unaffected. Similarly, DON modified the community  
24 structures of fungi, bacteria and protozoa to various extents but not that of nematodes. DON-

25 contaminated straw was found attractive for earthworms, and its presence stimulated their  
26 reproduction or cocoon hatching. The major conclusion is that DON briefly affected soil  
27 communities, disappeared over time and gave no observable advantage to soil-borne *F.*  
28 *graminearum* populations.

29

30 **Keywords:** mycotoxins, deoxynivalenol, soil-borne *Fusarium graminearum*, wheat straw,  
31 soil, microflora, soil fauna, community structure

32

### 33 **1. Introduction**

34 Mycotoxins are fungal secondary metabolites potentially harmful to humans and animals.  
35 Trichothecenes are among the most important mycotoxins produced in the field by the genus  
36 *Fusarium* and are considered as a great threat to humans and animals (Rocha et al., 2005;  
37 Tamburic-Ilincic et al., 2015; Mishra et al., 2016; Bilska et al., 2018). They are produced in  
38 the field during crop invasion by the pathogen. Their incidence in cereal crops is a huge  
39 challenge for the agricultural industry (Tanaka et al., 1988; Bottalico and Perrone, 2002;  
40 Oldenburg et al., 2017). Deoxynivalenol (DON) is one of the main trichothecene metabolites;  
41 it is found in cereal crops such as wheat (*Triticum aestivum*), rye (*Secale cereale* L.), barley  
42 (*Hordeum vulgare*), and maize (*Zea mays*) (Nielsen et al., 2012; Oldenburg et al., 2017;  
43 Janssen et al., 2018). DON (also known as vomitoxin) is a type-B trichothecene and is mainly  
44 produced by *F. graminearum* (Megalla et al 1986; Tanaka et al 1988). It is a commonly  
45 reported mycotoxin associated with Fusarium head blight (FHB) diseased cereals (Paul et al.,  
46 2005; Foroud and Eudes, 2009; De Almeida et al., 2016;). DON has very destructive effects  
47 on mammals, e.g. immunity reduction, protein biosynthesis damage, food refusal, diarrhea,  
48 vomiting, and other severe disease symptoms (Placinta et al., 1999; Fokunang et al., 2006;

49 Wild and Gong, 2010; Payros et al., 2016). The European Union has set the threshold level  
50 for DON in winter wheat to 1.250 mg kg<sup>-1</sup> (CE N°1116/2007). Beyer et al. (2007) reported  
51 that 4.3 % of Fusarium-damaged kernels reach or exceed this limit.

52 FHB is spread worldwide, and different approaches have been investigated to overcome the  
53 disease such as disease forecasting, cultivation of resistant varieties, use of fungicides,  
54 agricultural practices, and biocontrol agents (Schisler et al., 2002; De Wolf et al., 2003;  
55 Buerstmayr et al., 2009; Prandini et al., 2009; Mesterhazy, 2014; Lemmens et al., 2016;  
56 Sarrocco et al., 2019; Rojas et al., 2020). Despite all these experimental investigations,  
57 proposing a durable solution for FHB control is still a challenge for agriculture round the  
58 world (Venkatesh and Keller, 2019). Disease management is an ultimate tool to reduce related  
59 mycotoxins and food losses to make food secure for the increasing world population (Wang et  
60 al., 2019). Some mycotoxins like DON are reported to play an active role in fungal  
61 aggressiveness. They are not indispensable for initial infection by the fungus, but they favour  
62 the spread of FHB within a spike (Bai et al., 2002; Mesterházy, 2002; Mudge et al., 2006).

63 Crop residues are important components of arable soil functioning thanks to the nutrients they  
64 bring back to the soil. They stimulate a high diversity of decomposers in the process of litter  
65 decomposition and fuel multitrophic interactions among various soil inhabitants with  
66 noticeable consequences on biogeochemical cycles (Perez et al., 2008; Turmel et al., 2015).  
67 Potential mechanisms include fungus-driven nutrient transfer among litter species, inhibition  
68 or stimulation of microorganisms by specific litter compounds, and a positive feedback of the  
69 soil fauna due to greater habitat and food diversity (Rantalainen et al., 2004; Hättenschwiler et  
70 al., 2005; Sauvadet et al., 2016). The way the species richness of decomposer fungi, bacteria,  
71 as well as the soil fauna including protozoa, or their relative frequencies of occurrence (i.e.  
72 community structure) influence the decomposition of organic matter in arable soil is poorly  
73 known as compared to forest litter soil (Buee et al., 2009; Barbi et al 2014). Using different

74 sets of fungi isolated from plant litters, Deacon et al., (2006) showed a high degree of  
75 functional redundancy in assemblages of culturable decomposer fungi that could buffer the  
76 impact of external events on the decomposition process. Therefore, one may wonder how  
77 phytopathogenic fungi can be included in this food web during their saprotrophic phase and  
78 survive the dynamics of successions. One example of a very strong and effective interaction is  
79 provided by the common earthworms (*Lumbricus terrestris* L), which remove sources of  
80 phytopathogenic fungi (*Venturia inaequalis*) in orchards by grazing on the leaf litter (Holb et  
81 al., 2006).

82 Diseased crop residues (grains, straw and stubble) are colonised by the fungus and are the  
83 source of saprophytic survival during off seasons (Pereyra and Dill-Macky, 2008; Sarrocco et  
84 al., 2012; Leplat et al., 2016;). Current agriculture is moving towards reducing soil tillage  
85 practices to conserve the field soil structure and biotic activity (Bai and Shaner, 2004).  
86 However, this reduced tillage favours the survival of the fungus more than deep tillage (Sipila  
87 et al., 2012; Hofgaard et al., 2016). *Fusarium graminearum* survives as a saprotroph in crop  
88 residues and serves as a primary inoculum to the next crop when warm and moist climatic  
89 conditions lead to perithecium formation and ascospore dispersal (Parry et al., 2007; Trail,  
90 2009).

91 Crop residues appear as a very coveted resource, but *F. graminearum* is not such a good  
92 saprophytic competitor (Leplat et al., 2013) unless the mycotoxins it produces *in planta*  
93 provide it with a significant competitive advantage over the microbial and soil fauna  
94 communities. Few studies focus on the presence of mycotoxins in the soil and how they  
95 interact with the soil biota (Sarrocco et al., 2012; Venkatesh and Keller, 2019). *Lumbricus*  
96 *terrestris* earthworm has been found to prefer DON contaminated wheat straw over  
97 uncontaminated straw, which resulted in a decrease of the infectious potential associated with  
98 the presence of the pathogen, as well as a decrease in DON amounts (Wolfarth et al., 2011).

99 Other fauna components such as nematodes and collembolans also contribute to soil DON  
100 regulation (Wolfarth et al., 2016). The fate of mycotoxins in crop residues and in the soil  
101 remains to be addressed to understand the role of DON in the ecological habitat of  
102 microorganisms.

103 Based on this background, the main objective of this study is to understand the ecological role  
104 of DON during *F. graminearum* survival in crop residues by monitoring the survival of other  
105 soil biota including fungi, bacteria, protozoa, nematodes, and earthworms. Therefore, this  
106 study aims at testing the following hypotheses: (1) DON gives a competitive advantage to *F.*  
107 *graminearum* over fungal microflora; (2) DON increases the palatability of straw for  
108 earthworms and components of soil fauna; (3) the presence of DON stimulates soil  
109 microflora-microfauna interactions

## 110 **2. Materials and methods**

### 111 **2.1- Soil and straw collection**

112 The soil was collected in May 2010 from the Apk horizon of a Calcaric Cambisol (FAO  
113 World Reference Base Soil Classification system, WRB, 2014) on which a permanent  
114 meadow grows near a greenhouse area (Latitude: 47°19'1.349"; Longitude: 5°4'25.692") at  
115 the INRAE (National Research Institute for Agriculture, Food and Environment, Dijon,  
116 France) site. This soil was preferred to cultivated soil to avoid a recent history with wheat and  
117 DON. Briefly, the physico-chemical characteristics of this silty clay soil are as follows: 39.2  
118 % clay, 45.6 % silt and 15.2 % sand, 2.05 % organic matter, pH 8, C/N ratio 9.85. The surface  
119 of the soil was stripped over 2 cm to remove the grass and part of their root systems. The soil  
120 was then taken with a spade, to a depth of 15 cm and placed on metal trays on which it was  
121 crumbled by hand. The trays were placed in an oven at 17 ° C for 48 hours to partially dry the  
122 soil and facilitate sieving. It was sieved to 4 mm to remove gravels and crop debris. A

123 homogenous mixture of the soil was prepared by manual mixing. The soil was then stored in  
124 an incubator at 17°C for 2 weeks. When using it to prepare microcosms, the soil moisture was  
125 adjusted to 17 % w/w, *i.e.* 80 % of its water-holding capacity.

126 Air-dry straw was taken from winter wheat (*Triticum aestivum*) originating from a field of the  
127 INRAE Experimental Unit (Bretenières, France). The straw was shredded into approximately  
128 2-3 cm pieces.

## 129 **2.2- *Fusarium graminearum* inoculum**

130 A previously described DON-chemotype *F. graminearum* strain MIAE00376 (Leplat et al.,  
131 2016) was provided by the collection “Microorganisms of Interest for Agriculture and  
132 Environment” (MIAE, Agroécologie, INRAE Dijon, France).

133 Conidia were produced by the method adapted from Hassan and Bullerman (2009). Briefly,  
134 wheat bran liquid medium was prepared by adding 1% of wheat bran in distilled water and  
135 was autoclaved at 120°C for 20 min. Small plugs of 7-day-old cultures of *F. graminearum*  
136 strain MIAE00376 grown on potato dextrose agar (PDA) were added, and flasks were placed  
137 at 25°C on a rotary shaker (150 rpm) for seven days. The cultures were filtered through sterile  
138 cheesecloth (50 µm mesh size) to remove the mycelium mat and remaining pieces of bran.  
139 The conidial concentration was assessed under the microscope using a Malassez counting  
140 chamber. The concentration ( $6.7 \times 10^2$  conidia ml<sup>-1</sup>) was then adjusted with sterile distilled  
141 water to achieve a final concentration of 10<sup>3</sup> conidia g<sup>-1</sup> straw (see below).

## 142 **2.3- Collection of earthworms**

143 Adult *L. terrestris* specimens were caught from the permanent meadow near the greenhouse  
144 area where soil was collected, at the INRAE site in May 2010. The soil was thoroughly  
145 watered 2 days before catching earthworms, and then a new moderate watering using 0.5 %  
146 formalin in water was performed to compel earthworms to come out from the depth to the soil  
147 surface (Bouché, 1972). The collected adult *L. terrestris* were washed immediately twice

148 using tap water to remove the formalin from their skins. They were added to the soil of  
149 different containers and placed at 10°C for 3 weeks. At the start of the experiment,  
150 earthworms were added to the experimental soil at 17°C for 4 days.

#### 151 **2.4- Experimental design**

152 The study was performed in microcosms with soil- and *F. graminearum*-infected straw  
153 contaminated or non-contaminated with DON. The experiment had a completely randomized  
154 block design with three replications, five sampling times and including the following  
155 treatments:

- 156 1. SS: Straw at the Surface
- 157 2. SSD: Straw at the Surface + DON
- 158 3. SSE: Straw at the Surface + Earthworms
- 159 4. SSDE: Straw at the Surface + DON + Earthworms
- 160 5. SME: Straw incorporated to the soil + Earthworms
- 161 6. SMDE: Straw incorporated to the soil + DON + Earthworms

162 These microcosms were placed in an incubator at 17°C. Their moisture was measured  
163 regularly by weighing them and was adjusted by spraying water when needed. The sample of  
164 the representative homogenous DON-contaminated soil-straw mixture on the day of the  
165 experimental setting-up (T0) was considered as the starting point.

166 Samples were taken 0 (T0), 1 (T1), 2 (T2), 8 (T8), and 24 (T24) weeks after the setting-up of  
167 the experiment. At each sampling time, the earthworms were removed from the microcosms.  
168 The whole remaining contents (i.e. straw and soil) were mixed and freeze-dried and ground to  
169 powder to make a homogenous mixture of straw and soil, and preserved at -20°C until DNA  
170 extraction.

#### 171 **2.5- Microcosms preparation**

172 A transparent solution of DON was obtained by mixing 5 mg of DON powder (Sigma  
173 Aldrich, France - CAS No. 51481-10-8) in 4 ml of pure ethanol. The required concentration  
174 ( $66.7 \mu\text{g ml}^{-1}$ ) was prepared by dissolving the transparent solution of DON in sterile water.  
175 The water-holding capacity of the straw was found to be 3.5 ml of water in 1 g of straw dry  
176 weight (d.w.). The straw was inoculated with *F. graminearum* by spraying and mixing with  
177 1.5 ml of the conidial suspension ( $6.7 \times 10^2$  conidia  $\text{ml}^{-1}$ ) per gram of straw (d.w.). Then the  
178 straw was divided into two halves: one half was contaminated with DON by spraying and  
179 mixing with 1.5 ml of DON solution ( $66.7 \mu\text{g ml}^{-1}$ ) per gram of straw (d.w.), while the other  
180 half was moistened by spraying and mixing with distilled water to provide homogenous  
181 humidity to the whole straw. Finally, the whole straw contained  $10^3$  *F. graminearum* conidia  
182  $\text{g}^{-1}$  of straw (d.w.), and the DON-contaminated straw contained DON at a concentration of  
183  $100 \mu\text{g g}^{-1}$  of straw (d.w.).  
184 Two-L plastic jars (13 cm diameter  $\times$  15 cm height) were used as experimental units  
185 (microcosms). A hole was made in the lid of each jar to allow earthworms to breathe and let  
186 out the gases produced as a result of straw decomposition. This hole was covered with plastic  
187 gauze to avoid earthworms escaping.  
188 Each microcosm was filled with 1kg of soil (dry weight) and 10 g of *F. graminearum*-infected  
189 straw (dry weight) contaminated with DON or not. Ten g of straw contaminated with  $10 \mu\text{g}$  of  
190  $\text{DON g}^{-1}$  (d.w) were introduced into 1kg of soil (d.w.) to contaminate each microcosm with  
191  $1 \mu\text{g DON g}^{-1}$  soil-straw mixture, which is close to the threshold limit recommended by the  
192 European Union for unprocessed wheat grains ( $1.25 \mu\text{g of DON g}^{-1}$ ). The straw was either  
193 mixed with the soil or placed on its surface to form a layer. Four earthworms for a total mean  
194 biomass of  $13.7 (\pm 0.76)$  g were added to each required microcosm. This number of four  
195 earthworms per kg of soil was chosen so that there are enough earthworms for their role on

196 the fate of the straw is measurable and that, possibly, they can reproduce or decrease. At the  
197 same time, this number cannot be higher to prevent them from starving to death.

## 198 **2.6- Quantification of earthworms and nematodes**

199 At the different sampling times, the earthworms previously removed from the soil were  
200 washed twice with water to remove their outer soil and then gently surface-dried on tissue  
201 paper. They were counted, and the total weight of all the earthworms was measured for each  
202 microcosm and termed as their biomass.

203 Nematode extraction and counting were carried out using the elutriator described by  
204 Oostenbrink (1960). Briefly, nematodes were extracted from 250 g of soil or soil-straw  
205 mixture from each microcosm. The soil or soil-straw mixture was suspended in 1 L of water  
206 and sieved to 1 mm to remove the big fragments of straw. The sieved soil suspension  
207 containing nematodes was put in the elutriator using an upward flow of water (flow rate: 35 L  
208 h<sup>-1</sup>) for 15 min to accelerate the natural process of sedimentation of the coarse particles, and  
209 the soil fine particles were collected and sieved to 50 µm. The contents of the sieve were  
210 collected and transferred to a sieve containing a pre-moistened tissue paper. The sieve was  
211 placed in a large Petri dish containing an amount of water that could keep the permanent  
212 humidification of the sieve and ensure nematode migration. The nematodes migrated below  
213 the sieve into the Petri dish. After 48 h, the contents of the Petri dish were poured into a  
214 graduated cylinder up to a 25-ml volume. Then 2 ml were spread on a plate grid specific for  
215 counting the individuals under a dissecting microscope. This counting was performed twice  
216 for each sample. For each sample, 2 ml of the extracted nematode suspension were stored in  
217 microtubes at -20°C for DNA extraction.

## 218 **2.7- DNA extraction**

219 DNA was extracted from different substrates (soil, wheat straw, and soil-straw) following a  
220 procedure adapted from Edel-Hermann et al. (2004). Two g of freeze-dried and ground soil or

221 soil-straw mixture or 0.5 g of freeze-dried and ground straw were added to a 15-ml tube  
222 containing 4 g of 1.4-mm diameter silica beads, 5 g of 0.1-mm diameter ceramic beads, and  
223 eight 2-mm diameter glass beads. Then 8 ml of lysis buffer containing 100 mM Tris HCl (pH  
224 8.0), 100 mM EDTA (pH 8.0), 100 mM NaCl, and 2% sodium dodecyl sulfate (w/v) were  
225 added to each sample. The samples were shaken for 90 s at 4 m s<sup>-1</sup> in a Fast Prep-24  
226 Instrument (MP Biomedicals, Illkirch, France) and incubated for 30 min at 70°C with mixing  
227 after 15 and 30 min. Then they were centrifuged at 7,000 × g for 5 min at 15°C. The  
228 supernatants were recovered in 2-ml microtubes and incubated for 10 min on ice with 0.1  
229 volume of 5 M potassium acetate. After centrifugation at 14,000 × g for 10 min at 4°C, the  
230 nucleic acids in the supernatants were precipitated with one volume of ice-cold isopropanol  
231 for 15 min at -20°C. The precipitate was pelleted by centrifugation at 15,000 × g for 20 min at  
232 4°C, washed twice with 70% (v/v) ice-cold ethanol, air-dried, and dissolved in 200 µl of 10  
233 mM Tris HCl (pH 8.0). Out of 200 µl, only 100 µl of extracts of soil nucleic acids were used  
234 for purification, and the other 100 µl were frozen at -20°C for further use if needed.

235 The extracts of soil nucleic acids were purified twice by using a polyvinylpyrrolidone  
236 (PVPP) spin column to remove co-extracted humic acids, as described by Edel-Hermann et al.  
237 (2004). Then, the DNA extract was purified using a GeneClean Turbo kit (MP Biomedicals).

238 DNA extractions from the nematode-water suspensions were carried out by using the same  
239 procedure as the one used for the soil, with the following exceptions: 2 ml of nematode-water  
240 suspension were used instead of 2 g of soil, and 2 ml of 2-fold more concentrated lysis buffer  
241 were used for nucleic acid extraction. The dry DNA was dissolved in 30 µl of 10 mM Tris  
242 HCl pH 8.0 and purified using a GeneClean Turbo kit.

243 Ten-µl aliquots of purified DNA extracts were resolved by electrophoresis in a 0.8% agarose  
244 gel together with dilutions of calf thymus DNA (Bio-Rad). The gels were stained with  
245 ethidium bromide, photographed under a camera, and the staining intensities were measured

246 with Bio-1D<sup>++</sup> software (Vilber-Lourmat, Marne-la-Vallée, France). The DNA concentrations  
247 were calculated using a standard curve of 25-150 ng of calf thymus DNA plotted versus the  
248 staining intensity. DNAs were stored at -20°C until use.

## 249 **2.8- DON extraction and quantification**

250 The fate of DON during straw incorporation to the soil was monitored up to 24 weeks after  
251 the start of the experiment. DON was extracted and quantified at T = 0, 1, 2, 8, and 24 weeks  
252 for each treatment in the presence of earthworms, with the straw left on the soil surface (SSE  
253 and SSDE) or incorporated to the soil (SME and SMDE). It was also extracted and quantified  
254 at T = 0, 8, and 24 weeks in the absence of earthworms when the straw was left on the soil  
255 surface and contaminated (SSD) or not (SS) with DON (Table 1). The procedure was the one  
256 used by Bily et al. (2004) and recently adapted by Leplat et al. (2018). Briefly, 2 g of each  
257 sample were weighed and transferred to 50-ml tubes with 10 ml of an acetonitrile/ultrapure  
258 H<sub>2</sub>O mixture (84/16). Samples were shaken at 230 rpm for 3 h at 25°C before centrifugation  
259 (10 min, 4,500 rpm) to allow DON to transfer from the matrix to water. The supernatant was  
260 purified using an Immuno Affinity Column (IAC) Trichothecene P (R-Biopharm Rhône Ltd.,  
261 Saint-Didier-au-Mont-d'Or, France). The supernatant was eluted by gravity and DON was  
262 retained by anti-DON antibodies contained in the column gel. The retained mixture was  
263 washed twice with 3 ml of sterilised ultrapure water using gravity. Elution was done with 1.5  
264 ml of 100% pure methanol (MeOH). The organic phase was evaporated to dryness and  
265 dissolved in 200 µl of MeOH/ultrapure H<sub>2</sub>O (1 / 1) by vortexing vigorously for 1 min. The  
266 suspended residue was filtered through a 0.45-µm PFTE membrane (Merck Millipore,  
267 Billerica, USA). The extracts were maintained at -20°C until analysis by high-performance  
268 liquid chromatography with diode-array detection (HPLC-DAD).

269 DON detection and quantification was carried out using a Beckman Gold® HPLC system  
270 equipped with a programmable pump module 126 coupled to a 168-nm photodiode array

271 detector (Beckman Coulter, Fullerton, USA). An Ultrasphere® Octyl Analytical reverse-  
272 phase column (4.6 x 150 mm; particle size 5 µm) was used as an analytical column, and then  
273 a Pre-Column Ultrasphere® Octyl Guard (4.6 x 45 mm; particle size 5 µm) was used. The  
274 chromatographic system contained ultrapure H<sub>2</sub>O at pH 2.6 with H<sub>3</sub>PO<sub>4</sub> (eluent A) and  
275 acetonitrile (eluent B). The gradient started with 5% B. From 0 to 14 min, it linearly increased  
276 to 30% B, and then another linear increase to 90% B in the next 2 min. This phase was kept  
277 up to 18 min. The gradient was then linearly decreased to 5% B in 2 min. Finally, the column  
278 was equilibrated for 4 min before the next injection. The flow rate was 1 ml min<sup>-1</sup>. The  
279 injection volume was 20 µl. The detection wavelength was 220 nm and the retention time was  
280 8.32 min.

281 A calibration curve was prepared by spiking a matrix extract with a standard solution of  
282 DON. The detection limit was determined as the concentration with a signal-to-noise ratio of  
283 3:1. The quantification limit was at a signal-to-noise ratio of 10:1. The DON standard (ref  
284 [M125S100MEVC](#)) was purchased from Cluzeau Info Labo (C.I.L.) (33220 Sainte-Foy-La-  
285 Grande, France; <https://shop.cluzeau.fr/>).

## 286 **2.9- Quantification of *Fusarium graminearum*, fungi, and bacteria**

287 *Fusarium graminearum* and overall fungal and bacterial densities were quantified in all the  
288 samples using SYBR Green dye-based real-time Q-PCR carried out on an ABI PRISM 7900  
289 HT Sequence Detection System (Applied Biosystems TM, USA).

290 *Fusarium graminearum* was quantified by using the specific primer pair Fg16NF (ACA GAT  
291 GACAAGATTCAGGCACA)/Fg16NR (TTCTTTGACATCTGTTCAACCCA) (Nicholson et  
292 al., 1998), which amplifies a 284-bp fragment. One µl of DNA was mixed with 0.25 µM of  
293 each primer, 1 µg of T4 gene 32 protein (MP Biomedicals), 6.5 µl of Q-PCR SYBR Green  
294 ROX MIX (2X) (Thermo Fisher scientific Inc., USA), and DNase-RNase-free water up to a  
295 final volume of 13 µl. The real-time Q-PCR conditions consisted of an initial step of 10 min

296 at 95°C for enzyme activation, followed by 45 cycles of 15 s at 95°C (denaturation), 30 s at  
297 64°C (annealing), 30 s at 72°C (elongation), and 30 s at 78°C (data acquisition). Then a  
298 melting curve analysis was performed as follows: 95°C for 30 s, 70°C for 30 s, and then the  
299 temperature was increased from 70 to 95°C at a 2 % (°C min<sup>-1</sup>) ramp rate.

300 Fungal quantification was carried out by targeting a fungus-specific 348-bp fragment of 18S  
301 rDNA using the primer set FR1 (AICCATTC AATCGGTAIT)/FF390  
302 (CGATAACGAACGAGACCT) (Prévost-Bouré et al., 2011; Vainio and Hantula, 2000). For  
303 the PCR, 1 µl of DNA was mixed with 0.25 µM of each primer, 0.5 µg of T4 gene 32 protein,  
304 6.5 µl of QPCR SYBR Green ROX MIX (2X), and DNase-RNase-free water up to a final  
305 volume of 13 µl. The real-time Q-PCR conditions consisted of an initial step of 10 min at  
306 95°C, followed by 40 amplification cycles with 15 s at 95°C (denaturation), 30 s at 50°C  
307 (annealing), and 60 s at 72°C (elongation and data acquisition). Then a melting curve analysis  
308 was performed with cycling set as follows: 95°C for 15 s, 70°C for 15 s, and then a  
309 temperature increase from 70 to 95°C at a 2 % (°C min<sup>-1</sup>) ramp rate.

310 The bacterial quantifications were performed by targeting a bacterium-specific 194-bp  
311 fragment of 16S rDNA using the primers 341F (CCTACGGGAGGCAGCAG) and 534R, also  
312 referred to as 515R, (ATTACCGCGGCTGCTGGCA) (López-Gutiérrez et al., 2004). One µl  
313 of DNA was mixed with 0.25 µM of each primer, 0.5 µg of T4 gene 32 protein, 7.5 µl of Q-  
314 PCR SYBR Green ROX MIX (2X) and DNase-RNase-free water up to a final volume of 13  
315 µl. The real-time Q-PCR conditions consisted of an initial step of 10 min at 95°C for enzyme  
316 activation, a second step corresponding to the 35 PCR cycles with 15 s at 95°C (denaturation),  
317 30 s at 60°C (annealing), and 30 s at 72°C (elongation and data acquisition). Then a melting  
318 curve analysis was performed with cycling set as follows: 30 s at 95°C, 30 s at 72°C, and then  
319 a temperature increase from 72°C to 95°C at a 2 % (°C min<sup>-1</sup>) ramp rate.

320 Three technical replicates were performed for each biological sample of each of the three  
321 communities. A standard curve based on cycle threshold (Ct) values vs. known quantities of  
322 target DNA was generated for each real-time Q-PCR assay by using ten-fold dilution series of  
323 plasmid DNA containing the cloned specific region for each community corresponding to  $10^9$   
324 to  $10^2$  copies of target DNA per PCR reaction. Two repeats of the standard curve were  
325 assessed during each replication. The curve was used to quantify the amount of target DNA in  
326 the different DNA samples. The assay had a linear range of quantification, and there was a  
327 good correlation between the Ct values and the concentrations of cloned DNA ( $r^2 = 0.8373$ ,  
328  $0.9629$ , and  $0.9629$  for *F. graminearum*, fungi, and bacteria, respectively). Results were  
329 expressed as numbers of copies  $g^{-1}$  soil (d.w.).

330 Additionally, densities of culturable fungi and bacteria were estimated as colony-forming  
331 units (CFU) on the appropriate media. Briefly, 5 g of soil or soil-straw mixture were added to  
332 45 ml of sterile water and shaken for 20 min in a three-dimensional shaker at 700 oscillations  
333  $min^{-1}$ . Ten-fold dilutions were made for each sample from the stock suspension. Fungi were  
334 quantified on MEA (malt extract  $10 g L^{-1}$ , agar  $15 g L^{-1}$ ) supplemented with citric acid ( $250$   
335  $mg L^{-1}$ ), and antibacterial antibiotics (chlortetracycline  $50 mg L^{-1}$  and streptomycin sulphate  
336  $100 mg L^{-1}$ ). Bacteria were quantified on YPGA (yeast extract  $5 g L^{-1}$ , peptone  $5 g L^{-1}$ ,  
337 glucose  $10 g L^{-1}$ , agar  $15 g L^{-1}$ ) supplied with cycloheximide ( $50 mg L^{-1}$ ). Three replicates for  
338 bacteria and 5 replicates for fungi were performed.

### 339 **2.10- Fungal, bacterial, protozoan, and nematode community structures**

340 The structural changes in the microbial and microfauna communities during straw  
341 decomposition were determined by using terminal restriction fragment length polymorphism  
342 (T-RFLP) analysis.

343 Fungal community structures were assessed by targeting the internal transcribed spacer (ITS)  
344 of the ribosomal DNA (rDNA) with primers ITS1F (CTTGGTCATTTAGAGGAAGTAA)

345 (Gardes and Bruns, 1993) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al., 1990).  
346 Primer ITS1F was 5'-end-labeled with the fluorescent dye D3 (Beckman Coulter, Fullerton,  
347 CA, USA). PCR amplifications were performed in a final volume of 25  $\mu$ l by mixing 20 ng of  
348 DNA with 0.2  $\mu$ M of each primer, 200  $\mu$ M each of dATP, dCTP, dGTP, and dTTP, 6 U of  
349 *Taq* DNA polymerase (MP Biomedicals), 0.16 ng ml<sup>-1</sup> of bovine serum albumin (BSA), and  
350 PCR reaction buffer containing 2 mM MgCl<sub>2</sub>. DNA amplifications were performed in a  
351 Mastercycler (Eppendorf, Hamburg, Germany) with an initial denaturation of 5 min at 94°C  
352 followed by 35 cycles of denaturation (30 s at 94°C), primer annealing (30 s at 55°C), and  
353 extension (1 min at 72°C), and a final extension of 10 min at 72°C.

354 Bacterial community structures were assessed by targeting the 16S rDNA with primers 27F  
355 (AGAGTTTGATCCTGGCTCAG) (Edwards et al., 1989) and 1392R  
356 (ACGGGCGGTGTGTACA) (Braker et al., 2001). Primer 27F was 5'-end-labelled with the  
357 fluorescent dye D3 (Beckman Coulter). PCR amplifications were performed in a final volume  
358 of 25  $\mu$ l containing 20 ng of DNA with 0.2  $\mu$ M of each primer, 200  $\mu$ M of dNTP, 6 U of *Taq*  
359 DNA polymerase, and PCR reaction buffer containing 1.5 mM MgCl<sub>2</sub>. DNA amplifications  
360 were performed in a Mastercycler with an initial denaturation of 3 min at 94°C followed by  
361 30 cycles of denaturation (1 min at 94°C), primer annealing (1 min at 57°C), and extension (1  
362 min at 72°C), and a final extension of 10 min at 72°C.

363 In the case of the protozoan community structure, the amplification was performed using the  
364 group-specific PCR primers Kin24SF (TAGGAAGACCGATAGCGAACAAGTAG) 5'-end-  
365 labelled with the fluorescent dye D3 and Kin24SR (TTTCGGGTCCAAACAGGCACACT),  
366 which target the 24S rDNA of the flagellate kinetoplastids (Rasmussen et al., 2001). This  
367 group includes bacterivorous and fungivorous flagellates and is sensitive to the introduction  
368 of exogenous molecules in the soil (Ekelund et al., 2000). PCR amplifications were performed  
369 in a final volume of 25  $\mu$ l by mixing 20 ng of DNA sample with 0.2  $\mu$ M of each primer, 400

370  $\mu\text{M}$  of each dNTP, 6 U of *Taq* DNA polymerase, 0.16 ng ml<sup>-1</sup> of BSA, and PCR reaction  
371 buffer containing 3 mM MgCl<sub>2</sub>. DNA amplifications were performed in a Mastercycler with  
372 an initial denaturation step of 3 min at 94°C followed by 35 cycles of denaturation (1 min at  
373 94°C), primer annealing (1 min at 60°C), and extension (1 min at 72°C), and a final extension  
374 of 5 min at 72°C.

375 Nematode community structures were characterised using the specific primers NEMF1  
376 (CGCAAATTACCCACTCTC) 5'-end-labelled with the fluorescent dye D3, and S3  
377 (AGTCAAATTAAGCCGCAG), which target the 18S rDNA gene (Waite et al., 2003). PCR  
378 amplifications were performed in a final volume of 25  $\mu\text{l}$  by mixing 20 ng of soil DNA with  
379 0.3  $\mu\text{M}$  of each primer, 250  $\mu\text{M}$  of each dNTP, 6 U of *Taq* DNA polymerase, 16 ng ml<sup>-1</sup> of  
380 BSA, and PCR reaction buffer containing 2 mM MgCl<sub>2</sub>. DNA amplifications were performed  
381 in a Mastercycler with an initial denaturation step of 3 min at 94°C followed by 30 cycles of  
382 denaturation (1 min at 94°C), primer annealing (1 min at 53°C), and extension (1 min at  
383 72°C), and a final extension of 10 min at 72°C.

384 PCR products were controlled by electrophoresis in 2% agarose gels for fungi, protozoa and  
385 nematodes, and 1% agarose gels for bacteria. The PCR products were purified by using a  
386 MinElute PCR purification kit (Qiagen, Courtaboeuf, France) according to the manufacturer's  
387 instructions, with two final elutions of the PCR products in 2  $\times$  10  $\mu\text{l}$ . Purified amplicons were  
388 quantified by comparison with known quantities of the molecular-weight marker Smart  
389 Ladder (Eurogentec, Seraing, Belgium) in 2 % agarose gels for fungi, protozoa and  
390 nematodes, and 1 % agarose gels for bacteria. Then, 120 ng of purified amplicons were  
391 digested with 5 U of restriction enzyme in a final volume of 100  $\mu\text{l}$ . The restriction enzymes  
392 that yielded the greatest diversity were used for each community, i.e. *Hinf*I for fungi, *Hae*III  
393 for bacteria, *Alu*I for protozoa, and *Taq*I for nematodes. The digestion reactions were  
394 incubated for 3 h at 37°C, except for *Taq*I at 65°C.

395 The digested products were precipitated with 2  $\mu$ l of 2.5 mg ml<sup>-1</sup> of glycogen (Beckman  
396 Coulter), 10  $\mu$ l of 3 M sodium acetate (pH 5.2), and 250  $\mu$ l of ice-cold ethanol, and were  
397 centrifuged for 15 min at 12,000  $\times$  g at 4°C. The digested DNAs were rinsed twice with 200  
398  $\mu$ l of ice-cold 70% ethanol, and air-dried. The DNAs were re-suspended in 63  $\mu$ l of sample-  
399 loading solution (SLS, Beckman Coulter) and mixed with 30  $\mu$ l of a mixture containing 28.8  
400  $\mu$ l of SLS and 1.2  $\mu$ l of 600-bp size standard (Beckman Coulter). For each sample 30  $\mu$ l were  
401 deposited in three replicates in the wells of a 96-well plate and loaded onto a capillary  
402 electrophoresis sequencer CEQ™ 8000 (Beckman Coulter).

403 Analyses were run with the Frag 4-30 s method including a denaturation step of 2 min at  
404 90°C, injection at 2,000 V for 30 s, and separation at 4,800 V for 70 min. After  
405 electrophoresis, the length and the signal intensity of the fluorescently labelled terminal  
406 restriction fragments (TRF) were automatically calculated by comparison with the size  
407 standard using the CEQ 8000 Genetic Analysis System version 8.0.52. The 60 to 640 bp  
408 fragments corresponding to the size range of the standard were considered. The comparison of  
409 the TRF sizes between samples was automated by assigning them to discrete categories using  
410 the program Lis with an interval of 1.25 bp (Mougel et al., 2002). The T-RFLP analyses were  
411 performed in triplicate for each PCR product. Mean values for the intensity of the peaks found  
412 in at least two of the three analyses were considered for further statistical analyses of the  
413 microbial community structure.

#### 414 **2.11- Statistical analyses**

415 DON amounts in the different compartments over time, *F. graminearum*, fungal, and bacterial  
416 molecular biomass, as well as fungal, bacterial, earthworm, and nematode densities were  
417 compared between treatments and sampling times by analysis of variance (ANOVA) and  
418 Fisher LSD test (p=0.05) using XLSTAT-Pro version 7.1 (Addinsoft).

419 The communities characterised based on their TRF size and intensity measured from peak  
420 heights were compared by principal component analysis (PCA) using ADE-4 software  
421 (Thioulouse et al., 1997). PCA results were displayed as variations on a two-dimensional  
422 diagram for each community. The resulting significance of the structure was checked using  
423 Monte-Carlo tests ( $p < 0.05$ ) with 1,000 random permutations of the data.

424

### 425 **3- Results**

#### 426 **3.1- Fate of DON in wheat straw inside the soil**

427 The fate of DON during straw incorporation in the soil was monitored up to 24 weeks after  
428 the start of the experiment. At T0,  $0.831 \mu\text{g}$  of DON  $\text{g}^{-1}$  of soil-straw mix was detected (Table  
429 1),

430 i.e. 83% recovery of the amount added to the system, either due to adsorption on the matrix or  
431 inefficient extraction. Anyway, relatively to the initial value at T0, DON disappeared very  
432 rapidly when the straw was incorporated in the soil. At T1, the amount of DON was  
433 approximately half ( $0.349 \pm 0.034 \mu\text{g g}^{-1}$ ) that of T0 ( $0.831 \mu\text{g g}^{-1}$ ) in SMDE samples. The  
434 quantity of DON became significantly lower ( $0.033 \pm 0.02 \mu\text{g g}^{-1}$ ) at T2 and soon went below  
435 the quantification limit after T2. On the other hand, DON disappearance was very slow when  
436 the DON-contaminated straw was left on the soil surface (SSD and SSDE). DON started  
437 disappearing after 2 weeks, and was very low after 8 weeks ( $0.012 \pm 0.00 \mu\text{g g}^{-1}$ ) and not  
438 detected at 24 weeks.

439 Surprisingly enough, a low quantity of DON ( $0.105 \pm 0.02 \mu\text{g g}^{-1}$ ) was also found at T1 in the  
440 SSE microcosms (no DON, straw on the soil surface). It disappeared and dropped below the  
441 detection limit at T2. DON traces (below the limit of quantification) were found in non-  
442 contaminated straw (without DON addition) incorporated in the soil (SME).

443 Comparing DON quantities at T = 8 and 24 with DON-contaminated straw on the soil surface  
444 in the presence or absence of earthworms showed a link between the presence of earthworms  
445 and decreased DON quantities in the system. At T8, a significantly higher amount of DON  
446 ( $0.400 \pm 0.09 \mu\text{g g}^{-1}$ ) was found in the absence of earthworms (SSD) than in the presence of  
447 earthworms (SSDE) ( $0.012 \pm 0.00 \mu\text{g g}^{-1}$ ). DON dropped below the quantification limit  
448 ( $0.0018 \mu\text{g g}^{-1}$  of soil-straw mixture) or detection limit ( $0.00034 \mu\text{g g}^{-1}$  of soil-straw mixture)  
449 in all treatments after 24 weeks.

### 450 **3.2- Impact of DON on the microbial and fauna densities**

451 Densities were compared in the presence or absence of DON when the straw was left at the  
452 surface or incorporated in the soil in the presence of earthworms (SSDE, SSE, SMDE and  
453 SME) at T = 1, 2, 8, and 24 weeks, and in the absence of earthworms (SSD and SS) at T = 8  
454 and 24 weeks.

#### 455 **3.2.1 - *Fusarium graminearum* biomass in relation to DON**

456 The overall *F. graminearum* molecular biomass increased as early as T1, and then decreased  
457 with time (Fig. 1A). *F. graminearum* growth was particularly stimulated in the SMDE  
458 microcosms in week 1. Later, no impact of the different treatments was observed until week  
459 8. DON was found to support *F. graminearum* growth in week 24 in SMDE microcosms that  
460 contained DON-contaminated straw incorporated in the soil.

461 The DON-contaminated straw had no impact on *F. graminearum* biomass when the straw was  
462 left on the soil surface in the presence (SSDE) or absence (SSD) of earthworms throughout  
463 the experiment.

#### 464 **3.2.2 -Effect of DON on fungal and bacterial biomass**

465 Fungal and bacterial biomass increased after the microcosms were set up and then decreased  
466 from T2 until the end of the experiment (Fig. 1B and 1C). At T1, fungal densities were  
467 significantly higher in microcosms that incorporated straw in the soil (SME, SMDE).

468 Moreover, fungal as well as bacterial biomass values were significantly higher in the SMDE  
469 (DON-contaminated) microcosms than in the other microcosms. At T2, their biomass values  
470 significantly plummeted in the SMDE microcosms as compared to the other microcosms. This  
471 negative impact was still observed at T8. However, at T24, fungal and bacterial biomass  
472 values were again higher in the microcosms with DON-contaminated straw incorporated in  
473 the soil (SMDE) as compared to SME microcosms. On the other hand, the DON-  
474 contaminated straw left on the soil surface had no impact on fungal and bacterial biomass  
475 values in the presence of earthworms (SSDE) throughout the experiment. Fungal biomass was  
476 higher in the absence of earthworms (SSD) than in all other treatments at T24, while there  
477 was no difference at T8. Bacterial biomass was not affected by the DON-contaminated straw  
478 left at the soil surface in the presence (SSD) or absence (SSDE) of earthworms.

479 Densities of culturable microorganisms were also evaluated (Fig.2A and Fig.2B). Until T24,  
480 there was no significant effect of DON on the fungal and bacterial culturable densities whether  
481 DON-contaminated straw was incorporated in the soil (SMDE) or left at its surface (SSDE and  
482 SSD) in the presence or in the absence of earthworms. At T24, the fungal density is lower in  
483 the microcosms in which DON was present (SMDE and SSDE) compared to those in which  
484 there was no DON (SNE and SSE). Globally, the culturable fungal density increased after two  
485 weeks, unlike total molecular densities, but was not different from those at T0 and T24 (Fig.  
486 2A). As for fungi, at T24, the bacterial density was lower in the microcosms in which DON-  
487 contaminated straw was left at the soil surface (SSDE) compared to those in which non-  
488 contaminated straw was left at the soil surface. Globally, the culturable bacterial density  
489 increased in the first week and then decreased over time (Fig. 2B), which was more or less the  
490 same as total molecular biomass.

### 491 **3.2.3 -Effect of DON on earthworm and nematode densities**

492 The presence of DON had no impact on earthworm biomass (Fig. 2C). The cumulative  
493 earthworm biomass decreased remarkably up to T2 and then along with the disappearance of  
494 straw until T24. This drop in biomass was not related to the presence or absence of DON but  
495 seems to have been more dependent on food depletion. At T24, new-born earthworms were  
496 found in all the treatments, which indicates that earthworm reproduction or cocoon hatching  
497 was not affected by the presence of the mycotoxin. A higher number of new-born earthworms  
498 was found when DON-contaminated straw was left at the soil surface (SSDE) as compared to  
499 non-contaminated straw (SSE). Four new-born earthworms per microcosm ( $0.12 \pm 0.04$  g)  
500 were found in SSDE microcosms on average, vs. only 1 ( $0.045$  g) from the three biological  
501 replicates of the SSE microcosms. Conversely, no significant impact was observed when the  
502 straw was initially incorporated in the soil. Five new-born earthworms ( $0.174 \pm 0.053$  g) per  
503 microcosm were observed in the SMDE microcosms on average, and 4 ( $0.503 \pm 0.52$  g) in the  
504 SME microcosms. No earthworm died in the first 8 weeks, but mortality was observed at T24,  
505 and was slightly higher in the presence of DON. Earthworm mortality in the SSDE  
506 microcosms (straw left on the soil surface in the presence of DON) was 41 %, it was 33 % in  
507 the same DON-free microcosms (SSE), while it was 12 % in the SMDE microcosms (straw  
508 incorporated in the soil in the presence of DON) and 0 % in the absence of DON (SME).

509 The number of nematodes ( $2.4 \times 10^3 \pm 3.9 \times 10^2$ ) in the soil at the time of the experimental  
510 setting-up was considered as the starting point (T0) for all the treatments (Fig. 2D). The  
511 numbers of nematodes in all microcosms increased soon after T0, and the presence of DON had  
512 no influence on the nematode density in the first two weeks. Overall, a negative trend was  
513 observed on the nematode community in the presence of DON at T8. This trend became  
514 significant at T24 in the SSDE and SSD microcosms. Moreover, earthworms were also found to  
515 have suppressed nematodes at T24.

### 516 **3.3- Structural changes in the microbial and faunal communities**

517 The mean number of TRFs per soil sample was 69 for fungal communities, 115 for bacterial  
518 communities, 85 for protozoa communities, and 98 for nematode communities. The presence  
519 of DON in the system affected soil microbial communities according to the type of  
520 community and the location of the DON-contaminated wheat straw, i.e. whether it was  
521 incorporated in the soil or left at its surface.

522 The fungal community structure changed over time from T1 to T8 mainly when the straw was  
523 left at the surface, while it remained almost constant over time when the straw was  
524 incorporated in the soil (Fig. 3A). It is noticeable that the fungal community structure as a  
525 whole was not directly affected by the presence of DON, whether the straw was left at the soil  
526 surface or incorporated in it. In the same way, it is difficult to illustrate the direct role of  
527 earthworms on the fungal community structure, but a DON-earthworm interaction seems to  
528 have affected the fungal community along the first axis when the straw was left at the soil  
529 surface.

530 The bacterial community structure evolved over time in all six treatments (Fig. 3B). The  
531 presence of DON-contaminated straw had a significant effect on the bacterial community  
532 structure immediately after T0 and during the first two weeks, whether the straw was left at  
533 the soil surface or incorporated in it. Then, the differences in bacterial community structures  
534 started disappearing, and at T8, the differences between DON-contaminated and non-  
535 contaminated treatments had disappeared: DON disappeared or was degraded in the first  
536 eight weeks in the presence of earthworms (Table 1). The straw location, i.e. left at the soil  
537 surface (SSE and SSDE) or incorporated in it (SME and SMDE) only played a significant role  
538 on the community structure during the first two weeks. The comparison among the treatments  
539 in the presence or in the absence of DON when the straw was left at the soil surface in the  
540 presence or in the absence of earthworms (SS, SSE, SSD and SSDE) at T8 and T24 showed  
541 that the presence of the earthworms rather than DON modified the bacterial community

542 structure. The effect of DON in the SSD microcosms (straw was left at the soil surface in the  
543 absence of earthworms) was not clear throughout the experiment. Conversely, the community  
544 structures at T8 differed in the presence (SSDE) or absence (SSD) of earthworms when the  
545 straw was contaminated with DON. However, this effect was no longer found after 24 weeks.  
546 The protozoan community structure in the treatments with straw at the soil surface showed  
547 more variability in the biological replicates (Fig. 13C). The presence of DON showed a clear  
548 impact on the protozoan community structure throughout the experiment when the straw was  
549 left on the soil surface both in the presence and in the absence of earthworms (SSD and  
550 SSDE). DON did not affect the protozoan community structure when the straw was  
551 incorporated in the soil. The presence of earthworms had no significant impact on the  
552 protozoan community structure in the treatments with the straw left at the soil surface in the  
553 presence (SSE and SSDE) or absence (SS and SSD) of earthworms at T8 and T24.  
554 Changes in the nematode community structures were more explained by time than by DON  
555 (Fig. 3D). The nematode communities remained unchanged in the first two weeks, but then  
556 they changed along with the time factor, and this change continued till the end of the six  
557 months. The nematode community structure was not affected by the addition of straw in or on  
558 the soil until T24. However, at T24, it seems that the straw supply participated in the  
559 structuration of the nematode community along the axis 2, and this structure is more marked  
560 when the straw was incorporated in the soil than when it was left at the soil surface. Finally,  
561 the nematode community structure showed more variability in the biological replicates at the  
562 end of the experiment than in the first two weeks.

#### 563 **4- Discussion**

564 The fate of DON in winter wheat straw during the saprophytic survival of *F. graminearum*  
565 was monitored for up to six months in microcosms where the straw was left at the soil surface  
566 or incorporated in it in the presence of the whole soil biota.

567 DON disappeared from all the microcosms within the six months. Interestingly, the rate of  
568 disappearance of DON was considerably higher when the straw was incorporated in the soil  
569 than when it was left at its surface thanks to a greater exchange surface between the substrate  
570 and microorganisms leading to faster straw decomposition (Lupwayi et al., 2004) and  
571 microbial DON degradation (Ji et al., 2016; Vanhoutte et al., 2016). More specifically, *F.*  
572 *graminearum* showed the same kind of behaviour as the global fungal and bacterial  
573 communities and reacted to the presence of DON only where the straw was incorporated in  
574 the soil. *Fusarium graminearum* did not produce DON *in situ* or produced it in very low  
575 quantities that disappeared very rapidly during the saprophytic survival of the fungus.  
576 *Fusarium graminearum* was found to benefit from the presence of DON, and its biomass  
577 increased like the overall biomass of the fungal and bacterial communities. Contrary to other  
578 fungal populations in the fungal communities, the presence of DON had no negative effect on  
579 *F. graminearum* density as compared to the control. Similar results were observed with *F.*  
580 *culmorum* (Sarrocco et al., 2012). Although chemical processes partly explain DON  
581 disappearance (He et al., 2010; Guo et al., 2020), they are essentially biotic mechanisms  
582 especially involving the soil microflora and probably also the soil microfauna, which are  
583 suspected in the present study. Mycotoxin-degrading microorganisms including some able to  
584 use DON as a carbon source have indeed already been isolated from animal digestive tracts  
585 (Schatzmayr et al., 2006; He J et al., 2010; Vanhoutte et al., 2017). These are often anaerobic  
586 bacteria, and one of them, *Eubacterium* sp. strain BBSH 797, is marketed to detoxify cereals  
587 incorporated into animal diets (Karlovsy, 2011). However, other microorganisms have also  
588 been isolated from wheat heads (Ikunaga et al., 2011; Ito et al., 2012) and soil (He C.H. et al.,

589 2008; Sato et al., 2012, Ji et al., 2016; Vanhoutte et al., 2017). The modes of action of the  
590 various isolated microorganisms are related to degradation (Garda-Buffon and Badiale-  
591 Furlong, 2010; Sato et al., 2010; Ito et al., 2012; Ji et al., 2016) and / or detoxification by  
592 transforming DON into other smaller or non-toxic molecules (Völkl et al., 2004; Zhou et al.,  
593 2008; He J et al., 2010; McCormick, 2013). Mostly bacteria have been isolated, but fungi are  
594 also involved in DON degradation. For instance, *Aspergillus oryzae* and *Rhizopus oryzae*  
595 degraded 74 and 90 % of DON in 96 and 240 hours, respectively, in controlled conditions  
596 (Garda-Buffon and Badiale-Furlong, 2010). However, their degradation rate is probably lower  
597 under natural conditions, and although no microorganism has been isolated, several soil-borne  
598 microbial species likely produce mycotoxin-degrading extracellular enzymes, which explains  
599 the rapid disappearance of DON in our microcosms (McCormick, 2013). Furthermore,  
600 earthworms played a significant role in DON degradation. At T8, the DON concentration in  
601 the presence of earthworms was 40 times lower than in the earthworm-free microcosm with  
602 the straw left at the soil surface. This comparative assessment is in agreement with previous  
603 demonstrations that earthworms have a specific preference for DON-contaminated straw as  
604 compared to non-contaminated straw (Oldenburg et al., 2008; Wolfarth et al., 2011).  
605 Although this preference has not yet been explained, the choice may well be related to the  
606 earthworm gut microflora, which can degrade DON and therefore provide earthworms with  
607 specific trophic resources (Schrader et al., 2009). Actually, *L. terrestris* drives the whole soil  
608 microfauna, and subsequently the microflora, in the degradation process (Wolfarth et al.,  
609 2016; Meyer-Wolfarth et al., 2017). Although no insects or arthropods have been introduced  
610 into microcosms to date, the soil naturally hosts fauna components, including nematodes,  
611 enchytreids and small arthropods. These small animals are stimulated by the presence of  
612 earthworms and increase microbial activity by breaking down big pieces of straw into small

613 ones and burrowing them, thus increasing the accessibility of the substrate including DON to  
614 soil microorganisms (Hattenschwiler et al., 2005).

615 Changes in the community structures were monitored over six months to determine the impact  
616 of DON on the soil microbial and microfauna communities when it was present in the wheat  
617 straw. Bacterial and protozoan community structures were significantly affected in the  
618 presence of DON, while fungal and nematode communities showed a conditional reaction and  
619 no reaction to DON, respectively. Whether bacterial and protozoan populations were  
620 definitively favoured or suppressed by the presence of DON was not assessed, but the ratio  
621 between populations within each community changed; this revealed that some  
622 microorganisms are more susceptible to the toxin than others. The impact of the mycotoxin  
623 may be positive by favouring populations likely to degrade DON, as mentioned above  
624 (Ikunaga et al., 2011; Sato et al 2010); it may also be negative, but to our knowledge no lethal  
625 effect of DON on fungi or bacteria has been reported.

626 In the case of fungi, the community structures differed depending on whether the straw was  
627 left at the soil surface or incorporated in it. Our results are not contradictory to those obtained  
628 by Sarrocco et al., (2012). Indeed, although these authors demonstrated similar taxonomic  
629 profiles of fungal communities in DON contaminated residues and in uncontaminated  
630 residues buried for 7 days in soil, they did not assess the relative abundance of the different  
631 taxa and therefore, it is not known whether the fungal community structures were the same in  
632 the different residues they analyzed. In our case, the structure was affected by the presence of  
633 DON only in the absence of earthworms. The presence of earthworms globally affected the  
634 fungal community structure, as already mentioned (Wolfarth et al., 2016; Meyer-Wolfarth et  
635 al., 2017). The presence of earthworms might have masked the effect of DON on the fungal  
636 communities. On the one hand, certain fungal species are reported to multiply by degrading  
637 DON and other mycotoxins (Gardan-Bufferon and Badiale-Furlong, 2010; McCormick, 2013),

638 but the other hand, others declined because they were a choice food for earthworms  
639 (Bonkowski et al., 2000; Zirbes et al., 2011).

640 The difference in the bacterial community structures in the presence and absence of  
641 earthworms, also corresponding to different amounts of DON in these treatments, should be  
642 first attributed to the fauna activity and then to the mycotoxin.

643 The effect of DON on protozoa was related to the straw location. This was very clear  
644 throughout the whole experiment when the straw was left on the soil in the presence or in the  
645 absence of earthworms over the six months. As DON was no more detectable at this final  
646 stage, it can be assumed that the changes DON previously caused on the protozoan  
647 community were long-lasting, if not definitive. It would be worth checking for the possible  
648 erosion of protozoan diversity. When the straw was incorporated in the soil, the presence of  
649 DON had a lower impact on protozoa, probably because of a rapid degradation or  
650 detoxification of the mycotoxin by microbial and faunal activity (McCormick, 2013).

651 The results obtained in this study are original; it is the first time that the impact of a  
652 mycotoxin on soil protozoa has been highlighted. One study highlighted that protozoa were  
653 less able than bacteria to detoxify ochratoxin A in rumen fluid, but better efficacy was  
654 observed when bacteria, fungi and protozoa were combined (Mobashar et al., 2012).  
655 However, nothing is said about a possible negative impact of mycotoxins on these protists.

656 The nematode communities showed no response to the presence of DON in any treatment  
657 throughout the whole period of the experiment, whereas nematodes associated with  
658 collembolans and earthworms have been shown to play an important role in DON  
659 degradation, and even in the regulation of mycotoxin production by *Fusarium* sp. (Wolfarth et  
660 al., 2013; 2015). In our experiment, the community structure only changed over time after two  
661 weeks and because of straw supply at T24. Although it is likely that the incorporation and  
662 fragmentation of straw by earthworms was a structuring factor of the nematode community,

663 this hypothesis can not be tested in the absence of a SM (Straw incorporated to the soil) type  
664 of microcosm.

665 Earthworms are known bio-indicators of soil health and have a great importance in agriculture  
666 due to their ability to incorporate and degrade wheat crop residues and organic matter (Friberg  
667 et al., 2005; Kennedy et al 2013; Jiang et al 2018). Our results are in agreement with these  
668 previous findings, and they more specifically emphasize the role of *L. terrestris* in the  
669 degradation and incorporation of straw in the soil. Although the earthworm biomass decreased  
670 over time, probably because the food stocks depleted, the positive and original point was that  
671 this total biomass was not affected by DON-contaminated straw. On the contrary, DON-  
672 contaminated straw was found more attractive for earthworms, which is in agreement with  
673 other findings (Oldenburg et al., 2008; Wolfarth et al., 2016).

674 Furthermore, the presence of the new-born earthworms in all the treatments containing DON-  
675 contaminated straw showed that DON had no negative effect on earthworm reproduction or  
676 cocoon hatching. Even more, the presence of higher numbers of new-born earthworms when  
677 DON-contaminated straw was left at the soil surface showed that DON stimulated earthworm  
678 reproduction or increased hatching. Counting the number of earthworms at each sampling  
679 time revealed that earthworm mortality only occurred at the end of the six months. Overall  
680 more earthworms died when the straw was left at the soil surface. This mortality had no  
681 significant impact on the overall decrease in earthworm biomass, which did not significantly  
682 differ across treatments in the presence or absence of DON.

683 Nematodes are also a very important component of arable soil functioning and are considered  
684 as soil quality indicators (Schloter et al., 2003; Kapp et al., 2013; Du Preez et al., 2018). More  
685 than the direct role of nematodes in organic matter decomposition and in pathogen control  
686 (Hasna et al., 2007), it was the structure of the nematode community impacted by crop  
687 residues and the nature of the organic matter that determined the indicator value (Berry et al.,

688 2007). In our soil microcosms, the number of nematodes increased over time during straw  
689 decomposition while earthworm biomass decreased. There was no impact of DON on  
690 earthworm biomass in the first two weeks, but a negative trend related to the presence of  
691 DON was found at T8; it was significant at T24, suggesting a progressive more than a sudden  
692 poisoning effect. However, such an effect seems to be variable depending on the nematodes.  
693 The developmental process and egg hatching of *Caenorhabditis elegans* were reduced in  
694 liquid medium containing DON (Gowrinathan et al., 2011), while the population density of  
695 *Aphelenchoides saprophilus* increased when the soil received DON-contaminated wheat straw  
696 (Wolfarth et al., 2015). The outcome of nematode - earthworm interactions also seems  
697 variable. The nematode densities increased in the microcosms with the decrease in earthworm  
698 biomass; such a negative relationship between nematode biomass and earthworm biomass  
699 during the decomposition of an organic substrate has already been reported (Dominguez et al.,  
700 2003). Conversely, a positive relationship between nematodes, earthworms and collembolans  
701 resulted in a control of DON contents in wheat straw in the soil (Wolfarth et al., 2016).

702 The multiple interactions occurring in the studied system could have overshadowed a stronger  
703 impact of DON than the one revealed by the community structure fingerprints or the  
704 community molecular biomass. On the contrary, the classic microbiological methods revealed  
705 some variability among microorganisms in their response to the presence of DON, but they  
706 are not accurate enough and cannot ensure that the observed differences can be attributed to  
707 DON, to biotic interactions, or to technical limitations. Therefore, although molecular and  
708 classic microbiological assessments of the fate of bacteria and fungi in the crop residues in the  
709 presence or absence of DON occasionally gave different results, the two techniques are  
710 complementary. They both revealed similar trends, the main ones being that the microbial  
711 communities, including protozoa, were affected to various extents by the presence of DON  
712 but *F. graminearum* was not. All of these results are reassuring since DON present in the crop

713 residues is rapidly biodegraded without creating definitive disturbances within soil microflora  
714 and microfauna and without providing any particular advantage for *F. graminearum* survival.  
715 This is why the risk associated with the presence of DON in food and web goes first through  
716 the control of *F. graminearum*

717

## 718 **5-Conclusion**

719 This study provides a wide overview and a comprehensive knowledge on the fate of DON in  
720 crop residues in the soil and its impact on the soil microflora and fauna.

721 DON did not remain long in the straw and disappeared completely in all the treatments within  
722 six months. *F. graminearum* did not produce DON *in situ*, or DON was produced in very low  
723 quantities that did not compensate for its disappearance, whether through degradation or  
724 transformation into unmeasured molecules. The location of straw played a significant role, as  
725 DON disappeared more rapidly when the straw was incorporated in the soil than when it was  
726 left at its surface. The presence of earthworms enhanced DON disappearance.

727 The bacterial and fungal densities were negatively affected when DON-contaminated straw  
728 was incorporated in the soil, and when DON was present, it significantly changed the  
729 bacterial community structure. The effect on the fungal community structure was seen only in  
730 the absence of earthworms. *F. graminearum* was not affected by the presence of DON. In the  
731 case of protozoa, straw location was more important, and the effect of DON remained  
732 measurable throughout the experiment. The nematode community structure remained  
733 unaffected by the presence of DON.

734 Earthworm biomass was not affected by exposure to DON. Both earthworm reproduction and  
735 cocoon hatching were stimulated in the presence of DON when the straw was left on the soil  
736 surface. Nematode densities were negatively affected and their numbers were reduced, but the  
737 impact appeared late in the experiment.

738 In short, DON briefly impacted the soil communities, disappeared over time mainly through  
739 microbial and fauna activities, and gave no observable advantage to the soil-borne *F.*  
740 *graminearum* population. Thus, of the three hypotheses that our study aimed at testing, the  
741 first concerning the competitive advantage provided by DON to *F. graminearum* is refuted  
742 while those concerning the palatability of straw for earthworms and microfauna as well as the  
743 stimulation of biotic interactions in soil by DON are validated.

744

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752

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1058 **Table and Figure captions**

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1060 **Table 1:** Quantity of DON in the different treatments (SS=Straw at the Surface, SSD=  
1061 SS+DON, SSE= SS+Earthworms, SSDE= SSD+Earthworms, SME= Straw Mixed with the  
1062 soil+Earthworms, SMDE=SME+DON) at the different time points. ND = not determined;  
1063 Traces = below the limit of quantification (LOQ = 0.0018  $\mu\text{g g}^{-1}$  soil-straw mixture); 0 =  
1064 below the limit of detection (LOD = 0.00034  $\mu\text{g g}^{-1}$  soil-straw mixture).

1065 **Fig. 1:** Population dynamics (number of DNA copies) of A: *F. graminearum*, B: fungal  
1066 community, C: bacterial community in wheat straw in the presence or absence of DON and  
1067 earthworms, with straw left on the soil surface or incorporated in it. (SS=Straw at the Surface,  
1068 SSD= SS+DON, SSE= SS+Earthworms, SSDE= SSD+Earthworms, SME= Straw Mixed with  
1069 the soil+Earthworms, SMDE=SME+DON). The different treatments were compared at all the  
1070 sampling times by analysis of variance (ANOVA) and Fisher LSD tests ( $p=0.05$ ). Bars within  
1071 a week with the same lower case letters are not significantly different at  $P < 0.05$ . Time  
1072 periods with the same upper case letters are not significantly different at  $P < 0.05$ .

1073 (  Soil at T0,  SSE,  SSDE  SME,  SMDE  SS  SSD)

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1075 **Fig. 2:** Impact of DON on A: culturable fungi, B: culturable bacteria, C: earthworm biomass  
1076 and D: nematode densities in the microcosms with straw incorporated in the soil or placed on  
1077 its surface. (SS=Straw at the Surface, SSD= SS+DON, SSE= SS+Earthworms, SSDE=  
1078 SSD+Earthworms, SME= Straw Mixed with the soil+Earthworms, SMDE=SME+DON).

1079 The different treatments were compared at each sampling time by analysis of variance  
1080 (ANOVA) and Fisher LSD tests ( $p=0.05$ ). ). Bars within a week with the same lower case

1081 letters are not significantly different at  $P < 0.05$ . Time periods with the same upper case letters  
1082 are not significantly different at  $P < 0.05$ .

1083 (Soil at T0, SSE, SSDE, SME, SMDE, SS, SSD)

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1085 **Fig. 3:** Principal component analysis of the T-RFLP dataset in the two-dimensional diagram  
1086 from all the treatments for A: the fungal community structure, B: the bacterial community  
1087 structure, C: the protozoan community structure and D: the nematode community structure  
1088 Each number represents the mean of 3 biological replicates for one treatment at a specific  
1089 time period. The red numbers represent the presence of DON. SS=Straw at the Surface, SSD=  
1090 SS+DON, SSE= SS+Earthworms, SSDE= SSD+Earthworms, SME= Straw Mixed with the  
1091 soil+Earthworms, SMDE=SME+DON.

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1094 **Table 1**

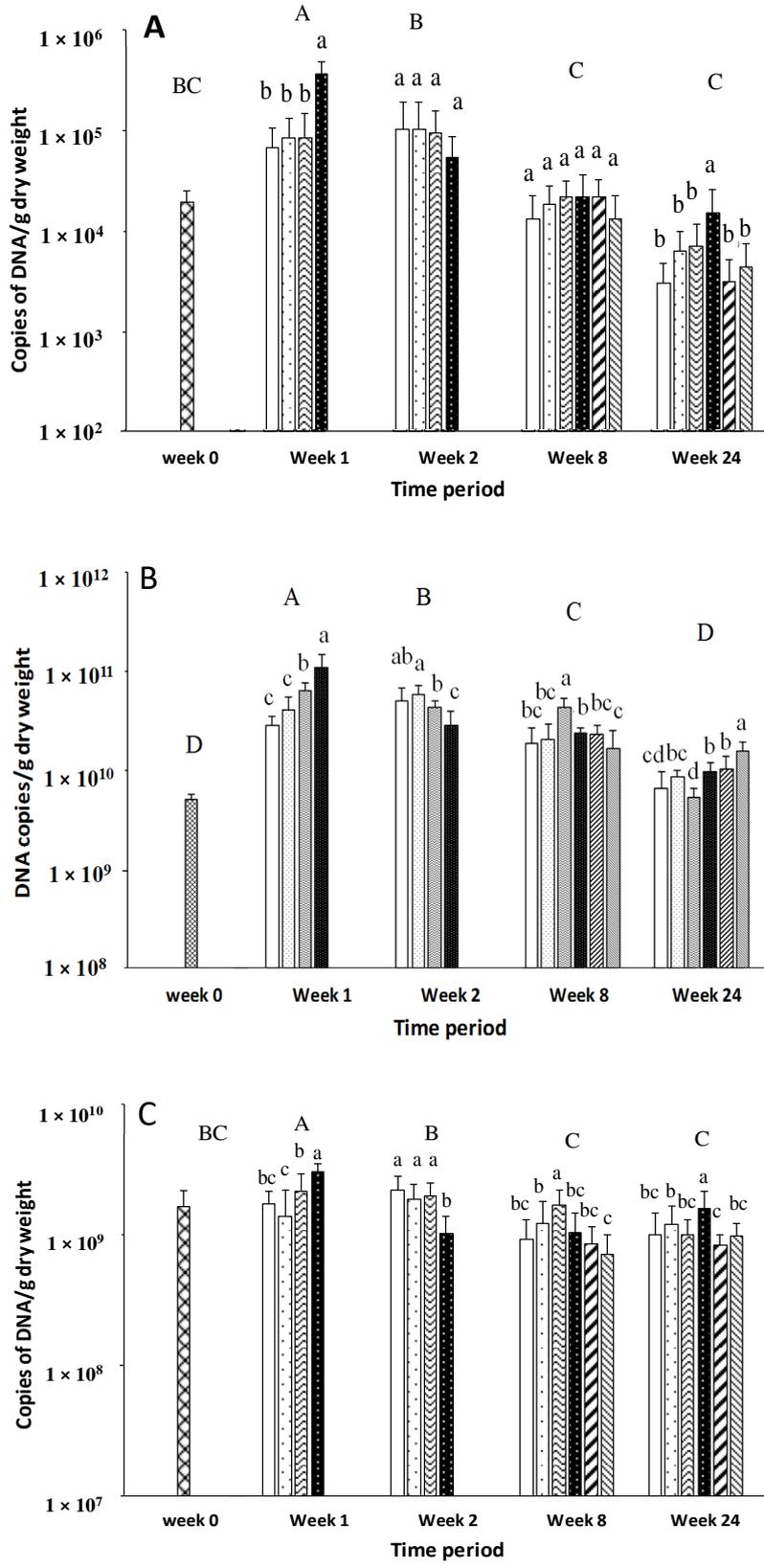
| Treatments | Quantity of DON (DON $\mu\text{g g}^{-1}$ soil-straw mixture) |         |         |         |         |
|------------|---|---------|---------|---------|---------|
|            | Week 0  | Week 1  | Week 2  | Week 8  | Week 24 |
| SME        | ND  | Traces  | 0       | 0       | Traces  |
| SMDE       | 0.831 a <sup>†</sup>  | 0.349 b | 0.033 c | Traces  | Traces  |
| SSE        | ND  | 0.105 a | 0.006 b | 0       | 0       |
| SSDE       | 0.831 a   | 0.889 a | 0.885 a | 0.012 b | 0       |
| SS         | ND  | ND      | ND      | 0       | 0       |
| SSD        | 0.831 a   | ND      | ND      | 0.400 b | 0       |

1095 <sup>†</sup>Values within a column followed by the same letter are not significantly different at  $P \leq 0.05$ .

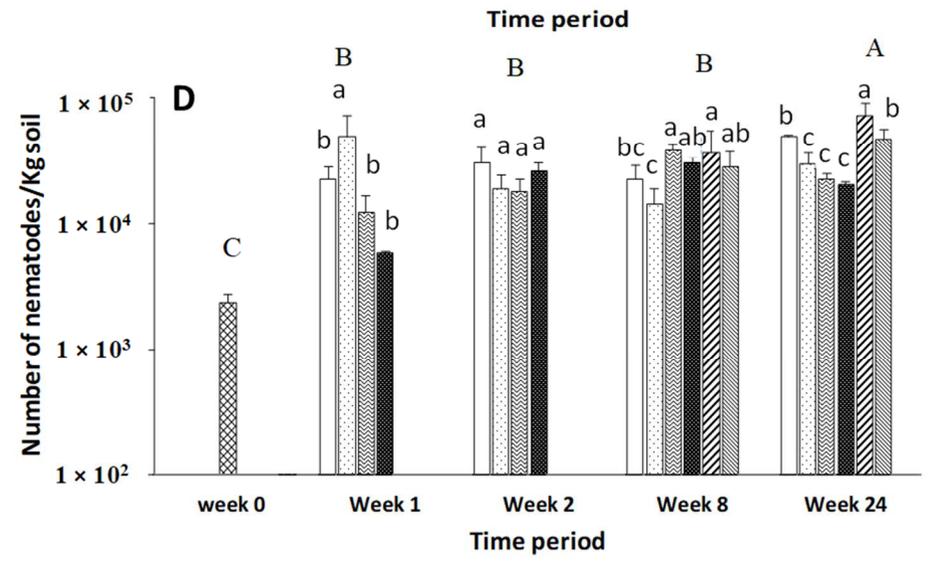
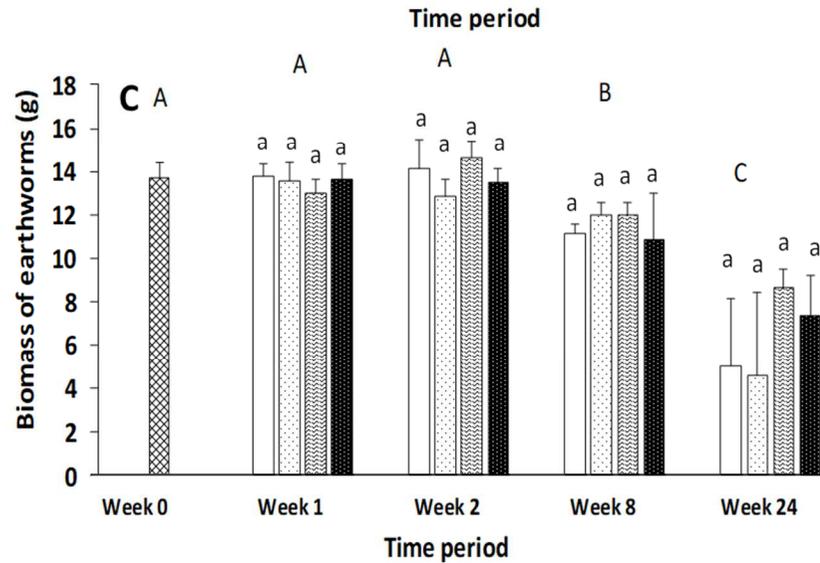
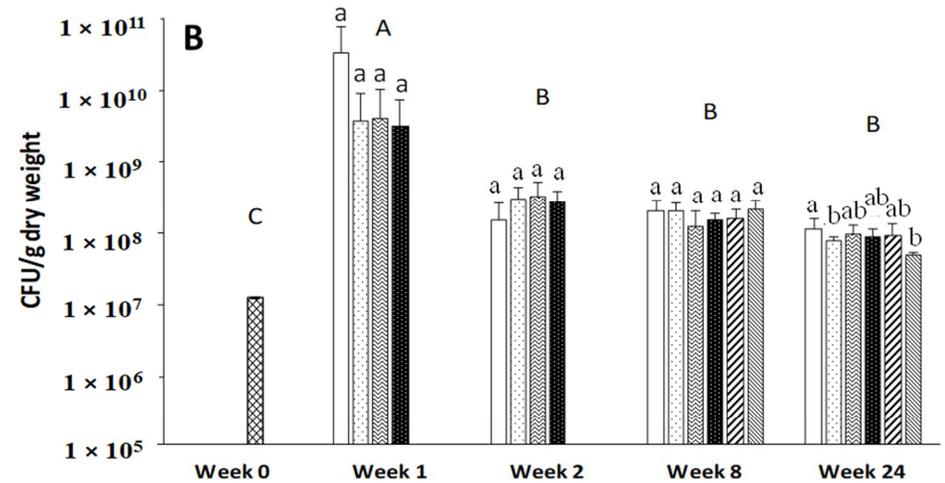
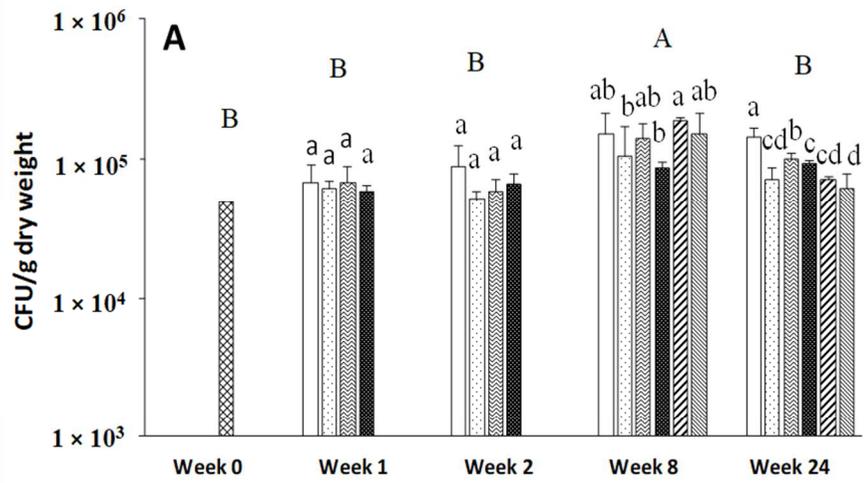
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Figure

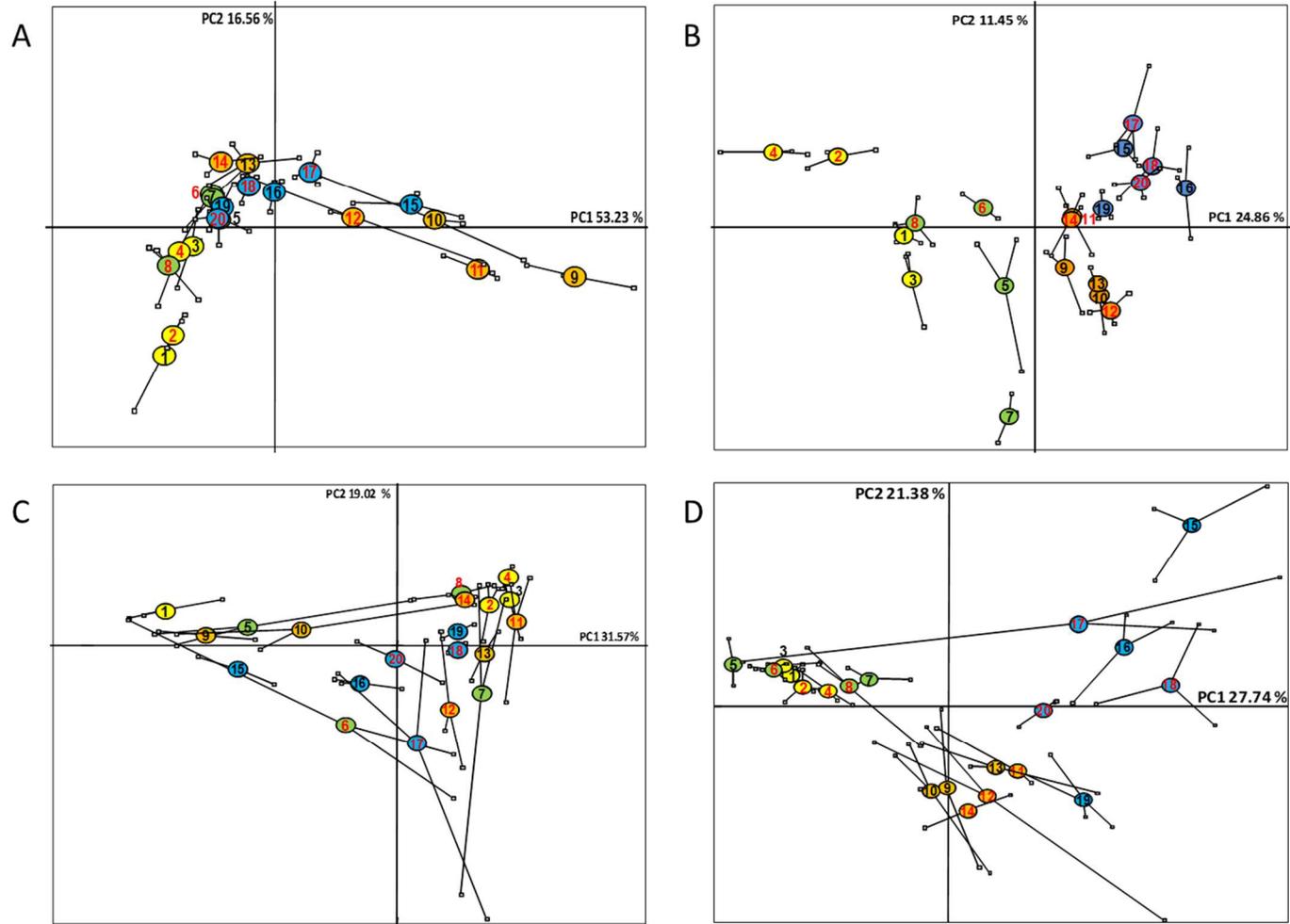


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1128 Figure

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| Treatments | Week 1 | Week 2 | Week 8 | Week 24 |
|------------|--------|--------|--------|---------|
| SS         |        |        | 9      | 15      |
| SSD        |        |        | 11     | 17      |
| SSE        | 1      | 5      | 10     | 16      |
| SSDE       | 2      | 6      | 12     | 18      |
| SME        | 3      | 7      | 13     | 19      |
| SMDE       | 4      | 8      | 14     | 20      |
|            |        |        |        |         |

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1132 Figure 3