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Quantification of *Aspergillus fumigatus* and enteric bacteria in European compost and biochar.

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1 **Quantification of *Aspergillus fumigatus* and enteric bacteria in European compost and**
2 **biochar**

3 Running head: *A. fumigatus* and enteric bacteria in compost and biochar

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27
28 **Abstract**

29 Although most potential human pathogens (PHPs) can be inactivated during composting, the risk
30 that such substrates represent for human health remains largely unknown due to the shortage of
31 information on presence and abundance of PHPs in finished composts. This study focused on the
32 assessment of *Salmonella* spp., *Listeria monocytogenes*, Shiga toxin-producing *Escherichia coli*
33 (STEC) and the opportunistic fungal pathogen *Aspergillus fumigatus* in different compost
34 commodities. A total of fifteen European composts, made from different waste types and processes,
35 were evaluated for the occurrence of the selected PHPs using molecular and traditional techniques.
36 The analyses were extended to five biochar because of their growing application in agriculture,
37 horticulture, floriculture and private gardening.

38 Enteric bacteria were detected by molecular methods in eight out of fifteen composts; however,
39 viable propagules were confirmed for *L. monocytogenes* only in two composts, and for STEC in
40 further three composts. No bacterial pathogens were found in biochar. Living *A. fumigatus* was
41 present in eleven composts and two biochars. None of the eighteen isolates contained SNPs relevant
42 for resistance to azole fungicides. The role of compost and biochar as a source of PHPs in the
43 environment and the risk for human health is discussed.

44
45
46 **Introduction**

47 Composting is a self-heating microbiologically driven process that allows the recycling of a variety
48 of organic materials with different origin (Ryckeboer *et al.* 2003; Stentiford and Bertoldi, 2010).
49 Municipal and green wastes or a mixture of those is common substrates but compost is also a mean
50 for recycling human sewage and animal wastes, although with increasing human hazard (Jones and
51 Martin, 2003).

52 The process includes a mesophilic phase (10-42°C) at the beginning of the process, a thermophilic
53 phase when temperature reaches 55-65°C for different extent of time, a further mesophilic stage and
54 a final maturing phase when the temperature declines and the material stabilizes. Most of the
55 potential human pathogens are eliminated during the thermophilic phase with 55°C for 3 days
56 (Anon, 1981) but different time-temperature combinations are applied depending on country
57 composting standards (Jones and Martin, 2003).

58 Composts in Europe are produced by following appropriate standard procedures and before
59 commercialization have to satisfy microbiological standards (e.g. levels of *E. coli* below 1000 CFU
60 per gram of fresh mass and absence of *Salmonella spp.*). Microbiological standards are also
61 recommended in the Draft Final Report on End-of-Waste for Compost and Digestate
62 (<http://ipts.jrc.ec.europa.eu/publications/pub.cfm?id=6869>), and expected in the future EU
63 harmonised compost quality regulation.

64 When composting processes are conducted in an inefficient manner, a substrate susceptible to re-
65 colonisation may be generated, and consequently compost could become a substrate maintaining a
66 number of enteric bacteria in the environment such as *Salmonella*, *Escherichia*, and *Listeria*, posing
67 human health issues (Jones and Martin, 2003).

68 To date, many studies have been carried out on the occurrence and survival of bacterial pathogens
69 in human and animal wastes and biosolids, and were extensively reviewed (Wiley and Westerberg
70 1969; Jones and Martin 2003; Sidhu and Toze 2009). However, despite the reported risk of plant
71 contamination by enteric pathogens when using composts (Islam *et al.*, 2004), there is a shortage of
72 information on the presence and abundance of these organisms in green and mixed composts
73 (Avery *et al.*, 2012). Indeed, microbiologist's attention is mainly focused towards the control and
74 inactivation of enteric pathogens to maintain their level under mandatory limits (Heringa *et al.*
75 2010; Shepherd *et al.* 2011; Singh *et al.* 2011).

76 Although the presence and abundance of the opportunistic fungal pathogen *Aspergillus fumigatus*
77 Fresenius cause of the so-called "aspergilloses" of which the most severe is represented by the

78 Invasive Aspergillosis (IA), have been reported in different types of compost, it has been rather
79 underestimated even though its role in the compost degradation process and its health implications
80 are widely recognized. This organism is a ubiquitous fungus normally inhabiting the soil and
81 decaying materials (Dagenais *et al.* 2009; Gisi, 2013) but it is well equipped to survive successfully
82 in a wide range of environments due to a number of features, recently discussed by Kwon-Chung
83 and Sugui (2013), first of all the wide growth temperature range. *A. fumigatus* is present in compost
84 samples at concentrations of 10^6 - 10^7 CFU/gdw (Millner *et al.* 1994), the spores are released to the
85 air during compost activities such as turning reaching concentrations of 10^4 - 10^7 /m³ (Recer *et al.*
86 2001; Wheeler *et al.* 2001).

87 According to O' Gorman (2011) the menace for IA coming from the airborne inoculum of *A.*
88 *fumigatus*, also originating from compost commodities, is highly underestimated.

89 Organic substrates, including them compost, have been claimed to be one of the environmental
90 sources for itraconazole resistant strains of *A. fumigatus* further contributing to hazard for human
91 health (Snelders *et al.* 2009; Verweij *et al.* 2009; Gisi 2013).

92 Biochars are obtained from plant and/or animal wastes transformed to carboniferous porous
93 material by pyrolysis processes (Beesley *et al.* 2011). Although biochar is considered safe for users,
94 thanks to the high temperatures used for its production, possible contaminations by human
95 pathogens may occur in later stages. Therefore, a proper storage and avoidance of cross
96 contamination must be taken into consideration.

97 In this study, a combination of relevant microbiological and molecular techniques have been
98 adopted to assay the occurrence of targeted *Salmonella spp.*, *L. monocytogenes*, Shiga toxin-
99 producing *E. coli* (STEC) and the opportunistic fungal pathogen *A. fumigatus*. The vitality and
100 concentration of enteric bacteria and *A. fumigatus* have been estimated by plating. Real time PCR
101 kits have been used as quick detection method of genomic and/or metagenomic DNA directly
102 extracted from compost or obtained after culture enrichment. While the presence of bacterial
103 pathogens have been confirmed by selective plating and real time PCR, the identification of *A.*

104 *fumigatus* was completed by macro-morphological assessment and by sequencing relevant gene
105 regions including ITS and β -tubulin (Samsom *et al.* 2007). The 14 α -sterol demethylase gene
106 *cyp51A* and the gene promoter were also sequenced in order to inspect whether strains resistant to
107 demethylation inhibitor (DMI) fungicides were present (Diaz Guerra *et al.* 2003; Chen *et al.* 2005;
108 Verweij *et al.* 2009; Howard *et al.* 2011). All testing were extended to five biochars to understand if
109 and to what extent these new soil amendments may embody a further environmental reservoir of the
110 targeted PHPs.

111 The aim of this study was to estimate the presence and abundance of targeted PHPs in organic
112 substrates such as compost and biochars, made from different waste types and processes. Because
113 there is a growing health concern linked to the increased recovery of *A. fumigatus* isolates resistant
114 to azole fungicides in clinical and environmental samples (Gisi 2013; Vermeulen *et al.* 2013), a
115 further objective of the study was to verify the potential contribution of compost and biochar as
116 environmental source for DMI resistant *A. fumigatus* strains as was recently hypothesised by some
117 medical researchers (Snelders *et al.* 2009; Verweij *et al.* 2009).

118

119

Materials and methods

120 *Composts and biochars, collection and storage*

121 Fifteen composts originated from six different European countries (Hungary, The Netherlands,
122 Spain, Italy, United Kingdom and Portugal) have been analyzed. Three types of composting
123 systems were selected: open outdoor composting; closed (in-vessel, turning) composting; combined
124 closed (first phase) and open (second phase) composting. Different types of waste were considered:
125 only green waste (garden and park waste, ERC 20 02 01); green waste and municipal waste (kitchen
126 and canteen waste, ERC 20 01 08); animal manure (manure, ERC 020106 and sludge, ERC
127 020301); agrifood waste (Olive mill pomace, ERC 02 03 01; Olive leaves, ERC 02 03 04). Specific
128 features of each substrate examined are listed in Table 1.

129 In their country of origin representative samples were taken from an approximately 1 m³ big bag of
130 compost, consisting of a composite sample obtained by pooling 5-6 individual subsamples taken
131 from a 20-30 m³ pile of sieved and ready to market compost.

132 The five biochars came from four different countries and, except for BIOCHAR 1 which was
133 obtained by the carbonization of animal bone, all others derived from plant wastes. Upon their
134 arrival (April-May) composts/chars were maintained in big bags stored under an outdoor canopy
135 and subsamples of 1kg (approx weight) were kept at 4°C for the following microbiological and
136 molecular analyses.

137

138 *Detection and identification of enteric pathogens*

139 Compost and biochar samples (25 grams) were collected in sterile Blender bags (VWR, Radnor,
140 PA, USA). *L. monocytogenes*, *Salmonella* spp., Shiga toxin-producing *E. coli* (STEC) were
141 detected by using real-time PCR kits (iQ-Check™, Bio-Rad, France) following manufacturers'
142 instructions with only minor modifications. In brief, for each pathogen, selective enrichment broth
143 at the appropriate incubation temperature, and DNA extraction protocol for environmental samples,
144 were followed. Simultaneously, 0.1 mL of samples were plated on Hektoen-Enter-Agar (Merck®,
145 Germany), and coliform bacterial colonies (typically orange-red surrounded by a zone of
146 precipitate) were enumerated after incubation at 35 °C for 24 h.

147 Selective platings were carried out for all samples to verify the presence of the targeted pathogens.
148 The procedures were as follows: 25 g of each sample was transferred from the container into a
149 sterile Blender bag (VWR, Radnor, PA, USA) together with 225 mL of 0.1% sterile peptone water
150 (Sigma-Aldrich, St. Louis, USA) and homogenized for 180 s in a Masticator (IUL instruments,
151 Barcelona, Spain). To enumerate *L. monocytogenes*, Oxford selective agar (Sigma-Aldrich, St.
152 Louis, USA) added with Oxford *Listeria* selective supplement (Sigma-Aldrich, St. Louis, USA)
153 were used. Two 100 µL homogenates were taken, and decimal dilution series were spread onto two
154 Oxford agar plates and incubated for 48 h at 35 °C. Similarly, homogenates of STEC positive

155 samples were seeded in duplicate onto CHROMagar™ STEC (CHROMagar, Paris, France) plates
156 were incubated at 37 °C for 24 hours. *Salmonella* spp. positive samples were checked by using
157 Xylose-Lysine-Desoxycholate Agar (XLD) plates after 24 h of incubation at 35 °C. For each
158 selective media, typical colonies appearance were counted according to the user's manual,
159 randomly selected (at least three colonies), and picked for further confirmation by using appropriate
160 real-time PCR kit as above.

161 All bacterial concentrations have been expressed as Colony Forming Units per gram dry weight of
162 substrate (CFU/gdw) for each compost/biochar.

163

164 *Isolation and identification of Aspergillus fumigatus*

165 Three subsamples of 0.5g (fresh weight) 10fold diluted in Ringer (Merck®, Germany) solution (2
166 tablets per litre), the flasks were incubated in a rotary shaker at 100 rpm for one hour and the broth
167 diluted ten-fold. One ml of suspension was spread in three 90 mm Petri dishes for each dilution
168 series containing 15 ml of Potato Dextrose Agar (PDA) amended with 60mg/l of streptomycin, and
169 plates sealed and incubated at 42°C (species optimum). After four days plates have been assessed
170 for the presence of typical dark green/grey colonies exhibiting *A. fumigatus* morphology. At least
171 one isolate was retained per each compost/biochar sample and purified through mono-hyphal
172 subculturing at least twice on PDA at 37°C. The first identification was carried out through
173 comparing the ability to growth at 10 and 50°C and assessing the colony and fungal structures
174 morphology with the reference CBS isolate 133.61 (Samson *et al.* 2007; Arendrup *et al.* 2010).

175 Cultures for DNA isolation were grown on cellophane disks for 7 days at 37°C, and mycelia
176 collected in 1.5 ml tubes. DNA of *A. fumigatus*-like colonies has been obtained using the EZNA®
177 FUNGAL DNA KIT starting from 100mg of fresh mycelia following manufactured instructions with the
178 exception that DNA was finally eluted in MilliQ autoclaved water instead of using kit elution
179 buffer. Isolate identification was confirmed by sequencing the rDNA ITS (White *et al.* 1990) and β -
180 tubulin (Glass and Donaldson, 1995). Each single reaction mixture contained: 1µl of template

181 DNA, 2µl of nucleotides mixture 2.5mM, 1µl of each primer prepared at a concentration of 10mM,
182 1.25µl of MgCl₂ 50mM, 2.5µl of 10XPCR buffer, 16 µl of MilliQ autoclaved water and 0.25 µl of
183 Taq Polymerase (Qiagen[®], Germany). PCR cycle for ITS and *benA* gene included a denaturing
184 stage of 95°C for 2 min and 35 cycles as follow: 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min
185 with a final elongation step at 72°C for 7 min.

186 To determine the presence of specific SNPs for DMI resistance among the pathogen isolates, the
187 *cyp51A* gene was amplified using high fidelity Taq and specific primer pairs (Chen *et al.* 2005).
188 Each reaction was composed of the following components: 1µl of template DNA, 2µl of nucleotide
189 mixture 2.5mM, 1µl of each primer prepared at a concentration of 10mM, 1.25µl of MgCl₂ 50mM,
190 2.5µl of 10XPCR buffer, 17.4 µl of MilliQ autoclaved water and 0.1 µl of *Pfu* Taq
191 (Invitrogen[®] Carlsbad, CA USA). For *cyp51A* gene identification, the cycles were: a denaturing
192 stage at 94°C for 2 min followed by 94°C for 30 sec, 56°C for 30 sec, 68°C for 2 min with 35
193 cycles, without final elongation. The *cyp51A* promoter was amplified using the protocol reported by
194 Mellado *et al.* (2001). The promoter PCR included a denaturation step at 94°C for two minutes
195 followed by 35 cycles at 94°C for 30s, 60°C for 30sec, 68°C for 2min.

196 ITS, β -tubulin and *cyp51A* amplicons were sequenced through Macrogen Europe sequencing
197 service, and sequences manually edited using BioEdit v. 7.9 (Hall 1999). The coding region was
198 compared with the *cyp51A* sequences present in the database to verify the existence of reported
199 mutations linked to DMI resistance. Confirmed *A. fumigatus* isolates were included in
200 AGROINNOVA culture collection and long-term stored in Tryptic Soy Broth (Merck[®], Germany)
201 25% glycerol at -20°C. All potentially hazardous materials were destroyed through autoclaving for
202 25min at 121°C.

203 The concentration of *A. fumigatus* was expressed as Colony Forming Units per gram of dry weight
204 (CFU/gdw) for each compost/biochar.

205

206 *DNA isolation and real time PCR for A. fumigatus direct detection*

207 Metagenomic DNA extraction was made with samples of 0.5 g of compost/char using NUCLEO SPIN
208 SOIL KIT (Macherey-Nagel GmbH & Co. KG) following manufacturers' instructions, with a final
209 elution step in MilliQ autoclaved water as for fungal DNA. Genesig[®] commercial kit adopted in
210 medical labs for the specific detection and quantification of *A. fumigatus* in clinical samples was
211 purchased by Primer Design[™] Ltd, UK. The kit uses a taq-Man probe developed for a gene of the
212 hypothetical protein AFUA_3G08890 to detect in 50 PCR cycles the presence/absence of *A.*
213 *fumigatus* in clinical samples with a cut-off Ct value of 39. Following manufacturers' instruction
214 the kit was first used on 1:20 diluted DNA obtained from mycelia and then applied to metagenomic
215 DNA extracted from compost and biochars.

216

217

Results and discussion

218

Compost and biochar characteristics

219 The main characteristics including percent organic carbon, C/N ratio, pH, provenances, input
220 materials and processes of 15 compost types and 5 chars screened in the study are summarized in
221 Table 1. Six composts each came from green waste (C2, C6, C7, C8, C11, C13) and municipal
222 waste (C3, C4, C9, C12, C14, C15), two from animal waste (C5, C10) and one from a combination
223 of olive pomace and sheep manure (C1). One char was animal based (CHAR1), the others plant
224 based. Most compost types were neutral (pH 6.5 to 7.5), some (C3, C6, C9, C10, C15) were slightly
225 basic (pH 7.5 to 8.1) and most chars strongly basic (pH > 9). Only a few compost types were rather
226 low in organic carbon (< 20%, C2, C5), all others ranged between 20 and 43%. The degradability of
227 organic substrates can be estimated by the C/N ratio; if >25, degradation is assumed to slow down.
228 All compost types showed rather favourable C/N ratios (10 to 20), some (C8, C9, C13, C15) even
229 below 10 indicating the presence of rather high nitrogen concentrations. No relation was found
230 between chemical properties and provenances of compost types.

231

232

Presence and abundance of enteric pathogens

233 Through real time PCR assays eighteen positive results were obtained for enteric bacteria: C6, C11,
234 C14 and CHAR3 for *L. monocytogenes*; C1, C2, C4, C5, C15, CHAR2 for *Salmonella* spp. and C1,
235 C2, C5, C10, C13, C14, C15, CHAR3 for STEC. Nevertheless, vital colonies were observed in only
236 five cases out of twenty samples (Table 2): *L. monocytogenes* was found in green compost in one
237 Spanish (C6) and one Italian (C11) sample with a concentration of 2.3×10^3 and 2.8×10^4
238 respectively. Shiga toxin-producing *E. coli* were detected in three composts made from municipal
239 and animal manure from Italy, Spain and Hungary (C10, C12, C13) with concentrations of 1.88 to
240 2.46×10^3 CFU/gdw.

241 Coliform bacteria were detected in nine of twenty samples between a minimum of 4.1×10^2
242 CFU/gdw in two Italian composts of municipal origin and a maximum of 1.94×10^5 CFU/gdw in C2
243 green compost from Netherland. Values are within the range of those already reported in other
244 studies on composts (Gong *et al.* 2005). Plate counts for *Salmonella* spp. were always negative (<10
245 CFU/gdw). In biochars targeted bacteria were not found (<10 CFU/gdw) (Table 2).

246 Although selective platings allowed to detect vital enteric bacteria in finished composts the
247 overestimation of their abundance linked to the aerosol dispersion should be also contemplated
248 according to what reported by Cevallos-Cevallos *et al.* (2012).

249 The detection of enteric bacteria in animal derived composts was expected as their presence and
250 survival are well known in biosolids (Sidhu and Toze, 2009) which represent similar matrices in
251 terms of input material for animal waste compost. The finding of enteric bacteria in green composts
252 is also not uncommon (Jones and Martin, 2003) although few data are available on their level and it
253 is still unclear which factor(s) correlate greatly with their presence and survival (Avery *et al.* 2012).

254 According to our results the detection of enteric pathogens in composts seem to be more linked to
255 handling, transport and/or external contamination (outdoor storage) rather than being a feature of
256 the material or process itself (Pietronave *et al.* 2004).

257 Detected concentrations are unlikely to cause contamination of vegetables growing on substrates
258 containing PHP's; however, considering that only few cells could cause illness they should not be

259 underestimated. Favourable conditions, in terms of humidity, temperature and lack of antagonistic
260 competitors could allow enteric bacteria to re-grow and re-colonise the substrates (Santamaria and
261 Torazos, 2003; Sidhu and Toze, 2009).

262 *Presence and abundance of A. fumigatus*

263 Viable propagules of *A. fumigatus* were found in eleven out of fifteen composts and in two biochars
264 out of five (Table 2). Interestingly, the fungus was not found (or below detection limit) in composts
265 made from animal manure (C5 and C10, both from Spain) or a combination of olive debris and
266 sheep manure (C1 from Spain) as well as in one compost from municipal waste (C15 from
267 Portugal) and three of five chars.

268 The highest concentration was detected in a British compost made from municipal waste (C14) with
269 6.15×10^5 CFU/gdw. In all compost samples made from green wastes (C2, C6, C7, C8, C11, C13)
270 and one from municipal waste (C4), the *A. fumigatus* load was intermediate: between 0.24 and 3.62
271 $\times 10^3$ CFU/gdw and comparable to those of biochar 5 (2.32×10^3 CFU/gdw). Low concentrations
272 were recorded in two composts from municipal waste (C3 and C9) and char 4, with estimated
273 concentrations lower than 10^2 CFU/gdw. In one case, *A. fumigatus* concentrations increased as a
274 consequence of longer storage and turning of the compost: in fact, the C12 sample represents the
275 same substrate as C3 but outdoor stored and periodically turned for a period of one year.
276 Concentrations lower than 10^2 CFU/gdw were obtained with the same methodology also for a soil
277 sample used as standard substrate in greenhouse experiments (data not shown). The *A. fumigatus*
278 concentrations observed in our study are within the range of those already reported in other
279 investigations on composts (Millner *et al.* 1994; Anastasi *et al.* 2005).

280 The highest concentrations of the fungus were detected in compost made with green wastes,
281 suggesting a close link between the presence of cellulose and lignocellulose substrates and *A.*
282 *fumigatus* abundance. In fact, *A. fumigatus* is a strong producer of cellulolytic enzymes (Liu *et al.*
283 2013).

284 While the link between the presence of the fungus with the type (mainly green) of compost appear
285 evident, the presence of *A. fumigatus* in biochar is most probably due to superficial proliferation of
286 the fungus likely as consequence of an airborne contamination.

287 On the other hand according to our results it does not seem to exist a connection between the
288 fungus level and the composting process because *A. fumigatus* was recovered from all types of
289 production methods.

290 Although real time PCR assays allowed confirming the identity of *A. fumigatus*, it was not possible
291 to detect its presence based on DNA extracted directly from compost, probably because of reaction
292 inhibitors and/or too low concentration of the fungus in the substrates.

293 Some protocols are available for the detection of *A. fumigatus* in water, air and clinical samples
294 (McDevitt *et al.* 2004; Bansod *et al.* 2008; Vesper *et al.* 2008; Serrano *et al.* 2011) but no studies
295 were carried out on its direct diagnosis in soils or composts. In agreement with O' Gorman (2011)
296 and Gisi (2013) further studies are needed to investigate the presence and abundance of *A.*
297 *fumigatus* in such types of environmental samples.

298

299 *Identification and characterisation of A. fumigatus strains.*

300 Eighteen fungal strains were retained from the isolations made on PDA at 42°C. Sequence analysis
301 confirmed full identity of all strains to the species *A. fumigatus*. Taq Man real time PCR further
302 confirmed their identity. Real time PCR assays failed to detect *A. fumigatus* presence in compost
303 samples when DNA was extracted directly from the organic substrates, despite several attempts of
304 sample dilutions up to 500 fold, probably because some humic acid compounds disturbed the PCR
305 reaction. DNA sequences of isolates were deposited in GenBank for the three regions assessed
306 (accessions KF921462-KF921475; KJ584392-95 for ITS; KF921476-KF921489; KJ584396-99 for
307 beta-tubulin; and KJ584374-90 for *cyp51A*).

308 All sequences shared 100% identity with *A. fumigatus* gene accessions present in the NCBI
309 database. However, there were some minor differences among isolates in the ITS and in the beta-

310 tubulin sequences: in four isolates, a T to C change at position 105 of the ITS sequence, and in one
311 other isolate a G to A change at position 203 of the beta-tubulin was found. Based on the analyses
312 of *cyp51A* gene, none of the eighteen isolates obtained from the compost samples carried any of the
313 known mutations for DMI resistance. However, isolate A11 showed several polymorphisms, but
314 only one translated in an amino acid change (E427K) (Table 3) which was reported previously in
315 either resistant or susceptible *A. fumigatus* isolates (Howard et al., 2011). In addition, isolates A56
316 and A57 showed an amino acid change (N>K) at the 248 position (Table 3), but this mutation has
317 not been reported among the ones linked to DMI resistance. Furthermore, none of the isolates had a
318 tandem repeat of 34 bp in the gene promoter (S1).

319 None of the *A. fumigatus* isolates from compost examined in this study contained relevant
320 mutations in the *cyp51A* gene, encoding DMI resistance. However, it cannot be ruled out that azole
321 resistant isolates may be detected in environmental samples especially when a larger study is
322 undertaken including more compost types and other habitats where *A. fumigatus* can grow and
323 sporulate (Gisi 2013). Two isolates (A56 and A57) showed an unknown mutation at the 248
324 position of the *cyp51A* protein sequence; whether or not this mutation may induce a reduced
325 sensitivity to DMI fungicides is currently under investigation.

326

327

Conclusions

328 Data on recovery and quantification of PHPs in green and mixed composts are either fairly limited
329 or outdated (Millner *et al.* 1977; Clark *et al.* 1983; Gong *et al.* 2005; De Clercq *et al.* 2007) and
330 missing for biochars, even though extended literature is available on the study of microbial
331 communities in composts and during their production process with different experimental and
332 technical approaches (Ryckeboer *et al.* 2003; Insham *et al.* 2003; Anastasi *et al.* 2005; Danon *et al.*
333 2008; Bonito *et al.* 2010; Neher *et al.* 2013). To our knowledge this work represents the first study
334 on the detection and quantification of four of the main PHPs in a reasonable wide number of

335 compost samples and it is definitely the first considering biochars. This combined approach was
336 adopted to have a broader, even if specific, view of PHPs inhabiting finished organic products.

337

338 Results of the analyses confirm the variable presence in compost of some enteric bacteria, but
339 mainly the consistent presence of *A. fumigatus*. This agrees with what is generally expected because
340 most bacterial pathogens are inactivated by composting while *A. fumigatus* is known to play an
341 active role in the process (Jones and Martin, 2003; O’Gorman 2011).

342 Among detected living PHPs, the presence of *L. monocytogenes* and Shiga toxin-producing *E.coli*
343 in compost, which could lead to crop contamination when contaminated compost is used in
344 agriculture, together with the abundance of the opportunistic fungus *A. fumigatus* in these organic
345 substrates may represent an health issue. It remains uncertain whether environmental exposure to
346 enteric pathogens by handling contaminated composts would present a tangible risk for humans
347 mainly through plant contamination.

348 To limit the health risk imposed by the potential presence of these pathogens, good agricultural
349 practices and proper handling of the substrates respecting strict hygienic rules by workers may be
350 good enough.

351 The detection of living *A. fumigatus* in variable concentrations in the majority of samples confirms
352 compost as being one of the major sources for this organism in the environment (O’ Gorman 2011).

353 Furthermore, the pathogen was detected for the first time in biochars posing questions on how and
354 where these product types should be used to limit the hazard of unintentional transmission of fungal
355 spores. In this study, we provide evidence that longer storage and turning of compost can increase
356 the concentration of *A. fumigatus* in the substrate. These findings highlight the need to widen future
357 studies to the dissemination of this organism within and outside compost facilities, in order to
358 identify the conditions favouring its dispersal and sporulation and to detect critical hazard points
359 during the process.

360 It is still an open question, whether *A. fumigatus* DMI resistant originates from medical treatments
361 (human and veterinary) spreading to the environment or vice versa. It can be assumed that
362 resistance emerges in all situations where azole (DMI) fungicides are used intensively at high
363 concentrations over a long period of time (Gisi 2013).

364 The use of compost and biochars in agriculture, horticulture, floriculture as well as for other
365 environmental applications is gaining more and more attention for a number of reasons. The major
366 value of such substrates are linked to their environmentally friendly features such as their effects for
367 long term crop plant fertilization and quality, favourable influence on soil structure, aeration, and
368 moisture, for suppressiveness of soil borne plant pathogens, for activation of nutrient cycling,
369 mineralization and bioremediation processes in the soil environment (Ahmad *et al.* 2007; Beesley *et*
370 *al.* 2010; Beesley *et al.* 2011). In addition, they can contribute to carbon sequestration (biochars).
371 Our results are of relevance for the ongoing discussion on regulatory aspect of these and similar
372 types of organic substrates for limiting the level of PHPs to reasonable levels in order to minimize
373 health hazard.

374 Further studies should be done for PHPs and *A. fumigatus*, in compost and biochar facilities as well
375 as in other relevant habitats of these organisms in order to understand their main environmental
376 sources. The presence and abundance of such organisms in commercial organic substrates
377 especially within horticultural and floricultural sectors should be considered in future studies along
378 with the contamination risk of vegetables by enteric bacteria. The development of reliable
379 molecular methods for the specific detection and quantification of living *A. fumigatus* inoculum in
380 soil, compost, biochar and similar substrates would be equally important. In this way, the origin and
381 migration of PHP's and *A. fumigatus* between different ecological niches (habitats) in the medical
382 (human and veterinary) and environmental (including agricultural, horticultural, floricultural)
383 sectors can and should be investigated in a more rational approach.

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Summary

386 Fifteen compost and five biochar were sampled in different European Countries. Enteric bacteria
387 were detected by molecular methods in eight out of fifteen composts; however, viable propagules
388 were confirmed for *L. monocytogenes* only in two composts, and for STEC in further three
389 composts. No bacterial pathogens were found in biochar. Living *A. fumigatus* was present in eleven
390 composts and two biochars. None of the eighteen isolates contained SNPs relevant for resistance to
391 azole fungicides. The role of compost and biochar as a source of PHPs in the environment and the
392 risk for human health is discussed.

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398 REFERTIL: Reducing mineral fertilisers and chemicals use in agriculture by recycling treated
399 organic waste as compost and biochar products).

400 All experimental materials were handled under a class 2 laboratory hood.

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