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

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Selma Franceschini[1] selma.franceschini@unito.it

Walter Chitarra[1] walter.chitarra@unito.it

Massimo Pugliese[1,2] massimo.pugliese@unito.it

Ulrich Gisi[1] u.gisi@bluewin.ch

Angelo Garibaldi[1] angelo.garibaldi@unito.it

Maria Lodovica Gullino[1,2] marialodovica.gullino@unito.it

[1]AGROINNOVA

Centre of Competence for Innovation in the Agro-Environmental Sector

Università degli Studi di Torino

Largo Paolo Braccini 2

10095 Grugliasco (TO) - Italia

Office: +39-0116708545

Fax +39 0116709307

[2]DISAFA

Department of Agricultural, Forestry and Food Sciences

Università degli Studi di Torino

Largo Paolo Braccini 2

10095 Grugliasco (TO) - Italia

Office: +39-0116708545

Fax +39-0116709307

Abstract

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

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

Introduction

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

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

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

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

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

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

Invasive Aspergillosis (IA), have been reported in different types of compost, it has been rather underestimated even though its role in the compost degradation process and its health implications are widely recognized. This organism is a ubiquitous fungus normally inhabiting the soil and decaying materials (Dagenais *et al.* 2009; Gisi, 2013) but it is well equipped to survive successfully in a wide range of environments due to a number of features, recently discussed by Kwon-Chung and Sugui (2013), first of all the wide growth temperature range. *A. fumigatus* is present in compost samples at concentrations of 10^6-10^7 CFU/gdw (Millner *et al.* 1994), the spores are released to the air during compost activities such as turning reaching concentrations of 10^4-10^7/m^3 (Recer *et al.* 2001; Wheeler *et al.* 2001).

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

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

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

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

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

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

Materials and methods

Composts and biochars, collection and storage

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

In their country of origin representative samples were taken from an approximately 1 m^3 big bag of compost, consisting of a composite sample obtained by pooling 5-6 individual subsamples taken from a 20-30 m^3 pile of siewed and ready to market compost.
The five biochars came from four different countries and, except for BIOCHAR 1 which was obtained by the carbonization of animal bone, all others derived from plant wastes. Upon their arrival (April-May) composts/chars were maintained in big bags stored under an outdoor canopy and subsamples of 1kg (approx weight) were kept at 4°C for the following microbiological and molecular analyses.

Detection and identification of enteric pathogens

Compost and biochar samples (25 grams) were collected in sterile Blender bags (VWR, Radnor, PA, USA). *L. monocytogenes*, *Salmonella* spp., Shiga toxin-producing *E. coli* (STEC) were detected by using real-time PCR kits (iQ-CheckTM,Bio-Rad, France) following manufacturers' instructions with only minor modifications. In brief, for each pathogen, selective enrichment broth at the appropriate incubation temperature, and DNA extraction protocol for environmental samples, were followed. Simultaneously, 0.1 mL of samples were plated on Hektoen-Entero-Agar (Merck®, Germany), and coliform bacterial colonies (typically orange-red surrounded by a zone of precipitate) were enumerated after incubation at 35 °C for 24 h.
Selective platings were carried out for all samples to verify the presence of the targeted pathogens. The procedures were as follows: 25 g of each sample was transferred from the container into a sterile Blender bag (VWR, Radnor, PA, USA) together with 225 mL of 0.1% sterile peptone water (Sigma-Aldrich, St.Louis, USA) and homogenized for 180 s in a Masticator (IUL instruments, Barcelona, Spain). To enumerate *L. monocytogenes*, Oxford selective agar (Sigma-Aldirich, St. Louis, USA) added with Oxford *Listeria* selective supplement (Sigma-Aldirich, St. Louis, USA) were used. Two 100 μL homogenates were taken, and decimal dilution series were spread onto two Oxford agar plates and incubated for 48 h at 35 °C. Similarly, homogenates of STEC positive

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

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

Isolation and identification of Aspergillus fumigatus

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

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

DNA, 2µl of nucleotides mixture 2.5mM, 1µl of each primer prepared at a concentration of 10mM, 1.25µl of $MgCl_2$ 50mM, 2.5µl of 10XPCR buffer, 16 µl of MilliQ autoclaved water and 0.25 µl of Taq Polymerase (Qiagen®, Germany). PCR cycle for ITS and *ben*A gene included a denaturing 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 with a final elongation step at 72°C for 7 min.

To determine the presence of specific SNPs for DMI resistance among the pathogen isolates, the *cyp*51A gene was amplified using high fidelity Taq and specific primer pairs (Chen *et al*. 2005). Each reaction was composed of the following components: 1µl of template DNA, 2µl of nucleotide mixture 2.5mM, 1µl of each primer prepared at a concentration of 10mM, 1.25µl of $MgCl_2$ 50mM, 2.5µl of 10XPCR buffer, 17.4 µl of MilliQ autoclaved water and 0.1 µl of *Pfu* Taq (Invitrogen®Carlsbad, CA USA). For *cyp51A* gene identification, the cycles were: a denaturing 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 cycles, without final elongation. The *cyp*51A promoter was amplified using the protocol reported by Mellado *et al.* (2001). The promoter PCR included a denaturation step at 94°C for two minutes followed by 35 cycles at 94°C for 30s, 60°C for 30sec, 68°C for 2min.

ITS, β-tubulin and *cyp51A* amplicons were sequenced through Macrogen Europe sequencing service, and sequences manually edited using BioEdit v. 7.9 (Hall 1999). The coding region was compared with the *cyp*51A sequences present in the database to verify the existence of reported mutations linked to DMI resistance. Confirmed *A. fumigatus* isolates were included in AGROINNOVA culture collection and long-term stored in Tryptic Soy Broth (Merck®, Germany) 25% glycerol at -20°C. All potentially hazardous materials were destroyed through autoclaving for 25min at 121°C.

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

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

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

Results and discussion

Compost and biochar characteristics

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

Presence and abundance of enteric pathogens

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

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

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

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

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

Detected concentrations are unlikely to cause contamination of vegetables growing on substrates containing PHP's; however, considering that only few cells could cause illness they should not be underestimated. Favourable conditions, in terms of humidity, temperature and lack of antagonistic competitors could allow enteric bacteria to re-grow and re-colonise the substrates (Santamaria and Torazos, 2003; Sidhu and Toze, 2009).

Presence and abundance of A. fumigatus

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

The highest concentration was detected in a British compost made from municipal waste (C14) with 6.15×10^{5} CFU/gdw. In all compost samples made from green wastes (C2, C6, C7, C8, C11, C13) and one from municipal waste (C4), the *A. fumigatus* load was intermediate: between 0.24 and 3.62 $\times10^{3}$ CFU/gdw and comparable to those of biochar 5 (2.32×10^{3} CFU/gdw). Low concentrations were recorded in two composts from municipal waste (C3 and C9) and char 4, with estimated concentrations lower than 10^{2} CFU/gdw. In one case, *A. fumigatus* concentrations increased as a consequence of longer storage and turning of the compost: in fact, the C12 sample represents the same substrate as C3 but outdoor stored and periodically turned for a period of one year. Concentrations lower than 10^{2} CFU/gdw were obtained with the same methodology also for a soil sample used as standard substrate in greenhouse experiments (data not shown). The *A. fumigatus* concentrations observed in our study are within the range of those already reported in other investigations on composts (Millner *et al.* 1994; Anastasi *et al*. 2005).

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

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

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

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

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

Identification and characterisation of A. fumigatus *strains.*

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

All sequences shared 100% identity with *A. fumigatus* gene accessions present in the NCBI database. However, there were some minor differences among isolates in the ITS and in the beta-tubulin sequences: in four isolates, a T to C change at position 105 of the ITS sequence, and in one other isolate a G to A change at position 203 of the beta-tubulin was found. Based on the analyses of *cyp51A* gene, none of the eighteen isolates obtained from the compost samples carried any of the known mutations for DMI resistance. However, isolate A11 showed several polymorphisms, but only one translated in an amino acid change (E427K) (Table 3) which was reported previously in either resistant or susceptible *A. fumigatus* isolates (Howard et al., 2011). In addition, isolates A56 and A57 showed an amino acid change (N>K) at the 248 position (Table 3), but this mutation has not been reported among the ones linked to DMI resistance. Furthermore, none of the isolates had a tandem repeat of 34 bp in the gene promoter (S1).

None of the *A. fumigatus* isolates from compost examined in this study contained relevant mutations in the *cyp*51A gene, encoding DMI resistance. However, it cannot be ruled out that azole resistant isolates may be detected in environmental samples especially when a larger study is undertaken including more compost types and other habitats where *A. fumigatus* can grow and sporulate (Gisi 2013). Two isolates (A56 and A57) showed an unknown mutation at the 248 position of the *cyp51A* protein sequence; whether or not this mutation may induce a reduced sensitivity to DMI fungicides is currently under investigation.

Conclusions

Data on recovery and quantification of PHPs in green and mixed composts are either fairly limited or outdated (Millner *et al.* 1977; Clark *et al.* 1983; Gong *et al.* 2005; De Clercq *et al.* 2007) and missing for biochars, even though extended literature is available on the study of microbial communities in composts and during their production process with different experimental and technical approaches (Ryckeboer *et al.* 2003; Insham *et al.* 2003; Anastasi *et al.* 2005; Danon *et al.* 2008; Bonito *et al.* 2010; Neher *et al.* 2013). To our knowledge this work represents the first study on the detection and quantification of four of the main PHPs in a reasonable wide number of compost samples and it is definitely the first considering biochars. This combined approach was adopted to have a broader, even if specific, view of PHPs inhabiting finished organic products.

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

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

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

The detection of living *A. fumigatus* in variable concentrations in the majority of samples confirms compost as being one of the major sources for this organism in the environment (O' Gorman 2011). Furthermore, the pathogen was detected for the first time in biochars posing questions on how and where these product types should be used to limit the hazard of unintentional transmission of fungal spores. In this study, we provide evidence that longer storage and turning of compost can increase the concentration of *A. fumigatus* in the substrate. These findings highlight the need to widen future studies to the dissemination of this organism within and outside compost facilities, in order to identify the conditions favouring its dispersal and sporulation and to detect critical hazard points during the process.

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

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

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

Summary

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

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

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