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The Compostable Plastic Poly(lactic) Acid Causes a Temporal Shift in Fungal Communities in Maturing Compost

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ABSTRACT

The compostable biopolymer, poly(lactic) acid (PLA), is increasingly being used as an alternative to conventional plastics for short shelf-life products, disposable bags and packaging, and in agriculture. Despite the increase in the amount of PLA entering composting systems, few studies have examined the impact of PLA degradation on the compost microbial community. Thermophilic fungi play an import role in the composting process as they secrete hydrolytic enzymes capable of breaking down an array of complex natural polymers. In this study, the impact of PLA hydrolysis on the compost fungal community was examined by terminal restriction fragment length polymorphism and 454 sequencing. At 25°C, the effect of PLA on the surrounding compost community was relatively small and no physical changes were observed to the PLA films. However, when incubated at 50°C, where physical disintegration of PLA was occurring, a clear divergence between the compost populations in the presence and absence of PLA was evident after 2 months but became closer to the population in the absence of PLA after 4 months indicating that, after causing an initial perturbation after 2 months, the population began to return to that seen in the absence of PLA. The only exception was in the population containing 50% (w/w) PLA film, which remained divergent after 4 months and was associated with a marked acidification of the compost. Thus, 454-pyrosequencing revealed that the presence of PLA caused a strong selection for a *Thermomyces* sp. that was present only at low abundance in the absence of PLA.

Introduction

Worldwide plastic production reached 311 million tons in 2014 (Plastics Europe 2016), with packaging accounting for the largest market sector (39%). Currently, plastics used for this sector are largely comprised of synthetic petroleum-based polymers including polyethylenepolypropylene, polyvinyl chloride, polystyrene, and polyethylene terephthalate, which are poorly degraded in the environment (Sabev et al. 2006; Shah et al. 2008; Shimao 2001; Sivan 2011).

Poly(lactic acid) (PLA) is a synthetic compostable biopolymer with a hydrolysable backbone that degrades at elevated temperatures abiotically releasing lactic acid (Bohlmann 2005; Drumright et al. 2000; Garlotta 2002; Lim et al. 2008; Williams 1982). PLA has favorable mechanical qualities comparable to petrochemical-based non-biodegradable (Auras et al. 2003; Lunt 1998). Global PLA production reached 180,000 tons in 2011 and is estimated to reach at least 800,000 tons p.a. by 2020 with the majority used for packaging (Nova Institute 2016). As global production of PLA is increasing with its main application being short-shelf-life compostable products, it is important to understand its impact on compost communities. Ghorpade et al. (2001) reported that PLA could efficiently be composted when added in amounts lower than 30% (w/w) as lactic acid released through hydrolysis at higher levels of suppressed microbial growth. However, the impact of PLA degradation on microbial diversity was not assessed (Ghoparde et al. 2001). Sangwan and Wu (2008) and Sangwan et al. (2009) used clone libraries to identify putative PLA degraders from the surface of PLA; we have previously shown that degradation at elevated temperatures is accelerated by the presence of microbes and that compost contains thermophilic fungi capable of degrading PLA (Karamanlioglu and Robson 2013;

Karamanlioglu et al. 2014). However, none of these studies have investigated the impact of PLA degradation on the surrounding microbial community, which may impact on commercial composting.

Materials and Methods

Polylactic Acid (PLA)

PLA films (Mw 160,000 ± 6000 g/mol, determined by gel permeation chromatography, results not shown) were cut from PLA food containers produced from semi-crystalline PLA (Ingeo™ Biopolymer, Grade 2003D, 96% L-isomer; obtained from NatureWorks LLC, USA and distributed through VegWare, Edinburgh, UK). According to the supplier, PLA resins contained 96% L-lactic acid isomer, a density of 1.24 g/cm^3, melting temperature of 145°C–170°C, glass transition temperature of 55°C–58°C, and 35% crystallinity.

Compost Source

Commercial compost in its cooling phase (average temperature 44°C) was purchased from The Compost Shop, UK. Compost had been screened to 10 mm to eliminate large particles but no further commercial pre-treatments were applied to the compost. Compost was sieved through a 2-mm mesh to remove particles >2 mm after purchase and stored at room temperature. Water holding capacity was determined to be 60%–64% by a modified method of Forster (1995) and the moisture content was adjusted to 30%–32% by adding sterile water through a plant spray prior to use.

PLA Amended Compost

PLA squares (1 × 1 × 0.02 cm) were cut from delicatessen containers and surface sterilized with 70% (v/v) ethanol. Sterility was checked by incubating PLA films on potato dextrose agar (PDA) and Luria-Bertani (LB) plates. PLA squares were mixed with compost to give 0, 10, 25, and 50% (w/w) PLA in rectangular plastic boxes (3 L) to give a final depth of ca. 7 cm and incubated at 25 and 50°C. Boxes were weighed periodically and water loss adjusted by the addition of sterile water using a plant spray.

pH Determination

The pH of compost was determined after mixing compost samples with distilled water (ratio 1:2) and standing for 1 h according to Forster (1995) and the pH of supernatant was measured with a pH meter (Metler Toledo, UK).

DNA Extraction from Compost

Total DNA from compost (0.25 g) and from PLA amended compost (excluding PLA film) were extracted using a Power Soil DNA extraction kit (Mo Bio Laboratories, UK) according to the manufacturer's instructions and quantified using a Nanodrop 1000 (Thermo Fisher Scientific, UK). For each compost sample, DNA was extracted from three replicates and DNA stored at −20°C in 10 mM Tris buffer (pH 8) until required.

Terminal Restriction Fragment Length Polymorphism (TRFLP) Analysis of Fungal Communities

Fungal community profiles in compost were analyzed by TRFLP as previously described (Karamanlioglu et al. 2014; Kawasaki et al. 2012). Fluorescently labeled fragments were separated and detected in-house by capillary electrophoresis (ABI Prism® 3100 Genetic Analyzer, Applied Biosystems Inc., Foster City, USA). The size of detected terminal restriction fragments (TRFs) was assessed by comparison with an internal size standard using Peak Scanner™ software (Applied Biosystems, California, USA), and aligned using the T-align program (T-align 2016).

T-align initially compared the replicate profiles within one sample and displayed TRFs with their abundance (peak area) after eliminating pseudo peaks (Smith et al. 2005). A single consensus profile was generated from the three replicates and TRFs displayed with their average peak areas and then normalized so that each peak area corresponded to the percentage of the total fluorescence (Smith et al. 2005).

Principal component analysis (PCA) was used to compare TRF profiles in the fungal communities and diversity index (H′) and evenness values (J′; Atlas and Bartha, 1986) were calculated using MVSP software (Ver. 3.12d, Kovach Computing Services).

454-Pyrosequencing

454-Pyrosequencing was used to identify the dominant fungal taxa in compost samples. 454-Pyrosequencing was conducted by MR DNA (Shallowater, TX, USA)

by amplicon pyrosequencing (bTEFAP) as described by Dowd et al. (2008). Total DNA concentration was adjusted to 30–40 ng/μl and subjected to initial polymerase chain reaction (PCR) using ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC; White et al. 1990) primers to amplify the ITS1-5.8S-ITS2 region of fungal rRNA genome using a HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA). Cycling conditions were as follows: Initial denaturation at 94°C for 10 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min. Amplicons were adjusted to equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA) according to the manufacturers protocol and subjected to pyrosequencing using a Roche 454 FLX Titanium Instrument following the manufacturer's instructions (MR DNA Lab, Shallowater, TX, USA). The average number of reads per assay was ca. 3000 and analyzed using the QIIME pipeline (Caporaso et al. 2010). Primers, barcodes, and short sequences <200 bp was removed and split libraries formed. Sequences were clustered at 97% similarity and assigned to operational taxonomic units (OTUs) using QIIME with UNITE + NCBI Genbank database according to instructions given on the QIIME website for fungal ITS analysis (QIIME 2016).

Results

Visual Changes to PLA Films During Incubation in Compost

To determine any physical changes to the PLA films during incubation in compost at 25 or 50°C, samples were removed periodically and visually compared with controls (not incubated with compost). When the PLA films were incubated with compost at 25°C, there were no visible signs of degradation or other physical changes in appearance and appeared identical to control films (figures 1*A* and 1*B*). However, films incubated in compost at 50°C became opaque and showed physical signs of disintegration after 2 months (figure 1*C*) and was no longer visible after 4 months.

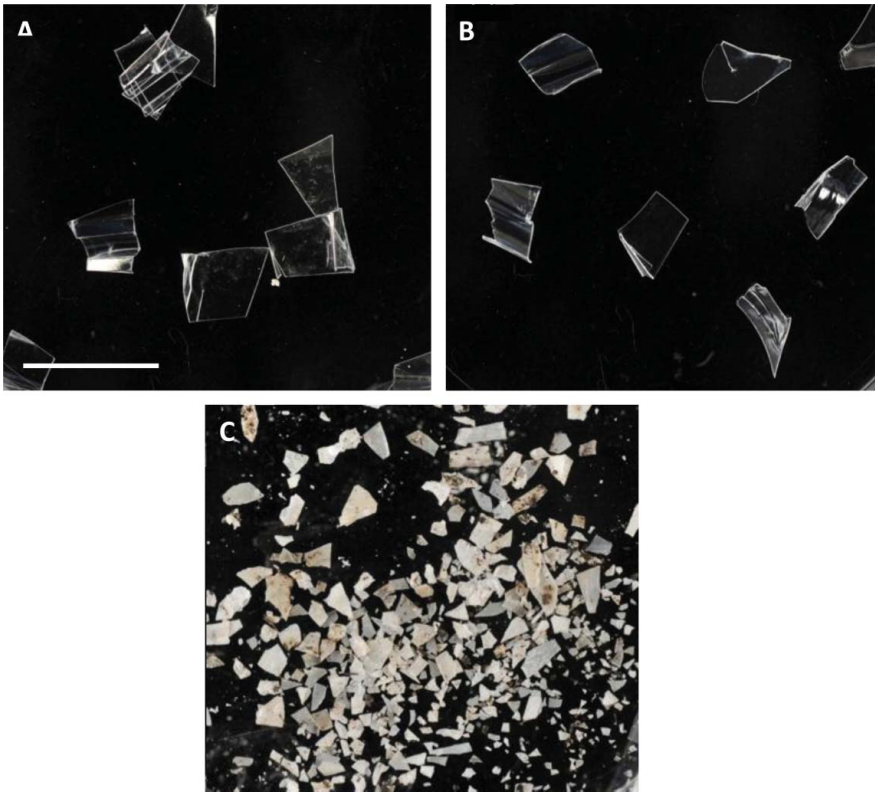


Figure 1. Physical changes in PLA films buried in compost. PLA films were mixed with compost and incubated at 25 and 50°C for up to 4 months. *A*, Films before incubation with compost. *B*, Films after 2 months incubation with compost at 25°C. *C*, Films after 4 months incubation with compost at 50°C. Scale bar represents 1 cm.

Effect of PLA on Compost pH

As PLA hydrolysis ultimately leads to the generation of its monomer, lactic acid, the pH of compost containing 0, 10, 25, and 50% (w/w) PLA films incubated at 25 or 50°C were measured before and after 2 and 4 months incubation. The initial compost had a pH of 7.1 ± 0.2 and no significant ($p > 0.05$) change was observed after 2 or 4 months incubation at 25°C with PLA films. Similarly, at 50°C, no significant ($p > 0.05$) change in pH was observed in compost in the presence of films with the exception of 50% (w/w) PLA films where the pH decreased to 3.3 ± 0.1 after 4 months (data not shown).

Influence of PLA on the Fungal Compost Community over 4 months

In order to determine the influence of PLA on the fungal compost community, compost samples were removed after 0, 2, and 4 months; total DNA was extracted and subjected to TRFLP analysis. TRF profiles from compost incubated at 25 and 50°C containing 0, 10, 25, and 50% (w/w) PLA films were subjected to principle component analysis (PCA) following normalization (figures 2*A* and 2*B*). Consequently, 50°C was chosen to represent the initial cooling phase when fungi first become dominant in the compost, and 25°C was chosen to represent the mature phase after cooling.

When PLA films were incubated in compost at 25°C, two principal components (PC1 and PC2) accounted for 48% of the total variability in the data set (figure 2*A*). The first cluster accounted for the compost community prior to incubation and after 2 months at 25°C without PLA; the second cluster accounted for the compost community in the presence of 10%–50% (w/w) PLA incubated for 2 months and 0 and 10% of PLA after 4 months; and the third cluster accounted for the community in the presence of 25 and 50% (w/w) PLA films incubated for 4 months at 25°C. PC1 mainly showed the difference between 25 and 50% (w/w) PLA film-containing compost incubated for 4 months at 25°C and the initial compost. PC2 explained 22% of the variation and showed the difference between compost samples containing 10%–50% PLA films incubated for 2 months from the other compost samples. PLA concentration effect was mainly observed after 2 months as compost with PLA grouped away from compost in the absence of PLA.

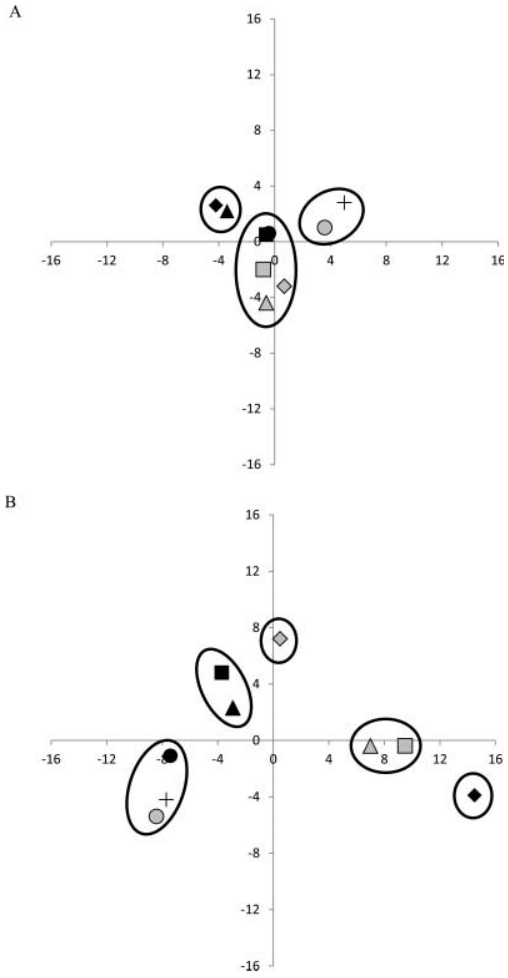


Figure 2. Principal component analysis (PCA) of TRFLP profiles of fungal communities isolated from initial compost (+) and compost after 2 months (grey symbols) and 4 months (black symbols) in the absence (○, ●) or presence of 10% (□, ■), 25% (△, ▲) or 50% (◇, ◆) (w/w) PLA incubated at either (*A*) 25°C or (*B*) 50°C.

When PLA films were incubated at 50°C, the first two principal components (PC1 and PC2) accounted for 88% of the total variability in the fungal community (figure 2*B*). PC1 accounted for 70% of the variation and four groups were observed. The first was the initial compost community and compost incubated for 2 and 4 months in the absence of PLA; the second group consisted of compost containing 10 and 25% PLA films incubated for 4 months and compost containing 50% PLA incubated for 2 months; the third group contained the profiles for compost containing 10 and 25% PLA incubated for 2 months; and the final group contained only compost containing 50% PLA films incubated for 4 months. Changes in the fungal community due to PLA concentration were most apparent after 2 months incubation as all compost

Table 1. Diversity index (H′), evenness (J′), number of TRFs of fungal communities in compost and % of TRFs only found in PLA film and/or PLA granule containing compost with respect to PLA concentration in compost (0%–50%).

		PLA concentration	Shannon diversity (H′)	Evenness(J′)	Number of TRFs	% of PLA unique TRFs
		0%, Initial compost	3.6	0.7	145	N/A
25°C, compost and PLA films	2 months	0%	3.0	0.6	131	
		10%	3.4	0.7	154	8
		25%	3.0	0.6	152	17
		50%	3.3	0.7	98	4
	4 months	0%	3.4	0.7	149	
		10%	3.6	0.7	169	7
		25%	3.8	0.7	192	11
		50%	3.8	0.7	207	14
50°C, compost and PLA films	2 months	0%	3.2	0.7	84	
		10%	2.6	0.6	97	16
		25%	2.7	0.5	129	21
		50%	2.6	0.6	52	8
	4 months	0%	3.7	0.8	137	
		10%	3.4	0.7	148	26
		25%	3.5	0.7	167	30
		50%	1.9	0.4	86	3

Note. Each compost sample had three replicates, which were pooled after PCR, and two technical replicates were aligned to represent each compost sample.

communities containing PLA clustered away from compost in the absence of PLA and the initial compost community. After 4 months incubation, with the exception of 50% (w/w) PLA film, populations were closer to the initial compost community and compost incubated in the absence of PLA for 4 months.

The effect of PLA concentrations on diversity of fungal communities in compost was assessed by calculating the number of TRFs, Shannon index (H′), and evenness values (J′) corresponding to population size, diversity, and evenness of populations in each compost community, respectively (table 1).

The number of TRFs in compost containing PLA films (10%–50% w/w) incubated at 25°C (≈ 154) were mostly similar with the initial compost (145) and compost in the absence of PLA (131) except with a decrease in compost with 50% PLA films; however, on the 4th month the number of TRFs was similar regardless of PLA concentration. The diversity and the evenness values were high ($H' = 3.0–3.8$; $J' = 0.6–0.7$, respectively) and similar for 4 months with the compost in the absence of PLA. On the 4th month of incubation the number of TRFs, diversity, and evenness increased compared to the 2nd month regardless of the PLA concentrations.

When PLA films were incubated at 50°C, a drastic decrease in TRF number was observed at 50% concentration, as the number of TRFs (52) was less than all the other compost samples in the presence and absence of PLA on the 2nd month (84–129). Diversity index decreased ($H \approx 2.6$) in compost with 10%–50% PLA films compared to initial ($H' = 3.6$) and compost with 0% PLA films ($H' = 3.2$); however, the sizes of populations in the communities were similar ($J' \approx 0.6$) with initial compost and compost with no PLA ($J' = 0.7$) incubated at 50°C for 2 months. The same trend was also observed on the 4th month of incubation; at 50% PLA film concentration in compost, the number of TRFs decreased drastically (86) compared to 0%–25% PLA containing compost samples (137–167). In addition, fungal diversity ($H' = 1.9$) and evenness ($J' = 0.4$) were less than compost having 0%–25% PLA ($H' = 3.4–3.7$; $J' = 0.7–0.8$), which had higher diversity and less variation of population sizes in the communities.

Meta-barcoding Analysis

In order to identify which fungal taxa in the compost community was affected by the degradation of PLA, meta-barcoding analysis was conducted on compost samples incubated at 50°C for 2 and 4 months in the presence and absence of 25% (w/w) PLA films (figure 3). A total of 5557 sequence counts (reads) were obtained with a saturation of >97% indicating the major components of the fungal community had been captured. The initial compost kept at 25°C had more OTUs than the rest of the samples and contained OTUs from 2 phyla: *Ascomycota* and *Zygomycota* (figure 3). The most dominant fungal group in the initial compost sample (50% relative abundance) was unidentified but belonged to phylum *Ascomycota*, while the second most abundant (32% relative abundance) belonged to *Ascomycota*,

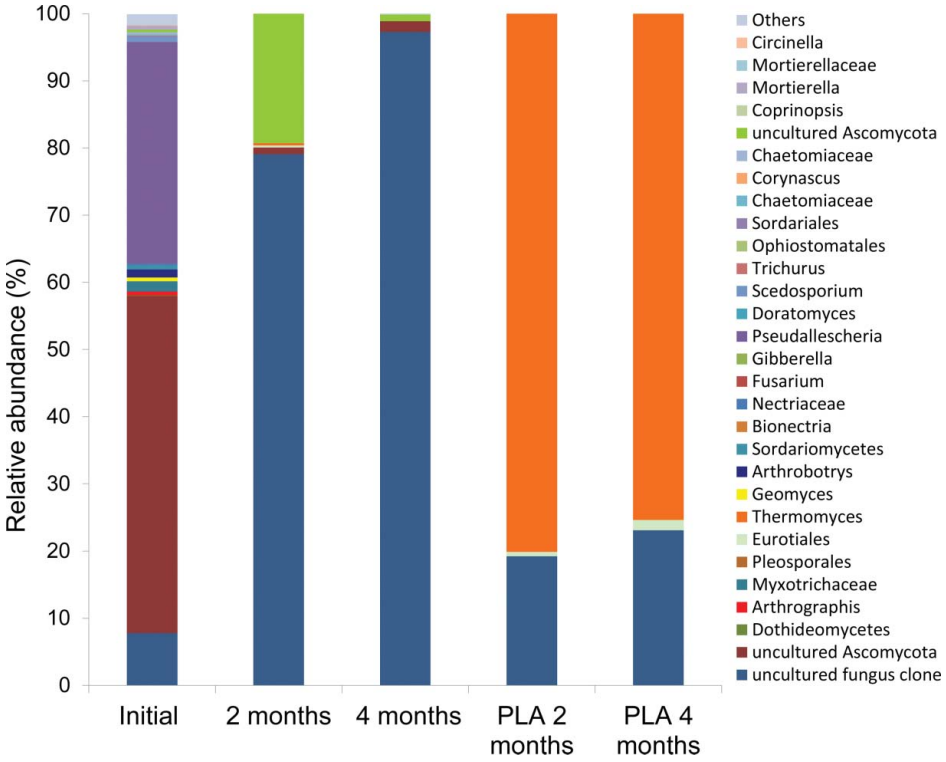


Figure 3. Relative abundance of fungal groups identified by meta-barcoding analysis from compost in the absence and presence of 25% (v/v) PLA films incubated at 50°C for 2 and 4 months. *Indicates molecular operational taxonomic units (mOTUs) unidentified beyond genus level.

Microascaceae, and genus *Pseudallescheria*. OTUs with low relative abundances were from the phylum *Zygomycota* (*Mortierellaceae*; genus *Mortierella* and *Mucoraceae*; genus *Circinella*). An uncultured fungal species, initially present with a relative abundance of 8% became the dominant species in the absence of PLA after 2 and 4 months incubation (79 and 97%, relative abundance, respectively) but had a lower abundance in the presence of PLA (18 and 23% relative abundance after 2 and 4 months incubation, respectively). In the presence of PLA, the dominant fungal group was identified as belonging to the genus *Thermomyces* (order *Eurotiales*) with a relative abundance of 80 and 75% after 2 and 4 months incubation, respectively. This clone was not detected in the original compost and found only after 2 months incubation in the absence of PLA at very low relative abundance (0.03%). PLA therefore significantly changed the dominant fungal species present in compost as detected by 454-pyrosequencing.

Discussion

PLA is a compostable plastic that is increasingly used commercially as an alternative packaging material for short-shelf-life products. Despite its growing use, few studies have investigated the impact of this material on the compost community and, in particular, on the fungal community. In this study, we investigated the influence of PLA degradation at 25 and 50°C on the fungal community using TRFLP. At 25°C, no visible sign of degradation of PLA films was evident; however, physical fragmentation and degradation were clearly seen when incubated in compost at 50°C after 2 months (figure 1) and was completely degraded by 4 months. PLA degradation is known to occur at temperatures close to its glass transition temperature (Tg, 55°C–62°C) in humid environments (Henton et al. 2005; Itävaara et al. 2002; Kale et al. 2007; Saadi et al. 2012; Weir et al. 2004). Polymer chains of PLA soften and become more flexible, which increases water absorption accelerating hydrolysis and microbial attachment (Agrawal et al. 1997; Henton et al. 2005; Kale et al. 2007; Sangwan and Wu, 2008; Vargas et al. 2009).

At 50°C, PLA films degraded rapidly and were disintegrated by 2 months. Hydrolysis of PLA leads to a progressive decrease in polymer chain length and ultimately the release of soluble oligomers and lactic acid

causing a decrease in the surrounding pH (Agarwal et al. 1998; Torres et al. 1996a). In this study, no change in the pH of compost was observed at 25°C confirming the lack of physical degradation observed at this temperature (figure 1). Tensile strength loss was also reported to be insignificant with PLA films even after a year when buried in compost, soil, or immersed in water at 25°C (Karamanlioglu et al. 2014). No change in pH was also observed in compost incubated in the presence of PLA at 50°C with the exception of the compost containing 50% (w/w) PLA films, where the pH decreased from 6.5 to 3.1 after 4 months (data not shown) and coincided with PLA disintegration (figure 1). Previously, we showed that lactic acid release causing a large decrease in pH was only observed after loss in tensile strength indicating that release of lactic acid occurs in the final stages of polymer hydrolysis (Karamanlioglu et al. 2014). However, since compost is not a sterile system, released PLA oligomers and lactic acid will be utilized by microorganisms as a carbon source. This may explain why a decrease in pH was not observed with PLA films at concentrations lower than 50%, as the released lactic acid may have been utilized by microbes present in the compost and insufficient to change the pH of the surrounding compost. In a study by Torres et al. (1996b), when PLA oligomers and lactic acid dimers were incubated with a fungus, *Fusarium moniliforme*, and a bacterium, *Pseudomonas putida*, separately, pH increase was observed in both cultures at 30°C. In another study, the pH in an abiotic reactor and sterile water decreased in the presence of PLA at temperatures of 40°C–60°C; however, pH increased in a biotic reactor under the same conditions due to utilization of released oligomers and lactic acid (Agarwal et al. 1998). In accordance with our study, Ghorpade et al. (2001) observed a decrease in pH when PLA was present in compost at a concentration of 30% (w/w) at 52°C but not at a concentration of 10% (w/w).

In order to determine if the presence of different concentrations of PLA influences the fungal community profile in compost, TRFLP profiles were compared by PCA (figure 2). PCA enables comparisons of complex data sets and is often used to analyze microbial TRFLP profiles (Dollhopf et al. 2001; Midgley, et al. 2007; Wang et al. 2004). At 50°C, where disintegration of PLA was occurring, much greater variation in populations was evident by PCA. In the absence of PLA, the variation in the population was low compared to the variation in the presence of PLA and so the initial population clustered with the population in the absence of PLA after 2 and 4 months incubation. In the presence of PLA, the greatest divergence to the population compared to the absence of PLA was seen after 2 months but became more similar to the population in the absence of PLA after 4 months. The only exception was in the population containing 50% (w/w) PLA film, which remained divergent and was the population in which the pH had significantly increased. This was also reflected in a lower number of detectable TRFs, diversity, and evenness in the compost community (table 1) as a result of the excess release of lactic acid.

Some TRFs appeared only in PLA containing compost and were not detected in compost without PLA (PLA unique TRFs; table 1). The percentage of PLA unique TRFs varied widely from 3 to 38% at 50°C suggesting a significant number of fungal species were under positive selection by the presence PLA, particularly at 50°C where hydrolysis was occurring. However, the total abundance of these PLA unique TRFs was never greater than 1% under any conditions suggesting they played a minimal role in PLA degradation and that selection for species already present at detectable levels in the population may be more important.

In order to obtain a more comprehensive view of the taxa affected by the presence of PLA, 454-pyrosequencing was employed to analyze the fungal community from compost in the presence and absence of 25% (w/w) PLA incubated at 50°C (figure 3). Compared to compost in the absence of PLA, the presence of PLA greatly affected the fungal composition of the compost with *Thermomyces* becoming the most dominant OTU. By contrast, in the absence of PLA, abundance of *Thermomyces* was very low as it was not identified in the initial compost community or after 4 months incubation and was only detected at low abundance (0.03%) after 2 months incubation, whereas in the presence of PLA, relative abundance increased to 80 and 75% after 2 and 4 months, respectively. Previously, *Thermomyces lanuginosus* was found to be the dominant organism that could be cultured from the surface of PLA when incubated at 50°C in both compost and soil and was shown to have the ability to degrade high molecular weight PLA (Karamanlioglu et al. 2014). Apart from the *Thermomyces* sp., an uncultured *Eurotiales* in the same order was also identified from compost in the presence of

PLA and was also not detected in the initial compost community suggesting that it may be another PLA degrader. The most abundant OTU in compost in the absence of PLA after 2 and 4 months incubation at 50°C was assigned to an unidentified clone (relative abundance of 79 and 97%, respectively) and was present at a lower abundance in the presence of PLA (19 and 23% after 2 and 4 months incubation, respectively). The inability to identify environmental fungal clones is problematic and arises due to the deposition of internal transcribed spacer (ITS) sequences to international DNA databases without taxonomic annotations or containing errors in taxonomic assignments (Hawksworth 2004; Vilgalys 2003) and also it has been estimated that the vast majority of fungal species have not been cultured (Hawksworth, 2001).

In this study, it was shown that fungal diversity and community structure in compost is affected by the presence of PLA at 50°C with a marked shift in community structure after 2 months but returning toward the initial structure after 4 months indicating PLA causes a temporal shift in the community structure but returns too close to the original as the PLA is degraded. 454-Pyrosequencing of compost samples indicated that PLA caused a strong selection for a *Thermomyces* sp. after 2 months, whereas it was only a relatively minor component of the community in the absence of PLA. Thus, PLA entering composting systems could have a major effect on shifting the fungal community profile; further, studies are required to evaluate the impact of this perturbation on the composting process and subsequent compost quality.

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