Fungal colonization of soil-buried plasticized polyvinyl chloride (pPVC) and the impact of incorporated biocides

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Plasticized polyvinyl chloride (pPVC) with or without incorporated biocides was buried in grassland and forest soil for up to 10 months. The change with time in viable counts of fungi on the plastic surface was followed, together with the percentage capable of clearing the two plasticizers dioctyl adipate (DOA) and dioctyl phthalate (DOP). With time fungal total viable counts (TVC) on control pPVC increased and the fraction able to clear DOA was considerably higher than the average estimated in both soil types. A total of 92 fungal morphotypes were isolated from grassland soil and 42 from forest soil with the greatest variety of fungal isolates observed on control pPVC. The incorporation of biocides into pPVC affected both fungal TVC and the richness of species isolated. The biocides NCMP [n-(trichloromethylthio)phthalimide], OBPA (10,10'-oxybisphenoxarsine) and OIT (2-n-octyl-4-isothiazolin-3-one) were the most effective in grassland soil, and TCMP [2,3,5,6-tetrachloro-4-(methylsulphonyl)pyridine] and NCMP the most effective in forest soil. In grassland soil, *Penicillium janthinellum* established as a principal colonizer and was recovered from all pPVC types. DOP clearers were found at much lower levels than DOA clearers, with *Doratomyces* spp. being the most efficient. At the end of 10 months the physical properties of the pPVC were altered; changes in stiffness were the most significant for heavily colonized grassland-buried pPVC samples, whereas in forest soil, the extensibility of the pPVC was affected more than the stiffness. These results suggest that fungi are important colonizers of pPVC buried in soil and that enrichment of soil fungi capable of clearing DOA occurs during colonization of the plastic surface. The results also demonstrate that incorporated biocides have a marked impact on the richness of species colonizing the pPVC surface.

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INTRODUCTION

Plastics possess a number of key characteristics, including inertness, flexibility and low production costs, that have led to their application in many areas of human life. According to recent reports, their production exceeds 140 million tonnes per year (Shimao, 2001). However, after use, a major proportion ends up either in landfill sites or as litter, creating a range of problems such as uncontrolled release of plasticizers from the polymer blend into the environment (Jonsson *et al.*, 2003).

Abbreviations: BBIT, n-butyl-1,2-benzisothiazolin-3-one; DCOIT, dichloro-octylisothiazoline; DOA, dioctyl adipate; DOP, dioctyl phthalate; NCMP, n-(trichloromethylthio)phthalimide; OBPA, 10,10'-oxybisphenoxarsine; OIT, 2-n-octyl-4-isothiazolin-3-one; pPVC, plasticized polyvinyl chloride; SEM, scanning electron microscopy; TCMP, 2,3,5,6-tetrachloro-4-(methylsulphonyl)pyridine; TVC, total viable count.

The GenBank/EMBL/DDBJ accession numbers for the rDNA sequences from the fungal isolates identified in this study are given in the text.

Polyvinyl chloride represents a significant proportion of the world's plastic production and has been extensively used due to its safety, effectiveness, manufacturing technology and cost (Lorenz, 1990). Plasticizers, fillers and stabilizers are often added to the polymer to produce the more flexible plasticized PVC (pPVC), extending its range of applications. Some of the most frequently used plasticizers are esters of phthalic acid (such as dioctyl phthalate, DOP) and adipic acid (such as dioctyl adipate, DOA) (Gumargalieva *et al.*, 1999; Roberts & Davidson, 1986).

Most studies on the microbial colonization and biodeterioration of plastics have focused on polyurethanes (Barratt *et al.*, 2003; Bentham *et al.*, 1987; Kay *et al.*, 1991), bacterial polyesters (Mergaert *et al.*, 1996) and nylon (Deguchi *et al.*, 1997). By contrast, there have been fewer studies focusing on the colonization of pPVC *in situ* (Upsher, 1984; Upsher & Roseblade, 1984; Webb *et al.*, 2000). Although there is no convincing evidence that the polymer backbone of PVC can be degraded by micro-organisms (Andrady, 1994), incorporated plasticizers in pPVC have shown varying degrees of

susceptibility to microbial degradation (Hamilton, 1983; Nalli *et al.*, 2002; Roberts & Davidson, 1986; Webb *et al.*, 2000). Of the plasticizers commonly incorporated into pPVC, phthalate plasticizers are of growing concern due to soil pollution leading to bioaccumulation and subsequent acute and chronic toxicity in microbes, plants and animals (Staples *et al.*, 1997a, b). Whilst evidence for the decomposition of phthalates by some bacteria has been reported (Eaton & Ribbons, 1982; Juneson *et al.*, 2001; Zeng *et al.*, 2002), there have to our knowledge been no reports that fungi can degrade phthalate plasticizers.

In order to delay microbial colonization and biodegradation of pPVC during a product's lifespan, a variety of biocides are often incorporated into the polymer blend (Jones *et al.*, 1996) or immobilized on the material surface (James & Jayakrishnan, 2003). However, when the plastics are disposed of, these biocides could have a negative effect by reducing colonization and degradation of plasticizers.

Soil is a rich microbial environment and natural habitat for fungi, where they play a major role in the decomposition of dead plant and animal materials (Thorn, 1997). Fungi play a major role in the biodegradation of organic materials due to their ability to secrete a variety of extracellular enzymes, and they actively invade and colonize substrates of different origins (Bennett & Faison, 1997). In this study we aimed to investigate the colonization of soil-buried pPVC *in situ* and to study the impact of incorporated biocides on the richness of isolated strains.

METHODS

Culture media and strain maintenance. Fungi enriched from soil-buried pPVC were maintained routinely on malt extract agar (MEA; Oxoid). For long-term storage, fungal spores or mycelium were harvested from agar plates in 20% (v/v) glycerol and frozen at -80°C . For initial fungal isolations, MEA was supplemented with ampicillin and streptomycin at $100\text{ }\mu\text{g ml}^{-1}$ to suppress bacterial growth.

To test the ability of fungal isolates to grow on plasticizers, a basal mineral salts medium (MSM) containing (per litre of deionized water) $7\text{ g K}_2\text{HPO}_4$, $3\text{ g KH}_2\text{PO}_4$, $0.1\text{ g MgSO}_4 \cdot 7\text{H}_2\text{O}$, $1\text{ g (NH}_4)_2\text{SO}_4$, $100\text{ ml soil extract}$ (Alef & Nanipieri, 1995), $15\text{ g bacteriological agar}$ (Oxoid) and 2 ml DOA or DOP as a carbon source was used. After autoclaving at 121°C for 15 min and cooling to approximately 55°C , an emulsion of the plasticizer was created within the medium using an UltraTurrax T25 homogenizer (Janke & Kunkel) at full power (24000 min^{-1}) for 1 min . Plates were poured immediately after homogenization. Fungal colonies able to grow on the plasticizers produced a zone of clearing in the agar.

pPVC samples. Sheets of pPVC were formulated as previously described (Webb *et al.*, 2000). Biocide-containing pPVC was prepared by adding the biocides during plastic manufacture. Plastics were manufactured to contain (per g plastic) $500\text{ }\mu\text{g dichloro-octyl-isothiazoline (DCOIT)}$, $500\text{ }\mu\text{g 2-n-octyl-4-isothiazolin-3-one (OIT)}$, $2000\text{ }\mu\text{g 10,10'-oxybisphenoxarsine (OBPA)}$ (all from Rohm & Haas), $2000\text{ }\mu\text{g 2,3,5,6-tetrachloro-4-(methylsulphonyl)pyridine (TCMP)}$, $2000\text{ }\mu\text{g n-butyl-1,2-benzisothiazolin-3-one (BBIT)}$ (Avecia Biocides) or $10000\text{ }\mu\text{g n-(trichloromethylthio)phthalimide (NCMP)}$

(Durham Chemical). Plastics were cut into $2.5 \times 8\text{ cm}$ pieces, washed in Vero detergent (Verila JSC), rinsed in distilled water and surface-sterilized by swabbing in 70% (v/v) ethanol.

Soil burial and isolation procedures. pPVC samples ($2.5 \times 8\text{ cm}$) were buried in two burial plots of $4 \times 4\text{ m}$ at the end of November. The first one was in a grassland soil in an area that had not been used for growing crops and had never been sprayed with pesticides or fertilizers, located approximately 7 km from Sofia in western Bulgaria. The second was located in a mixed deciduous-coniferous forest in the Vitosha mountains, approximately 15 km from Sofia. The top 40 cm layer was removed, mixed thoroughly several times and plastic samples placed vertically at a depth of 20 cm (from the surface to the top of the sample) in rows with a distance of 50 cm between rows. The average moisture content of the forest soil was always at least twice that of the grassland soil. Three pPVC pieces of each of the control and biocide-containing pPVC were taken at various time points; soil crumbs and debris were cleaned from the samples, which were placed in 25 cm diameter Petri plates containing $20\text{ ml } 0.9\%$ (w/v) NaCl and scraped thoroughly on both sides using a sterile scalpel blade. The pPVC samples and the suspension were aseptically transferred to a 50 ml centrifuge tube (Corning) and vortexed vigorously for 5 min . After serial dilution, $100\text{ }\mu\text{l}$ aliquots of diluted samples (10^{-1} – 10^{-3}) were plated in triplicate onto MEA for enumeration and onto DOA agar and DOP agar for estimating the overall proportion of plasticizer-degrading fungi. MEA plates were counted after 5 days of incubation at 25°C and plasticizer agar plates after 14 days.

Tensile strength measurements. Triplicate pieces of pPVC were cut using a dumb-bell cutting head (length 36 mm , width at the ends 10 mm and gauge length 22 mm). Tensile tests were performed on each dumb-bell by stretching the samples at a constant rate of 80 mm min^{-1} until breakage on an Instron 4301 tensiometer.

Scanning electron microscopy (SEM) of the surface of pPVC. Coupons, $1 \times 1\text{ cm}$, were cut from pPVC samples, placed in liquid nitrogen and freeze-dried for 24 h (Emitech 750). Samples were mounted and sputtered with gold for 2 min (Emscope SC 500) and observed with a Cambridge 360 scanning electron microscope (Cambridge Instruments).

Identification of recovered fungal isolates. Fungi were identified by PCR amplification and DNA sequencing of nuclear ribosomal 5.8S DNA and the internal transcribed spacers (ITS) using the fungal universal primers ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') as previously described (Webb *et al.*, 2000). Consensus sequences from the forward and reverse amplification (excluding primer sequences) were used to interrogate the EMBL fungal database (<http://www.ebi.ac.uk/>) using Fast3.

rDNA sequences. EMBL accession numbers for rDNA sequences from the fungal isolates identified in this study are as follows (sequences with which matches were made and the percentage match for each sequence is shown in parentheses). For the unidentified isolates, only the EMBL accession number is shown: GFI-1, AJ608979 (AY157495, 99.4%); GFI-2, AJ608961; GFI-3, AJ608986; GFI-5, AJ608966; GFI-7, AJ608975; GFI-11, AJ608977 (AF106532, 100%); GFI-21, AJ608972 (AF062819, 99.8%); GFI-22, AJ608988 (AY873967, 99.5%); GFI-24, AJ608991 (AF414339, 99.8%); GFI-25, AJ608968 (AY345347, 99.4%); GFI-27, AJ608982 (AF461746, 99.8%); GFI-32, AJ609125; GFI-33, AJ608981 (DQ093736, 100%); GFI-35, AJ608984 (AB103379, 99.8%); GFI-46, AJ608963 (AF033415, 99.3%); GFI-47, AJ608945 (AY73921, 99.8%); GFI-48, 608965 (AF033493, 100%); GFI-81, AJ608985; GFI-82, AJ608983; GFI-91, AJ608990 (AF150460, 100%); GFI-101, AJ608970 (AF136375); GFI-122, AJ608973 (AY378154, 99.8%); GFI-123,

AJ608969; GFI-125, AJ608962; GFI-143, AJ608964; GFI-145, AJ608974; GFI-146, AJ608980; GFI-147, AJ608976 (AF268185, 99.8 %); GFI-148, AJ608971 (AF414694, 99.8 %); GFI-149, AJ608967 (AC0271573, 99.6 %); GFI-156, AJ608990; GFI-157, AJ608978 (MRO301994, 99.8 %); GFI-185, AJ608987 (AF169303, 99.8 %); FFI-5, AJ608958 (AF474242, 99.7 %); FFI-6, AJ608957 (AY243948, 99.1 %); FFI-9, AJ608954; FFI-10, AJ608950; FFI-22, AJ608959 (AF033393, 100 %); FFI-25, AJ608956 (AF443917, 99.7 %); FFI-30, AJ608960 (AY345348, 99.3 %); FFI-42, AJ608947 (AF033493, 100 %); FFI-143, AJ608951; FFI-144, AJ608948 (AF414695/AF444421, 99.8 %); FFI-45, AJ608946 (AF033490, 100 %); FFI-46, AJ608949 (AF034451, 100 %); FFI-47, AJ608952 (AF033469, 99.6 %); FFI-48, AJ608953 (AF218786, 100 %); FFI-91, AJ608955 (CMA279446, 100 %). Matches were accurate as of October 2005. Subsequent additions to the database may identify isolates that could not be matched.

RESULTS

Viable counts of fungi from buried pPVC

During the course of the experiment in grassland soil the fungal total viable count (TVC) on control pPVC increased from $7 \times 10^2 \pm 3.5 \times 10^2 \text{ cm}^{-2}$ for the first 135 days to $8.5 \times 10^3 \pm 1.4 \times 10^2 \text{ cm}^{-2}$ after 300 days (Fig. 1a). With the exception of pPVC containing DCOIT, all other incorporated biocides suppressed total fungal counts over the period of the trial. The biocides NCMP and OBPA were most effective at causing a reduction in the fungal TVC, while DCOIT was the least effective, with TVC values increasing after 230 days, reaching final values similar to controls after 300 days (Fig. 1a).

When compared to pPVC buried in grassland soil, the TVC of fungi recovered from control pPVC buried in forest soil was initially similar. However, numbers did not increase substantially until 300 days, to give a final mean count of $1.05 \times 10^4 \pm 1.5 \times 10^2 \text{ cm}^{-2}$. On pPVC with incorporated biocides, counts remained low after 300 days with the exception of OBPA and BBIT, where numbers significantly increased (Fig. 1b).

Dynamics of DOA- and DOP-clearing fungi isolated from the surface of pPVC

The number of fungal colonies recovered from the surface of control pPVC that were able to clear DOA or DOP was expressed as a percentage of the total number of isolated fungal colonies (Fig. 2a, b). At the first sampling time (70 or 75 days) more than 50 % of the fungal colonies recovered from pPVC from grassland soil and over 65 % from pPVC from forest soil were able to clear DOA agar (Fig. 2a, b). The proportion of DOA-clearing fungi recovered from the pPVC surface increased thereafter (135 days) except for the last isolation (300 days), where the percentage fell to 40 % and 67 % on grassland and forest soils, respectively, despite an increase in the total numbers of fungi recovered on MEA (Fig. 1a). Likewise, the percentage of recovered isolates able to clear DOA from biocide-containing pPVC increased to 90–100 % after 135 days (data not shown).

Fig. 1. Total viable counts of fungi, recovered from pPVC buried in grassland soil (a) and forest soil (b). Error bars represent SEM, $n=3$.

By contrast, the percentage of fungal colonies recovered that were able to cause clearing on DOP agar was considerably lower than the percentage able to clear DOA agar. Initially 23 % of the isolated colonies from grassland control pPVC caused weak semi-transparent clearing on DOP agar and numbers subsequently decreased. After 200 days, fungi producing much stronger clearing on DOP agar were observed. Far fewer DOP-clearing fungi were recovered from control pPVC buried in forest soil.

Diversity of fungal species isolated from pPVC and their ability to clear plasticizers

A total of 92 distinctive fungal morphotypes that were able to clear DOA or DOP agar were recovered from pPVC buried in grassland soil. The greatest number of fungal species was recovered from control pPVC; the number of species recovered from biocide-containing pPVC was much lower (Fig. 3). In forest soil, the greatest variety of isolates

Fig. 2. Percentage of fungi recovered from pPVC buried in grassland soil (a) and forest soil (b) that could clear DOA (white bars) and DOP (black bars) agar. Error bars represent SEM, $n=3$.

was also recovered from control pPVC, although the diversity was lower compared to grassland soil. With the exception of TCMP, the incorporated biocides had less effect on species diversity recovered from pPVC buried in forest soil.

Fig. 3. Total number of fungal species recovered over the soil burial period on control and biocide-containing pPVC. White bars, grassland soil; black bars, forest soil.

Fig. 4. Change in Young's modulus (stiffness) (black bars) and strain (extensibility) (white bars) of control and pPVC with incorporated biocides, buried in grassland soil (a) and forest soil (b). Error bars represent SEM, $n=9$.

Change in physical parameters and weight loss from buried pPVC

Samples of pPVC recovered after 300 days were tested for changes in two tensile-strength parameters, stiffness (Young's modulus) and extensibility (breaking strain), compared to unburied controls (Fig. 4a, b). In pPVC buried in grassland soil (Fig. 4a) a greater change was observed in sample stiffness, which increased up to 60 % for control and DCOIT- and TCMP-containing pPVC and up to 20–40 % for the rest of the samples. Extensibility for grassland-buried pPVC changed less and was reduced by 17–33 % for all samples. In contrast, in pPVC buried in forest soil, extensibility of the samples changed more than stiffness, and in OBPA-, TCMP- and BBIT-containing pPVC it was reduced by 40 % (OBPA) and 30 % (TCMP and BBIT) respectively at the end of the experiment (Fig. 4b). The extensibility of the rest of the samples changed between 7 % and 20 %; their stiffness increased less when compared with grassland-buried pPVC and was in the range from 8 % for OIT-containing pPVC to 35 % for BBIT-containing pPVC.

The weight loss after 300 days was greater in control pPVC buried in grassland soil compared to biocide-containing

pPVC, where the samples lost a maximum of $4.00 \pm 1.56\%$ of their weight. pPVC containing TCMP and DCOIT lost $3.44 \pm 1.57\%$ and $2.64 \pm 0.92\%$ of their dry weights respectively whilst the rest of the biocide-containing plastics lost less than 1%. Weight loss from control pPVC pieces buried in forest soil after 300 days was similar to that of grassland-buried pPVC ($3.90 \pm 1.64\%$). OIT- and BBIT-containing pPVC lost $3.06 \pm 0.93\%$ and $1.66 \pm 1.06\%$ dry weight respectively, whilst the remaining biocide-containing plastics lost less than 1%.

After 300 days, SEM revealed that the surfaces of control pPVC buried in both grassland and forest soil were heavily colonized by a dense fungal mycelial network embedded in a slimy matrix (Fig. 5a, b). Following removal of surface material by scraping, SEM revealed subsurface penetration of fungal hyphae and disturbance of the plastic surface by numerous canals caused by fungal hyphal growth (Fig. 5c, d). In addition, deep spots of local damage of the pPVC were frequently found on pPVC buried in forest soil (Fig. 5e). The surface of biocide-containing pPVC appeared far less colonized than control pPVC from both grassland and forest. Biocide OBPA was most effective, with large areas free of hyphal growth (Fig. 5f).

Identification of fungal isolates on pPVC buried in grassland soil

Forty-two fungal isolates recovered from pPVC buried in grassland soil, which had been shown to clear DOA or DOP agar, were subjected to rDNA sequencing. The fungal isolates chosen for sequencing were either (a) species isolated at virtually all time points during the course of the trial; (b) species frequently recovered from control and/or some of the biocide-containing pPVC samples; (c) species isolated in low numbers but capable of strong clearing on DOA agar; or (d) species showing DOP clearing.

In grassland soil, the first DOA-clearing fungi recovered from control pPVC were *Geomyces vinaceus* (*Pseudogymnoascus roseus* – teleomorph), *Penicillium* spp., *Paecilomyces fumosoroseus*, *Mortierella hyalina* and the unidentified isolates GFI-2 (zygomycete) and GFI-3 (ascomycete) (Table 1). A similar pattern was also observed on pPVC with biocides TCMP and DCOIT. With time, the variety of *Penicillium* spp. on control pPVC increased and *P. janthinellum* established as one of the main colonizing species, followed closely by *P. roseopurpureum*. Strains isolated during the middle stages included isolate GFI123 (probable *Leptosphaeria* sp.), *Phoma exigua*, *Trichosporon porosum*, *Auxarthron conjugatum*, *Metarhizium anisopliae*, *Doratomyces stemonitis* and *Fusarium solani* (*Nectria haematococca* – teleomorph). A few zygomycetes were present at the initial isolation stages; however, their count and variety increased substantially during the later isolation stages. Most of these isolates had only weak DOA-clearing ability.

During the summer months (the middle of the experiment) the number of isolated species increased and included

Nectria gliocladioides, *Geomyces pannorum*, a new *Doratomyces* sp. (GFI-81) and another two new unidentified isolates clearing DOP agar (GFI-145 and GFI-146). On the last isolation stages, *Penicillium canescens*, *Myrothecium roridum* and two DOP-clearing fungi, *Paecilomyces carneus* and unknown strain GFI-156, were also isolated. Twelve DOA-clearing fungal isolates were recovered only from control pPVC during the later stages of the experiment; these included GFI-32 (probable *Cordyceps* sp.), *Zalerion varium* and *Trichoderma album*.

Identification of fungal isolates on pPVC buried in forest soil

Fifteen distinct fungal morphotypes from pPVC buried in forest soil were selected on the basis of DOA-clearing activity and identified by rDNA sequencing (Table 2). *Penicillium swiecickii* and *P. canescens* were the most frequent isolates recovered from most of the buried pPVC. Several basidiomycetes were isolated from control and DCOIT- and OIT-containing pPVC. However, some of the strong DOA-clearing fungi did not match sequences in the fungal database and were unidentified. The DOP-clearing unidentified isolate FFI-10 became established on control pPVC after 135 days of burial and was later recovered from pPVC with biocides BBIT and OIT. Ten isolates, belonging to the zygomycetes, *Trichoderma harzianum* (*Hypocrea lixii* – teleomorph) and two other *Trichoderma* spp., were recovered from the middle and later stages of the burial trial. *Neonectria ramulariae*, which displayed strong DOA clearing, was recovered throughout the trial but only from control pPVC.

DISCUSSION

This is believed to be the first detailed study investigating the colonization and degradation of soil-buried pPVC *in situ* and the influence of incorporated biocides on the soil microflora. Fungi were found to be important colonizers of pPVC, producing a dense mycelial network over the surface of the plastic readily visible by SEM, and with many of the recovered fungi able to grow on and clear DOA agar. Fungi were reported to be the principal colonizers and degraders of soil-buried polyurethane (Barratt *et al.*, 2003) and on air-exposed pPVC (Hamilton, 1983; Upsher & Roseblade, 1984; Webb *et al.*, 2000). Although in our study bacteria with an ability to clear DOA were initially recovered, their percentage declined over time (data not shown), more notably in grassland soil. The total TVC of fungi in both grassland and forest soil increased over the course of the experiment, indicating significant colonization of the buried pPVC samples.

The biocides incorporated into pPVC affected fungal colonization to different extents. Biocides OBPA and NCMP were found to have the greatest effect, significantly reducing both the TVC and the number of different species of the recovered grassland fungi, while biocides DCOIT and TCMP had little impact. In forest soil the most effective

Fig. 5. SEM of the surfaces of pPVC samples. (a, b) Control pPVC buried for 300 days in grassland soil (a) and forest soil (b); bars, 100 μm . (c, d) Control pPVC buried for 300 days in grassland soil (c) and forest soil (d) after scraping off the fungal material; bars, 25 μm . (e) Control pPVC buried for 300 days in forest soil showing localized damage; bars, 100 μm . (f) OBPA-containing pPVC buried in grassland soil for 300 days; bar, 100 μm .

biocides were TCMP, BBIT and OBPA, reducing the fungal TVC to just a few viable isolates per cm^2 , and only three

fungal morphotypes from TCMP-containing pPVC were recovered during the whole experiment. These biocides are

Table 1. Summary of the distribution of fungi isolated from control pPVC and pPVC containing various biocides buried for up to 300 days in grassland soil and their relative abilities to degrade the plasticizers DOA and DOP

Code	Name	Plasticizer clearing*		Recovered from (type of PVC)†						
		DOA	DOP	Control	DCOIT	BBIT	TCMP	OIT	NCMP	OBPA
GFI-47	<i>Penicillium janthinellum</i>	++	–	X	X	X	X	X	X	X
GFI-2	Unidentified zygomycete	+/-	–	X	X	X	X	X		X
GFI-125	Unidentified ascomycete	+/++	–	X	X	X	X	X	X	
GFI-46	<i>Penicillium roseopurpureum</i>	+/++	–	X	X	X	X	X	X	
GFI-48	<i>Penicillium canescens</i>	+	–	X	X	X	X	X	X	
GFI-143	Unidentified basidiomycete	++	–	X	X	X		X	X	X
GFI-5	Unidentified zygomycete	+/++	–	X	X	X			X	X
GFI-149	<i>Auxarthron conjugatum</i>	+	–	X	X	X		X	X	
GFI-25	<i>Geomyces</i> sp.	+	–	X	X	X	X			
GFI-123	Unidentified ascomycete	+/-	–	X	X	X	X			
GFI-101	<i>Metarhizium anisopliae</i>	+	–	X	X	X			X	
GFI-148	<i>Trichosporon porosum</i>	+/-	–	X	X			X		X
GFI-21	<i>Geomyces vinaceus</i>	+	+/-	X	X	X	X			
GFI-122	<i>Acremonium furcatum</i>	++	–	X	X	X				
GFI-145	Unidentified isolate	+	+	X	X	X				
GFI-7	Unidentified ascomycete	+/-	–	X	X		X			
GFI-147	<i>Phoma exigua</i>	+	+/-	X	X			X		
GFI-11	<i>Nectria gliocladioides</i>	++	–	X		X	X			
GFI-157	<i>Myrothecium roridum</i>	+/++	–	X		X	X			
GFI-1	Unidentified zygomycete	++	–	X			X		X	
GFI-146	Unidentified ascomycete	+/-	–		X	X	X			
GFI-33	<i>Psathyrella candolleana</i>	++	+	X		X				
GFI-27	<i>Paecilomyces fumosoroseus</i>	+	–	X			X			
GFI-82	<i>Doratomyces stemonitis</i>	+	+	X			X			
GFI-35	<i>Paecilomyces carneus</i>	++	+/-	X				X		
GFI-81	<i>Doratomyces</i> sp.	+	+	X						
GFI-3	Unidentified ascomycete	++	+/-	X						
GFI-32	Unidentified ascomycete	++	–	X						
GFI-185	<i>Zalerion varium</i>	++	–	X						
GFI-22	<i>Geomyces pannorum</i>	+++	+/-		X					
GFI-91	<i>Fusarium solani</i>	+++	+/-			X				
GFI-156	Unidentified ascomycete	++	+			X				
GFI-24	<i>Trichoderma album</i>	++	–						X	

*The extent of DOA or DOP clearing when grown on MSM-plasticizer agar was assessed according to the following criteria: ++, transparent clearing beyond or at the colony margin; +, semi-transparent clearing beyond or at the colony margin; +/-, weak clearing at the colony margin; –, growth without clearing.

†X indicates that the isolate was recovered from the pPVC.

effective broad-spectrum fungicides used in applications such as the manufacture of exterior paints (Shirakawa *et al.*, 2002) and coatings as well as plastics (Jones *et al.*, 1996). In forest soil, however, the apparent breach in the biocidal efficacy of BBIT, OBPA, DCOIT and OIT during the end of the trial led to a sharp rise in the fungal TVC; this may have been due to the higher levels of moisture recorded compared to grassland soil (data not shown) encouraging greater leaching and reduction in surface biocide concentration. Alternatively, the late increase in TVC may have reflected increased fungal activity in the forest soil during the autumn period.

Substantial enrichment of fungi capable of clearing DOA was observed on control pPVC over the first 135 days. This result suggests that fungi capable of clearing the plasticizer outcompeted fungi unable to clear the plasticizer over time. During the later stages, in spite of an overall increase in TVC, a decrease in the percentage of DOA-clearing fungi was recorded, probably due to conditioning of the pPVC surface with secondary byproducts and cell debris, enabling fungi with no DOA-clearing ability to grow on the surface of the pPVC. The appearance of non-DOA-clearing fungi at the end of a 96-week air-exposed PVC trial following initial colonization by DOA clearers, principally *Aureobasidium*

Table 2. Summary of the distribution of fungi isolated from control pPVC and pPVC containing various biocides buried for up to 300 days in forest soil and their relative abilities to degrade the plasticizers DOA and DOP

Code	Name	Plasticizer clearing*		Recovered from (type of pPVC)†						
		DOA	DOP	Control	DCOIT	BBIT	TCMP	OIT	NCMP	OBPA
FFI-45	<i>Penicillium swiecickii</i>	++	–	X	X	X		X	X	
FFI-42	<i>Penicillium canescens</i>	+/-	–	X	X		X	X		X
FFI-144	<i>Trichosporon (multisporum/laibachii)</i>	+/-	–	X	X			X		X
FFI-46	<i>Penicillium chrysogenum</i>	++	–	X	X		X			
FFI-10	Unidentified ascomycete	++	+	X		X		X		
FFI-143	Unidentified basidiomycete	++	–		X			X		X
FFI-47	<i>Penicillium coprophilum</i>	++	–			X			X	X
FFI-48	<i>Penicillium expansum</i>	++	–			X			X	X
FFI-9	Unidentified zygomycete	+	–	X			X			
FFI-91	<i>Neonectria ramulariae</i>	+/++	–	X					X	
FFI-25	<i>Trichoderma harzianum</i>	++	–				X			X
FFI-6	<i>Mucor hiemalis</i>	++	–					X		X
FFI-5	<i>Mucor fragilis</i>	++	–					X		
FFI-22	<i>Penicillium inflatum</i>	+	–	X						
FFI-30	<i>Geomyces</i> sp.	+	+							X

*The extent of DOA or DOP clearing when grown on MSM-plasticizer agar was assessed according to the following criteria: ++, transparent clearing beyond or at the colony margin; +, semi-transparent clearing beyond or at the colony margin; +/-, weak clearing at the colony margin; –, growth without clearing.

†X indicates that the isolate was recovered from the pPVC.

pullulans, has previously been reported (Webb *et al.*, 2000). Despite the strong reduction in TVC and fungal variety on pPVC containing OBPA (grassland soil), TCMP and NCMP (forest soil), all fungal species isolated from these samples were able to clear DOA and no biocide was able to completely prevent fungal colonization.

Penicillium janthinellum was found to be an important colonizer of pPVC and was one of the few strains recovered from pPVC containing biocide OBPA. *P. roseopurpureum* was also recovered frequently while *P. canescens* was recovered only during the later stages. The high frequency of recovered *Penicillium* spp. probably reflects their widespread and abundant distribution in temperate soils (Domsh *et al.*, 1980a, b); they were amongst the most commonly isolated from the surface of a number of polymeric materials exposed to the environment and from soil-buried polyurethane (Barratt *et al.*, 2003). While many of the recovered fungi were shown to be ascomycetes, a small number of zygomycetes and basidiomycetes were also recovered, many of which were poorly sporulating or non-sporulating and may have been under-represented on the isolation plates compared to the more abundantly sporulating ascomycetes. Moreover, it is well known that culture-based recovery techniques do not necessarily reflect either true diversity or abundance (Borneman & Hartin, 2000; Zak, 1992) and that the majority of fungal species may be difficult to recover in the laboratory due to their fastidious nutrient requirements. However, despite these limitations, a total of 92 species were recovered, although only those

capable of clearing plasticizer agar and recovered in high numbers or frequently during the course of the trial were subjected to rDNA sequencing for identification purposes. While many of the fungi recovered from the surface of the pPVC were capable of clearing DOA, few isolates were capable of clearing DOP. Two *Doratomyces* isolates were the most abundant recovered DOP-clearing fungi. Phthalate plasticizers have previously been shown to be far less susceptible to microbial hydrolysis compared to adipate plasticizers (Berk *et al.*, 1957; Eaton & Ribbons, 1982; Frankland *et al.*, 1990; Nalli *et al.*, 2002) and there have to our knowledge been no previous reports of phthalate ester clearing by fungi. As the majority of DOA-clearing fungi were unable to clear DOP, the two plasticizers must be hydrolysed by separate enzymic systems.

After 10 months of soil burial, the physical properties of the pPVC were significantly altered due to plasticizer utilization, with the largest changes correlating with those samples from which the greatest fungal TVCs were recovered. While stiffness was most affected in pPVC buried in grassland, extensibility of the samples was the most affected in pPVC buried in forest soil. This may reflect a higher level of plasticizer leaching in the forest soil due to the higher moisture content or be due to observed spots of local damage, of suspected fungal or insect origin, which were not seen on pPVC recovered from grassland soil.

In conclusion, we have shown that fungi are important colonizers of pPVC and that the surface of the plastic

selected initially for fungi with the ability to clear DOA, with non-DOA-clearing fungi appearing later after initial colonization. None of the incorporated biocides completely prevented colonization and growth on the pPVC surface; however some were able to significantly reduce both TVC and the number of species recovered, although the most effective biocides differed between the two soil types tested.

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