

**The University of Manchester
Faculty of Life Sciences**

**FUNGAL BIODEGRADATION OF
POLYVINYL ALCOHOL
IN SOIL AND COMPOST
ENVIRONMENTS**

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ABSTRACT

For over 50 years, synthetic petrochemical-based plastics have been produced in ever growing volumes globally and since their first commercial introduction; they have been continually developed with regards to quality, colour, durability, and resistance. With some exceptions, such as polyurethanes, most plastics are very stable and are not readily degraded when they enter the ground as waste, taking decades to biodegrade and therefore are major pollutants of terrestrial and marine ecosystems. During the last thirty years, extensive research has been conducted to develop biodegradable plastics as more environmentally benign alternatives to traditional plastic polymers. Polyvinyl alcohol (PVA) is a water-soluble polymer which has recently attracted interest for the manufacture of biodegradable plastic materials. PVA is widely used as a paper coating, in adhesives and films, as a finishing agent in the textile industries and in forming oxygen impermeable films. Consequently, waste-water can contain a considerable amount of PVA and can contaminate the wider environment where the rate of biodegradation is slow. Despite its growing use, relatively little is known about its degradation and in particular the role of fungi in this process. In this study, a number of fungal strains capable of degrading PVA from uncontaminated soil from eight different sites were isolated by enrichment in mineral salts medium containing PVA as a sole carbon source and subsequently identified by sequencing the ITS and 5.8S rDNA region. The most frequently isolated fungal strains were identified as *Galactomyces geotrichum*, *Trichosporon laibachii*, *Fimetariella rabenhorsti* and *Fusarium oxysporum*. *G. geotrichum* was shown to grow and utilise PVA as the sole carbon source with a mean doubling time of ca. 6-7 h and was similar on PVA with molecular weight ranges of 13-23 KDa, 30-50 KDa and 85-124 KDa. When solid PVA films were buried in compost, *Galactomyces geotrichum* was also found to be the principal colonizing fungus at 25°C, whereas at 45°C and 55°C, the principle species recovered was the thermophile *Talaromyces emersonii*. ESEM revealed that the surface of the PVA films were heavily covered with fungal mycelia and DGGE analysis of the surface mycelium confirmed that the fungi recovered from the surface of the PVA film constituted the majority of the colonising fungi. When PVA was added to soil at 25°C, and in compost at 25°C and 45°C, terminal restriction fragment length polymorphism (T-RFLP) revealed that the fungal community rapidly changed over two weeks with the appearance of novel species, presumably due to selection for degraders, but returned to a population that was similar to the starting population within six weeks, indicating that PVA contamination causes a temporary shift in the fungal community.

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DEDICATION

“Indeed, in the creation of the heavens and earth, and the alternation of the night and the day, and the (great) ships which sail through the sea with that which benefits people, and what Allah has sent down from the heavens of rain, giving life thereby to the earth after its lifelessness and dispersing therein every (kind of) moving creature, and (His) directing of the winds and the clouds controlled between the heaven and the earth are signs for a people who use reason”.

Holly Quran, Chapter 2 - Verses 164

Dedicated To My Lovely Family

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ABBREVIATIONS

CFU	colony forming units
DEPC	diethyl pyrocarbonate
DGGE	denaturing gradient gel electrophoresis
DH	degree of hydrolysis
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DOA	dioctyl adipate
DOP	dioctyl phthalate
EDTA	ethylenediaminetetraacetic acid
ESEM	Environmental scanning electron microscopy
HPLC	high performance liquid chromatography
l	Litre
ITS	internal transcript spacer
MD	mean doubling time
mg	Milligram
ml	Milliliter
mm	Millimeter
mM	Millimolar
MSA	mineral salts agar
MSM	mineral salts medium
Mw	molecular weight
OD	optical density
OPH	oxidized poly(vinyl alcohol) hydrolase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
PVA	poly(vinyl alcohol)
PVAc	poly(vinyl acetate)
PVADH	PVA dehydrogenase
PVC	poly(vinyl chloride)
rDNA	ribosomal DNA
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
s.d	Standard deviation of the mean
s.e	standard error of the mean
SEM	scanning electron microscopy
T-RFLP	terminal restriction fragment length polymorphism
UV	Ultraviolet
WHC	water holding capacity

1 CHAPTER ONE. INTRODUCTION

1.1 OVERVIEW

Currently it is estimated that over 140 million tonnes of synthetic plastics are produced annually worldwide (Shimao, 2001, Tokiwa *et al*, 2009; Sivan, 2011). A large proportion of these plastics are very stable and not easily degradable when they enter the ground as waste. There are very few natural enzymes that can biodegrade plastics and consequently the large volume of waste plastics entering the environment is problematic (Shah *et al.*, 2008) causing not only environmental pollution but also putting pressure on diminishing landfill site capacity (Hopewell *et al*, 2009). The longevity of plastics and synthetic polymers, the extent of biodegradation and their impact on the environment is therefore of major concern. More recently, research has focused increasingly on biodegradable synthetic plastics and plastic blends containing biodegradable components which will have a lower impact on the environment (Larry *et al.*, 1992; Tadros *et al.*, 1999; Eggins & Oxley., 2001; Corti *et al.*, 2002a; 2002b; Gu, 2003; Chiellini, 2003; Mueller, 2006; Chen *et al.*, 2007; Shah *et al.*, 2008) resulting in the development of some biodegradable plastics (Rivard *et al.*, 1995; Swift, 1997; Amass *et al.*, 1998 ; Chandra & Rustgi., 1998; Graaf & Janssen, 2000; 2001; Tokiwa & Calabria, 2007) and an understanding of the mechanisms by which they biodegrade (Shah *et al.*, 2008; Singh & Sharma., 2008).

Polyvinyl alcohol (PVA) is a synthetic water soluble polymer which is synthesised by the condensation of vinyl acetate units followed by replacement of the acetate group by a hydroxyl group (Finch, 1973). PVA is widely used as a

paper coating, in adhesives and films, as a finishing agent in textile industries, for manufacture of oxygen impermeable films and in the manufacture of environment-friendly plastic materials (Larking *et al.*, 1999; Solaro *et al.*, 2000). Waste-water can contain a considerable amount of PVA and could contaminate the environment as it is believed that PVA biodegrades quite slowly in the environment (Lee & Kim, 2003). The majority of studies on the biodegradation of PVA have focused on bacterial species where a limited number have been found to actively metabolise PVA (Watanabe *et al.*, 1976; Sakazawa *et al.*, 1981; Matsumara *et al.*, 1994; Hatanaka *et al.*, 1995; Mori *et al.*, 1996; 1997; Corti *et al.*, 2002b; Chiellini *et al.*, 2003; Chen *et al.*, 2007; Du *et al.*, 2007). However, relatively little work has been undertaken on the potential role of fungi in PVA biodegradation or of the effect of soil contamination on fungal communities.

1.2 PLASTICS

The most common of all synthetic polymers are plastics, which are commercially produced on a large scale with a wide variety of applications (Larry *et al.*, 1992). Since the first introduction of Bakelite on a large commercial scale in the 1950 s, numerous new plastic polymer chemistries have been commercialised with differing physical characteristics and uses (Rivard *et al.*, 1995) and have largely replaced natural materials in many applications (Scott, 1999; Singh & Sharma 2008). Many plastics are homopolymers containing a carbon carbon backbone that are considered to be resistant to microbial attack, or take decades to biodegrade (Mueller, 2006). Thus, plastic waste is one of the major environmental pollution challenges today having an effect on sensitive communities and harming

wildlife (Shah *et al.*, 2008).

1.2.1 Different types of plastic polymers

Plastics can be broadly categorized into two major groups; thermoplastics and thermoset plastics. Thermoplastics can be softened when heated and harden on cooling and therefore can be remoulded. Many thermoplastics are homopolymers containing a long carbon chain back bone which can interact with adjacent polymer molecules through intermolecular forces (Strong, 2008). Due to their homopolymer backbone, thermoplastics, which include polyethylene, polyvinyl chloride, polypropylene and polystyrene, are generally highly resistant to degradation or hydrolytic cleavage (Zheng *et al.*, 2005). By contrast, thermoset plastics are characterized by mixed heteropolymer backbone and form highly cross-linked structures. As a result, thermoset plastics breakdown on heating and cannot be remoulded (Strong, 2008). The heteropolymer backbone contains bonds which are susceptible to microbial enzymatic degradation such as ester or amide bonds and include polyesters, polyethylene terephthalates and polyurethanes (Zheng *et al.*, 2005).

1.2.2 Uses of plastics polymers

Synthetic plastics are widely used in a highly diverse number of industrial sectors worldwide. Around 30 percent of plastics are widely utilized as packaging materials for foods, pharmaceuticals, cosmetics, detergents and chemicals, because of their strength, lightness and superior physical and chemical properties such as resistance to water and microorganisms (Zheng *et al.*, 2005). Plastics also find extensive applications in agriculture, construction and the automotive industry. The

most common oil-based plastics are polyethylene (Lee *et al.*, 1991), polypropylene, polyurethane, polystyrene and the vinyl polymers (Chandra & Rustgi, 1998; Amass *et al.*, 1998; Shah *et al.*, 2008). A summary of the major plastic polymers and their structures are shown in Table 1.1.

1.2.3 Degradation of plastics

There is a considerable difference between plastics in their resistance to degradation and the mechanism of degradation (Hawkins, 1984). Degradation can be defined as any physical or chemical change in plastic caused by factors in the environment such as light, heat, moisture, chemical conditions or biological activity (Krzan *et al.*, 2006). These include processes that stimulate changes in polymeric properties such as cracking, erosion or discoloration which may result in bond scission followed by chemical transformations (Pospisil & Nespurek, 1997; Shah *et al.*, 2008). Many polymers are too large to be transported into the microbial cells and require depolymerisation fragments or monomers before they can be assimilated for growth (Shah *et al.*, 2008).

Hence, along with microorganisms, light, heat, moisture and chemical conditions have roles in initial breakdown and degradation of plastics.

1.2.3.1 Photo degradation

Photo degradation occurs in connection with the polymer's sensitivity and ability to absorb the deleterious aspect of tropospheric solar radiation such as UV-B (~ 295-315 nm) and UV -A radiation (~ 315-400 nm) which causes direct photo degradation (Gugumus, 1990; Pospisil & Nespurek, 1997, Shah *et al.*, 2008). This

high energy radiation activates the polymer's electrons and results in oxidation, cleavage and other degradation reactions.

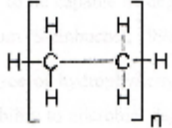
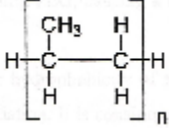
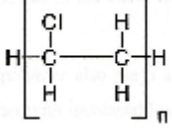
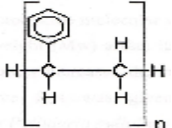
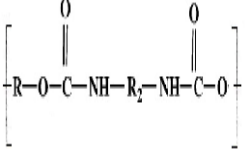
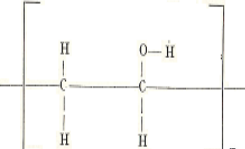
Name of Plastic	Uses	Structure
Polyethylene	Packaging and manufacture of films, fabrication of Bottles, tubes, pipes	
Polypropylene	Bottles, jugs, containers, medicine bottles, car seats, car batteries and bumpers	
Polyvinyl chloride	Raincoats, automobile seat covers, shower curtains, tanks, jugs, and containers, fabrication of bottles, garden hoses pipes	
Polystyrene	Packaging materials, laboratory ware, jugs, containers and disposable cups	
Polyurethane	Coatings, insulation, paints, tyres, furniture cushioning, life jackets and packing	
Polyvinyl alcohol	Adhesives, packaging include boxboard manufacture, paper bags, paper lamination, tube winding and remoisten able labels	

Table 1-1. Main types of synthetic polymers plastics and their uses (adapted from Shah et al., 2008).

1.2.3.2 Thermal degradation

When the molecules of a polymer deteriorate as a result of heating, thermal degradation takes place. Thermal degradation occurs when the polymer changes from a solid to a liquid form and the temperature at which this occurs depends on its molecular weight. Temperature may also affect the macromolecular framework of the polymer and the mobility and volume of the polymeric chains which can facilitate biological and chemical degradation. High temperatures trigger the molecular scission of the polymer's long chain backbone which react with one another and result in a change to the polymer properties and a reduction in molecular weight. Changes in the properties of the plastics include colour changes and cracking with plastics becoming brittle and more susceptible to microbial degradation (Olayan *et al.*, 1996).

1.2.3.3 Chemical degradation

Chemical degradation occurs when atmospheric pollutants and agrochemicals interact with the polymers and change their macromolecular properties. Some of the major forms of chemical degradation are outlined below.

1.2.3.3.1 Oxidative degradation

The oxidative degradation of plastics has been studied over several decades. Many different reactions are responsible for oxidative degradation (Hawkins, 1972) with the most common form involving interaction with atmospheric oxygen and ozone (Lucas *et al.*, 2008; Shelton, 1972). Oxygen reacts with the covalent bonds of the plastic polymer leading to changes in molecular structure or morphology (Winslow *et al.*, 1955). Oxidative degradation mostly occurs during long-term

aging and exposure of plastics to the atmosphere (Hawkins, 1984).

1.2.3.3.2 Degradation by hydrolysis

Hydrolysis is another form of chemical degradation and is dependent on water activity, temperature, pH and time (Lucas *et al.*, 2008). Plastics which are synthesized by condensation reactions are at risk of degradation by hydrolysis. Random scission of band along the back bone chain can occur and hydrolysis can be catalyzed by either bases or acids (Hawkins, 1984).

1.2.3.4 Mechanical degradation

Mechanical degradation happens as a result of compression, tension and shear forces which can range from pressure constraints during material installation, ageing, air and water turbulence or snow pressure and even bird damage. While mechanical degradation may not play a major role in biodegradation, it can activate or accelerate it (Briasoullis, 2004; 2006; 2007) and initiate molecular level degradation through synergy with other abiotic parameters such as temperature, solar radiation and chemicals (Lucas *et al.*, 2008).

1.2.3.5 Biodegradation of plastics

Biodegradation can be defined as a decomposition process through the action of microorganisms, which results in the recycling of the polymer carbon, followed by mineralization (that is the production of CO₂, H₂O and salts) of compounds and the creation of new biomass (Andrady, 2007; Amass *et al.*, 1998; Chandra and Rustgi, 1998; Lucas *et al.*, 2008) and is summarised in Fig 1.1. Biodegradation of both natural and synthetic plastics is processed by microorganisms such as fungi and bacteria (Shah *et al.*, 2008). Water-soluble

polymers as well as solid polymers can enter the environment and biodegrade in sediments, landfills, composts and soil. Biodegradation can be affected by various factors such as polymer characteristics, type of organism and chemical degradation (Howard, 2002).

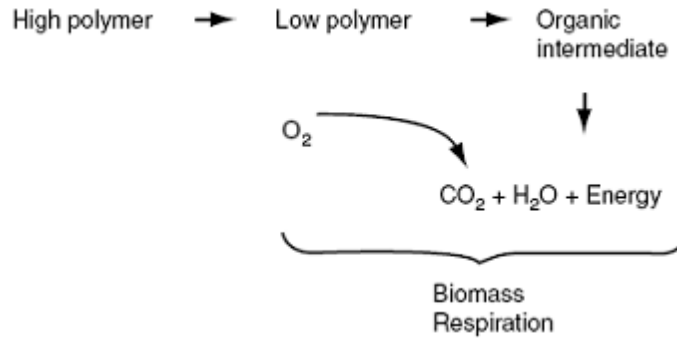


Figure 1-1. Biodegradation process in polymers (adapted from Andrady, 1994).

This in turn will lead to the recycling of carbon through mineralization and the creation of new biomass (Lucas *et al.*, 2008). Several stages are involved in the biodegradation of polymers, which may terminate at any stage (Lucas *et al.*, 2008). When a biodegradable polymer enters the environment, the end products are often carbon dioxide, water and salts during aerobic mineralization and in addition methane during anaerobic mineralization as illustrated by the equations below:

Eq. 1.1 for aerobic mineralization: $C(e) = CO_2 + C(c) + C(r)$

Eq. 1.2 for anaerobic mineralization: $C(e) = CO_2 + CH_4 + C(c) + C(r)$

Where $C(e)$ is total amount of carbon entering the environment and $C(c)$ is amount of carbon converted into biomass and $C(r)$ is the amount of residual carbon after biodegradation is completed. Based on these equations, complete biodegradation has taken place when $C(r) = 0$. These equations are applicable to polymers and all synthetic materials including organic chemicals such as pesticides (Swift, 1997). Soil is a rich microbial environment and in the biodegradation of organic materials, decomposition of dead plant and animal material occurs through the secretion of extracellular enzymes. Fungi represent a key group of microbes involved in the degradation of naturally occurring polymers and are also thought to play a major role in the biodegradation of plastics (Sabev *et al.*, 2006a). For example, *Nectria gliocladioides*, *Penicillium ochrochloron* and *Geomyces pannorum* were reported to be the principal colonizers and degraders of soil-buried polyurethane while little evidence of bacterial activity (Barratt *et al.*, 2003).

Degradation of polyester polyurethane by fungi is thought to be caused by the enzymatic action of esterases, proteases and/or ureases (Pathirana and Seal 1984a, b). The bacterium *Comamonas acidovorans* TB-35, which utilizes a polyester polyurethane as carbon source (Nakajima-Kambe *et al.*, 1999) appears to contain a cell-bound esterase that can hydrolyze polyester chains in polyurethane to diethylene glycol and adipic acid (Akutsu *et al.*, 1998). *Penicillium janthinellum* was found to be an important colonizer of pPVC. This fungal species can produce a dense mycelial network over the surface of plastics, and also affect the physical properties of pPVC due to plasticizer utilization (Sabev *et al.*, 2006b).

Biodegradation involves four stages, namely biodeterioration, biofragmentation, assimilation and mineralization (Lucas, 2008). There is relatively little known about the mechanisms involved in polymeric decomposition, however, it is known that the microorganisms involved in the process first invade the biodegradable fragments in the surface of the plastic (Ratajska & Boryniec, 1998) and enzymes produced by microorganisms are principally involved in the degradation of the plastic material. Biodeterioration is the stage in which the biodegradable materials are fragmented as a result of the joint action of microbes, decomposer organisms and/or abiotic factors (Eggins & Oxley, 2001; Walsh, 2001). The cohesiveness of the material is diminished when these fragments are removed making it more vulnerable to water penetration. Catalytic elements such as enzymes and free radicals are produced by these microorganisms which cleave the molecules of the polymers resulting in a decrease in their molecular weight. During this stage, oligomers, dimers and monomers are produced in the process of depolymerisation. Microbial cells can then take up these small molecular weight compounds and break down these products following transport across the cytoplasmic membrane (Lucas *et al.*, 2008). Assimilation then takes place in the cytoplasm to generate energy and new biomass. Mineralisation then occurs when intracellular metabolites such as CO₂, N₂, CH₄, water and various salts are in turn released in the environment (Lucas *et al.*, 2008).

One group of synthetic plastics which are most susceptible to biodegradation are polyesters, which are made of monomers polymerised through ester linkages. Ester-linkages are generally easy to hydrolyze and are susceptible to

specific esterases (Tokiwa & Calabria, 2004; Shima, 2001). Several synthetic polyesters have been demonstrated to be biodegradable. For example polycaprolactone is synthetic polyester that can easily be degraded by microorganisms (Shima, 2001; Kim & Rhee, 2003) and Barratt *et al* (2003) found fungi to be the predominant micro-organisms responsible for degradation of polyester polyurethane. Other commercially polymers used as biodegradable plastics include polylactic acid and polyvinyl alcohol.

1.2.4 Evaluation methods of polymer degradation

1.2.4.1 Scanning electron microscopy (SEM) observations

Changes in colour, roughness of the plastic surface, formation of cracks or holes and formation of biofilm on the surface of polymers can be used as a first indication of microbial attack (Shah *et al.*, 2008) and are readily observed on the surface of plastics under scanning electron microscopy (SEM) (Ikada, 1999; Fernandez *et al.*, 2008; Geweely & Ouf, 2011).

1.2.4.2 Weight loss quantification

The decrease in polymer mass is extensively used in degradation assessment and it can be verified by extraction or separation methods (Witt *et al.*, 2001).

1.2.4.3 Clear zone formation

This method is mostly applied to monitor degradation of a particular polymer and to assess degradation potential of a variety of microorganisms. In this method, polymer suspension is dispersed within the agar medium which is inoculated with microorganisms. When the potential polymer degrading

microorganisms produce extracellular enzymes degradation of the polymer suspension in the medium produces a clear halo around the colonies and is widely used as an indicator of the microorganisms ability to depolymerise the polymer (Augusta *et al.*, 1993; Lee *et al.*, 2005; Tokiwa *et al.*, 2009).

1.2.4.4 O₂ consumption /CO₂ evolution

The consumption of oxygen or the creation of carbon dioxide is one of the methods applied to assess biodegradation under laboratory conditions with the rate of oxygen consumption or carbon dioxide evolution being proportional to the rate of degradation (Bellina *et al.*, 2000).

1.2.4.5 Culture independent methods

As the majority of microorganisms cannot be cultivated readily on laboratory media (Hawksworth, 1991; Osborn *et al.*, 2000), culture independent techniques such as temperature gradient gel electrophoresis (TGGE), denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (TRFLP) can be used to enable an estimate of both community diversity and dynamics within complex microbial ecosystems during polymer degradation (Myers *et al.*, 1985; Muyzer *et al.* 1997; Muyzer and Smalla, 1998; Andoh, *et al.*, 2009 and Elliott *et al.*, 2012).

1.3 POLYVINYL ALCOHOL AND PVA-BASED COMPUNDS

1.3.1 Introduction

Polyvinyl alcohol (PVA) is a water-soluble polymer which has recently attracted interest for the manufacture of environment-friendly plastic materials

(Solaro *et al.*, 2000). PVA is widely used as a paper coating, in adhesives and films, as a finishing agent in the textile industries and in forming oxygen impermeable films (Larking *et al.*, 1999). Waste-water can contain a considerable amount of PVA and could contaminate the environment as it is believed that PVA biodegrades quite slowly (Lee & Kim, 2003). Efforts have been made to isolate PVA-degrading microorganisms with the most commonly isolated being *Pseudomonas* spp (Lee & Kim, 2003) although a limited number of bacteria have also been isolated from various sources such as activated sludge from waste-water treatment plants. The biodegradation of PVA blends with natural polymers has also been investigated. It was found that the hydrophobic property of gelatine seemed to have affected the biodegradation tendency of the PVA blend (Corti *et al.*, 2002a). The degree of PVA polymerization has also been shown to affect the degree of biodegradation and highly polymerised PVA samples were more difficult and took longer to degrade due to a combined action of extracellular and intracellular enzymes (Chen *et al.*, 2007).

1.3.2 Structure and properties

In 1924, Hermann and Haehnel first produced PVA by hydrolyzing polyvinyl acetate in ethanol with potassium hydroxide. The end product of this hydrolysis, which is carried out through an ester exchange with methanol in the presence of anhydrous sodium methylate or aqueous sodium hydroxide, yields PVA which is an odourless, translucent, and white or cream coloured granular powder. It is water-soluble but only slightly soluble in ethanol and insoluble in other organic solvents. A solution of 5% PVA has a pH of 5.0-6.0 and its molecular weight typically

ranges from 14,000 to 50,000 Da.

1.3.3 Manufacture and use of PVA

PVA is produced using vinyl acetate as its monomer in a polymerization process followed by partial hydrolysis in which the ester group in polyvinyl acetate is partially replaced with the hydroxyl group and in the presence of an aqueous solution of sodium hydroxide becomes fully hydrolysed (Fig 1.2). PVA is then precipitated, washed and dried subsequent to the gradual addition of the aqueous saponification agent.

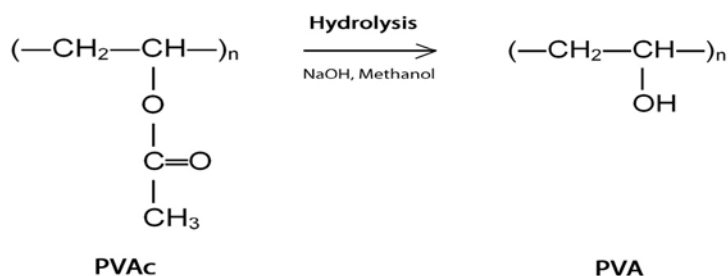


Figure 1-2. Hydrolysis of PVAc to PVA. Following polymerisation of polyvinyl acetate, the acetate group is removed by hydrolysis under alkaline conditions in methanol.

Most of the industrial processes are now based on pure ester interchange and the reaction is carried out in an anhydrous alcohol solution with a maximum of a few percent of water, with catalytic quantities of an alkali (Finch, 1973). For the production of PVA on an industrial scale only polyvinyl acetate is important. When the hydrolysis of polyvinyl acetate takes place in a low-boiling aliphatic alcohol for example methanol, it proceeds in a manner similar to the hydrolysis of monomolecular esters according to the following reaction scheme:

1) Ester interchange: PV-OAc (polyvinyl acetate) + ROH + NaOH (or acid) → PV-

OH (polyvinyl alcohol) +R-OAC

2) Direct hydrolysis: PV-OAC + NaOH (or acid) \rightarrow PV-OH + CH₃COONa (or CH₃COOH)

3) Hydrolysis of the ester formed in (1) R-OAC + NaOH (or acid) \rightarrow ROH + CH₃COONa (or CH₃·COOH)

The viscosity of the PVA formed by hydrolysis is determined by the degree of polymerization and by the branching of the polyvinyl acetate used (Finch, 1973). PVA is one of the key water-soluble polymer produced in the world and is also produced during the production cycles of petrochemical industries (Chen *et al.*, 2007). Major water-soluble polymeric materials are summarised in Table 1.2.

Class	Type	Acronym
Poly(carboxylate)s	Poly(malic acid) Poly(met	PMLA
	acrylic acid) Poly(aspartic	PMA
	acid)	PAsA
Poly(acrylics)	Poly(acrylic acid) Poly(acryl	PAA
	amide)	PAAm
Poly(ether)s	Poly(ethylene glycol)	PEG
	Poly(propylene glycol)	PPG
Poly(glutamic acid)		PGIA
Poly(hydroxylate)s	Poly(vinyl alcohol)	PVA

Table 1-2. Major water-soluble polymeric materials of potential environmental concern (Chiellini *et al.*, 2003).

Commercially available PVA is produced via different degrees of methanolysis, yielding different grades of PVA with varying levels of hydrolysis with a range of 70 to 99% (Chiellini *et al.*, 2003). PVA fibres can be utilized as a reinforcing material for buildings and as a brake pneumatic hose in agricultural machinery due to its high tensile strength. Partially hydrolysed PVA is quite commonly used as a moisture barrier for food supplement tablets and for foods that need to be protected from moisture. In manufacturing plastic materials which are environment-friendly, water-soluble PVA has drawn interest (Corti *et al.*, 2002a) because it can be used widely as coating for paper, in adhesives, and films. PVA is a very good barrier for fragrances, flavourings, oils and fats (Lee & Kim, 2003). PVA degradation is slow in the natural environment and its water solubility makes it persistent in waste water discharged from textile and dyeing factories, which can lead to pollution and accumulation in the environment (Larking *et al.*, 1999; Chen *et al.*, 2007). The molecular weight (MW) and degree of hydrolysis (DH) of PVA also have an impact on its characteristics and its degradation. Previous studies have reported that the rate of PVA degradation by mixed microbial cultures was affected by the degree of hydrolysis of the polymers. The biodegradation rate was faster in samples with a higher DH rather than samples with a low DH (Corti *et al.*, 2002b; Chen *et al.*, 2007). However, molecular weight differences had no significant effect on the rate of biodegradation of PVA samples (Chen *et al.*, 2007).

1.3.4 Degradation of PVA and PVA-based compounds

PVA's water solubility and biodegradability have given it the potential to be a suitable replacement for traditional non-degradable plastics made from fossil

fuels (Solaro *et al.*, 2000; Corti *et al.*, 2002a; Chiellini *et al.*, 2003). As PVA demonstrates excellent compatibility with some natural polymers (Jecu *et al.*, 2010), it is extensively utilized in the preparation of blends and composites containing natural renewable polymers such as starch, lignin (Pseja *et al.*, 2006; Fernandes *et al.*, 2006) and collagen (Sarti & Scandola, 1995). When blended with natural polymers, PVA improves the mechanical properties and performance of biopolymers due to its hydrophilic character and rheological properties (Jayasekara *et al.*, 2004). PVA-based materials are obtained by blending PVA with natural polymers of vegetable, animal and marine origin such as cellulose, lignin, starch, silk, and gelatin (Chiellini *et al.*, 2003). PVA based materials and PVA blends with natural biopolymers may facilitate the PVA biodegradation process improving its elimination from the environment (Jecu *et al.*, 2010).

1.3.4.1 Degradation of PVA

PVA is categorized as either partially or fully hydrolysed and is commercially manufactured from polyvinyl acetate in a continuous process (Chiellini *et al.*, 2003). Among vinyl polymers, PVA is the most readily biodegradable and it can be completely mineralized by microorganisms (Shimao, 2001).

In the past 40 years several studies have identified both single microorganisms (Suzuki *et al.*, 1973) and symbiotic or mixed cultures (Corti *et al.*, 2002b) with the ability to degrade PVA, which were isolated from soil and sludge samples (Sakazawa *et al.*, 1981; Corti *et al.*, 2002b; Du *et al.*, 2007; Chen *et al.*, 2007). Most of these isolated strains are bacteria such as *Pseudomonas sp* (Mori *et*

al., 1997; Watanabe *et al.*, 1976; Hatanaka *et al.*, 1995), *Alcaligenes faecalis* (Matsumara *et al.*, 1994), *Bacillus megaterium* (Mori *et al.*, 1996) although a *Penicillium sp* and *Flammulina velutipes* were previously reported to degrade PVA (Qian *et al.*, 2004; Tsujiyama *et al.*, 2011). Only a limited number of these strains can degrade PVA in pure culture (Chiellini *et al.*, 1999). The first report of complete PVA degradation was by a bacterial strain, *Pseudomonas O-3*, which used PVA as the only source of carbon and energy (Suzuki *et al.*, 1973). However, in many cases, PVA degradation requires the cooperative function of two strains, for example, *Pseudomonas sp.* VM15C and its symbiont *Pseudomonas sp.* VM15A (Sakazawa *et al.*, 1981; Shimao *et al.*, 1982; 1986), *Bacillus megaterium* and bacterial strain PN19 (Mori *et al.*, 1996), and *Sphingomonas sp.* SA3 and SA2 (Kim *et al.*, 2003). Sakazawa *et al.* (1981) isolated 30 symbiotic PVA utilizing cultures from around 1000 sludge, soil and waste samples from factories (Sakazawa *et al.*, 1981). In addition, a mixed microbial culture was isolated from paper mill sewage sludge which was capable of complete PVA biodegradation. None of the isolated strains could degrade PVA in pure culture (Corti *et al.*, 2002b; Chen *et al.*, 2007). The requirement for the presence of two strains for PVA degradation is due to the production of pyrroloquinoline quinone (PQQ) by one symbiont which is required by the other (Shimao, 2001). PQQ has been reported as a bacterial growth factor (Shimao, 2001). For example: in the symbionts of *Pseudomonas sp.* VM15C and VM15A, VM15C can degrade PVA, but it cannot grow alone in the presence of PVA as a sole carbon source and it requires PQQ for PVA degradation produced by VM15A (Shimao *et al.*, 1982). Another enzyme that

initiates PVA degradation and utilizes molecular oxygen and generates hydrogen peroxide during the reaction is the PQQ independent PVA oxidase (Sakai *et al.*, 1985). A further PVA degrading symbiotic relationship was also found for symbiotic strains *Sphingomonas sp.* OT3 and *Rhodococcus erythropolis* OT3. In addition to PQQ, *Sphingomonas sp.* OT3 requires an active catalyst to grow well on PVA medium (Vaclavkova *et al.*, 2007). PQQ is thought to act as a cofactor for PQQ-dependent PVA dehydrogenase (PVADH), an enzyme shown to catalyze the initial oxidation step in PVA degradation (Fig 1.3) in some microorganisms (Shimao *et al.*, 1982; 1986).

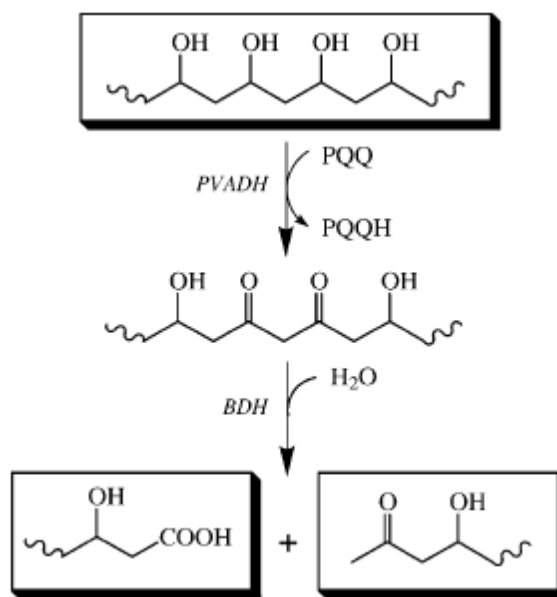


Figure 1-3. Biodegradation pathway of PVA as mediated by a PVA dehydrogenase PQQ-dependent in symbiotic bacterial culture (Chiellini *et al.*, 2003; Shimao *et al.*, 1986).

Further research identified an isolate from soil samples of a textile factory which can use PVA as a sole source of carbon and which did not require an additional partner during the PVA degradation process (Chiellini *et al.*, 2003; Du *et*

al., 2007) and was identified as a *Janthinobacterium* sp, WSH04-01. This strain was found to be capable of degrading 80% of the PVA present in cotton fabrics in 3h (Du *et al.*, 2007).

The first step in degradation is the cleavage of the carbon-carbon polymer backbone of PVA by extracellular enzymes (Chiellini *et al.*, 1999) followed by cellular uptake of the lower molecular weight PVA fragments with additional metabolism with the final steps of PVA degradation occurring inside the cells (Corti *et al.*, 2002b). Several enzymatic systems have been identified that catalyse the cleavage of the carbon-carbon polymer backbone of PVA. Cleavage of the polymer backbone is catalysed either by an oxidase or a dehydrogenase, followed by a hydrolase or aldolase reaction (Shimao, 2001). Watanabe *et al* (1976) purified and studied the properties of a PVA-degrading enzyme produced by a strain of *Pseudomonas*. Chandra and Rustgi (1998) studied the microbial degradation of PVA and its degradation using secondary alcohol peroxidases which had been isolated from a *Pseudomonas* strain isolated from soil. They found that the initial stage of biodegradation involved the enzymatic oxidation of secondary alcohol groups in PVA to ketone groups. Shimao *et al* (1982) investigated the production of PVA oxidase by a symbiotic mixed culture (Shimao *et al.*, 1982; Matsumura *et al.*, 1994; Shimao, 2001). PVA dehydrogenase from *Pseudomonas* sp. 113P3 was purified and characterized by Hatanaka *et al* (1995). Degradation was confirmed by both iodometric methods and gel permeation chromatography (Tokiwa *et al.*, 2000). A comparison of PVA-degradation rates among various microorganisms are listed in Table 1.3.

1.3.4.2 Biodegradation of PVA under different environmental conditions

The first studies on biodegradation of PVA were carried out in the presence of domestic or non-acclimated activated sludge (Chiellini *et al.*, 2003). Different conditions were selected to test degradability of PVA, including composting conditions, soil environment, aqueous environments (aerobic) and sewage sludge (Porter and Snider, 1976; Chiellini *et al.*, 2003). It was found that the efficient biological removal of the polymer was only achieved after long term exposure to PVA under conventional wastewater treatment conditions (Solaro *et al.*, 2000).

Organism (symbiont)	Initial PVA concentration	Degradation rate	Reference
<i>Sphingopyxis</i> sp	0.1%	>90% after 6 days	(Yamatsu <i>et al.</i> , 2006)
<i>Pseudomonas</i> O-3	0.5%	~100% after 5–7 days	(Suzuki <i>et al.</i> , 1973)
<i>Pseudomonas vesicularis</i> var. povalolyticus PH	0.1%	>90% after 5 days	(Hashimoto and Fujita, 1985)
<i>Pseudomonas</i> sp. VM15C (<i>Pseudomonas putida</i> VM15A)	0.5%	~100% after 6 days	(Sakazawa <i>et al.</i> , 1981)
<i>Sphingomonas</i> sp. SA3 (bacterial strain SA2)	0.5%	>95% after 4 days	(Kim <i>et al.</i> , 2003)
<i>Bacillus megaterium</i> BX1 (bacterial strain PN19)	0.1%	~100% after 10 days	(Mori <i>et al.</i> , 1996)
<i>Penicillium</i> sp. WSH02-21	0.5%	~100% after 12 days	(Qian <i>et al.</i> , 2004)
Gram-negative bacteria strain TK-2	0.5%	>95% after 4 days	(Tokiwa <i>et al.</i> , 2001)
<i>Alcaligenes faecalis</i> KK314	0.1%	>90% after 3 days	(Matsumura <i>et al.</i> , 1994)

Table 1-3. A comparison of PVA-degradation rates among various microorganisms.

1.3.4.2.1 Influence of pH on PVA biodegradation

pH has been shown to have an important effect on the ability of microbes to degrade PVA. Zhang (2009) showed the maximum rate of biodegradation by a mixed bacterial consortium of *Curtobacterium* sp. and *Bacillus* sp. was obtained when the initial pH was 8.0. This might be due to high degradation activity of secreted enzymes by the mixed bacterial culture in alkaline solutions (Zhang, 2009).

1.3.4.2.2 Biodegradation of PVA under composting conditions

Few studies have examined PVA degradation under composting conditions. Using compost extract as a microbial source demonstrated moderate PVA biodegradation (Liu *et al.*, 2009) but the degradation rate was faster than that obtained in soil.

1.3.4.2.3 Biodegradation of PVA in soil environments

Compared to other polymers such as polyhydroxybutyrate-hydroxyvalerate (PHB/HV) and polycaprolactone (PCL) that were extensively degraded by soil microorganisms (Kimura *et al.*, 1994), PVA biodegradation is much slower in the same soil environments (Chiellini *et al.*, 2000). In particular, a detailed investigation was carried out on PVA sheets buried in 18 different natural soil sites, with different compositions and climate conditions. However, after two years of incubation only very limited weight losses (less than 10%) were recorded under natural weathering conditions (Sawada, 1994; Chen *et al.*, 1997). The mineralization rate's dependence on the hydrolysis degree between two different commercial PVA samples with 88 and 98% degrees of saponification was also evaluated in soil burial experiment (Chiellini *et al.*, 1999; Chiellini *et al.*, 2003). The two PVA samples demonstrated small differences; the sample with the lowest HD showed a slightly larger propensity to microbial assimilation (Chiellini *et al.*, 1999).

1.3.4.2.4 Aerobic biodegradation of PVA in aqueous environment

Since PVA is a water-soluble polymer, many investigations have been carried out in aqueous media (Porter and Snider, 1976) and significant levels of

biodegradation were observed in the presence of acclimated PVA-degrading microorganisms (Chiellini *et al.*, 2003). Only moderate limited biodegradation of PVA-based blown films was detected (Chiellini *et al.*, 1999).

1.3.4.2.5 Biodegradation of PVA under anaerobic condition

An investigation of the anaerobic biodegradability of PVA was carried out by Matsumura *et al* (1993), using anaerobically preincubated microorganisms obtained from both river sediments and activated sludge from municipal sewage plants. Analysis demonstrated that PVA with low and high molecular weights were efficiently biodegraded, although low molecular weights were degraded more quickly preferentially by the anaerobic microorganisms (Matsumura *et al.*, 1993).

1.3.4.3 PVA blended materials

Due to the poor moisture and thermal resistance of some biodegradable polymers several methods have been applied to enhance their desired properties. Among the existing synthesized polymers, PVA possesses many useful properties, such as excellent chemical resistance, optical and physical properties (Sionkowska *et al.*, 2009), good film-forming capability, water solubility (Cinelli *et al.*, 2006) and excellent biocompatibility (Jiang *et al.*, 2004; Wan *et al.*, 2002). In a number of studies, PVA blended with other biodegradable polymers has been shown to enhance degradation. The most widely used filler of this kind is starch (Imam *et al.*, 2005; Zhao *et al.*, 2005) and is widely available at low cost making it an economically attractive filler not only for PVA (Pseja *et al.*, 2006) but also polyethylene and other plastics (Griffin, 1994; Kim *et al.*, 2003). Strength, flexibility and water resistance of starch products improved when PVA aqueous

solution was added (Cinelli *et al.*, 2006). For example, El-Mohdy (2007) modified blends of PVA (wheat starch or thermoplastic starch) by electron beam irradiation and showed that the presence of amylopectin increased water solubility. In general, starch is chemically modified in PVA blends, for example by etherification and methylation. Chemical modification of starch in most cases leads to markedly improved physico-chemical properties of PVA blends, but presents higher manufacturing costs (Julinova *et al.*, 2008). PVA blended with starch has been used as a new material for packaging (Fernandes *et al.*, 2006), whereas PVA blends with collagen can be used in biomedical applications (Sarti & Scandola, 1995; Fernandes *et al.*, 2006). However, as polysaccharides such as starch and dextran dissolve readily in water, they do not present mechanical and shape stabilities in fluids (Shi *et al.*, 2008). PVA blends with poly (acrylic acid) are attractive materials for use as a polymer electrolyte (Yang *et al.*, 2008). Moreover, PVA is widely used in the preparation of composites containing inorganic particles, such as perlite, montmorillonite, and graphite (Tian & Tagaya, 2008; Kaczmarek & Podgorski 2007; Sionkowska *et al.*, 2009).

In summary, PVA is a synthetic water soluble polymer that has found an increasing market in textiles and paper industries and more recently as a low oxygen permeable film and in building composites. As a result, PVA manufacture and use has grown over the last 10 years and environmental contamination is growing. Most studies however, have found PVA degradation in the environment to be relatively slow and only a limited number of bacterial species have been reported to be capable of degrading PVA as a sole carbon source. To date, the

potential of fungi to degrade PVA has been largely ignored and the overall aim of this study was to assess the overall potential of fungi in soils and compost to degrade PVA and to study the impact of PVA on fungal communities.

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1.5 EXPERIMENTAL AIMS

As little previous work has focused on fungi in relation to PVA degradation, this research investigates the diversity and extent of PVA degrading fungi in the environment and the influence of PVA on natural soil communities. This is addressed through the presentation of three results chapters which are organised in paper style and are outlined below.

- To determine the potential and the number of fungal species capable of degrading PVA in a range of environmental soils.

This aim is addressed in paper one. Broth culture was used to enrich fungal PVA degraders from a range of environmental soils to determine the capacity of fungi to degrade PVA and isolates identified by rDNA ITS sequencing.

- To investigate the rate and extent of degradation of PVA films in compost and to determine the principal fungal species involved in this process.

This aim is addressed in paper two. Fabricated films of PVA were buried in soil and compost and the rate of degradation determined over time.

- To investigate the effect of PVA contamination on the structure and diversity of fungal communities in soil and compost over time.

This aim is addressed in paper three. PVA was mixed with soil and compost and the influence of the presence of PVA over time on the fungal community structure

was evaluated using TRFLP which enables changes in microbial communities to be monitored in a culture independent manner.

1.6 ALTERNATIVE FORMAT

This PhD research is presented in an alternative style format according to the rules and regulations of the University of Manchester. The three results chapters are presented in the style of the intended journal of publication. However, some elements have been formatted for the purpose of the presentation of this thesis.

Below are summary of the three papers and the relative contributions of the authors.

Chapter 2:

Title: Isolation and characterization of polyvinyl alcohol degrading fungi from soils.

Authors: Somayeh Mollasalehi, Pauline S. Handley, Geoffrey D. Robson

Contribution: The work presented in this paper is wholly my own. Dr P Handley and Dr G Robson were my joint supervisors and helped with discussions during this research and to revise and provide comments on the draft paper I presented to them which I incorporated into the final version. The work was a continuation of a previous project carried out by an MPhil student, however they did not contribute to any of the research or the results presented.

Chapter 3:

Title: Fungal biodeterioration of polyvinyl alcohol films.

Authors: Somayeh Mollasalehi, Pauline S. Handley, Geoffrey D. Robson

Contribution: The work presented in this paper is wholly my own. Dr P Handley and Dr G Robson were my joint supervisors and helped with discussions during this research and to revise and provide comments on the draft paper I presented to them which I incorporated into the final version.

Chapter 4:

Title: Isolation and characterization of polyvinyl alcohol degrading fungi from soils.

Authors: Somayeh Mollasalehi, Pauline S. Handley, Geoffrey D. Robson

Contribution: The work presented in this paper is wholly my own. Dr P Handley and Dr G Robson were my joint supervisors and helped with discussions during this research and to revise and provide comments on the draft paper I presented to them which I incorporated into the final version. Dr Ashley Houldon provided advice on the post-analysis of the TRFLP data.

2 CHAPTER TWO

ISOLATION AND CHARACTERIZATION OF POLYVINYL ALCOHOL DEGRADING FUNGI FROM SOILS

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ABSTRACT

Polyvinyl alcohol (PVA) is a water soluble polymer that has a wide range of industrial applications principally in paper coating, textile sizing and in agriculture. Consequently, large quantities are discharged into the environment, contaminating water courses and soils. While regarded as biodegradable, biodegradation in the environment appears to be restricted and only a few bacterial species and consortia have been characterised that are capable of degrading PVA. Few studies have investigated the role of fungi in biodegradation. In this study, we used culture enrichment to isolate fungal degraders from eight soil samples which were shown to have very different fungal populations and dominant species revealed by denaturing gradient gel electrophoresis (DGGE). While all soils contained fungal degraders, the number of recovered species was restricted with the most common being *Galactomyces geotrichum* and *Trichosporon laibachii*. One thermophilic strain, *Talaromyces emersonii* was recovered at 50°C. Mean doubling times of the principle degraders varied from 6.9 h⁻¹ to 11.9 h⁻¹ on minimal medium with PVA as the sole carbon source. For *G. geotrichum*, a molecular weight range of 13-23 KDa, 30-50 KDa or 85-124 KDa had no significant effect on the growth rate (mean doubling time 6.3 to 6.9 h⁻¹) although there was an increased lag phase for the higher molecular weight PVA.

2.1 INTRODUCTION

Polyvinyl alcohol (PVA) is a water-soluble polymer which has recently attracted interest for the manufacture of biodegradable plastic materials (Solaro *et al.*, 2000). PVA is produced by the hydrolysis of polyvinyl acetate following polymerisation of vinyl acetate and has many valuable physicochemical properties including thermostability, solvent tolerance, high viscosity, flexibility, tensile and adhesive strength (Shimao, 2001; Kawai and Hu, 2009). PVA is widely used as a paper coating, in adhesives and films, as a finishing agent in the textile industry and in forming oxygen impermeable films (Amange and Minge, 2012; Chiellini *et al.*, 2003; Kawai and Hu, 2009; Larking *et al.*, 1999; Yamatsu, *et al.*, 2006). Due to the increasing utilization of PVA, large amounts of PVA are discharged into water systems every year (Kawai and Hu, 2009). Consequently, as a result of disposal and slow rate of biodegradation, waste water can contain a considerable amount of PVA that can contaminate the wider environment (Lee and Kim, 2003; Tang *et al.*, 2010). Despite its growing use, relatively little is known about degradation in the environment and the overall number of known PVA degrading microorganisms is relatively limited (Chiellini *et al.*, 2003; Corti *et al.*, 2002a; Zhang, 2009; Tang *et al.*, 2010).

The first PVA degrader to be characterized was the filamentous fungus *Fusarium lini* (Nord, 1936) although subsequently, the most common PVA degrading microorganisms reported are gram negative bacteria belonging to the genera *Pseudomonas* and *Sphingomonas* (Suzuki *et al.*, 1973; Kawagoshi *et al.*, 1997; Kawagoshi *et al.*, 1998; Lee and Kim, 2003; Kawai and Hu, 2009). Soils are

a rich microbial environment and fungi play a pivotal role in the decomposition of dead plant and animal tissue and a major role in nutrient recycling (Moore *et al.*, 2011). Due to the ability of fungi to secrete a diverse array of extracellular hydrolytic enzymes that degrade naturally occurring polymers in the environment, fungi are increasingly being recognized as playing an important role in the microbial biodegradation of several major synthetic polymers (Sabev *et al.*, 2006). For example, *Nectria gliocladioides*, *Penicillium ochrochloron* and *Geomyces pannorum* were reported to be the principal colonizers and degraders of soil-buried polyurethane (Barratt *et al.*, 2003; Cosgrove, 2007; 2010). While some fungi have been reported to be capable of degrading PVA, these studies are limited, have employed PVA blended with other materials such as starch or pre-treated PVA with an oxidising agent (Jecu *et al.*, 2010; Larking *et al.*, 1999; Qian *et al.*, 2004, Tsujiyama *et al.*, 2011). It is therefore unclear as to whether the ability of fungi to degrade PVA in the environment is widespread or limited to their potential contribution PVA degradation.

This study investigated the diversity and extent of PVA degrading fungi in the environment by enriching and isolating PVA degraders in broth culture using eight different soils from differing environments. We report that all soils contained a limited number of PVA degrading species that were enriched in PVA broth, with *Geomyces geotrichum* being the most commonly isolated. This suggests that while fungi are present in a wide range of environmental soils that are capable of degrading PVA, the number of species appears to be limited.

2.2 MATERIALS AND METHODS

2.2.1 Strain maintenance

Strains isolated in this study were routinely grown on potato dextrose agar (Formedium, UK) at 25°C. After confluent growth, spores were harvested in 0.05% (v/v) Tween 80 and filtered through four layers of sterile lens tissue (Whatman, UK). For long term storage, spore suspensions were mixed with an equal volume of glycerol and store at -80°C.

2.2.2 Soil samples

A total of eight soils were utilised in this study: two commercial, and six collected from a number of different sites in the UK (Table 2.1). Soils were stored in polythene bags at room temperature until required for a maximum of three month.

2.2.3 PVA samples

PVA of different molecular weights (13 – 23 KD, 30-50 KD and 85-125 KD) used in this study were obtained from Sigma-Aldrich, UK.

2.2.4 Growth of fungi in liquid culture

1 ml of ca. 1×10^6 spores/ml determined by using a haemocytometer was used to inoculate 50 ml of minimal medium (Crabbe *et al*, 1994) containing PVA or glucose as the sole carbon source in 250 ml flasks and incubated at 25°C on a rotary shaker (200 rpm) for up to 2 days. Growth was measured periodically by determining absorbance at 520 nm. Dry weights were determined by collecting biomass under vacuum onto Whatman no 1 filter paper and drying at 80°C to constant weight.

2.2.5 Enrichment of PVA degraders from soils

Three replicate 250 ml shake flasks containing 50 ml minimal salts medium (Crabbe *et al*, 1994) and 2 % (w/v) PVA (MW range 13-23 kD, Sigma, UK) as sole carbon source at pH 5.5 were each inoculated with 4 g of soil from different environments. Flasks were incubated on a rotary shaker (200 rpm) at 25°C for 7 days and 2 ml was transferred into fresh medium and incubated for a further 7 days. Minimal medium was prepared by adding 20 ml of 50X phosphate buffer (25 g of KH_2PO_4 and 50 g of K_2HPO_4 per litre, pH 5.5) to 970 ml of distilled water containing 1 g $(\text{NH}_4)_2\text{SO}_4$ and PVA (20g) and sterilised at 121°C for 15 min. When agar medium was required, 15 g agarose (Melford laboratories, UK) was included. After cooling, 10 ml of sterile (121°C, 15 min) 100X $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (50 g l^{-1}) solution and 1 ml of a filter sterilized (0.22 μm , Millipore, UK) 1000X trace elements solution (per litre; 150 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 27 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 26 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 22 mg ZnCl_2 , 28 mg of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ and 2 g of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$) was added. To enumerate and isolate PVA fungal degraders, samples were serially diluted and 0.1 ml spread evenly across the surface of 2% (w/v) PVA minimal medium agar plates (1.5 % w/v agarose) containing 50 $\mu\text{g ml}^{-1}$ chloramphenicol. As colony morphology on PVA media was insufficient to distinguish different colony morphotypes, colonies were transferred onto potato dextrose agar (PDA, Formedium, UK) and grouped by colony phenotype.

2.2.6 Determination of PVA concentration

PVA concentration in liquid media was determined in liquid cultures using an iodine-boric acid based colorimetric assay (Joshi *et al*, 1979) and quantified by

comparing to a standard PVA calibration curve (Appendix 2.1).

2.2.7 DNA extraction from fungal mycelia

Liquid cultures were harvested 48 h after inoculation and by centrifugation at 8000 x g for 15 min. The supernatant was discarded and the pellet flash frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, UK) according to the manufacturer's instructions and stored at -20°C until required.

2.2.8 Identifying the isolated strains using rDNA sequencing

Isolated fungi were identified by PCR amplification and DNA sequencing of nuclear ribosomal 5.8S DNA and internal transcribed spacer (ITS) regions using the fungal universal primers ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') as previously described (Webb *et al.*, 2000). PCR reagent concentrations were 0.25 µM of primers ITS-1 and ITS-4, 2 mM MgCl₂, 200 µM of each of the four dNTPs, 5 µl of 10 x NH₄ reaction buffer and 1.25 units of BIOTAQ DNA Polymerase (Bioline, UK) per 50 µl PCR reactions. Amplifications were carried out for 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, UK) according to the manufacturer's instructions and sequenced in house. Forward and reverse sequences were aligned and consensus sequence used to interrogate the NCBI (National Centre for Biotechnology Information) nucleotide database using the blastn algorithm.

2.2.9 DNA extraction from soil

Genomic DNA from soil was extracted using a PowerSoil DNA isolation Kit (MOBIO Laboratories, UK) according to the manufacturer's instructions, purified using a QIAquick PCR Purification Kit (Qiagen, UK) according to the manufacturer's instructions and stored at -20°C until required.

2.2.10 Denaturing gradient gel electrophoresis (DGGE) analysis

DGGE was used to compare the diversity of the fungal communities from the different soil samples alongside the individual isolated fungi. 50 ng of genomic DNA was used as the template for PCR amplification using primers GM2 (5'-CTGCGTTCTTCATCGAT-3') and B206c (5'-CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGGGAA GTAAAAGTCGTAACAAGG-3'), amplifies the ITS1- region of the fungal rDNA gene as previously described (Cosgrove *et al.*, 2007, 2010). PCR reactions contained 10 µM of primers GM2 and JB206c, 1.5 mM MgCl₂, 0.2 mM of each of the four dNTPs, 5 µl of 10 x NH₄ reaction buffer and 0.1 mg/ml bovine serum albumin per 50 µl PCR reaction. Amplification was carried out in 94°C for 5 min for initial denaturation; 20 'touchdown' cycles of 94 °C for 30 s, annealing for 30 s at 59°C to 49.5°C with annealing temperature being reduced by 0.5°C per cycle, extension at 72°C for 45 s; 30 cycles of denaturation at 95°C for 30 s, annealing at 49°C for 30 s and extension at 72°C for 45 s; and final extension at 72°C for 5 min. PCR products were separated by DGGE using a D-Code universal mutation detection system (Biorad, U.K.). DGGE gels measuring 16 cm x 16 cm x 1 mm contained 10% (v/v) bis-acrylamide in 1 x TAE (40 mM Tris base, 20 mM glacial

acetic acid, 1 mM EDTA). A perpendicular gel with a denaturant gradient of 25-55% was used. For all gels, 500 µg of PCR product was used per lane; gels were run in 1 X TAE buffer at a constant temperature of 65°C for 16.5 h at 42 volts. Gels were stained with SybrGold (Molecular Probes, Netherlands) for 1 h, washed for 10 min in 1 x TAE buffer and photographed under ultraviolet light.

2.3 RESULTS

2.3.1 Selection and identification of the dominant fungal PVA degrading strains from soils

In order to isolate putative PVA degrading fungi from soils, soils from different locations were used to inoculate minimal liquid medium containing PVA as the sole carbon source (Table 2.1). After incubation at 25°C for one week, 2 ml of each culture was used to inoculate a second flask of PVA medium to further enrich for putative PVA degrading fungi. A total of eight different soils, two commercial and six environmental, were used as the source of inoculum (Table 2.1.). Culture medium from the second enrichment flasks was serially diluted and plated onto PVA agar medium and viable counts (colony forming units) determined on these plates for seven consecutive days at 25°C (Fig 2.1). The number of colony forming units (CFU) detected increased up to day four but then remained stable. The total CFU following enrichment was similar regardless of the soil inoculum used and ranged from 1.7×10^6 to 7.9×10^6 ml⁻¹. To investigate if incubation temperature affected species enrichment on PVA medium, PVA broth was inoculated with commercial top soil and incubated at 25°C, 37°C, and 50°C (to isolate thermophiles) for one week and again used to inoculate a second PVA medium broth. Culture medium from the second flask was serially diluted after one week and plated onto PVA agar medium and incubated over seven days at the same temperature to determine CFU (Fig 2.2). In order to determine the efficiency of the enrichment procedure on PVA medium, culture broth from the second flask was also plated onto PDA medium (general fungal growth medium) and the total CFU's compared to the total CFU's obtained on PVA medium. The number of CFU's on

both media increased up to day four and then remained constant. The number of CFU's recovered from 25°C and 37°C on PVA medium were not significantly different, however, CFU's from 50°C was significantly lower ($P < 0.05$). When the CFU's on PVA medium were compared to PDA medium, the CFU's were found to be ca. 10% lower on PVA medium, indicating that growth on PVA medium was highly selective for PVA degrading fungi.

When culture broths were plated onto PVA media, fungal colony morphologies were similar due to lack of pigmentation and sporulation and therefore difficult to distinguish on this medium. In order to establish if individual colonies had the same or different morphologies, individual colonies were transferred onto potato dextrose agar (PDA) plates and incubated for 5 days. On this medium, pigmentation, sporulation and colony phenotypes enabled different morphotypes to be distinguished. A total of 27 different morphotypes were observed from all the enrichment cultures and at least three representatives of each morphotype were isolated and the ITS rDNA amplified using the ITS-1 ITS-4 primer set (Webb *et al*, 2000) to enable identification by comparison with the NCBI database. Amplified rDNA sequence lengths ranged from 308-551 bp for the 5.8S rDNA and ITS regions and identities against the NCBI database ranged from 98 – 100 % (Table 2.1, Fig 2.3). In many instances, the morphological differences were small and sequencing data indicated that they were the same species. For example, isolates C11, C21, C31, C51 and C61 differed slightly in their morphology but were all identified as *Galactomyces geotrichum* with identical ITS

sequences. In total twelve different fungal species were isolated as putative PVA degraders (Appendix Fig A2.4).

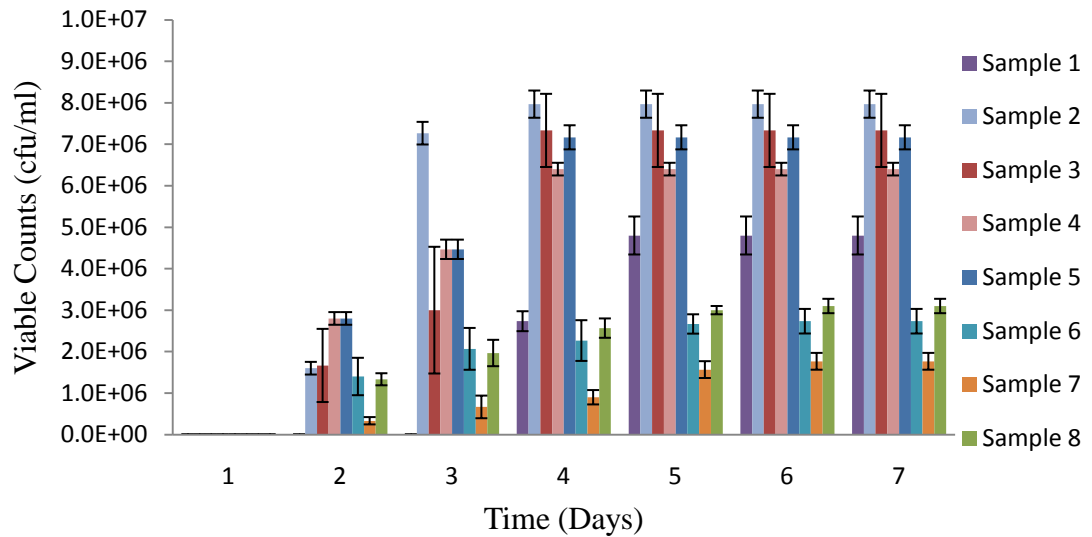


Figure 2-1. Changes in viable counts of putative PVA degrading fungi recovered from PVA broth cultures (13-23 MW, DH: 98%) inoculated with different environmental soils over 7 days at 25°C. PVA broth was inoculated with different soil inoculum and incubated at 25°C for one week and used to inoculate a second PVA broth culture and incubated a further week. Following the second incubation, PVA broth cultures were serially diluted and plated onto PVA agar and incubated at 25°C. Total colony forming units were counted over seven successive days. Results represent the means of triplicate samples \pm standard error of the mean (SEM). Soil sample details (source) are shown in Table 2.1

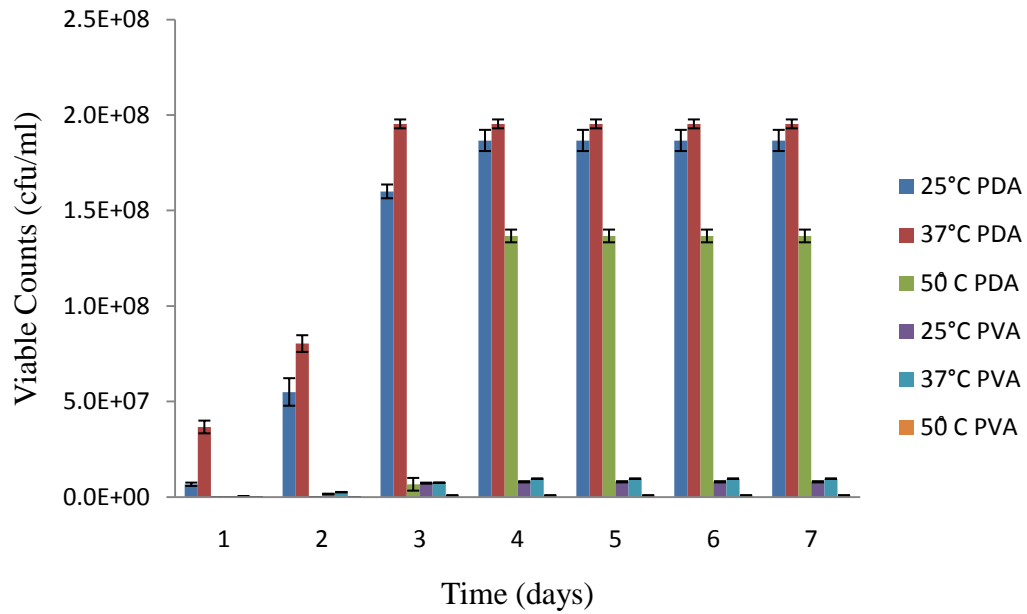


Figure 2-2. Changes in viable counts of putative PVA degrading fungi from PVA broth (13-23 MW, DH: 98%) inoculated with top soil (sample 2) at 25°C, 37°C and 50°C over 7 days. Results are the means of triplicate samples \pm standard error of the mean (SEM).

Soil sample	GPS coordinates	Isolate code	Temp (°C)	Strain (alternative name/anamorph)	Accession number & Percentage homology to closest match
Sample 1. Ashton, Manchester (Urban soil, pH 4.5).	53.283247,- 2.125071	S11	25	<i>Galactomyces geotrichum</i>	JX847746 (98%)
		S21	25	<i>Geotrichum candidum</i>	JX847747 (99%)
				<i>Galactomyces geotrichum</i>	
Sample 2. Top soil, IdealRange, UK (Commercial soil, pH 5.5).	NA	C11	25	<i>Galactomyces geotrichum</i>	JX847740 (99%)
		C21	25	<i>Galactomyces geotrichum</i>	JX847743 (99%)
		C31	25	<i>Galactomyces geotrichum</i>	JX847741 (100%)
		C51	25	<i>Galactomyces geotrichum</i>	JX847744 (99%)
		C61	25	<i>Galactomyces geotrichum</i>	JX847745 (99%)
		C41	25	<i>Trichosporon laibachii</i>	JX847742 (99%)
		S91	25	<i>Trichoderma viride</i>	JX847765 (99%)
		S61	25	<i>Umbelopsis isabellina</i>	JX847760 (100%)
		S371	37	<i>Mortierella isabellina</i>	JX847761 (99%)
		S375	37	<i>Candida tropicalis</i>	JX847763 (99%)
		S381	37	<i>Candida tropicalis</i>	JX847762 (99%)
	S502	50	<i>Issatchenkia orientalis</i> (<i>Candida krusei</i>)	JX847764 (99%)	
			<i>Talaromyces emersonii</i>		
Sample 3. University of Manchester (Urban soil, pH 4.5).	53.46471,- 2.228283	P11	25	<i>Galactomyces geotrichum</i>	JX847759 (96%)
Sample 4. Whitworth park, Manchester, (Grass land, pH4.2).	53.458697,- 2.228165	W11	25	<i>Trichosporon laibachii</i>	JX847757 (99%)
		W21	25	<i>Trichosporon laibachii</i>	JX847757 (100%)
Sample 5. Huddersfield (Deciduous woodland, pH 6).	53.604834,- 1.700279	H11	25	<i>Trichosporon laibachii</i>	JX847750 (99%)
		H21	25	<i>Fimetariella rabenhorstii</i>	JX847751 (99%)
		H31	25	(<i>Sordaria rabenhorstii</i>) <i>Coniochaeta ligniaria</i>	JX847752 (99%)
Sample 6. Holme Moss (Upland peat, pH 4.9).	53.531087,- 1.853095	O14	25	<i>Fimetariella rabenhorstii</i>	JX847755 (98%)
		O21	25	<i>Fimetariella rabenhorstii</i>	JX847756 (99%)
Sample 7. Malham Cove (Limestone grassland, pH 6).	54.067674,- 2.161345	MN21	25	<i>Fusarium oxysporum</i>	JX847754 (100%)
		M11	25	<i>Aspergillus fumigatus</i>	JX847753 (100%)
		M21	25	<i>Fusarium oxysporum</i>	JX847766 (99%)
Sample 8. Atras Edifield (Commercial loam, pH 5.6).	NA	A11	25	<i>Fusarium equiseti</i>	JX847748 (99%)
		A21	25	<i>Fusarium oxysporum</i>	JX847749 (99%)

Table 2-1. Fungal of PVA degraders recovered from eight soil samples by PVA broth enrichment. The percentage homologies to the top hit sequenced strains in the NCBI database are shown.



Figure 2-3. Phylogenetic analysis of the isolated fungal strains and closest matching strains from the NCBI database. Strains isolated and identified in this study are marked with star * or **.

2.3.2 Growth kinetics of putative isolated PVA fungal degraders

In order to investigate to what extent the isolated fungal strains were able to utilize PVA, six putative degraders were grown in minimal medium containing PVA as the sole carbon source and growth measured periodically over time by recording the optical density at a wavelength 520 nm (Fig 2.4). For all strains, negative controls consisting of minimal medium without PVA did not produce an OD over 0.05 indicating no growth in the absence of PVA (data not shown). All of the strains grew on PVA as a sole carbon source though at different rates and with different final optical densities. The specific growth rates and mean doubling times for each strain is shown in Table 2.2 on PVA and glucose for comparison. For all strains, growth on glucose was quicker than growth on PVA. The fastest growing strain on PVA was *Galactomyces geotrichum* with a specific growth rate of 0.10 h^{-1} (doubling time 6.9 h), and the slowest *Trichosporon laibachii* with a specific growth rate of 0.06 h^{-1} (doubling time 11.6 h).

2.3.3 Influence of PVA polymer length on the growth of *G. geotrichum*

As PVA is manufactured with a range of different polymer lengths, *G. geotrichum* was grown on minimal containing PVA as the sole carbon source with molecular weights in the range 13-23 KD, 30-50 KD, 85-124 KD (98 to 99% hydrolysis) to determine if polymer length influenced fungal growth and rate of utilization of PVA. The specific growth rate on all three molecular weight ranges was not significantly different ($P < 0.05$) and PVA concentration in the broth decreased during exponential growth at similar rates (Fig 2.5). But, PVA with

molecular weight 85-124 KD had longer lag phase than PVA with molecular weights 13-23 KD, 30-50 KD. Also, when molecular weight of the PVA increased the final OD decreased indicating that PVA of higher molecular weights were utilised and converted to biomass lower than PVA of lower molecular weight. Although, PVA with different polymer lengths were utilised with of *G. geotrichum* and polymer length was not limiting factor for PVA degradation (Fig 2.5).

2.3.4 Analysis of fungal community diversity in soils by denaturing gradient gel electrophoresis (DGGE)

As culture enrichment on PVA broth led to the isolation of only a small number of fungal species, some of which (e.g. *G. geotrichum*) were isolated from more than one soil, it suggested that either PVA degradation was limited to only a small number of species, or that the fungal communities of the soils were similar with low diversity. Denaturing gradient gel electrophoresis (DGGE) was used to compare the diversity and community profile of the eight soils used in this study (Fig 2.6). In all samples, the profiles clearly indicated that the species composition was highly divergent and that each soil contained different dominant species.

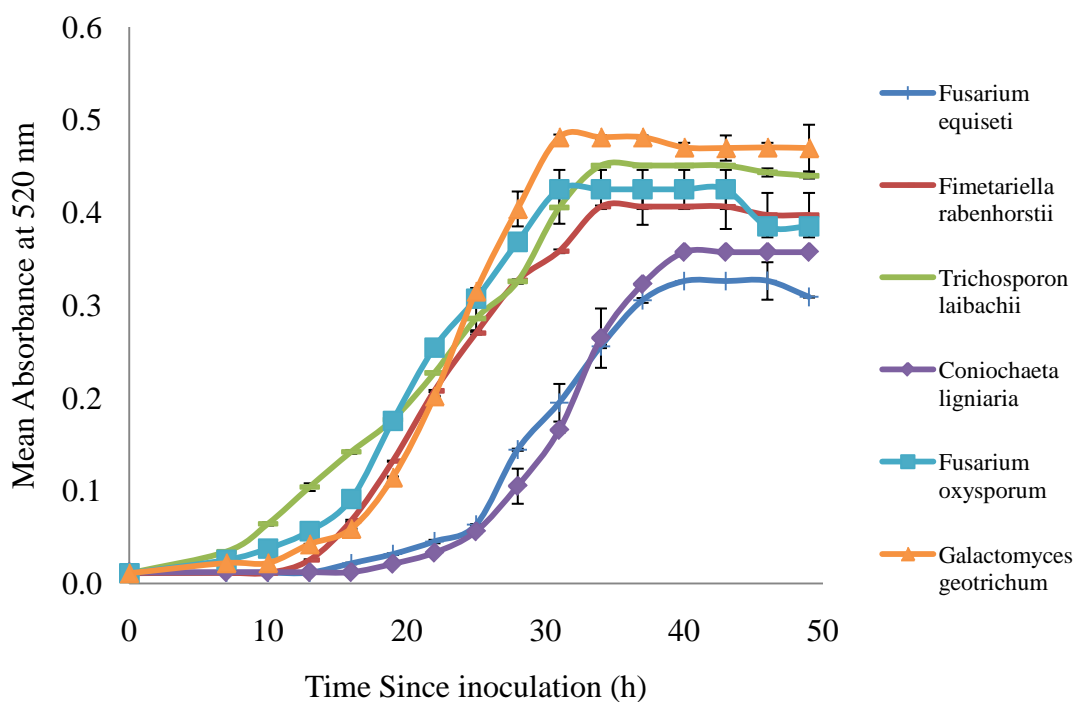


Figure 2-4. The growth of *Galactomyces geotrichum*, *Trichosporon laibachii*, *Fimetariella rabenhorstii*, *Fusarium oxysporum*, *Coniochaeta ligniaria* and *Fusarium equiseti* in 2 % (w/v) PVA (13-23 KD, 98 % DH) liquid minimal medium at 25°C. Data represents the mean of three replicates \pm SEM.

Strain	Specific growth rate (h ⁻¹)		Doubling time (h)		Yield (g/l)	
	PVA	Glucose	PVA	Glucose	PVA	Glucose
<i>Galactomyces geotrichum</i>	0.10 ± 0.01	0.15 ± 0.01	6.9 ± 0.01	4.6 ± 0.01	5.1 ± 0.1	12.3 ± 0.1
<i>Trichosporon laibachii</i>	0.06 ± 0.02	0.10 ± 0.03	11.6 ± 0.02	6.9 ± 0.03	4.9 ± 0.8	11.6 ± 0.2
<i>Fimetariella rabenhorstii</i>	0.07 ± 0.02	0.13 ± 0.02	9.9 ± 0.02	5.3 ± 0.02	4.8 ± 1.1	10.9 ± 0.9
<i>Fusarium oxysporum</i>	0.09 ± 0.03	0.13 ± 0.02	7.7 ± 0.03	5.3 ± 0.02	4.5 ± 0.1	10.5 ± 0.2
<i>Fusarium equiseti</i>	0.08 ± 0.09	0.13 ± 0.02	8.7 ± 0.06	5.3 ± 0.02	3.4 ± 1.0	10.1 ± 0.1
<i>Coniochaeta ligniaria</i>	0.10 ± 0.07	0.12 ± 0.02	6.9 ± 0.02	5.8 ± 0.02	2.9 ± 0.4	9.4 ± 0.1

Table 2-2. Comparison of the specific growth rate, mean doubling time and yields of selected PVA degraders grown on PVA or glucose as the sole carbon source. Strains were grown in liquid minimal medium containing 2 % (w/v) PVA (MW 13-23 KD) or 1 % (w/v) glucose at 25°C for up to 50 h. Specific growth rates and doubling times were calculated from the growth curves and yields from dry weights after 50 h. Data represents the means of three replicates ± SEM.

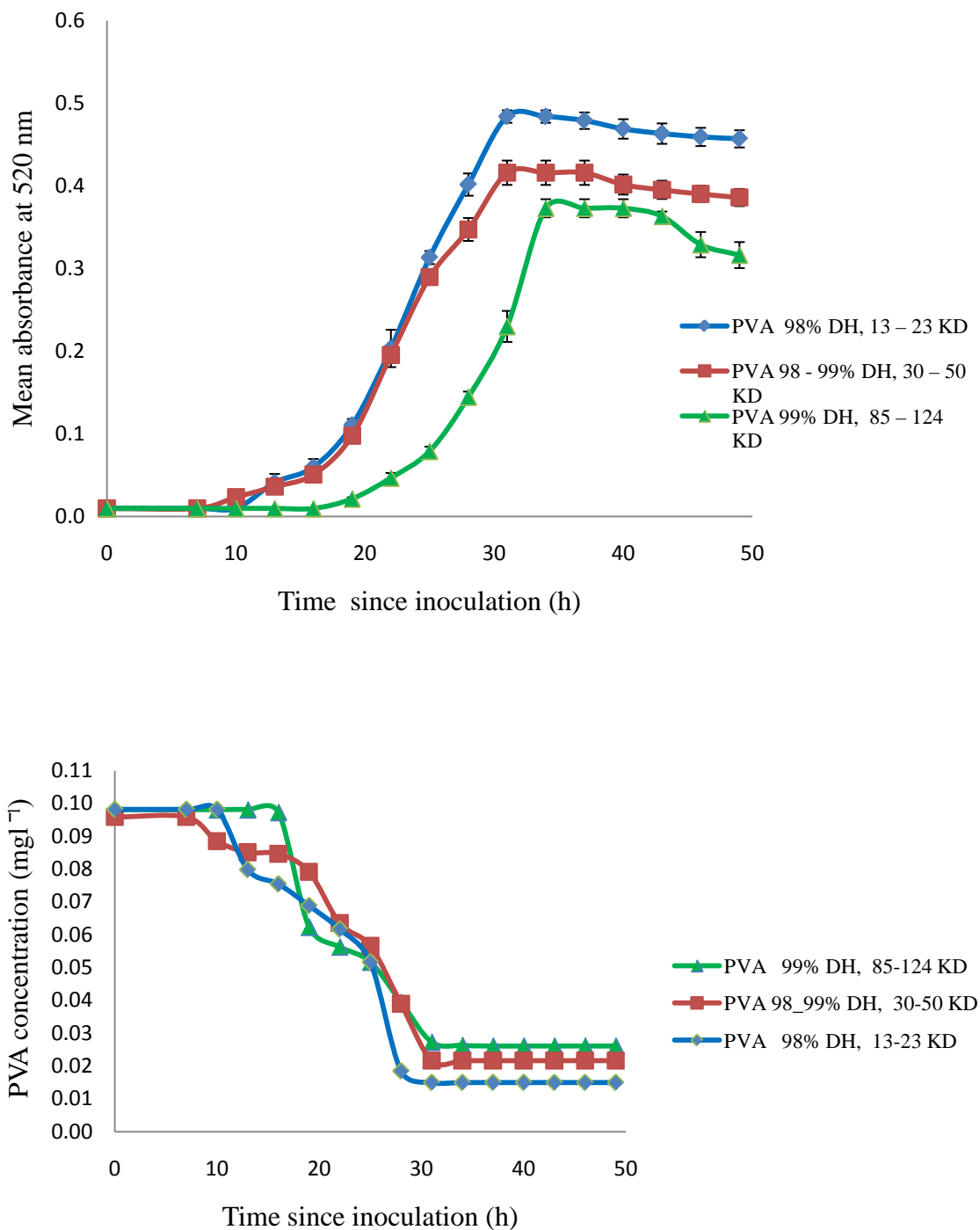


Figure 2-5. The growth curves of *G. geotrichum* in 2% (w/v) PVA (13-23 KD; 30-50 KD; 85-124 KD) minimal salts medium. *G. geotrichum* was grown in shake flask culture at 25°C for 50 h and the optical density (upper) and PVA concentration (lower) determined periodically by colorimetric method. Data represents the mean of three replicates \pm SEM.

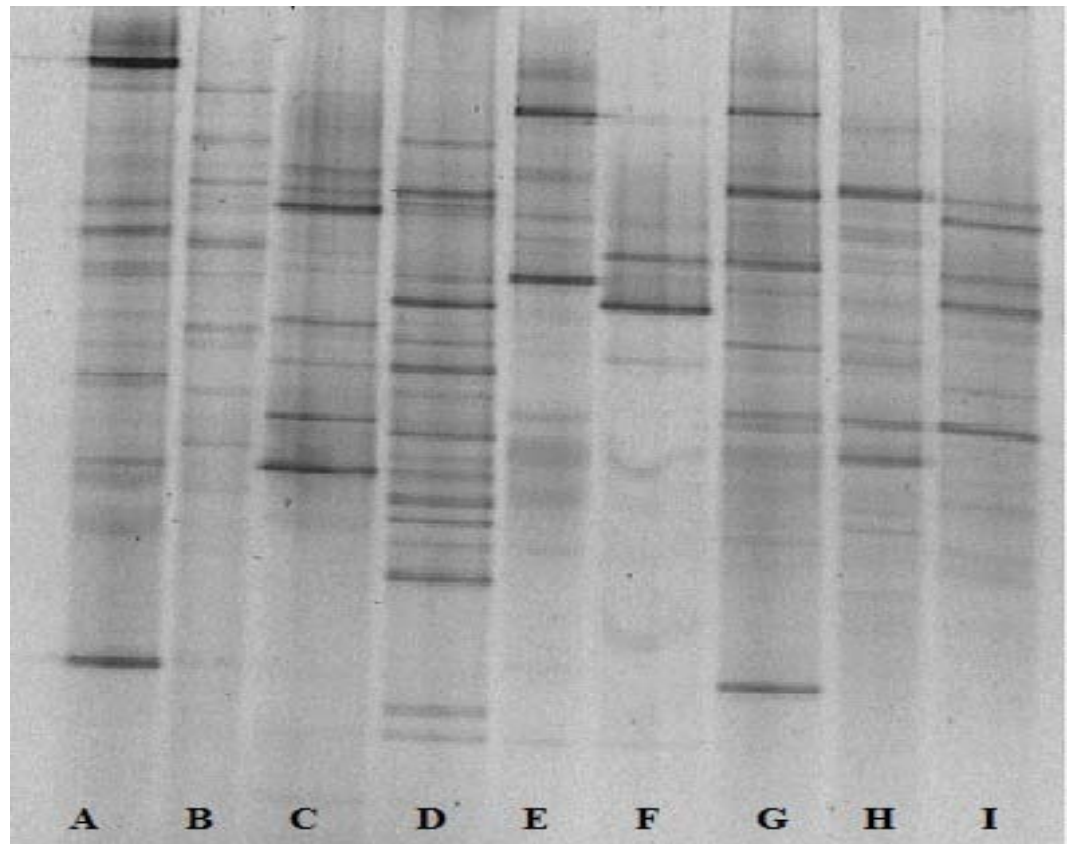


Figure 2-6. Fungal community profile of studied soils using DGGE. The diversity of the soils used for enriching PVA degraders were analysed by DGGE to determine the diversity and similarities of the fungal communities. Lane A=Atras Edifield (sample 8), lane B=Malham Cove (sample 7), lane C=IdealRange top soil (sample 2), lane D=The Compost Shop (compost), lane E=Holme moss (sample 6), lane F=Whitworth park (site 4), lane G=Ashton, Manchester (sample 1), lane H=Stopford garden (sample 3), lane I=Huddersfield (sample 5).

2.4 DISCUSSION

Previously, PVA degrading bacteria have been isolated from PVA contaminated soils, waste water and activated sludge (Solaro *et al.*, 2000; Lee & Kim, 2003; Chen *et al.*, 2007; Zhang, 2009) and the enzymes involved in the process of biodegradation have been identified (Chiellini *et al.*, 2003). Relatively few studies have been conducted on fungal species with *Geotrichum* sp (Mori *et al.*, 1996), *Fusarium lini* (Nord, 1936), *Pycnoporus cinnabarinus* (Larking *et al.*, 1999), *Phanerochaete chrysosporum*, (Mejia *et al.*, 2003), a *Penicillium* sp (Qian *et al.*, 2004) and *Flammulina velutipes* (Tsujiyama *et al.*, 2011) reported to be capable of degrading PVA. While a study of a number of tested fungal species including *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus flavus*, and *Aureobasidium pullulans* were reported to grow on PVA films, these were blended with starch and glycerol and no evidence for PVA degradation was presented (Jecu *et al.*, 2010). In this study, we isolated a number of fungal strains capable of degrading PVA as a sole carbon source from uncontaminated soil from eight different sites. The most frequently isolated fungal strains were identified as *Galactomyces geotrichum* (8 strains), *Trichosporon laibachii* (4 strains), *Fimetariella rabenhorsti* (3 strains) and *Fusarium oxysporum* (3 strains) and each was isolated from at least two of the sites. Thus, while PVA degrading fungi could be enriched from uncontaminated soils, only a limited number of PVA degrading fungal species were recovered despite DGGE confirming a diverse and different community profile in all of the eight sites tested. Therefore, as in the case of bacteria, PVA degradation is limited to only a relatively small number of fungal species. However, it has been estimated that only ca. 17% of fungal species can be readily grown in the laboratory (Hawksworth,

1991) and in addition, minimal medium was employed in this study to provide a strong selection pressure, further limiting the number of fungi that could potentially be recovered. Thus, the diversity of PVA degrading fungi in soils may be much higher. Nonetheless, as the most dominant and common soil fungi including *Aspergillus* spp. and *Penicillium* spp. were not enriched on PVA medium, it suggests that the ability to utilise PVA is not a common feature of filamentous fungi, perhaps reflecting that only a limited number of species have the enzymatic capability to degrade this polymer. Interestingly, a *Geotrichum* (*Galactomyces*) sp. was previously isolated from activated sludge from a textile factory in Japan as a fungal component of a PVA degrading microbial consortium. However, it appears that this strain was only capable of degrading polyvinyl oligomers released by PVA degrading bacteria rather than directly degrading PVA (Mori *et al*, 1996). As *G. geotrichum* was one of the most frequently isolated strains in this study, it suggests that *Galactomyces* sp. in general may have PVA or PVA oligomer degrading potential.

In order to further investigate the extent of fungal PVA degraders in soil, comparative enrichments were conducted for one soil at 25°C, 37°C and 50°C. 50°C was used for the selection of thermophilic fungal PVA degraders. The highest number of PVA degrading isolates was recovered at 25°C (23 strains) with only 3 strains recovered at 37°C. Only one thermophilic PVA degrader (*Talaromyces emersonii*) was recovered at 50°C. Thermophilic PVA bacterial degraders (*Geobacillus tepidamans*, *Brevibacillus brevis* and *Brevibacillus limnophilus*) have previously been recovered from PVA contaminated hot waste water from fabric

dyeing factories (Kim and Yoon, 2010).

To quantify and confirm the growth of putative PVA degraders on PVA as a sole carbon source, the specific growth rates of six degraders, including the most frequently isolated strains were determined in liquid cultures. While the specific growth rates of all strains were lower than that obtained on using glucose as a sole carbon source, nonetheless all strains showed relatively rapid growth on PVA with doubling times ranging from 6.9 h to 11.6 h. Previous studies on bacterial PVA degraders have reported little or no impact of PVA molecular weight on the rate of degradation (Corti *et al*, 2002a; 2002b; Fukae *et al*, 1994; Hatanaka *et al*, 1995; Solaro *et al*, 2000; Suzuki *et al.*, 1973) although differences in the mineralization profiles were apparent with different degrees of PVA hydrolysis (Corti *et al*, 2002a). In this study, the growth rate of the dominant isolated fungal strain *Galactomyces geotrichum* was not affected by the molecular weight of the polymer with similar specific growth rates (ca. 0.10 h⁻¹) on PVA with molecular weight ranges of 13-23 KDa, 30-50 KDa, and 85-124 KDa. Likewise, the rates of PVA utilisation appeared similar. However, for the highest molecular weight range, the lag phase was longer, potentially due to a longer time required for polymer degradation.

In this study we found that all of the eight soils contained at least one fungal species capable of degrading PVA indicating that while the potential for PVA degradation by fungi in soils is widespread, the number of fungi may be limited. Given the limited number of species in the soil, PVA contamination of soil may exert a strong selective pressure for those PVA degraders present. Due to the

limitations of culture based studies to accurately reflect changes in the fungal population in natural environments, further work studying the impact of PVA using non-culture-based molecular approaches would be valuable in determining the likely impact of PVA contamination of soils.

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APPENDIX 2.1

Spectrophotometric determination of PVA concentration in growth samples

In order to quantify the concentration of PVA during the growth of *G. geotrichum*, a colorimetric method was used based on the interaction between PVA and iodine in the presence of boric acid (Finley, 1961; Tebelev *et al.*, 1965; Pritchard and Akintola, 1972; Min *et al.*, 2012). This reaction produces a colour change from yellow to green in corresponding to PVA concentration (Fig A2.1) and a calibration curve constructed (between 0 and 0.1 mg ml⁻¹) by measuring the absorbance at 690 nm (Fig A2.2). The calibration curves obtained with PVA of molecular weight range 13-23 KD, 30-50 KD or 85-124 KD were identical (results not shown). This enabled the concentration of PVA in the culture broth to be determined during the growth of *G. geotrichum* with PVA as the sole carbon source (Fig 2.5).



Figure A2.1. Colour change observed with increasing concentrations of PVA from yellow to green in a colorimetric assay of PVA (0 to 0.1 mg ml⁻¹).

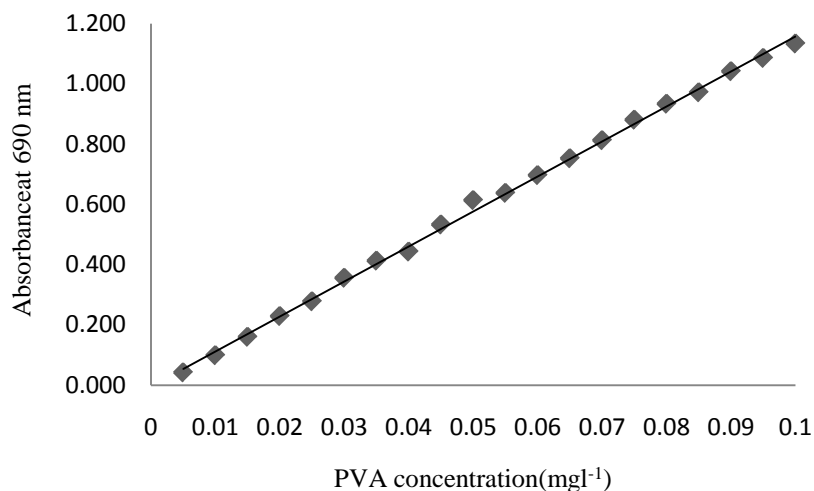


Figure A2.2. Calibration curves of PVA (MW 13-23 KD) using an iodine-based colorimetric assay.

Influence of initial PVA concentration on the stationary phase optical density of *Galactomyces geotrichum*

Our studies confirmed the ability of *Galactomyces geotrichum* to utilize PVA as a carbon source over a range of molecular weights from 13-124 KD to 85-124 KD. However, these experiments were conducted using a PVA concentration of 2% (w/v). In order to study the influence of changing the initial PVA concentration on the final optical density values in the stationary phase, *G. geotrichum* was grown in PVA broth (MW 13-23 KD, 98% hydrolysed) containing 0.5% to 5.0% (w/v) PVA (Fig A2.3). The mean OD₅₂₀ during the stationary phase for *G. geotrichum* was determined during the stationary phase and showed a linear relationship between the initial concentration of PVA and the final

stationary phase OD up to a concentration of 4% (w/v) suggesting that at higher concentrations, a nutrient in the medium other than carbon was limiting.

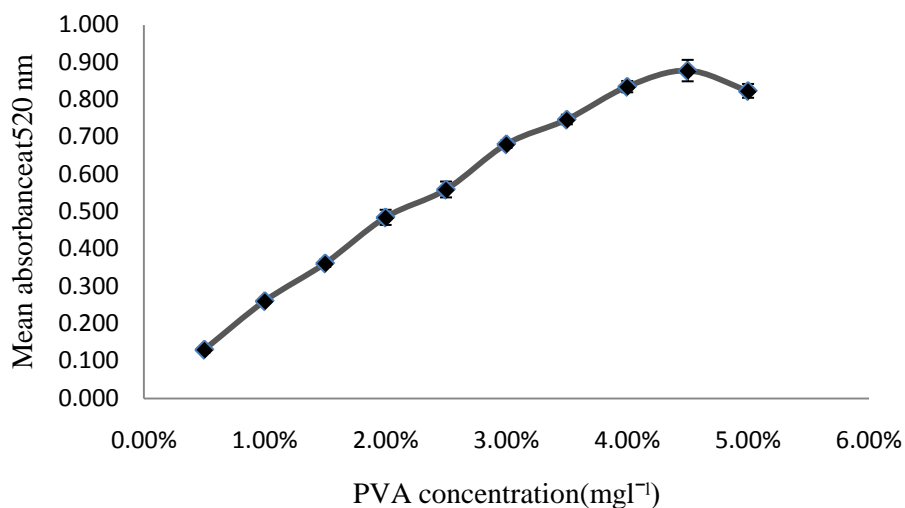


Figure A2.3. Influence of initial PVA concentration on final OD at stationary phase of *G. geotrichum*. *G. geotrichum* was grown in 50 ml PVA liquid culture medium at 25°C on a rotary shaker at 200 rpm until stationary phase (no further increase in OD) and the final OD determined. Data represents the mean of in different range of concentrations and data represent the mean of three replicates \pm SEM.

Fungal morphotypes isolated from soils on PVA agar

Fungi growing on PVA media which were isolated from PVA enrichment broth cultures inoculated using different soils were difficult to distinguish morphologically on this media. Individual colonies were therefore transferred onto potato dextrose agar and grouped into different morphotypes based on morphological similarity prior to ITS sequencing. Different morphotypes were distinguished and are shown in Figure A2.4

A



B



C



D



E



F



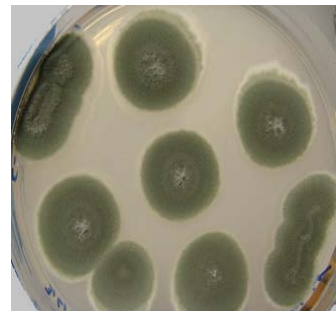
G



H



I



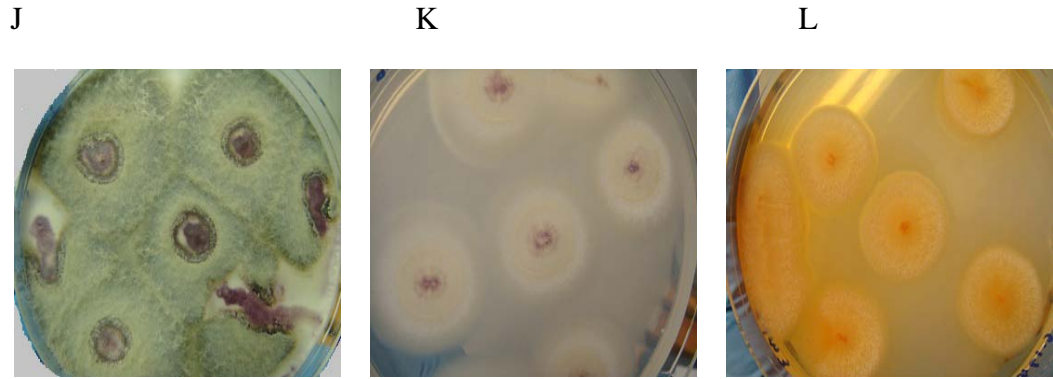


Figure A2.4. Morphological characteristics of dominant fungal strains isolated from PVA enrichment cultures growing on PDA agar plates. A (*Galactomyces geotrichum*), B (*Trichosporon laibachii*), C (*Candida tropicalis*), D (*Fusarium equiseti*), E (*Trichoderma viride*), F (*Issatchenkia orientalis*), G (*Talaromyces emersonii*), H (*Umbelopsis isabellina*), I (*Aspergillus fumigatus*), J (*Coniochaeta ligniaria*), K (*Fimetariella rabenhorstii*), L (*Fusarium oxysporum*).

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3 CHAPTER THREE

FUNGAL BIODETERIORATION OF POLYVINYL ALCOHOL FILMS

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ABSTRACT

Polyvinyl alcohol (PVA) is a biodegradable water soluble polymer that has a broad number of industrial applications. While mainly used as a coating for paper and as a sizing agent in the textile industry, PVA can be fabricated into films that are used as biodegradable bags for agricultural mulch and also increasingly in the domestic market for the collection of food waste. As a consequence, PVA is now entering commercial composting processes. However, relatively little research has been conducted on the microbiological degradation of PVA films in composts and in particular, in relation to fungi. In this study, solid PVA films were buried in compost for up to 12 weeks and the colonisation by fungi investigated at 25°C, 37°C, 45°C, 50°C and 55°C. *Galactomyces geotrichum* was the principal colonizing fungus which was recovered at 25°C followed by *Talaromyces emersonii* which was recovered at 45°C and 55°C. Other fungal colonizers were *Acremonium atrogriseum*, *Phialophora intermedia*, *Pichia manshurica*, *Candida tropicalis*, *Talaromyces emersonii*, *Aspergillus fumigatus*, *Talaromyces emersonii*, and *Geosmithia cylindrospora*. Fungal community analysis by DGGE confirmed that the fungi recovered and cultivated from the surface of the PVA film constituted the majority of the colonising fungi.

3.1 INTRODUCTION

Polyvinyl alcohol (PVA) is a water soluble polymer that is widely used for a number of applications including fibre and paper coating, textile sizing and adhesives (Kawai and Hu, 2009). Due to a growing interest in biodegradable polymers as environmentally more benign materials compared to recalcitrant conventional plastics, PVA is being formulated into films and plastics, usually blended with plasticisers such as glycerol or starch to increase its flexibility and widen its potential applications (Chai *et al*, 2009; Chiellini *et al*, 2003; Jecu *et al*, 2010; Tudorachi *et al*, 2000) and is currently used to manufacture water soluble bags for agricultural mulches, domestic food waste and sanitary bags (Chiellini *et al*, 2003; Shima, 2001; Tokiwa *et al.*, 2001). As a result of its broadened applications and the increase in composting of green waste, the amount of PVA entering composting systems is increasing (Shah *et al*, 2008).

During the composting process, organic matter is modified by microorganisms during biochemical decomposition (Tiquia *et al.*, 2005; 2010). Physico-chemical changes during composting are due to a succession of various microbial communities and compost communities change from mesophilic to thermophilic and finally return to a mesophilic population (de Bertoldi *et al*, 1983).

In this study, we investigated the degradation of PVA films in compost at mesophilic and thermophilic temperatures and isolated and identified the principal fungal colonizers.

3.2 MATERIALS AND METHODS

3.2.1 Fabrication of PVA plastic film

10 % (w/v) PVA (MW 13-23 KD, DH 98%) solution was sterilised at 121 °C for 15 min .After cooling, 20 ml of solution was poured into each of sterile petri dishes and left at room temperature inside a desiccators for 20 days. PVA coupons had a diameter of 88 mm and a thickness of 2 mm.

3.2.2 PVA burial

500 g of matured organic compost (Westland horticulture, UK) was added to polypropylene boxes (230 mm (width) x 150 mm (length) × 90 mm (depth)) and three replicate PVA sheets (88 mm diameter and 2 mm thick) were buried horizontally inside petri dishes to a depth of 0.5 cm and incubated at 25°C, 37°C, 45°C, 50°C and 55°C for up to 2 months. Water loss due to evaporation was calculated by measuring weight loss of the boxes weekly and brought back to the original weight by the addition of sterile water. The water holding capacity (WHC) was determined according to Barratt *et al* (2003). Control sheets were incubated in saturated sterile vermiculite and in sterile distilled water.

3.2.3 Enumeration of fungi

To enumerate fungal growth on the surface of the PVA sheets, PVA pieces were removed from polypropylene boxes and transferred individually into 50 ml universal tubes containing 10 ml sterile distilled H₂O and shaken for 2

minutes to remove loosely associated compost particles. PVA sheets were removed and placed in Petri-dish containing 5 ml sterile water. The surface was scraped with a sterile razor blade, vortexed briefly, to enumerate serially diluted and plated onto potato dextrose agar (Formedium, UK) and minimal salts medium (Crabbe *et al*, 1994) containing 2% (v/v) PVA (MW 13-23 KD, DH 98%, Sigma, UK) as sole carbon source. Agar media were supplemented with 50X filter sterilized (0.22 μ m, Millipore, UK) chloramphenicol (50 μ g/ml final concentration) to suppress bacterial growth. Plates were incubated for one week at 25°C, 37°C, 45°C, 50°C and 55°C and colony forming units recorded daily.

3.2.4 DNA extraction

Genomic DNA was extracted from compost using the PowerSoil DNA isolation kit (MoBio, UK) according to the manufacturer's instructions and stored at -20°C until required. Genomic DNA of isolated strains was extracted from mycelium or yeast cells grown in PDA broth culture (50 ml) in 250 ml conical flasks for 2 days at 25°C (250 rpm). Biomass was harvested by centrifugation at 8000x g for 15 min, the supernatant was discarded and the biomass frozen in liquid nitrogen and ground to a powder in a mortar and pestle. Genomic DNA was extracted according to manufacturer's instructions using a DNeasy® Plant Mini Kit (Qiagen, UK) and stored at -20°C until required.

3.2.5 ITS rDNA sequencing

Isolated fungi were identified by PCR amplification and DNA sequencing of nuclear ribosomal 5.8S DNA and internal transcribed spacer (ITS)

using the fungal universal primers ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') as previously described (Webb *et al.*, 2000) and PCR products sequenced in house. Sequences were used to interrogate the NCBI (National Centre for Biotechnology Information) database using the blastn algorithm (<http://www.ncbi.nlm.nih.gov>). Neighbour Joining trees were constructed using Mega 5.1 software (Molecular Evolutionary Genetics Analysis software, www.megasoftware.net) to align sequences. Putative identifies of isolates from rDNA sequence comparisons were further verified by comparing colony and conidial morphology with published descriptions.

3.2.6 Denaturing gradient gel electrophoresis (DGGE)

To investigate and compare changes in the fungal community profiles before and after PVA films burial, genomic DNA from compost and the surface of PVA sheets were subjected to DGGE using primers GM2 (5'-CTGCGTTCTTCATCGAT-3') and JB206c(5'CGCCCGCCGCGCGGGCGGGCGGGGGCACGGGGGG AAGTAAAAGTCGTAACAAGG-3'), which amplify the ITS1 region of fungal rDNA as previously described (Cosgrove *et al.*, 2007). In order to reduce impurities, genomic DNA from compost was purified using a QIAquick PCR Purification Kit (Qiagen, UK) according to the manufacturer's instructions prior to amplification.

3.2.7 Environmental scanning electron microscopy (ESEM)

PVA solid coupons were recovered from compost 21 day after burial and were subjected to ESEM (School of Chemistry, University of Manchester). Growth of fungal mycelia appeared as chains of branching on surface of PVA sheet. Also, PVA sample had been broken and partly disappeared after twelve weeks in 45°C and 55°C.

3.3 RESULTS

3.3.1 Physical changes to the PVA sheets during burial in compost

In order to investigate the impact of compost burial on the physical changes to PVA solid coupons, PVA samples were buried in compost and incubated at 25°C, 37°C, 45°C, 50°C and 55°C for a period of 21 days. Physical changes to the coupons were observed every three days and compared with two controls (coupons incubated in water-saturated sterile vermiculite or in sterile distilled water alone). Fungal colonization on the surface of PVA coupons were observed after 3 days and fungal accumulation increased over the 21 day incubation period (Fig 3.1 B). No physical damage was observed on PVA samples buried at 25°C and 37° C. However, PVA coupons appeared opaque, shrunken and partly disintegrated after incubation at higher temperatures (45°C, 50°C and 55°C) after 15 days incubation (Fig 3.1 C). Control coupons incubated in saturated sterile vermiculite and in sterile distilled water showed no physical changes or microbial growth during the period of experiment (Fig 3.1 A). PVA coupons after 21 days of incubation were subjected to ESEM and extensive growth of fungal mycelia appeared as chains of branching hyphae on the surface of the PVA coupons (Fig 3.1. D and E).

3.3.2 Viable counts of PVA colonizing fungi on PVA and PDA plates

To enumerate fungal colonization on the surface of the PVA coupons during compost burial at different temperatures, fungal biomass was recovered from the surface of the coupons after 3, 6, 9, 12, 15, 18 and 21 days, serially diluted and the colony forming units determined after incubation on PVA agar

(putative PVA degraders) and on PDA agar (total fungi) (Figure 3.2.). Total fungal CFU (on PDA agar) increased at all temperatures from day 3 to day 12 and remained constant thereafter. The highest fungal CFU's from the coupon surface were observed at 25°C and 37°C (ca. 2×10^7 CFU/cm²) with the lowest fungal CFU's observed at the higher temperatures ranging from ca. 7×10^6 /cm² at 45°C to ca. 2×10^6 /cm² at 55°C. CFU counts on PVA media were consistently lower at all temperatures compared to PDA media. At 25°C to 45°C, CFU's recovered were constant after 9 days ranging from 0.9×10^6 to 1.7×10^6 CFU/cm². CFU counts at 55°C were lower with a maximum of 1.1×10^5 CFU/cm². After 21 days incubation, the percentage of recovered CFU's on PVA medium compared to PDA medium varied depending on the incubation temperature (25°C = 6.8%, 37°C = 0.8%, 45°C = 15.4%, 50°C = 21.6% and 55°C = 3.4%). Colonies growing on PVA agar plates appeared morphologically similar to each other in colour due to lack of sporulation and it was not possible to distinguish between different morphotypes on this medium. Therefore, colonies from PVA plates were individually transferred onto PDA agar and after incubating for up to 5 days and were then grouped into different morphotypes depending on their colony phenotypes (Appendix Fig A3.1). Representative isolates of different colony morphotype were then subjected to ITS sequencing for comparison with the sequence database at NCBI.

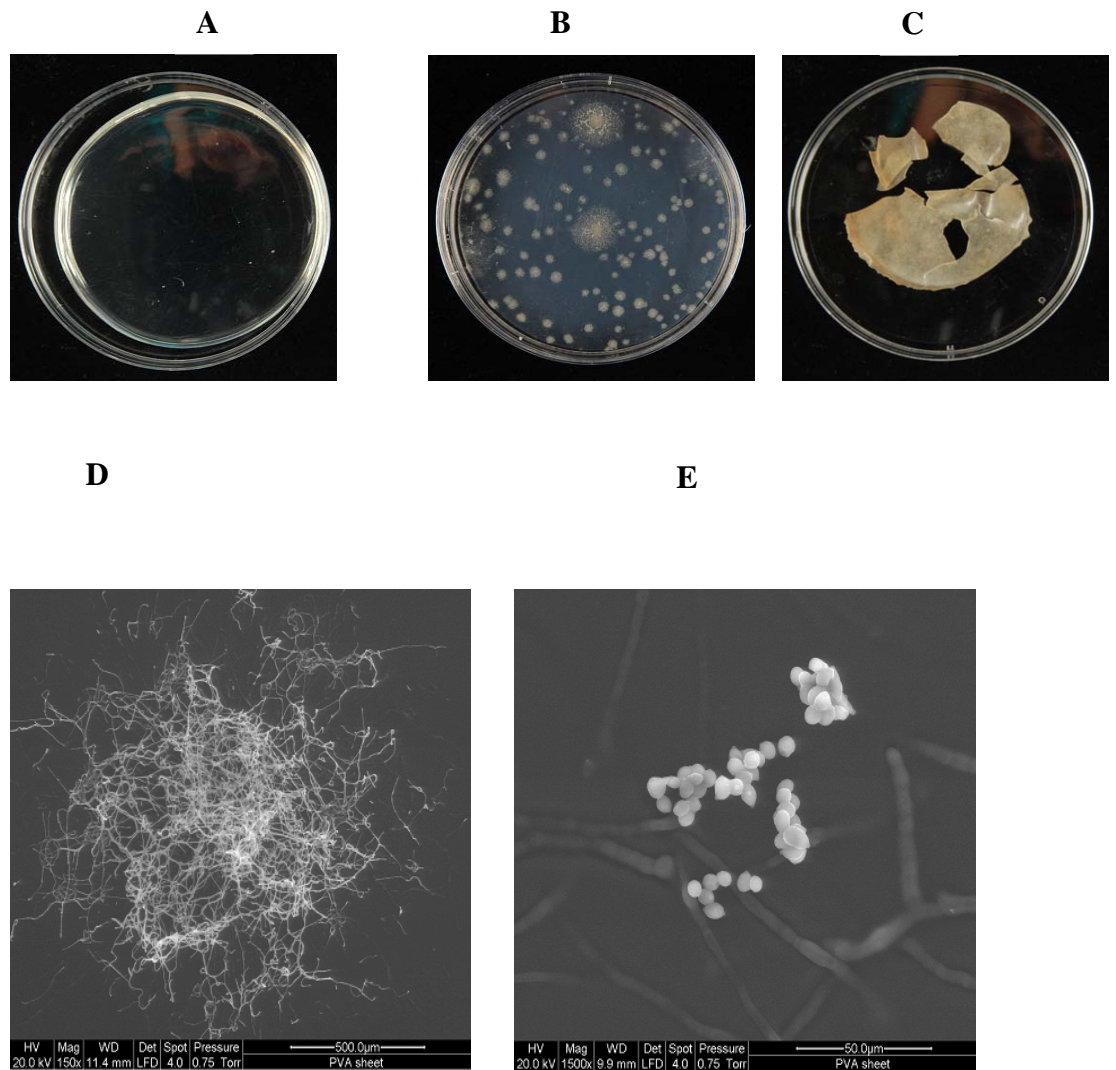


Figure 3-1. The effect of compost burial on PVA coupons. (A) PVA coupons before burial in compost, (B) PVA coupon after 9 days burial in compost at 50°C, (C) PVA coupon 60 days after burial in compost at 50°C. (D) ESEM micrograph (bar = 500 μm) of fungal mycelia 21 day after burial at 50°C, (E) ESEM micrograph (bar = 50 μm) of fungal mycelia 21 day after burial at 50°C.

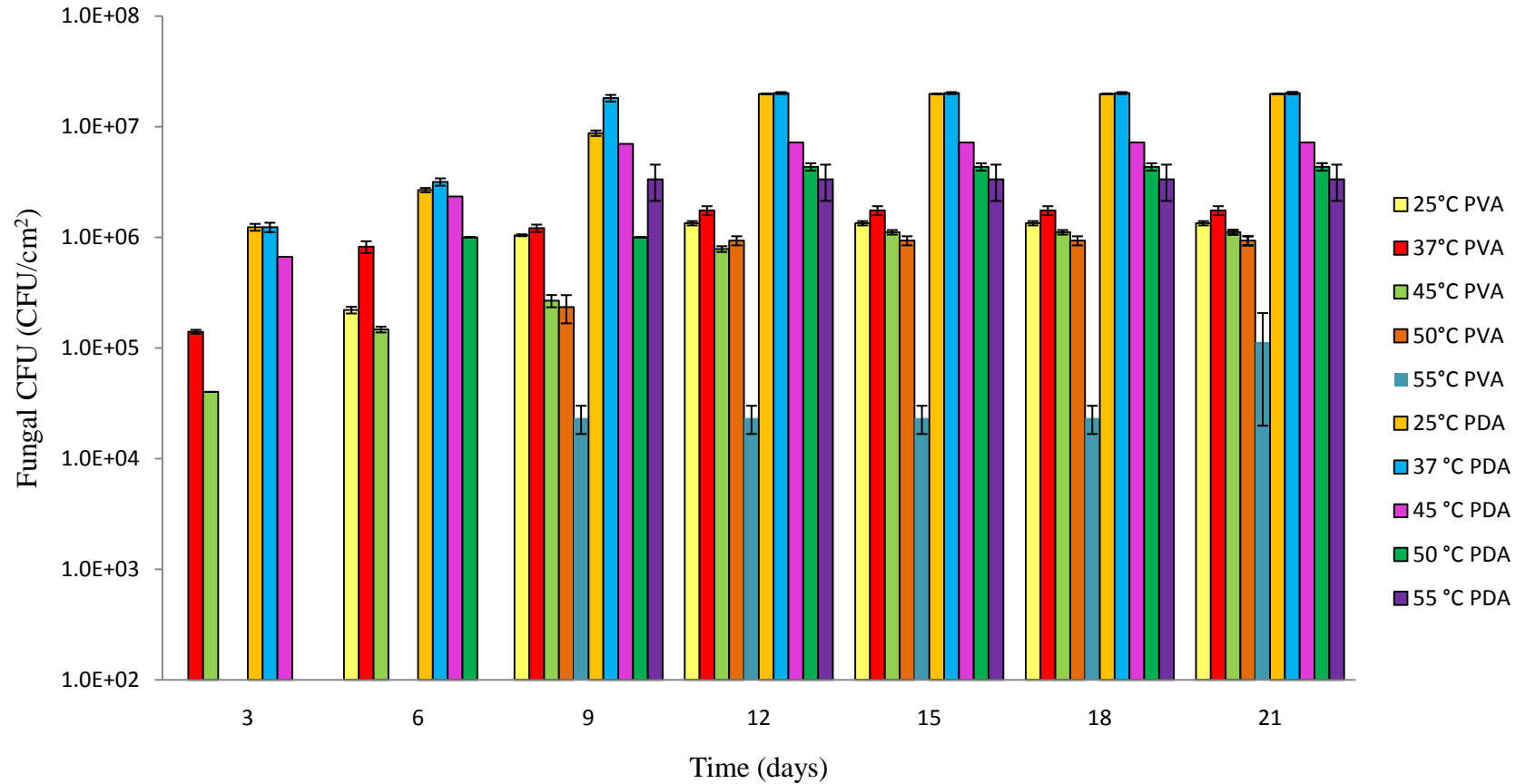


Figure 3-2. Changes in fungal colony forming units (CFU/cm²) recovered from surface of compost buried PVA coupons. PVA coupons were buried in compost and incubated at 25°C, 37°C, 45°C, 50°C and 55°C over 21 days. Biomass was removed from the surface and colony forming units (CFU) enumerated on PVA medium for PVA degraders (PVA as sole carbon source) and on PDA medium for total fungal counts (general fungal growth medium). Results are the means of triplicate samples \pm standard error of the mean (SEM) and expressed as CFU recovered per cm² of the PVA surface.

3.3.3 Identification of fungal isolates from the surface of compost buried PVA coupons

A total of fifteen distinct fungal morphotypes were recovered from the surface of PVA coupons buried in compost at 25°C, 37°C, 45°C, 50 °C and 55°C and ITS rDNA amplified using the ITS-1 and ITS-4 primer set to enable identification by comparison with the NCBI data base. Amplified rDNA sequence lengths ranged from 308-551 bp for the 5.8S rDNA and ITS regions and identities against the NCBI database ranged from 98 – 100 % (Table 3.1). At 25°C, four species were identified, *Acremonium atrogriseum*, *Phialophora intermedia*, *Pichia manshurica*, and *Galactomyces geotrichum*. Seven similar morphotypes were recovered but on sequencing were all found to be *Galactomyces geotrichum*. At 37°C, only *Candida tropicalis* was recovered, while at 45°C, two species, *Talaromyces emersonii* and *Geosmithia cylindrospora* were identified. At 50°C, one species, *Aspergillus fumigatus* was recovered and at 55°C only *Talaromyces emersonii* was recovered. Strains identified in this study were subjected to phylogenetic analysis to verify strain identity (Fig 3.3).

Isolate code	Temperature	Strain name	EMBL accession number	Percentage homology in NCBI data base
1125	25°C	<i>Acremonium atrogriseum</i>	JX847767	99%
1425	25°C	<i>Phialophora intermedia</i>	JX847774	99%
5125	25°C	<i>Galactomyces geotrichum</i>	JX847768	98%
6125	25°C	<i>Galactomyces geotrichum</i>	JX847775	99%
7125	25°C	<i>Pichia manshurica</i>	JX847776	98%
Co11	25°C	<i>Galactomyces geotrichum</i>	JX847770	100%
Co12	25°C	<i>Galactomyces geotrichum</i>	JX847771	98%
Co13	25°C	<i>Galactomyces geotrichum</i>	JX847769	100%
Co15	25°C	<i>Galactomyces geotrichum</i>	JX847772	98%
Co16	25°C	<i>Galactomyces geotrichum</i>	JX847773	98%
1137	37°C	<i>Candida tropicalis</i>	JX847777	99%
2145	45°C	<i>Talaromyces emersonii</i>	JX847778	100%
2245	45°C	<i>Geosmithia cylindrospora</i>	JX847781	100%
3150	50°C	<i>Aspergillus fumigatus</i>	JX847779	99%
5511	55°C	<i>Talaromyces emersonii</i>	JX847780	99%

Table 3-1. Putative identities of the fungal PVA degraders recovered from the surface of PVA buried in compost. Fungi recovered on PVA media from the surface of PVA sheets buried in compost and incubated at various temperatures were transferred onto PDA plates and grouped into different morphotypes. The ITS region of the rDNA from a representative of each morphotype was sequenced and the sequence used to interrogate the NCBI database.

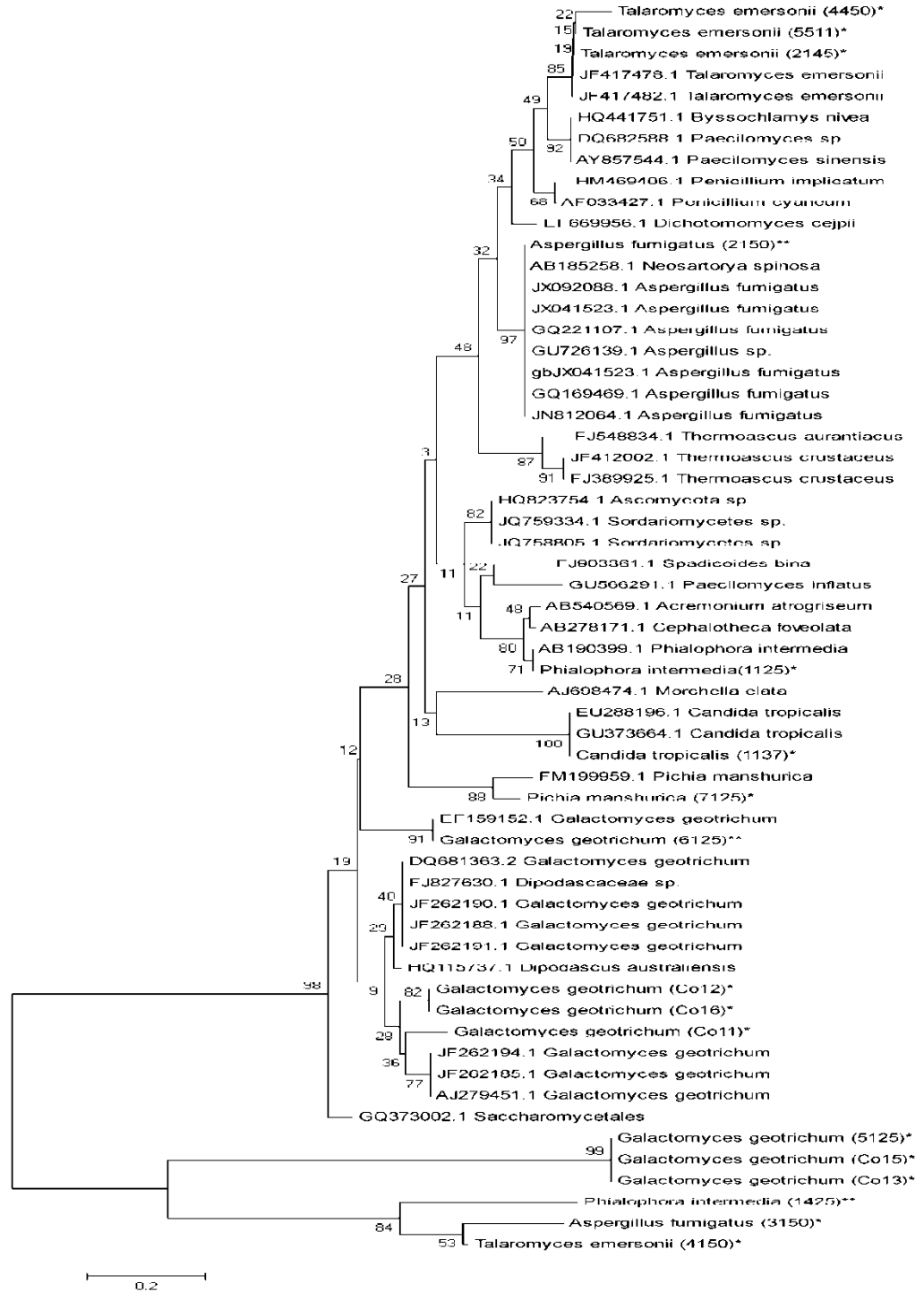


Figure 3-3. Phylogenetic analysis of the ITS rDNA sequences of fungal species isolated from the surface of PVA with the closest matching strains from the NCBI database. Strains isolated and identified in this study are marked with star *.

3.3.4 Analysis of the dominant fungal species colonising the surface of PVA coupons following burial in compost.

In order to compare the fungal community growing on the surface of PVA coupons with the community in the surrounding compost, DNA from the surface of the coupons and from the surrounding compost after 21 days incubation at temperatures between 25°C and 55°C was extracted and subjected to DGGE analysis following PCR amplification of the ITS region of the rDNA (Fig 3.4). Compared to the initial community in the compost prior to incubation, the number of DGGE bands decreased as incubation temperature increased to 55°C. Similarly, the fungal community profiles on the surface of PVA coupons were similar to the profile in the surrounding compost, except that the intensity of some bands had changed. No new DGGE bands were detected on the surface of PVA compared to the surrounding compost. This suggests that dominant organisms colonising the PVA surface were those already present in the compost community although the relative abundance changed on the surface compared to the surrounding compost.

To investigate this further, PVA degrading organisms isolated by cultivation from the PVA surface were individually subjected to DGGE analysis on the same gel as the initial total compost community (Fig 3.5). With the possible exception of *Galactomyces geotrichum* (lane B), none of the single PVA degrading isolate co-migrated with the dominant bands from the initial compost community suggesting that the majority of PVA degrading fungal strains recovered from the surface of PVA on PVA medium were not dominant members of the compost community.

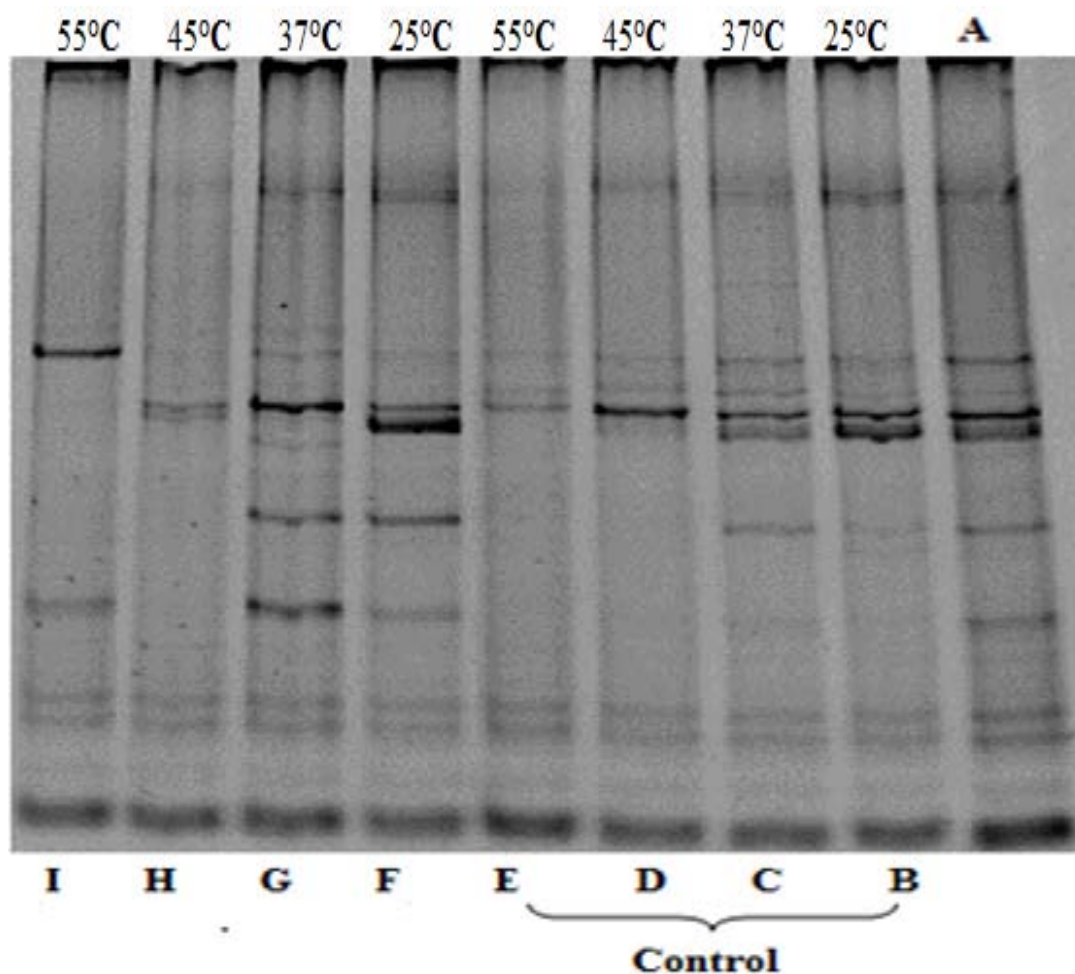


Figure 3-4. A comparison of the fungal community profile of compost and on the surface of PVA coupons after 21 days incubation at different temperatures by DGGE. Total community DNA from compost and from the surface of PVA buried in compost were used to amplify the ITS region of the rDNA and amplicons subjected to DGGE. Initial compost community (Lane A); compost community after 21 days incubation at 25°C, 37°C, 45°C and 55°C (Lanes B to E respectively). Fungal community profile from the surface of PVA after 21 days incubation buried in compost at 25°C, 37°C, 45°C and 55°C (Lanes F to I respectively).

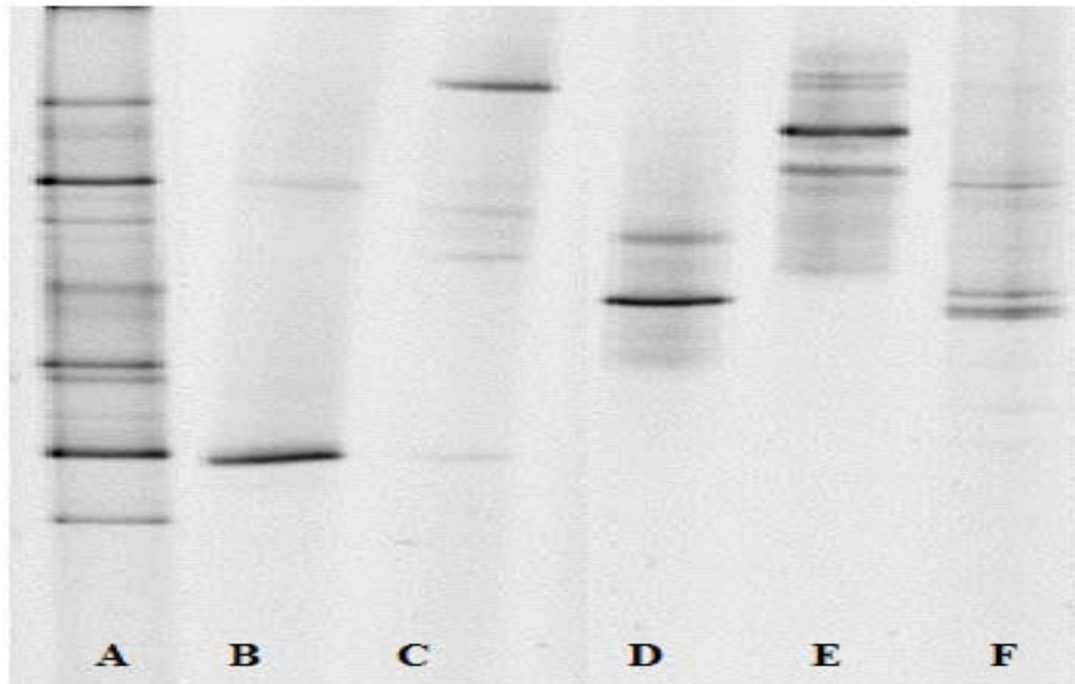


Figure 3-5. A comparison of the fungal community profile of compost with isolated fungal species recovered on PVA medium from the surface of PVA coupons buried in compost by DGGE. Total community DNA from compost and DNA from individual strains recovered by cultivation from the surface of PVA were used to amplify the ITS region of the rDNA and amplicons subjected to DGGE. Lane A, initial compost; Lane B *Galactomyces geotrichum* (isolated at 25°C); Lane C, *Talaromyces emersonii* (isolated at 45°C); Lane D, *Candida tropicalis* (isolated at 37°C); Lane E, *Geosmithia cylindrospora* (isolated at 50°C); lane F, *Aspergillus fumigatus* (isolated at 50°C).

3.4 DISCUSSION

The treatment of solid waste under composting conditions is increasingly used for the treatment and recycling of organic waste materials (Shah *et al.*, 2008). As larger volumes of biodegradable polymers are finding applications in industrial and domestic settings, these materials are increasingly entering composting waste streams; however, other than monitoring the degradation of these materials, there has been relatively little research on the microorganisms that colonise these polymers. Previous studies in soils, waste waters and sewage sludge has identified a small number of bacterial species including *Pseudomonas sp* (Watanabe *et al.*, 1976; Hatanaka *et al.*, 1995), *Alcaligenes faecalis* (Matsumara *et al.*, 1994) and *Bacillus megaterium* (Mori *et al.*, 1996a) that are capable of degrading PVA, although many require a symbiotic partner in co-culture (Corti *et al.*, 2002; Chen *et al.*, 2007). However, few studies have investigated microbial degradation of biodegradable polymers in composting systems. One study in compost isolated the thermophilic actinomycete *Thermomonospora fusca* which was thought to play an important role in the initial degradation of aliphatic-aromatic copolyesters (Kleeberg *et al.*, 1998).

In this study, PVA sheets were buried in mature compost and incubated at temperatures between 25°C and 55°C for 21 days. Examination of the surface of the sheets by light microscopy and ESEM revealed rapid and extensive growth of fungal colonies on the surface of PVA at all temperatures, suggesting fungi potentially play an important role in biodegradation of PVA. This was verified by an increase in CFU's recovered from the surface of the PVA sheets on media

containing PVA as the sole carbon source. While fungal growth was clearly visible on the surface of the PVA coupons, discolouration and partial disintegration was only seen at elevated temperatures suggesting a combination of higher temperature and fungal activity may have been responsible as control coupons incubated at equivalent temperatures in sterile water were unaffected. *Galactomyces geotrichum* was the principal colonizing fungus recovered from the surface of PVA coupons incubated at 25°C, while only thermophilic fungi were recovered at 45°C, 50 °C and 55°C with *Talaromyces emersonii* the principle organism recovered. *Geotrichum* species are common yeast-like fungi found in a variety of environments (Pimenta *et al.*, 2005) and are exploited industrially for lipase applications in detergents and for biodegradation of waste water textile dyes (Jadhav *et al.*, 2008; Phillips and Pretorius, 1991; Waghmode *et al.*, 2012). Previously, *G. geotrichum* were found to be the most frequently recovered strain in a number of different soils (Mollasalehi *et al*, unpublished) and was found to completely degrade PVA as a sole carbon source for growth and a related *Geotrichum* sp was also reported to be capable of degrading polyvinyl alcohol oligomers (Mori *et al.*, 1996b). As *G. geotrichum* was the major isolate recovered at 25 °C, it suggests that in temperate environments, it is the major organism selected when PVA is present and may therefore potentially also be useful in the removal of PVA from contaminated environments. PVA coupons incubated at 45°C and 55°C were degraded more rapidly compared to lower temperatures, whereas PVA sheets buried in sterile saturated vermiculite did not undergo any discernible physical changes. However, the fungal CFU's

recovered from the surface of the PVA sheet at 25° and 37° C was higher than that recovered at 45°, 50° and 55°C. DGGE profiles of compost and the surface of the PVA coupons after 21 days incubation revealed that the majority of the fungi detected on the surface were also dominant in the compost, suggesting that many fungi colonise PVA regardless of whether they are capable degrading it. This was also demonstrated by CFU's recovered from the PVA surface on PVA medium compared to PDA medium which were always lower (between 0.8% at 37°C and 21.6% at 50°C). In addition, with the exception of *G. geotrichum*, organisms recovered from the PVA surface on PVA medium were not visible in the compost community, suggesting they formed a minor component on the total fungal biomass on the PVA surface.

This study demonstrates that PVA entering the composting stream is susceptible to fungal degradation at all temperatures of the composting process and that while colonisation of the PVA surface by the dominant fungal species present in the compost occurs, selection for a small number of minor species capable of degrading PVA from within the fungal community plays an important role in the degradation of this polymer.

3.5 REFERENCES

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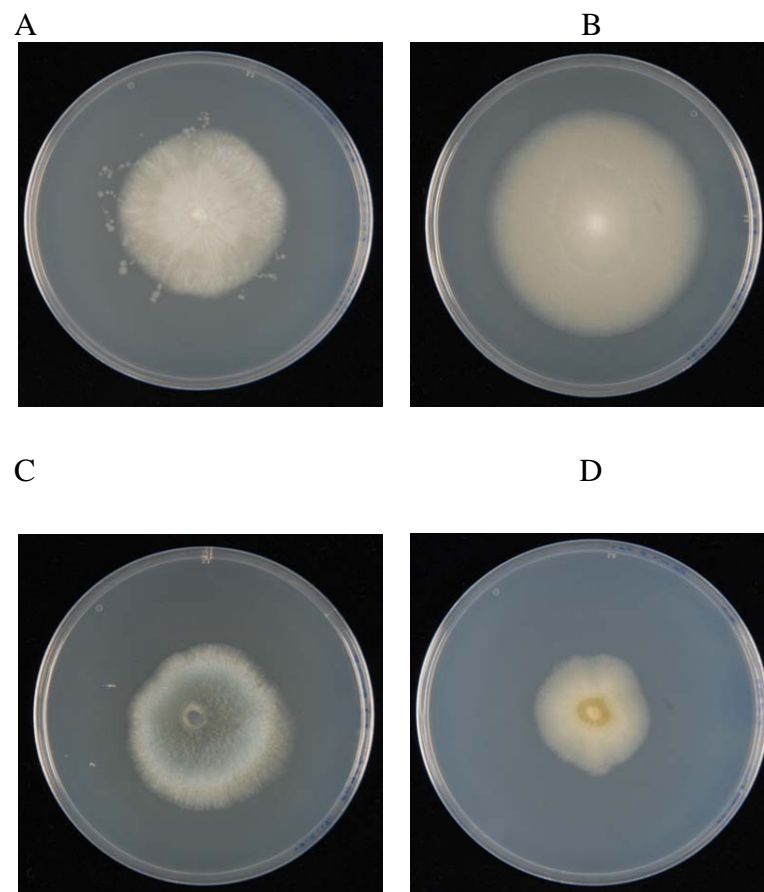
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APPENDIX 3.1

Fungal morphotypes isolated from compost on PVA agar

Fungi growing on PVA sheets transferred onto potato dextrose agar and grouped into different morphotypes based on morphological similarity prior to ITS sequencing. Different morphotypes were distinguished and are shown in Figure A3.1



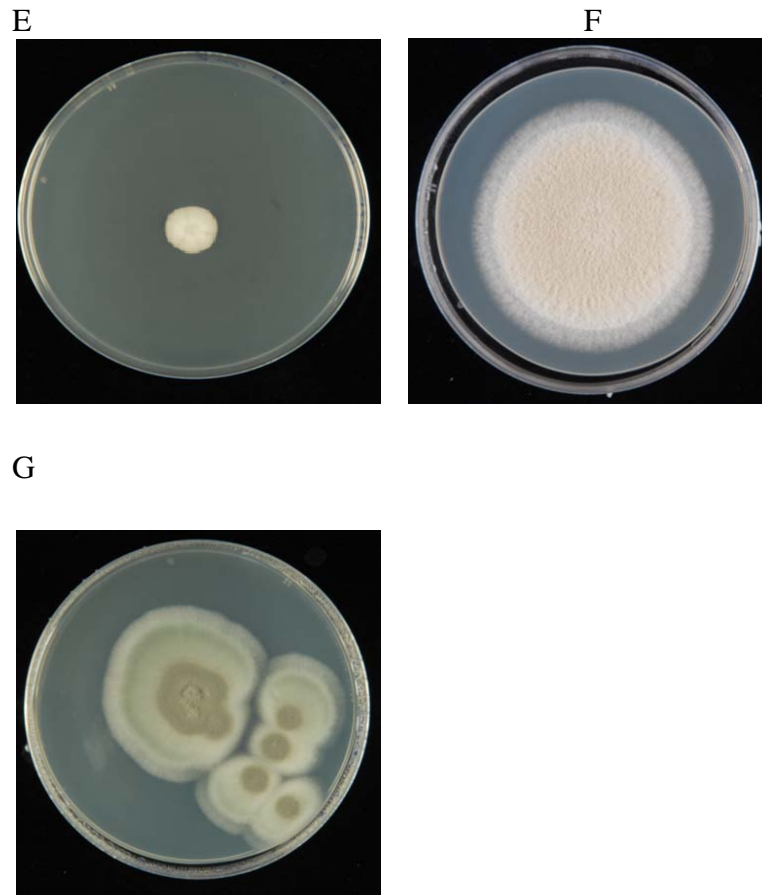


Figure A3.1. Morphological characteristic of dominant fungal strains isolated from studied soils on PDA agar plates. A is (*Galactomyces geotrichum*), B (*Phialophora intermedia*), C (*Acremonium atrogriseum*), D (*Pichia manshurica*), E (*Candida tropicalis*), F (*Talaromyces emersonii*), G (*Aspergillus fumigatus*).

4 CHAPTER FOUR

IMPACT OF THE WATER SOLUBLE POLYMER POLYVINYL ALCOHOL ON SOIL AND COMPOST FUNGAL COMMUNITIES

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ABSTRACT

The water soluble biodegradable polymer polyvinyl alcohol (PVA) is widely industrially used in paper coating and textile sizing as well a variety of other applications. While some individual microbes and consortia capable of degrading PVA have been characterised in the laboratory, there have been few studies that have examined its impact on naturally occurring microbial communities. In this study, terminal restriction fragment length polymorphism (TRFLP) was used to monitor changes in the fungal community profile in soil and compost over a six week period following the addition of PVA at 25°C and 45°C. In soil at 25°C, the community changed in the presence compared to the absence of PVA with the greatest shift in the community occurring after four weeks before returning to a profile similar to that seen in the absence of PVA after 6 weeks. In compost, the response to the presence of PVA was more complex. At both 25°C and 45°C, in the absence of PVA, the community shifted over 6 weeks, with greatest change visible after 2 weeks. In the presence of PVA, an increase in the number of TRFs was also observed but was lower than that seen in the absence of PVA. Overall, this study has shown that PVA causes a significant shift in the fungal community with a number of T-RF's detected only in the presence of PVA. However, these were minor components of the community and the presence of PVA did not cause a major shift in the dominant species. The greatest change was found after four weeks of incubation, however after six weeks the communities in the presence of PVA were more similar to that found in the absence of PVA suggesting that exposure to PVA causes a temporary shift in the fungal community.

4.1 INTRODUCTION

Polyvinyl alcohol is a water soluble biodegradable synthetic polymer which is used extensively in industrial applications such as the adhesive, textile and paper coating industries and in the production of biodegradable products including laundry bags and agricultural mulching films (Chiellini *et al.*, 2003; Shima, 2001; Tokiwa *et al.*, 2001). A number of bacterial strains have been reported to cause biodegradation of this polymer (Corti *et al.* 2002; Kim *et al.* 2003; Mori *et al.* 1996; Shima *et al.* 1984; Suzuki *et al.* 1973; Tokiwa *et al.* 2001; Vaclavkova *et al.* 2007), but few studies have focused on fungi or on the impact of this polymer on microbial communities in natural environments (Corti *et al.* 2002; Jecu *et al.* 2010; Qian *et al.* 2004; Tsujiyama *et al.* 2011). While a limited number of bacterial strains have been reported to be capable of degrading PVA, the majority of research has demonstrated that PVA is degraded more effectively by mixed consortia (Chen *et al.* 2007; Sakazawa *et al.* 1981). This has since been shown to be due to the production of growth factor(s) by a symbiotic partner(s) essential for the degradation of PVA by the PVA degrading strain (Hashimoto & Fujita, 1985; Mori *et al.* 1996; Shima, 2001; Vaclavkova *et al.* 2007). A number of enzymes have been identified that are responsible for the degradation of PVA in which the carbon-carbon polymer backbone is first cleaved by the action of either a dehydrogenase or an oxidase, followed by the action of an aldolase or hydrolase (Shima, 2001; Amange & Minge, 2012). Based on these studies, PVA is widely regarded as a biodegradable polymer. However, in reality the number of microbes capable of degrading PVA appears to be limited and PVA degradation in the environment does not occur easily

requiring an acclimation period during which PVA degraders are selected (Porter & Snider, 1976; Chiellini *et al* 1999; Chiellini *et al*, 2003). Moreover, no studies have been conducted to assess the overall impact of PVA on naturally occurring microbial communities.

Previously, we reported on the isolation of a limited number of fungal isolates from a diverse number of soil types through serial enrichment that were capable of degrading PVA (Mollasalehi *et al*, unpublished, Chapter 2). In this study, we used Terminal Fragment Length Polymorphism (TRFLP) to study the impact of PVA on the fungal community profile in soil and compost; non-culture based techniques that have been widely used to evaluate changes and composition of microbial communities within environmental samples. Such approaches are valuable as the majority of microorganisms cannot be cultivated on laboratory media (Hawksworth, 1991; Osborn *et al.*, 2000) and the method enable an estimate of both community diversity and dynamics within complex microbial ecosystems independent of conventional cultivation (Andronov *et al.*, 2009; Anderson and Cairney, 2004; Edwards and Zak, 2010; Ge and Zhang, 2011; Krummins *et al*, 2009; Liu *et al*, 1997; Muyzer *et al.* 1997; Muyzer and Smalla, 1998; Schutte *et al.*, 2008).

In this study, we report that PVA causes a marked shift in the fungal community population within two weeks. But, after six weeks the community profile begins to approach that of the initial population suggesting that transient PVA contamination in soils and compost may not have a lasting effect on the indigenous fungal community.

4.2 MATERIALS AND METHODS

4.2.1 PVA amended soil and compost

Commercial organic compost (The compost shop, UK) and top soil (Ideal Range, UK) were stored at room temperature until required. 50 ml of 10% (w/v) PVA (MW 13-23 KD, DH 98%) was mixed thoroughly with 250 g of compost or soil and incubated in a plastic box at 25°C (soil) and 25° and 45°C (compost). Controls contained soil or compost plus 50 ml sterile distilled water without PVA. The water content of soils and composts were evaluated weekly by weighing and sterile water was added to maintain a constant weight over the duration of the experiment. Water holding capacity (WHC) of the compost and soil were determined at the beginning and periodically according to Barratt *et al* (2003).

4.2.2 DNA extraction from soil and compost

Genomic DNA from soil and compost were extracted every two weeks using a PowerSoil DNA isolation Kit (MoBio laboratories, UK) and purified using a QIAquick purification kit (Qiagen, UK) according to the manufacturer's instructions. Genomic DNA was stored at -20°C until required.

4.2.3 Terminal restriction fragment length polymorphism (T-RFLP)

Extracted genomic DNA from soil and compost were amplified with fluorescently labelled primers, ITS4-HEX (hex-TCCTCCGCTTATTGATATGC) and ITS5-FAM (fam-GGAAGTAAAAGTCGTAACAAGG) at a concentration of 50 pmol/μl. PCR reagent concentrations were 50 mM MgCl₂, 10 mM of dNTPs, 5 μl of 10 x

reaction buffer supplied in BIOTAQ DNA polymerase kit, and 5 U/ μ l of BIOTAQ DNA Polymerase, 10mg/ml of 100 x BSA per 50 μ l PCR reaction. Amplification was carried out for 35 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 °C for 1 min. Amplified PCR products were purified (Qiaquick PCR purification kit, Qiagen, UK) and digested with 10 U/ μ l HhaI (Fermentas, UK, 10U/ μ l) for 2 h at room temperature and subjected to capillary electrophoresis in house enabling the digested dye labelled PCR digested fragments (T-RF'S) to be separated according to molecular weight.

4.2.4 Analysis of terminal restriction fragments (T-RF's)

The position and the peak areas of the T-RFs were analysed using PeakScanner v1.0 (Applied Biosystems, USA) and T-Align (Smith *et al* 2005). Light peak smoothing and a peak threshold of 30 fluorescence units were used for the analysis. Fragments which were less than 50 bp and greater than 500 bp were excluded from the analysis. Shannon Index diversity (H) and evenness (e) were used as species diversity indicators and were calculated for each sample as described by Tiquia (2005). Binary similarity matrices were subjected to principal component analysis (PCA) using MVSP multivariate analysis software (Kovach Computing Services, Anglesey, UK).

4.3 RESULTS

4.3.1 Influence of PVA on the soil and compost fungal community

As PVA is a water soluble polymer, the influence of the addition of PVA to the fungal community in soil at 25 °C and in compost at 25°C and 45°C was investigated by Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis. TRFLP is a non-culture-based technique enabling a fingerprint of the number of species and their relative abundance to be quantified. Soil and compost, amended with PVA or water, was incubated in containers at 25°C (soil) or 25°C and 45°C (compost) and triplicate samples removed for analysis prior to amendment (0 days, initial) and after 14, 28 and 42 days incubation. Following genomic DNA extraction, triplicate samples were pooled and TRFLP was performed to enable community profile changes to be quantified. The ITS4-ITS5 region of the fungal rDNA was amplified by PCR with the forward primer labelled with a fluorescent HEX dye to enable subsequent detection. PCR amplicons were digested overnight with Hha1 and digested amplicons separated by molecular weight by capillary electrophoresis. Electropherograms showing the distribution and normalized peak heights of the T-RF's from the soil and compost fungal communities in the presence and absence of PVA are shown in Figures 4.1, 4.2 and 4.3. In soil at 25°C, the profiles after 0, 2, 4 and 6 weeks in the absence of PVA were broadly similar and the number of TRF's ranged between 61 and 94 (Table 4.1). Following PVA addition, the electropherograms indicated that the community profile changed with a number of new peaks visible after four weeks (Fig 4.1) which was reflected by an increase in the number of TRF's which rose to 116 before

decreasing to 90 after 6 weeks (Table 4.1). However, the most abundant peaks remained unchanged indicating that PVA caused selection for a small number of additional fungi that peaked in week 4 for but had begun to disappear by week 6. PCA analysis confirmed that in the absence of PVA, the soil community changed little over six weeks whereas a marked shift was seen in response to PVA with the greatest difference in week 4 (Fig 4.4) before returning to a profile similar to that seen in the absence of PVA. This was also reflected in an increase in the Shannon index and Evenness in the presence of PVA at week four which indicates an increase in diversity in the fungal community (Table 4.1). The number of unique T-RF's (T-RF's only seen in the presence of PVA) rose from 7 after 2 weeks incubation to 76 after 4 weeks and declined to 17 after 6 weeks (Table 4.1).

Compost had initially a much lower number of detectable T-RF's (27) compared to soil (87) and the community profile was very different. In the absence of PVA at 25°C the number of TRF's rose significantly to 251 in week two before falling in weeks 4 and 6 to 161 and 123 respectively and this increase and subsequent decrease were clearly seen in the electropherograms (Fig 4.2, Table 4.2). In the presence of PVA, the number of TRF's also initially increased in week 2 but not as much as in the absence of PVA suggesting the presence of PVA repressed the increase in species diversity (157) before also falling in weeks 4 and 6 to reach a final level similar to week that seen in the absence of PVA (98). While some dominant peaks were retained in both the presence and absence of PVA, a number of new peaks were visible in the presence of PVA.

However, while the number of unique peaks in soil in the presence of PVA rose to a maximum of 76 (Table 4.1), in compost at 25°C the number of unique peaks seen only in the presence of PVA at 2, 4 and 6 weeks was 7, 6 and 4 (Table 4.2). Consequently, the largest change in the population was seen after two weeks in the absence of PVA as reflected in the Shannon index and Evenness (Table 4.2). PCA analysis of the electropherograms confirmed that the community initially changed the most after 2 weeks before returning close to the original profile by week 6, and that the profile in the presence of PVA was different to that seen in the absence of PVA (Fig 5.5). The number of unique T-RF's (T-RF's only seen in the presence of PVA) rose from 7 after 2 weeks incubation to 76 after 4 weeks and declined to 17 after 6 weeks (Table 4.2).

When the compost was incubated at 45°C, temperature had a marked effect on the community profile in both the presence and absence of PVA (Fig 4.3). In the absence of PVA, the number of TRF's increased from 27 to 150 after 2 weeks, remained similar at 159 after 4 weeks, before falling to 58 after 6 weeks (Table 4.2). In the presence of PVA, the number of TRF's also increased after 2 weeks though only to 89 compared to 150 in the absence of PVA. After 4 weeks, the number of T-RF's rose to 147 in the presence of PVA, similar to the numbers seen in the absence of PVA (159) before falling to 113 after 6 weeks, higher than that seen in the absence of PVA (58)(Table 4.2). Thus initially in compost at 45°C, PVA also depressed the increase in community diversity after 2 weeks as seen at 25°C. While the total numbers of T-RF's was similar after 4 weeks in the presence and absence of PVA, the number of unique T-RF's only seen in the

presence of PVA was highest (32) compared to weeks two and six (12 and 7 respectively, Table 4.2). This was confirmed in the PCA analysis which demonstrated the greatest differences in the structure of the fungal community was seen in the presence of PVA after 4 weeks (Fig 4.5) and also in the Shannon index (Table 4.2).

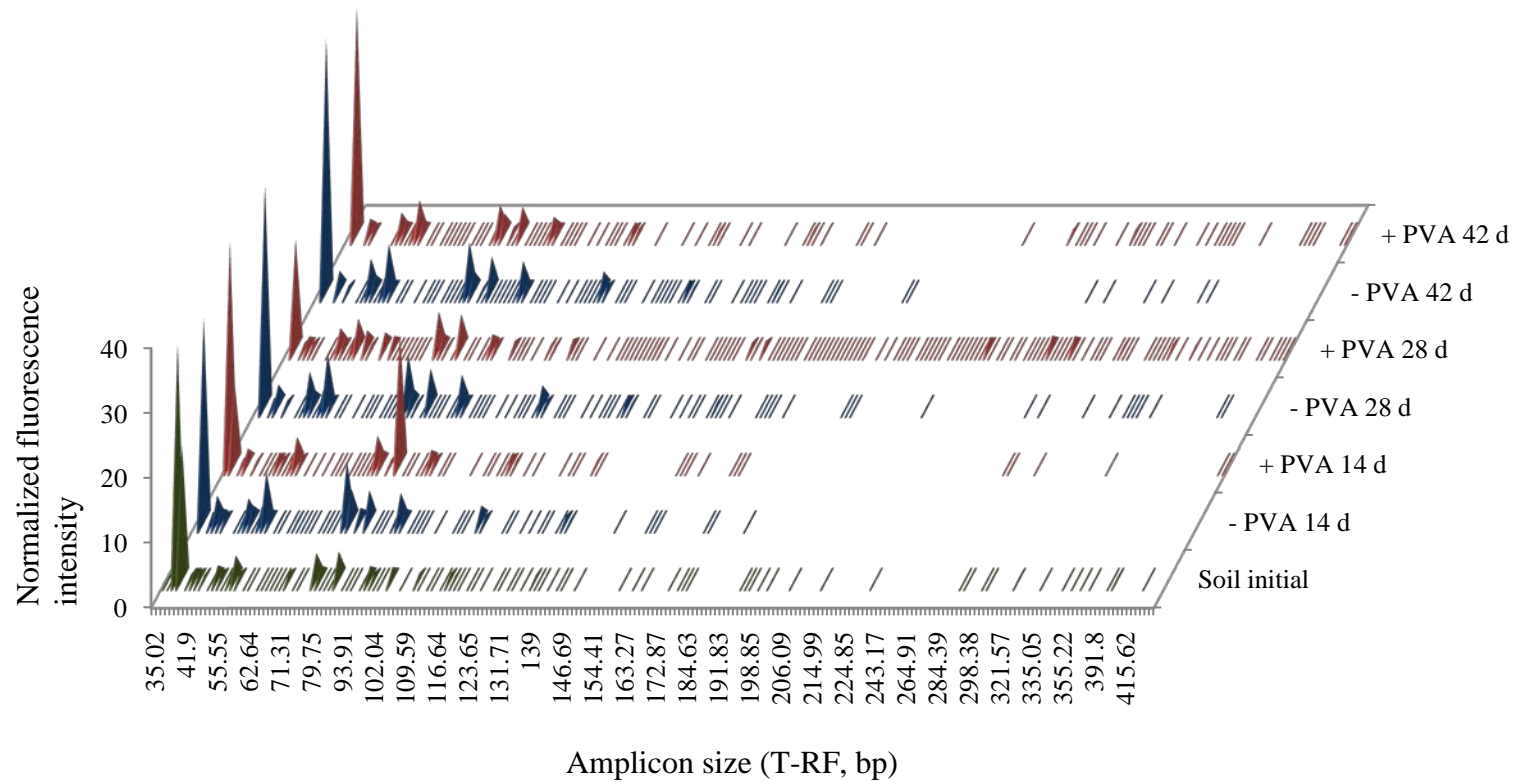


Figure 4-1. Effect of PVA on the fungal community profile of soil by TRFLP analysis. Commercial top soil was amended with either sterile water (blue) or PVA (red) and incubated in containers for 42 days at 25°C. Total genomic DNA was extracted from triplicate samples from the initial (green) soil prior to incubation and after 14, 28 and 42 days, pooled and the ITS4/ITS5 rDNA amplified by PCR. Following digestion with HhaI, HEX labelled amplicons were separated by capillary electrophoresis and individual amplicon peaks normalised against the total. Amplicons less than 35 bp were excluded from the analysis.

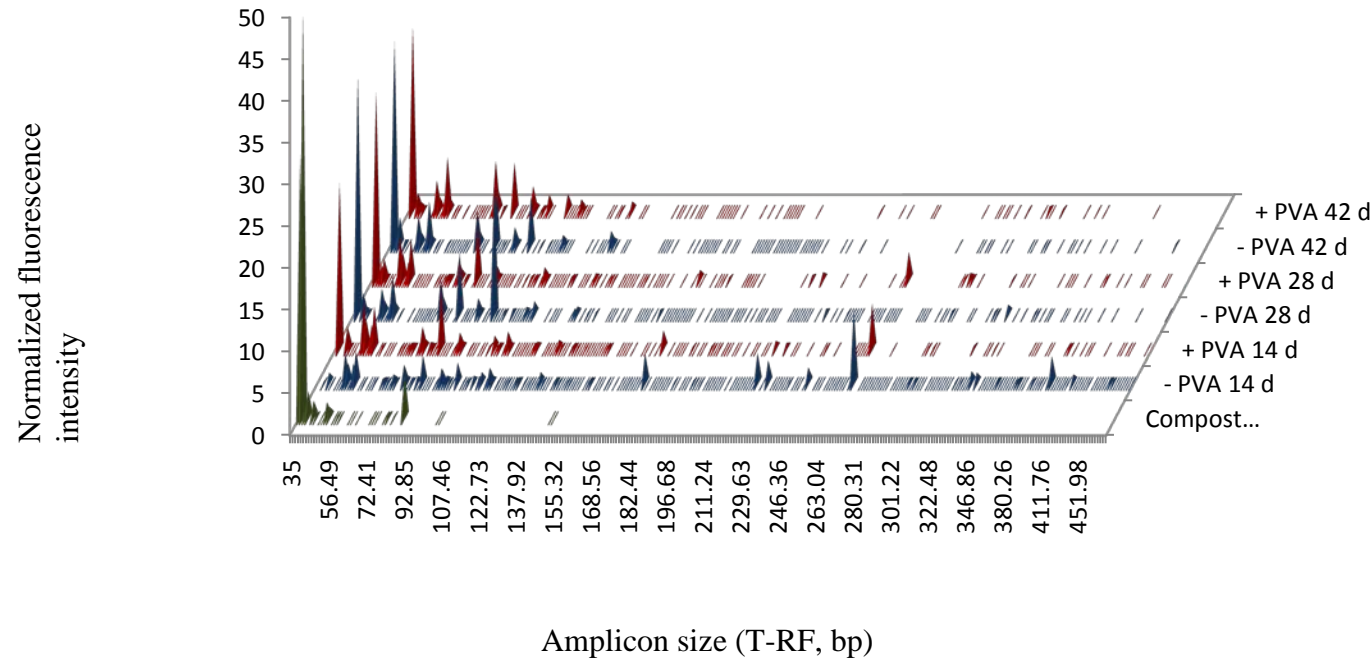


Figure 4-2. Effect of PVA on the fungal community profile of compost by TRFLP analysis. Commercial compost was amended with either sterile water (blue) or PVA (red) and incubated in containers for 42 days at 25°C. Total genomic DNA was extracted from triplicate samples from the initial (green) compost prior to incubation and after 14, 28 and 42 days, pooled and the ITS4/ITS5 rDNA amplified by PCR. Following digestion with HhaI, HEX labelled amplicons were separated by capillary electrophoresis and individual amplicon peaks normalised against the total. Amplicons less than 35 bp were excluded from the analysis.

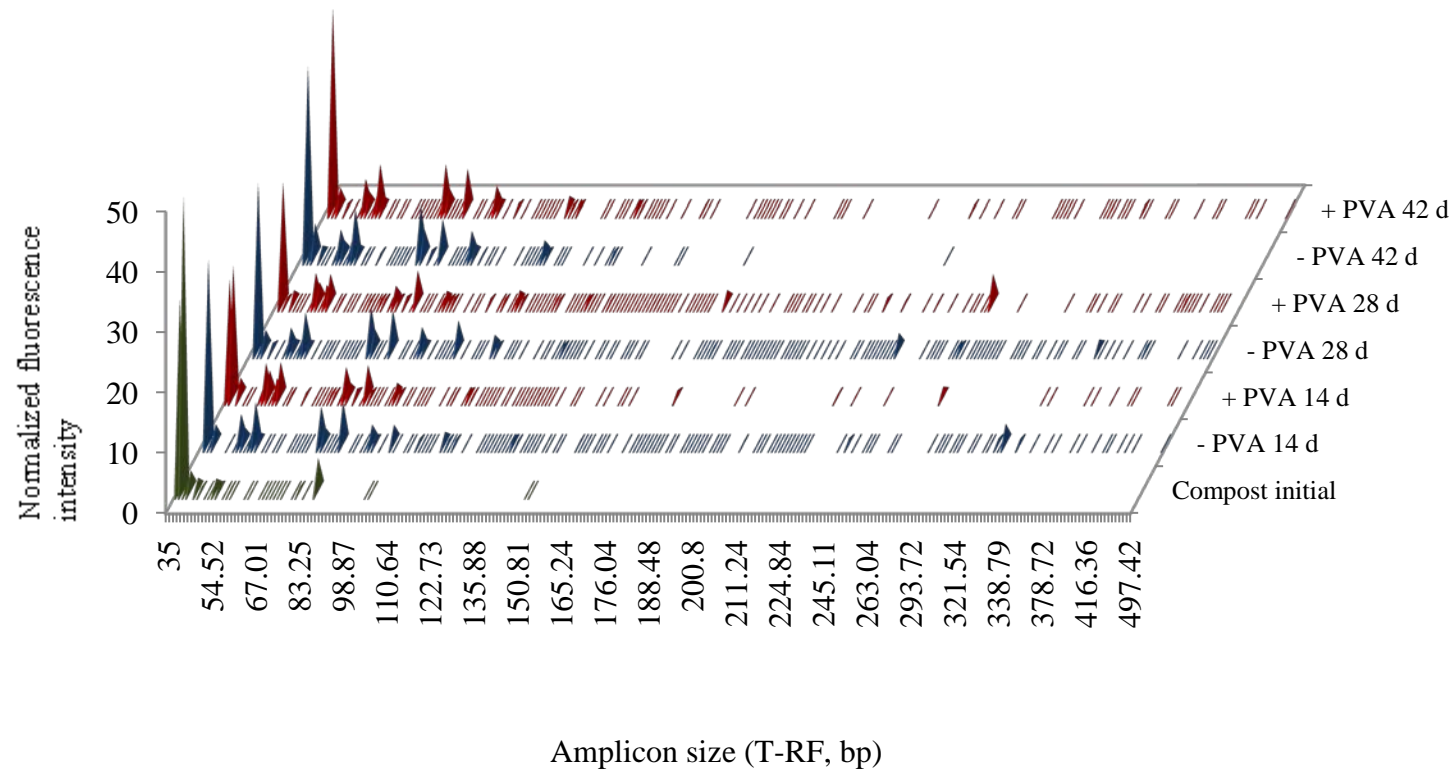


Figure 4-3. Effect of PVA on the fungal community profile of compost by TRFLP analysis. Commercial compost was amended with either sterile water (blue) or PVA (red) and incubated in containers for 42 days at 45°C. Total genomic DNA was extracted from triplicate samples from the initial (green) compost prior to incubation and after 14, 28 and 42 days, pooled and the ITS4/ITS5 rDNA amplified by PCR. Following digestion with HhaI, HEX labelled amplicons were separated by capillary electrophoresis and individual amplicon peaks normalised against the total. Amplicons less than 35 bp were excluded from the analysis.

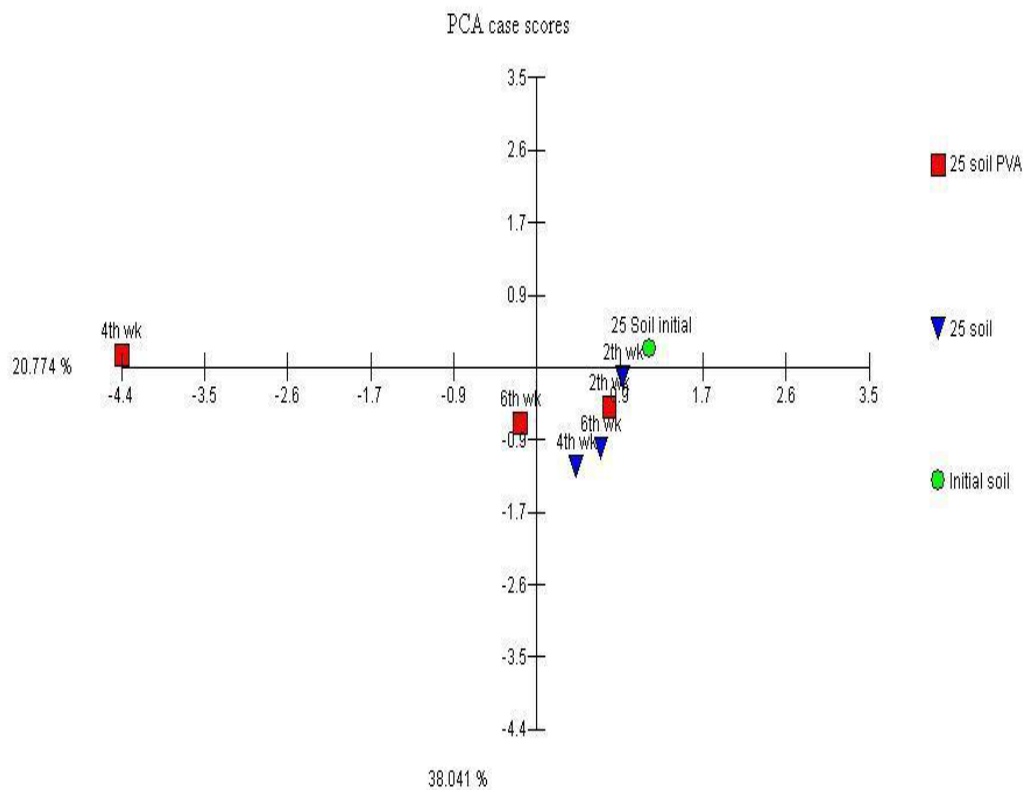


Figure 4-4. Principal Component Analysis (PCA) of T-RFLP data derived from amplified 18S rDNA from soil in the presence and absence of PVA and unpolluted soil. Soil was incubated at 25°C and TRFLP's analysed after 0, 2, 4 and 6 weeks. 20.774% and 38.041% represented the percentage of the total variance of each axis. PCA of variability was calculated by the presence/absence of TRFLP peaks by using binary analysis.

Samples	Week	Total TRFs	PVA unique TRF's	Shannon index (H')	Evenness
Soil initial	0	94	NA	2.57	0.57
Soil	+2	61	NA	2.74	0.67
Soil +PVA	+2	63	6	2.34	0.71
Soil	+4	88	NA	2.73	0.56
Soil +PVA	+4	157	76	3.60	0.58
Soil	+6	82	NA	2.57	0.61
Soil +PVA	+6	90	17	2.64	0.59

Table 4-1. Influence of PVA on the total number of TRF's, Shannon index (H') and evenness (E) of fungal communities in soil assessed by TRFLP following incubation 25°C over a six week period.

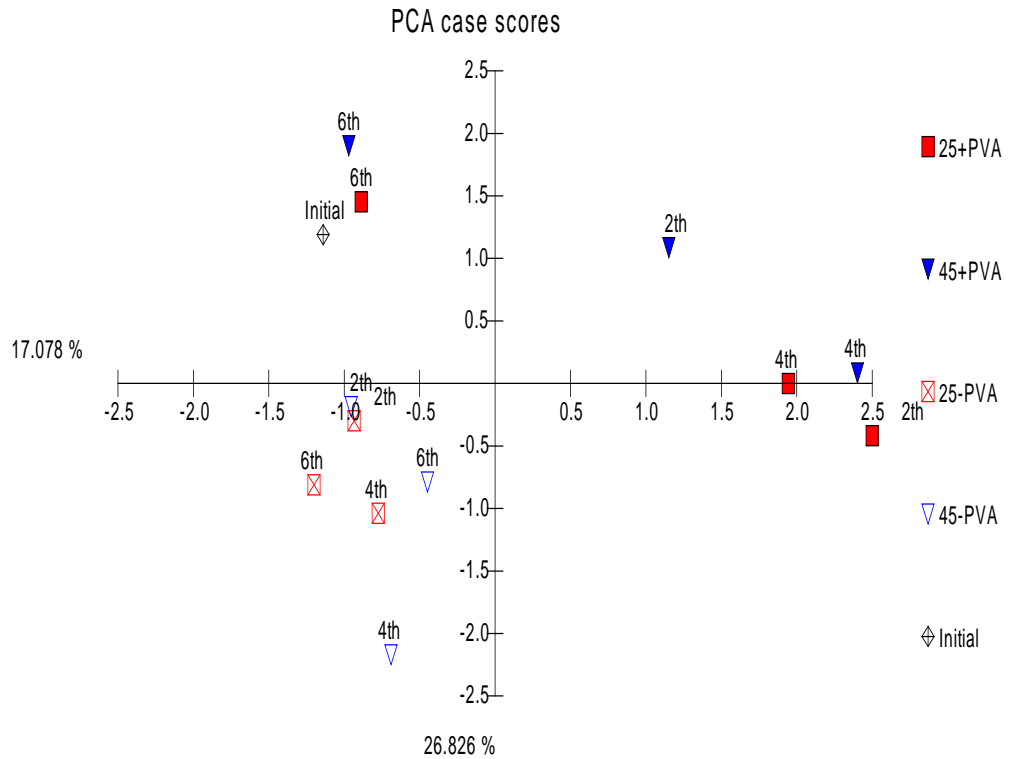


Figure 4-5. Principal Component Analysis (PCA) of T-RFLP data derived from amplified 18S rDNA from compost in the presence and absence of PVA. Compost was incubated at 25°C and 45°C and TRFLP's analysed after 0, 2, 4 and 6 weeks. 40.430% and 18.863% represented the percentage of the total variance of each axis. PCA of variability was calculated by the presence/absence of TRFLP peaks using binary analysis.

Samples	Week	Total TRFs	PVA unique TRF's	Shannon index (H')	Evenness
Compost initial	0	27	NA	1.48	0.45
25° Compost	+2	251	NA	4.36	0.79
25° Compost PVA	+2	157	7	3.65	0.72
25° Compost	+4	161	NA	3.07	0.60
25° Compost PVA	+4	132	6	3.43	0.70
25° Compost	+6	123	NA	3.06	0.64
25° Compost PVA	+6	98	4	3.07	0.67
45° Compost	+2	150	NA	3.06	0.61
45° Compost PVA	+2	89	12	2.92	0.65
45° Compost	+4	159	NA	3.17	0.63
45° Compost PVA	+4	147	32	3.56	0.71
45° Compost	+6	58	NA	2.70	0.66
45° Compost PVA	+6	113	7	2.86	0.61

Table 4-2. Influence of PVA on the total number of TRF's, diversity index (H') and evenness (E) of fungal communities in compost at 25°C and 45°C assessed by TRFLP following incubation over a six week period.

4.4 DISCUSSION

Conventional culture techniques are not sufficient to accurately profile microbial communities in the environment because only a small portion of microorganisms can be cultured in the laboratory (Bridge & Spooner, 2001). Non-culture based molecular techniques such as DGGE and TRFLP are commonly utilized methods for more accurately profiling of microbial communities although issues surrounding efficiency of DNA extraction and PCR bias also impact on accuracy (Anderson & Cairney, 2004; Clement *et al*, 1998; Denaro *et al*, 2005; Kirk *et al.*, 2004; LaMontagne *et al*, 2002; Peters *et al*, 2000; Suzuki & Giovannoni, 1996).

In this study, we aimed to evaluate the effect of polyvinyl alcohol (PVA) on fungal communities in soil at 25°C and in compost at 25°C and 45°C using TRFLP. TRFLP is known to be far more sensitive than DGGE and able to detect microbes at a lower abundance (Kennedy and Clipson, 2003). Previously, it was demonstrated that degradation of PVA appears to be limited to a relatively small number of fungi in soils (Mollasalehi *et al*, Chapter 2), as has also been demonstrated for bacteria. However, this study involved isolation of culturable fungi and therefore more fungal species may have been present that were unable to be recovered (Hawksworth, 2004). TRFLP analysis revealed that the soil fungal community in the absence of PVA at 25°C remained largely unchanged over the 6 week period. However, in the presence of PVA, the number of TRFs almost doubled after 4 weeks compared to the absence of PVA but then declined to levels equivalent to the absence of PVA after 6 weeks. This

was reflected in the number of new TRF's only found in the presence of PVA which rose from 6 after 2 weeks to 76 after 4 weeks before declining to 17 after 6 weeks. The appearance of numerous TRF's after 4 weeks in the presence of PVA was clearly visible on the electropherograms and PCA analysis revealed a clear separation of the population in the presence of PVA after 4 weeks while the population after 6 weeks more closely resembled the population seen in the absence of PVA. The data strongly suggest the selection of a number of additional species specifically in the presence of PVA but at low levels and that these declined by week 6 suggesting most of the PVA had been degraded and the population was returning to that found in the absence of PVA. In compost the situation was more complex as the population shifted considerably on incubation at either 25°C or 45°C in the absence of PVA. At 25°C, there was an initial proliferation of fungi in compost after week two which then decreased and remained relatively stable. An increase in fungi in compost at mesophilic temperatures has been observed previously and probably reflects the shift in temperature from the outdoor environment to 25°C enabling remaining readily utilizable substrates to be consumed (Tiquia, 2005). TRFLP analysis revealed that the presence of PVA appeared to select for a small number of additional fungi in the community in both soil and compost and that these appeared to be transient, presumably positively selected when PVA was present, but unable to be maintained once the PVA had been utilized. While the community profiles in the presence of PVA differed compared to those in absence of PVA, the most abundant members of the community were not affected and were largely

maintained in the presence of PVA. However, in compost at both 25°C and 45°C, the initial rise in the number of T-RF's and thus species detected after 2 weeks, was much lower in the presence of PVA suggesting that PVA entering composting waste may have an adverse effect on the diversity of the fungal community. The presence of PVA caused only a temporary appearance of specific fungal members in the profiles and largely returned to the profile observed in the absence of PVA. However, the profile at 45°C in compost appeared to cause a longer lasting shift in the profile. At 45°C, only thermophilic fungi are capable of actively growing and the number of species is restricted compared to mesophiles (Salar & Aneja, 2007), although, the number of detected TRFs at 45°C remained high. However, as TRFP does not distinguish between viable and non viable biomass, it may reflect that many of the mesophiles originally present were still present in the compost even if they were not viable or growing. Thus it is possible that PVA degradation may have occurred at a lower rate and that by week 6, sufficient PVA may still have been present to maintain positive selection for degraders.

Shifts in the microbial population following addition of a variety of contaminants are well documented, particularly for heavy metals and organic compounds including pesticides (Colores *et al.* 2000; Liu *et al.* 2012; Ge and Zhang, 2011; Gremion *et al.* 2004; Hamamura *et al.* 2006; Heilmann *et al.* 1995). Where these contaminants are toxic, selection of particular microbes is accompanied by decreases in the overall diversity, whereas non-toxic or environmental perturbations generally alter the microbial composition but do

not necessarily decrease diversity (Cruz *et al*, 2009; Esperschütz *et al*, 2007; Girvin *et al*, 2004; Li *et al*, 2008; Wu *et al*, 2012; Zhong *et al*, 2009). PVA is a non-toxic polymer and so unlikely to have a significant effect on the indigenous community if it is also not utilized by the majority of the organisms present.

Previously, it has been reported that only a relatively small number of fungal species appeared capable of degrading PVA in a number of different soils containing different fungal communities. In this study, only a small number of unique TRFs were transiently detected following addition of PVA suggesting positive selection for a small number of species capable of degrading PVA while the majority appeared largely unaffected. Thus, it appears that contamination with PVA causes transient selection for degraders but that the community returns largely to its original state prior to PVA exposure. The exception was in compost at 45°C, but this may have been due to a slower rate of degradation due to the more limited number of thermophilic compared to mesophilic species. Nonetheless, this study suggests that PVA does not cause major or permanent changes to soil or compost fungal communities and that PVA entering soil or into composting systems is degraded and while causing numerous small changes in the population, these changes are transient and once PVA is not degraded, the population returns to an equilibrium similar to that seen in the absence of PVA.

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5 CHAPTER FIVE

GENERAL CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

The following discussion focuses on the main conclusions and suggestions for potential future work following this present study.

Chapter 2. Isolation and characterization of polyvinyl alcohol degrading fungi from soils

Culture enrichment was used in this study to isolate fungal degraders from eight environmentally diverse soil samples. Previously, there was limited information available on PVA biodegradation mediated by microorganisms other than bacteria. In this study it has been confirmed that fungal species capable of degrading PVA are widespread, although for any one soil, the number of degrading species is limited. In addition, these soils were not previously contaminated with PVA, suggesting that PVA contamination of any soil environment will enrich for a limited number of degraders. The most common fungi recovered were *Galactomyces geotrichum* and *Trichosporon laibachii* although they were not recovered from every soil. All degraders isolated were capable of growing on PVA in monoculture, suggesting that each strain has the enzymatic capability to degrade and utilize this polymer.

While some authors have isolated single bacterial strains that can utilize PVA alone, most studies have indicated that PVA can only be degraded by mixed consortia where another strain is required to provide a growth factor(s) for growth and degradation (Chen *et al.* 2007; Hashimoto & Fujita, 1985; Mori *et al.* 1996; Sakazawa *et al.* 1981; Shima, 2001; Vaclavkova *et al.* 2007).

Furthermore, bacterial strains have previously been isolated from soils already contaminated with PVA and it is unknown whether uncontaminated soils have the pre-requisite bacterial strains for degradation. Thus, this study has shown that fungi may play an important role in removing PVA when it contaminates virgin environments.

The growth characteristics of the commonly isolated fungal strain *Galactomyces geotrichum* demonstrated that the molecular weight of PVA had little effect on the growth and utilization of the polymer, whereas bacterial strains have been shown to preferentially utilize lower molecular weight fractions (Solaro *et al.*, 2000). As strains were enriched for PVA degraders on mineral salts medium, it is possible that many more fungal strains have the capacity to utilize PVA but were unable to be recovered as they required additional complex nutrients. Further work should include enrichment on more complex media containing low concentrations of complex nutrients such as yeast extract or peptone.

Chapter 3. Fungal biodeterioration of polyvinyl alcohol coupons

PVA is increasingly being used for formulating biodegradable packaging and increasing quantities are likely to enter green composting systems. Therefore, this study investigated the fungal colonization of the surface of PVA films over 21 days in compost under different composting temperatures. Visible structural changes to the surface of PVA films were evident at all temperatures and environmental scanning electron microscopy (ESEM) revealed extensive surface damage and fungal colonization. *Galactomyces geotrichum* was found to

be the dominant colonizing organism recovered from surface of PVA at 25°C whereas *Talaromyces emersonii* was the sole species recovered at 45°C, 50°C and 55°C. Thus, the compost used in this study contained at least one mesophilic and one thermophilic fungal species capable of degrading PVA suggesting that PVA is actively utilized over a range of temperatures that are typically found at different stages of the composting process. However, as with the soil enrichment studies, the number of PVA degrading species appear very limited. It will therefore be necessary to conduct a much broader survey on a wide range of different composted materials to determine whether composts in general have an innate capacity for PVA degradation. In addition, it may be possible to enhance PVA degradation in composts by the addition of these two species at the start of the composting process and additional research on the effect of adding fungal spores of these species to the composting process and its impact on PVA degradation needs to be undertaken. Moreover, as PVA is formulated with other biodegradable plasticisers to form flexible films for packaging, such as glycerol, starch and collagen (Jecu *et al.*, 2010), the biodegradation of different formulated PVA films should also be studied.

Chapter 4. Impact of the water soluble polymer polyvinyl alcohol on soil and compost fungal communities

TRFLP is a non-culture based molecular technique which is widely used to assess the diversity of microbial communities and the impact of environmental perturbations on community structure and dynamics (Edwards and Zak, 2010;

Ge and Zhang, 2011; Krumins *et al*, 2009; Macdonald *et al*, 2009).

In this study, TRFLP was used to assess the impact of the addition of PVA on naturally occurring fungal communities in soil and compost. The TRFLP profiles revealed that the addition of PVA led to the selection of a small number of additional fungi after two to four weeks in both soil and compost but that by six weeks, these had largely disappeared, suggesting that these additional fungi were PVA degraders and that their disappearance was probably due to the utilisation of the PVA. As previous studies (Chapters 3 and 4) revealed that only a small number of fungi are capable of degrading PVA and as PVA is non-toxic, the addition of PVA had little impact on the major species present in both soil and compost.

While this technique is a valuable tool for monitoring changes in community diversity, it does not give information as to the species present. However, the advent of pyrosequencing, in which sequence information for all the major species present is generated, would potentially enable the identity of unique species appearing in response to PVA to be identified or at least to be phylogenetically analysed (Buee *et al*, 2009; Dumbrell *et al*, 2011; Handl *et al*, 2011; Nonnenmann *et al*, 2012). In addition, as this experiment examined only the response to a single addition of PVA, further work examining the effect of continuous contamination should be investigated to establish whether long term PVA contamination would cause more radical changes in the fungal community and whether such changes could be reversed in PVA contamination ceased.

Suggestions for future work

From the findings in this research, the following insights have been suggested for future research:

- Commonly isolated fungal strains were enriched on mineral salts medium, it is possible that many more fungal strains have the capacity to utilize PVA but were unable to be recovered as they required additional complex nutrients. Further work should include enrichment on more complex media containing low concentrations of complex nutrients such as yeast extract or peptone.
- Non cultures based techniques are valuable tool for monitoring changes in community diversity, but it does not give information as to the species present. By, the advent of pyrosequencing, in which sequence information for all the major species present is generated, would potentially enable the identity of unique species appearing in response to PVA to be identified or at least to be phylogenetically analysed
- This experiment examined only the response to a single addition of PVA, further work examining the effect of continuous contamination should be investigated to establish whether long term PVA contamination would cause more radical changes in the fungal community and whether such changes could be reversed in PVA contamination ceased.

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