FUNGAL COLONISATION AND CONTAMINATION OF CINEMATOGRAPHIC FILM: A THREAT TO FILM AND ARCHIVISTS

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FUNGAL COLONISATION AND CONTAMINATION OF CINEMATOGRAPHIC FILM: A THREAT TO FILM AND ARCHIVISTS

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Declaration of Authorship

I, Gavin David Bingley, declare that the thesis entitled *Fungal Conlonisation and Contamination of Cinematographic Film: A Threat to Film and Archivists,* and the work presented in this thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

• this work was done wholly or mainly while in candidature for a research degree at this University;
• where I have consulted the published work of others, this is always clearly attributed;
• where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
• I have acknowledged all main sources of help;
• where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
• parts of this work have been published as Bingley and Verran (2012) and Bingley et al. (2012)

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Abstract

Film archivists have expressed concern regarding the aerial release of fungal spores during inspection of mouldy cinematographic film. The aim of this study was to investigate this and identify of the contaminants causing this concern, and then to make recommendations to archivists regarding safe handling of such film. Eighteen films donated to the North West Film Archive, in Manchester, UK, were examined. During a simulated inspection procedure, spore release ranged from zero to several thousand spores per m^3 (i.e. above numbers considered 'safe'), with films that were the most visibly mouldy usually yielding the highest numbers of colonies. Predominant contaminants were *Aspergillus* and *Penicillium*. *A. versicolor* was the most common species isolated (8/15 isolates identified by CABI), and gelatinase assays indicated that this was also the most gelatinolytic species, perhaps explaining its high frequency of isolation, and indicating risk to film. No overtly pathogenic species were isolated, so the risk to archivists may be considered low.

Thus, informed recommendations for safe handling could be made. Wiping of lightly contaminated reels may remove superficial surface mould growth but cleaning of heavily contaminated reels is not recommended, because hyphal growth is likely to have penetrated the gelatine emulsion layer. In addition, high numbers of spores released could pose a health risk to archivists.

Although the contamination of some film was no longer viable, actively growing mould may cause further deterioration if reels are not stored in conditions not favourable to growth. Currently, assessment of viability is by culture, a method not usually available to archivists. An alternative method was explored by analysing microbial volatile organic compounds (MVOCs) released from fungi isolated from mouldy reels, using headspace solid phase micro-extraction (SPME) coupled with gas chromatography–mass spectrometry (GC-MS), to determine whether any compounds were common to isolates. Over 150 volatile compounds were detected from 16 fungal isolates, with 3 VOCs being the most common: 1-octen-3ol from 13 isolates, 3-octanone from 10 of the isolates and 3-octanol from 4 isolates. These three key chemical markers are indicative of viable fungal growth on cinematographic film, thus their detection would preclude the need for traditional microbiology laboratory culture methods. Development of 'one-shot ' screen printed sensors coupled with a hand held device proved possible, enabling in-can indication of viable mould, thus enabling films to be stored appropriately to halt further growth.

This study broadened the knowledge of contaminated cinematographic film in the hope that more valuable archive footage can be preserved.

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Published Journal Articles

1) Bingley, G., & Verran, J. (2012). Counts of fungal spores released during inspection of mouldy cinematographic film and determination of the gelatinolytic activity of predominant isolates. International Biodeterioration & Biodegradation, 1-7..... Page 263

2) Bingley, G.D., Verran, J., Munro, L., Banks, C. E. (2012). Identification of microbial volatile organic compounds (MVOCs) emitted from fungal isolates found on cinematographic film. Analytical Methods, 4, 1265-1271..... Page 260

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Chapter 1

Archiving the Recorded Image

1.0. Archive Material

Evidence of the cultural and intellectual development of the human race is contained in many types of archival objects, each composed of inorganic and organic materials. National archive material is considered to be of great historical and cultural importance not only to the countries to which they belong to but also to the global community, as it can show how society has developed and/ or indicate instances of great human achievement. Deterioration of recorded images is of particular concern; examples include textiles (Mitchell and Mcnamara, 2010), paper (Zotti et al., 2008), paintings (Ciferri, 1999) and photographs (Lourenco and Sampaio, 2009). A relatively recent addition to these materials are those which are used to store moving images, such as cinematographic film (cinefilm) (Abrusci et al., 2004a, 2004b, 2005, 2006, 2007, 2009), compact discs (CDs) and magnetic media (Cappitelli and Sorlini., 2005). Deterioration of these materials not only devalues the item financially but can also make it unusable. On a personal level, archive material used to record images, such as home movies, photographs or scrap books are used to document a person's life and can have a great personal meaning for the owner. Thus preservation and conservation of these materials is of concern. Deterioration of archival material is well documented, whether by chemical or biological mechanisms. However, the literature available on biodeterioration of moving image materials in particular is relatively sparse, thus will be the focus of this study.

1.1. Biodeterioration

Biodeterioration was described by Hueck (1968) as *'any undesirable change in the properties of a material caused by the vital activities of organisms'*, and archive materials are constituted of many different substrates which could potentially be growth media for microorganisms. Microorganisms that are airborne and present in dust pose the greatest threat with regards to biodeterioration, because prior to donation to archives, historic materials are often stored in lofts and cupboards and other places where dust becomes a problem. Items may also be subject to damp conditions, which enables growth of microorganisms and subsequently enhances the damage done to the materials involved. Human activities can also influence biodeterioration and the organisms involved. A major example of this is Lascaux Cave in France discovered in 1940 (Ciferri, 1999). The paintings in this cave were thought to be from 15,000 to 13,000 B.C and were painted in various minerals and animal fats, but despite their age they were in excellent condition. The cave was opened to the public for fifteen years but was quickly closed due to the

discovery of *Bracteacoccus minor*, a unicellular alga which was deteriorating the paintings. Human activity had brought in organic material and increased carbon dioxide levels, and combined with the electric lighting created adequate conditions for the growth of algae and fungi. This emphasises the need to control exposure of historical artefacts and archives to human contact, and take suitable precautions to ensure that further deterioration is not initiated.

1.1.1. Mechanisms of Biodeterioration

Allsopp et al. (2004) has suggested that there are several different types of biodeterioration and that one organism may cause more than one type.

- Physical or mechanical biodeterioration is where the growth or movement of the organism disrupts or distorts the material.
- Fouling or soiling (aesthetic biodeterioration) is where organisms grow on surface dirt but do not cause damage to the actual material but can still detract value (Allsopp et al., 2004).
- Metabolic products such as pigments, enzymes, and inorganic and organic acids can interact with and deteriorate the materials. Organisms growing on surface dirt may also produce chemical agents which have a deleterious effect on a material. This is known as biochemical dissimilatory biodeterioration (Allsopp et al., 2004).
- Biochemical assimilatory biodeterioration involves the organism utilising the material as a food or energy source. Fungal mycelia can then expand and penetrate a material, a process that is often aided by the production of enzymes such as cellulases and proteases, which aid in the breakdown of materials made of cellulose such as paper and wood.

1.2. Deterioration of Moving Image Storage Media

There have been many types of media for recording moving images. These were composed of different materials including: synthetic polymer and resin based media (e.g. compact disks (CDs)), magnetic media (e.g. floppy disks, hard drives, video and audio cassette tapes) and organic based materials (e.g. cinematographic film reels (Abrusci et al., 2004a)). These media are designed for long term storage of information, and are potentially susceptible to degradation and deterioration over time. This can be chemical and/or biological, and some mediums are more susceptible than others depending on their composition.

1.2.1. Computer storage media

Storage media used by computers have one thing in common: they are not human readable and require a computer and compatible hardware to convert information to visible form via a monitor (Frey and Susstrunk, 2000). This poses a problem when the formats become obsolete and transfer of data to a newer medium is often required e.g. from floppy disk to CDs. Transfer of large amounts of data requires significant time and expense in terms of purchasing new media carriers.

Limitations of the technologies during data transfer and subsequent storage could result in loss of information. Storage of data on digital media requires compression of which there are two types: lossy and lossless (Anon. (animemusicvideos), 2002). Lossless compression retains all the original data but can use a huge file space. Lossy compression compresses the information into small file sizes by removing data such as colours not visible to the human eye in videos and photos, or sounds in music not audible by the human ears, so that it appears that no data are lost. Repeated transfer to different storage devices can also cause some data loss (Krogh, (n.d.)) and could lead to degradation and loss of data and quality of files, in particular video and audio files.

In addition to technological limitations that could result in loss of information, physical, chemical and biological deterioration of these media are also of concern. Khan et al. (2008) investigated mould growth on floppy disks and CDs. Floppy disks comprise of polyester Mylar® (polyester and iron oxide) and polyvinyl chloride (PVC). These media do not contain organic components, thus it would be expected that this would limit microbial growth (Khan et al., 2008). Indeed, polyester Mylar® has been shown to be highly resistant to microbial attack and in tests remained essentially unchanged after a 36 month burial in 'bacteria-infested' soil (Szczepanowska and Wilson, 1998).

However, it has been observed that PVC and plasticisers could be potential substrates for microbial growth (Cappitelli and Sorlini., 2008), and it was observed by Khan et al. (2008), by the use of phase contrast fluorescence microscopy and culture methods, that bacteria and actinomycetes were able to grow on the surface of these materials. Thirty actinomycete and twenty three bacterial colony morphologies were isolated from mouldy floppy disks based on colony morphology of which 11 and 5 were selected respectively and identified to be primarily *Streptomyces, Bacillus* and *Micrococcus* and species. In addition to bacterial growth on the surface, fungal colonisation of floppy disks has been observed by McCain and Mirocha (1994). However, penetration of the materials is not necessary for deterioration to occur because colonisation on the surface of these materials still interferes with reading and writing on the media as

well as reproduction quality, thus valuable archive material could still be damaged or lost.

Like floppy disks, CDs are also composed of inorganic materials. The main components of a CD are polycarbonate plastic, a metallic layer and organic dyes (Khan et al., 2008). A *Geotrichum* like fungus was shown by Garcia-Guinea et al. (2001) to have grown into a CD by displacing the metal and polycarbonate particles, a process known as bioturbation (Gabet et al., 2003). Mould growth on the CD was suspected by visualisation with the naked eye of fungal bioturbation paths. Growth was confirmed by cutting the CD into pieces and placing a piece in a Petri dish, where the mould continued to grow. Due to the chemical stability of CDs and floppy discs it is expected that degradation of these materials will only occur after a long period of time (Khan et al., 2008). However, microorganisms do not have to utilise the materials for growth in order for them to pose a problem with data retrieval. Their presence on the surface of the media is enough to affect the read and write functions.

1.2.2. Videotapes

Videotapes were the primary recording medium for video prior to the invention of DVDs and recording onto hard disks. There are many types of videotape format some of which are already obsolete and some which never gained popularity. The most important to note are those 1 ½" tapes, which were used in the professional recording industry and also those that were widely used for home use. Two main competing technologies invented in 1975-1976 for domestic use were; Betamax (Sony) and VHS (JVC (Anon. (VideoOlson), n.d.), the latter of which became the dominant format in the videotape market (Owen, 2005). Betacam SP (Sony) was used for most TV stations and high end production houses (Anon. (VideoOlson), n.d.).

Videotapes were made of materials similar in composition to floppy disks i.e. a polyethylene terephthalate (PET) and polyvinyl chloride base which has been shown to be highly resistant to microbial attack (Müller et al., 2001), although there seems to be very few scientific papers describing biodeterioration of PET videotape. However, early videotape bases were made with cellulose acetate, which is potentially a substrate for microbial growth (Vassou, 2008) and Anon. (International Federation of Library Associations and Institutions), n.d.). In addition, cellulose acetate deteriorates over time, becomes brittle and shrinks, and these tapes were also susceptible to vinegar syndrome (i.e. formation of acetic acid from the breakdown of cellulose acetate) (Bigourdan et al., 2006). Thus cellulose acetate was only used from the mid-1930s to mid-1960s after which PET became the material of choice for video and audio tape (Schüller, 2008). The magnetic

coating attached to the base holds the information onto the videotape, and is made of metal particles such as iron oxide (Anon. (eNotes), n.d.), which determine the magnetic properties of the tape. Lubricants made of fatty acids, and carbon black are added to the coating to decrease the friction and wear of the tape, and enhance the durability and performance of the materials (Anon. (eNotes), n.d.). Binders provide structure and attach the metal particles along with other compounds such as lubricants, cleaners and anti-static particles to the PET base. The binder is made of polyester urethane and is the main limiting factor in determining the life of a videotape (Wheeler, 2002). Polyester urethane absorbs water and hydrolysis of this can lead to chemical deterioration of the tape, which causes the tape to become gummy or sticky (sticky-shed syndrome). Therefore, it is recommended that videotapes should be stored at lower temperatures and humidities than ambient air, in order to prevent this. There is information available regarding the storage of magnetic tape, thus archives are able to store the materials in conditions that prevent chemical and microbiological deterioration. It is recommended that conditions for storage of videotape in archives should be 11- 23^{o}C and 20-50% RH in order to prevent loss of magnetisation of particles and degradation of the binder and prevent microbial growth (Bigourdan et al., 2006 and Wheeler, 2002). However materials kept in the domestic environment are not likely to be preserved in the same way, so are susceptible to deterioration.

Fungal deterioration of video tapes is a relatively new problem of unknown significance. However, this is likely to become more of a problem in the future as the format becomes obsolete and the tapes are left for extended periods of time in places that are ideal for mould growth e.g. attics, lofts and cellars. Suspected mould growth on video tape can often be seen on the side of the reel through the clear plastic window (Fig.1) and is even more obvious when the tape is opened up (Fig.2), although fungal growth would only be confirmed by culture (assuming viability) and/ or molecular identification. This problem has been acknowledged in the media (Vassou, 2008) and there are numerous companies offering to either clean video tapes or transfer them to a digital medium such as DVD (Anon. (Eagle TV), 2011and Anon (video99), 2011). There is also information available on websites which offer advice on how to care for video tapes and remove mould growth if they are contaminated (Brothers, n.d. and Anon. (Harvard University), n.d.).

Due to format obsoletion and the development of digital technologies, many tapes in personal collections are being discarded (Casto, n.d.). Items such as films can be replaced with DVDs or Blu Ray etc. and personal videos can be transferred to digital formats using tools such as VHS to DVD converters (Rampur, 2011).

Videotapes that do remain in personal collections are often stored in less than ideal

conditions, thus archives play an important role in preserving videotapes and transferring the data to new media, before technology is no longer available for playback as these formats become obsolete.

Mould growth on videotape and other magnetic media is a problem that is likely to become more significant in the future. This is due to the relative youth of these materials and the microbiological resistance of the materials which means it may take some time before deterioration occurs (Khan et al., 2008). However, due to format obsoletion (Frey and Süsstrunk, 2000) and the possibility of long term storage of videotapes and other magnetic media, this is likely to be a greater, but still limited, problem in the future, and less in the present. Based on the small number of studies regarding biodeterioration of magnetic media, there does appear to be a lack of information available in the literature thus suggesting further research is needed.

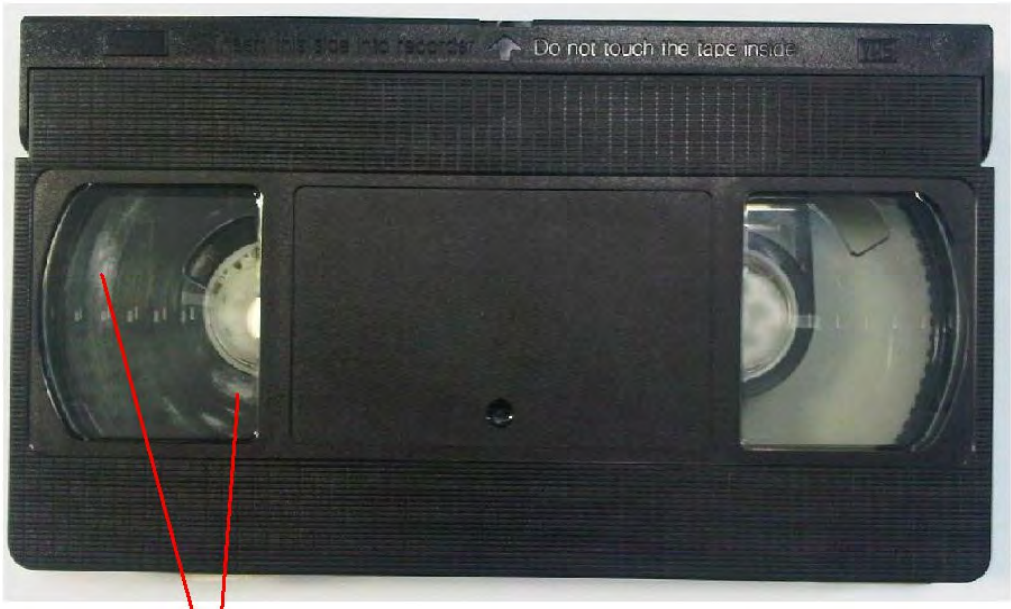

Fig.1. A VHS video tape that has suspected mould growth which can be seen through the clear plastic window.

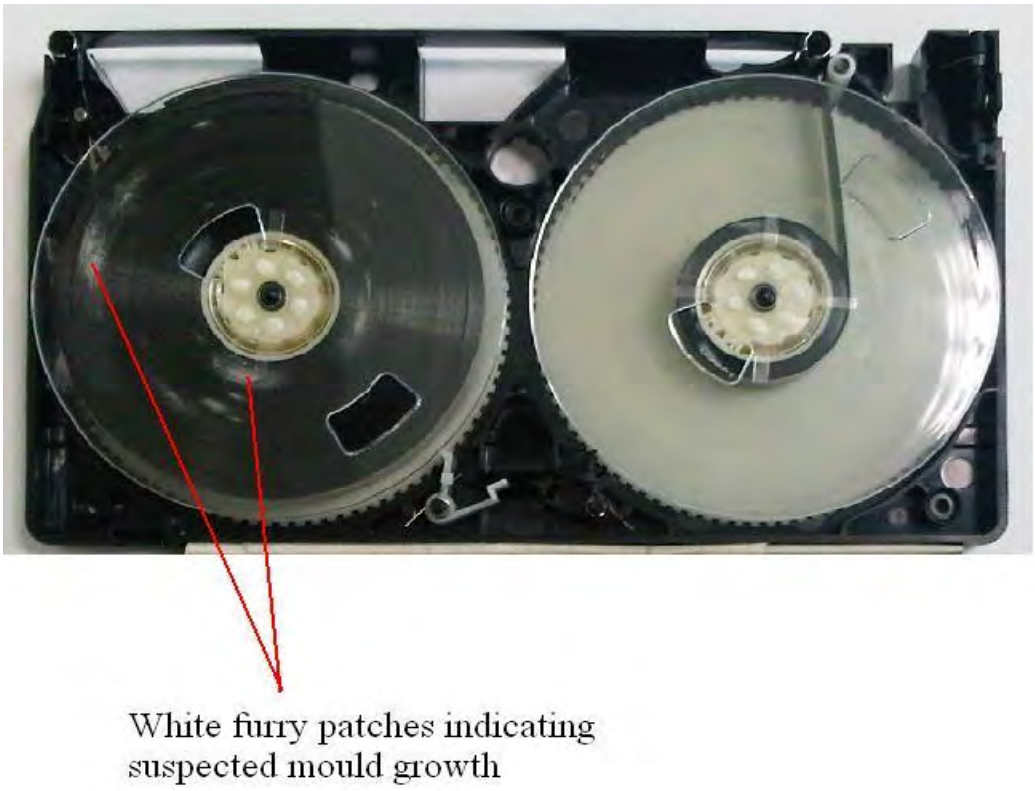

Fig.2. A VHS tape that has been opened up by removing the top half of the casing. White fungal mycelia can be seen growing on the reel.

1.2.3. Cinematographic Film

Another form of medium containing motion pictures is cinematographic film. Cinematographic film is a technology which has been around since the 1880s-1890s (Anon. (EarlyCinema.com), n.d.). Thomas Edison is credited with the invention of the first practical film in 1889 and also invented the Kinetoscope which was a primitive projector used to view moving film images (Nystrom, 2001). Cinematographic film has advantages over some other media formats: it requires cheaper and simpler technology to view, it is age resistant so it can be stored over long periods of time with relatively few resources, and can be used as an intermediate storage medium for transfer onto multiple digital formats (Anon. (Council on Library and Information Resources), 1997). However, unlike other media made of synthetic polymers, cinematographic film is made of organic components, which combined with their relative age to magnetic media means they are more susceptible to microbial deterioration (Abrusci et al., 2004a, 2004b, 2005, 2006, 2007, 2009). It is known that there is a problem concerning the biodeterioration of film stock, yet there is a lack of information currently available in the literature, especially with regards to health risks by exposure to mould spores when examining the reels and the identifications of the fungi responsible for deterioration. Therefore cinematographic film will be the storage medium which will be the main focus of this study.

Film is composed of three main generic layers (Lourenco and Sampaio, 2009): a photosensitive emulsion coating, a gelatine binder and a plastic polymer base support, which has been made of three different materials since the invention of film (Abrusci et al., 2005).

1.2.3.1. Film Bases

1) Cellulose nitrate (1889-1950)

Cellulose nitrate based film was in use between 1889 and 1950 and was ‘first used as a base film support as it was the oldest known and understood plastic...and is manufactured by the nitration of cotton linters' accomplished by mixing them with nitric and sulphuric acid then neutralising the acid with sodium bicarbonate

(Anon. (National Film and Sound Archive), 2008). Once the cellulose and nitric acids and sulphuric acids are mixed, the resulting polymer is mixed with a solvent such as camphor (a plasticiser) and cast on a highly polished flat bed to create a thin, flexible plastic film (Anon. (National Film and Sound Archive Australia (B)), n.d.). Under certain conditions nitrate film can break down to produce components that are toxic and oxidising resulting in combustion. Complete nitration of the cellulose produces gun cotton which is extremely

flammable and explosive even at low temperatures (Anon. (Nebraska State Historical Society), n.d.). Nitrate film is highly nitrated, and the addition of camphor increases this flammability, thus is only kept in specific archives which maintain the film under storage conditions which prevent fire and further chemical breakdown.

2) Cellulose Acetate (1948-2000)

This study however does not concentrate on cellulose nitrate reels but focuses on cellulose acetate films. Cellulose acetate film or 'safety film' introduced in the 1940's has advantages over nitrate film due to its resistance to burning (Anon. (National Film and Sound Archive Australia (B)), n.d.). The structure of cellulose triacetate is similar to cellulose nitrate except the nitro (NO_2) groups are replaced with acetyl groups. The cellulose fibres are mixed with acetic compounds and sulphuric acids to connect the acetyl groups to the cellulose. During early manufacture, it was impossible to remove all of the residual sulphuric acid and the acid would become trapped which would rapidly decompose the polymer. To remove the acid, the polymer was partially hydrolysed to remove some of the acetyl groups. This reduced the cellulose triacetate to cellulose diacetate which had poorer physical properties but was more chemically stable. Advances in manufacturing methods meant that acids could be removed without the removal of the acetyl group's thus increasing stability and physical properties. Plasticisers and cements in cellulose acetate films are acetone, methylene chloride and 1-4 dioxane. Triphenyl phosphate (TPP) is also added to the solvent mixture as an additive to remove solvents and manufacturing additives from the film base and act as a fire retardant which increases the temperature that is required for acetate to burn. Cellulose acetate bases were in use until the 1980s to 1990's where it was replaced by polyethylene terephthalate (Anon. (National Film and Sound Archive Australia (B)), n.d.) and Edge et al., 1991).

3) Polyester (Polyethylene terephthalate) (1990s-present)

Polyester like other esters is created by a condensation reaction between an alcohol and an acid (i.e. ethylene glycol and terephthalic acid) (Anon. (National Film and Sound Archive Australia (B)), n.d.). The ester groups in the polyester chain are polar so that the positive and negative charges of different ester groups are attracted to each other. The carbonyl oxygen atom has a slight negative charge and the carbonyl carbon atom has a slight positive charge allowing the ester groups of nearby chains to line up with each other forming strong linear structures. Unlike cellulose acetate and cellulose nitrate film bases, polyesters do not require plasticisers incorporated in them. The tough rigid structure of

polyester means that it is extremely resistant to tearing so is an ideal film base for the rigours of commercial cinema projection (Anon. (National Film and Sound Archive Australia (B)), n.d.). Despite having these properties and even being used for some stills in the mid 1950s, it is only since the mid 1990s that polyester has become the material of choice for the base of release print materials.

1.2.3.2. Determining Film Base Composition

There are several methods that are used to determine film base composition (Anon. (National Photographic Society, 1999). Identification of film bases is essential, to ensure proper handling and storage of the reels in order to preserve the reels and avoid risk to archivists (e.g. combustion of cellulose nitrate reels).

There are three non-destructive ways of finding out the base type of a film reel:

- Edge markings were used by some manufacturers to describe the film base: 'Nitrate' is cellulose nitrate film, 'Safety' is cellulose acetate and 'Estar' or 'Cronar' is polyethylene. This is not 100% reliable though, because film may have been copied onto a different film base, therefore the description would also be copied, thus describing the wrong film base (e.g. nitrate film may have been copied onto cellulose acetate film).
- Notch codes are small notches cut along one edge of the film border. Two were used by Kodak on film before 1949: a 'V' shaped notch indicated cellulose nitrate whilst a 'U' shaped notch indicated cellulose acetate.
- A polarisation test can be used to identify polyester (polyethylene) film. This requires placing the film between two filters or polarised sunglasses lenses and holding them up to the light. If the base shows to allow only dim light and no colours to pass though then this is cellulose nitrate or cellulose acetate. If there is a shimmering and rainbow-like patterns then indicates that the base is polyester.

Destructive methods can also be used to determine the film base but require hazardous chemicals which permanently damage the film, therefore only a small piece of film should be taken for these tests. These include:

- A 'float test' which uses trichloroethylene which is a known carcinogen. This test relies on difference in densities of film base types for identification. A small piece of film measuring 6mm is cut from the film border and placed in a test tube containing this chemical. If the film floats to the top it is cellulose acetate. If the film hovers in the middle it is polyester film. If the film sinks to the bottom it is

cellulose nitrate film.

- A diphenylamine test can be used to identify cellulose nitrate film. A small sample of film is placed on a microscope slide and a small drop of solution containing 90% sulphuric acid and diphenylamine is applied to the film for 60 seconds. If the film turns a deep blue it is cellulose nitrate, if the film remains clear or has a small tinge of blue then the film is cellulose acetate or polyester.
- A solubility test can be used to identify nitrate films (Anon. (National Film and Sound Archive Australia (B), n.d.). Nitrate film is soluble in a wide range of solvents such as ether, ethyl and methyl alcohol, whilst acetate and polyester films are not.

1.2.3.3 Emulsion Layer

In addition to the film base, there are other components which constitute the emulsion layer; a photosensitive emulsion coating (or sensitised layer) consisting of metallic silver particles on black and white images and dyes on colour images, and a binder which since the beginning of the 20th Century, has most commonly been based on gelatine. (Ball et al., 1998). Situated between the base and the emulsion layer is an anti-halation layer, which is usually a neutral density grey dye added to a layer of gelatine, and avoids the reduction of the sharpness of an image caused by scattering of light around bright objects (Abrusci et al. 2004a). There is also a protective overcoat that prevents physical damage to the image forming layers, e.g. scratches, which would impair image quality. A schematic diagram demonstrating the transverse section of film is illustrated (Fig.3).

Gelatine has many properties which make its use ideal for cinefilm (Abrusci et al., 2004b). Gelatine has hydrophobic properties, which enables the coating of several emulsion layers at the same time and also for chemicals to diffuse through different layers. In cinematographic film this is important as to allow diffusion of dyes (in colour film) or silver particles (black and white film). Gelatine can also be cross linked to increase the film stability and mechanical strength which increases resistance to higher temperatures and higher acid or alkaline conditions. Attempts have been made to replace gelatine with synthetic polymers to increase microbial resistance and reduce chemical deterioration but as of yet none have been 'satisfactory substitutes' (Abrusci et al., 2006). Gelatine is separated into grades and is classified on the strength of the gel formed. It is categorised from lowest to highest grades as: edible, pharmaceutical, photographic and technical (Anon. (Milligan and Higgins), 2009).

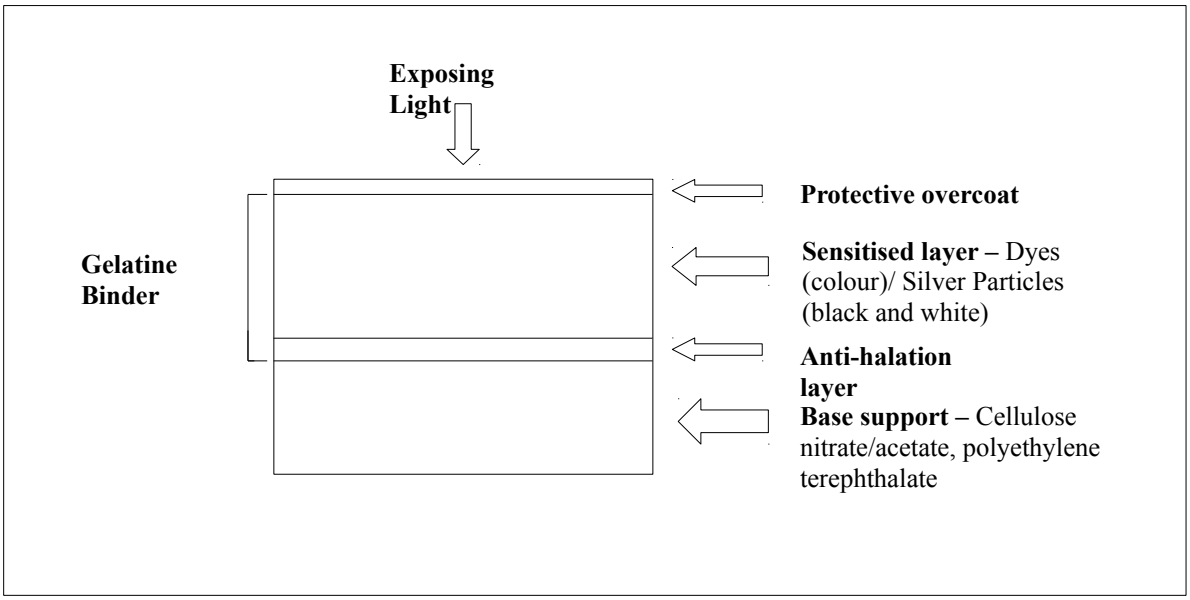

Fig.3. Cross section of a motion picture film showing the layer arrangement. The components within the gelatine binder constitute the emulsion layer (Not drawn to scale: approximate measurements of 1 frame of 16mm film: W=8mm D=16mm H=1mm (thickness of base and emulsion layer))(Adapted from Abrusci et al., 2004a).

Quality assurance and quality control testing requires specialist equipment to ensure gelatine meets specific physical, chemical and microbiological parameters (Anon. (PB Gelatins), n.d.). Photographic gelatine is made primarily with alkaline-processed bone type-B gelatine which is cross linked by chemical products to enhance strength and stability (Abrusci et al., 2006), and one of the strict specifications for photographic gelatine is that it meets a certain high gel strength indicated by a high 'bloom value'. The bloom value is measured by a gelometer during gelatine manufacture and is ‘the force in grams required to depress a standard plunger 4mm into the gel prepared from a 6.67% aqueous solution and cooled at 10^{o}C (Abrusci et al., 2006). It has been suggested (Bodner, M, (2009), North West Film Archive (NWFA), Personal communication) that lower grades of gelatine have been used for making photographic film and this may be the reason some film contains much more fungal growth than others. However, investigation of this is beyond the scope of this project.

1.2.3.4. Chemical Degradation of Film

Degradation of cinematographic film by microbes will be the focus of this study, but film can also be degraded by the breakdown or change in chemical components. The films used in this study were cellulose acetate, although swabs of cellulose nitrate films were provided by the British Film Institute (BFI).

Chemical degradation of cellulose nitrate films is a slow process primarily involving oxidation reactions. Nitrate film degrades because of its chemistry, as the polymer is made by substituting a strong acid onto an anhydro-glucose polymer chain (Vitale, 2009). These strong acids which are released when the film begins to deteriorate, can soften the gelatine, possibly assisting spoilage by microorganisms and also cause rusting of metal film cans (Ball et al., 1998). When deteriorating, nitrate film produces noxious nitric acid gases (nitric oxide, nitrous oxide and nitrous dioxide); and display amber discolouration which often makes the film soft, sticky or powdery with a rusty appearance (Anon. (National Film and Sound Archive Australia (B)), n.d.). 'Spoking' can also occur due to moisture loss which can cause the reel to become misshapen (Fig.4). Eventually the cellulose nitrate film gradually breaks down completely, but humidity and elevated temperatures increase the speed at which this occurs (Anon. (Kodak (A)), n.d.).

Fig.4. A cellulose nitrate film reel exhibiting chemical deterioration. Deteriorating films appear rusty, sticky or powdery and 'spoke' due to moisture loss.

Information regarding storage of cellulose nitrate reels from Kodak, states that if cellulose nitrate film is stored 'in large quantities of about 5,000 feet or more and in non approved storage cabinets without proper ventilation, it becomes a fire hazard' (Anon. (Kodak (A)) n.d.). However, Kodak notes that spontaneous combustion requires some 'pushing' and describes a laboratory study in which a 1,000 foot roll of film kept in a can, only combusted when exposed to a temperature of 41ºC for 17 days. However, the combustion of one film could create a catalytic effect which could lead to combustion of other reels resulting in major film losses. Thus, cellulose nitrate films should be stored in conditions which minimise the fire hazard, such as air conditioned vaults out of direct sunlight (BFI, 2012. Personal communication).

Deterioration of cellulose acetate differs from cellulose nitrate. If stored for long periods or in poor conditions such as heat and moisture, chemical degradation of cellulose acetate film results in ‘vinegar syndrome’ where the acetate breaks down to form acetic acid, which in turn causes further degradation of the film (Fig.5). Delamination can occur in rare circumstance, a process where the image forming layer separates from the polymer base. This renders the film completely unusable and occurs when the film is exposed to high humidity and temperature, such as in a damp cellar or near a leaky water heating system. Cellulose acetate films when deteriorated, release acetic acid gas, and appear to have red or blue discolouration, shrinkage, brittleness and presence of bubbles or crystals (Anon. (National Film and Sound Archive Australia (A), n.d.). Archives currently use 'A-D strips' to detect and measure the severity of vinegar syndrome (Anon. (Academy of Motion Picture Arts and Science), n.d.). These are essentially strips of litmus paper or pH strips, which change colour depending on the level of acetic acid produced from a deteriorating film. Flaws in the manufacturing process can also be responsible for causation of these processes.

Fig.5. A cellulose acetate film reel that has been severely affected by vinegar syndrome. (Anon. (National Film and Sound Archive Australia (C), n.d.)

Polyester film is the most recent development and has been in use since the 1990s (Abrusci et al., 2005). The chemistry of this film means that it is not prone to vinegar syndrome and is not flammable. This means it is highly stable and 'the life of a polyester film is more likely to be determined by the durability of the emulsion layer rather than the base' (Ball et al., 1998). Whilst chemical deterioration of polyester is unlikely, microbial contamination and subsequent colonisation may facilitate this process. Some microorganisms are able to attack synthetic polymers such as polyester (Cappitelli and Sorlini., 2008), and include fungal species such as *Aspergillus fumigatus,* which can produce depolymerases to cleave polyester chains (Scherer, 1996). This may also pose an additional problem, because polymers such as plastics have recently been used as conservation treatments and protective coatings of archive materials. These factors could mean that synthetic polymer materials such as the polyester film base could be susceptible to microbial attack in the future. Flemming (1998) described a range of biological mechanisms of action that can affect surface properties, i.e. the production of metabolites such as acids, enzymatic attack, physical penetration for example by fungal hyphae causing disruption of the materials surface, water accumulation and production of pigments. The binder and emulsion holding microorganisms close to the polyester might facilitate such damage, but there is no information in the literature currently describing deterioration of polyester film.

1.3. Substrates for Microbial Growth

1.3.1. Substrates in Film

Two organic compounds in cinefilm that could be potential growth substrates for microbes are the cellulose component in the cellulose acetate base and the gelatine in the binder. Abrusci et al. (2005) investigated the biodeterioration of cellulose acetates by bacteria and fungi, and in agreement with similar studies, it was found that cellulose acetates with a higher degree of acetylation were more resistant to microbial attack (Buchanan et al., 1993, and Sakai et al., 1996). Sakai et al. (1996) investigated the biodegradation of cellulose acetate by isolation of the bacterial species *Neisseria sicca* from soil, on medium containing cellulose acetate as the carbon source, and suggested that three different enzymes were needed to break down cellulose acetate i.e. esterases, lipases and cellulases. Thus, the cellulose triacetate and diacetate used in the manufacture of cinematographic film which have high degrees of acetylation should be much more resistant to microbial attack than the gelatine. The properties of photographic gelatine have been previously discussed. Gelatine unlike cellulose, requires only gelatinases (i.e. proteases to break down long gelatine polypeptides and peptidases to cleave short chains (Abrusci et al., 2004a)), and both bacterial and fungal species have been shown to degrade gelatine (Abrusci et al., 2005). Therefore, the gelatine binder is the more likely substrate in film to be subject to action by proteolytic enzymes produced by microorganisms. Lourenco and Sampaio (2009) investigated the differences in fungal growth between black and white and colour photographic film negatives, which are similar to cinematographic film in composition, as they both have a gelatine emulsion layer on which the image is imprinted. There was limited evidence to show that, when deliberately inoculated, fungi were able to grow more easily on colour films, because the silver particles in the black and white images impeded growth due to the antimicrobial properties of silver. It was also noted that black and white films also incorporated antimicrobials such as formaldehyde, which were incompatible with colour photographic materials.

Previous work at MMU (results not published) identified three species of fungi as contaminants of the film reels studied: *Aspergillus versicolor*, *A. candidus* and *Penicillium crustosum,* which are classed as hazard group A/1 in ATCC culture collections (Anon. (LGC), n.d.). Abrusci et al. (2005) isolated several species of *Aspergillus* from cinematographic film including *A. ustus*, *A. nidulans* and *A. versicolor.* Seven *Penicillium chrysogenum* strains as well as, *Alternaria alternata*, *Cladosporium cladosporioides*, *Mucor racemosus*, *Phoma glomerata* and *Trichoderma longibrachiatum* were also found.

The mechanisms of deterioration by these isolates was not explored.

It is likely that each film or group of films will be contaminated/ colonised by different species because it is likely that they would have been stored in different locations and conditions prior to donation to the archives

1.3.2 Materials Associated with Archived Film as Substrates for Microbial Growth

Archives also contain other materials of which cellulose is a key organic component, thus biodeterioration of cellulose based materials is of particular concern to museums and archives (Allsopp et al., 2004, Florian, 2002 and Valentin, 2007). These include wood, paper, maps, globes, paintings and structural materials in a building (Strzelczyk, 2004), which could all provide additional sources of fungal contamination. In addition to the cellulose acetate in cinematographic films, some of the films donated to the North West Film Archive (NWFA) were provided in cardboard boxes, where the additional cellulose provides an excellent nutrient source for fungi, particularly in potentially damp environments such as attics, chests, cupboards or cellars.

In addition to film reels, paper based archive material is stored at the NWFA. Paper is made of wood pulp which, although is primarily made of cellulose also contains lignin. Lignin is made of 'the same basic chemical elements as cellulose, namely carbon, hydrogen and oxygen' (Allsopp et al., 2004) although its chemical structure is very complex. This means it is less susceptible to biodegradation and as a result is the plants mechanism of resisting microbial attack. When paper is exposed to heat and light, the oxidation of lignin causes molecules to break down and the paper becomes acidified, causing it to become brittle and stained so it breaks down at a faster rate (Francois, 2009). As a result of this, documents are now printed on acid free paper which involves treating the paper with calcium or magnesium bicarbonate to neutralise the acids. This also prevents new acid from forming thus the paper is less susceptible to foxing caused by acid production of fungi (Fitzgerald, 2001). Old paper can also be de-acidified by treating with the chemicals mentioned previously.

Paper is also susceptible to deterioration by microorganisms, particularly fungi. Natural cellulose like wood is mainly crystalline with some amorphous sites (Allsopp et al., 2004). These are sites where molecules are in a random arrangement, thus are less stable and prone to degradation (Brent, (n.d.)). However, physical break up of cellulose fibres during the production of paper e.g. by grinding, increases the susceptibility of the material to microbial attack by creating more amorphous sites. Thus, endoglucanases produced by microorganisms can cut the polysaccharide chains at these amorphous sites

and degrade the material more easily (Lynd et al., 2002).

Mould growth on paper and cardboard is typically black, brown, dark green or pink (Fig.6), and it can affect the stability of the material, making it more brittle and more easy to damage (Fitzgerald, 2001). Spots often appear on paper, commonly known as 'foxing' and these can penetrate through several pages (Fig.7). Foxing is a term used to describe damage and discolouration in old paper used in books and manuscripts. It has been noted that some foxing is clearly fungal in origin, as hyphae can be seen to penetrate though several pages but other related forms of spotting could be chemical and due to oxidation of lignin as previously discussed (Buzio et al., 2004). Arai (2000) investigated foxing caused by fungi and found that the cellulose on paper fibres was not always essential for fungal growth, as foxing has also been found on silk, which is made of proteins and possibly polysaccharides but not cellulose. From this it was suggested that fungi were possibly using dust particles for nutrition, and it was established that the causation of the brown spots in some cases were not due to growth of fungal hyphae or iron deposits as was previously thought. Alternatively, it was thought to be caused by the fungal production of acids and by the oxidation of polysaccharides occurring under certain conditions after a long period of time (Strzelczyk, 2004), which can lead to degradation of the cellulose fibres.

In addition to the paper itself, there are also other materials involved with paper based materials which could provide substrates for microbial growth. Staff from the special collections of Manchester Metropolitan University (MMU) library observed that fungi more commonly grow down the centre of books close to the spine (Special collections staff, (2009), MMU, Personal Communication). It could be suggested this is because of the composition of the glue used for book binding. Animal glues were in common use before synthetic glues such as polyvinyl acetate (PVA), and animal derived adhesives were often created by hydrolysing collagen, accomplished by prolonged boiling of animal tissues (Pearson, 2003). Alternatively to collagen, Japanese glues are starch based adhesives made from rice and wheat (Anon. (Northeast Document Conservation Center), 2007). In special cases fish and rabbit glues have also been used (Strzelczyk, 2004). As these glues and the paper are derived from organic matter they are again potential food sources for saprophytic fungi, many of which produce multiple enzymes such as collagenases, amylases and cellulases (Mandrioli et al., 2004). Similarly, PVA glues may pose a similar problem in the future as they have been used in the past to coat paintings for cultural heritage purposes and are now showing chemical deterioration (Okada et al., 2011) although microbial deterioration is yet to be observed. In addition to glues, book covers made of leather may

also be substrates for microbial growth (Valentin, 2003). Valentin (2003) names several common environmental bacteria i.e. *Clostridium*, *Bacillus subtilis* and *Pseudomonas* spp. that are capable of producing biodeteriorating enzymes when growing on proteinaceous materials such as leather. Additionally, Valentin (2003) also suggests that leather may also be susceptible to fungal degradation, as treated leather has a pH in a range of 3-5 which is appropriate for fungal growth.

It should be noted that insects can also be the cause of book biodeterioration although, they will not be addressed in this project and are only of relevance here due to their relationship with fungi. There is a species of insect called *Liposcelis bostrychophila*, commonly known as a booklouse. Green (2008) investigated whether or not the type of fungi present on a book, had an effect on the nutritional substrate of this booklouse and found that there is a significant difference between which types of fungi the *L.bostrychophilia* feed on. Some volatile substances are produced by fungi and these may attract or repel insects, as different types of fungi have shown to affect the booklouses growth and egg laying potential. It may be suggested therefore that it would be better to target certain types of fungi for elimination as this could prevent problems with other colonisers of archive material.

Fig.6. A book which has been severely affected by mould presenting as the black patches on the right hand page. The left page is brown due to damp damage.

Fig.7. A book showing spots caused by foxing indicated by the brown spots on the right page.

1.4. Aerobiology

1.4.1. Sources of Contamination

Fungi pose a significant risk in comparison to bacteria with regards to deterioration of archival objects, as they can grow with relatively little available moisture (Mitchell and Mcnamara, 2010). There are several common sources of fungi which could potentially contaminate and deteriorate heritage objects (Florian, 2002). Contamination of materials during manufacture or exposure to the outdoor environment could be a source of deteriogenic fungi. However, the most likely sources of fungi are the surrounding air and may consist of culturable, non-culturable and non-viable organisms, or fragments of microorganisms and fungal spores (airspora). These particles may contain antigens and toxins, gases and other metabolic products (bioaerosols) (Adhikari et al., 2004, Florian, 2002). 'The most common conidial fungi are *Cladosporium* sp. which account for more than half of the airospora' (Florian, 2002). Three other genera are also abundant i.e. *Aspergillus* spp., *Penicillium* spp., and *Alternaria* spp., therefore due to their ubiquity are likely to be the causation of biodeterioration of archive materials (Muilenberg and Rogers, 2009). *Aspergillus* and *Penicillium* species have been the most commonly found contaminants in investigations into the aerobiology of libraries of different countries. These include Spain, Poland and Havana, all of which have different climatic conditions and varying microclimates within the archives themselves (Mandrioli et al., 2004).

1.4.2. Fungi in Indoor Environments

Indoor mould growth on food, plants, dust and mouldy objects also provides a source of cross contamination to objects in archives. Fungi which establish mould colonisation on objects are known as primary colonisers or storage moulds (Nielson, 2003). A water activity of approximately 0.75 -0.8 is adequate to establish colonies of fungal genera considered to be primary colonisers, such as *Penicillium* and *Aspergillus* (Florian, 2002), with the most common species being *P.chrysogenum* and *A.versicolor* (Nielson, 2003). Following primary colonisation, water activity may increase on a substrate due to metabolic functions of the fungi, thus leading to secondary colonisation by secondary colonisers which require a water activity of 0.8-0.9 such as *Alternaria*, *Cladosporium*, *Phoma* and *Ucladium* (Nielson, 2003).

The number of mould species present in buildings and subsequently the number of spores in the air could increase if buildings become damp (Lehtonen et al., 1993 and Pasanen et al., 1992). This would be a problem in archives as there are many substrates for

microbial growth present. Similarly, excess humidity and warmer temperatures could cause the same problem, as has been shown in archives in countries with more tropical climates (Silva et al., 2006). Thus it is important for archives to maintain climate controls for storage of materials, in order to prevent microbial growth on uncontaminated materials, or control microbial growth on materials that have been contaminated prior to donation to the archive.

1.4.3. Fungal Spores and Factors Affecting Their Release

The release of fungal spores during handling and inspection of mouldy materials, is a concern both to the archivists and the materials themselves and there are several factors that may influence this.

Variations in environmental conditions are known to correlate with airborne spore levels. Relative humidity (RH), amount of light, time of day and temperature could all affect microbial growth conditions and have all been shown to be highly influential in spore liberation (Nussbaum, 1991), thus leading to further fungal growth and spread of mould (O'Gorman and Fuller, 2008). Moisture is particularly important with regards to mould growth (Pasanen et al., 1992) and can be extremely damaging to archive materials. In living turgid cells of fungi, the osmotic potential caused by exposure and uptake of water causes the fruiting bodies to become turgid and burst; a process known as discharge by rounding off (Ingold, 1971). Alternatively, dehydration of cells can also cause spore release. The sudden loss of moisture from sap filled hyphae and the membranes of the fruiting bodies (e.g. sporangiophores in mucorales) causes the structures surrounding the spores to collapse and release them, which are then carried away by flowing air currents (Ingold, 1971). Light can also have an effect on spore discharge. Brook (1975) discovered that red, green and blue light influenced the rate of spore discharge by maturing the asci at a faster rate.

The dispersal of fungal spores not only causes problems with cross contamination of archive materials (Valentin, 2007), but also poses a health risk to handlers of contaminated materials (O'Gorman and Fuller, 2008) (described later).

There are some guidelines for what are deemed safe levels of spores in the indoor environment, but there are no internationally set safety regulations for aerobiology due to the daily and seasonal fluctuations of airborne microorganism concentrations (Crook, (2009), Health and Safety Executive (HSE), Personal communication). Some ideas of what should be considered to be safe levels are stated by agencies such as the American Conference of Government Industrial Hygienists (ACGHI) (100 – 1000 colony forming

units per cubic metre (CFUs m^3)) and the Korean indoor bio-aerosol guideline (800 CFUs m^3) (Jo and Seo., 2005). These do not take into account problems resulting from allergies or complications in immunocompromised people but, these levels are said not to pose a serious risk to health in the majority of people. With regards to allergies, it has been reported that higher incidences of asthma could be partially due to higher levels of spore numbers. Hu et al. (2006) found that homes of people who suffered from asthma harboured almost 8 times the recommended safe levels (7974 per m^3) compared to the control homes (980 per m^3). Thus one might propose that handling of the contaminated archive materials could be considered 'safe' if the CFUs were below 1000 per m^3 reflecting typical indoor levels.

These factors indicate that exposure to liberated spores needs to be minimised, and where possible contaminated materials should be handled in a way that prevents the release of spores into the surrounding air. This would to reduce both the risk of contamination other materials the health risks to handlers.

1.5. Associated microorganisms

There have been numerous studies that have isolated many different species of microorganisms from many different archive materials. However, this is not well documented in the case of cinematographic film. There are two main aspects to consider investigating the properties of isolates i.e. the mechanisms of biodeterioration and any health risks involved. Health risks associated with such microorganisms are well documented, although they rarely cause infection except in the immunocompromised. There are two main groups of enzymes deemed to be of importance for the deterioration of recorded image archive materials: cellulases and in particular gelatinases (proteases) for cinematographic film. One or both of these would be required for utilisation of cinefilm as a growth substrate.

As described previously, fungi pose the greatest threat to deterioration of heritage materials, due to their ability to thrive in materials with low moisture and nutrient content (Florian, 2002 and Mitchell and Mcnamara, 2010). Some fungal genera are predominant in the environment and of these, four are found in airspora worldwide: *Aspergillus, Penicillium, Cladosporium* and *Alternaria* (Florian, 2002).These have been found on many types of heritage material demonstrating their ubiquitous nature, with *Penicillium* spp. and *Aspergillus* spp. considered to be the most common biodeteriogens. In one study on fungal contamination of books and manuscripts by Shamsian et al. (2006) *Aspergillus* spp was the

most predominant genus and was isolated on 41% of materials. *Penicillium* spp. was the second most common and was found on 22.9% of materials. Isolation of a species from specific material may be due a variety of reasons. Other than being a specific deteriogen for a specific media, spores could have landed on the materials by chance. These may or may not be utilising the material as a growth substrate, but the potential ability of an organism to do so could be confirmed by enzyme assays, such as gelatinase (Kanemitsu et al., 2001) and cellulase (Kasana et al., 2008) assays. Thus, deterioration of materials would require the production of relevant enzymes depending on the material. Isolation of airborne fungi from materials on which they were not responsible for biodeterioration has been described in some studies. Most microorganisms can potentially contaminate materials but others can colonise the materials and utilise them as substrates for growth. For example Allsopp et al. (2004) noted that 'species of the fungal genera *Aspergillus*, *Penicillium*, and *Paecilomyces* had been isolated from, and were shown to grow on the surface of, floppy disks' although these are not plastic degrading organisms. Isolation of a fungal species could still be possible on materials that do not appear to show visible signs of contamination. Studies in Astan Quds Museum Library in Iran by Shamsian et al. (2006) found that 70.8% of the 56 books tested had fungal contamination although 29.2% had no visible signs of fungal growth or damage. This could potentially be a similar case for cinefilm and other media that record moving images. Some organisms could be present on the materials, but have not had, or a not having a deleterious effect on the material. To determine which of the fungi isolated from cinefilm pose a threat to deterioration of the organic components, gelatinase and cellulase enzymes could be performed as mentioned previously, enabling a profile of the properties of isolates to be assembled. Attempts could then be made to relate enzymatic activity to frequency of isolation and the extent of presence.

Previous studies on biodeteriogenic fungi on substrates that may be relevant to this project, have isolated many species. Peciulyte (2007) isolated many cellulolytic fungi from waste paper recycling, several of which have been shown to biodeteriorate archive materials. These included *Trichoderma* spp., *Penicillium* spp., *Fusarium* species, *Aspergillus* spp., *Cladosporium* and *Stachybotrys chartarum*. Abrusci et al. (2005) isolated many fungi from cinefilm and found several species of *Aspergillus* including *A. ustus*, *A. nidulans* and *A. versicolor*. Seven *Penicillium chrysogenum* strains were also found, as well as, *Alternaria alternata*, *Cladosporium cladosporioides*, *Mucor racemosus*, *Phoma glomerata* and *Trichoderma longibrachiatum*. Zyska (1997) isolated 84 genera of fungi and 234 species from the air of archives around the world and also from different library

materials including books, parchment, leather, glues, magnetic tapes, photographs, microfilms and wood. Although these could be common airborne contaminants some will have the ability to cause biodeterioration of the substrates onto which they deposit, given appropriate growth conditions.

As archives store materials in conditions which limit or prevent microbial and chemical deterioration, contamination and colonisation with fungi will be due to the conditions in which they were previously stored before donation to the archive, unless optimum storage conditions are not maintained within the archive. Growth of mould and subsequent damage to materials only occurs when conditions are adequate for spore germination, thus allowing biodeterioration to proceed.

There are several species of microorganisms which could be potentially problematic to conservation of archive materials and health of archivists.

1.5.1. Fungi

There are several species of fungi, which have been isolated from archives and could pose a threat to the preservation of moving image storage media. Some of these do present a health risk, although the risks are small unless the individual exposed is immunocompromised (Apuhan et al., 2011). As previously noted the greatest risk is from allergenic reactions (Gioulekas et al., 1997).

Alternaria spp. are fungi commonly isolated from plants, soil, food, and indoor building cellulolytic environments. It is primarily a plant pathogen and cellulose degrader, and infection of plants with *Alternaria* causes degradation of plant tissues, leading to diseases such as moldy-core disease of apples (Reuveni et al., 2007). Thus *Alternaria* is a cellulase producer which could pose a threat to archive material. Exposure to *Alternaria* spores have been linked with rhinitis (hay fever), sinusitis and allergic asthma (Salo et al 2006). Additionally, *Alternaria* has also been associated with ulcerated cutaneous infections in immunocompromised transplant patients (Lo Cascio et al., 2004).

Aspergillus species have also been isolated from archives and archive material including cinefilm (Abrusci et al., 2004a, Florian, 2002, Sterflinger and Pinzari, 2012) and members of this genus are ubiquitous in the environment. Studies have shown that *Aspergillus* spp. can grow on many different substrates including archival materials such as 'leather, cloth, fabrics and cellulose based materials such as books (Shamsian et al., 2006, Valentin, 2001). *Aspergillus* spp. are ubiquitous throughout the world and can be found on almost all types of substratum (Onions et al., 1981). They also produce a range of oxidative and hydrolytic enzymes to breakdown materials such as lignocellulose from plants (Baker,

2006), Their saphrophytic nature means this species is commonly found on deteriorating archive materials. *Aspergillus versicolor* is a commonly isolated biodeteriogen (Liang et al., 2011) and has been isolated from cinefilm in previous studies at MMU (unpublished) and from other studies found in the literature (Abrusci et al., 2004a, 2005). Gopinath et al. (2005) found *A.versicolor* exhibited a high degree of gelatinolytic activity which is a good indication as to why the species has been isolated from some severely contaminated cinefilm previously processed in our laboratories. These fungi were probably using the gelatine binder in the film as a nutrient source causing deterioration of the film. Other *Aspergillus* species that are ubiqitous in the environment and have been isolated from the air of archives are *A.niger*, *A.fumigatus* and *A.flavus* (Shabbir et al., 2007). However, previous studies did not isolate these from cinefilm.

The greatest risks posed by exposure to *Aspergillus* is allergic reactions. However, there are some species of *Aspergillus* that could be potentially problematic for individuals who are immunocompromised, although serious complications are rare for people with a healthy immune system (Apuhan et al.,2011).

Aspergillosis is a disease caused by this fungus, which usually only occurs in people with lung diseases or weakened immune systems (Anon (Centers for Disease Control and Prevention), 2012) and two species of particular concern are *A.fumigatus* and *A.flavus* (Perfect et al., 2001). Allergic bronchopulmonary aspergillosis (ABPA) is the development of an allergic reaction to *Aspergillus* spores which it is estimated that 5% of asthmatics may develop at some time in their lives (Anon. (University Hospital of South Manchester), n.d.). Another respiratory condition linked with exposure to *Aspergillus* species (primarily *A.fumigatus*), is known as farmers lung and as the name suggests predominantly affects farmers as they breathe in large amounts of spore containing dust (Pitt, 1994). This is an immunologically mediated inflammatory disease of the lungs and airways when spores are inhaled repeatedly over a long period and can lead to chronic coughs, weight loss, chest tightness, headache and malaise. If exposure is prolonged, continuous lung tissue loss can ensue and lead to permanent decrease in lung function or in the worst case respiratory failure. In rare cases when an individual is severely immunocompromised such as in an in intensive care unit, inhalation of fungal spores and subsequent germination can lead to invasive aspergillosis (Vandewoude et al., 2004). These can present in the form of fungal balls in the lungs known as aspergillomas and tissue necrosis as the hyphae invade the surrounding bronchioles. Symptoms can include fever, chronic cough or wheezing, and coughing up of blood (Brooks et al., 2007). If left untreated, this disease can become systemic and spread into other organs including the

eyes, kidneys, heart and skin. For example, *Aspergillus* sinusitis is also caused by fungal balls but in the sinus which can cause headache or discomfort of the face. The first reported case of invasive aspergillosis was in France in 1979 (Anon. (The Aspergillus Website), 2007) in which a 'fungal tumour' was found to be growing in the sinuses of a patient. At first treatment involved attempts to pull out the fungus but this only led to excessive bleeding. Successful treatment was only achieved by cauterisation, and cutting away of the affected area all 'without the benefit of anaesthetic'. In modern medicine several antifungal drugs are currently available to treat *Aspergillus* infections such as Amphotericin B and several azoles (Sugar, 1995). However due to development of drug resistance such as that of *A.fumigatus*, where mortality of invasive aspergillosis remains at 80-90% in immunocompromised patients (Chamilos and Kontoyiannis, 2005), initial exposure to these fungi in high concentrations should be still be avoided or limited.

Fortunately, there appears to be no evidence in the literature of any *Aspergillus* species being linked with illness caused by handling contaminated archive material, thus the threat posed to archivists is thought to be minimal.

Chaetomium is a cellulose degrading fungus (Allsopp et al., 2004) and plays a major role in the decomposition of cellulose based materials such as wood and paper (Hudson, 1986). This fungus is highly cellulolytic, and this activity increases under moist conditions. However, two species of this fungus: *C.globosum* and *C.cupreum* have been found to produce broad spectrum antifungal compounds which could prove useful in the biocontrol of some fungal crop diseases (Soytong et al., 2001). The *Chaetomium* fungus usually only causes disease in plants, and invasive infections caused by this fungus are extremely rare and has only occurred in patients who were immunocompromised with underlying illnesses such as those with leukaemia (Al-Aidaroos et al., 2007 and Barron et al., 2003) .

Cladosporium is another genus commonly found in the environment and due to its saprophytic nature is a cellulose degrader, thus produces cellulases (Abrha and Gashe, 1992). Cellulases produced by this mould indicate the potential of this organism to degrade cellulose components in film and other archive materials, and it has been isolated from materials such as paintings (Ciferi, 1999). *Cladosporium* species grow well on cellulose surfaces such as wood, moist window sills, and damp wallpaper and also other environments where the humidity is regularly above 50% such as tile grout and bathrooms, where the mould appears to be black due to melanin production (Fogarty and Tobin, 1996). Health risks caused by exposure to spores include the trigger of allergies such as asthma, or in rare cases severe inflammation in the lungs (sarcoidosis) also known as hypersensitivity

pneumonitis or 'hot tub lung' (Jacobs et al., 1986).

Penicillium species are a ubiquitous genus which are commonly found in many environments throughout the world and are able to colonise all types of organic materials (Rosas et al., 1993). *Penicillium* species are also known to be causative organisms of biodeterioration of many materials including leather and rubber in households (McGinnis et al., 1975), and cellulose based materials in archives (Guiamet et al., 2011). Common species in the environment include, *P.citrinum*, and *P.chrysogenum*, (a.k.a. *P.notatum*) and *P. brevicompactum* (Fallah et al., 2004). Many *Penicillium* species have been of benefit to human health, for example, *P.chrysogenum/ P.notatum* was the species first discovered by Alexander Fleming to produce penicillin (Deacon, 2006), which has been used to benefit millions and save many lives. Others such as *P.camemberti* and *P.roqueforti* are used in the food industry for example in the ripening of cheese (Drean et al., 2010). As with other opportunistic fungal infections, most *Penicillium* infections are found in immunocompromised hosts. However, infection with one species *P.marneffei*, is still important as this species is sometimes found in AIDS patients, and is even considered a marker for this disease in endemic areas (Cheng et al., 1998).

Stachybotrys chartarum is black mould and grows optimally at 23ºC on high cellulose containing materials such as paper and wood (Kuhn and Ghannoum, 2003). It is often isolated in water damaged buildings and materials and is known to pose a threat to health (Anderson et al., 2002). This genus was highlighted by archivists (Bodner, (2009), NWFA, Personal communication) and it was unknown if this fungus posed a threat to cinefilm. There are a number of adverse health effects that have been described. *Stachybotrys* can cause a condition known as stachybotrytoxicosis and produces a number of mycotoxins including macrocyclic trichothecenes, satratoxins and roridins which are immunosuppressive. Macrocyclic trichothecenes are highly toxic compounds which have been shown to inhibit protein synthesis in the cells of rats (Sorenson et al., 1987). They can also cause reduction of white blood cells (aleukia) which could potentially result in opportunistic infections or infection with other associated fungi (Kuhn and Ghannoum, 2003). However, despite the serious health risks described in the literature regarding *S.chartarum*, this fungus requires a much higher RH than other fungi to grow (93% compared to approximately 75%), and is a tertiary coloniser, which colonises after what are deemed 'less harmful' fungi such as *A.versicolor*, *Penicillium* spp. (primary colonisers) and *Cladosporium* spp. (secondary colonisers) (Kuhn and Ghannoum, 2003). Thus these species might theoretically only grow on cinematographic film after the materials had been water damaged.

Many cellulolytic and gelatinolytic moulds are very common in the environment, thus are likely to be isolated from film and cellulose based materials such as paper, all of which have the potential to cause deterioration. With the exception of immunocompromised individuals, most fungal species pose only a minimal threat to human health.

1.5.2. Bacteria

Whilst bacteria are not thought to pose a major threat to archival materials under 'normal' conditions due to their higher water requirements than fungi (Raschle, 1989), some bacteria have been isolated from cinematographic film, and have the capability to degrade gelatine (Abrusci et al., 2004a, 2005, 2007). Valentin (2001) noted that several potentially biodegradative/ opportunistic pathogenic bacteria species such as *Pseudomonas* are commonly present in a wide range of environments. *Clostridium,* and *Bacillus* spores could also be found on archive materials although their growth is restricted due to higher moisture requirements than fungi. Members of the Actinomycetes group can play a large part in the deterioration of historic objects (Strezelczyk, 2004) and are probably the only bacteria which are likely to cause major concern in biodegradation in archives. These bacteria are similar morphologically to fungi as they form branching, filamentous, mycelium and are even able to produce spores and tolerate low water regimes enabling survival in dry conditions such as archives and libraries. Actinomycetes species can also cause 'farmers lung' as previously described (Wild, 2012). Some bacteria have been isolated from cinematographic film such as the *Staphylococcus* species, i.e. *S.epidermidis, S.hominis*, *S.lentus*, *S.haemolyticus* and *S.lugdunensis* (Abrusci et al., 2005). These coagulase-negative staphylococci are part of the normal human flora and are found on the skin, nose and throat (Brooks et al., 2007). The occurrence on archive material therefore could be explained by direct human contact with the materials and the presence of skin flakes in dust. The health risks, and risks to archive materials are insignificant.

Although bacteria are known to produce many types of enzymes including cellulases and proteases, which aid in the biodeterioration of several types of material, they require a much higher RH and water activity (a_w) (usually >98) than fungi to grow (Raschle, 1989) (Water activity describes the amount of water needed for hydration of materials, where pure water is equal to 1.0 (Anon. (WaterActivity.org), 2011)). It has been found that bacteria only tend to grow well when there is flood damage and long lasting damp when paper has been soaked in water and provides an adequate substrate for bacterial biofilms to form. Thus overall, one can conclude that ‘under normal conditions,

bacteria do not play a significant part in the deterioration of historic objects' (Strezelczyk, 2004).

1.6. Consequences of Microbial Contamination and Growth

1.6.1. For Film

There are many mechanisms by which mould growth could damage film and this relates to the way in which fungi obtain their nutrients. Many fungal species are saphrophytic i.e. they break down decaying matter. Similarly to animals and most bacteria, fungi need organic nutrients to provide energy and a source of carbon for biosynthesis thus, are classed as chemoheterotrophs (Deacon, 1997). However, unlike animals they have a characteristic method of acquiring nutrition because they absorb nutrients through the cell wall and plasma membrane (Ingold, 1984). These nutrients are often in the form of complex polymers such as polysaccharides, proteins and lipids and need to be broken down into simpler molecules in order to pass through the cell wall and membrane. The hyphal tip of fungal mycelium (apex) is the area considered to be the main site of metabolic activity (Ingold, 1984) and continuously extends to new zones of substrate, during which the protoplasm needed for this extension of growth is supplied from behind. As the substrate is depleted the protoplasm is constantly moved around to areas where nutrients are still present so that extension of hyphae can continue and the colony will not die off. It has been shown that extracellular enzymes (depolymerases) are released mainly at areas of new cell wall growth (Deacon, 1997) and are released onto the growth substrate so that the molecules can be broken down absorbed into the fungal hyphae. This is because large molecules cannot pass out of pores in the mycelium onto the substrate, and a partially formed cell wall enables excretion to occur. This has been demonstrated in *Aspergillus niger*, where glucoamylase has been shown to be secreted exclusively from the hyphal tips (Deacon, 1997). In addition to extracellular enzymes, others may be membrane bound in the cell wall, and serve to degrade molecules further when they are absorbed into the hyphae.

Most fungi are only capable of absorbing small soluble molecules such as amino acids, monosaccharides, or peptides composed of 2 or 3 amino acids, thus absorption of nutrients is strongly dependent on enzymes. However, enzymes, like complex polymers, are large molecules so cannot diffuse far from the hyphal surface and as a result can only create localised zones of substrate degradation around the mycelia; thus the hyphae must

extend continuously into new zones (Deacon, 2006). For unicellular fungi i.e. yeasts, hyphae are not produced and depolymerase enzymes are not secreted. This is because they would not be able to move from the degraded substrate they had created, so instead they are found in environments containing simple soluble molecules, such as leaf and root surfaces or mucosal membranes (Deacon 2006), where the host breaks down the complex molecules making them easier to absorb. Once absorbed, translocation of nutrients and water depends on the hyphal structure. In Mucorales sp. the absence of septa allows movement of molecules to freely move from one part of the mycelium to another. In septate fungi, each cross wall (septum) contains a central pore in which streaming of nutrients can occur (Ingold, 1984). This is facilitated by tubes which interconnect the hyphae known as anastomoses, which provide short cuts for translocation across the mycelium (Gabriela Roca et al., 2005).

In addition to the utilisation of substrates for growth, the physical presence of the fungal mycelia may also affect the condition of the film. The hyphae may create a physical barrier to light passing through the slides during projection, which would be apparent when the film was projected. Fungal growth can also cause the gelatine to become etched or distorted, leading to permanent damage to the film, as at present there is no satisfactory method of restoration (Anon. (Parallels), 2011).

The mechanisms by which fungi obtain their nutrients is of concern when investigating biodeterioration of moving image media especially film. In addition to the physical presence of hyphae which can impede the projection of the film image, the growth of fungi on a film reel also causes permanent damage, due to enzymatic degradation of gelatine. Thus, growth should be prevented as there are little that can be done to restore the film, once fungal growth has occurred.

1.6.2. For Archivists

As well as damage to the materials themselves, potential health risks to archivists from handling mouldy materials may exist.

As previously discussed, microorganisms have the potential to be pathogenic and due to the presence of antigens also act as sensitisers, which can trigger allergic reactions in some vulnerable people (Muilenberg and Burge, 1996). Bioaerosol analysis is often undertaken to assess the risk factors which cause asthma allergic responses due to reaction to the conidial antigens (Sorenson, 1999), which can result in illnesses such as 'sick building syndrome' (Florian, 2002). Sick building syndrome occurs when people have been exposed to high concentrations of spores for extended periods of time in, for instance

buildings with damp problems (Assouline-Dayan et al., 2002). Working in damp or mouldy buildings has been shown to increase the risk of respiratory infections such as asthma and bronchitis and also increased fatigue (Nielson, 2003). Other symptoms include: mucous membrane irritation (e.g. eye, nose and throat), neurotoxic effects (e.g. headaches, and irritability) and other respiratory difficulties (e.g. chest tightness and wheezing) (Apter et al., 1994), which are alleviated when an individual leaves the building (Passarelli, 2009). Similar symptoms could be experienced by archivists if they are exposed to mouldy materials for a prolonged length of time.

Air sampling experiments performed by Buttner and Stetzenbach (1993) and Lehtonen and Reponen (1993) to simulate variations of airborne fungal spore counts in a residential indoor environment, gave counts of between 4 x 10^2 and 6.4 x 10^3 CFUs per m^3. Another study observed that the concentration of viable fungal spores in house dust to vary from 6 x 10^3 to 3.2 x 10^6 colony forming units (CFUs) per gram of dust (Korpi et al., 1997). This number however, could be considerably more in an attic, a cupboard or an environment where dust is allowed to settle for a longer length of time. Thus, it has been shown that indoor fungal spore concentrations could potentially be higher than what is recommended 'safe' levels by some health authorities of 800-1000 CFUs m^3 (Jo and Seo, 2005). Lehtonen and Reponen (1993) investigated the variations during everyday household activities such as cleaning, which releases fungal spores into the air. This would be similar when archivists handle and inspect mouldy and dusty archive material.

In addition to sensitisation by exposure to fungal spores, exposure to mycotoxins may also pose a potential health risk. Mycotoxins are produced by some species of fungi when they are growing in unfavourable conditions as these can inhibit the growth of other organisms. Aflatoxins are the most widely known mycotoxins which if ingested can produce severe allergic reactions or even in the case of aflatoxin B1 be carcinogenic and are produced by several *Aspergillus* species including *A. flavus* and *A. parasiticus* (Pildain et al., 2008). It is however, highly unlikely that aflatoxins would pose a problem to archivists as the materials would have to be ingested. Thus, most fungi are considered to be opportunistic pathogens, with infections and complications being rare and usually associated the immunocompromised or individuals who have encountered unusually high levels of exposure to spores.

1.6.3. Putting the Risks into Perspective

Despite the risks involved with handling mouldy materials, serious health problems caused by exposure to fungal spores are rare in healthy individuals (Apuhan et al., 2011).

Although there is the possibility that spores could be released in concentrations above levels deemed 'safe' when archivists handle mouldy materials, the likely risk is low. Safe handling practices, e.g. wearing protective clothing, face masks or inspecting materials in a closed or ventilated container, would reduce this risk further and minimise the likelihood of health complications.

Only a few species of fungi cause human disease and disease is usually linked with immunosuppressed individuals (Richardson, 2005). It is possible that certain individuals may have sensitivities to fungal antigens and may suffer from respiratory difficulties such as asthma or allergies. However, other daily household activities such as working in the garden for example, have been known to cause health problems due to allergic reactions (Anon. (Sky News), 2008) and could potentially expose someone to much larger concentrations of spores than working with contaminated archive material in addition to exposure to what are considered more dangerous species such as *A.fumigatus* (O'Gorman and Fuller, 2008). Thus, the risks posed by working with contaminated archive materials is considered to be low.

Some fungi can secrete toxigenic compounds which can lead to respiratory problems and more commonly allergic reactions. Thus, it is difficult to define safe concentrations of spores, as any adverse reactions would depend on the sensitivity of the person involved therefore it is more reasonable to make recommendations on best practices when dealing with fungi. Exposure to a higher concentration of spores could potentially increase the health risks involved, but whether an allergic reaction is triggered depends on the individual. Due to the ubiquity of fungal spores in the environment, exposure to varying levels of spores is unavoidable, however, most people do not suffer from any negative health effects and whilst all fungi are potentially pathogenic the risks to health are small. It is therefore difficult to make an assessment for handling contaminated film because health risks are dependent on the person involved rather than the fungi present.

The physical growth of the fungi and production of metabolites causes biodeterioration of archive materials, but it is exposure to the spores and toxins combined with the sensitivity of the host that determines the potential of health risks to archivists.

1.7. Preventing and Controlling Deterioration of Film Archive Materials

1.7.1. Archives

The purpose of film archives is to store materials and also maintain their condition. This is achieved by maintaining environmental conditions which prevent chemical deterioration and create conditions unfavourable to vegetative mould growth whilst preventing fungal spore germination, thus slowing deterioration.

Two archives have contributed to the materials and samples used in this study: the British Film Institute (BFI) and the North West Film Archive (NWFA).

The UK's largest archive is the BFI National Archive which contains more than 50,000 fiction films, over 100,000 non-fiction titles and around 625,000 television programmes (Anon. (British Film Institute), n.d.). The majority of the film reels are housed in bunkers in Gaydon, Warwickshire, which had been previously used to house nuclear warheads shortly after World War II (Fig.8). The protected, underground environment provides cool and safe conditions for the storage of film heritage, particularly the less stable cellulose nitrate films. Here, there are over half a million film reels (including approximately 140 million feet of cellulose nitrate film) (Everiss, 2008) many of which contain rare footage by directors such as Alfred Hitchcock (Sunday Mercury, July 21st, 1996 Page 11). Security is tight on site and the area is monitored by CCTV. Cellulose nitrate films are stored in cells separate from cellulose acetate films (Fig.9) so that in the event of ignition of the films, the spread of fire and subsequent damage will be restricted (Ewart, (2010), BFI, Personal communication). In the event of fire, water is released from the roof and flows down the outer sides of the vault preventing the fire from burning through the walls into other cells. The fire is then allowed to burn out in the cell which results in the loss of films in the affected cell only. The BFI copy cellulose nitrate films onto safety film reels and polyethylene reels. This means that the reels are no longer flammable but are still vulnerable to biodeterioration if they are not stored in conditions which prevent fungal growth.

Fig.8. Bunkers at the British Film Institute (BFI) Gaydon where film reels are stored.

Fig.9. The inside of a fireproof cell at BFI Gaydon, which house cellulose nitrate film reels.

The North West Film Archive (NWFA) at Manchester Metropolitan University (MMU) is the largest archive outside London (Hewitt, 2006) and only houses cellulose acetate not cellulose nitrate film reels due to safety restrictions.

Prevention of further chemical and microbial deterioration of film achieved by storing materials at a constant temperature below 10ºC, relative humidity (RH) below 70% (Child, 2011) and water activity (a_w) below 0.7. Most fungi will remain dormant in these conditions (Florian, 2002), preventing further deterioration of the materials. These conditions not only slow down or prevent chemical degradation, they restrict the growth of any fungi already contaminating the materials as well as preventing the germination of spores of fungi thought to be the predominant biodeteriorators of archive materials. These include *Aspergillus* spp. and *Penicillium* spp. which have optimal germinating water activities of 0.96 and 0.95a_w respectively (Pardo et al., 2005, 2006).

The conditions in which the archive material is stored is carefully defined and recommendations for film are available from film manufacturers such as Kodak (Anon. (Kodak (B), n.d.). Currently the standards for air conditioning units in buildings is 50% +/- 5% RH 20ºC +/- 2ºC (Valentin, 2007). Modern 'intelligent' buildings such as museums and archives contain excessive amounts of insulation. This often means that condensation is a problem due to lack of air flow and this creates ecosystems adequate for the development of microorganisms (Valentin, 2007). Valentin (2007) also noted that fungi can grow in a range from 60 – 90 % RH and bacteria at over 85 % RH as they need more water to grow. Conditions ideal for fungal growth and spore germination are 20ºC, 65% RH, 10% water content and 0.65 a_w in the materials (Maggi et al., 2000). Archives therefore need to store materials in controlled conditions which create environments unfavourable for fungal growth.

The NWFA stores their black and white film at 10ºC and 35% RH and colour film at 4ºC and 30% RH (Bodner, (2009), NWFA, Personal communication). Similarly films housed at BFI Gaydon are currently stored in bunkers maintained at 15ºC with less than 50% RH although the new vaults will store films at -15ºC with less than 5% RH.

Paper is stored in the archives at the NWFA at 18ºC with 50% RH (approx 7.4 water content (Valentin, 2003)). This process is to avoid damaging the materials, as moving directly from the outside environment to the colder, dryer conditions of the archives can be physically damaging, and can alter the chemical properties (such as colour dyes in colour film), which can also further deteriorate the material and image quality.

In addition to the vaults there is also an acclimatisation room used to store items for several days or weeks depending on the material, before moving to the designated vaults.

Mouldy films are also stored in this room for long periods, and may never be placed in the specialised storage areas (Bodner, (2009), NWFA, Personal communication).

There are many archives throughout the world in countries which experience different climatic conditions. Environmental temperature, humidity and even salinity of the air in archives situated near coastal regions are all factors which influence the microorganisms present and issues regarding biodeterioration (Gallo, 1993). Storage of film and paper even prior to arrival at the archives is important in preventing microbial contamination because treatment is often ineffective or unsuitable due to damage it may cause to materials. Allsopp et al., (2004) stated that mouldy materials should be copied onto new materials where possible, which is a practice currently employed at the BFI and NWFA. Archives now copy film reels onto digital media. However, prior to the widespread use of digital media, cellulose nitrate film reels were often copied onto cellulose acetate reels for safety reasons (Stark, (2009), BFI, Personal communication). Digital copies however, are of less good quality as digitalisation loses quality, therefore it is always best to have the original film (Anon. (Council on Library and Information Resources), n.d. and Rockwell, 2009).

1.7.2. Environmental Monitoring and Isolation of Microorganisms

There are many methods available for detecting microorganisms in the environment, but only those relevant to archiving that will be discussed. Traditional air sampling methods were performed by Maggi et al. (2000) using Millipore samplers to analyse the types of fungal spores present in the dust of archives. These were electrostatically charged to increase attraction of spores, which was achieved by rubbing them on the back with wool. These were then saturated with Sabouraud Maltose Broth and incubated. The use of an air sampler allows numbers of conidia present in the air to be estimated by trapping conidia and spores, culturing them on suitable media and counting them (Buttner and Stetzenbach, 1993, Florian, 2002, and Lehtonen and Reponen, 1993). Following isolation of fungal colonies by air sampling, colony morphology and growth characteristics on specific growth media can then be described. DNA extraction and sequencing can be used to identify fungal isolates to species level as has been shown by Michaelsen et al. (2006) who used these methods to identify fungal communities colonising paper materials. By using these methods, Maggi et al. (2000) found that different genera of fungi were predominant in the samples at different times of year. For example, *Cladosporium* species were dominant in the winter samples and *Penicillium* species dominated in the summer samples thus highlighting the changing risks potentially present, as some fungi will have greater

capability for deteriorating materials. In addition, colonisation by one fungal species may aid in the colonisation of other fungi. For example some *Aspergillus* species require low water activity (a_w) for spore germination but once vegetative growth has been initialised water will be produced by the fungi which may lead to colonisation and spore germination with fungi of different genera.

Various culture media can be used for the culturing of fungi such as malt extract agar (MEA) or potato dextrose agar (PDA). In the context of contaminated film, the fungi involved will be growing or present on a substrate with a relatively low nutrient content and will have also been growing on materials which have are not likely to have been kept at optimal temperature and moisture conditions for fungal growth. These are oligotrophic microorganisms and are '*able to grow in low concentrations or the apparent absence of nutrients by scavenging from the atmosphere or substratum on which they grow*' (Wainwright et al., 1993). MEA was selected for this study, being a commonly used, readily available and relatively cheap medium.

Detection of microbial volatile organic compounds (MVOCs) is an alternative method which could potentially be used for detection and even possibly identification of mould in archives (Canhoto et al., 2004). 'Electronic nose technology' which is currently used to detect volatile compounds produced by microorganisms. MVOCs are metabolic by-products of bacteria and fungi and are the cause of the musty, damp odour found where damp is present in buildings. They are detectable before any visible signs of mould growth, therefore could potentially indicate biocontamination problems in the early stages (Ruzsanyi et al., 2003). MVOCs known to be produced by microbial metabolism are alcohol, ketones, aldehydes, aromatic and chlorinated hydrocarbons, sulphur-based compounds, amines and terpenes (Foruk et al., 2001). Although electronic nose technology had been used to detect the presence of organisms in medical, environmental and food applications previously, Canhoto et al., (2004) used this device to detect microbial contamination of museum materials. The studies showed that different fungi produced different volatile compounds and that the device could distinguish between a few different types of organisms and even classify three types: *A.niger*, *A.flavus* and *P. chrysogenum*. Such a system could provide real time information of the contamination present in an archive, museum or library enabling precautionary measures to be taken, such as changing environmental conditions, to prevent damage from occurring.

1.7.3. Isolation of Microorganisms from Film

Methods have been used to detect the organisms present on film, although the literature currently available is limited, with only one reference having been found (Abrusci et al., 2005). One method previously developed at MMU laboratories was to use an air sampler in a polymer isolation glove box (Wolf Laboratories Ltd, York), specifically modified for this study by removing the gloves so that the user could wear nitrile gloves for increased dexterity. The film and equipment were then placed in the box and enclosed, and then the whole film was wound from one reel to another whilst an air sampler extracted a known volume (100 litres of air per minute) removing any spores that might have been released during this simulated inspection. A filter put over agar plates of Sabourand Dextrose Agar (SAB) and Malt Extract Agar (MEA) then concentrated this air flow onto the plates to attempt to isolate fungi, which could then be quantified, and key isolates identified. Abrusci et al. (2005), placed film directly onto agar and left them to incubate on either SAB with chloramphenicol for fungi, or Trypticase Soya Agar (TSA) for bacteria. Organisms present on the film would then be lifted off onto the agar, for subsequent culture and identification. This method however, is potentially destructive to the film because contact with the agar could perhaps transfer moisture and nutrient to the film providing further nutrition for the growth of microorganisms, and is less quantitative than the air sampling method.

1.8. Treatment of Contaminated Materials

Chemical and physical methods are available to decontaminate film and paper archive materials but these may not only damage the materials involved but also pose a risk to health by excessive release and dissemination of fungal spores. Due to the potential of chemical antimicrobials to damage materials and pose a risk to the health of archivists, physical removal of mould is often the only option, although this too could potentially cause damage to the material, therefore a decision tree is needed to enable careful handling of materials, and advise on the best course of action for removal of mould or disinfection of materials if possible. Chemical and physical methods have been used for removal of microorganisms from archive materials.

1.8.1. Biocides

Biocides are '*identified as speciality organic or inorganic chemicals that control, inhibit or kill the growth of different kinds of microorganisms*' (Arslan-Alaton, 2007) for example

algae, bacteria and fungi etc. Biocides could potentially be used to decontaminate or preserve a material or compound, and are often added during production especially if it is thought that they are at high risk of microbial deterioration. These could be for example; polymers, textiles, wood and disinfectants (D'Arcy, 2002). Some examples of biocides are; tributylin, halogenated phenolics (Arslan-Alaton, 2007), chlorhexidine and triclosan (Lear et al., 2006). Biocides such as formaldehyde and miconazole have been used in the manufacture of cinematographic film (Anon (RTBot), 2012) however, as mould has been found growing on film reels, the effectiveness and longevity of these biocides questionable.

All biocides have health risks associated with exposure and are not generally used in the preservation and decontamination of archive materials because of potential damage that would be caused to the materials. Several biocides however, have been used in libraries and archives for conservation of paper documents including formaldehyde, phenols and quaternary ammonium compounds (Velikova et al., 2011). Biocides used in library and archive environments have to meet certain requirements, the main requirements being; harmless for people, harmless to materials, absence of colour and smell, easy to use and time stable (Velikova et al., 2011). However, biocide use is restricted in libraries and archives due to regulations set by European and other global organisations, partly due to their destructive properties and also lack of specificity against target organisms, their eventual release into the environment and adverse effects on human health (Cappitelli and Sorlini., 2008). In addition, there could be an undetermined effect of the biocides on cinefilm, such as they could alter the film image, thus biocides are unlikely to be used in film archives.

1.8.2. Novel Methods of Removal

In addition to biocides, there are other antimcirobial compounds which could be used to treat mouldy materials. Antibiotics or antifungal compounds such as azoles, should not be used however, due to the potential of microorganisms to develop resistance so should be saved for medical use (Ghannoum and Rice, 1999). Thus alternative compounds and novel methods of removal and disinfection could be investigated. Essential oils are one example of an alternative to biocides for treatment of mouldy materials, and studies have been performed using materials from archives.

Rakotonirainy and Lavedrine (2005) tested several essential oils against moulds typically found in libraries and archive storage areas. It was discovered that the oils did not cause discolouration of the inks or the paper itself but did cause a slight change in pH,

although this was deemed to be relatively insignificant and limited to about one unit. The oils tested were found to have an effect the overall growth of fungi *in vitro* visible by a clear zone around oil soaked paper disks. Rakotonirainy and Lavedrine (2005) also used methods to test the effect of vapours on fungal growth and found them to have significant antimicrobial effects. The effect of vapours would have a greater potential use than direct application of the oils, due to both the decreased risk of damage to the materials and also the ability of the vapour to cover a larger surface area. This could reduce fungal contamination and consequently the risk to handlers.

It is well documented that essential oils have antimicrobial properties which can be fungistatic, fungicidal or either depending on the concentration used (Espinel-Ingroff et al., 2002). In eukaryotic cells, essential oils can act as prooxidants, affecting inner cell membranes and organelles such as mitochondria (Bakkali et al., 2008). Bakkali et al. (2008) mentions that the oils interfere with metabolic processes and chemical pathways within the cell but are not usually genotoxic. Essential oils also have bacteriostatic and bacteriocidal effects by interfering with metabolic pathways and reacting with proteins in the cytoplasmic membrane (Burt, 2004). Hammer et al. (1999) tested essential oils against many bacteria and also *Candida albicans*. By mixing the oil directly into the agar it was observed that the oils did have an inhibitory effect on some bacteria and also yeast. As essential oils seem to have a broad spectrum of effect both against eukaryotic and prokaryotic microorganisms, there seems to be scope here for further investigation into their potential use in archival material conservation.

If studies were performed using essential oils on cinematographic film, it would have to be investigated whether or not the fluid would penetrate the film enough to have a significant effect, and if the compound had a detrimental effect on the film. Methods for removal would be easy to demonstrate *in vitro*, but the effects of the oil vapours on the film would have to be investigated further.

1.8.3. Removal from Cinematographic Film

Due to the risks posed by chemical treatment of cinematographic film, physical methods are currently the only option available to clean mouldy film. If the mould is a light dry covering on the surface of film, and penetration of the gelatine emulsion is not visible, then the growth could be removed by cleaning with a cloth dampened with tepid water and air drying to prevent further growth. However, if the mould has penetrated into the gelatin emulsion layer, there is no method to remove this as the mould has already deteriorated the film image (Shriver and Spencer, 2005). In this instance copying the affected film reel and

digital restoration of the film may be the only option.

To remove visible contamination from film, conservationists at the NWFA used a process colloquially termed 'laminectomy'. This involved pouring a solution of art masking fluid (rubber solution, latex and ammonia) onto the edge of the film reel (top and bottom) (Section 2.1.1. and 2.2.2.), allowing it to set and then peeling it off. Preliminary work at MMU (unpublished) showed this to be effective at removing visible growth on the edges of the film, but spores were released back during removal of the coating, and considerable fungal mycelium was retained within the reels. This was indicated by an increase in the number of colonies isolated from subsequent air sampling post laminectomy, compared with pre-laminectomy. The laminectomy fluid also proved difficult to remove entirely, resulting in the potential for further damage to the film. Further investigation is therefore needed to determine the effectiveness of this method.

1.9. Summary

The literature available on the biodeterioration and biodegradation of moving media is minimal. Upon donation of film reels to the archive, archivists assess the condition of the film reels, and if they appear to be mouldy by visual examination i.e. white crusty growth or dusty patches, then they are stacked in the acclimatisation room where they will remain without further examination due to the perceived risk to the archivist of handling therefore valuable footage is lost.

Whilst there is a potential for health risks due to exposure of contaminated materials the risk is likely to be small for healthy individuals. However, this should not be ignored and contaminated materials should be handled taking safety into consideration. This demonstrates that further work is needed to assess these risks, and if possible determine the extent to which the fungi isolated from mouldy materials pose a risk both to the archivists, and to the substrates on which they are found. Recommendations could then be made for handling mouldy materials.

The following methods were initially proposed for this study to assess the fungal contamination of film. Visual examination of the film reels could be used to construct a chart, with varying levels of contamination described. Recommendations for safe handling could then be made for handling film at each contamination level. Enumeration of different colony morphologies would enable the frequencies of isolates to be determined. Isolates could then be identified to genus level, or species level where possible. In addition to traditional microbiological culture methods, alternative methods for detection of actively

growing mould on film reels using microbial volatile organic compounds (MVOCs) will also be explored.

It has been demonstrated that there is lack of information available regarding fungal contamination of cinematographic film and potential health risks associated with handling, thus there is a need for a simple method to assess risk to archivists enabling subsequent conservation and archiving of film. This study could enable archivists to handle such materials appropriately in order to prevent further deterioration of the materials, limit cross contamination, and minimise the health risks, allowing recovery of more valuable archive footage.

1.10. Aims

To assess the impact of fungal contamination of archival cinematographic film on film quality and archivists' health.

To develop a non-culture method for detection of contamination.

1.11. Objectives

1) To determine the number of fungal spores released during the inspection of cinematographic film.

2) To identify the fungi which cause biodeterioration of film.

3) To assess gelatinolytic and cellulolytic activity of enzymes produced by predominant fungal isolates.

4) To identify any microbial volatile organic compounds (MVOCs) are common to fungi isolated from cinematographic film.

5) To investigate the potential of a sensor for key MVOCs for detection of active fungal growth.

6) To assess the risk posed to archivists during the handling of film reels contaminated with mould.

7) To recommend optimal procedures for handling of mouldy film.

Chapter 2

Assessment of Fungal Growth on Moving Image Storage Media

2.0. Methods I: Sampling of Film

2.0.1. Film Reels Donated by the North West Film Archive (NWFA)

Eighteen film reels, donated to the study by the NWFA, had been given a reference number starting with 'RR' followed by a four digit number which related to the donor of the films. If the four digit number is the same for multiple reels, then the reels came from the same donor, and in case of several films being from the same donor the letter 'F' for 'film' and a number (1, 2, etc.) was added. All reels were donated with the donors permission and returned to the NWFA post analysis. The film reels came in two sizes known as gauges, which were 16mm and 8mm, and related to the width of the film (Table 1). Large spools for long films with 8mm and 16mm gauges measured 175mm in diameter, whilst the small spools for short 8mm films, which would typically be used for personal films/home movies, measured 7mm in diameter (Fig.10). All film reels were provided in metal cans except for reels belonging to the RR1491 collection, which were each held in cardboard boxes.

2.0.2. Film Reel Descriptions and Mould Categories

The films donated by the NWFA were contaminated to varying degrees ranging from heavy growth to a dusty appearance. Reels were given a qualitative, subjective 'mould category' ranging from 1-4 (4 being the most contaminated) based on how mouldy a film appeared visually. Category 1 had a light dust covering with no mycelial growth (Fig.11). No visible particles were released on inspection. Category 2 had a dusty appearance and a few white spots were sometimes visible, although there was no clearly visible mycelial growth (Fig. 12). Category 3 had a white crust covering the surface, with visible white spots of furry mould and mycelia growth on the image surface (Fig.13). Visible particles were released on inspection. Category 4 had a heavy granular covering of mould appearing as a white crust and obvious growth on the image surface with large fragments of mould falling off on inspection (Fig 14).

Approximate numbers of colony forming units (CFUs) released per cubic metre (m^3) for reels in each category were as follows: Category 1 = <100, Category 2 = >100, Category 3 = >250, Category 4 = >1000. These were based on the three sets of air sampling (methods described later, section 2.1.0.). Five films were classified as Category 1, four were Category 2, six were Category 3 and three were Category 4.

Table 1. Film reel gauges, type of film (colour/back and white) and the mould category that was initially assigned by visual inspection, and later associated with the number of spores released during simulated inspection. Approximate numbers of colony forming units (CFUs) released per cubic metre (m^3) for reels in each category were as follows: Category 1 = <100, Category 2 = >100, Category 3 = >250, Category 4 = >1000.

Film Name	Colour or Black and White Film (B/W)	Film Gauge (mm)	Mould Category
RR1491 F1	Colour	8	3
RR1491 F2	B/W	16	3
RR1491 F3	Colour	8	4
RR1494 F1	B/W	16	1
RR1494 F2	B/W	16	2
RR1494 F3	Colour	16	3
RR1494 F4	B/W	16	3
RR1514 F1	B/W	16	2
RR1514 F2	B/W	16	1
RR1440 F1	B/W	8	2
RR1440 F2	B/W	8	2
RR1440 F3	B/W	8	1
RR1470	B/W	16	3
RR1399	B/W	16	4
RR1549	Colour	16	2
RR1093	Colour	16	4
RR1533	B/W	8	1
RR1511	Colour	16	1

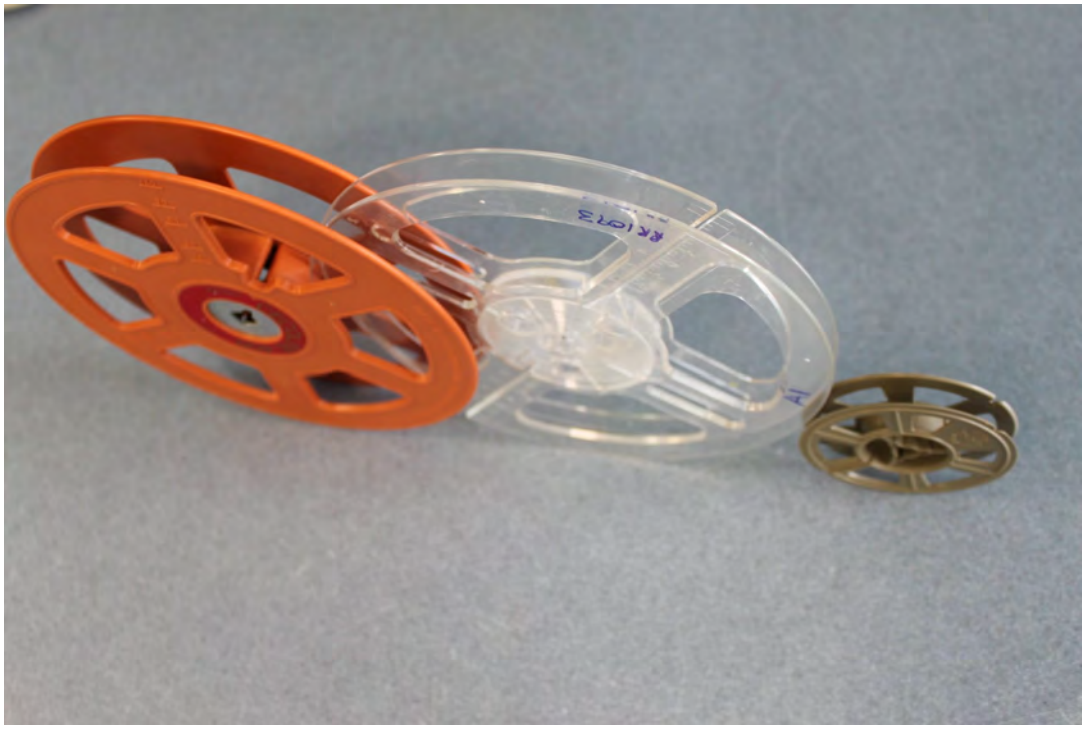

Fig.10. Different sizes of film spools. Left to right: large spool for 16mm films, large spool for 8mm films and small spool for 8mm home films.

Fig.11. Category 1 - a light dust covering with no mycelial growth.

Fig.12. Category 2 - a dusty appearance and a few white spots may be visible but no clearly visible mycelial growth.

Fig.13. Category 3- a white crust covering the surface, with visible white spots of furry mould and mycelia growth on the image surface.

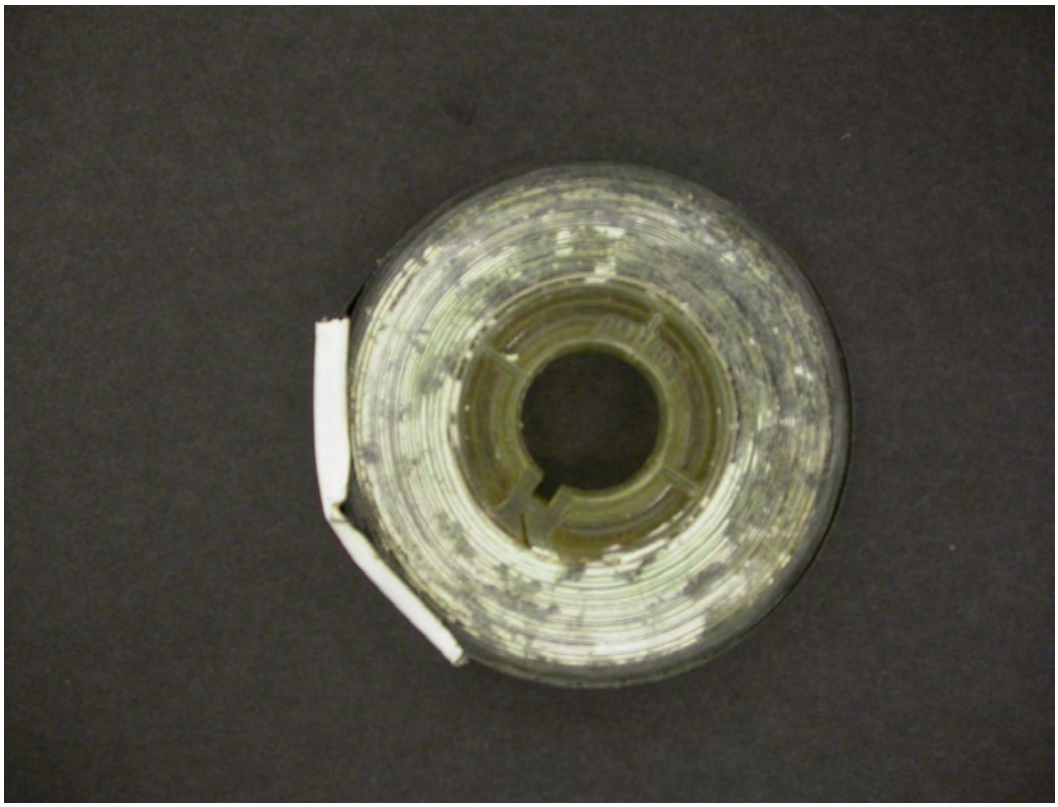

Fig.14. Category 4 - a heavy granular covering of mould appearing as a white crust and obvious growth on the image surface with large fragments of mould falling off on inspection. This particular reel also appeared to be covered with paint.

2.1.0. Sampling of Reels Provided by the North West Film Archive (NWFA)

The number of spores released during a simulated inspection of reels was investigated by air sampling. All inspections were performed in a polymer isolation glove box (Wolf Laboratories, York, UK(Model number: 8307030 (3ft)) approximately 2.3m^3 volume). This had been customised by removing the gloves at the wrists, so that manipulations in the box could be carried out with bare hands, but fungal spores released during inspection would be contained (Fig.15). This was chosen, rather than a biological safety cabinet level 2 (BSL2), because archivists have access to similar apparatus, thus making the simulation more accurate.

All fungal culture media, decontamination fluid (Trigene, Medichem, Kent, UK), and film, in-can, were placed in the glove box. The medium selected was malt extract agar (MEA) (Oxoid, Basingstoke, UK). Chloramphenicol supplement (Oxoid) solubilised in ethanol was added by syringe by sterile filtering (using a PALL Acrodisc 32mm with 0.2µm Supor membrane) into the MEA medium (6mL per litre of medium) after autoclaving to inhibit bacterial growth. For the 'simulated inspection' each film was mounted on spools at either end of a manual film spool winder so that 50cm of film was exposed and could be transferred from spool to spool under controlled conditions (Fig.16). An air sampler (Desaga Germ Sampler gs 100: Oklahoma City, US) was also placed in the glove box, so that the number of fungal spores released per unit volume over time during inspection could be assessed (Fig.17). This was set to measure 100L of air, over 60 seconds with a 5 second delay. When the air sampling began, with 50cm of film exposed, the film was wound forwards for 60 seconds, at approximately 90 turns per minute, after which the agar plate on the air sampler was removed, and replaced by a second plate (to give counts of an average of two plates (n=2)) during a repeated winding process where the film was re-wound back to its original spool. These two plates were taken as duplicates, although this is not strictly the case; on occasion the process was repeated, enabling four counts to be made. The film was then replaced in its can, the glove box sanitised with Trigene spray and left for 10 minutes before the next film was processed. Plates were incubated at 25^{o}C for 5 – 7 days. Colonies were counted, and calculated as counts per cubic metre (m^3) of air (Test 1).

It was thought that repeated inspection of the film might affect spore release, due to physical disturbance of film and mycelium, thus sampling and analysis for each film were repeated for a second time with 2 replicates, 7 days after the previous sampling (Test 2), and differences between number of spores released were noted. After the second sampling,

a ‘laminectomy’ (a process to reduce visible surface contamination)(described later, section 2.1.1.) was performed on selected reels which had been determined by visualisation and colony counts to have the greatest extent of contamination. All films were then also sampled for a third time at 14 days after test 1 (Test 3) so that the effectiveness of a laminectomy could be determined. For films RR1399, RR1093, RR1549 and RR1470 the number of colonies of each colony morphology type was also noted. Additionally, swabs were also taken of each reel on the day of each test, using a sterile swab moistened in sterile distilled water, to investigate whether any additional colonies not isolated by air sampling were present.

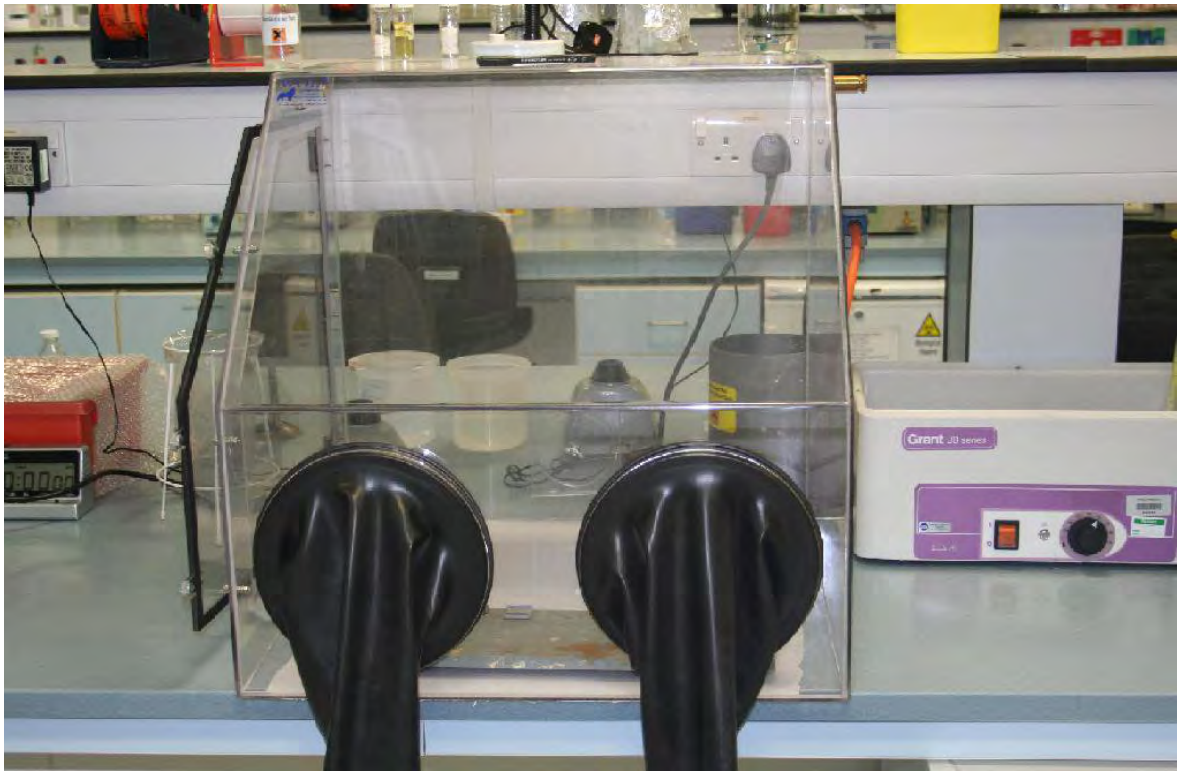

Fig.15. Isolation Glove Box (Wolf Laboratories, York, UK(Model number: 8307030 (3ft))) approximately 2.3m^3 volume) used when simulating film inspection. Access is provided via a side door (shown open on the left hand side) and rubber sleeves attached to front.

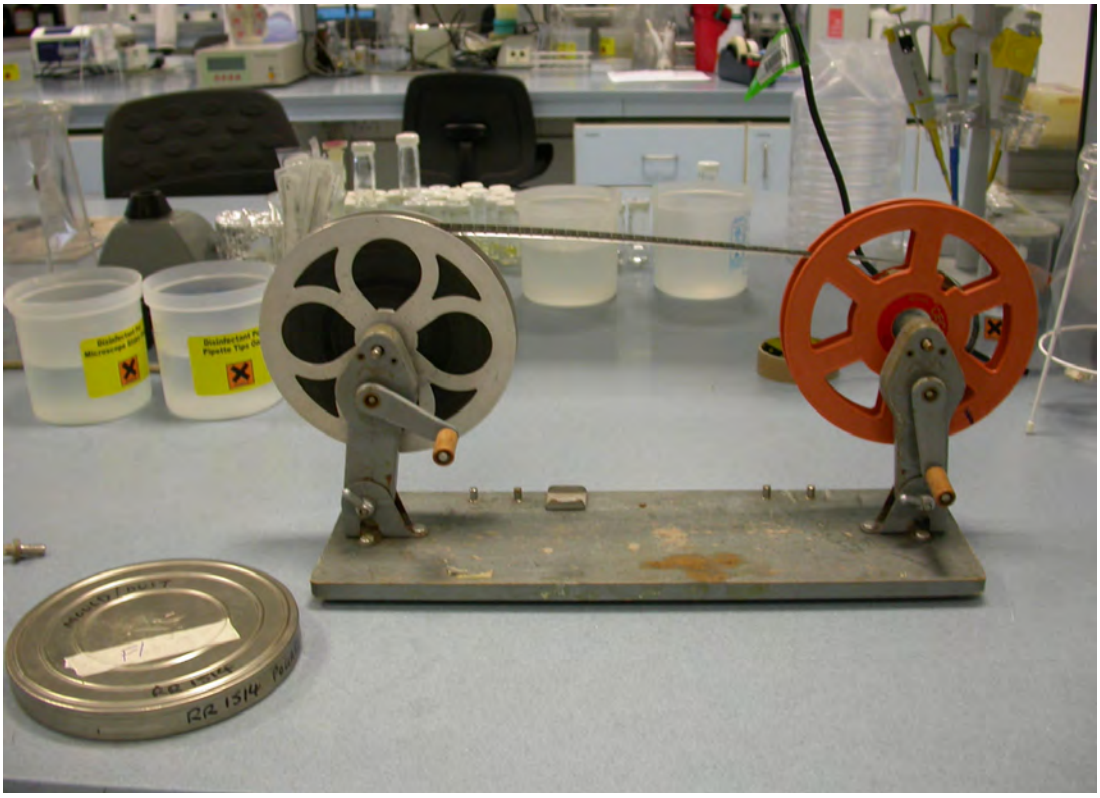

Fig.16. Spool winder with a film reel (left) and empty spool (right) used to wind the film. back and forth. (Bottom Left) Metal can containing film reel.

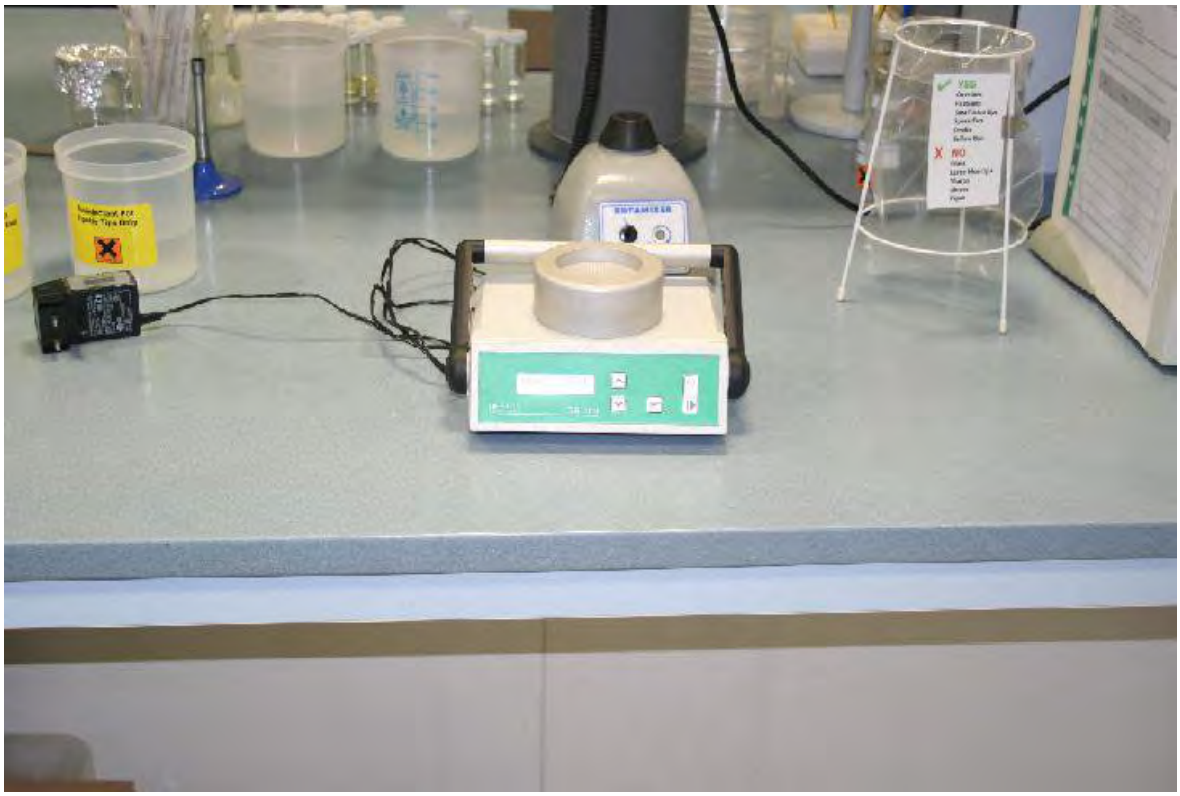

Fig.17. Air Sampler GS100 used for sampling with variable control up to 100L^3.

2.1.1. Proposed Method for Physical Removal of Mould Using Art Masking Fluid

Until December 2007 the NWFA had used a process which they termed ‘laminectomy’ to remove visible fungal growth from the outer edges of film reels. This process involves pouring art masking fluid i.e. a solution comprising of rubber, latex and ammonia onto the film, and peeling off after this sets to remove heavy fungal contamination off the film. However, at a conference attended by a member of the NWFA the risks posed by exposure to the mould were highlighted and this practice ceased (Bodner, (2009), NWFA, Personal communication). From that point film reels thought to be contaminated with mould growth were instead stored and not processed. To evaluate the effectiveness of a laminectomy in the laboratory, this technique (using art masking fluid from Winsor and Newton, London) was used on film reels RR1491 F3, RR1399 and RR1093 which visibly appeared to have the heaviest fungal growth of all the reels studied. An agar imprint was also made of the spool of RR1093 by pressing the spool onto an MEA plate and incubating the plate for 30^{o}C for 7 days, as the spool itself also appeared to be heavily contaminated. Sampling of these 3 reels, confirmed heavy contamination due to high colony counts in Test 1 and Test 2 of air sampling. Additionally, a laminectomy was also performed on the film RR1494 F4 as the NWFA had labelled this film with 'caution – very mouldy'. This reel however, appeared visually to be much less contaminated than the other 3 reels on which this process was also carried out. Laminectomies were carried out in a BSL2 safety cabinet because this gave greater access to the films than the isolation box. Laminectomies were done on the 3rd sampling (i.e. Test 3) of the selected films, so that the effectiveness of the laminectomy on reducing the number of spores released could be assessed. The film reels were placed flat on a work bench and a layer of laminectomy fluid was poured approximately 2mm thick to evenly cover the edge of the film (i.e. not on the film image). This was allowed to set for 2-3 hours at room temperature after which it was peeled off using sterile plastic disposable tweezers. The reels were then turned over and the process was then repeated on the other side of the films. Air sampling was repeated as before (Test 3) to measure any differences between the spores released before and after laminectomy. The percentage change between Test 2 and Test 3 of number of CFUs per m^{3} was then compared with other reels which had not had laminectomies to see if this process was efficient at reducing the spores release.

2.1.2. Analysis of Swabs Provided by The British Film Institute (BFI)

In addition to cellulose acetate reels, a limited study was also performed on 12 samples taken from cellulose nitrate reels. However, due to safety issues previously described (Chapter 1) reels were not provided to MMU. Thus, 12 dry swabs were provided by the British Film Institute (BFI, London) taken from 12 different cellulose nitrate film reels which had been identified by archivists as being mouldy. Details were provided as to where on the film reel the sampling had been carried out and the title of the film was also recorded (Table 2).

Swabs were placed into separate sterile universal bottles each containing 10 mLs of sterile distilled water and left to soak for one hour to lift spores from the swab. These were then vortex mixed with the swabs still present for approximately one minute and diluted to 10^{-3} to reduce the number of spores. Using 100µL volumes of each dilution, spread plates were then made in duplicate (n=2),onto MEA containing chloramphenicol solubilised in ethanol (previously described section 2.1.0.). These were left to incubate for 7 days at 30^{o}C and the colony forming units for predominant isolates were calculated using the following formula: colony count x pipetting factor x reciprocal of the dilution (i.e. multiply colony count by 10 as 100µl was added).

Predominant colonies were selected, subcultured and identified and given a code with ‘S’ for swab and then followed by a number. As with reels RR1093, RR1399, RR1470 and RR1549 the numbers of each colony present on each plate predominant isolates were counted and subsequently assayed for gelatinase production.

Table 2. Information provided by the BFI giving the title of the film and the location of the sampling on the reels.

Sample Number	BFI location	Title	Sample Taken From: Head/Tail/ Mid Reel/ Leader
1	2025462AA	Xivth Olympiad – The Glory of Sport	Tail
2	2025462AA	Xivth Olympiad – The Glory of Sport	Head- Printed Leader
3	2012272AA	France: Wonderful Debris of the Million Dollar Fire in Paris	Head- Tinted Tiles
4	2032153BB	Earthworm The	Tail
5	2007908AA	U-Boote Heraus	Head- Picture
6	K209	Training and Practical Hints	Head
7	C52-1	Kicking	Head
8	2042765AA	Antarctica: On the Great White Trail South	Head
9	2041629AE	Owd Bob (1924)	Head- Tinted Tiles
10	2012111AA	Firefighter's Love	Head- B/W Picture
11	201211AA	Firefighter's Love	Head- Tinted / Toned Title Letter
12	D12 C19	Earthworm The	Head

2.2. Results I: Colony Counts from Swabbing and Air Sampling of Reels

2.2.1. CFU Counts from Air Sampling of Film Reels

The number of spores released during three tests over three weeks was recorded (Table 3).

The recommended 'safe levels' for indoor spore exposure in the US is up to 1000 CFUm^3 of air (Jo and Seo, 2005). RR1399 and RR1093 released the highest number of spores of between 1.5 and 7 times the recommended safe limits. Swabbing of films labelled RR1440 failed to isolate any colonies and only a few colonies were isolated from films RR1494. RR1511 visually appeared to be contaminated with fungi but swabbing and air sampling of this reel failed to isolate any fungal colonies.

Several plates were discarded due to the growth of *Neurospora sp.* or *Rhizopus* species which had potential for lab infestation. These were from all reels belonging to the RR1440 collection and RR1514 F2. *Neurospora* was isolated from both by air sampling and swabbing and as a result only one set of air samples from films RR1440 and RR1514 F2 could be taken. Repetition of the simulated inspection revealed that the numbers of spores released varied with each sequential test. The percentage of all spores released during each test was then calculated to show how these numbers varied (Fig.18).

A general trend indicated that a small percentage of spores were released in Test 1, the largest percentage being released in Test 2 and on Test 3 the percentage of spores released decreased. Calculating total numbers of spores released from all films on each test, revealed that numbers decreased from the highest on Test 1 to lowest on Test 3. However, statistical analysis (t-test) showed that the P value between Test 1 and 2, Test 2 and 3 and Test 1 and 3 were all >0.05, showing that there was no significant difference in the spores released between each test, although spore numbers did appear to decrease with each subsequent sampling.

The mycology lab, microbiology research lab and the archives at the NWFA were sampled to ascertain the number fungal spores present in the air to compare with the results obtained from the films. In the microbiology research lab an average of 8 yeasts (n=3) were isolated per 100L air (80 per m^3) and no moulds. In the mycology lab, the NWFA work space and storage areas, and in vaults in the archive yielded only two colonies. In addition to the archives, the acclimatisation room where materials are kept before transfer into specific vaults was also sampled. One agar plate was used for air sampling near the area where the mouldy films were kept and the other was sampled at the opposite side of

the room. The plate sampled near to the mouldy reels isolated 40 CFUs per cubic meter compared to none at the opposite side of the room.

To compare the differences between spores released from sampling of black and white and colour film reels, the count of spores released during all three tests for each film was totalled. The average number of spores released for colour films and black and white films was calculated separately, with RR1511 and RR1470 being omitted as no spores were isolated from air sampling on any of the tests from these films, and gave a P value of >0.05, showing no significant difference.

Table 3**.** Average number of spores released from each film reels in different collections per m^3 of air (N=2 unless stated). Test 3 for reels RR1491 F3, RR1494 F4, RR1093 and RR1399 presents the results after a 'laminectomy' was performed (marked with an '*'). The percentage change between Test 2 and Test 3 is also shown. 'N/A' in the percentage change column indicates where no CFUs were isolated in Test 2 or Test 3.

	Average number of spores released from each film reel per m3 (N=2 unless stated)			
Film Name	**Test 1**	**Test 2**	**Test 3**	**Percentage Change Between Test 2 and Test 3 (2.dp.)**
RR1491 F1	146.67 +/- 37.86	693.33 +/- 457.64	96.67 +/- 41.63	86.06% decrease
RR1491 F2	86.67 +/- 32.15	376.67 +/- 218.25	260 +/- 145.26	30.97% decrease
***RR1491 F3**	853.33 +/- 446.36	2736 +/- 181.48	126.67 +/- 68.07	95.37% decrease
RR1494 F1	25 +/- 7.07	236.67 +/- 37.86	23.33 +/- 5.77	90.14% decrease
RR1494 F2	35 +/- 7.07	183.33 +/- 46.19	13.33 +/- 5.77	92.73% decrease
RR1494 F3	25 +/- 7.07	280 +/- 17.32	13.33 +/- 15.28	95.24% decrease
***RR1494 F4**	25 +/- 7.07	126.67 +/- 66.58	13.33 +/- 5.77	89.48% decrease
RR1514 F1	20 +/- 20	6.67 +/- 11.55	0	100% decrease
RR1514 F2	3.33 +/- 5.77	0	0	N/A
RR1440 F1	26.67 +/- 30.55	0	0	N/A
RR1440 F2	13.33 +/- 15.28	0	0	N/A
RR1440 F3	0	0	0	N/A
RR1470	23.33 +/- 5.77	16.67 +/- 11.55	356.67 +/- 125.03	2039.59 % increase
***RR1399**	5510 +/- 496.89	1853.33 (N=1)	6460 +/- 2021.19	248.56% increase
RR1549	56.67 +/- 25.17	16.67+/- 15.28	73.33 +/- 49.33	339.89 % increase
***RR1093**	3753.33 +/- 496.52	2696.67 +/- 1472.15	1476.67 +/- 1650.16	45.24% decrease
RR1533	3.33+/- 5.77	33.33 +/- 41.63	30+/- 17.32	9.99% decrease
RR1511	0	0	0	N/A

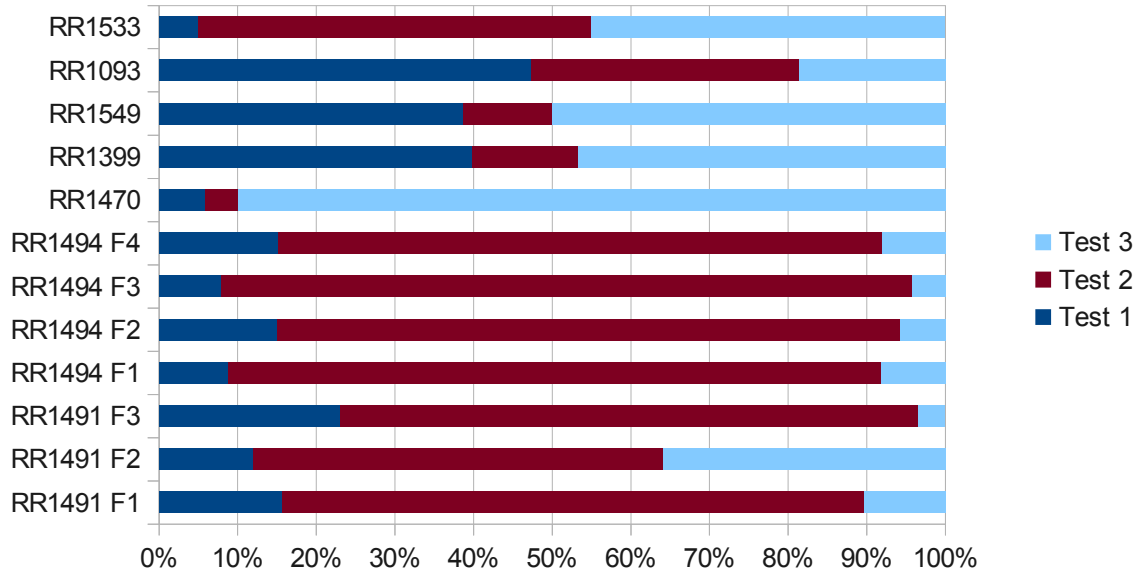

Fig 18. Representation of total percentage of spores released during 3 sets of air sampling with the percentage of spores released during each test indicated by a different coloured bar. Test 3 for reels RR1491 F3, RR1494 F4, RR1093 and RR1399 are post treatment with art masking fluid ('laminectomy'). Reel RR1514 F2 and all RR1440 reels are excluded from these results due contamination with *Neurospora sp.* so no further tests were carried out after test 1. RR1511 is also excluded as no spores were released during air sampling.

2.2.2. Effect of Physical Removal Attempts on CFU Counts

Four reels were selected for laminectomy for reasons previously mentioned (section 2.1.1.). Contamination of these heavily contaminated films had been confirmed from air sampling and swabbing. The agar imprint of the RR1399 spool (Fig.19) resulted in heavy growth on the MEA agar plate after incubation at 30^{o}C for 7 days (Fig.20)

The laminectomy fluid/art masking fluid (Winsor and Newton, London) is a white and thick substance comprising of rubber, latex and ammonia (Fig.21) which dries to form a clear rubber layer (Fig.22), which was peeled off and air sampling was performed. The percentage change in counts between test 2 (pre-laminectomy) and test 3 (post-laminectomy) was recorded and compared to films which had not undergone this process (Table 3).

There was a percentage increase in the numbers of spores released from films RR1399, RR1470 and RR1549. In all other cases, there was a reduction in counts, usually greater than 85%. There was insufficient data to determine whether the laminectomy decreased counts over and above just the physical winding of the film during air sampling and the numbers increased for RR1399, although on visualisation of the reel the films appeared to be cleaner than before the laminectomy (Fig.23 and Fig.24). However, winding through the film reel also appeared to remove some of the visible mould (Fig.25). Residual fluid on the reel also had an unknown effect on the film but would be undesirable. Difficulty in peeling the fluid off the reel was also observed, which has the potential to damage the film structure. Of the 4 films treated, RR1093 showed the least decrease in the number of spores released whilst RR1399 showed the greatest decrease.

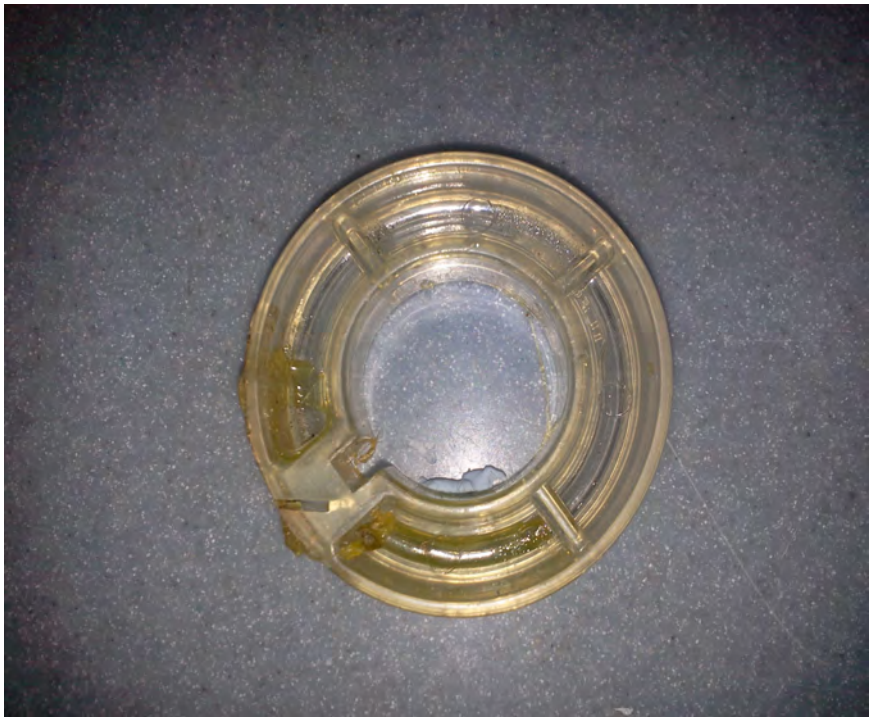

Fig 19. RR1399 spool which was cleaned with Trigene after pressing onto MEA.

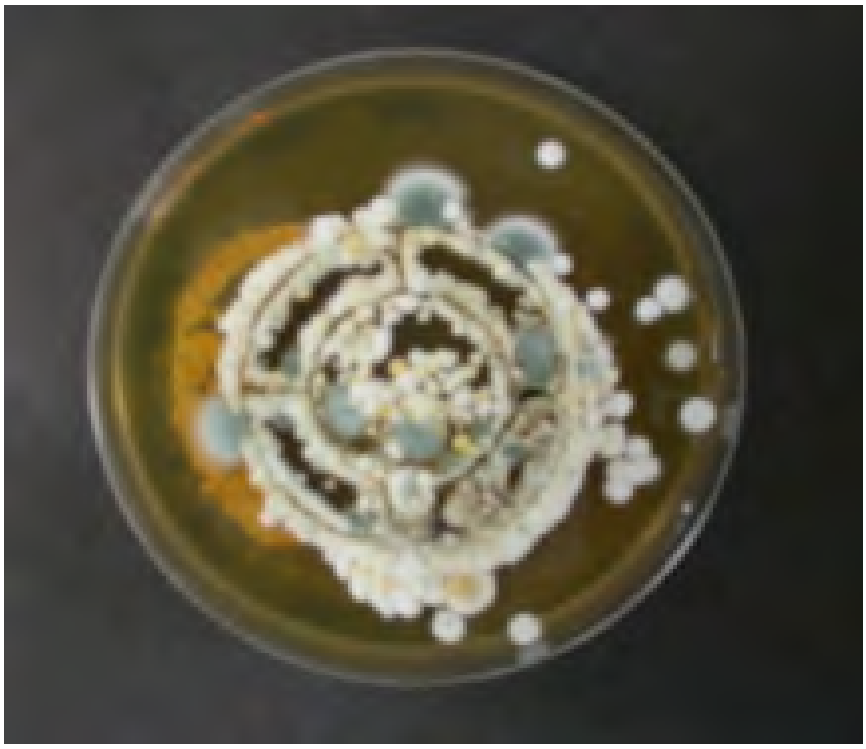

Fig.20. Pressing of the RR1399 spool resulted in heavy growth.

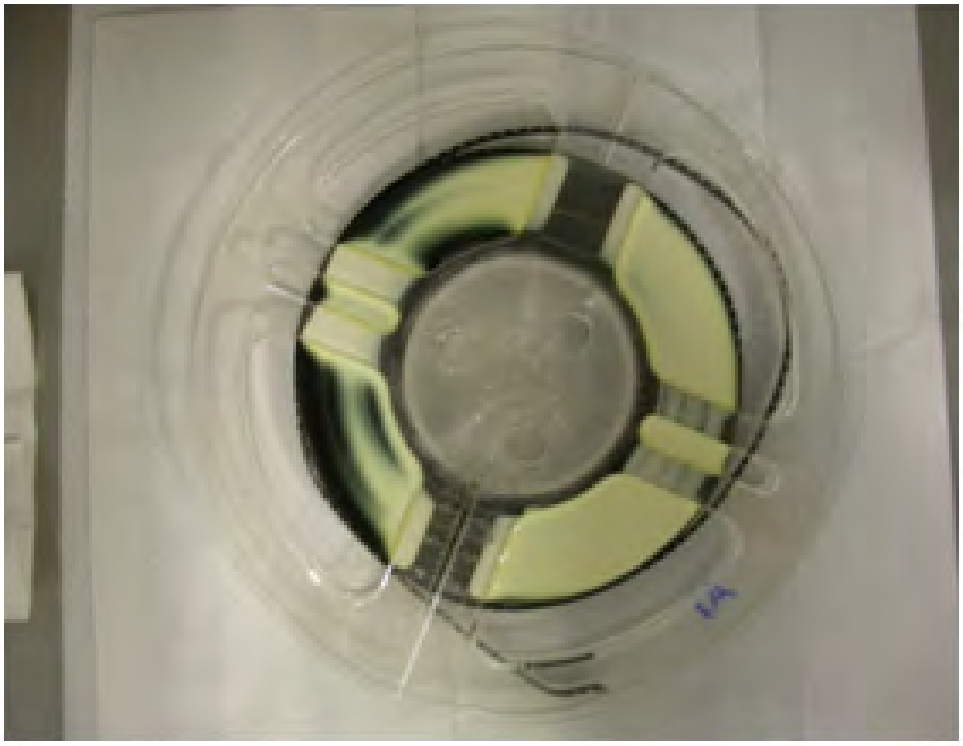

Fig.21. Reel RR1093 which was covered in art masking (laminectomy) fluid.

Fig.22. Reel RR1399 which has been covered in art masking fluid (laminectomy) and allowed to air dry.

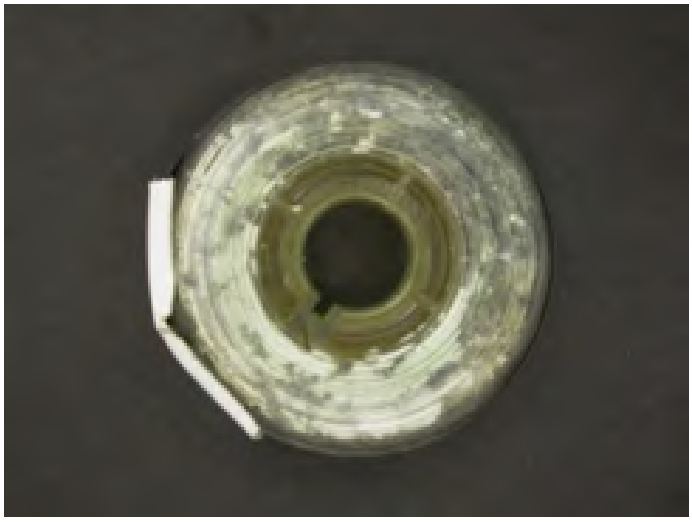

Fig 23. RR1399 before the air samplings showing heavy mould contamination.

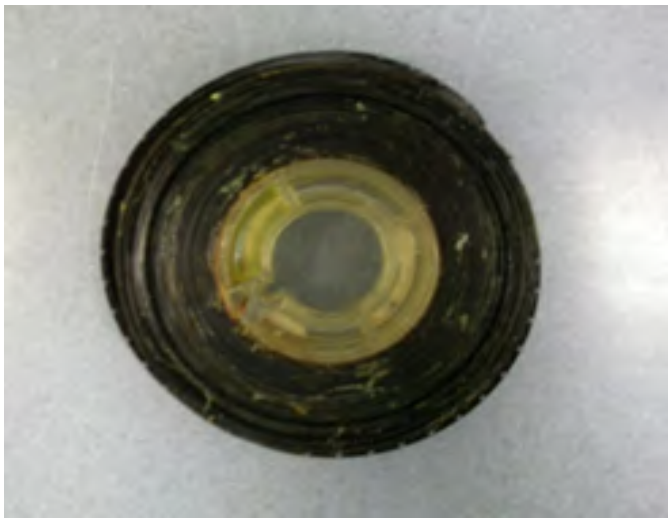

Fig.24. RR1399 after the laminectomy was performed. Some fluid remains due to difficulty in removal.

Fig.25. RR1399 after Test 1 and Test 2 before the laminectomy was performed. Most of the mould growth appears to have been removed by the physical winding of the film reel.

2.2.3. Counts to Measure Changes in Frequency of Isolates Released upon each Test

Data from four films (RR1093, RR1399, RR1549, RR1470) underwent further scrutiny to determine if the proportion of isolates varied in each test. Table 4 shows total combined counts of the most commonly found isolates and the change in the frequency of CFUs in each inspection. Thirty isolates in total were selected and later subcultured, identified and screened for gelatinase and cellulase production (described later).

RR1093 I4, RR1399 I1 and RR1399 I3 released above the recommended 'safe' limit of spore exposure of 1000 CFU/m^3 during inspection. RR1093 I6 to RR1093 I10 and RR1399 I7 (listed in Table 6) were not dominant and yielded minimal CFU counts so although all isolates were subcultured and identified, colony counts were not performed on these so were absent from Table 4.

2.2.4. Analysis of Swabs Provided by the British Film Institute (BFI)

Suspensions from swabs of cellulose nitrate films were used to estimate the total numbers of colony forming units per swab. A differential count for dominant isolates based on visualisation of different colony morphologies was also calculated. This provided a relative measurement of the contamination of the nitrate reels, to indicate which isolates were the most predominant on these films.

Counts were performed for isolates S2-S5 on 10^{-2} dilution, because 10^{-1} had too many colonies to count. S1 (later identified as a *Trichoderma* species) was present on all the swabs. However, the number of colonies for S1 could not be counted because this rapidly spreading organism covered the entire plates, often obscuring other colonisers.

Isolate S3 was present in the highest numbers, and showed the highest counts on swabs 1, 3 and 7, all of which had high total CFU counts of greater than 400 CFUs per mL, thus were determined to be the most heavily contaminated. This isolate represented approximate 74% of the total CFU counts for all 12 samples at 10^{-2} dilution. S2 represented 12%, S4 represented 6% and S5 represented 8%. All swabs yielded some growth, with some films yielding all five of the different colony morphologies that had been identified. Preliminary isolate identification to genus level of S1-S5 were made based on visualisation of colony morphology and microscopy (Fig.26), with S3 and S5 later identified to species level by CABI Microbial Services (shown later in Table 6. Section 2.4.2.).

Table 4. Numbers of CFUs of each isolate released during the three Tests. Counts were based on visualisation of differing colony morphologies which were later identified. RR1093 I4 and RR1399 I3 produced CFU counts above 1000 CFUs per cubic metre. Isolates RR1093 I6 to RR1093 I10 were only present in low numbers so although all were identified, colony counts were not performed so are excluded from the table.

	Average number of isolates per test (n=3) per m3		
Isolate Name	**Test 1**	**Test 2**	**Test 3**
RR1093 I1	53.33 +/- 5.77	46.67+/- 45.09	16.67 +/- 20.82
RR1093 I2	53.33 +/- 15.28	3.33 +/- 5.77	3.33 +/- 5.77
RR1093 I3	733.33 +/- 533.51	230 +/- 98.59	50 +/- 50
RR1093 I4	2296.67 +/- 548.57	1710 +/- 1213.47	1333.33+/- 1391.56
RR1093 I5	3.33+/- 5.77	3.33 +/- 5.77	0 +/- 0
RR1399 I1	920 +/- 511.18	1000 (N=1)	2140 +/- 608.28
RR1399 I2	100 +/- 65.67	280 (N=1)	273.33 +/- 61.1
RR1399 I3	3623 +/- 80.21	4240 (N=1)	253.33 +/- 102.63
RR1399 I4	10 +/- 10	0 (N=1)	440 +/- 124.9
RR1399 I5	23.33 +/- 20.82	10 (N=1)	120 +/- 20
RR1399 I6	396.67 +/- 20.82	220 (N=1)	386.67 +/- 61.1
RR1470 I1	3.33 +/- 5.77	0 +/- 0	0.26 +/- 5.77
RR1470 I2	13.33 +/- 15.28	0 +/- 0	0.53 +/- 5.77
RR1470 I3	3.33 +/- 5.77	3.33 +/- 5.77	0 +/- 0
RR1470 I4	0 +/- 0	0 +/- 0	5 +/- 15.28
RR1470 I5	0 +/- 0	0 +/- 0	22.89 +/- 160
RR1549 I1	3.33 +/- 5.77	0 +/- 0	0 +/- 0
RR1549 I2	26.67 +/- 15.28	6.67 +/- 5.77	0 +/- 0
RR1549 I3	13.33 +/- 15.28	0 +/- 0	0 +/- 0
RR1549 I4	0 +/- 0	6.67 +/- 5.77	1.05 +/- 23.09
RR1549 I5	0 +/- 0	0 +/- 0	4.74 +/- 62.45

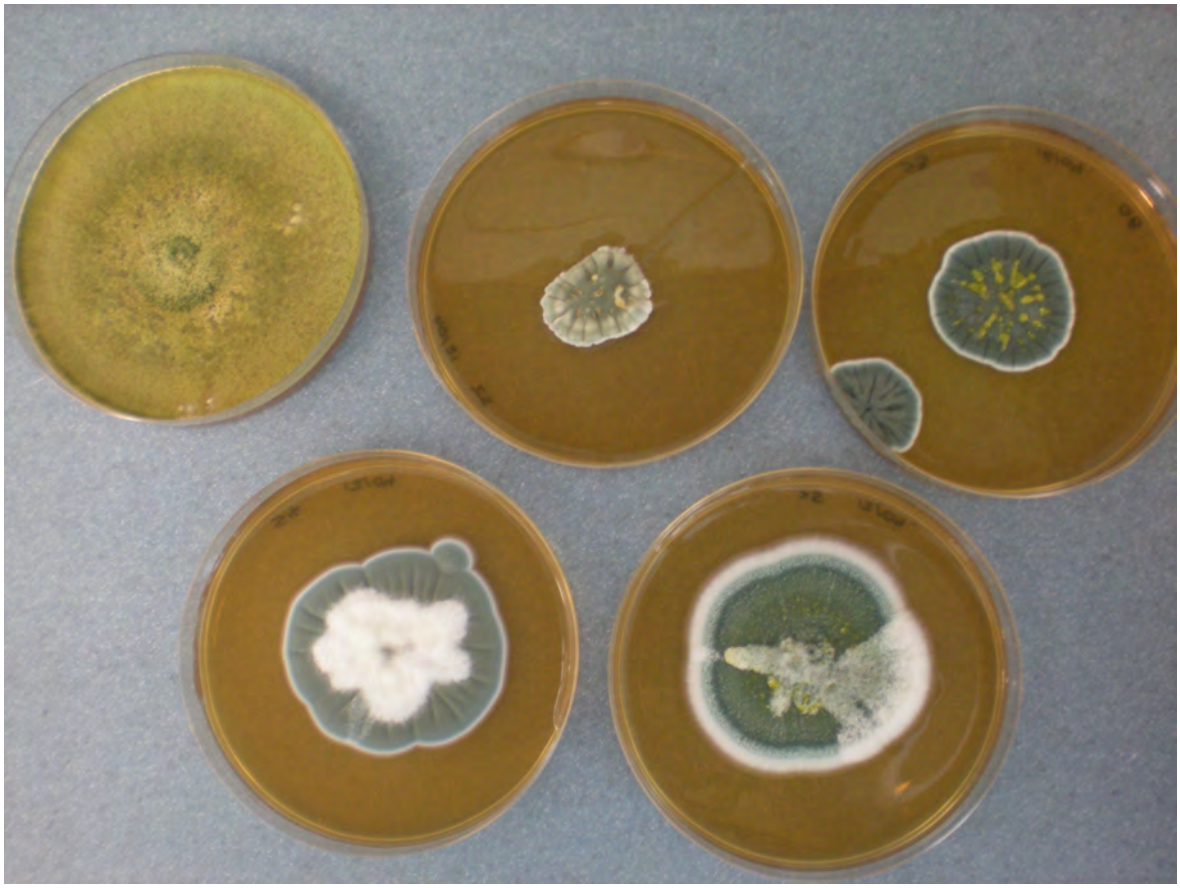

Fig.26. Isolates S1-S5 which resembled different colony morphologies These had been isolated from swabs taken by the BFI of cellulose nitrate reels. Preliminary identifications were made to genus level using microscopy. Identifications were S1=*Trichoderma* sp. and S2-S5 were *Penicillium* spp. Top row (left to right)= S1-S3, bottom row (left to right)= S4 and S5.

2.3. Methods II: Isolate Identification

2.3.1. Isolation and Identification of Fungi Found on Contaminated Film

After simulated inspection, film reels from the NWFA were swabbed both on the film image (frame) and the edge using a sterile swab moistened with sterile water and spread onto two plates of malt extract agar (MEA). After swabbing the edge of the film, film reels were partially unwound and samples were taken from film frames in the middle of the film which appeared to the naked eye to have visible mould growth. These were then grown at 25^{o}C for 5-7 days after which individual colonies were selected and subcultured to obtain pure cultures. Counts of colonies on plates used for swabs were not performed as this technique was only used to attempt to isolate different colonies which had not been picked up by air sampling. Isolates were also subcultured from colonies obtained from air sampling. Isolates were selected for subculturing either because they were predominant or represented the diversity of fungi present. All isolates from the air sampling and swabs of reels donated by the NWFA and S1-S5 from swabs of cellulose nitrate reels donated by the BFI were then identified where possible to genus level using light microscopy. Slide cultures were then prepared by pipetting 100µL of molten MEA agar onto a sterile microscope slide and allowed to cool. A sterile scalpel was used to cut the droplet in half and also inoculate fungi from plate culture into the cut by removing a small piece and placing it on the agar.. A cover slip was placed over the agar and the slides incubated for 24-48 hours at 25^{o}C in a humidity chamber (plastic boxes with moist tissue to avoid desiccation), after which they were examined under the light microscope at x10 or x40, magnification depending on the visibility of sporing structures. Identification was made to genus level with reference to images and keys in the literature (Barnett et al., 1972, Hoog et al., 1995, and Samson et al., 1996).

After preliminary genus identifications, several isolates also underwent molecular identification by CABI Microbial Services (Oxfordshire, UK) (n=15) by processing the samples using molecular sequencing of the internal transcribed spacer (ITS) region of the rRNA gene cluster. Results were then matched against reference sequences held in EMBL/GenBank (global databases) to establish identity. These were: RR1093 I1-I4, RR1399 I1-I6, RR1470 I1, RR1549 I2-I3, S3 and S5, and were selected based on their predominance on the selected reels.

2.4. Results II: Identification of Isolates

2.4.1. Identification of Fungal Isolates from Preliminary Investigations

By swabbing the film reels and performing simulated inspections using an air sampler, many colonies were isolated with varying morphologies (Fig.27). During preliminary work using air sampling of reels RR1491, RR1494, RR1514 and RR1533, only total CFU counts were recorded. From this sampling, twenty eight colonies were isolated representing different colony morphologies, and were subcultured and a preliminary identification made to genus level (Table 5).

Films labelled RR1491 were shown to be heavily contaminated and released the highest numbers of spores, although contamination was mainly restricted to a dominating genus i.e. *Aspergillus* spp. Films labelled RR1494 had lower colony counts but a wider range of species.

Molecular identification was used to identify predominant isolates with varying colony morphologies. This was performed using ITS2 and ITS4 primers and comparing the sequences with BLAST (Professor Malcolm Richardson, Wythenshawe, University Hospital of South Manchester). Identifications were:

RR1514 F1 I1 – *Penicillium chrysogenum*

RR1491 F1 I3 – *Aspergillus versicolor*

RR1491 F1 I15 - *Aspergillus versicolor*

RR1491 F2 I4 – *Stachybotrys chartarum*

RR1491 F3 I2 – *Arachnomyces glareosus*

The predominant colonies from films labelled RR1491 were either green and velvety or white, raised and furry in appearance and were identified by microscopy as primarily *Aspergillus* species, distinguished by the presence of a vesicle and radiating conidia on the conidiophore. (Fig.28). Four isolates produced red pigmentation in the malt extract agar (Fig.29) and through identification on culture slides were identified as *Aspergillus* species. Species such as *A. flavus* and *A. versicolor* are documented to produce red pigments (Assante et al., 1981) when cultivated on particular media such as MEA.

Other frequently seen green colonies were identified by microscopy to be *Penicillium spp.*(Fig.30), by visualisation of long conidiophore stipes arising from phialides at the top of metulae (Samson et al., 1996). *Cladosporium* was also isolated from RR1491 F1(Fig. 31). This film contained more genera of fungi than the other reels in the RR1491 collection although RR1491 F3 had higher colony counts.

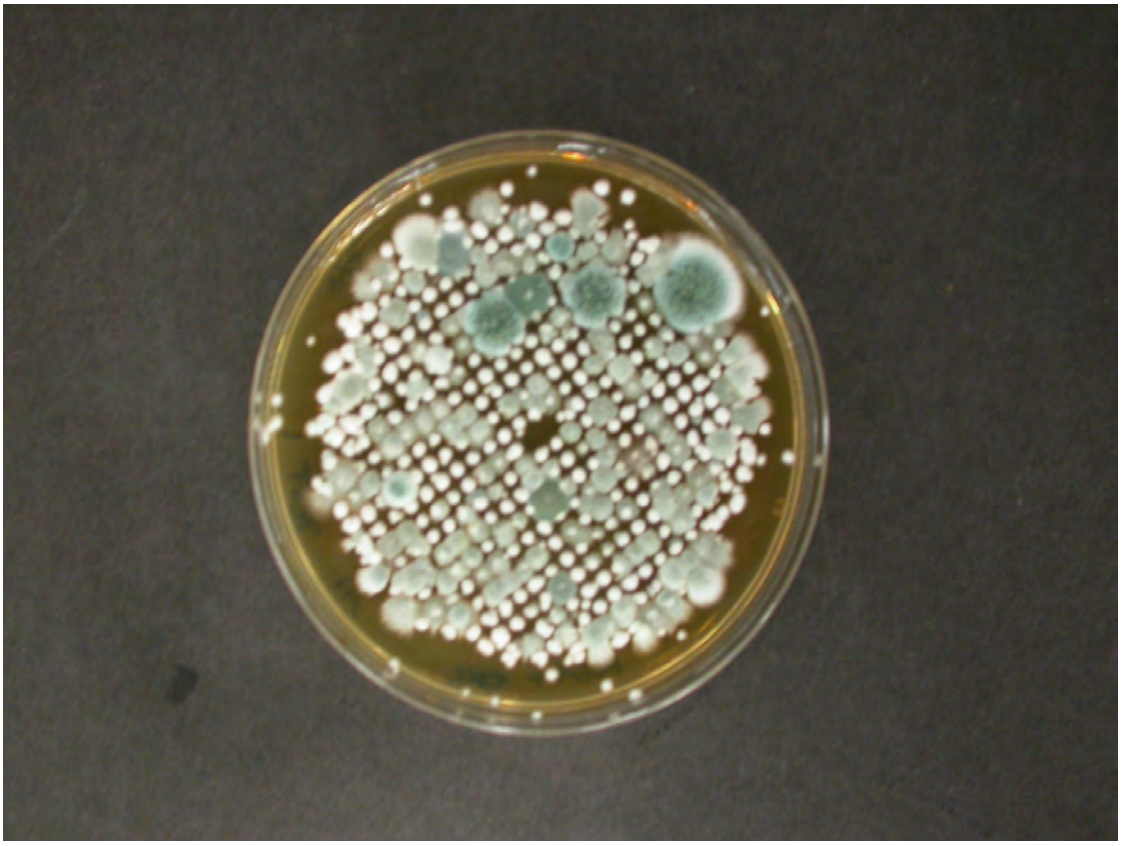

Fig.27. Air sampling of a heavily contaminated film from the RR1491 collection showing a wide range of colonies. The small colonies form a grid due to the position of holes on the air sampling plate. Colony sizes differ due to varying growth rates between genera and species.

Table 5. Preliminary studies of the range of isolates taken from air sampling and swabbing of film. A '*' in the 'Genus' column indicates isolates identified by molecular techniques.

Isolate Name	**Genus (and species if known)**
RR1491 F1 I1	*Aspergillus*
RR1491 F1 I3	**Aspergillus versicolor*
RR1491 F1 I4	*Aspergillus*
RR1491 F1I5	*Cladosporium*
RR1491 F1 I9	*Aspergillus niger*
RR1491 F1 I10	*Aspergillus*
RR1491 F1 I11	*Cladosporium* like
RR1491 F1 I12	*Aspergillus niger*
RR1491 F1 I13	*Penicillium*
RR1491 F1 I15	**Aspergillus versicolor*
RR1491 F2 I1	*Aspergillus*
RR1491 F2 I2	*Aspergillus niger*
RR1491 F2 I3	*Aspergillus*
RR1491 F2 I4	**Stachybotrys chartarum*
RR1491 F2 I6	*Aspergillus*
RR1491 F2 I8	*Aspergillus*
RR1491 F3 I1	*Aspergillus*
RR1491 F3 I2	**Arachnomyces glareosus*
RR1494 F2 I3	*Botrytis cinerea*
RR1494 F3 I1	*Penicillium*
RR1494 F3 I2	*Aspergillus*
RR1494 F4 I1	*Aspergillus*
RR1494 F4 I3	*Acremonium* like
RR1494 F4 I6	Unidentified
RR1514 F1 I1	**Penicillium chrysogenum*
RR1514 F1 I2	Unidentified
RR1533 I1	*Acremonium* like
RR1533 I3	*Cladosporium*

Fig.28. *Aspergillus versicolor* conidiophore (Photo: CABI Microbial Services) (x40)

Fig.29. Some colonies of *A.versicolor* also produced red pigment, which turned the medium red (MEA, Oxoid).

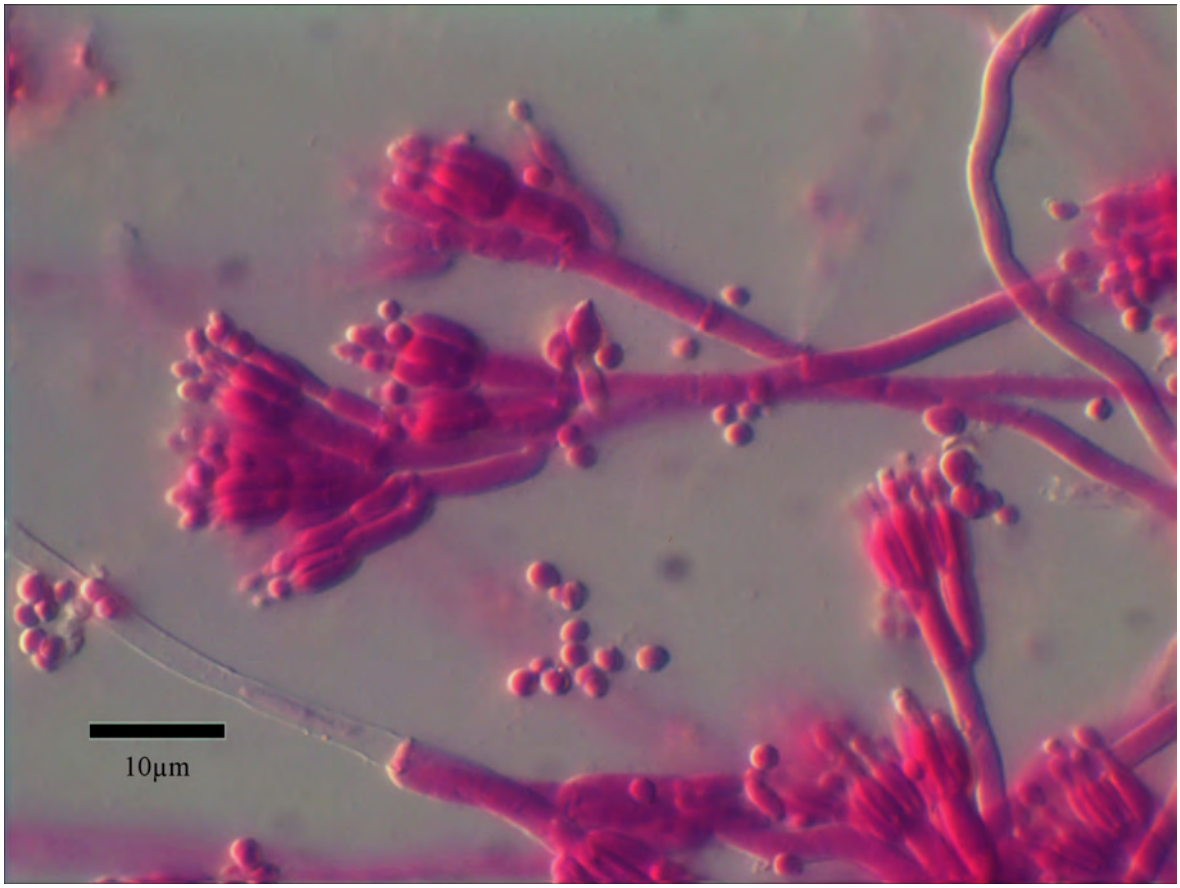

Fig.30. *Penicillium brevicompactum* conidiophore (Photo: CABI Microbial Services) (x40)

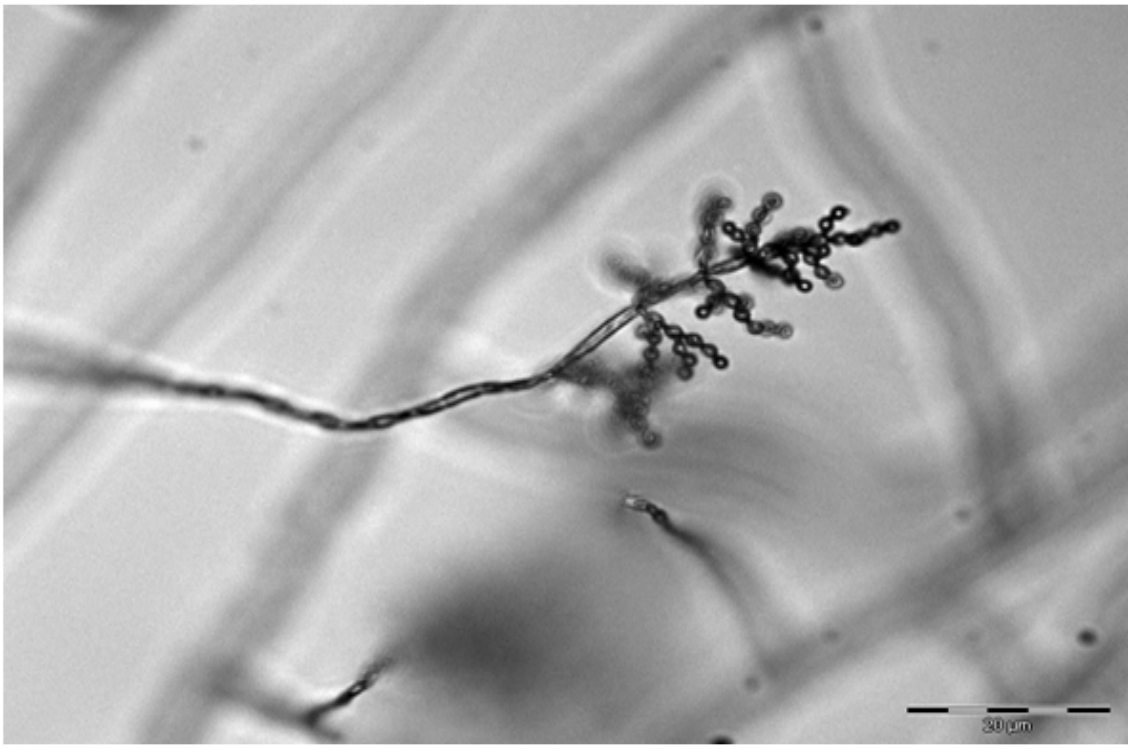

Fig.31. *Cladosporium* conidiophore (x40).

2.4.2. Further Identifications

Following the preliminary identifications of isolates, further studies were undertaken to describe isolates (n=33) in more detail for the subsequent film reels studied (i.e. reels RR1399, RR1093, RR1470 and RR1549) (Table 6) . *Aspergillus versicolor* ATCC 11730 was also described as this served as a control organism in subsequent enzyme assays, and this species had been frequently isolated from other studies at MMU (unpublished) and in the literature (Abrusci et al., 2004a, 2005). Isolates RR1491 F2 I4 (*Stachybotrys chartarum*) from preliminary sampling of reels and JWP1 I1 (*Alternaria* sp.) which was isolated from mouldy wall paper, were also described because they were selected for enzyme assays to extend the range of genera and colony morphologies tested. Initially, the genus of fungi was determined, and then the colony morphologies were described. Isolates were described by referring to the literature (Hoog et al., 1995, Samson et al., 1996 and Onions et al., 1981). Isolates RR1093 I1-I4, RR1399 I1-I6, RR1470 I1, RR1549 I2-I3, S3 and S5 were then selected based on their predominance on the selected reels and identified by CABI using molecular techniques for species determination.

Predominant genera with different colony morphologies, which were isolated from cellulose acetate films were *Aspergillus* species with *A.versicolor* being the species most frequently isolated (n=8/15) (Fig.32). *Penicillium* spp was the 2nd most predominant genus (Fig.33), and was the most frequently isolated from the cellulose nitrate swabs provided by the BFI. *P.chrysogenum* was the most frequently isolated *Penicillium* species on both the nitrate and acetate reels (n=5). All isolates listed (Table 6) were assayed for gelatinase and cellulase production.

After initial isolation from the film reel onto MEA, isolate RR1093 I5 (Unknown Ascomycete) became non-viable thus was discarded. After initial counts of RR1470 I4 and RR1470 I5 it was deemed that these were not predominant on the reels indicated by low CFU counts, thus were omitted from Table 6. Enzyme assays were not performed on these isolates.

Table 6. Descriptions of isolates from four contaminated cellulose acetate films (RR1093, RR1399, RR1470, RR1549) and RR1491 F2 I4 from preliminary sampling. JWP1 was isolated from mouldy paper. Isolates S1-S5 were isolated from swabs donated by the BFI of mouldy cellulose nitrate film reels. A '*' in the 'Isolate Name' column indicates the isolates identified by CABI. Identification of genera of some isolates was not possible due to lack of sporing structures so were labelled 'Unidentified'. All of these isolates were assayed for gelatinase and cellulase production.

Isolate Name	Identification	Colony Morphology on MEA
***(ATCC 11730)**	*Aspergillus versicolor*	Dark green with white edge and patches of various colours (hence 'versicolor').
JWP1	*Alternaria alternata*	Rapidly growing starting as black and becoming brown with age.
***RR1491 F2 I4**	*Stachybotrys chartarum*	Black/ brown flat colony with purple edge and protusions in centre. Slow growing.
***RR1093 I1**	*Penicillium chrysogenum*	Powdery blue/ green colony with white edge.
***RR1093 I2**	*Penicillium brevicompactum*	Powdery green colony white edge.
***RR1093 I3**	*Penicillium citreonigrum*	Slow mycelial growth with restricted colonies. Grey-green to yellow green.
***RR1093 I4**	*Penicillium chrysogenum*	Colony growing slowly, white, with dull grey green centre.
RR1093 I5	*Ascomycete* (unviable)	Grey /brown dense furry colony with rapidly expanding mycelia.
RR1093 I6	*Aspergillus*	Slow growing Orange to cinnamon brown colony with white outer edge.
RR1093 I7	*Nigrospora* like	White filamentous spreading colony on MEA with black centre. Produces light green exudate.
RR1093 I8	*Aspergillus*	Olivaceous dull grey/ green
RR1093 I9	*Penicillium*	Green with yellowy outer edge. Produces droplets of clear exudates.
RR1093 I10	*Penicillium*	Olivaceous dull grey/green.
***RR1399 I1**	*Aspergillus versicolor*	Green centre with red pigmented exudates.
***RR1399 I2**	*Aspergillus versicolor*	Colony white with red patches in centre.
***RR1399 I3**	*Aspergillus versicolor*	Very slow growing drab olive with bits of dull brown.
***RR1399 I4**	*Aspergillus versicolor*	Dull green raised colony. Dull greeny brown patches.
***RR1399 I5**	*Penicillium brevicompactum*	Dull green raised colony. White edge on new mycelia.
***RR1399 I6**	*Aspergillus versicolor*	Dull green/ olive colony with yellow outer edge.
RR1399 I7	*Aspergillus*	Colony slow growing and mainly green with white edge and bits of red pigmentation. Yellow exudates.
***RR1470 I1**	*Aspergillus versicolor*	Colony growing slowly,white.
RR1470 I2	*Aspergillus*	Olivaceous dull grey/ green colony.
RR1470 I3	Unidentified	Rapidly growing colony.
RR1549 I1	*Mucorales sp.*	Rapidly growing expanding Yellowy green colony forming a thick mat possibly due to abundant production of sporangiophores and fast growing myclelia.
***RR1549 I2**	*Aspergillus versicolor*	Red and white colony with mottled green patches. Produces red pigmentation in agar.
***RR1549 I3**	*Aspergillus versicolor*	White and cream slow growing colony with mottled green patches.
RR1549 I4	*Penicillium*	White slow growing colony with mottled green patches
RR1549 I5	*Penicillium*	White slow growing colony.
S1	*Trichoderma sp.*	Powdery white colony on MEA with patches of green. Very rapidly spreading.
S2	*Penicillium (or related species)*	Powdery blue/green with white edge on MEA. Produces clear exudate.
***S3**	*Penicillium chrysogenum*	Powdery green colony on MEA with clear exudate. Grows rapidly with yellow pigment in medium.
S4	*Penicillium*	Predominantly white colony with green centre. Slow growing but readily sporulating on MEA.
***S5**	*Penicillium chrysogenum*	Green with white edge on MEA with yellow exudate.

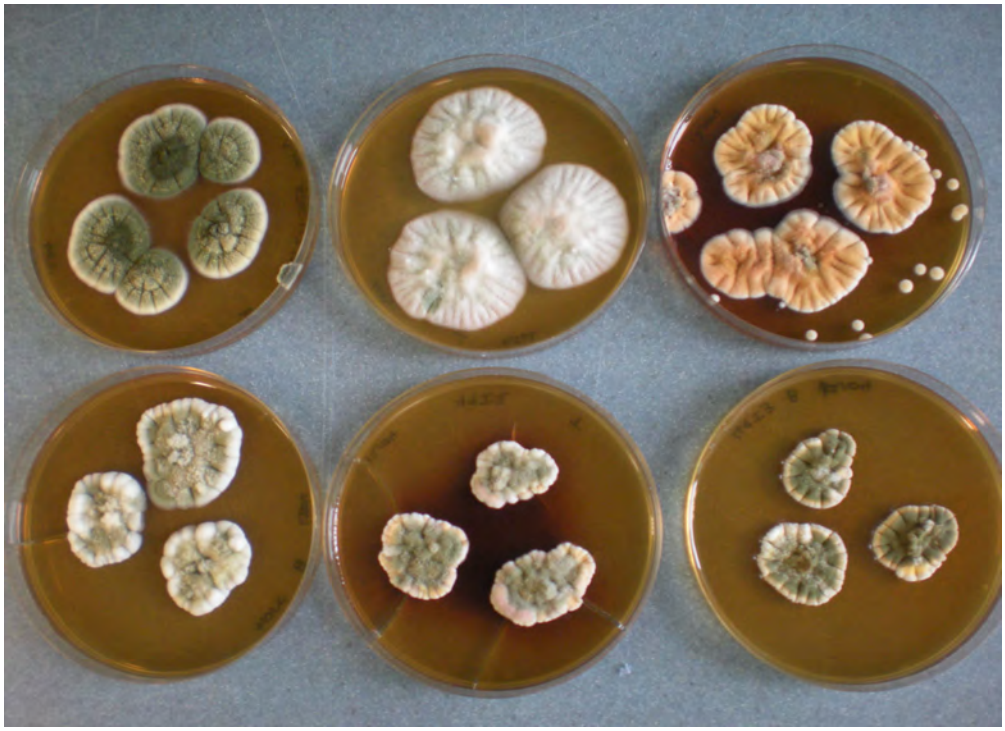

Fig.32. Different colony morphologies of *A.versicolor* which produces different colours hence the name 'versicolor'. Top (Left to right): ATCC11730, RR1399 I4 and RR1549 I2. Bottom (Left to right): RR1470 I1, RR1399 I2 and RR1549 I3.

Fig.33. Different colony morphologies of *Penicillium* spp on MEA which have been identified to species level by CABI. Top (Left to right): RR1093 I1 (*P.chrysogenum*), RR1093 I2 (*P.brevicompactum*), RR1093 I3 (*P.citreonigrum*). Bottom: (Left to right): RR1399 I5 (*P.brevicompactum*), S3 (*P.chrysogenum*), S5 (*P.chrysogenum*)

2.5. Methods III: Gelatinase Assays

2.5.1. Gelatine

Gelatine is thought to be the primary substrate for fungal growth on cinematographic film therefore any fungi that are able to utilise this will produce proteases, specifically gelatinases. The mechanism for this has been discussed previously (Chapter 1). Gelatine is an amorphous structure i.e. lacks a definitive form, and consists of covalently bonded collagen molecules. During the manufacture of gelatine from collagen, these bonds do not break which explains the gelling properties of gelatine when cooled (Abrusci et al., 2004b). Cross linking (hardening) agents are also necessary in photographic gelatine and have several beneficial properties. They prevent swelling of the gelatine which would affect the film image and are also resistant to chemicals involved in film processing. As a result of increased hardening, these agents also decrease the susceptibility of gelatin to microbial degradation. These agents can include formaldehyde, polymeric hardeners, polyacrylates (Abrusci et al., 2004b) or polyaldehyde which has also been used to harden gelatine to immobilise yeast cells (Parascandola, 1990), In cinematographic film, these agents need to give a higher dimensional stability than in other gelatine applications e.g. bakery products, canned hams, clarification of wines and juices by precipitating materials that cause cloudiness, in sweets, and the pharmaceutical industry for capsule based medicines.

Methods were developed to test key isolates for gelatinase production and attempts were made to quantify and assess the potential of an organism to damage film in terms of gelatinase activity i.e. working on the hypothesis that organisms with the highest activity will be the most destructive to film and be the most numerous on badly spoiled film.

2.5.1.1. Preparation of malt extract agar with gelatine for detection of hydrolysis

The method was modified from that of Kanemitsu et al. (2001), who used 5% agar to 0.8% gelatine to look for gelatinase production by enterococci. For fungi, the Oxoid manual suggests dissolving 5% MEA powder in distilled water. Dried gelatine powder ((Gelatin) Oxoid) was mixed with MEA before autoclaving at a ratio of 2.5: 97.5 (or 0.125g to 4.875g per 100mL distilled water). Gelatine at 2.5% of the total dried power was used because preliminary experiments had shown that this was the minimal amount required for zones of hydrolysis to be visible on the solid medium. After autoclaving, 20mL volumes of the medium were poured into Petri dishes which formed solid agar at room temperature.

Zones of hydrolysis around fungal isolates on this medium indicated gelatinase production. Single plugs of fungal isolates from cinematographic films, maintained on

MEA, were removed with an 8mm cork borer and placed centrally on gelatine/ MEA agar plates. These were incubated for 7 days at 30°C and visible zones of clearing appeared around isolates if gelatinolytic activity had occurred. Trichloroacetic acid (TCA) was mixed with sterile distilled water (1g per 100mL) and added by pipette onto the agar, as this was noted by Kanemitsu et al. (2001) to enhance visibility of gelatine hydrolysis, although visibility was still possible without it. Negative controls were MEA plates without gelatine.

Attempts were then made to quantify gelatinolytic activity, by growing the fungal isolates in broth cultures and assaying the supernatants.

2.5.1.2. Alternative method for detection of gelatinases

In addition to the MEA with added gelatine solid media, an assay using minimal media was also performed. It was thought that this medium would have advantages over MEA with gelatine due to the restricted availability of nutrients, therefore the fungi are more likely to utilise gelatine for growth rather than other nutritional components as would occur with MEA. The likelihood that gelatinase enzymes would be produced was therefore increased, thus making this medium more sensitive to detection of gelatinase producing isolates.

Gelatine selective agar was prepared based on the composition described by Kanemitsu et al. (2001). This was composed of Technical Agar no.3 (Oxoid, Basingstoke, UK) 1.2% w/v, Bacteriological Peptone (Oxoid, Basingstoke, UK) 0.02%w/v, sodium chloride 0.2% w/v, di-potassium hydrogen orthophosphate 0.1% w/v, magnesium sulphate 0.05% w/v, potassium chloride 0.05% w/v and 0.8% w/v gelatin (Oxoid). After autoclaving 20mL volumes were poured in Petri dishes.

Two sets of plates of gelatine selective agar were prepared; half contained all components except the gelatine (i.e. gelatine-ve) and half contained all components with added gelatine (i.e. gelatine+ve). Plugs of fungal isolates previously maintained on MEA were removed using an 8mm cork borer, placed in the centre of the plates and incubated for 7 days at 30°C. Visibility of gelatine hydrolysis by fungal isolates was only possible by the addition of TCA and additional visual enhancement was achieved using a light box (Fig.31).

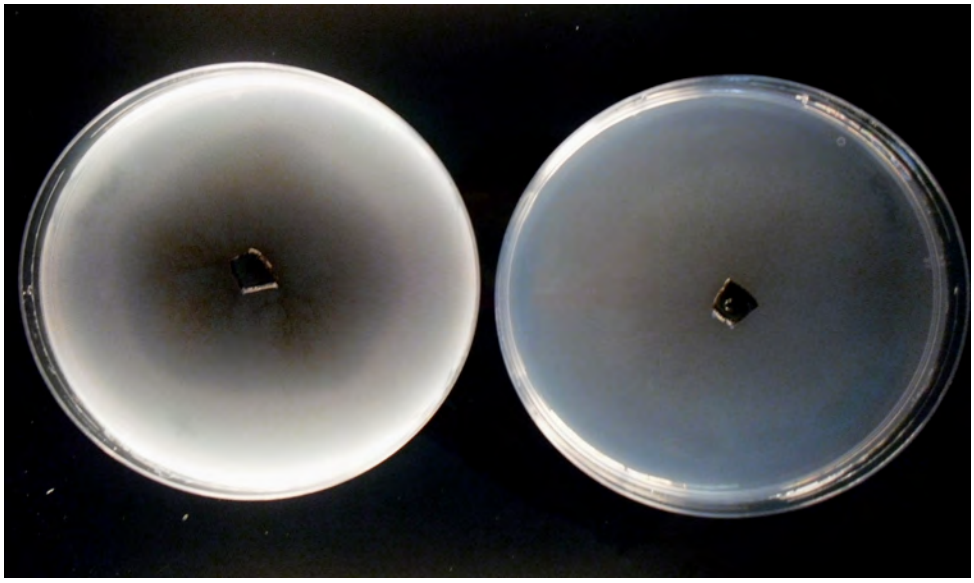

Fig.34. A plug of a fungal isolate cut with an 8mm cork borer and inoculated into the centre of gelatine selective agar and incubated for 7 days at 30ºC. The plates contained Technical Agar no.3 (Oxoid, Basingstoke, UK) 1.2% w/v, Bacteriological Peptone (Oxoid, Basingstoke, UK) 0.02%w/v, sodium chloride 0.2% w/v, di-potassium hydrogen orthophosphate 0.1% w/v, magnesium sulphate 0.05% w/v, potassium chloride 0.05% w/v, and 0.8% gelatine w/v. The plate on the left contains gelatine. Pipetting trichloroacetic acid onto the plate resulted in visibility of a clear zone around the isolate if gelatine had been hydrolysed (left).

2.5.1.3. Preparation of Broth cultures

Growth of microorganisms in broth culture, and subsequent extraction and assay of the supernatants for proteinase enzymes has been described previously (Kanemitsu et al., 2001). This method was used to induce gelatinase production and extract the enzymes for quantitative analysis. For preliminary tests gelatine was added to malt extract broth (MEB) in 5 different amounts: 2g, 3g, 5g, 10g and 15g per 100mL of distilled water. It was determined that 3g would be used subsequently, because this produced a broth which remained liquid at room temperature but solidified on refrigeration at 4ºC. MEB was also tested at different concentrations: 2.0g, 1.5g and 1g per 100mL. The final concentrations chosen were 2g of MEB with 3g of gelatine per 100mL, as this induced gelatinase production in the highest number of fungal isolates and produced good fungal growth.

Aspergillus versicolor (ATCC 11730) was used as a positive control because it has been found by Gopinath et al., (2005) to be highly gelatinolytic. It has been isolated from cinematographic film in previous studies (Abrusci et al., 2004a, 2004b) and growth of the test organism on the solid gelatine medium produced a zone of hydrolysis.

A.versicolor ATCC11730, *S.chartarum* (RR1491 F2 I4) and *Alternaria* (JWP1) were also incubated in MEB without gelatine and the supernatants assayed on solid media, to determine whether gelatinase production was inducible or constitutive.

To inoculate the broths, plugs of fungal mycelium were removed with an 8mm cork borer from cultures maintained on MEA. Spore suspensions were not used due to difficulties in removing or isolating spores from some cultures particularly isolates with wet spores (Yang and Heinsohn, 2007). Cultures were inoculated into 50 mL volumes of broth in shake flasks which each contained 1g MEB with 1.5g of gelatine. The optimum incubation time was determined by taking 1mL of supernatant on days 4, 5 and 6 days of growth, placing them in bijoux bottles and refrigerating the samples at 4ºC for 1 hour, after which they were removed to observe whether hydrolysis of gelatine had occurred i.e. the broth remained liquid. After 6 days all broths that contained gelatinase producing organisms remained liquid after this time thus indicating gelatinase hydrolysis. As a result, all isolates were subsequently incubated for 6 days at 30ºC in a rotary incubator set at 150 rpm, when spherical fungal balls of growth were apparent in the flasks (Fig.35).

Fig.35. Spherical balls of fungal growth seen in shake flasks after inoculation with a fungal isolate. and incubation on a rotary shaker (150rpm) at 30^{o}C.

2.5.1.4. Assaying of Supernatants

Three wells made with a 5mm cork borer were cut at 60° angles to each other approximately half way between the edge of the plate and the centre, to form an equilateral triangle and to ensure enough separation so that any clear zones would not overlap.

Proteinase K (Sigma) was used as a standard enzyme and served as a positive control to compare with the gelatinolytic activity of crude fungal enzyme extract (Kanemitsu et al., 2001). Sterile distilled water served as the negative control. The effect of Proteinase K on pure gelatine (15% w/v) was observed by dissolving gelatine in distilled water, autoclaving and dispensing into 20mL amounts in Petri dishes, which were then refrigerated at 4°C to ensure solidification of the medium. Three wells were cut into the medium in the same method as previously described, and inoculated with 40µl of Proteinase K at at concentration of 1000 µg per mL prepared in PBS to maintain salt concentration. This was placed back in the fridge because incubation at 30°C and leaving the plates at room temperature would have caused the medium to return to liquid state. After 24 hours the plates were removed from the fridge and Proteinase K had produced permanent liquidised gelatine at the point of inoculation whilst the area around the wells on the control plates containing sterile water had remained solid.

To investigate how a supernatant with gelatinolytic activity would be visualised on the MEA with added gelatine, 40µl of Proteinase K was added to wells on solid medium as described above. After 24 hours at 30°C and after addition of TCA zones of hydrolysis could be seen more clearly (Fig.33). This method was also applied to the gelatine selective medium but visualisation of hydrolysis zones was not possible without the addition of TCA, unlike the MEA and gelatine plates (Fig.34).

Assaying of supernatants was performed on the MEA and gelatine (2.5:97.5 v/v ratio) plates and gelatine selective medium (described previously, section 2.5.2.2.). Culture broths were decanted into 1 x 25mL sterile universal bottle per culture, and centrifuged at 150 rpm for 5 minutes to enable extraction of the supernatants without contamination by fungal mass.

Supernatants were sterile filtered using a Millipore filter (PALL Acrodisc 32mm with 0.2µm Supor™membrane) after which 40µl was pipetted into the 3 wells and incubated at 30°C for 24 hours. After addition of TCA the diameter of any zones of hydrolysis was then measured.

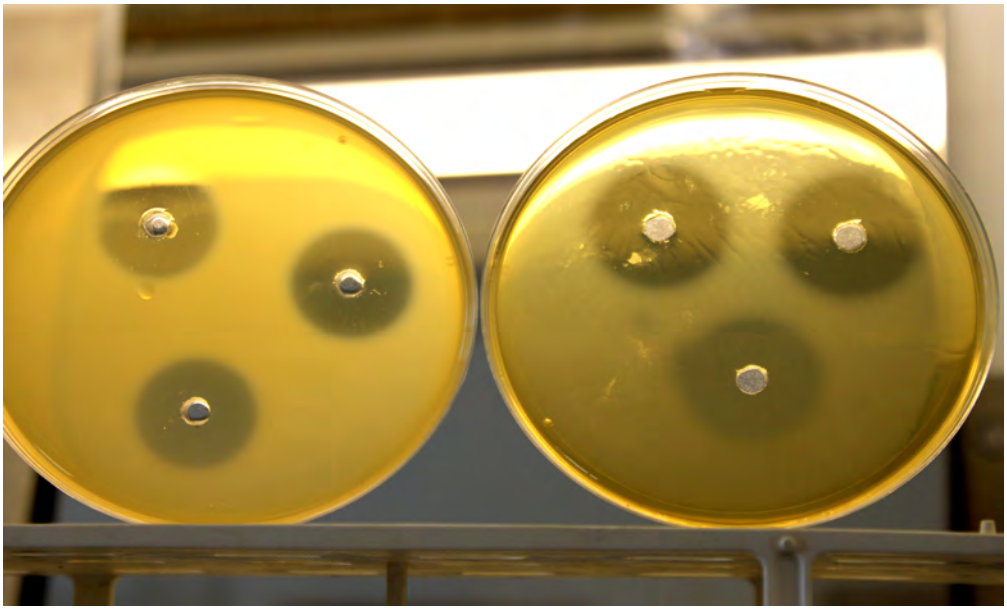

Fig.36. Malt extract agar with added gelatine. Three wells were cut at 60° angles with a 5mm cork borer. 40µl of sterile filtered supernatant or Proteinase K was then pipettted into the wells. A zone of hydrolysis indicated gelatinase production. The addition of trichloroacetic acid (TCA) enhanced visibility of the clear zones by increasing the visibility of gelatine in the agar (left).

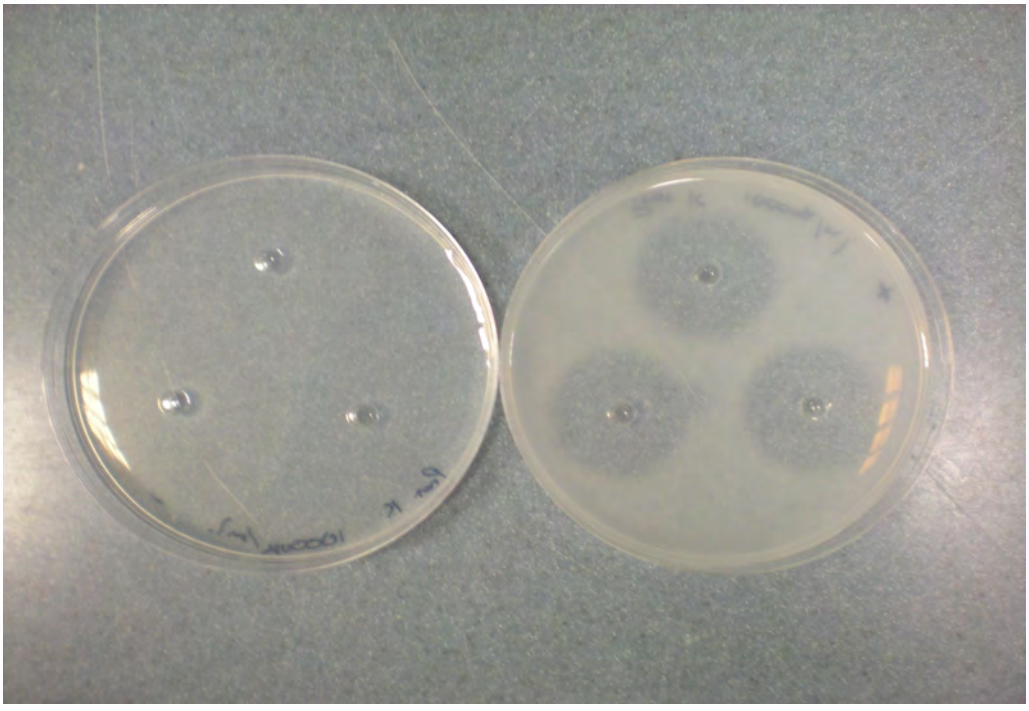

Fig.37. Two plates of gelatine selective medium. 40 µl of sterile filtered Proteinase K at a concentration of 1000 µg per mL of PBS, was pipetted into the wells of the plates (n=3) as a +ve control, and incubated at 30°C for 24 hours. The plates were flooded with trichloroacetic acid (TCA) which resulted in visibility of the hydrolysis zones (right). Visibility of gelatine hydrolysis was not possible on plates without TCA (left).

2.6. Results III: Gelatinase Assays

All isolates from reels RR1093, RR1399, RR1470 and RR1549 were assayed for gelatinase production (Table 7). *Aspergillus versicolor* (ATCC11730) was used as positive control due to isolation of *A.versicolor* from multiple mouldy reels in this study and from previous studies at MMU (unpublished). Its gelatinolytic activity has also been described in the literature (Gopinath et al., 2005). An *Alternaria* (JWP1) fungal isolate isolated from paper and a *Stachybotrys chartarum* (RR1491 F2 I4) isolated from preliminary experiments using film were also assayed to broaden the spectrum of fungal isolates tested. Isolates from swabs of cellulose nitrate reels sent from the BFI (S1-S5) were also assayed.

Initially all isolates were screened on malt extract agar (MEA) with added gelatine. At the optimum concentrations of 0.125% gelatine to 4.875% MEA w/v, nearly all fungal isolates that were shown to produce gelatinases in supernatants (indicated by hydrolysis of gelatine in the MEB with added gelatine broth cultures), also produced a zone of hydrolysis around the colony on solid media. However, some in some cases a zone of hydrolysis was not visible around a colony, even though they hydrolysed the gelatine in the broth cultures and assay of the supernatant resulted in zones of hydrolysis around the wells. These were JWP1, RR1093 I3, RR1399 I7, RR1470 I3 and S4. On the gelatine selective medium however, all isolates that were shown to produce gelatinase activity by hydrolysing the gelatine in the broth cultures also produced a zone of hydrolysis. The growth of isolates increased on gelatine selective medium compared to the gelatine from the negative control (Fig.38).

The most common isolates for reel RR1399 were I1, I2, I3 and I6 which were all identified as *A.versicolor*, presenting different colony morphologies. These all produced hydrolysis zones of similar size to the *A.versicolor* ATCC11730. These were 6cm on MEA with gelatine solid medium and 7 or 6 cm on gelatine selective medium. The most common isolates for reel RR1093 were I3 (*P.citreonigrum*) and I4 (*P.chrysogenum*). These produced clear zones of 6 and 5.5cm on MEA and gelatine medium respectively and 5 on gelatine selective medium which indicated a lower gelatinolytic activity than the *A.versicolor* isolates.

In experiments to deduce whether gelatinase enzymes were inducible or constitutive i.e. growth in MEB without gelatine, *A.versicolor* and *S.chartarum* produced zones of hydrolysis on both the MEA with added gelatine medium and the gelatine selective medium, whilst the *Alternaria* isolate did not. Thus, *A.versicolor* and *S.chartarum* produce constitutive protease enzymes which can degrade gelatine whilst *Alternaria* has the potential for gelatinase production in the presence of gelatine.

Table 7.Results of gelatinase assays from isolates taken from the film reels RR1093, RR1399, RR1470, RR1549, *A.versicolor* (ATCC 11730) and RR1491 F2 I4 from preliminary sampling. Isolates were assayed on 2 types of solid medium: gelatine and MEA and gelatine selective (minimal) medium. Confirmation of gelatinase production was by the appearance of a zone of hydrolysis around fungal isolates, that were made visible by the addition of trichloroacetic acid. The column labelled 'liquefaction of supernatant' indicates whether the gelatine and MEB broth remained liquid after refrigeration at 4ºC (a positive result indicates that gelatine has been hydrolysed by the production of enzymes). If a positive result was achieved for both solid and liquid media then supernatants were assayed for quantitative analysis. Some of the organisms hydrolysed the gelatine in the MEB and gelatine broth, although the supernatant failed to produce clear zones so was given a value of '0'. If further analysis of supernatants could not be carried out due to a negative results then 'N/A' was entered in the subsequent column. Isolates identified by molecular methods are indicated by a '*' in the 'Identification column.

Isolate Name	Identification	Gelatine Hydrolysis on MEA and Gelatine Agar (+/-)	Gelatine Hydrolysis on TA no.3 and gelatine	Gelatine Hydrolysis in Broth (+/-) after 7 days incubation	Size of hydrolysis zone from supernatant on MEA and gelatine (mm)	Size of Hydrolysis zone from supernatant on TA no.3 and gelatine (mm)
***(ATCC 11730)**	*Aspergillus versicolor*	positive	positive	positive	6	7
JWP1 I1	*Alternaria alternata*	negative	positive	positive	4.5	5.5
RR1491 F2 I4	*Stachybotrys chartarum*	positive	positive	positive	5	5
***RR1093 I1**	*Penicillium chrysogenum*	positive	positive	postive	5	5
***RR1093 I2**	*Penicillium brevicompactum*	positive	positive	positive	6	5
***RR1093 I3**	*Penicillium citreonigrum*	negative	positive	negative	6	5
***RR1093 I4**	*Penicillium chrysogenum*	positive	positive	positive	5.5	5
RR1093 I5	*Ascomycete* (unviable)	N/A	N/A	N/A	N/A	N/A
RR1093 I6	*Aspergillus* sp.	positive	positive	positive	6	6
RR1093 I7	*Nigrospora* like	positive	positive	positive	6	4
RR1093 I8	*Aspergillus* sp.	positive	positive	negative	6	6
RR1093 I9	*Penicillium* sp.	positive	positive	positive	5	6
RR1093 I10	*Penicillium* sp.	positive	positive	positive	6	5
***RR1399 I1**	*Aspergillus versicolor*	positive	positive	positive	6	6
***RR1399 I2**	*Aspergillus versicolor*	positive	positive	positive	6	7
***RR1399 I3**	*Aspergillus versicolor*	positive	positive	positive	6	6
***RR1399 I4**	*Aspergillus versicolor*	positive	positive	positive	4.5	7
***RR1399 I5**	*Penicillium brevicompactum*	positive	positive	positive	5	5
***RR1399 I6**	*Aspergillus versicolor*	positive	positive	positive	6	6
RR1399 I7	*Aspergillus* sp.	negative	positive	positive	6	4.5
***RR1470 I1**	*Aspergillus versicolor*	positive	positive	positive	4.5	6
RR1470 I2	*Aspergillus* sp.	positive	positive	negative	5	5
RR1470 I3	Unidentified	negative	positive	positive	6	5.5
RR1549 I1	*Mucorales* sp.	positive	positive	positive	6	3
***RR1549 I2**	*Aspergillus versicolor*	positive	positive	positive	5	6
***RR1549 I3**	*Aspergillus versicolor*	positive	positive	positive	6	5
RR1549 I4	*Penicillium* sp.	positive	positive	positive	5	6
RR1549 I5	*Penicillium* sp.	positive	positive	positive	5.5	4
S1	*Trichoderma* sp.	positive	positive	positive	7	7
S2	*Penicillium (or related species)*	positive	positive	negative	6	4
***S3**	*Penicillium chrysogenum*	positive	positive	positive	5	5
S4	*Penicillium* sp.	negative	positive	positive	5	4
***S5**	*Penicillium chrysogenum*	positive	positive	positive	4	5

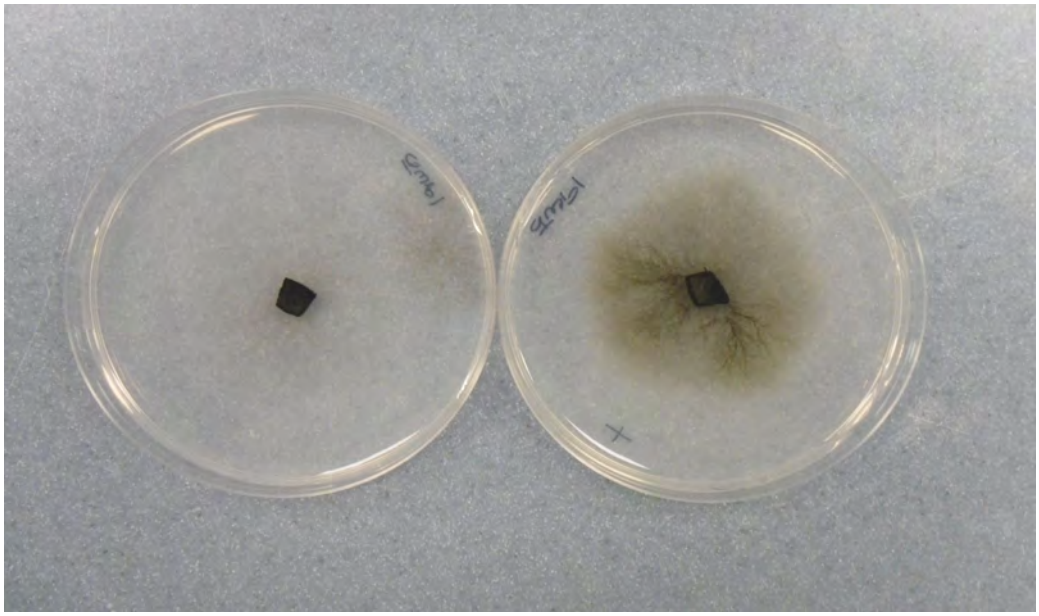

Fig.38. Gelatine selective medium containing Technical Agar no.3 (Oxoid, Basingstoke, UK) 1.2% w/v, Bacteriological Peptone (Oxoid, Basingstoke, UK) 0.02%w/v, sodium chloride 0.2% w/v, di-potassium hydrogen orthophosphate 0.1% w/v, magnesium sulphate 0.05% w/v, potassium chloride 0.05% w/v and on the right plate 0.8% gelatin (+ve) (Oxoid). After autoclaving 20mL volumes were poured in Petri dishes. After incubation for 7 days at 30^{o}C the *Alternaria* (JWP1) isolate shown here exhibited enhanced growth indicating the utilisation of gelatine for growth.

2.7. Methods IV: Cellulase Assays

2.7.1. Cellulose

In addition to gelatine, cellulose could be another potential substrate for fungal growth due to its presence in both cellulose acetate and cellulose nitrate film reels, and also in other materials e.g. cardboard boxes in which reels could be stored. However, it is not thought to be the primary substrate for microorganisms growing on cellulose acetate film reels due to the high degree of acetylation which make it more resistant to microbial attack (Buchanan et al., 1993, and Sakai et al., 1996). The availability of cellulose in nitrate film reels is unknown. Despite the predicted lack of availability of free cellulose on the film reels, cellulose assays were still performed *in-vitro* to determine the cellulolytic activities of isolates, as other materials are often present in archives which contain cellulose, and which could serve as a source of cross contamination to film reels.

Cellulose is a β-glucan and occurs primarily in nature in the cell walls of plants as well as some yeasts and fungi and is one of the most abundant biological compounds on earth with estimates being around 26.5 x 10^{10} tonnes (Allsopp and Gaylarde, 2004). These molecules are polysaccharides of D-glucose monomers linked by β-glycosidic bonds (Teather and Wood, 1982). Cellulose chains are held together with hydrogen bonds by Van der Waal's forces to form cellulose microfibrils (crystalline cellulose). As the cellulose chains are tightly packed, access by enzymes may be difficult thus various stages of attack by enzymes needs to occur.

Degradation of cellulose requires the actions of 3 types of enzyme which together are termed the cellulase enzyme complex: cellobiohydrolase which cleaves successive disaccharide units, endogluconase which separates the cellulose molecules into smaller fragments by attacking the centre of the chains (activated cellulose) and β-glucosidase which cleaves cellulobiose to glucose (Abrusci et al., 2004b and Deacon, 1997) (Fig.39). Production of the cellulase enzyme complex requires a lot of resources so as a result is tightly regulated. Thus, genes responsible for this production are suppressed in the presence of more readily utilised substrates such as cellobiose or glucose by a feedback system called catabolite repression (Deacon 2006). Cellulases find use in many biotechnological, and industrial applications such as bio-fuels (Wilson, 2009), paper and pulp industries (Sukmaran et al., 2005), pharmaceuticals (Bhat, 2000) and food waste management (Juwaied et al., 2011). Therefore a rapid screening process is useful to identify microorganisms that produce cellulases and to ascertain which produce enzymes with the greatest cellulolytic effect.

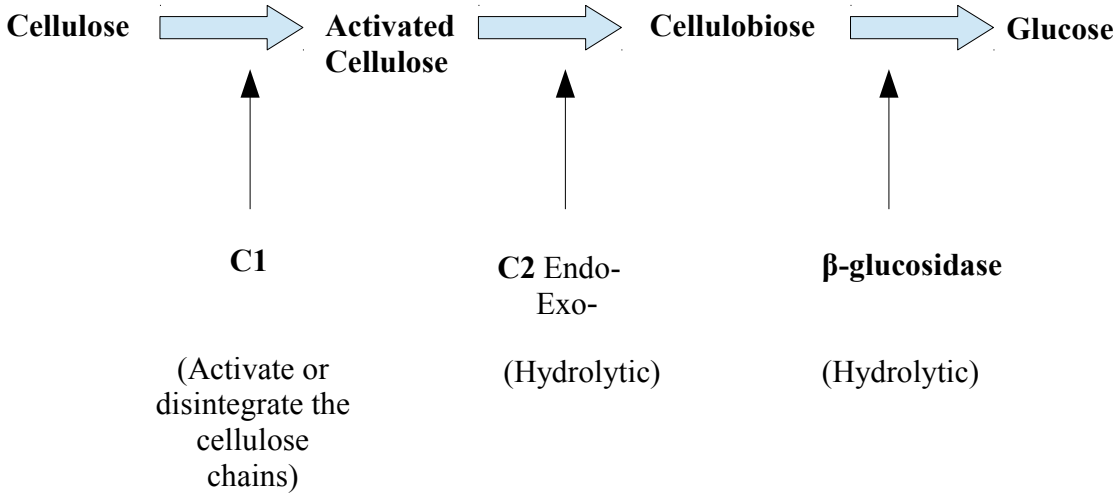

Fig.39. Multi-enzymatic system of the action of cellulases on the breakdown of cellulose to glucose. Breakdown of cellulose requires 3 types of enzyme: cellobiohydrolase which cleaves successive disaccharide units, endogluconase which separates the cellulose molecules into smaller fragments by attacking the centre of the chains (activated cellulose) and β-glucosidase which cleaves cellulobiose to glucose (Adapted from Abrusci et al., 2004b).

Screening for cellulase producing microorganisms is typically carried out on carboxymethylcellulose (CMC) sodium salt plates (Kasana et al., 2008) which after incubation with a microbial isolate, are stained to visualise a zone of cellulose hydrolysis if present. Two staining solutions have been used to visualise and measure CMC hydrolysis zones: Congo red and iodine. These were compared for speed of results and ease of hydrolysis zone visualisation by Kasana et al. (2008). A clear zone was only visible after 30-40 minutes using Congo red (flooding for 15-20mins then flooded with 1M NaCL for 15-20mins to remove excess dye), but a zone of hydrolysis could be seen around a cellulase producing isolate after flooding with Grams iodine within 3-5 minutes, thus it was suggested that the use of Grams iodine resulted in speedier results in addition to enhanced visibility of the clear zone around cellulase producing microorganisms i.e. *Bacillus* sp, *Pseudomonas* sp, *Streptomyces sannanensis* and *Penicillium chrysogenum*.
Each staining solution reacts with molecules in different ways. Congo red, used in several studies, (Apun, 1995, Kasana et al., 2008 and Peciulyte, 2007) interacts with intact β-D glucans e.g. those bonds present in cellulose which join the glucose monomers together (Teather and Wood, 1982). If these beta glucans are broken down by cellulases then Congo red will not bind to these molecules, and a zone of hydrolysis around the isolate will be visible (Teather and Wood, 1982). Iodine, used by Kasana et al. (2008) and Abu-Bakar et al. (2010) for cellulase assays, reacts with many polysaccharides and a positive reaction commonly results in a blue/ black colour due to the formation of blue/black complexes (Nakamura et al., 1998). No specific evidence could be found in the literature regarding the reaction of iodine with cellulose, because it is usually used for staining of starch.

Both indicators were investigated to determine the effectiveness of each, using *A.versicolor* (ATCC11730) as the test organism. Methods were developed to investigate cellulase production from fungi isolated from cellulose acetate films from the NWFA and cellulose nitrate film swabs from the BFI.

2.7.1.1. Reactions of Iodine with Various Nutrient Media

Iodine was tested first on a range of media because this was stated by Kasana et al. (2008) to be the most rapid method, and is a non-toxic chemical.

Starch agar was prepared to demonstrate iodine stain of a polysaccharide in culture medium. Two MEA (Oxoid) plates were made up using 50g per litre of distilled water as suggested by Oxoid. One plate also contained added starch at 0.5% (Steane, n.d.). In addition to other cellulase assays mentioned in the literature, two PDA (Oxoid) plates were made using 39g per litre as suggested by Oxoid with one plate also containing 0.5% starch. Corn meal agar (CMA, Oxoid) was also tested for reactions with iodine and compared with results on MEA because CMA is a well established mycological medium for the maintenance of fungal stock cultures and is low in nutrients used to indicate chlamydospore production in *Candida* sp. in (Anon. (Oxoid), n.d.). Two plates of CMA (corn meal extract from 50 grams of whole maize) were made with one containing CMC at 0.2% w/v. Low viscosity CMC (Sigma) was used because it has been shown to produce larger clear zones by Al-Tai et al. (1989) thus optimising the chance that enzyme production will be visible. Two plates of MEA agar were also made with one containing CMC at 0.2%. A plug of *A.versicolor* ATCC11730 which was being maintained on MEA, was removed using an 8mm cork borer and placed in the centre of each of the four plates (2x CMA and 2x MEA) and incubated at 25^{o}C for 5 days.

2.7.1.2. Reactions of Congo Red and Iodine with CMC selective agar

Two plates were made up of CMC selective agar each containing 20mL of growth medium to compare the reactions of both Congo red and iodine on CMC+ve and CMC-ve media. One plate contained Technical Agar no.3 (Oxoid) 1.2% w/v, Bacteriological Peptone (Oxoid) 0.02%w/v, sodium chloride 0.2% w/v, di-potassium hydrogen orthophosphate 0.1% w/v, magnesium sulphate 0.05% w/v and potassium chloride 0.05% w/v and the second plate contained all of the above, plus CMC 0.2% w/v.

An 8mm plug of *A.versicolor* was cut from pure culture with a cork borer and placed in the centre of each plate. These were then incubated at 25^{o}C for 5 days. One percent Congo red aqueous solution (Al-Tai et al., 1989) was then used to flood the plates and left for 30 minutes after which a clear zone could be seen. Subsequently 1 M sodium chloride was used to rinse off excess dye. Due to the relatively low nutrient content of CMC selective agar, colony growth was restricted, preventing spreading of the colony on the plate (Fig.40) thus being advantageous over MEA, PDA and cornmeal agar. Zones of clearing could then be measured as colonies were of comparable size.

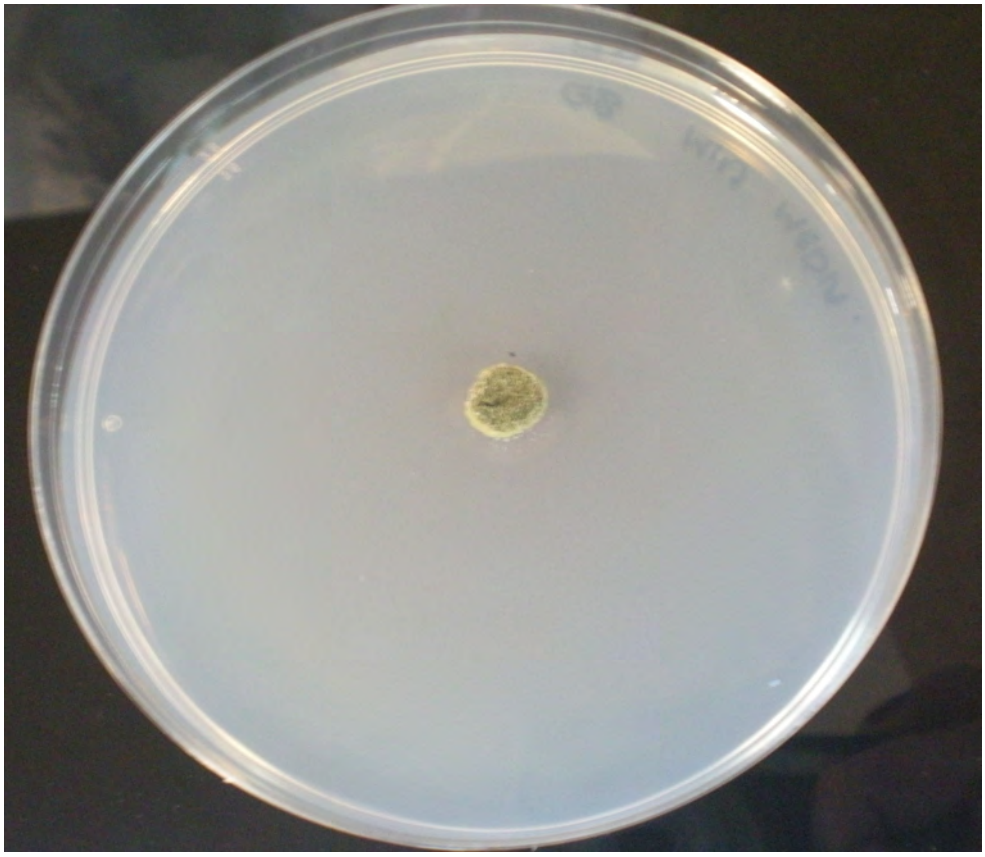

Fig.40. A plug of *A.versicolor* colony showing restricted growth on centre of a CMC selective agar plate after 5 days incubation at 30^{o}C. Colony growth was restricted due to low availability of nutrients compared with other nutrient media such as malt extract agar (MEA) potato dextrose agar (PDA) and corn meal agar (CMA).

To investigate the effects of iodine and Congo red, CMC selective agar plates were spot inoculated with *A.versicolor* (ATCC11730) and *Cladosporium* (highly cellulolytic and isolated from cinefilm) (Abrha and Gashe, 1992). Two sterile bijoux bottles each containing 5mL of sloppy agar (0.2g of Technical agar No.3 (Oxoid) and one drop of Tween 80 (Sigma) made in 100 mL of sterile distilled water) were inoculated with spores scraped from the top of fungal cultures that were maintained on malt extract agar (Oxoid). A sterile loop was used to inoculate a spot of each suspension onto opposite edges of the plate. As the fungus was inoculated by spores and not by a plug from the MEA, it was ensured that residual enzymes were not carried from the malt extract agar, and any enzymes that were produced were as a result of the utilisation of the CMC in the selective agar as a substrate. CMC+ve medium contained all the mineral salts, peptone and CMC sodium salt, whilst CMC-ve medium contained all other compounds minus the CMC sodium salt. The radii of clear zones were measured after 4, 5, 6, 7 and 8 days after inoculation and incubation at 30^{o}C. Fungi were inoculated onto 4 plates for each day measured: 1 Plate CMC +ve with Congo Red, 1 Plate CMC –ve with Congo red, 1 Plate CMC +ve with iodine and 1 Plate CMC –ve with iodine. This was to confirm previous findings that iodine was unsuitable for determination of cellulolytic activity.

2.8. Results IV: Cellulase Assays

2.8.1. Reactions of Iodine with Various Nutrient Media

When stained with iodine for 10 minutes, the MEA and PDA plates looked similar in appearance (Fig.41) and also appeared to look exactly the same as each other when the plates which contained added starch were also stained. Plates which contained added starch appeared blue/black whilst the nutrient media appeared red/brown.

When *A.versicolor* was grown in the centre of plates containing MEA with added starch, MEA (CMC-ve and CMC+ve) and CMA (CMC-ve and CMC+ve) and flooded with iodine; zones of clearance appeared around the isolates, indicating that *A.versicolor* produced extracellular enzymes to break down polysaccharides in the medium (Fig.42 and Fig.43). Zones of hydrolysis around isolates on CMC-ve plates indicated that iodine was not selectively reacting with cellulose, therefore zones of clearance were not indicative of cellulose breakdown by an organism. Thus iodine was not a suitable stain to use for assessing cellulase production. Iodine also reacted with components in the various nutrient media indicating that these too were not suitable for cellulase assays.

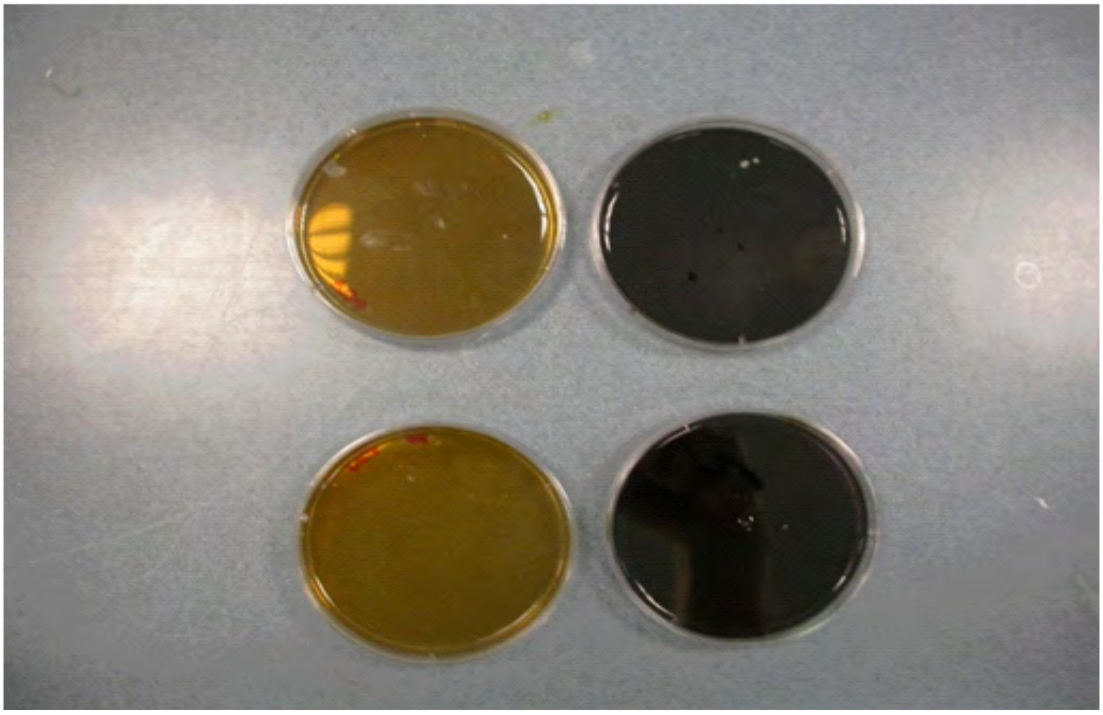

Fig.41. Top Left- PDA, Top Right- PDA with added starch. Bottom Left- MEA, Bottom Right- MEA with added starch. All plates were flooded with iodine for 10 minutes.

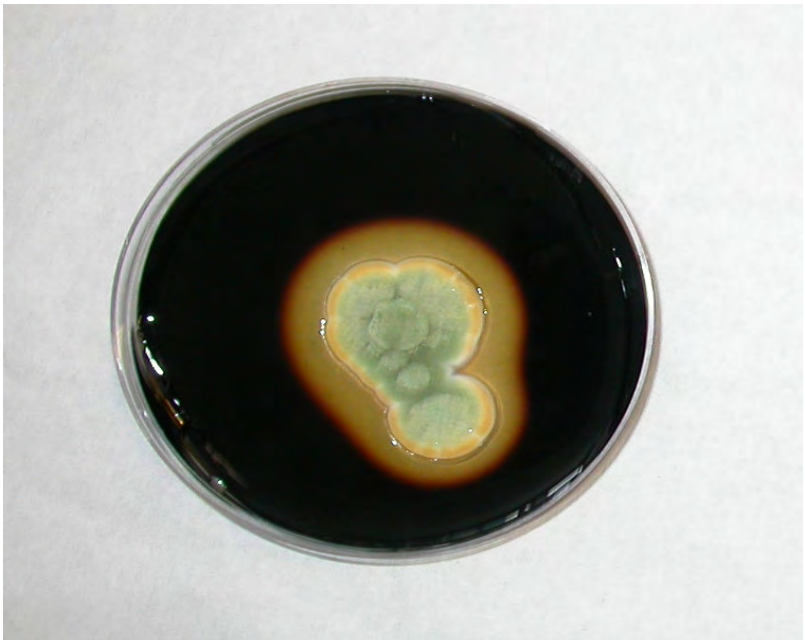

Fig.42. Malt extract agar and added starch (0.5%) with *A.versicolor* isolate grown in the centre of the plate for 5 days at 25^{o}C and then the plate was flooded with iodine.

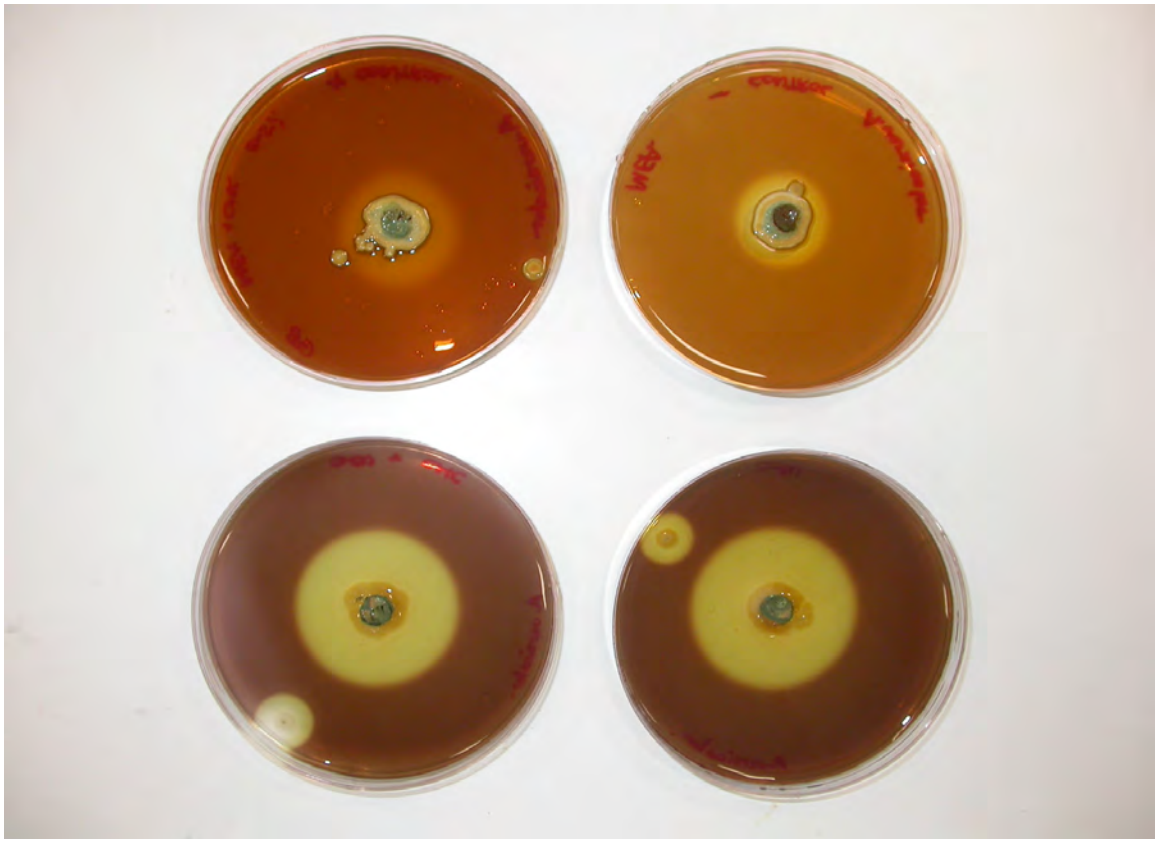

Fig.43. Comparison of clear zones produced by *A.versicolor* incubated for 5 days at 25°C on MEA plates (Oxoid) and corn meal agar plates (Oxoid) with added CMC (0.2%) and flooded with Iodine. Top Left- CMA(CMC-ve), bottom left- MEA (CMC-ve). Top right- CMA (CMC+ve), bottom right- MEA (CMC+ve).

2.8.2. Reactions of Congo Red and Iodine on CMC Selective Medium

As with assays on nutrient media, staining with iodine on CMC selective media also resulted in a zone of clearing on every plate, including those which did not contain CMC. A zone of hydrolysis could be seen around both the *A.versicolor* and *Cladosporium* isolate (Fig.44) which increased in size with time (Table 8). This further indicated that iodine did not differentiate breakdown products of CMC and other polysaccharides. Congo Red was then assayed for comparison to iodine and to evaluate suitability for detection of cellulases.

The Congo red turned the agar plate containing CMC a darker shade of red than the CMC free medium. After rinsing off of the excess dye with 1M NaCl, a zone of hydrolysis (yellow area) around the fungal isolate was seen on the plate containing CMC (but not the CMC free medium) which increased in size with time indicating that Congo red was selective for cellulose (Fig.45).

Congo red only produced zones of clearing around isolates on CMC+ve medium whilst iodine produced clear zones on both the -ve and +ve media (Table 8), thus indicating that Congo Red was suitable to investigate production of cellulases whilst iodine was not. As iodine reacted on plates which did not contain CMC it can be concluded that it reacted with another polysaccharide in the medium other than cellulose, thus in contradiction to work by Kasana et al. (2008) iodine was not considered suitable for cellulase assays. All assays for cellulase production were subsequently carried out on this CMC selective medium and stained with Congo red.

2.8.3. Cellulase Assays of Fungal Isolates

A zone of clearing could be seen around all of the isolates when flooded with Congo red. The radius of this zone was then measured in millimetres for quantitative analysis (Table 9) S1 (*Trichoderma* sp.) produced the largest zone of clearing with 20mm. In general, *Penicillium* spp. showed a higher cellulolytic activity than *Aspergillus* spp. with *Penicillum* spp. hydrolysis zones ranging between 16 and 20mm and *Aspergillus* zones ranging between 13 and 15.5mm. *Stachybotrys* and *Alternaria* showed the lowest cellulolytic activity with a radius of 13mm, however, growth rate of the colonies on CMC selective agar appeared to be slower than the *Aspergillus* and *Penicillium* isolates.

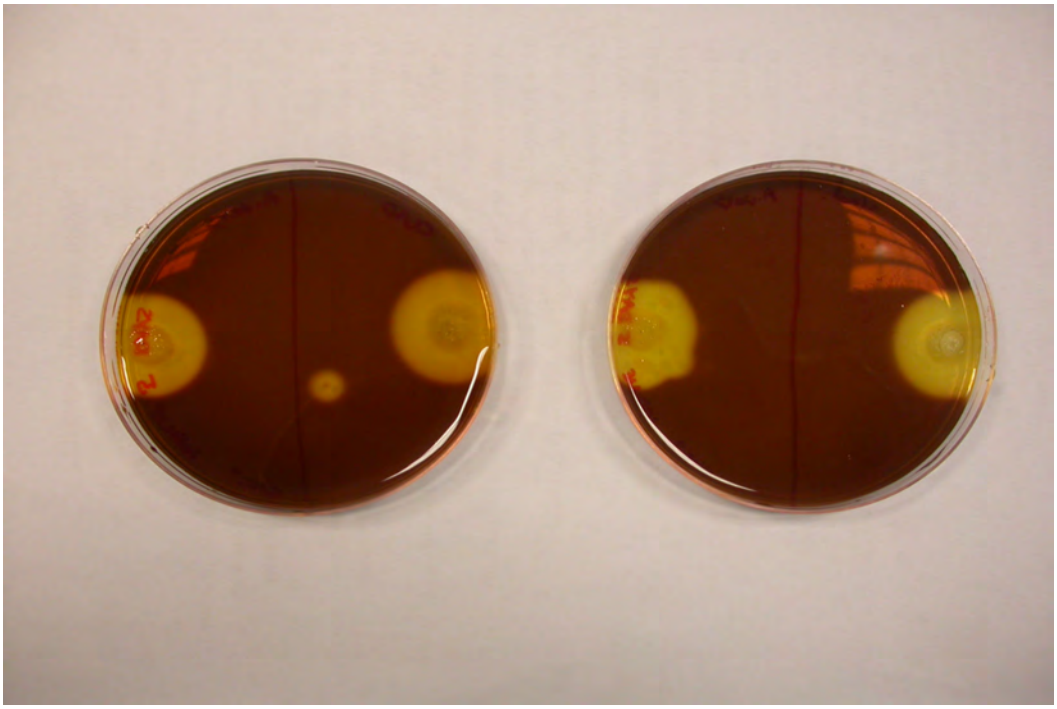

Fig.44. *Aspergillus versicolor* (left side of plates) and *Cladosporium* sp. (right side of plates) on CMC selective agar and stained with Iodine. Left=CMC-ve agar and Right=CMC+ve agar. A zone of hydrolysis was seen around the isolates on both the CMC-ve and CMC+ve plates indicating that the iodine was not reacting with cellulose.

Fig.45. CMC selective agar plates with a plug of *A.versicolor* in the centre incubated for 5 days at 25ºC and stained with Congo red. The left plate contains Technical Agar no.3 (Oxoid) with mineral salts and peptone, and the plate on the right contains Technical Agar no.3 (Oxoid) with mineral salts and peptone with added CMC 0.2% w/v. A zone of hydrolysis can be seen around the isolate on the cellulose medium indicating that Congo red stains cellulose.

Table 8. Radii of clear zones (mm) around spot inoculations of *A.versicolor* and *Cladosporium* which had inoculated on CMC+ve and CMC-ve media and stained with both Congo red and iodine for comparison.

	Congo Red				Iodine			
	A.versicolor		*Cladsosporium*		*A.versicolor*		*Cladsosporium*	
Day	CMC-ve	CMC+ve	CMC-ve	CMC+ve	CMC-ve	CMC+ve	CMC-ve	CMC+ve
4	0	10	0	8	5	5	7	7
5	0	12	0	10	12	12	14	14
6	0	16	0	15	15	15	17	14
7	0	20	0	17	19	19	18	18
8	0	21	0	18	22	22	20	20

Table 9. Radii of clear zones (mm) (highest to lowest) from cellulase assays of fungal isolates inoculated on carboxymethylcellulose (CMC) selective agar, incubated at 25°C for 5 days and then stained with Congo red. All isolates identified by molecular methods (CABI Microbiological Services) are indicated with a '*'.

Isolate Name	Identification	Radius of clear zone on CMC selective agar (mm) (n=3)
S1	*Trichoderma* sp.	21
RR1549 I4	*Penicillium* sp.	20
RR1549 I5	*Penicillium* sp.	20
*RR1093 I2	*Penicillium brevicompactum*	20
*S3	*Penicillium chrysogenum*	18
*RR1399 I5	*Penicillium brevicompactum*	17.5
*RR1093 I1	*Penicillium chrysogenum*	17
*RR1093 I4	*Penicillium chrysogenum*	17
RR1093 I7	*Nigrospora* like	17
S4	*Penicillium* sp.	17
RR1093 I9	*Penicillium* sp.	16
RR1093 I10	*Penicillium* sp.	16
RR1470 I3	Unidentified	16
*S5	*Penicillium chrysogenum*	16
*RR1093 I3	*Penicillium citreonigrum*	15.5
*RR1399 I3	*Aspergillus versicolor*	15.5
*RR1399 I4	*Aspergillus versicolor*	15.5
RR1399 I7	*Aspergillus* sp.	15.5
*(ATCC11730)	*Aspergillus versicolor*	15
RR1470 I2	*Aspergillus* sp.	15
RR1549 I1	*Mucorales* sp.	15
S2	*Penicillium* (or related species)	14
JWP1 I1	*Alternaria alternata*	13
RR1491 F2 I4	*Stachybotrys chartarum*	13
RR1093 I8	*Aspergillus* sp.	13
*RR1399 I1	*Aspergillus versicolor*	12.5
RR1093 I6	*Aspergillus* sp.	12
*RR1399 I2	*Aspergillus versicolor*	12
*RR1399 I6	*Aspergillus versicolor*	12
*RR1549 I2	*Aspergillus versicolor*	12
*RR1549 I3	*Aspergillus versicolor*	12
*RR1470 I1	*Aspergillus versicolor*	6.5
RR1093 I5	Ascomycete (unviable)	0

2.9. Methods V: Mould Growth on Film

2.9.1. Visual Analysis of Mouldy Film

To investigate whether mycelia and sporing structures were visible on contaminated cinematographic film, strips of RR1511 film taken from the end of the film reel were attached to a microscope slide using sellotape, which was fixed at each end of the film strip to the slide and was then examined at x10 magnification using a light microscope. Swabbing and air sampling of this film had indicated that no viable spores were present so the film was deemed 'safe' for projection, thus the film was also run through a projector and the reel was copied onto DVD by the NWFA, enabling still images to be captured using Cyberlink PowerDVD for Microsoft Windows®. This showed visually how mycelial growth appears when the film is viewed on a screen. Further microscopic analysis included viewing fungi on different layers and areas of 'test film' strips.

2.9.2. Inoculation of Film Strips

In addition to analysis of mouldy films donated by the NWFA, methods were developed to encourage and investigate fungal growth on film strips *in vitro*. Single frames of 'test film' supplied by the NWFA were used in each method. The film frames were examined in three areas: the gelatine binder and the dyes, the cellulose acetate layer, and the outer edges of the frame with holes used to move the film reel along in a projector (Fig.46). The test organism was *A.versicolor* ATCC11730 which had been shown to have a high gelatinolytic activity.

Three methods were investigated to attempt to inoculate test film:

1) Three plugs of fungal growth from MEA were placed at 60^{o} angles to each other approximately halfway between the edge of a malt extract agar plate and the centre, forming an equilateral triangle. A single frame of film was placed in the center (Fig.47).

2) One hundred microlitres of a spore suspension (of optical density corresponding to approximately 1 McFarland (0.257 OD at 600nm) or 4 x 10^{8} CFUs/mL) was inoculated on MEA to produce a fungal lawn and a frame of film was placed in the center of the plate.

3) A frame of film was placed on a microscope slide. 500µl of molten malt extract agar was dropped onto the film to create an agar overlay and also to fix the film onto the slide. A small piece of fungal mycelium was removed using a dissecting needle, then placed in the centre of the agar on the slide, and the slide placed in a humidity chamber.

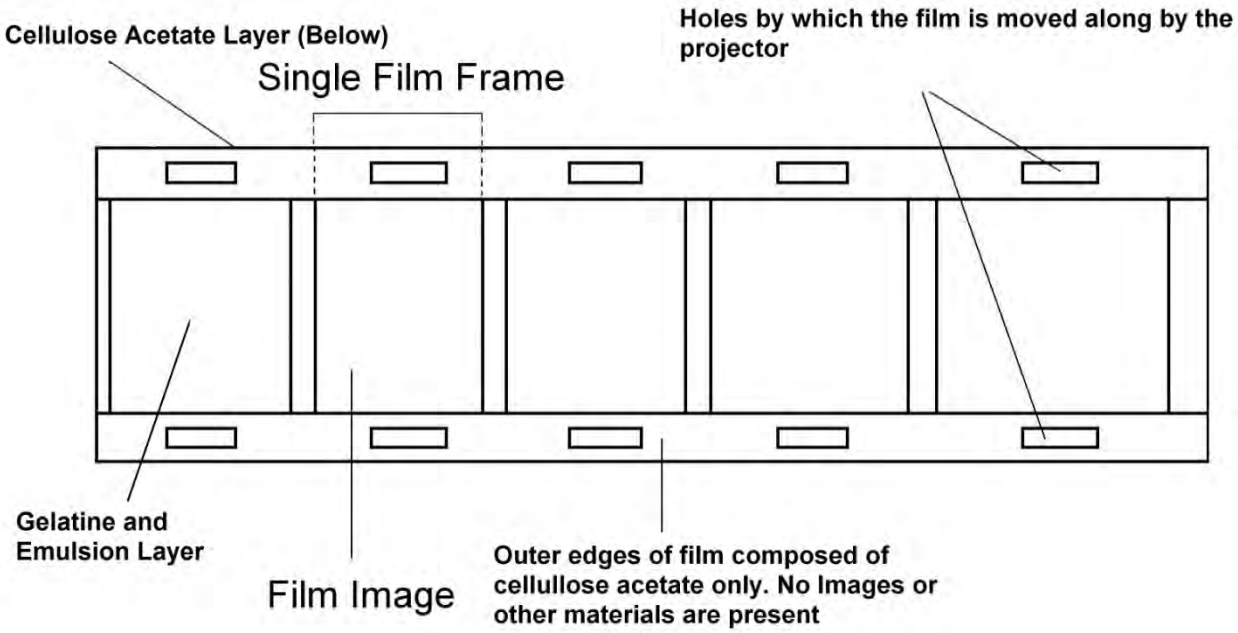

Fig.46. Complete film strip. Three areas of the 'Test Film' frames examined under the light microscope were: the gelatine binder and the dyes contained in the frame, the cellulose acetate layer, and the outer edges of the frame with holes used to move the film reel along in a projector.

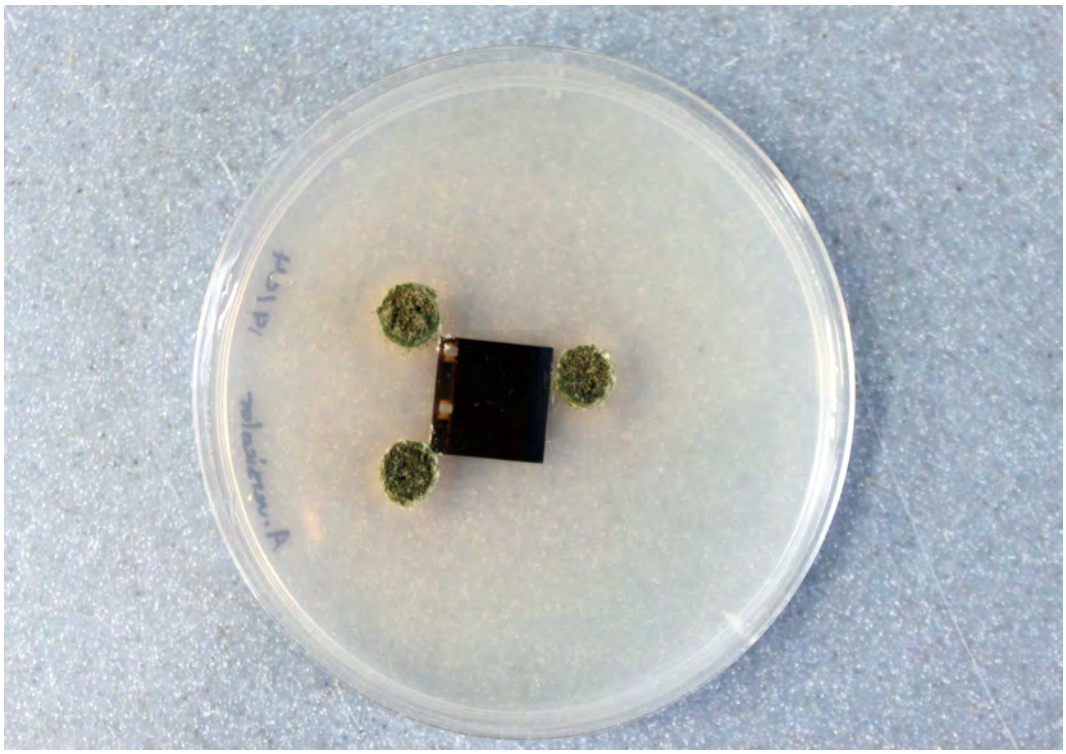

Fig.47. A frame of film was placed in the center of malt extract agar plates and minimal media plates (shown) and inoculated with three plugs of *A.versicolor* ATCC11730 which had been cut with an 8mm cork borer.

All plates were then incubated at 25°C for 7 days after which the film strips were removed from the agar using sterile tweezers. For methods 1 and 2, negative controls were individual frames of 'clean' test film placed on plates and slides in the same way as the methods listed however, these were left un-inoculated. In addition, frames of film were also placed on minimal medium composed of Technical Agar no.3 (Oxoid) 1.2% w/v, Bacteriological Peptone (Oxoid) 0.02%w/v, sodium chloride 0.2% w/v, di-potassium hydrogen orthophosphate 0.1% w/v, magnesium sulphate 0.05% w/v and potassium chloride 0.05% w/v, to determine whether *A.versicolor* was utilising the gelatine in the film for growth and not just the nutrients in the MEA. After removal from the agar in methods 1 and 2, frames were subsequently attached to a microscope slide by covering the frame with sellotape and all frames including those from method 3 were were viewed under a light microscope at x10 magnification.

2.10. Results V: Analysis of Mould Growth on Film

2.10.1. Visual Analysis of Mouldy Reels

Visualisation of mouldy film reels using a light microscope (x10) showed the presence of mycelial growth and sporing structures on the RR1511 film reel. On the first few frames at the beginning of the roll of film and also at the end of the film reel, the words 'safety film' could visualised with the use of a microscope, which refers to the term used for cellulose acetate film reels. Microscopic analysis focused on the letter 'Y' (Fig.48). Additionally scanning through the RR1511 under a light microscope (x10) and focusing on the image of a baby, as with the images from the beginning of the film reel, visible hyphal growth was also seen (Fig.49). Visual identification of the mould present was not possible and could not be cultured because spores were unviable for this reel. For comparison, reel RR1399 was also examined under a light microscope because this reel released high numbers of spores and visually appeared heavily contaminated. *Aspergillus versicolor* was a predominant contaminant of this reel, which was confirmed by visualisation of an *Aspergillus* conidiophore (Fig.50).

The still images of RR1511 taken using computer software, resemble the distorted images which were seen when the film reel was projected (Fig.51). Mycelia can be seen across the image as black lines which 'snake' across the frame causing considerable degradation of the film image. Deterioration and image distortion could be much more noticeable on heavily contaminated film reels.

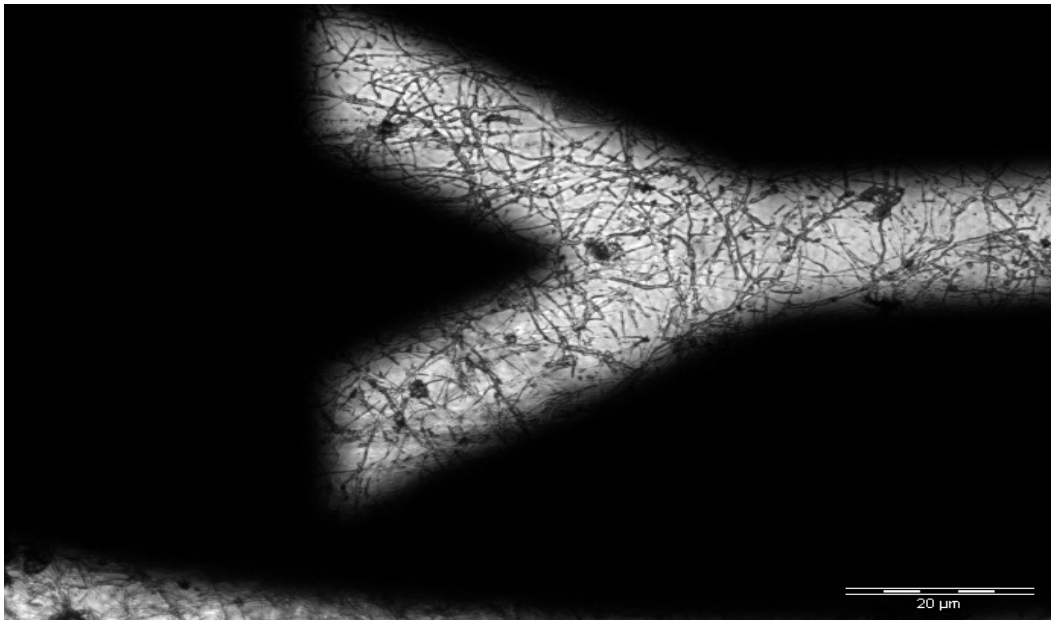

Fig.48. Light microscopy image (x10) of a 'Y' from the word 'safety' at the beginning of the cellulose acetate film reel RR1511. Mycelial growth is clearly seen.

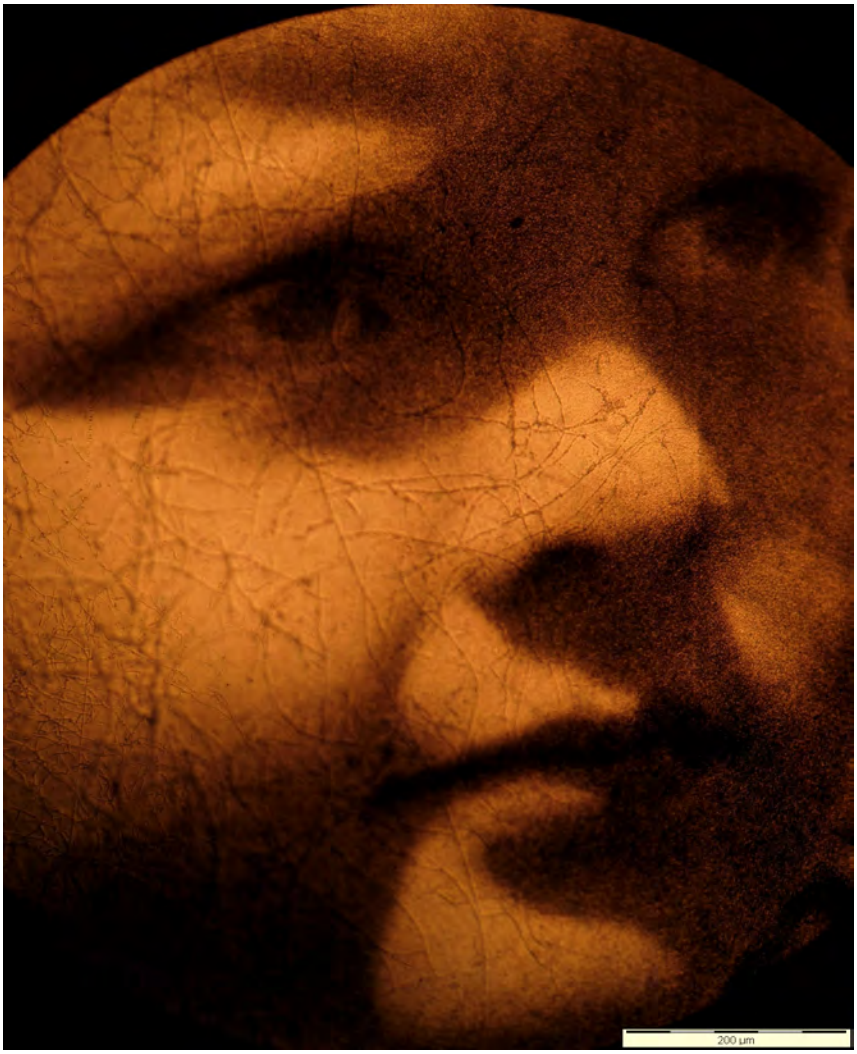

Fig.49. Light microscopy image (x10) of a baby's face from reel RR1511. Mycelial growth can be seen across the image.

Fig.50. Light microscopy image (x40) of reel RR1399 show showing the presence of an *Aspergillus* conidiophore. This reel was heavily contaminated and high number of CFUs were isolated from air sampling. The predominant coloniser of this reel was *A.versicolor*.

Fig.51. A single frame of RR1511 captured using Cyberlink PowerDVD for Microsoft Windows®. Mycelia are visible as black lines. Fungal growth significantly affected the quality of the image on a film which did not visually appear to be heavily contaminated nor were any isolates identified by culture.

2.10.2. Analysis of Fungal Growth on 'Test Film' Strips

After visualisation of fungi growing on RR1511, attempts were made to simulate this growth *in vitro*. For comparison with inoculated test film, individual frames of 'clean' test film were covered with sellotape and attached onto microscope slides. Microscopy indicated that there was no evidence of fungal growth (Fig.52). The dark flecks on the image were believed to be non-biological debris and imperfections in the cellulose acetate.

After placing the film strips on the agar and incubating with *A.versicolor* in the methods described (method 2, section 2.9.2), attempts to remove the film strips from the agar caused the emulsion layer consisting of gelatine and dyes, to separate from the cellulose acetate base (Fig.53). The cellulose acetate layer appeared as a yellow coloured layer whilst the gelatine and dye emulsion layer appeared blue. However, incubation of clean test film on MEA without inoculation of *A.versicolor* did not result in separation of the layers and image analysis under the microscope confirmed that the dyes were not distorted and the cellulose acetate layer remained intact (Fig.54), suggesting that separation was a result of film degradation by this species, not exposure to increased temperature and moisture in the medium. Partial separation of the cellulose acetate layer and emulsion layer was also observed (method 1, section 2.9.2.) (Fig.55). The emulsion layer remained fixed to the right half of the frame which appeared dark under a light microscope whilst it was left on the agar from the left side leaving only the cellulose acetate, which appeared lighter. *Aspergillus* conidiophores are apparent in the emulsion layer on the cellulose acetate layer. However, it is unclear whether the isolate was utilising the cellulose acetate for growth or had merely remained on the surface after emulsion layer was removed but the latter is more likely.

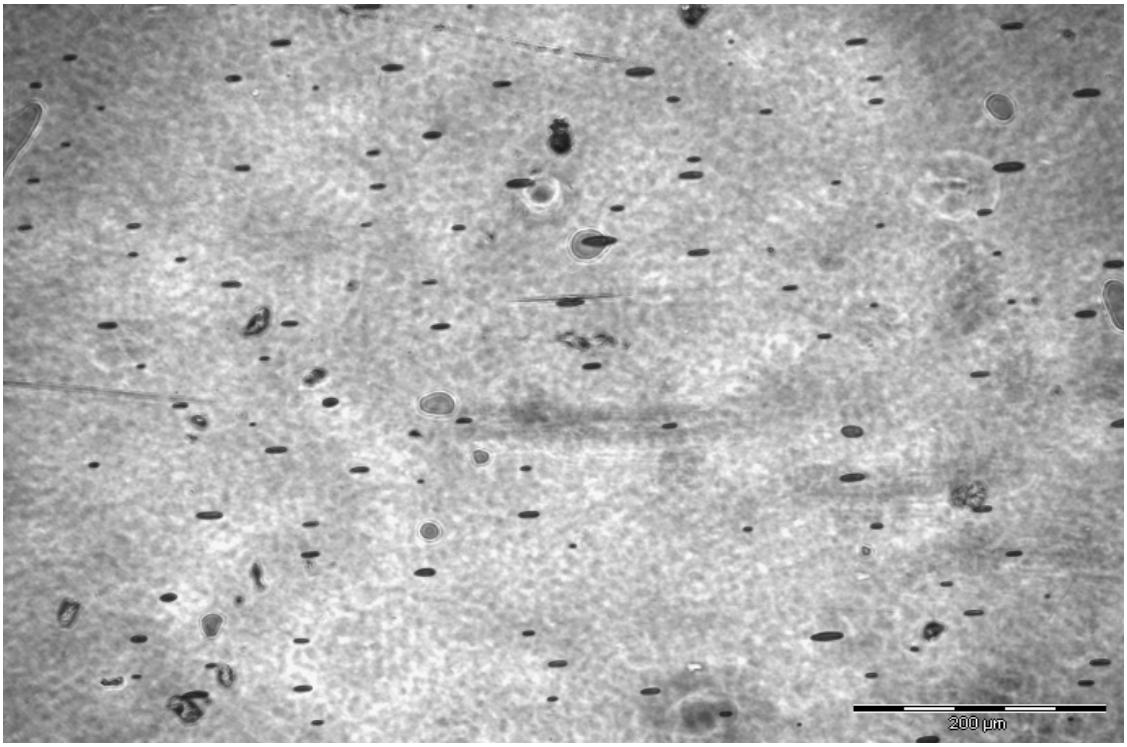

Fig.52. Image of clean test film (x10) showing evidence of some flecks on surface but no fungal growth.

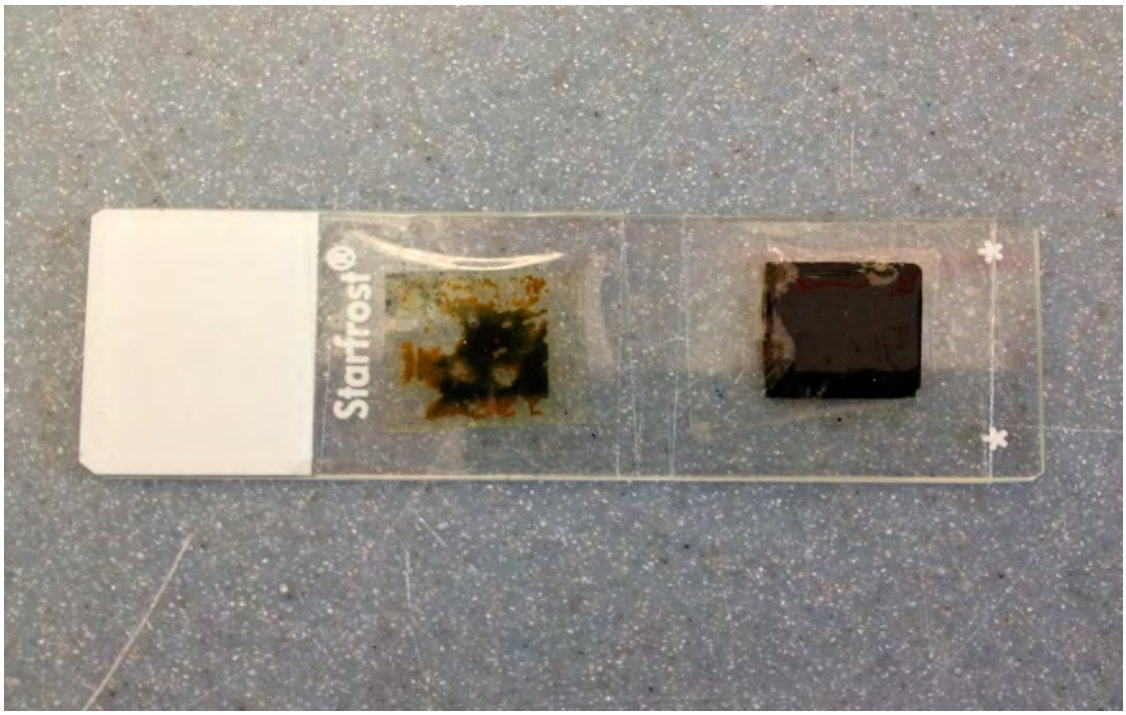

Fig.53. The separated cellulose acetate (yellow coloured frame on the left) which has separated from the emulsion (gelatine) layer (blue/ black frame on the right), These were examined under a light microscope.

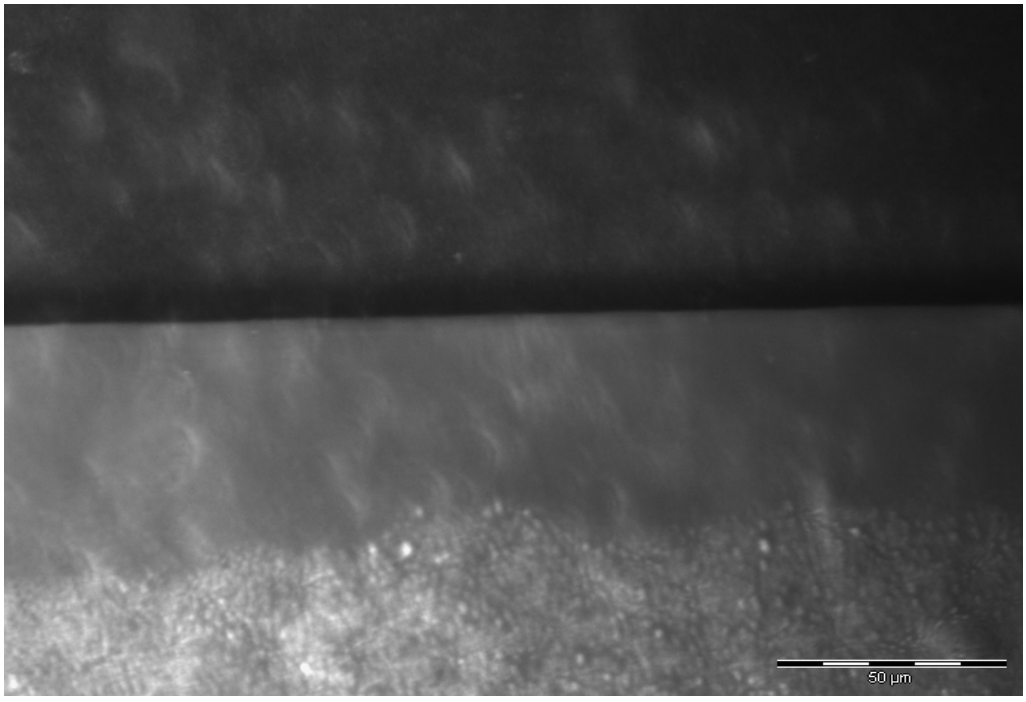

Fig.54. Image of clean Test Film (x10 magnification) after incubation. The light bottom half of the film is the film frame whilst the dark top half of the film is the edge of the film where no image would be present.

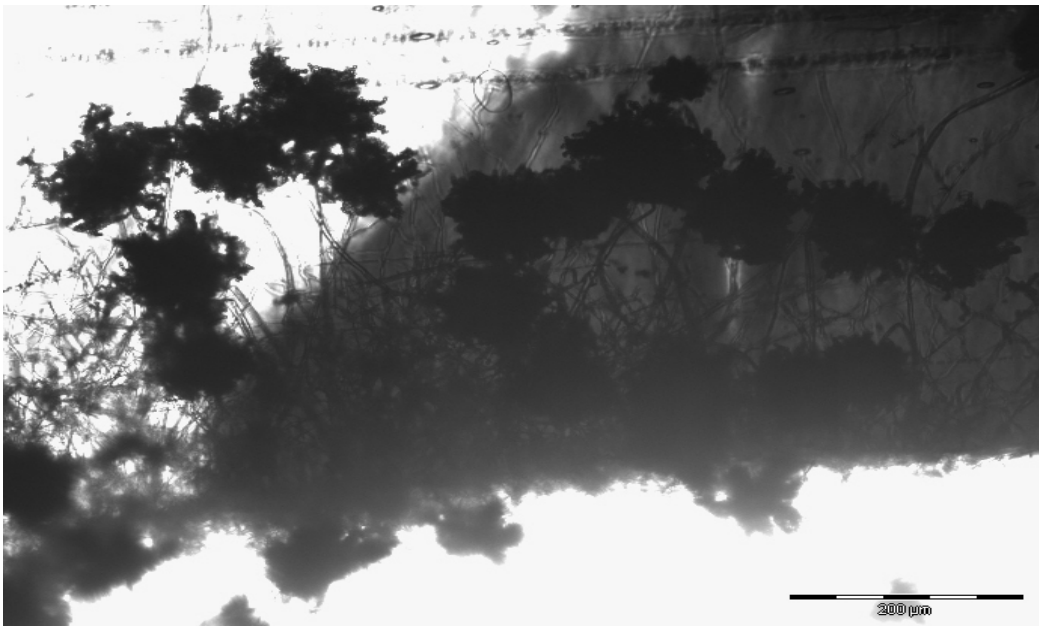

Fig.55. Light microscopy image (x10) of *Aspergillus* conidiophores on film test strip from method 1 showing partial removal of the gelatine from the acetate. The dark side on the right is the intact film strip, the left is the film (acetate) with the gelatine removed and the bottom is the edge of the film. The flecks in the cellulose acetate can be seen in the top left of the image.

Additionally, increased hyphal growth was observed on film frames which were placed on minimal media (Fig.56), due to gelatine being the only source of carbon on this media. On MEA, the media itself was preferential for growth rather than the film strip, due to increased availability of nutrients.

Removal of the film from the fungal lawn (method 2) also resulted in separation of the layers but in this case each layer remained intact. As with the previous method *Aspergillus* conidiophores can be seen on the cellulose acetate layer (Fig.57). The emulsion layer was placed on a microscope slide and stuck unaided due to moisture. Images were taken which showed wrinkle like patterns in the material and spore bearing structures (Fig.58 and Fig.59) possibly due to shrinkage and moisture when removed from the cellulose acetate support. Hyphal growth could also be seen on film frames which had been placed on inoculated minimal media (Fig.60).

Spot inoculating fungi onto an agar overlay (method 3) was not as successful as the lawn or plug methods for visualising fungal growth on film slides, because the drop of agar dried out despite being in a moisture chamber, thus restricting fungal growth. Growth of the fungi was restricted to the drop of agar only (Fig.61). Only mycelial growth occurred, which stopped prematurely due to the decrease in moisture and possibly depletion of nutrients, thus preventing the development of spore bearing structures. The mycelia growth did not reach the clean cellulose acetate (top right of the image), and separation of the layers did not occur. The ‘bubbles’ are droplets of agar which deposited when the initial drop of agar was placed on the slide. The black line is the top of the agar droplet.

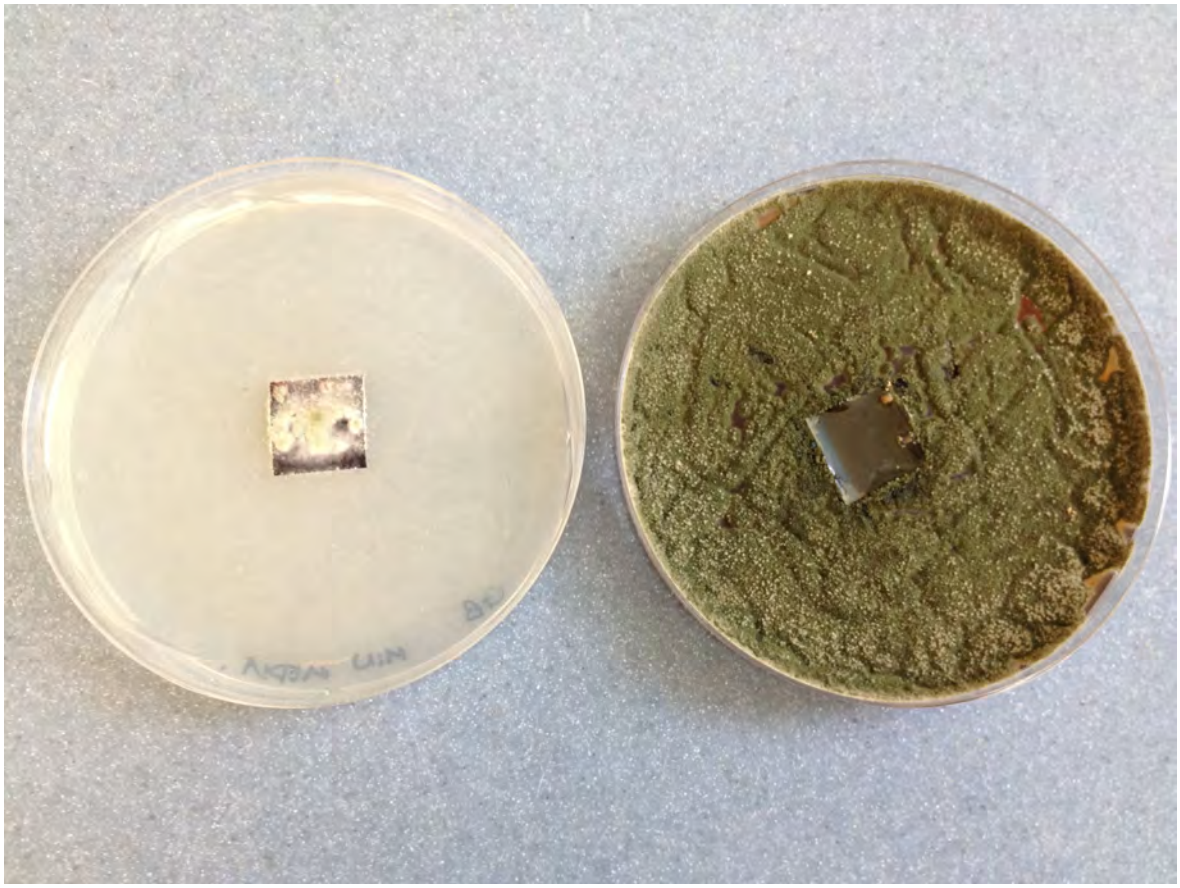

Fig 56. Plates of malt extract agar (MEA) (right) and minimal medium (left) which have been inoculated with a lawn of *A.versicolor* ATCC11730. A frame of test film was then placed in the centre and the plates were incubated for 7 days at 25°C after which the lawn could clearly be seen on the MEA. However, fungal growth could be seen growing on top of the film slide on minimal media but not on the MEA. This indicates that *A.versicolor* was capable of utilising the gelatine in the film for growth and will utilise this when no other nutrients are available but when grown on MEA, MEA is preferential for growth due to its high nutrient content. When frames were lifted off the media and viewed under a light microscope, mycelial growth could be seen on frames from both media, also indicating that the fungus was utilising the gelatine in the film for growth and not just the nutrients in the media.

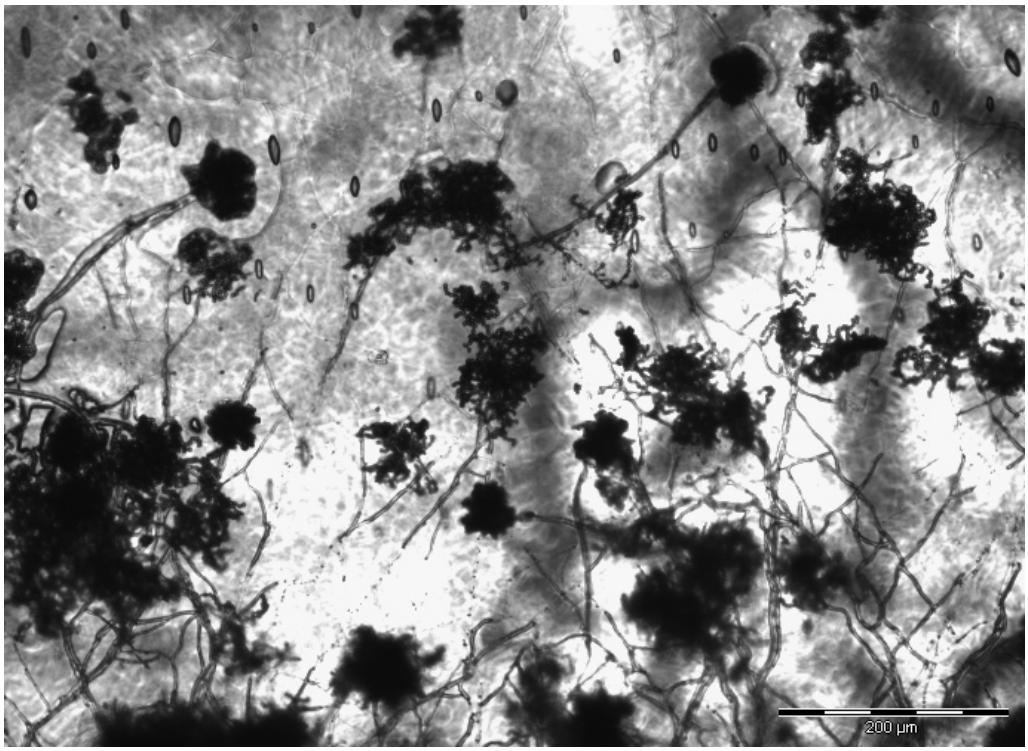

Fig.57. Image of the edge of the cellulose acetate under light microscope (x10) which has been separated from the emulsion layer, during the process of removal from the agar plate from method 2. *Aspergillus* conidiophores and mycelia can be seen.

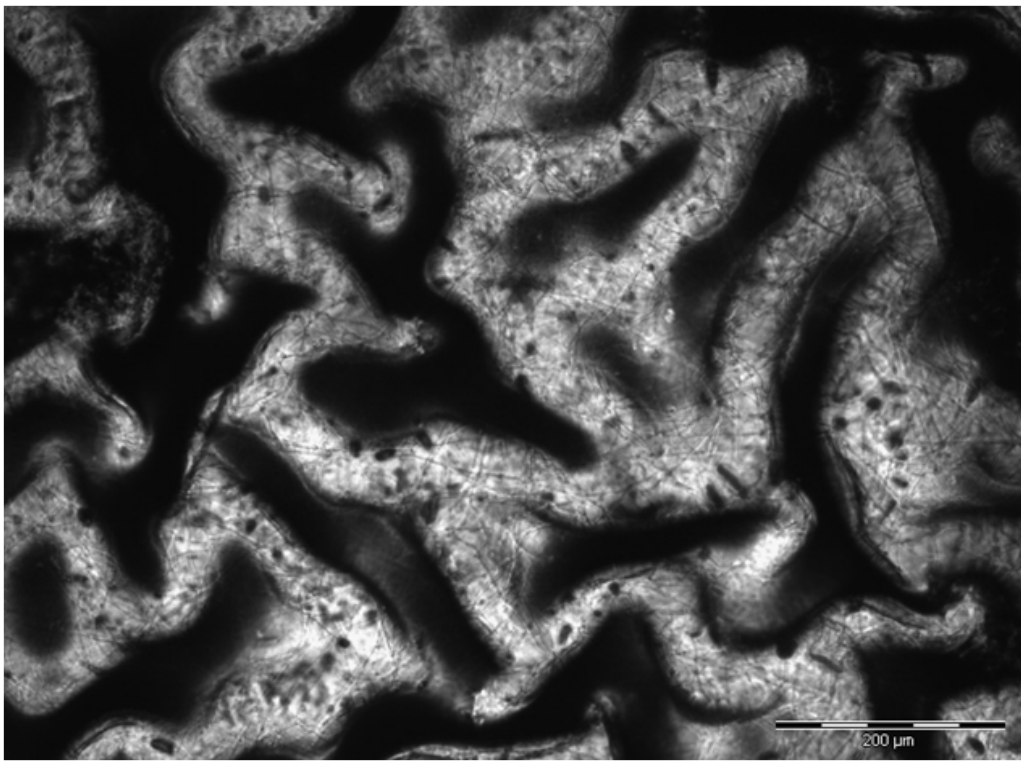

Fig.58. Emulsion layer as seen under a light microscope (x10 magnification). 'Wrinkles' can be seen in the emulsion layer with mycelia appearing as tracks and sporing structures appearing as black dots.

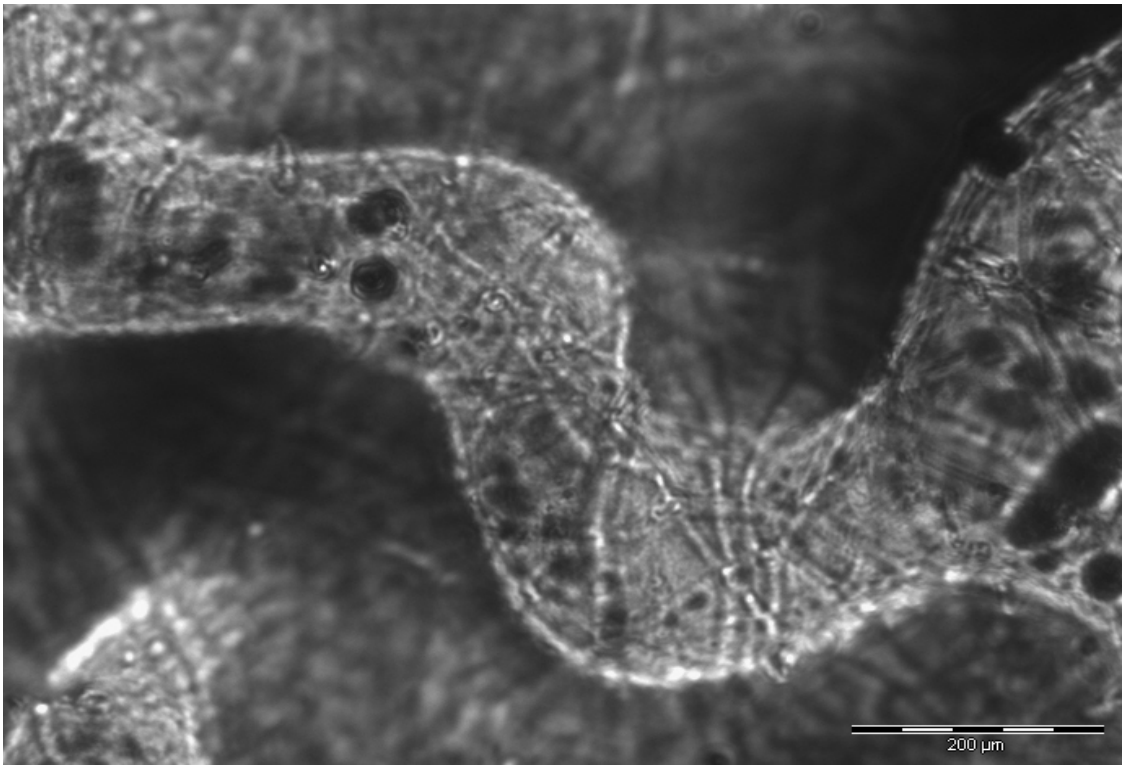

Fig.60. Emulsion layer as seen under a light microscope (x100 magnification). 'Wrinkles' can be seen in the dye with mycelia appearing as tracks and sporing structures appearing as black dots.

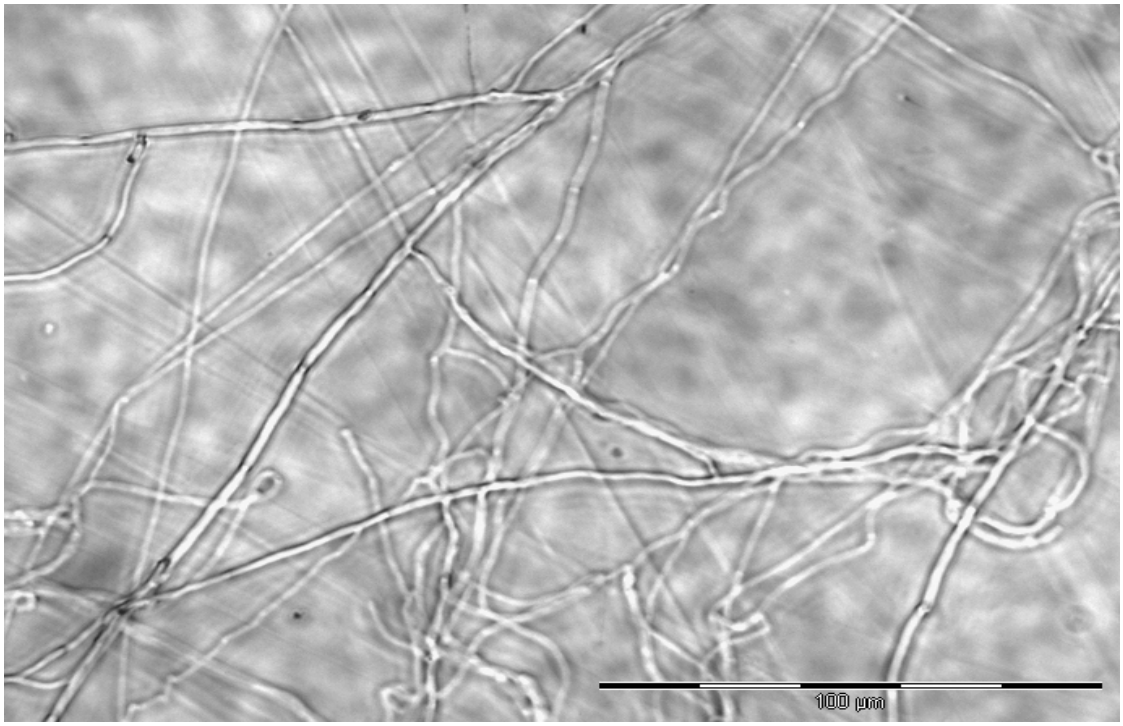

Fig.61. Test film which was placed on minimal media and inoculated with *A.versicolor* ATCC11730 and viewed using a light microscope (x40). Hyphae can be seen in the gelatine emulsion suggesting that this species was utilising the film for growth.

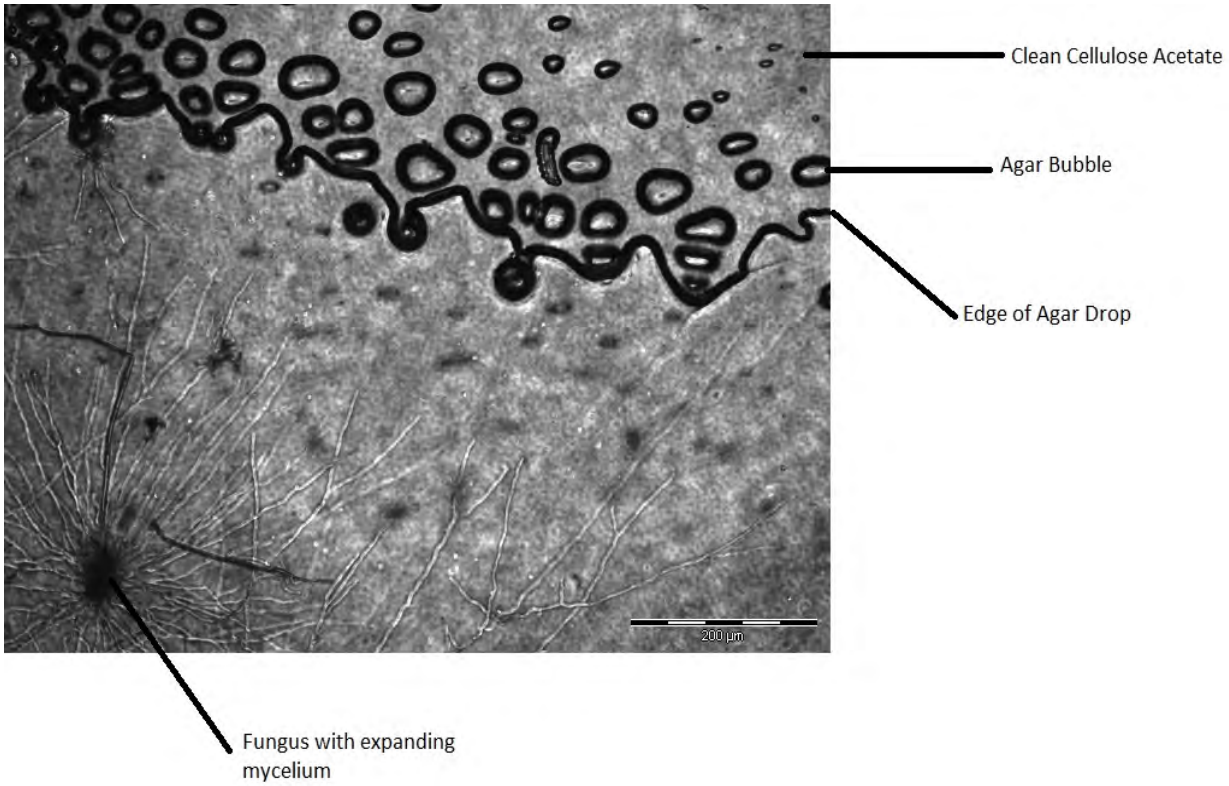

Fig.62. Image of test film strip with agar overlay which has been inoculated with *A.versicolor* ATCC11730. The mycelia appear to have only grown on the agar (bottom left) of the image and not the test strip itself on the top right.

2.11. Methods VI: Mould Growth on Videotapes

2.11.1. Mould on Videotape

Whilst fungal contamination of cinematographic film was the main focus of this project, mouldy videotapes were also investigated. Videotapes that visually appeared to harbour mould, were investigated to identify any microorganisms present. Likely mould growth was determined by visual appearance of white 'furry' patches resembling fungal mycelia which, could be seen through the clear plastic viewing window (Fig.63) or on the wound reels themselves when the plastic cases were removed (Fig.64).

Four videotape collections were studied. Videotapes were allocated numbers relating to their origin/collection. If there were multiple tapes in a collection, tapes were additionally allocated a number e.g. Collection 1 Tape 1 = C1 T1. The environment of origin was also noted (domestic/home or archive) (Table 10). All tapes except those in collection 4 were previously stored in cardboard cases, which could also have been additional substrates for mould growth.

To allow access to the magnetic tape, the plastic outer cases of videotapes were opened by removing the screws and the tightly wound reel was removed. The side of the wound reel which visually appeared colonised by mould growth, was pressed on plates of MEA (Oxoid), and incubated at 30^{o}C for 7 days to isolate fungal colonies. Identifications were then made to genus level using colony morphology and microscopy. After removal of the wound tape, the cassette tape cases were also swabbed with a moistened sterile swab.

Fig.63. Presumptive mould on a videotape can be see through the clear windows (above) or more clearly when the tape is removed from the cassette (below).

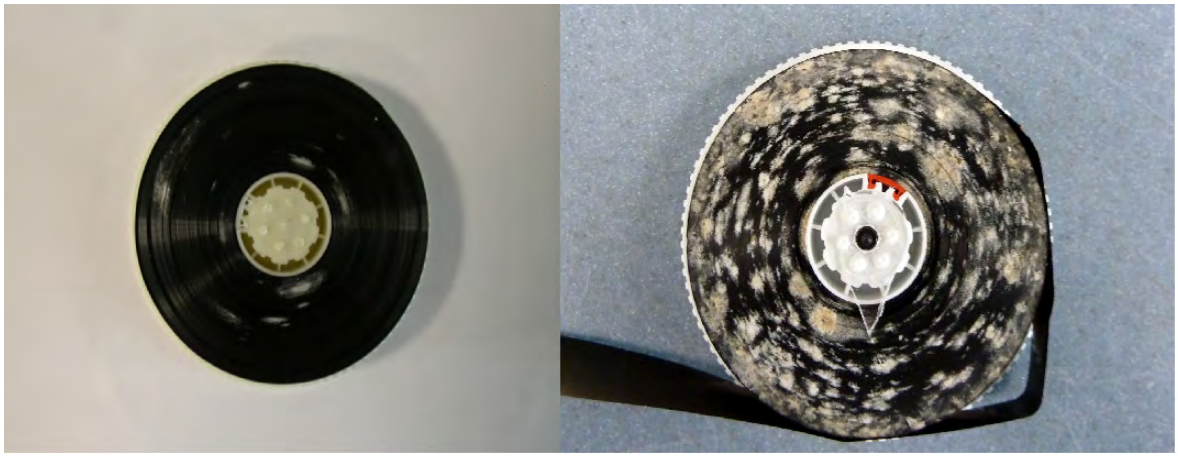

Fig.64. Videotape reels with the outer cases removed (left = C1 T1 and right=C4 T1). Mould growth was much more visible on heavily contaminated tapes.

Table 10. List of videotape collections and origin i.e. domestic or archive.

Collection	Number of Tapes	Origin	Other details
1	1	Domestic	Stored in cupboard which was rarely opened
2	1	Archive	British Film Institute (BFI)
3	5	Domestic	Stored in bookcase near where damp was coming through the wall then put in attic with varying humidity, temp etc
4	4	Domestic	Stored in dark cellar for many years

2.11.2. Inoculation of Videotape

To investigate whether videotape was a potential substrate for fungal growth, 5cm strips of 'clean' videotape from a separate collection which showed no visible signs of mould contamination, were disinfected by soaking them in 5% Trigene for 5 mins, dipping them in 70% ethanol and allowing it to evaporate, and finally rinsing in sterile distilled water by immersion before being allowed to air dry. The strips were then placed on MEA plates using sterilised tweezers and 8mm plugs of fungal growth were removed from cultures of *Stachybotrys* sp.and *Aspergillus* sp. isolated from collection 1and *Penicillium* sp. isolated from collection 4, and each was placed next to a 5cm strip of videotape (Fig.65) and incubated for 7 days at 30^{o}C then viewed using a stereomicroscope. Additionally strips were placed on minimal media containing Technical Agar no.3 (Oxoid) 1.2% w/v, Bacteriological Peptone (Oxoid) 0.02%w/v, sodium chloride 0.2% w/v, di-potassium hydrogen orthophosphate 0.1% w/v, magnesium sulphate 0.05% w/v and potassium chloride 0.05% w/v (Kasana et al., 2008).

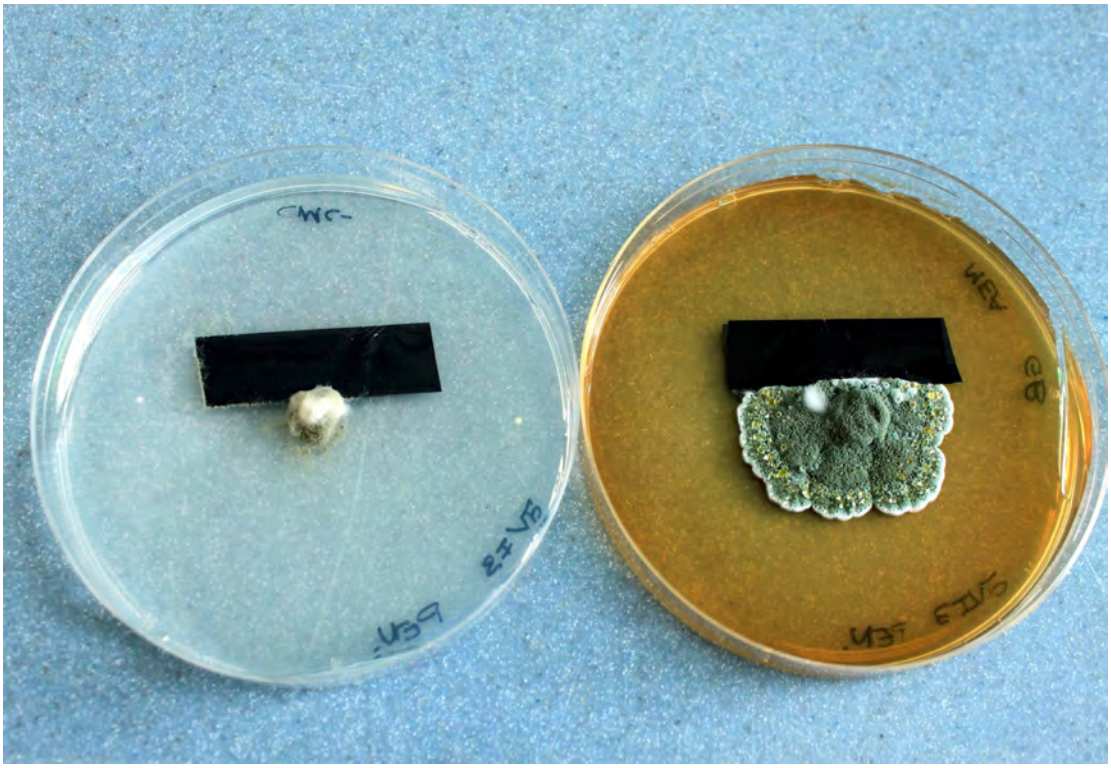

Fig.65. A *Penicillium* sp. isolated from videotape was inoculated onto different media. Left=*Penicillium* next to a piece of videotape on minimal media containing Technical Agar no.3 (Oxoid) 1.2% w/v, Bacteriological Peptone (Oxoid) 0.02%w/v, sodium chloride 0.2% w/v, di-potassium hydrogen orthophosphate 0.1% w/v, magnesium sulphate 0.05% w/v and potassium chloride 0.05% w/v. Right= A malt extract agar plate (MEA) inoculated with a plug of *Penicillium* next to a 5cm piece of videotape.

2.12. Results VI: Analysis of Mould Growth on Videotapes

2.12.1. Isolation and Identification of Fungal Colonies

Presses of videotapes resulted in various densities of fungal growth depending on the levels of contamination of the video (Fig.66-69).

Colonies with different morphologies were selected, isolated, subcultured and identified to genus level using slide cultures and light microscopy. The tape from collection 1 was only lightly contaminated with presumptive mould (Fig.66). Only single colonies were isolated from this tape: 1 *Aspergillus* from agar imprints (presumed *A. versicolor* based on colony morphology and comparison with identified isolates from cinefilm), 2 *Penicillium* sp. and 1 *Stachybotrys* sp. from swabs of the reel. The tape from collection 2 also presented only light contamination (Fig.67). A press of the reel yielded a few *Penicillium* colonies and a single unidentified isolate. Agar presses of the 5 heavily contaminated tapes from collection 3 yielded many colonies (Fig.68). Five different colony morphologies of *Aspergillus* (including a presumptive *A.niger*) were isolated, accounting for most of the contamination, as well as 3 colony morphologies of *Penicillium*. The 4 tapes from collection 4 were the most heavily contaminated (Fig.69). Due to the extensive growth of fungi on the plates it was difficult to isolate and identify individual colonies but *Penicillium* was the most predominant species present although swabbing of the cassette case also isolated some *Aspergillus* colonies including colonies of presumptive *A.niger*. Collections 3 and 4 each harboured their own typical range of contaminants.

The structural integrity of the tapes themselves did not appear to be affected by fungal growth. Growth on all tapes was limited to the edges of the tape and was not visible on the majority of the tapes' surfaces. The cardboard boxes on the tapes from collections 1-4 did not harbour any fungal growth.

2.12.2. Inoculation of Videotape

The fungal isolates grew better on MEA than minimal media as was shown with film (Fig.56). Neither the *Stachybotrys, Aspergillus or Penicillium* spp. appeared to utilise the tape as substrate for growth. Fungal growth across the tape surface was sparse with little vegetative growth, and the physical stability of the tape did not appear to be adversely affected when the strips of tape were removed from the agar and stuck to microscope slides for closer viewing under the stereomicroscope. Sporulating structures could not be seen on the tape inoculated with *Stachybotrys* (Fig.70), but were visible on the tape inoculated with *Aspergillus* (Fig.71) and *Penicillium*.

Fig.66. A press of the tape reel from collection 1 on MEA.

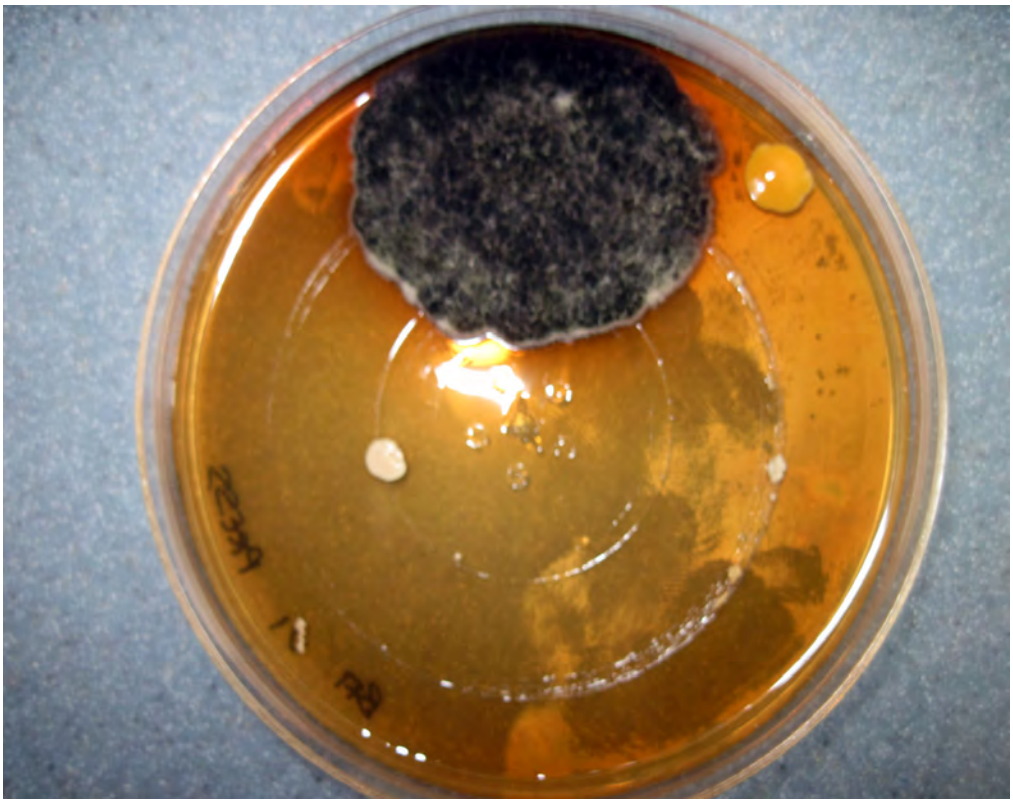

Fig.67. A press of the tape reel from collection 2 on MEA.

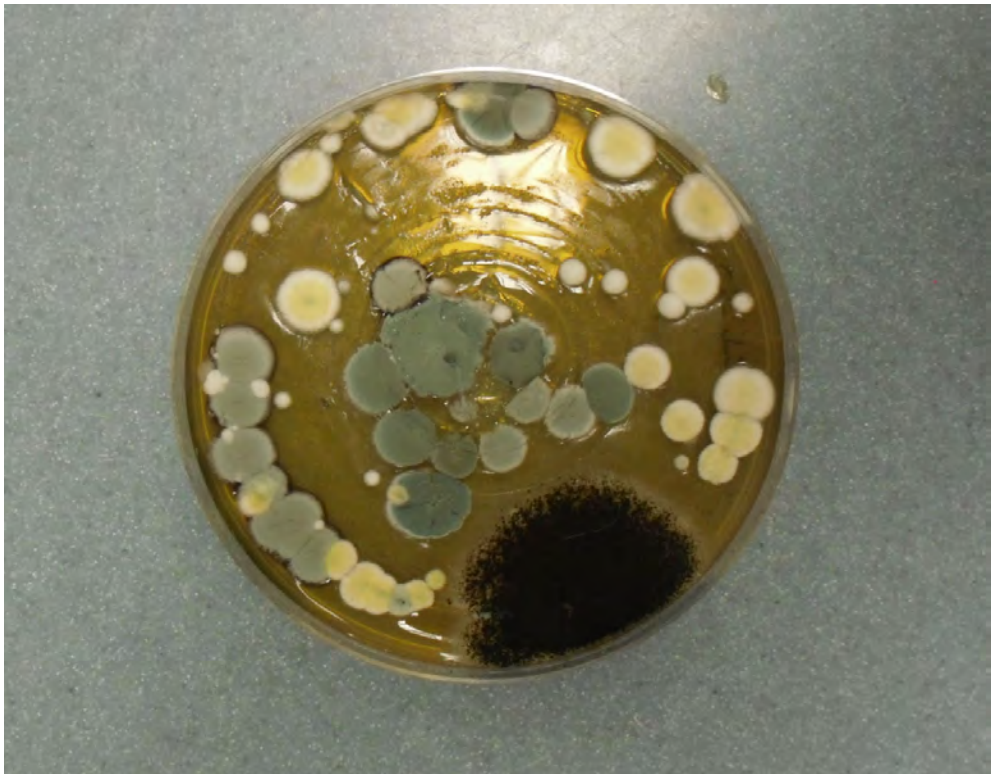

Fig.68. A press of a tape from collection 3 (typical results from this collection of 5 tapes) on MEA.

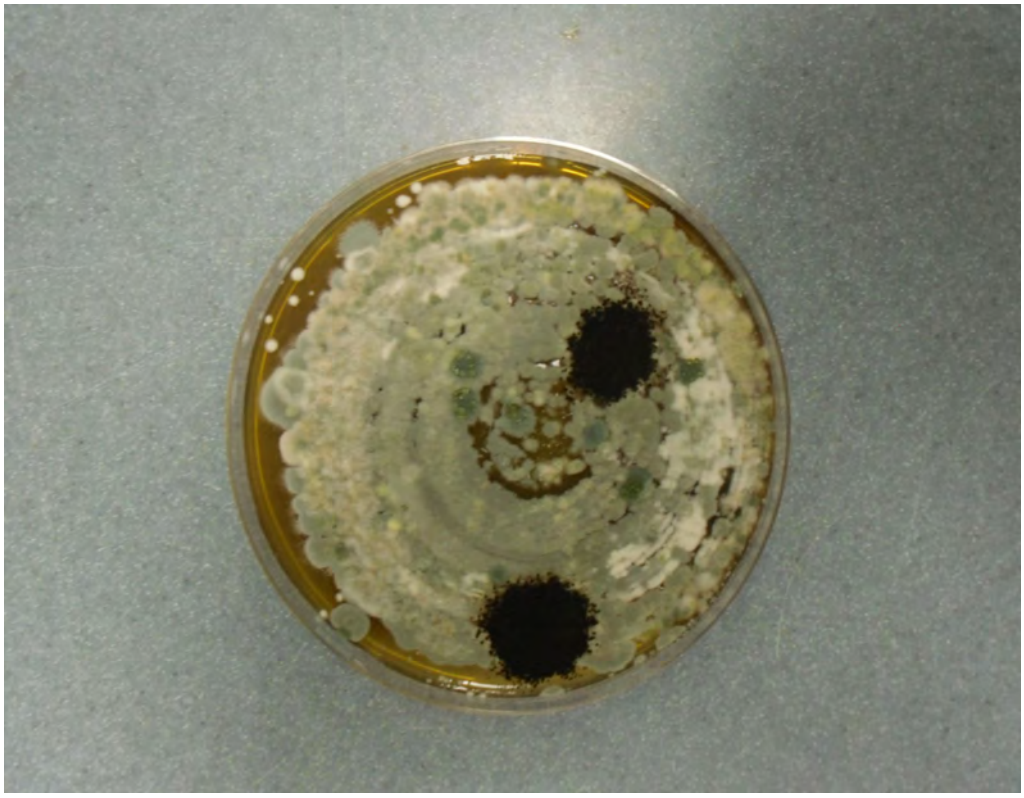

Fig.69. A press of a tape from collection 4 (typical results from this collection of 4 tapes) on MEA.

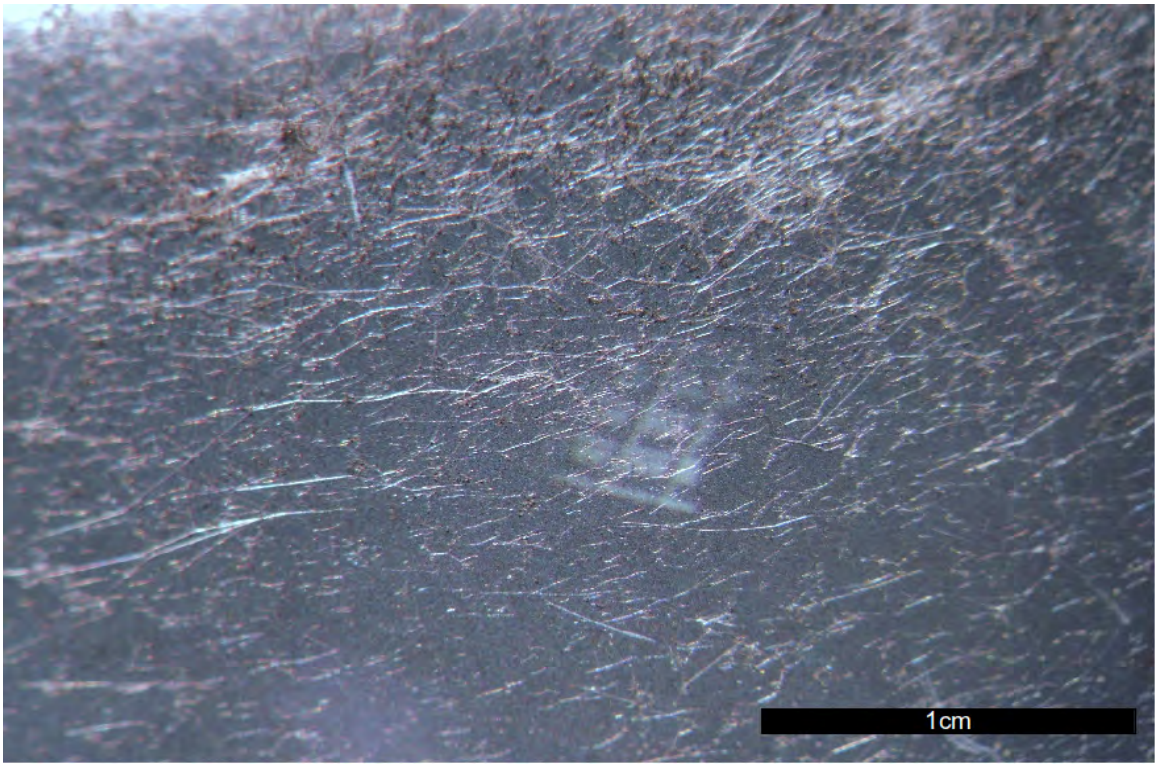

Fig.70. A piece of videotape, that had been placed on an MEA plate inoculated with a plug of *Stachybotrys chartarum* and incubated for 7 days at 30ºC, viewed under a bifocal magnifier. Sporulating structures were not visible.

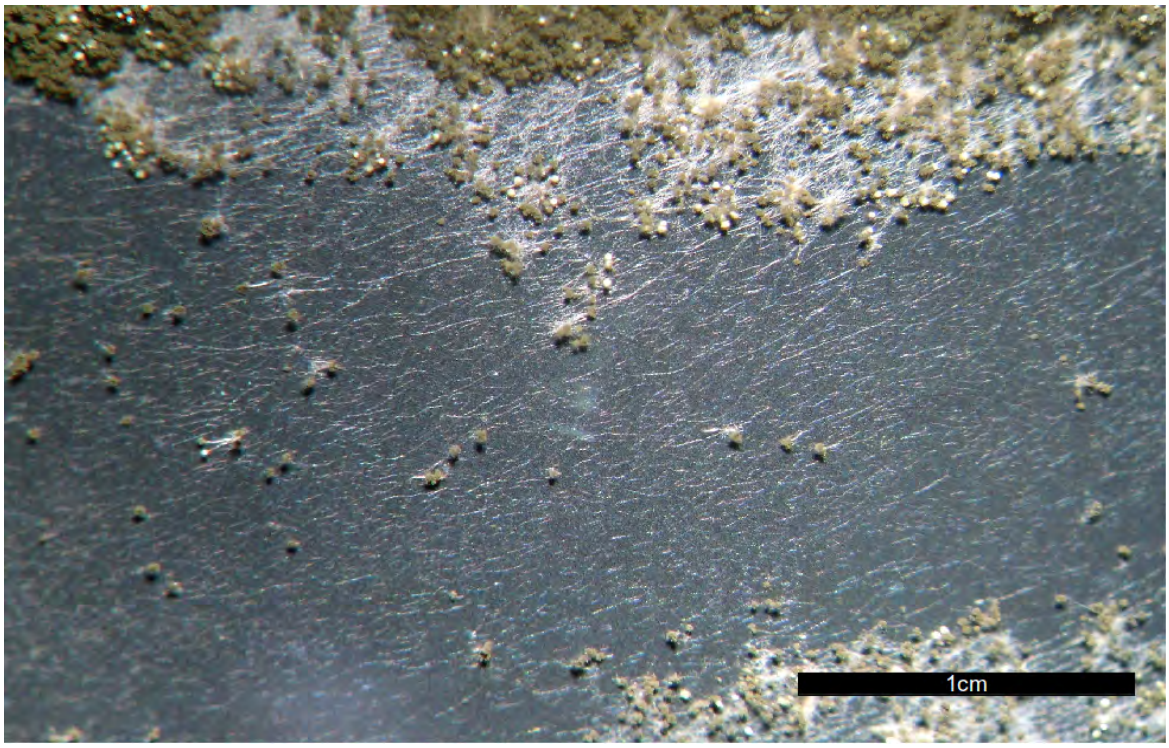

Fig.71. A piece of videotape that had been placed on an MEA plate inoculated with a plug of *A.versicolor* and incubated for 7 days at 30ºC, viewed under a bifocal magnifier. The edges of the tape at the top and bottom of the image were in contact with MEA. Sporing structures can be seen in the centre of the tape.

2.13. Discussion

2.13.1. Spore Counts and Evidence for Health Risks

As described in chapter 1, there was a concern expressed by archivists regarding the health risks posed by exposure to and inhalation of fungal spores, and there has been some indication that higher incidences of respiratory conditions such as asthma, could be partly due to exposure to higher concentrations of spores (Hu et al., 2006). There have been attempts by some agencies to define what are considered to be safe spore levels although these have some degree of variation. Seasonal fluctuations of spores in the environment may reach concentrations of above 1000m^3 but this would not be considered dangerous as individuals are exposed to these naturally in their life. There are discrepancies about what are considered 'safe' concentrations of spores depending on the source of the information. One internet source states that the European Union mould exposure standards of state <50m^3 is very low, <1000m^3 is medium and >10000m^3 is very high (Friedman, n.d.). With regards to exposure to healthy individuals, some environmental agencies state that the maximum indoor concentration of spores that is considered 'safe' from mixed common fungal species typical for a given location (e.g. *Cladosporium* sp.), is around 500-1000 CFUs/m^3 (Jo and Seo., 2005 and Friedman, n.d.) However, the World Health Organisation (WHO) suggests that a single fungal species considered to be either pathogenic or toxigenic at a concentration >50 spores per cubic meter is unacceptable. Additionally, spores of different genera vary widely in size, mass and toxicity (Friedman, n.d.) so evaluating the health risk posed by exposure to fungal spores in terms of numbers, is difficult, and there may still be problems with allergies or infections in the immunocompromised even at low concentrations. Thus, the health risks posed vary with each individual making a risk assessment for handling mouldy film reels difficult.

In this thesis, some of the film reels such as RR1399, RR1093 and RR1491 F3, released spores in concentrations above the 'safe' level of 1000 CFUs/m^3 during simulated inspection. In the case of *A.versicolor,* concentrations of spores from a single species were also released in higher levels than considered safe.

The results indicate that physical removal using art masking fluid (i.e. a 'laminectomy') was not an efficient method of reducing health risks. Colony forming unit counts of reels post laminectomy showed that in some cases such as RR1399, the number of spores released increased between test 2 and test 3, indicating that the laminectomy was not effective at reducing the number of spores. Peeling the laminectomy fluid off, could have disturbed spore bearing structures and therefore spores were isolated in higher

numbers during the subsequent air sampling. The laminectomy did appear provide an aesthetic cleaning effect because much of the visible debris on the top/bottom of the edges of the film is removed. However, any health related or cross contamination effects of decreasing spore release are limited, and the resulting dried fluid can be difficult to remove without possible damage to the film so it is not a well accepted practice and is not recommended. Indeed, the physical action of winding the film itself appeared to remove most of the exposed fungal growth, thus perhaps removing the need for addition of chemicals with an unknown effect on the film. However, it is highly likely that there will be growth on the parts of the film that do not come into contact with the laminectomy fluid i.e. the image surface and emulsion layer. Penetration by the fungal hyphae into the gelatine binder would mean that complete removal of the fungi by this method would not be possible, as the fluid only covers the film edges. Isolation of colonies by swabbing and subsequent air sampling confirmed that removal of the mould was not achieved by a laminectomy thus, there is no evidence for a reduced health risk for archivists regarding spore exposure using this method.

2.13.2. Predominant Fungal Genera

The predominant fungal genera isolated and identified from cinematographic films were *Aspergillus* and *Penicillum* species. The most probable reason for contamination and colonisation of films by these two predominant genera, is due to their ubiquity in the environment and ability to utilise many substrates for growth (Florian, 2002). In addition, Korpi et al. (1997) found that the dominating fungal genera in house dust were *Aspergillus* and *Penicillium* spp. Similarly to other findings in the literature (Abrusci et al., 2005) and with previous work carried out at MMU (unpublished) a particularly problematic species which had been isolated and identified using molecular methods was *A.versicolor* (n=8/32). Also similarly, the most frequently isolated *Penicillium* sp. was *P.chrysogenum* (n=4/32). Other isolates belonging to the same genera were identified (*Aspergillus* spp. n= 4/32, *Penicillium* spp. n=6/32) but species identification was not performed on these isolates.

The ATCC database classifies *Apergillus versicolor* and the *Penicillium* species isolated in this study as biosafety level 1 (Ulfig, 2003 and Anon. (LGC), n.d.), so they are not considered pathogenic to humans or hazardous to healthy individuals. An environmental species of particular concern with regards to health risks is *Stachybotrys chartarum,* due its potential for toxin production (Kuhn and Ghannoum, 2003), but it is still classified as biosafety level 1 on the ATCC database. However, as only single colonies

(or one spore) of this species were isolated from the cinematographic films used in this study from swabbing, it was not of particular concern in this instance. No other fungal species thought to pose a health risk of considerable concern were isolated.

The film reels donated to the NWFA were from private collections, and one might speculate that the owners may have stored them for long periods in attics, lofts, sheds or cupboards, all places in which they would come into contact with common household dust and possibly damp. Thus, if conditions were adequate for fungal growth, those fungal genera which are common in the environment would be likely to contaminate and subsequently colonise cinematographic film. From the results obtained, it would appear that the genera and species isolated from reels within one collection, were the same as other reels in that particular collection. For example, reel RR1093 (NWFA) and the swabs of nitrate reels kept at the BFI were predominantly contaminated with *Penicillium* spp., whilst films in the RR1399 were predominantly contaminated with *Aspergillus* spp. specifically *A.versicolor*. The predominance of one type may be due to cross contamination to other films in a collection once a reel is colonised, and also dependent on the fungi predominant in the environment in which that collection was stored. This could be investigated by sampling the domestic environment in which mouldy reels are found, and relate these findings to films. At the BFI in Gaydon, swabs of cellulose nitrate reels were taken which showed these were predominantly colonised with *Cladosporium* spp. as was the air of the vaults, which was ascertained with air sampling.

There were some instances where the film reels appeared to show some fungal growth but swabbing and air sampling failed to pick up any isolates. Abrusci et al. (2005) isolated many bacteria from the actinomycete family as well as *Bacillus* and staphylococcal species from the deteriorated film, but as the culture media used in this study was malt extract agar containing chloramphenicol, bacteria would be inhibited to some extent. However, the presence of hyphae on films such as RR1511 (visualised via microscopy), suggests that fungal growth was the cause of the damage, but either spores were no longer viable or the fungal structures were too strongly attached to the film. A similar case was true for reels RR1470 and RR1549, which released minimal numbers of spores although deterioration was rated as mould category 3 and 2 respectively (section 2.0. Table 1), again suggesting that the mould on these films was no longer viable. Also some microorganisms on the film may be un-culturable for example, it has been described in one study that the genetic diversity of cultivatable microorganisms found in soil is 200 times less than the total of those actually present (Pindi et al., 2009). It is estimated that only 2% of bacteria are culturable (Wade, 2002) compared with 10-20% of fungi

(Magnuson and Lasure, 2002). However, as predominant isolates from other reels were easily culturable and were common environmental fungi, it seems unlikely that those contaminating reels where no fungi were isolated, would be of a non-culturable species. As film reels were decades old, if contamination occurred in the past over time the mould may have depleted the nutrients and lost viability over time so this is the more likely reason for failure of isolation.

2.13.3. Substrate Utilisation and Enzyme Assays

2.13.3.1. Gelatinase Assays

Initially, gelatinase assays were performed on predominant fungal isolates from four reels (RR1399, RR1093, RR1549 and RR1470), and also additional isolates. There were some limitations to the different methods tested. On MEA and gelatine solid medium, a zone of hydrolysis was not always observed on isolates that were identified as gelatinase positive via liquefaction of the supernatant in broth culture after refrigeration due to gelatine hydrolysis. As MEA is a highly nutritious culture medium, gelatine may not have been the preferred growth substrate, therefore isolates would only utilise or begin to hydrolyse gelatine once the nutrients in the MEA had been depleted. This was indicated by the increased growth of colonies observed on MEA compared to minimal medium and enhanced gelatine hydrolysis on the latter. An example of this was the *Alternaria* isolate, which did not produce zones of hydrolysis on MEA and gelatine medium, but hydrolysed the gelatine in broth culture and produced a zone of hydrolysis when incubated on gelatine selective medium i.e. minimal nutritional medium where gelatine was the main nutrient source. When isolates were grown on solid media, all isolates which were shown to be gelatinolytic by other methods produced a zone of hydrolysis on gelatine selective medium compared with 98% of the isolates on the MEA with added gelatine. Thus gelatine selective medium is considered a more suitable medium for gelatinase assays.

RR1093 I3 did not liquefy the broth cultures but assay of the supernatant produced zones of clearance on the solid media. As the solid media had a lower concentration of gelatine than the broth (0.125% w/v gelatin and MEA media, 0.8% w/v gelatin selective media and 3% w/v in MEB and gelatine broth), enzymes may have been present in sufficient activity to hydrolyse the gelatine in liquid culture. RR1093 I1 produced a zone of hydrolysis around the isolate on solid media and liquefied the gelatine in the broth but no zone of clearance was visible around the wells. It is thought that this was an anomalous result, and even though the supernatant was assayed in 3 wells this was taken from the

same broth culture, so the cause is likely to be because of the media. However, as this was just one result and had little overall importance, this was not investigated further.

To investigate whether production of gelatinases was constitutive or inducible, three fungal isolates were grown in MEB without gelatine, and the supernatants subsequently assayed. The supernatants of *A.versicolor* and *S.chartarum* produced zones of hydrolysis on solid media, whilst *Alternaria* did not, thus indicating that protease (gelatinase) production may be constitutive or inducible, depending on the organism. Adding gelatine to the MEB was therefore necessary to induce gelatinase production in those isolates which would not otherwise have produced gelatinases.

Aspergillus isolates were more gelatinolytic than *Penicillium* isolates, with *A.versicolor* being the most frequently isolated and the most active. The increased gelatinolytic activity by this isolate suggests a possible reason for frequency of isolation from multiple film reels and high spore counts, as *A.versicolor* could utilise the gelatine more effectively for growth and outcompete other potential contaminants. Another study has shown that *A.versicolor* had a higher gelatinolytic activity, compared with that of other fungi including other *Aspergillus* species, and other species such as *Fusarium*, *Mucor* and *Penicillium* (Gopinath et al.,2005). Thus this species would be able to efficiently utilise the gelatine binder in the film as a nutrient source, and is therefore a likely predominant deteriogen for cinematographic film.

2.13.3.2. Cellulase Assays

Although gelatine is thought to be the main substrate for fungal growth on film reels due to the lack of availability of free cellulose in the acetate and nitrate bases, other materials containing cellulose may be present in an archive, which could be a source of cross contamination due to the presence of cellulolytic fungi. Two reels in the RR1491 collection were supplied in cardboard boxes instead of metal film cans, which could be an additional substrate for growth.

Two staining solutions were tested for their efficacy in cellulase assays: iodine and Congo red as, it was since suggested by Kasana et al. (2008) that iodine was more suitable due to reduced time for visualisation of hydrolysis zones and decreased toxicity. When a plate inoculated with *A.versicolor* was stained with iodine for 5 minutes, a clear zone was immediately apparent around the isolate. However, these zones of hydrolysis were present on both the medium which contained cellulose (CMC+ve) and the medium that didn't (CMC-ve) plates, indicating that *A.versicolor* was able to break down components other than CMC and that the iodine reacted with another polysaccharide in the agar, in addition

to cellulose. In comparison, no clear zones were observed on negative control plates (CMC-ve) when stained with Congo red, but they were visible around colonies on CMC+ve plates indicating that Congo red was reacting with intact cellulose. The Congo red also turned the agar plate containing CMC a darker shade of red than the CMC free medium whereas iodine did not reveal any of the intended products. Thus Congo red was considered a suitable stain for determination of cellulose hydrolysis.

The mechanism of action that effects a colour change is different for iodine and Congo red. Iodine is typically used to indicate the presence of starch (Rundle et al., 1944). Starch and cellulose are both polysaccharides based on glucose but bonding of the molecules give the compounds very different structural properties (Lamb and Loy., 2005). Starch molecules are made of glucose molecules joined by readily hydrolysed alpha bonds in linear chains (Bertoft and Akademi., 2004). In contrast cellulose is made of beta glycosidic bonds, which are much more difficult to hydrolyse (Mauseth, 2009). Iodine reacts with the straight chain portions of starch containing no fewer than six molecules of glucose, thus helices form, and iodine molecules assemble and become trapped, a phenomenon which produced the blue/black colour (Rundle et al., 1944). No evidence could be found in the literature of iodine reacting with cellulose molecules, but absorption of iodine could occur in cellulose complexes with decreased crystallinity, although a blue/black colour is not produced (Hessler and Power., 1954). Congo red does not react with intact starch molecules due to their hydrophobic properties, but reacts with cellulose molecules, which are hydrophilic (Sun et al., 2010), via hydrogen bonds (Lamb and Loy., 2005).

One of the limitations of the use of Congo red was the time taken for the test. Kasana et al. (2008) reported that it took 15-20 mins post-flooding with Congo red followed by 15-20 mins post-flooding with 1 M NaCl. However other studies used 15mins Congo red, 10-15mins NaCl (Apun., 1995) or 15 mins Congo red, 15 mins 1M NaCl (Teather and Wood., 1982). Thus in total 30 minutes is needed for a clear zone to become visible.

An additional concern is toxicity. Since Congo red is a benzidine based dye it has been postulated that it will be metabolised in the human body to benzidine (Kasana et al., 2008) which is a known carcinogen (Tanaka et al., 2004). Iodine would be safer to use as it is classified as an irritant (Anon. (Science Lab (A)), n.d.). However, the MSDS data for Congo red also classifies it as an irritant with no data available for the carcinogenic effects (Anon. (Science Lab (B)), n.d.). Thus if suitable protection is worn and the dyes are not

ingested both are suitable for use.

This comparative study indicates that, contrary to the findings of Kasana et al. (2008) iodine should not be used for detection of cellulases. Kasana et al. (2008) did not incubate isolates on CMC-ve medium as a control. Therefore it was not recognised that the clear zone would be visible using iodine on plates that did not contain CMC. Congo red only produced zones of clearing around isolates on CMC+ve medium, indicating its suitability for the detection of cellulases, in contrast to the less specific Gram's iodine. To date, Kasana's paper has been cited 17 times on the Web of Knowledge/ Web of Science (Anon. (Web of Science), n.d.) so it's impact and the use of its methods is likely to be limited.

All isolates were cellulase positive, which is not surprising considering cellulose is the most abundant carbon source on earth (Allsopp and Gaylarde., 2004) and most common environmental fungi are saphrophytes which degrade cellulose, with some also being phytopathogens (Sharma, 2004). *Penicillium* isolates had higher cellulolytic activity than *Aspergillus*.

2.13.3.3. Comparison of Cellulase and Gelatinase Assays

Cellulose is the most abundant natural polymer in the environment and represents approximately 40% of plant wall material (Deacon, 1997). Thus fungi have evolved to degrade this material, and play a pre-eminent role in cellulose breakdown, as indicated by all of the organisms isolated from cinematographic film showing cellulolytic activity. In addition, many fungi are also capable of producing gelatinases. Pisano et al. (1964) found that 13 out of 14 of the species of marine fungi tested produced gelatinase enzymes as our findings also showed. Additionally Abrusci et al. (2006) found that all of the fungal species isolated from cinematographic film were gelatinolytic to some extent, compared with only 6 out of 14 bacterial species tested in another study (Abrusci et al., 2004b). Thus there is a large number of fungi which have the potential to degrade cinematographic film.

The *Trichoderma* isolate (S1) showed the greatest gelatinolytic and cellulolytic activity and was found on all the swabs of cellulose nitrate films donated by the BFI, although this could be explained by its rapid growth rate on solid media compared with other isolates. However, this isolate was not isolated on films donated by the NWFA thus, it could be presumed that the species of fungi which contaminate and colonise film reels are related to the types of fungi present in environment where the film reels were stored. Sampling of reels from different locations would be a way to explore this further and

confirm these findings. *S.chartarum* was also capable of utilising cellulose and gelatine for growth however, the growth rate of this fungus was slow and this species requires a higher relative humidity than *Aspergillus* and *Penicillium* spp. (Florian, 2002). Thus this species was not as frequently isolated, as film reels would have had to be stored in an environment with a higher moisture content, in order for growth to occur. *A.versicolor* was shown to be gelatinolytic and cellulolytic and *Aspergillus* isolates in general were shown to be more gelatinolytic, whilst *Penicillium* spp. were more cellulolytic, explaining why the *Aspergillus* genus was the more predominant on the film reels. However as *Penicillium* spp. were the most predominant isolates on the BFI swabs and reel RR1093 from the NWFA (section 2.13.1.), this further suggests that the fungi present on mouldy film reels, correspond to the predominant isolates present in the environment where the reels were stored, in addition to their ability to utilise the film as a growth substrate.

2.13.4. Inoculation of Film

It has been demonstrated that fungi are able to produce gelatinases which would enable a species to deteriorate film if contamination and colonisation occurred. As stated previously, it is unknown how available the cellulose is in a film base (section 2.7.), for utilisation for growth. To demonstrate this in laboratory conditions, test film was inoculated with *A.versicolor* ATCC11730 as this species was frequently isolated from mouldy film reels and was shown to be both gelatinase and cellulase positive. When inoculated onto film strips, microscopic analysis indicated that the fungus had penetrated and grown in the gelatine emulsion layer and was visible on the cellulose acetate layer when the layers were separated. Hyphal growth also occurred on film strips placed on minimal media, which was more obvious on the lawn method. This could not have been due to nutrients in the medium, as the lawn grew much more on MEA and was not as visible on the minimal medium. However, fungal growth could be seen growing on top of the film slide on minimal media but not on the MEA (Section 2.10.2. Fig.70). This indicates that *A.versicolor* was capable of utilising the gelatine in the film for growth and will utilise this when no other nutrients are available but when grown on MEA, MEA is preferential for growth due to its high nutrient content.

On visual analysis of the separated layers, the cellulose acetate layer did not appear to be structurally deteriorated and mycelial presence and was restricted to surface growth, whilst degradation could clearly be seen on the emulsion layer, suggesting that the cellulose acetate was not being utilised for growth. Microscopic analysis revealed tracks in the emulsion layer, which contained hyphae and conidiophores. These tracks could not be

seen on the test film which had not been inoculated and incubated.

Microscopic analysis of a previously contaminated reel (RR1511) also revealed mycelial growth in the emulsion layer across the film frames, which could also be seen when the film was projected. However, no fungal colonies were isolated by swabbing or air sampling, indicating that it is the mycelium which damage film, whilst the spores pose a risk to archivists and other film.

2.13.5. Cinematographic Film Summary

Aspergillus and *Penicillium* species were the most predominant isolates from the reels studied and are common in the environment. In preliminary air sampling it appeared that fewer colony morphologies were isolated from heavily contaminated reels. This suggests that some fungal species, notably *A.versicolor*, were able to colonise the film and out compete other species for nutrients. This was indicated in gelatinase assays, where *Aspergillus* isolates were shown to have a greater gelatinolytic activity than *Penicillium* spp. *A.versicolor* has been shown in this study and other studies (Gopinath et al., 2005) to have a high gelatinolytic activity, so is able to utilise photographic gelatine for growth suggesting a reason for frequent isolation. Results from inoculation of film *in vitro* indicated that fungal mycelia penetrate and damage the emulsion layer, whilst leaving the cellulose acetate intact, because structural properties give cellulose acetate increased resistance to microbial degradation. Further studies should include more collections of reels from different archives from a range of climates. This would determine whether *A.versicolor* is a threat to cinematographic films throughout the world or whether it is limited to particular geographical locations. Whilst some reels released spore numbers in levels above what are considered safe, no pathogenic species which pose serious risk to health have been isolated in this work or in other studies. However, there is the possibility of an allergen risk, depending on the individual, and these risks can be reduced by wearing personal protective equipment (PPE) and carrying out inspections in ventilated cabinets/ glove boxes. Thus the greatest risk of mould contamination is to the film itself, whilst the risk to human health is low.

2.13.6. Videotapes

As was observed for film, videotapes that exhibited heavy contamination and colonisation appeared to have fewer morphologies of fungi whilst reels with less apparent contamination contained more. This was probably due to dominant growth by a few species which out-competed others for surface space to grow. Inoculation of malt extract

plates with presumptive mould growth from collection 1 did not always result in growth on the plate. This suggests that the mould could be dead/ unviable, unculturable or not fungal at all. DNA extraction and identification methods could confirm this.

The dominance of *Penicillium sp*. on the heavily contaminated tapes in collection 4 and to a lesser extent in collection 3, suggests that the videotapes were kept in a damp environment (e.g. a cellar). Some studies have shown that some *Penicillium* spp. require a higher water activity of 0.8-0.85 compared with 0.75-0.8 of *Aspergillus* spp. (including *A.versicolor*) (Adams and Moss, 2008, Anon. (International Commission on Microbiological Specification for Foods), 1996 and Chang, 1995). Although *Penicillium* spp. were more common on the videotapes studied than *Aspergillus* spp., it is impossible to say whether this would be the case for all mouldy videotapes, because this was only a small study. As with film reels, tapes in collections 3 and 4 were contaminated with the same fungal genera as other tapes in the same collection, which shows that isolates are likely to be found in high numbers in the environment in which the tapes were stored, and cross contamination of tapes can occur.

Although only a small number of videotapes were analysed in this study, results indicate that fungal contamination does occur on videotape thus further study is warranted. Physical structural damage to the tape did not seem to have occurred, and as attempts to inoculate the tapes on both nutrient and minimal media were unsuccessful, it can be concluded that the tape itself was not a substrate for fungal growth, and fungal growth may have occurred due to condensation between the plastic case and the tape reel. It has been shown that fungi can grow on materials but not utilise them as a substrate (Arai, 2000). This study showed that fungi growing on paper utilised dust and moisture rather than cellulose for growth, and fungi could grow on any material given adequate growth conditions. The same could be happening on videotape. This could be checked by cleaning the presumptive mould off and playing the tape. However, any remaining fungal growth or damage to the tape could potentially severely damage video players, and potentially cross contaminate other tapes if played through the same machine, thus was not attempted. Fungal contamination of videotapes may be a fairly short term issue for archives, since videotape technology is now out-dated, and data are now transferred to digital media but never the less, a potentially significant issue is posed in the short term. Contamination also poses a problem during transfer of tapes to digital media, as data quality could be lost. There have been few investigations on the contamination of videotape, but more studies are warranted to assess the extent of the problem and the nature of the contamination.

2.13.7. Alternative Methods for Detection of Mould on Media

There is a potential for mould growth on various visual media but, the extent to which this growth is actively occurring is not always obvious. Whilst microbiologists can isolate viable fungal colonies and identify the organisms present, archivists may not have access to these services. Thus film reels which are presumptively identified as being mouldy by visual inspection are not examined and potentially valuable and interesting footage is lost. Results have shown that fungi growing on cinefilm are common in the environment so are thought to pose only minimal risk to an archivists health. Thus actively growing mould is a threat to the film not to the archivist.

Active mould growth can cause more damage to materials and cross contaminate non-contaminated reels. Additionally, active mould growth could also pose a hazard due to toxic metabolite production (Bennett and Klich, 2003). In comparison, dead mould does not cause further damage to materials and if spores become unviable the risk of cross contaminating other materials also decreases. Thus, these are reasons why a means for archivists to detect active mould growth would be beneficial.

Due to the potential lack of microbiological expertise and specialist equipment, an alternative method is needed to detect active mould growth on film so that reels can be stored separately to avoid cross contamination of other reels. One method for achieving this could be by detection of microbial volatile organic compounds (MVOCs) which are compounds produced when fungi is actively growing (Fiedler et al., 2001). Methods to detect MVOCs were investigated.

Chapter 3

Detection of Fungi on Cinematographic Film by Analysis of Microbial Volatile Organic Compounds

3.0. Introduction

3.1. Microbial Volatile Organic Compounds (MVOCs)

The storage conditions of cinematographic film significantly affect microbial susceptibility to contamination and growth. Moisture damage and subsequent microbial growth can damage the film, and lead to further contamination of previously non-contaminated films and objects. In terms of health risks to archivists, allergies, irritation, infection and toxic effects have been related to fungal exposure (Sorenson, 1999). In some cases, visual inspection of archival objects may be sufficient to determine the presence of mould (Kuske et al., 2005). However, dust and other debris may appear similar to fungal growth to the untrained eye, and as a result films are not subsequently handled due to the fear of health risks thus resulting in the loss of potentially valuable footage (Bodner, (2009), NWFA, Personal communication).

Traditional microbiological methods could be used to confirm fungal growth and identify the fungi involved. Determination of viability and the extent of contamination commonly involve the measurement of spore levels using a device such as an air sampler (Florian, 2002), which concentrate spores onto a Petri dish containing growth media. Spores or hyphal fragments which land on the agar plate may germinate resulting in a colony which can subsequently be counted to give a relative measurement of mould contamination i.e. the greater the number of colonies, the greater the extent of contamination. These methods are often used when investigating mould growth in buildings but there are some limitations. Several investigations have shown that there may be no significant difference between spore levels in contaminated and non contaminated buildings (Sunesson et al., 1995). Some explanations for this are; fungal spores may be too large to penetrate some of the building materials such as wallpaper and insulation (Strom et al., 1994) and that fungi do not sporulate continuously (Nevalainen et al., 1991). Additionally, mould contamination may have occurred in the past but viability has been lost.

In addition to the limitations posed by traditional culture methods, archivists may not have access to specialist microbiological equipment or a trained microbiologist. Thus, a simple detection device that could detect mould growth without culture or specialist microbiologists would be valuable in these circumstances. The detection of microbial volatile organic compounds (MVOCs) could be a potential target using gas chromatography mass spectrometry (GC-MS).

The aims were to investigate whether detection of microbial volatile organic

compounds (MVOCs) could be used to assess active mould growth on contaminated materials: in this case on mouldy cinematographic film.

There are two commonly used GC-MS adsorbent techniques described in the literature for detecting MVOCs from fungi: headspace solid-phase microextraction (HS-SPME) and thermal desorption.

3.1.1. Headspace solid-phase microextraction (HS- SPME)

Solid phase microextraction (SPME) uses a fibre to concentrate volatile and non-volatile compounds thus removing the need for solvents or complicated apparatus. The key to the process is the fibre coating and by controlling the thickness and polarity of the fibre, compounds can be measured even if they are emitted in very low concentrations. Examples of fibres include polyacrylate (PA) and those suspended on a polydimethylsiloxane (PDMS) core (Kataoka et al., 2000) which among the newest of those is the divinylbenzene and carboxen coating, also known as DIV/CAR/PDMS (Alpendurada et al., 2000).

3.1.2. Thermal Desorption

Thermal desorption also preconcentrates VOCs onto an adsorbent material. However, in contrast to SPME, thermal desorption actively heats the sample so that volatile compounds are turned into gas (Prezant et al., 2008), which are then absorbed onto Tenax (Fischer et al., 1999) instead of PDMS. Tenax is a porous polymer resin based on 2,6 diphenylene oxide (Anon. (Scientific Instrument Services), n.d.), and has been used in studies to detect MVOCs from airborne fungi in compost (Fischer et al., 1999) and from *S.chartarum* cultivated on rice and gypsum board (Gao and Martin, 2002). In one study by Fischer et al. (1999), fungal colonies which had been incubated and grown on yeast extract sucrose agar (YES), were covered with a glass funnel to trap MVOCs including 1-octen-3-ol, and then heated to vapourise compounds which were then adsorbed onto Tenax (Fig.72) and analysed using GC-MS.

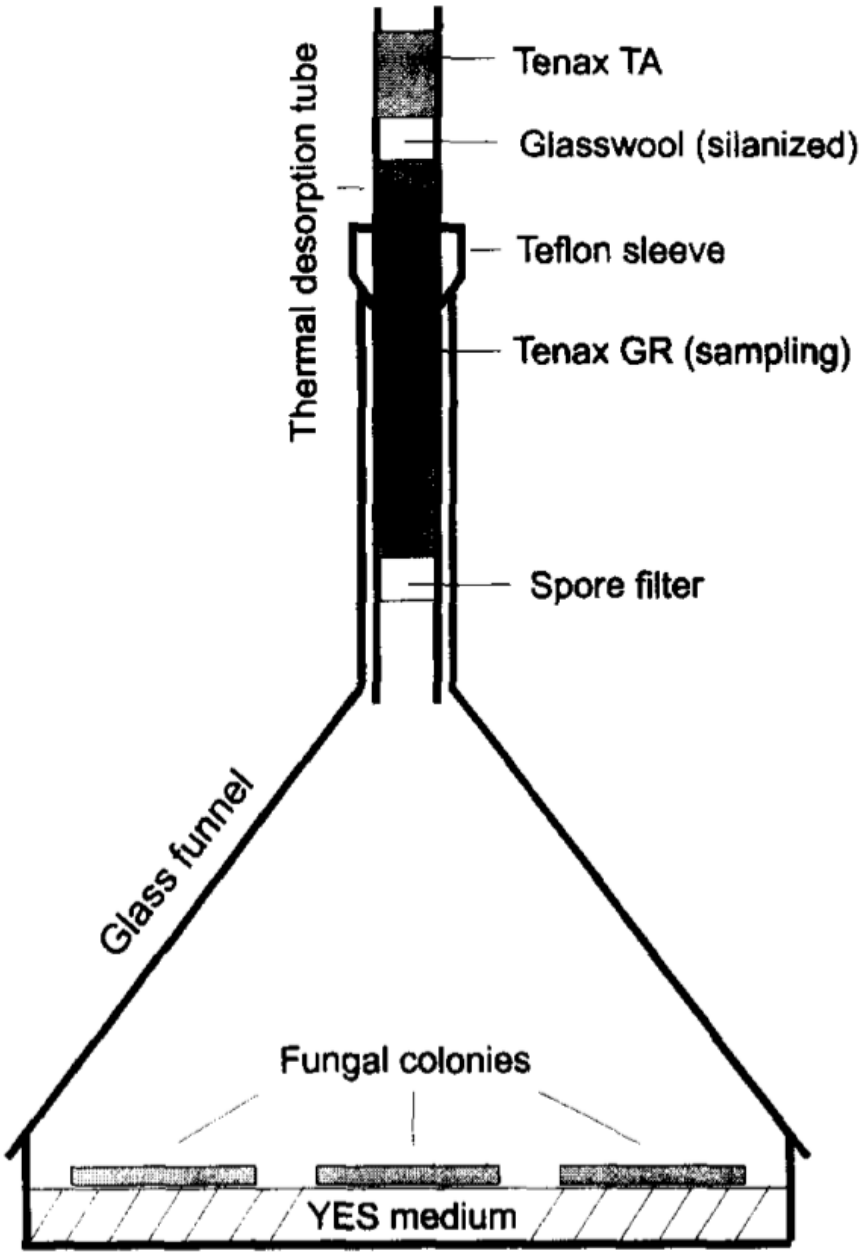

Fig.72. Mechanism of thermal desorption. Fungal colonies grown on yeast extract-sucrose medium were covered with a glass funnel to trap MVOCs. The sample is then heated which vapourises compounds, which are subsequently adsorbed onto Tenax, and analysed using GC-MS (Fischer et al., 1999).

3.2. Detection of fungal MVOCs

MVOCs are produced by fungi due to metabolic activities such as the breakdown of nutrients. For example, in mould ripened cheese this can be the breakdown of glycerides and fatty acids to methyl ketones, and the reduction of methyl ketones to alcohols (Moss et al., 1989). Lewis and Darnall (1970) have also shown that production of MVOCs could be part of the detoxification of toxic metabolites. For example, caproic acid (hexanoic acid) is a fatty acid found in coconut and palm oils and also milk fats (Refat et al., 2008). This acid has been shown to inhibit germination of spores and hinder oxygen uptake whilst preventing formation of the mycelium in several species of fungi including *Aspergillus niger* (Lewis and Darnall, 1970). In the presence of caproic acid, increased concentrations of the ketone 2-pentanone were noted, suggesting that production of this compound was part of the detoxification process of this acid. Similar processes could also occur for the production of other compounds.

Several MVOCs have been shown to be common markers of fungal growth although they cannot always be detected due to for example, the concentration of MVOCs being below the limit of detection or the selection of adsorbent fibre being inappropriate for the compounds requiring detection (Kataoka et al., 2000 and Bryant and McClung, 2011). This could be a problem when investigating MVOCs emitted from mouldy film reels, where moulds may no longer be actively growing due to long term storage or exhaustion of nutrients i.e. gelatine in the film for growth, or do not emit high concentrations of volatiles. Thus, detection of MVOCs is dependent both on the adsorbent used and the gas chromatography (GC) method used.

Moulds produce larger quantities and types of volatiles when growing on nutrient rich media in the laboratory compared to low nutrient substrates such as building materials (Wilkins et al., 2000) or possibly cinematographic film. When properly stored, film is a nutrient poor growth medium with little moisture content thus it could be assumed that similar growth would be observed. However, Wilkins et al. (2000) detected 2-methyl-1-propanol, 3-octanone and 1-octen-3ol from *Penicillium chrysogenum* growing on plasterboard, which were the same MVOCs found when using complex media (Wilkins and Larsen, 1995), suggesting that there is the possibility these will also be detected on mouldy film. This validates GC-MS with HS-SPME as a potential method for detecting fungal growth on mouldy film reels.

3.2.1. Factors affecting MVOC production

There have been several studies which aimed to determine which MVOCs could be used as markers of fungal growth and several biological factors have been shown to affect MVOC production. Investigations by Matsuoka et al. (1990) suggested that MVOC synthesis can affect spore germination and mycelial growth. Factors affecting MVOC synthesis include colony age (Black et al., 1999), relative humidity (RH) (Korpi et al., 1998), growth substrate and to a lesser extent temperature (Polizzi et al., 2011). MVOCs may differ between genera and also between different isolates of the same species (Black et al ., 1999). There are several factors that could affect MVOC production.

1) Growth substrate

The growth substrate has been shown to affect MVOC production and activator substances such as compounds in the growth medium may be necessary for production of some VOCs (Wilkins et al., 1995). Scotter et al., (2005) used Selected Ion Flow Tube-Mass Spectrometry (SIFT-MS) to detect volatiles from medically important fungi in real-time. This technique utilises chemical ionisation reactions of mass selected ions, which enables the characterisation of VOCs and has advantages over GC-MS methods as it is rapid, and can detect compounds of relatively low molecular weight whilst allowing for real-time quantitative monitoring without the need for time consuming manual GC-MS analysis. Scotter et al. (2005) analysed MVOCs from *A.flavus, A.fumigatus*, *Candida albicans*, *Mucor racemosus*, *Fusarium solani* and *Cryptococcus neoformans* grown on five types of media, including malt extract media, enabling comparison with other papers in the literature. It was shown that the metabolites produced from a single species can vary greatly, and depended on temperature, oxygen availability and growth substrate (Borjesson et al., 1992 and Scotter et al., 2005). Ethanol, acetaldehyde, acetone, methanethiol and crotonaldehyde were the specific identified volatiles targeted in this investigation. Of the five culture media investigated, growth on malt extract agar and Sabouraud's dextrose agar (SAB) resulted in a relatively moderate to high levels of VOCs being emitted compared with the moderate to no volatiles of the blood agar, brain-heart infusion broth and Columbia agar. Thus, malt extract agar seems to be an appropriate substrate to use for investing the MVOCs produced by fungi isolated from cinematographic films.

2) Stage of Mould Growth

The stage of mould growth has also been shown to influence which VOCs are emitted (Borjesson et al., 1992). MVOC production has also been measured at various growth

stages for a number of genera (Black et al., 1999). It was shown by Black et al. (1999) that *Alternaria alternata* and *Aspergillus versicolor* produced the most MVOCs at day two of growth whilst other isolates produced more at day ten. *Penicillium chrysogenum* produced the highest concentration of MVOCs. 2-octen-1-ol was produced by all isolates of *Cladosporium sphaerospernum, Aspergillus niger*, *A. versicolor*, *Penicillium chrysogenum* and *P.brevicompactum* and 1-octen-3-ol was detected from all isolates of the latter three species. Another study investigating MVOC production over time, was performed by Matysik et al. (2008). SPME and GC-MS was used by to detect MVOCs from wallpaper and synthetic media during different stages of mould growth. Monitoring of MVOC production took place over several weeks to investigate emission rates between the initial stage and later periods of growth. 2-pentanol and 2-pentanone were found to be common to all 6 species although 2-pentanol was only detected during the early growth stages. 1-octen-3-ol was detected in five of the species and was shown to emit a constant emission rate over the whole growth period. *A.versicolor* produced high amounts of 1,3-dimethoxybenzene whilst *Penicillium* species emitted high concentrations of 1-octen-3-ol and 3-octanol.

3) Moisture and RH

Moisture and RH have also been shown to affect concentrations of the MVOCs produced by mould growth. Strom et al. (1994) found that higher concentrations of these MVOCs were found in houses which contained above average levels of moisture although mould growth was not necessarily visible. This indicates that MVOC detection could potentially identify biocontamination of cinematographic film even when mould is not visible to the naked eye (this is a risk to the film rather than people). Similarly Korpi et al. (1998) also investigated moulds on various growth substrates in environments with differing RH values. It was found that growth of the fungi was greatest at over 90% RH (as shown in houses (Strom et al., 1994)), therefore the greatest number of MVOCs were emitted at these levels. Thermal desorption-gas chromatography and high performance liquid chromatography identified; 3-methyl-1-butanol, 1-pentanol and 1-hexanol, which were emitted at 90-92% RH whilst significant concentrations of 3-octanone was emitted at various RH values.

3.2.2. Potential Target MVOCs for Indicators of Growth of Target Moulds

There have been several studies on many fungi growing in various environments on many different materials. Strom et al. (1994) investigated whether MVOCs from both fungi and bacteria could be detected within buildings. Fifteen compounds were detected from houses in which people were reported to be suffering from sick building syndrome due to mould growth: 3-methylfuran, 3-methyl-1-butanol, 3-methyl-2-butanol, 2-methyl-1-propanol, 2-pentanol, 2-hexanone, 2-heptanone, 3-octanone, 1-butanol, 3-octanol, 1-octen-3-ol, 2-octen-1-ol, 2-methy-lisoborneol, geosmin and 2-isopropyl-3-methoxypyrazine. In agreement with Strom et al., (1994), Wilkins et al., (1995) identified several MVOCs 1-octen-3-ol, 3-octanol, 3-octanone, 2-hexanone, 2-heptanone and 2-heptanol as common to the *A.versicolor*, *P.chrysogenum* and *P.commune* strains investigated on various substrates indicating that some of these could be potential targets for detection from mouldy film.

Fiedler et al. (2001) used SPME to detect MVOCs from moulds on various substrata including; MEA, Conifer wood, Beech wood and yeast extract glucose chloramphenicol agar (YGC). Here, twelve fungal species were screened for MVOC production including *Aspergillus versicolor, P.brevicompactum* and *Penicillium chrysogenum* (which have also been isolated from cinematographic film in this study). Other fungal species that were analysed by Fiedler et al., (2001) which are commonly found in the environment were *A.fumigatus, A.niger*, *A.ochraceous*, *Trichoderma harzianum, P.expansum*, *Fusarium solani* and *Mucor* sp. Similarly to the work presented in this thesis, again malt extract agar was used by Fiedler et al. (2001) to maintain the cultures, thus indicating its suitability for analysis of fungi isolated from cinematographic film using SPME and GC-MS. Methods involved culturing the fungi in 500mL Erlenmeyer flasks and sampling the air space (head space) between the medium and the top of the bottle. SPME analysis involved inserting a fused silica fibre coated with a carbowax/divinylbenzene, polyacrylate, polydimethylsiloxane, or carboxen/polydimetylsiloxane phase, through the septum of the flask so that it was exposed to the volatiles in the cultures for 24-48, hours after which the fibre was retracted into the needle and removed from the flask for GC analysis. Fiedler et al. (2001) found that moulds grown on various nutrient media could potentially emit more than 150 volatile substances although no single compound was common to all species. Several compounds occurred in a large number of species including 1-octen-3-ol, 3-octanone, 2-methyl-1-butanol and 3-methyl-1-butanol as a result could be used as indicators of active mould growth on cinematographic film. Additionally, it was also found that the presence and concentration of MVOCs depended on the age of the mould culture. Similarly, Meruva et

al. (2004) used a closed system to investigate MVOCs from tobacco molds caused by *Aspergillus ornatus*, *Penicillium chrysogenum* and *Rhizopus stolonifer* and found 1-octen-3-ol, 2-octen-1-ol, 2-methyl-1-butanol, 3-methyl-1-butanol, 1-octene and 2-pentanone to be the prominent fungal markers identified.

Demytennaere et al. (2004) used HS-SPME analysis to examine volatiles produced by *Fusarium* species. Spores of *Fusarium* were inoculated onto small amounts of MEA in the bottom of 22 mL glass SPME (Supelco) vials. This provides an alternative to growing the cultures in broth and the headspace could be sampled directly after growth. It would also be relatively easy to minimise cross-contamination because no transfer of culture would take place, although it is unknown how well other fungal cultures would grow in sealed vials.

Thermal desorption was used by Fischer et al. (1999), who grew fungi on plates of solid Yeast extract-sucrose (YES) medium with an inverted airtight glass funnel over them which trapped any volatiles which were released by the fungi. Eleven fungal species were tested including *Aspergillus candidus, A. fumigatus, A.versicolor, Emericella nidulans, Paecilomyces variottii, Penicillium brevicompactum, P.crustosum, P.clavigerum, P.cyclpium, P.expansum,* and *P.glabrum*. 1-octen-3-ol, 3-octanone, 2-methyl-1-butanol and 3-methyl-1-butanol were found to be common from most isolates and correlates to work carried out by Fiedler et al. (2001). Limonene was also found to be common to all the fungi tested except *A.candidus and E.nidulans*.

Moularat et al. (2008) used thermal desorption with a flame ionisation detector (FID) to investigate whether MVOCs could be detected before mould growth was visible to the naked eye. An FID is sensitive to solutes which increase luminosity or impart colour to the flame, and is partially selective to aromatic hydrocarbons which impart strong luminosity (Scott, 2003). Hydrogen is used for combustion to create a small jet of flame. A cylindrical electrode surrounds the flame, and a relatively high voltage is applied between the electrode and the flame jet to collect ions that are formed in the flame. The current generated is amplified by a high impedance amplifier and the output is fed to a data acquisition system for analysis (Scott, 2003). *A.niger*, *A.versicolor* and *P.brevicompactum* were tested each on five different building materials. Six MVOCs were common to all the fungi on all the substrates; 1.3-Octadiene, 2-Methylfuran, 3-Methylfuran, 2-Ethylhexanoic Acid methyl, 1-octen-3-ol, 3-octanone, 2-methyl-1-butanol and 3-methyl-1-butanol, the latter four compounds of which have been mentioned in other literature (Fischer et al., 1999, Fiedler et al., 2001), indicating the potential use of these MVOCs as markers for fungal growth, with positive detection indicating active mould growth.

There are other technologies available which are similar to GC-MS that are used to detect MVOCs. Electronic noses (e-noses) mimic human olfaction and are based on the same processes as GC-MS i.e. they comprise of a sampling conditioning unit which extracts the volatiles from the headspace of the sample, a test chamber containing an array of sensors, and a processing unit which analyses data from the sensor and identifies patterns in volatiles produced. These are small kits that are custom built to detect a specific small range of volatiles and do not require the large machines required for GC. Canhoto et al. (2004) used electronic nose technology (E-nose) to see if mould could be detected on contaminated library paper. It was discovered that the electronic nose could differentiate between the control samples and paper which had been inoculated with fungi, and Schiffman et al. (2000) found that the e-nose could detect and differentiate between three fungi, *Aspergillus niger*, *A.flavus* and *Penicillium chrysogenum*. Elke et al. (1999) also used electronic nose technology to detect MVOCs from mouldy houses. 3-methylbutan-1-ol, hexan-2-one, and heptan-2-one were found to be the most reliable indicators of mould formation as well as 1-octen-3-ol, as mentioned previously. These findings could also have significance for cinematographic film, as it is likely that some of the same fungi which contaminate library paper are likely to contaminate film. Thus, e-nose technology could be used to detect MVOCs from similar fungi isolated from cinefilm, and has the potential to discriminate between non-contaminated and mouldy film.

3.2.3. Health Risks Associated by Exposure to MVOCs

Some MVOCs have been known to cause adverse health effects, most notably in cases of sick building syndrome (Wilkins et al., 1995). Several MVOCs commonly produced by fungi including 1-octen-3-ol, 3-octanol, 3-octanone, 2-methyl- 1-propanol, 2-heptanone, 1-pentanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 3-methyl-2- butanol, 2-hexanone, 2-butanone, 3-methyl-2-butanone, 2-pentanol, ethyl isobutyrate, and terpinen-4-ol, have been shown to be mutagenic based on results obtained by the Ames test (Nakajima et al., 2006), Walinder et al. (2008) investigated 1-octen-3 ol to test for adverse health effects and to see how fungal growth could produce some of the symptoms associated with mould exposure in volunteers. Volunteers reported symptoms of exposure to 1-octen-3-ol by several tests including measurement of blink frequency, the time required for dry spots to appear on the corneal surface after blinking, washing out of the nasal passages (nasal lavage), and monitoring of the nose and airways for mucous production. Subjects were exposed to either 10mg/m^3 of 1-octen-3-ol or clean air as control and were asked to give subjective ratings to some of the tests. It was shown that exposure to 1-octen-3-ol, resulted

in headache, nausea, smell and nasal irritation, eye irritation, increased blinking frequency throat irritation and higher nasal lavage biomarker levels of eosinophil cationic protein, myeloperoxidase and lysozyme (Walinder et al., 2008). Walinder et al. (2008) also noted that atopical asthmatics did not have more reactions due to exposure, whereas females experienced more smell and mucosal irritation.

These findings indicate the need for methods which can detect fungal growth on archive materials without the need for culture methods or specialist equipment, thus limiting exposure to MVOCs in order to protect the health of archivists, ensure safe handling and storage of mouldy materials.

3.2.3 Summary

Volatile organic compounds (VOCs) are produced as metabolites from fungi. There has been interest in microbial volatile organic compounds (MVOCs) with respect to cheese flavour, spoilage of foods such as grain, detecting of ripening of fruits and vegetables and in recent studies in connection with moulds in 'sick building syndrome'. More recently there has been concern with MVOCs regarding the museum environment and the dangers posed by allergens and toxins produced by mould such as *Stachybotrys chartarum* (McCrady, 2010). There is reference to MVOC production by fungi on various substrates in the literature (Fiedler et al., 2001) but no specific studies have been found concerning fungi found on mould contaminated cinematographic film.

There are many MVOCs produced by fungi but there is no single compound which is common to all fungal species, although it has been suggested that there are fourteen MVOCs common to general fungal growth from consistent isolation from a number of surveys (Ryan, 2005). These are; 3-methyl furan, 1-butanol, 3-methyl-1-butanol, 3-methyl-2-butanol, 2-pentanol, 2-hexanone, 2-heptanone, 3-octanone, 3-octanol, 1-octen-3-ol, 2-octen-1-ol, 2-nonanone, Borneol and Geosmin. Of these, 3 MVOCs appear to be common to many *Penicillium* and *Aspergillus* species, which are the genera that have been most frequently isolated on cinematographic film. These are 1-octen-3-ol, 3octanone and 3-octanol (Fischer et al., 1999, Fiedler et al., 2001 and Strom et al., 1994). Commonly detected MVOCs from gas sampling of *Aspergillus* and *Penicillium* species in other studies, which could have been potential targets for detection in this study are summarised (Table 11).

Table 11. Potential target MVOCs which could be used as markers for fungal growth. Most of the fungal isolates found on cinematographic film were *Aspergillus* and *Penicillium* sp. so identification of growth markers for these would be beneficial. Species from these genera which have been predominant on cinematographic film are listed. Additionally indicators of the growth *Stachybotrys chartatum* would be advantageous due to the health risks posed by this species.

Potential Target MVOCs	**Genus Indicators**	**Fungi Isolated from Cinefilm**
Geosmin	*Penicillium* sp.	*Aspergillus versicolor*
2-heptanone	*Aspergillus*/*Penicillium* sp.	*Penicillium brevicompactum*
Limonene	*Aspergillus*/*Penicillium* sp.	*Penicillium chrysogenum*
2-methyl-1-butanol	*Aspergillus*/*Penicillium* sp.	*Stachybotrys chartarum*
3-methyl-1-butanol	*Aspergillus*/*Penicillium* sp.	
2-pentanol	*Aspergillus*/*Penicillium* sp.	
2-pentanone	*Aspergillus*/*Penicillium* sp.	
3-octanol	*Aspergillus*/*Penicillium* sp.	
1-octen-3-ol	*Aspergillus*/*Penicillium* sp.	
3-octanone	*Aspergillus*/*Penicillium* sp.	

From the various studies described it appears that the alcohol 1-octen-3-ol is commonly detected from many fungal isolates. This compound has been found to be highly volatile and is produced in high quantities in *Penicillium* and *Aspergill*us species (Kaminski et al., 1974). When this compound was trapped and purified in one study it exhibited a strong fungal odour (Kaminski et al., 1974). It is indicated as a musty, earthy odour to human olfaction (Meruva et al., 2004 and Larsen and Frisvad, 1995) and can be detected in processes such as the rotting of fruit by *P.expansum* (Matysik et al., 2008). In addition to the common MVOCs frequently detected from various fungi, geosmin is also often reported in the literature as an indicator of the presence of mould (Nilsson et al., 1996). Mattheis et al., (1992) identified geosmin as a volatile collected from *Penicillium expansum*. Geosmin was also detected from *P. chrysogenum* by Matysik et al. (2008) but as it was only found on one of six isolates sampled, it would appear that this is not suitable to use as a general fungal growth indicator but could be a potential indicator for *Penicillium* species.

Wilkins et al. (2000) suggested that mould growth in buildings was limited to a few species so it would be beneficial to use their MVOCs to indicate general mould growth. The same observation has been made in this study from sampling of cinematographic film, where a few species appear to colonise the film and other minor species are assumed to be contaminants. Identification of these colonisers is the key to selection of fungal species to be investigated for MVOC production.

If common MVOCs can be detected from isolates found on mould contaminated cinematographic film, this demonstrates the potential use of a sensor which could distinguish between active mould growth on film and 'dead' mould. This will enable archivists to store materials in ways which prevent further mould growth and cross contamination of other non-mouldy films as well as protecting themselves from the health risks associated with handling fungi. Measurement of MVOCs could also enable detection of fungal development before there are visible signs of mould contamination, thus helping to protect the film.

GC-MS and headspace analysis have been widely described in the literature for analysis of fungal MVOCs (Canhoto et al., 2004, Fiedler et al., 2001 and Fischer et al., 1999). It appears that SPME coupled with GC-MS is the fastest, simplest method for MVOC detection and does not require thermal desorption, and is sensitive enough to detect MVOCs produced by fungi (Demyttenaere et al., 2003). Thus methods using these techniques were performed, to determine if any MVOCs could be used as markers of fungal growth on cinematographic film.

3.3. Principles of Gas Chromatography Mass Spectrometry (GC-MS)

Gas chromatography (GC) has become the main technique used for separation of volatile compounds. It is the separation of components between two phases i.e. the stationary phase (e.g. the SPME fibre and column) and the gas phase (e.g. the MVOCs). The sample is vaporised and carried by the mobile gas phase (i.e. the carrier gas) through the column. Samples separate (elute) into different components (solutes) based on their solubilities at a given temperature due to relative vapour pressures and affinities for the stationary bed (McNair and Miller, 1998). The compounds are then analysed by a detector and data system. A similar set up to the one used in this study is shown but the autosampler was not used and was replaced by a manual injection port (Fig.73) (Sánchez-Rodas et al., 2010). Additionally, a mass spectrometer detector was used in place of the Merlin fluorescence detector. Compounds move through the column at different speeds depending on their solubilities in the stationary or mobile phase. The time taken between the injection time and the sample producing a peak on the detector is known as the retention time (Schomburg, 1990).There are many benefits of using GC-MS i.e. fast analysis, efficient, high resolution, sensitive (parts per million (ppm) and parts per billion (ppb), non-destructive of test material, highly accurate quantitative analysis of components (relative standard deviations (RSDs) of 1-5%), only small samples are needed and the equipment is also relatively simple, reliable and cheap to use. Thus, GC-MS analysis coupled with HS-SPME is suitable for detecting MVOCs from fungal growth on contaminated cinefilm.

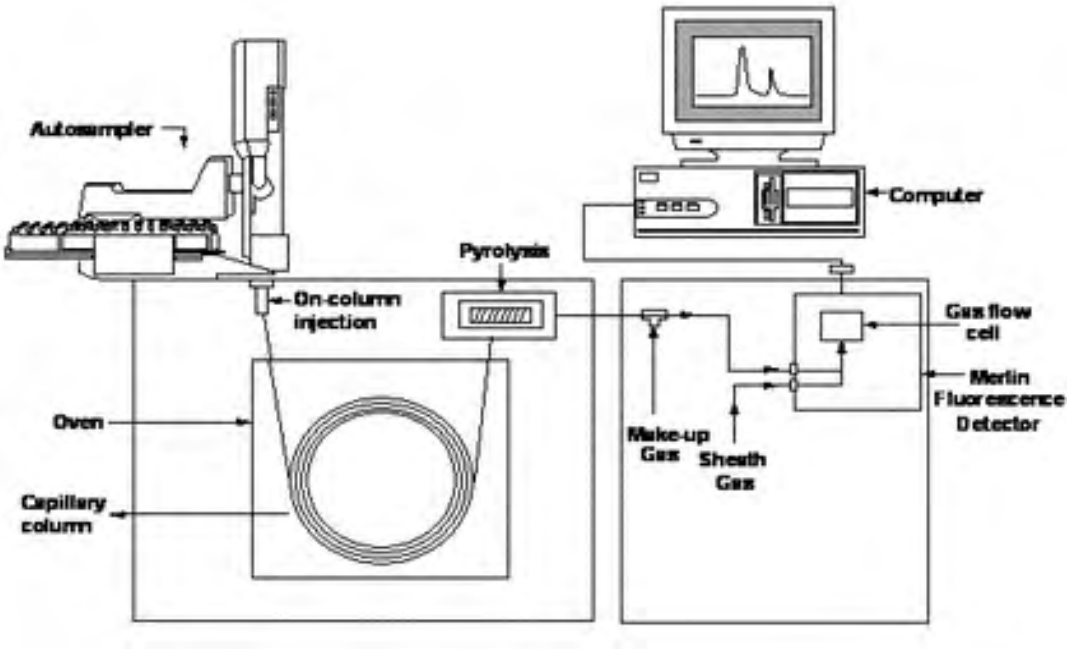

Fig.73. Schematic diagram of gas chromatograph showing how gases are analysed. A sample is injected via an autosampler, or the autosampler can be replaced by a manual injection port. Gases which have greater distribution in the mobile phase will run through the column at a faster rate where it will be detected and then recorded by a computer (Sánchez-Rodas et al., 2010).

3.3.1. Components of a GC-MS system

There are several instrumental components of the GC system which are described by McNair and Miller (1998) and Schomburg, (1990):

1) Carrier gas

The carrier gas is an inert gas which does not react with the sample and has two purposes; to carry the sample throughout the column and provide a suitable matrix for the detector to measure the sample components. The type of gas used varies depending on the detector used. Thermal conductivity and flame ionisation detectors use helium, a flame ionisation whilst electron capture detector uses either nitrogen, argon or methane.

2) Flow Controls

Control and measurement of carrier gas is essential for qualitative analysis and it is essential to have a constant flow rate so that retention times of compounds can be reproduced in later experiments. A two stage regulator reduces the carrier gas pressure from 2,500 pounds per square inch (psi) to 20-60 psi before it enters into the column. The flow is measured by a soap bubble flow meter and a digital electronic flow measuring device. A soap bubble is raised into the path of flowing gas and one bubble is timed though a defined volume with a stopwatch and calculated in mL/min.

3) Sample inlets and sampling devices

The sample inlet (Fig.74) permits a wide variety of gases, liquids and solids to be rapidly and quantitatively introduced into the carrier gas stream, by instantaneous injection into the column. The smallest sample size should always be used to obtain the best peak shape, although there is no standard size that should be used. In most cases the presence of other components will not affect the position and shape of a given solute although if there are more components present in the sample, the sample volume may have to be larger. For samples with only trace amounts of solutes, larger sample sizes may be needed so that the peaks will be larger.

If the sample is large and the concentration of volatiles is expected to be high, the sample inlet can be set to split, so as to control the volume of air being sampled. If the sample is small and the concentration of MVOCs is expected to be low, then splitless injection would be used as to maximise the level of volatiles detected in the sample. Injection takes place after the needle is put though a self sealing silicone septum, which prevents contamination from the surrounding air.

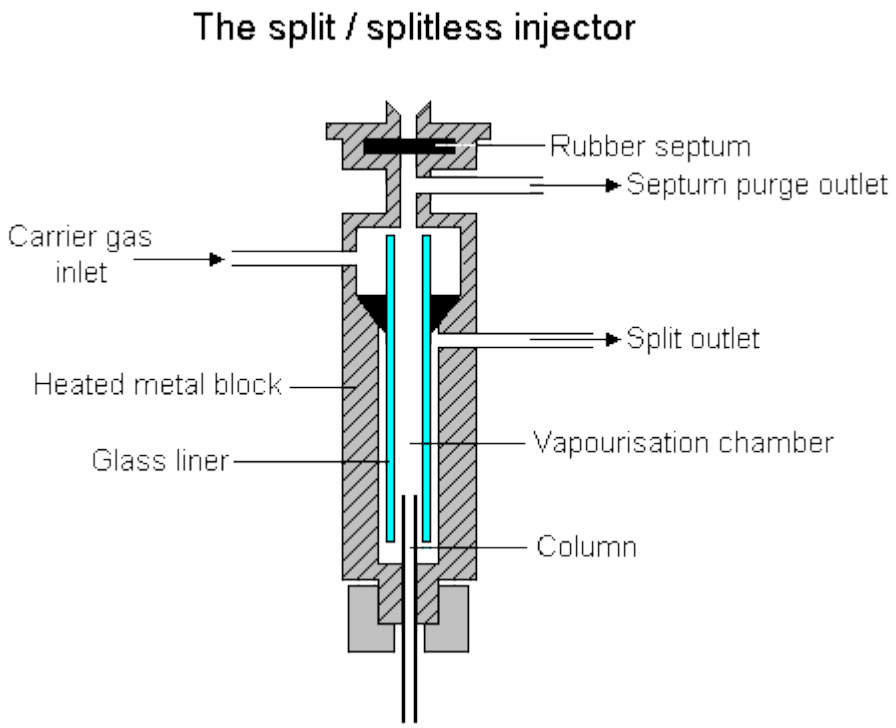

Fig.74. A split/ splitless inlet used to insert the sample into the column which is then mixed with the carrier gas (Anon. (Sheffield Hallam University), n.d.).

4) Columns

Columns are required for the separation of compounds. These are usually made of stainless steel due to its high strength (McNair and Miller, 1998). Two types of column are often used; packed columns and capillary columns (Schomburg, 1990). Packed columns are packed with porous support material based on diatomaceous earth, and coated with a thin film of silicon based liquid to prevent high polarity solutes being absorbed strongly (Schomburg, 1990). Capillary columns are fabricated from glass, preferably fused silica material (Schomburg, 1990) coated with a liquid stationary phase. The advantage of capillary columns over packed columns are they are easier to clean, as surfaces can be deactivated to prevent permanent absorption of VOCs. A disadvantage is that the peaks become distorted if the column is overloaded so samples with high concentrations must be diluted. Column temperature is extremely important as it will influence separation times and peaks of the components. The retention time of an analyte mainly depends on the boiling point of the sample, as GC programs are set to increase in temperature over time, and the time at which the column temperature reaches the boiling point of the sample corresponds to the point at which an analyte will be eluted from the needle. The GC column temperature should be kept above the dew point of the sample but not exceed the boiling point. The injection port temperature for insertion of the needle should be at least 50^{o}C hotter than the boiling point of the sample as to allow for flash vaporisation.

5) Detectors

Detectors sense the solutes in the column and output the results in the form of a chromatogram. There are three types of detectors with varying selectivity for compounds. A non-selective detector detects all components except the carrier gas, a selective detector will detect certain solutes and a specific detector will detect a single chemical compound. There are also two groups in which detectors are classified; concentration dependent detectors and mass flow dependent detectors. Concentration dependent detectors are the most probable detectors which will be used as they do not destroy the sample. A mass flow detector will usually destroy the sample and they are related to the rate at which the molecules enter the detector.

6) Data systems

GC columns produce peaks in real time so require a rapid accurate data system to read the signal sent by the detectors. These are computer based systems which calculate the start, end and apex of each peak of each solute and display the results via a graphical user

interface (GUI). Computer systems are flexible, reliable and can handle large amounts of data whilst taking into account various parameters.

All of these elements of the GC system make it a suitable method of detecting fungal MVOCs from cinematographic film and could enable archivists to determine if the film is contaminated without the aid of microbiologists to enable them to handle materials safely.

3.3.2. Headspace Analysis and Gas Sampling

In addition to the components of the GC-MS, instruments are needed to analyse samples in the gas phase. Analytes are extracted from the headspace of the sample and injected into the GC-MS system via the injector port for analysis. Two types of device for this purpose are; gas tight syringes to sample gas directly from the vials, and solid phase microextraction (SPME) needles which preconcentrate the samples, enabling detection of lower concentrations of MVOCs down to parts per billion (ppb).

3.3.2.1. Gas Tight Syringes

Gas tight syringes are the most commonly used pieces of equipment, which extract analytes from a needle accessible vial, in which the gases from the initial sample have been captured and concentrated. Upon insertion of the needle into the injection port on the GC, the carrier gas enters the needle and combines with the sample. The sample is then injected into the column though a flash vapouriser, and is best done quickly as a 'plug' of vapour to avoid detected analytes producing broad peaks on the GC spectrum (Anon. (Agilent Technologies, Inc), 2005). By using a gas syringe, the volume of gas extracted from the sample can be controlled. However, due to the low concentrations of MVOCs produced by fungi (ppb), extraction with a gas tight syringe may not be sensitive enough to detect analytes thus a more efficient method is needed.

3.3.2.2. Solid Phase Microextraction (SPME)

A SPME needle concentrates the volatiles onto the surface of a fused silica fibre by acting as a sponge during absorption and adsorption from the sample (Kataoka et al., 2000) (Fig.75). The needle is inserted through a self sealing rubber septum which acts as an airtight lid on a glass vial thus trapping the volatiles in the headspace. The fibre is exposed by pushing the plunger halfway down the manual holder. After a predetermined length of time during which the volatiles are adsorbed onto the fibre, the fibre is retracted and the needle is removed and injected into the GC-MS system for analysis of volatiles (Fig.76).

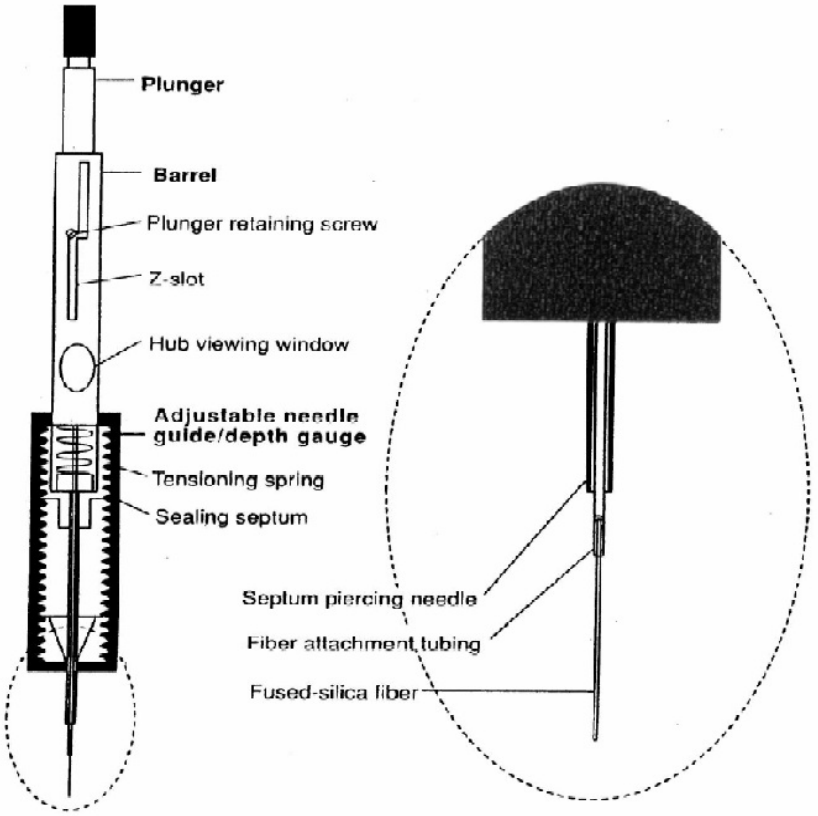

Fig.75. Commercial SPME fibre (Supelco, Dorset, UK). Once inserted into a GC vial, the plunger is pushed down to expose the fibre to the MVOCs. Once volatiles are absorbed the fibre is retracted and the needle is placed in the injector port of the GC-MS system (Kataoka et al., 2000).

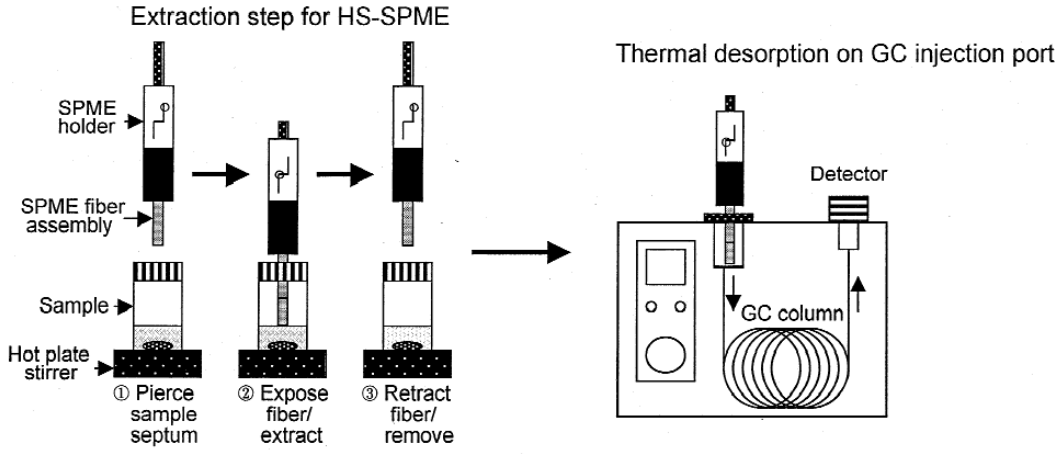

Fig.76. Extraction process of microbial volatile organic compounds by headspace analysis and SPME. The left hand side of the diagram shows the mechanism of extracting analytes using a SPME fibre (absorption), and the right side shows how compounds are injected into the GC-MS system (desorption) and analysed (Kataoka et al., 2000).

There are several coatings which can be used for extraction of VOCs, each having different properties such as thickness or pore size, which affects the affinity for different compounds. Volatile compounds have a higher affinity for coatings with a thicker coat as they are more sensitive than those with a thinner coat as a greater mass of analytes can be absorbed onto the coating. Polydimethylsiloxane (PDMS) can withstand high injection temperatures of up to 300^{o}C due to a backbone of Si-O-Si units rather than carbon which is contained in many other polymers. Silicon to silicon bonds also have far less energy than carbon-carbon bonds which also increases the stability of silicon polymers at higher temperatures. The bond lengths are also longer than other polymers such as polyethylene so the material is more flexible. (Anon. (The Titi Tudorancea Learning Center), n.d.), It is also non-polar and is often used for extraction of non-polar volatile flavour compounds (Kataoka et al., 2000), but can also be used to extract polar compounds after optimisation of extraction methods. Polyacrylate (PA) is a polar coating, so is used to extract analytes such as phenols and alcohols which polar compounds. Mixed fibre coatings containing divinylbenzene (DVB) and Carboxen (CAR: a porous activated carbon support) increase retention capacity of volatiles and can be used for extraction of volatiles with low molecular mass. CAR-PDMS coatings have been shown to have better extraction efficiency than 100μm PDMS (Kataoka et al., 2000). MVOCs emitted from stationary phases (i.e. solids such as MEA) can be immobilised by non-bonding, bonding, partially cross linking or high cross linking. There are currently seven types of fibres commercially available (Fig.77) with 50/30 DVB/CAR/PDMS having the strongest retention.

The use of SPME is deemed to be more suitable than a gas syringe for analysis of MVOCs produced by fungi, as they are expected to be emitted in low concentrations of parts per million (ppm).

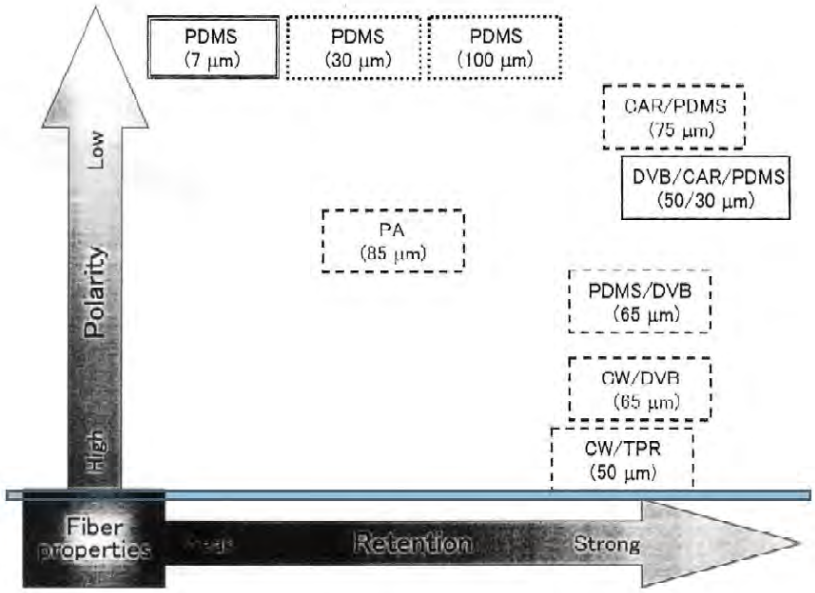

Fig.77. Properties of commercially viable SPME fibres indicating the strength of polarity and retention to molecules (Kataoka et al., 2000).

There are however, limitations to SPME and issues that need to be addressed before work is carried out (Hübschmann, 2008.). A worn septum in the injection port could result in the leakage of carrier gas and analytes, which subsequently means that the any compounds which may have leaked out will not be recorded. If oxygen enters the column through a damaged septum, it may react with the compounds which will then be recorded instead of the original compounds in the vials. Residual impurities and products may remain in the GC column after a previous sample has been run which will be detected in subsequent samples by resulting in a peak. To prevent this from happening the system is 'conditioned' by running a program without a sample being inserted. Any compound which is not from the column or SPME fibre is a residual compound and conditioning the system will elute these compounds. The injection port temperature needs to be calibrated according to which analytes are expected to be detected and the temperature should be high enough to instantly vaporise a liquid specimen. If the temperature is too low, there may not be any peaks resulting or peaks may be broad due to poor separation of compounds. Alternatively, if the temperature is too high in the injection port the compounds could decompose or have structural changes which would result in detection of compounds that were not present in the original sample. Also the time at which the sample is injected into the column affects retention time. For example, if the program is allowed to run for a few seconds before injection, then the retention time of the peak will be a few seconds later resulting in the recording of a slightly different time.

One major disadvantage of SPME is variability of peak sizes even at pre-determined concentrations of samples. It is expected that the concentration of a compound in a sample will produce a peak size proportional to the amount present. Running standard samples through the GC instruments with a gas syringe enables determination of peak sizes at various concentrations. However, for SPME internal standards are not used and SPME is generally not considered suitable for quantification of compounds, due to high variability between each sampling (Stadelmann, 2001). Another factor resulting in variability could be variability of instruments. Thus, the instrument's make, model, serial number, injection temperature, column temperature, carrier gas flow rates and pressure and detector type used should all be noted in the recording of results.

3.4. Competition Technologies

3.4.1. Detecting Chemical Deterioration of Cinematographic Film

Currently there is only one technology used to detect and measure the extent of cellulose acetate film degradation. This comprises of 'A-D Strips' priced at approximately £40 for 250 strips (Anon. (Academy of Motion Picture Arts and Science), n.d.) and manufactured by the Image Permanence Institute (IPI). Each strip contains an acid-base indicator that changes colour in the presence of acid vapour given off by the film during a chemical deterioration process of safety film known as 'vinegar syndrome'. The presence of acetic acid is caused by the breakdown of acetate to acetic acid, which causes the film to shrink, crumple and emit a strong vinegar odour (Anon. (Image Permanence Institute (B), n.d.). The strips measure about 38mm x 10mm and on colour change are compared to a 'reference pencil'. This pencil is printed with four bands of colour which are numbered 0-3 and correspond to four levels of acidity which are equivalent to approximate measurements of acid vapours in parts per million (ppmv= ml/m³); 0 = blue-green (1-2ppm), 1= green (3-5ppm), 2= yellow-green (6-8ppm) and 3=yellow (18-20ppm). The paper strips are placed into film cans for 24 hours and the colour is recorded by the archivists, thus indicating how far the deterioration has progressed and whether or not the storage conditions are adequate to preserve film reels. How quickly deterioration occurs depends on RH and temperature, and the rate at which this occurs increases rapidly after it has reached the autocatalytic point (Fig.78). The effects on the film indicated by each level of A-D colour change are described and recommended actions for storing the film are provided (Fig.79).

A-D strips are part of the environmental monitoring process which archives use to preserve materials. As stated previously, these strips are the only 'kit' used to indicate film deterioration. They do not give any indication of biological deterioration due to mould growth but are essentially pH indicators. Thus, there is clearly potential for the development of a simple kit to indicate the presence of viable fungal growth on archival film.

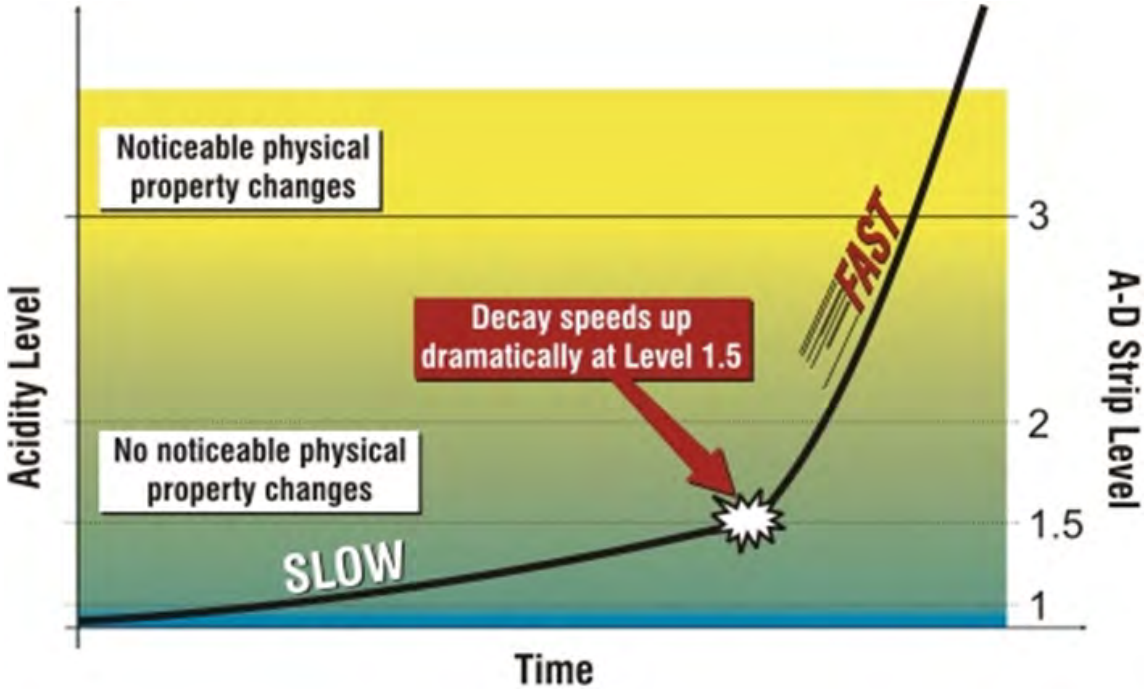

Fig.78. The correlation of the A-D strip levels and the acidity level of film. A rapid increase in deterioration happens after the film has reached the autocatalytic level of 1.5 (Anon. (Image Permanence Institute (A)), n.d.).

A-D Strip Level	Film Condition	Recommended Actions
0	Good—no deterioration	Cool or cold storage
1	Fair to Good—deterioration starting	Cold storage Monitor closely
1.5	Rapid decay starting—point of autocatalytic decay	Cold or frozen storage
2	Poor—actively degrading	Freeze Copying advisable
3	Critical—shrinkage and warping imminent, possible handling hazard	Freeze immediately Copy

Fig.79. Reference Pencil colours of A-D strips, describing the condition of film at each level of acetic acid and the recommended actions (Anon. (Image Permanence Institute (A)), n.d.).

3.4.2. Sensors and Transducers

There are some technologies currently available to detect VOCs that may be applicable or adaptable to detect fungi on cinematographic film. Similarly to SPME-GC-MS, the two main components of electronic sensors are the sensing system and the automated pattern recognition system.

Sensors are made of two basic functional units: a receptor part and a transducer part (Hulanicki et al., 1991). A receptor comes into contact with the analyte and transforms the chemical information into a form of energy which may be measured by the transducer. Signal transducers are required to transform one energy type to another for analysis i.e. changing the analyte on the receptor into electrical energy, so that the compound can be displayed digitally on a recorder such as a computer screen or hand held device (Hulanicki et al., 1991). Sensors are classified according to the principle of the transducer (Hulanicki et al., 1991) and several transducers are described by Mahmoudi (2009),which fall into four major classes: electrochemical, optical, thermal, and mass change e.g. piezoelectric (Mahmoodi, 2009 and Rocha-Gaso et al., 2009) (Table 12). Transducers can be combined to form an array of sensors or a single sensing device which can detect many types of MVOCs (Berna, 2010).

Volatile organic compounds (VOCs) coming into contact with the sensor array produces a signature or pattern, characteristic of that particular volatile compound. By subjecting the sensors and recognition system to different pure chemicals, a database of chemical signatures can then be created which will then ‘recognise’ the compounds should it be detected by the sensors again. Data analysis can then be performed using computer software and a visual display unit.

In order to develop a sensor for detecting mould growth on cinematographic film, it is necessary to discuss the range of sensor types currently available and some examples of their applications.

Table 12. Examples of transducers used in biosensor development (Mahmoudi, 2009).

Category	Principle	Examples
Electrochemical	(a) Potentiometric: dependents on changes in potential of a system at a constant current. (b) Amperometric: detects changes in current as a function of concentration of electroactive species.	Ion selective electrodes, ion selective field effect transistors. Solid electrolyte gas sensors, electronic noses.
Piezoelectric	Sensitive to changes in mass, density, viscosity and acoustic coupling phenomena.	Surface acoustic wave sensors.
Optical	Link changes in light intensity to changes in mass or concentration, therefore, fluorescent or colorimetric molecules must be present.	Optical fibres, surface plasmon resonance, absorbance luminescence.
Thermal	Detect changes in temperature.	Calorimetric sensors.

3.4.2.1. Electrochemical Sensors

Electrochemical sensors are based on ion selective electrodes which measure a change in ion concentration in a reaction (Mahmoudi, 2009). There are three main types of ion selective electrodes which are used in biosensors: normal glass pH electrodes, glass pH electrodes coated with a selective gas-permeable membrane, and solid-state electrodes consisting of a thin membrane of a specific ion conductor. There are two main types of electrochemical sensors: metal oxide sensors and conducting polymer sensors.

1) Metal Oxide Semiconductor (MOS) Sensors

Metal oxide semiconductors measure a charge on a surface by causing a current flow proportionate to the charge. MOS sensors are sensitive to gases (Berna, 2010) and gas samples are sensed by the change in the electrical resistance of the metal oxide semi-conductor' (Berna, 2010). Combustion reactions with the lattice oxygen species on the metal oxide particles on the sensor surface result in resistance changes, which presents as a peak on the analysis system, which will be bigger if the concentration of a compound is higher. MOS sensors are widely available commercially making them more heavily used than any other types of gas sensors (Schaller et al., 1998). Although there are many metal oxides which show sensitivity to gas, tin dioxide (SnO_2) combined with a catalyst such as platinum has been shown to be the most useful for a range of applications but zinc oxide (ZnO), titanium dioxide (TiO_2) and tungsten oxide (WO_3) gas sensors are also available. As well as the type of metal oxide in the sensor, the metal oxide film deposition is also important in terms of sensitivity and system performance (Kanan et al., 2009). The method of deposition of metal oxides on the film can include several techniques: 1) physical deposition such as sputtering (Mattox, 1998), 2) chemical vapour deposition- the substrate is placed inside a reactor and a chemical reaction occurs. The material then condenses on the substrate (Bishop, 2011), 3) electrodeposition- when two substrates are placed in a liquid electrolyte solution and application of a conducting electric current results in a formation of a layer of material (Fujita et al., 2006) and 4) physical application, specifically spraying for thin films (6-1000 nm) and screen printing and painting for thick films (10-300 μm) (Berna, 2010). Schaller et al. (1998) notes that thin films are more difficult to manufacture but offer significantly higher sensitivities than thick films. It is due to these difficulties with manufacture that means most commercially available MOS sensors are often based on thick film technologies.

There is evidence to show that metal oxide based 'electronic noses' have been able to differentiate between different species of mould on the basis of different VOCs (Kuske

et al., 2006). Such systems have already been used in the food industry to assess the freshness of sardines under cold storage (El-Barbri et al., 2007) and to test end product quality of meat loaf by the use of a Danish odour sensor system (DOSS) equipped with six metal oxide sensors (Modified Figaro 2000 series) (Hansen et al., 2005). An MOS sensor called FreshSense (IFL and Bodvaki-Maritech, Ko´pavogur, Iceland) has also been used to detect flavour characterisation of ripened cod roe: several volatile organic compounds including 1-octen-3-ol were detected (Jonsdottir et al., 2004) which has been noted as a marker of fungal growth (Fiedler et al., 2001). A Fox 3000 (Alpha M.O.S, Toulouse, France) has been used on treated wastewater to monitor and control water quality (Dewettinck et al., 2000), and also to monitor 'off'-flavours of alcoholic beverages such as 1-octen-3-ol (Regazzo-Sanchez et al., 2009). MOS sensors have also been used for detection of MVOCs of pathogenic bacteria in blood, serum, urine or food (Ivnitski et al., 1999), and also for detection of fungal toxins (aflatoxins) from contaminated milk (Paniel et al., 2010). A small device with 16 nanosensors which attaches to an iPhone has also been developed by NASA to detect substances such as ammonia, chlorine gas and methane (Glenn, 2012).

2) Conducting Polymer Sensor

Conducting polymer sensors are similar to MOS sensors in function however, instead of using metal oxides they use polymers which conduct electricity such as polypyrrole (Ppy), polyanaline (Pani) or polythiophene (PTh) (Bai and Shi, 2007). Like MOS sensors, the intensity and magnitude of the signal is usually representative of the concentration of the compound in the sample. Reference molecules such as pure compounds can be added at the same time as the substance to be analysed to improve accuracy. Conducting polymers for biosensing have a sensing element connected to a transducer which can recognise a physiochemical change (i.e. presence of a particular compound) which can then be detected on a processing unit (Dai et al., 2002). Another example of a conducting polymer gas sensor is the AromaScan A32S (Osmetech Plc, UK) which has been shown to identify unknown volatiles from wood decay more effectively than a PEN3 metal oxide sensor (Baietto et al., 2010).

Conducting polymer sensors have been used to detect *Alternaria* sp., *Botrytis cinerea* and *Celleotrichum gleosporioides* which cause blueberry fruit disease (Li et al., 2010), thus may be suitable for use in detection of MVOCs from fungi isolated from cinematographic film.

3.4.2.2. Polymer Coated Surface Acoustic Wave (SAW) Microsensors (Piezoelectric crystal sensors)

These devices are sensitive to changes in mass, density, viscosity and acoustics. 'As the acoustic wave propagates through or on the surface of the material, the velocity and /or amplitude of the wave are changed' (Mahmoudi, 2009). Changes in the velocity can then be measured using a sensor and signal processor in much the same way as a GC device. Substrates for piezoelectric devices include quartz, lithium tantalite, lithium niobate, silicon carbide and gallium arsenide. A surface acoustic wave (SAW) microsensor has much the same features of a GC although in a miniaturised form thus making it portable. In SAW microsensors, scrubbed ambient air is used as a carrier gas (Zhong, 2008). They also contain a miniature multiadsorbant preconcentrator, a column, a separation module for the analytes and an array of SAW microprocessors which analyses the VOCs by producing a characteristic fingerprint of each analyte. Uses for piezoelectric sensors are in the food industry with quartz crystal microbalance sensors being used to detect olive oil volatile compounds such as 1-octen-3-ol (Escuderos et al., 2011), and also for detection of MVOCs from pathogens (Rocha-Gaso et al., 2009).

3.4.2.3. Optical Sensors

Optical biosensors use long thin strands of pure glass and are usually based on luminescence, fluorescence and absorbance (Mahmoudi, 2009) via the production of electromagnetic radiation. They measure light radiation when an analyte is excited by thermal, electrical, or radiant energy.

There two types of optical sensors: 1) spectrophotometric sensors which are based on light absorption (e.g ultraviolet (UV)/ultraviolet visible (UV-VIS) and infra-red (IR) and 2) flourescence sensors which are based on light emission e.g. spectrofluorimeters and Ramen spectroscopy (Meurens, 2003).

Absorption techniques rely on the gradual loss in intensity of a beam of light radiations due to partial absorption by a particular analyte (Meurens, 2003).

Ultraviolet- visible (UV-VIS) spectroscopy uses wavelengths of light which are absorbed by a molecule and this absorption depends upon how tightly its various electrons are bound. Molecules which contain unshared electrons such as sulphur, bromine and iodine and unsaturated organic functional groups have less strongly held bonds which are relatively easily excited by radiation, so absorb UV and produce absorption peaks, which are useful for quantitative analysis (Meurens, 2003). Detection of UV-VIS absorption is carried out using a UV spectrophotometer which contains specific components, sources

and detectors for each spectral region of light. UV- ion mobility spectrometry (IMS), which analyses the mobility of ions in a carrier gas, has been used by Ruzsanyi et al., (2003) to detect microbial volatile organic compounds from mouldy bread using radioactive ^{63}Ni as a ligand. The volatile compounds detected included 1-octen-3-ol, 3-octanone and 3-octanol. Similarly Tiebe et al. (2009) used this method to indicate hidden mould growth in the indoor environment.

Infra-red (IR) sensors work by detecting absorption of different characteristic frequencies (or wavelengths) of IR radiation by different compounds or functional groups (Sherman Hsu, 1997). IR spectroscopy has been used in the food industry to control food authenticity and traceability (Meurens, 2003) and has been used in several applications such as distinguishing fruit types in fruit purée (Fügel et al., 2005), authentication of coffee bean varieties and detecting impurities in vegetable oils (adulteration) (Meurens, 2003)

In contrast to absorption techniques, fluorescence techniques analyse light emitted by electrons of the illuminated molecules of a particular analyte when they are excited by photons of light and has a high sensitivity and good selectivity for analytes thus is useful for quantitative work (Meurens, 2003). Compounds produce peaks of fluorescence intensity at wavelengths which are specific to that particular compound. Fluorescence spectroscopy has been used in the food industry to detect poultry skin used as a low cost filler in processed poultry products by measuring fluorescence of collagen (Swatland and Barbut, 1991), and to detect geographic origins of milk (Karoui et al., 2005),

3.4.3. Electronic Noses

Electronic noses (e-noses) operate in a similar principle to the human nose (Schaller et al., 1998) They comprise of a sensor array and pattern recognition software which compares the VOCs to a database of known compounds. Three elements are present: a sampling conditioning unit which isolates MVOCs from the headspace of the sample, a test chamber containing a sensor array, and a processing unit which analyses the data provided by the sensor array. They have advantages over GC for use in mobile applications, as they are often smaller thus making them portable, have direct detection, a fast response and are simple to use (Canhoto, 2005). There are several E-nose sensors commercially available (Table 12). E-nose technology does have some drawbacks. Many substances emitted from different sources can interfere with analysis (Canhoto, 2005), and thermal desorbers used to pre-concentrate volatiles (i.e. a SPME equivalent) are not currently commercially available for e-nose technology.

Table 13. Examples of commercially available electronic noses.

Reference	Manufacturer	Origin	Number of Sensors/ Sensor Type	Size of Instrument
A	Airsense analysis GmbH	Germany	10, MOS[1]	Laptop
A	Alpha MOS-Multi Organoleptic system	France	6-24, CP[2]. MOS. QCM[3]. SAW[4]	Desktop
A	AromaScan PLC	UK	32, CP	Desktop
A	Bloodhound Sensors Ltd.	UK	14, CP	Laptop
A	Cyranose,Cyrano Science Inc.	USA	32, CP	Palmtop
A	EEV Ltd. Chemical Sensor Systems	UK	8-28, CP. MOS. QCM. SAW	Desktop
A	Electronic Sensor technology Inc.	USA	1, GC[5]column. SAW	Desktop
A	Hewlett-Packard Co.	USA	QMS[6]	Desktop
A	HKR-Sensorsysteme GmbH	Germany	6, QCM	Desktop
A	Lennartz Electronic GmbH	Germany	16-40. MOS. QCM	Desktop
A	Nordic Sensor Technologies AB	Sweden	22, IR[7]. MOS. QCM	Laptop
A	Sawtek. Inc.	USA	2, SAW	Palmtop
B	MOSES II, Lenmartz Electronic	Germany	8 MOS, 8 QCM, 4 AGS[8]	Desktop
B	Fox, 2000, Alpha MOS	France	6 MOS	Desktop
B	Airsense Portable E-Nose (PEN2), Airsense Analytical	Germany	10 MOS	Portable Desktop
B	FreshSense, Bodvaki Maritech	Iceland	4 electrochemical for: CO, H_2, SO_2, NH_3	Unknown
B	zNose, Electronic Sensor Technology	USA	1 SAW	Palmtop/ Portable Desktop

Key to sensor types: 1. Metal oxide semiconductor. 2. Conducting polymer. 3. Quartz crystal microbalance. 4. Surface acoustic wave. 5. Gas Chromotography. 6. Quadrupole mass spectrometry. 7. Infrared. 8. Gas Sensors.

Key to references: A) Canhoto, 2005. B) Casalinuovo et al., 2006

3.4.3.1. Applications of Electronic Noses

There is an extensive range of applications for e-nose technology (Mahmoudi, 2009). These range from environmental monitoring (Canhoto, 2005 and Dewettinck et al., 2000), food science (Schaller et al., 1998, Casalinuovo et al., 2006 and Paolesse et al., 2006), medical applications (Pavlou et al., 2000) and detection of mould (Kuske et al., 2005). Electronic nose technology shows future potential in the detection of different microbial species because some MVOCs are specific to a certain species of group of organisms such as fungi and bacteria. Linking this technology to sensors which provide data in real time could enable early detection of the growth of microorganisms without the need for culture for example in the early detection of disease (Sahgal, 2008).

1) Medicine

Infection with microorganisms can often result in the emission of volatile odours which have the potential for detection with GC technology (Mahmoudi, 2009). Biosensors have the potential for use in disease diagnosis. Analysis of volatile compounds has been used to investigate human disease. It has been suggested that the detection of certain compounds from breath could be indicative of diseases such as diabetes (Boots et al., 2012 and Tanaka et al., 2004), and specific MVOCs specific to a particular bacterial genus could help in determining the cause of an infection without the need for culture (Zhu et al., 2010), which could enable more specific antibiotics to be used for treatment.

2) Food Industry

Currently the biggest area for development of electronic nose technology is the food industry (Mahmoudi, 2009). Detection of volatile odour compounds can be used for example, to detect the ripeness of fruit (Salim et al., 2005), the maturity of cheese (Trihaas et al., 2005) or whether a food such as fish is ‘off’ (Jonsdottir et al., 2004 and El-Barbri et al., 2007). This may replace or supplement the current use of human sensory panels and if data are provided in real time could reduce food wastage. For example by measuring MVOCs emitted from fruits as they begin to ripen and start to rot on supermarket shelves, foods can be rotated better reducing the amount that is discarded (von Radowitz, 2010).

3) Environmental Monitoring

There are also many ways in which e-nose technology could be used for environmental monitoring. These could include monitoring of wastewater (Canhoto and Magan, 2003) and importantly in the detection of mould. E-noses have been used to detect fungi. Kuske

et al. (2005) used e-noses to detect fungi in indoor environments. Visual inspection is the usual method to look for mould growth but this can a problem if the fungi are growing in hidden areas such as under surface wall coatings like paint, or in wall cavities. Hidden mould growth could still cause irritations in humans (Pasanen et al., 1998). E-nose technology could potentially be used to detect MVOCs before mould growth is apparent. This would clearly be useful in an archive environment as it would help prevent subsequent contamination and deterioration of material. There have also been attempts to use this technology to detect change on specific substrates. Canhoto et al. (2004) investigated the application of electronic nose technology to detect fungal VOCs on mould contaminated library paper. E-nose technology has also been used to test for chemical degradation of books by examining the VOCs released during the production of acid as the paper oxidises and deteriorates over time (Gill, 2009). There has been growing interest in work space monitoring (i.e. monitoring the air of the work environment) and the detection of aromatic and halogenated hydrocarbons (Dickert et al., 1999) from fungi in museums in archives. This could help prevent biodeterioration of materials by removing the problem once detected and protecting the archive material and health of handlers should high concentrations of these volatiles be detected. Results showed that different fungi produce slightly different volatiles in the headspace and Schiffman et al. (2000) found that e-nose technology could be used to detect and differentiate the three fungi tested, *Aspergillus niger*, *A.flavus* and *Penicillium chrysogenum*. However, for film, distinguishing between the species of fungi present on contaminated film may not always be necessary. It is necessary only to know whether the mould is viable/ unviable and whether or not it is actively growing, as active growth could damage the film further and lead to contamination of other materials.

3.4.3.2. Examples of commercial E-nose Sensors

1) Flexsmell

The FlexSmell project is a collaboration between many European universities and organisations to develop sensors which could sense for food freshness and quality traceability by detecting VOCs released from food (Anon. (FlexSmell), 2009). The sensors measure temperature, humidity and VOCs to enable detection of produce quality, and send the information wirelessly by radio signals. Poentially, this same technology could be used to detect MVOCs from mouldy cinematographic films and remote monitoring could be carried out in much the same way as currently happens in archives to measure temperature and humidity.

2) Olfactory Detector Outlet 'Sniffer Unit' – ODO II

The Olfactory Detector Outlet 'Sniffer Unit' – ODO II (Anon. (SGE), n.d.) is an olfactory system which can be coupled with a GC system and linked to a mass selective detector, which is designed to separate individual compounds from a complex mixture, for example essential oils, and transfer them through a nose cone through which the human nose can smell each component separately. The smell of each component is detected at the same time by the nose and GC so that the peak is produced on the GC. This is done by introducing a carrier gas at the exact point that the column flow is split between the GC and the nose cone olfactory detector. The air passing through the cone is humidified to prevent nasal membranes from drying out. The nose cone is an attachment which can be fitted to all commercially available GCs (Fig.80) and which can detect a range of volatile organic compounds such as 1-octen-3-ol, 3-octanone and 3-octanol so has a potential use for detecting MVOCs from mould on film.

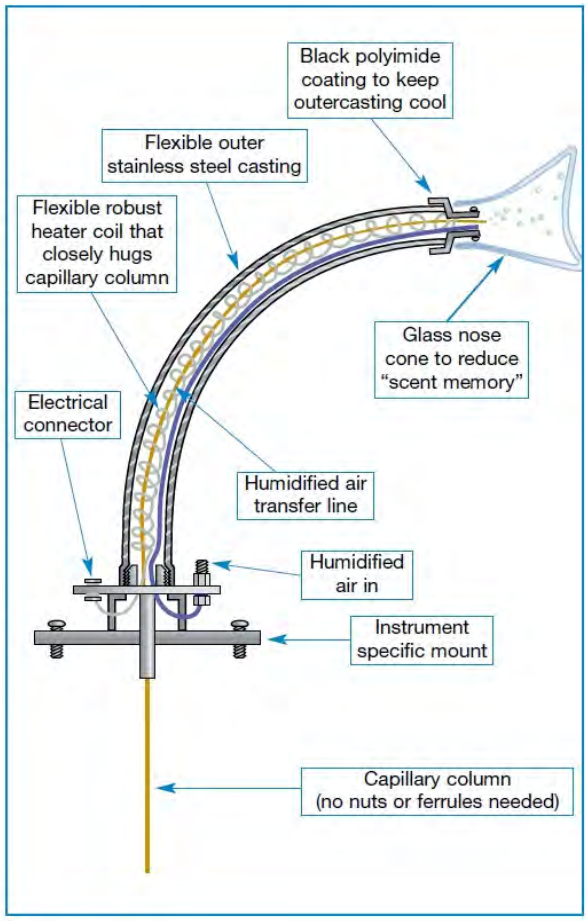

Fig.80. The Olfactory Detector Outlet 'Sniffer Unit' – ODO II (Anon. (SGE), n.d.).

3) Bloodhound Sensors

Keshri et al., (1998) investigated the use of a Bloodhound e-nose sensor (Bloodhound Sensors Ltd, Leeds, UK) to determine if the e-nose could provide early detection and differentiation of spoilage fungi by using the dominant odour volatiles; 3-methyl-1-butanol, 1-octen-3-ol and 3-octanone. This sensor incorporated 14 polymer sensors and it was discovered that whilst the sensor could potentially differentiate between the 5 fungi tested (*Eurotium rubrum*, *E.chevalleri*, *E.herbariorum*, *E.amsteoldami*, *Penicillium* sp, and *Wallemia sebi*), it could only detect volatiles produced at the later stages of growth, so was ineffective as an early detection system. This same system was used by Gibson et al. (2000) to detect disease causing bacteria in blood cultures and found that it was possible to differentiate between different species.

4) Moses II (MOdular SEnsor System)

The Moses II (Lennartz Electronic/MoTech) uses two arrays of tin oxide and quartz microbalance sensors and has been used to discriminate between different pollutants in water (e.g. pesticides, insecticides) and contaminants from industry (e.g. phenols, nitrobenzene and anilines) (Baby et al., 2000). This system can distinguish between compounds even at very low concentrations (1-500 ppm) possibly suggesting use for analysis of volatiles from fungi.

5) e-Nose 4000 (Neotronics)

The e-Nose 4000 has been used by Pinzari et al., (2004) to detect MVOCs in libraries and archives to assess its use as an early detection device for mould growth. This system uses a polypyrolle conducting polymer sensor array and was able to differentiate between different relative humidities of paper caused by three different species of mould.

6) AML AutoNose

The AML AutoNose (Anon. (Autonose Manufacturing Ltd), 2011) uses computer software called 'SenseLAB', to identify VOCs in 20-30 seconds. The gas in the headspace of a sample is analysed through a live RS232 serial interface to the E-Nose which is then connected to a laptop and used to analyse the data. This E-Nose uses an array of different sensors including conducting polymer, MOS and SAW sensors, to detect VOCs and display the information on the SenseLAB program. Applications include quality control in food processing, detection of bacterial infection (e.g. urinary tract, wound and chest infections) and detection of microbial contamination in industry e.g. paints, textiles and printing inks.

3.5. Criteria for Sensor Development

The information in the literature suggests that several MVOCs will have to be targets for future detectors of fungal growth on cinematographic film. These will have to be general indicator compounds and cover a wide range of common fungal genera as no single compound is unique to all fungi although, there are some which are common to many. Archivists would require a sensor that is simple to use, reproducible, and produces data which are easy to read and interpret without the need for specialist operators. Thus contaminated films could be stored appropriately to avoid cross contamination of non-contaminated films. Exposure to hazardous MVOCs which potentially pose a health risk would also be minimised. From analysis of the literature, three compounds appear to be frequently detected from various fungi: 1-octen-3-ol, 3-octanone and 3-octanol, thus these were considered to be good candidates for general markers of fungal growth. Methods were developed using SPME coupled with GC-MS to try to detect these compounds.

3.6. Methods I: SPME Analysis of Fungal Isolates

3.6.1. Selection of Fungal Isolates for SPME Analysis

A range of isolates previously obtained from contaminated film reels (n=16) were selected if enzyme assays had shown them to have a relatively high gelatinolytic and cellulolytic activity and, if relatively high numbers of spores had been isolated by air sampling compared with other isolates. Other isolates which were not from film reels were selected to enable a wider range of fungi to be investigated. Isolates found on films, were labelled with the film reel reference number followed by the isolate number. For example, isolates labelled with RR1399 I(x) (x= number allocated to the isolate) came from the RR1399 cellulose acetate film reel. S1 was a *Trichoderma* spp. isolated from a swab of a cellulose nitrate film provided from the British Film Institute (BFI) which had been placed in sterile water and 100µl spread plated onto MEA. The *Penicillium expansum, P.chrysogenum* and *Aspergillus versicolor* ATCC11730 were lab strains used for comparison to work in the literature such as Fiedler et al. (2001). The species *A.versicolor,* had also been isolated from cinefilm in previous work carried out at MMU and was shown to have a high gelatinolytic and cellulolytic activity. The *Cladosporium* isolate came from air sampling of cellulose nitrate vaults in BFI Gaydon. The JWP1 *Alternaria* spp. was isolated from a piece of mouldy wallpaper which had been donated at the preliminary stages of this study. In addition to the range of isolates, various substrates were also analysed (Table 14).

Table 14. Key to the samples used. For simplicity, numbers were allocated to a specific substrate and the letters were to each mould tested.

Key				
Substrate/ Sample			**Fungal Isolates**	
0	Blank		A	Mixed Contamination
1	Malt Extract Agar		B	*A.versicolor* ATCC11730
2	Malt Extract Broth + Gelatine		C	*P.expansum*
3	Test Film		D	*Cladosporium* sp.
4	Mouldy Film RR1093		E	*P.chrysogenum*
5	Inoculated Test Film		F	SI1 (*Trichoderma*)
			G	RR1399 I1 (*A.versicolor*)
			H	RR1399 I2 (*A.versicolor*)
			I	RR1399 I3 (*A.versicolor*)
			J	RR1399 I4 (*A.versicolor*)
			K	RR1399 I5 (*P.brevicompactum*)
			L	RR1399 I6 (*A.versicolor*)
			M	RR1491 F2 I4 (*Stachybotrys chartarum*)
			N	JWP1 (*Alternaria sp.*)
			O	RR1093 I1 (*P.chrysogenum*)
			P	RR1093 I2 (*P.brevicompactum*)
			Q	RR1093 I3 (*P.citreonigrum*)

3.6.2. Culture of Fungal Isolates on Different Media

Studies have shown that growth on MEA enables fungi to emit a high variety of volatiles (Scotter et al., 2005), therefore it was deemed to be a suitable substrate for investigating volatiles from fungal isolates found on cinematographic film. Molten MEA was dispensed into 8mL volumes in autoclaved 20 mL glass gas chromatography (GC) vials, which were placed at angles during cooling to produce agar slopes**.** Sterile loops were then used to inoculate the slopes with spores scraped from the surface of cultures that were maintained on MEA. The vials were then covered with aluminium foil to prevent contamination and enable growth and initially incubated for 3 days at 25^{o}C after which an airtight aluminium cap with a self sealing rubber septum was placed on the top of the vials (Fig.81). These were further incubated for 2 more days to enable accumulation of any MVOCs in the headspace during this additional growth time. Malt extract broth (MEB, Oxoid) with added gelatine (2g MEB 3g gelatine per 100 mL)was also used for comparison with MEA. Fifty millilitre volumes were placed in conical flasks and inoculated with a plug of MEA fungal cultures cut with an 8mm cork borer. These were incubated at 30^{o}C in shake culture at 150rpm for 10 days, as it was observed in preliminary experiments that broth cultures needed a longer time to grow compared with those on solid media. After this time, 10 mL of each broth culture was transferred to empty 20mL glass vials (n=2) and each was sealed with an airtight aluminium cap. In addition, strips of mouldy film were also sampled so sampling of minimal medium with added gelatine was not required. A sample of contaminated film RR1093 (measuring 2cm by 1cm) which was covered in mould previously shown to be non-viable, and clean 'test film' (2cm x 1cm) were cut off the main reels and placed directly into empty glass vials to investigate any volatiles emitted from the samples. A piece of test film was inoculated with mould by placing the film strips on MEA plates and inoculating them with 100µl of a mixed culture containing approx 3.89 x 10^{6} spores suspension of *Aspergillus versicolor* ATCC11730 and *Trichoderma* sp. (S1) (Table 14). These were incubated for 7 days at 25^{o}C after which a fungal lawn covered the medium and had grown onto the surface of the film as well as into the emulsion layer, confirmed by visualisation of hyphae under the light microscope (x10). The film was then removed from the layer using sterile tweezers and placed in a vial with an aluminium cap placed on top to seal in any volatiles which may have been produced. This was left for a further 2 days to allow diffusion of volatiles into the headspace which could then be sampled to see if active mould emitted volatiles when growing on film.

After 2 additional days SPME was performed again on all the samples to allow repeats and see if any additional volatiles were present in the headspace.

Fig.81. Vials containing samples for use with the Gas chromatography- Mass spectrometry for Solid Phase Micro Extraction- Head Space (SPME) Analysis. The three vials on the left contain fungal cultures grown on MEA slopes. The vial in the middle is un-inoculated MEA only and the three on the right are fungal cultures which have been grown in broth culture (MEA and gelatine) and transferred into the vials. Film strips in the GC vials are not included.

3.6.3. Solid phase microextraction (SPME) analysis

Vials were labelled with the name of the fungal species and the date (n = 2 for each species), then the aluminium cap was secured on, thus ensuring sampling was carried out on cultures of a known age. Vials containing the cultures were placed in water baths at 35^{o}C to equilibrate volatiles in the headspace. These were then subjected to SPME headspace analysis using a 50/30 divinylbenzene/carboxen on a polydimethylsiloxane bonded to a fused silica core (Supelco, Dorset, UK). Negative controls were: i) running the fibre through the GC-MS system without exposure to VOCs; ii) exposing the fibre to a piece of clean test film for comparison to both the inoculated and contaminated film; iii) un-inoculated MEA and un-inoculated MEB + gelatine were also recorded to determine which were emitted from the fungal isolates and which were from the different media.

The SPME needle was conditioned before use by inserting the needle into the GC-MS sampler and running the program in order to remove any volatile compounds absorbed on the fibre from previous experiments. The SPME needle was inserted into the vial through the self-sealing rubber septum into the headspace and was exposed to the VOCs (adsorption time). The SPME fibre was directly desorbed into the injector port on an Agilent 6850 series gas chromatography system coupled to an Agilent 5973 mass spectrometer which separated the headspace volatiles on a HP column (25m x 0.2mm, 0.5 um) coated with a 5% diphenyl – 95% polymethylsiloxane copolymer. Volatiles were then analysed using the Agilent Instrument 1 Enhanced ChemStation for Microsoft Operating systems.

3.6.4. GC Programs

Three programs were tested on the Agilent 6850 to investigate which was the most effective at separating and analysing MVOCs.

1) These settings were based on work by Demyttenaere et al. (2004). The absorption time was set at 15 minutes. The injector temperature was set at 250°C and the detector transfer line temperature was set at 280°C with a carrier gas (helium) flow rate of 1mL/min^{-1}. The initial temperature was maintained at 40°C for 2 minutes then increased (ramped) to 200°C at a rate of 10°C/ min^{-1} then from 200°C to 250°C at a rate of 15°C per minute and maintained at this temperature for 5 minutes. The total run time was 32 minutes.

2) These setting were based on previous work from MMU (unpublished) which were used to detect MVOCs from *Candida* spp. The absorption time was set at 15 minutes. The injector temperature was set at 250°C and the detector transfer temperature was set at 280°C with a carrier gas (helium) flow rate of 1 mL/min^{-1}. The initial temperature was maintained at 40°C for 2 minutes then ramped to 110°C at a rate of 5°C min^{-1} then from 110°C to 280°C at a rate of 10°C min^{-1} and maintained at this temperature for 5 minutes. Total run time was 48 minutes.

3) The settings were based on work by Fiedler et al. (2001). Absorption time was 15 minutes. The injector temperature was set at 220°C and the detector transfer line was 280°C with a carrier gas (helium) flow rate of 1 mL/min^{-1}. The initial temperature was 40°C for 7 minutes then ramped to 220°C at a rate of 10°C min^{-1} and then maintained at this temperature for 15 minutes. Total run time was 40 minutes.

For all programs, peaks of VOCs which were over 50% match quality (previously determined) on the Agilent Instrument 1 Enhanced ChemStation were recorded (a higher quality indicates a more probable identification of the volatiles queried). However, the fungal marker compounds 1-octen-3-ol, 3-octanone and 3-octanol, were recorded if a peak was produced but the quality was less than 50% because these were the target compounds. Retention time was confirmed by initially looking at the compounds profiles on the GC spectrum from multiple samples and later using chemical standards.

In addition, two fibre coatings were initially selected for comparison: 100µm PDMS (due to usage in previous studies of MVOCs from fungi) and 50/30 DVB/CAR/PDMS (Supelco, Dorset, UK).

3.7. Results I: Detected MVOCs

3.7.1. Detection of MVOCs from Fungal Isolates

After preliminary investigations of the 3 different G-C programs, it was ascertained that program 3 coupled with a 50/30 CAR/DVB/PDMS fibre would be used because this combination detected the most MVOCs including the target compounds; 1-octen-3-ol and, 3-octanol and 3-octanone.

The VOCs emitted from the malt extract broth with gelatine, the malt extract agar and the clean test film were recorded (Table 15).If the volatiles detected from the substrates which had not been inoculated by fungi were also detected from inoculated samples, then these were excluded from Table 16.

Data from culture on MEA were used for all isolates VOCs produced by fungal isolates grown in malt extract broth with gelatine were also recorded (substrate 2 Table 16) but this medium did not MVOCs from generate as many detectable volatile compounds as the MEA so in preliminary studies was only used to analyse *A.versicolor* ATCC11730 (B), *P.expansum* (C) and *P.chrysogenum* (E). In addition, the isolates grown in MEB and gelatine broth took longer to grow than the same isolates grown on MEA slopes in the vials i.e. 10+days in broth, 5-7 days in vials. There was also the added risk of contamination during transfer of broth from the shake flasks into the vials compared to the *in situ* growth on the MEA in vials.

Thus, after preliminary sampling all subsequent fungal samples were grown on MEA slopes in the vials because it resulted in the greatest number of MVOCs being produced by fungal isolates (substrate 1 Table 16).

The MVOCs from inoculated test film and contaminated film were also recorded.

The moulds grown on different nutrient media and test film together emitted a broad spectrum of more than 150 volatile substances belonging to a variety of chemical groups such as hydrocarbons and alcohols. There were >50 MVOCs that were common to more than one fungal isolate. However there were >100 MVOCs which were emitted from single isolates only. Due to the large number of MVOCs emitted (over 150) from inoculated media, only the compounds which were common to 2 or more fungal isolates were noted (Table 16). This was to determine which compounds could be considered to be common fungal growth markers.

Table 15. Volatile compounds emitted from uninoculated media and clean test film. Types of substrate were: 1)malt extract agar, 2)malt extract broth with added gelatine, 3) test film. These were subsequently not recorded from the spectra of inoculated samples if detected (Table 16).

Type of Substrate	**1**	**2**	**3**
Compound			
Adenosine			x
Benzaldehyde	x		
Benzene (isocyanomethyl)		x	
Benzenehexanamine			x
Furan,2-pentyl	x	x	
Morphine,3-0-[4-trifluoromethylphenyl]	x	x	x
N-ethyl-1,3-dithiosindoline	x	x	
Octane			x
Phenol,2,2-[1-methyl-1,2-ethanediyl]nitrilomethylidyne]bis	x		
Thieno[3,2-c]pyridine 5-Oxide Hemihyrdrate	x	x	
Tranylcypromine, pentaflourobenzoyl ester	x		
3-[4-N-N-Dimethylaminophenyl]propenoic acid,2- diethoxyphosphinyl]-ethyl ester	x		
5,6,8,9-tetramethoxy-2-methylpepero[3,4,5-JK]-9,10,dihydrophenanthracene	x		

Table 16. Summary of MVOCs emitted from multiple fungal isolates from various substrates.

Type of Substrate	4	5	1																2		
Fungi	A	B+F	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	B	C	E
Compound																					
Alloocime ne/ 2,2,-dimethyl-bicyclo(4.2.0) Oct-1(6)-ene		x					x														
alpha-amorphene		x					x	x		x											
alpha-chamigrene								x	x												
(+)-.alpha longipinene								x	x												
Benzene,2,3-disocyanato-1-methyl														x	x						
beta- Cubebene								x	x				x								
beta-funebrene									x												
beta.-Himachalene								x	x				x								
.beta.-sesquiphellandrene								x	x												
Bistrimethyl silyl N- acetyl Eicosasphinga-,aa-Dienine														x	x						
Calarene							x	x													
Camphor								x									x				
Eseroline, 7-bromo-methylcarbamate															x						x
Formamamidiniumacetate					x														x		
Furaltadone		x			x																
gamma-muurolene		x					x	x	x	x											
Heptane			x	x																	
Heptane, 6-Methyl-2-P-Tolyl								x	x												

Fungal species: A= *A.versicolor* with *Trichoderma*, B= *A.versicolor*, C= *P.expansum*, D= *Cladosporium*, E= *P.chrysogenum*, F= *Trichoderma*, G= *A.versicolor*, H= *A.versicolor*, I= *A.versicolor*, J= *A.versicolor*, K= *P.brevicompactum*, L= *A.versicolor*, M = *S.chartarum*, N= *Alternaria*, O= *P.chrysogenum*, P = *P. brevicompactum*, Q = *P.citreonigrum*

Substrates were: 1) malt extract agar 2) malt extract broth with added gelatine 4) mouldy film RR1093, 5) test film inoculated with a mixed culture of *A.versicolor* ATCC11730 and *Trichoderma* sp.

Table 16. Continued

Type of Substratum	4	5	1																2		
Fungi	A	B+F	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	B	C	E
Compound																					
Italicene		x					x	x	x				x								
Limonene														x				x			
Linalool							x										x				
Linalool oxide								x									x				
Longipinene								x	x												
Napthalene,1,2,3,4-tetrahydro-1,6-dimethyl-4(1-methylethyl)		x						x	x												
N,N-Dimethyl-Heptadeclamine			x		x																
Phenol, 2-methyl-5-(1-methylethyl)		x	x			x													x		
terpinene-4-ol							x									x					
trans-Caryophyllene								x	x	x											
Widdrene									x				x								
1-Methylallyyl(cyclooctatetraene)titanium							x			x					x				x		
1- Octen-3 ol		x	x	x	x	x	x	x	x	x	x	x	x				x	x	x		x
1,1,2,3,5,6-Hexamethyl-4-pentyl-2,4-cyclohexadiene													x					x			
1,2,2-Trimethyl-1-(P-Tolyl)-cyclopentane								x	x												
1,3-Dihydroxy-6-methoxy-1,2,3,4-tetrahydroquinolin-2-one					x										x		x				

Fungal species: A= *A.versicolor* with *Trichoderma*, B= *A.versicolor*, C= *P.expansum*, D= *Cladosporium*, E= *P.chrysogenum*, F= *Trichoderma*, G= *A.versicolor*, H= *A.versicolor*, I= *A.versicolor*, J= *A.versicolor*, K= *P.brevicompactum*, L= *A.versicolor*, M = *S.chartarum*, N= *Alternaria*, O= *P.chrysogenum*, P = *P. brevicompactum*, Q = *P.citreonigrum*

Substrates were: 1) malt extract agar 2) malt extract broth with added gelatine 4) mouldy film RR1093, 5) test film inoculated with a mixed culture of *A.versicolor* ATCC11730 and *Trichoderma* sp.

Table 16. Continued

Type of Substratum	4	5	1																2		
Fungi	A	B+F	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	B	C	E
Compound																					
2-methyl-5,12-dithianaphtho {2,3-b} quinoxaline			x													x			x		
2-(2,5-Dimethylphenyl)cyclohexanone								x	x												
3- Cyclohexen-1-ol,4-methyl-1-(1-methylethyl)							x	x									x				
3-Octanone		x	x		x	x	x	x		x	x	x	x				x				x
3-Octanol			x		x	x				x											x
(4-dimethylamino) phenyl-[4'-(N-methyl)(N-ethyl)amino]phenyl-imine																x	x				
7-Dicyanomethylene-7H-2-methoxy-9H-1,8-(1'propen-1'yl-3'-yliden)-benzo-cycloheptane						x													x		x

Fungal species: A= *A.versicolor* with *Trichoderma*, B= *A.versicolor*, C= *P.expansum*, D= *Cladosporium*, E= *P.chrysogenum*, F= *Trichoderma*, G= *A.versicolor*, H= *A.versicolor*, I= *A.versicolor*, J= *A.versicolor*, K= *P.brevicompactum*, L= *A.versicolor*, M = *S.chartarum*, N= *Alternaria*, O= *P.chrysogenum*, P = *P. brevicompactum*, Q = *P.citreonigrum*

Substrates were: 1) malt extract agar 2) malt extract broth with added gelatine 4) mouldy film RR1093, 5) test film inoculated with a mixed culture of *A.versicolor* ATCC11730 and *Trichoderma* sp.

Two compounds were detected from the greatest number of fungal isolates; 1-octen-3-ol and 3-octanone.1-octen-3-ol and are considered to be markers of fungal growth (Fiedler et al., 2001). Of the 16 isolates sampled, 1-octen-3-ol was detected on 13 of the isolates; *A.versicolor*, *P.expansum*, *Cladosporium*, *P.chrysogenum*, S1, RR1399 I1-I6, RR1093 I2 and RR1093 I3. This compound was not detected from RR1491 F2 I4 (*S.chartarum*) and, JWP1 (*Alternaria*) and RR1093 I1 (*P.chrysogenum*). 3-octanone was detected on 10 of the 16 isolates; *A.versicolor*, *Cladosporium*, *P.chrysogenum*, S1, RR1399 I1, RR1399 I3-I6 and RR1093 I2 and was not detected from *P.expansum*, RR1399 I2, RR1093 F2 I4, JWP1, RR1093 I1 and RR1093 I3. 3-octanol is considered to be another marker of fungal growth (Strom et al., 1994) in which emission was detected from 4 isolates; *A.versicolor*, *Cladosporium*, *P.chrysogenum and* RR1399 I3. All 3 of the volatiles considered to be markers of fungal growth were detected from *A.versicolor* (shown later).

There were other compounds which were detected from multiple fungal isolates. There were 27 volatile compounds common to 2 fungal isolates but these cannot be considered markers of general fungal growth as this is too small a proportion of the 16 isolates tested so will not be discussed. There were 5 compounds common to 3 different isolates; beta-Cubebene (G, H and L), beta- Himachalene (G,H and L), trans-Caryophyllene (G,H and I), 1,3- Dihydroxy-6-methoxy-1,2,3,4- tetrahydroquinolin-2-one (D, N and P), 3-Cyclohexan-1-ol,4-methyl-1-(1-methylethyl) (F,G and P). There were 2 compounds common to 4 different isolates; gamma muurolene (F,G,H and I) and italicene (F,G,H and L).

Stachybotrys chartarum (isolate RR1491 F2 I4, column M, Table 16) has been considered by some to pose a significant health risk (Hossain et al., 2004) and has been isolated on one occasion from cinematographic film (Manchester Metropolitan University (MMU), unpublished). None of the key fungal marker compounds (1-octen-3-ol, 3-octanone and 3-octanol) were detected from this species. The *Alternaria* (JWP1) isolate also did not emit these compounds.

Nine volatile compounds were detected from the 'test film' which had been inoculated with a mixed culture of *A.versicolor* (B) and *Trichoderma sp* (F).These were Alloocime ne/2,2,-dimethyl-bicyclo(4.2.0) Oct-1(6)-ene, alpha-amorphene, furaltadone, gamma muurolene, italicene, napthalene,1,2,3,4-tetrahydro-1,6-dimethyl-4(methylethyl), phenol,2-methyl-5-(1-methylethyl), and the two compounds which are considered to be fungal growth markers; 1-octen-3-ol and 3-octanone. However, these latter two compounds were not detected on mouldy film which had been previously contaminated upon donation to this study by the North West Archive (NWFA).

3.8. Methods II: *Aspergillus versicolor* growth markers

3.8.1. Differentiation of Growth Marker MVOCs from *A. versicolor* grown on MEA

From preliminary SPME analysis of fungi isolated from cinefilm and with reference to other studies in the literature, it appears that 3 MVOCs are common to many fungal species including 15 of the fungal isolates studied in this project. These were 1-octen-3-ol, 3-octanone and 3-octanol. The structure and details for these compounds of these is shown below (Fig.82) (Anon. (Chemical Book), n.d.). *A.versicolor* (ATCC11730) was shown to produce all three of the marker compounds thus was used as the test organism. This species had also been isolated from several film reels and was confirmed to be gelatinase positive by enzyme assays, thus could be actively involved in degradation. Approximate retention times for the three compounds emitted from a sample of *A.versicolor* are shown (Fig.83). Retention times were confirmed by using the mass spectrometry search function and also by running standard samples (Sigma-Aldrich) of the compounds through the GC-MS system. These were 11.29 for 1 octen-3-ol, 11.35 for 3-octanone and 11.58 for 3-octanol respectively (Fig.84). These were determined by injecting 1mL of gas at 0.02 % (v/v), from the headspace of vials using a 1mL gas syringe (Hamilton Bonaduz AG, Switzerland), and running program 3 described previously.

It was thought that the concentration of marker MVOCs may increase of decrease over the period of growth. As a preliminary study, 2 cultures of *A.versicolor* ATCC11730 were sampled over time to see if concentrations of the marker compounds changed over a period of growth.

For preparation of samples prior to analysis, a spore suspension of *A.versicolor* ATCC11730 which had previously been maintained on malt extract agar (MEA, Oxoid) was prepared in sterile water (equivalent to approx 2.45 $x10^8$ CFUs per mL or 0.15 OD at 600nm) and inoculated using a sterilised loop onto 2 MEA slopes, (8mL volumes at 60^o angles), which were made in 20mL glass gas chromatography (GC) vials. Aluminium foil was placed over the opening of the vials whilst fungal growth was occurring, to prevent build up of waste gases (e.g. CO_2), whilst enabling growth and preventing contamination. The point of inoculation was called ‘time 0’ because no growth had occurred and no volatiles were produced.

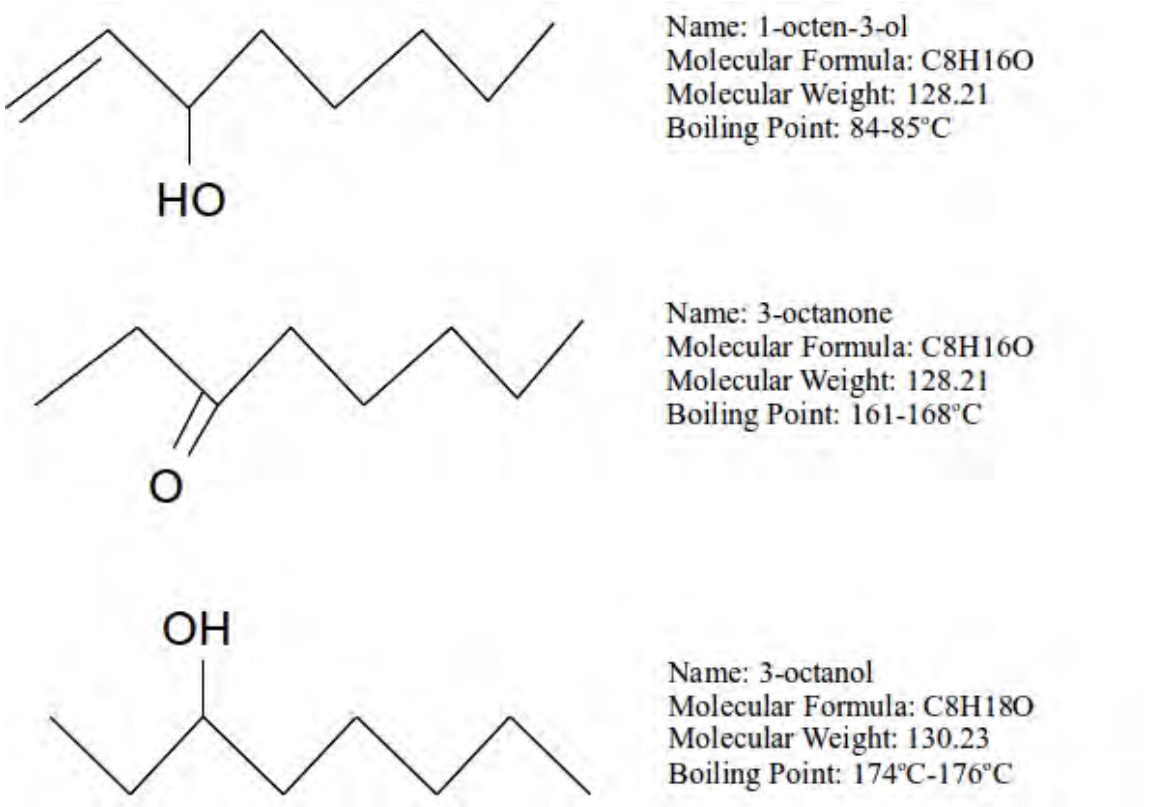

Fig.82. Structure and details of 1-octen-3-ol, 3-octanone and 3-octanol modified from www.chemicalbook.com (Anon. (Chemical Book), n.d.).

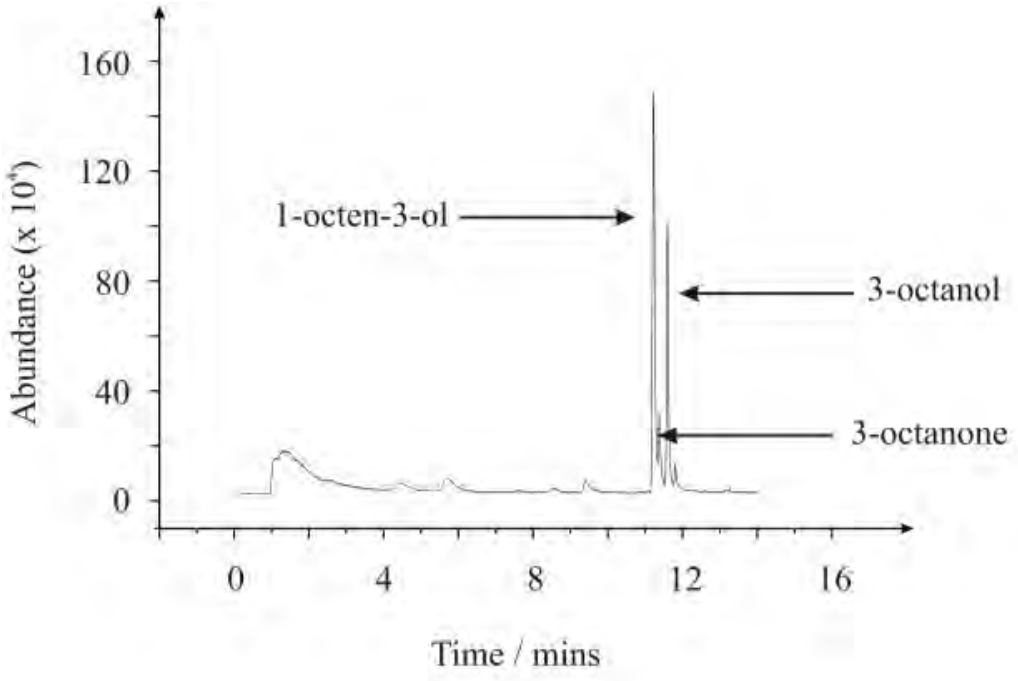

Fig.83. Typical chromatograph of *Aspergillus versicolor* displaying the chromatrographic peaks arising from 1-octen-3-ol, 3-octanone and 3-octanol.

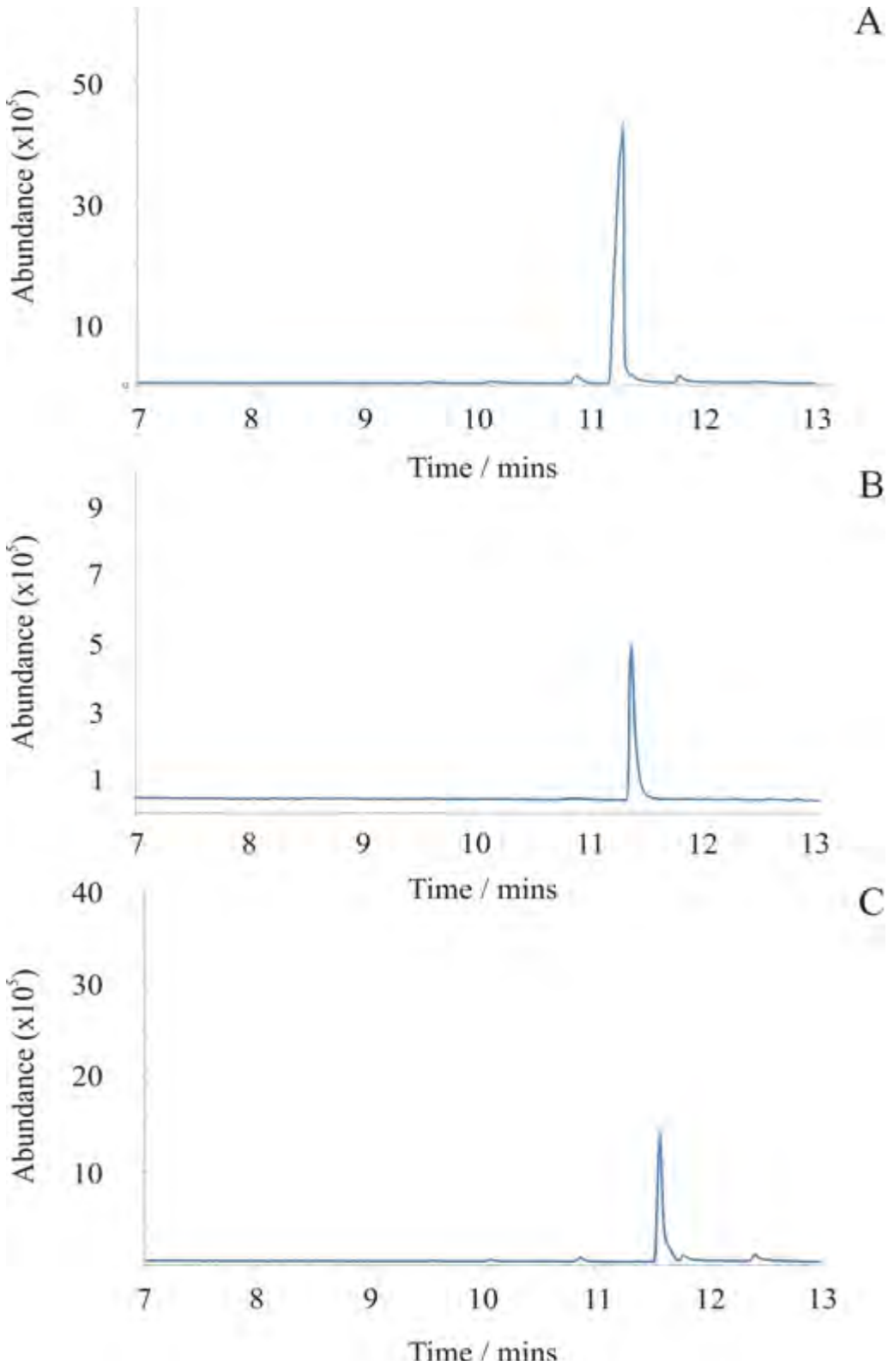

Fig.84. Chromatographs arising from 1-octen-3-ol (A), 3-octanone (B) and 3-octanol (C). These were 11.29 for 1 octen-3-ol, 11.35 for 3-octanone and 11.58 for 3-octanol respectively. These were determined by injecting 1mL of gas at 0.02 % (v/v), from the headspace of vials using a 1mL gas syringe (Hamilton Bonaduz AG, Switzerland).

On the first day of sampling (day 2 of growth), aluminium caps with a self sealing rubber septum which create an airtight seal, were placed on the two vials (results were in duplicate) thus trapping volatile compounds in the headspace. Prior to sampling, each of the two vials were placed in the water bath at 35^{o}C for 1 hour in order to equilibrate the volatiles in the headspace. During this time the GC column and SPME fibre were conditioned to remove any volatiles from previous sampling. After one hour, a 50/30 µm DVB/CAR/PDMS SPME fibre (Supelco) was inserted into the first of the two vials through the rubber on the aluminium cap and exposed to the volatiles in the headspace for 20 minutes. The needle was then retracted, and placed directly into the injector port on the Agilent 6850 system (Agilent Technologies) coupled to the Agilent 5973 Mass Selective Detector (Agilent Technologies) and analysed using the Instrument 1 Enhanced Chemstation. The settings for the GC were based on work by Fiedler et al. (2001) i.e. GC program 3 as described previously. The injector temperature was set at 220^{o}C and the detector transfer line was 280^{o}C with a carrier gas (helium) flow rate of 1 mL/min^{-1}. The initial temperature was 40^{o}C for 7 minutes then ramped to 120^{o}C at a rate of 10^{o}C min^{-1}, after which the program was stopped due to the retention time of all the target volatiles being less than 15 minutes. After allowing the GC-MS to cool back down to the initial temperature, the process was repeated for the remaining vial. After both of the vials had been sampled on a particular day, the airtight caps were removed and the vials were re-covered with aluminium foil until the next day of sampling. This process was repeated for 3, 6, 8, 10, 13, 15, 17, 20, 22, 24, 27, 29, and 31 days of growth. After 31 days of growth concentrations of volatiles were measured every 5 days at 35, 40, 45, 50 and 55 days of growth until the target volatiles were no longer detectable.

Following detection of the target MVOCs, attempts were made to quantify the amount of the compounds which were present in the headspace of the vials. The areas of the peaks for 1-octen-3-ol, 3-octanone and 3-octanol were measured and recorded for each sample for each day, and the average taken (n=2). This information gives a relative measurement of the amount of the compounds that had been absorbed onto the fibre in the 20 minutes of exposure time.

3.8.2 Differentiation of Growth Marker MVOCs from *A.versicolor* grown on film strips

In addition to measuring MVOCs produced over time from *A.versicolor* growing on MEA, the concentrations of 1-octen-3-ol, 3-octanone and 3-octanol were also measured over time from *A.versicolor* growing on inoculated cinematographic film. A spore suspension was made from an MEA culture of *A.versicolor* ATCC11730, which contained approximately 3.89 x10^6 CFUs per mL or 0.1OD at 600nm. A 100µl volume was pipetted onto a plate of MEA and spread plated. Two 5cm long strips of 16mm 'clean' test film which had been wiped with 70% ethanol and air dried to minimise contamination from spores and bacteria which may already have been present on the film, were placed on the plate using sterile forceps (Fig.85) and incubated for 3 days at 25°C until growth of *A.versicolor* was visible and the hyphae had penetrated into the gelatine emulsion layer on the film strips.

After the initial 3 days of growth, each strip was placed into a separate 20mL GC vial (n=2), and each was covered with an aluminium foil lid, before being placed into a moisture chamber (glass beaker with a tin foil lid) to prevent drying out of the film strips and to simulate the moist environment in which a film reel may be subjected to during storage, and which would propagate mould growth. On the 1st day of sampling (i.e. day 4), an aluminium cap with a self sealing rubber septum was placed on the two vials to trap volatiles in the headspace (Fig.86). These were then placed in a water bath for one hour at 35°C to equilibrate the volatiles in the headspace before sampling with the SPME fibre and GC-MS, using program 3 in the same way as described for *A.versicolor* on MEA. Sampling was carried out in duplicate, on days 4, 7, 9, 11, 14, 16, 18, 21, 23, 25, 28, 30, 32, 35, 40, 45, 50 and 55 days growth. After sampling, the airtight lids were removed from the vials and replaced with aluminium foil, and these vials which still contained the film strips were placed back in the moisture chamber until the next day of sampling. On the next day of sampling airtight aluminium caps were placed back on the vials and the process was repeated until the last day of sampling.

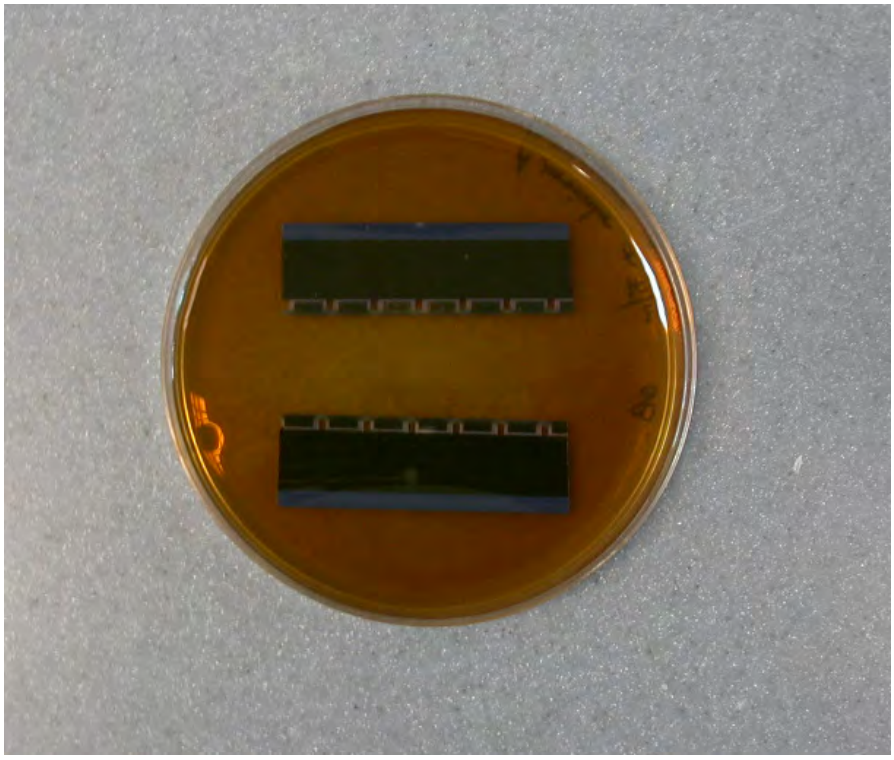

Fig.85. Two 5cm pieces of test film placed on an MEA plate on which a lawn of *Aspergillus versicolor* has been spread.

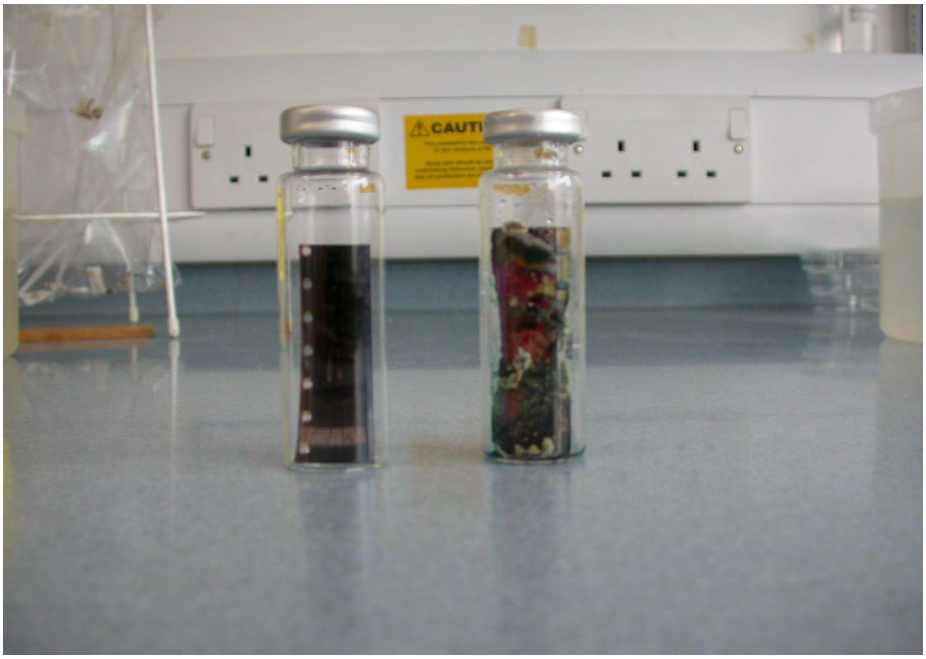

Fig.86. 5cm pieces of test film in a 20mL GC vial. Left- uninoculated film, right- film inoculated with *Aspergillus versicolor.*

3.9. Results II: *Aspergillus versicolor* growth markers

3.9.1. Differentiation of *A.versicolor* growth markers:1-octen-3-ol, 3-octanone and 3-octanol, when Grown on MEA

Analysis of the fungal growth markers 1-octen-3-ol, 3-octanone and 3-octanol was performed from day three of growth on MEA until day 55 when they could no longer be detected (see Appendix I). No volatile compounds were detected from *A.versicolor* using the 1mL gas syringe, thus analysis of compounds emitted from *A.versicolor* was performed using SPME. Attempts to quantify the amounts of the volatiles were attempted by analysing the Enhanced Chemstation spectrum and measuring the areas of the peaks of these three volatile compounds. As shown in Fig 82, 1-octen-3-ol appeared first on the spectrum, 3-octanone second and 3-octanol third. The areas of peaks were measured at days 2, 3, 6, 8, 10, 13, 15, 17, 20, 22, 24, 27, 29, 31, 35, 40, 45, 50 and 55 of growth (Fig 86). 1-octen-3-ol and 3octanone were detected at day 2 of growth, with 1-octen-3-ol being present in the highest concentration. 3-octanol was not detected until day 13 of growth. The general trend of the concentrations of the volatile compounds, was to increase until reaching a maximum concentration between 20 and 25 days of growth, after which the concentrations of the compounds decreased. The maximum concentration of 1-octen-3-ol was at 20 days of growth, 3-octanone was 24 days of growth and 3-octanol was 22 days of growth. 3-octanol was produced in lower concentrations than 1-octen-ol and 3-octanone on all days of analysis but was detectable for a longer period of time. 1-octen-3-ol could not be detected at 44 days of growth, 3-octanone at 50 and 3-octanol at 55 days of growth. No volatiles could be detected on the 55 day of sampling.

3.9.2. Differentiation of *Aspergillus versicolor* Growth Markers on Film Strips

Results for *Aspergillus versicolor* on film strips were recorded on days 4, 7, 9, 11, 14, 16, 18, 21, 23, 25, 28, 30, 32, 35, 40, 45, 50 and 55 days growth, when volatile compounds could no longer be detected (Fig 87) (See Appendix II). 1-octen-3-ol was detected from the 1st day of sampling (day 4) until day 50 of growth. 3-octanone was only detected from day 14 to day 28 of growth. 3-octanol was not detected at any time of growth on cinematographic film. The highest concentration of 1-octen-3-ol was detected at 28 days of growth; whilst 3-octanone was detected at the highest concentration on day 16 of growth. No volatile compounds could be detected at day 55 of growth. With the exception of the 1st sampling at day 4, the general trend of volatile emission by *A.versicolor* growing on film strips over time was similar to those released from cultures growing on MEA, except VOCs were emitted in much lower concentrations (approximately 4 -8 times less).

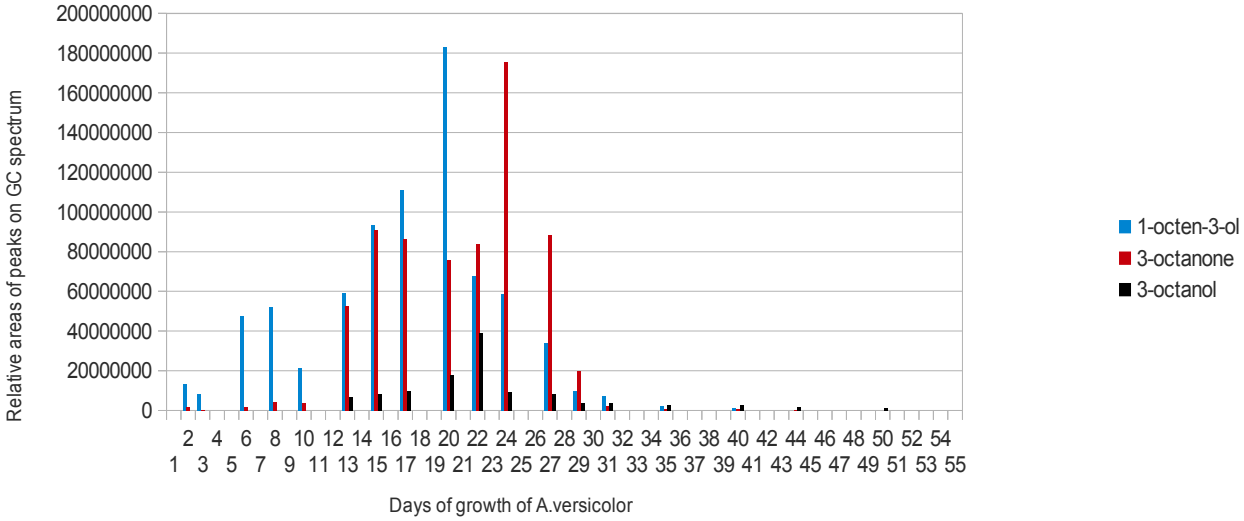

Fig.87. HS-SPME analysis of MVOCS released from *A.versicolor* grown on MEA showing relative areas of peaks for 1-octen-3-ol, 3-octanol and 3-octanone. Sampling was carried out on day 2, 3, 6, 8, 10, 13, 15, 17, 20, 22, 24, 27, 29, 31, 35, 40, 45, 50 and 55 of growth (see Appendix I).

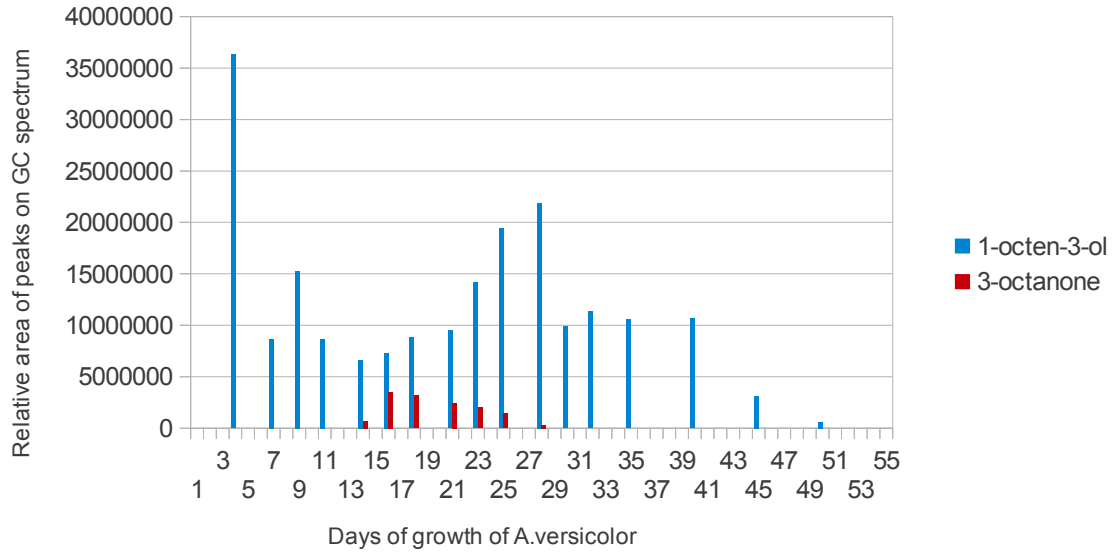

Fig.88. Relative area of peaks on GC spectrum of 1-octen-3-ol, and 3-octanone from analysis of *Aspergillus versicolor* inoculated on cinematographic film and sampled using GC-MS headspace analysis. Sampling was carried out on days 4, 7, 9, 11, 14, 16, 18, 21, 23, 25, 28, 30, 32, 35, 40, 45, 50 and 55 days of growth. 3-octanol was not detected throughout the period of sampling so is absent from the chart (see Appendix II).

3.10. Methods III: Limit of Detection (LOD)

3.10.1. Limits of detection of 1-octen-3ol, 3-octanol and 3-octanone

The limit of detection (LOD) for the three marker compounds was ascertained by analysing pure compounds of 1-octen-3ol, 3-octanol and 3-octanone (Sigma) at different concentrations. This determined the lowest concentration that could be detected by the GC-MS system and identified the limit of this technology for measuring MVOCs from fungi .

A SPME needle (Supelco) with a 50/30 Carboxen/divinylbenzene polydimethysiloxane adsorbent fibre was compared to a 1mL gas syringe (Hamilton Bonaduz AG, Switzerland) which does not have an adsorbent fibre, to analyse differences in peak sizes and accuracy of syringe types in quantification of MVOC concentrations. A 1mL gas syringe was chosen for comparison with SPME because preliminary sampling had shown this to detect volatiles at lower concentrations than smaller syringe sizes (results not presented) probably because of an increased volume of gas being sampled.

The pure liquid compounds of 3-octanone, 1-octen-3-ol and 3-octanol supplied by Sigma were designated 100% concentration, of which dilutions were then made in ethanol because the compounds are insoluble in water (Anon. (The Good Scents Company (A) and (B), n.d. and Anon. (Guidechem), n.d.). After preliminary sampling, it was ascertained that concentrations ranging from 0.03%-0.0005% would be analysed to determine the limit of detection for both the SPME needle and gas syringe (Table 16). Stock solutions for the range of concentrations from 0.03-0.001% were prepared in 100mL volumetric flasks and shaken vigorously to evenly distribute the compounds in the ethanol (methylated spirit/ ethanol denature (Fisher Scientific)). For concentrations of 0.000(x)% 1mL volumes were taken from concentrations of 0.00(x) and diluted in 100mL volumes (e.g. 1mL of 0.008 taken to 0.0008 in 100mL volumes with ethanol). Two millilitres samples of each compound at each concentration was transferred into 20mL GC vials. The GC vials were sealed with an airtight aluminium cap with a self sealing rubber septum thus trapping volatiles in the headspace.

The samples were then placed in a 35^{o}C water bath to equilibrate the volatile compounds in the headspace. Each dilution was sampled three times (n=3). For SPME, the adsorption time was 20 minutes, whereas for gas sampling the sample was taken and analysed immediately. Both types of needles were directly desorbed into the injector port on the Agilent 6850. As with previous MVOC analysis, the settings of GC were based on work by Fiedler et al. (2001). The injector temperature was set at 220^{o}C and the detector transfer line was 280^{o}C with a carrier gas (helium) flow rate of 1 mL/min. The initial

temperature was 40°C for 7 minutes then ramped to 120°C at a rate of 10°C min^{-1} after which the program was stopped due to the retention time of all the volatiles being less than 15 minutes. Pure methylated spirit served as a negative control and was run through the GC system after each dilution was sampled to ensure there was no residue of the 1-octen-3-ol, 3-octanone and 3-octanol compounds remaining in the column.

To evaluate variability when analysing mouldy film, samples were taken of *A.versicolor* ATCC11730 cultured on MEA (n=5 per syringe type). *A.versicolor* ($2.45x10^8$ CFUs per mL or 0.15 OD at 600nm) was inoculated onto 8mL of MEA slopes (60° angle) in 20mL GC vials which were then covered with aluminium foil and incubated at 30°C for 7 days. One hour prior to sampling, aluminium caps with a self sealing rubber septum, were clamped onto the vials to trap volatiles and vials were then placed in a water bath at 35°C to equilibriate the compounds in the headspace. After one hour the vials were sampled using SPME and a 1mL gas syringe GC-MS and the relative areas of peaks for 3-octanone were recorded.

Table 17. Dilution table for 1-octen-3-ol, 3-octanone and 3-octanol in 100mL for 0.03-0.001% concentrations and 1L for 0.0009-0.0001% concentrations when the volumes were diluted in ethanol (methylated spirit).

Total volume =100mL		
% Concentration	**Volume of 3-octanone (mL)**	**Volume of ethanol (mL)**
0.03	0.03	99.97
0.02	0.02	99.98
0.01	0.01	99.99
0.009	0.009	99.991
0.008	0.008	99.992
0.007	0.007	99.993
0.006	0.006	99.994
0.005	0.005	99.995
0.004	0.004	99.996
0.003	0.003	99.997
0.002	0.002	99.998
0.001	0.001	99.999
Total volume = 1 litre		
% Concentration	**Volume of 3-octanone (mL)**	**Volume of ethanol (mL)**
0.0009	0.009	999.991
0.0008	0.008	999.992
0.0007	0.007	999.993
0.0006	0.006	999.994
0.0005	0.005	999.995
0.0004	0.004	999.996
0.0003	0.003	999.997
0.0002	0.002	999.998
0.0001	0.001	999.999

3.11. Results III: Detection Limits

3.11.1. Limit of Detection of 3-octanone, 1-octen-3-ol and 3-octanol using SPME needle and a 1mL Gas Syringe needle

The limit of detection (LOD) for 3-octanone, 1-octen-3-ol and 3-octanol was determined using a 1mL gas syringe and a 50/30 DVB/CAR/PDMS SPME fibre for comparison (Table 18). The LOD with a 1mL gas syringe was at 0.01% concentration (100000 parts per billion (ppb)) for all three compounds. The LOD for SPME was at much lower concentrations. The LODs were as follows:

1-octen-3-ol - 0.002% (20000 ppb)

3-octanone – 0.0009% (9000 ppb)

3-octanol – 0.001% (10000 ppb)

All concentrations were sampled at least once with concentrations nearer to the limit of detection being sampled multiple times. The two dilutions above and below the limit of detection and the value considered to be the limit of detection were sampled three times. If the area of the peak on the GC spectrum did not decrease with decreasing concentrations, then that concentration was prepared again and re-sampled, with the original value being discarded.

Samples of 3-octanone were analysed using a 1mL gas syringe and a 50/30 DVB/CAR/PDMS SPME needle for comparison of the accuracy of sampling. Three vials containing 2mL of 3-octanone in ethanol were sampled for each needle type (n=3) at a concentration of 0.05%, 0.1%, 0.5% and 1% and an average taken (Table 19). The percentage variance for both the gas syringe and the SPME needle varied between approximately 10% and 20%.

The results for SPME were: 23007051.6 +/- 5757005.743 (25.02% variability)

No VOCs were detected using the gas tight syringe.

Table 18. The relative areas of peaks (n=2) of 3-octanone, 1-octen-3-ol and 3-octanol at concentrations of 0.03% (100000 ppb) to 0.0001% (100 ppb). The first value of '0' for each compound indicates the limit of detection. The limit is between this concentration and the dilution before it. If the value is '0' then the compound was not diluted further. A value of '0' for subsequent dilutions indicates that this concentration was sampled. A'-' indicates that no further sampling was carried out as the limit of detection had been determined.

Compound	1-octen-3-ol		3-octanone		3-octanol	
% Concentration	1mL Gas	SPME	1mL Gas	SPME	1mL Gas	SPME
0.03	85699174	19615556	2173422	62066275	5126172	47777460
0.02	7371222	10072871	12993907	98160612	4658041	29161307
0.01	4290106	12737393	802781	16194786	739457	61809011
0.009	0	7040402	0	8057454	0	11463349
0.008	0	2601973	0	9887308	0	4339406
0.007	-	7847421	-	9028615	-	7133613
0.006	-	4599918	-	7545608	-	4153113
0.005	-	2786421	-	8687788	-	3913465
0.004	-	1520366	-	6281988	-	8814998
0.003	-	1424667	-	4752583	-	8459853
0.002	-	620741	-	3952100	-	9069354
0.001	-	0	-	2016499	-	1150537
0.0009	-	0	-	1585125	-	0
0.0008	-	0	-	0	-	0
0.0007	-	-	-	0	-	0
0.0006	-	-	-	0	-	0
0.0005	-	-	-	0	-	0
0.0004	-	-	-	-	-	-
0.0003	-	-	-	-	-	-
0.0002	-	-	-	-	-	-
0.0001	-	-	-	-	-	-

Table 19. Standard deviation and percentage variance from sampling 2mL of 3-octanone (Sigma) in a 20mL GC vials at concentrations of 0.05, 0.1, 0.5 and 1% diluted in ethanol (n=3).

Needle Type	Concentration of 3-octanone (%)	Standard Deviation	% Variability (2 d.p.)
SPME	0.05	42684562.5 +/- 4311016.24	10.01
1 mL gas	0.05	2717408 +/- 339634.26	10.74
SPME	0.1	62145657 +/- 7247532.701	11.7
1 mL gas	0.1	7116436.5 +/-688168.34	9.67
SPME	0.5	380626209.3+/-70214081.51	18.45
1 mL gas	0.5	51989679 +/- 8360746.1	16.08
SPME	1	450622017.7 +/- 40364693.63	8.96
1 mL gas	1	64905338 +/- 12892355	19.86

3.12. Discussion

3.12.1. SPME of Fungal Isolates

No single MVOC was specific to all fungi. Some VOCs appear to be good indicators of fungal growth, although none is universally reliable as a detector (Korpi et al., 1998). 1-octen-3-ol, 3-octanone and 3-octanol were detected from many isolates thus were good indicators of fungal growth. As a fungal metabolite, 1-octen-3-ol is considered to be an important compound and serves a biological purpose. It is a self-inhibitor (Chitarra et al., 2005), and in high concentrations inhibits germ tube formation, whilst preventing germination of spores and asexual spore formation (microcycle conidation (Hanlin, 1994)), when spores are present in high density. It has also been shown to be attractant to mushroom eating insect pests (Combet et al., 2006). 1-octen-3-ol, 3-octanone and 3-octanol are the products of oxidative breakdown of linoleic acid (Chitarra et al., 2005 and Wilkins and Larsen, 1995), which is an unsaturated fatty acid representing between 64%-74% of the total fatty acids in mushrooms and is found in the mycelium and fruiting bodies of fungi (Combet et al., 2006). Fatty acids like linoleic acid serve as an energy store for spore germination, however the detailed metabolic pathway of production of MVOCs is unknown (Chitarra et al., 2005 and Combet et al., 2006).

The lowest amount of target analyte that could be detected using SPME GC-MS was explored on the compounds 1-octen-3-ol, 3-octanone and 3-octanol. Known amounts of these compounds were diluted with ethanol and analysed using the same program as described previously. The lowest levels that were measurable were: 1-octen-3-ol, 0.002 (v/v) %; 3-octanol, 0.001 (v/v) %; 3-octanone 0.0009 (v/v) % indicating that SPME was effective at detecting even low concentrations produced by fungi. Quantitative analysis was not possible however, due to the large variability of SPME and the size of the resulting peaks on the GC spectrum.

In agreement with Fiedler et al. (2001) and Scotter et al. (2005), MEA was found to be the best substrate to use, as growth on this resulted in the greatest number of detectable MVOCs being produced. SPME was found to be suitable for detection of MVOCs, as GC-MS analysis of *A.versicolor* growing on MEA slopes using a gas tight syringe failed to detect any volatiles.

The MVOCs from the isolates studied are the same as those from the literature although the method of sampling and adsorbent used could cause variation in the volatiles found. 1-octen-3-ol and 3-octanone were found from the lab strain of *Penicillium chrysogenum* Q176 tested and not the *P.expansum*, in agreement with the results found by

Kaminski et al. (1974) and Larsen and Frisvad, (1995). *A.versicolor* which has been isolated from cinematographic film in this study and in other studies at MMU (unpublished) and other studies (Abrusci et al., 2004a, 2005, 2006; Opela, 1992), is a fungus known to cause biodeterioration of many materials (Liang et al., 2011). This species produced 1-octen-3-ol, 3-octanone and 3-octanol as was also recorded by Sunesson et al., (1995) and Fiedler et al. (2001).

Stachybotrys chartarum did not emit any of the marker compounds at the time of testing. Other studies have shown that MVOCs from *S.chartarum* peak early and taper off to low or non-detectable concentrations after 4-6 weeks of growth (Anon. (Ohio University), n.d.). The isolate was sampled after one week of growth but repeated sampling over a longer time period could have detected more MVOCs specific to this isolate.

Analysis of the test film which had been inoculated with a mixed culture of *A.versicolor* and *Trichoderma* sp. identified 9 volatile compounds of which 2 were the fungal growth markers 1-octen-3-ol and 3-octanone. These were not detected from SPME analysis of mouldy film donated by the NWFA, but were detected from fungi that had been isolated from these films and were actively growing MEA. This indicates that these compounds can be detected from actively growing mould and not from inactive mould, thus demonstrating the potential for detecting active fungal growth on cinematographic film and differentiating actively growing mould from that which is considered 'dead' or non-viable. This may remove the need for traditional culture methods and reduce the risk of cross contamination of film and the health risks to archivists. Precautions could also be taken should actively growing mould be detected to ensure that these films are stored separately in order to avoid contaminating non-contaminated film reels.

3.12.2. Analysis of MVOCs from *Aspergillus versicolor* on Malt Extract Agar and Film Strips

Concentrations of the MVOCs were much higher from the mould growing on MEA than on film strips. The area and size of the peak on the GC spectrum is proportionate to the concentration of the compound in the headspace (Kenkel, 2002). The maximum relative peak area of 1-octen-3-ol and 3-octanone on MEA was approximately 160000000-180000000 whilst the maximum peak area of 1 octen-3-ol on film was approximately 40000000 (4 x less). In addition, 3-octanol was not detected from *Aspergillus versicolor* growing on film and 3-octanone was only detectable for a period of 14 days on film compared to 50 days on MEA. This suggests that nutrient availability of a substrate affects the concentrations of the MVOCs produced (Jellison et al., 2008). The film itself has fewer

nutrients than MEA, therefore an artificial inoculation method was needed using added nutrients, in order for the fungi to colonise the film in a shorter period of time.

The pattern of emission of 1-octen-3-ol and 3-octanone coincides with that of other fungi recorded in the literature and reflects the findings of Börjesson et al. (1992) that VOC synthesis is dependent on mould growth phase. Stoppacher et al. (2010) investigated the growth of *Trichoderma atroviride* over 120 hours and noted a higher concentration of 1-octen-3-ol highest in the 'middle' stages of growth (approx 50-80 hours) and the concentration of 3-octanone was the highest at the later stages of growth (approx 80-100 hours)**.** Similarly, although over a longer period of time, in this study *A.versicolor* on MEA also produced 1-octen-3-ol in higher concentrations than 3-octanone until the later stages of growth where, after 22 days 3-octanone was produced in higher concentrations. There appeared to be other similarities in the pattern of emissions. The concentration of volatiles increased after the initial stages of growth reaching a maximum peak in the middle stages, then declining until they were no longer detectable. It could be assumed that this emission pattern is linked to the function of the compounds. As has been previously noted (3.12.1.), high concentrations of 1-octen-3-ol prevent germination of spores when they are present in high numbers (Chitarra et al., 2005 and Combet et al., 2006). Thus, it could be suggested that in the middle stages of growth spore germination is inhibited to prevent overgrowth and rapid depletion of nutrients. As the nutrients are exhausted and mould growth begins to slow or the colony begins to die, the concentration of MVOCs decreases, thus enabling germination of spores once a new nutrient source is found.

The 1st peak on the spectrum for *A.versicolor* on film strips does not appear to follow this pattern and produced a peak with a relative larger area than for the other days of growth. It is assumed that this is due to the film being sampled on the same day as removal from the MEA, and concentrations of 1-octen-3-ol were high on the plate culture due to heavy fungal growth, which decreased after a few days as there was less growth on the film strip. After the 1st day of sampling, the pattern of MVOC emissions for *A.versicolor* on film was similar to that of *A.versicolor* on MEA. The concentration decreased by a large amount on the 2nd day sampling before increasing until 28 days of growth, then decreasing until no longer detectable at 50 days.

Although only a few samples were analysed for this preliminary study it does appear that there is a change in the concentration of MVOCs produced over time. It appears that the concentration of these compounds decreases to undetectable levels after nutrients have been depleted and the fungi begins to die, or when vegetative growth ceases. Thus, these findings indicate that MVOC detection would be a suitable method to

distinguish between dead and actively growing mould on cinematographic films.

3.12.3. Limit of Detection (LOD) and accuracy of quantification

Sampling of 3-octanone showed that both the SPME needle and gas syringe had a variability of between 10 and 20% which is similar to the 14% variation noted by Stadelmann (2001). This large percentage of variability indicates that the method is not accurate for quantification of VOCs as has been noted by Stadelmann (2001). However, this method is sensitive for detecting low concentrations of VOCs.

SPME sampling of *A. versicolor* at 7 days of growth resulted in an even higher percentage of variability of 25% compared with the 10-20% when sampling a pure compound. This could be due to the physiological state and growth of the organism (Insam and Seewald, 2010) in addition to the variability of the SPME technique.

In addition to the variability of the growth of the organism, the gas syringe and SPME needle also produced results with a high variability. HS-SPME does not use a known volume of air but instead relies on molecules being adsorbed onto the adsorption material. The vials were placed into water bath to equilibrate the volatiles in the headspace, but they may not have attached to the SPME fibre. The measurement of peaks was done manually in this instance, so repeated measurement of the same peak would not give exactly the same area but the variance of the human operator is undetermined. The high variability of the gas syringe could be due to low concentrations of the compound that was used, so equal amounts of the compound was not taken into the syringe during adsorption. Additionally, using the gas syringe is not as sensitive as the SPME fibre at these low concentrations, so could have added to the error. However, using higher concentrations of 3-octanol with SPME, would have saturated and possibly damaged the column.

The relative peak area size for compounds at the same concentration were much larger for the SPME needle than 1mL gas tight syringe (approximately 10-30 times), thus indicating that the SPME fibre was effective at preconcentrating the MVOCs before sampling with the GC-MS. For the LOD, the SPME was over 10 times more sensitive than the gas syringe.

3.13. Conclusions

It has been demonstrated via SPME GC-MS that there are three MVOCs that are common to fungal isolates found on cinematographic film, namely, 1-octen-3-ol, 3-octanone and 3-octanol. Quantification of these chemical markers indicate active mould growth suggesting MVOC detection can be used to distinguish between actively growing mould on film and 'dead' mould. A detection device for archivists could allow them to make decisions on safe handling of mouldy film reels in order to prevent contamination of non-mouldy film reels and protect the health of people handling the materials.

Chapter 4

Summary, Recommendations and Future Work

4.0 Discussion

4.1. Introduction

It has been shown that some species of mould are capable of growing on film. Once mould growth has occurred the damage to the film is permanent, thus prevention of growth should always be the primary aim of archives. However, in some instances mould growth may have already occurred due to reels being stored in sub-standard conditions prior to donation to the archives. Visual confirmation of mould growth is needed so that archivists can safely handle contaminated materials and avoid unnecessary health risks due to exposure to fungal spores. Microbiological isolation and culture can identify the presence of viable mould, but may be too costly for a few individual mouldy reels. In the event of flood damage resulting in large scale mould outbreaks, specialised commercial services may be required which can identify mould present, and advise on safe handling and clean up procedures. However, for single mouldy reels, culture and identification of the mould present may not be necessary, as the evidence suggests that pathogenic species are unlikely to be the predominant species present, thus the health risks posed by mould growth on film reels is considered to be low. Active mould growth is therefore a risk to film and identification of this activity will inform subsequent handling and storage and help preserve more film, as reels could be stored in conditions which prevent further growth. This chapter uses findings from previous work described in this thesis to provide an overview of procedures that may be taken. In addition, a novel method for determining mould growth by detection of MVOCs has been indicated.

4.2. Controlling Mould Growth

Due to the difficulties faced with removal of mould and handling of contaminated materials, preventing mould outbreaks from occurring is the most effective way to ensure preservation of materials. As discussed in chapter 1 (section 1.7), prevention of mould outbreaks requires regular monitoring of the storage conditions in an archive, in order to maintain an environment which is unfavourable for mould growth whilst preventing chemical deterioration. This includes monitoring RH, temperature and in some cases such as paper archives, materials can be stored in anoxic conditions which will prevent mould growth and additionally protect materials from insect infestation (Jacobs et al., 2001). The most commonly isolated environmental fungi such as *Aspergillus* (including *A.versicolor*) and *Penicillium* (including *P.chrysogenum* and *P.expansum*) species will grow optimally when the humidity reaches or exceeds 70-90% and the temperature is between 20-35^{o}C,

although much slower growth has even been observed around 0ºC (Deacon, 2006, Hocking, 2006 and Pasanen et al., 1991). Thus, materials should be kept in conditions lower than these temperatures and RH values. In addition, these conditions require monitoring to ensure they remain stable. In modern archives this is done continuously and automatically via sensors linked to computers which, warn an operator when optimal conditions in the archive change (Patkus, 2007).

As well as monitoring environmental conditions in an archive, it may also be beneficial to monitor microbial presence in the air. Biological materials in the air are often referred to in several different terms namely aerobiology (microbiological fragments and fungal spores) and bioaerosols (metabolites and MVOCs) (section 1.4.1.) (Florian, 2002). As noted previously concentrations of spores and MVOCs could be indicative of fungal growth it is visible. Thus, early detection could help archivists prevent mould growth, and preserve more materials.

General 'house keeping' measures such as regular dusting and cleaning of materials could prevent spore and dust build up. It has been shown in chapter 2, that winding through film reels in a ventilated cabinet a few consecutive times after long term storage, reduces at least to some extent, the number of fungal spores which will be released into the air during the next sampling. Thus, it is assumed that lower numbers of spores are also present on the film after multiple windings, and this property could be enhanced by performing this process in the presence of air suction, which would potentially remove more spores. Reducing the numbers of spores on a film reels will reduce the risks to archivists' health and the likelihood of cross contamination of other reels. Chemical agents, such as disinfectants and anti-microbial agents could also be used clean the floors and walls of archives. This would reduce the presence of microorganisms in the archive, thus reducing the chance of microbial growth occurring. However, microorganisms will always be present in an archive regardless of cleaning, particularly some fungal spores, which have been shown to have resistance to heat (Pitt and Hocking, 2009), radiation (Blank and Corrigan, 1995) and bleaching (Wilson et al., 2004). Thus, the focus should be on maintaining environmental conditions in the archive to prevent growth, and additionally checking the integrity of the building so that damage caused by leaks and flooding can be avoided.

4.3. Identifying Mould Growth

In modern archives, it is unlikely that mould growth will occur when the materials are in storage. However, film reels may already be contaminated upon donation to the archive thus visual inspection is needed so that if mould growth is present materials can be handled safely.

4.3.1. Visual inspection

Currently, the only way to determine whether a cinematographic film reel is contaminated with mould without culture methods, is by visual inspection. If a film reel is visibly mouldy it could potentially pose a problem both for deterioration of the film and a risk to handlers. If the film does not appear to have obvious fungal growth, the material present on the surface of the is most probably dust.

If mould growth has occurred on film reels, visible mould growth on the surface or edges of film reels can appear white, which is often either 'furry', crusty or granular in appearance due to the presence of fungal mycelia. Often, lighter mould growth is not visible on the outer surface of the film reel so if mould growth is suspected and if the equipment is available to archivists, film frames could also be checked under a microscope, magnifying glass or even a light source, to see if any hyphal filaments are evident across film frames.

If mould growth has occurred, fungal spores may also be present, which are produced continuously by fungi but especially in conditions of nutrient stress. These will remain on a material when conditions are no longer suitable for vegetative growth (Deacon, 2006). Dormant, viable mould will not cause further damage to the materials unless the appropriate conditions for growth are present, although it should be noted that spore release could contaminate other materials. Both unviable and viable spores may be present but viability is not confirmable without culture.

4.3.1.1. Safe Handling of Materials and Responses to Mould Growth

It is the vegetative growth of fungi which cause damage to film, and the release of spores which pose a risk to archivists. Microscopic analysis could enable archivists to determine whether mould growth has penetrated the gelatine emulsion layer. If this has not occurred extensively then the fungal growth is superficial (i.e. the suggested classification of category 1 and 2 (section 2.0.2.), then wiping the outer edges of the reel may be sufficient to remove visible growth, although Screen Heritage UK (SHUK) indicates that care should

be taken not to damage the film image (Anon. (The Screen Heritage UK), 2011). This process could enable the recovery of more archive footage, particularly from those reels which have been identified as being mouldy and consequently not processed. If reels are heavily contaminated with mould (i.e. the suggested classification of category 3 and 4 (section 2.0.2.)then one would not recommend inspecting the film further. Currently, there is no way to remove mould growth once it has penetrated the emulsion layer (BFI, 2012. Personal communication). Thus, the reels should be transferred to new cans and stored, which is already carried out at the NWFA and BFI, until a method becomes available for recovering more film.

When handling film, Screen Heritage UK (SHUK) suggests the use of clean, lint free cotton or inert vinyl gloves to avoid scratching the film. If there are concerns about direct physical contact with mould e.g. a skin irritation (e.g. rash) (Anon. (New York State Department of Health), 2011), nitrile or vinyl gloves could be more appropriate as these are often used in microbiology laboratories and offer better protection whilst eliminating the risk to latex allergies (Anon. (Latex Free Gloves), n.d.). In addition, a face mask may also be appropriate if an archivist is working in vaults containing many mouldy, deteriorating film reels as opening of cans may release large numbers of dust or fungal spores.

If resources are available to archivists, a recommendation for handling extremely mouldy film would be to perform inspections in an isolation glove box whilst air is extracted. Currently at the BFI in Berkhamsted, reels with light mould are inspected on a flat bed winder with extraction working above the films. For heavily contaminated reels, film cans are first opened on a down draft bench to remove loose spores and other debris, and then the films are inspected on a winder under a covered hood under which air is extracted (Allum et al., 2007). Ideally, inspections of mouldy reels would be performed in microbiological safety cabinets (Anon. (Public Health Agency of Canada), 2005) but these may not be financially viable, because mouldy reels are not normally handled in high numbers. There are 2 classes of cabinets that may be of use to archivists. Class I cabinets have unrecirculated airflow that carries air away from the operator which is filtered through a HEPA filter then released into the atmosphere. These provide good protection for the operator but do not protect the material within the cabinet from contamination. These would be adequate for handling contaminated cinematographic film, as the film reels themselves do not require protection from contaminants in the surrounding air but archivists would be protected from exposure to fungal spores when inspecting contaminated reels. Class II cabinets are designed for microbiological work involving BSL

2,3 and 4 microorganisms. They offer a higher level of protection and protect the operator, the materials inside and prevent contamination to the environment but are not necessary for handling mouldy film reels.

It has been shown in this thesis, that reels from a given collection appear to be contaminated by the same species as other reels in the same collection. This is most likely due to simultaneous or cross contamination of reels or environmental contamination, so steps should be taken to reduce the chances of this occurring when mouldy reels are initially donated to the archive. One suggestion would be to store contaminated films in separate sealed containers when they arrive at the archive, which is not a practice currently performed at the BFI or NWFA. This would prevent cross contamination of collections and minimise spore dispersal onto other materials. Additionally, this would also reduce the chance of fungal growth occurring on materials should ambient conditions change. However, failure of environmental systems is unlikely in well maintained archives due to the monitoring systems that are in place. Nevertheless it would seem to be good practice to separate mouldy films from others on receipt to the archive.

4.3.1.2. Responses to Large Scale Mould Growth

In addition to recommendations made regarding safe handling of small numbers of mouldy materials, information is available regarding response to larger outbreaks (Florian, 2002 and Price, 1996), such as in the case of flood damage. In all instances of mould growth on materials, finding the source of the contamination is crucial to preventing further growth, thus preventing further deterioration of materials. The reason for growth is typically due to exposure of materials to moisture. For materials which have been donated to archives and have been previously contaminated with mould, the source of the contamination was outside of the archive, so appropriate storage and handling should prevent further growth.

Mould growth in an archive is usually the result of an unexpected source of increased humidity such as broken ventilation systems, broken windows or damage to the structural work of buildings which allows moisture from the environment to get through. Removal of materials from this moisture source and subsequent drying will prevent any further mould growth and will inactivate mould presently growing if drying is sufficient. If the damage is due to flooding then the first 24 hours of exposure are critical to prevent a mould outbreak (Price, 1996).

Several treatments for materials contaminated with mould such as biocides, UV light and freeze drying, have been described by Florian (2002). However, these treatments are not recommended for cinematographic film due to adverse effects on the film image,

and freeze drying could lead to the formation of ice crystals, which could affect the gelatine. The application of art masking fluid ('laminectomy'. Section 2.1.1.) is not recommend for removal of growth on the edge of mouldy film reels due to difficulty in removal of the dried fluid, which could damage the structure of the film. This produces also the potential for spore dispersal onto other materials when the dried fluid is peeled off.

4.3.2. Culture

In addition to visual identification of mould growth, culture methods may be required determine whether spores and vegetative growth are/is viable or unviable. Also. it may not be possible to visually distinguish between mould growth and other debris such as dust and cobwebs which may be present on the surface of the film.

4.3.2.1. Mould Identification Services

If an outbreak is severe it may be necessary to consult professional services who will identify the mould present and clean up affected areas. In severe mould contamination, it may be of benefit to archivists to identify the mould present, so that handlers are not exposed to unnecessary risks. Some examples of professional identification services include: the Health Protection Agency (Anon. Health Protection Agency), 2012) and CABI Microbiological Services (Anon. (CABI), 2012), but there are also some other commercial services which additionally offer cleaning and removal of mould such as Mold Testing Lab (Anon. (Mold Testing Lab), n.d.), Munters Moisture Control Services (Anon. (Munters), n.d.), Solex Environmental Control Services (Anon. (Solex), n.d.), Specialist Mould Removal Services (Anon. (Specialist Removal Services), 2012) and Quartec (Anon. (Quartec), 2012).

The fungi most commonly isolated from cinematographic film described in this work and in other studies (Abrusci et al., 2004a, 2005, Opela, 1992), are ubiquitous in the environment, so the risk to health is low and expenditure on identification is probably not warranted. Most species isolated were classified as biohazard level 1 (discussed later) and are not recognised as human pathogens, thus are not listed in pathogen databases such as the American Biological Safety Association (ABSA) (Anon. American Biological Safety Association), n.d.). However, as with all environmental fungi, there is a chance of allergen risk or serious health risk in immunocompromised individuals (Apuhan et al., 2011). One might speculate that immunocompromised individuals are unlikely to be unknowingly working with mouldy materials in archives, thus the health risks posed by contaminated film reels may be considered to be low or absent to the vast majority of the population.

However, in the case of flood or water damage, other more potentially harmful organisms may be present in the archives and on materials. For example *Stachybotrys chartarum,* which produces mycotoxins and has been implicated in causing disease in humans (Hossain et al., 2004), requires a higher RH of 98% compared with the 70-90% of *Aspergillus* and *Penicillium* species to grow (Florian, 2002 and Pasanen et al., 1991) so is associated with water damaged materials and buildings (Anderson et al., 2002). Thus, in this case it may be necessary to consult these commercial services. However, as only a few isolated colonies of *S.chartarum* were isolated in this study (section 2.13.1.), probably due to slower growth than *Aspergillus* and *Penicillium* species it is unlikely to become the predominant coloniser of cinematographic film, due to being outcompeted by these two genera. Thus, these services are probably not needed for individually contaminated reels.

4.4. Risk Classification of Common Environmental Fungi

Some commercial services use a different system of risk classification from health organisations, possibly over exaggerating the risk of mould contamination. Some environmental organisms are classed as hazardous, despite their not being known pathogens.

Several health organisations including the Advisory Committee on Dangerous Pathogens (UK), the European Parliament and the The United States Department of Health and Centers for Disease Control (CDC) (US), rate microorganisms in biological hazard safety levels (BSL) ranging from 1-4 (Anon. (Belgian Biosafety Server), 2006). Descriptions for each level are:

level 1- Not known to consistently cause disease in healthy adults.

level 2- Associated with human disease, hazard = percutaneous injury, ingestion, mucous membrane exposure.

level 3- Indigenous or exotic agents with potential for aerosol transmission; disease may have serious or lethal consequences.

level 4-Dangerous/exotic agents which pose high risk of life threatening disease, aerosol transmitted lab infections; or related agents with unknown risk of transmission.

(Anon. UC Davis Health System), 2009)

Due to the health risks increasing with each level, recommendations for handling of micro-organisms differ, ranging from standard microbiological practices such as wearing personal protective equipment (PPE) for level 1, and working in BSL2 safety cabinets with level 2 organisms, to wearing full body suits and respiratory apparatus whilst working in

specialised facilities for level 3 and 4.

In contrast, for some commercial services and also on some websites, indoor moulds have been grouped into 3 hazard risk classes based on associated health risk. Some examples include:

Mold and Bacteria Consulting Laboratories (MBL) (Anon. Mold and Bacteria Consulting Laboratories), n.d.), Divinely Toxic Blog (Anon. (Divinely Toxic), n.d.), and Mould Facts (Anon. (Mould Facts), n.d.) Descriptions for these hazard classes are:

- Hazard class A- includes fungi or their metabolic products that are highly hazardous to health. These fungi or metabolites should not be present in occupied dwellings. Presence of these fungi in occupied buildings requires immediate attention.
- Hazard class B- includes those fungi which may cause allergic reactions to occupants if present indoors over a long period.
- Hazard class C- includes fungi not known to be a hazard to health. Growth of these fungi indoors, however, may cause economic damage and therefore should not be allowed.

The risk category for fungi isolated from cinematographic film differs between the two ranking systems. *Aspergillus versicolor* is ranked as hazard class A, conflicting with information listed in microbiological databases. The American Biological Safety Association (ABSA) database (Anon. (American Biological Safety Association), n.d.) lists the biological hazard risk group for known infectious agents but, *A.versicolor* is absent from this list. In addition, *A.versicolor* in ATCC culture collections is listed as BSL1 (Anon. (LGC), n.d.), indicating that this species is not classed as a pathogen or infectious agent. However, as with all fungi, an allergen risk has been recognised due to the presence of exoantigens, which are known to stimulate antibody production (Liang et al., 2011). Thus a commercial rating of hazard class B would seem more appropriate. *Penicillium* spp with a special note to *P.chrysogenum* are classified as hazard class B on the commercial websites. Only one species of this genus is listed by ABSA as BSL2 which is *P.marneffei* (not isolated in any studies of cinefilm). All *Penicillium* species isolated in this study including *P.chrysogenum*, *P.brevicompactum*, *P.citreonigrum* and *P.expansum* are classified as BSL1 (Anon. (LGC), n.d.). Although *Fusarium spp.* have not yet been isolated from cinematographic film in this thesis or in other studies, there is a significant discrepancy between the hazard class noted on Mold and Bacteria Consulting Laboratories (MBL) (Anon. Mold and Bacteria Consulting Laboratories), n.d.) and ABSA (Anon. (American Biological Safety Association), n.d.). Only one species is listed on the ABSA:

F.moniliforme (biohazard class 2). One study by Nucci and Anaissie (2007) describes superficial skin infections caused by *Fusarium* spp. but focuses on the main threat being to immunocompromised patients. However, commercial services have listed this genus as being hazard class A suggesting that the risks posed by this fungus is high. Interestingly *Stachybotrys chartarum* which is known as a toxic mould so is listed on websites as hazard class A, is also on listed in the database as BSL1.

There is only one genus of mould which has been isolated from cinematographic film where there does seem to be some agreement on classification. *Cladosporium* (unidentified species) was isolated from cellulose nitrate vaults at the BFI in Gaydon. *Cladosporium cladosporioides* and *C.sphaerospermum* are listed by commercial services as hazard class B and C respectively, and are listed in the ATCC culture collections as BSL 1 but are not listed on the ABSA database thus, are deemed to be essentially non-pathogenic.

All species of fungi are potentially capable of causing allergic reactions or respiratory problems and the risk is increased if this is due to exposure to high concentrations of spores over an extended period of time. Thus, one would recommend that all fungi should be placed into at least hazard class B using the hazard class system on MBL (Anon. Mold and Bacteria Consulting Laboratories), n.d.).

Fortunately, serious health problems caused by exposure to common environmental fungi growing indoors is rare (Chapter 1). However, exposure to increased numbers of fungal spores can pose an allergen risk or respiratory irritation in healthy individuals, thus heavily contaminated materials should still be handled with caution to minimise exposure to fungal spores and cross contamination of other materials.

4.5. Microbial Volatile Organic Compounds (MVOCs)

As explained previously (section 4.3.1.), visual inspection of reels is often the only method available to archivists to determine the presence of mould growth. However, without culture methods it is not possible to tell via visualisation, whether mould is actively growing on the reel, and failure to store reels in conditions which prevent further mould growth could enable further damage to occur. A method of achieving this is by detection of MVOCs.

Detection of MVOCs to indicate the presence of fungal growth has been discussed in detail in chapter 3, describing the first investigation of mould growth on cinematographic film using GC-MS (Bingley et al., 2012). The identification of 1-octen-3-ol, 3-octanone and 3-octanol as markers for fungal growth led to further development at

MMU. Identification of active mould growth by detection of MVOCs by archivists via a hand held sensor would be more practical, as specialist expertise and equipment would not be needed. One possible approach is to use screen printed sensors. These are 'economical one-shot disposable sensors' which are sensitive to the analysis of many target analytes. They are portable and allow for very rapid results (Banks, n.d.)(Fig.89).

For a compound to be detected, the molecules react with an element of the electrode known as the 'ink'. The inks consist of graphite particles, a polymer binder and other additives. Once target compounds are identified, the composition of the ink can be changed so that different compounds can be detected, which means that the sensor can be used for a variety of applications. When inserted into a reader, a peak is produced which is relative in size to the amount of compound that has reacted with the ink. This gives a relative measurement of how much of a particular analyte is present in the sample and has successfully been demonstrated for measuring concentrations of lead in drinking water (Yarnitzky et al., 2000).

A feasibility study has been completed, to determine whether these strips can be used for detection of active mould growth on film reels. Single strips which detect a particular compound (i.e. 1-octen-3-ol, 3-octanone and 3-octanol), can be placed inside a film can and removed shortly after. These can then be placed in a hand held sensor, which can confirm the presence of the selected MVOC, thus indicating whether mould is actively growing.

Detection of mould in damp buildings where vegetative growth is not visible is also another potential application although no evidence could be found in the literature for them currently being used for this. In addition, if a MVOC specific to *S.chartarum* could be identified using GC-MS with HS-SPME, a sensor for this organism could be developed so that any mould outbreaks concerning this organisms could be dealt with rapidly, without the need for culture of molecular identification. Modification of the ink, could allow detection of any compound thus, the detection of mould using a screen printed electrode is only one application: others such as measuring deterioration of cellulose acetate and cellulose nitrate reels as well as many other materials in archives are additional possibilities.

However detection of MVOCs does have drawbacks and does not indicate whether spores are viable, and this can only be confirmed by culture. Thus, even if MVOCs are not detected, safe handling procedures should still be followed if film reels visually appear mouldy to reduce health risks by exposure to increased numbers of fungal spores.

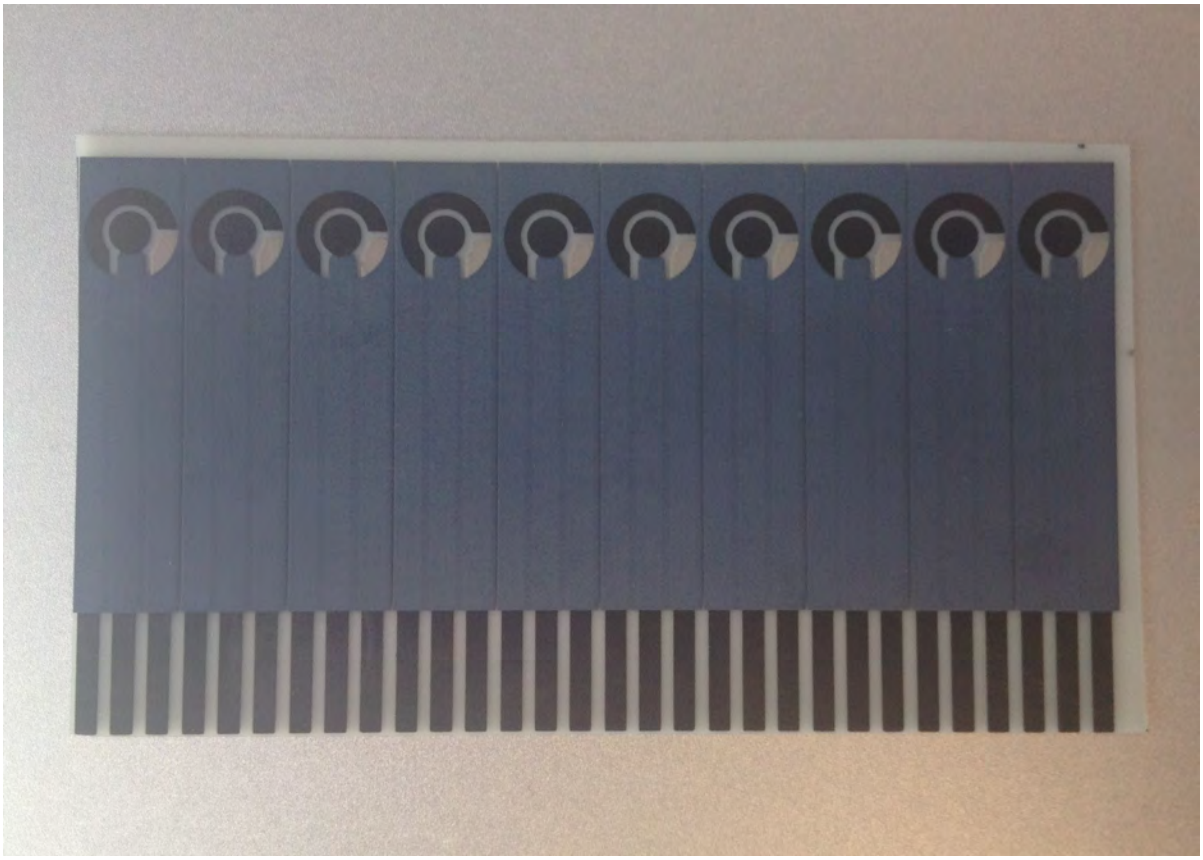

Fig.89. A strip of 10 individual screen printed electrodes. The composition of the ink (black circle at the top of the image) can be altered in composition to detect specific compounds. The bottom of the electrode can then be placed into either a hand-held sensor or into a computer for analysis of results.

4.6. Summary and Future Work

Initially, this work aimed to address the health risks potentially posed to archivists during the inspection of mouldy film reels. Before the potential risks had been highlighted, the NWFA and BFI cleaned reels which were only lightly contaminated by wiping off the mould, but are now storing mouldy reels without further investigation (Bodner, 2009. NWFA. Personal Communication and BFI Gaydon, May, 2012. Personal communication). The findings in this thesis suggest that the practice of cleaning lightly contaminated reels can resume, as health risks posed by the mould present on these reels is considered to be negligible.

Some reels which appeared mouldy and had been marked as 'very mouldy' by the NWFA, only released a few spores. For others, which appeared badly deteriorated, and where mycelium could be seen on the frames, no viable spores were recovered either from swabbing or air sampling. Active mould growth is therefore a risk to film due to the production of enzymes which degrade gelatine and deteriorate the film image but it is the spores which pose a risk to archivists.

There are no universally agreed guidelines on what are considered 'safe' background concentrations of spores (Swan et al., 2003), but some recommendations indicate that 800 to 1000 CFUs m^3 is not considered to pose a health risk to healthy individuals (Jo and Seo, 2001 and Levetin et al., 1995). Inspection of heavily contaminated reels, revealed that spores were released in numbers higher than the 800-1000 considered 'safe' for indoor levels. Thus, in terms of spore numbers, there is a potential health risk posed by handling heavily contaminated reels but they are considered to be low.

Air sampling of the old cellulose nitrate vaults at BFI Gaydon which contained many heavily deteriorated and possibly contaminated reels, recovered only a few colonies most of which were *Cladosporium* spp. Sampling of the new -5^{o}C vaults revealed mainly yeasts and a few *Cladosporium* colonies although in low numbers. The predominance of *Cladosporium* spp. in both cases, suggest that the source is the outside environment which is rural (Shelton et al., 2002). The new vaults contain considerably more film reels (n=6000-7000) (Fig.90 and Fig.91) compared with the old vaults (n=1100) (Section 1.7.1 (Fig 8 and Fig 9). Three areas of the new building were sampled: the corridor, a cellulose nitrate vault and a cellulose acetate vault (n=3), with 73 CFUs/m^3, 57 CFUs/m^3 and270 CFUs/m^3 isolated respectively. Thus, working in vaults which contain mouldy film reels is not considered to pose a health risk, and the risk occurs only when cans are opened and inspection of mouldy is performed.

There appears to be no evidence that pathogenic fungal species pose a significant contamination risk to film, and species present on the reels are overwhelmingly those which are typically common in the environment such as *Aspergillus* (i.e. *A.versicolor*) and *Penicillium* spp (i.e. *P.brevicompactum*, *P.citreonigrum* and *P. chrysogenum*), classified as BSL1.

A.versicolor was the most commonly isolated species in this study, and in other studies (MMU (unpublished), Opela, 1992 and Abrusci et al., 2005). It was the most gelatinoltyic compared with the other fungi isolated in this study, which offers a reason for its high frequency of isolation (8/15 isolates identified by CABI) (Bingley and Verran, 2012). In contrast the *Penicillium* species appeared to be more cellulolytic than gelatinolytic and species found were more diverse, thus no single species was predominant on cinematographic film.

The fungal isolates were common to the same reels in the same collection, presumably due to the organisms being present in the environment when the reels became colonised. This could be confirmed by sampling a domestic environment where mouldy reels are being stored, and comparing the isolates present on the mouldy reels with those from the air. However, this is a challenging experiment as mould contamination and subsequent growth could have happened decades ago, thus the organisms present in that environment could be unrelated to those that are on the reels.

A novel method to identify active mould growth other than visualisation and culture, is by detection of MVOCs, which are common to many fungal isolates. SPME coupled with GC-MS identified 1-octen-3-ol, 3-octanol and 3-octanone from many of the fungi isolated from cinematographic film, and screen printed electrochemical sensors made at MMU, were modified to detect these specific compounds. After use, these sensors can be placed in hand held monitors so specialist skills are not required, and the technology is more accessible and suited that of the needs of archivists. Thus, implementation of these sensors in the future could potentially save more film reels, and film reels found to harbour active mould growth could be placed in storage conditions which would halt further growth. There is also the possibility of these sensors being implemented for uses other than mould detection: a sensor to detect deterioration of materials such as cellulose acetate reels, would also be useful (BFI, Gaydon, 2012. Personal communication). Currently, common practice for determining the level of acetic acid present from a film suffering from vinegar syndrome, is by the use of A-D strips which measure pH and change colour depending on the amount of acid present. Detection and measurement of acid levels using an electrochemical sensor could potentially be a more accurate method of determining film

deterioration. Thus, there is potential for this technology to be used for a variety of applications in an archive in the future.

Preliminary studies on videotapes, identified a potential further area for study as the literature currently available is very thin. Agar imprints of heavily contaminated, exposed videotape isolated high numbers of colonies, which were mainly *Penicillium* spp. (species unidentified). The problems concerning videotapes are different from that of cinematographic film, as fungi do not appear to be utilising the videotape as a substrate but grow on the surface presumably utilising dust and available moisture. Similarly to videotape, mould on other media such as compact disks and floppy disks (Garcia-Guinea et al., 2001 and Khan et al., 2008) has been noted but has not been studied in detail. However, mould growth on these media is more likely to be a short term problem as these technologies are superseded by new technologies and data is transferred to digital media (Anon. (DAEFH), 2011), but nevertheless is still a potentially interesting area of study.

Future studies on cinematographic film, could include investigation of mouldy film reels found in other archives throughout the world which experience different climatic conditions. This would indicate whether the fungal species present (i.e. the predominance of *A.versicolor*), are the same globally, or whether they differ depending on the environment and conditions the reels are being stored in. Additionally, SPME with GC-MS could be used to analyse mould isolated from other archive materials such as videotapes and paper archive materials, to see if the 'one shot' screen printed strips could be applied to other areas, and potentially help conserve more valuable cultural heritage.

Fig.90. The newly built vaults at the BFI, Gaydon. In the event of combustion of nitrate film the sides of the vaults blow out into the trench. The reinforced concrete ensures only one vault of film reels would be lost due to fire in a single vault.

Fig.91. The new vaults at BFI, Gaydon which contain considerably more film reels (n=6000-7000) compared with the old vaults (n=1100).

4.7. Conclusion

The need to assess the potential risk of mould growth on cinematographic film in terms of film quality and archivists' health is pressing. Initial visual inspection of film reels can indicate the presence of mould. However, the amount of spore release on inspection by archivists cannot be predicted: some mould reels do not yield any viable spores. Fungi commonly present in the environment were isolated from cinematographic film, with *Aspergillus versicolor* being the most frequently isolated species with a higher a gelatinolytic activity than *Penicillium* spp. isolated from the same reels. No fungi considered to pose a serious health risk were isolated, so the risk to archivists from handling mouldy film reels may be considered to be low. However, simulated inspection of some reels released spores in higher numbers than those considered 'safe' by health organisations. It is therefore advisable that safe handling procedures should be recommended when handling heavily contaminated reels.

It has been demonstrated via SPME GC-MS that there are three MVOCs that are common to fungal isolates found on cinematographic film, namely, 1-octen-3-ol, 3-octanone and 3-octanol. Quantification of these chemical markers indicate active mould growth suggesting that MVOC detection can be used to distinguish between actively growing mould on film, and ‘dead’ mould. A detection device for archivists could allow them to make decisions on safe handling of mouldy film reels in order to prevent contamination of non-mouldy film reels and protect the health of people handling the materials. A prototype has been developed at MMU by modifying a screen printed sensor to detect these compounds.

It is the mycelial growth of fungi that causes damage to film reels but the release of fungal spores from contaminated reels that poses a health risk. The future implementation of the screen printed sensors that have been developed to detect mould growth based on findings in this thesis, could inform archivists activities and enable more films to be inspected, thus saving more archive footage, aiding in the preservation of valuable cultural heritage.

Appendix I: MVOCs of *A.versicolor* Grown on Malt Extract Agar

Relative areas of peaks measured on a gas chromatography spectrum for 1-octen-3-ol, 3-octanone and 3-octanol from HS-SPME sampling of *A.versicolor* grown on malt extract agar over 55 days. Two samples were taken for each day so results were in duplicate.

	1-octen-3-ol		Average	STDEV	% variability	3-octanone		Average	STDEV	% variability	3-octanol		Average	STDEV	% variability
Sample Number	1	2				1	2				1	2			
Days of Growth															
2	14548279	12587597	13567938.00	1386411.54	10.22	1813636	1524572	1669104.00	204399.11	12.25	0	0	0	0	0
3	8625329	7865747	8245538.00	537105.58	6.51	298321	223527	260924.00	52887.34	20.27	0	0	0	0	0
6	56554326	38755427	47654876.50	12585722.18	26.41	2038373	1540833	1789603.00	351813.91	19.66	0	0	0	0	0
8	55457763	48649627	52053695.00	4814079.13	9.25	4632682	3625787	4129234.50	711982.28	17.24	0	0	0	0	0
10	25086672	17253829	21170250.50	5538656.40	26.16	4563422	3273463	3918442.50	912138.76	23.28	0	0	0	0	0
13	64437287	53629127	59033207.00	7642523.23	12.95	61738324	43267898	52503111.00	13060563.48	24.88	7563722	5982733	6773227.5	1117928.0429	16.505101045
15	109235827	77473681	93354754.00	22459228.82	24.06	99325362	82263522	90794442.00	12064542.76	13.29	9783627	6503834	8143730.5	2319163.8712	28.477905441
17	127664682	94682928	111173805.00	23321621.91	20.98	92837485	80182375	86509930.00	8948514.10	10.34	10293746	8928238	9610992	965559.96656	10.046413175
20	212273648	153384649	182829148.50	41640810.53	22.78	84745631	67283736	76014683.50	12347424.37	16.24	16364898	19483743	17924320.5	2205356.449	12.303710196
22	72158273	63528292	67843282.50	6102318.09	8.99	93836271	73623538	83729904.50	14292560.57	17.07	42832763	34782632	38807697.5	5692302.2195	14.667972042
24	67362517	50172635	58767576.00	12155082.13	20.68	192643537	158274653	175459095.00	24302470.94	13.85	10287438	7898362	9092900	1689331.8404	18.578581535
27	38273696	29612635	33943165.50	6124294.97	18.04	98535284	78293745	88414514.50	14312929.49	16.19	8984638	7474536	8229587	1067803.3645	12.975175601
29	10803468	8627364	9715416.00	1538737.89	15.84	22729382	17263794	19996588.00	3864754.34	19.33	4145987	3200846	3673416.5	668315.61028	18.193297991
31	8427345	6382373	7404859.00	1446013.57	19.53	2478273	1772837	2125555.00	498818.58	23.47	4012826	3509625	3761225.5	355816.8394	9.4601304655
35	2517972	1925623	2221797.50	418853.99	18.85	863328	638873	751100.50	158713.65	21.13	2873657	2162746	2518201.5	502689.98892	19.96226231
40	1357654	1273384	1315519.00	59587.89	4.53	732364	591912	662138.00	99314.56	15.00	3383723	2419273	2901498	681969.13512	23.504036023
44	0	0	0.00	0.00	0.00	350263	262563	306413.00	62013.26	20.24	2138170	1628374	1883272	360480.20862	19.141165409
50	0	0	0.00	0.00	0.00	0	0	0	0	0	1279389	1042837	1161113	167267.5233	14.405791969
55	0	0	0.00	0.00	0.00	0	0	0	0	0	0	0	0	0	0

Appendix II- MVOCs of *A.versicolor* Inoculated onto Film Strips

Relative areas of peaks measured on a gas chromatography spectrum for 1-octen-3-ol, 3-octanone and 3-octanol from HS-SPME sampling of *A.versicolor* grown on inoculated film strips over 55 days. Two samples were taken for each day so results were in duplicate.

	1-octen-3-ol		Average	STDEV	%variability	3-octanone		Average	STDEV	%variability	3-octanol		Average	STDEV	% variability
Sample Number	1	2				1	2				1	2			
Days of Growth															
4	40007165	32695781	36351473.00	5169929.21	14.22	0	0	0.00	0.00	0.00	0	0	0.00	0.00	0.00
7	9931150	7422492	8676821.00	1773889.08	20.44	0	0	0.00	0.00	0.00	0	0	0.00	0.00	0.00
9	17530701	13062999	15296850.00	3159142.38	20.65	0	0	0.00	0.00	0.00	0	0	0.00	0.00	0.00
11	9728336	7656195	8692265.50	1465224.95	16.86	0	0	0.00	0.00	0.00	0	0	0.00	0.00	0.00
14	5681092	7533095	6607093.50	1309563.88	19.82	780743	598646	689694.50	128762.02	18.67	0	0	0.00	0.00	0.00
16	6356268	8243786	7300027.00	1334676.78	18.28	4004639	2974548	3489593.50	728384.33	20.87	0	0	0.00	0.00	0.00
18	9807276	7864523	8835899.50	1373733.82	15.55	3676708	2636649	3156678.50	735432.77	23.30	0	0	0.00	0.00	0.00
21	11731864	7282728	9507296.00	3146014.24	33.09	2983668	1928636	2456152.00	746020.28	30.37	0	0	0.00	0.00	0.00
23	16500803	11765701	14133252.00	3348222.73	23.69	2327356	1726537	2026946.50	424843.19	20.96	0	0	0.00	0.00	0.00
25	16412599	22436398	19424498.50	4259469.12	21.93	1689645	1138468	1414056.50	389740.99	27.56	0	0	0.00	0.00	0.00
28	24900166	18846476	21873321.00	4280605.25	19.57	277851	348373	313112.00	49866.58	15.93	0	0	0.00	0.00	0.00
30	10482184	9267527	9874855.50	858892.20	8.70	0	0	0.00	0.00	0.00	0	0	0.00	0.00	0.00
32	13327700	9368368	11348034.00	2799670.51	24.67	0	0	0.00	0.00	0.00	0	0	0.00	0.00	0.00
35	12376265	8727826	10552045.50	2579835.96	24.45	0	0	0.00	0.00	0.00	0	0	0.00	0.00	0.00
40	12558754	8726652	10642703.00	2709705.31	25.46	0	0	0.00	0.00	0.00	0	0	0.00	0.00	0.00
45	3467061	2726356	3096708.50	523757.53	16.91	0	0	0.00	0.00	0.00	0	0	0.00	0.00	0.00
50	720378	482938	601658.00	167895.43	27.91	0	0	0.00	0.00	0.00	0	0	0.00	0.00	0.00
55	0	0	0.00	0.00	0.00	0	0	0.00	0.00	0.00	0	0	0.00	0.00	0.00

Appendix III: Conference Abstract 1

Conference title: Biocides in Synthetic Materials

Date of conference: 28-29th September 2010

Conference Location: Berlin Germany

Gavin Bingley, Dr Gordon Craig, Mark Bodner, Professor Joanna Verran

Abstract

Cinematographic film is composed of 3 generic layers: a polymer based base support, a photosensitve emulsion coating and a binder based on gelatine. Studies were carried out on 18 film reels using air sampling, to quantify spores released from contaminated film during a simulated inspection process, in order to assess exposure of archivists to spores. Organisms present were identified and screened for gelatinase production, since gelatine is the major substrate for fungal growth on the film. The majority of fungi present were *Aspergillus* and *Penicillium* species, 16 out of 30 isolates of which produced gelatinase. For some films, released spore numbers exceeded the recommended 'safe' exposure levels of 1000CFUs/m^3. Some films appeared contaminated, but no fungal growth was detected post-inspection. However, hyphal growth was evident across film frames, indicating that the damage may have taken in the past. This study indicated a need for detection of fungal contamination of film, the presence of viable fungal spores, and safe handling recommendations for film archivists.

Appendix IV: Conference Abstract 2

Conference title: 15th International Biodeterioration and Biodegradation Symposium

Date of conference: 19-24th September 2011

Conference Location: Vienna, Austria

Gavin Bingley, Dr Craig Banks, Dr Gordon Craig, Mark Bodner, Professor Joanna Verran

Abstract

Studies were carried out on 18 cinematographic film reels contaminated with mould using air sampling, to quantify spores released from contaminated film using during a simulated inspection process, in order to assess exposure of archivists to spores. Organisms present were identified and screened for gelatinase production, since gelatine is the major substrate for fungal growth on the film. The majority of fungi present were *Aspergillus* and *Penicillium* species, of which 18 out of 27 isolates produced gelatinase. For some films, fungi released spore numbers which exceeded the recommended safe exposure levels of 1000 CFUs/m^3. Some films appeared contaminated, but no fungal growth was detected post-inspection. However, hyphal growth was evident across film frames, indicating that damage may have taken place in the past. Culture of fungi may not be possible in archives but detection of the product of fungal growth may be possible. Detection of microbial volatile organic compounds (MVOCs) by gas chromatography mass spectrometry (GC-MS) coupled with solid phase microextraction (SPME) could be an alternative to culture methods to determine if active mould growth is occurring. 1-octen-3ol, 3-octanone and 3-octanol are all said to be general indicators of fungal growth and have been detected by GC. Conservation and storage is essential in archives. A knowledge of the amount of fungi, identification of key fungal contaminants, production of MVOCs and their gelatinolytic potential will help development of methods to enable early detection of actively growing mould.

Bibliography

Abrha, B., Gashe, B. (1992). Cellulase production and activity in a species of *Cladosporium*. World Journal of Microbiology and Biotechnology, 8, 164-166.

Abrusci, C., Allen, N.S., Del Amo, A., Edge, M., Martin-Gonzalez, A. (2004). Biodegradation of Motion Picture Film Stocks. Journal of Film Preservation, 67, 37-54.

Abrusci, C., Marquina, D., Amo, A., Corrales, T., Catalina, F. (2006). A viscometric study of the biodegradation of photographic gelatin by fungi isolated from cinematographic films. International Biodeterioration and Biodegradation, 58, 142-149.

Abrusci, C., Marquina, D., Del Amo, A., Catalina, F., Collado, J., Platas, G. (2007). Biodegradation of cinematographic gelatin emulsion by bacteria and filamentous fungi using indirect impedance technique. International Biodeterioration and Biodegradation, 60, 137-143.

Abrusci, C., Marquina, D., Santos, A., Amo, A., Corrales, T., Catalina, F. (2009). Biodeterioration of cinematographic cellulose triacetate by *Sphingomonas paucimobilis* using indirect impedance and chemiluminescence techniques. International Biodeterioration and Biodegradation, 63, 754-759.

Abrusci, C., Martin-Gonzalez, A., Del Amo, A., Catalina, F., Collado, J., Platas, G. (2005). Isolation and Identification of Bacteria and Fungi from Cinematographic Film. International Biodeterioration and Biodegradation, 56, 58-68.

Abrusci, C., Martin-Gonzalez, A., Del Amo, A., Corrales, T., Catalina, F. (2004). Biodegradation of type-b gelatine by bacteria isolated from cinematographic films. A viscometric study. Polymer Degradation and Stability, 86, 283-291.

Abu-Bakar, N.K., Abd-Aziz, S., Hassan, M.A., Ghazali, F. M. (2010). Isolation and Selection of Appropriate Celluloytic Mixed Microbial Cultures For Cellulases Production from Oil Palm Empty Fruit Bunch. Biotechnology, 9, 73-78.

Adams, M.R., Moss, M. O. (2008). Food Microbiology (3rd ed.). Cambridge, England: The Royal Society of Chemistry.

Adhikari, A., Sen, M.M., Gupta-Bhattacharya, S., Chanda, S. (2004). Airborne viable, non-viable, and allergenic fungi in a rural agricultural area of India: a 2-year study at five outdoor sampling stations. Science of the Total Environment, 326, 123-141.

Al-Aidaroos, A., Bin-Hussain, I., Solh, H., Kofide, A., Thawadi, S., Belgaumi, A., Al Ahmari, A. (2007). Invasive *Chaetomium* Infection in Two Immunocompromised Pediatric Patients. Pediatric Infectious Disease Journal, 26, 456-458.

Al-Tai, A., Abdul-Nour, B.A., Abdul-Razzak, S. H. (1989). Cellulase Production from Actinomycetes isolated from Iraqi soils: Characterisation of a Cellulolytic *Streptomyces* Sp.Strain AT7. Journal of Islamic Academy of Sciences, 2, 109-112.

Allsopp, D., Seal, K.J., Gaylarde, C. (2004). Introduction to Biodeterioration. Cambridge: Cambridge University Press.

Allum, L., Bevan, K., Cleveland, D. (2007). Mould - Present Situations and Recommendations.

Alpendurada, M. F. (2000). Solid phase microextraction: a promising technique for sample preparation in environmental analysis. Jounal of Chromatography A, 889, 3-14.

Anderson, B., Nielson, K.F., Jarvis, B. B. (2002). Characterization of *Stachybotrys* from water-damaged buildings based on morphology, growth and metabolite production. Mycologia, 94(3), 392-403.

Anon. (n.d.). Nitrocellulose. Retrieved November 11, 2010, from http://www.fact-index.com/n/ni/nitrocellulose.html

Anon. (Autonose Manufacturing Ltd). (2011). Positively something to be sniffed at''. Retrieved July 6, 2011, from http://autonose.com/default.aspx

Anon. (Academy of Motion Picture Arts and Science). (n.d.). User's Guide For A-D Strips Film Base Deterioration Monitors. Retrieved June 26, 2012, from http://www.cwaller.de/english.htm?ea_d_strips.htm~information

Anon. (Agilent Technologies, Inc). (2005). GC Inlets An Introduction. Agilent User Manual. Retrieved from http://www.chem.agilent.com/library/usermanuals/public/5958-9468_041007.pdf

Anon. (American Biological Safety Association (ABSA)). (n.d.). Risk Group Classification for Infectious Agents - Fungi Search. Retrieved from http://www.absa.org/riskgroups/fungi.html

Anon. (Belgian Biosafety Server). (2006). International classification schemes for micro-organisms based on their biological risks. Retrieved May 30, 2012, from http://www.biosafety.be/ra/class/classint.html

Anon. (British Film Institute). (n.d.). What the archive contains. London. Retrieved July 21, 2012, from http://www.bfi.org.uk/archive-collections/about-bfi-national-archive/what-archive-contains

Anon. (CABI). (2012). CABI. Retrieved May 29, 2012, from www.cabi.org/

Anon. (Centers for Disease Control and Prevention). (2012). Aspergillosis. Retrieved February 14, 2012, from www.cdc.gov/fungal/aspergillosis/

Anon. (Chemical Book). (n.d.). Chemical Book. Chemical Book. Retrieved March 15, 2012, from www.chemicalbook.com

Anon. (Council on Library and Information Resources). (1997). Digitization as a Means of Preservation? European Commision on Preservation and Access. Retrieved June 22, 2010, from http://www.clir.org/pubs/reports/digpres/digpres1.html

Anon. (DAEFH). (2011). A digital agenda for European Film Heritage Study (DAEFH).

Anon. (Divinely Toxic). (n.d.). Divinely Toxic-Mycotoxins, Toxic Mold, *Candida* and You. Retrieved May 30, 2012, from http://divinelytoxic.blogspot.co.uk/p/types-of-mold.html

Anon. (Eagle TV). (2011). Welcome to Eagle TV- Video Production, Photography and Audio Visual Conversions. Retrieved July 27, 2011, from http://www.eagletv.co.nz/

Anon. (EarlyCinema.com). (n.d.). An Introduction to Early Cinema- Timeline. Retrieved July 21, 2012, from http://www.earlycinema.com/timeline/index.html

Anon. (FlexSmell). (2009). FlexSmell. Retrieved June 27, 2012, from http://www.flexsmell.eu/project-objectives.html

Anon. (Guidechem). (n.d.). 3-Octanol. Retrieved July 21, 2012, from http://www.guidechem.com/dictionary/589-98-0.html

Anon. (Harvard University). (n.d.). Library Preservation at Harvard. Retrieved August 18, 2011, from http://preserve.harvard.edu/

Anon. (Health Protection Agency (HPA)). (2012). Health Protection Agency. Retrieved May 29, 2012, from www.hpa.org.uk

Anon. (Image Permanence Institute (A)). (2010). A-D Strips. (I. P. Institute, Ed.). Image Permanence Institute. Retrieved August 27, 2010, from http://www.imagepermanenceinstitute.org/shtml_sub/cat_adstrips.asp

Anon. (Image Permanence Institute (B)). (2012). A-D Strips. Retrieved June 29, 2012, from https://www.imagepermanenceinstitute.org/imaging/ad-strips

Anon. (International Commission on Microbiological Specifications for Foods). (1996). Toxigenic fungi: *Aspergillus*. Microorganisms in Foods: Microbiological specifications of food pathogens, Volume 5. London, UK: Kluwer Academic/ Plenum Publishers.

Anon. (International Federation of Library Associations and Institutions). (n.d.). Typology and history. Retrieved July 27, 2011, from http://archive.ifla.org/VI/6/dswmedia/en/txt_magn.htm

Anon. (Kodak (A)). (n.d.). Storage and Handling of Processes Nitrate Film. Cinema and Television. Retrieved February 29, 2012, from http://motion.kodak.com/motion/Support/Technical_Information/Storage/storage_nitrate.htm

Anon. (Kodak (B)). (n.d.). Storage Information. Cinema and Television. Kodak. Retrieved June 25, 2012, from http://motion.kodak.com/motion/Support/Technical_Information/Storage/storage_info.htm

Anon. (LGC). (n.d.). LGC Advanced Catalog Search. Retrieved May 30, 2012, from http://www.lgcstandards-atcc.org/LGCAdvancedCatalogueSearch/tabid/961/Default.aspx?CollectionID=109

Anon. (Latex Free Gloves). (n.d.). Latex Free Gloves. Retrieved July 10, 2012, from http://latexfreegloves.net/

Anon. (Lenntech). (2009). Biocides. Retrieved June 23, 2012, from http://www.lenntech.com/biocides.htm

Anon. (Milligan and Higgins). (2009). Edible Gelatins and Technical Gelatins. Johnstown, NY: Milligans and Higgins. Retrieved September 17, 2009, from http://www.milligan1868.com/gelatins.html

Anon. (Mold Testing Lab). (n.d.). Mold Testing Lab. Retrieved May 29, 2012, from http://www.moldtestinglab.com/

Anon. (Mold and Bacteria Consulting Laboratories (MBL)). (n.d.). What is Mold? Mold in Homes and Their Hazard Classes. Retrieved May 30, 2012, from http://www.moldbacteria.com/learnmore/moldlist.html

Anon. (Mould Facts). (n.d.). Types of Mould. Retrieved May 31, 2012, from http://www.mouldfacts.ca/mouldtypes.html

Anon. (Munters). (n.d.). About Munters. Huntingdon, UK: Munters. Retrieved April 27, 2010, from http://www.munters.co.uk/en/uk/About-Munters/

Anon. (National Film and Sound Archive Australia (A)). (n.d.). Base polymer decomposition. Film Preservation Handbook. Retrieved July 20, 2012, from http://www.nfsa.gov.au/preservation/handbook/base-polymers-and-decomposition/base-polymer-decomposition/

Anon. (National Film and Sound Archive Australia (B)). (n.d.). Film Base Polymers. Film Preservation Handbook. Southeast Asia-Pacific Audiovisual Archive Association (SEAPAVAA). Retrieved July 20, 2012, from http://www.nfsa.gov.au/preservation/handbook/base-polymers-and-decomposition/film-base-polymers/

Anon. (National Film and Sound Archive Australia (C)). (n.d.). Vinegar Syndrome. Retrieved June 21, 2012, from http://www.nfsa.gov.au/preservation/glossary/vinegar-syndrome

Anon. (National Film and Sound Archive). (2008). Technical Glossary of Common Audiovisual Terms - Nitrate. Retrieved April 15, 2010, from http://www.nfsa.gov.au/preservation/audiovisual_terms/audiovisual_item.php?term=Nitrate

Anon. (National Photographic Society). (1999, September). Identification of Film-Base Photographic Materials. Conserve O Gram, 14/9. Washington DC, USA: U.S. Government Printing Office, Washington.

Anon. (Nebraska State Historical Society). (n.d.). Care of Nitrate and Acetate Films. Saving Your Treasures. Retrieved July 20, 2012, from http://www.netnebraska.org/extras/treasures/pdfs/Care of Nitrate and Acetate Film.pdf

Anon. (New York State Department of Health). (2011). Information About Mold. Retrieved July 2, 2012, from http://www.health.ny.gov/publications/7287/

Anon. (Northeast Document Conservation Center). (2007). Repairing Paper Artifacts.

Retrieved June 22, 2012, from http://www.nedcc.org/resources/leaflets/7Conservation_Procedures/03RepairingPaperArtifacts.php

Anon. (Oxoid). (n.d.). Dehydrated Culture Media- Corn Meal Agar. Retrieved July 21, 2012, from http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0103&org=19&c=UK&lang=EN

Anon. (PB Gelatins). (n.d.). Production Process. Retrieved July 21, 2012, from http://www.pbgelatins.com/about-gelatin/production-process/

Anon. (Parallels). (2011). Storage. (A. of C. and V. Laboratories, Ed.)Association of Cinema and Video Laboratories. Association of Cinema and Video Laboratories. Retrieved February 13, 2012, from www.acvl.org/avcl_manual/storage5.html

Anon. (Public Health Agency of Canada). (2005). Biological Safety Cabinets. Retrieved July 21, 2012, from http://www.phac-aspc.gc.ca/publicat/lbg-ldmbl-04/ch9-eng.php

Anon. (Quartec). (n.d.). Quartec. Retrieved May 29, 2012, from http://www.quartec-bio.co.uk/

Anon. (RTBot). (n.d.). E-6 Process. Retrieved June 25, 2012, from http://www.rtbot.net/e-6_process

Anon. (SGE). (n.d.). Olfactory Detector Outlet Sniffer Unit'' - ODO II. Retrieved March 9, 2011, from www.sge.com/root/pdfs_local/technical_articles/TA-0062-A.pdf

Anon. (Science Lab (A)). (n.d.). Material Safety Data Sheet Iodine (MSDS). sciencelab. Retrieved August 4, 2011, from http://www.sciencelab.com/msds.php?msdsId=9927547

Anon. (Science Lab (B)). (n.d.). Material Safety Data Sheet Congo red MSDS. Retrieved August 4, 2011, from http://www.sciencelab.com/msds.php?msdsId=9927502

Anon. (Scientific Instrument Services). (n.d.). Tenax TA Breakthrough Volume Data. (S. I. Services, Ed.). Scientific Instrument Services. Retrieved January 7, 2011, from http://www.sisweb.com/index/referenc/tenaxta.htm

Anon. (Sheffield Hallam University). (n.d.). Gas Chromatography. (S. H. University, Ed.). Sheffield Hallam University. Retrieved June 26, 2012, from http://teaching.shu.ac.uk/hwb/chemistry/tutorials/chrom/gaschrm.htm

Anon. (Sky News). (2008). Man Killed By Compost. (S. News, Ed.). Sky News. Retrieved July 21, 2012, from http://news.sky.com/skynews/Home/Sky-News-Archive/Article/20080641318945

Anon. (Solex). (n.d.). Loss Control Technology. Houston, Texas, USA: Solex. Retrieved April 27, 2010, from http://www.solexrobotics.com/ses/emergencyservices/ses.html

Anon. (Specialist Mould Removal Services). (2012). Specialist Mould Removal Services. Retrieved May 29, 2012, from http://specialistmouldremoval.co.uk/

Anon. (The *Aspergillus* Website). (2007). The First Reported Case of Aspergillosis. *Aspergillus* Newsletter. Retrieved June 22, 2012, from http://www.aspergillus.org.uk/updates/NewsletterOctober2009.html

Anon. (The Good Scents Company (A)). (2011). 3-octanone. (T. G. S. Company, Ed.). The Good Scents Company. Retrieved April 19, 2011, from http://www.thegoodscentscompany.com/data/rw1004231.html

Anon. (The Good Scents Company (B)). (2011). 1-octen-3-ol. Retrieved April 19, 2011, from http://www.thegoodscentscompany.com/data/rw1024051.html

Anon. (The Screen Heritage UK (SHUK). (2011). Screen Heritage Moving Image Collections Handbook. Retrieved May 29, 2012, from http://issuu.com/rpolding/docs/screenheritagemovingimagecollectionshandbook

Anon. (The Titi Tudorancea Learning Center). (n.d.). Silicone rubber. Retrieved July 21, 2012, from http://www.tititudorancea.com/z/silicone_rubber.htm

Anon. (UC Davis Health System). (2009). Biological Hazards- Recognition Evaluation and Control. Retrieved May 30, 2012, from http://www.ucdmc.ucdavis.edu/medresearch/medsp/includes/33BiologicalHazards.pdf

Anon. (University Hospital of South Manchester). (n.d.). What is Aspergillosis? Retrieved June 22, 2012, from http://www.uhsm.nhs.uk/nac/Pages/WhatisAspergillosis.aspx

Anon. (VideoOlson). (n.d.). Videotape Formats. Retrieved July 21, 2011, from http://www.ultimatewebdesigning.com/articles/formats.html

Anon. (WaterActivity.org.). (2011). What is water activity? Retrieved July 21, 2012, from http://www.wateractivity.org/theory.html

Anon. (Web of Science). (n.d.). Web of Science Citation Report - A Rapid and Easy Method for the Detection of Microbial Cellulases on Agar Plates Using Gram's Iodine. Web of Knowledge. Retrieved May 23, 2012, from http://apps.webofknowledge.com/CitationReport.do?product=WOS&search_mode=CitationReport&SID=W2hkLhAgaGekC2Hj9oO&page=1&cr_pqid=1&viewType=summary

Anon. (animemusicvideos). (2002). The Ins and Outs of Video Compression. Retrieved April 13, 2012, from http://www.animemusicvideos.org/guides/avtech/video3.htm

Anon. (eNotes). (n.d.). Videotape. Retrieved June 21, 2012, from http://www.enotes.com/videotape-reference/videotape

Anon. (video99). (2011). VHS/SVHS transfers to DVD from video99.co.uk. Retrieved July 27, 2011, from http://www.video99.co.uk/vhs_svhs_to_dvd_transfers.html

Apter, A., Bracker, A., Hodgson, M., Sidman, J., Leung, W.-Y. (1994). Epidemiology of the sick building syndrome. Journal of Allergy and Clinical Immunology, 94, 277-287.

Apuhan, T., Kucukbayrak, A., Hakyemez, I. (2011). Fungal sinusitis in immunocompromised hosts. African Journal of Microbiology Research, 5, 1577-1580.

Apun, K. (1995). Cellulase Production. Practical Biotechnology. Sarawak, Malaysia: National Centre for Biotechnology Education.

Arslan-Alaton, I. (2007). Degradation of a commercial textile biocide with advanced oxidation processes and ozone. Journal of Environmental Management, 82, 145–154.

Arai, H. (2000). Foxing caused by Fungi: twenty-five years of study. International Biodeterioration and Biodegredation, 46, 181-188.

Assante, G., Camarda, L., Locci, R., Merlini, L., Nasini, G., Papadopoulos, E. (1981). Isolation and Structure of Red Pigments from *Aspergillus flavus* and Related Species, Grown on Differential Medium. Journal of Agricultural and Food Chemistry, 29, 785-787.

Assouline-Dayan, Y., Leong, A., Shoenfeld, Y., Gershwin, E. (2002). Studies of Sick Building Syndrome. IV. Mycotoxicosis. Journal of Asthma, 39, 191-201.

Baby, R.E., Cabezas, M., Walsö de Reca, E. N. (2000). Electronic nose: a useful tool for monitoring environmental contamination. Sensors and Actuators B, 69, 214-218.

Bai, H., Shi, G. (2007). Gas Sensors Based on Conducting Polymers. Sensors, 7, 267-307.

Baietto, M., Wilson, A.D., Bassi, D., Ferrini, F. (2010). Evaluation of Three Electronic Noses for Detecting Incipient Wood Decay. Sensors, 10, 1062-1092.

Baker, S. E. (2006). *Aspergillus niger* genomics: Past, present and into the future. Medical Mycology, 44, 517-521.

Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M. (2008). Biological effects of essential oils - A review. Food and Chemical Toxicology, 46, 446-475.

Ball, S., Clark, S., Winsor, P. (1998). Collections Link - The anatomy of a film. Care of Photographic Materials and Related Media. Retrieved September 29, 2009, from http://www.collectionslink.org.uk/special_collection/photo_film/cine_film/anatomy_of_film

Banks, C. (n.d.). Screen Printing. Retrieved May 29, 2012, from http://craigbanksresearch.com/page4.html

Barnett, H., Hunter, B. B. (1972). Illustrated Genera of Imperfect Fungi (3rd Editio.). Minnespolis, Minnessota, U.S.A: Burgess Publishing Company.

Barron, M.A., Sutton, D.A., Veve, R., Guarro, J., Rinaldi, M., Thompson, E., Cagnoni, P.J., Moultney, K., Madinger, N. E. (2003). Invasive Mycotic Infections Caused by *Chaetomium perlucidum*, a New Agent of Cerebral Phaeohyphomycosis. Journal of Clinical Microbiology, 41(11), 5302-5307.

Bennett, J.W., Klich, M. (2003). Mycotoxins. Clinical Microbiology Reviews, 16, 497-516.

Berna, A. (2010). Metal oxide sensors for electronic noses and their application to food analysis. Sensors (Basel, Switzerland), 10, 3882-910.

Bertoft, E., Akademi, A. (2004). Analysing starch structure. Starch in Food. Cambridge: Woodhead Publishing Limited.

Bhat, M. K. (2000). Cellulases and related enzymes in biotechnology. Biotechnology Advances, 18, 355-383.

Bigourdan, J-L. Reilly, J.M., Santoro, K., Salesin, G. (2006). The Preservation of Magnetic Tape Collections: A Perspective. National Endowment for the Humanities Division of Preservation and Access.

Bingley, G., & Verran, J. (2012). Counts of fungal spores released during inspection of mouldy cinematographic film and determination of the gelatinolytic activity of predominant isolates. International Biodeterioration and Biodegradation, (In Press).

Bingley, G.D., Verran, J., Munro, L., Banks, C. E. (2012). Identification of microbial volatile organic compounds (MVOCs) emitted from fungal isolates found on cinematographic film. Analytical Methods, 4, 1265-1271.

Bishop, C. A. (2011). 18- Chemical Vapor Deposition/ Polymerization onto Webs. Vacuum Deposition onto Webs, Films and Foils (Second Edition) (319–330). Elsevier Inc.

Black, M.S. Horner, W.E. Morey, P. R. (1999). MVOC and VOC Emission Patterns from Multiple Strains of Indoor Fungi. Indoor Air 99 The 8th International Conference on Indoor Air Quality and Climate. Edinburgh, Scotland: Air Quality Sciences, Inc.

Blank, G., & Corrigan, D. (1995). Comparison of resistance of fungal spores to gamma and electron beam radiation. International journal of food microbiology, 26, 269-77.

Boots, A.W., van Berkel, J.B.N, Dallinga, J.W., Smolinska, A., Wouters, E.F., van, Schooten, F. J. (2012). The versatile use of exhaled volatile organic compounds in human health and disease. Journal of Breath Research, 6, 1-21.

Borjesson, T., Stollman, U., Schnurer, J. (1992). Volatile metabolites produced by six fungal species compared with other indicators of fungal growth on cereal grains. Applied and Environmental Microbiology, 58, 2599-2605.

Brent, R. (n.d.). Investigating Differences in Solubilility Between Crystalline and Amorphous Forms of Pharmaceuticals. Astrazeneca. Retrieved July 20, 2012, from https://docs.google.com/viewer?url=http://www.mmsconferencing.com/pdf/eyp/r.brent.pdf

Brook, P. J. (1975). Effect of Light on Ascospore Discharge by Five Fungi with Bitunicate Asci. New Phytologist, 74, 85-92.

Brooks, G.F., Carroll, K.C., Butel, J.S., Morse, S. A. (2007). Jawetz, Melnick and Adelberg's Medical Microbiology (24th ed.). Appleton and Lange.

Brothers, P. (n.d.). Salvaging Flooded Videotapes. Retrieved July 29, 2011, from http://www.amianet.org/resources/guides/Resource_Salvaging_Flooded_Videotapes.pdf

Bryant, R.J., McClung, A. M. (2011). Volatile profiles of aromatic and non-aromatic rice cultivars using SPME/GC–MS. Food Chemistry, 124, 501–513.

Buchanan, C.H., Gardner, R.M., Komarek, R. J. (1993). Aerobic biodegradation of celluose acetate. Journal of Applied Polymer Science, 74, 1709-1719.

Buttner, M.P., Stetzenbach, L. D. (1993). Monitoring Airborne Fungal Spores in an Experimental Indoor Environment To Evaluate Sampling Methods and the Effects of Human Activity on Air Samplin. Applied and Environmental Microbiology, 59, 219-226.

Buzio, R., Calvini, P., Ferroni, A., Valbusa, U. (2004). Surface analysis of paper documents damaged by foxing. Applied Physics A, 79, 383-387.

Canhoto, O. (2005). Applications of Volatile Fingerprint Sensor Arrays for Rapid Detection of Environmental and Microbiological Contaminants. Institute of Bioscience and Technology. University of Cranfield.

Canhoto, O., Magan, F. (2003). Potential for detection of microorganisms and heavy metals in potable water using electronic nose technology. Biosensors and Bioelectrics, 18, 751-754.

Canhoto, O., Pinzari, F., Fanelli, C., Magan, N. (2004). Application of electronic nose technology for the detection of fungal contamination in library paper. International Biodeterioration and Biodegradation, 54, 303-309.

Cappitelli, F., Sorlini, C. (2005). From Papyrus to Compact Disc: The Microbial Deterioration of Documentary. Critical Reviews in Microbiology, 31, 1-10.

Cappitelli, F., Sorlini, C. (2008). Microorganisms Attack Synthetic Polymers in Items Representing Our Cultural Heritage. Applied and Environmental Microbiology, 74, 564-569.

Casalinuovo, I.A., Di Pierr, D., Coletta, M., Di Francesco, P., (2006). Application of Electronic Noses for Disease Diagnosis and Food Spoilage Detection. Sensors, 6(11), 1428-1439.

Casto, R. (n.d.). How to Dispose of VHS Tapes. Retrieved January 2, 2012, from www.ehow.com/how_5121738_dispose-vhs-tapes.html

Chamilos, G., Kontoyiannis, D. P. (2005). Update on antifungal drug resistance mechanisms of *Aspergillus fumigatus*. Drug Resistance Updates, 8, 344-358.

Chang, J. C. S. (1995). Growth and Evaluation of Fungi (*Penicillium* and *Aspergillus* spp.) on Celing Tiles. Atmospheric Environment, 29, 2331-2337.

Cheng, N.C., Wong, W.W., Fung, C.P., Liu, C. Y. (1998). Unusual pulmonary manifestations of disseminated *Penicillium maneffei* infection in three AIDS patients. Medical Mycology, 6, 429-432.

Child, R. E. (2011). The Prevention and Treatment of Mould Outbreaks in Collections. Preservation Advisory Centre. Retrieved from www.bl.uk/blpac/pdf/mould.pdf

Chitarra, G.S., Abee, T., Rombouts, F.M., Dijksterhuis, J. (2005). 1-Octen-3-ol inhibits conidia germination of *Penicillium paneum* despite of mild effects on membrane permeability, respiration, intracellular pH, and changes the protein composition. FEMS Microbiology Ecology, 54, 67-75.

Ciferri, O. (1999). Microbial Degradation of Paintings. Applied and Environmental Microbiology, 65, 879-885.

Combet, E., Henerson, J., Eastwood, D.C., Burton, K. S. (2006). Eight-carbon volatiles in mushrooms and fungi: properties analysis and biosynthesis. Mycoscience, 47, 317-326.

Dai, L., Soundarrajan, P., Kim, T. (2002). Sensors and sensor arrays based on conjugated polymers and carbon nanotubes. Pure Applied Chemistry, 74, 1753-1772.

D'Arcy, N. (2002). Industrial Biocides Selection and Application. (D. Karsa, D.R., Ashworth, Ed.). The Royal Society of Chemistry.

Deacon, J. W. (1997). Modern Mycology (3rd ed.). Cambridge: Blackwell Science Ltd.

Deacon, J. W. (2006). Fungal Biology 4th Edition. Edinburgh, UK: Blackwell Publishing.

Demyttennaere, J.C.R., Morina, R.M., Kimp, N., Sandra, P. (2004). Use of headspace solid-phase microextraction and headspace sorptive extraction for the detection of the volatile metabolites produced by toxigenic *Fusarium* species. Jounal of Chromatography A, 1027, 147-154.

Demyttennaere, J.CR., Morina, R.M., Sandra, P. (2003). Monitoring and fast detection of mycotoxin-producing fungi based on headspace solid-phase microextraction and headspace sorptive extraction of the volatile metabolites. Journal of Chromatography A, 985, 127-135.

Dewettinck, T., Hege, K., Verstraete, W. (2001). The electronic nose as a rapid sensor for volatile compounds in treated domestic wastewater. Water Research, 35, 2475-2483.

Dickert, F.L., Hayden, O., Zenkel, M. E. (1999). Detection of Volatile Compounds with Mass-Sensitive Sensor Arrays in the Presence of Variable Ambient Humidity. Analytical Chemistry, 71, 1338-1341.

Drean, G., Mounier, J., Vasseur, V., Arzur, D., Habrylo, O., Barbier, G. (2010). Quantification of *Penicillium camemberti* and *P.roqueforti mycelium* by real-time PCR to assess their growth dynamics during ripening of cheese. International Journal of Food Microbiology, 138, 100-107.

Edge, M., Hayes, M., Mohammadian, M., Allen, N.S., Jewitt, T.S., Brems, K., Jones, K. (1991). Aspects of poly(ethylene terephthalate) degradation for archival life and environmental degradation. Polymer Degradation and Stability, 32, 131-153.

El-Barbri, N., Amari, A., Vinaixa, M., Bouchikhi, B., Correig, X., Llobet, E. (2007). Building of a metal oxide gas-sensor-based electronic nose to assess the freshness of sardines under cold storage. Sensors and Actuators B, 128, 235-244.

Elke, K., Begerow, J., Opperman, H., Kramer, U., Jermann, E., Dunemann, L. (1999). Determination of selected microbial volatile organic compounds by diffusive sampling and dual-column capillary GC-FID-a new feasible approach for the detection of an exposure to indoor mould fungi? Journal or Environmental Monitoring, 5, 445-452.

Escuderos, M.E., Sánchez, S., Jiménez, A. (2011). Application of a Quartz Crystal Microbalance (QCM) System Coated with Chromatographic Adsorbents for the Detection of Olive Oil Volatile Compounds. Journal of Sensor Technology, 1, 1-8.

Espinel-Ingroff, A., Fothergill, A., Peter, J., Rinaldi, M.G., Walsh, T. J. (2002). Testing Conditions for Determination of Minimum Fungicidal Concentrations of New and Established Antifungal Agents for *Aspergillus* spp: NCCLS Collaborative Study. Journal of Clinical Microbiology, 40, 3204-3208.

Everiss, B. (2008). British Film Institute National Archive Gaydon. Retrieved June 22, 2012, from http://harbury.villagebuzz.co.uk/viewtopic.php?f=16&t=57

Fallah, P., Burge, H.A., Gallup, J.M. (2004). Exposure potential of other common Penicillium species in indoor environments. Journal of Allergy and Clinical Immunology, 113, S223.

Fiedler, K., Schütz, E., & Geh, S. (2001). Detection of microbial volatile organic compounds (MVOCs) produced by moulds on various materials. International journal of hygiene and environmental health, 20, 111-21.

Fischer, G., Schwalbe, R., Moller, M., Ostrowski, R., Dott, W. (1999). Species-Specific Production of Microbial Volatile Organic Compounds (MVOC) By Airborne Fungi From A Compost Facility. Chemosphere, 39, 795-810.

Fitzgerald, R. (2001). Brittle Paper. Retrieved June 21, 2012, from http://faculty.msmary.edu/fitzgerald/brittle_paper.htm

Flemming, H. C. (1998). Relevence of biofilms for the biodeterioration of surfaces of polymeric materials. Polymer Degradation and Stability, 59, 309-315.

Florian, M. E. (2002). Fungal Facts - Solving fungal problems is heritage collections. Archetype Publications.

Fogarty, R.V., Tobin, J. M. (1996). Fungal melanins and their interactions with metals. Enzyme Microbiology Technology, 19, 311-317.

Foruk, M.J., Uhlman, S., Baker, D.B., Yang, H. (2001). Analysis of microbial contamination of a ventilation system detected by measurement of microbial volatile organic compounds. In E. Johanning (Ed.), Bioaerosols, Fungi and Mycotoxins: Health Effects, Assessment, Prevention and Control. New York: Boyd Printing.

Francois, C. (2009). What does Acid-Free’'Mean? Retrieved September 29, 2009, from http://www.wisegeek.com/what-does-acid-free-mean.htm

Frey, F., Susstrunk, S. (2000). Digital Photography-How Long Will It Last. International Symposium on Circuits and Systems, 5 (pp. 113-116). Geneva, Geneve, Switzerland.

Friedman, D. (n.d.). Mold Exposure Standards - Levels of allergenic or toxic mold and how much mold means a problem? Retrieved July 21, 2012, from http://www.inspectapedia.com/sickhouse/Mold_Standards.htm

Fügel, R., Carle, R., Schieber, A. (2005). Quality and authenticity control of fruit purées, fruit preparations and jams—a review. Trends in Food Science and Technology, 16, 433-441.

Fujita, N., Izaki, M., & Inoue, M. (2006). Fabrication of metal-oxide nano-composite films from aqueous solution by metal-oxide co-electrodeposition. Journal of Magnetism and Magnetic Materials, 300, 350–353.

Gabet, E.J., Reichman, O.J., Seabloom, E. W. (2003). The Effects of Bioturbation on Soil Processes and Sediment Transport. Annual Review of Earth and Planetary Sciences, 31, 249-273.

Gallo, F. (1993). Aerobiological research and problems in libraries. Aerobiologia, 9, 117-130.

Gao, P., Martin, J. (2002). Volatile metabolites produced by three strains of *Stachybotrys chartarum* cultivated on rice and gypsum board. Applied Occupational Environmental Hygiene, 17, 430-436.

Garcia-Guinea, J., Cárdenes, V., Martínez, A.T., Martínez, M. J. (2001). Fungal biourbation paths in a compact disk. Naturwissenschaften, 88, 351-354.

Ghannoum, M.A., Rice, L. B. (1999). Antifungal Agents: Mode of Action, Mechanisms of Resistance, and Correlation of These Mechanisms with Bacterial Disease. Clinical Microbiology Reviews, 12, 501-517.

Gibson, T.D., Hulbert, J.N., Prosser, O.C., Pavlou, A. K. (2000). Not to be sniffed at. Microbiology Today, 27. Reading, UK: Society for General Microbiology.

Gill, V. (2009). Sniff test to preserve old books. BBC News. Retrieved June 27, 2012, from http://news.bbc.co.uk/1/hi/8355888.stm

Gioulekas, D., Damialis, A., Papakosta, D., Spieksma, F., Giouleka, P., Patakas, D. (2004). Allergenic fungi spore records (15 years) and sensitization in patients with respiratory allergy in Thessaloniki-Greece. Journal of Investigational Allergology and Clinical Immunology, 14, 225-231.

Glenn, M. (2009). NASA turns iPhone into chemical sniffer. QJ.NET. Retrieved July 21, 2012, from http://www.qj.net/iphone/news/nasa-turns-iphone-into-chemical-sniffer.html

Gopinath, S.C.B., Anbu, P., Hilda, A. (2005). Extracellular enzymatic activity profiles in fungi isolated from oil-rich environments. Mycoscience, 46, 119-126.

Green, P. W. C. (2008). Fungal isolates involved in biodeterioration of book-paper and their effects on substrate selection by Liposcelis bostrychophil (Badonnel)(Psocoptera: Liposcelididae). Journal of Stored Products Research, 44, 258-263.

Guiamet, P., Borrego, S., Lavin, P., Perdomo, I., de Saravia, S. G. (2011). Biofouling and biodeterioration in materials stored at the Historical Archive of the Museum of La Plata, Argentine and at the National Archive of the Republic of Cuba. Colloids and Surfaces B. Biointerfaces, 85, 229-234.

Hammer, K.A., Carson, C.F., Riley, T. V. (1999). Antimicrobial activity of essential oils and other plant extracts. Applied Microbiology, 86, 985-990.

Hanlin, R. (1994). Microcycle conidiation. Mycoscience, 35, 113-123.

Hansen, T., Petersen, M.A., Byrne, D. V. (2005). Sensory based quality control utilising an electronic nose and GC-MS analyses to predict end-product quality from raw materials. Meat Science, 69, 621-634.

Hessler, L.E., Power, R. E. (1954). The Use of Iodine Absorption as a Measure of Cellulose Fiber Crytallinity. Textile Research Journal, 24, 822-827.

Hewitt, M. (2006). Welcome to North West Film Archive. Retrieved July 21, 2012, from www.nwfa.mmu.ac.uk/welcome.htm

Hocking, A. D. (2006). *Aspergillus* and related teleomorphs. Food Spoilage microorganisms (p. 474).

Hoog, G.S., Guarro, J. (1995). Atlas of Clinical Fungi. Centraalbureau voor Schimmelcultures/ Universitat Rovira i Virgili Reus, Spain.

Hossain, M.A., Ahmed, M.S., Ghannoum, M. A. (2004). Attributes of *Stachybotrys chartarum* and its association with human disease. Journal of Clinical Immunology, 113, 200-208.

Hu, F., Johnson, L., Kennedy, K., Portnoy, J.M., Barnes, C. S. (2006). Indoor Spore Levels in Asthmatic and Control Homes. Journal of Allergy and Clinical Immunology, 117.

Hudson, H.J. (1986). Lignin as a physical barrier to cellulase. Fungal Biology (p. 92). Cambridge University Press.

Hübschmann, H.-J. (2008). Handbook of GC/MS. John Wiley and Sons, Inc.

Hueck, H. J. (1968). The biodeterioration of materials - an appraisal. Biodeterioration of Materials, 6-12. Elsevier, London.

Hulanicki, A., Glab, S., Ingman, F. (1991). No Title. Pure and Applied Chemistry, 6, 1247-1250.

Ingold, C. T. (1971). Fungal Spores: Their Liberation and Dispersal. Oxford University Press.

Ingold, C. T. (1984). The Biolody of Fungi (5th ed.). London: Hutchinson & Co. Ltd.

Insam, H., Seewald, M. S. A. (2010). Volatile organic compounds (VOCs) in soils. Biology and Fertility of Soils, 46, 199-213.

Ivnitski, D., Abdel-hamid, I., Atanasov, P., & Wilkins, E. (1999). Biosensors for detection of pathogenic bacteria. Biosensors and Bioelectronics, 14, 599-624.

Jacobs, D., Jenner, S.J., Parker, A., French, D. (2001). Integrated Pest Management. The British Library. Retrieved May 31, 2012, from http://www.bl.uk/aboutus/stratpolprog/ccare/pubs/archivepubs/integratedpestmanagement.pdf

Jacobs, R.L., Thorner, R.E., Holcomb, J.R., Schwietz, L.A., Jacobs, F. R. (1986). Hypersensitivity Pnemonitis Caused by *Cladosporium* in an Enclosed Hot-Tub Area. Annals of Internal Medicine, 105, Retrieved May 31, 2012, from http://www.bl.uk/aboutus/stratpolprog/ccare/pubs/archive/integratedpestmanagement.pdf

Jellison, J., Goodell, B., Daniel, G. (2008). The Biology and Microscopy of Building Molds: Medical and Molecular Aspects. Development of Commercial Wood Preservatives (Vol. 982, pp. 182-197). ACS Symposium Series.

Jo, W.K., Seo, Y. J. (2005). Indoor and Outdoor bioaerosol levels at recreational facilities, elementary schools and homes. Chemosphere, 61, 1570-1579.

Jonsdottir, R., Olafsdottir, G., Martinsdottir, E., Stefansson, G. (2004). Flavor Characterisation of Ripened Cod Roe by Gas Chromatography, Sensory Analysis, and Electronic Nose. Agricultural and Food Chemistry, 52, 6250-6256.

Juwaied, A.A., Al-amiery, A.H., Abdumuniem, Z., Anaam, U. (2011). Optimisation of cellulase production by *Aspergillus niger* and *Trichoderma viride* using sugar cane waste. Journal of Yeast and Fungal Research, 2, 19-23.

Kaminski, E., Stawicki, S., Wasowicz, E. (1974). Volatile Flavor Compounds Produced by Molds of *Aspergillus, Penicillium*, and Fungi imperfecti. Applied Microbiology, 27, 1001-1004.

Kanan, S. M., El-Kadri, O. M., Abu-Yousef, I. a, & Kanan, M. C. (2009). Semiconducting metal oxide based sensors for selective gas pollutant detection. Sensors (Basel, Switzerland), 9, 8158-96.

Kanemitsu, K., Nishino, T., Kunishima, H., Okamura, N., Takemura, H., Yamamoto, H., Kaku, M. (2001). Quantitative determination of gelatinase activity among enterococci. Journal of Microbiolgical Methods, 47, 11-16.

Kasana, R.C., Dhar, R.S.H., Dutt, S., Gulati, A. (2008). A Rapid and Easy Method for the Detection of Microbial Cellulases on Agar Plates Using Grams's Iodine. Current Microbiology, 57, 503-507.

Kataoka, H., Lord, H., Pawliszyn, J. (2000). Applications of solid-phase microextraction in food analysis. Jounal of Chromatography A, 880, 35-62.

Kenkel, J. (2002). Analytical Chemistry for Technicians (Third Edition). CRC Press, USA.

Keshri, G., Magan, N., Voysey, P. (1998). Use of an electronic nose for the early detection and differentiation between spoilage fungi. Letters of Applied Microbiology, 27, 261-264.

Khan, M.R., Saha, M.L., Zuha, B. (2008). Bacteria and Actinomycetes Growing on Floppy and Compact Discs Under Ambient Conditions. Bangladesh Journal of Botany, 37, 7-14.

Korpi, A., Pasanen, A.L., Pasanen, P. (1998). Volatile Compounds Originating from Mixed Microbial Cultures on Building Materials under Various Humidity Conditions. Applied and Environmental Microbiology, 64, 2914-2919.

Korpi, A., Pasanen, A.L., Pasanen, P., Kalliokoski, P. (1997). Microbial Growth and Metabolism in House Dust. International Biodeterioration and Biodegradation, 40, 19-27.

Krogh, P. (n.d.). Backup Overview. American Society of Media Photographers. Retrieved July 29, 2011, from http://www.dpbestflow.org/backup/backup-overview

Kuhn, D.M., Ghannoum, M. A. (2003). Indoor Mold, Toxigenic Fungi, and *Stachybotrys chartarum*: Infectious Disease Perspective. Clinical Microbiology Reviews, 16, 144-172.

Kuske, M., Padilla, M., Romain, a. C., Nicolas, J., Rubio, R., & Marco, S. (2006). Detection of diverse mould species growing on building materials by gas sensor arrays and pattern recognition. Sensors and Actuators B: Chemical, 119, 33-40.

Kuske, M., Romain, A.C., Nicolas, J. (2005). Microbial volatile organic compounds as indicators of fungi. Can an electronic nose detect fungi in indoor environments? Building and Environment, 40, 824-832.

Lamb, J., Loy, T. (2005). Seeing red: The use of a biological stain to identify cooked and processed/ damaged starch grains in archaeological residues. Journal of Archaeological Science, 32, 1433-1440.

Lear, J. C., Maillard, J.-Y., Dettmar, P. W., Goddard, P. a., & Russell, A. D. (2006). Chloroxylenol- and triclosan-tolerant bacteria from industrial sources—susceptibility to antibiotics and other biocides. International Biodeterioration & Biodegradation, 57, 51–56.

Lehtonen, M., Reponen, T., Nevalainen, N. (1993). Everyday Activities and Variation of Fungal Spore Concentrations in Indoor Air. International Biodeterioration and Biodegradation, 31, 25-39.

Levetin, E., Shaughnessy, R., Fisher, E., Ligman, B., Harrison, J., Brennan, T. (1995). Indoor air quality in schools:exposure to fungal allergens. Aerobiologia, 11, 27-34.
Lewis, H.L., Darnall, D. W. (1970). Fatty Acid Toxicity and Methyl Ketone Production in Aspergillus niger. Journal of Bacteriology, 101(1), 65-67.

Li, C., Krewer, G., Ji, P., Scherm, H., Kays, S. J. (2010). Gas sensor array for blueberry fruit disease detection and classification. Postharvest Biology and Technology, 55, 144-149.

Liang, Y., Zhao, W., Xu, J., Miller, J. D. (2011). Characterization of two related exoantigens from the biodeteriogenic fungus *Aspergillus versicolor*. International Biodeterioration and Biodegradation, 65, 217-226.

Lo Cascio, G., Ligozzi, M., Maccacaro, L., Fontana, R. (2004). Utility of Molecular Identification in Opportunistic Mycotic Infections: a Case of Cutaneous *Alternaria infectoria* Infection in a Cardiac Transplant Patient. Journal of Clinical Microbiology, 42, 5334-5336.

Lourenco, M.J.L., Sampaio, J. P. (2009). Microbial deterioration of gelatin emulsion photographs: Differences of susceptibility between black and white and colour materials. International Biodeterioration and Biodegradation, 63, 496-502.

Lynd, L.R., Weimer, P.J., Zyl, W.H., Pretorious, I. S. (2002). Microbial Cellulose Utilisation: Fundamentals and Biotechnology. Microbiology and Molecular Biology Reviews, 55, 506-577.

Maggi, O., Persiani, A.M., Gallo, F., Valenti, P., Pasquariello, G., Sclocchi, M.C., Scorrano, M. (2000). Airborne fungal spores in dust present in archives: proposal for a detection method, new for archival materials. Aerobiologia, 16, 429-434.

Magnuson, J.K., Lasure, L. L. (2002). Fungal diversity in soils as assessed by direct culture and molecular techniques. Pacific Northwest National Laboratory: Richland, Washington, USA. P.

Mahmoudi, E. (2009). Electronic Nose Technology and its Applications. Sensors and Transducers, 107, 17-25.

Mandrioli, P., Caneva, G., Sabbioni, C. (2004). Cultural Heritage and Aerobiology: Methods and Measurement Techniques for Biodeterioration Monitoring (pp. 184-190). Kluwer Academic Publishers.

Matsuoka, Y., Takeawa, Y., Teraoka, T., H. I. (1990). Evaluation of antifungal volatile compounds on the basis of elongation rate of a single hypha. Applied and Environmental Microbiology, 56, 3779-3784.

Mattheis, J., Roberts, R. G. (1992). Identification of Geosmin as a Volatile Metabolite of *Penicillium expansum*. Applied and Environmental Microbiology, 58, 3170-3172.

Mattox, D. R. (1998). 6- Physical Sputtering and Sputter Deposition (Sputtering). Handbook of Physical Vapor Deposition (PvD) Processing: Film Formation, Adhesion, Surface Preparation and Contamination Control (343–405). New York, USA: William Andrew.

Matysik, S., Herbarth, O., Mueller, A. (2008). Determination of volatile metabolites originating from mould growth on wallpaper and selective media. Journal of Microbiological Methods, 75, 182-187.

Mauseth, J. D. (2009). Botany: an introduction to plant biology 4th Edition. London, UK: Jones and Bartlett Publishers.

McCain, J.W., Mirocha, C. J. (1994). Screening Computer Diskettes and Other Magnetic Media for Susceptibility to Fungal Colonisation. International Biodeterioration and Biodegradation, 255-268.

McCrady, E. (2010). Mold: Health Dangers for Museum Staffers. Retrieved July 4, 2012, from http://douglasfairbanks.org/2010/04/10/assessment-of-mold-problems/

McNair, H.M., Miller, J. M. (1998). Basic Gas Chromatography. Techniques in Analytical Chemistry (p. 200). John Wiley and Sons, Inc.

Mcginnis, M.R., Nilson, D.A., Ware, L. L. (1975). Mycotic Biodeterioration Associated with the Movement and Storage of Commercially Handled Household Goods. Mycopathologia, 57, 41-45.

Meruva, N.K., Penn, J.M., Farthing, D. E. (2004). Rapid identification of microbial VOCs from tobacco molds using closed-loop stripping and gas chromatography/ time-of-flight mass spectrometry. Journal of Industrial Microbiology Biotechnology, 31, 482-488.

Meurens, M. (2003). Spectrophotometric techniques. In M. Lees (Ed.), Food authenticity and traceability. Cambridge, England: Woodhead Publishing Limited.

Michaelsen, A., Pinzari, F., Ripka, K., Lubitz, W., Pinar, G. (2006). Application of molecular techniques for identification of fungal communities colonising paper material. International Biodeterioration and Biodegradation, 58, 133-141.

Mitchell, R., Mcnamara, C. J. (2010). Textiles. In P. Garside (Ed.), Cultural Heritage Microbiology: Fundamental Studies in conservation science (pp. 97-110). Washington, DC: American Society for Microbiology Press.

Moss, M.O., Jarvis, B., Skinner, F. A. (1989). Filamentous Fungi in Foods and Feeds. Society for Applied Bacteriology Symposium Series Number 18 (Vol. Supplement). Blackwell Scientific Publications.

Moularat, S., Robine, E., Ramalho, O., Oturan, M. A. (2008). Detection of fungal development in closed spaces through the determination of specific chemical targets. Chemosphere, 72, 224-232.

Muilenberg, M., Burge, H. (1996). Aerobiology. Pan American Aerobiology Association. Florida, U.S.A: CRC Press, Inc. Lewis Publishers.

Muilenberg, M.L., Rogers, C. A. (2009). Studies On Airborne Fungal Spores From Eight Historical And Modern Buildings. Annual Symposium of the Pan-American Aerobiology Association. Children's Mercy Hospitals and Clinics. Retrieved June 21, 2012, from http://www.paaa.org/Aerobiology2009Abs.pdf

Müller, R-J., Kleeberg, I., Deckwer, W.-D. (2001). Biodegradation of polyesters containing aromatic constituents. Journal of Biotechnology, 86, 87-95.

Nakajima, D., Ishii, R., Kagey, S., Onji, Y., Mineki, S., Morooka, N., Takatori, K., Goto, S. (2006). Genotoxicity of Microbial Volatile Organic Compounds. Journal of Health Science, 52(2), 148-153.

Nakamura, T., Vrinten, P., Hayakawa, K., Ikeda, J. (1998). Characterization of a Granule-Bound Starch Synthase Isoform Found in the Pericarp of Wheat. Plant Physiology, 118, 451-459.

Nevalainen, A., Pasanen, A.l, Nilninen, M., Reponen, T., Kalliokoski, P., Jantunen, M. J. (1991). The Indoor Air Quality in Finnish Homes with Mold Problems. Environment International, 17, 299-302.

Nielson, K. F. (2003). Mycotoxin production by indoor molds. Fungal Genetics and Biology, 39, 113-117.

Nilsson, T., Larsen, T.O., Montanarella, L., Madsen, J. O. (1996). Application of head-space solid-phase microextraction for the analysis of volatile metabolites emitted by *Penicillium* species. Journal of Microbiolgical Methods, 25, 245-255.

Nucci, M., Anaissie, E. (2007). *Fusarium* Infections in Immunocompromised Patients. Clinical Microbiology Reviews, 20(4), 695-704.

Nussbaum, F. (1991). Variation in the airborne fungal spore population of the Tuscarwas Valley. II. A comparison of arboreal and nonarboreal microenvironments. Mycopathologia, 116, 181-198.

Nystrom, J. E. (2001). History of sub-35mm Film Formats and Cameras. Retrieved September 23, 2009, from http://www.sci.fi/~animato/filmhist/filmhist.html

Okada, T., Kawanobe, W., Hayakawa, N., Tsubokura, S., Chujo, R., Fujimatsu, H., Takizawa, T., Hirai, T. (2011). Whitening of polyvinyl alcohol used as restoration material for Shohekiga. Polymer Journal, 43, 74-77.

Onions, A., Allsopp, D., Eggins, H. (1981). Smith's Introduction to Industrial Mycology (Seventh Ed., Vol. Seventh Ed). Edward Arnold Publishers Ltd.

Opela, V. (1992). Fungal and bacterial attack on motion picture film. Joint Technical Symposium, Session 5 (pp. 139-144). Ottawa, Canada: (FIAF).

Owen, D. (2005). The Betamax vs VHS Format War. Retrieved February 2, 2012, from www.mediacollege.com/video/format/compare/betamax-vhs.html

O'Gorman, C.M., Fuller, H. T. (2008). Prevalence of culturable airborne spores of selected allergenic and pathogenic fungi in outdoor air. Atmospheric Environment, 42, 4355-4368.

Paniel, N., Radoi, A., & Marty, J.-L. (2010). Development of an electrochemical biosensor for the detection of aflatoxin M1 in milk. Sensors (Basel, Switzerland), 10, 9439-9448.

Parascandola, P. (1990). Immobilization of Viable Yeast Cells Within Polyaldehyde-Hardened Gelatin Gel. Biotechnology Techniques, 4, 237-242.

Pardo, E., Lagunas, U., Sanchis, V., Ramos, A.J., Marin, S. (2005). Influence of water activity and temperature on conidial germination and mycelial growth of ochratoxigenic isolates of *Aspergillus ochraceus* on grape juice synthetic medium. Predictive models. Journal of the Science of Food and Agriculture, 85, 1681-1686.

Pardo, E., Malet, M., Marin, S., Sanchis, V., Ramos, A. J. (2006). Effects of water activity and temperature on germination and growth profiles of ochratoxigenic *Penicillium verrucosum* isolates on barley meal extract agar. International Journal of Food Microbiology, 106, 25-31.

Pasanen, A.L., Heinonen-Tanski, H.H., Kalliokoski, P. (1992). Fungal Microcolonies on Indoor Surfaces- An Explanation for the Base Level Fungal Spore Counts in Indoor Air. Atmospheric Environment, 26B, 117-120.

Pasanen, A.L., Kalliokoski, P., Pasanen, P. (1991). Laboratory Studies on the Relationship Between Fungal Growth and Atmospheric Temperature and Humidity. Environment International, 17, 225-228.

Pasanen, A.L., Korpi, A., Kasanen, J.P., Pasanen, P. (1998). Critical Aspects on the Significance of Microbial Volatile Metabolites as Indoor Air Pollutants. Environment International, 24, 703-712.

Passarelli, G. R. (2009). Sick building syndrome: An overview to raise awareness. Journal of Building Appraisal, 5, 55-66.

Patkus, B. L. (n.d.). Monitory Temperature and Relative Humidity. 2007.
Retrieved May 31, 2012, from
http://www.nedcc.org/resources/leaflets/2The_Environment/02TemperatureAndHumidity.php

Pavlou, A.K., Turner, A. P. F. (2000). Sniffing out the truth: clinical diagnosis using the electronic nose. Clinical Chemistry and Laboratory Medicine, 38, 99-112.

Pearson, C. L. (2003). Animal Glues and Adhesives. In A. Pizzi Mittal, K.L. (Ed.), Handbook of Adhesive Technology, (Second Edition). CRC Press, USA.

Peciulyte, D. (2007). Isolation of cellulolytic fungi from waste paper gradual recycling materials. Ekologija, 53, 11-18.

Perfect, J.R., Cox, G.M., Lee, J.Y., Kauffman, C.A., Repentigny, L., Chapmany, S.W., Morrison, V.A., Pappas, P., Hiemenz, J.W., Stevens, D. A. (2001). The Impact of Culture Isolation of *Aspergillus* Species: A Hospital-Based Survey of Aspergillosis. Clinical Infectious Diseases, 33, 1824-1833.

Pildain, M.B., Frisvad, J.C., Vaamonde, G., Cabral, D., Varga, J., Samson, R. A. (2008). Two novel aflatoxin-producing *Aspergillus* species from Argentinian peanuts. International Journal of Systematic Evolutionary Microbiology, 58, 725-735.

Pindi, P.K., Kishore, N., Reddy, S.R. (2009). Metagenomics: Principles, Methodology and Biotechnological Potentials. In A. Chauhan, A.K., Varma (Ed.), A Textbook of Molecular Biotechnology (p. 344). I.K. International Publishing House Pvt. Ltd.

Pinzari, F., Fanelli, C., Canhoto, O, Magan, N. (2004). Electronic Nose for the Early Detection of Moulds in Libraries and Archives. Indoor and Built Environment, 13, 387-395.

Pisano, M.A., Mihalik, J.A., Catalano, G. R. (1964). Gelatinase Activity by Marine Fungi. Applied Microbiology, 12, 470-474.

Pitt, J. I. (1994). The current role of Aspergillus and Penicillium in human and animal health. Journal of Medical and Veterinary Mycology, 32, 17-32.

Pitt, J, I., Hocking, A. D. (2009). Fungi and Food Spoilage (3rd ed.). Springer.

Polizzi, V., Adams, A., Picco, A.M., Adriaens, E., Lenoir, J., Van Peteghem, C., De Saeger, D., De Kimpe, N. (2011). Influence of environmental conditions on production of volatiles by Trichoderma atroviride in relation with the sick building syndrome. Building and Environment, 46, 945-954.

Prezant, B., Weekes, D.M., Mill, J. D. (2008). Sampling Methods Using Chemical Analysis. Recognition, Evaluation, and Control of Indoor Mold (p. 144). American Industrial Hygiene Association.

Price, L. O. (1996). Mold: managing a mold invasion: guidlines for a disaster response. Techinical Series No.1, 1. Philadelphia, U.S.A: Conservation Center for Art and Historic Artifacts. Retrieved from http://catalogue.nla.gov.au/Record/2848143

Rakotonirainy, M.S., Lavedrine, B. (2005). Screening for antifungal activity of essential oils and related compounds to control the biocontamination in libraries and archives storage areas. International Biodeterioration and Biodegradation, 55, 141-147.

Rampur, S. (2011). VHS to DVD Converter. Retrieved February 2, 2012, from www.buzzle.com/articles/vhs-to-dvd-converter.html

Raschle, P. (1989). Microbial Influence on Cellulosic Textiles and Microbiological Testing. International Biodeterioration, 25, 237-244.

Refat, M.S., El-Korashy, S.A., Kumar, D.N., Ahmed, A. S. (2008). FTIR, magnetic, H NMR spectral and thermal studies of some chelates of caproic acid: Inhibitory effect on different kinds of bacteria. Spectrochimica Acta, 70, 217–233.

Regazzo-Sanchez, J.A., Chalier, P., Chevalier-Lucia, D., Calderon-Santoyo, M Ghommidh, C. (2009). Off-flavours detection in alcoholic beverages by electronic nose coupled to GC. Sensors and Actuators B, 140, 29-34.

Reuvini, M., Hegkiv, N., Eshel, D., Prusky, D., Ben-Arie, R. (2007). Virulence and the production of endo -1,4-beta-glucanase by isolates of Alternaria alternata involved in the moldy-core disease of apples. Journal of Phytopathology, 155, 50-55.

Richardson, M. D. (2005). Changing patterns and trends in systemic fungal infections. Journal of Antimicrobial Chemotherapy, S1, i5-i11.

Rocha-Gaso, M.-I., March-Iborra, C., Montoya-Baides, Á., & Arnau-Vives, A. (2009). Surface Generated Acoustic Wave Biosensors for the Detection of Pathogens: A Review. Sensors, 9, 5740-5769.

Rockwell, K. (2009). Film: The Real Raw. Retrieved June 22, 2010, from http://www.kenrockwell.com/tech/real-raw.htm

Rosas, I., Calderon, C., Ulloa, M., Lacey, J. (1993). Abundance of Airborne *Penicillium* CFU in Relation to Urbanization in Mexico City. Applied and Environmental Microbiology, 59, 2648-2652.

Rundle, R.E., Foster, J.F., Baldwin, R. R. (1944). On the Nature of the Starch-Iodine Complex. Journal of American Chemical Society, 66, 2116-2120.

Ruzsanyi, V., Baumbach, J., Eiceman, G. (2003). Detection of the Mold Markers Using Ion Mobility Spectrometry. Dortmund, Germany and Las Cruces, USA: Institute of Spectrochemistry and Applied Spectroscopy1 and Department of Chemistry and Biochemistry2.

Ryan, T. (2005). 7.3. Total Fungi and Other Assessment Methods. In H. K. Hung, L-L., Miller, J.D., Dillon (Ed.), Field Guide for the Determination of Biological Contaminants in Environmental Samples (Second Edition). American Industrial Hygiene Association.

Sahgal, N. (2008). Microbial and Non-Microbial Volatile Fingerprints: Potential Clinical Applications of Electronic Nose of Early Diagnoses and Detection of Diseases. Cranfield Health. Cranfield, Bedfordshire: Cranfield University.

Sakai, K., Yamauchi, T., Nakasu, F., Ohe, T. (1996). Biodegradation of cellulose acetate by Neisseria sicca. Bioscience Biotechnology and Biochemistry, 60, 1617-1622.

Salim, S.N.M., Shakaff, A.Y.M., Ahmad, M.N., Adom, A.H., Husin, Z. (2005). Development of electronic nose for fruits ripeness determination. 1st International Conference on Sensing Technology (pp. 515-518).

Salo, P.M., Arbes, S.J., Sever, M., Jaramillo, R., Cohn, R.C., London, S.J., Zeldin, D. C. (2006). Exposure to Alternaria alternata in US homes is associated with asthma symptoms. Journal of Allergy and Clinical Immunology, 118, 892-898.

Samson, R.A., Hoekstra, E.S., Frisvad, J.C., Filten, O. (1996). Introduction to Food-Borne Fungi (5th ed.). Baarn, The Netherlands: Centraalbureau voor Schimmelcultures.

Sánchez-Rodas, D., Corns, W.T., Chen, B., Stockwell, P. B. (2010). Atomic Fluorescence Spectrometry: a suitable detection technique in speciation studies for arsenic, selenium, antimony and mercury. Journal of Analytical Spectrometry, 25, 933–946.

Schaller, E., Bosset, J.O., Escher, F. (1998). “Electronic Noses” and Their Application to Food. Lebensmittel-Wissenschaft und-Technologie, 31, 305-316.

Scherer, T. M. (1996). Biological and enzymatic mechanisms of polyester biodegradation by fungi. University of Massachusetts, Amherst.

Schiffman, S.S., Wyrick, D.W., Gutierrez-Osuna, R., Nagle, H. T. (2000). Effectiveness of an Electronic Nose for Monitoring Bacterial and Fungal Growth. In J. W. Gardner Persaud, K.C., (Ed.), International Symposium on Olfaction and Electronic Nose. Brighton, UK.

Schomburg, G. (1990). Gas Chromatography. New York, USA: VCH Verlagsgesellschaft mbH and VCH Publishers, Inc.

Schüller, D. (2008). Tape- Training for Audiovisual Preservation in Europe. (G. Boston, Ed.)Audio and video carriers. Culture 2000.

Scott, R. P. W. (2003). Chrom-Ed Book Series. Book 4 - Gas Chromatography Detectors. Libraryforscience, LLC.

Scotter, J.M., Langford, V.S., Wilson, P.F., McEwan, M.J., Chambers, S. T. (2005). Real-time detection of common microbial volatile organic compounds from medically important fungi by Selected Ion Flow Tube-Mass Spectrometry (SIFT-MS). Journal of Microbiological Methods, 63, 127-134.

Shabbir, A., Khan, M.A., Khan, A.M., Iqbal, M., Ahmad, F. (2007). Fungal Biodeterioration: A case study in the zoological museum of the Punjab University. Journal of Animal and Plant Sciences, 17, 90-92.

Shamsian, A., Fata, A., Mohajeri, M., Ghazvini, K. (2006). Fungal Contaminations in Historical Manuscripts at Astan Quds Museum Library, Mashhad, Iran. International Journal of Agriculture and Biology, 8, 420-422.

Sharma, P. D. (2004). Plant Pathology (1st ed., p. 49). Rakesh Kumar Rastogi for Rastogi Publications.

Shelton, B.G., Kirkland, K.H., Flanders, W.D., Morris, G. K. (2002). Profiles of Airborne Fungi in Buildings and Outdoor Environments in the United States. Applied and Environmental Microbiology, 68, 1743-1753.

Shenton, H. (2005). Addressing Cellulose Acetetate Microfim from a British Library Perspective. Google docs.

Sherman Hsu, C, P. (1997). Chapter 15- Infrared Spectroscopy. In F. Settle (Ed.), Handbook of Instrumental Techniques for Analytical Chemistry (1st ed., pp. 247-283). Prentice-Hall, Inc. Retrieved from http://www.prenhall.com/settle/html/chapters.html

Shriver, J., Spencer, M. (2005). Advice sought in cleaning film affected by mould and fungus. Retrieved September 14, 2010, from http://photo.net/film-and-processing-forum/00C9ip

Silva, M., Moraes, A.M.L., Nishikawa, M.M., Gatti, M.J.A., Vallim de Alencar, M.A., Brandao, L.E., Nobrega, A. (2006). Inactivation of fungi from deteriorated paper materials by radiation. International Biodeterioration and Biodegradation, 57, 163-167.

Sorenson, W. G. (1999). Fungal Spores: Hazardous to health. Environmental Health Perspectives, 107, 469-472.

Sorenson, W.G., Frazer, D.G., Jarvis, B.B., Simpson, J., Robinson, V. A. (1987). Trichothecene Mycotoxins in Aerosolized Conidia of *Stachybotrys atra*. Applied and Environmental Microbiology, 53, 1370-1375.

Soytong, K., Kanokmedhakul, S., Kukongviriyapa, V., Isobe, M. (2001). Application of *Chaetomium* species (Ketomium) as a new broad spectrum biolgical fungicide for plant disease control: A review article. Fungal Diversity, 7, 1-15.

Stadelmann, I. (2001). Extraction of Alcohols from Gasoline Using Solid Phase Microextraction (SPME). Chemistry. Virginia Polytechnic Institute and State University.

Steane, R. (n.d.). Starch Agar Experiment A. (biotopics.co.uk, Ed.). Retrieved July 21, 2012, from http://www.biotopics.co.uk/nutrition/stagab.html

Sterflinger, K., Pinzari, F. (2012). The revenge of time:fungal biodeterioration of Cultural Heritage in museums and libraries. (SFAM, Ed.)Environmental microbiology, 14, 559-566.

Stoppacher, N., Kluger, B., Zeilinger, S., Krska, R., Schumacher, R. (2010). Identification and profiling of volatile metabolites of the biocontrol fungus Trichoderma atroviride by HS-SPME-GC-MS. Journal of Microbiological Methods, 81, 187-193.

Strom, G., West, J., Wessen, B., Palmgren, U. (1994). Quantitative analysis of microbial volatiles in damp Swedish houses. In Flannigan, B., Flannigan, M.E.,
Verhoef, AP, Adan, O.C.G, Hoekstra, E.S., Samson, R.A. (Ed.), Health Implications of Fungi in Indoor Environments (pp. 291-305). Amsterdam: Elsevier.

Strzelczyk, A. B. (2004). Observations on aesthetic and structural changes induced in Polish historic objects by microorganisms. International Biodeterioration and Biodegradation, 53, 151-156.

Sugar, A. M. (1995). Minireview: Use of Amphotericin B with Azole Antifungal Drugs. Antimicrobial Agents and Chemotherapy, 39, 1907-1912.

Sukumaran, R.K., Singhania, R.R., Pandey, A. (2005). Microbial cellulases - Production, applications and challenges. Journal of Scientific and Industrial Research, 64, 832-844.

Sun, S., Mitchess, J.R., MacNaughtan, W., Foster, T.J., Harabagiu, v., Song, Y., Zheng, Q. (2010). Comparison of the Mechanical Properties of Cellulose and Starch Films. Journal of Biomacromolecules, 11, 126-132.

Sunesson, A.L., Vaes, W.H.J., Nilsson, C.A., Blomquist, G., Andersson, B., Carlson, R. (1995). Identification of Volatile Metabolites from Five Fungal Species Cultivated on Two Media. Applied and Environmental Microbiology, 61, 2911-2918.

Swan, J.R.M., Kelsey, A., Crook, B., Gilbert, E. J. (2003). Occupational and environmental exposure to bioaerosols from composts and potential health effects-A critical review of published data (Prepared by the Composting Association and Health and Safety Laboratory for the Health and Safety Executive) (p. 7).

Swatland, H.J., Barbut, S. (1991). Fluorimetry via a quartz-glass rod for predicting the skin content and processing characteristics of poultry meat slurry. International Journal of Food Science and Technology, 26(4), 373-380.

Szczepanowska, H., Wilson, W. (1998). Permanency of Reprographic Images on Polyester Film. Journal of the American Institute for Conservation, 39.

Tanaka, M., Anguri, H., Nonaka, A., Kataoka, K., Nagata, H., Kita, J., Shizukuishi, S. (2004). Clinical Assessment of Oral Malodor by the Electronic Nose System. Journal of Dental Research, 83, 317-321.

Teather, R.M., Wood, P. J. (1982). Use of Congo Red-Polysaccharide Interactions in Enumeration and Characterization of Cellulolytic Bacteria from the Bovine Rumen. Applied and Environmental Microbiology, 43, 777-780.

Tiebe, C., Miessner, H., Koch, B., Hübert, T. (2009). Detection of microbial volatile organic compounds (MVOCs) by ion-mobility spectrometry. Analytical and Bioanalytical Chemistry, 395, 2313-2323.

Trihaas, J., Vognsen, L., Nielson, P. V. (2005). Electronic nose: New tool in modelling the ripening of Danish blue cheese. International Dairy Journal, 15, 679-691.

Ulfig, K. (2003). Studies of Kerationlytic and Keratinophilic Fungi in Sewage Sludge by Means of a Multi-Temperature Hair Baiting Method. Polish Journal of Environmental Studies, 12, 461-466.

Valentin, N. (2001). Microbial contamination and insect infestation in Spanish Museums, Archives and Libraries. Coalition, 3, 5-7.

Valentin, N. (2003). Microbial contamination in museum collections: Organic materials. In C. Saiz-Jimenez (Ed.), Molecular Biology and Cultural Heritage (p. 85). Madrid, Spain: Swets and Zeitlinger.

Valentin, N. (2007). Microbial Contamination in Archives and Museums: Health Hazards and Preventative Strategies Using Air Ventilation Systems. (Experts' Roundtable on Sustainable Climate Management). The Getty Conservation Institute.

Vandewoude, K.H., Blot, S.I., Benoit, D., Colardyn, F., Vogelaers, D. (2004). Invasive aspergillosis in critically ill patients: attributable mortality and excesses in length of ICU stay and ventilator dependence. Journal of Hospital Infection, 56, 269-276.

Vassou, A.-M. (2008). Video memories under attack from mould. Computer Active. Computer Active. Retrieved July 21, 2012, from http://www.computeractive.co.uk/ca/news/1911088/video-memories-attack-mould

Velikova, T., Trepova, E., Rozen, T. (2011). The use of biocides for the protection of library documents: before and now. In A. Mendez-Vilas (Ed.), Science against microbial pathogens: communicating current research and technological advances (pp. 152-159). Saint Petersburg, Russia: Formatex.

Vitale, T. (2009). History, Science, Preservation and Treatment of Cellulose Nitrate Still Film. Video Preservation and Conservation. Retrieved June 21, 2012, from http://videopreservation.conservation-us.org/library/history_storage_of_cellulose_nitrate_film_v26.pdf

von Radowitz, J. (2010). Smart wrappers to sniff fruit freshness. The Sydney Morning Herald. Sydney, Austrailia. Retrieved July 21, 2012, from http://news.smh.com.au/breaking-news-world/smart-wrappers-to-sniff-fruit-freshness-20100426-tlv5.html

Wade, W. (2002). Unculturable bacteria-the uncharacterized organisms that cause oral infections. Journal of the Royal Society of Medicine, 95, 81-83.

Wainwright, M., Ali, T.A., Barakah, F. (1993). A Review of the Role of Oligotrophic Micro-organisms in Biodeterioration. International Biodeterioration and Biodegradation, 31, 1-13.

Walinder, R., Ernstgard, L., Norback, D., Wieslander, G., Johanson, G. (2008). Acute effects of 1-octen-3-ol, a microbial volatile organic compound (MVOC)-An experimental study. Toxicology Letters, 181, 141-147.

Wheeler, J. (2002). Videotape Preservation Handbook. Retrieved July 11, 2012, from http://www.media-matters.net/docs/resources/Traditional Audiovisual Preservation/WheelerVideo.pdf

Wild, L. G. (2012). Farmer's Lung. New Orleans, USA: emedicine from WebMD. Retrieved July 20, 2012, from http://emedicine.medscape.com/article/298811-overview

Wilkins, K., Larsen, K. (1995). Variation of Volatile Organic Compound Patterns of Mold Species from Damp Buildings. Chemosphere, 31(5), 3225-3236.

Wilkins, K., Larsen, K., Simkus, M. (2000). Volatile metabolites from mold growth on building materials and synthetic media. Chemosphere, 437-446.

Wilson, D. B. (2009). Cellulases and Biofuels. Current Opinion in Biotechnology, 20, 295-299.

Wilson, S.C., Brasel, T.L., Carriker, C.G., Fortenberry, G.D., Fogle, M.R., Martin, J.M.,

Wu, C., Andriychuk, L.A., Karunasena, E., Straus, D. C. (2004). An Investigation into Techniques for Cleaning Mold-Contaminated Home Contents. Journal of Occupational and Environmental Hygiene, 1, 442-447.

Yang, C.S., Heinsohn, P. A. (2007). Sampling and Analysis of Indoor Microorganisms. John and Wiley Sons inc.

Yarnitzky, C., Wang, J., Tian, B. (2000). Hand-held lead analyzer. Talanta, 51, 333-338.

Zhu, J., Bean, H.D., Kuo, Y.M., Hill, J.E. (2010). Fast detection of volatile organic compounds from bacterial cultures by secondary electrospray ionization-mass spectrometry. Journal of Clinical Microbiology, 48, 4426-4431.

Zong, Q. (2008). A Portable Gas Chromatograph with Tunable Separation and Microsensor Array Detection: Design, Characterization, and Environmental Health Applications. University of Michigan.

Zotti, M., Ferroni, A., Calvini, P. (2008). Microfungal biodeterioration of historic paper: Preliminary FTIR and microbiological analyses. International Biodeterioration and Biodegradation, 62, 186-194.

Zyska, B. (1997). Fungi Isolated from Library Materials: A Review of the Literature. International Biodeterioration and Biodegradation, 40(1), 43-51.

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Counts of fungal spores released during inspection of mouldy cinematographic film and determination of the gelatinolytic activity of predominant isolates

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ABSTRACT

Film archivists have expressed concern regarding the release of aerial spores during inspection of mouldy cinematographic film. This study investigated the release of fungal spores during a simulated inspection procedure, and identified the key contaminants, the aim being to make recommendations to archivists regarding safe handling of such film. Eighteen films (black and white or colour) donated to the North West Film Archive, in Manchester, UK, were examined. During simulated inspection, spore release ranged from zero to several thousand spores per m^3, with the films showing the most visible mould colonisation usually yielding the highest numbers of colonies. There was no significant difference between the number of spores released from black and white film in contrast with colour film reels. Major contaminants were of the genera *Aspergillus* or *Penicillium*. *A. versicolor* was the most common species isolated. Gelatinase assays were performed on predominant isolates, to investigate whether fungal species which released the most spores, were also the most gelatinolytic, thus posing the greatest threat to film preservation. However, this doesn't prove to be the case. Some films released spores in numbers greater than are deemed 'safe' levels; therefore caution is advised when dealing with very mouldy film reels.

1. Introduction

National and regional archive material encompassing books, papers and film, provides an invaluable record of social history, personal achievement and historic occasions for the population served (Gallo, 1993; Florian, 2002). On a personal level, material such as home movies represents a treasured narrative of life events. Deterioration of cinematographic film devalues its heritage status, and can irreversibly damage the content of the film, thus storage under appropriate conditions is of paramount importance, coupled with an effective means of monitoring the quality of the film. Film consists of several layers: an outer protective layer, image forming materials consisting of dyes in colour film and silver particles in black and white and an anti-halation layer to prevent the scattering of light around bright objects (http://motion.kodak.com/motion/uploadedFiles/US_plugins_acrobat_en_motion_newsletters_filmEss_04_How-film-makes-image.pdf). The image forming layers and anti-halation layer are held onto a base support by a gelatin layer and together these constitute the emulsion layer (Lourenco and Sampaio, 2009). Different materials have been used for the base support since the invention of cinematographic film, and some of these are susceptible to chemical deterioration. Cellulose nitrate (1900s–1960s) is highly flammable and releases nitric acid when decomposing, cellulose diacetate and later cellulose triacetate ('safety film') (1960s–1990s) was susceptible to vinegar syndrome i.e., the breakdown of acetate to form acetic acid; and polyethylene terephthalate (1990's-present) which has been shown to be very chemically stable. Chemical deterioration has been the focus of several publications, but the biological deterioration has received less attention (Abrusci et al., 2004b). The key substrate for microbial growth in cinematographic film is the gelatin binder. Gelatin is used to stabilise the film and can be cross-linked with other compounds to increase strength and tolerance towards higher temperatures, and due to these properties, gelatin has been used in all materials in photographic history (Abrusci et al., 2004b). Gelatin can be hydrolysed by extracellular enzymes produced by microorganisms. If the environmental conditions are appropriate, then microorganisms contaminating the film can multiply, using the gelatin as a growth substrate, and colonise the film. Fungi are a particular problem, because they can tolerate conditions of lower relative humidity than bacteria. The use of the gelatin layer as an energy source will enable fungal growth to occur on the outside of the reel, as well as on the film frames. Mycelial growth obscures the

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image, and damage to the gelatin binder also affects the image quality.

Previous studies have shown that a range of fungal species can colonise and deteriorate cinematographic film (Zyska, 1997; Abrusci et al., 2004a, 2004b, 2005, 2006, 2007, 2009; Lourenco and Sampaio, 2009). Studies by Zyska (1997) and Abrusci et al. (2004b) revealed the predominance of *Aspergillus* and *Penicillium* species in the library environment and their ability to colonise and contaminate a wide range of library materials. In a limited study by Lourenco and Sampaio (2009), it was found that colour film reels were more affected by the fungal deterioration by *Aspergillus* and *Penicillium* sp. than black and white reels, assumed to be due to the inhibition of growth caused by the silver particles present in black and white film. Genera found on cinematographic film are generally typical of those found in the outdoor and indoor environment (Florian, 2002), but it is interesting to note the predominance of certain species, such as *A. versicolor*. In one study, up to 50% of the isolates from cinematographic film were identified as this particular species (Opela, 1992) – which indicates that there might be some predilection for the particular substrate and/or enhanced gelatinolytic activity in this species. In addition, although the range of contaminants has been explored, no attempts have been made to quantify the contamination, other than descriptively.

Films are typically stored in-can. In the domestic environment, storage conditions provided by the ambient environment of cupboards, attics, trunk or sheds, may be particularly favourable for fungal growth – moisture and warmth being the key prerequisites. In film archives, storage conditions are carefully controlled, thus minimising the growth of fungal deteriogens.

Problems may arise when films are donated to archives. Typically, films are inspected, and transferred to a digital medium for ready access and viewing, whilst the film itself, which is of considerably better image quality, is archived. In some instances, the film may be treated to physically remove visible mould, for example by gentle suction. Conversely, films which present evidence of fungal growth may not be handled or inspected by archivists due to concerns regarding health and safety. It is then not possible to inspect the film, to describe its content, determine its condition or transfer the film to a more accessible medium. Such films are stored separately, in the hope that future technologies might enable decontamination, inspection, conservation and archiving (http://cool.conservation-us.org/byorg/abbey/an/an21/an21-7/an21-709.html). The authors could find no specific health and safety recommendations regarding handling of these items.

This paper describes a survey of cinematographic film donated to, and deemed 'mouldy' by, a regional archive. Efforts were made to identify the fungal contaminants, quantify the release of fungal spores into the air during a simulated inspection to see whether they pose a health risk to archivists, and determine whether predominant species were capable of degrading gelatin, thus posing a danger to the preservation of film stock.

2. Material and methods

2.1. Film reels

The North West Film Archive (NWFA), based at the Manchester Metropolitan University (MMU), is the largest UK archive outside London (www.nwfa.mmu.ac.uk). Set up in 1977, the NWFA has a collection of over 33,000 items, which include black and white film, colour film, photographs and paper. Most have been donated from sources across the North West of England. Films are stored under controlled conditions (black and white archived at 10 °C and 35% relative humidity; colour at 4 °C and 30% relative humidity). NWFA donated eighteen cellulose films for this study, having obtained prior permission from the film donors to do so. After use in our laboratory, the films were returned to the archive.

The film reels had been identified as 'mouldy' by the archivists and were mouldy upon donation to the archive (by members of the public). However, storage condition of films prior to donation is unknown. Film reels are given reference numbers by the archive and films with the same reference number have the same donor. Of the eighteen reels studied, three belonged to one donor (RR1491), four belonged to a second donor (RR1494), two belonged to a third donor (RR1514), three belonged to a fourth donor (RR1440) and the remaining six reels belonged to six individual donors (RR1470, RR1399, RR1549, RR1093, RR1533 and RR1511). Six films were colour and 12 were black and white.

2.2. Simulated film inspection, spore capture and cultivation

All inspections were performed in a polymer isolation glove box (Wolf Laboratories, York, UK (Model number: 8307030 (3 ft))) approximately 2.3 m^3 volume. This had been customised by removing the gloves at the wrists, so that manipulations in the box could be carried out with bare hands, but fungal spores released during inspection would be contained. This was chosen, rather than a biological safety cabinet level 2 (BSL2), because archivists have access to similar apparatus, thus making the simulation more accurate.

All fungal culture media (malt extract agar (MEA)) (Oxoid, Basingstoke, UK), decontamination fluid (Trigene, Medichem, Kent, UK), and film, in-can, were placed in the glove box. For the 'simulated inspection' each film was mounted on spools at either end of a manual film spool winder so that 50 cm of film was exposed and could be transferred from spool to spool under controlled conditions. An air sampler (Desaga Germ Sampler gs 100: Oklahoma City, US) was also placed in the glove box, so that the number of fungal spores released per unit volume over time during inspection could be assessed. This was set to measure 100 L air, over 60 s with a 5 s delay. When the air sampling began, with 50 cm of film exposed, the film was wound forwards for 60 s, at approximately 90 turns per minute, after which the agar plate on the air sampler was removed, and replaced by a second plate (to give counts of an average of two plates ($n = 2$)) during a repeated winding process where the film was re-wound back to its original spool. These two plates were taken as duplicates, although this is not strictly the case; on occasion the process was repeated, enabling four counts to be made. The film was then replaced in its can, the glove box sanitised with Trigene spray and left for 10 min before the next film was processed. Plates were incubated at 25 °C for 5–7 days. Colonies were counted, and calculated as counts per cubic metre (m^3) of air (Test 1). Films were re-sampled again after 7 (Test 2) and 14 days (Test 3), to determine the effect of repeated inspection on spore release.

After total colony counts were obtained via air sampling for Tests 1, 2 and 3, differential colony counts were also made for two representative heavily contaminated reels (RR1093 (colour), RR1399 (black and white)) and two representative lightly contaminated reels (RR1470 (black and white) and RR1549 (colour)). The aim of this procedure was to determine whether spores of certain species were released on first, second or third handling ('inspection').

2.3. Identification of fungal isolates and subsequent selection for gelatinase assays

Thirteen out of twenty one isolates from reels RR1093, RR1399, RR1470 and RR1549 were identified by CABI Microbial Services (Oxfordshire, UK) by processing the samples using molecular

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sequencing of the internal transcribed spacer (ITS) region of the rRNA gene cluster. Results were matched against reference sequences held in EMBL/GenBank (global databases) to establish identity. These isolates were subsequently used for gelatinase assays. These four reels were also swabbed after the 'inspection' procedure. However, the same colonies were isolated, thus were not identified further. These fungal isolates were deemed to represent film colonisers (arbitrarily differentiated as >500 CFUs per m^3) and film contaminants (<500 CFUs per m^3). In addition, species isolated from swabs of 12 cellulose nitrate reels provided by the British Film Institute (BFI, Berkhampsted, UK) were also assayed for gelatinase production. On receipt, these swabs were placed in 5 mL of sterile distilled water for 1 h then vortex mixed. The resultant suspensions were then serially diluted to 10^{-3} and 0.1 mL volumes were spread inoculated onto MEA. Three of five isolates from the swabs were selected, based on differing colony morphology and frequency of isolation, and identified. *Trichoderma harzianum* (S1) was present on all swabs, but counts could not be determined due to the spreading nature of colonies, S2 (*Penicillium chrysogenum*) was present in comparatively high numbers from the donated swabs, and was also present on all 12 reels (>500 CFUs per mL) and S3 (also *P. chrysogenum* but with a different colony morphology) was present on some of the reels in lower numbers than S2.

Aspergillus versicolor (ATCC 11730) was used as a positive control because it was reported by Gopinath et al. (2005) to be highly gelatinolytic, and the species has been isolated from cinematographic film in previous studies (Abrusci et al., 2004a,b).

2.3.1. Solid media for gelatinase assays

Gelatin agar was prepared based on composition of cellulose agar used by Kasana et al. (2008). This was composed of Technical Agar no. 3 (Oxoid, Basingstoke, UK) 1.2% w/v, Bacteriological Peptone (Oxoid, Basingstoke, UK) 0.02%w/v, sodium chloride 0.2% w/v, di-potassium hydrogen orthophosphate 0.1% w/v, magnesium sulphate 0.05% w/v, potassium chloride 0.05% w/v and 0.8% gelatin (Oxoid), which substituted cellulose. After autoclaving, 20 mL aliquots were poured in Petri dishes.

Two sets of plates of gelatin selective agar were prepared; half contained all components except the gelatin (i.e., gelatin −ve) and half contained all components with added gelatin (i.e., gelatin +ve). An 8 mm cork borer was used to remove plugs of culture on MEA. The plugs were placed in the centre of the plates and incubated for 7 days at 30 °C. Trichloroacetic acid (TCA) was mixed with sterile distilled water (1 g per 100 mL) and added by pipette onto the agar (noted by Kanemitsu et al. (2001) to enhance visibility of halos around the wells if the gelatine had been hydrolysed). Additionally visualisation was also improved by using a light box.

2.3.2. Liquid media for gelatinase assays

Growth of microorganisms in liquid culture (trypticase soy broth), with subsequent extraction and assay of the supernatants for proteinase activity for gelatinase activity of enterococci has been described in the literature (Kanemitsu et al., 2001). This was modified for fungi where 3 g gelatin (Oxoid) was mixed with 2 g malt extract broth ((MEB), Oxoid) per 100 mL of sterile distilled water.

To inoculate the broths, plugs of mycelial growth were removed from cultures maintained on MEA with an 8 mm cork borer. Spore suspensions were not used due to difficulties in removing or isolating spores from some cultures, particularly those with wet spores (Yang and Heinsohn, 2007). The plugs were inoculated into 50 mL volumes of liquid broth in shake flasks. One mL of the liquid was removed from the broths on days 4, 5 and 6 days of growth, refrigerated it at 4 °C for 1 h, after which, if the broth had remained liquid, hydrolysis of gelatin had occurred. After 7 days all broths that contained gelatinase producing organisms remained liquid after this time thus indicating gelatinase hydrolysis. Subsequently, all isolates were incubated for 6 days at 30 °C in a rotary incubator set at 150 rpm (Fig. 1).

Fig. 1. Broth cultures containing MEB and gelatine. If the broth remained a liquid after refrigeration hydrolysis of gelatine had occurred, thus gelatinase enzymes were present. The supernatants were then extracted and used in quantitative assays. Spherical balls of fungal growth seen in shake flasks after inoculation with a fungal isolate and incubation on a rotary shaker (150 rpm) at 30 °C.

2.3.3. Assaying of supernatants for gelatinase activity

After 6 days of growth, cultures were poured into 25 mL sterile universal bottles and centrifuged at 1500 rpm for 5 min, to enable separation of the supernatants from the fungal mass. Supernatants were sterile filtered into bijoux bottles using a Millipore filter (PALL Acrodisc 32 mm with 0.2 μm Supor™ membrane) after which 40 μl was pipetted into wells in gelatin medium plates (Fig. 2). Plates were incubated at 30 °C for 24 h after which TCA was added, and plates were examined for zones of hydrolysis.

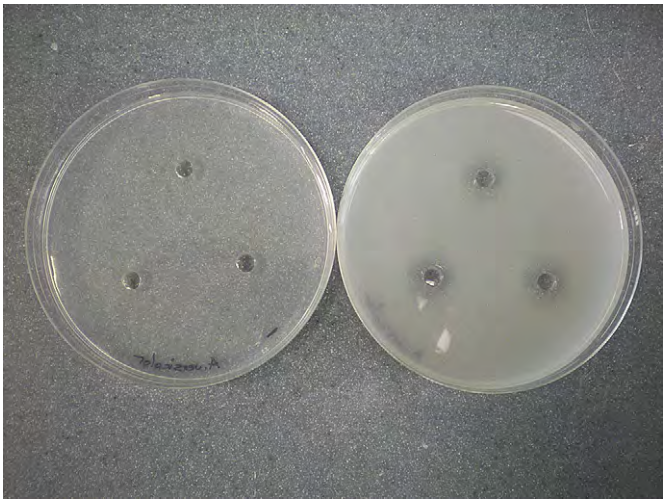

Fig. 2. Gelatin selective media plates with 3 wells containing 40 μl of supernatant (Left = gelatin −ve, right = gelatin +ve). Trichloroacetic acid (TCA) was added to make zones of hydrolysis of gelatin visible.

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Fig. 3. Photo of a film frame of a mouldy film picture of baby under microscope (×10) taken by Angelique Dudman (MMU).

3. Results

3.1. Total counts of spores released and frequencies of isolates

On visual inspection in the microbiology laboratory, some films presented obvious evidence of fungal growth, whilst others appeared dusty rather than mouldy (Figs. 3 and 4). The total counts of spores released during inspection (per m^3) for reels belonging to the collections RR1491, RR1494, RR1514, RR1440, RR1470, RR1399, RR1549, RR1093, RR1533 and RR1511 ranged from 0 to approx 4000 per m^3.

Fig. 4. Photo of mouldy film reel donated by the North West Film Archive (NWFA, Manchester, UK) taken by Mark Bodner (NWFA).

Although RR1511 appeared to be contaminated with fungi to the eye, air sampling of this reel failed to isolate any fungal colonies. Reel RR1514 F2 and all RR1440 reels were only sampled once due to infestation with *Neurospora* sp.

The sequential inspection process (Tests 1, 2, 3) revealed that the numbers of spores released varied, with the highest being on the second inspection (Fig. 5).

To compare the differences between spores released from sampling of black and white and colour film reels, the count of spores released during all three tests for each film was totalled. The average number of spores released for colour films and black and white films was calculated separately, with RR1511 and RR1470 being omitted as no spores were isolated from air sampling on any of the tests from these films. There was no significant difference in numbers of spores released from the two film types ($P > 0.05$).

3.1.1. Counts of different colony morphologies from selected reels

Colonies were counted for predominant isolates with different colony morphologies for reels RR1093, RR1399 which showed heavy contamination (>500 CFU m^3) and RR1470 and RR1549 which released fewer spores (<500 CFU m^3) (Table 1). In total twenty one colony morphologies were differentiated from air sampling.

Of the five isolates from reels RR1093 and six from RR1399, which were heavily contaminated and badly degraded, one isolate on each reel released spores in very high numbers, whilst comparable low numbers of spores were released from RR1470 and RR1549, which were not visibly heavily contaminated.

The same species were present in tests 1, 2 and 3, and the proportion of each colony morphology was also the same for each test.

3.2. Gelatinase assays

In total seventeen isolates were assayed for gelatinase production all of which were shown to have gelatinolytic activity. Sixteen unknown species were selected for identification by CABI Microbiological Services (Table 2). Isolates RR1399 I2 (*A. versicolor*), RR1399 I4 (*A. versicolor*) and S1 (*Trichoderma*), found on all the swabs of film reels donated from the British Film Institute, had the highest gelatinolytic activity (7 mm zone radius). *Aspergillus versicolor* ATCC 11730 (+ve control) also demonstrated a high gelatinolytic activity. Isolates RR1093 I4, RR1399 I1 and RR1399 I3 released the highest numbers of spores upon air sampling of each reel, but did not have higher gelatinolytic activities than other isolates on the same reel.

4. Discussion

4.1. Fungal isolates

It is not surprising to isolate common fungi such as *Aspergillus and Penicillium* sp. from an environmental surface, thus their presence on cinematographic film is not unexpected. The films that were examined which did not show obvious mould growth on the surface tended to yield low numbers of colonies of a range of different fungal species, indicating contamination rather than colonisation. Each film is an inert object susceptible to contamination and, potentially, subsequent colonisation, depending on storage conditions and nutrient availability. Thus the range of fungi theoretically able to contaminate the film is significant, and one might expect a wide range of isolates. This was the case for film that was contaminated rather than colonised. However, for

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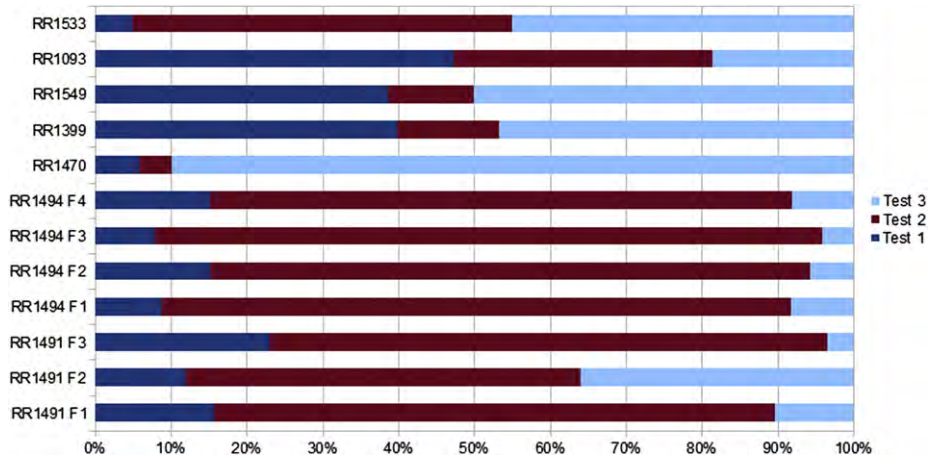

Fig. 5. Representation of the total numbers of spores released during 3 sets of air sampling with the percentage of spores released during each test indicated by a different coloured bar. Reel RR1514 F2 and all RR1440 reels were excluded from these results due contamination with *Neurospora* sp. so no further tests were carried out after test 1. RR1511 is also excluded as no spores were released during air sampling.

cinematographic film on which mould growth is evident to the naked eye, it appears that colonisation by a narrower range of species is likely.

Performing sequential inspection revealed differences in the number of spores released. The first inspection dislodged tightly packed spores from the tightly wound reel, which were released in high numbers on second inspection, with fewest spores released on the third inspection. With regards to numbers of spores released, the highest number of spores of between 1.5 and 7 times the recommended 'safe' limits of 1000 CFU m^3 of air (Jo and Seo, 2005) were released from RR1399 and RR1093 indicating there is a potential health risk associated with the handling of very mouldy film reels.

Although the average number of spores released for each reel was higher from colour reels than black and white, which mirrors that of a similar study on gelatin emulsion photographs (Lourenco and Sampaio, 2009), this was not significant.

Table 1
Frequencies of isolates (CFU's M^3) from reels RR1093, RR1399, RR1470 and RR1549.

Isolate name	Average number of isolates per test ($n = 3$) per m^3		
	Test 1	Test 2	Test 3
RR1093 I1	53.33 ± 5.77	46.67 ± 45.09	16.67 ± 20.82
RR1093 I2	53.33 ± 15.28	3.33 ± 5.77	3.33 ± 5.77
RR1093 I3	733.33 ± 533.51	230 ± 98.59	50 ± 50
RR1093 I4	2296.67 ± 548.57	1710 ± 1213.47	1333.33 ± 1391.56
RR1093 I5	3.33 ± 5.77	3.33 ± 5.77	0 ± 0
RR1399 I1	920 ± 511.18	1000	2140 ± 608.28
RR1399 I2	100 ± 65.67	280	273.33 ± 61.1
RR1399 I3	3623 ± 80.21	4240	253.33 ± 102.63
RR1399 I4	10 ± 10	0	440 ± 124.9
RR1399 I5	23.33 ± 20.82	10	120 ± 20
RR1399 I6	396.67 ± 20.82	220	386.67 ± 61.1
RR1470 I1	3.33 ± 5.77	0 ± 0	0.26 ± 5.77
RR1470 I2	13.33 ± 15.28	0 ± 0	0.53 ± 5.77
RR1470 I3	3.33 ± 5.77	3.33 ± 5.77	0 ± 0
RR1470 I4	0 ± 0	0 ± 0	5 ± 15.28
RR1470 I5	0 ± 0	0 ± 0	22.89 ± 160
RR1549 I1	3.33 ± 5.77	0 ± 0	0 ± 0
RR1549 I2	26.67 ± 15.28	6.67 ± 5.77	0 ± 0
RR1549 I3	13.33 ± 15.28	0 ± 0	0 ± 0
RR1549 I4	0 ± 0	6.67 ± 5.77	1.05 ± 23.09
RR1549 I5	0 ± 0	0 ± 0	4.74 ± 62.45

From these results, it was hypothesised that the dominant isolates were more capable of utilising the gelatin present in the film as a growth substrate. Isolates which were better able to utilise gelatin would therefore pose a greater threat to the destruction of film. To investigate this, isolates were screened for gelatinase production.

The accuracy of the counts obtained might be questioned. Differentiation of colony morphologies by an eye was difficult. Molecular identification of every colony on the plates from air sampling was not performed due to the low diversity of colony morphologies isolated. The methods used satisfied the aim of determining which fungal species released spores in the highest numbers. It was apparent in some cases that mycelium lad lost viability. In addition, other spores might not germinate. DNA isolation directly from the film might yield more information, but was beyond the scope of this work.

As noted previously there was a low diversity of species isolated in this study, There appears to be different fungal genera predominant for a given reel collection (i.e., from each different donor), presumably due to storage in the same environment prior to donation to the archive. In studies of this nature, analysis of more reels is always desirable.

4.2. Gelatinase assays

The pre-ponderance of *A. versicolor* on contaminated cinematographic film has been noted by other authors, working on archives in Spain (Abrusci et al., 2004b), but no reason was previously offered for this apparent selection. The gelatinase assays performed in this study, showed that the *A. versicolor* isolates had a higher gelatinolytic activity than the *Penicillium* species isolated, which could explain its frequent isolation from mouldy films.

Many of the isolates found were gelatinolytic (Abrusci et al., 2006), thus potentially able to colonise the surface of the film, given appropriate conditions for growth. However, the isolates which released the highest number of spores were not the most gelatinolytic i.e., RR1093 I4 and RR1399 I3. No evidence could be found in the literature indicating that *A. versicolor* does not sporulate as heavily as other *Aspergillus* species, although variation of levels of sporulation within a particular species such as *A. niger* has been noted in the literature (Nicolas-Santiago et al., 2006). This suggests that it is the hyphal growth on film frames that causes damage, not necessarily those moulds that produce the highest

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Table 2
Results of testing for gelatinase production from selected fungal isolates from film reels RR1093, RR1399, RR1470, RR1549 from air sampling of cellulose acetate film reels. The 'Gelatin Hydrolysis on Gelatin Selective Media' refers to when the isolates were inoculated onto the plates using an 8 mm cork borer and incubated for 7 days at 30 °C. The 'Gelatine Hydrolysis of Broth' indicates whether the broth remained liquid after refrigeration at 4 °C and the 'Size of Hydrolysis Zone' indicates the size of zones around wells after inoculation with supernatant ($n = 3$). Additionally *A. versicolor* ATCC 11730, and S1, S2 and S3 from swabs of cellulose nitrate reels were also assayed. Molecular identifications of fungal cultures were performed by CABI Microbial Services (Oxfordshire, UK).

Isolate name	Identification	Gelatin hydrolysis on Gelatin selective media	Gelatine hydrolysis in broth (±)	Size of hydrolysis zone (mm)
(ATCC 11730)	*Aspergillus versicolor*	Positive	Positive	7
RR1093 I1	*Penicillium chrysogenum*	Positive	Positive	5
RR1093 I2	*Penicillium brevicompactum*	Positive	Positive	5
RR1093 I3	*Penicillium citreonigrum*	Positive	Positive	5
RR1093 I4	*Penicillium chrysogenum*	Positive	Positive	5
RR1399 I1	*Aspergillus versicolor*	Positive	Positive	6
RR1399 I2	*Aspergillus versicolor*	Positive	Positive	7
RR1399 I3	*Aspergillus versicolor*	Positive	Positive	6
RR1399 I4	*Aspergillus versicolor*	Positive	Positive	7
RR1399 I5	*Penicillium brevicompactum*	Positive	Positive	5
RR1399 I6	*Aspergillus versicolor*	Positive	Positive	6
RR1470 I1	*Aspergillus versicolor*	Positive	Positive	6
RR1549 I2	*Aspergillus versicolor*	Positive	Positive	6
RR1549 I3	*Asoergillus versicolor*	Positive	Positive	5
S1	*Trichoderma harzianum*	Positive	Positive	7
S2	*Penicillium chrysogenum*	Positive	Positive	5
S3	*Penicillium chrysogenum*	Positive	Positive	5

numbers of spores. The fungi which release the most spores pose a risk to health, but it is the gelatinolytic potential of the organism that poses a risk to film.

With the limited number of publications in the area, deriving from different archives, the comment of Abrusci et al. (2004b) that 'more work should be done' should be re-iterated. If specific film deteriogens exist, then specific measures might be taken to detect, and minimise, their presence, or control their growth.

4.3. Evidence of health risks and recommendations for safer handling

The numbers of spores released from film varied considerably, with very high numbers being released from film that was visibly heavily contaminated. Repeated air sampling of the film reels revealed that the number of spores released increased on the second test but was greatly reduced on the third. This could suggest that repeatedly winding mouldy film reels whilst extracting the air above would reduce health risks caused by exposure to fungal spores. Inspection and cleaning would ideally be done in a BSL2 safety cabinet in the lab, but this equipment is not typically available to archives.

As with film reels analysed in other studies (Opela, 1992; Abrusci et al., 2005), no isolates of significant concern to health were isolated from the reels used in this study, thus the risk to handlers of exposure to mouldy film reels appears to be low. However, as with all fungi there is an allergen risk posed by exposure to conidial antigens (O'Gorman and Fuller, 2008), and some individuals may be more sensitive to these than others (Kurup, 2003), thus care should be taken when handling mouldy reels to limit exposure to spores. The majority of microorganisms are opportunist pathogens including those isolated in this study; however these are only likely to pose a risk in an immunocompromised individual (http://science.education.nih.gov/supplements/nih1/diseases/guide/understanding1.htm).

In future, one might recommend an arbitrary ranking of film in terms of visible mould growth, with subsequent behaviour of the archivists in terms of film handling modified depending on potential exposure to high numbers of spores. However, some films which appeared very mouldy such as RR1511, harboured no viable spores, with no growth being obtained during the simulated inspection. Thus it was concluded that the mould growth had occurred in the more distant past (the film was from the 1930s), and that viability had been lost. In these cases, the film will have been damaged, but the risk to the archivist is reduced. Potential allergenicity of non-viable fungal spores is unknown. In these cases, subsequent assessment of the viability of heavy contamination, perhaps by detection of volatile compounds (Canhoto et al., 2004; Kuske et al., 2005) will also inform subsequent handling.

5. Conclusions

The need to assess the potential risk of mould growth on cinematographic film in terms of film quality and archivists' health is pressing. Initial visual inspection of film reels can indicate the presence of mould. However, the amount of spore release on inspection by archivists cannot be predicted: some mould reels do not yield any viable spores. Fungi commonly present in the environment were isolated from cinematographic film, with *Aspergillus versicolor* being the most frequently isolated species with a higher a gelatinolytic activity than *Penicillium* spp. isolated from the same reels. No fungi considered to pose a serious health risk were isolated, so the risk to archivists from handling mouldy film reels may be considered to be low. However, simulated inspection of some reels released spores in higher numbers than those considered 'safe' by the health organisations. It is therefore advisable that safe handling procedures should be recommended when handling heavily contaminated reels.

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References

Abrusci, C., Martin Gonzalez, A., Del Amo, A., Corrales, T., Catalina, F., 2004a. Biodegradation of type-b gelatine by bacteria isolated from cinematographic films. A viscometric study. Polymer Degradation and Stability 86, 283–291.

Abrusci, C., Allen, N.S., Del Amo, A., Edge, M., Martin-Gonzalez, A., 2004b. Biodegradation of motion picture film Stocks. Journal of Film Preservation 67, 37–54.

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Abrusci, C., Martin-Gonzalez, A., Del Amo, A., Catalina, F., Collado, J., Platas, G., 2005. Isolation and identification of bacteria and fungi from cinematographic film. International Biodeterioration and Biodegradation 56, 58–68.

Abrusci, C., Marquina, D., Amo, A., Corrales, T., Catalina, F., 2006. A viscometric study of the biodegradation of photographic gelatin by fungi isolated from cinematographic films. Biodeterioration and Biodegradation 58, 142–149.

Abrusci, C., Marquina, D., Del Amo, A., Catalina, F., Collado, J., Platas, G., 2007. Biodegradation of cinematographic gelatin emulsion by bacteria and filamentous fungi using indirect impedance technique. International Biodeterioration and Biodegradation 60, 137–143.

Abrusci, C., Marquina, D., Santos, A., Amo, A., Corrales, T., Catalina, F., 2009. Biodeterioration of cinematographic cellulose triacetate by *Sphingomonas paucimobilis* using indirect impedance and chemiluminescence techniques. International Biodeterioration and Biodegradation 63, 754–759.

Canhoto, O., Pinzari, F., Fanelli, C., Magan, N., 2004. Application of electronic nose technology for the detection of fungal contamination in library paper. International Biodeterioration and Biodegradation 54, 303–309.

Florian, M.E., 2002. Fungal Facts – Solving Fungal Problems Is Heritage Collections. Archetype Publications.

Gallo, F., 1993. Aerobiological research and problems in libraries. Aerobiologia 9, 117–130.

Gopinath, S.C.B., Anbu, P., Hilda, A., 2005. Extracellular enzymatic activity profiles in fungi isolated from oil-rich environments. Mycoscience 46, 119–126.

Jo, W.K., Seo, Y.J., 2005. Indoor and outdoor bioaerosol levels at recreational facilities, elementary schools and homes. Chemosphere 61, 1570–1579.

Kanemitsu, K., Nishino, T., Kunishima, H., Okamura, N., Takemura, H., Yamamoto, H., Kaku, M., 2001. Quantitative determination of gelatinase activity among enterococci. Journal of Microbiological Methods 47, 11–16.

Kasana, R.C., Dhar, R.S.H., Dutt, S., Gulati, A., 2008. A Rapid and Easy Method for the Detection of Microbial Cellulases on Agar Plates Using Grams's Iodine. Current Microbiology 57, 503–507.

Kurup, V.P., 2003. Fungal allergens. Current Allergy and Asthma Reports 3, 416–423.

Kuske, M., Romain, A.C., Nicolas, J., 2005. Microbial volatile organic compounds as indicators of fungi. Can an electronic nose detect fungi in indoor environments? Building and Environment 40, 824–832.

Lourenco, M.J.L., Sampaio, J.P., 2009. Microbial deterioration of gelatin emulsion photographs: differences of susceptibility between black and white and colour materials. International Biodeterioration and Biodegradation 63, 496–502.

Nicolas-Santiago, S., Regaldo-Gonzalez, C., Garcia-Almendarez, B., Fernandez, F.J., Tellez-Jurado, A., Huerta-Ochoa, S., 2006. Physiological, morphological, and mannanase production studies on Aspergillus niger uam-gs1 mutants. Electronic Journal of Biotechnology 9, 51–60.

O'Gorman, C.M., Fuller, H.T., 2008. Prevalence of culturable airborne spores of selected allergenic and pathogenic fungi in outdoor air. Atmospheric Environment 42, 4355–4368.

Opela, V., 1992. Fungal and bacterial attack on motion picture film. FIAF, Joint Technical Symposium, Session 5, Ottawa, Canada.

Yang, C.S., Heinsohn, P.A., 2007. Sampling and Analysis of Indoor Microorganisms. John and Wiley Sons Inc..

Zyska, B., 1997. Fungi isolated from library materials: a Review of the literature. International Biodeterioration and Biodegradation 40, 43–51.

Websites

Anon., (Unknown). "Film Structure". Retrieved 9th January, 2012, from: http://motion.kodak.com/motion/uploadedFiles/US_plugins_acrobat_en_motion_newsletters_film_Ess_04_How-film-makes-image.pdf.

Anon., (Unknown). "Understanding Emerging and Re-emerging Infectious Disease", National Institutes of Health and National Institute of Allergy and Infectious Diseases. Retrieved 3rd April, 2012, from: http://science.education.nih.gov/supplements/nih1/diseases/guide/understanding1.htm.

Brothers, P., 1997. Mouldy Audiotape. Abbey Newsletter, vol. 21, No. 7. Retrieved 3rd December, 2009, from: http://cool.conservation-us.org/byorg/abbey/an/an21/an21-7/an21-709.html.

Hewitt, M., 2009. North West Film Archive. Retrieved 24th September, 2009, from: http://www.nwfa.mmu.ac.uk/.

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PAPER

Identification of microbial volatile organic compounds (MVOCs) emitted from fungal isolates found on cinematographic film†

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We report the detection of microbial contamination and growth of cinematographic film utilising headspace solid phase micro-extraction coupled with Gas Chromatography–Mass Spectrometry. Microbial Volatile Organic Compounds (MVOCs) are produced only when the mould is actively growing on the cine film. Over 150 volatile compounds were detected from 16 fungal isolates, with over 40 being common to 2 or more isolates. It was found that 1-octen-3ol was produced from 13 of the isolates analysed, 3-octanone from 10 of the isolates and 3-octanol from 4 isolates. These three key chemical markers are indicative of viable fungal growth on cinematographic film precluding the need for traditional microbiology laboratory culture methods. Such an approach would prevent valuable historical footage from being discarded due to health and safety concerns regarding spore inhalation, and would enable safe handling.

1. Introduction

The storage of conditions of cinematographic film greatly influences susceptibility to microbial contamination and growth. Moisture damage and subsequent microbial growth can be a risk to the health of archivists, damage films, and can lead to contamination of previously non-contaminated films and objects. Visual inspection of archival objects may be sufficient in some cases to determine the presence of mould.[1] In some cases, materials that appear mouldy may instead be heavily contaminated with dust. However, safe handling practices should still be observed as this cannot be confirmed without further microbiological investigation. It may also be difficult to determine whether the mould is actively growing, or has lost viability, without culture methods. Traditional methods used to detect the types of fungi involved and the extent of mould growth involve the measurement of spore levels using a device such as an air sampler which concentrates spores onto a Petri dish containing growth media.[2] Spores which land on the plate may germinate resulting in a colony. The number of colonies can then be counted to give a measurement of mould contamination *i.e.* the greater the number of colony forming units, the greater the extent of contamination.

Several microbial volatile organic compounds (MVOCs) have been shown to be common markers of fungal growth. Moulds produce larger quantities and types of volatiles when growing on nutrient-rich media in the laboratory compared to low nutrient substrates such as building materials, and perhaps cinematographic film.[3]

A range of MVOCs have been described in the literature. Mattheis *et al.*[4] identified geosmin as a volatile collected from *Penicillium expansum*. Geosmin is often reported to be an indicator of fungal growth.[5] 1-Octen-3-ol is an alcohol also produced by many moulds and is also indicated by olfaction as a musty odour.[6,7] 1-Octen-3-ol is highly volatile and is produced in large quantities by *Penicillium* and *Aspergillus* species.[8] Wilkins *et al.*[9] describes MVOCs as contributing to sick building syndrome and as a result of investigations to identify those compounds responsible, a volatiles 'hit list' was collated by Strom *et al.*[10] including 1-octen-3-ol, 2-hexanone, 2-heptanone and 2-heptanol, common to *A. versicolor*, *P. chrysogenum* and *P. commune*.[9] Wilkins *et al.*[3] suggested that mould growth in buildings is limited to a few species, thus it would be beneficial to recognise their specific MVOCs to indicate general mould growth. MVOC production has also been measured at various growth stages.[11] *Alternaria alternata* and *Aspergillus versicolor* produced most MVOCs at day 2 of growth whilst other isolates produced more at day 10. *Penicillium chrysogenum* produced the highest concentration of MVOCs: 2-octen-1-ol was produced by all isolates of *Cladosporium sphaerospernum*, *Aspergillus niger*, *A. versicolor*, *Penicillium chrysogenum* and *P. brevicompactum*, and 1-octen-3-ol were detected from all isolates of the latter three

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species. It was established that emission patterns seem to vary according to a wide range of factors such as a colony age, RH and temperature and that different isolates of the same species may emit various different MVOCs.

Solid Phase Micro-Extraction (SPME) and Gas Chromatography–Mass Spectrometry (GC-MS) was used by Matysik *et al.*[12] and Fiedler *et al.*[13] to detect MVOCs on wallpaper, other related materials and synthetic media during different stages of mould growth. The monitoring of MVOC production took place over several weeks to investigate emission rates between the initial stage and later periods of growth. 2-Pentanol and 2-pentanone were common to all 6 species tested, although 2-pentanol was only detected during the early growth stages. 1-Octen-3-ol was detected in five of the species and was emitted at a constant rate over the entire growth period. *A. versicolor* produced high amounts of 1,3-dimethoxybenzene whilst *Penicillium* species emitted high concentrations of 1-octen-3-ol and 3-octanol.

SPME involves inserting a fused silica fibre coated with a Carbowax/divinylbenzene, polyacrylate, polydimethylsiloxane, or Carboxen/polydimethylsiloxane phase, through the septum of a flask or GC vial so it is exposed to volatiles in cultures for a predetermined length of time after which the fibre is retracted into the needle and removed from the flask or vial for GC analysis. Using this approach, Fiedler *et al.*[13] found that moulds grown on various nutrient media could potentially emit more than 150 volatile substances although no single compound was common to all species. Several compounds occurred in a large number of species including 1-octen-3-ol, 3-octanone, 2-methyl-1-butanol and 3-methyl-1-butanol.

There are some MVOCs which have been known to cause adverse health effects. Walinder *et al.*[14] has shown the aliphatic 1-octen-3-ol to be a common VOC of microbial origin, and has been shown to cause genotoxicity effects in the library environment.[13,15] Walinder *et al.*[14] investigated 1-octen-3-ol to test for adverse health effects. Volunteers reported symptoms of exposure to 1-octen-3-ol by several tests including measurement of blink frequency by electromyography, the time required for dry spots to appear on the corneal surface after blinking, washing out of the nasal passages (nasal lavage), and monitoring of the nose and airways for mucous production. Subjects were exposed to either 10 mg m^{-3} of 1-octen-3-ol or clean air as control and were asked to give subjective ratings to some of the tests. During exposure to 1-octen-3-ol, headache, nausea, smell and nasal irritation were increased together with higher nasal lavage biomarker levels of eosinophil cationic protein, myeloperoxidase and lysozyme. Atopics did not have more reactions due to exposure, whereas females experienced more smell and mucosal irritation'.[14] Also eye irritation and blinking frequency were increased together with throat irritation, mild mucosal irritation for the eyes and airways, and a small decrease in vital capacity was also noted. These findings indicate the need to detect fungal growth without culture or specialist equipment in order to protect the health of archivists and ensure safe handling of materials.

The compounds 1-octen-3-ol, 3-octanone and 3-octanol are well documented indicators of fungal growth for many species of fungi found in the environment such as *Aspergillus* and *Penicillium* species.[10,13,16] In this paper we assess fungal isolates found on cinematographic films and lab strains of fungi found in other studies for the production of MVOCs;[1] such an approach would allow an indication to whether there is potential for MVOC monitoring as a method for determining actively growing mould on film, without the need for traditional culture methods preventing reels from being discarded unnecessarily and allowing film archivists to handle films safely without health worries.

2. Experimental

Fungal isolates

Mouldy film reels were donated to this study by the North West Film Archive (NWFA, Manchester, UK). A range of isolates previously obtained from contaminated film reels were selected if they had been shown in enzyme assays to have a high gelatinolytic and high cellulolytic activity, and if spores from these isolates were shown by air sampling of reels to be present in high numbers. Other isolates which were not from film reels were selected to enable a range of fungi to be investigated. All isolates were maintained on malt extract agar (MEA, Oxoid). Isolates which were found on films were labelled with the film reel reference number followed by the isolate number. The *Penicillium expansum*, *P. chrysogenum* and *Aspergillus versicolor* were lab strains used for comparison to other work.[13] *A. versicolor* had also been isolated and identified from previous work carried out at MMU and was shown to have a high gelatinolytic and cellulolytic activity. The *Cladosporium* isolate came from air sampling which had been carried out in cellulose nitrate vaults at the British Film Institute (BFI, Gaydon). The JWP1 *Alternaria* spp. was isolated from a piece of mouldy wallpaper which had been donated at the preliminary stages of this study. The 'S1' *Trichoderma* spp. was isolated from swabs of mouldy cellulose nitrate films reels, which had been sent by the BFI for analysis.

Preparation of fungal isolates on different media

Molten malt extract agar was dispensed into 8 mL volumes in autoclaved 20 mL glass gas chromatography (GC) vials, which were placed at angles during cooling to produce agar slopes. Sterile loops were then used to inoculate the slopes with spores scraped from the surface of cultures which were being maintained on MEA. The vials were then covered with aluminium foil to prevent contamination and enable growth and incubated for 3 days at 25 °C after which an airtight aluminium cap with a self sealing rubber septum was placed on the top of the vials (see Fig. 1). These were further incubated for 2 more days to enable accumulation of any MVOCs in the headspace produced during fungal growth. Malt extract broth (MEB, Oxoid) with added gelatine was also used to grow cultures for SPME analysis for comparison with MEA. Fifty millilitre volumes were placed in conical flasks and inoculated with spores of fungal isolates using a sterile loop. These were incubated for 10 days at 35 °C in shake culture at 150 rpm after which 10 mL only of each broth culture was transferred to empty 20 mL glass vials ($N = 2$) and each was sealed with an airtight aluminium cap (see Fig. 1). A sample of contaminated film RR1093 (measuring 2 cm by 1 cm) which was covered in mould previously shown to be non-viable and clean Test Film (2 cm × 1 cm) were cut off the main reels and placed directly into empty glass vials to investigate any volatiles emitted from the samples. A piece of test film was inoculated with mould

by placing the film strip on an MEA plate inoculated with a 100 μL of a mixed culture suspension containing *Aspergillus versicolor* and *Trichoderma* spp. (S1) (Table 1) containing approx. 3.89×10^6 spores and incubated for 7 days at 25 °C after which a fungal lawn covered the medium and had grown onto the surface of the film as well as into the emulsion layer, which was confirmed by visualisation of hyphae under the light microscope (×10). The film was then removed using sterile tweezers and placed in a vial with an aluminium cap placed on top to seal in any volatiles which may have been produced. This was left for a further 2 days to allow diffusion of volatiles into the headspace which could then be sampled to see if active mould emitted volatiles when growing on film. After 2 additional days SPME was performed again on the samples to allow repeats and see if any additional volatiles were present in the headspace.

Selection of fibre coatings for the solid phase microextraction (SPME) needle

There are several coatings which can be used for extraction of VOCs, each having different properties such as thickness or pore size, which affects the affinity for different compounds. Volatile compounds have a higher affinity for coatings with a thicker coat as they are more sensitive than those with a thinner coat as a greater mass of analytes can be absorbed onto the coating. Polydimethylsiloxane (PDMS) can withstand high injection temperatures of up to 300 °C, is non-polar and is often used for extraction of non-polar volatile flavour compounds, but can also be used to extract polar compounds after optimisation of extraction methods.[17] Polyacrylate (PA) is a polar coating, so is used to extract analytes such as phenols and alcohols which are polar compounds. Mixed fibre coatings containing divinylbenzene (DVB) and Carboxen (CAR: a porous activated carbon support) increase retention capacity of volatiles and can be used for the extraction of volatiles with low molecular masses. CAR–PDMS coatings have been shown to have better extraction efficiency than 100 μm PDMS. Kataoka *et al.*[17] describes seven types of fibres commercially available with 50/30 DVB/CAR/PDMS having the highest retention, thus was selected for analysis of MVOCs from fungal isolates from cinematographic film.

Fig. 1 Vials containing samples for use with the gas chromatography–mass spectrometry for Solid Phase MicroExtraction-HeadSpace (SPME) Analysis. The three vials on the left contain fungal cultures grown on MEA slopes. The vial in the middle is un-inoculated MEA only and the three on the right are fungal cultures which have been grown in broth culture (MEA and gelatine) and transferred into the vials.

Solid phase microextraction (SPME) analysis

Vials were labelled with the name of the fungal species and the date ($N = 2$), then the aluminium cap was secured on, thus ensuring sampling was carried out on cultures of a known age. Vials containing the cultures were placed in water baths at 35 °C to equilibrate volatiles in the headspace.[15] These were then subjected to SPME headspace analysis using a 50/30 divinylbenzene/carboxen on a polydimethylsiloxane bonded to a fused silica core (Supelco). Negative controls were: (i) running the fibre through the GC-MS system without exposure to VOCs; (ii) exposing the fibre to a piece of clean test film for comparison to both the inoculated and contaminated film; (iii) un-inoculated MEA and un-inoculated MEB + gelatine. The SPME needle was conditioned before use by inserting the needle into the GC-MS sampler and running the program in order to remove any volatile compounds absorbed on the fibre from previous experiments. The SPME needle was inserted into the vial through the self-sealing rubber septum into the headspace and was exposed to the VOCs (adsorption time). The SPME fibre was directly desorbed into the injector port on an Agilent 6850 series gas chromatography system coupled to an Agilent 5973 mass spectrometer which separated the headspace volatiles on a HP column (25 m × 0.2 mm, 0.5 μm) coated with a 5% diphenyl – 95% polymethylsiloxane copolymer. Volatiles were then analysed using the Agilent Instrument 1 Enhanced ChemStation for Microsoft Operating systems. The settings of GC were based on work by.[13] Absorption time was 15 minutes. The injector temperature was set at 220 °C and the detector 280 °C with a carrier gas (helium) flow rate of 1 mL min^{-1}. The initial temperature was 40 °C for 7 minutes then ramped to 220 °C at a rate of 10 °C min^{-1} and then maintained at this temperature for 15 minutes. Total run time was 40 minutes. Peaks of VOCs which were over 50% match quality (previously determined) on the Agilent Instrument 1 Enhanced ChemStation were recorded (a higher quality indicates a more probable identification of the volatiles queried). However, the fungal marker compounds 1-octen-3-ol and 3-octanone, were recorded if a peak was produced but the quality was less than 50% because these were the target compounds. Fig. 2 depicts a typical chromatogram of *Aspergillus versicolor* displaying the peaks arising from the identified alcohols.

3. Results and discussion

Data from culture on MEA was used for all isolates since it yielded the greatest number of MVOCs produced by fungal isolates. VOCs produced by fungal isolates grown in malt extract broth with gelatine have been recorded but will not be discussed, because preliminary testing revealed that this medium did not generate as many detectable volatile compounds. The moulds grown on different nutrient media and the substrates themselves together emitted a broad spectrum of more than 150 volatile substances belonging to a variety of chemical groups such as hydrocarbons and alcohols. Sixteen fungal isolates were

Table 1 Key to the samples used. For simplicity, numbers were allocated to a specific substrate and the letters were to each mould tested. Abbreviations for the SPME needles used are also included

Key					
Substrate/sample		Fungi		SPME needle type	
0	Blank	A	Mixed contamination	100 μm PDMS	100 μm polydimethylsiloxane
1	Malt extract agar	B	*A. versicolor*	50/30 DIV/CAR/PDMS	50/30 Divinylbenzene/carboxen/ polydimethylsiloxane
2	Malt extract broth + gelatine	C	*P. expansum*	65 μm CAR/DIV	65 μm Carbowax/divnylbenzene
3	Test film	D	*Cladosporium*		
4	Mouldy film RR1093	E	*P. chrysogenum*		
5	Inoculated test film	F	Sl (*Trichoderma*)		
		G	RR1399 I1 (*Aspergillus*)		
		H	RR1399 I2 (*Aspergillus*)		
		I	RR1399 I3 (*Aspergillus*)		
		J	RR1399 I4 (*Aspergillus*)		
		K	RR1399 I5 (*Penicillium*)		
		L	RR1399 I6 (*Penicillium*)		
		M	NB4 (*Stachybotrys chartarum*)		
		N	JWP 1 (*Alternaria*)		
		O	RR1093 I1 (*Penicillium*)		
		P	RR1093 I2 (*Aspergillus*)		
		Q	RR1093 (*Aspergillus*)		

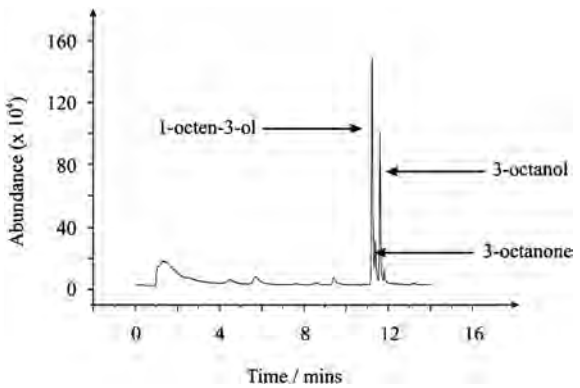

Fig. 2 Typical chromatograph of *Aspergillus versicolor* displaying the chromatrographic peaks arising from 1-octen-3-ol, 3-octanone and 3-octanol.

investigated for MVOC production (Table 1). The negative controls were the malt extract broth with gelatine, the malt extract agar and the clean test film. These were also analysed for emission of volatiles in order to determine which were emitted from the fungal isolates and which were from the different media (Table 2). If the volatiles detected from the substrates which had not been inoculated by fungi were also detected from inoculated samples then these were excluded from Table 3. Volatiles produced by fungi growing on various substrates were recorded (Table 3). The isolates grown in MEB and gelatine broth took longer to grow than the isolates grown on MEA slopes in the vials *i.e.* 10+ days in broth, 5–7 days in vials. There was also the added risk of contamination during transfer of broth from the shake flasks into the vials compared to the *in situ* growth on the MEA in vials. Thus all subsequent fungal samples were grown on MEA slopes in the vials. It is found that there were >50 MVOCs that were common to more than one fungal isolate. However there were >100 MVOCs which were emitted from single isolates only. Due to the large number of MVOCs emitted (over 150) from inoculated media, only the compounds which were common to 2 or more fungal isolates were noted (Table 3). This was to determine which compounds could be considered to be common fungal growth markers.

Table 2 Compounds emitted from various substrates on which fungi were grown. For 'Type of Substrate': 1 = malt extract agar, 2 = malt extract broth with added gelatine, 3 = sample of clean cinematographic film

Type of substrate	1	2	3
Compound			
Adenosine			X
Benzaldehyde	X		
Benzene (isocyanomethyl)		X	
Benzenehexanamine			X
Furan, 2-pentyl	X	X	
Morphine, 3-0-[4-trifluoromethylphenyl]	X	X	X
N-Ethyl-1,3-dithiosindoline	X	X	
Octane			X
Phenol, 2,2-[1-methyl-1,2-ethanediyl]nitrilomethylidyme]bis	X		
Thieno[3,2-*c*]pyridine 5-oxide hemihydrate	X	X	
Tranylcypromine, pentafluorobenzoyl ester	X		
3-[4-*N*-*N*-Dimethylaaminophenyl]propenoic acid, 2-diethoxyphosphinyl]-ethyl ester	X		
5,6,8,9-Tramethoxy-2-methylpepero-3,4,5-JK]-9,10,dihydrophenanthracene	X		

Table 3 Compounds emitted from various fungal isolates grown on different substrates. For 'Type of Substrate': 1 = malt extract agar, 2 = malt extract broth with added gelatine, 4 = a sample of a film reel donated by the North West Film Archive that was already contaminated by fungi (RR1093), 5 = cinematographic film inoculated by fungi isolated from contaminated film reels. For 'Fungi' (sp. = species): A = various contaminants, B = *Aspergillus versicolor*, C = *Penicillium expansum*, D = *Cladosporium* sp., E = *Penicillium chrysogenum*, F = *Trichoderma* sp., G = *Aspergillus* sp., H = *Aspergillus* sp., I = *Aspergillus* sp., J = *Aspergillus* sp., K = *Penicillium* sp., L = *Penicillium* sp., M = *Stachybotrys chartarum*, N = *Alternaria* sp., O = *Penicillium* sp., P = *Aspergillus* sp., Q = *Aspergillus* sp

Type of substrate	4	5	1															2		
Fungi	A	B + F	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	B	C	E
Compound																					
Alloocimene/2,2,-dimethyl-bicyclo(4.2.0) oct-1(6)-ene		X					X														
α-Amorphene		X					X	X		X											
α-Chamigrene								X	X												
(+)-α-Longipinene								X	X												
Benzene, 2,3-disocyanato-1-methyl														X	X						
β-Cubebene								X	X				X								
β-Funebrene									X												
β-Himachalene								X	X				X								
β-Sesquiphellandrene								X	X												
Bistrimethylsilyl *N*-acetyl eicosasphinga-, aa-dienine														X	X						
Calarene						X	X														
Camphor							X										X				
Eseroline, 7-bromo-methycarbamate															X						X
Formamami diniumacetate					X														X		
Furaltadone		X			X																
γ-Muurolene		X					X	X	X	X											
Heptane			X	X																	
Heptane, 6-methyl-2-*p*-tolyl								X	X												
Italicene		X					X	X	X				X								
Limonene														X				X			
Linalool							X										X				
Linalool oxide								X									X				
Longipinene								X	X												
Naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4(1-methylethyl)		X						X	X												
N,*N*-Dimethyl-heptadeclamine			X		X																
Phenol, 2-methyl-5-(1-methylethyl)		X	X			X													X		
Terpinene-4-ol							X									X					
trans-Caryophyllene								X	X	X											
Widdrene									X				X								
1-Methylallyyl(cyclooctatetraene)titanium							X			X					X				X		
1-Octen-3-ol		X	X	X	X	X	X	X	X	X	X	X	X				X	X			X
1,1,2,3,5,6-Hexamethyl 1-4-penthyl-2,4-cyclohexadiene													X					X			
1,2,2-Trimethyl-1-(*p*-tolyl)-cyclopentane								X	X												
1,3-Dihydroxy-6-methoxy-1,2,3,4-tetrahydroquinolin-2-one					X										X		X				
2-Methyl-5,12-dithianaphtho{2,3-*b*} quinoxaline			X													X			X		
2-(2,5-Dimethylphenyl)cyclohexanone								X	X												
3-Cyclohexen-1-ol, 4-methyl-1-(1-mrthylethyl)							X	X									X				
3-Octanone		X	X		X	X	X	X		X	X	X	X				X				X
3-Octanol			X		X	X				X											X
(4-Dimethylamino)phenyl-[4′-(*N*-methyl)(*N*-ethyl)amino]phenyl-imine																X	X				
7-Dicyanomethylene-7*H*-2-methoxy-9*H*-1,8-(1′ propen-1′ yl-3′-yliden)-benzo-cycloheptane						X													X		X

Two compounds were detected from multiple fungal isolates and are considered to be markers of fungal growth i.e. 1-octen-3-ol and 3-octanone.[13] Of the 16 isolates sampled, 1-octen-3-ol was detected on 13 of the isolates; *A. versicolor*, *P. expansum*, *Cladosporium*, *P. chrysogenum*, S1, RR1399 I1-I6, RR1093 I2 and RR1093 I3. This compound was not detected from NB4 and, JWP1 and RR1093 I1. 3-octanone was detected on 10 of the 16 isolates; *A. versicolor*, *Cladosporium*, *P. chrysogenum*, S1, RR1399 I1, RR1399 I3-I6 and RR1093 I2 and was not detected from *P. expansum*, RR1399 I2, NB4, JWP1, RR1093 I1 and RR1093 I3. 3-octanol is considered to be another marker of fungal growth[10] in which emission was detected from 4 isolates; *A. versicolor*,

Cladosporium, *P. chrysogenum and* RR1399 I3. *A. versicolor* produced all three of the volatiles considered to be markers of fungal growth as shown in Fig. 2; these marker compounds were identified using the mass spectrometry search function and also by running standards through the GC-MS to confirm the retention times match (see below). There were other compounds which were detected from multiple fungal isolates. There were 27 volatile compounds common to 2 fungal isolates but these cannot be considered markers of general fungal growth as this is too small a proportion of the 16 isolates tested so will not be discussed. There were 5 compounds common to 3 different isolates; β-cubebene (G, H and L), β-himachalene (G, H and L), *trans*-caryophyllene (G, H and I), 1,3-dihydroxy-6-methoxy-1,2,3,4-tetrahydroquinolin-2-one (D, N and P), 3-cyclohexan-1-ol, 4-methyl-1-(1-methylethyl) (F, G and P). There were 2 compounds common to 4 different isolates; γ-muurolene (F, G, H and I) and italicene (F, G, H and L). *Stachybotrys chartarum* (isolate NB4, column M, Table 3) has been considered by some to pose a significant health risk[18] and has been isolated from cinematographic film (Manchester Metropolitan University (MMU), unpublished). None of the key fungal marker compounds (1-octen-3-ol, 3-octanone and 3-octanol) were detected from this species. The *Alternaria* (JWP1) isolate also did not emit these compounds.

Nine volatile compounds were detected from the 'Test Film' which had been inoculated with a mixed culture of *A. versicolor* (B) and *Trichoderma* sp. (F). These were Alloocimene/2,2,-dimethyl-bicyclo(4.2.0) oct-1(6)-ene, α-amorphene, furaltadone, γ-muurolene, italicene, naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4(methylethyl), phenol, 2-methyl-5-(1-methylethyl), and the two compounds which are considered to be fungal growth markers; 1-octen-3-ol and 3-octanone. These were not detected on mouldy film which had been previously contaminated upon donation to this study by the North West Film Archive (NWFA). Identification of the marker compounds were achieved using the mass spectrometry search function and along with chemical standards run to confirm retention times. Shown in the ESI† are the chromatograms of the chemical standards for 1-octen-3-ol, 3-octanone and 3-octanol which have retention times of 11.29, 11.35 and 11.58 respectively. These were determined by injecting 1 mL of gas at 0.02% (v/v), from the headspace of vials using a 1 mL gas syringe (Hamilton Bonaduz AG, Switzerland), and running the program described previously.

No single MVOC was specific to all fungi however; some VOCs appear to be good indicators of fungal growth, although none is universally reliable as a detector.[19] 1-Octen-3-ol, 3-octanone and 3-octanol were detected from many isolates and thus were good indicators of fungal growth.

The lowest amount of target analyte that could be detected using SPME GC-MS was explored on the compounds 1-octen-3-ol, 3-octanone and 3-octanol. Known amounts of these compounds were diluted with ethanol and analysed using the same program as described previously. The lowest levels that were measurable were: 1-octen-3-ol, 0.002 (v/v) %; 3-octanol, 0.001 (v/v) %; 3-octanone 0.0009 (v/v) % indicating that SPME is effective at detecting even low concentrations produced by fungi. Quantitative analysis is not possible in this study due to the large variability of SPME and the size of the resulting peaks on the GC spectrum but could be made possible with the introduction of an internal standard.

In agreement with Fiedler *et al.*[13] and Scotter *et al.*,[20] MEA was found to be the best substrate to use, as growth on this resulted in the greatest number of detectable MVOCs being produced. *Stachybotrys chartarum* did not emit any of the marker compounds at the time of testing. The isolate was sampled after 1 week of growth but repeated sampling over a longer time period could have detected more MVOCs specific to this isolate. The MVOCs from the isolates studied are the same as those found in the literature although the method of sampling and adsorbent used could cause variation in the volatiles found. 1-Octen-3-ol and 3-octanone were found from the lab strain of *Penicillium chrysogenum* tested and not the *P. expansum*, in agreement with the results found by Kaminski *et al.*[8] and Larsen and Frisvad.[7] *Aspergillus versicolor* which in some studies has been isolated from cinematographic film,[21] is a fungus known to cause biodeterioration of many materials,[22] produced 1-octen-3-ol, 3-octanone and 3-octanol as was also recorded by Sunesson *et al.*[23] and Fiedler *et al.*[13]

Analysis of the test film which had been inoculated with a mixed culture of *A. versicolor* and *Trichoderma* spp. identified 9 volatile compounds of which 2 were the fungal growth markers 1-octen-3-ol and 3-octanone. These were not detected from SPME analysis of mouldy film donated by the NWFA, but were detected from fungi that had been isolated from these films and were actively growing MEA. This indicates that these compounds can be detected from actively growing mould and not from inactive mould, thus demonstrating the potential for detecting active fungal growth on cinematographic film and differentiating actively growing mould from that which is considered 'dead' or non-viable. This may remove the need for traditional culture methods and reduce the risk of cross contamination of film and the health risks to archivists. Precautions could also be taken should actively growing mould be detected to ensure that these films are stored separately in order to avoid contaminating non-contaminated film reels.

4. Conclusions

We have demonstrated *via* SPME GC-MS that there are three MVOCs that are common to fungal isolates found on cinematographic film, namely, 1-octen-3-ol, 3-octanone and 3-octanol. Quantification of these chemical markers indicate active mould growth suggesting MVOC detection can be used to distinguish between actively growing mould on film and 'dead' mould. A detection device for archivists could allow them to make decisions on safe handling of mouldy film reels in order to prevent contamination of non-mouldy film reels and protect the health of people handling the materials; this is currently underway.

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References

1 M. Kuske, A. C. Romain and J. Nicolas, *Build. Environ.*, 2005, **40**, 824–832.
2 M. E. Florian, *Fungal Facts – Solving Fungal Problems in Heritage Collections*, Archetype Publications, 2002, p. 146.

3 K. Wilkins, K. Larsen and M. Simkus, *Chemosphere*, 2000, 437–446.
4 J. Mattheis and R. G. Roberts, *Appl. Environ. Microbiol.*, 1992, **58**(9), 3170–3172.
5 T. Nilsson, T. O. Larsen, L. Montanarella and J. O. Madsen, *J. Microbiol. Methods*, 1996, **25**, 245–255.
6 N. K. Meruva, J. M. Penn and D. E. Farthing, *J. Ind. Microbiol. Biotechnol.*, 2004, **31**, 482–488.
7 T. O. Larsen and J. C. Frisvad, *Mycol. Res.*, 1995, **99**(10), 1153–1166.
8 E. Kaminski, S. Stawicki and E. Wasowicz, *Appl. Microbiol.*, 1974, **27**(5), 1001–1004.
9 K. Wilkins and K. Larsen, *Chemosphere*, 1995, **31**(5), 3225–3236.
10 G. Strom, J. West, B. Wessen and U. Palmgren, *Quantitative Analysis of Microbial Volatiles in Damp Houses*, ed. B. Flannigan and R. Samson, Elsevier, 1994.
11 M. S. Black, W. E. Horner and P. R. Morey, MVOC and VOC Emission Patterns from Multiple Strains of Indoor Fungi, in *Indoor Air 99. The 8th International Conference on Indoor Air Quality and Climate*, Air Quality Sciences, Inc., Edinburgh, Scotland, 1999.
12 S. Matysik, O. Herbarth and A. Mueller, *J. Microbiol. Methods*, 2008, **75**, 182–187.
13 K. Fiedler, E. Schutz and S. Geh, *Int. J. Hyg. Environ. Health*, 2001, **204**, 111–121.
14 R. Walinder, L. Ernstgard, D. Norback, G. Wieslander and G. Johanson, *Toxicol. Lett.*, 2008, **181**, 141–147.
15 O. Canhoto, F. Pinzari, C. Fanelli and N. Magan, *Int. Biodeterior. Biodegrad.*, 2004, **54**, 303–309.
16 G. Fischer, R. Schwalbe, M. Moller, R. Ostrowski and W. Dott, *Species-Specific Chemosphere*, 1999, **39**(5), 795–810.
17 H. Kataoka, H. Lord and J. Pawliszyn, *J. Chromatogr., A*, 2000, **880**, 35–62.
18 B. D. Nelson, *Stachybotrys Chartarum: The Toxic Indoor Mould*, 2001 11/01, [cited 2009 17th September], http://www.apsnet.org/online/feature/Stachybotrys/.
19 A. Korpi, A. L. Pasanen and P. Pasanen, *Appl. Environ. Microbiol.*, 1998, **64**(8), 2914–2919.
20 J. M. Scotter, V. S. Langford, P. F. Wilson, M. J. McEwan and S. T. Chambers, *J. Microbiol. Methods*, 2005, **63**, 127–134.
21 C. Abrusci, N. S. Allen, A. Del Amo, M. Edge and A. Martin-Gonzalez, *J. Film Preservation*, 2004, **67**, 37–54.
22 Y. Liang, W. Zhao, J. Xu and J. D. Miller, *Int. Biodeterior. Biodegrad.*, 2011, **65**, 217–226.
23 A. L. Sunesson, W. H. J. Vaes, C. A. Nilsson, G. Blomquist, B. Andersson and R. Carlson, *Appl. Environ. Microbiol.*, 1995, **61**(8), 2911–2918.