CRANFIELD UNIVERSITY

SCHALE FRAZER

CHARACTERISATION OF *STACHYBOTRYS CHARTARUM* FROM WATER DAMAGED BUILDINGS

CRANFIELD HEALTH
APPLIED MYCOLOGY GROUP

PhD Thesis
Academic Year: 2008 - 2011

Supervisor: Dr Dave Aldred
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This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

ABSTRACT

Fungal contaminated buildings and related adverse human health implications have long been a topical issue throughout the world and concern is mounting with regards to the presence of more toxigenic fungi found in buildings and the associated health risks. These risks are compounded when homes are affected by water damage as a result of water intrusion problems, in particular flooding. With the ever changing climate and unpredictable weather conditions the frequency of flash flooding has increased in recent years and is set to increase and subsequently more homes will inevitably be effected by mould contamination. The present study initially aimed to determine the types of fungi commonly detected in buildings in the United Kingdom with varied levels of water intrusion problems via a small survey using various sampling techniques and particularly aimed to determine the conditions by which growth of the toxigenic fungi *Stachybotrys chartarum* could occur. *Penicillium, Cladosporium* and *Aspergillus* species were the most commonly detected fungi in buildings with relatively moderate levels of water intrusion problems; *Stacybotrys chartarum* was only detected in building with more severe water intrusion problems.

The main body of the present study aimed to characterize the effects of water activity (a_w) and temperature on *S. chartarum* isolates including the conditions for germination, germ tube extension, mycelia growth, sporulation and toxin production *in vitro* on potato dextrose agar (PDA) growth medium. Overall, germination of two *S. chartarum* isolates (IBT 7711 and IBT 14915) was significantly influenced by a_w and temperature and was fastest at 0.997-0.98 a_w between 15 and 30°C with complete germination within 24 h. Germ tube extension was found to be most rapid at similar a_w levels and

25-30°C. Mycelial growth rates were optimal at 0.997 a_w between 25 and 30°C, with very little growth at 37°C. Sporulation was optimum at 30°C at 0.997 a_w. However, under drier conditions, this was optimum at 25°C. Differences were clearly observed in the interaction ranges of water activity and temperature for germination, germ tube extension, mycelial growth, sporulation and for toxin production. This is valuable information and may help in understanding the role of this fungal species in damp buildings and conditions under which immune-compromised patients may be at risk when exposed to such contaminants in the indoor air environment.

A range of control agents butylated hydroxyanisole (BHA), propyl gallate (PG, tebuconazole (Teb) and silver nitrate (SN) were examined for their ability to control the growth of *S. chartarum* isolates IBT 7711, 14915 and a field isolate (Denbigh) using conventional plating methods *in vitro* on potato dextrose agar (PDA) and a more rapid automated method *in vitro* in potato extract broth (PEB). BHA was also used to control *S. chartarum* growth *in situ* on building materials and with supplementation in matt emulsion paint. Minimum inhibitory concentrations (MIC) obtained for *S. chartarum* isolates IBT 7711, 14915 *in vitro* on PDA were determined 200 mg l^{-1}. The same isolates produced MICs of 1000 mg l^{-1} for PG.

Antifungal susceptibility tests using Bioscreen Opical density (OD) growth measurements with a range of antifungal compounds revealed varied levels of effectiveness against *S. chartarum* isolates IBT 7711, 14915 and field isolate (Denbigh). Silver nitrate was found to be the most effective antifungal compound producing MIC values of <3 mg l^{-1} for all tested isolates. Butylated hyroxyanisole (BHA), propyl gallate

(PG) and tebuconazole (Teb) also showed promising levels of antifungal activity against *S. chartarum* growth.

Extracellular enzyme production for isolates of *S. chartarum* showed significant effects of water activity with optimal levels where water was freely available. The level of extracellular production also found to vary between *S. chartarum* isolates. Haemolytic activity was also demonstrated for the *S. chartarum* isolates 7711, 14915 and Denbigh. Interestingly the non macrocyclic trichothecene producing isolates produced significantly higher levels of haemolytic activity than the macrocyclic trichothecene producing isolates, which is a serious finding as 60% of the *S. chartarum* isolates detected in water damaged building are of this type.

Keywords:

Temperature, Water Activity, Antioxidants, Pulmonary haemorrhage, Mould

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ABBREVIATIONS

ABPA:	Allergic bronchopulmonary aspergillosis
AFS:	Allergic fungal sinusitis
AIHA:	American Industrial Hygiene Association
ANOVA:	Analysis of Variance
API:	Analytical profile index
APIZYM:	Analytical profile index for enzymes
ATCC:	American type culture collection
AUC:	Area under curve
a_w:	Water activity
BALF:	Bronchoalveolar lavage fluid
BCA:	Bicinchoninic acid
BHA:	Butylated hydroxyanisole
BPW:	Buffered peptone water
BRE:	Building Research Establishment
BSA:	Bovine serum albumen
CA:	Cellulose agar
CDC:	Centres for Disease Control and Prevention
CEC:	Commission of European Communities

CFU:	Colony forming units
CYA:	Czapek Yeast Autolysate
DOF:	Degrees of freedom
DMSO:	Dimethyl sulphoxide
^{o}C:	Degrees centigrade
ELISA:	Enzyme Linked Immunosorbant Assay
fa:	Fractional area (derived from optical density measurements)
GRAS:	Generally regarded as safe
HBEC:	Human brain endothelial cells
HP:	Hypersensitivity pneumonitis
IAQ:	Indoor air quality
IBT:	Culture Collection of Fungi, Mycology Group, BioCentrum-DTU, Technical University of Denmark
IgE:	Immunoglobulin E
IgG:	Immunoglobulin G
IH:	Immediate or Type I hypersensitivity
IPH:	Idiopathic pulmonary haemosiderosis
LCL:	Lower confidence level
LPB:	Lining papered plasterboard
LPM	Lambert-Pearson Model
MEA:	Malt extract agar

Mg l^{-1}:	Milligram per litre
MIC:	Minimum Inhibitory Concentration
MSE:	Mean squared error
MVOC:	Microbial Volatile Organic Compound
MW:	Molecular weight
NIC:	Non-inhibitory concentration
OD:	Optical density
OPP:	Office of Pesticide Programs
PEB:	Potato extract broth
PBS:	Phosphate buffered saline
PBS-T:	Phosphate buffered saline plus Tween
PDA:	Potato dextrose agar
PPB:	Painted plasterboard
PG:	Propyl gallate
RAUC:	Relative area under curve
RMSE:	Root mean square error
RTD:	Rate of time to detection
SAS:	Surface air sampler
SBS:	Sick Building Syndrome
SG:	Satratoxin G
SN:	Silver nitrate

SS:	Sum of squares
SSE:	Sum of squared errors
StdErr:	Standrd error
Teb:	Tebuconazole
TLV:	Threshold limit values
TNF:	Tumour necrosis factor
TTD:	Time to detection
UCL:	Upper confidence level
VOC:	Volatile organic compound
v/v	volume/volume
WC:	Water closet
WHO:	World Health Organisation
w/v	weight/volume

1 INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

There is an ever increasing concern related to fungal, or mould growth within the indoor environment, especially within water damaged buildings, and it is speculated that there may be a direct relation to the ill-health sometimes reported by occupants of affected dwellings. This association has gathered momentum with many reports in the literature showing incidences of people with health problems associated with their mouldy and damp living or working conditions and has been referred to as Sick Building Syndrome (SBS) (Burge, 2004; Crook and Burton, 2010). The UK population spend a large proportion of time (>80%) indoors (Singh, 2005); with the development of new energy efficient buildings, ever changing weather conditions and the potential for increased frequency of flooding due to climate change, the occurrence of mould growth along with the associated health problems are likely to exacerbate if they are not understood. Problems associated with moulds are not new; in fact they have been problematic for many centuries, being reported even in the Bible (Leviticus Chapter 14, 33-48). In the Old Testament law the Lord gave Moses and Aaron regulations about houses affected by the spread of mildew. These regulations declared homes with green or red spots on their walls unclean and that immediate attention was required for its removal. Media focus on this issue increased as a result of the death of 12 infants in Cleveland, Ohio between 1993 and 1998 who were diagnosed with Pulmonary haemosiderosis, a rare condition characterized by spontaneous pulmonary haemorrhage, often associated with toxigenic fungi in water damaged homes or people with iron-deficiency anaemia (Levy and Wilmott, 1986). The Centres for Disease Control and Prevention (CDC) concluded that there was an association between water damaged homes and the presence of various

fungi, including those that can produce highly toxic metabolites in the indoor environment. The victims' homes were found in particular to have higher quantities of airborne conidia from the toxigenic fungi *Stachybotrys. chartarum* than those detected in the surrounding non symptomatic homes (CDC, 1994; CDC, 1997; CDC, 2000; Dharmage *et al.*, 2002; Miller *et al.*, 2003; Green *et al.*, 2005; Roussel *et al.*, 2008).

In England it has been estimated that there are 5.2 million properties at risk of flooding annually with an estimated cost of £1 billion (Environment Agency, 2009; Murphy *et al.*, 2009). Climate change has led to an increase in rainfall in northern Europe, Russia and Canada while parts of Africa and India have become drier (Zhang *et al.*, 2007). As a result of the increased rainfall many homes have become severely water damaged after being affected by flooding from burst riverbanks and drainage systems collapse. Subsequently household materials can become saturated with surplus amounts of flood water, which in some cases, remains undisturbed for weeks. These conditions are ideal for some organisms to proliferate and have the potential to remain problematic long after the water has subsided and materials appear to be dry.

In 2005, hurricanes Katrina and Rita hit southern States of USA. This caused the flooding of thousands of homes throughout the country. As a result of this flooding, many of these homes were affected by extensive mould growth due to the water intrusion which remained undisturbed for many weeks. After hurricane Katrina, a large proportion of homes were reported to be affected by *Stachybotrys*, although it is likely that other dematiaceous fungi were present that were misidentified as *Stachybotrys* (Solomon *et al.*, 2006; Bloom *et al.*, 2009) (Plate 1.1).

Plate 1.1Flooding in New Orleans resulting from Hurricane Katrina, 2005 (KatrinaDestruction.com, 2011)**.**

Mould growth in many of these buildings after hurricane Katrina can also be attributed to the type and quality of building construction as many building in USA are timber framed and ideal for mould growth when saturated with water in flood conditions.

Many homes in Northern Europe also suffered a similar fate as a result of flooding in summer 2007 (Plate 1.2). But, interestingly, there are no reports in the literature on the presence of *Stachybotrys* in U.K homes as a result of the 2007 floods. This may indicate

a large difference in risk perception in terms of this fungus in the UK compared to the USA; after Katrina, for example, a large amount of information on the prevalence of *Stachybotrys* was made available.

Plate 1.2 Torrential rainfall resulting in the Tewkesbury Borough UK floods, July 2007
(Tewkesbury Borough Council, 2007)

Fungal growth as a result of condensation is another growing problem for occupants within many of the UK housing sectors, including those in social housing where heating and ventilation issues are known to aggravate the problems. It is stated that 1.5m dwellings in England are unfit for habitation of which 15% are due to lack of ventilation (English House Condition Survey, 2009).

Condensation occurs when humidity levels reach saturation point, this causes water vapour to condense onto cold surfaces, which if ignored leads to mould growth and ultimately degradation of the building structure. (Plate 1.3)

Plate 1.3 Condensation in a domestic dwelling

Newly constructed energy efficient buildings and residences are also thought to be susceptible to mould infestation. This is primarily due to poor construction practices, substandard material use, and increased air tightness of new construction techniques which can allow moisture to become trapped in exterior walls, creating an environment conducive to mould growth. Advances in technology have lead to improvements in the heating and insulation of homes but these have partially exacerbated the air quality problems due to related condensation and ventilation issues in addition to the introduction of airborne particles from new types of floorings, furnishings and cleaning products (Passon Jr. *et al.*, 1996; Brownson, 2000; Montz Jr. and Passon Jr., 2001; Crump *et al.*, 2009).

Fuel poverty has also been found to be a contributory factor influencing mould growth in homes. Individuals particularly at risk include those on low income, the elderly and diabled people who commonly have depts to fuel companies and are unable to sufficiently heat their homes. Many homes as a result suffer deterioration by mould growth as a reult of damp and condensation problems.

1.2 Associated health concerns

1.2.1 Exposure to fungi

Due to the ubiquitous nature of fungi, contact is unavoidable even in the indoor environment. Exposure can be via ingestion, inhalation or skin contact and can result in many different health effects including, allergic reactions, infections, neurological effects and toxic effects. Carcinogenic effects of some mycotoxins have also been reported but presently there is no evidence of carcinogenicity of these toxins or any other compounds produced by species of *Stachybotrys* (National Toxicology Program, 2004). The health concerns associated with many of these organisms depends on many factors and certain individuals are much more susceptible than others. Factors contributing to the occurrence of ill-effects include: the types of organisms present, whether individuals are immune-compromised, exposure time, their age and their sensitivity. It is estimated that about 10% of the population have allergic antibodies to fungal antigens of which 5% would be expected to show clinical illnesses (American College of Occupational and Environmental Medicine, 2002).

While all mould growth should be immediately addressed in the indoor environment, those that produce macrocyclic trichothecenes are of particular concern as they have been reported to cause severe adverse health effects in both humans and animals (Kuhn and Ghannoum, 2003; Kováčiková *et al.*, 2007).

Based on associated health risks, some countries have put indoor moulds into three hazard groups (Mold and Bacteria Consulting Laboratories, 2011).

Hazard Class A: Indicates fungi or metabolites that are highly hazardous to heath. Occupied dwellings found within this hazard class require immediate attention. Examples of fungi within this group include: *Aspergillus niger* (found in flower pot soil), *Aspergillus fumigatus* (found in cellars and basements), *Stachybotrys chartarum* and *Fusarium sp*. (found on wallpaper).

Hazard Class B: Indicates indoor fungi that may cause allergic reactions to occupants over a long exposure period. Examples of fungi within this group include: *Trichoderma* sp, (found on wallpaper), *Cladosporium cladosporioides* (found in kitchens and bathrooms) and *Penicillium chrysogenum* (found on mattresses and carpets).

Hazard Class C: Indicates non hazardous fungi with very little health risk, although they may cause economic damage to the building and should be removed. Examples of fungi within this group include: *Ulocladium botrystis* (found in kitchens and bathrooms) and *Wallemia sebi* (found on mattresses and carpets)

1.2.2 Mechanisms of associated health related illnesses

β-D (1→3) glucans

The structural cell-wall components of most fungi and some bacteria contain β-D (1→3) glucans. Exposure to these components by sensitive individuals has been reported to have immunomodulating properties which may affect the respiratory health of those exposed (Douwes, 2005). They account for up to 60% of the dry weight of the cell wall and consist of glucose polymers with varied relative molecular mass that are linked to proteins, lipids and carbohydrates such as mannan and chitin. Most research on the effects of these cell wall components have focused on orally or intravenously administered glucans and a few studies have investigated the exposure of humans to mould or glucans. Rylander (1996) found that exposure to pure β-glucan (particulate curdlan 210 ng m^{-3}) for four hours at concentrations similar to those sometimes found in indoor air, did not significantly affect lung function although some exposure-related irritation was produced in the nose and throat. Beijer *et al* (1999) found an increased blood level of tumour necrosis factor alpha (TNF-α) when test subjects were exposed to another β-glucan (grifolan in saline at about 30 ng m^{-3}) for three hours. These reports highlight the potential health implications from these fungal components but short term exposures cannot be compared directly with exposure to measured levels in buildings, as they rarely exceed 100 ng m^{-3} of β-glucan, therefore further work on exposure levels of β-glucans needs to be investigated (WHO guidelines (2009).

Allergy

Allergy, also known as atopy, is a rapid response mediated by the immune system to environmental allergens including fungi. This can result in inflammatory responses that

can occur in sensitive individuals which are characterized by excessive activation of mast cells and basophils by the antibodies Immunoglobulin E (IgG) or Immunoglobulin (IgG).

Immediate or Type I hypersensitivity (IH) is an IgE mediated immune response triggered by contact with fungal spores and hyphal fragments from species including *Penicillium, Aspergillus, Cladosporium, Stachybotrys* and *Alternaria*, and many environmental proteins. Affecting about 40% of the population, it is the most common form of hypersensitivity to moulds and can lead to asthma, rhinitis (hay fever) or atopic dermatitis. Severity of the allergy varies from person to person and depends on the level of exposure and sensitivity to allergens. Sensitive individuals generally suffer the same allergic reactivity against other allergens such as dust mites, and pollens (Bush and Peden, 2006; Karvala *et al.*, 2008; Myatt *et al.*, 2008; Xu *et al.*, 2008).

Extrinsic allergic alveolitis (EAA) is an inflammation of the alveoli within the lung caused by hypersensitivity to inhaled organic dusts. An intense immune reaction resulting from the interaction between inhaled fungal proteins and fungal-directed cell mediated IgG antibodies. It is characterised by very high serum levels of specific IgG proteins or exposure by inhalation to very large quantities of fungal (or other) proteins (Weltermann *et al.*, 1998; Miyazaki *et al.*, 2008; Sakamoto, 2008; Bogaert *et al.*, 2009; Engelhart *et al.*, 2009; Lacasse *et al.*, 2009;). EAA usually results from occupational exposure and affects individuals working very closely or in an environment with very high levels of allergens. Other examples include Farmers Lung which is a hypersensitivity reaction induced by the inhalation of biological dusts coming from hay

dust, mould spores or other agricultural products. (American College of Occupational and Environmental Medicine, 2002).

Allergic bronchopulmonary aspergillosis (ABPA) is a condition which occurs in allergic individuals who have had previous bronchial illnesses or airway damage that impair normal drainage e.g. bronchiectasis. Susceptible individuals experience fungal colonization, without the invasion of adjacent tissues, in these areas by *Aspergillus* sp. Colonisation produces no adverse health effects unless the subject is allergic to the specific fungus which would subsequently result in an ongoing allergic reactivity to the fungal proteins released directly into the body (Greenberger, 2002; Shah and Panjabi, 2002; Soubani and Chandrasekar, 2002; Bogacka, 2008; Chen and Hollingsworth, 2008; Galwa *et al.*, 2008; Shah *et al.*, 2008). Allergic bronchopulmonary mycosis is a term that has also been suggested for this condition as it may be caused by fungi other than *Aspergillus* spp.

Allergic fungal sinusitis (AFS) is a condition similar to ABPA and occurs in individuals with poor drainage problems and occurs when fungi e.g. *Aspergillus* or *Curvularia* species, colonise the sinus (Kuhn and Swain Jr., 2003; Yusuf Mohamed *et al.*, 2008).

1.2.3 Immunosuppression

It has been reported that various agents, present in the air of buildings with water intrusion problems, can suppress the immune system which subsequently can lead to an increased susceptibility to infections (Åberg *et al.*, 1996; Pirhonen *et al.*, 1996; Kilpeläinen *et al.*, 2001). People living or working in these environments have been found to have increased frequencies of common respiratory infections and many

microbes or the toxins they produce have been shown to have immunosuppressive effects *in vitro* (Piecková and Jesenská, 1996; Piecková and Jesenská, 1998).

1.2.4 Infection

Infection of immune compromised individuals i.e. those with cancer or with human immunodeficiency virus by common moulds including *Aspergillu*s species is well known. In the indoor environment opportunistic ubiquitous moulds find suitable hosts after indoor exposure of such individuals. *Aspergillus* species, reported to be the most aggressive of these fungi, give rise to infections also in patients with less severe airway disease, such as cystic fibrosis, asthma and chronic obstructive pulmonary disease (Geisler and Corey, 2002; Lednicky and Rayner, 2006).

1.2.5 Health effects of *Stachybotrys*

A disease known as Stachybotrytoxicosis has been attributed to toxin producing *Stachybotrys* species which have been found to produce cytotoxic effects in test animals (Jarvis, 1991). This disease was first reported in the Soviet Union in 1931 where horses suffered irritation of the throat, mouth, lips and nose followed by swollen glands and subsequent death after exposure to *Stachybotrys sp* contaminated feed. Necropsies performed on the fatalities found extensive internal haemorrhaging on all major organs especially the digestive tract (Forgacs, 1965; Forgacs, 1972). The same disease was also reported at a Hungarian farm where 100 sheep died after being exposed to bedding straw contaminated with *S. chartarum* (Harrach *et al.*, 1983). More recently Gyula (2005) reported a case of stachybotryotoxicosis in a domestic dog that had also been exposed to *S. chartarum* on bedding straw. Test guinea pigs exposed to the same bedding straw experienced changes similar to those observed in the dog which included

purulent-necrotic inflammation of the mucous membrane of the mouth and catarrhal rhinitis.

S. chartarum species have also been implicated as possible causes of adverse health effects to humans with cases of abdominal pain, nausea, vomiting, chills and diarrhoea being in affected individuals. Particular concerns related to *S. chartarum* exposure have been recently due to a disease suffered by humans which is very similar to stachybotryotoxicosis called Idiopathic pulmonary haemosiderosis (IPH). First reported in Cleveland, Ohio 1993-1994, 10 infants, previously in good health, were found suffering from the disease which resulted in each of them coughing up blood. All infants required paediatric intensive care admission after suffering severe haemorrhaging after which one died. The infants were all African American males except for one female and the conditions at which they were living were reported to be water damaged with growth of moulds including *S. chartarum* (CDC, 1994).

1.2.6 Neurotoxicity

Exposure to trichothecene mycotoxins produced by *S. chartarum* in water damaged buildings has been reported to cause many neurological effects including loss/lack of concentration, memory loss, fatigue and dizziness (Johanning *et al.*, 1996; Gordon *et al.*, 2007). In a study conducted by Karunasena *et al* (2010) satratoxin H was determined to cause the activation of inflammatory and apoptopic pathways in human neurological tissues, at levels similar to those found in water damaged buildings. Cells tested included Human brain endothelial cells (HBEC) which form the structure of the blood brain barrier, and astrocytes which act as macrophages in neural tissues and neurons. Their findings concluded that neural cells were unable to repair extensive

cellular damage from cytotoxic events that induce apoptosis or severe inflammation. There is however no study in the literature that has determined a definitive link between people living in damp buildings and neurological effects.

1.3 Microorganisms in the indoor environment

There are many organisms which live and breed in the indoor environment including fungi, dust mites, protozoa and bacteria, of which some have the potential to affect health and the quality of life. Many of the microrganisms are heterotrophic, requiring organic matter as their principal source of nutrients and thus obtain their nutrients from the everyday material used to build and furnish homes including wood panelling, plaster boards, wallpaper, carpet and wood furnishings (Plate 1.4) Many microorganisms produce particular enzymes that are able to break down the large organic molecules to their constituent smaller absorbable molecules (monomers) and are therefore associated with the particular materials on which they grow best. Some fungi, including *S. chartarum* for example, produce enzymes that are able to break down many constructional and finishing materials in buildings that contain natural organic polymers e.g. cellulose and starch (Flannigan and Miller, 2001).

Plate 1.4 Mould affected home

1.3.1 Fungi/moulds

Fungi are some of the most common microorganisms detected in the indoor environment. They are eukaryotic organisms that grow as long, tangled strands or hyphae which give rise to visible masses called mycelium. The mycelia generally have a rough cottony texture and can be seen with the un-aided eye. They contain typical organelles e.g. ribosome, mitochondria, an endomembrane system, a cytoskeleton and one or more nuclei. They are heterotrophic organisms which lack chloroplasts and thus cannot carry out photosynthesis and therefore acquire the nutrients needed for growth through absorption by secreting enzymes into the extracellular environment. These extracellular enzymes hydrolyze complex organic compounds into simpler more easily absorbable ones like glucose and amino acids. Some fungi are also capable of producing toxic secondary metabolites (mycotoxins) on water damaged building materials which have been reported to cause serious adverse health effects in humans and animals.

Together with bacteria, fungi decompose vast amounts of dead organic matter that would otherwise accumulate and make the earth uninhabitable (Kuhn and Ghannoum, 2003; Pommerville, 2004).

Fungi, including yeasts, comprise many different species that can enter buildings on clothing, through open windows and doors, and through ventilation systems. They are commonly detected in the indoor environment on painted walls and ceilings of poorly ventilated houses or those with water intrusion problems and include *Aspergillus, Cladosporium, Penicillium, Alternaria and Ulocladium* species, all of which can find suitable conditions for growth. Kuhn and Ghannoum (2003) investigated water damaged buildings and determined the most frequent isolated species in one study were *Penicillium* (96%), and *Cladosporium* (89%). A study which investigated the indoor air of 500 rooms in 128 dwellings in France, found that 86.16% of the total fungi isolated there were *Cladosporium, Penicillium* or *Aspergillus* spp (Roussel *et al.*, 2008). The highly toxic fungus *Stachybotrys chartarum* has also been found to thrive on many of the materials used to build homes and have particularly been found growing on gypsum board, paper, lint and fibreboard in buildings that have been affected by water intrusion (Andersen *et al.*, 2002). Numerous studies have demonstrated the conditions needed for extensive fungal growth and toxin production on building materials within the indoor environment and many of these conditions are similar to those found in improperly ventilated or water damaged homes (Billups *et al.*, 1999; Tsai *et al.*, 2001; Andersen *et al.*, 2002; Menetrez and Foarde, 2004; Nielsen *et al.*, 2004). The very worrying factor is that many common indoor moulds and the toxins that they produce have still not been fully characterized; therefore the true health implications associated with their growth in the indoor environment are not fully known or understood (Green *et al.*, 2005).

1.3.2 Penicillium

Penicillium species are some of the most commonly found fungal genera in buildings. These saprotrophic fungi naturally found in soil, can be found wherever organic material is available and have been frequently detected in air samples as well as on paint and wallpaper (Plate 1.5). Optimal temperatures for growth range between 25-30^{o}C with maximum growth rates occurring between 28 and 35^{o}C (Flannigan and Miller, 2001; Samson *et al.*, 2002). Some species are known mycotoxin producers and have allergenic properties e.g. *Penicillium chrysogenum.* Identification to the species level can be difficult and the spores are very similar to *Aspergillus* spores. *Penicillium* is often associated with water damaged buildings. It has been reported by Gravesen *et al* (1999) that most cellulose based materials in water damaged buildings are vulnerable to attack by indoor fungi with *Penicillium* species being among the most common particularly on gypsum boards. In their study *Penicillium* sp were recovered on 68% of 72 tested samples.

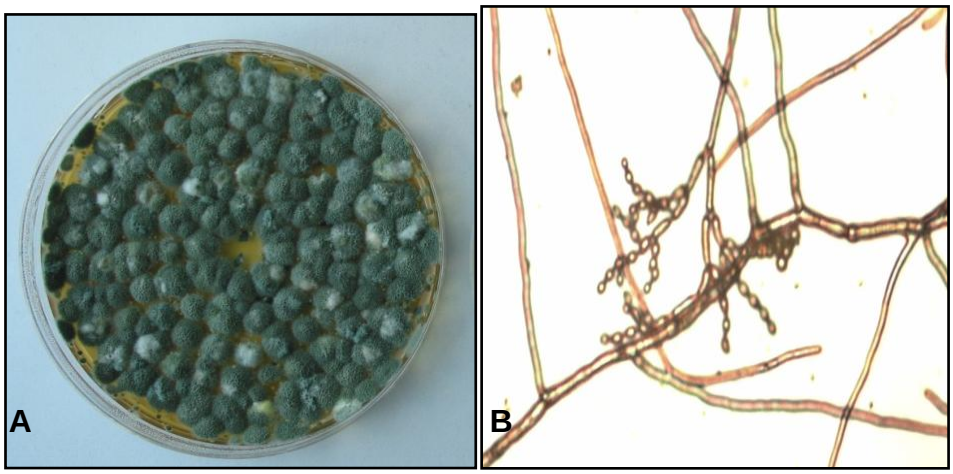

Plate 1.5 *Penicillium* sp (A) growth on MEA (B) micrograph x400

1.3.3 Aspergillus

These species are found on many different textiles and organic materials such as soil, compost, stored grain, wood and paper, and their moisture requirements vary widely with some preferring dryer conditions (Plate 1.6). Optimal temperature for growth is 30-40^{o}C with a maximal temperature for growth between 37-45^{o}C (Flannigan and Miller, 2001; Samson *et al.*, 2002; Pommerville, 2004). *Aspergillus* spp are often found growing on water damaged building materials and their dry spores are easily aerosolised making these organisms a common cause of respiratory irritation and infection. Their spores are similar to those of *Penicillium* species making identification indistinguishable through non-viable analysis, and as such, are often classified as *Penicillium/Aspergillus* spp. Species, such as *A. niger*, *A. versicolor*, *A. fumigatus*, and A. *flavus*, are some of most commonly found fungi detected on water damaged buildings accounting for up to 56% of the organisms detected on contaminated building materials (Gravesen *et al* (1999). These species are also commonly detected when water damaged carpeting is present, and feed well on the jute backing, the glues in the carpet padding, and on any organic dust that accumulates. The elevation of any one species of this genus in viable samples can be an indication of the growth of that mould inside a building.

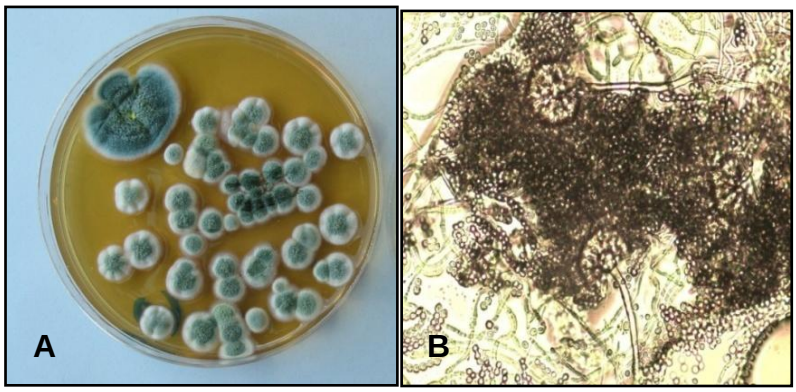

Plate 1.6 *Aspergillus sp (*A) growth on MEA (B) micrograph x400

1.3.4 Cladosporium

Cladosporium species are some of the most common mould found in outdoor and indoor environments. They are commonly found on many indoor surfaces and grow well on moist window sills. Hunter and Bravery (1989) investigated mould growth on gypsum boards in water damaged homes and reported the presence of *Cladosporium* sp in 70% of the mould patches tested. Airborne spores of these species are known allergens, and in large amounts they can cause hay fever and severely affect people with asthma or other respiratory diseases. The distinctive appearance of this mould yields olive to brown pigmentation with dry spores that easily become airborne through disturbance. The mould grows relatively fast with an optimal temperature for growth occurring between 20-28^{o}C and may look velvety or woolly (Plate 1.7). *Cladosporium* species are often used in the media as the marker organisms for the daily allergy level reports. High levels of *Cladosporuim* species indoors may not be a problem as long as the levels are less than the outdoor levels. They produce no major mycotoxins of concern but prolonged exposure may weaken the immune system (Flannigan and Miller, 2001; Samson *et al.*, 2002; Pommerville, 2004).

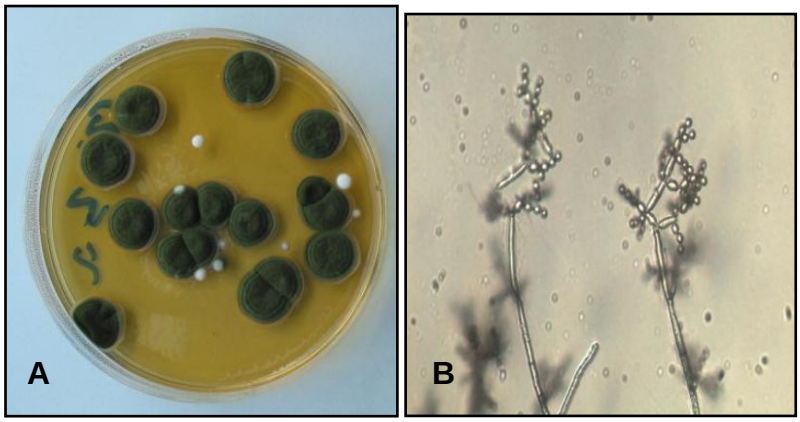

Plate 1.7 *Cladosporium sp* (A) **growth on MEA (B) micrograph x400 (B)**

1.3.5 Alternaria

Species of *Alternaria* are ubiquitous plant pathogens found in soil, seeds, plants and water damaged buildings (growing on carpets, textiles and horizontal surfaces such as window frames). Gravesen *et al* (1999) determined the presence of *Alternaria* sp in 8% of 72 water damaged building materials tested. This fungus ranges in colour from dark olive green to brown with a velvety texture and long soft hairs. The spores are club-shaped and can be individual or form long chains and easily become airborne through agitation (Plate 1.8). The optimum temperature reported for growth is between 25 and 30 °C, while the maximum growth temperature is between 32 and 35^{o}C (Magan and Lacey, 1988; Flannigan and Miller, 2001; Samson *et al.*, 2002; Pommerville, 2004;)

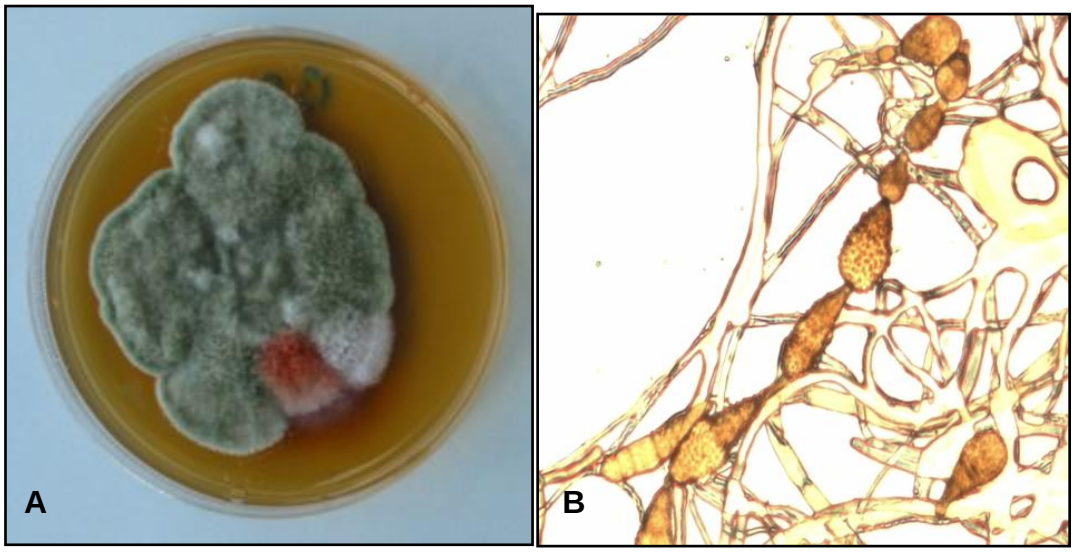

Plate 1.8 *Alternaria sp* (A) growth on MEA (pink area is a contaminant) (B) micrograph x400

1.3.6 Fusarium

Found in soil and on many plants, this mould requires very wet conditions to grow and is a producer of trichothecene toxins which affect the circulatory, alimentary, skin and nervous systems (Plate 1.9). Hunter and Bravery (1989) reported the presence of *Fusarium* sp in 13% of tested samples from water damaged buildings. Exposure to *Fusarium* species can lead to haemorrhagic syndrome (symptoms include nausea, vomiting, dermatitis, and extensive internal bleeding). Some *Fusarium* species have allergenic properties and they are also often associated with eye, skin and nail infections. High moisture requirement for growth mean that this organism is often found in buildings with water intrusion problems. Optimum temperature for growth is 25^{o}C with a maximum temperature between 34-37^{o}C (Flannigan and Miller, 2001; Samson *et al.*, 2002; Pommerville, 2004).

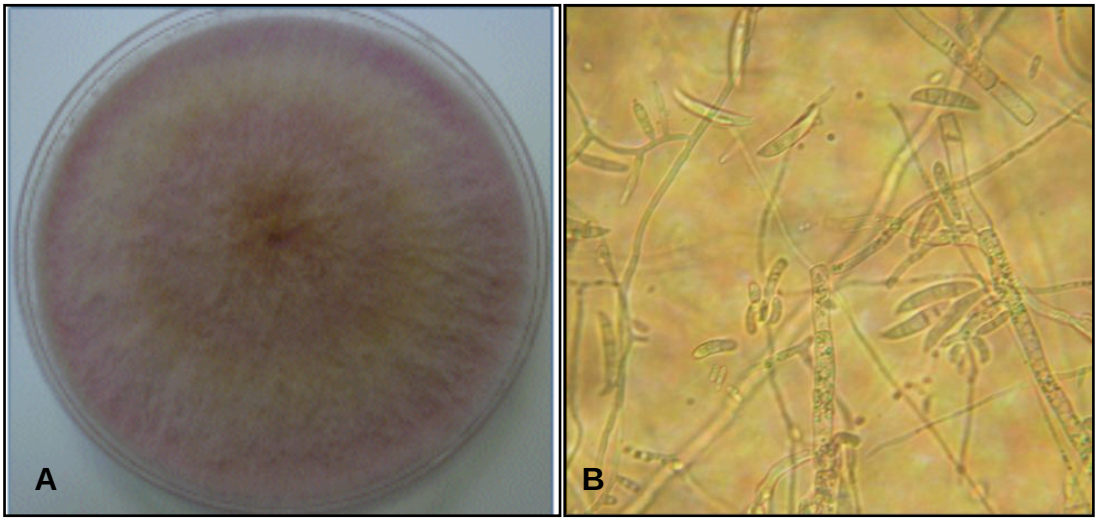

Plate 1.9 *Fusarium sp* (A) growth on MEA (B) Micrograph x 400 (Kung'u, 2006)

1.3.7 Trichoderma

Trichoderma are a genus of fungi also known as opportunistic avirulent plant symbionts. Species of this genus are present in all soils and are the most prevalent culturable fungi. They have also been detected growing on gypsum boards in water damaged buildings (Gravesen *et al.*, 1999). During growth, on media such as potato dextrose agar (PDA), colonies first appear as white mycelium, this is followed by the production of compact or loose conidia, usually green or yellow in colour, within one week (Plate 1.10). Optimum temperatures for growth are between 25-30°C with no growth at 35°C or greater. A yellow pigment may also be secreted into the agar and some species have been found to produce a characteristic sweet or 'coconut' odour (Flannigan and Miller, 2001; Samson *et al.*, 2002; Pommerville, 2004).

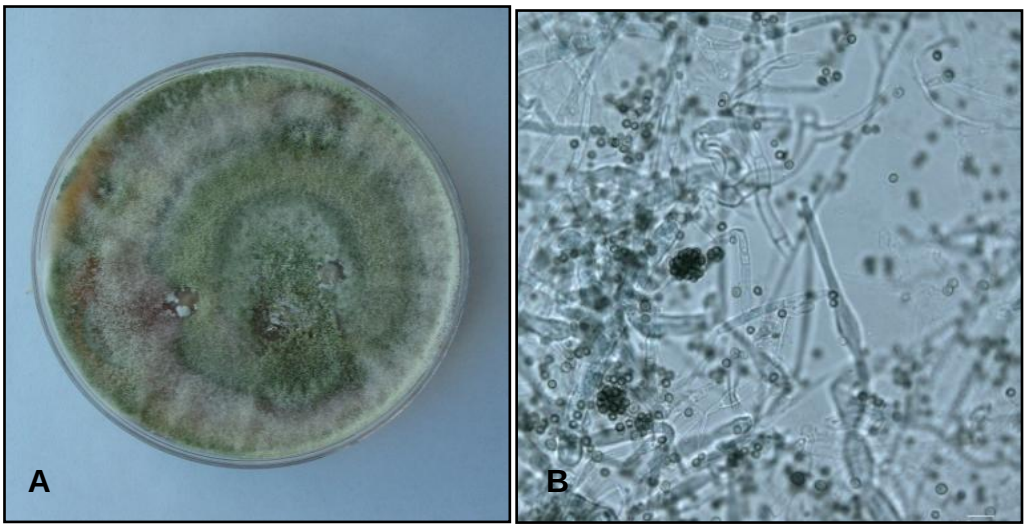

Plate 1.10Trichoderma sp (A) growth on MEA (B) micrograph x400

1.3.8 Stachybotrys chartarum

Proposed by Corda (1837) *Stachybotrys chartarum* (also known as *S. atra* and *S. alternans*) is a greenish black wet slimy mould (Plate 1.11). These organisms produce asexual spores (conidia) by conidium producing cells known as phialides on fertile hyphae by mitosis and colonies may also have a powdery surface during sporulation (Jong and Davis, 1976). Significant studies have been carried out which have contributed to further understanding this particular species. Bisby (1945) presented a detailed review of the earlier literature on this genus and described them as having brightly coloured or dark hyphae, phialophores and phialides with accumulating clusters of dark conidia (slime spores). Jong and Davis (1976) also presented a detailed description of *Stachybotrys* sp and compared its close similarity to the *Memnoniella* spp. In this very detailed investigation it was reported that the two fungi differed in the ways in which they produced conidia. *Stachybotrys* was found to produce slimy heads of conidia whereas *Memnoniella* produced chains of conidia. The times at which this occurred also differed.

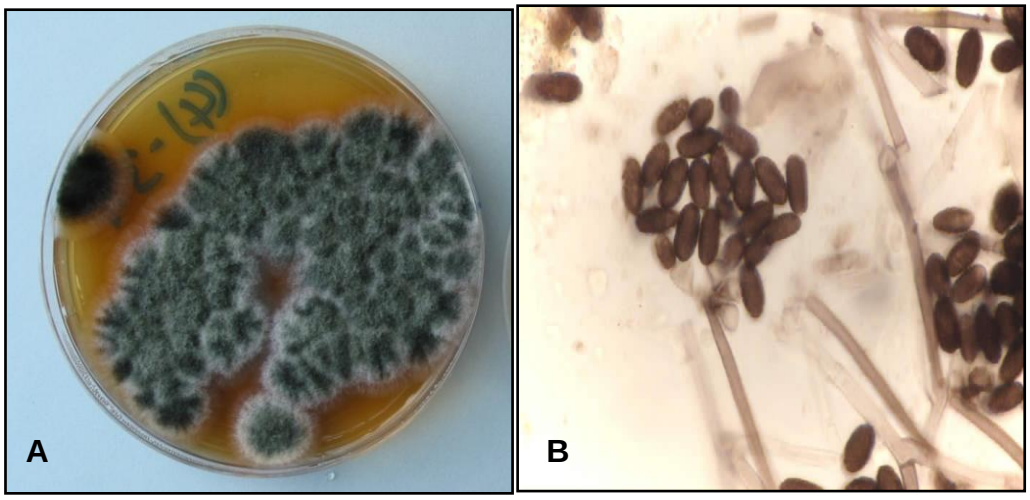

Plate 1.11 *Stachybotrys chartarum* plated on MEA (left) and micrograph x 400 (Right) (Florida Mould Inspectors, 2009)

This very important report documented the characterisation and re-identification of 50 fungal strains from the American Type Culture Collection (ATCC) and included detailed illustrations of the fungal species including colour, shape and other features used to definitively distinguish different species of *Stachybotrys* and *Memnoniella*. There are very limited numbers of reports in the literature that state the growth conditions required for *S. chartratum* isolates. Optimal growth conditions are reported to be high moisture levels with temperature variability, and infestation often results from the build up of moisture due to condensation, excessive humidity, water leaks, or flooding (Peltola *et al.*, 2001; Haverinen-Shaughnessy *et al.*, 2008; Roussel *et al.*, 2008).

Indoor isolated strains of *S. chartarum* have been found to grow well on wet material with high cellulose content such as wallpaper, gypsum board, fibreboard, drywall, paper, wood, dust and lint. Nielsen *et al* (1998) investigated a heavy infestation of *S. chartarum* on water damaged gypsum boards in a domestic residences in Copenhagen and found these strains to be highly toxic macrocyclic trichothecene producers. Gravesen *et al* (1999) determined the presence of *S. chartarum* in 19% of 72 water damaged building materials tested. Boutin-Forzano *et al* (2004) investigated the effect of wall relative humidity of 458 samples from 100 dwellings. Results were very conclusive with *S. chartarum* growth detected on samples containing high levels of humidity. Previously Ayerst (1969) investigated the effects of moisture and temperature on the growth of fungi and determined high water requirements of *S. chartarum.*

1.4 Microorganisms and water activity

Water availability is the primary requirement essential for microbial growth, with specific free water availability requirements needed for each and every one. The free water in a material is the absorbed water that is loosely held in spaces by the weak forces of capillary action (Flannigan and Miller, 2001). The availability of water also termed water activity can be reduced with the addition of salts or sugars to substrates. This causes a reduction in the availability of water to microbial cells. Other factors that can affect the availability of water in a substance include dissolved substances which can interact with water through interactions with chemical groups on un-dissolved ingredients like proteins and starches through ionic bonds, van der Waals forces, hydrophobic interaction and hydrogen bonds which reduce the humidity above the sample at equilibrium by combining to increase the energy required to cause the water to evaporate and results in a fall in the available water. The lower the water activity the less likely microbial cells will grow although there are some exceptional microbes called osmophiles that can grow at low water availability.

1.4.1 Definition of Water activity

Water activity (a_w) is defined as the vapour pressure of water above a sample divided by that of pure water at the same temperature (at equilibrium) and represents the energy status of the water in a system. Pure distilled water has a water activity of exactly one.

Formula $a_w = P/ Po$

P: is the % vapour pressure of water in a substance, Po: is the % vapour pressure of pure water at the same temperature

1.4.2 Water activity requirements for microorganisms in the indoor environment.

Microorganisms vary in their water activity requirements, higher a_w substances tend to support more microorganisms. Bacteria usually require a water activity of at least 0.91 a_w, and fungi at least 0.7 a_w. Fungi can be grouped according to their ability to grow in low a_w conditions (Grant *et al.*, 1989) (Plate 1.12 and Table 1.1). **Primary colonisers:** Species capable of growth below 0.8 a_w. **Intermediate colonisers:** Species capable of growth between 0.8 and 0.9 a_w. **Tertiary colonisers:** Species requiring at least 0.9 a_w for growth met mainly by conditions of incoming water, and to a lesser extent high humidity and condensation.

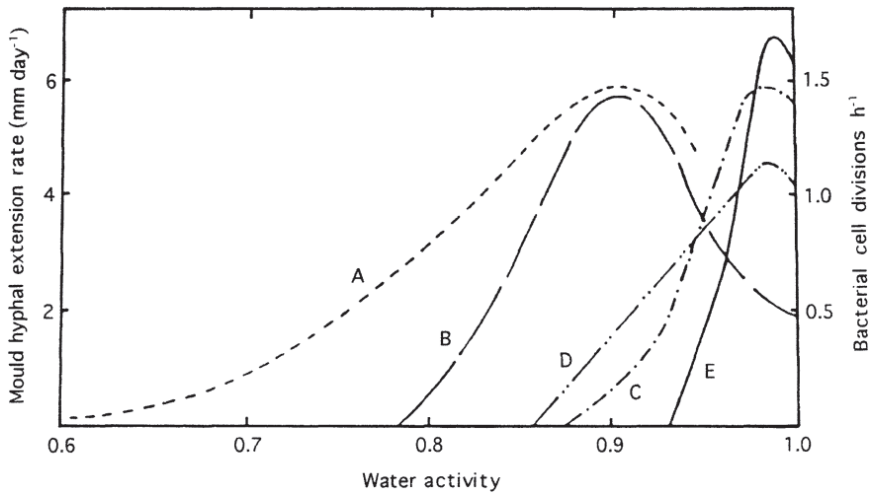

Plate 1.12 Relationship between water activity and growth rate of fungi and bacteria.
(Flannigan and Miller, 2001)

A: *Xeromyces bisporus;*
B: *Eurotium herbariorum (A. glaucus)* } Fungi
C: *Aspergillus niger;*

D: *Staphylococcus aureus;* } Bacteria
E: *Salmonella* sp.

Table 1.1 Moisture level requirements for microorganisms on building materials (WHO, 2009)

Moisture level	Category of microorganism
Low (< 0.80 a_w)	**Primary colonisers**
	Aspergellus citri
	Aspergillus (Eurotium) amstelodami
	Aspergillus niger
	Penicillium aurantiogriseum
	Penicillium chrysogenum
	Penicillium griseofulvum
	Wallemia sebi
Intermediate (0.80-0.90 a_w)	**Intermediate colonisers**
	Aspergillus flavus
	Aspergillus versicolor
	Cladosporium species
	Rhizopus sp
High (> 0.90 a_w)	**Tertiary colonisers**
	Alternaria alternata
	Aspergillus fumigatus
	Epicoccum sp
	Fusarium moniliforme
	Rhizopus sp
	Stachybotrys chartarum
	Trichoderma sp
	Ulocladium consortiale

1.5 Metabolites and compounds produced by fungi

1.5.1 Microbial Volatile Organic Compounds (MVOCs)

MVOCs are organic chemical compounds that have high enough vapour pressure under normal conditions to significantly vaporise and enter the atmosphere. They have been reported to be associated with many health effects which are dependent on the exposure period, concentration and toxicity of the pollutant (Environmental Protection Agency, 2011).

During microbial growth MVOC production is sometimes driven by the competition for resources in an environment where nutrient availability is poor. Moulds subsequently produce metabolites including carbon dioxide, water and many MVOCs including aldehydes, alcohols, ketones, and hydrocarbons. These MVOCs are usually responsible for the musty mouldy odour that is noticed by occupants of affected dwellings (Piecková and Jesenská, 1999). Many organisms produce distinctive patterns of MVOCs which are being used as tools to determine the presence of specific toxic organisms but this has proved difficult as they have been reported to produce different patterns of MVOCs on different substrates (Claeson, 2006). MVOCs such as 1-butanol, 3-methyl-1-butanol (isoamyl alcohol), 3-methyl-2-butanol and thujopsene (an essential oil) were found to be produced when *S. chartarum* cultures (from water-damaged homes in Cleveland) were grown on rice, but only 1-butanol was produced when the same cultures were grown on gypsum boards (Gao *et al.*, 2002; Betancourt *et al.*, 2006). Wilkins *et al* (2000) reported methoxybenzene (also known as anisole) to be a unique MVOC specific to *S. chartarum* and this was also shown by Mason *et al* (2010) who determined that this MVOC was produced by *S. chartarum* while growing on many

building materials including gypsum wallboard and ceiling tiles. This was an important finding and could be a very useful tool for determining *S. chartarum* contamination in buildings without destructive sampling. It is important to note that not all MVOCs are of microbial origin. Other pollutants of indoor air quality include those that are generated outdoors by industry and traffic which diffuse into buildings through doors, windows or ventilation systems. Other indoor sources include VOCs released from cleaning products, furnishings toiletries and those that are involved in the combustion of tobacco, fuels and candles.

1.5.2 Mycotoxins

'Mycotoxin' is a general term used to describe the toxic secondary metabolites produced by many fungi. They have been found to have a variety of toxic effects when exposed to humans and animals via ingestion of food upon which fungal moulds have grown or by the inhalation of fungal spores or fragments coated in mycotoxins in the environment. It has been reported that mycotoxin production by a particular mould is often a result of competition between other organisms and also a response to specific environmental conditions including temperature, water activity, pH and availability of food. Importantly, this means that the amount of toxin produced by a fungus is often dictated by the prevailing environmental conditions (Davis, 2001). Examples of mycotoxins include: Aflatoxins produced by *Aspergillus* species including *Aspergillus flavus* and *Aspergillus parasiticus* that are capable of growth on a variety of foods and organic substances. These commonly studied mycotoxins have been found to be toxic to the liver cells and have carcinogenic properties (Kuhn and Ghannoum, 2003). Sterigmatocystin produced by the fungi *Aspergillus nidulans* and *A. versicolor* are potent liver carcinogens. These toxic metabolites are closely related to the aflatoxins but

appear to occur less frequently. A study conducted by Tuomi *et al* (2000) on toxins in water damaged buildings determined sterigmatocystin as the most prevalent toxins being detected on one in five samples of material from which species of *Aspergillus* were recovered. Ochratoxins produced by some *Aspergillus* and *Penicillium* species are reported to be carcinogenic and can cause acute toxicity to mammalian kidneys.

1.5.3 Trichothecenes

History and general structure

The term trichothecene comes from trichothecin, which was one of the first members of the family to be identified (Bennett and Klich, 2003). They make up a family of closely related compounds called sesquiterpenoids and are non-volatile low-molecular weight (MW 250-550) compounds (Wannemacher and Wiener, 1997). Trichothecenes are produced by species of *Fusarium, Myrothecium. Trichoderma, Verticimonosporium, Cephalosporium, Memnoniella* and *Stachybotrys* and are identified by a characteristic tetracyclic 12, 13-epoxytrichothecene skeleton and an olefinic bond with various side chain substitutions (Plate 1.13). They are cytotoxic protein synthesis inhibitors to most eukaryotic cells and affect rapidly proliferating tissues once in systemic circulation (Ciegler, 1975; Chapman, 2003; Widestrand *et al.*, 2003; Abid-Essefi *et al.*, 2004; Doi *et al.*, 2008).

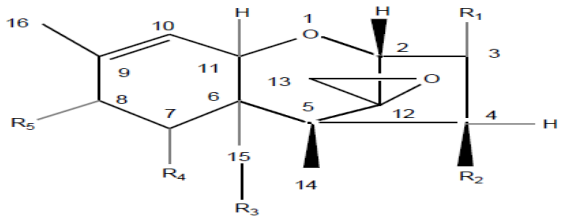

Plate 1.13 The general structure of the tetracyclic trichothecene nucleus.

1.5.3.1 Trichothecene groups

Trichothecenes are divided into four groups and are classified as macrocyclic or non macrocyclic depending on the presence of a macrocyclic ester or an ester-ester bridge between C-4 and C-15 (Bennett and Klich, 2003).

Type A trichothecenes (Non macrocyclic): Highly cytotoxic and can be isolated from species within the fungal genera *Memnoniella, Trichoderma, Trichothecium, Myrothecium, Stachybotrys,* and *Fusarium* spp.

Type B trichothecenes (Non macrocyclic): Produced only by *Fusarium* species, are characterized by a C_8-keto group and are 10% as toxic as Type A trichothecenes. Examples in this group include nivalenol and Deoxynivalenol (vomitoxin), which are highly toxigenic and have been noted for producing a wide range of immunological disturbances both in humans and animals (Foroud and Eudes, 2009).

Type C Trichothecenes (Macrocyclic trichothecenes): Produced by *Stachybotrys* and *Myrothecium* spp, are characterized by the ring from R2 to the R3 alcohol group and are at least 10 times more cytotoxic than the type A trichothecenes

Type D trichothecenes: these are characterized by C-7, 8 or C-9, 10 epoxy group

1.5.3.2 Macrocyclic trichothecenes

The macrocyclic trichothecenes, produced by *S. chartarum,* include satratoxins F, G, and H, verrucarrins B and J, isosatratoxins F, G and H and roridin E and roridin L-2. Studies performed on mammalian cells have found satratoxin G to be the most cytotoxic (Yang *et al.*, 2000).

Non macrocyclic trichothecenes include trichodermol (roridin C), trichodermin and trichoverrins. Studies have found that many *S. chartarum* isolates, irrespective of geographical origin, produce the same mycotoxins and that non toxin producers can become producers of toxins when grown on specific culture medium when enriched with glucose (El-Maghraby *et al.*, 1991).

1.5.3.3 Chemotypes of *S. chartarum*

It is reported that about one-third of *S. chartarum* isolates produce macrocyclic trichothecenes and about two-thirds produce the less toxic non-macrocyclic trichothecenes. Isolates are generally divided into two chemotypes and produce different patterns of VOCs (Koster *et al.*, 2003).

Chemotype S: Macrocyclic trichothecene producers e.g. satratoxins (produced by 40% of *S. chartarum* strains, (Plate 1.14 A and B).

Chemotype A: Non macrocyclic trichothecene producers e.g. atranones and dolabellanes (Plate 1.14 C and D), (produced by 60% of *S. chartarum* strains) (Hinkley *et al.*, 2000).

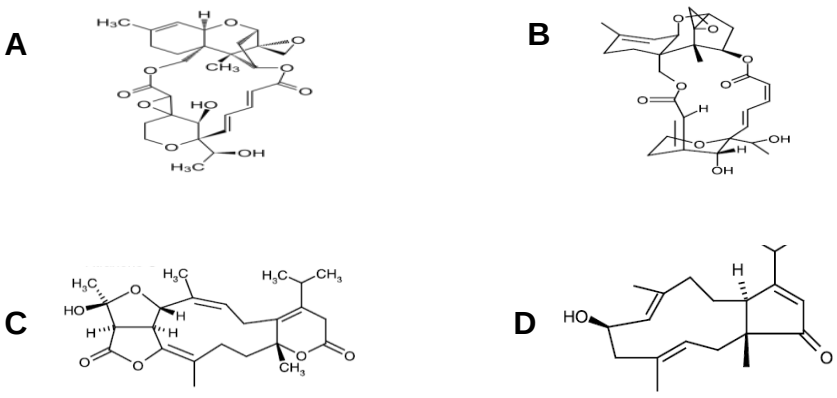

Plate 1.14 General structures of (A) Satratoxin G, (B) Satratoxin H, (C) Atranone C and (D) Dolabelladiene

Literature has so far not reported any strains that produce both chemotype groups, but it has been suggested that this may be due to insufficient methods for their detection. Some atranone producing isolates of *Stachybotrys* previously thought to be of the *chartarum sp* have not been found to correspond to any of the two chemotypes and have been given the name *S. chlorohalonata*. These species produce a green halo of extracellular pigment around the colonies when grown on Czapek Yeast Autolysate *aga*r (CYA) medium (Andersen *et al.*, 2002).

1.5.4 Stachylysin

S. chartarum isolates are also capable of producing cytotoxic metabolites known as stachylysins. These metabolites are secreted from mycelia and conidia and are capable of lysing red blood cells through haemolytic activity (Gregory *et al.*, 2004; McGinnis, 2004). Vesper and Vesper (2002) determined haemolytic alterations when eight strains isolated from homes in Cleveland and Texas from infants suffering from Pulmonary haemorrhage (PH) were examined in a laboratory setting. It was reported that only one of these isolates produced stachylysin when grown on Tryptic soy broth, but stachylysin production was found from all isolates when they were grown on the same media with the addition of 0.7% sheep's blood.

1.5.4.1 Yellow Rain Controversy

It is speculated that the Soviet Union and its client states used aircraft launched rockets, spray tanks and exploding canisters containing trichothecenes as biological weapons between 1974 and 1981 in Cambodia, Afghanistan and Laos. Air attacks were said to consist of sticky yellow material that sounded like rain as it fell from the sky termed "yellow rain", which rapidly dried to a powder. It is documented that these attacks

resulted in more than 981 deaths in Cambodia, 3,042 deaths in Afghanistan, and 6,310 deaths in Laos (Spyker and Spyker, 1983; Watson *et al.*, 1984; Heyndrickx *et al.*, 1984; Dashek *et al.*, 1986; Katz and Singer, 2007).

It has been suggested that trichothecenes are easily absorbed through the pulmonary mycosa and gut due to their lipophilic nature. Effects have been found to be less rapid when they are absorbed through the skin in the form of dust or powder but can be increased if they are dissolved in solvents like dimethyl sulfoxide **(**DMSO) (Wannemacher and Wiener, 1997).

Absorption of many toxins, including the T-2 toxin (produced by *Fusarium* spp) has rapid effects when administered systemically and peaks in the blood within an hour. The dose of inhaled T-2 toxin has an equal or lesser effect than the systemic one where exposure to high concentrations of aerosolized mycotoxins are lethal within 1-12 hours on mice, guinea pigs and rats (Marrs *et al.*, 1986; Stark, 2005; Pestka, 2007).

1.6 Indoor air quality

For many years indoor air quality and fungi have been issues in housing, with a vast range of associated health problems reported (Andersson *et al.*, 1997; Peat *et al.*, 1998; Davis, 2001; Seppänen and Fisk, 2004; Sahakian *et al.*, 2008)**.** The distribution levels of airborne indoor fungi are usually around 10% of outdoor levels. A higher level of particular types of fungi, detected in the indoor environment when compared to outdoor levels, is indicative of contamination indoors. The quality of air in the indoor environment can be greatly influenced by temperature and humidity but can be also affected by many other factors including the presence of volatile organic compounds

VOCs, particulate matter and biological particles e.g. bacteria, fungi and pollen. Research is mounting which suggests that occupants of many homes which have been flooded, have damp problems, or have inadequate ventilation systems, are potentially at risk of ill health from related air quality problems (Lu *et al.*, 2009). Studies have shown that many fungi thrive in these favourable conditions in the indoor environment and subsequently produce large numbers of spores and other allergens, which once airborne, can be inhaled and deposited on the mucosal surface of the upper airways and eyes and can cause hay fever-like symptoms or other more serious health problems (Singh, 2005).

1.6.1 Aerosolisation

Many factors affect the aerosolisation of fungi into the indoor air from a contaminated source including air flow and relative humidity. Many organisms have specially adapted techniques which facilitate their chances of survival. Many fungi, for example, produce spores that can be dispersed in to the air over long distances from their reproductive structures by forcible ejection, which also ensures that the spores are fully removed from the reproductive structures (Elbert *et al.*, 2007; Trail, 2007; Yafetto *et al.*, 2008). High humidity in some cases stimulates active release mechanisms that depend on rupture of turgid cells, while low humidity can cause tissue desiccation which triggers another class of release mechanism. Studies on these processes for spore release have mainly been carried out in controlled experiments and have generally been conducted under equilibrium conditions in simple systems, and the impact of the indoor microenvironment has not been considered (Ingold, 1971; Schmale III *et al.*, 2005).

1.6.2 Indoor air quality regulations

Various institutions world-wide have proposed guidelines for the detection of indoor fungi but currently there are no universally accepted techniques for indoor fungal detection. Guidelines include a report published in Europe by the Commission of the European Communities (CEC) on biological particles in indoor environments with the strategy and methodology for investigating indoor environments, and a manual outlining methods for microbiological sampling published by The American Industrial Hygiene Association (AIHA). There are also no universally accepted threshold limit values (TLVs) for airborne concentrations of spores and no health-based exposure limits for indoor biological agents have been recommended (Samson *et al.*, 2002). Recently WHO (2009) produced an extensive review which set guidelines intended for worldwide use, to "protect public health under various environmental, social and economic conditions and to support the achievement of optimal indoor air quality". This review outlined objectives for indoor air quality management with a detailed review of the many factors affecting indoor air quality and health. In England and Wales there are however regulatory requirements for domestic ventilation aimed at preventing fungal growth. These regulations, covered under a series of Approved Documents, have been published by the UK Government (HM Government, 2010).

1.6.3 Risk indicators

The presence of *Aspergillus fumigatus*, *Stachybotrys chartarum or* some *Penicillium* species have been reported to be significant risk indicators for measuring indoor air quality (Nieminen *et al.*, 2002). Allergenic *Penicillium* species, for example, are very common in some indoor environments and, at high concentrations; these can become

problematic for sensitive individuals. Although these organisms are good indicators of fungal contamination, they are not considered accurate indicators of indoor air quality as there are many other non-viable propagules capable of causing allergic responses that are not detectable by the conventional methods. The level of disease causing micro-organisms in the indoor environment deemed as safe was suggested by Wright *et al* (1969), who proposed that the total number of micro-organisms should not exceed 1700 cfu m^{-3} of air under normal conditions. Gravesen (1979) reported allergic symptoms where levels of *Cladosporium* spp and *Alternaria* spp in air were 3000 and 100 cfu m^{-3} of air and Nathanson (1995) has suggested that unacceptable levels of air spora in the office environment may be as low as 50 cfu m^{-3} of air if only one species is present and 150 cfu m^{-3} of air if a mixture, but these levels are very stringent and do not correlate with the majority of indoor air quality assessments. It has been proposed that if indoor air contains a level of viable micro-organisms exceeding 1000 cfu m^{-3} of air, then an investigation into the problem is perhaps required. The probability of there being an active source of contamination within the indoor environment is also increased if more microbial propagules are recovered from the air in an indoor environment compared to a local outdoor air sample, which would signal the need for further investigation. For example when an indoor air sample is found to be composed entirely of *Penicillium* species, compared to an outdoor sample composed of phyllosphere/soil organisms this is an indication that there is probably a contaminated source within the building.

The levels of airborne *S. chartarum* spores, however, have been much more difficult to determine, and have been the topic of extensive debate for many years especially due to the increase in cases of people suffering respiratory ailments associated with affected dwellings. Due to the growth characteristics of *Stachybotrys* species and its production

of wet slimy spores, literature has reported that it is highly unlikely that these will easily become aerosolised. This has been disputed as spores of *S. chartarum* have been isolated from the air in homes sampled in Southern California, London, central Scotland, and in Cleveland, Ohio, USA. Detection of *S. chartarum* in water damaged buildings however is problematic.

It is reported that *S. chartarum* spores are often non viable on growth medium and therefore the true levels of exposure are very hard to determine with conventional methods (National Toxicology Program, 2004). Wu *et al* (2003) also reported the loss of viability of airborne *S. chartarum* spores but also determined a retained toxigenic effect. Brasel *et al* (2005) reported that toxins produced by *S. chartarum* were not only found on conidia, but also on many smaller particles e.g. fungal fragments, substrates or debris which have the potential to become airborne and persist over a longer period of time. These smaller particles were likely to have been saturated with *S. chartarum* toxins during growth and once dried were easily aerosolised through slight mechanical movement. Recently Gottschalk *et al* (2008) detected the presence of highly toxic metabolites produced by *S. chartarum* from the indoor air of a water damaged building. This was a significant finding as these toxins have rarely been isolated in the indoor environment.

1.6.4 Sampling methods for the detection of indoor fungi

There are various methods used to examine the levels of fungal contaminations within buildings, but there are currently no universally accepted methods used for the detection of indoor fungi which has made it extremely difficult to draw comparisons between different locations and determining the true health implications associated with indoor

fungal growth. Many of the methods used for indoor fungal detection have been reviewed by Portnoy *et al* (2004) who proposed a standardised guideline for the detection of indoor fungi. The aim of this guideline was to establish a foundation by which all fungal investigators and remediation companies alike could adhere to enabling a general consensus for indoor fungal analysis. Current methods used for the detection indoor fungal contaminants include:

Visual examination: This is one of the primary methods used to measure the extent of contaminant fungi on building materials. This method enables rapid identification of some fungi based on their morphological structures as formed on indoor substrates.

Air sampling: these methods can be divided into volumetric and non-volumetric of which both have their limitations. The non-volumetric method involves the use of sedimentation plates consisting of media that are left open in the environment under investigation for 15-60 minutes. Plates are then closed and the resultant fungal growth after 5-10 days at 25^{o}C is counted and identified. A bias towards small fungal propagules e.g. *Aspergillus* and *Penicillium spp* have been reported to be drawbacks to this method. The volumetric method involves the use of impact devices, including the (Andersen, 1958) air sampler (AAS) and the Surface Air Sampler (SAS), which draw a measured volume of air from the surrounding environment on to microbiological growth media. The resultant fungal growth is counted and identified after 5-7 days incubation at 25^{o}C (for general indoor fungi) and 37^{o}C (for opportunistic pathogens) and are expressed as CFU m^{-3} or CFU $20l^{-1}$ of air dependent on device used.

Direct plating/Bulk sampling involves the use of representative pieces of material suspected of fungal contamination. Small pieces of material are plated directly on to

growth media where viable organisms present can be cultured and identified after 5-10 days incubation at the relevant temperature. Problems faced with this method are that fast growing organisms e.g. *Aspergillus, Penicillium* spp, yeasts and bacteria are capable of out competing more slower growing species and therefore can mask a true representation of the total fungal flora present on the material under investigation. To combat this problem selective media supplemented with antibacterial and antifungal agents are used e.g. chloramphenicol and miconazole.

Swab sampling:

Surface swab samples are used to derermine the level of fungal contamination on a surface under investigation. They are collected by brushing swabs over suspected areas which are then suspended in liquid and diluted as necessary and plated out on specific growth medium in the required conditions. Subsequant growth is then quantified and identified to the genus and the concentrations are expressed in terms of colony forming units per swab or colony forming units per area sampled (CFU cm^2).

Aims and objectives:

- To survey for fungal contamination homes with a varied range of water intrusion i.e. ventilation/condensation problems to more chronically water damaged dwellings.
- To better understand the occurrence, dynamics of growth and toxin production for important toxigenic fungi, principally *Stachybotrys chartarum*, in the built environment.
- To investigate the use of alternative methods e.g. Bioscreen, to develop rapid methods for measuring the effects of water activity and temperature on *S. chartarum* growth *in vitro* as well as antifungal susceptibility.

The work programme will be organized in three broad phases:

a) *In vitro* phase: To characterize growth for a range of biotic and abiotic conditions in the laboratory. Studies will investigate temperature, water activity, substrate type, presence of toxins and interactions between competing fungal species in the context of damp and energy efficient conditions. Studies will be carried out on agar media, and toxin production will be assessed by chromatographical methods or by toxicity assays.

b) *In vivo* phase: To characterize the development of fungal contamination on water saturated building materials in environmental chamber conditions, investigating in particular the relationship between *S. chartarum* species and temperature, water activity and substrate type. The use of antifungal compounds to control fungal growth on building materials will also be investigated.

c) Toxin and extracellular metabolite production: To determine the affect of water activity and temperature on the production of satratoxin G by *S. chartarum* isolates. To determine the effect of water activity on the extracellular enzyme production by isolates of *S. chartarum*. To determine of the haemolytic activity of *S. chartarum* isolates.

2 MATERIALS AND METHODS

2.1 Initial survey

The initial aim of the research was to carry out a small survey on buildings with varying extents of water damage to determine the typical types of flora associated with them, and to specifically determine how often the toxic fungus *S. chartarum* could be isolated. Buildings surveyed in this study included those with minor water intrusion problems e.g. condensation problems, those with moderate water intrusion problems including water leaks or plumbing problems, and those with severe water damage i.e. flooded buildings or with extensive roof leakage (Table 2.2). As there are no standard methods outlined for sampling indoors a range of procedures were used for the detection of the associated flora including: air sampling, swabbing, contact plating and bulk sampling.. These procedures were used in conjunction with specialised, selective agar media to identify the general fungal population and specific fungal genera from the samples and included malt extract agar (MEA), potato dextrose agar (PDA) and cellulose agar (CA). The nutrient rich general purpose growth medium MEA was used to detect all fungal species present in the environments under investigation. Some MEA along with PDA were also supplemented with chloramphenicol (an antibiotic to suppress bacterial growth) and miconazole (an anti-fungal, used particularly to suppress faster growing fungi in mixed cultures and thus allow the growth and detection of *S. chartarum*).

CA, a nutrient limited medium, was specifically used to isolate *S. chartarum* based on its cellulytic properties and thus prevent the overgrowth of other highly competitive fungal species. 82 mm Agar plates and 55 mm contact plates were made up for all media preparations. These detection procedures were also compared for their

usefulness. In order to obtain access to relevant homes suitable for the survey an advertisement, which explained the details of the research and the types of homes being investigated i.e. water damaged or those suffering from ventilation problems, were posted on to the university internet and in the Cranfield University magazine. This resulted in 4 responses which were investigated along with others found independently.

2.1.1 Preparation of miconazole stock solution (1.6μg ml^{-1})

A stock solution of miconazole was first prepared by adding 160μg of miconazole (Fischer, UK) to 100ml of acetone (Fischer, UK). This solution was then filter sterilized using a 0.2μm filter and stored at room temperature until required.

2.1.2 Preparation of PDA supplemented with 0.1g l^{-1} chloramphenichol + 1.6μg l^{-1} miconazole

PDA was prepared according to the manufacturer's instructions. 39g of potato dextrose agar, (Oxoid UK), along with 0.1g of chloramphenicol, (Sigma UK), were added to a liter of water and sterilized at 121˚C for 15 minutes. After sterilization the agar was cooled to 50˚C followed by the aseptic addition of 1ml of the previously prepared miconazole solution.

2.1.3 Preparation of MEA

MEA was prepared by adding 50g of Malt extract agar (Oxoid, UK) to a litre of water and sterilized by autoclaving at 121˚C for 15 minutes. The addition of 0.1 g l^{-1} of chloramphenicol before autoclaving was also required for supplemented preparations of MEA.

2.1.4 Preparation of modified CA

Modified CA was prepared by adding all the constituents shown in Table 2.1. The mixture was sterilized by autoclaving at 121˚C for 15 minutes.

Table 2.1 Modified cellulose agar preparation

Constituents	Supplier	g 1^{-1}
$NaN0_3$	Sigma	0.5
K_2HP0_4	BDH	10
$MgS0_47H_20$	Fischer	0.5
KCl	Fischer	0.5
$Fe_2(SO4)_37H_2O$	BDH	0.1
Alpha-Cellulose	Sigma	12
Agar No.1	Oxoid	20

2.1.5 Preparation of buffered peptone water (BPW)

BPW (Oxoid, UK) was prepared according to the manufacturers' instruction. 20g of BPW was added to 1 litre of water. Once fully dissolved the solution was dispensed into universal bottles in (9.9 ml, and 2 ml) volumes and then sterilized by autoclaving at 121˚C for 15 minutes.

2.1.6 Procedure for sampling

a) Visual Assessment: Notes and photographic capture of areas showing visible signs of fungal contamination and/or water damage was taken. These areas were selected for mycological examination, including surfaces and air samples.

b) Air Sampling: Viable microbial contaminants in air were trapped using an agar plate impacter **(**Burkard, Hertfordshire, England) comprising of a pump and a sieve plate containing 100 precision drilled holes. This instrument was used at 1 minute intervals with a pump flow rate of 20 l min^{-1} to draw in air from the environment onto an agar plate containing growth medium previously loaded into it (Plate 2.2A). All agar plates obtained from air sampling were incubated at 25 ± 2^{o}C for 1 week with examination at 3, 5 and 7 days. This was followed with identification, at least to genus level, of any subsequent fungal growth from samples after enumeration.

c) Swab sampling: Swabs (Fischer, UK) (Plate 2.2C) were taken of floor, wall and wood surfaces (where appropriate), using individually sealed, sterile swabs initially wetted (where appropriate) in 2 ml of BPW along with a pre-cut 5 x 5cm plastic stencil (25 cm^2) (used for quantitative sample measurement) (Plate 2.1). Swab sampling involved sweeping the swab across the test area, first in a horizontal direction, then in a vertical direction, which was then placed into a universal bottle containing 2ml of BPW before being transported back to the Cranfield University lab for further analysis. Replicate swabs were taken where appropriate. The initial 2 ml swab sample suspensions, taken at the survey sites, were later 100 fold serially diluted in BPW and were then spread plated onto replicate MEA, PDA and CA plates (if applicable) followed by incubation at 25

± 2°C for 1 week with examination at 3, 5, and 7 days. After the incubation period the inoculated media plates found with countable growth were recorded and further examined. Fungal species present were also further enumerated and identified at least to genus level. To determine a count per original surface area of sample (in cm^2) the appropriate dilution factor was applied to the plate counts obtained.

Plate 2.1 Swab sampling in progress at BRE Victorian stable block, Watford

d) Contact plate sampling: Contact plates (Fischer, UK) with a 22 cm^2 surface area consisting of MEA, PDA and CA were used alongside swabs to obtain a direct sample of the contaminated surface under investigation. (Plate 2.2B)

Replicate samples, where appropriate, were obtained by firmly pressing individual sterile plates onto the surface under test and holding for 5 seconds.

Contact plates were incubated and read in the same way as those obtained for the air and swab samples Final counts were expressed as per 22 cm^2.

e) **Bulk samples:** Samples of affected materials e.g. wallpaper; brick dust, plaster and wood were removed from the site for further investigation.

Weighed samples (typically 0.1 gram) were placed in 2 ml of BPW and then 100 fold serially diluted as for swab samples. Results were expressed as CFU G^{-1} of the original sample.

A **B** **C**

Plate 2.2 Equipment used for survey work (A) Air sampler, (B) Contact plates, (C) Swab

2.1.7 Fungal isolates

Two isolates of *S. chartarum* were used in this study. *S. chartarum* IBT 7711 was chosen as it is a good representative macrocyclic trichothecene producer (chemotype S) and *S. chartarum* IBT 14915 was chosen as it is a good representative non-macrocyclic producing isolate (chemotype A). These isolates were kindly provided by the Mycology Group, Systems Biology Department, Technical University of Denmark. Two Field isolates of *S. chartarum* were also used in some experiment; an isolate obtained from a wallpaper sample taken from Denbigh Hospital, north Wales and an isolate obtained from a paint sample taken from the naval training base near Portsmouth.

Table 2.2 Surveyed locations and sampling methods used

Surveyed location	Date	Sampling methods used	media used
Five Springs, Marsh Farm, Luton	06/03/08	Air sampling Swab sampling Contact plates Bulk sampling	MEA & PDA (supplemented with chloramphenicol & miconazole) CA
Henson Close, Cranfield University	18/03/08	Air sampling Swab sampling Contact plates Bulk sampling	MEA & PDA (supplemented with chloramphenicol & miconazole) CA
Victory Housing trust, Sheringham, Norfolk	04/08	Air sampling Contact plates	MEA (supplemented with chloramphenicol & miconazole)
Stable block, BRE, Watford (historical building under renovation)	06/12/07 04/04/08	Air sampling Swab sampling Contact plates Bulk samples	MEA & PDA (supplemented with chloramphenicol & miconazole)
Denbigh Hospital, Wales (disused psychiatric hospital)	16/05/08	Bulk sampling	MEA & PDA (supplemented with chloramphenicol & miconazole)
High Street, Cranfield	27/06/08	Swab sampling Bulk sampling	PDA (supplemented with chloramphenicol & miconazole)
Home in Cricklewood, London	12/09/08	Swab sampling Bulk sampling	MEA & PDA (supplemented with chloramphenicol & miconazole)
HMS Victory/Portsmouth (disused naval base)	04/2010	Air sampling Bulk sampling	MEA & PDA (supplemented with chloramphenicol & miconazole)

2.2 Influence of water activity and temperature on germination, growth and sporulation of *S. chartarum* isolates

2.2.1 Introduction

Fungal colonisation on materials in the indoor environment is influenced by key abiotic factors including water activity (a_w) and temperature. In ideal conditions many organisms including *Penicillium*, *Aspergillus* and *Eurotium* species have been implicated in human health problems in the indoor environment, especially asthma (Ayerst, 1969; Flannigan and Morey, 1996; Dharmage *et al.*, 2002; Diette *et al.*, 2008). Many fungi can cause problems under wet conditions, but it is important to understand the marginal conditions under which establishment may occur and also those conditions at which sporulation occur. These are very important factors that can be used to help determine the risk from mould species such as *Stachybotrys chartarum* under condensation conditions, excessive humidity, water leaks, or flooding as there is surprisingly little knowledge of the effect of interacting conditions of a_w x temperature on the life cycle of *Stachybotrys* species in particular.

As the abiotic factors are considered to be the most important factors known to affect the development of fungal growth in indoor environments, this experiment aimed to determine the minimal and optimal conditions for germination, germ-tube extension mycelia growth and conidia production of *S. chartarum* isolates which could be used to further understand the important growth characteristics of this species.

In this experiment two strains of *S. chartarum* were used 7711 and 14915. Both strains were point inoculated onto PDA (modified with glycerol to 0.99, 0.98, 0.95 and 0.92a_w) and incubated at 10, 15, 20, 25, 30 and 37^oC ± 2^oC for 11 days. The effect of water activity was then determined using JMP 7 and Statistica 10 data analysis software.

2.2.2 Preparation of media

The PDA was modified with the addition of the non-ionic solute glycerol to obtain water activity levels of 0.997, 0.98, 0.95 and 0.92 a_w. The water activity of all media was determined with a Thermconstanter (NovaSina Sprint). Autoclaved media were poured into 9 cm diameter sterile Petri plates (15 ml/plate)

2.2.3 Preparation of inocula

Fungi were grown on PDA for 7 days at 25˚C ± 2^{o}C to obtain heavily sporulating cultures. Plates were then flooded with 10 ml sterile water and the spores were removed by gentle agitation with a sterile inoculating loop. The suspended spores were next filtered through glass wool into a sterile universal glass bottle. 2ml of these stock spore suspensions were added to 4ml sterile distilled water previously modified with glycerol to the required water availability level. The final water activities of the treatments were 0.997, 0.98, 0.95 and 0.92 a_w and the final concentration of the spores was in the range of 1-5 x 10^6 spores ml^{-1}.

2.2.4 Germination

A 0.1 ml aliquot of the spore suspension (1-5 x 10^6 spores ml^{-1}) was spread over the surface of PDA plates with a sterile bent glass rod (Magan, 1988). Petri dishes of the same water activity treatments were enclosed in polyethylene bags and incubated at 15, 20, 25, 30 and 37˚C ± 2^{o}C. Experiments were carried out with three sub-samples per treatment. On a 12 hourly basis agar discs were aseptically removed from each replicate plate using a sterile cork borer (1 cm diameter), placed on a glass slide, stained with lactophenol/methylene blue and examined microscopically. Fifty single spores per disc (150/treatment) were examined for evidence of germination (germination rate per 50

spores assessed). Spores were considered to have germinated when the germ tube was equal to or greater than the diameter of the spore (Magan, 1988). Mean germ tube lengths were also measured every 12 hours for all treatments for a maximum of 72 hrs.

2.2.5 Mycelial growth

A 3µl aliquot of the spore suspension (1-5 x 10^6 spores ml^{-1}) was point inoculated at the centre of three replicate PDA plates for all treatments. The final water activities of the treatments were 0.99, 0.98, 0.95 and 0.92 a_w. Plates of the same water activity were sealed in polyethylene bags and incubated at 10, 15, 20, 25, 30 and 37˚C ± 2^{o}C for 14 days. The replicates were examined every two days, and two diameters at right angles were measured for each colony. The temporal mycelial extension data were used to determine the rates of growth (mm day^{-1}) by linear regression.

2.2.6 Sporulation assessment

A 3µl aliquot of the spore suspension (1-5 x 10^6 spores ml^{-1}) was point inoculated at the centre of three replicate PDA plates for all treatments. The final a_w of the treatments were 0.997, 0.98, 0.95 and 0.92 a_w. Plates of the same a_w were sealed in polyethylene bags and incubated at 15, 20, 25, 30 and 37˚C ± 2^{o}C for 14 days. Replicate plates were each flooded with 10 ml sterile water and spores were agitated into solution using a sterile inoculating loop. The spore solution was then filtered into sterile universal bottles through a sterilised funnel with glass wool to remove all mycelia/hyphal fragments and retain the spores. The total number of spore was determined by using a haemocytometer. To determine spores mm^{-1} the total number of spores was divided by the total area of the colony on the agar plate (mm^2).

2.3 Determination of the effect of water activity on enzyme production by *S. chartarum* isolates

2.3.1 Introduction

S. chartarum is a heterotrophic organism that is able to grow and thrive in many conditions. This is due to the production of extracellular enzymes which it secretes into its surroundings to break down complex organic materials to simpler, more utilisable components. These enzymes include proteases, which can break down proteins into their constituent amino acids, lipases which break down lipids into fatty acids, and amylases that break down starch to simple disaccharides. These smaller molecules are then absorbed into the hypha and enter the myelial complex through the cell wall via endocytosis (Clegg and Mackean, 2000)**.**

Fungal isolates:

One representative strain of each chemotype of *S. chartarum* IBT-7711 (macrocyclic trichothecene producer) and *S. chartarum* 14915 (non-macrocyclic trichothecene producer) and a field isolate (Denbigh) were used in all experiments. The isolates were maintained on PDA.

2.3.2 Preparation of potato extract broth

Potato extract broth (PEB) was prepared by adding 4g potato extract and 20g l^{-1} of D-glucose to distilled water. Broths were then modified to the required water activity levels 0.99, 0.98, 0.95, 0.93 and 0.887a_w with the addition of glycerol. These were sterilised by autoclaving at 121$^oC \pm 2^oC$ for 15 minutes.

2.3.3 Determination of extracellular enzyme production by *S.chartarum* during initial growth in potato extract broth using APIZYM.

An initial experiment was performed to determine the extracellular enzymes produced by *S. chartarum* isolates during initial growth. 100 µl of a 1-5x10^5 ml^{-1} spore suspension of *S. chartarum* isolates 7711, 14915 and Denbigh were initially grown in 10 ml of potato extract broth modified with glycerol to 0.99, 0.98 and 0.95a_w for 6 days at 25°C before mycelium was removed by centrifugation at1300 rpm and filtration through Whatman No.1 filter paper was carried out. Each filtrate was then assayed on APIZYM test kits (Biomeriux, France), which were used for the rapid evaluation of 19 enzymatic activities (Plate 2.3 and Table 2.3). In principle the kit worked by semi quantitatively measuring the contact between the substrates in the support strips and any specific enzymes in the filtrate. Each cupule of the APIZYM strip was inoculated with 65µL of the culture filtrate and incubated at 37°C ± 2°C for 4 hours. After incubation one drop (30 ml) of ZYM A reagent and an equal amount of ZYM B reagent were added simultaneously. The colours became visible within 30 minutes

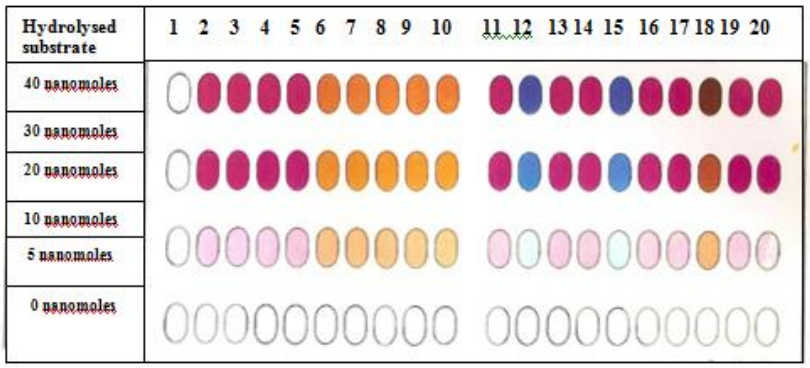

Plate 2.3 APIZYM Colour chart used to determine the concentration of substrate hydrolysed by the extracellular enzyme detected in the sample filtrate. (1-20) denotes the assayed enzymes listed in Table 2.3

Table 2.3 Enzymes and substrates assayed through the APIZYM system

Enzyme	Substrate	Cupule number
Control	Control	1
Phosphate alkaline	2-naphtyl phosphate	2
Esterase(C4)	2-naphtyl butyrate	3
Esterase lipase	2-naphtyl caprylate	4
Lipase(C14)	2-naphtyl myristate	5
Leucine arylamidase	L-leucyl-2-napthylamide	6
Valine arylamidase	L-valyl-2-napthylamide	7
Cystine arylamidase	L-cystyl-2-napthylamide	8
Trypsine	N-benzoyl-Larginine-2-napthylamide	8
Chymotrypsine	N-glutaryl-phenyalamine-2-napthylamide	10
Acid phosphotase	2-naphtyl phosphate	11
Napthol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate	12
α-galactosidase	6-Br-2-naphtyl-α-D-glucopyranoside	13
β–galactosidase	2-naphtyl-β-D-glucopyranoside	14
β–glucoronidase	Napthol-AS-BI-glucopyranoside	15
α–glucosidase	2-naphtyl-α-D-glucopyranosidase	16
β–glucosidase	6-Br-2-naphtyl-B–β-D-glucopyranoside	17
N–acetyl–β–glucosamidase	1-naphtyl-N-acetyl-b-D-glucosaminide	18
α–mannosidase	6-Br-2-naphtyl-α-D-manopyranoside	E19
α–fucosidase	2-naphtyl-α-L-fucopyranoside	E20

2.3.4 Effect of water activity and time on the quantitative production of enzymes by *S. chartarum* isolates.

A further experiment was used to more accurately quantify the levels of extracellular enzymes produced by *S. chartarum* isolates using a method previously described (Marín *et al.*, 1998). Cultures of *S. chartarum* 7711, 14915 and Denbigh were initially grown on PDA and 1-5 x 10^6 spores ml^{-1} suspensions of each were prepared as previously described. A 0.1 ml volume of each spore suspension was spread onto the surface of PDA plates modified with glycerol to 0.99, 0. 98 and 0.95a_w. A total of 12 replicates were used for each a_w treatment and incubated at 25°C ± 2°C. Thus three replicates could be destructively sampled after 48, 72, 96 and 120 h. Treatments of the same a_w were kept in sealed polyethylene bags. Six agar discs (5mm diameter) were removed from each replicate using a surface sterilised cork borer. The agar discs were placed in 4ml potassium phosphate extraction buffer (Sigma, 10mM; pH7.2). The bottles were shaken for 1 hour at 4°C ± 2°C. The washings were decanted into 1ml plastic Eppendorf tubes and centrifuged for 10 min at 13000 rpm and the supernatant used for quantitative analysis of esterase, acid phosphatase and alkaline phosphatase (Table 2.4) and total protein analysis.

2.3.5 Quantitative analysis of esterase, acid phosphatase and alkaline phosphatase

To determine the amount of enzyme in the extract, calibration curves were prepared using p-nitropnenyl (Sigma) at a range between 0-4mg ml^{-1} at pH 4.8, 7.5 and 8. The level of enzyme activity from the *S. chartarum* 7711, 14915 and Denbigh potassium phosphate extracts were determined using specific 4-nitrophenyl substrates at the required pH level. Enzyme activity was measured by the increase in optical density at

405 nm caused by the liberation of 4-nitrophenol upon enzyme hydrolysis of the substrate, three minutes after stopping the reaction with 1M Na_2CO_3 (BDH, UK). To allow rapid assays of multiple samples a Varioscan plate reader was used. The reaction mixture consisted of a 40 μl substrate solution, 40 μl enzyme extract and 20μl of appropriate buffer (Table 2.4).

2.3.6 Determination of total protein activity

To determine the total protein concentration in the *S. chartarum* 7711, 14915 and, Denbigh potassium phosphate extracts, a bicinchoninic acid protein assay (BCA) (Thermo Scientific, UK) was used. A calibration curve was produced with albumen standards at a range of 0-2500 μg. From the calibration curve the absorbance values obtained for the enzyme extracts were used to calculate their specific activity in mmol 4-nitrophenol released min^{-1} μg^{-1} protein. A purple complex is formed with bicinchoninic acid when proteins in the sample reduce alkaline Cu (II) to Cu (I). The protein concentration in the extract is directly proportional to the absorbance reading at 550 nm (Alam *et al.*, 2009). Using a BCA protein assay procedure, the total protein activity of the potassium phosphate extracts were measured using a microtitre plate reader (Thermo Scientific, UK). 50 parts BCA reagent A was first mixed with 1 part reagent B to obtain the working reagent. This was followed by the addition of 10μl of each potassium phosphate extract (in duplicate) into appropriate wells on a microtitre plate (Thermo Scientific, UK). 10 μl of potassium phosphate buffer was used for blank control wells. 200μl of the working reagent was added to each well and the plates were gently shaken to mix the well contents. Microtitre plates were incubated at 37^{o}C for 30 minutes. The plates were then cooled at room temperature and read at 540 nm.

Table 2.4 Assayed hydrolytic enzymes, substrates and buffers.

Enzyme	Substrate	Concentration (mM)	Buffer	pH
Esterase	p-nitrophenol butyrate (Sigma, UK)	10	0.2M MOPS	7.5
Acid phosphatase	4-nitrophenol phosphate disodium (Sigma, UK)	1.9	0.6M acetate	4.8
Alkaline phosphotase	4-nitrophenol phosphate disodium (Sigma, UK)	2	25mM acetate	8.5

2.3.7 Determination of extracellular enzyme production by *S. chartarum* during initial growth on PDA using APIZYM.

Quantitative analysis of the extracellular enzyme activity determined inconclusive results therefore extracts from section 2.3.4 were semi quantitatively analysed using APIZYM test kits. Each couple of the APIZYM strip was inoculated with 65µl of the culture extracts in potassium phosphate and analysed as in section 2.3.3.

2.4 Determination of the haemolytic activity of *S. chartarum* isolates

2.4.1 Introduction

As previously stated, metabolites produced by *S. chartarum* have been associated with health effects to those exposed. It has been reported that many of these metabolites, known as haemolysins, can damage vascular tissue resulting in haemorrhaging. Pulmonary haemosiderosis, one of the many associated illnesses associated with this organism results in haemorrhaging of red blood cells in the lungs or airway passages that have been exposed to fungal spores. The haemolytic metabolite thought to be the cause of this illness, known as stachlysin, has been investigated previously by Vesper (2002) who investigated the effects on earthworms of haemolysins produced by indoor fungi. They reported that earthworms injected with stachylysin showed leakage of haemoglobin from their vascular system and subsequently died, while those that were injected with heat inactivated stachylysin survived indicating that the active stachylysin had a toxic effect.

To determine the haemolytic activity of the different isolates of *S. chartarum* 7% sheep's blood agar was used. Plates were incubated at 25 and 37^{o}C to determine their growth capabilities at these two temperatures. 25^{o}C was chosen as it was found to be the optimum growth temperature and 37^{o}C was chosen as it is the human body temperature. Isolates of *S. chartarum* 7711, 14915 and Denbigh were initially grown on PDA for 7-10 days. A 1-5 x 10^{6} spore suspension was then prepared for each isolate, and was used in subsequent experiments.

2.4.2 Preparation of sheep's blood agar.

Blood agar base (Oxoid, UK) was prepared according to the manufacturer's instructions and sterilised by autoclaving at 121^{o}C for 15 minutes. Once cooled to 50^{o}C 7% of defibrinated sheep's blood (Sigma, UK) was added and mixed. 20 ml petri-plates were then poured. 3µl of each spore suspension were then added to the centre of 6 plates for each strain. Triplicate plates for each strain were then incubated at 25 and 37^{o}C ± 2^{o}C for 14 days and were examined for growth on various occasions over the incubation period, for a clearance zone indicating lysis of red blood cells which was captured photographically. Lysis types include:

Alpha haemolysis (α-haemolysis): is the term used when the organism under observation displays a greenish colour present under or around the colony. This is caused by hydrogen peroxide produced by the organism, which oxidizes haemoglobin to green methaemoglobin.

Beta haemolysis (β-haemolysis): is the term used when the organism under observation causes complete lysis of the red blood cells in the media. This results in a yellow or transparent clearing under and around the colony.

Gamma haemolysis (γ-haemolysis): is the term used when no haemolysis is induced by the organism being tested and the area around and under the colony is unaffected. Triplicate control plates of *Streptomyces pyogenes* were also plated on the prepared media and used as control plates for both incubation temperatures. *Streptomyces pyogenes* was used as these organisms produce haemolytic activity on blood agar of the Beta type so would be used a reference plate for haemolytic activity on the blood agar plates.

2.5 Satratoxin G production by strains of *S. chartarum* under varied water activities and temperatures

2.5.1 Introduction

S. chartarum growth in water damaged buildings is a growing problem and has generated major concern with regards to the associated adverse health effects experienced by individuals who have been exposed to the metabolites including the highly toxic macrocyclic trichothecene Satratoxin G (SG) produced by these fungi (Peat *et al.*, 1998; Sahakian *et al.*, 2008; Lu *et al.*, 2009). It is reported that the acute toxicity of these metabolites varies among species exposed but they can affect rapidly proliferating tissues by inhibiting DNA and protein synthesis once they enter systemic circulation (Wannemacher and Wiener, 1997). Previously, Nielsen *et al.* (1998) showed that the trichothecene mycotoxins satratoxin G and H were both present on water damaged gypsum boards in Denmark and that growth occurred only at >0.95 a_w on gypsum board building materials. Metabolite profiling has suggested that production of satratoxins occurs in many *Stachybotrys* strains isolated from water-damaged buildings in Finland (Nielsen *et al.*, 2002). There is thus a clear need here for improved understanding of the role of the environment in growth and toxigenesis of *S. chartarum*.

The objective of this study was to determine the effects of a_w and temperature on growth and SG production by two strains of *S.chartarum* considered to be a type S (IBT 7711), a macrocyclic trichothecene producer, and type A (IBT 14915), a non-producer.

Pure standards of the macrocyclic trichothecenes produced by *S. chartarum* species including satratoxins were not available from any of the major suppliers which limited the type of analysis that could be performed for their detection. An SG ELISA was thus

kindly performed by James J. Pestka and Jae-Kyung Kim from the Centre for Integrative Toxicology, Michigan State University, East Lansing, MI 48824, United States, who determined the levels of satratoxin G production by isolates of *S. chartarum* grown on PDA at various water activities and temperatures at the Cranfield laboratories. SG was quantified by a competitive SG ELISA which uses polyclonal antibodies that have been raised against SG. Any SG produced by the *S. chartarum* extracts bind to the immobilised polyclonal antibodies in the microtitre plate well. This is then revealed with the addition of a substrate which binds to polyclonal antibody-SG complex producing a blue colour which is proportional to the amount of SG detected in the sample (Chung *et al.*, 2003).

2.5.2 Medium

To determine the influence of water activity on mycelial growth, experiments were carried out on PDA which was modified with the addition of glycerol to obtain a_w levels of 0.99, 0.98, 0.95 and 0.92a_w. The water activity of all media was determined with a Thermconstanter (Novisina). Autoclaved media were poured into 9 cm diameter sterile plastic Petri dishes (20 ml/plate).

2.5.3 Preparation of inocula

Each fungal strain of *S. chartarum* was grown on PDA for 7-10 days at 25˚C ± 2^{o}C to obtain heavy sporulating cultures. Spores were then suspended in sterile distilled water containing one drop of a wetting agent (Tween 80). Stock spore suspensions (2ml) were added to 4ml sterile water previously modified with glycerol to the required water availability level. The final water activities of the treatments were 0.99, 0.98, 0.95 and 0.92 a_w and the final concentration of the spores was in the range of 1-5x10^6 spores ml^{-1}.

2.5.4 Inoculation and incubation

3µl of each fungal spore suspension was point inoculated at the centre of triplicate PDA plates for all treatments. The final water activities of the treatments were 0.99, 0.98, and 0.95 a_w and the final concentration of the spores was in the range of 1-5 x 10^6 spores ml^{-1}. Plates of all tested water activities were sealed in polyethylene bags and incubated at 15, 20, 25, 30 and 37˚C ± 2^{o}C for 14 days.

2.5.5 Sample preparation for transport

Representative plugs from each of the tested conditions were aseptically removed from each plate using a sterile cork borer, and then placed in appropriately labelled tubes. All plugs were freeze dried using a Cool Safe freeze drier (Scanvac) and were weighed. These were then stored at -20˚C until further required.

2.5.6 Satratoxin G extraction

Freeze dried samples were soaked in 4 ml phosphate buffered saline (PBS) (pH 7.2, 10 mM) overnight at 4°C ± 2^{o}C. 1.5 ml of each of the PBS and *S. chartarum* IBT 7711 (Chemotype S) mixtures were added to appropriately labelled Eppendorf tubes. This

was repeated for *S. chartarum* IBT 14915 (Chemotype A). Samples were centrifuged for 15 min (13000 rpm at 4°C) and the supernatants used for the SG ELISA.

2.5.7 Satratoxin G ELISA

SG polyclonal antibodies (100 µl) diluted (0.5 µg ml^{-1}) in PBS (pH 7.2, 10 mM) were incubated in 96-well ELISA plates (NUNC) overnight at 4°C. Plates were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBS-T) and blocked with 300 µl of 1% (w/v) bovine serum albumin in Deionised water (BSA), and then incubated for 60 min at 37 °C ± 2°C. After washing the plates four times with PBS-T, 50 µl of "standard" or "samples" with 50 µl SG-horseradish peroxidase conjugate diluted (0.5 µg ml^{-1}) in BSA was added and plates were incubated at room temperature for 60 min. Plates were washed seven times with PBS-T and the bound peroxidase was determined after incubation for 30 min at 25°C ± 2°C with 100 µl/well of K-Blue Substrate (Neogen, Lansing). The reaction was terminated with 100 µl/well of 2M sulfuric acid stopping reagent and the plate was read at 450 nm by a Vmax Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA). All samples were run in triplicate.

2.6 Determination of the effect of antioxidants on mycelial growth of *S. chartarum* isolates

2.6.1 Introduction

Antioxidants are widely used in the food industry as preservatives to reduce the peroxidation, (degradation) of unsaturated lipids and other materials in foods due to humidity, oxygen and metals and are generally regarded as safe (GRAS) at low concentrations (Soobrattee *et al.*, 2005). They are naturally produced by many vegetables and fruit and are reported to exhibit a wide range of biological effects including antibacterial, anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, anticarcinogenic and vasodilatory actions (Middleton Jr. *et al.*, 2000). Well known antioxidants include Vitamin C and E. Butylated hydroxyanisole (BHA and propyl gallate (PG) are some of the most commonly used synthetic antioxidants and have been reported to vary in their antifungal abilities (Plate 2.4).

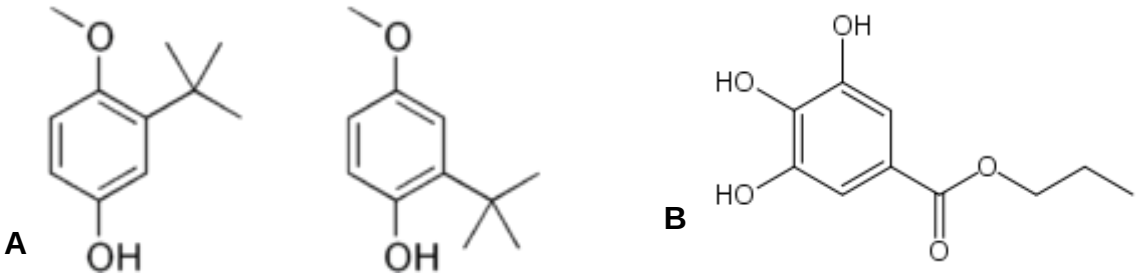

Plate 2.4 General structure of (A) Butylated hydroxyanisole and (B) Propyl gallate

Most reports have shown BHA and PG to be effective at inhibiting the germination, vegetative growth and toxin production of many microorganisms but little is known about their mechanism of action. It has been suggested that reduced oxygen tension in

the growth medium created by antioxidants may be one of the reasons for the inhibition of growth and mycotoxin production by aerobic moulds. Antioxidants have also been reported to play a role in altering cell permeability causing the disruption of the cell membrane by interacting with the cell membrane proteins (Fung *et al.*, 1977). Although antioxidants are normally considered as food preservatives they were investigated as possible control agents against *S. chatarum* growth as they are generally non toxic to humans.

2.6.2 Mycelial growth

A 3µl aliquot of *S. chartarum* 7711 and 14915 spore suspension (1-5 x 10^6 spores ml^{-1}) were point inoculated at the centre of three replicate PDA plates for all treatments. The final concentration of antioxidant treatments for BHA were 1000, 800, 600, 400, 200, 100, 50, 25, 12.5 and 2.5 mg l^{-1}. For PG the concentrations used were 1000, 800, 600, 400, 200 and 100 mg l^{-1}. Plates were sealed in polyethylene bags and incubated at 25˚C for 11 days. The replicates were examined every two or three days, and two diameters at right angles were measured for each colony. The temporal mycelial extension data were used to determine the rates of growth (mm day^{-1}).

2.7 Determination of the growth rate of *S. chartarum* using the Bioscreen

2.7.1 Introduction

A novel approach for studying *S. chartarum* was investigated. The Bioscreen (Labsystems, Helsinki, Finland) (Plate 2.5), is an automated microbiology growth analysis system which uses kinetic measurements of turbidity of 200 samples simultaneously, using microtitre plates that are used to gather growth data under set experimental conditions and time points. The data is then further analysed using specific data analysis packages for growth rate determination.

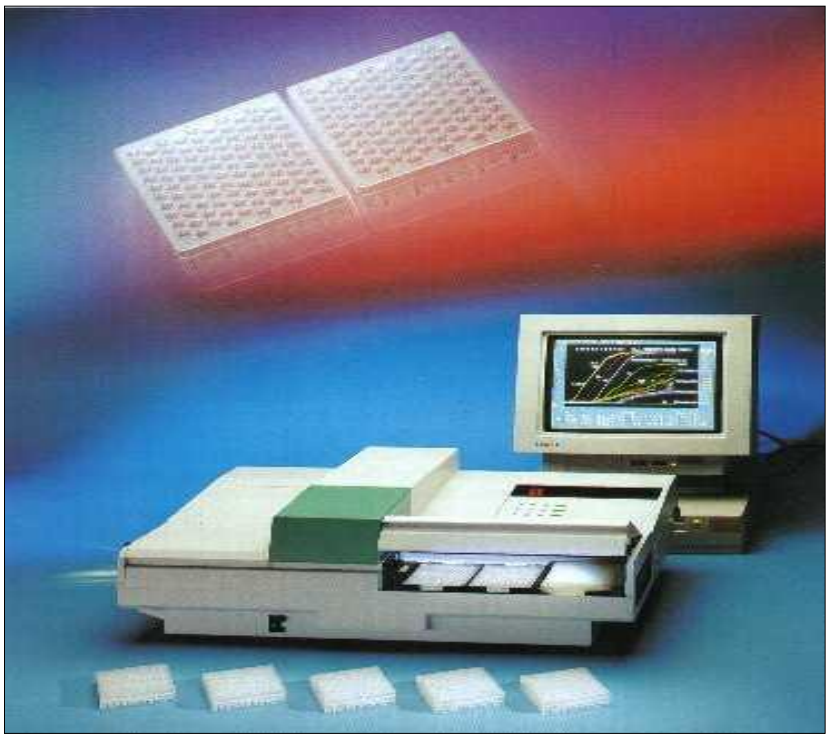

Plate 2.5 Bioscreen plate reader

2.7.2 Determination of the growth rates of different inocula of *S. chartarum* isolates using the Bioscreen instrument

To determine whether the Bioscreen could be used as a means of measuring fungal growth an initial experiment was set up which measured the growth rate of *S. chartarum* isolates 7711 and 14915 at various inocula levels. This was used to generate a calibration curve, which was used as the basis for further experiments using the bioscreen.

2.7.3 Preparation of microtitre plates

PEB at 0.99a_w was prepared as in section 2.3.2. 200µl of PEB was added to all wells on two microtitre plates. From 7-10 day old cultures of *S. chartarum* 7711 and 14915, 200µl of spore suspension ($5x10^6$ spores ml^{-1}) was added to the first 10 wells on the microtitre plate. 200µl was then taken from the first row and 2-fold diluted across the microtitre plate. The last row was left blank (media only). The microtitre plates were placed in the Bioscreen reader for 7 days at 25^oC, with continuous shaking at medium setting, reading OD at 600nm with 30 minute intervals.

2.8 Determination of the effect of water activity and temperature on the growth rate of *S. chartarum* isolates using the Bioscreen

2.8.1 Introduction

The bioscreen was chosen as a novel way of rapidly measuring the effect of multiple parameters i.e. water activities (0.99, 0.98, 0.95, 0.93 and 0.887a_w) and temperatures (10, 20, 25, 30, and 37^oC) on the growth rate of *S. chartarum* isolates 7711 and 14915 in PEB. Optical density measurements were read automatically every 30 minutes over a 5 day period. Data were analysed using bespoke software on Microsoft Excel. Growth

rates were determined by calculating the time taken for the optical density for each well of the microtitre plates to reach a set OD value e.g. 0.2. From this a list of times to detection (TTD) was generated by the software. These data were then converted into rate of time to detection (RTD) in hours using the formula 1/TTD*60, which were plotted against the tested water activities. Linear regression analysis was used to generate growth rates for each experiment, which were subsequently used to generate graphs for the growth rates of the *S. chartarum* isolates 7711 and 14915 across all of the tested water activities and temperatures.

2.8.2 Preparation of microtitre plates

PEB was prepared as in section 2.3.2. 200µl of PEB modified to 0.99a_w was added to all the wells in columns 1 and 2 of a microtitre plate, 200µl of PEB modified to 0.98 a_w was added to all wells in column 3 and 4, 200µl of PEB modified to 0.95 a_w was added to all wells in columns 5 and 6, 200µl of PEB modified to 0.93 a_w was added to all the all wells in column 7 and 8 and lastly 200µl of PEB modified to 0.887 a_w was added to all the wells in columns 9 and 10. From 7-10 day old cultures of *S. chartarum* 7711 and 14915, spore preparations were made at $1x10^4$ spore ml^{-1}. 50µl of the *S. chartarum* 7711 spore suspension was added to all wells in columns 1, 3, 5, 7 and 9. Columns 2, 4, 6, 8 and 10 were controls for each water activity. This was repeated on a separate microtitre plate for *S. chartarum* 14915 (Table 2.5). Microtitre plates were placed in the Bioscreen reader for 5 days at 10, 20, 25, 30 and 37^oC, with continuous shaking, at medium setting, reading OD at 600nm with 30 minute intervals.

Table 2.5 Microtitre plate layout for the measurement of the effect of various water activites and temperatures on the growth of *S. chartarum* isolates 7711 and 14915.

1	2	3	4	5	6	7	8	9	10
0.99a_w *S.chartarum*	0.99a_w Neg control	0.98a_w *S.chartarum*	0.98a_w Neg control	0.95a_w *S.chartarum*	0.95a_w Neg control	0.93a_w *S.chartarum*	0.93a_w Neg control	0.887a_w *S.chartarum*	0.887a_w Neg control

2.9 Determination of the effect of tebuconazole and other antifungals on the growth of *S. chartarum* isolates using the Bioscreen

2.9.1 Introduction

The Bioscreen was chosen as a novel approach to determine the effect of tebuconazole, and silver nitrate on the growth of *S. chartarum* isolates in PEB as it was a means of rapidly measuring a wide range of concentrations at one time. Optical density measurements were read automatically every 30 seconds over a 5 day period at 25^{o}C for *S. chartarum* isolates 7711, 14915 and Denbigh. Tebuconazole is a triazole fungicide used agriculturally to treat plant pathogenic fungi (Plate 2.6). Though the U.S. Food and Drug Administration consider this fungicide to be safe for humans, it may still pose a risk. It is listed as a possible carcinogen in the United States Environmental Protection Agency Office of Pesticide Programs (OPP) Carcinogen List with a rating of C (possible carcinogen). Its acute toxicity is moderate. According to the World Health Organization Toxicity Classification, it is listed as III, which means slightly hazardous (Sergent *et al.*, 2009).

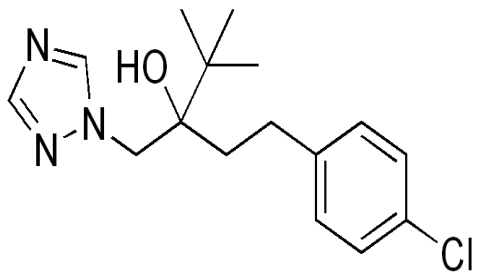

Plate 2.6 General structure of Tebuconazole

The effects of silver nitrate (Plate 2.7) result from silver ions readily combining with sulphydryl, carboxyl, phosphate, amino, and other biologically important chemical groups which can lead to the inhibition of respiratory processes and interruption of cell wall synthesis resulting in loss of essential nutrients. They interact with a wide range of molecular processes within microorganisms resulting in a range of effects from inhibition of growth, loss of infectivity to cell death. The mechanism depends on both the concentration of silver ions present and the sensitivity of the microbial species to silver. Contact time, temperature, pH and the presence of free water all impact on both the rate and extent of antimicrobial activity. However, the spectrum of activity is very wide and the development of resistance relatively low (Fuhrmann and Rothstein, 1968; Furr, 1994; Industrial Microbiological Services Ltd, 2005).

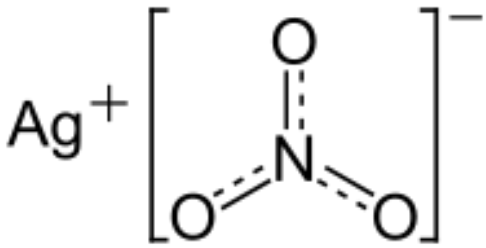

Plate 2.7 General structure of Silver nitrate

The susceptibility of the three isolates of *S. chartarum* used in this study to tebuconazole were examined using a modification of the Lambert-Pearson susceptibility test (Lambert and Pearson, 2000) in conjunction with the Bioscreen. Using multiple half-fold dilutions of the inhibitor and a standard inoculum of each *S. chartarum* isolate, the level of inhibition was observed as a reduction in the area of the OD/incubation time curve relative to an untreated control. From theory, as the concentration of an inhibitor increases the more pronounced the effect on visible growth of the microbes. The

minimum inhibitory concentration (MIC) is defined as the minimum concentration of inhibitor required to prevent visible microbial growth i.e. failure to show turbidity. The non-inhibitory Concentration (NIC) is the threshold concentration below which normal, visible, growth occurs, even in the presence of the inhibiting substance. The analysis of the data was straightforward: a plot of fractional area (*fa*, derived from optical density measurements) against concentration gave a curve which was modelled, simply, as an exponential decay. A plot of the log of inhibitor concentration (x) vs. *fa* allowed the NIC and MIC to be defined as the intercept of the line tangential to the inflexion point with the *fa* = 1 and *fa* = 0 boundaries, Equations 1.1, 1.2 and 1.3 respectively.

Equation 1.1 $$fa = e^{-\left(\frac{x}{P_1}\right)^{P_2}}$$

Equation 1.2 $$NIC = P_1 e^{\left(\frac{1-e}{P_2}\right)}$$

Equation 1.3 $$MIC = P_1 e^{\left(\frac{1}{P_2}\right)}$$

where P_1 is the concentration of the preservative at $fa = 1/e$, (e = 2.7182..) and P_2 is a slope parameter

The area under each OD/time curve was calculated using the simple trapezoidal rule. The relative area under the curve was defined as RAUC = (Area of test – Area of negative control) / (Area of positive control – Area of negative control). This gave a range of 1 for full, uninhibited growth to zero –complete inhibition.

2.9.2 Preparation of BHA, PG, tebuconazole and silver nitrate stock solutions

A 20 mg ml^{-1} stock solution of BHA was prepared in ethanol, A 200 mg ml^{-1} stock solution of PG was prepared in ethanol, a 250 mg ml^{-1} stock solution of tebuconazole was prepared in sterile water and a 10 mg ml^{-1} stock solution of silver nitrate was also prepared in sterile water.

2.9.3 Preparation of linear dilutions of BHA stock solution (20mg ml^{-1})

5ml of PEB was added to 11 sterile dilution tubes then the appropriate volume of BHA stock solution was added to each dilution tube and mixed (Table 2.6). 50 µl of ethanol was added to the 11th tube and used for the control wells. 200µl of PEB (0.99 $a_{w)}$ was added to all wells in columns 2-9 on a microtitre plate. This was followed by the addition of 400µl of each previously prepared linear dilution of BHA to the wells in the 10^{th} column down the microtitre plate. These were then 2 fold diluted across the microtitre plate by pipetting 200µl from column 10 into the wells in column 9. This was repeated across the microtitre plate to column 2. The remaining 200µl was discarded. 200µl of the PEB control was next added to all wells in the 1st column on the microtitre plate. 50µl of a 1-5x10^4 spore ml^{-1} suspension, made from 7-10 day old cultures of *S. chartarum* 7711 were then added to the first 5 wells in the first column followed by all the remaining wells in columns 2-10. This was repeated on a separate plate for *S. chartarum* 14915 (Plate 2.8). Microtitre plates were placed in the bioscreen reader for 5 days at 25^oC, with continuous shaking, on medium setting, reading OD at 600 nm with 30 minute intervals.

Table 2.6 BHA solution preparation table

Dilution tube	Volume of BHA stock solution added to 5ml PEB (μL)	Concentration of BHA (mg l-1)
1	50	200
2	45	180
3	40	160
4	35	140
5	30	120
6	25	100
7	20	80
8	15	60
9	10	40
10	5	20
11	50μl of ethanol	Control

1	2	3	4	5	6	7	8	9	10
Pos con									200
Pos con									180
Pos con									160
Pos con									140
Pos con									120
Neg con									100
Neg con									80
Neg con									60
Neg con									40
Neg con									20

Plate 2.8 Microtitre plate layout for the analysis of a matrix of antifungal concentrations on *S. chartarum growth*.

2.10 Determination of the effect of water activity, temperature and BHA on the growth of *S. chartarum* isolates on building materials

2.10.1 Introduction

Plasterboard is one of the predominant building materials used in the construction industry. It is composed of a gypsum inner core with starch and additives that are sandwiched between paper linings. In normal dry conditions plasterboard surfaces are not conducive for extensive fungal growth but in the event of water intrusion these materials easily become saturated with water sometimes for long periods, which permits fungal growth to occur. Because these materials contain sufficient amounts of nutrients, fungal spores are capable of germination on them. The growth of *S. chartarum* is of particular concern as this organism favours the high cellulose content in these building materials and is able to utilise cellulose in the core material as well as the paper lining as a food source by producing extracellular enzymes that facilitate its growth and proliferation in favourable conditions (Andersson *et al.*, 1997). Fungal growth on plasterboard is very much dependent on the pre-treatment applied to the plasterboard. Some plasterboard types can contain a combination of many different types of antifungal components to prevent mould growth e.g. Aquaboards (used in areas where there is water present) contain the water resistant additive silicone oil and a biocide. Most plasterboard commonly used for rooms in a building are the standard type which does not contain any antifungal components and thus are more susceptible to mould growth. Paint can contain many specifically added antifungal compounds which can also impede fungal growth but most standard emulsion paints do not contain any antifungal components and can actually enhance fungal growth due to constituent organic compounds and sugars. In this experiment standard plasterboard samples treated with BHA at 0, 10, 50 and 200 mg l^{-1} were inoculated with *S. chartarum* isolate*s* 7711

and 14915. The effect of these materials on *S. chartarum* growth rate was measured using a modified British Standard protocol (2005), which measured the growth by a grading system between 0 and 5 (0= no growth and 5= extensive growth). Growth measurements were performed on replicate samples for each treatment every 10 days for a period of 30 days.

2.10.2 Preparation and inoculation of Plasterboard blocks

Plasterboards (Homebase, UK) were cut in to 5 x 5 cm blocks, a selection of these plasterboard blocks were covered on one side with 1200 grade lining paper (Homebase, UK) using Evo stick PVA glue (Homebase, UK), another selection of the plasterboard blocks were painted on one side with matt emulsion paint (Homebase, UK), Plate 2.9. The prepared blocks were then wrapped in aluminium foil and sterilised by autoclaving at 121^{o}C for 15 minutes.

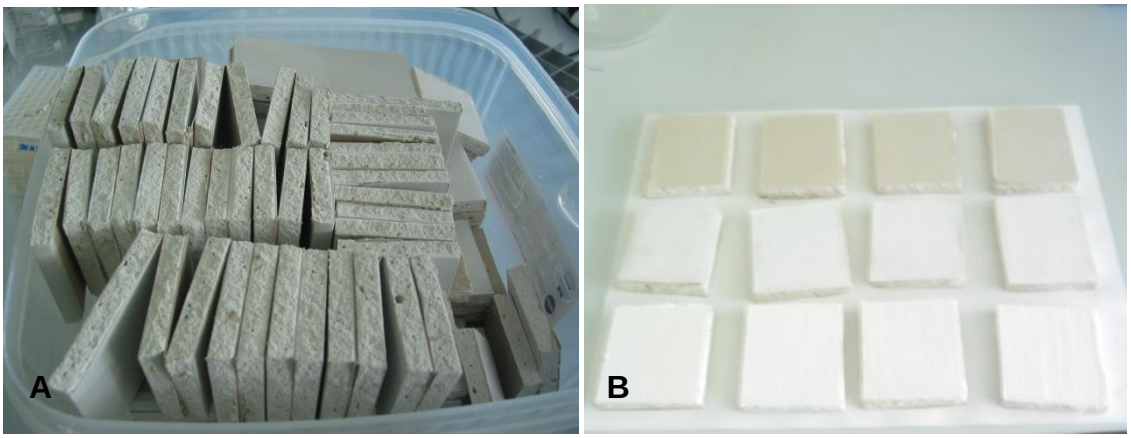

Plate 2.9 (A) Plasterboard samples cut to 5x5cm (B) Prepared plasterboard samples (top row) unmodified plaster board (middle row) painted plasterboard sample (lower row) lining papered plasterboard samples

Solutions of BHA were prepared to 2000, 500 and 100 mg ml^{-1} in ethanol. 100 μL of each BHA concentration were spread over appropriately labelled plasterboard samples

using a sterile L-shaped glass rod. A 100 µl aliquot from each *S. chartarum* 7711, 14915 and Denbigh spore suspension, at 1-5 x 10^6 spores ml^{-1}, were each evenly spread over appropriately labelled plasterboard samples using a sterile L-shaped glass rod and were incubated at 15 and 25^oC and 0.99 and 0.95a_w for 40 days. The extent of fungal coverage on the plasterboard samples was measured every 10 days and a modified BS2011 standard growth coverage rating system was applied which grades the extent of fungal coverage growth from 0 for no fungal coverage to 5 for full fungal coverage.

2.11 Determination of the effect of BHA in paint on the growth of *S. chartarum* isolates on plasterboard.

In this experiment painted plasterboard (PPB) samples supplemented with BHA at 0, 1000, 5000 and 10000 mg l^{-1} were inoculated with strains of *S. chartarum*. The effect of these treatments on the growth coverage was measured using the same modified BS2011 rating system previously described in section 2.10.1. Samples were performed in triplicate and growth coverage on the plasterboard samples were measured every 10 days over a 30 day period.

2.11.1 Preparation of BHA concentrations in matt emulsion paint

Approximate weights of BHA were first dissolved in 20ml of ethanol then added to 80 ml of matt emulsion paint to make BHA concentrations of 1,000, 5,000 and 10,000 mg l^{-1}. Pre sterilised plasterboard samples cut into 5 x 5cm blocks were covered on one side with the emulsion paint/BHA solutions at the appropriate concentrations. 100 µl of a 1x10^6 spore ml^{-1} Concentrations of BHA added to 20 ml of ethanol then to 80 ml of emulsion paint.

3 RESULTS

3.1 Initial survey

3.1.1 Marsh Farm, Luton

The occupant of this high-rise 2 bedroom flat in Marsh Farm, Luton complained of mould problems in the bedroom that she occupied. She explained that on numerous occasions there were incidents of water intrusion coming from the ceiling of her bedroom which ran down the affected walls. On visual examination the walls showed approximately 10% fungal coverage but was significantly worse on the skirting boards. Although there was no strong mouldy smell found, we were advised by the occupant that a strong mouldy smell accumulated within the premises if the windows were not regularly opened. It was also noted that the mother did not suffer from any respiratory problems although her child suffered from mild asthma. Swab sampling of the walls and skirting board were performed along with contact plates and air samples which were taken in the main bedroom with visible mould growth (Plate 3.1) and the adjacent child's bedroom, which showed no visible signs of mould growth. Colony forming unit (CFU) counts from the air samples taken in the main bedroom (Figure 3.1A) showed the detection of *Penicillium* sp at levels of 516 m^{-3} and *Cladosporium* sp at 316 m^{-3} on MEA. Only *Cladosporium* sp were detected on PDA and CA at levels of 550 m^{-3} and 283 m^{-3} respectively. Air samples taken from the child's bedroom (Figure 3.1B) detected *Penicillium* spp only on MEA at levels of 116 CFU m^{-3}. There was no growth recovered on PDA or CA. Swab samples taken of the skirting board which were added to MEA plates (Figure 3.2A**)** recovered *Penicillium, Cladosporium* and yeast spp at levels of 16, 98 and 120 CFU cm^{-2} respectively. PDA and CA recovered solely

Cladosporium spp at levels of 140 and 148 CFU cm^{-2} respectively. Swab samples taken from the wall of the main bedroom, which showed less visible fungal growth, recovered the same range of fungi as those obtained from the skirting board but at lower levels (Figure 3.2B). MEA recovered mixed fungal species of *Penicillium*, *Cladosporium* and Yeasts spp at levels of 2, 49 and 41 CFU cm^{-2} respectively. *Cladosporium* spp were solely recovered on PDA and CA at levels of 54 and 77 CFU cm^{2} respectively. Contact plates taken from the main bedroom wall and skirting board showed confluent *Penicillium* growth.

Plate 3.1 Fungal growth on bedroom wall and skirting board, Marsh Farm, Luton

From the results it was clear that the main contamination problem within the flat came from the walls within the main bedroom. As the tenant reported that there had previously been water leaking down the affected walls (which were outside facing) it was assumed that this condition allowed condensation to build up on the walls forming

conditions favourable for mould growth. It was also noticed that this room was not maintained in a very clean state which could also be contributing to the problems.

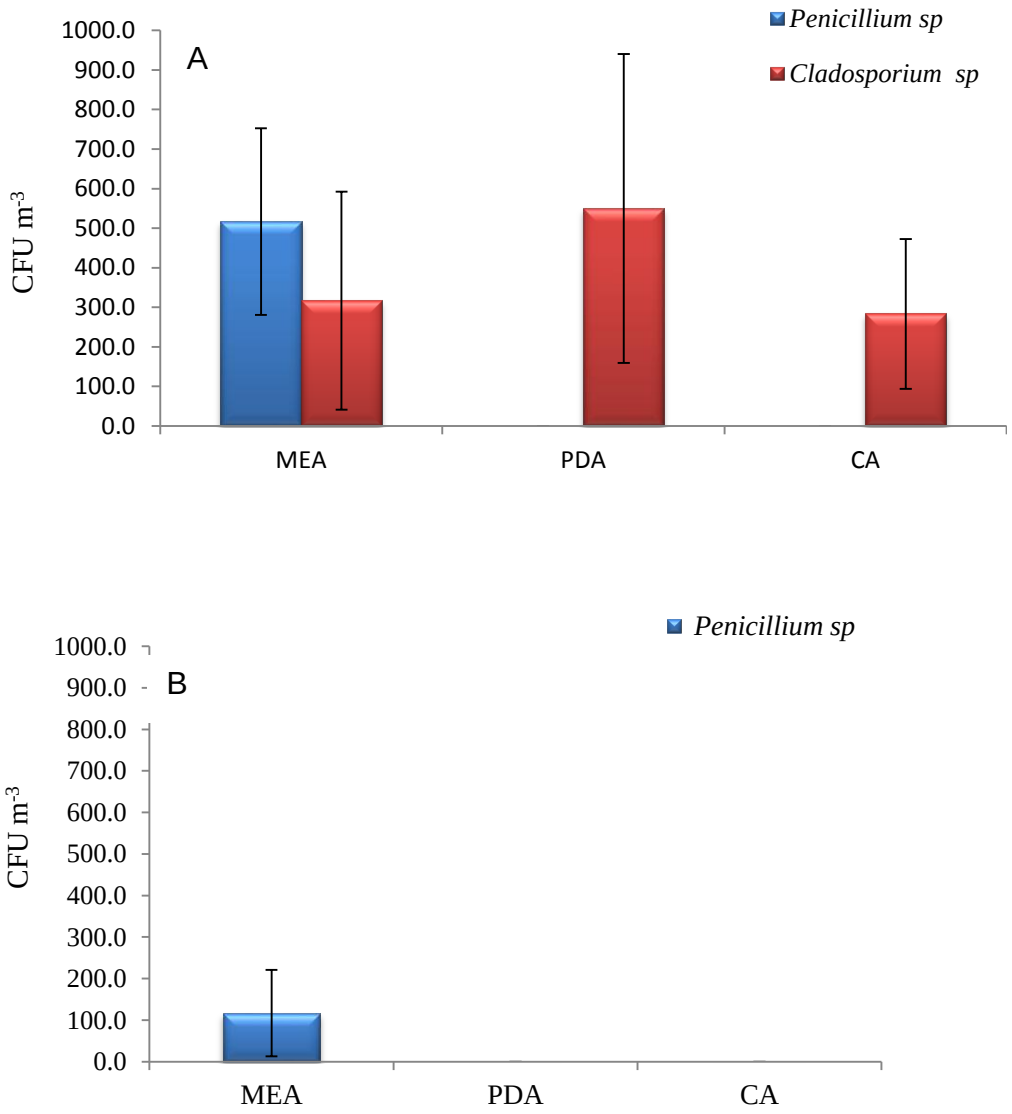

Figure 3.1 Fungal species recovered from air samples taken in (A) main bedroom and (B) child's bedroom, Marsh Farm, Luton

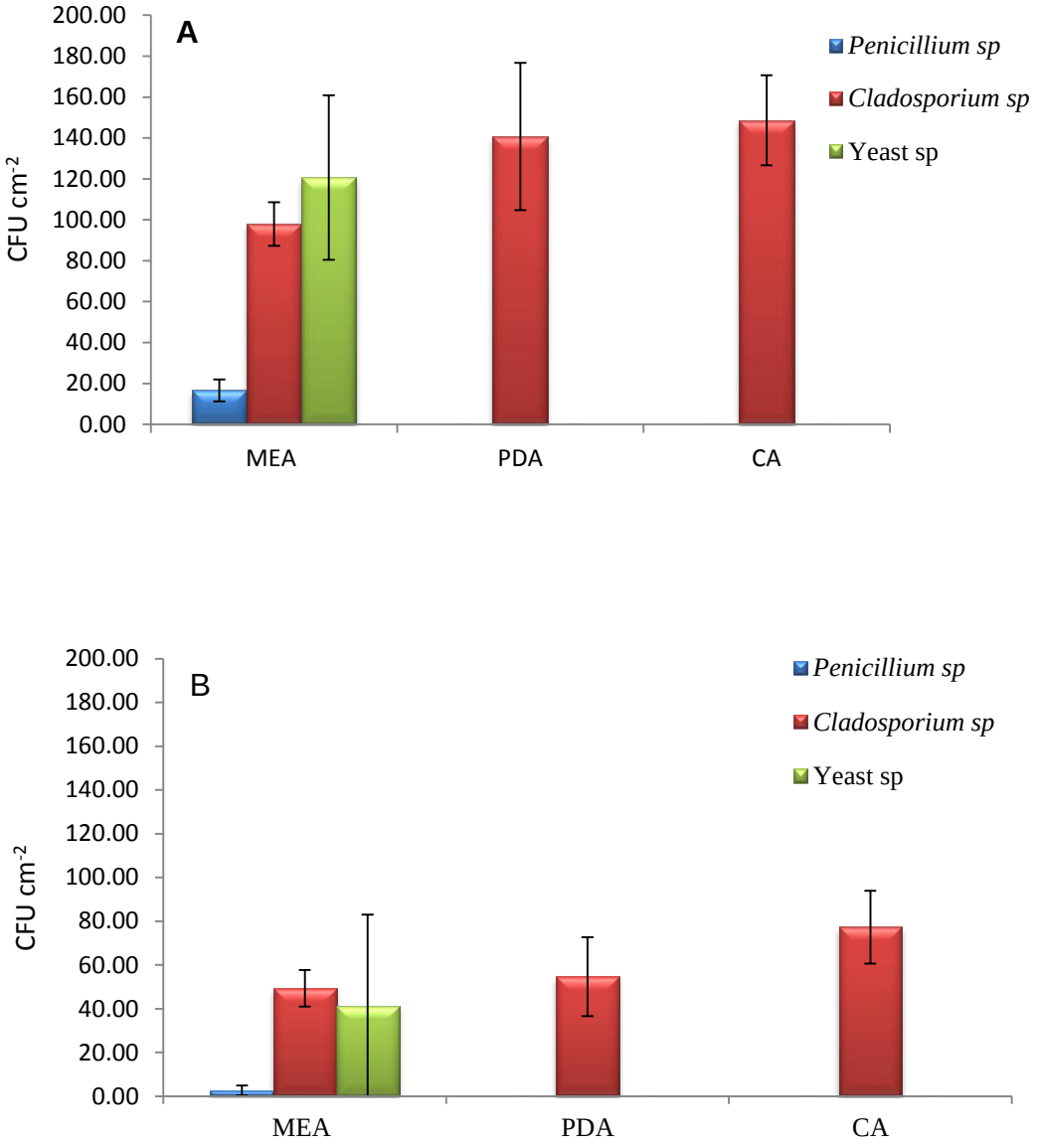

Figure 3.2 Fungal growth recovered on various media from swab samples taken from the main bedroom (A) Skirting board (B) Wall, Marsh Farm, Luton

3.1.2 Henson Close, Cranfield University campus

The occupant of this house complained of mould problems throughout her home. On visual examination, the walls throughout house showed approximately 5% of mould growth. The WC showed approximately 20% higher levels of mould growth on the window sill (Plate 3.2). Sampling of the walls and skirting board within the WC were performed along with air samples which were also taken in the passage directly outside the WC. Air samples taken in the WC room recovered fungal growth on MEA, PDA and CA. MEA recovered a mixed population of *Penicillium, Cladosporium* and yeast sp at levels of 716 CFU m^{-3}, 50 CFU m^{-3} and 667 CFU m^{-3} respectively. PDA recovered yeast sp at levels of 17 CFU m^{-3} and CA recovered *Cladosporium* spp at levels of 1417 CFU m^{-3} (Figure 3.3A). Air samples taken in the passage outside the WC recovered fungal growth on MEA and CA (Figure 3.3B). MEA recovered a mixed population of fungal sp including *Penicillium, Cladosporium* and yeast spp at levels of 83 CFU m^{-3}, 16 CFU m^{-3} and 216 CFU m^{-3} respectively. No fungal growth was recovered on PDA and 215 CFU m^{-3} of *Cladosporium* spp were recovered on CA. Swab samples taken from the window sill in the WC recovered mixed fungal growth on MEA and PDA. *Penicillium* and *Cladosporium* spp were recovered on MEA at 141 CFU cm^2 and 318 CFU cm^2 respectively. PDA recovered *Cladosporium, Eurotium* and pink yeast spp at levels of 1, 206 and 100 CFU cm^2 respectively (Figure 3.4A). Swab samples taken from the wall of the WC recovered *Penicillium* spp at levels of 376 CFU cm^2 on MEA. *Cladosporium* spp were recovered on PDA and CA at levels of 4 CFU cm^2 and 678 CFU cm^2 respectively (Figure 3.4B).

Overall the house did not show very high levels of mould growth although there was minor visible growth on some walls which could be easily removed with cleaning fluids and regular ventilation.

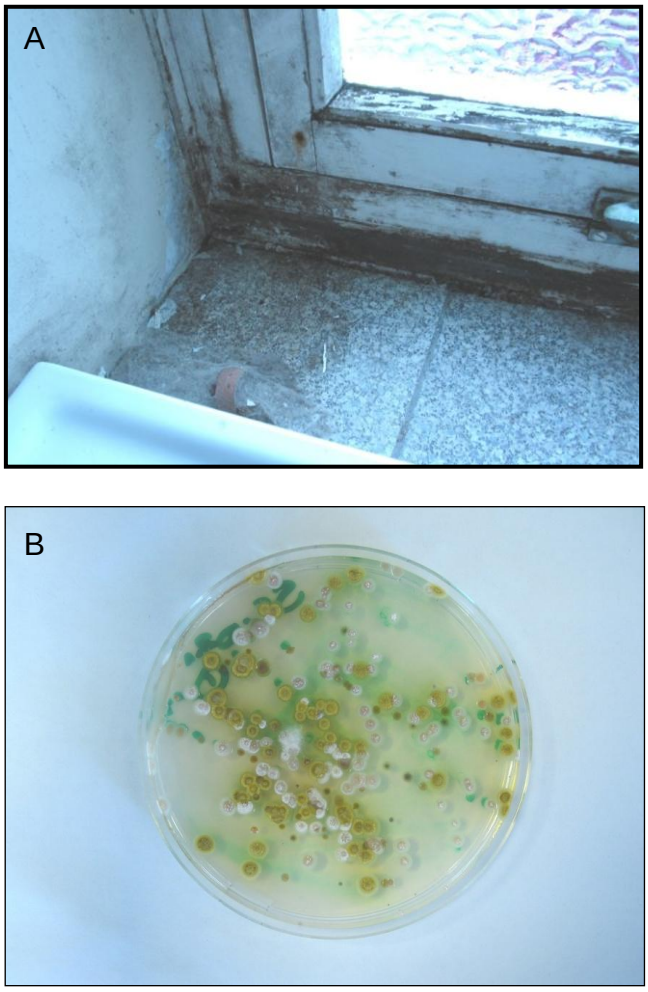

Plate 3.2 Fungal growth on the (A) window sill of the WC (B) *Eurotium* and yeast spp recovered on PDA from swab samples taken from the window sill in the WC, Henson Close, Cranfield.

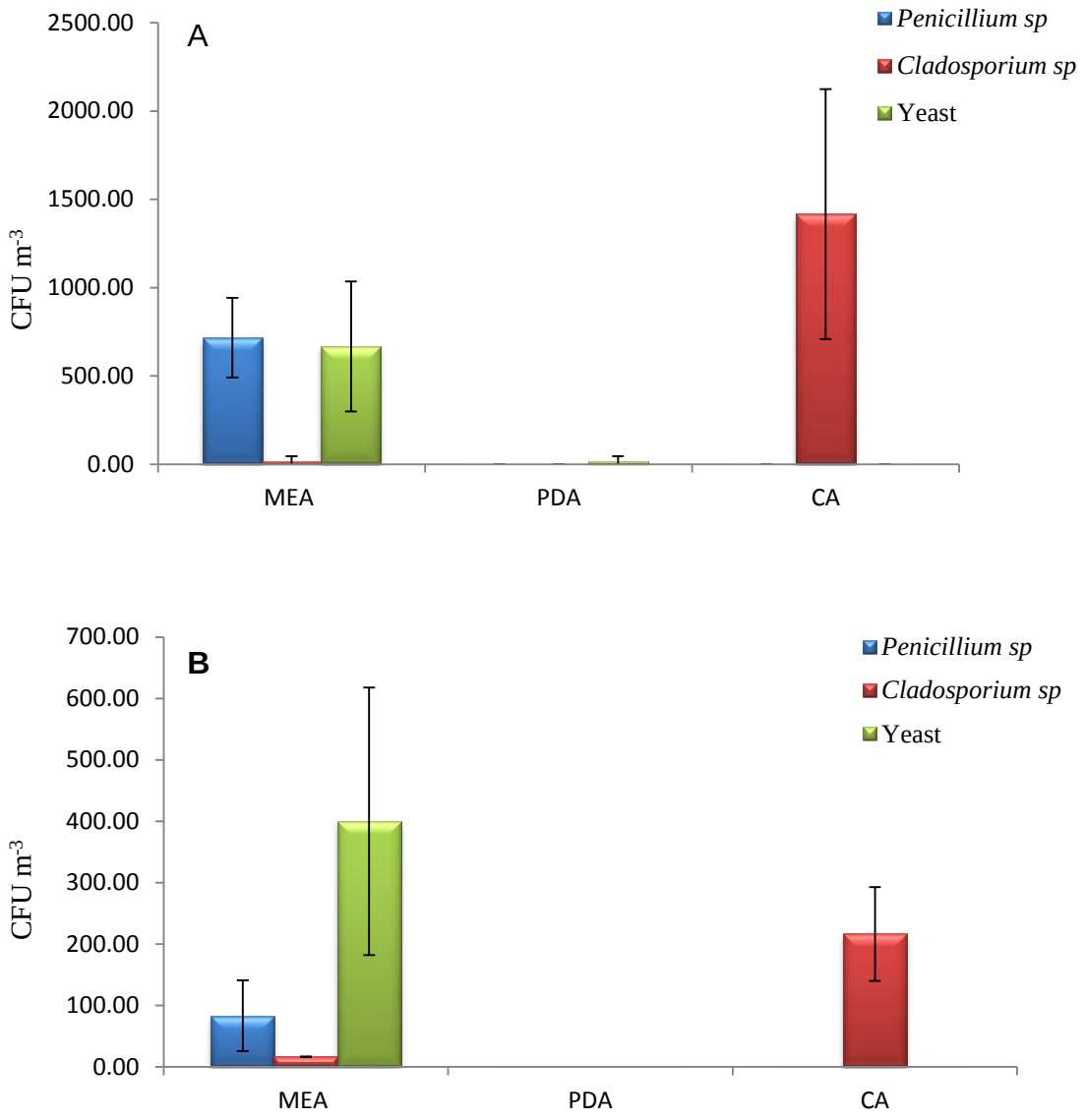

Figure 3.3 Fungal growth recovered on MEA, PDA and CA from air samples taken in (A) WC (B) passage, Henson Close, Cranfield

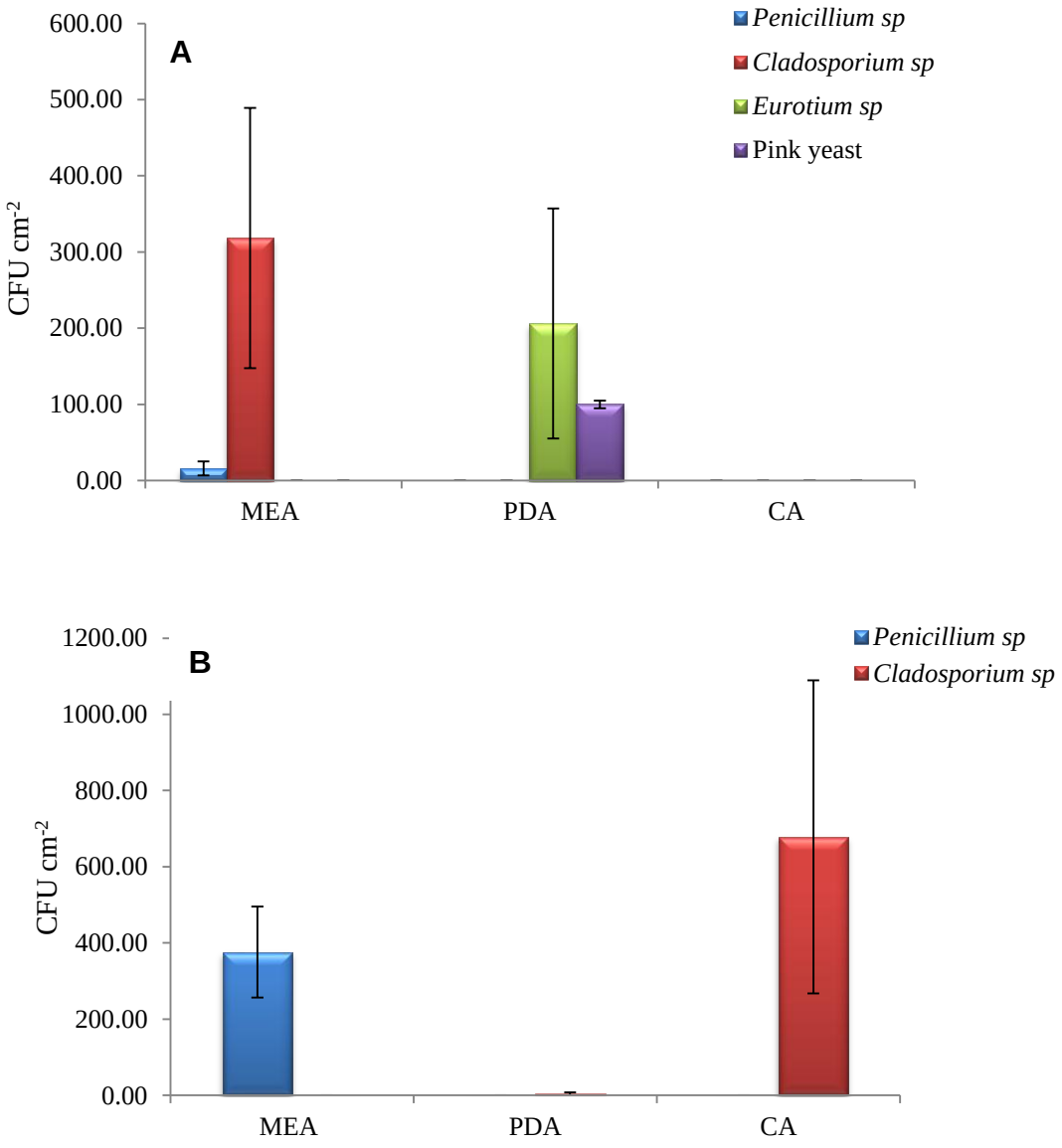

Figure 3.4 Fungal growth from swab samples taken from (A) window sill (B) Wall in the WC, Henson Close, Cranfield

3.1.3 Victory House, Sheringham

This survey was initiated as a result of complaints made by the occupant, with no prior allergies or respiratory problems, living in the building. He described an unusual 'fermentation', 'mouldy', 'sweet' type smelling odour within the building which was causing ill-health effects of the respiratory type. The problem had been occurring for several years. Structural and cosmetic changes to the dwelling three years prior allegedly was the cause and was the approximate time that the occupant first noticed the odour, which subsequently appeared intermittently with severity increasing during warmer weather. The main bedroom of the dwelling was reported to be the main focus of the problem, which was no longer used by the occupant. However, the odour was also reported to be present, to a lesser extent, in other parts of the house. A visual inspection of the dwelling was carried out, including, as far as possible, an inspection of the under floor space in the main bedroom. Air samples were taken from:

- The main bedroom (upstairs)
- Under the floor in the main bedroom
- The adjacent bedroom (upstairs)
- The living area on the ground floor
- The garden

The outdoor air sample was taken as there was no evidence of contamination from visual inspection, therefore it was assumed that the indoor fungal levels would be influenced by the outdoor environment. MEA contact plates were also used to take samples of surface from the various locations listed below as there were no visible signs of mould contamination.

- The main bedroom floor
- The under floor area (in the same area as the under floor air sample)
- A specific area of an under floor joist showing a grey deposit.

The air sample results (Figure 3.5) showed a mixture of typical 'outdoor' fungi consisting of phylloplane species (species present on plants), namely *Cladosporium*, *Epicoccum* and *Fusarium* species on MEA. The exterior garden sample fungal counts were at levels of 270 CFU m^{-3}, the side bedroom 46 CFU m^{-3}, the main bedroom 29 CFU m^{-3} and the under floor area 33 CFU m^{-3}. The results obtained here were clearly influenced by the outdoor counts. Mixed fungal species typical of the outdoor environment including *Fusarium* spp (Plate 3.3 A) were recovered on MEA from the air sample taken in the garden. The same species were recovered on MEA from air samples but at much lower levels (Plate 3.3 B).

The MEA contact plates taken inside the dwelling recovered very low levels of phyloplane species. 0.5 CFU cm^{-2} was recovered from the main bedroom, 0.7 CFU cm^{-2} was recovered from the under floor area and there was no fungal growth recovered from the joist. (Figure 3.6)

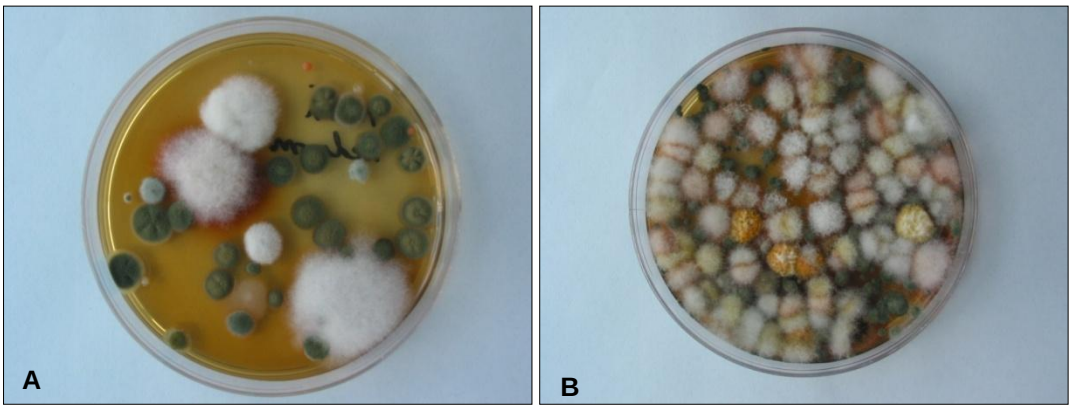

Plate 3.3 Mixed fungal species detected from an (A) interior and (B) exterior air sample on MEA

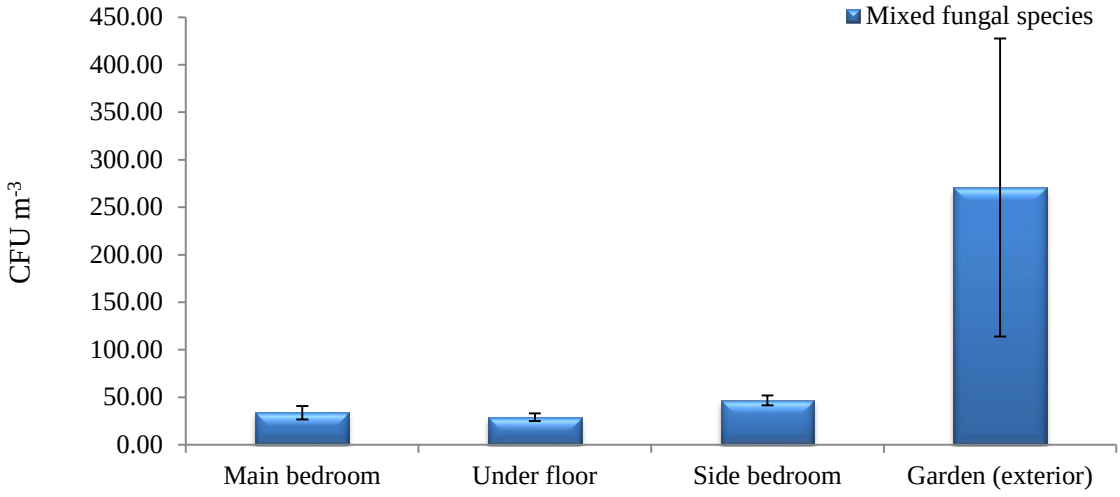

Figure 3.5 Fungal growth recovered on MEA from air samples, Victory Housing trust, Sheringham

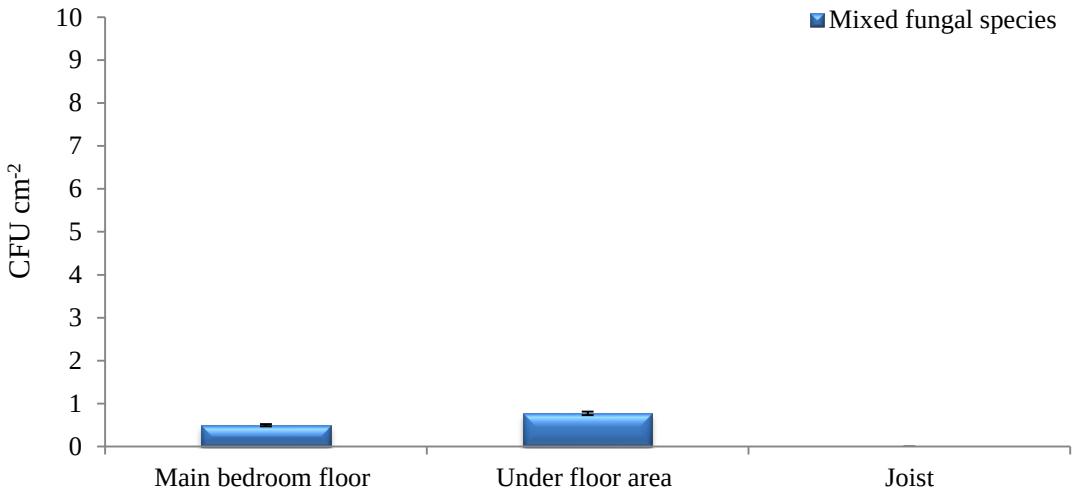

Figure 3.6 Fungal growth recovered on MEA contact plates, Victory Housing trust, Sheringham

3.1.4 Cranfield High Street

The occupant reported mild mould growth in ground floor flat (Plate 3.4). Minor mould growth, found on the bedroom window sill skirting boards and bathroom, were examined by swab sampling. Laboratory analysis of the swab samples taken from the window sill in both the bedroom and bathroom revealed *Cladosposrium* spp on PDA at levels of 22 CFU and 228 CFU cm^2 respectively including high levels of yeasts and bacteria. Swab samples taken from the bathroom blinds revealed predominantly confluent levels of yeasts.

Plate 3.4 Fungal contamination on the bedroom window sill and bathroom blinds, Cranfield High Street

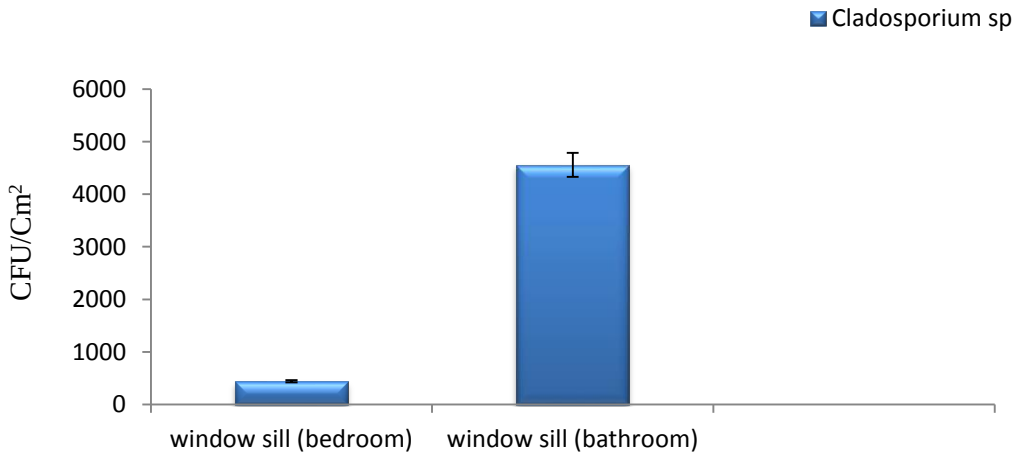

Figure 3.7 Fungal growth recovered from swab samples on PDA, Cranfield High Street

3.1.5 Cricklewood

This ground floor flat was in the process of an insurance claim and had suffered severe water intrusion due to water leakage from an above flat over a short period of time e.g. not chronic. This situation commonly occurs in homes; therefore an investigation to determine whether *S. chartarum* could be detected in these circumstances was conducted. Very minor mould growth was found, this was probably due to the swift action of the remediation company whom had installed drying equipment throughout the building. Although little visible mould growth was found, swab and bulk samples were taken from the main water damaged areas. All of the bulk samples that were taken showed growth of *Penicillium* spp on MEA. Bulk sample 4 taken from the wallpaper recovered 63000 CFU G^{-1}, sample 5 recovered 3000 CFU G^{-1}, and sample 6 recovered 9500 CFU G^{-1} (Figure 3.8). Confluent growth of yeast was recovered from the same samples on PDA (not shown). Swab samples taken from these areas recovered confluent growth of yeast on MEA and PDA which was probably due to contamination.

Plate 3.5 Visible fungal growth on the ceiling and wall, Cricklewood

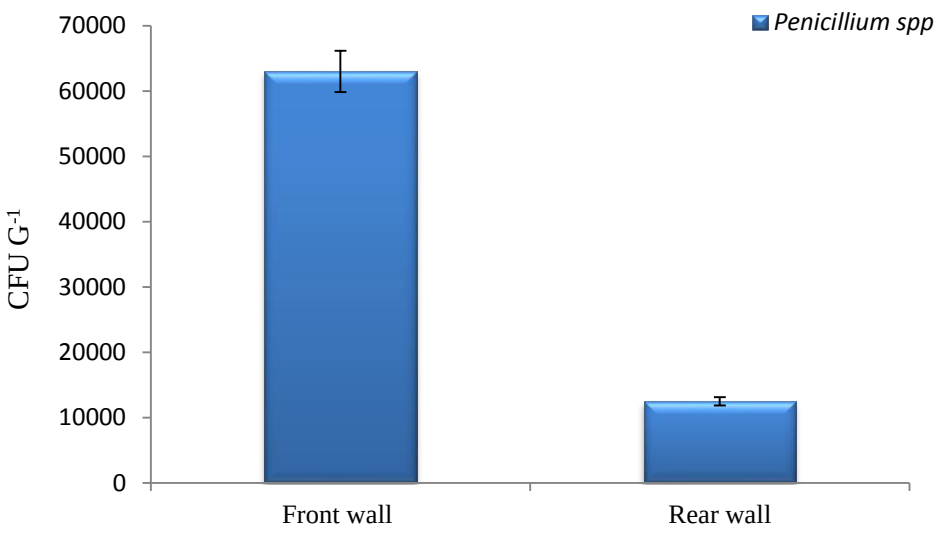

Figure 3.8 Fungal growth from bulk samples, Cricklewood

3.1.6 Victorian stable block, Building Research Establishment (BRE) Refurbishment Project Watford

This property had suffered chronic water damage for up to a decade and was being stripped for refurbishment. Samples were taken from this property before and after the internal fittings were removed. The surveys were performed in order to determine the levels of fungal growth and damage within the main fabric of the building material and to determine the level of mould contamination in the brickwork after all the plasterboard had been stripped. The information gathered from these surveys was necessary as any contaminated material could potentially become problematic after refurbishment of the building. Visual examination of the walls in the upstairs rooms, particularly in Room 1 and 2, and to a less extent in Room 3, showed that they were heavily infested by mould growth. Room 1 was also tested after the interior fittings were removed (Plate 3.6). CFU counts of the air samples taken in the Victorian stable block (Figure 3.9) recovered *Penicillium* spp on MEA in room 1 at levels of 6700 CFU m^{-3}, room 2 at 9250 CFU m^{-3}, room 3 at 5650 CFU m^{-3}, room 4 at 4000 CFU m^{-3}, and the downstairs area at 5400 CFU m^{-3}. The external air samples recovered mixed fungal spp on MEA at levels of 1400 CFU m^{-3}. Swab samples (Figure 3.10) taken from the south facing wall in room 1 recovered mixed fungal species of *Penicillium* and *Cladosporium* spp at levels of 407 CFU cm^{-2} on MEA and 684 CFU cm^{-2} on CA. The east facing wall in room 1 recovered mixed fungal species of *Penicillium* and *Cladosporium* spp at levels of 14 CFU cm^{-2} on MEA only. The north facing wall in room 1 recovered mixed fungal species of *Penicillium* and *Cladosporium* spp at levels of 72 CFU cm^{-2} on MEA, 115 CFU cm^{-2} on PDA and 22 CFU cm^{-2} on CA. The wood roof member in room 2 recovered species of *Penicillium* at levels of 10989 CFU cm^{-2} on MEA only. The wood on the east facing

wall in room 2 recovered mixed fungal species of *Penicillium* and *Cladosporium* spp at levels of 136 CFU cm^{-2} on MEA and 18 CFU cm^{-2} on CA. Various plasterboard samples (Bulk 1-9) were taken from rooms 1 and 2 where fungal contamination was visible at varying degrees (Plate 3.6 A and D), and a general dust sample (sample G), recovered mixed fungal spp when plated on MEA: at levels of 8333.3 CFU G^{-1}, 900 CFU G^{-1}, 2000 CFU G^{-1}, 1333.3 CFU G^{-1}, 48333.3 CFU G^{-1}, 126000 CFU G^{-1}, 92666.7 CFU G^{-1}, 17333.3 CFU G^{-1}, 13000 CFU G^{-1}, and 6500 CFU G^{-1} respectively. Bulk samples 2, 4, 6 and 8, taken from these locations recovered *Cladosporium* spp when plated on PDA, at levels of 333.3 CFU G^{-1}, 333.3 CFU G^{-1}, and 333.3 CFU G^{-1} and 8666.7 333.3 CFU G^{-1} respectively. Bulk samples 5, 7, and 8, recovered *Cladosporium* spp when plated on CA, at levels of 466.7 CFU G^{-1}, 10333.3 CFU G^{-1}, and 27333.3 CFU G^{-1} (Plate 3.7 and Figure 3.11).

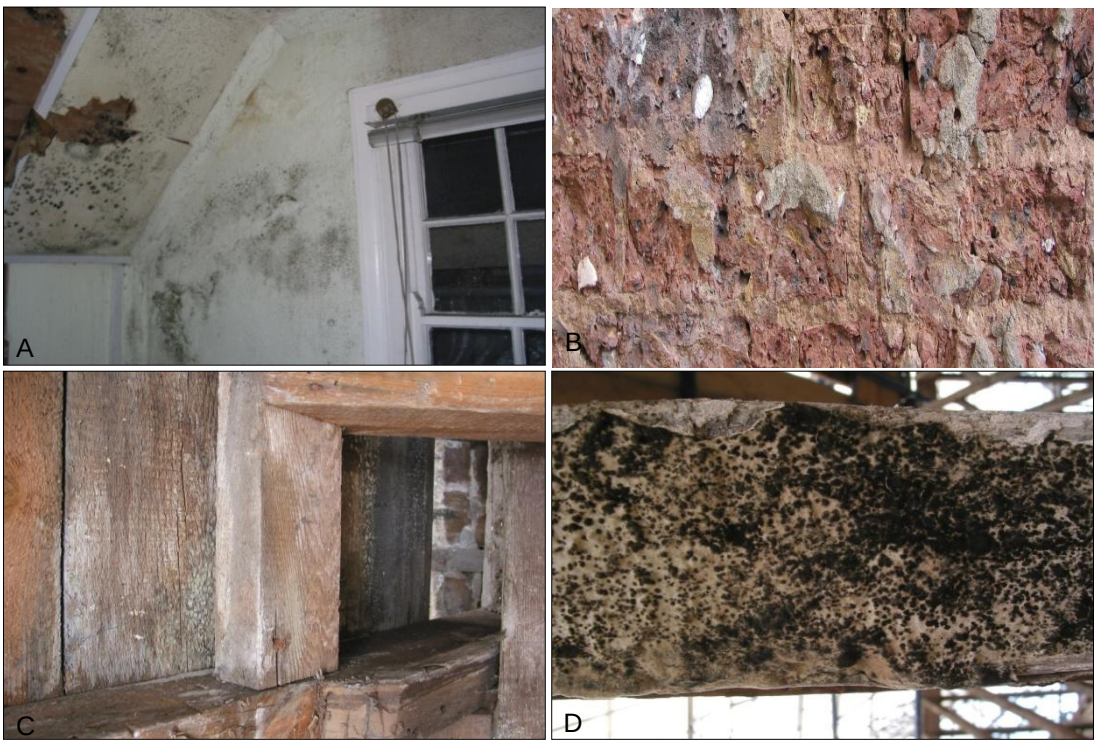

Plate 3.6 Visual fungal growth observed in the Victorian stable block, Watford (A) Room 1, east and north facing walls (B) Room 1, south facing brickwork (C) Room 2 clocktower frame, (D) Room 2, wooden roof member with plaster.

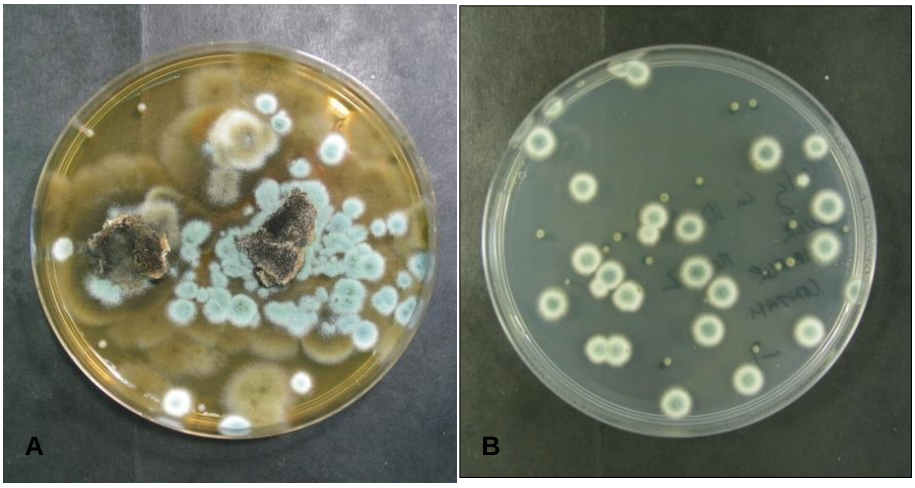

Plate 3.7 Fungal growth determined from plasterboard samples taken from rooms 1 and 2 in the Victorian stable block, Watford (A) Mixed fungal growth on MEA from direct plating and (B) Growth of *Cladosporium* sp (small green spots) and *Penicillium* sp on PDA from a serially diluted bulk sample.

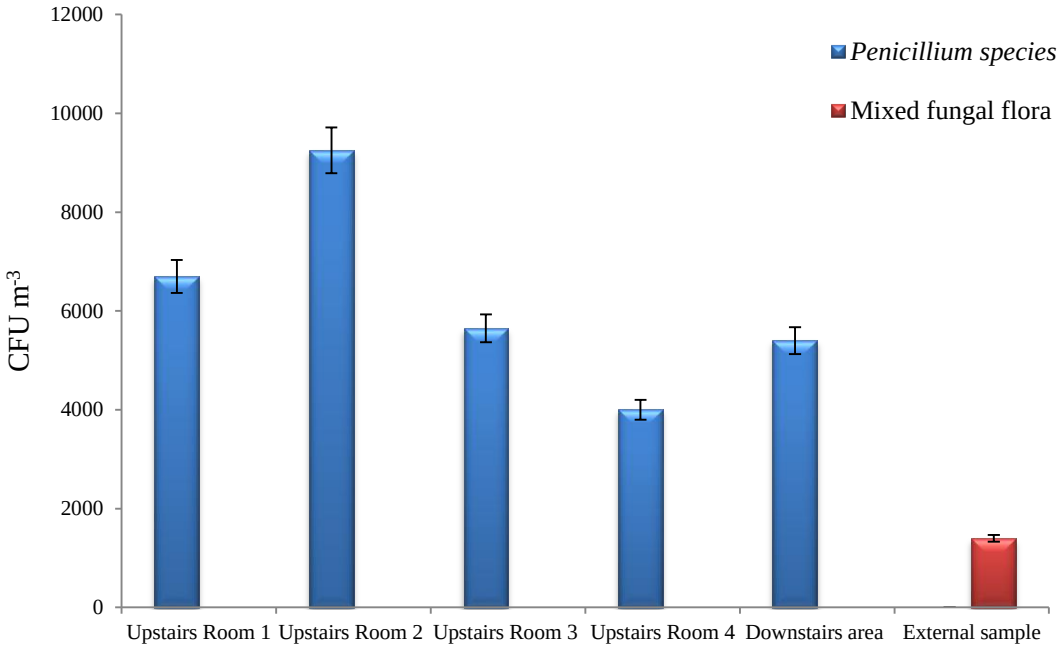

Figure 3.9 Fungal growth on MEA from air samples, Victorian stable block, Watford

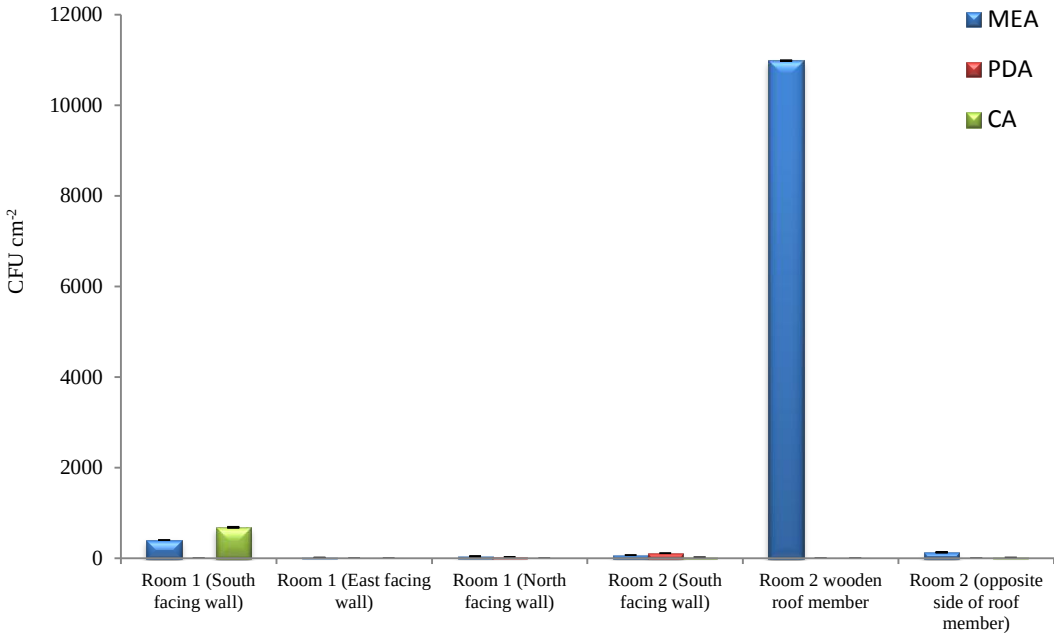

Figure 3.10 Fungal growth from swab samples taken at Victorian stable block, Watford

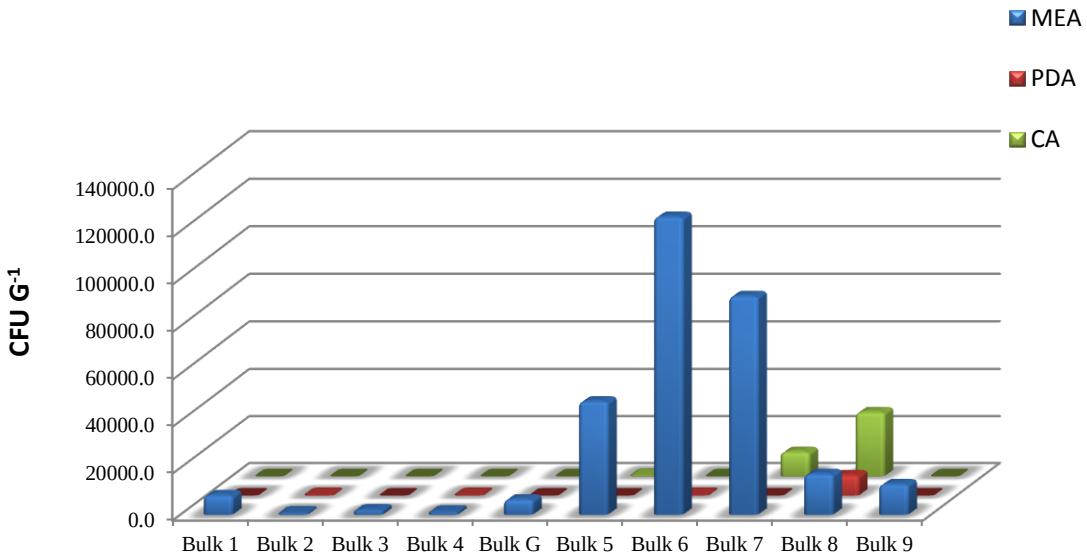

Figure 3.11 Fungal growth from bulk samples plated on MEA, PDA and CA, Victorian stable block, Watford

3.1.7 Denbigh Hospital, Wales

The disused psychiatric hospital, Denbigh was chosen as an ideal site for survey as it had suffered severe water damage over the years, after being abandoned around 20 years ago, and was now being used for military training. Since being abandoned, the building had gradually deteriorated as is evident in (Plate 3.8) and there were a number of parts of the building where water had been freely entering for many years resulting in permanent damp. This location therefore represents the highest level of water damage to be expected in a building, other than perhaps during a flood. Many materials within the rooms of the two main buildings showed various levels of mould growth from which representative bulk samples were taken. Bulk samples taken from Denbigh Hospital recovered a range of fungal spp when plated on MEA. *Cladosporium* spp were recovered from bulk samples Plasterboard (B1 and B7), Wallpaper (B4) and Paint (B6) at levels of 7667, 1667, 1333, and 9667 CFU G^{-1}, and respectively. *Penicillium* spp were recovered from Wood above the door frame (B8) at levels of 6000 CFU G^{-1}, *Stachybotrys* spp were recovered from Wallpaper (B9) at levels of 104000 CFU G^{-1} (Plate 3.9), *Penicillium* and *Stachybotrys* spp were recovered from Wood panelling (B10) at levels of 333 and 667 CFU G^{-1} respectively. *Penicillium* and *Eurotium* spp were recovered from ceiling tile (B12) at levels of 1333 CFU G^{-1}. Overgrowth of *Trichoderma* spp was recovered from Wood from the ceiling (B11) on MEA and PDA and therefore the CFU counts were not possible from these plates. No growth was recovered from Ceiling (B3) on MEA (Figure 3.12).

Plate 3.8 (A) Entrance to building 1, (B) Severely infested room in building 1 (C) water damaged room in building 2 (D) infested wall in building 2, Denbigh Hospital, north Wales

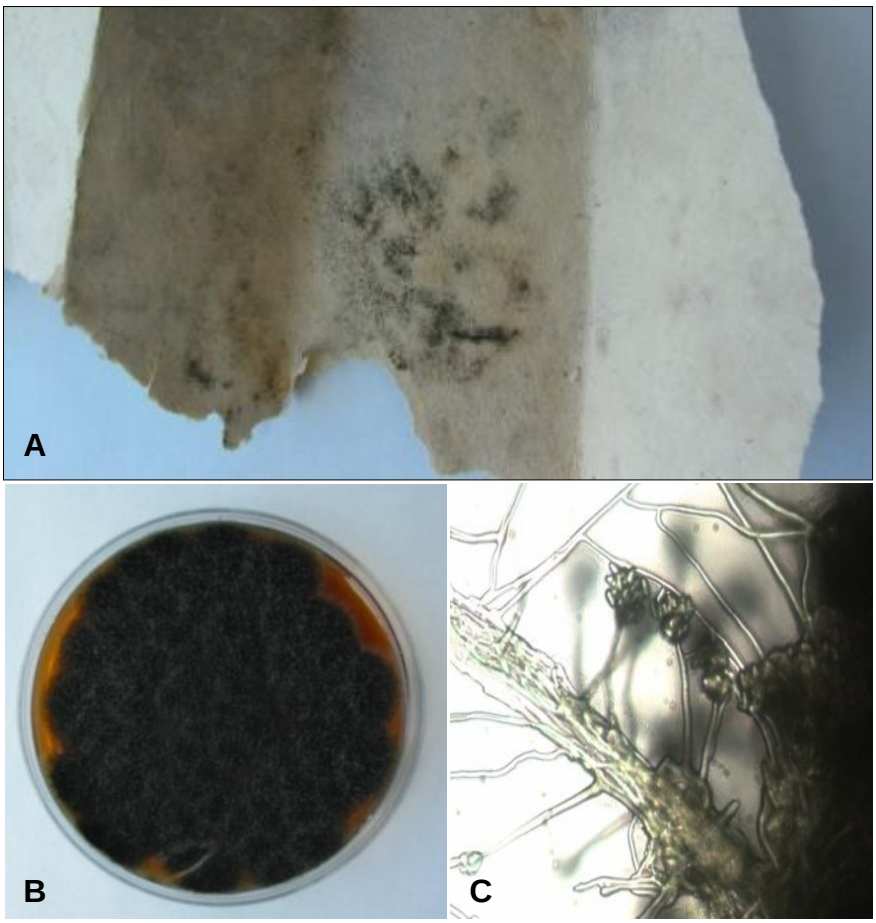

Plate 3.9 *S. chartarum* detected from Denbigh Hospital, Wales (A) Wallpaper sample with S. *chartarum* growth (B) *S. chartarum* growth determined on MEA from the plated wallpaper sample. (C) Denbigh *S. chartarum isolate* x400.

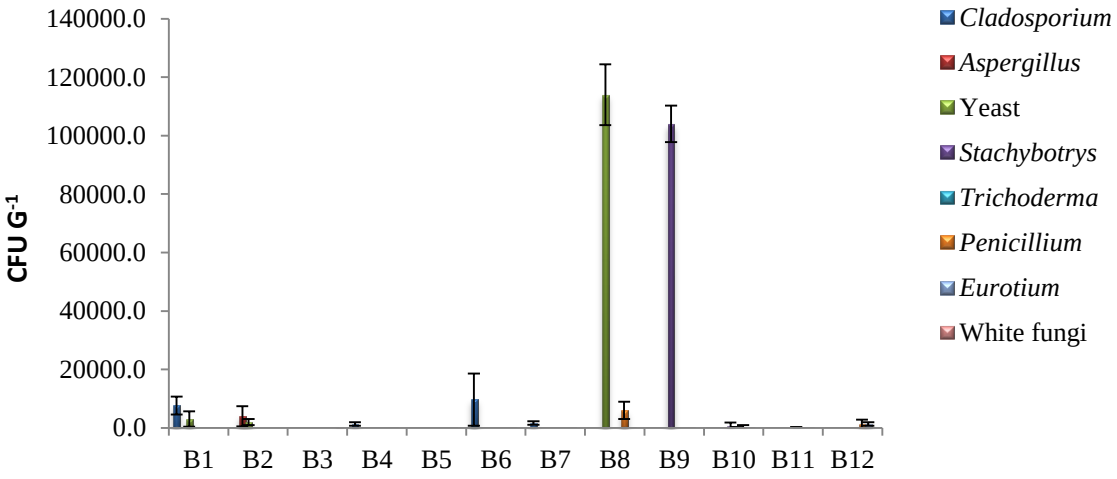

Figure 3.12 Fungal growth from bulk samples plated on MEA, Denbigh Hospital, north Wales

3.1.8 'HMS Mercury' (disused naval training facility near Portsmouth)

This building had been abandoned for about 10 years and was believed to have suffered from severe flooding due to a burst pipe, at some point in the past (Plate 3.10). Air samples taken from the foyer recovered *Penicillium* sp on MEA and PDA at levels of 5733 and 8850 CFU m^{-3} of air respectively. *Penicillium* spp were also recovered in the corridor at levels of 5083 and 8816 CFU m^{-3} of air respectively (Figure 3.13). Representative bulk samples were taken throughout the building and taken back to the laboratory at Cranfield University for further analysis (Plate 3.11).

The foyer area was the area from which the building was entered. This area showed extensive amounts of dark coloured fungal growth on the walls. Samples were taken from different parts of the wall for a full representation of the fungal diversity growing there. Bulk samples 1 and 2 taken from the foyer wall revealed the presence of *Alternaria* and *Aspergillus sp* at levels of 16550 and 51 CFU G^{-1} respectively. Bulk sample 4 taken from a small black patch on the lower portion of the foyer wall and bulk sample 5 taken from the top section of the foyer wall revealed the presence of *S. chartarum* sp at levels of 139000 and 3 CFU G^{-1} respectively. The next area investigated was the corridor which showed some fungal growth. Bulk sample 3 taken in this area revealed the presence of an unidentified white species which produced high levels of white mycelium when grown on MEA and PDA at levels of 1150 CFU G^{-1}

Bulk sample 8 taken of the ceiling panels in this area revealed high levels of *Penicillium* and *Aspergillus* sp at levels of 14500 and 7500 CFU G^{-1} respectively. The final area sampled was the stairway. Bulk samples 6 and 7 taken from this area revealed the presence of *Penicillium* and *Aspergillus* sp at levels of 280 and 270 CFU G^{-1} respectively (Figure 3.14).

Plate 3.10 Visual fungal growth observed at 'HMS Victory', Portsmouth (A) Foyer (B), Corridor (C), Stairway.

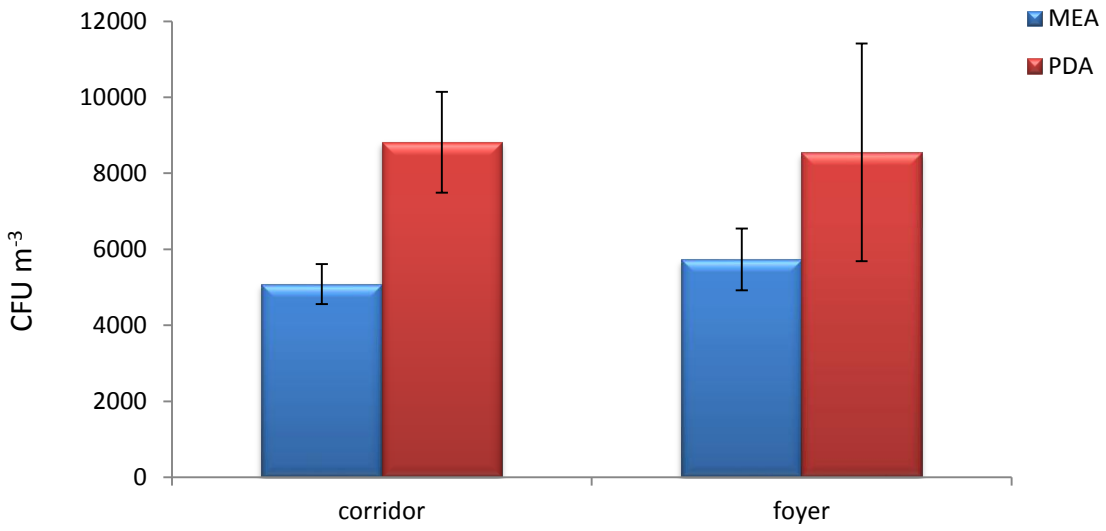

Figure 3.13 *Penicillium* growth recovered on MEA and PDA from air samples taken at HMS Victory. Portsmouth

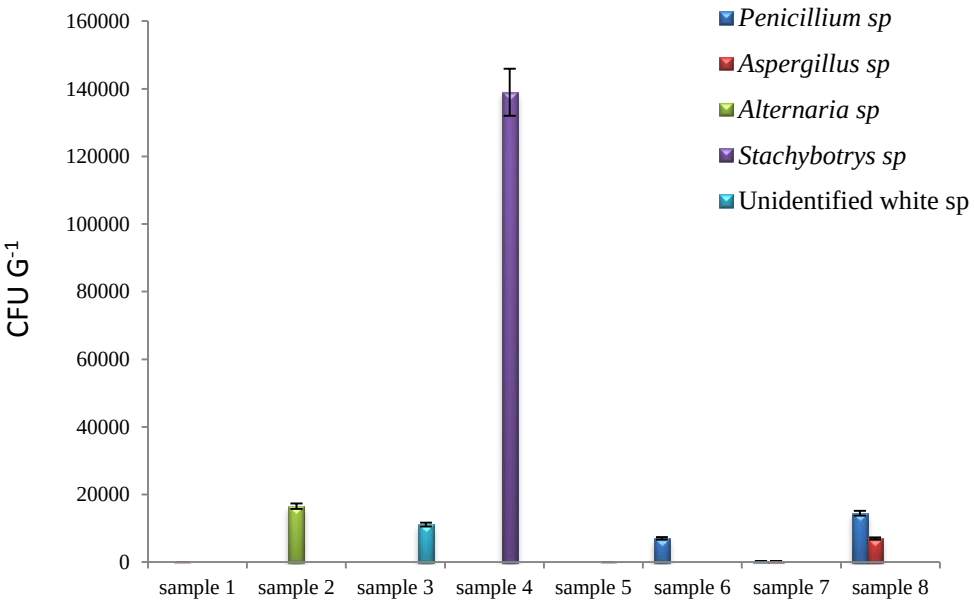

Figure 3.14 Fungal growth from bulk samples plated on to MEA

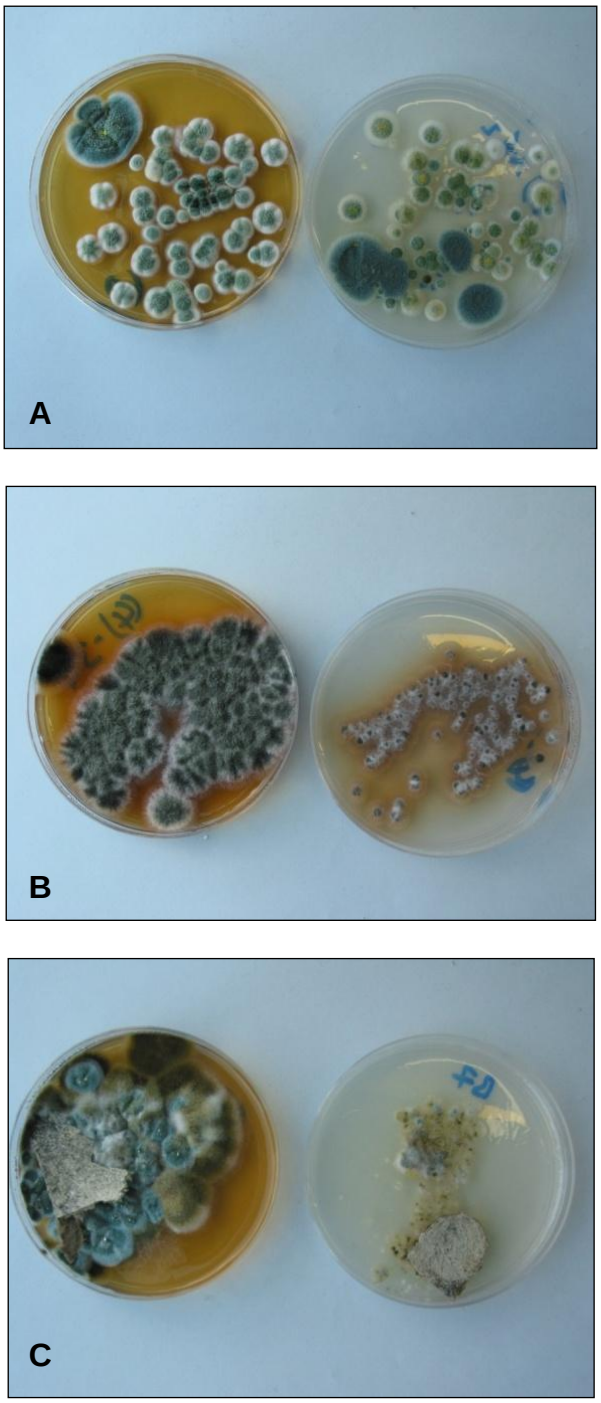

Plate 3.11 Fungal growth from plated bulk samples on MEA (left) and PDA (right) after 4 days incubation at 25°C. (A) *Aspergillus* sp, (B) *Stachybotrys* sp, (C) *Penicillium sp* and *Aspergillus* sp.

3.1.9 Comparisons of the surveyed locations

By comparing the average fungal counts determined at the surveyed locations using the various sampling methods, there is clearly a relationship between fungal contamination and extent of water damage. Buildings with minor water intrusion or ventilation problems showed much lower levels of fungal contamination with *Penicillium* and *Cladosporium* species predominant. The number and diversity of fungal contamination increased with water intrusion severity (Figure 3.15).

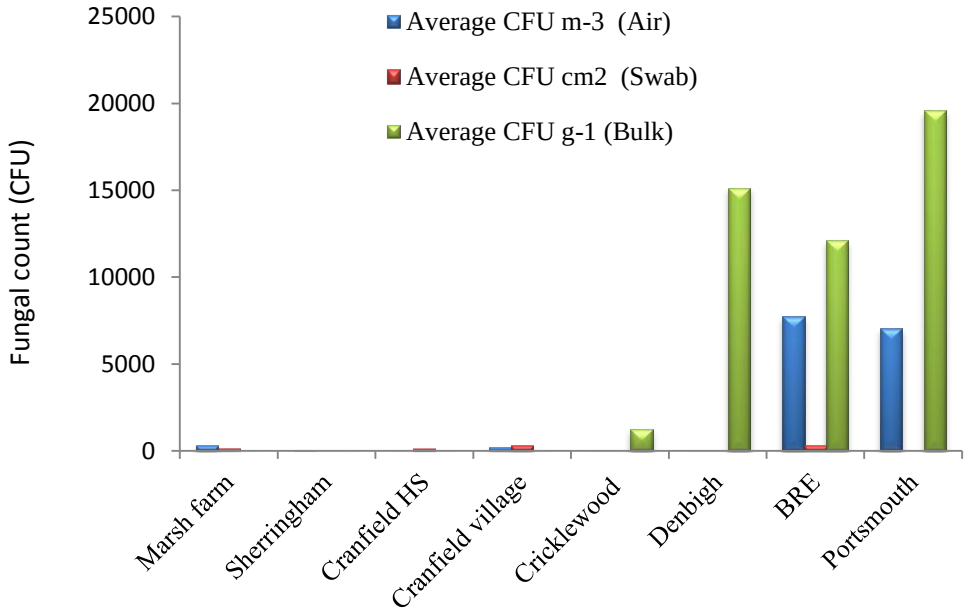

Figure 3.15 Comparison of the average fungal CFUs obtained from the surveyed locations.

3.2 Influence of temperature and a_w on the germination, germtube extension, growth and conidial production of *S. chartarum*

3.2.1 Germination and germ tube extension

The effect of temperature and water activity on the germination of *S. chartarum* 7711 and 14915 conida revealed that water activity and temperature significantly affected the rate of germination (Plate 3.12). Temperature showed effects at the extreme temperatures 15 and 37^{o}C which interacted with the lower a_w levels. High rates of germination was observed between 0.95 and 0.99a_w with 100% germination within 24 hours between 20-30^{o}C for both isolates (Figure 3.16 and Figure 3.17).

High rates of germ tube extension occurred for both isolates between 15 and 30^{o}C with freely available water at levels >0.95a_w. Germ tube extension rates significantly reduced across all temperatures at 0.92 a_w over the 72 hour period for both isolates (Figure 3.19). Contour plots revealed faster rates of germination and germ tube extension occurred over a wider range of temperatures and water activities for *S. chartarum* isolate 7711 than isolate 14915 (Figure 3.18 and Figure 3.20).

Statistical analyses (ANOVA) of the effect of water activity, temperature, strains and their two- and three-way interactions showed that all these factors were statistically significant (Table 3.1 and Table 3.2) (P = 0.05).

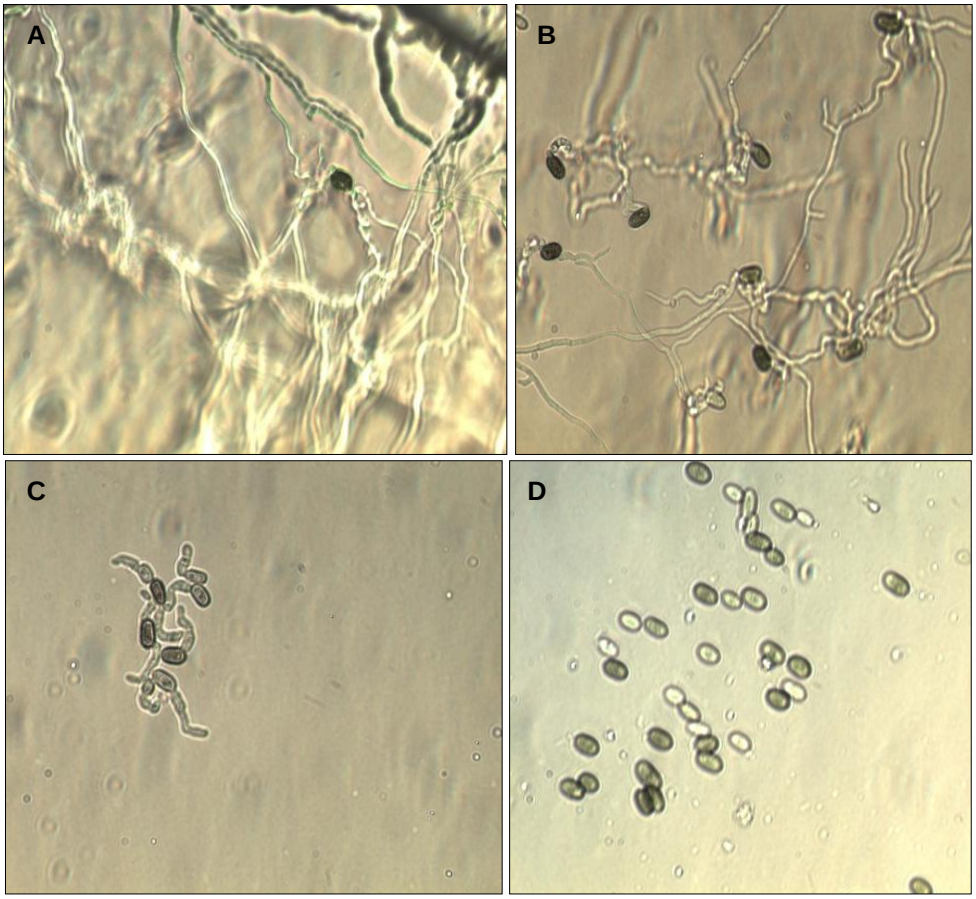

Plate 3.12 The effect of water activity on the germination of *S. chartarum* 14915 conidia after 24 hours incubation on PDA at 25°C. (A) 0.997a_w (B) 0.98a_w (C) 0.95 a_w (D) 0.92 a_w

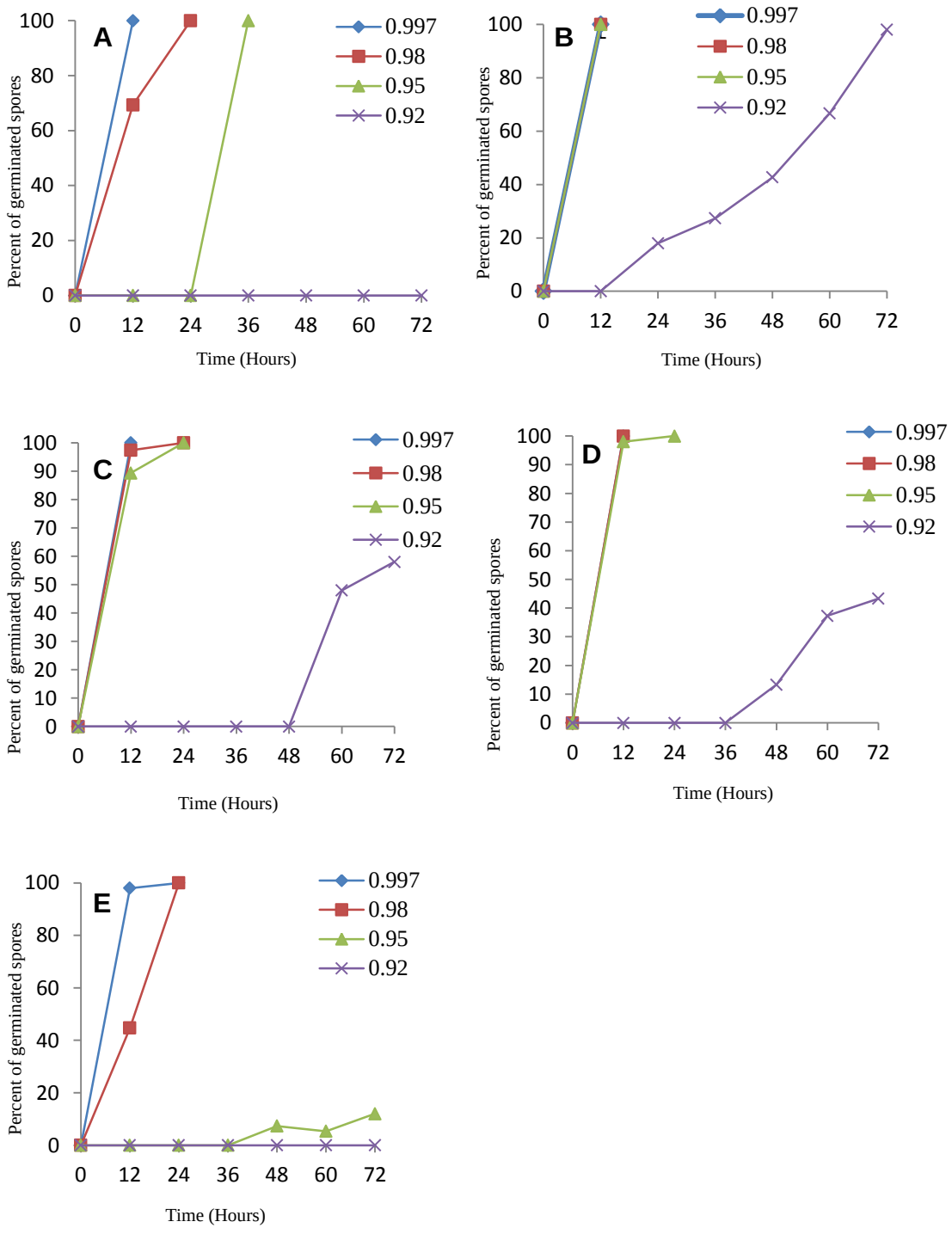

Figure 3.16 The effect of water activity and temperature and time on the germination of *S. chartarum* IBT 7711 on PDA at (A) 15°C, (B) 20°C, (C) 25°C, (D) 30°C, (E) 37°C.

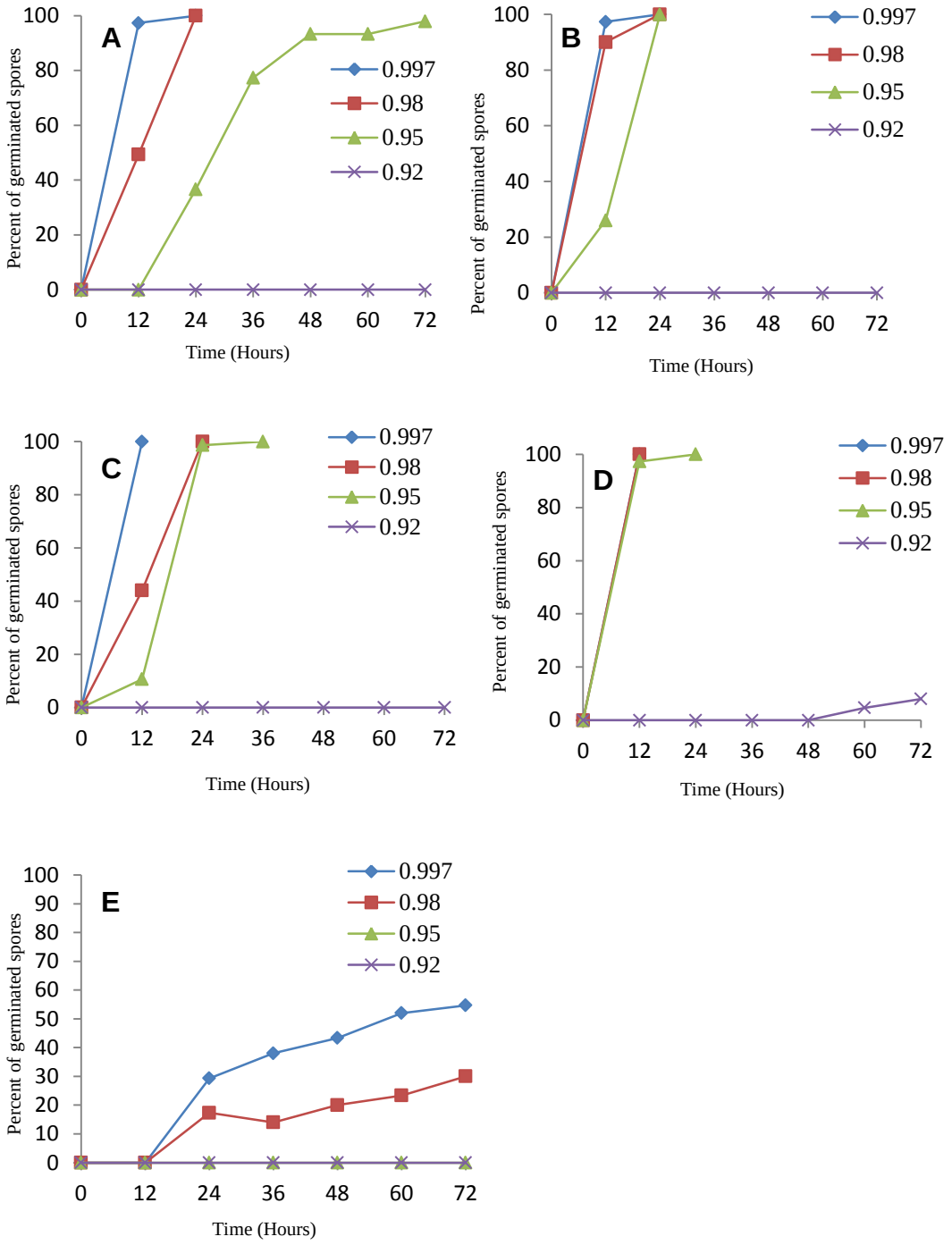

Figure 3.17 The effect of water activity, temperature and time on the germination of *S. chartarum* IBT 14915 on PDA at (A) 15°C, (B) 20°C, (C) 25°C, (D) 30°C, (E) 37°C.

Table 3.1 Analysis of variance of the effect of water activity, temperature and their interaction on the germination rate of *S. chartarum* isolates 7711 and 14915

EFFECT	SS	DOF	MS	F	P
Intercept	15120836	1	15120836	2294.518	0.000000
aw	6979330	3	2326443	353.027	0.000000
temp	6584880	4	1646220	249.806	0.000000
Strain	7635	1	7635	1.159	0.284991
aw*temp	7195503	12	599625	90.990	0.000000
aw*Strain	256106	3	85369	12.954	0.000001
Temp*Strain	1471863	4	367966	55.837	0.000000
aw*temp*Strain	1940739	12	161728	24.542	0.000000
Error	527199	80	6590		

DOF: degree of freedom, SS: sum of squares, MS: mean square, F: F value, P: probability, Probability at confidence 0.95.

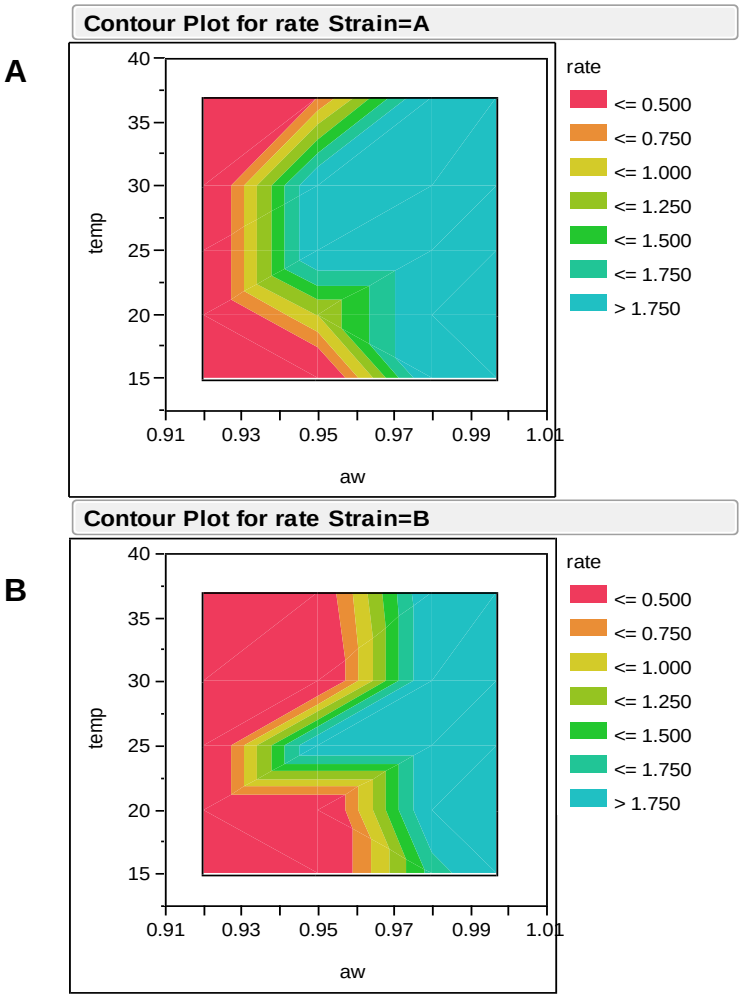

Figure 3.18 Contour plots showing the effect of temperature and water activity on the rate of germination by *S. chartarum* (A) 7711 and (B) 14915 on PDA.

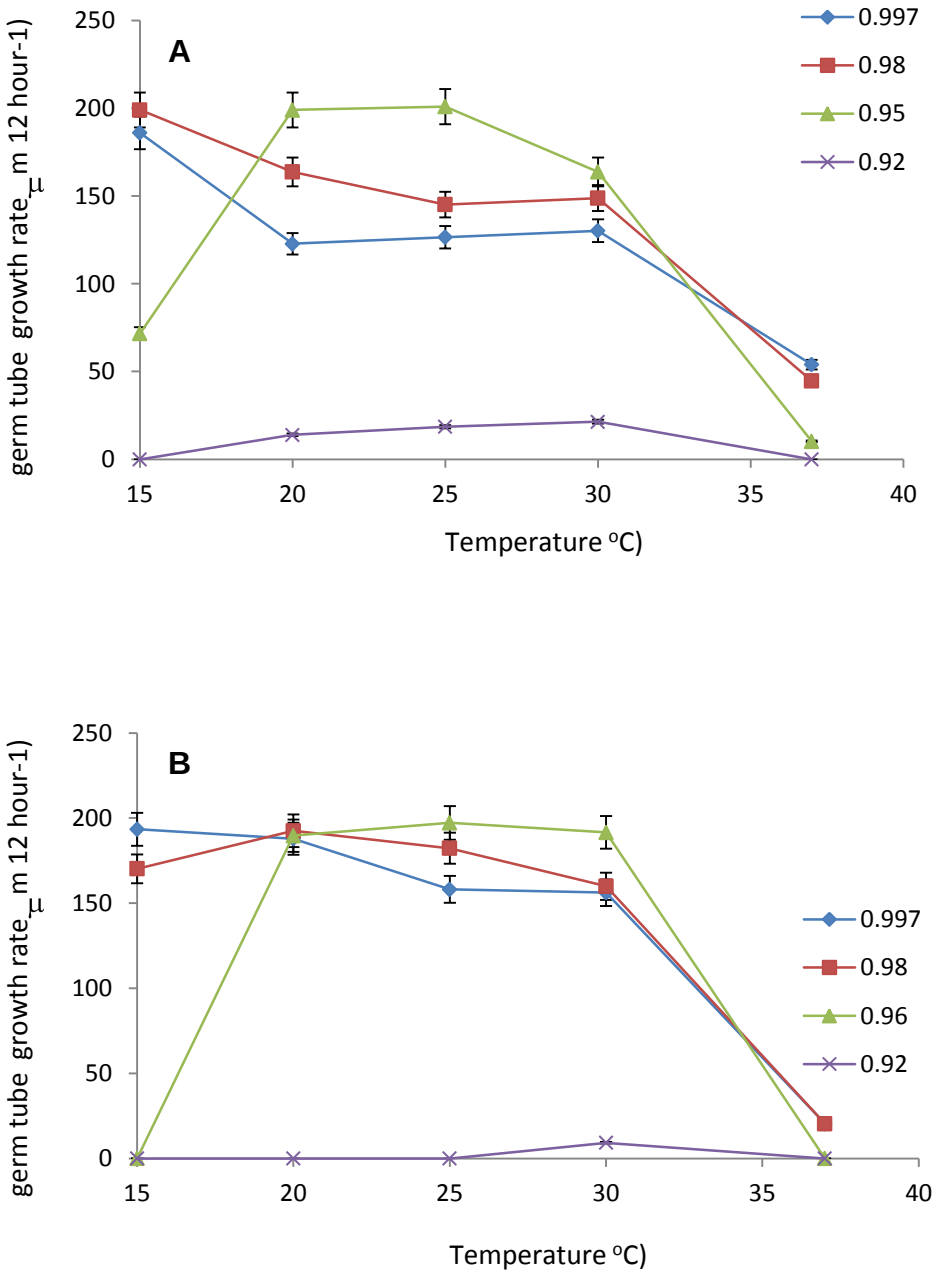

Figure 3.19 The effect of temperature and water activity on the rate of germ tube extension. *S. chartarum* 7711(A) and 14915 (B) over a 72 hour period.

Table 3.2 Analysis of variance of the effect of water activity, temperature and their interaction on the germ tube extension rate of *S. chartarum* isolates 7711 and 14915.

EFFECT	SS	DOF	MS	F	P
Intercept	1463250	1	1463250	35177.62	0.00
aw	206739	3	68913	1656.72	0.00
temp	84349	4	21087	506.96	0.00
Strain	1430929	1	1430929	34400.58	0.00
aw*temp	731848	12	60987	1466.18	0.00
aw*Strain	207798	3	69266	1665.21	0.00
Temp*Strain	80757	4	20189	485.37	0.00
aw*temp*Strain	736760	12	61397	1476.02	0.00
Error	3328	80	42		

DOF: degree of freedom, SS: sum of squares, MS: mean square, F: value, P: Probability at confidence 0.95.

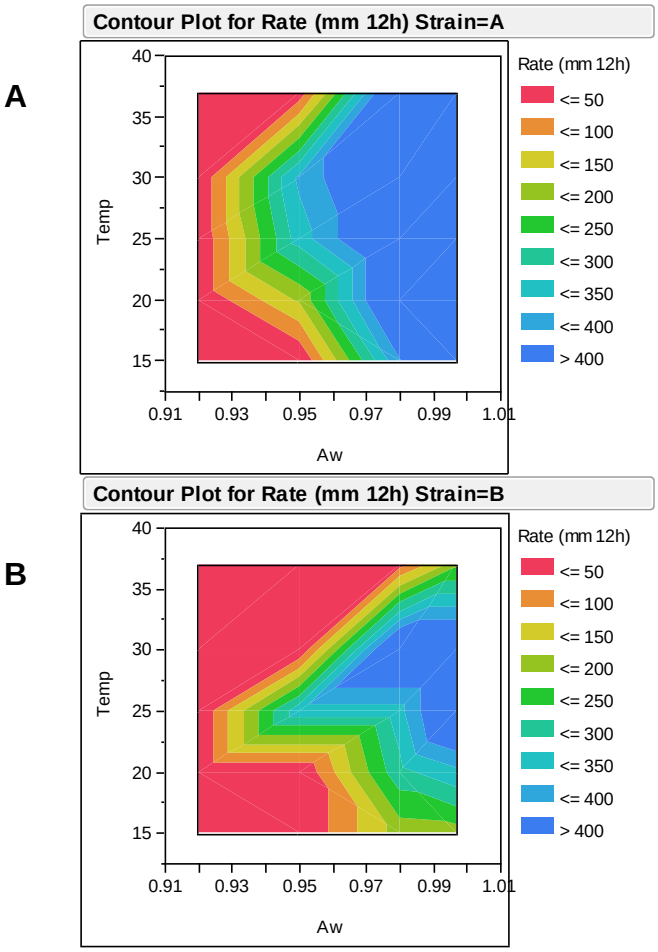

Figure 3.20 Contour plot showing the effect of temperature and water activity on the rate of germ tube extension by *S. chartarum* (A) 7711 and (B) 14915 on PDA.

3.2.2 Influence of temperature and a_w on the mycelial growth rate *in vitro* on PDA.

The initial experiment, performed to determine the effect of water activity on four isolates of *S. chartarum* at 25^{o}C incubation, revealed optimal growth rates for all strains at 0.99a_w with *S. chartarum* 14915 displaying the fastest growth rate at 1.69 mm day $^{-1}$ then the Portsmouth isolate at 1.59 mm day^{-1} followed by strain 7711 at 1.46 mm day $^{-1}$ and lastly the Denbigh isolate at 1.14 mm day $^{-1}$. All strains showed no or very little mycelial growth at 0.92 a_w (Figure 3.21).

The main experiment, which measured the effect of a range of water activities and temperatures on the mycelial growth rates of *S. chartarum* isolates 7711 and 14915, showed optimal growth rates of 1.637 and 2.055 mm day^{-1} respectively at 0.99a_w and 25^{o}C. Both isolates showed a shift in the optimal growth temperature from 25 to 30^{o}C when a slight water stress was imposed from 0.99 to 0.98a_w. Minimal conditions observed for growth were measured at 0.98a_w and 10^{o}C for both isolates. At 37^{o}C incubation both isolates showed slight mycelial growth with freely available water (Figure 3.22 and Plate 3.13).

Contour plots also revealed rates of mycelia growth occurred optimally between 15 and 30^{o}C at 0.99a_w for both *S. chartarum* isolates 7711 and 14915 (Figure 3.22).

Statistical analyses (ANOVA) of the effect of water activity, temperature, strains and their two- and three-way interactions showed that all these factors were statistically significant (Table 3.3 and Table 3.4) (P = 0.05).

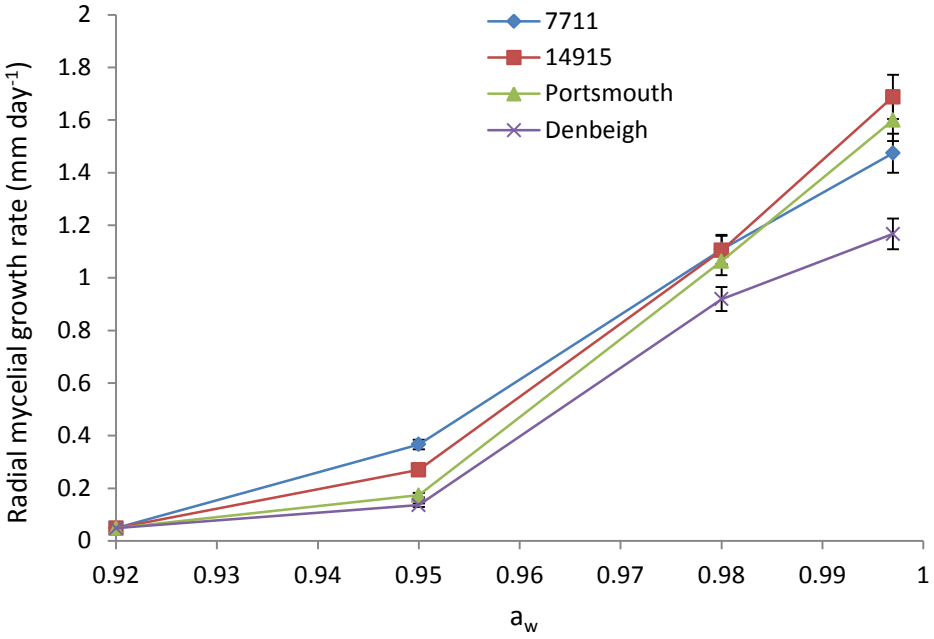

Figure 3.21 The effect of water activity on the mycelial growth of four strain of *S. chartarum* at 25°C for 10 days.

Table 3.3 Analysis of variance of the effect of water activity on the growth rate of *S. chartarum* isolates 7711, 14915, Portsmouth and Denbigh.

	SS	DOF	MS	F	P
Intercept	23.78622	1	23.78622	3070.997	0.000000
aw	16.47757	3	5.49252	709.130	0.000000
Strain	0.31611	3	0.10537	13.604	0.000003
Error	0.31756	41	0.00775		

DOF: degree of freedom, SS: sum of squares, MS: mean square, F: F value, P: Probability at confidence 0.95.

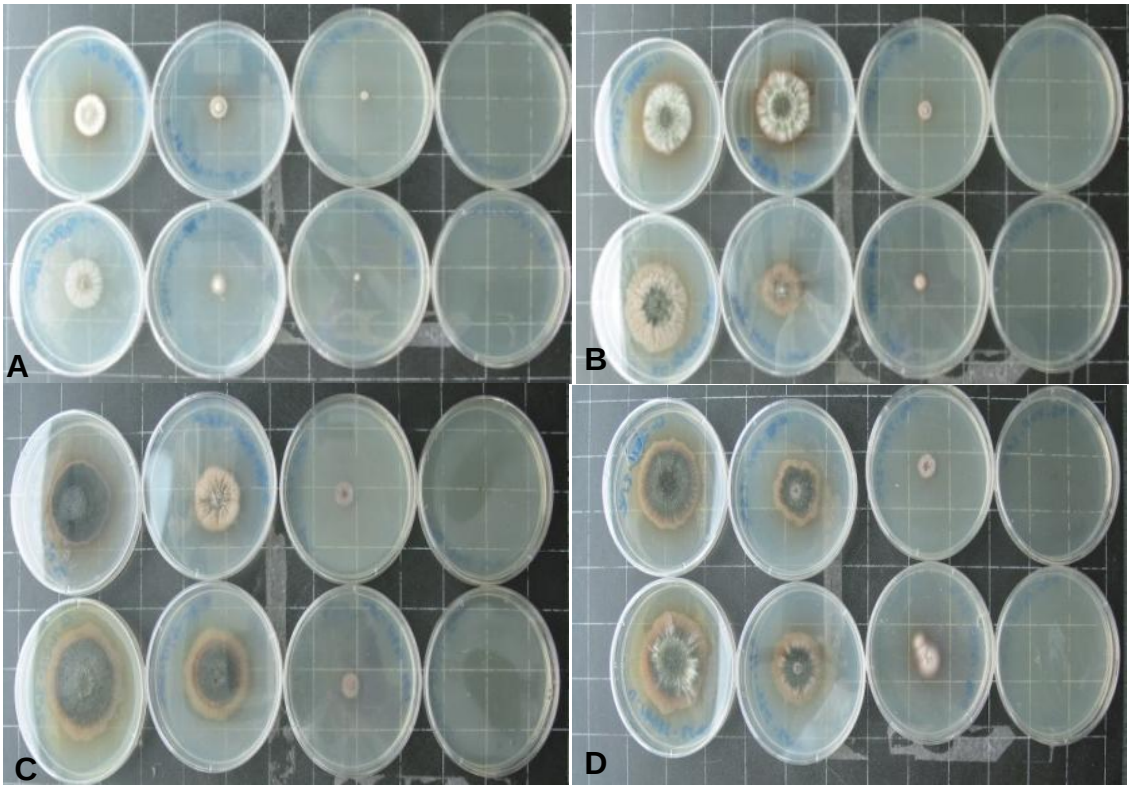

Plate 3.13 The effect of water activity and temperature on the growth of *S. chartarum* isolates. Top row of plates: Isolate 7711 (0.99, 0.98, 0.95 and 0.92a_w) bottom row of plates: Isolate 14915. Left to right: 0.99, 0.98, 0.95 and 0.92a_w. (A) 15^{o}C, (B) 20^{o}C, (C), 25^{o}C (D), 30^{o}C.

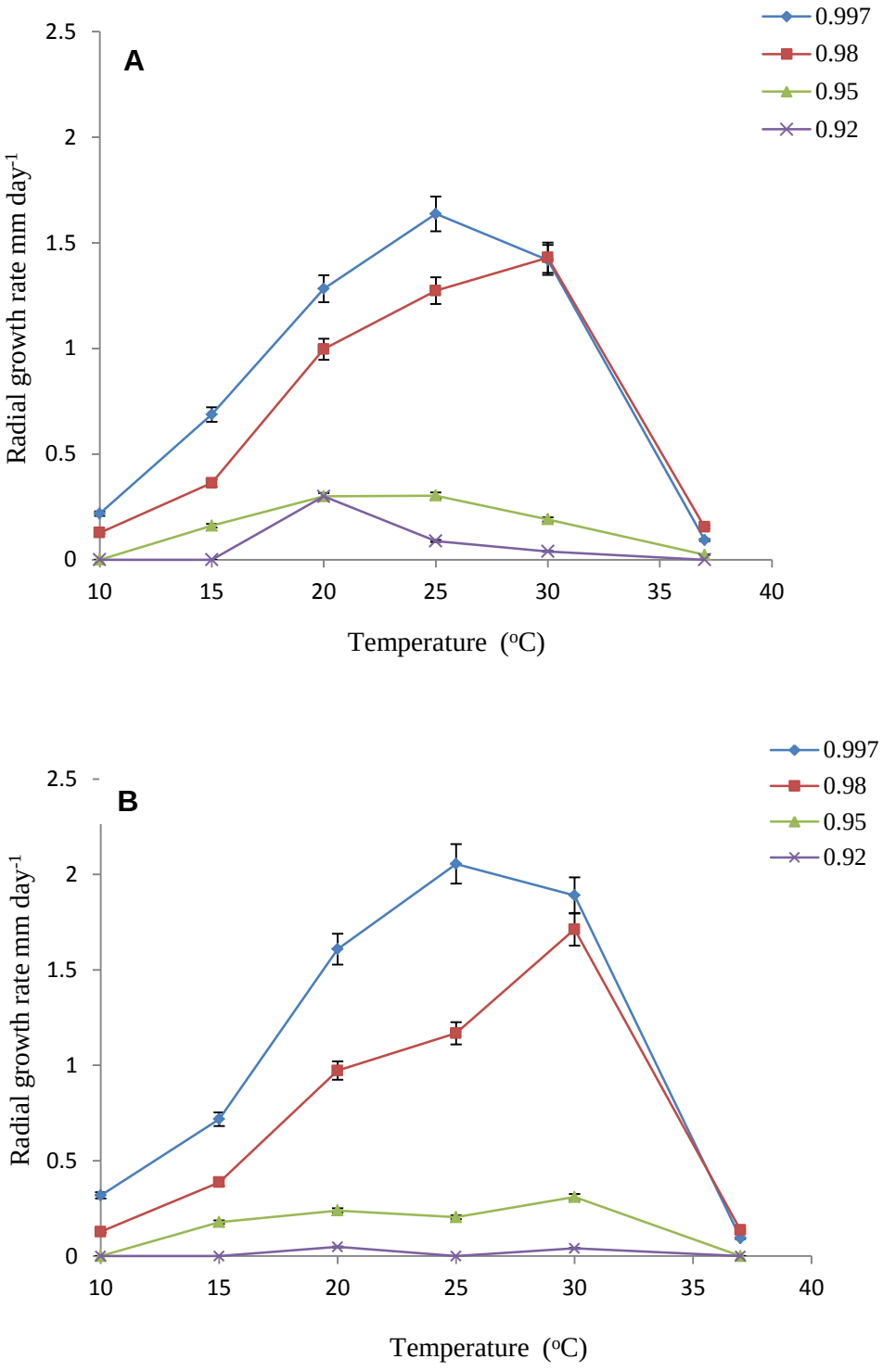

Figure 3.22 The effect of temperature and water activity on the mycelial growth rate of *S. chartarum* (A) 7711 and (B) 14915 over 14 days incubation on PDA.

Table 3.4 Analysis of variance of the effect of water activity (a_w), temperature and their interaction on the mycelia growth rate of *S. chartarum* isolates 7711 and 14915 on PDA.

	SS	DOF	MS	F	P
Intercept	155.9101	1	155.9101	56186.07	0.00
aw	104.4037	3	34.8012	12541.49	0.00
temperature	52.4472	4	13.1118	4725.16	0.00
Strain	0.8992	1	0.8992	324.03	0.00
aw*temp	38.1830	12	3.1819	1146.68	0.00
aw*strain	1.3592	3	0.4531	163.28	0.00
temp*strain	0.9076	4	0.2269	81.77	0.00
aw*temp*strain	1.0361	12	0.0863	31.12	0.00
Error	0.2220	80	0.0028		

DOF: degree of freedom, SS: sum of squares, MS: mean square, F: F value, P: Probability at confidence 0.95.

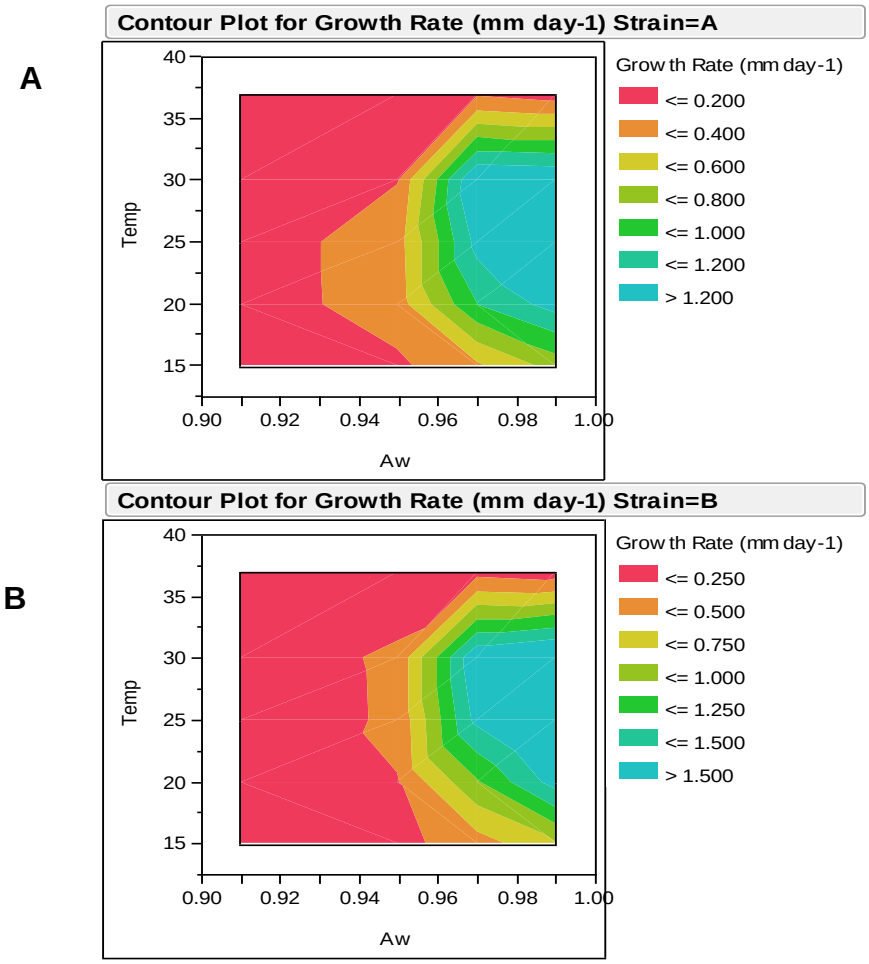

Figure 3.23 Contour plot showing the effect of temperature and water activity on the mycelia growth rate of *S. chartarum* (A) 7711 and (B) 14915 on PDA.

3.2.3 Influence of temperature and a_w on the sporulation of *S. chartarum* isolates *in vitro* on PDA

By the end of the 14 day experimental period, PDA plates that were incubated at 25–30^oC and 0.98-099a_w had full fungal coverage. With freely available water, optimum conditions for maximum sporulation were observed at 30^oC, of around 4.0 log^{10} conidia per mm^2. Interestingly, at 0.98 and 0.95 a_w, maximum spore production was at 25^oC. Maximum sporulation at 0.95a_w occurred at lower temperatures (20–25^oC) for isolate 7711 than for isolate 14915 (25–30^oC), where there was no production at 20^oC. Slight spore production was observed at an incubation temperature of 15^oC and freely available water but there was no spore production observed at 37^oC under any of the water availability conditions (Figure 3.24). Contour plots revealed sporulation occurred optimally between 15 and 30^oC and 0.99a_w for both *S. chartarum* isolates 7711 and 14915 (Figure 3.24).

Statistical analyses (Table 3.5) of the effect of water activity, temperature, strains and their two- and three-way interactions showed that all these factors were statistically significant (P = 0.05).

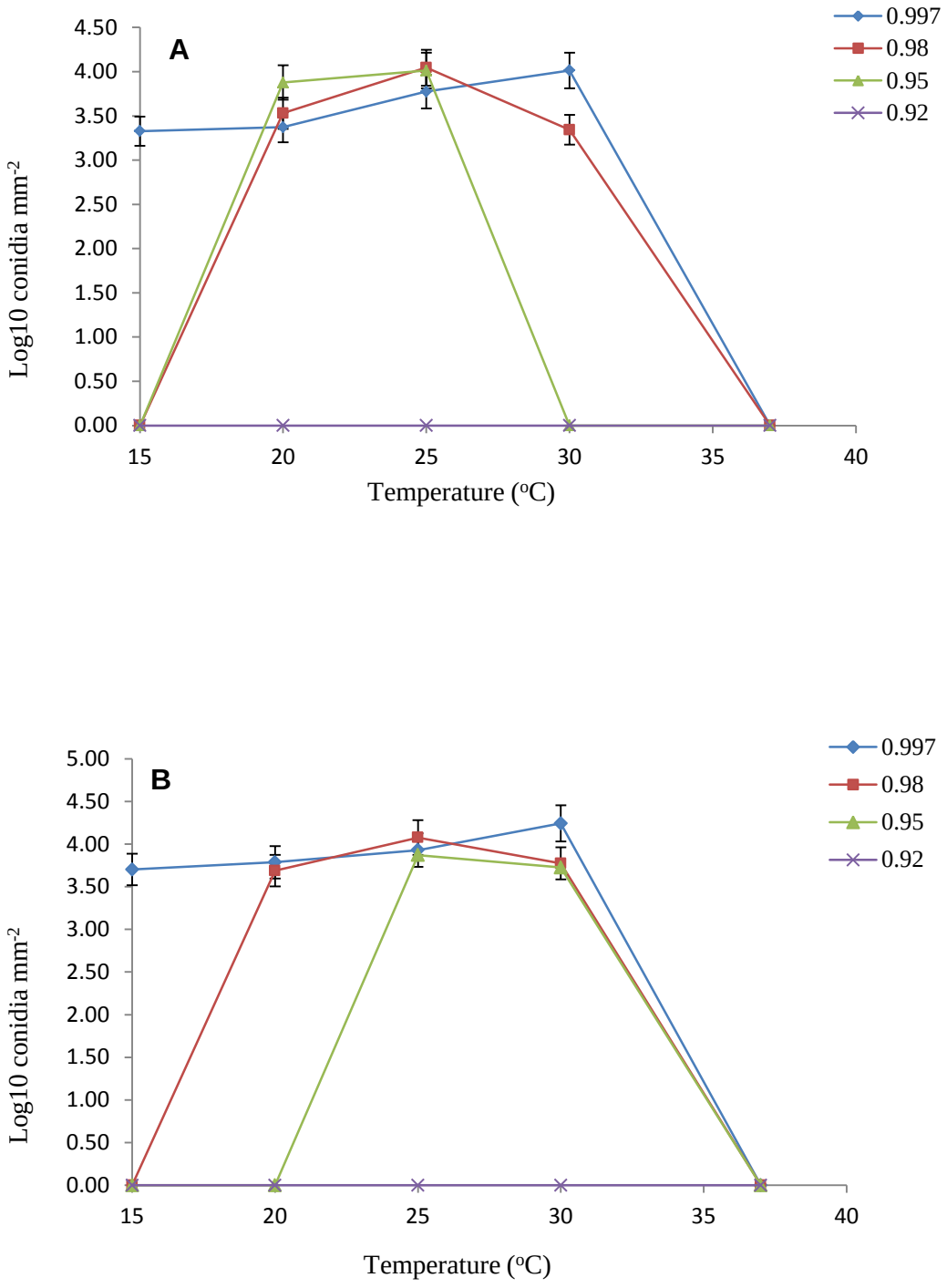

Figure 3.24 The effect of temperature and water activity on conidia production by *S. chartarm* (A) IBT 7711 and (B) IBT 14915 on PDA.

Table 3.5 Analysis of variance of the effect of water activity (a_w), temperature and their interactions on the rate of conidia production by *S. chartarum* isolates 7711 and 14915.

EFFECT	SS	DOF	MS	F	P
Intercept	12299310	1	12299310	1867.866	0.000000
aw	5230461	3	1743487	264.779	0.000000
temp	7922730	4	1980682	300.801	0.000000
Strain	225548	1	225548	34.253	0.000000
aw*temp	7048769	12	587397	89.207	0.000000
aw*Strain	765129	3	255043	38.733	0.000000
Temp*Strain	809275	4	202319	30.726	0.000000
aw*temp*Strain	1048944	12	87412	13.275	0.000000
Error	526775	80	6585		

DOF: degree of freedom, SS: sum of squares, MS: mean square, F: F value, P: Probability at confidence 0.95.

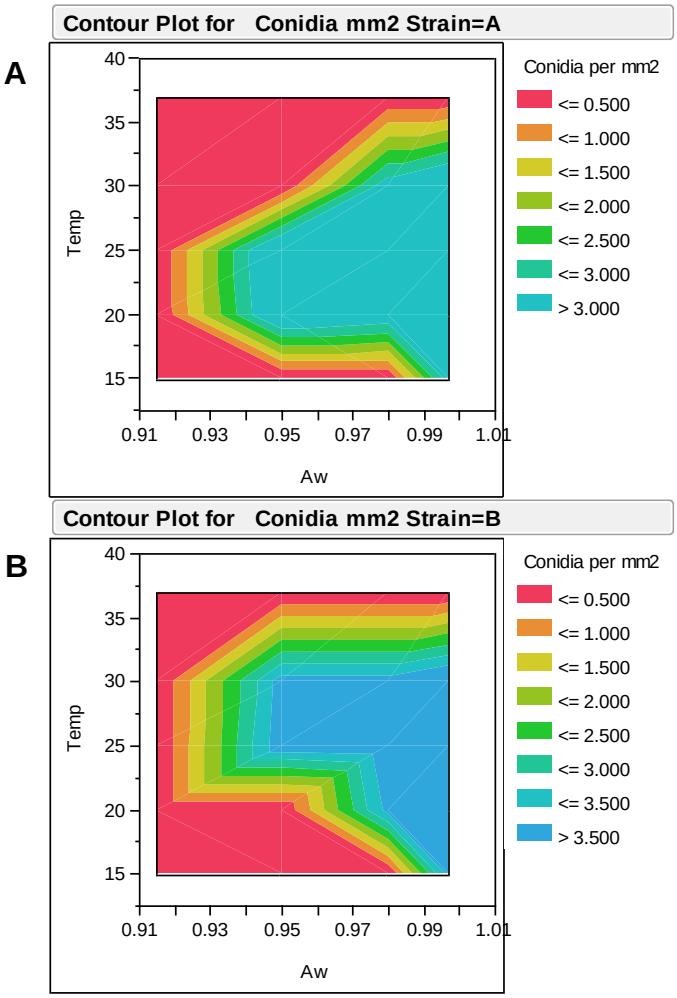

Figure 3.25 Contour plot showing the effect of temperature and water activity on the rate of conidia production by *S. chartarum* (A) 7711 and (B) 14915 on PDA.

3.3 Determination of the effect of water activity and time on the extracellular enzyme production by *S. chartarum* isolates using APIZYM.

Water activity showed significant effect on the extracellular enzyme production by *S. chartarum*. Both isolates 7711 and 14915 produced esterase, acid phosphatase and napthol-AS-BI-phosphohydrolase across all of the tested water activities. Phosphate alkaline was solely produced at 0.997a_w for isolate 14915. Higher levels of acid phosphase and napthol-AS-BI-phosphohydrolase was observed at 0.98 and 0.99a_w of 40 nanomoles were produced for strain 7711 and slightly lower levels of acid phosphatase was determined for strain 14915. Lower levels of esterase were produced across all tested water activities at the level of 5 nanomoles. 5 nanomoles of phosphate alkaline was detected for strain 14915 only.

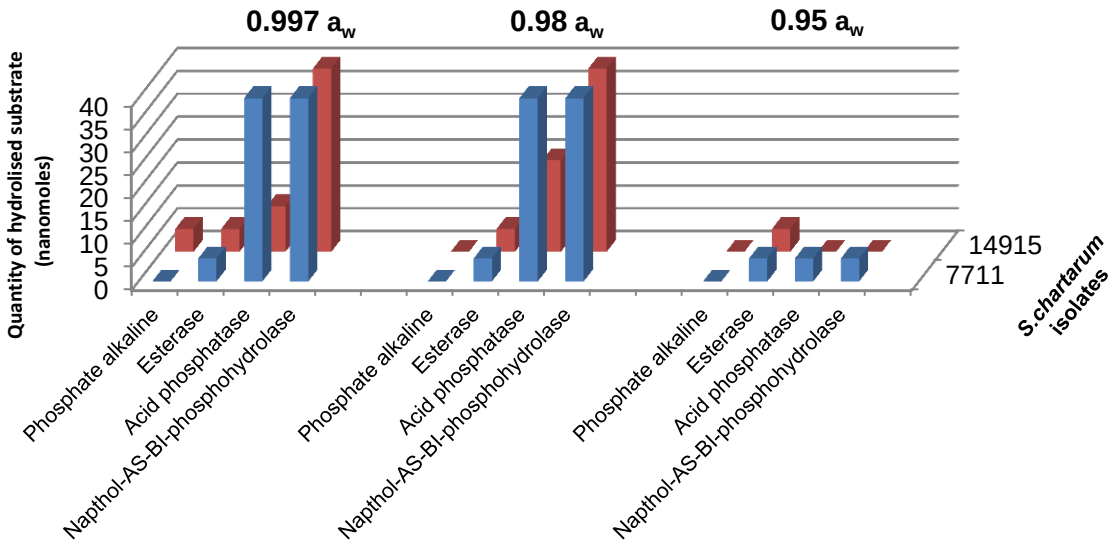

Figure 3.26 The effect of water activity on extracellular enzymes production by *S. chartarum* isolates after 6 days incubation in potato extract broth at 25°C.

The effect of time on the extracellular enzyme production by three isolates of *S. chartarum* grown on PDA at 0.99a_w using the APIZYM system was also determined. Isolate 7711 produced phosphate alkaline and napthol-AS-BI-phosphohydrolase at levels of 20 nanomoles within the first 48 hours of growth which declined to 5 nanomoles after 96 hours of growth. The isolate obtained from Denbigh produced high levels of phosphate alkaline and napthol-AS-BI-phosphohydrolase between 48- 96 hours of growth which then declined to 5 nanomoles after 120 hours. Isolate 14915 produced low levels (5 nanomoles) of esterase after 96 hours of growth. After 120 hours the same isolate displayed no esterase production but the production of low levels of phosphate alkaline and acid phosphatase was detected (Figure 3.26).

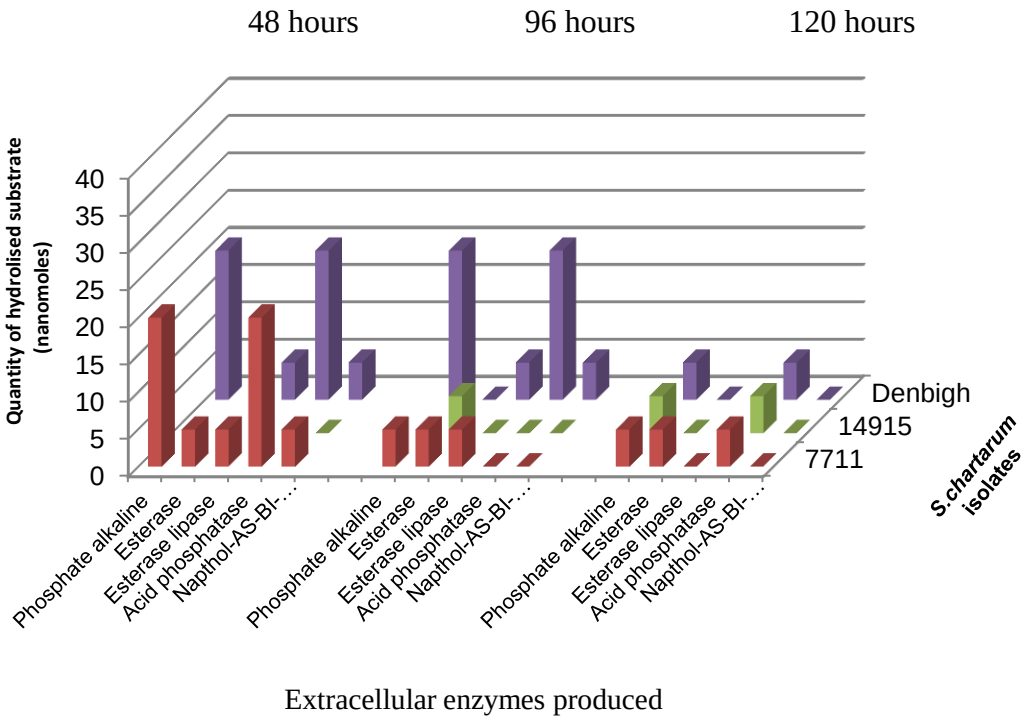

Figure 3.27 The effect of time on the extracellular enzyme production by *S. chartarum* isolates 7711, 14915 and Denbigh grown on PDA at 0.997a_w and 25°C.

3.4 Determination of the effect of water activity and time on the total protein production by *S. chartarum* isolates

Very little difference was observed between isolates in the levels of total protein production over time. It was however interesting to find when water stress was imposed slightly higher levels of protein were produced for all isolates from 72 hours onwards (Figure 3.28).

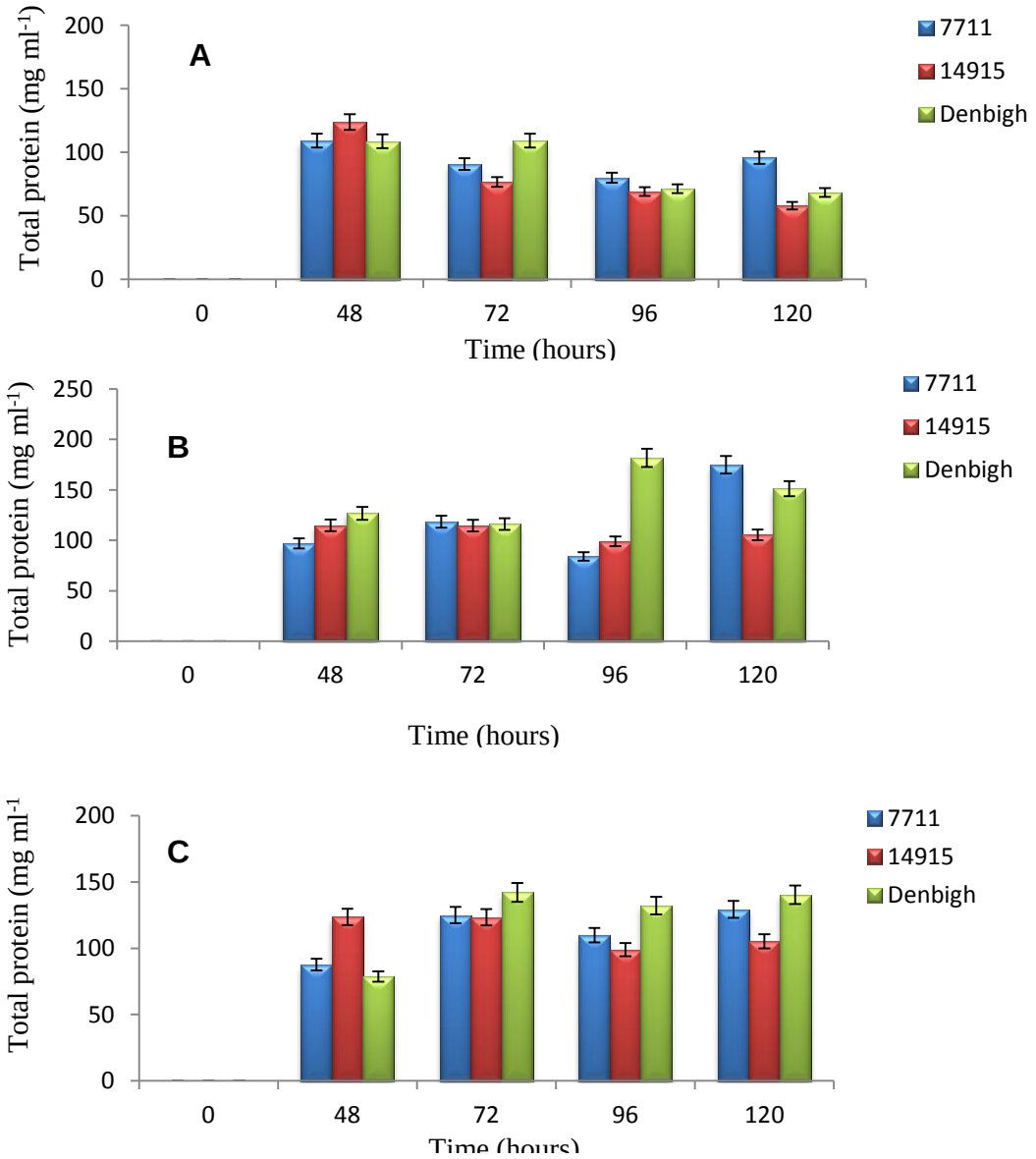

Figure 3.28 Total protein production by *S. chartarum* strains *in vitro* on PDA at 25°C. (A) 0.997a_w, (B) 0.98 a_w, (C) 0.95 a_w

3.5 Determination of haemolytic activity of *S. chartarum* isolates

All three *S. chartarum* isolates showed haemolytic activity of the beta type when grown on 7% sheep's blood agar. Clear zones under and around the colonies varied among the tested isolates. Visual examination of the plates determined that strain 14915 produced the largest clear zone around the colony followed by isolate 7711 and lastly Denbigh. When grown at 37^{o}C both isolates 7711 and Denbigh showed slight growth but no haemolytic activity. It was also noticed that theses isolates grew in yeast like form when incubated on PDA at this temperature (Plate 3.14).

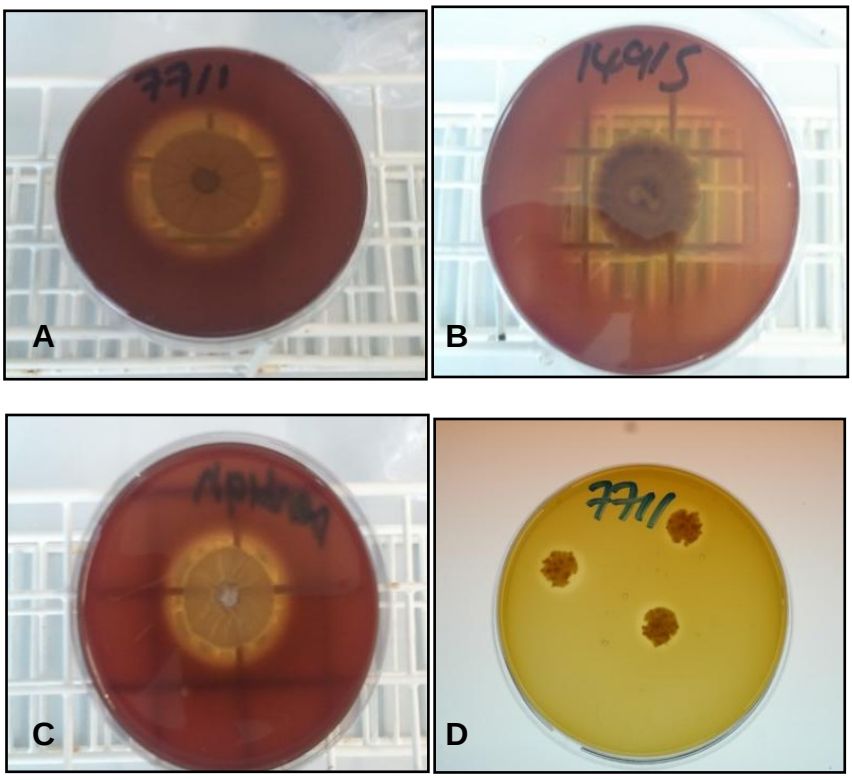

Plate 3.14 Growth of *S. chartarum* isolates on 7% sheep's blood agar after 14 days incubation at 0.99a_w. (A) IBT 7711 at 25^{o}C, (B) 14915 at 25^{o}C, (C) Denbigh at 25^{o}C, (D) 7711 at 37^{o}C.

3.6 Influence of temperature and a_w on satratoxin G production by strains of *S. chartarum*.

Satratoxin G production was determined to be significantly affected by a_w and temperature. Strain 7711 produced 100 times more SG than strain 14915. Isolate 7711 (Chemotype S) showed maximum SG production of 271160.1 ng $g^{-1}$of freeze dried sample at 20^{o}C and 0.98a_w. Isolate 14915 (Chemotype A) showed maximum SG production of 1621.7 ng/g of freeze dried sample at 25^{o}C and 0.98a_w. Both strains showed very little SG production between 30 and 37^{o}C at all tested a_w levels, compared to the 15, 20 and 25^{o}C levels (Figure 3.29). These results are clearly reflected in the contour plots which also show levels of SG production occurred optimally at 20^{o}C and 0.98a_w for *S. chartarum* isolate 7711 (Figure 3.30).

Statistical analyses (Table 3.6) of the effect of water activity, temperature, strains and their two- and three-way interactions showed that all these factors were statistically significant (P = 0.05).

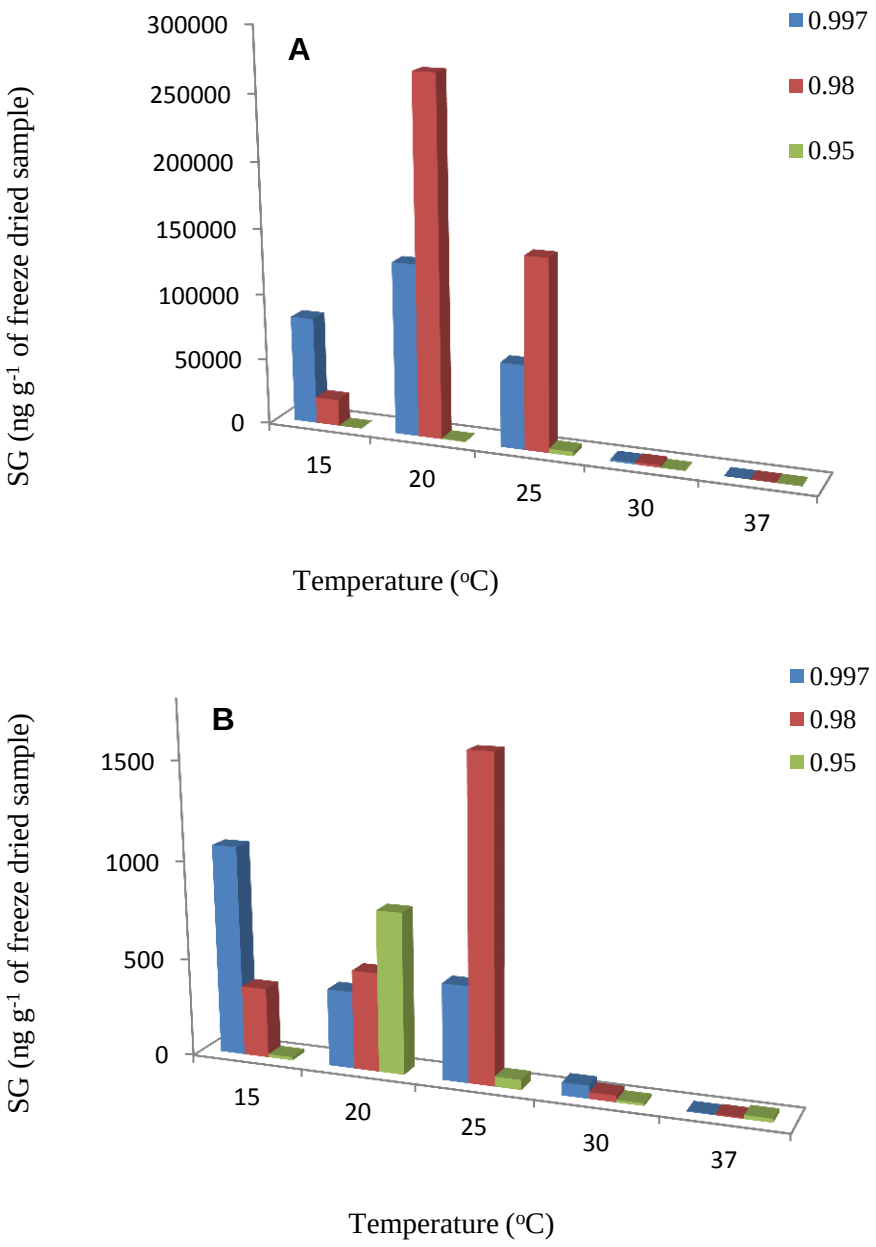

Figure 3.29 The influence of temperature and a_w on satratoxin G production by *S. chartarum* isolates (A) 7711 and (B) 14915.

Table 3.6 Analysis of variance of the effect of water activity, temperature and their interaction on the Satratoxin G production of S. *chartarum* isolates 7711 and 14915.

EFFECT	SS	DOF	MS	F	P
Intercept	4.748603E+10	1	4.748603E+10	248.9101	0.000000
aw	2.474729E+10	2	1.237365E+10	64.8596	0.000000
temp	5.157038E+10	4	1.289259E+10	67.5798	0.000000
Strain	4.607600E+10	1	4.607600E+10	241.5191	0.000000
aw*temp	4.104497E+10	8	5.130622E+09	26.8935	0.000000
aw*Strain	2.438232E+10	2	1.219116E+10	63.9031	0.000000
Temp*Strain	5.065593E+10	4	1.266398E+10	66.3815	0.000000
aw*temp*Strain	4.079112E+10	8	5.098890E+09	26.7271	0.000000
Error	1.049267E+10	55	1.907758E+08		

DOF: degree of freedom, SS: sum of squares, MS: mean square, F: F value, P: probability at confidence 0.95.

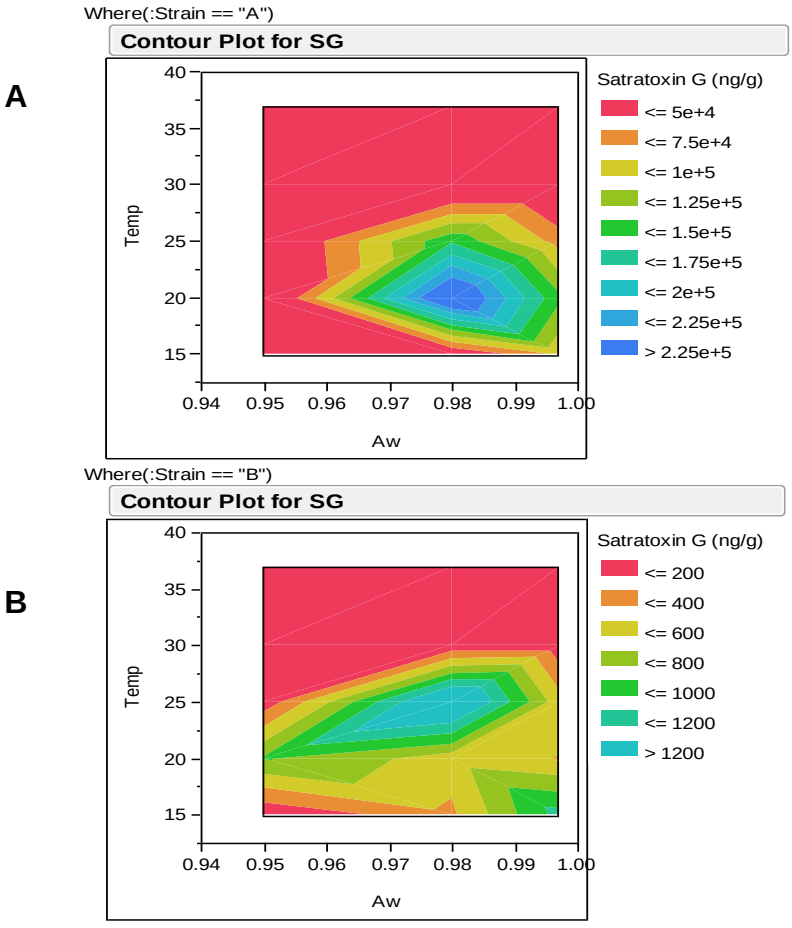

Figure 3.30 Contour plots of Satratoxin G (ng g^{-1} freeze dried sample) production by *S. chartarum* (A) 7711 and (B) 14195.

3.7 Determination of the effect of antioxidants on the mycelial growth and sporulation of *S. chartarum* isolates on PDA at 25°C

3.7.1 Effect of BHA.

BHA was found to be very effective in inhibiting the growth of both isolates 7711 and 14915, MIC for growth was measured at <200 mg l^{-1}. Low levels (approx 2.5mg l^{-1} of BHA were found to have a stimulatory effect on the growth of both strains. (Figure 3.31)

BHA had some effect on the growth and sporulation of both strains of *S. chartarum*. Both strains showed a complete inhibition of growth at 200mg l^{-1}. At 50 mg l^{-1} strain 7711 had complete inhibition of sporulation (Plate 3.15).

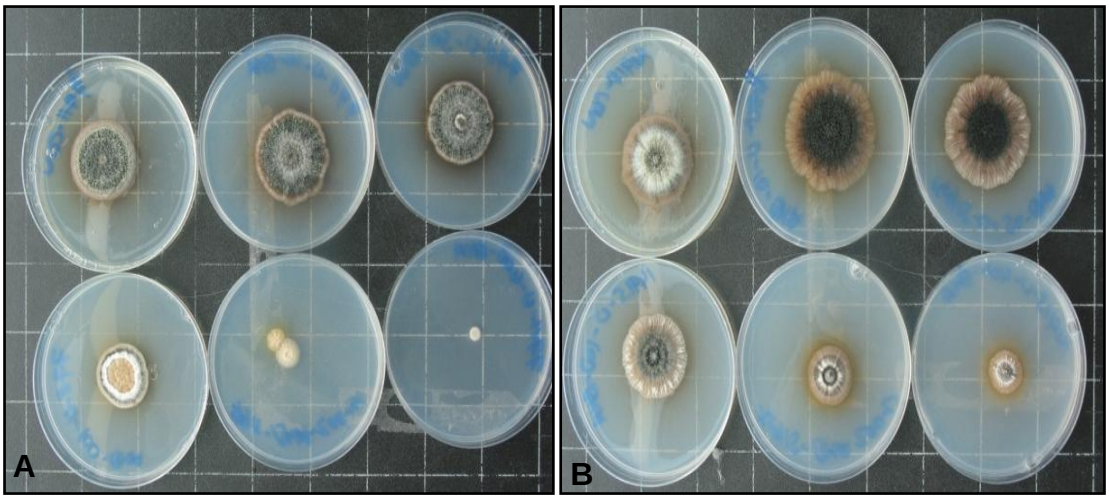

Plate 3.15 The effect of BHA on the growth rate of *S. chartarum* (A) 7711 and (B) 14915 on PDA after 14 days incubation at 0.99a_w and 25°C. Top row: Control, 2.5 and 12.5mg l^{-1} of BHA. Bottom row: 25, 50 and 100mg l^{-1} of BHA.

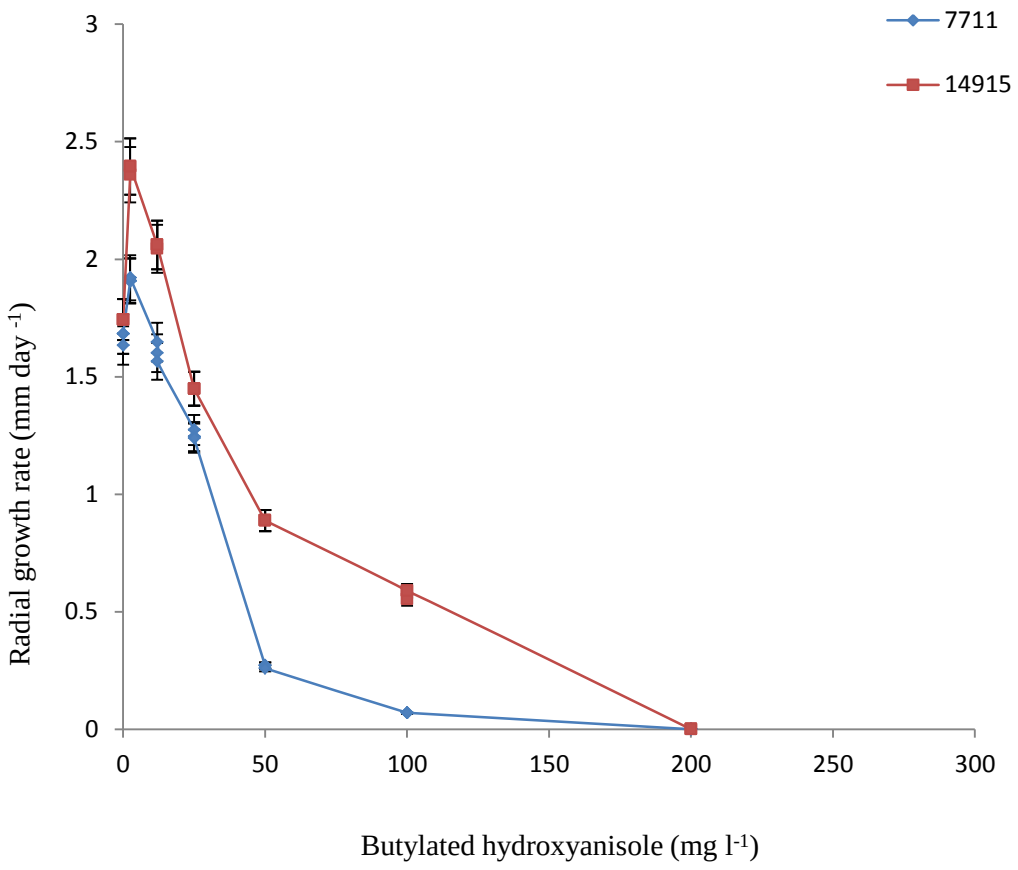

Figure 3.31 The effect of BHA on the radial growth rate of *S. chartarum* 7711 and 14915 on PDA at 0.99a_w and 25°C.

3.8 Effect of propyl gallate

Propyl gallate had some effect on the growth and sporulation of both strains of *S. chartarum*. Strain 7711 showed complete inhibition of sporulation at 200 mg l^{-1} and complete inhibition of growth at 800 mg l^{-1}. Strain 14915 showed complete inhibition of sporulation at 400 mg l^{-1} and complete inhibition of growth at 1000 mg l^{-1} (Plate 3.16 and Figure 3.32).

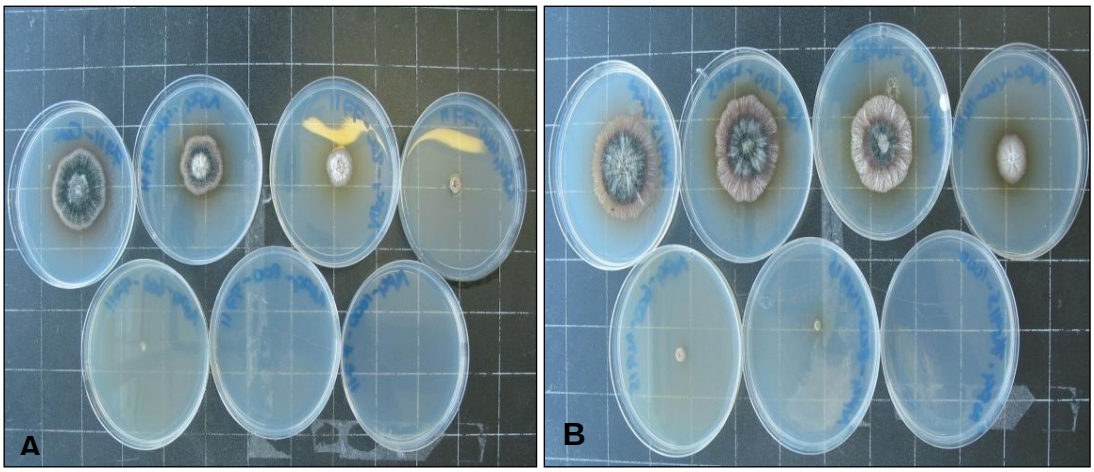

Plate 3.16 The effect of propyl gallate on the growth of *S. chartarum* (A) and (B) 14915 after 11 days on PDA at 0.997a_w and 25°C. Top row: Control, 100, 200, 400. Bottom row: 600, 800, 1000mg l^{-1} of propyl gallate

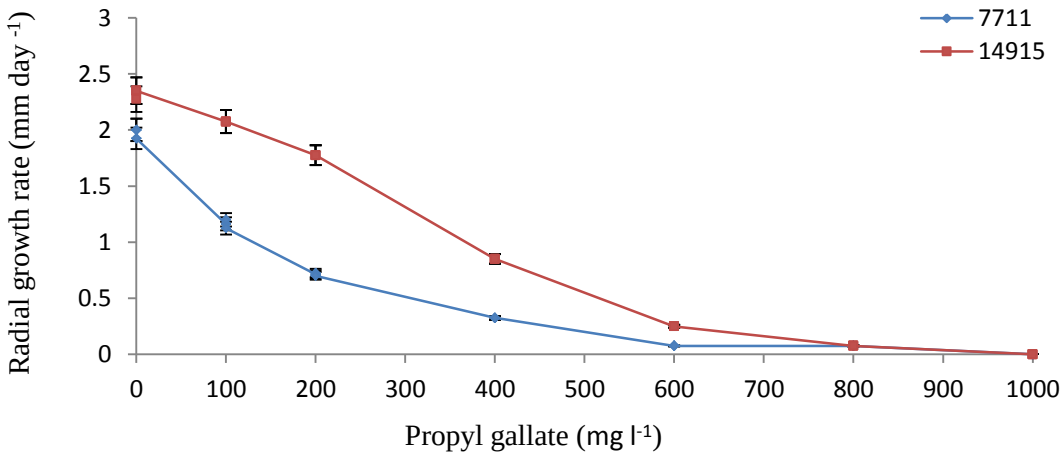

Figure 3.32 The effect of propyl gallate on the radial growth rate of *S. chartarum* isolates

3.9 Determination of growth rate of *S. chartarum* using the Bioscreen

The bioscreen proved to be be a reliable means of measuring the *S. chartarum* growth at varied inocula levels (P = 0.01), (Figure 3.33 and Table 3.7).

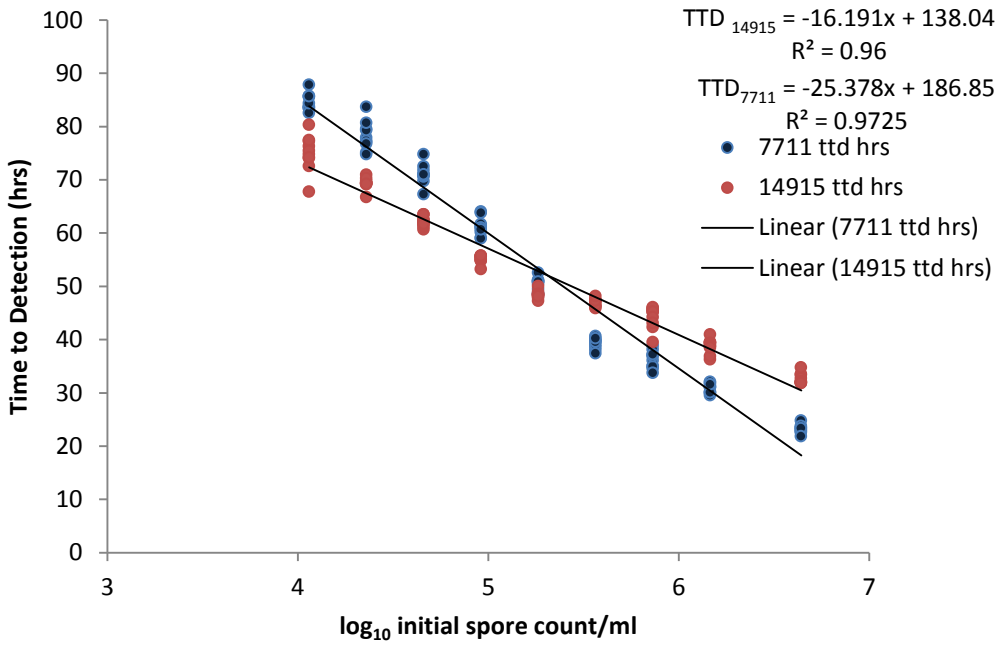

Figure 3.33 Effect of varied inocula levels on the growth of *S. chartarum* isolate 7711 and 14915 after 5 days incubation in PEB at 0.997a_w and 25°C.

Table 3.7 Analysis of variance of varied inocula levels for *S. chartarum* isolate 7711 and 14915

ANOVA
isolate 7711

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	37925.02245	37925.02	3110.589	1.87694E-70
Residual	88	1072.916301	12.19223		
Total	89	38997.93875			

ANOVA
Isolate 14915

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	15437.83554	15437.84	2110.968	2.73365E-63
Residual	88	643.5577551	7.313156		
Total	89	16081.39329			

DOF: degree of freedom, SS: sum of squares, MS: mean square, F: F value, Significant F: Probability at confidence 0.95.

3.10 Determination of the effect of water activity and temperature on the growth rate of *S. chartarum* using the Bioscreen

Growth rates measured using the bioscreen showed both isolates to be significantly influenced by water activity and temperature (Plate 3.17). 0.93a_w was found to be the minimum a_w for growth. Isolate 14915 proved to have a faster growth rate over all tested temperatures and water activities. Both isolates showed no growth at 10^oC and 37^oC after 5 days incubation. These results show that the bioscreen is a very useful tool for monitoring the effect of temperature and water activity on *S. chartarum* growth. Optimum growth rates for both isolates were determined at 30^oC between 0.93 and 0.99a_w. Isolate 7711 showed a slightly faster growth rate compared to isolate 14915 at optimal conditions of 30^oC and 0.99a_w at 0.320 and 0.314 OD per hour respectively (Figure 3.34). Contour plots revealed the highest rates of growth occurred at 30^oC and 0.99a_w for both *S. chartarum* isolates 7711 and 14915 (Figure 3.35).

Statistical analyses (Table 3.8) of the effect of water activity, temperature, strains and their two- and three-way interactions showed that all these factors were statistically significant (P = 0.05).

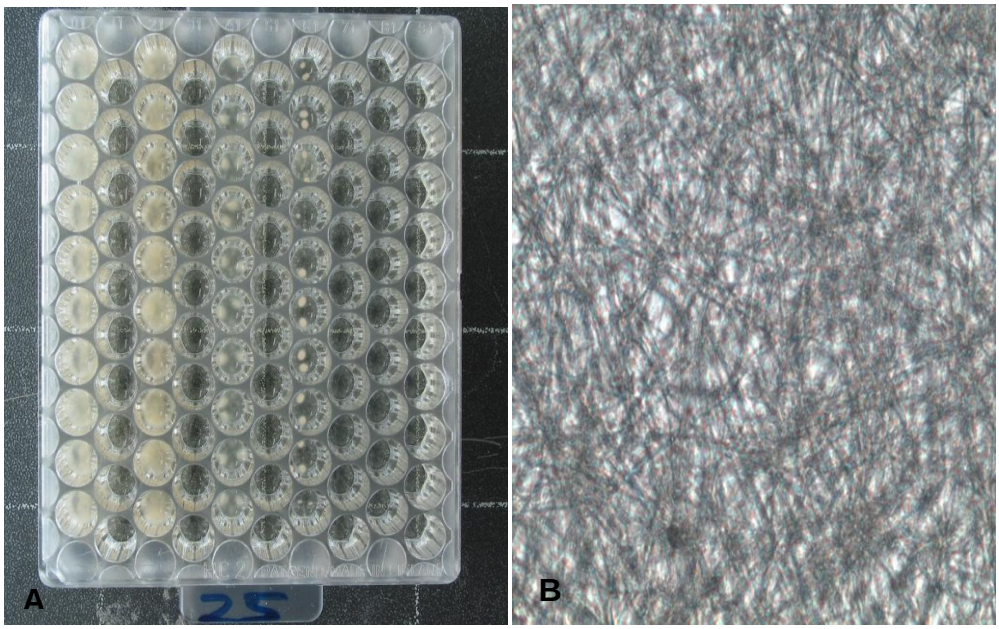

Plate 3.17 (A) Microtitre plate showing the effect of water activity on the growth of *S. chartarum* 7711 after 5 days incubation at 25°C in PEB. Left to right: Columns 1 and 2 (0.997a_w), Columns 3 and 4 (0.98a_w), Columns 5 and 6 (0.95a_w), Columns 7 and 8 (0.93a_w), Columns 9 and 10 (0.887a_w). Wells in columns 1, 3, 5, 7 and 9 inoculated with *S. chartarum*, wells 2, 4, 6, 8 and 10 control blanks. (B) Mycelial growth of *S. chartarum* 7711 (x400) observed in the wells of the microtitre plate after 5 days incubation at 25°C in PEB.

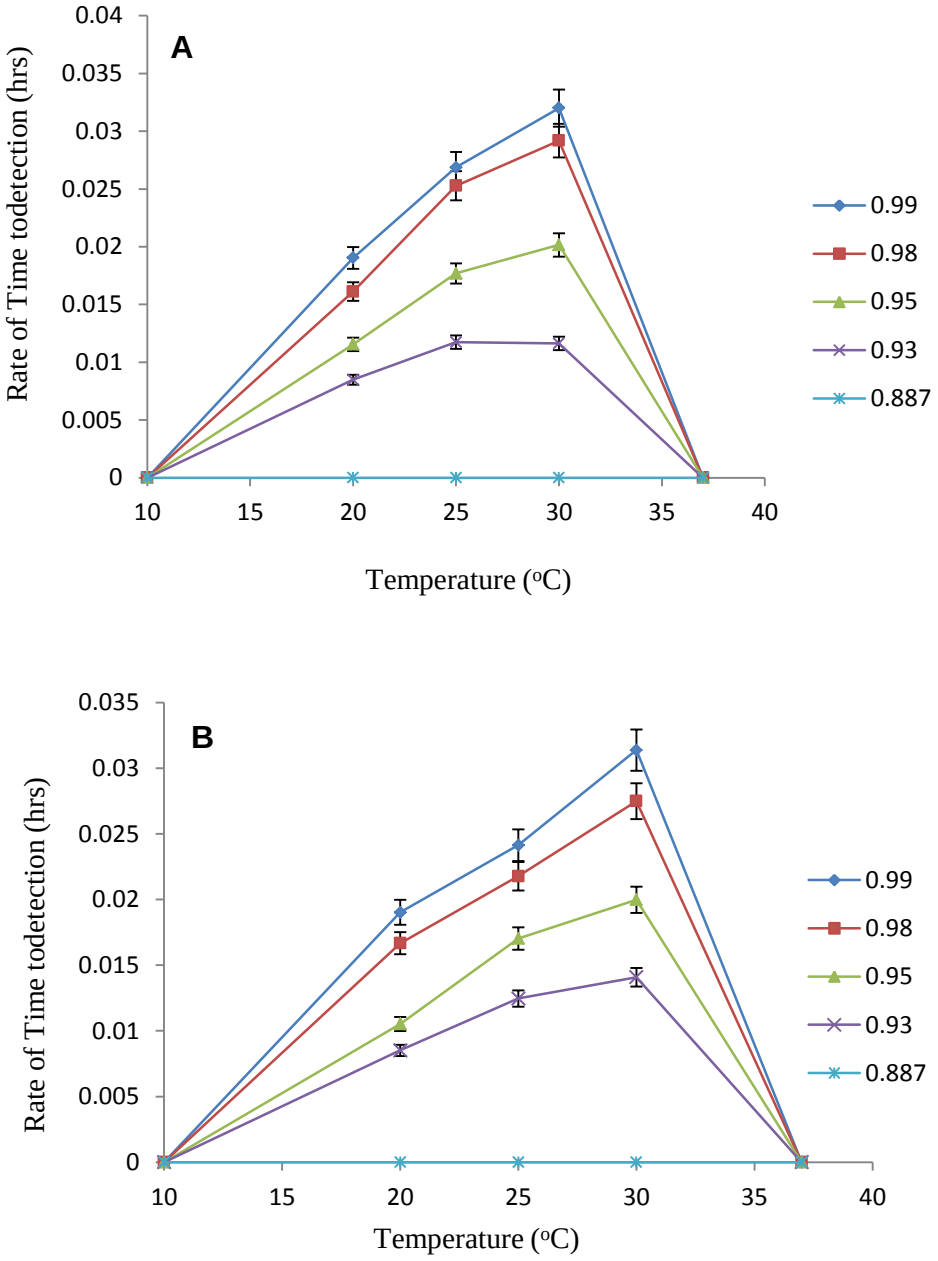

Figure 3.34 The effect of temperature and water activity on the growth rate of *S. chartarum* (A) 7711 and (B) 14915 after 5 days incubation in PEB at 0.99a_w and 25°C.

Table 3.8 Analysis of variance of the effect of water activity (a_w), temperature and their interaction on the growth rate of *S. chartarum* isolates 7711 and 14915 using the bioscreen.

EFFECT	SS	DOF	MS	F	P
Intercept	380089131	1	380089131	5616.386	0.000000
aw	290014737	4	72503684	1071.350	0.000000
temp	204457045	4	51114261	755.290	0.000000
Strain	432821	1	432821	6.396	0.011782
aw*temp	142928939	16	8933059	131.999	0.000000
aw*Strain	2839064	4	709766	10.488	0.000000
Temp*Strain	1433020	4	358255	5.294	0.000357
aw*temp*Strain	8248516	16	515532	7.618	0.000000
Error	30386092	449	67675		

DOF: degree of freedom, SS: sum of squares, MS: mean square, F: F value, P: Probability at confidence 0.95.

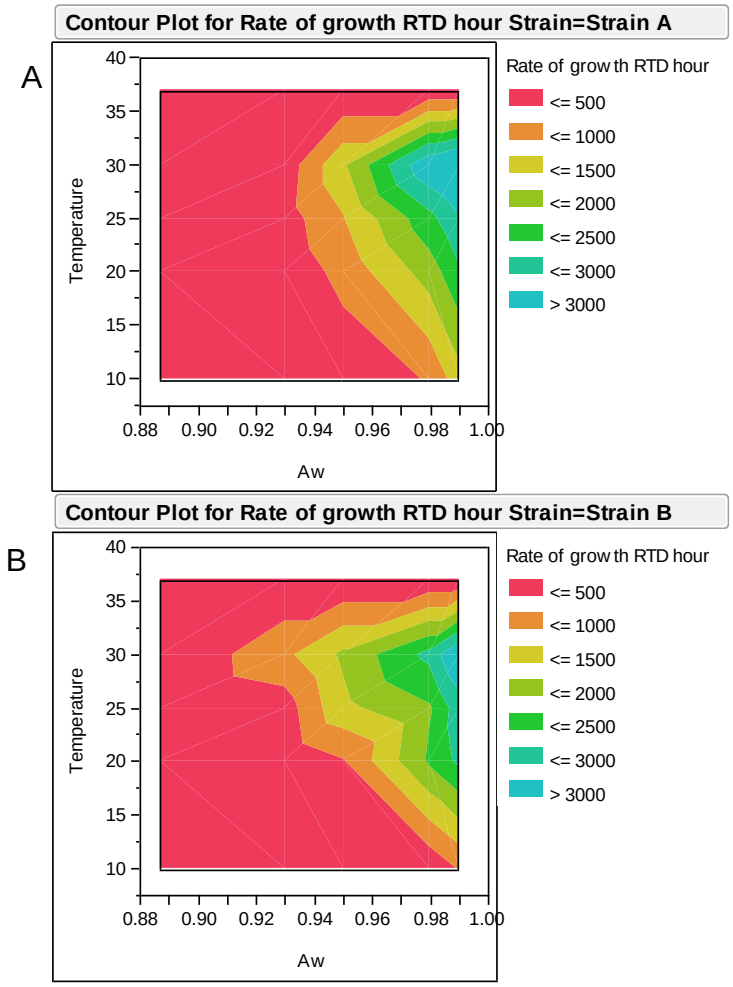

Figure 3.35 Contour plots showing the effect of temperature and water activity on the growth rate of *S. chartarum* (A) 7711 and (B) 14915 after 5 days incubation in PEB at 0.99a_w and 25°C.

3.11 Determination of the effect of tebuconazole and other inhibitors on the growth rate of *S. chartarum* using the Bioscreen.

It was noted that at high concentrations of tebuconazole, the initial turbidity of the well increased (Figure 3.36). However, as the incubation time increased (but before growth commenced) the OD generally decreased exponentially, suggesting a settling effect. This made background correction a time dependent phenomenon. As such OD data at concentrations of tebuconazole greater than 15mg l^{-1}, which in all cases showed no growth, were not used in the analyses of MIC. Strain 7711 and the Denbigh strain both showed a very high degree of similarity, whereas strain 14915 showed completely different behaviour. This difference was also observed with the OD/time plots themselves (Figure 3.38). The reason for the difference could be the presence of a resistant population. The shapes of the OD curves for strain 14915 suggest the same growth rates, with presumably increasing lag times with increasing Tebuconazole concentration whereas the Denbigh strain (and strain 7711 not shown) suggests a reduction in growth rate with increasing inhibitor concentrations (Figure 3.37). For *S. chartarum* isolates Denbigh and 7711 the relative AUC data were fitted using the standard Lambert-Pearson model (Equation1.1), Table 3.9 gives the results.

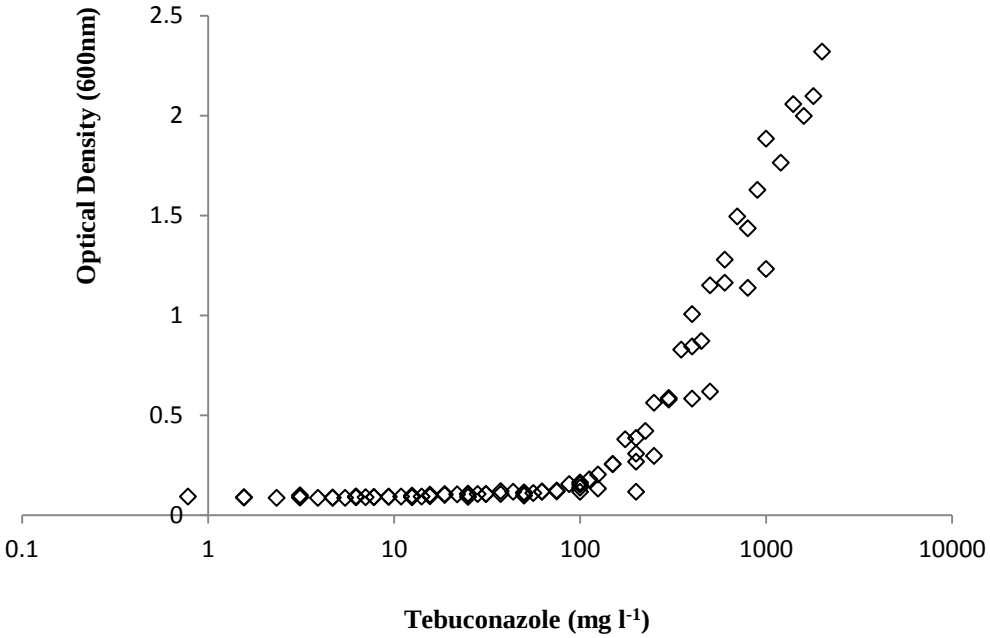

Figure 3.36. The initial optical density at 600nm with respect to tebuconazole concentration. The increase is due to the insolubility of tebuconazole in the growth media.

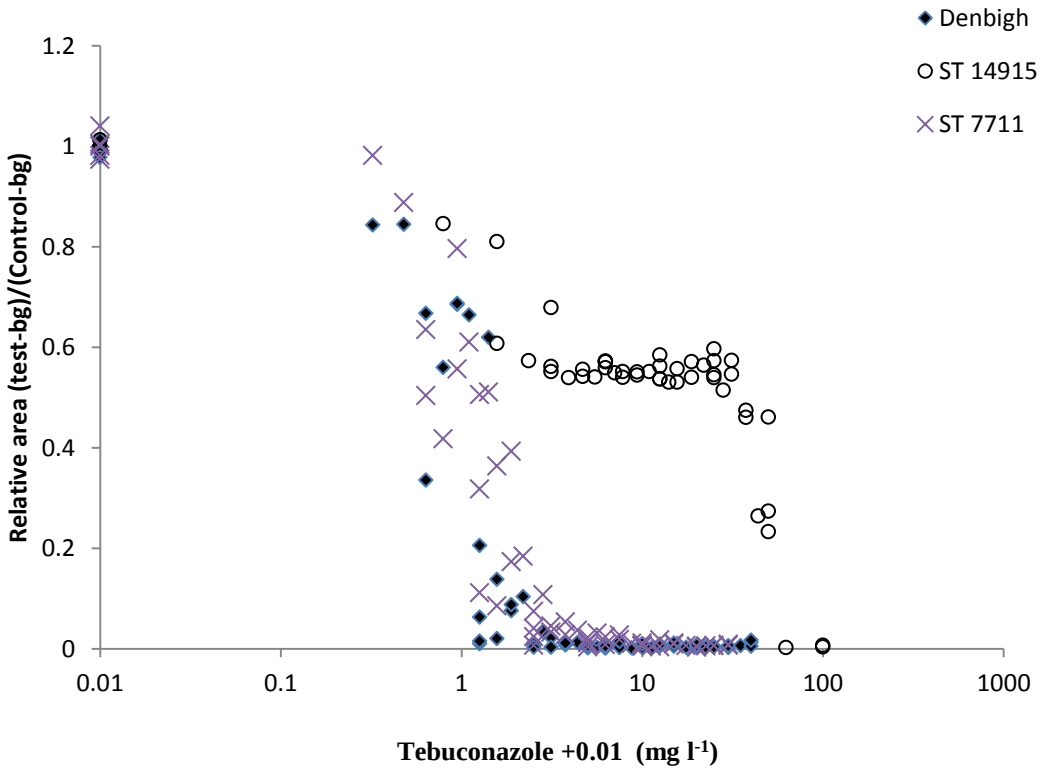

Figure 3.37 Effect of tebuconazole on the visible growth of three isolates of *S. chartarum*: Denbigh - filled diamonds; 14915 - open circles; 7711 – crosses.

The standard LPM could not be used with the data for strain 14915 as it shows a biphasic inhibition pattern. The MIC is estimated from the graph to be 100 mg l^{-1} tebuconazole.

Table 3.9 Modelled parameters for the effect of tebuconazole on *S. chartarum* isolates 7711 and 14915.

Strain	Parameter	Estimate	ApproxStdErr	LCL	UCL	MIC	NIC
Denbigh	P1	1.080	0.055	0.971	1.192	1.978	0.382
	P2	1.652	0.238	1.228	2.269		
	SSE	DFE	MSE	RMSE			
	0.7052	63	0.0112	0.106			
7711	P1	1.331	0.0611	1.212	1.456	2.785	0.179
	P2	1.354	0.139	1.102	1.664		
	SSE	DFE	MSE	RMSE			
	0.4363	60	0.0075	0.0867			

Units: P1, MIC, NIC: mg l^{-1}

P_1 is the concentration of the preservative at $fa = 1/e$, ($e = 2.7182..$) and P_2 is a slope parameter. LCL: lower confidence level, UCL: upper confidence level, MIC: minimum inhibitory concentration, DFE: degree of freedom, SSE: sum of squares, MSE: mean squared error, RMSE: root-mean-square error.

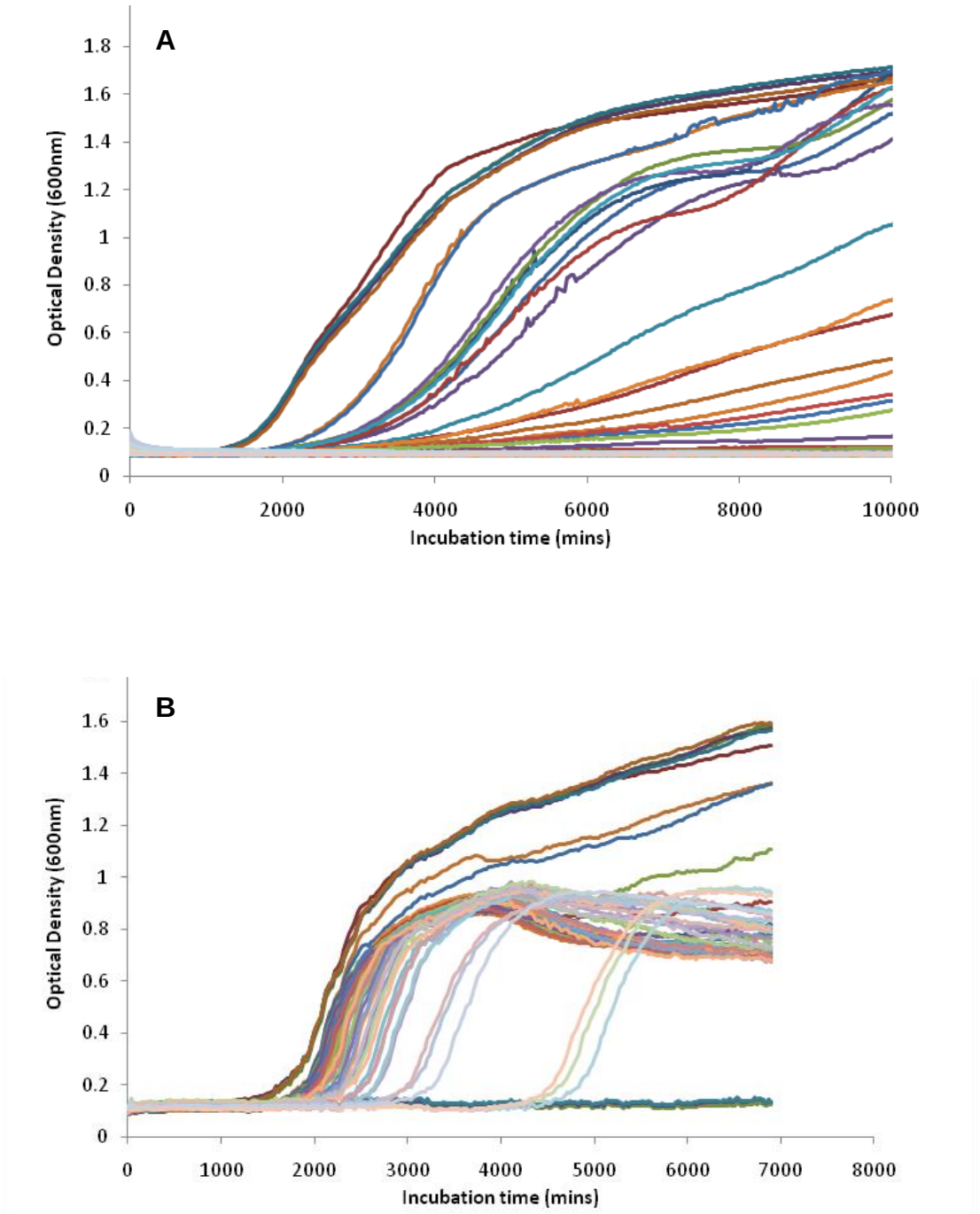

Figure 3.38. Optical density/incubation time plots for *S. chartarum* isolates (A) Denbigh and (B) 14915 in the presence of tebuconazole.

3.11.1 Butylated hydroxy anisole

The OD/time profiles for all the strains were generally similar. An analysis of the AUC data using the LPM suggested that the Denbigh and 7711 strains were similar in their response to BHA, but that 14915 gave a different profile. However, the variability in the Denbigh data was quite high and this is reflected in an RMSE value approximately 3 times as high as the other two fits (Figure 3.39). Table 3.10 gives the parameters obtained from the fitting procedure.

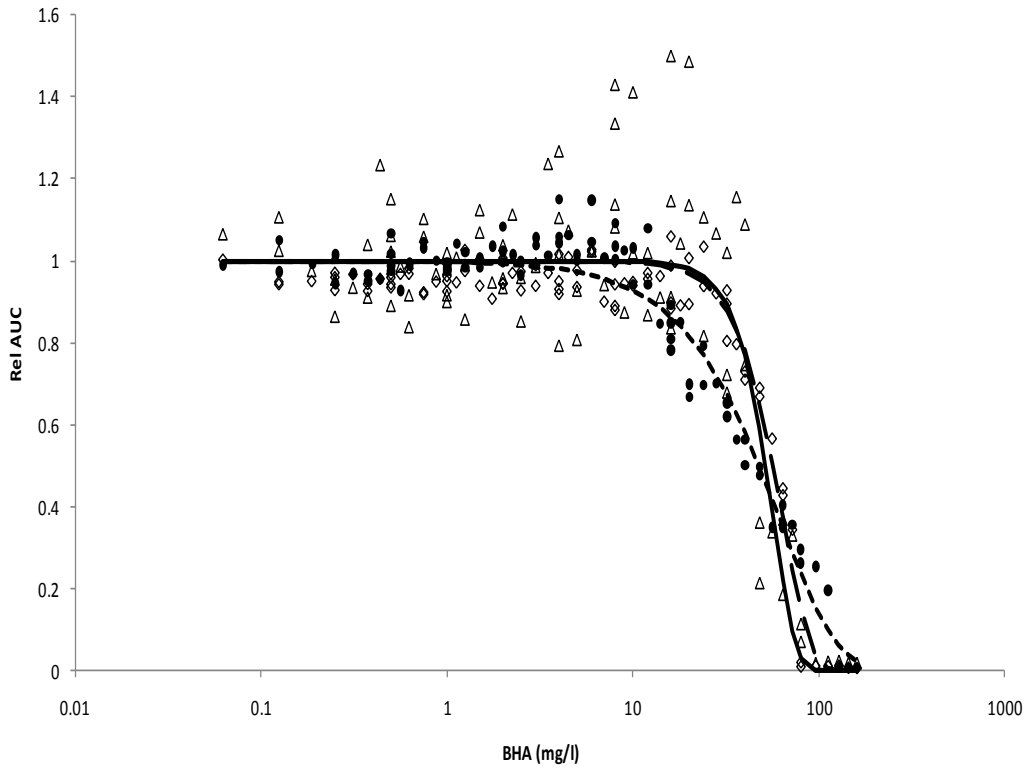

Figure 3.39. Effect of BHA on the visible growth of three isolates of *S. chartarum*: Denbigh - open triangles; 14915 – filled circles; 7711 – Open diamonds. Fitted models are Denbigh – solid line; 14915 long dashed; 7711 – short dashed.

Table 3.10 Modelled Parameters for the effect of BHA on three isolates of *S. chartarum*.

Strain	Parameter	Estimate	ApproxStdErr	LCL	UCL	MIC	NIC
7711	P1	64.173	1.245	61.776	66.744	90.116	35.808
	P2	2.945	0.214	2.555	3.415		
	SSE	DFE	MSE	RMSE			
	0.2952	92	0.0032	0.0566			
14915	P1	61.762	2.088	57.722	66.231	124.963	18.400
	P2	1.419	0.076	1.294	1.561		
	SSE	DFE	MSE	RMSE			
	0.3495	93	0.0038	0.0613			
Denbigh	P1	57.183	2.913	45.849	64.707	74.707	36.123
	P2	3.741	0.781	2.656			
	SSE	DFE	MSE	RMSE			
	2.3261	93	0.0250	0.1581			

Units: P1, MIC, NIC: mg l^{-1}

P_1 is the concentration of the preservative at $fa = 1/e$, ($e = 2.7182..$) and P_2 is a slope parameter. LCL: lower confidence level, UCL: upper confidence level, MIC: minimum inhibitory concentration, DFE: degree of freedom, SSE: sum of squares, MSE: mean squared error, RMSE: root-mean-square error.

3.11.2 Propyl gallate

An analysis of the AUC data using the LPM suggested that isolates 7711, 14915 and Denbigh were similar in their response to propyl gallate, with the MIC of the Denbigh isolate less than that obtained for isolates 14915 and 7711 (Figure 3.40). Table 3.11 gives the parameters obtained from the fitting procedure.

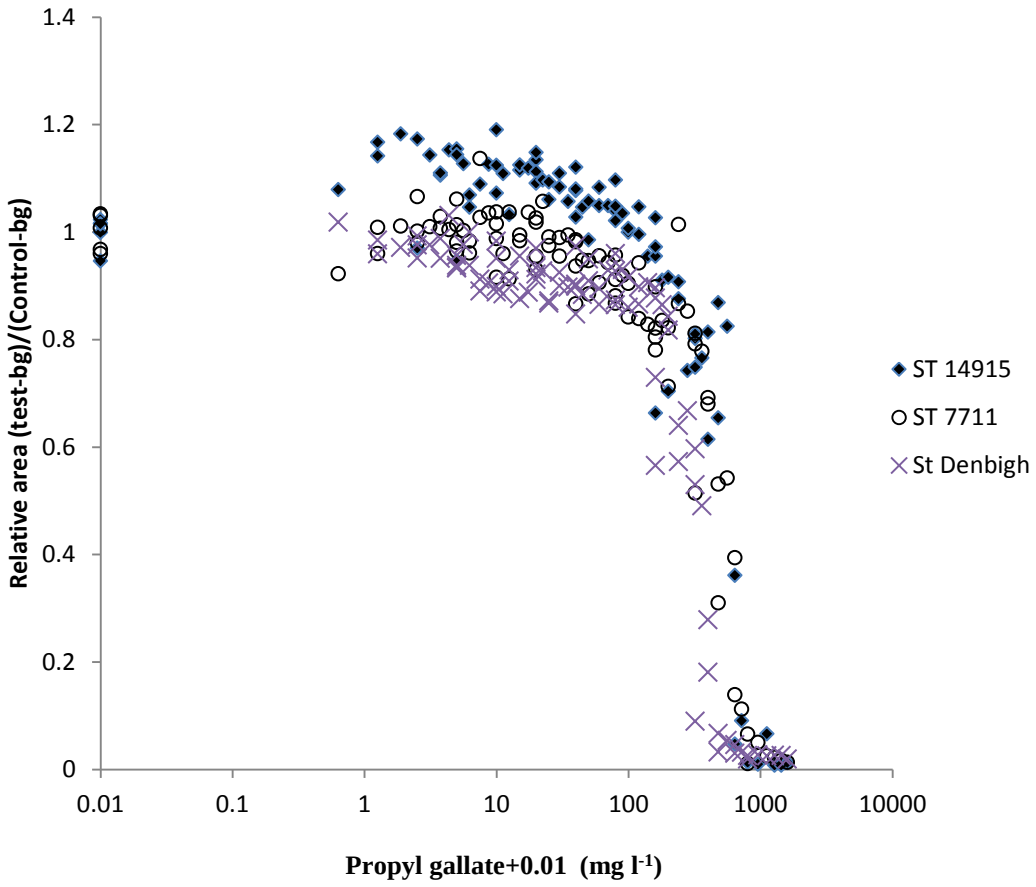

Figure 3.40 Effect of propyl gallate on the visible growth of three isolates of *S. chartarum*: 14915-filled diamonds; 7711 - open circles; Denbigh – crosses.

Table 3.11 Modelled parameters for the effect of propyl gallate on three isolates of *S. chartarum*.

Strain	Parameter	Estimate	ApproxStdErr	LCL	UCL	MIC	NIC
7711	P1	563.5	19.9	528.1	603.8	986.1	215.4
	P2	1.79	0.13	1.52	2.13		
	SSE	DFE	MSE	RMSE			
	0.518	93	0.0056	0.0746			
14915	P1	605.2	23.3	561.4	655.5	863.6	328.4
	P2	2.81	0.37	2.12	4.02		
	SSE	DFE	MSE	RMSE			
	1.130	88	0.0128	0.113			
Denbigh	P1	349.6	12.8	326.3	376.2	586.8	143.5
	P2	1.93	0.16	1.59	2.38		
	SSE	DFE	MSE	RMSE			
	0.645	88	0.0073	0.0856			

Units: P1, MIC, NIC: mg l^{-1}

P_1 is the concentration of the preservative at $fa = 1/e$, ($e = 2.7182..$) and P_2 is a slope parameter. LCL: lower confidence level, UCL: upper confidence level, MIC: minimum inhibitory concentration, DFE: degree of freedom, SSE: sum of squares, MSE: mean squared error, RMSE: root-mean-square error.

3.11.3 Silver ion

Solutions of silver nitrate above 10 mg l^{-1} show a slight increase in the initial OD. The increase was deemed not to affect the analyses significantly. The OD/time profiles for all the strains were generally similar. An analysis of the AUC data using the LPM suggested that the Denbigh and 7711 isolates were similar in their response to silver nitrate, but that 14915 gave a different response (Figure 3.41). Table 3.12 gives the parameters obtained from the fitting procedure.

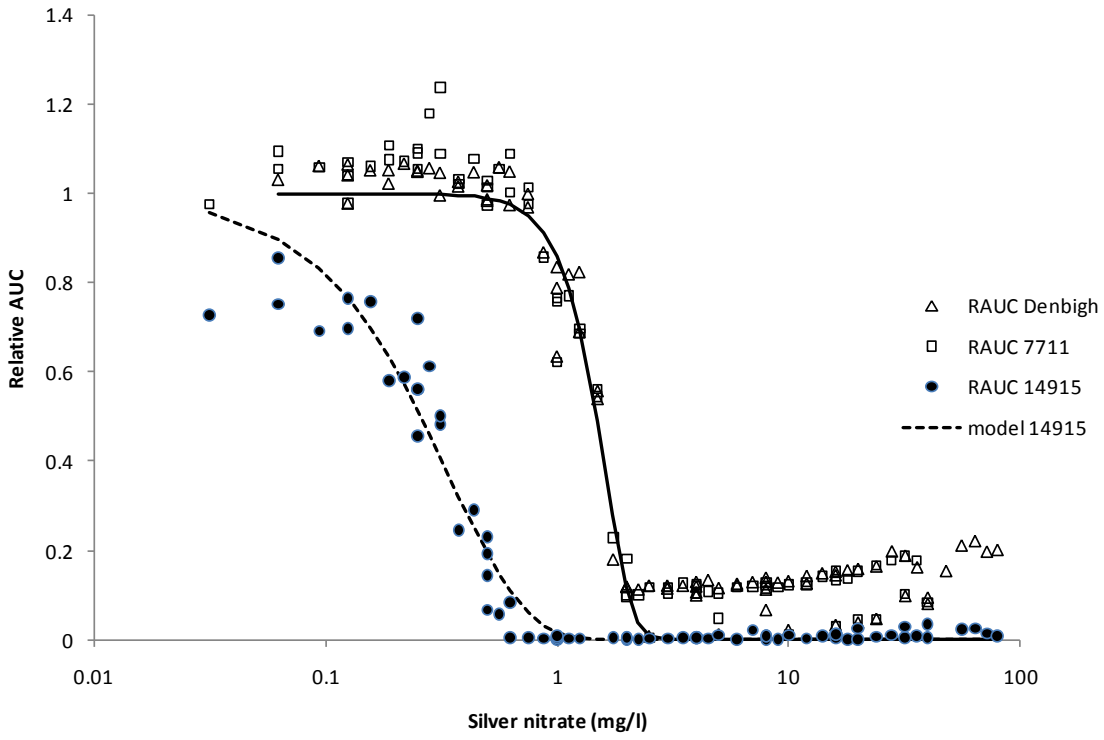

Figure 3.41 Effect of silver nitrate on the visible growth of three isolates of *S. chartarum*: Denbigh - open triangles; 14915 – filled circles; 7711 – open squares. Fitted models are Denbigh – solid line; 14915 dashed.

Table 3.12 Modelled parameters for the effect of silver nitrate on three isolates of *S. chartarum*

Strain	Parameter	Estimate	ApproxStdErr	LCL	UCL	MIC	NIC
7711	P1	1.635	0.040	1.560	1.715	2.176	1.001
	P2	3.499	0.332	2.965	4.184		
	SSE	DFE	MSE	RMSE			
	0.352	63	0.0056	0.0747			
14915	P1	0.341	0.010	0.322	0.360	0.731	0.092
	P2	1.310	0.077	1.158	1.487		
	SSE	DFE	MSE	RMSE			
	0.268	84	0.0032	0.0565			
Denbigh	P1	1.642	0.036	1.573	1.715	2.136	1.044
	P2	3.796	0.352	3.179	4.607		
	SSE	DFE	MSE	RMSE			
	0.268	53	0.0051	0.0712			

Units: P1, MIC, NIC: mg l^{-1}

P_1 is the concentration of the preservative at $fa = 1/e$, ($e = 2.7182..$) and P_2 is a slope parameter. LCL: lower confidence level, UCL: upper confidence level, MIC: minimum inhibitory concentration, DFE: degree of freedom, SSE: sum of squares, MSE: mean squared error, RMSE: root-mean-square error.

3.12 Determination of the effect of antioxidants on the growth rate of *S. chartarum* on building materials (Preliminary experiment)

A preliminary experiment was conducted to determine the best conditions for measuring fungal growth on plasterboard samples. In this experiment two different procedures were used. Box 1 contained dry plasterboard samples and box 2 contained plasterboard samples that had been pre soaked in sterile water for 5 minutes (to simulate a flooding event). 100 μl of a 1 x 10^6 spore ml^{-1} solution of *S. chartarum* isolates 7711 and 14915 were spread over the appropriately labelled plasterboard samples and incubated at 25°C for 21 days. Results showed growth only occurred on pre water saturated plasterboard. (Plate 3.18).

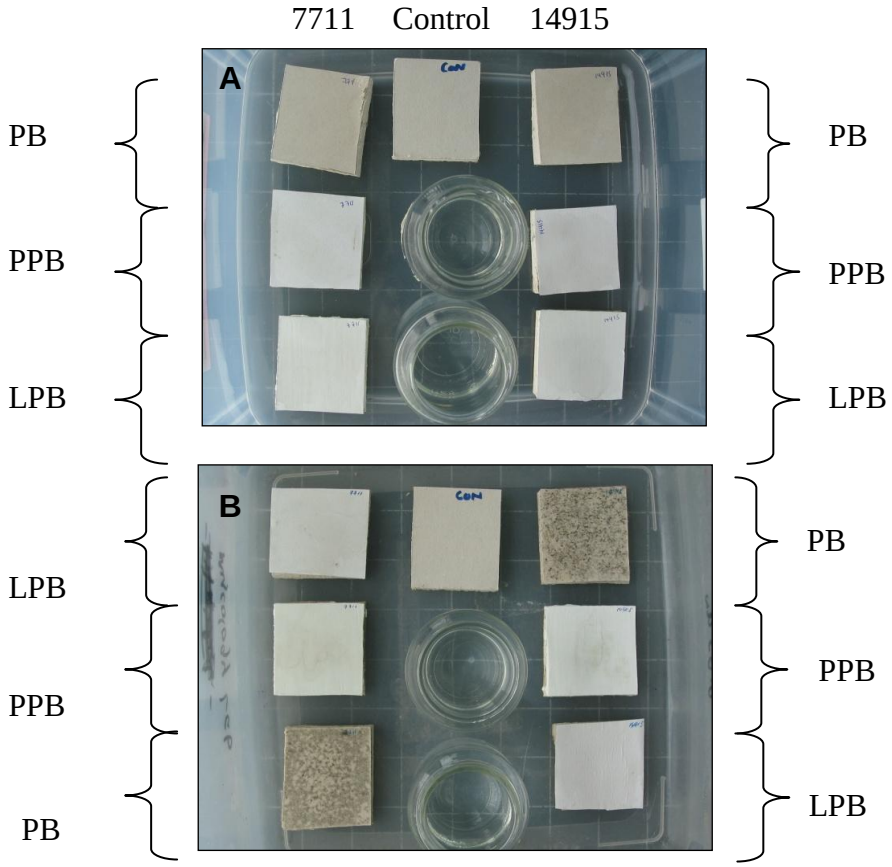

Plate 3.18 The effect of water saturation of plasterboard samples on the growth of *S. chartarum* isolates after 21 days at 25°C and 0.99a_w. (A) Plasterboard samples unsaturated with water before inoculation, (B) Plasterboard samples saturated with water before inoculation. PB = Plasterboard, PPB = Painted plasterboard, LPB =Lining papered plasterboard.

3.13 Determination of the effect of antioxidants on the growth of *S. chartarum* on building materials

The BHA concentrations used on the plasterboard samples showed no significant effects on the growth of the *S. chartarum* isolates with a combination of water activities (0.96 and 0.99 a_w) and temperature of 25^oC after 40 days incubation (Plate 3.19 and Figures 3.42 and 3.43). Chamber temperature, however, significantly affected the growth of S. *chartarum* IBT 7711 particularly on PB and LPB surfaces at 15^oC incubation (Figure 3.42 C and D). Isolate IBT 14915 was not affected under these same conditions and grew well on all of the tested surfaces (Figure 3.43 C and D). Both isolates generally grew best on PPB under all test conditions and LPB was found most inhibitory on *S. chartarum* growth.

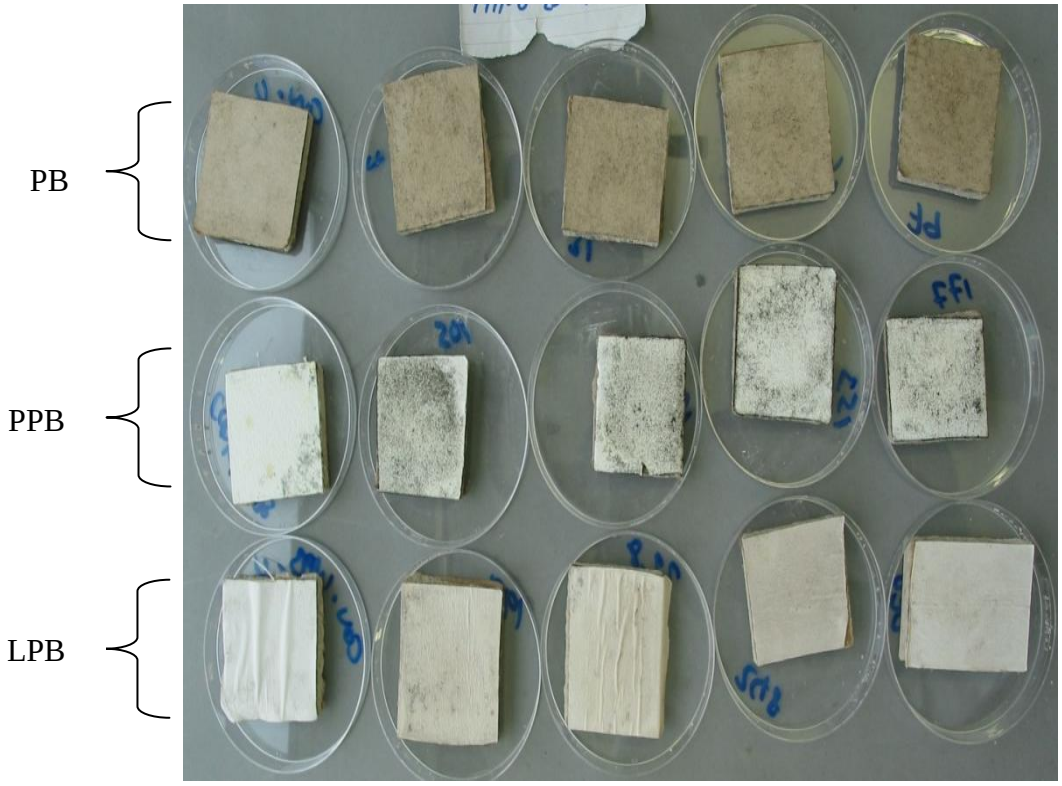

Plate 3.19 *S. chartarum* 7711 growth on plasterboard samples treated with BHA after 40 days incubation. Chamber humidity at 0.99a_w and 25^oC. Left to right: Control, 0, 10, 50 and 200mg l^{-1}.

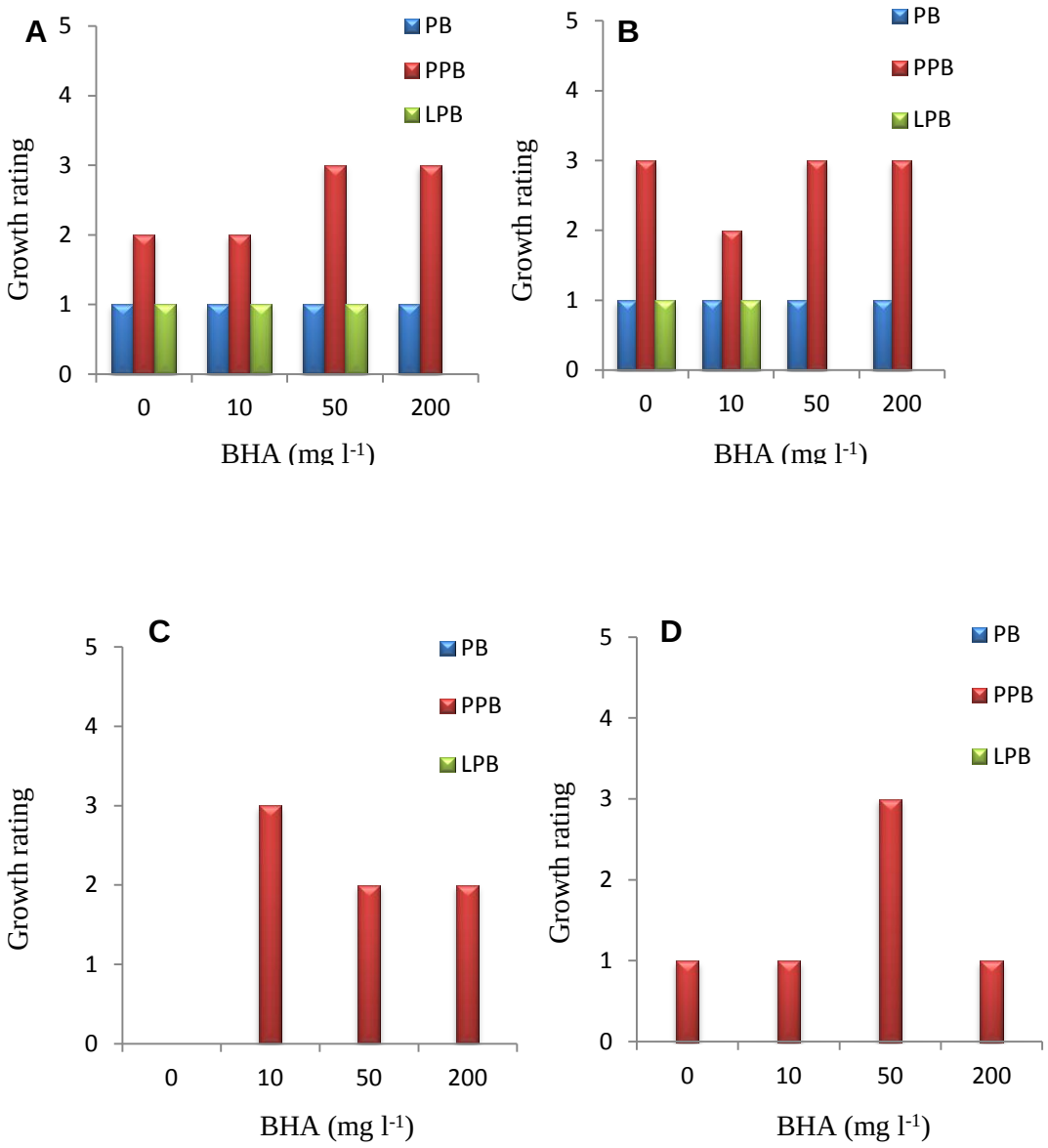

Figure 3.42 Effect of temperature, water activity and BHA of the growth of *S. chartarum* 7711 on Plasterboard (PB) Painted plasterboard (PPB) and Lining papered plasterboard (LPB) samples after 40 days incubation. (A) 0.99a_w and 25°C (B) 0.95a_w and 25°C (C) 0.99a_w and 15°C (D) 0.95a_w and 15°C. (0-5) growth rating (0 no growth- 5 extensive growth)

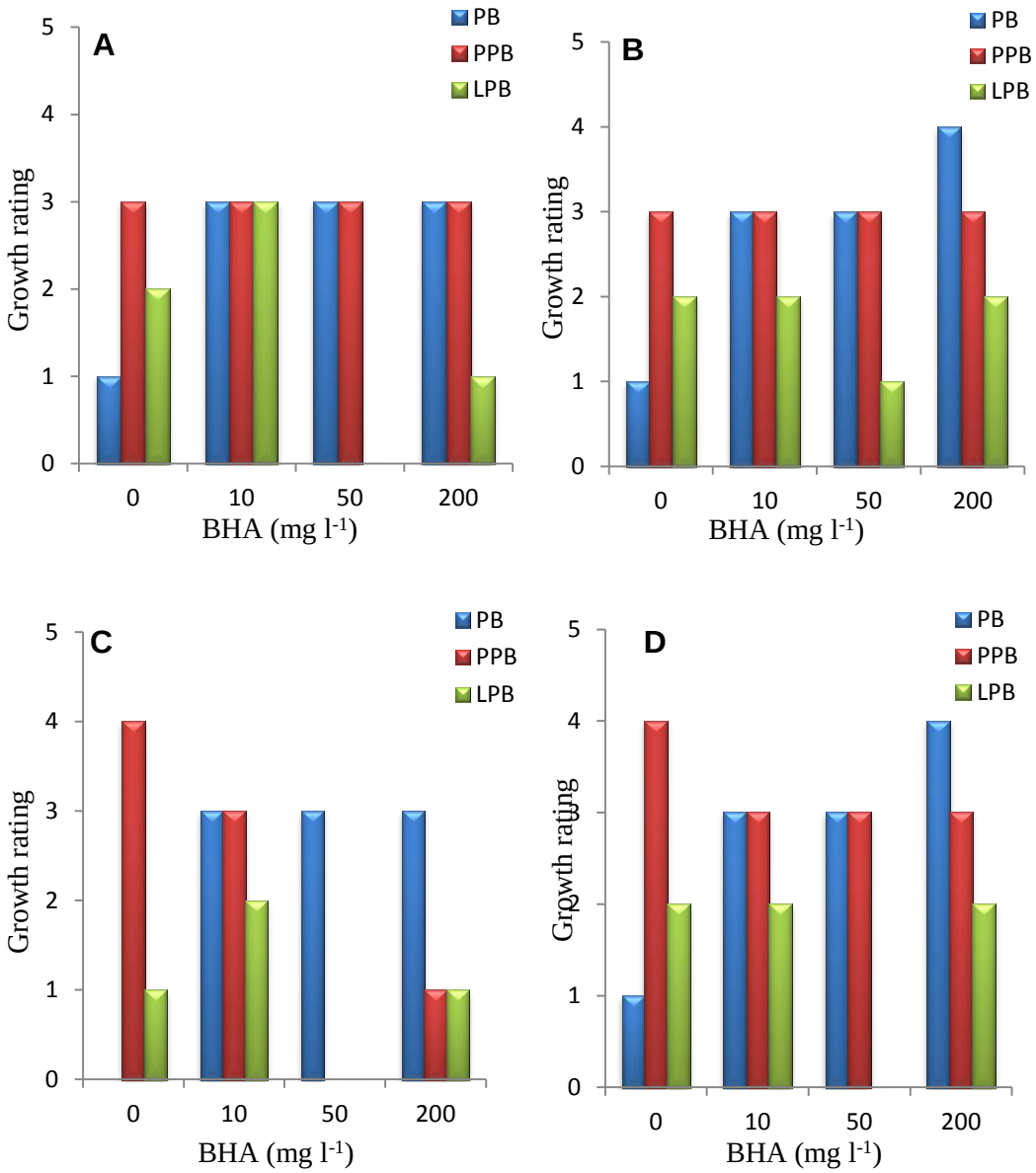

Figure 3.43 Effect of temperature, water activity and BHA of the growth of *S. chartarum* 14915 on plasterboard (PB) painted plasterboard (PPB) and lining papered plasterboard (LPB) samples after 40 days incubation. (A) 0.99a_w and 25°C (B) 0.95a_w and 25°C (C) 0.99a_w and 15°C (D) 0.95a_w and 15°C. (0-5) growth coverage (0 no growth- 5 extensive growth)

3.14 Determination of the effect of butylated hydroxyanisole in matt emulsion paint on the growth rate of *S. chartarum* on building materials.

In the previous experiment the maximum concentration of BHA added directly to the plasterboard samples was ineffective at controlling fungal growth therefore a small experiment was devised which involved the addition of BHA directly to the paint before being spread over selected plasterboard samples. In this experiment higher concentrations of BHA (1000, 5000 and 10,000 mg l^{-1}) were added to the paint. Results revealed concentrations of 10,000 mg l^{-1} completely inhibited the growth of all isolates on the supplemented painted plasterboard samples (Figure 3.44 and Plate 3.20).

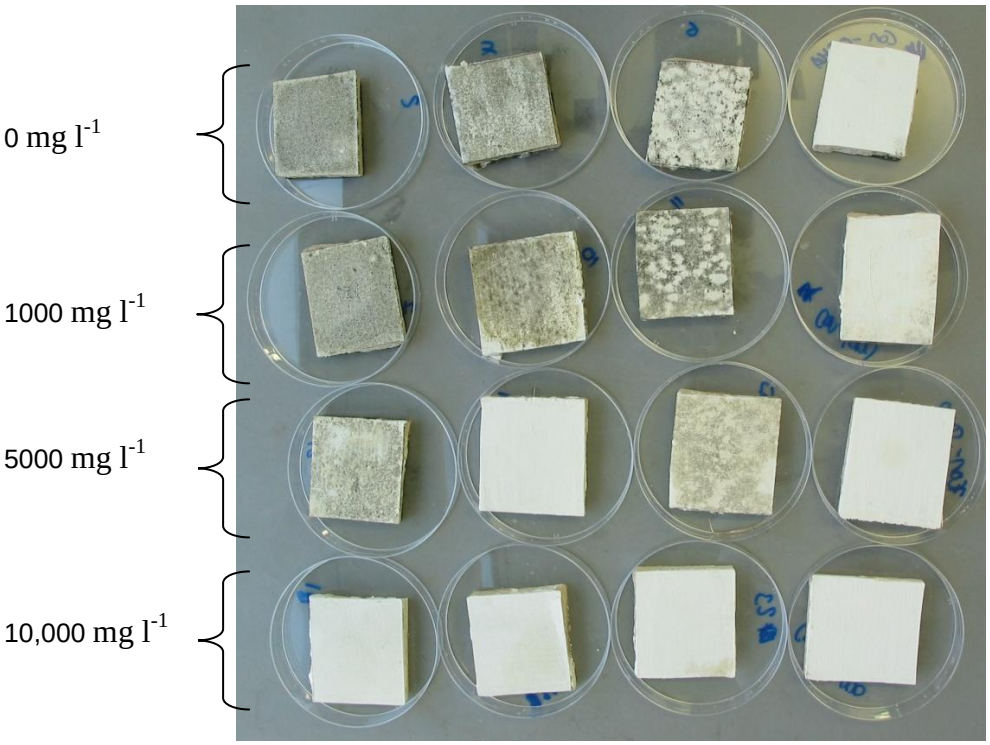

Plate 3.20 The effect of BHA in paint, Top to bottom: (0, 1000, 5000 and 10000 mg l^{-1}) on the growth of *S. chartarum*. Across: 7711, 14915, Denbigh and negative control, after 30 days incubation at 25°C and 0.99a_w.

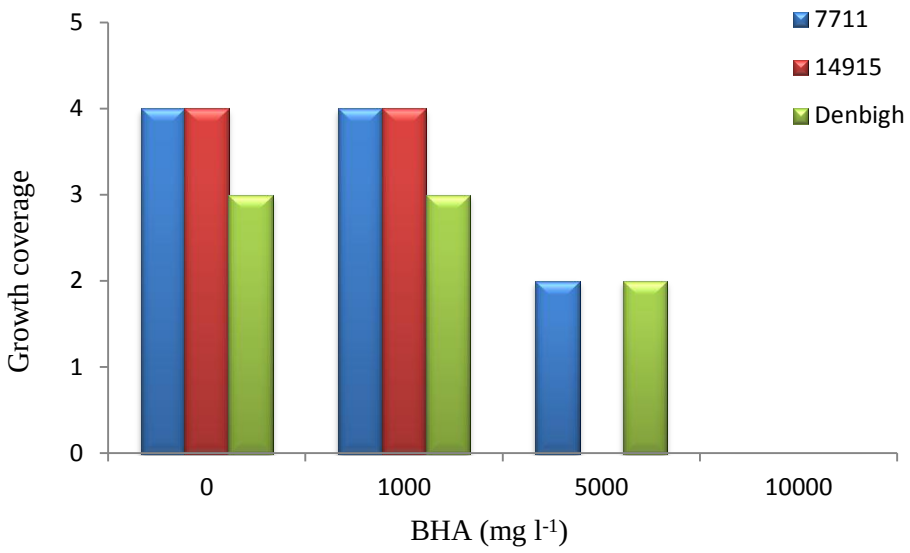

Figure 3.44 Level of *S. chartarum* growth coverage observed after 30 days on BHA treated painted plasterboard samples at 0.99a_w and 25°C. (0-5) growth coverage.

4 DISCUSSION

4.1 Overall summary of survey work

Sampling methods

There are currently no universally accepted methods for determining the level of fungal contamination in buildings; therefore the mini survey performed using various methods was timely and important. The sampling methods used for the analysis of fungi in the locations surveyed showed varied levels of effectiveness.

The air sampler, in conjunction with selective media, gave an indication of the levels and range of fungal conidia within the air of the dwelling under investigation. No *Stachybotrys* sp were detected in any of the surveyed locations when this method was applied. This is in line with a report by Miller et al (2003) who reported that up to 90 percent of field-collected *S. chartarum* spores cannot be cultured. So a true representation of the level of possible *S. chartarum* conidia in air may have been overlooked as a result. Another important factor which may have attributed to the low levels of detection could be due to the fact that *S. chartarum* produce wet slimy spores that are not designed to be transported by air, so unless the cultures were old and dry they would not have been able to release spores in to the air. In these cases the old dry spores are usually non viable and are not culturable.

Swab sampling proved to be a useful sampling tool as samples taken from many surfaces in affected areas could undergo further testing in the laboratory environment without the need for destructive sampling which is not always possible. A problem encountered with this method was the occurrence of high levels of yeast and bacterial

growth from swab samples plated on media. Chilling the samples during transit may have prevented this from occurring.

Contact plates were found to be a poor choice for the determination of fungal species in heavily contaminated buildings although they may be useful in buildings where there is no visible contamination on apparently clean surfaces. In theory these plates seemed an easy method as they required no further laboratory work after sampling but the resultant fungal growth on these plates was very difficult to interpret as most plates produced confluent growth from a very early stage during the incubation period, or dominant, fast growing species masked other slow growing ones.

The collection of bulk samples was found to be the most useful method for investigation as, on further analysis in the laboratory, it showed how associated fungi can gron on these materials.

The chosen media used for the surveys varied in their effectiveness. MEA proved to be the most useful media which was able to support the growth of a large range of fungal species when used in conjunction with the various sampling methods. Detected organisms included *Penicillium, Cladosporium, Alternaria, Eurotium, Trichoderma* and *S. chartarum*. PDA with the addition of chloramphenichol and miconazole proved to be more selective and supported mainly *Cladosporium* sp. Most notably, *Penicillium* species were largely inhibited on this medium although some *Penicillium* species were also supported on some occasions. *S. chartarum* growth was also supported on this medium but grew slightly slower on this medium than on MEA.

CA was found to be a very poor media and did not readily support the growth of any species. It did however, very poorly, support the growth of some *Cladosporium* sp. This

rich cellulose medium was initially chosen to grow selectively for *S. chartarum* as suggested by Chapman (2003) but laboratory analysis using this media proved fruitless as *S. chartarum* isolates showed only slight mycelia growth on this medium and failed to sporulate in comparison to MEA and PDA (Plate 4.1).

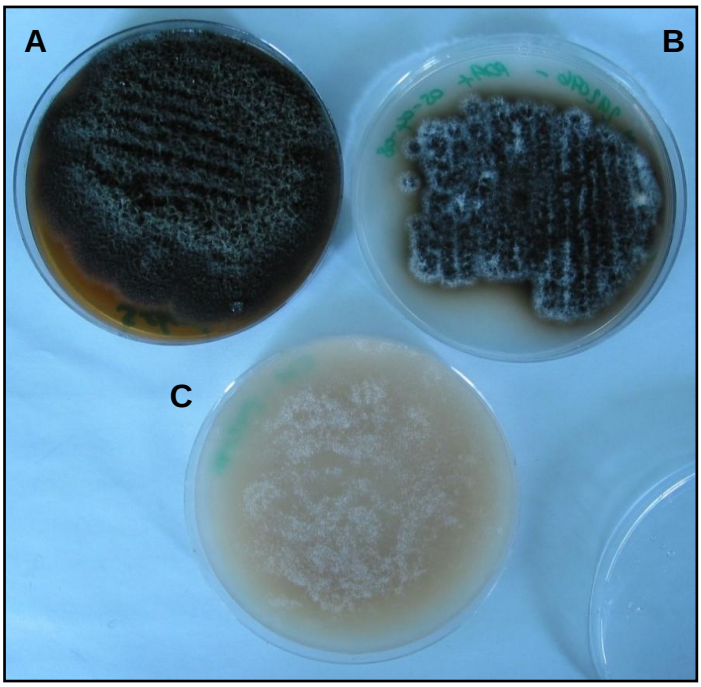

Plate 4.1 Growth of *S. chartarum* isolate 7711 after 10 days incubation at 25°C and 0.99a_w (A) MEA, (B) PDA, (C) CA.

Survey

From the locations surveyed it was determined that most were affected by mould due to condensation or ventilation problems. The main organisms found within these premises included *Penicillium* and *Cladosporium* spp which is in line with most reports within the literature (Pasanen *et al.*, 1992; Ren *et al.*, 2001; Green *et al.*, 2003). The extensively water damaged, partially derelict hospital in Denbigh showed a much wider range of fungal sp with *Stachybotrys sp* being predominant on the wallpaper in certain

areas. Plated samples of fungal infested wallpaper revealed the presence of *Stachybotrys* sp. Gravesen (1979) also investigated the level of fungal contamination in water damaged buildings and found most materials were susceptible to fungal contamination especially those with biodegradeable cellulose components. Comparison of the counts of the fungal species detected in the surveyed locations revealed that there was an increase in both numbers and diversity as the severity of water damage increased. A similar observation was described by Andersson et al (1997) who reported on the presence of *Stachybotrys chartarum* as significant colonizers in water-damaged buildings along with *Penicillium* and *Aspergillus* species. Air and swab samples taken at both Marsh Farm and Henson Close, where ventilation/ condensation problems were observed, showed relatively low CFU counts compared to the high CFU counts obtained from the extensively water damaged Victorian stable block. (Figure 3.15). This was also evident from the bulk samples taken from various locations.

4.2 Influence of temperature and a_w on the germination and germ tube extension of *S. chartarum in vitro* on PDA

Germination and germ tube extension were significantly affected by water activity and temperature. Optimal germination rates occurred across all temperatures with freely available water with 100% germination of conidia within 24 hours for both *S. chartarum* isolates 7711 and 14915. Water activity proved to have a significant effect on the rate of germination and substantially decreased at $0.95a_w$. Germination also occurred at 37^oC with freely available water along with slight germ tube extension, but no further mycelia growth was observed (Figures 3.16 and 3.17). These results are comparable to those reported by Ayerst (1969) who determined optimal conditions for the germination of *S. atra* (as *S. chartarum* was then named) conidia at 30^oC and >0.95 a_w on malt agar.

High rates of germ tube extension occurred when water was freely available between 20 and 30^oC for both isolates. When water stress was imposed the rate of germ tube extension decreased significantly. This was also observed at temperatures above 30^oC, a phenomenon also reported by Ayerst (1969) with *Aspergillus* sp. Pardo et al (2005a; 2005b) investigated the effect of water activity and temperature on conidial germination and mycelial growth of ochratoxigenic *Penicillium verrucosum* on barley meal extract agar and also found a significant effect. In these studies strains with the lowest germination rates showed the slowest growth rates and both germination and growth were significantly affected by a_w.

There are limited reports in the literature regarding the effects of water activity and temperature on the germination and germ tube extension rates of *S. chartarum* isolates.

This information is important and shows that *S. chartarum* is able to rapidly germinate over a wide range of temperatures including those typical of occupied buildings. In the inevitable event of flooding UK buildings potentially have a heightened risk of infestation by *S. chartarum* which may persist for long periods of time.

4.2.1 Influence of temperature and a_w on the mycelia growth rate *in vitro* on PDA.

The minimum conditions in which both *S. chartarum* isolates 7711 and 14915 could actively produce mycelial growth *in vitro* was 0.95 a_w and 15^oC on PDA. At higher a_w levels between 0.98 and 0.99a_w mycelial growth was observed at a slightly lower temperature of 10^oC (Figure 3.22). Previously Ayerst (1969) reported the minimal a_w for mycelia growth at 0.94 a_w with an optimal temperature of 23^oC for a *Stachybotrys atra* (as *S. chartarum* was then named) isolate from the U.S Army.

These results show that the various abiotic factors in combination have a significant effect on the growth capabilities of *S. chartarum* species with water availability being a key factor. Both isolates displayed optimal growth rates at 25^oC and 0.99$a_{w,}$ growth rates decreased significantly at 37^oC. This is in line with reports by Grant *et al* (1989) who determined an optimal temperature of 30^oC for growth of *S. atra* isolates.

Both isolates displayed signs of slight mycelial growth at 0.92 a_w but this was regarded as initial germ tube extension as no further mycelia growth was determined after the incubation period (Figure 3.22). The results further strengthen the case for concern over the possible growth of *S. chartarum* in water damaged buildings and demonstrate that after the event of flooding this organism may still persist despite fluctuations in UK seasonal temperatures.

4.2.2 Influence of temperature and a_w on the sporulation of *S. chartarum* isolates *in vitro* on PDA

The spore production of *S. chartarum* isolates, grown *in vitro* on PDA, showed a significant effect of temperature and water availability. Both isolates 7711 and 14915 showed spore production between 15 and 30^{o}C with freely available water. Mycelial growth of both isolates was reduced by almost 70–80% at 0.95 $a_{w,}$ However, spore production was still at the levels that occur with freely available water which was an interesting finding. This suggests that spore production may occur during periods of stress as a means of survival. No conidia were produced by either of the tested isolates at 10^{o}C over the incubation period with freely available water which demonstrated that these organisms require higher temperatures for spore production along with high levels of water availability. These are significant findings and should have implications for remediation of damp buildings and surfaces and suggests that aerosols of such spores could represent a health hazard under a relatively wide range of a_w and temperature conditions (Figure 3.24).

There is very little information regarding the effect of water activity and temperature on spore production by strains of *S. chartarum*. The exception is the study by Harrington (2002) who observed fast growth and sporulation on potato dextrose, cornmeal, rice extract, and 2% oatmeal agars by 10 *S. chartarum* isolates on 18 fungal growth media under constant light, alternate light/dark, and constant dark for 10 days at 23-24 ºC.

4.3 Determination of the extracellular enzyme production by *S. chartarum* isolates.

Quantitative analysis, using the APIZYM test kit, of the extracellular enzyme production by isolates of *S. chartarum* grown in PEB at 25^{o}C for six days revealed the production of phosphate alkaline, esterase, esterase lipase, acid phosphatase and napthol-AS-BI-phosphohydrolase at varying levels. Similar results were reported by Nabrdalik (2007) who investigated extracellular enzyme expression on building materials by *Stachybotrys, Cladosporium* and *Alternaria* species. Saibi *et al* (2010) investigated the extracellular enzyme production by *Stachybotrys microspora* and showed β−glucosidase activity at varying quantities depending on the carbon food source source. Picart *et al* (2008) reported on extracellular enzyme activity by *Stachybotrys* isolates and found non tricothecene producing isolates produced high levels of cellulases in mineral media supplemented with rice straw. Tweddell *et al* (1994) investigated chitinase and β-1,3-glucanase production by *Stachybotrys elegans* and also reported a relationhip between expression and the presence of carbon. Most of the reports in the literature state that many cellulytic organisms including *Stachybotrys chartarum* regulate the expression of extracellular enzymes by a process known as catabolite repression which allows organisms to quickly adapt to a preferred carbon source e.g glucose which requires less energy. In this process catabolic enzymes are switched on and off dependent on the carbon source (Morissette *et al.*, 2006). Therefore the extracellular enzyme activity demonstrated by *S. chartarum* isolates 7711, 14915 and Denbigh was most probably representative of the choice of growth medium used. Further experimentation using the same media supplemented with cellulose may have yielded a wider range of extracellular enzymes. Water activity was found to

significantly affect the extracellular enzyme production by the tested isolates. The highest level of extracellular enzyme production was observed at $0.99a_w$. This was dramatically reduced at $<0.95a_w$ (Figure 3.26). Alam *et al* (2009) reported similar effects of water activity on the extracelluar enzyme activity of *Aspergillus* species. These findings coincide with fact that *S. chartarum* growth is limited at lower a_w levels which would imply that low extracellular enzyme expression hinders the organisms ability break down complex substrates necessary for growth.

Analysis of the extracts in potassium phosphate using specific substrates attached to the indicator nitrophenyl proved inconclusive as no enzyme production was detected for any of the isolates. Potassium extracts fom PDA plates modified to $0.99a_w$ were thus analysed using the APIZYM test kit. Results revealed time was a contributory factor in the levels of extracellular enzymes produced by the tested isolates. Highest levels were observed by isolates 7711 and Denbigh after 48 hours of growth on PDA at 25^oC, this dramatically decreased after 120 hours. A delay in the production of extracellular enzymes was observed by isolate 14915 with low levels of extracellular enzyme production after 96 hours incubation at the same temperature (Figure 3.27). This implies that different strains of *S. chartarum* are able to adapt to their environment better than others by secreting extracellular enzymes necessary for growth. This could present significant future health risk to those affected by water damage as the macrocyclic trichothecene producing isolates seem to quickly adapt in these conditions with much higher levels of extracellular enzymes production than that shown by non macrocyclic trichothecene isolates.

4.4 Determination of haemolytic activity of *S. chartarum* isolates

Haemolytic activity was found to be produced by all of three *S. chartarum* isolates 7711 14915 and field isolate Denbigh, (Plate 3.14). It was interesting that from visual examination the non macrocyclic trichothecene producing isolate 14915 appeared to show the highest haemolytic activity compared to the other two isolates. This was also observed by Yike *et al* (2007) who showed that non macrocyclic trichothecene producing isolates of *S. chartarum* produced more than a 6-fold higher content of stachylysin compared to the macrocyclic trichothecene producing isolates. This is very significant as two thirds of *S. chartarum* isolates detected in water damaged buildings are of the non macrocyclic trichothecene producing type and can represent a major health risk to those exposed (Hinkley *et al.*, 2000). All three isolates also grew on sheep's blood agar at 37^{o}C in yeast like form but at a much slower rate. This is an interesting finding and could signify that *S. chartarum* isolates could be invasive and pathogenic at normal human body temperature, which further strengthens the case of a possible relation with pulmonary haemorrhaging. Piecková *et al* (2009) reported the haematotoxic and inflammation-inducing properties of *S. chartarum* on rats and showed a significantly higher total cell count in bronchoalveolar lavage fluid (BALF) samples indicating inflammation. Stachylysin production was also observed in all of the tested *S. chartarum* isolates in this study and it was reported that this highly immunogenic metabolite has the capabilities of lowering hematocrit, the proportion of blood volume occupied by red blood cells.

4.5 Influence of temperature and a_w on Satratoxin G production by *S. chartarum* isolates.

Water activity and temperature showed a significant effect on the levels of satratoxin production by both isolates. Isolate 7711 showed optimal production of SG at 20^{o}C at 0.98a_w. This was interesting as it was neither at the optimum growth temperature or water activity previously determined for this isolate. Isolate 14915 showed optimal SG production at 25^{o}C and 0.98a_w which was also not the optimal growth condition previously determined for this isolate (Figure 3.29). Toxin production by these strains was previously investigated by Andersen *et al* (2002) who determined strain 7711 to be a tricothecene producer and 14915 to be a non trichothecene producer. The satratoxin G ELISA assay determined that both isolates were only able to produce very low levels of satratoxin G at 30^{o}C which was very interesting especially between 0.98 and 0.99a_w as the level of growth and sporulation was very rapid at this temperature (Figure 3.22 and Figure 3.24). These results are significant and showed that high levels of growth and sporulation of a trichothecene producing isolate does not necessarily equate to high levels of toxin production. Low levels of SG production by these isolates under these conditions may be due to the energy requirements and survival. Nielsen *et al* (1998) measured the satratoxin H production using both isolates 7711 and 14915 on new and old gypsum boards and determined similar results to those found in this investigation. It thus appears that these organisms produce the same comparative levels of similar toxins on different substrates. There has been surprisingly little information on the ecophysiology of SG production by isolates of *S. chartarum*. The available literature in relation to this mould and its relationship with causing human health problems was reviewed by Miller *et al* (2003). They suggested that presence of *S. chartarum* was

probably not the cause of putative neurological damage which was associated with exposure to this mould. Karunasena *et al* (2010) determined satratoxin H was able to cause the activation of inflammatory and apoptopic pathways in human neurological tissues, at levels similar to those found in water damaged buildings. Their findings concluded that neural cells were unable to repair extensive cellular damage from cytotoxic events that induce apoptosis or severe inflammation. The results have shown that the conditions for SG production are much narrower than those for growth in terms of a_w and temperature conditions, especially for the toxigenic strain. The most interesting findings here were that optimum conditions for SG production were different from those for growth (20 vs 25-30^oC, respectively). Production was also limited to about 0.96 a_w in the SG producing strain. Production of SG by isolate 14915 was very low and varied much more with environmental conditions. The contour plots show clearly that no SG toxin is produced at $\geq 30^oC$. This is important and suggests that in damp building environments the risk of exposure to the SG toxin may be much higher at $<25^oC$ and >0.95 a_w. The observation that the non-producer isolate 14915 produced low amounts of SG could reflect the high sensitivity of ELISA method employed in this study. The data for SG production was however more variable and the contour plots reflect this. From the results it was clearly shown that environmental factors have a profound effect on growth and SG production by strains of *S. chartarum*. The optimum a_w and temperature conditions for growth and SG production were shown to be different for the first time. The contour maps developed in this study will be beneficial for determining practically, the conditions which represent (i) risk from growth of *S. chartarum* and (ii) risk from SG production and exposure in damp buildings by this environmentally important fungus (Figure 3.30).

4.6 Determination of the effect of antioxidants on mycelial growth and conidia production on *S. chartarum* isolates on PDA

The antioxidants butylated hydroxyanisole (BHA) and propyl gallate (PG) had varying effects on the growth rate of *S. chartarum* isolates when grown on PDA in optimal conditions. BHA showed antifungal activity against isolates 7711 and 14915 producing MIC values of 200 mg l^{-1} for both isolates (Figure 3.31). At 100 mg l^{-1} conidial production for isolate 7711 was inhibited while isolate 14915 still showed signs of conidia production at >100 mg ml^{-1} (Plate 3.15). PG also showed antifungal activity against isolates 7711 and 14915 with an MIC of 1000 mg l^{-1} for both isolates (Figure 3.32). Both isolate 7711 and 14915 showed complete inhibition of spore production at 200 mg ml^{-1} and 400 mg l^{-1} respectively (Plate 3.16). Fabri *et al* (1997) showed varying levels of effectiveness on strains of *Penicillium, Aspergillus and Stachybotrys* sp using concentration of BHA at 1000 mg l^{-1}. There have been numerous reports on the effects of antioxidants on the fungal species but there is little information on the effects of these compounds on *S. chartarum* isolates. Giridhar and Reddy (2001) reported the effectiveness of BHA on the growth of *Penicillium, Aspergillus and Stachybotrys* sp reporting inhibition at 100 mg l^{-1}. PG was also found to inhibit fungal growth but at much higher concentrations of 1000 mg l^{-1}. Razavi-Rohani and Griffiths (1999) determined the antifungal activity of BHA against various fungal strains with MICs ranging from >12 mg l^{-1} for *Fusarium, Penicillium* and *Aspergillus sp* to 460 mg l^{-1} for *Candida sp*. Chang and Branen (1975) found mycelium growth and aflatoxin production of *Aspergillus parasiticus* was totally inhibited at >250 mg l^{-1} of BHA. Ahmad and Branen (1981) reported that 200 mg $l^{-1}$of BHA in a glucose and salt broth caused 100% growth inhibition of *Penicillium* and *Aspergillus sp.* These results are

very interesting as they show that some antioxidants can be used at low concentrations to control the growth of these toxigenic fungi. It was interesting to find the variability between *S. chartarum* isolates with regards to their spore production when under the influence of the antifungal agents at the same concentration. This is important information with regards to remediation of contaminated environments and demonstrates antioxidants can be useful and safe means of controlling *S. chartarum* growth and sporulation in water damaged buildings.

4.7 Determination of *S. chartarum* growth rates using the Bioscreen C

The Bioscreen was found to be a very effective means of measuring the growth rate of *S. chartarum* isolates in liquid culture. Previously this machine has been used routinely for bacteriological growth measurements but there have been no published reports that it has been used before for *S. chartarum*. Microbial analysis of the growth in each of the inoculated wells of the bioscreen microtitre plate revealed the presence of mycelial growth which was proportional to OD measurement obtained over time. The initial innocula experiment gave very reproducible results and serial dilutions of *S. chartarum* levels across the microtitre plate produced results that could be used as a calibration curve for future experimental results (Figure 3.33).

4.7.1 Determination of the effect of water activity and temperature on growth rate of *S. chartarum* using the Bioscreen

Growth rates of the two *S. chartarum* isolates 7711 and 14915, when analysed using the Bioscreen showed a significant influence of temperature and water activity when grown *in vitro* on PEB. Optimum growth rates were observed at 30^{o}C and 0.99a_w for both isolates. Growth was not determined at 10^{o}C and 37^{o}C for either isolate, nor at 0.887a_w. Note these experiments were only run over a 5 day period and therefore time could have been a factor in the growth rate determination under these conditions (Figure 3.33). These results were very similar to those determined with the same isolates on PDA although optimum mycelia growth on this was determined to be at 25^{o}C and 0.99a_w (Figure 3.34). This indicates that isolates of *S. chartarum* can respond slightly differently to varying growth medium, and experimental set-up.

These results are very significant and show the Bioscreen is a very useful and accurate tool for measuring the effect of water activity and temperature on the growth rates of multiple samples over a much shorter time period than conventional plating methods.

4.7.2 Determination of the effect of tebuconazole and other inhibitors on the growth rate of *S. chartarum* isolates using the Bioscreen

The Bioscreen proved to be very useful in determining the effect of different antifungal agents on the growth rate of *S. chartarum* isolates 7711, 14915 and the field isolate from Denbigh Hospital (Table 4.1). Tebuconazole showed antifungal activity towards *S. chartarum* isolates 7711 and Denbigh with MIC values at levels of 2.8 and 1.9 respectively. MIC values obtained for isolate 14915 were not calculated but estimated at levels of 100 mg l^{-1} as the growth pattern was found to be biphasic under this antifungal

agent. BHA showed antifungal activity towards *S. chartarum* isolates 7711, 14915 and Denbigh with MIC values at levels of 90.1, 124.9 and 74.7 mg l^{-1} respectively. Propyl gallate showed antifungal activity on *S. chartarum* isolates 7711 14915 and Denbigh but with less effect. MIC values determined were at levels of 986.1, 863.6 and 586.8 mg l^{-1} respectively. Silver nitrate showed antifungal activity on *S. chartarum* isolates 7711 14915 and Denbigh with MIC values at levels of 2.1, 0.7 and 2.1 mg l^{-1}. These results for BHA and PG were similar to those determined on PDA and show that the Bioscreen is a very useful and rapid tool that can be used to determine the susceptibility of fungi to antifungal agents. There are no reports on the effects of tebuconazole on *S. chartarum* growth but the antifungal capabilities of this compound have been demonstrated with similar results on numerous fungi. Snelders *et al* (2009) reported the antifungal capabilities of tebuconazone on *A. fumigatus* with MIC values ranging from 2 and >16 mg l^{-1}. Eckert *et al* (2010) investigated the use of tebuconazole against Phoma stem canker, caused by the coexisting related fungal pathogens *Leptosphaeria* genus, and reported complete inhibition of germination of conidia at 4 mg l^{-1}. No reports were found regarding the effect of silver nitrate on *S. chartarum* growth but various reports are available on the effects on various other microrganisms. Xu *et al* (2009) reported the promising activity of silver nitrate against 260 isolates of pathogenic fungi including *Fusarium, Aspergillus* and *Alternaria* species and found 90% inhibition of growth at <2 mg l^{-1}. Woo *et al* (2008) reported the effect of silver ions on *Staphylococcus* and *Eschericia* coli and determined reductions of more than 5 $\log_{10}$ CFU ml^{-1} at 0.2 mg l^{-1}.

Table 4.1 Comparisons of the MICs determined for *S. chartarum* isolates using the Bioscreen.

Antifungal	*S. chartarum isolate*	MIC	NIC
Tebuconazole	7711	2.78	1.79
	14915	-	-
	Denbigh	1.98	0.38
BHA	7711	90.11	35.81
	14915	124.96	18.4
	Denbigh	74.71	36.12
PG	7711	986.1	215.4
	14915	863.6	328.4
	Denbigh	586.8	143.5
Silver nitrate	7711	2.176	1.001
	14915	0.731	0.092
	Denbigh	2.136	1.044

MIC = Minimum inhibitory concentration (mg l^{-1}), NIC = Non inhibitory concentration (mg l^{-1})

To my knowledge, this is the first study to look at the effect of antifungal compounds on *S.chartarum* growth using the Bioscreen. The antifungal agents varied in their level of activity against the tested isolates, variability was also observed between isolates with the same antifungal agent. This was an interesting finding as colonisation of *S. chartarum* on surfaces may persist in the case of more resistant strains. A solution for this could be the use of a combination of antifungal agents to deter infestation of building materials.

4.8 Determination of the effect of water activity, temperature and BHA on the growth of *S. chartarum* isolates on building materials

The concentrations of BHA used as a means to control the growth on building materials showed very little effect in controlling the growth of *S. chartarum* on the plasterboard (PB), lining papered (LPB) and painted plasterboard (PPB) samples. It was assumed that this was due to the composition of the plasterboard samples under investigation which may have absorbed the BHA therefore minimising the antifungal activity on the surface. An alternative explanation could be that the concentrations of BHA were too low to have any significant affect on *S. chartarum* growth. Incubation temperature and chamber water activity to a lesser extent did however have an effect on *S. chartarum* growth. Isolate 7711 proved to be very sensitive to the incubation temperature of 15^{o}C under both of the tested water activity conditions when grown on PB and LPB but grew well on the painted surface under the same conditions (Figure 3.42). Isolate 14915 grew well under all of the tested conditions and proved to be less sensitive than isolate 7711 (Figure 3.43). LPB samples proved to have the greatest resistance to fungal growth followed by the untreated PB samples. The PPB| samples were found to be least effective at inhibiting *S. chartarum* growth and a slight stimulation of growth of *S. chartarum* isolates was observed on these surfaces (Plate 3.19). This may have been due to the nutrient content that is sometimes found in emulsion paints which may have been utilised by the colonising fungi as an organic food source (Becker *et al.*, 1986). These results are in line with reports in the literature that have shown that optimum growth on buildings for *S. chartarum* occurs at 20 to 30°C with freely available water and a water activity <0.96a_w produces less growth of *S. chartarum* (Gravesen *et al.*, 1999; Lillard, 2004). It was however interesting to discover that isolates of *S. chartarum* varied in their

sensitivities to environmental conditions when grown on plasterboard. This can be problematic for the control of this organism after water intrusion in affected buildings. Langhorst *et al* (2006) reported on the complexity involved in controlling the growth of microorganisms on building materials and highlighted the many factors that needed to be considered for a safe and durable treatment method. This report explains the problems associated with the vast majority of antimicrobials that work by leaching or moving from the surface on which they are applied to control microorganisms. Some of these products have proved dangerous as they can contact the skin and potentially affect the normal skin bacteria, cross the skin barrier, and/or have the potential to cause rashes and other skin irritations. They can also allow for adaption and the formation of resistant microorganisms which could be potentially more dangerous. The study also investigated the use of a different and novel antimicrobial technology in the building materials industry which does not leach but instead remains permanently affixed to the surface on which it is applied (Langhorst *et al.*, 2006). This bound antimicrobial technology, an organofunctional silane, was reported to have a mode of action that relies on the chemistry remaining affixed to the substrate thus killing microorganisms as they contact the surface to which it is applied. When BHA was supplemented in paint results showed growth of all three S. *chartarum* isolates were completely inhibited with 10,000 mg l^{-1} of BHA (Plate 3.20). From these results it was clear that the concentration of BHA required to control the growth of *S. chartarum* is much higher *in vivo* than *in vitro*. There are no reports in the literature regarding the use of antioxidants in paints to control fungal growth in buildings. Menetrez *et al* (2008) evaluated the antifungal capabilities of a range of commercially available paint products, also known as encapsulants that prevent the return of mould. Antifungal activities at varying levels

were observed against *S. chartarum* colonisation. The results achieved are significant findings and show antioxidants, which are safe alternatives to other irritable antifungal compounds, can be supplemented in paints to control *S. chartarum* growth.

5 CONCLUSIONS AND FUTURE WORK

5.1 Conclusion

The initial objective of this work was to determine the frequency and conditions by which *S. chartarum* can persist in water damaged buildings. The next main objective was to determine the ecophysiological characteristics of *S. chartarum* under varying levels of water activity and temperature. Methods for controlling the growth of *S. chartarum* were also investigated using various antifungal compounds *in vitro* and *in vivo.*

This section highlights the main findings from the various phases carried out in this research project.

(A) Determination of the frequency of *S .chartarum* species in water damaged buildings

- The diversity of fungal species detected in the surveyed locations was significantly influenced by the extent of water damage. *Penicillium*, *Cladosporium* and *Aspergillus* species were the most commonly detected fungal species in buildings with mild levels of water intrusion problems. *S. chartarum* was only detected in buildings with severe water intrusion problems. Which suggest that *S. chartarum* could potentionally be a future problem in homes that have been affected by extensive water damage.
- The main infested materials included wood panelling, wooden surfaces, plasterboard, wallpaper and ceiling tiles which are common materials used in UK buildings.

- No *S. chartarum* was detected from air sampling which suggests that many conidia may have been non-culturable. However, conidia and other non culturable fragments in the air may still be dangerous to occupants of affected building as they may be contaminated by the toxic metabolites.
- Swab sampling in conjunction with selective media was an effective method for determining the levels of fungal contamination without destructive measures to the fabric of the building under investigation. A drawback of this method was that large levels of yeasts were detected with this method which sometimes masked the true diversity of fungal species.
- Bulk sampling in conjunction with selective media proved effective methods for the detection of fungal contamination within buildings. A drawback to this sampling method is that it is not always possible to destructively take pieces of material from affected buildings.

(B) Influence of water activity, temperature, and their interaction on *S. chartarum* germination, germ tube extension, mycelia growth and conidia production.

- Germination, germ tube extension and mycelial growth for *S. chartarum* isolates 7711 and 14915 occurred over a wide range of temperatures under sufficient water availability ranges but was optimal between 20 and 30^{o}C. These rates were reduced when water stress was imposed at the extreme temperatures 15 and 37^{o}C. This suggest that colonisation of *S. chartarum* can occur under many conditions when appropriate food sources are available.

- Conidia production was also expressed over a wide range of temperatures and interestingly at lower a_w levels which suggest this organism can potentially be problematic in buildings during the drying out period.

(C) Influence of water activity, temperature and their interaction on SG production by *S. chartarum* isolates.

- Optimal SG for the macrocyclic trichothecene producing isolate S. *chartarum* isolate 7711 was highest at sub optimal conditions for growth which suggests that this organism could be expressing SG as a mechanism for survival under stressful conditions.
- The non macrocyclic trichothecene producing isolate 14915 was able to produce much lower levels of SG under similar conditions.
- Both *S. chartarum* isolates 7711 and 14915 showed little or no SG production at 30^oC which suggests that this organism utilises most of its energy for growth and sporulation at this temperature as a mechanism for its survival.

(D) Influence of water activity, temperature, and their interaction on *S. chartarum* extracellular enzyme production.

- Optimal levels of extracellular activity was observed by *S. chartarum* isolates 7711 and 14915 at 25^oC with freely available water. This expression dramatically reduced when water stress was imposed. This suggests that water is a key factor for the expression of extracellular enzymes for this organism. This correlates with *S. chartarum* growth characteristics under the same conditions and demonstrates that enzyme expression plays a key role.

- When *S. chartarum* isolates 7711, 14915 and Denbigh were grown in optimal conditions with freely available water, isolate 14915 showed much lower extracellular enzyme activity over the hour period compared to the other two isolates. This suggests that macrocyclic producing isolates may adapt to their environments faster than non macrocyclic tricothecene producing isolates which could have serious health implications to those exposed while living in affected water damaged buildings.

(E) Rapid methods for the characterisation of *S. chartarum* isolates

- The Bioscreen proved to be a very useful tool for rapid measurements of growth and antifungal susceptibility on *S. chartarum* isolates in liquid culture, and showed comparable results to those obtained through convential methods but within a shorter period of time.

(F) Control of *S. chartarum in vitro* and *in situ*

- Silver nitrate and tebuconazole were the most effective antifungals at controlling the growth of *S. chartarum* isolates 7711, 14915 and Denbigh agents *in vitro* with MICs determined at levels of <3 mg l^{-1}. Isolate 14915 however showed a biphasic growth pattern when treated with tebuconazole and gave an estimated MIC at levels of 100 mg l^{-1}. This biphasic growth may be a mechanism of adaptibility of non macrocyclic trichothecene producing isolates which could be a problem during the remediation of affected buildings.

- BHA and PG also showed inhibitory effect on the *S. chartarum* isolates *in vitro* with MICs at levels of 200 and 1000 mg l^{-1} respectively.
- BHA was ineffective at controlling the growth of *S. chartarum* isolates on plasterboard samples at MIC levels obtained *in vitro*. Higher concentrations of antioxidant may thus be required to control *S. chartarum* growth *in situ*.
- A reduction in incubation temperature from 25 to 15^{o}C dramatically reduced the growth of *S. chartarum* isolate 7711 on plasterboard samples except for painted surfaces. Isolate 14915 was able to grow normally under both conditions which shows some isolates may be more better adapted to harsher conditions than others which could enhance their chances of survival in water damaged buildings.
- Growth *S. chartarum* isolates 7711, 14915 and Denbigh was completely inhibited on plasterboard samples with BHA treated painted surfaces at 10,000 mg l^{-1}. This shows that this non toxic antifungal agent has the potential for use, as a safe alternative, to control *S. chartarum* colonisation compared to conventional antifungal agents which may contain dangerous heavy metals.

(G) Haemolytic activity of *S. chartarum* isolates

- Both macrocyclic and non macrocyclic tricothecene producing isolates *of S. chartarum* showed haemolytic activity when grown on 7% sheeps blood agar. This shows that there is a great potential health risk to those exposed as 60% of *S. chartarum* isolates detected in water damaged buildings are non macrocyclic tricothecene producers.

Suggestions for future work

(1) Effect of water activity and temperature on VOC profile for *S.chartarum* species

(2) Gene expression of *S. chartarum* toxins under varied abiotic conditions

(3) Competitiveness of *S. chartarum* species with other indoor fungal species under varied abiotic conditions

(4) Expression of extracellular enzymes on cellulose based media and building materials under varied abiotic conditions

6 REFERENCES

Åberg, N., Sundell, J., Eriksson, B., Hesselmar, B. and Åberg, B. (1996), "Prevalence of allergic diseases in schoolchildren in relation to family history, upper respiratory infections, and residential characteristics", *Allergy: European Journal of Allergy and Clinical Immunology,* vol. 51, no. 4, pp. 232-237.

Abid-Essefi, S., Ouanes, Z., Hassen, W., Baudrimont, I., Creppy, E. and Bacha, H. (2004), "Cytotoxicity, inhibition of DNA and protein syntheses and oxidative damage in cultured cells exposed to zearalenone", *Toxicology in Vitro,* vol. 18, no. 4, pp. 467-474.

Ahmad, S. and Branen, A. L. (1981), "Inhibition of mold growth by butylated hydroxyanisole", *Journal of Food Science,* vol. 46, no. 4, pp. 1059-1063.

Alam, S., Shah, H. U. and Magan, N. (2009), "Water availability affects extracellular hydrolytic enzyme production by *Aspergillus flavus* and *Aspergillus parasiticus*", *World Mycotoxin Journal,* vol. 2, no. 3, pp. 313-322.

American College of Occupational and Environmental Medicine (2002), *Adverse Human Health Effects Associated with Molds in the Indoor Environment*, available at: http://www.acoem.org/guidelines.aspx?id=850 (accessed 23/02/11).

Andersen, B., Nielsen, K. F. and Jarvis, B. B. (2002), "Characterization of *Stachybotrys* from water-damaged buildings based on morphology, growth, and metabolite production", *Mycologia,* vol. 94, no. 3, pp. 392-403.

Andersson, M. A., Nikulin, M., Köljalg, U., Andersson, M. C., Rainey, F., Reijula, K., Hintikka, E. L. and Salkinoja-Salonen, M. (1997), "Bacteria, molds, and toxins in water-damaged building materials", *Applied and Environmental Microbiology,* vol. 63, no. 2, pp. 387-393.

Ayerst, G. (1969), "The effects of moisture and temperature on growth and spore germination in some fungi", *Journal of Stored Products Research,* vol. 5, no. 2, pp. 127-141.

Becker, R., Puterman, M. and Laks, J. (1986), "Effect of porosity of emulsion paints on mould growth", *Durability of Building Materials,* vol. 3, no. 4, pp. 369-380.

Beijer, L., Thorn, J. and Rylander, R. (1999), "Inhalation of (1→3)-β-D-glucan in humans", *Proceedings of the 1998 Beltwide Cotton Conferences,* January 5-9 1999, San Diego, CA, pp. 251-254.

Bennett, J. W. and Klich, M. (2003), "Mycotoxins", *Clinical Microbiology Reviews,* vol. 16, no. 3, pp. 497-516.

Betancourt, D. A., Dean, T. R., Menetrez, M. Y. and Moore, S. A. (2006), "Characterization of microbial volatile organic compounds (MVOC) emitted by *Stachybotrys chartarum*", *Proceedings of the A and WMA Indoor Environmental Quality: Problems, Research and Solutions Conference,* Vol. 1, 17-19 July, Durham, NC, pp. 143-152.

Billups, R. A., Tilton, K. S. and Warden, P. S. (1999), "Identification of *Stachybotrys chartarum* utilizing various media and two temperature settings", *Proceedings of the First NSF International Conference on Indoor Air Health: Impacts, Issues and Solutions,* 3-5 May, Denver, NSF International, Ann Arbour, MI, pp. 166-174.

Bisby, G. R. (1945), "*Stachybotrys* and *Memnoniella*", *Transactions of the British Mycological Society,* vol. 28, pp. 11-12.

Bloom, E., Grimsley, L. F., Pehrson, C., Lewis, J. and Larsson, L. (2009), "Molds and mycotoxins in dust from water-damaged homes in New Orleans after hurricane Katrina", *Indoor Air,* vol. 19, no. 2, pp. 153-158.

Bogacka, E. (2008), "Mould allergy: diagnosis and treatment", *Polski Merkuriusz Lekarski,* vol. 24, no. Suppl. 1, pp. 11-14.

Bogaert, P., Tournoy, K. G., Naessens, T. and Grooten, J. (2009), "Where asthma and hypersensitivity pneumonitis meet and differ: noneosinophilic severe asthma", *American Journal of Pathology,* vol. 174, no. 1, pp. 3-13.

Boutin-Forzano, S., Charpin-Kadouch, C., Chabbi, S., Bennedjai, N., Dumon, H. and Charpin, D. (2004), "Wall relative humidity: a simple and reliable index for predicting *Stachybotrys chartarum* infestation in dwellings", *Indoor Air,* vol. 14, no. 3, pp. 196-199.

Brasel, T. L., Douglas, D. R., Wilson, S. C. and Straus, D. C. (2005), "Detection of airborne *Stachybotrys chartarum* macrocyclic trichothecene mycotoxins on particulates smaller than conidia", *Applied and Environmental Microbiology,* vol. 71, no. 1, pp. 114-122.

British Standards (2005), *Environmental Testing. Tests. Test J and Guidance: Mould Growth,* BS EN 60068-2-10:2005, British Standards, London.

Brownson, K. (2000), "Hospital air is sick", *Hospital Material Management Quarterly,* vol. 22, no. 2, pp. 1-8.

Burge, P. S. (2004), "Sick building syndrome", *Occupational and Environmental Medicine,* vol. 61, no. 2, pp. 185-190.

Bush, R. K. and Peden, D. B. (2006), "Advances in environmental and occupational disorders", *Journal of Allergy and Clinical Immunology,* vol. 117, no. 6, pp. 1367-1373.

CDC (2000), "Update: Pulmonary hemorrhage/hemosiderosis among infants - Cleveland, Ohio, 1993-1996", *Journal of the American Medical Association,* vol. 283, no. 15, pp. 1951-1953.

CDC (1997), "Update: Pulmonary hemorrhage/hemosiderosis among infants - Cleveland, Ohio, 1993-1996", *Morbidity and Mortality Weekly Report,* vol. 46, no. 2, pp. 33-35.

CDC (1994), "Acute pulmonary hemorrhage/hemosiderosis among infants - Cleveland, January 1993-November 1994", *Morbidity and Mortality Weekly Report,* vol. 43, no. 48, pp. 881-883.

Chang, H. C. and Branen, A. L. (1975), "Antimicrobial effects of butylated hydroxyanisole (BHA)", *Journal of Food Science,* vol. 40, no. 2, pp. 349-351.

Chapman, J. A. (2003), "*Stachybotrys chartarum* (chartarum = atra = alternans) and other problems caused by allergenic fungi", *Allergy and Asthma Proceedings,* vol. 24, no. 1, pp. 1-7.

Chen, T. H. and Hollingsworth, H. (2008), "Allergic bronchopulmonary aspergillosis", *New England Journal of Medicine,* vol. 359, no. 6, pp. e7.

Chung, Y. J., Jarvis, B. B., Tak, H. and Pestka, J. J. (2003), "Immunochemical assay for Satratoxin G and other macrocyclic trichothecenes associated with indoor air contamination by *Stachybotrys chartarum*", *Toxicology Mechanisms and Methods,* vol. 13, no. 4, pp. 247-252.

Ciegler, A. (1975), "Mycotoxins: occurrence, chemistry, biological activity", *Lloydia, vol. 38, no. 1, pp. 21-35.*

Claeson, A. S. (2006), *Volatile organic compounds from microorganisms - identification and health effects* (unpublished Medical Dissertation thesis), Umeå University, Umeå.

Clegg, C. J. and Mackean, D. G. (2000), *Advanced Biology: Principles and Applications,* 2nd ed, John Murray, London.

Corda, A. C. I. (1837), "Icones fungorum hucusque cognitorum", *Abbildungen der Pilze und Schwaemme I, .*

Crook, B. and Burton, N. C. (2010), "Indoor moulds, Sick Building Syndrome and building related illness", *Fungal Biology Reviews,* vol. 24, no. 3-4, pp. 106-113.

Crump, D., Dengel, A. and Swainson, M. (2009), *Indoor Air Quality in Highly Energy Efficient Homes - a Review,* NHBC Foundation, Amersham, UK.

Dashek, W. V., Mayfield, J. E., Llewellyn, G. C., O'Rear, C. E. and Bata, A. (1986), "Trichothecenes and yellow rain: possible biological warfare agents", *BioEssays*, vol. 4, no. 1, pp. 27-30.

Davis, P. J. (2001), "Molds, toxic molds, and indoor air quality", *California Research Bureau Note*, [Online], vol. 8, no. 1, pp. http://www.library.ca.gov/crb/01/notes/v8n1.pdf-1-18.

Dharmage, S., Bailey, M., Raven, J., Abeyawickrama, K., Cao, D., Guest, D., Rolland, J., Forbes, A., Thien, F., Abramson, M. and Walters, E. H. (2002), "Mouldy houses influence symptoms of asthma among atopic individuals", *Clinical and Experimental Allergy*, vol. 32, no. 5, pp. 714-720.

Diette, G. B., McCormack, M. C., Hansel, N. N., Breysse, P. N. and Matsui, E. C. (2008), "Environmental issues in managing asthma.", *Respiratory Care*, vol. 53, no. 5, pp. 602-615; discussion 616-617.

Doi, K., Ishigami, N. and Sehata, S. (2008), "T-2 toxin-induced toxicity in pregnant mice and rats", *International Journal of Molecular Sciences*, vol. 9, no. 11, pp. 2146-2158.

Douwes, J. (2005), "(1 → 3)-β-D-glucans and respiratory health: a review of the scientific evidence", *Indoor Air*, vol. 15, no. 3, pp. 160-169.

Eckert, M. R., Rossall, S., Selley, A. and Fitt, B. D. L. (2010), "Effects of fungicides on *in vitro* spore germination and mycelial growth of the phytopathogens *Leptosphaeria maculans* and *L. biglobosa* (Phoma stem canker of oilseed rape)", *Pest Management Science*, vol. 66, no. 4, pp. 396-405.

Elbert, W., Taylor, P. E., Andreae, M. O. and Pöschl, U. (2007), "Contribution of fungi to primary biogenic aerosols in the atmosphere: wet and dry discharged spores, carbohydrates, and inorganic ions", *Atmospheric Chemistry and Physics*, vol. 7, no. 17, pp. 4569-4588.

El-Maghraby, O. M. O., Bean, G. A., Jarvis, B. B. and Aboul-Nasr, M. B. (1991), "Macrocyclic trichothecenes produced by *Stachybotrys* isolated from Egypt and Eastern Europe", *Mycopathologia*, vol. 113, no. 2, pp. 109-115.

Engelhart, S., Rietschel, E., Exner, M. and Lange, L. (2009), "Childhood hypersensitivity pneumonitis associated with fungal contamination of indoor hydroponics", *International Journal of Hygiene and Environmental Health*, vol. 212, no. 1, pp. 18-20.

English House Condition Survey (2009), *Annual Report 2007: Decent Homes and Decent Places*, available at: http://www.communities.gov.uk/publications/corporate/statistics/ehcs2007annualreport (accessed 25/03/11).

Environment Agency (2009), *Flooding in England:a National Assessment of Flood Risk*, Environment Agency, Bristol, UK.

Environmental Protection Agency (2011), *An Introduction to Indoor Air Quality (IAQ)*, available at: http://www.epa.gov/iaq/voc.html (accessed 08/06/11).

Fabbri, A. A., Ricelli, A., Brasini, S. and Fanelli, C. (1997), "Effect of different antifungals on the control of paper biodeterioration caused by fungi", *International Biodeterioration and Biodegradation*, vol. 39, no. 1, pp. 61-65.

Flannigan, B. and Miller, J. D. (2001), "Microbial growth in indoor environments", in Flannigan, B., Samson, R. A. and Miller, J. D. (eds.) *Microorganisms in Home and Indoor Work Environments: Diversity, Health Impacts Investigation and Control*, Taylor & Francis, London, pp. 35-67.

Flannigan, B. and Morey, P. R. (1996), *Control of Moisture Problems Affecting Biological Indoor Air Quality*, ISIAQ-Guideline TFI-1996, International Society of Indoor Air Quality and Climate, Milan.

Florida Mould Inspectors (2009), *Stachybotrys chartarum*, available at: http://www.floridamoldinspectors.us/Stachybotrys%20spores%201.jpg (accessed 23/02/11).

Forgacs, J. (1972), "Stachybotryotoxicosis", *Microbial Toxins*, vol. 8, pp. 95-128.

Forgacs, J. (1965), "Stachybotrytoxicosis and moldy corn toxicosis", in Wogan, G. N. (ed.) *Mycotoxins in Foodstuffs*, MIT Press, Cambridge, Mass, pp. 87-104.

Foroud, N. A. and Eudes, F. (2009), "Trichothecenes in cereal grains", *International Journal of Molecular Sciences*, vol. 10, no. 1, pp. 147-173.

Fuhrmann, G. F. and Rothstein, A. (1968), "The mechanism of the partial inhibition of fermentation in yeast by nickel ions", *Biochimica et Biophysica Acta - Biomembranes*, vol. 163, no. 3, pp. 331-338.

Fung, D. Y. C., Taylor, S. and Kahan, J. (1977), "Effects of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) on growth and aflatoxin production of *Aspergillus flavus*", *Journal of Food Safety*, vol. 1, pp. 39-51.

Furr, J. R. (1994), "Antibacterial activity of Actisorb Plus, Actisorb and silver nitrate", *Journal of Hospital Infection*, vol. 27, no. 3, pp. 201-208.

Galwa, R. P., Gupta, P. and Mumtaz, H. A. (2008), "Clinical significance of high-attenuation mucus in patients with allergic bronchopulmonary aspergillosis.", *RadioGraphics*, vol. 28, no. 5, pp. 1460.

Gao, P., Korley, F., Martin, J. and Chen, B. T. (2002), "Determination of unique microbial volatile organic compounds produced by five *Aspergillus* species

commonly found in problem buildings", *American Industrial Hygiene Association Journal,* vol. 63, no. 2, pp. 135-140.

Geisler, W. M. and Corey, L. (2002), "*Chlamydia pneumoniae* respiratory infection after allogeneic stem cell transplantation", *Transplantation,* vol. 73, no. 6, pp. 1002-1005.

Giridhar, P. and Reddy, S. M. (2001), "Phenolic antioxidants for the control of some mycotoxigenic fungi", *Journal of Food Science and Technology,* vol. 38, no. 4, pp. 397-399.

Gordon, W. A., Cantor, J. B., Spielman, L., Ashman, T. A. and Johanning, E. (2007), "Cognitive impairment associated with toxigenic fungal exposure: a response to two critiques", *Applied Neuropsychology,* vol. 13, no. 4, pp. 251-257.

Gottschalk, C., Bauer, J. and Meyer, K. (2008), "Detection of satratoxin G and H in indoor air from a water-damaged building", *Mycopathologia,* vol. 166, no. 2, pp. 103-107.

Grant, C., Hunter, C. A., Flannigan, B. and Bravery, A. F. (1989), "The moisture requirements of moulds isolated from domestic dwellings", *International Biodeterioration,* vol. 25, no. 4, pp. 259-284.

Gravesen, S. (1979), "Fungi as a cause of allergic disease", *Allergy,* vol. 34, no. 3, pp. 135-154.

Gravesen, S., Nielsen, P. A., Iversen, R. and Nielsen, K. F. (1999), "Microfungal contamination of damp buildings - examples of risk constructions and risk materials", *Environmental Health Perspectives,* vol. 107, no. Suppl. 3, pp. 505-508.

Green, B. J., Sercombe, J. K. and Tovey, E. R. (2005), "Fungal fragments and undocumented conidia function as new aeroallergen sources", *Journal of Allergy and Clinical Immunology,* vol. 115, no. 5, pp. 1043-1048.

Green, C. F., Scarpino, P. V. and Gibbs, S. G. (2003), "Assessment and modeling of indoor fungal and bacterial bioaerosol concentrations", *Aerobiologia,* vol. 19, no. 3-4, pp. 159-169.

Greenberger, P. A. (2002), "Allergic bronchopulmonary aspergillosis", *Journal of Allergy and Clinical Immunology,* vol. 110, no. 5, pp. 685-692.

Gregory, L., Pestka, J. J., Dearborn, D. G. and Rand, T. G. (2004), "Localization of Satratoxin-G in *Stachybotrys chartarum* spores and spore-impacted mouse lung using immunocytochemistry", *Toxicologic Pathology,* vol. 32, no. 1, pp. 26-34.

Gyula, S. (2005), "Domestic occurrence of stachybotryotoxicosis in dogs. Case report", *Magyar Allatorvosok Lapja,* vol. 127, no. 12, pp. 727-732.

Harrach, B., Bata, A., Bajmocy, E. and Benko, M. (1983), "Isolation of satratoxins from the bedding straw of a sheep flock with fatal stachybotryotoxicosis", *Applied and Environmental Microbiology,* vol. 45, no. 5, pp. 1419-1422.

Harrington, B. J. (2002), "Growth and sporulation of *Stachybotrys chartarum* on different media and under different light/dark incubation conditions", *Abstracts of the General Meeting of the American Society for Microbiology,* vol. 201, pp. 452.

Haverinen-Shaughnessy, U., Hyvärinen, A., Putus, T. and Nevalainen, A. (2008), "Monitoring success of remediation: seven case studies of moisture and mold damaged buildings", *Science of the Total Environment,* vol. 399, no. 1-3, pp. 19-27.

Heyndrickx, A., Sookvanichsilp, N. and Van den Heede, M. (1984), "Detection of trichothecene mycotoxins (yellow rain) in blood, urine and faeces of Iranian soldiers treated as victims of a gas attack", *Archives Belges,* vol. Suppl, pp. 143-146.

Hinkley, S. F., Mazzola, E. P., Fettinger, J. C., Lam, Y. F. and Jarvis, B. B. (2000), "Atranones A-G, from the toxigenic mold *Stachybotrys chartarum*", *Phytochemistry,* vol. 55, no. 6, pp. 663-673.

HM Government (2010), *The Building Regulations 2010: Ventilation,* Approved Document F, HM Government, London.

Hunter, C. A. and Bravery, A. F. (1989), "Requirements for growth and control of surface moulds in dwellings.", in Flannigan, B. (ed.) *Airborne Deteriogens and Pathogens,* The Biodeterioration Society, Kew, UK, pp. 174-182.

Industrial Microbiological Services Ltd (2005), *Technical White Paper: Antimicrobial Activity of Silver,* available at: http://www.autospec.co.za/productmedia/flowcrete/b01490/datasheets/accreditations/antimicrobial.pdf (accessed 08/06/11).

Ingold, C. T. (1971), *Fungal Spores: Their Liberation and Dispersal,* Clarendon Press, Oxford.

Jarvis, B. B. (1991), "Macrocyclic trichothecenes", in Sharma, R. P. and Salunkhe, D. K. (eds.) *Mycotoxins and Phytoalexins in Human and Animal Health,* CRC Press, Boca Raton, FL, pp. 361-421.

Johanning, E., Biagini, R., Hull, D., Morey, P., Jarvis, B. and Landsbergis, P. (1996), "Health and immunology study following exposure to toxigenic fungi (*Stachybotrys chartarum*) in a water-damaged office environment", *International Archives of Occupational and Environmental Health,* vol. 68, no. 4, pp. 207-218.

Jong, S. C. and Davis, E. E. (1976), "Contribution to the knowledge of *Stachybotrys* and *Memnoniella* in culture", *Mycotaxon,* vol. 3, no. 3, pp. 409-485.

Karunasena, E., Larrañaga, M. D., Simoni, J. S., Douglas, D. R. and Straus, D. C. (2010), "Building-associated neurological damage modeled in human cells: a mechanism of neurotoxic effects by exposure to mycotoxins in the indoor environment", *Mycopathologia,* vol. 170, no. 6, pp. 377-390.

Karvala, K., Nordman, H., Luukkonen, R., Nykyri, E., Lappalainen, S., Hannu, T. and Toskala, E. (2008), "Occupational rhinitis in damp and moldy workplaces", *American Journal of Rhinology,* vol. 22, no. 5, pp. 457-462.

KatrinaDestruction.com (2011), *Photo Essays of Hurricane Katrina*, available at: www.katrinadestruction.com (accessed 23/02/11).

Katz, R. and Singer, B. (2007), "Can an attribution assessment be made for Yellow Rain? Systematic reanalysis in a chemical-and-biological-weapons use investigation", *Politics and the Life Sciences,* vol. 26, no. 1, pp. 24-42.

Kilpeläinen, M., Terho, E. O., Helenius, H. and Koskenvuo, M. (2001), "Home dampness, current allergic diseases, and respiratory infections among young adults", *Thorax,* vol. 56, no. 6, pp. 462-467.

Koster, B., Scott, J., Wong, B., Malloch, D. and Straus, N. (2003), "A geographically diverse set of isolates indicates two phylogenetic lineages within *Stachybotrys chartarum*", *Canadian Journal of Botany,* vol. 81, no. 6, pp. 633-643.

Kováčiková, Z., Piecková, E., Tátrai, E., Pivovarová, Z. and Mataušic-Pišl, M. (2007), "Use of the *in vitro* model for the evaluation of toxic effects of metabolites produced by fungi", *WIT Transactions on Biomedicine and Health,* vol. 11, pp. 79-84.

Kuhn, D. M. and Ghannoum, M. A. (2003), "Indoor mold, toxigenic fungi, and *Stachybotrys chartarum*: infectious disease perspective", *Clinical Microbiology Reviews,* vol. 16, no. 1, pp. 144-172.

Kuhn, F. A. and Swain Jr., R. (2003), "Allergic fungal sinusitis: diagnosis and treatment", *Current Opinion in Otolaryngology and Head and Neck Surgery,* vol. 11, no. 1, pp. 1-5.

Kung'u, J. (2006), *The Mould Fusarium: How Does It Affect Our Lives?*, available at: http://www.moldbacteria.com/newsletters/2006/aug2006.html (accessed 17/06/11).

Lacasse, Y., Selman, M., Costabel, U., Dalphin, J. C., Morell, F., Erkinjuntti-Pekkanen, R., Mueller, N. L., Colby, T. V., Schuyler, M., Jomphe, V. and Cormier, Y. (2009), "Classification of hypersensitivity pneumonitis: a hypothesis", *International Archives of Allergy and Immunology*, vol. 149, no. 2, pp. 161-166.

Lambert, R. J. W. and Pearson, J. (2000), "Susceptibility testing: accurate and reproducible minimum inhibitory concentration (MIC) and non-inhibitory

concentration (NIC) values", *Journal of Applied Microbiology*, vol. 88, no. 5, pp. 784-790.

Langhorst, M., Curtis White, W. and Monticello, R. A. (2006), "Antimicrobial treatment on wallboard paper where economy meets functionality", *International Nonwovens Technical Conference, INTC 2006,* 25-28 Sept, Houston, TX, pp. 417-425.

Lednicky, J. A. and Rayner, J. O. (2006), "Uncommon respiratory pathogens", *Current Opinion in Pulmonary Medicine,* vol. 12, no. 3, pp. 235-239.

Levy, J. and Wilmott, R. W. (1986), "Pulmonary hemosiderosis", *Pediatric Pulmonology*, vol. 2, no. 6, pp. 384-391.

Lillard, S. (2004), *Stachybotrys chartarum*, available at: http://www.mold-help.org/content/view/429/0/ (accessed 13/06/11).

Lu, Z., Lu, W. Z., Zhang, J. L. and Sun, D. X. (2009), "Microorganisms and particles in AHU systems: measurement and analysis", *Building and Environment*, vol. 44, no. 4, pp. 694-698.

Magan, N. (1988), "Effect of water potential and temperature on spore germination and germ tube growth *in vitro* and on straw leaf sheaths", *Transactions of the British Mycological Society*, vol. 90, no. 1, pp. 97-107.

Magan, N. and Lacey, J. (1988), "Ecological determinants of mould growth in stored grain", *International Journal of Food Microbiology*, vol. 7, no. 3, pp. 245-256.

Marín, S., Sanchis, V., Ramos, A. J. and Magan, N. (1998), "Effect of water activity on hydrolytic enzyme production by *Fusarium moniliforme* and *Fusarium proliferatum* during colonisation of maize", *International Journal of Food Microbiology*, vol. 42, no. 3, pp. 185-194.

Marrs, T. C., Edginton, J. A. G., Price, P. N. and Upshall, D. G. (1986), "Acute toxicity of T2 mycotoxin to the guinea-pig by inhalation and subcutaneous routes", *British Journal of Experimental Pathology*, vol. 67, no. 2, pp. 259-268.

Mason, S., Cortes, D. and Horner, W. E. (2010), "Detection of gaseous effluents and by-products of fungal growth that affect environments (RP-1243)", *HVAC and R Research*, vol. 16, no. 2, pp. 109-121.

McGinnis, M. R. (2004), "Pathogenesis of indoor fungal diseases", *Medical Mycology*, vol. 42, no. 2, pp. 107-117.

Menetrez, M. Y. and Foarde, K. K. (2004), "Emission exposure model for the transport of toxic mold", *Indoor and Built Environment*, vol. 13, no. 1, pp. 75-82.

Menetrez, M. Y., Foarde, K. K., Webber, T. D., Dean, T. R. and Betancourt, D. A. (2008), "Testing antimicrobial paint efficacy on gypsum wallboard contaminated with *Stachybotrys chartarum*", *Journal of Occupational and Environmental Hygiene*, vol. 5, no. 2, pp. 63-66.

Middleton Jr., E., Kandaswami, C. and Theoharides, T. C. (2000), "The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer", *Pharmacological Reviews*, vol. 52, no. 4, pp. 673-751.

Miller, D. J., Rand, T. G. and Jarvis, B. B. (2003), "*Stachybotrys chartarum*: cause of human disease or media darling?", *Medical Mycology*, vol. 41, no. 4, pp. 271-291.

Miyazaki, Y., Tateishi, T., Akashi, T., Ohtani, Y., Inase, N. and Yoshizawa, Y. (2008), "Clinical predictors and histologic appearance of acute exacerbations in chronic hypersensitivity pneumonitis", *Chest*, vol. 134, no. 6, pp. 1265-1270.

Mold and Bacteria Consulting Laboratories (2011), *Mold in Homes and Their Hazard Classes*, available at: http://www.moldbacteria.com/learnmore/moldlist.html (accessed 23/02/11).

Montz Jr., W. E. and Passon Jr., T. J. (2001), "What seems to be the problem", *Engineered Systems*, vol. 18, no. 6, pp. 68-74.

Morissette, D. C., Seguin, P. and Jabaji-Hare, S. H. (2006), "Expression regulation of the endochitinase-encoding gene sechi44 from the mycoparasite Stachybotrys elegans", *Canadian journal of microbiology*, vol. 52, no. 11, pp. 1103-1109.

Murphy, J. M., Sexton, D. M. H., Jenkins, G. J., Booth, B. B. B., Brown, C. C., Clark, R. T., Collins, M., Harris, G. R., Kendon, E. J., Betts, R. A., Brown, S. J., Humphrey, K. A., McCarthy, M. P., McDonald, R. E., Stephens, A., Wallace, C., Warren, R., Wilby, R. and Wood, R. (2009), *UK Climate Projections Science Report: Climate Change Projections.* Met Office Hadley Centre, Exeter, UK.

Myatt, T. A., Minegishi, T., Allen, J. G. and MacIntosh, D. L. (2008), "Control of asthma triggers in indoor air with air cleaners: a modeling analysis", *Environmental Health*, vol. 7, pp. article 43.

Nabrdalik, M. (2007), "Enzymatic activity of moulds occurring in human environment", *Mikologia Lekarska*, vol. 14, no. 3, pp. 195-200.

Nathanson, T. (1995), *Indoor Air Quality in Office Buildings: a Technical Guide. A Report of the Federal Provincial Advisory Committee on Environmental and Occupational Health*, available at: http://dsp-psd.pwgsc.gc.ca/Collection/H46-2-93-166Erev.pdf (accessed 29/01/10).

National Toxicology Program (2004), *Stachybotrys chartarum (or S. atra or S. alternans) [CAS No. 67892-26-6]. Review of Toxicological Literature* , available at:

http://ntp.niehs.nih.gov/ntp/htdocs/Chem_Background/ExSumPdf/Stachybotrys.pdf (accessed 09/03/11).

Nielsen, K. F., Hansen, M. Ø., Larsen, T. O. and Thrane, U. (1998), "Production of trichothecene mycotoxins on water damaged gypsum boards in Danish buildings", *International Biodeterioration and Biodegradation,* vol. 42, no. 1, pp. 1-7.

Nielsen, K. F., Holm, G., Uttrup, L. P. and Nielsen, P. A. (2004), "Mould growth on building materials under low water activities. Influence of humidity and temperature on fungal growth and secondary metabolism", *International Biodeterioration and Biodegradation,* vol. 54, no. 4, pp. 325-336.

Nielsen, K. F., Huttunen, K., Hyvärinen, A., Andersen, B., Jarvis, B. B. and Hirvonen, M. -. (2002), "Metabolite profiles of *Stachybotrys* isolates from water-damaged buildings and their induction of inflammatory mediators and cytotoxicity in macrophages", *Mycopathologia,* vol. 154, no. 4, pp. 201-205.

Nielsen, K. F., Thrane, U., Larsen, T. O., Nielsen, P. A. and Gravesen, S. (1998), "Production of mycotoxins on artificially inoculated building materials", *International Biodeterioration & Biodegradation,* vol. 42, no. 1, pp. 9-16.

Nieminen, S. M., Kärki, R., Auriola, S., Toivola, M., Laatsch, H., Laatikainen, R., Hyvärinen, A. and Von Wright, A. (2002), "Isolation and identification of *Aspergillus fumigatus* mycotoxins on growth medium and some building materials", *Applied and Environmental Microbiology,* vol. 68, no. 10, pp. 4871-4875.

Pardo, E., Lagunas, U., Sanchis, V., Ramos, A. J. and Marín, S. (2005a), "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,* vol. 85, no. 10, pp. 1681-1686.

Pardo, E., Ramos, A. J., Sanchis, V. and Marín, S. (2005b), "Modelling of effects of water activity and temperature on germination and growth of ochratoxigenic isolates of *Aspergillus ochraceus* on a green coffee-based medium", *International Journal of Food Microbiology,* vol. 98, no. 1, pp. 1-9.

Pasanen, A. L., Juutinen, T., Jantunen, M. J. and Kalliokoski, P. (1992), "Occurrence and moisture requirements of microbial growth in building materials", *International Biodeterioration and Biodegradation,* vol. 30, no. 4, pp. 273-283.

Passon Jr., T. J., Brown, J. W. and Mante, S. (1996), "New and emerging pathogens, part 6. Sick-building syndrome and building-related illness.", *Medical Laboratory Observer,* vol. 28, no. 7, pp. 84-86, 88, 90 passim; quiz 98-99.

Peat, J. K., Dickerson, J. and Li, J. (1998), "Effects of damp and mould in the home on respiratory health: a review of the literature", *Allergy: European Journal of Allergy and Clinical Immunology,* vol. 53, no. 2, pp. 120-128.

Peltola, J., Andersson, M. A., Haahtela, T., Mussalo-Rauhamaa, H., Rainey, F. A., Kroppenstedt, R. M., Samson, R. A. and Salkinoja-Salonen, M. S. (2001), "Toxic-metabolite-producing bacteria and fungus in an indoor environment", *Applied and Environmental Microbiology,* vol. 67, no. 7, pp. 3269-3274.

Pestka, J. J. (2007), "Deoxynivalenol: toxicity, mechanisms and animal health risks", *Animal Feed Science and Technology,* vol. 137, no. 3-4, pp. 283-298.

Picart, P., Diaz, P. and Pastor, F. I. J. (2008), "Stachybotrys atra BP-A produces alkali-resistant and thermostable cellulases", *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology,* vol. 94, no. 2, pp. 307-316.

Piecková, E., Hurbánková, M., Černá, S., Lišková, A., Kováčiková, Z., Kolláriková, Z. and Wimmerová, S. (2009), "Inflammatory and haematotoxic potential of indoor *Stachybotrys chartarum* (Ehrenb.) hughes metabolites", *Arhiv za Higijenu Rada i Toksikologiju,* vol. 60, no. 4, pp. 401-409.

Piecková, E. and Jesenská, Z. (1999), "Microscopic fungi in dwellings and their health implications in humans", *Annals of Agricultural and Environmental Medicine,* vol. 6, no. 1, pp. 1-11.

Piecková, E. and Jesenská, Z. (1998), "Molds on house walls and the effect of their chloroform-extractable metabolites on the respiratory cilia movement of one-day-old chicks *in vitro*", *Folia Microbiologica,* vol. 43, no. 6, pp. 672-678.

Piecková, E. and Jesenská, Z. (1996), "Ciliostatic effect of fungi on the respiratory tract ciliary movement of one-day-old chickens *in vitro*", *Folia Microbiologica,* vol. 41, no. 6, pp. 517-520.

Pirhonen, I., Nevalainen, A., Husman, T. and Pekkanen, J. (1996), "Home dampness, moulds and their influence on respiratory infections and symptoms in adults in Finland", *European Respiratory Journal,* vol. 9, no. 12, pp. 2618-2622.

Pommerville, J. C. (2004), *Alcomo's Fundementals of Microbiology,* 7th ed, Jones and Bartlett, Boston.

Portnoy, J. M., Barnes, C. S. and Kennedy, K. (2004), "Sampling for indoor fungi", *Journal of Allergy and Clinical Immunology,* vol. 113, no. 2, pp. 189-199.

Razavi-Rohani, S. M. and Griffiths, M. W. (1999), "The antifungal activity of butylated hydroxyanizole and lysozyme", *Journal of Food Safety,* vol. 19, no. 2, pp. 97-108.

Ren, P., Jankun, T. M., Belanger, K., Bracken, M. B. and Leaderer, B. P. (2001), "The relation between fungal propagules in indoor air and home characteristics",

Allergy: European Journal of Allergy and Clinical Immunology, vol. 56, no. 5, pp. 419-424.

Roussel, S., Reboux, G., Bellanger, A. P., Sornin, S., Grenouillet, F., Dalphin, J. C., Piarroux, R. and Millon, L. (2008), "Characteristics of dwellings contaminated by moulds", *Journal of Environmental Monitoring,* vol. 10, no. 6, pp. 724-729.

Rylander, G. (1996), "Airway responsiveness and chest symptoms after inhalation of endotoxin or (1→3)-β-D-glucan", *Indoor and Built Environment,* vol. 5, no. 2, pp. 106-111.

Sahakian, N. M., Park, J. H. and Cox-Ganser, J. M. (2008), "Dampness and mold in the indoor environment: implications for asthma", *Immunology and Allergy Clinics of North America,* vol. 28, no. 3, pp. 485-505.

Saibi, W., Abdeljalil, S. and Gargouri, A. (2010), "Carbon source directs the differential expression of β-glucosidases in Stachybotrys microspora", *World Journal of Microbiology and Biotechnology,* , pp. 1-10.

Sakamoto, T. (2008), "Allergic effects and laboratory assessment of mold exposure", *Japanese Journal of Allergology,* vol. 57, no. 8, pp. 949-959.

Samson, R. A., Hoekstra, E. S., Seifert, K. A., Thrane, U. and Frisvad, J. C. (2002), "Identification of the common food-borne fungi", in Samson, R. A. and Hoekstra, E. S. (eds.) *Introduction to Food- and Airborne Fungi,* 6th ed, Centraalbureau voor Schimmelcultures, Utrecht, pp. 1-282.

Schmale III, D. G., Arntsen, Q. A. and Bergstrom, G. C. (2005), "The forcible discharge distance of ascospores of *Gibberella zeae*", *Canadian Journal of Plant Pathology,* vol. 27, no. 3, pp. 376-382.

Seppänen, O. A. and Fisk, W. J. (2004), "Summary of human responses to ventilation", *Indoor Air,* vol. 14, no. Suppl. 7, pp. 102-118.

Sergent, T., Dupont, I., Jassogne, C., Ribonnet, L., van der Heiden, E., Scippo, M. -., Muller, M., McAlister, D., Pussemier, L., Larondelle, Y. and Schneider, Y. -. (2009), "CYP1A1 induction and CYP3A4 inhibition by the fungicide imazalil in the human intestinal Caco-2 cells-Comparison with other conazole pesticides", *Toxicology letters,* vol. 184, no. 3, pp. 159-168.

Shah, A., Kala, J., Sahay, S. and Panjabi, C. (2008), "Frequency of familial occurrence in 164 patients with allergic bronchopulmonary aspergillosis", *Annals of Allergy, Asthma and Immunology,* vol. 101, no. 4, pp. 363-369.

Shah, A. and Panjabi, C. (2002), "Allergic bronchopulmonary aspergillosis: a review of a disease with a worldwide distribution", *Journal of Asthma,* vol. 39, no. 4, pp. 273-289.

Singh, J. (2005), "Toxic moulds and indoor air quality", *Indoor and Built Environment*, vol. 14, no. 3-4, pp. 229-234.

Snelders, E., Huis In't Veld, R. A. G., Rijs, A. J. M. M., Kema, G. H. J., Melchers, W. J. G. and Verweij, P. E. (2009), "Possible environmental origin of resistance of Aspergillus fumigatus to medical triazoles", *Applied and Environmental Microbiology*, vol. 75, no. 12, pp. 4053-4057.

Solomon, G. M., Hjelmroos-Koski, M., Rotkin-Ellman, M. and Hammond, S. K. (2006), "Airborne mold and endotoxin concentrations in New Orleans, Louisiana, after flooding, October through November 2005", *Environmental Health Perspectives*, vol. 114, no. 9, pp. 1381-1386.

Soobrattee, M. A., Neergheen, V. S., Luximon-Ramma, A., Aruoma, O. I. and Bahorun, T. (2005), "Phenolics as potential antioxidant therapeutic agents: mechanism and actions", *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 579, no. 1-2, pp. 200-213.

Soubani, A. O. and Chandrasekar, P. H. (2002), "The clinical spectrum of pulmonary aspergillosis", *Chest*, vol. 121, no. 6, pp. 1988-1999.

Spyker, S. M. and Spyker, D. A. (1983), "Yellow rain: chemical warfare in Southeast Asia and Afghanistan", *Veterinary and Human Toxicology*, vol. 25, no. 5, pp. 335-340.

Stark, A. A. (2005), "Threat assessment of mycotoxins as weapons: molecular mechanisms of acute toxicity", *Journal of Food Protection*, vol. 68, no. 6, pp. 1285-1293.

Tewkesbury Borough Council (2007), *Tewkesbury Borough Floods 2007*, available at: http://www.tewkesbury.gov.uk/index.cfm?articleid=3436 (accessed 23/02/11).

Trail, F. (2007), "Fungal cannons: explosive spore discharge in the *Ascomycota*", *FEMS Microbiology Letters*, vol. 276, no. 1, pp. 12-18.

Tsai, S. M., Yang, C. S. and Heinsohn, P. (2001), "Comparative studies of fungal media for recovery of *Stachybotrys chartarum* from environmental samples", in Johanning, E. (ed.) *Bioaerosols, Fungi and Mycotoxins: Health Effects, Assessment, Prevention and Control*, Fungal Research Group Foundation, New York, pp. 330-334.

Tuomi, T., Reijula, K., Johnsson, T., Hemminki, K., Hintikka, E. L., Lindroos, O., Kalso, S., Koukila-Kähkölä, P., Mussalo-Rauhamaa, H. and Haahtela, T. (2000), "Mycotoxins in crude building materials from water-damaged buildings", *Applied and Environmental Microbiology*, vol. 66, no. 5, pp. 1899-1904.

Tweddell, R. J., Jabaji-Hare, S. H. and Charest, P. M. (1994), "Production of chitinases and β-1,3-glucanases by Stachybotrys elegans, a mycoparasite of Rhizoctonia solani", *Applied and Environmental Microbiology,* vol. 60, no. 2, pp. 489-495.

Vesper, S. J. and Vesper, M. J. (2002), "Stachylysin may be a cause of hemorrhaging in humans exposed to *Stachybotrys chartarum*", *Infection and Immunity,* vol. 70, no. 4, pp. 2065-2069.

Wannemacher, R. W. J. and Wiener, S. L. (1997), "Trichothecene mycotoxins", in Sidell, F. R., Takafuji, E. T. and Franz, D. R. (eds.) *Medical Aspects of Chemical and Biological Warfare,* TMM Publications, Washington, DC, pp. 655-676.

Watson, S. A., Mirocha, C. J. and Hayes, A. W. (1984), "Analysis for trichothecenes in samples from southeast Asia associated with 'yellow rain'", *Fundamental and Applied Toxicology,* vol. 4, no. 5, pp. 700-717.

Weltermann, B. M., Hodgson, M., Storey, E., DeGraff Jr., A. C., Bracker, A., Groseclose, S., Cole, S. R., Cartter, M. and Phillips, D. (1998), "Hypersensitivity pneumonitis: a sentinel event investigation in a wet building", *American Journal of Industrial Medicine,* vol. 34, no. 5, pp. 499-505.

WHO (2009), *WHO Guidelines for Indoor Air Quality : Dampness and Mould,* WHO, Copenhagen.

Widestrand, J., Lundh, T., Pettersson, H. and Lindberg, J. E. (2003), "A rapid and sensitive cytotoxicity screening assay for trichothecenes in cereal samples", *Food and Chemical Toxicology,* vol. 41, no. 10, pp. 1307-1313.

Wilkins, K., Larsen, K. and Simkus, M. (2000), "Volatile metabolites from mold growth on building materials and synthetic media", *Chemosphere,* vol. 41, no. 3, pp. 437-446.

Woo, K. J., Hye, C. K., Ki, W. K., Shin, S., So, H. K. and Yong, H. P. (2008), "Antibacterial activity and mechanism of action of the silver ion in *Staphylococcus aureus* and *Escherichia coli*", *Applied and Environmental Microbiology,* vol. 74, no. 7, pp. 2171-2178.

Wright, T. J., Greene, V. W. and Paulus, H. J. (1969), "Viable microorganisms in an urban atmosphere", *Journal of the Air Pollution Control Association,* vol. 19, no. 5, pp. 337-341.

Wu, Z., Tsumura, Y., Blomquist, G. and Wang, X. -. (2003), "18S rRNA gene variation among common airborne fungi, and development of specific oligonucleotide probes for the detection of fungal isolates", *Applied and Environmental Microbiology,* vol. 69, no. 9, pp. 5389-5397.

Xu, J., Liang, Y., Belisle, D. and Miller, J. D. (2008), "Characterization of monoclonal antibodies to an antigenic protein from *Stachybotrys chartarum* and its

measurement in house dust", *Journal of Immunological Methods*, vol. 332, no. 1-2, pp. 121-128.

Xu, Y., Pang, G. R., Gao, C. W., Zhao, D. Q., Wang, B. L., Zhou, L. T., Sun, S. T., Du, L. X. and Chen, Z. J. (2009), "Comparison of the activities of silver nitrate with those of three antifungal agents against ocular pathogenic fungi *in vitro*", *Chinese Journal of Ophthalmology*, vol. 45, no. 8, pp. 730-735.

Yafetto, L., Carroll, L., Cui, Y., Davis, D. J., Fischer, M. W. F., Henterly, A. C., Kessler, J. D., Kilroy, H. A., Shidler, J. B., Stolze-Rybczynski, J. L., Sugawara, Z. and Money, N. P. (2008), "The fastest flights in nature: high-speed spore discharge mechanisms among fungi", *PLoS ONE*, vol. 3, no. 9, pp. e3237.

Yang, G. H., Jarvis, B. B., Chung, Y. J. and Pestka, J. J. (2000), "Apoptosis induction by the satratoxins and other trichothecene mycotoxins: relationship to ERK, p38 MAPK, and SAPK/JNK activation", *Toxicology and Applied Pharmacology*, vol. 164, no. 2, pp. 149-160.

Yike, I., Rand, T. and Dearborn, D. G. (2007), "The role of fungal proteinases in pathophysiology of *Stachybotrys chartarum*", *Mycopathologia*, vol. 164, no. 4, pp. 171-181.

Yusuf Mohamed, H. A., Jaffar, A. and Abdul Rehman, H. M. (2008), "Allergic fungal sinusitis", *Emirates Medical Journal*, vol. 26, no. 2, pp. 121-123.

Zhang, X., Zwiers, F. W., Hegerl, G. C., Lambert, F. H., Gillett, N. P., Solomon, S., Stott, P. A. and Nozawa, T. (2007), "Detection of human influence on twentieth-century precipitation trends", *Nature*, vol. 448, no. 7152, pp. 461-465.

7 PUBLICATIONS

ORAL PRESENTATION

Frazer S., Aldred D., Magan N., 2010. Characterisation of *Stachybotrys chartarum* from water damaged buildings. Cranfield Health Postgraduate Conference, September 2010

POSTER PRESENTATIONS

Frazer S., Aldred D., Magan N., 2009. Characterisation of Toxigenic Mould Contamination in Flood Damaged and Energy Efficient Buildings. Cranfield Health Postgraduate Conference, September 2009.

Frazer S., Aldred D., Magan N., 2011. Characterisation of toxigenic fungi in water damaged buildings. IEH Annual UK Review Meeting on Outdoor and Indoor Air Pollution. 2011

PUBLICATIONS

Frazer S., Magan N., Aldred D., 2011. The Influence of Water Activity and Temperature on Germination, Growth and Sporulation of *Stachybotrys chartarum Strains.* Mycopathologia DOI 10.1007/s11046-011-9394-x

Frazer, S; Pestka, J; Kim, J; Medina, A; Aldred, D; Magan, N., 2011. Impact of environmental factors on growth and satratoxin G production by strains of *Stachybotrys chartarum*. Submitted to Mycopathologia.

Frazer S., Lambert R., Aldred D., 2011. Tebuconazole and Other Inhibitors on the growth of *Stachybotrys chartarum*. Due to be submitted August 2011