Significance of Fungi in Water Distribution Systems. (EPG/1/9/69)

Final Report to DWI

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CAB INTERNATIONAL

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Contents

Summary

Objective 1 *To develop, validate and carry out limited performance testing of isolation and culture media and techniques for the detection and quantification of filamentous fungi in water distribution systems.*

Propagule suspensions of thirteen fungal strains were incubated in stirred phosphate buffer for 24 hours, the fungi were then recovered by plating out serial dilutions using up to six different media for each fungus. Viable propagules were recovered for all the fungi. The proportion of propagules recovered varied widely between the fungi. There was no single optimum combination of medium and incubation conditions for recovery of all the fungi. Of the incubation conditions, the longest and warmest conditions produced the highest recovery rates in most of the fungi. Irrespective of incubation conditions, no one medium was responsible for high or low recoveries across the entire range of fungi. Ten of the fungi showed recovery levels which suggest that growth may have occurred in the buffer or that propagules were clumped initially but became more evenly dispersed after 24 hours in the stirred buffer. Recommendations for routine enumeration of fungi in water systems include limiting the incubation conditions to 6 days at 22°C but using an additional close range of low dilutions in conjunction with a tenfold dilution series. Any growth of fungi within a system will be incorporated in the count but the potential for fungal growth in water systems would require further study.

Objective 2 *To carry out limited performance testing of trial media and to investigate the possibility of using enrichment and baiting techniques for the detection and quantification of fungi. To compare results from direct plating and filter methods.*

Two samplings were conducted. Some 141 fungal taxa have been identified. Differences between media, plating techniques and temperature of incubation are apparent. The selected range of conditions used was essential to isolate the many diverse fungal strains. A number of isolates have been found in treated waters only. Scanning Electron Micrographic examination of filters indicates that fungi are equally present as both spores and hyphal fragments.

Objective 3 *To prepare a database of fungi isolated from water distribution systems to include any known health implications.*

A database has been prepared which is ready for transferral.

Objective 4 *To provide results from the above in a format suitable for publication in the Standing Committee of Analysts "Blue Book" series.*

This present report is provided in the appropriate Blue Book format and is available in electronic format.

1. Introduction

Microorganisms are present in source waters used for producing drinking water and may pass through treatment processes. Also, they may arise as contaminants during repair or renovation of water mains or through seepage from the surrounding soil into service reservoirs (Wood *et al.*, 1983). The microbiological examination of drinking water has of necessity focused primarily on the occurrence of bacteria, many of which are indicators of faecal contamination. Extensive monitoring is routinely undertaken to ensure that these bacteria are not present in water supplies and so to ensure that water quality is maintained. Recently, more recognition has been given to other microorganisms, including fungi, about which little is known regarding their implications for health but which may nonetheless act as opportunist pathogens, potential toxin producers or contribute to deterioration in water quality through the production of undesirable tastes, odours and discolouration (Kelstrup *et al.*, 1977). There is now a small number of studies on fungi in water supplies (Anonymous, 1995; Astier *et al.*, 1995; Baylet *et al.*, 1981; Hinzelin & Block, 1985; Nagy and Olson, 1982 & 1985; Niemi *et al.*, 1982; Nyström *et al.*, 1992; Popovska, 1983; Rosenzweig and Pipes, 1987 & 1988 and West, 1986). However, it is premature to regard such studies as exhaustive because the isolation programmes normally use only very limited techniques coupled with media which tend to isolate common, heavily sporulating soil-borne fungi. In the United Kingdom, mycological techniques and media are not mentioned at all in the current industry standard methods handbook, the 'blue book' (Anonymous, 1994), and media and techniques used routinely by water companies to investigate microbiological problems and customer complaints are also limited by the emphasis on bacteria.

Only a small range of selective media have been used to investigate the occurrence of fungi in distribution systems. The most frequent being Sabouraud agar (dextrose-based) which was developed for use in clinical, rather than environmental mycology. Nagy & Olson (1985) found no difference in isolation frequency of filamentous fungi or yeasts from pipe surfaces when using Czapek (sucrose-based), Nutrient (complex nutrients) or Sabouraud agars this may merely indicate that the media were all equally nutrient-rich and therefore selected similar common heavily sporulating fungi. Similarly, neopeptone-dextrose-rose bengal-tetracycline agar was developed for the isolation of fungi from soils, water systems and sewage (Cooke, 1987) and whilst not routinely used in the United Kingdom it is recommended in the USA (Anonymous, 1995). This however, is a very wide variety of substrates from which to isolate using one medium, which is again very rich in nutrients. Overgrowth of fungal colonies by bacteria on isolation plates or filters (Hinzelin & Block, 1985) reduces the recovery of fungi, so antibacterial compounds are usually added to the incubation medium, with streptomycin and chloramphenicol being most commonly used. However, the nature and/or concentrations of some antibacterial substances may inhibit growth of a number of fungi (Buck, 1975; Klein & Wu, 1974). Fungi can also be overgrown by other more rapidly growing fungi, so rose bengal is often added to inhibit growth and improve quantification. This compound however, is by definition toxic to fungi and will often cause under-representation of the variety of species present. Media containing rose bengal must also be stored and incubated in the dark to prevent increased toxicity. All of the media used are far removed from the nutrient conditions which prevail in water distribution systems, they all contain large quantities of free sugars which would be unlikely to occur in water distribution systems. They all

therefore favour the growth of rapid growing fungi normally present as spores (and possibly dormant), rather than indigenous fungi which may have been growing in low nutrient drinking water. Media and techniques employed in the USA (Anonymous, 1995) are more detailed than those in the United Kingdom but the same criticisms of media choice can be levelled in that slow growing fungi present will be missed. Furthermore, oomycetes and other zoosporic fungi are not considered at all.

From a mycological viewpoint, there is a clear need to improve techniques used for sampling fungi from water systems. What is required is a medium capable of supporting the growth of oliogotrophic fungi (assuming distribution systems can be considered to be oligotrophic) and for a comparison to be made with the high carbohydrate media currently in use. A 'distribution system extract' agar, as suggested by Nagy & Olson (1982), might be the ideal isolation medium. West (1986) used media without antibiotics or growth inhibitors on account of the high quality of the water in the transmission and distribution systems in Nevada. Selective media exist for the isolation of potentially toxigenic fungi (Pitt *et al.*, 1983; Beuchat, 1984; Abarca *et al.*, 1988) and for the estimation of mycotoxin levels in solid culture media (Cotty, 1988) and these could be considered for use in fungal isolations from drinking water.

The significance of fungi in water systems is very poorly understood. Some fungi, such as species of Pythium (Middleton, 1943) require an aquatic environment for the motile stages in their life cycle, other fungi are from genera known to contain opportunistic human pathogens and for example include species in the genera *Aspergillus* (Campbell, 1994) and *Phoma* and *Pyrenochaeta* (Punithalingam, 1979). Yet other fungi have been implicated in odour problems, a study by Nyström *et al.* (1992), found that 2,4,6-trichloroanisole (TCA) was the major odour-causing compound in drinking water derived from groundwater (both chlorinated and non-chlorinated), and that there was a significant increase in TCA concentration within the distribution system. They concluded that formation of TCA during distribution was probably due to microbial methylation of 2,4,6-trichlorophenol (TCP), fungi of the genera *Acremonium, Penicillium* and *Phialophora* were found to be capable of producing TCA by methylation of TCP. The TCP was itself formed either by chlorine disinfection or by natural halogenation. A great number of fungi produce mycotoxins (Abarca *et al.*, 1988; Beuchat, 1984; Cole & Cox, 1981 and Miller *et al.*, 1991), but there is little information on the likelihood of these secondary metabolites being produced under the conditions found in distribution systems or whether they would be in sufficient quantities to be significant to human health. The chlorine demand of fungal spores is one to two orders of magnitude greater than that of coliform bacterial cells and hence, if fungi are present, the chlorine residual in the distribution system may be reduced (Rosenzweig *et al.*, 1983). Fungi and yeasts may also contribute to the build up of biofilms which again may reduce residual chlorine efficacy. In addition, sediments in the mains and also pits, joints or cracks in pipework may provide micro-habitats where fungi may be protected from disinfectant in the bulk water, effectively reducing the biocidal efficacy of the disinfectant and meaning that treatment strategies would require re-evaluation. However, the parameters which affect the proliferation of fungi in water supplies and distribution systems have not been investigated and information is required on the factors which promote fungal growth and influence the production of secondary metabolites.

Before any of the concerns regarding the significance of fungi in distribution systems can begin to be addressed, improved sampling techniques for fungi are required. Current methods recommended in the United Kingdom will only detect fast growing fungi or those already present in sufficiently large quantities to be the obvious source of a nuisance. The media and culture techniques employed will miss slow growing fungi as well as those specialist genera which are specifically found in water, and will in fact inhibit the growth of many fungi.

This programme aims to assess methods for both the quantitative recovery of fungi and the isolation of the maximum variety of fungi. The more frequently employed media used by United Kingdom and the USA water researchers have been compared to experimental and routine media used elsewhere. Unlike bacteria there are only a few readily identified fungal pathogens and even fewer species whose significance as mycotoxin producers are understood. Therefore it is not possible to devise media and methods which will only detect fungi with health implications as is possible for bacteria. Media were initially used without antibiotics as these themselves can inhibit growth and recovery rates but it was found early on that overgrowth by bacteria was a greater problem particularly for nutrient-rich media and so were included where necessary. Enrichment and baiting techniques have been designed and implemented to both increase the number of fungal propagules for isolation and to selectively encourage the growth of more fastidious organisms. The methods developed provide systems which estimate the number of propagules present and detect as many different fungi as possible thus allowing their importance to health to be assessed through the database which has been created as part of this project.

2. Materials and Methods

2.1. General

Eight media were selected for investigation in all objectives. The media and the reasons for their selection are shown in Table 1. Ingredients and preparation details are provided in Appendix 1 as are details of routine culture media.

2.2. Objective 1

To develop, validate and carry out limited performance testing of isolation and culture media and techniques for the detection and quantification of filamentous fungi in water distribution systems.

Table 1. Media selected for study.

Medium	Reason selected
Modified Neopeptone Glucose Rose Bengal Aureomycin Agar (NGRBA)	Used in USA for analysis of potable waters (Anonymous, 199?)
Sabouraud Dextrose Agar (SDA)	Used widely in UK food and water industries (e.g Hinzelin & Block, 1985)
Half Strength Potato Carrot Agar (PCA/2)	Used at IMI for water studies involving fungi
Half Strength Cornmeal (CMA/2)	Used at IMI for water studies involving fungi

Oomycete Selective Medium (OSM)	Used at IMI for water studies involving fungi
Wort Agar (WA)	General medium which may have applications in this area
Low Redox Potential Medium (LRM)	To select for fungi able to grow at low oxygen concentrations
Aspergillus flavus and *A. parasiticus* agar (AFPA)	Designed to detect aflatoxin-producing fungi (Beuchat, 1984)

2.2.1 *Approach*

Media employed in standard methods in the UK as well as in other European countries and the USA for isolations from water distribution systems were compared with experimental media. Samples were separately seeded with fungal strains representative of genera known to be present in water systems. Performance of the different media was assessed by comparing numbers of each fungal strain recovered on each different medium used.

2.2.2 *Procedure*

Thirteen fungal strains were selected for recovery and growth studies (Table 2). It had also been the intention to use the sewage fungus *Leptomitus lacteus* (Roth) C. Agardh but a viable culture was not available.

Table 2. Experimental strains.

Fungus name and IMI isolate number	Reason selected	Routine growth medium
Acremonium strictum W. Gams IMI 214832	Genus reported from water samples and associated with off flavours	PCA
Alternaria alternata (Fr.) Keissl. IMI 325055	Genus reported from water samples	PCA
Aspergillus flavus Link IMI 91856	Genus reported from water samples and a potential mycotoxin species	CZA
Aureobasidium pullulans (de Bary) G. Arnaud IMI 45533	Genus reported from water samples	MA
Cladosporium herbarum (Pers.) Link IMI 49630	Genus reported from water samples	MA
Epicoccum nigrum Link IMI 333106	Genus reported from water samples	MA
Fusarium culmorum (W.G. Sm.) Sacc. IMI 314383	Genus reported from water samples and a potential mycotoxin species	SNA
Fusarium solani (Mart.) Sacc. IMI 339173	Genus reported from water samples	SNA
Mucor racemosus Fresen. IMI 199611	Genus reported from water samples	MA
Penicillium brevicompactum Dierckx IMI 17456	Genus reported from water samples and a potential mycotoxin producer	CZA
Phialophora lagerbergii (Melin & Nannf.) Conant IMI 209404	Genus reported from water samples and associated with off flavours	PCA
Pythium ultimum Trow IMI 82514	Oomycete genus capable of growth in aquatic environments	OA
Trichoderma viride Pers. IMI 238903	Genus reported from water samples and a secondary metabolite producer	PCA

Stock cultures were grown on the routine media shown in Table 2 using 9 cm Petri dishes at room temperature. Phosphate buffer was chosen as the medium for seeding with fungal propagules (spores and hyphal fragments), which while not necessarily representing distribution systems provided a standard for comparing media.

Propagule suspensions were prepared for each fungal strain by adding approximately 10 ml phosphate buffer (3.5 g Na_2HPO_4 and 1.5 g KH_2PO_4 in 1 l distilled water, sterilized by autoclaving at 121°C for 15 minutes, final pH 7.2) containing 0.5% ($^{w}/_{v}$) Tween 80 as a wetting agent to a stock culture and scraping the culture surface with a sterile inoculating loop. The suspended propagules were pipetted off and washed by centrifugation (three times at 3000 rpm for 3 minutes and resuspended in approximately 5 ml phosphate buffer each time). Suspensions were adjusted to give between 10^7 and 10^{11} propagules ml^{-1} depending on the growth of each fungus. A 1 ml aliquot from each suspension was plated immediately onto PCA/2 as a precautionary viability check. For each fungal strain 1 ml of propagule suspension was added to 99 ml of phosphate buffer to give a final concentrations of between 10^5 and 10^9propagules.ml^{-1}. These were then stirred at room temperature for 24 hours to simulate residence time in a distribution system. After which, each was serially diluted tenfold with phosphate buffer to give dilutions of 10^0 to 10^{-6} and 1 ml of each dilution was incorporated into pour-plates of CMA/2, LRM, NGBRA, PCA/2 and SDA in 9 cm Petri dishes replicated five times. The potential mycotoxin-producing fungi (*A. flavus*, *F. culmorum* and *P. brevicompactum*) were additionally plated onto AFPA. *P. ultimum* was additionally plated onto OSM. Plates were incubated at 12 and 22 °C in the dark and counted after 3 days and again after 6 days.

2.3. Objective 2

To develop, validate and carry out limited performance testing of sampling and enrichment methods for the detection and quantification of filamentous fungi as planktonic organisms in water distribution systems

2.3.1 Approach

Samples of surface waters and treated tap waters were used. They were examined for the presence of fungi by using a variety of isolation techniques including direct plating onto agar media, concentration prior to plating by using filters, baiting of the samples with a range of substrates and adding aliquots of the samples to enrichment broths. A selected range of media, incubation temperatures and sampling times were used. The techniques were assessed in terms of the range of fungi detected, and in conjunction with Objective 1 in terms of quantifying fungi in water systems by a most probable number technique.

2.3.2. Procedure

Water samples were obtained from ten local surface waters and ten treated tap water supplies, the details of which are included in Table 4, and in all represent seven water companies. One litre samples were taken using pre-sterilised bottles dosed with sodium thiosulphate, following the methods recommended in Report 71. Two sets of samples were taken from each site, the first during March 1996, the second during July 1996. Each sample was treated as follows;

I. Aliquots of 1 ml were plated onto each of the eight media listed in Table 1 in duplicate 9 cm petri dishes. A plate of each medium was incubated at 12 and 22°C. Counts were made at 3, 5, 7 and 10 days and isolates were identified.

II. 100 ml aliquots were passed through pre-sterilised 0.45µm millipore filters, separate filters were plated onto SDA and CMA/2 in 9 cm petri dishes and incubated at 12 and 22°C, these were counted after 3, 5, 7 and 10 days and the isolates identified. A further filter was plated onto PCA/2 and incubated at 5°C to detect psychrophilic fungi, counts were made after 10 and 21 days and the organisms isolated and identified. The second round of sampling was slightly modified in that all eight media in Table 1 were used at both 12 and 22°C but PCA/2 at 5°C was not used.

III. Two 100 ml aliquots were placed in separate conical flasks and each was baited with several 0.5 cm squares of sterile grass and snake skin as well as hemp seeds in order to isolate zoosporic fungi. One flask was incubated at 12°C and one at 22°C for up to 5 days. Baits with suspected colonies were removed aseptically and plated on to OSM in 9 cm petri dishes for identification.

IV. Using tap waters only, 10 ml aliquots were added to 90 ml SDA and PCA broths in duplicate (to prepare broths simply omit the agar component of the recipes given in Appendix 1). One set was incubated at 22°C for 48 hours and the other set was incubated at 12°C for 72 hours both with shaking. After the incubation period, 1 ml aliquots were then separately plated onto their equivalent agar media and incubated at the respective temperatures. Counts were made at 3, 5, 7 and 10 days and the organisms isolated and identified.

It was initially proposed that this sampling exercise be performed three times to catch seasonal variations. However, point II described above was enlarged as indicated from the initial sampling to include plating of filters onto the all eight test media. It was consequently felt that adequate data would be obtained from just two sampling exercises.

As part of the second sampling it was also decided to observe the nature of the fungal propagules retained on the isolation filters by using scanning electron microscopy (SEM). For each sample tested a 1 cm square piece of filter was mounted on a stub with double-sided adhesive tape, sputter coated with gold for 3 minutes at 25 milliamps and examined at a magnification of x1000 (at approximately 15 kV). The entire piece of filter was examined and counts made of fungal spores and hyphal fragments.

2.4. Objective 3

To prepare a database of fungi isolated from water distribution systems to include any known health implications.

A database has been produced according to the format agreed in the Six Month Report, details of which appear in Appendix 2.

3. Results and Discussions

3.1. Objective 1

In total 13 fungal strains were examined. The results are shown in Table 3. Viable propagules were recovered for all of the fungi.

Table 3. Recovery of fungal propagules from phosphate buffer in stirred flasks incubated for 24 hours at room temperature. Figures are propagules.l^{-1}. The data shown for each treatment is from the greatest dilution which did not produce any replicates giving a zero count or failing which the greatest dilution giving the lowest mean count from the highest number of replicates not returning a zero count.

	3 days		6 days	
	12°C	22°C	12°C	22°C
Acremonium strictum concentration in flask 1.2×10^9, viable count 1.8×10^8				
NGRBA	$0.3 \pm 0.1 \times 10^5$	$5.6 \pm 3.9 \times 10^8$	$5.2 \pm 4.0 \times 10^8$	$5.8 \pm 3.7 \times 10^8$
SDA	$0.4 \pm 0.2 \times 10^5$	$7.0 \pm 2.2 \times 10^8$	$4.6 \pm 2.7 \times 10^8$	$7.2 \pm 2.4 \times 10^8$
LRM	$0.1 \pm 0.02 \times 10^5$	$4.8 \pm 0.8 \times 10^8$	$4.4 \pm 3.2 \times 10^8$	$4.8 \pm 0.8 \times 10^8$
PCA/2	$0.1 \pm 0.02 \times 10^6$	$6.8 \pm 2.2 \times 10^8$	$4.1 \pm 4.7 \times 10^8$	$1.6 \pm 0.6 \times 10^9$
CMA/2	$0.1 \pm 0.1 \times 10^5$	$2.8 \pm 1.8 \times 10^9$	$5.6 \pm 4.5 \times 10^8$	$3.2 \pm 2.2 \times 10^9$
Alternaria alternata concentration in flask 5.1×10^5, viable count 9.0×10^4				
NGRBA	0	$0.2 \pm 0.2 \times 10^4$	$0.1 \pm 0.2 \times 10^4$	$0.2 \pm 0.2 \times 10^4$
SDA	0	$0.1 \pm 0.1 \times 10^4$	$0.4 \pm 1.0 \times 10^3$	$0.2 \pm 1.0 \times 10^5$
LRM	0	$0.2 \pm 1.0 \times 10^5$	0	$0.2 \pm 1.0 \times 10^5$
PCA/2	0	0	0	$0.2 \pm 0.1 \times 10^4$
CMA/2	0	$0.3 \pm 0.2 \times 10^4$	0	$0.4 \pm 0.2 \times 10^4$
Aspergillus flavus concentration in flask 7.2×10^8, viable count 2.0×10^7				
NGRBA	0	$0.6 \pm 0.2 \times 10^7$	0	$0.7 \pm 0.3 \times 10^7$
SDA	0	$1.8 \pm 0.8 \times 10^7$	0	$1.8 \pm 0.8 \times 10^7$
LRM	0	$1.2 \pm 0.5 \times 10^7$	0	$1.2 \pm 0.5 \times 10^7$
PCA/2	0	$2.4 \pm 1.1 \times 10^7$	0	$6.0 \pm 6.8 \times 10^7$
CMA/2	0	$0.6 \pm 0.1 \times 10^7$	$0.2 \pm 0.5 \times 10^4$	$1.2 \pm 1.3 \times 10^7$
AFPA	0	$0.7 \pm 0.3 \times 10^7$	0	$0.7 \pm 0.3 \times 10^7$
Aureobasidium pullulans concentration in flask 2.5×10^6, viable count 6.2×10^5				
NGRBA	0	$1.0 \pm 0.2 \times 10^7$	$0.9 \pm 0.4 \times 10^7$	$0.4 \pm 0.2 \times 10^7$
SDA	0	$0.1 \pm 0.1 \times 10^7$	$0.1 \pm 0.1 \times 10^7$	$0.2 \pm 0.1 \times 10^7$
LRM	0	$0.3 \pm 0.4 \times 10^5$	$0.2 \pm 0.1 \times 10^6$	$0.3 \pm 0.3 \times 10^7$
PCA/2	$0.1 \pm 0.1 \times 10^6$	$0.1 \pm 0.1 \times 10^7$	$0.1 \pm 0.01 \times 10^7$	$0.7 \pm 0.5 \times 10^7$
CMA/2	0	$1.1 \pm 0.3 \times 10^7$	$0.7 \pm 0.3 \times 10^7$	$1.1 \pm 0.3 \times 10^7$

Table 3. continued

Cladosporium herbarum concentration in flask 1.0×10^6, viable count 2.0×10^6				
NGRBA	0	0	$0.4 \pm 1.0 \times 10^6$	$0.2 \pm 0.4 \times 10^4$
SDA	0	$0.1 \pm 0.3 \times 10^5$	$0.1 \pm 0.1 \times 10^6$	$0.1 \pm 0.1 \times 10^6$
LRM	0	$0.2 \pm 1.0 \times 10^6$	0	$0.2 \pm 0.5 \times 10^6$
PCA/2	$0.2 \pm 0.1 \times 10^5$	$6.7 \pm 4.2 \times 10^7$	$0.3 \pm 0.1 \times 10^5$	$0.1 \pm 0.1 \times 10^6$
CMA/2	0	$0.4 \pm 0.4 \times 10^6$	$3.2 \pm 3.2 \times 10^7$	$0.1 \pm 0.04 \times 10^7$
Epicoccum nigrum concentration in flask 3.4×10^5, viable count 3.0×10^5				
NGRBA	0	0	$9.8 \pm 19.2 \times 10^8$	$0.4 \pm 0.6 \times 10^7$
SDA	0	$0.2 \pm 0.5 \times 10^7$	$0.2 \pm 1.0 \times 10^5$	$2.0 \pm 5.0 \times 10^7$
LRM	0	$8.8 \pm 12.2 \times 10^8$	0	$8.8 \pm 12.2 \times 10^8$
PCA/2	0	$2.4 \pm 1.8 \times 10^8$	0	$3.2 \pm 1.6 \times 10^8$
CMA/2	0	$2.0 \pm 1.1 \times 10^8$	$4.6 \pm 6.0 \times 10^8$	$2.6 \pm 1.5 \times 10^8$
Fusarium culmorum concentration in flask 3.1×10^8, viable count 2.5×10^6				
NGRBA	0	$0.1 \pm 0.1 \times 10^6$	$0.3 \pm 0.3 \times 10^7$	$0.3 \pm 0.3 \times 10^6$
SDA	$0.1 \pm 0.1 \times 10^4$	$0.1 \pm 0.1 \times 10^7$	$1.1 \pm 1.6 \times 10^7$	$0.8 \pm 0.3 \times 10^5$
LRM	$0.1 \pm 0.1 \times 10^4$	$0.4 \pm 0.3 \times 10^5$	$0.4 \pm 0.4 \times 10^5$	$0.1 \pm 0.1 \times 10^7$
PCA/2	$0.2 \pm 0.4 \times 10^4$	$0.1 \pm 0.03 \times 10^5$	$0.1 \pm 0.1 \times 10^6$	$0.1 \pm 0.03 \times 10^5$
CMA/2	$0.1 \pm 0.1 \times 10^5$	$0.3 \pm 0.2 \times 10^6$	$0.4 \pm 0.3 \times 10^6$	$0.4 \pm 0.1 \times 10^6$
AFPA	0	$0.2 \pm 0.2 \times 10^4$	$0.1 \pm 0.1 \times 10^5$	$0.1 \pm 0.1 \times 10^5$
Fusarium solani concentration in flask 1.8×10^6, viable count 3.6×10^5				
NGRBA	0	$0.1 \pm 0.02 \times 10^7$	$0.2 \pm 0.4 \times 10^4$	$0.1 \pm 0.02 \times 10^5$
SDA	0	$0.1 \pm 0.02 \times 10^5$	$0.2 \pm 1.0 \times 10^4$	$0.1 \pm 0.02 \times 10^5$
LRM	0	0	0	$0.2 \pm 0.4 \times 10^4$
PCA/2	0	$0.3 \pm 0.2 \times 10^5$	$0.1 \pm 0.1 \times 10^5$	$0.1 \pm 0.02 \times 10^6$
CMA/2	0	$0.1 \pm 0.1 \times 10^5$	$0.1 \pm 0.1 \times 10^5$	$0.1 \pm 0.03 \times 10^6$
Mucor racemosus concentration in flask 8.8×10^5, viable count 3.0×10^4				
NGRBA	$0.1 \pm 0.02 \times 10^7$	$0.1 \pm 0.04 \times 10^7$	$0.1 \pm 0.02 \times 10^7$	$0.1 \pm 0.04 \times 10^7$
SDA	$0.1 \pm 0.1 \times 10^7$	$3.2 \pm 0.9 \times 10^7$	$1.1 \pm 1.4 \times 10^9$	$3.1 \pm 0.7 \times 10^9$
LRM	$0.1 \pm 0.01 \times 10^7$	$3.8 \pm 6.0 \times 10^7$	$2.9 \pm 3.0 \times 10^7$	$2.0 \pm 5.0 \times 10^7$
PCA/2	$0.1 \pm 0.1 \times 10^6$	$0.1 \pm 0.1 \times 10^6$	$0.3 \pm 0.3 \times 10^7$	$1.1 \pm 2.2 \times 10^7$
CMA/2	$0.1 \pm 0.1 \times 10^6$	$0.1 \pm 0.04 \times 10^7$	$0.3 \pm 0.1 \times 10^6$	$0.1 \pm 0.04 \times 10^7$
Penicillium brevicompactum concentration in flask 4.2×10^8, viable count 7.5×10^7				
NGRBA	$0.2 \pm 0.1 \times 10^6$	$9.8 \pm 1.3 \times 10^7$	$7.0 \pm 4.3 \times 10^7$	$9.8 \pm 1.3 \times 10^7$
SDA	$0.2 \pm 0.1 \times 10^6$	$5.0 \pm 3.4 \times 10^7$	$7.6 \pm 2.0 \times 10^7$	$5.2 \pm 3.8 \times 10^7$
LRM	$0.3 \pm 1.0 \times 10^4$	$1.2 \pm 0.5 \times 10^8$	$4.8 \pm 2.6 \times 10^7$	$1.2 \pm 0.5 \times 10^8$
PCA/2	$0.3 \pm 0.2 \times 10^6$	$7.8 \pm 2.6 \times 10^7$	$6.4 \pm 2.7 \times 10^7$	$8.0 \pm 3.6 \times 10^7$
CMA/2	0	$7.8 \pm 2.6 \times 10^7$	$3.4 \pm 2.1 \times 10^7$	$1.1 \pm 0.3 \times 10^8$
AFPA	$0.9 \pm 0.3 \times 10^7$	$7.6 \pm 2.5 \times 10^7$	$5.4 \pm 2.7 \times 10^7$	$8.4 \pm 3.2 \times 10^7$
Phialophora lagerbergii concentration in flask 2.1×10^9, viable count 5.1×10^8				
NGRBA	0	$0.3 \pm 0.3 \times 10^6$	$0.4 \pm 0.1 \times 10^5$	$2.6 \pm 1.6 \times 10^7$
SDA	0	$0.2 \pm 0.2 \times 10^5$	$0.3 \pm 0.4 \times 10^5$	$0.4 \pm 0.8 \times 10^7$
LRM	0	$0.1 \pm 0 \times 10^5$	$0.1 \pm 0.02 \times 10^5$	$0.1 \pm 0 \times 10^5$
PCA/2	$0.1 \pm 0.3 \times 10^4$	$0.1 \pm 0.1 \times 10^6$	$0.2 \pm 0.1 \times 10^7$	$0.1 \pm 0.1 \times 10^7$
CMA/2	0	$0.2 \pm 1.0 \times 10^3$	$0.3 \pm 0.2 \times 10^6$	$0.2 \pm 0.4 \times 10^6$

Table 3. continued

Pythium ultimum	concentration in flask 1.6×10^6, viable count 2.3×10^5			
NGRBA	0	$0.2 \pm 0.1 \times 10^6$	$0.3 \pm 0.1 \times 10^6$	$0.8 \pm 1.0 \times 10^6$
SDA	0	$0.6 \pm 0.9 \times 10^7$	$0.3 \pm 1.0 \times 10^6$	$0.4 \pm 1.0 \times 10^6$
LRM	0	$0.4 \pm 1.0 \times 10^4$	$0.1 \pm 0.2 \times 10^6$	$0.2 \pm 0.2 \times 10^7$
PCA/2	0	$0.2 \pm 0.1 \times 10^5$	$0.1 \pm 0.04 \times 10^5$	$0.2 \pm 0.1 \times 10^7$
CMA/2	0	$0.3 \pm 1.0 \times 10^4$	$0.1 \pm 0.3 \times 10^7$	$0.2 \pm 0.3 \times 10^5$
OSM	0	$0.1 \pm 0.1 \times 10^5$	0	$0.6 \pm 0.9 \times 10^7$
Trichoderma viride	concentration in flask 1.2×10^5, viable count 5.0×10^4			
NGRBA	0	$0.1 \pm 0.03 \times 10^7$	$0.7 \pm 0.3 \times 10^7$	$0.4 \pm 0.9 \times 10^7$
SDA	0	$0.4 \pm 0.3 \times 10^6$	$0.2 \pm 0.1 \times 10^5$	$1.1 \pm 2.3 \times 10^8$
LRM	0	$0.5 \pm 0.3 \times 10^7$	$0.1 \pm 0.1 \times 10^6$	$1.1 \pm 0.3 \times 10^7$
PCA/2	0	$0.1 \pm 0.02 \times 10^6$	$0.1 \pm 0.01 \times 10^7$	$0.4 \pm 0.3 \times 10^7$
CMA/2	$0.2 \pm 1.0 \times 10^5$	$0.2 \pm 0.1 \times 10^6$	$0.2 \pm 0.1 \times 10^5$	$0.7 \pm 0.2 \times 10^6$

Phialophora lagerbergii was recovered at the lowest levels overall with the highest recovery on modified NGRBA after 6 days at 22°C representing only 5% of the initial viable count and recoveries after all the other treatments were 1% or less.

Alternaria alternata and *Fusarium solani* were also recovered at relatively low levels. *A. alternata* was recovered on LRM after 3 days at 22°C and on both SDA and LRM after 6 days at 22°C at only 22% of the initial viable count while recoveries after all the other treatments were 5% or less. *F. solani* was recovered on NGRBA after 6 days at 22 °C at a somewhat anomalously high level representing 278% of the initial viable count but on both PCA/2 and CMA/2 after 6 days at 22°C at much lower level of 28% and all the remaining treatments gave recoveries that were only 0-8%.

Aspergillus flavus, *Cladosporium herbarum* and *Fusarium culmorum* were all recovered at generally low but somewhat erratic levels. *A. flavus* showed virtually no recovery at 12°C with only CMA/2 after 6 days producing a non-zero result and this represented less than 0.5% of the initial viable inoculum, most other treatments yielded recoveries of 30-90% with the exception of PCA/2 which yielded 120% after 3 days and later 300% after 6 days at 22 °C. *C. herbarum* showed virtually no recovery after 3 days at 12°C and after the other treatments was recovered at levels of 0-50% with the exceptions of PCA/2 after 3 days at 22 °C and CMA/2 after 6 days at 12 °C where the recoveries were both in excess of 1000% of the initial inoculum. *F. culmorum* was recovered on NGRBA and SDA after 6 days at 12 °C at levels representing well in excess of 100%, and on both SDA after 3 days at 22 °C and LRM after 6 days at 22°C at 40% but all other treatments yielded only 0-16% of the initial viable count.

Penicillium brevicompactum showed the closest to an idealised normal recovery pattern, after 3 days at 12 °C only 0-12% of the initial viable count was recovered but after 6 days at 12 °C 64-101% was recovered, whereas after 3 days at 22 °C the recovery was 67-160% which after 6 days at 22 °C was virtually unchanged at 69-160%.

Acremonium strictum, *Aureobasidium pullulans*, *Epicoccum nigrum*, *Pythium ultimum* and *Trichoderma viride* all showed very low recovery of the initial viable count after 3 days at 12 °C but after the other treatments they generally showed recoveries vastly in excess of 100% with the following exceptions; *A. pullulans* on LRM after 6 days at 22 °C and after 3 days at 12 °C where the recoveries were respectively 5% and 32% of the initial viable count, *E. nigrum* was not recovered on NGRBA after 6 days at 22 °C nor on LRM nor PCA/2 after 3 days at 12°C and on SDA after 3 days at 12 °C was only recovered at 7% of the initial viable inoculum, *P. ultimum* on LRM, PCA/2 and CMA/2 after 3 days at 22°C and on CMA/2 after 6 days at 22 °C was recovered at 10% or less of the initial viable inoculum and *T. viride* on SDA and CMA/2 after 6 days at 12°C was recovered in both cases at 40%.

Mucor racemosus was recovered after all treatments vastly in excess of the intial viable inoculum.

As perhaps might be expected from such a diverse selection of fungi, the proportion of propagules recovered varied widely between the fungi. There was no single treatment (i.e. combination of growth medium, incubation time and temperature) which gave optimal recovery for all the fungi. Nonetheless some general points can be made; of the incubation conditions, the longest and warmest (which was 6 days at 22°C) produced the highest recovery rates in most of the fungi, whereas the shortest and coldest (which was 3 days at 12°C) produced the lowest recovery rates for all the fungi. Irrespective of incubation conditions, no one medium was responsible for high or low recoveries across the entire range of fungi. Ten of the fungi showed some or all recoveries greatly in excess of 100% of the initial viable inoculum, which suggests that growth may have occurred in the phosphate buffer or that propagules were clumped together in initial suspensions but became more evenly dispersed after 24 hours in the stirred buffer.

It was felt that the data obtained was too erratic to enable meaningful analysis of variance or most probable number estimates to be calculated and would be unlikely to add any further information. This may be a consequence of fungal growth or propagule dispersal which caused some dilution series to show inconsistent concentrations. It graphically illustrates the problems of attempting to quantify filamentous fungi by straightforward counting techniques.

3.1.1. Recommendations

If enumeration of fungal propagules must be attempted from field samples it will best be achieved by using the same range of media but the incubation conditions can be limited to just 3 and 6 days at 22°C. Obviously samples should be processed within a very short time after collection and certainly within 24h unless maintained in cold storage. It may be preferable to use a closer range of low dilutions such as a series of fivefold or even twofold dilutions in conjunction with a tenfold dilution series as there can sometimes be a sudden cut-off in counts from the 10^0 to the 10^{-1} dilution. The problem of recovering higher numbers than initially seeded is unlikely to affect field sampling as propagules in field samples should naturally be well dispersed unlike laboratory conditions where they are freshly harvested and initially at extremely high densities, besides which it is highly unlikely that the presence and quantities of fungi within a water system could ever be related to the exact natural inoculum source. Any growth of fungi within a system will be

incorporated in the count but the potential for fungal growth in water systems would require further study.

3.2. Objective 2

Table 4 summarizes the numbers of taxa isolated from each site at each sampling time. Fungi were present in all samples and in total 141 different fungal taxa were isolated. Similar numbers of taxa were isolated at each sampling, i.e. 98 were isolated at the first and 87 at the second, however 44 of those isolated at the second sampling had already been isolated in the first sampling. Hence, only about 50% of the taxa of the second sampling were newly isolated. Assuming that any further samplings would also only isolate 50% of the previous sampling then the 141 isolated by two samplings represents about 70% of a potential total of about 200 taxa and to isolate 95% would require 6 samplings. This would be a very conservative estimate of both total diversity and requisite sequential samplings as it relies on the unlikely assumptions that all fungi are present in equal numbers and that they are all equally likely to be isolated by the methods used.

Table 4. Numbers of different fungal taxa obtained from each site and sample

N°	Surface water or treated water sample	Sample site	Number of different taxa in first sample	Number of different taxa in second sample (number of taxa in common with first sampling)	Number of different taxa in both samples combined
1	Surface	River Thames near Staines Bridge	22	14 (7)	29
2	Surface	River Crane at Twickenham	18	16 (7)	27
3	Surface	Sunningdale	28	11 (6)	33
4	Surface	Virginia Water Lake	20	24 (6)	38
5	Surface	Basingstoke Canal	21	18 (5)	34
6	Treated	Thames Water region	5	3 (0)	8
7	Treated	Mid Surrey Water region	3	3 (0)	6
8	Treated	Three Valleys Water region	6	9 (2)	13
9	Treated	East Surrey region	5	4 (1)	8
10	Treated	Southern Water region	13	5 (1)	17
11	Surface	River Medway, Kent	21	15 (6)	30
12	Surface	Henley Park Lake, Surrey	17	15 (4)	28
13	Surface	Stream, 937515	15	20 (5)	30
14	Surface	Pond, Royal Holloway College	23	18 (5)	36
15	Surface	River Mole, near Box Hill, Surrey	19	23 (8)	34
16	Treated	Rickmansworth, Three Valleys region	0	6 (0)	6
17	Treated	North Surrey Water Region	1	6 (0)	7
18	Treated	Harpenden, Three Valleys region	2	7 (0)	9
19	Treated	IMI, North Surrey Water region	7	7 (1)	13
20	Treated	Severn Trent Water region	1	6 (1)	6
		All samples	98	87 (44)	141

The above 50% discovery rate in the second sampling is evident even if just the treated waters are considered; 46 different taxa were isolated from treated waters overall, of which 30 were isolated at the first sampling, whereas the second sampling added a further 16 taxa.

The average number of taxa present in the surface water samples was 32 (from Table 4), this is about three times greater than the average of the treated water samples which was 9. This expected lower number of taxa in treated waters was relatively consistent at between 6 and 17 taxa.

Table 5 illustrates taxa isolated by different methods and different media. In terms of total numbers of different taxa, the largest number of taxa were isolated on SDA followed by NGRBA, these two media together accounted for 99 of the 141 taxa isolated. To some extent this was expected as both SDA and NGRBA are media known to select for common, heavily sporing organisms. SDA isolations produced 3 *Aspergillus* and 14 *Penicillium* spp. out of 74 isolates (23%) and NGRBA isolations produced 6 *Aspergillus* and 16 *Penicillium* spp. out of 59 isolates (37%). Whereas the next medium in terms of numbers of taxa, OSM, produced only 1 *Aspergillus* and 4 *Penicillium* spp. from 50 isolates (12%). These are the genera which are particularly over represented using current techniques.

Table 5. Comparison of numbers of different taxa obtained using direct plating, incubated filters and baiting methods and a range of media. Results from all samples combined (figures in brackets are numbers of taxa also isolated by other methods).

Medium	Baiting	Direct plating	Incubated Filters	All methods
AFPA	na	28	13	35
CMA/2	na	31	22	41
LRM	na	15	12	25
NGRBA	na	52	16	59
OSM	44	12	7	50
PCA/2	na	31	17	38
SDA	na	51	48	74
WA	na	35	12	42
TWA*	na	4	2	6
All media	44 (34)	109 (61)	72 (53)	141

* Tap water agar, only used in the second round of sampling

SDA and LRM appeared to select the same number of taxa by both isolation methods (Table 5), whereas numbers isolated on AFPA, NGRBA OSM, PCA/2 and WA (and to a lesser extent CMA/2) were much higher for direct plating than for incubated filters. None of the taxa isolated on AFPA showed the characteristic red pigmentation in the medium indicating toxin production.

Regardless of medium a larger range of fungi was isolated by direct plating than from incubated filters or by baiting (Table 5). However when looking at taxa isolated solely by one method, 61 of the 109 isolated by direct plating were also isolated by at least one of the other methods leaving 48 taxa which were isolated solely by direct plating, likewise 19 were isolated solely from incubated filters and 10 solely by baiting giving a total of 77

taxa which were isolated by only one method. Hence, incubated filters contributed 25% and baiting 13% of the taxa isolated by a single method, so that one method alone should not be employed.

With treated waters where fewer propagules are present the figures for isolations by direct plating and incubated filters are closer to parity; overall 30 taxa were isolated by direct plating from treated waters compared to 26 by incubated filters.

The nature of the fungal propagules present in the samples was investigated by examination of filters by using SEM (Table 6) which showed that there was a slightly higher number of hyphal fragments than spores, suggesting that viable hyphae were present in the distribution systems.

Table 6. Numbers of propagules observed on 1 cm^2 pieces of filter paper used in the isolation of fungi from treated water samples determined by SEM.

Site	Number of spores	Number of hyphal fragments
1	0	4
6	3	2
7	2	10
8	3	3
9	4	4
16	0	0
17	2	1
18	0	1

The full list of fungi isolated is presented in Table 7. This represents the most comprehensive study of fungi from water samples. Some general points can be made on the 141 taxa isolated; of the well-represented genera *Aspergillus* species were mostly isolated by direct plating at 22°C on NGRBA, *Cladosporium* species were isolated by virtually any treatment except that only one isolation was made by baiting, *Fusarium* species somewhat unexpectedly were often isolated using baits at 12°C, *Mucor* species were often isolated from incubated filters at 22°C, *Penicillium* species were isolated on virtually any medium but were isolated predominantly by direct plating (it may be that filtered sampling selects against *Penicillium* species possibly due to charges on the conidia or to the hydrophobic nature of the conidial coats of this genus but there is no evidence to support this), *Phoma* species were mostly isolated at 22°C and *Trichoderma* species were isolated by virtually any treatment. It is difficult to make general points on the more numerous but lesser-represented genera other than to note that OSM was used in many isolations and at the same time largely selected against *Aspergillus* and *Penicillium* species. Incubation at 5°C appeared to select only psycrotolerant rather than psychrophilic strains as these were also isolated at the other temperatures used, and hence 5°C was not used for the second sampling. It is important to note that the range of fungi obtained would not have been so great if only a more limited selection of media and treatments had been used. Indeed, despite the great number of isolations made (858 in total) it is surprising that only one *Verticillium* was obtained and two important genera, namely *Phytophthora* and *Pythium*, were absent. This would suggest that if even more isolations were made then yet more fungal taxa would be identified.

Table 7. List of all fungal taxa isolated with synopsis of methods and media.

Name	Sites from which isolated on first sampling	Sites from which isolated on second sampling	Isolation method	Isolation medium	°C	Bait
Absidia cylindrospora		2 5 14	direct filter	LRM NGRBA PCA/2 SDA	12 22	
Absidia glauca	11 15	14	baited filter	CMA/2 SDA OSM	22	grass
Acremonium spp.	4 5 8 11 15	4 8 11	baited direct filter	AFPA CMA/2 NGRBA OSM PCA/2 SDA	12 22	snake skin
Acremonium simplex		8	direct	NGRBA	12	
Alternaria sp.		14	baited	OSM	12	hemp seed
Alternaria alternata	10 11	11 14	baited direct filter	OSM PCA/2 WA	22	grass snake skin
Alternaria infectoria	3	4	direct	NGRBA SDA	22	
Apiospora montagnei	14		direct	AFPA SDA	12	
Arthrinium phaeospermum	4 11 13 14		direct filter	AFPA CMA/2 NGRBA PCA/2 SDA WA	12 22	
Arthrographis cuboidea	2	11	baited direct filter	NGRBA OSM SDA	12 22	hemp seed
Ascochyta spp.		15 16 18	baited direct filter	LRM NGRBA OSM PCA/2	12 22	grass
Aspergillus niger		15	direct	SDA	22	
Aspergillus aculeatus	3 4		direct	NGRBA	22	
Aspergillus clavatus	3 4	12	direct filter	NGRBA	22	
Aspergillus flavus	1 3 4 5 6		direct	NGRBA WA	22	
Aspergillus fumigatus	4	13 17	direct	OSM WA	22	
Aspergillus japonicus	3		direct	NGRBA	22	
Aspergillus parvulus	12		direct	NGRBA	22	
Aspergillus terreus	9 12		direct filter	CMA/2 LRM PCA/2 SDA	22	
Aspergillus versicolor	13 18	11	direct filter	NGRBA SDA	22	
Asteroma sp.		4	direct	NGRBA	22	
Asteromella sp.		12	direct	NGRBA	22	
Aureobasidium sp.		16	filter	SDA	22	

Table 7. continued

Beauvaria bassiana	3		direct	WA	22	
Beauvaria brongniartii	1		direct	OSM	22	
Botrytis cinerea	4 15	2 5 8 11 14 20	baited direct filter	NGRBA OSM PCA/2 SDA WA	12 22	grass
Chaetocladium brefeldii	1		direct	SDA	12	
Chaetomium globosum	10		filter	SDA	22	
Cladosporium spp.	14	10 15 17	direct filter	CMA/2 NGRBA SDA WA	12 22	
Cladosporium cladosporioides	3 4 10 11 14	3 7 8 11 14 15 16 18 19	direct filter	AFPA CMA/2 LRM NGRBA PCA/2 SDA WA	5 12 22	
Cladosporium herbarum	10 12	1 4 6 8 9 10 11 13 16 18 19 20	baited direct filter	AFPA CMA/2 NGRBA OSM PCA/2 SDA WA	12 22	hemp seed
Cladosporium sphaerospermum	20	20	filter	SDA	12 22	
Cyclothyrium sp.		12	direct	SDA	22	
Cylindrocarpon magnusianum	1		baited	OSM	12	hemp seed
Cytospora spp.	1 2 12 15	15	direct filter	AFPA CMA/2 NGRBA SDA WA	22	
Discosporium sp.	13		direct	AFPA CMA/2 SDA	12	
Embellisia sp.		4	direct	AFPA PCA/2	22	
Epicoccum nigrum	14	4 5 12 14	baited direct filter	CMA/2 NGRBA OSM PCA/2 SDA TWA	5 12 22	grass hemp seed snake skin
Eupenicillium sp.		15	direct	WA	22	
Exophialia jeanselmei	18		filter	CMA/2	22	
Fusarium spp.		1 4 14	baited filter	AFPA LRM OSM	12 22	snake skin
Fusarium aquaeductuum var. *aquaeductuum*		13	baited	OSM	12	hemp seed
Fusarium avenaceum	1 15	1 2 5 11 13	baited direct filter	AFPA CMA/2 LRM NGRBA OSM PCA/2 TWA WA	12 22	hemp seed snake skin
Fusarium culmorum	15	3 4 5 11 12 13 14 15	baited direct filter	AFPA CMA/2 LRM OSM NGRBA PCA/2 SDA	12 22	grass snake skin
Fusarium diamini		10	baited	OSM	12	grass snake skin
Fusarium equiseti		3	direct	NGRBA	12	
Fusarium flocciferum		4	direct	PCA/2	12	

Table 7. continued

Fusarium moniliforme		2	direct	LRM	12	
Fusarium oxysporum		6 7 14	direct filter	CMA/2 PCA/2 SDA	12 22	
Fusarium cf *oxysporum*		6 10	baited direct	CMA/2 OSM PCA/2 SDA	12 22	hemp seed snake skin
Fusarium solani		1 8	baited direct filter	LRM OSM	22	grass
Fusarium torulosum		4	direct	CMA/2	22	
Geotrichum sp.		15	baited	OSM	12	hemp seed
Geotrichum candidum	1 2 3	2 12	baited direct filter	AFPA LRM OSM SDA WA	12 22	grass snake skin
Gliocladium sp.		13	baited	OSM	22	snake skin
Gliocladium roseum	19	2 3	baited direct filter	AFPA OSM SDA	22	hemp seed
Gongronella butleri	2	2	direct	NGRBA	12 22	
Hormonema dematioides	12		direct	WA	12	
Leptodontidium sp.		13	baited	OSM	12	snake skin
Leptodothiorella sp.	14		filter	SDA	22	
Leptosphaeria coniothyrium	4 11		direct	CMA/2 SDA	22	
Leptosphaeria fuckelii		13 15	direct	NGRBA	22	
Mauginiella spp.	15 19		baited direct	NGRBA OSM	12 22	
Micromucor isabellina		4	direct	SDA	22	
Micromucor ramannianus	3 5 14		direct filter	AFPA SDA WA	12 22	
Microsphaeropsis sp.	5		direct	SDA	22	
Microsphaeropsis olivacea	3 12		baited direct	CMA/2 NGRBA OSM	22	hemp seed
Mortierella alpina	10		filter	SDA	22	
Mortierella elongata	10		filter	SDA	22	
Mortierella zychae	14		direct filter	PCA/2	22	
Mucor spp.		2 4 5	baited filter	PCA/2 OSM	22	grass hemp seed
Mucor circinelloides	13	4 12	direct filter	NGRBA PCA/2 SDA	12 22	
Mucor circinelloides f. *janssenii*	3 4		filter	SDA	22	
Mucor fuscus	1		baited	OSM	22	snake skin
Mucor hiemalis	1 2 3 8 10 12 14 15	1 2 3 4 5 12 13 14 15	baited filter direct	AFPA CMA/2 LRM NGRBA OSM PCA/2 SDA WA	5 12 22	grass

Table 7. continued

Mucor plumbeus		14	filter	WA	22	
Mucor racemosus	1 2 11 12 13	1 2 11 13	direct filter	CMA/2 LRM NGRBA OSM PCA/2 SDA WA	12 22	
Mucor racemosus f. *sphaerosporus*		1 15	filter	AFPA CMA/2 LRM NGRBA OSM SDA WA	12 22	
Mucor strictus	15		filter	SDA	22	
Neurospora sp.		4	filter	SDA	22	
Not identified	1 2 3 4 5 6 10 11 12 13 14 15 19	1 2 3 4 5 7 9 11 12 13 14 15 17 18 19 20	baited direct filter	AFPA CMA/2 LRM NGRBA OSM PCA/2 SDA WA	12 22	grass hemp seed snake skin
Oomycete		16	filter	SDA	22	
Paecilomyces lilacinus		15	direct	NGRBA	22	
Penicillium sp.	1		filter	SDA	22	
Penicillium aurantiogriseum	2 4 5 15	2 4 5 13 15	baited direct filter	AFPA CMA/2 LRM NGRBA OSM PCA/2 SDA WA	12 22	hemp seed
Penicillium brevicompactum	2 3 4 5 11 13 14 15	12 14 15	baited direct filter	AFPA CMA/2 LRM NGRBA OSM SDA WA	12 22	hemp seed
Penicillium chrysogenum	1 2 5 6 9	1 4 8 9 10 11 12 13 14 15 18	direct filter	AFPA CMA/2 LRM NGRBA PCA/2 SDA TWA WA	12 22	
Penicillium citrinum	1 2 3 6 7 8 9 11 12 13 14 15	3 13	direct	AFPA CMA/2 LRM NGRBA PCA/2 SDA WA	12 22	
Penicillium corylophilum	5 19		direct	AFPA NGRBA	22	
Penicillium crustosum		5	direct	NGRBA	12	
Penicillium echinulatum	1		filter	SDA	22	
Penicillium expansum	2 5 12 14	1 12	direct filter	LRM PCA/2 SDA	12 22	
Penicillium glabrum	11 13	14 15	direct filter	NGRBA SDA WA	12 22	
Penicillium griseofulvum	2		direct	SDA	22	
Penicillium hirsutum	15		direct	AFPA	22	
Penicillium janczewskii	1 2 3 4 6 8 10 11 14 15	3 18	direct filter	AFPA NGRBA OSM SDA WA	12 22	
Penicillium minioluteum	19		direct	NGRBA PCA/2 SDA	12 22	
Penicillium ochrosalmoneum		13	direct	NGRBA	12	
Penicillium pinophilum		13	direct	NGRBA	22	
Penicillium purpurogenum	1 2 3 9 10 11 12 14 15	13	direct filter	AFPA CMA/2 LRM NGRBA PCA/2 SDA WA	12 22	

Table 7. continued

Penicillium raistrickii	1 3		direct	NGRBA WA	22	
Penicillium simplicissimum	1 3		direct	CMA/2 NGRBA	22	
Penicillium solitum	15		direct	NGRBA	22	
Penicillium spinulosum	2 4 7 11 12 13	2 17	filter direct	AFPA NGRBA OSM PCA/2 SDA TWA WA	12 22	
Penicillium waksmanii	3	4	direct	SDA WA	22	
Periconiella sp.		5	direct	WA	12	
Phialophora fastigiata	5 7 9 17 19		direct filter	CMA/2 PCA/2 SDA	5 22	
Phoma spp.	3 4 5 8 11 12 13 14 15	1 4 5 8 11 12 13 15 17 19	baited direct filter	AFPA CMA/2 NGRBA OSM PCA/2 SDA TWA WA	12 22	grass hemp seed
Phoma exigua	10 11 13		baited direct	OSM SDA WA	12 22	hemp seed
Phoma jolyana	11	8	baited direct	OSM PCA/2	12	hemp seed
Phoma leveillei	3 5 14	19 20	baited direct filter	CMA/2 NGRBA OSM SDA WA	22	grass snake skin
Phoma macrostoma	5 10		baited direct filter	OSM SDA	12 22	hemp seed
Phoma medicaginis	14		direct	CMA/2	22	
Phoma nebulosa	11 13		baited direct	NGRBA OSM PCA/2 SDA	22	grass
Phoma sect. *Phyllostictoides*	13		direct	WA	22	
Phomopsis spp.	11	13 19	direct filter	LRM SDA WA	22	
Pilidium concavum		5 14	direct baited	SDA OSM	22	hemp seed
Pithomyces sacchari		5	direct	SDA	12	
Pseudeurotium zonatum	1		direct	NGRBA	22	
Pycnidiophora dispersa	5		direct	AFPA	22	
Pyrenochaeta sp.	4		direct	CMA/2	22	
Rhizopus stolonifer	5	1 2 5 9 12 15 16 19 20	direct filter	AFPA CMA/2 LRM NGRBA OSM PCA/2 SDA WA	12 22	
Saprolegnia ferax	5		baited	OSM	12	grass
Saprolegnia parasitica	4 5 12		direct filter	CMA/2 SDA	22	
Saprolegnia subterranea	3		direct	CMA/2	22	
Scopulariopsis acremonium		3	filter	AFPA	22	
Scopulariopsis brevicaulis		3 11	direct filter	LRM	22	
Seimatosporium lichenicola	14		filter	CMA/2	22	

Table 7. continued

Sporothrix spp.	8 12		baited direct	OSM PCA/2	22	grass snake skin
Sporotrichum pruinosum	1		direct	SDA	22	
Stereum sp.		18	direct filter	SDA	22	
Syncephalastrum racemosum	12 14		direct	CMA/2 SDA	22	
Trichoderma spp.	3	15	direct filter	NGRBA WA	12 22	
Trichoderma harzianum	1 2 3 4 5 11 13 15 19	1 2 3 4 5 11 13 14 15 17	baited direct filter	AFPA CMA/2 NGRBA OSM PCA/2 SDA WA	12 22	grass hemp seed snake skin
Trichoderma koningii	1 4 14	1 2 4 5 12 15	baited direct filter	AFPA CMA/2 LRM NGRBA OSM PCA/2 TWA SDA WA	12 22	hemp seed snake skin
Trichoderma polysporum	14	2	baited filter	OSM WA	22	hemp seed
Trichoderma pseudokoningii	2 3 5 11		direct filter	AFPA CMA/2 NGRBA SDA WA	22	
Truncatella angustata	10		filter	SDA	22	
Ulocladium atrum		4	filter	AFPA	22	
Ulocladium botrytis	14		direct	PCA/2	12	
Verticillium sp.		15	baited	OSM	12	hemp seed
Zygorhynchus moelleri	3 5	4	direct filter	LRM SDA	12 22	

Figure 1 shows isolates from surface waters at 22°C, under these conditions CMA/2 appears to recover the largest number of cfu and for all media there is little increase beyond 7 days incubation. At 12°C however (Fig. 2), CMA/2, WA, SDA and NGRBA appear to give similar results. The selective media LRM, OSM, AFPA recover, not surprisingly, much fewer propagules than the more broad spectrum agars. These figures must however, be cross-referred to the taxa results because as previously noted NGRBA selects particularly for large numbers of *Aspergillus* and *Penicillium* species and although OSM recovers low total numbers of propagules the diversity of taxa is high.

Figure 3 shows the number of cfu isolated from treated waters when incubated at 22°C. As could be predicted these are much lower than from surface waters, but again CMA/2 appears to be isolating somewhat larger numbers of cfu than PCA/2, NGRBA and SDA which are some way behind, followed by the more selective media, the high number after 10 days on WA is probably anomalous. Total numbers recovered from treated waters at 12°C were lower still and could not be easily illustrated on charts.

Whilst it is not possible to make quantitative estimates of individual fungi, the present study has shown that a carefully selected range of isolation strategies and media combined with an intensive identification effort has yielded the most comprehensive listing of fungal

Figure 1

Surface water 22C Direct plating

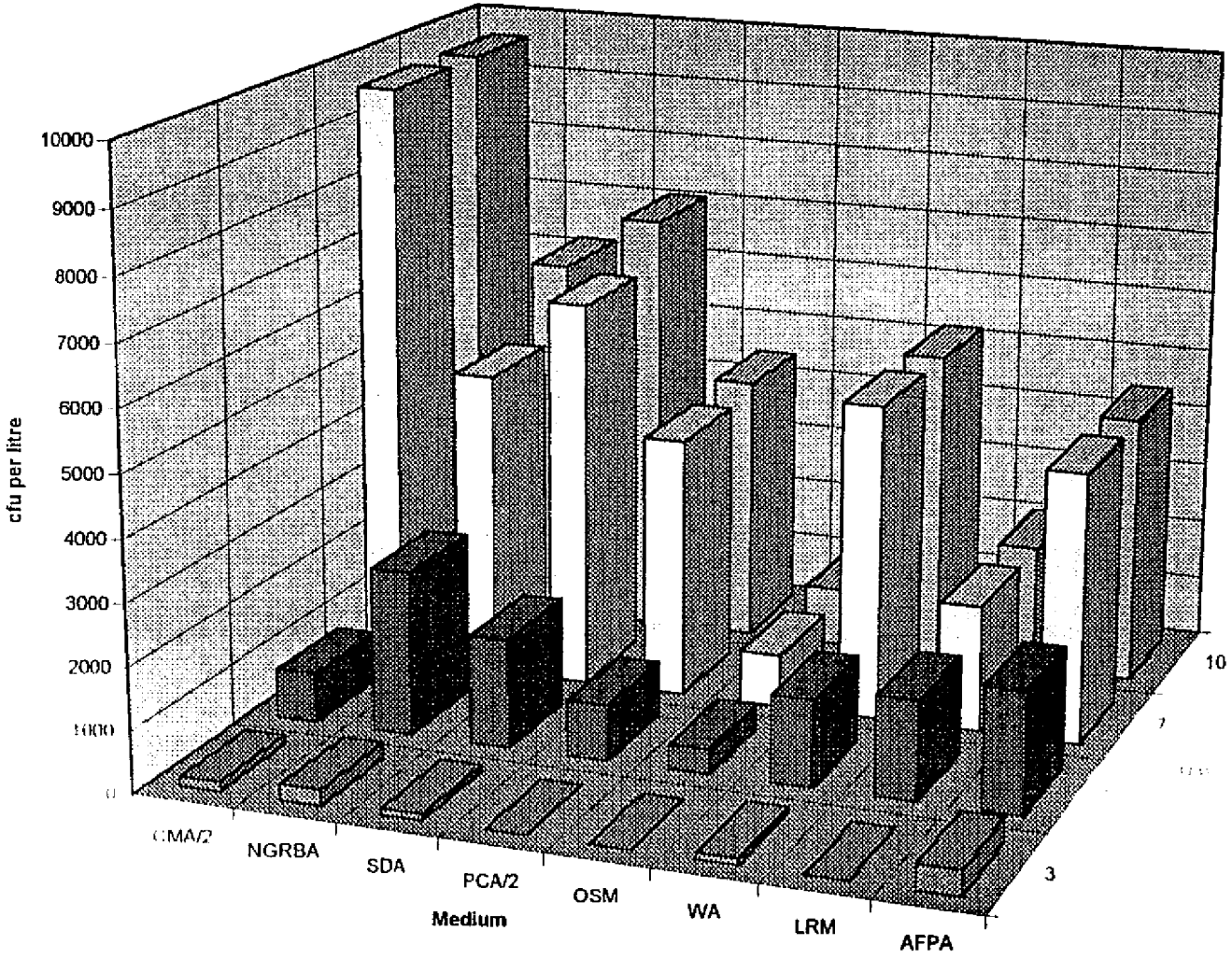


Figure 2

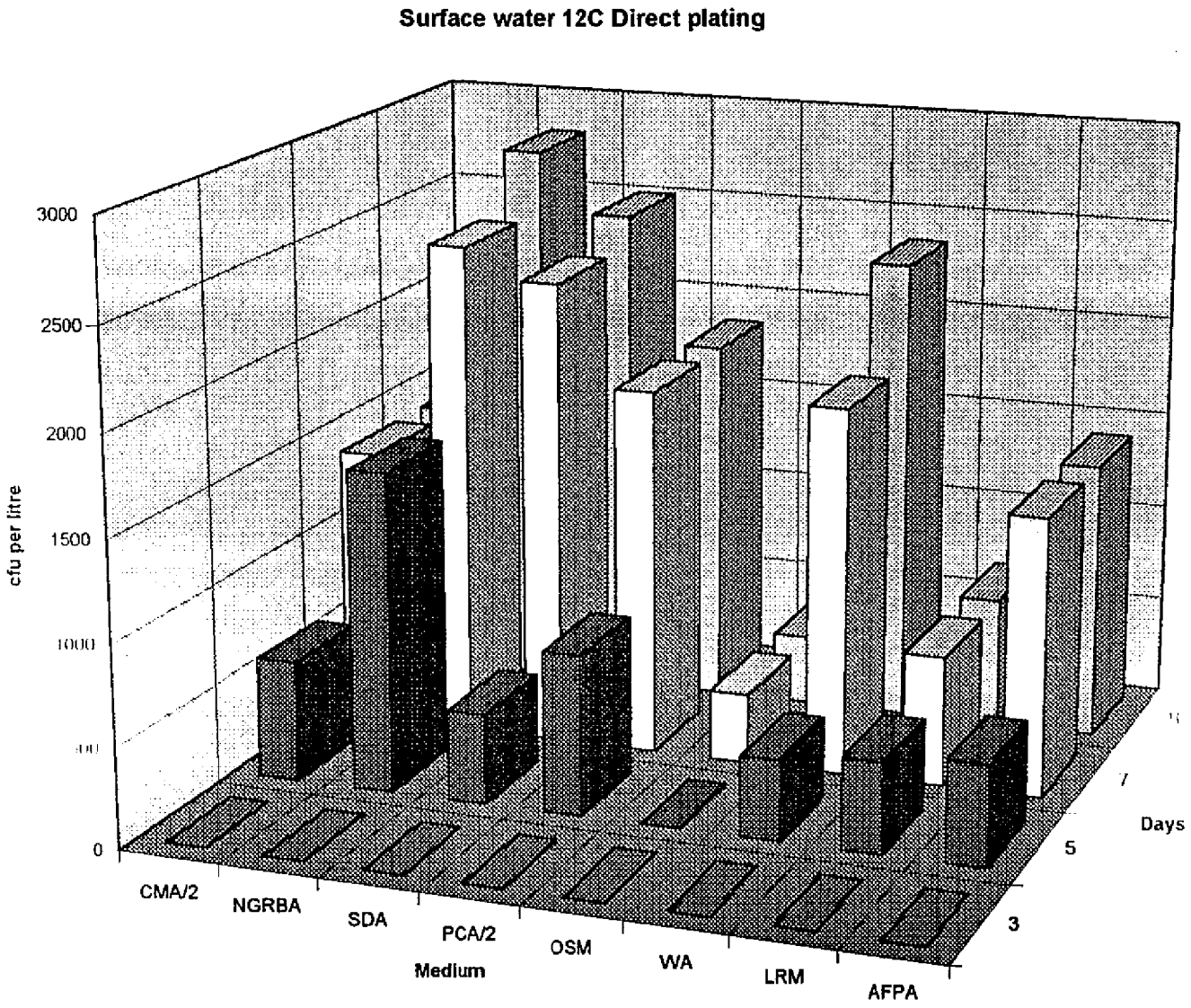
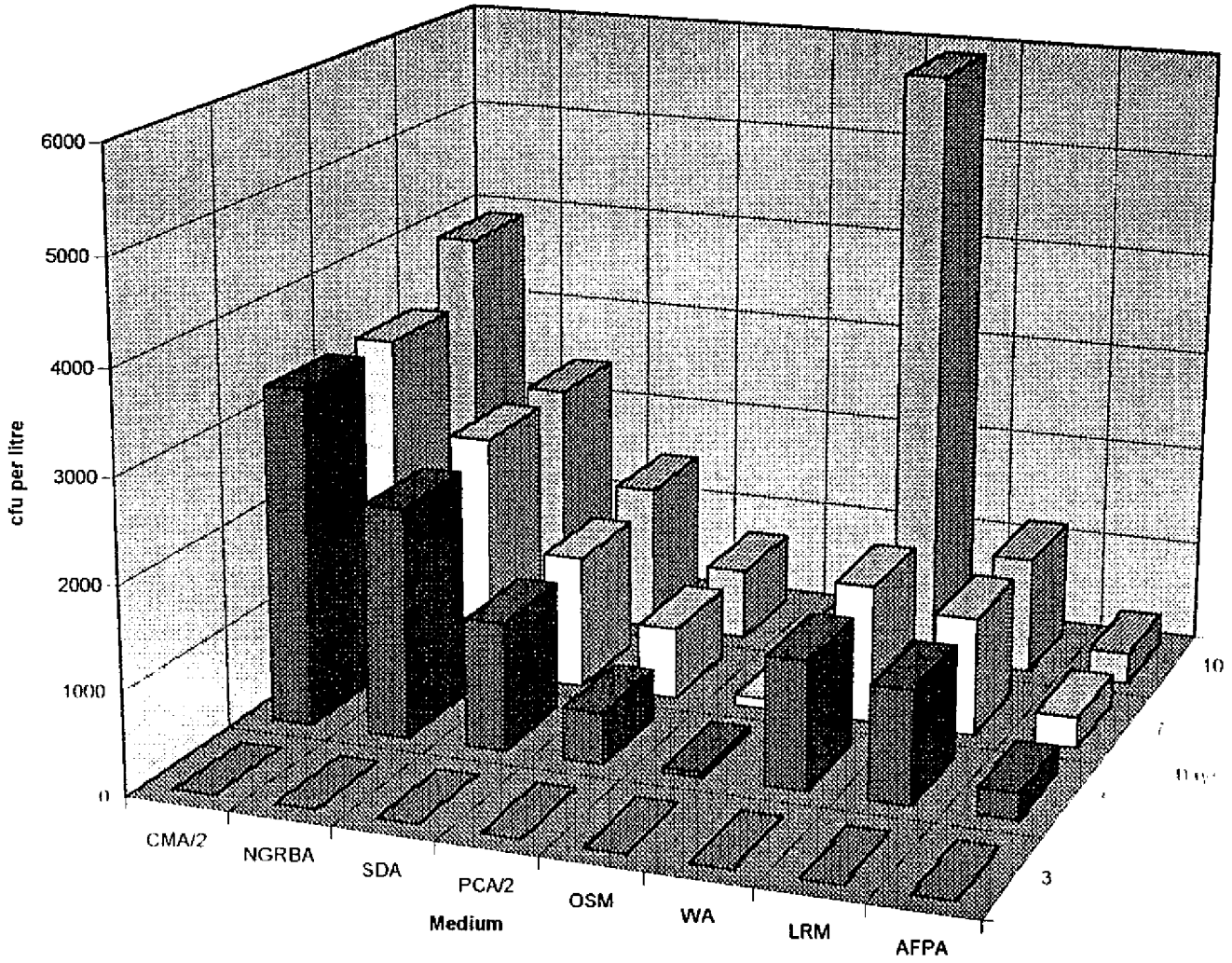

Surface water 12C Direct plating
3000
2500
2000
1500
1000
500
0
cfu per litre
CMA/2
NGRBA
SDA
PCA/2
OSM
WA
LRM
AFPA
Medium
3
5
7
Days

Figure 3

Tap water 22C Direct plating



taxa from water samples. However, the significance of the majority of the fungi isolated to water quality is as yet unknown. Although 95 taxa were isolated solely from the surface water samples, the 46 isolated from the treated water samples are perhaps of most concern, these are listed in Table 8, and include 16 isolated solely from treated waters. Furthermore, the presence of hyphal fragments on filters (Table 6) suggests that fungi are resident or capable of growth in water distribution systems possibly within biofilms. Figures 1-3 illustrate the number of colony forming units (cfu) from all samples isolated on the various media by direct plating.

Table 8. Fungal taxa isolated from treated waters

Fungi isolated solely from treated waters	Fungi isolated from both surface and treated waters
Acremonium simplex	*Acremonium* spp.
Aureobasidium sp.	*Alternaria alternata*
Chaetomium globosum	*Ascochyta* spp.
Cladosporium sphaerospermum	*Aspergillus flavus*
Exophialia jeanselmei	*Aspergillus fumigatus*
Fusarium cf *oxysporum*	*Aspergillus terreus*
Fusarium diamini	*Aspergillus versicolor*
Mortierella alpina	*Botrytis cinerea*
Mortierella elongata	*Cladosporium* spp.
Oomycete	*Cladosporium cladosporioides*
Penicillium minioluteum	*Cladosporium herbarum*
Stereum sp.	*Fusarium oxysporum*
Truncatella angustata	*Fusarium solani*
	Gliocaldium roseum
	Mauginiella spp.
	Mucor hiemalis
	Not identified
	Penicillium chrysogenum
	Penicillium citrinum
	Penicillium corylophilum
	Penicillium janczewskii
	Penicillium purpurogenum
	Penicillium spinulosum
	Phialophora fastigiata
	Phoma spp.
	Phoma exigua
	Phoma jolyana
	Phoma leveillei
	Phoma macrostoma
	Phomopsis spp.
	Rhizopus stolonifer
	Sporothrix spp.
	Trichoderma harzianum

3.2.1. Recommendations

Routine assessment of fungi in water samples will best be achieved by limiting media to just SDA and OSM, with isolation methods to include both direct plating and incubated filters, also baits should be used in conjunction with OSM. Incubation temperature can be limited to just 22°C. Although these conditions will isolate a smaller number of fungi than the full set of conditions used in the present project, it is considered that they will still

achieve a sufficiently large range of taxa on which to assess the water sample. Notwithstanding the recommendations in Part 3.1.1. which take greater account of the variation due to different viabilities of fungi, quantitative sampling using CMA/2 at 22°C for about 7 days would appear to provide the best practical set of standard conditions for most of the fungi isolated.

3.3. Objective 3

The database has been prepared following the outline given in Appendix 2 and will be transferred in conjunction with the production of this present report.

3.4 Objective 4

This present report is in a format similar to the Blue Book style and is available in wordprocessor format as a Microsoft 'Word for Windows' file if required.

4. References

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Appendix 1. Media formulations.

For all media Oxoid No. 3 agar was used and unless otherwise stated preparation was by adding the ingredients to the water, dissolving them by heating and then sterilizing the media by autoclaving at 121°C for 15 minutes.

1. SABOURAUD DEXTROSE AGAR (SDA)
   - 65 g Sabouraud dextrose agar (Oxoid CM41, which contains; 10 g mycological peptone, 40 g glucose, 15 g agar).
   - 1 l distilled water

2. HALF-STRENGTH CORN MEAL AGAR (CMA/2)
   - 8.5 g Corn meal agar (Oxoid CM103, which contains; 1 g corn meal extract, 7.5 g agar)
   - 11.5 g agar
   - 1 l distilled water

3. HALF-STRENGTH POTATO CARROT AGAR (PCA/2)
   - 10 g grated carrot
   - 10 g grated potato
   - 20 g agar
   - 1 l tap water

   Boil the potatoes and carrots in half the water for 30 minutes, add the agar to the second half of the water and heat to dissolve, combine the two halves and sterilize.

4. WORT AGAR (WA)
   - 50 g Wort agar (Oxoid CM247, which contains; 15 g malt extract, 0.78 g peptone, 12.75 g maltose, 2.75 g Dextrin, 2.35 g glycerol, 1 g K_2HPO_4, 1 g NH_4Cl, 15 g agar)
   - 1 l distilled water

5. OOMYCETE SELECTIVE AGAR (OSM)
   - 17 g Corn meal agar (Oxoid CM103, which contains; 2 g corn meal extract, 15 g aga
   - 23 g agar
   - 20 g sucrose
   - 10 mg $MgSO_4.7H_2O$
   - 10 mg $CaCl_2$
   - 1 mg $ZnCl_2$
   - 0.02 mg $FeSO_4.7H_2O$
   - 0.02 mg $CuSO_4.5H_2O$
   - 0.02 mg MoO_3
   - 2 mg thiamine hydrochloride
   - 25 ml Benlate stock suspension (0.5 g of Benlate in 500 ml distilled water)
   - 975 ml distilled water

   Add the thiamine after sterilizing. This medium must be stored in the dark and used within 36 hours.

   * Benlate = 1-butyl carbonyl-2-benzimadazole carbamic acid methyl ester.

6. LOW REDOX MEDIUM (LRM)

29 g Thioglycollate liquid broth (Oxoid CM391, which contains; 0.5 g L-cystine, 2.5 g NaCl, 5.5 g glucose, 5 g yeast extract, 15 g pancreatic digest of casein, 0.5 g sodium thioglycollate)
12 g agar

7. MODIFIED NEOPEPTONE-GLUCOSE-ROSE BENGAL AUREOMYCIN AGAR (NGRBA)

5 g neopeptone
10 g glucose
5 ml Chloramphenicol solution (150 mg chloramphenicol in 10 ml methanol)
5 ml Pencillin-G solution (150 mg in 10 ml distilled water)
20 g agar
990 ml distilled water
Add the Penicillin solution after autoclaving.
This medium is recommended in USA water methods it has been modified to remove Rose Bengal and uses amended antibiotics.

8. *ASPERGILLUS FLAVUS* AND *A. PARASITICUS* AGAR (AFPA)

20 g yeast extract
10 g bacteriological peptone
0.5 g ferric ammonium citrate
0.1 g Chloramphenicol
1 ml Dichloran solution (0.2 g 2,6-dichloro-4-nitroaniline in 10 ml methanol)
15 g agar
1 l distilled water
Adjust the pH to 4.5 before sterilizing.

9. TAP WATER AGAR (TWA)

15 g agar
1 l tap water

ROUTINE MEDIA

CZAPEK DOX AGAR (CZA)

50 ml solution A (40 g $NaNO_3$, 10 g KCl, 10 g $MgSO_4.7H_2O$, 0.2 g $FeSO_4.7H_2O$ in 1 l distilled water)
50 ml solution B (20 g K_2HPO_4 in 1 l distilled water)
1 ml $ZnSO_4$ solution (1 g $ZnSO_4.7H_2O$ in 100 ml distilled water)
1 ml $CuSO_4$ solution (0.5 g $CuSO_4.5H_2O$ in 100 ml distilled water)
30 g sucrose
900 ml distilled water
20 g agar
Dissolve the agar in the water then and add the remaining components and autoclave immediately at121°C for 20 minutes.

MALT EXTRACT AGAR (MA)

20 g Malt extract (Oxoid L39)
20 g Agar
1 l tap water

OAT MEAL AGAR (OA)

30 g ground oat meal
20 g agar
1 l tap water
Add the oat meal to half the water and heat for 1 hour, dissolve the agar in the other half of the water, pass the cooked oat meal through a fine strainer and mix with the dissolved agar, top up the volume to 1 l and autoclave at 121°C for 20 minutes.

POTATO CARROT AGAR (PCA)

20 g grated carrot
20 g grated potato
20 g agar
1 l tap water
Boil the potatoes and carrots in half the water for 30 minutes, add the agar to the second half of the water and heat to dissolve, combine the two halves and sterilize.

SYNTHETIC NUTRIENT WEAK AGAR (SNA)

1 g KH_2PO_4
1 g KNO_3
0.5 g $MgSO_4.7H_2O$
0.5 g KCl
0.2 g glucose
0.2 g sucrose
20 g agar
1 l distilled water
Dissolve all the components except the agar, adjust the pH to 6-6.5, then add the agar and sterilize.

Appendix 2. Database structure.

The data on which this report is based has been maintained in various electronic formats. The DWI database described here, and associated spreadsheet files, contain a compilation of that information. It may be used as a reference source for information on a particular species, mycotoxins, diseases etc, or may be used as the basis of an on-going catalogue of fungal isolates and related information.

The database was created using Microsoft Access version 2.0 and you will need this software to interrogate the relational data-sets.

The key data set in the database is the list of fungal names. Names are used in the literature in connection with reports of fungi isolated from distribution systems, or allied species known to produce mycotoxins or be associated with human disease.

The same taxon may be known by several names, either because of synonymy (multiple publications of the same taxon), or because the species may be referred to by a perfect (teleomorph) or imperfect (anamorph) state. The fungal name data is therefore self-referential to facilitate extraction of appropriate information regardless of a particular name. It should be noted that the synonymy is not exhaustive and IMI accepts no responsibility for the accuracy of the data.

Figure 4 shows the database structural relationships, the main tables of the database are;

Table 'TBL-Fungi'

This is the principle table and contains 380 names of fungi used in the literature or isolated from water samples at IMI.

Table 'TBL-Fungal Relations'

This table contains references to any related name for the organism and the nature of that connection, eg teleomorph, synonym etc.

Table 'TBL-Bibli'

This table contains 112 full bibliographic literature references to reports of fungi isolated from potable water, or reports of odour and taste producing organisms.

Table 'TBL-IMI Isolates'

This table contains 858 records of fungi isolated at IMI during the project. The table contains the following fields:-

NAME	- Name of the fungus
IMI	- IMI accession number
SAMPLE	- Code for location from which sample was taken
TREATMENT	- Direct isolation or from filtered water

MEDIUM	- Isolation medium
TEMPERATURE	- Growth temperature
BAIT	- Bait for oomycetes
LOCATION	- Location from which sample was taken
SOURCE DATE	- Date of sample

Table 'TBL-Disease'

This table contains 37 records of diseases associated with numerous fungal species noted in this report. The table contains the following fields:-

GENUS	- The genus associated with the disease
SPECIES	- Specific species associated with the disease
DISEASE	- Description of the disease
TISSUE/SITE	- The site affected by the disease

Tables 'TBL-Isolation Methods/TBL-Isolation Report Methodology'

These two tables summarise data on the isolation methods and sample history from 62 literature reports of fungi isolated from drinking water.

Table 'TBL-Mycotoxins'

This table contains 167 records of fungal toxigenic secondary metabolites produced by genera reported here.

Table 'TBL-Odours'

This table contains 33 records of odour forming compounds produced by species reported here.

Table 'TBL-Tastes'

This table contains 26 records of taste producing compounds produced by species reported here.

Figure 4. Database relationships