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ORIGINAL ARTICLE

Potential for preventing spread of fungi in air-conditioning systems constructed using copper instead of aluminium

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Abstract

Aims: As copper has been previously suggested as an antimicrobial surface, we tested the effectiveness of copper as an antifungal surface which could be used in air-conditioning systems as an alternative to aluminium.
Methods and Results: Coupons of copper (C11000) and aluminium were inoculated with fungal isolates (*Aspergillus* spp., *Fusarium* spp., *Penicillium chrysogenum* and *Candida albicans*) for various time periods. Culture on potato dextrose agar and an *in situ* viability assay using the fluorochrome FUN-1 were used to determine whether spores had survived. The results showed increased die off of fungal isolates tested compared to aluminium. In addition, copper also prevented the germination of spores present, thereby reducing the risk of the release of spores.
Conclusions: Copper offered an antifungal surface and prevented subsequent germination of spores present. FUN-1 demonstrated that fungal spores entered into a viable but not culturable (VBNC) state on copper indicating the importance of using such methods when assessing the effect of an antifungal as culture alone may give false results.
Significance and impact of study: Copper offers a valuable alternative to aluminium which could be used in air-conditioning systems in buildings, particularly in hospital environments where patients are more susceptible to fungal infections.

Introduction

Airborne fungal spores can pose problems in buildings, especially in hospital environments where patients are susceptible to invasive fungal infections. Infections can be life threatening, and measures to control these infections have proved to be difficult even when air filtration systems are put in place. One route of spread of fungal spores is through air-conditioning systems which are becoming more widespread in buildings and hospital intensive care units (Perdelli *et al.* 2006). Increasingly, more time, estimated at over 90%, is spent in indoor environments in developed countries and may contribute to sick building syndrome (Li *et al.* 2007). Previous research has found that using heating, ventilation and air-conditioning (HVAC) systems improved the air quality of offices but stressed the importance of maintaining the systems (Parat *et al.* 1997). In a review of the role of ventilation systems in transmission of airborne infectious agents in buildings, a lack of conclusive evidence in the literature available for citation is noted and indicates further studies are necessary in hospital and work place environments (Li *et al.* 2007). The review did however conclude that there is sufficient evidence to demonstrate a link between ventilation and the spread of airborne infectious agents in buildings.

There are indications that fungi have mechanisms to tolerate metal ions including entrapment within cell wall components, altering metal uptake, chelation or precipitation by secreted metabolites and intracellular binding by metallothioneins (Gadd 1993). Recent studies have demonstrated copper ion tolerance in aquatic fungi by means

of initiation of an antioxidant defence system (Azevedo *et al.* 2007).

Aluminium is widely used in the construction of HVAC systems, particularly in the design of fins within air-conditioning units, because of its heat transfer characteristics, reduced corrosion and reduced weight. However, there has been little regard as to potential health problems this may cause. Indeed, the use of aluminium fins within air-conditioning units offers no protection from the spread of fungal spores and may in fact increase their numbers. An alternative material that shares many of the advantages of aluminium is copper, with the additional advantage that copper has the ability to kill bacteria and viruses when used as an antimicrobial surface (Wilks *et al.* 2005, 2006; Noyce *et al.* 2006, 2007; Weaver *et al.* 2008). While copper ion solutions are known to be fungicidal, it is surprising that using copper as an antifungal solid surface has not been investigated extensively to our knowledge. Consequently, the potential of copper as an alternative to aluminium fins offering an antifungal property within air-conditioning systems is presented.

Methods

Culture preparation

Cultures of each of the fungal isolates were grown on potato dextrose agar (Merck) at 25 (±2)ºC until confluent growth with spores was observed. Plates were checked as they were growing for contamination. Spores and mycelia were scraped from the plates using a sterile loop and suspended in 1 ml of fungal diluent, GHD (glucose HEPES diluent) (2% glucose and 10 mmol l^{-1} sodium HEPES salt, pH 7·2). The number of spores per ml was counted using ×100 magnification in well slides.

Preparation of coupons

Sheets (0.5-mm thickness) of copper (unified numbering system, UNS, C110000) and aluminium were cut into 10 mm × 10 mm coupons. Coupons were washed in acetone and placed in 70% ethanol until ready for use. Before use, coupons were flamed and placed in sterile Petri dishes.

Inoculation of coupons

Duplicate coupons for each exposure time were analysed using culture or *in-situ* staining; 20 μl of spore suspension was pipetted over the coupons and exposed for various times (0, 3, 6, 24, 48, 96, 120, 168 and 576 h) at ambient temperature and humidity.

Culture analysis

After exposure time, coupons were placed in 5 ml of GHD using 20–30 2-mm diameter glass beads and vortexed for 1 min. Dilution series were prepared in GHD, and 100 μl of each diluent spread over potato dextrose agar (PDA) in triplicate. Plates were sealed and incubated at 22 (±2)°C for up to 7 days (until colonies could be counted). The numbers of colonies on each plate was counted by eye, and the numbers per coupon (cm^2) was calculated.

Epifluorescence microscopy

Duplicate coupons, after the required exposure time, were dried in sterile air (Class II microbiology safety cabinet). After drying, 50 μl of FUN-1 (Invitrogen) was pipetted over the coupon, and the coupons incubated in a humid chamber at 22 (±2)°C for 1·5 h. Coupons were tipped to remove dye and gently washed with filter sterile deionized water. After drying, coupons were visualized using an episcopic differential interference contrast microscope equipped with epifluorescence (EDIC/EF microscopy) (Nikon Eclipse Model ME600; Best Scientific, UK) with a wide band TRITC filter at ×400 magnification (Keevil 2003). Images were taken (at least 10 fields, over 200 spores) using a digital camera (Model Coolsnap CF; Roper Industries, UK) connected to a computer with digital image analysis software (IMAGE-PRO PLUS, ver. 4.5.1.22; Media Cybernetics, UK). Spores were counted using Image-Pro for total spores (green and orange/red fluorescent) and metabolically active spores (orange/red fluorescent). The number of metabolically active spores per coupon (cm^2) was then calculated from the number per field of view multiplied by the number of fields counted.

Results

Culture analysis

Copper affected the survival of all the fungal species tested with the exception of *Aspergillus niger* where no significant die off was seen even after 576 h (24 days) exposure (Table 1). Fastest die off occurred with *Candida albicans*, *Fusarium culmonium*, *Fusarium oxysporium* and *Fusarium solani*, and *Penicillium chrysogenum*, all of which showed a total die off after 24 h exposure to copper (Table 1). *Aspergillus* species were more resilient to copper. *Aspergillus flavus* and *A. fumigatus* required 120 and 144 h exposure, respectively, before total die off was observed.

Table 1 Mean (Geomean) spore counts from plate cultures and viable stain counts (FUN-1) per cm^2 for fungi exposed to copper (C11000) and aluminium coupons. Spores were exposed for different time periods and then either cultured on potato dextrose agar (PDA) or stained and viewed *in situ* under epifluorescence microscope

		Aspergillus flavus		*Aspergillus fumigatus*		*Aspergillus niger*		*Fusarium culmonium*		*Fusarium oxysporium*		*Fusarium solani*		*Penicillium crysogenum*		*Candida albicans*	
Exposure time(h)	Coupon	Plate counts (PDA)	FUN-1 counts	Plate counts (PDA)	FUN-1 counts	Plate counts (PDA)	FUN-1 counts	Plate counts (PDA)	FUN-1 counts	Plate counts (PDA)	FUN-1 counts	Plate counts (PDA)	FUN-1 counts	Plate counts (PDA)	FUN-1 counts	Plate counts (PDA)	FUN-1 counts
0	C11000	325 000	1076	6123 724	699	193 192	805	278 497	244	7454 997	1439	583 324	338	1485 735	206	29 716 632	782
3								1322 876	2105	1224 745	21 502	35 178	4401	874 300	33 576	86 603	1110
6								113 661	5344	100 871	6791	4330	24 460	610 533	26 609	5000	898
24		200 000	1	2329 968	5295			0	89	0	2187	0	2326	0	0	0	0
48		6124	6089	932 309	3459			0	134	0	32	0	141			0	7
96			1640		3319	91 108	8569										
120		0	0	50	0	116 348	12 805	0	0	0	0	0	0	0	0	0	0
576						241 644											
0	Aluminium	325 000	3443	6123 724	535	193 192	805	278 497	244	7454 997	1439	583 324	338	1485 735	206	29 716 632	782
3										3872 983	36 777	750 000	10 154	7232 738	32 768	22 660 538	71 842
6										3992 180	5996	612 372	11 666	5645 795	17 015	26 720 778	41 777
24		264 575	11 440	2050 598	1114			50 000	1260	1085 703	19 938	117 017	3389			5972 437	21 576
48		158 114	8302		10 707			335 410	557	2554 408	15 703		2093	162 019	951	4225 961	5151
96				2550 000	9872	164 851	3735										
120		382 426	6339	2550 000	22 862			24 875	732	220 794	12 033	25 000	759	52 440	537	3046 514	9472
576						291 725											

Epifluorescence microscopy

Comparing the survival of *Aspergillus* species spores on copper with aluminium using FUN-1 viability stain indicated a similar survival on both coupon types (Table 1). It was noted that although the survival of the spores was not significantly different on copper compared with aluminium, when the coupons were studied, the spores were not seen to be germinating on copper over the test period, whereas on aluminium, rapid germination was observed, with the spread of hyphae over the coupons (Fig. 1).

Fusarium species showed a reduced survival of spores on copper compared with aluminium, and after 120 h no viable spores were observed on copper (Table 1). This was longer survival compared with the culture analysis results which showed no growth after 24 h on copper. Again, when observed using EDIC/EF microscopy, no germination of spores was observed on copper coupons but was observed on aluminium coupons. This prolonged survival on aluminium observed using FUN-1 stain was also seen with *Penicillium chrysogenum* and *Candida albicans* (Table 1).

To investigate further the effect of copper coupons on the growth of *A. niger* compared with aluminium, PDA plates were spread with *A. niger* culture, and coupons of copper or aluminium were placed on the plates. After incubation for up to 10 days at 22(±2)°C, plates were observed for the inhibition of growth of *A. niger* (Fig. 2). The growth of *A. niger* was inhibited around the copper coupons, whereas growth occurred up to and over the aluminium coupons.

Discussion

HVAC systems are becoming more common across the world, and there is a lack of evidence as to the impact of these systems on human health. Most studies suggest that the use of air-conditioning units can increase the occurrence of sick building syndrome if units are not properly maintained (Parat *et al.* 1997; Perdelli *et al.* 2006). In hospital environments, the use of air filtration systems

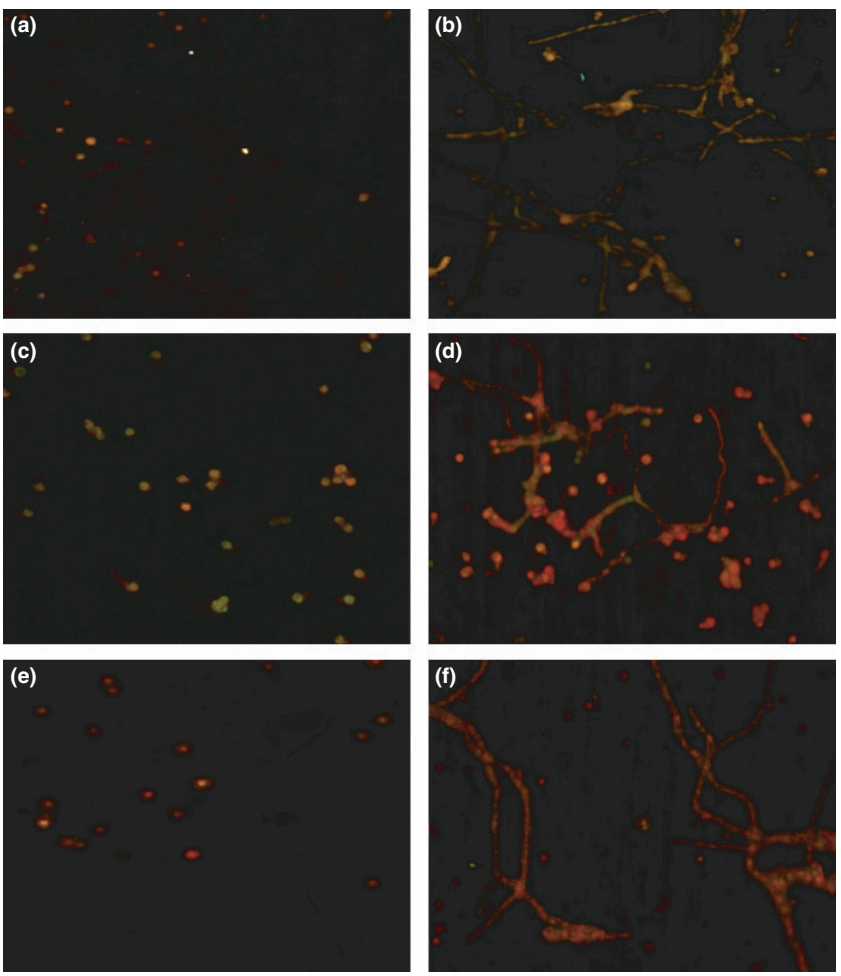

Figure 1 *Aspergillus niger* spores after 7 days exposure on copper (C11000) (a) and aluminium (b); *Aspergillus flavus* after 4 days exposure on copper (c) and aluminium (d); and *Aspergillus fumigatus* after 4 days exposure on copper (e) and aluminium (f) assessed using epifluorescence microscopy. Spores or hyphae stained orange to red are metabolically active and those remaining green to yellow are not active.

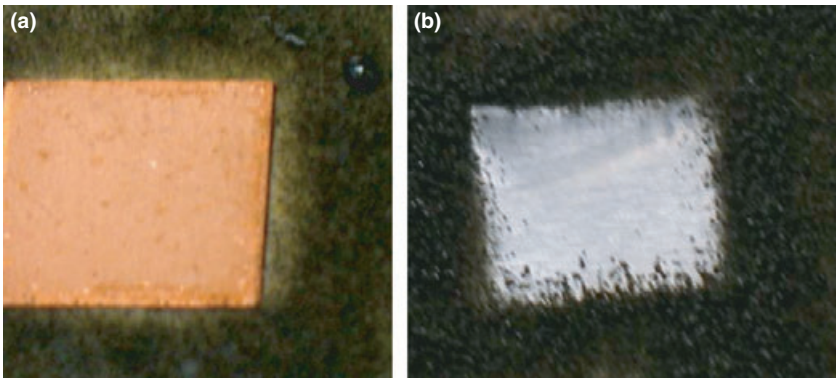

Figure 2 Inhibition of *Aspergillus niger* growth on copper and aluminium coupons after 10 days. Spore suspensions of *A. niger* (100 μl) were spread over PDA plates, and coupons of copper and aluminium were placed onto surface. Plates were incubated at 22(±2)°C for 10 days. Growth occurred onto the aluminium coupon (b), whereas growth was inhibited surrounding the copper coupon (a).

can only have a benefit to patients if the units are ran at high efficiency (Perdelli *et al.* 2006; Falvey and Streifel 2007).

All of the fungal isolates tested, with the exception of *A. niger*, showed the reduced survival and growth seen by culture analysis on copper surfaces compared to aluminium. *Aspergillus* spp. often have the highest prevalence in air samples, and, of the species present, *A. niger* appears most often (Anaissie *et al.* 2003). The results presented here indicate that *A. niger* has a more resilient nature to surface exposure, and this might explain its high rate of occurrence in previous studies. Anaissie *et al.* 2003 concluded that airborne moulds in hospitals originate from hospital water supplies and not contamination by outside air. They found higher incidence of moulds from air samples taken in moist environments e.g. bathrooms and conclude the origin is from the water supply. It may be also an increased survival and growth of moulds present from air in the moist environment which accounts for this increased occurrence. If this is the case, it could also be true in the circulating water within HVAC systems and dehumidifiers.

When *in situ* epifluorescence microscopy was used with FUN-1 staining, a clear difference was observed in the morphology of fungal isolates on copper compared with aluminium. The lack of germination of spores and die off of hyphae on copper indicates that copper has a fungi static effect as well as a fungicidal effect. This is particularly important in the case of *A. niger*. This was the only fungal species investigated that showed extended resilience to exposure to copper surfaces, because the spores remained viable over long time periods and could be isolated and cultured on the PDA growth medium. By contrast, the *in situ* epifluorescence microscopy showed that there was no germination on copper surfaces, unlike on aluminium, and cultures on PDA were incapable of colonizing exposed copper surfaces. This indicates that copper surfaces are ultimately fungicidal for the majority of fungal species investigated and still offer an important fungistatic benefit in the case of *A. niger*. It is unclear why *A. niger* spores may be more resilient to copper. There have been suggestions that copper tolerance of fungi is linked to the production of acid phosphatases during mycelia growth in the presence of up to 1-mM cupric ion to produce insoluble metal phosphates (Tsekova *et al.* 2002). Indeed, a survey of *Aspergillus, Penicillium, Acrophialophora* and *Alternaria* isolates found maximum production of acid phosphatase to occur in cultures of *A. niger* (Tarafdar *et al.* 1988). Tsekova *et al.* (2002) suggested that, as a periplasmic enzyme, a part of the alkaline phosphatase exists in soluble form and may be secreted into the extracellular medium where it may participate in the precipitation of metal ions as insoluble acid phosphates. The other part of enzyme is attached to the membrane and is located near the cell walls where it may participate in the processes of copper (II) uptake, causing precipitation of metal ions away from the sensitive cellular sides. At higher copper concentrations, the metal ion appears to inhibit the phosphatase activity. Of course, phosphatase-linked copper tolerance in mycelia may not be case with spores, and perhaps the spore coat of *A. niger* is just more resistant to copper uptake than other species, restricting the permeation of copper to fungistatic rather than fungicidal levels. Although there have been no reports of copper binding to *A. niger* spores, Yang *et al.* (2004) reported excellent sorption of ^{241}Americium by *A. niger* spores.

The use of the FUN-1 staining offers an important advantage over culture analysis in rapid turnaround of results (<1 day compared with 7–14 days using culture analysis). Previously, fluorescent probes have been evaluated for viability assessment in fungi and yeasts (Brul *et al.* 1997; De Vos and Nelis 2006; Vanhee *et al.* 2008). It has been reported that using fluorescent viability assays detected more fungi in dialysis water compared to plate counting, possibly indicating the presence of a viable but not culturable (VBNC) state in fungi (De Vos and Nelis 2006; Vanhee *et al.* 2008). The use of FUN-1 as a viability

stain in fungi has been investigated previously, although the authors of the report only evaluated its potential use as a viability assay, and no enumeration was undertaken(Brul *et al.* 1997).

Incorporating copper into HVAC systems in place of aluminium could offer the potential for a method of controlling fungal spore growth and survival and thus reduce the risk of infection and spread of fungal related diseases. This would be particularly important where cleaning regimes were ineffective because of the tortuous configuration of the HVAC system making access difficult, or the nature of the cleaning products used were not completely effective at removing or killing fungal spores, or cleaning was practiced only occasionally.

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References

Anaissie, E.J., Stratton, S.L., Dignani, M.C., Lee, C.K., Summerbell, R.C., Rex, J.H., Monson, T.P. and Walsh, T.J. (2003) Pathogenic molds (including *Aspergillus* species) in hospital water distribution systems: a 3-year prospective study and clinical implications for patients with hematologic malignancies. *Blood* **101**, 2542–2546.

Azevedo, M.M., Carvalho, A., Pascoal, C., Rodrigues, F. and Cassio, F. (2007) Responses of antioxidant defenses to Cu and Zn stress in two aquatic fungi. *Sci Total Environ* **377**, 233–243.

Brul, S., Nussbaum, J. and Dielbandhoesing, S.K. (1997) Fluorescent probes for wall porosity and membrane integrity in filamentous fungi. *J Microbiol Method* **28**, 169–178.

De Vos, M.M. and Nelis, H.J. (2006) An improved method for the selective detection of fungi in hospital waters by solid phase cytometry. *J Microbiol Methods* **67**, 557–565.

Falvey, D.G. and Streifel, A.J. (2007) Ten-year air sample analysis of *Aspergillus* prevalence in a university hospital. *J Hosp Infect* **67**, 35–41.

Gadd, G.M. (1993) Interaction of fungi with toxic metals. *New Phytol* **124**, 25–60.

Keevil, C.W. (2003) Rapid detection of biofilms and adherent pathogens using scanning confocal laser microscopy and episcopic differential interference contrast microscopy. *Water Sci Technol* **47**, 105–116.

Li, Y.Y., Leung, G.M., Tang, J.W., Yang, X., Chao, C.Y.H., Lin, J.Z., Lu, J.W., Nielsen, P.V. *et al.* (2007) Role of ventilation in airborne transmission of infectious agents in the built environment - a multidisciplinary review. *Indoor Air* **17**, 2–18.

Noyce, J.O., Michels, H. and Keevil, C.W. (2006) Potential use of copper surfaces to reduce survival of epidemic meticillin-resistant *Staphylococcus aureus* in the healthcare environment. *J Hosp Infect* **63**, 289–297.

Noyce, J.O., Michels, H. and Keevil, C.W. (2007) Inactivation of Influenza A virus on copper versus stainless steel surfaces. *Appl Environ Microbiol* **73**, 2748–2750.

Parat, S., Perdrix, A., Fricker-Hidalgo, H., Saude, I., Grillot, R. and Baconnier, P. (1997) Multivariate analysis comparing microbial air content of an air-conditioned building and a naturally ventilated building over one year. *Atmos Environ* **31**, 441–449.

Perdelli, F., Sartini, M., Spagnolo, A.M., Dallera, M., Lombardi, R. and Cristina, M.L. (2006) A problem of hospital hygiene: The presence of *Aspergilli* in hospital wards with different air-conditioning features. *Am J Infect Control* **34**, 264–268.

Tarafdar, J., Rao, A. and Bala, K. (1988) Production of phosphatates by fungi isolated from desert soils. *Folia Microbiol* **33**, 453–457.

Tsekova, K., Galabova, D., Todorova, K. and Ilieva, S. (2002) Phosphatase activity and copper uptake during growth of *Aspergillus niger*. *Process Biochem* **37**, 753–758.

Vanhee, L.M.E., Nelis, H.J. and Coenye, T. (2008) Enumeration of airborne bacteria and fungi using solid phase cytometry. *J Microbiol Methods* **72**, 12–19.

Weaver, L., Michels, H.T. and Keevil, C.W. (2008) Survival of *Clostridium difficile* on copper and steel: futuristic options for hospital hygiene. *J Hosp Infect* **68**, 145–151.

Wilks, S.A., Michels, H. and Keevil, C.W. (2005) The survival of *Escherichia coli* O157 on a range of metal surfaces. *Int J Food Microbiol* **105**, 445–454.

Wilks, S.A., Michels, H.T. and Keevil, C.W. (2006) Survival of *Listeria monocytogenes* Scott a on metal surfaces: Implications for cross-contamination. *Int J Food Microbiol* **111**, 93–98.

Yang, Y., Liu, N., Luo, S., Liao, J., Jin, J., Zhang, T. and Zhao, P. (2004) Sorption of ^{241}Am by *Aspergillus niger* spore and hyphae. *J Radioanal Nucl Chem* **260**, 659–663.

