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EVALUATION OF STRATEGIES FOR THE DECONTAMINATION OF EQUIPMENT FOR *GEOMYCES DESTRUCTANS*, THE CAUSATIVE AGENT OF WHITE-NOSE SYNDROME (WNS)

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Abstract: White-nose syndrome is an emerging infectious disease that has led to a dramatic decline in cave-hibernating bat species. White-nose syndrome is caused by the newly described fungal pathogen *Geomyces destructans*, which infects the ear, muzzle, and wing membranes of bats. Although the exact mechanism by which the fungus causes death is not yet understood, *G. destructans* leads to a high mortality rate in infected animals. While the primary mechanism of infection appears to be bat-to-bat transfer, it is still unclear what role human activity may play in the spread of this pathogen. Here we evaluate the effectiveness of decontamination protocols that can be utilized by speleologists to reduce the likelihood of spreading this dangerous pathogen to naïve bats or uninfected hibernacula. Our results show that pre-cleaning to remove muds and/or sediments followed by the use of commercially available disinfectants can effectively remove *G. destructans* from caving fabrics. Alternatively, immersion in water above 50 °C for at least 20 minutes effectively destroys the fungal spores. These results have allowed the development of a decontamination protocol (http://www.fws.gov/WhiteNoseSyndrome/cavers.html) that, when appropriately followed, can greatly reduce the likelihood of the human mediated transfer of *G. destructans* from an infected to uninfected site.

Introduction

In 2006 the bat population in Howe Caverns, a commercial cave in New York, USA, contained a large number of dead or dying bats (Blehert et al., 2009). A common link between all of the dying bats was that they had a white, powder-like substance around their muzzles, ears, and wing-membranes; this white substance caused the disease to be named White-nose Syndrome (WNS). WNS is believed to cause a greater than 70% mortality rate (range 30 to 99%) in bat populations of infected hibernacula and has been associated with a mass bat die-off in the northeastern US, with an estimated 5.5 million deaths (USFWS, 2012). To date, eighteen states have either confirmed WNS [demonstrating bats with histological evidence of a WNS infection: (Meteyer et al., 2009)] and/or bats that test positive for the presence of the etiological agent, the fungus *Geomyces destructans*. These states are Connecticut, Delaware, Kentucky, Maine, Maryland, Massachusetts, Missouri, New Hampshire, New Jersey, New York, North Carolina, Ohio, Oklahoma, Pennsylvania, Tennessee, Vermont, Virginia, and West Virginia. The fungus is also now affecting bat populations in Canada, including the provinces of New Brunswick, Nova Scotia, Ontario, and Quebec. As of December 2011, more than two hundred bat hibernacula have been affected by WNS, which has resulted in a significant decrease in cave-hibernating bat populations in the Northeast (Kunz and Tuttle, 2009). At the current rate of both mortality and spread, WNS is likely to cause the regional extinction of the little brown bat *Myotis lucifugus* within the next twenty years (Frick et al., 2010).

Koch's postulates were recently satisfied for *G. destructans* (Lorch et al., 2011), demonstrating that *G. destructans* was the sole agent of WNS. While this paper demonstrated that *G. destructans* could be spread directly through bat-to-bat contact, airborne movement of the pathogen was inconclusive, and the role of fomites was not investigated. Whatever the mechanisms of transport, Chaturvedi et al. (2010) demonstrated that a single strain of *G. destructans* was responsible for WNS in over one hundred bats from geographically diverse regions. These investigators used random amplification of polymorphic DNA (RAPD) to demonstrate that all tested isolates had identical RAPD patterns using differential primer sets. Further evidence suggests that *G. destructans* may be an introduced pathogen is provided by the discovery of morphologically and genetically similar *Geomyces* strains from France

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(Puechmaille et al., 2010). This European fungus has since been confirmed in three additional countries: Germany, Switzerland, and Hungary (Wibbelt et al., 2010). However, DNA sequencing across a range of genes will be needed to confirm that this strain is the source of the WNS epidemic. The high mortality rates caused by WNS in the US have not been observed among European bats infected with *G. destructans*. Although the susceptibility of European bat species to WNS has not been fully evaluated, they do demonstrate different hibernation patterns, which may reflect the influence of past epidemics on the populations (Puechmaille, personal communication, 2010).

Past epizootics demonstrate the role human activity can play in the spread of animal pathogens, including foot-and-mouth disease virus (Savill et al., 2006) and the chytrid fungus *Batrachochytrium dendrobatidis* (Daszak et al., 2000). In these epidemics, human activity has contributed to the spread of the pathogen, while altered behavior and disinfection have been shown to limit or prevent its dissemination (Savill et al., 2006, Webb et al., 2007). At this time, we have no understanding of *G. destructans* viability in the environment or how many fungal cells or spores are sufficient to infect a bat or colonize a cave environment. Until this information is available, it is critical that decontamination protocols are used to eliminate the possibility of WNS spread by human activity. In this paper we assess potential physical and chemical methods to decontaminate the fabrics and materials used by speleologists. Our results demonstrate that safe and effective methods of disinfection are possible to limit the anthropomorphic spread of *G. destructans*.

MATERIALS AND METHODS

FUNGAL STRAINS AND GROWTH CONDITIONS

The following strains were obtained from the American Type Culture Collection (ATCC; Manassas, Virginia): *Geomyces pannorum* var *pannorum* (ATCC 16222), *Aspergillus brasiliensis* (née *niger*, ATCC 9642) and *Penicillium pinophilum* (ATCC 9644). These species were grown in *Geomyces* media (40 g dextrose, 10 g peptone L^{-1}) to which 2% agarose was added as a gelling agent for solid media. *Geomyces destructans* was obtained from a little-brown bat collected in Hell Hole Cave, West Virginia, sent to the SCWDS and confirmed by cell morphology, PCR, and an invasive histology diagnostic of WNS. Growth was carried out at room temperature (RT) for all strains except *G. destructans,* which was grown at 7 °C. Mueller-Hinton agar for disk-diffusion assays was obtained from BD Difco/BBL (Franklin Lakes, New Jersey).

SPORE SUSPENSIONS

Conidiospores (spores) from all fungi were harvested from fungal plates in liquid *Geomyces* media using the American Society for Testing and Materials protocol G21-09 (ASTM, 2002), with 0.1% sodium dioctyl sulfosuccinate (DSS) as a wetting agent. DSS was tested for toxicity against each of the fungal strains and demonstrated no inhibitory effect on growth or spore germination. Spore suspensions were counted using a hemocytometer and resuspended in liquid *Geomyces* media to a stock concentration of 1×10^6 spores mL^{-1}. Once made, all spore suspensions were stored at 4 °C and used within four days.

SURVIVAL ASSAYS

Three assays were carried out on spore suspensions to determine spore killing (sporicidal) or fungal growth inhibition (fungistatic) activity of treatments. All assays were carried out in triplicate and the average of each treatment was recorded.

Sporocidal assay – Suspensions of spores at 1×10^3, 1×10^4 and 1×10^5 spores mL^{-1} were filtered onto a 25-mm-diameter 5.0 μm PFTE membrane (Cat # LSWG02500, Millipore, Billerica, Massachusetts; *Geomyces* spores are ~17 mm Ø) using a sterile glass membrane filtration unit (Millipore Cat # XX1002502). While still in the filtration unit, the membranes were exposed to chemical treatment for ten minutes, washed three times with 10 mL sterile distilled water, then aseptically removed and placed on culture media for growth. Following all treatments, the membrane plates were incubated until colonies were observed: up to six days for *G. pannorum* and nine days for the slower growing *G. destructans.* Germination/outgrowth rates were calculated as the number of colonies divided by the total number of viable spores applied; viability was determined from untreated controls.

Disk diffusion assays – Approximately 1×10^4 spores of *G. pannorum* or *G. destructans* were streaked across the entire surface of either a Mueller-Hinton or *Geomyces*-media plate. The agar plate was allowed to dry until surface moisture was no longer evident, and a 6 mm sterile Whatman paper disk (Whatman, Piscataway, New Jersey) soaked in a single test chemical was placed directly on the plate, using aseptic techniques. Plates were allowed to incubate until confluent fungal growth was observed, and the diameter of the zone of inhibition was measured. To examine the effectiveness of disinfection on *G. pannorum* in the presence of other materials, a top agar containing *Geomyces* media, 1% agar, and 2% of the test material (organic material, muds and sediment) was added. This top agar had been autoclaved and was poured over the surface of *Geomyces*-media plates to a depth of 2 mm. Testing was then carried out using the standard disk-diffusion assay. The organic material, mud and sediment (weathered sandstone) was obtained of a Kentucky cave within the Ste. Genevieve Formation.

CHEMICALS TESTED FOR POTENTIAL EFFECTIVENESS AGAINST *GEOMYCES* SPP.

Lab grade chemicals were obtained from Sigma-Aldrich (St. Louis, Missouri). The off-the-shelf chemicals were obtained from local retail stores. Household bleach

(Clorox, 6% HOCl) was diluted 1:10 (0.6%) prior to use in all the assays described. The chemical formulations for products evaluated were obtained from publically available material safety data sheets (MSDS). Initial screening was carried out using the disk diffusion assay (Bauer 1966) on both Mueller-Hinton and *Geomyces* media plates.

Heat Treatments of Spores

Dry-Air Treatments

Suspensions of 10^3–10^4 *G. pannorum* or *G. destructans* spores were placed on membrane filters as previously described, placed in sterile petri dishes and exposed to air temperatures of 50 °C, 60 °C, 70 °C, 80 °C, 90 °C, or 100 °C for fifteen minutes. The membrane filters were then placed on *Geomyces* media and incubated at 7 °C for three weeks.

Submersion Treatments

One mL of a 3×10^5 per mL spore suspension of *G. pannorum* or *G. destructans* was placed in conical centrifuge tubes and immersed in water at 30 °C, 40 °C, 50 °C, 60 °C, or 70 °C for twenty minutes. A 100 µL aliquot of the suspension was spread on an SDA plate and incubated at 7 °C for forty days, with colony numbers, in a randomly placed 4 × 4 cm grid, recorded at days seven and forty. All heat treatments were done in replicates of three.

Autoclaving

Preparations of *G. pannorum* and *G. destructans* spores and hyphae were heated to 121 °C at 15 psi (103 kPa) for fifteen minutes using an AMSCO 3013 autoclave sterilizer (STERIS Corporation, Mentor, Ohio). All heat treatments were done in replicates of three. A variety of caving equipment, such as oversuits, helmets, and ropes were treated in the autoclave under the same conditions.

Surface Treatment Assays

New nylon caving fabrics appeared to carry a charge from the nylon or contained water-repellent coatings, preventing the attachment or penetration of spore suspensions. As a result, these materials were not used. Instead a 1000-denier Cordura caving oversuit manufactured by Warmbac (Glastonbury, England) that had been heavily used for four years was used in the surface-treatment assays. One-inch tubular webbing, Sterling 11 mm HTP polyester rope, and Sterling 11 mm Superstatic nylon rope were obtained from Sterling Rope (Biddeford, Maine).

Surface Sterilization Assays

Treatment assays were carried out according to the standard protocol for testing the resistance of synthetic polymeric materials to fungi (ASTM G21-09; ASTM, 2002). Briefly, sections of test fabric were cut into roughly 3 × 3 cm squares, while rope and webbing sections were cut into approximately 3 cm pieces using a heated 1 mm wire. All samples were autoclaved prior to treatment, with sterile control samples demonstrating no fungal growth. Surfaces were sprayed with 250 µL of a 1×10^6 spores per mL suspension, allowed to air-dry for one hour to overnight, depending on the material, and were then exposed to the treatment. After exposure, the material was washed twice by immersion in 500 mL of sterile distilled water, allowed to air-dry, and then either pressed or rolled (in the case of rope) onto the surface of solid *Geomyces* media to deposit spores. The total number of colonies per plate was counted to determine the number of colonies divided by the number of viable applied spores. In the case of adherent fungi, which were not transferred from the fabric to the media, the remaining material was placed in 40 mL *Geomyces* media. Any growth indicated spore survival following disinfection.

Rope-Strength Testing

To test the strength of safety equipment following treatment, 4 m lengths of rope or 1 m sewn webbing loops were treated under the conditions deemed most effective at decontaminating ropes: washing in a front-loading washing machine with the manufacturer's recommended concentration of Woolite detergent, rinsing with water, soaking in a 1:64 dilution of Lysol IC for ten minutes, and rinsing twice in water. That concentration of Lysol IC was used to chemically stress the material, although we showed that a 1:128 dilution is sufficient for disinfection. To test whether this altered the strength of the rope or webbing, three replicate samples were subjected to one or five rounds of treatment (Woolite wash, rinse, treat with Lysol IC, rinse twice). The strength of these materials was then tested at Sterling Rope on a custom-built pull apparatus using a 60 kN hydraulic ram according to the Cordage Institute standard 1801 and calibrated by American Calibration, Inc. (ISO/IEC 17025 accredited, Crystal Lake, Illinois). Pull data was captured by means of a load cell attached to a PAXS strain gage meter (Red Lion Controls, York, Pennsylvania) with a sample rate of 20 Hz. Statistical analysis was performed on grouped samples using a paired *t*-test with a *p*-value of 0.05.

Results

In order to determine the effectiveness of all treatments, we began by autoclaving *G. pannorum* and *G. destructans* cultures. These tests demonstrated that autoclaving at 15 psi (103 kPA) for fifteen minutes killed 100% of *Geomyces* spores and hyphae, even at fairly high concentrations (e.g., 1×10^7 spores per mL). Most caving equipment did not withstand autoclave conditions: ropes became permanently fixed in shape, oversuits became friable, and the lining and cradle fabrics of helmets melted, although the plastics retained their shape. Given the gross physical changes, no attempt was made to determine how autoclaving affected the strength of these fabrics.

Spores of *G. pannorum*, *A. brasiliensis*, and *P. pinophilum* were all resistant to killing by baking (dry heat) above 50 °C (Table 1). At 50 °C, germination and growth of *G.*

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Table 1. Number of viable spores following 15 minutes of dry-air heating at 50 °C.

Species	Spores/mL	Treatment			
		Control[a]	5 min	10 min	15 min
Geomyces pannorum	1×10^4	TNTC	TNTC	TNTC	21
	1×10^3	TNTC	TNTC	NG	NG
Aspergillus brasiliensis	1×10^4	TNTC	TNTC	TNTC	TNTC
	1×10^3	TNTC	TNTC	TNTC	TNTC
Penicillium pinophilum	1×10^4	TNTC	TNTC	TNTC	TNTC
	1×10^3	TNTC	TNTC	TNTC	2

[a] Untreated spore suspension.
TNTC = Too numerous to count.
NG = No growth.

pannorum spores was 82.7% of the untreated controls. At the same temperature, 35.3% of *G. destructans* spores were able to germinate by day seven. When the treatments were increased to 70 °C, no *G. destructans* spores germinated. Nonetheless, the spores of *G. destructans* were not as resistant to immersion in hot water. When *G. destructans* spore suspensions were treated at 50 °C for even two minutes, germination of spores after seven days was eliminated (Fig. 1), while unheated controls had abundant growth (data not shown). Exposure to 40 °C and 45 °C also reduced the viability of *G. destructans* spores (Fig. 1), but by day forty, viable colonies were observed. Repeated

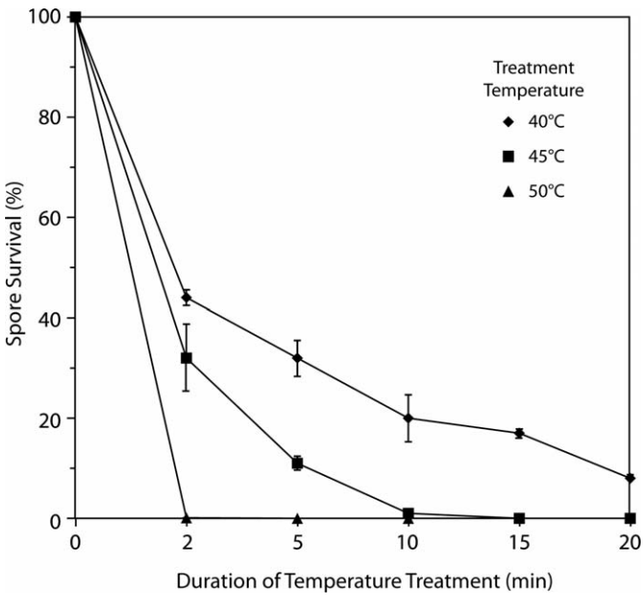

Figure 1. Effects of immersive heating on spore suspensions of *G. destructans*. Spore suspensions were heated in a water bath, then plated on culture media and incubated at 6°C for seven days. The average spore growth (percentage) compared to the original inoculum of three separate assays is shown. Error bars indicate the standard deviation from this average. Inhibited growth of *G. destructans* was confirmed for up to forty days post-treatment.

assays demonstrated that only immersion at ≥ 50 °C for a minimum of twenty minutes resulted in sterilization of *G. destructans* spores.

Commercially available cleaning agents, disinfectants, and chemicals (Table 2) were screened for their ability to either kill *Geomyces* spores (sporicidal) or limit the germination and growth of the fungus (sporostatic). In all, forty-four commercially available disinfection products were screened against *G. pannorum, A. brasiliensis*, and *P. pinophilum* (Table 2). As expected, some traditional disinfecting agents, such as hydrogen peroxide (H_2O_2), crystal violet, and iodine demonstrated good inhibition of fungal growth. Other effective chemicals contained quaternary ammonium compounds as a disinfectant, including Lysol IC (*n*-alkyl (C8–18) dimethylbenzylammonium chloride), Formula 409 [*n*-alkyl (C12-16) dimethylbenzyl ammonium chloride], and Oust Surface and Air cleaner (*n*-Alkyl dimethyl benzyl ammonium saccharinate). Lysol IC comes in concentrated form (20 to 25%), and our work demonstrates that a 1:128 dilution (0.15 to 0.20% final concentration) of Lysol IC is the minimum effective concentration. Agents containing a detergent also appeared to be effective, including Dawn, Ivory, and Palmolive products, all of which contain ionic and non-ionic detergents. Agents that contain both a disinfectant and a detergent, such as Clorox Toilet Bowl Cleaner (disinfectant: NaClO 1 to 5%), Tide with Bleach (disinfectant: benzenesulfonic acid 10%), Woolite (disinfectant: benzenesulfonic acid), and Dawn Antibacterial Dish Soap (disinfectant: Triclosan, undisclosed percent), are also effective against the growth of *Geomyces* species.

Other chemicals demonstrated less effectiveness. Alcohols did not demonstrate strong activity against *G. pannorum,* either directly (chemical-grade 70% alcohol) or in the following products: Purell Hand Sanitizer, Quick Care, Listerine, Exo Balance, and Cal Stat. Acids and bases also failed to demonstrate any significant effect against *Geomyces* growth, including those found in Grease Lightning, Cascade Gel, Jump Ultra Fabric Softner, and food-grade vinegar. Finally, Tilex Fresh Shower, which contains the cation-chelating agent ethylenediaminetetraacetic acid (EDTA), Seventh Generation Fabric Softner,

Table 2. Resistance of fungal spores to various disinfectants based on Kirby-Bauer disk-diffusion assays.

Disinfectant/ Chemical Agent	Zone of Inhibition, mm		
	G. pannorum	*Aspergillus*	*Penicillium*
crystal violet stain	39	20	28
Dawn Simple Pleasures	35	27	32
Chlorox Toilet Bowl Cleaner	35	28	24
Palmolive pure and clean	34	24	18
Tide with Bleach	34	35	25
Oust Surface and Air	34	10	14
Meyer's Clean Day	34	24	27
Hydrogen Peroxide (3%)	34	NZ	NZ
Dawn Dish Liquid	33	27	33
anti-bacterial hand soap	31	21	13
Woolite	31	NZ	20
Penguin Sport-Wash	30	25	26
Formula 409	29	25	13
Ivory Hand soap	28	NZ	12
iodine	25	12	NZ
Cascade Gel	25	NZ	NZ
Pine-Sol	25	9	29
Hibiclens	24	18	14
Scope	22	NZ	NZ
Vick's Early Defense	18	17	16
70% ethanol	18	4	10
Scotchguard Oxy Pet Spot Stain Cleaner	17	NZ	NZ
household bleach (1:10)	16	NZ	18
decolorizer	14	26	NZ
Lysol IC Disinfectant Cleaner (1:128)	15	NZ	NZ
Tabasco	13	–	–
Vesphene Disinfectant	13	–	–
Hydrogen Peroxide (0.3%)	12	–	–
SporiCLEAN	11	–	–
Grease Lightning	9	–	–
vinegar	9	–	–
Cascade Actionpack	NZ	–	–
Purell Hand Sanitizer	NZ	–	–
silver nitrate	NZ	–	–
Quick Care	NZ	–	–
Listerine	NZ	–	–
Cal Stat	NZ	–	–
Tilex Fresh Shower	NZ	–	–
Jump Ultra Fabric Softner	NZ	–	–
Seventh Generation Fabric Softner	NZ	–	–
PureGreen 24	NZ	–	–
Virkon (1%)	NZ	–	–
Exo Balance	NZ	–	–

NZ = No zone of inhibition (growth up to disk).
Dashes indicate test not done.

which contains the palm-oil extract dihydrogenated palmoylethyl hydroxyethylmonium methosulfate, and Virkon, which contains potassium peroxymonosulfate, all failed to demonstrate any effect on fungal growth. Based on some discussion within the WNS scientific community, we also examined products that contained silver as a disinfectant [PureGreen24 (colloidal silver) and silver nitrate (1% $AgNO_3$)], as well as enzymatic cleaners such as SporiCLEAN; however, none of these products demonstrated significant antifungal activity.

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Table 3. Comparative resistance of *G. pannorum* and *G. destructans* spores to disinfectants based on the disk-diffusion assays.

	Zone of Inhibition, mm		
	Geomyces pannorum		*Geomyces destructans*
Treatment	Day 7	Day 30	Day 30
control	NZ	NZ	NZ
Lysol	23	23	31
bleach (1:10)	67	45	NG
ethanol (70%)	11	NZ	7
Woolite	27	24	44
Formula 409	27	24	31

NZ = No zone of inhibition (growth up to disk).
NG = Fungus was completely cleared from the plate.

Following this preliminary screen, the agents chosen for further study either demonstrated effectiveness against *G. pannorum* (Woolite, Formula 409) or had known activity against fungi (Lysol IC, household bleach, and alcohol). A number of agents that demonstrated strong antifungal activity in the *G. pannorum* assays were not examined due to practical disinfection issues, such as staining of materials (crystal violet and iodine), the concentrations necessary to generate antifungal activity (Dawn Dish Soap), or potential damage to fabrics (Clorox Toilet Bowl Cleaner). Before additional, more extensive testing was carried out, Lysol IC, Woolite, Formula 409, household bleach, and 70% alcohol were also tested for their effectiveness against the pathogen *G. destructans* in a disk-diffusion assay. The results for *G. destructans* and *G. pannorum* were similar, although *G. destructans* appeared to be more susceptible to the same chemical treatments as *G. pannorum* (Table 3), confirming the choice of these compounds.

Disk-diffusion assays only demonstrate the susceptibility of an organism to grow in the presence of a particular disinfectant; they do not demonstrate the effectiveness of that agent as an exposure-based disinfectant. We therefore used the filtration assay to test the effectiveness of exposure to Formula 409, Woolite, Lysol IC, bleach, and 70% ethanol in preventing *G. pannorum* and *G. destructans* germination. Due to the sub-leathal damage that can occur from such treatments, *G. destructans* spores were allowed to recover for up to forty days following exposure (Russell, 1990). The results (Table 4) demonstrated that direct exposure to household bleach (1:10), Formula 409, and Lysol IC (1:128) completely inactivated *Geomyces* spores following exposure. Woolite demonstrated a reduced level of sporicidal activity (Table 3), suggesting that the activity of this agent in disk-diffusion assays is fungistatic. Alcohol did not demonstrate any effective killing of fungal spores in these assays (Table 4).

While these approaches demonstrate the effectiveness of chemical disinfection in the laboratory, they do not necessarily translate to efficient disinfection in the field. We therefore examined factors that may affect the effectiveness of these disinfectants under real-world conditions.

Cave explorers use a number of different fabrics due to their durability and abrasion resistance, as well as critical life-support equipment, including rope, webbing, and harnesses. The structure of these fabrics, with multiple niches for spore attachment, protection, and chemical absorption, may affect the penetration and effectiveness of chemical disinfectants. Therefore, we took fabrics used by cavers and examined disinfection efficiency following the direct application of fungal spores to the surface. Approximately 2.5×10^4 spores per cm^2 were applied to ballistic nylon (used under cave conditions), and treatment was carried out with Formula 409, household bleach (1:10), and Lysol IC (1:128) for 5, 10, and 15 minutes. The results (Table 5) demonstrated that fungal spores are effectively and reproducibly disinfected on these surfaces with no remaining viable spores when treatment is applied for a minimum of 10 minutes. Woolite greatly reduced the number of viable spores, but did not act as a disinfectant in this assay, while alcohol had comparatively little activity.

During exploration, caving fabrics become covered in mud, clay, or other sediments and organic debris, which could limit the effectiveness of disinfecting agents against fungal spores (Russell, 1990). To this end, organic material,

Table 4. Sporicidal activity of disinfectants against *G. destructans* compared to *G. pannorum* following a 10-minute exposure.

		Treatments						
Species	Days after Treatment	spores/mL	control	Formula 409	Woolite	Lysol (1:100)	bleach (1:10)	ethanol (70%)
G. pannorum	4	1×10^4	56	49	26	13	0	6
	5	1×10^4	TNTC	53	32	TNTC	0	35
	6	1×10^4	TNTC	TNTC	TNTC	TNTC	0	TNTC
G. destructans	7	1×10^4	TNTC	0	0	0	0	177
	8	1×10^4	TNTC	0	0	0	0	TNTC
	9	1×10^4	TNTC	0	1	0	0	TNTC

TNTC = Too numerous to count.

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Table 5. Effectiveness of Disinfectants against *G. pannorum* in the Presence of Various Cave Sediments.

	Disk Diffusion				Fabric Treatment (Rope)			
	Zone of Inhibition, mm				Colonies			
Disinfectant	Control	Clay	Sand	Silt	Control	Clay	Sand	Silt
Bleach (1:10)	67	12	NZ	NZ	TNTC	0	11	9
Lysol IC (1:128)	23	NZ	12	12	TNTC	0	0	0
Formula 409	27	32	NZ	NZ	TNTC	0	0	0

TNTC = Too numerous to count.
NZ = No zone of inhibition (growth up to disk).

mud, and sediment were added to the disk-diffusion assays to determine how great an effect the presence of these materials could have on disinfection. The results (Table 5) demonstrate that cave detritus can have a dramatic impact on the effectiveness of these disinfectants. We therefore repeated our cave fabric disinfection protocol with mud applied along with the fungal spores. The results (Table 5) demonstrated that these substances also dramatically reduced the effectiveness of disinfection on these surfaces, arguing for a pre-cleaning treatment prior to disinfection.

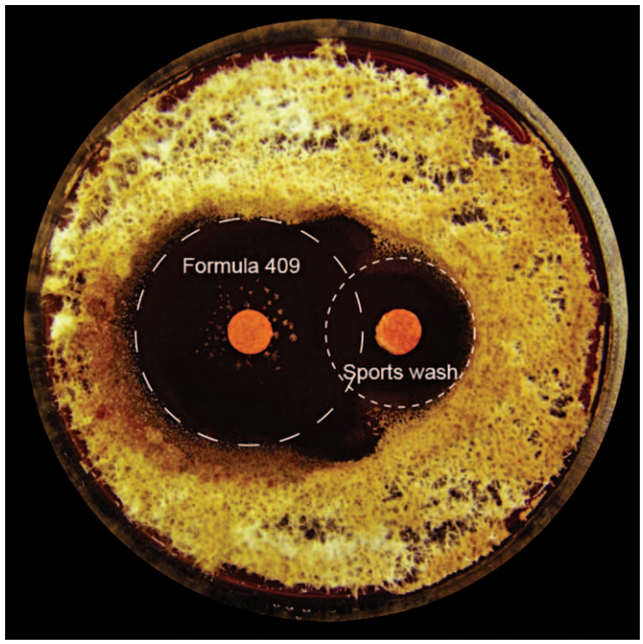

Figure 2. Synergistic effects of multiple treatments on the growth of *G. pannorum*. Disk diffusion assays were set up using various combinations of disinfecting agents. The combination of Formula 409 (left disk) and Penguin Sport-Wash (right disk) is shown. While a zone where each chemical prevents the growth of the fungus around each disk is observed, the combination of the two chemicals generates a greater zone of inhibition than each alone (larger zone between the two disks).

To test this, we carried out a disk diffusion assay to determine if specific detergents and disinfectants were compatible; such combinations can often be problematic as cationic disinfectants and anionic detergents can bind to each other, precipitate, and negate the effect of both products. In these assays we compared the inhibition of fungal growth by combinations of both Woolite and Meyer's Clean Day washing detergents with Formula 409, household bleach (1:10), and Lysol IC (1:128). The results (for example, Fig. 2) suggested that these products lead to a greater inhibition of fungal growth together than each does individually. It therefore appears that a pre-wash with a detergent such as Woolite can remove inhibitory substances while contributing to overall disinfection.

In addition to fabrics, speleologists use a number of pieces of critical life support equipment, including harnesses, webbing, and ropes. The nylon in this equipment gives the ropes or webbing strength; however, it is also susceptible to ionic attack, by substances such as bleach, which can damage the structural integrity and strength of the material. Consequently, we wanted to examine the ability of disinfection protocols to remove or kill spores on this material without harming its strength. Given the negative potential effects of bleach on nylon, we examined the ability of Woolite (which is traditionally used by speleologists to wash ropes) and Lysol IC (which can be made up in large volumes) to disinfect sewn webbing and nylon ropes. Treatment was carried out by washing in Woolite, rinsing with sterile water, soaking in a 1:64 dilution of Lysol IC for ten minutes, and rinsing twice in sterile water. The results demonstrated 100% killing of 2.5×10^5 spores applied to rope and webbing samples (Table 5). A higher concentration of Lysol IC (1:64) was used to chemically stress the material, although a 1:128 dilution has been shown to be 100% effective in this assay. To test whether this altered the strength of the webbing or rope, we treated samples of one-inch tubular webbing, Sterling 11 mm HTP rope, and Sterling 11 mm Superstatic rope, for one or five rounds of treatment. The strength of these materials was then tested, and the average breaking strength (in kN) was recorded (Fig. 3). Similar rope tests have not been carried out on samples returned to other rope manufacturers despite repeated requests.

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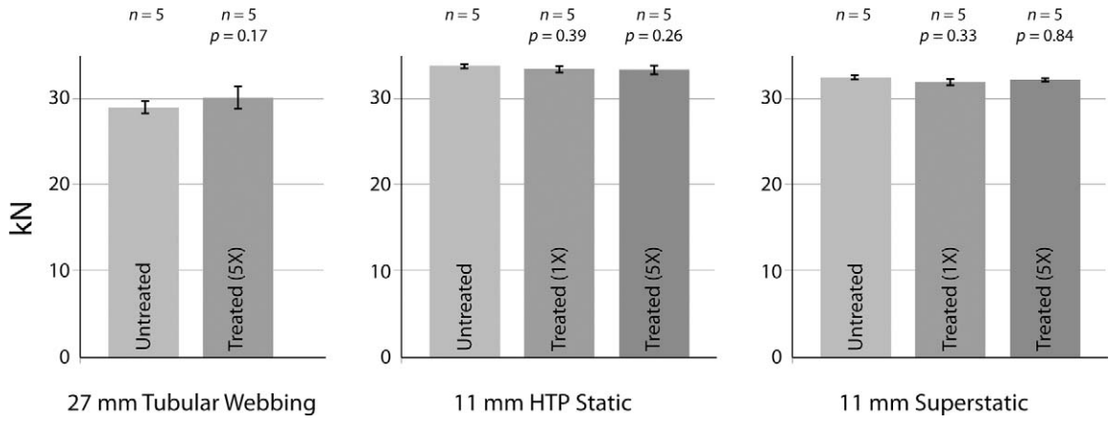

Figure 3. Rope and webbing post-treatment strength tests. Samples of rope and webbing were treated in triplicate with either one or five rounds of chemical treatment. The samples were then subjected to strength tests to determine the average breaking strength (in kN). The standard deviation of the combined results is shown as error bars. Based on a paired t-test ($p = 0.05$) there was no significant difference between the strength of the treated and untreated samples.

DISCUSSION

We carried out our preliminary assays in the non-pathogenic *G. pannorum* due to the ease of cultivation and comparatively short generation time for this species in the laboratory (five days to confluent growth versus approximately one month for *G. destructans*); however, to confirm the effectiveness of our decontamination protocols, we repeated key assays with *G. destructans.* Our results demonstrated a strong correlation between the effectiveness of these agents in *G. pannorum* and *G. destructans,* arguing for the appropriateness of *G. pannorum* as a model organism for preliminary assay development. We also chose to concentrate our assays on spores, the more resistant form of the *G. destructans* fungus (Deacon, 2005). Conidiospores are made by fungi for dispersal of the parent organism and, as such, are intrinsically resistant to environmental stressors such as UV and desiccation (Deacon, 2005, Gottlieb, 1950, Russell, 1990). Indeed, this can be seen by the relative resistance of these spores to both heat treatments and alcohols, and that heat stress actually promotes the germination and outgrowth of fungal spores (Table 1), even in the case of this psychrophilic fungi (Deacon, 2005, Russell, 1990).

Our results demonstrate that certain compounds, such as Formula 409 or bleach, are viable means of *G. destructans* disinfection to prevent the spread of WNS. Given the ineffectiveness of alcohols, protocols that suggest cavers should wipe exposed skin with alcohol swabs or alcohol-based hand sanitizer should be met with skepticism. The fact that most speleologists wear gloves that can be decontaminated suggests that standard personal hygiene practices (hand washing, bathing) likely represent a much more practical approach to limiting the spread of WNS on human skin.

In addition to chemical treatments, immersive heating of materials able to withstand submersion could be useful for reducing contamination by *G. destructans* spores. It is important to note that such conditions are not normally achieved in a standard washing machine, although sterilizing washing machines that can heat and maintain the temperature above 50 °C for twenty minutes would be a practical alternative. Given the difficulty in establishing and maintaining dry-air temperatures above 70 °C without laboratory-type controlled ovens or autoclaves, this mechanism of disinfection is not advised for anyone without access to, and training in the use of, such equipment. Regardless of these practical considerations, if heat treatment is the primary mechanism of disinfection, it is also important to consider the risks of contaminating external surfaces of containers, instruments, or surrounding surfaces. Limiting contact of contaminated gear or subsequent disinfection of such surfaces with effective chemical agents would help mitigate this risk.

Our chemical-disinfection experiments demonstrate the effectiveness of a number of agents that are known to be sporostatic but not sporicidal, including quarternary ammonium compounds and detergents (Russell, 1990). These results actually align well with those for bacterial spores and sporicidal agents (Russell, 1990 and references therein) and the work of Chaturvedi et al. (2011) against *G. destructans*. The effectiveness of these agents may well be due to a phenomenon by which these agents become attached to the spore coat and are hard to remove (other than by neutralization; Russell, 1990), suggesting that these agents do not act specifically on the spores, but rather act on germ tube formation during germination (Deacon, 2005). In all our assays, the presence of a long-chain alkyl group in the most effective antifungal agents suggests that such chemical structures play an important role in

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preventing the growth of *Geomyces* spp. (Table 2). The specificity of such activity may help to identify whether *Geomyces*-restrictive antifungal agents could be identified for the in situ treatment of hibernating bats.

There were some differences between the results found in the disk-diffusion assay and the spore assay, specifically as they relate to bleach. These differences can probably be accounted for by adsorption of the reactive chlorite ions (ClO_2^-) to reagents in the media. This effect was amplified by the addition of clays, sand, and silt to the media. Mud consists of insoluble clay particles that remain after speleogenesis. The composition of these muds depends on the rock formation from which they were derived. In the Ste. Genevieve Formation (used in these assays), these muds are primarily silica (SiO_2), but also contain kalsilite ($KAlSiO_4$), iron (Fe_2O_3) and aluminum (Al_2O_3) oxides (Preston and Denver, 1967). Such clays contain numerous reactive surfaces, such as hydroxyl (OH^-) or siloxane [$(SiO^-)_n$] groups, which can be bound by reactive ions (Sposito et al., 1999). Indeed, such clays are often used as adsorption agents in a variety of settings, including hydrocarbon extraction and absorptive chemical liners (Sposito et al., 1999). It is therefore likely that such compounds adsorb the reactive ions of disinfectants, negating some of the antifungal properties of these agents. Although disk-diffusion assays yielded similar results for both *G. pannorum* and *G. destructans*, the sporicidal activity of agents differed for the two fungi, possibly due to differences in the spore coats. This assay involved treating spores with the agent of interest, thoroughly washing the spores, then culturing the washed spores. Differences in spore coats could result in differential adherence of certain compounds to the spores of the two species examined.

The chemicals chosen in the development of these protocols (Table 2) were based on their commercial availability and ability to be included in a disinfection protocol that could be carried out by untrained personnel in the field. Chemicals were also selected that could disinfect without damaging the potentially life-supporting materials of recreational cavers. Our data demonstrate the effectiveness of protocols that can be used on caving equipment to decontaminate *Geomyces* fungal spores from surfaces in a short time (~15 minutes) and that the chemicals did not significantly alter the strength of Sterling rope (Fig. 3). Our protocols and results led to the development of the USFWS standard decontamination protocols (http://www.fws.gov/WhiteNoseSyndrome/cavers.html) and demonstrate the ability to kill all of 2.5×10^4 spores, an effective kill rate of greater than 99.995%, although it is possible that this kill rate may actually be higher.

In the food and pharmaceutical industries, hygiene protocols aim not to sterilize pharmaceuticals and cosmetics, but to remove sufficient microorganisms to prevent human infections (Baird et al., 2000). As such, much lower concentrations of disinfectants, used for longer periods, may reduce the fungal spore burden down to a level that is considered safe (Gupta et al., 2001). At this time, no information is available about spore loading during human visits to a cave or the minimum infective dose of *G. destructans* to initiate WNS in bats or contaminate the cave environment. The spread of WNS along bat migration routes (Frick et al., 2010) and the lack of numerous geographic epicenters may also suggest that human-vectored transport of *G. destructans* may be rare. Nonetheless, until the exact mechanism of *G. destructans* transport and environmental survival is known, it is critical to remove the potential impact of human transport. However draconian the proposed decontamination measures seem, a high level of adoption within the caving community can ensure continued access to both popular recreational sites and demonstrate to landowners and management agencies the commitment of cavers to both protect the fragile underground wilderness and care for the only other mammalian species that penetrates as deeply into this subterranean world.

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