Vol. 57: 255–260, 2003 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published December 29

Fungicidal effects of chemical disinfectants, UV light, desiccation and heat on the amphibian chytrid *Batrachochytrium dendrobatidis*

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ABSTRACT: The efficacy of a number of disinfection treatments was tested on *in vitro* cultures of the fungus *Batrachochytrium dendrobatidis*, the causative agent of chytridiomycosis in amphibians. The aim was to evaluate the fungicidal effects of chemical disinfectants, sterilising ultraviolet (UV) light, heat and desiccation, using methods that were feasible for either disinfection in the field, in amphibian husbandry or in the laboratory. The chemical disinfectants tested were: sodium chloride, household bleach (active ingredient: sodium hypochlorite), potassium permanganate, formaldehyde solution, Path-X^{TM} agricultural disinfectant (active ingredient: didecyl dimethyl ammonium chloride, DDAC), quaternary ammonium compound 128 (DDAC), Dithane, Virkon, ethanol and benzalkonium chloride. In 2 series of experiments using separate isolates of *B. dendrobatidis*, the fungicidal effect was evaluated for various time periods and at a range of chemical concentrations. The end point measured was death of 100% of zoospores and zoosporangia. Nearly all chemical disinfectants resulted in 100% mortality for at least one of the concentrations tested. However, concentration and time of exposure was critical for most chemicals. Exposure to 70% ethanol, 1 mg Virkon ml^{-1} or 1 mg benzalkonium chloride ml^{-1} resulted in death of all zoosporangia after 20 s. The most effective products for field use were Path-X^{TM} and the quaternary ammonium compound 128, which can be used at dilutions containing low levels (e.g. 0.012 or 0.008%, respectively) of the active compound didecyl dimethyl ammonium chloride. Bleach, containing the active ingredient sodium hypochlorite, was effective at concentrations of 1% sodium hypochlorite and above. Cultures did not survive complete drying, which occurred after <3 h at room temperature. *B. dendrobatidis* was sensitive to heating, and within 4 h at 37°C, 30 min at 47°C and 5 min at 60°C, 100% mortality occurred. UV light (at 1000 mW m^{-2} with a wavelength of 254 nm) was ineffective at killing *B. dendrobatidis* in culture.

KEY WORDS: *Batrachochytrium dendrobatidis* · Disinfectant · Ultraviolet light · Didecyl dimethyl ammonium chloride · DDAC · Desiccation · Sodium hypochlorite

INTRODUCTION

The amphibian chytrid *Batrachochytrium dendrobatidis* is a highly virulent fungal pathogen of amphibians, causing death both in the wild and in captivity in Australia, New Zealand, USA, Central America, South America, Spain and Germany (Berger et al. 1998, Pessier et al. 1999, Mutschmann et al. 2000, Bosch et al. 2001, Bradley et al. 2002). *B. dendrobatidis* has probably been introduced into new countries and regions by the movement of infected amphibians, or in contaminated water or soil containing zoospores (Berger et al. 1999, Daszak et al. 2000, Speare et al. 2001a). The listing of amphibian chytridiomycosis on the World Organisation Animal Health (Office Internationale des Epizooties) Wildlife Dis-

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eases List in 2001 (S. Haig pers. comm.) highlighted the significance of this disease. To prevent transmission of *Batrachochytrium dendrobatidis* by fomites, effective protocols are needed for disinfection in the field of equipment such as boots, nets and measuring instruments, and also in captive husbandry and the laboratory (Speare et al. 2001b). Chloramine, chlorhexidine, bleach and ethyl, methyl and isopropyl alcohols have been recommended on empirical grounds (NNPWS 2000), but no testing of effectiveness has been done. The need to evaluate a range of chemical disinfectants and feasible physical protocols was one of 119 recommendations recently proposed to lessen the risks of chytridiomycosis to wild amphibian populations (Speare et al. 2001b).

Disinfectants play a role in at least 3 situations: (1) to prevent the spread of amphibian disease in the wild by cleaning equipment that comes into contact with amphibians, such as nets and toe-clipping instruments, as well as by cleaning items that come into contact with water, such as boots; (2) in captive husbandry, as a crucial part of quarantine in cleaning equipment and enclosures before reuse, and (3) in the laboratory, to prevent contamination and to kill unwanted cultures. An ideal disinfectant would work rapidly, be safe for the human operator, have minimal impact on equipment, have a low risk of contaminating the environment, be easily available, and any residual concentration would have no effect on amphibians.

Because a resistant resting spore has not been detected for *Batrachochytrium dendrobatidis* (Longcore et al. 1999), this study evaluates the ability of a disinfectant to kill zoospores and zoosporangia only.

MATERIALS AND METHODS

The physical techniques tested were desiccation, ultraviolet (UV) light, and heat. The chemical disinfectants tested were sodium chloride, household bleach (active ingredient: sodium hypochlorite), potassium permanganate, formaldehyde solution, Path-X™ agricultural disinfectant (active ingredient: didecyl dimethyl ammonium chloride, DDAC), quaternary ammonium compound 128 (active ingredient: DDAC), Dithane, Virkon, ethanol and benzalkonium chloride. Because there is no standard technique for evaluating the fungicidal effect of disinfectants on *Batrachochytrium dendrobatidis*, several series of experiments were performed using marginally different protocols. Initially, Series 1 was carried out by L. Berger at the Australian Animal Health Laboratory, Geelong, in 2000, and was subsequently followed by Series 2 using a modified, more detailed protocol, by M. L. Johnson at James Cook University, Townsville, in 2001/2002.

Protocol for Series 1. *Batrachochytrium dendrobatidis* Strain 98-1810/3 isolated from a wild adult of *Nyctimystes dayi* from Tully, Queensland, was used for all *in vitro* experiments in Series 1. The culture was isolated and maintained in 10 ml culture flasks (TPP, CSL Biosciences) using TGhL liquid medium (16 g tryptone, 2 g gelatin hydrolysate, 4 g lactose in 1000 ml deionised water) at 23°C in a humidified incubator following routine methods (Longcore et al. 1999).

Chemical disinfectants: Cultures were grown as monolayers in 96-well plates by adding 100 µl of 4 d old liquid medium cultures, each well containing a total of 10 000 zoosporangia. Zoosporangia were counted using a haemocytometer. Following incubation for 4 d, the monolayers were exposed to disinfectants by replacing the media with 100 µl of 70% ethanol (Univar), or 1 mg Virkon ml^{-1} (Antec International; active ingredients are potassium peroxy sulphate compound, sulphamic acid, alkyl benzene sulphate, alkyl hexameta phosphate, malic acid, and sodium chloride) or 1 mg benzalkonium chloride ml^{-1} (Apex). Each treatment agent was diluted to the appropriate concentrations in TGhL liquid medium and vortexed. For each disinfectant, *Batrachochytrium dendrobatidis* was exposed for 20 s or 5 min in replicates of 8 wells. At the end of the timed period, treatments were removed by inverting the plates and discarding the supernatant. Each well was then washed 3 times with 100 µl media, a final volume of media was added, and the plates were incubated for 1 wk at 18 to 20°C. Plates were examined for growth and the presence of motile zoospores on an inverted microscope over a period of 4 wk. Eight wells were washed with fresh media and kept as controls.

Heat: Flasks of liquid cultures were incubated at various temperatures to determine the time required to kill all zoospores and zoosporangia. Five ml of 4 d old culture, with ca. 50 000 zoosporangia, was added to 25 cm^2 plastic tissue-culture flasks (Corning), which were then placed in incubators at 23, 26, 32, 37, 47, and 60°C, or immersed in water at 100°C. Prior calibration of incubators showed that they varied <1°C from the set temperature. After some pilot trials, zoospores and zoosporangia in flasks were exposed to the 7 temperatures for a range of times: 2, 5 or 30 min, 1, 2, 4, 15, 24, 48 or 96 h. Two flasks were used for each temperature-time point. Flasks were then incubated at 23°C for 2 wk and checked for growth. Growth was obvious by the opacity of the media and a layer of zoosporangia attached to the surface of the plastic. Flasks containing proliferating cultures were discarded, whereas flasks with no observable growth were incubated for another 2 wk.

Desiccation: To test the effect of drying, monolayers of culture were grown in a 24-well plate with 20 000 zoosporangia per well. After 3 d, media was removed

from 18 wells and the plate was left open in a biohazard laminar flow hood at 22°C. Media was removed and replaced with fresh media in 6 control wells. After 1 h, media was replaced on 12 dried wells, and after 5 h media was replaced for the remaining 6 wells. Plates were monitored for growth and the presence of motile zoospores for 2 wk using an inverted microscope.

Protocol for Series 2. *Batrachochytrium dendrobatidis* Strain 98-1469/10 (Amphibian Research Centre, Melbourne, Victoria), isolated from a captive juvenile *Limnodynastes dumerilii*, was maintained *in vitro* in 10 ml culture flasks using TGhL liquid medium at 23°C in a humidified incubator. Prior to disinfection experiments, cultures were inoculated into 30 ml culture flasks and grown for 3 to 4 d, or until the cultures contained numerous active zoospores. Aliquots were then removed, zoospores counted using a haemocytometer, and diluted to a final concentration of 4×10^5 zoospores ml^{-1} in liquid medium. A total of 200 µl of this dilution was aliquoted into each well of 96-well flat-bottomed plates, and placed at 23°C to grow for 3 to 4 d prior to treatment with various test disinfection agents. Each treatment agent was diluted to the appropriate concentrations in medium and vortexed. Using staggered starts, at each time point a multi-channel pipette was used to add each dilution of the treatment agent to 8 wells, after removing 180 µl of medium per well. Each treatment was removed by inverting the plates and discarding the supernatant. Each well was then washed 3 times with medium, and a final volume of 200 µl of medium was added per well. The 96-well plates were then incubated at 23°C for 4 d. Each well was scanned using an inverted Olympus microscope to check for active zoospores and growth of zoosporangia. The gross morphology of the zoosporangia was also examined and recorded. Each 96-well plate was scanned every 72 to 96 h over a period of 4 wk. If the presence of new growth was indeterminate, or there was no activity apparent and the zoosporangia were still attached and intact, 20 µl of medium was removed from each well and inoculated into another 96-well plate containing 180 µl of fresh medium. These new cultures were then observed for growth over time. If growth or activity by zoospores was apparent, then the treatment was deemed ineffective.

Chemical disinfectants: The following chemical disinfectants were tested: sodium chloride, household bleach (active ingredient: 4% sodium hypochlorite), potassium permanganate (Soul Pattinson), formaldehyde solution (Asia Pacific Speciality Chemicals; active ingredient: 37% formaldehyde with 9.5% methanol), Path-X™ agricultural disinfectant (NutriTech Solutions; active ingredient: 12% DDAC), quaternary ammonium compound 128 (Wade's Enterprises; active ingredient: 7.68% DDAC), and Dithane (Bayer; active ingredient: mancozeb in petroleum oil). Sodium chloride was used at 10, 5, 2.5 and 1% w/v for 5, 2 and 1 min, respectively. In a second experiment an additional exposure time of 30 s was used. Each experiment had 8 replicates, for each concentration tested, at each time point, and untreated wells were included as controls. For household bleach (sodium hypochlorite), cultures were exposed to 4, 2, 1, 0.4, 0.2 and 0.1% sodium hypochlorite for 10, 5, 2 and 1 min, and 30 s. In a second experiment, lower concentrations were tested: 0.2, 0.1, 0.05 and 0.01% at 10, 5, and 1 min, and 30 s exposure. Each of the above experiments were replicated 8 times per concentration of sodium hypochlorite, and per time point, and untreated controls were included. Each experiment was repeated at least twice. Path-X™ was tested at 1×10^{-1}, $\times 10^{-2}$, $\times 10^{-3}$, $\times 10^{-4}$ and $\times 10^{-5}$ dilutions each for 5, 2 and 1 min, and 30 s. Quaternary ammonium compound 128 was tested undiluted, and at dilutions of 1×10^{-1}, $\times 10^{-2}$, $\times 10^{-3}$, and $\times 10^{-4}$ each for 5, 2 and 1 min, and 30 s. Dithane was tested at dilutions of 1×10^{-2}, $\times 10^{-3}$, $\times 10^{-4}$, $\times 10^{-5}$ and $\times 10^{-6}$ each for 5, 2 and 1 min, and 30 s. Potassium permanganate was tested at 2, 1, 0.1, 0.01 and 0.001% w/v each for exposure times of 10, 5, 2 and 1 min. Formaldehyde solution was tested at full strength, and diluted to 10, 1, 0.1, 0.01 and 0.001% each for exposure times of 10, 5, 2 and 1 min.

UV light: Cultures in 96-well plates were exposed to UV light of 1000 mW m^{-2} at a wavelength of 254 nm for a range of times. A 180 µl volume of culture supernatant was removed from each well (20 µl was left to ensure the cultures did not dry out) and the plates placed in a biohazard laminar flow hood and exposed to UV light for 3, 2 and 1 h and for 30, 15, 10, 5 and 1 min. Controls were left unexposed. A second experiment was conducted using 5×20 µl aliquots of culture suspension per 60 mm petri dish. Lids were removed and the dishes were exposed to UV light for the same times as above.

Desiccation: All media were removed from cultures in 96-well plates, which were left with the lids off in a biohazard laminar flow hood for 30, 20, 15, 10, 5, 2, and 1 min at 22°C, before adding 200 µl of TGhL liquid medium per well. In a second experiment, wells were dried for 6, 5, 4, 3 and 1 h, and 30 min. Based on the results observed in the above experiments, a final experiment was performed with exposure times of 1 h, 1 h 20 min, 1 h 40 min, 2 h, 2 h 20 min, 2 h 40 min, and 3 h.

Heat: Exposure of *Batrachochytrium dendrobatidis* to 37°C was repeated due to a different strain being used in the second series of experiments and the importance of the Series 1 results. The incubator was monitored for temperature variation, before and during the experiment, and it remained constant. Duplicate flasks containing 5 ml of 4 d old cultures were exposed for 2, 5, and 30 min, 1, 2 and 4 h. After expo-

Table 1. *Batrachochytrium dendrobatidis.* Summary of effect of chemical disinfectants on zoospores and zoosporangia after exposure at listed concentrations and times (from Series 1 and 2). DDAC: didecyl dimethyl ammonium chloride

Chemical/product	Active ingredient (%)	Exposure time (active ingredient)	100% kill
Sodium chloride	10	5 min, 2 min	Yes
	10	1 min, 30 s	No
	5	5 min	Yes
	5	2 min, 1 min, 30 s	No
	2.5 to 1	5, 2, 1 min, 30 s	No
Household bleach	4 to 1	10, 5, 2 and 1 min, 30 s	Yes
(active ingredient: sodium hypochlorite)	0.2 to 0.01	10 min	Yes
	0.4	5, 2, 1 min and 30 s	No
	0.4	10, 5, 2 and 1 min, 30 s	No
Potassium permanganate	2	10 min, 5 min	Yes
	2	2 min, 1 min	No
	1	10 min	Yes
	1	5, 2 and 1 min	No
	0.1 to 0.001	10, 5, 2, 1 min	No
Formaldehyde solution	1	10 min, 5 min	Yes
	1	2 min, 1 min	No
	0.1	10 min	Yes
	0.1	5, 2 and 1 min	No
	0.01 to 0.001	10, 5, 2, 1 min	No
Path-X™ agricultural disinfectant	1×10^{-2} to 1×10^{-3}	5, 2 and 1 min, 30 s	Yes
(active ingredient: DDAC)	1×10^{-4}	5 min, 2 min	Yes
	1×10^{-4}	1 min, 30 s	No
	1×10^{-5} to 1×10^{-6}	5, 2, and 1 min, 30 s	No
Quaternary ammonium compound 128	Full strength to 1×10^{-3}	5, 2, and 1 min, 30 s	Yes
(active ingredient: DDAC)	1×10^{-4} to 1×10^{-6}	5, 2, and 1 min, 30 s	No
Dithane	1×10^{-2} to 1×10^{-6}	5, 2, and 1 min, 30 s	No
Virkon	1 mg ml^{-1}	5 min, 20 s	Yes
Ethanol	70%	5 min, 20 s	Yes
Benzalkonium chloride	1 mg ml^{-1}	5 min, 20 s	Yes

sure, flasks were examined for zoospore activity and then incubated at 23°C. After 4 d, these cultures were used to inoculate new flasks, which were incubated at 23°C and checked regularly for growth and activity over 2 wk. Actively growing cultures were discarded, whereas cultures showing no discernable growth or activity were incubated for up to a further 4 wk. If no activity was recorded within this time they were recorded as dead.

RESULTS

All chemical disinfectants resulted in 100% mortality for at least 1 of the concentrations tested (Table 1), apart from Dithane, which was ineffective at all concentrations. However, the concentration and time of exposure was critical for most chemicals. Exposure to 70% ethanol, 1 mg Virkon ml^{-1} or 1 mg benzalkonium chloride ml^{-1} resulted in death of all zoosporangia after 20 s exposure. Zoosporangia that had been killed with ethanol appeared shrivelled.

Desiccation in Series 1 (using cultures in 24-well plates) resulted in 100% mortality after 1 or more hours. Zoosporangia appeared shrivelled, and the walls were less defined and less refractile. In Series 2 (using cultures in 96-well plates), drying for 3 h caused 100% mortality, but survival occurred after shorter drying times (Table 2). Exposure to UV light for 24 h did not kill cultures of *Batrachochytrium dendrobatidis* (Table 2), yet they were sensitive even to mild heat, with 100% mortality in flasks kept at 32°C and above

Table 2. *Batrachochytrium dendrobatidis.* Efficacy of UV light and desiccation on zoospores and zoosporangia after exposure for various times (from Series 1 and 2)

Method	Exposure time	Effective?
UV light	24, 12, 2 and 1 h, 30, 15, 10, 5 and 1 min	No
Desiccation	6, 5, 4 and 3 h	Yes
	2 h 40 min to 1 h	No

Table 3. *Batrachochytrium dendrobatidis.* Times at different temperatures to kill 100% of the fungus in culture (from Series 1 and 2)

Temperature (°C)	Time at which all zoosporangia were killed
100	1 min
60	5 min
47	30 min
37	4 h
32	96 h
26	No death
23	No death

(Table 3). Mortality was dependent on time of exposure, and at 37°C and above, death occurred after short exposure times.

DISCUSSION

This study has shown that a number of physical measures and chemical disinfectants can cause 100% mortality of *Batrachochytrium dendrobatidis in vitro.* Disinfection treatments, concentrations and times of exposure were selected with practical considerations in mind. Ideal treatments would not be harmful to the environment, and would be inexpensive and practical to apply. For disinfection of equipment and personnel, short exposure times are preferable, particularly in the field environment. Therefore, a range of concentrations was tested for each reagent over a range of relatively short exposure times.

The fungus is highly sensitive to heat, and died within 4 h at 37°C, and within 30 min at 47°C. The practical implications of this temperature sensitivity are that hot water at a temperature safe for humans (<60°C for a minimum of 5 min) can be used for disinfecting aquarium equipment and tanks by immersion (boiling water is not required). The sensitivity of *B. dendrobatidis* to temperatures of 32°C and above implies that mammals have little risk of being infected with *B. dendrobatidis*. Temperature sensitivity also suggests that subjecting infected amphibians to temperatures of 37°C may be useful as part of a treatment regime. Desiccation killed cultures of *B. dendrobatidis*, although small amounts of residual water appeared to allow survival up to 3 h. Complete desiccation appeared to take longer in the smaller wells of 96-well plates than in the 24-well plates. Drying, as a strategy to sterilise equipment, would have to be long enough to ensure that all water had evaporated. A combination of heat and drying is recommended; however, this combination was not specifically tested. UV light at a wavelength and intensity capable of killing viruses and bacteria did not kill cultures of *B. dendrobatidis*, and is therefore not recommended for its disinfection.

Several chemical disinfectants we tested can be recommended for use. Ethanol (70%) and Virkon (1%) are both commonly used in laboratories for cleaning surgical equipment and surfaces, and both killed *Batrachochytrium dendrobatidis* within 30 s. Seventy percent ethanol could also be used in the field to kill *B. dendrobatidis* zoosporangia on instruments used for amphibian studies, such as scissors and weighing equipment. Ethanol should cause no damage to these items. Larger pieces of field equipment could be disinfected with 2% sodium hypochlorite (household bleach) or 0.1% DDAC. Sodium hypochlorite at concentrations above 1% active ingredient was rapidly effective; at 0.4% it required a minimum 10 min exposure time; and at concentrations below 0.4% it was ineffective. Sodium hypochlorite can be inactivated by organic debris and this may reduce the disinfectant action of concentrations that are effective *in vitro*. DDAC was a highly effective disinfectant at dilutions of 1/1000 of the original product. Field workers can therefore carry small amounts of concentrated solution to dilute with water collected from natural water bodies. DDAC is promoted as being environmentally safe and is used in agriculture and in forestry (Xiao & Kreber 1999), but extensive test data do not appear to be publicly available. Care must be taken to avoid environmental contamination with the use of any disinfectant in the field. Dithane was tested, since this product is used extensively as a fungicide by the banana industry in north Queensland, and frogs are often inadvertently transported with produce (G. Marantelli pers. comm.). It had no disinfectant effect at exposure times of 5 min or less. Formaldehyde solution was effective at 1% for at least 5 min, or at 0.1% for at least 10 min; lower concentrations were ineffective. Due to its toxicity at effective concentrations, formaldehyde solution is not recommended as a disinfectant for *B. dendrobatidis*. Sodium chloride and potassium permanganate were tested since they are easily obtained and sometimes used as disinfectants in the aquarium industry (Needham & Cross 1988). Sodium chloride was effective only at high concentrations for exposure times of 5 min or greater. The concentrations required (5 to 10%) would probably cause damage to equipment. Potassium permanganate was effective at 1 to 2% for an exposure time of 10 min. This solution may be useful for amateur herpetologists when disinfecting aquaria and equipment by immersion.

These results are also relevant to the epidemiology of chytridiomycosis; for example, the disease appears unlikely to persist in hot, dry climates, but UV levels may not be significant for survival.

Acknowledgements. Thanks to Gerry Marantelli, Alastair Freeman and Keith McDonald for providing infected frogs from which we obtained the fungal cultures used in this study.

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Editorial responsibility: Peernel Zwart, Utrecht, The Netherlands

Submitted: April 14, 2003; Accepted: August 18, 2003
Proofs received from author(s): December 10, 2003