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Antifungal efficacy of F10SC veterinary disinfectant against *Batrachochytrium dendrobatidis*

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Abstract

The Infectious disease chytridiomycosis, which is caused by the fungal pathogen *Batrachochytrium dendrobatidis,* has been identified as one of the most important drivers of amphibian declines and extinction. *In vitro B. dendrobatidis* is susceptible to a range of disinfectants, but not all have been tested on animals and some that have been proven effective have harmful side effects on the surrounding environment or the animals being treated. We tested the efficacy of F10SC veterinary disinfectant to treat *B. dendrobatidis* in experimentally infected tadpole and juvenile *Sclerophrys gutturalis* and tadpoles of *Sclerophrys poweri* and *Amietia hymenopus*. The minimum inhibitory concentration for F10SC on *in vitro B. dendrobatidis* ranged between 1:7000 for 5-min contact time and 1:10000 for 10-min contact time. Based on the survival data of test animals the no observed effect concentration for 15-min contact time was estimated to be 1:2000 dilution for juveniles, and 1:10000 for tadpoles. In *S. gutturalis* juveniles an 86% infection clearance rate was achieved after five 15-min doses of 1:3000 dilution. A 100% clearance was achieved in *A. hymenopus* tadpoles after seven 15-min doses of 1:10000 dilution, and after nine doses of the same treatment in *S. poweri* tadpoles. F10SC has the benefit of being a concentrated compound that provides a treatment protocol which is nontoxic to tadpoles and post-metamorphic individuals, has a short half-life and is effective against *B. dendrobatidis* during short contact times, but further testing on different species of amphibians is advised.

Key words: amphibian disease, treatment, F10SC, chytridiomycosis, *Amietia*, *Sclerophrys*.

Introduction

Emerging infectious diseases have become recognized as one of the most significant threats to humans, wildlife, and plants over the last three decades and are often driven by large-scale anthropogenic environmental change.[1–3] It is therefore in the interest of public health and conservation that disease control strategies be developed for example by testing disinfection protocols. Disinfection of animals and equipment is important for biosecurity and reduction of cross-contamination during research, as well as for captive

husbandry, and as a quarantine procedure or part of a conservation management strategy for species threatened by disease.[4] Disinfectants can also be used as treatments for pathogenic organisms, including fungal infections.[5,6]

One emerging infectious disease of amphibians in particular, chytridiomycosis has received a lot of attention from conservation strategists.[7–9] Chytridiomycosis has been implicated as the driver of population declines in over 200 species on a global scale and has been associated with the extinction of some species.[10] This disease is caused by the presence of the chytrid fungus, *Batrachochytrium dendrobatidis* in the skin of all three amphibian orders.[11–14] Chytridiomycosis causes death by disruption of normal physiological skin functions, including cutaneous respiration and osmoregulation, resulting in fatal electrolyte imbalances.[15] Because *B. dendrobatidis* can remain infective in the environment without an amphibian host for up to 7 weeks and up to 4 weeks in deionized or tap water,[16] its persistence presents a major threat to biosecurity in *ex situ* conservation. Effective disease control should therefore include both the treatment of infected animals and disinfection of the environment in which they are maintained.[17] Recently, a congeneric chytrid fungus *B. salamandrivorans* has been discovered in salamanders and newts in Asia and Europe.[18,19] Its invasive nature and pathogenicity implies that similar disease management strategies, including treatment protocols need to be developed for *B. salamandrivorans*.

In vitro B. dendrobatidis is susceptible to elevated temperature, drying, salt, and a broad range of antibiotics and chemicals. The most widely used disinfectant is bleach (sodium hypochlorite), but it is harmful to live amphibians. Other disinfectants recommended for use include Path-X™ agricultural disinfectant, Virkon, benzalkonium chloride, ethanol,[20] Betadine antiseptic liquid, F10SC veterinary disinfectant, and TriGene virucidal disinfectant cleaner.[21] These compounds are effective over short contact times and ideal for surface decontamination but not all are safe for use on animals. Furthermore, the concentrations that kill 100% of the zoospores and prevents growth and encystment vary between agents.[22] Elevated temperature has shown promise as a treatment, but species differences in tolerance preclude this as a consistently viable treatment.[23] A treatment combining formalin and malachite green was followed by the death of five frogs during the first 48 h of treatment.[24] Chloramphenicol has been proven to be an effective treatment but issues related to human health eliminate it as a viable treatment.[25,26] Two independent studies used itraconazole to successfully clear infection in juvenile frogs[27] and anuran tadpoles,[28] respectively, but resultant depigmentation in tadpoles limits its application. Other antifungals that have successfully cleared infection in post-metamorphic frogs include voriconazole spray and terbinafine hydrochloride in ethanol baths.[29,30]

An agent selected to disinfect equipment or treat animals should not have any harmful side effects, either on the environment or the animals being treated. There is therefore a need to develop treatment protocols using compounds that are effective at low concentrations and over short contact times without harming the environment or host and showing efficacy over all amphibian life stages. F10SC veterinary disinfectant (F10SC) is a quaternary ammonium and biguanidine compound generally used as a surface acting biocidal. Due to its high concentration, inherently low toxicity and biodegradable nature, it is widely used within the veterinary profession in clinical practice, referral hospitals, zoos, laboratories, and various other institutions (www.healthandhygiene.co.za/f10sa). F10SC has been proven to be effective against *B. dendrobatidis in vitro* at a concentration of 1:3000[21] and has been used as a prophylactic treatment for dermatomycoses in quarantined amphibians.[31] However, F10SC had not been experimentally tested as a treatment option for *B. dendrobatidis* infection, highlighting the need for clinical trials.

Here we report on the toxicity characteristics of F10SC to *B. dendrobatidis* and three anuran host species, as well as its efficacy as a treatment option in both tadpoles and juvenile toads. It is possible to test clearance success by first infecting individuals through exposure to fungal zoospores,[22,32] followed by subsequent treatment with the test compound. Moreover, infection status can accurately be diagnosed in the keratinised superficial skin layers of post-metamorphic individuals and in the jaw sheaths of tadpoles as a way of gauging the efficacy of the antifungal test compound.[15]

Methods

Ethics approval was obtained from the North-West University Animal Ethics Office (ethics number NWU-00013-10-S4). We followed a phased approach to ensure that the minimum number of animals was exposed to lethal concentrations of F10SC, and that the selected concentration(s) ultimately killed *B. dendrobatidis* without harming the experimental animals. The toxicity levels of F10SC for *B. dendrobatidis* were determined, and these parameters used to guide toxicity trials on the experimental animals. A broad range of effective concentrations (able to kill *B. dendrobatidis*) were first tested on individual animals before safe concentrations (animal survives exposure) were replicated on the test subjects.

Batrachochytrium dendrobatidis culture

We used an isolate of *B. dendrobatidis* (MG04) that was obtained from a wild *Amietia fuscigula* during a disease survey of the Western Cape. This isolate belongs to the

global panzootic lineage (GPL) and thus has a high infection potential and virulence.[33] In sum, 1 ml *B. dendrobatidis* culture was passaged into tissue culture flasks (25 cm^2 growth surface) containing 14 ml 1% tryptone broth and kept at 4°C .[12,34] Four days prior to zoospore harvesting the cultures were incubated at 21°C to stimulate zoospore production. Cultures were first inspected with an inverted microscope (Nikon Eclipse TS100-F) to determine if zoospores were present, before zoospore density was quantified using a hemocytometer.

F10SC toxicity test on *Batrachochytrium dendrobatidis*

The efficacy of F10SC on *B. dendrobatidis* was determined by establishing the minimum inhibitory concentration (MIC) defined as the lowest concentration of an antimicrobial drug that will inhibit the visible growth of a microorganism after overnight incubation.[35] The fungicidal tests were carried out in accordance with Section 5.2 of SANS 636–2013 guidelines for disinfectants based on quaternary ammonium compounds but with the following modifications.[36] In sum, 200 μl of broth containing approximately 10,000 *B. dendrobatidis* zoospores was transferred to each of 96-well plates, the plates sealed with Parafilm and incubated at 21°C for 4 days. The supernatant in each well was then removed and replaced with a chosen dilution of F10SC. The *B. dendrobatidis* plates were assigned to six treatments according to the dilution of F10SC (1:3000, 1:5000, 1:7000, 1:10000, 1:15000, and 1:30000), plus a control that received autoclaved distilled water. Each treatment was tested at four different time intervals (range 5–120 min) and was replicated 12 times. Microscopy using an inverted microscope established culture viability at the various time intervals. A culture was presumed nonviable if no motile zoospores were seen after 30 s of observation. The F10SC was then removed and replaced with 1% tryptone broth, and again observed for the presence/absence of motile zoospores after 24 h of incubation at 21°C. We assumed that if motile zoospores only were sensitive to F10, a burst of zoospores originating from mature sporangia present in the culture at the time of the exposure, would be observed the day following incubation. Alternatively, we assumed that a persistent loss of motile zoospores indicated that the culture was not viable, since not only motile zoospores but also mature sporangia were inhibited. The respective F10SC treatments (concentration and exposure time) were deemed effective at inhibiting *B. dendrobatidis*.

Collection and husbandry of test animals

Three anuran species known to be susceptible to *B. dendrobatidis* infection were included in this study.[37,38] Guttural toads, *Sclerophrys gutturalis,* is a robust species under captive conditions and an imminent experimental model of chytridiomycosis (Weldon, unpublished data). Power's toad, *Sclerophrys Poweri,* is closely related to *S. gutturalis* and allowed us to test if subtle differences in toxicity to F10SC and treatment of *B. dendrobatidis* exist within a genus. The Phofung river frog, *Amietia hymenopus,* is an important long-term indicator of chytridiomycosis in nature and one of the few species known to experience seasonal mortality in South Africa.[37] Egg strings of *S. gutturalis* (four clutches) were collected from ponds in the Potchefstroom area, North West Province (26.679976, 27.095241) on 01/11/2013 (permit no. 028NW-11). Three egg clutches of *S. poweri* were collected from a farm dam near Ventersdorp, North West Province (26°20′41.8″S 26°46′37.7″E) on 08/12/2014 under the same permit. Tadpoles of *A. hymenopus* were collected from Mont-Aux-Sources, KwaZulu-Natal (28°44′54.0″S 28°52′39.5″E) on 03/12/2014 (permit no OP526/2014). Only tadpoles under Gosner stage 30 were collected as these have a reduced likelihood of being infected with *B. dendrobatidis*.[37]

Temperature was adjusted at 20°C and 15°C for the toads and frogs, respectively, for the duration of the experiment and lighting was regulated on a 12-hour day/night cycle. The eggs were hatched in aerated shallow trays (42× 28 cm). Tadpoles were housed in glass aquaria (30×40 cm) in aerated aged borehole water. Toad tadpoles were reared on ground Tetra tabimin pellets, and three quarter water changes were made every other day. *A. hymenopus* tadpoles were fed ground Marcus Rohrer Spirulina tablets and received three quarter water changes daily. Once their tails were resorbed juveniles were kept individually in plastic tubs containing wetted tissue paper substrate (8×13 cm). Juveniles were fed 5–8 pinhead crickets 3 times a week and the substrate replaced twice weekly.

F10SC amphibian toxicity trials

F10SC toxicity was determined in three steps on the test animals by establishing the highest concentrations where no adverse effect was observed (NOEC, no observed effect concentration). First a range finding test was performed by testing a wide range of concentrations with large steps between consecutive concentrations on single animals. In doing so it is possible to find those concentrations that are safe to use while preventing the unnecessary death of groups of animals.[39] Tadpoles from all three species were exposed in plastic cups containing 100 ml of F10SC dilution, while juvenile *S. gutturalis* exposures were performed in petri dishes filled with 20 ml of F10SC dilution. Aged borehole water replaced the test compound in the control groups. Animals were exposed for 5 min to the following F10SC concentrations during the range finding test: 1:100, 1:500, 1:1000,

1:5000, and 1:10000. Second, replicated trials were conducted on a narrower range of F10SC concentrations identified by the range finding test in order to yield an even more precise safe concentration for experimental animals. These concentrations were more diluted than the lowest lethal concentration, but more concentrated than the highest safe concentration. Third, selected concentrations were tested on *S. gutturalis* juveniles and tadpoles and *S. poweri* tadpoles during 5, 15, and 30 min exposures and replicated in 10 animals. Tests for each concentration started with the shortest contact time and only progressed to a longer contact time if all test subjects survived.

Observable differences in behavior from control animals were interpreted as signs of discomfort and used as humane endpoints during the exposures. For tadpoles, the endpoints included any change in the frequency or intensity of movement, sporadic and intense surfacing, and turning upside down. Endpoints for juveniles included increased mucus production, cutaneous erythema, and any change in posture or behavior in attempts to escape the solution.[40] Experiments were terminated when humane endpoints were reached and the animals euthanized with Ethyl 3-aminobenzoate methanesulfonate salt (MS222). Animals that survived exposure were kept for a further 7 days to observe any potential adverse effects, after which they were euthanized with MS222. All specimens were preserved in 70% ethanol.

B. dendrobatidis clearance trials

Tadpole inoculation and treatment

Tadpoles of *S. poweri* and *A. hymenopus* between developmental stages 31 and 33 were randomly selected from the various clutches and assigned to two treatment groups, a positive control (exposed to *B. dendrobatidis* but not treated with F10SC) and a negative control group, each of 20 animals, thus totaling 80 tadpoles for each species. Tadpoles were individually housed in plastic cups containing 200 ml aged borehole water. Zoospores were harvested and quantified using the procedures described above; density varied between 0.7×10^5 – 1×10^5 zoospores per ml. Prior to *B. dendrobatidis* exposure, cups were decanted so that ~50 ml water remained. In sum, 500 μl inoculation culture was then pipetted into each cup. Control animals received a similar dose of filtered *B. dendrobatidis* culture that was passed through a sterile 0.2 μm single use syringe filter unit. Cups were refilled with aged borehole water after 30-min exposure time. Tadpoles were inoculated every other day for a total of five doses. Seven days after the last dose F10SC treatment commenced.

Tadpoles were treated in petri dishes filled with 20 ml of either 1:10000 F10SC solution for 15 min or aged borehole water (control group), after which they were returned to their cups. One group received seven daily treatments, and another group received nine daily treatments. The third group served as a positive control (previously exposed to *B. dendrobatidis*), while the fourth group served as negative control (no prior *B. dendrobatidis* exposure). These were exposed to aged borehole water for nine consecutive days. The treatment group that received seven F10SC treatments received aged borehole water on days eight and nine. Seven days after the last F10SC treatment the animals were euthanized with MS222.

Juvenile inoculation and treatment

Recently metamorphosed *S. gutturalis* juveniles were randomly assigned to three treatment groups, a positive control and a negative control group, each of 30 animals, thus totaling 150 animals. Animals were inoculated with *B. dendrobatidis* in petri dishes containing 15 ml borehole water. Zoospore density varied between 5.8×10^5 and 6.3×10^5 zoospores per ml. Treatment and positive control animals received five doses of 500 μl *B. dendrobatidis* culture every other day, while negative control animals received a similar dose of filtered *B. dendrobatidis* culture. Animals were returned to their tubs after 3 hours of exposure. Seven days after the last dose F10SC treatment commenced.

Animals were treated in petri dishes containing 15 ml aged borehole water for 10 min with a 1:3000 F10SC dilution before being returned to their tubs. One group received only one treatment, while another two groups received three and five daily treatments, respectively. A fourth group served as a positive control (previously exposed to *B. dendrobatidis*), while the last group served as a negative control (no prior *B. dendrobatidis* exposure) and were exposed to aged borehole water for five consecutive days. The treatment groups that received fewer than five F10SC treatments received aged borehole water on the remaining treatment days. Seven days after the last F10SC treatment the animals were euthanized. Juveniles that died during the experiment were placed in 1.5 ml micro tubes with 70% ethanol. All surviving animals were preserved in 70% ethanol filled micro tubes after euthanasia.

Infection diagnosis

A noninvasive technique was used to screen for *B. dendrobatidis* in the juvenile toads before administering the first F10SC treatment. A sterile cotton swab was streaked 10 times over the ventral surface of the hind feet, thighs, and belly region. Gloves were changed between individuals. Swabs were stored dry at 4°C until they were tested for *B. dendrobatidis* with quantitative polymerase chain reaction

(qPCR). The oral discs of preserved tadpole specimens and the hind limbs of preserved juvenile specimens were excised using a sterile surgical blade and cutting stage. The blade and dissecting stage were wiped with 96% ethanol and paper towel between each specimen and replaced between treatment groups. DNA from the pretreatment swabs and posttreatment tissue samples was subjected to qPCR for the diagnosis of *B. dendrobatidis* using a modified protocol of Boyle et al.[34] For DNA extraction we used 50 μl PrepMan Ultra, the samples were vortexed for 5 min, and centrifuged for 3 min. We included only 0.4 μl BSA per sample in the Mastermix, which reduced the added distilled water to 9.475 μl, and the last two steps in the qPCR cycle were repeated 60 times.

Results

F10 toxicity to *Batrachochytrium dendrobatidis*

After incubating *B. dendrobatidis* for 3 days in 96 well-plates, a white biofilm was visible at the bottom of each well, and active growing *B. dendrobatidis* cultures were observed upon microscopic examination in all the experimental and control wells. A series of F10SC dilutions was then allowed to react with the live *B. dendrobatidis* cultures, and their viability recorded at regular time intervals. Death of the fungal colonies (complete inhibition of zoospores) was confirmed after a 24-h post-exposure incubation period. *B. dendrobatidis* survival time progressively decreased with increasing concentrations of the test substance. It took less than 5 min contact time for F10SC to kill at least 99.9% of *B. dendrobatidis* at concentrations ≥1:7000, which represents at least a Log-4 reduction of the original zoospore density in the wells (Table 1). The MIC for 5 min contact time was estimated to be 0.0143% F10SC or a 1:7000 dilution. Concentrations of 1:10000 and 1:15000 required 10 min of contact time to kill *B. dendrobatidis*, resulting in an estimated MIC for 10-min contact time of 0.0067% F10SC. And 1:30000 was the lowest concentration tested and took up to 120 min to kill all of the *B. dendrobatidis* colonies in the test wells.

Table 1. Fungicidal efficacy of F10SC against *Batrachochytrium dendrobatidis*.

Dilution	Contact time	Percentage kill of *B. dendrobatidis*
1:3000 or 0.033% F10SC	5 min	99.9%
1:5000 or 0.02% F10SC	5 min	99.9%
1:7000 or 0.0143% F10SC	5 min	99.9%
1:10000 or 0.01% F10SC	10 min	99.9%
1:15000 or 0.0067% F10SC	10 min	99.9%
1:30000 or 0.0033% F10SC	120 min	99.9%

F10 toxicity to frogs

The initial exposure of test animals to F10SC was done on a broad range of concentrations in order to minimize the time taken to find a safe dose and thus reduce the total number of test animals. These range finding tests demonstrated that survival in all three of the test species decreased as F10SC became more concentrated (Fig. 1). The tolerance levels for juvenile *Sclerophrys gutturalis* was found to be almost 20 times higher than that of tadpoles of the same and other species. Differences in tolerance between tadpole species were negligible, with toxicity experienced at a 1:5000 F10SC dilution. Toxicity became much more acute (40x shorter survival time) when concentrations increased to 1:3000 and higher. In contrast to tadpoles, juvenile toads tolerated concentrations as high as 1:500, with the 1:100 dilution being toxic. The majority of test animals that displayed humane endpoint symptoms were im-

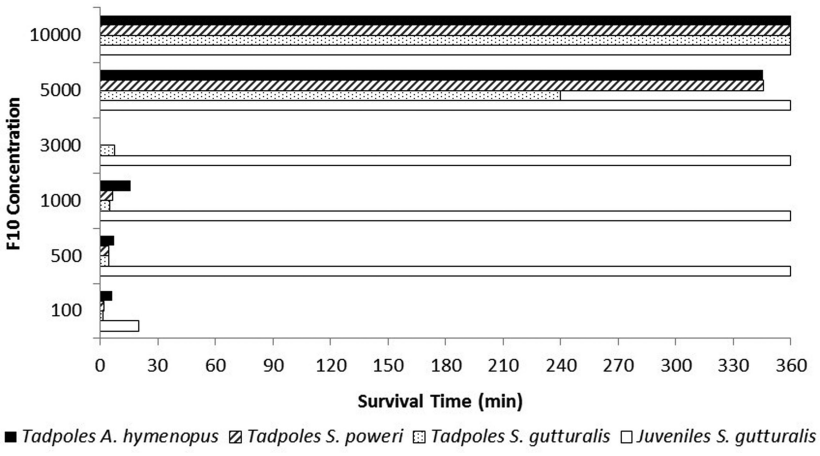

Figure 1. Survival time of different life stages of three species when exposed to a range of F10SC concentrations. Contact time was kept constant at 5 min. Individuals that survived up to 360 min post-exposure survived for 7 days before being euthanized.

Table 2. Survival rates of *Sclerophrys gutturalis* and *Sclerophrys poweri* following different exposure times to a range of F10SC concentrations.

		Survival proportion		
Species	Concentration	5 min	15 min	30 min
Juveniles				
S. gutturalis	1:1000	0.8*	0.0	not tested
S. gutturalis	1:2000	1.0	1.0	0.0
S. gutturalis	1:3000	1.0	1.0	0.0
Tadpoles				
S. gutturalis	1:5000	1.0*	0.2*	not tested
S. poweri		1.0*	0.3*	not tested
S. gutturalis	1:6000	1.0*	0.3*	not tested
S. poweri		1.0*	0.4*	not tested
S. gutturalis	1:7000	1.0*	0.8*	not tested
S. poweri		1.0*	not tested	not tested
S. gutturalis	1:10000	1.0	1.0	0.8*
S. poweri		1.0	1.0	0.7*

*Animals that displayed symptoms of humane endpoints were euthanized immediately following exposure time.

mediately euthanized. Those animals that died before euthanasia could be administered all died in under 5 min and were from the highest concentration treatments (1:100 – 1:1000). The mechanism for intolerance of F10SC is unknown but may stem from a sensitivity to high concentrations of its quaternary ammonium compounds. Generally, a shorter exposure duration (contact time between test compound and animal) and lower concentration of F10SC resulted in a higher proportion of test animals surviving the exposures. *Sclerophrys gutturalis* juveniles survived 5 and 15 min exposures below a 1:1000 concentration, but 30 min of exposure to the same concentrations was toxic (Table 2). Concentrations lower than 1:3000 were not tested on juveniles since a 100% survival rate had already been obtained at concentrations as high as 1:2000. Similar sensitivity to exposure duration and dose concentration were observed for tadpoles of both *S. gutturalis* and *S. poweri*. Both species demonstrated 100% survival after 5-min exposure to all concentrations tested (1:5000-1:10000), whereas 15 min of exposure resulted in only 20–80% survival when exposed to concentrations higher than 1:10000. However, tadpoles displayed symptoms of distress during all exposures above 1:10000 concentration regardless of exposure time and were euthanized immediately following exposures. Only tadpoles that were exposed to a 1:10000 concentration for up to 15 min survived without displaying any distress symptoms; 30 min of exposure at this same concentration resulted in 80% survival of *S. gutturalis* tadpoles and 70% survival of *S. poweri* tadpoles. Based on the survival data the NOEC for 15-min contact time was estimated to be 1:2000 dilution for juvenile toads and 1:10000 for tadpoles. No NOEC testing was performed on *Amietia hymenopus* because they displayed similar trends in the range finding test, and in order to reduce the number of animals used to the absolute minimum as required by the AnimCare ethics committee.

Host susceptibility and survival

Forty of the 150 juvenile *S. gutturalis* from the three treatment groups and positive control group (exposed to *B. dendrobatidis*) died before the F10SC treatment commenced. At the time when the diagnostic skin swabs were taken (7 days after the last *B. dendrobatidis* exposure), 11 animals from the exposure groups had died, and by the following day a further 29 animals had died (including three from the negative control group). The remaining 73.3% of the exposure animals were treated with F10SC. Animal numbers stabilized during treatment with F10SC, but nine more animals from the positive control group (no F10SC treatment) died. All of the remaining negative control animals were alive at the end of the experiment. Exposure to *B. dendrobatidis* did not result in 100% infection in any of the treatment or positive control groups. Instead, infection prevalence ranged between 71 and 93% (mean 83%, SD 9.93). The negative control group remained *B. dendrobatidis* free midway through the experiment and at the end of the experiment.

No deaths occurred in either of the two tadpole species (*S. poweri* and *Amietia hymenopus*) following *B. dendrobatidis* exposure and prior to F10SC treatment. Infection status was not determined in tadpoles before commencement of F10SC treatment in order to prevent possible damage caused by focused swabbing of their small mouthparts (loci of infection). All positive and negative control animals survived the duration of the experiment.

Clearance of *Batrachocytrium dendrobatidis*

A dose dependent response to the 1:3000 F10SC treatment was observed in *Sclerophrys gutturalis* juveniles that tested positive for *B. dendrobatidis* before the treatment regime (Fig. 2A). After a single dose, 40% of *B. dendrobatidis* infections were still present. Infection clearance rate was higher in animals that received repeated doses: 88 and 86% in the three- and five-dose groups, respectively. The negative control animals remained uninfected, while the positive control animals retained their infection. The animals that remained infected after F10SC exposure had a 3 times lower infection intensity (mean genomic equivalent (GE) ± standard error (SE) = 1.554 ± 1.990) than the positive control group (mean GE ± SE = 4.852 ± 7.325). Treatment of tad-

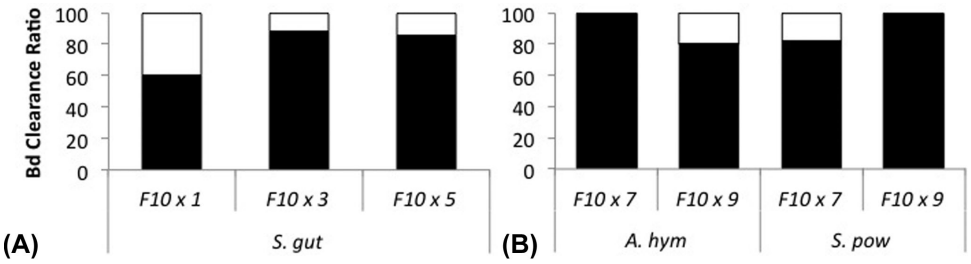

Figure 2. F10SC clearance success of *Batrachochytrium dendrobatidis* by dose frequency in (A) juveniles of *Sclerophrys gutturalis*; (B) tadpoles of *S. poweri* and *Amietia hymenopus*. Shaded area represents proportion of animals that were *B. dendrobatidis* −, while white area represents proportion of animals that remained *B. dendrobatidis* +.

poles resulted in a 100% clearance rate in both species, but efficacy according to dose frequency was variable (Fig. 2B). In *A. hymenopus* complete efficacy was achieved in the seven-dose group, while 20% of the animals that received nine doses remained infected. In *S. poweri* 18% of animals remained infected after seven doses, while complete efficacy was achieved in the nine-dose group. Infection status in the control groups remained unchanged. Infection intensity in the positive control group of *S. poweri* was 40 times higher (mean GE ± SE = 7.20 ± 11.77) than in the animals that remained infected after treatment (mean GE ± SE = 0.18 ± 0.08). Similar infection intensity was found in the positive control and the nine-dose groups of *A. hymenopus* (mean GE ± SE = 0.19 ± 0.225 and 0.16 ± 0.17, respectively).

Discussion

Concentrations of F10SC as low as 1:30000 effectively inhibited the growth of *B. dendrobatidis* as the MIC resulted in a Log-4 reduction in zoospore density in compliance with SANS 636–2013 guidelines for disinfectants based on quaternary ammonium compounds.[36] MIC was found to be time dependent, thus for each concentration to inhibit *B. dendrobatidis* a specific contact time is required. However, practical application of the antifungal has to be considered when deciding on a concentration and exposure time. Two hours of contact time at 1:30000 dilution can place a huge time constraint on certain applications, whereas exposures of up to 10 min at 1:15000 are more practical for general disinfection purposes. Inhibition of *B. dendrobatidis* at these low concentrations (i.e., small volumes), and the fact that it is biodegradable means that F10SC can be recommended for disinfection during field studies.

Prolonged or over exposure to normally safe concentrations can be just as toxic to the host animal as shorter exposures to more concentrated F10SC solutions. When applied at the right concentration and for the correct contact time F10SC is nontoxic and safe to use on both juvenile toads and tadpoles. However, distinct differences exist in the tolerance levels of these two life stages with the NOEC values for tadpoles being 5 times lower than that of juveniles for the same exposure times (15 min NOEC = 1:2000 in juveniles and 1:10000 in tadpoles). Although comparable results were obtained for sensitivity to F10SC in all three of our tadpole species (*Sclerophrys gutturalis*, *S. poweri*, and *Amietia hymenopus*), other species may show greater variability in sensitivity. The aim of this research was to test whether *B. dendrobatidis* infection in our three test species can be cleared with F10SC and not to establish a generic treatment for all amphibians. Treatment of other species, especially members of other orders of amphibians may require different concentrations.

Our results indicate that survival of test animals is not only influenced by the test substance concentration but also by contact time. Concentrations that appear to be safe for use during short contact times can be lethal when contact time is extended. Results for survival as a factor of contact time were also constant for *S. gutturalis* and *S. poweri* tadpoles. In such cases a higher MIC with a shorter contact time is advisable. Based on the survival data, the NOEC for 15 min contact time was estimated to be 1:2000 dilution for juvenile toads and 1:10000 for tadpoles. Importantly, our 7-day observation following F10SC exposure only allowed for establishing acute toxicity of the test substance and did not account for any possible latent toxicity that may result from the exposure or treatment including deleterious effects on tadpole metamorphosis. Previous studies on the use of F10SC to treat various ailments in four frog species (including *S. gutturalis*) with F10SC have demonstrated that full recoveries were made even after 3 months of observation.[31,41] The lack of long-term complications following treatment suggest a lack of systemic toxicity for the species treated.[31] However, to confirm any latent toxic effects resulting from F10SC treatment would require a grow-out study of treated tadpoles and histological examination of specimens.

Our infection protocol did not achieve a 100% infection rate in the exposed individuals before starting with the treatment regime. Obtaining less than complete infection post-exposure to *B. dendrobatidis* is not uncommon,[6,22]

and infection rates are known to be *B. dendrobatidis*-dose dependent and variable between species.[28] A few juvenile *S. gutturalis* that had been exposed to *B. dendrobatidis* died almost 3 weeks into the experiment before F10SC treatment commenced. Whether mortality was directly due to *B. dendrobatidis* or another cause is not clear. The fact that more deaths were sustained by the positive control group (no F10SC treatment) during the treatment phase suggests that *B. dendrobatidis* was involved in the deaths. Moreover, no more deaths occurred in any of the groups that received F10SC treatment. However, swabbing seemed to have an adverse effect on infected animals since almost three times this number of animals died within 24 h following swabbing, and even a few negative control animals died. Tadpoles appeared more resilient to *B. dendrobatidis* since neither *S. poweri* nor *A. hymenopus* tadpoles suffered any deaths before F10SC treatment commenced. In contrast to early metamorphosed amphibians, a lack of significant deaths in tadpoles due to *B. dendrobatidis* infection has been widely documented.[42,43]

Successful treatment requires an effective concentration that is safe to use on the test animals. For this reason, we lowered the test concentration of the NOEC for juveniles from 1:2000 to 1:3000 in order to reduce the risk of adverse effects even further and because of previous anecdotal evidence that this concentration was safe for use on amphibians.[31] We used a test concentration of 1:10000 in our treatment trials with tadpoles as indicated by the NOEC test and because no lower concentrations were tested. One dose of F10SC was sufficient to clear *B. dendrobatidis* infection in some of the *S. gutturalis* juveniles (60%) in the one-dose treatment group. Higher clearance rates were only achieved after three to five repetitive treatments (88 and 86%, respectively). Significantly, a 100% clearance was achieved after seven repetitive treatments in *A. hymenopus* tadpoles and after nine treatments in *S. poweri* tadpoles. Because we do not have infection data for tadpoles before starting with the treatment trials and 100% infection was not achieved in juveniles, clearance rates in tadpoles is probably an overestimation and should be interpreted with caution. However, the fact that tadpoles from the positive control group remained positive suggests that a significant portion of the rest of the exposed tadpoles were infected before treatment commenced. We did not test more than five treatments on juvenile *S. gutturalis,* but the evidence from the tadpole trials suggest that higher clearance can likely be achieved if a higher number of treatments are administered. Being able to clear infection in tadpoles and post-metamorphic individuals is especially beneficial in aquatic and semi-aquatic amphibian species where the same habitat is shared between life stages. Inhibition of infection intensity, even if not 100%, as observed in the animals that remained infected after F10SC treatment could prevent individuals of some species from becoming moribund, since *B. dendrobatidis* infection load is a known driver of pathogenicity.[44]

F10SC is a concentrated compound that provides a treatment protocol that is nontoxic, has a short half-life, and is effective against *B. dendrobatidis* during short contact times. However, latent toxicological effects are yet to be determined, and follow-up studies focusing on the efficacy of this compound on different species of amphibians, as well as on different sized individuals is necessary in order to comprehend the generic application of F10SC as a treatment for chytridiomycosis caused by *B. dendrobatidis*. These developments will not only benefit captive populations but wild populations as well, especially when conservation goals include establishing disease-free populations through reintroduction programs.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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