

Laboratory tests of antifungal agents to treat tadpoles against the pathogen *Batrachochytrium dendrobatidis*

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ABSTRACT: The fungus *Batrachochytrium dendrobatidis* (*Bd*), which is the etiological agent of the disease chytridiomycosis, is threatening both wild and captive amphibians. While there are some methods of treating amphibians in captivity, no method has yet been shown to be a promising treatment for amphibian populations in natural habitats. Here we present the results of a laboratory experiment in which we tested 2 antifungal agents that might be used to treat amphibians in the field. As a first step towards the goal of developing mitigation methods, we tested the efficiency of these agents in reducing *Bd* prevalence and loads (zoospore counts) in the laboratory. We exposed naturally infected tadpoles of the midwife toad *Alytes obstetricans* to different concentrations of the antifungal agents for 7 d. We found that Virkon Aquatic[®] affected neither *Bd* prevalence nor loads. At 0.625 ml l⁻¹ of General Tonic[®], prevalence was reduced to 60%, and infected animals had greatly reduced burdens. However, tadpole length was reduced by 19% and mass by 32% on average compared to the control group, suggesting a negative effect on fitness. Tadpole survival was not affected at 0.625 ml l⁻¹ or 1.25 ml l⁻¹, but was reduced to 60% at 2.5 ml l⁻¹. Keeping animals in a dilution of General Tonic[®] for 7 d at a concentration of 0.625 ml l⁻¹ might be an easy way to reduce zoospore counts in large numbers of animals at relatively low cost.

KEY WORDS: Amphibian · *Alytes obstetricans* · Chytrid fungus · Treatment · General Tonic[®] · Mitigation · Virkon Aquatic[®]

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INTRODUCTION

Newly emerging diseases can pose serious threats to humans, wildlife, and biodiversity (Daszak et al. 2000, Jones et al. 2008, Fisher et al. 2012). Even though it is unusual for a pathogen to drive its host to extinction, there are some examples where an emerging pathogen has caused host extinction (Smith et al. 2009). One of the most fatal diseases for wildlife capable of driving hosts to extinction is chytridiomycosis, a disease of amphibians caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*; Berger et al. 1998, Lips et al. 2006, Skerratt et al. 2007).

Chytridiomycosis is now considered a major threat to amphibian species and populations; hence, successful amphibian conservation depends on effective treatment of this disease (Woodhams et al. 2011).

While it is possible to treat captive amphibians against infection with *Bd*, treating amphibians in the wild remains a challenge (Woodhams et al. 2011). A treatment protocol must meet different demands to be successful in the field (Berger et al. 2010, Speare & Young 2010): (1) it should be highly effective against *Bd*; (2) it should be effective after a single application; (3) it should act rapidly; (4) it should be able to treat large numbers of amphibians, as whole

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populations need to be treated in the field; (5) it should have a high safety margin; (6) it should be inexpensive; (7) the equipment for the treatment method must be readily available; and (8) it should be able to cure amphibians in a late stage of the disease. Most of these criteria are also fundamental for treatments in the laboratory. For a treatment in the field, however, numbers 2 and 4 above are essential—but also challenging.

To date, multiple attempts have been undertaken to find a suitable method of treating amphibians against *Bd*. This research has resulted in several reports including treatment protocols using elevated temperature, various antifungal agents, antifungal skin bacteria or peptides of amphibians, natural predators, or salt (Woodhams et al. 2011). As it is often the case with antifungal treatments (Denning & Hope 2010), most of these treatment methods have profound disadvantages because some are toxic for amphibians or not tolerated by them, they are too labor-intensive for the treatment of large numbers of animals, or they are simply not effective against *Bd* (Berger et al. 2009, Garner et al. 2009a, Martel et al. 2011, Woodhams et al. 2012). Currently, no known treatment fulfills the criteria mentioned above and has been tested successfully in natural habitats. Many methods that could be used for captive amphibians are unlikely to be useful as anti-*Bd* treatments for amphibian populations in natural habitats, as they do not meet criteria numbers 2 (effective after a single application), 4 (treat large numbers of amphibians rapidly), and 5 (safe for the environment). Hence, we need to explore new treatments that are promising for use in natural populations. Additionally, many treatments have been developed to treat adult amphibians; however, in many species, substantial mortality occurs shortly after metamorphosis (Garner et al. 2009b). If it were possible to clear infection in tadpoles or at least reduce the pathogen load of tadpoles shortly before metamorphosis, then metamorphosing amphibians would likely survive (Briggs et al. 2010, Vredenburg et al. 2010, Kinney et al. 2011). Hence, there is a need for treatments which can be used for tadpoles.

Here we tested 2 antifungal agents that could potentially be used for the mitigation of *Bd* in natural habitats: Virkon Aquatic® (Du Pont, Sudbury, Suffolk, UK) and General Tonic® (Tetra GmbH, Melle, Germany). Both agents were developed to treat large numbers of captive fish against pathogens (including viruses, bacteria, and fungi). We chose these agents because application is very simple even for large numbers of animals and because they are compar-

tively cheap and widely available. Virkon Aquatic® has the same active substances as Virkon S, a disinfectant that is recommended for the disinfection of field equipment at sites with *Bd* (Webb et al. 2007) and is not harmful to tadpoles and zooplankton (Schmidt et al. 2009).

Our study species was the common midwife toad *Alytes obstetricans*, which is highly susceptible to chytridiomycosis (Bosch et al. 2001, Pasmans et al. 2010, Tobler & Schmidt 2010, Böll et al. 2012). In *A. obstetricans*, mortality occurs usually shortly after metamorphosis (Tobler & Schmidt 2010). We therefore focused our treatment on the aquatic life stage of the species to make sure that the animals entered metamorphosis either with no infection or with a low infection burden. We treated naturally infected tadpoles of the midwife toad with different concentrations of General Tonic® and Virkon Aquatic®.

MATERIALS AND METHODS

Study site

In early March 2011, we collected 176 tadpoles of *Alytes obstetricans* after hibernation from Chalkofen, a site in Switzerland in the canton Baselland (7.76647° E, 47.4775° N, 395 m asl). The site is located on a south-exposed hillside with sparsely growing trees and bushes around several ponds. The site includes 4 small ponds of about 3 to 16 m² in size and about 0.5 to 0.8 m in depth. Three of the 4 ponds are located close together, while the fourth is at a distance of about 50 m. Two of the 4 water bodies are densely vegetated, whereas two have no aquatic vegetation. We found tadpoles in one of the 2 vegetated water bodies as well as in the vegetation-free pond located 50 m away. We chose this site because we knew that (1) *A. obstetricans* is abundant at that site, (2) tadpoles hibernate there, and (3) amphibians at the site are positive for *Bd* (among the 176 tadpoles collected in March 2011, *Bd* prevalence = 77%, mean \pm SE *Bd* zoospore count = 96 \pm 10.43).

Laboratory procedure

We transported the tadpoles to the laboratory and placed them individually in clear plastic 1.5 l containers with clear plastic covers filled with 1 l of tap water. Holes in the covers enabled circulation of air. Containers were placed on a shelf and randomly allocated to a treatment. Tadpoles were fed ad libitum

with fish food (Sera Spirulina Tabs) 3 times wk^{-1} throughout the course of the experiment. The water was changed before starting the experiment and thereafter twice a week. The room was equipped with full spectrum sunlight lamps set for a 12 h day length. Room temperature was kept at 19 to 21°C. Six days after capture, the tadpoles were staged (Gosner 1960), weighed to the nearest 0.01 g, measured from the snout to the beginning of the tail muscle to the nearest 0.1 mm, and swabbed over the mouthparts with a sterile rayon-tipped plain swab with a plastic applicator (Copan). Further data on infection, size, and Gosner developmental stage were recorded 1 wk after the experiment was terminated. To avoid cross contamination, gloves were changed between handling different animals, and fresh containers were used for the weighing of each individual. Swabs were analyzed for the presence of *Bd* with real time PCR (rt-PCR, see below). Among those tadpoles that tested positive for *Bd* (136 out of 176), 64 were chosen randomly for use in the experiment. Hence, before starting the experiment, *Bd* prevalence was 100% in all treatment groups.

Antifungal agents

We selected Virkon Aquatic® and General Tonic® as possible agents to treat amphibians against *Bd*. According to the manufacturer, Virkon Aquatic® is an oxygen-based disinfectant used for cleaning and disinfecting surfaces associated with aquaculture and has been tested for its efficacy against a wide range of fish pathogens including viruses, bacteria, and fungi (Antec International and DuPont Animal Health Solutions, www.antecint.co.uk/MAIN/virkaqua.htm). The active substance in Virkon Aquatic® is potassium peroxomonosulfate triple salt. In the environment, it degrades to potassium and sulfate ions. General Tonic® was developed to treat captive fish against bacterial infections and ectoparasites and to treat lesions. According to the manufacturer, the active substances are ethacridine lactate, acriflavine, aminoacridine hydrochloride and methylene blue (Tetra GmbH, www.tetra.net).

Experimental treatments

Each antifungal agent was tested in 6 different concentrations (including a control), with 5 replicates for each concentration and 7 replicates for the control. Concentrations were chosen according to rec-

ommendations of the manufacturer. Because concentrations depended on the antifungal agent, we did not treat the experiment as a factorial experiment with the factors 'antifungal agent' and 'concentration' but treated the tests of the 2 antifungal agents as separate experiments. For Virkon Aquatic®, the concentrations used were 1, 2, 3, 4, and 10 mg l^{-1} ; the recommended normal concentration for treatment against different bacteria and viruses is 2 mg l^{-1} . For General Tonic®, the concentrations used were 0.625, 1.25, 2.5, 3.75, and 5 ml l^{-1} ; the recommended normal concentration is 1.25 ml l^{-1} . The different concentrations were achieved by pipetting the corresponding amount of the agent into 1 l of water and stirring well. Tadpoles were kept individually for 7 d in the corresponding dilution and were observed daily. Animals showing any abnormality in behavior, pigmentation, or body shape during the experiment were put into fresh tap water and were removed from the experiment. Dead animals were stored in 98% ethanol. After 7 d, the water was changed, and tadpoles were kept an additional week before swabbing. During this week, *Bd* infection could regenerate after exposure to the agent such that the risk of false negatives could be minimized.

DNA extractions and rt-PCR

Upon completion of the experiment, the tips of the swabs were cut off, put in 60 μl of PrepMan Ultra (Applied Biosystems), and extracted, applying the bead-beating protocol of Boyle et al. (2004). These extractions were diluted 1/10 and amplified following the rt-PCR protocol of Boyle et al. (2004). Samples were run in duplicate with negative controls and 4 dilutions of standards (100, 10, 1, 0.1 zoospore genomic equivalents, GE). If the results of the 2 PCR wells were inconsistent, the analysis was repeated. Values of GE are corrected for the 1/10 dilution.

Response variables and statistical analyses

We tested 2 main features of the antifungal agents: (1) efficiency against *Bd* (taking *Bd* prevalence and *Bd* zoospore counts as response variables) and (2) side effects on the tadpoles (taking survival and size of the tadpoles as response variables). We measured side effects because we knew from other studies that a treatment can eliminate *Bd* efficiently but could have unwelcome side effects on amphibians (Garner et al. 2009a, Woodhams et al. 2012).

Analysis of efficiency

To analyze differences in prevalence after treatment among different concentrations of the antifungal agents, we used a logistic regression with binomial errors, including prevalence as the dependent variable and concentration of the antifungal agent as the categorical explanatory variable (prevalence before the treatment was 100%). We also tested for differences in GE after treatment among different concentrations of the antifungal agents using linear regression. In this analysis, the log-transformed GE after treatment was the dependent variable and the concentration of the antifungal agent was the continuous explanatory variable. Before starting the experiment, GE varied by chance among different concentrations of General Tonic®, with higher GE at high concentrations (linear regression with GE as the dependent variable and concentration as continuous explanatory variable, $p = 0.024$). To account for variation in GE at the beginning of the experiment, we included GE before the experiment as a covariate in the statistical analysis. We did the analyses separately for General Tonic® and for Virkon Aquatic®.

Analysis of side effects on tadpoles

To analyze whether mortality varied among treatments, we used a logistic regression with binomial errors. Survival was the dependent variable and concentration of the antifungal agent was the categorical explanatory variable. We tested for an effect of both agents on the size of tadpoles. We used principal component analysis to combine mass and length of the tadpoles after the experiment into a single variable (Schmidt et al. 2012). We used the first principal component axis (PC1) for further analyses. PC1 explained 83% (in the experiment where we used General Tonic® as the antifungal agent) and 96% (in the experiment where we used Virkon Aquatic® as the antifungal agent) of the variance. Both mass and length were positively correlated with PC1 in General Tonic® ($r = 0.70$ and $r = 0.70$, respectively) and in Virkon Aquatic® ($r = 0.70$ and $r = 0.70$, respectively). We tested for an effect of the anti-

fungal agents on PC1 using a linear regression where PC1 was the dependent variable and the concentration of the agent was the categorical explanatory variable. We did both analyses separately for General Tonic® and for Virkon Aquatic®. All statistical analyses were done in R version 2.15.0 (R Development Core Team 2012).

RESULTS

General Tonic® significantly reduced the prevalence of *Bd* (logistic regression, $p = 0.018$), though not to 0% (Fig. 1, Table 1). Prevalence was reduced to 60% at the lowest concentration (Fig. 1). Among those that remained infected, tadpoles treated with General Tonic® had significantly lower zoospore counts at all concentrations compared to the control group (linear regression, $p = 0.011$; Fig. 1, Table 1).

There was a significant effect of the treatment with General Tonic® on tadpole survival (logistic

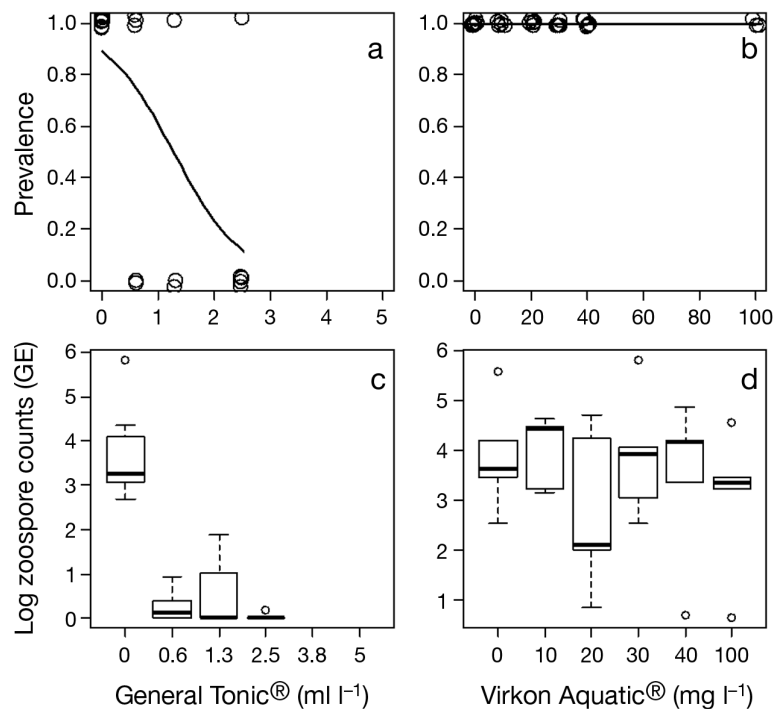


Fig. 1. *Alytes obstetricans*. (a,b) Effect of different concentrations of antifungal agents on prevalence of *Batrachochytrium dendrobatidis* (*Bd*) in tadpoles. Symbols represent individual tadpoles and are slightly offset such that all data points are visible. The line shows prevalence as predicted by logistic regression. (c,d) Infection loads of tadpoles after a 7 d treatment with different concentrations of General Tonic® or Virkon Aquatic®. Concentration '0' indicates control groups. Black line: median; box: interquartile range (IQR); whiskers: $1.5 \times$ IQR; circles: outliers. GE: genomic equivalents

Table 1. *Alytes obstetricans*. Effects of the antifungal agents General Tonic[®] and Virkon Aquatic[®] on zoospore counts and prevalence of *Bd* in tadpoles. At the beginning of the experiment, prevalence was 100% in all treatment groups and remained 100% in all Virkon Aquatic[®] groups. NA indicates that animals were removed from the experiments because of mortality or side effects. There are no p-values for prevalence among those concentrations where prevalence remained 100% (– in the table). p-values are for the contrast between control and the respective concentration of the antifungal agent. *p < 0.05

Concentration (ml l ⁻¹)	Effects on prevalence p-value	Effects on zoospore counts p-value
General Tonic[®]		
0	–	–
0.625	0.122	<0.001*
1.25	0.026*	0.013*
2.5	0.005*	0.006*
3.75	NA	NA
5	NA	NA
Virkon Aquatic[®]		
0	–	–
10	–	0.872
20	–	0.164
30	–	0.983
40	–	0.598
100	–	0.288

Table 2. *Alytes obstetricans*. Side effects of antifungal agents on tadpoles. High concentrations of General Tonic[®] had a mortality effect and a significant effect on size (as PC1 scores, calculated by combining mass and length in a principal component analysis). Virkon Aquatic[®] caused no side effects. NA means that animals were removed from the experiments because of mortality or side effects. There are no p-values for mortality among those treatments where no mortality was observed. p-values are for the contrast between control and the respective concentration of the antifungal agent. *p < 0.05

Concentration (ml l ⁻¹)	Effects on mortality p-value	Effects on fitness (PC1) p-value
General Tonic[®]		
0	–	–
0.625	1	0.008*
1.25	1	0.009*
2.5	0.033*	0.04*
3.75	0.002*	NA
5	<0.001*	NA
Virkon Aquatic[®]		
0	–	–
10	–	0.691
20	–	0.196
30	–	0.265
40	–	0.182
100	–	0.969

regression, $p = 0.004$; Fig. 2, Table 2). Survival was 100% up to a concentration of 1.25 ml l⁻¹ but was reduced to 60% at 2.5 ml l⁻¹, 40% at 3.75 ml l⁻¹ and 0% at 5 ml l⁻¹. Tadpoles started to be lethargic in the General Tonic[®] treatment at a concentration of 2.5 ml l⁻¹ or above. Tadpoles did not exhibit any abnormal behavior at lower concentrations. We did not detect changes in pigmentation. Mass and length of tadpoles, as summarized by PC1, did not differ among treatments at the beginning of the experiment (linear regression, $p < 0.900$). Tadpole size (PC1), was significantly affected by the experimental treatment (linear regression, $p = 0.045$). An analysis of variance (ANOVA), where General Tonic[®] concentration was treated as the categorical explanatory variable, showed that PC1 was significantly lower at all concentrations than in the control (Table 2).

Prevalence of *Bd* in tadpoles treated with Virkon Aquatic[®] remained 100% at all concentrations. Virkon Aquatic[®] had no significant effect on zoospore counts (linear regression, $p = 0.577$) (Fig. 1, Table 1). Virkon Aquatic[®] had no effect on tadpole mortality or on PC1 (Fig. 2; $p = 1.0$ and $p = 0.581$, respectively). Tadpole survival was 100% for all concentrations. We registered no abnormal behavior and found no changes in pigmentation.

DISCUSSION

Outbreaks of chytridiomycosis have led to the extinction of amphibian species and populations (Berger et al. 1998, Bosch et al. 2001, Vredenburg et al. 2010). Therefore, chytridiomycosis is considered to be a major threat to the survival of amphibians (Daszak et al. 2000, Fisher et al. 2012). Although methods are available to treat amphibians in captivity, most of the published methods cannot be used to treat amphibian populations in natural habitats. For example, Garner et al. (2009a) showed that itraconazole cleared *Bd* infection but led to depigmentation of tadpoles. Thus, there is an urgent need to develop a method to treat amphibians in natural habitats against *Bd* (Woodhams et al. 2011). As a first step towards that goal, we tested the efficiency of antifungal agents that seemed promising for use in natural habitats. In our experiment, we tested whether the antifungal agents General Tonic[®] and Virkon Aquatic[®] were effective at clearing infection or reducing infection load and whether they had side effects on tadpoles of *Alytes obstetricans*, a species known to be susceptible to *Bd* (Bosch et al. 2001, Tobler & Schmidt 2010).

Virkon Aquatic[®], whose formulation is based on the same active substance as Virkon S which is often used and recommended for the disinfection of field

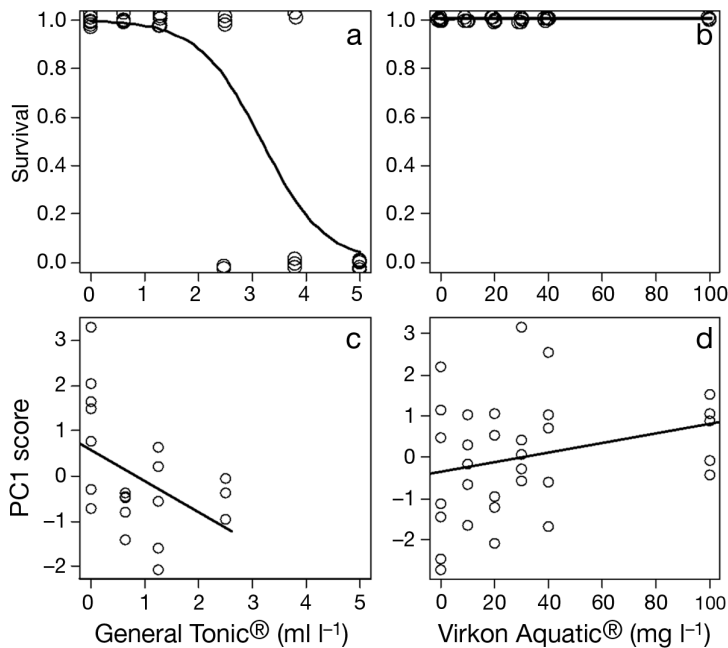


Fig. 2. *Alytes obstetricans*. (a,b) Effects of antifungal agents on tadpole survival. Based on the results of the logistic regression (shown by the line), predicted survival was 100% up to a General Tonic[®] concentration of 1.25 ml l⁻¹, while in Virkon Aquatic[®], survival was 100% among all concentrations. Symbols represent individual tadpoles and are slightly offset such that all data points are visible. (c,d) Effects of antifungal agents on tadpole fitness. Fitness is represented by PC1 scores, calculated by combining mass and length in a principal component analysis. General Tonic[®] showed a significant negative effect

equipment (Webb et al. 2007), did not reduce *Bd* prevalence or load in individually held tadpoles in the laboratory. We therefore exclude it from further discussion and focus on the antifungal agent General Tonic[®].

Although General Tonic[®] did not completely clear all tadpoles from *Bd*, it did reduce *Bd* loads significantly (Fig. 1). General Tonic[®] may therefore be used to treat amphibian larvae against *Bd* infection. It is very easy to apply, even for large numbers of animals. At a concentration of 0.625 ml l⁻¹, General Tonic[®] reduced prevalence to 60%, and infected animals had greatly reduced pathogen burdens. Survival was not affected at 0.625 ml l⁻¹ or 1.25 ml l⁻¹, but was reduced to 60% at 2.5 ml l⁻¹. General Tonic[®] should be used carefully because it had an effect on size (PC1) of tadpoles. The long-term consequences of reduced body size on individual fitness (e.g. breeding success) are unknown but should be the focus of further study. Interestingly, the negative effect on PC1 was not detected in an experiment where animals were kept in groups in mesocosms (C. C. Geiger, B. R. Schmidt, F. C. Origi, unpubl. data).

General Tonic[®] reduced loads but not prevalence. In our opinion, this is an important result because loads determine whether infection leads to chytridiomycosis and death of individuals. Vredenburg et al. (2010) showed that amphibian mortality begins once infection loads reach a critical threshold of 10 000 zoospores (the threshold may vary among amphibian species). The models of Briggs et al. (2010) and Mitchell et al. (2008) showed that reducing *Bd* infection loads might help populations to persist with endemic *Bd* infection. Thus, reducing *Bd* infection loads with General Tonic[®] could help amphibians to survive despite the presence of *Bd*. To be helpful in preventing deaths at metamorphosis, it would seem that General Tonic[®] would have to be applied when tadpoles are approaching metamorphosis. We do not claim that General Tonic[®] is a better treatment than other established and successful agents such as itraconazole (Garner et al. 2009a, Woodhams et al. 2011, Brannelly et al. 2012); General Tonic[®] simply has other advantages compared to itraconazole, e.g. application is very simple which is an interesting feature for a treatment in natural habitats. Itraconazole is more effective in reducing *Bd* infection in the laboratory; however its application is labor intensive and thus less attractive for field treatment

In summary, our experiment demonstrates that General Tonic[®] may be used to treat amphibian larvae against *Bd* infection. Future research will include experimental tests of efficiency of General Tonic[®] as a treatment against *Bd* infection in mesocosms and natural ponds where side effects of this agent on the amphibians and the pond ecosystem will be tested. These experiments will show whether General Tonic[®] might be used to treat amphibian populations in natural ponds against the emerging pathogen *Bd* or whether side effects of this agent are too strong to make it recommendable for application in the environment.

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