Short Communication

Trypan Blue Dye is an Effective and Inexpensive Way to Determine the Viability of *Batrachochytrium dendrobatidis* Zoospores

Taegan A. McMahon[1] and Jason R. Rohr[2]

[1]*University of Tampa, Tampa, FL*
[2]*University of South Florida, Tampa, FL*

Abstract: *Batrachochytrium dendrobatidis* (*Bd*) has been implicated in hundreds of amphibian declines and is the focus of a vast amount of research. Despite this, there is no reported efficient way to assess *Bd* viability. Discriminating between live and dead *Bd* would help determine the dose of live *Bd* zoospores and whether factors have lethal or sublethal effects on *Bd*. We tested whether trypan blue, a common stain to discriminate live and dead cells, could be used to assess *Bd* viability. We show that the proportion of live zoospores (zoospores that excluded the trypan blue dye) matched the proportion of known live zoospores added to cultures. In contrast, all of the zoosporangia stages of *Bd* stained blue. These results demonstrate that trypan blue can be used to determine the viability of *Bd* zoospores but not zoosporangia. We recommend using trypan blue to report the number of live zoospores to which hosts are exposed.

Keywords: chytrid fungus, amphibian decline, chytridiomycosis, viability stain

Chytridiomycosis, a disease caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), has spread around the world and has been implicated in the decline or extinction of hundreds of amphibian species (Skerratt et al. 2007). Its populations appear to be influenced by climate (Rohr et al. 2008; Rohr and Raffel 2010; Liu et al. 2013; Raffel et al. 2013), habitat (Raffel et al. 2010; Becker and Zamudio 2011; Murray et al. 2011; Liu et al. 2013), densities of amphibian and non-amphibian hosts (Briggs et al. 2010; Vredenburg et al. 2010; McMahon et al. 2013), human movements (Rohr et al. 2011; Liu et al. 2013), and other microbes (Harris et al. 2009). Despite a tremendous amount of research focused on this pathogen, one challenge that remains is that there is no re-

ported accurate, inexpensive, and easy way to determine the viability of *Bd*. To determine *Bd* densities, some researchers count only the moving zoospores (infectious stage) while others count total zoospores (live and dead). Neither method is very accurate at estimating *Bd* densities because live zoospores can remain still for several minutes (personal observation) and dead zoospores can look like stationary, living zoospores. Hence, determining the viability of *Bd* is important for accurately determining the number of live *Bd* zoospores to which hosts are exposed, which, in turn, should improve experimental consistency and replication.

Additionally, discriminating between live and dead zoospores would facilitate determining whether a factor has a lethal (survival) or sublethal (growth) effect on *Bd*. For example, researchers are exploring chemicals that might cure amphibians of *Bd* infections and thus, they are

exposing *Bd* to antifungal chemicals and pesticides (Berger et al. 2009; Garner et al. 2009). These studies quantified *Bd* density, but it is unclear whether any tested chemical reduced population growth (a sublethal effect) or directly killed *Bd*. A dye that differentially stains live and dead *Bd* would allow researchers to distinguish between lethal and sublethal effects of factors.

The stains SYBR 14 and propidium iodide have been used to determine zoospore viability (Stockwell et al. 2010). However, these fluorescent dyes are expensive, difficult to use, and require access to an inverted microscope with a mercury lamp, which many laboratories do not have. In addition, very high concentrations of *Bd* are needed to accurately assess the proportion of live versus dead zoospores (Stockwell et al. 2010), which, in many cases, is not experimentally feasible.

A possible alternative to using fluorescent stains is trypan blue, an inexpensive, quick, and easy to use dye that is absorbed by dead cells and excluded by live cells (Strober 2001). Trypan blue has been used to determine viability in fungi (Bhadauria et al. 2010), but its ability to accurately determine the viability of *Bd* cells has not been determined. Here, we tested whether trypan blue dye could effectively determine the viability of *Bd* zoospores and zoosporangia (stage that produces zoospores).

To accomplish this goal, we made separate *Bd* cultures for each replicate in our experiment. Each culture was made by adding 1 mL of *Bd* stock (isolate SRS 812 isolated from *Rana catesbeiana*) to 10 mL of 1% tryptone broth for 14 days at 23°C. Each culture was then split in half and one half was killed to create live and dead cultures. We used two killing methods to ensure that our results were not a function of the method used to kill *Bd*. Some of the *Bd* cultures were killed by exposure to heat, whereas others were killed by being flash frozen in liquid nitrogen.

For each replicate (heat killed: $n = 19$ and flash frozen: $n = 4$), the live and dead cultures were mixed together to create 0, 25, 50, 75, and 100% live *Bd* samples. A 10 μL aliquot from each of these solutions was mixed with 10 μL of trypan blue dye (0.4% trypan blue in phosphate buffered saline). After 1 min of trypan blue exposure, we recorded the number of blue and clear/white zoospores and zoosporangia in each culture using a hemocytometer and a compound microscope (at 100× magnification; all counts were done double blind).

To confirm that the heat and flash freezing treatments actually killed the zoospores and zoosporangia in the "dead" cultures, a 1 mL aliquot of each dead culture was grown on 1% tryptone agar for 8 days at 23°C. The plate was then inspected under a microscope at 5× magnification to check for the presence of moving zoospores. To ensure that the living cultures had live zoospores, all living cultures used were visually inspected for moving zoospores prior to the beginning of each trial.

Statistics were analyzed with R statistical software (R Development Core Team 2010). Significance was attributed when $P < 0.05$. A paired *t* test was used to determine if there was a difference in the proportion of zoospores that were not stained blue and the expected proportion of live *Bd* in the culture.

For each of the cultures, heating and freezing successfully killed (heat killed $n = 19$ and flash frozen $n = 4$) all the *Bd* because we observed no living zoospores growing in culture after 8 days. For the cultures receiving trypan blue, there was no difference between the proportion of zoospores that were not stained blue and the expected proportion of live *Bd* in the culture no matter the method of killing *Bd* (heat killed: $t = -1.17$, df = 92, $P = 0.25$, Fig. 1a; flash frozen: $t = -0.3775$, df = 15, $P = 0.771$; Fig. 1b). We expected that each live culture would contain a small number of dead zoospores from the initial inoculate. Indeed, we found that trypan stained a portion of the zoospores in each of the live cultures blue, demonstrating that trypan blue can dye zoospores that died naturally, as well as those that were heat-killed or flash frozen. Surprisingly, all zoosporangia stained blue.

All of the zoosporangia in this experiment were unexpectedly blue, which usually indicates a dead cell. However, in this case, it is highly unlikely that all of the zoosporangia were dead given that these were healthy cultures containing millions of live zoospores. Zoosporangia are bounded by a cell wall, which may be porous or permeable (e.g. discharge tubules) and may not exclude the dye. Nevertheless, although trypan blue was not an effective way to determine the viability of *Bd* zoosporangia, it was effective at determining the viability of zoospores. This demonstrates that live cells may not always exclude trypan blue and it is important to verify the effectiveness of the dye for each cell type.

Determining the viability of zoospores should be useful for discriminating between lethal and sublethal (e.g. reduced *Bd* growth) effects of a factor on *Bd*. For example, Raffel et al. (2013) demonstrated that different temperatures and temperature shifts affected *Bd* growth in culture, but they were unable to differentiate between reduced growth and mortality. Additionally, researchers have been testing various chemicals as a way to clear amphibians of *Bd* infections (Berger et al. 2009; Garner et al. 2009). Some
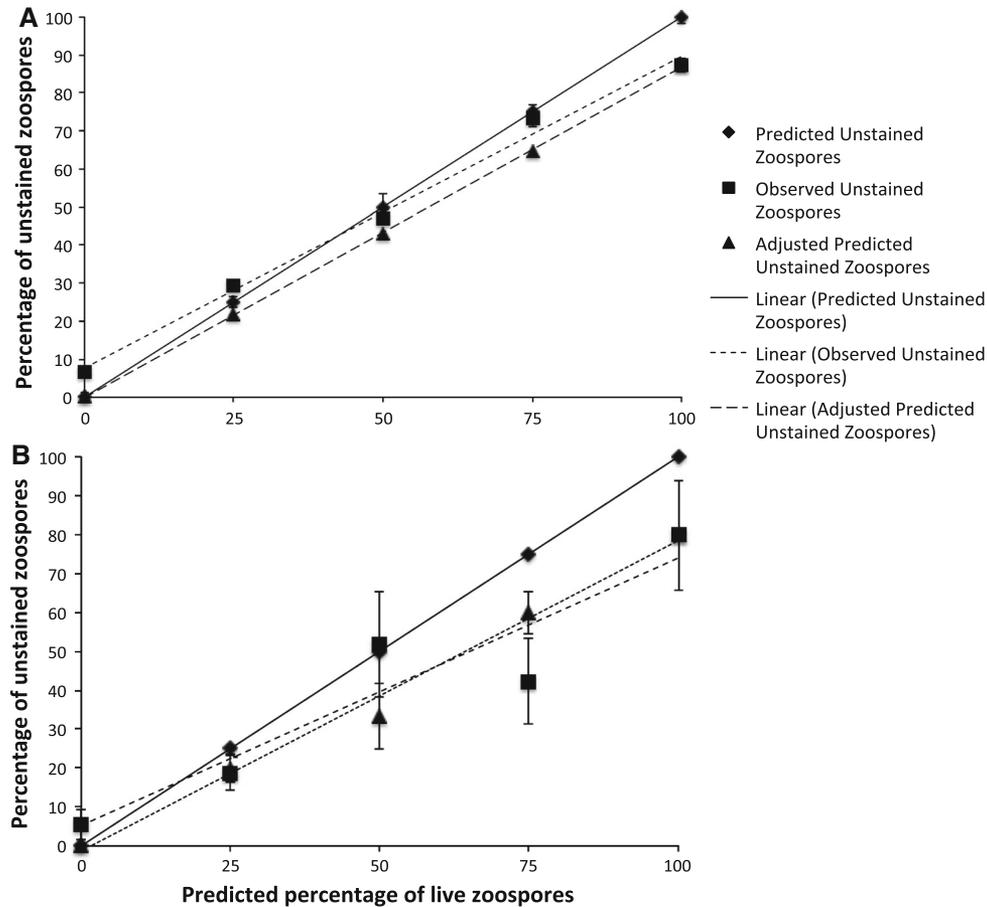
Figure 1. Each replicate was a mixture of live (0, 25, 50, 75, and 100% live culture) and **a** heat-killed *Bd* cultures ($n = 19$) or **b** flash frozen *Bd* cultures ($n = 4$) exposed to trypan blue dye. Shown are mean $\pm$ SEM of the percentage of unstained zoospores observed and the predicted percentage of unstained zoospores and posthoc adjustment to the predicted percentage of unstained zoospores. The posthoc adjustment was to account for the fact that even the "100% live culture" was expected to have some dead cells because some zoospores are always dead in a mature culture. We multiplied the predicted percentage of unstained zoospores (0, 25, 50, 75, and 100% live) by the proportion of unstained zoospores in the "100% live culture" to account for the estimated proportion of dead zoospores in each dilution. When you account for the estimated proportion of dead cells in the "100% live culture," the fit improves. Statistical analyses were only conducted on the unadjusted expected values so that our analyses were not circular (i.e. based on a trypan blue adjustment when our goal was to validate the use of trypan blue).

of these studies use *Bd* abundance in culture as a proxy for chemical-induced death, but stalled growth can appear the same as death. If a chemical simply slows *Bd* growth, the zoospores may still be infectious and exposure to live zoospores would obviously have a more negative impact on amphibians than dead zoospores.

Trypan blue is an ideal way to determine the viability of *Bd* zoospores because it is effective, quick, inexpensive, and requires minimal equipment. We encourage researchers to use trypan blue to report the number of live zoospores to which they expose hosts. This will increase exposure consistency among experiments and laboratories,

will make experiments more replicable, and will help distinguish between lethal and sublethal treatment effects.

References

Becker CG, Zamudio KR (2011) Tropical amphibian populations experience higher disease risk in natural habitats. *Proceedings of the National Academy of Sciences of the United States of America* 108:9893–9898

Berger L, Speare R, Marantelli G, Skerratt LF (2009) A zoospore inhibition technique to evaluate the activity of antifungal compounds against *Batrachochytrium dendrobatidis* and unsuccessful treatment of experimentally infected green tree

frogs (*Litoria caerulea*) by fluconazole and benzalkonium chloride. *Research in Veterinary Science* 87:106–110

Bhadauria V, Miraz P, Kennedy R, Banniza S, Wei Y (2010) Dual trypan–aniline blue fluorescence staining methods for studying fungus–plant interactions. *Biotechnic & Histochemistry* 85:99–105

Briggs CJ, Knapp RA, Vredenburg VT (2010) Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proceedings of the National Academy of Sciences of the United States of America* 107:9695–9700

Garner TW, Garcia G, Carroll B, Fisher MC (2009) Using itraconazole to clear *Batrachochytrium dendrobatidis* infection, and subsequent depigmentation of *Alytes muletensis* tadpoles. *Diseases of Aquatic Organisms* 83:257–260

Harris RN, Brucker RM, Walke JB, Becker MH, Schwantes CR, Flaherty DC, et al. (2009) Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME Journal* 3:818–824

Liu X, Rohr JR, Li YM (2013) Climate, vegetation, introduced hosts and trade shape a global wildlife pandemic. *Proceedings of the Royal Society B—Biological Sciences* 280:20122506

McMahon TA, Brannelly LA, Chatfield MWH, Johnson PTJ, Maxwell JB, McKenzie VJ, et al. (2013) Chytrid fungus *Batrachochytrium dendrobatidis* has nonamphibian hosts and releases chemicals that cause pathology in the absence of infection. *Proceedings of the National Academy of Sciences of the United States of America* 110:210–215

Murray KA, Retallick RWR, Puschendorf R, Skerratt LF, Rosauer D, McCallum HI, et al. (2011) Assessing spatial patterns of disease risk to biodiversity: implications for the management of the amphibian pathogen, *Batrachochytrium dendrobatidis*. *Journal of Applied Ecology* 48:163–173

R Development Core Team (2010) *R: A Language and Environment for Statistical Computing*, version 281. Vienna: R Foundation for Statistical Computing.

Raffel TR, Halstead NT, McMahon T, Romansic JM, Venesky MD, Rohr JR (2013) Disease and thermal acclimation in a more variable and unpredictable climate. *Nature Climate Change* 3:146–151

Raffel TR, Michel PJ, Sites EW, Rohr JR (2010) What drives chytrid infections in newt populations? Associations with substrate, temperature, and shade *EcoHealth* 7:526–536

Rohr JR, Raffel TR (2010) Linking global climate and temperature variability to widespread amphibian declines putatively caused by disease. *Proceedings of the National Academy of Sciences of the United States of America* 107:8269–8274

Rohr JR, Halstead NT, Raffel TR (2011) Modelling the future distribution of the amphibian chytrid fungus: the influence of climate and human-associated factors. *Journal of Applied Ecology* 48:174–176

Rohr JR, Raffel TR, Romansic JM, McCallum H, Hudson PJ (2008) Evaluating the links between climate, disease spread, and amphibian declines. *Proceedings of the National Academy of Sciences of the United States of America* 105:17436–17441

Skerratt LF, Berger L, Speare R, Cashins S, McDonald KR, Phillott AD, et al. (2007) Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth* 4:125–134

Stockwell MP, Clulow J, Mahony MJ (2010) Efficacy of SYBR 14/ propidium iodide viability stain for the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms* 88:177–181

Strober W (2001) Trypan blue exclusion test of cell viability. *Current Protocols in Immunology*: A.3B.1–A.3B.2.

Vredenburg VT, Knapp RA, Tunstall TS, Briggs CJ (2010) Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proceedings of the National Academy of Sciences of the United States of America* 107:9689–9694