Quantifying *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* Viability

Alexa Lindauer, 1 Tiffany May, 1 Gabriela Rios-Sotelo, 1 Ciara Sheets, 1 and Jamie Voyles 1

1Department of Biology, University of Nevada, Reno, 1664 North Virginia Street, Reno, NV 89557

Abstract: The disease chytridiomycosis is responsible for global amphibian declines. Chytridiomycosis is caused by *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* (Bsal), fungal pathogens with stationary and transmissible life stages. Establishing methods that quantify growth and survival of both life stages can facilitate research on the pathophysiology and disease ecology of these pathogens. We tested the efficacy of the MTT assay, a colorimetric test of cell viability, and found it to be a reliable method for quantifying the viability of *Bd* and *Bsal* stationary life stages. This method can provide insights into these pathogens’ growth and reproduction to improve our understanding of chytridiomycosis.

Keywords: *Batrachochytrium dendrobatidis*, *Batrachochytrium salamandrivorans*, MTT assay, Pathogen, Disease, Amphibian declines

Chytridiomycosis is an amphibian disease caused by the fungal pathogens *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* (Bsal; Berger et al. 1998; Longcore et al. 1999; Martel et al. 2013). Both pathogens have caused amphibian declines and are considered threats to biodiversity (Skerratt et al. 2007; Wake and Vredenburg 2008; Stegen et al. 2017). Although the pathogenesis of Bsal is less understood (Van Rooij et al. 2015), development of lethal chytridiomycosis from Bd has been linked with increases in infection intensity (i.e., Bd loads; Voyles et al. 2009; Vredenburg et al. 2010). As such, investigations on Bd and Bsal growth have been key to understanding the biology of this disease (Woodhams et al. 2008; Voyles et al. 2017).

Both Bd and Bsal have complex life histories (Longcore et al. 1999; Martel et al. 2013). Motile Bd and Bsal zoospores encyst and develop into zoosporangia. Stationary zoosporangia produce zoospores and release them into the environment or back onto the host (Longcore et al. 1999; Berger et al. 2005; Martel et al. 2013). Since increases in zoospore production are not always proportional to increases in zoosporangia growth rate (e.g., at temperatures below the Bd thermal optimum; Woodhams et al. 2008; Voyles et al. 2012), understanding differences in growth and reproduction of specific life stages is important to understand the infectivity of these pathogens and the trade-offs they face under different conditions.

Multiple methods have been used to measure Bd and Bsal growth in vitro (Piotrowski et al. 2004; Martel et al. 2013). Zoospore production can be measured by counting motile zoospores using a hemocytometer, and stains (e.g., trypan blue, SYBR-14, propidium iodide) can improve count accuracy (Stockell et al. 2010; McMahon and Rohr 2014). Lag, exponential, and stationary phases of Bd and
Bsal growth can be measured by reading optical density (OD) at 490 nm (Rollins-Smith et al. 2002, Piotrowski et al. 2004; Rollins-Smith et al. 2006). However, OD measurements lack specificity because they do not differentiate between living and dead cells.

We tested the efficacy of an MTT assay in measuring Bd and Bsal growth and viability. The MTT assay is a reliable colorimetric test for cell viability that has been used in unicellular fungi and mammalian cell lines (Levitz and Diamond 1983; Freimoser et al. 1999). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow tetrazolium salt that is reduced to purple MTT–formazan crystals in metabolically active cells (Mosmann 1983; Liu et al. 1997). The color change can be quantified by solubilizing the formazan crystals and reading culture absorbance at 570 nm, the most sensitive wavelength for this assay (Altman 1976).

We conducted experiments to (1) optimize MTT concentration and incubation time for Bd, (2) test the efficacy of the assay using serial dilution, and (3) apply the assay to quantify Bd and Bsal growth and viability over time. In addition, we measured zoospore production and zoosporangia growth by counting zoospores and reading OD_{570} to relate the MTT assay to other accepted quantification methods.

We revived Bd and Bsal isolates (Bd MYLF-16343, Bd NMBF-04, Bsal AMFP13/1) from cryopreservation (Boyle et al. 2003) and passaged them following established protocols (Longcore et al. 1999; Martel et al. 2013). Specifically, we cultured the pathogens in TGhL media in tissue culture flasks at 18°C for 7–9 days for Bd and at 10°C for 3–4 days for Bsal until we observed zoospore release. We then harvested zoospores by scraping cells from the flasks and filtering cultures through sterilized filter paper to remove zoosporangia and debris (Voyles 2011). We inoculated 100 μL diluted zoospore filtrate into 96-well plates (Bd concentration: 23 × 10^4 zoospores/mL; Bsal concentration: 48 × 10^4 zoospores/mL) and used heat-killed zoospore filtrate, heat-killed for 10 min in a 40°C water bath, as a negative control. We incubated the plates at temperatures within the pathogens’ optimal ranges (Bd: 17.5°C, Bsal: 10°C; Piotrowski et al. 2004; Martel et al. 2013).

To determine an optimum MTT concentration and incubation time, we added either 10 μL or 20 μL 5 mg/mL MTT in sterile PBS to 100 μL Bd culture and stopped the reaction after 30-min, 1-h, 2-h, or 24-h incubation (Mosmann 1983; Hansen et al. 1989). At each time point, we solubilized the formazan crystals by adding 140 μL sodium dodecyl sulfate in dimethylformamide solution (20% SDS/50% DMF w/v) and homogenizing gently (Hansen et al. 1989). We then measured OD at 570 nm (Biotek ELx800 Absorbance Reader). We fit asymptotic regression curves using the “nlme” package (Pinheiro et al. 2018) in R v3.4.3 (used for all analyses; R Core Team 2018). We corrected OD values by subtracting mean heat-killed OD from live well readings and compared incubation times and concentrations using t tests.

To test the efficacy of the MTT assay, we conducted a serial dilution experiment and measured Bd viability on the day of peak zoospore production. We inoculated 100 μL actively growing culture into sterile flat-bottom 96-well plates as described above and serially diluted the cultures in 50 μL TGhL media. We repeated the same dilution with heat-killed cultures as a negative control. We added 20 μL 5 mg/mL MTT, incubated for 2 h, solubilized the formazan product, and recorded OD at 570 nm. We fit a linear model to corrected OD_{570} to determine whether the MTT colorimetric signal was directly proportional to cell density.

To determine the viability of Bd and Bsal cultures over time, we used the MTT assay to quantify culture growth every other day for 12 days. On each sampling day, we used the optimized MTT assay (as described above) to measure viability in randomly selected wells. To compare the MTT assay to widely accepted methods for measuring Bd and Bsal growth and reproduction, we measured OD_{490} before initiating the MTT assay, and we quantified zoospore production using a hemocytometer. For Bd cultures, we compared OD with and without the addition of MTT over time using ANCOVA.

We found that MTT effectively stains Bd and Bsal, visibly staining viable zoosporangia purple. Asymptotic regression models (P < 0.001 for all parameters of both concentrations) show that OD_{570} readings of MTT-assayed cultures increased over 24 h, reached an asymptote after 4 h, and differed by MTT concentration (Fig. 1). For wells exposed to 20 μL 5 mg/mL MTT, we did not detect a significant difference between OD_{570} readings at 2 and 24 h (t test, t_{8} = -1.84, P = 0.10). Wells incubated with 20 μL 5 mg/mL MTT had higher OD_{570} readings than wells incubated with 10 μL 5 mg/mL MTT (2 h: t_{8} = -4.2687, P = 0.003; 24 h: t_{8} = -4.1104, P = 0.003). We did not observe a reduction of MTT in purified zoospores on day 0 for either pathogen at these MTT concentrations.

We found that OD readings of the MTT assay are directly proportional to Bd density (Fig. 2). OD readings
increased linearly with increasing cell density for live Bd cultures assayed with MTT ($F_{(1,44)} = 604.5$, $P < 0.001$, $R^2 = 0.93$).

We found that the MTT assay effectively measures Bd and Bsal viability over time. Reduction of MTT by live Bsal zoosporangia increased until peak zoospore release on day 8, after which zoosporangia viability decreased (Fig.3). Bd zoosporangia viability increased through the period of peak zoospore release and plateaued on days 10–12 (Fig.4). Culture growth as measured by OD 490 without the MTT assay also increased over time and reached stationary phase by day 12. The MTT assay produced a higher colorimetric signal over time than OD490 measurements without MTT (Fig. 4; ANCOVA, $F_{(3108)} = 360.4$, $P < 0.001$; assay/day, $t = 5.42$, $P < 0.001$).

Our results suggest that the MTT assay is an effective tool for quantifying Bd and Bsal viability over time. Using a 2-h MTT incubation, the MTT assay is an efficient way to collect Bd and Bsal viability data during reproductive cycles. This method improves on measurements of OD490 alone because it can quantify growth of a specific life stage and it amplifies the OD signal (Fig. 4). Moreover, this method allows researchers to capture lag, exponential, stationary, and decay phases of pathogen growth (Fig. 3). When paired with measurements of zoospore production, this assay may help resolve other aspects of pathogen growth and reproduction.

The MTT assay will allow investigators to measure pathogen viability under ecologically relevant conditions, which can help improve understanding of pathogen growth in vivo. For example, amphibians contend with changes in ambient temperatures, which likely influences
pathogen growth (Richards-Zawacki 2009; Rowley and Alford 2013). Using the MTT assay, pathogen growth and viability can be modeled in vitro to assess their responses to dynamic thermal environments (Woodhams et al. 2008; Voyles et al. 2012). In addition, MTT assays could provide an effective method for quantifying Bd or Bsal viability in the presence of inhibitory compounds such as antimicrobial peptides produced in frog skin glands, or antifungal metabolites produced by the amphibian skin microbiome (Rollins-Smith et al. 2002, 2006; Harris et al. 2009). Applying the MTT assay to a range of experimental Bd and Bsal research questions can help improve our understanding of the ecology of chytridiomycosis.

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**Data Availability**

Datasets are available from the corresponding author upon request.

**Compliance with Ethical Standards**

**Human and Animal Rights** This study did not involve humans or animals.

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