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Interactions between two key amphibian defenses to Batrachochytrium dendrobatidis in Panamanian glass frogs (Espadarana prosoblepon)

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Interactions between two key amphibian defenses to *Batrachochytrium dendrobatidis* in Panamanian glass frogs (*Espadarana prosoblepon*)

by

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Abstract

Research on the amphibian pathogen *Batrachochytrium dendrobatidis (Bd)*, the causative agent of the lethal disease chytridiomycosis, has advanced from assessments of pathogenicity and species susceptibility to more specialized questions concerning the complex interactions between the pathogen, species-specific immune responses, and the environment. Our work examines the potential for interactions between the two most important innate immune defenses of frogs against *Bd*: secretions of antimicrobial peptides and communities of commensal cutaneous bacteria. While both defenses have been studied individually, little data are available to examine interactions between these defenses. We conducted our study with field captured Panamanian glass frogs (*Espadarana prosoblepon*) and used a norepinephrine injection to induce a stress response and the release of peptides in skin secretions. We quantified the peptides from these secretions using an altered BCA assay. We also collected samples of cutaneous bacteria before and after injection to determine if the bacterial community changed after exposure to skin secretions. We used the bacteria samples to isolate and purify unique bacterial types based on colony morphology, then used challenge assays to understand the effect of isolated bacterial types on *Bd* growth *in vitro*. We also exposed several of the isolated bacteria to the skin peptides to analyze their susceptibility to inhibition to the peptide. We found that the overall amount of culturable cutaneous bacterial morphotypes decreased after the frogs were injected, although we did not observe a statistical difference in the amount of peptides secreted from saline and norepinephrine injected frogs. Every bacterial species we collected inhibited *Bd* growth to some degree, which is a higher proportion than typically found in previous studies. Growth in the presence of the frog skin peptides for three bacteria was dependent on the morphotype, which is indicative of different individual molecular interactions. Overall, these data provide useful information for the developing conceptualization of the *Bd*-host system, which can assist conservationists in preparing the most effective course of action in preventing disease-induced amphibian declines.

Introduction

Amphibian chytridiomycosis is an emerging infectious disease caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) that infects a wide variety of frog species and can be lethal. Currently, the population status of over one-third of amphibian species is threatened, and *Bd* has been responsible for causing population declines and extinctions of over 200 species. Thus, this fungus poses a demonstrated and significant threat to global biodiversity (Harris et al. 2009). By 1997, researchers confirmed *Bd* fungus as a major factor in global declines due partly to the abundance of *Bd*-naïve populations before pathogenic *Bd* spread. Even though *Bd* now is considered endemic to many areas, frog populations are continuing to show significant susceptibility (Berger et al. 2016). *Bd* spreads by forming zoosporangia on the surface of amphibian skin, disrupting the skin membrane, which fills with zoospores, and eventually burst. The release of these new motile zoospores infect available areas of the keratinized skin of both the current host and other nearby hosts. Theories to explain the lethality of *B* propose that either the fungus releases harmful chemicals that kill the frogs, or that the disruption of the skin interferes with the transfer of necessary ions, inducing enough stress to cause cardiac arrest (Berger et al. 2005).

Due to the high conservation priority of *Bd* management, many strategies have been proposed for active steps toward disease prevention, including reduction of host density, active chemical treatment of individual hosts, re-introduction of hosts with artificial selection for resistance, promotion of existing post-disease populations, alteration of the physical environment in *Bd*-susceptible areas, active treatment of hosts with *Bd* predators, and alteration of the microbiome of individual hosts to promote the growth of beneficial bacteria (Woodhams et al. 2011). However, more research into these management strategies will provide a more solid understanding of the disease ecology and dynamics of *Bd* in the wild. An important step towards examining these potential solutions is to increase our understanding of characteristics that make some frogs resistant to *Bd* and why those are effective in current environmental conditions.

On the level of an individual frog, resistance to *Bd* can occur due to inherent immune defenses present in amphibians. Much research has been done on the innate amphibian immunity, but these responses are continuously changing as a result of more frequent hypothalamic-pituitary axis stimulation caused by environmental degradation and climate change (Rollins-Smith 2017, Rollins-Smith et al. 2011). Although adaptive immunity to *Bd* has not been demonstrated frequently, research suggests that adaptive immunity in frogs could alter and inform conservation approaches to *Bd* prevention (Woodhams et al. 2011). Our study aims to examine two key innate amphibian defenses to *Bd*, host secretion of antimicrobial peptides and a cutaneous microbiome of beneficial bacteria, and how they change after an induced stress-response.

Research on host defenses in frogs has historically focused on the secretion of antimicrobial peptides. These peptides are collected through induction of a stress response, and are characterized as α-helical, positively charged, short peptides which insert themselves into microbial membranes (e.g. Conlon et al. 2004, Nutkins and Williams 1989). Multiple studies have shown their potential in inhibiting *Bd* growth, and have demonstrated differences in *Bd* inhibition specific to a particular frog species (e.g., Woodhams et al. 2007, Rollins-Smith et al. 2005). For example, researchers have examined altering peptide structure to change antimicrobial effectiveness, which could have implications for *Bd* prevention (Conlon et al. 2007). Based on the amount of peptides, researchers have also quantified skin secretions to predict susceptibility of different frog species to *Bd*

infection (Woodhams et al. 2006). Our study aims to collect antimicrobial peptides from frogs with saline injections and norepinephrine injections to quantify the amount of peptides released under different stress conditions.

Similar to stress-induced responses in antimicrobial peptides, environmental changes may alter the cutaneous microbiome of amphibian skin communities. Because the microbiome has immune properties that may help prevent *Bd* infection, environmentally-induced alterations to the microbiome could alter susceptibility to infection (Rollins-Smith et al. 2011, Woodhams et al. 2011). Work on beneficial cutaneous bacteria has shown promising results for *Bd* resistance and probiotic inoculation of hosts has been proposed as a conservation strategy worthy of continued research (e.g. Bletz et al. 2013, Woodhams et al. 2011, Harris et al. 2009). Some bacteria are capable of inhibiting *Bd* growth due to the secretion of secondary metabolites, and multiple types of assays have been developed to challenge *Bd* with commensal bacteria to quantify any inhibition of fungal growth (e.g. Bell et al. 2013, Harris et al. 2006). Our study used a spectrophotometric assay to test for *Bd* survival in the presence of metabolites secreted from bacteria collected at different growth phases, as phase may impact the efficacy of the secretion collection.

Several studies have examined the antimicrobial peptides of frogs and their ability to inhibit *Bd* growth, as well as the composition of a cutaneous microbiome and which bacteria may also inhibit *Bd*. These two mechanisms have been suggested as the main way frogs may be resistant to *Bd* infection (Holden et al. 2015). However, few studies have examined how these two defenses interact with one another. Myers et al. (2012) show that the antimicrobial peptides secreted by Mountain Yellow-Legged Frogs (*Rana mucosa*) also have antimicrobial action against commensal and *Bd*-preventative bacteria, and different quantities of peptides led to differential inhibition in the bacteria. However, they selected bacteria known to inhibit *Bd* and used samples of antimicrobial peptides that were collected from different frogs than the cutaneous bacteria samples, so it is possible that antimicrobial peptides and bacteria collected from the same frogs would have a different relationship. Further, Holden et al. (2015) showed that raising frogs in lab conditions—a common practice for studies on antimicrobial peptide and bacterial defenses to *Bd*—can lead to decreased secretion of antimicrobial peptides, and that the peptides frogs secrete in the wild can differ across geographic regions. While they also examined bacterial growth and abundance across differential antimicrobial peptide secretion, they did not characterize the composition and diversity of the cutaneous microbiome and how it changes after a mass secretion of antimicrobial peptides. Keuneman et al. (2013) also showed how geography of frogs and the particular species can provide slightly different microbiome composition, suggesting that frogs of the same species and same geographic site would be most likely to have similar microbiomes.

Overall, our study aims to examine how the microbiome changes in composition after a mass peptide secretion event caused by the induction of a stress-response. We use frogs from the same geographic site (in a *Bd*-affected area of Panama) to avoid differential molecular composition of antimicrobial peptides and radically different strains of the same bacterial species across frogs. Further, our study aims to assess how the growth of these isolated bacteria capable of inhibiting *Bd* growth are affected *in-vitro* by the presence of high concentrations of antimicrobial peptides collected from the same frogs as the bacteria. Together, this work will provide valuable information about the complicated relationships between the two main immune defenses frogs have to *Bd*, which may inform management strategies.

Methods

Bacterial and Peptide Sample Collection in Panama

Panamanian Glass Frogs (*Espadarana prosoblepon*) were collected along a 200 m transect on the Quebrada Jordinal. This species of frog was chosen based on its abundance and exposure to *Bd*. We used 24 frogs from this transect for our study. Each frog was collected in a sterile bag and the time and location of capture were recorded. The frogs were transported to an off-site location overnight and were released after sample collection the following morning. The frogs were returned to the transect location where they were removed. All weather and location data for the site and day were also recorded.

Secreted peptides were collected from the frogs for quantification and further experimentation. Before collecting these antimicrobial peptides, each frog was injected according to their treatment group and mass. Control group frogs were injected with 1 mL of Phosphate Buffered Saline (PBS) solution while treatment frogs were injected with 1 mL of a norepinephrine bitartrate-PBS salt solution at a concentration of 0.01 mL/gram individual body weight concurrent with previously used methods (Conlon et al. 2007; Rollins-Smith et al. 2005; Nutkins and Williams 1989). The injected frogs were then placed in a bath of High Performance Liquid Chromatography (HPLC) water for 10 minutes. After 10 minutes, the frogs were removed and the HPLC water was run through a primed column to store the peptides for later quantification.

To analyze the composition, diversity, and changes to the culturable microbiota before and after peptide secretion, sterile swabs were used to collect cutaneous bacteria. Prior to injection with PBS or norepinephrine, the skin of each frog was rinsed with HPLC water and then was rubbed in entirety with a sterile swab to collect a bacterial sample representative of the frogs' cutaneous microbiome. After the injection and subsequent water bath, each frog was rinsed and swabbed again to collect a sample representative of the cutaneous microbiome post-injection and exposure to potential antimicrobial peptides. Each bacterial swab was stored in 20%-glycerol Trypticase Yeast Soy Extract (TYSE) solution.

All peptide samples and bacterial swabs were frozen (-80℃) in a cryoprotectant for 11 months. They were transported to the University of Nevada, Reno, where subsequent experimentation was conducted. The peptides were isolated using liquid chromatography and quantified using a BCA assay in which Bradykinin Acetate salt was used to generate a comparative standard curve. The mean quantity of peptides collected from the saline and norepinephrine injected frogs was compared using a Student's t-test.

Generation of Bacterial Cell-Free Supernatant

For challenges against *Bd*, secreted bacterial metabolites were collected from the skin microbiome samples based on procedures outlined by Bell et al. (2013). The bacterial swab samples were thawed and homogenized to produce a representative sample, then 20 uL of each sample was spread onto a petri dish with R2A low-nutrient agar, incubated at ambient temperature and observed daily. Each morphologically distinct colony that grew on the R2A plate was transferred to an individual sterile R2A agar plate for subsequent isolation and characterization. If the primary isolation plate produced morphologically distinct colonies, each distinct colony was transferred to a new individual R2A dish for consecutive isolation. This was repeated until each

morphologically distinct colony from each spread dish was successfully streaked onto an individual dish. After isolation, the bacteria were compared across spread samples to find recurring bacterial morphotype. Based on morphology, the bacteria were grouped into 12 groups of similar appearing types and each group was labelled alphabetically ("A" – "L"). For preliminary experimentation, the groups were assumed to contain bacteria of the same species, and samples were sent for 16s sequencing for identification.

A representative from each bacterial group was selected for characterization of that group's growth in liquid Tryptone Gelatin Hydrolysate Lactose (TGhL) media. The representative isolate was grown in TGhL media in four wells of a 12 well cell culture plate and compared to four wells of just TGhL media as a negative control. The optical density (spectrophotometry; 450 nm) of each well was taken every three hours until the cultures plateaued, and a representative growth curve was developed for each bacterial type to determine the time points at which given optical densities are achieved.

To conduct an analysis of metabolites secreted at different parts of the bacterial growth curve, supernatant was generated during the exponential phase and stationary phase of each representative bacteria. Once the time points of full optical density and half of the full optical density were determined for each bacterial type, each type was grown again in 8 wells of a 12 well culture plate. At the determined timepoint for each bacteria to have achieved half of its respective full optical density, the media of four of the wells from the plate was collected. The media was then centrifuged to pellet bacteria, and the supernatant was collected and filtered through a 0.22 µm filter to remove all bacterial cells. The cell-free supernatant of half optical density for each bacterial type was then refrigerated (4℃) for use in the *Bd* challenge assays. When the cultures for each bacterial type reached the time point of full optical density for its respective bacteria, the media in the four remaining culture wells was collected and processed to obtain bacterial cell-free supernatant in the same process as that of the half optical density treatment media and stored at 4℃ for later use.

Bd—Supernatant Challenge Assay Experiment

Bd was prepared for experimentation by growing cultures from an isolated stock that was passaged regularly and stored as a refrigerated culture (4℃). The original stock was isolated from *Colostethus panamensis* frogs in Campana Panama in November of 2016 and was passaged into fresh TGhL media 6 times before aliquots were removed for this experiment. The *Bd* was grown in TGhL at 20℃ for 4-7 days before each plate was set up to attain the optimum density of zoospores. The culture was then filtered using 10 micron paper to remove existing zoosporangia and generate a culture of motile zoospores in TGhL.

To examine the interaction between the metabolites produced by our 12 bacterial types and *Bd* growth, a challenge assay was run to compare *Bd* growth in the presence and absence of bacterial metabolites. A 96-well cell-culture plate was set up for each bacterial type, and a perimeter of wells with 100 µL of TGhL media was used to prevent evaporation in the treatment wells. Ten replicates were then made for each treatment group in every plate, which were characterized as follows:

1) Positive Control: 50 µL of *Bd* zoospore culture pipetted into 50 µL of TGhL

2) Negative Control: 50 µL of *Bd* zoospore culture killed by heating in a boiling water bath for a minimum of ten minutes, pipetted into 50 µL of TGhL
3) Stationary Phase Supernatant: 50 µL of *Bd* zoospore culture in 50 µL of cell-free supernatant collected at the time point indicative of the bacteria's full optical density
4) Growth Phase Supernatant: 50 µL of *Bd* zoospore culture in 50 µL of cell-free supernatant collected at the time point indicative of half of the bacteria's potential optical density
5) Diluted Stationary Phase Supernatant: 50 µL of *Bd* zoospore culture in 25 µL of cell-free supernatant collected at the time point indicative of the bacteria's full potential optical density and 25 µL of distilled water
6) Low Nutrient Control: 50 µL of *Bd* zoospore culture in 50 µL of distilled water to represent *Bd* growth in nutrient-deprived conditions

Challenge plates for each representative bacterial type were incubated (20℃) and observed daily. During observation, optical density readings were taken for each well as above, and the density of motile zoospores in each well was quantified using a semi-quantitative Zoospore Density Index. We assigned a score (0-4) for the number of zoospores visible in one microscope view of the well at 100x magnification (where 0 = no visible motile zoospores; 1 = 0-5 motile zoospores; 2 = 5-100 motile zoospores; 3 = 100-200 motile zoospores; 4 = over 200 motile zoospores).

Bacteria – Peptide Challenge Assay Experiment

After determining which secreted bacterial metabolites inhibited *Bd*, we assessed whether the same bacteria that secreted these metabolites could be inhibited by the peptides we collected from frogs. Bacteria types "A," "B," and "E," were used for experimentation with the antimicrobial peptide samples because they were the three most abundant types on our frogs and all showed inhibition of *Bd* growth according to our challenge assay. To study the peptide impact on beneficial bacteria, we challenged these bacteria with a sample of the highest concentration of peptides from frog 24 (160617_24), which was injected with norepinephrine. To prepare the bacteria for the antimicrobial peptide challenge, the stock cultures were removed from the freezer (-80℃) and grown in an overnight culture of R2A liquid media shaken at ambient temperature.

When the bacteria were grown to their growth phase, they were removed from incubation and were used to set-up a 96-well challenge plate with a perimeter of wells of 100 µL liquid R2A media to prevent dehydration of the treatment wells. For each of the three bacterial types, two treatment groups of eight replicates were prepared as follows:

1) 100 µL R2A media inoculated with 10 µL of bacterial culture to be used as a positive control for bacterial growth and

2) 100 µL R2A media and 20 µL of diluted peptide sample from the quantification assay, inoculated with 10 µL of bacterial culture.

As negative controls for the bacterial treatments, two groups of six wells were prepared consistent with:

1) 100 µL only R2A media and

2) 100 µL R2A media and 20 µL of diluted peptide sample.

The plate was shaken at low speed and at ambient temperature for 50 hours and the optical density (BioTek microplate reader with Gen 5 software; 450 nm) was recorded every 2 hours. We compared the optical density at 50 hours using a two-way ANOVA to test for differences between bacterial types and the effect of the peptide (statistical analysis performed in JMP version 13.0).

Results

Bacterial Community Changes with Peptide Secretion

More bacterial morphological types were present on frogs before injection than after injection, regardless of the injected solution (Table 1). The specific morphotype(s) of culturable bacteria present either changed or remained the same depending on the frog and the bacteria in question. On several frogs, no bacteria were isolated from pre-injection or post-injection swabs. On one frog (14) bacteria was isolated from the post-injection swab but not the pre-injection swab, and on several frogs bacteria was isolated from the pre-injection swab but not the post-injection swab. There was no significant difference in the mean quantity of the peptides collected from the saline and norepinephrine injected frogs (Figure 1; $P > 0.05$). Each frog collection yielded more than 169 ug/mol of peptides (Appendix Table S1).

Inhibition of Bd Growth using Bacterial Metabolites

The *Bd* growth in the presence of bacterial secreted metabolites was recorded by optical density readings in order to generate a representation of change in growth over time (Table 2; Appendix Figure S1.A-L.). Nine of the twelve bacterial types had metabolites that were able to prevent any growth of *Bd* (relative to the positive control treatment) over the 12-day observation period. Metabolites from two other bacterial types were able to reduce *Bd* growth without fully inhibiting it based on optical density data (relative to the positive control treatment), and the data from the zoospore recordings shows that all tested bacterial supernatants caused a decrease in the zoospore score relative to the positive control (Table 2; Appendix Figure S2.A-L).

We were able to successfully collect both stationary phase and exponential phase supernatants from five bacterial types ("C," "D," "F," "G," and "H") for use in our secreted metabolite and bacterial phase analysis. In addition to these supernatants, we recorded the growth of *Bd* in the presence of stationary phase supernatant that was diluted by half with media to normalize for the amount of bacteria. In all five bacteria from which we successfully collected these supernatants, the stationary phase caused more growth inhibition than the growth phase supernatant (Appendix Figure S1.A-L.).

Bacteria – Peptide Challenge Assay Experiment

To determine if the growth of three bacterial types which were abundant and capable of inhibiting *Bd* changed in the presence of the peptides we collected from glass frogs, we grew the bacteria in the presence and absence of peptides from frog 24 (160617_24) and compared their optical density after 50 hours at ambient temperature. Bacteria types "A" and "B" showed increased optical density of their growth wells in the presence of peptides (relative to positive

controls in the absence of peptides). Bacteria E showed a decreased optical density in the presence of peptides relative to a positive control in the absence of peptides (Figure 2). Statistically, there was a significant interaction between bacteria type and treatment ($F_{2,2} = 112.0$, $P < 0.001$). The main effect of media treatment was also significant ($F_{1,2} = 97.5$, $P < 0.0001$) but the main effect of bacteria type was not significant ($F_{2,2} = 2.8$, $P = 0.07$), likely due to the strength of the interaction.

Discussion

In order to further understand *Bd* interactions and the innate immune defenses of frogs, we collected and characterized bacteria under different injection-induced stressors. We then used our bacteria to generate different metabolites with which to challenge *Bd* growth. Finally, we examined whether a selection of the bacteria we collected were susceptible to inhibition from the skin peptides the frogs secreted post-injection. This work shows how the most popularly studied Bd-relevant immune defenses of frogs interact with each other to inform conservation strategies.

We observed changes to bacterial community composition before and after injection-induced stress for individual frogs. The number of different bacteria we collected is consistent with previous work (Becker et al. 2015). However, in a different study, Bell et al. (2013) were able to collect and culture a much higher diversity of bacteria from individual frogs in their study by using similar methods to ours. The freezing and transporting steps that we took, may have altered the amount of culturable bacteria we obtained. While the diversity of culturable bacteria tended to decrease after injection, there were several instances in which few or no bacterial types were observed before injection and more were observed after injection, which implies our sampling method does have imperfect detection of culturable bacteria. Therefore, we did not find evidence that bacterial communities were negatively impacted by injection-induced stress responses in these frogs.

The amount of antimicrobial peptides we collected was not different between the saline-injected frogs and the norepinephrine-injected frogs. This suggests that the frogs may not have differed in the amount of stress they were experiencing between treatment groups. One possibility is that the frogs in the norepinephrine injection group were not injected with enough norepinephrine to induce an elevated stress response. However, we based our injections on the concentrations used by Conlon et al. (2007), who saw noticeable peptide secretion as a response of injection. Another explanation is that the collection and handling of the frogs—including injection—was enough to induce a stress response regardless of the type of injection. Due to our consistency with previous norepinephrine work and our collection of observable peptides from both treatment groups, handling-induced stress is a more likely explanation for a lack of difference between the groups. Another potential factor could have been the short time in which the frogs were placed in a water bath (10-15 minutes) for peptide collection. A longer soaking period may have allowed us to collect more peptides and further test for differences between treatment groups.

Our results show that most of the commensal bacteria we collected from the surface of frogs were capable of inhibiting *Bd* growth *in-vitro*. These data are consistent with many previous studies showing *Bd* inhibition from commensal bacteria (e.g. Woodhams et al. 2007, Harris et al. 2009, Bell et al. 2013). However, the high proportion of our culturable bacteria that are capable of inhibiting *Bd* based on our metabolite assay is notable, and may show an increase in *Bd*-inhibitory bacteria in frogs from *Bd*-established regions over time. Our results contribute to theories of

microbiome evolution that supports the emergence of adaptive immunity (e.g. Woodhams et al. 2011) in *Bd*-exposed areas of Panama, although more studies are needed to analyze recent microbiomes and provide further evidence of changes over time.

The bacteria that we challenged with peptides collected from one of our frogs showed differential growth responses in the presence of the peptides. Myers et al. (2012) showed that the response of bacteria depends on the concentration of peptides, but generally showed a consistent response for all bacterial types at each concentration of peptide. Two of the bacteria we tested appeared to show improved growth in the presence of the peptides, while one of the bacteria showed decreased growth in the presence of the same peptides. The reasons for the differential expression could be due to different molecular interactions with the peptides. For example, if the bacteria differed in whether they were gram-positive or gram-negative then their exterior membranes would differ and they would logically show differential expression to the same peptides (as in Conlon et al. 2007). These results highlight the importance of using chemical equipment to characterize the identity of the collected peptides and of sequencing the bacteria to get a better understanding of the potential molecular interactions between each individual bacterial type and the peptides.

Another interesting result of our work is that the supernatant collected from bacteria in their stationary phase inhibited *Bd* more consistently than the supernatant collected from the same bacteria in their exponential phase. It is well known that bacteria produce and secrete different compounds in their different growth phases (Drew and Demain 1977), and work has been done to analyze the genetic regulation involved in specific metabolite pathways between growth phases (e.g. Romeo 1998, Al-Qadiri et al. 2008). The lack of inhibitory power from supernatant collected before stationary phase suggests that it is important for researchers to characterize the standard growth curves for the bacteria they are using before collecting metabolites to ensure that enough growth has occurred to allow for the secretion and accumulation of the metabolites in question; otherwise, false negatives for the inhibitory power of some bacteria may be produced.

The bacterial phase results hold several implications when analyzing host-microbiome environment interactions as well. For example, changes to the environment of a bacterial host may directly lead to changes in microbiome structure and composition, which has been shown to occur in amphibians (Rebollar et al. 2018). Further, the state of the individual host may be able to impact the growth phase of the bacteria inside or on it. Korem et al. (2015) showed that the community of gut microbiota *in-vivo* in mice had differential growth-phase profiles depending on the behavior and diet of the individual host mouse. The state of the host may also play a role in the community structure of the bacteria, which could determine its growth in a more biofilm-like fashion or a more planktonic fashion *in-vivo*. The metabolic gene expression of bacteria can heavily depend on which of these growth structures the bacteria would be utilizing, which would further impact the presence of secondary metabolites from bacterial secretion *in-vivo* (Miller et al. 2016). Combined, these points suggest that the interpretation of bacterial ability to inhibit *Bd* growth in the wild may not be as predictable based on *in-vitro* experimentation as has previously been proposed. Future studies on microbiomes and their relationships to amphibian immunity, stress, and *Bd* susceptibility should therefore collect as much possible data on the underlying variables that could impact the bacterial community's structure and ability to secrete inhibitory metabolites.

Overall, this study contributes to an ever-developing conceptualization of *Bd*-host interactions and their dependence on environmental conditions. Our focus on stress-induced

peptide secretion and its interactions with the cutaneous microbiome of frogs in a *Bd*-prone area also informs about how the system may change with more frequent stress-responses as a result of climate change and habitat degradation. Conservationists will be able to use this information in developing the most effective plans for the prevention of consecutive *Bd*-induced amphibian declines.

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Bibliography

Al-Qadiri, H. M., Al-Alami, N. I., Lin, M., Al-Holy, M., Cavinato, A. G., and Rasco, B. A. 2008. Studying of the bacterial growth phases using Fourier Transorm Infrared Spectroscopy and multivariate analysis. Journal of Rapid Methods & Automation in Microbiology. 16(1): 73-89.

Becker, M. H., Walke, J. B., Murrill, L., Woodhams, D. C., Reinert, L. K., Rollins-Smith, L. A., Burzynski, E. A., Umile, T. P., Minbiole, K. P. C., and Belden, L. K. 2015. Phylogenetic distribution of symbiotic bacteria from Panamanian amphibians that inhibit growth of the lethal fungal pathogen *Batracochytrium dendrobatidis*. Molecular Ecology. 24: 1628-1641.

Bell, S. C., Alford, R. A., Garland, S., Padilla, G., Thomas, A. D. 2013. Screening bacterial metabolites for inhibitory effects against *Batrachochytrium dendrobatidis* using a spectrophotometric assay. Diseases of Aquatic Organisms. 103: 77-85.

Berger, L., Hyatt, A. D., Speare, R., and Longcore, J. E. 2005. Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. Diseases of aquatic organisms. 68: 51-63.

Berger, L., Roberts, A. A., Voyles, J., Longcore, J. E., Murray, K. A., and Skerratt, L. F. 2016. History and recent progress on chytridiomycosis in amphibians. Fungal Ecology. 19: 89-99.

Bletz, M.C., Loudon, A. H., Becker, M. H., Bell, S. C., Woodhams, D. C., Minbiole, K. P., and Harris, R. N. 2013. Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. Ecological Letters. 16(6): 807-820.

Conlon, J. M., Al-Ghaferi, N., Abraham, B., and Leprince, J. 2007. Strategies for transformation of naturally-occurring amphibian antimicrobial peptides into therapeutically valuable anti-infective agents. Methods. 42: 349-357.

Conlon, J. M., Kolodziejek, J., and Nowotny, N., 2004. Antimicrobial peptides from ranid frogs: taxonomic and phylogenetic markers and a potential source of new therapeutic agents. Biochem. Biophys. Acta. 1696: 1-14

Drew, S. W. and Demain, A. L. 1977. Effect of primary metabolites on secondary metabolism. Ann. Rev. Microbiol. 31: 343-356.

Harris, R. N., James, T. Y., Lauer, A., Simon, M. A., and Patel, A. 2006. Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. EcoHealth. 3: 53-56

Harris, R.N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., Lam, B. A., Woodhams, D. C., Briggs, C. J., Vredenburg, V.T., and Minbiole, K. P. 2009. Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. The International Society for Microbial Ecology Journal. 3: 818-824.

Holden, W. M., Reinert, L. K., Hanlon, S. M., Parris, M. J., and Rollins-Smith, L. A. 2015. Development of antimicrobial peptide defenses of southern leopard frogs, *Rana sphenocephala*, against the pathogenic chytrid fungus, *Batrachochytrium dendrobataidis*. Developmental and Comparative Immunology. 48(1): 65-75.

Keuneman, J. G., Parfrey, L. W., Woodhams, D. C., Archer, H. M., Knight, R., and Mckenzie, V. J. 2013. The amphibian skin-associated microbiome across species, space and life history stages. Molecular Ecology.

Korem, T., Zeevi, D., Suez, J., Weinberger, A., Avnit-Sagi, T., Pompan-Lotan, M., Matot, E., Jona, G., Harmelin, A., Cohen, N., Sirota-Madi, A., Thaiss, C. A., Pevsner-Fischer, M., Sorek, R., Xavier, R. J., Elinav, E., and Segal, E. 2015. Growth dynamics of gut microbiota in health and disease inferred from singe metagenomic samples. Science. 349(6252): 1101-1106.

Miller, C. L., Romero, M., Karna, S. L. R., Chen, T., Heeb, S., and Leung, K. P. 2016. RsmW, *Psedomonas aeruginosa* small non-coding RsmA-binding RNA upregulated in biofilm versus planktonic growth conditions.

Myers, J. M., Ramsey, J. P., Blackman, A. L., Nichols, A. E., Minbiole, K. P. C., and Harris, R. N. 2012. Synergistic inhibition of the lethal fungal pathogen *Batrachochytrium dendrobatidis*: the combined effect of symbiotic bacterial metabolites and antimicrobial peptides of the frog Rana mucosa. Journal of Chemical Ecology. 38(8): 958-965.

Nutkins, J. C. and Williams, D. H. 1989. Identification of highly acidic peptides from processing of the skin prepropeptides of *Xenopus laevis*. Eur. J. Biochem. 181: 97-102.

Rebollar, E. A., Gutiérrez-Preciado, A., Noecker, C., Eng, A., Hughey, M., Medina, D., Walke, J. B., Borenstein, E., Jensen, R. V., Belden, L. K., and Harris, R. N. 2018. The skin microbiome of the neotropical frog *Craugastor fitzingeri*: inferring potential bacterial-host-pathogen interactions from metagenomic data. Frontiers in Microbiology. 9(466): 1-12.

Rollins-Smith, L. A. 2017. Amphibian immunity-stress, disease, and climate change. Developmental & Comparative Immunology. 66: 111-119.

Rollins-Smith, L.A. Ramsey, J. P., Pask, J. D., Reinert, L. K., Woodhams, D. C. 2011. Amphibian immune defenses against chytridiomycosis: impacts of changing environments. Integrated Comparative Biology. 51(4): 52-562.

Rollins-Smith, L.A., Reinert, L. K., O'Leary, C. J., Houston, L. E., and Woodhams, D. C. 2005. Antimicrobial peptide defenses in amphibian skin. Integrative and Comparative Biology. 45: 137-142.

Romeo, T. 1998. Global regulation by the small RNA-binding protein CSRA and the non-coding RNA molecule CsrB. Molecular Microbiology. 29(6): 1321-1330.

Woodhams, D. C., Ardipradja, K., Alford, R. A., Marantelli, G., Reinert, L. K., and Rollins-Smith, L. A. 2007. Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. Animal Conservation. 10: 409-417.

Woodhams, D. C., Bosch, J., Briggs, C. J., Cashins, S., Davis, L. R., Lauer, A. L., Muths, E., Puschendorf, R., Schmidt, B. R., Sheafor, B., Voyles, J. 2011. Mitigating amphibian disease: strategies to maintain wild populations and control chytridiomycosis. Frontiers in Zoology. 8: 8.

Woodhams, D.C., Voyles, J., Lips, K. R., Carey, C., and Rollins-Smith, L. A. 2006. Predicted disease susceptibility in a Panamanian amphibian assemblage based on skin peptide defenses. Journal of Wildlife Diseases. 42: 207-218.

Woodhams, D.C., Vredenburg, V. T., Simon, M., Billheimer, D., Shakhtour, B., Shyr, Y., Briggs, C. J., Rollins-Smith, L. A., and Harris, R. N. 2007. Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana mucosa*. Biol. Conservation. 138: 390-398.

Table 1. The bacterial type (characterized by morphology and given an alphabetical identity "A"-"L") found on that frog skin before and after injection. The quantity of peptides collected after injection were quantified using an altered BCA assay and are shown for each individual frog. Rows 1-12 were injected with saline and rows 13-24 were injected with norepinephrine.

Frog	Pre-injection Bacteria	Post-injection Bacteria	Amount of Peptides (ug/mL)	Norepinephrine Treatment
1	C,E	C,J	201.13	-
2	A	I	195.95	-
3			260.03	-
4	C		191.92	-
5	A		243.55	-
6	B		364.47	-
7	A	A	216.65	-
8	B,C,D	B,E,J	169.22	-
9	B,E		184.33	-
10	E		241.92	-
11	A	A	178.64	-
12			187.57	-
13	A	A,H	261.29	+
14		J,K	214.35	+
15	E,F,G,K	E,K,L	169.22	+
16	A		207.88	+
17			253.79	+
18	A	A	194.11	+
19	A	B	241.92	+
20	E,F,H		217.04	+
21	C,D,E,H,J		249.26	+
22	A,J	A	177.58	+
23	B,H	H	266.77	+
24	A	E	300.40	+

Table 2. Summary table for the *Bd* challenge assay experimental results. "Gross Abundance" represents the number of bacterial swabs (pre- and post-injection) that produced each bacterial type. "Inhibitory OD" represents whether the stationary-phase metabolites prevented growth of *Bd* relative to the positive control based on recordings of the optical density of the well treatments, and "Inhibitory Zoospore Index" represents an analysis of the inhibition based on the zoospore index of the full supernatant wells in comparison to the positive controls (N=10 wells for each treatment). + = complete inhibition, +/- = incomplete inhibition, - = no inhibition, N.d. = not done due to insufficient collection of bacterial secretions.

Bacterial Type	**Gross Abundance**	**Proportion of Frogs Observed**	**Inhibition of Bd Growth**	**Inhibition of Zoospores**
A	15	0.417	+	+
B	6	0.208	+	+
C	5	0.167	+	+
D	2	0.083	+	+
E	9	0.333	+	+
F	2	0.083	+/-	+
G	1	0.042	+	+
H	5	0.167	+	+
I	1	0.042	+/-	+
J	5	0.208	+	+
K	3	0.083	+	+
L	1	0.042	N.d.	N.d.

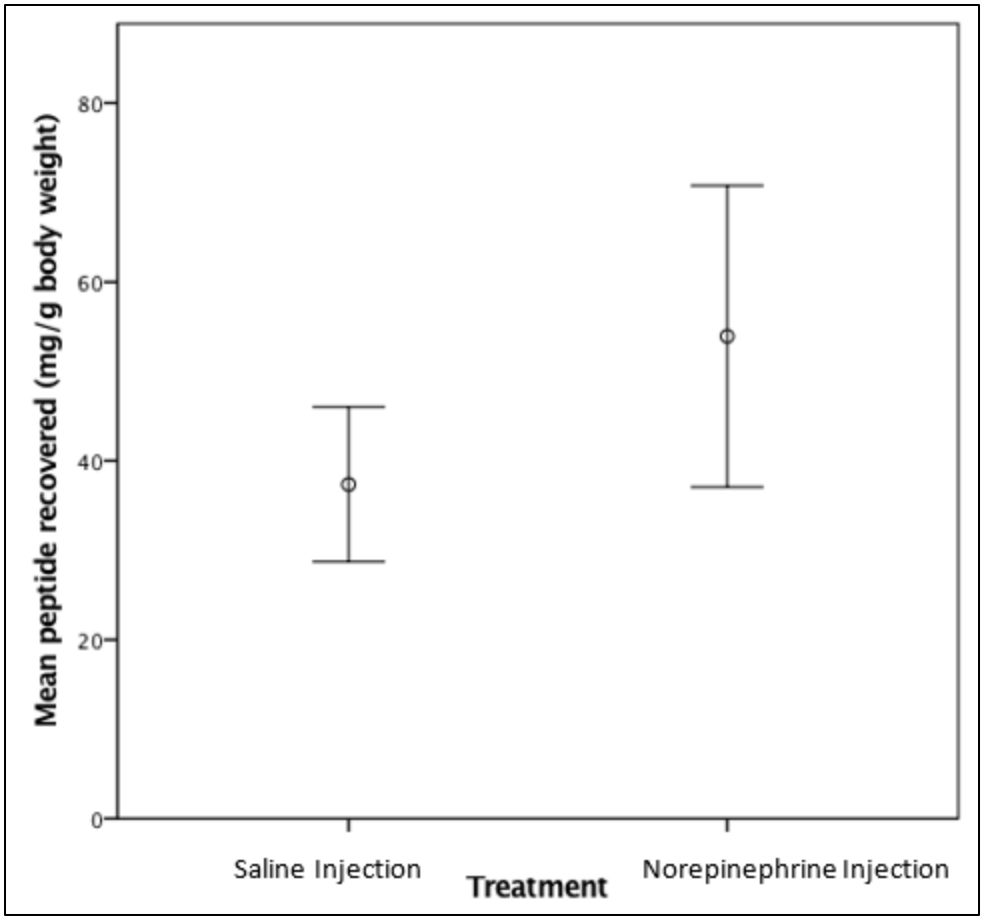

Figure 1. The mean amount of peptides collected through HPLC from glass frogs injected with saline and norepinephrine shown with error bars representing 95% CI. Quantified using BSA with bradykinin acetate salt standard, N = 24 frogs.

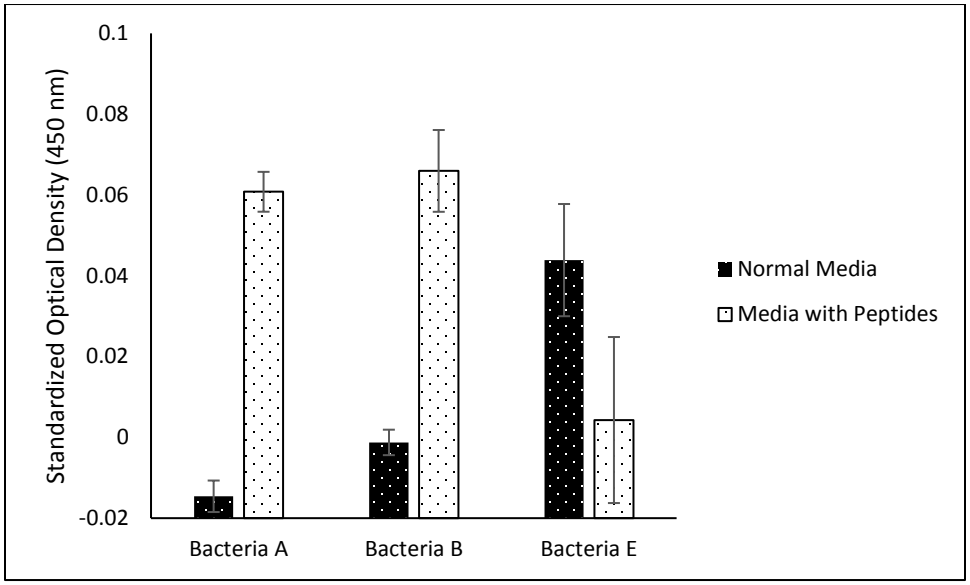

Figure 2. Growth in bacterial types "A," "B," and "E" in the presence and absence of peptides. Optical density values represent the optical density after 50 hours of growth at ambient temperature and were standardized against negative control values. N = 8 wells for each treatment and bacteria. Bars represent ± 1 standard error of the mean.

Appendix/Supplemental Information

Table S1. Assumed antimicrobial peptides collected from all frog samples. The metrics recorded for the individual frogs are represented, as well as the quantification of peptides collected from each frog based on a BCA assay with a Bradykinin Acetate Salt standard.

Individual	Injection	Life stage	Sex	Bd	SVL (mm)	Mass (g)	Date	Concentration (µg/ml)	Notes
160617_01	Saline	adult	M		26.8	0.90	2016.06.17	201.1	
160617_02	Saline	adult	M		23.6	0.90	2016.06.17	195.9	
160617_03	Saline	adult	M		27.3	1.20	2016.06.17	260.0	
160617_04	Saline	adult	M		24.6	1.20	2016.06.17	191.9	
160617_05	Saline	adult	M		24.3	1.20	2016.06.17	243.5	
160617_06	Saline	adult	M		26.8	1.10	2016.06.17	364.5	
160617_07	Saline	adult	M		28.2	1.20	2016.06.17	216.7	
160617_08	Saline	adult	M		29.6	1.20	2016.06.17	169.2	
160617_09	Saline	adult	M		26.2	1.20	2016.06.17	184.3	
160617_10	Saline	adult	F		25.6	1.10	2016.06.17	241.9	
160617_11	Saline	adult	M		27.4	0.80	2016.06.17	178.6	
160617_12	Saline	adult	M		27.1	1.10	2016.06.17	187.6	
160617_13	Norepinephrine	adult	M		26.3	1.10	2016.06.17	261.3	
160617_14	Norepinephrine	adult	M		28.0	1.10	2016.06.17	214.3	
160617_15	Norepinephrine	adult	M		25.1	1.00	2016.06.17	169.2	
160617_16	Norepinephrine	adult	M		28.3	1.20	2016.06.17	207.9	
160617_17	Norepinephrine	adult	M		27.6	1.20	2016.06.17	253.8	Mortality
160617_18	Norepinephrine	adult	M		26.8	1.20	2016.06.17	194.1	
160617_19	Norepinephrine	adult	M		28.8	1.30	2016.06.17	241.9	Mortality
160617_20	Norepinephrine	adult	F		28.2	1.10	2016.06.17	217.0	
160617_21	Norepinephrine	adult	M		25.1	0.70	2016.06.17	249.3	
160617_22	Norepinephrine	adult	M		27.1	1.20	2016.06.17	177.6	
160617_23	Norepinephrine	adult	M		26.4	1.00	2016.06.17	266.8	
160617_24	Norepinephrine	adult	M		26.3	1.10	2016.06.17	300.4	

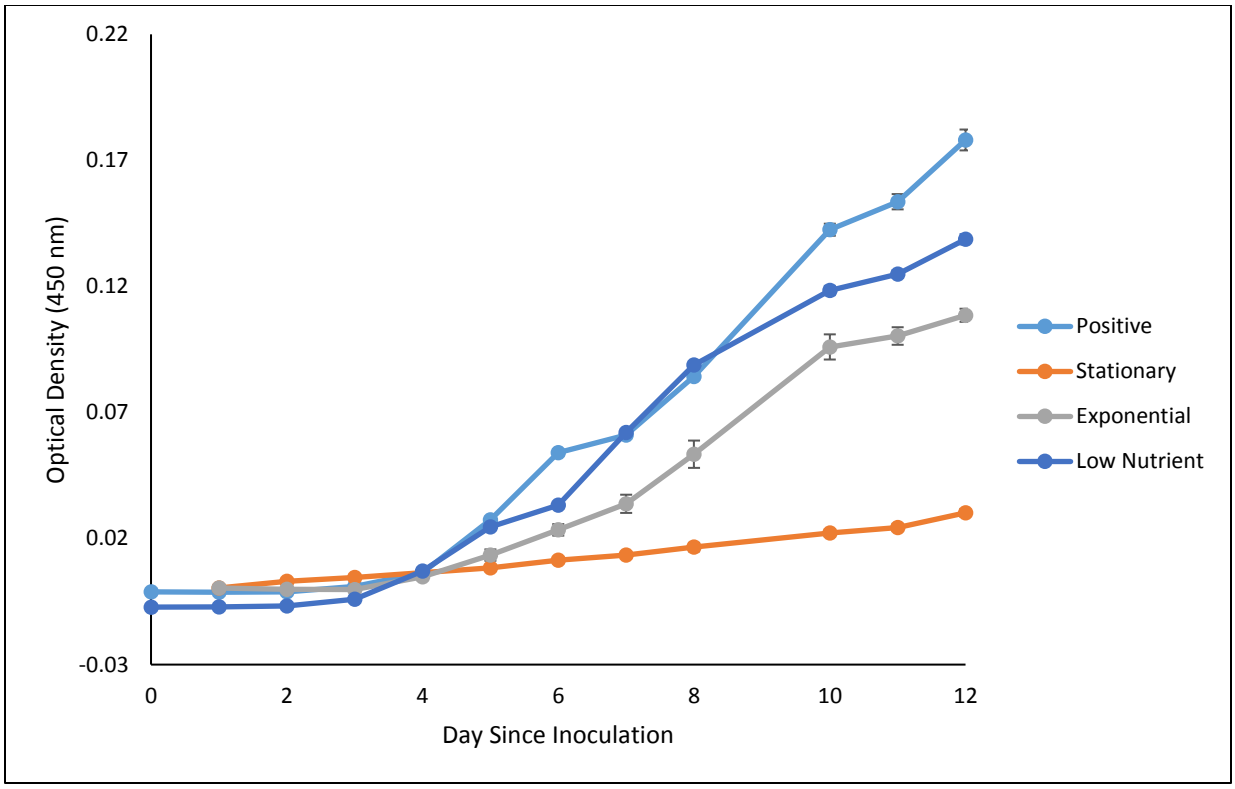

Figure S1.A. Growth of *Bd* in the presence of secreted metabolites from Bacteria "A". Each data point represents 10 wells (N=10) and the error bars represent the ± standard error of the mean. Most error bars are too small to noticeably discern from the data markers. "Positive" refers to *Bd* growth in media only, "Stationary" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "Exponential" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's growth phase, and "Low Nutrient" refers to *Bd* growth after inoculation into distilled water.

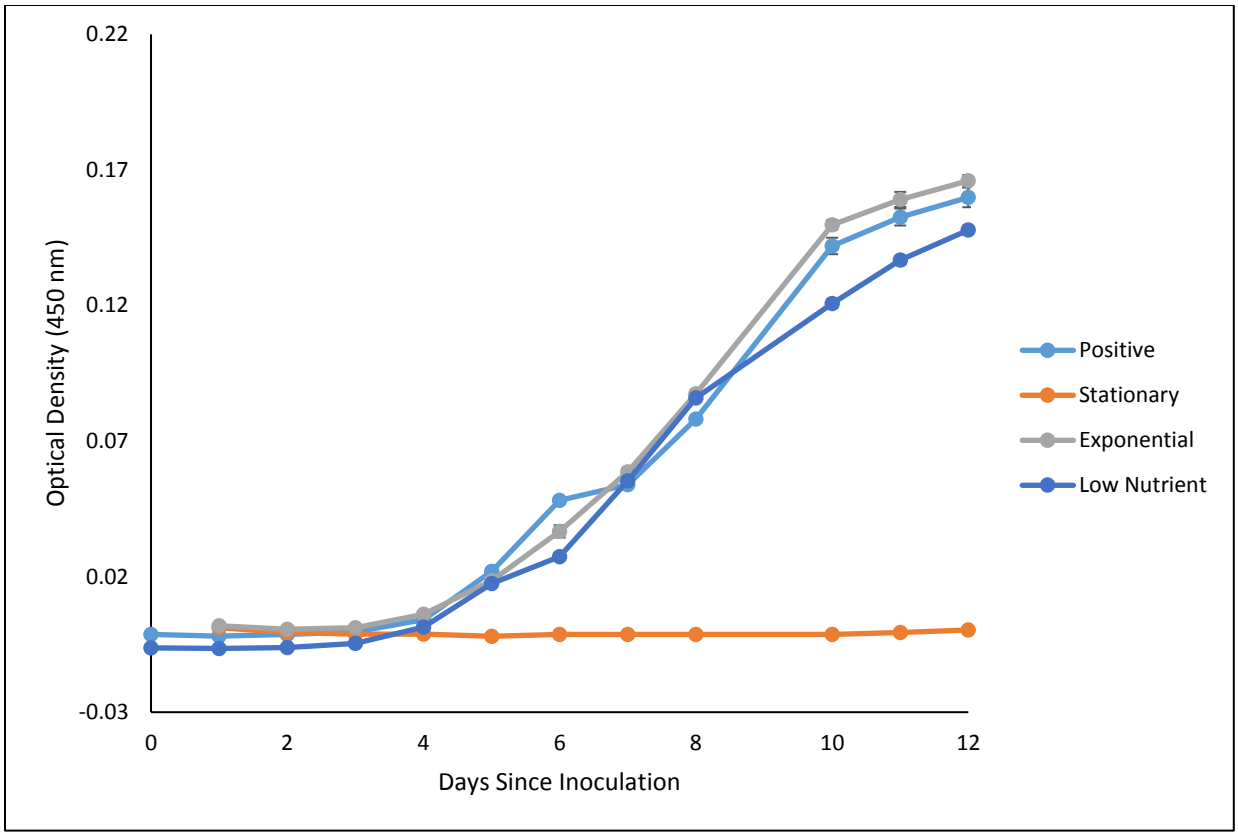

Figure S1.B. Growth of *Bd* in the presence of secreted metabolites from Bacteria "B". Each data point represents 10 wells (N=10) and the error bars represent the ± standard error of the mean. Most error bars are too small to noticeably discern from the data markers. "Positive" refers to *Bd* growth in media only, "Stationary" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "Exponential" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's growth phase, and "Low Nutrient" refers to *Bd* growth after inoculation into distilled water.

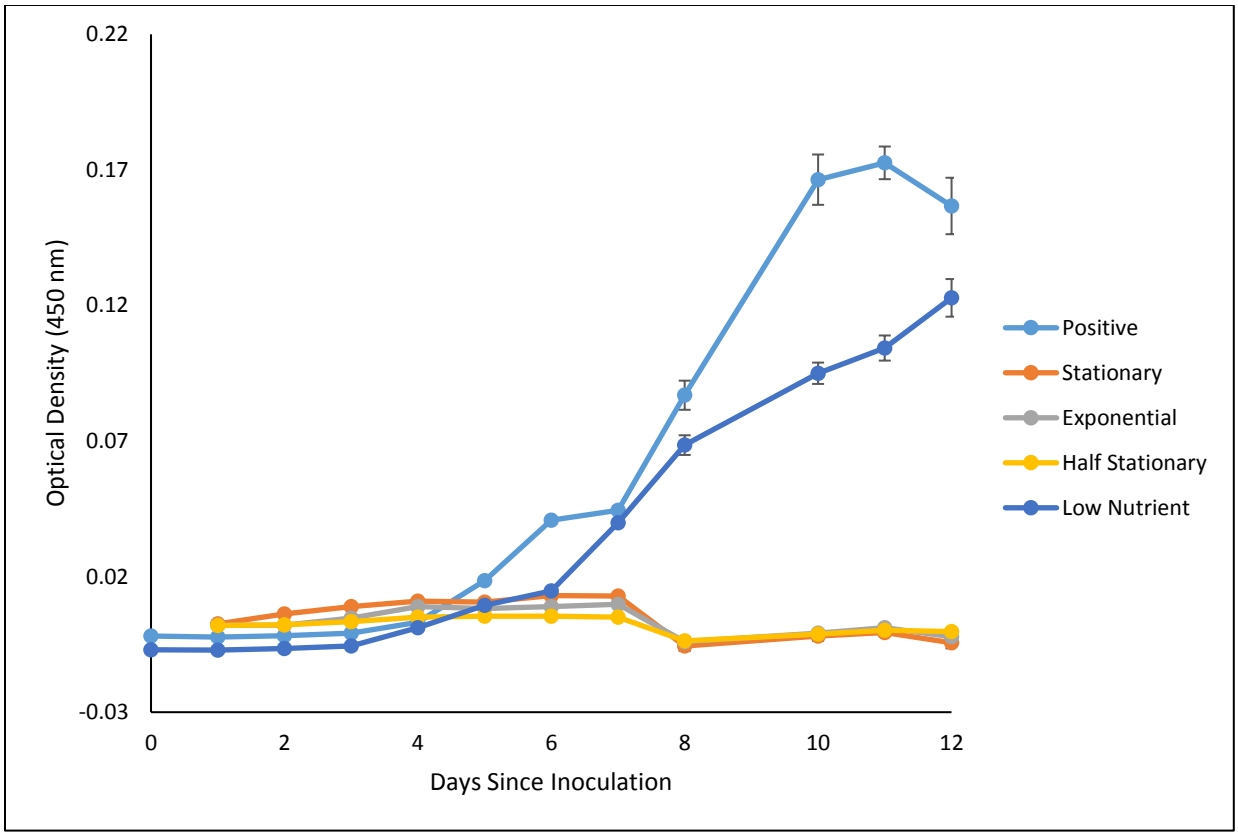

Figure S1.C. Growth of *Bd* in the presence of secreted metabolites from Bacteria "C". Each data point represents 10 wells (N=10) and the error bars represent the ± standard error of the mean. Most error bars are too small to noticeably discern from the data markers. "Positive" refers to *Bd* growth in media only, "Stationary" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "Exponential" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's growth phase, "Half Stationary" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's stationary phase and diluted by 50%, and "Low Nutrient" refers to *Bd* growth after inoculation into distilled water.

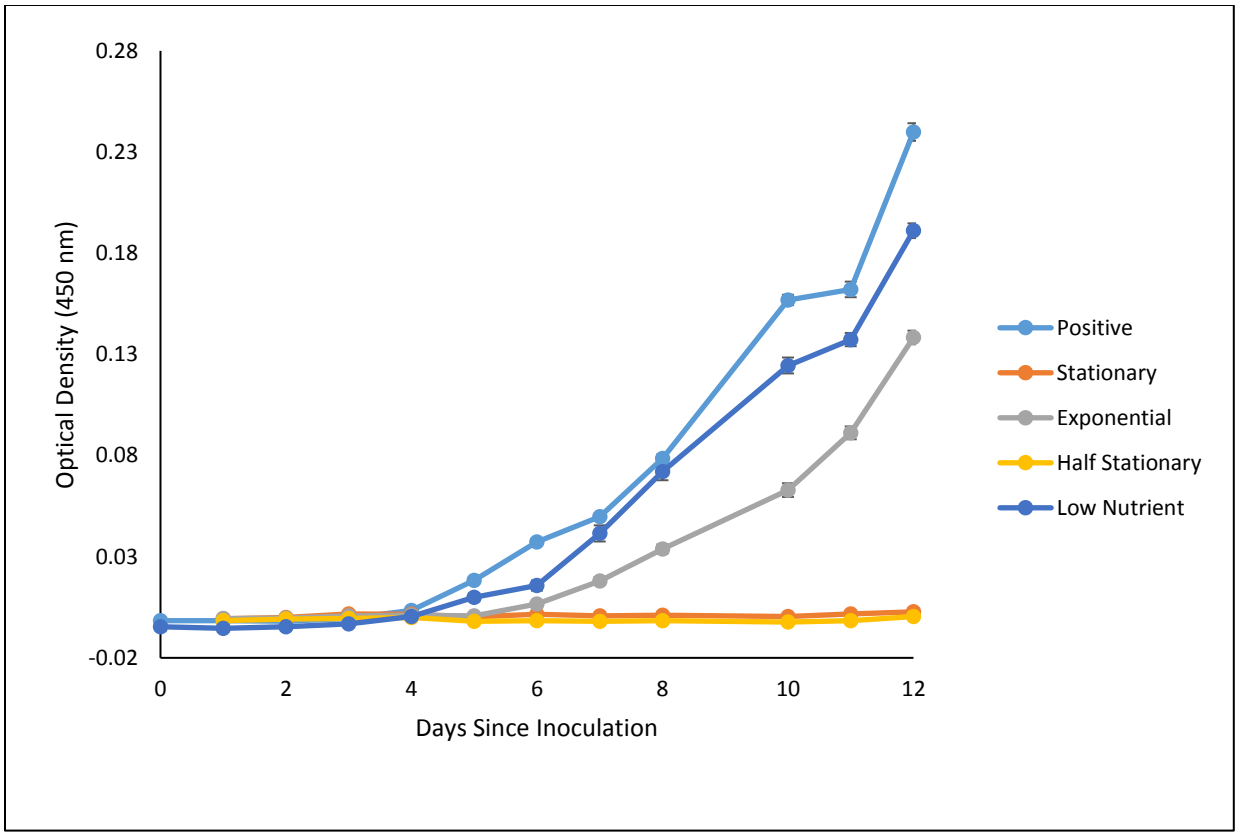

Figure S1.D. Growth of *Bd* in the presence of secreted metabolites from Bacteria "D". Each data point represents 10 wells (N=10) and the error bars represent the ± standard error of the mean. Most error bars are too small to noticeably discern from the data markers. "Positive" refers to *Bd* growth in media only, "Stationary" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "Exponential" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's growth phase, "Half Stationary" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's stationary phase and diluted by 50%, and "Low Nutrient" refers to *Bd* growth after inoculation into distilled water.

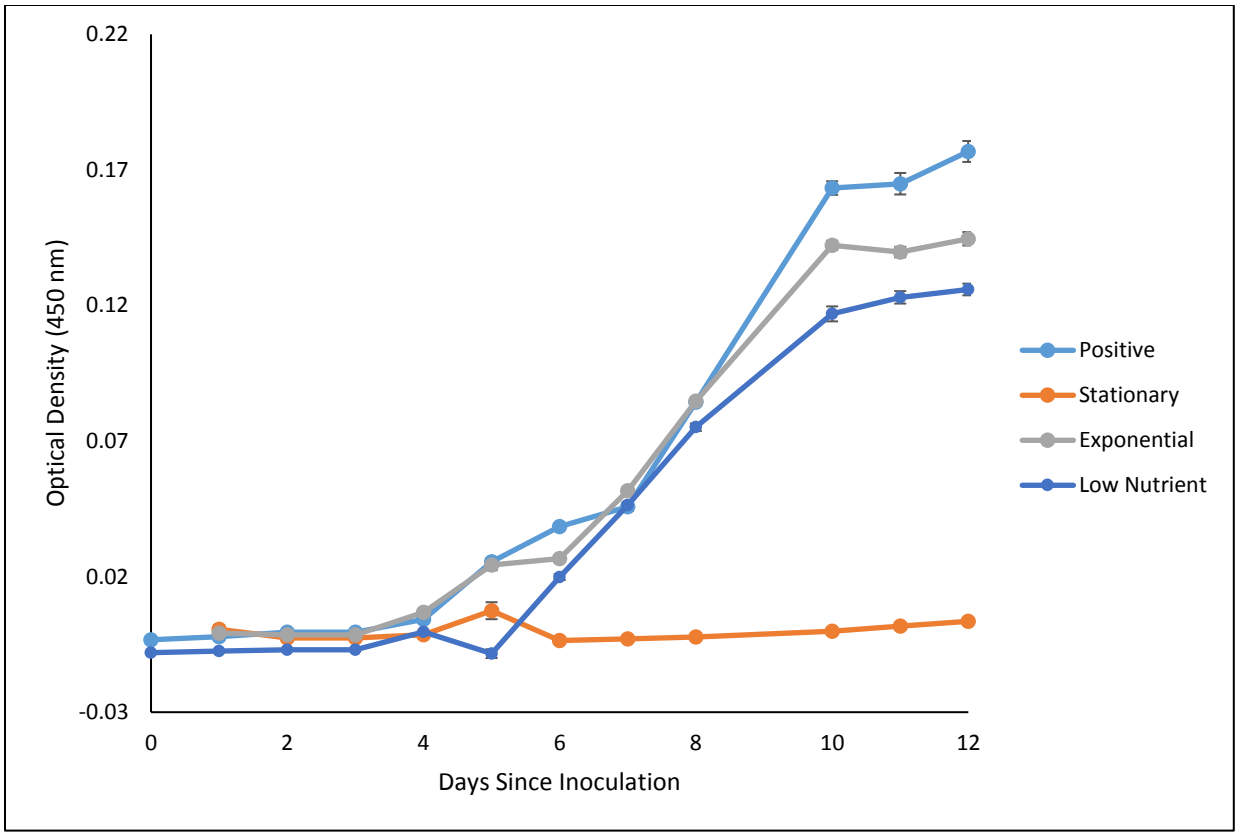

Figure S1.E. Growth of *Bd* in the presence of secreted metabolites from Bacteria "E". Each data point represents 10 wells (N=10) and the error bars represent the ± standard error of the mean. Most error bars are too small to noticeably discern from the data markers. "Positive" refers to *Bd* growth in media only, "Stationary" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "Exponential" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's growth phase, and "Low Nutrient" refers to *Bd* growth after inoculation into distilled water.

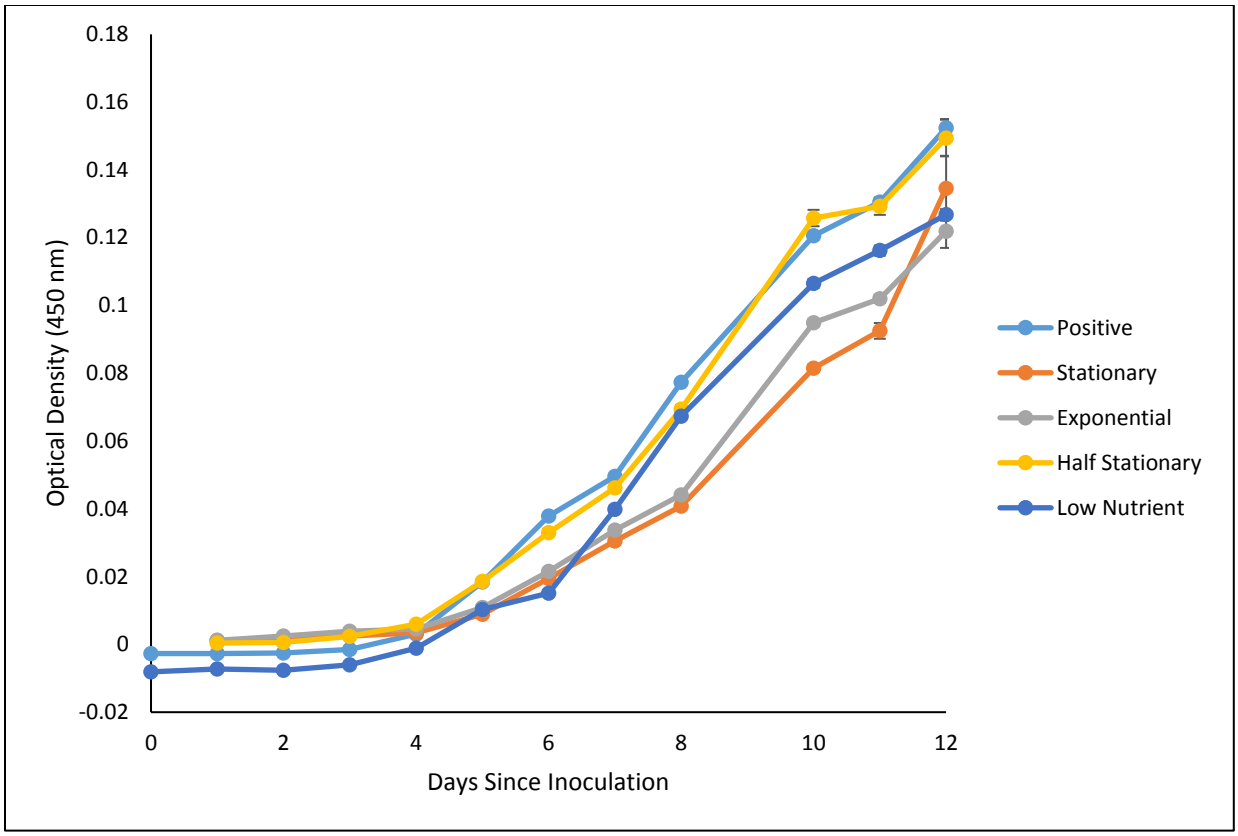

Figure S1.F. Growth of *Bd* in the presence of secreted metabolites from Bacteria "F". Each data point represents 10 wells (N=10) and the error bars represent the ± standard error of the mean. Most error bars are too small to noticeably discern from the data markers. "Positive" refers to *Bd* growth in media only, "Stationary" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "Exponential" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's growth phase, "Half Stationary" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's stationary phase and diluted by 50%, and "Low Nutrient" refers to *Bd* growth after inoculation into distilled water.

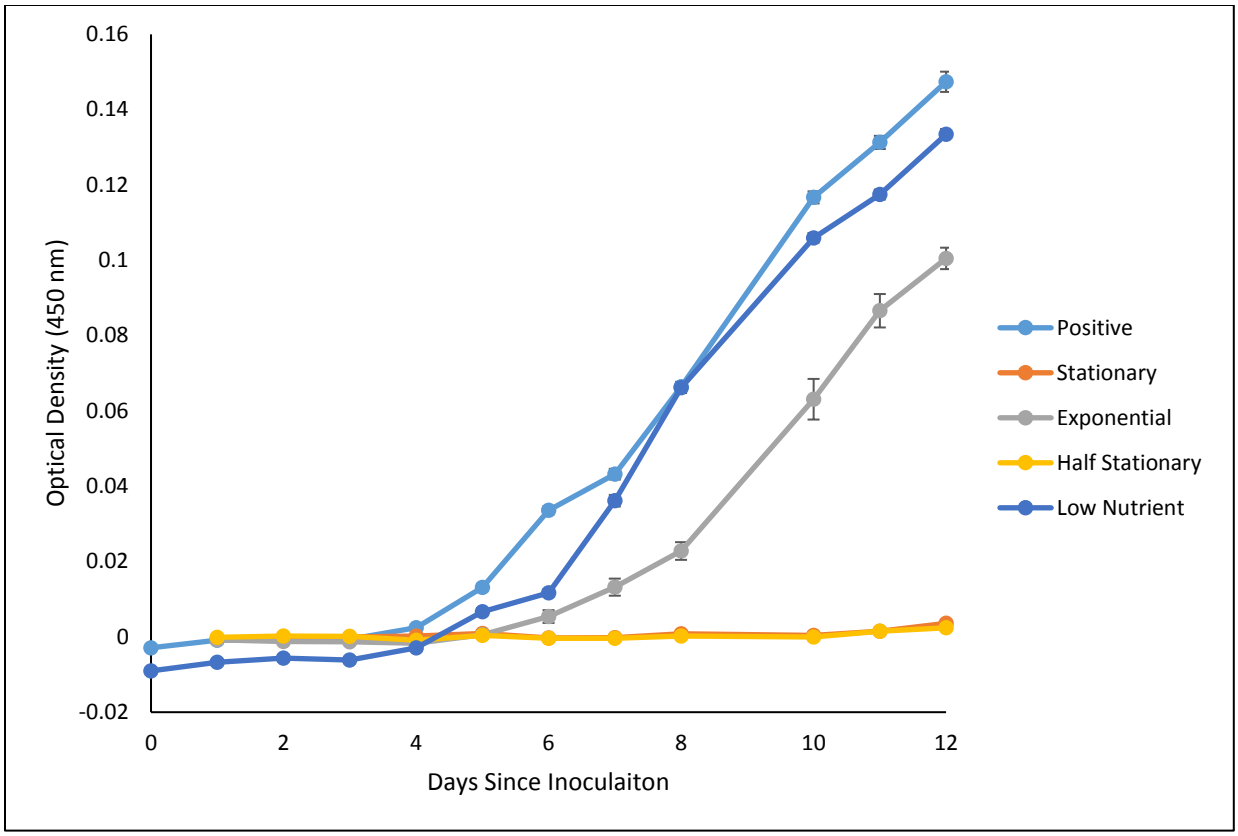

Figure S1.G. Growth of *Bd* in the presence of secreted metabolites from Bacteria "G". Each data point represents 10 wells (N=10) and the error bars represent the ± standard error of the mean. Most error bars are too small to noticeably discern from the data markers. "Positive" refers to *Bd* growth in media only, "Stationary" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "Exponential" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's growth phase, "Half Stationary" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's stationary phase and diluted by 50%, and "Low Nutrient" refers to *Bd* growth after inoculation into distilled water.

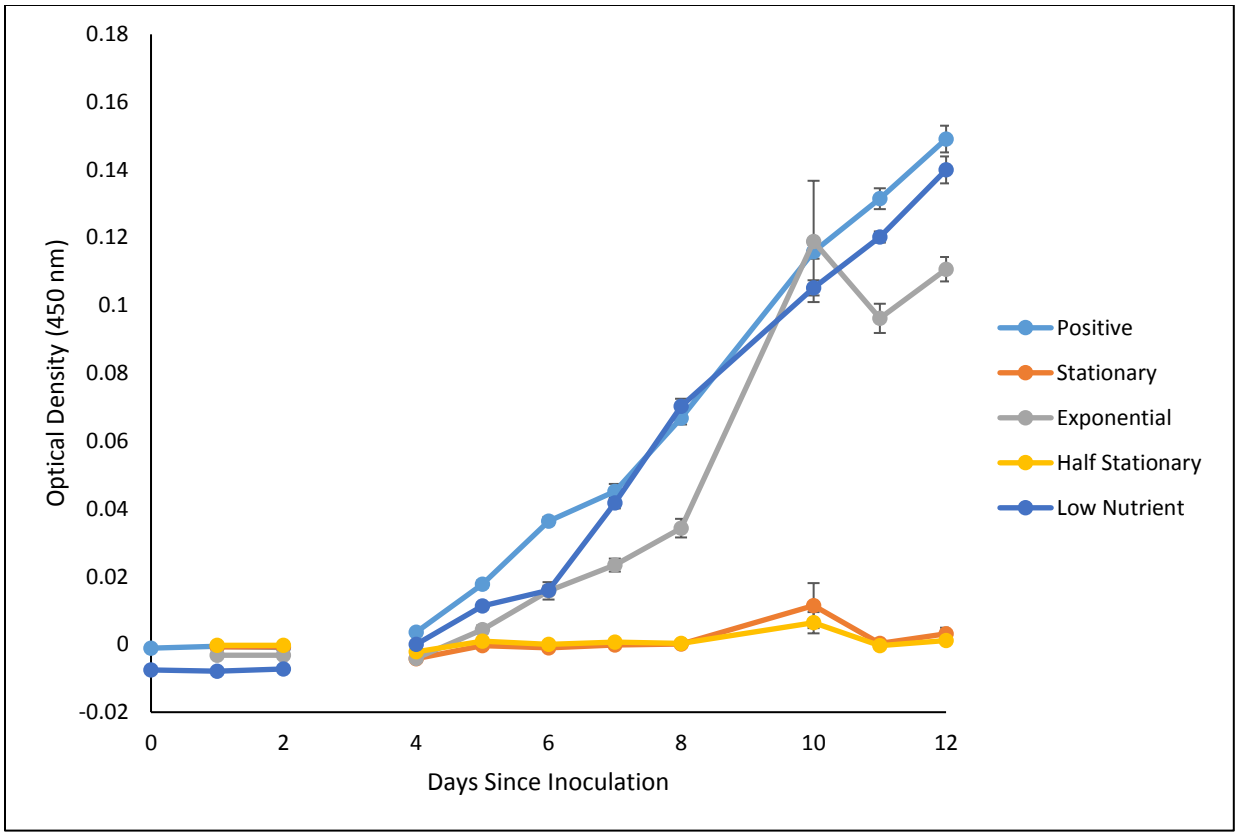

Figure S1.H. Growth of *Bd* in the presence of secreted metabolites from Bacteria "H". Each data point represents 10 wells (N=10) and the error bars represent the ± standard error of the mean. Most error bars are too small to noticeably discern from the data markers. "Positive" refers to *Bd* growth in media only, "Stationary" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "Exponential" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's growth phase, "Half Stationary" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's stationary phase and diluted by 50%, and "Low Nutrient" refers to *Bd* growth after inoculation into distilled water. The data for Day 3 was lost for this experiment.

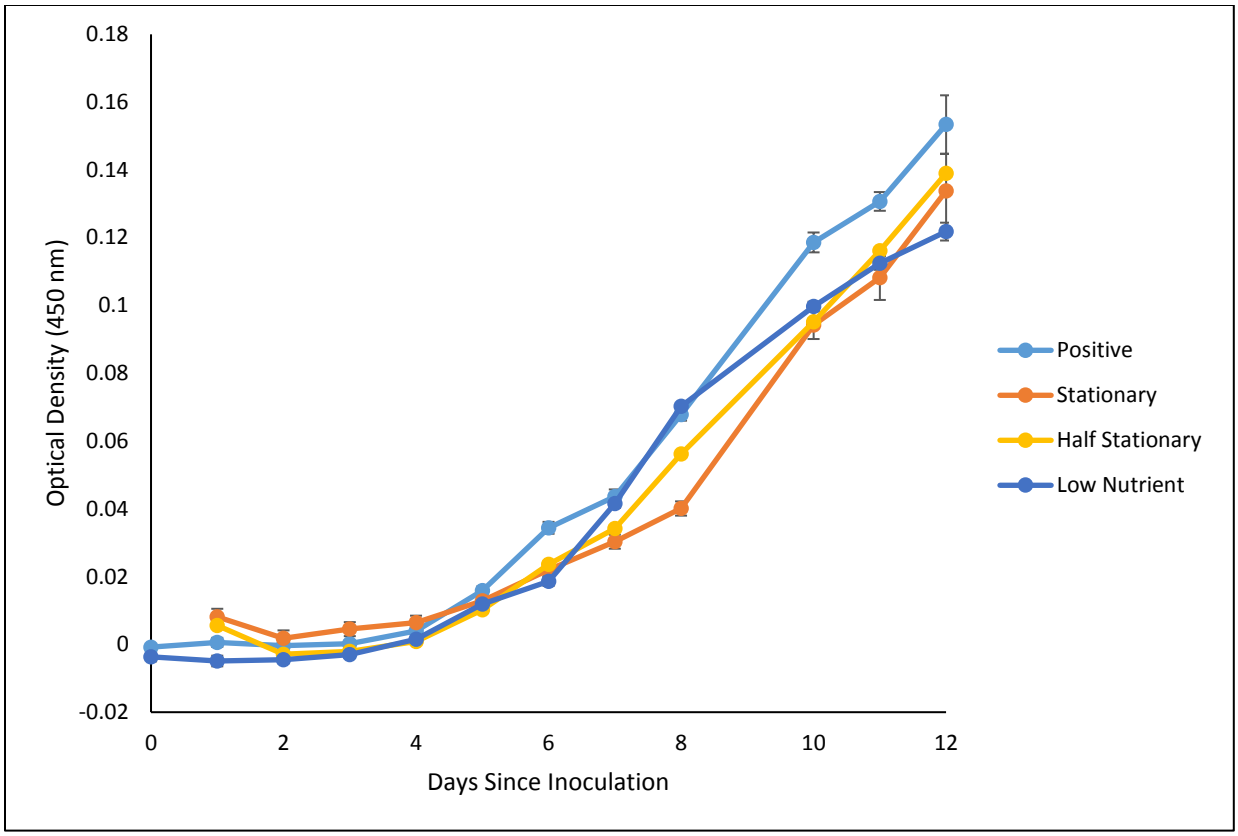

Figure S1.I. Growth of *Bd* in the presence of secreted metabolites from Bacteria "I". Each data point represents 10 wells (N=10) and the error bars represent the ± standard error of the mean. Most error bars are too small to noticeably discern from the data markers. "Positive" refers to *Bd* growth in media only, "Stationary" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "Half Stationary" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's stationary phase and diluted by 50%, and "Low Nutrient" refers to *Bd* growth after inoculation into distilled water.

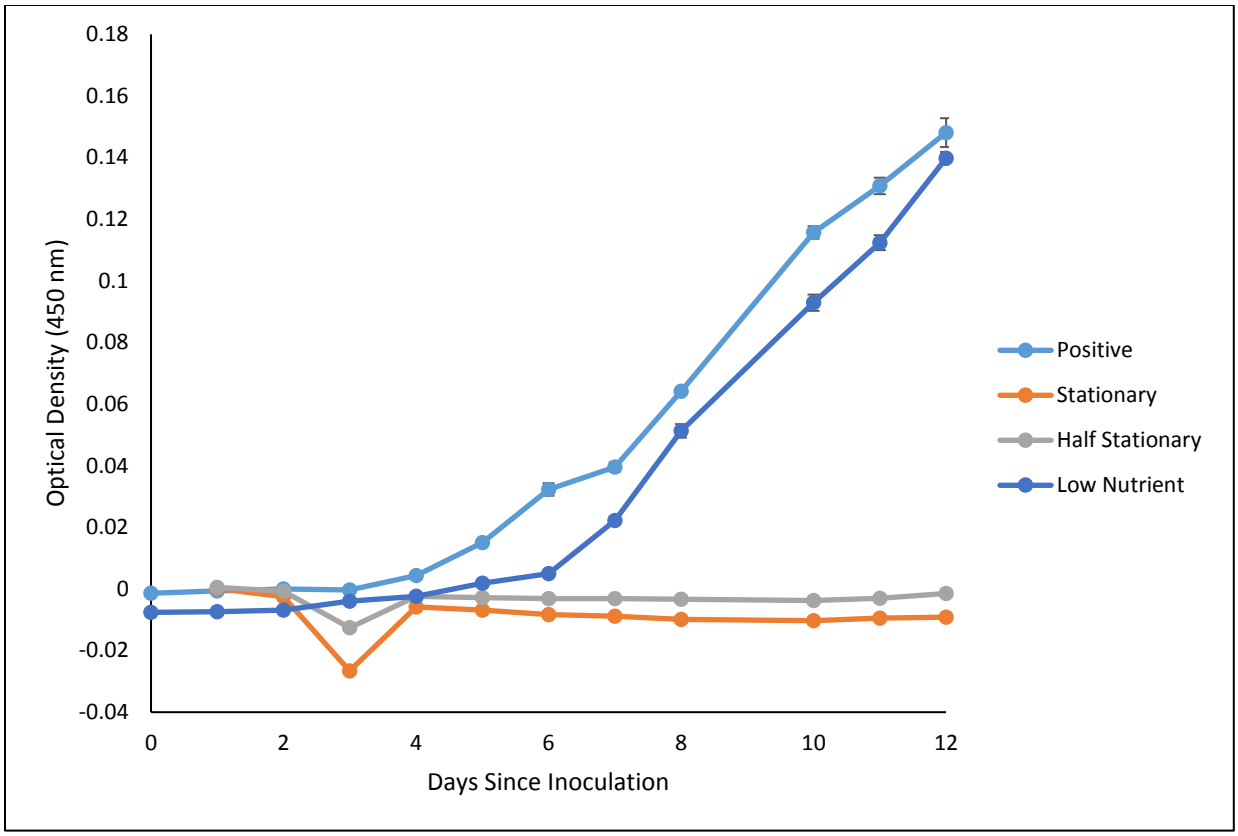

Figure S1.J. Growth of *Bd* in the presence of secreted metabolites from Bacteria "J". Each data point represents 10 wells (N=10) and the error bars represent the ± standard error of the mean. Most error bars are too small to noticeably discern from the data markers. "Positive" refers to *Bd* growth in media only, "Stationary" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "Half Stationary" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's stationary phase and diluted by 50%, and "Low Nutrient" refers to *Bd* growth after inoculation into distilled water.

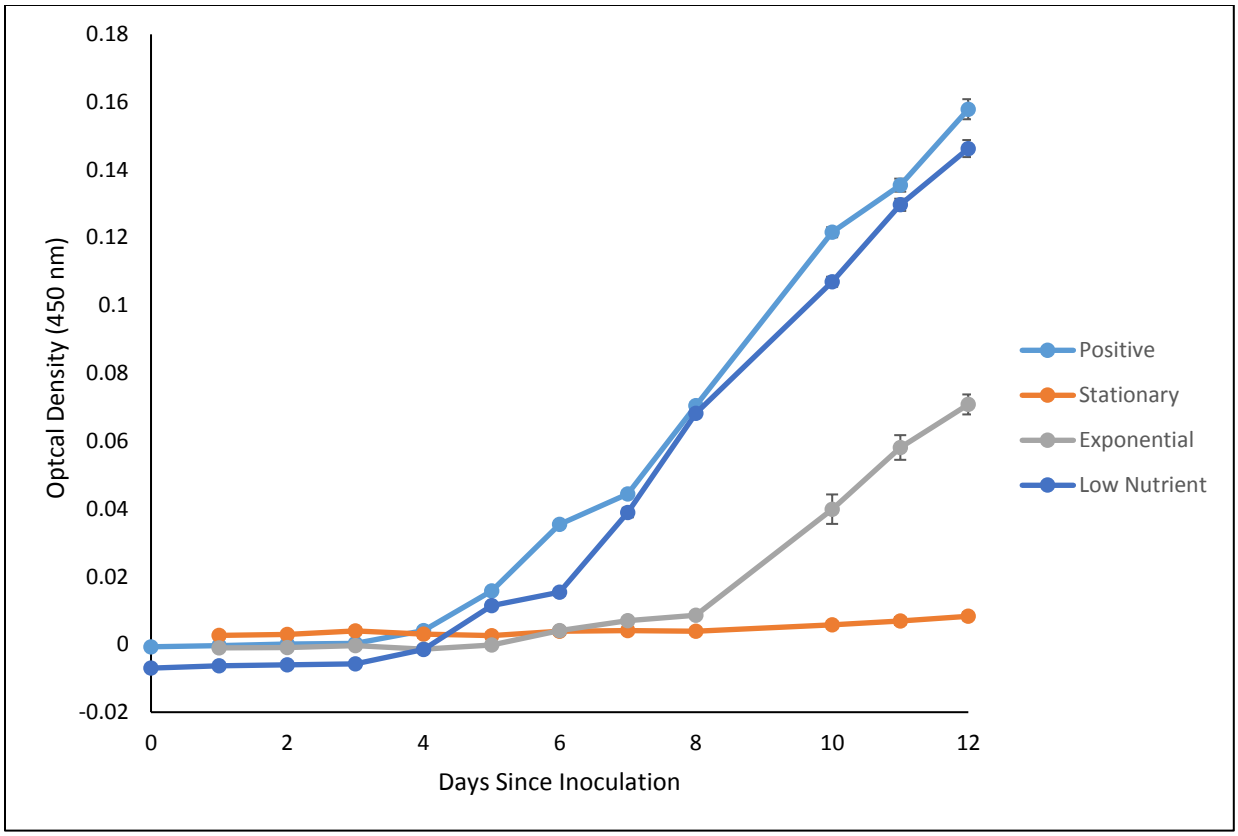

Figure S1.K. Growth of *Bd* in the presence of secreted metabolites from Bacteria "K". Each data point represents 10 wells (N=10) and the error bars represent the ± standard error of the mean. Most error bars are too small to noticeably discern from the data markers. "Positive" refers to *Bd* growth in media only, "Stationary" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "Exponential" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's growth phase, and "Low Nutrient" refers to *Bd* growth after inoculation into distilled water.

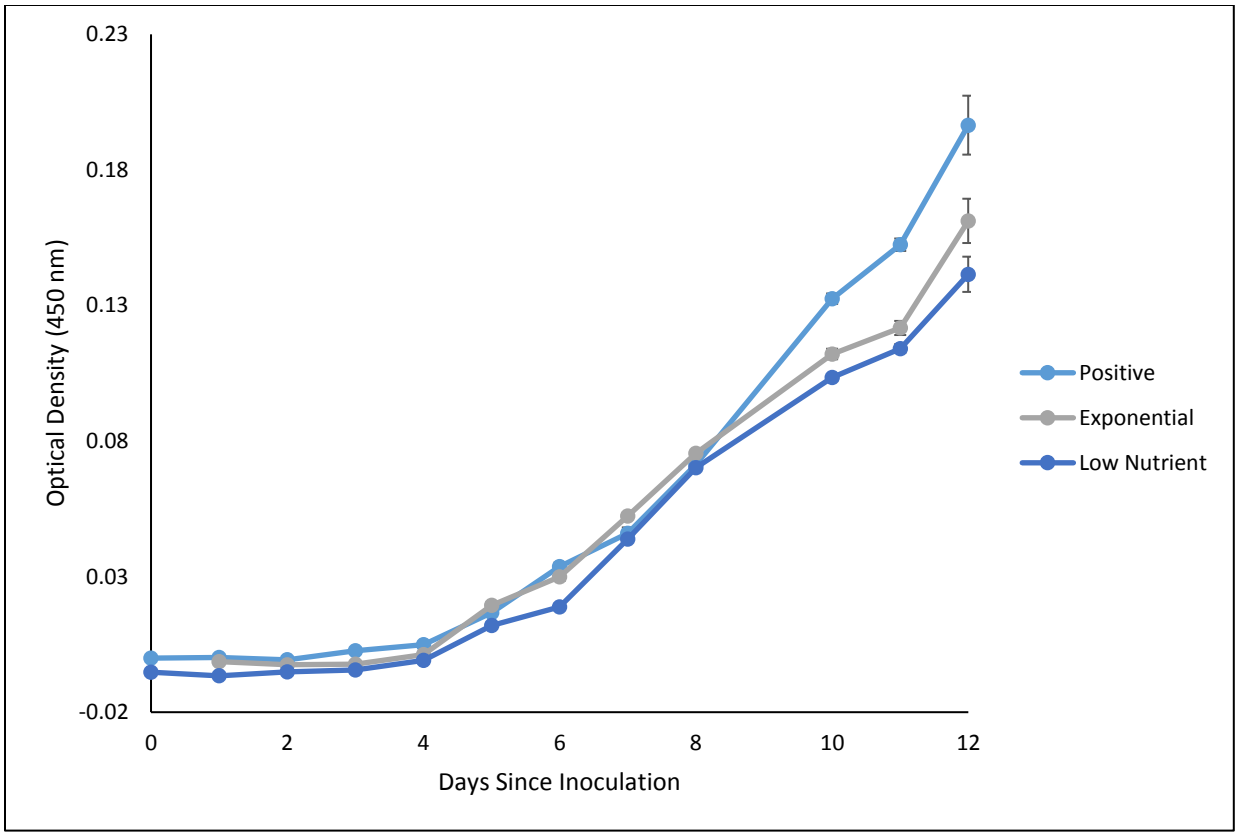

Figure S1.L. Growth of *Bd* in the presence of secreted metabolites from Bacteria "L". Each data point represents 10 wells (N=10) and the error bars represent the ± standard error of the mean. Most error bars are too small to noticeably discern from the data markers. "Positive" refers to *Bd* growth in media only, "Exponential" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's growth phase, and "Low Nutrient" refers to *Bd* growth after inoculation into distilled water.

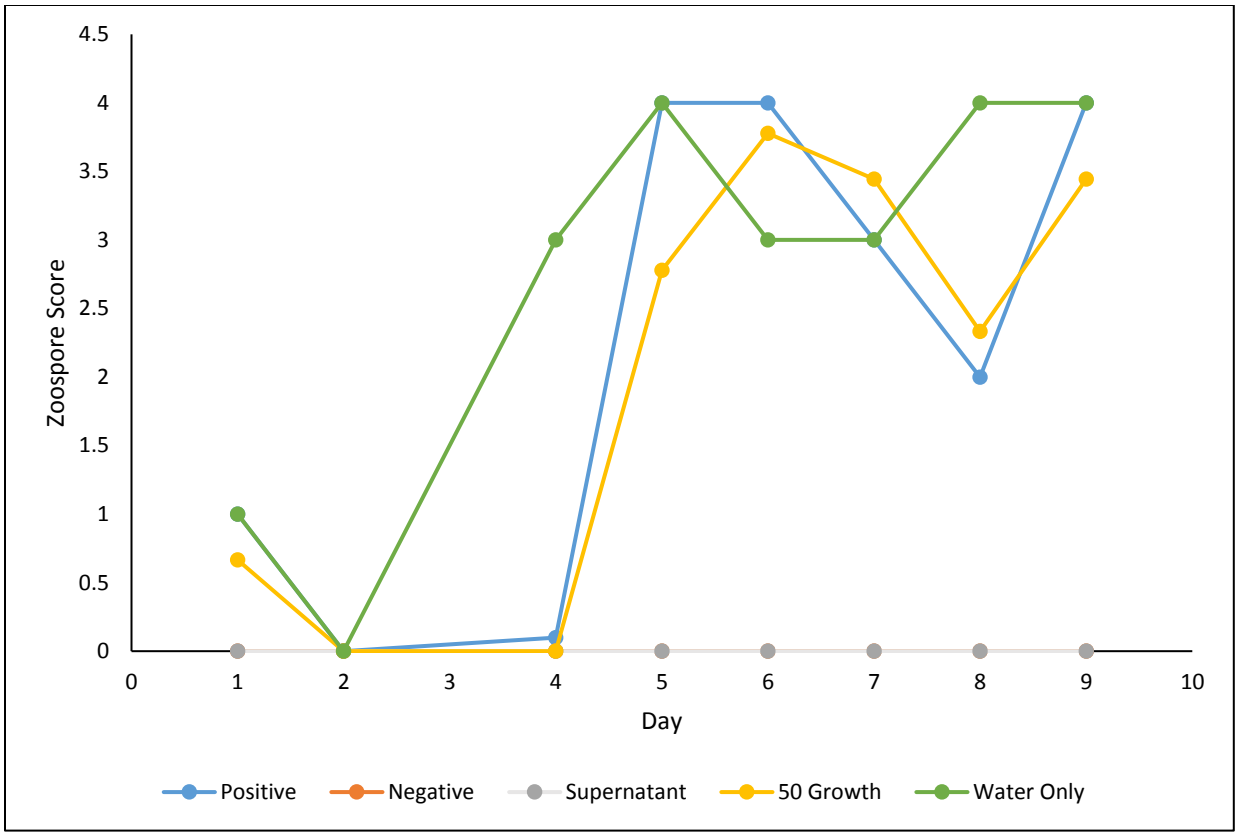

Figure S2.A. Zoospore Index results for *Bd* grown with supernatant collected from bacteria "A." Each data point represents 10 wells (N=10). "Positive" refers to *Bd* growth in media only, "Negative" refers to heat-killed *Bd* zoosporangia in media, "Supernatant" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "50 Growth" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's growth phase, and "Water Only" refers to *Bd* growth in distilled water. Overlapping data series may not be discernable.

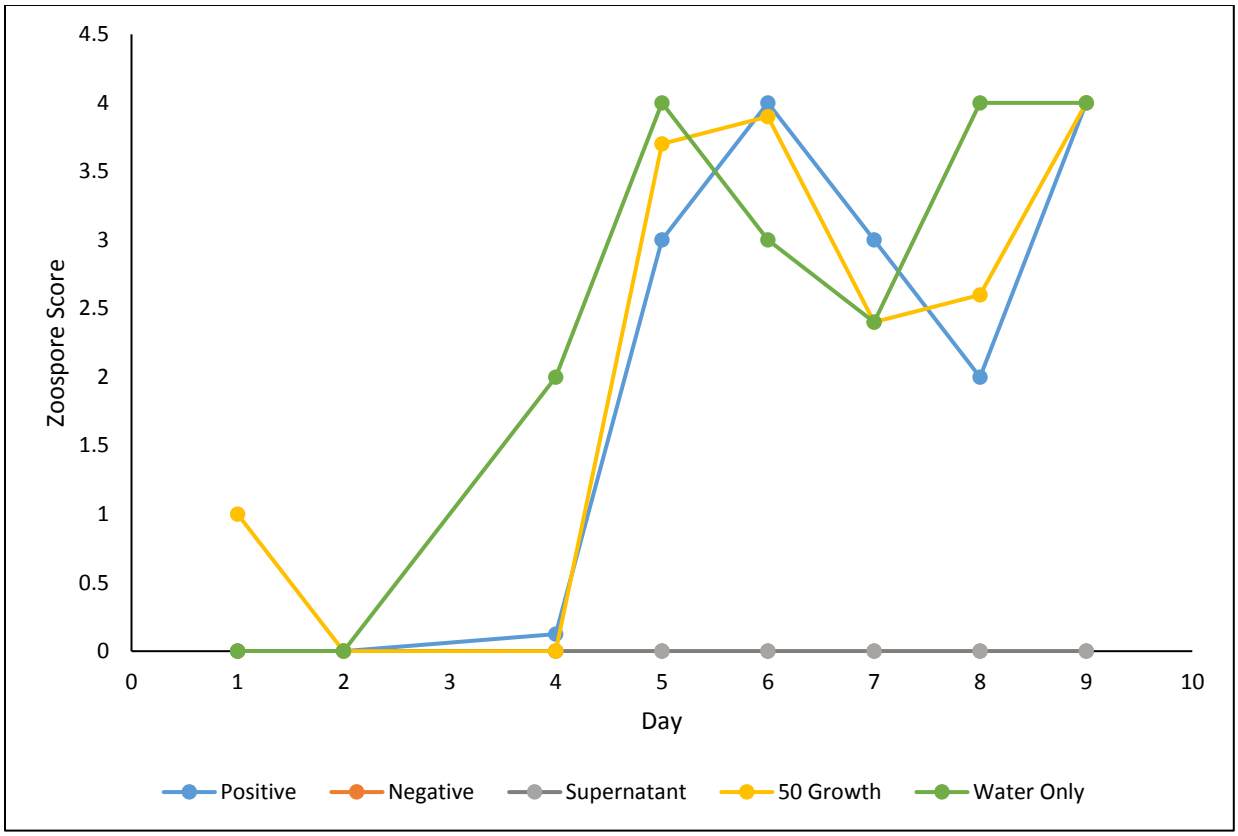

Figure S2.B. Zoospore Index results for *Bd* grown with supernatant collected from bacteria "B." Each data point represents 10 wells (N=10). "Positive" refers to *Bd* growth in media only, "Negative" refers to heat-killed *Bd* zoosporangia in media, "Supernatant" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "50 Growth" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's growth phase, and "Water Only" refers to *Bd* growth in distilled water. Overlapping data series may not be discernable.

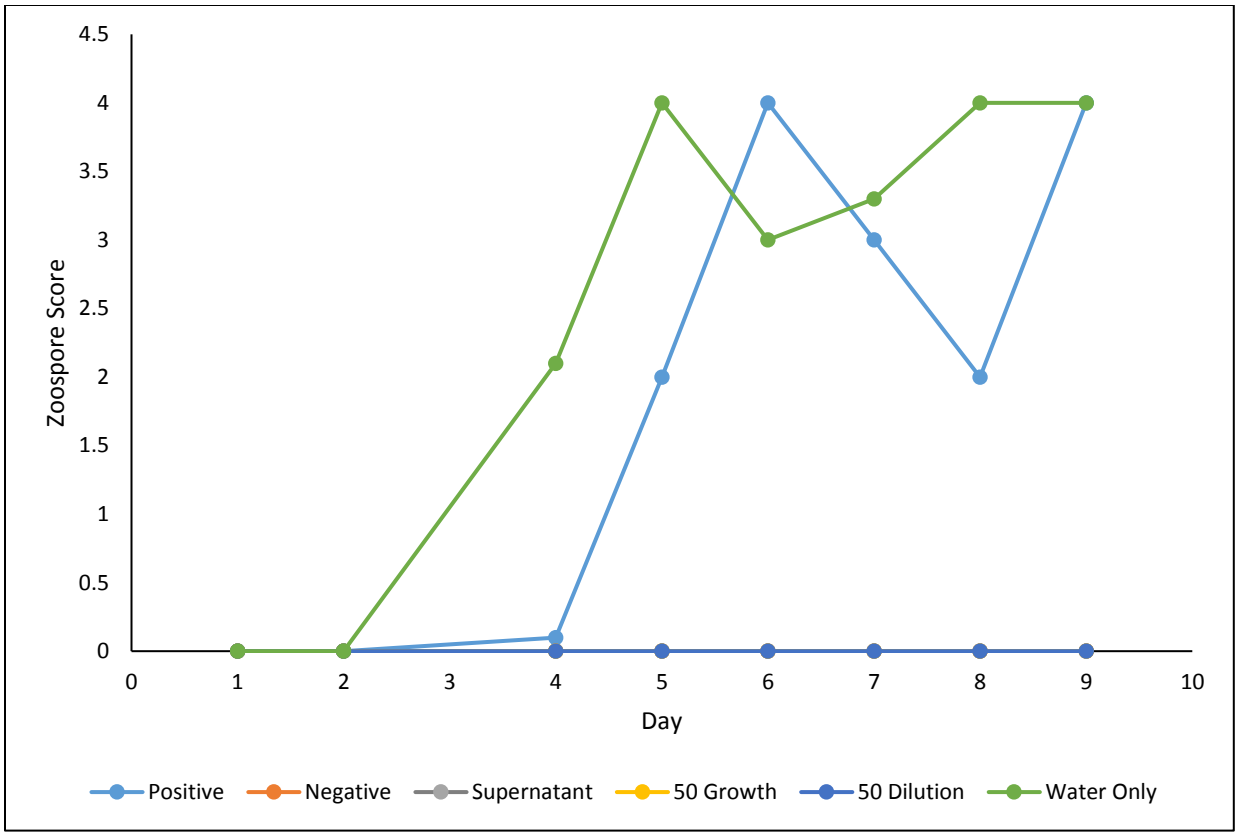

Figure S2.C. Zoospore Index results for *Bd* grown with supernatant collected from bacteria "C." Each data point represents 10 wells (N=10). "Positive" refers to *Bd* growth in media only, "Negative" refers to heat-killed *Bd* zoosporangia in media, "Supernatant" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "50 Growth" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's growth phase, "50 Dilution" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's stationary phase and diluted by 50%, and "Water Only" refers to *Bd* growth in distilled water. Overlapping data series may not be discernable.

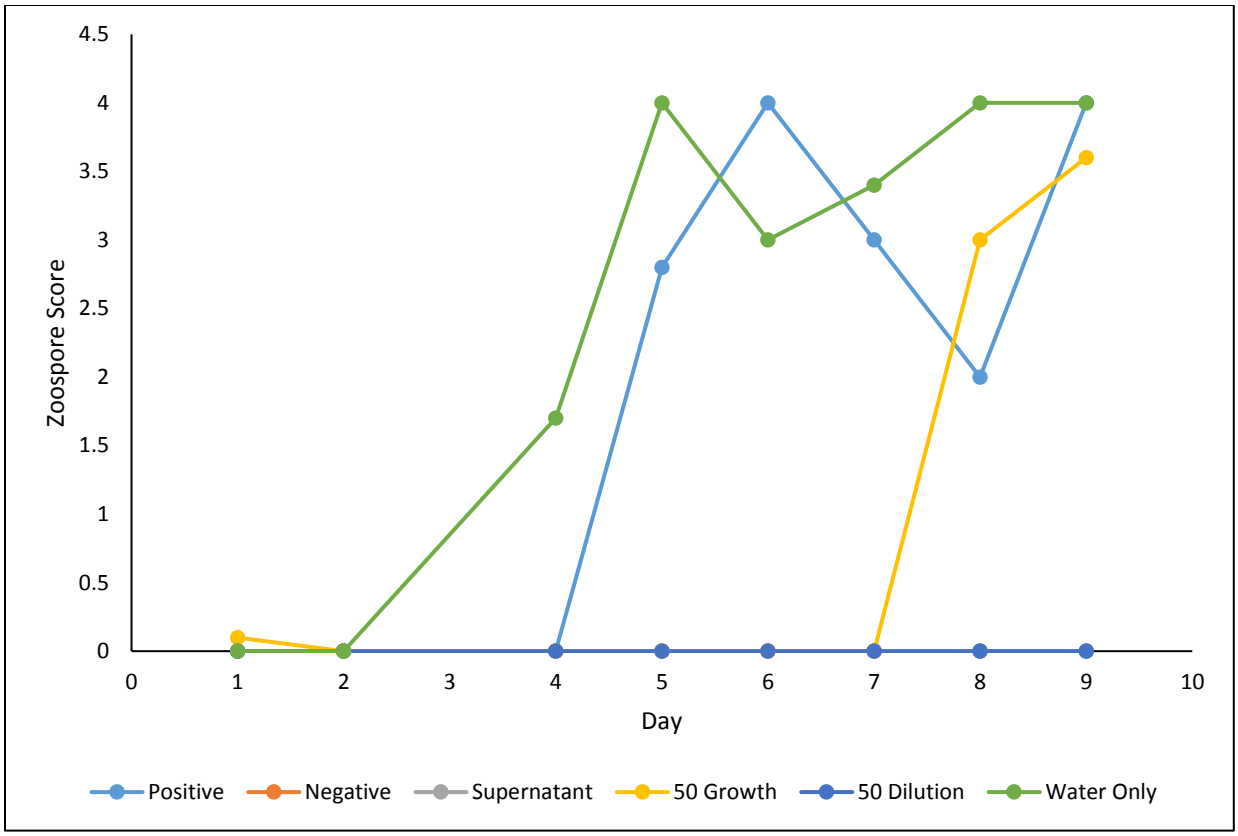

Figure S2.D. Zoospore Index results for *Bd* grown with supernatant collected from bacteria "D." Each data point represents 10 wells (N=10). "Positive" refers to *Bd* growth in media only, "Negative" refers to heat-killed *Bd* zoosporangia in media, "Supernatant" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "50 Growth" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's growth phase, "50 Dilution" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's stationary phase and diluted by 50%, and "Water Only" refers to *Bd* growth in distilled water. Overlapping data series may not be discernable.

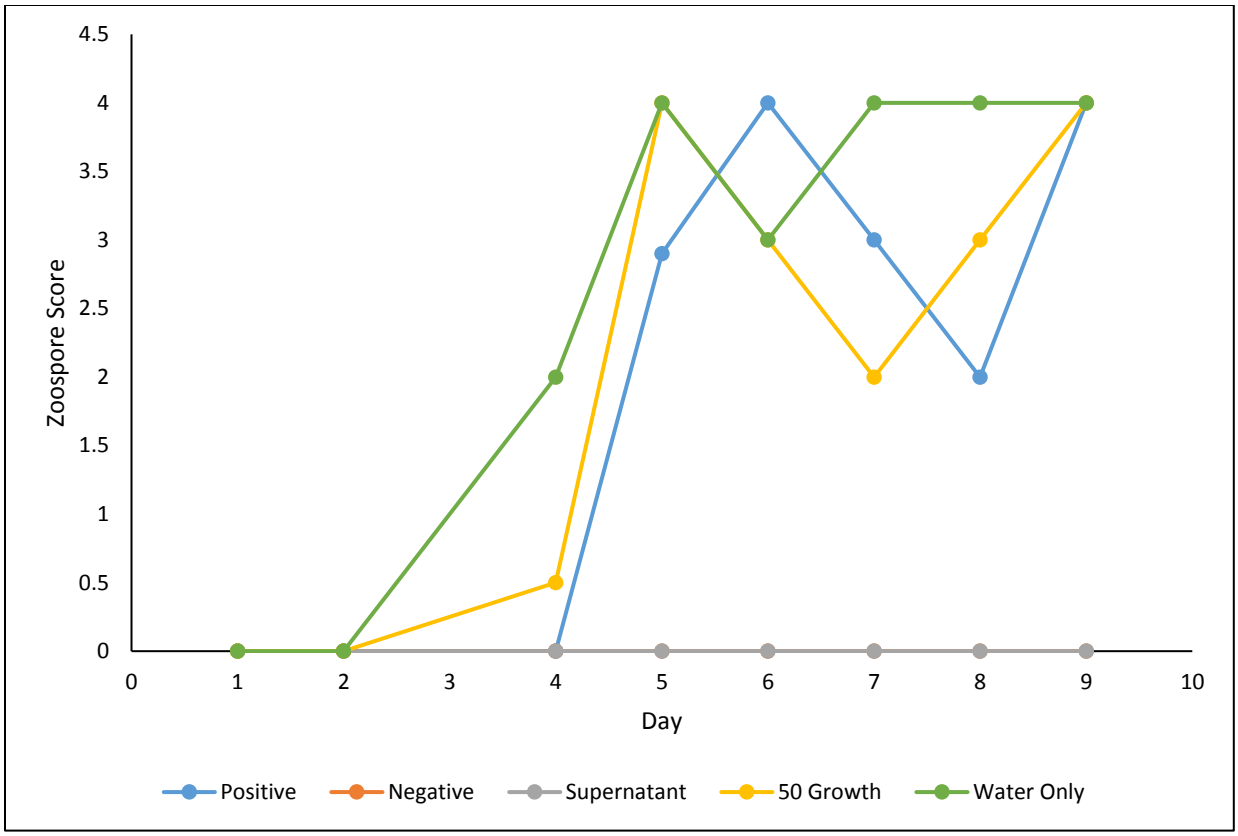

Figure S2.E. Zoospore Index results for *Bd* grown with supernatant collected from bacteria "E." Each data point represents 10 wells (N=10). "Positive" refers to *Bd* growth in media only, "Negative" refers to heat-killed *Bd* zoosporangia in media, "Supernatant" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "50 Growth" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's growth phase, and "Water Only" refers to *Bd* growth in distilled water. Overlapping data series may not be discernable.

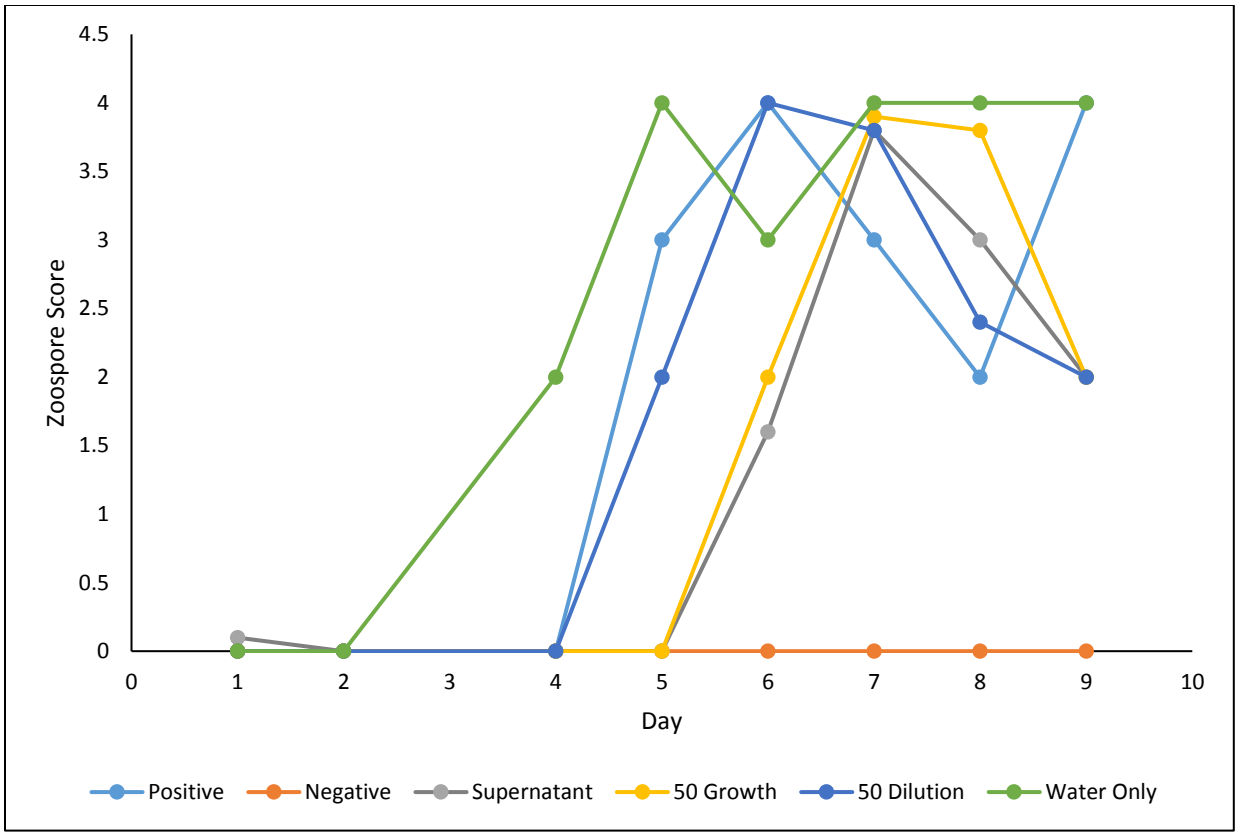

Figure S2.F. Zoospore Index results for *Bd* grown with supernatant collected from bacteria "F." Each data point represents 10 wells (N=10). "Positive" refers to *Bd* growth in media only, "Negative" refers to heat-killed *Bd* zoosporangia in media, "Supernatant" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "50 Growth" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's growth phase, "50 Dilution" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's stationary phase and diluted by 50%, and "Water Only" refers to *Bd* growth in distilled water. Overlapping data series may not be discernable.

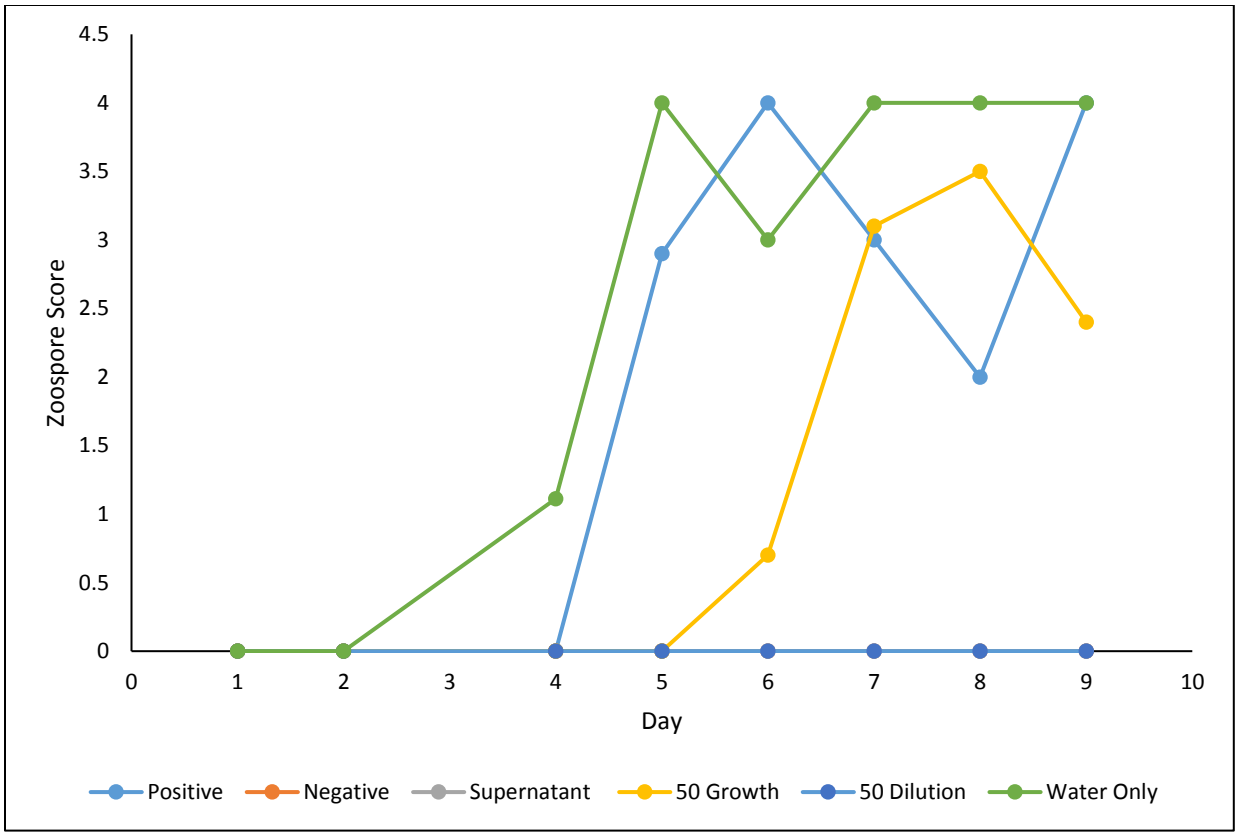

Figure S2.G. Zoospore Index results for *Bd* grown with supernatant collected from bacteria "G." Each data point represents 10 wells (N=10). "Positive" refers to *Bd* growth in media only, "Negative" refers to heat-killed *Bd* zoosporangia in media, "Supernatant" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "50 Growth" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's growth phase, "50 Dilution" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's stationary phase and diluted by 50%, and "Water Only" refers to *Bd* growth in distilled water. Overlapping data series may not be discernable.

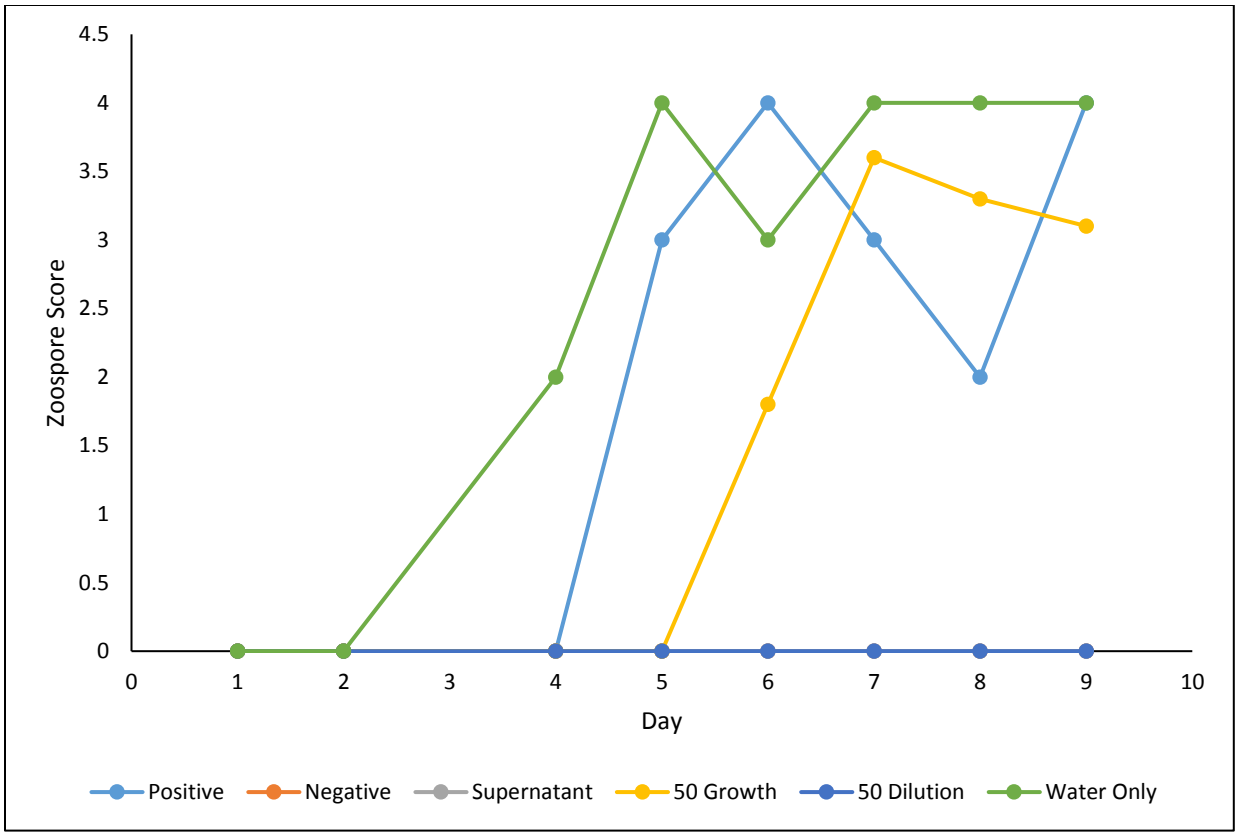

Figure S2.H. Zoospore Index results for *Bd* grown with supernatant collected from bacteria "H." Each data point represents 10 wells (N=10). "Positive" refers to *Bd* growth in media only, "Negative" refers to heat-killed *Bd* zoosporangia in media, "Supernatant" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "50 Growth" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's growth phase, "50 Dilution" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's stationary phase and diluted by 50%, and "Water Only" refers to *Bd* growth in distilled water. Overlapping data series may not be discernable.

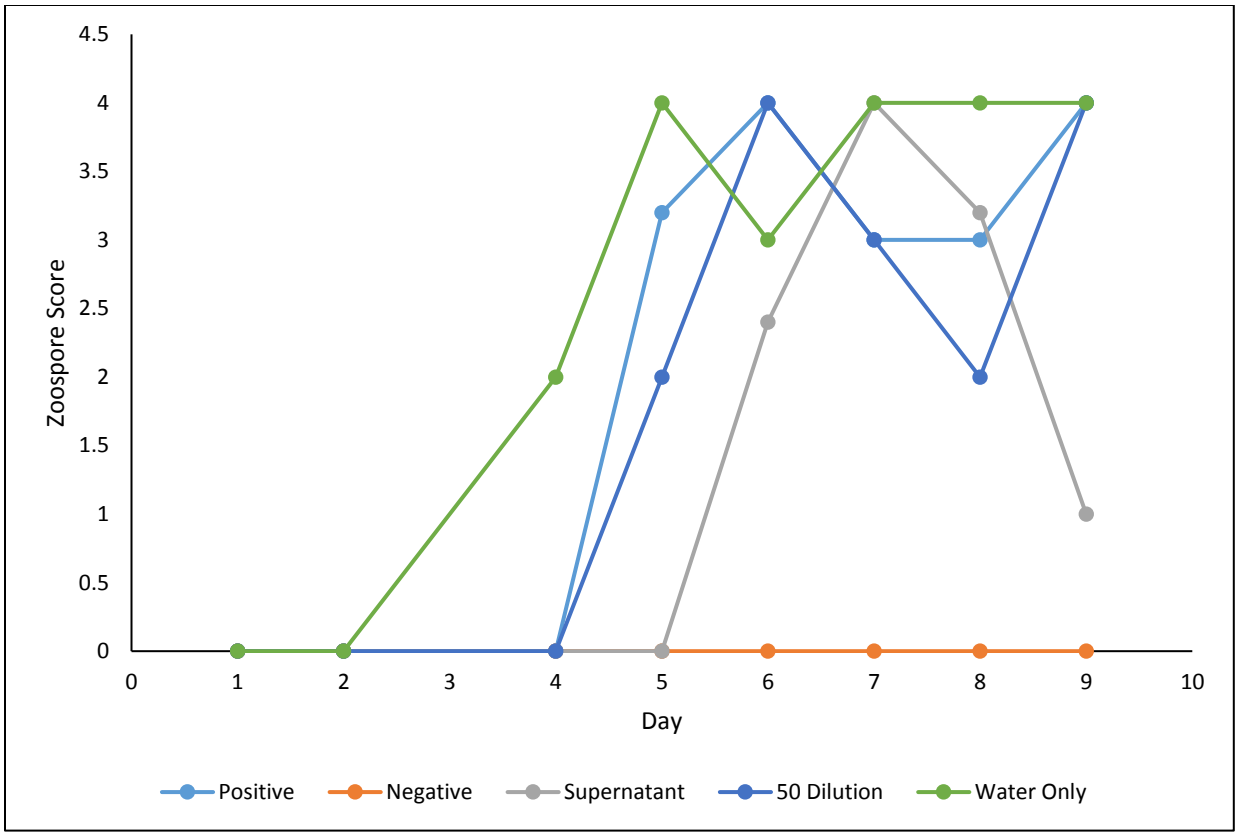

Figure S2.I. Zoospore Index results for *Bd* grown with supernatant collected from bacteria "I." Each data point represents 10 wells (N=10). "Positive" refers to *Bd* growth in media only, "Negative" refers to heat-killed *Bd* zoosporangia in media, "Supernatant" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "50 Dilution" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's stationary phase and diluted by 50%, and "Water Only" refers to *Bd* growth in distilled water. Overlapping data series may not be discernable.

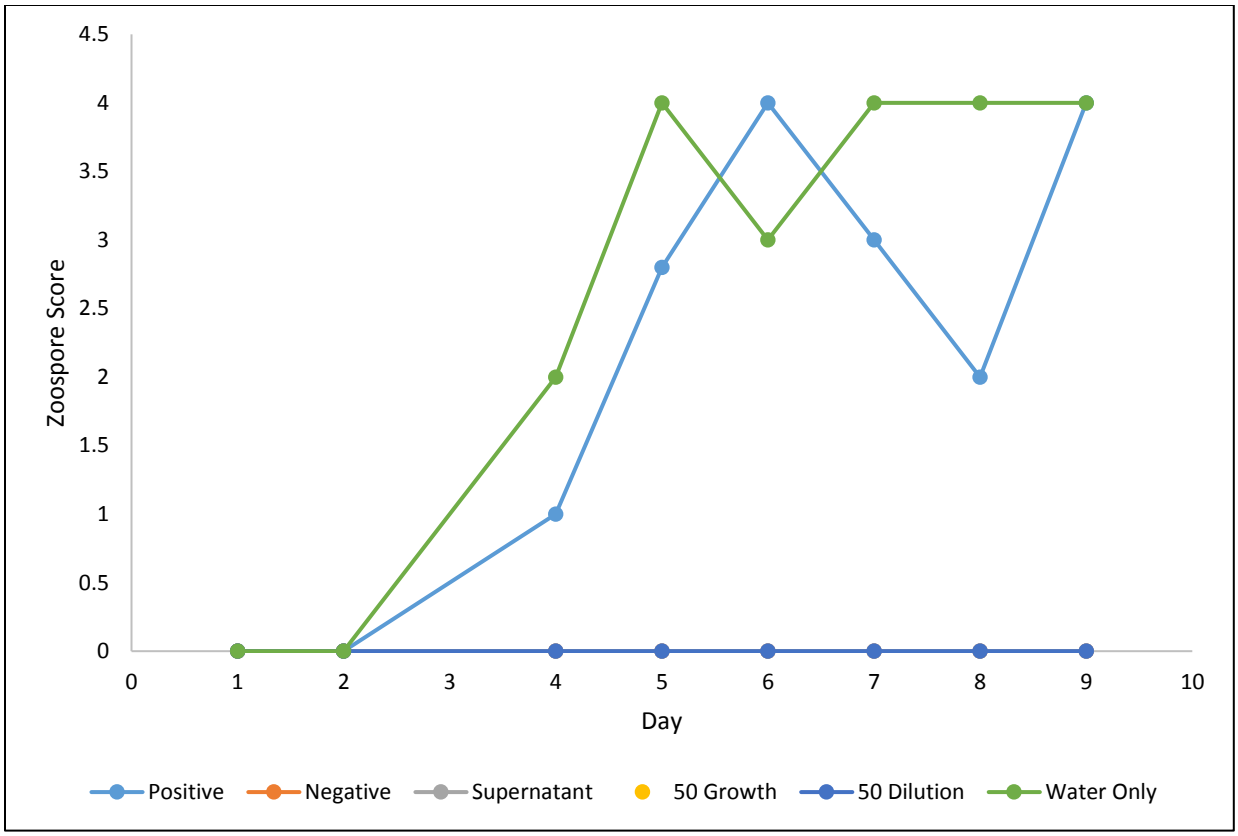

Figure S2.J. Zoospore Index results for *Bd* grown with supernatant collected from bacteria "J." Each data point represents 10 wells (N=10). "Positive" refers to *Bd* growth in media only, "Negative" refers to heat-killed *Bd* zoosporangia in media, "Supernatant" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "50 Growth" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's growth phase, "50 Dilution" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's stationary phase and diluted by 50%, and "Water Only" refers to *Bd* growth in distilled water. Overlapping data series may not be discernable.

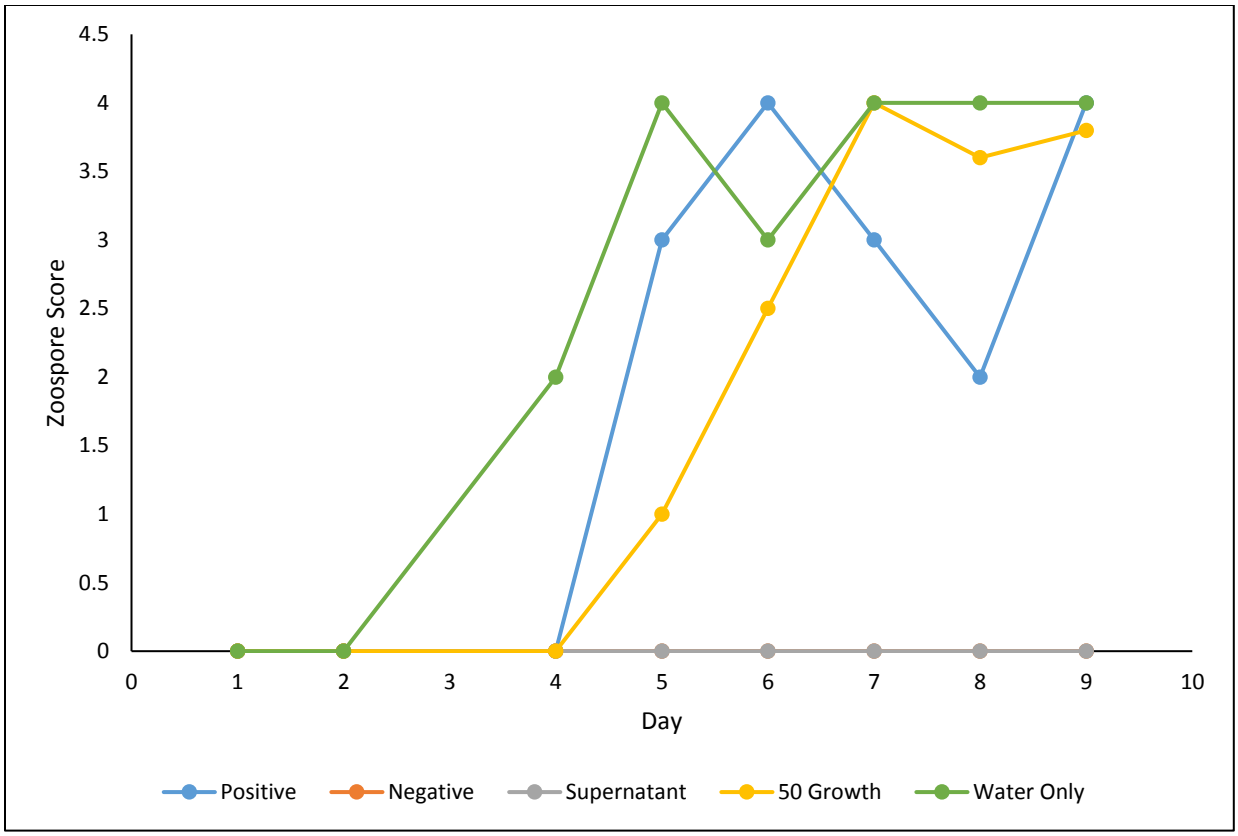

Figure S2.K. Zoospore Index results for *Bd* grown with supernatant collected from bacteria "K." Each data point represents 10 wells (N=10). "Positive" refers to *Bd* growth in media only, "Negative" refers to heat-killed *Bd* zoosporangia in media, "Supernatant" refers to *Bd* growth in media with cell-free supernatant collected during the bacteria's stationary phase, "50 Growth" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's growth phase, and "Water Only" refers to *Bd* growth in distilled water. Overlapping data series may not be discernable.

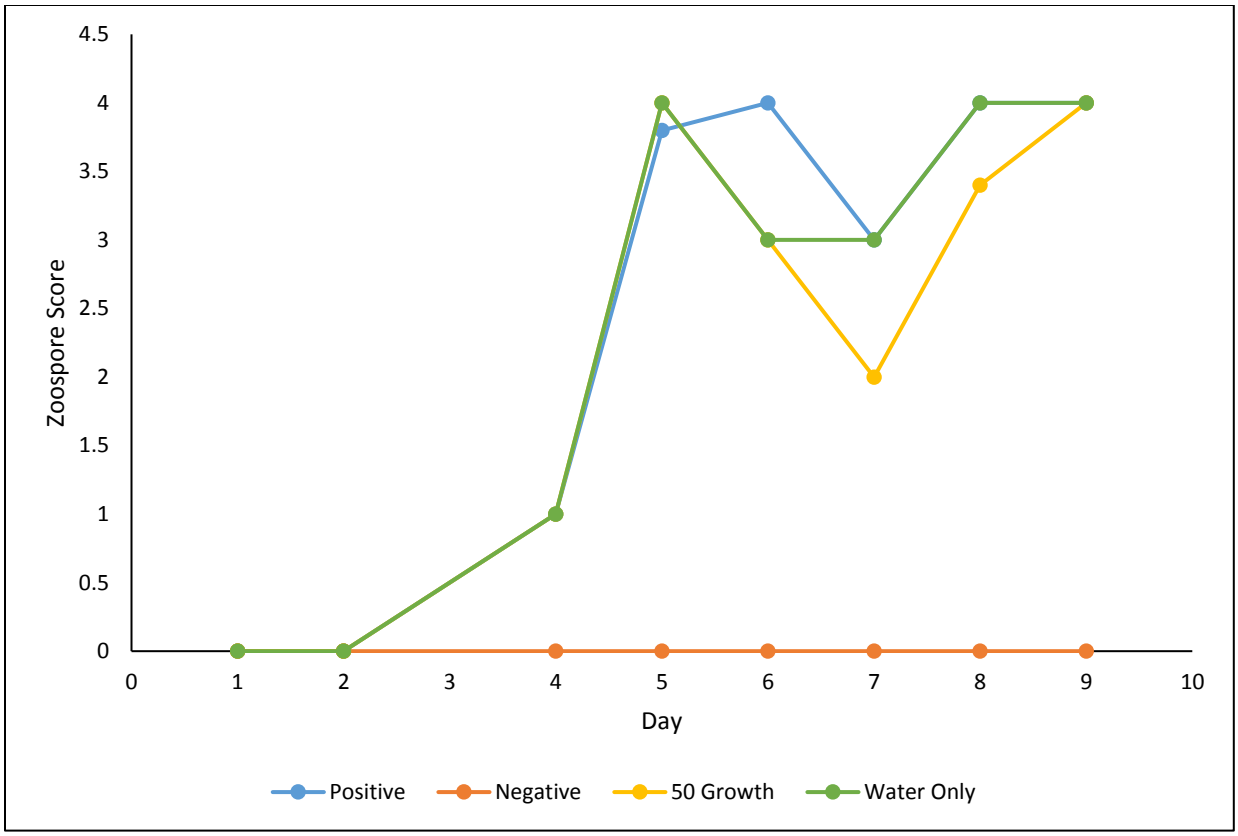

Figure S2.L. Zoospore Index results for *Bd* grown with supernatant collected from bacteria "L." Each data point represents 10 wells (N=10). "Positive" refers to *Bd* growth in media only, "Negative" refers to heat-killed *Bd* zoosporangia in media, "50 Growth" refers to *Bd* grown in media with cell-free supernatant collected during the bacteria's growth phase, and "Water Only" refers to *Bd* growth in distilled water. Overlapping data series may not be discernable.