Faculty of Science

INHIBITION OF *PSEUDOGYMNOASCUS DESTRUCTANS*, THE CAUSATIVE AGENT OF WHITE-NOSE SYNDROME, BY ENVIRONMENTAL MICROORGANISMS

2017 | ROBYN LYNNE MCARTHUR

B.Sc. Honours thesis - Biology

THOMPSON RIVERS UNIVERSITY

Inhibition of *Pseudogymnoascus destructans*, the causative agent of white-nose syndrome, by environmental microorganisms

by

Robyn Lynne McArthur

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

BACHELOR OF SCIENCE (HONS.)
in the
DEPARTMENT OF BIOLOGICAL SCIENCES
(Cellular, Molecular, Microbial Biology)

This thesis has been accepted as conforming to the required standards by:
Naowarat (Ann) Cheeptham (Ph.D.), Thesis Supervisor, Dept. Biological Sciences
Soumya Ghosh (Ph.D.), Co-supervisor, Dept. Biological Sciences
J. Norman Reed (Ph.D.), Examining Committee member, Dept. Chemistry

Dated this 2nd day of June, 2017, in Kamloops, British Columbia, Canada

ABSTRACT

White-nose syndrome, a disease that affects hibernating bats, has caused mass mortality in many bat populations since it was first detected in North America in 2006. This disease has both ecological and economic impacts, since bats are relied upon by the agricultural sector for natural pest-control. White-nose syndrome is caused by the psychrophilic fungus *Pseudogymnoascus destructans*, which is believed to be an invasive species in North America. Although biological and chemical agents able to inhibit the growth of *P. destructans* have been found, none of these have been put into widespread use, so the search for inhibitory agents continues. Environmental microorganisms are a diverse source of antibiotics and antifungals; therefore, the goal of this project was to isolate bacteria and fungi from three unique environments and screen these microorganisms for inhibitory activity towards *P. destructans*. Infected plant (rotten leaves, bark, wood) and mushroom samples collected from the area around Bush Lake and Timber Lake, British Columbia were plated on Sabouraud dextrose agar plates and incubated at 25°C to promote microbial growth. Additionally, fungi were isolated from dwarf mistletoe plants and soil samples collected at a mushroom farm in Summerland, British Columbia. Morphologically distinct bacterial and fungal colonies were isolated in pure culture and screened for inhibitory activity towards *P. destructans*. Ninety-three bacterial isolates were pre-screened using a direct contact streak assay, with 30 exhibiting inhibitory activity towards *P. destructans*. These 30 bacterial isolates plus 77 fungal isolates were then grown in V8 broth and the broth supernatant was collected and filtered before being used in a Kirby-Bauer style agar diffusion assay. Thirteen bacteria and seven fungi exhibited inhibitory activity towards *P. destructans* in the agar diffusion assay. Further characterization of the positive candidates could lead to the production of a novel biological control agent that could be used for treatment and/or prevention of WNS.

Thesis Supervisor: Associate Professor Naowarat (Ann) Cheeptham

ACKNOWLEDGEMENTS

I would like to thank Dr. Naowarat (Ann) Cheeptham for her kindness, support, and guidance while acting as my supervisor. I would also like to thank Dr. Soumya Ghosh for his feedback and enthusiasm throughout this project and Dr. Norman Reed for acting as a member of my examining committee. I am grateful to Christine Petersen, Jennifer Petersen, Jennifer Barden, Lucas Hampel, Keegan Koning, and Brian Callow (What the Fungus Mushroom Farm, Summerland, BC) for their assistance with sample collection. I would like to thank Dr. Cori Lausen for her support and Dr. JP Xu of McMaster University for his guidance and providing *Pseudogymnoascus destructans* samples. I would also like to thank the TRU Department of Biological Sciences for some of the supplies used in this project and Dr. Louis Gosselin for his role in the Honors program. I would like to acknowledge the funding provided through Dr. Cheeptham's and Lausen's USFWS grant (F16AP00028).

TABLE OF CONTENTS

ABSTRACT ..... **II**

ACKNOWLEDGEMENTS ..... **III**

LIST OF FIGURES ..... **V**

LIST OF TABLES ..... **VI**

INTRODUCTION **1**

MATERIALS AND METHODS **7**

GROWTH AND CULTURE OF *PSEUDOGYMNOASCUS DESTRUCTANS* 7

ISOLATION OF *PD* CONIDIA 8

COLLECTION AND PURIFICATION OF ENVIRONMENTAL BACTERIAL AND FUNGAL ISOLATES 8

PRE-SCREENING OF BACTERIAL ISOLATES FOR ANTI-*PD* ACTIVITY 9

PREPARATION OF MICROBIAL SUPERNATANTS FOR ANTI-*PD* BIOASSAY 9

SCREENING OF MICROBIAL SUPERNATANTS WITH AN ANTI-*PD* BIOASSAY 10

RESULTS **11**

PRE-SCREENING OF BACTERIAL ISOLATES FOR ANTI-*PD* ACTIVITY 11

SCREENING OF MICROBIAL SUPERNATANTS WITH AN ANTI-*PD* BIOASSAY 13

DISCUSSION **20**

LITERATURE CITED **26**

APPENDICES **30**

LIST OF FIGURES

FIGURE 1. THE SPREAD MAP OF REPORTED SUSPECT AND CONFIRMED CASES OF WHITE-NOSE SYNDROME BY COUNTY/DISTRICT FOR EACH BAT HIBERNATION PERIOD FROM WINTER 2006 TO MAY 9, 2017 (U.S. FISH AND WILDLIFE SERVICE, 2017). 1

FIGURE 2. A LITTLE BROWN BAT WITH FUNGAL GROWTH CHARACTERISTIC OF WHITE-NOSE SYNDROME ON THE MUZZLE AND WINGS (A) (MORIARTY, 2009) AND A SCANNING ELECTRON MICROGRAPH OF *P. DESTRUCTANS* WITH CONIDIA COLORED PURPLE (B) (SPRINGER, 2013). 2

FIGURE 3. THE FOUR TYPES OF *P. DESTRUCTANS* INHIBITION EXHIBITED BY BACTERIAL ISOLATES DURING THE PRE-SCREENING EXPERIMENTS. ANY ISOLATES CREATING A ZONE OF INHIBITION WERE SUBJECTED TO FURTHER TESTING USING THE ANTI-*PD* BIOASSAY 11

FIGURE 4. ZONES OF INHIBITION CREATED IN *P. DESTRUCTANS* FUNGAL LAWNS BY FILTERED BROTH SUPERNATANT OF THREE FUNGAL ISOLATES COLLECTED SEVEN DAYS AFTER BROTH INOCULATION. THE PHOTOGRAPHS WERE TAKEN ON DAY 12 (#123) AND DAY 10 (#156 AND #158) AFTER THE PLATE WAS INOCULATED. .. 14

FIGURE 5. ANTI-*PD* BIOASSAY PLATE WITH A *P. DESTRUCTANS* FUNGAL LAWN AND INHIBITORY ZONES CREATED BY FILTERED BACTERIAL SUPERNATANT COLLECTED SEVEN DAYS AFTER BROTH INOCULATION. THE PHOTOGRAPH WAS TAKEN 10 DAYS AFTER THE PLATE WAS INOCULATED. BACTERIAL ISOLATES CREATING ZONES OF INHIBITION ARE LABELED WITH THEIR ASSIGNED SCREENING NUMBER. CONTROLS ARE LABELED WITH A LETTER TO INDICATE THEIR IDENTITY. B=10% BLEACH, P=1.5% PEROXIGARD, W=STERILE WATER, V8=STERILE V8 BROTH. 15

FIGURE 6. ANTI-*PD* BIOASSAY PLATE WITH A *P. DESTRUCTANS* FUNGAL LAWN AND INHIBITORY ZONES CREATED BY FILTERED BACTERIAL SUPERNATANT COLLECTED 14 DAYS AFTER BROTH INOCULATION. THE PHOTOGRAPH WAS TAKEN 10 DAYS AFTER THE PLATE WAS INOCULATED. BACTERIAL ISOLATES CREATING ZONES OF INHIBITION ARE LABELED WITH THEIR ASSIGNED SCREENING NUMBER. CONTROLS ARE LABELED WITH A LETTER TO INDICATE THEIR IDENTITY. B=10% BLEACH, P=1.5% PEROXIGARD, W=STERILE WATER, V8=STERILE V8 BROTH. 17

FIGURE 7. ZONES OF INHIBITION CREATED IN *P. DESTRUCTANS* FUNGAL LAWNS BY FILTERED BROTH SUPERNATANT OF SEVEN FUNGAL ISOLATES COLLECTED 14 DAYS AFTER BROTH INOCULATION. THE PHOTOGRAPHS WERE TAKEN 10 DAYS AFTER THE PLATE WAS INOCULATED. 18

FIGURE 8. COMPARISON OF AVERAGE INHIBITORY ZONE DIAMETERS (MM) CREATED IN *P. DESTRUCTANS* FUNGAL LAWNS BY BACTERIAL AND FUNGAL FILTERED BROTH SUPERNATANT COLLECTED SEVEN (GREY) AND 14 (BLUE) DAYS AFTER BROTH INOCULATION. ERROR BARS REPRESENT STANDARD ERROR (N=3). 20

LIST OF TABLES

TABLE 1. INHIBITION TYPE FOR BACTERIAL ISOLATES AND CONTROLS (WATER, BLEACH, PEROXIGARD) CREATING INHIBITORY ZONES IN *P. DESTRUCTANS* FUNGAL LAWNS ON DAY 10 AND DAY 15 AFTER INOCULATION..... 12

TABLE 2. THE NUMBER OF ISOLATES CREATING EACH TYPE (COMPLETE, SUBSURFACE, SURFACE, OR PARTIAL SURFACE) OF INHIBITORY ZONE IN *P. DESTRUCTANS* FUNGAL LAWNS ON DAY 10 AND DAY 15 AFTER INOCULATION. 13

TABLE 3. AVERAGE DIAMETER (MM), TYPE, AND LENGTH OF PRESENCE FOR INHIBITORY ZONES CREATED IN *P. DESTRUCTANS* FUNGAL LAWNS BY BACTERIAL OR FUNGAL FILTERED BROTH SUPERNATANT COLLECTED SEVEN DAYS AFTER BROTH INOCULATION. INHIBITORY ZONES WERE MEASURED 10 DAYS AFTER THE PLATE WAS INOCULATED (EXCEPT ISOLATE 123 AND THE CORRESPONDING CONTROLS THAT WERE MEASURED ON DAY 17) AND MONITORED UNTIL DAY 21. THE HORIZONTAL LINES IN THE TABLE SEPARATE THE RESULTS FROM DIFFERENT SCREENING PLATES. 16

TABLE 4. AVERAGE DIAMETER (MM), TYPE, AND LENGTH OF PRESENCE FOR INHIBITORY ZONES CREATED IN *P. DESTRUCTANS* FUNGAL LAWNS BY BACTERIAL OR FUNGAL FILTERED BROTH SUPERNATANT COLLECTED 14 DAYS AFTER BROTH INOCULATION. INHIBITORY ZONES WERE MEASURED 10 DAYS AFTER THE PLATE WAS INOCULATED AND MONITORED UNTIL DAY 21. THE HORIZONTAL LINES IN THE TABLE SEPARATE THE RESULTS FROM DIFFERENT SCREENING PLATES. 18

INTRODUCTION

White-nose syndrome (WNS) is a disease that has led to the mass mortality of hibernating insectivorous bats in North America since it was first documented in winter 2006/2007 (U.S. Fish and Wildlife Service, 2017). In the 10 years since it was first detected in New York, WNS has spread across Eastern North America into 31 U.S. states and five Canadian provinces (New Brunswick, Nova Scotia, Ontario, Prince Edward Island, Quebec) (Figure 1). Additionally, on March 11, 2016 a bat found in Washington State was submitted to the U.S. Geological Survey (USGS) National Wildlife Health Centre for testing and was confirmed to have WNS (Haman et al., 2016). This is the westernmost case to date, with a distance of over 2000 km between Washington and the previous westernmost case.

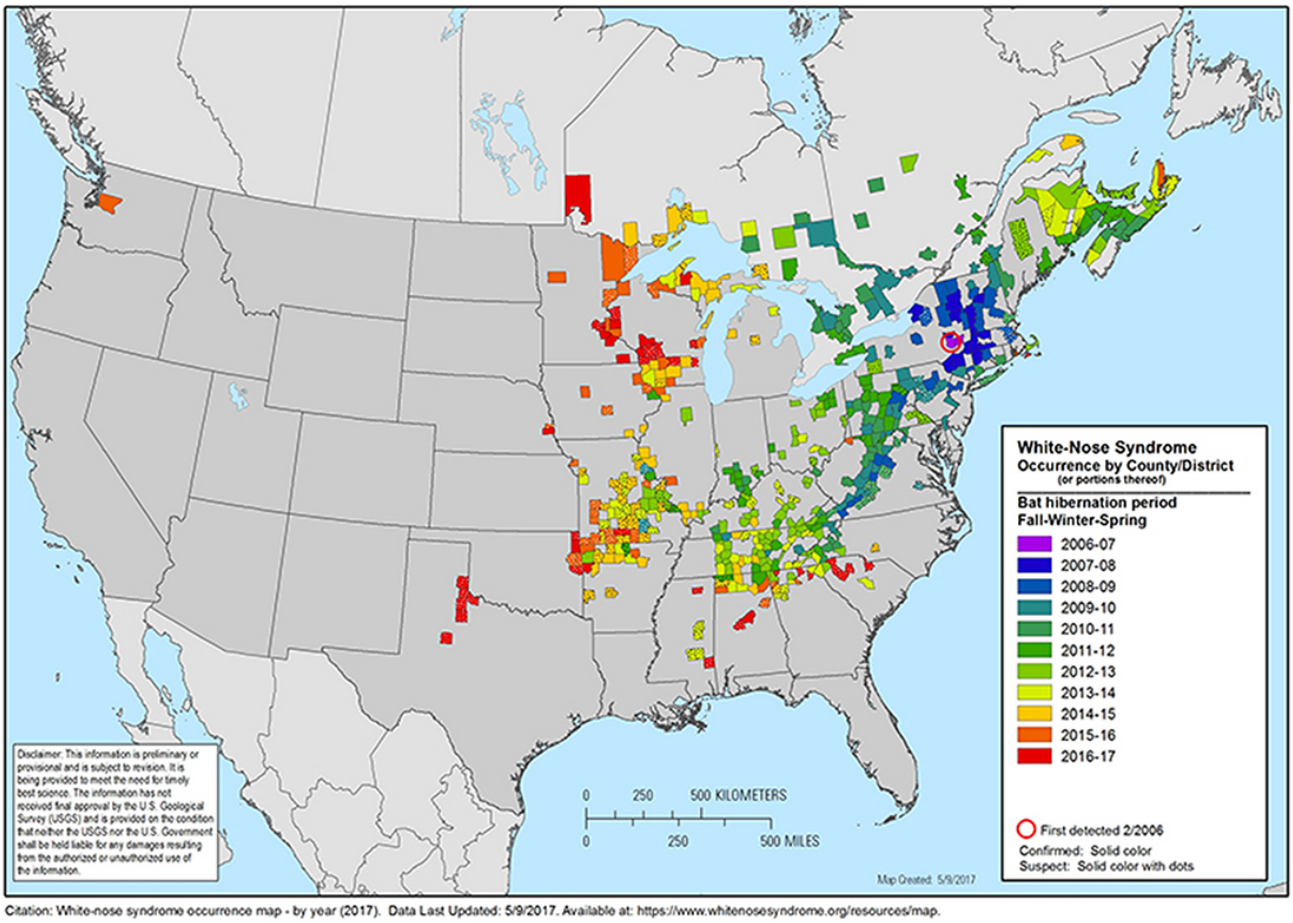

Figure 1. The spread map of reported suspect and confirmed cases of white-nose syndrome by county/district for each bat hibernation period from winter 2006 to May 9, 2017 (U.S. Fish and Wildlife Service, 2017).

Although WNS is primarily transmitted from bat to bat during hibernation, the causative agent may also be transmitted by humans on clothing or gear, which may explain the sudden appearance of WNS in Washington State (U.S. Fish and Wildlife Service, 2017). In North America, eight bat species (including two endangered species, and one threatened species) have been confirmed with diagnostic symptoms of WNS and the causative agent of WNS has been found on an additional seven bat species (including one endangered species).

One of the symptoms of WNS is fungal growth on the wings, ears, and muzzles of the bats (Gargas et al., 2009) (Figure 2). The causative fungus was identified by Gargas *et al.* (2009) based on small subunit (SSU) and internal transcribed spacer (ITS) rRNA gene sequencing and named *Geomyces destructans. G. destructans* was further confirmed as the cause of WNS by Lorch *et al.* (2011), who demonstrated that healthy little brown bats (*Myotis lucifigus*) would develop WNS if exposed to pure cultures of *G. destructans* or if they had direct contact with infected bats. However, Minnis and Lindner (2013) conducted a phylogenetic analysis of *Geomyces* and relatives based on DNA sequence data from five genes/regions and concluded that *Geomyces* and *Pseudogymnoascus* are genetically distinct, and *G. destructans* was renamed *Pseudogymnoascus destructans* (*Pd*) based on its placement in their phylogenetic tree.

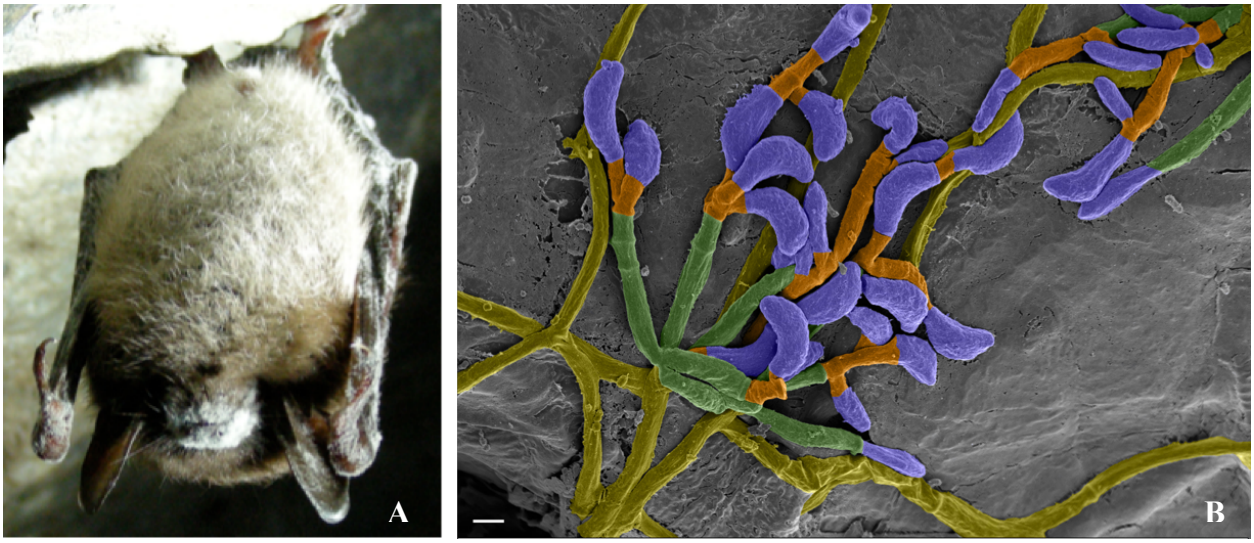

Figure 2. A little brown bat with fungal growth characteristic of white-nose syndrome on the muzzle and wings (A) (Moriarty, 2009) and a scanning electron micrograph of *P. destructans* with conidia colored purple (B) (Springer, 2013).

Microscopically, *Pd* has septate hyphae with unique curved conidia produced on branched conidiophores and hyphae (Chaturvedi et al., 2010; Verant et al., 2012) (Figure 2). *Pd* is classified as a psychrophilic fungus since its optimum temperature for growth is 12.5°C-15.8°C, with no growth observed at or below -10°C or above 19.0°C-19.8°C, depending on the strain. Although *Pd* grows very slowly on artificial media, the ability to do so indicates that *Pd* may be a facultative, rather than obligate, pathogen (Gargas et al., 2009; Reynolds and Barton, 2014).

Pd infection involves penetration and colonization of the sub-cutaneous tissue of the bat muzzle, wings, ears, and/or tail by fungal hyphae, which is promoted by the secretion of various enzymes (O'Donoghue et al., 2015). Although the *Pd* secretome includes peptidases, lipases, glycosidases, and redox enzymes, one of the most important proteins for virulence is Destructin-1, a serine endopeptidase with the ability to degrade collagen. The ability of Destructin-1 to cleave cross-links in collagen, an important component of mammalian connective tissue, is believed to cause structural instability and promote deep tissue penetration in the bat host. Another virulence factor that plays a key role in WNS is the secretion of riboflavin (vitamin B_2) by *Pd*, which accumulates in bat skin lesions due to decreased metabolism and perfusion during hibernation (Flieger et al., 2016). Riboflavin is cytotoxic at high concentrations since it can lead to the production of free radicals and cause oxidative stress, apoptosis, and necrosis during arousal from hibernation. This worsens skin lesions, especially in the bat wings, which are required for thermoregulation, water regulation, gas exchange, and immune function (Flieger et al., 2016; Warnecke et al., 2013). The disruption of the wing tissue therefore leads to increased levels of carbon dioxide in the blood, which stimulates arousal from torpor to correct the CO_2 levels and blood pH (Verant et al., 2014).

Although cycles of torpor and arousal are normal during bat hibernation, increased arousal duration and/or frequency caused by WNS can lead to mortality due to a lack of food available during hibernation coupled with the high energy demand and fat depletion associated with arousal (Reeder et al., 2012; Thomas et al., 1990). For the majority of the hibernation period, bats are in torpor, meaning that body temperature will drop to near ambient (2°C-8°C), metabolic rate will be highly reduced (may be <5% of basal metabolic rate), and the innate and adaptive

immune systems will be less active (Flieger et al., 2016; Reeder et al., 2012). When in torpor, bats are able to conserve their energy stored as body fat and survive through the winter without eating, since insects are scarce (Reeder et al., 2012). Periodically during hibernation bats will briefly arouse from torpor, body temperature will increase to approximately 37°C, metabolic rate will increase, and bats may become active (Thomas et al., 1990). Although arousal periods may allow bats to re-activate their immune systems, arousal is responsible for 80-90% of energy consumption during hibernation (Reeder et al., 2012). Research indicates that WNS both increases metabolic rate during hibernation, as well as decreases the length of torpor bouts by approximately half due to increased arousal frequency (Reeder et al., 2012; Verant et al., 2014). This, in addition to wing damage, leads to increased energy usage, water loss, dehydration, electrolyte depletion, starvation due to depletion of fat reserves, and ultimately, death (Verant et al., 2014; Warnecke et al., 2013).

Interestingly, although *Pd* has been found on hibernating bats throughout Europe, no mortality due to WNS has been recorded (Puechmaille et al., 2011). The lack of WNS-associated mortality in Europe along with an absence of closely related *Pseudogymnoascus* species in North America suggests that *Pd* may have originated in Europe, where co-evolution with European bats may have occurred before it became an invasive species in North America (Minnis and Lindner, 2013; Puechmaille et al., 2011). The high degree of sequence polymorphism in European *Pd* isolates compared to the lack of variation detected in North American *Pd* isolates, as well as the sharing of a common *Pd* haplotype between North American and some European *Pd* populations provides further support for a European origin for WNS and introduction of *Pd* into North America (Leopardi et al., 2015). Due to the distance between Europe and North America, it is most likely that this inter-continental *Pd* movement was a result of anthropogenic activity. Recent research suggests that *Pd* is also present beyond Europe, into the West Siberian Plain of Russia, and although *Pd* is highly prevalent in Europe and Russia, bats have developed tolerance to the pathogen in these areas (Zukal et al., 2016).

In North America, there is evidence that some bat populations are developing resistance or tolerance to *Pd* that allows them to persist with the fungus; however, some species are still at risk of extinction due to the large environmental reservoirs of *Pd* that exist in some hibernacula

(Frick et al., 2017). Hoyt *et al.* (2015a) demonstrated that *Pd* conidia can remain viable for over five years under laboratory conditions, suggesting that conidia shed into the environment by infected bats may stay viable in hibernacula for long periods of time and prevent recolonization of these sites. Additionally, Reynolds and Barton (2014) found that *Pd* is capable of secreting enzymes used for saprotrophic growth, indicating that it may be possible for *Pd* to grow in hibernacula outside of a bat host.

Although bats in North America may be slowly developing resistance or tolerance to *Pd*, bats have a very low reproductive rate so populations will take a long time to recover from the WNS-associated mass mortality that has occurred over the last decade (Bat Conservation International, 2017). This may have lasting impacts, since bats play a crucial role in ecosystems and act as a form of natural pest control. Insectivorous bats in North America consume a large number of forest and crop pests, which reduces crop/forest losses, pesticide application, and the likelihood of insects becoming pesticide resistant (Boyles et al., 2011). This is estimated to save the agricultural sector an estimated 3.7 billon dollars per year; however, this number could be as high as 53 billion dollars per year. Bats also add to the biodiversity of ecosystems, help maintain ecosystem stability, consume insects that are human/animal pathogens, and redistribute nutrients through their guano (Kunz et al., 2011). Bat guano acts as a natural fertilizer rich in nitrogen and phosphorous and is the primary source of organic matter that supports some cave ecosystems.

Since North American bats are highly susceptible to WNS and they play such a crucial role in the ecosystem, a portion of WNS research over the past decade has focused on finding fungicides or fungistatic agents that can be used for treatment or prevention of WNS. Despite the ability of chemical agents such as bleach, Lysol, antifungals, fungicides, and some biocides to kill or inhibit the growth of *Pd*, these agents would likely harm the cave ecosystem or the bats, so have little application in hibernacula (Chaturvedi et al., 2011; Shelley et al., 2013). Thus, most studies of this nature have focused on potentially non-toxic natural products, such as cold-pressed terpeneless orange oil, 1-octen-3-ol (mushroom oil), and 1-hexanol, all of which are able to inhibit *Pd* growth and have been proposed as treatments for infected hibernacula (Boire et al., 2016; Padhi et al., 2017).

There have also been a number of studies testing bacteria against *Pd* for the purpose of finding a biological control agent against WNS. For example, 6 bacterial isolates from bat skin swabs belonging to the genus *Pseudomonas* and 36 Actinobacteria (mostly *Streptomyces* spp.) isolated from WNS-free bats inhibited the growth of *Pd in vitro* and therefore have biological control agent potential (Hamm et al., 2017; Hoyt et al., 2015b). However, the most promising biological control agent to date is *Rhodococcus rhodochrous* strain DAP96253, a Gram-positive bacterium that produces volatile organic compounds with fungistatic activity towards *Pd* (Cornelison et al., 2014). This treatment is advantageous because of its contact-independent nature and was able to successfully treat 75 bats suffering from WNS (Gill, 2016).

Overall, less research has been conducted to investigate the use of fungal agents against *Pd*; however, one fungal species and one compound derived from *Candida albicans* have also shown biological control potential. Zhang *et al.* (2015) found that strain WPM 39143 of *Trichoderma polysporum*, collected in a cave air sample, exhibited fungicidal activity specific to *Pd.* Trans, trans-farnesol, a quorum-sensing compound produced by *C. albicans*, was also able to inhibit the germination of *Pd* conidia and inhibit hyphal growth under laboratory conditions (Raudabaugh and Miller, 2015).

Due to the limited amount of research in this area and the need for a reliable biological control agent against *Pd*, we chose to screen bacteria and fungi isolated from the area around Bush Lake and Timber Lake, B.C., as well as fungi isolated from dwarf mistletoe plants and a mushroom farm in Summerland, B.C. for inhibitory activity towards *Pd*. The choice to screen bacteria and fungi from these locations was based on the success of previous research that involved screening microorganisms for anti-*Pd* activity, as demonstrated above, as well as the knowledge that environmental microorganisms have long been a source of novel antibiotics and antifungal agents (Wohlleben et al., 2016). For example, Magnusson *et al.* screened over 1200 environmental isolates of lactic acid bacteria for antifungal activity and found that 10% of the isolates had inhibitory activity towards *Aspergillus fumigatus* and some also had activity towards other molds (Magnusson et al., 2003). There are also numerous examples of fungal biocontrol agents active against fungal plant pathogens, nematodes, weeds, and insects (Perotto et al., 2013). In general, fungi may be used for biocontrol because they can compete with pathogenic

fungi for nutrients and space or produce inhibitory substances. For example, the use of fungi to control *Fusarium* head blight, a fungal disease of wheat crops, and die-back syndrome of the cosmopolitan common reed caused by the fungal pathogen *Gibberella fujikuroi* have been investigated with promising results.

In order to obtain pure bacterial and fungal cultures that could be used in the screening assays, morphologically distinct colonies in mixed microbial cultures grown from plant and mushroom samples (Bush Lake and Timber Lake, B.C), dwarf mistletoe plants, and mushroom compost samples (What the Fungus Mushroom Farm, Summerland, B.C.) were streaked for pure culture. *Pd* was grown until mature and a conidia isolation procedure was carried out to obtain conidia for inoculating the screening plates. Bacteria were pre-screened for inhibitory activity towards *Pd* using a direct contact streak assay and isolates producing zones of inhibition were subjected to further testing using a Kirby-Bauer agar diffusion assay, along with the fungal isolates. To the extent of our knowledge, this is the first study to screen bacteria and fungi from the abovementioned environments for inhibitory activity towards *Pd*, as well as only the second study to test fungi against *Pd*. The long-term goal of this study is the production of a novel biological control agent for the treatment or prevention of WNS.

MATERIALS AND METHODS

Growth and culture of *Pseudogymnoascus destructans*

Stock plates of *Pseudogymnoascus destructans* were prepared by plating small agar plugs taken from a previously-grown stock culture of *Pseudogymnoascus destructans* M3906-2 on Sabouraud Dextrose Agar (SDA) (Product # M063, HiMedia, India) containing 34 µg/mL of chloramphenicol (Product # BP904, Fisher Scientific, Fairlawn, NJ) to inhibit bacterial growth. Plates were parafilmed, inverted, and placed inside of a plastic container in a 15°C incubator for approximately two months. These stock plates were used to inoculate SDA plates and YM broth (Product # 271120, Difco, Sparks, MD) for the creation of additional *Pd* cultures. All *Pd* cultures were grown at 15°C and YM broth cultures were grown shaking at 110 rpm. YM broth cultures of *Pd* were spread on SDA plates (34 µg/mL chloramphenicol) and allowed to grow for approximately 11 days at 15°C to produce conidia.

Isolation of *Pd* conidia

Pd conidia were harvested from cultures of *Pd* that were approximately 11 days old (see above) using the protocol described by Cornelison *et al.* (2014), with minor adjustments suggested by Hoyt *et al.* (2015b). The *Pd* colonies on each plate were submerged in 10 mL of Conidia Harvesting Solution (CHS) (0.05% Tween 80, 0.9% NaCl) for five minutes before the colonies were vigorously scraped with a sterile loop to dislodge conidia. The CHS from each plate was filtered through sterile glass wool and centrifuged at 4000 rcf for 10 minutes. The supernatant was removed and conidia were washed in phosphate buffered saline (PBS, pH 7), resuspended in PBS, and filtered through sterile glass wool. A 10 µl sample of the final conidia suspension was removed and pipetted into a hemocytometer to count the conidia. The approximate concentration of conidia in the suspension was determined using the following equation: Average # of conidia in 1 square x dilution factor x 10^4 = # of conidia/mL. Conidia suspensions were stored in PBS at 4°C until needed.

Collection and purification of environmental bacterial and fungal isolates

Mushrooms from the phylloplane, tree bark, and infected plant wood and leaves were collected from the area around Bush Lake near Lac le Jeune Road (Lat 50.531823; Lon 120.452856) and Timber Lake Road (Lat 50.572227; Lon 120.450068), B.C. These samples were laid down on Sabouraud Dextrose Agar (SDA) plates, which were incubated at 25°C until bacterial and fungal growth was observed. Morphologically distinct bacterial and fungal colonies were streaked for pure culture on SDA (34 µg/mL chloramphenicol) and plates were placed at 25°C until growth was observed (two to six days, depending on the organism). Plates were restreaked again if necessary until pure cultures were obtained. Pure cultures were parafilmed and stored at 4°C until testing.

Fungi subcultured from dwarf mistletoe plants collected in the Kamloops area and isolated in pure culture were obtained from Lucas Hampel (Ross-Friedman Lab, Thompson Rivers University) and stored at 4°C.

Three mushroom compost samples were collected at What the Fungus Mushroom farm in Summerland, B.C. One sample was taken from the exterior of the compost pile, while the other

two samples were taken from the interior of the pile. Samples were collected with a trowel, placed in plastic zip-top bags, and transported in a Styrofoam container kept at approximately 4°C to the Thompson Rivers University microbiology laboratory, where they were stored at 4°C. Ten grams of mushroom compost was added to 250 mL Erlenmeyer flasks containing 50 mL of isolation solution (2% Tween20, 0.9% NaCl). The flasks were placed shaking at 120 rpm at 37°C for three hours. The flasks were then taken off of the shaker and left undisturbed for 10 minutes before 1000 µl of supernatant was collected. The supernatant was serially diluted and plated on SDA. Plates were incubated at 25°C until fungal growth was observed. Morphologically distinct fungal colonies were streaked for pure culture on SDA (34 µg/mL chloramphenicol) and plates were placed at 25°C until growth was observed and stored at 4°C.

Pre-screening of bacterial isolates for anti-*Pd* activity

SDA medium was prepared according to the manufacturer's instructions and 85 µl of 100 mg/mL chloramphenicol dissolved in ethanol (Product # 676829, Sigma Aldrich, USA) and 1 mL of conidia suspension at approximately 1 x 10^6 conidia/mL were added per 250 mL of SDA after autoclaving for final concentrations of 34 µg /mL and 4000 conidia/mL, respectively. The medium was mixed and poured into large bioassay plates (Nunc®, 245 mm x 245 mm x 25 mm) or individual petri plates and allowed to cool in the laminar flow hood. For individual petri plates, bacterial isolates were streaked in the center of each plate using a sterile pipet tip. The large bioassay plates were divided into 36 squares and approximately 31 bacterial isolates were streaked on each plate using the previously mentioned technique. Sterile water was used as a negative control and 10% bleach (London Drugs, prod by RW packaging Ltd, Win, MB), and 1.5% peroxigard (Bayer, Toronto, Canada) were used as positive controls. 40 µl of each of these substances was pipetted onto sterile 8 mm paper discs (Toyo Roshi Kaisha Ltd., Japan), which were air dried and placed on plates with sterile forceps. All plates were then parafilmed and inverted at 15°C. Plates were observed from day 7 to day 26, with photographs being taken when confluent *Pd* growth was observed on the surface of the plate.

Preparation of microbial supernatants for anti-*Pd* bioassay

Modified V8 broth medium (20% low sodium V8 vegetable cocktail supernatant, 1% Pharmamedia, 0.5% calcium carbonate, pH 6) was prepared, separated into 5 mL aliquots in glass test tubes, and autoclaved at 121°C for 15 minutes. A sterile loop was used to pick a

bacterial colony or scrape fungal hyphae from each pure culture and inoculate V8 broth. Tubes were incubated shaking at 110 rpm at 15°C for 14 days. On both day 7 and day 14 after inoculation, 1 mL of broth was removed from each test tube and stored in a sterile microfuge tube. Broth containing bacteria was centrifuged at 4°C at 14000 rpm for 20 minutes, and broth containing fungi was centrifuged at 4°C at 14000 rpm for five minutes. Supernatants were then filtered through VWR sterile syringe filters (0.2 µm cellulose acetate membrane, NA catalog #28145-477) to ensure removal of bacteria and fungi. Filtered supernatants were used immediately or stored at -20°C overnight. Once the filtered supernatants had been tested, they were stored at -20°C.

Screening of microbial supernatants with an anti-*Pd* bioassay

SDA medium was prepared as per the manufacturer's instructions and autoclaved for 15 minutes at 121°C. After the medium had cooled to approximately 50°C, 85 µl of 100 mg/mL chloramphenicol dissolved in ethanol and 50-100 µl of conidia suspension at approximately 4.9 x 10^7 conidia/mL were added per 250 mL of SDA. The concentration of chloramphenicol and conidia in the medium were 34 µg /mL and 9800-19600 conidia/mL, respectively. The medium was then gently mixed, poured into bioassay plates (Nunc®, 245 mm x 245 mm x 25 mm), and allowed to solidify in a laminar flow hood. Before the medium was added, grids were drawn on the bottom surface of each plate to allow 30 bacterial supernatants plus controls (6 x 6 grid) or 26 fungal supernatants plus controls (6 x 5 grid) to be tested at one time. 50 µl of filtered supernatant or control substance was pipetted onto each 8 mm paper disc (Toyo Roshi Kaisha Ltd., Japan) and these were allowed to dry before being placed on the bioassay plates with sterile forceps. Sterile water and V8 broth were used as negative controls, and 10% bleach and 1.5% peroxigard were used as positive controls. Plates were then parafilmed and placed upright at 15°C. After one to two days, plates were inverted. Plates were observed from day 7 to day 21, with photographs being taken when confluent *Pd* growth was observed on the surface of the plate. Isolates creating zones of inhibition were recorded and the zones diameters were measured in three directions with an electronic Vernier caliper (Guangxi China, Mainland) 10 days after plates were inoculated with *Pd*. The average diameters of inhibitory zones were calculated from the three zone diameter measurements taken for each zone using the Average function in Excel. The standard deviation of the measurements was calculated using the STDEV.S function in

Excel for each inhibitory zone. Average zone diameters were visualized as a bar chart in Excel, with error bars representing the standard deviation calculated for each zone.

RESULTS

Pre-screening of bacterial isolates for anti-*Pd* activity

A direct contact streak assay was used to pre-screen bacterial isolates for anti-*Pd* activity in order to increase the efficiency of screening with the subsequent anti-*Pd* bioassay. *Pd* growth was visible in the agar seven days after the plate was inoculated, and some inhibitory zones were also starting to become visible at this time. By day 10, confluent *Pd* lawn growth was observed on the agar surface and zones of inhibition were clearly visible. Fifteen days after inoculation, some zones of inhibition were no longer visible or had become reduced in size or intensity. Four types of *Pd* inhibition were observed on the screening plates: complete, subsurface, surface, and partial surface inhibition (Figure 3). Inhibition was considered to be complete when no conidial germination leading to *Pd* colonies was observed within or on the agar surface in the area surrounding the bacterial streak. Subsurface inhibition was considered to be a lack of *Pd* germination in the agar, with some *Pd* colony growth observed on the surface of the plate. Surface inhibition was considered to be a lack of *Pd* colony growth on the agar surface surrounding the bacterial streak with *Pd* germination and/or growth observed within the agar. Partial surface inhibition was considered to be a weaker form of surface inhibition, with more *Pd* growth observed in the agar or some minimal *Pd* growth observed on the agar surface.

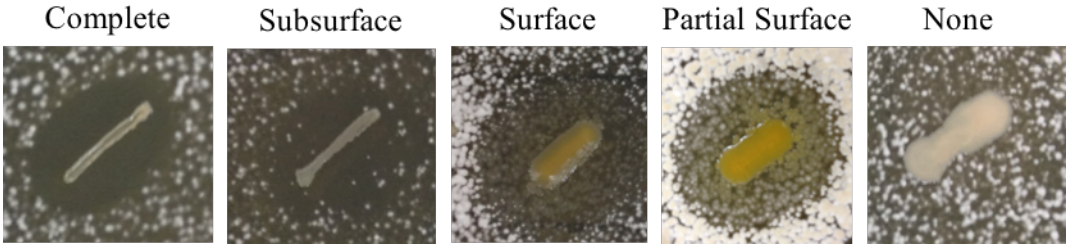

Figure 3. The four types of *P. destructans* inhibition exhibited by bacterial isolates during the pre-screening experiments. Any isolates creating a zone of inhibition were subjected to further testing using the anti-*Pd* bioassay.

The type of *Pd* inhibition observed ten and fifteen days after plate inoculation was recorded for each bacterial isolate creating a zone of inhibition, as well as the negative (water) and positive (10% bleach and 1.5% peroxigard) controls (Table 1, summarized in Table 2). Water did not create a zone of *Pd* inhibition. Bleach created a zone of surface inhibition, which decreased in size by day 15. Peroxigard created a zone of complete inhibition. One bacterial isolate (#47) created a zone of complete inhibition that was visible on both day 10 and day 15 after inoculation. Zones of subsurface inhibition that persisted for 15 days were created in *Pd* lawns by isolate numbers 14, 37, 38, 51, and 53. All other inhibitory zones created by bacterial isolates were zones of surface inhibition, with some decreasing in size, becoming zones of partial surface inhibition, or disappearing altogether by day 15 after inoculation.

Table 1. Inhibition type for bacterial isolates and controls (water, bleach, peroxigard) creating inhibitory zones in *P. destructans* fungal lawns on day 10 and day 15 after inoculation.

	Inhibition type	
Assigned screening number	**Day 10**	**Day 15**
3	Surface	Surface
10	Surface	None
12	Surface	Partial surface
13	Surface	Partial surface
14	Subsurface	Subsurface
19	Surface	None
20	Surface	Partial surface
21	Surface	Partial surface
32	Surface	Partial surface
34	Surface	Surface
35	Surface	Surface
36	Surface	Surface
37	Subsurface	Subsurface
38	Subsurface	Subsurface
41	Surface	Surface
47	Complete	Complete
51	Subsurface	Subsurface
53	Subsurface	Subsurface
54	Surface	Surface
55	Surface	Surface
56	Surface	Surface

70	Surface	Partial surface
71	Surface	Partial surface
77	Surface	Partial surface
80	Surface	Surface
81	Surface	Surface
84	Surface	Partial surface
86	Surface	None
89	Surface	None
90	Surface	Partial surface
Water	None	None
Bleach	Surface	Surface
Peroxigard	Complete	Complete

Table 2. The number of isolates creating each type (complete, subsurface, surface, or partial surface) of inhibitory zone in *P. destructans* fungal lawns on day 10 and day 15 after inoculation.

	Number of isolates creating inhibitory zone in *P. destructans* fungal lawn	
Type of inhibition	**Day 10**	**Day 15**
Complete	1	1
Subsurface	5	5
Surface	24	10
Partial surface	0	10

Screening of microbial supernatants with an anti-*Pd* bioassay

An anti-*Pd* bioassay was used to determine if inhibitory activity would persist in the absence of bacterial cells and to screen fungal isolates for inhibitory activity towards *Pd.* Bacteria exhibiting anti-*Pd* activity in the pre-screening experiment, as well as all of the fungal isolates, were grown in V8 broth cultures for a total of 14 days. On both day 7 and day 14 after the broth was inoculated with the microbial isolates, a sample was aliquoted into a microfuge tube, centrifuged, and the broth supernatant was filtered and used for the anti-*Pd* bioassay. The plates were photographed and the inhibitory zone diameters were measured on day 10 after plate inoculation.

The positive controls (10% bleach and 1.5% peroxigard) created zones of inhibition in the *Pd* lawn. The negative controls (water and sterile V8 broth) did not create inhibitory zones. Fungal isolate numbers 123, 156, and 158 created zones of inhibition in the *Pd* lawn when supernatant collected seven days after broth inoculation was used (Figure 4). Isolate number 123 created a small zone of partial surface inhibition, number 156 created a larger zone of partial surface inhibition, and number 158 created a small zone of complete inhibition.

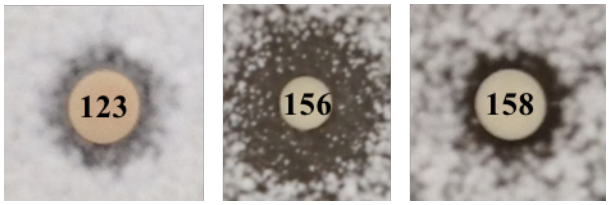

Figure 4. Zones of inhibition created in *P. destructans* fungal lawns by filtered broth supernatant of three fungal isolates collected seven days after broth inoculation. The photographs were taken on day 12 (#123) and day 10 (#156 and #158) after the plate was inoculated.

Bacterial isolate numbers 3, 13, 21, 32, 36, 55, 56, 81, 84, and 90 all created zones of surface inhibition in the *Pd* fungal lawn when broth supernatant collected on day 7 was used (Figure 5). The largest zones, which were all over 33 mm in diameter (Table 3), were created by isolates 3, 13, and 90; however, bacterial growth was observed around the paper discs containing the filtered broth supernatant from these isolates. All zones of inhibition created by bacterial broth supernatants collected on day 7 were over 18 mm in diameter (Table 3). Apart from isolate numbers 3, 13, and 90, no zones of inhibition created by bacteria were still present on day 21 after plate inoculation. However, these three zones of inhibition persisted due to unwanted bacterial growth on the plate surface from the supernatant. The zone diameters created by filtered fungal broth supernatant numbers 123, 156, and 158 measured 11.7 mm, 20.6 mm, and 11.1 mm, respectively. However, only fungal isolate number 123 still had an inhibitory zone present after 21 days of *Pd* growth, although the zone size was greatly reduced by day 21.

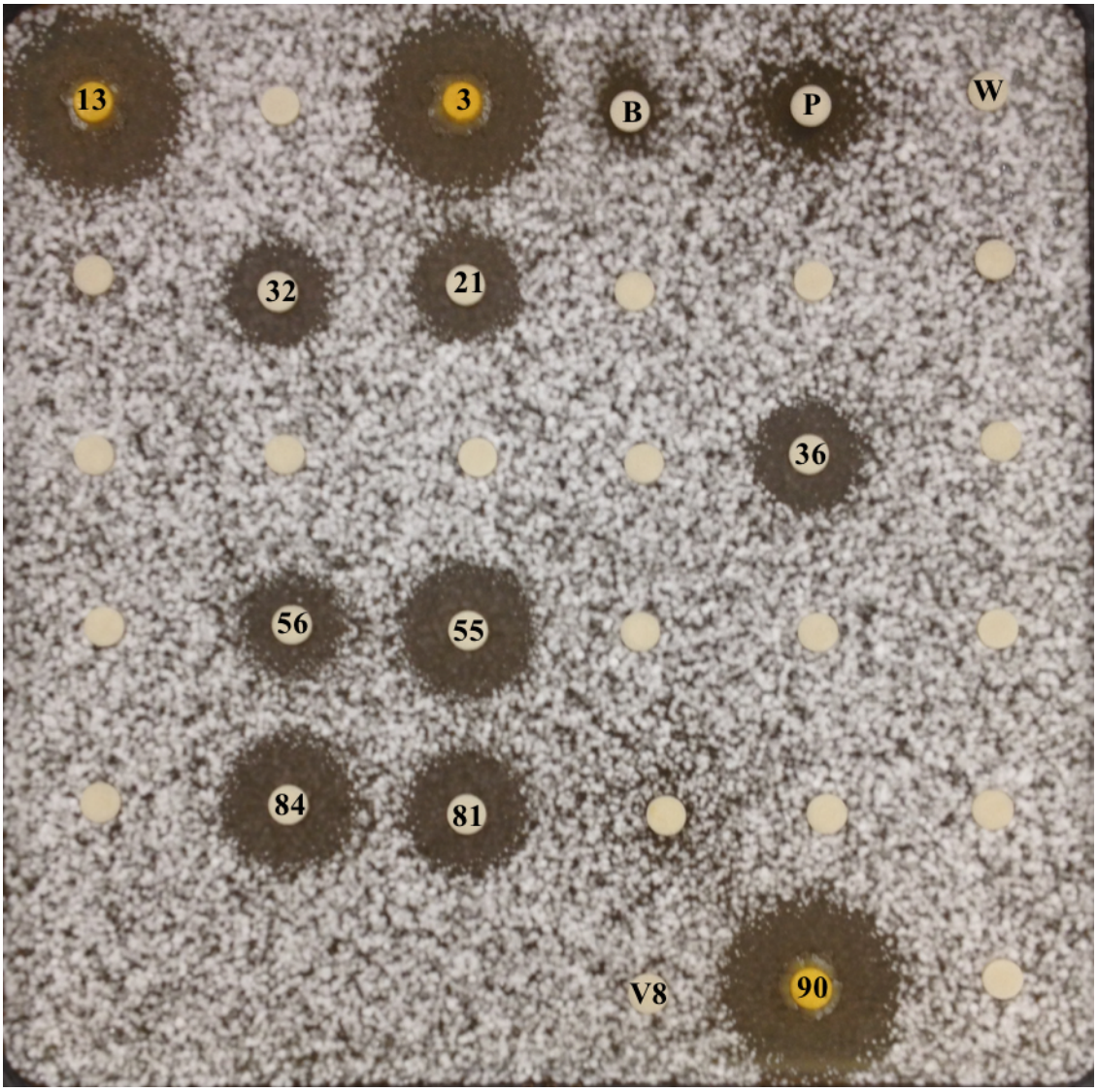

Figure 5. Anti-*Pd* bioassay plate with a *P. destructans* fungal lawn and inhibitory zones created by filtered bacterial supernatant collected seven days after broth inoculation. The photograph was taken 10 days after the plate was inoculated. Bacterial isolates creating zones of inhibition are labeled with their assigned screening number. Controls are labeled with a letter to indicate their identity. B=10% bleach, P=1.5% peroxigard, W=sterile water, V8=sterile V8 broth.

Table 3. Average diameter (mm), type, and length of presence for inhibitory zones created in *P. destructans* fungal lawns by bacterial or fungal filtered broth supernatant collected seven days after broth inoculation. Inhibitory zones were measured 10 days after the plate was inoculated (except isolate 123 and the corresponding controls that were measured on day 17) and monitored until day 21. The horizontal lines in the table separate the results from different screening plates.

Screening number	Average inhibitory zone diameter (mm)	Inhibition type	Zone still present on day 21
bleach	11.2	surface	no
peroxigard	12.0	complete	no
3	33.7	surface	yes
13	34.9	surface	yes
21	21.6	surface	no
32	20.4	surface	no
36	20.9	surface	no
55	26.0	surface	no
56	18.0	surface	no
81	24.4	surface	no
84	25.2	surface	no
90	33.6	surface	yes
bleach	0.0	none	no
peroxigard	10.0	surface	yes
123	11.7	partial surface	yes
bleach	13.7	surface	no
peroxigard	14.7	complete	yes
156	20.6	partial surface	no
158	11.1	complete	no

When the broth supernatant was collected on day 14 after broth inoculation, bacterial isolate numbers 3, 13, 14, 21, 32, 34, 36, 55, 56, 80, 81, 84, and 90 all created a zone of surface inhibition in the *Pd* fungal lawn (Figure 6). All of the average bacterial inhibitory zone diameters were greater than 16 mm, with the largest diameter being 28.8 mm (Table 4). Peroxigard (1.5%) created a zone of complete inhibition measuring 14.0 mm in diameter and bleach (10%) created a zone of surface inhibition measuring 13.3 mm in diameter. Sterile water and sterile V8 broth, which were used as negative controls, did not create inhibitory zones in the *Pd* lawn.

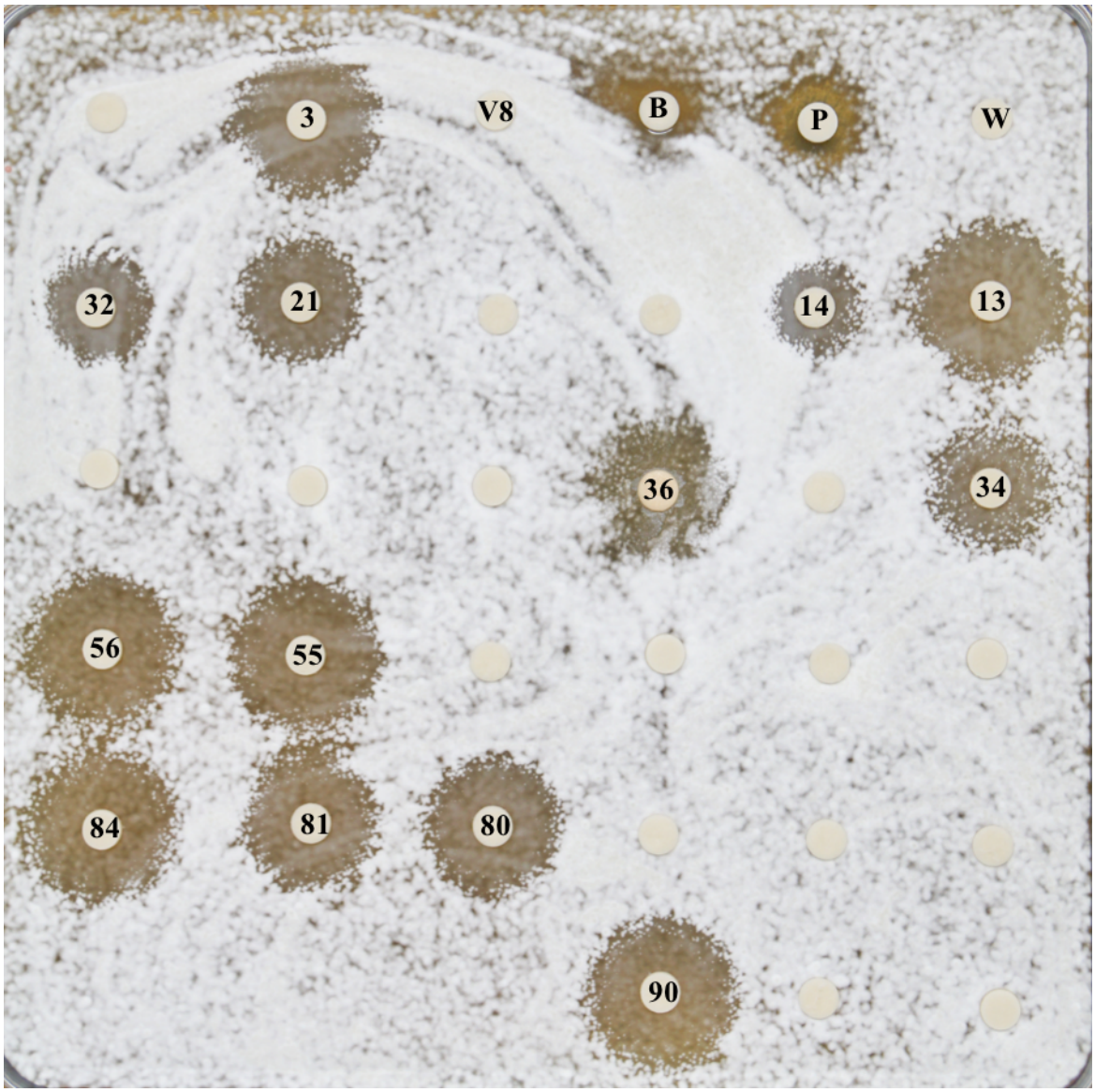

Figure 6. Anti-*Pd* bioassay plate with a *P. destructans* fungal lawn and inhibitory zones created by filtered bacterial supernatant collected 14 days after broth inoculation. The photograph was taken 10 days after the plate was inoculated. Bacterial isolates creating zones of inhibition are labeled with their assigned screening number. Controls are labeled with a letter to indicate their identity. B=10% bleach, P=1.5% peroxigard, W=sterile water, V8=sterile V8 broth.

The filtered broth supernatants from fungal isolate numbers 123, 124, 125, 127, 139, 156 and 158 also created zones of *Pd* inhibition when the broth was collected 14 days after inoculation (Figure 7). Isolate numbers 124 and 158 created zones of complete inhibition measuring 13.0 mm and 14.4 mm in diameter, respectively (Table 4). Isolate number 123 created a zone of surface inhibition with a 25.5 mm diameter. The fungal broth supernatant from fungal isolate numbers 125, 127, 139, and 156 created zones of partial surface inhibition with zone diameters of 12.8 mm, 13.2 mm, 13.0 mm, and 19.9 mm, respectively.

Figure 7. Zones of inhibition created in *P. destructans* fungal lawns by filtered broth supernatant of seven fungal isolates collected 14 days after broth inoculation. The photographs were taken 10 days after the plate was inoculated.

Of the zones of inhibition created by broth supernatants collected 14 days after inoculation, only isolate number 36 still had a zone of inhibition by day 21 of *Pd* growth (Table 4). However, this zone was reduced in size and had an irregular shape by day 21. Peroxigard had a zone of complete *Pd* inhibition that persisted until day 21 but did decrease in size; however, the zone of surface inhibition created by bleach did not persist until day 21.

Table 4. Average diameter (mm), type, and length of presence for inhibitory zones created in *P. destructans* fungal lawns by bacterial or fungal filtered broth supernatant collected 14 days after broth inoculation. Inhibitory zones were measured 10 days after the plate was inoculated and monitored until day 21. The horizontal lines in the table separate the results from different screening plates.

Screening number	Average inhibitory zone diameter (mm)	Inhibition type	Zone still present on day 21
bleach	13.3	surface	no
peroxigard	14.0	complete	yes
3	25.1	surface	no
13	28.8	surface	no
14	16.4	partial surface	no
21	24.3	surface	no
32	20.8	surface	no
34	22.1	surface	no
36	19.3	surface	yes
55	27.8	surface	no
56	27.6	surface	no
80	24.3	surface	no
81	23.7	surface	no
84	27.9	surface	no
90	28.0	surface	no
bleach	23.2	surface	no
peroxigard	17.4	complete	yes
123	25.5	surface	no

124	13.0	complete	no
125	12.8	partial surface	no
bleach	16.6	surface	no
peroxigard	14.7	complete	yes
127	13.2	partial surface	no
139	13.0	partial surface	no
bleach	27.0	surface	no
peroxigard	15.1	complete	yes
156	19.9	partial surface	no
158	14.4	complete	no

In order to compare the size of inhibitory zones created in *Pd* fungal lawns by broth collected on day 7 versus day 14 after inoculation, a bar chart of average zone diameters was created in Excel, with the error bars representing standard deviation (Figure 8, see also Supplementary Table 2). For isolate numbers 14, 34, 80, 124, 125, 127, and 139, no inhibition was observed when day 7 supernatant was used; however, inhibitory zones were observed when day 14 supernatant was applied to the plates. The day 7 supernatant created a larger zone of inhibition than the day 14 supernatant for isolate numbers 3, 13, 36, 81, 90, and 156. The day 14 supernatant created a larger zone of inhibition than the day 7 supernatant for isolate numbers 21, 32, 55, 56, 84, 123, and 158.

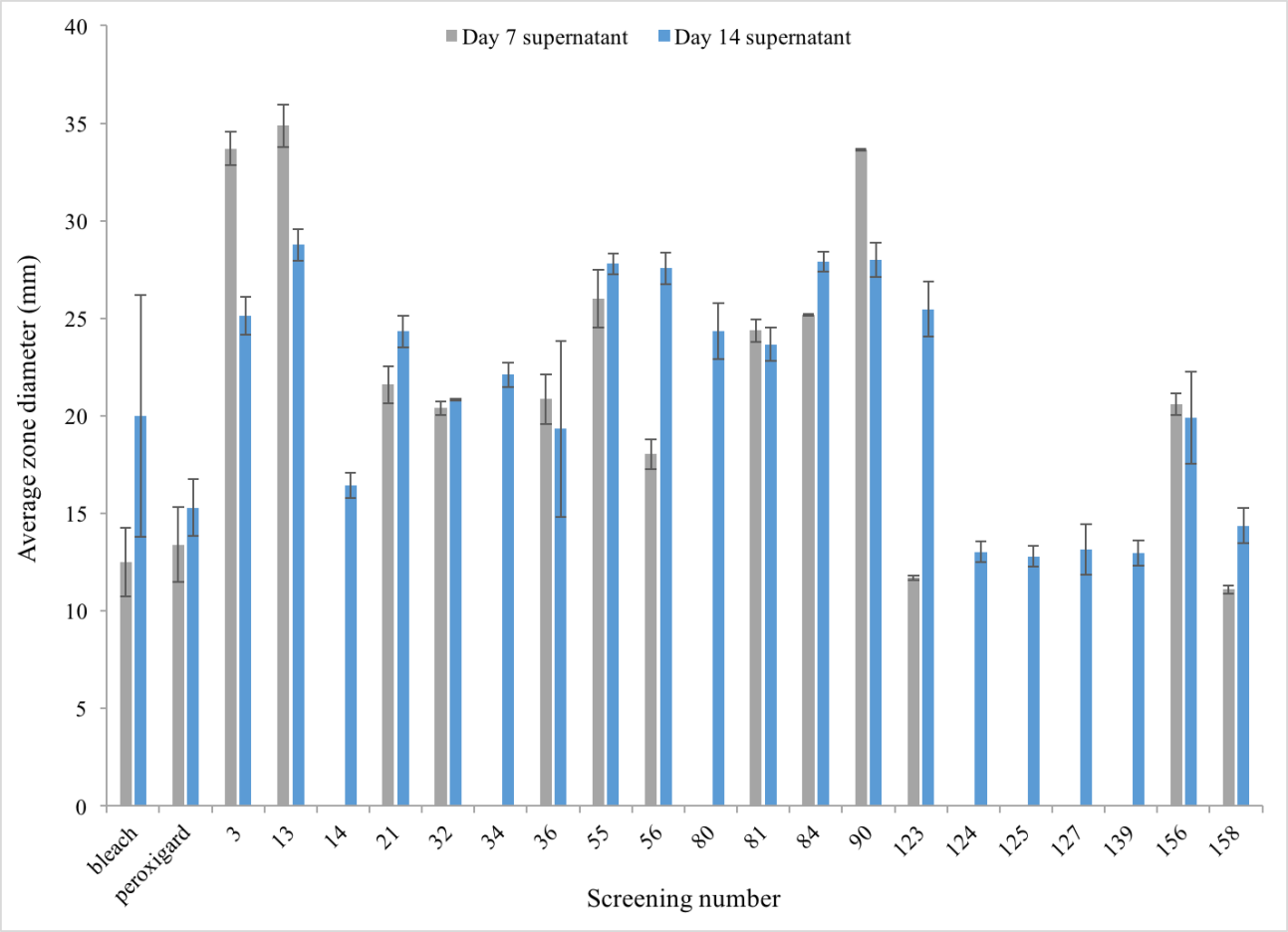

Figure 8. Comparison of average inhibitory zone diameters (mm) created in *P. destructans* fungal lawns by bacterial and fungal filtered broth supernatant collected seven (grey) and 14 (blue) days after broth inoculation. Error bars represent standard error (n=3).

DISCUSSION

The goal of this study was to screen bacteria and fungi for inhibitory activity towards *Pd* in order to identify candidates that could have potential for use as a biological control agent. The ecological and economic importance of bats combined with the high death tolls due to WNS and the continued spread of the disease across North America exemplify why this is an important area of research. Initially, 93 bacterial isolates collected in the areas near Bush Lake and Timber Lake, B.C. were pre-screened for anti-*Pd* activity using a direct contact streak assay. Thirty of the 93 isolates created a zone of inhibition in the *Pd* fungal lawn, which is a relatively high percentage of the isolates (32%) compared to previous studies. Hamm and colleagues (2017) isolated 632 Actinobacteria from bats, with only 36 isolates (6%) showing inhibitory activity

towards *Pd* and Hoyt and colleagues (2015b) isolated 133 bacterial morphotypes from bats, with only six isolates (4.5%) observed with anti-*Pd* activity. This suggests that although bats may harbor some bacteria able to inhibit *Pd* growth, our sample location may be a better source of bacterial anti-*Pd* agents. However, it should be noted that bacterial isolates were not genetically identified in this study, so it is possible that some of the isolates are identical, leading to a potential overestimation of the percentage of bacterial strains from our sampling location exhibiting anti-*Pd* activity.

Although the types of inhibition varied, all 30 of the bacteria creating inhibitory zones in the pre-screening experiment were subjected to further testing using a Kirby-Bauer style agar diffusion assay, which is referred to as an anti-*Pd* bioassay. The fungal isolates were not pre-screened, since a direct contact assay led to the overgrowth of the fungal isolates being tested due to a difference in the growth rates of *Pd* and the fungal isolates. Therefore, the fungi were only screened for anti-*Pd* activity using the anti-*Pd* bioassay, which involved growing bacteria and fungi in V8 broth, collecting and filtering the supernatants on day 7 and day 14 of growth, and spotting the filtered supernatants on paper discs that were placed on the surface of agar seeded with *Pd* conidia. Since the supernatant was filtered, the assay tested for inhibitory substance production and *Pd* inhibition in the absence of microbial cells. This is a novel assay for anti-*Pd* activity screening, since, to the extent of our knowledge, no other published studies have used the seeded agar technique or filtered microbial broth supernatant for screening microorganisms for anti-*Pd* activity.

Of the bacteria that produced anti-*Pd* activity in the pre-screening experiment, 10 exhibited anti-*Pd* activity when the day 7 filtered supernatant was tested against *Pd*, and the same 10 isolates plus an additional three of the original 30 positive candidates exhibited anti-*Pd* activity when the day 14 filtered supernatant was used. The reduction in the number of candidates exhibiting anti-*Pd* activity when progressing from the pre-screening experiment to using the filtered broth supernatant could be a result of using different media to grow the bacteria in the two experiments. With the exception of isolate number 14, the bacterial supernatants that produced zones of surface inhibition in the anti-*Pd* bioassay corresponded to bacteria that produced large zones of surface inhibition in the pre-screening experiment. The largest zones of inhibition observed in our study when either filtered day 7 supernatant or filtered day 14 supernatant were

used were both produced by isolate number 13 and measured 34.9 mm and 28.8 mm in diameter, respectively. The zones of inhibition produced by Actinobacteria tested by Hamm and colleagues (2017) ranged from 1 mm to 45 mm in diameter, with most of their isolates producing zones of inhibition less than 30 mm in diameter, although the difference in screening methods makes it difficult to accurately compare studies. In this study, all of the zones of inhibition created by bacterial broth supernatants were larger than the zones of inhibition produced by the positive controls (bleach and peroxigard), however the zones created by the positive controls persisted for longer periods of time than those produced by the bacterial supernatants, which only lasted for one to two days after confluent *Pd* growth was observed. Although it is difficult to determine why the zones of inhibition did not persist without knowing the mechanism of *Pd* inhibition, it is possible that *Pd* degrades the inhibitory substance over time or the inhibitory substance is only able to slow the growth or germination of *Pd* rather than stopping germination completely.

During the fungal isolate screening, five of the 53 fungi collected from the area around Bush Lake and Timber Lake, B.C., none of the 16 fungi collected from the mushroom farm in Summerland, B.C., and two of the eight fungi collected from dwarf mistletoe plants exhibited anti-*Pd* activity. Of the seven positive fungal candidates, only three created an inhibitory zone when filtered day 7 supernatant was used, but all seven displayed anti-*Pd* activity when filtered day 14 supernatant was used. When testing day 7 supernatant, the largest inhibitory zone was created by fungal isolate number 156 (20.6 mm diameter). Both isolate number 156 and number 123 created inhibitory zones larger than the zones created by the positive controls when day 7 supernatant was used. The largest inhibitory zone created by a fungal supernatant collected on day 14 was 25.5 mm in diameter (isolate number 123). However, most of the positive fungal candidates created inhibitory zones that were smaller than the zones created by positive controls when the day 14 supernatant was used.

Overall, bacteria appear to be a better source of anti-*Pd* activity than fungi, based on this study. A greater number of bacterial isolates produced anti-*Pd* activity compared to the fungal isolates and the zones of inhibition produced by bacteria tended to be larger than those produced by fungi. The ability to use a pre-screen and the ease of working with bacteria compared to fungi also favors future studies investigating bacteria for anti-*Pd* activity over fungi. Additionally, collecting bacterial and fungal broth supernatant on day 14 rather than day 7 should be used for

future studies since a greater number of isolates exhibited activity when day 14 supernatant was used and a greater proportion of the inhibitory zones were larger when day 14 supernatant was used, compared to day 7 supernatant. It is possible that a greater variety of metabolites with anti-*Pd* activity are produced closer to day 14 or that the amounts of these metabolites increase over time, leading to increased efficacy of the day 14 supernatants. However, there may be a day in between day seven and 14 or after day 14 that is optimal, which would require further research, such as the completion of a fermentation time course experiment.

The appearance of the three types of inhibitory zones (complete, subsurface, surface) in the two screening assays was likely dependent on whether the anti-*Pd* activity was fungistatic (*Pd* germination and growth slowed/inhibited) or fungicidal (*Pd* conidia killed) (Shelley et al., 2013), the mechanism of growth inhibition, and the rate of agar diffusion if a secreted inhibitory substance was responsible for inhibition. However, none of these factors were examined during this study, so the reasons for the varying types of inhibition cannot be determined. The zone of complete inhibition could have been caused by fungistatic or fungicidal activity, but the zones of surface inhibition were likely due to fungistatic activity since conidial germination did occur and the zones of inhibition did not persist. Unlike the zones of inhibition created on the agar surface, the zones of complete and subsurface inhibition did not appear to have any conidial germination. If zones of inhibition were due to fungistatic activity, it is possible that this was caused by the binding of an inhibitory agent to the conidia coat and the subsequent inhibition of conidial germination by the bound agent acting on germ tube formation (Shelley et al., 2013). Little is known about the mechanisms of *Pd* inhibition by other biocontrol agents; however, the sensitivity of *Pd* to amphotericin B and certain azole antifungals is caused by the inhibition of ergosterol (an important fungal cell membrane component) biosynthesis or the binding of the antifungal to ergosterol in the cell membrane (Chaturvedi et al., 2011). Shelley *et al.* (2013) also found that effective antifungal agents contained a long-chain alkyl group in their chemical structure, indicating that chemical structure may play a key role in growth inhibition of *Pd*.

Another key factor affecting the type and size of inhibitory zone observed is the ability of the inhibitory substance to diffuse through the agar. Diffusion through agar can depend on many factors, such as concentration of the substance, amount of the substance, depth of the agar, size of the molecules, and the incubation temperature (Koch, 1999). When bacteria are streaked on

the agar surface or a paper disc containing a substance is placed on the agar, diffusion occurs both vertically and radially from the source of the substance. Koch (1999) determined that the greater the radial distance from the source, the lower the concentration of the substance will be at the bottom surface of the agar at that point and the more time it will take for the concentration of the substance to equalize between the top and bottom surface of the agar. Keeping this in mind, it may be possible that the substance(s) being produced by certain bacteria or fungi have varying abilities to diffuse through the agar, affecting the size or type of inhibitory zone observed. For instance, if the conidia near the agar surface were exposed to high concentrations of a substance diffusing from the agar surface and the conidia near the bottom of the agar were exposed to a very low concentration of this substance due to unequal diffusion through the agar, this may lead to a zone of surface inhibition, since conidia near the agar bottom may not experience the same effects as the conidia near the surface and may still be able to germinate. This may give misleading results, since the agar diffusion ability of the substance may determine the level of anti-*Pd* activity observed, rather than the potency of the agent itself. This is a limit of the method used, since rather than having one layer of conidia on the agar surface, the conidia are dispersed throughout the agar, meaning that any anti-*Pd* agent must diffuse from the surface of the agar to the bottom surface of the plate at a high enough concentration to kill or inhibit conidial germination in order to create a zone of complete inhibition. In future studies, this could be avoided by spreading conidia on the agar surface rather than embedding them in the agar, although fungal lawns created using this method are not as uniform.

Further experiments could also be conducted to determine if the observed anti-*Pd* activity was fungicidal or fungistatic. For example, a germule suppression assay could be used to examine conidia during germination (Cornelison et al., 2014), or a sporicidal assay could be used to determine if spores can germinate after being soaked in an inhibitory agent (Shelley et al., 2013). It may also be interesting to view *Pd* spores using a scanning electron microscope to determine if inhibition involves any distortion of the spores, something that hasn't been done in any other studies involving *Pd*. Additionally, the isolates with strongest anti-*Pd* activity (13, 90, 3, 55, 84) should undergo further testing such as whole-genome sequencing, transcriptomic analysis, and metabolic profiling in order to determine if they could be used for the biological control of *Pd*. Ideally, if the active compounds produced by these isolates could be identified, then potentially the corresponding genes could be transformed into *Escherichia coli* and the compound(s) could

be produced on a large scale. Testing the compound(s) for safety for use on bats or in bat hibernacula would also need to be conducted before any novel biological control agent could be used in the field.

In summary, 93 bacterial isolates and 77 fungal isolates were screened for anti-*Pd* activity with 30 bacterial isolates and seven fungal isolates exhibiting anti-*Pd* activity. Our results indicate that microorganisms isolated from the areas around Bush Lake and Timber Lake B.C. have a high level of antimicrobial activity and are promising candidates in the search for a novel biological control agent for *Pd*, the causative agent of WNS. To the extent of our knowledge, this study is the first to use embedded *Pd* conidia and microbial broth supernatants to screen microorganisms for anti-*Pd* activity. Additionally, this is the first study to screen bacteria and fungi from Bush Lake and Timber Lake B.C., a mushroom farm, and dwarf mistletoe plants, as well as one of the only studies to screen fungi for anti-*Pd* activity.

LITERATURE CITED

Bat Conservation International (2017). Bats Are: Threatened. Available from http://www.batcon.org/why-bats/bats-are/bats-are-threatened [accessed 15 May 2017].

Boire, N., Zhang, S., Khuvis, J., Lee, R., Rivers, J., Crandall, P., Keel, M.K., and Parrish, N. (2016). Potent Inhibition of Pseudogymnoascus destructans, the Causative Agent of White-Nose Syndrome in Bats, by Cold-Pressed, Terpeneless, Valencia Orange Oil. PLOS ONE *11*, e0148473.

Boyles, J.G., Cryan, P.M., McCracken, G.F., and Kunz, T.H. (2011). Economic Importance of Bats in Agriculture. Science *332*, 41–42.

Chaturvedi, S., Rajkumar, S.S., Li, X., Hurteau, G.J., Shtutman, M., and Chaturvedi, V. (2011). Antifungal Testing and High-Throughput Screening of Compound Library against Geomyces destructans, the Etiologic Agent of Geomycosis (WNS) in Bats. PLOS ONE *6*, e17032.

Chaturvedi, V., Springer, D.J., Behr, M.J., Ramani, R., Li, X., Peck, M.K., Ren, P., Bopp, D.J., Wood, B., Samsonoff, W.A., et al. (2010). Morphological and Molecular Characterizations of Psychrophilic Fungus Geomyces destructans from New York Bats with White Nose Syndrome (WNS). PLOS ONE *5*, e10783.

Cornelison, C.T., Keel, M.K., Gabriel, K.T., Barlament, C.K., Tucker, T.A., Pierce, G.E., and Crow, S.A. (2014). A preliminary report on the contact-independent antagonism of Pseudogymnoascus destructans by Rhodococcus rhodochrousstrain DAP96253. BMC Microbiol. *14*, 246.

Flieger, M., Bandouchova, H., Cerny, J., Chudíčková, M., Kolarik, M., Kovacova, V., Martínková, N., Novák, P., Šebesta, O., Stodůlková, E., et al. (2016). Vitamin B2 as a virulence factor in Pseudogymnoascus destructans skin infection. Sci. Rep. *6*.

Frick, W.F., Cheng, T.L., Langwig, K.E., Hoyt, J.R., Janicki, A.F., Parise, K.L., Foster, J.T., and Kilpatrick, A.M. (2017). Pathogen dynamics during invasion and establishment of white-nose syndrome explain mechanisms of host persistence. Ecology *98*, 624–631.

Gargas, A., Trest, M.T., Christensen, M., Volk, T.J., and Blehert, D.S. (2009). Geomyces destructans sp. nov. associated with bat white-nose syndrome. Mycotaxon *108*, 147–154.

Gill, J. (2016). Bat disease treatment a step forward, but not a cure, researcher says. Available from http://www.cbc.ca/news/canada/new-brunswick/white-nose-syndrome-nov-nin-si-1.3843537 [accessed 15 May 2017].

Haman, K., Hibbard, C., and Lubeck, M. (2016). Bat with white-nose syndrome confirmed in Washington state. Wash. Dep. Fish Wildl.

Hamm, P.S., Caimi, N.A., Northup, D.E., Valdez, E.W., Buecher, D.C., Dunlap, C.A., Labeda, D.P., Lueschow, S., and Porras-Alfaro, A. (2017). Western Bats as a Reservoir of Novel Streptomyces Species with Antifungal Activity. Appl. Environ. Microbiol. *83*, e03057-16.

Hoyt, J.R., Langwig, K.E., Okoniewski, J., Frick, W.F., Stone, W.B., and Kilpatrick, A.M. (2015a). Long-Term Persistence of Pseudogymnoascus destructans, the Causative Agent of White-Nose Syndrome, in the Absence of Bats. EcoHealth *12*, 330–333.

Hoyt, J.R., Cheng, T.L., Langwig, K.E., Hee, M.M., Frick, W.F., and Kilpatrick, A.M. (2015b). Bacteria Isolated from Bats Inhibit the Growth of Pseudogymnoascus destructans, the Causative Agent of White-Nose Syndrome. PLOS ONE *10*, e0121329.

Koch, A.L. (1999). Diffusion through agar blocks of finite dimensions: a theoretical analysis of three systems of practical significance in microbiology. Microbiol. Read. Engl. *145 (Pt 3)*, 643–654.

Kunz, T.H., de Torrez, E.B., Bauer, D., Lobova, T., and Fleming, T.H. (2011). Ecosystem services provided by bats. Ann. N. Y. Acad. Sci. *1223*, 1–38.

Leopardi, S., Blake, D., and Puechmaille, S.J. (2015). White-Nose Syndrome fungus introduced from Europe to North America. Curr. Biol. *25*, R217–R219.

Lorch, J.M., Meteyer, C.U., Behr, M.J., Boyles, J.G., Cryan, P.M., Hicks, A.C., Ballmann, A.E., Coleman, J.T.H., Redell, D.N., Reeder, D.M., et al. (2011). Experimental infection of bats with Geomyces destructans causes white-nose syndrome. Nature *480*, 376–378.

Magnusson, J., Ström, K., Roos, S., Sjögren, J., and Schnürer, J. (2003). Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. FEMS Microbiol. Lett. *219*, 129–135.

Minnis, A.M., and Lindner, D.L. (2013). Phylogenetic evaluation of Geomyces and allies reveals no close relatives of Pseudogymnoascus destructans, comb. nov., in bat hibernacula of eastern North America. Fungal Biol. *117*, 638–649.

Moriarty, M. (2009). Little brown bat with white-nose syndrome in Greeley Mine, Vermont.

O'Donoghue, A.J., Knudsen, G.M., Beekman, C., Perry, J.A., Johnson, A.D., DeRisi, J.L., Craik, C.S., and Bennett, R.J. (2015). Destructin-1 is a collagen-degrading endopeptidase secreted by Pseudogymnoascus destructans, the causative agent of white-nose syndrome. Proc. Natl. Acad. Sci. *112*, 7478–7483.

Padhi, S., Dias, I., and Bennett, J.W. (2017). Two volatile-phase alcohols inhibit growth of Pseudogymnoascus destructans, causative agent of white-nose syndrome in bats. Mycology *8*, 11–16.

Perotto, S., Angelini, P., Bianciotto, V., Bonfante, P., Girlanda, M., Kull, T., Mello, A., Pecoraro, L., Perini, C., Persiani, A.M., et al. (2013). Interactions of fungi with other organisms. Plant Biosyst. - Int. J. Deal. Asp. Plant Biol. *147*, 208–218.

Puechmaille, S.J., Wibbelt, G., Korn, V., Fuller, H., Forget, F., Mühldorfer, K., Kurth, A., Bogdanowicz, W., Borel, C., Bosch, T., et al. (2011). Pan-European Distribution of White-Nose

Syndrome Fungus (Geomyces destructans) Not Associated with Mass Mortality. PLOS ONE *6*, e19167.

Raudabaugh, D.B., and Miller, A.N. (2015). Effect of Trans, Trans-Farnesol on Pseudogymnoascus destructans and Several Closely Related Species. Mycopathologia *180*, 325–332.

Reeder, D.M., Frank, C.L., Turner, G.G., Meteyer, C.U., Kurta, A., Britzke, E.R., Vodzak, M.E., Darling, S.R., Stihler, C.W., Hicks, A.C., et al. (2012). Frequent Arousal from Hibernation Linked to Severity of Infection and Mortality in Bats with White-Nose Syndrome. PLOS ONE *7*, e38920.

Reynolds, H.T., and Barton, H.A. (2014). Comparison of the White-Nose Syndrome Agent Pseudogymnoascus destructans to Cave-Dwelling Relatives Suggests Reduced Saprotrophic Enzyme Activity. PLOS ONE *9*, e86437.

Shelley, V., Kaiser, S., Shelley, E., Williams, T., Kramer, M., Haman, K., Keel, K., and Barton, H.A. (2013). Evaluation of strategies for the decontamination of equipment for Geomyces destructans, the causative agent of white-nose syndrome (WNS). J. Cave Karst Stud. *75*, 1–10.

Springer, D.J. (2013). Original false-colored scanning electron micrograph of G. destructans on a Little Brown Bat (Myotis lucifugs).

Thomas, D.W., Dorais, M., and Bergeron, J.-M. (1990). Winter Energy Budgets and Cost of Arousals for Hibernating Little Brown Bats, Myotis lucifugus. J. Mammal. *71*, 475–479.

U.S. Fish and Wildlife Service (2017). About white-nose syndrome. Available from https://www.whitenosesyndrome.org/about-white-nose-syndrome [accessed 15 May 2017].

Verant, M.L., Boyles, J.G., Jr, W.W., Wibbelt, G., and Blehert, D.S. (2012). Temperature-Dependent Growth of Geomyces destructans, the Fungus That Causes Bat White-Nose Syndrome. PLOS ONE *7*, e46280.

Verant, M.L., Meteyer, C.U., Speakman, J.R., Cryan, P.M., Lorch, J.M., and Blehert, D.S. (2014). White-nose syndrome initiates a cascade of physiologic disturbances in the hibernating bat host. BMC Physiol. *14*, 10.

Warnecke, L., Turner, J.M., Bollinger, T.K., Misra, V., Cryan, P.M., Blehert, D.S., Wibbelt, G., and Willis, C.K.R. (2013). Pathophysiology of white-nose syndrome in bats: a mechanistic model linking wing damage to mortality. Biol. Lett. *9*, 20130177.

Wohlleben, W., Mast, Y., Stegmann, E., and Ziemert, N. (2016). Antibiotic drug discovery. Microb. Biotechnol. *9*, 541–548.

Zhang, T., Chaturvedi, V., and Chaturvedi, S. (2015). Novel Trichoderma polysporum Strain for the Biocontrol of Pseudogymnoascus destructans, the Fungal Etiologic Agent of Bat White Nose Syndrome. PLOS ONE *10*, e0141316.

Zukal, J., Bandouchova, H., Brichta, J., Cmokova, A., Jaron, K.S., Kolarik, M., Kovacova, V., Kubátová, A., Nováková, A., Orlov, O., et al. (2016). White-nose syndrome without borders: Pseudogymnoascus destructans infection tolerated in Europe and Palearctic Asia but not in North America. Sci. Rep. *6*, 19829.

APPENDICES

Supplementary Table 1. A summary of bacterial and fungal samples collected from Bush and Timber Lakes, What the Fungus mushroom farm, and dwarf mistletoe plants and their inhibitory activity towards *P. destructans* in the bacterial pre-screening and/or the anti-*Pd* bioassay.

Sample location	Isolate numbers & total number of isolates	Number of bacteria pre-screened	Bacterial positive candidate numbers (pre-screen)	Bacterial positive candidate numbers (supernatant screen)	Number of fungi screened (supernatant screen)	Fungal positive candidate numbers
Bush Lake & Timber Lake, B.C.	1-93 (bacteria) 125-177 (fungi) Total: 146	93	3, 10, 12, 13, 14, 19, 20, 21, 32, 34, 35, 36, 37, 38, 41, 47, 51, 53, 54, 55, 56, 70, 71, 77, 80, 81, 84, 86, 89, 90	3, 13, 14, 21, 32, 34, 36, 55, 56, 80, 81, 84, 90	53	125, 127, 139, 156, 158
What the Fungus Mushroom Farm, Summerland, B.C.	101-116 (fungi) Total: 16	n/a	n/a	n/a	16	n/a
Dwarf mistletoe plants	117-124 (fungi) Total: 8	n/a	n/a	n/a	8	123, 124

Supplementary Table 2. Comparison of average diameter (mm) of inhibitory zones created in *P. destructans* fungal lawns by bacterial or fungal filtered broth supernatants collected either seven or 14 days after broth inoculation. Inhibitory zones were measured 10 days after the plate was inoculated with *Pd* spores. The bleach and peroxigard preceding the list of isolate numbers correspond to the positive controls on the same plate as those bacterial or fungal candidates.

Screening number	Average inhibitory zone diameter (mm) for day 7 supernatant	Average inhibitory zone diameter (mm) for day 14 supernatant
bleach	11.2	13.3
peroxigard	12.0	14.0
3	33.7	25.1
13	34.9	28.8
14	n/a	16.4
21	21.6	24.3
32	20.4	20.8
34	n/a	22.1
36	20.9	19.3
55	26.0	27.8
56	18.0	27.6
80	n/a	24.3
81	24.4	23.7
84	25.2	27.9
90	33.6	28.0
bleach	0.0	23.2
peroxigard	10.0	17.4
123	11.7	25.5
124	n/a	13.0
125	n/a	12.8
bleach	n/a	16.6
peroxigard	n/a	14.7
127	n/a	13.2
139	n/a	13.0
bleach	13.7	27.0
peroxigard	14.7	15.1
156	20.6	19.9
158	11.1	14.4