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OBJECTIVE LENS

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WELCOME TO *FINE FOCUS*.

This is an exciting milestone! As you read this, our first issue of *Fine Focus*, I invite you to reflect on the countless hours over many months spent by dozens of dedicated undergraduate students who worked to bring this new journal to fruition. The journal is entirely run, from promotion to print, by an interdisciplinary team of undergraduate students (although we have an Editorial Board of over four dozen experts in various subdisciplines of microbiology who complete double-blind external reviews on all manuscripts). The student names are included here to let you see these dynamic individuals, who represent not only biology students, but marketing, graphic design, legal studies, art, nursing, chemistry, and technology majors as well. Each team shared the common vision of bringing this journal to an international audience of undergraduate microbiology students performing research, as well as their mentors, all of whom are invited to submit manuscripts.

Fine Focus is much more than simply a new peer-reviewed journal geared towards undergraduates in microbiology, however. The students who manage and publish *Fine Focus* participate firsthand in marketing, promoting, designing, and selecting/reviewing papers that will enhance undergraduate education through research. The *Fine Focus* staff members are consummate professionals who have proven their passion for interdisciplinary immersive learning, and these ideals are reflected in our cover logo. I envision the coming years to bring a great variety of outstanding articles to the undergraduate microbiology research community through our journal, and I am thrilled to lead *Fine Focus* towards this and many other long-term goals. Thank you in advance for any and all comments and suggestions you may have which could improve *Fine Focus* as we move forward. Many of you have already done so, either at ASM, ASMCUE, CUR, Sigma Xi, or ADSA. We expect our second issue to be in print and available online in July of 2015 and are continually reviewing submissions for this next issue. Until then, we wish you the best in your undergraduate research progress, and look forward to receiving your manuscript!

ACKNOWLEDGEMENTS

The Editorial staff of *Fine Focus* would like to acknowledge the following individuals for their assistance and support in helping to bring our new journal to production and print, and/or for advertising/promoting *Fine Focus*:

Delores Brown Indiana Academy of Science
David Concepcion Ball State University
Fritz Dolak Ball State University
Daniel Y.C. Fung Kansas State University
Kelly Gull ASM
Eric Harvey Ball State University
Hans Kellogg Ball State University
Susan Merkel ASM & Cornell University
Phil Mixter Washington State University
Antony Schwartz NIH
Gail Stoutamoyer Ball State University

Funding provided by the Ball State University Provost's Immersive Learning Grant Program, and by generous contributions from Dr. Indira Komanapalli, Ph.D.

THE OCCURRENCE OF MYXOMYCETES FROM A LOWLAND MONTANE FOREST AND AGRICULTURAL PLANTATIONS OF NEGROS OCCIDENTAL, WESTERN VISAYAS, PHILIPPINES

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MANUSCRIPT RECEIVED 22 SEPTEMBER, 2014; ACCEPTED 19 DECEMBER, 2014

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KEYWORDS

- abundance
- amoeboid eukaryotes
- diversity
- fruiting body
- sclerotia

ABSTRACT

Higher floral and faunal biodiversity is expected in multi-species-covered mountainous forests than in mono-typic agricultural plantations. To verify this supposition for cryptogamic species like the plasmodial slime molds, a rapid field survey was conducted for myxomycetes and substrates in forest floor litter and agricultural plantation were collected in Negros Occidental, Philippines. Morphological characterization identified a total of 28 species belonging to the genera *Arcyria*, *Ceratiomyxa*, *Collaria*, *Comatricha*, *Craterium*, *Cribraria*, *Diderma*, *Didymium*, *Hemitrichia*, *Lamproderma*, *Physarum*, *Stemonitis*, *Trichia* and *Tubifera*. The myxomycete species *Arcyria cinerea* was the only abundant species found both in the agricultural and forested areas. The majority of collected species were rarely occurring. In terms of species composition, more myxomycetes were recorded in the mountainous forest (27) compared to agricultural sites. Furthermore, aerial leaf litter collected in the forests had the highest number of records for fruiting bodies but in terms of species diversity, twigs yielded higher value based on Shannon index. Findings in this study verify that a habitat with more heterogenous plant communities yields higher species of myxomycete assemblages. This research is the first study to report myxomycetes from Negros Occidental.

INTRODUCTION

Myxomycetes, commonly known as true slime molds, are acellular, phagotrophic, eukaryotic organisms under the Kingdom Protista (9). These organisms have been known to exhibit both fungal and protozoan characteristics but through an amoeboid phase, they feed on other microorganisms, including bacteria and yeast (9). These microorganisms are distributed worldwide and usually occur on dead substrata such as bark, twigs, and dried leaves of plants (29). Several studies regarding the taxonomy and ecology of myxomycetes have been conducted but most of this research was carried out in temperate regions, such as North America (35, 36), South America (16), and Europe (8,10). Numerous studies have also been completed in the tropics, such as Costa Rica (30), Puerto Rico (23) and Mexico (17). Despite the number of the studies that were executed and the high potential of biodiversity

in tropical systems, little is known about them particularly in the tropical Southeast Asia such as the Philippines.

Thus, myxomycete profiling in the Philippines is still considered incomplete. As a result, there is limited knowledge on the ecology and taxonomy of myxomycetes found in such tropical areas where there are abundant forested areas serving as excellent habitats for myxomycetes (25). Previous studies on Philippines myxomycetes in the late 1970s and early 1980s encompassed the most comprehensive listing for the country (24). However, most of these publications during that time were merely extensive annotated lists. But in recent years, myxomycete diversity and ecology studies in the Philippines has progressed, as several reports have accounted on myxomycete distribution and occurrences in several habitat types, e.g. in selected forest parks (7, 19), in coastal forests (12, 18), and lowland mountain forests (3,4). These papers surveyed different forest habitats in the Luzon main island, which is just a small portion of the large archipelagic geography of the Philippines. No previous reports had ever documented myxomycetes on the scatter islands of the Visayas region, or reported myxomycete occurrence in large agricultural plantations in the country. As such, the findings from this research paper on myxomycete composition in lowland forests will serve as a baseline reference for the profiles of myxomycetes in a local scale of Negros Occidental, but will also contribute to understanding their distribution in the whole of the Philippines.

Thus, the objectives of this paper are to (1) collect myxomycetes using opportunistic sampling methods in the field, and moist chamber cultures; (2) determine the collected species of myxomycetes; (3) measure the sampling strategy used in the survey; (4) assess the occurrence of each myxomycete species; (5) calculate the species diversity of the myxomycetes from the different substrates; and (6) compare the similarities of myxomycete assemblages between mountainous forests and agricultural plantations.

MATERIALS AND METHODS

STUDY SITES AND ITS COLLECTING LOCALITIES

Field survey and substrate collection were carried out during May 2013 in Negros Occidental, Western Visayas. The province is part of the whole Negros Island, the third largest island in the Philippines. It is estimated that the province is approximately 375 kilometers long from north to south with basically volcanic vegetation, making its arable land ideal for cultivation of economically important crops, especially sugarcanes (http://www.negros-occ.gov.ph). Based on the Philippine Atmospheric, Geophysical and Astronomical Services Administration – Department of Science and Technology (PAG-ASA DOST) climatological data, the whole area is characterized as having two distinct seasons: dry from December to May and wet from June to November. Along the study area, two different habitat types, namely, a lowland forested area and an agricultural plantation were chosen. The descriptions of each habitat types and its collecting localities (Fig. 1) are further described below.

A. Forested Areas (Mt. Kanlaon National Park, 10° 24.787N, 123° 07.982E). This area rises to a height of 2,465 m (7987 ft.) and is located in the province of Negros Occidental, Western Visayas. It is characterized by low serrated mountain ranges. The forest can be

described as a moist tropical disturbed rainforest dominated with large dipterocarp trees and non-vascular plants. Within this forest area, six sites were randomly selected along an established 1000 m accessible forest trail that serves as a transect.

B. Agricultural Area (Sugarcane Plantations, *Saccharum officinarum*). Only sugarcane plantations along the road in the northwest part of the province were selected for this study. Three collecting localities characterized as dry and with extensive light exposure were selected to collect substrates that were subjected for moist chamber cultures. Dried substrata are ideal for the preparation of moist chambers, as these substrates retain spores better. The collecting localities are: Silay City (SC1, 10°46'46.18"N 123°0'24.80"E), Bacolod City (SC2, 10°42'29.30"N 122°58'56.72"E), and Bago City (SC3, 10°32'37.29"N 122°51'47.51"E).

FIELD COLLECTION OF MYXOMYCETE SPECIMENS

Fruiting bodies of myxomycetes directly observed in the field were immediately placed in clean compartmentalized plastic collecting boxes. These specimens were brought back to the laboratory and after several days of air drying, the specimens was glued in herbarium trays and placed inside matchbox-sized herbarium boxes for permanent storage.

MOIST CHAMBER PREPARATION FROM SUBSTRATE COLLECTED IN THE TWO HABITAT TYPES

Ninety samples each of ground leaf litter (GL), aerial leaf litter (AL), twigs (TW), 60 samples each of ferns (F), and 30 samples of vines (V) from mountain forests, and 90 sugarcane leaf litter (SC) from agricultural plantations were collected, accounting for a total of 450 substrates used for this study. The collected substrates were placed inside dry paper bags, labeled, and transported back to the laboratory. Collection of samples was done following the methods described by Stephenson (36). Samples of ground floor litter were gathered at 3–5 m regular intervals. These samples consisted of mixtures of leaves. Twigs < 1.0 cm in diameter in size were also collected. Samples of aerial litter were collected from dead twigs or leaves still attached to branches of plants and trees. The specimens were wrapped gently in paper before being transported to the laboratory, where they were placed in small boxes for storage to prevent insects from getting into the samples. The samples were air dried for one week to prevent the growth of molds. To avoid pseudoreplication, a single moist chamber (MC) was prepared for each substrate collected. The moist chambers used consisted of disposable plastic Petri dishes, 10 cm in diameter and 4 cm deep, lined with filter paper. Samples were moistened with sterile distilled water. After 24 h, excess water was removed up to the point adequate enough for the chamber to be moist, and the pH of each of the substrate was checked using a pH meter (Sartorius PB-11). Following the incubation conditions of Dagamac *et al.* (3), moist chambers were kept at room temperature (22–25°C) in diffuse daylight. When necessary, a small amount of water was added to each culture to maintain moist conditions.

DETERMINATION OF MYXOMYCETE SPECIES

The specimens obtained from the moist chamber cultures were identified using a dissecting microscope three times per week (two day intervals) for a period of up to three months, by comparing the color, size, and structure of the myxomycete plasmodia,

types of fruiting bodies (e.g. sporangium, aethalium, pseudoaethalium, and plasmodiocarp), and spores of myxomycetes in the descriptions stated in the standard monographs of myxomycetes (21, 32). Web-based electronic databases, e.g. Eumycetozoan Project (http://slimemold.uark.edu), were also utilized for verification of some morphological features. Nomenclature used for the identified myxomycetes follows the names used in Nomenyx (http://nomen.eumycetozoa.com). For specimens that could not be fully identified with strong certainty due to some malformed specimens but with distinguishing character enough to separate as a species, the abbreviation "cf" was used in the taxon name. All specimens listed herein are deposited in the myxomycete herbarium of the Fungal Biodiversity and Systematics Group of the Research Center for the Natural and Applied Sciences at the University of Santo Tomas in Manila, Philippines.

EVALUATION OF DATA

To evaluate the sample effort of the myxomycete survey in this study, an individual-based rarefaction curve was established. Using the rarefaction formula that computes for a number of estimators for species richness of the free downloadable program EstimateS (Version 9, Colwell 2013, 100 randomizations), species accumulation curves were initially constructed. In accordance with Unterseher *et al.* (39), the Chao2 estimator was then chosen as the best estimator to use and was calculated using the classical settings of EstimateS. The estimated value for the sampling effort in the study area was then determined using the formula of Ndiritu *et al.* (22) by dividing the actual number of species recorded by the mean number of species expected as estimated by the Chao 2 estimator. Additionally, a hyperbolic regression in the form of Coleman rarefaction curve according to the Michaelis–Menten formula $y = ax/(b+x)$, where x represents the number of samples, y is the number of species recorded and the parameter a giving an estimate for the maximum number of species to be expected at this kind of substrate resulting in a very close curve shape (Magurran 20) was applied to the dataset.

For the assessment of species occurrence of myxomycetes, species composition was initially determined for the collection site. Occurrence refers to the frequency of the presence of a particular species of myxomycetes in a positive MC. A moist chamber positive for having a fruiting body of a particular species was considered as one positive collection. A collection was then considered as a single unit. The number of collections reflects the abundance of myxomycetes in Negros Occidental and was expressed as relative abundance. The relative abundance for every species of myxomycetes were then calculated and reported as Abundance Index (AI) by Stephenson *et al.* (1993). Each species were then categorized as: (1) abundant if their relative abundance (RA) is>3% of the total collections, (2) common if RA is >1.5% but <3% of the total collections, (3) occasionally occurring if RA is >0.5% but <1.5% of the total collections, and (4) rare if the myxomycetes had an RA of < 0.5% of the total collections.

To further determine the myxomycetes diversity for the different substrates, species diversity was also calculated using three different diversity indices provided in Magurran (20). Shannon diversity index (HS) measures species diversity with respect to both species evenness and richness. This index assumes that individuals are randomly sampled from an infinitely large community and that all species are represented in the sample (14). The Gleason Index (HG) measures the species diversity in relation to species richness. Richness is defined as the number of different species

found in a biota. Pielou's species evenness index (E), on the other hand, quantifies how equal communities are in a given sampling area. These indices are computed as follows:

Equation 1: Shannon Index of Diversity (HS) = $-\_i(pi \ln pi)$, where *pi* = the total number of individuals in the ith species.

Equation 2: Gleason Index (HG) = Np – 1/ln Ni, where Np = the total number of species and Ni = the total number of individuals in the ith species.

Equation 3: Pielou's index of species evenness (E) E = HS/Hmax where HS = Shannon Index of Diversity and Hmax= the maximum value of HS.

The similarities of myxomycete assemblages between the mountainous forest and agricultural plantation were also compared by Sorensen's coefficient of community index and the Percentage Similarity index. The equation for Sorensen's coefficient is based on the presence or absences of species.

Equation 4: Coefficient of Community (CC = 2c/(a+b)), where a = total number of species in the first habitat, b = total number of species in the second habitat, and c = no. of species common to both habitat.

The value of CC ranges from 0 – 1 where 0 is if there are no species present in both habitat and 1 when all species are present in both habitat. On the other hand, the Percentage Similarity (PS) index considers not only the presence or absence of species but their relative abundance. The PS value was computed as follows:

Equation 5: PS = Σ min (A, B, ... X)
where min = the lesser of the two percentage compositions of species A, B, C, ... X in the two communities.

RESULTS

Using the combined opportunistic sampling in the field and moist chamber culture preparation, a total of 193 records of myxomycetes were noted for this survey. From these 193 records, 42 were fruiting body records in the field and 151 were recovered either as plasmodia or fruiting body records in the moist chamber cultures. In terms of the field survey, there were no field specimens that were observed in the agricultural plantations. Moreover, a higher yield of myxomycetes was noted among moist chambers in forest litter than agricultural litter. Only two bright-spored myxomycetes species (*Arcyria cinerea* and *Tubifera ferruginosa*) were recorded in the sugarcane litter.

A total of 32 morphospecies were identified from the 193 myxomycete records. However, four of these species were only determined to the genus level (*Arcyria*, *Comatricha*, *Didymium*, and *Stemonitis*) because they were recovered from moist chambers wherein most of the fruiting bodies were already withered. The list of species presented hereafter has a total of 28 species belonging to 14 genera. To evaluate the sampling effort used in this study, an individual based species accumulation curve was constructed using the software estimates and showed that the mean Chao 2 estimator reached a constant value of 59 (Fig. 2). Using the formula of Ndiritu *et al.* (22) to calculate the exhaustiveness of the sampling effort for the whole study, our results gave us a computed sampling effort of 54.2% for the present study.

Assessing the occurrence of the 28 determinable myxomycete species, two species were reported to be abundant,

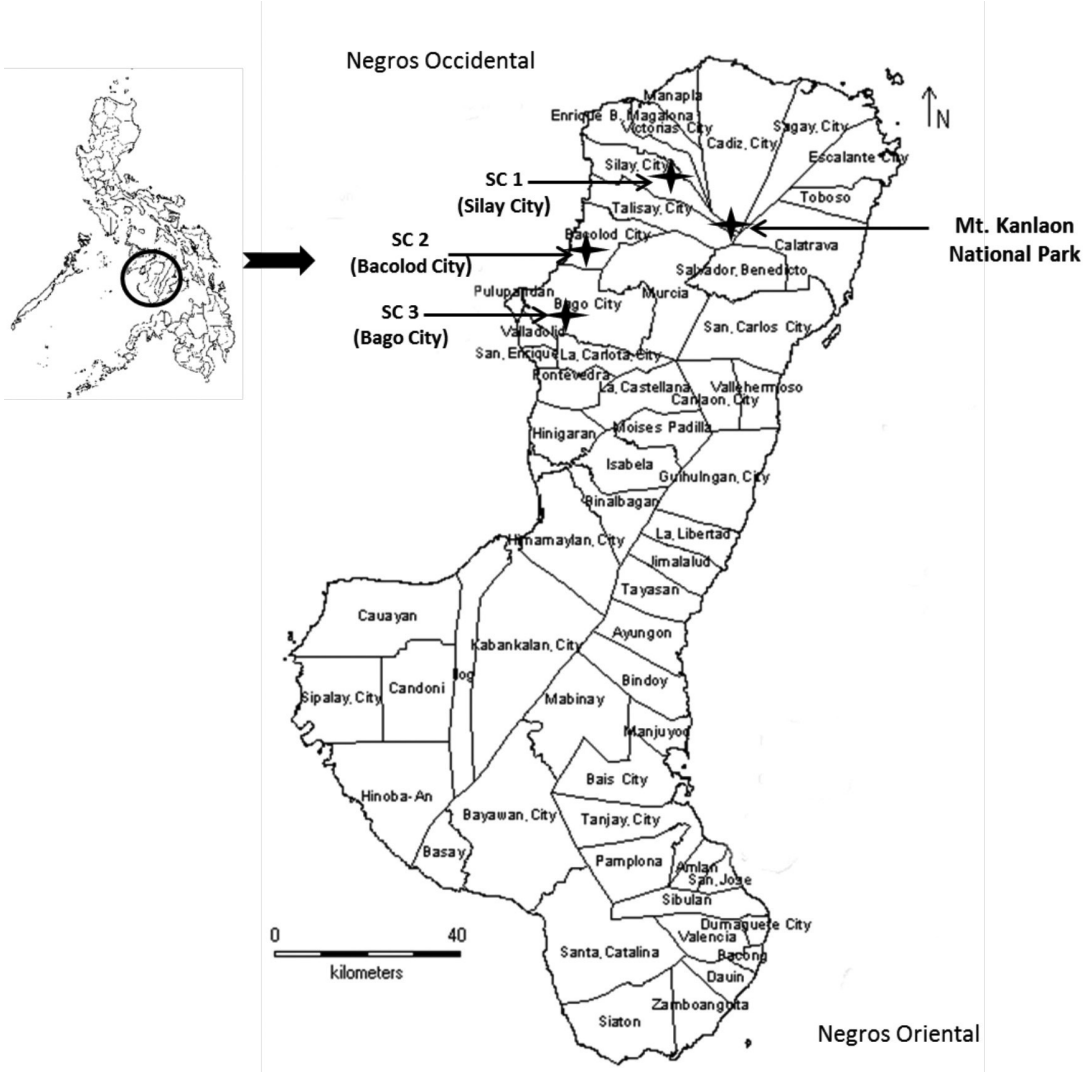

Fig. 1. Study sites: Mt. Kanlaon National Park (forested areas) and the agricultural plantation (SC) in Negros Occidental, Western Visayas, Philippines, May 2013.

namely *Arcyria cinerea* and *Didymium nigripes*. Nine species were common, four were occasional and 13 were reported to be occurring as rare (Table 1). Comparing the composition of myxomycetes from the different substrates collected from two habitat types, 27 species were found in forested areas that were characterized to have heterogenous plant litter and only two species were accounted in the sugarcane plantations.

In terms of productivity of the microhabitats tested in this study by using the moist chambers, 121 of the 450 MCs (27%) were positive for growth of myxomycetes either as plasmodia or as fruiting body. All of the moist chambers prepared had a relatively acidic mean pH condition. Highest percent yield

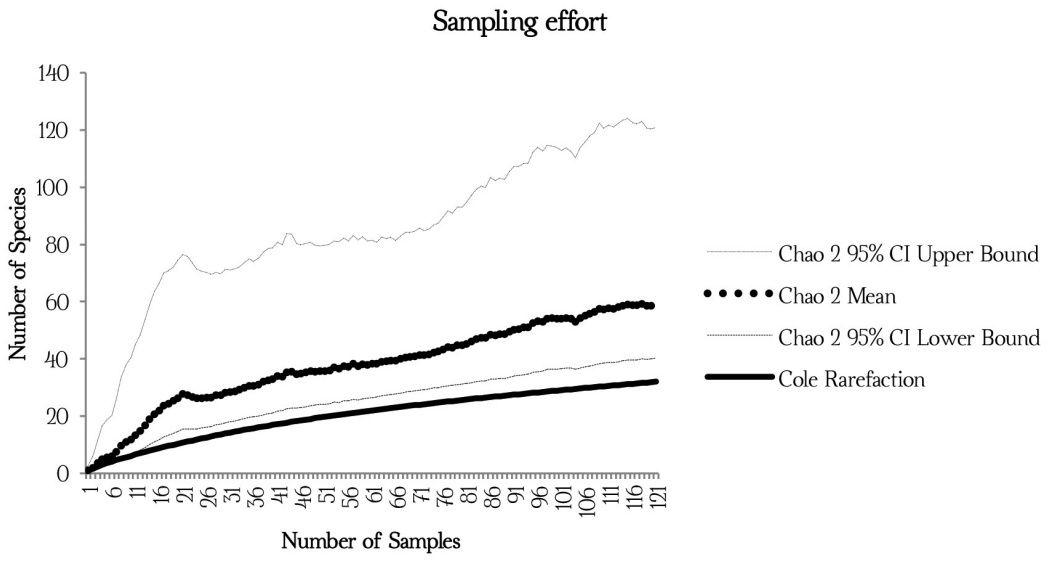

Fig. 2. Individual based species accumulation curve smoothed by Cole rarefaction for the myxomycetes collection in Negros Occidental.

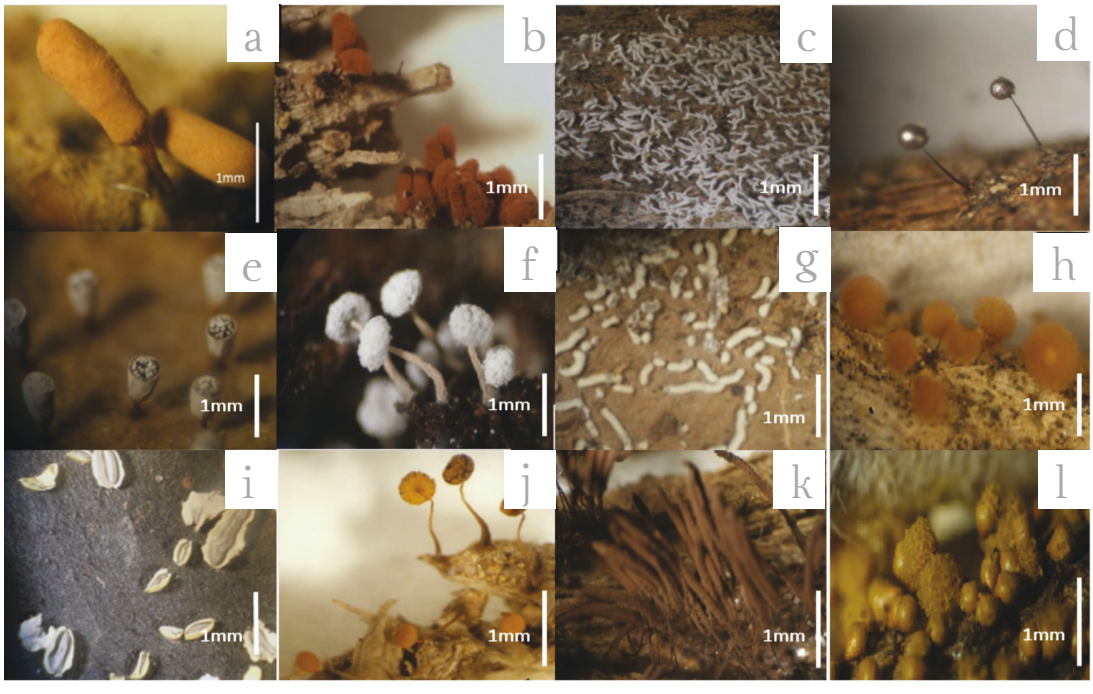

Fig. 3. Some representative myxomycetes collected in Negros Occidental: (a) *Arcyria cinerea*, (b) *Arcyria denudata*, (c) *Ceratiomyxa fruticulosa*, (d) *Collaria arcyrionema*, (e) *Craterium leucocephalum var. cylindricum*, (f) *Didymium squmolosum*, (g) *Diderma effusum*, (h) Hemitricha *calyculata*, (i) *Physarum bogoriense*, (k) *Stemonitis fusca*, and (l) *Trichia decepiens*.

Table 1. Occurrence of myxomycetes in Negros Occidental showing the number of records accounted from the rapid field survey and the use of moist chamber cultures

Species	Number of Records							
	Field Collection	Moist Chamber Cultures						Total
		AL	GL	F	TW	VN	S	
Abundant								
Arcyria cinerea (Bulls.) Pers.	1	25	10	10	5		2	53
Didymium nigripes (Link) Fr.	4	2						6
Common								
Arcyria denudata (L.) Wetts.	4							4
Ceratiomyxa fruticulosa (O.F. Mull.) Macbr.	3				2			5
Craterium leucophaeum var. *cylindricum* (Pers ex J. F. Gmel) Ditmar	3							3
Diderma effusum (Schwein.) Morgan	2	2				1		5
Hemitrichia calyculata (Speg.) M.L. Farr.	4							4
Lamproderma scintillans (Berk& Broome.) Morgan.	1	1			2			4
Physarum album (Bull.) Chevall.	3	1						4
Physarum viride (Bull.) Pers.	2		2		1			5
Stemonitis fusca Roth	1	1			1			3
Occassional								
Cribraria cancellata (Batsch) Nann. – Bremek.	2							2
Cribraria microcarpa (Schrad) Pers.	2							2
Didymium iridis (Ditmar) Fr.	1	1						2
Physarum melleum (Berk. & Broome.) Massee.	2							2
Rare								
Arcyria afroalpina Rammeloo		1						1
Collaria arcyrionema (Rostaf.) Nann. – Bremek. Ex Lado.					1			1
Comatricha nigra (Pers. Ex J. F. Gmel) J. Schroet	1							1
Didymium squamolosum (Alb. & Schwein.) Fr.	1							1
Hemitrichia serpula (Scop.) Rostaf. Ex Lister.	1							1
Physarum bogoriense Racib.	1							1
Physarum cinereum (Batsch) Pers.						1		1
Physarum compressum Alb. & Schwein.	1							1
Physarum echinosporum Lister				1				1
Physarum nucleatum Rex	1							1
Stemonitis axifera (Bull.) T. Macbr.					1			1
Trichia decepiens (Pers.) T. Macbr.	1							1
Tubifera ferruginosa (Batsch) J.F.Gmel.							1	1

was observed from the aerial litter (72%), and consequently had the most number of records of myxomycetes (Table 2). The other substrates, such as the twigs and vines had the next highest number of percent yield. However, these substrates had a relatively low number of determinable records due to the fact that most of the substrates were recorded as positive culture because of the appearance of plasmodium during the incubation period. These MCs were unsuccessful in developing into fruiting bodies (Table 2). Lowest percentage yield (18%) was observed in sugarcane litter randomly collected in the agricultural plantations of the study area. Moreover, among the six substrates collected in the two types of habitats (forest and agricultural), twigs had the highest species diversity as calculated using Gleason index (Hg

=2.12) and ferns had the highest species evenness based from Pielou's Evenness index (E =1.00). However, using the Shannon index that considers species diversity and species evenness, twigs gave the highest value (Hs = 0.75). Comparing the assemblages of myxomycetes between the two habitat types, a CC value of 0.08 and PS value of 0.48 were computed in the study (Table 3). These results show that species similarities between the two sites were only 8.3%, which is relatively low since the only species of myxomycetes that was present in both sites was *Arcyria cinerea*.

Table 2. Statistics of the different substrate types used in the moist chamber

Substrate Type	Cultures prepared	Positive for myxo	Percent Yield	Number of determinable records	mean pH value	Hg	E	Hs
Mountainous forest								
Aerial litter	90	65	72	34	5,82	1.99	0.31	0.47
Ground litter	90	19	21	12	5,99	0.40	0.18	0.13
Twigs	90	35	38	13	5,86	2.12	0.61	0.75
Vines	30	11	37	2	5,68	1.44	0.10	0.30
Ferns	60	13	22	11	6,20	0.42	1.00	0.13
Agricultural plantation								
Sugarcane	90	16	18	3	5,91	1.44	0.50	0.15

DISCUSSION

In terms of biodiversity, the Philippines is considered to be one of the most diverse countries in the Asia Pacific basin. However, microbial diversity assessments in the country are under-investigated. This particularly holds true among the less explored fungus-like protists like the myxomycetes where a generally tropical condition would seem to be favorable for their growth and development (15). In fact, in recent years, most investigations on myxomycetes in the Philippines were concentrated only among the forest vegetation and coastal habitats of the Luzon main island (3,18). Thus, findings in this research paper are the first intensive diversity report for the Visayas group of islands of the Philippine archipelago.

PRODUCTIVITY OF MYXOMYCETES IN MOIST CHAMBER CULTURES

The use of a moist chamber culture in assessing the occurrences of myxomycetes was a vital component for this study. Current studies from arid environments in China (28) and submerged plant materials in the Big Thicket National Preserve (40) employed the usage of this technique to recover species of myxomycetes not easily seen on the field. In our study, a total of 450 moist chambers were prepared wherein only 27% were positive for myxomycete growth. After almost 15 weeks of incubation, 11% showed positive results for fruiting bodies, while 16% were positive for plasmodial growth. This now shows that the majority of

the plasmodium specimens were not able to develop to fruiting bodies. In comparison to related local studies, a similarly low yield was observed by Dagamac *et al.* (5), wherein 17.5% yielded plasmodia and only 5.1% yielded fruiting bodies from different bark samples collected from the Luzon Islands. However, Kuhn *et al.* (13) had a percentage yield of 51%, or 214 out of 420 moist chamber cultures containing 40% positive for plasmodia and 23% positive for fruiting bodies in six highland areas in Luzon. Substrates collected in a protected ecopark by Macabago *et al.* (19) had a percentage yield of 51%, or 121 out of 240 moist chambers. It seems now that most studies of myxomycetes in the Philippines that used moist chambers always supported a higher level of plasmodium yield than recovering fruiting body phenologies. Perhaps the fast dessication of most of the moist chambers during the incubation time can be a factor here, since a suitable moist environment is needed to allow for the plasmodium to successfully develop into fruiting bodies. It is important to note that in doing myxomycetes biodiversity and distribution studies by means of the moist chamber technique, fruiting bodies are more important as compared to plasmodium or sclerotia, since most species identification is based on the determinable morphologies of the fruiting body (11, 31). Moreover, among the six substrates collected in our study, aerial leaf litter yielded the highest level of success. The highest productivity yield from aerial leaf litter was also recorded from other studies in the tropics, including Rojas & Stephenson (27) who reported 93% in the Coco's Island, Costa Rica, and in a more recent comparative species listing of dela Cruz *et al.* (6) between substrates collected in the tropics and the temperate ecoregions. This may be attributed to specimen exposure to open air where aerial litter has a higher potential in catching spores. This supposition was already demonstrated by the studies from Schnittler & Stephenson (30) where the authors noted that slight breeze can cause the myxomycetes spores to be dispersed more than one kilometer from the starting point. Perhaps aerial litter from our study has a higher probability to trap spores dispersed by wind.

SPECIES COMPOSITION OF MYXOMYCETES IN NEGROS OCCIDENTAL

In this study, 28 morphospecies of myxomycetes were collected from lowland montane forests and sugarcane plantations in the northern part of Negros Occidental. This number is similar in comparison to other lowland montane vegetation area studies conducted in the Luzon main islands, including Mt. Arayat National Park (3) and in Mt. Makulot (1), which reported 30 and 28 morphospecies of myxomycetes, respectively. Albeit this number of morphospecies is not yet reflective of the overall number of myxomycetes that can be accounted in Negros Occidental as was suggested by the 54.2% sampling effort for this study, findings from this research paper serve as a good starting basis for future directives in understanding the distribution of myxomycetes in a local setting. To expand the sampling effort, it is recommended to increase the distance covered during intensive surveys and to add other substrates, i.e. barks of living deciduous trees, dung of herviborous animals, and inflorescences where myxomycetes can also thrive.

In terms of species composition in the whole study area, *Arcyria cinerea* was noted to be the only abundant species found in both the agricultural and forest habitats, with the other species occurring relatively rarely. Stephenson (35) had the highest percentage yield of *A. cinerea* in moist chambers (85% in the upland temperate forest of Southwestern Virginia, USA). Similar results in terms of occurrence were also obtained from the studies conducted by Rojas *et al.* (26) in the northern Neotropics and

Kuhn *et al.* (12) in Anda island in Pangasinan, Philippines. Our findings now support other previous results that also showed *Arcyria cinerea* to be of cosmopolitan distribution worldwide, since it is widely known to be tolerant to many environments.

MYXOMYCETE DISTRIBUTION IN AGRICULTURAL LITTER IS MORE LIMITED THAN IN FOREST LITTER

Most of the related studies on myxomycetes in the Philippines always used litter from the forest floor. To the best of our knowledge, the findings in this paper are the first report for the Philippines attempting to evaluate myxomycetes in a sugarcane plantation where the decaying litter and vegetation is generally specific and to compare it to the myxomycete communities in forest litter where decaying litter is more heterogenous. In contrast to related studies in the Paleotropics, our findings seems to contradict the observations of Tran *et al.* (38), which intensively evaluated distribution of myxomycete assemblages in agricultural ground litter and the forest floor. Their results showed a relatively higher productivity among agricultural litter than the forest floor litter during both the rainy and dry seasons. Perhaps the smooth surface of the sugarcane leaf is not a favorable spore trap for other myxomycetes species in contrast to the pubescent surfaces of the three agricultural litters used in Thailand (banana, mango and corn plantations). Nonetheless, findings from our study supports the theory that diversities of plant communities and litter heterogeneity (37) in a study area influence the composition of myxomycete assemblages, as evident from a higher number of myxomycete occurrences in the forest floor litters in Negros Occidental.

MYXOMYCETES FROM NEGROS OCCIDENTAL AS BASELINE INFORMATION

An understanding of the distribution for myxomycetes in the Philippines is still far from complete. Many ecological factors and/or unexplored landscapes in the country still need to be investigated. Despite the findings presented in this study, it is still significant to note the limitations of a descriptive study like this are associated with the sampling efforts in collecting the substrata used in the study. The most noteworthy contribution of this paper relates to the fact that it increases the knowledge about the local ecology of myxomycetes in an ecoregion of the world where investigations about myxomycete diversity is still considered to be in its infancy.

ACKNOWLEDGEMENTS

The authors are grateful to the Department of Environment and Natural Resources (DENR, PAMB), Negros Occidental, Region VI for the gratuitous permit granted for the collection and field survey in Mt. Kanlaon Nation Park. Moreover, JRA, DLA, JBA and TEDC are indebted to the Philippine Society of Microbiology (PSM) for the undergraduate thesis subsidy grant used for this research study. NHAD would also like to thank the Deutscher Akademischer Austauschdienst (DAAD) for the scholarship grant. Furthermore, the authors would like to thank Angelica Rea – Maminta and Sittie Aisha Macabago for all the technical assistance and to the anonymous reviewers that helped in improving this manuscript.

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RELATIONSHIPS BETWEEN ALGAL BIOMASS AND DIVERSITY WITH STREAM SIZE AND ADJACENT LAND USE

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MANUSCRIPT RECEIVED 15 SEPTEMBER, 2014; ACCEPTED 30 OCTOBER, 2014

ABSTRACT

Land use adjacent to waterways, such as development or agriculture, alters hydrological patterns leading to increases in runoff and nutrient input. Forests and wetlands, as natural land cover types, reduce water movement and allow infiltration into soil. We measured algal biomass and diversity in order to quantify the influence neighboring land cover types have on streams in Northeastern Indiana. In the study area, cultivated crops were the dominant land cover type, with open development and deciduous forest following. Emergent wetland area had the greatest influence on algal biomass, with increases in wetland area decreasing biomass. However, open development, low intensity development, grassland, shrub, and forested wetlands added to increases in biomass. Conversely, forested wetlands reduced algal richness, while open development and pastures increased richness. Because open development (i.e. dominated by turf grass, lawns, parks, golf courses) was the second most common land cover type and positively influenced both algal biomass and richness, management of those properties will likely have direct impact on nutrient flow into streams. Additionally, adding functional wetlands dominated by emergent herbaceous plants will directly impact future algal biomass.

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KEYWORDS

• algae
• eutrophication
• nutrient flow
• chlorophyll
• runoff

INTRODUCTION

Land use and cover types can directly influence water infiltration and runoff patterns within a watershed (22). Decreases in natural vegetation often results in increases in surface runoff (15). Additionally, the complexity of layers within the vegetation cover can influence infiltration of water into soil (26). Developed land cover types have decreased in tree cover in the U.S., while increasing in impervious surfaces (18). Both changes in tree and impervious land cover have direct impacts on runoff volume and soluble nutrient content (2). Assessing the anthropogenic changes to land cover types may be important in identifying potential water quality issues (20).

In addition to decreases in infiltration and increases in runoff, there is a resultant increase in sedimentation and nutrient content associated with anthropogenic land cover changes (i.e. simplified vegetative structure, impermeable surfaces) (7,15,17,23). Degradation of freshwater

has been clearly linked to increased nitrogen and phosphorous fertilization (24). Increases in human population densities have resulted in increases in nitrate and suspended solids within streams (1). While agricultural fertilization may add to eutrophication, urban development imparts major influence on biologically available phosphorus (4). Increased nutrient content within streams leads to human and environmental health issues including increases in disease vector populations and toxic algal blooms (11,13,16).

In the Midwest region of the United States, agricultural land dominates the land cover types, with cultivated crops and pasture lands making up 80–90% of land cover (6). In Indiana major changes in land cover between 2001 and 2006 were focused around the state's larger urban areas (i.e. Indianapolis, Fort Wayne, Evansville) (8). While there is minimal change in land cover types in Indiana, those persistent land cover types adjacent to streams may have sustained and continual influence on water quality. The objectives of this study were to 1) quantify algal diversity and biomass within streams in Northeastern Indiana, 2) compare the influence of stream channel size and land use on algal communities, and 3) test the hypothesis that neighboring land use types influence algal diversity and biomass.

MATERIALS AND METHODS

We selected thirteen sites in Adams, Huntington, and Wells Counties, Indiana, based on size and access from public property (i.e. road crossings; Fig. 1). Each stream was measured at the sampling location from bank to bank. Six small streams (< 8m channel width), four medium streams (≥ 8 and < 20m width) and three large streams (≥ 20m width) were selected (Fig. 1). Monthly from May to August 2013, we collected 40 mL of water from the center of the channel for each stream. Samples were immediately separated into 20 mL containers, each for chlorophyll a biomass and diversity assessments.

We used methods described by EPA (5) for chlorophyll a extraction. Briefly, the 20 mL samples were centrifuged (9000 RPM, 20 minutes), aqueous layer decanted, and 10 mL of acetone was added and mixed with the pellet on a vortex. The acetone solution was stored for 24 hours at 5°C, centrifuged (7000 RPM, 5 minutes), and aqueous layer decanted for spectrophotometry. Using the equation presented by EPA (5), we calculated µg/L of chlorophyll a in the streams. For diversity, we identified algal morphospecies as a rapid assessment tool within three random microscope views per sample (100 µL per slide, 100x magnification). Morphospecies were defined based on shape (i.e. spherical, filamentous), motility (i.e. non-motile, flagellated), and colonial status (unicellular, colonial). We used morphospecies richness (counts of different morphospecies) as a measure for algal diversity.

Land use adjacent to the stream were assessed using 2011 National Land Cover Data (10). In ArcMap (version 10.1, ESRI Inc., Redlands, CA), land use were categorized in a 0.5 km buffer around the stream channel 2 km upstream from the sample location (including all tributaries within the 2 km buffer). The area of land within the assessment buffers for each land use type were calculated. Additionally, the number of tributaries within the assessment buffer were counted for each stream.

Stream size classes were compared using one-way analysis of variance (ANOVA) for total land area within assessment buffers and num-

ber of tributaries, as well as repeated measures ANOVA for algal richness and biomass. Tukey HSD was used as a post-hoc multiple comparison test. Pearson correlations were used to identify relationships between algal richness, algal biomass, and number of tributaries. Multiple regression with reverse variable selection was used to identify relationships between stream size and land use types, with biomass and morphospecies richness. All analyses were conducted in R (version 3.1.1, The R Foundation for Statistical Computing, Vienna, Austria).

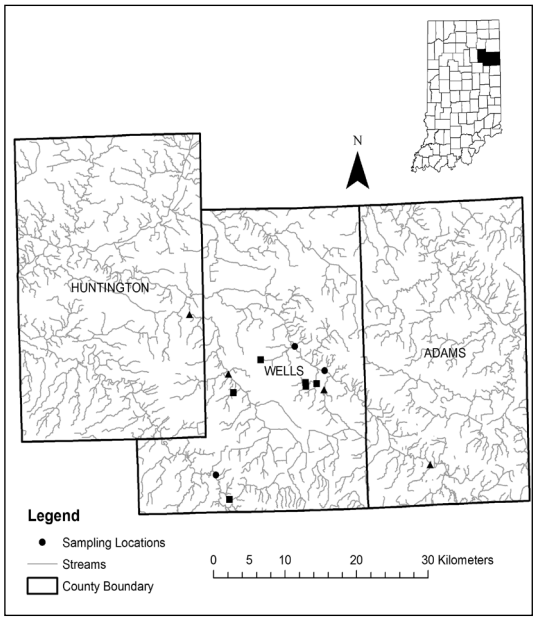

Fig. 1. Sampling locations within Adams, Huntington, and Wells Counties, Indiana. Squares indicate small stream sampling locations (<8 m channel width), triangles indicate medium streams (>8 and <20 m width), and circles indicate large streams (>20 m width).

RESULTS

Large streams had significantly more tributaries within the 2 km upstream from the sampling location than small streams ($F_{2,10} = 8.19$, $p = 0.008$). However, the total area within the assessment buffers were not significantly different between the size classes ($F_{2,10} = 1.06$, $p = 0.382$). Stream size classes were not significantly different in chlorophyll a biomass ($F_{2,6} = 1.59$, $p = 0.279$, Table 1) using repeated measures over the months. However, stream sizes were significantly different in monthly algal richness, with large and medium streams having greater numbers of morphospecies than small streams ($F_{2,6} = 13.48$, $p = 0.006$, Table 1). Algal biomass and richness were not significantly correlated ($r = 0.34$, $p = 0.259$). Mean algal biomass was significantly correlated with number of tributaries upstream ($r = 0.61$, $p = 0.026$), while richness was not correlated with tributaries ($r = 0.36$, $p = 0.223$). Using different models defining the relationship between algal biomass and total phosphorus (12,25), estimated mean phosphorus ranged from 0.003 (± 0.002) to 0.057 (± 0.018) mg/L.

We encountered thirteen of the 20 land cover types in the land assessment buffers (Table 2). Cultivated crop agriculture dominated the land cover types accounting for 77.2% of the area within the study. Open development and deciduous forest lands were distant second and third in area, accounting for 6.7% and 6.5%, respectively. Land cover types provided significant multiple regression models for both algal richness and biomass

(Table 2). For algal richness, open developed land and pasture had positive influence, while woody wetlands had negative influence. For algal biomass, open and low developed land, grass, shrubs, and woody wetlands had a positive influence. Emergent wetlands had the strongest influence and negatively impacted algal biomass.

Table 1. Algal biomass measured in µg/L chlorophyll a (SE) and species richness (SE) per size class over four sampling periods.

	May		June		July		August	
Size	Biomass	Richness	Biomass	Richness	Biomass	Richness	Biomass	Richness
Small	15.32	0.00	20.63	0.67	23.32	0.67	19.63	0.67
	(4.34)		(10.48)	(0.33)	(8.09)	(0.21)	(2.83)	(0.21)
Medium	54.20	1.00	39.35	2.00	27.86	0.67	20.66	2.67
	(39.72)	(1.00)	(17.63)	(1.00)	(6.25)	(0.33)	(5.13)	(0.33)
Large	246.19	1.50	23.28	2.25	39.99	1.75	48.39	2.00
	(142.14)	(0.65)	(13.39)	(0.48)	(2.82)	(0.48)	(11.45)	(0.91)
All	105.24	0.83	27.75	1.64	30.39	1.03	29.56	1.78
	(41.32)	(0.14)	(2.08)	(0.20)	(1.54)	(0.08)	(2.58)	(0.22)

DISCUSSION

Land use cover type changes can greatly influence runoff and fertilization of local streams (2,15,18,22,26). The majority of changes occur as shifting from natural land cover with complex vegetative layers to simplified layers (17,20,23). Within the study area in Northeastern Indiana, little land cover has changed recently; agriculture has dominated. Dominance of both agricultural and developed land, both anthropogenic simplifications of the natural land cover, greatly alter stream nitrogen and phosphorus contents (1,7,24).

Stream size classes were designated as arbitrary categories based on channel width at the sampling location. As evidenced by the significant correlation between algal biomass and number of tributaries two km upstream from the sampling point, a categorical index based on tributaries may have been a more appropriate size category. The land cover/tributary interaction on the landscape can have substantial impact downstream (19). However, since our buffer production began with a 500 m buffer around all streams and then used a two km buffer, the area of land that was potentially draining into the sample stream channel didn't differ between size classes. The tributary influence would have been included in the arbitrary size class categorization (large streams with more tributaries than small).

While nitrogen and phosphorous are im-

Table 2. Land use types occurring within stream buffers (0.5 km x 2 km upstream) with mean number of tributaries (SE) and mean ha area (SE) for small (< 8 m), medium (≥ 8 m, < 20 m), and large (≥ 20 m) width streams.

		Land Use Cover Types*												
Stream	Count of		Developed				Forest				Planted/Cultivated		Wetlands	
Size	Tributaries	Water	Open	Low	Medium	High	Deciduous	Evergreen	Shrub	Grass	Pasture	Crops	Woody	Emergent
Small	1.2	0.64	13.20	1.61	1.04	1.03	11.69		0.07	2.91		224.68	0.19	0.17
	(0.3)	(0.26)	(1.84)	(1.35)	(0.95)		(4.55)			(0.79)		(35.17)		(0.11)
Medium	3.5	2.84	27.95	2.04	0.46		26.21		0.59	5.46	26.93	365.12	8.58	1.89
	(0.9)	(1.75)	(6.87)	(1.58)			(10.43)		(0.49)	(1.23)	(16.77)	(22.75)	(8.05)	(1.13)
Large	5	4.18	45.15	23.69	8.16	4.16	46.79	2.79	0.80	13.95	4.85	316.27	5.31	7.36
	(1.1)	(2.10)	(9.65)	(16.54)		(3.83)	(31.64)	(2.52)	(0.44)	(9.08)		(84.87)	(2.76)	(2.66)
All	2.8	2.14	25.11	6.84	2.50	1.43	24.26	0.64	0.40	6.24	9.40	289.03	3.96	2.36
	(0.6)	(0.77)	(4.61)	(4.26)	(1.89)	(0.98)	(8.17)	(0.60)	(0.19)	(2.24)	(5.85)	(29.61)	(2.53)	(1.02)

Table 3. Multiple regression equations for predicting algal richness and biomass with adjacent land cover type area within a 0.5 km wide buffer 2 km upstream from sampling location.

Dependent	Equation	F	df	P-value	R^2
Richness (count)	1.05 + 0.06*DevelopedOpen + 0.08*Pasture − 0.20*WetlandsWoody	8.11	3,9	0.006	0.73
Biomass (µg/L)	7.11 + 2.91*DevelopedOpen + 1.76*DevelopedLow + 2.79*Grass + 6.73*Shrub − 10.57*WetlandsEmergent + 2.14*WetlandsWoody	33.69	6,6	< 0.001	0.97

portant nutrients in driving algal population size, phosphorus is considered the more important of the two in freshwater systems (3). Because of this, we wanted to use published models for predicting algal biomass to inversely estimate stream phosphorus content. Due to differences in systems and model techniques, we estimated a very broad range of phosphorus (0.003–0.057 mg/L) (12,25). Even with this broad range, the estimated phosphorus content of the streams were well below the benchmark values set by the Indiana State Department of Agriculture of 0.3 mg/L (9). While there are limitations to interpreting this conversion of algal biomass to estimated total phosphorus, it does indicate that the streams we sampled were well below the State's benchmark value.

In our study area, combined agriculture (pasture and cultivated crops; 80% of area) and combined development (open, low, medium, and high; 10% of area) were the clearly dominant land cover types. However, agriculture was not included in either algal richness or biomass models. Likely this is due to the ubiquitous nature of agriculture as a dominant land cover within the buffers for every stream. Changes in open developed land for algal richness, as well as both open and low intensity for algal biomass, significantly added positively to the multiple regression models. While phosphorus is often intentionally added to agricultural fields, total biologically available phosphorus during baseline flow rates in agricultural streams appears similar to urban streams (1,4,24). Increasing urban development dramatically increases coverage of impermeable surfaces, decreasing water infiltration, and increases total biologically available phosphorus in streams (2,4,17,20). The influence of open and low intensity land use in the two models fits with an expectation of a potential increase in phosphorus with increasing area.

The effect of wetlands on algal biomass and richness may be explained through absorption of nutrient-rich runoff from the dominating agricultural land use. However, emergent herbaceous wetlands have limited phosphorus retention capacity, while forested wetlands may serve as phosphorus sinks (14,21). Our results may indicate interactions between natural systems: stream channel and natural wetlands. The two strongest influences within both models were negative associations with woody and emergent wetlands. Both are common wetland types, when wetlands occur, within the study region. Within our buffers, wetlands only accounted for 1.7% of land area. Even though they were not a dominant land type, wetlands play a substantial and dramatic role in the algal richness and biomass responses within the study streams.

Our results highlight the importance of responsible land management near waterways to maintain high water quality. As part of the Indiana Nutrient Reduction Strategy (9), there is a focus on urban residential fertilizer application in addition to the typical focuses on agriculture and sewer management. Open development land use (i.e. dominated by turf grass, lawns, parks, golf courses) has strong positive influence on algal richness and biomass, which could have future water quality implications if land uses change with increases in urban and suburbanization within the region.

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IDENTIFICATION OF DIATOMS IN A HEALTHY PENNSYLVANIA STREAM COMPARED TO THREE DOWNSTREAM SITES IMPACTED BY ABANDONED MINE DRAINAGE

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MANUSCRIPT RECEIVED 29 APRIL, 2014; ACCEPTED 30 MAY, 2014

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KEYWORDS

- diatom
- abandoned mine
- drainage
- borehole
- *Eunotia exigua*

ABSTRACT

Life in a healthy stream can be severely impacted by changes in pH and other water quality parameters. This study reveals differences in diatom diversity and water quality characteristics in a central Pennsylvania stream. One healthy site was compared to three nearby sites affected by abandoned mine drainage during a July sampling in 2013. Permanent slides were made and microscopically assessed for diatom identification. The healthy stream contained eleven diatom genera while the site most impacted by mine drainage showed only one diatom, *Eunotia exigua*. Data were analyzed for Shannon diversity index and species richness. Water samples showed differences in pH, aluminum, sulfate, and iron. This work demonstrates the use of diatoms as bioindicators of stream health.

INTRODUCTION

Abandoned mine drainage (AMD) is a prominent source of pollution in currently and previously mined areas throughout the United States. AMD impacted water is saturated with metals such as iron and may be very low in pH making it an inhospitable environment for the majority of aquatic life. Hughes borehole is a source of AMD pollution that flows into the Little Conemaugh River near Portage, Pennsylvania. The borehole was drilled in the 1920s to release water from miles of flooded underground coal mines in the area. The borehole was capped in 1950s only to blow out due to underground pressure some twenty years later. Since then, water with a pH as low as 3.08 has been bubbling out of the borehole at a rate of 800–3500 gallons per minute and blanketing the surrounding six acres with a reddish brown iron precipitate. (2)

Mine drainage occurs in areas where water comes in contact with exposed rocks that have a high concentration of sulfide minerals. Pyrite, also known as fool's gold, is a common mineral found with coal in the eastern United States. The oxidation of pyrite and other sulfide-rich minerals causes the release of sulfuric acid and metal ions. If a stream has a limited buffer capacity, the pH will continue to decrease, thus increasing the oxidation reactions and the precipitation of metals. When the temperature of the water increases in the summer months, gases such as oxygen become less soluble and salts become more soluble. (1)

Diatoms are unicellular, photosynthetic algae which can survive in a wide variety of aquatic environments. Each diatom species has a specifically shaped silica cell wall, called a frustule, which is used for microscopic identification. Diatom species are found in two different microenvironments, they are either suspended in water (planktonic) or growing on a substrate (benthic). Environmental factors such as pH, light availability, and temperature may cause teratology in frustule morphology,

and particularly in harsh environments can have an effect on overall growth characteristics of the diatom. These factors, most importantly temperature, can have an effect on solubility of salts and gases found within waters especially those impacted with AMD, thus leading to large overall changes in water chemistry. Fluctuations in water chemistry throughout the year due to temperature change can have an effect on diatom species present as well as seasonal variation in diatom populations. Each diatom species has specific growth parameters and morphology giving us the ability to identify them by their frustule, making them good bioindicators of water quality. (6)
This study was undertaken to assess diatom diversity in a healthy stream and three sites downstream from the AMD outflow. The first site is the healthy stream, 40 m upstream of the AMD discharge with a pH of 7.12. The second site is at Hughes borehole, 5 m below the source of AMD discharge due to safety fencing, with a pH of 3.36. The borehole sits uphill and is completely devoid of vegetation. The third site is a naturally formed settling pond, 50 m below the discharge, where the flow of polluted water slows and has a pH of 3.24. The fourth site is at a bridge 600 m below the AMD discharge; roughly 30 m from where the healthy and low pH waters mix, with a pH of 6.68. Fig. 1 illustrates the four sampling sites.

MATERIALS AND METHODS

Sampling was conducted in July 2013. Diatoms were collected by harvesting biofilms from benthonic sediments by scraping a three centimeter square area into a sterile 15mL polypropylene disposable centrifuge tube (Fisherbrand). Two samples per site were gathered and processed in a ventilation hood by placing 25–30 mL of the sample into a 150mL beaker on a hotplate, then adding 10–15mL nitric acid (Flinn Scientific Inc.). Samples were then boiled to remove organic matter, leaving behind only diatom frustules per Sgro and Johansen (7). Centrifugation, decanting of liquid waste, and suspension in 10–15mL of distilled water was performed six times. Permanent slides were created by suspending diatom frustules in 70% denatured ethyl alcohol (Fisher Chemical) until a cloudy suspension was achieved. Samples were diluted to approximately 400 frustules per field of view on low magnification (100X total magnification) to allow clear observation on permanent slides. Approximately 1mL of solution was placed on a glass coverslip and the alcohol was allowed to evaporate overnight, leaving behind only diatom frustules fixed to the coverslip. Coverslips were permanently mounted on slides using naphrax mounting medium (Brunel). Slide sets of 24 slides were created for use in laboratories such as general microbiology and water ecology.

Diatoms were identified to the genus level using an online database, Diatoms of the United States, (8) and a diatom identification text (6). From each site, 400 total diatoms were identified under oil immersion (1000X total magnification). Diatom images were captured with a Zeiss Axiostar Plus light microscope and SPOT imaging system with an in-sight camera and edited with SPOT version 5.0 software (SPOT Imaging Solutions).

Water analysis was sent to G and C Coal Analysis Lab (Summerville, PA) for testing of the suspended solids, dissolved metals and pH. These data were used in conjunction with the Shannon diversity statistical analysis data. Relationships between water quality and diatom diversity were examined.

RESULTS

Diatom samples from the healthy stream showed the most genera present, eleven in total. The healthy stream site contained low species diversity due to one genus (*Navicula*) dominating the population (Table 1). Shannon diversity index data for this site showed a fairly low diversity rating of 0.855 and a low richness (evenness) rating of 0.356. Diatoms of the genus *Navicula* made up 78% of the population in this location followed by *Surirella* at 12% and *Eunotia* at 3.5%. One other notable genus found in the sample, *Staurosirella*, was present in trace amounts but only found at this particular site (Fig. 2).

The Hughes borehole sampling site contained only one genus, *Eunotia* sp. Within in this genus however, we noted morphological size differentials. We observed either short and fat or long and narrow frustule morphology (Fig. 3). At all other sampling sites *Eunotia* was observed as being uniform in length and width. Because only one species was present, the Shannon diversity index was 0 and evenness could not be calculated.

The settling pond site exhibited the poorest water quality from which we sampled but showed eight genera of diatoms in total. The population here was dominated by *Eunotia* sp. which accounted for 79.9% of the population. *Pinnularia* sp and *Navicula* sp. made up 15.4% and 2.45% of the population respectively. The remaining species of diatoms were present in trace amounts (Fig. 4). Shannon diversity index data from this site were similar to that of the healthy stream with a lower diversity score of 0.670 and a similar evenness score of 0.344.

The bridge sampling site 600m downstream showed the most diverse and evenly distributed population of all four sites. The Shannon diversity score here was 1.01 and this was the most evenly distributed population with a richness score of 0.439. *Navicula* sp. made up 73.2% of the sample but three other genera made up more than 5% of the population, *Surirella* (9.97 %), *Eunotia* (6.81 %), and *Planothidium* (6.08%). (Fig. 5)

Water quality data, comparing pH, suspended solids, total iron, sulfate, and total aluminum concentration in mg/L (Table 2) were taken at each site. The healthy stream (pH 7.12), had the lowest measured concentrations of suspended solids, total iron, sulfate and total aluminum at 8 mg/L, 0.16 mg/L, .68 mg/L and 40.9 mg/L respectively. At the bridge site (pH 6.68), water quality data was very similar to that of the healthy site; total suspended solids were 9 mg/L, total iron was 1.58 mg/L, aluminum was 70.9 mg/L and sulfate was 1.16 mg/L.

At Hughes borehole and settling pond sites extreme amounts of metals were detected. At Hughes borehole (pH 3.36), iron was found at a concentration of 82.45 mg/L and aluminum is was found at 571 mg/L as well as total suspended solids with a concentration of 10 mg/L and sulfate of 10.25 mg/L. At the settling pond (pH 3.24), sulfate concentrations are 9.64 mg/L much higher than both the healthy stream and bridge sites. Iron was 76.67 mg/L and aluminum at 9.64 mg/L slightly lower than the borehole site whereas the settling pond showed a higher concentration of total suspended solids at 14 mg/L.

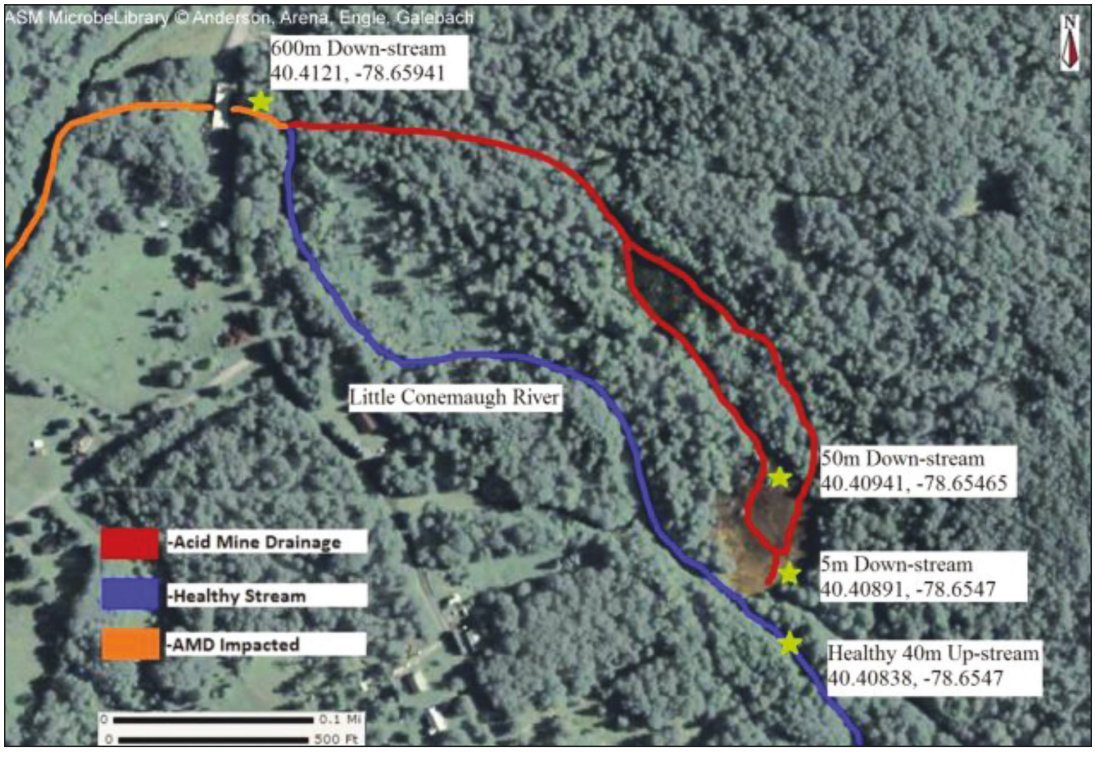

Fig. 1. This map shows the healthy stream and three downstream sites impacted by abandoned mine drainage.

Table 1. Summary of diatom and diversity findings at a healthy stream compared to three sites impacted by abandoned mine drainage.

Site	Shanon Diversity Index	Evenness	Number of diatom genera identified
Healthy	0.855	0.356	11
Hughes Borehole (5 m downstream)	0	0	1
Settling Pond (50 m downstream)	0.670	0.344	8
Bridge (600 m downstream)	1.01	0.439	11

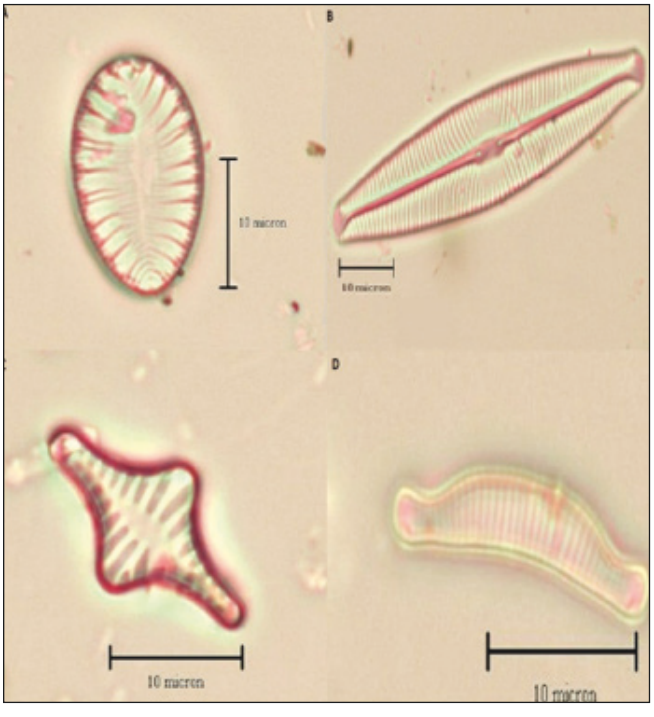

Fig. 2. On the right, the total number of diatoms counted in our healthy stream sample. On the left, diatoms from the genera *Surirella* (top left), *Navicula* (top right), *Staurosirella* (bottom left) and *Eunotia* (bottom right).

Genera	Healthy	Percent in Population
Eunotia	14	3.5
Navicula	313	78.25
Surirella	48	12
Cocconeis	1	.25
Diatoma	1	.25
Fragilaria	6	1.5
Meridion	2	.50
Melosira	7	1.75
Planothidium	7	1.75
Ulnaria	1	.25
Staurosirella	1	.25
Total	401	

Table 2. Water quality characteristics from the four sampling sites.

Site	pH	Suspended solids (mg/L)	Total Iron (mg/L)	Sulfate (mg/L)	Total Aluminum (mg/L)
Healthy	7.12	8	0.16	0.68	40.9
Hughes Borehole (5 m downstream)	3.36	10	82.45	10.25	571
Settling Pond (50 m downstream)	3.24	14	76.67	9.64	564.6
Bridge (600 m downstream)	6.68	9	1.58	1.16	70.9

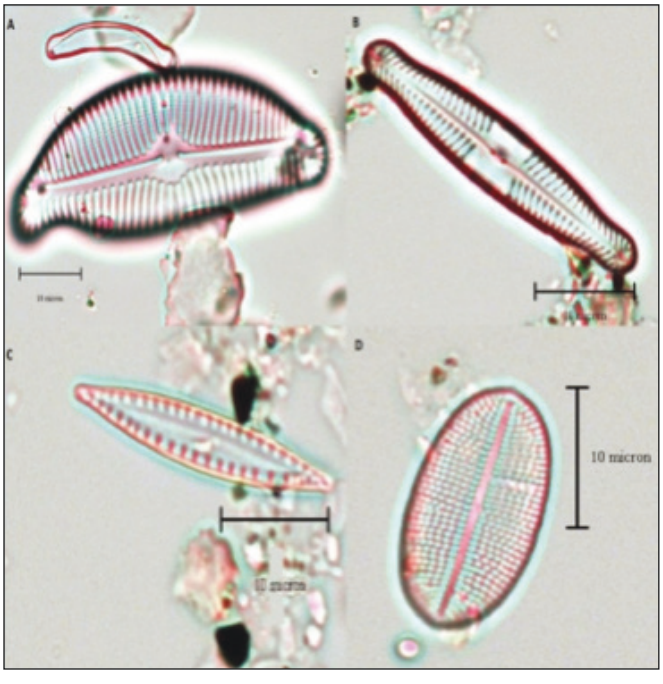

Fig. 4. On the right, the total number of diatoms counted in the settling pond sample. On the left, diatoms from the genera *Encyonema* (top left), *Pinnularia* (top right), *Pseudostaurosira* (bottom left), *Cocconeis* (bottom right)

Genera	Healthy	Percent in Population
Eunotia	326	79.9
Navicula	10	2.45
Encyonema	1	.24
Pinnularia	63	15.4
Surirella	2	.49
Cocconeis	4	.98
Diatoma	2	.49
Tabellaria	1	.24
Total	409	

DISCUSSION

We hypothesized an association between diatom distribution and water quality data, predicting that AMD impacted sites would exhibit reduced diatom diversity compared to the healthy stream.

The healthy stream site essentially served as a positive control site for comparisons and showed eleven genera of diatoms present. This was to be expected considering it had a neutral pH of 7.12 and was low in dissolved metals, with aluminum measured at 0.68 mg/L and iron at 0.16 mg/L, both within normal ranges for stream health

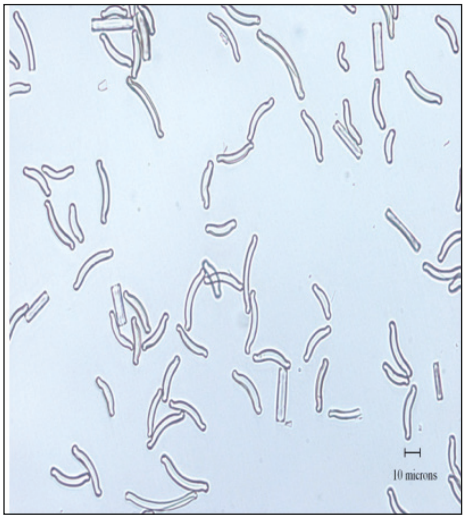

Fig. 3. *Eunotia* sp. found at Hughes borehole.

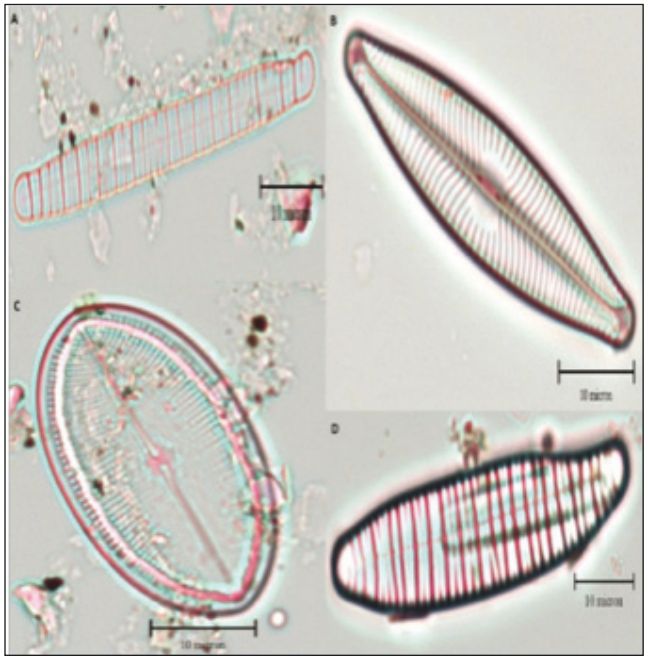

Fig. 5. On the right, the total number of diatoms counted at the bridge site. On the left, diatoms from the genera *Diatoma* (top left and bottom right), *Navicula* (top right), and *Cocconeis* (bottom left).

Genera	Healthy	Percent in Population
Eunotia	28	6.81
Navicula	301	73.2
Surirella	41	9.97
Diatoma	6	1.45
Tabellaria	1	.242
Fragilaria	3	.729
Meridion	1	.242
Synedra	1	.242
Melosira	4	.97
Planothidium	25	6.08
Total	412	

(4). The dominant diatom at this site was *Navicula* sp. making up 78% of the diatom population. *Navicula* was also found at the other three sampling locations; this not surprising considering *Navicula* can survive in a wide range of environments but will tend to thrive where water quality is the highest, making it valuable for assessing water quality and health of aquatic environments (3). The genus *Staurosirella* was also observed at the healthy site, but was not found at any other sites, leading us to hypothesize that it could be used as a bioindicator of good water quality. *Staurosirella* has been shown to thrive in moderate light and nutrient situations similar to the healthy stream we sampled from (5).

The two heavily AMD-impacted sites, Hughes borehole and the settling pond, are fairly similar in water quality. Both have an extremely acidic pH; the settling pond being slightly more acidic with a pH of 3.24 compared to a pH of 3.36 at the borehole. The two locations were also very high in concentrations of total dissolved solids and sulfates both of which are characteristic of AMD impacted waters that come in contact with organic sulfur compounds such as pyrite (FeS_2). As the AMD water emerges at the borehole, the slight increase in temperature changes the solubility of oxygen, removing it from solution and producing the iron precipitate found at the borehole and outlying areas.

There is a large "terrace" of precipitate as the water moves about 50m to the settling pond. Bottom dwelling organisms such as diatoms are particularly sensitive to AMD precipitates so we were surprised to find diatoms growing at both locations.

The only genus found at Hughes borehole was *Eunotia*, most likely *Eunotia exigua*. However, there were variations in morphology between *Eunotia* frustules found at the borehole and those found at our other three sites. *Eunotia* frustules at the other three sites all appear to be consistent in length, but what we saw at the borehole was individual diatoms within the population varying greatly in length but still showing all other morphological characteristics of the genus. We believe this morphological variability is due to lack of nutrients present within the borehole environment specifically the availability of silica, a known limiting factor of diatom growth. It is otherwise possible that we have two separate species of *Eunotia* here. Further research into this phenomenon is ongoing using DNA sequencing and other molecular techniques.

At the settling pond site we observed eight different species, which was unexpected considering the similar water chemistry to the borehole. *Eunotia* sp. dominated this population making up 79% of the total diatoms found in our sample. *Eunotia* at the settling pond did not show variable morphology like we observed at the borehole. Possible explanations for this increased diatom diversity include changes in nutrient availability, inflow of diatoms from surrounding unimpacted environments, or creation of microenvironments from increased organic matter. Another factor possibly contributing to the increased diatom growth at the settling pond could be due to a slight decrease of dissolved metals in solution. The precipitate found near the borehole appears to be coarse and quickly accumulates as the waters hit the surface. As this water makes its way 50m downstream to the settling pond the precipitate appears more as a mud or fine silt. This in conjunction with the micronutrients being added to this sampling site could be a possible explanation for the increased diversity of diatoms observed.

The final site, the bridge, showed the most evenly distributed population of diatoms. The water at this site was still moderately impacted by AMD; white aluminum and orange iron precipitate was clearly be observed. Of the eleven species present, there were very few genera that differed from the settling pond site. The precipitate observed at the bridge, like the settling pond, was very fine in texture. The population was mainly *Navicula* sp. (73.2 %), but three other genera made up over 5% of the population. This possibly shows that the genera present, specifically *Surirella*, can survive in harsh environments at the borehole and settling pond sites but don't thrive in that environment like at the bridge site where water quality was more suitable for life.

The Shannon diversity index measures overall species diversity, as well as richness or the evenness of the population. A site with a low richness score is often dominated by one or two main genera with the others only being present in trace amounts. Shannon diversity data in the healthy stream and settling pond sites were very similar as both populations are dominated by *Navicula* and *Eunotia*. Each site contained different diatom genera, but the ecosystem structure appears to be similar based on Shannon diversity calculations at each site. This suggests that the particular diatom genera present may be a better indicator of water quality than the diversity and richness of the aquatic life present. The borehole, not surprisingly, had a diversity and richness

score of zero due to only one genus being present. The bridge site, however, showed the most evenly distributed and diverse population of diatoms according to the Shannon diversity data. The population at the bridge site was still dominated by one genus, *Navicula*, but a few other diatoms emerged in numbers significant enough to balance the population. Although the bridge site was impacted by the AMD discharge, a healthy aquatic ecosystem was achieved according to Shannon diversity index and higher number of genera observed. This suggests that even a slight change in water chemistry and substrate can have a huge effect on aquatic life, meaning only a small amount of remediation is needed at AMD impacted sites to make a huge difference in overall stream health and ecology.

Samples for this study were collected in mid-July when water temperatures are warmer than other times of the year, particularly at the settling pond. We know from sampling in the winter months that some seasonal variation in diatom diversity does occur. Changes in temperature can impact water quality factors such as the solubility of salts. With warmer temperatures, more nutrients are found in solution which can be filtered and used by diatoms. Changes in temperature can also impact the solubility of gases such as oxygen. In the summer months oxygen can be removed from the solution more quickly due to decreased solubility. This leads to the reaction and precipitate formation occurring closer to the borehole and thus better living conditions in the settling pond conducive to increased diatom diversity. During the winter, this reaction may occur more slowly and have an effect on downstream diatom growth. Future research will focus on the amount of seasonal variation within our sites as well as the overall effects of precipitate formation on diatom growth.

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ACKNOWLEDGEMENTS Funding: NSF grant 1226175

ISOLATION AND CHARACTERIZATION OF HALOTOLERANT 2,4-DICHLOROPHENOXYACETIC ACID DEGRADING BACTERIA FROM SULFIDIC, LOW SALINITY SALT SPRINGS

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MANUSCRIPT RECEIVED 30 APRIL, 2014; ACCEPTED 30 MAY, 2014

ABSTRACT

The bacterial communities at two sulfidic, low salinity springs with no history of herbicide contamination were screened for their ability to grow on 2,4-dichlorophenoxyacetic acid (2,4-D). Nineteen isolates, closely matching the genera *Bacillus*, *Halobacillus*, *Halomonas*, *Georgenia* and *Kocuria*, showed diverse growth strategies on NaCl-supplemented and NaCl-free 2,4-D medium. The majority of isolates were halotolerant, growing best on nutrient rich broth with 0% or 5% NaCl; none of the isolates thrived in medium with 20% NaCl. The *tfdA* gene, which codes for an α – ketoglutarate dioxygenase and catalyzes the first step in 2,4-D degradation, was detected in nine of the salt spring isolates. The *tfdAa* gene, which shows ~60% identity to *tfdA*, was present in all nineteen isolates. Many of the bacteria described here were not previously associated with 2,4-D degradation suggesting these salt springs may contain microbial communities of interest for bioremediation.

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KEYWORDS

- 2,4-D
- *tfdA*
- *tfdAa*
- salt springs
- halotolerant bacteria

INTRODUCTION

Bacteria have tremendous potential to degrade organic compounds and study of the metabolic pathways involved is a key component to more efficient environmental remediation. One well-studied organic acid degradation pathway is that of 2,4–dichlorophenoxyacetic acid (2,4-D) (4, 12, 13, 15, 19, 23). The broadleaf herbicide 2,4-D was developed during World War II by British scientists to help boost production of grass crops like corn, wheat, and rice (23). Since the late 1940's it has been one of the most popular herbicides used by industrial farms and homeowners for the control of broadleaf weeds (30).

The 2,4-D degradation pathway of *Cupriavidus necator* JMP134 (B-*Proteobacteria*; formerly *Ralstonia eutropha*) has received considerable attention and serves as a model system for microbial degradation (2, 3, 26). The first gene in the catabolic pathway, *tfdA*, codes for an α – ketoglutarate dioxygenase (6, 7). The remainder of the pathway is comprised of at least five genes, e.g. *tfdBCDEF*, that continue to break down the molecule for use in central metabolism (29). Members of the *a-*, *B-* and γ-*Proteobacteria* are known to degrade 2,4-D and several unique degradation pathways have been identified. In the B- and γ- *Proteobacteria* three classes of *tfdA* genes are found while the *a-Proteobacteria* carry *tfdAa* that has ~60% identity to the *tfdA* gene of JMP134 (9, 30). A novel *cadABC*-based degradation

pathway which codes for 2,4-D oxygenase subunits (15) has also been found in *a-Proteobacteria* (10).

Although there has been extensive research conducted on 2,4-D degraders in the soil (12 ,13 ,15, 23), there is less work on the removal of 2,4-D in aquatic environments of varying salinity. Maltseva *et al.* (18) isolated three 2,4-D degrading bacteria from the family *Halomonadaceae* in the soil of a high salinity, alkaline lake that was heavily polluted with 2,4-D and other organic acids. Although these isolates came from an extreme environment, and were moderately halophilic and alkaliphilic, they still utilized the well-known *tfdA*-based degradation pathway of JMP134 (18).

The objective of this study was to isolate and characterize 2,4-D degrading bacteria from salt springs at Big Bone Lick State Park in Boone County, KY. The park and surrounding area contain numerous natural springs but this area does not have a history of 2,4-D contamination. The isolates obtained, including some genera not previously associated with 2,4-D degradation, were examined for their ability to grow on 2,4-D in the presence and absence of NaCl and screened for commonly occurring 2,4-D degradation genes. Isolation and characterization of 2,4-D degrading bacteria from novel environments, such as the salt springs in Big Bone Lick State Park, not only expands information on the diversity of herbicide degrading bacteria but also provides useful information for bioremediation of contaminated saline environments.

MATERIALS AND METHODS

SITE DESCRIPTION AND SAMPLE COLLECTION

Salt spring samples were collected from Big Bone Lick State Park located in Boone County, KY. The area surrounding the park is a rural semi-agricultural region; there are several salt-springs inside the park's boundary and many more springs throughout the surrounding area. Two springs were the focus of this study because they are easily accessed from the park visitor center. Spring samples representing both spring water and water mixed with spring sediment were collected from the small spring (N 38° 53' 4.582", W 84° 45' 10.648") and large spring (N 38° 53" 5.899", W 48° 45" 9.928") in sterile polypropylene containers, transported to IU Southeast in a cooler (~1.5 h) and used immediately for plating experiments.

PLATING AND ISOLATE PURIFICATION

A dilution series of the spring samples was done using sterile phosphate buffered saline with dilutions spread onto Artificial Sea Water nutrient broth (ASW) (17) agar plates with 5, 10 or 20% NaCl to assess levels of halotolerant heterotrophs and MMO minimal medium plates (13) supplemented with 125 ppm 2,4-D / 5% NaCl to assess levels of 2,4-D degraders. Plates were incubated for two weeks at 28°C. Single colonies were transferred to MMO 125 ppm 2,4-D / 5% NaCl for purification and after several plate transfers they were grown in Difco Marine broth (Beckton Dickinson & Co., Sparks, MD) and stored at -80°C in 15% glycerol.

GROWTH ASSESSMENT ON 2,4-D

Purified isolates were streaked to MMO 2,4-D plates with and without 5% NaCl and incubated at 28°C for 14 days. At regular intervals growth of the isolates was assessed visually by two independent observers and growth was scored as none (-), poor (+), moderate (++) or good (+++).

DNA EXTRACTION, PCR AND 16S rRNA GENE CHARACTERIZATION

Genomic DNA from individual isolates was extracted using the MoBio Microbial DNA Extraction Kit (mobio.com). The 16S rRNA gene was amplified using 27f and 1492r primers (25), and isolates were screened for *tfdA* using the TVU and TVL primers of Hogan *et al.* (8). Screening for *tfdAα* was done using Alpha1 (5'-ACS GAG TTC KSC GAC ATG CG-3') and Alpha2 (5'-GCG GTT GTC CCA CAT CAC-3') primers using the PCR conditions described by Itoh *et al.* (9). Promega GoTaq® Green Master Mix was used for PCR with reaction mixtures prepared according to manufacturer's protocols and thermal cycling was performed using a TECHNE Flexigene thermal cycler. The 16S rRNA gene products were purified using the MoBio Ultraclean™ PCR Cleanup Kit and DNA sequencing using primers 27f, 1492r and 519f (25) was performed at the IUPUI Core Sequencing Facility using Applied Biosystems 3100 Genetic Analyzers and Big Dye Terminator chemistry v3.1 (perkinelmer.com). Sequences were edited using Chromas (http://technelysium.com.au) and compiled in BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Full or partial sequences were used to query the Genbank database using the Basic Local Alignment Search Tool (BLAST) (1).

SALT TOLERANCE

Each isolate was transferred to 2ml of ASW nutrient broth (17) containing NaCl ranging from 0, 5, 10, 15 or 20% and incubated at 25°C at 200 rpm for 48 hours. Longer incubations of 72 and 96 hours did not yield increases in optical density. Optical density was measured using a Biotek ELx 800 plate reader at 630nm.

SALT SPRING CHEMISTRY

Spring water temperature, pH, salinity, total dissolved solids and conductivity were measured using a Hach SensION 156 multi-parameter meter on 9 Sept 2006, 8 Mar 2007, 30 Sept 2009, 16 Oct 2011, 5 Feb 2010 and 10 Oct 2010. No pH data was collected from the springs during the 5 Feb 2010 sampling trip as the pH probe malfunctioned on this date.

RESULTS

SALT SPRING CHARACTERISTICS

This study focused on two salt springs located within the borders of Big Bone Lick State Park in Boone County, KY. During six visits to the springs between 2006-2010 spring characteristics and water chemistry such as pH, temperature and salinity were measured (Table 1). The springs are shallow (~13 cm at the deepest point) and located no more than 100 meters from one another. In general, chemistry between the two springs was similar which, along with their proximity, suggests a common groundwater source.

	Small spring	Large spring
Temperature (°C)	16.10 ± 3.28	13.88 ± 2.42
pH	7.34 ± 0.22	7.49 ± 0.35
Salinity (‰)	7.32 ± 0.53	7.42 ± 0.27
TDS (mg/L)	6.91 ± 1.16	7.18 ± 0.23
Conductivity (µs/cm)	10.76 ± 0.86	10.14 ± 0.70

[1]Values are mean ± SEM. All measurements n=6 except pH where n=5.

Table 1. Water characteristics of two Big Bone Lick State Park salt springs.

ASSESSMENT OF CULTURABLE BACTERIAL COMMUNITIES OF THE SPRINGS

Spring samples representing water only and water mixed with sediment were plated onto artificial seawater medium (ASW) supplemented with 5, 10 or 20% NaCl to assess heterotrophic halotolerant bacterial populations (Table 2). CFUs were observed at all three salt concentrations although there was generally a reduction in CFU/mL as the salt concentration increased. Sediment samples from both springs contained larger bacterial populations at each salt concentration compared to water-only spring samples. Samples from both springs yielded CFUs on MMO 2,4-D medium supplemented with 5% NaCl (Table 2). The sediment containing small spring sample had the most 2,4-D degrading bacteria with counts ranging from 1-2 orders of magnitude more than the other three spring samples.

ISOLATION AND IDENTIFICATION OF PUTATIVE 2,4-D DEGRADING ISOLATES

Nineteen isolates transferred from the original 2,4-D plates and which produced small transparent colonies on media with 2,4-D as the sole carbon source at 28°C were chosen for further characterization. Using 16S rRNA gene sequence data the closest match to each isolate was determined from Genbank (Table 3). Eleven of 19 isolates closely matched the genus *Bacillus* with *Halobacillus* as the next most common genus (4 isolates) followed by *Halomonas* (2 isolates). Eleven of the sequences were nearly full length (1389 nts or greater) and all identities to database sequences were 99% with the exception of isolate 19 (*Halomonas ventosae*) which showed 98% identity.

GROWTH ASSESSMENT ON 2,4-D

To assess growth on 2,4-D as a sole carbon

Table 2. CFU/mL estimates of halotolerant bacteria in two Big Bone Lick State Park salt springs.

	Small spring		Large spring	
Growth medium	Water	Water + Sediment	Water	Water + Sediment
	CFU/mL	CFU/mL	CFU/mL	CFU/mL
5% NaCl ASW[1]	570	1.4×10^5	3.1×10^5	4.2×10^5
10% NaCl ASW	100	3.1×10^4	160	1.6×10^4
20% NaCl ASW	330	7.9×10^5	40	170
MMO 2,4-D 5%NaCl	60	8.1×10^5	70	730

[1]Artificial sea water agar-based medium supplemented with NaCl.

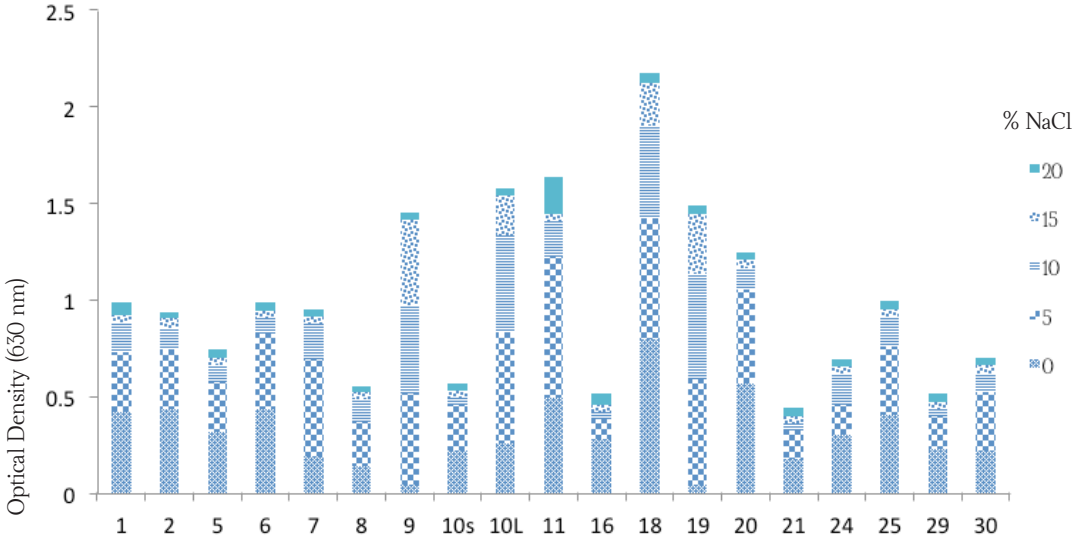

Fig. 1. Salt tolerance estimates of isolates from Big Bone Lick State Park. Each bar shows the optical density at 630nm across five salt concentrations in ASW broth.

Table 3. Identity, *tfdA* gene presence and growth characteristics of 2,4–D degrading halotolerant isolates.

Isolate number	Species/Accession number	Sequence length	% Identity	*tfda*[1]	*tfda–α*[2]	Growth 2,4–D	Growth 2,4–D + 5% NaCl
1	*Bacillus hwajinpoensis* / FR695443	1454	99		*	+++	+++
2	*Georgenia muralis* / AB455495	1406	99	*	*	+++	+++
5	*Bacillus hwajinpoensis* / FR695443	1469	99	*	*	++	++
6	*Bacillus hwajinpoensis* / FR695443	1440	99	*	*	++	++
7	*Bacillus baekryungensis* / AY505507	1452	99	*	*	++	++
8	*Bacillus aquamaris* / KF443807	958	99	*	*	+	++
9	*Halomonas sp.* / GU212640	1450	99	*	*	+	++
10s	*Halobacillus trueperi* / KJ174505	1470	99	*	*	+++	+++
10L	*Halobacillus trueperi* / DQ157162	1461	99	*	*	+	–
11	*Halobacillus alkaliphilus* / JQ068929	1467	99	*	*	+	+
16	*Bacillus sp.* / DQ084469	1453	99		*	+++	++
18	*Kocuria sp.* / HM579811	1389	99		*	–	++
19	*Halomonas ventosae* / GQ903444	795	98		*	–	++
20	*Bacillus baekryungensis* / JN210568	913	99		*	++	++
21	*Halobacillus alkaliphilus* / JQ068929	1465	99		*	+++	+
24	*Bacillus aquimaris* / KF769538	1436	99		*	+++	++
25	*Bacillus baekryungensis* / JN210568	1437	99		*	+	–
29	*Bacillus sp.* / HM587911	629	99		*	+++	+++
30	*Bacillus sp.* / HQ677199	687	99		*	+++	+++

[1]Amplified with primers from Hogan *et al.* (8).[2] Amplified with primers from Itoh *et al.* (9).

source each of the nineteen isolates was streaked onto MMO medium containing 125 ppm 2,4-D with or without 5% NaCl. Over the course of 14 days each isolate was visually examined independently by two investigators and the level of growth was scored as none (–), poor (+), moderate (++) or good (+++). Five isolates (represented by the genera *Bacillus*, *Georgenia*, *Halobacillus*) showed good growth (+++) on MMO 2,4-D plates with or without NaCl and four isolates showed moderate growth (++) on both medium types (Table 3). Interestingly, isolates 18 & 19 (represented by *Kocuria* and *Halomonas*, respectively) showed no growth (–) on MMO 2,4-D medium but good growth (+++) on MMO 2,4-D medium supplemented with NaCl. Numerous attempts to grow the nineteen isolates in MMO 2,4-D broth failed to yield observable growth. Additional attempts to encourage growth with casamino acids and to design liquid medium optimized to spring conditions also failed.

PRESENCE OF *TFDA* AND *TFDA*-LIKE GENES

The nineteen spring isolates were screened by PCR for two types of genes commonly found in 2,4-D degrading bacteria. DNA extracts from nine of the isolates were positive for the presence of *tfdA* and all nineteen isolates were positive for amplification of *tfdAa* (Table 3).

SALT TOLERANCE OF THE ISOLATES

Salt tolerance of each isolate was determined by inoculating ASW medium supplemented with 0, 5, 10, 15 or 20% NaCl. Fig. 1 shows the optical density at 630nm for each isolate across the five salt concentrations. Eighteen of the nineteen isolates showed a preference for media with 0% or 5% NaCl. ASW without added NaCl supported growth of all the isolates above 0.1 OD units with the exception of isolates 9 and 19 (both *Halomonas*). The two *Halomonas* isolates showed a similar growth pattern with good growth at 5, 10 and 15% NaCl. Although isolate 18 (*Kocuria* sp.) grew poorly on MMO 2,4-D medium without salt (Table 3), it grew the best of the nineteen isolates in 0% ASW and also had the highest combined optical density across the salt concentrations of any isolate (Fig. 1).

DISCUSSION

The objective of this study was to undertake a culture-based approach to examine 2,4-D degrading bacteria in salt springs at Big Bone Lick State Park. The springs in the park offer a unique environment for microbial growth. Low salinity, sulfur-enriched groundwater feeds the springs throughout the year and mineral deposits regularly appear in the spring's water and outer edges as white precipitate. This work represents the first attempt to examine 2,4-D degrading microbial communities in these springs. Although the springs sampled are of low salinity previous culture-based studies have identified large populations of moderate halophilic and halotolerant bacteria in spring water and sediment samples. With this in mind, the current study was designed to isolate 2,4-D degrading bacteria that could tolerate at least 5% NaCl.

Although the salt springs sampled in this

study have no known history of 2,4-D contamination, samples from two salt springs at Big Bone Lick State Park showed an abundance of colonies on agar-based plates with 2,4-D as the sole carbon source. Ka *et al.* (12) demonstrated that successive 2,4-D applications in agricultural soil can greatly enrich the soil microbial community for 2,4-D degrading bacteria. Although the Big Bone Lick springs were not obviously treated or heavily contaminated with 2,4-D, low level 2,4-D groundwater contamination from agricultural runoff cannot be ruled out as an enrichment source.

Varied growth levels on 2,4-D medium with and without salt were observed in the nineteen spring isolates chosen for further characterization. While some isolates grew equally well on 2,4-D medium with or without NaCl, others showed a clear preference for salt-free or salt-containing medium. These diverse growth strategies make sense given that the isolates described here were enriched on 2,4-D agar-based medium rather than 2,4-D supplemented broth which may select only the fastest growing isolates. Allowing the initial 2,4-D containing plates to incubate for 14 days may have also provided slow growing isolates time to become established. Novel slow growing 2,4-D degrading bacteria have been found in pristine environments using an enrichment strategy involving organic supplements and low initial levels of 2,4-D (14).

Two of the nineteen isolates described here were identified as closely matching the genus *Halomonas*. Maltseva *et al.* (18) previously isolated three moderately halophilic, alkaliphilic bacteria from alkali lakes heavily contaminated with 2,4-D and numerous other aromatic compounds. These isolates were members of the Halomonadaceae family and contained 2,4-D degradation pathways that matched the *tfdA* pathway from *Cupriavidus necator* JMP134. As *tfdA* was found in only one of the two *Halomonas* isolates described here, more work is clearly needed to determine the nature of degradation in the non-*tfdA* containing *Halomonas* isolate. *Halomonas* is a metabolically diverse genus well known for its halotolerance and biodegradative potential. For example, *Halomonas organivorans*, isolated from the soil of Isla Cristina, Spain grows on phenol and a wide-range of other organic acids (5).

Interestingly, seventeen of the nineteen isolates described here were Gram positive bacteria. *Bacillus* was the most common genus found (eleven isolates) and several of these contained the *tfdA* gene. *Bacillus* sp. have been previously reported to carry *tfdA* (8) but these isolates did not degrade 2,4-D. The *Bacillus* isolates in this study closely match recently described halotolerant species such as such as *Bacillus aquamaris*, *Bacillus hwajinpoensis* and *Bacillus baekryungensis* (27, 28) and thus these isolates should be screened more fully for their degradative potential.

Isolates closely matching *Halobacillus* were also identified as 2,4-D degraders in this study and three out of the four isolates contained the *tfdA* gene. The genus *Halobacillus* was first proposed in 1996 (24) and now contains nearly 20 species with interesting properties such as production of protective carotenoids and novel salt-tolerant enzymes of interest to biotechnology (11). This is the first report identifying 2,4-D degradation genes in this genus.

Two additional Gram positive isolates closely matched *Kocuria* sp. and *Georgenia muralis*. *Kocuria* are commonly found on the skin of humans and some species have recently emerged as pathogens in immunocompromised patients (22). *Georgenia muralis* was first isolated from

a medieval wall painting in the church St. Georgen in Austria in 2002 (16). A novel finding of this study is that *Georgenia muralis* and *Kocuria* sp. have not previously been shown to degrade 2,4-D. The *Georgenia muralis* isolate described here not only grew well on 2,4-D medium with or without added NaCl but also contained the *tfdA* gene, which has not previously been reported.

All nineteen of the isolates from Big Bone Lick State Park were positive for *tfdAa*. *tfdAa* is found in *Proteobacteria* such as *Bradyrhizobium* and *Sphingomonas* sp. (9, 10) and thus it is interesting that the diverse collection of isolates described here, none of which are *a-Proteobacteria*, carry this gene. Additional primer sets designed to detect novel *tfdA*-like genes were recently described (30) and it would be informative to screen the isolates described here with these primers to further examine *tfdA* gene diversity.

Nine of the nineteen isolates in this study contained both *tfdA* and *tfdAa* raising the possibility that multiple degradation pathways exist in these isolates. It has been suggested that the *tfdA* gene products serve other cellular roles besides the degradation of herbicides (8) and it would be worthwhile to screen the Big Bone Lick salt spring isolates for other degradation abilities.

One area to enhance this study lies in designing minimal broth that will support growth of the salt spring isolates. Numerous attempts to grow the isolates in minimal liquid medium with 2,4-D as the sole carbon source failed to stimulate growth. Additionally, liquid medium designed to replicate spring water chemistry also yielded poor growth. Development of minimal broth supporting growth of the isolates described here will allow confirmation of 2,4-D degradation and provide a means for quantitative determination of 2,4-D disappearance (14).

In summary, this study has identified the salt springs at Big Bone Lick State Park as a potentially novel source of 2,4-D degrading bacteria. Since degradation pathways in the salt spring isolates appear to match those associated with *tfdA* and *tfdAa*, it would be informative to sequence these genes to determine their relationship to other known 2,4-D degradation pathways. Horizontal gene transfer of 2,4-D degradation pathways has been proposed based on incongruent phylogenies of 16S rRNA genes compared to *tfdA* (20). More recently, a transposon containing both *cadAB* and tfd associated genes, but not *tfdA* or *tfdAa* was found in a 2-methyl-4-chlorophenoxyacetic acid (MCPA) degrading *Sphingomonas* isolate (21). Understanding the role gene transfer plays in the evolution of moderately halophilic bacteria would be an exciting area of future research.

ACKNOWLEDGEMENTS

M.G.W. was supported by an Indiana University Southeast Undergraduate Research Fellowship for part of this work. Thanks to Todd Young, naturalist at Big Bone Lick State Park for his help with access to the salt springs, sampling and information about the park's history.

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BDELLOVIBRIO BACTERIOVORUS PROTECTS *CAENORHABDITIS ELEGANS* FROM BACTERIAL PATHOGENS

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MANUSCRIPT RECEIVED 29 APRIL, 2014; ACCEPTED 14 JUNE, 2014

ABSTRACT

Bdellovibrio bacteriovorus is a naturally predatory bacterium that multiplies inside Gram negative prey bacteria. There is much interest in using *Bdellovibrio* as a living antibiotic to control infections by Gram negative pathogens. In recent years *Caenorhabditis elegans* has proven to be an attractive animal model of bacterial pathogenesis for a range of pathogens. We have used the *C. elegans* animal pathogenesis model to examine the ability of *B. bacteriovorus* to protect nematodes from four bacterial pathogens. In all cases, nematodes treated with *B. bacteriovorus* and the pathogen survived at a significantly higher level than nematodes treated with the pathogen alone. Treatment with *B. bacteriovorus* alone was nontoxic to the worms. We monitored the persistence of *E. coli* K-12 and *E. coli* OP50 in both *B. bacteriovorus* treated nematodes and control nematodes. *E. coli* K-12 levels were significantly lower in *B. bacteriovorus* treated nematodes than in control nematodes one day after *Bdellovibrio* exposure and *E. coli* K-12 was eliminated from the worm gut two days faster in *B. bacteriovorus* treated nematodes. *E. coli* OP50 also demonstrated significantly lower levels in *B. bacteriovorus* treated nematodes and faster elimination from the worm gut. The successful use of *B. bacteriovorus* as a therapeutic agent in *C. elegans* indicates that it may be useful as a living antibiotic in other animal systems.

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KEYWORDS

- *Bdellovibrio bacteriovorus*
- *Caenorhabditis elegans*
- pathogenesis, biocontrol
- infection model

INTRODUCTION

Bdellovibrio bacteria are intriguing because they naturally reproduce inside other Gram negative bacteria. The *Bdellovibrio* life cycle involves attachment to and penetration of prey cells, elongation inside the prey periplasm using prey components for growth, fragmentation into multiple cells, and finally, lysis of the prey cell (1). Because *Bdellovibrio* lyses prey as it multiplies, and because it cannot infect eukaryotic cells, there is growing interest in using *Bdellovibrio* as a "living antibiotic" (2).

Numerous researchers have demonstrated in vitro killing of pathogens by *Bdellovibrio,* (3, 4, 5, 6) supporting the idea of using *Bdellovibrio* to control infections. Additionally, *Bdellovibrio* has been shown to attack prey within bacterial biofilms and reduce biofilm biomass (7, 8, 9). Two studies have put the living antibiotic concept into practice, demonstrating protection against *Aeromonas hydrophila* infection in fish and protection against *Proteus penneri* infection in shrimp through the use of *Bdellovibrio*

(10, 11). Fish and shrimp mortality was significantly lower when the animals swam in water containing both the pathogen and *Bdellovibrio* as compared to animals in water containing only the pathogen. However, it was not determined whether the mechanism of *Bdellovibrio* protection was simply a reduction of the pathogen level in the water, the killing of the pathogen within the animal, or a combination of the two. Until recently, the use of *Bdellovibrio* as an *in vivo* treatment for infection has been an intriguing, but theoretical option. In 2011 Atterbury *et al.* demonstrated *Bdellovibrio* could be used therapeutically to control *Salmonella* infection in chickens without negative effects on the birds (12). This was the first study to demonstrate in vivo efficacy of *Bdellovibrio* as a treatment for bacterial infection. Here we continue the use of *Bdellovibrio* as an *in vivo* therapeutic agent, but in the *C. elegans* bacterial pathogenesis model.

In 1999 Tan *et al.* first reported the use of the nematode *C. elegans* as an animal model for bacterial pathogenesis (13). Since then numerous researchers have demonstrated that this system can be used for multiple bacterial pathogens including *Pseudomonas aeruginosa, Salmonella enterica, Serratia marcescens,* and *Staphylococcus aureus* (14, 15). Genes identified in *C. elegans* as important in pathogenesis have been confirmed in mouse models of pathogenesis, validating the use of *C. elegans* as a pathogenesis model (16). Using *C. elegans* as an animal model for pathogenesis is attractive for numerous reasons such as low cost, short generation time, complete genome sequence and ease of genetic manipulation (17). When *C. elegans* are maintained in the laboratory they are grown on Petri plates containing lawns of nonpathogenic *E. coli* OP50 as their food source and the worms typically live two weeks (18). When grown on a pathogen instead of OP50, worm survival is greatly reduced (16).

Our lab has taken advantage of the well-studied *C. elegans* bacterial pathogenesis model system to examine the use of *Bdellovibrio* to protect *C. elegans* from bacterial infection. In this study, we first established an infection in the nematode and then examined the curative effect of a brief exposure to *Bdellovibrio*. We show that worms treated with both *Bdellovibrio* and a pathogen live significantly longer than worms treated with the pathogen alone. We also demonstrate that bacterial levels are lower and cleared faster in *Bdellovibrio* treated worms than control worms. This work demonstrates that *Bdellovibrio* can be used as a therapeutic treatment for bacterial infections in a well-defined animal model.

MATERIALS AND METHODS

NEMATODE AND BACTERIAL STRAINS

Wild type *C. elegans* N2 worms were used in all nematode assays. Worms and nonpathogenic *E. coli* OP50 were supplied by the Caenorhabditis Genetics Center (Minneapolis, MN). Worms were grown on nematode growth medium (NGM) with *E. coli* OP50 as the food source (18). Pathogens tested were *E. coli* K-12, *Enterobacter aerogenes* ATCC 13048, *Pantoea agglomerans* LS005, and *Salmonella enterica serovar Typhimurium* LT2 (19). *B. bacteriovorus* HD100 was used for all biocontrol assays (20). *E. coli* HB101 was used as the non-pathogenic control in the biocontrol assays since our early work in this system used *B. bacteriovorus* 109J, which does not infect *E. coli* OP50, but does infect *E. coli* HB101.

However, all the experiments described here used *B. bacteriovorus* HD100, which does infect both *E. coli* OP50 and *E. coli* HB101. *B. bacteriovorus* HD100 was cultured using *E. coli* K-12 as prey according to standard protocols (21). *B. bacteriovorus* prey lysates were checked microscopically for active, motile *B. bacteriovorus* cells and an absence of prey cells. Prey lysates contained approximately 6 x 10^8 *B. bacteriovorus* cells per ml. The persistence assays utilized kanamycin-resistant *E. coli* K-12 derivative strain JW1863-1 (22), supplied by the *E. coli* Genetic Stock Center (New Haven, CT) and ampicillin-resistant *E. coli* OP50-GFP strain DB15, kindly supplied by J. Ewbank (Centre d'Immunologie de Marseille-Luminy, Marseille, France).

PATHOGENICITY ASSAY

Bacteria were grown overnight in LB broth and 50 µl culture was spread on 60 mm diameter NGM plates. Plates were incubated for two days at 25°C to establish bacterial lawns. *C. elegans* were reared on NGM with lawns of *E. coli* OP50 as the food source. One-day old adult worms were placed on NGM plates containing lawns of bacteria. Worm survival was monitored daily for the next nine days. Worms were considered dead when they did not respond to gentle prodding with a platinum wire. Surviving adult worms were transferred daily to fresh bacterial lawn plates to separate them from newly hatched juvenile worms. Each trial measured the survival of 30 worms per treatment.

BIOCONTROL ASSAY

Bacteria were grown overnight in LB broth and 50 µl culture was spread on NGM plates. Plates were incubated for two days at 25°C to establish bacterial lawns. *C. elegans* were reared on NGM with lawns of *E. coli* OP50 as the food source. One day old adult worms were placed on NGM plates containing lawns of a pathogen or nonpathogenic *E. coli* HB101. After exposing the worms to the pathogen or HB101 for 48 hours (32 hours for *E. coli* K-12), worms were washed three times in Ca/HEPES buffer (21) to remove external bacteria. *E. coli* K-12 treated worms were exposed to *E. coli* for 32 hours instead of 48 hours because a 48 hour exposure to *E. coli* K-12 was too toxic and killed the majority of the worms. Washed worms were suspended in 1 ml of an active *B. bacteriovorus* prey lysate or 1ml of Ca/HEPES buffer for 15 minutes. A 15 minute exposure to *B. bacteriovorus* was chosen because this is the time required for *B. bacteriovorus* to attach to prey cells (2). Then the worms were pelleted and placed on NGM plates containing lawns of the nonpathogenic *E. coli* HB101. Worms were transferred to new *E. coli* HB101 plates daily and worm survival was monitored daily for the next seven days. Each trial measured the survival of 40-50 worms per treatment.

E. COLI PERSISTENCE IN *C. ELEGANS*

Nematodes were exposed to an antibiotic-resistant strain of *E. coli* (32 hour exposure for kanamycin-resistant *E. coli* K-12 derivative JW1863-1 or 48 hour exposure for ampicillin-resistant *E. coli* OP50-GFP strain DB15) followed by three washes in Ca/HEPES buffer. The washed worms were suspended for 15 minutes in either 1 ml of an active *B. bacteriovorus* prey lysate or 1 ml of Ca/HEPES buffer, then pelleted and placed on NGM plates with *E. coli* HB101 lawns. Worms were transferred daily on to fresh *E. coli* HB101 plates as described above for the biocontrol assays. Numbers of internal bacteria persisting in the nematodes after *B. bacteriovorus* or buffer exposure were determined daily using the protocol of Garsin *et al.* (23) with the following modifications. Briefly, 5 worms were placed on a LB agar plate containing the appropriate antibiotic (50 µg/ml) and washed twice with 4 µl

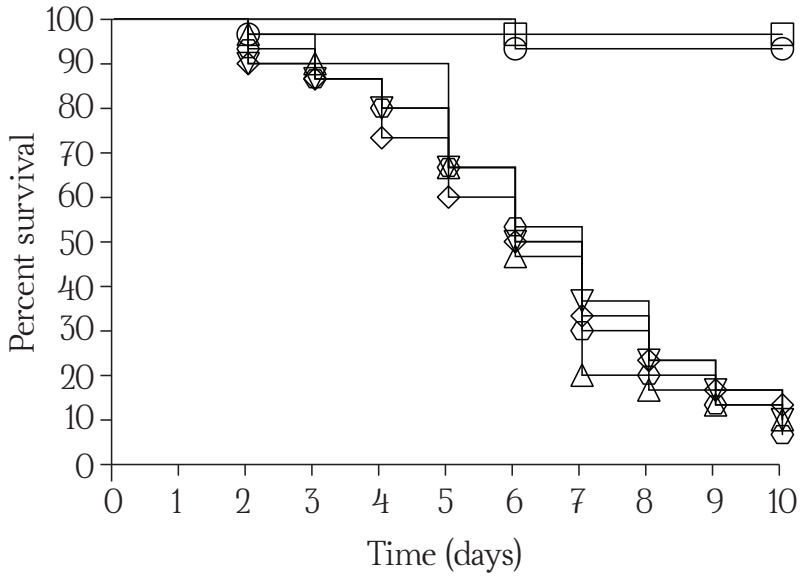

Figure 1. Survival curves for *C. elegans* exposed to *E. coli* OP50 (□), *E. coli* HB101 (○), *E. coli* K-12 (▽), *E. aerogenes* (△), *P. agglomerans* (◇), and S. *enterica* (⬡). Data are from one trial representative of two independent trials.

M9 medium to remove surface bacteria. Washed worms were suspended in 20 µl M9 medium and ground with a pestle. 30 µl of M9 medium was added to the worm solution to bring the total volume up to 50 µl; the solution was diluted in Ca/HEPES buffer and plated on LB agar containing the appropriate antibiotic (50 µg/ml) for bacterial enumeration.

STATISTICS

Kaplan-Meir survival analysis followed by pairwise logrank tests (24, 25, 26) was used to analyze *C. elegans* survival over time. The Mann Whitney test was used to analyze *E. coli* persistence data. Data analyses were performed using GraphPad Prism® 4 (27). The significance level for all statistical analyses was set at $a = 0.05$.

RESULTS

PATHOGENICITY ASSAY

We tested the pathogenicity of four species of bacteria, comparing them to the standard, nonpathogenic *E. coli* OP50 routinely used to maintain *C. elegans*. All four species tested were pathogenic when compared to *E. coli* OP50, greatly reducing worm survival (Fig. 1). The pairwise comparisons examining worm survival between the four pathogens indicated that all four pathogens were similar in pathogenicity (p=0.9926). We also tested *E. coli* HB101 and found it to be nonpathogenic. Worm survival on *E. coli* HB101 was not significantly different from worm survival on *E. coli* OP50 (p=0.5482). Worms grown on all four pathogens survived significantly less than worms grown on *E. coli* OP50 (p<0.001) and worms grown on all four pathogens survived significantly less than worms grown on *E. coli* HB101 (p<0.001). We proceeded to use *E. coli* HB101 as the *C. elegans* food source when monitoring worm survival in our biocontrol assays rather than *E. coli* OP50 since our early work in this system used *B. bacteriovorus* strain

Table 1 P values for pairwise comparisons in the biocontrol assay survival curves.

Pathogen	Comparison					
	HB101 vs. HB101 +Bd[a]	HB101 vs. Pathogen	HB101 vs. Pathogen +Bd	HB101 +Bd vs. Pathogen	HB101 +Bd vs. Pathogen +Bd	Pathogen vs. Pathogen +Bd
E. coli K-12	0.4958	<0.0001	0.0047	<0.0001	0.0412	<0.0001
E. aerogenes	0.4402	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
P. agglomerans	0.7376	<0.0001	0.0207	<0.0001	0.0098	<0.0001
S. enterica	0.7318	<0.0001	0.1901	<0.0001	0.3292	<0.0001

[a]Bd indicates *Bdellovibrio*

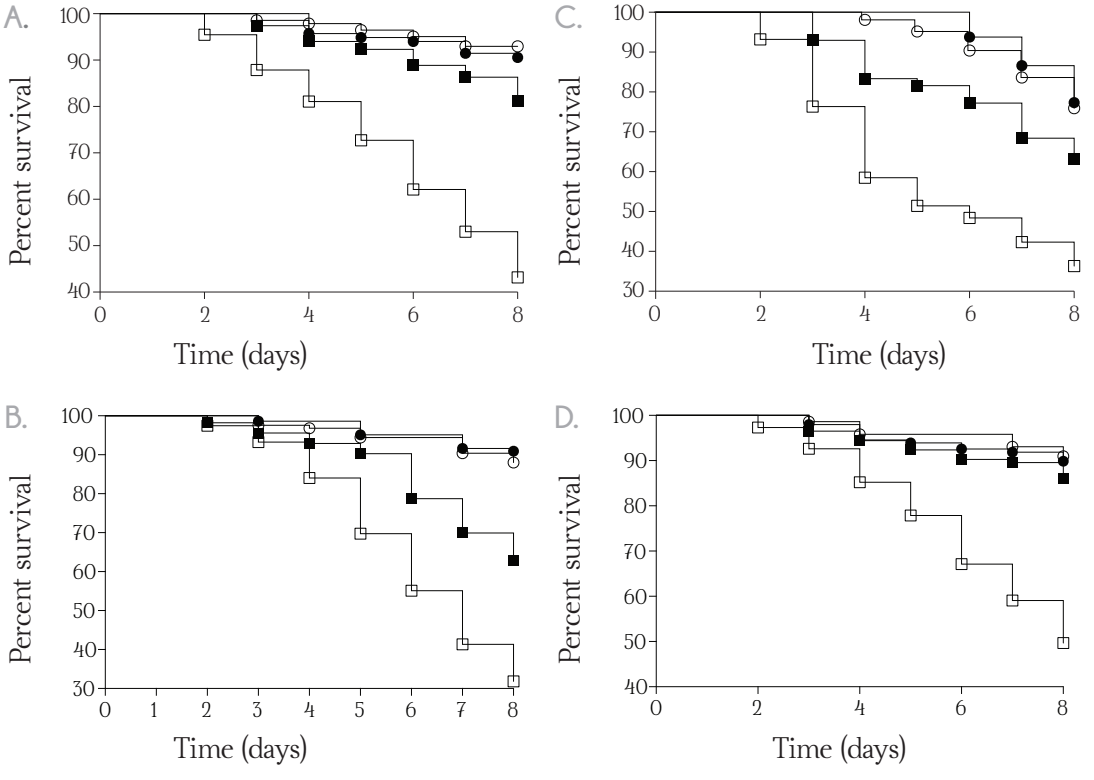

Fig. 2. Survival curves for *C. elegans* exposed to (a) *E. coli* K-12 (b) *E. aerogenes* (c) *P. agglomerans* and (d) *S. enterica*. Worms were treated with nonpathogenic *E. coli* HB101 (○), HB101 and *Bdellovibrio* (●), pathogen (□), or pathogen and *Bdellovibrio* (■). Worms were exposed to *Bdellovibrio* or control buffer on day one. Data are from three independent trials for each pathogen.

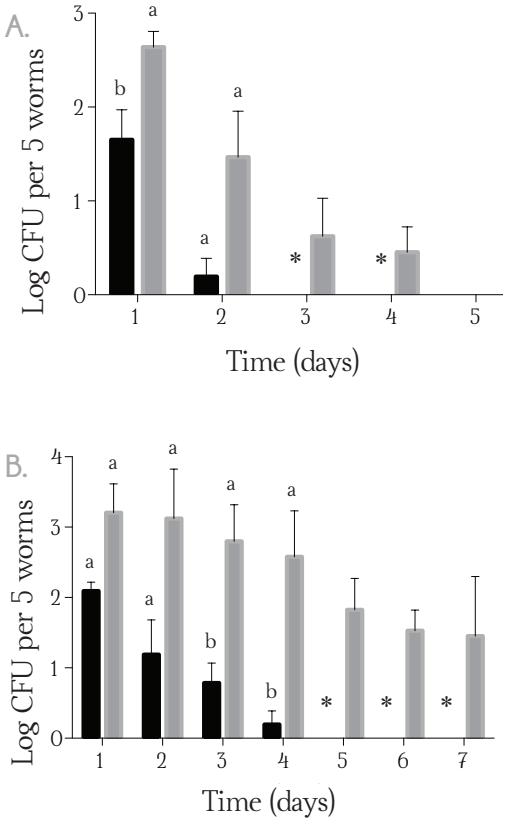

Fig. 3. Persistence of antibiotic-resistant derivatives of (a) *E. coli* K-12 and (b) *E. coli* OP50 within *C. elegans* treated with *Bdellovibrio* (black bars) or control buffer (grey bars). Worms were treated with *Bdellovibrio* or control buffer on day zero. Values with the same letter for a single time are not significantly different ($p \leq 0.05$). Asterisks indicate values with zero variance and thus these days were excluded from analysis. Log transformed data are from four independent trials and error bars indicate standard error.

109J, which did not prey on *E. coli* OP50.

BIOCONTROL ASSAY

To determine whether *B. bacteriovorus* could protect nematodes from bacterial pathogens, we established infections in the nematodes, briefly treated infected worms with *B. bacteriovorus*, placed worms on non-pathogenic *E. coli* HB101, and monitored worm survival for seven days. For all four pathogens tested, worm survival was significantly improved when worms were treated with *B. bacteriovorus* as compared to the pathogen alone (Fig. 2). For each pathogen, the pairwise comparison between worms treated with the pathogen alone and worms treated with both the pathogen and *Bdellovibrio* was highly significant (Table 1). Worm survival was unaffected by *B. bacteriovorus* treatment when worms were grown on nonpathogenic *E. coli* HB101 (Table 1), demonstrating that *B. bacteriovorus* is nontoxic to worms. *Bdellovibrio* and pathogen treated worms had significantly longer survival than worms treated with the pathogen alone. However, for three of the four pathogens, *Bdellovibrio* treatment was unable to restore the same level of worm survival as with the nonpathogenic *E. coli* HB101 control, and there were still significant survival differences between control worms and pathogen plus *Bdellovibrio* treated worms. *S. enterica* infection was the only one completely rescued by *Bdellovibrio* with no significant difference in survival curves between control worms and *S. enterica* plus *Bdellovibrio* treated worms (Table 1).

E. COLI PERSISTENCE IN *C. ELEGANS*

We also monitored the persistence of one of the four pathogens (a kanamycin-resistant derivative of *E. coli* K-12) as well as ampicillin-resistant *E. coli* OP50 in both

Bdellovibrio treated and control worms. One day after exposure to *Bdellovibrio* or a control buffer, *E. coli* K-12 levels were significantly lower in worms treated with *Bdellovibrio* compared to control worms (Fig. 3A). Levels of pathogenic *E. coli* K-12 decreased to undetectable levels in worms three days after *Bdellovibrio* treatment, while it took five days for pathogenic *E. coli* to drop below detectable levels in control worms. *E. coli* OP50 showed a similar trend in that bacterial levels were lower in *Bdellovibrio* treated worms, although a significant difference between *Bdellovibrio* treated and control worms was not detected until three days after *Bdellovibrio* treatment (Fig. 3B). *E. coli* OP50 was also cleared to undetectable levels faster in *Bdellovibrio* treated worms and *E. coli* OP50, unlike *E. coli* K-12, persisted in the control worms for the entire seven day experiment. The limit of pathogen detection was five CFU per five worms.

DISCUSSION

While many have used *C. elegans* as a model for bacterial pathogenesis, we have extended that model to investigate control of four bacterial pathogens by *Bdellovibrio*. The non-vertebrate *C. elegans* has many advantages as an animal model for *Bdellovibrio* infection control studies including short life span, ease of manipulation, low cost, consumption of bacteria as food, and absence of ethical concerns. Our work in *C. elegans* supports and extends earlier work using *Bdellovibrio* as a therapeutic agent to control bacterial infections in chickens (12). Interestingly, the one log reduction in S. enterica by *Bdellovibrio* in chickens is similar to the reduction in *E. coli* K-12 levels we demonstrated in *C. elegans* (Fig. 3A). In agreement with the chicken study, our work demonstrated improved animal health with a single, discrete dose of *Bdellovibrio*. Using *Bdellovibrio* to control infection is often compared to bacteriophage therapy with *Bdellovibrio* having the advantage of a wider prey range than phage (2). Indeed, similar to our results, one group has demonstrated the ability of phage to protect *C. elegans* from *Salmonella* infection (28) confirming the robustness of the *C. elegans* model.

Our pathogenicity assay results demonstrate a clear difference in nematode survival between the four pathogens tested and the two non-pathogenic *E. coli* strains (Fig. 1). This highly significant survival difference is also reflected in the biocontrol assay comparing the HB101 treated worms with the pathogen treated worms (Fig. 2). Although *E. coli* K-12 is typically considered to be nonpathogenic in animal models and our referring to *E. coli* K-12 as a pathogen may seem inaccurate, others have also demonstrated that *E. coli* K-12 is pathogenic in *C. elegans* (29). *E. coli* OP50 is the strain typically used as a nonpathogenic food source for *C. elegans*; however we have demonstrated that *E. coli* strain HB101 is also nonpathogenic. Similar nematode survival curves between OP50 and HB101 have also been demonstrated by researchers examining the effect of bacterial nutrition on *C. elegans* lifespan (30). Interestingly, when survival is examined beyond ten days, worms live longer on HB101 compared to survival on OP50 (30).

Although *Bdellovibrio* provided intermediate protection from most pathogens, the significant improvement in survival along with the complete protection of *Salmonella*

treated worms clearly demonstrates the protective ability of *Bdellovibrio* in this system (Fig. 2 and Table 1). The variation in *Bdellovibrio* protection of *C. elegans* from pathogens may be due to the difference in bacterial colonization of the worms. *S. enterica* serovar Typhimurium kills worms through a persistent intestinal colonization while *E. coli* kills through a non-persistent intestinal colonization (16). The ability of *S. enterica* to multiply within and distend the worm intestinal lumen, establishing a persistent infection after the worms are no longer being fed *S. enterica* cells (31), may provide a more concentrated source of pathogen cells to support increased *Bdellovibrio* growth and predation, leading to complete recovery from infection. Interestingly, these data suggest that the more numerous the pathogen cells are in the host, the more effective *Bdellovibrio* treatment may be for resolving the infection.

We followed the persistence of two *E. coli* strains in this system using antibiotic-resistant derivatives of *E. coli* K-12 and *E. coli* OP50 to examine the effect of *Bdellovibrio* on *E. coli* clearance from the worm. Pathogenic *E. coli* K-12 levels were significantly lower in *Bdellovibrio* treated worms one day after treatment and *E. coli* K-12 was cleared from the worms two days quicker in *Bdellovibrio* treated worms (Fig. 3A). This marked reduction in pathogenic *E. coli* levels by *Bdellovibrio* was enough to significantly improve worm survival, but not enough to restore worm survival back to the level seen in non-pathogen treated control worms (Table 1). Our results are based on a single, 15 minute exposure of the worms to *Bdellovibrio* and increased survival may occur with longer or repeated exposures of the worms to *Bdellovibrio.* We chose a 15 minute exposure to allow time for *Bdellovibrio* to attach to prey cells and begin invasion of the prey cell (2). Even without *Bdellovibrio* treatment, *E. coli* K-12 was cleared from the worms, in agreement with earlier research demonstrating that pathogenic *E. coli* does not establish a persistent infection in worms (16). Levels of nonpathogenic *E. coli* OP50 were also significantly lower and cleared faster in *Bdellovibrio* treated worms (Fig. 3B). However, unlike *E. coli* K-12, nonpathogenic *E. coli* OP50 was able to persist in the control worms for seven days. The levels of *E. coli* OP50 we detected in control worms on day one agree closely with those found by others investigating viable *E. coli* OP50 counts in *C. elegans* lysates (30), validating our work in this system.

C. elegans appears to be an ideal model system for refining and exploring the use of *Bdellovibrio* as a therapeutic agent. Since *C. elegans* is a bacteriovore, exposure of the worms to pathogenic bacteria is simple and easy. The lower growth temperatures favored by *C. elegans* (20–25°C) compared to birds and mammals coupled with *Bdellovibrio's* optimal growth temperature of 28°C makes *C. elegans* an attractive animal system to investigate the use of *Bdellovibrio* as a biocontrol agent. We administered *Bdellovibrio* as a liquid treatment for precise, controlled dosing, but worms could also be treated with *Bdellovibrio* through placement on plaque plates (17) containing both the pathogen and *Bdellovibrio.* Our work prepares the way for future experiments with *C. elegans* and *Bdellovibrio* to examine additional pathogens, dosage and frequency of *Bdellovibrio* treatment, persistence of *Bdellovibrio* in worms, effect (if any) of *Bdellovibrio* on worm morphology, as well as other variables.

While an intriguing hypothesis, the use of *Bdellovibrio* as a feasible therapeutic agent has only been demonstrated *in vivo* in chickens against *Salmonella* (12). Here we extend

that work by demonstrating significantly increased nematode protection from four different pathogens through *Bdellovibrio* treatment. In addition to being a well-studied pathogenesis model, *C. elegans* are much more tractable than chickens and our results lay the groundwork for future *Bdellovibrio* biocontrol studies in *C. elegans*. The presence of *Bdellovibrio* as a member of a healthy gut community in children (32), along with its lack of toxicity in birds and nematodes, suggests that it holds potential for therapeutic use. Our demonstration of protection by *Bdellovibrio* against multiple bacterial pathogens in the well-studied *C. elegans* pathogenesis model strengthens the validity of *Bdellovibrio* as a promising, future therapeutic agent.

ACKNOWLEDGEMENTS

This work was funded by the Richard A. Henson School of Science and Technology at Salisbury University. Thanks to Rebecca Pinekenstein for her work in the early stages of this research. Thanks to Jonathan Ewbank for supplying the *E. coli* OP50-GFP strain DB15. Thanks to J. Martin Bland, Dept. of Health Sciences, University of York, York, UK for information on the logrank tests.

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NICOTINE: ITS STIMULATING AND INHIBITORY EFFECTS ON ORAL MICROORGANISMS

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MANUSCRIPT RECEIVED 15 SEPTEMBER, 2014; ACCEPTED 30 OCTOBER, 2014

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KEYWORDS

- dental caries
- periodontal disease
- biofilm
- nicotine
- tobacco
- *Lactobacillus casei*
- *Actinomyces viscosus*
- *Actinomyces naeslundii*
- *Rothia dentocariosa*
- *Enterococcus faecalis*
- *Candida albicans*

ABSTRACT

Tobacco users are much more susceptible to dental caries and periodontal diseases than non-tobacco users. Research suggests that this increased susceptibility may be due in part to nicotine, a primary active component of tobacco. Five bacterial species and one yeast species commonly found in the human oral cavity, *Lactobacillus casei, Actinomyces viscosus, Actinomyces naeslundii, Rothia dentocariosa, Enterococcus faecalis,* and *Candida albicans* respectively, were utilized to investigate if any correlation existed between exposure to various concentrations of nicotine ranging from 0 to 32 mg/ml and the growth of each microorganism. The minimum inhibitory concentration (MIC), minimum biofilm inhibitory concentration (MBIC), and planktonic growth were measured. The MIC was determined to be 16 mg/ml for all organisms except *E. faecalis,* which had an MIC of 32 mg/ml. Nicotine had a varying effect on planktonic growth across the different species. A distinct upward trend in biofilm formation was found in *A. viscosus, L. casei, E. faecalis,* and *C. albicans* through 8 mg/ml. Nicotine also enhanced *R. dentocariosa* biofilm formation in all concentrations through 8 mg/ml but was most enhanced at 1 mg/ml. Alternatively, *A. naeslundii* exhibited a complete downward trend through 32 mg/ml. The MBIC was found to be 16 mg/ml in all organisms studied. These findings further support research suggesting that the increased susceptibility to oral health diseases experienced by tobacco users may be caused in part by an upregulation in biofilm formation of these oral pathogens.

INTRODUCTION

Periodontal disease is a condition that causes inflammation of the gums including diseases such as gingivitis, in which gums become swollen and bleed easily. Periodontitis, another form of periodontal disease, is an advanced form of gingivitis in which the gums pull away from the teeth and form pockets where dental plaque begins to grow. This infection can lead to both severe tissue and bone damage due to the increased exposure to previously protected areas. Periodontal disease is also

closely linked with dental caries. The occurrence of both periodontal disease and dental caries can be associated with several factors. Some of the most common risk factors include smoking, diabetes, autoimmune disorders, genetics, and obesity (14). The use of tobacco products remains the largest risk factor for periodontal disease. Environmental tobacco smoke has been associated with the increased likelihood of caries formation in children (2). Recent studies in this laboratory demonstrated increased biofilm formation of *S. mutans* with increasing nicotine concentrations (20). The average nicotine content in a single cigarette has been found in amounts up to 2 mg (12). A recent study tested over 40 smokeless tobacco products, such as snuff and chew, and it was discovered that they contained anywhere from 3.6–25.3 mg of nicotine per gram of smokeless tobacco (33). Nicotine contents of cigars have been found to vary greatly, ranging from 5.9 mg per cigar to 335.2 mg per cigar (18). Studies have demonstrated that when smoking cigarettes, an average of approximately 1 mg of nicotine per cigarette is absorbed by the body, however, the amount of nicotine within oral biofilm of smokers has not yet been explored (5). This study used the following opportunistic pathogens, commonly found in the human oral cavity, to investigate possible effects on growth and biofilm formation.

LACTOBACILLUS CASEI

Lactobacillus casei is a Gram-positive, facultative anaerobic bacterium. They are nonmotile and have a rod shape. Like other strains of lactobacilli, *L. casei* produces a significant amount of lactic acid, allowing it to remain viable under various pH levels. Poor diet, weak tooth enamel, previous plaque buildup, and acidic conditions contribute to an optimal environment for *L. casei* to thrive (22). *L. casei* alone lacks adequate strength to adhere to tooth enamel, however, when co-cultured with *Streptococcus mutans, L. casei* gains the ability to synthesize glucans thus improving tooth enamel adhesion. Because of this, *L. casei* along with *S. mutans* have been found to be the most prevalent bacteria leading to dental caries (19).

ACTINOMYCES VISCOSUS

Actinomyces viscosus is a facultative anaerobic, Gram-positive bacterium that is often isolated from the oral cavity, especially from subgingival plaque and along the teeth and gums. *A. viscosus* is rod-shaped and filamentous. Distinct characteristics of the filamentous shape include branching with swollen, rounded, or clavate ends (7). *A. viscosus* is a severely cariogenic bacterium that acts as an agent in promoting and initializing carious lesions (15). Studies have shown that the bacterium has adhesins which allow it to bind to complementary receptors on a substrate. *A. viscosus* binds to salivary acidic proline-rich proteins (PRPs) adsorbed onto the apatitic surfaces of the tooth. Cryptic segments exposed in adsorbed molecules are identified by the adhesins, which allow *A. viscosus* to efficiently attach to teeth while planktonically suspended in saliva (1).

ACTINOMYCES NAESLUNDII

Actinomyces naeslundii is a Gram-positive bacterium characterized by its rod shape and prominent fimbriae that allow it to bind to the tooth surface. *A. naeslundii* is facultatively anaerobic, nonmotile, non-spore forming, and commonly grows in the oral cavity. (6) *A. naeslundii* is also a major component of dental plaque and it is known to cause dental caries, root canal infections, and periodontal disease (27). It is one of the specific species of the genus *Actinomyces* capable of causing the chronic bacterial infection, actinomycosis,

in humans (8). *A. naeslundii* plays a role in actinomycosis by initiating infections in carious teeth, as well as causing infections in places where the mucosal integrity of the tooth is compromised (32). These infections are the precursor of the actinomycosis, however, the bacterium itself exhibits very low pathogenic properties. These properties are enhanced by the presence of microorganisms such as species of *Prevotella*, *Staphylococcus*, and *Streptococcus* (35).

ROTHIA DENTOCARIOSA

Rothia dentocariosa is a facultative anaerobic, Gram-positive, pleomorphic bacterium that is commonly found in either filamentous or coccoid forms (24). It is common in the oral cavity, and has been associated with many types of bacterial infections. Although *R. dentocariosa* was first isolated in dental caries, endocarditis is the most common infection caused by the organism. Cases have also been reported associating *R. dentocariosa* with intrauterine fetal death (16,25,28). *R. dentocariosa* is commonly associated with dental caries and periodontal disease. Studies have demonstrated the ability of *R. dentocariosa* to induce the release of TNF-*a* by macrophages, thereby eliciting inflammatory responses within the gingiva (36).

ENTEROCOCCUS FAECALIS

Enterococcus faecalis is a facultative anaerobic, Gram-positive cocci bacterium that is typically found in pairs or short chains. The most common locations for this microorganism in humans are the gastrointestinal tract, the vagina, and the oral cavity. *E. faecalis* is considered to be the most prevalent bacterium found in root canal infections (23). It is able to resist nutritional deprivation in part due to its ability to utilize a number of energy sources including malate, citrate, lactate, carbohydrates, and a number of amino acids. It is also able to withstand extremely basic environments, high salt concentrations, desiccation, and the presence of many antibiotics (17). These attributes allow *E. faecalis* to thrive in deep root canals. Additionally, scientists recently discovered *E. faecalis* has the ability to suppress lymphocyte function as well as produce lytic enzymes which aid in destruction of gingival tissues (9).

CANDIDA ALBICANS

Candida albicans is a ubiquitous pleomorphic fungus that colonizes the gastrointestinal, epithelial, and mucosal tissues of over 70% of the human population (26). Whether *C. albicans* takes on hyphal, pseudohyphal, or yeast form is dependent upon environmental factors such as temperature, pH, and presence of serum. *C. albicans* is an opportunistic pathogen that is often the cause of infection in immunocompromised patients, such as those with HIV/AIDS or indwelling medical devices. Imbalances in normal body flora due to antibiotic and steroid use have also been shown to provoke the overgrowth of *Candida*, causing candidiasis (21). Oral candidiasis, commonly known as thrush, can exacerbate already existing oral diseases. *C. albicans* has also been found within dental caries and is a contributor to periodontal disease (26). Many proteins within saliva have been found to promote adherence of *C. albicans* to gingival tissues (10). Additionally, tissues already colonized by streptococcal strains greatly enhance the ability of *C. albicans* to colonize the oral cavity (10).

MATERIALS AND METHODS

MICROBIAL STRAINS AND MEDIA

One yeast and five bacterial strains were utilized for this investigation. All microorganisms were obtained from the American Type Culture Collection (ATCC), Manassas, VA. The bacterial strains used were: *Lactobacillus casei* (ATCC 393), *Rothia dentocariosa* (ATCC 17931), *Enterococcus faecalis* (ATCC 29212), *Actinomyces naeslundii* (ATCC 12104) and *Actinomyces viscosus* (ATCC 43146). The yeast strain used was *Candida albicans* (ATCC 10231). Four of the bacterial strains were grown in tryptic soy broth + 1% sucrose (TSBS; Difco Laboratories, Detroit, MI), while *Actinomyces naeslundii* was grown in brain heart infusion broth (BHI; Difco). All bacteria were incubated at 37°C in 5% CO2 for 24 or 48 hours. *C. albicans* was grown in yeast peptone dextrose broth (YPD; Difco) at 30°C in 5% CO2 for 48 hours. The design of the experiment is consistent with other studies that have been both published and conducted in this laboratory (20).

MINIMUM INHIBITORY CONCENTRATION AND PLANKTONIC GROWTH QUANTIFICATION

Nicotine dilutions were prepared using a 1 g/ml nicotine stock solution (Sigma Chemical Co., St. Louis, MO) in TSBS or BHI for the bacteria and in YPD for the yeast. An initial dilution of 32 mg/ml of nicotine was prepared and a 1:2 serial dilution series was made through 0.25 mg/ml. Additionally, a control was prepared containing only 200 µl of media with no nicotine. Diluted aliquots of 190µl were transferred into a sterile 96-well microtiter plate along with 10 µl of an overnight culture of the respective organism. The bacterial microtiter plates, excluding *A. naeslundii*, were incubated at 37°C in 5% CO2 for 24 hours and *C. albicans* and *A. neaslundii* were incubated under the same conditions for 48 hours. After incubation, the absorbance of each well in the plates was measured in a spectrophotometer (SpectraMax 190; Molecular Devices, Inc., Sunnyvale, CA) at an optical absorbance of 595 nm (OD595) to determine the minimum inhibitory concentration (MIC). (20) To quantify the planktonic growth, 120µl aliquots of planktonic culture fluid was removed from each well into corresponding wells in a fresh 96-well microtiter plate and the absorbances determined in a spectrophotometer at 595 nm. Absorbance values greater than .05 units of the control would be considered a significant change. Anything equal or less to .05 absorbance units would be considered insignificant.

MINIMUM BIOFILM INHIBITORY CONCENTRATION

Ninety six-well plates of the organisms in varying concentrations of nicotine were prepared as described above. Following incubation, the remaining planktonic culture fluid was disposed of and 200µl of 10% formaldehyde (Fisher Scientific Co., Pittsburg, PA) was added to each well for 30 minutes at room temperature. The plate was then washed in deionized (DI) water before adding 200µl of 0.3% crystal violet (Sigma) to each well. The plate was allowed to incubate for an additional 30 minutes and then washed in DI water. 200µL of isopropanol (Fisher) was then added to each well to allow extraction of the crystal violet from the biofilm cells. Following a one-hour incubation in isopropanol at

room temperature, the optical density of the biofilm was recorded at 490 nm. Using this method, both *R. dentocariosa* and *A. naeslundii* were unable to form tight biofilms in their 96-well plates. Therefore, instead of utilizing formaldehyde, crystal violet and isopropanol, the biofilm of *R. dentocariosa* and *A. naeslundii* were resuspended in 200µl of a 0.9% saline solution and the optical density was measured at 595 nm.

STATISTICAL ANALYSES

All experiments were carried out in quadruplicate wells at least three times. Means, standard deviations, and statistical significance were calculated using Excel. Student t-tests were used to determine p values, which were considered significant if $P<0.05$. Data was transferred to SigmaPlot 12.0 to graph and further analyze results.

Table 1 Minimum inhibitory concentration (MIC) and minimum biofilm inhibitory concentration (MBIC) of nicotine on microorganisms

Organisms	MIC (mg/ml)	MBIC (mg/ml)
L. casei	16	16
A. viscosus	16	16
A. naeslundii	16	16
R. dentocariosa	16	16
E. faecalis	32	16
C. albicans	16	16

RESULTS

Table 1 indicates the MIC and MBIC of each microorganism tested. All organisms had an MIC of 16 mg/ml of nicotine except *E. faecalis*, which had an MIC of 32 mg/ml of nicotine. Similarly, the MBIC of all organisms tested was 16 mg/ml of nicotine.

The total growth of the organisms in each nicotine concentration is presented in Fig. 1. This data represents the total of biofilm and planktonic growth. *L. casei*, *A. viscosus*, *R. dentocariosa*, and *C. albicans* had an increase in growth through 8 mg/ml of nicotine. *E. faecalis* growth increased through 16 mg/ml but growth of all other organisms was decreased at this concentration of nicotine. *A. naeslundii* growth decreased through 8 mg/ml and demonstrated complete inhibition beginning at 16 mg/ml. Additionally, all organisms displayed a decrease in total growth at 32 mg/ml of nicotine.

Fig. 2 denotes the planktonic growth of each organism in nicotine. Decreased growth occurred at 32 mg/ml of nicotine in all organisms tested. Additionally, *L. casei*, *A. viscosus*, and *R. dentocariosa* demonstrated statistically significant inhibition of planktonic growth at 16 mg/ml. An increase in growth occurred at 8 mg/ml of

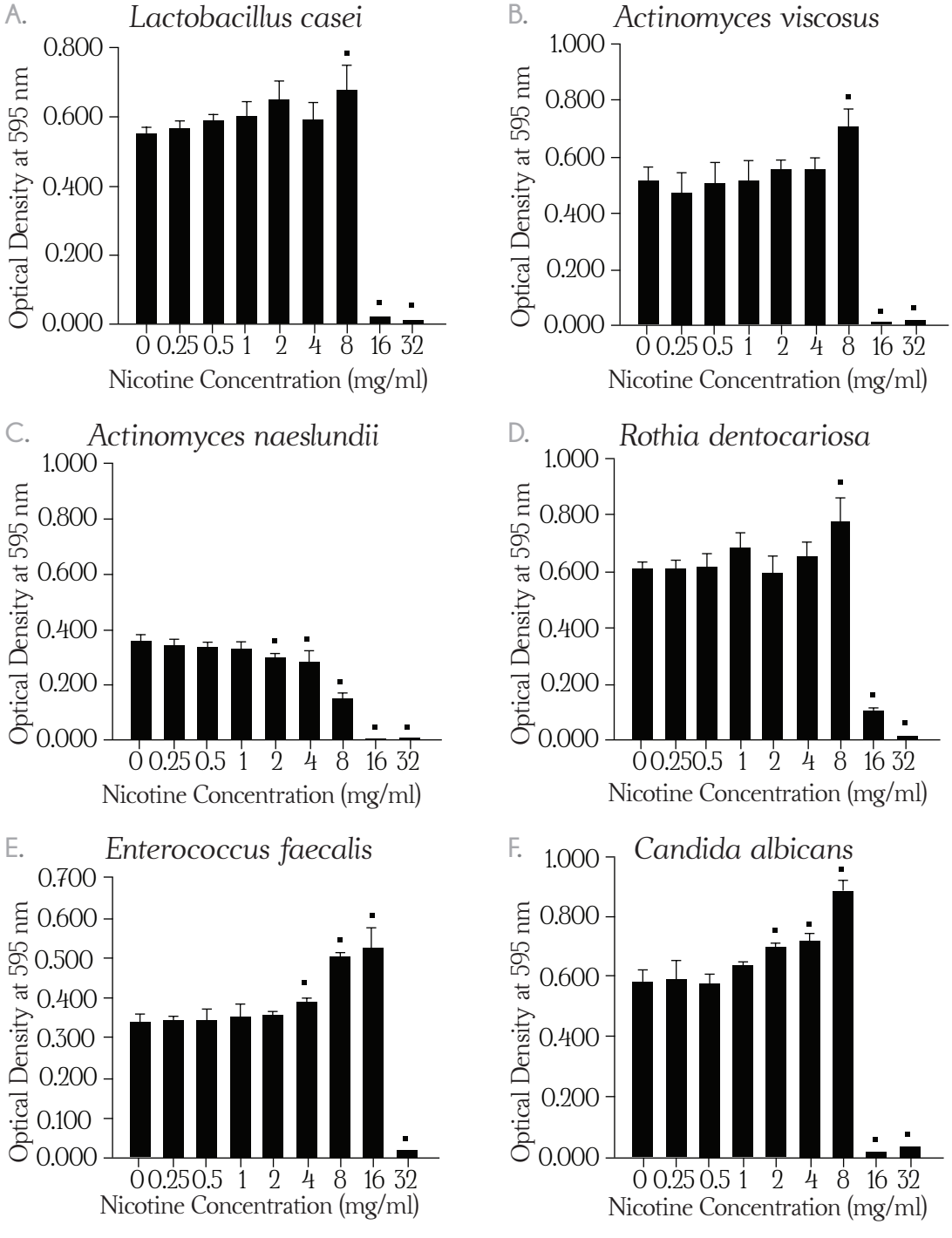

Fig. 1: Total growth of nicotine-treated microorganisms in quadruplicate wells. After incubating with various concentrations of nicotine the OD was measured at 595 nm. The OD value is represented as (mean + SD). Each experiment was repeated at least three times. Asterisks indicate statistical significance ($P < 0.05$) compared to the 0 mg/ml nicotine control.

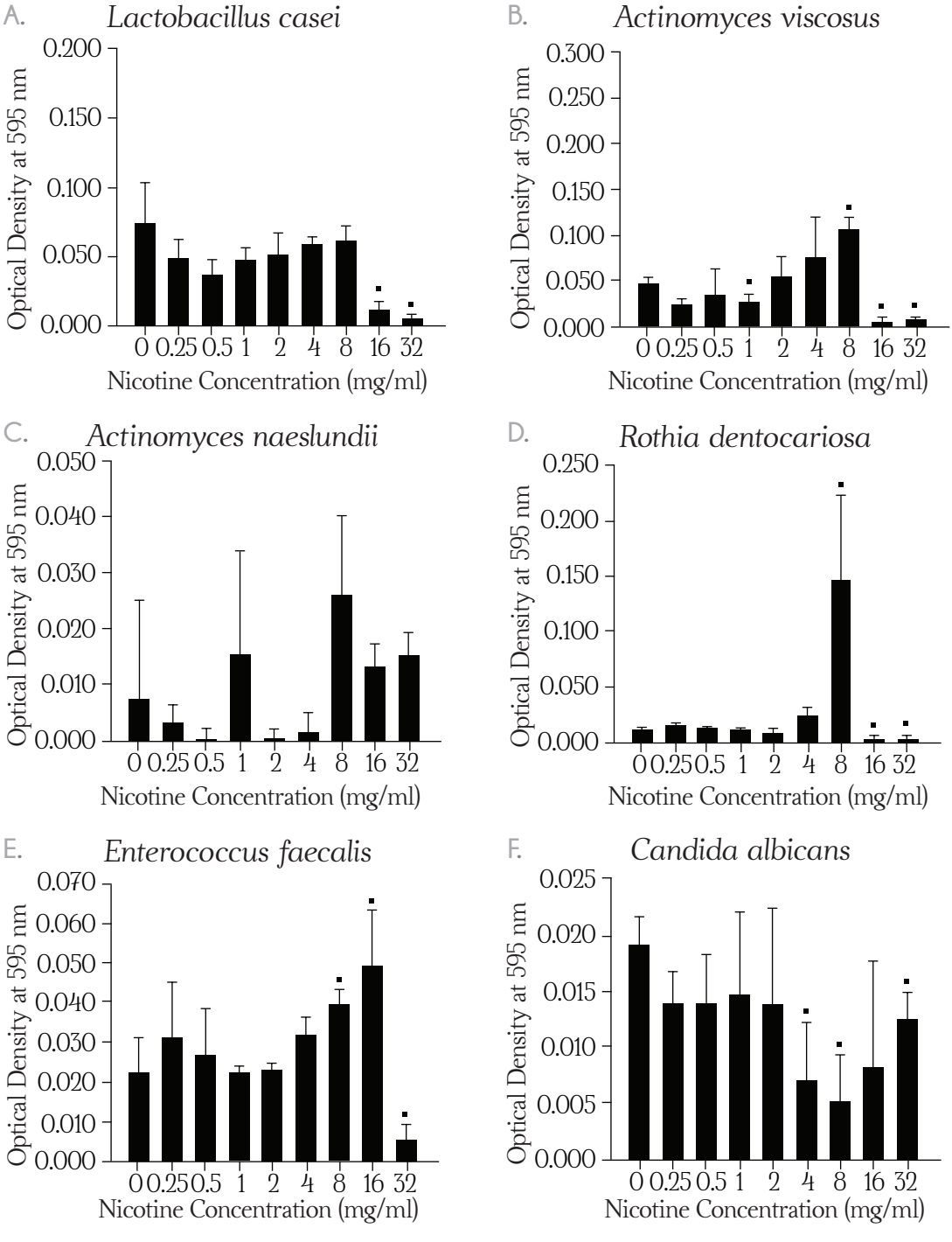

Fig. 2: Planktonic growth of nicotine-treated microorganisms in quadruplicate wells. After incubating with various concentrations of nicotine for 48 hours, the supernatant was removed and the OD of the supernatant was measured at 595 nm. The OD value is represented as (mean + SD). Each experiment was repeated at least three times. Asterisks indicate statistical significance ($P < 0.05$) compared to the 0 mg/ml nicotine control.

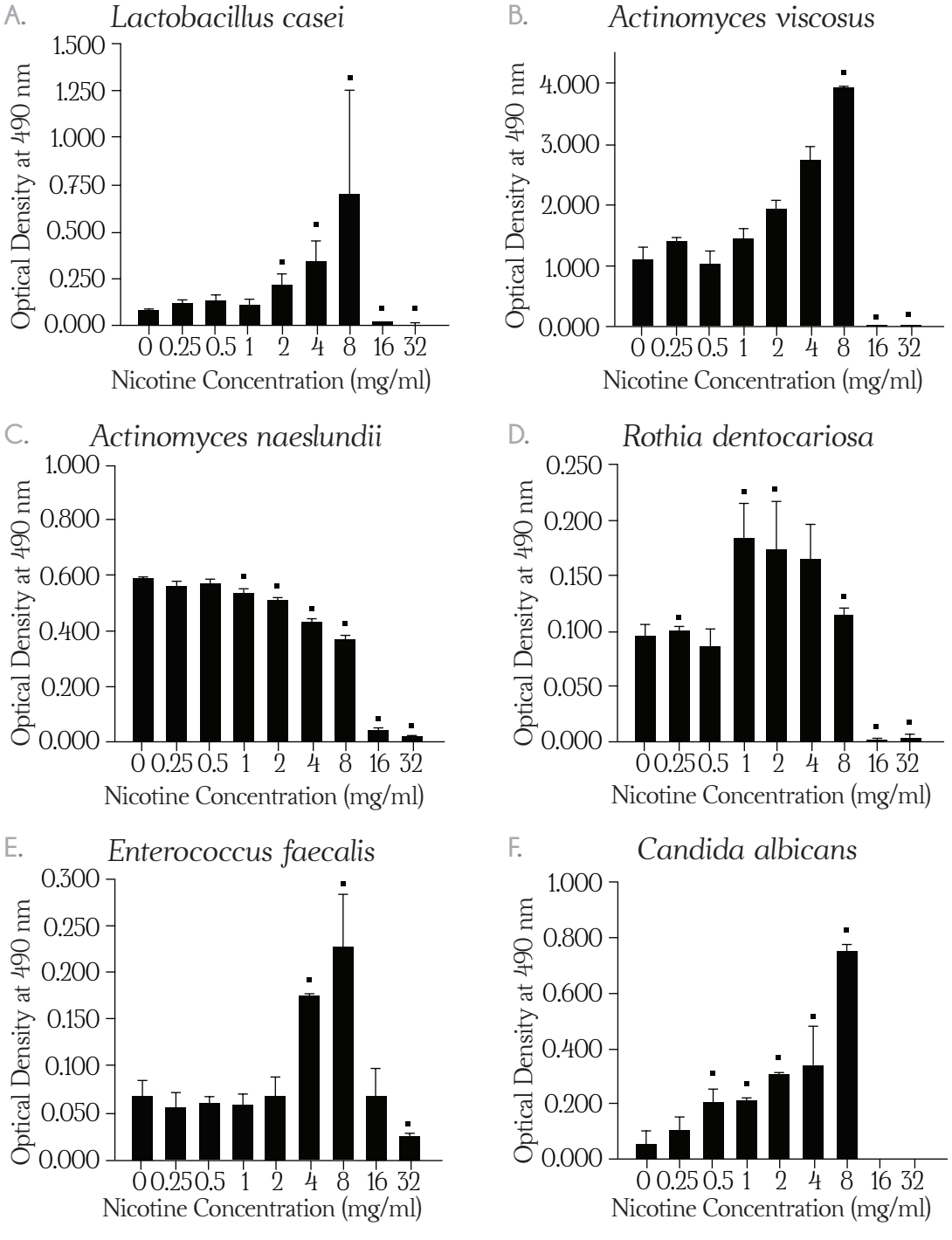

Fig. 3: Biofilm formation of nicotine-treated microorganisms in quadruplicate wells. After incubating with various concentrations of nicotine for 24 or 48 hours, the biofilm was either stained with crystal violet or suspended in saline and the OD was assessed at 490 nm or 595 nm, respectively. The OD value is represented as (mean + SD). Each experiment was repeated at least three times. Asterisks indicate statistical significance ($P < 0.05$) compared to the 0 mg/ml nicotine control.

Table 1 indicates the MIC and MBIC of each microorganism tested. All organisms had an MIC of 16 mg/ml of nicotine except *E. faecalis*, which had an MIC of 32 mg/ml of nicotine. Similarly, the MBIC of all organisms tested was 16 mg/ml of nicotine.

The total growth of the organisms in each nicotine concentration is presented in Fig. 1. This data represents the total of biofilm and planktonic growth. *L. casei*, *A. viscosus*, *R. dentocariosa*, and *C. albicans* had an increase in growth through 8 mg/ml of nicotine. *E. faecalis* growth increased through 16 mg/ml but growth of all other organisms was decreased at this concentration of nicotine. *A. naeslundii* growth decreased through 8 mg/ml and demonstrated complete inhibition beginning at 16 mg/ml. Additionally, all organisms displayed a decrease in total growth at 32 mg/ml of nicotine.

Fig. 2 denotes the planktonic growth of each organism in nicotine. Decreased growth occurred at 32 mg/ml of nicotine in all organisms tested. Additionally, *L. casei*, *A. viscosus*, and *R. dentocariosa* demonstrated statistically significant inhibition of planktonic growth at 16 mg/ml. An increase in growth occurred at 8 mg/ml of nicotine for *A. viscosus* and *R. dentocariosa*. Moreover, *E. faecalis* planktonic growth was enhanced at 8 mg/ml and 16 mg/ml of nicotine. Significant inhibition was observed in 1 mg/ml of nicotine for *A. viscosus* and for 4, 8, and 32 mg/ml of nicotine in *C. albicans*. *A. naeslundii* along with other bacteria in this study depict a high sensitivity to nicotine. The planktonic readings varied between trials, which made interpretations of planktonic response to nicotine inconsistent at times and difficult to interpret.

Biofilm formation of the organisms in each nicotine concentration is indicated in Fig. 3. An upward trend in biofilm growth through 8 mg/ml of nicotine was observed for *L. casei*, *A. viscosus*, *E. faecalis*, and *C. albicans*. *R. dentocariosa* exhibited the most growth at 1 mg/ml of nicotine but also had increased growth in 0.25, 2, and 8 mg/ml. Inhibition of biofilm formation for all organisms was indicated at 16 and 32 mg/ml of nicotine. Additionally, *A. naeslundii* demonstrated statistically significant inhibition beginning at 1 mg/ml and continuing through 32 mg/ml of nicotine.

DISCUSSION

Significant inhibition occurred with all tested organisms in nicotine concentrations of 16 and 32 mg/ml except *E. faecalis*, providing a MIC of 32 mg/ml for *E. faecalis* and a MIC of 16 mg/ml for the remaining organisms. Similar studies demonstrate other oral microorganisms, most notably *S. mutans*, also have a MIC of 16 mg/ml. (7)(20) It is possible that the MIC exists at nicotine concentrations between 8 and 16 mg/ml but further studies are required to determine the validity of this supposition. Research by Huang *et al.* (2014) also demonstrated stimulatory effects by nicotine on *Streptococcus gordonii* (34). Another study investigating nicotine effects on oral microorganisms like *Actinobacillus actinomycetemcomitans*, *Porphymonas gingivalis*, *Fusobacterium nucleatum* and *S. gordonii* demonstrated no effect could be observed in concentrations less than 1 mg/ml (13). The research conducted with these microorganisms is consistent with this study and demonstrates significant variations in activity at higher concentrations of nicotine. Alternatively, Pavia *et al.* (2000) were able

to demonstrate that nicotine concentrations between 100 and 250 ug/ml reduced growth of *Escherichia coli, Listeria monocytogenes, Candida albicans, Klebsiella pneumoniae* and *Cryptococcus neoformans* (27).

All concentrations of nicotine exerted some inhibition of planktonic growth, particularly at 16 and 32 mg/ml, when compared to the non–nicotine–treated controls for both *L. casei* and *C. albicans*. Previous research suggests that planktonic bacteria are more susceptible to various chemicals and antibiotics and so it is possible that the presence of nicotine induced this effect on the microorganisms studied (4). No reports were found on planktonic yeasts relating to chemical susceptibility, but it is plausible that similar effects as those observed in planktonic bacteria might also exist in yeast. All species displayed planktonic inhibition at 32 mg/ml. Furthermore, at concentrations of 16 mg/ml both *R. dentocariosa* and *L. casei* were significantly inhibited. *C. albicans* growth was also inhibited in nicotine concentrations of 4 and 8 mg/ml. *L. casei, A. viscosus, R. dentocariosa, E. faecalis,* and *C. albicans* demonstrated no statistically significant differences in non–nicotine treated planktonic organisms compared to those treated with nicotine concentrations up to 2 mg/ml. *A. viscosus, R. dentocariosa* and *E. faecalis* had planktonic growth enhancement in concentrations of 16, 8, and 8 through 16 mg/ml, respectively. All OD values measured from the planktonic growth were much lower than OD values recorded from biofilm growth. Visual analysis of the microtiter plates also signified greater biofilm growth when compared to planktonic growth. It is known that bacteria prefer existing in biofilms rather than as unicellular planktonic cells and it has been suggested that nearly 90% of bacteria exist as biofilms in nature. (31) The present study further supports this theory as well as extends the observation to *C. albicans* due to its perceived preference to grow as a biofilm rather than as a planktonic yeast phase. As Fig. 3 indicates, a notable upward trend in the biofilm formation of *L. casei, A. viscosus, A. naeslundii, E. faecalis* and *C. albicans* was demonstrated through 8 mg/ml of nicotine. Biofilm growth was most notably enhanced in 8 mg/ml of nicotine in the aforementioned organisms. *R. dentocariosa* presents a unique demonstration of enhancement in concentrations of nicotine ranging from 0.25 to 8 mg/ml but was most prominently enhanced at 1 mg/ml. In addition, studies have shown that an increase in adhesion to surfaces occurs over time when *C. albican, S. mutans,* and *S. pneumonaiae* are exposed to cigarette smoke condensate (CSC). (4)(29) CSC is a crude aqueous extract of tobacco and contains approximately 2.4% nicotine by weight along with many other chemical components of tobacco. It is plausible that one of the attributing factors to the increase in adhesion is due to the nicotine contained within the CSC, which would thus account for the results observed in this study. Additionally, Antunes *et al.* 2012 (3) found that oxidative stress caused increased biofilm formation of *P. aeruginosa*. This study identified hydrogen peroxide as one causative agent of oxidative stress in cigarette smoke. Other research has indicated nicotine can also act as an oxidative stressor, which might account for the increased biofilm formation seen in many of the nicotine concentrations used in the current study (11). The MBIC of nicotine for all organisms studied was determined to be 16 mg/ml as indicated in Fig. 3. As with the MIC, it is possible the MBIC also exists at some concentration between 8 and 16 mg/ml of nicotine, but further studies are needed to explore this probability.

In this present study, concentrations at which enhanced growth was demonstrated correspond to average nicotine concentrations found in many of the tobacco

products discussed previously. It is believed that the findings of this study support the hypothesis that the increased risk of oral health issues faced by many tobacco users is caused in part by the stimulation of oral microorganism biofilm formation by nicotine and other tobacco components.

This study can be used to provide confirmation that further research on the relationship between oral microorganisms and nicotine is needed. Under similar growth conditions, the organisms could be exposed to nicotine intermittently throughout a specified length of time, much like how a smoker smokes periodically throughout the day. This study emulates the nicotine exposure which occurs to the oral flora of a smoker. Additionally, because microbes often exhibit different behaviors when interacting with multiple species, it would be beneficial to conduct a study which assesses the biofilm formation of multiple bacterial species in the mouth. This would more closely simulate an in vivo environment in an attempt to simulate dental caries, oral biofilm, periodontal disease and other diseases present in the mouth. Furthermore, investigating the types of interactions which occur between nicotine, proline-rich proteins, and bacteria would allow for a more thorough understanding of the specific mechanisms which cause biofilm enhancement in the presence of nicotine.

ACKNOWLEDGEMENTS

This study was financially supported by the Center for Research and Learning, Indiana University Purdue University at Indianapolis through a Multidisciplinary Undergraduate Research Institute (MURI) grant. Assistance and advice provided by mentors Drs. Richard Gregory, Fengyu Song, Angela Bruzzaniti, and Jack Windsor was greatly appreciated. Further thanks are also extended to Ruijie Huang, Grace Gomez, and Landon Howell for additional support. The authors declare no conflicts of interest exist.

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PERSPECTIVE

UNDERGRADUATE RESEARCH IN THE SCIENCES AS A SERIES OF TRANSFORMATIVE OPPORTUNITIES

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EXPECTATIONS OF UNDERGRADUATES

Undergraduate students tend to find, from the moment of their arrival on campus until graduation, that they are held to a long series of ever-increasing professional expectations. Some are curricular standards set by the university, while others are evolving objectives the students decide for themselves. Students anticipate they should engage during lecture and laboratory courses, maintain high academic standings throughout their college career, and, potentially, pursue part-time employment. But perhaps one of the most pronounced expectations of an undergraduate is for the student to become socially and professionally involved on campus. The idea of campus "involvement" can be both vague and intimidating, especially to a new student.

Universities have numerous and varied organizations; these can be academic, social, faith-based, or service-oriented in nature, to name only a few. When a student is faced with many opportunities but has limited time, it can be a challenge to decide which of these commitments are worth pursuing with limited time. It is in an undergraduate student's best interest to choose activities that complement his or her area of study while promoting personal and professional growth. However, a student may be more interested in finding activities which build lasting, meaningful relationships with peers. This fundamental choice does not have to be a mutually exclusive one. None of the elements mentioned above are missing from undergraduate research experiences, which is why commitment to extended study outside of the classroom is one of the most valuable uses for an undergraduate's time. This is especially true of students majoring in biology. Research allows students to apply broad concepts learned in the classroom to original research problems in the field or laboratory setting, all of which enhances content comprehension, professional development, and peer interaction.

CONTENT COMPREHENSION AND TECHNICAL SKILL

The most immediate benefit of an undergraduate research experience is the ability to translate what is learned in the laboratory to one's understanding of scientific concepts learned in the classroom. A recent study by Hunter *et al.* indicated a common gain for students after an undergraduate research experience was perceiving "increased relevance of coursework" (2007). In a science lecture, broad and sometimes overgeneralized ideas are taught first, and eventually the finer details are covered. Research, however, begins by trying to answer a very specific question or solve a particular problem. For example, my first research experience involved determining the effects of different concentrations of carvacrol (a bactericidal extract from oil of oregano) on *Bacillus cereus*, a toxigenic bacterium associated with foodborne illness and ocular infections. Using a nematode model, *Caenorhabditis elegans*, I was able to quantify the effects of *Bacillus* toxins because the nematodes would ingest the bacteria and become infected. Although I had no background in cell biology or genetics at that point in my college career, my research advisor was able to build from my knowledge of basic biology and teach me about the organism I was studying.

Often, I encountered information while working in the lab before I had taken a course which covered those ideas—part of my *Bacillus* project involved transforming the bacterium with a specific plasmid vector that my advisor and I had designed. When I took genetics a few semesters later, I studied how bacteria are naturally competent. Research for me became a balance of relating concepts from the classroom to my project, and relating

my research back to the classroom to realize the real-world implications of what I was learning. This learning style does not stress memorization as much as application, which is more valuable considering scientific "facts" may change with breakthroughs (AAAS, 2011). Translating knowledge between the lab and the classroom allowed me to appreciate the complexity and importance of what I was studying, while giving me a better, more complete understanding of some of the more challenging theories.

> "RESEARCH FOR ME BECAME A BALANCE OF RELATING CONCEPTS FROM THE CLASSROOM TO MY PROJECT, AND RELATING MY RESEARCH BACK TO THE CLASSROOM..."

As classroom content is applied to a real-world setting, students performing research also begin to increase their technical skill set in the lab. Some of the first aspects of my research experience were becoming oriented with the lab and learning proper execution of basic bench skills, such as using aseptic technique or performing polymerase chain reaction (PCR). Bench work and instrumentation revealed the reality of research: it can often be tedious. But the practical experience was worthwhile in learning what the process of designing, executing, and analyzing an experiment is like from start to finish. One of the most valuable skills learned in research is the ability to troubleshoot problems when they arise. In the early phases of my *Bacillus* study, one nematode was to be placed in an individual well with agar on a 96 well plate. Then, each individual nematode could be studied separately as the *Bacillus* toxins began to take effect. Isolating a microscopic animal, however, turned out to be extraordinarily difficult. It was hard to avoid picking up multiple nematodes at a time, so the methodology for the project had to be amended. While this may sound like it would have been a frustrating experience, it was actually exciting and eye-opening. The difference between a real undergraduate research experience and a "canned" lab experiment that a student encounters in a basic biology class is that no one knows the "right" way to execute a research project. This gives the student ownership of the entire experiment and the freedom to be creative when adjusting for problems encountered during the process, and the end results are that much more rewarding when the project is completed.

The three-hour labs designed for a classroom setting may give students some practice in bench techniques, but these skills are only applied to a piece of an overall research experiment. In an immunology lab, I read through a three-part protocol that stated parts one and two had been done for the students. This is not a criticism of the immunology course; it simply illustrates that students have a limited perspective of the goals in a research experiment and the process involved to acquire the end results in a short lab period. Furthermore, students may find it difficult to imagine application of methods they are using to solve real problems, even if they understand the concept being illustrated in a classroom lab. This is the advantage of undergraduate research: students are exposed to the scientific method from beginning to end, including the planning of the project and

the presentation of results. After participating in undergraduate research, I no longer look at a graph and see only the data, but also have an appreciation for the months and years of work that producing only one figure required. I have a more realistic conception of what research is like, and I am more able to understand how other scientists arrived at their results and conclusions because I have a sense of what they may have done in their own processes. In fact, one study indicated students who perform research–based activities rather than lab–based activities gain confidence in interpreting data (Brownell, 2012). When I have questions about a concept in microbiology, I can imagine how a scientist may have approached discovering an answer because I have been exposed to a number of techniques and instruments used in my field. Research enables a student to think critically within his or her own field rather than simply accepting facts in a classroom without being able to put the "pieces" together in a broader understanding of the world.

Performing undergraduate research shifts a student's outlook on aspects of his or her own specific area of study. But the research process may also give students a new appreciation for other natural sciences as well, primarily because students will discover that subsets of science are not separated by as distinct of boundaries as course curricula may indicate. While I primarily use techniques I have learned in microbiology courses in the research lab, I also find myself referring to knowledge I acquired in chemistry or physics classes to execute my project. For example, purifying the plasmids necessary for transformation of *Bacillus* requires a number of reagents. I relied on general chemistry knowledge to make these solutions at appropriate concentrations. And while not a topic I studied directly, having knowledge of the laws that govern forces and energy because of my physics education also helps me to understand the living systems in which I was interested. Physics, chemistry, and biology all build upon each other, something that is not stressed in lecture. Therefore, it was difficult for me to see how necessary my understanding of all of these disciplines was until I had research experience.

Undergraduate research gives a student appreciation for all of the "core curricular" sciences, but for students studying microbiology, research also allows for a better understanding of the relationship between the various disciplines within biology. For example, my research in environmental microbiology involved taking measurements such as pH, dissolved oxygen, water table height, and temperature of the water in which the algae of interest was growing. These data were considered when studying nutrient effects on algae because they can influence algal metabolism, as well as the presence of other microbes, and interactions between these communities also impact algal biomass and metabolism. Evaluating the influence of the environment on microorganisms helped me appreciate that the toxigenic bacterium I was studying in the food microbiology lab also changed depending on the conditions in which the cells grew, even though my work was in the laboratory and not in the field. I appreciated more the ability to carefully control variables and I became a more conscientious scientist. While working with *Bacillus*, I learned the importance of handling samples with precise, sterile techniques, and this training prepared me to more efficiently process hundreds of water samples in the environmental microbiology lab. My involvement in two laboratory projects has exposed me to the details within a subdiscipline, but has also enabled me to think critically about the broader concepts and implications of the subjects I am studying, and the problems and diagnoses I will make in my future training and career.

"SOFT" SKILLS AND PROFESSIONAL DEVELOPMENT

Developing a deeper understanding of the biological sciences through research is a critical and valuable undergraduate experience, and a student undertaking a research project might expect this to be an outcome of the process. What students may find surprising is that they also grow interpersonal skills immensely while engaging in research. Communication of scientific concepts becomes more comfortable as a student has more practice both reading and writing scientific literature. Utilizing primary literature—peer-reviewed publication of original scientific findings—is helpful in learning background information for a project, but it also adjusts a student to thinking and speaking in scientific terminology. As scientific studies produce information much faster than editions of textbooks can be produced, relying on scientific articles for supplemental detail of a broader classroom concept can be a critical piece of an undergraduate science education (Hoskins, 2007). The first time I read a seven-page piece of primary literature about *Bacillus*, I spent several hours deciphering the dense writing. I found later that this was a valuable investment of my time; I became more confident in speaking about my research to professors and other students because I understood the "language." With enough practice, I could read a scientific article as fast as I could read anything else, and this gave me a sense of belonging to the scientific community.

The more a student reads primary literature, the better he or she will be able to compose a poster presentation, oral presentation, or manuscript in the future, and the more insightful their questions will become. Likewise, delivering an oral or poster presentation requires much practice to convey the essential information to an interdisciplinary audience. Successfully transferring the salient aspects of your work to a mixed audience involves not only a thorough understanding of your project on all levels, but a realization for how to "teach" and engage your audience as well. This concept is becoming more important with each passing year as new specialty areas develop within each subdiscipline of the life sciences. Without consideration of the audience at hand when rehearsing a presentation, the implications of a student's finding may be lost on those who are not familiar with the jargon of a subspecialty. It is critical that a student presents his or her findings in a way that allows the scientific community to learn from the results and build from them in future studies. With careful preparation, especially in the background content of a presentation, a student can successfully and confidently convey findings from a study without overestimating the audience's background, and without running overtime, two of the most common errors among students and experienced researchers alike. As a student gains more experience presenting, these presentations become less rehearsed and more of a conversation between the student and the audience. This is an exciting transformation, because students can begin to share ideas with peers about each other's projects, and they become more interested and engaged in each other's work as the conversation progresses. I encountered this at the 2014 Indiana Academy of Science conference, where a professor was presenting a poster on her study of the nervous system of the same nematode model which I used for my *Bacillus* project. As the conversation progressed, I was both learning from this professor and offering valuable information for her; it was a discussion that felt more collegial than instructional, which is atypical compared to most of my interactions with professors. Communicating and sharing ideas in this way builds a sense of fellowship between students and professors, so the student starts to feel less

like a science major and more like a scientist through this process of contributing and collaborating.

Collaboration is, in fact, an important piece of the research process. Even if a student is working on an individual project, he or she will often rely on peers who have more research experience for advice and wisdom. This student-centered learning, with the advising professor assuming the role of a facilitator rather than an instructor, builds students' prowess in the lab and willingness to give input as to the direction of the research projects discussed. Teamwork in the lab makes the research projects more successful, but it also allows a students to form valuable friendships with others of their own discipline. Another research experience which I undertook relied heavily upon collaboration. During the summer of 2013, I studied in the Bonanza Creek Experimental Forest in Fairbanks, Alaska for three weeks with a professor and graduate student. We were assessing the effects of warming and nutrient addition on algal biomass and metabolism. This experiment had many components, and at times, it was difficult to keep the "big picture" in mind when I was focused on my comparatively small set of data. I was able to rely on the graduate assistant for help when I was trying to make sense of the results. She helped me have a better appreciation for the role of algae as primary producers, and I was able to keep the end goal of the experiment in mind because of her explanations. I began to see her as a mentor, but also as a friend, because we worked very closely over the course of those three weeks. But these friendships form regardless of the length or location of the project. I interact with students working in the same research labs as I on a more regular basis than many other students. Not only do we collaborate on our research together, but we have many of the same classes together as well, so some of the best connections I have had with peers during my college career have resulted from research experiences.

My relationships with my faculty advisors have also grown and become more valuable than I anticipated as I have become more involved with research. At the beginning, I was being told what to do and how to work at the bench. I was being taught in the traditional way I was used to in a classroom, although it was one-on-one interaction. As my skills grew and I relied on my professors less for technical instruction, I felt more confident in expressing my take on the data or my ideas for amending the methodology. My advisors respected what I had to offer; I felt trusted and accepted as a scientist, even while I was still their student. Beyond that, my advisors have been incredible resources to me in realms outside of the research laboratory. They have written recommendation letters for me and edited my research presentations and posters, but they've also given me advice throughout my undergraduate career, which has been what I value most about out interactions. I can share experiences I'm having in class or in the process of applying for medical school, and they encourage me and give me a sense of what to expect as I move forward in my college years. Having a faculty member support me as I work to accomplish my goals has increased my confidence and improved my work, and has been easily the best aspect of my undergraduate research experiences.

By mentoring undergraduate students, faculty engage in service to their profession by training future scientists. Of course, the student is helping further that research project, but there is a great deal of commitment to the training of that student and investment in that student's future given by the most dedicated faculty before those results emerge. "Service to the profession" has been heavily emphasized in my own

> MY ADVISORS RESPECTED WHAT I HAD TO OFFER; I FELT TRUSTED AND ACCEPTED AS A SCIENTIST, EVEN WHILE I WAS STILL THEIR STUDENT.

research training; for example, I have been especially encouraged to be on a journal editorial board someday so that I can aid my peers in science by critiquing their manuscripts. As an undergraduate, students are prepared for this service through research training. Students will often read and discuss primary literature with each other or an advisor and learn how to critique an author's work thoroughly while still communicating the errors respectfully. Following an advisor's example, more advanced students can also facilitate the training of some of the novice students in bench technique and general concept comprehension. Commentary on each other's poster presentations and talks also models a professional conference, in which a scientist would field questions from colleagues and engage in dialogue about the study. In addition to professional service, students also serve their community through volunteerism. Our lab community, for example, organizes a fundraiser for Next Generation Nepal, which is a non-profit dedicated to returning trafficked children to their families. We use the "penny war" method of collecting donations and involve the science professors and students in the process. While not directly connected to our lab work, this collaboration for a greater cause on the part of a few research students has allowed us to contribute to society in both an academic and social capacity.

PERSONAL GROWTH AND IDENTITY

As students begin to build relationships with peers and faculty who are also involved in research, these students are engaging in a socialization process into the scientific community. Students in an undergraduate research experience are integrating the role "scientist" as part of their identity, and they are learning that a scientist is so much more than someone who executes an experiment. As I enter into my last year of undergraduate research, I find that I begin to take on the role of a peer mentor while still being guided by advisors and other students. Mentorship is so closely connected to research because science involves a great deal of collaboration to be successful. The characteristics that I have appreciated in my own advisors—patience, enthusiasm, and respect—I have attempted to implement in my own attitude when working with other students. For example, when consulting a lab partner on methodology for the *Bacillus* project, I noticed that she had difficulty recalling some of the math concepts from general chemistry. I was able to find a new way of explaining the calculations that she hadn't heard before which made sense to her. At the same time, my lab partner organized the methodology into a list and was able to walk me through what needed to be done. She saw the bigger picture of the project and how we needed to progress through each phase, whereas I was focused on the details of a particular step. We both assumed different roles in the partnership and were able to teach each other different aspects of the same research project, which was valuable leadership practice. In the future, my career as a physician will require a great deal of patience and commitment to mentorship of medical students and resident physicians. These partnerships are most successful when the members rely on each other's strengths, even though one is the "mentor" and one is the "mentee." It is difficult to be engaged and

invested in one's own learning if one does not have an active hand in the learning process. As a physician, I would expect my mentees to offer input regarding the subject material and I, as a mentor, would be willing to let the students take ownership over solving the problem at hand with guidance from me. I know this method has worked for me while I have been a mentee, and I think it is important to deviate from the traditional lecture-based learning to some degree so the students feel like a valued member of the class or group. This is what research does, and I believe my peer mentorship experience from research will translate easily to the medical field.

Beyond mediating discussion and encouraging my peers in science, research has increased my interest in developing methods of communicating scientific findings to the general public. Through my involvement as an editor for *Fine Focus*, I collaborate regularly with a marketing team, while my role is primarily for handling manuscript submissions. The interdisciplinary project has revealed to me the importance of packaging content in a way that is appealing and understandable for a target audience. This is a new concept to me; I am familiar with marketing products, but the intricacies of marketing information have become a more immediate challenge to me as someone striving to publish in the sciences. The frustration that scientists can feel when their findings are lost on an under-informed audience is expressed by Volpes' The Shame of Science Education (1984):
Public understanding of science is appalling. The major contributor to society's stunning ignorance of science has been our educational system. The inability of students to appreciate the scope, meaning, and limitations of science reflects our conventional lecture-oriented curriculum with its emphasis on passive learning.

I would argue that while the public may have a limited view of some current scientific studies, scientists also have a minimal understanding of how to convey that information to a broad audience. Scientists write and talk for other scientists in the system of publication that currently exists. These are valuable data and analyses, but it is not for everyone. I would argue that undergraduate participation in research begins to encourage students to think about science from other perspectives so that the student can communicate to individuals of various educational backgrounds. For example, an ecology professor of mine once played an NPR interview of a paleontologist; this is a perfect example of an instance in which language had to be carefully tailored to speak to a particular audience, and this particular interviewee did so effectively. Undergraduates may, in their future careers, encounter situations in which they need to convey findings to the media or other public entities. Collaboration in research is a small step in developing these communication skills, because students are only working with other science majors. Nonetheless, students are bound to encounter diversity even within the sciences, and this student-centered, active learning process is excellent practice for conveying scientific content to a number of audiences.

To be certain, communicating scientific concepts is both exciting and challenging for any student new to research. A student is bound to encounter road blocks throughout the research process which will require critical thinking and problem solving, especially when the original methods fail to produce acceptable results. These frustrations are combatted by the desire to satiate one's own curiosity as to how living systems operate, which develops as one becomes more attached to the research project, and more empowered in knowing that research can allow these discoveries to be

had. This desire for understanding motivates a student to be flexible as he or she copes with the challenges associated with running an experiment. I felt tested when attempting to run a successful polymerase chain reaction (PCR) for my *Bacillus* experiment. PCR, like other tools in the arsenal of a microbiologist, involves sensitive reactions and is time-sensitive as well. It was important for me to be precise when working with small volumes of DNA, primers, and reagents. Even though I knew I had handled the samples carefully, it still took several attempts to generate copies of the plasmid I needed, and I was embarrassed I might have had poor technique. My advisor reassured me I was doing well, and that successful PCR is determined by a variety of factors, some of which may have been outside my control. With this in mind, I was able to be more patient with myself as I made more attempts at PCR, and this shift in attitude has translated over to my classroom work as well. I am less likely to get frustrated if, for example, I am trying to solve a chemistry problem that I don't understand. Instead, I look for creative approaches to the question and persist until I find an explanation for the concept that makes sense to me. This patience and flexibility is crucial to the mindset of a college student, because balancing schoolwork can be difficult. Training in perseverance through the research process helps a student better face this obstacle.

Once I was able to solve problems on my own in the lab, I began to feel more ownership over the project which had been assigned to me. I was more comfortable working without supervision and I felt responsible for performing quality work, even though there would be no "grade" assigned to my research. This intrinsic motivation is harder to feel in a classroom setting. Classroom learning is passive, and students may not know how to integrate information that seems surface-level (Lopatto, 2009). The knowledge a student gains in a lecture doesn't feel as though it "belongs" to the student because it is so readily given. But new knowledge generated in research almost has an emotional attachment associated with it, because the student knows first-hand the work required to discover this information. In this way, research is its own reward, and it fosters a desire for understanding in other realms of a student's life.

The personal satisfaction and comprehension of scientific content are only gained, however, if the student is producing original results at the end of the research process. A research project which does not add new knowledge to the scientific community does injustice to both the student and fellow scientists. A typical classroom science lab, when the results are known at the process is designed to "work", is helpful in illustrating a concept but does little to prepare a student for the reality of research as a career, in which results are elusive and methodology often needs revision (Chmielewski, 2009). Furthermore, if a student is not striving to solve unanswered problems through research, the student does not have new information to publish, and the opportunity to grow scientific writing and presenting skills is lost. One way to ensure that a student is building on prior studies but is developing novel results is by reading primary literature. Consulting scientific journal articles, whether for a course or for research, begins to feel more like participating in a dialogue than tedious work. I became more interested in scientific discovery as my research progressed, making me more willing to ask questions of my teachers and advisors when I was confused. Throughout primary and even secondary education, there is this fear associated with "being wrong" which can prevent students from engaging in classroom conversation. This anxiety quickly becomes outweighed during undergraduate education by the

desire to know more as a student becomes more involved with research.

This internal drive brought on by research has allowed me to overcome the fears associated with the risk of trying something unusual. Adapting the attitude that a new experience will enable me, even if it may seem intimidating at first, has been a direct lesson of my undergraduate research experiences. I have learned not to feel anxious when I don't know what to anticipate from a class or a job, because I have experience encountering "the unexpected" in the lab. For example, in the *Bacillus* experiment, the plasmids were designed with the addition of the *gfp* gene,

> "... UNDERGRADUATE PARTICIPATION IN RESEARCH BEGINS TO ENCOURAGE STUDENTS TO THINK ABOUT SCIENCE FROM OTHER PERSPECTIVES..."

so that once the bacteria transformed the vector, fluorescence would be an indicator of expression of products on the vector. We used a flow cytometer to measure fluorescence, and we expected stressed *Bacillus* to express a particular gene on the vector and therefore fluoresce. We also expected our control bacteria not to fluoresce because they did not have the vector with *gfp*. However, our control *Bacillus* did fluoresce. The experiment was repeated, because it was assumed that we mislabeled our samples or some other aspect of the methodology went wrong. But again, the control bacteria fluoresced. Making sense of the unexpected was challenging and exciting, and it was concluded that when stressed, *Bacillus* must produce a primary metabolite that fluoresces. An experience that could have been frustrating ended up being enlightening, and it has allowed me to readily embrace new challenges.

BROAD IMPACTS

Engaging in an undergraduate research experience is a large undertaking. Scientific discovery involves active learning and adapting to new findings, a process initially uncomfortable to students accustomed to lecture-style lessons and rigid syllabi. Yet these challenges enable a student to grow in ways that a standard course could not allow. Students learn the complexity of the scientific method, and are able to appreciate and understand published literature after going through the process themselves. Students collaborate with faculty and peers to better communicate their findings and learn from the experience of others. Students come to realize that they are more capable in understanding and performing science than they could have known. The contributions which undergraduate research students make to the body of scientific knowledge are rewarding and stimulate further interest and motivation in scientific work. In my own experience, research has allowed me to feel immersed in the process of doing science and has made me more invested and interested in my own education. My undergraduate career would have been incredibly different without research as a tool to enhance my core understanding of science and improve my confidence in professional settings. I highly encourage all students participate in an undergraduate research experience to realize their full potential as a scholar and scientist.

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