Abstract (Bold faced words are listed in the glossary)

Title: Determining the microbial drivers of the phosphorus cycle (resubmission)

In a cell, **DNA** becomes **RNA**, a molecule that controls the synthesis of proteins. Proteins then construct all other compounds within a cell, including **extracellular enzymes**. The soil microbial community, the microscopic bacteria and fungi living in soil, produces extracellular enzymes to degrade materials in soil to obtain a nutrient when it is needed. Through the production of such enzymes, the soil microbial community also provides nutrients to plants and plays a role in controlling plant growth. In Southeast Ohio, soils are deficient in the essential element phosphorus, and it has been reported that changes in phosphorus availability have incurred measureable changes in the activity of extracellular enzymes. However, we poorly understand the mechanism that controls this activity; previous studies have pointed to internal control at the level of RNA or external changes in the environment through the production of enzymes. Because RNA is easily degraded and protein synthesis only occurs when there it is needed, quantifying the RNA of **genes** representing extracellular enzymes provides a snapshot of the soil microbial community's response to changes in nutrient availability. DNA and proteins may remain in soil long after the gene or protein is needed so observing their presence does not reflect the immediate needs of the community. This study aims to address the following question: *How does the availability of phosphorus alter the genes expressed by the soil microbial community in low-phosphorus ecosystems*? I plan to observe the differences in the amounts genes created by the soil microbial community in response to phosphorus additions to a low-phosphorus forest ecosystem**.** This research will fill a critical gap in understanding how the availability of phosphorus affects the performance of the soil microbial community performs in forested ecosystems. The data generated will also be useful to inform computer models to better predict how ecosystems will respond to changes in resource availability. I plan to publish my findings in the academic journal *Soil Biology & Biogeochemistry*, and I will present at the Ohio University Student Research and Creativity Expo in Spring of 2018.

Project Narrative

Phosphorus is an essential nutrient for plant growth. However, human activities that have increased acid deposition (i.e. **acid rain**) which has caused a decrease in the amount of phosphorus available to plants. Changes in phosphorus availability also influence the availability of carbon and nitrogen in the soil and has been linked to climate change (11). These effects are especially visible in Southeast Ohio, where older, low-phosphorus soils mean that the region has few means of regaining phosphorus consumed by plants or washed from the soil (26). Plants in this region rely on interactions with the soil microbial community to mitigate the phosphorus deficit and help strike a balance between phosphorus, carbon, and nitrogen. Therefore, by producing extracellular enzymes, the soil microbial community is also the principle driver of the phosphorus cycle, which impacts the cycling of nitrogen and carbon, as well (2, 18). Additionally, although some plants can produce extracellular enzymes from their roots, (8, 15, 17), because of their small size, microorganisms have a greater capacity to use enzymes to obtain nutrients from small soil particles that are not physically accessible to plants (20, 27).

Previous Work in this Research Area - Researchers have traditionally performed phosphorus deficiency studies in tropical or agricultural systems, but phosphorus deficiency and its effects are not restricted to

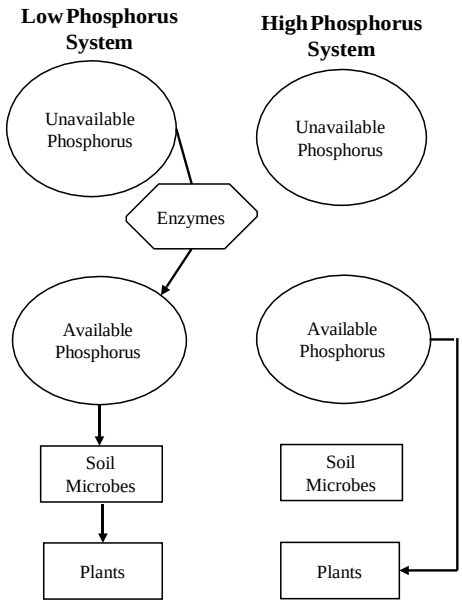

Fig 1. Conceptual diagram of microbial enzyme requirements in low and high phosphorus systems

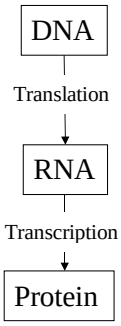

Fig 2. The Central Dogma of Molecular Biology

following genes will be included: phoD and phyA, which represent extracellular enzymes that act on unavailable organic phosphorus in soils to increase its availability to plants and microbes (10, 14); xynB and ypdH, which represent extracellular enzymes that break down cellulose, a material in plant cells, to increase the availability of carbon to plants and the soil microbial community (3, 12); and pepZ and nagA, which represent enzymes that degrade oligosaccharides, another plant material that decays in the soil, to increase the availability of nitrogen (4, 16). I chose these genes because they represent the enzymes our lab has reported on in previous publications (6, 7).

I expect to observe significant changes in the amounts of these genes when phosphorus availability increases. Alternatively, if I observe no significant changes gene expression levels, I can assume that a quality of the external environment inhibits the production of new enzymes**.** To test this prediction, I will use **reverse transcription qPCR** to quantify the relative amounts of these genes in soil (20).

The study sites are located in Southeastern Ohio. These forested sites are naturally low in phosphorus (70 mg P kg^{-1}) when compared to forests in Northern Ohio (188 mg P kg^{-1}) (7). In 2009, 18 treatment plots were established across three forests. Since then, each forest has been subjected to two treatments - control (naturally-occurring soil conditions) and elevated phosphorus (at least 3x natural phosphorus levels through the addition of fertilizer).

In June of 2017, Twelve soil samples were collected from each of the 18 plots and pooled into one bag for a total of 18 combined samples. Because soil samples taken even a few feet from each other can differ, I have taken multiple samples from each plot and pooled them together to obtain a sample that encompasses the variety of soil within a plot, and therefore, the variety of the soil microbial community. Small stones and roots were removed with a 2mm sieve, and the samples were transported to the lab on dry ice. Once in the lab, the samples were kept at -80˚ C until RNA extraction (5).

From July to September 2017, I isolated RNA from the microbial community in the 18 soil samples using the MoBio RNA PowerSoil Total RNA Isolation Kit per the manufacturer's instructions (5). I have completed all RNA isolations, and the RNA isolates are frozen at -80 ˚C in. This fall, I will use the Agilent BioAnalyzer at the Ohio University Genomics Facility to assess the success of my isolations.

This fall, I will also design **primers**, small chains of DNA that selectively bind to regions of interest on a strand of DNA or **cDNA** to select and amplify the genes during qPCR. The company Integrated DNA Technologies provides a free webtool for this purpose. Primers will also be designed for the genes representing DNA polymerase in bacteria and fungi. DNA polymerase is an internal enzyme critical for DNA synthesis that should be present in every microbe in all soil samples, which will provide a baseline for RNA synthesis and protein production occurring in each sample. Because DNA polymerase is so ubiquitous, I will use it as the **reference gene** in this study; during qPCR its presence will form the standard curve against which the presence of the study genes will be compared (9).

The samples will then be **reverse transcribed** using the BioRad iScript cDNA synthesis kit. The cDNA will be used in qPCR at the Ohio University Genomics Facility using the PowerUP SYBR Green qPCR kit from ThermoFisher Scientific. In Winter of 2018, I will analyze difference in the quantity of each gene using a two-way ANOVA in *R* statistical program, a freely available software package, per DeForest et al. 2012 (6). My findings will be part of my Master's thesis, and I plan to present at the Ohio University Student Research and Creativity Expo in Spring 2018, as well as publish in the academic journal *Soil Biology and Biogeochemistry* that summer. See Table 1 on page 5 for a general timeline.

Table 1: Timeline of Events

Activity	2017							2018							
Sampling															
RNA Isolation & BioAnalyzer															
Primer Design															
Reverse Transcritption & qPCR															
Data Analysis															
OU Student Expo															
Thesis & Manuscript Prep.															
Thesis Defense & Graduation															
Month	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A

Significance **-** The method I have chosen will allow me to selectively observe the quantities of the genes representing enzymes that help microbes and plants acquire phosphorus, nitrogen, and carbon. It has been shown that differences in nutrient availability are reflected by differences in enzyme production (6, 7). Like all proteins, RNA controls the synthesis enzymes, and changes in enzyme activity may be caused, or even predicted, by different amounts of the genes that represent those enzymes. Thus, this method will provide the quantitative evidence to support my prediction or its alternate.

More broadly, human activity has altered phosphorus, nitrogen, and carbon availability, through several means, and this has impacted the global cycling of these elements and plant growth (11, 18). The presence each of these elements affects the availability of other elements, so understanding how their cycling is linked to soil microbial community is crucial. For example, in climate change models, the inclusion of the microbial gene expression data will allow for a more complete picture of global nutrient cycling and inform nutrient control of plant growth (19). Because extracellular enzymes are the major mechanism that cycles nutrients through ecosystem, this study will elucidate the link between gene presence, extracellular enzyme activity, and changes in nutrient cycling in response to phosphorus deficiency. Previous studies have not shown this comprehensive ‘genes-to-ecosystems’ connection between phosphorus limitation and the soil microbial community (7, 18, 22); therefore, my research will clarify the connection between microbial genes and ecosystem processes and will provide a deeper understanding of the role the soil microbial community plays in both regional and global forests.

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Budget

	Cost	Quantity	Total
Sampling & Extraction			
Whirl-Pak Sampling Bags, 58 mL, 500 (FisherSci)	$44.80	1	$44.80
Phenol: chloroform: isoamylalcohol, 400 mL (FisherSci)	$128.00	1	$128.00
20% SDS Solution (FisherSci)	$48.67	1	$48.67
PowerSoil Total RNA Isolation Kit, 25 rxn (MoBio)	$317.00	1	$317.00
Filter tips (VWR)	$142.00	1	$142.00
Ohio University Genomics Facility			
Use of Agilent BioAnalyzer at OUGF, per 12 samples	$53.00	1.5	$79.50
Use of qPCR machine, per plate at OUGF	$25.00	7	$175.00
Primer Design			
Primers + shipping (Integrated DNA Technologies)	$12.00	14	$168.00
Reverse Transcription & qPCR			
iScript cDNA synthesis kit, 25 rxn (BioRad)	$150.00	1	$150.00
PowerUP SYBR Green qPCR Kit, 1 mL (Thermofisher)	$83.00	2	$166.00
96-well PCR plates, 25 ct (Thermofisher)	$131.00	1	$131.00
Plate seals, 100 ct (Thermofisher)	$142.00	1	$142.00
Subtotal			**$1,691.97**
Items Purchased by the DeForest Lab			
Whirl-Pak Sampling Bags, 58 mL, 500	$44.80	1	$44.80
Phenol: chloroform: isoamylalcohol, 400 mL (FisherSci)	$128.00	1	$128.00
20% SDS Solution	$48.67	1	$48.67
PowerSoil Total RNA Isolation Kit, 25 rxn (MoBio)	$317.00	1	$317.00
Filter tips (VWR)	$150.00	1	$150.00
96-well PCR plates, 25 ct (Thermofisher)	$131.00	1	$131.00
Plate seals, 100 ct (Thermofisher)	$142.00	1	$142.00
			($961.47)
Total Funds Requested			**$730.50**

Budget Justification

Sampling & RNA Isolation

WhirlPak bags are the standard in the field of soil science, and our lab has been using them to sterilely collect and transport soil samples for years. They are small plastic bags that are sterile until they are opened, and twist shut to minimize sample loss. Phenol: Chloroform: Isoamyl alcohol is required as part of the MoBio PowerSoil extraction protocol but is not included in the kit. This reagent allows for the separation of RNA and cellular debris in an early step of the RNA isolation process. 20% SDS solution will be used as a detergent to remove RNA-degrading enzymes from work surfaces. PowerSoil Total RNA Isolation Kit, 25 rxn (MoBio) has been shown by several researchers to provide quality RNA from a small amount of soil for use in qPCR (5). Filter tips are required for working with RNA as they help prevent contamination by providing a barrier between the sample and the pipetman.

Ohio University Genomics Facility: BioAnalyzer and qPCR machine

Funding is also requested for the use of equipment in the Ohio University Genomics Facility, and pricing is based on cost listed as of May 2017. This facility offers quality molecular services, right on campus, for an affordable price. The Agilent BioAnalyzer provides data on the quality and quantity of RNA in a sample. With each use, the BioAnalyzer allows the user to quantify 12 samples at a time, and OUGF director will allow me to perform one and a half uses to stay under budget. I will also use the Facility's qPCR machine. For this project, I will use the machine 7 times; each time different sample and primer combinations will be quantified.

Primer Design

Sets of forward and reverse primers will be designed using the IDT webtool to select for genes encoding extracellular enzymes and the reference gene. Primers attach to and select the region of DNA or cDNA in which a researcher is interested, allowing the researcher to work with only that region, a basic tool for molecular biology. Primers attach to both the beginning and the end of the region of interest, so I am requesting funding for 14 primers, two for each of the seven genes I have described.

Reverse Transcription & qPCR

Funding is requested for the kits for the reverse transcription of RNA into cDNA and the qPCR set up. The iScript cDNA synthesis kit is commonly converts RNA into cDNA for qPCR and more stable storage. The PowerUP SYBR Green qPCR kit contains all the solutions required for qPCR except for the primers: an enzyme that cuts the cDNA at the place specified by the primers (i.e. at each gene), buffers to ensure that the reaction continues at the correct temperature and pH, and a dye that attaches to the DNA and fluoresces. As the reaction progresses, the dye will increasingly fluoresce. The qPCR reads the amount of light produced by each gene at certain points in the reaction. The amount of light produced for each gene can be compared to that of the reference gene. This kit is very affordable when compared to similar products.

Samples will be processed in 96-well plates and must be sealed with special seals. 96-well plates (plastic rectangular pieces with small wells to hold samples) are a staple of molecular biology. While many companies make such plates, the Thermofisher 96-well PCR plates have been specifically designed to fit in the qPCR machine at the OUGF, the Stratagene MXP.

Items Purchased by the DeForest Lab

Items for sampling and extraction as well as 96-well plates and plate seals have already been acquired by our lab.

Curriculum Vitae Removed to Maintain Applicant Privacy

Glossary

Acid rain: Precipitation containing nitrogen in the form of nitric or sulfuric acid, caused by human activity**.** Acidifies the soil, increases soil N, and decreases soil P.

cDNA: "Complementary" or "copy" DNA, a double stranded DNA molecule formed from an RNA molecule. Typically used because it is more chemically stable than RNA. Normally, DNA is transcribed into RNA, but the formation of cDNA from RNA requires different enzymes.

DNA (Deoxyribonucleic Acid): The carrier of genetic information. This is a molecule present in all cells, living and dead, and is the first molecule involved in the formation of proteins.

Extracellular enzyme: A protein created by a cell that acts on materials around it to perform a task like helping the cell obtain nutrients or changing DNA into RNA. Enzymes themselves can be both intracellular (inside the cell) or extracellular (sent outside of the cell).

Gene: a sequence of DNA or RNA molecules that represent a protein. Genes start as DNA, are transcribed into RNA when they are needed, and then become proteins, which are the building blocks of life. The genes I refer to in this text represent the extracellular enzymes necessary to make phosphorous, nitrogen, and carbon available. I could be isolating the DNA of these genes, but isolating the RNA means that I am obtaining information and generating data on the genes that are being transcribed and the associating proteins that are being made exactly at the time of sampling.

Protein: A building block of life. Proteins are formed from RNA. They then go to form all other structures within a cell. An example of a protein is an enzyme.

Primers: sections of DNA designed to bind to a desired part of a DNA sequence. Allows for preferential amplification of the desired sequence as part of a common and well-established method in molecular biology.

Reference gene: a gene that is selected to provide a standard curve with which all genes of interest are compared in a qPCR reaction. The reference gene must be present in all cells to provide an accurate comparison. In this study, I will use the gene that represents DNA polymerase, an enzyme with in the cell related to the production of DNA.

Reverse transcription (RT): a technique in molecular biology in which the starting material, RNA, is turned into double stranded DNA using an enzyme, reverse transcriptase.

Quantitative PCR (qPCR): a technique in molecular biology in which gene(s) of interest are exponentially amplified and quantified with fluorescence as the reaction runs to the completion of the reagents. At the point at which fluorescence is detectable, a value is given and compared to the fluorometric value given by the reference gene in the same reaction. A high value indicates a low starting DNA concentration; a low value indicates a high starting concentration. In this study, I am using reverse transcription qPCR, or RT qPCR, which combines a reverse transcription step as described above and quantitative PCR to calculate the amount of the RNA of a gene that is present in the system.

RNA (Ribonucleic acid): generally, RNA acts as an intermediate step between DNA sequences and protein synthesis. DNA → RNA → Protein. RNA is more easily degraded than DNA or proteins in soils, and so isolating the RNA generates data for the exact time of sampling.

The Central Dogma of Molecular Biology: within a cell, DNA is transcribed into RNA, which is translated into proteins. In other words, DNA becomes RNA, and RNA controls the synthesis of proteins.

Copy of Reviewer Comments
Summer funding cycle 2017

Applicant's Name: XXXXX
Decision: Reject-Resubmit at Full Committee / Advance to Full Committee / Accepted at Full Committee
Overall Comments: Members at full committee agreed that you addressed the comments made on the previous submission well. However, the change in direction of the project also resulted in new comments and critics on the central question as well as the budget.
Compliance issues (formatting)- Literature is not double spaced. Fonts are different throughout (should be a 12-point font throughout). But that does not help her have an advantage. This should be change in a resubmission however.
Abstract- Within the abstract, there were words that needed to be defined within the glossary, or the abstract should have been written more simply for the study. Overall, it gave a solid overview of the project.
Narrative- The question that the author asks: 1) the author's aim is to test the community, but the author is checking the genes (not the taxonomy of the community); 2) if the author changes the aim to meet the methodology, we need to know what genes would be tested. The methodology must address the aim.
Your use of "community" seems to be unclear as it could take the project in two separate directions depending on the meaning. If referencing to the microbial community in the context of it's individual, taxonomical parts (species, genus, etc.) then the methodologies don't seem to provide a way to identify those parts as the methods focus on the genes solely.
If you refer to the "community" in terms of the microbiome as a whole and are looking more at the transcriptome of the community then the specific genes and why you selected them should be well detailed.
Be sure that your references go after the narrative.
Methods- Even though your methodology was changed, the questions that you are asking are still not be answered by the methods that you choose. Your title, what you are trying to test, etc., do not match.
Budget and Budget Justification- The budget is quite extensive; it includes nearly everything that is necessary, but also everything that is already in the lab. The budget justification is rather limited in comparison to the extensive amount of information in the actual budget. The budget justification could be more simplified in the terminology that was used.
Strengths: Good job addressing the comments of the last review. Glossary was done quite well.
Weaknesses: There still seems to be some vagueness in the question that the author is asking. However, is good at addressing an important knowledge gap.
PLEASE NOTE: If you choose to resubmit this grant for future funding cycles, THIS reviewer feedback sheet MUST be included in your resubmission. Please see the Request for Proposals on the GSS website for more details.

Resubmission summary (For resubmission Fall 2017)

Again, I would like to thank the review committee for their critique of my Original Works Grant. Although the goal of this project has not changed since my first submission in Spring of 2017, I have reframed the proposal to more clearly represent these goals.

Compliance

The literature is now double spaced and in the correct place, and all headers are in 11-point font.

Abstract

The abstract has been edited for jargon, and some terms (now bold faced) have been added to the glossary. The abstract has also been re-written to emphasize the most important points of the project.

Narrative

This study does not aim to address changes in community composition that may occur with changes in nutrient availability; however, we aim to elucidate how the genes expressed by the soil microbial community change with the addition of phosphorus. The names of these microbial genes and their functions have been included in the methods section of the project narrative.

Methods

I have made very few changes to the methods for the Fall 2017 submission of my Original Works Grant. As I explained in the section above, the goal of this project is to determine the changes that occur within the soil microbial community at a genetic level in response to the addition of phosphorus fertilizer to soils that are naturally low in phosphorus. In response to the reviewers' comments on the lack of clarity in the title of my proposal, the phrase "microbial drivers" uses "microbial" as an adjective and "drivers" refers to the genetic changes that may be observed within the community. The methods I have chosen will allow me to quantify the differences in gene expression, which is the goal of this project.

Budget & Budget Justification

I have expanded the budget justification to include all items listed in the budget and explain what each item does. An effort was made to remove the technical jargon from this section.

Copy of Reviewer Comments
GSS Funding Period: Spring 2017

Applicant's Name: XXXXX
Grant Title: Determining the Microbial Drivers of the Phosphorus Cycle

Introductory Comments:

- While your current project suggests a promising project, GSS cannot fund your proposal at this time. As with each term, we receive a number of submissions, all of which go through a peer review process. At this time, your proposal was not among those selected by the committee to advance to the final round for review. We encourage you to continue working on your proposal and to consider resubmitting it for funding in the future. Below are suggestions that should be incorporated into your revised proposal.

Abstract:

- The abstract states the research questions as "which microorganisms are the primary drivers of the phosphorus cycle in phosphorus limited soils?" This is not further developed or refined in the narrative, causing problems because there is not a clear connection between the data generated from the methods and this questions.

Narrative:

- The narrative focuses on the specific enzyme phytase, successfully arguing that more attention should focus on this enzyme. That said, it is unclear if this enzyme is conserved across the tree of life or if it differs to a substantial degree among groups of microorganisms - and if so do we know enough about that variation to pick it out of transcriptome data? Maybe we do, but this was not articulated in the proposal.

- The question from the abstract also concerns the specific taxa responsible for P-cycling. Whether taxonomic information can be obtained from metatranscriptomics is unclear, so it should be explained how this is expected to work. Then, if you can obtain taxonomic information, how is that linked to variable levels of phytase expression? In other words, can you pair the gene-expression data from the metatranscriptome to taxonomic information somehow?

- It is stated that ancillary soil data will be available. How will this be used? Correlated with phytase expression levels or taxonomic abundances?

- Overall the study has obvious potential. The research question needs clarification so that the methods can be described as they directly apply to the question/hypothesis. This will likely require consolidating some of the background material, and expansion of the methods. By further outlining your expected results in connection with the ultimate goal of your project the significance of this research will be better demonstrated.

Budget and Budget Justification:

- There are no sequencing charges in the budget despite the statement "I plan to sequence using the Illumina MiSeq" in the narrative. Given that MiSeq runs are expensive this expense should be there - even if funded in some other way.

Curriculum Vitae:

- The curriculum vitae demonstrates appropriate experience and skills suited for the study proposed.

Resubmission Summary (For resubmission Summer 2017)

To begin, I would like to thank the review committee for their insightful and thorough critique of my original proposal. Most importantly, the reviewers' comments made it clear that not only did was the budget for this project unsustainable by funding from the Graduate Student Senate alone, the original methods did not answer the research question in s succinct or efficient manner. This project has been re-scoped to broaden its impact as well as altered for resubmission of a more relevant and supportable proposal. Further, this project can now be entirely funded by the GSS OWG. Reviewer comments by section are addressed below.

Abstract

The original research question was a poorly worded and badly edited on my part. This mistake has been corrected, and the research question is now addressed properly throughout the proposal.

Narrative

1) The reviewer is correct to point out that, although the enzyme of interest is produced by many lifeforms, the transcriptomic differences between samples may have been difficult to observe, and neither of these points was well articulated in the original submission. It has also been decided that just focusing on one enzyme limits the scope of this project, and the proposal has been adjusted to include others.

2) It became clear to me after the first submission of this proposal that the transcriptomic data would be insufficient to determine taxonomy. This has been corrected throughout the proposal by changing the research question and altering the methods.

3) The purpose of the ancillary data was unclear in the original submission, and this data is no longer needed to answer the research question.

4) The scope of this project has refocused for resubmission. The methods have been changed and parts have bene expanded to more accurately address the altered research question "how does the availability of phosphorus affect the *functioning* of the microbial community?".

Budget

Upon further consideration, I have decided to answer my research question using RT qPCR instead of NexGen Sequencing. This change ensures that this project, although slightly smaller in size, can be funded on one Original Works Grant and easily completed during the remaining year of my MS degree, while not significantly decreasing its potential impact or advancement of knowledge.