Microorganisms and enzymes driving glyphosate degradation is Lake Erie

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Ohio Water Resources Center

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Abstract:
Glyphosate is the most widely applied herbicide in the world. It is present in measurable concentrations in bodies of water that drain watersheds wherever the chemical is applied. Because the glyphosate chemical structure includes phosphorus and nitrogen, the two most important nutrients in most freshwater ecosystems, the contribution of this chemical to eutrophication has been of increasing interest. Bacterial biodegradation of glyphosate has been observed in bacterial cell culture and is presumed to occur in nature, but the gene pathways that drive this metabolism in the environment are unclear. Only a subset of the microbial population can degrade glyphosate and the known pathways result in the production of different breakdown chemicals, which are also only usable by particular microbial taxa. These processes must be better understood to obtain a full picture of Lake Erie's nutrient cycles and to specifically understand its recent algal bloom formation. We propose to expose naturally occurring Lake Erie microbial communities to glyphosate and its primary breakdown product AMPA, through microcosm experiments, and use a metatranscriptomic approach to measure the expression of genes involved in the microbial response to glyphosate exposure. This study will determine the microorganisms and degradation pathways responsible for the metabolism of glyphosate and its breakdown products. We will compare these experimental results to publicly available gene expression libraries collected directly from Lake Erie to confirm the importance of experimentally identified genes in the natural system. These results will resolve the pathways and chemical forms through which glyphosate is converted to nutrients that directly contribute to harmful algal bloom formation in Lake Erie.

1) Problem and research objectives

Glyphosate, the active ingredient in the Roundup, is the most widely applied herbicide in the world (Murray *et al.*, 2003, Nadin, 2007, Benbrook, 2016). The usage of this herbicide has grown significantly with the development of crops that are resistant to the chemical. With the availability of Roundup Ready soybeans in 1996 and corn in 1998, demand for this chemical has grown considerably (Dill, 2005). Additionally, glyphosate was marketed as a safer alternative to other herbicides, such as atrazine, that had been linked to significant negative health effects. Glyphosate was also believed to become deactivated in the soil shortly following application, further driving arguments for its safety (Baylis, 2000). Increasingly though, the widespread use of glyphosate has been questioned due to safety and environmental concerns (Williams *et al.*, 2000, Annett *et al.*, 2014, Cuhra, 2015, Mesnage & Séralini, 2018, Gonçalves *et al.*, 2019).

Harmful algal blooms (HABs), driven by widespread eutrophication, are among the most significant global environmental concerns and is also of particular concern in Lake Erie where large-scale blooms of the toxic cyanobacterium *Microcystis* have been occurring annually since the 1990s (Steffen *et al.*, 2014, Watson *et al.*, 2016). The sources of the nutrients that drive HAB formation have received intensive study, and their identification and control are understood to be a multifaceted problem (Glibert, 2017). There is increasing concern that growing glyphosate concentrations are contributing to eutrophication and HAB formation in lakes and coastal areas throughout the United States (Saxton *et al.*, 2011, Hébert *et al.*, 2019). While this chemical is best known for its herbicidal effects, it also includes phosphorus (P) and nitrogen (N) in its chemical structure. In recent years glyphosate P is an increasing proportion of field-applied P nationally, growing to greater than 1% of fertilizer-applied P in western Ohio watersheds and greater than 15% of fertilizer-P in some areas nationally (Hébert *et al.*, 2019). While in freshwaters eutrophication studies have long focused on P, the importance of N in HAB formation is increasingly appreciated (Paerl & Scott, 2010, Chaffin *et al.*, 2013, Paerl *et al.*, 2014, Davis *et al.*, 2015), and the possibility that glyphosate-N may contribute to Lake Erie HABs has also been suggested (Saxton *et al.*, 2011).

Additionally, there is significant evidence indicating field-applied glyphosate runs off agricultural fields and accumulates in near-shore areas of Lake Erie (Bullerjahn and Mckay, pers comm, Spiese *et al.*, 2018). Glyphosate and its primary breakdown product aminomethylphosphonic acid (AMPA) are present in measurable quantities in near-shore Lake Erie and its tributaries in the weeks after spring application (Saxton *et al.*, 2011). During peak concentrations dissolved glyphosate concentrations range from 0.2-2% of total dissolved P. This glyphosate does not persist in the body of the lake or its tributaries; rather, concentrations drop below detection in the weeks after initial run-off. Significant evidence supports the

assertion that the observed drop in glyphosate concentration is primarily the result of microbial biodegradation of the chemical (Borggaard & Gimsing, 2008, Saxton *et al.*, 2011, Sviridov *et al.*, 2015).

Two microbial enzymes are known to be able to breakdown glyphosate, C-P lyase, and glyphosate oxidoreductase. These two pathways are performed by different subsets of the microbial community and result in the production of distinctive chemical intermediates that are utilized by unique downstream microbes, including potentially HAB-forming cyanobacteria (Saxton *et al.*, 2011). Whether either of these pathways in employed in Lake Erie is not known. Despite growing regional and national interest in the influence of glyphosate to nutrient loading, both, the contribution of glyphosate to the eutrophication of Lake Erie and the biodegradation pathways by which this chemical becomes available to phytoplankton in this environment are poorly understood.

Application of herbicides such as glyphosate are not currently considered in nutrient-loading regulations or models. As current application trends continue, and this chemical grows to be a larger proportion of field-applied P it is of critical importance that systems managers and researchers have a complete understanding of how this chemical contributes to nutrient loads, how it is processed through the environment, and how it becomes available to HAB cyanobacteria. Understanding the pathways by which glyphosate breakdown takes place will allow systems managers to better understand how this chemical contributes to P and N loading to the lake.

Objective 1) Determine the enzymatic pathways responsible for microbial response to glyphosate addition in Sandusky Bay.

We hypothesized that glyphosate has significant impact on community gene expression and that genes involved in glyphosate degradation will be expressed. To address all three objectves we performed experiments in which water, collected from Sandusky Bay, was exposed to glyphosate, AMPA, and several nutrients or nutrient combinations (PO_4^{3-}, NH_4NO_3, PO_4^{3-} + NH_4NO_3). Following exposure, gene expression in the exposed microbial communities was investigated via a metatranscriptomic approach. We will be able to resolve the specific gene families that Lake Erie microbes use to respond to glyphosate exposure.

Objective 2) Resolve the microbial taxa that respond to glyphosate addition.

In addition to identifying the gene pathways stimulated by glyphosate exposure, using the results described in objective 1, we determined the microbial taxa performing these functions. *We hypothesized that different subsets of the population will respond to each individual source of N and P, with a unique population responding to glyphosate addition*. This will be accomplished by comparing the community transcriptional profile following glyphosate

application to transcription when exposed to the individual nutrient amendments. In addition, we performed a second experiment in which glyphosate and several other pesticides were added to water collected from Actin Lake, a reservoir near Oxford, OH. The effect of these pesticides on aquatic microbial community structure was investigated using 16S rRNA amplicon sequencing and differential fluorescence fingerprints.

Objective 3) Determine the prominence of the identified enzymatic pathways in natural systems

Using publicly available transcriptomic libraries collected from Sandusky Bay and elsewhere in Lake Erie, we will examine the *in situ* prevalence of the genetic pathways and did microorganisms identified in objectives 1 and 2*. Because of the high agricultural usage in the Sandusky watershed and reported glyphosate exposure in Sandusky Bay we hypothesize that genes and organisms identified in objectives 1 and 2 will be active here, though likely at lower levels than in our experimental samples.*

Methods:

Sample water was amended to final concentrations of 1 µM glyphosate, AMPA, PO_4^{3-}, NH_4NO_3, PO_4^{3-} + NH_4NO_3, as well as unamended controls in one experiment. This concentration is in the upper range of glyphosate and AMPA observed in nature and has been shown to drive biological change in previous experiments (Saxton *et al.*, 2011). In a second experiment, several herbicides (Glyphosate, Simazine, Acetochlor, Propazine, Metolachlor, and Atrazine) or insecticides (Aldicarb, Carbofuran, Chlorpyrifos, Terbufos and Carbaryl) were added, as well as unamended controls. In both experiments sample water will be distributed into 1.2L polycarbonate bottles for incubation. Incubations were performed in triplicate for 48h at *in situ* light and temperature levels in lit, shaking, and temperature-controlled incubators. Following incubation, samples for nucleic acid sequence analysis were filtered onto 0.2 µm pore size polycarbonate filters. Following filtration, samples were frozen and stored at -80°C until extraction. RNA was extracted and DNA was extracted using the Qiagen Powerwater total DNA isolation kit.

Figure 1) Glyphosate exposure gene expression experiment sample bottles

2) Principal findings and results for each objective

The timely completion of these objectives has been materially impacted by the COVID-19 pandemic in two significant ways, resulting in delays not remedied by the initial no-cost extension; 1) Lab closures and field work delays and, 2) Supply chain issues. Miami University closed research lab facilities for several months in March 2020. Because of this, we were not able to complete field sampling or perform experiments planned for spring/summer of 2020. Further, COVID-19 related issues resulted in delay in the planned experiments from May 2021 to August. Supply chain issues have also been impactful. Reagents needed for RNA extraction have been subject to significant delays since March 2020 as these materials have been diverted to the COVID-19 testing effort. Additionally, the availability of nucleic acid sequencing reagents has been delayed. Samples for transcriptomic analysis have been waiting at the sequencing facility for several months. Data are expected in early February.

Objective 1) Determine the enzymatic pathways responsible for microbial response to glyphosate addition in Sandusky Bay.

Upon receipt from the sequencing facility, RNA samples will be sequenced on the Illumina NextSeq platform with a target of 20-30 million reads per library. To investigate changes in transcription profiles between treatments, metatranscriptome libraries will be (1) assembled *de novo* and (2) mapped to publicly available reference genomes of likely glyphosate-degrading microorganisms identified in the *de novo* assemblies. rRNA sequences will be identified and removed using SortMeRNA (Kopylova *et al.*, 2012). De novo assembly will be performed using Trinity (Haas *et al.*, 2013), Oases (Schulz *et al.*, 2012), and IDBA-MT (Leung *et al.*, 2013) and the quality of each assembly will be assessed using transrate. Identification and functional assignment of protein coding genes is as above. Statistical comparisons in expression profiles between treatments will be performed in R using DESeq2 (Love *et al.*, 2014). Transcripts will be mapped to metagenome contigs and referenced genomes using Bowtie 2 (Langmead & Salzberg, 2012). Protein coding genes will be identified using prodigal (Hyatt *et al.*, 2010), and their function will be assigned using BLASTx (Gish & States, 1993) by comparing predicted proteins against the NCBI COGs (Tatusov *et al.*, 2000), KEGG (Kanehisa *et al.*, 2015) and UniProtKB (Boutet *et al.*, 2016) databases. Within bins, metabolic pathways will be mapped using KEGG and pathways of interest will be carefully curated

Objective 2) Resolve the microbial taxa that respond to glyphosate addition.
While Objective 2 will be completed as described as the primary dataset becomes available in February 2022, it became apparent that the various COVID-19 related issues had the potential of significantly delaying the completion of our primary research objectives we set about designing and completing secondary experiments that could address at least one of our objectives. To this end, a second experiment was performed with similar methods as the first. In this experiment, water acquired from Acton Lake was amended with several herbicides (Glyphosate, Simazine, Acetochlor, Propazine, Metolachlor, and Atrazine) or insecticides (Aldicarb, Carbofuran, Chlorpyrifos, Terbufos and Carbaryl). Actin Lake is in a heavily agricultural area on the borders of Butler and Preble counties in southwest Ohio. While funds and supplies (due to COVID-19 supply chain issues described above) were not available to perform the experiment as was done for glyphosate a second experiment was planned to investigate the impacts of these agricultural chemicals on algal and heterotrophic bacterial communities. We hypothesized that exposure to these chemicals would cause measurable changes to the heterotrophic bacterial community and that herbicides would have a larger impact on the algal community than insecticides.

Impact of pesticides on algal communities:

To investigate how exposure to the above listed herbicides and insecticides we used a fluoroprobe fluorometer to determine the concentration of pigments associated with several broad algal groups (diatoms, green algae, cryptophytes, and cyanobacteria (bluegreen algae). We observed, that while certain chemicals caused changes in algal concentration when compared to the control treatment, these trends did not extend across either the herbicide or insecticide group (Figure 2).

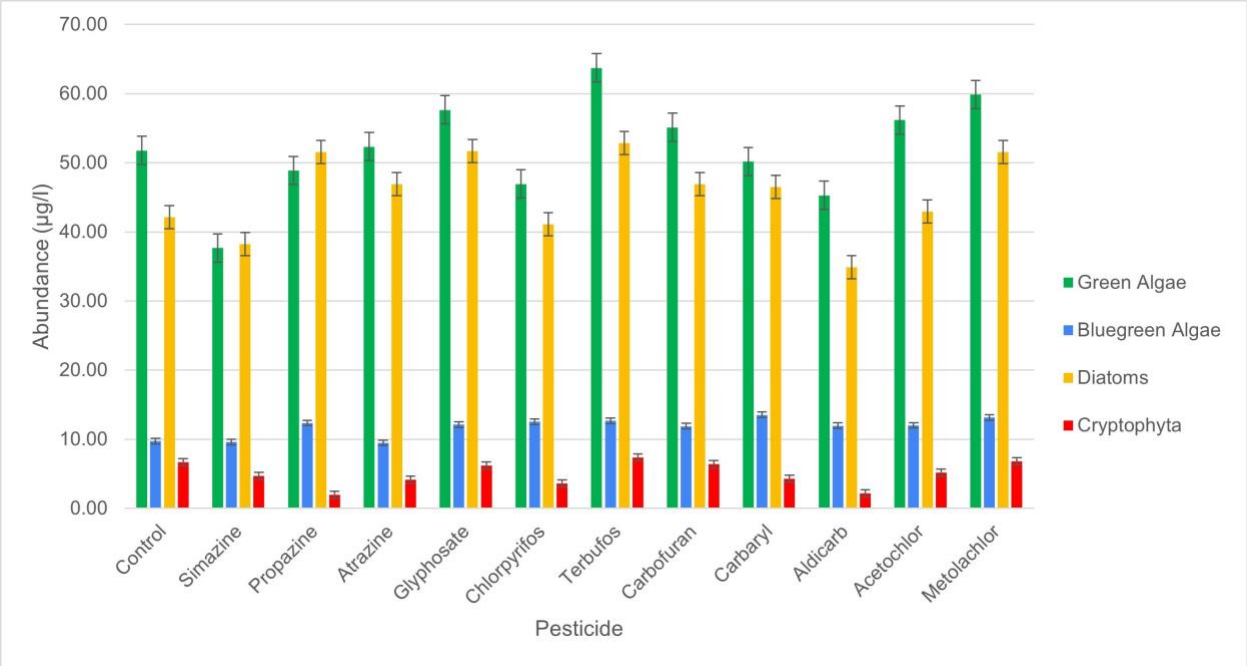

Figure 2) Algal conccentration with pesicide treattment

Impact of pesticides on heterotrophic bacterial communities:

To study the impact of pesticide treatment on bacterial communities generally, 16S rRNA gene amplicons were sequenced on an Illumina MiSeq platform. When looking generally at the bacterial communities across all treatments using nonmetric multi-dimensional scaling analysis no clustering of 16S libraries was observed (Figure 3). This indicates that broadly, the bacterial communities did not change according to a trend associated with our treatments. However, when we used a negative binomial distribution method to investigate changes in abundance of particular taxonomic groups significant differences were observed when compared to the control incubations. Results for the glyphosate incubation are showed in Figure 4, many taxa showed higher abundance in the glyphosate treatment with log2 fold changes greater than 5. While many groups were represented, several species from the family *Burkholderiaceae* were observed. Interestingly, this group has also been seen to respond to glyphosate treatment in the gut of the freshwater invertebrate *Daphnia* (Suppa *et al.*, 2020). This effort is providing critical targets for the transcriptomic dataset arriving February 2022.

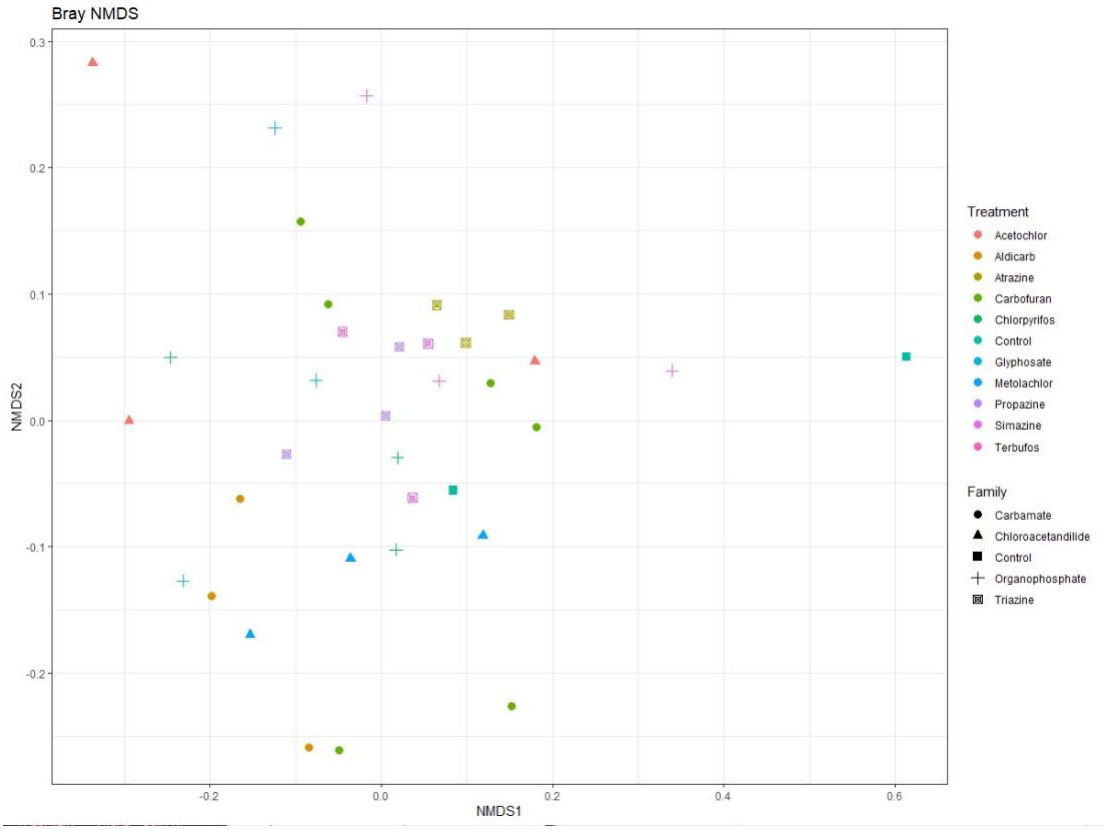

Figure 3) NMDS showing 16S rRNA libraries of Acton Lake (OH) bacterial communities exposed to pesticides

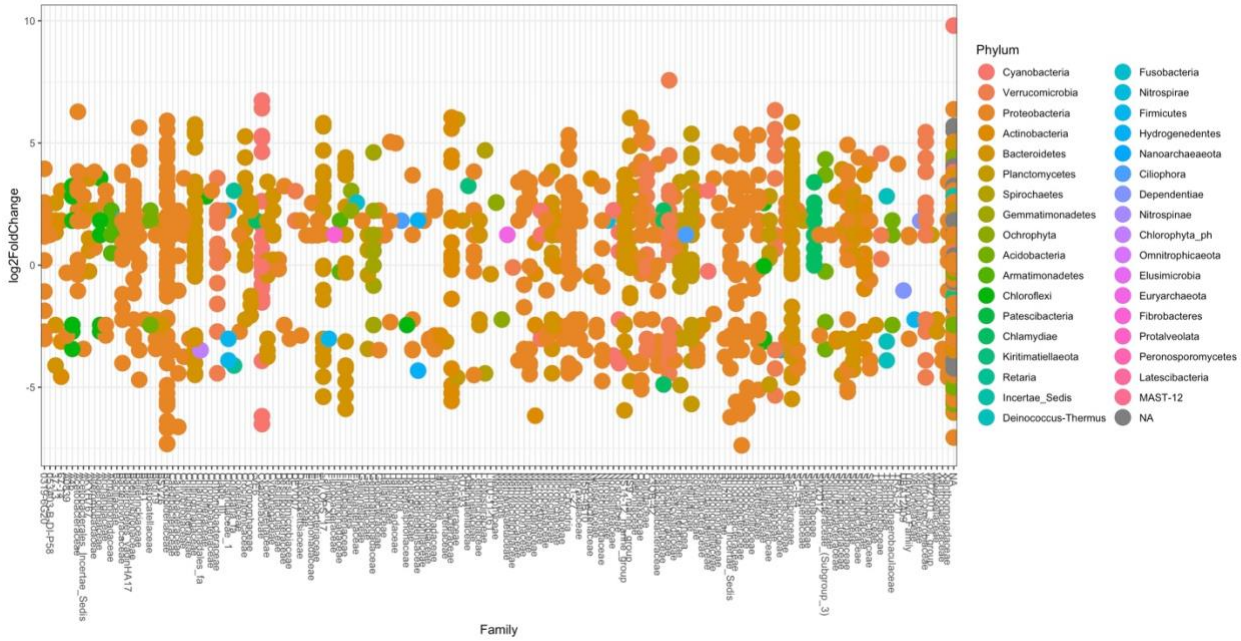

Figure 4) Negative binomial distribution analysis showing bacterial taxa with higher or lower abundance in glyphosate treated samples (compared to control)

Objective 3) Determine the prominence of the identified enzymatic pathways in natural systems

Metatranscriptomic libraries originating from samples collected throughout the Great Lakes have been identified and downloaded in anticipation of the main dataset associated with this project becoming available in early February 2022.

3) Significance

This work is the first study investigating microbial gene expression in response to glyphosate exposure in aquatic systems. With the forthcoming data resulting from this project, we will be able to determine the gene pathway or multiple pathways used to degrade glyphosate in Lake Erie. These results will provide substantial evidence as to the forms through which glyphosate-derived nutrients enter this system and the likelihood of downstream impacts on harmful algal blooms and hypoxia.

Additional work associated with this project has described how aquatic microbial communities respond to pesticide exposure. These data are highly novel and will provide critical understanding of how pesticide treatment impact the ecology of agriculturally impacted ecosystems, and how microbial communities may modulate these impacts.

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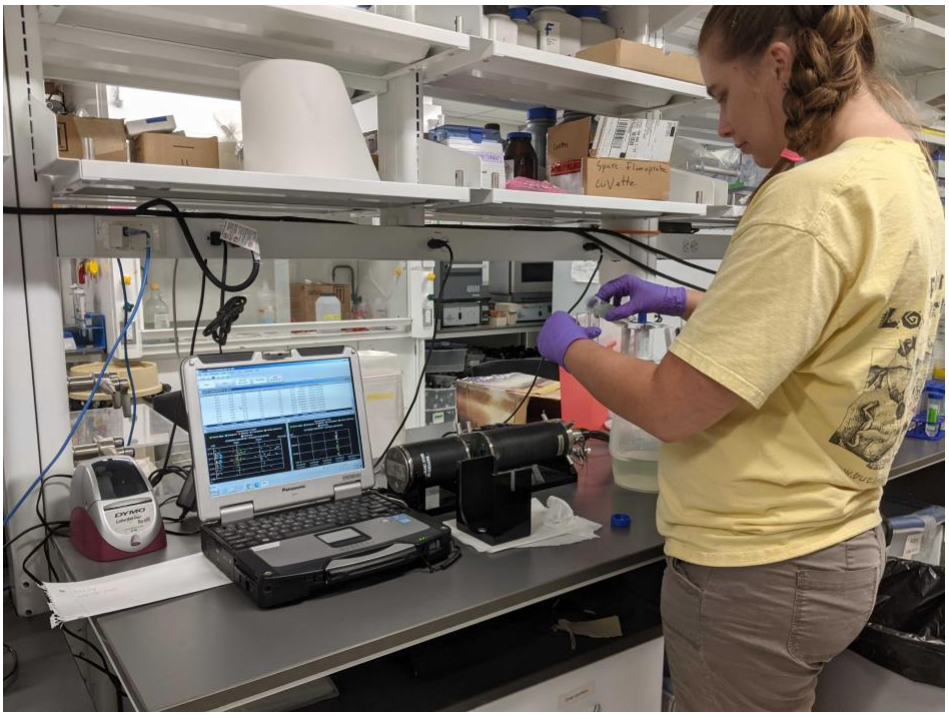
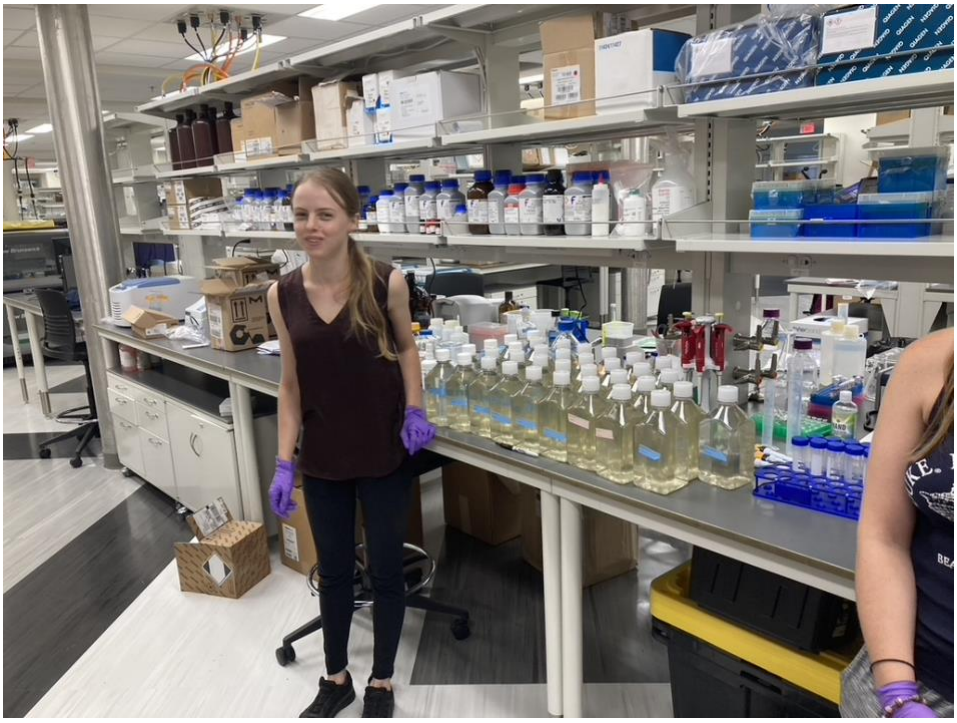
Ph.D student Oluwaseun Olubodun extracting RNA



Undergraduate student Emma Jones using a fluoroprobe to investigate algal community structure



Undergraduate student Helena Hitch with sample bottles during experiment break down