# Characterizing Metabolic Behavior of Aquatic Microbial Communities Impacted by Septic Pollution

Alona Chester, Nora Demers

## Abstract

The overutilization of septic tanks poses a threat to the aquatic ecosystem by introducing nutrients and potentially harmful pollutants, altering the behavior and composition of the microbiome. To investigate the characterization and impact of these chemicals on microbiota in these systems, this study utilized Biolog EcoPlates (Biolog, Inc., Hayward, CA, USA) to observe patterns in carbon utilization among these microbial communities to assess larger patterning in relation to proximity to septic tanks. Four sites in the San Carlos Park community were chosen due to the high volume of septic tank utilizing properties. Two are canals running alongside septic properties, while the other two were lakes with one encircles with septic properties and the other two surrounded by houses running on city sewer. An additional site outside the San Carlos Park community at FGCU Outfall was taken for comparison. Sample sets display differences in substrate utilization between locations and substrate types but there was no significant difference between samples depending on seasonal weather patterns. Samples taken at FGCU Outfall outside of the septic community displayed significantly lower rates of substrate utilization across all groups and had very little overlap with other clusters in PCA analyses. The lack of seasonal patterning within the microbial community and high substrate utilization are behaviors consistent with prior investigations on the potential impact of septic pollution. These results indicate that observing patterns in microbial communities using EcoPlates may detect long term symptoms of septic pollution within communities based on those characterizations.

## Introduction

Microbiota are ubiquitous and essential to the health and wellbeing of all ecosystems. These organisms contribute to vital functions like nutrient cycling, nitrogen fixation, and energy flow through the various trophic levels (Cavicchioli et al. 2019; Gavrilescu et al. 2015). The microbiome of aquatic systems, however, are threatened by the overutilization of septic tanks which have been found to load pollutants like nitrogen and phosphorous into groundwater systems if they placed close to the water table or without adequate distance from one another (Lusk et al. 2017; Beal et al. 2017).

The addition of septic pollution has been found to contribute to eutrophication and alter the natural selective pressures that maintain community structure and seasonal variation (Lusk et al. 2017; Beal et al. 2017; Qiu et al. 2020). Using the Biolog EcoPlates (Biolog, Inc., Hayward, CA, USA), I aimed to characterize the aquatic microbiome of a community primarily utilizing septic tanks, identify where variation exists between those sample sites, and assess the EcoPlate's ability to observe these trends.

## Methods

The selected sites for sampling are in the community of San Carlos Park, Florida, which predominantly utilizes septic tanks for human waste disposal. Lake San Carlos was taken from an isolated pond upstream of the nearby canal systems but surrounded by properties utilizing septic tanks. Caloosa/Phlox and Cypress/Phlox were located along different sections of the same canal system running directly alongside properties using septic tanks. Woodbriar Lake is surrounded by houses attached to city sewer systems rather than septic tanks. FGCU Outfall was a control site collected on campus away from any source of septic pollution.

The samples were taken in 50 mL sterilized tubes. The first set of samples were taken April 14th amidst Southwest Florida's dry season. The second set of samples were taken on July 21st amidst the wet season in Southwest Florida. The samples were poured into a sterile 25 mL well in order to utilize an 8 channel multipipette to distribute 125 µL into each well of the Biolog EcoPlate (Biolog, Inc., Hayward, CA, USA). The samples incubated to ~25°C. Every 24 hours the plate was scanned at 570 nm by the Dynex MRX Revelation 4.22 (DYNEX Technologies, Inc., Chantilly, VA, USA) for 120 hours.

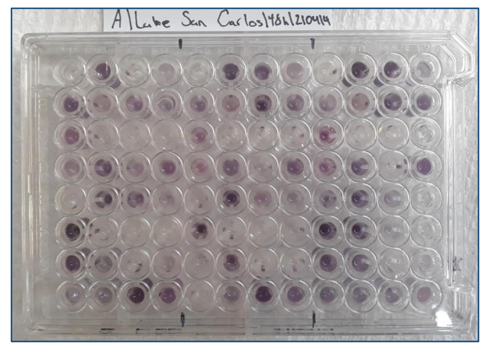


Photo: EcoPlate Coloration at 48 Hours for Lake San Carlos sample.

Samples were assessed with PAST Software to generate a dendrogram, as well as with multi-sample permutation tests with multiple independent variables were studied using R to generate PCA analyses and examine eigenvalues.

## Results

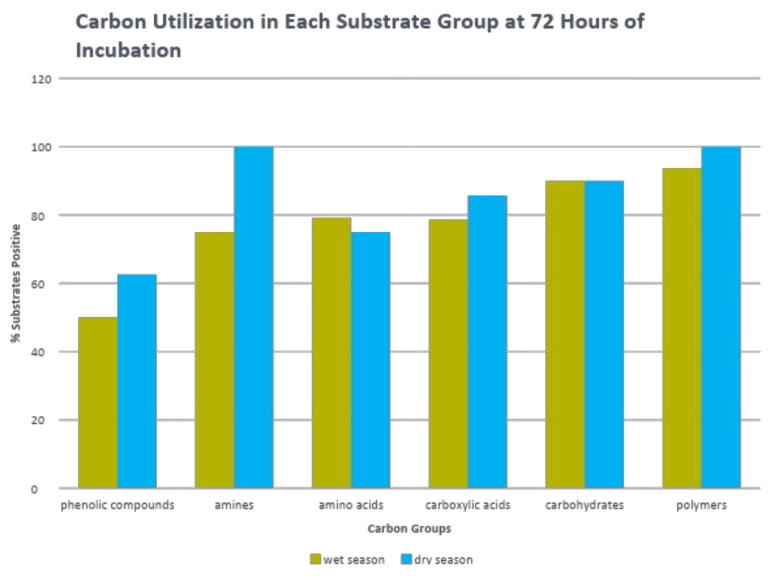


Figure 1*: The percentage of carbon utilization in each substrate type at 72 hours of incubation*. Measurements derived from the dry season and wet season are indicated with green and blue bars respectively. All samples had greater than 50% of the substrates in use. A table attached to the figure notes the percentages of each substrate used listed. No statistical difference was indicated based on season for any location (p>0.05).

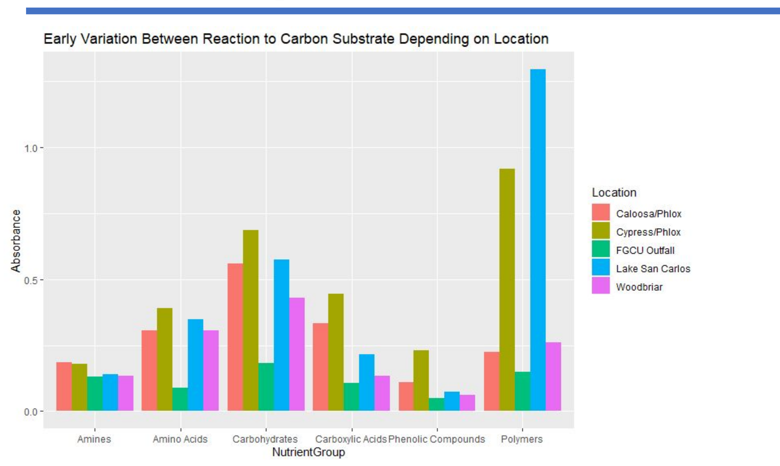


Figure 2: *The average absorption per substrate type in each location at 24 hours.* This figure displays early variation in reaction between groups. Little reaction occurs in phenolic compounds. Amines, carboxylic acids, and amino acids are statistically the same (p>.05). Polymers are highly favored by both Lake San Carlos and Cypress/Phlox.

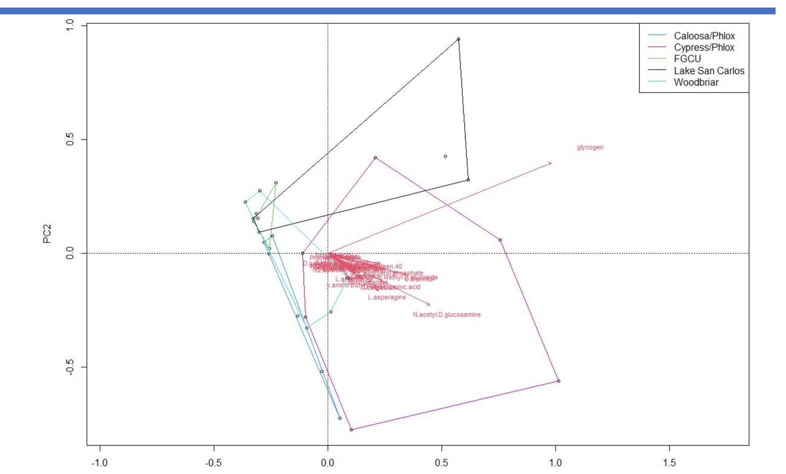

Figure 3: *PCA analysis at 24 hours indicates variation fueled by a few specific substrates*. The vectors for glycogen and N-acetyl-D-glucosamine drives variation. Eigenvalues at this stage are all less then 1, so not yet significant. ANOVA values indicate there is no differences between sites (p=0.0814). Proximity to p=0.05 may indicate sites are diverging.

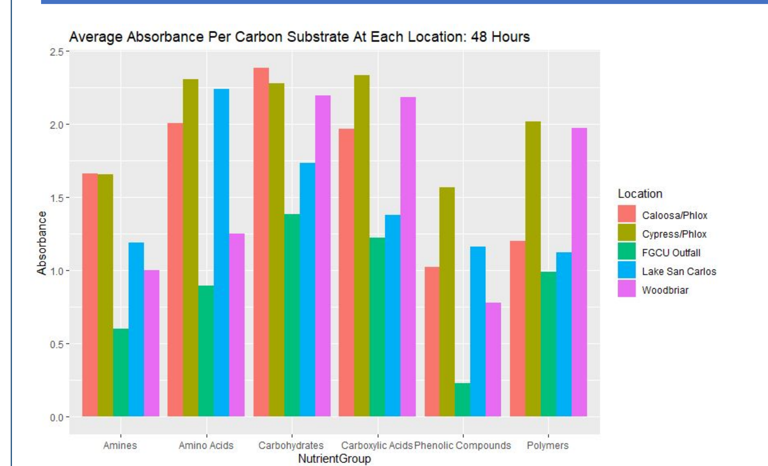


Figure 4: *The average absorption per substrate type in each location at 48 hours.* A large increase is seen between the 24 and 48 hours. Cypress/Phlox has the highest absorption across all groups barring carbohydrates. FGCU Outfall has the lowest absorption across all groups. There is no difference in metabolism between amines, amino acids, carboxylic acids, and polymers (p>0.35). Initial high polymer metabolism in Lake San Carlos is overtaken by other sites.

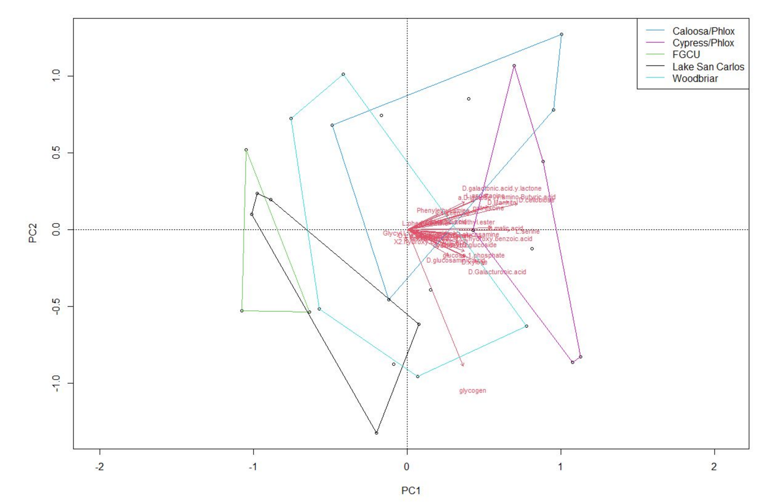

Figure 5: *PCA analysis at 48 hours shows clusters separating along PC1.* The eigenvalue for PC1 is 3.34. Glycogen continues to be a source of variance for the groups, along with D-cellobiose, y amino butyric acid, D. mannitol, L-serine, and several other high consumption carbon substrates. ANOVA tests indicate that there are significant differences between all sites at 48 hours (p<0.05).

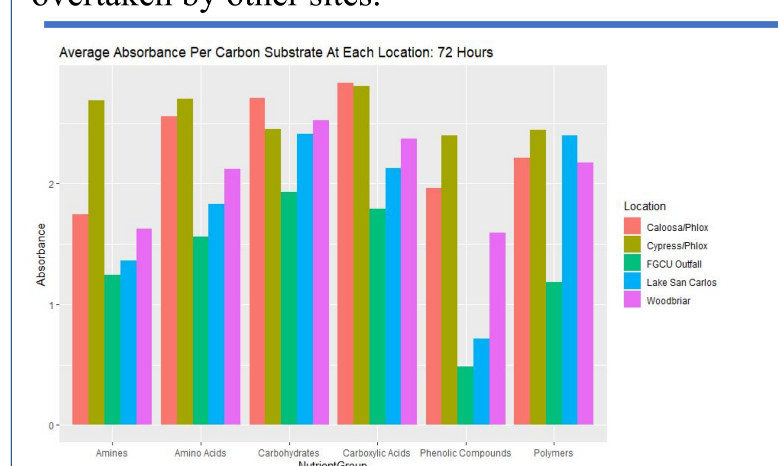


Figure 6: *The average absorption per substrate type in each location at 72 hours.* FGCU Outfall sees its greatest increase at this interval, though it still has the lowest values among all groups. Phenolic compounds continue to have the slowest use of all substrate types. There is no statistical difference between amines, amino acids, and carboxylic acids (p>0.15). At this time carbohydrates and polymers have no difference (p=0.92).

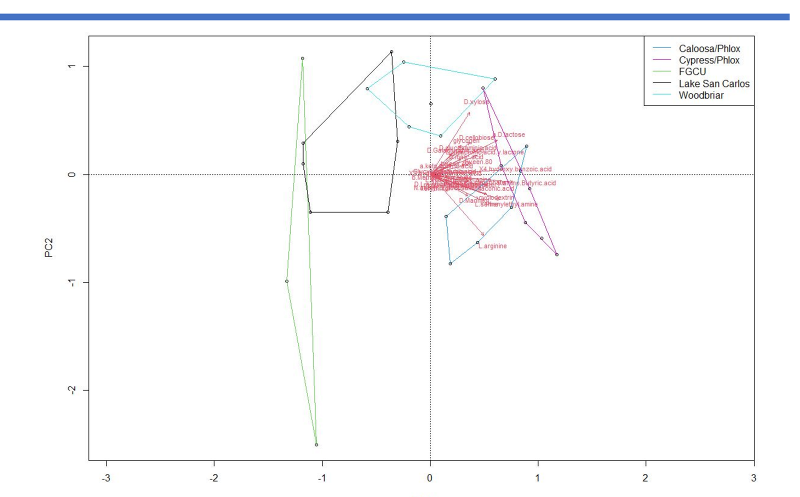

Figure 7: *PCA analysis at 72 hours shows distinct separation occurring along PC2 with the FGCU Outlook sample*. PC1 and PC2 had an eigenvalue of 3.87 and 1.16, respectively. Caloosa/Phlox and Cypress/Phlox samples begin overlapping, supporting the ANOVA value indicating no difference (p=0.07). Variation is driven by L-arginine, D-xylose, a,D-lactose, y-amino butyric acid, and phenylethylamine.

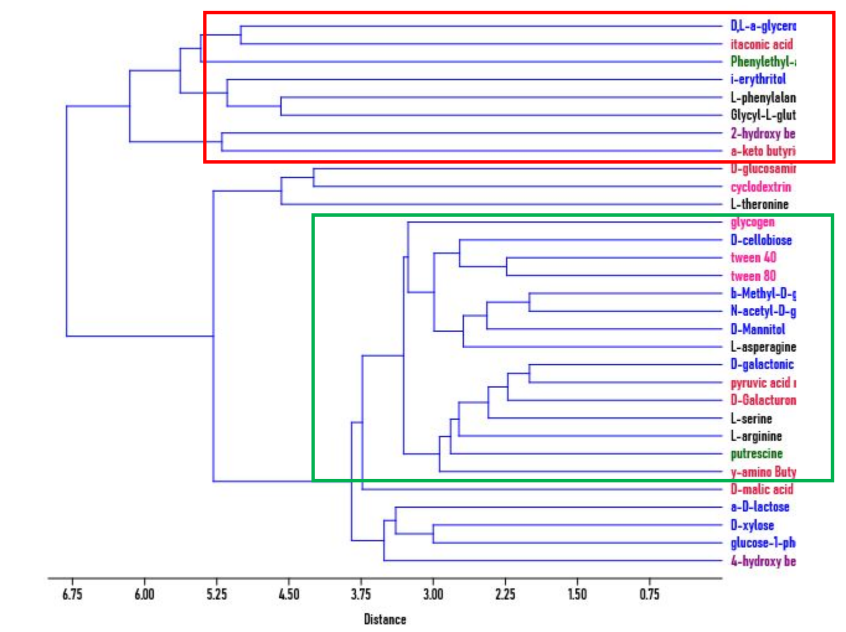


Figure 12*: Community Cluster Analysis for San Carlos Park*. Color combinations: Phenolic compounds= purple, Amines=green, Amino acids= black, Polymers=Pink, Carboxylic acid=red, Carbohydrates=blue. This dendrogram was created using data all four sites in the San Carlos Park community. Low use compounds are grouped with the red square. High use substrates are marked with the green square. Both the high use substrates and low use substrates are comprised of substrates from different groups, displaying how there were distinct rankings on which metabolites were metabolized first

## Discussion

All data matched expectations that there would be no difference between wet and dry season values and that having increased nutrient would result in higher metabolic rates among septic community locations. A lack of seasonal variation is expected due to the influx of nutrients during all seasons that would limit the effect of rainfall (Qiu et al. 2020) This characterization could be useful in the long-term assessment of pollution on microbial patterns in aquatic environments using the EcoPlates.

The evidence showing Lake San Carlos was more like FGCU Outfall than Woodbriar Lake was unexpected due to proximity to septic properties to the former location This can be clarified with future nutrient samples from those sites or utilizing16S rRNA sequencing to find full community structure.

Initial variation at 24 hours is present mostly in the metabolism of glycogen and N-acetyl-D-glucosamine in Cypress/Phlox and Lake San Carlos. Glycogen is a common metabolite in more than 50 species of bacteria, and its increased accumulation in cells has been noted to indicate increased presence of carbon sources. N-acetyl-D-glucosamine is also very common as a dissolved metabolite in aquatic systems and is a common source for nitrogen uptake (Goh, Y. J., & Klaenhammer, T. R. 2014). It's also notable that N-acetyl-D-glucosamine has been indicated to have a relationship with the hydrolysis of polymers (Riemann, L. & Azam, F. 2002). This displays a relationship that may implicate differences in carbohydrate presence that may help characterize the septic affected microbiome.

48 hours is the interval with the largest increase in absorption. Polymer metabolism was overtaken by the carbohydrate group. Phenolic compounds had the least absorption, driven by the nonreaction of 2-hydroxybenzoic acid (salicylic acid). Variation is seen occurring with many different compounds with different nutrients. From the PCA analysis it is visible that the locations are beginning to diverge. At 72 hours the PCA analysis shows all the groups clustered separate from one another. At this point the ANOVA values indicate that there is no difference between Cypress/Phlox and Caloosa/Phlox. While the PCA does overlap for those two location, they have different shapes and overlap is limited.

From this study, higher substrate utilization and a lack of seasonal variation are both characteristic of this septic tank community. Further analysis of this data can be achieved by studying the relationship specific pollutants can have on the community by introducing specific nutrients added with sceptic pollution like nitrogen and phosphorous to water samples from that community to display specific responses to those nutrients. Other ways to expand research includes the assessment of glycogen storage in presence of excess carbohydrates. The ability of the EcoPlate to define all this information makes it a viable option for detecting long-term septic pollution. This results in a viable method for assessing the presence and effects of long-term septic pollution.

**>EcoPlates can measure the distinct characteristics to assess effects of septic pollution.**

## References

Beal, C. D., Gardner, E. A., & Menzies, N. W. (2005). Process, performance, and pollution potential: A review of septic tank–soil absorption systems. *Soil Research*, *43*(7), 781-802.
Cavicchioli, R., Ripple, W. J., Timmis, K. N., Azam, F., Bakken, L. R., Baylis, M., ... & Webster, N. S. (2019). Scientists' warning to humanity: microorganisms and climate change. *Nature Reviews Microbiology*, 17(9), 569-586.
Gavrilescu, M., Demnerová, K., Aamand, J., Agathos, S., & Fava, F. (2015). Emerging pollutants in the environment: present and future challenges in biomonitoring, ecological risks and bioremediation. *New Biotechnology*, 32(1), 147-156.
Goh, Y. J., & Klaenhammer, T. R. (2014). Insights into glycogen metabolism in Lactobacillus acidophilus: impact on carbohydrate metabolism, stress tolerance and gut retention. Microbial cell factories, 13(1), 1-12.
Lusk, M. G., Toor, G. S., Yang, Y. Y., Mechtensimer, S., De, M., & Obreza, T. A. (2017). A review of the fate and transport of nitrogen, phosphorus, pathogens, and trace organic chemicals in septic systems. *Critical Reviews in Environmental Science and Technology*, 47(7), 455-541.
Qiu, H., Gu, L., Sun, B., Zhang, J., Zhang, M., He, S., ... & Leng, X. (2020). Metagenomic Analysis Revealed that the Terrestrial Pollutants Override the Effects of Seasonal Variation on Microbiome in River Sediments. *Bulletin of Environmental Contamination and Toxicology,* 105(6), 892-898.
Riemann, L., & Azam, F. (2002). Widespread N-acetyl-D-glucosamine uptake among pelagic marine bacteria and its ecological implications. *Applied and environmental microbiology*, 68(11), 5554-5562.