Naturalake Biosciences' Studies on Sediment Reduction

An overview on Naturalake Biosciences' studies of sediment reduction with MD Pellets and MuckBiotics from 2017 to 2019

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Abstract

Over time, many ponds and lakes will experience a build-up of soft organic sediments (muck). The accumulation of muck depth leads to problems such as high algal or aquatic plant growth and decreased water volume. Muck accumulated in water resources can be difficult to remove and often leads to the release of nutrients such as inorganic phosphorus and nitrogen. High levels of phosphorus in a water resource are known to select for cyanobacterial blooms, which in some cases, may be detrimental to human health. There are a few products currently available to decrease the depth of muck in a water resource through biological means, however, the efficacy of these products has yet to be established. Naturalake Biosciences has developed an experimental laboratory setup and methods to test muck reduction. This lab setup and methods were used to assess the efficacy of Naturalake Biosciences' MD Pellets and MuckBiotics products to reduce pond muck. Fifteen-gallon standard aquarium tanks with added pond muck were used, with or without washed sand or clay layers, and with pond water over the muck to a specified height. The tanks also contained two submerged powerhead pumps to provide lateral water movement over the sediment. Measurements of muck depth and water quality were taken weekly. The lab tanks with added MD Pellets or MuckBiotics showed sediment reduction that was greater than that of the Control tanks. This lab setup may be more representative of the water movement over the sediments in a pond and may explain why typical bucket tests or tests with muck in 5-gallon aquariums without pumps do not give satisfactory results. In our experiments with MD Pellets or MuckBiotics, we observed nutrient levels reduction with decreases in sediment depth. We also observed differences in the microbial community in the tanks treated with MD Pellets or MuckBiotics compared to the Control tanks. The Naturalake Biosciences lab setup and methods for assessing biological reduction of pond muck appeared to be more accurate than previous methods for assessing the efficacy of biological muck reducing products.

1. Introduction

The accumulation of nutrients over time in water resources leads to a buildup of decaying organic matter from plants, algae, bacteria, and in many cases, animals. This leads to the formation of nutrient-rich sediments known as muck. The accumulation of muck can lead to a wide variety of negative impacts on water resources, including reduced water depth for recreational uses, increased growth of problem algae and plants due to nutrients contained within muck, poor water clarity, and increased odors. Due to widespread contamination of nutrients, the accumulation of muck is evident in many water resources around the world. Muck is traditionally difficult to reduce or remove and can lead to nutrient release into a water column. Because of this, Naturalake Biosciences began studying techniques for the biological reduction of muck. This led to the development of our Muck Degrading (MD) Pellets and later the development of MuckBiotics.

1.1 Eutrophication and Algae Blooms

The accumulation of nutrients (eutrophication) in lakes, rivers, and ponds has been a rapidly accelerating global problem due to continuous pollution and increases in global temperatures. A study released in 2019 on 71 lakes found algae bloom intensity has increased in 68% of the lakes studied (Ho, 2019). The rise of global temperature and elevated nutrient levels were considered to be the two primary factors that led to an increase in these problem algae (Ho, 2019). The enrichment of nutrients taking place is quickly becoming a worldwide threat to public and environmental health. Eutrophication leads to increased prevalence of cyanobacterial blooms.

Algae growth can be helpful in lowering soluble nutrients. However, in eutrophic water bodies, excess growth leads to aesthetic concerns and odor issues. In addition, visual identification of harmful algae in water resources is unreliable, and therefore, without testing, it can be difficult to determine if an algal bloom is harmful or not. Many water resources experience temporary or permanent closures due to harmful algal blooms. Annually, in the United States, negative effects from harmful algae blooms cost over 4 billion dollars (USD) (Ho, 2019). These economic impacts are caused by effects on aquatic food production, recreation and tourism, and on drinking water supplies (Ho, 2019).

1.2 Eutrophication Process and Nutrient Cycling in Ponds, Lakes, and Rivers

The process of eutrophication in water bodies typically occurs after the continued addition of excess nutrients for an extended period. Nutrients such as nitrogen and phosphorus are often retained in sediments within water bodies through natural mechanisms for binding phosphorus, such as adsorption to inorganic particulates as well as uptake by bacteria, algae, and macrophytes (Withers, 2008). Over time, these nutrients lead to an increase in abundance of all life forms present in the water column. These life forms begin dying over time, and decaying bacteria, algae, and macrophyte material begin to accumulate in a nutrient-rich sediment layer known as muck. Changes in temperature, agitation, pH, and oxidation-reduction conditions can lead to a release of soluble nutrients into the water column. In cases with a relatively low nutrient sediment layer, most of the nutrient-enriched sediments are a necessary nutrient source for diverse life in the water body. However, high concentrations of nutrients in the sediment layer increase the likelihood of nutrient release due to changes in pond conditions (Withers, 2008).

1.3 Source of Nutrients in Lakes, Ponds, and Rivers

1.3a External Nutrients. Nutrients in lakes, ponds, and rivers come from a variety of different sources, which can lead to the growth of organisms such as algae. These nutrients can be categorized into internal and external sources of nutrients. Runoff is the most commonly talked about and studied external nutrient source for water bodies. Information on the contribution of runoff on cyanobacterial blooms has led to increased examination of the use of fertilizer and its impacts on the environment. This information has led to numerous phosphorus bans in fertilizer, such as the statewide ban in Wisconsin on lawn fertilizer, enacted in 2010. These bans are limited in effectiveness as phosphorus-containing fertilizers are still available for purchase, and other nutrients, especially nitrogen, are still widely available in fertilizers. In some cases, it is possible to appeal for exceptions from the ban (Novac, 2010). Leaf litter and agricultural waste are still widely present nutrient contaminants in water resources and limited strategies are available for controlling these types of nutrients. In urban areas, grass clippings and landscaping debris often migrate to waterways, leading to nutrient contamination as well.

Wastewater treatment effluent is another commonly discussed form of external nutrient loading in waterways. Due to increasing populations, the demand for wastewater treatment is likely to continue to increase. Because of this, many government agencies have been enacting increasingly strict effluent limits on wastewater treatment facilities. Government regulations are guided by affordability of treatment; therefore, many smaller wastewater facilities which cannot afford treatments capable of producing very low levels of effluent nutrients are likely to have less strict effluent limits. Wastewater treatment plants are considered a point source of nutrients, which means a large volume of pollution is being discharged in a single location. This leads to more detrimental impacts on local water bodies but less widespread impacts of pollution. Most of the phosphorus in wastewater treatment plant effluent tends to be in the form of soluble reactive phosphorus (SRP), meaning it is more easily available to plants and algae compared to particulate phosphorus (Jarvie, 2005).

Septic leakage is a widespread issue for nutrient contamination as well, particularly in rural areas. Contamination from phosphorus, ammonia, and nitrate is frequently observed from septic leakage. Many people with septic systems are unaware if their systems are functioning properly and measurements are not taken frequently enough to confirm proper septic function

(Yintao, 2007). It is likely that leakage from septic systems is a significant contributor to nutrient enrichment in most rural areas.

In addition to these forms of external nutrient loading, it is important to also consider the water source of a water body. Lake- and river-fed water bodies tend to receive most of their nutrient loading from upstream sources, as opposed to rain-fed ponds that receive most of the nutrient loading from direct runoff. Groundwater-fed ponds tend to receive elevated levels of high soluble contaminants that are not filtered out well by soil, such as nitrate. Soil has a large capacity for adsorption and ion exchange reactions with ammonia and phosphate (Bleam, 2017), but soil nitrifiers are often capable of converting the bound ammonia to nitrate, which then can flow more freely.

1.3b Internal Nutrients. Internal nutrient cycling refers to the binding, accumulation, and resuspension of nutrients in a water column. In addition to competition between different organisms, competition between certain chemical processes and organisms is also taking place.

Studies of phosphorus recycling in rivers and ponds have shown that phosphorus pollution will often be retained in water bodies, even in high flow-through systems such as rivers (Withers, 2008). This effect has multiple implications, but indicates phosphorus is likely to stay relatively close to its source when it flows into a water body. Phosphorus has been known to precipitate out during the process of calcite formation, which can act as a temporary sink to dissolved phosphorus and has potential to rerelease into systems if changes to ORP or pH take place (Hamilton, 2009). Iron and aluminum frequently form precipitates when exposed to phosphate, which often dissolve in low oxygen conditions. Phosphorus tied up in organic forms, such as phospholipids, can also be maintained in the bottom of a water body. Often, organic phosphorus is present in the form of mono and diesters (Bleam, 2017). Organic matter from dead and decaying life forms contain high levels of nutrients. These can easily be released into water bodies due to environmental changes. One exception to this rule is nutrients contained in dead bacterial cell walls. Bacteria have a difficult-to-break-down peptidoglycan cell wall that may prevent the release of absorbed nutrients. The bacterial cell wall has been observed to remain intact for as long as 69 days (Jorgensen, 2003), indicating the nutrients in the bacteria were unlikely to be released during this time period. In addition, the nutrients in bacterial cell walls are more likely to be released slowly, cause less spikes in bioavailable nutrients, and are less likely to lead to significant algal blooms.

Calcite and appetite formation can also lead to the reduction of phosphorus in water bodies. Many substances, such as zeolite, can also perform ion exchange reactions that remove ammonia and other compounds from the solution. The suspension of particulates due to physical agitation can lead to the release or binding of nutrients in a water body, depending on water conditions and the saturation of nutrients in a system (Palmer-Felgate, 2011). Populations of macroinvertebrates, such as various snails or worm species, lead to mild agitation of the aerobic surface layer and improve oxygenation and sediment turnover in sediments (Covich, 1999). These macroinvertebrates lead to faster degradation of high-organic sediments and can contribute to an increase of soluble nutrients in the water column due to waste by-products (Covich, 1999).

A study on the effects of internal nutrient loading was conducted in 2011. The water body in question experienced nutrient loading, followed by a phytoplankton crash, which caused the water body’s sediments to convert from a nutrient sink to a nutrient source between March and June (Palmer-Felgate, 2011). Once nutrients are bound in a water body’s sediment layer, they form a nutrient-enriched sediment layer that often contain high organics known as muck. In cases where a thick sediment layer is present, anaerobic digestion is the primary cause of sediment degradation and nutrient release.

Anaerobic digestion occurs in the deep sediment layers, leading to the production of methane, carbon dioxide, and hydrogen sulfide. In addition, phosphorus release due to anaerobic digestion appears to be the main cause of release of sediment phosphorus in many cases, compared to dissolution of ferric sulfates (Prairie, 2001).

Muck is a mixture of decaying organics (such as dead plants, algae, and bacteria) and inorganic particulates such as sand and silt. Muck usually contains high levels of organic carbon, nitrogen, and phosphorus which accumulate over time. Due to muck's tendency to release nutrients in varied conditions, its presence can often be the source of repeated algal blooms, despite the reduction of incoming nutrients. Muck tends to contain high volumes of water trapped in organic matter and is therefore much closer to the density of water than inorganic sediments. Muck's organic contents often leads to the depletion of oxygen and the anaerobic digestion of organic carbon, which leads to the release of nutrients such as phosphorus and nitrogen (Palmer-Felgate, 2011). Because of the problems associated with muck volume and nutrient content, many water bodies must occasionally dredge the bottom muck layer in order to prevent loss of water depth and to reduce the frequency of harmful algal blooms.

Dredging is a commonly considered process to remove enriched sediments from the bottom of water bodies. This process normally takes place through the mechanical scooping or vacuuming of pond sediments to remove muck accumulation. Dredging has been shown to be an effective nutrient control in water resources but can have negative impacts. First, dredging provides much agitation to sediments on the bottom. This suspension of sediments can cause concerns, temporarily increasing nutrient diffusion in a water body. This may lead to short-term negative impacts, but overall is better than leaving nutrient-rich sediment. In sediments with toxic contaminants, it is also possible that this increased agitation can risk exposing toxic waste.

In 1978, release of DDT from water bodies was occasionally observed during dredging, and likely applies to other contaminants as well (Peterson, 1978).

1.4 Competition Between Plants, Algae, and Bacteria

The competition of organisms in water bodies is one of the most significant factors in rapid growth of harmful algae. When nutrient levels are low in a body of water, diverse growth and competition between organisms is encouraged. As low levels of nutrients enter the system, diverse organism growth is increased initially, but eventually a niche-filling organism is likely to take over due to favorable conditions for rapid growth (Withers, 2008). Cyanobacteria tend to be faster growing than most green algae due to their simple cell structure and therefore tend to compete more favorably in conditions with high temperatures and high levels of available nutrients. This is especially true of planktonic cyanobacteria such as *Microcystis*. It is worth noting that different environmental conditions, such as light exposure, pH, temperature, nutrient levels, and DO levels all significantly contribute to the selection of different organisms in lakes and ponds.

In cases where sufficient dissolved organic carbon is available, bacteria can compete well with algae and plants for inorganic nutrients. This is common in oligotrophic conditions with organic carbon but limited nitrogen and phosphorus. As bacteria are not light limited, they can thrive in conditions when large algal blooms are taking place as bacteria do not need to compete for light. Bacteria are known to uptake significant amounts of phosphorus and ammonia from waterways, though typically are not likely to use nitrate as a nitrogen source. Bacteria have been measured taking up a wide range of nutrients in water bodies. Studies have shown the contribution of bacteria in phosphorus uptakes to be as low as 5%, to over 90% of the total phosphorus uptake in ponds (Kirchman, 1994). In addition, bacteria appear to uptake between 30 to 60% of all available nitrogen in many cases. Bacterial membranes contain high levels of phosphorus in phospholipids. DNA content also contains significant amounts of phosphorus in bacterial cells (Kirchman, 1994). Bacteria have been monitored taking up urea and nitrate as nitrogen sources, but typically will fail to compete effectively for these nutrients compared to plants and algae. In these cases, if low levels of soluble organic carbon are available, bacteria can form symbiotic relationships with algae to obtain organic carbon leaked from autotrophic energy production (Rier, 2002). However, in conditions with moderately elevated dissolved organic carbon, bacteria are capable of uptaking these nutrients in addition to amino acids and ammonia (Kirchman, 1994). If excessive levels of soluble organic carbon are present, occasional bacterial blooms can occur, which can lead to turbid water, encourage the growth of pathogens, and interfere with the growth of submerged aquatic plants.

Plant life is one of the most effective controls for long-term nutrient contamination. Constructed wetlands use plants to effectively use up phosphorus and nitrogen entering larger water bodies. Shoreline plants also provide some capacity to absorb nutrient runoff from lawns and agricultural fields, as well as limit erosion. In addition, aquatic plants compete directly for nutrients and light with algal populations. Fast growing plants that can thrive in limited light conditions tend to be the most effective competitors with algae. This includes plants such as *Hydrilla*, watermilfoil, *Vallisneria*, coontail, and others.

Competition for light appears to be the most significant factor between algae and submerged plant growth in eutrophic conditions. In eutrophic environments, in many cases sufficient nutrients exist to fuel both the growth of algae and plants. Macrophytes (plants) grow significantly more slowly than algae, so in cases with highly elevated nutrients in the water column, large algal blooms can quickly occur. However, if sufficient light and nutrients are available, plant growth will often continue. This was studied in 2004 where the competition between algae and duckweed quantified the impacts of algae on duckweed growth with different levels of shading. When shading on algae was limited, a 60% reduction of duckweed growth was observed. It appeared the algae were outgrowing the duckweed with sufficient nutrients, and their presence reduced the growth of duckweed by 60 to 62% (Roijakers, 2004). Since duckweed grows on the surface of water bodies, it was not light limited and therefore continued to grow, which eventually led to duckweed outcompeting algae due to their access to sunlight. Submerged macrophytes were also observed to be unable to effectively compete with algae when algal blooms occur due to light limitation (O’Hare, 2018). In many cases, despite having sufficient access to nutrients, light appears to be the dominant factor in competition between plants and algae. Because of this, aquatic dye is often applied to reduce light penetration into water resources. This is likely an effective control on some benthic algae, as well as submerged plants, but would likely lead to improved conditions for planktonic or surface-dwelling autotrophs. Therefore, in cases of attempting to reduce the prevalence of harmful algal blooms, it is unlikely limiting light penetration through water bodies is effective and control of nutrient contamination is more likely to lead to long-term success, particularly when high levels of nutrients are already available in water bodies. In general, it is safe to assume that high levels of available nutrients will favor organisms with faster growth rates, such as bacteria and cyanobacteria.

1.5 Development of “Pond in a Tank” Testing with MuckBiotics and MD Pellets

Development of Naturalake Biosciences’ “Pond in a Tank” setup began in 2012. This testing was necessary for our research due to the inability to effectively replicate pond conditions between different sites. This testing is designed to minimize variables and allow for side-by-side testing of various additives and to monitor the effect of different environmental conditions on nutrient levels in the water column. Previous attempts to replicate successful field studies in laboratories using muck in 5-gallon buckets, small wading pools, or other means, were not accurate representations of pond ecosystems. More accurate representations of pond ecosystems and data collection allows for a better understanding of the MD Pellets or MuckBiotics effect on reducing sediments in real-world scenarios. In our studies, we performed analysis for sediment and water column nutrients, changes in sediment depth, changes in water clarity, and changes in lifeform diversity. The primary variable in our studies was the addition of “MD Pellets” (2017–2018) and “MuckBiotics” (2019) to analyze whether muck volume reduction was occurring without any potential detrimental effects, such as nutrient release, to an aquatic environment. Studies are currently ongoing. This paper summarizes our findings currently, describes methods and modifications throughout our testing, and provides analysis of our data. We also contrast our in-lab findings compared to various completed and ongoing field studies.

2. 2017 Tanks

2.1 Methods

The tanks used in testing were 15-gallon standard glass Aqueon aquarium fish tanks. The tanks were cleaned before the addition of sediment and water. A 5 cm layer of soft pond sediment was then spread on the bottom of the tanks. Soft pond sediments were collected from Pond 1 for all tanks. No initial samples were taken for analysis.

Water from Pond 1 was added to all of the tanks to bring the water level to 15 cm above the soft sediment layer. Each tank contained an air stone, placed 1 cm above the sediment layer, and powered by a Marina 50 aerator. The aerator was turned on for 12 hours per day, during the time that the tank light was turned off. Each tank had its own Aqueon 19-watt fluorescent overhead aquarium light source that was on for 12 hours and off for 12 hours each day. Each tank also had two Korelia Nano 240 gph pumps on either side of the tank. The pumps were angled to the side of the tank and slightly upward in order to induce a water flow current throughout the entire tank. The pumps were turned on for 15 minutes every 6 hours and the controller switched between coinciding pumps every 10 seconds. A testing grid was constructed above the tank, totaling 50 equal-area sample sites for muck depth measurements (2-inch squares). Only 10 of the sample sites were used for muck depth measurements.

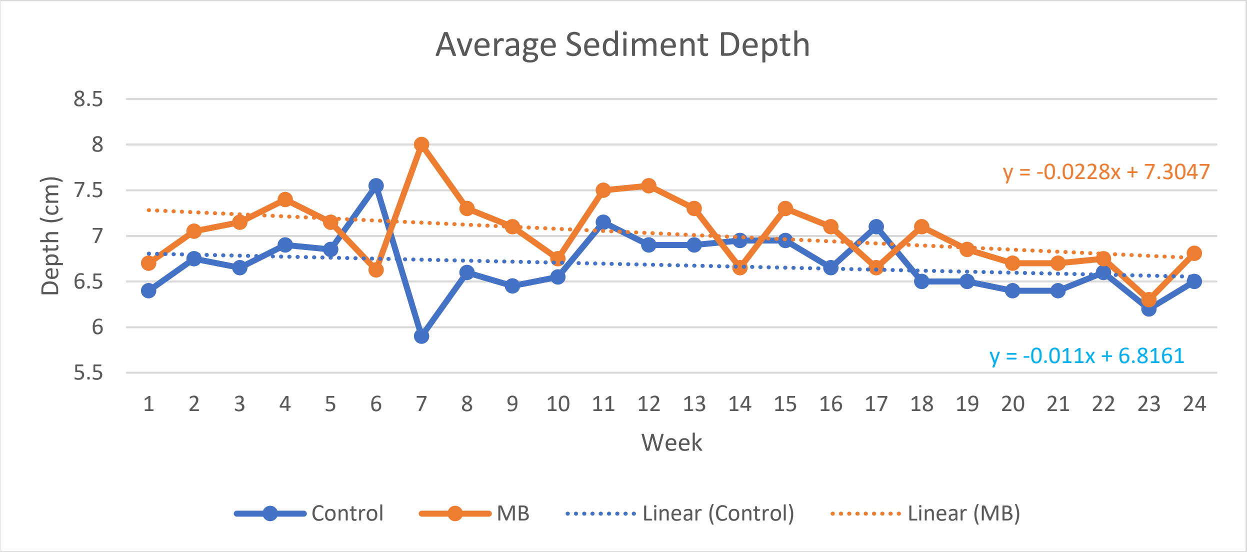
Weekly measurements began after the initial set up of the tanks. Visual observations and photos were taken of each tank. Weekly water quality measurements were conducted from water collected one inch above the sediments in each tank. Water quality analysis was conducted in the Naturalake Biosciences' laboratory. Water quality measurement parameters included general water quality using Hach test kits, pH, DO, and temperature. The Hach HQ40D portable meter, along with LDO101 and PHC101 field probes, were used to measure pH, DO, and temperature. Ten equal-area sample sites within the testing grid were used as a representative sample and measured for water depth over the sediments, as well as total depth of the water and soft sediments. Soft sediment depth was calculated for each sample site. Water levels were maintained at 15 cm for the duration of the testing. The tanks were tested once a week for the 24-week treatment testing period.

The tanks that were given no additional treatment products acted as controls for our testing. The treatment tanks were given an application of MD Pellets spread throughout the tanks in order to mimic a field dose of 50 lbs per acre per month. All water quality measurements were taken before the application of MD Pellets.

Upon the conclusion of the 24-week testing period, final measurements, observations, and images were taken. The remaining water was drained from the tank, and a final soft sediment sample was taken for analysis. 500 mL of the top layer of soft sediment was sent to Eurofins TestAmerica, Chicago for nutrient analysis. Thirty mL of soft sediment was frozen, and then sent to the University of Wisconsin Biotech Center (UWBC) located in Madison, WI for DNA extraction and 16S rRNA sequencing analysis.

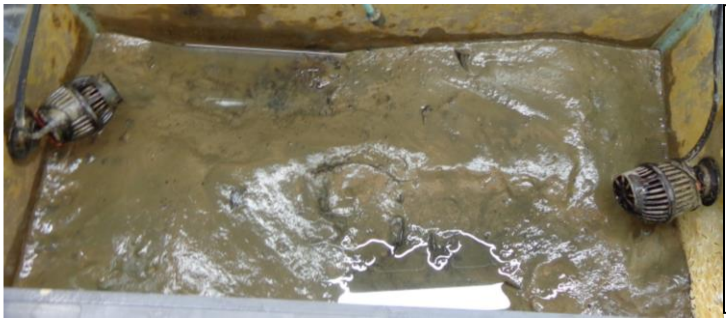
2.2 Results

To show that sediment reduction using MD Pellets can be achieved in laboratory settings, it was necessary to set up fully functional laboratory-scale ponds. More accurate representations of pond ecosystems and data collection allows for a better understanding of the MD Pellets effect on reducing sediments in real-world scenarios.

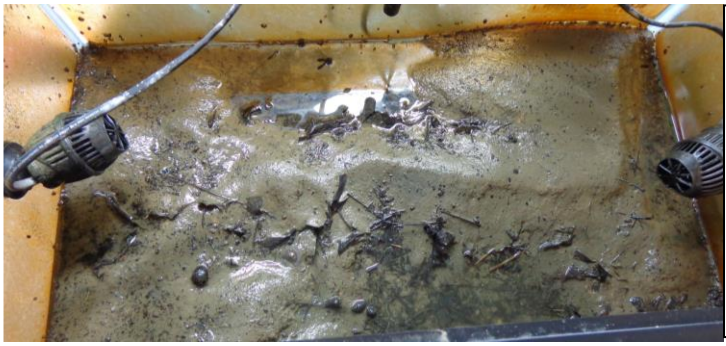


In the 2017 test we saw a larger downward slope in the MD Pellets tank compared to the Control tank. This faster sediment depth decrease may have been more apparent in the MD Pellets tank had the whole leaves been screened out of the sediments before the sediments were added to the tanks. The whole leaves were visually observed to collect gas bubbles and may have inflated the sediment height of both tanks to different degrees. Visual observations suggested that measuring more sites may have resulted in further separation in the control versus the MD Pellets tank slopes. This test was conducted using a field dose of 50 lbs per acre per month of MD Pellets and the test ran for 24 weeks.

2.2a Sediment Texture, Color, and Odor Changes When Dosed with MuckBiotics. The tanks in the lab testing were drained at the end of the test and the texture of the sediments was observed. There were noticeable differences between the Control tanks and the tanks treated with MD Pellets. The Control tanks' sediments had a soft semiliquid texture compared to the grainy and firm texture of the sediments from the MD Pellets tanks. The Control tanks' sediments also were darker brown or black in color and had a sulfur or anaerobic odor compared to the sediments from the MD Pellets tanks. This altered texture and color is similar to what has been reported and observed in several field studies (See Appendix B).



The 2017 Control tank ended with deeper pits and higher levels of soft sediments on top of a layer of leaves. These top sediments were semiliquid and hard to scoop because they would not hold a shape.



The 2017 MD Pellets tank ended with a flatter sediment layer overall. The sediments were firmer and had a sandy consistency. These sediments had leaves mixed throughout and were uniform all the way down, rather than the two distinct layers observed in the Control tank.

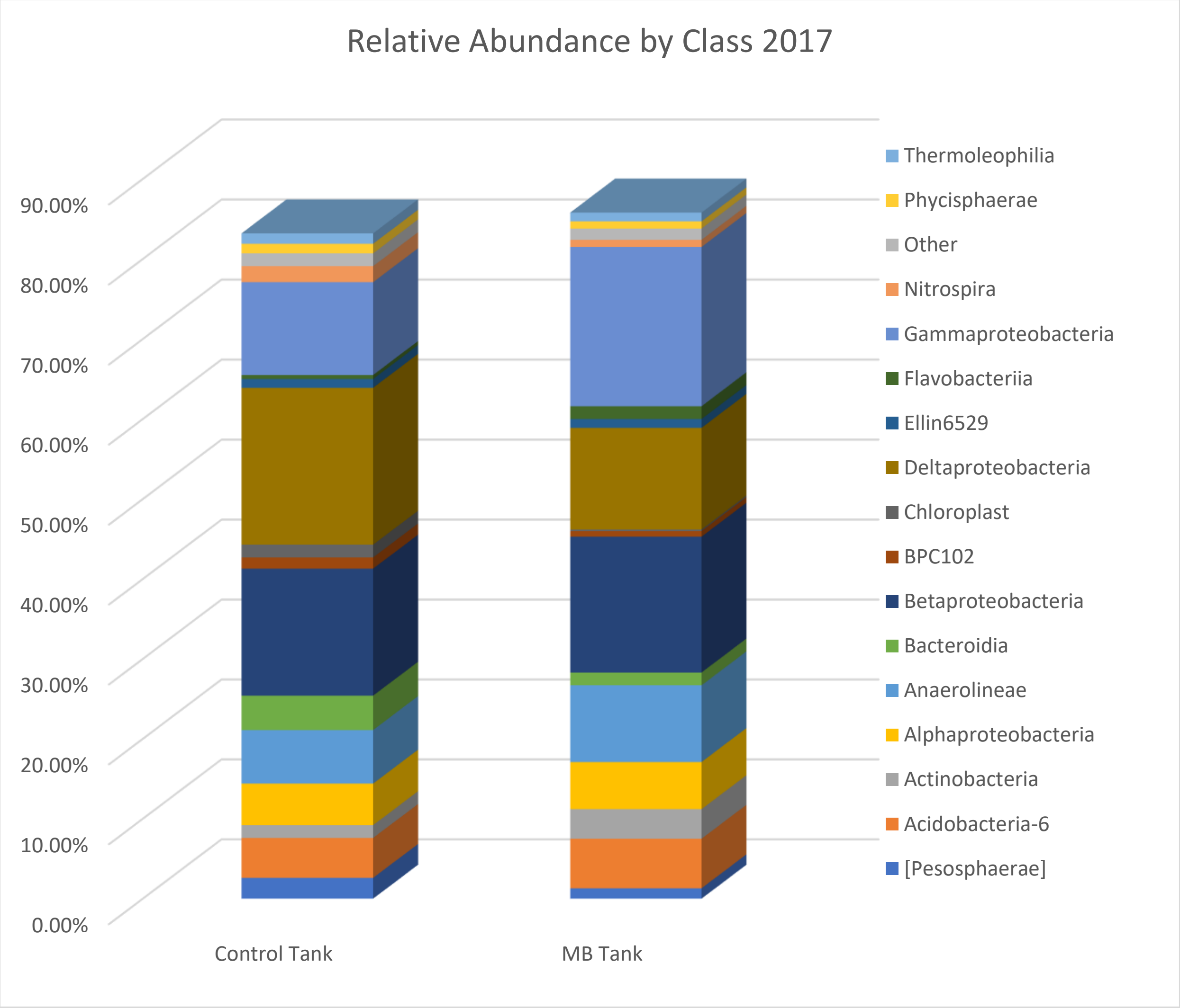
2.2b MD Pellets Induces Changes in Sediment Microbial Community. To better understand how MD Pellets may contribute to soft sediment reduction, the topmost light brown layer of sediment was removed from the lab tanks and sent for DNA analysis. In 2017, the UWBC performed 16S rRNA analysis to determine the bacterial and archaea relative abundances in the sediment using an ABI 3730xl DNA Analyzer (Sanger sequencing). In 2018 and early 2019, the UWBC analyzed the sediment sample using the Illumina NovaSeq 6000 platform. This is a metagenomic platform that allowed sequencing from the eukaryotes as well as the prokaryotes and archaea.

2.2c Methods for Interpreting DNA Analysis. Only sequence entries that comprised at least 1% of the total prokaryotic community in at least one of the tanks were investigated. The entries that are lower than 1% of the total community are probably too low to be significant to the metabolic pathways going on in the sediment of the tanks (Esteban et al., 2015). Although rare bacteria have the potential to alter the environment, they were not investigated since the very low abundance OTUs could possibly be due to sequencing artifacts as well as by rare microbes (Rundell et al., 2014). The DNA extraction was performed using both wet mass and dried mass of the sediment samples. The two methods had some differences, but the dry mass values were used in this analysis because they tended to have higher bacterial biomass (DNA) than the wet values. This may also indicate that DNA analysis from these samples contained a larger population of the total bacterial community in the tanks and could be more representative than the DNA extracted using the wet mass.

Sequences were evaluated by phylum and then by class. Statistical calculations of diversity such as Shannon index, Berger–Parker, or other diversity algorithms were not assessed here and they may not matter since both tanks were in similar conditions, and only the top aerobic layer of each was used for analysis. Such indices may become more relevant if lower layers with different oxidation reduction potentials are explored within a tank. As mentioned before, the "enriched" bacteria in the tanks were not compared to the original pond sediment collected from the pond in this study. This will be added to later studies.

2.2d DNA Analysis General Results. When sediment is collected from the pond, a founding population is captured along with the chemical and physical properties of the sediment (Rundell et al., 2014). All of this contributes to the establishment of niches and propagation of specific bacteria. Over time, the community diverges significantly from the founding population (Esteban et al., 2015). A shift in bacterial communities can provide important insights into environmental changes in the sediments (Wan et al., 2017).

Microorganisms assigned to unclassified bacteria existed in higher proportions in freshwater sediment samples than in soil samples (Chen et al., 2016). This is possibly because freshwater sediments have not been as extensively studied as soil or even marine sediments, although this may begin to change.



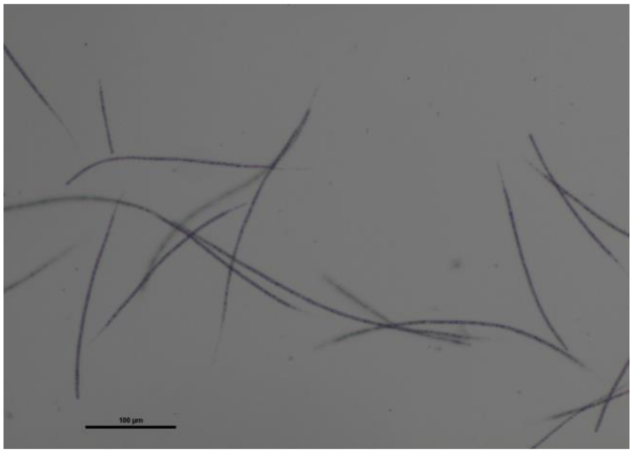
Overall, in 2017, the bacteria in the sediments indicated that the sediment in the MD Pellets tank became less anaerobic. There were also less cyanobacteria present in the MD Pellets tank compared to the Control tank. The Control tank had more *Deltaproteobacteria* and *Nitrospira* than the MD Pellets tank. Both bacterial classes are important for nutrient release in the sediments.

The class Chloroplast was 8-fold higher in the Control tank than in the MD Pellets tank. The class Chloroplast is generally enriched in the surface layer (Zhou et al., 2017), which is reasonable since these are all photoautotrophic cyanobacteria. The high abundance in the Control tank might be why the water nutrients were low compared to the MD Pellets tank that had clear water. Light intensity may have also been a factor in the 2017 lab testing.

The class Flavobacteria was 3.2-fold higher in the MD Pellets tank than in the Control tank. Flavobacteria are mostly aerobic and degrade proteins and polysaccharides (Zhou et al., 2017). The class Actinobacteria was 2.3-fold higher in the MD Pellets tank than in the Control tank. Actinobacteria are considered a biomarker for leaf litter (Rundell et al., 2014). This abundance of Actinobacteria was not unexpected since there were high levels of leaves in the lab tanks since the muck used in 2017 was not screened.

Good resolution down to the genus level was not achieved for the majority of the sequences. Only seven entries were named. This should change as metagenomics becomes more widely used and more research is compiled. Specific genus and species of bacteria have been studied experimentally and more is known amount the organisms' metabolic capabilities and environmental niches. Based on the phylum and class distributions between the two tanks, it seems that the Control tank sediments were more anaerobic than the sediments in the MD Pellets tank. This may indicate that the Control tank had higher amounts of easily usable organic carbon substrates. This may have made more favorable conditions for methane generation and possibly methane oxidation in the top aerobic layers. For example, the Control tank had higher amounts of *Syntrophobacter* and *Syntrhophus*, which are known to make precursor compounds to methane generation such as acetate (Jackson et al., 1999). On the other hand, the MD Pellets tank had more *Methylocaldum*, an aerobic methane oxidizer (Takeuchi et al., 2014).

2.2e Cyanobacteria Much Lower in MD Pellets Tank.



The largest difference was the Control tank contained the phylum Cyanobacteria at rates five-fold higher than the MD Pellets tank. The Control tank ended with a cyanobacterial bloom that mostly contained *Aphanizomenon,* which was identified microscopically. During this time, the MD Pellets tank had clear water. Cyanobacteria are photoautotrophs that are known to be positively correlated with total nitrogen, nitrite, and nitrate (Kragelund et al., 2011). There is the possibility that both tanks should have experienced a cyanobacterial bloom, but that the Control tank was the only one exposed to high enough illumination and UV radiation levels due to its closer position to the lab windows. It is also possible that the MD Pellet tank was lacking some other nutrient that was present in the Control tank. A possible nutrient could have been soluble ammonium released from the sediments. The Control tank had previously experienced a drop in DO and a corresponding increase in ammonium in the water column. This was not observed in the MD Pellet tank.

The phylum Nitrospirae was 2.2-fold higher in the Control tank compared to the MD Pellets tank. Members of this phylum are involved in the nitrogen cycle, particularly the oxidation of ammonium to nitrite to nitrate. The soluble ammonium in the MD Pellets tank was high from weeks 2 to 13, then lower than the Control tank, but still around 0.15 ppm. This amount of ammonium in pond water is considered higher than normal. There was possibly less soluble ammonium in the Control tank during this time because the sediment or water added to the Control tank had a more established nitrogen cycle.

2.3 Summary

In summary, for the 2017 lab tank test, sediment reduction was achieved in the laboratory set up. The sediment 16S rRNA analysis show that there is a difference in the bacterial community between the Control tank and the tank treated with MD Pellets and may partially explain the observed sediment reduction observed in the test. There were also visual observations of reduced cyanobacterial levels in the tank treated with MD Pellets as compared to the Control tank.

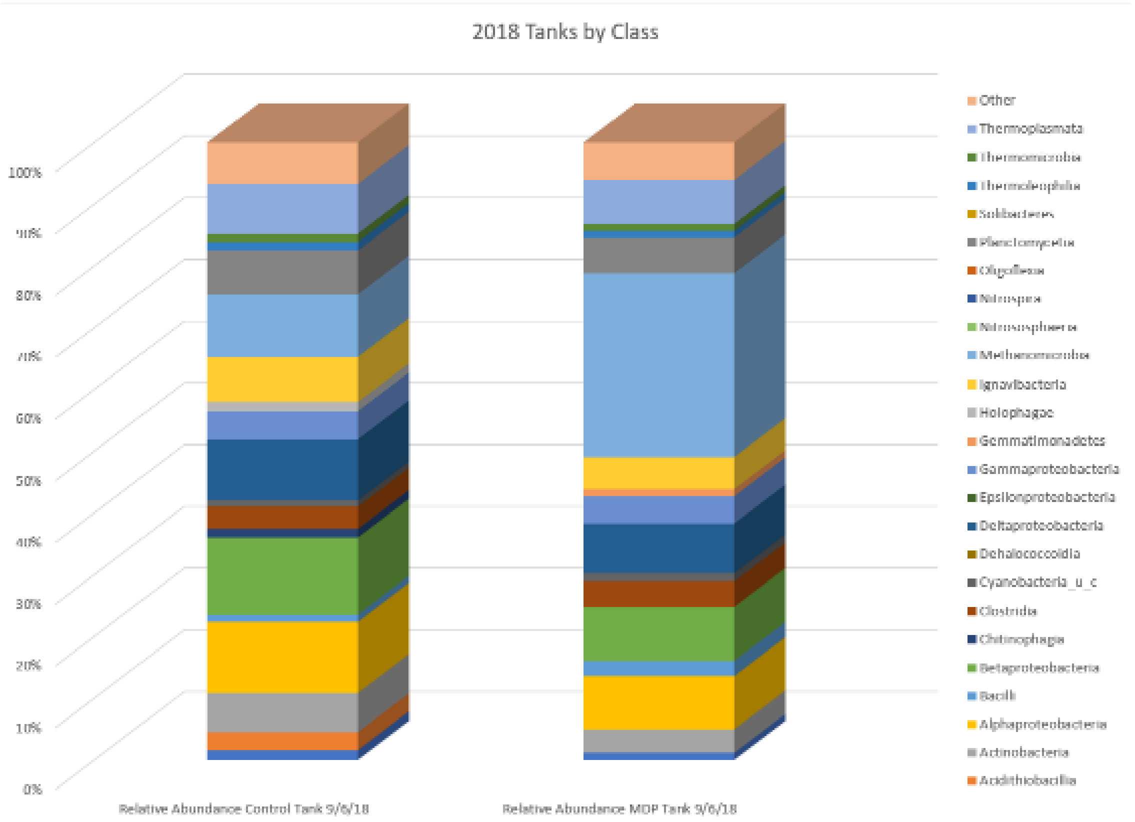
3. 2018 Tanks

3.1 Tank and Testing Modifications

In 2018, the same general methods were used for the "pond in a tank" to observe if newly available metagenomic platforms would provide more complete data. Slight modifications in the methods were made. Testing was reduced from 24 to 12 weeks since the measurements became relatively stable after 12 weeks in the previous tank studies. An initial composite pond sediment sample was taken for analysis prior to the addition of the pond sediment into the tanks. 30 mL of soft sediment was frozen and then sent to the UWBC for DNA extraction and Illumina NovaSeq 6000 metagenomic analysis. After 12 weeks of testing, a 30 mL composite soft sediment sample was taken for all MD Pellets and Control tanks. All samples were frozen, and then sent to the UWBC for DNA extraction and Illumina NovaSeq 6000 metagenomic analysis.

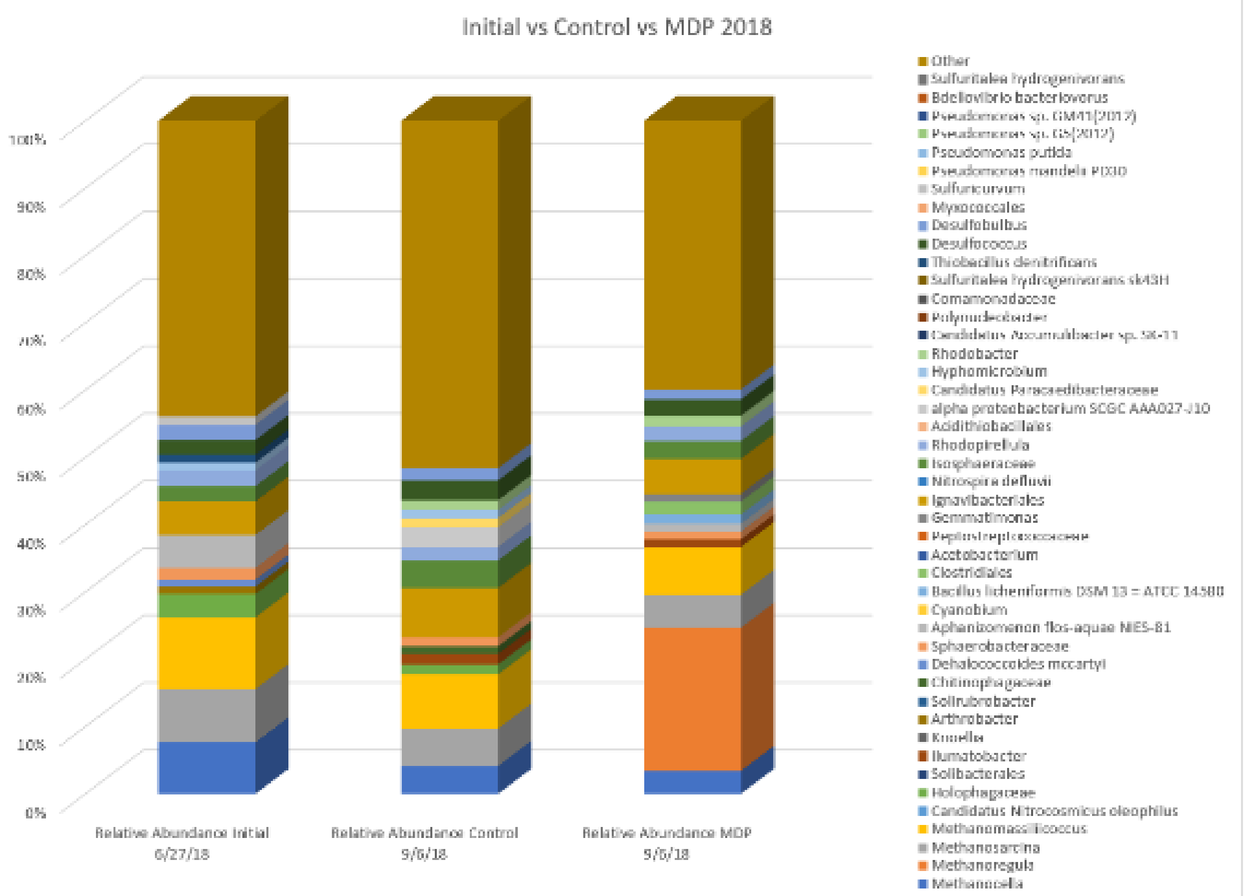
3.2 Results

3.2a MuckBiotics Induces Changes in Sediment Microbial Community. To better understand how MD Pellets may contribute to soft sediment reduction, the topmost light brown layer of sediment was removed from the lab tanks and sent for DNA analysis. In 2017, the UWBC performed 16S rRNA analysis to determine the bacterial and archaea relative abundances in the sediment using an ABI 373oxl DNA Analyzer (Sanger sequencing). In 2018 and early 2019, the UWBC analyzed the sediment sample using the Illumina NovaSeq 6000 platform. This is a metagenomic platform that allowed sequencing from the eukaryotes as well as the prokaryotes and archaea.



In general, it's more informative and accurate to look at the genera within the bacterial and archaeal communities. In 2018, access to metagenomic platforms for more complete analysis was made available. The Control tank and the MD Pellets tank do appear to be different in microbial composition. The MD Pellets tank had 2.9x more *Methanomicrobia* than the Control tank and even more than the initial starting muck. This generally indicates a high amount of methane-producing microbes that could also be an indicator of complete organic substrate digestion. The Control tank had 10x more Chitinophagia than the MD Pellets tank. This may have indicated more aerobic conditions or a larger aerobic layer (Li et al., 2017). There were also 3.7x more Gemmatimonadetes in the MD Pellets tank. This class includes organisms that are aerobic polyphosphate-accumulating organisms (Green et al., 2010). This also indicates that the MD Pellets tank may have had more aerobic areas in the sediments than the Control tank. Only the Control tank had Acidithiobacillia detected. This may indicate more acidic conditions in the sediment of the Control tank (*Acidithiobacillus thiooxidans*. (n.d.)).

Looking at the genera in the 2018 tanks, we see that the Control tanks may still have been in the hydrolysis or denitrifying phase of anaerobic metabolism. There were 1.8x more *Acetobacterium*, 1.4x more *Ignavibacteriodes,* and 1.6x more *Comamonadacea* in the Control tank. *Acetobacterium* is a homoacetogenic bacteria that can use organic acids (Balch et al., 1977; Eichler & Schink, 1984). These bacteria may contribute to formation of acetate for methanogenesis. *Ignavibacteriales* are found in environments with high nitrate (Iino et al., 2009) and can also shift from denitrification to dissimilatory nitrate reduction to ammonium (DNRA) (Grießmeier et al., 2017). *Comamonadaceae* are known denitrifiers with PHA-degrading capabilities. They use a wide variety of organic acids and very few carbohydrates (Willems et al., 1991). The higher abundance of these three genera would make it more likely that the nitrate was



depleted or near depleted, and more organic carbon substrates were becoming soluble. However, this also indicates that there was not as complete a digestion of carbon substrates as in the MD Pellets tank, since there were much less methanogens observed in the Control tank than in the MD Pellets tank.

It could have been just by chance that the MD Pellets tank started with higher *Methanoregula*, however, this does not explain why there was no *Methanoregula* present in the Control tank and about 21.3% relative abundance of this archaeon in the MD Pellets tank. The high relative abundances of methanogens such as *Methanocella*, *Methanosarcina*, and *Methanomassiliicoccus*, in addition to *Methanoregula*, indicate more complete digestion of organic carbon substrates in the MD Pellets tank. The 9.6x more *Clostridiales* in the MD Pellets tank may indicate that there were higher levels of plant decomposition and low nitrate conditions (Conrad et al., 2013; Grießmeier et al., 2017). This would also indicate that the MD Pellets tank had lower levels of organic acids remaining in the sediment. This was confirmed by lower amounts of TVS:TS in the MD Pellets tank (5.8:40) compared to the Control tank (5.8:34). The MD Pellets tank also had lower TN and TP than the Control tank, further indicating more complete digestion of organic matter. Unlike aerobic heterotrophs that have C:N:P,of 100:10:1; anaerobic heterotrophs have COD:N:P 1000:7:1 (high-strength waste) and 350:7:1 (low loadings) (Gerardi, 2003). Generally, a C:N value of 25:1 is suggested for optimal gas production (Gerardi, 2003) under anaerobic conditions. This higher carbon requirement is probably due to the low amount of energy gained though anaerobic metabolism than aerobic. The Control tank had 10.77 C:N and MDP had 12.73 C:N so the MD Pellets tank may have been further along to methanogenesis than the Control tank.

3.3 Summary

In summary, for the 2018 lab tank test, the sediment metagenomic analysis was more insightful than just the 16S rRNA analysis and showed that there is a difference in the bacterial community between the Control tank and the tank treated with MD Pellets. The differences in relative abundance of the bacteria genera may partially explain the observed sediment reduction observed in the test. There were also visual observations of reduced photosynthetic biomass levels in the tank treated with MD Pellets as compared to the Control tank.

4. Early 2019

4.1 Modifications

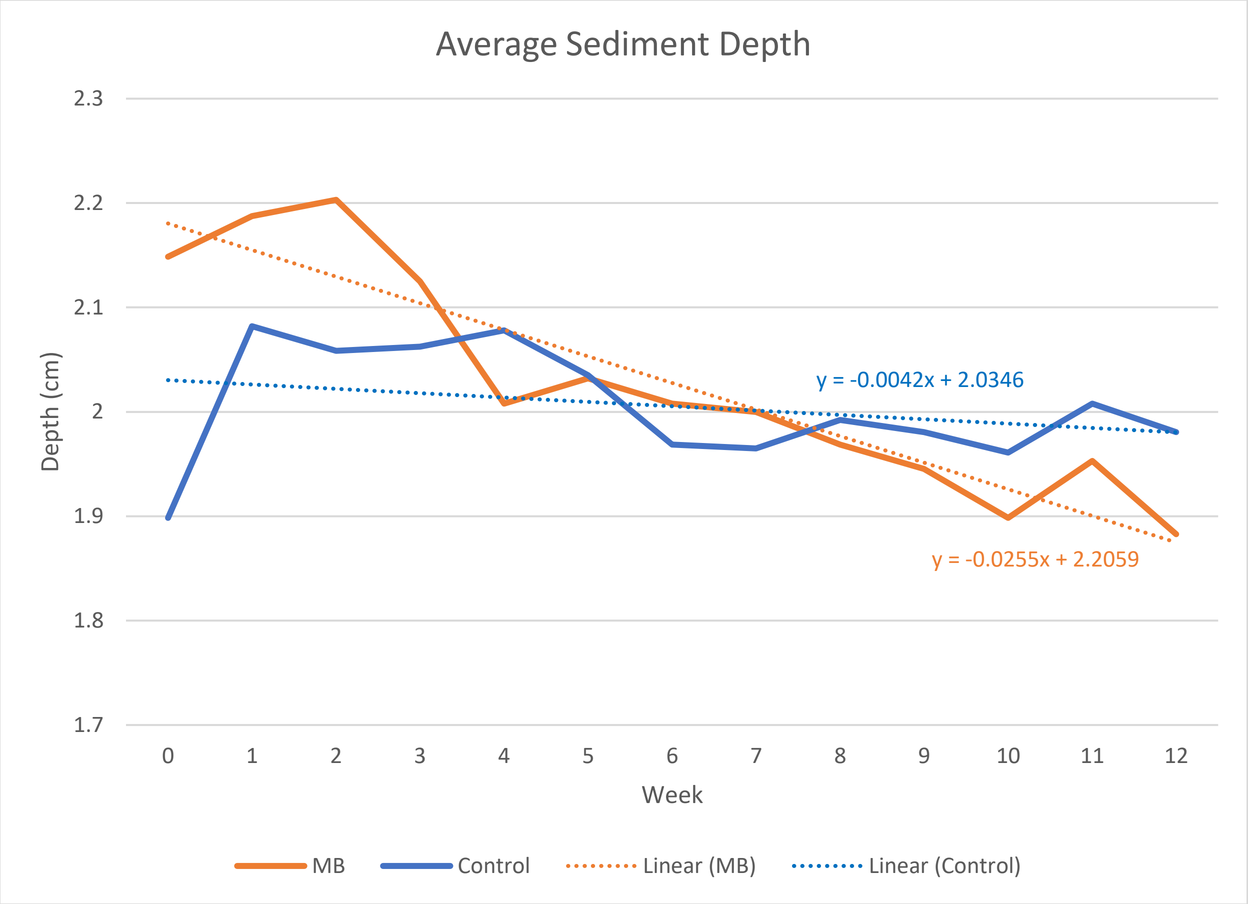
In 2019, the sediment profile was modified with the addition of sand and clay layers in order to provide a more realistic comparison to sedimentary layers found in ponds. Sand was washed with tap water before use and a 3 cm layer of sand was applied to the bottom of the tank. Sand was washed in order to remove debris and fine particulates prior to being applied within the tanks. A clay bentonite layer measuring 0.5 cm was applied on top of the sand layer. Soft pond sediments were collected from Pond 1 for all tanks and were screened in the field with a metal cooling rack with 1 cm squares to remove leaves, sticks, snails, acorns, and visible filamentous algae. An initial composite pond sediment sample was taken for analysis prior to the addition of the pond sediment into the tanks. A 500 mL sample of soft pond sediment was sent to Eurofins TestAmerica, Chicago for nutrient analysis. A 30 mL sample of soft sediment was frozen and then sent to the UWBC for DNA extraction and Illumina NovaSeq 6000 metagenomic analysis. After the collection and screening of pond sediments in the field, sediments were frozen prior to their application into the tanks. The freezing of pond sediments was intended to reduce the amount of plant and algae growth as well as the prevalence of benthic worms that were observed in previous tank studies. After the pond sediments were thawed out, a 2 cm layer of soft pond sediment was spread on top of the sand and clay bentonite layers. Due to Pond 1 being frozen during this testing period, dechlorinated tap water was used to bring the tanks water level to 15 cm above the sediments. Also, the tanks were covered on all sides with aluminum foil, making the overhead light the sole light source for each tank. The front pane of foil was removed only during weekly testing periods. A modified testing grid was constructed above the tank, totaling 32 equal-area sample sites for muck depth measurements (3-inch squares). All 32 of the sample sites were used for muck depth measurements.

After the set up was completed, the tanks were allowed to run with the aerator and pumps cycling for one month as an adjustment period for the pond system before any measurements were made or products were applied. The aquarium light was not turned on until the start of the 12-week testing period in order to limit algae and plant growth prior to our testing. Also, the pumps were now turned on for 30 minutes every 6 hours and the controller switched between coinciding pumps every 10 seconds. During the 12-week testing period, MuckBiotics was spread throughout the tanks in order to mimic a field dose rate of 12.5 lbs per acre per month. Water quality analysis now included OD580, which is the optimal wavelength for our tank's bacteria.

All other testing parameters remained consistent with the updated 2018 methods. After the 12-week testing period, a final water and soft sediment sample was taken for analysis from all tanks. A 500 mL sample of soft sediment was sent to Eurofins TestAmerica, Chicago for nutrient analysis. A 30 mL sample of soft sediment was frozen and then sent to the UWBC for DNA extraction and Illumina NovaSeq 6000 metagenomic analysis.

4.2 Results

Testing was switched from the MD Pellets to a new tablet formulation named MuckBiotics.



In the early 2019 lab tank testing, the sediment depth of the tank treated with MuckBiotics reduced much faster and ended at a lower soft sediment depth than the Control tank. In this test, the MuckBiotics were applied at a field dose of 12.5 lbs per acre per month. The sediments were frozen once, before being added to the tanks. This appeared to have killed more of the worms, plants, and algae than in the previous years. This also made it easier to measure the water depth and soft sediment depth compared to previous years. The slower growth of plants and algae may have led to increased water nutrients in both tanks during this testing period.

Test Tank Control-2 Sediment Depths (01/08/19)

1.75	2	2	2	2.25	2.5	1.5	0
1.5	0	0	2	2	2	2	2.5
1.5	2.5	1.5	2	1.5	1.75	1.5	1.75
2	3	3	2	2.25	1.75	2	2.25

Test Tank TR13-1 Sediment Depths (01/08/19)

2.25	3	2.5	2.75	2.25	3	2	2
2.5	1	2	2.75	3	1.75	2	2.5
2.5	1	0	2	2.25	0	2	2.5
2.75	2	2.75	3.25	2	2	2	2.5

Test Start

Test Tank Control-2 Sediment Depths (04/02/19)

1.75	2	2	2	2	2.25	2.25	2
2	0.5	0	2	2.5	2.5	2.75	2.25
1.75	1	1.25	2	1.75	1.75	2.25	2.5
2	2.25	2.5	2.5	2	1.75	2.25	2.5

Test Tank TR13-1 Sediment Depths (04/02/19)

2	2	3	2.5	2	2.25	2	1.75
2	1.75	1	2	3.25	2.5	2	2
2	1	0	1.25	1.75	1.75	1	2
2.25	2	2	3	1.75	0.25	2	2.25

Test End

Average sediment depth start = 1.82

Average sediment depth end = 1.961

Average sediment reduction = -7.7%

Average sediment depth start = 2.148

Average sediment depth end = 1.883

Average sediment reduction = 12.3%

The sediment depth maps of the early 2019 Control tank (above left) and MuckBiotics tank (above right) show that there was not as much redistribution of the sediments as in the previous lab tank tests. This is due to better positioning of the side powerhead pumps than before. Having less direct sediment disruption may have also led to increased soft sediment reduction.

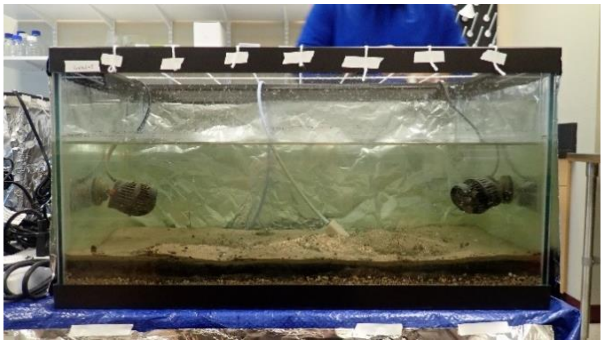
4.2a Sediment Nutrients. Adding MuckBiotics to a system lead to a decrease in the nutrient content of the sediments in the lab tanks. This was observed primarily in the early 2019 lab tank testing and also corresponded with sediment reduction. The MuckBiotics tank had higher levels of total solids than the Control tank and higher levels of total solids than either the pond or the stored muck. There were also lower levels of total volatile solids in the MuckBiotics tank than in the Control tank or the stored muck. The level of nitrogen in the sediments of tanks treated with MuckBiotics tended to decrease compared to the Control tanks. The levels of total phosphorus in the sediments also decreased below that of the Control tank in the early 2019 lab tanks test.

Sediment Early 2019		**Pond**	**Stored Muck**	**MB**	**Control**
analyte	units	5/21/2018	6/27/2018	4/3/2019	4/3/2019
Total Solids	%	36	38	51	44
Total Volatile Solids	%	9.2	9.8	5.3	6.7
Total Organic Carbon	mg/Kg	29000	30000	12000	13000
Total Nitrogen	mg/Kg	1200	1100	980	1200
Total Kjeldahl Nitrogen	mg/Kg	1200	1100	980	1200
Nitrate	mg/Kg	2.6	<1.1	<0.76	<0.89
Total Phosphorus	mg/Kg	340	370	240	400
Orthophosphate	mg/Kg	36	18	8.2	7.5
ratio TVS:TS		0.26	0.26	0.10	0.15

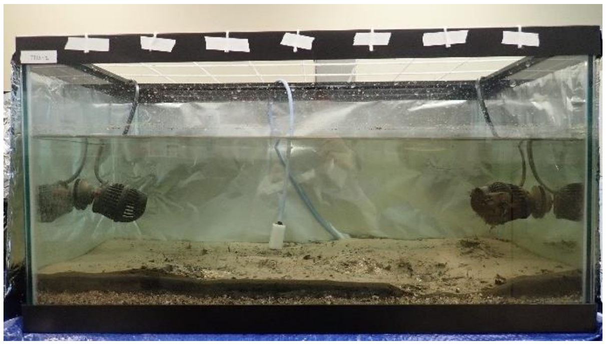
The pond sediments used in this testing had relatively low levels of total volatile solids so the lower dose of 12.5 lbs per acre per month was used for MuckBiotics rather than the 50 lbs per acre per month that was used in the previous tests with MD Pellets. In the MuckBiotics tank, the total organic nitrogen, total nitrogen, and total phosphorus all decreased. There was also an increase in total solids and a decrease in total volatile solids in this tank. This is consistent with what we have seen in field studies treated with MD Pellets (supplementary material). The ratio of total volatile solids to total solids (TVS:TS) showed that the MuckBiotics tank had fewer organic solids remaining than the Control tank.

4.2b MuckBiotics Improves Water Clarity. When MuckBiotics was applied to test tanks in the lab, or field, we saw improved water clarity. This was measured in the lab with both visual observations and absorbance of the water column using a spectrophotometer (Genesys 20, Thermo Scientific) and optical density of 580 nm (OD_{580}), which is generally where bacteria have the highest absorbance in pond water. Even when there was a slight cloudiness visible to the naked eye, the cloudiness appeared to be caused by bacteria in the tanks dosed by MuckBiotics and not due to algal cells.

Control



MuckBiotics

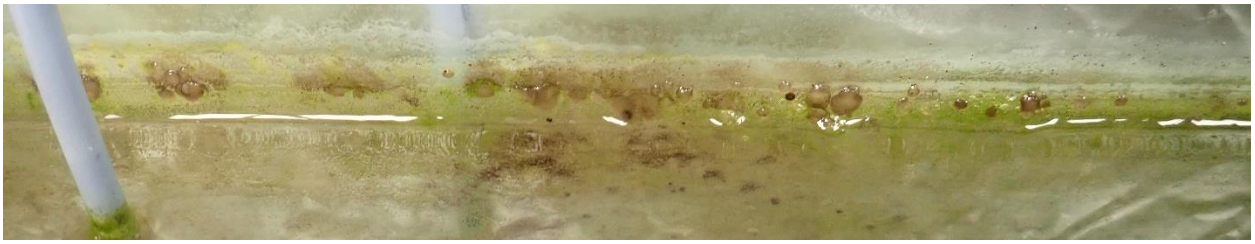


In the early part of 2019, MuckBiotics was tested in our lab tank setup (above). At the beginning of the test period (01/08/19), the tanks had clear water and no plant or algae growth after the one-month acclimatization time.

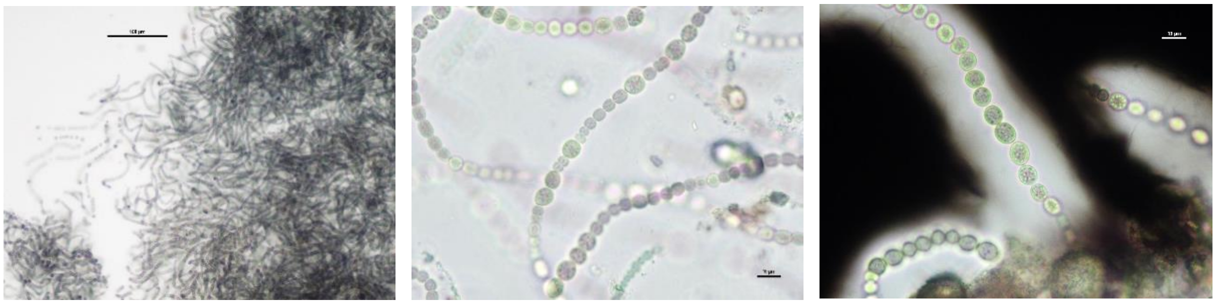
Control

MuckBiotics

At the end of the 12-week test period, for the early 2019 test, all the tanks still had very clear water. However, the Control tank had a buildup of cyanobacteria at the back of the tank by the aerator. In the lab tank testing in 2017, 2018, and early in 2019, it was observed that the Control tank had visibly more plant and green filamentous algae growth than the tanks dosed with MD Pellets or MuckBiotics. In both tanks, the filamentous green algae were *Pithophora* and *Chara*, and there were small growths of small pondweed.

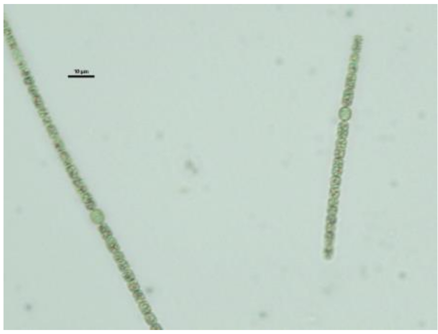


Above is a closeup of the brown spheres of cyanobacteria attached in great numbers to the back of the Control tank. These brown/green spheres were the cyanobacterium *Nostoc* (pictures below). The MuckBiotics tank did not have these spheres of *Nostoc* present. *Nostoc* is a filamentous cyanobacterium that can fix nitrogen and is known to form these macroscopic colony structures from excreted extracellular polymeric substances (picture below on right).

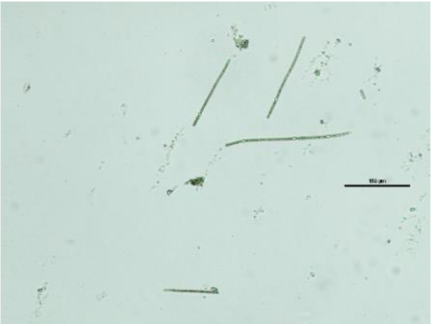


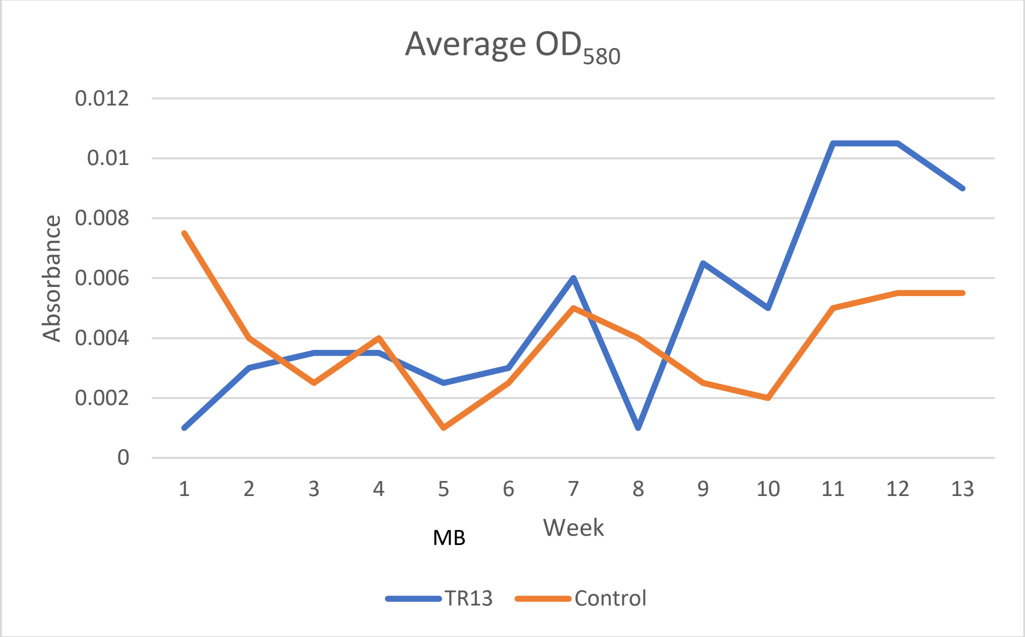
The Control tank in the early 2019 testing also had *Aphanizomenon* present along with free bacteria in the water column. There were low amounts of both *Aphanizomenon*, or other algae, and free bacteria in the MuckBiotics tank (pictures below). However, the MuckBiotics tank did have higher levels of filamentous green algae *Pithrophora*, along with *Chara* and small pondweed.

Control



MuckBiotics

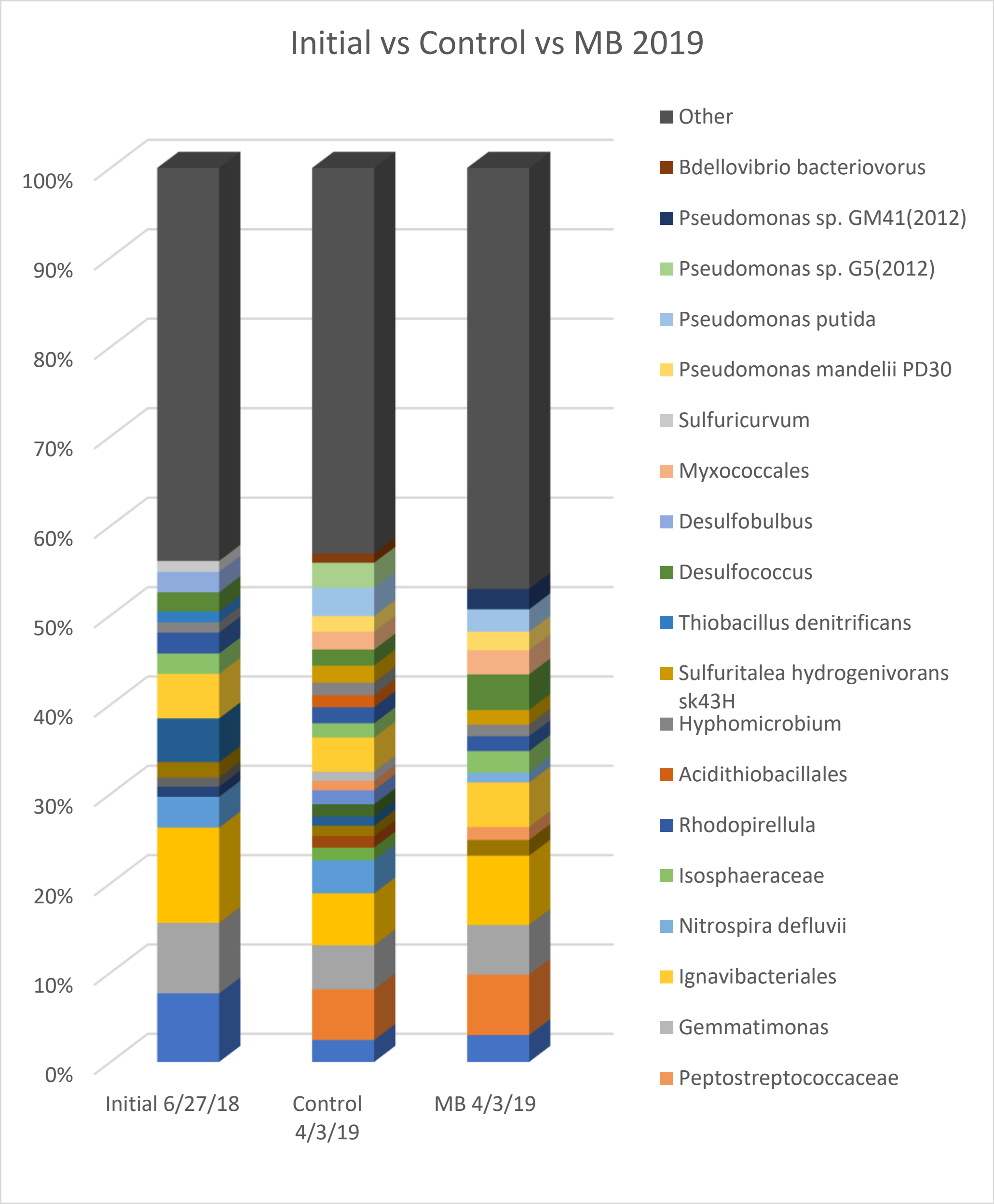




(Early 2019 OD_{580} Graph – There is a spike of bacterial growth in the MuckBiotics tank, however, the amount of bacteria present was not enough to observe any cloudiness in the water using the naked eye. Clear water can lead to the production of more zooplankton, as was observed in the MD Pellets tank in the 2017 lab testing.)

The optical density at 580 nm (OD_{580}) as a measurement of water clarity could be related to the amount of soluble COD in the water column. The Control tank in the early 2019 testing had OD_{580} values that generally go up and down throughout the test. This was also observed in the 2018 lab tank testing. This could imply there is always some amount of COD increase into the water column, either from the sediments or from the photosynthetic biomass growing in these tanks. This may imply there were some additional COD release in the tanks that may have enhanced suspended bacterial growth. It is possible that the improvement of water clarity, observed in the field with MD Pellets, is due to the formation of a biofilm over the surface of the sediments. Less sediment resuspension may result in less nutrient release into the water column and lower levels of bacterial and planktonic algal growth.

4.2c DNA Results.



Looking at the genera in the 2019 lab tanks, the Control tank and the MuckBiotics tank appeared to be very different. Both tanks were also lower in organics and reducing power than the Initial muck. Initial muck appeared to have more nitrate- and sulfate-reducing bacteria than the test tanks. This is expected as more of the organics were decomposed. This is indicated in the higher levels of *Sulfuricurvum* in the Initial muck than in the test tanks. The test tanks ended with sediments that were more anaerobic than the Initial muck. This is shown by the lower levels of

Myxococcales in the Initial compared to the test tanks. *Myxococcales* is a strict anaerobe that can form spores (Huntley et al., 2010). Spores of *Myxococcales* may have washed into the pond with runoff and then germinated within the tanks as the sediments became more anaerobic over time.

The Control tank had higher Shannon diversity than the MuckBiotics tank. This may indicate that higher diversity does not promote sediment depth reduction. This indicates that the capabilities of the microbial community may be more important in promoting sediment depth reduction, presumably through degradation of organic compounds.

The Control tank had 11.2x more *Acidithiobacillales* than the MuckBiotics tank. *Acidithiobacillales* metabolize sulfur (*Acidithiobacillus thiooxidans*. (n.d.)) and may indicate that the Control tank sediments had more reducing power remaining. The Control tanks may have still been primarily in the hydrolysis of organic matter (3.6x more *Clostridiales*) phase. There may have also been more primary producers in the Control tanks such as *Aphanizomenon*. *Aphanizomenon flos-aquae* NIES-81 was present in the Control tank at 1.6x more than the MuckBiotics tank. It is possible that in the Control tanks, excess phosphorus was used by the abundance of both green algae and cyanobacteria, whereas in the MuckBiotics tank, this was used mostly by green algae. In the sediments, however, the MuckBiotics tank had lower levels of total phosphorus and total nitrogen than the Control tank. The increased amounts of primary producers in the Control tank may hinder the breakdown of other complex carbohydrates such as cellulose or lignin, in the sediments. Primary producers may have also been adding to the buildup of sediments, as portions of the biomass die over time.

The MuckBiotics tank had more sulfur oxidizers (4.0x more *Sulfuricurvum* than Control). *Sulfuricurvum* is found in environments with high sulfur and low oxygen, also low COD (Chen et al., 2009; Krayzelova et al., 2015). This may indicate that the MuckBiotics tank had more anaerobic sediments that contributed to the breakdown of complex carbohydrates faster than the

Control tank (Krayzelova et al., 2015). The MuckBiotics tank did have lower TOC than the Control tank. It is possible that the MuckBiotics tank started out with lower amounts of organic carbon than the Control tank, leading to better compacting sediment particles that exclude more water. Although the MuckBiotics tank had more sulfate reducers and more methane producers than the Control tank, there may have been physical separation of these competing metabolisms with sulfate reducers, such as *Desulfococcus* (2x more than Control), in the upper layers or in more exposed areas of the sediment (sides of tank or dips in the sediment) (Platen, Temmes, & Schink, 1990). There was possibly faster degradation of organic matter in the MuckBiotics tank and generation of substrates for methanogens. There was 1.8x more *Acetobacterium* in the MuckBiotics tank than in the Control tank. *Acetobacterium* is a homoacetogenic bacterium that may act in nature as syntrophic partners of methanogens (Eichler & Schink, 1984). We did not observe any known common bacterial human pathogens in the 2018 and early 2019 test sediments.

4.3 Summary

The early 2019 tests used more accurate methods and showed the greatest difference in results. High amounts of muck reduction and a decrease in sediment nutrients were observed in the test compared to the Control tank. Visual observations also noted lower levels of cyanobacteria and green algae in the MuckBiotics tank versus the control. The metagenomic analysis of the sediments showed a difference in the microbial community between the MuckBiotics tank and the Control tank, indicating that the MuckBiotics tank sediment may have had more complete digestion of organic matter and that this level of digestion was completed faster than in the Control tank.

5. Late 2019

5.1 Modifications

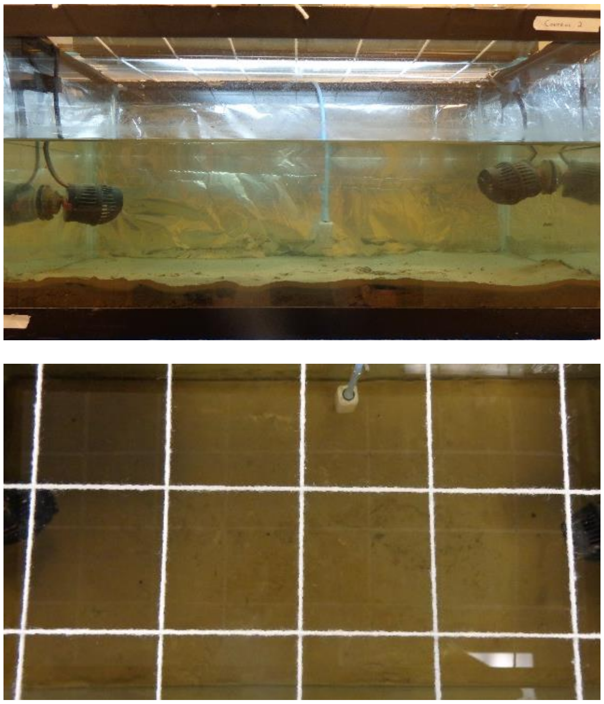
Minimal modifications were made to the methods for the late 2019 tank testing. Both the sand and clay bentonite layers were washed with water prior to being applied in layers on the bottom of the tanks. After the collection and screening of pond sediments in the field, sediments were now frozen for 2 days, allowed to thaw, and then frozen again for another 2 days prior to thawing out and being spread on top of the sand and clay bentonite layers. About equal mass of pond sediments were added in order to produce a 2 cm layer. Sediments were allowed to dry and stabilize on the bottom of the tanks for a week prior to the addition of Pond 1 water. Pond 1 water was added to the tanks weekly to maintain a water level 15 cm above the sediments. Weekly water quality samples were now sent to Eurofins TestAmerica, Chicago for general water quality analysis. All other testing parameters remained consistent with the updated early 2019 methods. A complete and comprehensive description of the late 2019 methods is provided in the appendix.

5.2 Results

Week 0 lab tanks – 07/09/19

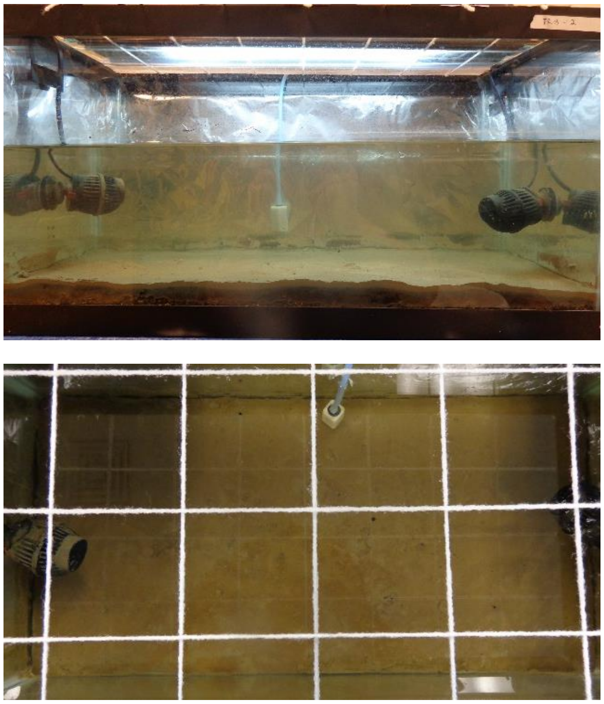
Control 1

Control 2



MB-1

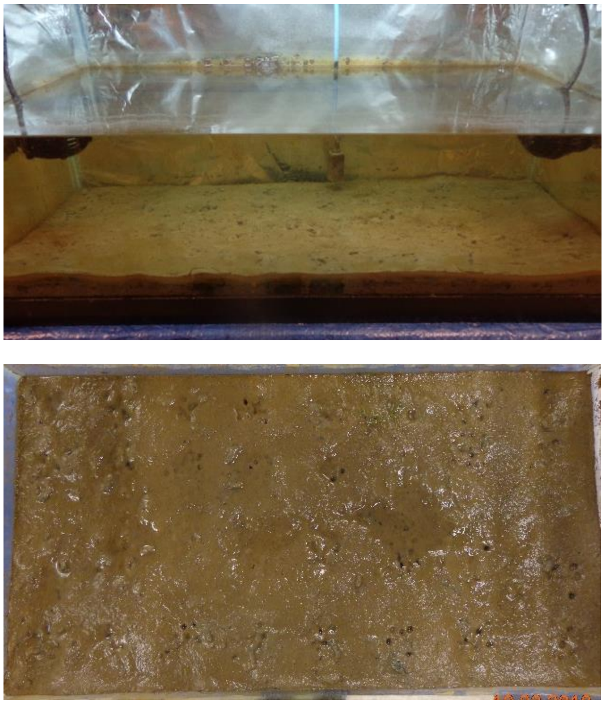
MB-2



Week 12 lab tanks – 10/01/19

Control 1

Control 2



MB-1

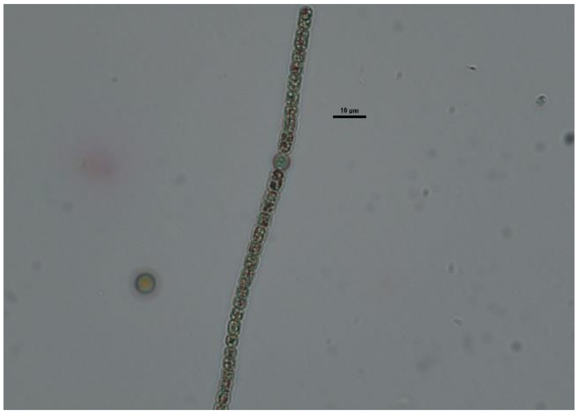
MB-2

All of the tanks started off with clear water and very low to no growth of rooted plants or filamentous algae. The tanks in this test did not develop any of the divots in the sediment that were present throughout the previous lab tank tests. By the end of the test period, all of the tanks still had mostly clear water. MB-1 was the only tank to develop somewhat cloudy water at the end of the test, however, the level of bacteria was not high enough to be noticeable in a pond or lake. The lower levels of plant/algae growth compared to previous years made it easier to measure water and sediment depth.

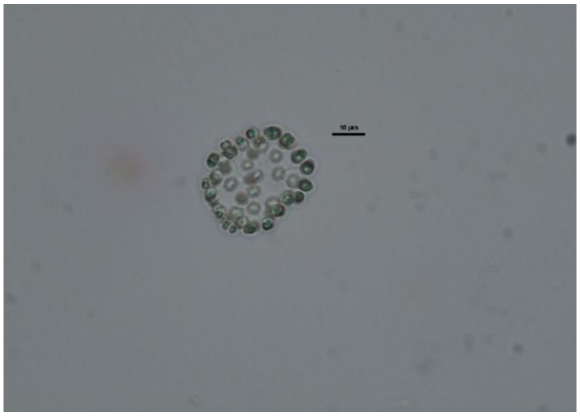
At the end of this lab test the sediment texture in both Control tanks were similar and the sediment texture in both MuckBiotics tanks were similar. The MuckBiotics tanks sediment had a semiliquid brown top aerobic layer of muck, which was collected for analysis, and the bottom layer was firmer and resisted being pressed down on. The sediment in both Control tanks was semiliquid and puddling-like, more so than the aerobic layer in the MuckBiotics tanks. The Control tanks aerobic layer was darker brown than the aerobic layer in the MuckBiotics tanks and the remaining layers were softer and squishier than the remaining layers of the MuckBiotics tanks.

Microscopic

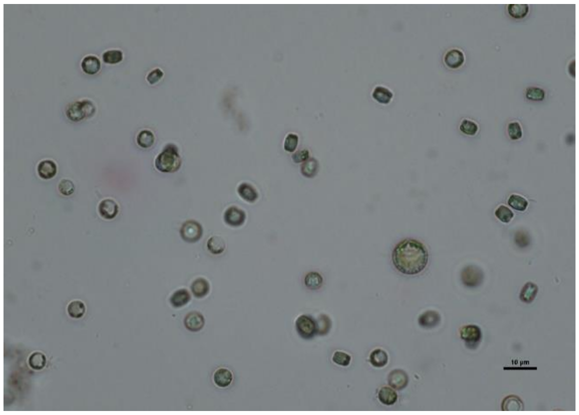
Control 1



Control 2

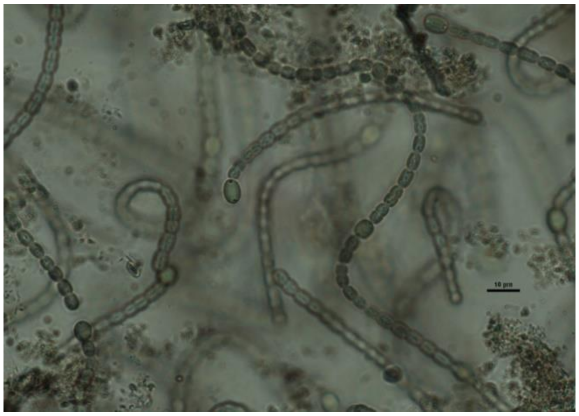


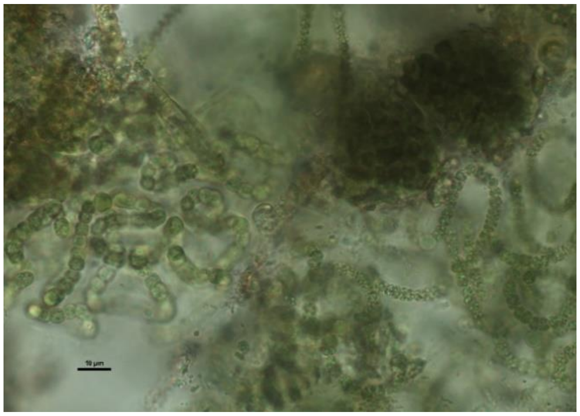
MB-1



MB-2

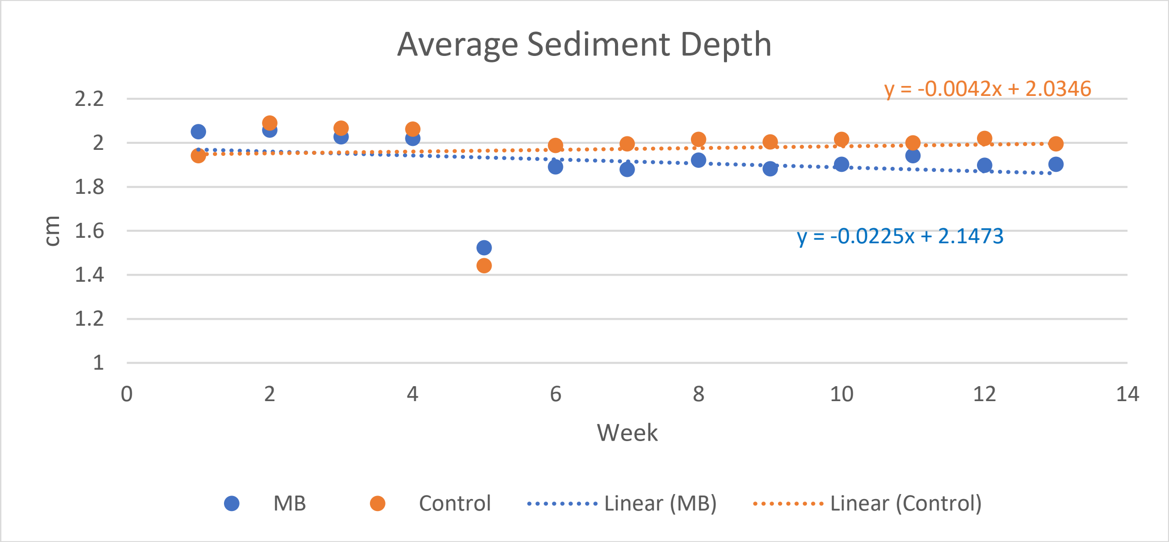
Aphanizomenon (Control 1 and MB-2) was present in the water of all tanks along with free bacteria. There may have been less of this cyanobacterium than in previous lab tank tests. There were more green algae and diatoms present than in previous lab tank tests. *Dictyosphaerium* (Control 2) was present in all of the tanks. The MuckBiotics tanks may have had higher levels of small diatoms (MB-1) than the Control tanks, although they were observed in all tanks. All of the tanks had lower levels of *Pithophora*, *Chara,* and rooted plants than in previous lab tank testing. This may have been due to freezing the sediments twice instead of just once before addition to the test tanks. *Pithophora* was observed in all tanks, mostly attached to the side pumps. This may have been because the pumps were reused from the early 2019 lab tank tests, which had high levels of *Pithophora* growth. There were visually lower levels of *Pithophora*, *Chara.* and rooted plants in the MuckBiotics tanks than in the Control tanks.





Both Control tanks again ended with these brown/green spheres of *Nostoc* at the water surface growing above the air stone. This attached cyanobacterial growth was not observed in either MuckBiotics tanks. *Nostoc* is a filamentous cyanobacterium that can fix nitrogen and is known to form these macroscopic colony structures from excreted extracellular polymeric substances.

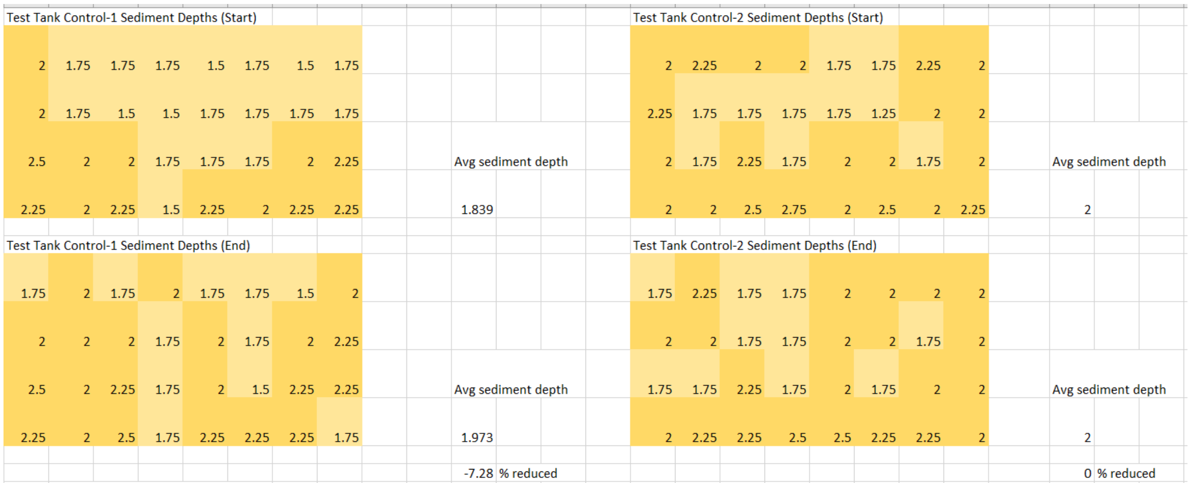
5.2a Sediment Depth.



Every month, the MuckBiotics tanks were dosed with tablets using a field dose of 12.5 lbs per acre. The MuckBiotics tanks had consistently decreasing sediment depths throughout the test. The downward trend was faster than that of the Control tanks. Killing off more of the macro life in the sediments led to more consistent sediment decrease than with unfrozen sediments used in 2017 or once frozen sediments used in early 2019. Freezing the sediments appeared to decrease the levels of benthic worms observed in previous testing. Freezing the sediments also tended to slow plant and macro algae growths. This also led to easier measurement of water depth and total depth, which included soft sediment depth. The slower growth of plants and algae should contribute to increased water nutrients in all of the tanks over that of previous lab tank tests.

Control 1

Control 2



Test Tank Control-1 Sediment Depths (Start)

2	1.75	1.75	1.75	1.5	1.75	1.5	1.75		
2	1.75	1.5	1.5	1.75	1.75	1.75	1.75		
2.5	2	2	1.75	1.75	1.75	2	2.25	Avg sediment depth	
2.25	2	2.25	1.5	2.25	2	2.25	2.25	1.839	

Test Tank Control-1 Sediment Depths (End)

1.75	2	1.75	2	1.75	1.75	1.5	2		
2	2	2	1.75	2	1.75	2	2.25		
2.5	2	2.25	1.75	2	1.5	2.25	2.25	Avg sediment depth	
2.25	2	2.5	1.75	2.25	2.25	2.25	1.75	1.973	
								-7.28	% reduced

Test Tank Control-2 Sediment Depths (Start)

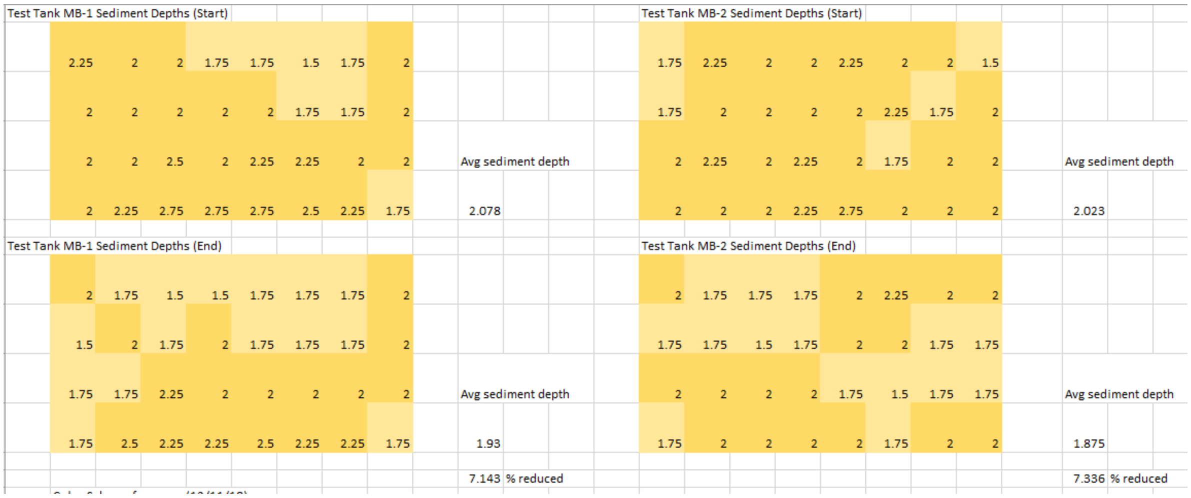
2	2.25	2	2	1.75	1.75	2.25	2		
2.25	1.75	1.75	1.75	1.75	1.25	2	2		
2	1.75	2.25	1.75	2	2	1.75	2	Avg sediment depth	
2	2	2.5	2.75	2	2.5	2	2.25	2	

Test Tank Control-2 Sediment Depths (End)

1.75	2.25	1.75	1.75	2	2	2	2		
2	2	1.75	1.75	2	2	1.75	2		
1.75	1.75	2.25	1.75	2	1.75	2	2	Avg sediment depth	
2	2.25	2.25	2.5	2.5	2.25	2.25	2	2	
								0	% reduced

M-1

MB-2



Test Tank MB-1 Sediment Depths (Start)

2.25	2	2	1.75	1.75	1.5	1.75	2
2	2	2	2	2	1.75	1.75	2
2	2	2.5	2	2.25	2.25	2	2
2	2.25	2.75	2.75	2.75	2.5	2.25	1.75

Avg sediment depth 2.078

Test Tank MB-1 Sediment Depths (End)

2	1.75	1.5	1.5	1.75	1.75	1.75	2
1.5	2	1.75	2	1.75	1.75	1.75	2
1.75	1.75	2.25	2	2	2	2	2
1.75	2.5	2.25	2.25	2.5	2.25	2.25	1.75

Avg sediment depth 1.93

7.143 % reduced

Test Tank MB-2 Sediment Depths (Start)

1.75	2.25	2	2	2.25	2	2	1.5
1.75	2	2	2	2	2.25	1.75	2
2	2.25	2	2.25	2	1.75	2	2
2	2	2	2.25	2.75	2	2	2

Avg sediment depth 2.023

Test Tank MB-2 Sediment Depths (End)

2	1.75	1.75	1.75	2	2.25	2	2
1.75	1.75	1.5	1.75	2	2	1.75	1.75
2	2	2	2	1.75	1.5	1.75	1.75
1.75	2	2	2	2	1.75	2	2

Avg sediment depth 1.875

7.336 % reduced

The sediment depth maps (above) show that there was not much redistribution of the sediments as in previous tank tests. This is due to better positioning of the side powerhead pumps and taping them in place. Less sediment resuspension due to the pumps in the tanks could have led to less nutrient release into the water column. Interestingly, Control 1 had a 7.3% increase in average sediment depth from the beginning to the end of the test, while the average sediment depth in Control 2 had minimal change from the beginning to the end of the test. MB-1 and MB-2 both had an average reduction of about 7% from beginning to end of the test. This was similar to the average sediment reduction observed in test Pond 1.

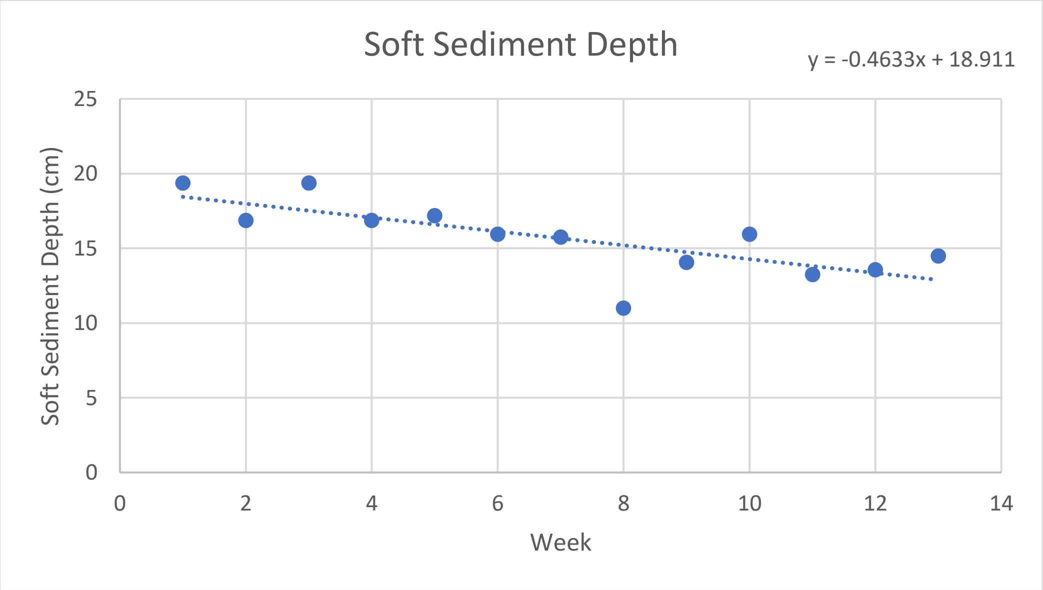
5.4 Case Study: Pond 1 Summer 2019

Treatment was started in June of 2019 at Pond 1, the same pond that acted as the source for soft sediment and pond water for tank testing 2017–2019. Testing was conducted with a similar procedure as in the laboratory tank testing in order to act as a field comparison. Pond 1 was approximately 0.15 acres in size. An initial composite sediment sample was taken using an Ekman dredge prior to the start of the testing period. MuckBiotics was applied at a 12.5 lbs per acre dose rate at week 0, week 4, and week 8 during the treatment period.

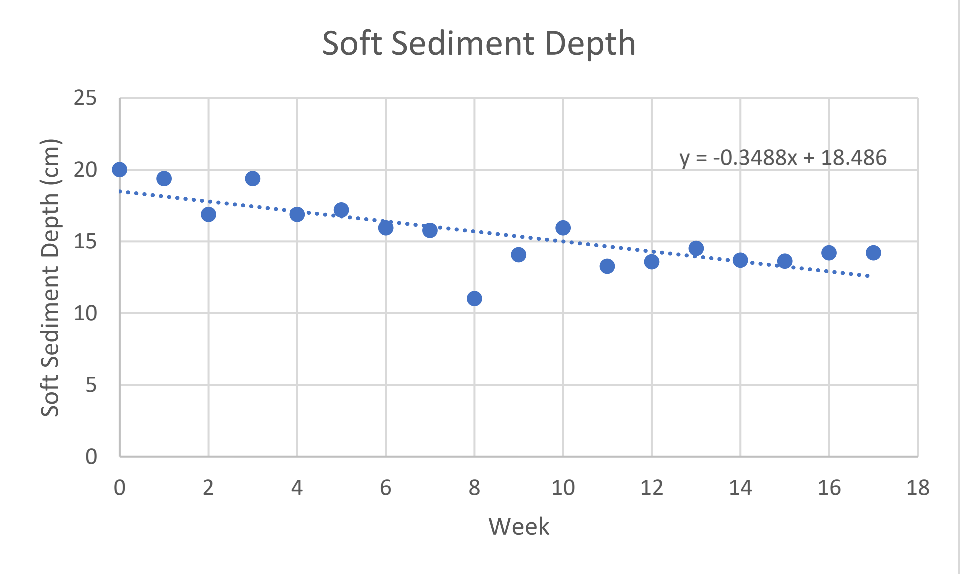
Measurements and observations were taken weekly for a 12-week treatment period. Water quality was measured from a one-foot depth at the center of the pond and all water quality analysis was conducted either at the Naturalake Biosciences' lab or Eurofins TestAmerica, Chicago. Water quality measurement parameters included OD580, which is the optimal wavelength for our tank's bacteria, general water quality, pH, DO, and temperature. The Hach HQ40D portable meter, along with LDO101 and PHC101 field probes, were used to measure pH, DO, and temperature. Eight representative sample sites were distributed throughout the pond for sediment depth measurements. The eight sites were measured for water depth as well as a total depth for water and soft sediments. When present, algae samples were taken from the surface of the water. A final composite sediment sample was taken using an Ekman dredge at the end of the 12-week testing period. All sediment samples were analyzed by Eurofins TestAmerica, Chicago.

5.5 Results

5.5a Pond 1 Soft Sediment Depth.



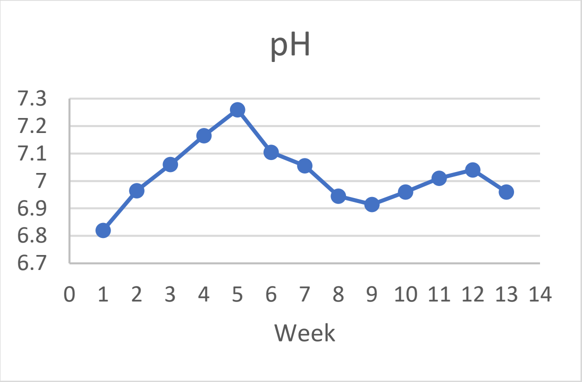
The soft sediment depths of Pond 1 during the 12 weeks of treatment show that there was a noticable decline in soft sediment depth during treatment with MuckBiotics. Treatments were applied on weeks 1, 5, and 9. The average soft sediment depth went from 20 cm on week 0 to 13.6 cm on week 13. This is about a 30% reduction in average depth of soft sediments in this pond over 3 treatment months with MuckBiotics.



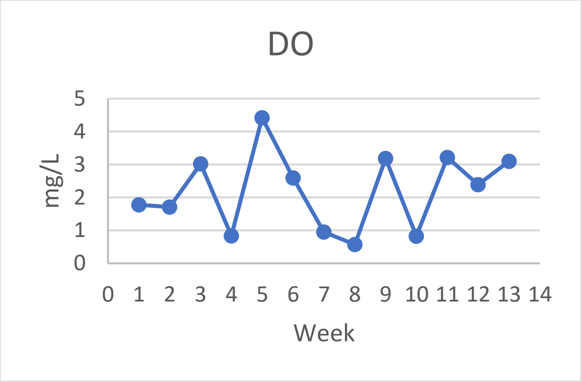
Pond 1 was monitored for an additional 4 weeks after treatment ended (weeks 14–17). During these 4 weeks the pond sediments did not continue to decrease, but also did not increase. The sediment depth appeared to hold steady during the 4 weeks without additional MuckBiotics treatments.

5.5b Water Quality.

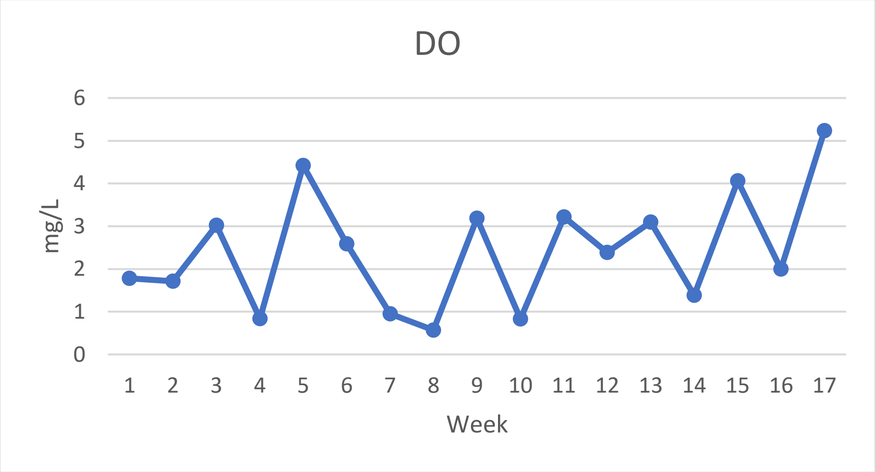
Pond pH



Pond DO



The pH of the pond water ranged from pH 6.8 to pH 7.3 over the course of the test period. This is pretty typical for the pH of most ponds. The pond water dissolved oxygen (DO) ranged from <1 mg/L to 4.3 mg/L and tended to flucuate between less than 1 mg/L and 3 or 4 mg/L over the test time. This is not that typical for a healthy pond. Due to the physical parameters of this pond (small surface area and shallow depth) it is possible that the DO levels were highly influenced by rainfall events and runoff from the surrounding area. The flux in DO levels continued during weeks 14 to 17, which was after the testing period.

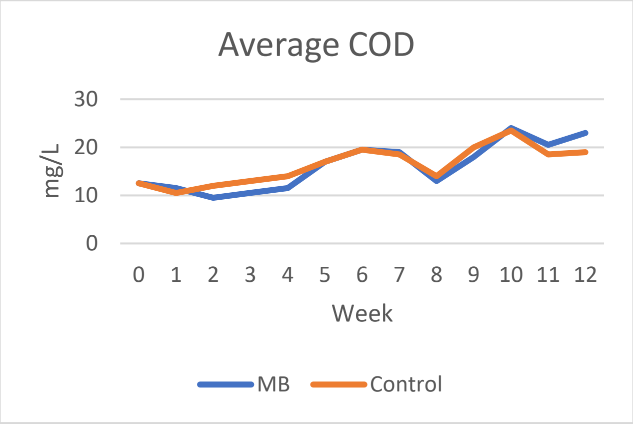


The DO in this pond seems to be cyclic between very low one week (1–2 mg/L) and higher the next week (3–4 mg/L). It does not appear that treatment with MuckBiotics affected this.

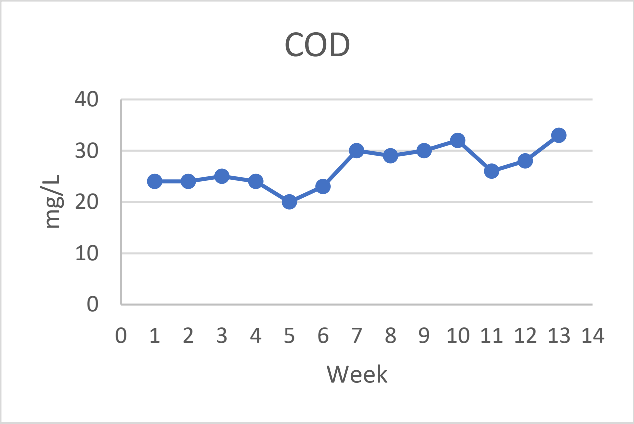
The lab tank’s pH and DO were pretty consistant throughout the test. The tank pH varied between pH 7.7 and pH 8.4 while tank DO varied between 8 mg/L and 9 mg/L.

The pond temperature varied from 20°C to 26°C for the entire observation time. In weeks 2 to 7 when most of the treatment with MuckBiotics occurred, the temperature was around 25°C. The temperature of the lab tanks was very consistent between 20 to 21°C for the entire test time.

Tank COD

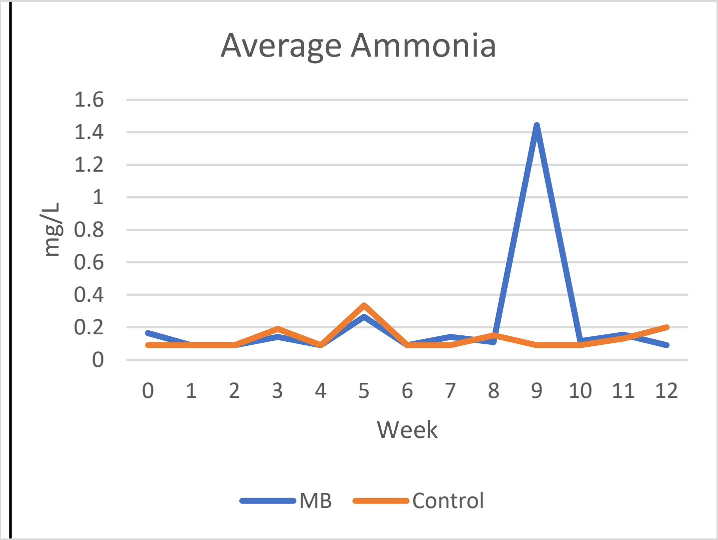


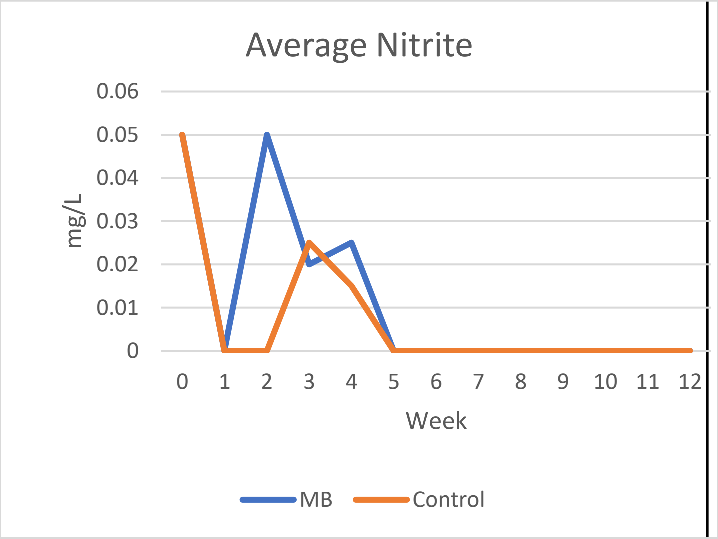
Pond COD

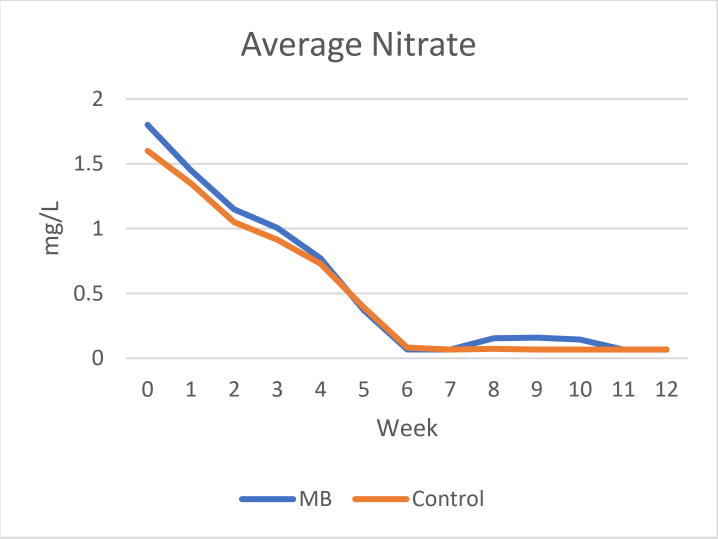


The lab tanks all had COD water levels that remained pretty low throughout the testing. The pond also had soluble COD levels that remained low, although the values were all a little higher than those of the lab tanks.

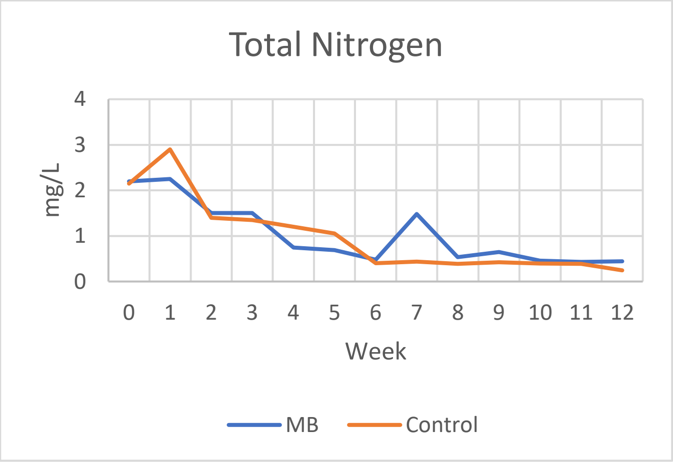
5.5c Tank Nitrogen.

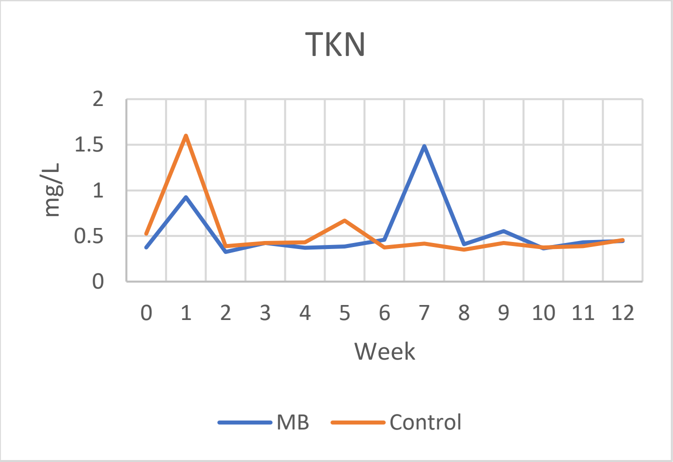






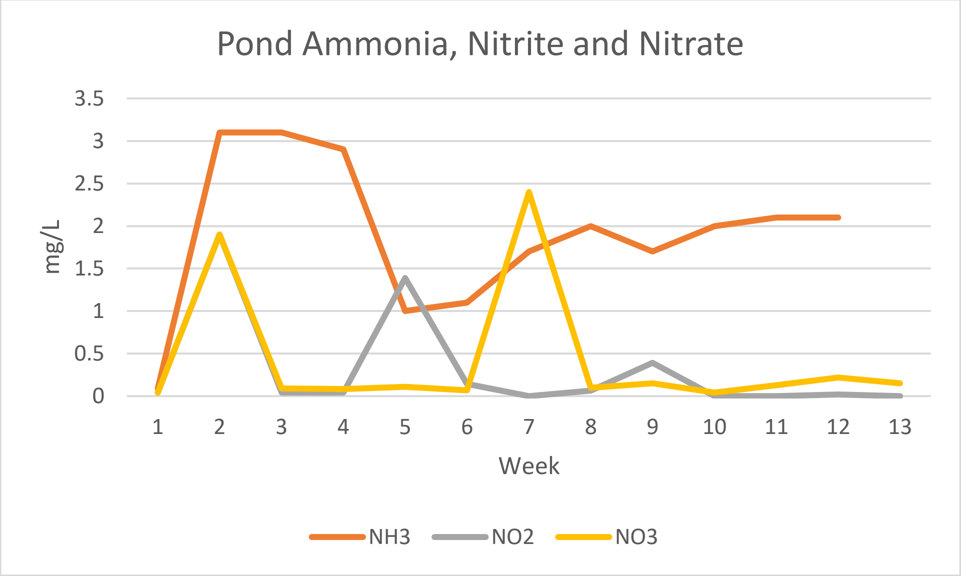
The tank MB-2 had a spike of ammonia on week 9. The levels of nitrite and nitrate were initially a little high in all of the tanks, possibly from sediment denitrification, and then at near zero from week 6 on for all of the tanks.



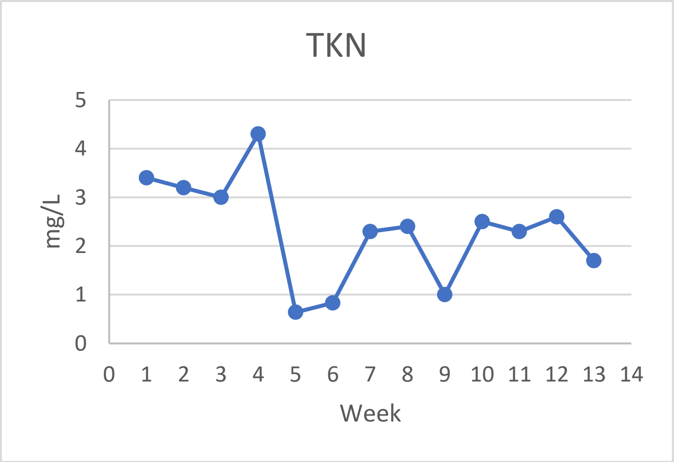


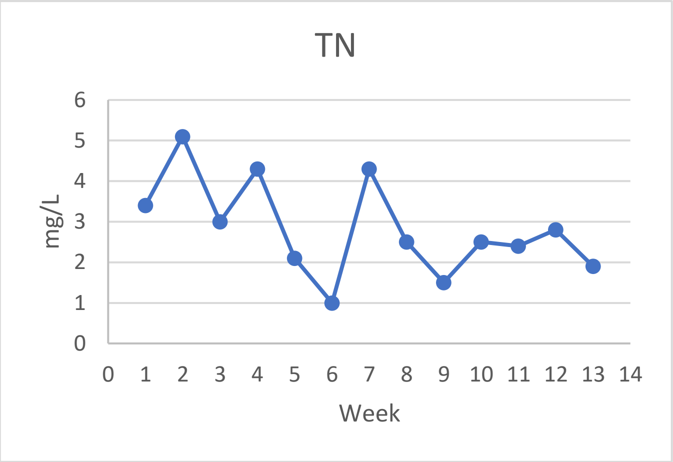
The tank total nitrogen (TN) mostly decreased through the entire test. The total Kjehdal nitrogen (TKN) in the tanks mostly responded to inorganic nitrogen, however, the week 7 spike may have been due to proteins.

5.5d Pond Nitrogen.



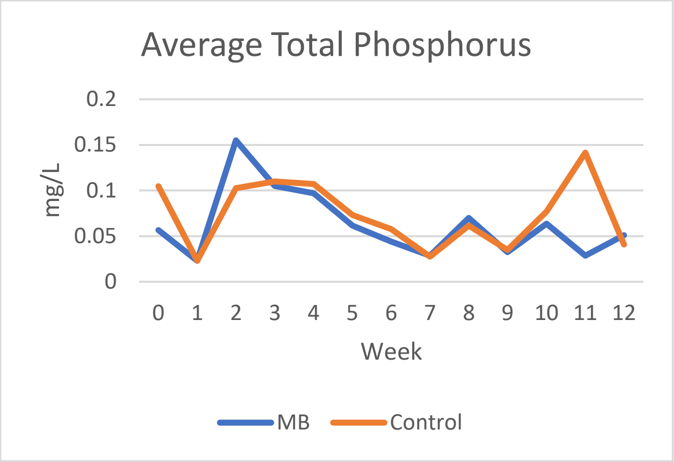
The pond water had a drop in ammonia and nitrite and the rise of nitrate in weeks 5–7 is consistent with nitrification. The low nitrate and nitrite with increasing ammonia may correlate with increasing COD at this time. The increasing COD may correlate with DO flux and pH.

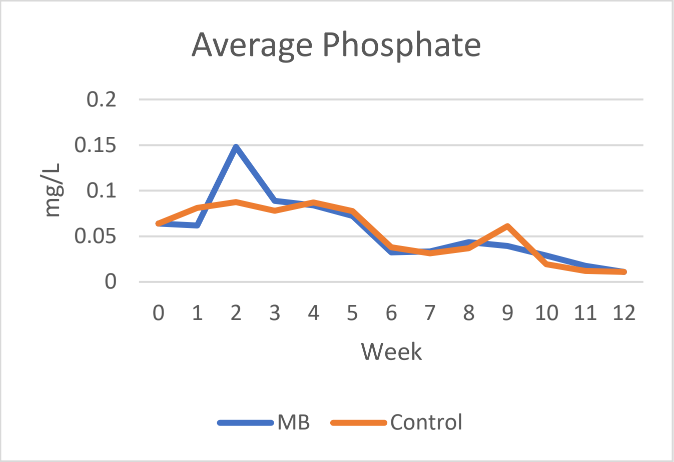




There may have been more inorganic nitrogen in the pond during the starting weeks (week 0–6), then more protein later on, since the total nitrogen and TKN look about same from week 6 on.

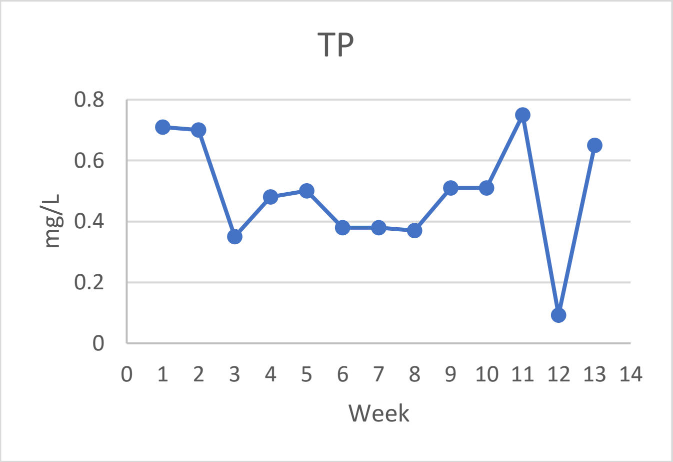
5.5e Tank Phosphorous.

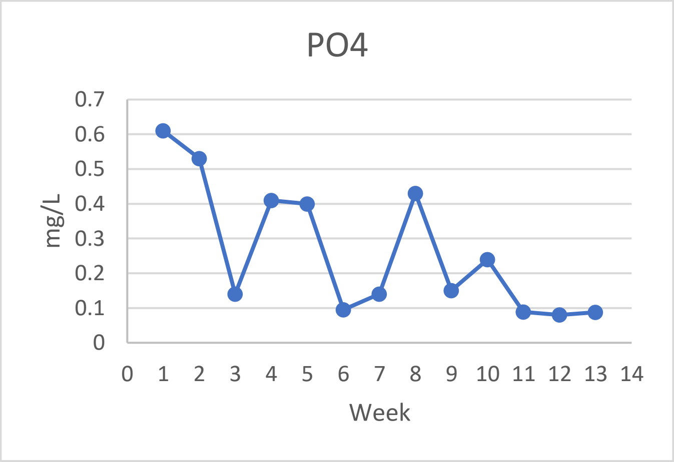




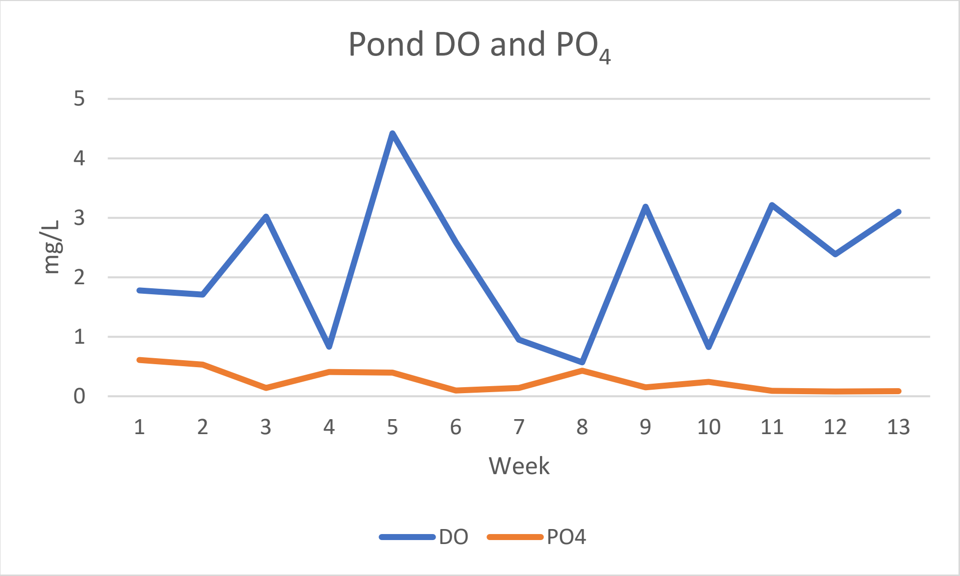
The tank phosphate mostly decreased throughout the test period. The tank's total phosphorus (TP) did not decrease as evenly, but did more or less decrease over the test period. There was a TP spike in Control 2 at week 10 and 11 for unknown reasons. Control 1 had a small spike in orthophosphorus (OP) at week 9 for unknown reasons. All tanks except Control 2 had a small spike in TP at week 8. There was no obvious reason for these spikes based on the data collected.

5.5f Pond Phosphorous.





The pond initially had TP and PO_4 relatively similar to each other, however, by week 8, the pond phosphate began to shift away from the TP pattern.



DO may be the controlling parameter for water PO_4 levels or PO_4 release from sediments in this pond.

5.5g Sediment Nutrients.

Analyte	Pond 5/22/19	Frozen Muck 6/3/19	MB Avg. 10/2/19	Control Avg. 10/2/19	Pond 6/17/19	Pond 9/16/19	Pond 10/14/19
Total Solids	37	51	37	39	31	29	24
Total Volatile Solids	7.1	6.7	5.3	6.05	8.8	8.5	9.4
Total Organic Carbon	25,000	17,000	21,500	24,000	37,000	12	40,000
Total Nitrogen	1,400	1,600	935	1,250	1,400	2,100	1,900
Total Kjeldahl Nitrogen	1,400	1,600	935	1,250	1,400	2,100	1,900
Nitrate	<1.1	<0.78	1.8	1.7	<1.3	<2.3	<2.7
Total Phosphorus	170	150	455	390	130	390	440
Orthophosphate	7.8	12	6.65	9.5	6.1	<3.6	<22
Aluminum	19,000	15,000	15,500	14,500	16,000	16,000	22,000
Calcium	5,600	4,800	4,650	4,250	4,000	3,500	5,100
Copper	40	31	33.5	30	28	33	42
Iron	20,000	16,000	19,000	16,500	15,000	16,000	22,000
Magnesium	3,900	3,300	3,900	3,550	3,600	3,700	5,100
Sulfate			45	59			200
pH			7.2	7.35			6.5

Freezing the pond sediments twice before adding to the test tanks caused an increase in the sediment total solids. However, the lab tanks total solids all decreased below this point (51%) to closer to the initial pond muck total solids (37%). The pond at the end of the season lost solids after the treatment stopped. In early 2019, the lab tanks from that test time started with total solids at about 37%, but all tanks except MB-2 ended with total solids that were higher than the initial. The early 2019 MB-2 tank ended with total solids that were similar to the start.

The tank total volatile solids (TVS) in the frozen muck decreased slightly and continued to slightly decrease by the end of the tank test. In the pond, the TVS increased slightly by the start of MuckBiotics applications, but held steady until the end of applications and then increased slightly by October. In the early 2019 tanks, the TVS decreased over the test for both the MuckBiotics and Control tanks. The tank MB-1 had much less TVS while MB-2 had TVS similar to the starting levels. Tank MB-2 did not perform as well as MB-1 in sediment reduction or in nutrient profile.

The total organic carbon (TOC) content decreased in the frozen muck compared to the initial muck from the pond. The TOC in the pond seemed to increase over the course of the season. Some time in 2019 Test America changed their TOC analysis method due to the closing of their Nashville lab. A new method that was more comparable to the old method will be used from now on. The pond on the September sample time, unfortunately, used the leachate method, which is very different from the method formerly used. Between the MB tanks and Control tanks at the end of the test, it appears that TOC decreased in the MB tanks to roughly half the level left in the Control tanks. In the early 2019 tanks, the TOC stayed about the same for MB-2, but decreased for all of the other tanks. TOC decreased the most in MB-1.

The total nitrogen (TN) and total Kjeldahl nitrogen (TKN) were equal for all sediment samples. TN increased in the frozen muck but then decreased at the end of the tank tests. The MuckBiotics tanks reduced more TN than the Control tanks (especially MB-2). This is consistent with what we have seen with previous lab tanks testing with MuckBiotics and also MD Pellets. In the pond, the TN increased during the treatment with MuckBiotics. It is unknown if the TN generally increases at the end of summer in this pond. In the early 2019 lab tank tests, the TN and TKN were equal for all sediments. The TN remained about the same as the start in both Control tanks. TN increased in MB-2 while decreasing below starting levels in MB-1.

The total phosphorus (TP) levels decreased in the muck with freezing. In the pond, the TP levels gradually increased over the season and ended at levels much higher than the beginning. This sometimes happens in ponds with algae blooms or high levels of aquatic plants or leaves that die and sink by fall. The tanks also saw an increase in TP by the end of the test. The levels were about the same as what the pond ended at. The MuckBiotics tanks ended with higher levels of TP than the Control tanks, but had lower levels of phosphate. In the early 2019 lab tanks tests, the TP remained about the same as the start in all tanks, except MB-1, where it decreased by the end of the testing. The levels of metals didn’t really change in either the lab tanks testing or in the pond over the course of the test.

For the first time, we looked at sulfate levels as a way to gauge if sulfate reduction may have taken place. It is possible that there was more sulfate reduction in the MuckBiotics tanks than in the Control tanks, and more sulfate reduction in the tanks than in the pond. The pH of the pond tanks sediments remained around pH 7, while the pond sediments were at pH 6.5. The pond may be starting to have sulfate reduction, or may have had more fermentation and other acid generation within the sediments by October.

The ratio of total volatile solids to total solids (TVS:TS) decreased in the frozen muck and remained about the same throughout the lab test (around 0.16). In the pond, TVS:TS started off low (0.19) and then increased until October (0.39). The early 2019 lab tanks test saw the TVS:TS ratio decrease over the course of the test, mostly by decreasing TVS for most of the tanks. The total organic carbon to total nitrogen ratio decreased in the frozen muck and increased in the pond. The ratio of TOC:TN increased in all of the tanks over the course of testing, however, it increased more in the MB tanks than in the Control tanks. In the early 2019 lab tanks test, there was a decrease in the TOC:TN ratio for most of the tanks. The total Kjeldahl nitrogen to total phosphorus (TKN:TP) ratio increased in the frozen muck and then decreased dramatically in the tanks to levels lower than intial, probably due to increase in TP, by the end of the test. In the pond, the TKN:TP ratio increased at the start of the treatment, then decreased by the end of the treatment (also by increasing TP) and decreased further by October (by further increase in TP). The lab tank tests in early 2019 saw no real change in the ratio of TKN:TP over the course of the test.

5.6 Conclusions

The lab tanks show sediment reduction and, for the first time, a lack of phosphate increase in the tank water. This lab experimental setup is in its final form and can be used to explore other issues in ponds. The lab tanks were similar to what was observed in the pond, aside from the DO flux and wider temperature range observed in the pond. The pond also experienced sediment reduction with MuckBiotics, however, the scum of algae remained present and the water did not gain clarity as is generally reported with MD Pellets in the field.

6. Discussion

6.1 Using MuckBiotics Increases Water Clarity

In the field and laboratory testing, consistent improvements in water clarity were observed when MD Pellets or MuckBiotics were applied. Increased water clarity provides benefits to water bodies in terms of use and health. Clear water promotes better aesthetics for homes around the pond, it allows fishermen to see the fish, is more appealing for people to swim in, and generally has lower odors than more turbid waters. Clear water may help promote zooplankton, which can increase fish productivity for a water body, and also reduce the chances of algal blooms, including harmful algal blooms (HABs). Improved light penetration into the water column also enhances submerged aquatic plant growth that can increase phosphorus uptake and increase oxygen at the sediment surface (Søndergaard, Jensen, & Jeppesen, 2003). Increased water clarity also allows for UV light to reach a greater depth than in turbid water, allowing for more disinfecting of pathogenic microbes. This allows for safer water and is especially a concern in drinking water reservoirs.

It is possible that addition of MD Pellets or MuckBiotics promoted higher growth of bacteria over that of algae. This was supported in the tank tests where in every year, the Control tanks had visibly higher levels of free cyanobacteria in the tank water compared to the tanks treated with MD Pellets or MuckBiotics (See 2017 8x higher Chloroplast in Control, 2017 tank pictures, 2019 Control tank pictures).

In the lab tank test conducted in early 2019, the tanks treated with MuckBiotics mostly only had bacteria in the tank water. Bacteria may cause a bloom; however, bacteria can also form flocs once the nutrients have been depleted and these flocs will sink out of the water column resulting in clear water. The treated tanks also had visibly lower levels of the filamentous green alga *Pithophora*, along with *Chara*, and small pondweed. It is known that small benthic invertebrates in pond sediments may consume some algal cells or resting structures, and the activity of these organisms may have contributed to the lower plant and algal biomass observed in tanks treated with MD Pellets or MuckBiotics.

6.2 Using MuckBiotics Decreases Water Column Nutrients

When MD Pellets or MuckBiotics have been used in the field, we received anecdotal reports of lowered water column nutrients along with improved water clarity. Lower water column nutrients are desirable in lakes and ponds since it limits both bacterial and algal blooms that cause high turbidity and may be harmful to humans and aquatic life. Low water column nutrients and increased water clarity can also promote submerged plant growth and may limit the amount of floating plants, such as duckweed, watermeal, and water hyacinth, just to name a few, that can impede human use of ponds and other waterways. Zooplankton such as rotifers, cyclops, and daphnia, as well as filter feeders such as stalked ciliates, also help clear the water of bacteria and algal cells. Lower water column nutrients and subsequent microbial respiration can lead to increased dissolved oxygen (DO) levels in a pond. This may also help to promote more zooplankton and macrofauna diversity that help to clear the water and cycle incoming nutrients.

In the lab tank testing, it was observed that a biofilm formed over the sediments, stabilizing the particles from resuspension (Fang et al., 2015) when the pumps were on. This biofilm was generally more obvious in the tanks treated with MD Pellets or MuckBiotics than in the Control tanks, although the biofilm may have been slower to develop. The resuspension of sediments into the water column can release nutrients and metals into the overlying water (Cheng et al., 2016). Biofilms can alter sediment properties through altered size, morphology, density, and stability (Fang et al., 2015) and can bind fine-grained sediment, thus changing the sediment surface to become more resistant to shear stress and lower erosion (Fang et al., 2017). Even if the community structure of a biofilm and its morphology are different in various environments, the viscoelastic properties are consistent and the method by which biofilms stabilize the sediments is the same (Fang et al., 2017). Biofilms generally initiate in an area of low shear stress and then spread towards high shear stress areas (Thomen et al., 2017). MD Pellets or MuckBiotics may stimulate the natural biofilm formation in the tanks and in the field, which is generally not under static conditions, and limit the transfer or release of sediment nutrients into the water column (Cheng et al., 2016).

6.3 Using MuckBiotics Decreases Sediment Nutrients

An increase in total solids in the sediment samples was observed after MD Pellet or MuckBiotic use, both in the lab tank testing and also in field sites, and comparatively, a decrease in sediment nutrients: total volatile solids (TVS), total nitrogen (TN) or total Kjeldahl nitrogen (TKN), total phosphorus (TP), and orthophosphorus (OP). Lower sediment nutrients decrease the risk of nutrients being released from the sediments into the overlying water, mainly due to sudden high flows, turbulence from storms, or other activities that lead to sediment disturbance (Reddy et al., 1999). This lowers the amount of algal blooms that would feed from these released nutrients. Lower sediment nutrients also limit the amount of rooted submerged aquatic plants that will grow in a pond. Some species of submerged aquatic plants can grow very quickly in ponds that have high sediment nutrients and they can obstruct swimming, fishing, and boating activities in the pond.

The complete mechanism is still being researched, however, it is possible that MD Pellets and MuckBiotics aid in the breakdown of organic compounds, may enhance the rate of organic compound break down, may aid in the breakdown of recalcitrant organic compounds, or may aid in some other way, such as through limiting the occurrence of buildup of inbound organic matter.

Faster breakdown of organic compounds may aid the microbes in the sediment by supplying a limiting nutrient such as nitrogen or phosphorus to the growing cells. From the initial pond muck until the end of the lab tank tests, we have observed that the sediments used have been very high in total organic carbon and lower in both nitrogen and phosphorus. This indicates that most of the organic matter is probably from terrestrial sources, which was also visually observed in the pond with high levels of oak leaves. The pond sediments had a C:N ratio of 24, which is generally higher than the assumed ratio of 6.6 for organic matter from phytoplankton (Hou et al., 2013). This indicates that much of the organic carbon in this pond is more difficult to decompose than ponds that have organic carbon from phytoplankton or submerged aquatic plants. Pond sediments with lower C:N ratios or higher levels of total volatile solids (TVS) will likely be composed of easier-to-degrade organic substrates. The resulting sediment reduction is expected to be higher than what was observed in the lab tanks. The TN:TP ratios were all below 4 and indicate that nitrogen is limiting in this sediment (Hou et al., 2013). Complex carbon polymers such as cellulose, lignin, or long chain fatty acids may require degradation by specialized organisms (Lewin et al., 2016). When degraded, lignin and other complex polymers tend to release nitrogen, which may be released into the water (Hou et al., 2013), unless utilized by other organisms. It is possible that MD Pellets and MuckBiotics aids in using this released nitrogen before it was released into the water column since no appreciable levels of ammonia, nitrite, or nitrate were detected in the lab tank tests.

In the lab tank tests, the tanks treated with MD Pellets or MuckBiotics tended to show a larger decrease in sediment total phosphorus than the Control tanks. This is sometimes observed in the field but tends to be confounded by environmental factors such as sudden heavy rains bringing in more particles, time of sample collection that sometimes was after fall plant die-off, or mechanical disruptions to the sediments in the area studied. The organic forms of phosphorus generally include nucleic acids, phospholipids, and slowly decomposable inositol phosphates (Reddy et al, 1999; Martinova, 1993). These more refractory organic phosphorus compounds are not released during mineralization and are permanently buried in the sediments (Søndergaard, Jensen, & Jeppesen, 2003). Generally, in the sediments under aerobic conditions, phosphorus can bind with Fe^{3+} to form $Fe_2(PO_4)_3$ and at the same time, dissolved phosphorus in the overlying water can be adsorbed by $Fe(OH)_3$ in the sediment (Hou et al., 2013). In shallow lakes, the entire water column is generally oxic (Søndergaard, Jensen, & Jeppesen, 2003), and so retention of phosphorus is generally possible as long as the Fe:P ratio is between 15 and 20 (by weight) (Søndergaard, Jensen, & Jeppesen, 2003; Parsons et al., 2017). Anoxic conditions in the sediment can result in phosphorus release to the overlying water (Parsons et al., 2017). In general, the phosphorus release is lake specific (Søndergaard, Jensen, & Jeppesen, 2003). In some cases, shallow lakes with high oxygen may have their sediment surface layer saturated with phosphorus and will still get a release of phosphorus into the water column as phosphorus transported upwards will pass through the saturated oxic layer (Søndergaard, Jensen, & Jeppesen, 2003). The effects of bioturbation by fish or benthic invertebrates on sediments can release phosphorus to the water (Parsons et al., 2017) but these effects may also be countered by water column reduction of phosphorus by phytoplankton and bacteria (Søndergaard, Jensen, & Jeppesen, 2003). Bacteria then contribute to terminal phosphorus burial by production of refractory organic compounds that become part of particulate matter in sediments (Reddy et al., 1999). Some bacteria also have luxury uptake and storage of phosphorus (Reddy et al., 1999) that can contribute to lowered levels of reactive phosphorus in the water column.

6.4 Using MuckBiotics Decreases Soft Sediment Depth

In the field, it has been reported that MD Pellets and MuckBiotics applications can reduce soft sediment depth. This soft sediment is presumed to be mostly composed of organic matter. We have also observed soft sediment reduction in our lab tank tests using MD Pellets or MuckBiotics. Reducing the soft sediment in a pond also increases the water volume of that pond. This increase in water allows for more dilution effect for nutrients that may be released or incoming. Decreasing the amount of soft organic sediment also decreases the internal nutrient reserve of a pond. This may allow for more available sites for phosphorus binding capacity. Reduction of nitrogen and phosphorus in the pond system may transition algal blooms from harmful cyanobacteria, or other toxin-producing algae, to less harmful green algae.

MD Pellets and MuckBiotics may be changing the microbial community of the sediments, resulting in reduction of sediment organic matter. Decreasing organic matter excludes more water from the sediment particles, resulting in compaction of sediments and decreased soft sediment depth. This may indicate the addition of MD Pellets or MuckBiotics to a pond imparts some missing metabolic connection that when present allows for the faster degradation of organic substrates in the sediments.

Anaerobic degradation of organic compounds is generally assumed to be slower than aerobic digestion. This highly depends on the type of organic matter available. Small, labile compounds may be decomposed at similar rates for aerobic and anaerobic conditions; however, more resistant compounds may degrade more slowly under anaerobic conditions (Lee, 1992) unless specialized microbes are present. There is also the problem of build-up of inhibitory metabolic waste products that may be slow to get rid of in sediments (Lee, 1992). Terrestrial organic matter consists of complex structures such as lignin, tannin, and cellulose. These complex compounds become buried deeper in the sediment as easy-to-degrade compounds at the surface are degraded (Mermillod-Blondin, 2011; Garrity, Bell & Lilburn, 2005). Sulfate-reducing bacteria are capable of degrading more complex substrates, including long chain and aromatic hydrocarbons (Nho et al., 2018). In sediments with high loads of organic matter, the sulfate-reducing bacteria become sulfate limited, leading to the accumulation of fermentation products (acetate or hydrogen) for the methanogenic population (Laanbroek et al., 1987). Methanogens generally use simple substrates for methanogenesis (Nho et al., 2018) although they are generally not found in the same environment as sulfate reducers. It is possible for sulfate reduction and methanogenesis to occur near the same physical space if they are both within a biofilm (Grießmeier et al., 2017) that would provide a niche with low concentrations of toxic intermediates. In general, an increase in methanogens is considered an indicator for more complete degradation of organic matter (Conrad et al., 2013), which was observed in the lab tanks that were treated with MD Pellets or MuckBiotics.

Fine, soft, and pliable sediment was observed in the Control tanks of our lab tank testing. This type of sediment is also observed in the field before MD Pellet or MuckBiotics applications have been made to a pond. This type of fine sediment generally has an overall higher level of organic content relative to coarse, more sandy textured sediment (Fang et al., 2015). As organic matter is broken down, the sediments begin to become composed of more inorganic solids. Inorganic solids tend to be more dense than organic matter (Avnimelech et al., 2001) and are less susceptible to resuspension than the fine organic floc. At the end of the lab tank tests, it was observed that the tanks treated with MD Pellets or MuckBiotics had a change in sediment texture from soft and pliable to sandy-like, more firmly compacting granules. This has also been observed in some field studies. Some of these results may be confounded by algae or primary producers that could keep adding simple organic carbon to the sediments. In the field, it has been observed that addition of MD Pellets and MuckBiotics may be able to enhance degradation to the point where at least some of this additional input is not an issue. It has also been suggested that that over time, algal blooms become less frequent or appear to have a lower intensity than before MD Pellets or MuckBiotics were applied to a pond.

Benthic burrowing organisms can also contribute to nutrient release and reduction (Mermillod-Blondin, 2011). The mixing of sediments through biological processes is known as bioturbation (Biles et al., 2002). Bioturbation by macrofauna may enhance decomposition by increasing the porosity of sediments and allowing oxygen to penetrate further down (Lee, 1992). Worms are known to construct tubes and burrows and irrigate these structures, increasing oxygen in the nearby sediments (Mermillod-Blondin, 2011). Bioturbation can also lead to the release of nutrients from deeper layers of sediment and also from the pore water, along with animal excretion, or sediment resuspension (Mermillod-Blondin & Lemoine, 2010; Martinova, 1993; Biles et al., 2002). Bioturbation could also affect the vertical distribution of algal resting stages and move these resting states to the sediment surface, thereby exposing them to germination cues in the water, such as increasing nutrient concentrations, light, or other conditions that could promote rapid proliferation (Ståhl-Delbanco & Hansson, 2002). Some burrowing worms may also eat the soft organic floc and lead to a release of inorganic nitrogen due to the breakdown of the protein fraction of bacterial EPS (Valk et al., 2016).

6.5 Future Research

The past and current experiments have established a viable lab experimental setup and method to examine sediment reduction. The lab testing from summer 2019 has shown that this lab setup is comparable to what is observed within a small pond (data pending). This lab setup should also be tested on other types of ponds and ponds with higher organic content in the sediments (total volatile solids). Testing to compare various muck reduction products, including liquid and powder products, could be conducted using this lab experimental setup to explore what happens to the water and sediments when applied to pond sediments. For example, it would be beneficial to understand if there were limiting nutrients available to the cyanobacteria that allowed proliferation in the Control tanks but not in the treated tanks.

7. Conclusions

MD Pellets and MuckBiotics do not cause the sediment to float to promote sediment reduction in a pond or in our lab tank setup. This means sediments and the nutrients contained within will not simply be moved downstream or out of the system. MD Pellets and Muckbiotics promote the digestion of organic compounds in the sediments that lead to reduction of soft sediment depth, decrease of available nutrients in the sediments that remain, and promote increased water clarity. Through the lab tank testing, it has been observed that MD Pellets and MuckBiotics enhances degradation of easily usable, and some complex, carbon substrates and also may aid in complete digestion to methanogenesis of such compounds.

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Appendix A: Complete Final "Pond in a Tank" 2019 Methods

The tanks used in testing were 15-gallon standard glass Aqueon aquarium fish tanks. The tanks were cleaned before the addition of sediment and water. The sand was washed with tap water before use and a 3 cm layer of sand was applied to the bottom of the tanks. A washed, clay bentonite layer measuring 0.5 cm was applied on top of the sand layer. Sand and clay were washed in order to remove debris and fine particulates prior to being applied within tanks. A 2 cm layer of soft pond sediment was then spread on top of the sand and clay bentonite layers. Soft pond sediments were collected from Pond 1 for all tanks and were screened in the field with a metal cooling rack with 1 cm squares to remove leaves, sticks, snails, acorns, and visible filamentous algae. The screened pond sediments were frozen for 2 days, allowed to thaw, and then frozen again for another 2 days prior to thawing out and being spread on top of the sand and clay bentonite layers. The freezing of pond sediments was intended to reduce the amount of plant and algae growth as well as the prevalence of benthic worms that were observed in previous tank studies. An initial composite pond sediment sample was taken for analysis prior to the addition of the pond sediment into the tanks. A 500 mL sample of soft pond sediment was sent to Eurofins TestAmerica, Chicago for nutrient analysis. A 30 mL sample of soft sediment was frozen and then sent to the University of Wisconsin Biotech Center for DNA extraction and Illumina NovaSeq 6000 metagenomic analysis.

Sediments were allowed to dry and stabilize on the bottom of the tanks for a week. Pond water from Pond 1 was added to the tanks to bring the water level to a height of 15 cm above the sediment layer.

Each tank contained an air stone, placed 1 cm above the sediment layer and powered by a Marina 50 aerator. The aerator was turned on for 12 hours per day, during the time that the tank light was turned off. Each tank had its own Aqueon 19-watt fluorescent overhead aquarium light source that was on for 12 hours and off for 12 hours each day. Tanks were entirely covered on all sides with aluminum foil in order to block all light besides the controlled light source directly above each tank. Each tank also had two Korelia Nano 240 gph pumps on either side of the tank. The pumps were angled to the side of the tank and slightly upward in order to induce a water flow current throughout the entire tank. The pumps were turned on for 30 minutes every 6 hours and the controller switched between coinciding pumps every 10 seconds. A testing grid was constructed above the tank, totaling 32 equal-area sample sites for muck depth measurements (3-inch squares). After the setup was completed, the tanks were allowed to run with the aerator and pumps cycling for one month as an adjustment period for the pond system before any measurements were made or products were applied.

Weekly measurements began after the one-month adjustment period. Visual observations and photos were taken of each tank. Weekly water quality measurements were from water collected 1-inch above the sediments in each tank. Water quality analysis was either conducted in the Naturalake Biosciences' laboratory or sent to Eurofins TestAmerica, Chicago. Water quality measurement parameters included OD580, which is the optimal wavelength for our tank's bacteria, general water quality, pH, DO, and temperature. The Hach HQ40D portable meter, along with LDO101 and PHC101 field probes, were used to measure pH, DO, and temperature. The 32 equal-area sample sites were measured for water depth over the sediments and total depth of water and soft sediments. Soft sediment depth was calculated for each sample site. Water levels were maintained at 15 cm for the duration of the testing. The tanks were tested once a week for the 12-week treatment testing period.

The tanks that were given no additional treatment products acted as controls for our testing. The treatment tanks were given an application of 0.280 g of MuckBiotics tablets spread throughout the tanks in order to mimic a field dose of 12.5 lbs per acre per month. MuckBiotics was applied at week 0, week 4, and week 8 during the testing period. All water quality measurements were taken before the application of MuckBiotics.

Upon the conclusion of the 12-week testing period, final measurements, observations, and images were taken. The remaining water was drained from the tank and a final soft sediment sample was taken for analysis. A 500 mL sample of soft sediment was sent to Eurofins TestAmerica, Chicago for nutrient analysis. A 30 mL sample of soft sediment was frozen and then sent to the University of Wisconsin Biotech Center for DNA extraction and Illumina NovaSeq 6000 metagenomic analysis.

Lake
and Pond
Solutions Co.

CASE STUDY: TARGETED MUCK REDUCTION WITH MD PELLETS

NOVEMBER 2016

PROJECT OVERVIEW

Mallard Lake is a 6.5 acre private stormwater lake located in SE Wisconsin. It has three main fingers and three small islands located on the southern portion of the lake. The maximum depth is 10.9' while the average depth is 5.1'. Despite having a 12-diffuser summer aeration system and a SolarBee for winter aeration, the lake saw extensive fish kills during the winters of 2009 and 2014.

Following the 2014 fish kill, total phosphorus and dissolved phosphorus in the lake quadrupled to 0.280 ppm and 0.110 ppm, respectively. As expected, algae growth exploded including difficult to control species like hydrodictyon, pithophora, and spirogyra.

Mallard Lake Stats

Acreage	6.50
Max Depth	10.9'
Average Depth	5.1'
< 3' Deep	20.40%
3 - 6' Deep	46.10%
6 - 9' Deep	27.20%
> 9' Deep	6.30%
Avg. Dissolved P	0.127 ppm
Avg. Total P	0.228 ppm


Hydrodictyon growth on Mallard Lake (2014)

In 2015, spring sampling showed another staggering increase in total and dissolved phosphorus (double from the previous spring). The thought was that decaying fish from the previous year had charged lake sediments with high amounts of phosphorus. The HOA undertook a nutrient reduction program using aluminum sulfate in an attempt to significantly reduce phosphorus levels. Although the treatment was successful by dropping phosphorus and limiting algae growth, it was short lived as phosphorus soared even higher by the fall.

During the winter of 2015, Mallard Lake HOA and LPS decided on a plan to look at the soft sediment accumulation and reduction as a way to reduce in-lake nutrient loading.

METHODS

Due to the sheer size of the pond, 3 key areas of excessive soft sediment accumulation where chosen as test sites (see inset picture on right). The NW area was 0.16 acres in size, the NE area was 0.17 acres in size, and the South area (near the outflow) was 0.09 acres in size.

On March 28th, 2016, LPS took soft sediment readings in each area using a custom marked 10-foot pole with a sediment disc. Random locations were chosen and depth of soft sediment was recorded.

An aggressive 42#/acre rate of Aquafix MD Pellets was chosen and applied monthly for five months (May – September) in order to achieve accelerated results. Pellets were hand spread and all algae treatments were performed at least 3 days out from any MD Pellet application to limit adverse reactions.

On September 15th, 2016, LPS took post season soft sediment readings in each area. Random locations were again chosen and depth of soft sediment was recorded and averaged.

RESULTS

In the NW and South areas, average soft sediment was reduced by 5.85" – 14.5". Those same areas also saw reductions in each minimum and maximum reading. The NE area did not show as much of an average reduction (3") although the minimum reading did decrease. It should be pointed out that the NE end of this area is where the most extensive muck in the lake was found (42"). No aeration is present there and it's theorized that oxygen demand was too great to achieve dramatic results. The control site, located at the SW end of the lake, saw no significant changes to soft sediment levels.

SUMMARY

Aquafix MD Pellet applications of 42#/acre resulted in reductions of 3" – 14.5" over the span of all test areas. These results were confirmed by two separate Mallard Lake HOA members who performed their own soft sediment testing. In their sampling, they showed average reductions of 5.58" with maximum reductions up to 18".

Mallard Lake Muck Depths

NW LOBE

DATE	MIN	MAX	AVG	Readings
3/28/2016	9	21	14.25	15,9,21,12
9/15/2016	0	18	8.4	18,6,0,6,12

NE LOBE

DATE	MIN	MAX	AVG	Readings
3/28/2016	6	42	16.5	15,9,18,9,6,42
9/15/2016	0	42	13.5	0,9,0,6,42,24

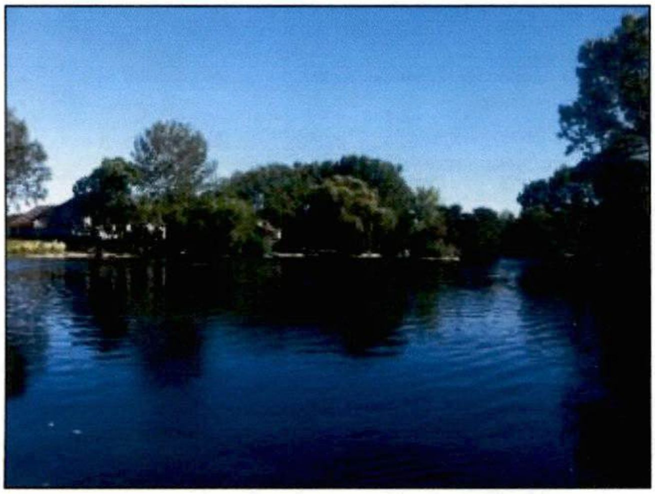
S LOBE

DATE	MIN	MAX	AVG	Readings
3/28/2016	21	24	22.5	21,24
9/15/2016	6	12	8.0	12,6,6

SW LOBE (Control)

DATE	MIN	MAX	AVG	Readings
3/28/2016	8	17	12.4	8,14,12,17,11
9/15/2016	7	17	12.8	7,12,17,13,15

Data collected by Lake and Pond Solutions Co. (2016)



"I'm encouraged as overall we saw an improvement on all the test areas. I'm sure we'll never win the war but we're putting up a good battle." – Kurt L. (Mallard Lake HOA)

"These results are encouraging, especially when we know that if we did nothing, the muck layer would be thicker in the fall than in the spring." – Ron L. (Mallard Lake HOA)

The Mallard Lake HOA in conjunction with Lake and Pond Solutions Co. are looking to expand the test areas in 2017.

AQUA DOC®
LAKE & POND MANAGEMENT

A Study of Naturalake Bioscience's MD Pellets on Two Small Northeast Ohio Ponds

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Naturalake
Biosciences

Introduction and Goal:

Introduction

Pelletized bacterial products are an exciting and relatively new technique to reduce benthic organic build-up found in ponds via enhancement of decomposition processes. One product in particular, Naturalake Bioscience's MD pellets seem to have heightened potential to decrease these materials. The benefit of this is a potential substitute to expensive and aggressive dredging operations which have historically been the only effective way of organic sediment removal from waterbodies.

Goal

The goal of this study was to assess the effectiveness of MD pellets to reduce benthic organic substrate in two Northeast Ohio ponds (Figures 1 & 2).

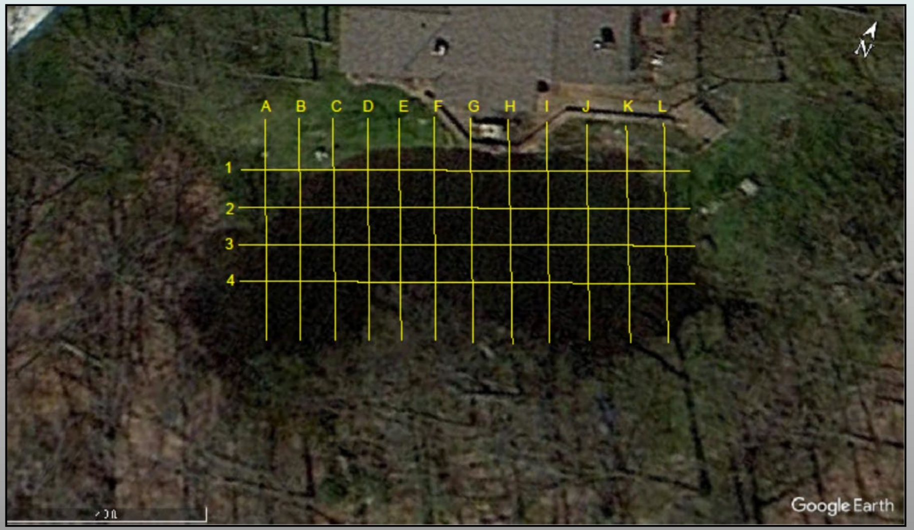

Figure 3: Pond 2 gridded for sediment probing.

Table 1: Water and sediment depth data example for Pond 2 (pre-treatment) .

	ft	A	B	C	D	E	F	G	H	I	J	K	L	M
1	Depth		0.50	1.00	2.50	3.50	3.00	3.50	3.00	3.00	2.50	1.00		
	Sed.		1.50	1.00	0.50	1.00	1.00	1.00	1.50	1.50	2.00	1.50		
2	Depth		1.00	1.00	4.00	4.00	4.00	4.50	4.00	4.50	4.00	3.50	0.50	
	Sed.		2.50	3.00	1.00	0.50	0.50	1.00	2.00	2.00	2.00	1.50	3.00	
3	Depth	0.50	2.00	3.50	4.00	4.50	4.50	5.00	4.50	4.50	3.50	3.00	0.50	
	Sed.	2.00	1.50	1.00	1.00	0.50	1.00	1.00	1.50	1.50	2.00	2.00	2.50	
4	Depth		0.50	2.00	2.00	2.00	2.50	2.50	2.00	2.00	2.00	1.00	0.50	
	Sed.		2.00	1.00	1.50	1.00	0.50	1.00	1.00	1.00	1.00	2.00	2.00	

Avg Water Depth =	2.70	ft
Avg Sediment Depth =	1.40	ft
Total Sediment (1.4 ft sed. x 0.09 SA) =	0.13	ac-ft
Total Sediment (0.13 ac-ft x 1615 yd^3/ac-ft) =	208	yd^3

Figures 1 & 2: Images of the two sample ponds used during this study. Both have aeration and are surrounded by sources of organic materials.

Table 2: bathymetric information relevant to the study pre- and post- treatment for Pond

Pre-Treatment	Post-Treatment
0.05 SA	0.05 SA
Max Water Depth = 5.0 ft	**Max Water Depth = 6.0 ft**
Average Water Depth = 2.5 ft	**Average Water Depth = 2.8 ft**
Average Sediment Depth = 1.7 ft	**Average Sediment Depth = 0.90 ft**
136 yds^3 sediment	**73 yds^3 sediment**

Table 3: bathymetric information relevant to the study pre- and post- treatment for Pond

Pre-Treatment	Post-Treatment
0.09 SA	0.09 SA
Max Water Depth = 5.0 ft	Max Water Depth = 5.0 ft
Average Water Depth = 2.7 ft	Average Water Depth = 2.7 ft
Average Sediment Depth = 1.4 ft	**Average Sediment Depth = 1.1 ft**
208 yds^3 sediment	**156 yds^3 sediment**

Methods:

Bathymetry

- Pond size determined through the use of a range finder and satellite imagery.
- Sediment build-up determination accomplished through the use of a sediment probe along individualized standard grids (30 minimum; Figure 3).
- Probing was conducted at the same gridded location pre as well as post treatment.

Treatment

- Treatment rates of MD pellets were 2.5 lbs initial and 1.25 lbs thereafter for Pond A and 5.0 lbs initial and 2.5 lbs thereafter for Pond B. Treatments were applied on a biweekly basis from June until October.

Analysis

- Post-data collection, water and sediment depth were recorded in depth profiles at each corresponding transect where color coding indicated trends in water and sediment depth (Table 1).

Results and Conclusions:

Results

- Organic sediment build-up in Pond A was found to have been reduced by approximately 63 yds^3 (46% reduction; Tables 2 and 3) while Pond B was found to have been reduced by 52 yds^3 (25% reduction).
- Both reductions were found to be statistically significant via Welch two sample t-test (Pond 1: $t = 4.13$, p-value = 0.0002, $\alpha = 0.05$; Pond 2: $t = 2.75$, p-value = 0.007, $\alpha = 0.05$)
- The average sediment depths of the two ponds were reduced by 0.8 ft and 0.3 ft for Pond A and B respectively.
- The max depth of Pond A also was shown to increase by 1.0 ft with a measurable increase in average depth as well (0.3 ft). Pond B did not show any measurable change in max depth or average depth.

Conclusions

Based on the findings of this study, it would appear as though the use of MD pellets effectively reduced benthic organic substrate from the two test ponds. Additional studies should be conducted however to determine sources of variability from one water body to another and to create necessary models to allow for effective rate changes.