INV22 Engineered and Replaceable Sea-Sponge Bio-Filtration Module for Harvesting of Algae, Carbon, Nitrogen and Phosphorus with Downstream Bacterial Denitrification to Mitigate Harmful Algal Blooms

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Executive Summary

The Indian River Lagoon (IRL) is a critical estuary on Florida's east coast. It supports high biodiversity and is a major economic driver for the region (OneLagoon.org). It is also negatively impacted by human activities. As a result, the IRL was added to the National Estuary Program in 1991. The Department of Environmental Protection (DEP) has implemented Basin Management Plans (BMAPs) and calculated Total Maximum Daily Loads (TMDL) for specific pollutants, including nutrients in different parts of the lagoon. Despite this, the IRL continues to suffer from a century of increasing human impacts.

Harmful algal blooms (HABs) can devastate the local ecosystem. They are caused by an array of factors including eutrophication, rising temperatures and loss of filter feeding organisms. Since 2011, the IRL has experienced repeated HABs. This increase in severity and frequency of HABs has been identified as a potential cause of the decrease in seagrass cover throughout the IRL. HABs may be controlled by directly removing the phytoplankton and by removing nutrients that fuel the blooms.

Utilizing natural processes can lead to environmentally friendly solutions. Sponges are remarkably efficient filter feeders. They can remove a wide range of particles, reducing turbidty and increasing water quality. Sponges convert dissolved organic nitrogen (DON) into dissolved inorganic nitrogen (DIN), including higher proportions of nitrate and nitrite. This can increase the efficiency of bacterial bioreactors by providing more easily accessible forms of nitrogen for final breakdown and removal. The purpose of this study was to prevent HABs by removing particulates, including photosynthetic organisms and nutrients using a novel, modular system that paired a bioengineered sponge biofilter with a bacterial bioreactor.

Over the course of the experiment, two species of sponges were successfully reared, both in the lab and *in situ*. The sponges were found to enrich nutrients, in particular DIN, providing a concentrated influent for the bacterial bioreactor. A major inefficiency was the lack of carbon for the bacteria to efficiently break down the nutrients. To address this, a carbon dosing module was added, which increased the removal efficiency. Several iterations needed to be performed during the field demonstration optimization. A rain shield was added to maintain optimal conditions in the sponge module. A shade was installed to control macroalgal growth. Sponge nurseries were modified to facilitate production of bioengineered sponges. At peak performance, the system was removing more than 90% of nutrients, >70% of phycoerythrin and >50% of chlorophyll activity.

This method holds great promise for improving water quality and controlling HABs as shown in this initial pilot study. Improvements to efficiency and robustness will be investigated in future experiments. In conclusion, the modular biofiltration and denitrification system was able to dramatically decrease photosynthetic activity and nutrient concentrations.

Introduction/Background

Location

The Indian River Lagoon (IRL) is approximately 251 km long and occupies 40% of Florida's east coast. The IRL consists of the Mosquito Lagoon in the north, the Banana River Lagoon (BR), and the IRL itself (OneLagoon.org). Brevard County, in the central part of the lagoon, has the largest area within its borders (Gao 2009). The IRL system spans tropical and subtropical climates and habitats, supporting a high level of biodiversity (Gao 2009). Additionally, it is a major economic driver, providing jobs, housing, industry and recreational opportunities. It is estimated that the IRL system generates $7.6 billion annually, and each dollar spent on restoration provides a $33 return to the economy (OneLagoon.org).

In the northern and central extents of the IRL system, 90 miles between the Ponce de Leon and Sebastian ocean inlets restrict circulation and flushing with long freshwater residence times on the order of 300 days (Smith 1993). This long residence time for water contributes to nutrient pollution, with freshwater terrestrial runoff and pollutants accumulating before eventual discharge to the coastal Atlantic Ocean. Contributing to issues related to residence times, anthropogenic activities, and changing land use have increased the supply of fresh water and pollutants to this system. Since the early 1900s, the IRL watershed has more than doubled in size from ~560,000 acres to 1,500,000 acres (Osborn 2016). Associated changes to land use and increasing coastal populations have led to increased stress from nutrient loading and eutrophication.

As the IRL watershed became more populated, numerous management, engineering and restoration activities were carried out to address water quality issues. Notable steps included the designation of a priority water body needing restoration and protection in 1987 and its addition to the National Estuary Program (NEP) in 1991. In 1990, the Indian River Lagoon System and Basin Act was passed, which banned the discharge of primary treated sewage directly into the IRL, leading to the beneficial reuse of reclaimed water and the establishment of centralized water treatment facilities. Since 1992, DEP has maintained lists of water bodies that do not meet standards outlined in section 303 of the Clean Water Act. Implementation of Basin Management Action Plans (BMAPs) and Total Maximum Daily Loads (TMDLs) have helped define the maximum amount of a pollutant (N or P) that a waterbody can receive while still meeting water quality standards. In the IRL, nutrient targets were developed based on evidence of a decrease in seagrass distribution to determine allowable loadings of nitrogen and phosphorus (Gao 2009). Despite improved management of external nutrient loading, the IRL suffers the consequences of a century of human development. For example, the accumulation of fine-grained, organic-rich sediments, also called muck, has added internal recycling of nutrients as a major source of dissolved nutrients. These internal releases of dissolved nitrogen and phosphorus now contribute an estimated 25 to 40% of the total internal plus external loading (Fox and Trefry 2023).

Harmful Algal Blooms: Problems and Potential Solution

Harmful algal blooms (HAB) resulting from eutrophication, warming water temperatures, and destruction of filter-feeding organism habitats devastate the impacted ecosystem and the local community. In the shallow lagoons of south Texas, *Aureoumbra lagunensis* blooms were associated with a loss of seagrass and benthic invertebrates (Gobler et al 2013). This brown tide-producing species is particularly successful in estuarine environments because of its broad salinity tolerance, ability to inhibit grazing by zooplankton, and to utilize low levels of inorganic nutrients (Gobler and Sunda 2012). Once restricted to the Gulf of Mexico, *A. lagunensis* has been documented in the Indian River Lagoon in connection with the brown tide events dating back to 2011 (Gobler et al 2013). Figure 1 shows the Chlorophyll A distribution for the 2012 brown tide event and the average for 1996-2010. These bloom events continue to increase in intensity and frequency and can dramatically impact the ecosystems where they occur.

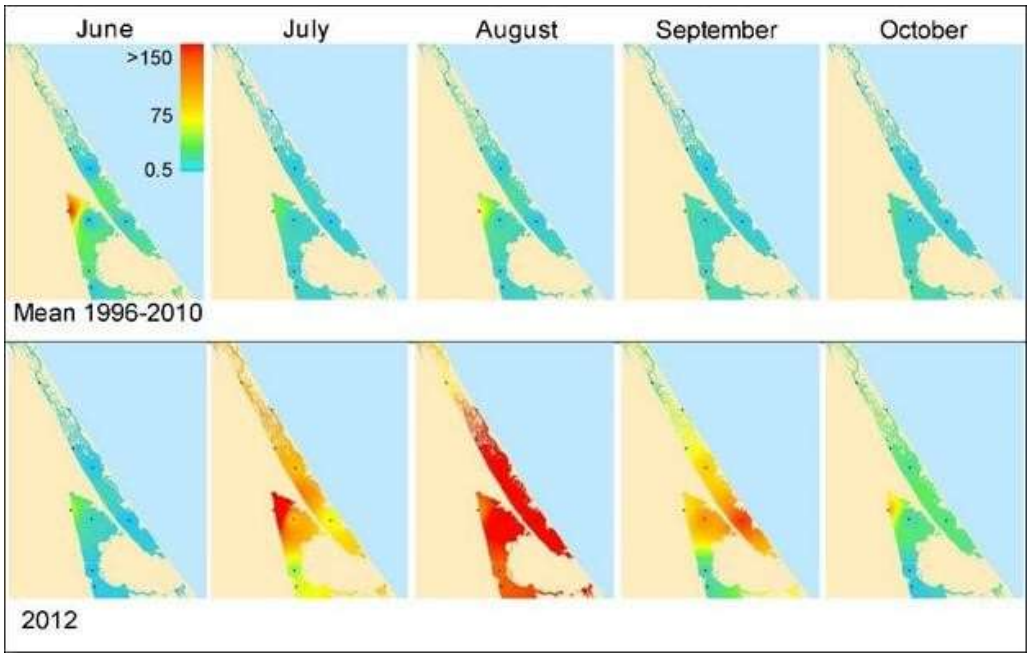

Figure 1: Chlorophyll a distribution in the northern IRL and Mosquito Lagoon. The top panels represent the mean data for all years 1996–2010, while the bottom panel represents the brown tide bloom of 2012. Chlorophyll *a* values ranged from 0.6 (lightest blue) to 300 µg L−1 (darkest red). From Gobler et al. 2013.

In 2011, a change in algal bloom characteristics was reported, dominated by nanoplanktonic species, including a new dominant taxon, *Aureoumbra lagunensis* (Phlips et al. 2021). At the same time, record decreases in seagrass abundance were reported, with a 58% decrease in IRL seagrasses between 2011 and 2019 (Morris et al. 2022). These simultaneous increases in algal biomass and loss of seagrass biomass follow a conceptual model of a regime shift or a change in stable states (Phlips et al. 2021). This shift coincided with an increase in dissolved nutrient concentrations not explained by a change in external nutrient loading. This suggests a change in the internal nutrient dynamics and biogeochemistry within the IRL, as well as benthic biomass

disruption. Internal nutrient sources include mobilization from sediments and benthic biomass disruption (Fox and Trefry 2018; Phlips et al. 2021). Other estuaries have experienced similar problems with cascading trophic impacts, including more frequent adverse impacts such as hypoxia, despite no appreciable increase in external nutrient loading (Buskey et al. 2001; Kemp et al. 2005). Beyond some threshold, the trophic state of an estuary can become self-stabilizing, where decreased external loading of nutrients alone may be insufficient to restore an estuary to a lower trophic state. Instead, restoring ecosystem services, including filter-feeding organisms coupled with bacterial processes, may be essential in restoring degraded estuarine ecosystems.

The potential for sponges to mitigate algal blooms has been examined in the Florida Bay (Peterson et al. 2006). After several mass sponge mortality events in the past several decades, over 90% of sponges in the lagoon were lost. This decline in the sponge population coincided with an increase in widespread algal blooms independent of nutrient loading and other factors that increase algal blooms. As a result of laboratory and in-situ grazing experiments, the five species of sponges examined were able to graze the bloom-forming species *Synechococcus elongatus*, *Cyclotella choctawatcheen* and *Prorocentrum hoffmanianum* at rates that would prevent blooms when restored to their historical abundance. This finding demonstrates the potential for sponge restoration in estuarine environments to reduce the abundance of harmful algal bloom species like *A. lagunensis* to sub-bloom quantities.

In addition to direct removal of HAB species, blooms may be controlled by removing the excess nutrients that facilitate excess growth. Once water quality improves, coupled filtration and bacterial (e.g., coupled nitrification-denitrification) processes should recover through natural succession; however, breaking feedback loops to facilitate water quality improvements will likely benefit from intervention. Removing nutrients from IRL and other systems can be accomplished using various nutrient treatment systems, including bioreactors that create conditions to promote and support these natural processes.

Organisms

Sponges are metazoans and some of the simplest multicellular animals (Pechenik 2014). As the only members of the phylum Porifera, there are approximately 10,000 species found in all climates, 98% of which are marine. A diverse group, the number of sponge species has been shown to outnumber corals and algae on coral reef systems (Diaz and Rützler 2001). Sponges lack nerves and musculature, creating an amorphous and asymmetrical body shape. Sponges are sessile organisms, and most sponges rely on suspension feeding, although some species of carnivorous sponges have been discovered in the deep sea. While they have no specialized reproductive, digestive, sensory, or excretory organs, sponges contain at least 20 morphologically different cell types that undertake the same biological and ecological functions.

Sponges are remarkably efficient filter feeders. Studies of filtration rates in sponges tend to be highly specific, focusing on a small number of species, locations, and parameters. Filtration rates have been shown to vary based on a variety of factors, including species, climate, abundance, growth form, seasonal variation in food availability, and morphological variation (Larsen and

Riisgård 2021). In feeding trials, sponges have been found to remove cyanobacteria, dinoflagellates and diatoms. In some studies, sponges have been found to create a layer of plankton-deficient water over the benthic community as a result of their suspension feeding. Typical Florida sponge filtration rates range from 141 – 5604 ml/h/g tissue dry weight. Sponges retain particles ranging in size from 0.1 - 50 µm, thereby decreasing turbidity and improving water quality (Osinga et al. 1999).

Sponges have close associations with many varieties of cyanobacteria and dinoflagellates (Bell 2008). This relationship helps them assimilate carbon and nitrogen through filter feeding on plankton, dissolved organic matter (DOM) and possibly even viruses and dissolved carbon. While this relationship may be analogous to the relationship between zooxanthellae and corals, the interactions between sponges and their symbionts have been less studied. On coral reefs, it has been estimated that sponges with bacterial symbionts make up approximately 28 - 58% of the sponge diversity; however, these relationships are less common in shallower waters and estuaries (Diaz and Rützler 2001). In one of the few studies examining this relationship, Wilkinson estimated that photosynthetic organisms provide 48 - 80% of the sponge's energy requirement (Wilkinson 1987). In the highly productive coral reef ecosystem, this accounted for only 10% of overall primary production. However, this production may have a greater impact on other less productive environments.

Sponges assimilate carbon and nitrogen from the water column through the filter-feeding of plankton, DOM, and possibly viruses (Figure 2; Bell 2008). Sponges can be a net sink for prokaryotes through filter-feeding, thereby transferring carbon through trophic levels (Diaz and Rützler 2001; Bell 2008). In addition to feeding on plankton, sponges can remove dissolved organic carbon from the water column, possibly through interactions with their symbiotes. Because most of the carbon available in the world's ocean exists in the form of dissolved organic carbon, the ability of sponges to directly uptake and utilize this form of carbon represents a likely significant and often overlooked contribution to carbon cycling in marine waters.

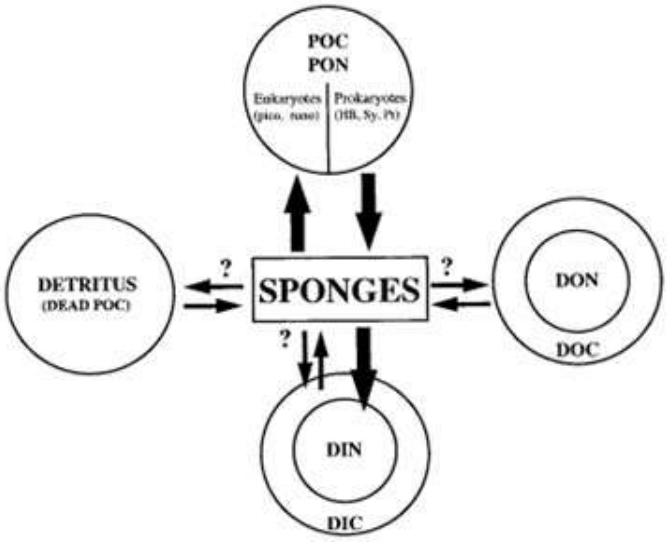

Figure 2: Cycling of carbon and nitrogen in the water column by sponges. There are four major compartments: Particulate organic carbon (POC) and nitrogen (PON), Dissolved organic carbon (DOC) and nitrogen (DON), Dissolved inorganic carbon (DIC) and nitrogen (DIN), and detritus. Established relationships are represented by thick arrows. Some Caribbean sponges have been found to be a net sink for prokaryotes (i.e., heterotrophic bacteria, *Synechoccocus*, *Prochlorococcus*) and a net source for eukaryotes. The net release of DIN has been measured for various reef sponges. Other detected or expected but not assessed relationships are represented by thin arrows and question marks (Diaz and Rützler 2001).

While most organisms excrete ammonium or amino-rich compounds, sponges can excrete nitrate or nitrite into the water column (Corredor et al. 1988; Bell 2008). It is suspected that this process occurs because of interaction with symbiotic bacteria. In addition, the consumption of nitrifying bacteria by sponges may aid in the breakdown of ammonia, allowing sponges to release forms of nitrogen into the environment that are more readily available to other organisms (Bell 2008). In Belize, a mutualistic relationship occurs between red mangroves (*Rhizophora mangle)* and two fouling sponges (*Tedania ignis* and *Haliclona implexiformis*) partially based on the sponges' ability to supply the mangrove with dissolved inorganic nitrogen. This oxidized waste nitrogen is readily denitrified by bacteria.

Coupling sponges with a bacterial bioreactor has the potential to aid bioreactor performance by increasing its ability to remove the less labile organic nitrogen species. Sponges and their symbiotic bacteria can break down organic forms of nitrogen that otherwise pass through most bacterial-based bioreactors. The resulting ammonium can then be oxidized by symbiotic bacteria, providing an idealized influent for downstream bacteria. Using these natural filters that convert DON to DIN has the potential to aid in the removal of organic nitrogen species.

In the natural environment, aerobic nitrifying bacteria from the genus Nitrosomonas and Nitrobacter sequentially oxidize reduced forms of nitrogen to nitrate and then nitrite. In deeper

layers of sediment and in suboxic microzones, less specific, anaerobic, denitrifying bacteria obtain energy from the oxidation of organic matter using nitrate or nitrite as the terminal electron acceptor or oxidizing agent. In healthy sediments, an aerobic surface layer of sediment houses nitrifying bacteria that convert ammonium to nitrate or nitrite. Nitrate and nitrite diffuse downward into suboxic sediments, which are utilized by anaerobic bacteria to yield nonbioavailable nitrogen gas in the presence of a carbon source. As the benthos experiences lower oxygen availability due to changes in the trophic structure of the estuary, nitrification can become inhibited.

Restoring or preserving the natural co-occurrence of these processes is essential for improving or maintaining water quality in coastal systems. To supplement naturally occurring nitrogen removal, these processes can be promoted in bioreactors that provide ideal environments for these bacteria. Such bioreactors are used frequently in freshwater applications, with fewer examples applied in brackish and seawater, at least partially due to lower specific growth rates and metabolic activity of nitrifying bacteria in marine versus freshwater environments (Ramaswami et al. 2019). Nevertheless, there are examples of the successful use of biological treatment in brackish or marine environments, namely in recirculating aquaculture systems (RAS) or large aquariums (e.g., Hamlin et al. 2008). Despite successful use, results from a RAS indicate that the maximum nitrification rates in saltwater systems were considerably lower than in freshwater systems. For example, one study reported 37% lower efficiency in salt water (Nijhof and Bovendewur 1990; Chen et al. 2006). This study aimed to improve performance towards removal of TDN by pre-treating water using natural filtration carried out by sponges.

Justification & Purpose

The purpose of these experiments is to prevent harmful algal blooms by removing particulates, algae and nutrients using a modular system consisting of bioengineered sponges paired with a bacterial bioreactor. This project aimed to utilize naturally occurring filter feeders and bacterial processes to treat lagoon water in a flow-through bioreactor system. The controlled environment provides an idealized environment for these organisms, allowing them to efficiently filter and remove nutrients from the system.

Methods

Methods are fully laid out in the QAPP (Task 1), which was submitted and approved previously and is attached as appendix 3.

Results

Sponge Collection

Sponges were collected in January 2023 from a Brevard Zoo Restore Our Shores oyster gardening facility (Figure 3). These fragments were placed in a sponge nursery to recover from stress from the collection. We had observed in a previous collection that sponges produced sulfur-smelling compounds when stressed, and this continued for a couple of days after removal from the oyster

cages or other original habitats. This stress negatively impacted the sponges, particularly in a lab setting, with no or limited water exchange. After a period of recovery, a portion of sponges were taken to the Florida Institute of Technology Aqualab, and additional sponges were left to grow in the field.

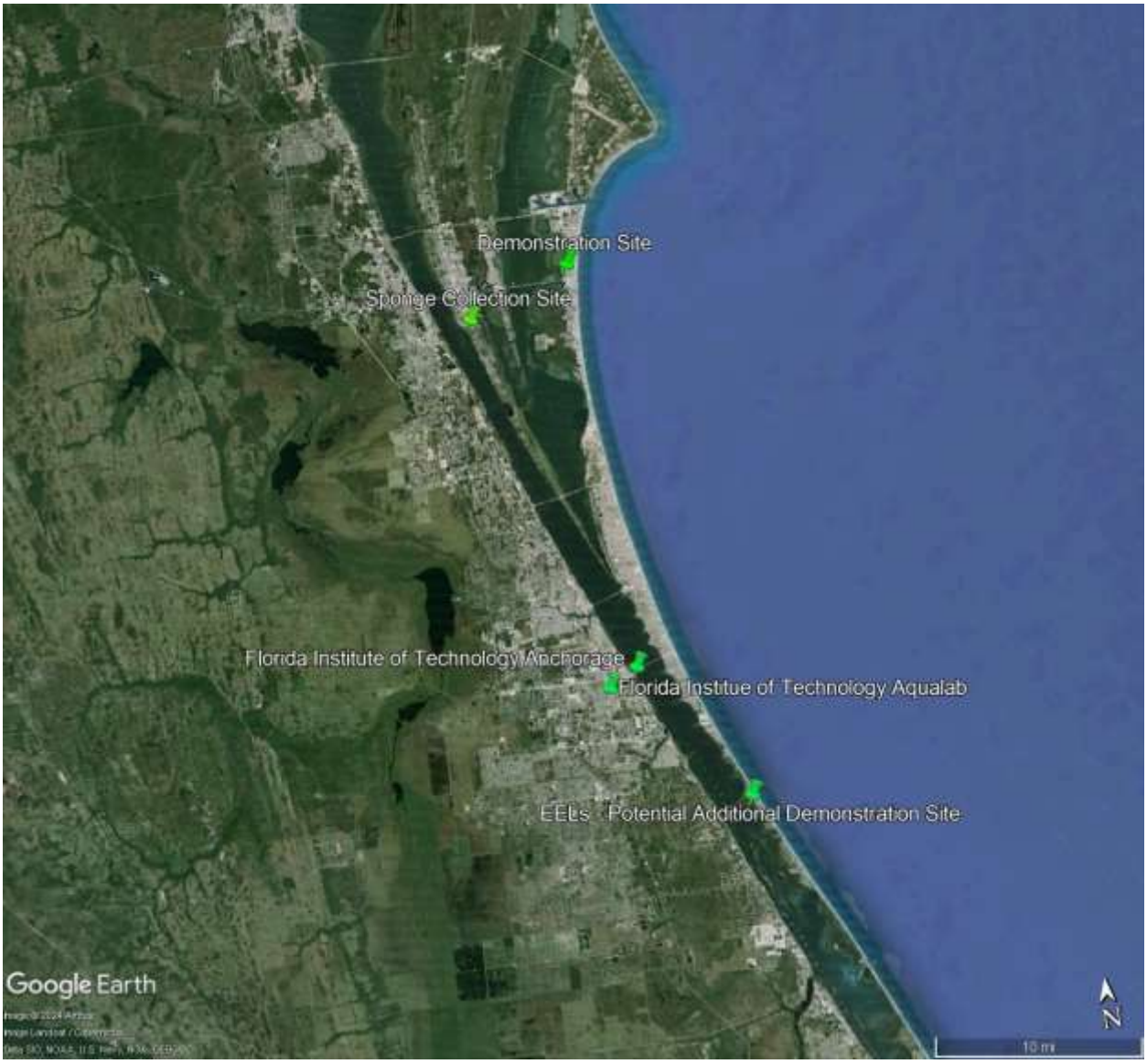

Figure 3: Map of project locations including sponge collection site, Aqualab and demonstration location. For greater detail, please refer to Appendix 2.

The original sponge nursery was left to grow *in situ* until July 2023. Sponges had strong growth through this period in all parts of the nursery and grew outside the cage (Figure 4). Because of the success of this method, additional nurseries were constructed based on the same design, just smaller in scale. Some sponges were collected to return some fragments to the lab to replace those lost during culture experiments. Additional sponges were collected and put into the new

nurseries and placed at three sites along the length of the lagoon to determine the best location for the field demonstration. Sites included the site of the original collection, the Anchorage and in the IRL near the Sebastian Inlet.

Figure 4: First version of the sponge nursery for growing sponges *in situ*. This nursery had been stocked for six months when the photo was taken. In these images, the excurrent siphons or pores can clearly be seen. Water samples for nutrient analysis were taken from these large pores to determine how sponges change the concentration and composition of nutrients.

One final sponge collection was undertaken in January 2024. Sponges were collected at the demonstration site and the original collection site. Newly redesigned sponge nurseries were installed (Figure 5). These improved upon the original design. The sides were left open so that the sponges could be installed more easily without taking them out of the water. It had been observed that sponges had a greater tendency to float if they were exposed to air while fragmented. Air trapped in sponges may negatively impact their ability to pump water as well (Osinga et al. 1999). Additionally, the space between shelves was increased to allow more room for growth, while still protecting sponges from predation and loss. As with previous collections, after a few days of acclimation, a subset of sponges were removed and returned to Aqualab. The rest were left to be used in the field demonstration and to grow out for future experiments.

Figure 5: Redesigned sponge nursery. This shows two nurseries. Note that the open design makes loading easier and provides plenty of room for growth.

Upon returning the collected sponge specimens to the laboratory, several individuals were selected to be processed for identification. The first step of this process included recording initial observations, including size, color and condition. Sponges were then portioned for microscopic examination. In order to examine the sponges microscopically, a 3 - 5 cm piece of each sponge was removed using a scalpel blade. Each portion was then placed on a gridded slide and macerated with a small blade. This process broke down the skeletal structure of the sponge so that individual spicules could be observed under the scope. Three to five intact spicules were photographed for each sample to be used for taxonomic identification (Figure 6). Once spicules were photographed, the images were analyzed using Image J software to get a measurement of each spicule. The average spicule size from the samples used in the laboratory portion of this experiment was 0.641 cm. Spicules were also noted to have one pointed end and one rounded or blunt end.

Based on morphological observations paired with microscopic spicules observations and measurement, the sponges used in the laboratory and field portions of this experiment were determined to be from the genus Halichondria. Several members of the family Halichondriidae have been identified in the Indian River Lagoon, including *Amorphinopsis atlantica*, *Halichondria melanodocia*, *Halichondria magniconulosa*, *Hymeniacidon heliophile*, and *Hymeniacidon perlevis*. Members of this family are typically found in shallow coastal waters with a worldwide distribution. (Erpenbeck and van Soest 2000)

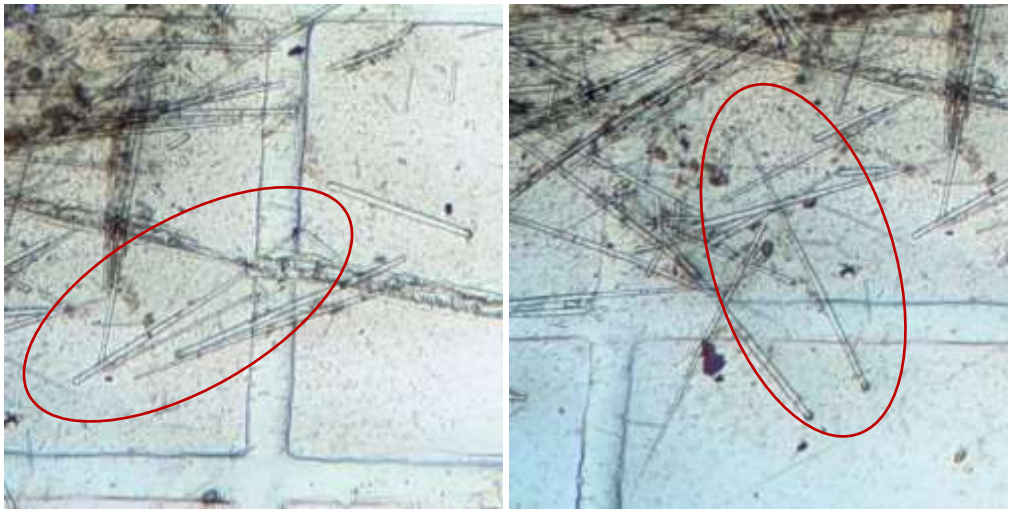

Figure 6: Photographs of spicules used for sponge identification under 40x magnification. Intact spicules have one rounded end, and one pointed end and averaged 0.641 cm. These shape and size characteristics help to identify the collected sponges as members of the family *Halichondria*.

Laboratory Sponge Culture

There is a dearth of information on cultivating sponges in the lab. We initially collected sponges in the Fall of 2022 for a pilot study. Although these sponges survived until power was lost in a hurricane after approximately two months, they did not thrive. To perform the necessary experiments to investigate and optimize the modular system before field deployment, we needed to determine how to cultivate these sponges in the lab.

In the initial sponge collection, water from the IRL was filtered to collect plankton, which was continuously fed to the laboratory sponges to maintain their natural diet. However, the volumes needed to collect an adequate dose of plankton to sustain the sponges were not practical, and the sponge health quickly declined. Additional groupings of sponges were offered a commercially available coral diet known as Reef Roids several times a week. This rate and diet slowed the deterioration of the sponges; growth was minimal.

Sponges are filter-feeding organisms. Their filtration is limited by the size of the incurrent siphons. These sponges have relatively small incurrent pores. Better growth was observed in the lab when sponges were fed every two to three days rather than daily. This may have been due to the symbiotic microbes in the sponge holobiont needing time to fully digest and utilize the food, as shown by a buildup of undigested food when sponges were fed daily.

A feeding trial was developed to compare several sponge diets and determine which had the best impact on sponge growth (Figure 7). A lab culture system was developed that consisted of sponges in separate two L cubes resting in a water bath. The water bath contained bottle caps to

provide surface area for the biofilter to break down excess nutrients in the system. A feeding trial was conducted to identify the best foods for sponge survival and growth. Sponges were fed four different diets, Reef Roids, a mix of brown and green cultured phytoplankton (*Isochrysis* and *Nannochloropsis*), the phytoplankton mixture supplemented with a growth medium (Marine Broth 2216), and the growth medium by itself. Twice a week, flow to the sponge system was halted, and five individuals were dosed with each diet. Water flow remained off for two hours to give sponges time to filter feed before flow was resumed. Every two weeks, growth was tracked via photographs taken from above and from the side. Images were then analyzed using ImageJ. This experiment continued for three months, and while a decrease in surface area was observed across all treatments, new growth was also observed. The sponges used in the trial all survived six months using phytoplankton and marine broth food.

Sponges in the Aqualab were found to need additional supplements as well. Sponges play an important role in silica cycling, particularly as a silica sink (Bell 2008). Sponges directly consume silica from the water column for spicule and skeletal construction. In addition to this direct uptake, some polar sponge species have been able to utilize silica from the digestion of diatoms (Cerrano et al. 2004). Because silica is a growth requirement for some sponges, a commercially available silica test kit was purchased from an aquarium company. Initial testing revealed the silica content of the system to be almost zero. A silica supplement for aquarium sponges, Sponge Excel by Brightwell Aquatics, was purchased, and manufacturer instructions were followed to slowly increase the levels of silica found in the system. Silica was dosed several times a week, and the water was tested to determine the sponges' level of consumption. Once the system reached a level of silica dosing and frequency where excess silica was still present without being depleted, silica testing became weekly, and a maintenance dosing schedule was developed.

Sponges have a three dimensionally complex shape and grow in a non-linear fashion (Figure 8). To determine whether growth was occurring in the lab, a way to measure changes in size was developed. Traditionally, this has been done using water displacement and other disruptive or destructive techniques (Morganti 2019). Because sponges have a soft body and the water content can change with handling, this method may be confounded and become complicated. In coral restoration, both a 2D and 3D approach have been used to track coral growth and restoration success (Figure 9; Koch et al 2021). While the use of 3D scanning technology can provide a more accurate result of surface area, likely due to the scanners' ability to interpret curved surfaces, the more common 2D methods are significantly faster and more cost-effective. Both methods provide consistent results for measuring coral growth; therefore, both methods were employed when examining the sponges to determine the best fit for the project's needs and scope.

Figure 7: Laboratory grow out system for testing cultivation of sponges. The system consists of 20 two-liter cubes in a water bath. The water bath maintained a consistent temperature and provided an area for biofilter media. Additional breakdown of excess nutrients occurred in a system sump containing additional biofilter media. Water was supplied to each cube through a rate controlled system. When sponges were added, all effort was taken to ensure they remained immersed in water, and sufficient time was given for them to acclimate to lab conditions (temperature, salinity, pH). The final image shows three of the four foods (l - r): phytoplankton, marine broth and phytoplankton + marine broth.

Figure 8: New growth on sponges in the lab. Note the presence of clear siphons extending from the surface of the sponges in both the whole sponge fragment and the close-up views. If sponges continued to be healthy, these siphons would fill in with similarly colored, spongy material and grow to match the older body.

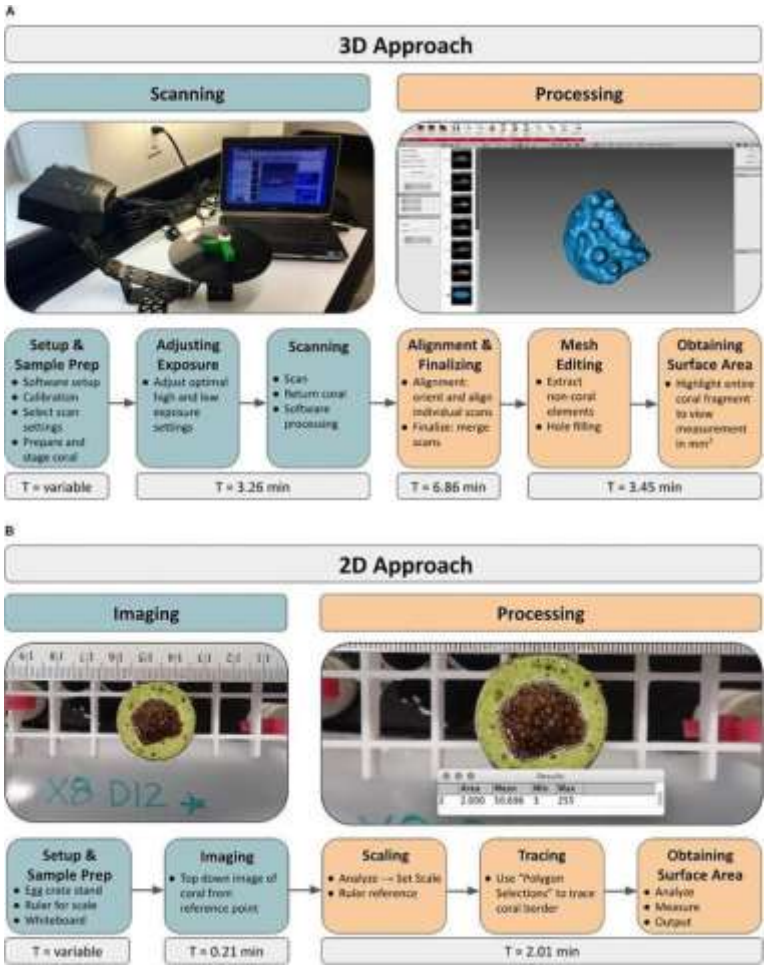

Figure 9: (A) Workflow depicts major steps involved in both the 3D scanning approach and the 2D photography approach (B). The initial Setup and Sample Prep time ranges from 0.29 to 0.63 min in the 2D approach and from 4.75 to 6.20 min in the 3D approach (Kock et al 2021).

The first method attempted was 3D photogrammetry using a laser scanner (Figure 10). Sponges were removed from their grow out aquaria into a transfer vessel and taken to Camid for 3D scanning. Sponges were placed in various clear vessels and scanned in both air and water. Although it was possible to scan in water through a glass container, the results were inconsistent, and results were not robust enough to use. After several attempts to standardize the measurement method, it was put aside in favor of a simpler and more reproducible method.

Figure 10: First attempts to measure sponges using 3D scanning techniques.

The second method utilized a two image approach with an object of known size (Figure 11). In this method, an insert was made to fit into the grow out cubes. This insert could be removed from the lab system without disturbing the sponges. The insert was placed on a flat surface, and an object of known size (an air stone) was placed next to the sponge. One image from the top and one from the side were taken. These images were then analyzed using Image J to determine the size of the sponges and how that changed over time. This method minimized stress on the sponges and proved to be robust and reproducible.

Figure 11: Measuring the sponges using the 2D approach. This method uses an object of known size (an air stone) and two images, one from above and one from the side, to measure changes in size over time. It has proven to be a simple, reproducible and robust method for measuring sponge growth. Because of the modifications to the lab system, sponges are removed from their growing cubes and measured without ever being removed from the water.

Laboratory Experimental Setup

The proposed field system was mimicked in the lab with a scaled version of the proposed system. A series of separate two L tanks were arranged in a stepped gridded pattern (Figure 12). influent water was pumped from a ~60 gallon holding tank into the top cell in each column. Drain ports near the top of each cell allowed overflow to drain through a PVC pipe into the bottom of the next cell. This strategy achieved an up-flow through each reactor cell. In the top row, each tank received a sponge fragment of approximately the same size. The third row of columns 1 - 4 was packed with bottle caps, initially with no supplemental aeration or carbon. Bottle caps were used to provide substrate for the bacteria in the bioreactor. It was hypothesized that sponges would break down particles to provide a readily available and labile carbon source while also increasing concentrations of dissolved nitrogen that would subsequently be removed by the bacterial bioreactor module (bottle caps). Rows two and four were left empty in each treatment train. The fifth column contained sponges in row one, with no bottle caps. Due to the large volume of water required, the system was recirculating, and nutrients were added periodically through algal additions to support sponge growth. As a result, the influent water varied in composition from lagoon water with DIN at >2000 µM (2539 ± 17 µM) with TDN composed of less than 1%

ammonium, 68% nitrate plus nitrite and 31% dissolved organic nitrogen. This composition is typical of aerobic recirculating aquarium systems. Typical water from the lagoon has an average DIN at 6 µM with 5.3% of the TDN present as ammonium, 1.3% as nitrate and 93.3% as DON. Following treatment using the laboratory sponge module, TDN increased, consistent with the breakdown of particulate material and release of dissolved nitrogen. This was mostly observed as an increase in DON with a small but insignificant increase in DIN. This suggests an increase in associated dissolved carbon species. Nevertheless, despite an aerobic cell, in the absence of a supplemental carbon source, there was no significant decrease in DIN.

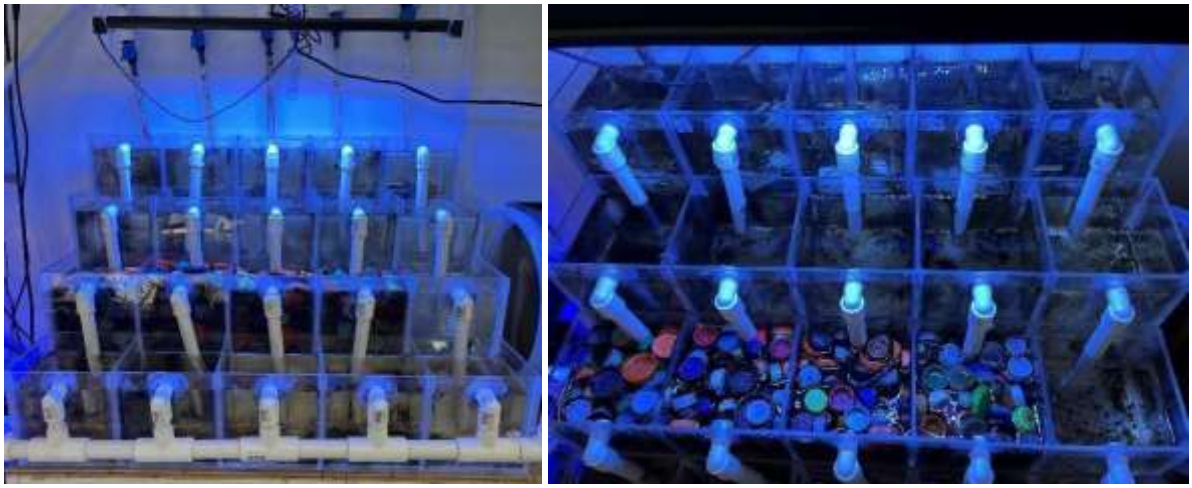

Figure 12: Laboratory setup. Water enters at each of the top cells; then, incrementally, gravity feeds to each of the cells in front of them. An overhead view shows bottle caps in four of the cells with a control blank on the right.

To further evaluate the need for supplemental carbon in conditions more similar to the field environment, a separate laboratory experiment was carried out to test the treatment efficiency under aerobic and anaerobic conditions, with and without supplemental carbon (Tables 1 & 2).

Table 1: Aerobic and Anaerobic (w/o supplemental carbon dosing)

		Influent		**Effluent**		**Percent Decrease**	
Sample ID	Date	DIN (µM)	DIP (µM)	DIN (µM)	DIP (µM)	DIN	DIP
Aerobic, no supplemental carbon	5/24/2023	**50.53**	3.60	**59.64**	4.48	-18%	-24%
Anaerobic, no supplemental carbon	5/24/2023	**50.53**	3.60	**9.71**	3.92	81%	-9%

		Influent		**Effluent**		**Percent Decrease**	
Sample ID	Date	DIN (µM)	DIP (µM)	DIN (µM)	DIP (µM)	DIN	DIP
Aerobic, no supplemental carbon	6/02/2023	33.84	2.76	**52.57**	2.82	-55%	-2%
Anaerobic, no supplemental carbon	6/02/2023	33.84	2.76	**9.69**	4.37	71%	-58%

Table 2: Aerobic and Anaerobic (with carbon dosing (sucrose)

		Influent		**Effluent**		**Percent Change**	
Sample ID	Date	DIN (µM)	DIP (µM)	DIN (µM)	DIP (µM)	DIN	DIP
Aerobic Sugar	5/24/2023	**50.53**	3.60	**0.47**	0.05	99%	99%
Anaerobic Sugar	5/24/2023	**50.53**	3.60	**0.61**	0.48	99%	87%

		Influent		**Effluent**		**Percent Change**	
Sample ID	Date	DIN (µM)	DIP (µM)	DIN (µM)	DIP (µM)	DIN	DIP
Aerobic Sugar	6/02/2023	33.84	2.76	**0.53**	0.25	98%	91%
Anaerobic Sugar	6/02/2023	33.84	2.76	23.53	1.52	30%	45%

In aerobic cells without the use of supplemental aeration, there was no significant decrease in DIN concentrations. This is likely related to the absence of anaerobic denitrification reactions. In aerobic environments, aerobic bacterial mineralization of particulate nitrogen produces ammonium that can be subsequently oxidized to nitrate or nitrite. Nevertheless, this is not expected to decrease DIN concentrations; simply change speciation from ammonium to nitrate plus nitrite. In contrast, in anaerobic cells without supplemental oxygen, there was an approximately 75% decrease in DIN related to anaerobic denitrification. Based on the pretreatment using sponge modules that produce nitrate (e.g., Corredor et al. 1988; Bell 2008), the field bacterial bioreactor system was designed to be suboxic or anaerobic. Adding a supplemental carbon source improved the efficiency of aerobic test cells, with mixed results for anaerobic cells. Based on these laboratory data, the field system was initially designed without the use of supplemental aeration or carbon, relying instead on metabolites from the upstream sponge module.

A carbon dosing system was added to the lab system to improve the removal of DIN (Figure 13). A sugar solution is added by a peristaltic pump at a C/N molar ratio of 9. The sugar was added to two of the bottle cap modules. Within days of adding the carbon, biofilms in the dosed modules had changed appearance dramatically (Figure 14). They had become thick, opaque and cohesive.

The un-dosed modules maintained the thin, dark colored, mat-like biofilm all bioreactor modules had previously contained.

Figure 13: Lab setup after sugar dosing system was added. The white bottle in the upper left holds the sugar solution. Inside the black box are peristaltic pumps, adding microdoses of the sugar solution. The dosing is maintained at a C/N molar ratio of 9. The sugar is being supplied to the two cubes on the left through flexible silicone tubing.

Figure 14: Close-up view of the bottle cap bioreactor modules in the lab setup. The left two cubes have had sugar added (indicated by the tubing), and the right are as initially set up. In the closer view, the differences in biofilm are clear. The sugar-added films are thicker, more opaque and cohesive than the thin, dark biofilms that haven't had sugar added.

Totipotent Cells

Sponges have remarkable plasticity and are constantly remodeling their structures through disorganization and reorganization to improve fitness or in response to damage or disturbance (Lavrov & Kosevich 2014; Coutinho et al. 2017; Riesgo et al. 2022). Because of their primitive nature, sponge cells can dissociate into primitive stem cells, also called totipotent or pluripotent cells. These cells can reaggregate, differentiate and regrow into new sponges (Eerkes-Medrano et

al. 2015; Ereskovsky et al. 2021; Riesgo et al. 2022). Sponges are known to regenerate whole bodies from a reduced number of cells or small fragments (Eerkes-Medrano et al. 2015, Ereskovsky et al. 2021). The most common method of releasing these totipotent cells is by crushing or squeezing them through a fine mesh; however, they may also be released during fragmentation or other physical disturbance (Osinga et al. 1999; Lavrov & Kosevich 2014; Eerkes-Medrano et al. 2015; Coutinho et al. 2017; Ereskovsky et al. 2021).

Totipotent cells released by crushing sponges have been challenging to cultivate into a functional sponge by previous authors (Eerkes-Medrano et al. 2015). However, these cells are also released during fragmentation and disturbance and have been shown to aid healing and increase growth rate (Coutinho et al. 2017). Sponges had rapid growth for the first few days after being fragmented and brought into the lab (Figure 15). This was observed by the rapid increase in new siphons and the overall size of the sponges. Sponges in the field also regenerated rapidly in the sponge nurseries. The use of smaller fragments to rapidly produce bioengineered sponge filters is promising and will be used in future field implementations and investigated further in next generation modular systems.

Figure 15: Rapid growth and reattachment of fragmented sponges highlighted by red circles

An initial test to release totipotent cells was performed. The sponge was crushed, and the product was placed in a tank containing artificial seawater with a bubbler. The seawater replicated the conditions that the sponge had been immersed in prior to crushing. Despite some initially promising signs that totipotent cells had been produced, no settlement was observed.

A second test to release totipotent cells was performed. In this test, a healthy sponge was pressed through nylons and coarse plankton mesh to try and produce totipotent cells. Both methods

produced visible changes in water color and clarity (Figure 16). The cells were dosed into a dish with a plankton mesh bottom and a pump that pulled water into the plankton mesh bottom. Additional material was carefully poured into a dish with plankton mesh that did not have a pump installed, using gravity to have cells settle onto the mesh. Cells showed initial aggregation in the tank, and some sponge growth was observed (Figure 17). Despite these promising initial results, growth was slow, and few cell aggregations formed.

Figure 16: Production of totipotent cells. A sponge was macerated through a fine mesh to release cells. The green color of the water clearly shows the presence of macerated sponge. The solution was poured through a funnel into containment dishes in a larger water bath tank.

Figure 17: Settled and aggregated sponge cells from the release of crushed cells.

Sponge Field Cultivation

Some sponges from the January 2023 collection were left in the field to grow and produce more sponge material for use in experiments. These sponges had grown to sufficient volume by the

summer of 2023 to use in additional experiments. Prior to removal, samples were collected for nutrient analysis. One sample was collected from the open water; the second sample was collected from the excurrent siphon of the sponge (Figure 4). The sponge habitat was raised near the surface and stabilized to prevent too much movement. A syringe was carefully placed in the excurrent siphon, and a water sample was gently and slowly collected to avoid damage to the sponge or accidental collection of sponge material. The sample was handled as in the QAPP, filtered and returned to the lab for analysis. As expected, sponges had higher nutrients than the ambient water (Table 3).

Table 3: Nutrient analysis collected from the sponge excurrent siphon and the open water.

Sample	Ammonium (µg/L)	Nitrate+Nitrite (µg/L)	DIN (µg/L)	DON (µg/L)	TDN (µg/L)	PO4 (µg/L)	Silica (mg/L)
Open	27.07	3.08	30.16	906.89	937.05	15.96	6.22
Sponge	100.51	4.69	105.20	1023.18	1128.38	35.83	6.66

Three additional sponge nurseries were placed in July 2023. These nurseries were smaller than the original and were intended for acclimation and grow out. One was placed next to the first nursery at the Brevard Zoo Restore Our Shores oyster grow site; one was placed at Florida Institute of Technologies Anchorage; the final was placed on a dock in a canal near Sebastian Inlet. Despite initial promising results, none of the sponges survived in their new location, and the sponges in the original nursery died back to nearly nothing. This may have been due to many things that are not mutually exclusive. One possibility is the historic high temperatures from the summer of 2023. The water quality in the new sites may have been non-conducive to sponge survival because of low salinity or high sedimentation, although this does not explain the lack of positive results at the first site. Finally, the sponge fragments may have been unable to compete with fouling organisms that recruited in high numbers to the nursery cages. This may have been exacerbated by stress from transplant to a new site with more variable, less ideal conditions.

The sponges from the January 2024 collection that were left to grow in the field in the redesigned nursery are still alive in June 2024 (Figure 18). In particular, the nursery that sits deeper in the lagoon has had sponge growth that has extended outside of the cage. The shallower nursery has had less growth and lower survival. It also had greater growth of algae and other fouling organisms.

Figure 18: Side and top views of the third iteration of the sponge nursery after six months of immersion. This clearly shows the growth possible in the second species of sponge, as figure 4 shows the growth possible in the first species of sponge.

Iterative Design and Installation of Field Demonstration Modules

The initial concept for the field demonstration system consisted of two modules: a sponge module and a biofilter module (Figure 19). A submersible pump would provide lagoon water from mid-water depth in the IRL. The lagoon water would first enter the sponge module to aid in the filtration of particles and nutrient breakdown. Then, it would travel into the bioreactor for further nutrient breakdown and removal. The polished water would be returned to the lagoon.

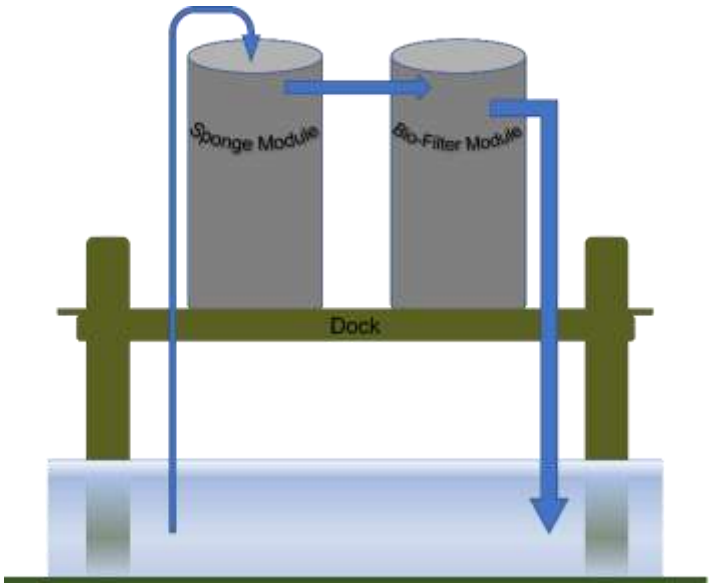

Figure 19: Schematic of the modular system as initially designed.

Iterative optimization

The first iteration of the Modular Biofiltration Denitrification System was built according to the initial concept. The field demonstration unit utilizes readily available High Density Polyethylene (HDPE) Lab Pack Drums (Figure 20). The initial design of the sponge module had an open top to allow sunlight. The sponges are a type that have a well developed microbiota, including photosynthetic microbes. The system was installed on February 8, 2024, and cycled without sponges for six days. On February 14, sponges were placed into the sponge filter scaffolding.

Figure 20: Image of the field demonstration of the modular system. The first image was taken during the build. The second image is the system as initially installed. Each module has a 55 gallon (208 liter) capacity. The pumping rate is 75 gallons/hour (300 l/hr). The image on the left shows the initial dry test fit of the system components, and on the right is the first iteration of the demonstration system as initially installed.

The second iteration was implemented to address a concern that rain would negatively impact the salinity in the module by causing it to change too quickly. To combat this, a clear lid was installed on February 17. It was designed to allow light and air to enter the module while preventing rain.

The third iteration was implemented to address excessive algae growth. After one month, the sponge module had accumulated a heavy growth of turf and string macroalgae. This was negatively impacting the sponges. On March 29, a shade cloth (50%) was installed on the clear lid (Figure 21). This shading controlled the algae within one week of installation. Subsequent recovery, color intensification and growth of sponges indicated the efficacy of this modification.

Figure 21: Field system with rain and sunshade installed.

The fourth iteration was implemented when it was determined that a dosing system was needed to supply the bioreactor with a carbon source for more complete nitrogen removal. Initial testing of the field system indicated that nutrient removal was not occurring at peak efficiency (Table 5). This parallels results from lab testing. In response, a carbon dosing system was installed on April 8 (Figure 22). An additional 55 gallon (208 L) lab pack drum was used as the weatherproof protection for a peristaltic pump, controllers and a 15 gallon (57L) sugar bucket. The carbon dosing was set to achieve a C:N molar ratio of 18 based on complementary experiments in INV21. After the sugar dosing was added, Nitrogen removal improved (Table 6), and the biofilms in the bioreactor changed dramatically.

Figure 22: Field system with the dosing module installed.

System Design

Overview - Modular Biofiltration Denitrification System

The modular biofiltration denitrification system is designed to remove the nutrients from natural waters that fuel algal blooms. This is ultimately achieved by biological processes. Therefore, each module of the system is designed to create an environment that supports a specific set of organisms. In this way, nutrients can be converted in a stepwise process into forms that phytoplankton no longer utilizes. The modular design of the system allows for scaling up or down or adding new modules to further improve efficiency (Figure 23). Drawings of specific elements of the system are included in Appendix 1.

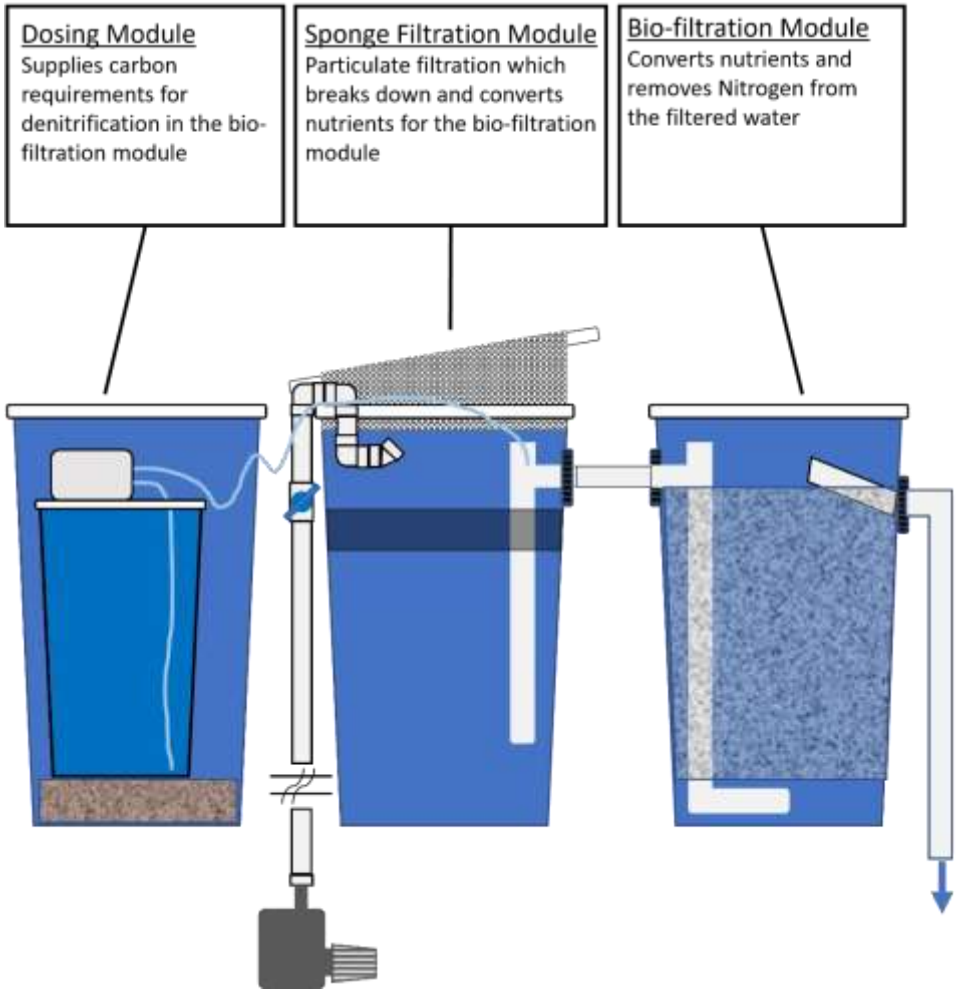

Figure 23: Schematic of the final design of the Modular Biofiltration and Denitrification System. For full details on the system, please see Appendix 1. The final demonstration system consists of three modules: a sponge filtration module, a biofiltration module and a dosing module.

Sponge Module

The sponge module of the system is designed to provide the living sponge filter with an environment suitable for all its biological requirements. These include a continuous exchange of lagoon water, adequate sunlight, sufficient air exchange and a substrate on which to attach and grow. Many of the design elements were incorporated to allow for continued optimization of the system while minimizing the challenges of operating in the marine environment.

A continuous supply of lagoon water is essential for the sponge filter to thrive. Marine organisms are accustomed to a stable environment. The continual flow of water into the system helps to maintain steady water temperatures, salinity, and pH. It also supplies a steady supply of silica and trace elements required for growth. The plankton, particles and their associated nutrients, which

are contained in the water supply, feed the sponges, which in turn condition the water for the biofiltration module.

Biofouling, such as barnacles, oysters, algae etc., is one of the largest challenges to the continuous pumping of natural waters. Therefore, a comprehensive plan was developed to manage biofouling. A submersible pump was utilized to avoid burnout due to loss of prime, which often occurs with other types of pumps. Therefore, the pump and the water supply line had to be maintained free of biofouling. This was accomplished by building two identical pumping systems and swapping them out weekly. Drying out while not in the water killed any freshly settled organisms before they could grow large enough to impact pumping performance.

To create a convenient swap out method for the pumps, they were attached to a fixed length of rigid sch40 PVC pipe. The length was set to maintain the pump mid water column. The power cord for the pump was attached to the intake pipe at a height which kept it well clear of the water line while leaving enough length to reach the power plug. Wash down rated waterproof plugs were utilized to ensure a safe connection out on the dock. Additionally, the shore power at the stanchion was wi-fi enabled, which would send an alert of any disruptions in the power supply that would impact water flow.

The pumps that were used are very durable and have been proven to provide years of continuous operation in marine aquaria. The size of the pumps was determined by several criteria. They needed to be able to provide the required volume with the head pressures expected from pumping up onto a dock. They needed to provide a consistent flow rate even if the head pressure changed with fluctuating tidal levels. The flow rate needed to be adjustable for system optimization.

The water flow to the system was managed near the discharge point of the supply line. A ball valve was used to adjust the flow rate. Flow rates were verified by pumping into a bucket at the same height as the sponge module while monitoring the time required to fill the bucket. Both pumping systems were set to the same flow rate, and reference marks were made on the valves. Flow rates were chosen based on volume turnover and residence time requirements of each module.

The outflow from the pump to the sponge module was designed to aid in easy weekly swap outs, as well as provide suitable water flow for the sponges within the module. The U bend at the top of the supply line acted as a hook to hang the pump from the sponge module. The forty five degree upward bend at the discharge point ensured that the most turbulent water from the outflow would be projected away from the sponges. This is important since, in the natural environment, the sponges are mostly subjected to laminar flow with a relatively low velocity. Additionally, the inflow water is projected to the center of the air - water interface in the tank. This is beneficial for sufficient gas exchange between the water and the air. In tanks such as this, there is a tendency for a layer of proteins, lipids and algae to form, which inhibits gas exchange.

Continuous mixing of the surface by the inflow of turbulent water helps minimize this effect, which is also beneficial for the penetration of sunlight.

Some sunlight is essential for these sponges, which host a variety of symbiotic cells. Sponges and many other marine organisms in the photic zone must use energy to minimize the accumulation of algal biofouling, which may smother sponges, especially in eutrophic waters. Sunlight is rapidly attenuated with depth in the water. The abundance of wavelengths and intensity of light in shallow waters fuels rapid algal growth. The sponge filter is situated near the top of the sponge module to minimize the shading by the tank walls, which would reduce the number of sunlight hours the sponge receives. A covering of 50% shade cloth was used to minimize algal growth while still allowing sufficient light for the sponges. The shade cloth was installed over the rain shield.

To minimize salinity reduction from rainfall events, a rain shield was installed. The shield was constructed from clear acrylic. It was pitched at a ten-degree angle to shed water. The oval shape allows for more overhang where there is a larger gap between it and the tank. The rain shield is ten centimeters above the tank at the highest point to allow for adequate ventilation and access. In keeping with the modular design of the system, its attachment is fabricated from a standard HDPE drum lid and closure ring system. HDPE was selected over the more common steel lid and closure ring in order to avoid corrosion issues. Most of the surface area of the original drum lid was removed so as not to inhibit sunlight, air exchange and access to the vent pipe.

The vent at the top of the T fitting of the discharge pipe is a convenient place to collect water samples downstream of the sponge filter. Since it draws from the bottom of the tank, the discharge pipe ensures that the water must pass the sponge filter before exiting the tank. All flow out of the tanks utilizes a gravity feed for energy efficiency and system simplicity. The size and configuration of those water lines was designed to allow for sufficient drainage, taking into account extremely low head pressure and the potential for biofouling accumulation. The vent is also a convenient place for the dosing system to introduce the carbon source for the biofiltration module.

Dosing Module

The dosing module of the system is designed to provide the biofilter with the carbon source necessary to completely convert Nitrogen to an inert gas. In keeping with the modular design of the system, all of its components are contained within a standard 55 gallon HDPE Lab Pack Drum. These include the equipment housing, the dosing solution reservoir and ballast weight, which is provided by clean sand.

The equipment housing is a moisture resistant plastic case with strain-relief ports for wiring and tubing to pass through. Within the equipment housing are several components. There is a power converter that plugs into the 120v AC shore power supply and provides 12v DC power for the rest of the equipment. The sensitive electronics are contained within a moisture proof box inside the

equipment housing. Within the electronics box is a programmable control board. This maintains a precise dosing concentration by regulating the peristaltic dosing pumping.

The dosing pump pulls the solution from a reservoir. This is a 14 gallon HDPE Lab Pack Drum with a plastic lid. It can hold several weeks' worth of dosing solution. Flexible silicone tubing is immersed in the solution and weighted near the end to maintain its vertical position. This tubing transports the dosing solution to the pump, and another piece of tubing brings it from the pump to the Biofilter.

Biofilter Module

The biofilter module of the system is designed to provide an environment that will convert nutrients to the point where they don't fuel algal blooms. Bacteria are the means by which this is achieved. Bacteria are extremely varied in their nutrient requirements. Therefore, if enough biodiversity can be maintained among the bacterial colonies, nutrient cycles can be achieved, which render those nutrients unavailable for algal growth. In order to maintain this environment, there are physical and chemical considerations.

One of the main physical objectives is to maximize the surface area available for bacterial colonization. Repurposed plastic bottle caps fit this need nicely. Their ridges and threads increase the surface area of each cap. Due to their shape and varying sizes, they pack together without nesting, creating a fairly dense biofilter media with lots of interstitial water space. The biofilter media was first packed into nylon mesh bags, which were doubly secured with plastic cable ties. This precaution was taken to ensure that loose plastic was not inadvertently released into the environment. The biofiltration module is nearly filled with this biofilter media.

Water transfer through the filter media is the other main physical objective. The water supply and discharge lines were designed to support this. The supply line discharges at the bottom of the tank. It is split into three outlet ports by a PVC cross coupler sitting horizontally on the tank bottom. This ensures that the inflow pressure is spread out at the same height and close to the center of the tank. The outflow pipe is constructed in a similar manner, near the top of the tank, at the desired water height. The discharge pipe exits below the tank's water line. This ensures that water flows freely as it approaches the outlet drains. This creates a low-pressure zone at the surface around the center of the tank, which draws water up through the filter media. This is balanced by the waters' tendency to take the easiest path along the walls, thereby maximizing flow through the biofilter media.

Understanding the numerous chemical considerations for the biofilter is crucial to achieve the targeted results. The flow rates were adjusted to achieve a water residence time that was optimized for nutrient removal. The dosing system provided the carbon essential for nitrogen removal.

Field Results

Lagoon water from the test site contained, on average, 4.9 ± 2.4 uM ammonium and 1.2 ± 0.8 uM nitrate plus nitrite (N+N) for 6.0 uM total DIN (Table 4). Concentrations during deployment of the field system varied, peaking at 11.7 uM DIN on April 22nd, decreasing to relatively low concentrations during May (the monitoring phase). During the monitoring phase, DIN decreased to 1.7 uM on May 9.

Table 4: Average concentrations of dissolved ammonium, nitrite plus nitrate (N+N), DIN (ammonium plus N+N), and phosphate (DIP) in the lagoon at the project site (influent concentrations) over the course of the field deployment.

Ammonium (uM / µg/L)	Nitrate + nitrite (uM / µg/L)	DIN (uM / µg/L)	DIP (uM / µg/L)
4.9 ± 2.4 / 69 ±34	1.2 ± 0.8 / 17 ±11	6 ± 3 / 84 ±42	0.3 ± 0.1 / 9 ±3

When first deployed, the field system was operated with the sponge module followed by a bacterial bioreactor module operated without a supplemental carbon source or oxygen. Based on lab data, the combined system yielded sufficient nitrification without the use of supplemental aeration. Between February 14 and April 8 there was no supplemental carbon or oxygen added (Table 5). During this initial deployment, DIN increased (both ammonium and N+N) from 5.8 ± 2.1 uM in the influent to 9.1 ± 3.1 uM in the sponge module to 11.8 ± 3.3 uM in the effluent. Based on results from the initial field deployment and laboratory systems, a supplemental carbon source (sucrose) was added to the bioreactor cell on 4/8/2024. Almost immediately, the bacterial bioreactor began to substantially decrease both ammonium and N+N levels (Table 6). As expected, the sponge module increased DIN compared to the influent, followed by subsequent removal in the bacterial bioreactor module (using a supplemental carbon source and no additional aeration). When fully functional between 4/12/2024 and 4/29/2024, DIN increased from 8.6 ± 3.2 uM in the lagoon to 11.0 ± 3.7 uM in the sponge module, as expected. This was followed by a decrease to only 2.5 ± 1.4 uM in effluent after treatment in the bacterial bioreactor module. The complete system yielded an approximately 70% removal of DIN during April, peaking at 94% removal one week after carbon dosing began. The increase in biofilms in the bioreactor was notable, and performance was best during this active growth phase. Much like what was seen in the lab, the biofilms became thick, white, opaque and cohesive to the point of being mucusy. The biofilm was so cohesive it could be scooped from the water and maintained structural integrity in a bag after collection. After the first week, performance declined as the bioreactor became congested by biofilms (Figure 24). The absence of active aeration and, thereby, mixing in the bioreactor module likely led to channeling and decreased performance over time. In the future, this can be addressed to maintain adequate surface area and flow within the bioreactor cell. Additionally, adequate mixing would help mitigate the variable influent DO that results from changes occurring outside of the system.

Table 5: Dissolved nutrient concentrations (DIN and DIP) at the influent, in the sponge module and in effluent leaving the bioreactor module between February 14 and April 8 before supplemental carbon dosing was used.

	Influent		Sponge		Effluent		Change	
Date	DIN (µM / µg/L)	DIP (µM / µg/L)	DIN (µM / µg/L)	DIP (µM / µg/L)	DIN (µM / µg/L)	DIP (µM / µg/L)	DIN (%)	DIP (%)
2/14	2.7 / 38.5	0.20 / 6.3	5.4 / 75.9	0.25 / 7.7	5.1 / 71.4	0.26 / 8.2	85	31
2/17	6.7 / 93.3	0.33 / 10.1	8.6 / 120.4	0.37 / 11.3	9.2 / 128.9	0.30 / 9.4	38	-7
2/28	4.2 / 58.6	0.24 / 7.5	4.7 / 66.1	0.21 / 6.5	11.0 / 154.5	0.46 / 14.3	164	89
3/4	9.9 / 139.8	0.56 / 17.4	13.2 / 185.2	0.74 / 23.1	15.3 / 214.1	0.92 / 28.3	53	63
3/11	6.8 / 95.5	0.66 / 20.4	13.0 / 182.8	0.89 / 27.7	16.2 / 226.3	1.15 / 35.6	137	75
3/20	4.9 / 68.9	0.54 / 16.8	7.1 / 99.4	0.48 / 14.7	10.6 / 148.2	0.71 / 22.0	115	31
3/29	6.9 / 97.4	0.24 / 7.3	11.4 / 159.1	0.58 / 18.0	16.1 / 225.5	0.88 / 27.3	132	275
4/8	3.9 / 55.3	0.25 / 7.7	9.3 / 130.2	0.36 / 11.1	11.0 / 154.3	0.48 / 14.9	179	94

Table 6: Dissolved nutrient concentrations (DIN and DIP) at the influent, in the sponge module and in effluent leaving the bioreactor module between April 12 and 29 after supplemental carbon dosing was initiated on 4/8/2024.

	Influent		Sponge		Effluent		Change	
Date	DIN (µM / µg/L)	DIP (µM / µg/L)	DIN (µM / µg/L)	DIP (µM / µg/L)	DIN (µM / µg/L)	DIP (µM / µg/L)	DIN (%)	DIP (%)
4/12	11.8 / 165.7	0.30 / 9.2	8.6 / 120.1	0.39 / 11.9	**0.7 / 10.0**	0.16 / 4.9	-94	-47
4/19	5.2 / 73.5	0.40 / 8.3	8.9 / 124.7	0.26 / 7.8	1.8 / 25.8	0.25 / 7.6	-65	-8
4/22	11.7 / 164.2	0.39 / 12.3	17.4 / 244.1	0.26 / 8.0	4.4 / 61.4	0.27 / 8.3	-63	-32
4/29	5.4 / 75.9	0.23 / 7.0	9.1 / 127.7	0.23 / 7.3	2.9 / 41.0	0.26 / 8.1	-46	15

Figure 24: Growth of biofilms in the modules after sugar was added. The biofilm has become thick, opaque and cohesive.

Monitoring

The monitoring phase was pre-selected to take place during May 2024 to allow as much time as possible for iterative optimization of the system. These laboratory and field iterations led to the development of a field system that removed >70% of the DIN without the use of supplemental aeration. Unfortunately, in May, N+N was below the Pace Labs practical quantification limit in all cases, and no change could be determined (Table 7). Concentrations of ammonium were also low and below the Pace PQL on 5/9/24.

Water temperature in the area increased throughout the month of May from ~20C to almost 30C. Within the treatment system, temperature increased by ~1C between influent and effluent due to heating of the blue barrels exposed to direct sunlight. This slight increase in temperature was anticipated and was not expected to influence the performance of the system. Salinity did not change between influent and effluent due to the use of rain shields and the relatively short (~1 hour) residence time that limited evaporation. Instead, salinity followed patterns for the region ranging from ~20 to 23 ppt, well within the preferred range for the sponges. Within the system, oxygen decreased continuously from the inflow through the sponge module to the bioreactor cell/effluent. A decrease in oxygen was expected and was incorporated into the system's design to achieve anaerobic denitrification in microzones within biofilms in the bioreactor module. Consistent with a decrease in DO and microbial respiration that releases CO_2, pH decreased throughout treatment due to the reaction of CO_2 with water to form carbonic acid. Consistent with results for dissolved nutrients (during April when N+N was detectable) with an increase in dissolved nitrate, the oxidation-reduction potential (ORP) was consistently positive and >100 mV in the sponge module. This is indicative of an overall oxidizing environment that promotes nitrification. Despite the presence of dissolved oxygen in the bioreactor module, ORP was negative and suggested an overall reducing environment. This was consistent with the intended design of the system, where the bioreactor module would facilitate anaerobic denitrification and the removal of nitrate/DIN as observed when dissolved nutrient concentration in the influent was sufficient to be quantified.

Another benefit of this system is the natural filtration of water, where sponges filter particles, including algae. Overall, concentrations of chlorophyll *a* decreased by about 50% from inflow to outflow during the treatment process when the system was working optimally. This filtration provides the second control mechanism for HABs, which is direct removal by sponges.

Table 7: Dissolved nutrient concentrations (DIN separated by ammonium (amm.) and nitrate plus nitrite and DIP) in influent and in effluent leaving the treatment system during the monitoring phase from May 9 to May 30.

	Inflow			Outflow			Change		
Date	Amm. (µg/L)	Nitrate+ Nitrite (µg/L)	Ortho-P (µg/L)	Amm. (µg/L)	Nitrate+ Nitrite (µg/L)	OrthoP (µg/L)	Amm, (%)	Nitrate+ Nitrite (%)	Ortho-P (%)
5/9/2024	37[I]	21[I]	12	100	17[I]	17	ND	ND	42
5/16/2024	100	<15[T]	3.8	100	32[I]	5.5	0	ND	45
5/23/2024	51	<15[T]	11	110	25[I]	18	116	ND	64
5/30/2024	77	<15[T]	3.8	94	16[I]	8	22	ND	111

MDL was 15 ug/L NOx. [I] indicates values above the detection limit but below the PQL of 50 µg/L.

Conclusions (Recommendations and Future Plans)

The modular biofiltration and denitrification system had the ability to convert and remove significant quantities of nutrients during the iterative optimization phase. However, the timing of the greatest reduction varied depending on the nutrient species. Further optimization of the system would aim to reproduce the largest changes more consistently and at the same time. Significant improvements to water quality could, therefore, be achieved.

The primary task of the bioreactor with carbon dosing was to remove dissolved inorganic nitrogen (DIN). However, the concentration of dissolved organic nitrogen (DON) was roughly an order of magnitude greater than that of dissolved inorganic nitrogen (DIN), representing a potential source of underutilized nutrients. The system was very productive at converting DON to DIN prior to carbon dosing, which shows potential for even greater nitrogen removal. Data from April 8 (prior to additional carbon) showed a 27% reduction in DON and a 179% increase in DIN. After carbon was added (April 12), >90% of the DIN was removed. However, DON was not converted to DIN by the bioreactor during this initial active-growth phase of biofilms in the bioreactor. Refining the bioreactor by adding additional stages may increase nitrogen removal by maximizing conversion of DON to DIN and removal of concentrated DIN. The new system could also be designed to mitigate the buildup of biofilms and the subsequent reduction in surface area.

The Sponge filter contributed to the conversion from DON to DIN. The greatest conversion (41%) was seen on February 28, just before some of the sponge biomass was removed for laboratory testing. This indicates that greater sponge biomass may be preferable. Sponge growth in the module was not as vigorous as anticipated. To provide a more favorable environment for sponge growth, the addition of a sediment settlement module may be effective. This would most likely result in even greater reductions in chlorophyll and phycoerythrin than the ~50% currently observed.

The new settlement module and the bioreactor could be designed in such a way as to incorporate a means to continuously remove high moisture content solids such as biofilms and muck. These could then be desiccated utilizing solar energy facilitating disposal. In this way, any nutrients, pollutants or particles contained in those biofilms and muck would be permanently removed from the lagoon.

The addition of plant or macroalgae-based modules could further improve system performance. The release of sugars from seagrasses could help fuel the bioreactor module without the need to dose the system with carbon. In addition, seagrass mats could be produced for replanting efforts. An algal turf scrubber could help scavenge any remaining nutrients from the effluent before it returns to the natural water body.

In conclusion, preliminary testing has shown great promise for nutrient removal and provided proof of concept for the modular biofiltration and denitrification system. At its peak, algal activity was decreased by approximately 50% and >90% of DIN was removed by the system. Analysis of the current data shows a need for additional iterative changes to further optimize performance. In future phases, the system will be refined through the design changes as suggested above. The efficacy of changes will be monitored through intense water quality monitoring, leading to further optimization and enhanced system efficiency.

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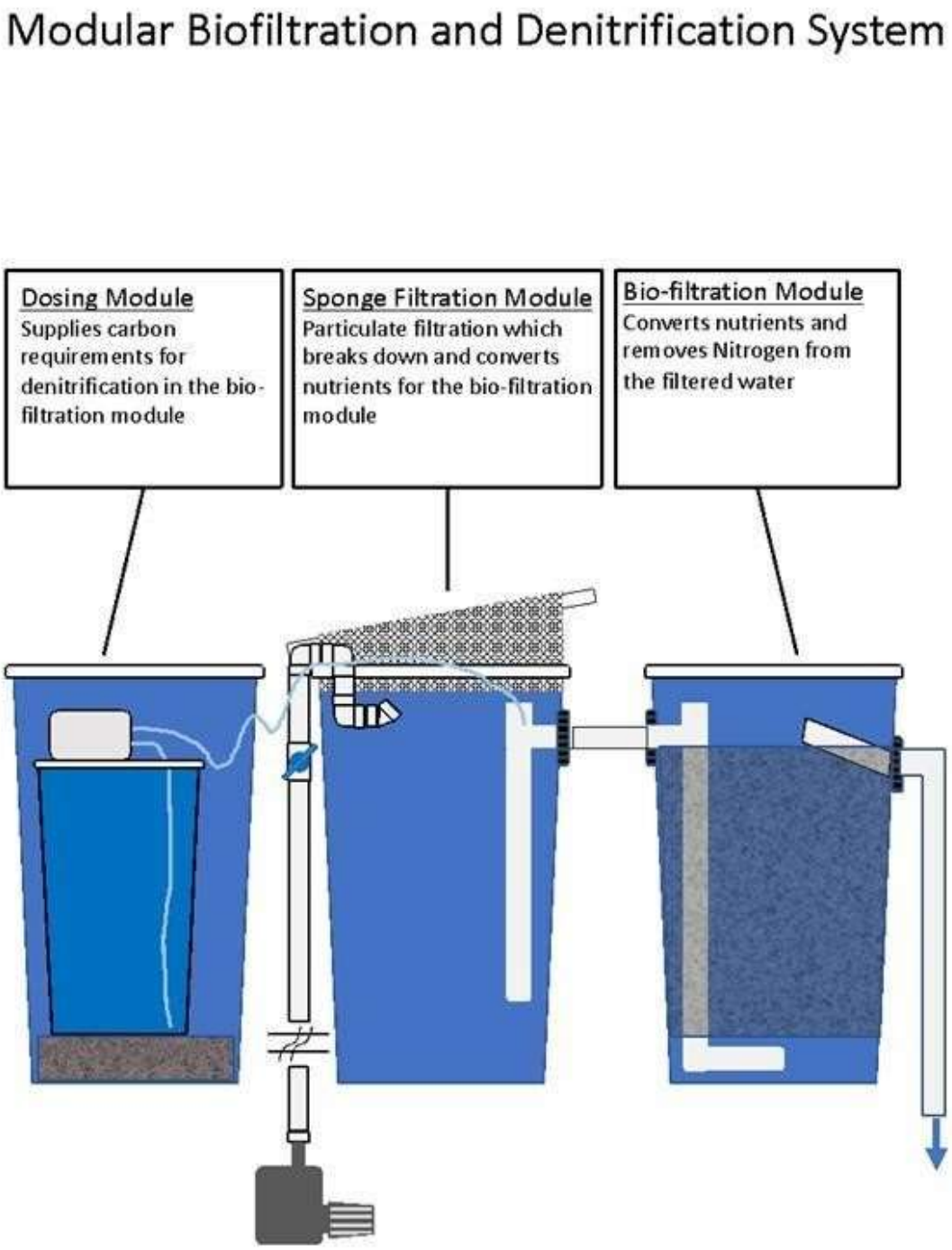
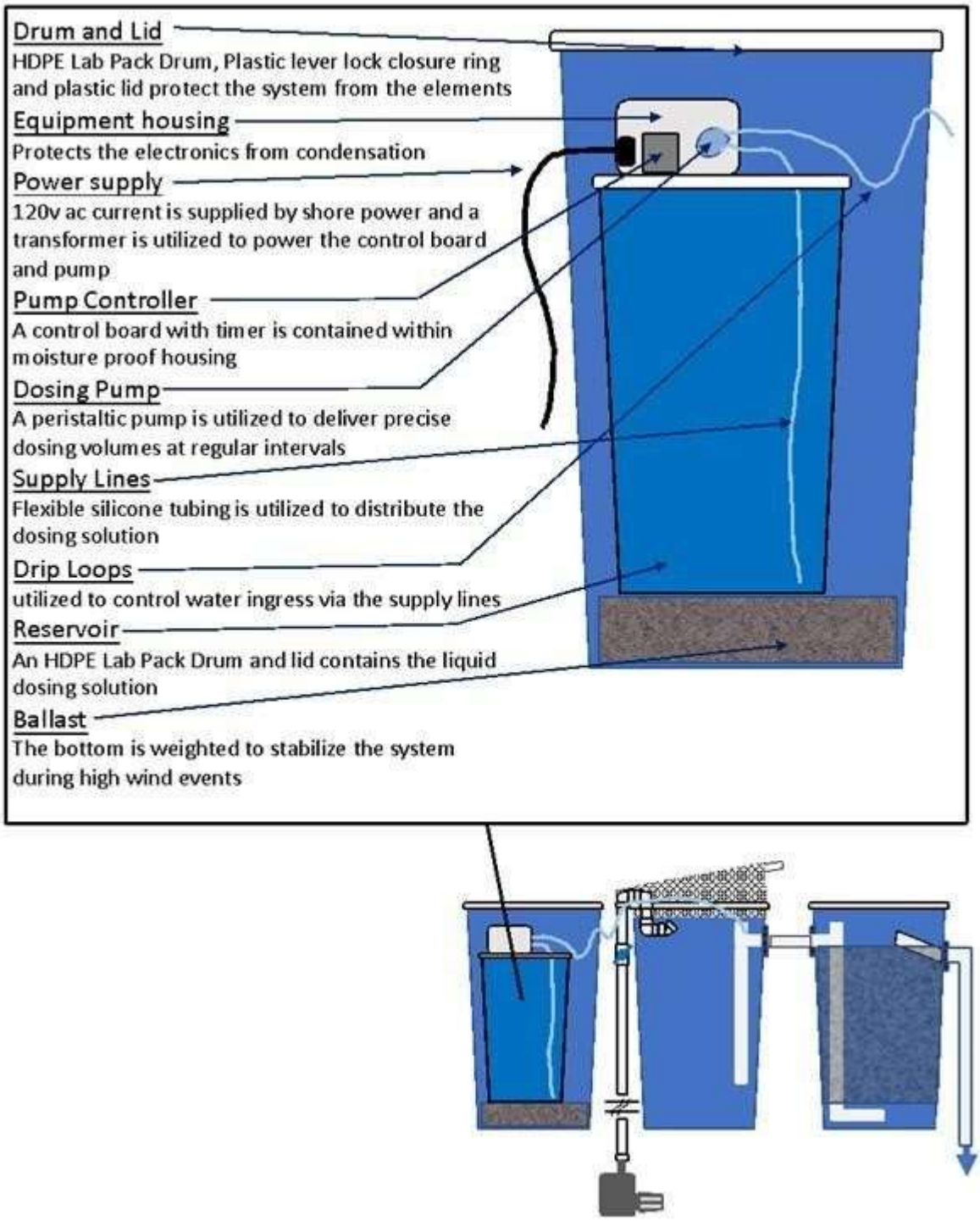
Appendices

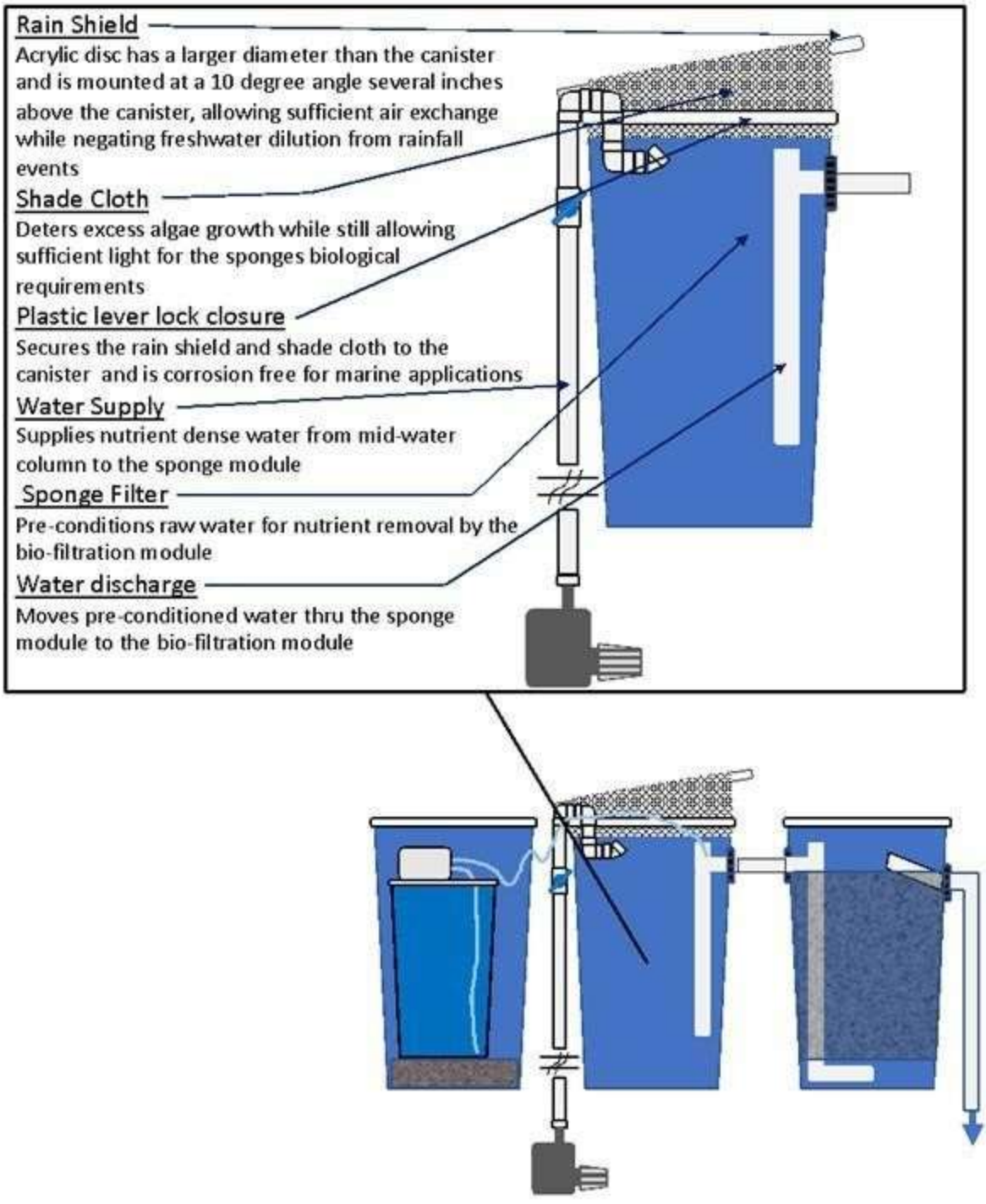
Appendix 1: Full Schematic of the Modular Biofiltration and Denitrification System



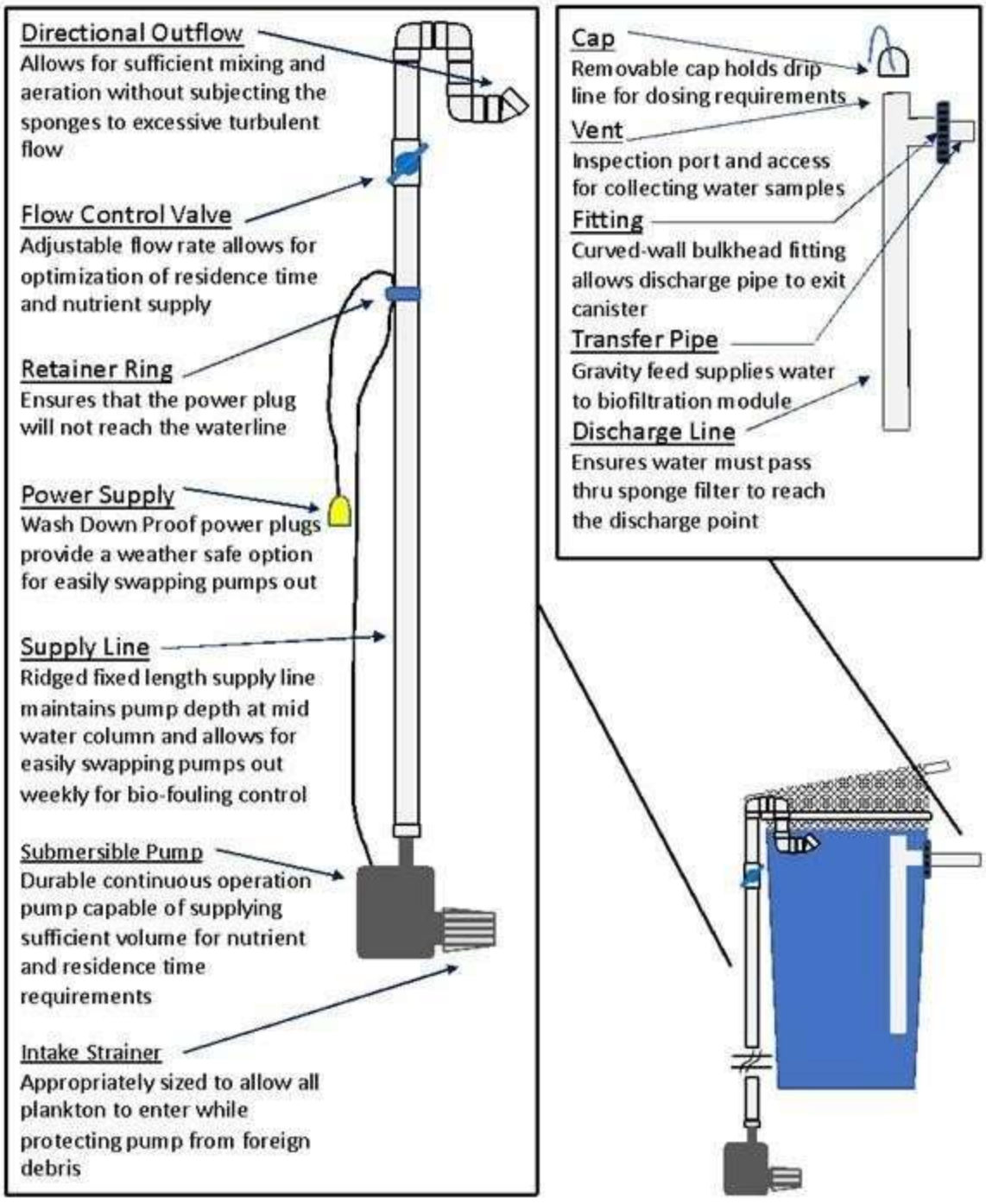
Dosing Module Canister Schematic



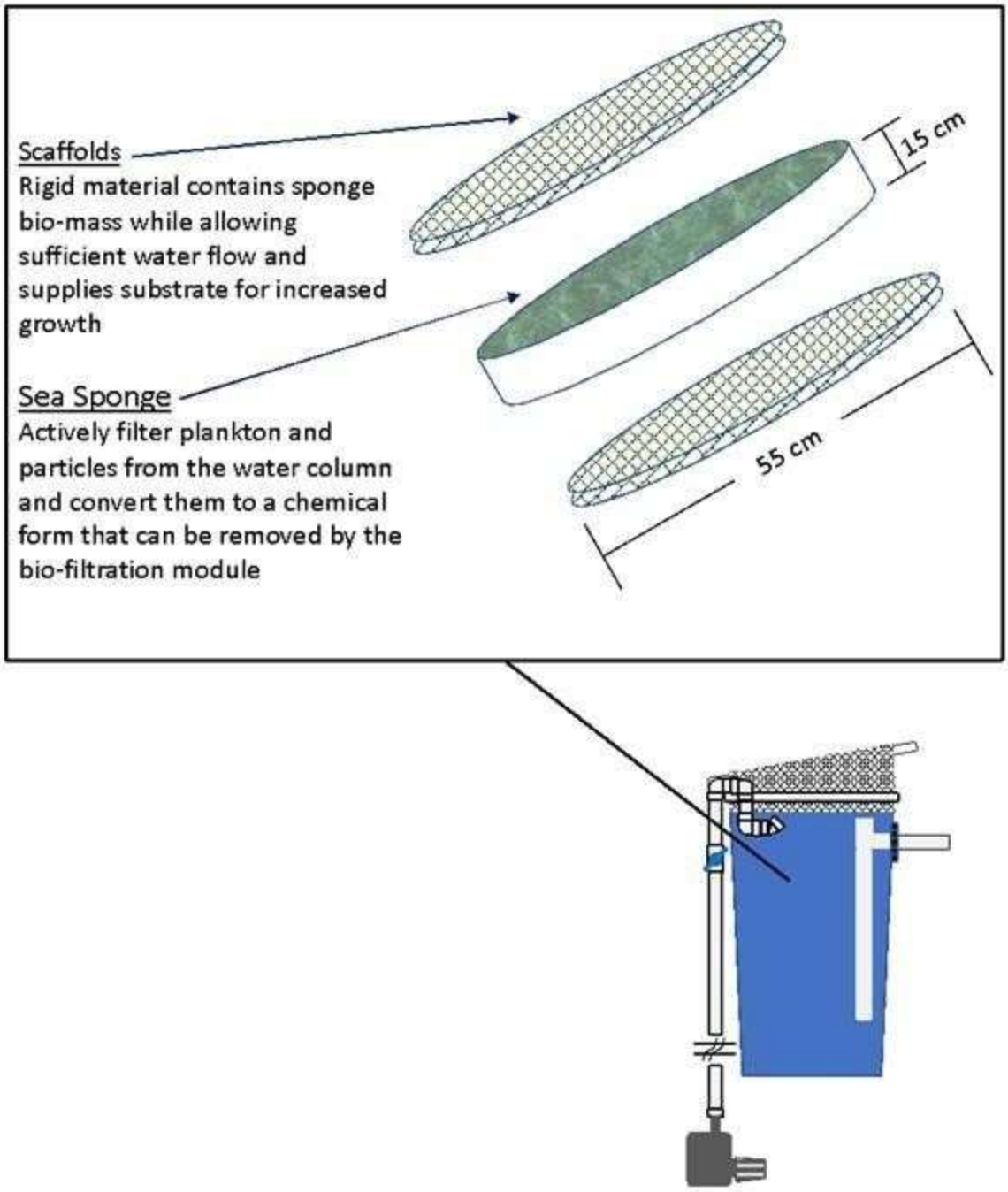
Sponge Module Canister Schematic



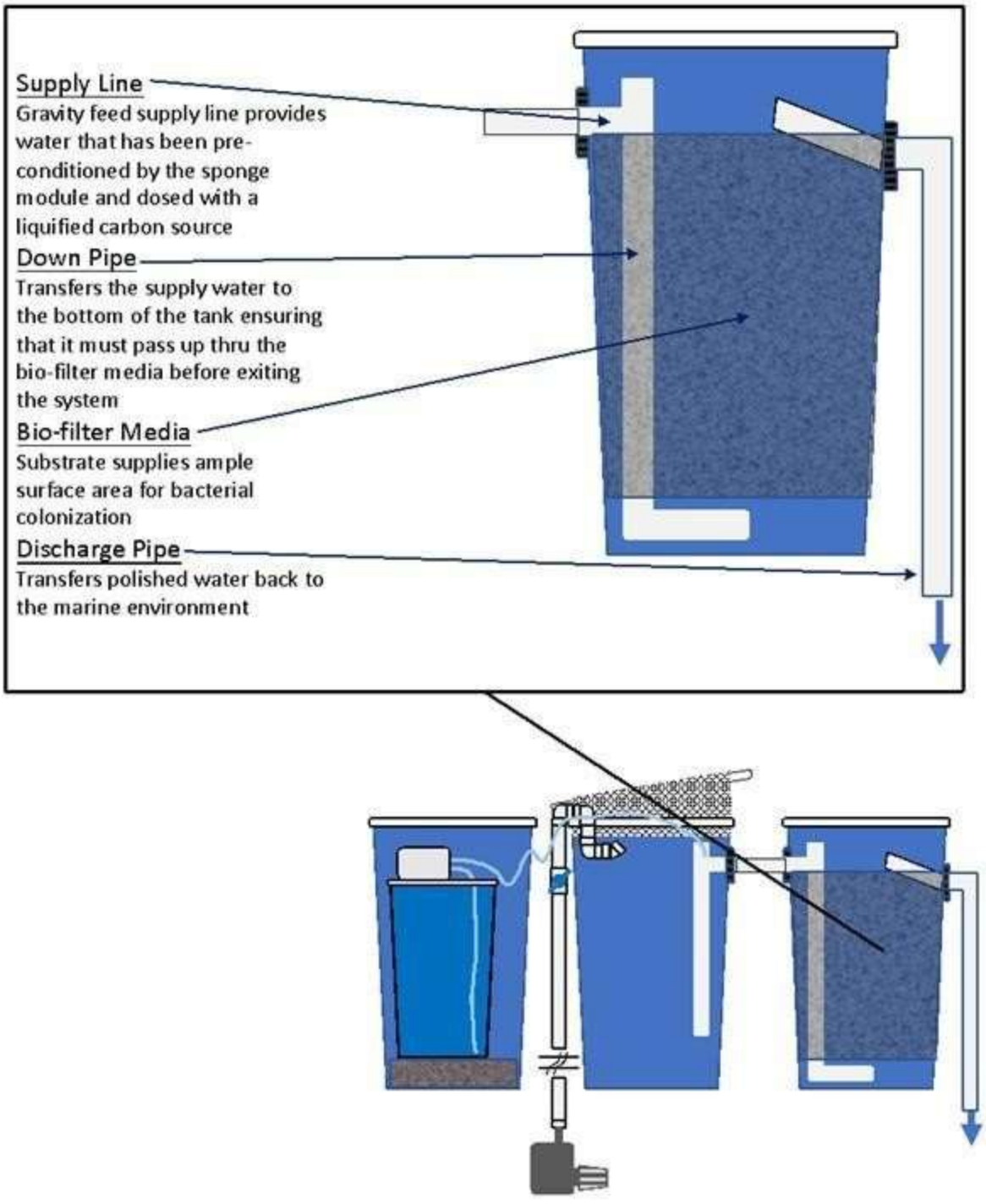
Sponge Module Plumbing Schematic



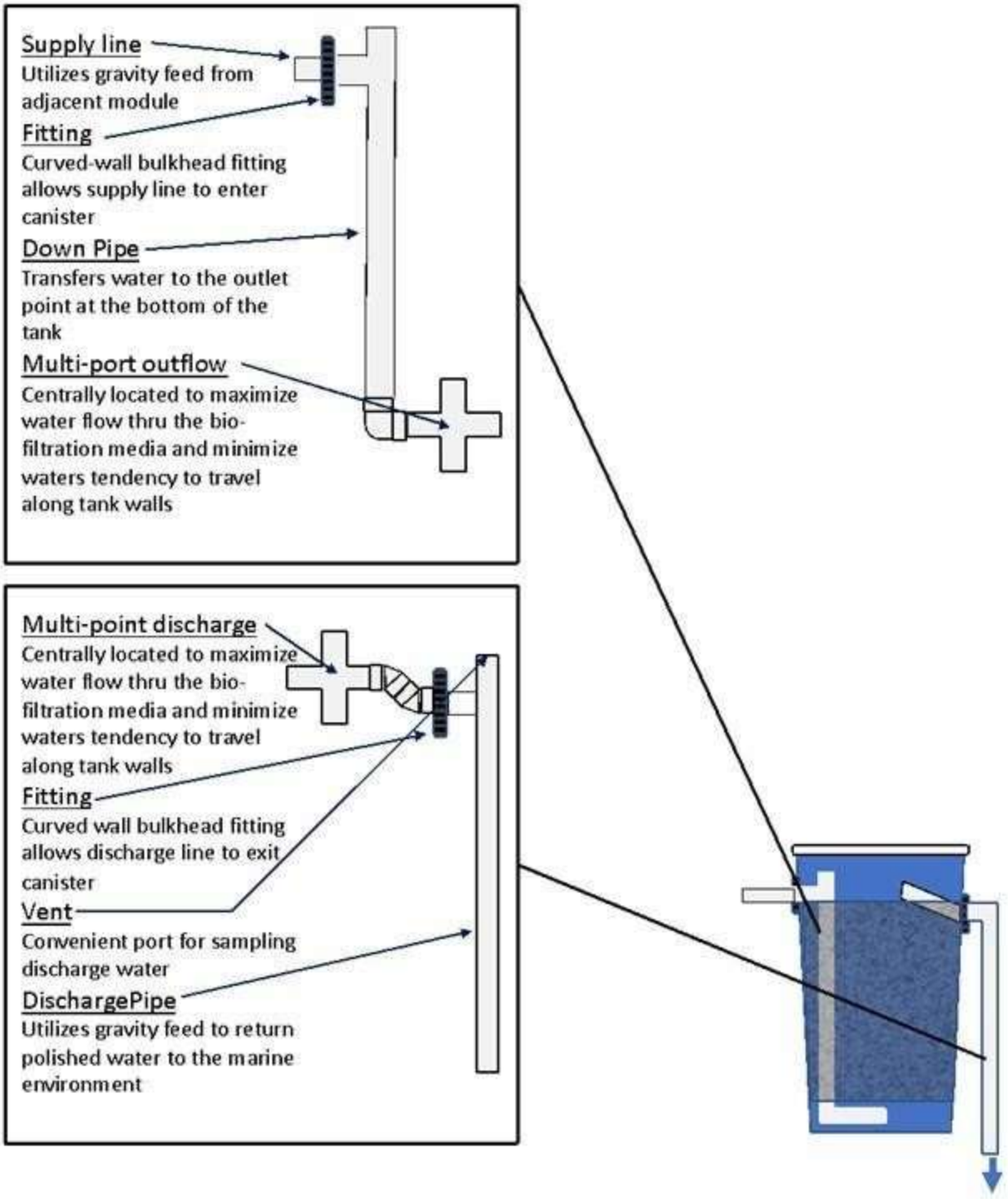
Sponge Filter Exploded Diagram



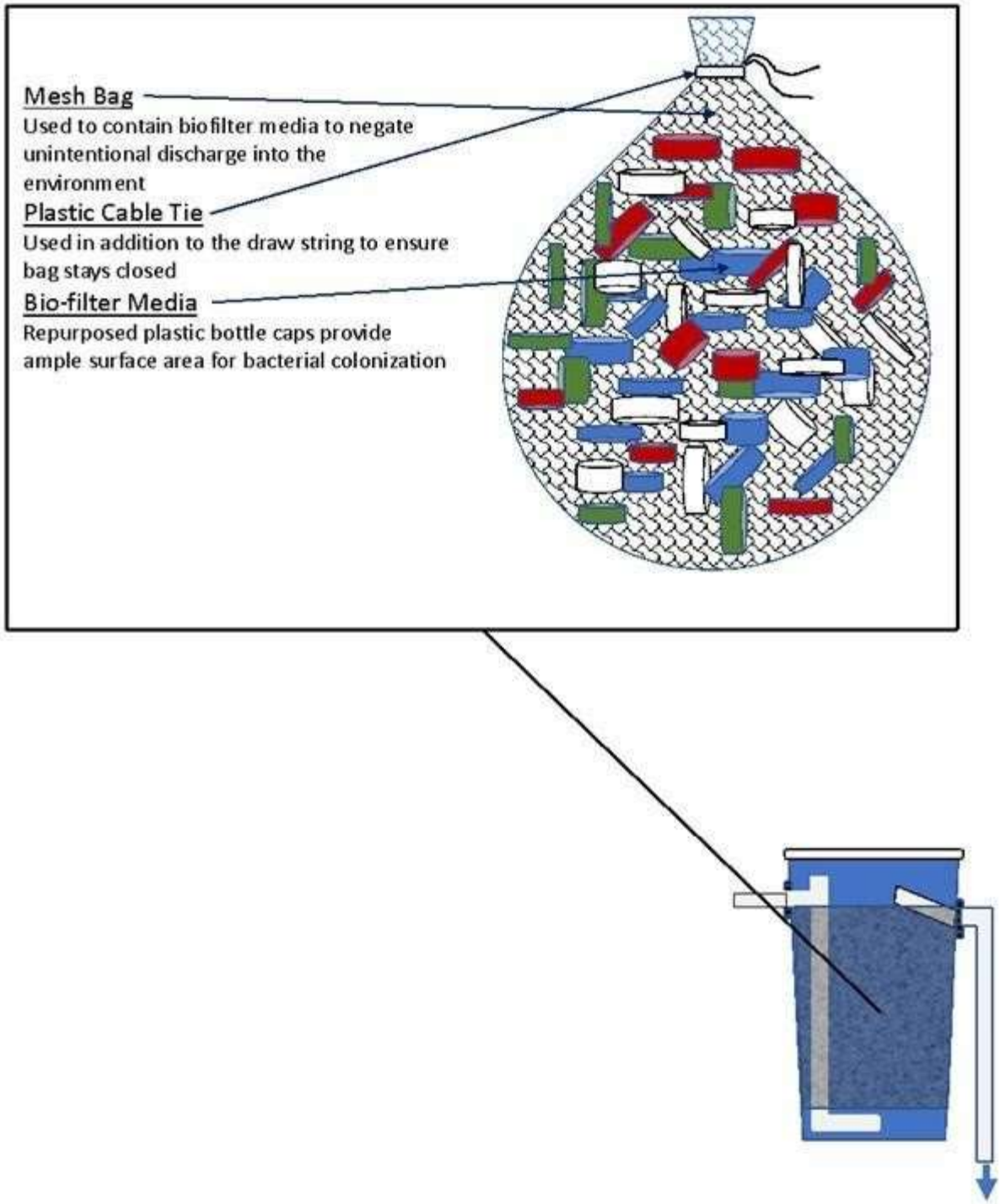
Biofiltration Module Canister Schematic



Biofilter Module Plumbing Schematic



Biofilter Exploded Diagram



Appendix 2: General project information

1. Project Timeline and Budget Summary:

The project was originally scheduled as summarized in Table 1a, with a total cost of $292,000.00
Table 1a: Original project timeline including start and end dates as well as deliverable due dates.

Task/ Deliverable No.	Task or Deliverable Title	Task Start Date	Task End Date	Deliverable Due Date/ Frequency
1	Quality Assurance Project Plan	7/1/2021	5/15/2023	
1a	Draft Quality Assurance Project Plan			2/15/2023
1b	Final Quality Assurance Project Plan			4/15/2023
2	Design, Construction and Optimization	7/1/2021	11/1/2024	
3	Permitting	7/1/2021	11/1/2023	
4	Monitoring	9/1/2023	1/1/2024	
5	Final Report	7/1/2021	5/15/2024	
5a	Draft Final Report			2/15/2024
5b	Final Report			4/15/2024

Work on design, construction and optimization began immediately following approval of the QAPP on 6/9/2023. The timeline was adjusted using a no cost extension on 6/18/2024 to allow results from monitoring to be received, prior to submission of the draft final report. This change would also facilitate review after submission of the draft final report. The updated timeline is summarized in Table 2a

.

Table 2a: Amended project timeline includes start dates, end dates and deliverable due dates.

Task/ Deliverable No.	Task or Deliverable Title	Task Start Date	Task End Date	Deliverable Due Date/ Frequency
1	**Quality Assurance Project Plan**	**7/1/2021**	**5/15/2023**	
1a	**Draft Quality Assurance Project Plan**			**2/15/2023**
1b	**Final Quality Assurance Project Plan**			**4/15/2023**
2	**Design, Construction and Optimization**	**7/1/2021**	**6/15/2024**	
3	**Permitting**	**7/1/2021**	**6/15/2024**	
4	**Monitoring**	**9/1/2023**	**6/15/2024**	
5	**Final Report**	**7/1/2021**	**9/30/2024**	
5a	**Draft Final Report**			**6/30/2024**
5b	**Final Report**			**8/31/2024**

Table 3a: Project budget summary table.

Category Totals	Grant Funding, Not to Exceed, $
Supplies	$28,555.88
Salaries	$135,960.00
Fringe	$37,044.14
Indirect Cost (44.87%)	$90,439.98
Total:	**$292,000.00**

There was no change to the budget table over the course of the project. Details regarding the final project cost will be provided by our Office of Sponsored Programs and will accompany the final invoice.

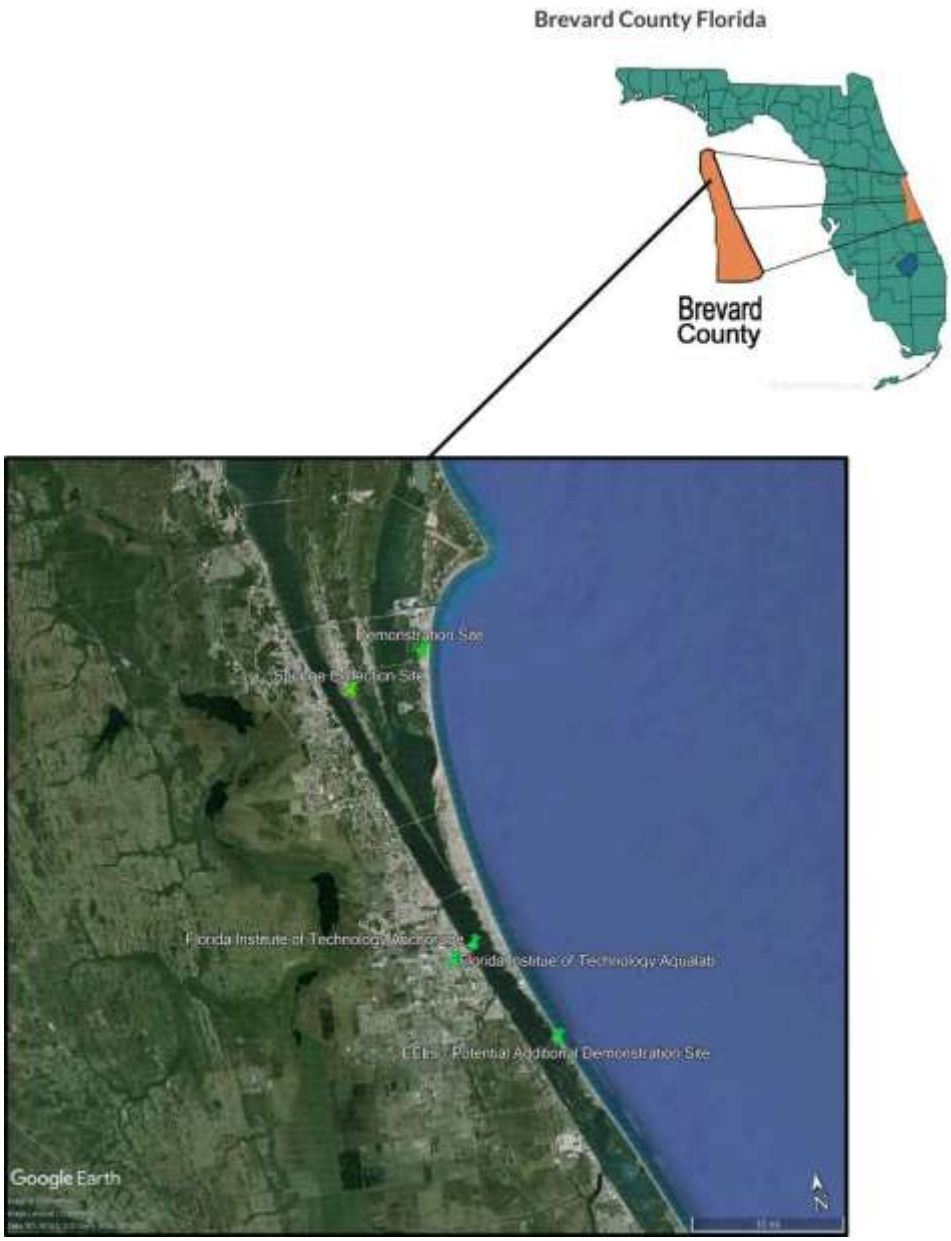


Figure 1a: Project locations in the Indian River Lagoon on the east coast of Florida.

Appendix 3: QAPP

See attached pdf of the executed QAPP.

Appendix 4: Monitoring Data - July

Table 1. FIT monitoring water chemistry data

FIT data	Ammonium (µg/L)			Nitrate + Nitrite (µg/L)			Orthophosphate (µg/L)		
Sample Date	In flow	Out flow	% change	In flow	Out flow	% change	In flow	Out flow	% change
07/09/24	76	313	309	11	25	123	6	24	277
07/16/24	183	272	48	17	9	-45	10	18	82
07/23/24	143	158	10	19	17	-7	5	7	43
07/30/24	54	138	154	23	27	15	2	3	46

Table 2. FIT monitoring water quality data

Sample Date	ODO (% SAT)			ODO (MG/L)		
	In flow	Out flow	% change	In flow	Out flow	Change (mg/L)
07/09/24	56.43±0.86	28.5±0	-49	3.65±0.06	1.84±0	-1.81
07/16/24	70.58±0.28	12.55±0.065	-82	4.57±0.02	0.81±0	-3.77
07/23/24	38.35±0.27	21.45±0.065	-44	2.48±0.02	1.39±0	-1.09
07/30/24	61.38±0.17	30.23±0.11	-51	3.91±0.01	1.91±0.01	-2.00

Table 3. FIT monitoring water quality data

Sample Date	Turbidity (NTU)			pH		
	In flow	Out flow	% change	In flow	Out flow	% change
07/09/24	4.67 ± 0.14	4.12 ± 0.11	-12	8.46 ± 0	8.34 ± 0.01	-1
07/16/24	4.67 ± 0.3	5.61 ± 0.06	20	8.48 ± 0	7.94 ± 0.01	-6
07/23/24	3.32 ± 0.18	11.33 ± 0.24	241	8.4 ± 0	8.24 ± 0.01	-2
07/30/24	3.15 ± 0.08	5.69 ± 0.38	81	8.33 ± 0	7.86 ± 0.01	-6

Table 4. FIT monitoring water quality data

Sample Date	PE (RFU)			CHLOROPHYLL (RFU)		
	In flow	Out flow	% change	In flow	Out flow	% change
07/09/24	2.27 ± 0.22	0.74 ± 0.01	-67	0.67 ± 0.07	0.3 ± 0	-56
07/16/24	0.96 ± 0.05	0.48 ± 0.01	-50	0.35 ± 0.01	0.22 ± 0	-38
07/23/24	0.7 ± 0.02	0.82 ± 0.01	16	0.26 ± 0.01	0.3 ± 0	13
07/30/24	0.95 ± 0.03	0.23 ± 0.01	-76	0.33 ± 0.01	0.15 ± 0	-53

Table 5. FIT monitoring water quality data

Sample Date	Temperature (˚C)			Salinity (PSU)		
	In flow	Out flow	Change (˚C)	In flow	Out flow	Change (PSU)
07/09/24	31.77±0	31.85±0	0.08	23.14±0	23.05±0	-0.09
07/16/24	31.35±0	31.94±0.01	0.59	24.01±0	24.01±0	0.00
07/23/24	31.27±0.03	31.26±0	-0.01	24.38±0.01	24.38±0	0.00
07/30/24	32.14±0.01	32.65±0	0.51	24.78±0.01	24.84±0	0.06