DEPARTMENT OF ENVIRONMENTAL PROTECTION

Final Report

DEP Agreement No.:	INV21		
Grantee Name:	Florida Institute of Technology (FIT)		
Grantee Address:	150 W. University Blvd, Melbourne, FL 32901		
Grantee's Grant Manager:	Austin Fox	**Telephone No.:**	(321) 674-7463
Reporting Period:	Final Report 7/1/2021 to 6/30/2024		
Project Number and Title:	An innovative bioreactor utilizing repurposed materials to remove nitrogen and phosphorus in the Indian River Lagoon		

Tasks 1-5a are complete as of 6/30/2024.

Task 1 (Complete):
The QAPP was approved by DEP on May 18, 2023. The final deliverable for Task 1, the completed QAPP package, was submitted on 6/29/2023.

Task 2 (Complete):
Design, construction, and optimization of the scalable bioreactor are complete. Deliverables 1) An electronic copy of the final design; 2) Dated color photographs of the final constructed fixed film bioreactor; and 3) Summary and results of laboratory and field optimization experiments (dates completed, sampling and analysis conducted, and results of any sampling and analysis, along with interpretation of those results) are included in the accompanying final report.

Task 3 (Complete):
Permit exemption requests were submitted to FDEP and Army Corps. Exemption waivers were received from both FDEP and Army Corps based on the nature of this project. FDEP File No.: 444910-001, Army Corps File No.: SAJ-2024-00643 (NPR-JAZ). Final deliverables are included in the accompanying final report and appendices.

Task 4 (Complete):
Monitoring is complete with samples collected for third party (certified lab) analysis on 5/9/24, 5/16/24, 5/23/24 and 5/30/24. Final deliverables are included in the accompanying detailed scientific report and appendices.

Task 5 (Complete):
The draft final report (Deliverable 5a) summarized the results of the project including all tasks in the Grant work plan. Deliverable 5b, the Final Report addressing comments was submitted on 8/19/2024 and Resubmitted on 10/4/2024 .

INV21: An innovative bioreactor utilizing repurposed materials to remove nitrogen and phosphorus in the Indian River Lagoon

Final Report Submitted to Florida Department of Environmental Protection

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Florida Institute of Technology (Florida Tech), Melbourne Florida, USA

August 2024

Executive Summary:

Bioreactors can be used to promote natural bacterial processes in a controlled environment where coupled nitrification-denitrification contribute to removal of bioavailable forms of nitrogen from the water column. These bacteria are naturally present in lagoon water and sediments; however, degraded water and sediment quality have resulted in conditions that limit the potential for nitrogen removal. Creating a habitat (bioreactor) that maintains ideal growing conditions promotes these natural processes to restore nitrogen assimilation that should be occurring naturally in the lagoon. To create these conditions, bacteria need a suitable surface to adhere to; this is, in nature, the grains of sand both above and below the aerobic-anaerobic interface (Natori et al., 2024).

This project was intended to design and build a scalable, coupled nitrification-denitrification bioreactor, specifically for use in the Indian River Lagoon System. The underlying principle behind the design relies on amplifying natural processes. This is accomplished by creating an environment ideally suited for both aerobic nitrifying bacteria and anaerobic denitrifying bacteria. This was accomplished first in the laboratory where a series of experiences helped to optimize performance before constructing and testing a scalable field scale system.

Laboratory experiments were used to test the efficiency of a supplemental anaerobic cell (oxygen concentrations), carbon sources, carbon concentration (C:N) and residence times. Prior to each experiment, bacteria were cultivated for approximately one month under test conditions (oxygen concentrations, carbon source, C:N ratios and residence times). This pre-culture or establishment period allowed for the bacterial cultures equilibrate to experimental conditions prior to sampling.

The bioreactor media was created using repurposed plastic bottle caps composed of high-density polyethylene (HDPE) and polypropylene (PP) with a surface area to volume ratio of 300-400 m^2/m^3. These same materials, HDPE and PP, are used in commercially available growth media (e.g., bioballs). Bottle caps were selected for this application following an intensive study of different growth media used to promote nitrification-denitrification in the IRL system (Gering, 2021).

Both aerobic and anaerobic bacteria utilize carbon as a metabolite in nitrification and denitrification. To promote these processes, supplemental carbon can increase rates of nitrogen removal (Tam et al., 1992). Four carbon sources were considered: molasses, sucrose, methanol and acetic acid based on past use (e.g., Khanitchaidecha et al., 2010; Tam et al., 1992; Isaacs and Henze, 1995; Zou et al., 2022; Yan et al., 2022). Sucrose is ideally suited for use in the lagoon system as it is naturally produced by seagrasses (e.g., Sogin et al., 2022). All carbon sources yielded significant decreases in ammonium concentrations over the 1-hour incubation period. The sucrose and molasses solutions decreased ammonium concentrations by >80% (81% and 87%). Acetic Acid and methanol removed <50% (39% and 23%) of the ammonium. With the highest efficiency towards nitrogen removal, sucrose was selected for further experiments to refine C:N ratios and residence times. Sucrose is also

an ideal carbon source for use in IRL because it is naturally produced and stored in the marine environment by seagrasses and environments where coupled nitrification-denitrification occurs naturally (Sogin et al., 2022).

Carbon to nitrogen ratios were refined by testing molar ratios ranging from 3 to 35 in flow-through bioreactors. By varying carbon additions relative to average influent nitrogen concentration, molar C:N ratios were maintained at 3, 9, 18 and 35. At a C:N ratio of 3, DIN decreased by 54%. Increasing the C:N ratio to 9 resulted in 98% removal of DIN. Further increasing the carbon dosing and the C:N ratio resulted in no additional removal of nitrogen and no substantial increase in P removal. Achieving 98% nitrogen removal suggests that the 8-hour residence time is longer than necessary and further experiments were carried out to identify the minimum required residence time using sucrose as a carbon source and a C:N ratio of 18. This ratio was selected to account for any spikes in influent nitrogen concentrations.

At normal influent concentrations, residence times between 8 hours and 5 minutes each removed at least 93% of the DIN and most of the DIP. With residence times between 2 and 8 hours the system achieved 98% removal of DIN. Decreasing the residence time did slightly decrease the total % removal, with 96% removal of DIN at residence times between 30 and 90 minutes. With a residence time of just 5 minutes, the system still achieved 93% removal of DIN. Despite a lower overall removal percentage, the system with a 5-minute residence time treats 96 times more water (8*60 = 480 minutes / 5 minutes = 96x) than a system with a residence time of 8 hours. The ideal residence time balances the % removal and throughput to achieve the greatest total removal of nitrogen.

Based on results from laboratory testing, a field system was designed with a focus on scalability. The primary bioreactors were constructed in 55-gallon plastic drums that can be connected in series to increase treatment capacity. Water was supplied from IRL using a pump set to 50 gallons per hour achieve an ~1-hour residence time (and remain within the permit limits). Carbon dosing was accomplished using peristaltic pumps set to achieve the C:N ratio of 18 based on results from laboratory experiments and typical influent nitrogen concentrations. During the May 2024 monitoring period, samples from the field system were collected weekly and transported to Pace labs for analysis. Overall, influent concentrations of ammonium and nitrite plus nitrite ranged from 63 to 160 µg/L and 23 µg/L to below detection, respectively (Pace Labs practical quantification limit for ammonium and nitrate are 0.05 mg/L (50 µg/L)). Median influent ammonium was 118 ± 49 µg/L (8.4 ± 3.5 µM). After treatment, all values for ammonium and nitrate plus nitrite were below detection (between the detection limit and zero). Clearly the system is effective and was limited only by influent nitrogen concentrations.

These data and findings clearly demonstrate the potential for bacterially mediated processes (e.g., nitrification-denitrification) to remove significant quantities of nitrogen from coastal systems. Ideally, these processes can be promoted in the natural environment by restoring and promoting healthy benthic habitats; however, this project demonstrated the ability to stimulate these processes in a self-contained and scalable bioreactor system. Our hope is that these systems can be used to disrupt feedback loops, thereby helping these naturally

occurring process to become once again self-sustaining in targeted areas throughout IRL. Although we identified decreased percent removal with shorter residence times, the total nitrogen removal was inversely related to residence time and further decreasing residence time may be advantageous. Moving forward, we would recommend testing residence times less than 5 minutes to determine a maximum treatment capacity and determine the size of a system required to treat for example, an entire streamflow.

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1. Background / Introduction:

Globally, coastal systems have experienced declining water quality often attributed to eutrophication, altered coastal land use and hydrology plus changes in rainfall patterns and other stressors. These perturbations are often associated with increasing human population and development of coastal areas. Although there are many stressors, nutrients and eutrophication are in many cases the dominant driver of ecosystem level changes. As a result, management agencies, where they exist have in many cases developed nutrient load reduction strategies. In the state of Florida these strategies are encompassed in Basin Management Action Plans (BMAPs) with similar management strategies used elsewhere, for example, watershed implementation plans (WIPs) in Chesapeake Bay. These plans reference regulatory terms from the U.S. Clean Water Act that describes the maximum amount of a pollutant (N or P) that a waterbody can receive while still meeting water quality standards. The total loading is divided into three categories, a Waste Load Allocation (WLA), a Load Allocation (LA) and a margin of safety (MOS). The WLA is the sum of all point sources while the LA includes all non-point sources and background concentrations. A margin of safety accounts for uncertainty in how changing pollutant loading will influence water quality (EPA).

$$TMDL = \sum WLA + \sum LA + MOS$$

These management targets have helped to guide investment and development of load reduction plans such as BMAPs and WIPs. Although TMDLs are based on loading, the underlying principle of these management strategies relies on an estimate of a systems assimilative capacity. In coastal systems, assimilation or mitigation of nutrient loading occurs in several ways. Ideally, a sustainable fishery helps to remove nutrients through biomass harvest. Additionally, in healthy systems bacterial populations carry out coupled nitrification-denitrification, phosphorus is sequestered onto oxic sediment particles and bound in mineral forms, some nitrogen and phosphorus are buried in sediments as refractory organic matter and some is discharged to the coastal ocean (Nixon et al., 1996). In healthy estuaries these ecosystem services are capable of assimilating external nutrient loading while mitigating adverse impacts. As ecosystem health declines, a series of feedback loops help to sustain alternate stable states with one of the consequences being a diminished ability to assimilate nutrients without experiencing adverse impacts (Kemp et al., 2009). One of these impacts, hypoxia and anoxia can decrease the efficiency and overall role of sediment denitrification as a sink for nitrogen (Cornwell et al., 1999). As a result of these feedback mechanisms, restoration and loss trajectories may be non-linear with respect to changes in nutrient loading (Harris et al., 2015). This is the combined result of the loss of ecosystem services including coupled nitrification-denitrification, bioturbation / bio irrigation, and decreased biomass harvest.

Locally, IRL has experienced degraded water quality over the past decade with a notable shift coinciding with the super bloom in 2011. Following this shift, algal blooms have become more frequent and less predictable based on rainfall and external nutrient loading (Phlips et al., 2021). This change brought about renewed interest in the importance of internal sources

of nutrients to IRL. Recent studies suggest that benthic fluxes of dissolved nitrogen and phosphorus account for 20-40% of the total internal + external loading (Fox and Trefry, 2023). The contribution from internal sources likely increased post 2011 because of a loss of plant biomass, with 58% of seagrasses lost from IRL between 2011 and 2019 (Morris et al., 2022). By pumping oxygen across the sediment water interface, seagrasses can contribute to maintaining oxic sediments thereby promoting aerobic bacterial processes including nitrification. Other studies have reported higher rates of coupled nitrification-denitrification in vegetated compared to unvegetated sediments (e.g., Chen et al., 2021). This results from symbiotic relationships between aquatic plans and sediment bacteria with root and rhizome systems supplying oxygen and labile organic carbon to sediments (Racchetti et al., 2017; Aoki and McGlathery 2017, 2018). The loss of seagrasses and other habitats likely decreases the capacity of an estuary to assimilate nutrients without experiencing adverse impacts. As a result, the assimilative capacity and the underlying assumption of a TMDL are dynamic and vary over time with a healthy estuary able to assimilate larger quantities of nutrients. By restoring sediment conditions, we can help to restore these ecosystem services over large expanses of an estuary contributing to an increase in assimilation capacity. When estuaries become overwhelmed with organic matter or nutrients, instances of hypoxia/anoxia become more prevalent, and denitrification can become less efficient via decreased nitrification. In eutrophic systems such as the IRL, hypoxia/anoxia can decrease the efficiency and overall role of denitrification as a sink for nitrogen (Cornwell et al., 1999). In these cases, it may become necessary to supplement natural nutrient removal. Bacterial process responsible for nitrogen removal, are utilized in active and passive water treatment systems such as wastewater treatment plants and bioswales using bioactive media BAM.

As we seek methods to address nutrient loading into IRL, BMAPs list projects aimed at decreasing nutrient loads. These projects include upgrades to wastewater infrastructure / bioreactors with active biological treatment. At the other extreme, the use of bioactive media promotes nitrogen cycling in passive environments and is dependent on conditions at each location. Both extremes are typically designed for use in freshwater and mitigating upstream nutrient loading; however, 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 recirculating aquaculture systems 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 eta l., 2006). This is at least partially due to a lower specific growth rates and metabolic activity of nitrifying bacteria in marine versus freshwater environments (Ramaswami et al., 2019). Nevertheless, bioreactors can still be a valuable method to decrease nutrient loading from stormwater.

To date, most active bioreactors are not used to treat natural water for the reasons stated above, but also because impaired coastal waters have significantly lower nitrogen concentrations than aquaculture systems, aquariums or wastewater and their efficiency is limited by influent nitrogen concentrations. Regardless, biological treatment systems are a viable option to remove nitrogen and phosphorus from natural waters by promoting bacterial processes. From a management perspective, these treatment strategies normally help to mitigate nutrient loading as part of either the WLA or the LA; however, in developing

bioreactors for use in natural water, this knowledge can help restore ecosystem services through targeted restoration efforts that will improve the assimilative capacity of the estuary. Viewing the underlying mechanisms that TMDLs represent as dynamic, restoration of bacterial nutrient cycling will increase assimilative capacities and the TMDL itself.

In the Indian River Lagoon Basin, Central Indian River Lagoon BMAP, FDEP proposed a DO and nutrient TMDL for Crane Creek that receives drainage from Melbourne and Melbourne Village (WBID 3085A; FDEP, 2013). Drainage from Crane Creek is encompassed in the Central IRL BMAP (WBID 2963A). To address eutrophication in tributaries to IRL, we have designed and built a coupled, nitrification-denitrification bioreactor, specifically for use in the Indian River Lagoon System. The goal of this effort was to 1), establish a scalable bioreactor for treatment of stormwater and lagoon water and 2) leverage data to better understand the assimilative capacity of IRL.

The underlying principle behind the design relies on amplifying natural processes. This is accomplished by creating an environment ideally suited for 1) aerobic nitrifying bacteria and 2) anaerobic denitrifying bacteria. These processes should ideally be carried out in lagoon sediments; however low concentrations of dissolved oxygen in porewater have likely shrunk the thickness of oxic sediments with a loss of habitat for aerobic nitrifying bacteria. This is supported by data showing a decrease in the relative abundance of dissolved nitrate versus other nitrogen species since the 2011 super bloom (SJRWMD data). This is also supported by increased concentrations of dissolved phosphorus after the 2011 super bloom (Phlips et al., 2021) that could have resulted from lower sorption capacities and release of phosphorous from sediments as they experience more frequent hypoxia (Pant and Reddy, 2011; Sorensen and Dahl, 2024). Creating a habitat for these aerobic bacteria, an oxic surface, would allow for recruitment of naturally occurring, nitrifying bacteria from influent water and sediment particles. In sediments, nitrogen cycling is largely controlled by oxygen but also by the availability of labile organic carbon and it is common in bioreactors to supplement with a labile carbon source. Many passive systems use wood chips or similar cellulose-based carbon that also acts as a surface for bacteria to grow. Unfortunately, these media deteriorate over with system losing efficiency. Other studies have supplemented inert media with readily bioavailable carbon sources including vinegar, molasses, methanol, and sugars (e.g., Hamlin et al., 2008). Developing this system for IRL, we previously tested media including biochar, zeolite, perlite, wood chips, sand, commercially available bio balls and an alternate repurposed plastic media, bottle caps (Gering and Fox, 2021). This study aimed to improve efficiency of that system while also compressing the system into a modular, scalable unit.

2. Project Timeline and Budget Summary:

The project was originally scheduled as summarized in Table 1, with a total cost of $169,850.

Table 1. 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 2.

Table 2. Amended 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	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			10/15/2024

The original project budget is summarized in Table 3. The budget was modified after Lapin Services was identified as a vendor and not a subcontractor. The modified budget (no change in cost) is summarized in Table 4.

Table 3. Original task and budget table including a breakdown of budget by task and budget category.

Task No.	Budget Category	Budget Amount
1	Contractual Services	$0
	Supplies/Other Expenses	$0
	Salaries	$990.00
	Fringe	$24.10
	Indirect Cost	$455.03
	Total for Task:	**$1,469.12**
2	Contractual Services	$81,818.18
	Supplies/Other Expenses	$10,950.35
	Salaries	$28,060.68
	Fringe	$4,569.998
	Indirect Cost	$30,772.31
	Total for Task	**$156,165.00**
3	Contractual Services	$0
	Supplies/Other Expenses	$0
	Salaries	$330
	Fringe	$8.03
	Indirect Cost	$151.68
	Total for Task	**$489.71**
4	Contractual Services	$8182.00
	Supplies/Other Expenses	$1095.04
	Salaries	$660
	Fringe	$16.07
	Indirect Cost	$794.69
	Total for Task	**$10,747.00**
5	Contractual Services	$0
	Supplies/Other Expenses	$0
	Salaries	$660
	Fringe	$16.07
	Indirect Cost	$303.35
	Total for Task	**$979.42**

Table 4. Revised task and budget table including a breakdown of budget by task and budget category.

Task No.	Budget Category	Budget Amount
1	Salaries	$990.00
	Fringe	$24.10
	Indirect Cost (44.87%)	$455.02
	Total for Task:	**$1,469.12**
2	Contractual Services	$63,516.19
	Supplies/Other Expenses	$10,945.73
	Salaries	$28,060.68
	Fringe	$4,570.00
	Indirect Cost	$48,052.46
3	**Total for Task**	**$155,145.08**
	Salaries	$330.00
	Fringe	$8.03
	Indirect Cost	$151.67
	Total for Task	**$489.70**
4	Contractual Services	$6351.62
	Supplies/Other Expenses	$1094.56
	Salaries	$660.00
	Fringe	$16.07
	Indirect Cost	$3,644.45
5	**Total for Task**	**$11,766.69**
	Salaries	$660.00
	Fringe	$16.07
	Indirect Cost	$303.35
	Total for Task	**$979.42**
	Total Budget	**$169,850.00**

This project was completed within budget and no major modification were required. A complete breakdown of the actual budget will be supplied by our office of sponsored programs. This project built upon prior efforts and significantly improved the efficacy of this conceptualized system while also leading to the development and implementation of a mobile and scalable system. Services provided by the contractor Lapin Services provided valuable but also facilitated their adoption of this project as an available service. With a maximum removal rate of 3 $kg/m^3/day$ demonstrated in the lab, this system is a viable option for the treatment of industrial effluent (e.g., dredge material) and stormwater.

3. Methods

For a complete list of laboratory methods, standard operating procedures and quality assurance steps, please refer to the submitted and approved QAPP.

4. Concept, Design and Laboratory Optimization:

4.1 Source of Water

Tributaries, including Crane Creek, transport stormwater runoff containing nutrients from urban sources including, sewage, leaking septic systems and excess fertilizer applications (Bradshaw et al., 2020). Crane Creek that receives drainage from Melbourne and Melbourne Village experienced flows ranging from 6 to 434 cubic feet per second (cfs) between 4/1/2023 and 6/15/2024 with mean and median discharge at 28 and 21 cfs, respectively (Figure 1 and Figure 2.). High discharge associated with storm events was short lived with flow returning to median values within ~1 day after rainfall. Prior data from Trefry and Fox, 2021 indicates that upstream Crane Creek receives a relatively large fraction of the total dissolved nitrogen (TDN; 64.8 ±18.5 µM or 900 ± 260 µg/L) as dissolved inorganic nitrogen (DIN; 37% of DIN, 8% ammonium and 29% nitrate plus nitrite). Dissolved inorganic nitrogen includes ammonium and nitrate plus nitrite, chemical species that are readily utilized by nitrifying and denitrifying bacteria. In this tributary, nitrate concentrations were negatively correlated with the log of flow with higher concentrations of DIN at lower flow rates. This suggests that Crane Creek is an ideal location for a flow through bioreactor system treating base flow and stormwater.

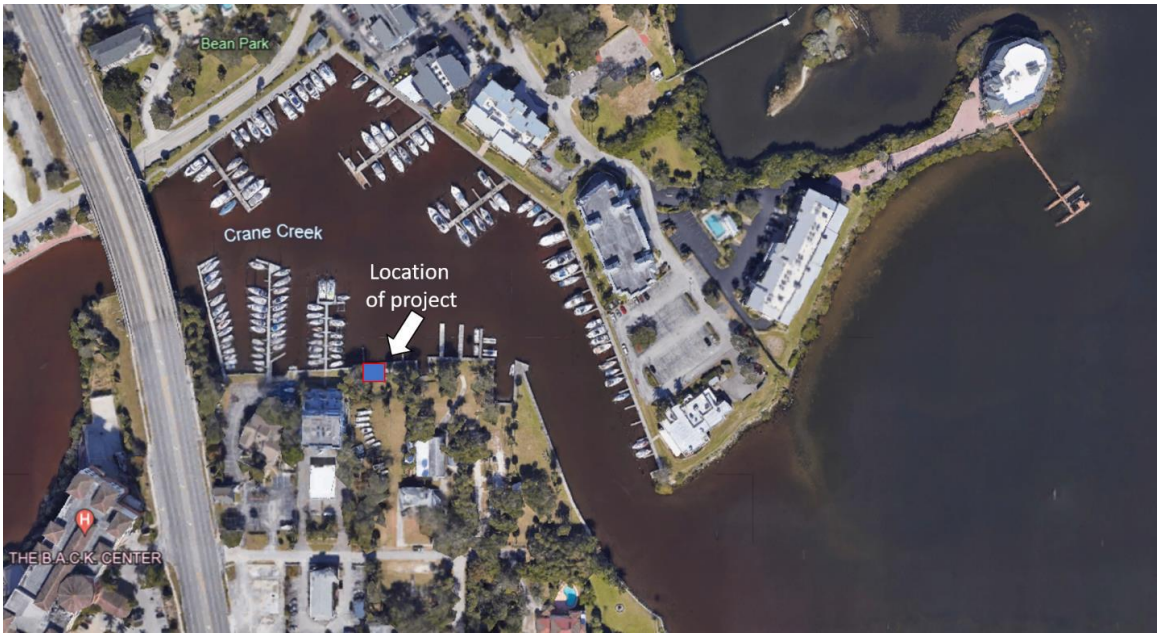

Figure 1. Map showing the location of the Florida Tech Anchorage.

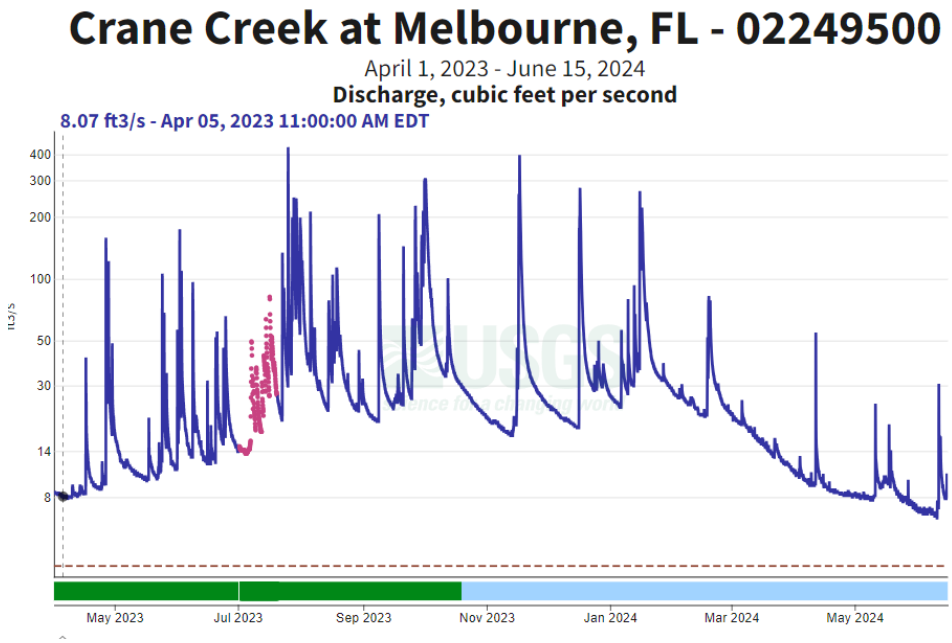

Figure 2. Discharge from Crane Creek (ft^3/sec) from April 2023 to June 2024 (USGS).

To carry out laboratory experiments, water was collected from Crane Creek at the Florida Tech Anchorage located at 28.077°N, -80.601°W (Figure 1). Water was obtained bi-weekly to support laboratory experiments and was immediately transferred to the Marine and Environmental Chemistry Laboratory at Florida Tech's main campus. On campus, water was aerated using microporous diffusers for up to 3 days in an influent/holding tank.

4.2 Bioreactor Media (Bottle Caps)

This system was developed to promote natural bacterial processes, coupled nitrification-denitrification, that together contribute to removal of bioavailable forms of nitrogen from the water column. These bacteria are naturally present in lagoon water and sediments; however, degraded water and sediment quality have resulted in conditions that limit the potential for nitrogen removal. Creating a habitat (bioreactor) that maintains ideal growing conditions promotes these natural processes to restore nitrogen assimilation that should be occurring naturally in the lagoon. To create these conditions, bacteria need a suitable surface to adhere to; this is, in nature, the grains of sand both above and below the aerobic-anaerobic interface (Natori et al., 2024).

Repurposed plastic bottle caps composed of high-density polyethylene (HDPE) and polypropylene (PP) were used as growth media. These same materials, HDPE and PP, are used in commercially available growth media (e.g., bioballs). Bottle caps were selected for this application following an intensive study of different growth media used to promote nitrification-denitrification in the IRL system (Gering, 2021). The prior effort identified bottle caps as the most efficient and cost-effective media when coupled with a supplemental carbon source. To determine the surface area (SA) to volume (V) ratio, a representative set

of bottle caps were carefully measured and modeled in 3D to calculate 3-dimensional surface area of bottle caps (e.g., Figure 3). Bioreactors were then loaded with bottle caps and the total caps per volume (L) were recorded.

Figure 3. 3D-model of a bottle cap used in the study.

Using the 3-dimensional surface area of bottle caps and the packing density (number per dm^3), the SA/V ratio was 300-400 m^2 of available surface area per m^3 of bioreactor space. This is about 80% as much as commercially available media (up to 500 m^2/m^3). Despite a slightly lower SA/V, this plastic was repurposed and acquired at no cost while promoting public education and outreach to the community without the need to manufacture additional materials.

4.3 Redox Environment and Aeration

In addition to a suitable surface, nitrifying and denitrifying bacteria require different redox environments. Nitrification is a two-step process characterized by the oxidation of ammonium nitrogen to nitrite by Nitrosomonas bacteria, followed by the oxidation of nitrite to nitrate carried out by Nitrobacter bacteria in aerobic environments ($NH_4 \rightarrow NO_2 \rightarrow NO_3$) (Puopolo and Savaglio, 2012). Subsequently, heterotrophic bacteria reduce nitrate (NO_3) to nitrite (NO_2) then to inert nitrogen (N_2) gas in anaerobic conditions (denitrification) ($NO_3 \rightarrow NO_2 \rightarrow NO \rightarrow N_2O \rightarrow N_2$) (Hamlin et al., 2008). Normally, these reactions are carried out successively using an aerobic cell followed by an anaerobic cell; however, in this system, nitrification and dentification occur simultaneously in an aerobic cell with anaerobic conditions within the fixed biofilms (Zeng et al., 2003). Recent studies have shown simultaneous nitrification-denitrification (SND) has achieved significant removal of nitrogen and carbon from wastewater, and in removing micropollutants (James and Vijayanandan, 2023). In a fixed film bioreactor, aerobic bacteria on the surface of biofilms, utilize oxygen available from the water column for nitrification, and anaerobic bacteria carry out denitrification in anaerobic micro zones within the biofilm (Figure 4).

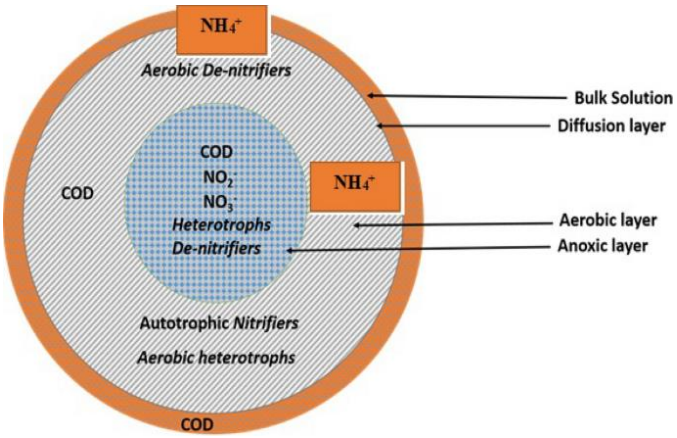

Figure 4. Fixed biofilm layers of nitrifying and denitrifying bacteria (James and Vijayanandan, 2023).

4.4 Carbon Source

Both aerobic and anaerobic bacteria utilize carbon as a metabolite in nitrification and denitrification. To promote these processes, supplemental carbon can increase rates of nitrogen removal (Tam et al., 1992). Sometimes, supplemental carbon is supplied through consumable media such as wood chips or maize (Saliling et al., 2007). Unfortunately, these media can degrade over time leading to a loss of efficiency with no long-term solution. Fortunately, there has been some research into the efficacy of supplemental carbon sources that we were able to use as a starting point (Figure 5).

Carbon source	C/N ratio (mg-C/mg-N)	Nitrogen removal efficiency (%)	Reference
Acetate	1.5	90-100	[2]
Methanol	1.5	90-100	
Acetate	5.0	~100	[3]
Glucose	5.0	~100	
Acetic acid	1.1	~100	[9]
Ethanol	0.6	~100	
Acetic acid	4.3	98	
Ethanol	2.35	91	[10]
Methanol	2.9	93	
Ethanol	2.5	~100	[11]
Succinate	2.5		
Molasses	6[a]	92	[6]
Corncobs	1.5[a]	90	[7]

[a] mg-COD/mg-N ratio

Figure 5. Table from (W. Khanitchaidecha et al., 2010) of nitrogen removal efficiencies and C/N ratios requirements observed using various carbon sources. NOTE: 1 mg C/ mg N = 1.17 mol C / mol N.

To develop the laboratory bioreactor, four carbon sources were considered: molasses, sucrose, methanol, and acetic acid based on past use (e.g., Khanitchaidecha et al., 2010; Tam et al., 1992; Isaacs and Henze, 1995; Zou et al., 2022; Yan et al., 2022). In past studies molasses used at a C:N mass ratio of 7 has provided efficient decreases in total ammonium and nitrate (Samocha et. al., 2007). Molasses, a byproduct of sucrose production is relatively inexpensive and contains micronutrients that can aid in bacterial growth. Sucrose, as a carbon source is relatively inexpensive and has been reported to achieve >95% removal of ammonium with C:N ratios ≥ 1 and a retention time of 6 hours (Cheng & Chen, 1994). Sucrose is ideally suited for use in the lagoon system as it is naturally produced by seagrasses (e.g., Sogin et al., 2022). Methanol is the most commonly used carbon source in many terrestrial applications including wastewater treatment plants (WRF, 2019). Methanol was reported to achieve 85% removal of NH_4-N during a 5-hour retention time (Tam et al., 1992). Acetic acid has also been used as an effective carbon source to treat groundwater (Mohseni-Bandpi et al., 1999).

Another consideration for carbon sources used in a scalable and transportable field system include the logistics, safety of transport and storage as well as potential permitting for hazardous materials (i.e., methanol or acetic acid). For transport and storage, the carbon source would ideally have a high carbon density. For example, concentrated versions of these carbon sources (e.g., molasses, granular sucrose, concentrated methanol and glacial acetic acid) yield differing carbon contents per unit mass and volume. Molasses, granular sucrose, methanol and acetic acid are ~24%, 42%, 38% and 40% carbon by weight, respectively. The densities for molasses, sucrose, methanol and acetic acid are: 1.6 g/cm^3, 1.58 g/cm^3, 0.79 g/cm^3 and 1.05 g/cm^3, respectively. Related to safety, molasses and sucrose are both edible and considered safe where concentrated methanol and acetic acid are hazardous materials. Granular sucrose being safe, naturally occurring in the environment and having the highest carbon content (m/m) and the highest density of carbon (m/v) make sucrose an ideal carbon source for environmental water treatment systems.

4.5 Residence Time:

The bacterially mediated nitrification and denitrification reactions require the correct bacteria, sufficient surface area, a mix of both aerobic and anaerobic zones, a supply of nitrogen and carbon, and sufficient reaction time for the successive nitrification-denitrification reactions to occur. Past studies have used a wide variety of residence/treatment times and the maximum nitrification rates in saltwater systems are reported to be considerably lower than in freshwater systems (Nijhof and Bovendewur 1990; Chen eta l., 2006). This is partially due to a lower specific growth rates and metabolic activity of nitrifying bacteria in marine environments and can result in the need for a longer residence time (Ramaswami et al., 2019). Nitrogen removal efficiency can be reported as percent removal that is independent of residence time (flow / time) or total removal that is often reported as mass of nitrogen/m^3/day. Gering, 2021 identified a maximum percent reduction for nitrogen (>90%) using residence times ranging from 8 to 48 hours and a combination of aerobic and anaerobic treatment cells. Other studies have used various residence times for example Cheng and Chen, 1994 used 6 hours. Building upon prior efforts, this study aimed

to identify an ideal ratio of residence time to removal efficiency (as percent) to maximize nitrogen removal (kg/m^3/day).

4.6 Experimental Setup:

The laboratory bioreactor was developed in two phases based on existing bioreactor technologies and considerations discussed above. These experimental designs were used to test the efficiency of a supplemental anaerobic cell (oxygen concentrations), carbon sources, carbon concentration (C:N) and residence times. Prior to each experiment, bacteria were cultivated for approximately one month under test conditions (oxygen concentrations, carbon source, C:N ratios and residence times). This pre-culture or establishment period allowed for the bacterial cultures equilibrate to experimental conditions prior to sampling. This establishment period eliminated artificially low removal efficiencies that could result if bacterial communities were not fully established.

The first set of experiments utilized a sequencing/sequential batch bioreactor (SRB). This type of bioreactor is commonly used in municipal water treatment with a distinct aerobic followed by anaerobic treatment cells. This type of bioreactor is (1) filled with influent, (2) allowed to react (in an aerobic cell) over a designated residence time (3) decanted to the next bioreactor cell, typically an anaerobic cell where denitrification takes place. In municipal water treatment facilities, there are of often mixing and settling phases; however, the fixed film used in this system eliminated the additional need for settling. In the lab, bioreactor cells were made from PVC tubes with volumes of ~2L (Figure 6 and Figure 7). Each tube contained a mechanical stirrer to homogenize water within the system and a HOBO U26 DO datalogger to track oxygen consumption. Tubes were packed with pre-incubated bacterial cultures grown on the bottle cap media and then filled with influent water brought to 100% DO saturation and dosed with supplemental carbon for treatment. An initial water sample was retrieved, and the chambers were sealed. Reactors were incubated for set residence times, opened and samples retrieved. If a subsequent phase was to be carried out, water was transferred to the anaerobic cell and the process repeated. This setup was used to investigate oxygen consumption and to identify the most effective carbon source to be used in subsequent optimization experiments.

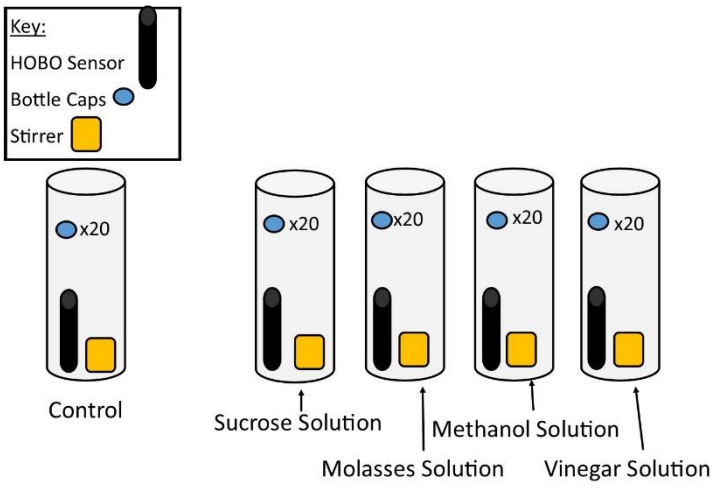

Figure 6. Schematic of batch bioreactors.

Figure 7. Images captured of batch bioreactor experiment A) Chambers, B) Chamber #1 showing packed bottle caps, C) The DO Logger and mechanical stirrer used inside the chambers during the experiment, and D) the set-up of the bottles to take samples and dose correct C:N ratios of carbon.

Following batch reactor experiments, the second set of experiments utilized a flow through fixed film bioreactor aimed at carrying out simultaneous nitrification and denitrification (SND). The flow-through treatment system was used to maximize nitrogen removal by maintaining optimal environmental conditions including oxygen levels and continuously supplying carbon and nitrogen to bacterial cultures (Figure 8). The laboratory scale bioreactor system consisted of influent/holding tanks (10-gallon glass aquariums) that were continuously filled with water from Crane Creek. Influent water was aerated using ambient air and microporous diffusers to maintain oxygen at levels consistent with the source water from Crane Creek. To build bioreactor cells, initially a 10-gallon aquarium was subdivided using acrylic panels into 4, 2.5-gallon (9.46 L) bioreactor cells (Figure 8 and Figure 9). The water level in each cell was adjusted to achieve the desired total bioreactor volume by changing the height of the drain port. Total volumes were adjusted (scaled) for logistical purposes related to transport of site water to the lab at the Florida Tech main campus. Influent water was pumped from influent holding tanks into each bioreactor cell using Atlas Scientific EZO-PMP embedded dosing pumps controlled using Arduino Uno R3 (A000066) microcontrollers programmed using Arduino IDE version 2.3.2. Pumps were calibrated using the manufacturer methods and programming. Flow through rates were determined (mL/min) based on the desired residence time and the total volume of each cell. For example, to achieve a 20-minute residence time in a bioreactor cell containing 5 L of water, influent water was pumped at 250 mL/min (250 mL/min * 20 min = 5000 mL or 5L). In the lab, SND bioreactor cells were supplied with oxygen using microporous diffusers to maintain aerobic oxygen levels while also maintaining a homogenous environment within each bioreactor cell. The supplemental carbon source was dosed using separate Atlas Scientific peristaltic pumps at rates determined based on influent nitrogen concentrations and a desired C:N ratio.

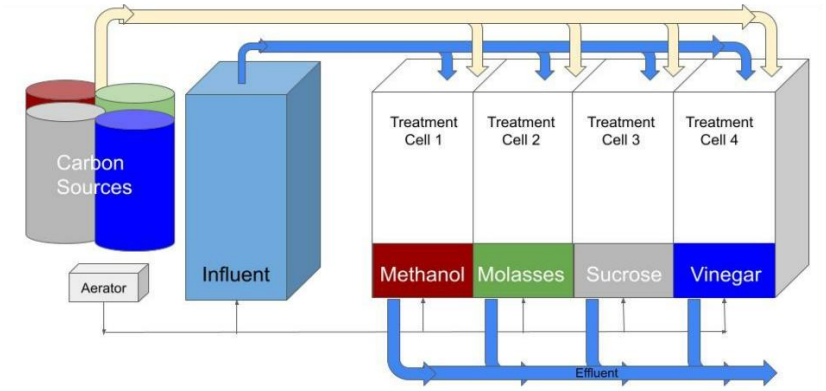

Figure 8. Schematic diagram of flow-through treatment tank for carbon source testing.

Figure 9. Image of experimental set-up for flow-through bioreactor.

5. Results / Discussion:

The two laboratory systems described above were utilized to optimize the performance of a bioreactor system under laboratory conditions. These data were then used to refine the design and construction of an effective, scalable field system. To optimize performance, the carbon source, C:N ratios and residence times were varied to find ideal values to maximize nitrogen removal efficiency. Because the performance of bioreactors is dependent on multiple variables, these test parameters are intrinsically linked. As a result, changing one parameter can influence the others. To best address this issue, we approached these tests in an order that would limit these effects and the first test of carbon sources was repeated once idealized C:N ratios and residence times were determined.

5.1 Carbon Source, Batch Reactors (Initial)

Bottle cap media in batch reactors were supplied with lagoon water and periotic additions of solutions (batch reactions) containing one of 4 carbon sources (sucrose, methanol, molasses and acetic acid). Carbon solutions were formulated to achieve equal molar carbon concentrations of 60 mM so that the volume added from carbon dosing was the same across treatments. Following an establishment period where bacterial cultures had fully developed in each carbon source, caps were transferred into batch reactors for experiments (Figure 6 and Figure 7). Bioreactors were filled with aerated lagoon water (100% DO saturation) from Crane Creek and dosed with the appropriate carbon solution to achieve a C:N molar ratio of 3:1 (influent nitrogen to added carbon). Following carbon additions, chambers were sealed with a DO datalogger and stirrer and were incubated for a one-hour under aerobic conditions.

Table 5: Concentrations of Ammonium (NH_4^+), Nitrate + Nitrite (NOx), and Dissolved Inorganic Nitrogen (DIN) in mg/L, before (initial) and after (final) treatment relative to relaying Carbon Sources (t-test, α = 0.05).

	Initial NH_4	Initial NO_x	Initial DIN	Final NH_4	Final NO_x	Final DIN	Avg. % NH_4 reduced	P-Value (NH_4)
Control	0.085 ± 0.017	0.064 ± 0.017	0.15 ± 0.028	0.093 ± 0.017	0.13 ± 0.061	0.22 ± 0.044	-10%*	0.002
Methanol	0.085 ± 0.017	0.069 ± 0.013	0.15 ± 0.029	0.066 ± 0.016	0.14 ± 0.047	0.21 ± 0.031	23%	0.002
Sucrose	0.085 ± 0.017	0.069 ± 0.013	0.15 ± 0.029	0.015 ± 0.002	0.12 ± 0.034	0.13 ± 0.036	81%	0.018
Acetic Acid	0.096 ± 0.002	0.047 ± 0.001	0.14 ± 0.002	0.059 ± 0.004	0.061 ± 0.010	0.12 ± 0.006	39%	0.002
Molasses	0.096 ± 0.002	0.047 ± 0.001	0.14 ± 0.002	0.013 ± 0.001	0.076 ± 0.010	0.089 ± 0.011	87%	<0.001

*negative reduction indicates an increase in NH4. Molecular weight of N: 14.0067 g.

All carbon sources yielded significant decreases in ammonium concentrations over the 1-hour incubation period (Table 5). The sucrose and molasses solutions decreased ammonium concentrations by >80% (81% and 87%). Acetic Acid and methanol removed <50% (39% and 23%) of the ammonium. In each aerobic chamber, nitrate concentrations increased, likely resulting from aerobic nitrifying bacteria. Oxygen consumption was tracked in these experiments (oxygen was not added during the batch reactor experiment) using HOBO U26 dissolved oxygen dataloggers. The highest oxygen consumption was in cells using sugar and molasses with lower oxygen demand in reactors using acetic acid and methanol (Figure 10). These data reflect the increased oxygen demand from aerobic nitrifying bacteria, the first essential step in coupled nitrification-denitrification. Based on the remaining oxygen after incubations, and increased nitrate concentrations, a separate anaerobic chamber or longer residence times would be needed to complete the denitrification step.

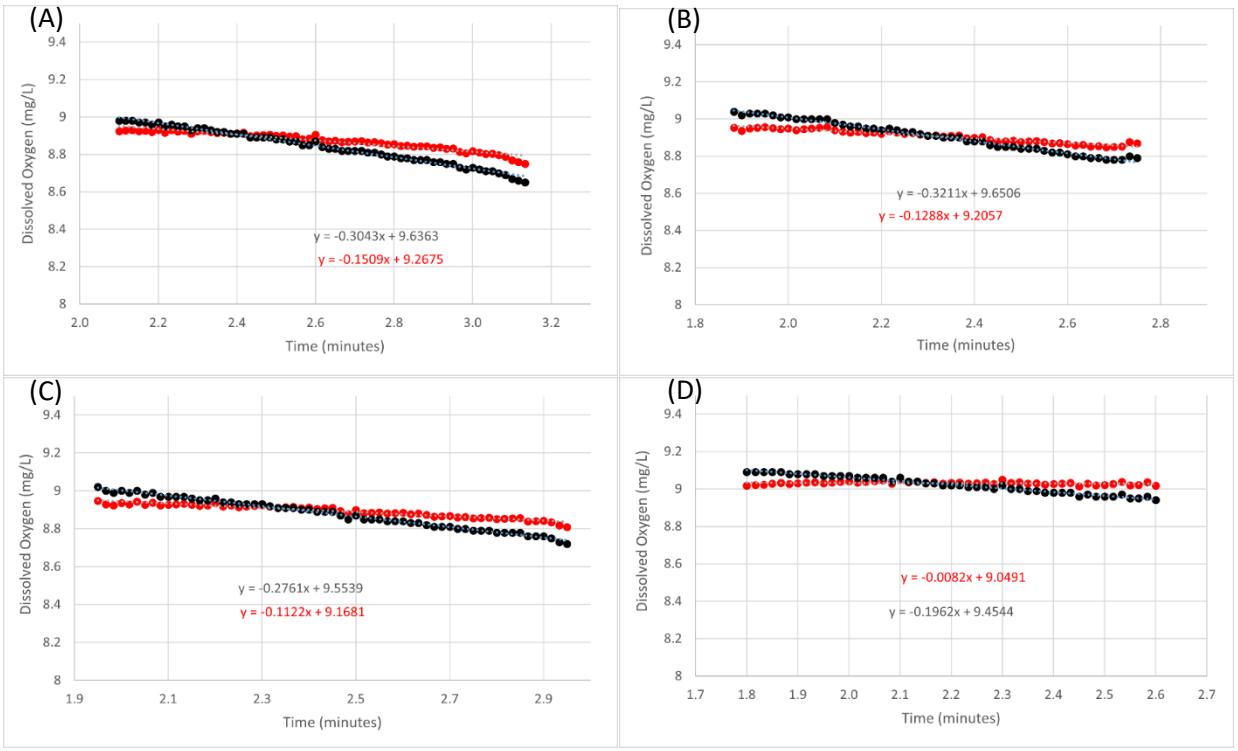

Figure 10. Concentrations of dissolved oxygen (mg/L) over time in batch reactors using the various carbon sources (A) Sucrose, (B) Molasses, (C) Methanol and (D) Acetic Acid during the chamber experiment. The black slope is uncorrected relative to temperature; The red slope is temperature adjusted.

With the highest efficiency towards nitrogen removal, sucrose was selected for further experiments to refine C:N ratios and residence times. Sucrose is also an ideal carbon source for use in IRL because it is naturally produced and stored in the marine environment by seagrasses and environments where coupled nitrification-denitrification occurs naturally (Sogin et al., 2022).

5.2 C:N Ratios, Flow Through Bioreactor

Carbon to nitrogen ratios were refined by testing molar ratios ranging from 3 to 35 in flow-through bioreactors. For this experiment, sucrose was utilized based on results from the carbon source experiments. Bacterial cultures were grown in the flow systems for a >1-month acclimatation period where lagoon water and carbon was dosed continuously at an ~8-hour residence time with redox conditions maintained using microporous diffusers and ambient air (Figure 11). By varying carbon additions relative to average influent nitrogen concentration, molar C:N ratios were maintained at 3, 9, 18 and 35. These values were selected for testing based on results from prior investigations (e.g., Gering, 2021; W. Khanitchaidecha et al., 2010). Following the acclimatization period, samples were obtained from each experimental cell with differing C:N ratios.

Figure 11. Images of flow through carbon concentrations (%) dosed into the flow through bioreactor for different carbon to nitrogen ratios.

Table 6: Concentrations of Dissolved Inorganic Nitrogen (DIN) and dissolved inorganic phosphorus (DIP) in mg/L, before (initial) and after (final) treatment using varied C:N ratios plus the average percent removal of DIN and DIP plus statistical p-values (paired, t-test, α = 0.05).

C:N molar ratios	Initial DIN	Initial DIP	Avg. Final DIN	Avg. Final DIP	Avg. Percent removal DIN	Avg. Percent removal DIP	P-Value (DIN)	P-Value (DIP)
35	0.61 ± 0.073	0.15 ± 0.032	0.013 ± 0.002	0.058 ± 0.03	98%	65%	<0.001	<0.001
18	0.61 ± 0.073	0.15 ± 0.032	0.015 ± 0.005	0.062 ± 0.038	98%	63%	<0.001	<0.001
9	0.61 ± 0.073	0.15 ± 0.032	0.016 ± 0.007	0.064 ± 0.039	98%	60%	<0.001	0.003
3	0.61 ± 0.073	0.15 ± 0.032	0.29 ± 0.22	0.12 ± 0.061	54%	25%	<0.019	0.147

Molecular weight of N, P: 14.0067 g, 30.97 g.

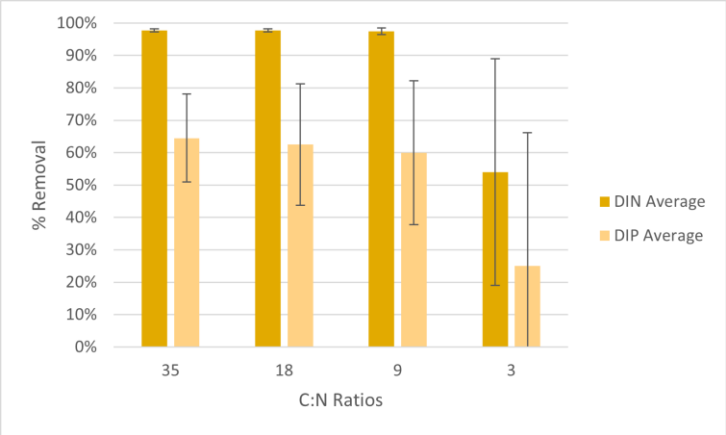

Figure 12. Average percent DIN removal in flow-through treatment with varying C:N ratios.

Molar carbon to nitrogen ratios ranging from 3 to 35 (using sucrose) decreased nitrogen concentrations by at least 50%. At a C:N ratio of 3, DIN decreased by 54%. Increasing the C:N ratio to 9 resulted in 98% removal of DIN (Table 6; Figure 12). Further increasing the carbon dosing and the C:N ratio resulted in no additional removal of nitrogen and no substantial increase in P removal. This suggests that a C:N ratio of >3 increased system efficiency; however, there was no added benefit to increasing above 9. These data demonstrate a minimum carbon dosing to reach the maximum efficiency while enabling an efficient use of supplemental carbon. Minimizing carbon additions will contribute to lowering the operating cost and logistics of a scalable system. Achieving 98% nitrogen removal suggests that the 8-hour residence time is longer than necessary and further experiments were carried out to identify the minimum required residence time using sucrose as a carbon source and a C:N ratio of 18. This ratio was selected to account for any spikes in influent nitrogen concentrations.

5.3 Residence Times, Flow Through Bioreactor

Efficient residence times for nitrogen removal were identified using flow-through bioreactors. Bacterial cultures were grown in the flow systems for a >1-month acclimatation period where lagoon water from Crane Creek and sucrose were dosed continuously at varying residence times ranging from 8 hours to just 5 minutes (Figure 13). Based on results from prior experiments to determine an ideal C:N ratio, a ratio of 18 was used based on average influent N concentrations while allowing periods of elevated nitrogen input. Redox conditions were maintained using microporous diffusers and ambient air.

Figure 13: Images of flow bioreactor with varying residence times with the same carbon solution.

Table 7: Concentrations of Dissolved Inorganic Nitrogen (DIN) and dissolved inorganic phosphorus (DIP) in mg/L, before (initial) and after (final) treatment using different residence times plus the average percent removal of DIN and DIP and statistical p-values (paired, t-test, α = 0.05).

Residence Times (min)	Initial DIN	Initial DIP	Avg. Final DIN	Avg. Final DIP	Avg. Percent removal DIN	Avg. Percent removal DIP	P-Value (DIN)	P-Value (DIP)
480	0.47 ± 0.12	0.067 ± 0.0093	0.013 ± 0.0038	0.0068 ± 0.0093	98%	91%	0.01	0.001
360	0.47 ± 0.12	0.067 ± 0.0093	0.015 ± 0.005	0.0050 ± 0.0050	97%	93%	0.012	0.001
240	0.47 ± 0.12	0.067 ± 0.0093	0.012 ± 0.0011	0.0040 ± 0.0034	98%	95%	0.012	0.002
120	0.47 ± 0.12	0.067 ± 0.0093	0.012 ± 0.0017	0.0028 ± 0.0015	98%	96%	0.011	0.003
90	0.54 ± 0.10	0.048 ± 0.0040	0.022 ± 0.0024	0.0028 ± 0.00031	96%	94%	0.006	0.001
60	0.54 ± 0.10	0.048 ± 0.0040	0.021 ± 0.0064	0.0087 ± 0.0093	96%	83%	0.007	0.003
30	0.54 ± 0.10	0.048 ± 0.0040	0.021 ± 0.0032	0.0028 ± 0.00031	96%	94%	0.006	0.001

15	0.41 ± 0.040	0.056 ± 0.0050	0.022 ± 0.0021	0.0043 ± 0.0015	94%	92%	0.002	0.001
10	0.36 ± 0.012	0.063 ± 0.014	0.022 ± 0.0024	0.0087 ± 0.00093	94%	86%	<0.001	0.01
5	0.41 ± 0.020	0.067 ± 0.00062	0.027 ± 0.0056	0.0071 ± 0.00031	93%	90%	<0.001	<0.001

480 min = 8 hours, 360 min = 6 hours, 240 min = 4 hours, 120 minutes = 2 hours. Molecular weight of N, P: 14.0067 g, 30.97 g.

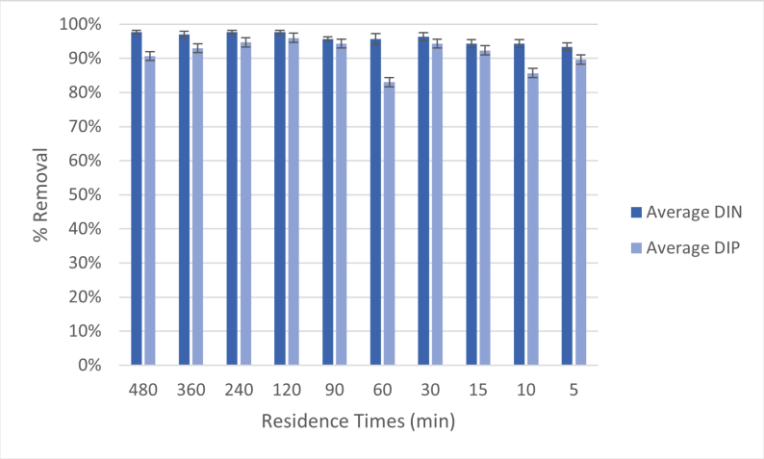

Figure 14: Average percent DIN removal in flow-through treatment with varying residence times.

Residence times between 8 hours and 5 minutes each removed at least 93% of the DIN and most of the DIP (Table 7; Figure 13: Images of flow bioreactor with varying residence times with the same carbon solution.

Figure 14). With residence times between 2 and 8 hours the system achieved 98% removal of DIN. Decreasing the residence time did slightly decrease the total % removal with 96% removal of DIN at residence times between 30 and 90 minutes. With a residence time of just 5 minutes, the system still achieved 93% removal of DIN. Despite a lower overall removal percentage, the system with a 5-minute residence time treats 96 times more water (8*60 = 480 minutes / 5 minutes = 96x) than a system with a residence time of 8 hours. The ideal residence time balances the % removal and throughput to achieve the greatest total removal of nitrogen. For example, in a 1L treatment cell, if influent water contained 30 µmol/L (420 µg/L) and the system achieved 98% removal over an 8-hour residence time, the system has removed 29.4 µmoles (411.6 µg) of nitrogen in 8 hours. With 93% removal at a 5-minute residence time, the system would remove 2,678 µmoles (37,498 µg) of nitrogen in the same 8 hours. To that end, a lower % removal at a shorter residence time can be more efficient.

5.4 Carbon Source, Flow Through Bioreactor

Having determined that the appropriate C:N ratio using sucrose in water from Crane Creek was >3, we took this opportunity to re-evaluate the alternate carbon sources using the flow-through system with a stable redox environment, a shorter residence (30 minutes) time and a more appropriate C:N ratio (18). Consistent with prior test, bacterial cultures were grown using the respective carbon sources for approximately 1 month before conducting experiments (Figure 15).

Figure 15. Images of flow bioreactor with varying carbon sources and the corresponding solutions for an equal C:N molar ratio.

Table 8: Concentrations of Dissolved Inorganic Nitrogen (DIN) and dissolved inorganic phosphorus (DIP) in mg/L, before (initial) and after (final) treatment using varied Carbon Sources plus the average percent removal of DIN and DIP plus statistical p-values (paired, t-test, α = 0.05).

	Initial DIN	Initial DIP	Final DIN	Final DIP	Avg. % removal DIN	Avg. % removal DIP	P-Value (DIN)	P-Value (DIP)
Methanol	0.45 ± 0.050	0.069 ± 0.0065	0.035 ± 0.026	0.020 ± 0.0090	93%	71%	0.001	0.001
Sucrose	0.45 ± 0.050	0.069 ± 0.0065	0.018 ± 0.0022	0.0077 ± 0.0034	96%	89%	0.002	0.0004
Acetic Acid	0.45 ± 0.050	0.069 ± 0.0065	0.019 ± 0.0069	0.014 ± 0.0040	96%	79%	0.002	0.001
Molasses	0.45 ± 0.050	0.069 ± 0.0065	0.017 ± 0.0032	0.012 ± 0.0059	96%	83%	0.002	0.0003

Molecular weight of N,P: 14.0067 g, 30.97 g.

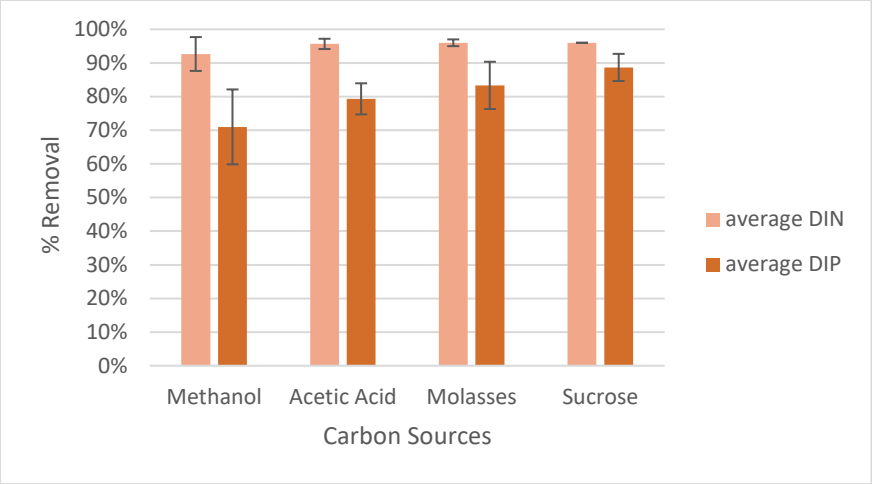

Figure 16. Average DIN and DIP percent removal in flow-through treatment from testing various carbon sources.

Treatment using methanol, sucrose, acetic acid and molasses removed between 93% and 96% of the DIN and 71% to 89% of the DIP (Table 8; Figure 16). Overall, the higher C:N ratio used in the flow-through system resulted in enhanced nitrogen and phosphorus removal compared to the initial experiments using batch reactors. This repeated experiment with carbon sources confirming that sucrose was still the optimal choice as a carbon source removing 96% of DIN and 89% of DIP.

5.5 Maximum Treatment Capacity

The ability of a bioreactor to remove nitrogen is dependent on a combination of the percent removal and residence time as discussed above. One key difference between the use of a bioreactor to treat stormwater and natural surface waters compared to wastewater, aquarium water, or recirculating aquaculture systems is a striking difference in influent nitrogen concentrations. For example, domestic wastewater typically contains 7-28 mg/L of dissolved inorganic nitrogen (EPA) compared to 0.256 ± 0.143 mg/L (18.3 ± 10.2 µM) at Crane Creek. The much lower concentrations of DIN in stormwater compared to wastewater limits the ability of the system to remove nitrogen simply because there is less to remove. To test the treatment capacity of the bottle caps system, for potential applications where influent nitrogen is higher (e.g., industrial effluent), influent water was spiked with additional nitrogen (Figure 17). These additions yielded influent nitrogen concentrations ranging from 6 to 30 mg/L (444 to 23376 µM), consistent with values frequently reported for wastewater (Table 9).

Table 9: Concentrations of dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphorus (DIP) in mg/L, before (influent) and after (effluent) treatment using varied residence times plus the average percent change of DIN and DIP.

		Influent		Effluent		% Change	
Res. time (min)	**Date**	**DIN (mg/L)**	**DIP (mg/L)**	**DIN (mg/L)**	**DIP (mg/L)**	**DIN**	**DIP**
60	5/21/24	8.34	0.0028	3.31	0.0084	60%	-200%
60	5/22/24	6.22	0.015	2.69	0.015	57%	2%
60	5/29/24	8.62	0.24	3.61	0.12	58%	51%
60	6/3/24	33.3	0.50	19.2	0.073	42%	86%
5	6/4/24	30.1	1.25	14.6	0.034	52%	97%

Molecular weight of N, P: 14.0067 g, 30.97 g.

Using a 5-minute residence time, a C:N ratio of 12 and 30 mg/L influent DIN, the system removed 52% of the DIN or 15.6 mg/L. It is possible that with a longer residence time the percent removal would be increased. Nevertheless, the ideal residence time balances the % removal and throughput to achieve the greatest total removal of nitrogen. In this case 15.6 mg/L were removed in 5 minutes. With the volume fraction of water in the bioreactor (75%; the other 25% is occupied by media) this is 11.7 mg/L (of bioreactor volume)/5 minutes. This is ~3 kg/m^3/day. This value is consistent with those reported for bioreactors used in wastewater etc.; however, this system is optimized for use in brackish and marine systems. The bioreactor also removed up to 97% of DIP; however, during the first experimental run with spiked nutrient concentrations, DIP increased during treatment (Table 9). The increase occurred when influent DIP was low, possibly related to sorption of phosphate onto iron rich particles originating either from Crane Creek or through the addition of a nutrient spike. Within the bioreactor, iron can become reduced in anaerobic micro zones and desorb phosphate. This is one possible geochemical explanation. Nevertheless, phosphorus removal increased over time.

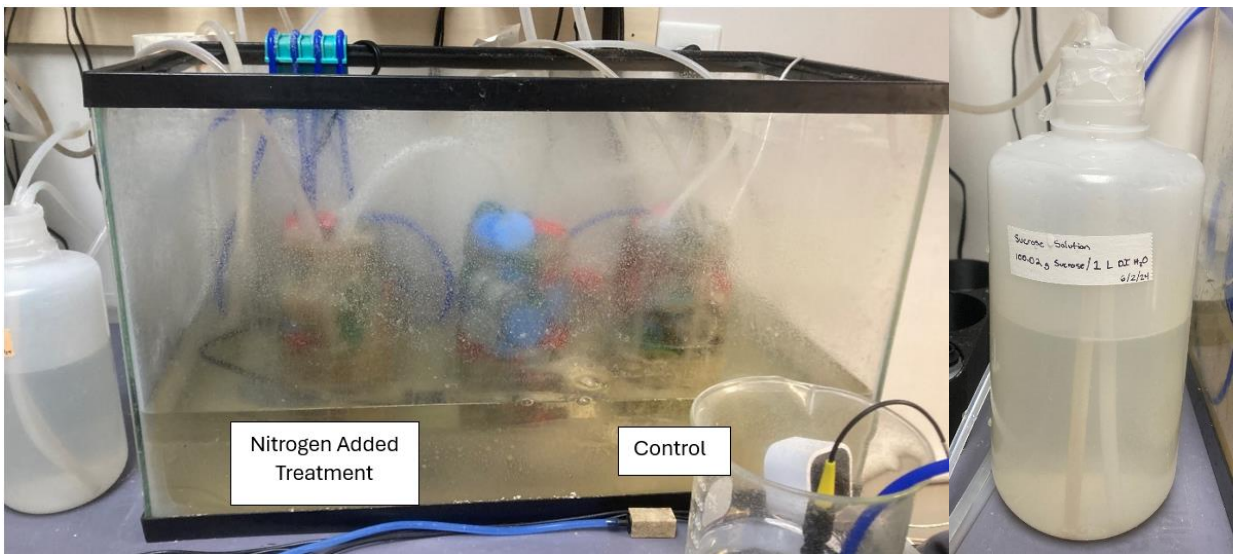

Figure 17. Images of flow bioreactor with higher nitrogen input and a control and a sucrose solution being dosed with C:N molar ratio of 12.

5.6 Designing the Field System

The laboratory system was initially established with multiple bioreactor cells to facilitate a multi-stage treatment process. Many denitrification bioreactors take advantage of an aerobic cell and a separate anaerobic cell to facilitate the stepwise oxidation of nitrogen compounds in an aerobic cell followed by reduction to N_2 gas in a later anaerobic cell. Following extensive laboratory testing, the primary bioreactor was condensed into a single SND bioreactor cell. The Field System was designed based on findings from laboratory experiments with specific design elements to facilitate scalability and variations in the composition of influent water. Water (~50 gph) was pumped into the field treatment system from a dock extending into Crane Creek from the Florida Tech Anchorage. The bioreactor system provides oxygen and sucrose that are otherwise naturally produced in the estuary by seagrasses, while surface area on inert bottle caps (media) provides substrate. Following treatment in the reactor, where supplemental oxygen and sucrose are completely consumed, water is discharged to the lagoon. This effluent contains significantly (>90%) less DIN. The system requires only 1 treatment cell; however, the modular design allows for increased residence times or throughput by bringing barrels 2 through 4 online if inflow nitrogen concentrations increase or if there is a desire to increase the total throughout (Figure 18). The field system was contained within a trailer, on land with ¾ inch intake and discharge hoses retrieving and discharging water to the lagoon (Figure 20. Photos **of the field system that were constructed at the Florida Tech Anchorage.**

The scalable design, with the exception of the oxygen system, focused on readily available materials. Bioreactors were constructed in 55-gallon plastic drums model #S-9945BLU and were filled with repurposed plastic bottle caps made from high density polyethylene (HDPE) and polypropylene (PP). Piping within the system was constructed using PVC and vinyl tubing (e.g., Model # PVC 04007 0600). The supplemental carbon solution was produced using sucrose ($C_{12}H_{22}O_{11}$) available from local vendors (Domino Foods Inc.), dissolved into regular tap water or site water. The concentration of carbon solution can be varied depending on anticipated influent nitrogen concentrations and desired pump rates. Sucrose has a high carbon density, 42% carbon by mass of added solids, an important consideration for a large-scale system. Also, storage and handling of sucrose is safe compared to other carbon sources that did not perform as well (e.g., methanol). For this system, carbon solution was stored in a 55-gallon drum (model #S-9945BLU); however, carbon dosing and storage can be easily modified to the scale of the project and influent nitrogen concentrations. For example, the molar concentration of the carbon solution can be increased, or even dry sucrose could be directly added to bioreactors if volume storage is an issue. Carbon dosing was accomplished to achieve a C:N ratio of 18 using a combination of Atlas Scientific EZO-PMP embedded dosing pumps and Kamoer KHPP260 peristaltic pumps. Kamoer pumps were run using L298N motor drivers with all pumps controlled using Arduino Uno R3 (A000066) and R4 (ABX00087) microcontrollers programmed using Arduino IDE version 2.3.2. Kamoer pumps were calibrated in the lab with a known volume per duty cycle. Atlas Scientific pumps were calibrated using the manufacturer techniques and programming. Carbon was dosed into each bioreactor cell (Figure 18).

The specific surface area of inert HDPE and PP media (bottle caps) is 300-400 m^2/m^3. Each 55-gallon bioreactor cell contained 0.2 m^3 of media with a total surface area of 70 m^2 (per cell) equivalent to a flat surface 8.3 x 8.3 m (27 x 27 feet). Water was pumped from IRL into a holding tank using a Danner Manufacturing Inc. Supreme Aqua-Mag Model 7 pump reduced to a flow of ~50 gph based on the original permit and anticipated longer residence time (8 hours). In the holding tank, water was super oxygenated using the Oxsolve system to promote bacterial decomposition of dissolved organics releasing DIN to achieve more complete nitrogen removal. From the holding tank water was gravity fed into bioreactor cells. The modular system requires only a single cell; however, the multicell system allows for the simultaneous evaluation of multiple residence times and or scaling to treat higher nitrogen concentrations or volumes of water. Bioreactor cells were connected using 1-1/2" PVC piping drained from the top of the upstream cell, through a pipe to the bottom of the subsequent bioreactor cell (Figure 18 and Figure 19). This design element created an upward flow through each bioreactor cell, mitigating channeling where water would circumvent the bioactive media. Water level in the complete system was regulated by the height of a gravity fed discharge pipe in this case returning treated water to IRL. If there is residual nitrogen remaining after cell 1, the identical bioreactor cell 2 provides additional treatment. This step is not necessary but contributes to optimization of the system and refinement of the residence time for a single cell system. Following optimization, monitoring evaluated the performance of the single cell bioreactor.

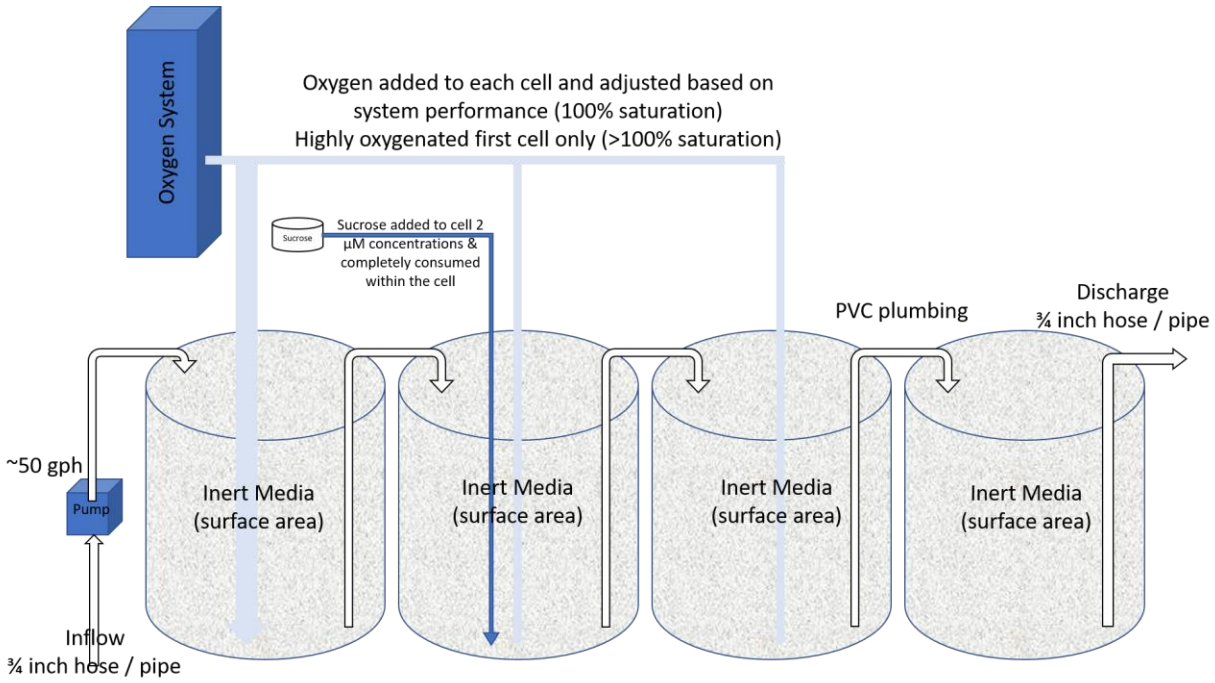

Figure 18. Simple schematic of the field-scale bioreactor design including 4 independent bioreactors that can be connected to either increase the residence time for influent water containing higher nitrogen concentrations or used individually to increase throughput.

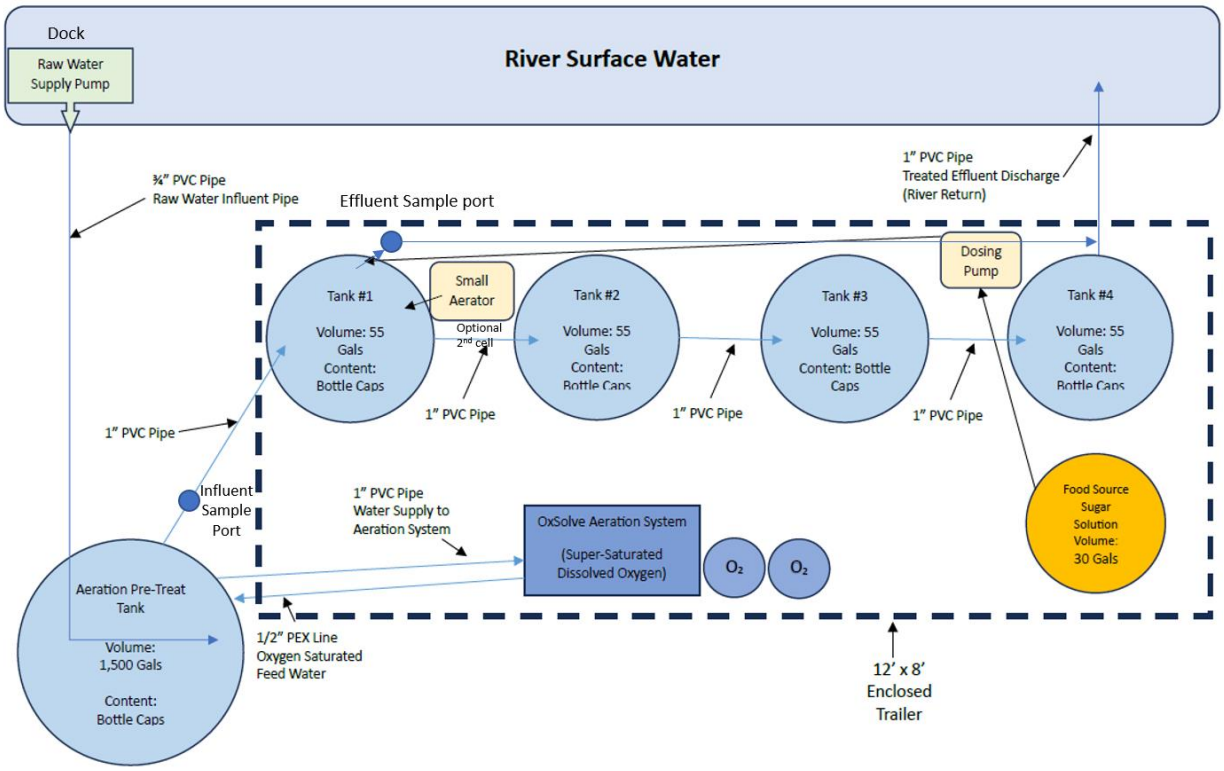

Figure 19. Top-view of field-scale bioreactor design with blue arrows indicating movement of water from Crane Creek, through the system of the Aeration Pre-Treatment Tank to Tank #1, Tank #2, Tank #3, Tank #4, and back into Crane Creek.

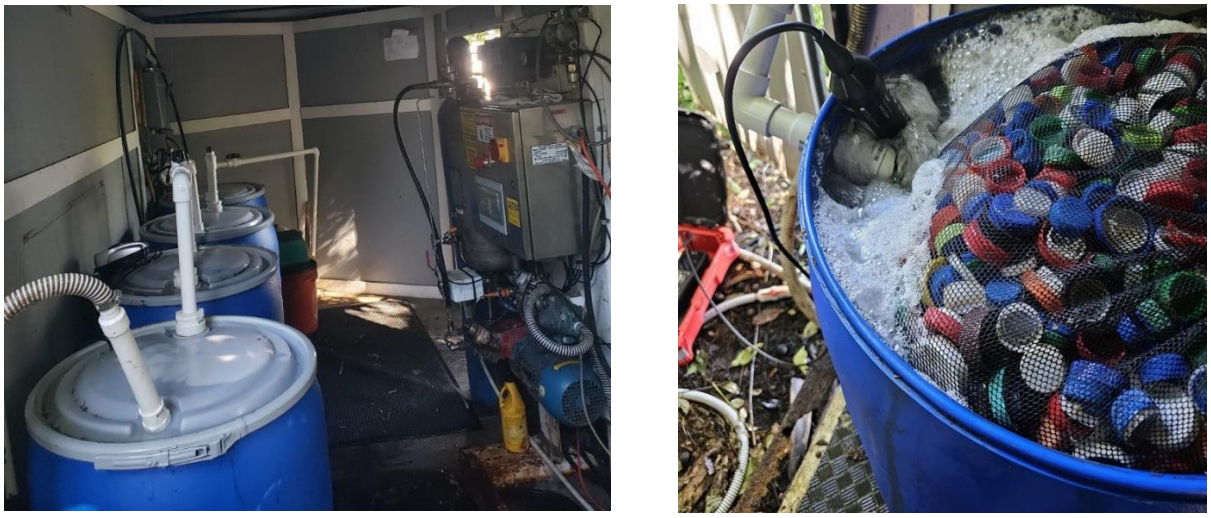

Figure 20. Photos of the field system that were constructed at the Florida Tech Anchorage.

6. Permitting the Field System (Task 3):

Reviewing possible permit requirements for a shore-based system discharging treated water from IRL into IRL, we identified Army Corps Statewide permits and FDEP surface water discharge permits for this project. Researching requirements and discussing them with agency staff, we determined this project would likely fall under a *de minimis* exemption for FDEP and a permit waiver request was submitted. After review, we received a permit exemption from FDEP: FDEP File No.: 444910-001. After receiving this exemption, Army Corps was contacted regarding additional permitting requirements. After forwarding the FDEP information to the Jacksonville District North Permits Branch Cocoa Section, we were provided with a letter indicating that no permit would be required from Army Corps: Army Corps File No.: SAJ-2024-00643 (NPR-JAZ). Task 3, permitting, is complete.

7. Monitoring the Field System (Task 4)

Monitoring of the field system was completed at the Florida Tech Anchorage with assistance from Lapin Services with nutrient samples sent to Pace labs as outlined in the QAPP. The field system was operated based on findings from the laboratory optimization. Sucrose was used as the supplemental carbon source, dosed to maintain a C:N ratio of 18. The residence time was 1-hour.

During the monitoring period, May 2024, samples were collected weekly and transported to Pace labs for analysis. Results were used to evaluate the performance of the field scale bioreactor system. Overall, influent concentrations of ammonium and nitrite plus nitrite ranged from 63 to 160 µg/L and 23 µg/L to below detection, respectively (Pace Labs practical quantification limit for ammonium and nitrate are 0.05 mg/L (50 µg/L)). Median influent ammonium was 118 ± 49 µg/L (8.4 ± 3.5 µM). After treatment, all values for ammonium and nitrate plus nitrite samples were below detection (between the detection limit and zero). As a result, percent changes were calculated based on initial concentrations and the detection limit and reported as a minimum treatment efficiency (Table 10). As discussed above, these efficiencies were limited only by the influent nitrogen concentrations.

Table 10. Concentrations of Ammonium, Nitrate + Nitrite, and Ortho-P in µg/L, before (Inflow) and after (Outflow) treatment at Anchorage System.

	Inflow			Outflow			Percent Change		
Date	Ammonium (µg/L)	Nitrate + Nitrite (µg/L)	Ortho-P (µg/L)	Ammonium (µg/L)	Nitrate + Nitrite (µg/L)	Ortho-P (µg/L)	Ammonium	Nitrate + Nitrite	Ortho-P
5/9/2024	90	18 I	120	35 U	15 U	36	>61%	>17%	70%
5/16/2024	160	23 I	99	35 U	15 U	90	>78%	>35%	9%
5/23/2024	160	15 U	74	35 U	15 U	52	>78%	>0%	30%
5/30/2024	63	15 U	84	35 U	15 U	84	>44%	>0%	0%

*Pace Labs detection limit for Ammonium and Nitrate + Nitrite: 0.050 mg/L. Red indicates values below the practical quantification limit (PQL); Blue indicates values not detected (below the method detection limit). Data qualifiers I: The reported value is greater than or equal to the laboratory method detection limit but less

than the laboratory practical quantitation limit. U: Indicates that the compound was analyzed for but not detected. The value is reported for informational purposes only and shall not be used in statistical analysis.

Supplemental data for temperature, salinity, DO, pH, ORP and Chlorophyll *a* were collected during the monitoring phase to put nutrient reductions into context. Water temperature increased throughout May from 26.8 °C on May 9, to 29.8 °C on May 30 (Table 11). Within the bioreactor, there was no significant change in water temperature. Salinity at the Florida Tech Anchorage at the mouth of Crane Creek is variable due to the mixing of stormwater from Crane Creek with higher salinity lagoon water (Table 12). Overall, there was no consistent trend for salinity as it passed through the system; however, small variations in salinity between the dock, inflow and outflow likely reflect changes in salinity in the creek over a matter of hours and the residence time for water within in the system. pH was tracked because nitrification produces protons and can result in a decrease in pH; in contrast, denitrification consumes protons and can result in an increase in pH. The net result in the SND bioreactor should be no net change in pH; however, a decrease was expected and observed based on mineralization of OM and the production of carbonic acid ($CO_2 + H_2O = HCO_3^- + H^+$;

Table 13). Bacterial processes in the bioreactor explain the observed decrease in pH between the dock the bioreactor.

Table 11. Temperature at the dock where water was pumped from Crane Creek, in influent water and in discharged effluent plus weather conditions during each field visit.

Temperature (Celsius)				
Date	**Dock**	**Inflow**	**Outflow**	**Weather**
5/9/2024	26.8	26.3	26.6	Partly Cloudy, Roughly 80 degrees Fahrenheit, Humid
5/16/2024	27.5	26.5	26.9	Sunny, Light wind, low tide, Humid
5/23/2024	28.5	26.8	27.4	Sunny, Light wind, Roughly 80 degrees Fahrenheit, Not humid
5/30/2024	29.8	29.8	29.7	Clear and Sunny, Roughly 87 degrees Fahrenheit, Humid

Table 12. Salinity at the dock where water was pumped from Crane Creek, in influent water and in discharged effluent plus weather conditions during each field visit.

Salinity			
Date	**Dock**	**Inflow**	**Outflow**
5/9/2024	21.04	17.57	16.6
5/16/2024	25.12	21.91	17.02
5/23/2024	27.19	26.79	24.51
5/30/2024	22.31	23.72	23.27

Table 13. pH at the dock where water was pumped from Crane Creek, in influent water and in discharged effluent plus weather conditions during each field visit.

pH			
Date	**Dock**	**Inflow**	**Outflow**
5/9/2024	7.81	7.40	7.37
5/16/2024	7.62	7.52	7.53
5/23/2024	7.70	7.45	7.50
5/30/2024	7.25	6.91	7.07

Dissolved oxygen and the oxidation reduction potential (ORP) were also monitored at the dock where water was pumped into the system, in the influent tank and in treated effluent. The influent tank was supplied with supplemental pure oxygen using the Oxsolve system to pre-treat water with oxygen and break down additional organic materials thereby increasing influent DIN. This system resulted in an increase in the ORP (a measure of the redox environment) from 117 ± 28 at the dock to 217 ± 89 at the influent port despite no significant increase in DO (2.37 mg/L) (Table 14 and Table 15). Microbubble aeration within each bioreactor cell led to an increase in DO in the effluent (5.63 mg/L) despite a significant drop in ORP to 14 ± 67. This low ORP in the presence of oxygen indicates reducing chemical reactions in the presence of oxygen, a good indicator that both aerobic nitrification and anaerobic denitrification are occurring as is indicated by the removal of DIN.

Table 14. Oxidation Reduction Potential (ORP) at the dock where water was pumped from Crane Creek, in influent water and in discharged effluent plus weather conditions during each field visit.

ORP (mV)			
Date	**Dock**	**Inflow**	**Outflow**
5/9/2024	79.1	91.9	-63.9
5/16/2024	101.2	213.5	-12.9
5/23/2024	136.3	276.8	44.6
5/30/2024	150.2	285.9	90.0

Table 15. Dissolved Oxygen (DO) at the dock where water was pumped from Crane Creek, in influent water and in discharged effluent plus weather conditions during each field visit.

DO (mg/L)			
Date	**Dock**	**Inflow**	**Outflow**
5/9/2024	4.00	2.59	5.18
5/16/2024	2.89	2.27	5.60
5/23/2024	3.80	1.80	5.94
5/30/2024	4.12	2.81	5.83

In addition to removing dissolved inorganic nitrogen, there was a decrease in chlorophyll concentrations between the dock and effluent, demonstrating this systems ability to mitigate algal biomass (Table 16). In some cases, chlorophyll appeared to have increased slightly within the treatment system (<4%); this is likely related to the residence for water in the system and variable conditions at the dock. Although prior lab efforts identified a decrease in turbidity, no consistent trend was identified for turbidity in influent or effluent at the field site (turbidity was not monitored at the dock) (Table 17). This is likely due to the highly variable turbidity in this location within Crane Creek and the residence time within the system.

Table 16. Concentrations of chlorophyll *a* at the dock where water was pumped from Crane Creek, in influent water and in discharged effluent plus weather conditions during each field visit.

Chl *a* (ug/L)			
Date	**Dock**	**Inflow**	**Outflow**
5/9/2024	19.06	10.52	6.47
5/16/2024	11.90	7.99	4.42
5/23/2024	13.36	4.74	4.85
5/30/2024	29.07	17.52	18.07

Table 17. Turbidity at the dock where water was pumped from Crane Creek, in influent water and in discharged effluent plus weather conditions during each field visit.

Turbidity (NTU)			
Date	**Dock**	**Inflow**	**Outflow**
5/9/2024	NA	15.0	12.2
5/16/2024	NA	2.76	8.98
5/23/2024	NA	2.68	4.79
5/30/2024	NA	16.3	3.61

In addition to removing nutrients, and algae (chlorophyll), the bottle caps system was previously demonstrated to remove microplastics (Gering, 2021). The rapidly colonizing bacteria cultures grow on microplastic particles and increase their density causing them to sink and accumulate in the sludge produced within the bioreactor. This sludge can then be collected and discarded. Other research efforts have demonstrated that plastics used in water treatment can help to sequester and remove organic contaminates based on miscibility.

8. Summary and Conclusions:

Overall, both the lab scale and field systems performed extremely well with respect to nutrient removal and demonstrated potential other benefits including decreasing chlorophyll concentrations. Testing four different carbon sources that have been previously used in similar systems, sucrose was identified as the most efficient towards nutrient removal achieving 96% removal of DIN and 89% removal of phosphate (DIP). Sucrose is also

an ideal choice as it is naturally produced in the environment by seagrasses and is certainly used by bacteria naturally present in sediments throughout the IRL system. The efficiency of sucrose in this bioreactor demonstrates the potential broader implications of seagrass losses that can influence ecosystem services such as coupled nitrification-denitrification. Beyond constrained bioreactor systems, lessons learned from this study can help us better understand the benefits of restoration projects, including seagrass restoration.

Tests to determine the minimum amount of supplemental carbon required identified a minimum C:N ratio of 9 to sustain treatment efficiency >90% while minimizing consumables and allowing for maximum scalability. Testing the minimum required residence time found >90% removal of DIN in only 5 minutes. This is a substantial improvement over the previous residence time of 8 hours facilitating the treatment of 96 times more water and 96 times more nitrogen. With short residence times and high nitrogen removal from spiked laboratory experiments, this system achieved removal of >3 kg N/m^3/day. This removal capacity is impressive and on par with values reported for many freshwater systems including wastewater treatment (e.g., Sharp et al., 2020; Frijters eta al., 2000). Working with a contractor Lapin Services DBA Oxsolve, the system is scalable and constructed in such a way that it can be readily implemented in other locations.

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