UF UNIVERSITY of FLORIDA
Institute of Food and Agricultural Sciences
Fort Lauderdale Research and Education Center

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Final report

Effects of nanobubble oxygenated water technologies on managing water quality, microbial community structure and cyanobacterial harmful algal blooms

PI: H. Dail Laughinghouse IV, Ph.D.[1]
Co-PIs: Jéssica A. Moretto Altarugio, Ph.D.[1]
Ashley R. Smyth, Ph.D.[2]
Cooperating researcher: David E. Berthold, Ph.D.[1], Forrest Lefler Ph.D.[1]
[1]Fort Lauderdale Research and Education Center, University of Florida / IFAS, 3205 College Avenue, Davie, FL 33314;
[2]Tropical Research and Education Center, University of Florida/IFAS, 18905 SW 280th St., Homestead, FL 33031

Description

Increased nutrients in water bodies due to point and nonpoint sources can favor the excessive growth of cyanobacteria (blue green algae) and eukaryotic algae, causing harmful algal blooms (HABs). In fresh waters, many cyanobacteria can produce secondary metabolites, including taste and odor compounds and toxins, which can harm people, animals, aquatic ecosystems, potable water supplies, recreational activities, and the economy. Also, cyanobacterial blooms can deplete oxygen, nutrients and block the sunlight needed for other organisms to survive in the water.

The use of nanobubble technologies has emerged in the past decade as a novel technology for water treatment; as nanobubbles have been cited to inactivate cyanobacteria without inducing significant lysis, since both cyanobacterial cells and nanobubbles are negatively charged, electrostatic repulsions occur between them. Although some studies using nanobubbles have been conducted, there is still lack of information on the fundamentals and application of this technology for cleaning waters. This project seeks to determine effects of nanobubbles *in situ* on microbial community structure (i.e., bacteria and cyanobacteria), HABs, nutrients and nitrogen fate.

How This Proposal is Different: Characterization of nanobubbles in ponds is challenging because of presence of other colloids of similar size that interfere in the process and affect the size and distributions of nanobubbles. Therefore, it is crucial that research be done in waters of various characteristics to reflect this. As a result, this proposal seeks to investigate the effects of oxygen nanobubble technology to manage nutrients, microbial community structure and HAB-formers in four different ponds.

Potential Benefit to the Golf Industry: The presence of HABs and impaired waters in golf courses is aesthetically displeasing and a potential health threat. The use of nanobubble technology can remediate waters in golf courses by improving water quality and mitigating HABs. An added benefit is that the improved water that can be used for irrigation.

Deliverables:

- An annual brief report will be submitted by November 10, 2023.
- Final report will be submitted by March 31, 2024.
- Monies are requested to attend a State or National Conference to present these data to the scientific community.
- Results may be presented in a peer-reviewed journal article and at UF/IFAS or Florida Sea Grant extension/outreach events.

INTRODUCTION

Cyanobacteria are the earliest group of oxygenic photosynthetic microorganisms and are crucial to the aquatic environment. They are the base of the food chain and are essential to several biogeochemical cycles. Cyanobacteria community composition and structure vary spatially and seasonally, due to temperature, light, nutrients, presence of aquatic plants and other factors (Almanza et al. 2019). Despite their importance in the environment, they are known for producing toxic blooms (cyanobacterial harmful algal blooms – cyanoHABs) that pose a serious and ongoing threat to ecosystems, wildlife, human health, and recreational activities (Carvalho et al 2008; Paerl & Paul 2012). CyanoHABs have been intensifying and altering their populational structure in some areas as a result of increased nutrients, particularly nitrogen and phosphorus, as well as climate change. Therefore, testing methods to control cyanoHABs and improving water quality are necessary.

In the past years, nanobubbles have become a novel method for treating water and wastewater, removing contaminants from sediments and soils, and other environmental applications (Argawal et al., 2011; Soyluoglu et al., 2022). However, there is still lack of information on the fundamentals and application of this technology for remediating nutrients and controlling cyanoHABs.

Nanobubbles are pockets of gas filled cavities that can be found attached on a surface or dispersed in liquid and hence are referred to as surface or bulk nanobubbles. Because of their small size (below 1000 nm - a millionth of a meter), they have a large surface area per unit volume, with a corresponding concentration that can get as high as a hundred million to ten trillion bubbles per milliliter of liquid (Atkinson et al., 2019). Nanobubbles are generated by using different techniques, including creating pressure difference below a certain critical value that promotes cavitation (cavity formation), and are also negatively charged in the pH range that is common in the environment (2 to 12) (Temesgen et al., 2017). They are stable in liquid for an extended period (Atkinson et al., 2019). Sonication, electrolysis, and the use of membranes to force gases of specific sizes into a moving liquid are a few more examples of approaches (Phan et al, 2020). This method has been utilized to create nanobubbles from a variety of gases, the most prevalent of which are oxygenated nanobubbles.

In particular, the rupture/collapse of oxygenated nanobubbles has been shown to create reactive oxygen species (ROS) such as peroxides and hydroxyl radicals (Temesgen et al., 2017; Ahmed et al., 2018; Atkinson et al., 2019), which can inactivate cyanobacteria without inducing significant lysis. This is due to both cyanobacterial cells and nanobubbles being negatively charged, so electrostatic repulsions occur between them (Henderson et al., 2008). It has been indicated that the impact of ROS on living cells is dependent on their concentration. It is expected that nanobubbles reduce microorganisms in treated waters and improve water quality. Infusion of oxygenated nanobubbles into water can also have other potential applications on nutrients and fate, thus changing the water that is used for irrigation.

OBJECTIVES

- Determine effects of nanobubble on microbial community structure.
- Determine effects of nanobubble on HAB-forming species.
- Evaluate nutrients and nitrogen fate to determine if this technology has the potential for broader applicability for nutrient control.
- Assess whether nanobubbles are generating peroxides in water (ROS).

MATERIALS AND METHODS

Experimental set-up:

The study was carried out on test ponds at the University of Florida – IFAS, Fort Lauderdale Research and Education Center (Davie, FL). Four ponds were selected to conduct the experiment (figure below). The experiment was conducted from 7/5/23-10/31/23.

Figure 1: Google image of ponds. A) first pond with treatment; B) second pond with treatment; C) Pond control; D) third pond with treatment.

Water sampling

During each sampling event, sub-surface waters were collected to characterize the water column microbial (including algal) community structure and nutrient concentrations. 60 samples were promised, however, a total of 87 samples were collected and analyzed.

Independent sub-surface waters were taken from three sites in each pond that represent the overall pond water. The nanobubble treatment was run on Ponds A, B, and D over a period of 14 days. A separate pond was the negative control (Pond C). On day zero, sampling was done before turning on the nanobubble machine.

Sampling occurred at various intervals from day 186 to 303 (Julian calendar) across the ponds. For Pond A, samples were collected at day 186 (time zero; on this day the machine was turned on, 193 (7 days after turning on the machine), 206 (14 days after turning on the machine and on this day the machine was turned off), 216 (30 days after starting the experiment), 248 (60 days after starting the experiment). For Pond B, samples were collected at day 206 (time zero; on this day the machine was turned on), 216 (7 days after turning on the machine), 220 (14 days after turning on the machine; on this day the machine was turned off), 235 (30 days after starting the experiment), 263 (60 days after starting the experiment). In pond C, samples were collected weekly from day 186 (time zero) to 303. In pond D, samples were collected at day 248 (time zero; on this day the machine was turned on), 256 (7 days after turning on the machine), 263 (14 days after turning on the machine), 277 (30 days after starting the experiment), 303 (60 days after starting the experiment). In the results section, DAT was used for the days after the start of the experiment/treatment.

Environmental parameters:

In-situ measurements of water temperature, dissolved oxygen, chlorophyll a (chla), phycocyanin, turbidity, conductivity, and pH were taken using a YSI EXO3. Also, total dissolved oxygen (DO, mg L^{-1}), before and after turning on the equipment, were recorded at each sampling event.

Water samples were also filtered in the laboratory for chlorophyll a (chla), extracted and analyzed (Yepremian et al., 2017).

Nutrients and metals analysis

A total of 87 samples were analyzed for Nitrate, Phosphorus, Aluminum, Boron, Calcium, Chloride, Copper, Iron, Potassium, Magnesium, Manganese, Molybdenum, Sodium, Sulfate, and Zinc (Baird & Bridgewater, 2017; Luenam, 2017).

Nitrogen Removal via Denitrification

A total of 108 samples were collected for denitrification estimates by measuring nitrogen gas (N_2) production or consumption following methods in Loeks and Cotner, 2020.

Analysis for N_2, O_2, and Ar concentrations were conducted using a Membrane Inlet Mass Spectrometer (Kana et al. 1994).

The N_2 saturation ratio was calculated based on expected concentrations, pond solubility, salinity, and temperature. A saturation value greater than 1 indicates that the samples are supersaturated.

Hydrogen Peroxide measurements

Water samples were collected before the nanobubbles exposure (time 0 – the day the machine was turned on), and also 7,14, 30 and 60 days after starting the experiment and untreated control. The nanobubble machine was run on each treatment pond for a period of 14 days. Hydrogen peroxide concentrations were measured using a colorimetric method (Kinley et al., 2015).

Characterization of the phytoplanktonic and bacterial communities:

Water samples were filtered onto a 0.22 µm MCE filter until clogging. eDNA was extracted from the filters using a Qiagen Blood Tissue kit with modified protocols by Djurhuus et al (2017). The extracted DNA was used for total bacterial and cyanobacterial community analysis, the V3-V4 region of 16S rRNA was amplified using the primers 515F-Y and 926R. Amplicon libraries were sequenced using paired-end (2×250 bp) Illumina Novaseq (Novogene) and the bioinformatic analyses were processed with R v4.0.0 (R Core Team). Amplicon sequences were demultiplexed and assigned to specific sample IDs based on their MIDs at Novogene using an in-house bioinformatic pipeline. DADA2 (Callahan et al., 2016) was used to process raw sequences in R v4.0.0 (R Core Team). Paired-end reads were filtered, trimmed, and merged under strict criteria. Cleaned and merged reads were dereplicated and subsequently

analyzed for detection and removal of potential chimeras using DADA2. Non-chimeric sequences were pooled together to define amplicon sequence variants (ASVs).

Taxonomic assignment of ASVs was based on naive Bayesian classifying method (Wang et al., 2007) and the 16S rRNA database CyanoSeq (Lefler et al., 2023) with SILVA 138.1 (Quast et al 2013) used as reference. Archaeal, chloroplast, eukaryotic, and mitochondrial ASVs were removed prior to downstream analyses.

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RESULTS

Nutrients and trace metals including phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), sodium (Na), chloride (Cl), boron (B), iron (Fe), manganese (Mn), sulfate, copper (Cu), zinc (Zn), aluminum (Al), and molybdenum (Mo) were analyzed over 60 days for Pond A, Pond B, Pond C, and Pond D. Results are presented in Table 1, 2, 3 and 4. Despite the thorough analysis, no statistically significant variances were detected in these components throughout the duration of the study.

Table 1: Result of analysis of Nutrients and Trace Metals in Pond A over a 60-Day experiment.

Days after treatment	P (ppm)	K (ppm)	Mg (ppm)	Ca (ppm)	Na (ppm)	Cl (ppm)	B (ppm)	Fe (ppm)	Mn (ppm)	sulfate (ppm)	Cu (ppm)	Zn (ppm)	AL (ppm)	Mo (ppm)
0	0.01	1.85	1.16	37.3	13	14.1	0.01	0.01	0.01	0.848	0.01	0.01	< 0.015	< 0.015
0	0.01	1.82	1.18	37.5	12.9	14.3	0.01	0.01	0.01	0.71	0.01	0.01	< 0.015	< 0.015
0	0.01	1.8	1.18	36.6	12.9	13.1	0.01	0.01	0.01	0.644	0.01	0.01	< 0.015	< 0.015
7	0.01	1.97	1.24	37.9	12.6	14.7	0.01	0.01	0.01	1.31	0.01	0.064	< 0.015	< 0.015
7	0.01	1.75	1.19	41.2	12.4	16.3	0.01	0.01	0.01	0.743	0.01	1.15	< 0.015	< 0.015
7	0.01	1.63	1.14	36.4	12.2	13.4	0.01	0.01	0.01	0.665	0.01	0.162	< 0.015	< 0.015
14	0.01	1.59	1.04	29.9	11.8	14.8	0.01	0.01	0.01	0.83	0.01	0.01	< 0.015	< 0.015
14	0.01	1.62	1.05	32	11.9	12.3	0.01	0.01	0.01	0.983	0.01	0.01	< 0.015	< 0.015
14	0.01	1.42	1.03	34.4	12	11.6	0.01	0.01	0.01	0.971	0.01	0.01	< 0.015	< 0.015
30	0.01	1.48	1.13	37.5	12.1	14	0.01	0.01	0.01	1.72	0.01	0.01	< 0.015	< 0.015
30	0.01	1.49	1.14	41.3	12.2	12.4	0.01	0.01	0.01	1.2	0.01	0.066	< 0.015	< 0.015
30	0.01	1.24	1.03	40.2	11.2	13.6	0.01	0.01	0.01	0.857	0.01	0.167	< 0.015	< 0.015
60	0.01	1.2	1.15	40.7	12.7	14.3	0.01	0.01	0.01	2.37	0.01	0.01	< 0.015	< 0.015
60	0.01	1.14	1.1	38.5	12.5	12.2	0.01	0.01	0.01	2.15	0.01	0.01	< 0.015	< 0.015
60	0.01	1.12	1.05	35.8	12.3	14.4	0.01	0.01	0.01	2.06	0.01	0.01	< 0.015	< 0.015

Table 2: Result of analysis of Nutrients and Trace Metals in Pond B over a 60-Day experiment.

Days after treatment	P (ppm)	K (ppm)	Mg (ppm)	Ca (ppm)	Na (ppm)	Cl (ppm)	B (ppm)	Fe (ppm)	Mn (ppm)	sulfate (ppm)	Cu (ppm)	Zn (ppm)	AL (ppm)	Mo (ppm)
0	0.01	0.351	0.522	16.6	3.13	3.81	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
0	0.01	0.065	0.53	20.1	2.62	3.42	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
0	0.01	1.54	4.03	18.2	36.7	66.8	0.01	0.01	0.01	6.77	0.01	0.01	< 0.015	< 0.015
7	0.01	0.354	1.28	34.9	7.68	8.69	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
7	0.01	0.387	1.24	34.6	6.96	9.63	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
7	0.01	0.294	1.27	35.6	7.61	10.1	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
14	0.01	0.174	0.587	29.1	2.62	4.06	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
14	0.01	0.3	0.672	29.6	3.77	5.58	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
14	0.01	0.163	0.605	25.5	3.12	4.32	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
30	0.01	0.056	0.68	31.3	3.02	2.86	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
30	0.01	0.068	0.641	28.7	2.83	2.76	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
30	0.01	0.083	0.641	30.8	2.83	4.32	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
60	0.01	0.263	0.768	35.6	3.26	3.01	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
60	0.01	0.235	0.684	35.6	3.22	3.79	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
60	0.01	0.237	0.742	36.2	3.21	2.81	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015

Table 3: Result of analysis of Nutrients and Trace Metals in Pond C over a 60-Day experiment.

Days after treatment	P (ppm)	K (ppm)	Mg (ppm)	Ca (ppm)	Na (ppm)	Cl (ppm)	B (ppm)	Fe (ppm)	Mn (ppm)	sulfate (ppm)	Cu (ppm)	Zn (ppm)	AL (ppm)	Mo (ppm)
0	0.01	0.031	0.554	29.4	4.91	5.44	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
0	0.01	0.01	0.569	28.7	4.77	4.2	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
0	0.01	0.01	0.563	31.1	4.77	5.19	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
7	0.01	0.033	0.507	28.4	4.22	8.14	0.01	0.01	0.01	0.03	0.01	3.33	< 0.015	< 0.015
7	0.01	0.01	0.521	29.8	4.32	5.09	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
7	0.01	0.01	0.491	26.2	4.25	4.39	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
14	0.01	0.102	0.629	23.1	5.27	5.49	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015

14	0.01	0.103	0.458	25.1	3.92	3.8	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
14	0.01	0.031	0.473	22.6	4.12	4.61	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
21	0.01	0.01	0.45	31	3.53	2.75	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
21	0.01	0.136	0.504	27.7	4.18	5.25	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
21	0.01	0.041	0.505	29.2	3.99	4.17	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
28	0.01	0.174	0.587	29.1	2.62	4.06	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
28	0.01	0.3	0.672	29.6	3.77	5.58	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
28	0.01	0.163	0.605	25.5	3.12	4.32	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
35	0.052	0.062	0.593	32.3	9.49	3.75	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
35	0.01	0.01	0.472	29.5	3.59	3.95	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
35	0.01	0.01	0.471	30.7	3.47	3.18	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
42	0.01	0.01	0.482	28.7	3.46	3.13	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
42	0.01	0.01	0.508	27.6	3.36	3.63	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
42	0.01	0.01	0.466	30.5	3.41	4.03	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
49	0.01	0.01	0.562	36.4	3.7	4.25	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
49	0.01	0.01	0.484	30.2	3.49	3.52	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
49	0.01	0.01	0.486	33	3.43	2.52	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
56	0.01	2.98	0.813	42.5	10.6	7.29	0.01	0.01	0.01	0.989	0.01	0.01	< 0.015	< 0.015
56	0.01	0.142	0.623	37.3	4	2	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
56	0.01	0.048	0.575	38	3.66	4.12	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
63	0.01	0.219	1.44	37.9	9.52	16.2	0.01	0.01	0.01	0.74	0.01	0.01	< 0.015	< 0.015
63	0.01	0.043	0.89	41.5	6.28	21.7	0.01	0.01	0.01	0.048	0.01	0.01	< 0.015	< 0.015
63	0.01	0.01	0.501	37.6	3.22	3.22	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
70	0.01	2.09	3.44	14	12.7	20.5	0.01	0.01	0.01	8.12	0.01	0.01	0.036	< 0.015
70	0.01	0.254	1.54	47.6	8.6	14.3	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
70	0.01	0.173	1.49	44.5	8.93	14.4	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015

Table 4: Result of analysis of Nutrients and Trace Metals in Pond D over a 60-Day experiment.

Days after treatment	P (ppm)	K (ppm)	Mg (ppm)	Ca (ppm)	Na (ppm)	Cl (ppm)	B (ppm)	Fe (ppm)	Mn (ppm)	sulfate (ppm)	Cu (ppm)	Zn (ppm)	AL (ppm)	Mo (ppm)
0	0.01	0.149	1.35	39.4	7.84	10.3	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
0	0.01	0.217	1.29	35.1	7.72	11.4	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
0	0.01	0.206	1.22	33.1	7.46	8.69	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
7	0.01	0.237	1.32	41.4	7.72	11.7	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
7	0.01	0.186	1.47	40.4	8.35	12.8	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
7	0.01	0.227	1.52	44.9	8.73	15.2	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
14	0.01	0.232	1.51	48.8	8.16	11.7	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
14	0.01	0.324	1.59	46.3	7.46	11.4	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
14	0.01	0.318	1.55	47.5	8.21	11.1	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
30	0.01	0.211	1.32	48.3	6.63	8.64	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
30	0.01	0.186	1.17	42	6.2	8.95	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
30	0.01	0.237	1.33	48	6.71	17.3	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
60	0.01	0.175	1.46	45.5	8.46	12.6	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
60	0.01	0.036	0.744	41.3	4.68	6.53	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015
60	0.01	0.046	0.49	39.2	3.33	2.96	0.01	0.01	0.01	0.03	0.01	0.01	< 0.015	< 0.015

The study analyzed environmental parameters across all ponds over time. Turbidity levels remained stable throughout the study. However, dissolved oxygen (DO) showed a slight decrease 60 days after starting the experiment in Ponds B and D, while Ponds A and C maintained consistent DO levels. Also, conductivity exhibited a slight increase in all ponds 60 days after starting the experiment. Meanwhile, temperatures decreased gradually in all ponds over time (Figure 2).

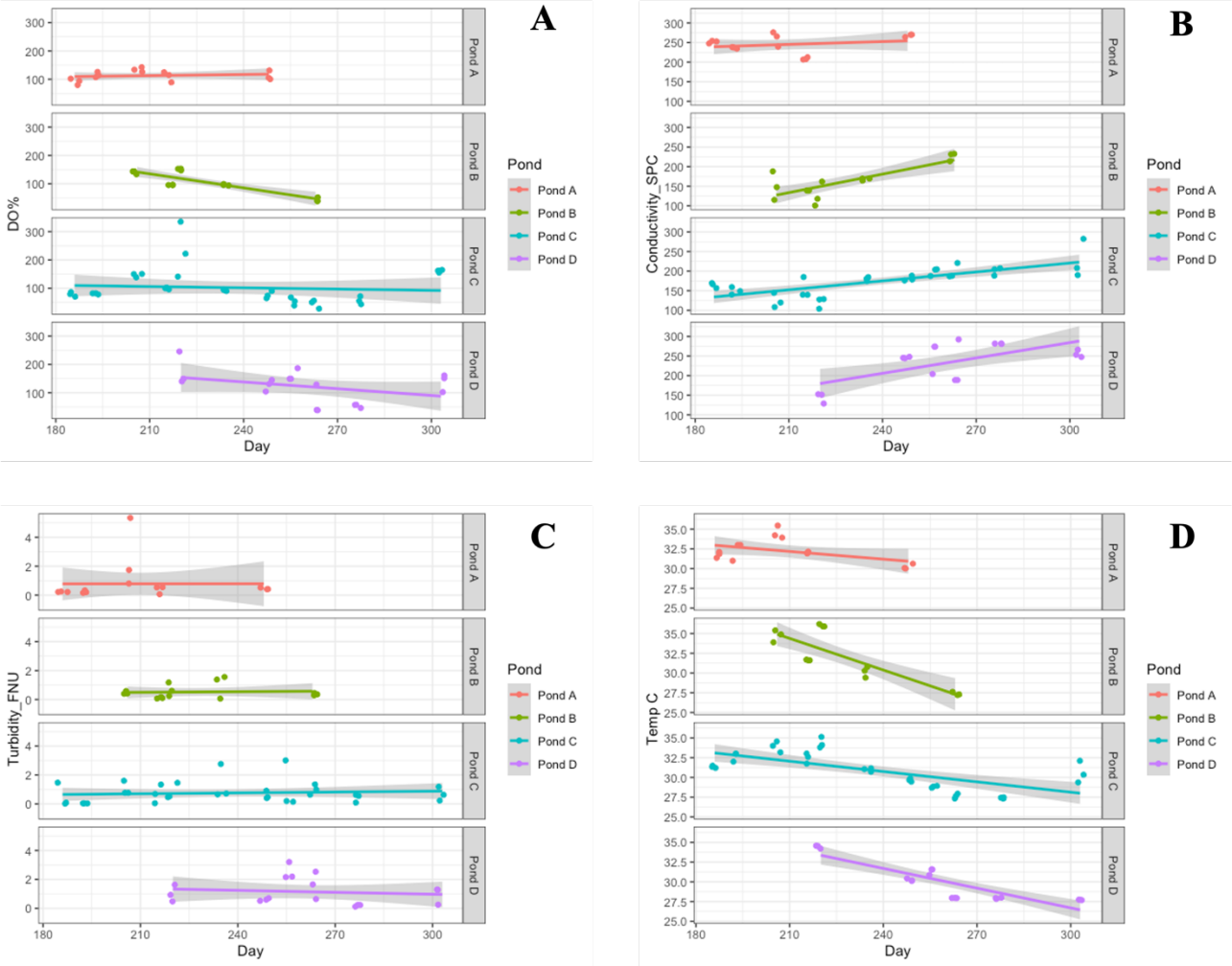

Figure 2: Water quality parameters of sampled ponds over a 60-Day experiment. A) Dissolved Oxygen (%); B) Conductivity (SPC); C) Turbidity (FNU); D) Temperature (C).

Chlorophyll a increased 60 days after starting the experiment in Pond A. There was no statistical difference in nitrate in all ponds. Total dissolved solids (TDS) slightly increased 60 days after starting the experiment in Pond C and Pond D.

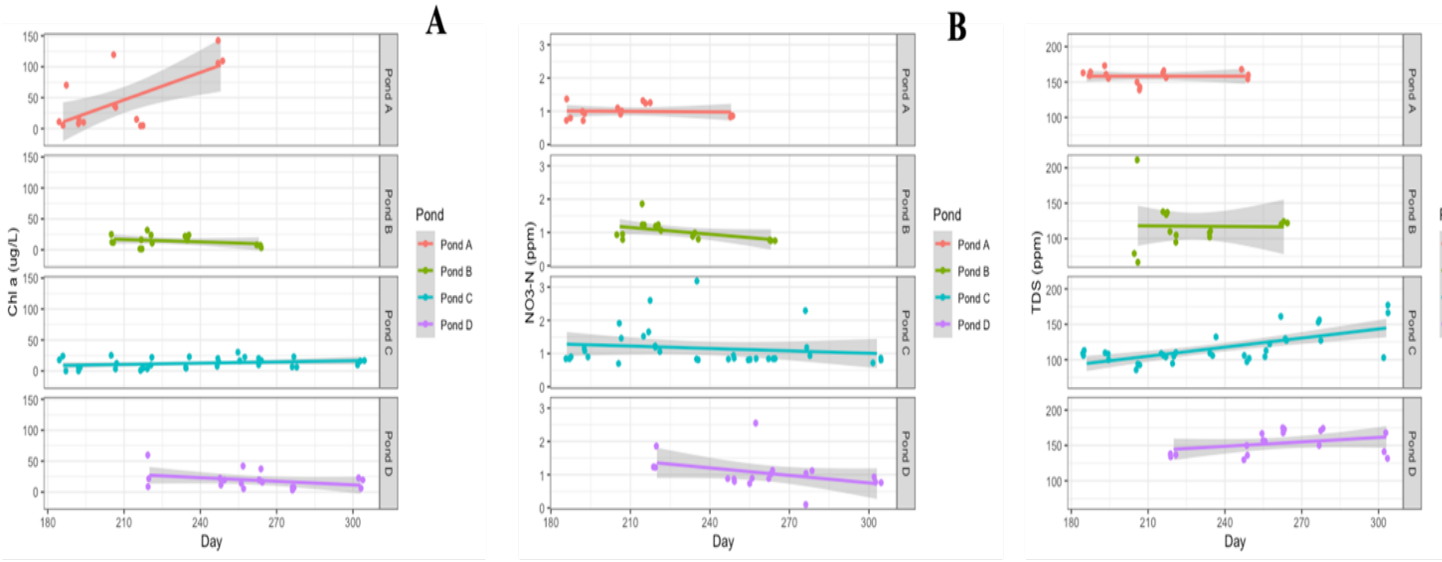

Figure 3: Water parameters of sampled ponds over a 60-Day experiment. A) Chlorophyll-a (ug/L); B) Nitrate NO_3^-N (ppm); C) Total dissolved solids – TDS (ppm).

The data show that when nanobubbles are introduced into water, they do not lead to the production of hydrogen peroxide. This finding is significant because peroxides have varied effects on aquatic ecosystems and water quality. By analyzing both nanobubble-treated ponds and negative control ponds (those without nanobubble treatment), the levels of hydrogen peroxide were extremely low, close to zero (Figure 4). As mentioned before, the water samples were collected before the nanobubbles exposure (time 0), and also 7, 14, 30 and 60 days after starting the experiment in treated ponds and untreated control. The nanobubble treatment was carried out Ponds A, B, and D over a period of 14 days.

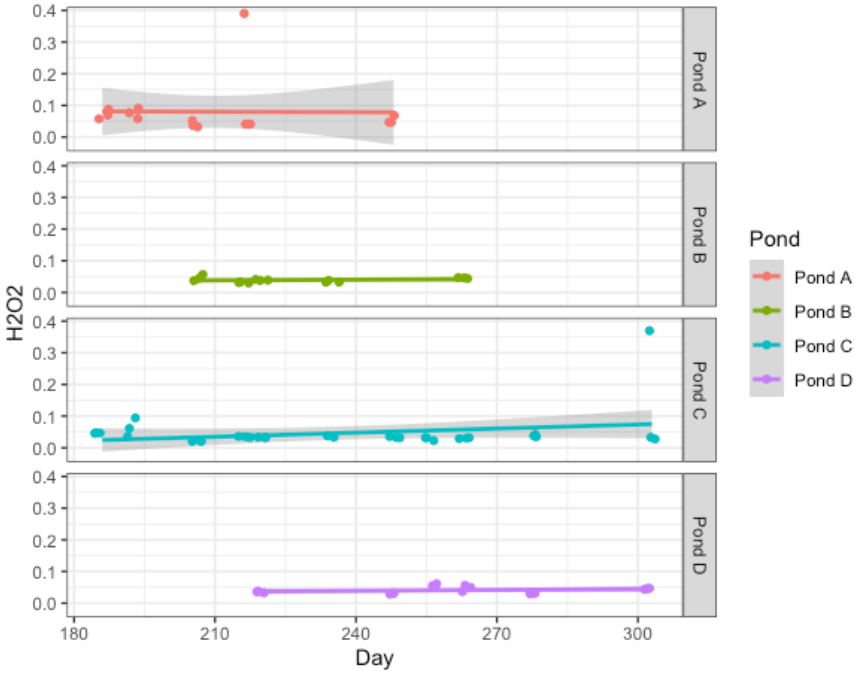

Figure 4: Results of hydrogen peroxide in ponds over a 60-Day experiment. Day mentioned on the x-axis, corresponds to the days of the year (Julian day) in which the experiment was conducted in each pond.

The bacterial community in Ponds B, C and D, were primarily composed of Gammaproteobacteria, Verrucomicrobiae, and Actinobacteria, while Pond A had a higher abundance of Cyanophyceae (Figure 5). There were significant differences in the bacterial communities throughout time in all ponds.

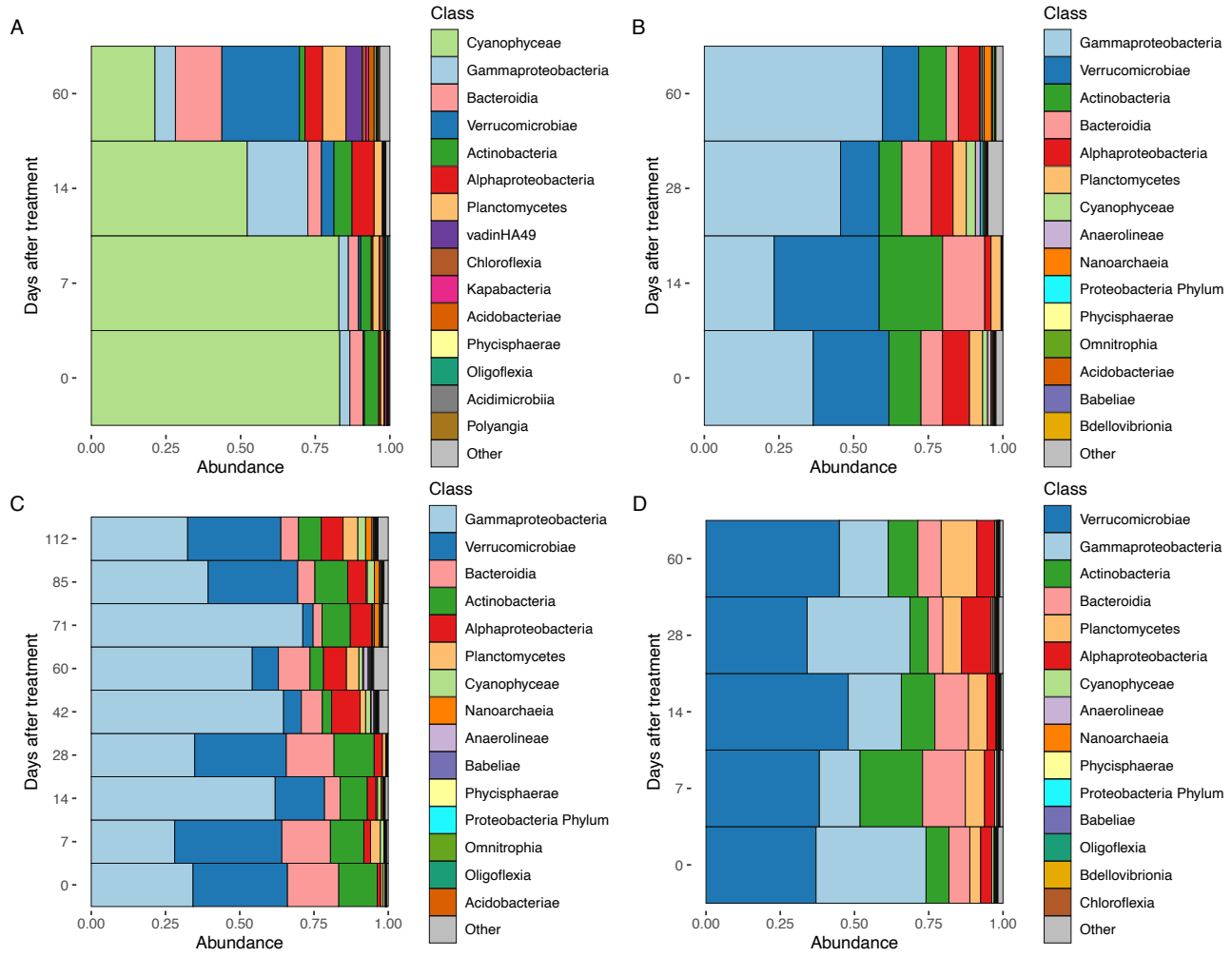

Figure 5: Class-level bacterial community analysis. A) Pond A, B) Pond B, C) Pond C, and D) Pond D.

In Pond A, the cyanobacterial community was initially denominated by *Cyanobium*, however, there was a significant reduction of *Cyanobium* over the time, accompanied by an increase in bloom-forming cyanobacteria (e.g., *Microcystis*) 60 days after starting the experiment (Figure 6A). In Ponds B, C, and D, the bacterial community was largely comprised of uncultured bacteria (FukuN18) and members of the Comamonadaceae family (Figure 6). These findings illustrate dynamic changes in bacterial populations over time, with notable differences in composition between ponds.

We observed a temporal change in the community structure individually in each of the ponds (p= 0.001), however, when we compared treatment and control, there was no significant difference in the community structure between Pond C (control) and Pond B (treatment) (p=

0.237) and also between Pond C (control) and Pond D (treatment) (p= 0.06) during their respective treatment days (Figure 6).

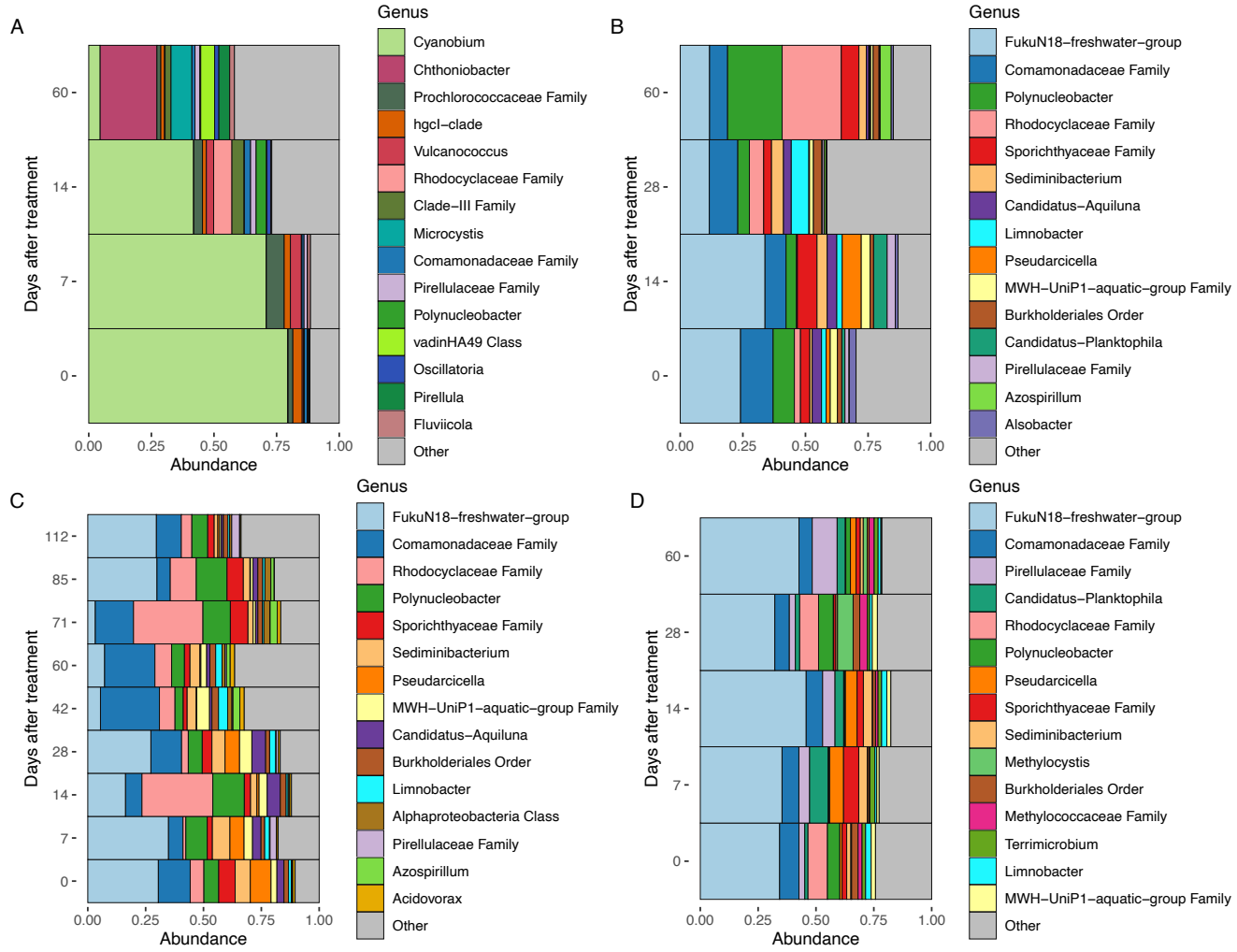

Figure 6: Genus-level bacterial community analysis. A) Pond A, B) Pond B, C) Pond C, and D) Pond D.

Before treatment, ponds A and D were undersaturated with respect to N2, suggesting net nitrogen fixation was occurring in these ponds. However, by 28 days after starting the experiment, the N saturation ratio was near 1, indicating the ponds were reaching equilibrium. The data at 14 days after starting the experiment suggested that nanobubbles may have displaced N_2 gas, leading to a significant decrease in the N_2 saturation ratio in Pond B. Interestingly, this trend was not observed in Ponds A and D. However, this may indicate that data from the 14 days after starting the experiment samples were not able to tell us sufficient insight into the dynamics of denitrification and nitrogen fixation but rather capture the physical effects of adding the nanobubbles (Figure 7).

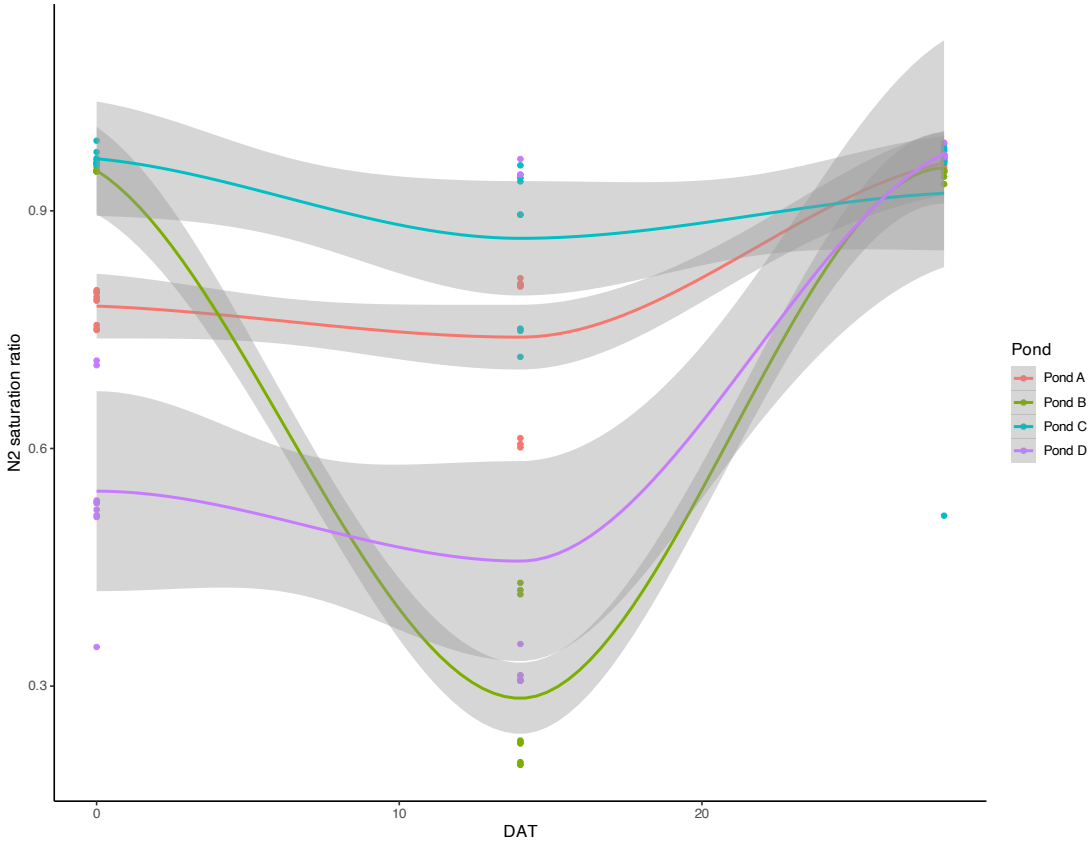

Figure 7: Nitrogen saturation ratio in day after treatment.

CONCLUSION:

In summary, our study carried out over approximately four months on nutrient levels, environmental factors, and bacterial communities in four ponds yielded interesting findings. Despite analysis, there were no significant changes in nutrient and metal concentrations over time in each pond. While some environmental parameters remained stable, others showed slight fluctuations, such as a decrease in dissolved oxygen and an increase in conductivity. Notably, nanobubble treatment did not result in hydrogen peroxide production in any ponds. The increase in chlorophyll-a levels 60 days after starting the experiment and the simultaneous decrease in the abundance of cyanophytes can be explained by the potential change in the dominance of other algal species after treatment. Bacterial communities exhibited a temporal shift, however, comparisons between treated and control ponds showed consistent community structures. These data suggest that nanobubble treatment did not have an observable impact on the overall structure of the bacterial community. The results suggest an initial occurrence of net nitrogen fixation in ponds A and D. Nanobubbles may have displaced N_2 gas, leading to a decrease in the

N_2 saturation ratio (significantly in Pond B). Yet, after the nanobubble treatment stopped, equilibrium was reached in all ponds.

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