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Influence of Nutrients and the Native on E. Coli Survival in the Beach Environment

Brigid Meyers
University of Wisconsin-Milwaukee

INFLUENCE OF NUTRIENTS AND THE NATIVE COMMUNITY ON *E. COLI* SURVIVAL IN THE BEACH ENVIRONMENT

by

Brigid Meyers

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ABSTRACT

INFLUENCE OF THE NATIVE COMMUNITY AND NUTRIENTS ON *E. COLI* SURVIVAL IN THE BEACH ENVIRONMENT

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Brigid Meyers

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E. coli is used as an indicator for water quality to determine if water poses a health risk for pathogens. Past research has shown that *E. coli* is present in high numbers in freshwater beach sands distinct from fecal pollution events, yet the precise mechanism for their persistence is not well understood. Persistent *E. coli* populations in sand can resuspend into adjacent water and lead to increased beach closures when no threat is present. This work identifies factors that influence the survival of *E. coli* in sand using laboratory microcosms to replicate beach conditions. Microcosms were deployed to examine the effect of genetic background, competition with autochthonous microbes, and increased nutrient sources on *E. coli* survival.

E. coli strains can be characterized into phylogroups, genetic divergences through evolution of the strain. *E. coli* phylogroup B2 is commonly recovered from mammals, while phylogroup B1 is commonly recovered from the environment. Survival was comparable between the distinct phylotypes, however, having a deficient stress response greatly reduced survival. In the absence of the native community under nutrient conditions comparable to those observed in sand, *E. coli* cell densities remained within an order of magnitude of initial concentrations after 5 weeks of incubation. Increasing carbon and nitrogen concentrations resulted in higher *E. coli* levels over

time, with increased nitrogen associated with higher levels of survival in the first 2 weeks, and increased carbon providing an advantage at later time points. However, the highest survival was found with the addition of both carbon and nitrogen. Native sand seeded with fresh *Cladophora* maintained higher concentrations of *E. coli*, compared to sand containing decayed *Cladophora* or no *Cladophora*. Our findings demonstrate persistent *E. coli* populations in sand can be affected by the availability of carbon and nitrogen, the ability to regulate stress, and the presence of algae mats (i.e., *Cladophora*). Further, native microbial communities may modulate survival by outcompeting *E. coli* for nutrients.

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CFU	Colony Forming Units
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
FIB	Fecal Indicator Bacteria
MPN	Most Probable Number
qPCR	quantitative polymerase chain reaction
RWQC	Regional Water Quality Committee
TC	Total Carbon
TN	Total Nitrogen
TOC	Total Organic Carbon
µg	Microgram
USEPA	United Stated Environmental Protect Agency

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Chapter 1-Introduction

1.1 *E. coli* as a fecal indicator

The Federal Beach Environmental Assessment and Coastal Health (BEACH) Act was signed into law in 2000, an amendment to the Clean Water Act (CWA), which enforced the monitoring of recreational waters for pathogens and pathogen indicators (33 U.S.C. §1313 et seq. (2000)). The BEACH Act requires public notification when water exceeds or is likely to exceed water quality standards. Surface water quality monitoring uses single indicator organisms, fecal indicator bacterium (FIB) (33 U.S.C. §1313 et seq. (2000)), to determine if water is unsafe due to fecal pollution (US EPA, 2000). Commonly used FIB are *E. coli* for freshwater and Enterococci for marine waters (United States Environmental Protection Agency, 2012).

E. coli is a rod-shaped, gram-negative bacterium (Blount, 2015). The primary environment of *E. coli* is the intestines of warm-blooded animals, found at a concentration of 10^6-10^9 cells per gram of fecal matter in humans, and is classified as a fecal coliform (Blount, 2015; Savageau, 1983). *E.* coli was traditionally expected to have a two-day half-life outside of the host (Blount, 2015; Leclerc et al., 2001; Petersen & Hubbart, 2020; Savageau, 1983). Therefore, the isolation of *E. coli* from the environment, in this case water, indicated a recent fecal pollution event had occurred, and recreational water was potentially hazardous to swim in. Governing authorities implemented the widespread use of *E. coli* as a water quality metric (Donna Francy et al., 1993; United States Environmental Protection Agency, 2012; World Health Organization, n.d.). Current water quality criteria in the United States declares that an *E. coli* colony-forming unit (CFU) count above 235/100 mL in a single sample or geometric mean of 126/100 mL for 30 days is a risk for swimmers due to fecal pollution (United States Environmental Protection Agency, 2012).

E. coli monitoring in freshwater systems is reliant on culture-based methods, such as membrane filtration, where colony forming units (CFU) are obtained per volume of water, or most probable number (MPN) a substrate-based method that relies on a variety of tests to estimate the number of bacteria present (United States Environmental Protection Agency, 2012). Additional methods include quantitative polymerase chain reaction (qPCR) in which deoxyribonucleic acid (DNA) from the sample is extracted, amplified, and then quantified which results in copy numbers of *E. coli* (United States Environmental Protection Agency, 2012;Walker et al., 2017). In many cases, *E. coli* is a good indicator when there are overt sanitation concerns. However, when *E. coli* is present and there is no recent contamination, culture and qPCR based approaches may overestimate the extent to which fecal pollution is present in the water due to persistent *E. coli* populations in the secondary environment (i.e., beach sand) (Cloutier & McLellan, 2017; Rumball et al., 2021; Savageau, 1983).

1.2 *E. coli* in freshwater beach sands

E. coli has been identified to have a biphasic lifestyle which alternates between a primary habitat, the gut of endotherms, and a secondary habitat, the external environment (Savageau, 1983). *E. coli* is shed into secondary environments in the form of fecal matter, with gulls being a major contributor to freshwater beaches (Converse et al., 2012; Ishii et al., 2007). *E. coli* can establish populations in host independent environments, such as soil, sediment, or water (Savageau, 1983). Beach sand has been identified as a reservoir for *E. coli* burdens by providing ideal moisture conditions and UV protection through particle attachment which has led to consistent and sustained populations (Beversdorf et al., 2007; Whitman et al., 2014; Whitman & Nevers, 2003).

Sand has been identified to contain higher *E. coli* concentrations than recreational waters, with studies showing can contain 13X higher *E. coli* concentrations (Whitman & Nevers, 2003; Vogel et al., 2016). Berm sand, defined as wave-washed sand, is the focus of this study due to the high concentration of FIB, compared to other beach zones (Beversdorf et al., 2007; Byappanahalli, Whitman, Shively, Sadowsky, et al., 2006; Cloutier et al., 2015; Ishii et al., 2010). In addition, there is a strong correlation between adjacent water samples, suggesting the two *E. coli* reservoirs are linked (Cloutier et al., 2015). Current *E. coli* monitoring does not account for populations that have established in beach sand which can result in an overestimation of the extent of fecal pollution present in recreational water.

Select *E. coli* isolated from sand is genetically distinct from host-specific strains which has suggested that there are *E. coli* strains better suited for survival in beach sand (Dufour & Ballentine Richard, 1986; Savageau, 1983; Vogel et al., 2016). Phylogenomic analysis of *E. coli* from freshwater beaches has identified that there are distinct patterns between human and beach strains of *E. coli*. Of the eight phylogroups, B1 is the most abundant and B2 is the least abundant phylotype recovered (Martinson et al., 2019; Rumball et al., 2021; Tenaillon et al., 2010). This further identifies that certain *E. coli* populations are better suited for establishment and accumulation in sand. This study aims to further our understanding of the mechanism for persistent *E. coli* populations in freshwater sand, specifically investigating environmental and genetic factors, to aid beach management.

Freshwater beach sand and water quality are interconnected because of sand-water interaction, with research suggesting that there is a link between the two reservoirs (Cloutier et al., 2015). *E. coli* loading into freshwater beach sand can occur through direct input such as gull droppings (Converse et al., 2012; Ishii et al., 2007). Loading can also occur indirectly as *E. coli*

populations in water can transport into sand through wave-induced infiltration during low energy wave conditions (Wu et al., 2017). In addition, accumulated *E. coli* concentrations in sand can resuspend into the water column through high wave-water interaction such as high precipitation events or high energy waves (Vogel et al., 2016). Resuspension of *E. coli* populations from the sand can lead to increased concentrations in adjacent recreational water, without any threat to human health being present. This work aims to understand the mechanism for persistent *E. coli* populations in sand to assist in addressing *E. coli* in recreational waters.

1.3 Potential drivers for survival

The primary habitat and secondary habitat have many distinctions including nutrient availability**,** chemical parameters, and temperature (Petersen & Hubbart, 2020). The secondary habitat contains more stressors than the gut of endotherms, such as nutrient limitations and seasonal variation in temperatures (Savageau, 1983). It is suggested that persistent *E. coli* populations in the secondary environment can regulate stress response in order to adapt to environmental changes, with previous studies identifying persistent populations in soil having a conserved general stress response regulation(Somorin et al., 2016). The beach environment is complex, with a wide range of abiotic and biotic components that are suspected to modulate *E. coli* survival with major influencing factors suggested to be the native community and nutrient availability (Baker et al., 2020; Petersen & Hubbart, 2020; Rumball et al., 2021).

The native bacterial community of freshwater beach sands is abundant with a single sand grain harboring approximately 10^4 to 10^5 cells and FIB, *E. coli* or Enterococci, represent only 2 colony-forming units (CFU) per gram of sand (Cloutier et al., 2015; Probandt et al., 2018). In the absence of the native bacterial community, *E. coli* from different genetic backgrounds and host

sources are found in higher concentrations in lake water, soils, and beach sand whose native community was removed (Baker et al., 2020; Flint, 1989; Rumball et al., 2021). This suggests that the native community modulates *E. coli* survival in freshwater beach sands through predation or competition for nutrients.

Observational studies of the relationship between nutrients and *E. coli* have been conducted on the Eastern and Western shorelines of Lake Michigan, which identified a positive correlation between *E. coli* concentrations and nutrient concentrations (Cloutier et al., 2015). In addition, environmental survival experiments using microcosms or growth chambers have been carried out. In these experiments, sand seeded with nutrient sources prolonged *E. coli* survival and in some cases even modulated growth. For example, the addition of plankton and modified sand with increased available nutrients (autoclaved) in benchtop microcosms has shown to prolong survival as well as autoclaved sand in field microcosms (Alm et al., 2006; Byappanahalli, Whitman, Shively, Ting, et al., 2006; Rumball et al., 2021).

Cladophora is found along the shorelines of beaches in the Great Lakes region, it is a nuisance alga due to the pungent odor it produces while decaying. Through the decaying process, *Cladophora* releases nutrients into adjacent water and sediment (Zhang et al., 2021). *Cladophora* has also been identified to harbor bacteria including *E. coli*, which contains higher FIB concentrations than EPA limits (Olapade et al., 2006). *E. coli* has been identified to persist in dried algal matts stored at 4°C for 6 months and after being rehydrated, populations have been shown to exhibit a 4 log CFU/g increase in cell density (Whitman et al., 2003). When *E. coli* was added into *Cladophora* leachate, lake water seeded with *Cladophora*, and sand seeded with *Cladophora* growth was observed over a 4-day period (Byappanahalli et al., 2003; vanden Heuvel et al., 2010; Whitman et al., 2003). The presence of *Cladophora* has been shown to

promote *E. coli* growth over a short period in previous work. This study will examine the effects of long-term exposure, the effects of state of decay, and well as nutrient availability.

1.4 The scope of this work

Fecal pollution in recreational waters is monitored through *E. coli*, as laid out by the BEACH Act and Recreational Water Quality Criteria. Recent research has identified that *E. coli* can be recovered from secondary environments, in this case freshwater beach sand, without fecal pollution being present. *E. coli* populations can accumulate in sand, resuspend into the water column, and lead to higher counts in water without any threat present. Despite the identification of *E. coli* populations in freshwater beach sand, the mechanism for persistence is not well understood. This study explores factors influencing *E. coli* survival in freshwater beach sand through laboratory microcosms with *E. coli* from distinct genetic backgrounds and the addition of nutrients. Our objective was to examine i) the role of genetic background, ii) the effect of increased nutrients and iii) the role of the native community on *E. coli* survival in sand.

We tested two known phylotypes of *E. coli,* including one that is commonly host-associated (B2) and one that is frequently detected in beach sand and therefore likely suited to survive in the secondary environment (B1). We also tested an *E. coli* strain with a mutation in the gene responsible for regulating the stress response (*rpoS*), a known mechanism *E. coli* employs to survive in harsh conditions. There were no observable differences in decay rates between distinct phylogroups, however we determined the *rpoS* deficient strain survived poorly in native sand. We tested for the influence of both simple and complex nutrient sources on *E. coli* survival through microcosms with glucose and ammonia chloride as a nutrient source, simple, as well as the addition of *Cladophora*, complex. For simple sources we increased carbon, increased nitrogen, and both above observed levels in beach sand. It was determined when both

carbon and nitrogen were added to sand, at least four-fold above concentrations observed baseline concentrations, *E. coli* survival was prolonged. Complex nutrient sources were examined through the addition of *Cladophora.* We tested the influence of fresh, decayed, and varying concentrations of *Cladophora* on *E. coli* survival These experiments identified that fresh *Cladophora* has a higher influence on *E. coli* cell densities due to the high potential to provide nutrients into the sand, compared to decayed *Cladophora*. Previous work has identified that the native community of beach sand modulates *E.* coli survival, we tested whether this occurred through predation or competition using sand with and without the microbial community present. Our findings suggest that competition of the native community results in reduced *E. coli* survival.

1.6 Significance

Current monitoring techniques for monitoring fecal pollution does not account for persistent *E. coli* populations in freshwater sand. These populations can resuspend into the water column leading to higher concentrations in adjacent water with no threat present. The overarching goal of this work is to improve current knowledge of drivers for persistent *E. coli* populations in sand to aid beach management techniques. We examined the influence of nutrients, including localized sources such as stranded *Cladophora*, on *E. coli* populations which can assist with understanding *E. coli* fluctuations in water. We found the major determinant of *E. coli* survival in freshwater beach sand was the addition of nutrients, specifically carbon and nitrogen concentrations higher than observed in beach sand. This work provided the framework for identifying pollution sources that can promote *E. coli* survival in sand through the characterization of carbon and nitrogen content, which can be incorporated into beach

management techniques. Through this improved knowledge, we can begin to understand *E. coli* fluctuations in water due to sand populations resuspension into water.

Chapter 2-Experimental Design and Methodology

2.1 *Sample collection and processing*

Sand to be deployed in laboratory microcosms was collected from Atwater Beach in Milwaukee County, Wisconsin (43°05'25.1"N, 87°52'24.2"W) from the berm in a sterile 2L wide mouth round bottle. Berm sand, defined as exposed but wave-washed sand, was chosen due to its high concentration of *E. coli*, relative to other beach zones, and the notion that samples are positively correlated with water samples (Beversdorf et al., 2007; Byappanahalli, Whitman, Shively, Sadowsky, et al., 2006; Cloutier & McLellan, 2017; Ishii et al., 2010). Sand was stored at 4°C and used within a week of collection. *Cladophora* was collected on the north section of Bradford Beach in Milwaukee County, Wisconsin (43°03'51.4"N 87°52'11.5"W) in a sterile 1L wide mouth round bottle on August 31, 2021. Fresh *Cladophora* was used within 1 week of collection, while decayed *Cladophora* was stored at 4°C for 13 weeks to allow for decomposition.

2.2 *Isolates used in microcosm experiments*

Isolates used in these experiments included *E. coli* strains belonging to different genetic backgrounds: phylotype B1, phylotype B2, and a *rpoS* deficient strain. The phylotype B1 *E. coli* strain was isolated from the beach environment at Point Beach in Manitowoc, Wisconsin (Rumball et al., 2021). *E. coli* phylotype B2 is strain ECOR66 (Ochman & Selander, 1984) which was isolated from a mammalian source. Phylogroups were verified through methods described by Clermont et. al. The *rpoS* deficient *E. coli* strain ZK1000 (Bohannon,' et al., 1991) was kindly provided by the Finkle lab at the University of Southern California.

2.3 *Microcosm experiments*

Laboratory microcosms were deployed to monitor *E. coli* dynamics to investigate the influences on survival. The experimental design was adapted from Alm et. al 2006, in which *E. coli* was inoculated into either Luria Broth (LB) or M9 minimal media depending on the specific microcosms set up and shaken at 37°C for 18 hours. *E. coli* inoculated into M9 included cells to be seeded into increased carbon and nitrogen treatments and the *rpoS* deficient strain, all other cells were inoculated into LB. Cells were spun down and washed three times with sterile water and resuspended, seeded into sand, and homogenized to give a final concentration of approximately 10^6 cells per gram (g) of sand. Exact cell counts were determined for each initial time point. Petri dishes containing 35 g sand were then placed in a loosely closed container with a sterile reservoir containing water, to maintain consistent moisture content, and incubated until enumeration. At each processing point, 4 g of sand was harvested and eluted in 40 ml of sterile water and *E. coli* was enumerated, following the 1:10 ratio of sand to water for *E. coli* extraction as described previously (Boehm et al., 2009).

Microcosms included testing the influence of genetic background, nutrients, and the native microbial community on *E. coli* survival. All microcosms involved the deployment of phylotype B1 as the model *E. coli* to monitor, except the experiment investigating the role of genetic background where additional strains were tested. We employed B1 because it is the predominant phylotype isolated from the beach environment. Previous beach surveys and *in-situ* microcosms collected sand moisture and temperature data, which allowed for the high throughput laboratory microcosms in this study to accurately replicate beach conditions with a temperature of 15-16°C and a moisture content of 20-24% (Beversdorf et al., 2007; Cloutier et al., 2015; Rumball et al., 2021).

The role of genetics on *E. coli* survival was examined by deploying phylotype B1, phylotype B2, and a *rpo*S deficient strain into native sand and monitoring their decay over a 4 week period. The phylotype B1 has a high frequency of isolation from the sand and B2 a low frequency of isolation, suggesting they may have different abilities to survive in the external environment. The *rpo*S deficient strain was selected to investigate the role that regulating stress response has on *E. coli* survival in sand.

The influence of nutrients on *E. coli* proliferation in beach sand was investigated through the addition of varying concentrations of simple carbon and nitrogen sources as well as complex environmental sources. Field surveys of Atwater and Bradford Beach were conducted for available carbon and nitrogen, testing nutrients eluted from sand (FIG S.1). Based on field survey data, we were able to determine the amount of carbon and nitrogen in sand collected from Atwater beach to be 144.5 μg C/g and 1.60 μg N/g (Table S.1). We then determined the amount of glucose and ammonia chloride required to bring the levels to 2X, 4X, and 10X above the baseline (Table 1). Nutrient additions were added in separate and combined microcosm setups (i.e., there is a 2X C, 2X N, and 2X CN) that were then monitored for 6 weeks, with enumeration weekly.

Table 1 Additional nutrient contents for carbon and nitrogen microcosm experiment to increase levels over TOC and TN in beach sand baseline measurements.

Sand treatment	Nutrient composition µg /g [1]	
	Total Organic Carbon	Nitrogen
0X	0.00	0.00
2X	144.5	1.59
4X	433.6	4.79
10X	1300.0	14.4

[1]In micrograms per gram of sand and are indicative of **additional** nutrients in treatments, above what was determined through the Atwater beach survey.

To investigate the influence of prolonged exposure to complex environmental nutrient sources on *E. coli* survival, *Cladophora* was seeded into native sand. The first deployment entailed seeding fresh *Cladophora* into native sand at 12.5% (wet weight *Cladophora/*wet weight sand) and 0% for 9 weeks, with enumeration weekly in September of 2021. The second deployment was run for approximately 13 weeks and utilized the same *Cladophora*, which was in varying stages of decomposition. The decayed *Cladophora* was seeded into native sand at a concentration of 12.5%, 6.25%, 1.25%, and 0% (w/w) and *E. coli* was monitored weekly for 6 weeks in December of 2021. In the second deployment Total Carbon (TC), Total Organic Carbon (TOC), and Total Nitrogen (TN) concentrations of un-inoculated microcosms, just *Cladophora* seeded into native sand, were measured at the initial and endpoint of the microcosm.

The role of the native community was investigated by inoculating *E. coli* in native and baked sand over a 5 week period. Native sand was collected from Atwater Beach in August of 2021 and was un-altered. A portion was baked at 550°C for 5 hours in a muffle furnace, then rinsed three times with sterile water, and stored at room temperature in a sterile sealed container until use. Through the baking process, the microbial community was removed.

2.4 *Nutrient Analysis*

Nutrient profiles of TC, TOC, and TN were analyzed with the Shimadzu TOC-TL analyzer with the TN extension. For nutrient sources collected in a liquid state, the sample was processed on a 0.22 μm filter. For samples collected in a solid-state, the solid matter was eluted in water through vigorous shaking, filtered, and then analyzed. Carbon samples were diluted to 10^{-2} concentration in sterile water. TOC and TN samples were acidified with a 10% HCl solution, at a 0.05% (v/v) concentration. 9 mL of the filtered sample was then ran on the Shimadzu TOC-TL analyzer with potassium hydrogen phthalate and potassium nitrate standards, for carbon and nitrogen.

2.5 *Data Analysis*

The data visualization and statistical analyses in this paper were conducted in R, version 4.1.1 (R. Core Team, 2018) using core packages. Raw CFU counts were log-transformed to CFU per gram of sand. Decay coefficients were determined using the first-order exponential decay equation $\ln(C/C_0) = kt$, where C is the CFU at a given time point, C_o is CFU at time 0, k is the decay rate in days $^{-1}$, and t is the incubation time. A two-way analysis of variance (ANOVA) was used to evaluate if there were significant differences in *E. coli* decay strategies, in which CFU was linearized following the first-order decay equation, $\ln(C/C_0)$ and enumeration points were categorical variables. A Post-Hoc Tukey Honestly Significant Difference (HSD) analysis in R-Studio was determined to examine the differences in treatment on *E. coli* decay, a P-value of ≤ 0.05 was deemed significant.

Chapter 3-Results

3.1 *Stress response more important for survival than genetic background*

Isolates from phylotype B1 and B2 persisted throughout the 4 week incubation and experienced a three order of magnitude loss. In contrast, the *rpo*S deficient strain experienced a rapid 3 order of magnitude loss after 1 week of incubation and was no longer enumerable after 3.5 weeks. After 3.5 weeks of incubation in native sand average, exponential decay rates of -0.24, -0.26, and -0.43 day^{-1} were observed for B1, B2 and *rpo*S deficient strain (FIG 1). Phylotype B1 and B2 survived 4 weeks, with final average concentrations of $4.84x10^2$ and $3.57x10^2$ CFU/g sand. Phylotype B1 had a slightly lower decay rate than B2, but this difference was not significant ($p = 0.70$) and survival was within an order of magnitude. The decay rate of *rpo*S was significantly faster than that of the phylogroups B1 and B2 ($p<0.0001$).

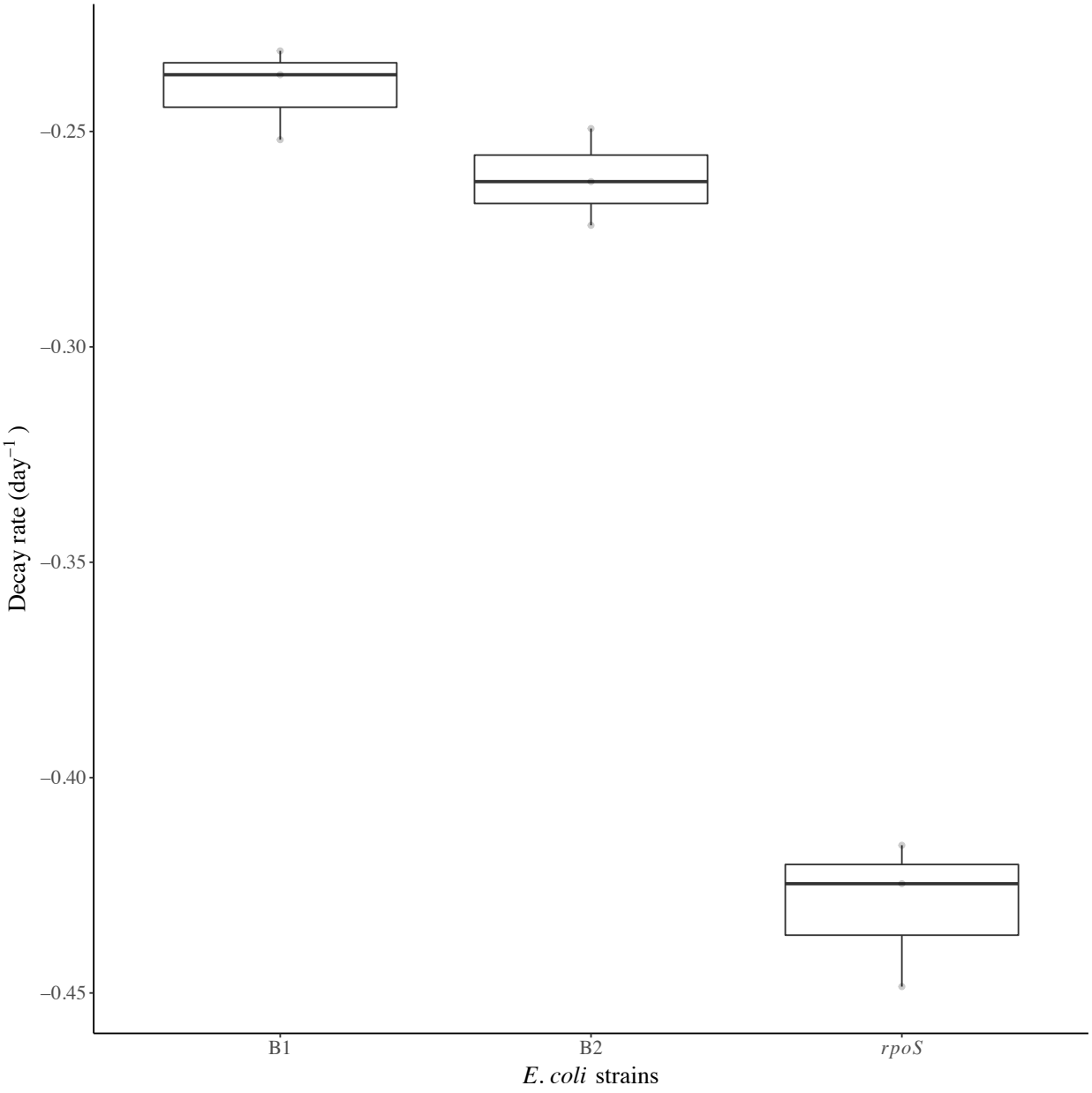

FIG 1 Decay rates of *E.* coli from different genetic backgrounds including phylotype B1, phylotype B2, and a *rpoS* deficient strain. Decay rates are determined from initial and after 24 days of incubation in native sand, from transformed CFU data. Box plots are representative of transformed CFU triplicate data (n=3).

3.2 *Effect of nutrient concentration on* E. coli *decay*

We determined the available nutrient concentrations in beach sand from field surveys, then determined the amount of carbon (C) and nitrogen (N) required to create 2X, 4X, and 10X amendments, and then deployed microcosms with the additional N, C, or N and C to assess if N or C were limiting for survival (FIG 2, FIG S2-S4). *E. coli* placed in increased concentrations of combined carbon and nitrogen contained higher cell densities than all other treatments over a 6-week incubation. Treatments with 10X combined N and C yielded significantly higher residual CFU counts than other 10X treatments, sole C and N, as well as no additional, 0X ($p<0.0001$). Average exponential decay rates after 6 weeks of incubation across the 2X, 4X, and 10X combined carbon and nitrogen treatments were -0.16, -0.12, and -0.09 day^{-1}, respectively, with final average *E. coli* concentrations in combined carbon and nitrogen treatments of $7.00x10^3$, $9.18x10^4$, and $2.38x10^5$ CFU/g sand. In contrast, decay rates for no nutrient addition averaged -0.20 day^{-1}. These results suggest longer persistence with increasing nutrients, particularly above a two-fold increase in baseline levels.

The addition of C alone did not appear to reduce decay rates until later time points after 3 weeks. This effect was most pronounced in the 4X and 10X treatments, but when considering the decay rates over time, there was not a significant difference between C and N treatments, even in the 10X treatment. However, 10X C treatment did show significantly slower decay than no nutrient addition ($p=0.004$).

E. coli seeded with only N initially contained higher cell densities than the control and carbon addition at 4X and 10X, but this effect was only observed for the first 2 weeks and was not significant. Average *E. coli* retention after 2 weeks of incubation was similar to no addition

and lower than treatments with an analogous amount of increased carbon alone. Decay coefficients for each treatment are shown in Table S2.

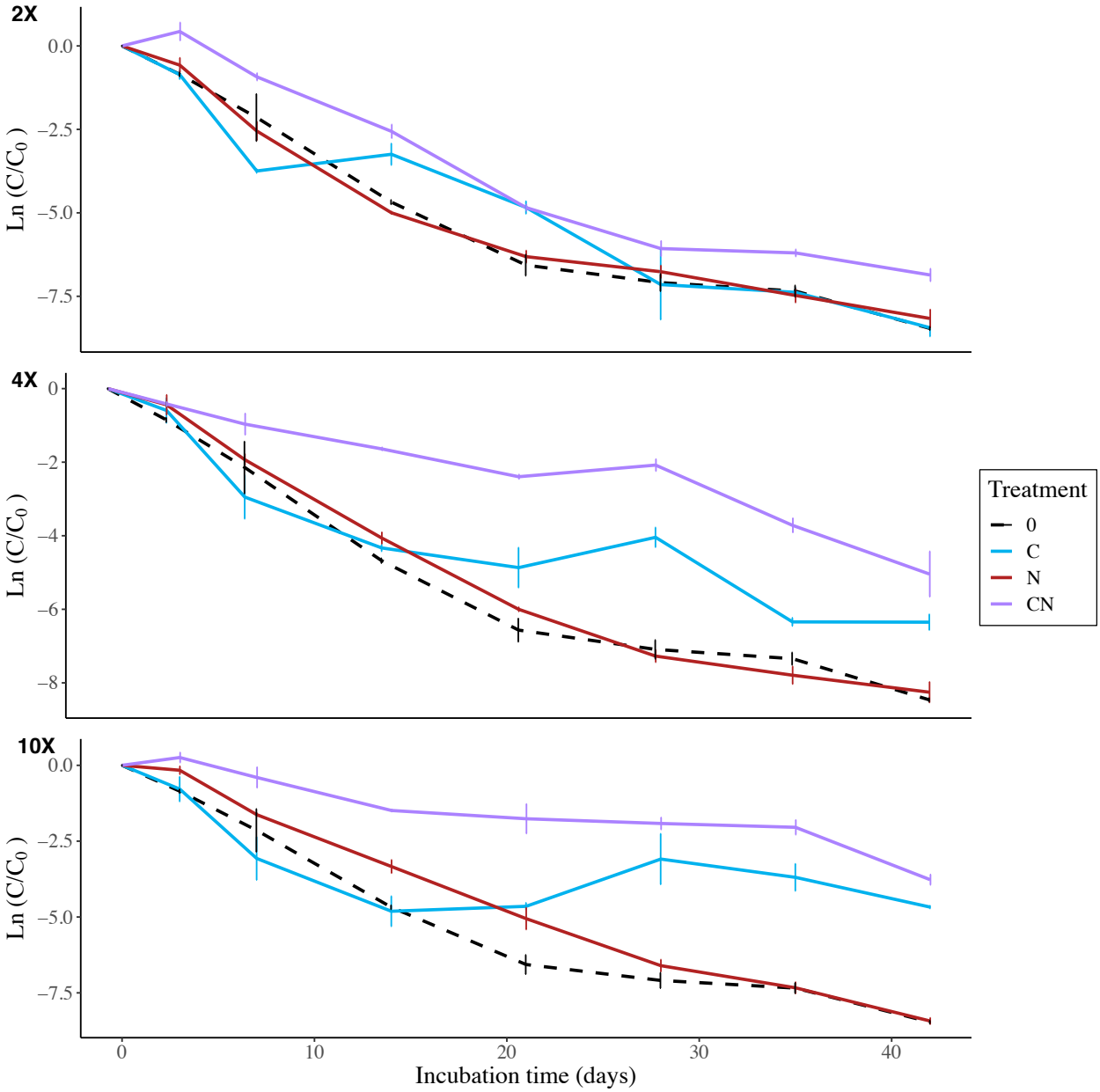

FIG 2 Natural log of average colony-forming units (CFU) per gram of sand (dry weight) normalized to the initial time point of *E. coli* phylotype B1 in increased carbon and nitrogen concentrations, grouped by fold increase. Error bars represent the standard deviation of the CFU triplicates. The top graph is the 2X treatment, the middle is 4X treatment, and the bottom is the 10X treatment. Carbon is indicated by the solid blue line, nitrogen is the solid red line, and carbon and nitrogen is represented by the solid purple line. Baseline microcosms, 0X increase, were inoculated with *E. coli*, but did not contain any carbon or nitrogen to serve as a control. Baseline treatments are the same within each of the 3 graphs indicated by the black dashed line.

3.3 Cladophora *addition prolongs survival*

The addition of fresh *Cladophora* into native sand resulted in longer *E. coli* survival than microcosms with *Cladophora* that had been held for 3 months (FIG 3, S.5). Cell densities in fresh *Cladophora* dropped one order of magnitude after 6 weeks, averaging an exponential decay of -0.05 day^{-1}, and only experienced 2 order of magnitude loss after 9 weeks of incubation. In contrast, the 12.5% decayed *Cladophora* microcosm experienced higher average rates of decay, -0.16 day^{-1}, and cell densities dropping 3 orders of magnitude after 6 weeks. *E. coli* decay in sand with fresh *Cladophora* sand was significantly less than in sand with decayed ($p=0.024$).

Overall, we noted a dose-response relationship with respect to the amount of *Cladophora* added. In the decayed *Cladophora* microcosms, after 2 weeks of incubation the cell densities of the less concentrated treatments, 1.25% and 0%, were an order of magnitude lower than the 6.25% and 12.5% treatments. The distinction between higher and lower concentrations was observed from 2 to 4 weeks and ceased at 6 weeks, suggesting shorter-term effects as nutrients were depleted. Final average exponential decay rates were very similar with -0.16, -0.17, and -0.19 day^{-1}, for 0%, 1.25%, and 6.25% decayed *Cladophora*, respectively.

We measured the change in nutrients over time in the decayed *Cladophora* microcosm (FIG. 4). TOC measurements in the decayed *Cladophora* decreased on average 60 percent over time and did not display carbon concentration changes proportional to the amount of *Cladophora* added, with all values being within range of one another. In contrast, TN measurements increased between the initial and end time points of the microcosm with values increasing proportionally to the amount of *Cladophora*.

Further, there were differences in *E. coli* cell density between the 0% *Cladophora* treatment between the microcosms, although the experimental design remained the same. The

difference between the two controls was the date of sand collection, the "fresh" treatment sand was collected in August and the "decayed" in November. The changes in collection date resulted in lower cell decay rates in sand collected in the colder month, November. *E. coli* in August sand experienced a four-order of magnitude loss and in contrast, November sand underwent a three-order magnitude loss, after 42 days of incubation ($p<0.0001$). Final average *E. coli* concentrations were $1.70x10^2$ and $5.55x10^3$ CFU/g sand.

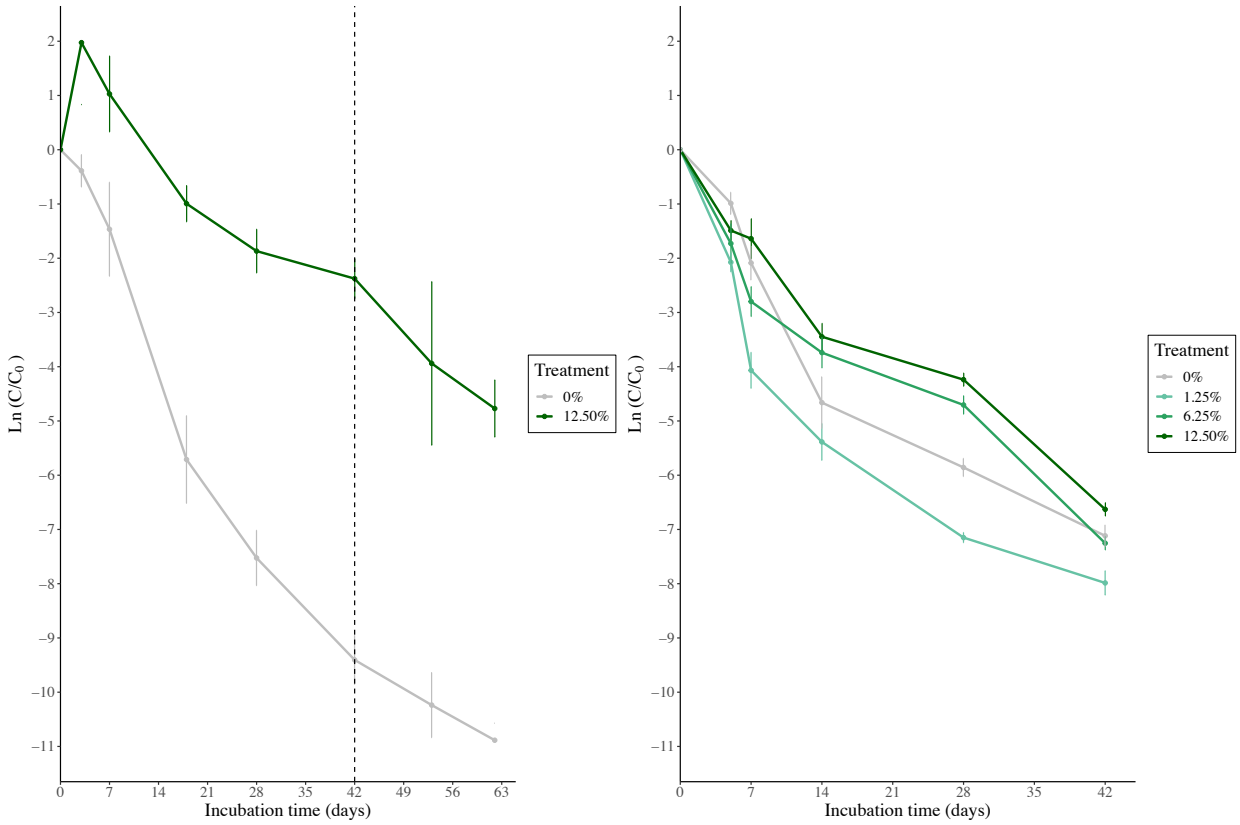

FIG 3 Natural log of average colony-forming units (CFU) per gram of sand (dry weight) normalized to the initial time point of *E. coli* with *Cladophora* seeded into native sand. Fresh *Cladophora* incubated for 62 days (left) at 12.5% (w/w) concentration and decayed *Cladophora* incubated for 42 days (right) at 12.5%, 6.25%, and 1.25% (w/w) concentrations. The dashed line in fresh *Cladophora* depicts the extent to which decayed *Cladophora* microcosm was incubated, for comparison. Baseline microcosms, 0%, were seeded with no *Cladophora* to serve as a control, indicated by grey color. Error bars represent the standard deviation of the CFU triplicates.

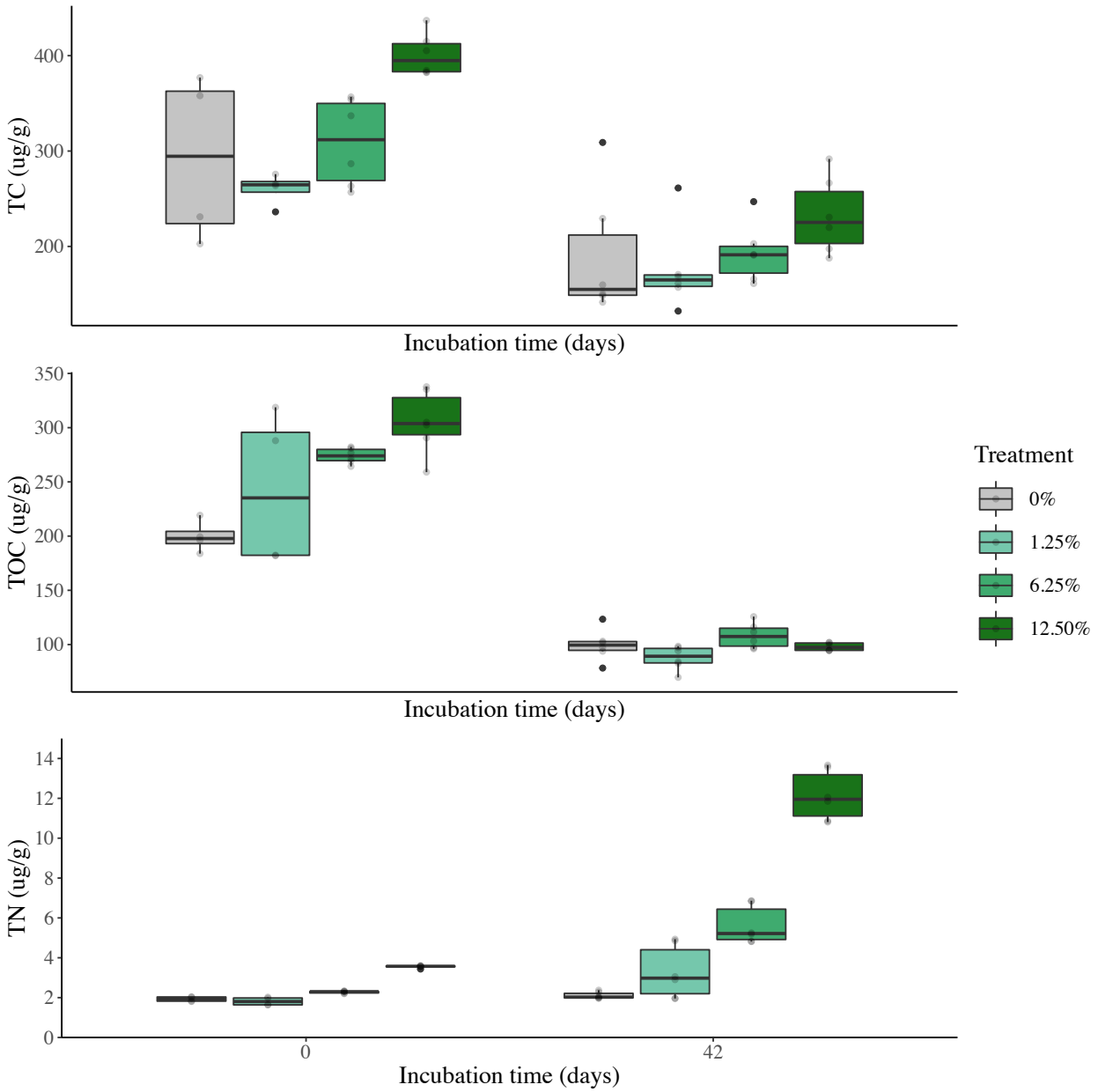

FIG 4 Total Carbon (TC), Total Organic Carbon (TOC), and Total Nitrogen (TN) concentrations overtime for decayed *Cladophora* in control, un-inoculated microcosm at T=0 and T=42. Carbon concentrations decreased throughout the microcosm, while nitrogen concentrations increased. Grey color boxplots indicate no *Cladophora* addition, and the green gradient indicates increasing *Cladophora* concentration. Boxplots are representative of the triplicates processed in technical duplicates. (n=6). Points in the boxplot represent data values, with black points depicting outliers of the sample. TC and TOC decreased over time for all treatments and TN increased over time for all treatments.

3.4 *Native community limits* E. coli *survival*

Overall cell densities of *E. coli* B1 in both native and baked sand decreased over 5 week incubation in sand, with native sand decaying at a faster rate (FIG 5). The native microbial community is absent in baked sand and contained only small differences in nutrient composition. Native sand nutrient concentrations were 1% higher in TC, 13.7% higher in TOC, and 24.7% lower in TN, relative to baked sand (Table S1). *E. coli* in baked sand was reduced only one order of magnitude after 5 weeks, with an average decay rate of -0.07 day^{-1}. In contrast, cell densities in native sand decreased 3 orders of magnitude after 5 weeks, averaging a decay rate of -0.22 day^{-1}, which was significantly faster ($p<0.0001$). The final average concentrations were $1.22x10^5$ and $8.79x10^2$ CFU/g sand, for baked and native sand, respectively.

We also directly compared the decay constants in experimental treatments in native sand with and without nutrients to the baked sand treatments. *E. coli* concentrations were observed over range of microcosm experiments seeded with nutrients including fresh *Cladophora*, 10X CN, concentrated decayed *Cladophora*, and 4X CN listed in descending order of nutrient concentrations. Average exponential decay rates were -0.07, -0.09, -0.15, and -0.10 day^{-1}after 4 weeks of incubation, respective to the order outlined prior. The amount of nutrients added is positively correlated with decreased decay. In contrast, *E. coli* decay rates in native sand without seeding in nutrients were higher, ranging from -0.21 to -0.34 day^{-1}after 4 weeks of incubation (Table S1). This demonstrates that sand seeded with increased nutrients prolongs *E. coli* survival, which we hypothesize is due to decreased competition with the native community.

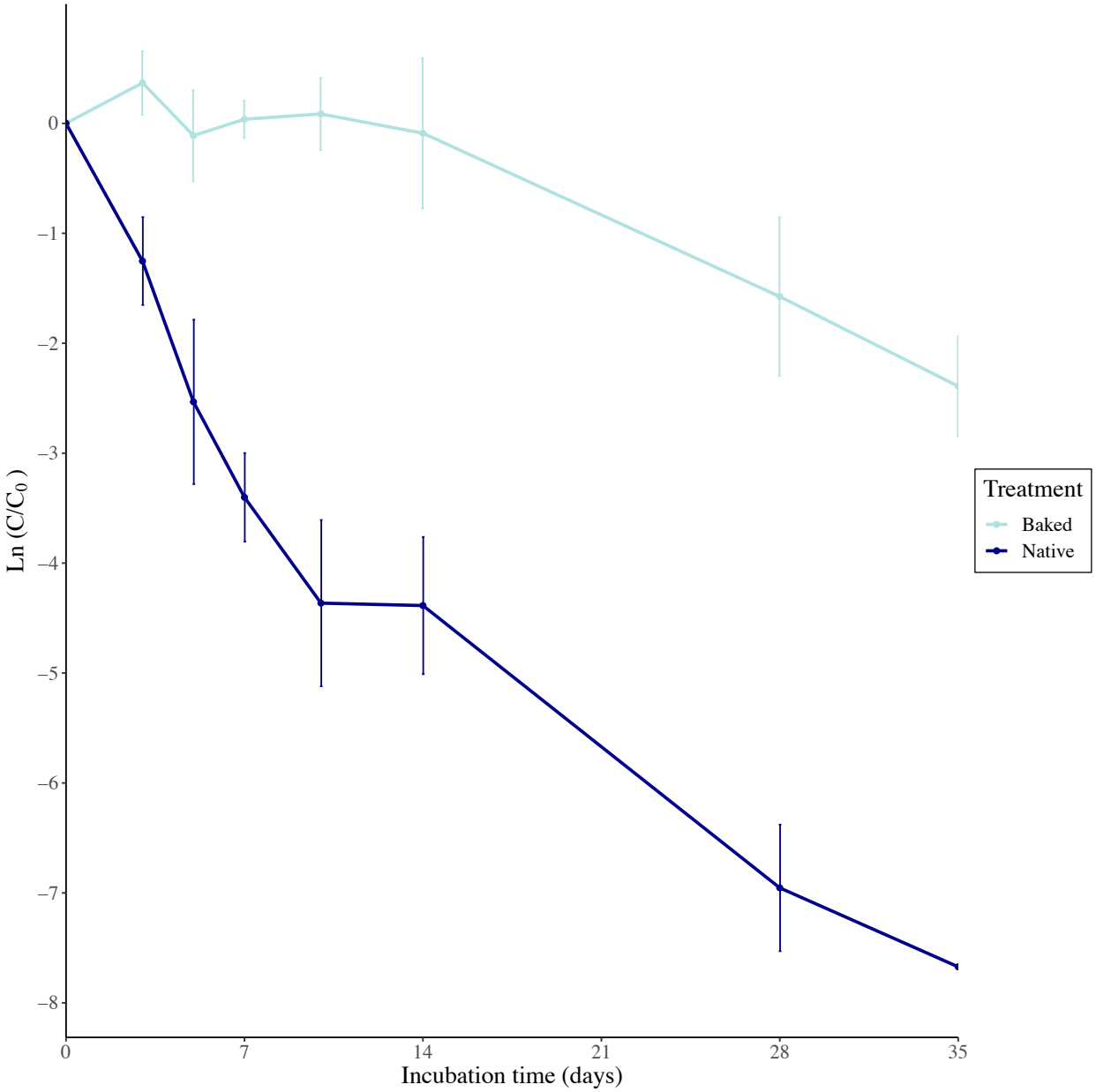

FIG 5 Natural log of average colony-forming units (CFU) per gram of sand (dry weight) normalized to the initial time point of *E. coli* phylotype B1in baked and native sand treatments. Sand treatments contain similar nutrient profiles, with the major distinction beng the removal of the native community in baked sand. Error bars represent the standard deviation of the CFU triplicates.

Chapter 4- Discussion

It is well established that there are *E. coli* populations surviving in freshwater beach sands distinct of fecal pollution events, yet the precise mechanism for their persistence is not well understood. These populations pose a risk for higher *E. coli* concentrations in adjacent water bodies and can lead to increased beach closures (Cloutier et al., 2015; Vogel et al., 2016). This research identified that *E. coli* in freshwater beach sands reduces 3 order of magnitude after 4 weeks. This suggests that there is an accumulation of cells within beach sand due to consistent recovery of *E. coli* from freshwater beach sand. We investigated the influence of both genetic and environmental factors on *E. coli* survival in freshwater beach sand, with an emphasis on understanding the effect of different nutrient sources that prolong *E. coli* survival. Our findings reveal a strong association between *E. coli* survival and nutrient availability, specifically when carbon and nitrogen concentrations are higher than what is observed in beach sand.

4.1 *Genetic background and survival*

Phylogenomic analysis of *E. coli* strains isolated from beach surveys have determined that phylotype B1 is the most frequently isolated, suggesting it preferentially survives in the beach environment (Rumball et al., 2021; Walk et al., 2007). While phylotype B2 is less commonly isolated from the beach environment and is more commonly found in the host environment, the gut of endotherms (Martinson et al., 2019; Rumball et al., 2021). Despite the distinction in the frequency of isolation between phylotypes, after 5 weeks of incubation in native sand cell densities were within an order of magnitude. This suggests that phylogenetic background is not a major determinant for the long-term (weeks) survival of *E. coli* in the sand. Differential loading of phylogroups into sand may contribute to the observed differences

between the recovery and survival of distinct phylotypes (i.e. B1 may have higher loading into beach sand than B2). This study only examined the decay rates of one representative isolate from each phylotype. *E. coli* is a genetically diverse bacteria and it would be expected that isolates within each phylotype may show different decay dynamics but within a similar range of those stated in this paper.

E. coli survivors have been suggested to have the ability to regulate response for adaption to nutrient limitation, competition, and predation with the native community (Cloutier & McLellan, 2017; Rumball et al., 2021; Wanjugi et al., 2016; Whitman et al., 2014). Under carbon and nitrogen limitation levels of sigma factor *rpo*S, stress/stationary phase regulation, is heightened in *E. coli* with a higher level of expression observed under carbon limitation (Mandel & Silhavy, 2005). Consistent with these findings, other reports have shown a positive correlation between increased C/N ratios and *rpo*S expression (Kumar & Shimizu, 2010). Persistent *E. coli* in the secondary environment, specifically soil, have a conserved general stress response regulation (Somorin et al., 2016, 2017). *E. coli* strains deficient of these responses survive poorly under laboratory conditions, reduced moisture conditions, and under increased predation compared to strains that can regulate *rpoS* (Somorin et al., 2016, 2017). We examined the importance of stress regulation in the context of the beach environment where we showed that *E. coli* without the ability to regulate stress response could not survive in the sand (>22 days), whereas *E. coli* with an intact stress response generally showed only a 50% reduction in many of our experiments at 6 weeks. Further, the survival of *E. coli* without rpoS regulation in sand was half than of was observed in the soil environment (Somorin et al., 2016, 2017).

4.2 *Influence of nutrients on survival*

Beach surveys have been conducted on the Eastern and Western shorelines of Lake Michigan, which identified a positive correlation between *E. coli* numbers and nutrient concentrations (Cloutier et al., 2015). This correlation was further explored through laboratory microcosms of sand seeded with plankton, *Cladophora*, and autoclaved sand as well as *in-situ* microcosms deployed at a Lake Michigan beach with autoclaved sand, which demonstrated prolonged *E. coli* survival (Alm et al., 2006; Byappanahalli et al., 2003; Byappanahalli, Whitman, Shively, Ting, et al., 2006; Rumball et al., 2021; vanden Heuvel et al., 2010; Whitman et al., 2003). In this work, we built upon co-occurrence studies and examined the specific drivers of carbon, nitrogen, combined C and N, and an ecologically relevant nutrient source, *Cladophora*, on *E. coli* survival over a span of about 6 weeks.

We determined the C/N ratios of beach sand was 90:1. Our carbon and nitrogen combined nutrient treatments maintained these ratios. Treatments with only nitrogen had decreased C/N ratios and treatments with only carbon had increased C/N ratios relative to the ratio in beach sand. Previous research on C/N ratios have that identified lower ratios, 10:1 and 20:1 relative to 40:1, were associated with reduced *E. coli* cell loss in compost when the compost was at 20% moisture content, which is similar to the moisture content of berm sand (Mandel & Silhavy, 2005; Somorin et al., 2016). Our work observed an opposite pattern, in which sources with high C/N ratios, ≥90:1, prolonged *E. coli* survival over the baseline while sources with low C/N ratios, <90:1, did not influence *E. coli* survival. Differential survival between our findings and previous work might be attributed to *E. coli* storing carbon under nitrogen limitation as well as the ability to metabolize the specific carbon source.

Microcosm experiments in this study with *Cladophora* are distinct from those previously reported (Byappanahalli et al., 2003; Whitman et al., 2003) where native sand was seeded with dried algal matts over (3 days). We seeded native sand with fresh and decayed *Cladophora* over a prolonged period (>6 weeks) and determined available nutrient concentrations of decayed *Cladophora* seeded sand and liquid input. The fresh *Cladophora* input TOC concentration was 25.4 times lower and TN concentration was 14 times lower, relative to decayed *Cladophora.* This suggests that fresh *Cladophora* has more potential to release nutrients over time, while decayed *Cladophora* likely released most nutrients prior to input into the sand. Our *Cladophora* experiments showed that fresh *Cladophora* has a higher influence on survival than decayed, due to the suspected higher potential to provide nutrients to sand.

Cladophora contains a complex mix of macronutrients, micronutrients, trace elements, and heavy metals, as compared to glucose and ammonia chloride which was used in our increased carbon and nitrogen microcosms (Nutautaitė et al., 2021). The potential of fresh *Cladophora*, 12.5% (w/w), for carbon and nitrogen release into sand was approximated by averaging carbon and nitrogen concentrations of dried *Cladophora* of tissue collected along the shorelines of the Great Lake beaches and determining the maximum amount per gram of sand in our microcosms (Higgins et al., 2008). Through this, we identified fold higher C and N concentrations than our simple sources with 12.5% *Cladophora* having a maximum potential of 33,875 µg C/g and 824.29 µg N/g, which was 26X and 57X more concentrated than our simple sources (Table 1, S3, S4). Despite the distinctions in complexity and concentration we observed similar decay rates between our fresh *Cladophora* microcosm and our 10X CN after 6 weeks, -0.05 and -0.09 respectively. This suggests that simple nutrient sources have a greater impact in smaller doses than complex sources on *E. coli* survival in beach sand.

4.3 *Microbial communities and survival*

The beach environment has an abundance of life present, with a single grain of sand harboring 10^4 to 10^5 cells. *E. coli* and Enterococci are only a small portion of all microbial life present in sand, representing 1 to 2 CFU per gram of sand. Previous work has demonstrated that *E. coli* survival in sand is modulated by the native community either through competition for nutrients or predation (Baker et al., 2020; Cloutier et al., 2015; Rumball et al., 2021). We observed that when *E. coli* was placed in sand with similar nutrient profiles, cell densities were higher in the sand substate with the native community absent. We also observed distinctions in *E. coli* decay rates when sand was amended with nutrients with minimal cell loss in sources with higher nutrient composition, fresh *Cladophora* and 10X CN, and reduced cell loss in mid-level nutrient sources, decayed *Cladophora* and 4X CN. This suggests that competition for nutrients is more influential than predation, as shown by the correlation between increased nutrients and reduced *E. coli* decay.

The effect of microbial communities is further shown through the variation of the control between *Cladophora* microcosms, with higher rates of decay associated with a warmer collection date. Previous research has identified temperature as a driving force for soil microorganisms with an optimal growth temperature of 15°C, which is within the range of observed internal temperatures of beach sand during summer months (Borowik & Wyszkowska, 2016; Rumball et al., 2021). In contrast, *E. coli* can persist at a wide range of temperature gradients, 4-20°C, as observed through beach surveys and microcosm experiments (Rumball et al., 2021; Sampson et al., 2006; Vogel et al., 2016). Beach sand provides optimal temperature conditions in the summer for the native community, which results in competition for nutrients against *E. coli* populations.

We propose that the mechanism in which the native community modulates *E. coli* survival is competition due to the proportional difference of *E. coli* to that of microbial life in beach sand.

4.4 *Nutrient pollution and implications for beach managers*

Microcosm experiments investigating the limiting nutrient for *E. coli* in sand identified that combined concentrated carbon and nitrogen additions have the highest influence on survival, nitrogen addition increasing short term persistence (< 2 weeks), carbon additions influencing long term survival (6 weeks) but to a lesser extent than combined sources. The short-term influence of nitrogen is further supported by the addition of decayed *Cladophora*, which we determined was a nitrogen donor to the sand, where *E. coli* survival was prolonged in the short term. We also identified the form of available nutrients is more influential than the concentration, determining that simple sources promote survival in smaller doses compared to complex sources. The analysis of nutrient pollution to beaches, can determine high-risk sources, which then can assist in our understanding of *E. coli* fluctuations at the beach (i.e., if a carbon and nitrogen heavy source reaches the beach there will likely be heightened *E. coli* concentrations in both sand and water). Through this understanding, beach management techniques can be improved by developing strategies for targeting and mitigating problematic sources, which can then reduce *E. coli* concentrations in sand and loading in adjacent water.

Chapter 5- Conclusions

E. coli populations can be isolated from freshwater sand, yet the mechanism for their persistence is unknown. *E. coli* concentrations in freshwater sand can be resuspended into the water column through wave water interaction, such as high rain events or strong waves. Fluctuations of *E. coli* in water can be further understood by identifying drivers for persistent populations in the sand. This work identified that nutrient pollution containing high carbon and nitrogen concentrations, above what is observed at the beach, has the greatest impact on *E. coli* survival in beach sand. Beach management techniques can incorporate the characterization of nutrient pollution to the beach for carbon and nitrogen concentrations and identify sources that would likely prolong *E. coli* survival (i.e., 10X CN). Common pollutant sources in freshwater beaches can include beach wrack, stormwater runoff, agriculture runoff, combined sewage overflows (CSO), and riverine inputs. The above-mentioned sources can be characterized for carbon and nitrogen composition, compared to beach values, and sources higher than that observed in sand can be prioritized to mitigate *E. coli* survival. Additional methods to reduce *E. coli* survival in sand can include the removal of fresh *Cladophora* mats from the shoreline as well as beach grooming techniques. *E. coli* concentrations in beach sand and therefore adjacent water will ideally be reduced through the targeting and mitigating of high-risk nutrient pollution tot the beach environment.

Chapter 6- References

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Appendix A- Supplemental Information for Chapter 3

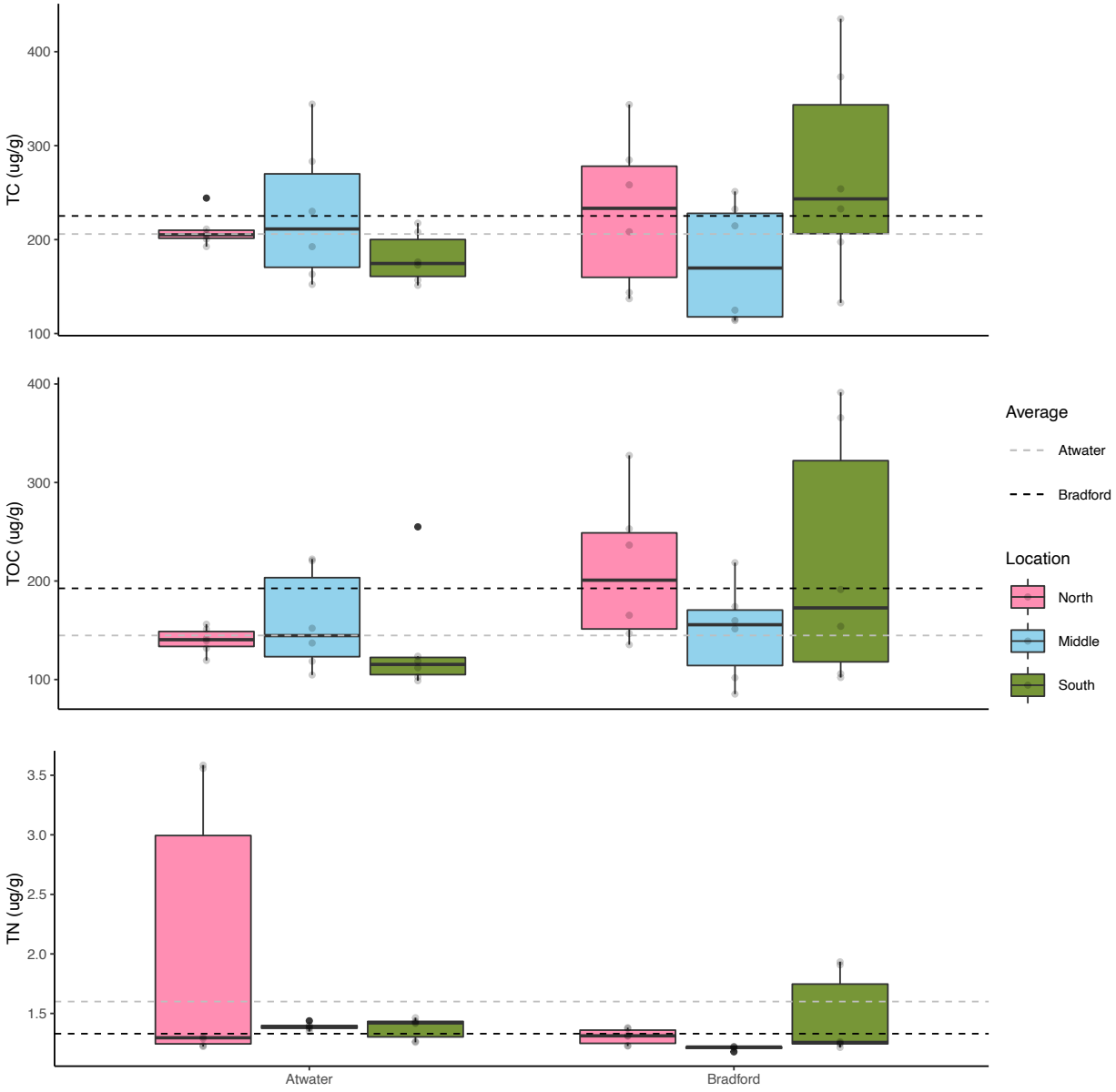

FIGURE S.1: TC, TOC, and TN concentrations of Atwater beach and Bradford beach surveys from the north, middle and southern sections of along the berm reported in µg nutrient per g of beach sand. Each boxplot is representative of the biological triplicates and their technical duplicates (n=6). Points in the boxplot represent actual values with black indicating outliers. Dashed horizontal lines are indicative of averages for the Bradford beach and Atwater beach, black and grey respectively.

TABLE S.1 Nutrient Profile of Beach sand

Sand	Mean (SD)[1] µg/g		
	Total Carbon	**Total Organic Carbon**	**Total Nitrogen**
Native Sand, Bradford Beach	225 (92.3)	193 (90.8)	1.33 (0.72)
Native Sand, Atwater Beach	206 (48.4)	145 (44.3)	1.60 (0.22)
Baked Sand, Atwater Beach	203 (21.8)	127.3 (13.5)	2.12 (.057)

[1]Beach samples were taken in trisect along the berm of the beaches, standard deviation is representative of samples processed in biological triplicates and technical duplicates (n=6) for each trisect (n=18)**.** Baked sand was processed in biological triplicate and technical duplicates (n-6)

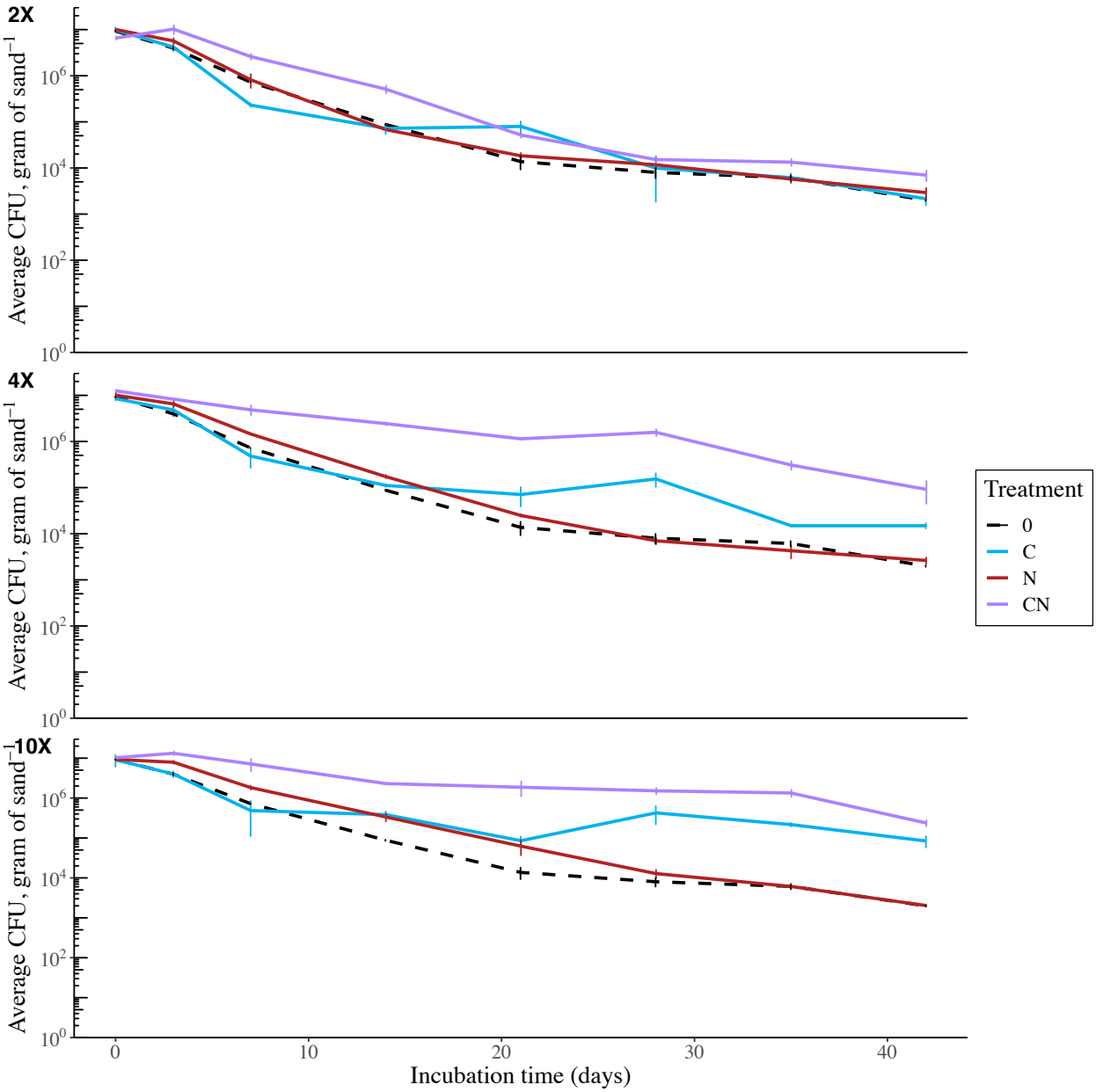

Figure S.2: Average colony forming units (CFU) per gram of sand (dry weight) of *E. coli* phylotype B1 in increased carbon and nitrogen concentrations, grouped by **fold increase.** Error bars represent the standard deviation of the CFU triplicates. The top graph is the carbon treatment, middle is nitrogen treatment, and bottom is the carbon and nitrogen treatment. Baseline microcosms, 0X increase, were inoculated with *E. coli*, but did not contain any carbon or nitrogen to serve as a control. Baseline treatments are the same within each of the 3 graphs, indicated by the black dashed line.

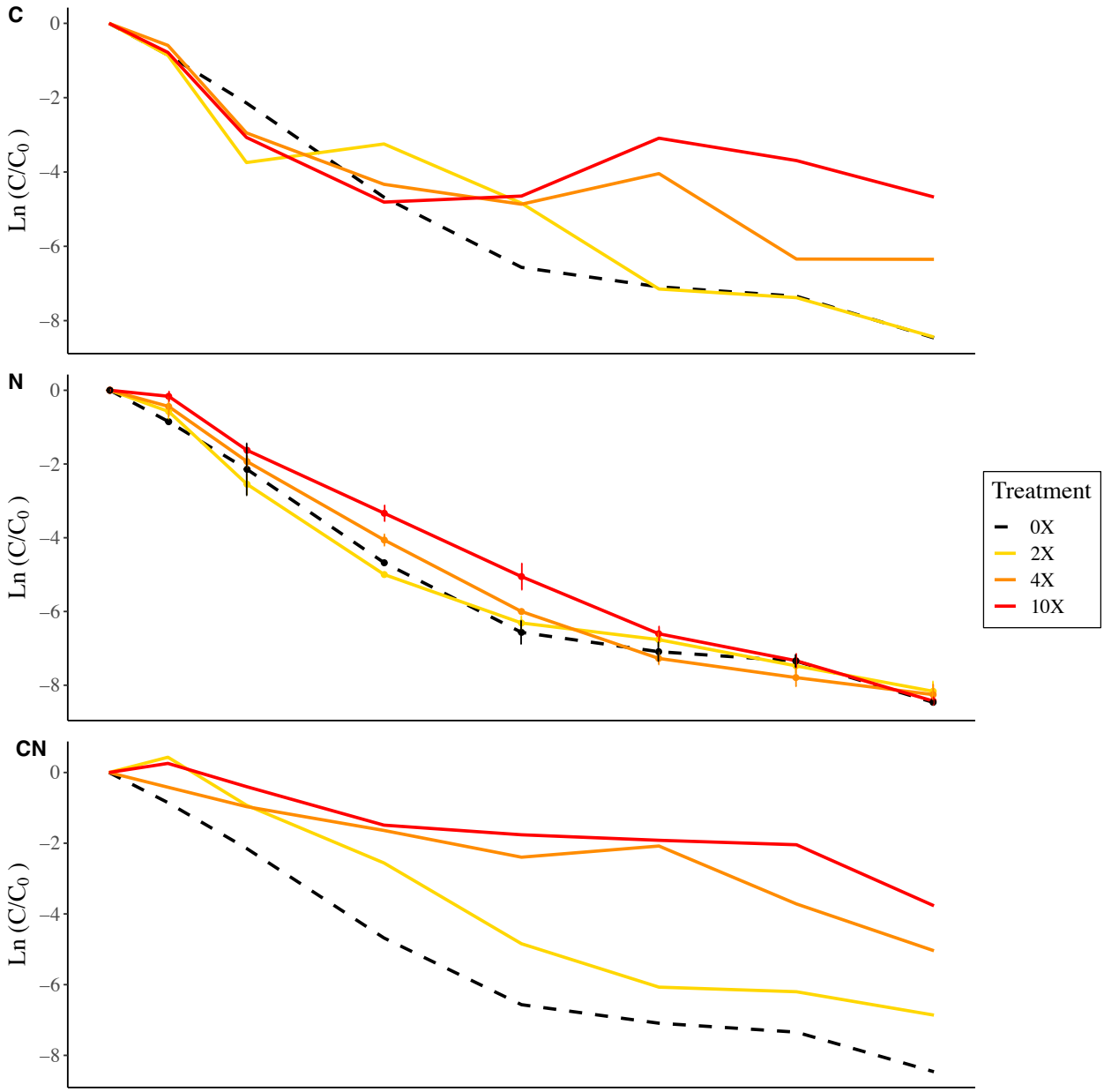

Figure S.3 Natural log of average colony forming units (CFU) per gram of sand (dry weight) normalized to the initial timepoint of *E. coli* phylotype B1 in increased carbon and nitrogen concentrations, grouped by nutrient treatment. Error bars represent the standard deviation of the CFU triplicates. The top graph is the carbon treatment, middle is nitrogen treatment, and bottom is the carbon and nitrogen treatment. Baseline microcosms, 0X increase, were inoculated with *E. coli*, but did not contain any carbon or nitrogen to serve as a control. Baseline treatments are the same within each of the 3 graphs, indicated by the black dashed line.

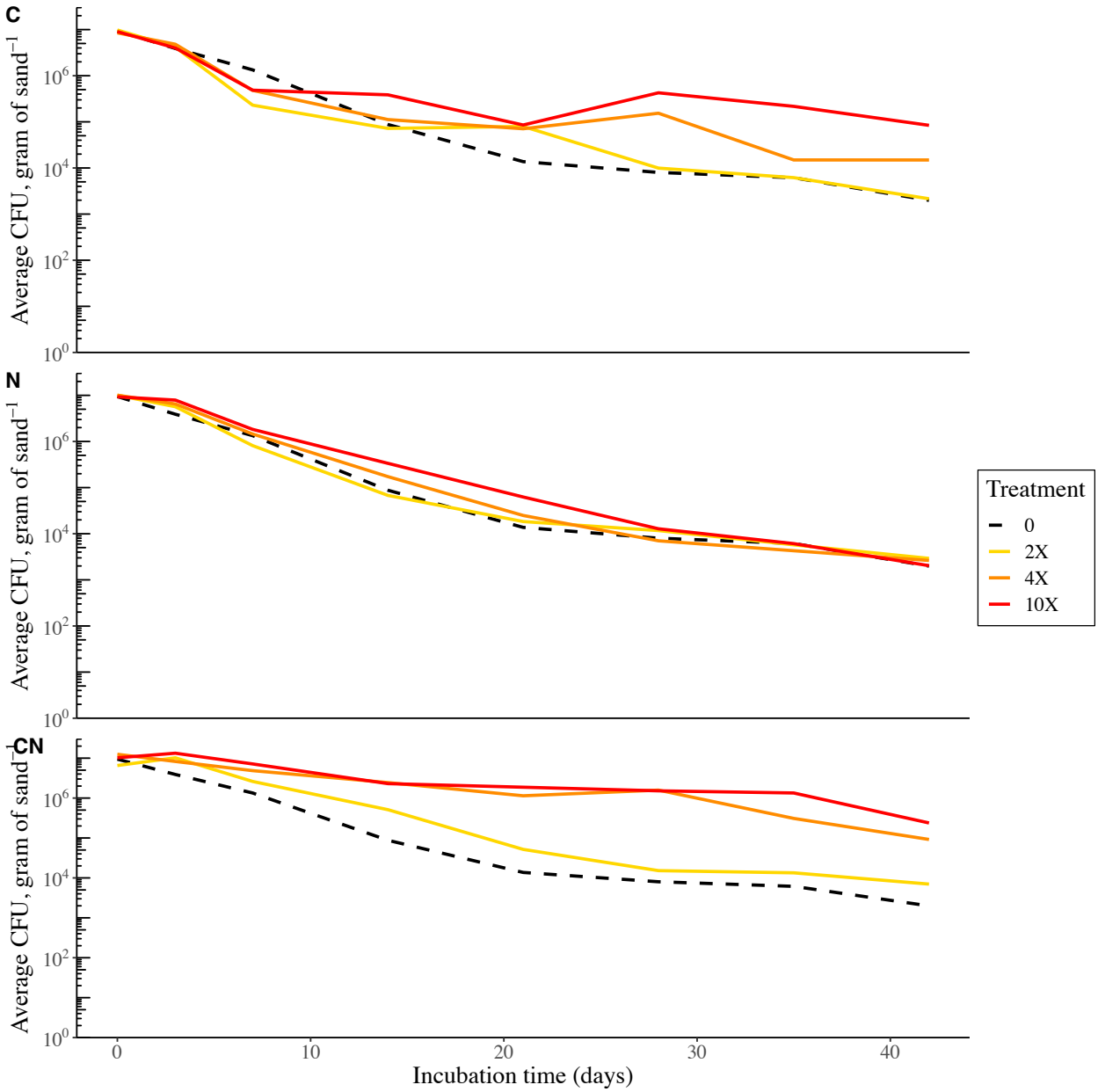

Figure S.4: Average colony forming units (CFU) per gram of sand (dry weight) of *E. coli* phylotype B1 in increased carbon and nitrogen concentrations, grouped by nutrient treatment. Error bars represent the standard deviation of the CFU triplicates. The top graph is the carbon treatment, middle is nitrogen treatment, and bottom is the carbon and nitrogen treatment. Baseline microcosms, 0X increase, were inoculated with *E. coli*, but did not contain any carbon or nitrogen to serve as a control. Baseline treatments are the same within each of the 3 graphs, indicated by the black dashed line.

Table S.2 Decay rates of *E. coli* phylotype B1 throughout microcosms at 28 days

Nutrient Treatment or Sand Type	Mean (day -1) Decay rate
12.5% Fresh Cladophora	-0.07
12.5% Decayed Cladophora	-0.15
6.25% Decayed Cladophora	-0.17
1.25% Decayed Cladophora	-0.26
2X C	-0.34
4X C	-0.19
10X C	-0.15
2X N	-0.35
4X N	-0.35
10X N	-0.31
2X CN	-0.29
4X CN	-0.10
10X CN	-0.09
Baked Sand	-0.11
Native sand, July	-0.23
Native sand, September	-0.27
Native Sand, August	-0.25
Native Sand, November	-0.21
Native Sand, November II	-0.34

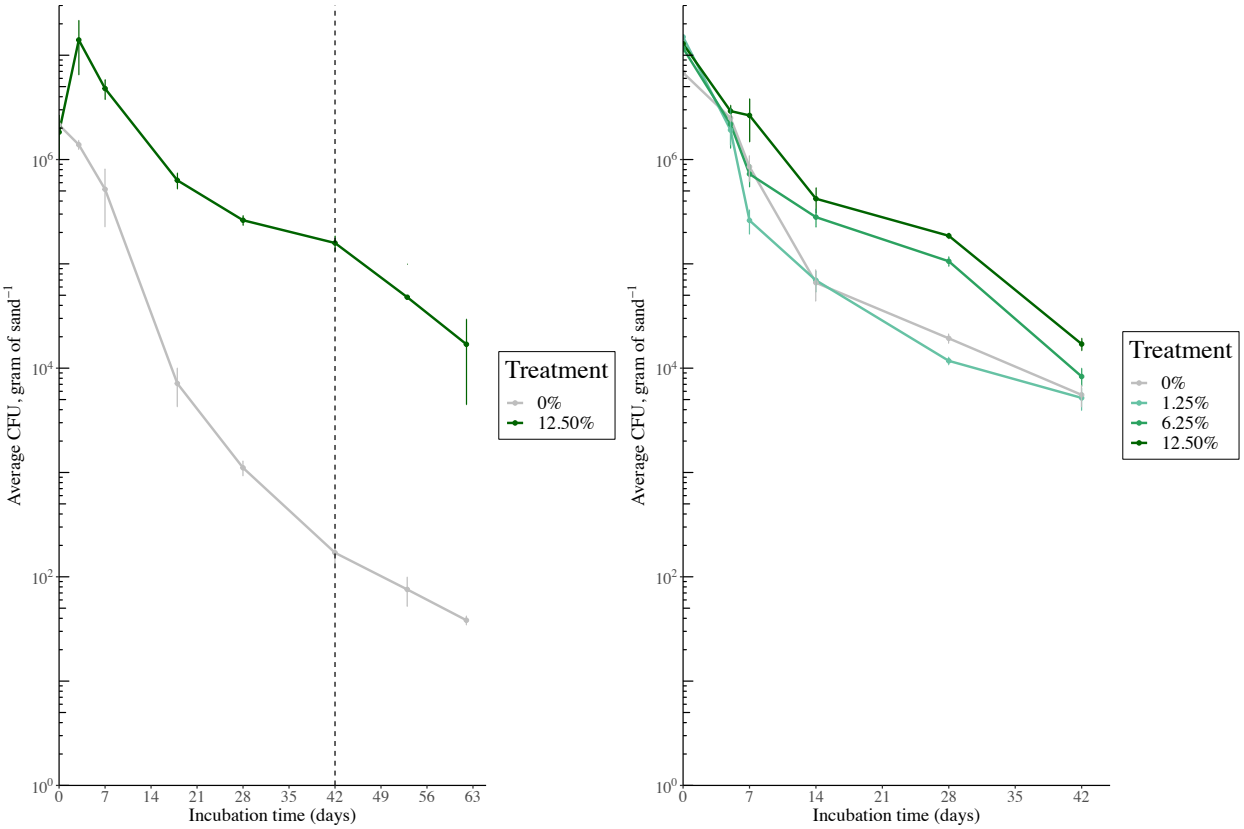

Figure S. 5 Average colony forming units (CFU) per gram of sand (dry weight) of *E. coli* phylotype B1 with *Cladophora* seeded into native sand. Fresh *Cladophora* incubated for 62 days (left) at 12.5% (wt/wt) concentration and decayed *Cladophora* incubated for 42 days (right) at 12.5%, 6.25%, and 1.25% (wt/wt) concentrations. Dashed line in fresh *Cladophora* depicts the extent in which decayed *Cladophora* microcosm was incubated, for comparison. Baseline microcosms, 0%, were seeded with no *Cladophora* to serve as a control, indicated by grey color. Error bars represent the standard deviation of the CFU triplicates.

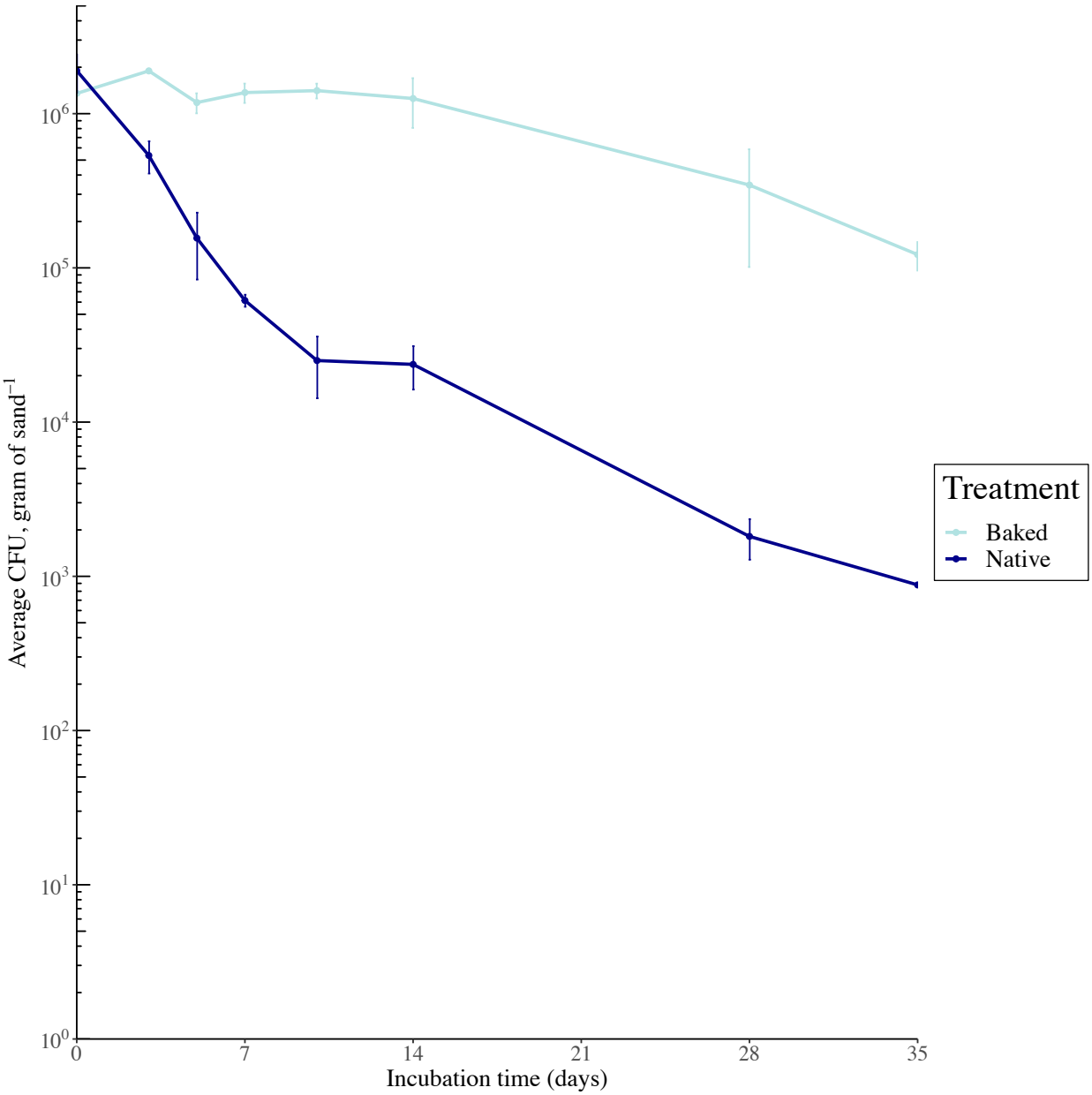

Figure S. 6 Average colony forming units (CFU) per gram of sand (dry weight) of *E. coli* phylotype B1 in baked and native sand treatments. Sand treatments contain similar nutrient profiles, with the major distinction is the removal of the native community in baked sand. Error bars represent the standard deviation of the CFU triplicates.

Table S.3: Summary of *Cladophora* Tissue from Higgins et. al 2008

Lake	Average Mean (SD) % Dry Mass	
	Carbon	Nitrogen
Huron	25.1 (2.3)	2.9 (0.5)
Erie	29.1 (2.7)	2.0 (0.4)
Ontario	28.4 (2.8)	2.4 (0.5)
All Lakes	27.1 (1.6)	2.4 (0.7)

Table S.4: Potential nutrient capacity of *Cladophora* treatments based on Higgins et. al 2008

	Amount added (µg) per sand (g)		
Treatment	**Cladophora**	**Carbon [1]**	**Nitrogen [2]**
12.50%	125000	33875.0	824.29
6.25%	62500	16937.5	412.15
1.25%	12500	3387.5	82.43

[1]Based on assumption that *Cladophora* tissue carbon content is 27.1% of its dry mass.
[2]Based on assumption that *Cladophora* tissue nitrogen content is 2.4% of its dry mass.

Appendix B- Characterization nutrient profile of beach sand and environmental inputs

Summary

This work included collection and processing of environmental samples for available nutrients TC, TOC, and TN including beach sand and nutrient sources. Beach surveys were conducted for available nutrients which determined there was only statistical difference between Atwater beach and Bradford beach TOC content. The purpose of the survey was to determine the baseline of available carbon and nitrogen in beach sand so we could determine the amount needed for fold increases, see section 2.3 and 3.2. Environmental samples collected included fresh *Cladophora,* decayed *Cladophora*, stormwater, and lake water. It was determined that lake water and fresh *Cladophora* had similar and low concentrations of available carbon and nitrogen. While decayed *Cladophora* and Stormwater had more available nutrients with variation in carbon and nitrogen concentrations.

Methods

Sand was collected in November and December, to test for available nutrient concentration within 24 hours of sampling. Nutrient analysis was conducted by eluting 16g of sand in 40mL of sterile water, shaken vigorously, and the eluent was analyzed for TC, TOC, and TN as described in Section 2.4. Stormwater and Lake water was directly measured for available nutrients. We were able to determine the amount of available carbon and nitrogen concentrations in the input liquid to both the fresh and decayed microcosms through eluting *Cladophora* in water and analyzing the eluent.

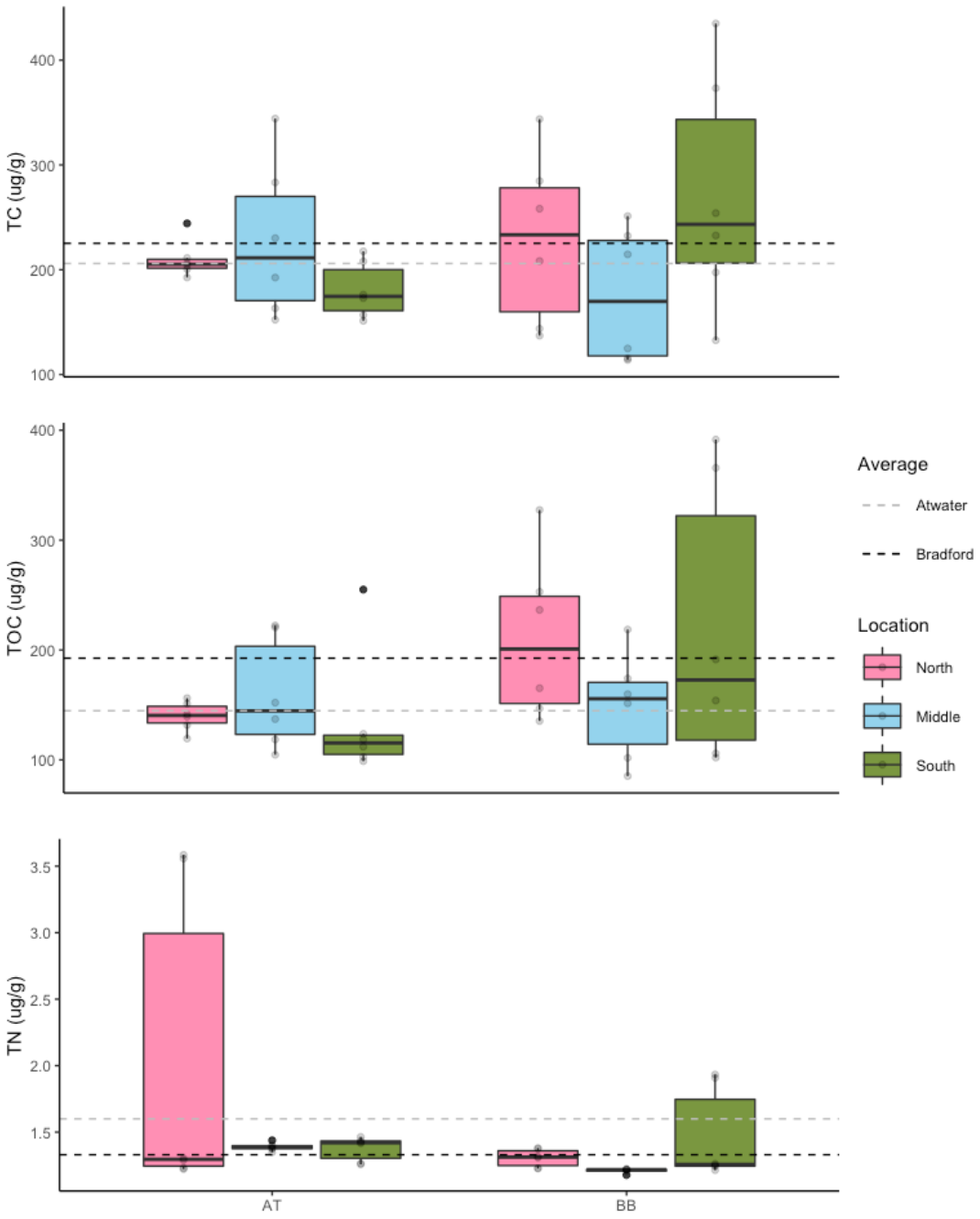

FIGURE 1: TC, TOC, and TN concentrations of Atwater beach and Bradford beach surveys from the north, middle and southern sections of along the berm reported in µg nutrient per g of beach sand. Each boxplot is representative of the biological triplicates and their technical duplicates (n=6). Points in the boxplot represent actual values with black indicating outliers. Dashed horizontal lines are indicative of averages for the Bradford beach and Atwater beach, black and grey respectively.

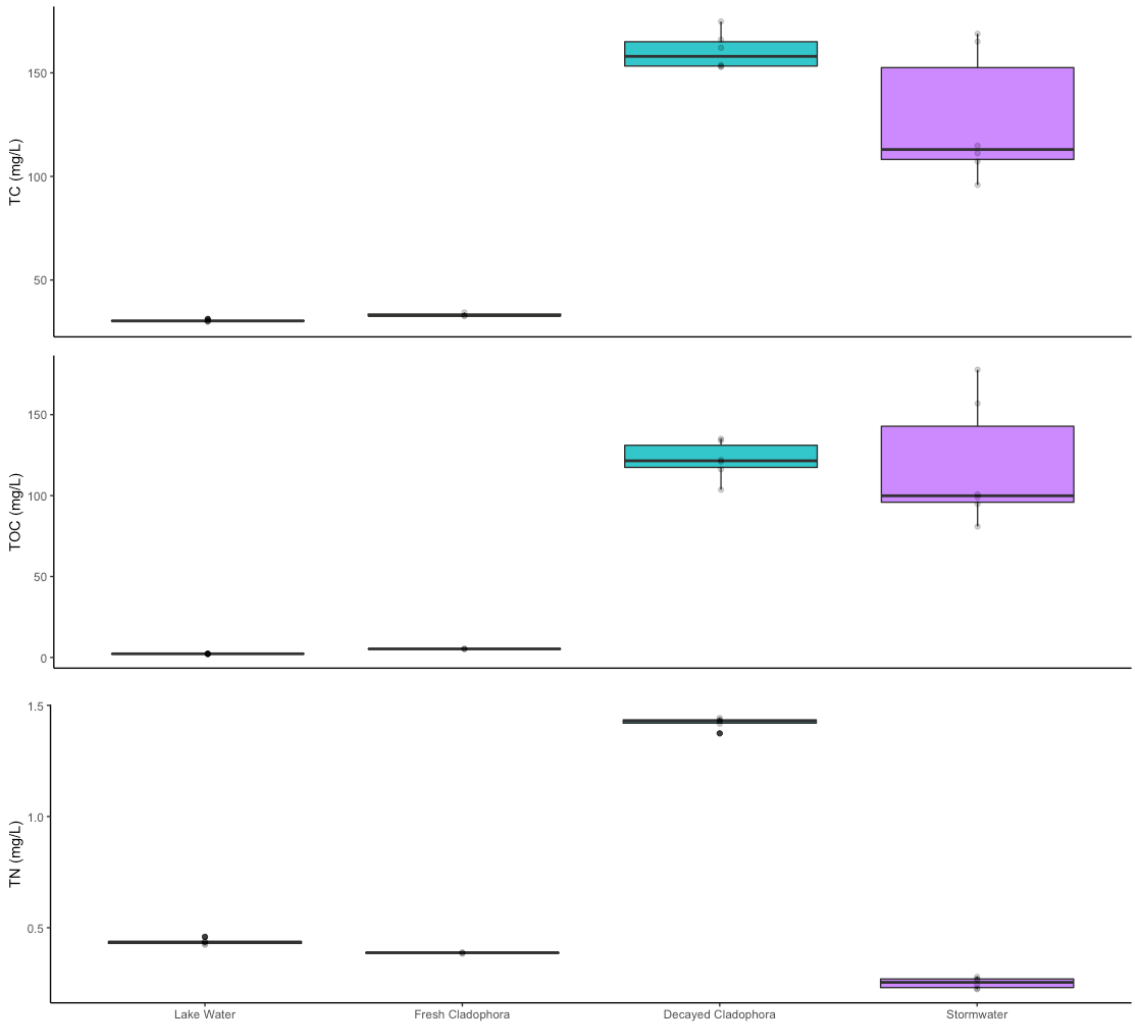

Figure 2: Total Carbon, Total Organic Carbon, and Total nitrogen concentrations in lake water, *Cladophora*, Decayed, *Cladophora,* and Stormwater collected in Milwaukee, WI.

Table1: Nutrient source collection data

Nutrient Source	Sample Information	
	Date Collected	Location
Lake Water	9-Jun-21	Bradford Beach
Cladophora	31-Aug-21	Bradford Beach
Decayed *Cladophora*[1]	1-Dec-21	Bradford Beach
Stormwater	25-Oct-21	Locust street

[1]The is the same sample collected on August 31, 2021, and remained in the fridge at 4°C until use which resulted in the *Cladophora* being in variable stages of decay.

Table 2: Carbon and Nitrogen profile of environmental nutrient sources.

Nutrient Source	**Mean (SD) mg/L**		
	TC	**TOC**	**TN**
Cladophora	32.2 (0.95)	5.29 (0.039)	0.387 (0.0033)
Decayed Cladophora	182 (5.9)	134 (2.6)	5.40 (0.0071)
Lake water	30.3 (0.47)	2.24 (0.026)	0.436 (0.012)
Stormwater	179 (20.0)	155 (39)	2.35 (0.031)

Appendix C-Nutrient limitation experiments

Summary

In the pursuit of identifying if carbon or nitrogen is the limiting for *E. coli* survival, we conducted a series of microcosm experiments with reduced concentrations. The first experiment examined *E. coli* survival at carbon and nitrogen concentrations ranging from 0 to 1:1,000 to 1:100,000 of a typical M9 media in microcosms with baked sand, using native sand as a control. *E. coli* survival between carbon and nitrogen concentrations were within an order of magnitude, despite differential carbon and nitrogen concentrations. Significant findings in this experiment are differential survival in native and baked sand, see section 3.4. To further examine the influence of carbon and nitrogen, we deployed a microcosm in native sand to examine the dynamic range with nitrogen and carbon in separate petri dishes, ranging from M9 concentrations to 1:10,000 of M9. The results determined that despite the more encompassing range of carbon and nitrogen concentrations, exponential decay coefficients were comparable.

Methods

Preliminary experiments determined a survival strength level of M9 carbon and nitrogen concentrations, through suspension in a liquid medium in which *E. coli* did counts remained stable. The survival strength was determined to be 1:1,000 of a typical M9 media. From this, carbon and nitrogen concentrations were further diluted to a 1:100,000 of a typical M9 media. Microcosms were deployed with 1:1,000CN, 1:100,000C, 1:100,000N, 1:100,000 CN, No CN in baked sand with *E. coli* from phylotype B1. *E. coli* phylotype B1 was deployed in native sand to serve as a control which was deployed for 5 weeks. We then conduced microcosm, in which we deployed *E. coli* from phylotype B1, in native sand at a range carbon and nitrogen

concentrations, ranging from typical M9 media to 1:10,000 of M9 media to examine the dynamic range. This microcosm was conducted for 10 days, with initial and endpoint concentrations.

Table 1. Carbon and nitrogen concentrations of modified M9 media treatments

Treatment	Nutrient Concentration (mM/L)	
	Carbon	Nitrogen
M9	1.32E-01	2.33E-02
1:10 M9	1.32E-02	2.33E-03
1:100 M9	1.32E-03	2.33E-04
1:1,000 M9	1.32E-04	2.33E-05
1:10,000 M9	1.32E-05	2.33E-06
1:100,000 M9	1.32E-06	2.33E-07

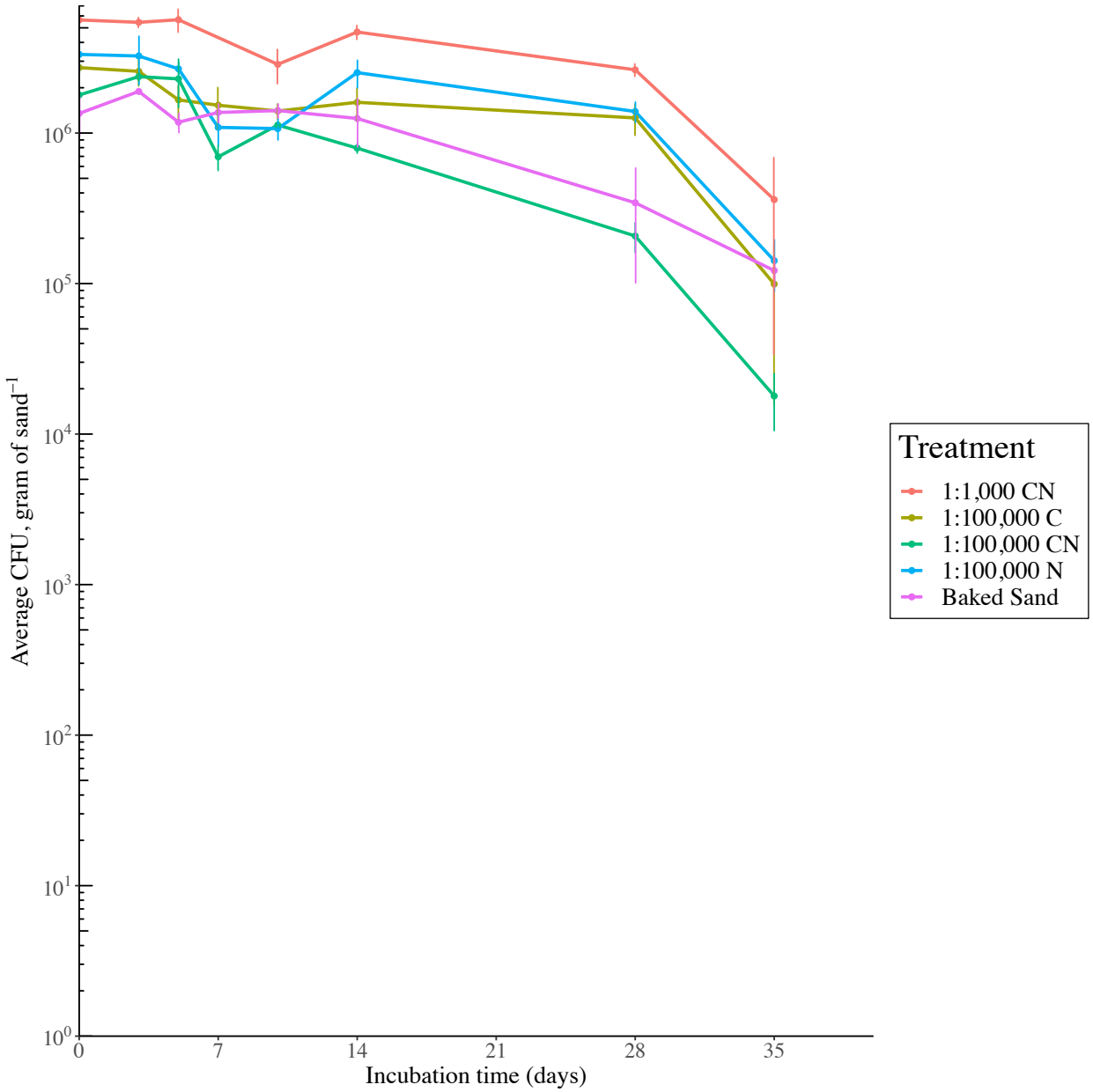

Figure 1: Average colony forming units (CFU) per gram of sand (dry weight) of *E. coli* phylotype B1 in decreased carbon and nitrogen concentrations. Error bars represent the standard deviation of the CFU triplicates.

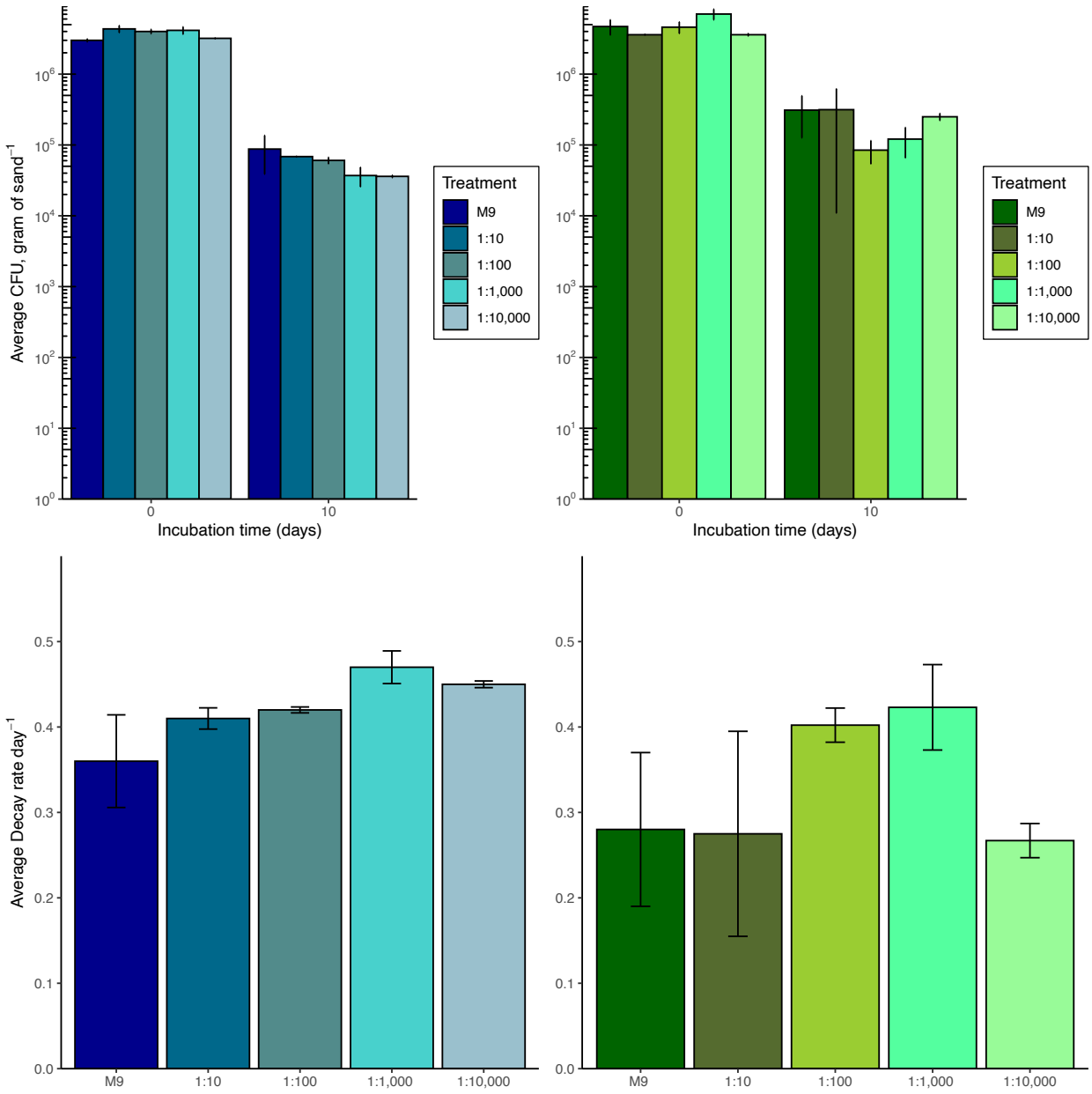

Figure 2: Average CFU data overtime (top) and decay rates after ten days (bottom) of carbon (left) and nitrogen (right) in modified M9 media treatments. Error bars represent the standard deviation of the microcosms processed in duplicate.

Appendix D- Preliminary experiments on reporter constructs

Summary

Reporter constructs were obtained from Horizon Discovery to monitor for carbon, nitrogen, phosphorous, and universal stress response through green fluorescent protein (GFP) tied to the gene expression of *csiD, glnA, phoA, and uspA*, respectively. Reporter constructs were in *E. coli* K12 strain MG1655 which is a laboratory strain. To accurately mimic beach conditions, reporter constructs were placed in *E. coli* phylotype B1, a beach strain. Preliminary experiments included comparing growth curves of the K12 and B1 promoters, which we determined transformations did not alter endpoint optical density. Fluorescent measurements of a constituently expressed GFP and the reporter constructs were compared, which showed the reporters GFP expression was 40X lower in promoters. Reporter extraction from the sand, following protocol outlined in section 2.3, was experimented with. However, cells were unable to concentrated to obtain a measurable GFP signal due to low cell concentration which resulted in a failure to pellet cells. Future experiment is needed to validate reporter constructs for use of sensing for nutrient limitation and cellular stress.

Methods

Plasmid DNA was extracted from *E. coli* k12 with the QIAprep Spin Miniprep Kit, B1 competent cells were constructed and GFP plasmid was introduced following the protocol outlined by (Seidman, Struhl, Sheen, & Jensen, 2001). Growth curves of reporter constructs were determined by measuring optical density at 600 (OD_{600}) every hour over a 24-hour period with the Synergy H4 Multi-mode Reader, in triplicate for each reporter construct. Fluorescence was measured using an a Tecan plate reader from the top with excitation and emission wavelengths of 395nm and 509nm using *E.* coli JM109, a constituently express GFP strain, for baseline

comparison after 18 hours of incubation at 37°C . Reporter inoculation into the sand from the sand followed protocol outlined in section 2.3, in which cells are inoculated into sand at a 10^6 concentration. To measure GFP from sand, we attempted to concentrate cells through centrifugation of eluent at 10,000rpm however, due to low concentration of cells ($<10^8$) there was no observable pellet. As a result of unsuccessful pelleting, cells were not able to be concentrated and GFP measurements were unable to be obtained.

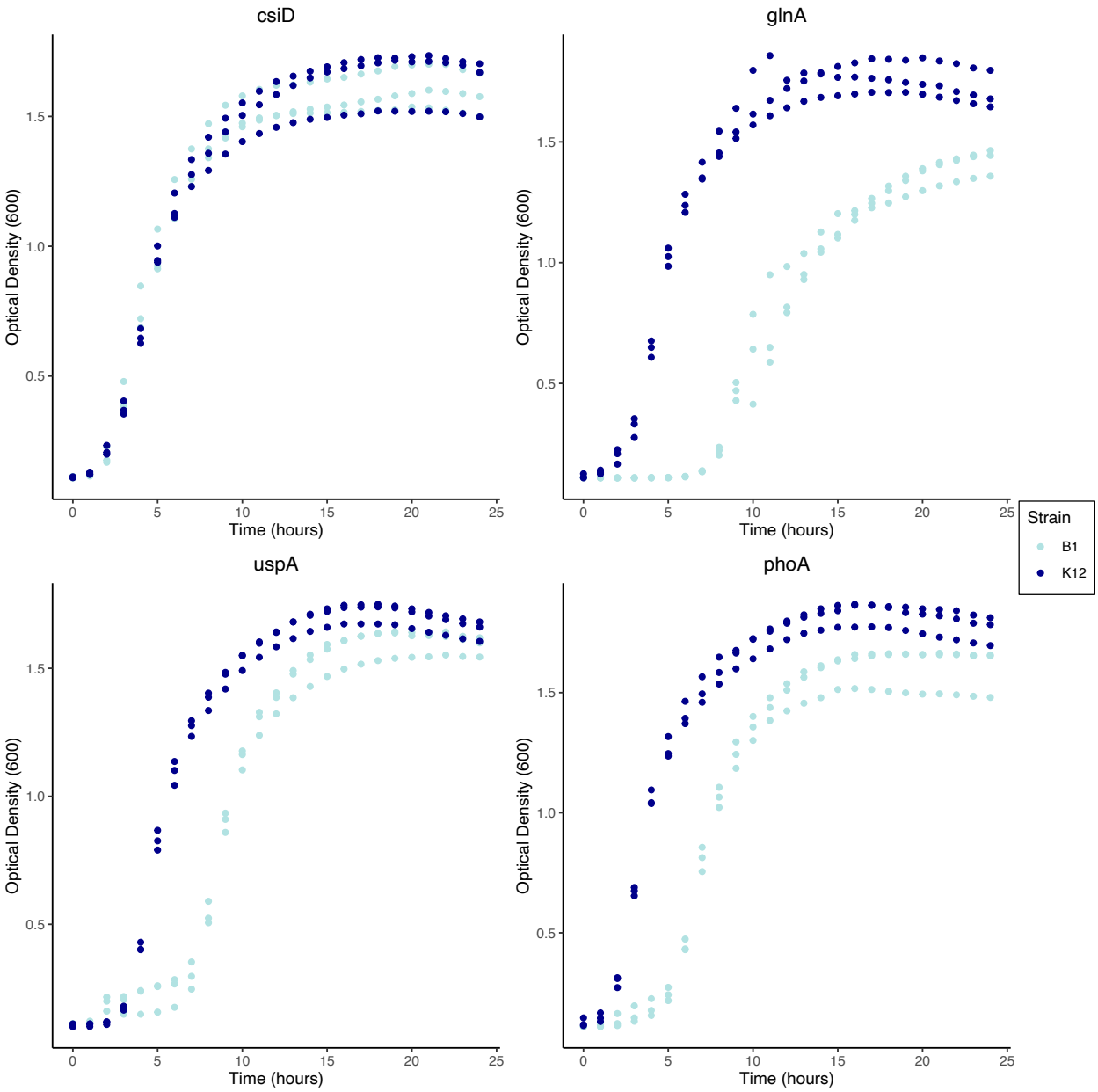

Figure 1: Growth curves of reporter constructs glnA, csiD, uspA, and phoA over a 24-hour period. Plots include reporters in different *E. coli* strains of K12, dark blue, and B1, light blue. Optical density at 600 absorbance is plotted against hour, to obtain the growth over time.