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Physical and Biological Constraints on the Abundance of Cyanobacteria in the James River Estuary

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Physical and biological constraints on the abundance of cyanobacteria in the James River Estuary

A thesis submitted in partial fulfillment of the requirements for the Master of Science in Biology at Virginia Commonwealth University

by

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Acknowledgment

Table of Contents

List of Tables

List of Figures

Abstract

PHYSICAL AND BIOLOGICAL CONSTRAINTS ON THE ABUNDANCE OF
CYANOBACTERIA IN THE JAMES RIVER ESTUARY

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A thesis submitted in partial fulfillment of the requirements for the Master of Science in Biology
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The tidal-fresh James River experiences recurring blooms of toxin-producing
cyanobacteria, including *Microcystis aeruginosa*. However, cyanobacteria cell densities in the
James are relatively low. Our purpose was to identify key factors suppressing cyanobacteria
growth in the face of eutrophication. A mesocosm experiment was designed to test the effects of
light, mixing and grazing on cyanobacteria abundance, with nutrients held constant. We
predicted that toxic cyanobacteria would be most abundant under stagnant conditions, with
enhanced light, with no bivalve grazers present. Abundances of indicator gene copies and

phytoplankton counts supported this hypothesis. However, chlorophyll-a, phycocyanin, and the toxin microcystin were all found to be most abundant under mixed conditions with ambient light. Statistically, light and mixing were important in controlling toxic cyanobacteria abundance, with little to no effect observed for bivalve grazers. Our findings suggest that toxin production may be regulated by factors independent from those driving algal growth and cyanobacteria abundance.

Introduction

Algal growth in estuaries is stimulated by anthropogenic nutrient loading, resulting in eutrophic conditions liable to produce blooms of cyanobacteria (Anderson et al. 2008). Enhanced cyanobacteria growth can result in hypoxic conditions, and several common forms of cyanobacteria produce toxic secondary metabolites (cyanotoxins) and are thereby classified as harmful algae (Paerl and Otten 2013a). Over the past several decades these harmful algal blooms (HABs) have become ubiquitous and prolific, affecting coastal systems worldwide (Anderson et al. 2008). Toxic HABs result in impaired water quality and deleterious effects on living resources (Carmichael 1997, Ibelings and Havens 2008). The cyanotoxin microcystin (MC) is a hepatotoxin known to cause damaging health effects to humans and biota (de Figueiredo et al. 2004). Thus the World Health Organization has established recommended guidelines for consumption and exposure (WHO 2003). Microcystin is common in freshwater systems and readily accumulates in the tissues of a wide range of aquatic organisms (Ibelings and Havens 2008, Wood et al. 2014). Physiological responses of organisms exposed to MC may include liver damage, behavioral changes, reduced fecundity and mortality (Ibelings and Havens 2008, Wood et al. 2014).

The tidal fresh James River Estuary is eutrophic and highly productive, receiving nutrient inputs from agriculture, wastewater and urban runoff (Smock et al. 2005, Wood et al. 2014). Thus the tidal fresh James often experiences blooms of toxic cyanobacteria (Marshall and Egerton 2012). Microcystin persists seasonally in this segment of the river, with peak toxin

concentrations occurring in late summer and early fall (Wood 2014). Several forms of cyanobacteria are known to produce MC (Hisbergues et al. 2003), but the primary species of concern in this system is *Microcystis aeruginosa* (Wood et al. 2014). Cyanobacteria account for a large proportion of phytoplankton cell densities in the James, but due to their small cell size contribute less than 10% to total biomass (Marshall and Alden 1990, Marshall and Burchardt 1998, Wood et al. 2014). In other freshwater systems, cyanobacteria blooms comprising ≥ 80 % of phytoplankton biomass have often been reported (Downing et al. 2001, Vanderploeg et al. 2001, Chen et al. 2003, Agha et al. 2012), raising the question: why are cyanobacteria not more abundant in the tidal fresh James River?

Extensive research has been devoted to the causes of cyanobacteria blooms, with nutrients as a primary focus (Carmichael 1997, Anderson et al. 2008). However, other physical and biological factors also promote or suppress bloom development and toxin production (Paerl and Otten 2013a). The abundance of cyanobacteria is thought to be bolstered by high solar irradiation, weak mixing forces, and low grazing rates (Reynolds 1994, Paerl and Otten 2013a, b).

Cyanobacteria are diverse, exhibiting unique light requirements and strategies during light competition (Oliver 1994, Huisman and Hulot 2005). Some species of cyanobacteria, including *M. aeruginosa,* may gain a competitive advantage by the formation of gas vesicles for buoyancy (Oliver 1994, Walsby et al.1997). Under unmixed conditions, buoyant cyanobacteria congregate at the surface of the water column, creating scums beneath which shaded conditions further enable cyanobacteria dominance (Visser et al. 1996, Kardinaal et al. 2007).

Turbulence also plays a role in light availability, both by increasing light attenuation through the suspension of particulate matter, and by rendering buoyancy regulation by

cyanobacteria ineffective (Oliver 1994, Reynolds 1994). Furthermore, diatoms and green algae have lower light requirements than cyanobacteria and thus tend to dominate turbulent systems where light is limited (Huisman et al. 2004). The proliferation of cyanobacteria is thought to occur during periods of low flow and weak mixing, when light availability is enhanced and buoyancy regulation allows for congregation of cyanobacteria at the surface (Oliver 1994, Harris and Baxter 1996, Sherman et al. 1998, Paerl and Otten 2013a).

Herbivory is also known to impact phytoplankton abundance and community structure (Urrutia-Cordero et al. 2015). However, the effectiveness of cyanobacteria reduction can vary between grazer species, and empirical studies of grazer controls on cyanobacteria have led to conflicting results (Paerl and Otten 2013a). Bivalve filter feeders consume phytoplankton directly, but toxin production by some cyanobacteria can reduce bivalve grazing rates (Wood et al. 2014).

The tidal fresh James River is turbulent, and features light conditions that are favorable for algal growth (Bukaveckas et al. 2011). Zooplankton grazing rates are low in this segment of the river, but fish and shellfish also graze phytoplankton in this system (Bukaveckas et al. 2011, Wood et al. 2014). The objective of this study was to improve our understanding of factors controlling cyanobacteria abundance in the James River Estuary. Mesocosm experiments were conducted to independently manipulate light, mixing and grazing, and to measure their effects on cyanobacteria abundance and toxicity. We predicted that toxic cyanobacteria would be most abundant under stagnant conditions, with enhanced light and no grazers present; this would indicate that cyanobacteria in the tidal fresh James are constrained by turbulence, light limitation, and grazing.

Methods

Study Site

Experiments were performed at the Virginia Commonwealth University (VCU) Rice

Rivers Center mesocosm facility in Charles City County, VA. The facility contains 24 circular

2000-L fiberglass tanks (1.8 m diameter) that were filled to a depth of 80 cm by pumping water

directly from the James River. The water intake point is the Rice Rivers Center Research Pier,

which draws surface water (~1 m depth) from a nearshore deep-water location. The intake is

within the tidal freshwater segment of the James, approximately 75 miles upstream from the

confluence with the Chesapeake Bay (JMS 75). Each tank was fitted with a fiberglass cover to

reduce incident solar radiation and atmospheric inputs. Openings within the covers allowed

sufficient solar radiation to create light conditions corresponding to a 2.5 m water column (mean

depth of the tidal fresh segment =3.1 m) based on average light attenuation observed in this

segment of the James (Bukaveckas et al. 2011).

Experimental Design

Three 10-day experiments were conducted in June, August and October 2014, using the

same experimental protocol. The 10-day period was selected to allow enough time for algal

growth to occur, while remaining within the known residence time of the river (mean=15.6 days,

May-Oct; Wood et al. 2014). By repeating the experiment in early summer, late summer and fall,

we sought to account for seasonal changes in phytoplankton and zooplankton communities. Each

experiment employed a full factorial design (3 factors, 2 levels, 3 replicates). The three treatment factors were solar irradiation (ambient or enhanced), turbulence (mixed or stagnant), and grazing by bivalve clams (presence or absence of *Rangia cuneata*). Each treatment was replicated in three independent tanks, with treatments distributed among the mesocosms in a randomized block design.

Light was manipulated by the presence or absence of the aforementioned mesocosm lids. Ambient light treatments were mostly covered (shaded); enhanced light treatments were open (unshaded). Turbulence was simulated using submersible aquarium pumps (capacity 1120 L/h). Mesocosms containing pumps (4 per tank) represented mixed conditions and, tanks without pumps represented stagnant conditions. To simulate a grazing effect, *R. cuneata* clams were collected from a nearby location in the James immediately prior to each experiment. Five clams of approximately equal sizes (between 4-7 cm) were then suspended within mesh cages in each of the grazing treatment tanks, but not within the non-grazing tanks.

In order to remove the effects of nutrient availability on phytoplankton growth, nutrients (dissolved inorganic nitrogen; DIN; and dissolved inorganic phosphorous; DIP) were added to each tank throughout each experiment. Nutrient additions of 0.125 mg/L/d DIN and 0.03 mg/L/d DIP corresponded to annual average loading rates for the tidal fresh James (Bukaveckas and Isenberg 2013). Nitrogen additions were derived from NH_4Cl and $NaNO_3$, phosphorous additions from Na_2HPO_4. Nutrient additions were performed every 48 hours (after sample collection). In prior mesocosm experiments this rate of nutrient loading was sufficient to remove the limiting effects of nutrients on phytoplankton growth in the tanks (Wood 2014).

Dependent Variables

Dependent variables included chlorophyll-a (a measure of algal biomass), phycocyanin (a cyanobacteria-specific pigment), and microcystin (an algal toxin produced by cyanobacteria). Samples were also collected for microscopic examination and genetic analysis of phytoplankton community composition during the experiments. Genetic analysis was performed to measure copies of the *16S rRNA* gene associated with cyanobacteria, as well as the *16S rRNA* gene associated with the genus *Microcystis*, and the *mcyD* gene, which encodes for the production of microcystin (Rinta-Kanto et al. 2009). These data were used to assess changes in the abundance of cyanobacteria, the relative proportion of *Microcystis*, and the presence of toxic strains. Microscopic counts were performed to identify and enumerate the dominant phytoplankton taxa.

Water quality and zooplankton abundance were monitored within each of the tanks. Water quality parameters included turbidity, temperature, dissolved oxygen, pH and nutrients. Nutrients were analyzed for: total nitrogen (TN), total phosphorous (TP), DIN, and DIP. Concurrent *in situ* water quality data in the tidal fresh James River were available from continuous monitoring stations operated by VCU at the Rice Center Pier (http://gis.vcu.edu/riceriversrealtime/Pier.aspx), and from the Chesapeake Bay Interpretive Buoy System (http://buoybay.noaa.gov). Weekly measurements of chlorophyll-a, microcystin, and nutrient data from the tidal fresh James were available from a nearby VCU monitoring station at river-mile seventy-five (JMS 75).

Data Collection

At the beginning of each experiment (day 0), and at 48-hour intervals, a YSI 6600 multiparameter sonde was used to measure phycocyanin concentrations (as relative fluorescence

units; RFU), along with water quality parameters (temperature, dissolved oxygen, turbidity, pH).
All sonde readings were taken at mid-day. Identical, calibrated YSI sondes were also deployed in
three randomly selected mesocosms on day 0 to measure water quality parameters at 15 minute
intervals for the duration of the experiment. Water samples (2-L) were collected on day 0 and
every 48 hours for the duration of the experiment. A representative sample of the water column
was obtained using 1.2 m PVC pipe and a rubber stopper. The PVC pipe was first submerged
vertically into the tank with one end remaining out of the water. Suction was created by closing
off the unsubmerged opening with the stopper, and the whole pipe was then removed to obtain a
depth-integrated sample. On day 0 initial values were obtained from 3 randomly selected tanks
from among the 24 used in each experiment. Water samples were filtered for chlorophyll-a and
samples from days 0, 6, and 10 were tested for microcystin concentration. Due to logistical
constraints, samples for nutrients, algal genetics, zooplankton and phytoplankton analysis were
collected on day zero (3 tanks) and day 10 (all tanks). A 200 mL sample was collected for
phytoplankton counts and immediately preserved with 1.5 ml Lugol's and 1 ml formalin. For
zooplankton samples, 2 L water samples were filtered through a 23 μm mesh sieve and preserved
with 1.5 ml Lugol's.

Sample Analysis

To measure chlorophyll-a (CHLa), water was filtered through Whatman glass filters (0.5
μm), and extracted for 18 hours in buffered acetone before fluorometric analysis (Turner TD
700; Arar and Collins 1997). Microcystin analysis was conducted with commercial ELISA test
kits (Abraxis). Genetic analysis was performed using the methods of Wood et al. (2014) and are
briefly summarized here. Samples were filtered (0.45 μm) and extracted using the Mo Bio
PowerWater DNA Isolation Kit (Carlsbad, CA) from one randomly selected mesocosm from

each treatment group. This analysis used quantitative PCR (qPCR) via Bio-Rad CFX 96 Real Time system and SsoAdvanced SYBR Green qPCR Supermix (BioRad Hercules, CA). The following primer sets were used to amplify extracted DNA: CYAN 108F[38] and 377R[39] (*16s rRNA*), *MICR* 184F and 431R [40] (Microcystis-specific *16s rRNA*), and *mcyD* F2 and R2[41] (toxin producing strains). Nutrient concentrations (TN, DIN, TP, DIP) were measured via Skalar segmented flow analyzer using standardized methods (APHA 1992). For zooplankton counts, a sub-sample from each filtered sample was counted using a Nikon SMZ800 stereomicroscope (~100-800 individuals counted per sample). Zooplankton were identified as rotifers (phylum Rotifera), cladocerans (order Cladocera), copepods (order Copepoda), or as juvenile copepods (nauplii). Phytoplankton counts were performed by Dr. Matthew Semcheski at Old Dominion University, where a 0.1 mL aliquot of each sample was counted using a Palmer-Maloney counting chamber. Phytoplankton counts were conducted without replication.

Statistical Analysis

A three-way ANOVA was run for each dependent variable (CHLa, phycocyanin, microcystin) to test for treatment effects in each experiment. Time was not considered a factor, and statistical tests were performed on the mean values across sampling dates (excluding day 0). Normality was tested by way of QQ plots, and some data sets were log transformed in order to meet assumptions for normal distribution (Table 3). All three-way ANOVA tests had a sample size of 24 and 7 degrees of freedom. After analyzing the results of the three-way ANOVA, it became apparent that the presence of bivalve grazers (*Rangia*) had minimal effects on the dependent variables. Of the nine three-way ANOVAs performed (3 experiments x 3 dependent variables), *Rangia* only appeared as a statistically significant factor twice, both times as an interactive effect. Thus in subsequent analyses *Rangia* and non-*Rangia* treatments were pooled,

8

resulting in four groups upon which further statistical analyses (two-way ANOVA) were performed. All two-way ANOVA tests had sample sizes of 24 and 4 degrees of freedom. When interactive effects of light and mixing were indicated by the two-way ANOVA, subsequent non-parametric two-group analyses (Wilcoxon rank-sum) were run to identify the significantly different groups (n=6 per group). Two-group non-parametric tests were also performed on the genetics data and phytoplankton counts, where logistical constraints prevented adequate replication to support a multi-way ANOVA. For all tests a significance level of $\alpha=0.05$ was used. All statistical analyses were performed using JMP (Pro 11.1).

Results

Initial water quality conditions in the mesocosms were similar to concurrent conditions in the tidal fresh James River, and reflected the expected seasonal patterns for these variables (Table 1). Initial CHLa values (Mean = 20.3, 32.8, and 24.4 µg/L for June, August and October respectively) as well as initial MC concentrations (Mean = 0.20, 0.81, 0.30 µg/L, respectively) were comparable to *in situ* values measured concurrently at a nearby station (JMS 75). Highest starting concentrations of CHLa and MC occurred during the August experiment. Initial phycocyanin values in the mesocosms (4.7 to 10.5 RFU) were higher than *in situ* values (1.8-3.8 RFU) during each of the experiments. Water quality conditions within the mesocosms were generally similar to those measured concurrently in the James Estuary at JMS 75, with the exception of turbidity (Table 2). Mesocosm turbidity maximums were low (0.0-3.7 NTU) in comparison to the range of values measured in the James over the course of the experiments (17.8 – 51.5 NTU). In all experiments turbidity values began near the maximum of the range before quickly decreasing in the first few days of the experiment. Temperature conditions were similar in the mesocosms and the estuary with higher values observed in June and August (25-30 ^{o}C) and lower values in October (15-23 ^{o}C). Dissolved oxygen and pH values were marginally higher in the mesocosms (pH = 7.7-9.3, DO = 7.9-12.0 mg/L) than in the James (pH = 7.6-8.8, DO = 7.2-11.0). Dissolved oxygen and pH in the mesocosms were higher in June and August than in October.

Chlorophyll-a

Although starting CHLa concentrations were generally similar in each month, we observed large differences in the magnitude of response to treatments across experiments (Figure 1). The largest increase in CHLa, and the clearest effect of treatments occurred during the October experiment. From a starting CHLa concentration of 24.4 µg/L, mean values (across dates) reached 60-70 µg/L in mesocosms with mixing and ambient light (with or without *Rangia*). Among other treatments in this experiment, mean CHLa concentrations were 30-40 µg/L. Statistical analyses of these data revealed a significant interaction effect between light and mixing (Table 3). By comparison, the August experiment yielded small increases in CHLa (up to 45 µg/L from a starting concentration of 32 µg/L) and no statistically significant effects of treatments. In June all treatments showed lower mean CHLa values relative to initial values, though there was a statistical difference between treatments with higher CHLa at ambient light relative to enhanced light.

Microcystin

Despite the lack of response in CHLa, highest mean MC concentrations occurred during the June experiment (Figure 2). In this experiment, MC concentrations increased from a starting value of 0.20 µg/L to almost 5 µg/L in the treatment with ambient light and mixing. In other treatments, MC increased to 1.5-2.0 µg/L, with the exception of the stagnant, enhanced light treatment (mean ~ 0.5 µg/L). Similar patterns were observed during the August and September experiments with highest MC levels occurring under mixed and ambient light conditions. Although initial MC concentrations were higher in August (0.81 µg/L), the observed increases during the experiment were < 1 µg/L, with a peak toxin concentration of 1.39 µg/L. As in June,

11

August maximum MC concentrations occurred under mixed conditions with ambient light, while all stagnant and enhanced light treatments exhibited mean MC concentrations that declined from the initial value. October mean MC levels were the lowest observed in all three months, as concentrations in all October treatments stayed near the initial value (mean = 0.30 µg/L). Results of statistical analysis suggest that increases in MC concentrations in the mesocosms were primarily driven by light and mixing, as primary effects in June and August and as interactive effects in October (Table 3).

Phycocyanin

The largest increases in phycocyanin occurred during the August experiment, when phycocyanin levels increased from a starting value of 10 RFU to mean values ranging from 20 to 25 RFU across treatments. In August there were no significant differences between the treatments. In June and October, phycocyanin levels were lower (5-10 RFU) but showed differing responses among treatments. In both experiments, ambient light treatments exhibited significantly higher phycocyanin than enhanced light treatments (Table 3). In June, phycocyanin levels increased in ambient light treatments, while mean phycocyanin levels fell below initial values in enhanced light treatments. The October experiment showed an increase in mean phycocyanin under mixed, ambient light conditions (by 6.6 RFU), which mirrored the CHLa increases (Figure 1). All other treatments in October remained near or fell below initial values. Statistical analysis of the phycocyanin data showed significant effects from light and mixing in both June (primary effect) and October (interactive effect; Table 3).

Genetics

Highest initial values for cyanobacteria gene copies, *Microcystis* gene copies, and *mcyD* gene copies occurred in October (Figure 4). Treatment effects were most apparent in August when all three genetic markers increased in response to stagnant and enhanced light conditions. In June and October, increases in the abundance of *mcyD* gene copies were also observed under stagnant and enhanced light conditions, but these were not accompanied by increases in cyanobacteria or *Microcystis* gene copies. In June, increases in *mcyD* gene copies were observed under stagnant conditions for both the ambient and enhanced light treatments. Statistical tests indicate that mixing and grazers had significant effects (Figure 4), but lack of replication precluded testing for interactive effects.

Phytoplankton Counts

Initial values for cyanobacteria abundance were lowest in June (2.3×10^3 cells/mL), while exhibiting higher levels in August (2.8×10^5 cells/mL) and October (2.6×10^5 cells/mL). Increases in cyanobacteria cell densities were most apparent in August, particularly under stagnant conditions (Figure 5). From starting values of 2.8×10^5 cells/mL, final values in stagnant treatments reached 8.0×10^5 cells/mL (ambient light) and 1.1×10^6 cells/mL (enhanced light). During the June experiment, cyanobacteria cell densities remained similar to initial values. Statistical tests indicated that mixing had a significant effect on cyanobacteria cell densities, however the impact of mixing reduced cyanobacteria in August, while increasing cell densities in October. The dominant species of cyanobacteria in initial treatments for all months was *Pseudanabaena,* which remained the most abundant species in the majority of treatments, even when community composition changed dramatically (data not shown). The treatments that

showed the highest levels of MC in all three experiments (June, mixed, ambient) were dominated by *Aphanizomenon*.

Zooplankton

Zooplankton communities in the mesocosms were initially dominated by rotifers, which comprised on average 89% of initial abundance across the three experiments (Figure 6). In both June and August, zooplankton community composition changed during the course of the experiment, with larger zooplankton (copepods, nauplii, and cladocerans) becoming more abundant in all treatments. These differences were most apparent under stagnant conditions. In the June experiment, in tanks with no mixing, the percentage of rotifers dropped from 89% to 24%. In August the percentage of rotifers in the stagnant tanks dropped from 99% to 11%. As the abundance in rotifers dropped, there were corresponding increases in the abundance of cladocerans (300 ind/L, June experiment), copepods (245 ind/L August experiment) and nauplii (430 ind/L August experiment). In October, rotifers were the dominant species in all treatments for the entire experiment. The abundance of rotifers increased during the October experiment under enhanced light conditions (mixed and stagnant). The peak total abundance for zooplankton occurred in October, with maximum concentrations approaching 3000 ind/L. Statistical analysis found light to be a significant primary effect on total zooplankton abundance in October, while no significant effects on total zooplankton were found in other months (Figure-7).

Nutrients

Equal amounts of DIN and DIP were added to all treatments, simulating *in situ* loading rates. Initial values for DIN were highest in June (0.10 mg/L) and lower in August and October (~0.03 and 0.04 mg/L respectively). Treatments exhibited differing uptake rates of DIN. In June

and October, DIN uptake was significantly higher in the enhanced light treatments as compared to ambient light treatments (Figure 8). In August, greater DIN uptake occurred in the mixed treatments as compared to stagnant treatments. DIN accumulated in all treatments during experiments in August and October (i.e. final values were greater than initial values), but DIN uptake during June was greater than or equal to loading rates under enhanced light conditions. Initial values for DIP were similarly low for all experiments (≤ 0.02 mg/L), and with the exception of one outlier, DIP accumulated in all tanks across all three experiments. In June and August, DIP uptake was highest in the enhanced light treatments (Figure 8). Statistical tests indicated a primary effect of mixing on DIN in August, and no statistical effects on DIP in October (Figure 8). All other statistical tests on nutrient concentrations indicated light as a statistically significant factor (Figure 8). Total nitrogen and total phosphorous accumulated in all treatments.

Discussion

Our primary concern in this experiment was the persistence of microcystin in the James River. Empirical evidence shows elevated MC levels *in situ* tend to occur in late summer or early autumn (Wood 2014), coinciding with elevated *in situ* CHLa and phycocyanin (unpublished monitoring data). This relationship is explained by the fact that toxin-producing cyanobacteria are a component of overall cyanobacteria, which is in turn a component of the larger algal community. When the amount of CHLa rises in this system, cyanobacteria represent a portion of those algae, and thus more toxin producing cyanobacteria may be present. However we did not observe the same predictable, uniform response in the mesocosms. There were marked seasonal differences in the amount of toxic cyanobacteria overall, and responses of some related variables occurred under different treatment conditions. This suggests that the production of MC may be regulated by different factors than the abundance of CHLa and the proportion of cyanobacteria in the algal community.

In order to identify the physical and biological factors that control the abundance of toxic cyanobacteria in the James (and thus the concentration of MC), we tested the effects of light availability, mixing forces and the presence of bivalve grazers. Our hypothesis was that the greatest amount of toxic cyanobacteria would occur under enhanced light with weak mixing forces (in the absence of grazers). This hypothesis was most strongly supported by the data from phytoplankton counts and genetic analysis. During the August experiment, the greatest

abundances of *mcyD* gene copies and *16s rRNA* gene copies associated with cyanobacteria and *Microcystis* occurred under stagnant, enhanced light conditions. In the June and October experiments, the greatest abundances of *mcyD* gene copies were observed under stagnant conditions with the highest abundances in October occurring in the enhanced light treatments. Thus the greatest number of gene copies indicating toxin producing strains of cyanobacteria (*mcyD*) occurred under stagnant conditions in all months. These findings are supported by previous work that shows dominance by cyanobacteria occurs during periods of low flow (Harris and Baxter 1996), and that *Microcystis* is a poor competitor for light in well mixed waters (Huisman and Hulot 1995). According to the phytoplankton count data, cyanobacteria were most abundant in the mesocosms under enhanced light during the August and October experiments. This is supported by the work of Huisman et al. (2004) who found that cyanobacteria were outcompeted by green algae under low solar irradiation. August phytoplankton counts also indicated greater cyanobacteria under stagnant conditions, although the October phytoplankton counts did not.

Conversely, CHLa, phycocyanin, and MC data did not exhibit the same predictable pattern of response. The greatest CHLa values were measured during the October experiment under mixed conditions with ambient light. The lack of chlorophyll response under enhanced light conditions could be explained in part by variation in the CHLa content within individual algal cells, which decreases when exposed to high light intensity (Beale and Appleman 1971). However, we also expected to observe the highest phycocyanin and the highest MC under enhanced light with stagnant conditions, but our results showed that these variables responded to mixed conditions and ambient light as well. The buoyancy regulation of some species of cyanobacteria (including *Microcystis*) suggests that stagnant conditions should allow for

17

dominance of the algal community, but empirical evidence has not always supported this assumption. For example, the implementation of artificial mixing in eutrophic lakes has produced inconsistent results for the mitigation of cyanobacteria blooms (Visser et al. 1996, Oberholster et al. 2006). In a mesocosm experiment, Pinckney et al. (1999) determined that the highest biomass of cyanobacteria resulted under well-mixed conditions.

Though the presence of bivalve grazers had minimal impact on our dependent variables, all tanks were subject to grazing by zooplankton. Zooplankton were not under direct experimental control, but zooplankton abundance increased over the course of each experiment, and exhibited differential responses to the treatment conditions. In the June and August experiments, zooplankton community structure shifted from a rotifer-dominated community to greater abundances of copepods and cladocerans. These changes in community structure may be due to the removal of the zooplankton from predatory controls (e.g., in the absence of planktivorous fishes; Urrutia-Cordero et al. 2015) or changes in food quantity and quality within the mesocosms (i.e., settling of particulate inorganic matter). This shift to larger primary filter feeders with greater filtering capacity could potentially have impacted cyanobacteria abundance (Ka et al. 2012). Although cyanobacteria are thought to be a poor food source for zooplankton, prior work has shown that copepods are effective in suppressing the growth of *Microcystis* (Urrutia-Cordero et al. 2015). In October, zooplankton communities remained dominated by rotifers throughout the experiment, with the greatest increases in zooplankton abundance occurring under enhanced light. This could explain in part why CHLa was higher under ambient light during the October experiment. Ka et al. (2012) determined that zooplankton could in some cases effectively reduce cyanobacteria, with the exception that no species was shown to consume *Microcystis aeruginosa*.

Experimental factors influenced the growth of algae, the proportion of cyanobacteria within the algal community, and the production of MC. While these three variables are related (cyanobacteria as a component of algae, and toxin producers as a subset of cyanobacteria) the highest levels of each variable did not coincide. CHLa, phycocyanin and MC all responded to ambient light and mixing, but peak levels of these variables occurred during different months. CHLa was highest in October, while the August experiment resulted in the highest phycocyanin. The highest levels of MC occurred in June, when the other two variables were relatively low, suggesting that the production of toxins is regulated by different factors than those that favor the growth of algae and cyanobacteria. While these aforementioned variables differed between seasons, the genetics work and phytoplankton counts responded differently to the treatments. Gene copies of cyanobacteria, Microcystis, and *mcyD* showed the greatest response to stagnant, enhanced light treatments. For cyanobacteria and *Microcystis* this response occurred only in August, while for *mcyD* this response occurred in all months. Similarly, phytoplankton counts revealed the highest cyanobacteria under enhanced light treatments during August and October. These results clearly indicate that high levels of MC may occur during periods of relatively low CHLa and phycocyanin, and under conditions in which copies of the *mcyD* gene that encodes for toxin production may not be abundant. The latter finding concurs with the work of Beversdorf et al. (2015) who found that the presence of microcystin genes were not a good indicator of toxins in the environment. Because our genetic sampling was limited, further inquiry may be necessary to obtain clear resolution on our genetics results.

Conclusion

Our goal was to determine the response of toxic cyanobacteria to treatment conditions (light, mixing and grazers), while simulating the river's constant loading rate of nutrients. Our hypothesis was that the greatest abundance of toxic cyanobacteria would occur under enhanced light, with stagnant conditions, when no bivalve grazers were present. Statistical evidence pointed to a limited role for bivalve grazers, with light and mixing producing a significant response in our variables. However we encountered issues measuring variables that ought to have been supportive of one another. In particular, the genetics data did not corroborate our other results. This conflict is an important finding, because it tells us that the gene copies we were measuring may not be accurate predictors of toxin production. Thus how we interpret our results depends upon which variables we choose to rely. If we disregard the genetics work for argument's sake, we are left with several other variables that measure toxic cyanobacteria in some form (chlorophyll-a, phycocyanin, microcystin). Although these variables showed peak growth in different months, they were mutually supportive in their response to treatment combinations. All three showed the greatest response under ambient light and mixing. These results do not support our hypothesis, but they are robust in their support of one another.

Another interesting finding comes from the phytoplankton count data, which reveals that the highest toxicity occurred in mesocosms that were dominated by genera of cyanobacteria other than *Microcystis*. Certain aspects of our hypothesis were based upon the assumption that

the primary toxin producing species of cyanobacteria in the tidal fresh James River is the buoyant *Microcystis aeruginosa*. Our results indicate that this assumption should be reconsidered, as the mesocosms were more often dominated by *Pseudanabaena* in the majority of tanks and *Aphanizomenon* in those tanks with the highest MC levels.

Tables

Mesocosm Initial Values (mean ± std err)		
Chlorophyll-a (µg/L)	Microcystin (µg/L)	Phycocyanin (RFU)
June	20.3 ± 3.28	0.20 ± 0.02	4.7 ± 0.05
August	32.8 ± 1.14	0.81 ± 0.03	10.5 ± 0.10
October	24.4 ± 3.40	0.30 ± 0.02	8.1 ± 0.03
Concurrent Ranges in the Tidal Fresh James River		
June	(27.9, 43.6)	(0.07, 0.68)	(1.3, 1.8)
August	(29.5, 50.9)	(0.50, 0.75)	(2.8, 3.8)
October	(33.1, 62.4)	(0.06, 0.37)	(1.5, 2.3)

Table 1. Initial mesocosm values for chlorophyll, microcystin, and phycocyanin (mean ± SE) for experiments conducted in June, August and October. Also shown are ranges of values for the same variables measured in in the James River Estuary over the duration of each experiment at a nearby monitoring station (JMS 75).

Mesocosm Water Quality Ranges			
Temperature (°C)	pH	Turbidity (NTU)	Dissolved O_2 (mg/L)
June	(27.1, 30.5)	(8.43, 9.31)	(0.03, 10.0)	(7.89, 12.04)
August	(26.0, 29.2)	(8.46, 9.27)	(3.7, 11.2)	(8.84, 12.22)
October	(15.2, 22.8)	(7.73, 9.09)	(1.1, 15.7)	(9.27, 11.81)
Concurrent Ranges in the Tidal Fresh James River			
June	(27.0, 30.5)	(7.73, 8.78)	(17.8, 48.4)	(7.20, 11.02)
August	(27.1, 28.9)	(7.72, 8.62)	(19.9, 51.5)	(7.78, 10.24)
October	(17.9, 21.6)	(7.61, 8.11)	(23.9, 34.4)	(8.81, 10.91)

Table 2. Ranges of water quality parameters measured in the mesocosms (top) and within the James River (bottom) over the course of each experiment. Mesocosm water quality derived from continuous measurement in three randomly selected tanks. James River water quality obtained from the Chesapeake Bay Interpretive Buoy System.

Chlorophyll

Month	Mean	Standard Deviation	Sum of Squares	Mean Square	F- Ratio	p-value	Primary Effects	Interactive Effects
3-Way ANOVA (Light, Mixing, Rangia)								
June	13.0	4.5	349.72	49.96	7.35	0.0005	-	L*M, M*G
August	38.7	8.3	697.64	99.66	1.83	0.15	-	-
October*	44.0	13.8	0.29	0.04	8.19	0.0003	-	L*M
2-Way ANOVA (Light, Mixing)								
June			156.66	52.22	3.46	0.004	L	-
August			471.03	157.01	2.86	0.06	-	-
October*			0.27	0.09	17.63	<.0001	-	L*M

Microcystin

Month	Mean	Standard Deviation	Sum of Squares	Mean Square	F- Ratio	p-value	Primary Effects	Interactive Effects
3-Way ANOVA (Light, Mixing, Rangia)								
June*	1.7	1.9	2.24	0.32	13.68	<0.0001	L, M	-
August*	0.7	0.5	1.58	0.23	27.69	<0.0001	L, M	-
October*	0.3	0.1	0.82	0.19	96.29	<0.0001	L	M*G
2-Way ANOVA (Light, Mixing)								
June*			2.11	0.70	27.83	<.0001	L,M	-
August*			1.57	0.52	74.72	<.0001	-	L*M
October*			0.80	0.27	169.75	<.0001	L,M	-

Phycocyanin

Month	Mean	Standard Deviation	Sum of Squares	Mean Square	F- Ratio	p-value	Primary Effects	Interactive Effects
3-Way ANOVA (Light, Mixing, Rangia)								
June*	6.5	3.8	1.82	0.26	286.62	<0.0001	L, M	-
August	21.7	2.9	78.17	11.17	1.52	0.231	-	-
October*	8.8	3.5	0.55	0.08	181.12	<0.0001	-	L*M
2-Way ANOVA (Light, Mixing)								
June*			1.82	0.61	701.81	<0.0001	L, M	-
August			47.55	15.85	2.14	0.13	-	-
October*			0.54	0.18	390.82	<0.0001	-	L*M

Zooplankton

Month	Mean	Standard Deviation	Sum of Squares	Mean Square	F- Ratio	p-value	Primary Effects	Interactive Effects
3-Way ANOVA (Light, Mixing, Rangia)								
June*	1306.9	504.0	0.36	0.05	2.64	0.051	-	-
August*	1287.0	735.3	0.31	0.04	0.76	0.63	-	-
October*	2095.2	958.3	0.55	0.08	2.94	0.035	L	-
2-Way ANOVA (Light, Mixing)								
June Rotifers			1.25E+06	4.18E+05	6.49	0.003	M	-
August Rotifers*			12.32	4.11	15.71	<0.0001	-	L*M
October Rotifers*			0.51	0.17	5.19	0.0082	L	-
June Cladocerans*			4.30	1.43	6.82	0.002	-	L*M
August Cladocerans*			4.85	1.62	7.99	0.001	L	-
October Cladocerans*			10.86	3.62	11.42	0.0001	M	-
June Nauplii*			1.28	0.43	12.32	<0.0001	L, M	-
August Nauplii			8.15E+05	2.72E+05	9.94	0.0003	M	-
October Nauplii*			6.20	2.07	9.93	0.0003	M	-
June Copepods*			10.74	3.58	21.44	<0.0001	L, M	-
August Copepods*			9.23	3.08	23.34	<0.0001	-	L*M
October Copepods*			1.62	0.54	1.25	0.32	-	-

Table 3. ANOVA tables for CHLa, MC, phycocyanin, and zooplankton in the June, August and October experiments. 3-Way ANOVAs (light, mixing, *Rangia*) for each variable shown, as well as 2-way ANOVA tables with pooled grazer treatments (light, mixing). Statistical reporting is for the global ANOVA test. Data sets that were log transformed are indicated by *. 3-way ANOVA for zooplankton performed on total abundance. Significance level = 0.05. Primary effects were only considered in the absence of interactive effects.
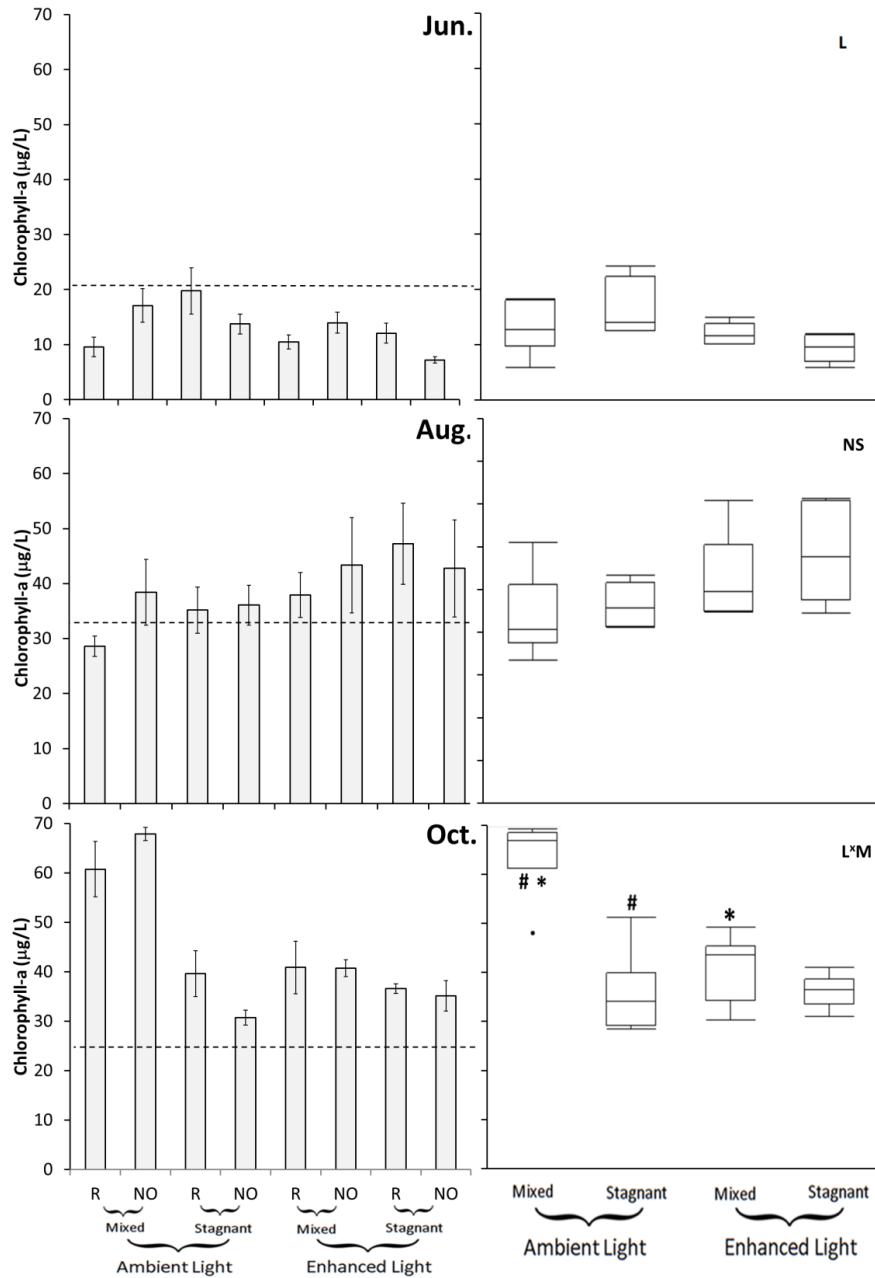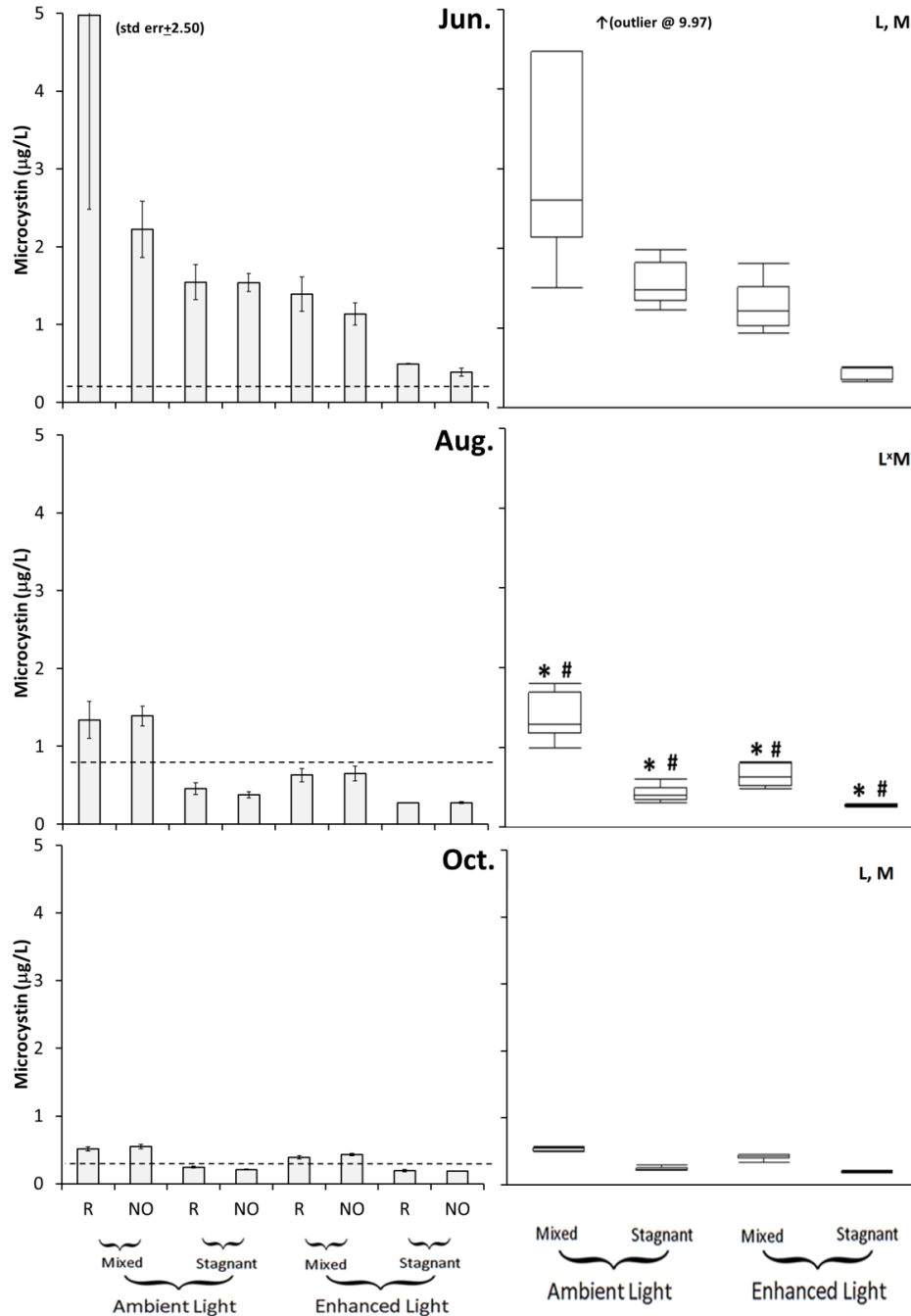
Figures

Figure 1. Mean chlorophyll-a levels (+ SE) for each treatment during experiments performed in June, August and October. *Rangia* treatments indicated by R, non-*Rangia* treatments indicated by NO. Initial values (day 0) are indicated by the dotted horizontal line. Box and whisker plots show median, IQR, and range for data pooled across the grazer treatments. Statistically significant factors indicated by initial in the top right corner of each plot (M=mixing, L=light, NS=no significant factors). Interactive effects in the two-way ANOVA are indicated by 'x' (i.e., $L^x M$). Significant differences resulting from t-tests in the presence of interactive effects are indicated by *, #. Statistical summaries are given in Table-3.
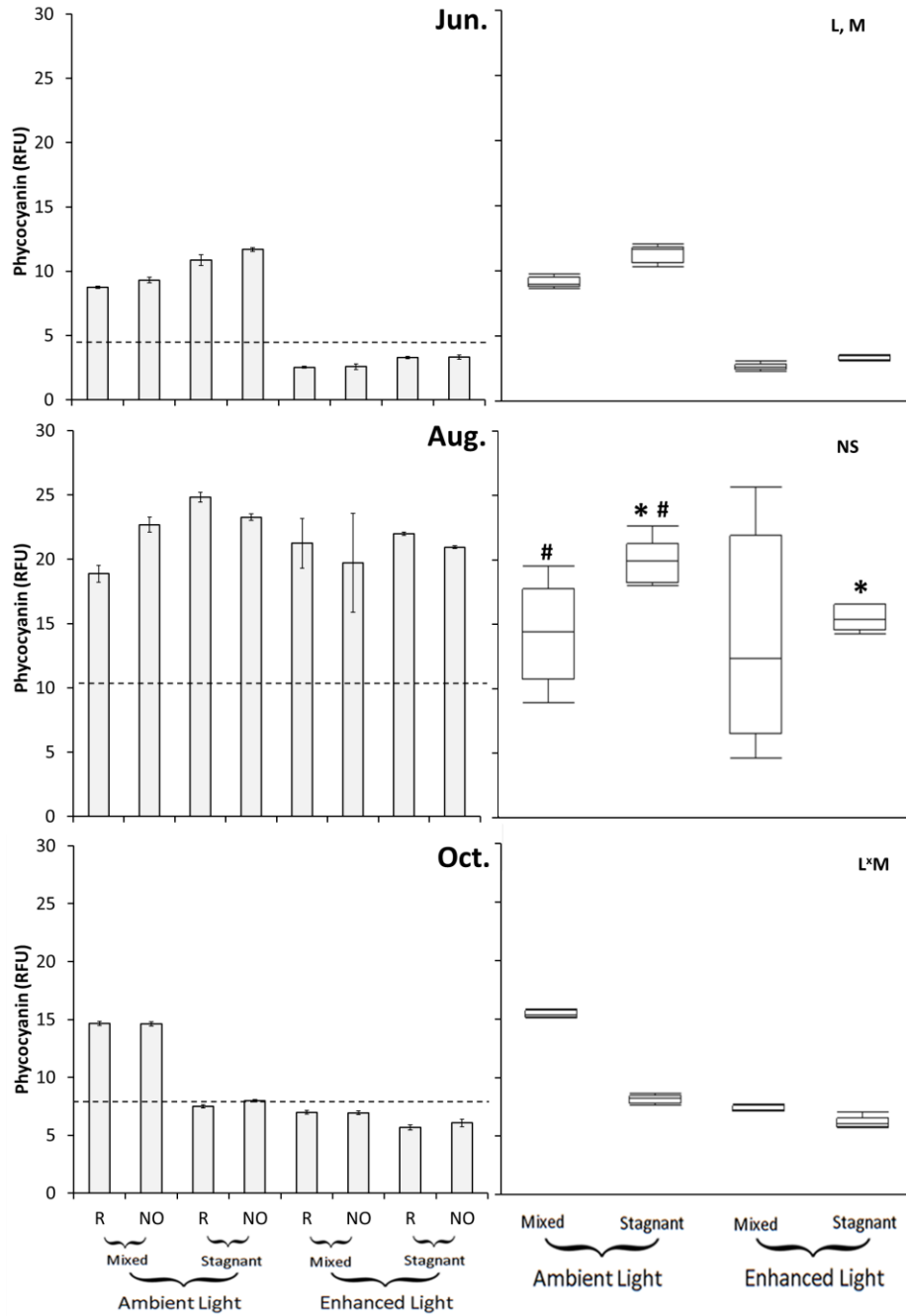
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Figure 2. Mean microcystin levels ($\pm$ SE) for each treatment during experiments performed in June, August and October. *Rangia* treatments indicated by R, non-*Rangia* treatments indicated by NO. Initial values (day 0) are indicated by the dotted horizontal line. Box and whisker plots show median, IQR, and range for data pooled across the grazer treatments. Statistically significant factors indicated by initial in the top right corner of each plot (M=mixing, L=light, NS=no significant factors). Interactive effects in the two-way ANOVA are indicated by 'x' (i.e., L^{x}M). Significant differences resulting from t-tests in the presence of interactive effects are indicated by *, #. Statistical summaries are given in Table-3.
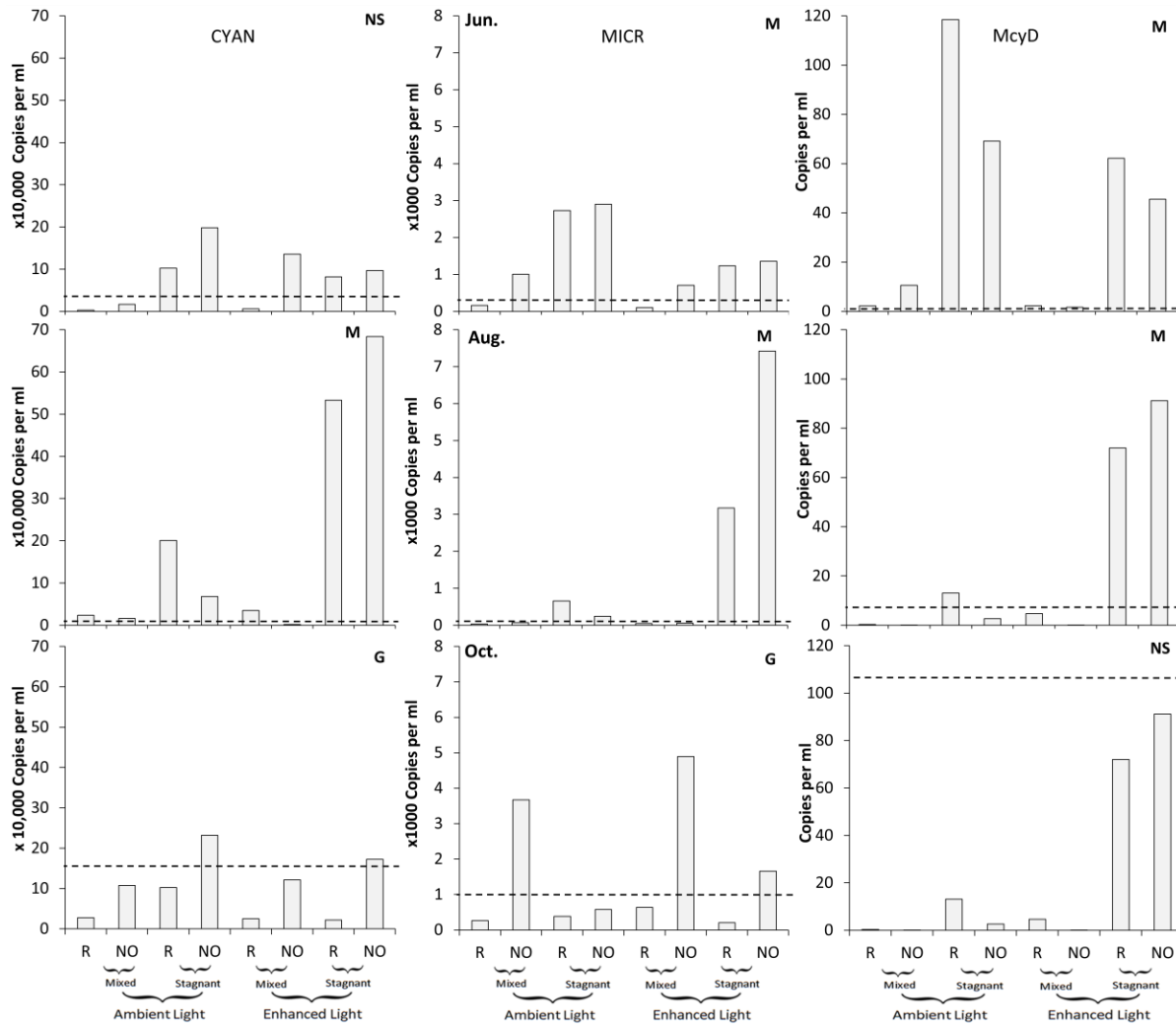
Figure 3. Mean phycocyanin levels ($\pm$ SE) for each treatment during experiments performed in June, August and October. *Rangia* treatments indicated by R, non-*Rangia* treatments indicated by NO. Initial values (day 0) are indicated by the dotted horizontal line. Box and whisker plots show median, IQR, and range for data pooled across the grazer treatments. Statistically significant factors indicated by initial in the top right corner of each plot (M=mixing, L=light, N=no significant factors). Interactive effects in the two-way ANOVA are indicated by 'x' (i.e., L^xM). Significant differences resulting from t-tests in the presence of interactive effects are indicated by *, #. Statistical summaries are given in Table-3.

Figure 4. Final values for gene copies of cyanobacteria (CYAN), *Microcystis* (MICR), and microcystin (mcyD) for each treatment during each experiment performed during June, August and October. Initial values indicated by the horizontal dotted line. *Rangia* treatments indicated by R, non-*Rangia* treatments indicated by NO. Statistically significant factors indicated by initial in the top right corner of each plot (M=mixing, L=light, G=grazers, N=no significant factors).
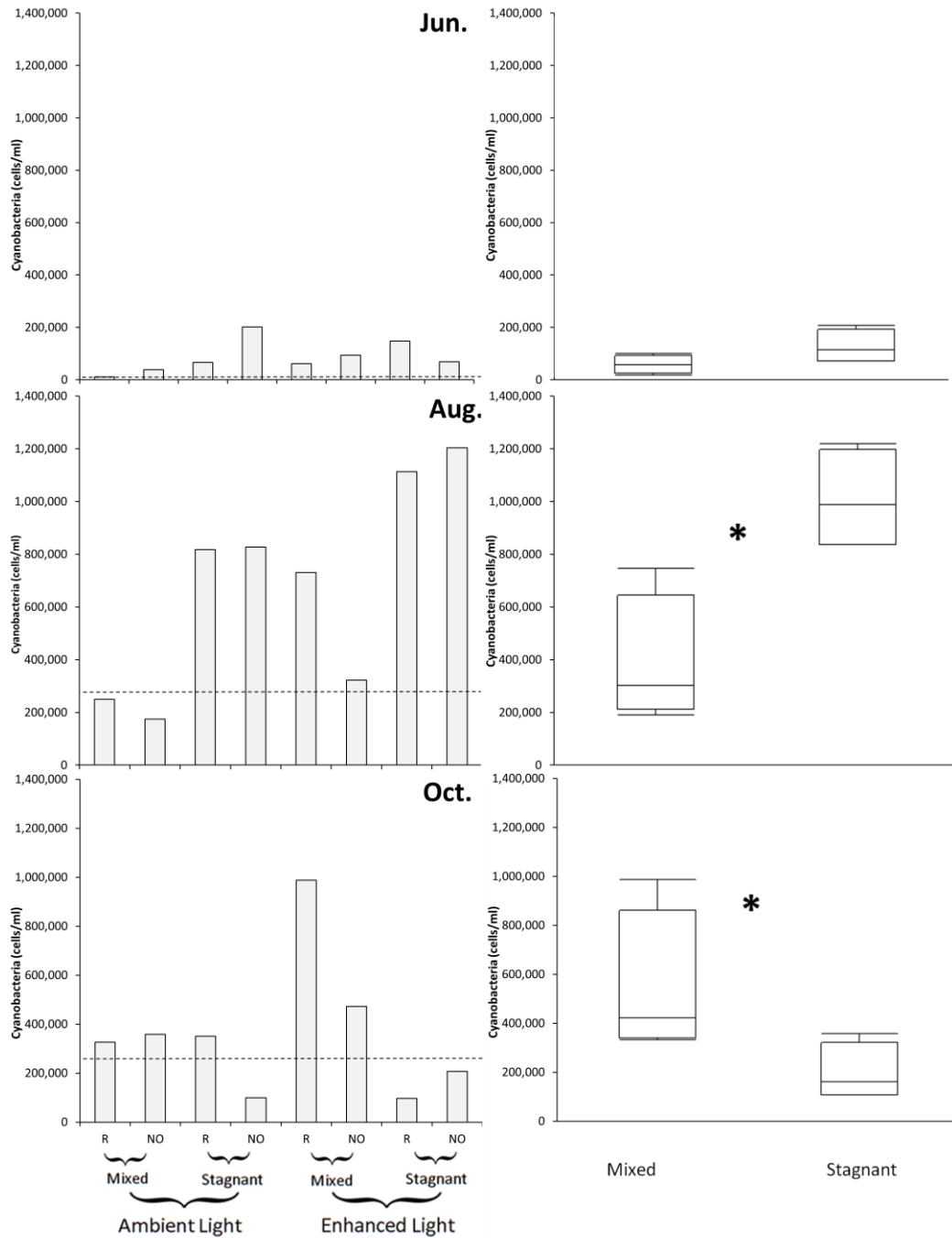
Figure-5. Final values for cyanobacteria cell densities for each treatment during each experiment performed during June, August and October. Initial values (day 0) indicated by the dotted horizontal line. *Rangia* treatments indicated by R, non-*Rangia* treatments indicated by NO. Box and whisker plots for each month indicate median, IQR, and range for all mixed treatments and all stagnant treatments. Significant effect of mixing indicated by *.
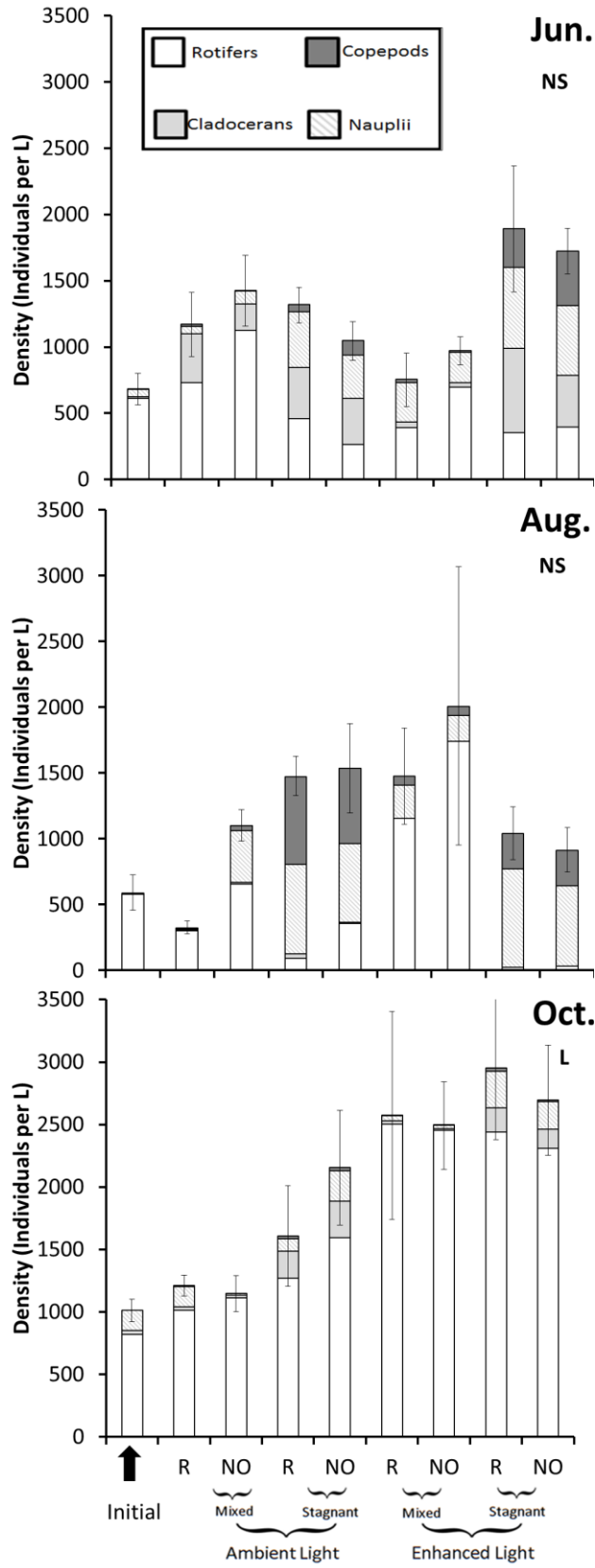
Figure 6. Final zooplankton abundance ($\pm$ SE) in each treatment for each experiment performed during June, August and October. Columns are divided by species. *Rangia* treatments indicated by R, non-*Rangia* treatments indicated by NO. Initial values shown by the far left column in each plot. Statistically significant factors indicated in the top right corner of each plot. (M=mixing, L=light, G=grazers, N=no significant factors). Statistical summaries in Table-3.
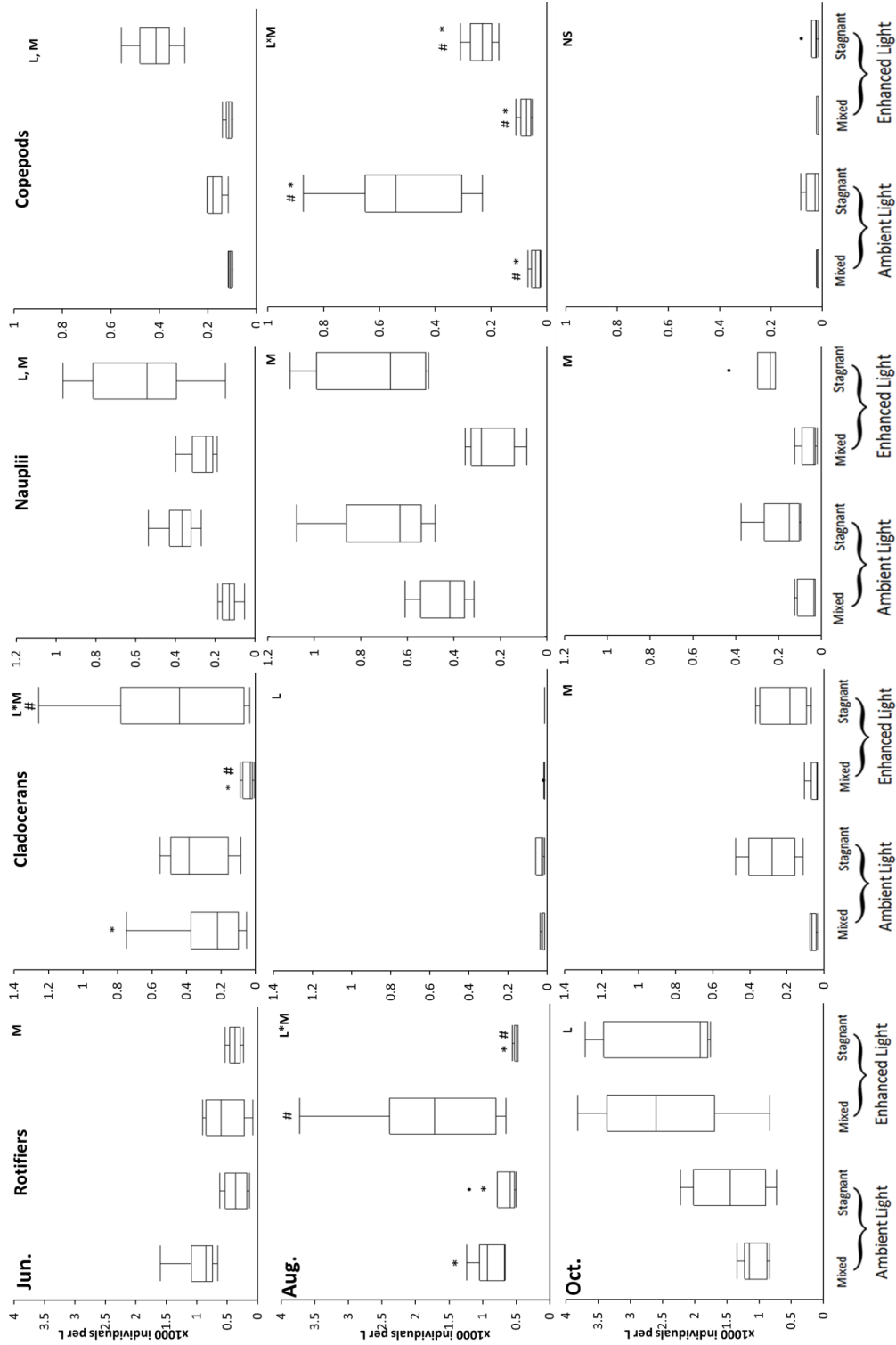
Figure 7. Box and whisker plots showing the median, range and IQR of treatment zooplankton abundances during mesocosm experiments in June, August and October. Data pooled across *Rangia* and non-*Rangia* treatments. Statistically significant effects are shown at the top right of each plot (M=mixing, L=light, G=grazers, NS= no significant effects). Location of significant differences in the presence of interactive effects are indicated by *, #. Statistical summaries provided in Table 3.
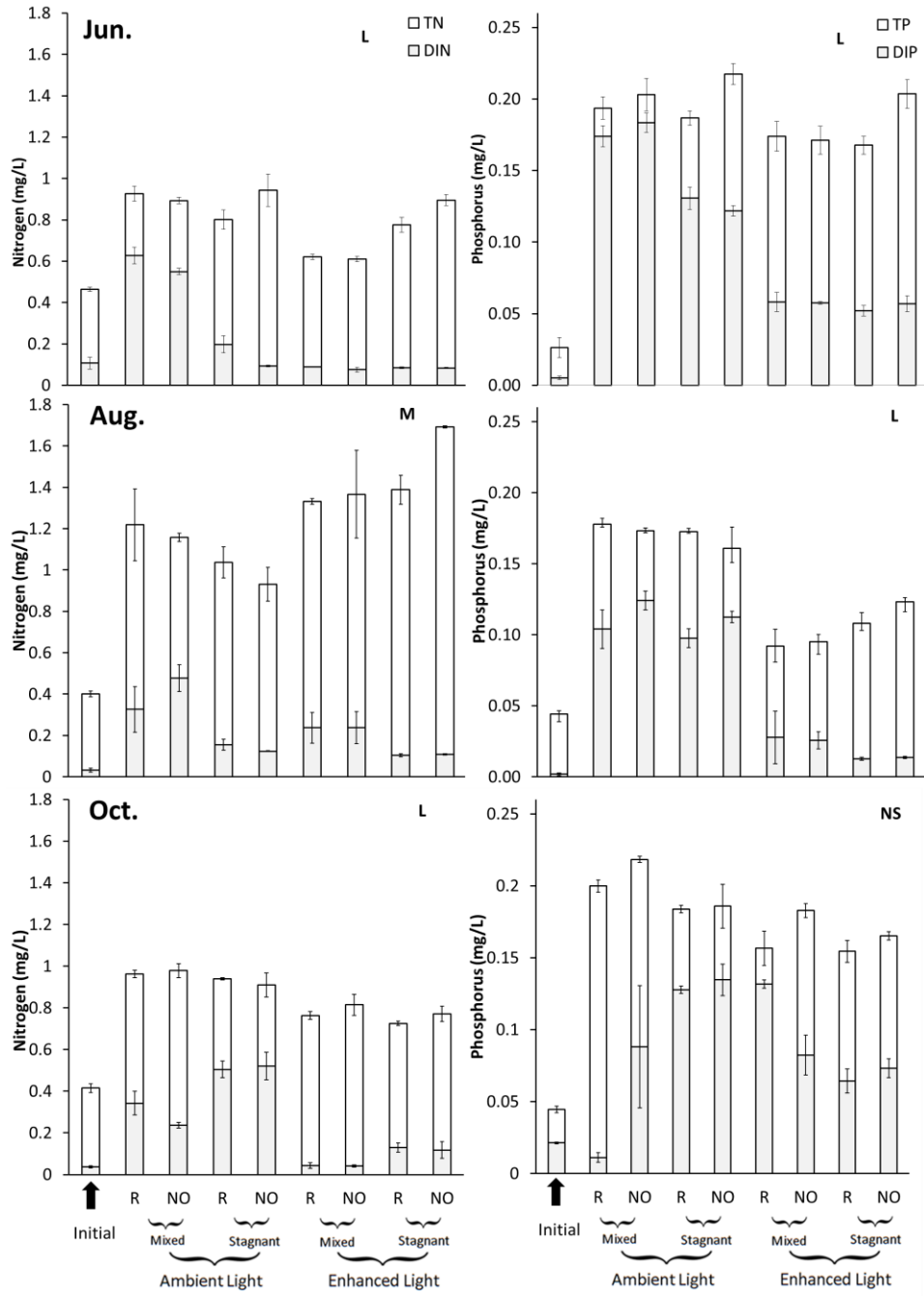
Figure 8. Nutrient concentrations ($\pm$ SE) in each treatment during experiments conducted during June, August and October. Nitrogen shown in column at left, phosphorous in the right column. Each plot shows total nutrients (unshaded) and dissolved inorganic nutrients (shaded). Initial values indicated by the far left column within each plot. *Rangia* treatments indicated by R, non-*Rangia* treatments indicated by NO. Statistically significant factors indicated in the top-left corner of each plot (L=light, M=mixing, NS= no significant effects).

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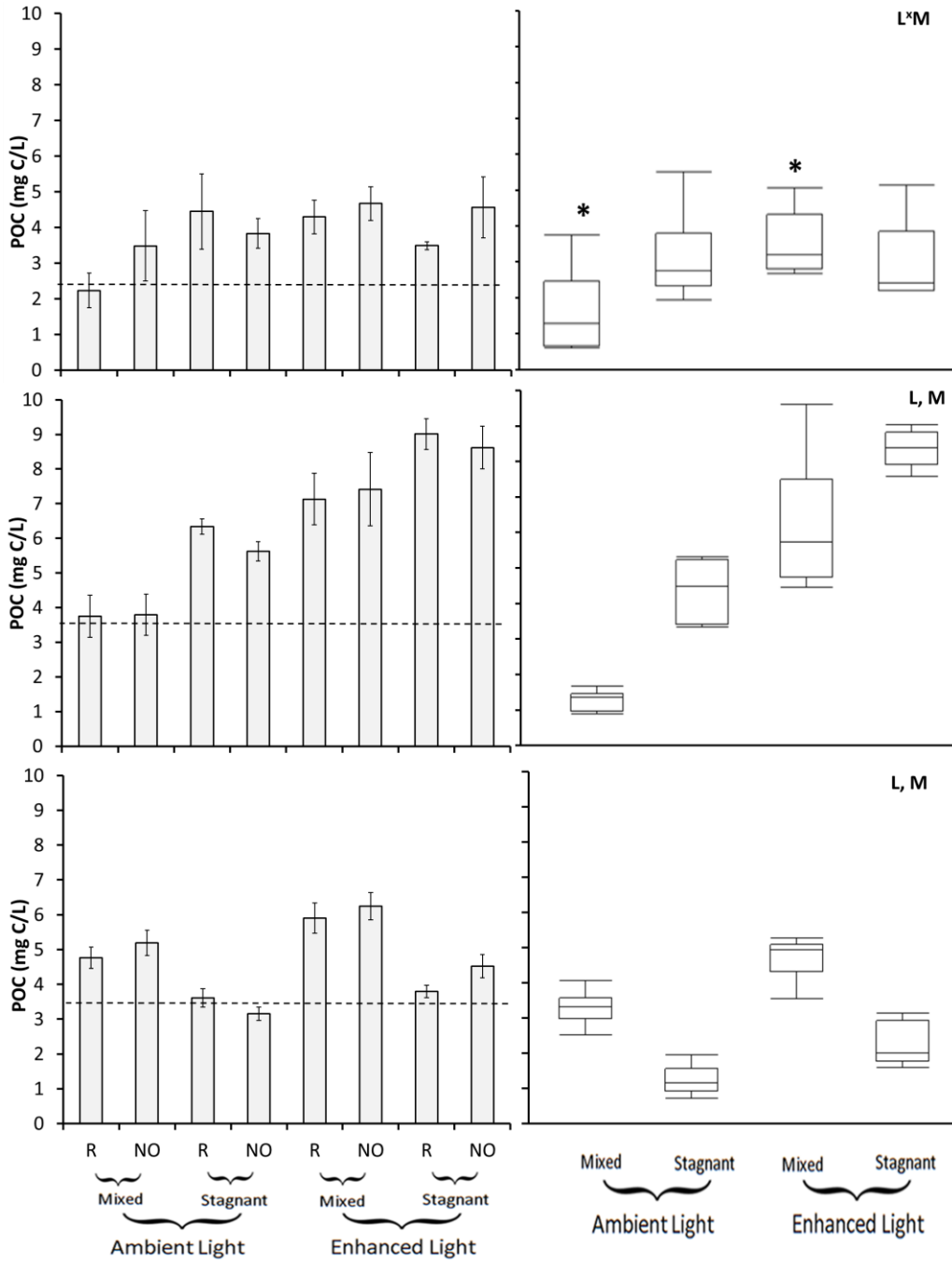
Figure 9. Mean particulate organic carbon levels ($\pm$ SE) for each treatment during experiments performed in June, August and October. *Rangia* treatments indicated by R, non-*Rangia* treatments indicated by NO. Initial values (day 0) are indicated by the dotted horizontal line. Box and whisker plots show median, IQR, and range for data pooled across the grazer treatments. Statistically significant factors indicated by initial in the top right corner of each plot. Location of significant differences in the presence of interactive effects are indicated by *, #.
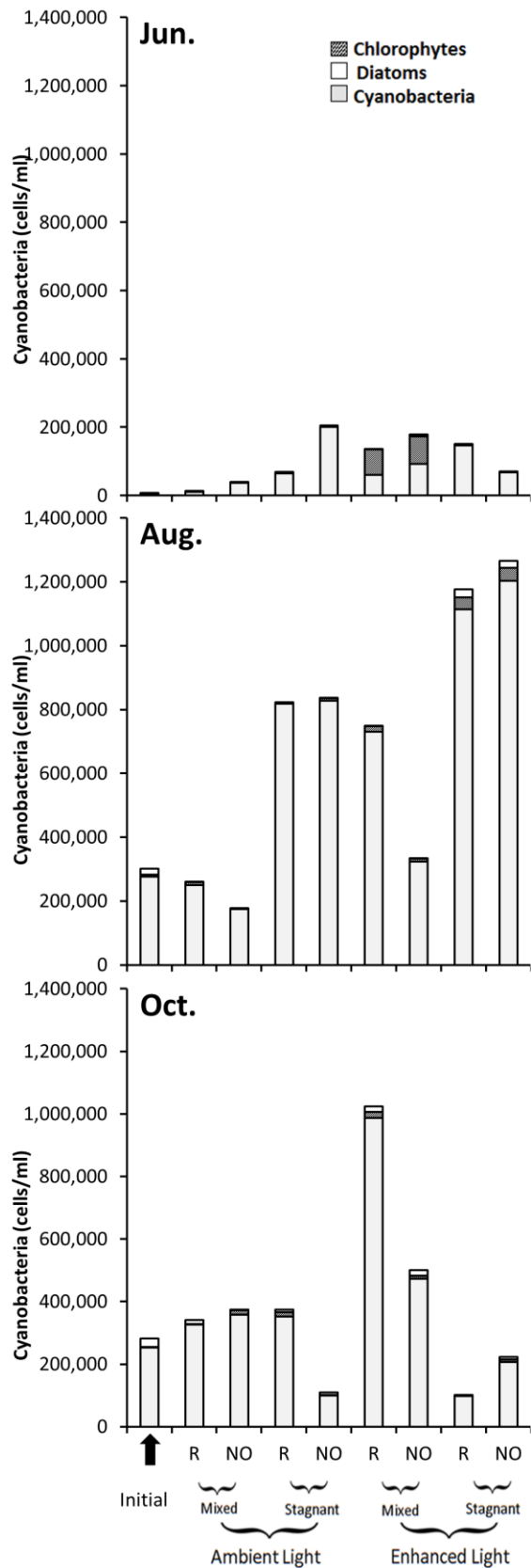
Figure 10. Final phytoplankton abundance ($\pm$ SE) in each treatment for each experiment performed during June, August and October. Columns are divided by species. *Rangia* treatments indicated by R, non-*Rangia* treatments indicated by NO. Initial values shown by the far left column in each plot.