The effects of two common urban pollutants and increased nutrient concentrations on algal biofilms in a Midwestern stream

BIOS 35502: Practicum in Environmental Field Biology

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Abstract

Biofilms serve as important biological filters by removing nutrients from stream ecosystems. Copious amounts of nutrients in bodies of water often lead to hypoxic events and decreases in biodiversity and fishery yields. However, there is evidence that urban pollutants have negative effects on biofilm growth. This study examined the combined effect of nutrient and urban pollutants on biofilms in a forested Midwestern stream. Results from chlorophyll *a* analysis revealed that there was no effect of urban pollutants on biofilm growth nor was there an interaction between nutrients and pollutants.

Introduction

Biofilm, commonly called periphyton or *Aufwuchs*, is an overarching term that describes the collection of heterotrophic and autotrophic microorganisms (i.e. algae, bacteria, and fungi) and an extracellular polymeric substance growing on inorganic and organic surfaces (Azim et al. 2005; Tank et al. 2006). Biofilms are critical to ecosystem function as they are key in nutrient absorption and spiraling, organic carbon cycling, primary production, and respiration (Steinman et al. 2006; Bunch and Bernot 2010; Peterson et al. 2001; Reisinger et al. 2016).

Headwater streams are streams at the top of the watershed where respiration rates are typically higher than production rates (Vannote et al. 1980). Biofilms in headwater streams are major contributors to primary production and the base of the food chain; therefore, the health of the biofilm can help determine the health of the stream (Meyer 1994). In addition, biofilms in headwater streams can act as filters, removing the nutrient pollution from the water before it travels downstream (Alexander et al. 2000; Peterson et al. 2001; Tank et al. 2006). These streams are highly connected to adjacent terrestrial systems, as recognized in both classic and recent studies (Peterson et al. 2001; Vannote et al. 1980), meaning pollution on land can quickly become pollution within water. As a result, it is essential to understand how headwater streams, such as those in the agricultural Midwestern United States, process nutrients prior to downstream export so that dead zones can be prevented and local stream health can be protected.

Excess nutrients from agricultural fields and wastewater leach into streams and rivers, causing immediate problems within the stream ecosystem and problems for downstream ecosystems (Tank et al. 2006). Nitrogen in particular has often been found to result in eutrophication and loss of species diversity (Carpenter et al. 1998; Tank et al. 2006). When nutrient-rich water travels downstream, it accumulates in watershed basins like the Gulf of

Mexico or the Great Lakes where it can cause large hypoxic “dead zones” (Vitousek et al. 1997; Carpenter el al. 1998; Rabalais et al. 2002; David et al. 2010). These “dead zones” have extremely low oxygen levels and cause both environmental and economic problems for the regions, such as disturbing the natural flora and fauna and decreasing fisheries (Vitousek et al. 1997; Rabalais et al. 2002).

Though agricultural lands are a substantial source of excess nutrients to stream systems, many streams contain inputs from urban environments as well. Urban inputs include pharmaceuticals and personal care products, some of which can be nearly ubiquitous within streams, such as caffeine (Bunch and Bernot 2010). While some studies have examined the effects of either nutrients or pharmaceuticals on algal biofilms, very few have looked at their combined interactions on biofilms (Rosi-Marshall et al. 2013). However, it is likely that urban pollutants are found in unusually high concentrations in streams near agricultural areas, rather than streams near cities, due to the use of septic tanks (Bunch and Bernot 2010). This is means that areas with higher nutrient inputs also are more likely to have higher levels of urban pollutants.

Biofilms in headwater streams are vitally important in removing excess nutrients, but there is research that indicates urban pollutants have the potential to negatively affect biofilm’s growth and its ability to act as a filter (Alexander et al. 2000; Peterson et al. 2001; Tank et al. 2006; Rosi-Marshall et al. 2013). For this reason, this study analyzed how the biomass of biofilm changed in the presence of commonly found urban pollutants with and without the addition of nutrients. Chlorophyll *a* concentrations were used as an indicator for biofilm biomass (i.e. health), which is a common procedure for autotrophic biofilms (Tank et al. 2006; Steinman et al. 2006). I hypothesized that the nutrients would increase the biofilm’s biomass, while the

pharmaceuticals would decrease the biomass, as shown in previous studies (Sanderson et al. 2009; Rosi-Marshall et al. 2013).

Materials and Methods

Sample Site

Sampling took place at the University of Notre Dame Environmental Research Center (UNDERC East), a property consisting of 7500 acres within the Ottawa National Forest on the border of Wisconsin and the Upper Peninsula of Michigan (46° 13’ N, 89° 32’ W). It contains 26 aquatic habitats which make up approximately 16% of the total area of the property. The watershed of the streams and creeks is nearly completely forested. The sampling location was Tenderfoot Creek, which starts at Tenderfoot Lake and flows north to Lake Superior (Figure 1).

The sample site had an average flow rate of 0.40 m/s and a depth of 0.43 m. The bottom of the stream consisted of mixed substrate with fine sediment, gravel, and cobble. Direct sunlight reached the stream site for the majority of the daylight hours. Photosynthesis was occurring at the bottom of the stream during the time of the experiment, as shown by biofilm growing on inorganic benthic matter and the presence of macrophytes. This indicated ample sunlight was able to reach the bottom of the stream.

Pharmaceutical Diffusing Substrata

Pharmaceutical diffusing substrata (PhaDS) bioassays were constructed based on previous methodology for nutrient diffusing substrates (Tank et al. 2006) as well as an adjusted methodology for PhaDS (Bunch and Bernot 2010). Previously, nutrient diffusing substrate bioassays have been used to determine if the growth of biofilms in freshwater ecosystems was

nutrient limited. In the case of this experiment, nutrient limitation was tested in combination with the potential effects of urban pollutants. The PhaDS were created using 1 oz Polycon cups with lids that had a 0.022 m diameter circular hole through the top. The cups held agar which was amended with each nutrient (nitrogen and/or phosphorous) and/or urban pollutant (caffeine and/or ibuprofen) treatment (Table 1). The control PhaDS contained agars with nutrients alone, pollutants alone, or neither nutrient nor pollutant (Table 1). Treatments and controls were replicated 4 times for a total of 60 samples. The nutrients used were nitrogen (N) and phosphorous (P), as these are commonly found in streams and known to be limiting factors of biofilm growth (Tank et al. 2006). The pollutants used were caffeine (C) and ibuprofen (I), which are prevalent in headwater streams (Bunch and Bernot 2010; Li et al. 2015). Small fritted glass discs (0.027 m in diameter) were placed on top of the agar in order to provide a solid surface on which the biofilm could grow. The cups were organized using a random number generator and glued onto metal baking sheets using rubber silicon glue and doubly secured with zip-ties. The baking sheets were staked to the bottom of Tenderfoot Creek in a run, allowing for constant flow over the cups (Figure 2). The cups were left to incubate in the stream 17 days, from June 17 through July 3, 2016. This time period was long enough to ensure biofilm growth and short enough to prevent sloughing (Francoeur et al. 1999). Since temperature is often the limiting factor in biofilm growth (Francoeur et al. 1999), summer was the ideal season for the experiment. At the end of the sampling period, PhaDS were removed from the stream, and the glass discs were immediately placed in individual plastic bags, frozen at -20°C, and kept out of light.

Biomass Analysis

Biofilm biomass was measured using chlorophyll *a* concentration (Steinman et al. 2006). In a dimmed room, the growth on each glass disc was brushed off with a toothbrush and water, creating a biofilm slurry. The resulting slurry from each disc was then individually filtered through pre-weighed 47 mm glass fiber filters (Whatman GF/F, GE Healthcare Life Sciences, Piscataway, NJ, USA).

In order to determine chlorophyll *a* concentrations, the filters were frozen for 24 hours at -20°C, and chlorophyll *a* was extracted with basic methanol. Using the lowest volume necessary to completely cover the filters, the chlorophyll *a* was extracted in an opaque film canister. The precise amount of basic methanol used was recorded. The samples remained in a refrigerator for 24 hours. Chlorophyll *a* concentrations were measured on a fluorometer (Turner Designs, Sunnyvale, CA, USA), using standards that were verified with a fluorescence spectrophotometer (Spectronic Instruments, Rochester, NY) following previous methodologies (Wetzel and Likens 2000).

Statistics

A two-way analysis of variances (ANOVA) was performed with the pollutant and nutrient combinations as the factors. After, a Shapiro-Wilk normality test was used to determine if a transformation or a non-parametric test was necessary. While a logarithmic transformation did not result in normal distribution, Levene's Test for homogeneity of variance revealed that the variance was homogenous ($p > 0.1$), and so the issue of normality was dismissed. The Tukey Honest Significant Difference (HSD) test was used as a post hoc test to determine the differences among treatments and controls.

Results

Analysis of the biofilms indicated that the nutrients had an effect on the density of chlorophyll *a*. The amendment of nutrients to the PhaDS had a positive influence on the chlorophyll *a* density ($F_{3,45} = 33.629$, $p < 0.001$) (Figure 3A, 3B). The inclusion of nitrogen in the agar resulted in the highest chlorophyll *a* density compared to the other treatments and controls (Tukey HSD, $p < 0.001$) (Table 2; Figure 4A). The phosphorous amendment did not result in higher chlorophyll *a* densities than the controls (Tukey HSD, $p > 0.05$) (Figure 4A). Amendments of pollutants neither increased nor decreased the chlorophyll *a* density ($F_{3,45} = 2.777$, $p > 0.05$) (Figure 3A). Nitrogen amendments resulted in chlorophyll *a* densities twice as large as those from the phosphorous treatments (N = 6606 ± 269 $\mu g \bullet m^{-2}$; P = 2845 ± 340 $\mu g \bullet m^{-2}$) (Table 2).

Discussion

Nutrient-amended substrates enriched chlorophyll *a* production and, therefore, biofilm density, supporting this study's hypothesis. When looking strictly at the nutrient additions, it is clear that the stream was nitrogen limited, as the addition of nitrogen into the PhaDS produced more biofilm (Figure 4A). The amendment of phosphorous to the agars did not have an effect on the chlorophyll *a* concentration, revealing that the stream already has generous amounts of phosphorous present (Figure 4A). This result is supported by previous research that found streams are nutrient limited when the canopy is open (Johnson et al. 2009) and phosphorous limited when the watershed has a high agricultural presence (Francoeur 2001; Reisinger et al. 2016). Unlike in agricultural areas, the watersheds around UNDERC are most likely low in pollutants and nutrients since they are on private property and surrounded by the Ottawa

National Forest. Taking this into consideration, previous studies support the finding that this protected Midwestern stream is nitrogen limited (Sanderson et al. 2009).

The introduction of urban pollutants had no effect on the biofilm growth (Figure 4B), rejecting my original hypothesis and previous research that examined similar chemical water pollutants (Rosi-Marshall et al. 2012; Lawrence et al. 2012). There is a lack of evidence to support that caffeine and ibuprofen harm biofilms (Figure 3A and 3B). Despite increases in urban development and personal care products within the environment, urban pollutants like ibuprofen and caffeine, on their own, will not cause lower biomasses in the coming years (Wellesley et al. 2015). If the results of this experiment showed that the two urban pollutants negatively affected biofilm growth, the implications would be that urban pollutants lead to a biofilm that is less healthy and therefore less able to process nutrients. These increasingly unhealthy biofilms would lead to larger and more frequent hypoxic events in watershed basins. However, this research provides evidence this will not be the case.

One limitation to the current research presented in this paper is that only autotrophic biofilms were examined. Heterotrophic biofilms are also affected by nutrient concentration changes, and it is likely that they would be affected by urban pollutants as well (Johnson et al. 2009). Currently, data from this project are being processed and plan to examine both the heterotrophic and autotrophic biofilm growth through organic biomass using an ash-free dry mass method. It is expected that there will be similar patterns of increased biomass in the nitrogen treatments and no difference in the pollutant treatments, which will corroborate the current chlorophyll *a* data.

Further experimentation and analyses of the effects of typical urban pollutants on biofilms are needed. The information within this field is minimal, and the amount of pollutants in

water supplies is expected only to grow (Carpenter et al. 1998; Jekel et al. 2013). In addition, very few regulations exist to control the amount of pharmaceuticals and other chemicals allowed to enter waterways, and water treatment plants do not have methods to remove most pharmaceutical and personal care product pollutants (Lawrence et al. 2012; Edwards and Kjellerup 2013). It is possible that different pollutants or combinations of pollutants could be detrimental to biofilm growth. A study completed by Rosi-Marshall et al. found that a combination of multiple pollutants (including antibiotics, antihistamines, and caffeine) resulted in lower biofilm growth (2012). Considering that biofilms are composed of both algae and bacteria (Azim et al. 2015), it is probable that antibiotics have a strong effect on their growth. Likewise, there is potential that there are long term effects of urban pollutants on biofilms, but due to time restraints, only a short term experiment was conducted. Future experiments that look at biofilms over a long time scale and with more combinations of pharmaceuticals should be conducted. It is important to fully understand the potential implications of urban pollutants on the biofilms as they are the basis of many stream food chains and act as critical filters for nutrients in streams (Bunch and Bernot 2010; Peterson et al. 2001; Reisinger et al. 2016).

Understanding the consequences of urban pollutants on biofilms is vitally important to getting a clear picture of how to prevent hypoxia and promote stream health, which in turn promotes biodiversity and the economics surrounding fisheries (Vitousek et al. 1997; Carpenter et al. 1998; Alexander et al. 2000; Rabalais et al. 2002; David et al. 2010). With climate change and urbanization, these urban pollutants will only increase and the biofilms will become more critical for ecosystem health (Carpenter et al. 1998; Wellesley et al. 2015). Research from this study implies that ibuprofen and caffeine do not have an effect on biofilm growth, but more research is needed in the field in order to determine which pharmaceuticals or pollutants have

effect on the growth of biofilm in order to implement appropriate controls on the pollutant inputs.

Acknowledgments

I would like to thank Sara Benevente for being my mentor as well as guiding and assisting me throughout the process of this experiment. In addition, immense thanks is owed to Esmeralda Martínez and Jennifer Lam for helping in the initial set up of the experiment. I would also like to thank Brittany Hanrahan and Brittni Bertolet in assistance in data analysis and Catherine McQuestion for help with statistics. Likewise, valuable advice was given by Dominic Acri and Audrey Thellman during the final portion of the project. Lastly, this entire experiment would not have been made possible were it not for the generous Hank family and the Bernard J. Hank Family Endowment which funded my research and allowed me to stay at UNDERC this summer, so I am extremely grateful to the Hank family for giving me this opportunity.

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Tables and Figures:

Table 1. Approximate concentrations of agar for each pharmaceutical diffusing substrata bioassay. A total of 60 bioassays were constructed, with 4 replicates per treatment and control.

		Nutrients			
		Nitrogen	Phosphorous	Nitrogen + Phosphorous	Control
Pollutants	Ibuprofen	N: 50.6 g/L I: 34 mg/L	P: 60.8 g/L I: 34 mg/L	N: 50.6 g/L P: 60.8 g/L I: 34 mg/L	I: 34 mg/L
	Caffeine	N: 50.6 g/L C: 65 mg/L	P: 60.8 g/L C: 65 mg/L	N: 50.6 g/L P: 60.8 g/L C: 65 mg/L	C: 65 mg/L
	Ibuprofen + Caffeine	N: 50.6 g/L I: 34 mg/L C: 65 mg/L	P: 60.8 g/L I: 34 mg/L C: 65 mg/L	N: 50.6 g/L P: 60.8 g/L I: 34 mg/L C: 65 mg/L	I: 34 mg/L C: 65 mg/L
	Control	N: 50.6 g/L	P: 60.8 g/L	N: 50.6 g/L P: 60.8 g/L	-

Table 2. Chlorophyll *a* concentrations from glass discs of each treatment and control (n=4 replicates) before logarithmic transformation. Values are means and standard error ($\mu g \bullet m^{-2}$).

		Nutrients			
		Nitrogen	Phosphorous	Nitrogen + Phosphorous	Control
Pollutants	Ibuprofen	6606 ± 269	2845 ± 340	8787 ± 1158	2935 ± 404
	Caffeine	6144 ± 914	3440 ± 242	100017 ± 2716	2313 ± 371
	Ibuprofen + Caffeine	7469 ± 894	3725 ± 599	8920 ± 931	DNE
	Control	6384 ± 1367	2737 ± 309	7902 ± 1759	3096 ±203

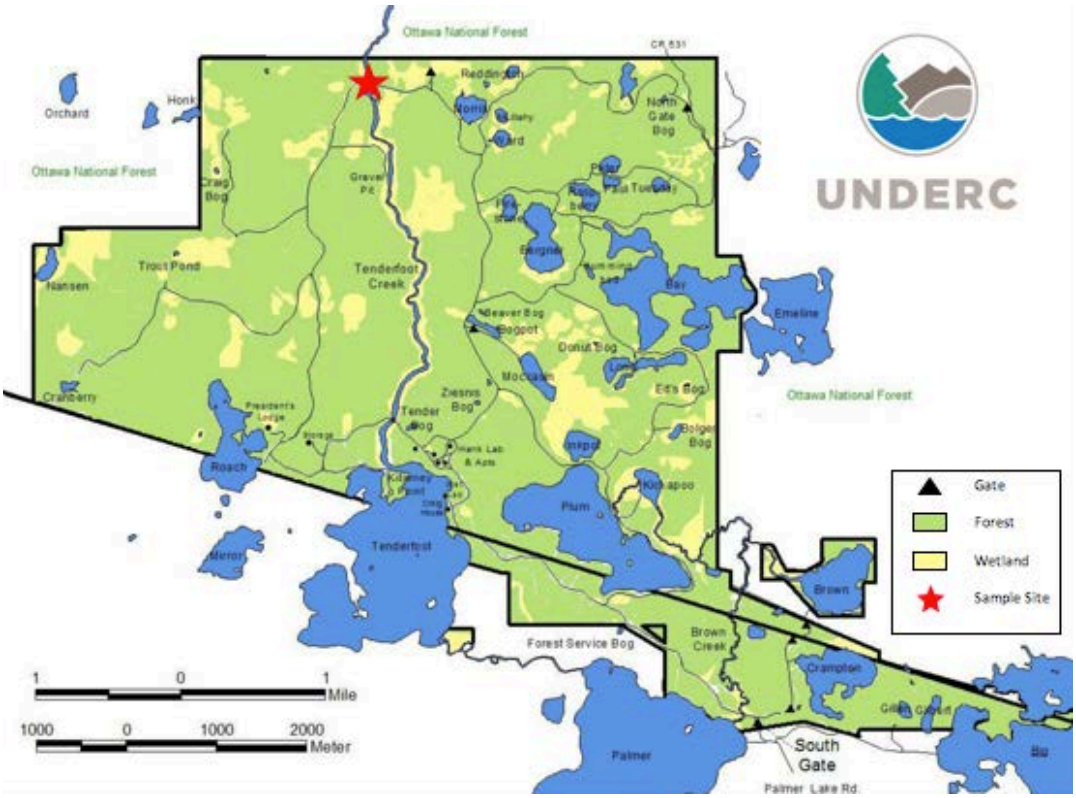

Figure 1. Map of the University of Notre Dame Environmental Research Center (UNDERC) property. The property is located in the Ottawa National Forest on the border of Northern Wisconsin and the Upper Peninsula of Michigan and is the location for the study.

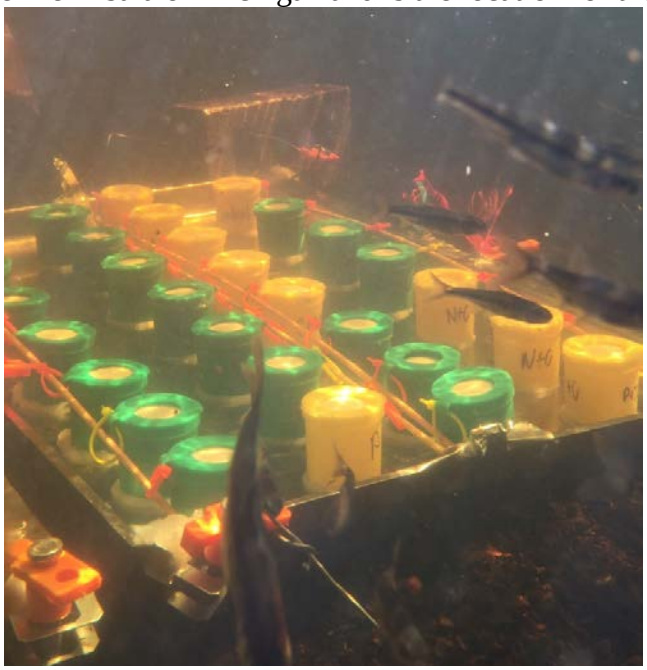

Figure 2. Pharmaceutical diffusing substrata bioassays (N=60) placed in Tenderfoot Creek at UNDERC during incubation for 17 days in the summer of 2016.

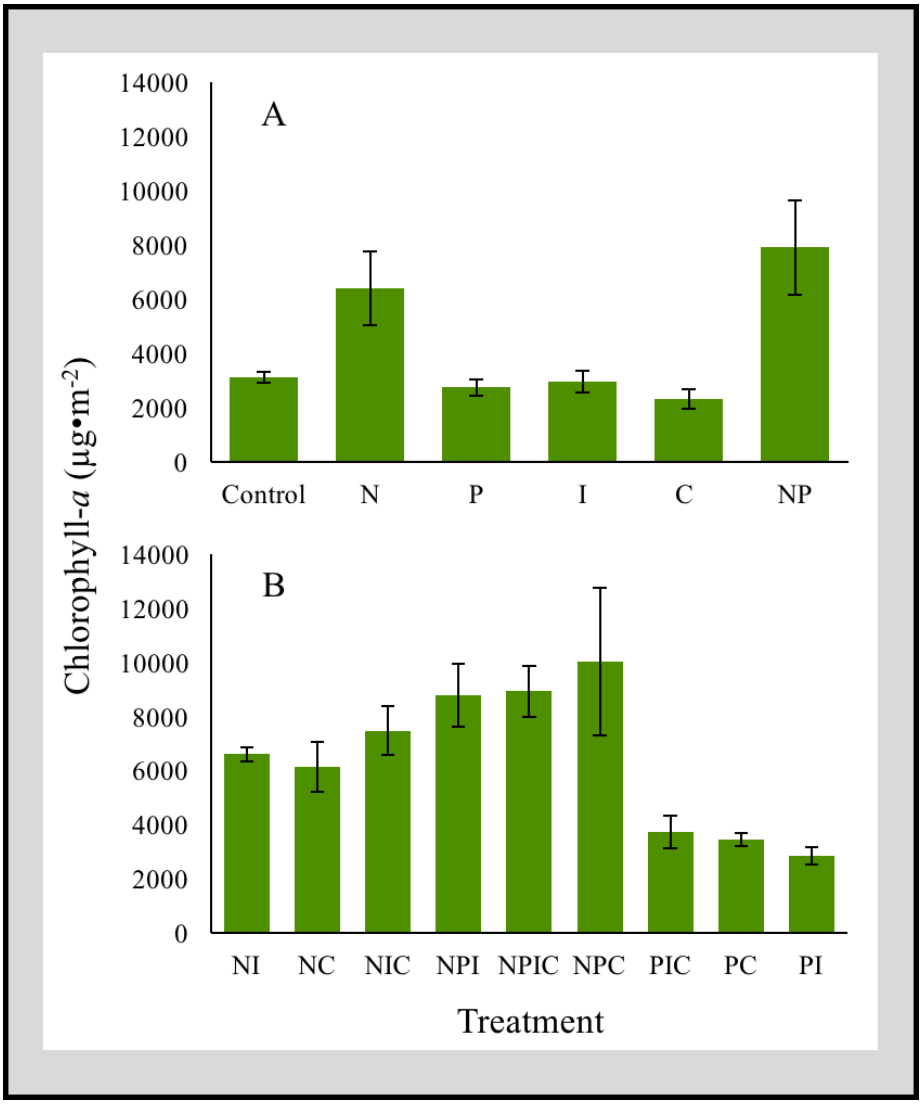

Figure 3. Chlorophyll-*a* density of bioassays for (A) controls and (B) nutrient and pollutant treatments. Values are means with standard error bars (n = 4 replicates). A two-way ANOVA comparing the experimental (i.e. nutrient and pollutant) treatments revealed that nutrients had significant effects on chlorophyll-*a* density. There was no significant interaction between nutrients and pollutants (nutrient treatments, $F_{3,45} = 33.629$, $p < 0.001$; pollutant treatments, $F_{3,45} = 2.77$, $p > 0.05$; interaction $F_{8,45} = 0.522$, $p > 0.05$).

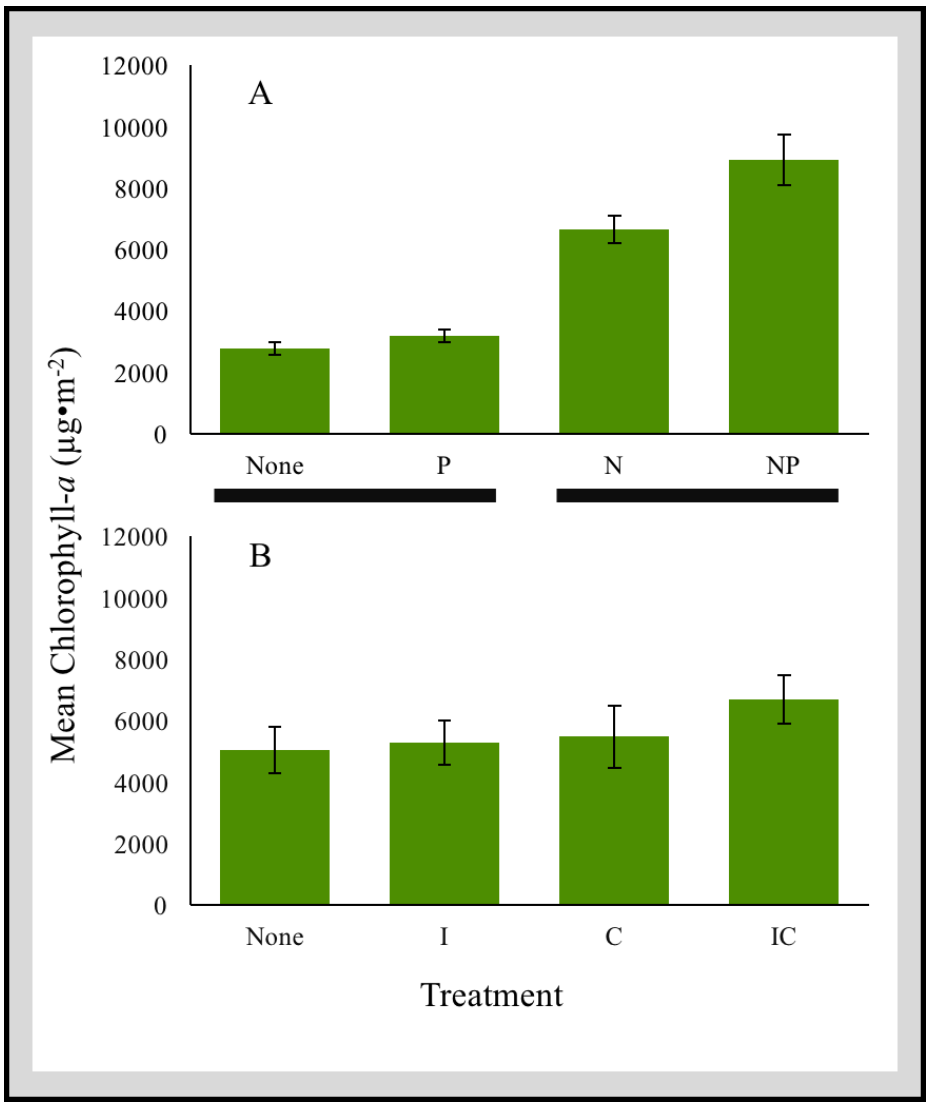

Figure 4. The mean chlorophyll-*a* densities for the bioassays of pharmaceutical diffusing substrata different in the (A) nutrient and (B) pollutant treatments. Groups of means connected by horizontal lines are not significantly different from one another (Tukey HSD test, $p < 0.05$). No significant difference found between pollutant treatments (ANOVA, $F_{3,45} = 2.77$ $p > 0.05$). Values shown are means with standard error bars (n = 28 for IC and n = 32 for all others). Nitrogen treatments resulted in higher chlorophyll *a* concentrations than all other treatments.