INTERACTING EFFECTS OF LIGHT AND PHARMACEUTICALS ON STREAM BIOFILMS

YASHOMA BOODHAN

Cary Institute of Ecosystem Studies, Millbrook, NY 12545 USA

MENTOR SCIENTISTS: DRS. EMMA ROSI-MARSHALL[1] AND ALEXANDER J. REISINGER[1]
[1] *Cary Institute of Ecosystem Studies, Millbrook, NY 12545 USA*

Abstract. Pharmaceuticals and personal care products (PPCPs) are detected in streams and water supplies worldwide, and are known to affect stream biofilms. The algae, bacteria, and fungi that make up biofilms are important contributors to stream ecosystems because they maintain water quality by regulating excess nutrients and serve as a food source for consumers. Although there has been recent research on the effects of PPCPs on biofilms in aquatic ecosystems, the cumulative effect of different levels of light intensity and a pharmaceutical mixture has not yet been examined. To investigate the interacting effects of light and a mixture of PPCPs, we conducted two experiments – one in artificial streams testing biofilm colonization on artificial substrates and one in a natural stream using pharmaceutical diffusing substrates (PhaDS) – and quantified the response of stream biofilms by examining gross primary production (GPP), community respiration (CR), algal pigment diversity, and chlorophyll-a (chl-*a*) concentrations. From the PhaDS experiment, we found a significant effect of light on GPP, and significant effects of light and pharmaceuticals on CR. We saw that pharmaceuticals increased CR in stream biofilms, which signifies reduced microbial activity. For both experiments, we saw a difference in chl-*a* concentrations between open and shaded streams, but in an opposite direction of our initial hypothesis. Chl-*a* concentrations in open versus shaded streams indicated that other factors, for example biofilm community composition, need to be considered. Our results indicate that pharmaceuticals and altered light intensities have the potential to change stream biofilm processes which can affect organisms that depend on biofilms for energy and the overall water quality of our freshwater streams.

INTRODUCTION

The global population is becoming increasingly urban, and the majority of humans currently live in urban areas (Grimm et al. 2008). As the urban population expands, water quality in urban streams is of increasing concern. Urbanization near bodies of water can change the physical, chemical, and biological conditions of stream ecosystems (Walsh et al. 2005, Chadwick et al. 2006). As areas become more urbanized, the amount of light received by stream ecosystems changes as well. In addition, urban streams typically display elevated concentrations of nutrients and contaminants (Kolpin et al. 2002, Chen et al. 2012). One class of contaminants commonly found in stream ecosystems is pharmaceuticals and personal care products (PPCPs). The effects of PPCPs on stream ecosystem function remains largely unknown, although an increasing body of research recognizes their adverse effects on stream biota (Cunningham et al. 2006, Rosi-Marshall and Royer 2012, Rosi-Marshall et al. 2013).

Compounds like veterinary drugs, diagnostic agents, food supplements, cosmetics, fragrances, and sun-screen are often grouped together as PPCPs (Ellis 2006), and are commonly found at low concentrations in surface waters (Kolpin et al. 2002). There are multiple pathways for these compounds to enter streams: effluent from waste water treatment plants, waste from manufacturing facilities, discharge from sewer overflows, and agricultural runoff (Ellis 2006, Silva et al. 2011, Sim et al. 2011, Rosi-Marshall and Royer 2012). The ecological effects of PPCPs can differ when they are tested singularly as opposed to in a mixture (Backhaus et al. 2011), and PPCPs tend to be detected in mixtures in the environment (Kolpin et al. 2002, Rosi-Marshall and Royer 2012). As urbanization, commercial activities, demand for medications, and concern for personal care, hygiene, and health increase, the concentrations and potential risks associated with PPCPs in water systems also increase (Ellis 2006).

The presence and combinations of PPCPs in stream ecosystems are alarming and can affect multiple components of streams, including invertebrate species and biofilm development (Hoppe et al. 2012, Rosi-Marshall and Royer 2012, Drury et al. 2013). Biofilms are matrices consisting of algae, bacteria, and fungi (Lock et al. 1984, Sabater et al. 2007, Férard 2013) that help maintain water quality, and contribute to nitrogen, phosphorus, and carbon cycling (Paul et al. 1991, Davey and O'toole 2000, Battin et al. 2003, Pace and Lovett 2013). Biofilms also serve as an important food source for primary consumers (Pace and Lovett 2013). The productivity and overall activity of biofilms positively correlate with light availability (Schnurr et al. 2016), reflecting the autotrophic component of biofilms. These biofilm communities have repeatedly been shown to be affected by PPCPs. For example, caffeine, ciprofloxacin, and diphenhydramine suppressed algal biomass by 22%, 4% and 18% respectively, and suppressed biofilm respiration by 53%, 91% and 63% respectively (Rosi-Marshall et al. 2013). The pharmaceutical compound cimetidine suppressed biofilm respiration by 51% (Rosi-Marshall et al. 2013). In addition, fluoxetine suppressed autotrophic growth by 77%, photosynthetic efficiency by 48%, and respiration by 55% (Robson et al. unpublished data). The antibiotic sulfamethoxazole has been shown to alter the physical structure of biofilm, increase biofilm biomass and thickness (Bruchmann et al. 2013), and alter photosynthetic pigments, but indicated no relative shift in pigment composition (Johansson et al. 2014). Carbamazepine has been shown to increase algal biomass and alter biofilm architecture as well (Lawrence et al. 2005).

Because biofilms represent the base of stream food webs, altered biofilm development can affect many other factors of stream ecosystems. As algal biofilms represent a major component of autotrophic biomass in streams, constrained algal growth can limit algal activity and result in higher nutrient concentrations (Hill et al. 1995, 2001). Biofilms have been used to test the effects of PPCPs on stream ecosystems because they are an integral part of stream ecosystems, and can serve as indicators for other potential implications to aquatic ecosystems (Rosi-Marshall et al. 2013). It is important to explore the combined effects of a PPCP mixture (Rosi-Marshall and Royer 2012) and light because these factors can alter stream biofilms and water quality. Due to the ecological role of stream biofilms, understanding their responses to PPCPs and altered light can provide insight about the potential consequences of these treatments on stream ecosystems at large.

We performed two separate experiments, the first in artificial streams using artificial substrates and the second in a natural stream using pharmaceutical diffusing substrates (PhaDS), to examine the effects of a PPCP mixture and varying light conditions on biofilm gross primary production (GPP), community respiration (CR), algal pigment diversity, and chlorophyll-a (chl-*a*) concentration. We conducted the experiments in controlled, artificial conditions, and in natural conditions to ask whether the effects of light and a PPCP mixture in artificial streams will hold in a natural stream. We hypothesized that biofilms exposed to more light will have higher chl-*a* content, and more variation in algal pigments. We also hypothesized that biofilms exposed to the pharmaceutical mixture will have suppressed algal biomass and altered pigments, but that the pharmaceuticals will have the same effect on biofilms in both open and shaded light conditions.

METHODS

Artificial Streams and Sampling

The artificial stream experiment was conducted from June 9th, 2016 to June 30th, 2016 using 16 fiberglass artificial streams (4m x 15.5cm x 15cm) located in the greenhouse facility on site of the Cary Institute of Ecosystem Studies in Millbrook, NY. Out of the 16 artificial streams, eight were open to natural light and eight were shaded using AgFabric sunblock shade cloth for ~60% reduction in light. We used four replicate streams for each treatment, in a 2x2 factorial design. The factors included were light (open vs. shaded) and pharmaceutical addition (+pharms vs. no pharms).

We filled each artificial stream with 60L of groundwater. To provide representative microbial and invertebrate communities, each artificial stream contained eight artificial substrates, constructed by wrapping ~100g of material in nylon mesh, and closing each end of the mesh using cable ties. These substrates incubated for five

days in Wappingers Creek, and eight of them were added to each artificial stream. Of the eight constructed substrates added to each stream, four contained only sand and four contained sand and 2% organic matter (ground red maple leaves with stems). Each stream also had five leaf packs which were incubated in Wappingers Creek for 28 days, 30 colonized rocks harvested from Wappingers Creek on the initial day of the experiment, and 12 inert un-colonized rocks. We added 16 fritted glass disks to each stream (close together in a 2x8 layout) which we used to quantify algal colonization.

We covered the artificial streams with mesh to contain any emerged invertebrates and left the streams to adjust for 24 hours prior to the commencement of the experiment. After 24 hours, we dosed the +pharms streams with 10mL of a pharmaceutical mixture and continued to do so every two days. The mixture contained caffeine (target in-stream concentration: 1.8 µg/L), ciprofloxacin (0.14 µg/L), cimetidine (0.07 µg/L), acetaminophen (3.5 µg/L), sulfamethoxazole (0.03 µg/L), carbamazepine (0.02 µg/L), diphenhydramine (0.3 µg/L), and fluoxetine (0.02 µg/L). These concentrations are similar to concentrations found in urban streams in Baltimore, MD (Lee and Rosi-Marshall, unpublished data).

We collected two fritted glass disk samples every three days, with the first samples taken on the third day of the experiment. On every collection date, we collected one disk to quantify algal biomass and the other disk for a qualitative measure of algal pigment diversity. We wrapped the samples in aluminum foil immediately after they were collected from the streams, labeled them and stored them frozen until analysis.

Field Site and PhaDS Construction

One way to quantify the effect of PPCPs on stream biofilms is through the use of PhaDS. This method is inexpensive, easily replicated, and effective. It has provided greater understanding of the effects of pharmaceuticals on biofilm development and activity. This method has been used previously to test the effects of singular pharmaceuticals and pharmaceutical mixtures in varying concentrations on stream biofilms (Rosi-Marshall and Royer 2012, Rosi-Marshall et al. 2013, Costello et al. 2015, Robson et al., unpublished data). PhaDS can be easily moved to different areas of light in order to test the effects of pharmaceuticals on biofilm in varying light conditions.

We selected Wappingers Creek on the Cary Institute of Ecosystem Studies campus in Millbrook, NY as the field site for PhaDS deployment. Our artificial streams contained leaf packs incubated in this stream and colonized rocks which were harvested from this stream, making it the ideal location for the purposes of our experiment.

We made and deployed 40 PhaDS using a modified method based on the procedure for the construction of nutrient diffusing substrates (Tank et al. 2006, Costello et al. 2015). Briefly, we made a 2% by weight agar mixture containing either no additional chemicals (control PhaDS) or a mixture of PPCPs (+pharms PhaDS) aimed at mirroring the concentrations from the artificial stream experiment. To achieve the appropriate PPCP concentration diffusing out of the PhaDS, we added 5.5 mL of a secondary intermediate stock solution and 1 mL of a primary intermediate stock solution to the agar mixture. Target concentrations (µg/L) for acetaminophen, sulfamethoxazole, fluoxetine, caffeine, cimetidine, diphenhydramine, ciprofloxacin, and carbamazepine in each cup were ~506.54, 6.67, 2.47, 589.80, 16.6, 78.35, 11.97, and 6.17 respectively. The target flux rates (ng h^{-1} cm^{-2}) for acetaminophen, sulfamethoxazole, fluoxetine, caffeine, cimetidine, diphenhydramine, and ciprofloxacin were ~3.21, 0.02, 0.01, 2.41, 0.06, 0.39, and 0.07 respectively. The flux rate for carbamazepine could not be calculated because its diffusion coefficient was unknown. All calculations for the target flux rates were completed under the assumption that the temperature of Wappingers Creek was 20°C during deployment. After the agar solution in the polycon cups solidified, we placed fritted glass disks on the surface of the agar and closed the polycon cups. We secured the fully constructed polycon cups on L-bars using cable ties and silicon glue. We wrapped the entire assembly in plastic wrap and kept it refrigerated until deployment (~24 hours).

We deployed the fully constructed PhaDS on the streambed of Wappingers Creek on July 12th, 2016 and left them there for ~14 days to allow for biofilm colonization. PhaDS were deployed in open and shaded areas of the stream and oriented parallel to the stream flow. We deployed 20 PhaDS (10 +pharms, 10 control) in each habitat (open and shaded). We also deployed HOBO light meters on the stream bank to monitor light intensity over the deployment period. We used temperature data from the Environmental Monitoring Program at the Cary Institute of Ecosystem Studies to track temperature over the course of deployment. The average temperature of the stream during the time of deployment was 21.3°C.

After the ~14-day deployment period, we collected the PhaDS and transported them to the laboratory in a cooler. Immediately upon return to the laboratory, we began incubations to measure GPP and CR using the traditional light-dark incubation method. After completing the incubations, we wrapped half of the disks in each treatment group in aluminum foil and stored them frozen until extraction for high performance liquid chromatography (HPLC) analysis. We placed the other fritted glass disks in film canisters and stored them frozen until extraction for chl-*a* analysis (see detailed methods below).

Chlorophyll-a

We measured chl-*a* using the methanol extraction method and a fluorometer (Holm-Hansen et al. 1965, Marker 1972). Briefly, we submerged the colonized fritted glass disks in 10mL of basic methanol solution (1L methanol, 1mL 1M NaOH) in film canisters. The film canisters were left at room temperature for ~24 hours. After ~24 hours, we pipetted 5mL of the resulting solution into fluorometer tubes. We diluted samples as necessary using basic methanol. We then measured fluorescence using a Turner Designs Model TD-700 fluorometer. After the initial reading, we acidified samples by adding 50 μL of 0.3M HCl to each sample. We re-read fluorescence one-hour post acidification. The acidification step allows for the quantification and correction for any phaeophytin in the biofilm. Ultimately, this method allowed us to calculate chl-*a* concentrations ($\mu g\ cm^{-2}$).

Qualitative Measure of Algal Pigment Diversity

High performance liquid chromatography (HPLC) can be used to separate different biofilm algal pigments. HPLC is an increasingly popular analytical separation technique used for the qualitative and quantitative analysis (Mackey et al. 1996, Brotas and Plante-Cuny 2003) of freshwater photosynthetic pigments. An influx of literature proposing, reporting on, and comparing different HPLC analysis methods have been published (Wright 1991, Mackey et al. 1996, Mendes et al. 2007), and HPLC analysis has been useful in providing information for the studying of microphytobenthos communities (Brotas and Plante-Cuny 2003) and phytoplankton taxonomy (Irigoien et al. 2004).

We used an Ultimate 3000 high-performance liquid chromatography machine with an Acclaim[TM] C18 column to examine algal pigments in biofilms that colonized the fritted glass disks. Briefly, samples were extracted using 10 mL of a basic methanol solution (1L methanol, 1mL 1M NaOH) in dimmed lighting and left in the refrigerator for ~24 hours. After ~24 hours, 2mL of solution was transferred to amber HPLC vials. The vials were kept in the refrigerator and a set was inserted every 5 hours. The HPLC sampler kept vials at a temperature of 15°C. We ran and analyzed all samples using an algal pigment gradient on Chromeleon software (Table 1). We randomized samples in their respective sequences.

The HPLC provided us with a unique chromatogram from each sample, and each chromatogram had a variety of peaks with varying heights and retention times. Each peak on the chromatograms represented either an algal pigment or an algal pigment degradation product. We used the resulting peak heights for all sample peaks to create two-dimensional non-metric multi-dimensional scaling (NMDS) ordination plots to assess algal pigment diversity across treatments and time points.

Gross Primary Production (GPP) and Community Respiration (CR)

We measured GPP and CR on fritted glass disk substrates using the light-dark incubation method. Briefly, we placed fritted substrates in 50mL centrifuge tubes, filled the tubes with stream water from Wappingers Creek of known dissolved oxygen (DO) concentration and capped the tubes underwater to ensure there were no air bubbles present inside the tubes. To measure CR (μgO_2 cm^{-2} h^{-1}), we incubated the tubes in a cooler covered with a black opaque plastic bag to ensure darkness for 2-4 hours. We included three tubes, which were filled with only stream water, to be used as blanks to correct for any GPP or CR occurring in the water column. After 2-4 hours, we measured DO concentrations using a ProODO meter. To measure production, we removed the water from the tubes and added fresh water from Wappingers Creek. Then, we let the tubes incubate in the light for 2-4 hours before measuring DO concentration again. We calculated GPP (μgO_2 cm^{-2} h^{-1}) as:

$$NPP + CR = GPP$$

In the above equation, gross primary production (GPP), or the rate at which oxygen is being produced overall before any consumption, is the sum of the net primary production (NPP), the rate at which oxygen is being made available while there is consumption, and community respiration (CR), the rate at which oxygen is being consumed.

Statistics

All statistics were performed using R software in R Studio. We used a two-way ANOVA test to analyze chl-*a* across treatment groups for samples taken from the artificial streams and the PhaDS. We also used a two-way ANOVA test to analyze GPP and CR across treatment groups for samples taken from the PhaDS. We also analyzed chl-*a* for the artificial streams samples using a mixed-effects model, which considers both fixed (light and PPCP treatments) and random effects (sampling time points). To analyze pigment diversity, we used the peak heights from the resulting HPLC chromatograms to represent algal 'species' and then used two-dimensional non-metric multi-dimensional scaling (NMDS) ordination plots to identify differences in algal diversity across treatments.

RESULTS

Artificial Streams

A two-way ANOVA test showed that the concentrations of chl-*a* on the fritted glass disk substrates were significantly higher in shaded streams than in open streams ($p<0.001$), but did not differ between pharmaceutical and control streams ($p>0.05$, Table 2, Figure 1). Based on our mixed-effects model, chl-*a* concentrations were significantly affected by light conditions ($p<0.001$), but not drug conditions ($p>0.05$, Table 2). Based on our linear mixed-effects model we saw that fixed effects (light and pharmaceutical treatments) accounted for 16.3% of chl-*a* variation whereas the varying sample collection time points only explained 2.8% of chl-*a* variation. Both the two-way ANOVA test and mixed-effects model indicated that there was no significant interaction between light and drug treatments in relation to chl-*a* ($p>0.05$, Table 2). Due to outlying data points caused by an unknown error, chl-*a* data from the fourth collection time point (day 12) were not included in the analyses, but results remain largely unchanged if data from this time point is included.

Analysis of HPLC algal pigment data for samples in the first and seventh collection time points (days 3 and 21 respectively) using NMDS ordination plots revealed differences in algal pigment variability across treatment groups. On day 3, the pharmaceutical treatment groups were separate from the control groups (Figure 2). In general, shaded treatments had a wider variety of pigments than open treatments. Overall, the NMDS ordination seems to indicate that PPCPs were the driving factors for algal pigment community diversity on day 3. For day 21 of the experiment, the spread of the algal pigment communities on the NMDS ordination plot indicated that the

pigment communities of the open treatment group contrasted those of the shaded treatment group (Figure 3). In general, the shaded communities are seen having more algal pigment variability than open communities, shown by their larger size on the NMDS ordination plot. Overall, the NMDS ordination seems to indicate that light conditions were the driving factors for algal pigment community diversity on day 21.

PhaDS

Chl-*a*, GPP, and CR were all analyzed using two-way ANOVA tests (Table 2). Chl-*a* concentrations were reduced by pharmaceuticals ($p<0.001$) but did not differ between open and shaded areas ($p>0.05$, Table 2, Figure 4). In contrast, GPP was not affected by pharmaceuticals ($p>0.05$) but was higher in PhaDS incubated in the open area of the stream ($p<0.001$, Table 2, Figure 5). Finally, rates of CR were affected by both drug and light conditions. Rates of CR were higher in PhaDS that received the pharmaceutical treatment ($p<0.001$) and that were incubated in open areas ($p<0.001$) of Wappingers Creek (Table 2, Figure 6).

Analysis of HPLC algal pigment data using a NMDS ordination plot revealed differences in algal pigment diversity across treatment groups. The algal pigments of the open treatment group contrasted those of the shaded treatment group because these treatment groups were separate from each other on the NMDS ordination (Figure 7). Shaded treatments had a wider variety of algal pigments than open treatments, shown by the the larger polygon size on the NMDS ordination plot. It appears that pharmaceutical treatments reduced algal pigment diversity relative to the control treatments, but more formal statistical analysis is required to confirm whether or not the pharmaceutical effect is significant.

DISCUSSION

Artificial Streams

We hypothesized that open treatments would have higher chl-*a* concentrations than shaded treatments because more light would fuel biomass production. We saw that biofilms in shaded streams had significantly higher chl-*a* than biofilms in open streams. This may because pigments exposed to higher light intensities can experience photo bleaching or loss of chl-*a* pigment. High light intensities have been found to result in photosaturation in algae and other organisms (Powles 1984, Sforza et al. 2015). Photosaturation can occur when organisms are moved from a low light zone to a fully sunlit zone (Powles 1984). In our experiment, we harvested biofilm from Wappingers Creek, an area that is not exposed to full sunlight. The intensely lit environment for artificial streams in the open treatment might not have been ideal for the existing biofilm community and may have altered chl-*a* production and the production of other pigments.

PhaDS

To explore whether the trends we observed from the artificial streams would be the same in natural, unregulated conditions, we measured the responses of stream biofilms using PhaDS. We saw differences in rates of CR, chl-*a*, GPP and algal pigments diversity across treatment groups.

The differences in CR between open and shaded treatments and control and +pharms treatments are statistically significant and ecologically important. Our data indicates that open streams tend to have a higher CR than shaded streams. We found a significant increase in CR for +pharms treatments. A higher rate of respiration may have resulted from the death of certain microbes in the presence of PPCPs. We saw that GPP and CR were significantly affected by light conditions while chl-*a* concentrations were not. This may be due to the sensitivity of processes like GPP and CR. A study which tested the effects of pharmaceuticals on biofilms has reported that processes such as GPP may serve as better metrics than chl-*a* of stream biofilm response (Rosi-Marshall et al. 2013) because these processes tend to be more sensitive to ecological stressors.

We saw a significant difference in chl-*a* concentrations between pharmaceutical treatments and control treatments. Our data indicates that pharmaceuticals significantly suppressed algal biomass. We saw that biofilms from the control PhaDS had on average almost four times the chl-*a* concentration of biofilms from +pharms PhaDS. This drastic reduction in the biomass of biofilms can have rippling effects on the aquatic ecosystem. For example, algae has been shown to control nutrient cycling (Paul et al. 1991, Chen et al. 2015) and remove contaminants (Bai and Acharya 2016), and therefore reduced chl-*a* or algal biomass will also reduce water quality. Similarly, algae are an excellent food source for a variety of stream organisms, and reductions in chl-*a* driven by PPCPs can affect multiple levels of aquatic food webs (Davey and O'toole 2000, Pace and Lovett 2013).

Our results for GPP indicated that open treatments had significantly higher GPP than shaded treatments, even though chl-*a* concentrations in open treatments was generally lower than those of shaded treatments. The communities of algae in the open treatment biofilms might be very active but may not produce a lot of biomass or have algae communities that produce biomass containing a wider variety of pigments other than chl-*a* compared to communities in the shaded treatments.

Another possible explanation is that while chl-*a* pigment might be lacking in a particular treatment, there might be an abundance of another pigment that we didn't test for, which can also account for some algal biomass. When examining the resulting chromatograms from the HPLC run, we noticed that there was an abundance of two pigments in open treatments, in addition to chl-*a*, that were suppressed in shaded treatments. We believe that these pigments might be two different forms of chlorophyll-c (Wright 1991, Mendes et al. 2007), and may account for a large amount of the biomass in open treatments. Based upon the GPP, chl-*a*, and algal pigment results, it appears that PPCPs may shift the algal community away from chl-*a* and towards other pigments.

We noticed a contradiction between relative chl-*a* concentrations when using the fluorometer chl-*a* methanol extraction method and peak height as a measure of chl-*a* from HPLC analysis (Figure 8). The results from analyzing peak height for the peak we identified to be chl-*a* based on past literature (Wright 1991, Mendes et al. 2007), indicated that open treatments had more chl-*a* than shaded treatments. These results, although contradictory to the results from the fluorometric method, are what we hypothesized. The HPLC analysis results also support the common finding that more light promotes more biomass growth (Schnurr et al. 2016) while reduced light hinders biomass growth (Steinman 1992, Hill et al. 2001) and as a result, chl-*a* concentrations. Studies have indicated little variation between photosynthetic pigment concentrations measured using other methods, such as fluorometry, spectrophotometry, and HPLC analysis (Murray et al. 1986, Sabina 2011). The discrepancy between the two values we measured could be attributed to the handling of the colonized fritted glass disks for the purposes of measuring rates of CR and GPP. In addition, we did not use the same the colonized fritted glass disks to measure chl-*a* on the fluorometer and using HPLC analysis.

Cumulative Findings

Our findings indicate that altered light conditions and exposure to pharmaceuticals can have significant and unpredictable ecological effects. Surprisingly, we found that biofilms incubated in our shaded treatments had more chl-*a* than those in open treatments exposed to full sun. This finding may be due to the source algal community harvested from Wappingers Creek. The biofilms in the forested Wappingers Creek might be adapted to shaded areas.

We did not see the same drastic reduction in chl-*a* concentration due to pharmaceuticals in the artificial streams that we saw from the PhaDS. This could be attributed to the fact that biofilms developing on the PhaDS had a continuous and more direct dose of pharmaceuticals than the biofilms developing on the disks in the artificial streams, which were farther away from the dosing point and received doses every two days. Additionally, the fact that pharmaceuticals were added to the water column in the artificial streams may allow biofilms colonizing disks

in these streams to have some degree of protection from PPCPs, based upon limited diffusion from the water column through the boundary layer of the disks (Lee et al. unpublished data).

Results from the NMDS ordination of HPLC peak height data indicated that there were differences between the algal pigment diversity of open treatments and shaded treatments which might be indicative of different algal communities (Figures 2, 3, and 7). For both experiments, we saw that shaded treatments had a wider variety of algal pigments compared to open treatments. This difference may be because the algal communities from the shaded treatments were well adapted to shaded areas, like the area of Wappingers Creek from which colonized rocks and incubated leaf packs were collected. Algal communities that are well adapted to shaded areas may not thrive as well in open areas. For shaded areas receiving more light than usual due to the removal of trees, this is of grave concern.

A recent study found that PPCPs can alter clarity of stream water, and as a result, the amount of light being received by biofilms. The addition of PPCPs to artificial streams after a 3-week pre-dosing period led to a reduction in sestonic biomass relative to un-treated control streams. This reduction in seston stimulated benthic biomass, with the authors hypothesizing that the reduction in seston led to an increase in light availability for benthic biofilms (Lee et al. unpublished data). These observations suggest that pharmaceuticals have the potential to alter light conditions and drastically change the environment for developing and well-established biofilms. In our experiment, we were able to observe significant changes in developing stream biofilms due to light conditions and pharmaceutical addition. However, persistent, long-term exposure to PPCPs, in the presence of other contaminants such as nutrients, can lead to cumulative stress and toxicity (Ellis 2006) which can eventually lead to more changes in stream ecosystems. Repeating our study, but using fully developed biofilms at the start of the experiment would allow us to test more chronic effects of PPCPs.

The findings of this study contribute to our understanding of the rippling effects of PPCPs and changing light conditions on our freshwater ecosystems. Stream biofilms are essentially at the beginning of the food web for aquatic ecosystems, and altering stream biofilms processes though pharmaceuticals or light alteration may influence nutrient cycling and consumers that depend on stream biofilms. Since pharmaceuticals are likely to interact with other stressors like nutrients (Rosi-Marshall et al. 2013), more research needs to be done on the combined effects of these contaminants and altered light conditions on our freshwater ecosystems. We found that both PPCPs and light availability appear to alter algal pigment diversity, which may explain some of the contrasting results we found with GPP and chl-*a*. Overall, the results of this study confirm that light intensity controls biofilm development, but that PPCPs also can affect various aspects of stream biofilms, including metabolic activity and the pigment diversity.

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APPENDIX

TABLE 1. High-performance liquid chromatography analytical gradient reflecting experimental conditions.

Time (min)	Flow (ml/min)	B (%)	C (%)	D (%)	Conditions
0	0.200	100	0	0	Injection
2	0.300	0	100	0	Linear gradient
2.6	0.300	0	90	10	Linear gradient
13.600	0.300	0	65	35	Linear gradient
18.000	0.300	0	31	69	Linear gradient
23.000	0.300	0	31	69	Hold
25.000	0.300	0	100	0	Linear gradient
26.000	0.300	100	0	0	Linear gradient
34.000	0.300	100	0	0	Hold

Solvent B: Methanol with 0.5 ammonium acetate buffer, pH 7.2 (80:20).

Solvent C: Acetonitrile with HPLC grade water (90:10).

Solvent D: HPLC grade ethyl acetate (100%).

TABLE 2. Resulting p-values for measured endpoints. Factor 1 is drug treatments (+pharms versus no pharms) and factor 2 is light treatments (open versus shaded). We considered $p<0.05$ to be significant.

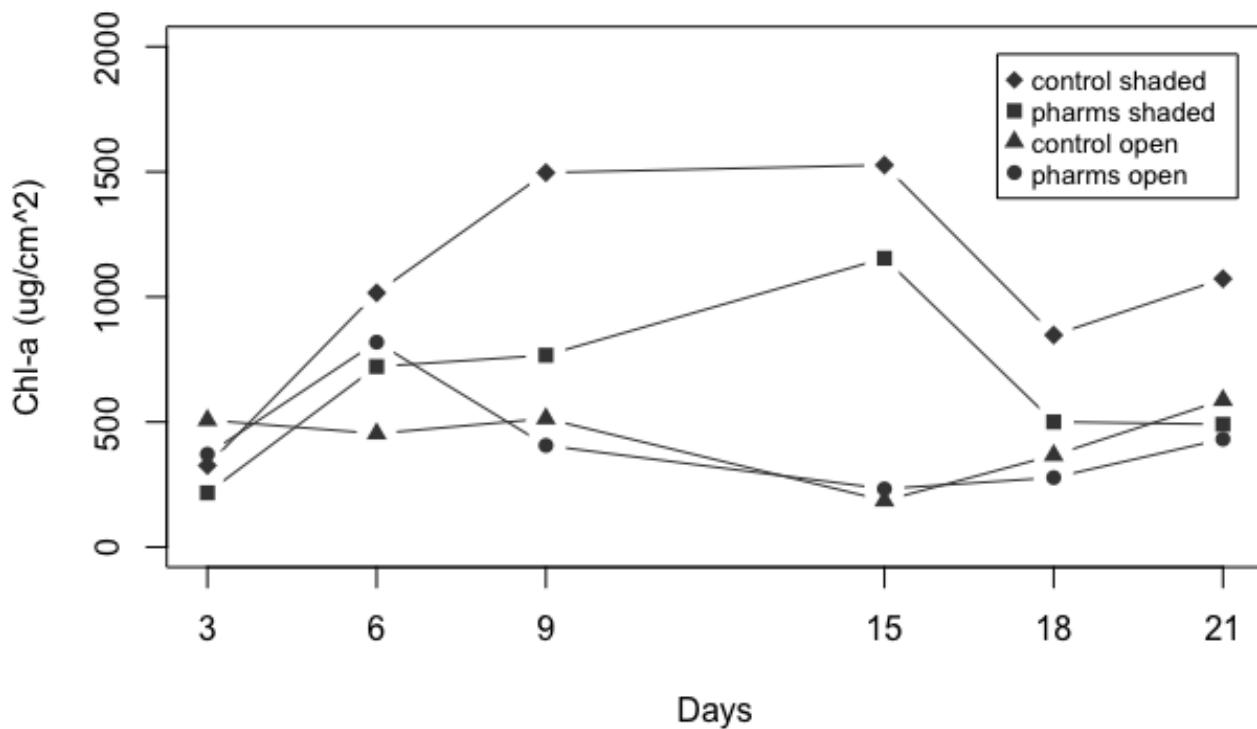|	Artificial Streams		Pharmaceutical-diffusing substrates (PhaDS)		
	Chl-*a*		Chl-*a*	GPP	CR
	ANOVA	Mixed-effects model	ANOVA	ANOVA	ANOVA
Factor 1	0.0809027	0.0744954	8.31 e-10	0.3482	0.0004384
Factor 2	0.0006901	0.0003515	0.3006	1.009 e-07	3.641 e-10
Factor 1: Factor 2	0.0971141	0.0903220	0.8966	0.6397	0.7986572

FIGURE 1. Chlorophyll-a (chl-*a*) concentrations (µg cm^{-2}) from colonized fritted glass disks in the artificial streams over time. Chl-*a* concentrations were higher on fritted glass disks in shaded streams than open streams (p<0.001), regardless of PPCP treatment (p>0.05). Each collection date represents the average from four replicate streams. Day four has been excluded due to outlying data.
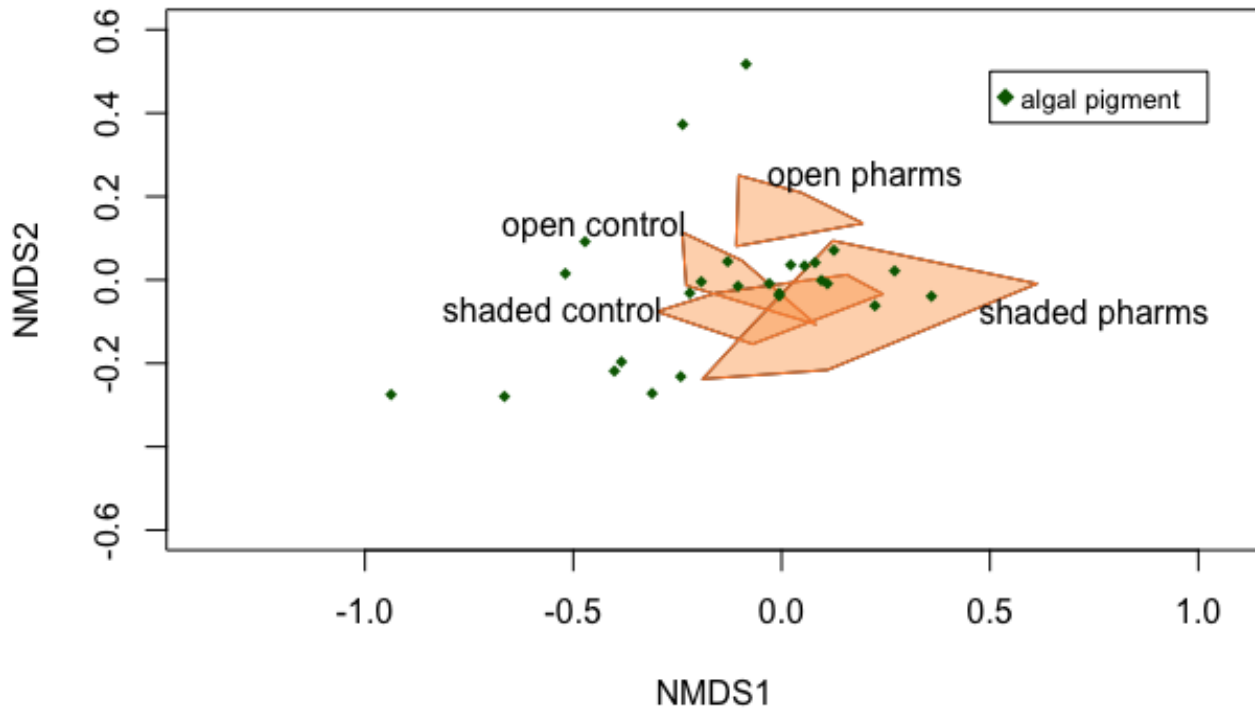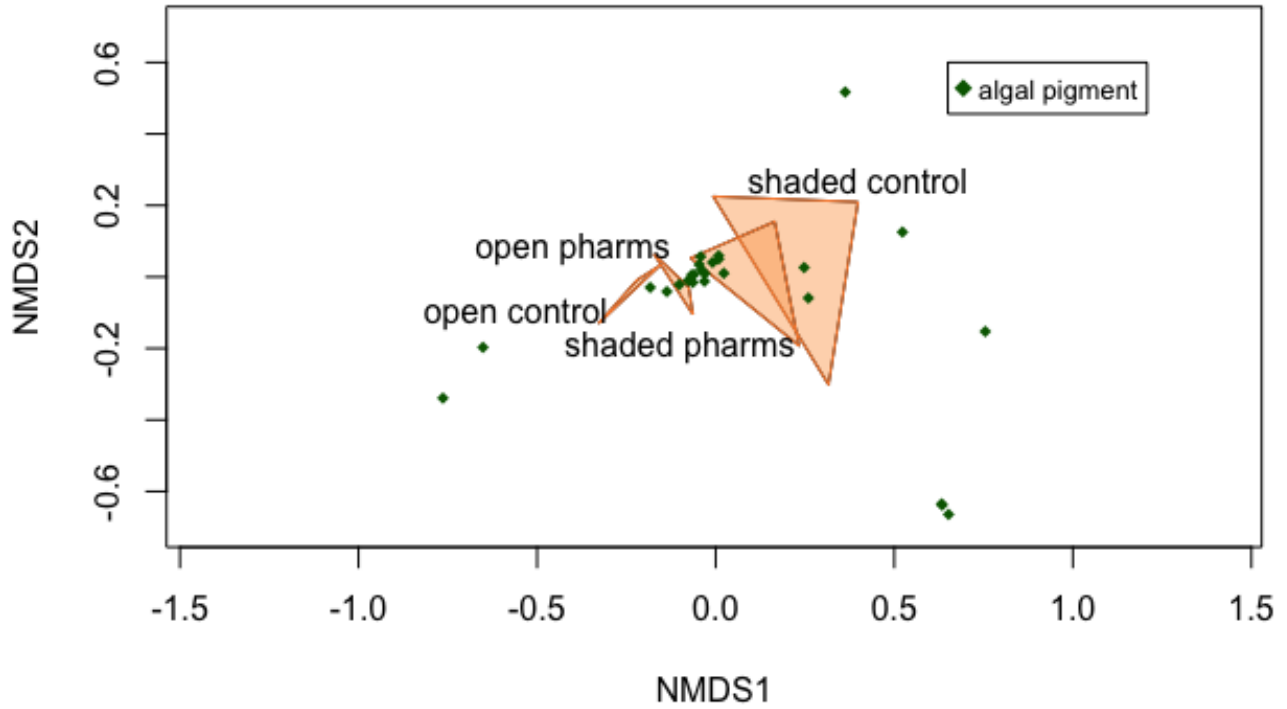
FIGURE 2. Two-dimensional non-metric multi-dimensional (NMDS) ordination of high performance liquid chromatography (HPLC) results for colonized fritted glass disks collected from the artificial streams on day 3. Green points represent the different algal pigments and polygons are drawn to enclose three to four replicate disks from each treatment. Pharmaceutical treatments appear to have different algal pigment communities relative to control treatments.

FIGURE 3. Two-dimensional non-metric multi-dimensional (NMDS) ordination of high performance liquid chromatography (HPLC) results for colonized fritted glass disks collected from the artificial streams on day 21. Green points represent the different algal pigments and polygons are drawn to enclose three to four replicate disks from each treatment. Shaded treatments appear to have different algal pigment communities relative to open treatments and more variability of pigments as well.
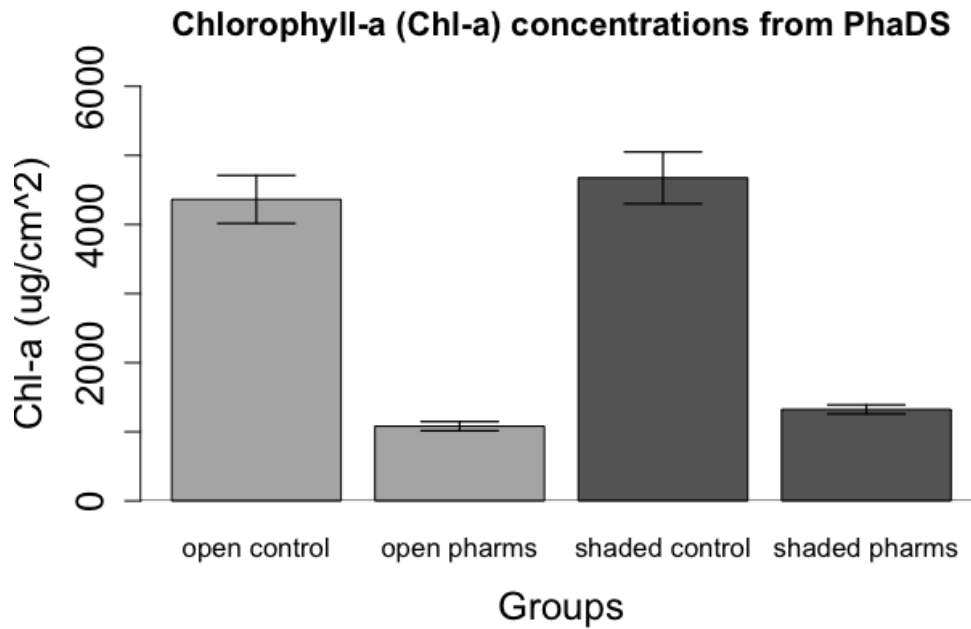
FIGURE 4. Average chlorophyll-a (chl-*a*) concentrations ($\mu g\ cm^{-2}$) from colonized fritted glass disks of the pharmaceutical diffusing substrates (PhaDS). Each bar represents the average of five replicates and error bars denote +/- one SE. There was a significant difference between +pharms and no pharms treatments ($p<0.001$).
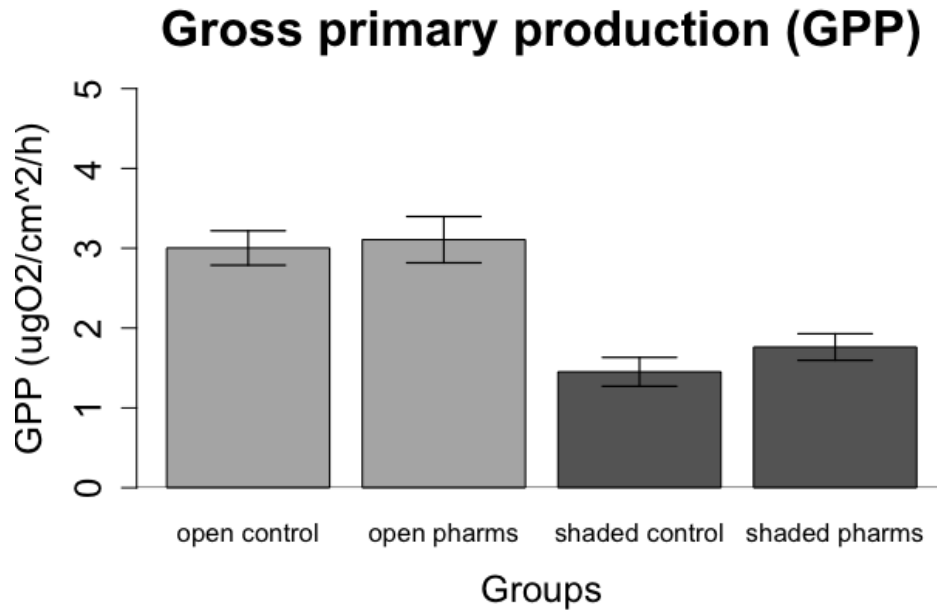
Gross primary production (GPP)

FIGURE 5. Average gross primary-production (GPP; $\mu g \ O_2 \ cm^{-2} \ h^{-1}$) for all treatments from the pharmaceutical diffusing substrates (PhaDS). Each bar represents the average of five replicates and error bars denote +/- one SE. There was a significant difference between open and shaded treatments ($p < 0.001$).
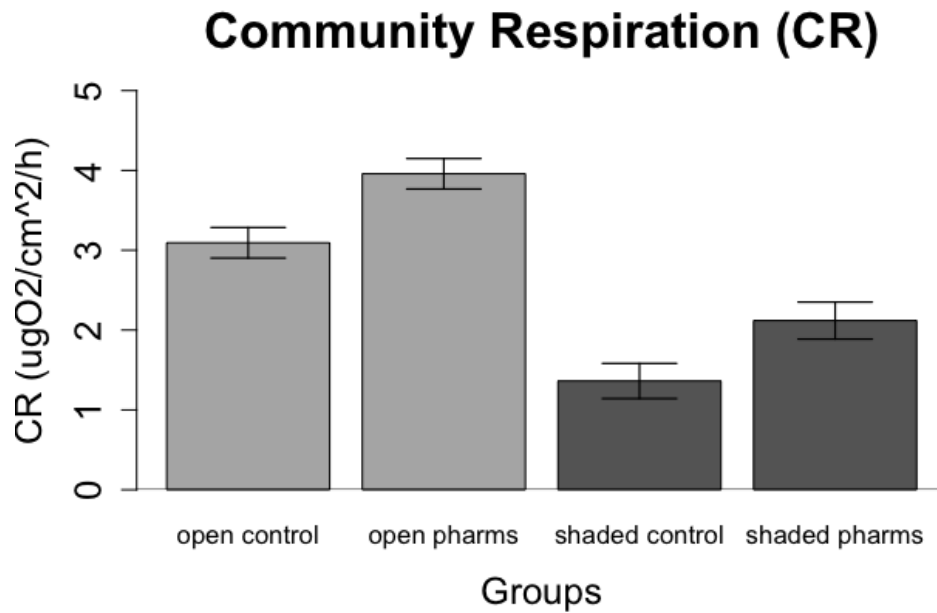
Community Respiration (CR)

FIGURE 6. Average community respiration (CR; μg O_2 cm^{-2} h^{-1}) for all treatments from the pharmaceutical-diffusing substrates (PhaDS). Each bar represents the average of five replicates and error bars denote +/- one SE. There was a significant difference in CR between open and shaded treatments ($p<0.001$) and +pharms and no pharms treatments ($p<0.001$).
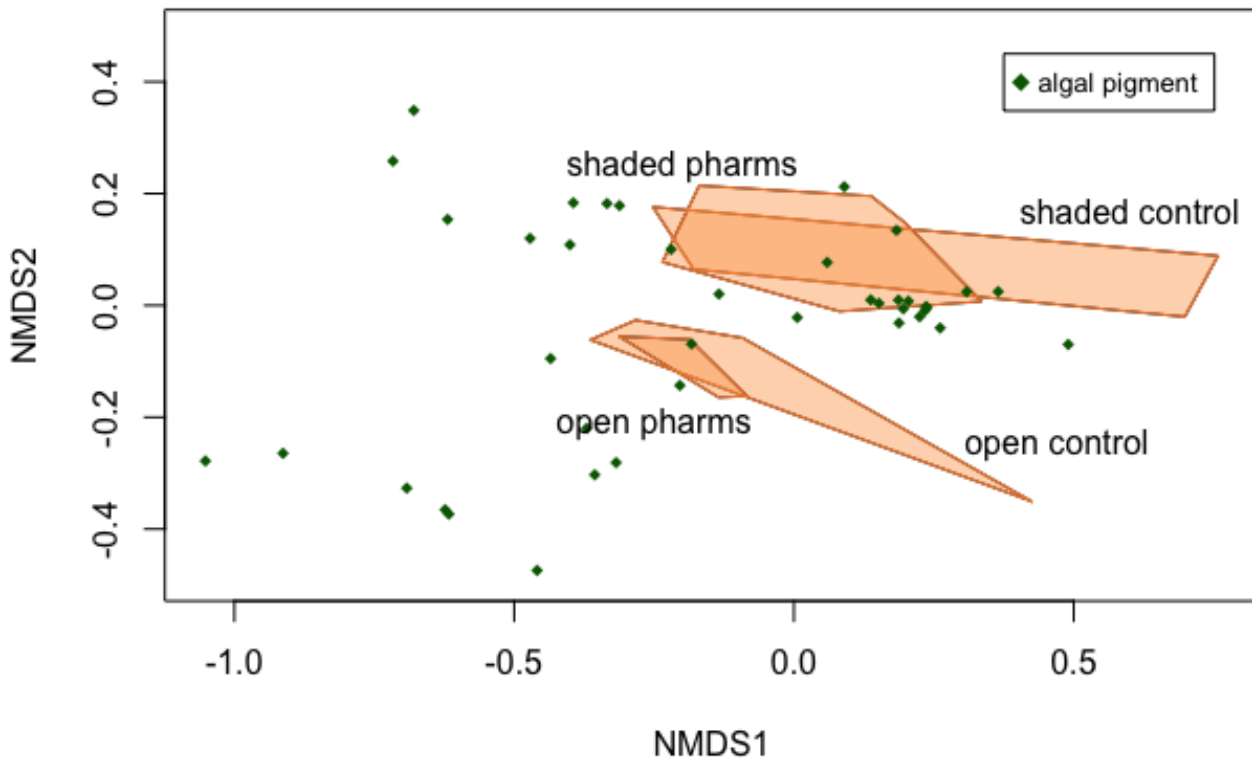
Algal pigment diversity across treatments

FIGURE 7. Two-dimensional non-metric multi-dimensional (NMDS) ordination of high performance liquid chromatography (HPLC) results for colonized fritted glass disks from the pharmaceutical diffusing substrates (PhaDS). Green points represent the different algal pigments and polygons are drawn to enclose five replicate disks from each treatment. Shaded and open treatments have different pigment communities. Pharmaceutical treatments appear to have reduced algal pigment diversity relative to control treatments.
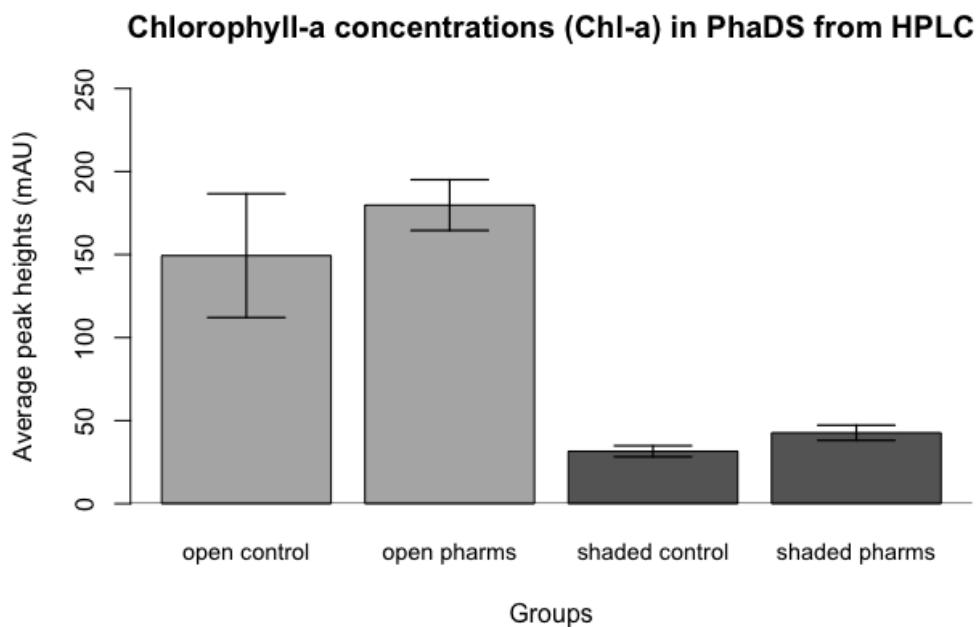
Chlorophyll-a concentrations (Chl-a) in PhaDS from HPLC

FIGURE 8. Average peak height for the putative chlorophyll-a taken from the resulting HPLC chromatographs of the pharmaceutical diffusing substrates (PhaDS). Each bar represents the average peak height from five replicates with error bars denoting +/- one SE.