INVESTIGATING INTERACTIONS AMONG PHARMACEUTICAL COMPOUNDS ON STREAM BIOFILMS

SHELBY SERVAIS
Mount Saint Mary's University, 16300 Old Emmitsburg Rd, Emmitsburg, MD 21727

MENTOR SCIENTISTS: DRS. EMMA ROSI-MARSHALL AND HEATHER BECHTOLD
Cary Institute of Ecosystem Studies, Box AB, Millbrook NY, 12545

Abstract. Pharmaceutical and personal care products (PPCPS) are any products used by people for health, or cosmetic reasons or for veterinary purposes. Trace amounts of PPCPs can be found in aquatic ecosystems via wastewater effluent, septic systems, factory effluent, and agricultural runoff. We measured the effect of two common pharmaceuticals, ciprofloxacin and diphenhydramine, individually and in combination on the gross primary production (GPP) and community respiration (CR) of stream biofilms in Wappinger's Creek, NY. Although we found no significant response of biofilm to diphenhydramine or ciprofloxacin (10, 25,100, and 1000 ng/L) after 4 or 24 hours of exposure, longer duration experiments results in significant effects on biofilms. Ciprofloxacin significantly decreased CR of biofilm that had been grown on sponges before exposure to the compound (pre-colonized). Diphenhydramine significantly decreased both GPP and CR of biofilm on pre-colonized disks. The combination of the two PPCPs lead to a significant decrease in GPP of the biofilm on the pre-colonized disks, and a significant decrease in CR of the biofilm on both new and pre-colonized sponges. It appears that ciprofloxacin more strongly affects the heterotrophic biofilm community, while diphenhydramine more strongly affects both components of the community, especially on well-developed biofilms (i.e., pre-colonized discs).Due to the effectiveness of ciprofloxacin and diphenhydramine at suppressing biofilm activity it was difficult to observe synergistic effects when both compounds were present. These data suggest that the functioning of stream biofilms may altered by these compounds.

INTRODUCTION

North America has one of the highest rates of pharmaceutical consumption in the world (Robinson et al. 2006). These PPCPs are products used for human health, cosmetic or veterinary reasons. Unfortunately, their presence in the environment continues after their use. There are several pathways that lead to the release of these compounds into the environment. Wastewater treatment plants (WWTP) are not designed to completely remove these compounds and consequently PPCPs enter the environment in biologically active forms (Rosi-Marshall and Royer 2012). Septic systems are also not designed to remove PPCPs; however, the removal of some PPCPs can be 90% or more as the effluent moves through soil horizons (Conn et al. 2012). Many pharmaceuticals are not completely broken down by the body and therefore are excreted into sewage (Kolpin et al. 2002). In addition, improper disposal of PPCPs, for example, flushing an expired prescription down the toilet, can result in their entry to wastewater treatment plants and the environment. Industrial sites that manufacture PPCPs may also release compounds into the environment (Philips et al. 2010; Larson et al. 2007; Fick et al. 2009).

PPCPs are present in streams in low concentrations; however, as a result of chronic exposure aquatic organisms may face long-term effects when PPCPs enter streams (Rosi-Marshall and Royer 2012). Pharmaceuticals are a pollutant of concern because they can have persistent effects on biological processes even at low concentrations (Nilsen et al. 2007).

As PPCPs are becoming more widely consumed and released into the aquatic environment, the long-term effects have become a relevant area of study. In addition, PPCPs are typically present in mixtures (Rosi-Marshall and Royer 2012). Understanding the potential synergistic effects is crucial to understanding

environmental risks. Studies have shown that the affect of these compounds differs when they are applied singularly or as a part of a mixture (Backhaus et al. 2011). A synergistic effect occurs when two compounds, when present simultaneously, have a greater net effect then the sum of effect if the compounds acting alone. This experiment looked specifically at diphenhydramine and ciprofloxacin to observe how these compounds affect biofilm individually and in combination.

We studied two pharmaceutical compounds, diphenhydramine and ciprofloxacin, which are commonly used water soluble compounds. Diphenhydramine (2-diphenylmethoxy-N, N-dimethyl) is an antihistamine that is used to target H1 receptors in order to alleviate the symptoms of allergies (PubMed 2011). Diphenhydramine can be found in antihistamine medications like Benadryl ™. It has been found to absorb to sediments and has the potential to accumulate at concentrations up to a thousand times higher than its concentration in the water (Monterio and Boxall 2010). Ciprofloxacin (1-cyclopropyl-6-fluoro-1,4-dehydro-4-oxo-7(1-piperazinyl)-3-quinoline-carboxacylic acid) belongs to the fluroquinolone class of pharmaceuticals and is used to treat and prevent infections caused by bacteria (Githinji et al. 2011, Wilson et al. 2003). Ciprofloxacin is used for both human and veterinary purposes due to its ability to inhibit bacterial multiplication by disrupting DNA replication and the repair processes (Martins et al. 2012). Ciprofloxacin has a broad spectrum of antibacterial activity and is highly effective against gram-negative bacteria (Githinji et al. 2011). Studies have shown that there are high levels of ciprofloxacin in hospital effluent (Martins et al. 2012). Ciprofloxacin is genotoxic, and while there is research available on its acute toxicity, little is known regarding the long-term chronic effects of exposure (Martins et al. 2012). Martins and colleagues found that ciprofloxacin does induce a toxic effect on bacteria, microalgae, and macrophytes (2012).

Stream biofilms are a community of microorganisms that colonize on surfaces in streams such as rocks, sediment, and organic matter. This community consists of both autotrophs (algae and diatoms) and heterotrophs (fungi and bacteria) (Wetzel 1993). Biofilms are an important component of the stream community affecting ecosystem services such nutrient retention and stream health (Bechtold et al. 2012). Due to its ecological significance, understanding the effects of PPCPs on stream biofilm function will provide insight into the potential ecosystem consequences of PPCPs in stream environments. Diphenhydramine is thought to target productivity of the autotrophic component of biofilm while ciprofloxacin should target the respiration of the heterotrophic component of biofilm (Rosi-Marshall et al. 2013)

In this experiment, we examined the individual short-term effects of each of the two PPCPs on biofilm GPP and CR at increasing concentrations. We hypothesized that as the concentration of diphenhydramine and ciprofloxacin was increased there would be a decreased response in biofilm GPP and CR respectively. For the second part of this project we investigated how long-term exposure of the two compounds, both separately and combined, affected the GPP and CR of stream of biofilm at different stages of colonization on different substrates. We hypothesized that there would be a difference in the biofilm response to the pharmaceuticals based upon the degree of colonization. We also hypothesized that we would observe an interaction between ciprofloxacin and diphenhydramine when biofilms were exposed to them in combination.

METHODS

We deployed fritted glass disks on June 4th, 2012 for 10-14 days in order to allow for the colonization of biofilm. They were held in rows using clips which were then glued to L-bars and were secured to the stream bed of Wappinger's creek, NY. After stream incubation, we exposed biofilm to concentrations of 0, 10, 25, 100, and 1000 ng L^{-1} of each compound. These concentrations were based off a study of Lopper and colleagues (2006) and commonly reported in natural waters. We placed the biofilm in 50 mL centrifuge tube chambers which were filled using filtered stream water which was dosed according to the

treatments mentioned above, measured DO, and capped and removed all air bubbles from the chambers. Then we incubated the tubes in the light for approximately four hours in-stream. We included five blank samples in the incubation which was used to correct for any changes background dissolved oxygen (DO). After the incubation we measured final DO in order calculate the total change in oxygen divided by time. To measure CR we saved each substrate and we replenished the water, still at the appropriate dosages, measuring starting DO and incubated the tubes in the dark for approximately two hours underwater in the stream. Following the dark incubation we measured dissolved oxygen concentration again. We used the combination of light and dark incubations to estimate gross primary productivity (GPP) and community respiration (CR) respectively. In order to increase the length of time we exposed the biofilm to the pharmaceutical, we also performed a 24 hour incubation following the first light/dark incubation. We dosed the biofilm again and incubated the chambers in-stream overnight.

Due to the lack of response observed after a 4 hour and 24 hour exposure time we decided to increase the length of exposure even further. Therefore, we implemented Pharmaceutical Diffusing Substrates (PhaDS) to increase the exposure time. We filled 30 mL polyethylene cups with a 2% by weight agar solution amended with 0.015 M L^{-1} pharmaceutical treatments. There were four PhaDS treatments: a control treatment with no added drug, a 0.015 M diphenhydramine treatment, a 0.015 M ciprofloxacin treatment, and a mixture of the two drugs. We attached The PhaDS to L-bars and left to rest on the bottom of Wappinger's creek for 10 days. For each of the four treatments we used four different substrate types in order to represent different type of biofilm communities (Figure 1). We placed new sponges and disks, those that have never been used, onto the PhaDS and deployed them in the stream to incubate for ten days. The other types of substrates included pre-colonized sponges and disks. We achieved this by placing previously colonized and new disks and sponges in the stream without the PhaDS for 7 days and 6 days respectively. After the incubation period, we brought the PhaDS to the laboratory and separated the glass or sponge substrate from the agar cups. We then placed the substrates in 50 mL centrifuge tubes and filled them underwater, using filtered stream water, to remove air bubbles. We then incubated these in the light for approximately four hours. We included five blank samples in the incubation and used to these to correct for changes DO. After the incubation we measured DO in each tube. We measured CR by incubating the substrates in the dark for 2 hours with replenished filtered stream water.

RESULTS

Estimated GPP after a four hour exposure to diphenhydramine was highest in the 25 ng/L dose followed by 100, 0, 10, and 1000 ng/L doses in decreasing order. A linear regression line was fit to the dose-response curve and resulted in an R^2 of 0.24 ($y=-0.0115x+43.272$). For the increasing doses of diphenhydramine experiment the estimated GPP after the 24 hour exposure was highest for the 10 ng/L dose followed by the 25, 100, 1000 and 0 ng/L doses in decreasing order. A linear regression line was fit to the dose-response curve and resulted in an R^2 of 0.067 ($y=1.1119x+30.74$).

For the increasing doses of ciprofloxacin experiment the estimated CR, amount of oxygen consumed, after four hour exposure was greatest for 100 ng/L followed by 0, 25, 1000, and 10 ng/L in decreasing order. A linear regression line was fit to the dose-response curve and resulted in a R^2 of 0.048 ($y=0.0028x-9.7589$). For the increasing doses of ciprofloxacin experiment the estimated CR, amount of oxygen consumed after a 24 hour exposure was greatest for 1000 ng/L followed by 0, 100, 10 and 25 ng/L in decreasing order. A linear regression line was fit to the dose-response curve and resulted in a R^2 of 0.702 ($y=-0.0067x-9.9856$).

Prior to placement on PhaDS GPP and CR was measured for substrates that had been placed in the stream to colonize biofilm. Disks have a greater GPP than sponges and sponges have a higher CR than disks. P-values of 0.92 and <0.0001 were obtained using a Student's t-test to compare the GPP and CR of disks and sponges respectively.

For pre-colonized disks the GPP, amount of oxygen produced was highest on those that were exposed to the control PhaDS (55.7 mg $O_2/m^2/h$). The second highest GPP estimated was for the ciprofloxacin treatment (31.7 mg $O_2/m^2/h$). Both the diphenhydramine and the combined treatment (diphen+cipro) had a GPP estimated of 0 mg $O_2/m^2/h$. When compared to the control the diphenhydramine and both drugs combined treatments had p-values $p<0.001$. Ciprofloxacin compared to the control had a p-value of 0.0038. These data demonstrate that there was a significant effect of exposure to both diphenhydramine and ciprofloxacin on rates of GPP compared to the controls.

For pre-colonized sponges the CR, amount of oxygen consumed, was highest rate was measured on the control PhaDS (2,304 mg $O_2/m^2/h$). The second highest CR measured was for the diphenhydramine (42.4 $O_2/m^2/h$) and ciprofloxacin (40.3 $O_2/m^2/h$) treatments. The combined treatment (diphen+cipro) had an estimated CR of 0 mg $O_2/m^2/h$. When compared to the control the diphenhydramine, ciprofloxacin, and both drugs combined treatments all had p-values of $p<0.001$. These data demonstrate that there were significant effects of diphenhydramine and ciprofloxacin exposure on CR of pre-colonized biofilms compared to the control.

We observed an effect of ciprofloxacin that increased the GPP on uncolonized disks and was significantly different from control GPP (0.02). However, the diphenhydramine and the combined treatment (diphen+cipro) had an estimated GPP of 0 mg $O_2/m^2/h$ that was lower than but not significantly different from the control (0.32 and 0.32). For uncolonized sponges the rates CR not significantly different between diphenhydramine, ciprofloxacin and the control. However, the rates of CR on the combined treatment (diphen+cipro) had an estimated CR of 0 mg $O_2/m^2/h$, which was significantly from the control ($p< 0.001$). The rates of GPP and CR were much lower on the uncolonized disks and sponges were much lower than the colonized disks and may have limited our capacity to examine the effects of the drugs during the 6 day incubations.

DISCUSSION

Our data demonstrate that the effects of diphenhydramine and ciprofloxacin are manifested during chronic exposure. Short-term incubations (4 and 24hour) with these drugs did not significantly affect the rates of GPP or CR on biofilms. In contrast, our long-term chronic exposures to diphenhydramine and ciprofloxacin, especially on colonized biofilms, resulted in significant reductions in the rates of primary production and respiration. We also hypothesized that we would observe an interaction between ciprofloxacin and diphenhydramine when they were combined. Although an interaction between diphenhydramine and ciprofloxacin was observed in the uncolonized sponge treatment, we were unable to establish an interaction because the effects of the drugs were similar to the drugs by themselves (e.g. complete suppression of GPP by diphenhydramine alone and in combination with ciprofloxacin) Since in most cases the diphenhydramine or ciprofloxacin treatments resulted in a significant suppression of GPP or CR it is hard to determine if any synergistic effect is occurring.

We also found that the degree of colonization influenced the response to the pharmaceuticals and potentially our ability to detect responses. There were differences in the rates of GPP and CR in response to the pharmaceuticals on the pre-colonized substrates. In the past, uncolonized substrates have been used for these types of studies (Tank et al. 2006, Rosi-Marshall et al. 2013). However, in this experiment the pre-colonized substrates had a much greater response in both CR and GPP to the pharmaceuticals than the new disks. While they cannot be directly compared as all the results are relative to the control of each trial, it is interesting to note that there is an approximately ten-fold increase in the amount of oxygen either being produced or consumed by the pre-colonized substrates than the new substrates. By pre-colonizing the substrates a community of biofilm developed and can indicate how well-developed biofilms in streams may respond to short-duration pulses of pharmaceutical exposure typical of combined

sewer overflow releases of wastewater. In contrast, using uncolonized substrates allows for biofilms to develop in response to pharmaceuticals and may provide insight into how biofilm grow under chronic exposure. Therefore the two preparation methods address different questions. The pre-colonized substrates help explain what happens when a pristine stream ecosystem is exposed to PPCPs. The new substrates help explain what happens to a stream ecosystem that has been exposed regularly to PPCPs.

Our experiment also allowed us to compare what types of biofilms develop on the two different substrate types, without exposure to pharmaceuticals. GPP and CR of pre-colonized biofilm show sponges and disks target different communities. Cellulose sponges provide a food source for heterotrophic organisms while fritted glass disks provide a stable substrate for autotrophic organisms. While both types of organisms, heterotrophs and autotrophs, are present on both substrate types there is a stronger presence of heterotrophs on sponges and autotrophs on disks. Since there is a difference consideration should be taken when determining the type of substrate necessary for an experiment. This finding has been shown elsewhere (Rosi-Marshall et al. 2013) and demonstrates the utility of using two substrates for investigation of the effects on biofilm function.

Our results confirm previous research that PPCPs have the potential to influence the functioning of aquatic biofilms and suggest that chronic exposure may strongly suppress important functions of stream biofilms. Biofilm is an integral part of the stream ecosystem and the effects of PPCPs and other contaminants on this habitat should consider. Our findings further demonstrate that colonized biofilms may be strongly suppressed by exposure to contaminants such as diphenhydramine and ciprofloxacin. Although we were not able to establish the effects of the drugs in combination in this study, it is known that these and other PPCPs are typically found together in aquatic ecosystems and further research exploring synergistic effects will be needed to fully assess the consequences of these compounds in aquatic ecosystems.

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APPENDIX

TABLE 1. Community respiration estimates of biofilm on pre-colonized and uncolonized sponges. Community respiration is represented in $O_2/m^2/h$ consumed.

	Pre-colonized	Uncolonized
Control	2305	20.69
Diphenhydramine	42.36	20.73
Ciprofloxacin	40.33	16.10
Diphen+Cipro	20.00	0.51

TABLE 2. Gross primary production estimates of biofilm on pre-colonized and uncolonized disks. GPP is represented in $O_2/m^2/h$ produced.

	Pre-colonized	Uncolonized
Control	55.68	0.86
Diphenhydramine	0	0
Ciprofloxacin	31.71	4.59
Diphen+Cipro	0	0

FIGURE 1. An overview of the methods for preparing new substrates and pre-colonized substrates.

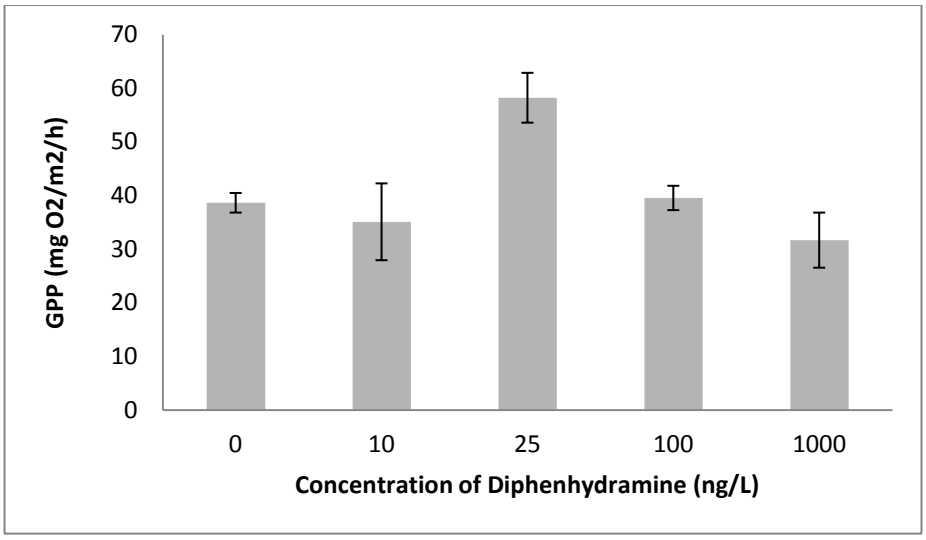

FIGURE 2. Biofilm gross primary productivity response to increasing doses of diphenhydramine for a four hour incubation. Disks that were placed in the stream and then were pulled and exposed to 0, 10, 25, 100, and 1000 ng/L doses of ciprofloxacin for light dark incubations in-stream. Bars represent the average of five replicate treatments.

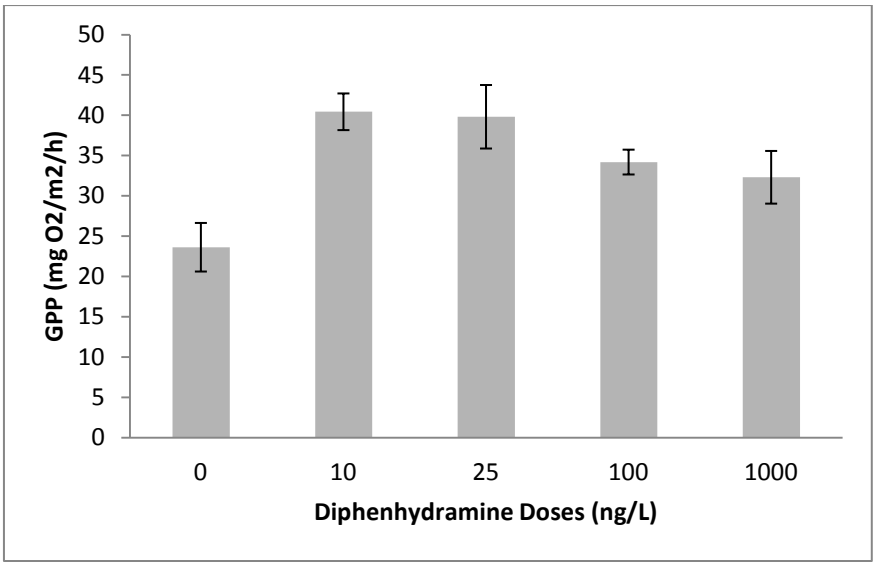

FIGURE 3**.** Biofilm gross primary productivity response to increasing doses of diphenhydramine for a twenty four hour incubation. Following the four hour exposure disks were left in 0, 10, 25, 100, and 1000 ng/L solutions of diphenhydramine in-stream overnight. Following the exposure light dark incubations were performed in-stream. Bars represent the average of five replicate treatments.

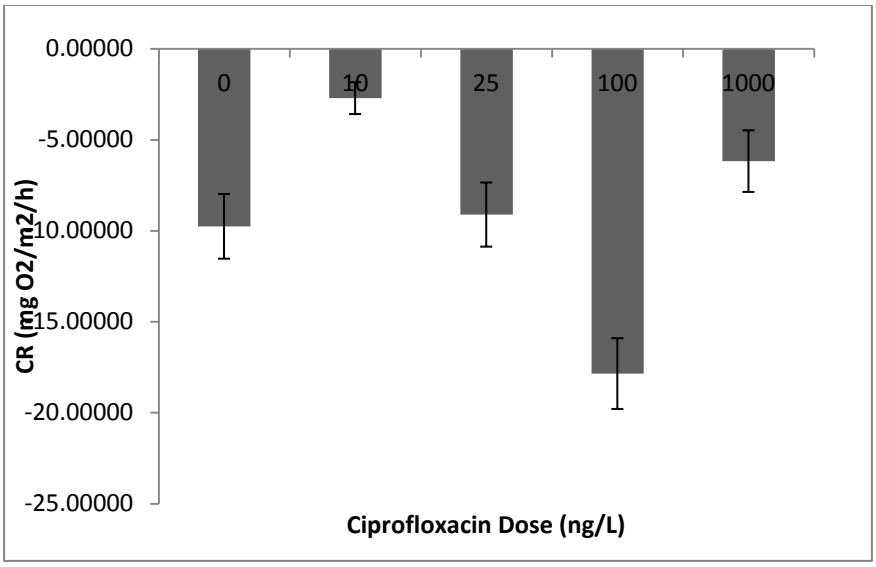

FIGURE 4. Biofilm community respiration response to increasing doses of ciprofloxacin for a four hour incubation. Disks that were placed in the stream for 12 days were pulled and exposed to 0, 10, 25, 100, and 1000 ng doses of ciprofloxacin for light dark incubations in-stream. Bars represent the average of five replicate treatments.

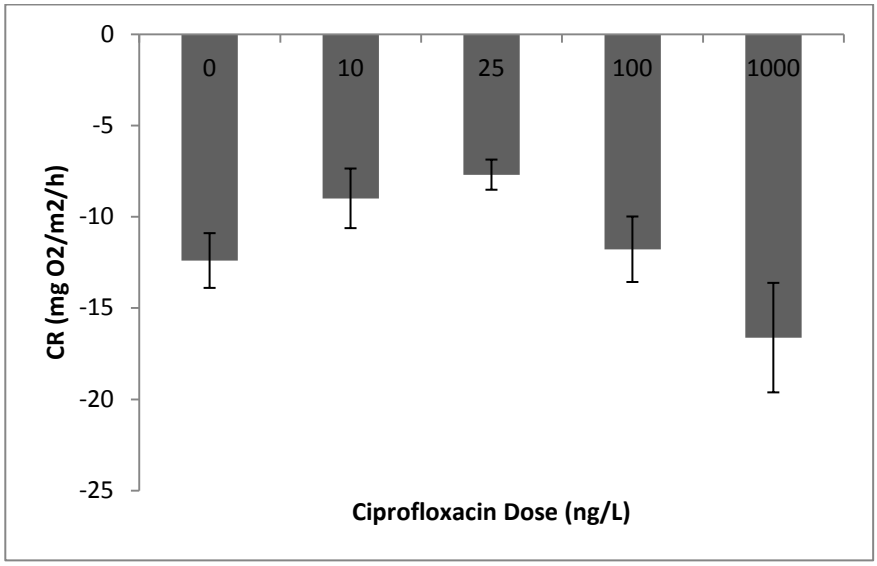

FIGURE 5. Biofilm community respiration response to increasing doses of ciprofloxacin for a twenty four hour incubation. Following the four hour exposure disks were left in 0, 10, 25, 100, and 1000 ng solutions of ciprofloxacin in-stream overnight. Following the exposure light dark incubations were performed in-stream. Bars represent the average of five replicate treatments.

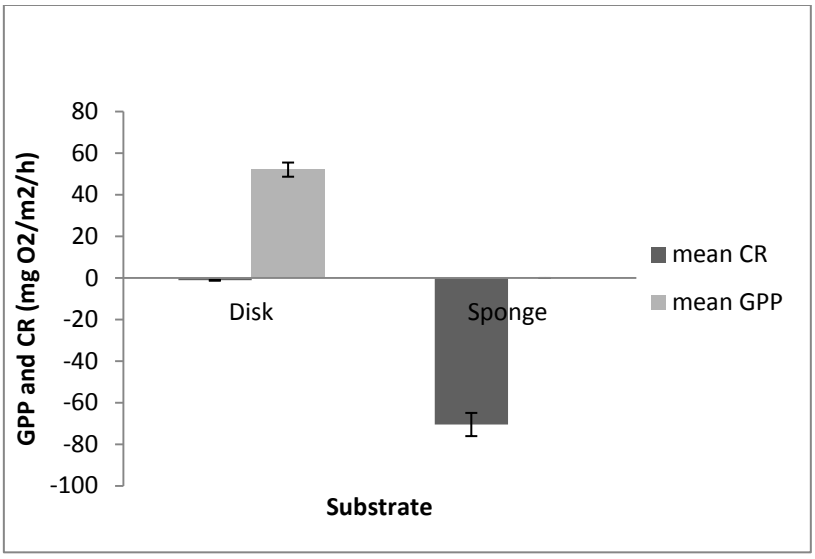

FIGURE 6. Mean CR and GPP of disk and sponge substrates that had been placed in Wappinger's Creek. GPP and CR were estimated using light/dark incubations.

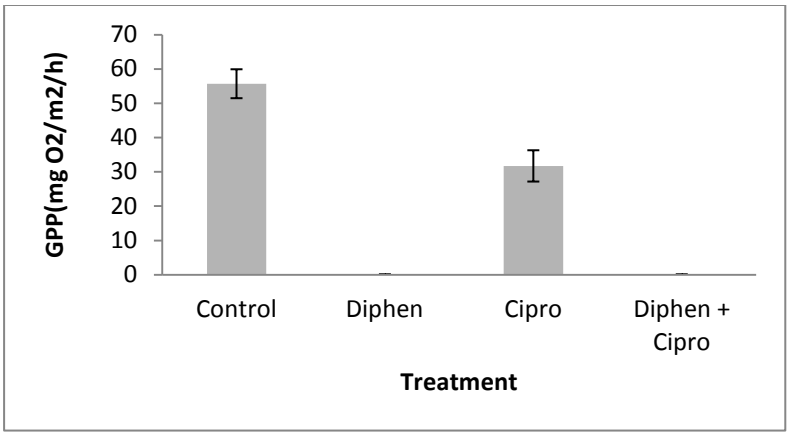

FIGURE 7. Pre-colonized disk GPP after PhaDS exposure. GPP was estimated using light/dark incubations of pre-colonized sponges that were placed on PhaDS with treatments of diphenhydramine, ciprofloxacin, and a combination of the two. Prior to the treatment stream biofilm colonized on the substrate for seven days. Control bar represents the average of ten replicate measurements while the Diphen, Cipro, and Diphen+Cipro bars represent the average of five replicate measurements.

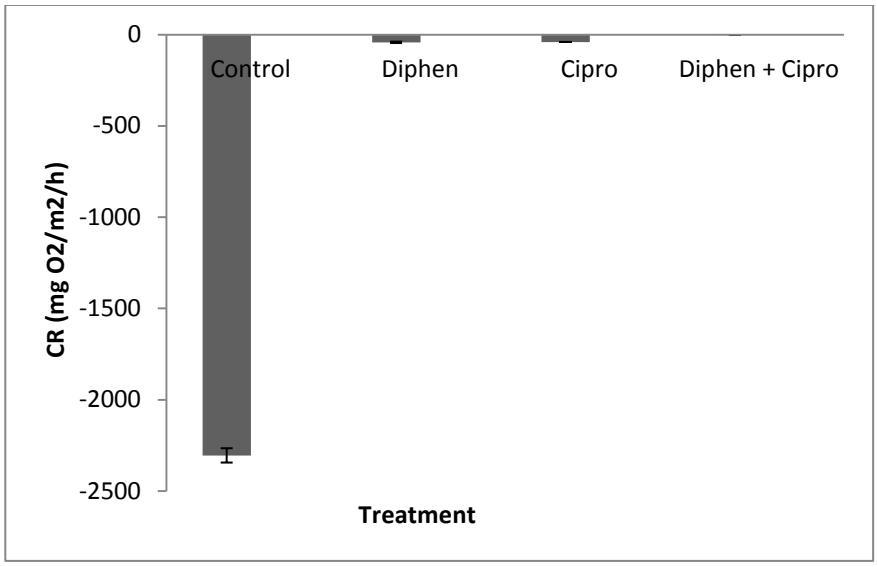

FIGURE 8. Pre-colonized sponge CR after exposure to PhaDS. CR was estimated using light/dark incubations of pre-colonized sponges that were placed on PhaDS with treatments of diphenhydramine, ciprofloxacin, and a combination of the two. Prior to the treatment stream biofilm colonized on the substrate for seven days. Control bar represents the average of ten replicate measurements while the Diphen, Cipro, and Diphen+Cipro bars represent the average of five replicate measurements.

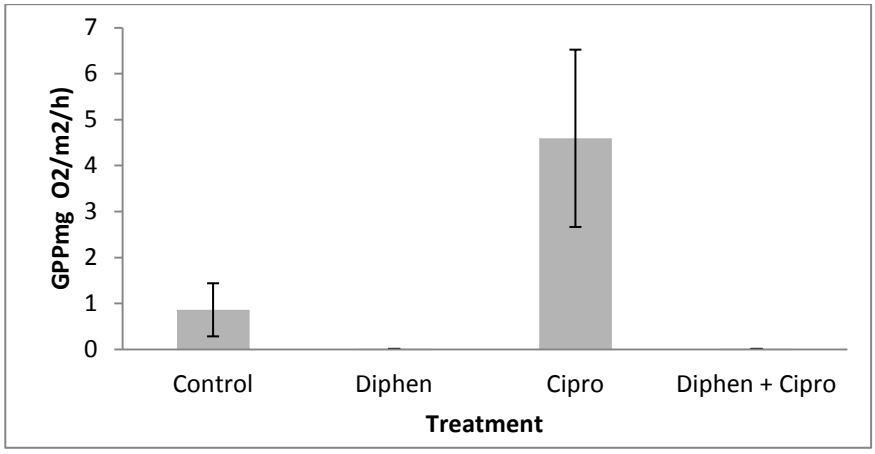

FIGURE 9. New Disk GPP after exposure to PhaDS. GPP was estimated using light/dark incubations of new disks that were placed on PhaDS with treatments of diphenhydramine, ciprofloxacin, and a combination of the two. These disks were left with the PhaDS to colonize biofilm for ten days. Control bar represents the average of ten replicate measurements while the Diphen, Cipro, and Diphen+Cipro bars represent the average of five replicate measurements.

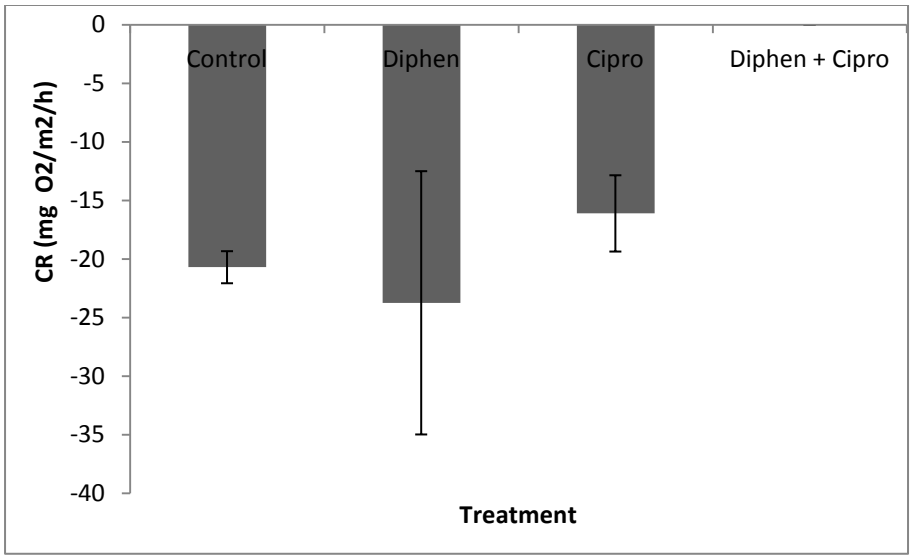

FIGURE 10. New Sponge CR after exposure to PhaDS. CR was estimated using light/dark incubations of new sponges that were placed on PhaDS with treatments of diphenhydramine, ciprofloxacin, and a combination of the two. These disks were left with the PhaDS to colonize biofilm for ten days. Control bar represents the average of ten replicate measurements while the Diphen, Cipro, and Diphen+Cipro bars represent the average of five replicate measurements.