NATIONAL WATER RESEARCH INSTITUTE

NWRI GRADUATE FELLOW SEMI-ANNUAL PROGRESS REPORT

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Project Title:	Antibiotic resistance genes in stormwater biofilters: implications for hotspot identification
Graduate Fellow:	Megyn B. Rugh
Advisor:	Dr. Jennifer Jay
Institution:	UCLA

Background and Introduction

In Southern California, several stormwater treatment infrastructures are being built to capture and treat stormwater for reuse. Biofilters (also called bioretention systems, low-impact development systems, or natural treatment systems) capture and treat diverted stormwater through vertical soil filtration (1). The stormwater is also treated through sedimentation, increased retention time, and biological uptake of certain compounds (2). The treated water is collected in an underdrain system for potential reuse instead of being washed into the ocean via storm drains.

Although biofilters work well to remove ammonia, total suspended solids, total phosphorus, and heavy metals (3, 4), biofilters have not yet been well-studied for efficacy in removing pollutants that are harmful to human health, such as pathogens and toxic chemicals (5). It is not clear whether biofilters sufficiently remove emerging contaminants like antibiotic-resistant bacteria (ARB) and antibiotic-resistant genes (ARGs). Studies have shown that stormwater carries ARGs into the ocean and other nearby water bodies (6, 7). Additionally, biofilters can accumulate both commensal (friendly) and pathogenic bacteria from stormwater, as well as antibiotics and metals, which are both contaminants that can increase the total amount of ARGs. To combat the increasing mortality rates and economic burdens associated with ARB (8, 9), the environmental transport of ARGs must be understood.

The study goals include testing if there are higher or lower concentrations of ARGs within biofilters after stormwater inundation, identifying what type of media (soil, sand, compost, biochar) works best for ARG removal, and if the addition of heavy metals will result in an increase of ARGs within biofilters (co-selection). To explore these goals, this study analyzes five existing biofilters on UC campuses and controlled batch microcosm experiments. For both the UC biofilters and laboratory microcosm experiment, the following genes are quantified: genes that confer resistance to beta-lactams (bla_{SHV}), tetracyclines (*tet*(W)), fluoroquinolones (*qnrA*), macrolides (*erm*(F)) and sulfonamides (*sul*1 and *sul*2); the mobile genetic element *intI*1 (the integron *intI*1); *HF183 Bacteroides* 16S rRNA genetic marker (indicates human fecal pollution); and 16S rRNA (proxy for total cells).

Progress to Date

Experimental Design

A batch microcosm experiment was conducted from November 2018 to March 2019. The microcosm was designed to mimic a natural treatment system media bed. Each bottle received two grams of Kellogg Garden AMEND (sieved 2 mm) and eight grams of sand (coarse 20-30 mm). Compost and sand are commonly used together as biofilter media. This garden soil was selected because it is composed of both compost and manure fertilizer.

To represent a wet-weather event, each bottle received a mixture of copper and sewage (heavy metals and fecal indicator bacteria are common stormwater contaminants) that was suspended in phosphate buffer saline solution. These copper concentrations were chosen based on previous research that indicated ARG and heavy metal co-selection can occur at this range (10). After the bottles were shaken for 24 hours, the supernatant was drained to imitate percolation within a stormwater biofilter. There were seven different experimental conditions, tested in triplicate, as shown in Table 1:

Table 1. Experimental conditions

	Sewage	Copper
A: Control	0 mL	0 mL
B: Control	0 mL	25 ppb
C: Control	4 mL	0 mL
D: Low Cu^{2+}	4 mL	5 ppb
E: Medium Cu^{2+}	4 mL	10 ppb
F: High Cu^{2+}	4 mL	25 ppb
G: Very High Cu^{2+}	4 mL	40 ppb

Data Collection and Analysis

Time points were taken on days 0, 1, 7, 14, 21, and 105. The microcosm was kept in the dark at room temperature. The soil samples from each time point were stored at -20°C until subsequent DNA extraction. Following DNA extraction, qPCR was performed for *sul*1, *sul*2, and *intI*1. Samples and calibration standards were run in triplicate.

Results

The following preliminary results are outlined in Figures 1 and 2:

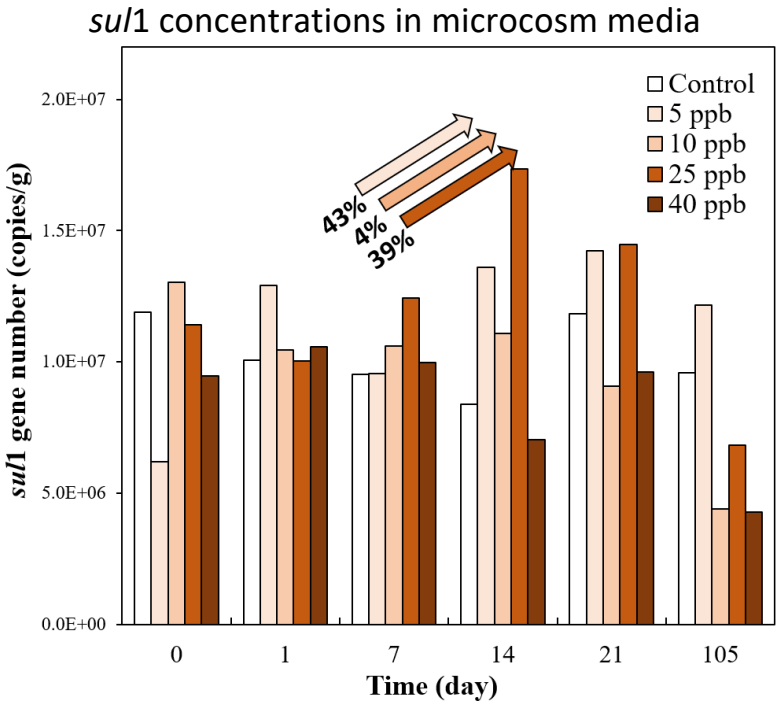

Figure 1. *sul*1 concentrations

The highest number of *sul1* gene copies were found in two experimental conditions: D, at 5 ppb copper and sewage on day 21, and F, 25 ppb copper with sewage on day 14. There was an increase between days 7 and day 14: when *sul*1 concentrations were measured on day 14, experimental conditions D and F increased by 43 percent and 39 percent from their day 7 time point levels, respectively. The included control represents experimental condition C. The control's *sul*1 levels decreased at every time point until an increase was observed on day 21. By the last time point, there is a decline in *sul*1 gene copies in all the experiments containing the three highest copper doses. However, treatment without copper and treatment with only 5 ppb copper still had similar *sul*1 concentrations as day 21.

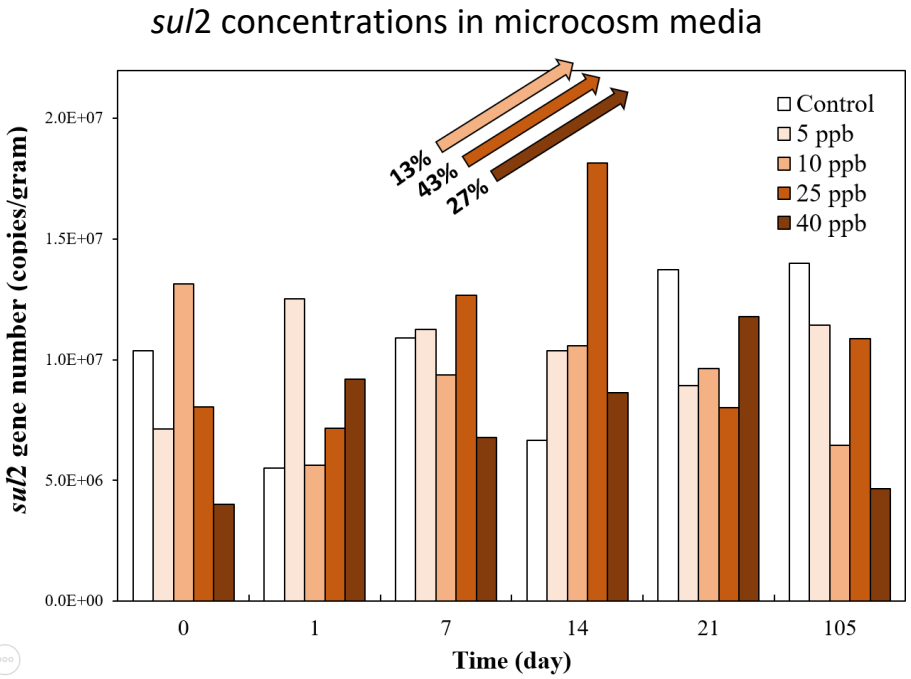

Figure 2. *sul*2 concentrations

The biggest increase of *sul*2 gene copies were found in three experimental treatments: E, 10 ppb copper and sewage; F, 25 ppb copper and sewage; and G, 40 ppb copper with sewage. These elevated *sul*2 concentrations were measured on day 14, with experimental conditions E, F, and G increasing by 13 percent, 43 percent, and 27 percent from their day-7 time point levels, respectively. The control graphed represents experimental condition C. By the last time point, there is a decline in *sul*2 gene copies in experimental condition E and G, but F remains more elevated. Treatment without copper remained at the same level as day 21, and treatment with only 5 ppb copper increased in *sul*2 concentrations from day 21.

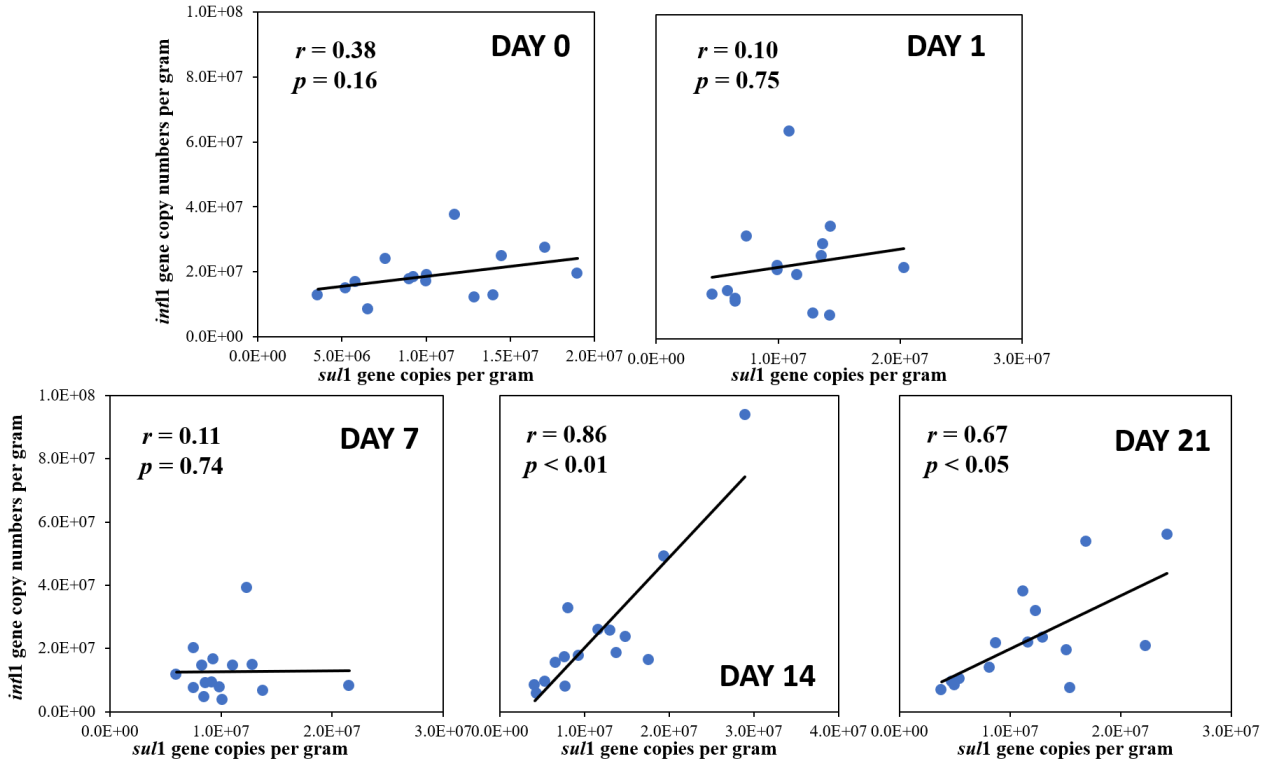

Figure 3. Day 14 results divided by time point

For all combined data, *intI*1 and *sul*1 did not appear to be correlated. However, when divided by time point, day 14 shows that *intI*1 and *sul*1 are highly correlated. This may explain the increase of both *sul*1 and *sul*2 from days 7 and 14.

Conclusions

The most striking results of the microcosm occurred on day 14. At this time point, experiments that included treatment with copper had large increases in *sul*1 and *sul*2 genes compared to day 7 values. Because the control treatment had decreased levels of ARGs, the increase in *sul*1 and *sul*2 in experimental conditions D, E, F, G may be explained by copper co-selection. Under pressure from the heavy metal contaminant, bacteria that have a mechanism of resistance to copper would be selected to survive within the microcosm. ARGs are often located on the same plasmids as metal-resistance genes, so ARGs can increase when a metal is present (10). These findings are supported by a previous study that found that sublethal doses of heavy metals within a river catchment were positively correlated with ARGs (11).

There might be a critical time period in biofilters (and other soil systems) where conditions cause increased horizontal gene transfer (HGT) rates. HGT occurs when bacteria receive DNA from other bacteria or from free DNA from the environment, as opposed to vertical gene transfer (from parent to offspring). HGT contributes to the spread of antibiotic resistance, because pathogenic bacteria can uptake ARGs from harmless environmental bacteria. The *intI*1 gene is located on class 1 integrons, which can carry *sul*1 along with other resistance genes (12). Thus, *intI*1 facilitates dissemination of *sul*1 and elevated *intI*1 levels are associated with increased rates of horizontal gene transfer (10, 13). In this microcosm, *intI*1 and *sul*1 are highly correlated on day 14. Past research has shown that the abundance of *sul*1 and *intI*1 within soils are significantly correlated (10).

Overall, the ARGs seemed to decline by the last time point. Biofilters may serve as a reservoir for ARGs and allow ARB/ARGs to proliferate for a certain amount of time, but eventually the ARGs quantities are reduced. This agrees with a study by Heuer et al who found that *sul*1 in soil decrease after 175 days (14).

Next Steps

The microcosm was completed and no more time points will be taken. The project's remaining work includes completing qPCR experiments for the remaining genes and molecular markers. The qPCR data will need to be calculated, analyzed, and correlated. The remaining soil from all the experimental conditions is undergoing acid digestion for metal analysis. This will determine the amount of copper that adsorbed to the soil and the amount that was dissolved in the supernatant. New data analysis and correlations can be determined with these results.

Another component of this project is measuring ARGs within soil samples from seven existing UC biofilters. This February, our collaborators sent us 56 samples from winter and spring time points. We have spent the last two months extracting DNA from the samples (in triplicate). When we complete the DNA extractions, we will begin qPCR experiments to test for the same suite of genes. Additionally, our collaborators are analyzing the biofilter soil samples for heavy metals so potential correlations with ARGs may be discovered.

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