UNIVERSITY OF WISCONSIN - LA CROSSE

Graduate Studies

EFFECT OF PHOSPHORUS ON MICROBIALLY INDUCED CORROSION TO COPPER METAL AND POSSIBLE IMPLICATIONS FOR DRINKING WATER QUALITY

A Manuscript Style Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science

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December 2021

EFFECT OF PHOSPHORUS ON MICROBIALLY INDUCED CORROSION TO COPPER METAL AND POSSIBLE IMPLICATIONS FOR DRINKING WATER QUALITY

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ABSTRACT

Wenzel, E.R. Effect of phosphorus on microbially induced corrosion to copper metal and possible implications for drinking water quality. MS in Microbiology, December 2021, 59 pp. (B. Bratina)

Microbial corrosion is defined as the degradation of industrial material by microbiological activity. In home drinking water systems, corrosion of piping material can lead to hazardous levels of copper or lead in potable water. Current monitoring and remediation practices focus mainly on chemical factors while giving little attention to biological ones. A grade of copper pipe commonly referred to as DHP (C12200) is commonly used for home water distribution. This grade of copper pipe contains trace amounts of phosphorus left over from the smelting process. If microbial corrosion under phosphorus limited conditions is able to liberate copper-bound phosphorus it could be a new source of phosphorus that microbes could use for growth. This study examined the potential of microbially induced corrosion to release phosphorus for microbial utilization using two grades of copper coupons, DHP and Pure (C10100). The phosphorus amount in each environment was also manipulated and used as control groups. Biofilms were grown on copper coupons housed in "biofilm chambers", which were supplied with fresh nutrient bulk water weekly. Corrosion to coupons were assessed using scanning electron microscopy of used coupon surfaces and detection of total copper in spent bulk water by microwave plasma atomic emission spectroscopy. Utilization of phosphorus from corroded copper was assessed by secondary production and comparison of carbohydrate and protein amounts in biofilm scraped from copper coupons. Microbial corrosion occurred on coupons in phosphorus limited environments, regardless of coupon metal grade. Pitting was only observed on coupons from phosphorus limited environments. Bulk water total copper concentrations were significantly greater in phosphorus limited chambers and remained constant from week to week. Biofilm carbohydrate and protein assays produced unexpected results that did not agree with findings in other literature. Microbial utilization of phosphorus from corroded copper was inconclusive at the tested conditions. It is suspected that using a mixture of carbon source may affect phosphorus-based carbohydrate and protein trends.

ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Bonnie Bratina for assisting with designing and performing our experiment and guiding me through the many steps necessary in bringing this paper to completion. Thank you to my committee members Dr. Paul Schweiger, Dr. Eric Strauss, and Dr. Kris Rolfhus for their scientific/creative input through the design and writing process. I am grateful for the UW - La Crosse Research, Service, and Educational Leadership (RSEL) grant for the financial aid, which allowed this research to take place. Thank you to the members of the UW - La Crosse Microbiology Department for their support and Dr. Reineke and Dr. Elfessi of the Statistics Consulting Center for assisting with statistical analysis of our data set.

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INTRODUCTION

Easy access to clean drinking water is a great accomplishment of developed countries but can be taken for granted. In the United States, laws such as the Safe Drinking Water Act of 1974 have been enacted to maintain the safety and quality of drinking water. Despite regulations, water quality issues still occur, such as the crisis that happened in Flint, Michigan where lead concentrations far exceeded potable water standards after switching source water. (https://www.theguardian.com/news/2018/jul/03/nothing-to-worry-about-the-water-is-fine-how-flint-michigan-poisoned-its-people)

Today, almost all regulations focus on water quality within distribution systems or at the treatment plant. A notable exception is the Lead and Copper rule, which is enforced at end user taps (1). Many issues associated with water quality can be traced back to environments that support corrosive water. The corrosive action is heavily dependent on the material that is being corroded (2, 3, 4, 5). In the United States in 2001, a survey found that 90% of homes used copper piping for drinking water (6). For many homeowners, corrosion of piping leads to deterioration of water quality and has the potential to violate the Lead and Copper Rule established by the EPA. Corrosion of piping used for drinking water is usually noticed by odor, odd color, or taste. If corrosion is aggressive in copper piping it can release enough copper into the bulk water to lead to a phenomenon known as “Blue-Green” water, which is recognized by the blue-green

stain it leaves on fixtures (7, 8, 9). Aside from being aesthetically displeasing, this indicates that the longevity of the piping system is in danger. Additionally, mold growth can result from pinhole leaks in piping and can be a major concern for homeowners who would need to cover the expenses of repair.

Drinking water corrosion is heavily controlled and monitored by chemical means, but a commonly overlooked factor is microbially induced corrosion that is set in motion by biofilms. Microbial induced corrosion refers to the degradation of industrial material via microbiological activity from bacteria, archaea, and fungi. A biofilm is a gelatinous mass of microorganisms that attach themselves to the surface of materials. Under the right circumstances, such as stress, caused by nutrient limitation, disinfectants or antimicrobial agents, biofilm formation can take place. Biofilm can lead to the corrosion of metal materials in drinking water systems, deteriorating water quality.

Corrosion in premise plumbing systems is the result of abiotic and biotic conditions within the system. Premise plumbing is generally defined as the portion of a plumbing system between the end user taps and connection line to the service main. Abiotic corrosion, in general, results from the chemistry of the influent water dissolving away at the piping causing metal release. Biotic corrosion, in general, is the result of microbial activity and can result in pin hole leaks and metals release. Under ideal conditions, a corrosion product called scale will form on the surface of the pipe acting as a boundary layer between the pipe and bulk water preventing continued corrosion. Various models have been proposed to try and explain the interplay of corrosion and scale formation at the pipe-water interface but no one model is sufficiently able to explain

the phenomena (10, 11, 12, 13, 14). It is likely that portions of each model explain corrosion and scale formation at different times and under various conditions.

Scale is created in potable water systems when metal ions form insoluble complexes with chemicals in the bulk water and precipitate onto the surface of the pipe (10, 11). The formation of scale is natural in potable water systems and is an important process. Because scale forms layers on pipe, it acts as a barrier between the bulk water and the pure elemental surface of the pipe. This helps prevent corrosion to the pipe by creating a chemically more stable metal complex that is less reactive with the bulk water. Oxygen is a common element that metal ions will bind with to precipitate out of solution as oxides. Copper, for instance, will form cuprous oxide scale (Cu_2O) at neutral pH and in the presence of oxygen (13, 14). Three processes help explain the formation of scale on metal: speciation reactions, electron transfer reactions, and mass transfer processes (10). Speciation reactions are based on the equilibrium of metal species at the pipe-water interface and in the bulk water. Speciation reactions are heavily impacted by bulk water flow or stagnation and are based on precipitation, dissolution, complexation, and acid-base reactions at those locations. Electron transfer reactions are electrochemical processes caused by the transfer of electrons from the oxidation of metal by chemical species. Strong oxidizing species such as oxygen and chlorine impact the rate and prevalence of this process. Mass transfer processes deal with flux of metal ions from the pipe and scale into the bulk water. This process is suspected of being the rate-limiting step in copper corrosion releasing soluble copper into the bulk water.

Copper pipes are manufactured in two ways, either they are smelted from ores extracted from the ground, or copper is recycled from scrap. Various techniques are then

used to produce copper pipes, resulting in different grades and alloys of pipe. The most common grade of pipe used in non-electrical engineering is DHP or Deoxidized High Phosphorus copper (13). DHP copper contains trace amounts of phosphorus remaining from the smelting process, usually ranging from 0.015-0.04% w/w (13). Phosphorus is purposefully added during the smelting process to bind with oxygen that would otherwise cause the copper metal to become brittle and prone to breaking if it were allowed to remain in the finished pipe.

A logical question is whether phosphorus bound in copper metal can be liberated from its copper matrix to the environment. A recent study examined the phosphorus composition in scale on the surface of DHP copper using X-ray photoelectron spectroscopy (14). The study found that phosphorus was concentrated in a cuprous oxide phosphate layer on the surface of the pipe; showing that phosphorus trapped in DHP copper metal can be mobilized from the pipe matrix to the environment. However, these results were obtained when the copper pipe had been exposed to 770 K and 0.1 Torr of oxygen. Clearly, these circumstances are not present within potable water systems; however, it showed that phosphate has the potential to form during DHP copper scale formation and become bioavailable.

In potable water, most bacteria are sessile (attached to surfaces) while very few are planktonic (free floating). To remain sessile, some microbes form a structure called a biofilm. A biofilm is a large structure made of extracellular polymeric substances (EPS) that hold microbes within it. Biofilms are not unique to potable water; they are ubiquitous and can be found in many environments. Rarely are biofilms composed of a single bacterial species; typically, they hold a wide range of species and metabolisms (2).

The formation of biofilms can be characterized by five developmental stages (3). In the first stage, reversible attachment, microorganisms collect on a surface where they have the potential to accumulate. Suspended solids or corrosion byproducts can also become associated in this early structure. Microorganisms are reversibly bound to the surface and can be swept away or detached during this stage. Interestingly, aquatic humic substances (humic and fulvic acids) have been suggested to play an important role in mediating the first stage of biofilm development (4, 10). The second stage is EPS production. Microorganisms are now irreversibly attached to the surface in a monolayer and they begin excreting EPS. In the third stage of biofilm development, cells become multilayered communities within a single EPS structure. In the fourth stage of biofilm maturation, water channels begin to form within the biofilm, making the biofilm somewhat resemble a kitchen sponge, with the microbes inside of the sponge and the sponge structure made of EPS. In the fifth stage of biofilm development, cells are released from the mature biofilm (erosion) to colonize new surfaces and begin the cycle again. In premise plumbing, the time development of each stage is dependent on a multitude of factors, but only a few have been well characterized. For example, in non-chlorinated systems, piping material is most influential on biofilm development (2, 3). Also, older pipes with rougher surfaces lead to faster establishment of biofilms than smooth, younger pipes. Interestingly, the difference in time to establish has little effect on the final mature biofilm consortium diversity and presumably EPS structure (2).

The defining feature of biofilm is EPS. EPS is a large structure of secreted biological compounds composed of carbohydrates, proteins, lipids, and nucleic acids. The ratio of these four constituents within biofilm will change depending on nutrient

conditions. For example, when limited for phosphate, a biofilm is more carbohydrate based and contains less protein. The opposite is true for ample phosphorus conditions, protein becomes a greater constituent of biofilm and carbohydrate becomes less. A recent study examined biofilm growth in annular reactor systems supplemented with three different concentrations of phosphate: 3, 30, and 300 μg/L (15). Biofilm growth was found to increase in the 30 and 300 μg/L tests but not the 3 μg/L test. Measurements of total protein and total carbohydrates within the biofilms found more protein and less carbohydrate in biofilms from the 30 and 300 μg/L amended phosphate tests than the 3 μg/L test.

EPS structure in biofilms is heterogeneous; during biofilm maturation microenvironments set up within the biofilm. Microenvironments are pockets within the same EPS structure that provide different living conditions allowing the biofilm to support a wide variety of microbial metabolisms. In essence, this allows the community of microbes within the biofilm to be more genetically and metabolically diverse in their nutritional needs. The advantage to this is that the microbes are able to utilize each other's by-products as resources and recycle them amongst themselves. This leads to an accumulation of resources within a small area as compared to the oligotrophic bulk water environment.

Biofilms provide many advantages to the microbes they harbor. EPS can be used as a storage system for nutrients. It chelates metals in the water, allowing microbes within the biofilm to survive in high metal concentrations commonly seen in corrosive systems. Bacterial growth and replication will occur faster in biofilm than in planktonic suspension. It provides protection from disinfection agents such as chlorine, significantly

raising the amount of disinfection agent needed for removal. Effective disinfection of microorganisms in drinking water is a major concern to public safety. Biofilm can provide a sanctuary that pathogenic bacteria can shelter in to escape disinfection. If an erosion or sloughing event were to occur, the biofilm and any pathogenic bacteria it may harbor would be released to the bulk water used for drinking.

Not all biofilms are the same, given structure and composition of EPS is heavily dependent on the environmental conditions of the system. Microorganisms will not be evenly dispersed within a biofilm and there are few microbes in direct contact with the metal pipe. Because of a biofilm's heterogeneity, nutrients are not shared equally resulting in enzymatic expression within a biofilm being temporally and spatially distinct (16). Expression will be temporally distinct during biofilm life cycle stages and spatially distinct in different microenvironments within the same biofilm.

Biofilms that establish in potable water systems are normally composed of a wide variety of bacterial species (17). However, some phyla, such as Proteobacteria are common to potable water systems. Sulfate reducing bacteria and sulfur oxidizing bacteria belong to the Proteobacteria phylum and are commonly found in potable water prone to microbially induced corrosion (11, 18, 19). Sulfate reducing bacteria are anaerobic bacteria that reduce sulfate to sulfide, a corrosive waste product. Sulfur oxidizing bacteria are aerobic bacteria that oxidize sulfide to sulfate. Fungi and Archaea have also been found in biofilms, although their contribution to the biofilm system is not well understood (2, 17).

The precise mechanism of microbial induced corrosion (MIC) is debated, but many studies indicate that acid metabolites, metal-binding exopolymers, and waste

products such as hydrogen sulfide are primarily responsible for MIC (8, 19, 20, 21, 22). Recently, reports have begun looking at the role extracellular ligands and enzymes have to play in MIC. Likely, it is a combination of factors that allows MIC to occur. Acid metabolites have been the most studied as they are suspected to cause MIC due to their chemical nature (3, 8, 18). Exopolymers such as humic acids and xanthan have been implicated as metal sequestering polysaccharides (21). Extracellular ligands, such as siderophores, have been implicated in destabilizing metal scales, leading to MIC (22). Siderophores are metal scavenging compounds excreted by microbes to take up metal ions, usually iron. Certain compounds such as acylated homoserine lactones, proteins, and peptide have also received attention as biofilm forming and MIC signaling compounds (16, 23). One such potential compound is the enzyme bacterial alkaline phosphatase (BAP).

BAP is an exoenzyme secreted by a variety of bacterial species that removes phosphate esters from organic compounds. BAP concentration is inversely proportional to phosphate concentration and thus will have a higher activity in low phosphate environments (24, 25, 26). In *E. coli*, BAP is expressed under phosphorus-limited conditions as part of the *Pst* system. The *Pst* system produces transport proteins and exoenzymes to scavenge phosphate from the environment when external phosphate concentrations are below 20 μM (25). At external phosphate concentrations above 20 μM, the low affinity, constitutively expressed *Pit* system will transport phosphate and BAP will be repressed to its basal level. Using the *Pit* system, cells rely on the proton motive force for energy to take up phosphate from the environment. In extremely phosphate limited environments, BAP can constitute as much as 6% of *E. coli* total

protein production (26). The large degree to which BAP can be expressed and the fact that it is expressed extracellularly makes it a convenient enzyme to measure.

The importance of BAP to this experiment is that it provides an easily quantifiable measure of phosphorus nutritional needs of a biofilm. As stated above, biofilms are heterogeneous, there will be differing nutritional needs and living environments within the same EPS structure. Measurement of a microbial community response is far more informative than assumption of phosphorus condition based on a known phosphorus addition.

A recent experiment tested the impact that corrosion has on nutrient release from a metal (27). The experiment focused on the potential for bacterial growth due to phosphorus, carbon, and nitrogen release from iron metal. Iron is another metal that contains trace amounts of phosphorous (0.03 – 0.2% wt/wt). The researchers exposed cast iron metal to various pH concentrations along with three different phosphorus chemical species to assess their stability in solution. They concluded that corrosion of 0.2% phosphorus-containing iron would be a significant source of phosphorus for microbes to use for growth. This experiment, however, was purely chemical. Microbes were not grown in these systems and potential bacterial populations that could be supported by the corroding metal were derived mathematically. We modified this experiment, primarily directed towards a biological perspective, to determine if phosphorus contained in copper can be a phosphate source for microbes sheltered within biofilms.

The purpose of this experiment is to examine if phosphorus limitation will promote microbial induced corrosion of copper and in the process liberate copper bound

phosphorus for microbial growth. We hypothesize that biofilm formation and microbial induced corrosion of copper will occur in phosphorus-limited environments and corrosion of DHP copper will release phosphorus that can be used for bacterial growth. Two objectives were used to address the above hypothesis:

1) To determine if phosphorus limitation causes biofilm formation and subsequently microbial induced corrosion to the copper surface.
2) If microbial induced corrosion occurs, to determine if phosphorus can be released during corrosion and become available for assimilation by microbes.

MATERIALS AND METHODS

Experimental Design

Two variables were manipulated for this experiment; the concentration of phosphorus in the bulk water and the copper metal grade used as a surface for biofilm attachment and microbial induced corrosion. A two by two factorial design was used to create four different treatment conditions:

1) Phosphorus ample environment with copper grade C12200 coupon (PA DHP)
2) Phosphorus ample environment with copper grade C10100 coupon (PA Pure)
3) Phosphorus limited environment with copper grade C12200 coupon (PL DHP)
4) Phosphorus limited environment with copper grade C10100 coupon (PL Pure)

Each treatment condition was performed in replicates of four, for a total of sixteen treatments. Each treatment was housed in its own "Biofilm Chamber" which was used to contain the bulk water and six suspended copper coupons. Bulk water was a minimal nutrient broth designed to promote rapid growth of biofilm. Although this does provide more nutrients than would be found in drinking water, it has the benefit of having a known composition, which helped control nutrient variation. Each chamber received an identical biofilm source inoculum that was collected from well water. Spent bulk water effluent was collected weekly and analyzed for copper concentration and alkaline phosphatase activity. After 5 weeks of growth, three coupons were collected from each biofilm chamber and biofilm was tested for total protein, total carbohydrate, microbial productivity, biofilm bound copper, and biofilm alkaline phosphatase activity.

Biofilm Chamber Rig

The biofilm chamber was constructed of PVC and plastic materials; the only metal used in the chamber was the copper coupons. Biofilm chambers were constructed using 3'' diameter PVC tubing, 3'' diameter PVC dome cap, ¾'' plastic quick connect male valve, plastic pegs, 3'' diameter PVC flat cap, and 3'' long plastic tubing stuffed with cotton. A schematic of a biofilm chamber is shown in FIG 1.

Sixteen chambers were constructed and placed around a rig used to hold the biofilm chambers in place. A picture of the biofilm chamber rig system is shown in APPENDIX A. Prior to use, each biofilm chamber rig was cleaned with soapy water and disinfected with 1-Step cleaning solution (Northern Brewer, St. Paul, MN).

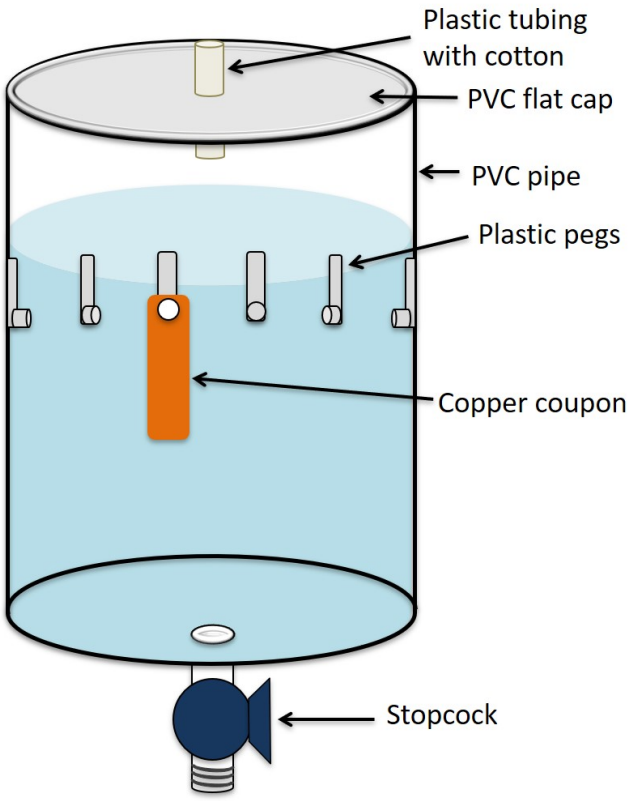

Figure 1. Biofilm chamber diagram: A total of sixteen chambers were constructed.

Copper Coupons

Both Pure (C10100) and DHP (C12200) copper coupons were manufactured as sheet cold rolled strips, measuring 1/2 x 3 x 1/16 inch, and purchased from Metal Samples Co. Munford, AL (APPENDIX B). Copper coupons were chosen for experimentation over copper tubing for two reasons, convenience, and chemical consistency. Copper coupons have a small profile as compared to traditional tubing, allowing the biofilm chamber size to be scaled down, thus requiring less bulk water and lab space. Additionally, the copper coupons have a known chemical composition (TABLE 1) and were simpler to collect biofilm sample and perform microscopy. Coupons were separately packaged and shipped directly to the lab after manufacturing,

avoiding any handling or transport contamination that could occur with store bought copper tubing.

Table 1. Elemental Composition of Copper Coupon Metal Grades[a]

Element	Total Composition (%)	
	C10100 (Pure)	C12200 (DHP)
Cu	>99.99	>99.99
O_2	0.00010	NT[b]
Sb	0.00010	NT
As	0.00010	NT
Bi	0.00010	NT
Cd	0.00010	NT
Fe	0.00010	NT
Pb	0.00010	NT
Mn	0.00001	NT
Ni	0.00010	NT
P	0.00020	0.02400
Se	0.00010	NT
Ag	0.00170	NT
S	0.00030	NT
Te	0.00010	NT
Sn	0.00010	NT
Zn	0.00010	NT

[a] Values are reported as described in the Certificate of Conformity provided by Metal Samples Co. for each metal grade manufacturing batch ID.
[b] Not Tested

Coupon Activation

Coupon activation was performed on all coupons to clean and chemically roughen the surface of the copper coupons for enhanced biofilm attachment. Coupon activation was modified from the method described by Fang, Hu, and Ong (15). The protocol for coupon activation is as follows; each coupon was gently shaken in 0.01 M HCl for 30 seconds, removed, and then rinsed with DI to remove the acid from the coupon. The coupon was then gently dipped in 100% methanol to drive off water from the coupon surface and air dried for approximately 10 minutes to allow the methanol to evaporate.

Following coupon activation, each coupon was placed in its respective biofilm chamber on the plastic pegs and then bulk water was added to the biofilm chamber.

Bulk Water

A modified R2B solution (28) was prepared weekly and used as the bulk water, which completely submerged the copper coupons. Two different R2B solutions were prepared from dry powder to create phosphorus ample and phosphorus limited environments (TABLE 2). The phosphorus ample bulk water had a phosphorus concentration of 30 mM. The phosphorus limited bulk water had a phosphorus concentration less than 0.01 mM. Biologically available phosphorus was determined by the presence of extracellular phosphatase enzyme, which will only be present in phosphorus limited environments. The phosphatase assay was used as a check to ensure phosphorus was either limited or ample to microbes in their chamber.

Phosphorus limited bulk water was prepared by weighing 1.86 g of the stock dry powder described in TABLE 2 and dissolving in 6 L of deionized water in a disinfected carboy. Phosphorus ample bulk water was prepared by weighing 2.15 g of stock dry powder described in TABLE 2 and dissolving in 6 L of deionized water. Both solutions were pH adjusted to 7.0 s.u. ± 0.5. The exact amount of dry stock weighed, and final pH of the solution, were recorded for each bulk water replacement.

Table 2. R2B Batch Dry Powder Composition

Chemical	Amount in stock (g)
Yeast Extract	0.015
Casamino Acids	0.300
Dextrose anhydrous	0.300
Soluble Starch	0.300
Sodium Pyruvate	0.180
Potassium Phosphate (K_2PO_4)[a]	9.000
Magnesium Sulfate anhydrous	0.300
Potassium Chloride[b]	7.650
Protease Peptone	0.300

[a] Chemical was not added to phosphorus limited stock
[b] Chemical was not added to phosphorus ample stock

Bulk Water Replacement

Bulk water was replaced on a weekly basis by discarding the stagnant bulk water in the biofilm chamber out through the bottom quick connect valve. A portion of the spent bulk water was collected for alkaline phosphatase and total copper analysis at this time. Bulk water was collected in a 10 mL conical tube by taking small aliquots of bulk water as it exited through the quick connect. A portion of the collected bulk water was used immediately for the alkaline phosphatase assay and the remainder was preserved with 70% trace metals grade nitric acid to a final pH lower than 2.0 s.u. for later use in total copper analysis.

Once the spent bulk water was drained from the biofilm chamber, 600 mL of freshly prepared bulk water was added to the biofilm chamber using a PVC replacement tube, which extended to the bottom of the biofilm chamber (FIG 2). Bulk water was then added through the PVC replacement tube to minimize disturbance of the biofilm that would have otherwise occurred if the bulk water were added directly into the biofilm

chamber. The PVC replacement tube was cleaned and disinfected with 1-Step cleaning solution and a DI water rinse prior to use in each biofilm chamber.

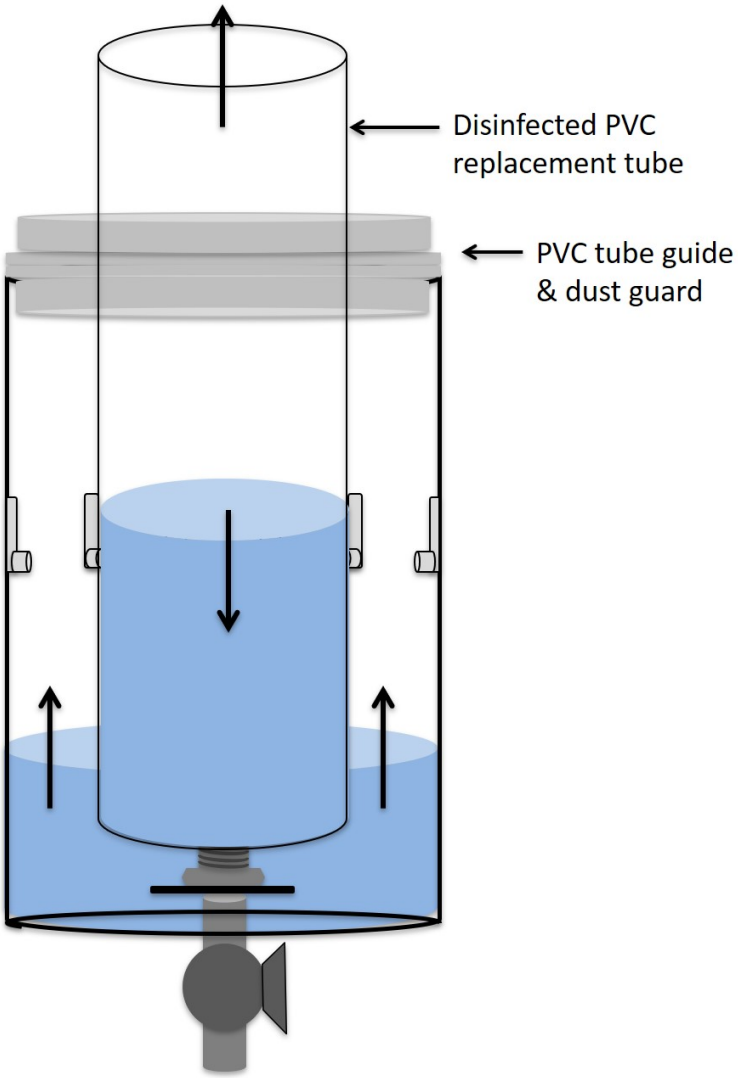

Figure 2. Bulk water replacement PVC tube. The PVC replacement tube and guide/dust guard were disinfected with 1-Step cleaning solution and then placed on the biofilm chamber as shown. The PVC tube is lowered through the guide to the bottom of the chamber followed by bulk water being poured down the center of the PVC replacement tube. The PVC replacement tube is then slowly lifted out of the biofilm chamber, releasing the water from the replacement tube into the biofilm chamber.

Inoculum

Cowley Hall well water from the University of Wisconsin - La Crosse was used as the bacterial source to inoculate the biofilm chambers. Twenty liters of well water was collected in a cleaned and disinfected carboy and then filtered through a 0.22 μm Millipore Durapore Membrane filter. The effluent water was discarded, and the cells collected on the filter were scraped into 20 mL of 0.9% saline solution from the filter using a sterile cell scraper. The solution was then mixed, and 1 mL of the solution was dispensed into each biofilm chamber.

Experiment Timetable

The experiment was conducted over a five-week period. After inoculating the bulk water of the biofilm chambers containing the activated coupons, the chambers were allowed to equilibrate for one week at room temperature. At the end of week 1, bulk water samples were collected and fresh bulk water completely replaced seven-day old bulk water in the biofilm chambers. Bulk water sample collection and replacement continued to week 4. At the end of 5 weeks the final bulk water sample was collected and three coupons were removed from the chamber for biofilm collection.

Biofilm Sample Collection

Biofilm samples were collected by removing a coupon from its biofilm chamber with a flame sterilized tweezers and placing it in a Nesco 4 oz Whirlpak bag with 2.5 mL of sterile 0.9% saline and 0.01 M magnesium chloride solution. The copper coupon was wetted with the saline magnesium chloride solution and then a sterile rubber policeman was used to scrape biofilm off of the coupon and into the Whirlpak bag. Once scraping was completed, an additional 2.5 mL of 0.9% saline and 0.01 M magnesium chloride

solution was used to wash the coupon, collecting the wash in the Whirlpak bag. This process was repeated with an additional two coupons from the same chamber being scraped into the same Whirlpak bag, comprising one sample. Each sample was homogenized by mixing the closed Whirlpak bag on a vortex and then transferring to ice water prior to use. The final volume of the biofilm sample was 10 mL. Each scraped copper coupon, after it was air dried for 1 hour, was stored at room temperature in the paper envelope it was shipped in for later SEM analysis.

Bulk Water Effluent Tests

Bacterial Phosphatase Assay (BAP): The bacterial phosphatase assay using a 96-well plate and 96-well plate reader was used to quantify the amount of extracellular phosphatase enzyme produced by bacteria in the chamber. Samples for the phosphatase assay were collected weekly from bulk water effluent, which had been in contact with copper coupons and biofilm for seven days. The test was conducted following the protocol described by Fang and Metcalf (26) with a few modifications as described below which allow for the assay to be performed in a 96- well plate.

Chromogenic substrate para-nitrophenylphosphate (pNPP) was prepared to a stock strength of 10 mM in sterile 0.5 M Tris-HCl (pH 8.0) and 0.01 M magnesium chloride. In a 96-well clear bottom plate, 100 µL of bulk water sample was added to the proper well locations in triplicate. Control wells contained deionized water and was aliquoted in triplicate to the proper wells on each plate. To the control and sample wells, 100 µL of pNPP was aliquoted to the wells using a multichannel pipette. The 96-well plate was then incubated at 37°C for 30 minutes. Following 30 minutes of enzymatic

reaction, the 96-plate was read on a plate reader at 410 nm to measure the formation of the yellow colored product, para-nitrophenol. The concentration of para-nitrophenol was calculated using its extinction coefficient: 15,460 $M^{-1}cm^{-1}$.

Total Copper: Microwave Plasma Atomic Emission Spectroscopy (MP-AES) was used to quantify the amount of copper released from copper coupons to the bulk water effluent. Bulk water effluent was collected after exposure to copper coupons and biofilm every seven days. Approximately 10 mL of bulk water effluent sample, remaining after an aliquot was removed for the BAP assay, had 70% trace metals grade nitric acid added to a final concentration of 1%. Unfiltered samples were then analyzed on an Agilent 4100 nitrogen based MP-AES (29).

Biofilm Tests

Anthrone Test: The anthrone test was used to quantify the amount of carbohydrate in the biofilm on the copper coupons. Glucose was used as a reference chemical to generate a standard curve. The test was conducted following the protocol from Jermyn (30) with a few modifications as described below.

Anthrone solution was prepared using 200 mg of anthrone reagent in 80% sulfuric acid, not 20 mg, as specified by Jermyn (30). Test tubes containing a mixture of anthrone solution with sample or standard were not vortexed to mix. Test tubes were gently rocked back and forth to mix, minimizing bubbling. Sample and standard solutions were given 10 minutes for bubbles to dissipate from cuvette walls prior to reading absorbance at 630 nm. Sample carbohydrate concentration was determined from a standard curve and then converted to total amount of carbohydrate scraped form the copper coupons.

Bradford Test: The Bradford test (31) was used to quantify the amount of protein in the biofilm on the copper coupons. Bovine serum albumin was used as a reference chemical to generate a standard curve. The test was conducted following the method with a few modifications as described below.

Coomassie Brilliant Blue G-250 (2.5 mL) was added to 13x100 mm glass test tubes. To the appropriate test tube, 0.2 mL of biofilm sample or standard was added and then gently vortexed to mix. The solutions reacted for 5 minutes at room temperature and then the absorbance of each solution was read at 595 nm in a disposable plastic cuvette. Absorbance measurements were taken within an hour of setting up the reactions. Sample protein concentration was determined from a standard curve and then converted to total amount of protein scraped from the copper coupons.

Microbial Productivity: Productivity was estimated based on the incorporation of radiolabeled tritiated leucine by the bacteria in each biofilm sample. The test was conducted following the protocol from McDonough, Sanders, Porter, and Kirchman (32) with a few modifications as described below.

A biofilm sample (4.5 mL) was aliquoted into a 15 mL plastic conical tube and then 0.5 mL of 0.01 M magnesium chloride in 0.5 M Tris-HCl (pH 7.4) was added. Tritiated leucine (25 nCi) was added to each sample, vortexed gently to mix, and incubated at 15°C for 30 minutes. Addition of 0.5 mL of 10% formaldehyde to each tube was used to stop the reaction. Trichloroacetic acid was added to a final concentration of 5% and the samples placed in an 80°C water bath for 15 minutes. After 15 minutes the samples were placed on ice for 30 minutes to precipitate macromolecules. The precipitate was collected using a filtration system fitted with 0.2 μm Nucleopore filter

(Whatman) supported by a GN6 filter (Whatman). The Nucleopore filters were transferred to a scintillation vial with 10 mL of scintillation cocktail liquid. Samples were analyzed using a Perkin Elmer Tri-carb 3110TR alpha counter. Productivity was calculated as total active cells.

Biofilm Bacterial Alkaline Phosphatase Assay: A 0.5 mL aliquot of biofilm sample was analyzed for BAP activity. The same procedure was used as described above for the bulk water analysis.

Biofilm Total Copper: A 2.0 mL aliquot of the biofilm sample was acidified with 70% trace metals grade nitric acid to a final concentration of 1% nitric to be used for MP-AES copper analysis as described above.

SEM Copper Coupon Analysis

Scraped copper coupons were analyzed for signs of microbial induced corrosion by SEM performed with a Zeiss EVO HD with Bruker EDS. Coupons were analyzed using a standard scanning approach of designating six evenly spaced regions to the coupon. Each region was scanned at low magnification (approximately 300x) to characterize the surface. If a unique feature was observed at low magnification, the feature was scanned at higher magnification (approximately 2500x) for better detail. Two scraped coupons were randomly selected from the three collected for each treatment and examined as described above.

Statistical Analysis

Two-way univariate analysis of variance (ANOVA) using SPSS statistical software was used for statistical analysis of the data. Data was collected in replicates of four. The anthrone test had a large outlier associated with the data. The Shapiro-Wilk

test for normality was performed on the anthrone data and did not reject the null hypothesis at a 5% level. A Kruskal-Wallis analytical test was then conducted to examine effects and results were found to be the same as ANOVA testing; meaning the outlier did not have an outsized effect on the data and ANOVA results could be used for analysis. The Shaprio-Wilk test for the other analytical procedures did not reject the null hypothesis so ANOVA analysis was appropriate to apply to the data sets.

RESULTS

Bulk Water Effluent

Spent bulk water from each biofilm chamber was collected on a weekly basis and analyzed for bacterial phosphatase activity (BAP) and total copper concentration. BAP activity was used as a measure for biofilm phosphorus need, primarily to ensure that R2B bulk water supplied to each biofilm chamber was correctly made and delivered to the proper chamber. Biofilm in need of phosphate will produce more phosphatase enzyme and thus have a greater BAP activity than a biofilm meeting its phosphate requirement. Data show that effluent BAP activity from Week 0 to Week 4 for phosphorus limited environments (PL) steadily increased while phosphorus ample environments (PA) decreased then remained low (FIG 3).

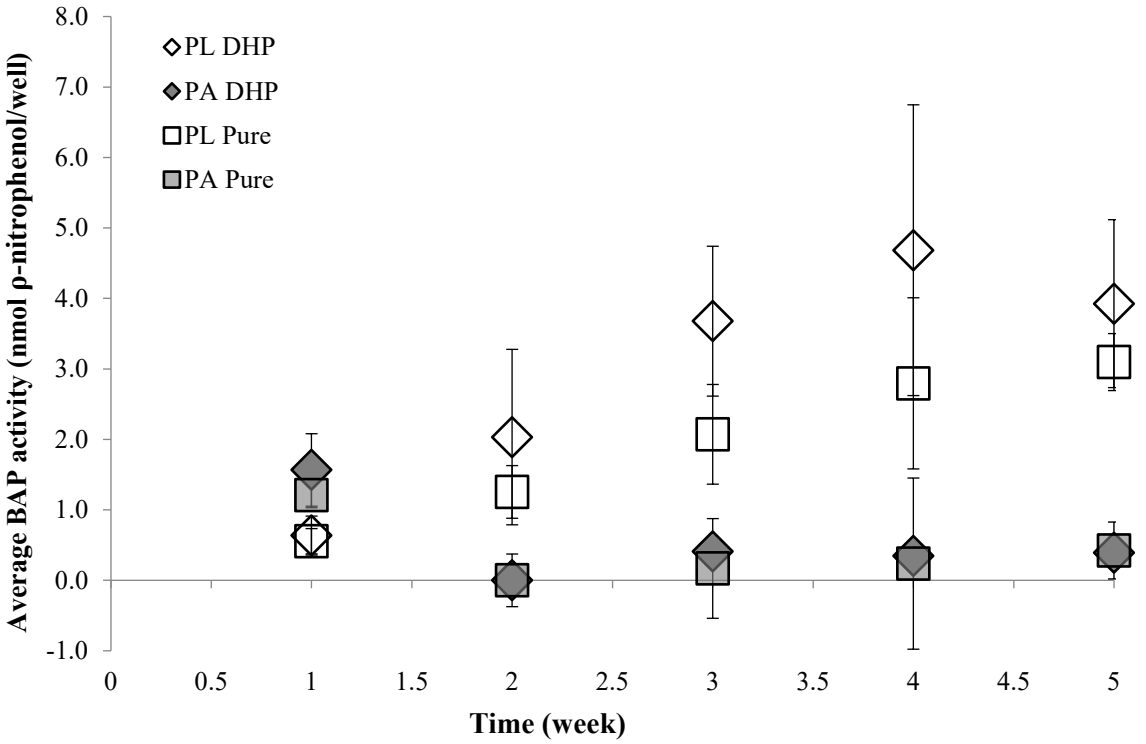

Figure 3. Comparison of extracellular bacterial phosphatase activity in biofilm chamber effluent. Effluent from chambers with copper grade DHP are depicted by (◊), effluent from chambers with copper grade Pure are depicted by (□). Effluent from phosphorus ample chambers are depicted by filled symbols, effluent from phosphorus limited chambers are depicted with open symbols. Error bars represent standard deviation. n = 4.

Total copper was analyzed in spent bulk water from each biofilm chamber as a measure of copper release from the coupon due to corrosion. Effluent total copper concentration from Week 0 to Week 4 for PL chambers slightly increased and had more total copper than PA chambers, which remained low during the entire experiment (FIG 4). During Week 1 biofilm chamber bulk water replacement it was noticed that PL biofilm chamber bulk water had a faint light blue tinge, and the walls of the biofilm chambers were stained blue, especially at the water line. Staining of the walls of the biofilm chambers increased with time. PA chambers did not have a faint light blue tinge to the bulk water or staining of the chamber walls.

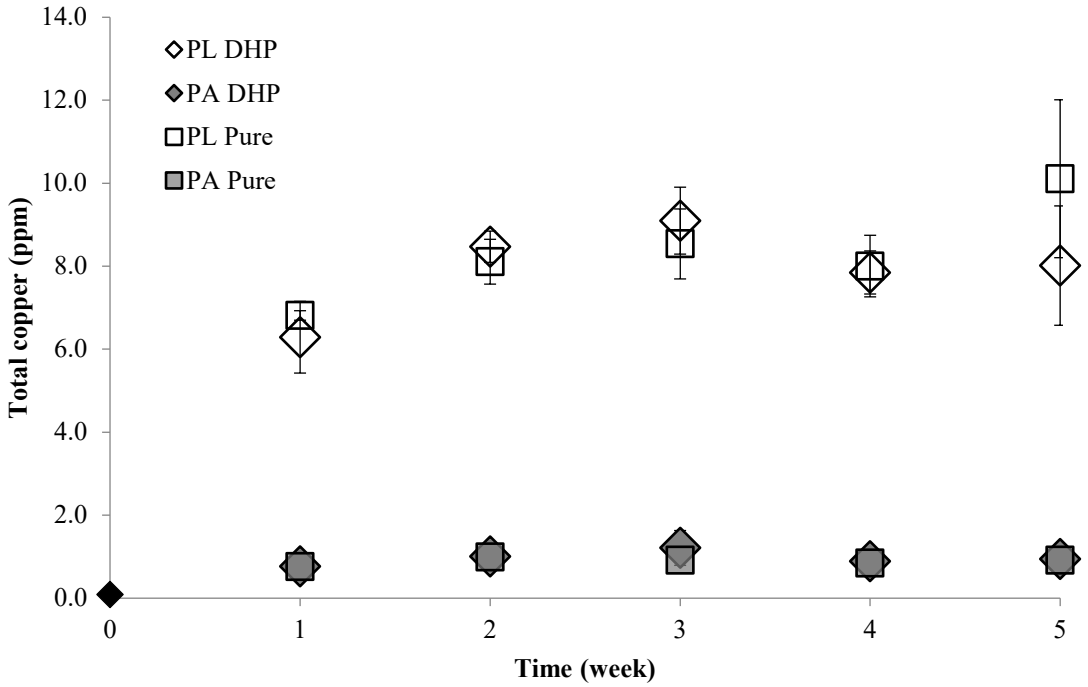

Figure 4. Comparison of total copper in biofilm chamber effluent. Effluent from chambers with copper grade DHP are depicted by (◊), effluent from chambers with copper grade Pure are depicted by (□). Effluent from phosphorus ample chambers are depicted with solid symbols, effluent from phosphorus limited chambers are depicted with open symbols. The modified R2B solution used as bulk water is depicted by the filled diamond. Error bars represent standard deviation. n = 4.

Coupon Biofilm

Copper coupons were scraped to remove all of the biofilm that had attached to the coupon surface for analysis and is here on referred to as "coupon biofilm". BAP activity, total copper, microbial productivity, total carbohydrate, and total protein were assayed on coupon biofilm samples.

BAP activity of coupon biofilm samples was assayed to assess if phosphorus conditions within the biofilm were different than the bulk water in contact with the

biofilm. If the biofilm became thick enough, nutrient conditions could differ between microbes within the biofilm in contact with the bulk water and microbes residing in the biofilm underside, leading to a different expression of phosphatase enzymes. Statistical analysis of biofilm phosphatase activity showed no significant difference between PL DHP and PL Pure treatments (FIG 5). Treatments PA DHP and PA Pure were statistically different from one another and from PL DHP and PL Pure. Treatments that were phosphorus limited had the lowest BAP activity followed by PA DHP with intermediate activity and PA Pure with the greatest activity.

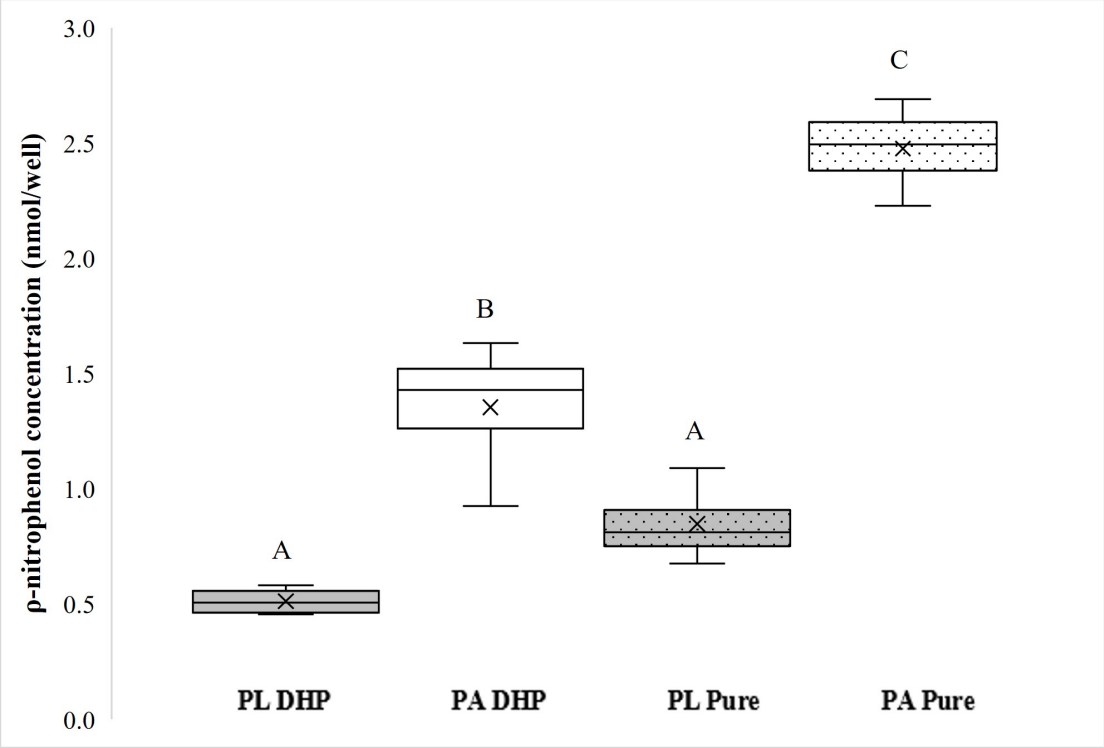

Figure 5. Comparison of biofilm associated bacterial phosphatase activity from coupon biofilm in the different biofilm chambers after 5 weeks of growth. Treatment data means are shown as solid black lines inside the box and the 25th and 75th percentiles are shown at the box bottom and top. Whiskers mark the 5th and 95th percentile. n = 4.

A total copper assay was performed on the coupon biofilm samples to assess the concentration of copper attached to the biofilm. Statistical analysis showed that biofilm bound copper did not differ significantly between treatments PL DHP, PL Pure, and PA DHP (FIG 6). The PA Pure treatment did significantly differ from the other three treatments having the lowest amount of copper. While replacing the bulk water during week 2 sampling, what appeared to be corrosion product was observed near the coupon hole on some of the coupons in the PA chambers. The suspected corrosion product on the PA chamber coupons looked like blue-green specks attached to the coupon surface. PL coupons did not show any visible signs of suspected corrosion product on the coupon surface.

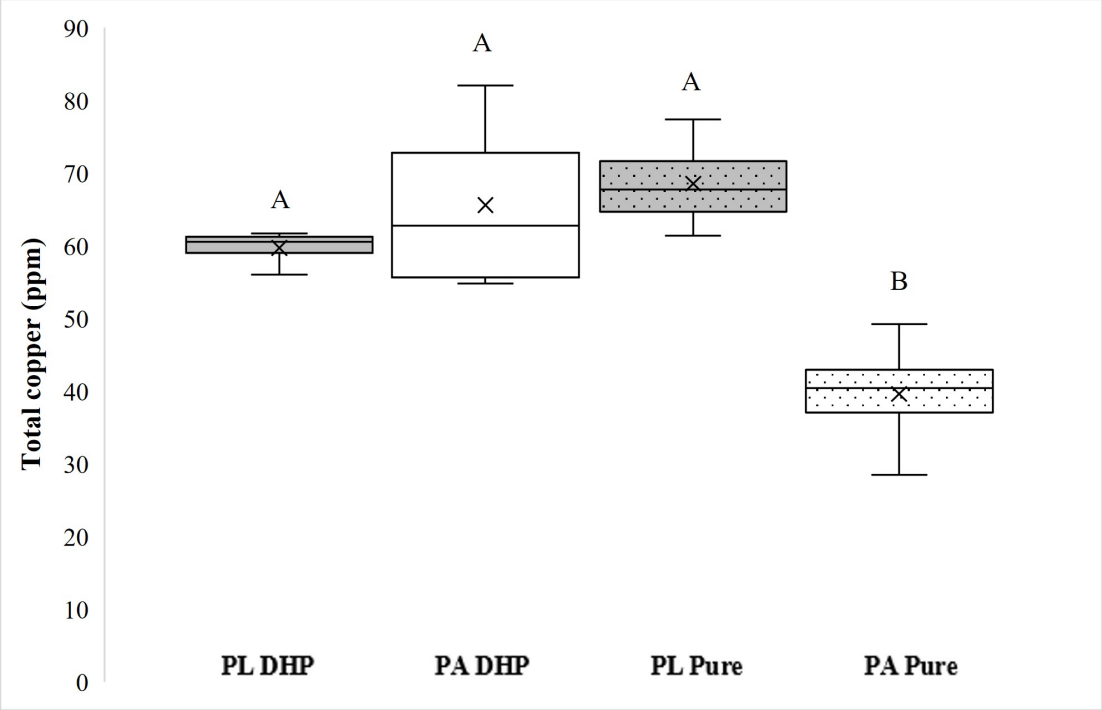

Figure 6. Comparison of total biofilm bound copper collected from coupon biofilm in the different biofilm chambers after 5 weeks of growth. Treatment data means are shown as solid black lines inside the box and the 25th and 75th percentiles are shown at the box bottom and top. Whiskers mark the 5th and 95th percentile. n = 4.

A total carbohydrate analysis was performed on the coupon biofilm samples to assess the amount of EPS production by bacteria in the biofilm. Comparison of EPS production is a method for determining phosphorus utilization. EPS production, under a steady carbon concentration, should increase under phosphorus limited conditions as compared to phosphorus ample conditions. Analysis showed no statistical difference in carbohydrate amount between phosphorus environment and metal grade treatments (FIG 7). While replacing the bulk water during week 1 sampling, it was noticed that PA chambers contained an orangish-brown fluff deposited at the bottom of the chambers. PL chambers contained what appeared to be a thinner layer of the orangish-brown fluff than the PA chambers. This orangish-brown fluff is suspected to be sloughed biofilm. During coupon collection a portion of fluff from the bottom of the chamber was inadvertently suspended and attached to the surface of one of the PA Pure coupons. That PA Pure treatment sample had a high carbohydrate amount and is shown in Figure 7 as the outlier data point, lending support to the idea that the fluff was sloughed biofilm.

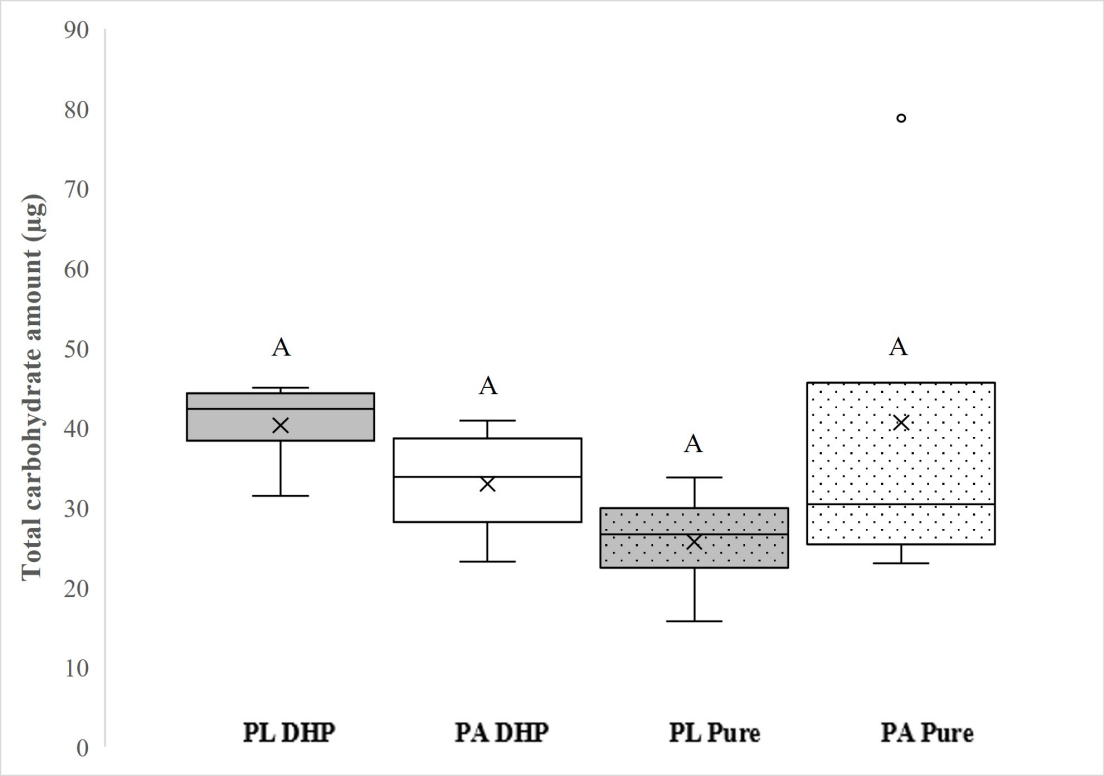

Figure 7. Comparison of biofilm associated carbohydrate from coupon biofilm in the different biofilm chambers after 5-weeks of growth. Treatment data means are shown as solid black lines inside the box and the 25th and 75th percentiles are shown at the box bottom and top. Whiskers mark the 5th and 95th percentile. The outlier is shown as an open circle outside of the whiskers. n = 4.

A total protein analysis was performed on the coupon biofilm samples to assess the amount of protein production by bacteria in the biofilm. Protein can be used as a proxy of bacterial growth. Comparison of protein production is a measure for determining phosphorus utilization, as growth will be restricted under phosphorus limitation. Protein production should decrease under phosphorus limited conditions as compared to phosphorus ample conditions. Statistical analysis showed that protein production was the only parameter that had two main effects, as it was influenced by the combination of phosphorus amount and grade of copper metal (FIG 8). Essentially, total protein was

related to the combination of phosphate amount and copper metal grade. Phosphorus ample environments resulted in more biofilm associated protein than phosphorus limited and, for a given phosphorus level, copper metal grade Pure resulted in more biofilm associated protein than DHP. PL DHP treatment had statistically the least amount of protein production, while PA DHP and PL Pure were similar, and PA Pure had the greatest amount of protein production. The outlier sample from the total carbohydrate analysis did not show an outlier in the total protein analysis. Indicating the fluff at the bottom of the chamber that attached to the coupon was primarily carbohydrate based and potentially was sloughed EPS.

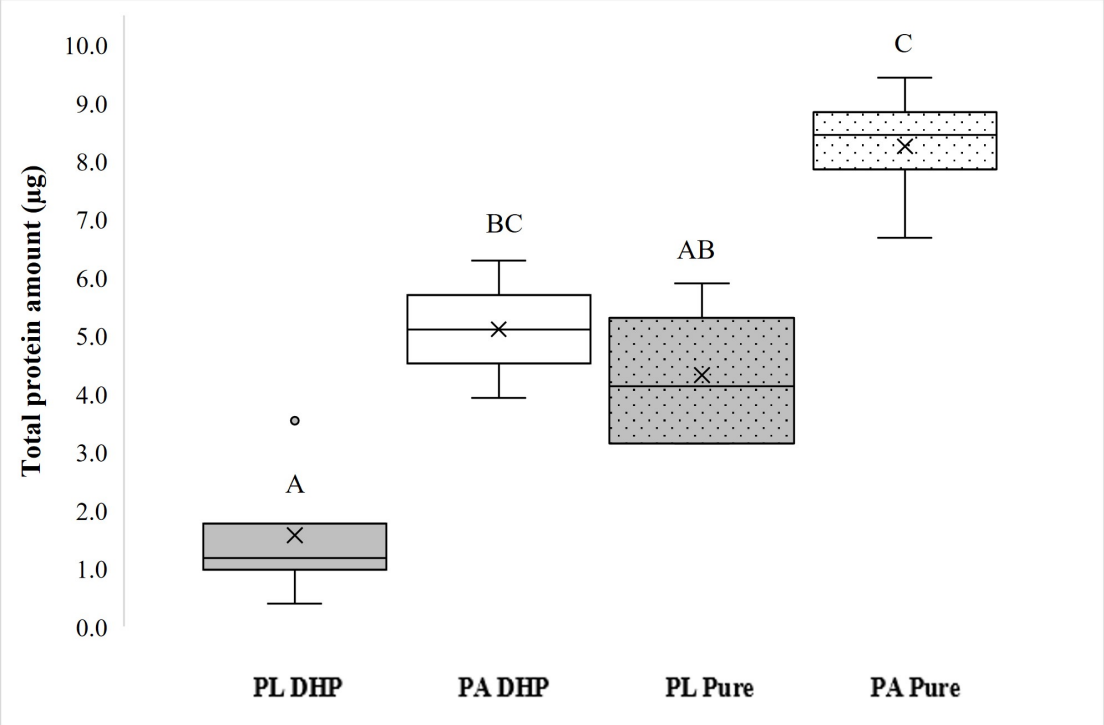

Figure 8. Biofilm associated protein amount collected from biofilm scraping of copper coupons in biofilm chambers after 5-weeks of growth. Treatment data means are shown as solid black lines inside the box and the 25th and 75th percentiles are shown at the box bottom and top. Whiskers mark the 5th and 95th percentile. The outlier is shown as an open circle outside of the whiskers. n = 4.

Coupon Microscopy

Appendix C shows scraped biofilm chamber coupons and activated and non-activated coupons. At a macroscopic level, there are clear differences in coupon coloration based on the treatment type. PA Pure was most similar in color to the activated and non-activated coupons. PL DHP and PA DHP were similar to each other being brownish-tan in color. PL Pure was also brownish-tan in color but had a colorful shimmer to its surface not seen in any of the other coupons.

Surface characterization of copper coupons was done using SEM to examine surface features of used copper coupons after the biofilm had been removed and of unused copper coupons. Microbial induced corrosion was inferred by the presence of pitting on the coupon surface. Coupons from each of the treatment trials and unused coupons that were activated and non-activated were analyzed by SEM to characterize their surface.

Microbial induced pitting of treatment coupons was suggested by surface features that had a distinct circular shape, burrowing into the coupon, vaguely resembling the look of a mine shaft (10, 33, 34). Analysis of unused activated coupons compared to activated coupons shows that the activation process does not produce pitting or pitting-like structures on the copper coupon surface. Topology of activated coupons showed uniform "mound" like features covering the surface of the coupon (FIG 9 A & B). Non-activated coupon topology had a uniformly flaky appearance with little three-dimensional structure (FIG 9 C & D).

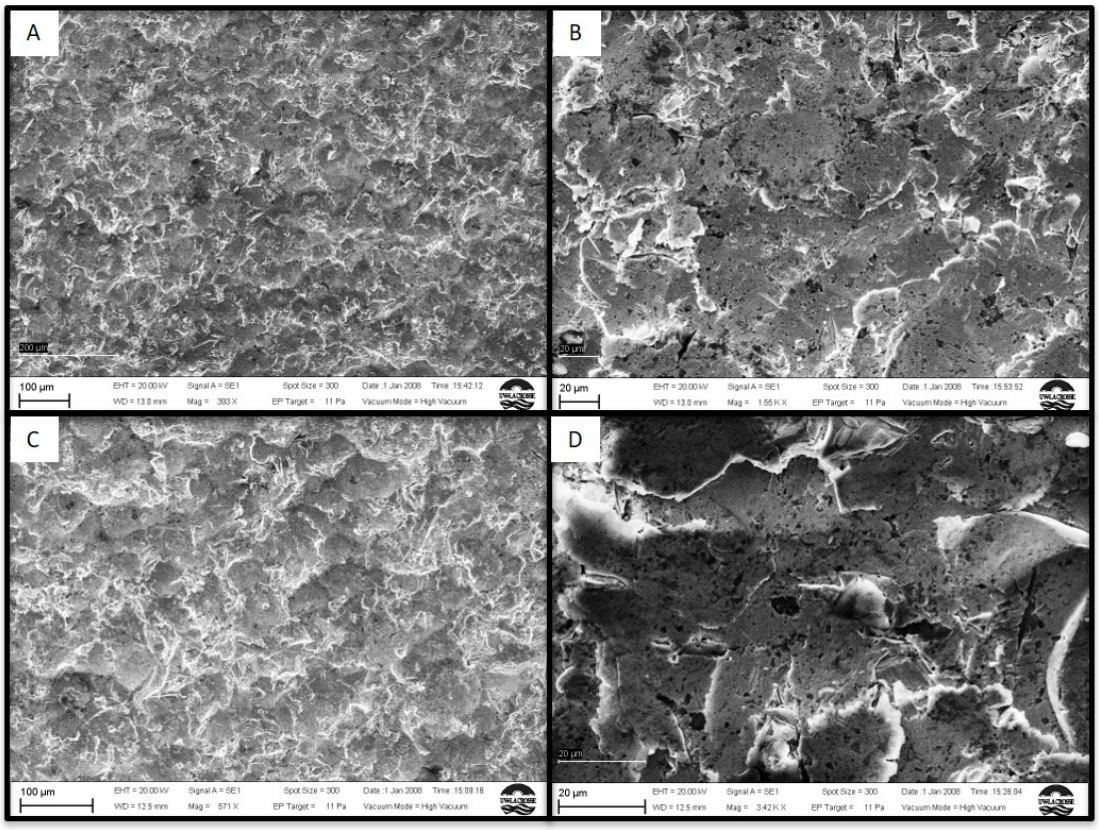

Figure 9. Comparison of activated and non-activated unused copper coupon surface features. Activated coupon is shown in panes A and B at 400x and 1,500x magnification, respectively. Non-activated coupon is shown in panes C and D at 550x and 3,500x, respectively.

Surface topology of coupons in treatment PA Pure did not show obvious characteristics of microbially induced corrosion nor were patches of biofilm observed on the surface. Unique among this treatment was the color pattern on the coupon surface (FIG 10, Pane A), which looked similar to marbling that you would see in a counter top. This feature was not ubiquitous throughout a single coupon but was found in patches on the coupon surface. Surface topology of coupons from treatment PA Pure did not greatly differ from activated and non-activated coupons.

Surface topology of coupons in treatment PA DHP also did not show obvious characteristics of microbially induced corrosion nor were patches of biofilm observed on

the surface. In general, the coupon surface looked similar to the activated coupon surface.

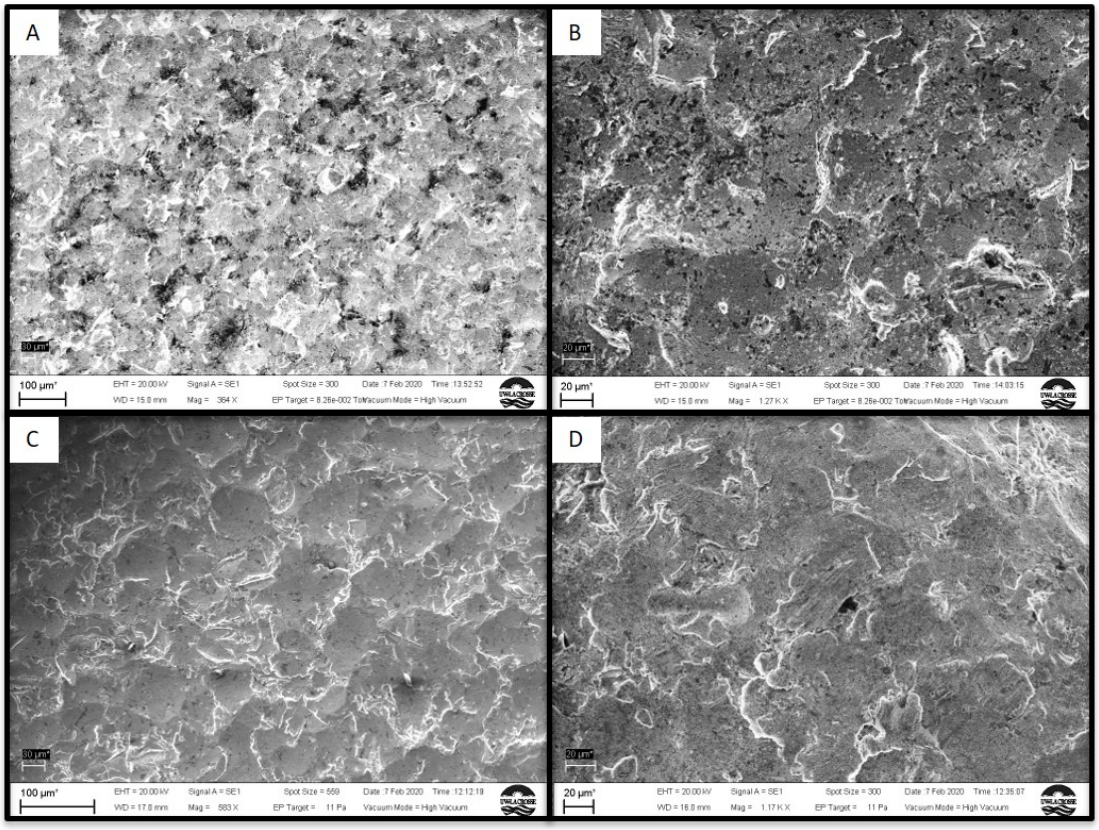

Figure 10. Comparison of treatment PA Pure and treatment PA DHP coupon surface features. Coupons from treatment PA Pure are shown in panes A and B at 350x and 1,300x magnifications, respectively. Coupons from treatment PA DHP are shown in panes C and D at 550x and 1,200x magnifications, respectively.

Coupon surface topology of PL Pure and PL DHP showed obvious characteristics of microbially induced corrosion (FIG 11). In general, most of the coupon surface looked similar to activated coupon surface but patches of biofilm or biofilm corrosion products were obvious. Pits were observed on coupons from treatment PL Pure that were approximately 25 µm in diameter with what appeared to be corrosion debris and/or biofilm surrounding it. Pitting was observed on coupons from treatment PL DHP that

were approximately 20 µm in diameter with what appears to be corrosion debris and/or biofilm surrounding it (Figure 11, indicated by red arrows).

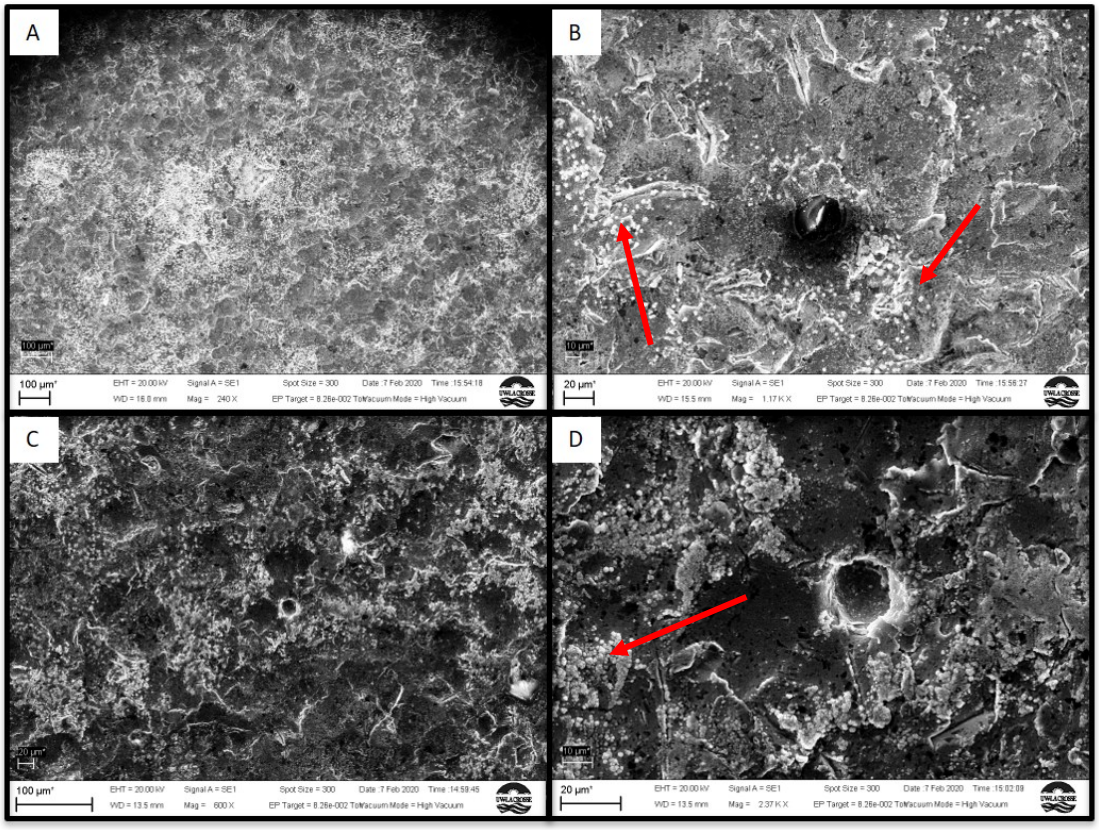

Figure 11. Comparison of treatment PL Pure and treatment PL DHP coupon surface features. Coupons from treatment PL Pure are shown in panes A and B at 250x and 1,200x magnifications, respectively. Coupons from treatment PL DHP are shown in panes C and D at 600x and 2,400x magnifications, respectively. Red arrows indicate areas that appear to contain corrosion debris and/or remnant biofilm.

The microbial productivity of each coupon biofilm was measured to compare the active population size within each of the biofilm samples from the different treatment chambers. This provides an idea of the carrying capacity of each of the treatments. Replicates of each treatment varied greatly for this assay. Statistical analysis showed that PA Pure had the greatest microbial activity on average while the other three treatments

were statistically similar (FIG 13). Interestingly, the outliers in assays for bacterial productivity and total protein for treatment PL DHP were from different coupons.

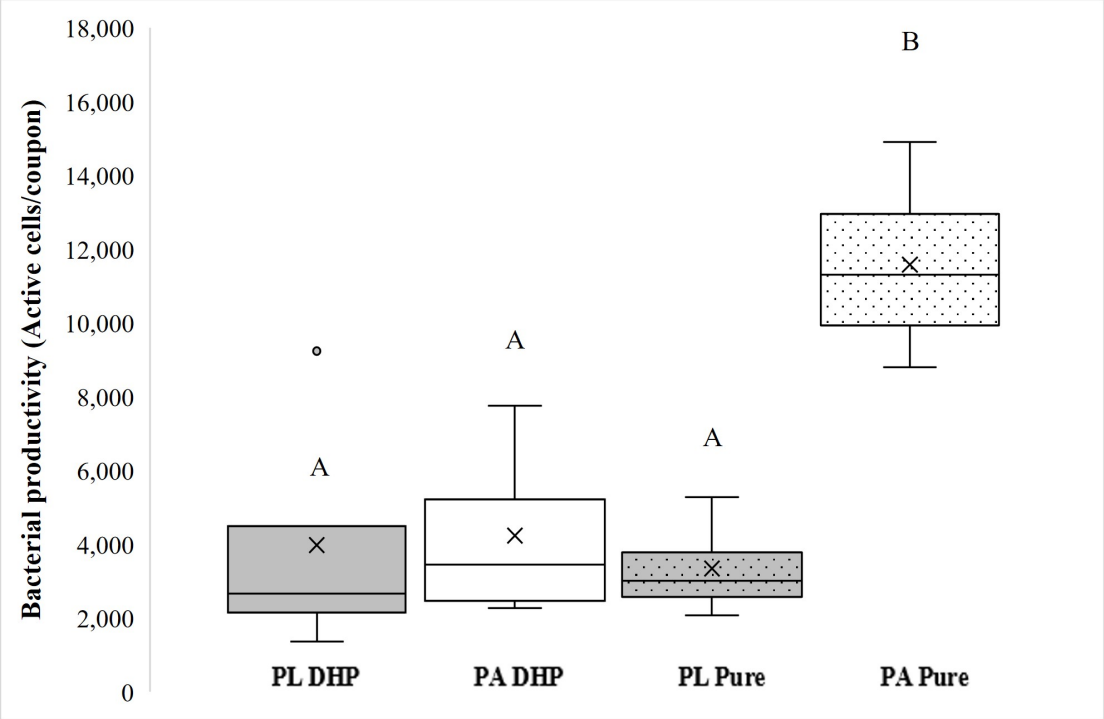

Figure 12. Comparison of bacterial productivity of active cells within coupon biofilm from the different biofilm chambers after 5 weeks of growth. Treatment data means are shown as solid black lines inside the box and the 25th and 75th percentiles are shown at the box bottom and top. Whiskers mark the 5th and 95th percentile. The outlier is shown as an open circle outside of the whiskers. n = 4.

DISCUSSION

Phosphorus availability and copper metal composition affect formation of biofilms and the potential for microbial induced copper corrosion to occur. Our hypothesis was that bacterial cells experiencing phosphate limitation would form more biofilm on the coupon surface and cause more corrosion to the coupon than cells that were not limited. The assumption being that biofilms form in response to an environmental stress; in our case limited phosphate. Formation of biofilm in a stress-inducing environment is advantageous to microbes because biofilm acts as a "sanctuary". It provides microenvironments, protects from anti-microbial agents, and can store nutrients. It also allows for a diverse bacterial consortium to establish within one structure. Clustering of microbes benefits the individual by concentrating interactions into a localized area versus spreading out over an undefined range. Beneficial interactions could be things such as nutrient recycling, which benefit individual microbes by allowing one microbe's waste product to become another's nutrient source.

Over the course of the experiment, more copper was released from coupons in the phosphorus limited chambers than those in the phosphorus ample chambers, regardless of the coupon metal grade. Interestingly, effluent data for the BAP bulk water (FIG 3) correlates with total copper effluent data, effluent from phosphorus limited chambers had greater BAP activity and bulk water copper concentrations than effluent from phosphorus ample chambers. This suggests that phosphorus-limited bacterial metabolism may contribute to copper corrosion. However, phosphate is a strong metal chelator and could potentially precipitate dissolved corroded copper in the bulk water. This would have implications towards the phosphorus ample chambers where dissolved corroded copper

could interact with free phosphate in the bulk water and precipitate out of solution, "hiding" it from analyses. To prevent this from skewing total copper results, sample collection involved sampling from multiple biofilm chamber water levels; with the intent of including precipitated metals that could have accumulated at the bottom of biofilm chambers.

SEM imaging further suggests that specific biofilm metabolic activity can contribute to copper coupon corrosion in a phosphorus limited environment. Imaging of scraped coupons showed pits in coupons from phosphorus limited biofilm chambers but not phosphorus ample biofilm chambers (FIG 10 and FIG 11). The absence of visible corrosion signs on coupons from phosphorus ample biofilm chambers implies that copper was not released to the bulk water. This could mean that there wouldn't be free copper in the bulk water to precipitate with phosphate because little copper was released to the bulk water from coupon corrosion for phosphate to bind with.

While there was little copper in the bulk water of phosphorus ample chambers, biofilm for all treatments contained a considerable amount of biofilm bound copper. As a reference point, the Lead Copper Rule enforced by the federal government sets the maximum copper level at 1.3 ppm. For comparison, converting total copper results to reflect proportional surface area to volume ratios realistically encountered in drinking water piping gives the following: PL DHP 6.0 ppm, PA DHP 6.4 ppm, PL Pure 6.8 ppm, and PA Pure 4.1 ppm. This is three to five times greater than allowed by the Lead Copper Rule. These data agree with current literature showing that biofilm and EPS components can sequester copper metal (2, 10). Common components of EPS are carboxylic acids, phenolics, and tannic acid functional groups, that can chelate metals

trapping them in the biofilm matrix. However, this finding appears to conflict with total carbohydrate results. Carbohydrate amounts did not statistically change between any of the treatments while total copper for PA Pure was statistically less than the other treatments. If EPS and biofilm matrix components were solely responsible for copper corrosion, we would expect to see similar total copper results given the amount of carbohydrate is similar across treatments. That does not seem to be the case here.

Our second hypothesis was if microbial corrosion did occur, as observed in the phosphorus limited chambers, phosphorus released from DHP copper metal corrosion would be available for microbial assimilation. Phosphorus utilization from corroded copper was determined by comparing protein and carbohydrate amount. If phosphorus from corroded DHP coupons was available for microbial use, we would expect to see greater protein and less carbohydrate in treatment PL DHP as compared to PL Pure. Literature has suggested that cells having access to adequate amounts of phosphorus will produce more protein and less carbohydrate, whereas the reverse is true for cells having limited access to phosphorus (15, 35, 36).

As expected, biofilm from phosphorus ample environments had more protein than biofilm from phosphorus limited environments. Unexpectedly, biofilms from coupon grade Pure had more protein than biofilms from coupon grade DHP. This would suggest that, while corrosion did occur in treatment PL DHP, phosphorus that was bound in copper metal was not used by microbes. It is unclear at the tested conditions as to why phosphorus was unavailable for use. This result seems to conflict with biofilm BAP data. BAP results show that biofilm from treatment PL DHP had low phosphatase activity, suggesting that phosphorus was available for use. Additionally, treatment PL DHP

showed visible signs of pitting to the coupon's surface. Taken together, these two results would suggest that corrosion to the coupon released phosphorus for use, which refute the protein results.

Surprisingly, biofilms across treatments had carbohydrate amounts that were statistically similar. We expected to see PL Pure biofilm with the greatest amount of carbohydrate and PA DHP biofilm with the least amount. Carbohydrate results suggest that phosphorus in treatment PL DHP was unable to be utilized by microbes. The fact that carbohydrate amounts across all treatments are the same is surprising given the amount of phosphorus between the treatments varied so greatly. Upon closer inspection of the literature, correlations between carbohydrate and protein were obtained when various phosphorus environments were supplemented with a single low concentration carbon source. R2B bulk water was a complex medium containing several carbon sources and other growth factors, which may be why we did not see the expected correlations from the literature. It has been speculated that the increase in EPS with phosphorus addition may be due to a shift from a phosphorus-limited to a carbon-limited environment (15). Considering R2B is a complex solution, this may mean that carbohydrate and protein are inadequate measures of utilization of the phosphorus released by copper corrosion at the tested conditions.

In summary biofilm formation took place in all of the treatment conditions regardless of phosphorus availability. Physical signs of corrosion to the coupon surfaces were observed in phosphorus limited environments, but not in the phosphorus ample environments. Bulk water total copper concentrations support this finding and confirm

our hypothesis that phosphorus limitation will lead to microbially induced copper corrosion.

Corrosion that occurred in the phosphorus limited environments, specifically to the DHP coupons, was unable to release sufficient copper-bound phosphorus for microbial use to be detected at the tested growth conditions based on comparing protein and carbohydrate trends as reported in the literature. Our use of a complex medium, rather than a single low concentration carbon source, may have affected protein and carbohydrate trends correlated with phosphorus concentration. Future experiments should aim to devise a different, ideally more direct, method for determining phosphorus utilization from corroded DHP copper.

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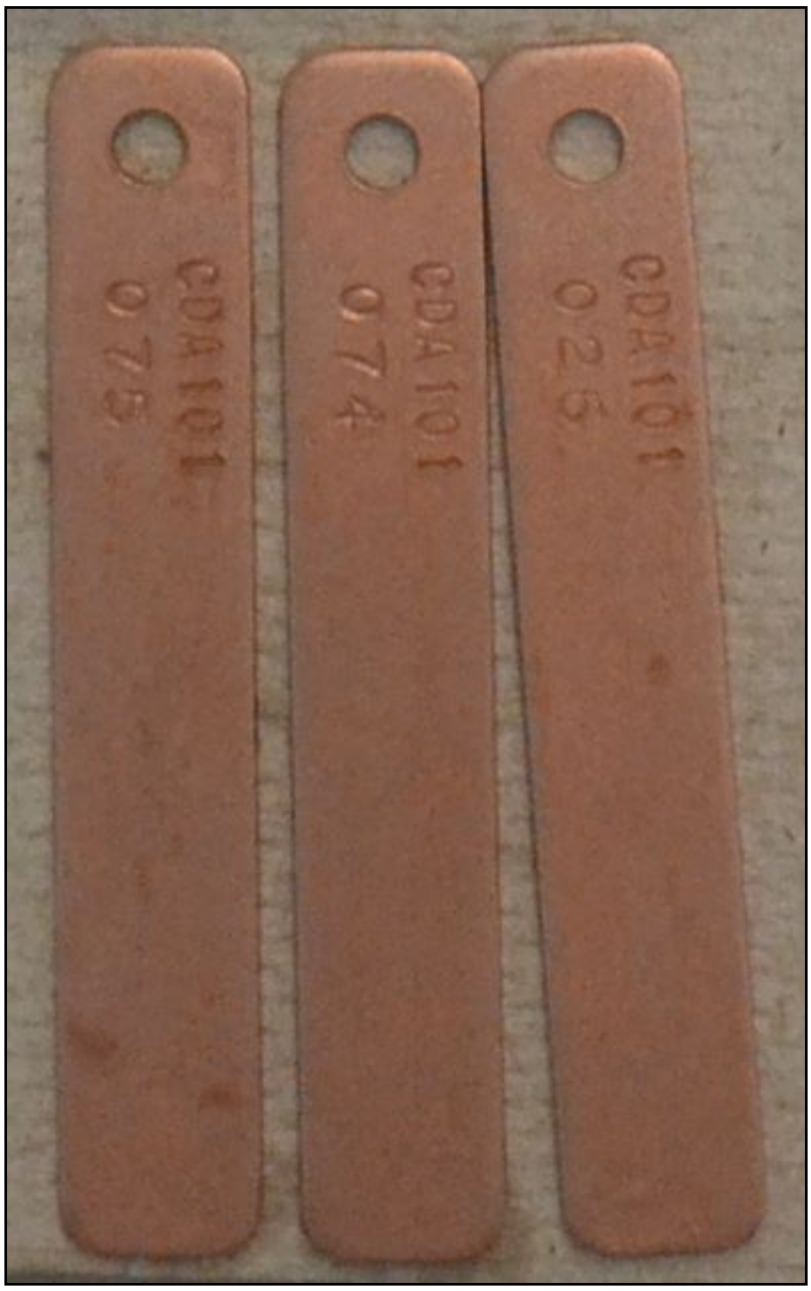
APPENDIX A

BIOFILM CHAMBER HOUSING SET UP

Biofilm chamber rig set up. The rig system used for experimentation was incubated in a laboratory room at room temperature (approximately 20°C).

APPENDIX B

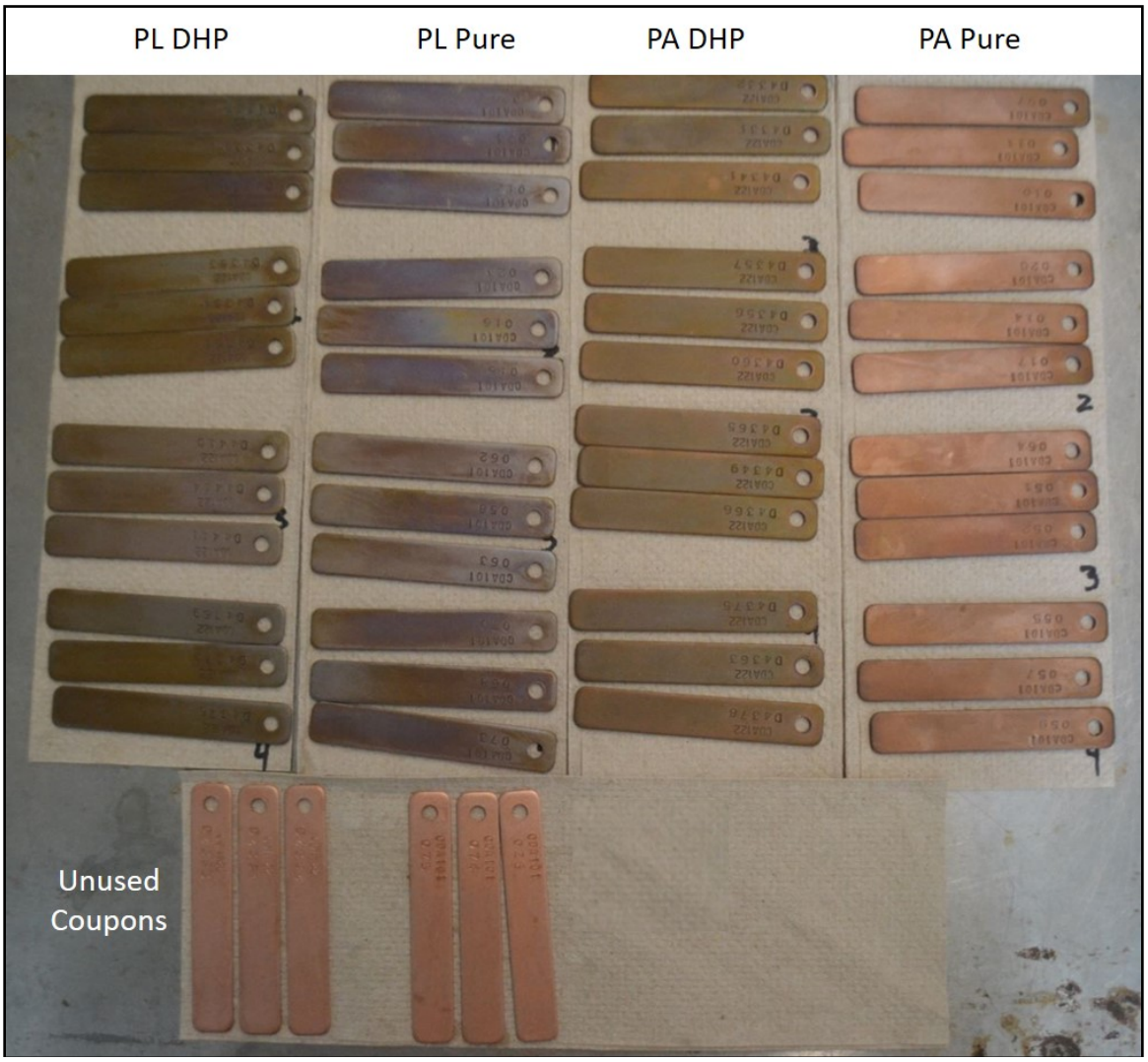
METAL SAMPLES CO. COPPER COUPONS



Copper coupons used as a biofilm growth surface.

APPENDIX C

SCRAPED COPPER COUPONS FROM BIOFILM CHAMBERS



Scraped copper coupons collected from biofilm chambers after five weeks of biofilm growth