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Effect of Distribution System Materials and Water Quality on Heterotrophic Plate Counts and Biofilm Proliferation

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Abstract The biofilms on pipe walls in water distribution systems are of interest since they can lead to chlorine demand, coliform growth, pipe corrosion, and water taste and odor problems. As such, the study described in this paper is part of an AWWARF and Tampa Bay Water tailored collaboration project to determine the effect of blending different source waters on the water quality in various distribution systems. The project was based on 18 independent pilot distribution systems (PDS), each being fed by a different water blend (7 finished waters blended in different proportions). The source waters compared were groundwater, surface water, and brackish water, which were treated in a variety of pilot distribution systems, including reverse osmosis (RO) (desalination), both membrane and chemical softening, and ozonation-biological activated carbon (BAC), resulting in a total of 7 different finished waters. The observations from this study consistently demonstrated that unlined ductile iron was more heavily colonized by a biomass than galvanized steel, lined ductile iron, and PVC (in that order) and that the fixed biomass accumulation was more influenced by the nature of the supporting material than by the water quality (including the secondary residual levels). However, although the bulk liquid water cultivable bacterial counts (i.e. heterotrophic plate counts or HPCs) did not increase with a greater biofilm accumulation, the results also suggested that high HPCs corresponded to a low disinfectant residual more than a high biofilm inventory. Furthermore, temperature was found to affect the biofilms, plus the AOC was important when the residual was between 0.6 and 2.0 mg Cl_2/l. An additional aspect of the current study was that the potential of the exoproteolytic activity (PEPA) technique was used along with a traditional so-called destructive technique in which the biofilm was scrapped off the coupon surface, resuspended, and cultivated on an R2A agar. Both techniques indicated similar trends and relative comparisons among the PDSs, yet the culturable biofilm values for the traditional method were several orders of magnitude lower than the PEPA values.

Key words: Pipe material, biofilm biomass quantification, drinking water, PEPA

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One of the major concerns of drinking water producers is to provide bacteriologically safe water to the public. Controlling bacterial growth in North America largely depends upon maintaining a disinfectant residual throughout the distribution systems. The disinfectant role is to provide a residual in the distribution systems to control any microbiological degradation in the water quality and protect against possible contamination. A biofilm can harbor coliform organisms, as well as viruses and opportunistic pathogens, like *Cryptosporidium* oocysts [1]. Also, a high bacterial population in potable water can be associated with an increased possibility of waterborne disease, taste and odor problems, corrosion, and the need to maintain a higher disinfectant residual [2]. Moreover, coliform bacteria have been associated with a high abundance of heterotrophic bacteria and biofilms, increasing the potential health risk [3, 4, 5, 6]. It is also thought that suspended cells in the liquid phase originate from a biofilm through detachment processes during the distribution of drinking water and that control of suspended cell concentrations can be assisted by minimizing biofilm cells. However, biofilm-associated microorganisms have been shown to be less susceptible to disinfectants than planktonic microorganisms [7, 8]. As such, the increasing use of monochloramines in North America, to prevent the production of harmful disinfection byproducts, is an interesting alternative to free chlorine for controlling bacterial growth, since monochloramines are thought to penetrate deeper into a biofilm matrix [9].

In the past, controlling bacterial regrowth in distribution systems has focused on limiting the nutrient levels (AOC

and BDOC) and use of a secondary residual (free and combined chlorine). The effect of distribution system materials on heterotrophic plate counts and biofilm proliferation has been more extensively studied during the past decade, and recent evidence suggests that biofilm growth is closely associated with the corrosion of pipe materials, through an increase in disinfectant demand by corrosion products. Consequently, the corrosion potential of pipe materials influences the bacterial regrowth in distribution systems and ultimately the downstream water quality [10]. Recent studies have also shown that the densities of the fixed bacterial biomass are dependent on the pipe material and that gray iron supports more biofilm cells than plastic-based materials in different oligotrophic drinking water environments [11]. As such, it has been concluded that the bacteria fixed on gray iron would appear to be more protected from the chlorine residual in the water than those fixed on noncorroding materials, implying that pipe reactivity is an important promoting factor of bacterial regrowth in the distribution system. Accordingly, since the pipe material seems to have a strong influence on bacterial regrowth, the current paper investigates the interrelationships between HPC and biofilm proliferation.

MATERIALS AND METHODS

Pilot Distribution Systems

This study was part of an AWWARF and Tampa Bay Water tailored collaboration project to determine the effect of blending different treated source waters on the water quality of distribution systems. The project analysis was carried out at the University of Central Florida, Civil and Environmental Engineering Department, based on 18 independent pilot distribution systems (PDSs), each fed by a different water blend (7 finished waters blended in different proportions). The source waters compared were groundwater, surface water, and brackish water that were treated in a variety of pilot distribution systems, including RO (desalination), both membrane and chemical softening, and ozonation - BAC, to produce a total of 7 different finished waters. The pilot distribution system (PDS) lines were constructed from actual member government distribution systems. The first fourteen lines (PDS 1– 14) were made of a combination of PVC, lined ductile iron, unlined cast iron, and galvanized steel pipe sections attached in series in that order (upstream to downstream), referred to as the "hybrid" lines. The four remaining lines (PDS 15– 18) were made of a single material (unlined ductile iron for PDS 15, lined ductile iron for PDS 16, PVC for PDS 17, and galvanized steel for PDS 18), referred to as the "single-material" lines. Each PDS had a hydraulic retention time of about 5 days. Chloramines were used for both primary disinfection and as the secondary residual in the PDSs.

Incubation Conditions

Coupons cut from existing pipes excavated from the ground (PVC, lined and unlined ductile iron, and galvanized steel, all from utilities in the Tampa Bay area) were incubated in these lines for a 3-month period (corresponding to a single operational phase of the project). Coupons of each material being study were incubated in the "hybrid" lines, while coupons of the same material as the line were incubated in the "single-material" lines. The coupons, taken from existing pipes, were sometimes extensively corroded, in which case they were referred to as "aged" coupons. The coupons were always used in duplicate. Duplicate coupons were also provided for both assays (HPC and PEPA). The coupons were all 3.0 cm in diameter and affixed to a peg made from a PVC welding rod. The coupons were then placed inside a cradle consisting of a 3-inch diameter PVC pipe cut lengthwise. The cradles had holes for the coupon pegs and were placed inside a 4-inch diameter PVC pipe connected to the end of each PDS for incubation of the coupons. Before being placed inside the cradles, the outer side of the coupons was taped with 1-inch-wide Teflon tape to expose only the inner surface of the coupons to the water. The operation of the pipe cradle was similar to that of the PDSs. Specifically, it normally operated at a flow of 4.7 gpd and was flushed at 1 fps once a week.

Sampling Conditions

The coupon holder was carefully removed from the cradles, with the coupons still affixed, then transported inside a closed PVC container. The humidity in the container was elevated by placing a wet sponge inside, to prevent any desiccation of the biofilms. Upon arrival in the laboratory, the coupons were analyzed during the next 24 h.

HPC Enumeration of Biofilm

The coupons were rinsed twice very carefully with a Phosphate Buffer Solution (PBS). The biofilms were manually detached from the coupons using a sterile weighing spatula (sterilized by 70% Ethanol) into 4 ml of sterile PBS, then homogenized using a tissue blender (Tissue Tearor™, Biospec Products, Inc., Racine, WI, U.S.A.) at 3,000 rpm for 2 min. Next, the sample was serially diluted and two plates per dilution spread on an R2A agar plate (ref. 1826-17-1, Difco Laboratories, Detroit, MI, U.S.A.) and incubated for 7 days at 25°C. The typical dilutions used were 10^{-3} and 10^{-4}. Finally, after incubation and enumeration, the results were expressed as cfu/cm^2, taking into account the dilutions used and the surface area of the scraped coupons. The results obtained with this technique are hereinafter referred to as the BFHPCs for the biofilm HPCs. This spread plate technique on an R2A agar is outlined in *Standard Method 9215C* [12].

Potential of Exoproteolytic Activity Assay

The protocol used was similar to the one previously described by Laurent and Servais [13], except that it was slightly modified to use much less expensive batch reactors for the biofilm coupons, facilitating high sample volumes at a reasonable cost [10]. The batch reactors were cylindrical PVC cups made from 1¼ inch schedule 40 PVC pipe and a flat 1¼ inch PVC cap for the bottom. The height of the cup was about 3 to 4 inches. Upon arrival in the laboratory, the coupons were detached from the coupon holder with caution and new Teflon tape affixed over the used Teflon tape to prevent any interactions between the bacterial biomass deposited on the outer part of the coupon and the reagent. To avoid dessication, the coupons were analyzed as soon as possible. Both duplicate coupons were then placed in autoclaved PVC containers covered by aluminum foil. When the experiment was ready to be carried out, 8 ml of a solution of non-fluorescent L-leucyl-β-naphtylamide (LL-βN, Sigma, St Louis, MO, U.S.A.) at a saturating concentration of 1 mM was poured into the container to submerge the coupon. The concentration was used by Somville and Billén [14]. At this point, the enzymatic hydrolysis reaction that releases the fluorochrome β-naphtylamine from the LL-βN molecule occurred due to the action of the bacterial exoenzymes in the coupon biofilm. Thereafter and at 10-min intervals, 2 ml subsamples were removed and the associated fluorescence measured at 410 nm under 340 nm excitation, then those samples were returned to the PVC container.

A standard curve correlating the fluorescence intensity to the βN concentration was created for each set of samples. After conversion using the standard curve, the βN concentration was plotted against time and the slope, i.e. the βN concentration increase (nmol of βN produced per min and per cm^2), was obtained by simple linear regression on the linear portion of the response curve, which was then converted into the biomass expressed in μg C/cm^2 by multiplying by 6.57 (reciprocal of the slope of the correlation straight line established by Laurent and Servais [13]). Finally, the cells/cm^2 was calculated based on considering an average carbon content of 20×10^{-15} g C/cell [10].

Water Quality Analyses

Most water quality analyses for the bulk liquid were carried out with respect to *Standard Methods* [12]. The residual concentrations of free and total chlorine were measured on site using a portable spectrophotometer (Hach 46700; precision ±0.03 mg Cl_2/l) and recommended reagents (Hach No. 21055-69, N,N-diethyl-*p*-phenylenediamine (DPD); Hach No. 21056-69, DPD and potassium iodide).

The sampling bottles and 40-ml vials for the AOC analysis were muffled at 525°C for 5 h after cleaning, and the AOC measured using the rapid method of LeChevallier *et al*. [10], except that plate counts were used to enumerate the bacteria rather than the ATP fluorescence, in conjunction with *Standard Methods 9217* (1995) and the method of van der Kooij [15].

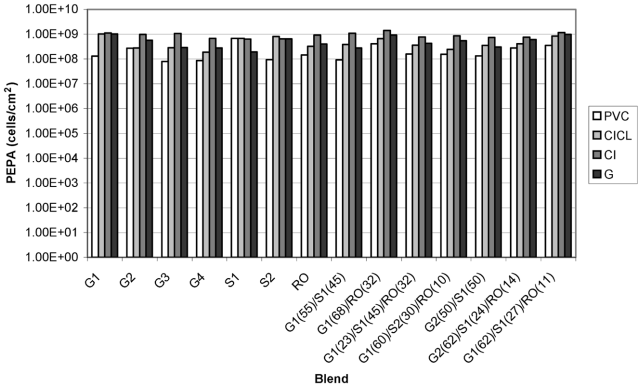

Fig. 1. PEPA end of Phase 2 (sort by blends).

RESULTS AND DISCUSSION

The results summarized the four operational phases of the TBW-AWWARF project, i.e. twelve months of study, and consistently demonstrated that unlined ductile iron was more heavily colonized by a biomass than galvanized steel, lined ductile iron, and PVC (in that order). Figure 1 presents a representative example of this observation, for the second phase of the study regarding the hybrid lines. In the hybrid data, the secondary residual was equal for all four coupons in a single hybrid PDS. Thus, in this case, the cast iron had a higher biomass inventory (as much as one order of magnitude) than the PVC, even when the residual was equal. Holden *et al.* [16] documented similar results when comparing biofilm inventories on cast iron and medium-density polyethylene (MDPE). It should also be noted

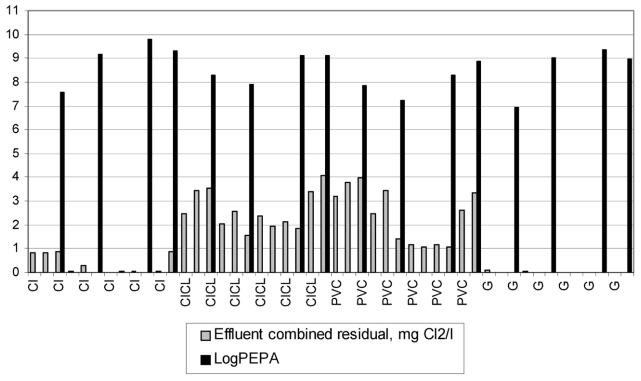

Fig. 2. LogPEPA and effluent combined residual vs. material pure lines only.

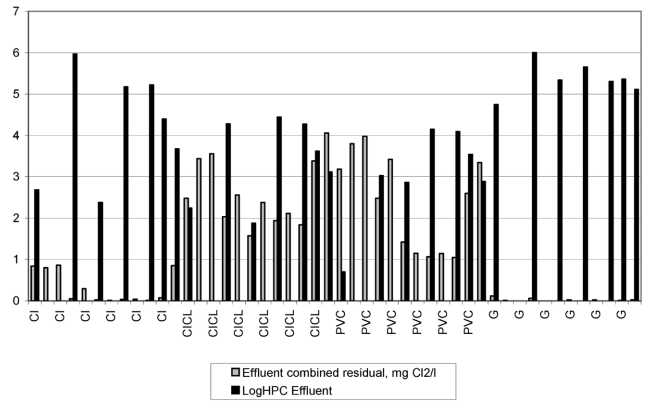

Fig. 3. LogHPC and effluent combined residual vs material - pure lines only.

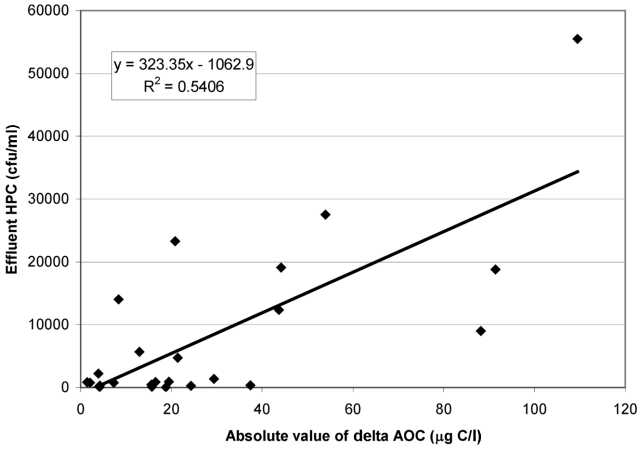

Fig. 5. HPC effluent vs. absolute value of delta AOC.

that even when the hybrid lines were fed with different blends, this did not seem to have an effect on the observed trend. Therefore, the fixed biomass accumulation was more influenced by the nature of the supporting material than the water quality.

Figure 2 presents the combined results of the four phases of the study for the pure lines (fed using same blend, yet consisting of different single materials). The trend in Fig. 2 was the same (e.g., PVC lowest, CI greatest) when the average values were compared, even though the combined residual levels were different from one line to the next. The fixed biomass inventory was fairly insensitive to the residual levels, which were much lower for the unlined metals.

In contrast, the bulk liquid bacterial counts (i.e. heterotrophic plate counts or HPCs) varied inversely with the disinfectant residual (Fig. 3). The highest HPCs were observed in the unlined cast iron and galvanized steel lines, which had very low residuals.

An analysis of both the hybrid and pure PDS data throughout the length of the study showed that the combined residual had a significant effect on the bulk liquid HPCs, particularly when the levels were below 0.6 mg Cl_2/l (Fig. 4). As such, HPCs above 100,000 cfu/ml were not seen if the residuals were above 0.6 mg/l (chloramines were being used), yet very low counts (<100 cfu/ml) were sometimes seen in systems with a negligible residual, and lower counts consistently seen with residuals in excess of 0.6 mg/l. Below residual levels of 0.6 mg Cl_2/l, the HPCs were high (10^5 or greater being common) and this seemed to be true regardless of the AOC concentration or stability.

However, when residuals were between 0.6 and 2.0 mg, the Cl_2/l HPC counts correlated directly with the AOC stability (and more loosely with the AOC concentration). Waters showing changes in the AOC level (between the influent and effluent) greater than 37 µg C/l tended to have elevated HPC counts (>10^4 cfu/ml; Fig. 5). The data were ambiguous above 2.0 mg Cl_2/l of residual with respect to the AOC, since there was not a broad range of influent

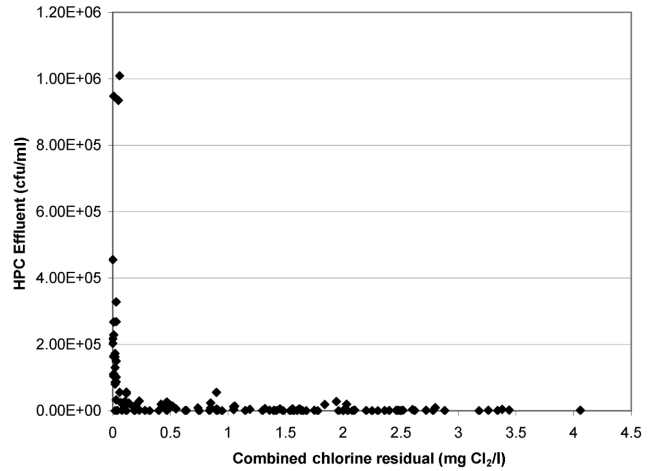

Fig. 4. HPC effluent vs. effluent combined residual.

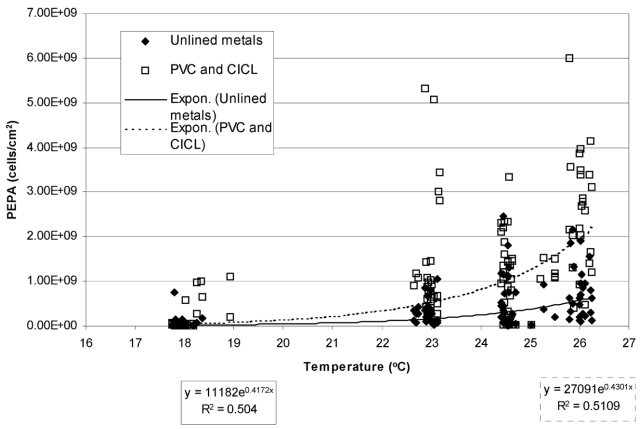

Fig. 6. PEPA vs. average temperature (influent/effluent).

AOC values in that data set, and none above 109 μg C/l. The AOC and HPCs seemed stable with a residual above 2.0 mg Cl_2/l, yet whether this would have remained true for a higher AOC finished water could not be evaluated. With the biofilm inventory, there was no significant difference in the hybrid lines, even though the HPCs varied from 100 to 100,000 cfu/ml. There did appear to be a correlation in the pure lines, yet, due to the confounding effect of the material and residual concentration, this correlation could not be exclusively attributed to either the residual or the material. Low residuals significantly increased the probability of high HPC counts, while the effect of the material on the HPC counts was inconclusive, and probably more a result of the residual consumption associated with the material.

The effect of temperature was also investigated, and it was observed that the fixed biomass was sensitive to an increase in temperature (Fig. 6). An increase of 5°C (from 18 to 23°C) led to increases of about one order of magnitude in the biofilm inventory, while an increase from 23 to 26°C multiplied the fixed cell densities by a factor of 5 to 10. Meanwhile, the effect of temperature on the HPCs was inconclusive. Even though a depletion of the residual was observed at higher temperatures, no straightforward correlation was found between temperature and the HPCs. However, the impact of higher temperatures driving an increased consumption of the combined residual levels did affect the HPCs in a very significant way.

In addition to the PEPA technique, a further aspect of the current work was that the biofilm was scrapped off from an identical coupon surface for the pure material lines, resuspended, and cultivated on an R2A agar (BFHPC). As such, Fig. 7 (second phase, representative of the other phases) shows that the BFHPC technique yielded much lower estimates for the biofilm inventory on the coupons (2 to 4 orders of magnitude lower than the PEPA results). However, the trend observed as regards the effect of the material on biofilm accumulation, i.e., that the cast iron was more heavily colonized than the PVC, was the same using both techniques, thereby strengthening the conclusion that the material was the major factor in determining the biofilm inventory.

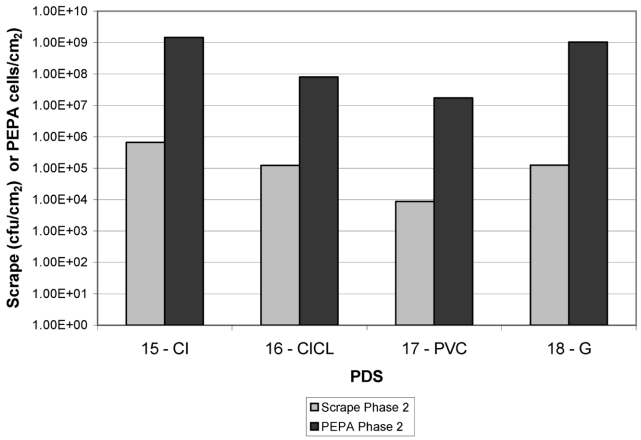

Fig. 7. PEPA and BFHPC vs. material pure lines only.

Biofilm bacteria are thought to be more resistant to disinfectant due to a physiological state associated with low growth rates or increased diffusional resistance created by extracellular polymers [4, 5]. Previous work by de Beer *et al.* [17] using chlorine-sensitive microelectrodes demonstrated that chlorine did not fully penetrate into the biofilm matrix, as the chlorine reacted with the biofilm constituents faster than it could diffuse into the biofilm. This phenomenon has also been documented in more recent studies [18, 19]. As such, this resistance to disinfectant levels can explain the insensitivity of the biofilm inventory to the combined residual observed in the current study.

In terms of the bulk liquid HPCs, the effect of the materials was most significant in terms of the material impact on the residual levels. The unlined metals depleted the residual levels, resulting in elevated HPCs, whereas any intrinsic effect of the material was of a much smaller magnitude than the residual effect and not observable, if present.

The major findings of the experiments discussed herein are summarized as follows: Firstly, the biofilm inventory was a function of the material and relatively insensitive to the secondary residual levels or variations in the water quality. Secondly, the HPC counts could not be directly correlated to the biofilm inventory. The HPCs were most significantly impacted by the residual. As the secondary residual decreased from 0.6 to 0 mg Cl_2/l, the probability of a high HPC count (>100,000 cfu/ml) increased very significantly. Thirdly, the AOC stability affected the HPC proliferation when the residual was between 0.6 and 2.0 mg Cl_2/l. Lastly, unlined metals and a higher temperature (summer time) resulted in a significantly greater residual consumption, which also increased the probability of a high HPC count if the residual consumption was nearly complete.

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