PERGAMON

Water Research 36 (2002) 3898–3910

WATER RESEARCH

www.elsevier.com/locate/watres

Minimizing biofilm in the presence of iron oxides and humic substances

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Abstract

Based upon circumstantial evidence linking elevated coliform bacteria counts in drinking water distribution systems with unlined cast iron pipe, it was hypothesized that adsorption of humic substances by iron oxide containing corrosion products (CPs) can stimulate and/or support biofilm development. Using porous media consisting of iron-oxide-coated glass beads (IOCBs) or actual iron CPs, experiments were performed to evaluate the effectiveness of different corrosion control and disinfection treatments in reducing biofilm when humic substances were the carbon source. Free chlorine was the most effective treatment in minimizing biofilm. Addition of phosphate alone did not significantly reduce biofilm using the CPs, but there was weak evidence it did using the IOCBs. The combination of free chlorine and phosphate was more effective at minimizing biofilm than free chlorine alone when CPs were the media. The presence of humic substances was a major factor when considering biofilm minimization based on results of experiments using both types of iron oxide media. The combination of humic substances and CPs led to an increase in biofilm biomass when free chlorine was not present, similar to conditions that could occur at distribution system dead-ends. Treatment to raise the pH to 9 did not reduce biofilm in experiments using both media, and actually increased biofilm in the experiment using CPs under the conditions tested.

Keywords: Biofilm; Humic substances; Iron oxides; Corrosion products; Corrosion control; Chlorine

1. Introduction

The possible interactions between biofilm, humic substances and iron oxide containing corrosion products (CPs) has become of great interest to the drinking water industry because adsorption of humic substances to iron oxides may promote biofilm growth, resulting in an increase of coliform and heterotrophic plate count (HPC) bacteria in drinking water. Many older drinking water distribution system pipes are composed of unlined steel, cast or ductile iron pipes that are subject to corrosion in the drinking water environment. Several studies of full-scale distribution systems have found circumstantial evidence relating unlined ferrous pipes with an increase in positive coliform samples [1–3]. Presence or absence of coliform bacteria is the primary measurement parameter currently used in the US to predict microbial safety of drinking water in the distribution system [4].

Laboratory- and pilot-scale studies have demonstrated that coliform bacteria in drinking water can result from detachment of these organisms from biofilm on pipe surfaces [5]. Coliform bacteria have been found to be more abundant in the presence of ferrous metal CPs [5] and in corrosion tubercles [6]. Laboratory studies have shown that biofilm in drinking water environments are capable of utilizing humic substances (sometimes referred to as natural organic matter or NOM) as their primary carbon source at concentrations as low as 500 μg C/L [7]. Humic substances are a primary source of organic carbon found in drinking water [8] and will readily adsorb to iron oxides similar to those formed during ferrous pipe corrosion [9–11]. It has been hypothesized that adsorption of humic substances to iron oxides may create a nutrient-rich environment

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0043-1354/02/$ - see front matter
PII: S0043-1354(02)00088-X

that promotes biofilm growth, resulting in an increase in heterotrophic and coliform bacteria in drinking water.

Treatment processes to control lead and copper corrosion [12] appear to have benefits other than reducing the concentration of these metals in drinking water. The reaction between chlorine and iron oxide CPs can reduce chlorine concentration in drinking water while also affecting the ability of chlorine to act on biofilm [13,14]. Common corrosion control treatments for lead and copper include addition of phosphate or alkaline pH adjustment. These treatments have been shown to reduce corrosion and the number of positive coliform samples in drinking water [2,15], although the mechanism by which organism control is gained is unknown.

One hypothesis that results from these findings involves the well documented adsorption of humic substances by iron oxides and the role that adsorbed humic substances could play in promoting biofilm growth. Factors that can influence surface sites available for adsorption of humic substances to iron oxides include the type of iron oxide [16], solution pH [10], concentration of divalent cations such as Ca^{2+} and Mg^{2+} [17], and the presence of other compounds, such as phosphate, that compete with humic substances for attachment sites on iron oxides [10].

An additional factor that may play an important role in the interaction of humic substances, biofilm and CPs lies in the structural change of humic substance molecules when adsorbed to an iron oxide surface. Based on experimental studies, Gu et al. [10] speculated that in the process of attaching to iron oxides humic substances may unfold and maximize the points of interaction between humic substances and iron oxide. Although not proven, it may be possible that such unfolding or collapse of the molecule could expose to the biofilm easily degradable functional groups on the humic substance molecule such as amines or carbohydrates.

Because corrosion control and humic substances may play an important role in biofilm development, we investigated interactions between corrosion control treatments, humic substances, iron oxides, and biofilm. The goals were to determine if adsorption of humic substances to iron oxides promoted biofilm development and if commonly practiced corrosion control and disinfection treatment techniques could help minimize biofilm.

2. Materials and methods

2.1. Experimental setup

Two experiments were performed, the first used iron-oxide-coated beads (IOCBs) as the media in packed columns and the second used iron CPs. Physical characteristics of these media are described in more detail below. Each experiment tested identical treatments (the term "treatment" was used to describe the feed solution) except the CPs experiment did not contain a pH 8 treatment. Table 1 presents a description of the treatments investigated. Small diameter columns containing the iron oxide media were used and in each experiment there were two replicate columns for each treatment. Experiments were operated at room temperature (20–22°C) until a pseudo-steady-state was achieved with respect to biofilm growth (56 days minimum), based on consistent measurements for effluent total direct cell counts (TDCs) and ultraviolet

Table 1
Description of treatment types for each of the two experiments

Treatment	Treatment description
Time 0	+Fe[a], time zero, sample media for biofilm following humics loading and inoculation
−Fe	Uncoated glass bead media, +humics[b], pH 7.3[c]
−Humics	+Fe, –humics[d], pH 7.3
pH 7.3	+Fe, +humics, pH 7.3
pH 8	+Fe, +humics, pH 8
pH 9	+Fe, +humics, pH 9
Cl_2	+Fe, +humics, chlorine[e], pH 7.3
$Cl_2 + PO_4$	+Fe, +humics, chlorine, phosphate[f], pH 7.3
PO_4	+Fe, +humics, phosphate, pH 7.3

[a] +Fe—Columns contained iron oxide media, IOCBs first experiment, CPs second experiment.
[b] +Humics—Humic substances in feed solution, ~2.85 mg C/L.
[c] pH adjusted using HCl or NaOH.
[d] –Humics—No humic substances in feed solution.
[e] Chlorine—Added to column influent feed, effluent residual concentration of 0.15–0.2 mg/L free chlorine.
[f] Phosphate—Phosphoric acid in feed solution, 3 mg/L as phosphate.

light absorption at 254 nm (UV_{254}). TDCs were determined using DAPI (4′,6-diamidino-2-phenylindole, Sigma) stain [18] and epifluorescent microscopy (Olympus BH-2 microscope, Melville, NY). Twenty fields or 200 cells minimum were counted per sample. UV_{254} was measured using a spectrophotometer (Milton Roy Spectronic 601) and a 5-cm-path-length quartz sample cell. Intensive sampling of column influent and effluent was performed during the last 10–14 days of the experiment, followed by sampling the media for biofilm biomass at the experiment's conclusion.

2.2. Corrosion products (CPs) characteristics

CPs were obtained from the inside of a cast iron pipe cut from a greater Boston, MA, drinking water distribution system. Details of the CPs' physical characteristics are presented in Table 2. Following removal from inside the pipe, CPs were dried under a nitrogen gas atmosphere and then gently crushed and processed for particle size classification using US Standard sieve sizes. Particle sizes were selected so that the "specific" surface area of CPs was approximately the same as the "specific" surface area of IOCBs on a per unit volume basis. Specific surface area was estimated for the CPs based on measured headloss through the porous media [19,20]. Surface area of the CPs was also determined by multi-point BET and nitrogen gas. Density of the material was measured using a laboratory pycnometer. The sorption site density of the CPs was estimated at an equilibrium pH of 7.3 by titration of the media under a nitrogen atmosphere from pH 11 to pH 3 using HCl [21]. Powder X-ray diffraction analysis was utilized to identify primary iron oxides composing the CPs. Based on scanning electron microscopy (SEM) pictures, the surface of CPs was very irregular and consisted of numerous crystalline structures.

2.3. Iron-oxide-coated beads (IOCBs) characteristics

Glass beads with a nominal diameter of 0.5 mm (Biospec Products, Inc., Bartlesville, OK) were coated with iron oxides using a technique described elsewhere [22–25]. Pycnometry was utilized to determine the density of coated glass beads and the specific surface area was calculated based on their measured diameter. SEM pictures indicated that the surface of IOCBs appeared amorphous and did not exhibit the distinct crystalline structure seen in CPs. Results of transmission electron microscopy-electron diffraction (TEM-ED) analyses revealed the iron oxide coating to have a mixture of crystalline and poorly crystalline structures, the latter being many randomly oriented crystallites. Details for the IOCBs' physical characteristics are presented in Table 2.

2.4. Column reactors

Column reactors were PTFE tubing, 1 cm in diameter with a volume of 8 cm^3. End fittings and retaining screens were stainless steel. All tubing, fittings, unpacked columns, and feed solutions were initially sterile. Because autoclaving IOCBs or CPs would have altered their original surface characteristics, these media were not sterile, even though aseptic techniques were used in their preparation and handling. Columns were operated in an upflow mode and effluent dripped into a collection channel. Feed solutions were pumped from 20-L glass carboys, through 0.2-μm-pore-size sterile inline cartridge filters (Pall Gelman SuporCap 50) using positive displacement tubing pumps (Masterflex) at a nominal flow rate of 0.35 mL/min. Stainless steel chlorine contact columns, 30 min detention time, preceded reactors that received chlorine treatment. Prior to starting the CPs experiment, the media was rinsed with a once-through flow of sterile reagent grade water (Nanopure) until the

Table 2
Physical characteristics for CPs and IOCBs

Media	Size	Density (g/cm^3)	Specific surface area (cm^2/g)	BET surface area (cm^2/g)	Sorption site density (meq/L)	Predominant iron oxides
CPs	Equal weights of: Nos. 10–12, Nos. 12–16[a]	3.87	67.4	2.611×10^5	0.145	Goethite, ferrihydrite, iron hydroxide, ferroxyhite, lepidocrite[b]
IOCBs	0.5-mm nominal diameter	2.48	39.0	673.3 (640.1)[c]	0.0106	Hematite, ferrihydrite[d]

[a] US standard sieve size nominal openings: No. 10—2.00 mm, No. 12—1.70 mm, No. 16—1.18 mm.
[b] Determined by powder X-ray diffraction.
[c] BET area for uncoated glass beads.
[d] Determined by TEM-ED analysis.

UV_{254} absorption (a surrogate measurement for organic carbon) of the column effluent stabilized.

2.5. Feed solutions

Feed solutions consisted of sterile Nanopure water containing humic substances derived from Eliot Silt Loam Soil (International humic Substances Society-BS102M), sodium bicarbonate for alkalinity (30 mg/L as $CaCO_3$), and sufficient nitrogen (as nitrate) and phosphorous (as phosphate) to create carbon limiting growth conditions based on stoichiometric calculations (refer to Table 1). Organic carbon concentration of the feed solution was determined as non-purgeable organic carbon (NPOC) using a Shimadzu TOC-5000A (Shimadzu Scientific Instruments, Columbia, MD) total organic carbon analyzer. Feed solutions that contained phosphate were amended by addition of high performance liquid chromatography (HPLC) grade phosphoric acid. Separately fed chlorine solutions were made using sterile Nanopure water and liquid sodium hypochlorite. The pH of all feed solutions was adjusted prior to their use and during the experiment as required. Prior to each use all glass carboys were baked at 500°C for 4 h to combust any organic carbon. Care was taken to minimize introduction of extraneous organic carbon into feed amendment solutions.

2.6. Column loading and inoculation

Prior to inoculation columns were "loaded" with a 50 mg C/L humic substances solution (pH 7.3) for a period of 24 h. NPOC measurements of the humic substances solution confirmed that adsorption had occurred. Columns were then inoculated with a mixed population of heterotrophic bacteria by pumping the effluent of a biologically active carbon (BAC) column through the reactors for a period of 24 h on a once-through basis. The BAC columns treated dechlorinated City of Bozeman, MT, drinking water and had been in operation for a period of over 5 years. At the end of the inoculation period one pair of columns (Time 0 treatment) were sampled for initial biofilm biomass.

2.7. Influent and effluent sampling

The column influent and effluent were sampled and analyzed for pH, PO_4, NO_3, Cl, SO_4, Cl_2, and dissolved organic carbon (DOC). In addition, column effluent was sampled for heterotrophic plate counts (HPCs) and TDCs. pH was measured using flow cells made of Teflon® with a pH probe insert, allowing pH to be measured without exposing liquid to the atmosphere. PO_4, NO_3, Cl and SO_4 were measured by HPLC using a Dionex DX-500 instrument according to Standard Method 4110B [26]. Total Fe was determined using Hach Method 8147–FerroZine Method and free chlorine using the DPD (*N*,*N*-diethyl-*p*-phenylenediamine) colorimetric method, Standard Method 4500-Cl G, and a Hach DR-2000 spectrophotometer. DOC was determined as NPOC after filtration of the sample through a 0.2-μm-pore-size nylon filter. HPCs were determined by spread plating serially diluted samples on R2A medium (Difco) using three plates per dilution and incubating at room temperature for 7 days. TDCs were determined using DAPI stain and epifluorescent microscopy as described earlier. Effluent cell biomass was determined by multiplying the total number of cells per unit volume (TDCs) or colony forming units per unit volume (HPCs) by a carbon per cell value based on cell size measurements and a previously determined cell carbon per volume relationship [27].

2.8. Biofilm sampling and biomass quantification

At the conclusion of an experiment media were removed for determining biomass using a modification of the potential exoproteolytic activity (PEPA) assay [27] and HPCs. TDCs and cell sizing were performed for biofilm from uncoated glass beads (negative Fe treatment) for calibration of the PEPA assay [27]. Approximately one gram of media was aseptically removed from the top and bottom 1.5 cm of the columns. Samples were divided evenly along the column's axis, taking an equal amount of media for the PEPA assay and an equal amount for HPCs and TDCs. Split-samples were plated for all HPC measurements. Biofilm biomass using HPCs (HX) was determined by multiplying the colony forming units per gram of media by the measured carbon per cell value (37.53 fm) [27]. Biomass was normalized using two parameters for the media: (1) per gram of media, and (2) per square centimeter of specific surface area.

2.9. Data analysis

Biofilm specific growth rates were determined by assuming the biofilm had reached a pseudo-steady-state with respect to growth, and all biomass leaving the reactors was from growth and detachment, allowing the specific growth rate to be calculated knowing biofilm biomass in the column. The term carbon removal rate was used to describe overall removal of organic carbon across the column and was not the true carbon uptake by biofilm cells. Using influent and effluent DOC concentrations, flow rate, and biofilm biomass, a specific carbon removal rate (q) was calculated. Statistical analyses were performed using the computer software MiniTab™, Version 13.3.

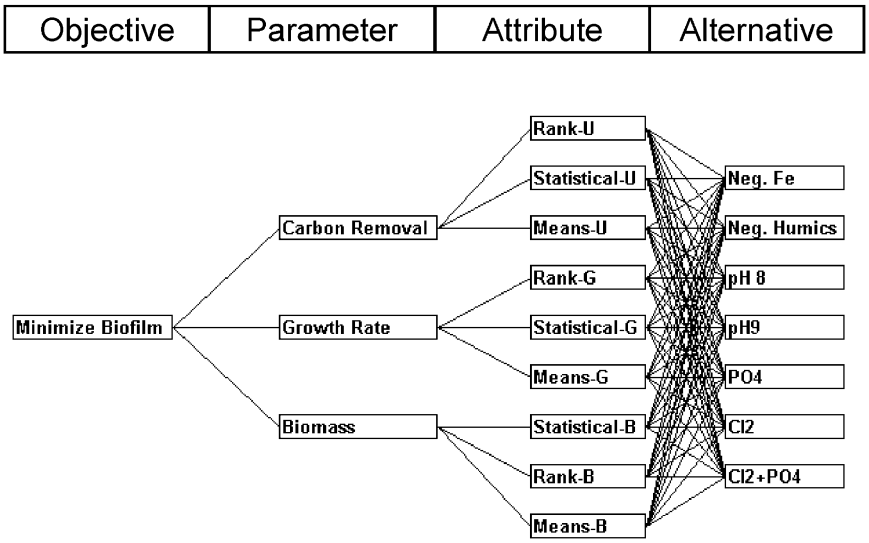

Fig. 1. Hierarchy used to evaluate alternatives for all scenarios. CPs experiment did not include the pH 8 treatment alternative.

2.10. Analysis of alternatives for minimizing biofilm formation

Alternative treatments were ranked using a weighted hierarchy process called Simple Multiattribute Rating Technique (SMART), implemented by using the computer software Criterium Decision Plus, Version 3.0.3 (InfoHarvest, Inc., Seattle, WA). The analysis consisted of defining a goal and selecting rating criteria for alternatives. The result was a hierarchy of elements (criteria and subcriteria) that were rated to assist in selection of alternatives that were the most effective in minimizing biofilm. The hierarchy diagram shown in Fig. 1 was utilized to evaluate two scenarios for each experiment; one using HPCs to determine biofilm biomass (HX) and effluent biomass, and the other using biofilm biomass measured using PEPA (EX) and effluent biomass using TDCs.

The selected goal of the treatment alternatives was to minimize biofilm, e.g., a drinking water supplier would perform these treatments with the goal of minimizing biofilm in the distribution system. Alternatives consisted of the corrosion control treatments, pH 8, pH 9, PO_4, Cl_2 and $Cl_2 + PO_4$, and control treatments, negative Fe and negative humics (refer to Table 1). Criteria and attributes for each alternative were evaluated using the rules summarized in Table 3. The basic premise was that less biofilm, lower growth rates and lower carbon removal rates equated to a higher rating with respect to the goal of minimizing biofilm formation.

The three basic parameters determined for each alternative were compared with those of the pH 7.3 control treatment to determine if the difference of the means was statistically significant. Statistical comparisons were made using one-way ANOVA with the exception of biomass, where there were not sufficient data points for an ANOVA.

The analyses resulted in a decision score and its estimated uncertainty. Final decision scores could range from 0 to 1.0, the decision score for the pH 7.3 control alternative always being 0.500. A decision score <0.500 would indicate the alternative ranked lower than the pH 7.3 control and scores >0.500 indicated the alternative ranked higher than the control. An uncertainty analysis provided a measure of variability in decision scores. The uncertainty analysis assumed a normal distribution with the rating value as its mean and a standard deviation based on an estimate of the possible range for the mean value. For the uncertainty analysis, the mean, lower 5-percentile and upper 95-percentile values were computed for each alternative. Using the uncertainty analysis, a percentage of the time a particular alternative was better than all others was computed (direct comparison). Alternatives that resulted in being better $>5\%$ of the time were considered as possibly significant in meeting the objective, minimizing biofilm.

3. Results

3.1. Iron, pH and chlorine demand

In both the IOCBs and CPs experiments there was no measurable total iron in column effluents, indicating media remained stable during the course of the experiments. At pH 7.3 there was no significant change in pH across the columns in both experiments. At pH 8 and pH 9 there were statistically significant differences

Table 3
Rules used to rate criteria for analysis of alternatives

Rating of	By	Rating rule
Minimize biofilm	Biomass	50% greater than value given to growth or carbon removal
	Growth	Neutral, compared to biofilm biomass
	Carbon removal	Neutral, compared to biofilm biomass
Biomass, growth, or carbon removal	Statistical	50% greater than value given to means
	Means	Neutral, compared to statistical
	Rank	50% greater than value given to means
Alternative	Statistical	Up to 2 times weight of mean's rating if statistically different, greater or less than neutral
	Means	50% greater or less than neutral if means different
	Rank	Rank with respect to all other alternatives, control as neutral, weighted by the absolute difference between the alternative and the control

between influent and effluent pH ($p < 0.05$). The mean pH shift was 0.30 and 0.69 units downward in the IOCBs' pH 8 and pH 9 treatments, respectively, and 1.20 units downward in the CPs' pH 9 treatment.

Free chlorine demand during the last 2 weeks of the CPs experiment was 2.59 mg/L, $\approx$40% greater than that measured for the IOCBs experiment. This was expected based on the greater number of reactive surface sites measured for CPs compared to IOCBs. In the CPs experiment, free chlorine demand for the $Cl_2 + PO_4$ treatment was greater than for the Cl_2 only treatment, and the difference (0.26 mg/L) was significant ($p = 0.000$). The difference between the same two treatments for the IOCBs experiments (0.07 mg/L) was not significant. Based on free chlorine measurements following contact columns and prior to reactor columns, the difference in demand was attributable to reactions within the $Cl_2 + PO_4$ column and not the demand of humic substances. Free chlorine demand of humic substances after 30 min of contact time was consistent for both experiments and averaged 0.36 mg Cl_2/mg humics C.

3.2. Parameters for analysis of alternatives

Mean values for the three basic parameters utilized for comparison of alternatives are presented in Tables 4–6 for biofilm biomass, specific growth rates and specific carbon removal rates, respectively. Values shown for these parameters represent the mean for data from both replicate columns. Results of statistical analysis of the replicate means are also presented. Probability values < 0.05 would indicate the means for the two replicates were statistically different at a 95% confidence level. Where significant variability existed between replicate means, data was evaluated by each individual replicate to assure that combining data for the two replicates did not give an erroneous interpretation.

3.3. Comparison of treatment alternatives—IOCBs experiment

Decision scores that resulted from analysis of alternative treatments are presented graphically in Fig. 2 for parameters determined using HX and EX. The Cl_2 treatment resulted in the highest decision score for both methods of determining biomass (HX and EX). The $Cl_2 + PO_4$ and negative humics alternatives also had high scores for both methods of determining biomass, and the PO_4 alternative could be considered as a less significant but still important factor (better than other alternatives 5% of the time) when using biomass as EX. When EX was the basis for biomass measurement there was little difference between pH 8, pH 9 and negative Fe alternatives. If Cl_2 and $Cl_2 + PO_4$ alternatives were removed from the analyses (data not shown), the negative humics alternative ranked above others, followed by the PO_4 treatment; PO_4 treatment would be an important factor using EX biomass as the basis. The negative Fe alternative ranked lower than the control when using HX. Both pH 8 and negative Fe were essentially the same as the control when using EX. The consistently low ranking of negative Fe indicated the relative unimportance of the type of iron oxides on IOCBs.

3.4. Comparison of treatment alternatives—CPs experiment

Decision scores from the analysis of alternative treatments for the CPs experiment are presented in Fig. 3. Three treatment alternatives that had the highest

Table 4
Mean biomass values by treatment type and experiment for combined data from replicate columns

Column group No	Treatment	HPCs (HX)		Enzyme assay (EX)	
		(μg C/g)	(μg C/cm^2)	(μg C/g)	(μg C/cm^2)
IOCBs experiment					
12	Time 0	0.553	0.01419	0.397	0.0102
13	−Fe	3.937	0.09679	29.864	0.7342
14	−Humics	0.608	0.01559	2.75	0.0706
15	pH 7.3	5.819	0.14929	18.920	0.4854
16	pH 8	5.745	0.14739	25.707	0.6595
17	pH 9	6.110	0.15673	10.810	0.2773
18	Cl_2	0.0007	0.00002	0.050	0.0013
19	$Cl_2 + PO_4$	0.0003	0.00001	0.018	0.0005
20	PO_4	1.632	0.04187	18.231	0.4677
CPs experiment					
21	Time 0	3.066	0.0455	0.78	0.0117
22	−Fe	0.830	0.02045	21.07	0.5181
23	−Humics	2.088	0.03095	36.42	0.5405
24	pH 7.3	5.465	0.08110	112.18	1.6648
25	pH 9	11.585	0.17195	135.50	2.0109
26	Cl_2	0.161	0.00240	4.03	0.0598
27	$Cl_2 + PO_4$	0.211	0.00315	0.92	0.0136
28	PO_4	7.691	0.11415	92.92	1.3790

Table 5
Results of one-way ANOVA between replicates for specific growth rates (h^{-1}) by treatment type and experiment

Column group	Treatment	Mean for HPC/HX	HPC/HX *p*-value	Mean for TDC/EX	TDC/EX *p*-value
IOCBs experiment					
13	−Fe	0.01465	0.103	0.00611	0.000
14	−Humics	0.05702	0.632	0.03891	0.015
15	pH 7.3	0.00333	0.977	0.00495	0.224
16	pH 8	0.00472	0.023	0.00427	0.000
17	pH 9	0.01300	0.877	0.02120	0.788
18	Cl_2	0.00266	0.384	0.01463	0.001
19	$Cl_2 + PO_4$	0.02080	0.182	0.04857	0.630
20	PO_4	0.00438	0.196	0.00398	0.000
CPs experiment					
22	−Fe	0.011090	0.000	0.006590	0.081
23	−Humics	0.006570	0.014	0.002223	0.074
24	pH 7.3	0.007137	0.546	0.001711	0.000
25	pH 9	0.041841	0.007	0.002474	0.023
26	Cl_2	0.000105	0.002	0.004519	0.005
27	$Cl_2 + PO_4$	0.000181	0.992	0.015066	0.002
28	PO_4	0.014012	0.314	0.022030	0.002

Mean specific growth rate values are for combined data from replicate columns.

decision scores, regardless of biomass measurement method, were Cl_2, $Cl_2 + PO_4$, and negative humics, but not necessarily in that order. When HX was used, the $Cl_2 + PO_4$ and Cl_2 treatments scored higher than all other alternatives, with negative humics ranking third. Considering the range of uncertainty in decision scores, as shown in Fig. 3, the Cl_2 and $Cl_2 + PO_4$ treatments using HX scored much higher than other alternatives. When EX biomass data were analyzed, $Cl_2 + PO_4$ and negative humics scored significantly higher than other alternatives. The significant difference between $Cl_2 + PO_4$ and Cl_2 indicated that phosphate combined

Table 6
Mean specific carbon removal rates (q, h^{-1}) by treatment and experiment for combined data from both replicates

Column group	Treatment	Mean for q-HX	q-HX p-value	Mean for q-EX	q-EX p-value
IOCBs experiment					
13	−Fe	0.3181	0.000	0.02965	0.687
14	−Humics	−0.0524	0.759	−0.05832	0.914
15	pH 7.3	0.1972	0.701	0.06130	0.251
16	pH 8	0.1756	0.828	0.04457	0.009
17	pH 9	0.0875	0.362	0.04915	0.408
18	Cl_2	−1007	0.476	−2.92	0.715
19	$Cl_2 + PO_4$	−68	0.994	−1.19	0.970
20	PO_4	0.4016	0.003	0.05626	0.217
CPs experiment					
22	−Fe	0.8498	0.345	0.0458	0.948
23	−Humics	0.2712	0.903	0.0140	0.763
24	pH 7.3	1.4788	0.416	0.0255	0.009
25	pH 9	0.8774	0.673	0.0128	0.087
26	Cl_2	0.1220	0.605	0.0249	0.623
27	$Cl_2 + PO_4$	−0.5556	0.386	−0.5823	0.619
28	PO_4	1.0504	0.808	0.0216	0.418

p-Values were determined using one-way ANOVA between replicates for each treatment.

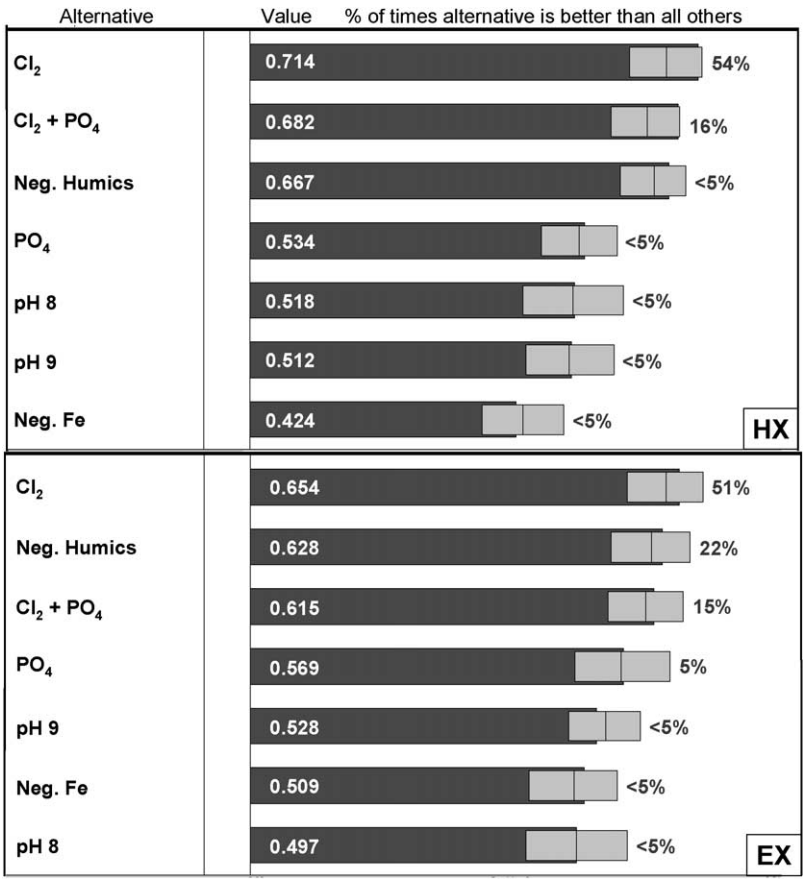

Fig. 2. Decision scores for comparison of alternative treatments for IOCBs experiment. Top-HX for biofilm biomass, bottom-EX for biofilm biomass. Error bars indicate the uncertainty in decision scores. Horizontal scales are not equal.

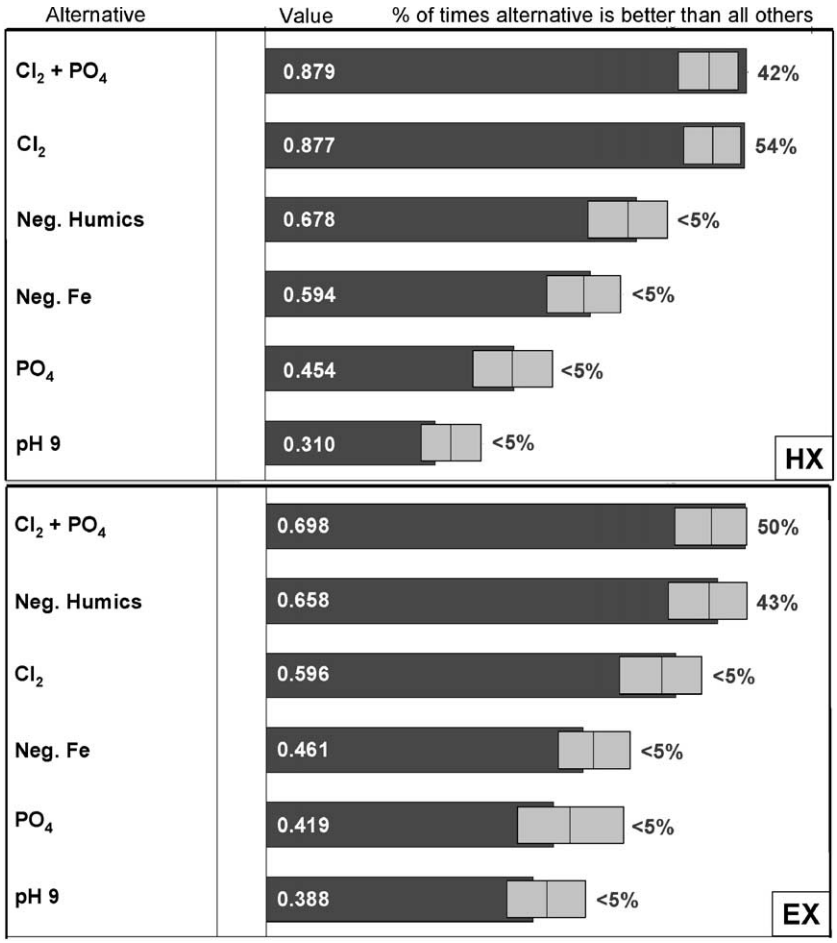

Fig. 3. Decision scores for comparison of alternative treatments for CPs experiment. Top-HX for biofilm biomass, bottom-EX for biofilm biomass. Error bars indicate the uncertainty in decision scores. Horizontal scales are not equal.

with chlorine was important in reducing EX biomass for CPs, a finding weakly supported by the IOCBs experiment. When the Cl_2 and $Cl_2 + PO_4$ alternatives were removed from the analyses (results not shown), the negative humics alternative ranked above the others followed by the negative Fe treatment; negative Fe treatment was ranked highest when HX biomass was the basis for parameters. pH 9 and PO_4 treatments ranked consistently lower than the pH 7.3 control.

4. Discussion

Any comparison of IOCBs and CPs experiments must take into account differences in the iron oxides. CPs consisted of well-formed crystalline structures, whereas iron oxides on the glass beads had a very fine, microcrystalline structure, only discernible using TEM techniques. The greater number of reactive surface sites measured for CPs indicated their greater potential for reactivity with humic substances, chlorine and phosphate. CPs had higher adsorption of humic substances prior to column inoculation and higher chlorine demand than did IOCBs.

The potential effectiveness of chlorine for minimizing biofilm was demonstrated in both experiments, regardless of the method used to measure biomass. Columns receiving $Cl_2 + PO_4$ treatment in the CPs experiment had a greater chlorine demand across the column than did the Cl_2 only treated columns. To meet the higher demand the chlorine dose had to be greater and the resultant chlorine concentration entering the columns was higher than in the Cl_2 only columns. The higher free chlorine concentration entering the $Cl_2 + PO_4$ column may have been responsible for the higher ranking of the alternative, particularly since the PO_4 only treatment in the CPs experiment did not rank high with respect to the goal of biofilm minimization. A closer look at the CPs' chlorinated treatments when EX was the basis for biomass indicated that $Cl_2 + PO_4$ treated biofilm had a

higher specific growth rate than Cl_2 treated biofilm, but biomass and specific removal rate were less. Based on these results phosphate may have been of some benefit when combined with chlorine. Review of influent and effluent phosphate data for both experiments (data not shown) indicated only a small amount of phosphate was being removed in the columns for both the Cl_2+PO_4 and PO_4 treatments, confirming that sufficient phosphate was present in the feed solutions to meet any adsorption that might occur on the media.

In the IOCBs experiment, the PO_4 treatment alternative showed some importance, ranking behind only Cl_2, Cl_2+PO_4 and negative humics. These results indicate that phosphate was of more importance in meeting the goal of minimizing biofilm on less reactive iron oxides (IOCBs) than on more reactive iron oxides (CPs). Phosphate appeared to be more effective at reducing carbon removal (biodegradation plus adsorption) in the CPs experiment than in the IOCBs experiment, but specific growth rate and biomass were greater. The relative ranking of the PO_4 alternative in the CPs experiment indicates that other factors, specifically chlorine, humic substances and iron, were more important in meeting the goal. Given CPs' high adsorptive capacity for humic substances, it could be possible that humic substances occupied most reactive surface sites, blocking phosphate adsorption. The opposite situation could have occurred for IOCBs making phosphate a better alternative for less reactive iron oxides. However, this possibility was not investigated further during this work.

The presence of humic substances was a significant factor when considering the goal of minimizing biofilm. Decision scores for negative humics were high in all but one scenario, CPs-HX. When chlorine treatments were not considered in the analysis of alternatives, negative humics became the most significant (highest scoring) alternative for both experiments. Where chlorine is not present, humic substances in water may become a critical factor when considering options to minimize biofilm, particularly in the presence of iron oxides. An example of this condition could exist in dead-end pipes of drinking water distribution systems where free chlorine concentrations can be low or non-existent. Assuming that positive coliform measurements and high HPC counts result from biofilm growth and detachment, humic substances in water at dead-ends would become an important factor in biofilm and coliform regrowth.

The presence of iron was an important factor for CPs, ranking behind only the Cl_2+PO_4, Cl_2 and negative humics alternatives. This relative high ranking of the negative Fe alternative helps provide support to the hypothesis that reactive iron oxides are important when considering the minimization of biofilm, and the combination of humic substances and iron oxides create a favorable situation for biofilm. The low ranking of negative Fe for the IOCBs experiment demonstrated that iron oxides with lower reactivity and less potential for adsorption of humic substances were not as important in meeting the goal of minimizing biofilm.

The EX biomass measurements for Time 0 and negative humics treatments (see Table 4) may provide information regarding the importance of adsorbed humic substances. In the IOCBs experiment there was slightly more EX biomass in the negative humics treated column (2.75 μg C/g) when compared to the Time 0 treatment (0.397 μg C/g). In comparison, the negative humics treatment for the CPs experiment had significantly more biofilm (36.42 μg C/g) than did the Time 0 treatment (0.780 μg C/g). The negative humics treatments developed and supported biofilm over the course of the experiment. This result brings into question how low the carbon concentration must be to prevent biofilm from developing. The only sources of carbon in the negative humics feed were those introduced via carbon contamination of chemicals used for nitrate, phosphate, bicarbonate and acid, or from BAC water during initial inoculation (see values below). Every precaution was taken to minimize any contamination from these and other sources.

The significant amount of EX biomass for the CPs' negative humics treatment compared to the same treatment in the IOCBs experiment may be related to the greater amount of humic substances initially adsorbed by CPs compared to IOCBs (≈ 9 times greater). However, CPs from drinking water pipes are known to contain substantial amounts of organic carbon. Measurement of organic carbon in CPs derived from pipes of four different drinking water systems ranged from 3.2 to 11.9 mg C/g [28]. Based on the measured value for q-EX ($0.0140\,h^{-1}$), the initially adsorbed humic substances on CPs could have provided $\approx 7\%$ of the organic carbon removed during the 56-day experiment. The remainder had to come from the negative humics feed solution (an average requirement of 161 μg C/L) or the organic carbon originally in the CPs (11.3 μg C/g CPs/d–633 μg C/g CPs/56 days). The CPs' negative humics feed solution had an average DOC concentration of 450 μg C/L. Approximately 36% of the feed solution DOC would have had to be biodegradable to meet the 161 μg C/L required for biodegradation/adsorption over the course of the CPs experiment. Although initial washing of the CPs prior to start of the experiment was effective in removing any readily soluble organic carbon, CPs could have easily contained sufficient organic carbon to support the biofilm biomass.

Comparison of EX biomass for negative Fe and negative humics treatments of both experiments provided information regarding the importance of both CPs and humic substances. Comparing biomass based on the specific surface area, the CPs' negative humics treatment (0.541 μg cell C/cm^2) had approximately the same

amount of biomass as the negative Fe treatment (0.518 μg cell C/cm^2). In the IOCBs experiment, the negative Fe treatment resulted in greater biomass (0.0968 μg cell C/cm^2) than the negative humics treatment (0.0156 μg cell C/cm^2). Major differences between the two experiments were the amount of humic substances initially adsorbed to the iron oxides and/or native organic carbon in CPs. Since the experiments did not investigate these factors in more detail, it can only be concluded that the presence of CPs can lead to more biofilm biomass when humic substances are not present than the case where humic substances are present without CPs. This points out that simply removing humic substances from water in a system that has unlined ferrous metal pipes may not be sufficient to minimize biofilm, at least as long as biofilm can utilize organic carbon in CPs. Removing CPs and lining older unlined pipes may be required to limit biofilm development and related problems.

The high free chlorine demand of the CPs created different conditions within the columns when compared to the IOCBs experiment. An example of the effect of chlorine on biofilm biomass helps explain this point. Columns were operated in an upflow mode; the bottom of the column was the influent end. In the IOCBs experiment the bottom of chlorine-treated columns had more biofilm than did the top, but exactly the opposite was true for the CPs experiment. Free chlorine dose for CPs columns (mean = 1.45 mg/L) had to be higher (48% higher) than for IOCBs columns (mean = 0.50 mg/L). The higher chlorine concentration entering the CPs columns limited biofilm development at the bottom or influent end. After the chlorine concentration had been reduced by reaction of chlorine with CPs (and possibly biofilm), biofilm was able to develop and grow resulting in more biofilm biomass at the top of the columns. The free chlorine concentration entering IOCBs columns was apparently not high enough to limit biofilm development at the column's influent end (bottom). Reduction in biodegradable carbon within the column would be a likely explanation for lower biomass at the top of the IOCBs chlorinated columns, although carbon removal across the columns was not measurable.

Biomass distribution in chlorinated columns of the two experiments may provide insight into biofilm development in drinking water distribution systems with extensive iron corrosion. The chlorine residual leaving a US surface water treatment facility would typically be at a concentration high enough to overcome the chlorine demand exerted by iron CPs and provide a residual at the most distant reaches of the system. If water quality conditions changed unexpectedly, such as an increase in organic carbon content after a heavy rainfall event [29], the initial chlorine demand of the system would also increase, causing residuals to decrease as water moved further from the treatment point. Biofilm could develop more rapidly at those points most distant from the treatment plant where little or no residual remained. Other conditions that could affect the amount of chlorine added to a system would be attempts to minimize chlorine disinfection byproducts by lowering chlorine dose. On the other hand, if a distribution system had less initial chlorine demand, a situation similar to the IOCBs experiment, the initial chlorine dose could be low enough to allow greater biofilm development nearer to the source of treatment and a reduction in biofilm (and biodegradable organic carbon) as water moved towards the ends of the system. This latter situation has been shown to occur in some drinking water systems [30]. In a study of 95 full-scale drinking water distribution systems Volk and LeChevallier [8] concluded that to minimize coliform occurrences free chlorine residuals should be >0.5 mg/L, biodegradable DOC <0.32 mg/L and temperature $<15^{\circ}$C. Although influent to IOCBs columns had a free chlorine concentration of 0.5 mg/L, biodegradable DOC exceeded 0.32 mg/L and temperature was $>15^{\circ}$C, and biofilm was able to grow and develop as predicted by full-scale results. When humic substances were present, even with relatively high free chlorine residuals, biofilm minimization was not achieved. Maintenance of a chlorine residual in a drinking water distribution system may not prevent positive coliform measurements [1], but it is generally agreed that a free chlorine residual is necessary for protection against microbial contaminants [4].

In the CPs experiments the pH 9 treatment was one of the least effective in meeting the goal of minimizing biofilm, ranking low because of high specific growth rates and greater biomass. In the IOCBs experiment the pH 9 treatment ranked very close to the PO_4 treatment. Raising pH was detrimental to minimization of biofilm development for the CPs experiment. Several studies have demonstrated that raising solution pH reduces the amount of humic substances that can adsorb to iron oxides by reducing possible adsorption sites [10,21]. Specific carbon removal rates for the pH 9 treatment were typically low compared to the control, possibly because of reduced adsorption to iron oxide surfaces, but overall biofilm was not minimized by alkaline pH adjustment. It was also noted that pH 8 and 9 treatments were the only treatments where there was a statistically significant drop in pH across the columns. Several researchers have shown that when humic substances adsorb to iron oxides the solution pH tends to increase due to release of hydroxyl groups from the iron oxide [10,11,16]. The fact that pH dropped across the columns could possibly indicate that humic substances were not being adsorbed. As this work did not investigate further the pH 9 treatment results, it is not known why the alternative did not rank higher. It has been shown that hydrophilic organic carbon can be

extracted from CPs using NaOH [28], making one possibility be that raising pH increased release of organic carbon from CPs and stimulated biofilm growth, but this is truly conjecture.

5. Conclusions

The results of this project provided increasing but not conclusive support of the hypothesis that adsorption of humic substances by iron oxides can stimulate and/or support biofilm development. The presence of free chlorine was the most effective treatment in minimizing biofilm. The presence of humic substances was a major factor when considering biofilm minimization based on results of both experiments, the negative humics alternative ranking either second or third behind the Cl_2 or $Cl_2 + PO_4$ treatments. The combination of humic substances and CPs led to an increase in biofilm biomass when free chlorine was not present, a condition similar to that which can occur at distribution system dead-ends. Phosphate addition was not a major factor in either the IOCBs or CPs experiments. There was weak evidence that phosphate reduced biofilm in the IOCBs experiment and that the $Cl_2 + PO_4$ treatment was more effective at minimizing biofilm than the Cl_2 treatment when CPs were the media. Results of the chlorine treatments demonstrate how differences in chlorine demand by pipe surfaces would impact spatial distribution of biofilm in a drinking water distribution system. Based on the results of this project, treatment to raise pH may not have the expected effect of minimizing biofilm when CPs are already present in a distribution system.

Many drinking water utilities have aging distribution systems containing unlined cast iron pipes. These utilities are faced with decisions regarding replacement or lining of unlined pipe, what corrosion control treatment to implement, and what disinfectant they should utilize in the distribution system. This work provides a better understanding of the complex relationships between iron oxides (CPs), corrosion control treatment, chlorine disinfection and biofilm. Chlorine was shown to be effective in control of biofilm when residuals can be maintained. However, biofilm growth utilizing humic substances that adsorb to iron oxides was shown to be an important factor to consider when attempting to minimize biofilm.

Acknowledgements

The authors wish to thank Dr. Don Reasoner of the US Environmental Protection Agency for his support throughout the course of this project. The authors acknowledge and thank the International humic Substances Society for supplying the Elliot Silt Loam soil. The US Environmental Protection Agency through its Office of Research and Development funded and collaborated in the research described here under assistance agreement number CR-826927010 to Montana State University. It has not been subjected to Agency review and therefore does not necessarily reflect the views of the Agency, and no official endorsement should be inferred. During this research the Center for Biofilm Engineering was supported by cooperative agreement EED-8907039 between the National Science Foundation and Montana State University.

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