PERGAMON

Water Research 36 (2002) 4391–4405

WATER RESEARCH

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Chlorination of model drinking water biofilm: implications for growth and organic carbon removal

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Received 1 November 2001; received in revised form 1 March 2002; accepted 19 March 2002

Abstract

The influence of chlorine on biofilm in low organic carbon environments typical of drinking water or industrial process water was examined by comparing biomass and kinetic parameters for biofilm growth in a chlorinated reactor to those in a non-chlorinated control. Mixed-population heterotrophic biofilms were developed in rotating annular reactors under low concentration, carbon-limited conditions (<2 mg/L as carbon) using three substrate groups (amino acids, carbohydrates and humic substances). Reactors were operated in parallel under identical conditions with the exception that chlorine was added to one reactor at a dose sufficient to maintain a free chlorine residual of 0.09–0.15 mg/L in the effluent. The presence of free chlorine resulted in development of less biofilm biomass compared to the control for all substrates investigated. However, specific growth and organic carbon removal rates were on the average five times greater for chlorinated biofilm compared to the control. Observed yield values were less for chlorinated biofilm. Although chlorinated biofilm's specific organic carbon removal rate was high, the low observed yield indicated organic carbon was being utilized for purposes other than creating new cell biomass. The impacts of free chlorine on mixed-population biofilms in low-nutrient environments were different depending upon the available substrate. Biofilms grown using amino acids exhibited the least difference between control and chlorinated kinetic parameters; biofilm grown using carbohydrates had the greatest differences. These findings are particularly relevant to the fundamental kinetic parameters used in models of biofilm growth in piping systems that distribute chlorinated, low-carbon-concentration water. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Biofilm; Chlorine; Kinetic parameters; Drinking water

1. Introduction

Because drinking water and industrial water distribution systems are typically extensive and complex, numerical models have been developed to help predict biofilm growth, substrate utilization and chlorine decay. Several of these include numerous variables and algorithms to model the large number of interactions that exist between biofilm cells and their water environment, often leading to reliance upon parameters that cannot be easily or reliably determined, therefore, limiting extensive application of the models. Research and experience have demonstrated the ability of biofilm in water pipelines to survive and apparently overcome the bactericidal effects of disinfectants such as chlorine. A more basic understanding of chlorine's effect on parameters such as biofilm specific growth rate and yield could simplify and improve modeling efforts, particularly if those effects could be ascertained for a variety of nutrients, substratum and disinfectant conditions.

Past research has shown that the presence of free chlorine results in less accumulation of biomass within biofilm compared to non-chlorinated systems [1], but

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PII: S0043-1354(02)00148-3

Nomenclature

A	surface area available for biofilm development (cm^2)
C_v	cell carbon per unit volume (μg C/μm^3)
F	volumetric flow rate (mL/h)
L	cell length (μm)
q	specific carbon removal rate for biofilm (h^{-1})
S	substrate concentration in reactor effluent (μg C/mL)
S_0	substrate concentration in reactor influent (μg C/mL)
V	cell volume (μm^3)
W	cell width (μm)
X	planktonic cell biomass in reactor effluent (μg C/mL)
X_0	planktonic cell biomass in reactor influent (μg C/mL)
Y_{obs}	observed yield for biofilm (μg cell C) (μg substrate C)$^{-1}$
μ_b	specific growth rate for biofilm (h^{-1})

there appears to be no consensus on how chlorine influences kinetic parameters for biofilm such as specific growth rate. Van der Wende et al. [1] determined the growth rate for biofilm to be greater in rotating annular reactors using low concentrations (0.8 mg/L dose and 0.2 mg/L effluent residual) free chlorine compared to non-chlorinated reactor systems. In contrast, utilizing tritiated thymidine uptake to estimate the activity of biofilm cells attached to cast iron surfaces, Servais et al. [2,3] found that thymidine uptake decreased with increasing free chlorine concentration.

Several deterministic models have been developed to estimate biofilm growth in the presence of free chlorine [2–6,7]. Each model presents a different approach for determining the effects of free chlorine on biofilm growth and substrate utilization. The SANCHO model uses first-order kinetics to describe decay of chlorine concentration in a distribution system and then adjusts the activity of chlorinated biofilm by reducing the maximum substrate uptake rate and the maximum rate of exoenzymatic hydrolysis of macromolecules, the latter two functions being described by Michaelis–Menten saturation kinetics [5]. The model developed by Dukan et al. [4] also incorporates a chlorine decay function to determine concentration and then uses chlorine concentration to determine a rate of bacterial mortality in a prescribed fraction of the biofilm, effectively reducing biomass. Stewart et al. [7] used a similar approach whereby chlorine reduced a fraction of the biofilm biomass without any adjustment to growth rate or yield, effectively reducing substrate uptake and biofilm biomass. These differences point out the apparent ambiguity regarding how chlorine affects biofilm, yet proper understanding of this subject is extremely important to biofilm modeling efforts.

Another parameter influencing biofilm growth and the consumption of chlorine is natural organic matter (NOM). The fraction of NOM available to biofilm as organic carbon is defined as biodegradable dissolved organic carbon (BDOC); the BDOC fraction is variable depending on substrate type and environmental conditions. The BDOC in water supplies, consisting primarily of humic substances, amino acids and carbohydrates, provides nutrients for biofilm in a distribution system and reacts with disinfectants. The SANCHO model uses the reaction of chlorine with NOM to determine chlorine residuals in the distribution system. Humic substances comprise 50–75% of the NOM found in surface waters [8,9]. Pedogenic and aquagenic proteinaceous compounds make up 5–10% of NOM in water [10], while carbohydrates make up from 1% to 30% of the total dissolved organic carbon (DOC) [11]. Many treatment processes utilizing chemical coagulation are not effective in the removal of low molecular weight compounds such as amino acids [12], and only partially effective in removal of higher molecular weight humic substances [13]. Therefore, these compounds can make their way into water distribution systems and serve as biodegradable substrates for biofilm growth.

To address the divergence in approaches for estimating chlorine's effects on biofilm accumulation and kinetics in the presence of varying types of NOM, experiments were performed under conditions simulating the low-nutrient environments typical of most drinking and industrial water systems. Comparisons were made between kinetic parameters for biofilm exposed to free chlorine disinfection to those from a control situation where no chlorine was present. In addition, three different substrate classes were investigated, each representing a major group of BOM found in natural water supplies.

2. Materials and methods

Eight experiments were performed over the course of the project; three replicates using amino acids as substrate, three using carbohydrates and two using humic substances. For each experiment two reactors were operated in parallel under identical conditions, the only exception being that one reactor received chlorine

(chlorinated reactor) and the other served as a non-chlorinated control.

2.1. Reactors

Model drinking water system biofilms were grown in continuous flow, rotating annular reactors [1,14]. Reactors were constructed of polycarbonate and consisted of a stationary outer cylinder with a rotating inner drum forming an annular space between the inner drum and the outer cylinder. Twelve removable polycarbonate slides ($\sim 30\,cm^2$ per slide) were located in grooves machined into the inside wall of the outer cylinder. Slides could be pulled from the reactor for sampling biofilm at any time without interrupting reactor operation.

Rotation of the inner drum (30 revolutions per minute) simulated the hydraulic shear stress at the wall of a 4-in-diameter (102-mm-diameter) pipe with a flow velocity of 1.0 ft/s (0.3 m/s). Total flow rate to each reactor was 5 mL/min, making the hydraulic detention time ≈ 2.1 h, a residence time that minimized planktonic growth of suspended heterotrophic organisms [16]. The lower portion of the reactors was immersed in a water bath maintained at $20 \pm 1^\circ C$ utilizing an external cooling device.

Influent flow to each reactor consisted of the substrate of interest, a nitrate/phosphate solution and dilution water (referred to as reactor dilution water). All feeds to reactors were delivered using variable-speed peristaltic pumps (Masterflex, Cole-Parmer, Vernon Hills, IL) and silicone tubing (Masterflex). Reactor dilution water was effluent from a biologically active carbon (BAC) filter column treating dechlorinated City of Bozeman, MT, drinking water. The BAC filter had been in continuous operation for a period of over 5 years. The BAC filter removed BDOC from dilution water and supplied a continuous source of indigenous, heterotrophic bacteria acclimated to a low-carbon drinking water environment. The mixed-population of heterotrophic bacteria in reactor dilution water also served as initial inoculum for biofilm in reactors. Annular reactors, tubing and feed solutions were sterile at the start of each experiment. Operation and sampling of the reactors followed aseptic technique throughout the experiments.

Two additional reactors were operated over the course of the experiments to assess bioavailability of organic carbon in reactor dilution water. The negative-control reactor consisted of a rotating annular reactor that received as its influent only reactor dilution water (BAC treated City water) and nitrate/phosphate supplement (no substrate added). Startup and operation of the negative-control reactor was identical to all other control reactors used in the experiments. The second reactor consisted of columns to measure BDOC according to the method of Ribas et al. [17]. BDOC columns were acclimated to reactor dilution water for over 6 months prior to sampling influent and effluent for DOC. The mean DOC of reactor dilution water was $1014 \pm 394\,\mu g$ C/L. There was little net change in DOC across the negative-control annular reactor ($-11 \pm 38\,\mu g$ C/L). The mean BDOC of reactor dilution water as measured using BDOC columns was $32 \pm 38\,\mu g$ C/L, a value that is extremely low. Because reactor dilution water had very low concentrations of utilizable organic carbon, its DOC concentration was assumed non-biodegradable in calculations used to determine utilization of added substrate group by biofilm in reactors.

2.2. Substrates and chlorine

Amino acids, carbohydrates and humic substances were the three substrate groups utilized for experiments. Amino acids and carbohydrates experiments utilized equimolar concentrations as carbon of the following compounds:

- Amino acid group—L-glutamic acid, L-aspartic acid, L-serine and L-alanine (Sigma, St. Louis, MO).
- Carbohydrate group—D(+)glucose, D-galacturonic acid, D(+) galactose and D(–)arabinose (Sigma, St. Louis, MO).

All feed solutions were made using reagent grade water (Nanopure, Barnstead/Thermolyne, Dubuque, IA) in glass carboys that had been combusted at 500°C for 4 h to remove residual carbon. Glass carboys and water were autoclaved prior to use, then sterile substrate or nitrate/phosphate solutions were added. Nitrate (KNO_3) and phosphate ($KH_2PO_4 + K_2HPO_4$) were fed at sufficient concentrations to create carbon limiting growth conditions based on stoichiometric calculations. Chlorine solutions were prepared by adding bleach ($\approx 5.25\%$ sodium hypochlorite) to autoclaved Nanopure water.

A concentrated humic substances stock solution was prepared by adding International Humic Substances Society (IHSS) Elliot Silt Loam Soil (BS102 M) to a 0.1 N sodium hydroxide solution, mixing for 24 h, and then centrifuging at 10,000*g* at 4°C to remove particulate matter. The concentrated humic substances stock solution (pH $\sim$ 11) was not autoclaved and was stored in the dark at 4°C. Following addition of humic substances stock solution to sterile Nanopure water the pH was adjusted to ≈ 7 using 2 N HCl.

2.3. Reactor operation

Substrate organic carbon was controlled to provide approximate influent concentrations of 500, 1000 and 2000 micrograms as carbon per liter (μg C/L). Each

reactor was fed an initial substrate concentration of $\approx 500\,\mu g$ C/L (nominal carbon level) until reactors reached a pseudo-steady state based on total direct cell counts (TDCs) in reactor effluent. Substrate concentration was increased to $1000\,\mu g$ C/L (nominal carbon level) and reactors operated for ≈ 1 week before chlorine addition was initiated. Chlorine dose was slowly increased until a free chlorine residual of 0.09–0.15 mg/L was obtained in the effluent. Reactors were operated until another pseudo-steady state was reached at the $1000\,\mu g$ C/L nominal carbon level, usually a period of 4–6 weeks. Next the substrate concentration was increased to $2000\,\mu g$ C/L (nominal carbon level). Without an immediate increase in chlorine dose, reactors were operated at the $2000\,\mu g$ C/L nominal carbon level for ≈ 1 week, then chlorine dose was increased to obtain a consistent free chlorine residual. Reactor operation at the $2000\,\mu g$ C/L nominal carbon level continued until a pseudo-steady state was again reached. Total duration of a single experiment was from 2 to 4 months.

2.4. Reactor sampling

The sampling protocol for reactors during their operation included samples of reactor dilution water and effluent for TDCs, and free chlorine concentration for chlorinated reactor effluent. Once a pseudo-steady state was reached at each nominal carbon level, four to five samples were taken over a period of 7–10 days, followed by sampling biofilm for TDCs. Data collected at pseudo-steady state was used to calculate kinetic parameters and biofilm biomass. During the pseudo-steady-state sampling period, reactor dilution water, effluent, substrate feed and nitrate/phosphate feed were sampled for total organic carbon (TOC) and DOC using the non-purgeable organic carbon (NPOC) analysis method. NPOC analyses were performed according to Standard Method 5310 B [18] using a Shimadzu TOC 5000A (Shimadzu Scientific Instruments, Columbia, MD) with high sensitivity catalyst and an auto-sampler. Filters for DOC determination were sterile 0.2-μm pore-size nylon syringe filters (Fisher Scientific, Pittsburgh, PA), prewashed with 30 mL of 0.1 N HCl then 120 mL of Nanopure water using 30 mL sterile, disposable syringes that were also used for the sample. The first 10 mL of the filtered sample was discarded. All glassware used for carbon analyses was soaked in concentrated sulfuric acid with NoChromix (Godax Laboratories, Tacoma Park, MD) for 12 h, rinsed six times using Nanopure water, followed by combustion at 500°C for 4 h. TDCs were made using epifluorescence microscopy (Olympus BH-2 microscope [Olympus, Melville, NY] at $1000\times$ magnification, minimum of 30 fields counted per sample) and 4′,6-diamidino-2-phenylindole (DAPI) stain (Sigma, St. Louis, MO) [19]. Organic carbon measurements were not made for the first two replicates of the amino acids experiment because TOC equipment was not available. Chlorine concentration was determined according to Standard Method 4500-Cl G [18] using the DPD (*N*,*N*-diethyl-*p*-phenylenediamine) colorimetric method.

Biofilm in reactors was sampled by removing a slide from the reactor, aseptically scraping biofilm from the slide into sterile phosphate buffer solution, homogenizing at 20,000 rpm for 1 m using a tissue homogenizer (IKA Labortechnik, Janke & Kunkel, Germany), then analyzing the sample for TDCs.

2.5. Carbon removal calculations

Total organic carbon entering the reactor was calculated based on flow rates for the individual feeds and their associated DOC measurements. Effluent organic carbon concentration was the measured DOC. An estimate of substrate carbon in reactor effluent was made by assuming the BDOC of reactor dilution water was negligible and that any organic carbon in the nitrate–phosphate feed was non-biodegradable. Total carbon removal across the reactors was the difference between substrate carbon in the influent and that in the effluent.

2.6. Cell volume and carbon estimates

Reactor influent and effluent samples used for TDCs, DOC and TOC measurements were also used for determining cell carbon per cell volume estimates. Mean carbon per cell was the difference between TOC and DOC measurements, divided by the number of cells as determined using TDCs. Cell volume was determined from digital images of cells stained with DAPI, gathered from random fields of view using an Olympus BH-2 microscope (Olympus, Melville, NY) and a charge coupled device (CCD-Optronics 470 T, Goleta, CA) camera linked to a personal computer running Image-Pro Plus™ software (ver 3.0, Media Cybernetics). Cell length and width were measured for a minimum of 200 cells per sample utilizing the digital images and the free software ImageTool (ver 1.27), developed at the University of Texas Health Science Center at San Antonio, Texas. Cell volume for the predominantly cocci and rod shaped cells was calculated using the formula $(\pi/4)W^2(L - W/3)$ [20]. Mean carbon per unit volume (C_v) was calculated using mean cell volume, TDCs, and the difference between TOC and DOC. A general relationship between cell volume and C_v was developed using the Generalized Pareto function [21,22], described as follows:

$$\alpha^{-1}[1 - \kappa(V - \beta)/\alpha]^{1/\kappa - 1}, \tag{1}$$

$$\alpha = 0.0078, \quad \beta = -0.0122, \quad \kappa = 0.4992, \quad r^2 = 0.91,$$
$$\text{Fit Std Error} = 41.8,$$

where V is the cell volume and parameters of the function are α, β and $\kappa(\kappa < 0, \kappa > 0)$. The Generalized Pareto function as an extreme-value function suitable for data with a highly skewed distribution, the data in this case being skewed to the right. Function parameters were estimated by least squares regression of the data (TableCurve 2D software, ver. 4, Jandel Scientific, San Rafael, CA). Using derived parameters, the Generalized Pareto function was applied to each measured cell size (and calculated volume) for an individual sample (n per sample >200) to estimate a carbon per cell value for each measured cell, and the mean of all carbon per cell values was multiplied by TDCs to estimate cell biomass as carbon.

2.7. Data analyses

Mean values for biofilm biomass and growth related kinetic parameters were compared between experiments using analysis of variance (ANOVA) techniques to determine if means were not statistically equal. Specific growth and specific carbon removal rates were natural log transformed to provide homogeneity of variance prior to ANOVA analysis. All tests were performed at a significance level $(\alpha) = 0.05$ using standard statistical software (Minitab Release 12.1, State College, PA). Standard (two-variable) correlation was performed using the Kendal tau test and the software WQHYDRO [21].

Data collected during the pseudo-steady-state time period (TDCs, cell size measurements, and DOC) were used to estimate kinetic parameters for biofilm in the reactors. The analysis assumed reactors were continuously stirred tank reactors (CSTRs) with biofilm on wetted surfaces of the reactor, biofilm specific growth rates had reached a pseudo-steady state with respect to growth, and negligible planktonic cell growth. Using cell biomass (based on TDCs and carbon per cell) in reactor influent and effluent and assuming the difference was the result of balanced growth and detachment of biofilm biomass, a specific growth rate (μ_b) was calculated for biofilm based on biofilm biomass within the reactor as follows:

$$\frac{F}{A}\left(\frac{X - X_0}{X_b}\right) = \mu_b. \tag{2}$$

Using influent and effluent DOC concentrations, volumetric flow rate, and biofilm biomass as carbon, a specific carbon removal rate (q) was calculated using the following relationship:

$$\frac{F(S_0 - S)}{X_b A} = q. \tag{3}$$

The term "carbon removal rate" was used to describe overall removal of DOC across the reactor and was an estimate of carbon utilization by biofilm cells. An observed yield (Y_{obs}) was calculated based on the difference in biomass between reactor influent and effluent, and DOC removal across the reactor, determined using the following relationship:

$$\frac{X_0 - X}{S_0 - S} = Y_{obs}. \tag{4}$$

Derivations of the above kinetic relationships are presented elsewhere [1,14].

Specific parameters for chlorinated reactor biofilm were compared to those of the control using a weighted ranking technique. The analysis was performed using a weighted hierarchy process called Simple Multiattribute Rating Technique (SMART), implemented using the computer software Criterium Decision Plus, Version 3.0.3 (InfoHarvest, Inc., Seattle, WA). To facilitate analysis, a decision goal or objective was defined and criteria selected for rating alternatives. The objective was to determine the effects of chlorination on a parameter taking into account data from all substrate groups, carbon levels and experiments. Alternatives were the control and chlorinated reactor biofilms. Fig. 1 presents hierarchy details for evaluation of a single parameter by substrate group, in this case specific growth rate for amino acids, with a description of each element of the hierarchy shown in the table at the top of the figure. For example, to analyze the effect of chlorine on specific growth rate, the detailed hierarchy shown in Fig. 1 was repeated for each substrate group and combined to form a larger hierarchy as shown in an abbreviated format in Fig. 2.

Rating of alternatives by attribute (Stats and Means) followed set criteria for each. The Stats (statistics) attribute was rated proportional to the actual p-value from comparison of means from the two alternatives. If no p-value was available the rating was neutral (each alternative received an equal rating value). The Means attribute was rated proportionally to the magnitude of the difference between means for the two alternatives. All other hierarchy elements were rated neutral and served to organize the hierarchy. The basic premise was that when parameters for chlorinated biofilm were compared to those of the control, less biofilm biomass, lower specific growth rates, lower specific carbon removal rates or lower observed yield equated to a negative ranking score, and a positive ranking score when a parameter value was greater than the control.

The analyses resulted in weighted decision scores and their estimated uncertainties. Final decision scores could range from 0 to 1.0, the decision score for the control alternative always being 0.500. A decision score <0.500 for the chlorinated alternative indicated the parameter being evaluated was less than the control, and scores >0.500 indicated the parameter was greater than for 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

Goal	Substrate	Experiment	Carbon Level	Parameter	Attribute	Alternative

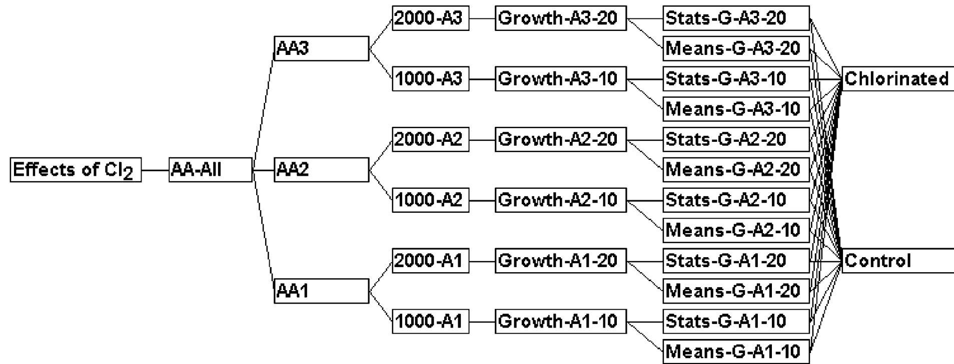

Fig. 1. Hierarchy diagram illustrating components used for analyzing the effects of chlorine on a single parameter (specific growth rate) for a substrate group (amino acids experiments). The table above the diagram identifies the name for each level of the hierarchy. AA = amino acids; AA1 = amino acids experiment 1, typical; A3-20 = amino acids replicate experiment 3 at the 2000 carbon level, typical; G = growth; Stats = statistics.

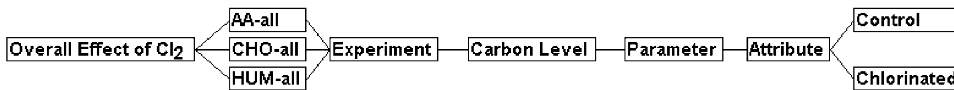

Fig. 2. General hierarchy for determining the effects of chlorine on a single parameter using all substrate groups. See Fig. 1 for a more detailed hierarchy by substrate group. AA = amino acids; CHO = carbohydrates; HUM = humic substances.

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 5th-percentile and upper 95th-percentile decision score values were computed for each alternative. Using uncertainty results, a percentage of the time a particular alternative was better than the other was computed (direct comparison). If an alternative resulted in being better $>5\%$ of the time the result was considered possibly significant with respect to the objective of determining the effects of chlorine. For example, if the decision score for observed yield for the chlorinated alternative was >0.500 and better 50% of the time, then it could be concluded that the effect of chlorine was to make observed yield for chlorinated biofilm greater than for the control.

3. Results

3.1. Cell carbon per unit volume

A significant correlation ($p<0.01$) was found to exist between cell volume and cell carbon per volume (C_v). When a power function of the form $C_v = aV^b$ was fit to the data, as has been done by others [23,24], the predicted C_v at low cell volume values became unreasonably high (919–1365 femtograms of carbon per cubic micron [fg C/μm^3] at a cell volume of 0.01 μm^3). In contrast, the Generalized Pareto function predicted a more reasonable value of 535 fg C/μm^3 at 0.01 μm^3. Mean C_v measured in this study was 262 ± 167 (mean ± standard deviation, $n = 61$) fg C/μm^3. This value falls within the range of cell carbon per volume reported by others for a variety of environments: 133–400 fg C/μm^3 [23], 350 fg C/μm^3 [25], 560 fg C/μm^3 [20] and 380 fg C/μm^3 [26].

3.2. Total carbon removal by substrate group

Chlorine addition had little impact on the total removal of amino acids as a fraction of influent amino acids concentration expressed as carbon (0.96-control vs. 0.90-chlorinated) and had the greatest effect on the fraction of carbohydrates removed (0.66-control vs. 0.17-chlorinated). Addition of chlorine reduced total removal of humic substances (0.51-control vs.

Table 1
Mean values for free chlorine dose, residual and demand by substrate group and nominal carbon level

Substrate[a]	Nominal carbon level (μg C/L)	Free Cl_2[a]		
		Dose (mg/L)	Residual (mg/L)	Demand (mg/L)
AA	1000	1.02	0.09	0.93
AA	2000	2.09	0.10	1.99
CHO	1000	0.44	0.14	0.30
CHO	2000	0.47	0.14	0.33
HUM	1000	0.81	0.15	0.66
HUM	2000	1.06	0.11	0.95

[a] AA = amino acids; CHO = carbohydrates; HUM = humic substances; Cl_2 = chlorine.

Table 2
Mean biofilm biomass (X_b) by substrate type, experiment and nominal carbon level for the two reactor types (control and chlorinated)

Experiment[a]	Nominal carbon level (μg C/L)	Biofilm biomass (X_b)[b]	
		Control (μg cell C/cm^2)	Chlorinated (μg cell C/cm^2)
AA1	1000	1.06 (0.065)[c]	1.15 (0.089)
AA2	1000	0.117 (0.004)	0.243 (0.006)
AA3	1000	3.58 (0.154)	3.58 (0.007)
AA1	2000	2.43 (0.345)	4.56 (0.056)
AA2	2000	0.130 (0.004)	0.080 (0.004)
AA3	2000	2.98 (0.033)	1.38 (0.082)
CHO1	1000	0.518 (0.019)	0.038 (0.003)
CHO2	1000	0.461 (0.025)	0.036 (0.004)
CHO3	1000	0.458 (0.023)	0.008 (0.000)
CHO1	2000	0.497 (0.106)	0.007 (0.001)
CHO2	2000	0.477 (0.043)	0.018 (0.001)
CHO3	2000	3.65 (0.203)	0.014 (0.001)
HUM1	1000	0.949 (0.059)	0.030 (0.002)
HUM2	1000	4.05 (0.239)	0.443 (0.017)
HUM1	2000	2.16 (0.120)	0.235 (0.001)
HUM2	2000	4.29 (0.171)	1.49 (0.105)

[a] AA = amino acids; CHO = carbohydrates; HUM = humic substances.
[b] Biomass based on TDCs, measured cell sizes and conversion of calculated cell volume to cell carbon.
[c] Standard error of the mean, typical.

0.41-chlorinated) but the difference was not as great as that measured in carbohydrate experiments. Free chlorine demand for a given substrate group (see Table 1) appeared to have a relationship to total carbon removal. Chlorine had the greatest effect on total carbon removal for the substrate group that had the lowest free chlorine demand, the carbohydrates group. Chlorine had the least effect on total carbon removal for amino acids, the substrate with the highest free chlorine demand, even though a free chlorine residual was measured. The results indicate the importance of substrate type in overall organic carbon removal by biofilm.

3.3. *Biofilm parameters*

Tables 2–5 present the mean parameter values for biofilm biomass (X_b), specific growth rate (μ_b), specific carbon removal rate (q) and observed yield (Y_{obs}), respectively. The mean parameter values and their associated p-values from the comparison of means between reactor types were used to rank attributes for each alternative by specific parameter. Insufficient data points were available for a statistical comparison of means for biofilm biomass. Results of the ranking and analysis are discussed below. Sufficient data were available to compare specific growth rate and observed yield between replicate experiments, and p-values for that comparison are shown in Tables 3 and 5. There was considerable variability between specific growth rates for replicate carbohydrates experiments at the 1000 μg C/L nominal carbon level, but all other experiments and substrates had good repeatability between replicate experiments. Comparison of observed yield between replicate experiments showed much more variability than for specific growth rate. The degree of variability between replicate experiments seen in this work was not unexpected since mixed-populations of bacteria indigenous to drinking water were used in all experiments. Because of variability between experiments for some parameters, the analysis technique allowed the results of each individual experiment to be ranked separately, i.e., the chlorinated reactor parameter was always compared to the control reactor parameter for a given substrate group, replicate experiment and carbon level.

Table 3
Mean biofilm specific growth rate (μ_b) by substrate type, experiment and nominal carbon level for the two reactor types (control and chlorinated)

Experiment[a]	Nominal carbon level (μg C/L)	Specific growth rate (μ_b)		
		Control (h^{-1})	Chlorinated (h^{-1})	p (reactor type)
AA1	1000	0.0202 (0.0036)[b]	0.0134 (0.0020)	0.154
AA2	1000	0.0864 (0.0199)	0.0460 (0.0078)	0.053
AA3	1000	0.0071 (0.0023)	0.0329 (0.0051)	<0.0005
	p (replicates)	<0.0005	0.014	
AA1	2000	0.0128 (0.0014)	0.0161 (0.0013)	0.124
AA2	2000	0.0845 (0.0051)	0.1068 (0.0164)	0.282
AA3	2000	0.0140 (0.0028)	0.0058 (0.0004)	0.007
	p (replicates)	<0.0005	<0.0005	
CHO1	1000	0.0101 (0.0001)	0.0586 (0.0116)	0.006
CHO2	1000	0.0165 (0.0025)	0.0779 (0.0200)	0.003
CHO3	1000	0.0166 (0.0027)	0.1216 (0.0458)	0.041
	p (replicates)	0.160	0.287	
CHO1	2000	0.0170 (0.0059)	0.1788 (0.0277)	0.001
CHO2	2000	0.0538 (0.0097)	0.0497 (0.0161)	0.829
CHO3	2000	0.0031 (0.0007)	0.0648 (0.0341)	0.082
	p (replicates)	0.0030	0.008	
HUM1	1000	0.0033 (0.0006)	0.0796 (0.0103)	<0.0005
HUM2	1000	0.0009 (0.0002)	0.0067 (0.0014)	0.001
	p (replicates)	0.007	<0.0005	
HUM1	2000	0.0031 (0.0008)	0.0085 (0.0011)	0.002
HUM2	2000	0.0010 (0.0001)	0.0031 (0.0004)	<0.0005
	p (replicates)	0.054	<0.0005	

[a] AA = amino acids; CHO = carbohydrates; HUM = humic substances.
[b] Standard error of the mean, typical.

3.4. *Effects of chlorine on biofilm by parameter*

Results of the analysis by parameter using the combination of data from all substrate groups are presented graphically in Fig. 3. Compared to control reactors, addition of chlorine resulted in greater specific growth rates (e.g., the decision score for the chlorinated alternative was >0.500), greater specific carbon removal rates, lower observed yield and lower biofilm biomass. Given the calculated uncertainty in the decision score for

Table 4
Mean substrate concentration (reactor influent) and specific carbon removal rates (q) by substrate type, experiment and nominal carbon level for the two reactor types (control and chlorinated)

Experiment[a]	Nominal carbon level (μg C/L)	Substrate conc. (μg C/L)	Specific carbon removal rate (q)		
			Control (h^{-1})	Chlorinated (h^{-1})	p (reactor type)
AA3	1000	991	0.048 (0.006)[b]	0.811 (0.272)	0.013
AA3	2000	1776	0.102 (0.006)	0.208 (0.012)	<0.0005
CHO1	1000	774	0.267 (—)[c]	1.176 (—)[c]	—[c]
CHO2	1000	1017	0.247 (—)[c]	0.339 (—)[c]	—[c]
CHO3	1000	954	0.233 (0.032)	5.311 (0.845)	0.001
CHO1	2000	N/A[d]	N/A	N/A	N/A
CHO2	2000	1924	0.415 (0.031)	2.365 (0.754)	0.236
CHO3	2000	1946	0.076 (0.001)	3.724 (0.214)	<0.0005
HUM1	1000	877	0.035 (0.027)	1.362 (—)[c]	—[c]
HUM2	1000	1090	0.018 (0.004)	0.115 (0.010)	0.069
HUM1	2000	1969	0.089 (—)[c]	0.587 (—)[c]	—[c]
HUM2	2000	1783	0.039 (0.008)	0.079 (0.023)	0.112

[a] AA = amino acids; CHO = carbohydrates; HUM = humic substances.
[b] Standard error of the mean, typical.
[c] Insufficient data points for statistics.
[d] N/A—Data not available.

each alternative as shown in Fig. 3, the differences noted above were most likely significant, a conclusion based on the percent of the time one alternative was better than the other; alternatives being the control and chlorinated biofilm. Results presented in Fig. 3 verify a general trend observed in data shown in Tables 2–5; chlorination increased specific growth and carbon removal rates, and reduced observed yield and biomass. If this trend had not been significant for a substrate group the difference in the decision scores would not have been as great and the percent of the time one alternative was greater than the other would have been less for one and greater for the other.

Table 6 presents a breakdown of decision scores by parameter and alternative for each substrate group. Also shown in Table 6 is the difference in decision scores (Δ score) for each alternative. The Δ score does not measure the magnitude of the difference between parameters; it does provide a measurement of the strength of the difference taking into account the relative magnitude of the difference and the probability that the two parameters being compared are not equal. The magnitude of the Δ score indicates how strong the effect of chlorine was on a particular parameter for one substrate group when compared to that of the other substrate groups; and a relative indicator of chlorine's effect on individual parameters when compared within a substrate group. The observations noted above and shown in Fig. 3 were consistent for all substrate groups except amino acids where the magnitude of the Δ scores for the growth and biomass parameters was low, showing the strength of any effect chlorine had on these two parameters was very low. It should be noted that the chlorine residual in the chlorinated reactor during the amino acids experiment was similar to residuals used in experiments for other substrates (refer to Table 1). Type of substrate present influenced how strongly chlorine effected kinetic parameters.

Amino acids had the highest chlorine demand of the three substrate groups (see Table 1), indicative of the

Table 5
Mean observed yield (Y_{obs}) by substrate type, experiment and nominal carbon level for the two reactor types (control and chlorinated)

Experiment[a]	Nominal carbon level (μg C/L)	Observed yield (Y_{obs})		
		Control (g/g)	Chlorinated (g/g)	p (reactor type)
AA3	1000	0.077 (0.017)[b]	0.037 (0.010)	0.007
AA3	2000	0.119 (0.018)	0.028 (0.002)	<0.005
CHO1	1000	0.038 (0.001)	0.050 (0.010)	0.267
CHO2	1000	0.079 (0.016)	0.070 (0.012)	0.654
CHO3	1000	0.072 (0.008)	0.041 (0.018)	0.158
	p (replicates)	0.159	0.331	
CHO1	2000	N/A[c]	N/A	N/A
CHO2	2000	0.128 (0.023)	0.020 (0.006)	0.001
CHO3	2000	0.041 (0.010)	0.042 (0.024)	0.680
	p (replicates)	0.011	0.457	
HUM1	1000	0.197 (0.067)	0.058 (0.008)	0.057
HUM2	1000	0.047 (0.008)	0.048 (0.010)	0.680
	p (replicates)	0.008	0.582	
HUM1	2000	0.020 (0.003)	0.014 (0.001)	0.024
HUM2	2000	0.029 (0.004)	0.043 (0.006)	0.092
	p (replicates)	0.136	0.005	

[a] AA = amino acids; CHO = carbohydrates; HUM = humic substances.
[b] Standard error of the mean, typical.
[c] N/A—Data not available.

known high reactivity of nitrogen compounds with chlorine [27]. Reaction of amino acids with chlorine did not impact growth and development of biofilm under conditions evaluated in these experiments, indicating that the reaction between amino acids and free chlorine had little effect on overall development of biofilm.

3.5. *Effects of chlorine on biofilm parameters by substrate type*

A comparison of Δ scores between substrate groups for a specific parameter provided a relative indication of which substrate group's biofilm was most strongly affected by chlorination. Observed yield was most strongly impacted by chlorination when amino acids were the substrate group, and there was little difference in strength of the overall effect on observed yield for humic substances and carbohydrates even though true differences existed in observed yield values for these two substrates. Specific growth rates and specific carbon removal rates for humic substance experiments were most strongly affected by chlorination (they had the greatest Δ score values for those parameters). In terms of chlorine demand, humic substances had demand values between amino acids on the high end and carbohydrates on the low end (see Table 1). A substrate group's chlorine demand did not correlate to the magnitude of the Δ scores for chlorine's ultimate effects on specific growth rate and a similar observation was noted for specific carbon removal rates.

Chlorination most strongly affected biomass when carbohydrates were the substrate and least when amino

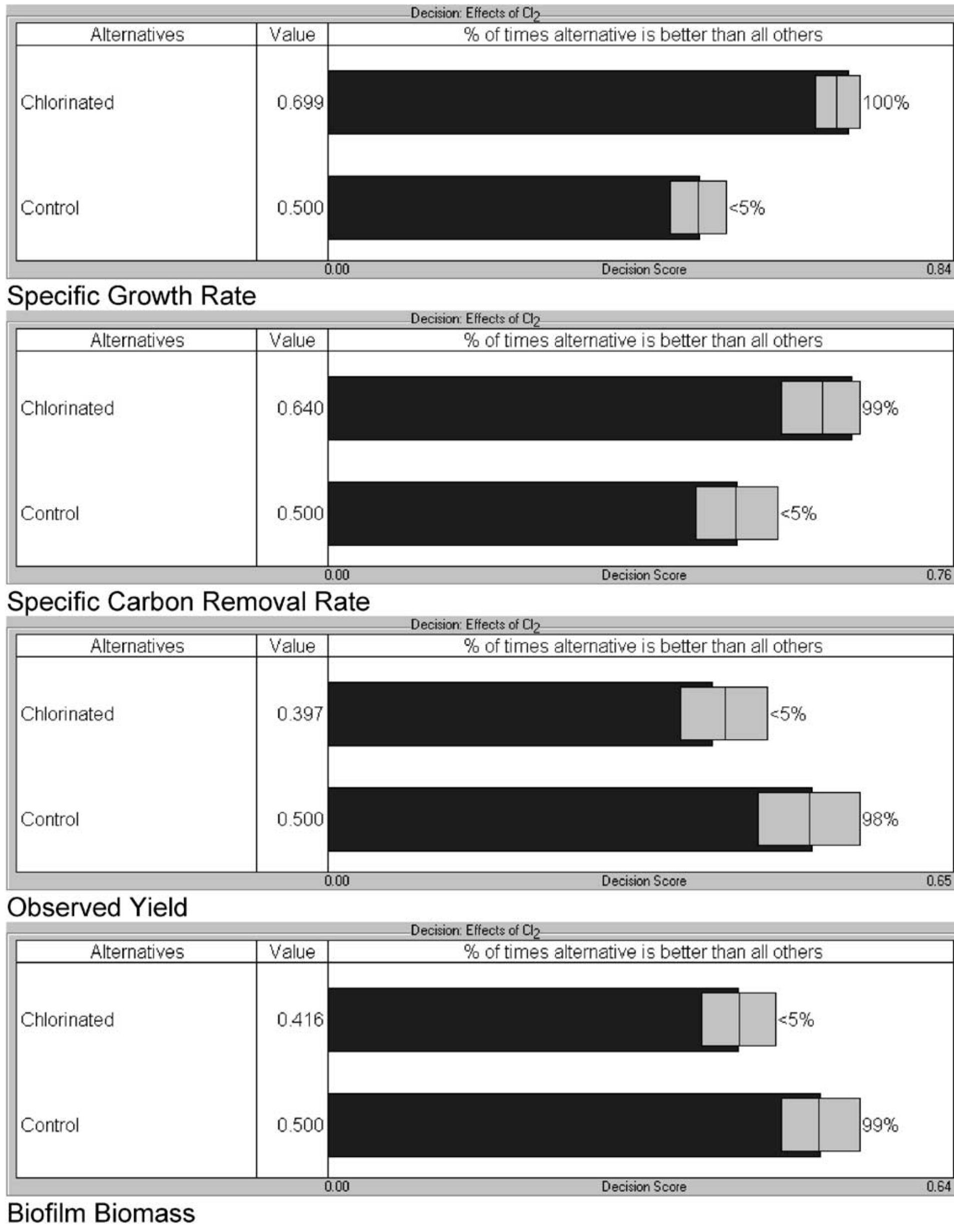

Fig. 3. Graphical presentation of decision scores and degree of uncertainty for analysis of specific parameters when all substrates were combined. Boxes at right end of bar columns represent estimated uncertainty in decision score; range is 5th–95th percentile and bar inside box is the mean.

acids were the substrate. Carbohydrates had the lowest chlorine demand of the three substrates investigated (see Table 1) and the chlorinated carbohydrates reactor had the lowest total carbon removal. These factors may have played a role in the large reduction of biomass when chlorine was present in the carbohydrates experiments. Chlorine species may be more reactive with biofilm if added chlorine does not immediately react with substrates.

Biofilm biomass and total carbon removal appeared to be related to the chlorine demand of the substrate group; high demand resulted in high biomass and high total carbon removal. However, biofilm kinetic parameters for specific growth and carbon removal rates did not have the same correlation to chlorine demand. The type of substrate had an effect on these parameters, indicating that chlorine demand or chlorine concentration may not be a good predictor of chlorine's effects on

Table 6
Decision scores by substrate group for each parameter and alternative

Parameter	Alternative	HUM[a]		CHO		AA	
		Score	Δ Score	Score	Δ Score	Score	Δ Score
Growth	Control	0.167	0.111	0.167	0.090	0.167	−0.002
	Chlorinated	0.278		0.257		0.165	
Carbon	Control	0.167	0.058	0.167	0.049	0.167	0.032
	Chlorinated	0.225		0.216		0.199	
Yield	Control	0.167	−0.033	0.167	−0.031	0.167	−0.040
	Chlorinated	0.134		0.136		0.127	
Biomass	Control	0.167	−0.021	0.167	−0.056	0.167	−0.008
	Chlorinated	0.146		0.111		0.159	

Note: Absolute value of Δ score (=score[Chlorinated]−score [Control]) provides an indication of how strong the effect of chlorine was on chlorinated biofilms when compared to the control.

[a] AA = amino acids; CHO = carbohydrates; HUM = humic substances; Δ = difference.

these parameters. Chlorine's presence significantly modified specific growth and specific carbon removal rates when humic substances and carbohydrates were the substrate groups, but the change was to increase these parameters rather than retard them as has been assumed in many biofilm models.

4. Discussion

The approach used for this study was to compare kinetic parameters and biomass for a mixed-population biofilm with and without the presence of free chlorine under conditions relevant to a distribution system carrying low organic-carbon-content water. The result that indicated less biofilm biomass in the chlorinated reactor than in the control reactor was expected. However, higher specific growth and specific carbon removal rates for chlorinated reactor biofilm when compared to the control were surprising.

Compared to the control reactor, there was less biofilm biomass and lower total carbon removal by the chlorinated reactor biofilm. Although specific carbon removal rates were greater for chlorinated biofilm, the fact that there was much less biofilm biomass resulted in lower overall total carbon removal and a higher substrate concentration within the reactor's liquid phase. Higher substrate concentration in the chlorinated reactors may have influenced specific growth and specific carbon removal rates by increasing the concentration gradient between the liquid phase and biofilm, resulting in greater mass transport rates. Analysis of data for each carbon level (not shown) indicated that there was no distinct correlation or relationship (such as a hyperbolic or positive linear function) between specific growth rate or specific carbon removal rate and effluent substrate concentration [28]; in most cases a zero-order relationship was found. Without evidence of a direct, positive correlation between substrate concentration and these kinetic parameters it would be difficult to conclude that higher substrate concentration was a major factor leading to greater specific growth and specific carbon removal rates for biofilms exposed to chlorine.

Interactions between a particular substrate group and free chlorine are complex and may affect mechanisms employed by biofilm to eventually utilize substrate. For example, amino acids react with chlorine quite rapidly, forming *N*-chloro compounds such as organochloramines [29–31] or creating the corresponding aldehyde or nitrile [31]. The high demand for chlorine in amino acid experiments indicated high reactivity of amino acids with added chlorine. Organochloramines formed by reaction of chlorine with amino acids have little disinfection capability [32] and could possibly explain why there was nearly as much biofilm biomass in the chlorinated reactor as the control. However, the free chlorine residual in the reactors was the same for all experiments, thus reducing the possibility that low disinfection capability of organochloramines was a reason for high biomass in the chlorinated reactor. Glutamic acid, one of the amino acids used in this work, has been reported to have a low demand for chlorine [30] and little interference with the chlorination process [32]; added glutamic acid may have been directly available to biofilm in chlorinated reactors. Data presented in Table 3 indicate only a marginal difference between biofilm specific growth rates in the control and chlorinated reactors receiving amino acids. Despite the potential

interactions between amino acids and chlorine, chlorinated biofilm cells were able to grow on the oxidation products. Overall carbon removal fractions for amino acids experiments were 0.96 and 0.90 in the control and chlorinated reactors, respectively, demonstrating amino acids' high potential for biodegradation by biofilm in drinking water or other low-carbon environments regardless of the presence of free chlorine.

Free carbohydrates do not typically react with chlorine as has been shown in measurements of their trihalomethane (THM) formation potential [33]. As shown in Table 1 there was little difference between mean chlorine demand at the 1000 and 2000 μg C/L nominal carbon levels for carbohydrate experiments indicating chlorine demand was not a function of substrate concentration. It would be logical to postulate that in chlorinated reactor receiving carbohydrates chlorine was more reactive with biofilm, thereby decreasing biofilm development. There was much less biofilm in the chlorinated carbohydrates reactor (0.02 micrograms of cell carbon per square centimeter [μg cell C/cm^2]) compared to the control reactor (1.01 μg cell C/cm^2).

Chlorine interactions with humic substances may reduce their molecular weight and make them more available to biofilm, leading to an increase in biofilm growth rates. Chlorine demand for humic substances was less than for amino acids but more than for carbohydrates (see Table 1). In a study of the effects of chlorine on humic substances, it was found that there was a decrease in phenols, methoxyl carbon, aryl-carbon and ketones, and an increase in carbohydrate and alkyl carbon [34]. Chlorination of humic substances can also rupture the aromatic ring structure of carbon compounds, creating haloacetic acids, dicarboxylic acid and α-chloropropionic acid [35,36]. These products of chlorination are typically of lower molecular weight and more biodegradable, therefore, chlorination has the potential to increase bioavailability of humic substances. Chlorination products may be more available to microorganisms than non-chlorinated humic substances because lower molecular weight compounds require less cell energy for their uptake and utilization.

The ability of the extracellular polymeric substance (EPS) matrix to bind humic substances may have influenced biofilm growth rates when humic substances were the substrate. The distinctive yellow–brown color of humic substances that had attached to or become part of the biofilm was clearly visible when sample slides were pulled from reactors receiving humic substances as the substrate. Marshall [37] cites work by himself and others showing that when humic substances and other hydrophobic compounds adsorb to surfaces, biofilm develops at those surfaces and uses the adsorbed molecules as nutrients. Wolfaardt et al. [38,39] have demonstrated the ability of EPS to accumulate nutrients for subsequent biofilm cell utilization and that a variety of available ion exchange sites exist within the biofilm EPS matrix where nutrients can be adsorbed.

Based on the above information regarding humic substances, one hypothesis would be that attachment of humic substance molecules to the biofilm matrix could make the biofilm growth rate a function of how fast biofilm cells can break down attached humic substances rather than a function of humic substances concentration in the bulk fluid. In this hypothesis the interaction of chorine with attached organic substances could offer some protection against the effects of chlorine by serving as sacrificial compounds for oxidation by free chlorine [40], and at the same time chlorine could make adsorbed humic substances more biodegradable.

As shown by the relative magnitude of decision scores in Table 6, the specific growth and specific carbon removal rates for biofilm in chlorinated reactors were higher than the control, while observed yield was lower. The only exception to this observation was specific growth rates for the amino acids experiments. Production of EPS, proposed by several researchers as a method used by biofilm to protect against the effects of hypochlorous acid [15,41,42] or other compounds such as glutathione [43] that can afford protection against the effects of chlorine could require uptake and conversion of large amounts of organic carbon and other nutrients. Lower observed yield in chlorinated reactors supports the concept that the larger amount of substrate carbon removed per unit of biofilm biomass was not necessarily going into production of more biomass but could have been used to produce compounds required for protection and survival. Since the work reported here did not attempt to investigate further some of the underlying mechanisms, the possible explanations given are conjecture.

Based on results of this work it can be stated that the presence of chlorine in a drinking water environment does not retard specific growth and specific carbon removal rates as has been the premise of several biofilm models. On the contrary, specific growth and specific carbon removal rates are nearly equal to or greater than those rates seen without the presence of chlorine, dependent upon the type of substrate group available to biofilm. Yield is the kinetic parameter that can account for carbon that is taken up but not used for creation of new biomass. In the chlorinated drinking water environments that were investigated it can be generally concluded that yield will be lower than in a non-chlorinated environment, resulting in less biofilm biomass even when specific carbon removal rates are quite high. Type of substrate and its potential interactions with chlorine can be key to estimating biomass development. Amino acids, a substrate that has high demand for chlorine, resulted in nearly the same amount of biomass as in the control, whereas carbohydrates, a

substrate that has a low chlorine demand, resulted in the least biofilm biomass in the presence of chlorine. Phenomenological models of biofilm in drinking water systems should consider the type of substrate groups present and how biofilm responds to those groups when chlorine is present. Biofilm models that assume the presence of chlorine slows down biofilm's growth and uptake mechanisms may be giving the appropriate answer, but for the wrong reasons.

5. Conclusions

Based upon the results of this research, biofilm exposed to free chlorine in low-nutrient environments similar to that found in drinking and industrial water systems can actively reproduce and take up necessary carbon compounds required not only to sustain high growth rates but also to presumably support a variety of mechanisms required to protect cells within the biofilm community from the effects of free chlorine. The type of substrate present in water and its basic interaction with chlorine will determine to a great extent the biofilm development. Since water supplies contain a complex mixture of organic compounds that are available as substrates, a host of different mechanisms are most likely employed by biofilm to survive the deleterious effects of free chlorine. Knowledge of actual relationships between basic kinetic parameters provides a better understanding of the true effects of free chlorine on drinking water biofilm and will help improve models used to estimate biofilm growth in chlorinated water distribution systems.

Acknowledgements

This work was supported by the Center for Biofilm Engineering at Montana State University-Bozeman, a National Science Foundation supported Engineering Research Center (Cooperative agreement EEC-8907039), and by the American Water Works Association Research Foundation.

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