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Chloride Enhances DNA Reactivity with Chlorine under Conditions Relevant to Water Treatment

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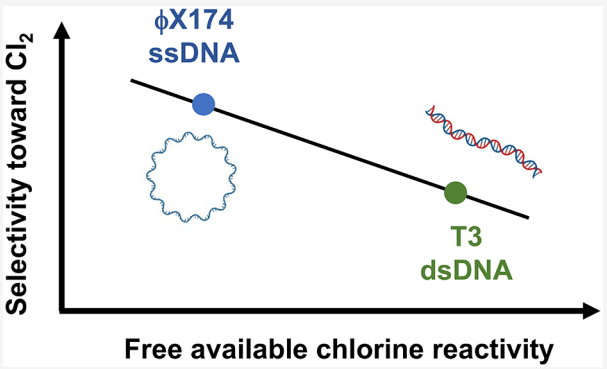
ABSTRACT: Free available chlorine (FAC) is widely used to inactivate viruses by oxidizing viral components, including genomes. It is commonly assumed that hypochlorous acid (HOCl) is the chlorinating agent responsible for virus inactivation; however, recent studies have underscored that minor constituents of FAC existing in equilibrium with HOCl, such as molecular chlorine (Cl_2), can influence FAC reactivity toward select organic compounds. This study measures the FAC reaction kinetics with dsDNA and ssDNA extracted from representative bacteriophages (T3 and ϕX174) in samples augmented with chloride. Herein, chloride enhances FAC reactivity toward dsDNA and, to a lesser extent, toward ssDNA, especially at pH < 7.5. The enhanced reactivity can be attributed to the formation of Cl_2. Second-order rate constants were determined for reactions of ssDNA and dsDNA with HOCl and Cl_2. DNA chlorination kinetics followed the reactivity-selectivity principle, where the more-reactive nucleophilic species (ssDNA, ∼100× more reactive than dsDNA) reacted less selectively with electrophilic FAC species. The addition of chloride was also shown to enhance the inactivation of bacteriophage T3 (dsDNA genome) by FAC but did not enhance the inactivation of bacteriophage ϕX174 (ssDNA genome). Overall, the results suggest that Cl_2 is an important chlorinating agent of nucleic acids and viruses.



KEYWORDS: *deoxyribonucleic acid, ssDNA, dsDNA, bacteriophages, free available chlorine, chloride, pH*

INTRODUCTION

Viruses are a major cause of waterborne and foodborne diseases globally.[1,2] Free available chlorine (FAC) is used in a wide variety of applications to control viruses and other pathogens. More than 70% of water treatment plants in the United States use FAC to inactivate pathogens at various stages of treatment, making FAC the most widely used water disinfectant in the United States and Canada.[3] The proliferation of aquatic viral infections in the Asian aquaculture industry, which accounts for 90% of global aquaculture production (by volume), has popularized the use of chlorine-based disinfectants in five countries.[4] FAC is directly added to conventional swimming pool water and, to a lesser extent, to saltwater pools, to inactivate pathogens and provide a disinfectant residual.[5] Furthermore, FAC is the most popular postharvest disinfectant of produce, meat, and fish in the United States.[6,7]

Hypochlorous acid (HOCl; pK_a = 7.54) is regarded as the FAC species responsible for disinfection; HOCl inactivates viruses by oxidizing viral components.[8,9] Previous work suggests that viral inactivation can be driven by reactions involving proteins or nucleic acids.[8−11] For example, Nuanualsuwan et al. concluded that poliovirus inactivation by FAC is driven by genome damage,[12] whereas Wigginton and Kohn concluded that bacteriophage MS2 inactivation by FAC could be attributed to reactions with the genome and capsid proteins.[13] These differences in mechanisms may be driven by the virus structure, including the accessibility of highly reactive regions of the proteins and nucleic acids to FAC. For nucleic acids, FAC halogenates nucleotides with rate constants ranging from 6.4 M^{-1} s^{-1} (for adenosine, pH ∼ 7) to 2.1 × 10^4 M^{-1} s^{-1} (for guanosine, pH ∼ 7).[14−16] The higher-order nucleic acid structure impacts FAC reaction rates, with single-stranded DNA (ssDNA) having FAC rate constants >100× larger than double-stranded DNA (dsDNA) rate constants.[17]

To date, researchers have assumed that HOCl is the chlorinating agent that initiates viral damage, and FAC reaction kinetics determined for viral genomes are based on that

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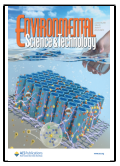

assumption.[18−20] Studies have underscored that minor constituents of FAC, molecular chlorine (Cl_2) and dichlorine monoxide (Cl_2O), which exist in equilibrium with HOCl (eqs 1 and 2),[21,22] can influence the chlorination rates of several classes of organic compounds[23−27]

$$HOCl + Cl^- + H^+ \rightleftharpoons Cl_2 + H_2O \qquad \log K_{Cl_2} = 2.72 \tag{1}$$

$$2HOCl \rightleftharpoons Cl_2O + H_2O \qquad \log K_{Cl_2O} = -2.06 \tag{2}$$

In particular, Cl_2 and Cl_2O play key roles in FAC reaction kinetics of aromatic compounds such as (chloro)phenols,[24,27] aromatic ethers,[23] and nitrogen-containing heterocycles;[28,29] FAC reactivity thereof cannot be explained if Cl_2 and Cl_2O are not considered.[23,24,27−29] Using ionones as model alkenes, Lau et al. showed that as the FAC reactivity of compounds increases, the selectivity of the compounds toward Cl_2 and Cl_2O decreases in a manner consistent with the reactivity-selectivity principle.[24,30] Furthermore, at chloride concentrations >1 mM and pH 7, reactions with Cl_2 and Cl_2O can contribute up to 63% of the total FAC reactivity for α-ionone[24] and 83% for 3-methyl-anisole.[23] Omitting Cl_2 and Cl_2O from kinetic modeling may therefore yield "rate constants of dubious validity."[24] This is particularly true for disinfection of chloride-containing waters, such as drinking water (up to 7.1 mM chloride)[31] and seawater aquaculture systems (up to 530 mM chloride).[32]

Given that dsDNA and ssDNA are common virus genome types and exhibit different reactivities with FAC,[17] we examined the reactions of viral dsDNA and ssDNA with FAC in the presence of chloride. We systematically varied the pH and chloride concentrations in samples to determine whether Cl_2 and Cl_2O make appreciable contributions to the overall FAC reactivity of DNA. We further compared the magnitude of the effect that chloride has on the FAC reactivity toward dsDNA and ssDNA. We extended this approach to determine whether chloride has an effect on viral inactivation by FAC by measuring inactivation rates of bacteriophages T3 and ϕX174. Given that the selectivity toward Cl_2 is anticipated to decrease with increasing FAC reactivity,[30] we examined whether the reactivity-selectivity principle holds across biomolecules and viruses.

MATERIALS AND METHODS

Reagent Preparation. Phosphate buffer solutions (5 mM, pH: 6−9) were prepared by dissolving mono- and disodium phosphate (Fisher Scientific) in MilliQ water (Millipore Sigma). The pH varied by <0.1 throughout the experiments. Buffer solutions were augmented with sodium chloride or sodium perchlorate (Sigma-Aldrich), filter-sterilized (0.22 μm polyethersulfone; Millipore Sigma), and heat-treated to inactivate nuclease (95 °C; 30 min).[17] FAC stock solutions were prepared by diluting 10% w/v sodium hypochlorite (Sigma-Aldrich) in Milli-Q water (Millipore Sigma) and were standardized spectrophotometrically (ε_{292nm} = 365 M^{-1} cm^{-1}).[33,34] Chlorine residuals were measured using *N,N*-diethyl-*p*-phenylenediamine indicator solution (Ricca Chemical) according to EPA Method 4500. FAC was quenched using ultrapure Tris−HCl (Invitrogen); controls showed that Tris−HCl did not affect quantitative polymerase chain reaction (qPCR) assays. Reagents used were of reagent grade or higher and prepared in chlorine-demand-free glassware. Bromide concentrations measured in our reagents were on par with those in other studies (Text S4 and Table S15).

Viral Genome Preparation. Experiments were conducted using genomes extracted from two bacteriophages, *Escherichia coli (E. coli)*bacteriophage T3 (ATCC 11303-B3) and *E. coli* bacteriophage ϕX174 (ATCC 13706-B1). T3 was chosen as the dsDNA genome virus; the T3 virion contains a nonsegmented ∼38.2 kbp dsDNA genome. ϕX174 was chosen as the ssDNA genome virus; the ϕX174 virion contains a circular ∼5.4 kbp ssDNA genome. Bacteriophage propagation and purification were described previously.[9,35] Briefly, T3 and ϕX174 were propagated by an agar overlay technique using *E. coli* ATCC 11303 and *E. coli* 13706 as the respective hosts. Phage stocks were purified using a sucrose gradient and buffer exchanged into 5 mM phosphate with 100 kDa centrifugal ultrafilters (0.22 μm polyethersulfone; Millipore Sigma). The purified stocks (>10^{10} plaque forming units/mL) were stored at 4 °C prior to extraction.

Genomes were extracted using a Maxwell 16 Viral Total Nucleic Acid Purification Kit (Promega) and eluted into ultrapure nuclease-free water according to manufacturer instructions. As a control, the bacteriophage T3 genome was extracted using the QIAmp UltraSens Virus Kit (Qiagen) according to manufacturer instructions.[36] Control experiments showed the same FAC reactivity for genomes extracted using either kit (Figure S1). Extracted nucleic acid mass concentrations were quantified using Qubit high-sensitivity dsDNA (for T3 genome) and ssDNA (for ϕX174 genome) assay kits (Invitrogen). Extract purity was verified using a nanodrop spectrophotometer; 260/280 ratios for extracts ranged from 1.6 to 1.8. Nucleic acid mass concentrations (in ng/μL) were converted to gene copy concentrations (in gene copies (gc)/μL) using a copy number calculator (SciencePrimer). Extracts were stored on ice prior to experiments, which were initiated within 1 h of genome extraction.

Reaction Conditions. The reaction times required to achieve 2 log reduction of extracted viral DNA in the presence of 5 mg-Cl_2/L (0.071 mM) of FAC, a dose within the range typically applied to water by treatment plants,[3] for dsDNA are on the order of minutes, whereas those for ssDNA are on the order of seconds.[17] To measure reaction kinetics at these time scales, two experimental setups, described previously,[9] were used: (1) a batch reactor setup and (2) a continuous quench-flow setup. Briefly, in the batch reactor setup, equal volumes of a 10 mg-Cl_2/L FAC buffer solution and a ∼2 × 10^7 gc/μL viral genome buffer solution were mixed in a beaker, resulting in a 5 mg-Cl_2/L and ∼1 × 10^7 gc/μL viral genome reaction. At each timepoint, reaction vessels were sacrificed and quenched with Tris-HCl buffer (final 50 mM concentration). In the continuous quench-flow setup, syringe pumps dispensed a 10 mg-Cl_2/L FAC buffer solution and a ∼2 × 10^7 gc/μL viral genome buffer solution at equal rates (125 μL/min). These buffers were mixed in a mixing tee, resulting in a 5 mg-Cl_2/L and ∼1 × 10^7 gc/μL viral genome reaction. The mixture flowed through sample loops of various volumes; reaction times were based on the combined flow rate and loop volume. Samples were quenched with Tris-HCl (50 mM final concentration) in a mixing tee at the end of the sample loop. Experiments were performed at room temperature (∼25 °C). Given that ionic strength can influence FAC reactivity of organic compounds[28] and can influence DNA configuration,[37] we maintained constant ionic strength across variable chloride conditions by augmenting reactions with sodium perchlorate

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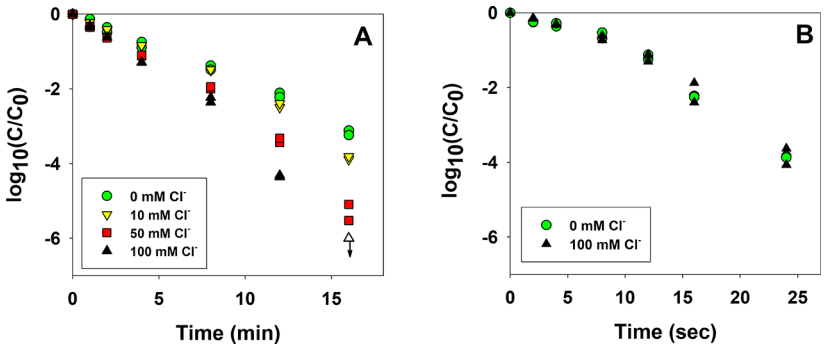

Figure 1. Reactivity of the (A) T3 dsDNA genome (region A amplicon) and (B) ϕX174 ssDNA genome (region A amplicon) with FAC (5 mg-Cl_2/L, pH = 7.5) at increasing chloride (Cl^-) concentrations. Unfilled symbols with downward arrows represent data points that were below the limit of detection. Experimental duplicates are shown for each condition as separate data points.

such that $[ClO_4^-]$ + $[Cl^-]$ = 10 or 100 mM. Quenched samples were stored on ice prior to genome quantification by qPCR.

qPCR Assays. Samples were analyzed by qPCR within 3 h of collection; the hold time, tris-chloramine species, and chloride concentration did not affect sample cycle threshold values (Figures S8 and S9). To determine if FAC reactivity is consistent across each genome, two disparate regions (referred to as A and B) that were ∼500 bases (ssDNA) and ∼500 base pairs (dsDNA) in length were analyzed for each of the genomes. Analysis details, adapted from Qiao et al.,[35] are included in Text S1 and Table S1.

Data Analysis. FAC reaction kinetics with DNA were modeled based on eq 3:

$$\ln(C/C_0) = -k_{obs} \times t \quad (3)$$

Here, C represents the genome concentration (gc/μL) at a reaction time, C_0 represents the initial genome concentration (gc/μL), k_{obs} represents the pseudo-first-order reaction rate constant (s^{-1}), and t represents the reaction time (s). FAC concentration varied by <10% throughout the experiments. To facilitate comparison with previously published data, k_{obs} was normalized by the 0.071 mM FAC concentration and the amount of bases (b) present in each normalized amplicon and is referred to as the apparent rate constant, k (M^{-1} s^{-1} b^{-1}). Second-order rate constants for DNA reactivity with HOCl, OCl^-, Cl_2, and Cl_2O (k_{HOCl}, k_{Ocl^-}, k_{Cl_2}, and k_{Cl_2O}, respectively) were determined from k, as described in Supporting Information, Text S3.

For some reaction conditions, deviations from pseudo-first-order kinetics were observed; statistical differences between conditions were determined both by comparing k values for DNA degradation and by comparing samples collected at the longest reaction times. Unpaired student t-tests were used to determine statistical significance. The null hypothesis was rejected when $p < 0.05$. P-values are listed in Tables S2−S5 and S7−S14.

RESULTS AND DISCUSSION

Effect of Chloride on Viral Genome Chlorination. To determine the effect of chloride on viral genome FAC reaction kinetics, we suspended genome extracts in solutions augmented with 0−100 mM sodium chloride (pH = 7.5) at constant ionic strength. 100 mM chloride concentrations are around those expected for saltwater pools (up to ∼103 mM chloride concentration),[38] higher than the reported levels for groundwater subject to saltwater intrusion (up to ∼40 mM chloride concentration)[39] and lower than those anticipated for seawater aquaculture ponds (∼530 mM chloride concentration).[31] A pH of 7.5 falls within the pH range for surface water (6.5−8.5).[40]

The reaction between FAC and naked viral genomes followed pseudo-first-order kinetics across the first 90% decay of both the T3 dsDNA and ϕX174 ssDNA regions regardless of the chloride concentration in the sample (Figures 1 and S2). However, after 99% decay, ϕX174 ssDNA degradation rates accelerated, while T3 dsDNA degradation rates remained constant. Conservatively, we determined reaction rate constants for dsDNA by modeling the entire data set collected. For ssDNA, we modeled the first 90% of degradation for select conditions (Table S6) which yielded log-linear R^2 values greater than 0.97.

Overall, ssDNA reactivity with FAC was greater than dsDNA reactivity with FAC. Within each genome and condition tested, there were no statistical differences in FAC reactivity for region A and B amplicons, and the discussion below is based on apparent rate constants averaged across the two amplicons (Tables S2−S5). For samples that were not augmented with chloride (Figures 1 and S2; 0 mM Cl^-), the apparent rate constant was 96× greater for ssDNA (k = 10.6 M^{-1} s^{-1} b^{-1}) than that for dsDNA (k = 0.109 M^{-1} s^{-1} b^{-1}) (Figures 1 and S2; Table S6). The greater sensitivity of ssDNA to FAC compared to dsDNA FAC sensitivity is expected; nucleobases in single-stranded genomes are more accessible to FAC than those in double-stranded genomes, resulting in greater FAC reaction rates for ssDNA than those for dsDNA.[17]

Increasing the chloride concentration in samples increased the FAC reactivity of T3 dsDNA amplicons (Figures 1A and S2; Table S6). Observed apparent rate constants for the dsDNA samples augmented with 10 mM (k = 0.119 M^{-1} s^{-1} b^{-1}), 50 mM (k = 0.158 M^{-1} s^{-1} b^{-1}), and 100 mM (k = 0.195 M^{-1} s^{-1} b^{-1}) chloride were, on average, 8.1, 44, and 77% higher than those that were not augmented with chloride (k = 0.110 M^{-1} s^{-1} b^{-1}). Statistical comparisons (provided in Tables S2 and S3) show that the increase in the reaction kinetics of dsDNA and FAC in the presence of chloride is statistically significant. Conversely, there was no statistical difference between apparent rate constants for samples not augmented with chloride and those augmented with 100 mM chloride for ϕX174 ssDNA (Figures 1B and S2; Tables S4 and S5).

Aqueous Cl_2 is a more potent chlorinating agent than HOCl and forms in FAC solutions in the presence of chloride (eq 1).

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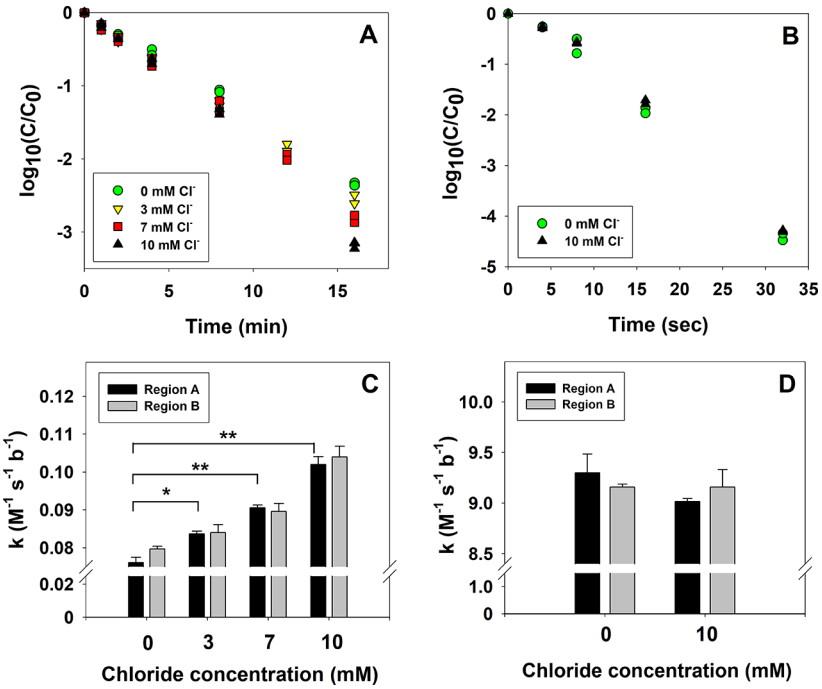

Figure 2. Reactivity of the T3 dsDNA genome (A,C) and ϕX174 ssDNA genome (B,D) with FAC (5 mg-Cl_2/L, pH = 7.5) at chloride (Cl^-) concentrations relevant to water treatment. (A,B) Reactivity of region A of the genomes as measured by qPCR (experimental duplicates) and (C,D) apparent reaction rate constants measured for regions A and B of the genomes. Error bars on (C,D) represent the range of duplicate measurements. In (C), statistical significance was determined by comparing the k values calculated from inactivation curve slopes, with * marking $0.01 < p < 0.05$ and ** marking $p < 0.01$. In (D), there were no statistical differences between conditions and reactivity between regions A and B.

In the results presented here, dsDNA FAC reactivity increased with increasing chloride concentration (Figures 1 and S2) and, by extension, with increasing Cl_2 in solution. This trend suggests that dsDNA is reactive with Cl_2. Our differing results for ssDNA and dsDNA also imply that, much like previous observations for aromatic ethers,[23] DNA reactivity with Cl_2 follows the reactivity-selectivity principle. Specifically, the presence of 100 mM chloride did not enhance the ssDNA FAC reaction kinetics; ssDNA is 96× more reactive with FAC than dsDNA. As the more reactive biomolecule, ssDNA is less selective in the chlorine species it reacts with,[30] and hence, chloride, and by extension, Cl_2, did not enhance ssDNA FAC reactivity under the conditions tested.

The ssDNA and dsDNA exhibited accelerating kinetics with FAC, especially at higher chloride concentrations. The accelerating kinetics were more pronounced with the ssDNA than with the dsDNA. Accelerating reactivity with FAC was observed in naked bacterial dsDNA in the context of antibiotic resistance gene research and attributed to the numerous mechanisms by which chlorine can modify DNA nucleotides.[41−45] Most notably, He et al. postulated that DNA chlorination is a two-step process, consisting of N-chlorination of nucleotide bases occurring prior to denaturation and irreversible C-chlorination of unpaired nucleotide bases after denaturation.[41] The disruption of hydrogen bonds and chlorination of bases within amplicons were hypothesized to accelerate their reactivity toward FAC.[41] That study did not measure the chlorination of ssDNA, and it is unclear why ssDNA would be more affected by this mechanism than dsDNA. A higher-order ssDNA structure could contribute to the effect. Unlike T3 dsDNA, which has a linear structure, the ϕX174 genome is circular. ssDNA is also more prone to secondary structure formation than dsDNA,[46] and accelerating degradation rates may be a function of increased availability of nucleotides to FAC as secondary structure is disrupted. Salts, which can stabilize ssDNA and dsDNA to different extents,[47] can further contribute to the effect.

Chlorine Reaction Kinetics with DNA under Chloride Conditions Relevant to Water Treatment. The results presented above demonstrate that chloride levels of 10−100 mM can enhance reaction kinetics with FAC. In drinking water treatment, the EPA Secondary Drinking Water Standard for chloride is 7.1 mM (250 mg/L).[31] Wastewater effluent chloride levels can reach 8.9 mM (320 mg/L)[48] due to water softener use,[49] and surface water concentrations of up to 14.2 mM (500 mg/L) chloride have been reported due to road salt runoff.[50] To determine the effect of chloride on viral genome FAC reactivity under conditions relevant to drinking water and wastewater treatment, we suspended viral genomes in solutions augmented with 0−10 mM sodium chloride at constant ionic strength (pH = 7.5).

Much like trends observed for elevated chloride concentrations, T3 dsDNA amplicons were more sensitive to FAC than ϕX174 ssDNA amplicons at chloride concentrations relevant to drinking water treatment (Figure 2C,D). For each genome and under each condition, there was no statistical difference between region A and region B reactivities, and the following discussion is based on apparent rate constants averaged across the two amplicons (Figures 2C,D and S3; Tables S7−S10). Our T3 dsDNA apparent rate constant (k = 0.079 M^{-1} s^{-1} b^{-1}) fell within the 0.020−0.39 M^{-1} s^{-1} b^{-1} range of previously reported dsDNA FAC reactivity constants measured by qPCR (pH = 7−8).[41,51,52] In samples not augmented with chloride, we observed that the ϕX174 ssDNA is, on average, 117× more susceptible to FAC than T3 dsDNA (Figures 2 and S3; Table S6).

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Increasing the chloride concentrations by up to 10 mM increased T3 dsDNA reactivity with FAC but did not affect ϕX174 ssDNA FAC reactivity. Apparent rate constants for the dsDNA samples augmented with 3 mM (k = 0.085 M^{-1} s^{-1} b^{-1}), 7 mM (k = 0.092 M^{-1} s^{-1} b^{-1}), and 10 mM (k = 0.103 M^{-1} s^{-1} b^{-1}) chloride were 9.0, 18, and 32% higher than those in samples without added chloride (k = 0.078 M^{-1} s^{-1} b^{-1}), respectively. Statistical tests comparing the rate constants showed that the chloride-augmented conditions were statistically different from the no added chloride condition (Tables S7 and S8). Conversely, there was no statistical difference in apparent rate constants between samples not augmented with chloride and those augmented with 10 mM chloride for ϕX174 ssDNA amplicons (Figure 2D and Tables S9 and S10). The consistency of the effect of chloride on reaction kinetics of T3 dsDNA and FAC and lack of an effect of chloride on reaction kinetics of ϕX174 ssDNA and FAC further suggest that Cl_2 is reactive with DNA and that DNA reactivity with Cl_2 follows the reactivity-selectivity principle.

Effect of Ionic Strength on DNA FAC Reactivity. We maintained two distinct ionic strengths in the data sets collected for chloride concentrations relevant to water treatment (lower ionic strength: 10 mM added salts) and elevated chloride concentrations (higher ionic strength: 100 mM added salts). The data collected for samples not augmented with chloride (0 mM Cl^-) at the lower and higher ionic strength conditions allow us to compare DNA FAC reactivity across multiple ionic strengths.

At higher ionic strengths, the ssDNA reaction kinetics exhibited more acceleration than dsDNA (Figures 1B, 2B, S2, and S3), potentially due to the impact of ionic strength on ssDNA structure.[53,54] For example, ssDNA is more prone to hairpin formation at higher ionic strengths. Benevides et al. showed that the addition of 100 mM salts to naked ϕX174 ssDNA solutions resulted in extensive hairpin formation within the genome.[55] Given that hairpins correlate with hydrogen bonding in ssDNA, and He et al. implicate disruption of hydrogen bonds with genome degradation acceleration,[41] hairpins may contribute to ssDNA FAC reaction rate acceleration at higher ionic strengths.

Our results suggest that ionic strength further influences DNA FAC reactivity. On average, the apparent rate constants for dsDNA and ssDNA at the lower ionic strength condition were 29.0 and 12.2% lower than those at the higher ionic strength condition (Table S6), respectively. We note that there was no statistically significant difference in DNA reactivity with FAC when samples were supplemented with 10 mM nitrate in lieu of 10 mM perchlorate (Figure S7). These results suggest that higher ionic strengths promote amplicon degradation. Salt-dependent electrostatic effects play a major role in nucleic acid stability and reactivity[56] and predicting how increased salt concentrations (and hence ionic strength) affect genome behavior in solution is nontrivial. For example, Maity et al. showed that increased ionic strengths have a destabilizing effect on dsDNA past a certain threshold;[57] destabilized dsDNA would be more reactive with FAC in accordance with our observations. Ionic strength can also affect observed virus inactivation levels. For example, increasing solution conductivity from 30 to 300 μS increased the inactivation of dsDNA virus adenovirus by ~2 log after a 1 min FAC contact time (pH 8).[58] Mechanistically, FAC reactions with dsDNA drive adenovirus inactivation,[10] implying that dsDNA FAC reactivity increases with increased solution conductivity. Interestingly, the discrepancy of ionic strength effects between ssDNA and dsDNA was observed previously, albeit for UV photolysis.[35]

Effect of pH and Chloride on DNA FAC Reaction Kinetics. To determine the influence of pH on the reactivity of DNA with FAC in the presence of chloride, we measured the FAC reaction kinetics with T3 dsDNA and ϕX174 ssDNA at pH values ranging from 6.0 to 9.0 and samples augmented with either 0 or 10 mM chloride. For perspective, the typical pH of drinking water ranges from 6.5 to 8.5,[59] wastewater pH ranges from 6.0 to 8.0,[60] bottled alkaline water pH ranges from 8.0 to 9.0,[61] and pH values as low as 4.2 have been reported in produce postharvest disinfection facilities.[6]

FAC reactivity with T3 dsDNA increased with decreasing pH (Figures 3, S4, and S5). At pH values of 6.0, 6.8, and 7.5,

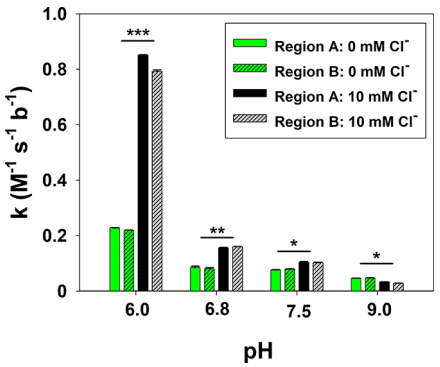

Figure 3. Reactivity of the T3 dsDNA genome (region A) with FAC (5 mg-Cl_2/L) at varying pH values for samples augmented with 0 or 10 mM chloride. Statistical significance was determined by comparing the apparent reaction rate constants, with * marking $0.01 < p < 0.05$, ** marking $0.001 < p < 0.01$, and *** marking $p < 0.001$. Error bars represent the range of experimental duplicates.

we observed pseudo-first order kinetics throughout the tested doses. At a pH value of 9.0, FAC reactivity accelerated over the course of the experiment, potentially due to structural changes in dsDNA and partial denaturation under alkaline pH conditions.[62] We therefore used only the first 90% of the observed amplicon degradation to calculate rate constants for the pH 9 data. For each genome and under each tested condition, there were no statistical differences in FAC reactivity for region A and B amplicons, and the discussion below is based on apparent rate constants averaged across the two amplicons (Tables S9–S12). Apparent rate constants for samples not supplemented with chloride at pH = 6.0, 6.8, and 7.5 were 4.8×, 1.8×, and 1.7× higher than the apparent rate constant at pH = 9.0 (Table S6), respectively. For samples supplemented with 10 mM chloride, apparent rate constants at pH = 6.0, 6.8, and 7.5 were 25×, 5.0×, and 3.3× higher than the apparent rate constant at pH = 9.0 (Table S6), respectively. This trend of increasing reactivity with decreasing pH is expected as FAC speciation shifts toward HOCl with decreasing pH (eq 4), and HOCl is a stronger oxidant than OCl^-.

$$HOCl \rightleftharpoons OCl^- + H^+ \qquad pK_{a,HOCl} = 7.54 \tag{4}$$

The average apparent rate constants for samples supplemented with 10 mM chloride were higher than those that were not supplemented with chloride at pH < 9.0, and the magnitude of the difference increased with decreasing pH.

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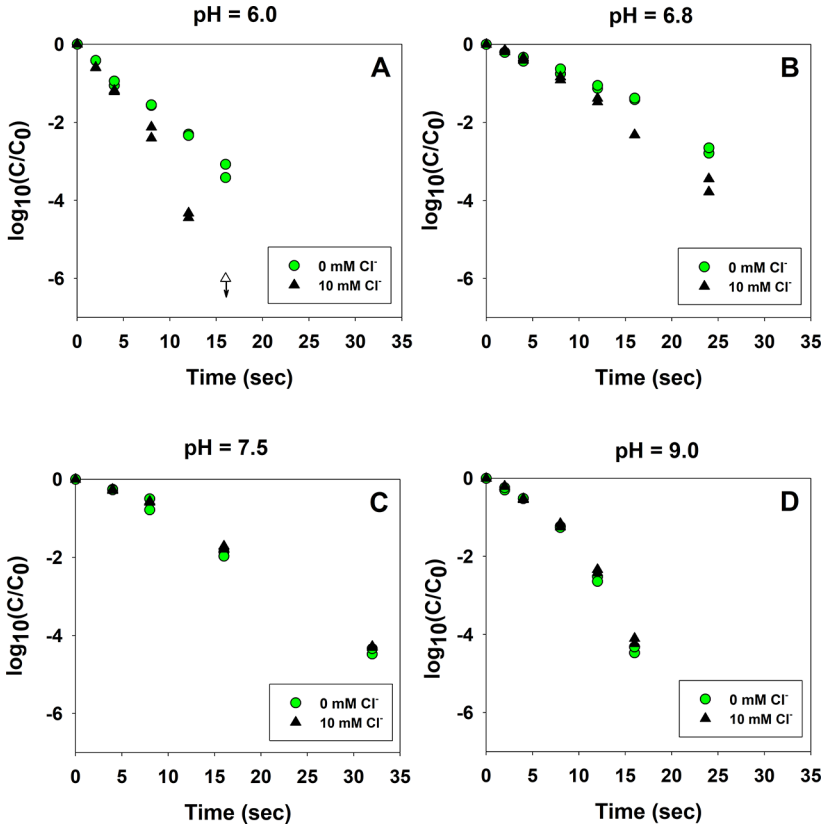

Figure 4. Reactivity of the ϕX174 ssDNA genome (region A amplicon) with FAC (5 mg-Cl_2/L) at (A) pH = 6.0, (B) 6.8, (C) 7.5, and (D) 9.0 for samples augmented with 0 or 10 mM chloride. Unfilled symbols with downward arrows represent data points that were below the limit of detection. Experimental duplicates are shown.

Control experiments confirmed that these differences were not due to trace contaminants present in the salts used (Text S2). At pH = 6.0, 6.8, and 7.5, the apparent rate constants in the presence of 10 mM chloride were 3.7×, 1.9×, and 1.3× greater than those in samples that were not augmented with chloride, respectively, and differences between the apparent rate constants were statistically significant (Table S6). In contrast to the trends at pH 6.0, 6.8, and 7.5, at pH 9, we observed 1.4× slower dsDNA reaction kinetics in samples augmented with 10 mM chloride than those not augmented with chloride; the difference was statistically significant (Tables S11 and S12). dsDNA stability could influence the pH 9.0 results, and changes in DNA nucleotide speciation (e.g., pK_a of guanosine = 9.2)[62] could affect FAC reactivity.

In contrast to the T3 dsDNA, ϕX174 ssDNA FAC reactivity did not increase with decreasing pH in the absence of chloride (Figures 4 and S6). Although we would expect the susceptibility of DNA to FAC to correlate with HOCl concentrations, pH-dependent changes in ssDNA structure could also affect FAC reactivity. d'Souza and Kool showed that the melting temperature of certain ssDNA oligonucleotide sequences changes as a function of pH,[63] implying that the folding of ssDNA can be pH dependent. Structural changes in ssDNA could therefore change the accessibility of nucleotides to FAC and affect reactivity; conversely, dsDNA is considered to be stable between pH 5 and up to a pH of ~9.[62] Interestingly, at pH = 9.0, we observed accelerating degradation rates for ϕX174 ssDNA amplicons, which is consistent with the trend observed for T3 dsDNA.

In the presence of 10 mM chloride, FAC reactivities with ϕX174 ssDNA at pH = 6.0 and 6.8 were 1.8× and 1.4× greater compared to samples not augmented with chloride (Table S6), respectively. The differences between the two conditions were statistically significant (Tables S9 and S10). At pH = 7.5 and pH = 9.0, the addition of 10 mM chloride to samples did not affect ssDNA FAC reactivity (Tables S9 and S10).

Taken together, the FAC reaction kinetic results for T3 dsDNA and ϕX174 ssDNA across the pH range of 6.0−9.0 further define the role of Cl_2 in FAC reactions. Aqueous Cl_2 concentrations increase with decreasing pH (eq 1). As such, if Cl_2 is reactive with DNA, the magnitude of the effect of chloride on FAC reactivity is anticipated to increase with decreasing pH, as demonstrated for both T3 dsDNA and ϕX174 ssDNA. The magnitude for the effect of chloride is greater for T3 dsDNA than ϕX174 ssDNA. For T3 dsDNA, chloride enhances chlorination rates at pH values up to 7.5, whereas for ϕX174 ssDNA, FAC reactivity is only enhanced at pH $\leq$ 6.8. This trend agrees with the reactivity-selectivity principle[30]—ssDNA is more reactive than dsDNA with FAC and is thus less selective in the chlorine species it reacts with.

Quantifying the Contribution of Chlorine Species to DNA Reactivity with FAC. The FAC reactivity data obtained at multiple pH values allows us to probe the contribution of HOCl, OCl^-, Cl_2O, and Cl_2 to T3 dsDNA and ϕX174 ssDNA FAC reactivity. Here, we calculated initial estimates of second-order reaction rate constants for the reactions of HOCl, OCl^-, Cl_2O, and Cl_2 (k_{HOCl}, k_{OCl^-}, k_{Cl_2O}, and k_{Cl_2}) with the ssDNA and dsDNA regions by adapting models previously developed

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for anthropogenic contaminants.[24] Details of the calculations are provided in Text S3.

Overall, our model (Text S3) fits the measured data well under most conditions, but we observed considerable deviation from the measured values under certain conditions, especially at pH = 9.0 (Figures S11 and S13). Our model assumed that DNA speciation is independent of pH. A study by Prutz et al., however, suggested that guanosine, as a monomer, has a pK_a of 9.2.[15] If this pK_a consistent when the base is incorporated into DNA, DNA reactivity with FAC could be affected and this can help explain the discrepancy of our results at pH 9. In addition to speciation, the complex structure of DNA relative to anthropogenic contaminants is likely a factor in the deviation of the model from the measured data. DNA is composed of a sequence of four molecules, whereas this type of model has previously been applied to single-molecule contaminants. Furthermore, DNA structure may change as a function of pH and change functional group susceptibility to FAC.[62,63] Previous chlorination studies of small organic contaminants had a generally well-defined and singular mechanism (e.g., electrophilic aromatic substitution).[26] For DNA, a broader range of chlorination mechanisms are plausible (e.g., N-chlorination, electron transfer, and electrophilic substitution)[41,45] and are likely to proceed in parallel, which further complicates kinetic assessments of chlorination rates.

Despite its limitations, the model reveals important insights into the reactivity of DNA with FAC. For example, the models indicate that HOCl and Cl_2 contributed to FAC reactivity with ssDNA and dsDNA. The second-order reaction rate constants are greater for Cl_2 than those for HOCl for both ϕX174 ssDNA and T3 dsDNA (Table 1). Specifically, the fitted k_{Cl2} values were 6.1×10^4-fold and 1.7×10^5-fold larger than the k_{HOCl} values for ϕX174 ssDNA and T3 dsDNA, respectively. These values indicate that the selectivity of ssDNA toward Cl_2 is lower than the selectivity of dsDNA toward Cl_2 (Figure S11), likely due to the higher overall reactivity of ssDNA to FAC in comparison to dsDNA. Our results follow the same trend observed for several classes of organic compounds, where compounds with higher k_{HOCl} values exhibit relatively lower k_{Cl_2} values, as recently reviewed by Rose et al.[26]

Table 1. Predicted Second-Order Rate Constants for HOCl Reactivity (k_{HOCl}), OCl^- Reactivity (k_{OCl^-}), and Cl_2 Reactivity (k_{Cl_2}) with ϕX174 ssDNA and T3 dsDNA[a]

	k_{HOCl} (M^{-1} s^{-1} b^{-1})	k_{OCl^-} (M^{-1} s^{-1} b^{-1})	k_{Cl_2} (M^{-1} s^{-1} b^{-1})
ϕX174 ssDNA	12.2 ± 2.3	14 ± 11	$(7.5 \pm 2.5) \times 10^5$
T3 dsDNA	0.25 ± 0.05	not quantified[b]	$(4.3 \pm 0.5) \times 10^4$

[a]Uncertainties denote 95% confidence intervals. [b]Inclusion of k_{OCl^-} did not improve the model fit.

The model suggests that OCl^- also contributes to ssDNA FAC reactivity (Table 1), albeit with high uncertainty. Including Cl_2O as a reactant did not improve model fits (Text S3). Cl_2O is a key chlorinating agent for several classes of compounds including antipyrine, dimethenamid, and aromatic ethers,[23,28,64] but Cl_2O does not contribute significantly to FAC reactivity with other compounds (e.g., chlorophenols).[25]

Effect of Chloride on Bacteriophage Inactivation Kinetics. Our results demonstrate that chloride affects the FAC reactivity of extracted ssDNA and dsDNA viral genomes. In intact viruses, viral genomes are enclosed in a protein capsid. To verify that the observed differences in viral genome FAC reactivity are relevant to viral inactivation, we suspended bacteriophage ϕX174 (ssDNA genome) and bacteriophage T3 (dsDNA genome) in 5 mM phosphate buffer augmented with either 100 mM chloride or 100 mM perchlorate (pH = 7.5). Under both conditions, bacteriophage T3 inactivation by FAC was more rapid than bacteriophage ϕX174 inactivation by FAC (Figure S12). This finding is likely due to the larger size of T3 compared to that of ϕX174 and thus the larger number of protein and genome targets in each infectious particle. T3 was inactivated more rapidly by FAC in the chloride condition, whereas chloride did not affect ϕX174 FAC inactivation (Figure S12). This result implies that Cl_2 has a greater effect on dsDNA T3 inactivation than that on ssDNA ϕX174 inactivation and is consistent with the viral genome reactivity trend observed. In addition to reacting with viral genomes, FAC reactions with viral capsid proteins contribute to viral inactivation;[8,9] to our knowledge, the role that chloride plays in viral protein reactivity with FAC has not been described.

■ ENVIRONMENTAL IMPLICATIONS

In this study, we demonstrate that chloride is not an inert species to be overlooked during viral genome chlorination. Our results demonstrate that (1) solution ionic strength affects viral DNA chlorination kinetics, (2) decreasing pH and increasing chloride concentrations promote DNA FAC reactivity, and (3) Cl_2 is reactive with DNA. We further provide initial estimates for k_{HOCl} and k_{Cl_2} for both ssDNA and dsDNA and find that DNA chlorination follows the reactivity-selectivity principle, with Cl_2 contributing more to the FAC reactivity of the less-reactive dsDNA.

Our results suggest that Cl_2 reactions should not be ignored when predicting DNA FAC reactivity in treatment scenarios where chloride concentrations could be elevated, including, but not limited to, brackish groundwater chlorination,[39] wastewater treatment,[48] and water reuse.[65−69] The significance of the effect of chloride on DNA FAC reactivity complicates experimental design and casts doubts on previously reported FAC reaction kinetics. First, conventional experimental setups do not adequately track chloride inputs into reaction systems. Practices such as adjusting buffer pHs with HCl, purifying viral stocks with cell culture-grade phosphate-buffered saline (which contain 137 mM NaCl; Gibco), and using low-grade salts that could have residual chloride can substantially increase chloride concentrations in reactions. Given that chloride can increase DNA reactivity with FAC, studies likely overestimate DNA reactivity with HOCl, especially at low pH. Second, chloride is frequently used to maintain constant ionic strength in reactions.[8,26] While maintaining constant ionic strength is commendable, especially given our results which show that ionic strength does influence DNA FAC reaction kinetics, chloride may accelerate DNA FAC reactivity. Rather, higher-purity salts (ACS grade and above) that do not influence chlorine speciation, such as perchlorate or nitrate, and thus would not result in an overestimation of DNA reactivity with HOCl, should be used. Salt grade may be particularly important when considering bromide: brominating agents (BrCl, BrOCl, and Br_2O) are reactive with anthropogenic contaminants[26] and may also influence DNA reactivity. In fact, bromide concentrations in the salts used within our study are

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on par with those reported previously[70] and may be significantly higher in lower-grade salts.

Within this study, we showed that the addition of chloride not only affects the FAC reactivity of extracted ssDNA and dsDNA viral genomes but can also affect viral inactivation. In intact viruses, genomes are enclosed in a protein capsid. Future research should consider whether capsid amino acids are more susceptible to Cl_2 than to HOCl and whether Cl_2 is more likely to penetrate the capsid than HOCl. The effect of chloride on viral inactivation sheds light on differences perceived in chlorine reactivity with viruses at various ionic strengths. Previous publications that compared viral reactivity with FAC at various ionic strengths used chloride salts to adjust the ionic strength. As examples, these studies hypothesized that changes in reactivity at various ionic strengths can be attributed to effects of salt on the virus itself,[71] ion potentiating effects,[72] and viral aggregation.[58] Our results point to Cl_2 accounting for FAC reactivity differences associated with changes in chloride concentrations.

Finally, the results presented here have implications beyond FAC disinfection of viruses. For example, extracellular bacterial DNA carrying antibiotic resistance genes is of interest in wastewater and surface water treatment.[41] Furthermore, blood chloride concentrations normally range between 96 and 106 mM,[73] and in vitro studies focus on the effects of endogenously produced HOCl on proteins and extracellular nucleic acids in blood matrices.[74,75] The extent to which Cl_2 contributes to degradation of these biomolecules is unclear and warrants further investigation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c03267.

qPCR methods used; trace contaminant control experiments; calculations for k_{HOCl}, k_{Cl_2}, and k_{Cl_2O}; modeled second-order rate constants and p values for statistical tests; DNA FAC reactivity for region B amplicons; and control experiments (PDF)

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Notes

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