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DISINFECTION EFFICACY OF WATERBORNE ENTERIC VIRUSES UNDER LOW CHLORINE DOSES: INVESTIGATION BASED ON INFECTIVITY AND GENOMIC INTEGRITY OF A VIRAL SURROGATE

BADANIA NAD EFEKTYWNOŚCIĄ DEZYNFEKCJI NISKIMI DAWKAMI CHLORU ENTEROWIRUSÓW PRZENOSZONYCH DROGĄ WODNĄ

The efficiency of disinfection by chlorine at low doses for waterborne enteric viruses was investigated using MS2 as the surrogate based on its changes in infectivity and genome integrity during contact with free chlorine added with the doses of 0.1, 0.5 and 1.0mg/L. The effect of co-existing bacteria was also tested by choosing E.coli as the indicating bacterial species. The results obtained clearly demonstrated the stronger resistance of MS2 than E.coli against chlorine, the adversary effect of co-existing bacterial species and the involvement of combined chlorine formed during reaction of free chlorine with the constituents of MS2 and E.coli; and may thus be used as important reference for water treatment plants practicing lower chlorine doses for alleviating the unpleasant tastes and hazardous disinfection byproduct formation with chlorination.

1. Introduction

Chlorination stays as the most widely used water disinfection process all over the world mainly due to its low cost and generally well recognized effectiveness. The major disadvantage of chlorination is the generation of chlorinated organic compounds that may be carcinogenic or harmful to humans. Residual chlorine or chloramines in wastewater after disinfection treatment may also impose detrimental effects to the natural aquatic environment [1]. As consumers pursue for higher quality of drinking water, the unpleasant taste brought by the residual chlorine in the water continues to be a concerning issue for water production industries.

In order to control the generation of disinfection by-products and improve the taste of the water, many drinking water treatment plants have started to implement chlorination at low doses based on the disinfection efficiency confirmed with general bacteria, coliform group or *E.coli*, the major fecal pollution indicators whose concentrations in the finished water are regulated in the current drinking water standards of most countries. However, this approach exposes the water supply to the risk of waterborne diseases caused by viruses. In recent years, many countries have had the experience of suffering from diseases caused by waterborne viruses, such as adenovirus, echovirus and norovirus [2-4]. These viral types have the common feature that they are much stronger than bacteria in regarding their resistance against chlorine. Therefore, the evaluation of the performance of disinfection under lower chlorine dosages against all likely waterborne pathogenic agents, especially the effects on waterborne viruses is important.

Inactivation of viruses through disinfection can be achieved by either destructing the protective capsids or degrading the genomic material within the viral particles. Conventional evaluation method with the plaque assay provides direct information on the infectivity changes of viruses with disinfection and has been thus used. Compared to this method, real-time polymerase chain reaction (Real-time PCR), a laboratory technique of molecular biology, enables investigation of bacteria or viruses from genomic aspect, and is thus considered equally important.

The major objective of this study was to investigate the disinfection efficiency of waterborne enteric viruses under low chlorine doses. For this purpose, MS2, a F-specific coliphage species originated from animal feces was used as the viral surrogate, and the disinfection efficiency was evaluated according to the infectivity and genomic integrity of MS2 analyzed with plaque assay and real-time PCR, respectively. The effect of co-existing bacteria on disinfection was also examined by using *E. coli* as the model bacterial species to co-exist with MS2.

2. Materials and methods

2.1. Preparation and enumeration of MS2

Escherichia coli phage MS2 (NBRC 102619) was cultivated by using the host bacterium of *Escherichia coli* (*Migula 1895*) *Castellani and Chalmers 1919* (NBRC 13965). MS2 seed was propagated in LB Broth Base culture medium with exponentially cultured *E. coli* in shaking water bath at 37 ºC for 24 hours. Propagated MS2 was centrifuged at 3500 rpm for 10 min and filtered by 0.45 μm cellulose acetate filter. The filtrate was further purified using 50 kDa Centriprep centrifugal filter unit. MS2 stock solution was then stored in 0.85% NaCl solution at 4 ºC. MS2 was enumerated through plaque assay and real time Reverse Transcription Polymerase Chain Reaction (real time RT-PCR) for the infective count and the genomic integrity assessed based on the DNA-copies amplified, respectively. Plaque assay was done by pouring 1 mL of sample in the mixture of LB Broth Base single layer agar and *E. coli* onto a petri dish. The petri dish was then inverted and cultivation was done under 37 ºC for 24 hours. Plaque counts within 30 - 300 were taken as PFU/mL. Samples for real time RT-PCR were boiled to extract the

phage RNA. The extracted RNA was then reverse-transcripted by using ReverTra Ace® qPCR RT Kit (TOYOBO Co., Ltd.) into DNA-copies. Real time RT-PCR was finally done to enumerate the equivalent viral counts of MS2 by using the corresponding primers (Forward: 5'-CGT TCA CAG GCT TAC AAA GTA ACC T-3'; Reverse: 5'-CCA ACA GTC TGG GTT GCC AC-3') (Applied Biosystems, Life Technologies Co., Ltd) [5, 6].

2.2. Preparation and enumeration of *E. coli*

The seed of *E. coli* was propagated in LB Broth Base culture medium in shaking water bath at 37 ºC for 4 - 6 hours. The exponentially cultured *E. coli* was centrifuged at 3500 rpm for 10 min. The supernatant was discarded and the settled *E. coli* was rinsed by 0.85% NaCl for 3 times to remove the culture medium. The obtained *E. coli* stock solution was stored in 0.85% NaCl solution under 4 ºC prior to use for chlorination. *E. coli* was enumerated through plate count assay and real time PCR for the infective count and genomic integrity, respectively. Plate count assay was done by cultivating 1 mL of sample on the MacConkey single layer agar in a petri dish. The petri dish was then inverted and cultivation was done under 37 ºC for 24 hours. Colony counts within 30 - 300 were taken as CFU/mL. The DNA of *E. coli* in the samples was extracted and purified by using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc.). The extracted DNA was then enumerated through real time PCR by using the corresponding primers (com1: 5'-CAG CAG CCG CGG TAA TAC-3'; com2: 5'-CCG TCA ATT CCT TTG AGT TT-3').

2.3. Chlorination experiment

Chlorination was done under chlorine doses of 0.1, 0.5 and 1.0 mg/L by using sodium hypochlorite, NaOCl as the free chlorine standard. 8 to 10 samples were taken along 2-hour reactions. Experiments were carried out in ultrapure water at 20 ºC. Two working systems were applied, namely the single-target-system (STS) and the mixed-target-system (MTS). STS consisted of only one type of targets, either MS2 or *E. coli*; while the MTS consisted of the mixture of both. The initial concentration of MS2 in STS was 10^7 PFU/mL; while the initial concentration of *E. coli* in the STS was 10^7 CFU/mL. The MTS contained 10^7 PFU/mL of MS2 and 10^7 CFU/mL of *E. coli*. At each contact time, 10 mL of sample from each disinfection reactor was taken for quantification with plaque assay and real time RT-PCR in a 15 mL sterilized disposable centrifuge tube added in advance with 0.01 M sodium thiosulfate, $Na_2S_2O_3$ to quench the chlorine reaction. At the same time, another 20 mL of sample was taken for measurement of the free and total residual chlorine by using the N, N-diethyl-p-phenylenediamine (DPD) method with a commercially available analytical kit and the Pocket Colorimeter for Free and Total Chlorine (HACH Co., Ltd.). All chemical reagents used were with reagent grade or higher grade. Control experiments were done with the ultrapure water without addition of MS2 and *E.coli* at each chlorine dosage, as well as ultrapure water with either MS2 or *E.coli* but without chlorine dosing.

3. Results and discussion

3.1. Changes of infectivity

The time profiles revealing the changes of infectivity of MS2 and *E. coli* in both STS and MTS under chlorine dose of 0.1 mg-Cl_2/L assessed with the plague assay are shown in **Figure 1**. The results under 0.5 and 1.0 mg Cl_2/L are shown in **Figure 2**.

From these figures, it could be seen that the inactivation rates of MS2 and *E. coli* in both systems of STS and MTS increased as the chlorine doses increased. Comparing the results between MS2 and *E.coli* in STS, the inactivation rates of the former were lower, indicating that MS2 was more resistant to chlorine than *E. coli*. Only under the lowest chlorine dose of 0.1 mg/L, the inactivation rate of *E. coli* in the STS decreased after the first 10 seconds. Complete inactivation of both targets in STS under this dose was achieved similarly within 1 minute.

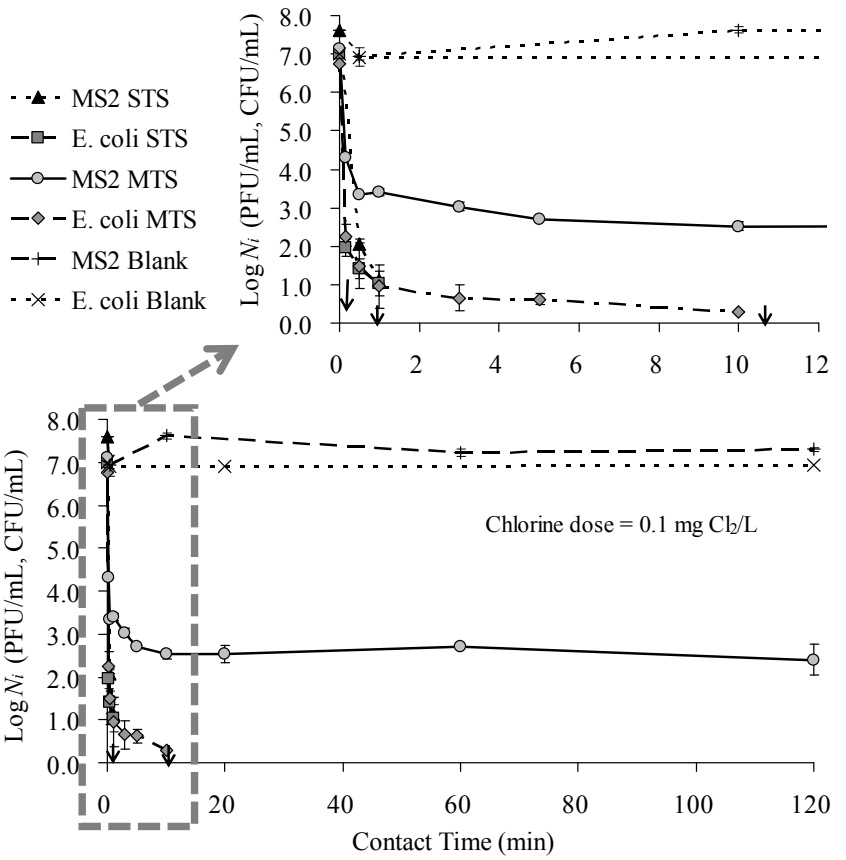

Fig. 1. Changes of infectivity of MS2 and E. coli in both STS and MTS under chlorine dose of 0.1 mg Cl_2/L assessed by plague assay. Plots after the arrow marks are not shown since infective ones were not detected.

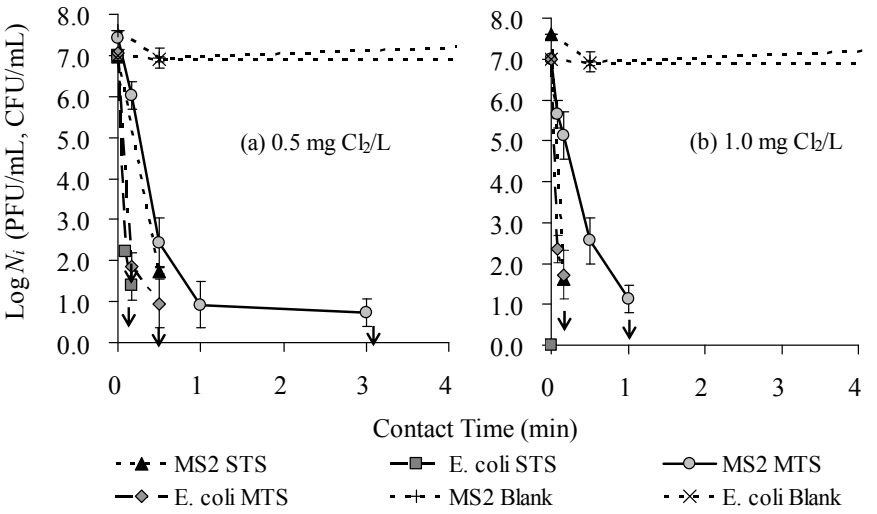

Fig. 2. Changes of infectivity of MS2 and E. coli in both STS and MTS under chlorine doses of 0.5 mg Cl_2/L (a) and 1.0 mg Cl_2/L (b) assessed by plague assay. Plots after the arrow marks are not shown since infective ones were not detected.

In the MTS, complete inactivation of MS2 was achieved under higher chlorine doses but not under 0.1 mg/L. Under this dose, the maximum inactivation of MS2 was around 4.5 log and it was achieved only after nearly 10 minutes of reaction. No further changes in the infectivity of MS2 afterwards were observed. On the other hand, *E. coli* was completely inactivated within 10 minutes under the same chlorine dose. The free residual chlorine was totally consumed in 5 minutes of reaction, leaving only the combined chlorine species in the rest of the 2 hours. The concentration of combined chlorine generated at this dose was only able to fully inactivate *E. coli* but not to further inactivate the remaining infective MS2.

The lower MS2 inactivation rate in the MTS as compared to the STS was caused by the presence of *E. coli* in the system. *E. coli* that was much larger than MS2 had most probably hindered the MS2 particles from getting in contact with the chlorine species. From other studies, it was found that, the constituents of the *E. coli* outer layers (outer membrane proteins, pili proteins and inner membrane proteins) were more reactive with chlorine species as compared to the constituents from MS2 capsid. This might be another factor leading to higher inactivation rate for *E. coli* as compared to *MS2*.

As compared to the effect given by co-existing *E. coli* to the inactivation rate of MS2, the effect of co-existing MS2 to the inactivation of *E. coli* was much less significant. Significant decrease of the inactivation rate of *E. coli* in the MTS occurred only under the chlorine dose of 0.1 mg/L. The main factor would be similar to the findings from STS, given by the limitation of available chlorine species for the oxidizing reaction.

3.2. Changes of genome integrity

The time profiles revealing the changes of genome integrity of MS2 in both STS and MTS assessed with real-time PCR are shown in **Figure 3**. The degradation rate of MS2

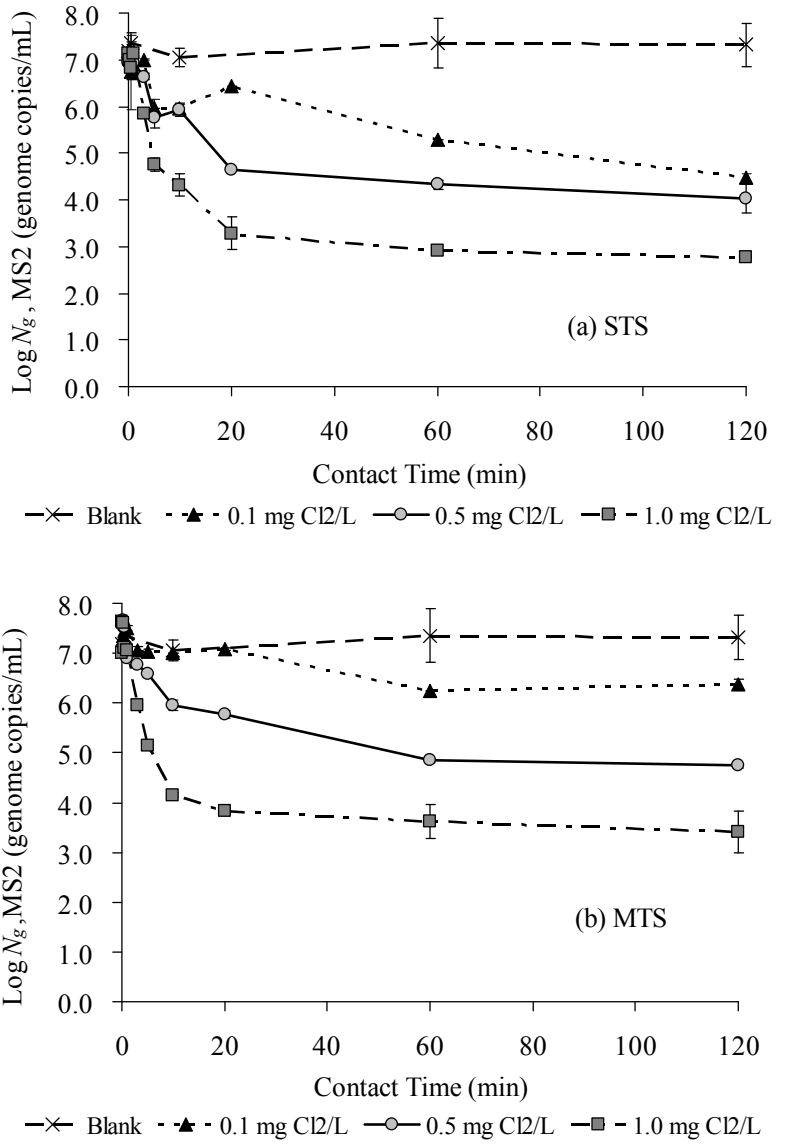

Fig. 3. Changes of genome integrity of MS2 in both STS (a) and MTS (b) assessed by real-time PCR under the chlorine doses of 0.1, 0.5 and 1.0 mg Cl_2/L.

genome was much lower as compared to its inactivation rate shown earlier in figures 1 and 2 under each chlorine dose. In the reaction system of STS, the genome degradation of MS2 at the end of 2 hours was around 2.8 log, 3.3 log and 4.5 log under the chlorine doses of 0.1, 0.5 and 1.0 mg/L, respectively. In the MTS, however, the respective genome degradation turned lower to around 0.8 log, 2.5 log and 3.9 log. Within the 2

hours' reaction, complete degradation of the detected gene sequence was not able to be achieved under any of the investigated three chlorine doses. It is thus conceivable that the main factor of the inactivation of MS2 (infectivity loss) was not attributed to the destruction of the genomic material by chlorine. This agreed with other studies proposing that chlorine has higher reactivity with the protein material (amino acids) of viral particles as compared to the genomic material (nucleotide monomers) [7].

The degradation of MS2 genome in the beginning of the reaction was in a more gradual trend especially under the lowest chlorine dose of 0.1 mg/L. This was similar to the initial lag (shoulder) effect given by the organic matter in the water to the disinfection of microorganisms and the tailing effect caused by the shielding big particles. However, the factor that brought to the happening of the initial lag in this study was the initial reaction of chlorine with the capsid of MS2. Before approaching the genomic material of the viral particle, the chlorine species would first react with the protective coat of the virus. This eventually reduced the available chlorine species to be reacted with the genomic material and hence the reaction rate (degradation rate of genome), giving the tailing effect. However, under higher chlorine doses, the initial lag was less significant. The genome degradation started with higher rates then gradually faded with tailing effect. The tailing effect was most probably given by the competing relationship between the remaining capsid constituents and the genomic material.

Comparisons of genome integrity of MS2 in STS and MTS under chlorine doses of 0.1, 0.5 and 1.0 mg/L at certain time points are shown in **Figure 4**. The total amount of MS2 genome degraded at the end of 2 hours' reaction in the MTS under the chlorine dose of 0.1 mg/L decreased by 2 log due to the presence of *E. coli* as compared to STS. However, the decrement in the amount of degraded genome in the MTS under two higher doses were not as significant, with only around 0.7 log and 0.6 log for doses of

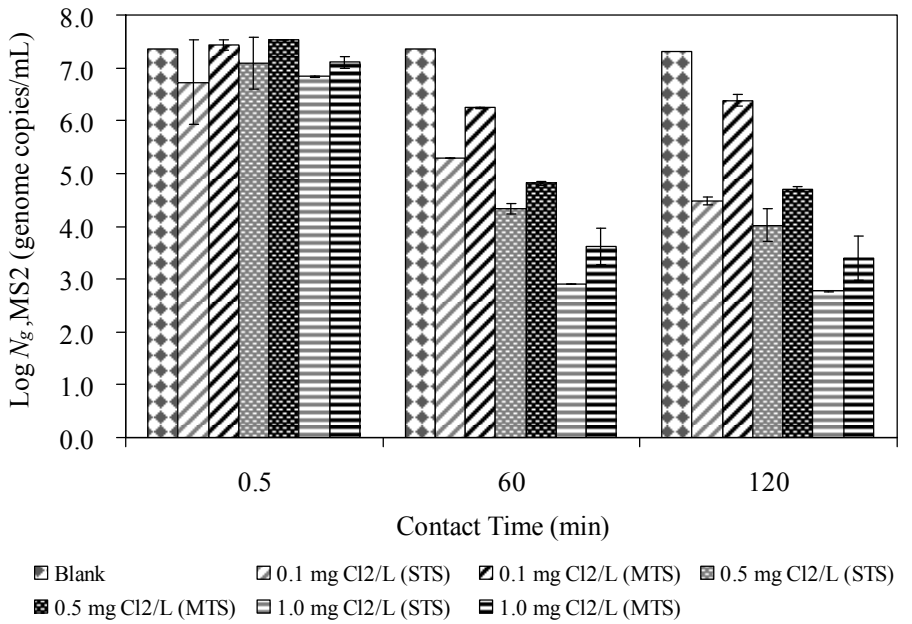

Fig. 4. Comparisons of genome integrity of MS2 in STS and MTS under chlorine doses of 0.1, 0.5 and 1.0 mg/L at certain time points.

0.5 and 1.0 mg/L, respectively. More obvious reduction in the degradation of MS2 genome in MTS under the dose of 0.1 mg/L comparing to higher doses was most probably due to relatively more significant and preferential consumption of free chlorine by *E. coli* at the dose. After all, the reduction of MS2 genomic degradation rate induced by the co-existing *E. coli* was lower compared to the reduction in the inactivation rate (infectivity loss) of MS2 under the same dose.

Regarding the genome integrity of *E. coli*, as shown in **Figure 5**, no observable changes under all three working chlorine doses appeared. This was because of the absence of reaction between chlorine species with the fragments of *E. coli* gene sequence detected by the applied real time PCR primers.

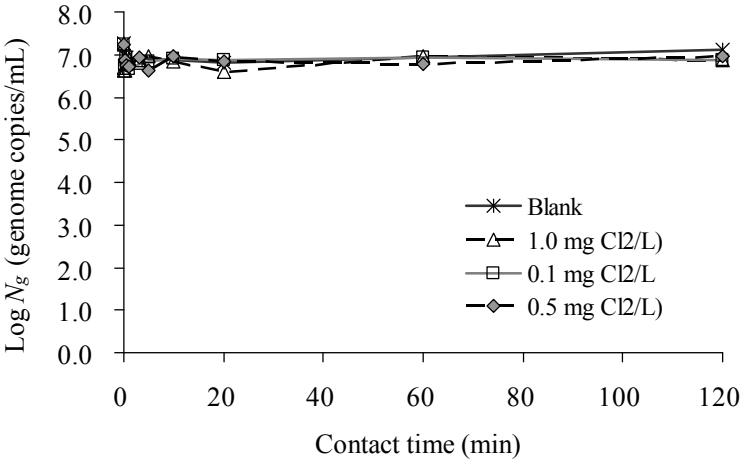

Fig. 5. Changes of genome integrity of E.coli in STS under chlorine doses of 0.1, 0.5 and 1.0 mg/L.

3.3. Relationship of chlorine species with the changes of Infectivity and Genome Integrity

Time-scale relationships of the changes of free chlorine consumption and combined chlorine concentration with the changes of infectivity and genome integrity of MS2 and *E. coli* in MTS under the free chlorine dose of 0.1 mg/L are shown in **Figure 6**. The free chlorine consumption was in an ascending order of MS2 STS < *E. coli* STS < MTS. Free chlorine consumed by the reaction with the constituents of organisms can form combined chlorine species and other byproducts. The combined chlorine formed is able to disinfect with a lower reaction rate than free chlorine. In consequence, the concentration of combined chlorine in the system is the balance between the generation from free chlorine and the loss due to oxidization. In the initial few minutes of the reaction, consumed free chlorine was mostly converted to combined chlorine. The reaction was dominated by the more reactive free chlorine species, resulting in the net production of combined chlorine species.

However, as free chlorine concentration decreased, its reactivity eventually decreased too. The combined chlorine consumption gradually overcame the generation, resulting in net consumption (loss) by the reaction in the later part of chlorination, especially after the complete consumption of free chlorine. Under working dose of 0.1 mg/L, the free chlorine available was not able to sustain within the 2 hours' reaction in any of the systems; which vanished in 5 min, 20 min and 60 min for the MTS, *E. coli* STS and MS2 STS, respectively. After the disappearance of the free chlorine, the combined chlorine reacted with a lower rate to further oxidize the constituents of the organisms. Under higher chlorine doses, the combined chlorine concentration in the later part of the reactions still showed a decreasing trend (net consumption). The decrement of combined chlorine concentration started even when the free residual chlorine was still above 0.1 mg/L. This may suggest the possibility that combined chlorine was more reactive with the constituents of the inner layers of organisms (MS2 or *E. coli*) comparing to the outer layer constituents.

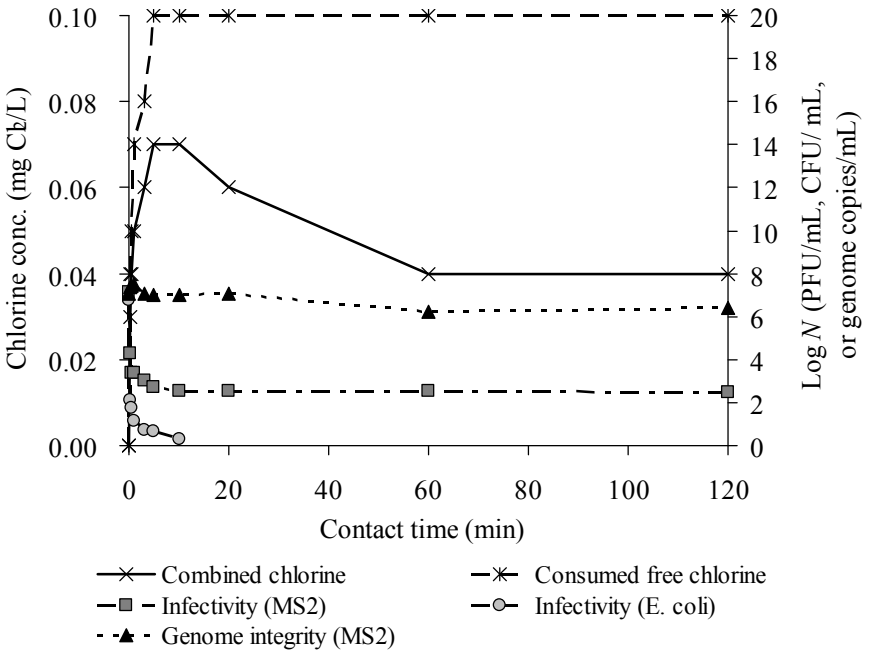

Fig. 6. Time-scale relationships of the changes of free chlorine consumption and combined chlorine concentration with the changes of infectivity and genome integrity of MS2 and E. coli in MTS under the free chlorine dose of 0.1 mg/L.

From the results of the single *E. coli* system (*E.coli* STS), the combined chlorine concentration showed no significant changes after reaching the maximum concentration in the beginning of the reactions, except under the dose of 0.1 mg/L where there was a slight decrease of concentration after the free chlorine was completely consumed. On the other hand, the decreasing trend of the combined chlorine concentration was observed under all working doses for the single disinfection system of MS2 (MS2 STS). This may

indicate that combined chlorine had low reactivity with the inner layer constituents of *E. coli* but high reactivity with the inner layer constituents of MS2.

Infectivity of both MS2 and *E. coli* showed more significant relationship with the free chlorine as compared to the combined chlorine. On the other hand, the changes of genome integrity of MS2 were also related to the combined chlorine. As could be seen from Figure 6, the genome degradation of MS2 continued slowly even after the complete consumption of free chlorine. Similarly, in other working systems of MS2, the reduction in its genome integrity revealed the tendency to follow the changes in the concentrations of combined chlorine.

4. Conclusions

MS2 was more resistant to chlorination as compared to *E. coli* through the infectivity analysis. The presence of *E. coli* could greatly reduce the inactivation rate of MS2 by chlorine. The dose of 0.1 mg/L could only cause 4.5 log inactivation of MS2 under the co-existence of 10^7 PFU/mL of MS2 and 10^7 CFU/mL of *E. coli*. The degradation rate of MS2 genome was lower than its inactivation rate. The chlorine doses of 0.5 and 1.0 mg/L were able to completely inactivate both MS2 and *E. coli* under the investigated condition of this study. However, complete genome degradation of MS2 under these chlorine doses were not able to be reached no matter if *E.coli* coexisted or not. The free chlorine and combined chlorine species showed different significance towards the changes in infectivity and genome integrity of MS2.

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