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Enhanced virus inactivation by copper and silver ions in the presence of natural organic matter in water

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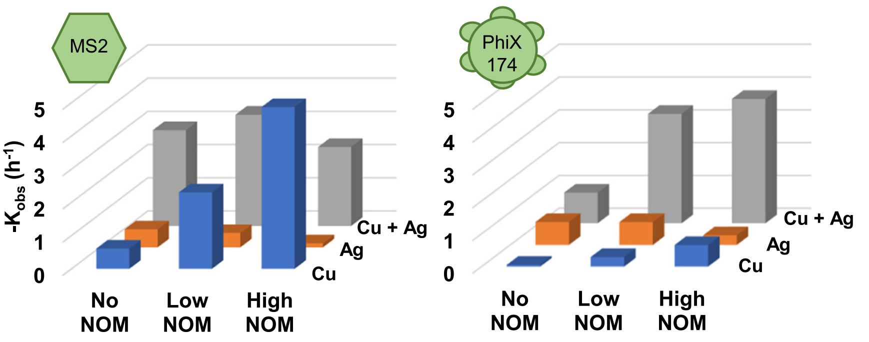
HIGHLIGHTS

- Virus inactivation by Cu was significantly enhanced by NOM due to the production of ROS.
- Virus inactivation by Ag was significantly reduced by the presence of high NOM in water.
- Combined Cu and Ag ions synergistically inactivated MS2 except in water with high NOM.
- Meanwhile, for PhiX 174 synergism of Cu and Ag was observed in water with low or high NOM.

GRAPHICAL ABSTRACT



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ABSTRACT

Natural organic matter (NOM) is present in water matrix that serves as a drinking water source. This study examined the effect of low and high NOM concentrations on inactivation kinetics of a model RNA virus (MS2) and a model DNA virus (PhiX 174) by copper (Cu^{2+}) and/or silver (Ag^{+}) ions. Cu and Ag are increasingly applied in household water treatment (HHWT) systems. However, the impact of NOM on their inactivation kinetics remains elusive despite its importance for their application. The presence of NOM in water led to faster virus inactivation by Cu^{2+} but slower by Ag^{+}. The fastest inactivation kinetics of MS2 ($K_{obs} = 4.8\ h^{-1}$) were observed by Cu in water containing high NOM (20 mg C/L). Meanwhile, for PhiX 174, the fastest inactivation kinetics (av. $K_{obs} = 3.5\ h^{-1}$) were observed by Cu and Ag synergism in water containing high NOM. Altogether, it can be concluded that the combination of Cu and Ag is promising as a virus disinfectant in treatment options allowing for multiple hours of residence time such as safe water storage tanks.

1. Introduction

The burden of waterborne disease remains a global threat to public health and economic prosperity. To date, 44 % of the global population lacks access to safe drinking water (WHO, 2021). Fecally contaminated water can contain harmful organisms such as deadly waterborne viruses. For example, rotavirus (waterborne virus) might cause fatal diarrhoeal disease and hepatitis A or E, other waterborne viruses, cause liver disease (WHO, 2008, 2017, 2019). Hence, it is vital to provide consumers with water treatment systems with adequate protection from waterborne viruses.

To that end, metals such as Cu and Ag have been increasingly investigated as additives to existing filters for enhancing virus removal. Cu and//or Ag has been added to existing household water treatment to improve virus removal in treated water (Brown and Sobsey, 2010; Ehdaie et al., 2020; Friedman, 2018; Jackson et al., 2020; Van der Laan et al., 2014). Cu and Ag were also combined with other disinfection methods such as UV light to accelerate the disinfection of viruses in water (Butkus et al., 2004; Kim et al., 2008).

Multiple factors can impact the antiviral efficiency of Cu and Ag. For example, water parameters such as pH, organic carbon, temperature,

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dissolved oxygen determine the oxidation state and the speciation of metals (Allen et al., 1980; Allen and Hansen, 1996; Lemire et al., 2013). Viruses can exhibit different sensitivity to different Cu or Ag chemical species (Kim et al., 2015; Minoshima et al., 2016; Nieto-Juarez et al., 2010; Sunada et al., 2012). Despite the importance of water composition in studying Cu and Ag antiviral efficiency, a systematic assessment of physiochemical parameters impacting inactivation kinetics remains elusive.

In our previous study, we demonstrated the significant impact of pH on Cu and Ag inactivation kinetics of two model conservative model viruses: MS2 (ssRNA) and PhiX 174 (ssDNA) (Soliman et al., 2020). Another factor is to assess the role natural organic matter (NOM) can play in Cu and Ag antiviral efficiency. The presence of NOM in source water for household water treatment (HHWT) systems is inevitable, hence its examination is crucial for Cu and Ag application (Venis and Basu, 2020). Yet, to our knowledge, the effect of NOM on virus inactivation by Cu and Ag ions has not been reported.

Both Cu and Ag have an affinity to bind with ligands in NOM, changing the metal speciation (Hou et al., 2013; Lemire et al., 2013). NOM contains functionalities such as thiols, hydroxyls, aldehydes, etc. that can form organic-Ag complexes, AgCl, Ag_2S or over lengthy periods reduce Ag to silver nanoparticles (Akaighe et al., 2011; Thalmann et al., 2016). The formation of soluble or insoluble Ag solids has been reported as an inert disinfectant compared to Ag^+ (Choi et al., 2009; Singh et al., 2020; Xiu et al., 2011, 2012). Similarly, in freshwater bodies, most of Cu is bound to NOM which reduces its environmental toxicity (Buck et al., 2007). Moreover, Cu binding to NOM was reported to result in the production of reactive oxygen species (ROS) (Pham et al., 2012). The presence of ROS can result in rapid inactivation of MS2 bacteriophage as observed in many studies (Kim et al., 2015; Nguyen et al., 2013; Nieto-Juarez et al., 2010; Sagripanti et al., 1993; Yamamoto, 1969). Hence, it is possible that viricidal efficiency of Ag or Cu would be reduced by the presence of NOM. Although, the possibility of ROS production by Cu-NOM interaction suggests the opposite. Nonetheless, the antiviral efficiency or possible synergism of Cu and Ag combination when combined with NOM cannot be anticipated.

Therefore, we evaluated the antiviral efficiency of Cu and/or Ag in the presence and absence of NOM using MS2 and PhiX 174 bacteriophages as a conservative model RNA and DNA virus, respectively. Cu and Ag were tested individually and combined to examine possible synergies in the presence of NOM. Two levels of NOM were added to water, resembling low NOM (2 mg C/L) and high NOM (20 mg C/L). The chemical speciation of Cu and/or Ag in water w/o NOM was calculated using CHEAQS Next -chemical equilibrium program (Simonin, 2017). Possible ROS production was examined through scavenger experiments and measurement of hydrogen peroxide as a step in the ROS chain.

2. Materials and methods

2.1. Chemical reagents and microbial stocks

All chemicals were reagent grade and used without further purification as described in Soliman et al. (2020). Experimental solutions were prepared using Milli-Q water. Sodium phosphate buffer (PB; 1 mM, pH 7) contained sodium phosphate monobasic monohydrate ($NaH_2PO_4.H_2O$), sodium phosphate dibasic heptahydrate ($Na_2HPO_4.7H_2O$) mixed at concentration of 0.58 mM and 0.42 mM. PB buffer was autoclaved at 121 °C for 20 min and stored at 4 °C until further use. Potassium iodide (KI, 0.1 M) solution was prepared by dissolving KI (99 %, Sigma) in MilliQ water and 0.01 M molybdate solution by dissolving Ammonium molybdate tetrahydrate (99.98 %, Sigma) in MilliQ water.

NOM of ground water origin was obtained from Dutch company Vitens. NOM was recovered from ion exchange brine resulting in concentrated NOM stock, 89.9 mg/ L measured as dissolved organic carbon which contained 88 % humic substance; of which 69.3 % was fulvic acid and 10.7 % humic acid (Caltran et al., 2020). The 5 NOM fractions are measured and reported by Caltran et al. (2020). The used NOM stocks were prepared through diluting the concentrated NOM stock in MilliQ water, filter sterilized through sterile 0.22 μm syringe filters (PES, VWR) and stored at 4 °C until used.

Purified MS2 and PhiX 174 stocks were used as conservative virus models. The culturing and purification of both phages is described thoroughly in Soliman et al. (2020). In brief, MS2 stock was produced by infecting host bacterium *Escherichia coli* C3000 (ATCC 15597) and PhiX 174 by infecting *E. coli* WG5 (DSM 18455). Following overnight replication, the harvested lysate was centrifuged and filter sterilized using 0.22 μm sterile syringe filters. MS2 was purified in iodixanol gradient (OptiPrep™-Stemcell) and PhiX 174 in cesium chloride by centrifugation for 20 h (32,000 rpm, 4 °C, Beckman coulter optima L-90 K ultracentrifuge). The recovered phage was washed twice using 100 kDa Amicon® Ultra columns and stored in PB buffer (pH 7) at 4 °C.

2.2. Impact of NOM on MS2 and PhiX 174 inactivation

The examination of NOM effect on MS2 and PhiX 174's inactivation by metals was conducted by comparing inactivation kinetics under 3 conditions: no NOM (PB buffer), low NOM (PB buffer +2 mg C/L) and high NOM (PB buffer +20 mg C/L). Although the presence of bacteriophages in PB buffer constituted an organic content, its concentration compared to added NOM was negligible.

In addition, the stability of MS2 and PhiX 174 was examined without adding metals (control conditions). This aimed to account for any possible bacteriophage decay in the test water not caused by the metals. Overall, each bacteriophage was examined in 12 settings (3 controls, 3 with Cu, 3 with Ag and 3 with Cu and Ag). Each setting was tested in triplicate beakers.

In a sterile glass beaker, a 60 mL of test water (no NOM or low NOM or high NOM) containing MS2 and PhiX 174 at a final concentration $\sim 10^6$ pfu/mL was added. For metal conditions, aliquots of Cu, Ag or Cu and Ag were added to a final concentration of 1 mg/L for Cu and 0.1 mg/L for Ag. All beakers were kept at 25 °C in a dark incubator and stirred continuously at 60 rpm (2mag-Magnetic-Drive) for 6 h. At time intervals 0, 0.17, 0.5, 1, 3 and 6 h, a 5 mL aliquot of sample was withdrawn using sterile syringes and added to a sterile tube containing neutralizing solution. Samples containing Cu were neutralized using 150 μL EDTA (0.1 M) and Ag samples using 150 μL of sodium thiosulfate (0.1 M). Control samples and Cu and Ag samples were neutralized using 150 μL EDTA and 150 μL of sodium thiosulfate. The addition of neutralizer to control samples aimed to test the effect of neutralizing solutions on phage stability. All samples were diluted in PBS buffer and enumerated for each phage in duplicates.

Phage enumeration followed the ISO-10705, where a 1 mL sample was assayed using the double agar layer (DAL) method. MS2 samples were enumerated using *Salmonella typhimurium* WG49 as a host and PhiX 174 using *E. coli* WG5 as host. Agars and cultures used followed the ISO- 10705 part 1 and 2. Glass beakers used were sterilized, acid washed (10 % nitric acid and 12.5 % Hydrochloric acid), wrapped in aluminium foil and covered from the top with a sterile petri-dish and foil to ensure dark conditions.

2.3. Role of ROS

The interaction between Cu and NOM can lead to production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) (Pham et al., 2012). Measurements of H_2O_2 followed the Ghormley triiodide method (Ghormley and Stewart, 1956) as described by Romero et al. (2011). H_2O_2 samples were obtained from replicates of the inactivation experiments. Every 0.5 h, a 1 mL sample was drawn, filtered through 0.45 μm filters and directly measured for H_2O_2. To quantify H_2O_2, 200 μL of sample was rapidly mixed with 1000 μL of KI solution and 20 μL of the molybdate solution in 2 mL disposable cuvette (VWR). Absorbance was recorded UV–Vis Spectrophotometer (GENESYS 10S) at 350 nm after reading stabilized. The concentration of H_2O_2 was determined based on a calibration curve of

H_2O_2 stock solution (Merck) of different concentrations, as shown in Supplementary S1.

To distinguish between inactivation kinetics caused by metals versus the additional inactivation by ROS, scavenger experiments were added. The 12 settings mentioned above were repeated, each in triplicate beakers, with the addition of reagent grade tempol (Sigma-Aldrich—Germany) as ROS scavenger. Tempol is a known redox-cycling nitroxide and used as potent ROS scavenger to protect from oxidative damage (Li et al., 2016; Wilcox, 2010; Wilcox and Pearlman, 2008). In order to determine the appropriate tempol dosage, possible tempol toxicity towards MS2 or PhiX 174 (Li et al., 2016) and time related stability of tempol (Matsumoto et al., 2009) were taken into account. Prior to the scavenger experiments,

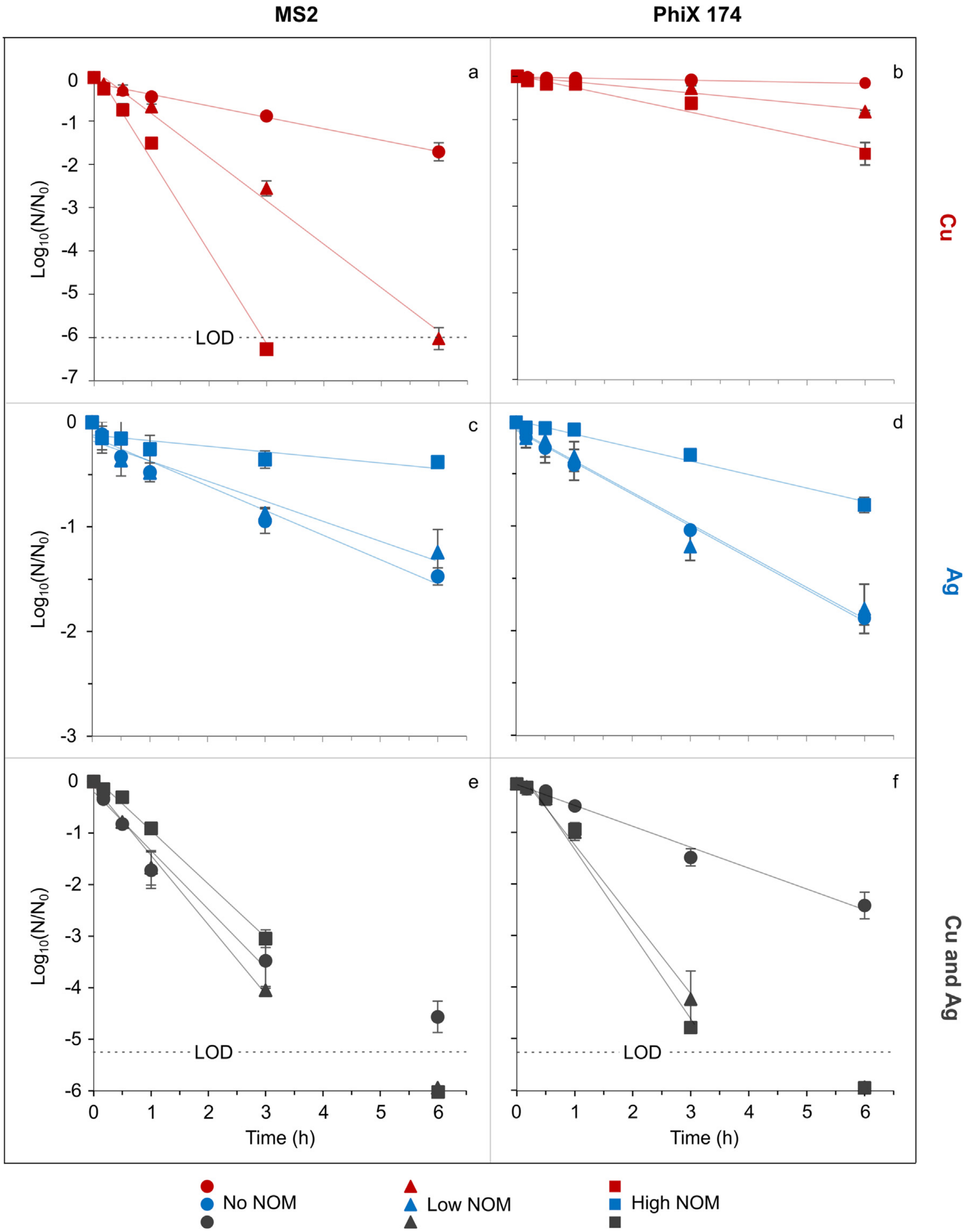

Fig. 1. Inactivation of MS2 (a, c, and e) and PhiX 174 (b, d, and f) by Cu (a and b), Ag (c and d) and their combination Cu and Ag (e and f) in No NOM water (PB buffer), low NOM water (PB + 2 mg C/L) and high NOM water (PB + 20 mg C/L). Concentration of metals used were Cu (1 mg/L) and Ag (0.1 mg/L) at solution pH 7 and 25 °C. Limit of detection (LOD) was 1 pfu/mL. Reported results represent the average values and standard deviations of triplicate tests. Inactivation rate constants: Kobs (h-1) ± standard deviation (SD) values are mentioned in the text and calculated based on the slope on linear regression lines. R2 values were > 0.96 for all Kobs values considered.

different doses of tempol and dosing intervals were tested (Supplementary S2-S4). Based on the results, it was determined that 1 mM of tempol dosed every 1.5 h would provide maximum protection of MS2 or PhiX 174 without toxic effect towards either bacteriophage (as shown by the stability in tempol control test). Therefore, inactivation experiments were replicated with the addition of 1 mM of tempol every 1.5 h. Experimental procedure, sampling intervals, neutralizing and phage enumeration were repeated exactly as described in the previous section. In addition, pH was measured in the beginning and experiment's end to ensure pH stability in presence of tempol.

2.4. Chemical analysis

NOM was measured as total organic carbon (TOC) using a TOC-V CPH analyser (Shimadzu). Cu and Ag measurements were analysed using ICP-OES (Spectro Arcos eop). Moreover, samples from NOM beakers were analysed for all detectable elements to evaluate any additional ions present in water by adding NOM stock. Furthermore, we used CHEAQS Next -chemical equilibrium program- to calculate the free ionic concentrations (Ag^+ and Cu^{2+}) in the water matrices used. Metal – organic complexation was evaluated using model 7, in CHEAQS Next (Simonin, 2017). Input for NOM included the measured concentration as mg C/L and measured fraction of fulvic acids as 0.69.

2.5. Data analysis

The measured concentration (pfu/mL) of MS2 or PhiX 174 at time t (N_t) and at time 0 h (N_0) were used to calculate the $\log_{10}$ inactivation values. To analyse inactivation kinetics, linear regression analysis was conducted with 95 % confidence interval to calculate the slope of $\mathrm{Ln}(N_t/N_0)$ versus time, known as first order inactivation rate constant: K_{obs} (h^{-1}). Measured K_{obs} (h^{-1}) of control experiments was compared against zero (no slope) to evaluate the stability of MS2 and PhiX 174 in control conditions. Other conditions' K_{obs} (mean value, SD and df) were compared against each other for significant difference using an unpaired *t*-test.

3. Results

3.1. Impact of NOM on MS2 and PhiX 174 inactivation

The results of control (metal free) experiments showed stability of MS2 and PhiX 174 in the test waters w/o NOM (Supplementary S5). As depicted in Fig. 1, presence of NOM with Cu enhanced inactivation kinetics of MS2 and PhiX 174 compared to inactivation by Cu without NOM. MS2's $\log_{10}$ inactivation by Cu reached 1.7 ± 0.2 after 6 h without NOM, ≥ 6 logs after 6 h with low NOM and ≥ 6 logs after only 3 h with high NOM (Fig. 1 a). Hence, increasing NOM accelerated the MS2's inactivation by Cu. Less pronounced effect of Cu (w/o NOM) was observed on PhiX 174 compared to MS2 (Fig. 1 b). For example, in presence of high NOM, Cu inactivated 1.7 ± 0.3 $\log_{10}$ of PhiX 174 after 6 h.

Inactivation kinetics of MS2 and PhiX 174 followed the first order Chick Watson model (Watson, 1908) as shown in Fig. 1. The K_{obs} values are depicted in Fig. 2 as 'No Tempol' columns. MS2's K_{obs} by Cu was significantly ($p < 0.0001$) higher in presence of high NOM ($K_{obs} = -4.9 \pm 0.3\ h^{-1}$) and low NOM ($K_{obs} = -2.3 \pm 0.1\ h^{-1}$) compared to no NOM ($K_{obs} = -0.6 \pm 0.04\ h^{-1}$). Similarly, PhiX 174's K_{obs} by Cu was significantly higher ($p < 0.0001$) in the presence of high NOM ($K_{obs} = -0.7 \pm 0.1\ h^{-1}$) compared to no NOM ($K_{obs} = -0.05 \pm 0.01\ h^{-1}$).

On the other hand, presence of NOM with Ag did not enhance inactivation kinetics of MS2 or PhiX 174 (Fig. 1 b and c). K_{obs} of Ag were statistically similar ($p > 0.005$) without NOM and with low NOM for both bacteriophages (Fig. 2 c and d). Moreover, the 6 h Ag's $\log_{10}$ inactivation of MS2 and PhiX 174 were ≤ 2 without NOM and with low NOM. This value reduced to only 0.4 and 0.8 logs for MS2 and PhiX in presence of high NOM. As observed in Fig. 2 c and d, presence of high NOM with Ag significantly ($p < 0.001$) reduced K_{obs} for MS2 and PhiX 174.

So far, the RNA and DNA bacteriophages MS2 and PhiX 174 were affected in a similar manner by presence of NOM with Cu (enhanced inactivation) or Ag (reduced kinetics). However, for the combination of Cu and Ag, NOM had a different effect on inactivation kinetics of MS2 from PhiX 174. Statistically similar ($p = 0.44$) inactivation of MS2 was observed by Cu and Ag without NOM and with High NOM (Figs. 1 and 2 e). However, significantly higher ($p < 0.001$) inactivation of MS2 was observed with low NOM. Meanwhile, statistically similar ($p = 0.32$) inactivation of PhiX 174 by Cu and Ag was observed with low and high NOM. Overall, the inactivation of PhiX 174 by Cu and Ag with NOM was significantly higher ($p < 0.0001$) than that without NOM (Figs. 1 and 2 f). Although all inactivation kinetics followed the first order Chick Watson model, MS2 inactivation by Cu and Ag without NOM followed a biphasic pattern (Fig. 1e), as also reported in our previous study (Soliman et al., 2020).

3.2. Contribution of ROS to inactivation kinetics

Tempol, a scavenger for ROS, was used to evaluate possible production of ROS and its contribution to the inactivation kinetics. Fig. 2 provides an overview of the K_{obs} in presence or absence of tempol.

MS2 and PhiX 174's K_{obs} by Cu with NOM (low or high) was significantly ($p < 0.001$) reduced after adding tempol (Fig. 2 a and b). In contrast, tempol did not affect the K_{obs} of MS2 or PhiX 174 by Cu without NOM. Hence, tempol effect on K_{obs} of Cu was only limited to the presence of NOM. This suggests that combining Cu and NOM leads to production of ROS and subsequently higher inactivation kinetics of MS2 and PhiX 174.

Moreover, H_2O_2 was detected when Cu was combined with NOM, but not without NOM (Fig. 3a). The measured H_2O_2 in Cu-NOM water demonstrated ROS production by Cu- NOM interaction. Higher H_2O_2 was measured in presence of high NOM with Cu, compared to low NOM. This suggests more production of ROS in presence of higher NOM, leading to faster inactivation kinetics.

H_2O_2 was not detected with Ag, regardless of NOM presence. Nevertheless, significant reduction ($p < 0.05$) in Ag's K_{obs} of MS2 and PhiX 174 without NOM or with low NOM was observed after adding tempol (Fig. 2 c and d). However, this does not suggest production of ROS by Ag and low NOM since K_{obs} was also reduced by tempol without NOM.

For Cu and Ag combination, significant ($p < 0.05$) reduction in MS2 and PhiX 174's K_{obs} was observed after adding tempol to water containing NOM (Fig. 2 e and f). Tempol had no effect ($p > 0.05$) on K_{obs} by Cu and Ag without NOM for either bacteriophage. This suggests production of ROS when Cu and Ag were combined with NOM. Moreover, H_2O_2 measurement in water containing Cu and Ag with NOM (Fig. 3 b), supports production of ROS when Cu and Ag were combined with NOM.

3.3. Effect of NOM on synergy of Cu and Ag

The examination of Cu and Ag combination versus their individual use aimed to identify possible synergistic effect of the metal combination w/o NOM. To that end, we compared the K_{obs} experimentally obtained by using both metals (observed) against the mathematical sum of K_{obs} obtained from each metal (estimated) w/o NOM. If the observed K_{obs} is significantly higher than estimated K_{obs} by Cu and Ag in the same water matrix, it is considered to show synergism.

For MS2, Cu and Ag synergism ($p < 0.05$) was observed without NOM and with low NOM only (Fig. 4a). On the other hand, for PhiX 174, strong ($p < 0.0001$) Cu and Ag synergy was observed with low and high NOM (Fig. 4b). The observed synergy for MS2 and absence of synergy for PhiX 174 by Cu and Ag (without NOM) was also reported in our previous study (Soliman et al., 2020).

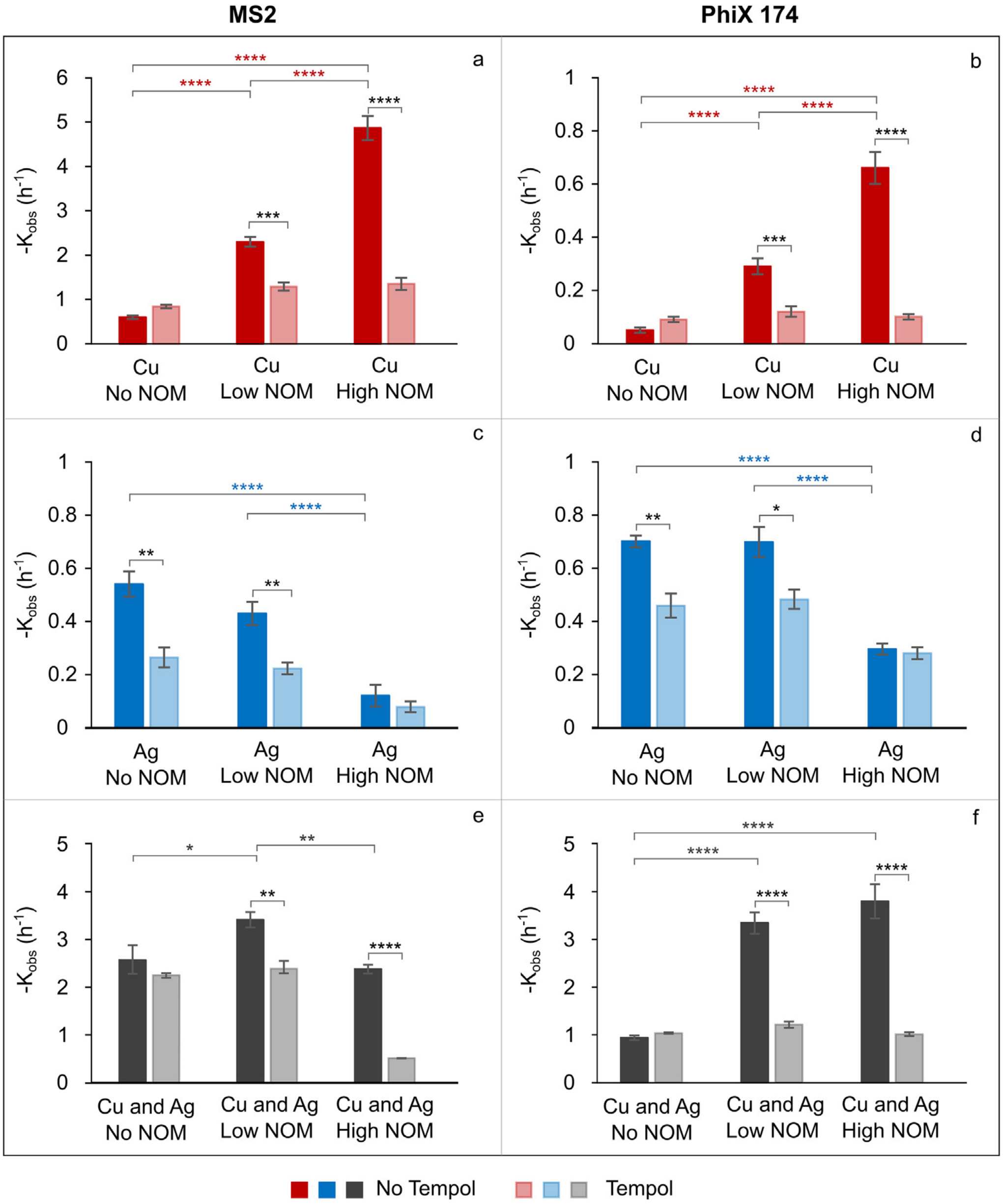

Fig. 2. Inactivation rate constant Kobs (h-1) of MS2 (a, c, and e) and PhiX 174 (b, d, and f) w/o tempol scavenger resulting from inactivation by Cu (a and b), Ag (c and d) and Cu and Ag (e and f) tested in no NOM water (PB buffer), low NOM water (PB + 2 mg C/L) and high NOM water (PB + 20 mg C/L). Statistical significance between experimental sets (calculated as *p* value) is expressed by asterisk (*), whereas (*) is used for $P \leq 0.05$, (**) for $P \leq 0.01$, (***) for $P \leq 0.001$ and (****) for $P \leq 0.0001$. Kobs (h-1) was calculated based on the slope of linear regression of Ln (Nt/N0) versus time (h). R2 values were > 0.96 for all Kobs values considered. Reported results represent the average values and standard deviations of triplicate tests.

Ag alone. The mathematical addition of the two parts adds up to the estimated K_{obs} by Cu + Ag.

3.4. Speciation of metal ions

The examination of metal speciation in water w/o NOM was conducted using CHEAQS Next as chemical speciation model. Speciation of Cu and Ag was simulated individually and combined. The results are depicted in Fig. 5, featuring speciation of Cu when added to water individually (Fig. 5a) and when combined with Ag (Fig. 5 c). Similarly, speciation of Ag is depicted in Fig. 5 b and d.

Without NOM, 23 % of total Cu was present as dissolved Cu (Fig. 5 a). This percentage decreased to 20 % and 2 % in presence of low and high NOM respectively. This decrease was accompanied by increase in organic bound Cu in presence of NOM. 16 % of Cu was organic bound when water contained low NOM and 92 % of Cu was organic bound in the presence of high NOM. So, increasing NOM in water lead to increase in the percentage of organic bound Cu. Speciation of Cu was not affected by the presence of Ag (w/o NOM) as depicted in Fig. 5 c.

The percentage of dissolved Ag^+ ions decreased from 100 % -without NOM- to 85 %, with low NOM, and down to 24 % with high NOM (Fig. 5 b). The reduction in Ag^+ in presence of NOM was due to the formation of organic bound Ag (15 % and 76 % respectively). Hence, presence of low NOM had negligible effect on the speciation of Ag. However, when Ag was combined with Cu, the speciation of Ag in presence of NOM differed (Fig. 5 b and d). Organic bound Ag reduced to 1 % in presence of low NOM and 16 % with high NOM. Hence, presence of Cu reduced the binding of Ag to NOM and enhances its dissolution.

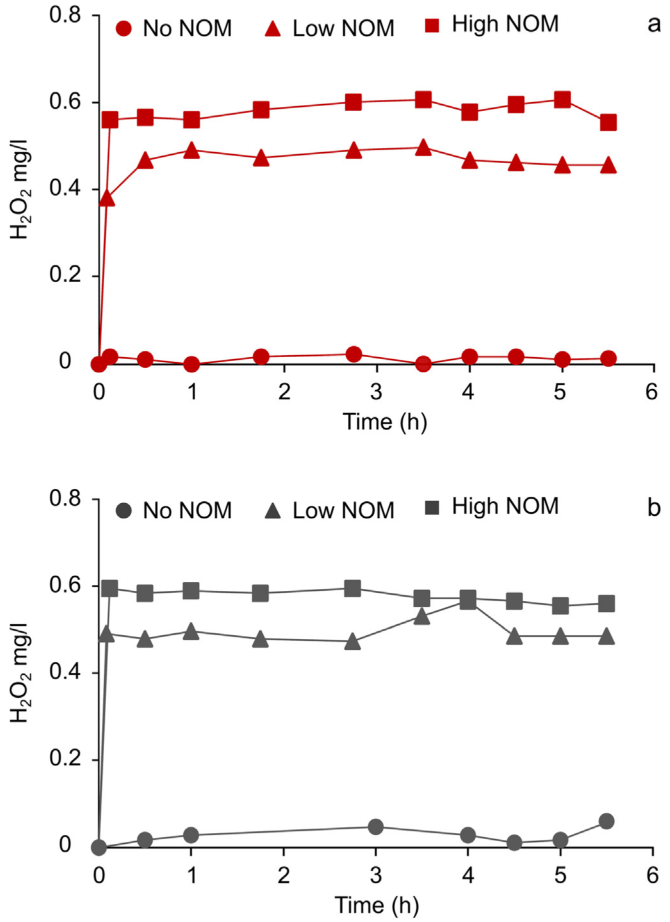

Fig. 3. Measured H2O2 in (a) Cu or (b) Cu and Ag combined with no NOM, low NOM, or high NOM waters.

4. Discussion

4.1. Copper and NOM

Without NOM, Cu had pronounced effect on MS2 but not on PhiX 174 (Soliman et al., 2020). The presence of NOM in water led to enhanced Cu inactivation of MS2 and PhiX 174. Higher NOM led to faster inactivation kinetics for both bacteriophages.

Environmentally, most Cu in water bodies or in wastewater is bound to organics (Buck et al., 2007; Coale and Bruland, 1990). Here, we observed that chemical interaction between Cu and NOM led to the formation of organic bound Cu, especially with high NOM (Fig. 5; 92 %). Hence this leaves less dissolved free Cu^{2+} available for interaction with MS2 or PhiX 174. Although previous studies described availability of dissolved Cu^{2+} as key element in enhancing inactivation kinetics by Cu, we observed accelerated inactivation with higher organic bound Cu instead.

Cu binding to NOM is the starting point of a chemical interaction producing ROS (Pham et al., 2012). In the presence of oxygen, Cu^{2+} is reduced by NOM's reducing moieties to Cu^{1+}. Then, oxygen rapidly interacts with Cu^{1+} producing superoxide which immediately reacts producing H_2O_2 as described by the following Eqs. (1)–(4) (Pham et al., 2012; Xing et al., 2020). Moreover, interactions could lead to production of organic radicals or toxic Cu^{+3} (Xing et al., 2020).

$$Cu(\mathrm{II}) + \mathrm{R} \leftrightharpoons Cu(\mathrm{I}) + R^{+} \text{ where R is the reducing organic moieties.} \tag{1}$$

$$Cu(\mathrm{I}) + O_2 \longrightarrow Cu(\mathrm{II}) + O_2^{\bullet\,-} \tag{2}$$

$$Cu(\mathrm{II}) + O_2^{\bullet\,-} \longrightarrow Cu(\mathrm{I}) + O_2 \tag{3}$$

$$Cu(\mathrm{I}) + O_2^{\bullet\,-} \longrightarrow Cu(\mathrm{II}) + H_2O_2 \tag{4}$$

As observed in our results, interaction between NOM (low or high) and Cu led to production of H_2O_2. Higher H_2O_2 was measured in higher NOM water, similar to literature reports by Pham et al. (2012) and Xing et al. (2020). Moreover, tempol scavenging experiments showed that production of ROS was responsible for enhancing Cu inactivation kinetics in presence of NOM. It has been also reported by others that combination of Cu and ROS enhances inactivation of MS2 and PhiX 174, compared to Cu alone (Kim et al., 2015; Nguyen et al., 2013; Nieto-Juarez et al., 2010; Sagripanti et al., 1993). ROS causes protein oxidation and RNA damage of MS2 accelerating its inactivation (Fitts et al., 1999; Kim et al., 2010).

Despite the similarity in MS2 and PhiX 174 response to Cu disinfection in NOM water, significantly higher kinetics were observed for MS2 than PhiX 174. This could be due to higher susceptibility of MS2 to certain ROS species or radicals compared to PhiX 174. For example, MS2 is more susceptible to hydroxyl radicals ($OH^{\bullet}$) and H_2O_2 than PhiX 174 (Mattle et al., 2015; Timchak and Gitis, 2012). The higher resistance of PhiX 174 to ROS, compared to MS2, could be explained by the hypothesis that oxidative damage of PhiX 174 targets the capsid protein, and secondary to it the genome, while for MS2 the genome is the primary target (Kohn et al., 2016; Mattle et al., 2015; Rodriguez et al., 2014). MS2's capsid is porous and thinner than the capsid of PhiX 174, which might allow oxidants to penetrate

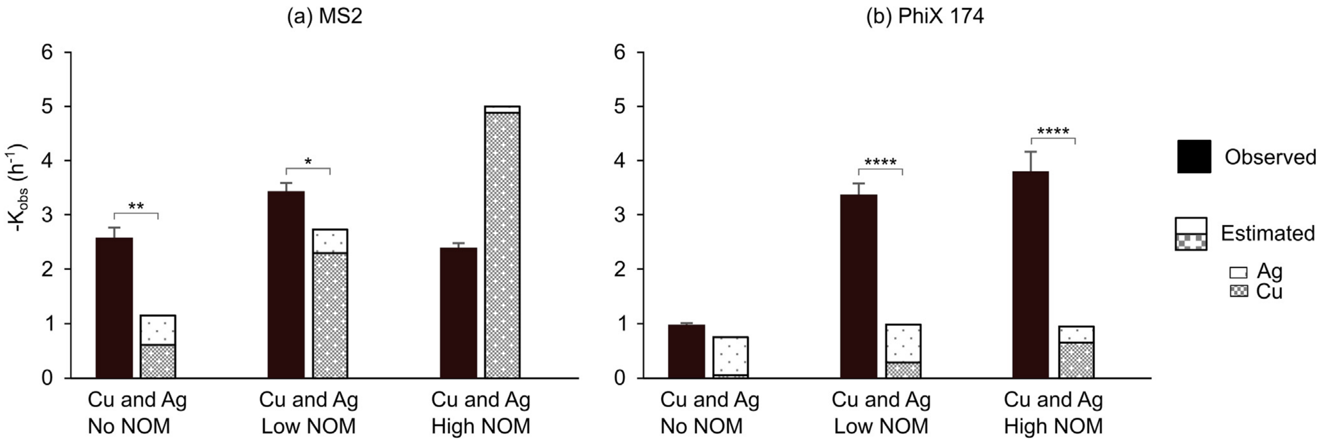

Fig. 4. Inactivation rate constants (Kobs; h-1) of (a) MS2 and (b) PhiX 174. Dark black columns are the Kobs experimentally obtained by Cu and Ag ions combined, referred to as observed. Light grey dotted column part is Kobs by Cu alone and white dotted column part is Kobs of Ag alone. The mathematical addition of the two parts adds up to the estimated Kobs by Cu + Ag.

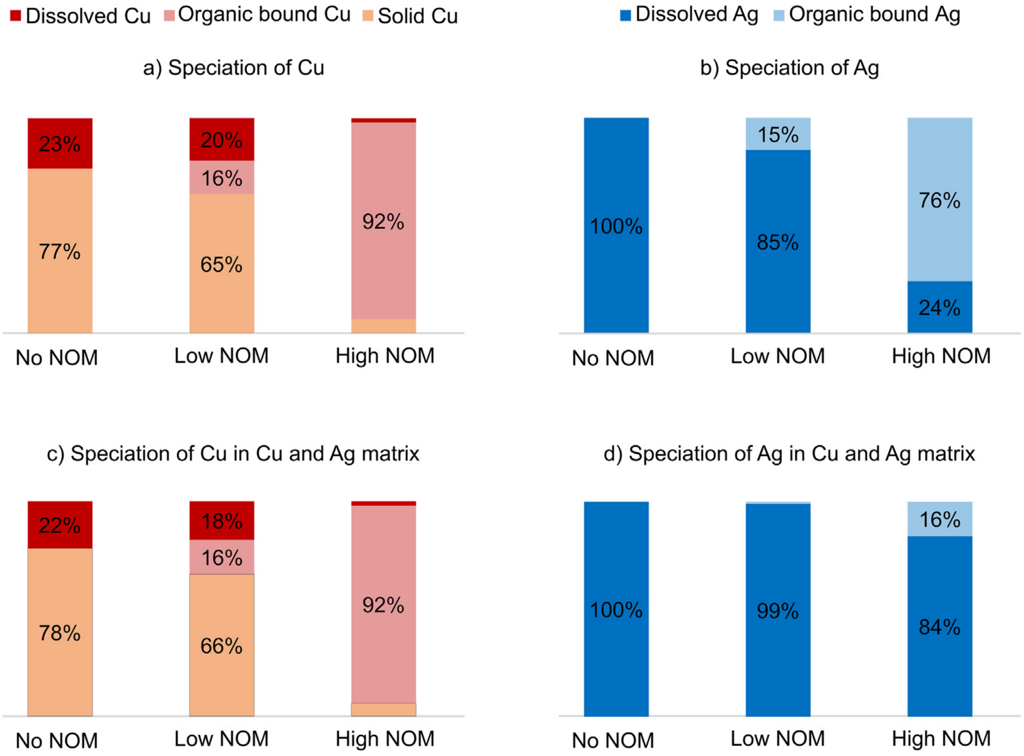

Fig. 5. CHEAQS Next simulation (a) Cu or (b) Ag speciation when added individually each water matrix and (c) Cu or (d) Ag speciation in water matrices containing Cu and Ag combined.

the capsid more readily causing faster damage to the genome (Mattle et al., 2015).

4.2. Silver and NOM

Unlike Cu, the presence of NOM did not enhance inactivation kinetics of MS2 or PhiX 174 for Ag. In fact, slower inactivation kinetics of MS2 and PhiX 174 were observed by Ag with high NOM. High NOM (20 mg C/L) led to a loss of 76 % of Ag^+ by binding to NOM. This strongly suggest that complexation of Ag with NOM competes against viruses, hence leads to slower inactivation kinetics. In case of Ag and low NOM, K_{obs} of MS2 and PhiX 174 were like that without NOM. Moreover, only 15 % of Ag was organic bound in presence of low NOM (2 mg C/L). Therefore, it is likely that loss of free dissolved Ag^+ by 15 % was not significant enough to have noticeable impact on inactivation kinetics of either MS2 or PhiX 174. Complexation of Ag with NOM has been reported by multiple studies (Hogstrand and Wood, 1998; Miller and Bruland, 1995; Reinfelder and Chang, 1999). In fresh water, organic complexation of Ag reduces it toxicity (Hogstrand and Wood, 1998; Reinfelder and Chang, 1999). This emphasizes that inactivation kinetics of Ag is dependent on the availability of free ionic Ag^+.

We also observed no H_2O_2 in the presence of Ag and NOM. To our knowledge, Ag has not been reported to produce ROS when interacting with MS2 or PhiX 174. Only in bacterial studies, Ag can produce ROS as part of the cell defence mechanism following the interaction between Ag and enzymes in the bacterial cell wall (Lemire et al., 2013; Park et al., 2009). However, MS2 and PhiX 174 are non-enveloped (McKenna et al., 1992) and don't possess enzymes, regulatory or defence mechanism. Hence, production of ROS due to sole interaction between MS2 and PhiX 174 with Ag is unlikely.

4.3. Combined Cu, Ag and NOM

Cu and Ag's inactivation kinetics of MS2 was only enhanced in presence of low NOM (2 mg C/L) but not in presence of high NOM (20 mg C/L). On the other hand, high NOM and low NOM led to a similar, yet significant increase in PhiX 174's K_{obs} by Cu and Ag compared to K_{obs} without NOM.

The similarity in PhiX 174's K_{obs} between low and high NOM can be explained by the combined effect of ROS production and Ag-NOM complexation. Less free Ag^+ ions were available in water containing higher NOM but higher H_2O_2 was produced. Hence, it is possible that the balance between H_2O_2 and Ag^+ ions availability led to equivalent inactivation kinetics in presence of low and high NOM. It would have been expected that the higher H_2O_2 with high NOM compensates for the loss in Ag^+ concentration, hence resulting in similar K_{obs} in both NOM waters for MS2. Yet, that was not the case.

The variability between MS2 and PhiX 174 in reaction could be due to a production of radicals or intermediates that can be more toxic to PhiX 174 than MS2. The reaction between Ag^+ and H_2O_2 in water can result into a cascade of interaction possibly producing organic free radicals and intermediates such as bound hydroxyl radicals or Ag(II) species (He et al., 2011; Henglein, 1979). The possible production of these species and their potential effect on MS2 versus PhiX 174 is unknown and cannot be judged based on the measurement in this study. Measurements of H_2O_2 were used as indicative of ROS production but not as conclusive representation or measurement of ROS. Hence, it is possible that other radicals, uncovered by this study, were produced and responsible for the inactivation rates observed by Cu and Ag in presence of NOM. Yet, it cannot be judged with certainty under the scope of the current study. Further investigation is needed to evaluate the full cascade of ROS species produced in each condition.

4.4. Synergy of Cu and Ag

Without NOM, Cu and Ag had synergistic effect on MS2 (observed K_{obs} 2.5 times > estimated K_{obs}) but not on PhiX 174. With NOM (low or high) strong synergy (observed K_{obs} 3–4 times > estimated K_{obs}) of Cu and Ag was observed for PhiX 174. Meanwhile, for MS2, in presence of high NOM with Cu and Ag; observed K_{obs} was 0.5 times lower than the estimated K_{obs}, indicating slower kinetics and lack of synergy. Overall, the highest inactivation kinetics of PhiX 174 (av. $K_{obs} = 3.5\ h^{-1}$) was observed by Cu and Ag combination in presence of NOM (low or high). Meanwhile, the highest inactivation kinetics of MS2 ($K_{obs} = 4.8\ h^{-1}$) was observed using Cu alone with high NOM.

It is likely that the observed Cu and Ag synergy in disinfecting PhiX 174 stems from interaction between Cu and NOM producing ROS, followed by its interaction with Ag^+. The interaction of Ag^+ ions and H_2O_2 in water was reported by Pedahzur et al. (1995) to possess synergistic and long lasting effect in disinfecting *E.coli*. This suggests that simultaneous presence of Cu, Ag and ROS is crucial for fast inactivation kinetics of PhiX 174.

Meanwhile, Cu and Ag's synergistic effect on MS2 was only observed without NOM and with low NOM. The two conditions in which free Ag^+ ions were available as 100 % and 99 %. Yet, the inactivation observed (Fig. 4) under these conditions with added synergy was lower than while using Cu alone with high NOM ($K_{obs} = 4.8\ h^{-1}$). Hence, for MS2 the fastest inactivation did not lie in synergy of Cu and Ag but rather in high ROS with Cu alone. However, the structural reason for this observation is unclear based on the data available. Especially, since similar H_2O_2 was measured in water containing Cu and high NOM to that containing Cu, Ag, and high NOM. Yet, slower kinetics were observed for MS2 in the latter. Future structural study is needed to investigate the full speciation of ROS and their structural damage of MS2 and PhiX 174 in presence of NOM, Cu and/or Ag.

Finally, the observed high $\log_{10}$ inactivation values in this study highlight the potential of Cu (1 mg/L) and Ag (0.1 mg/L) in providing safe drinking water, complying with the WHO highly protective (5 logs) virus removal. Although contact time is in scale of hours, it is promising for (household) safe storage treatment where longer contact time is possible. More research is needed to examine Cu and Ag antiviral efficiency in more conclusive water matrix (e.g., rich in ions) to enable their application.

5. Conclusions

- Presence of NOM in water reduced the availability of free ionic Cu^{2+} and Ag^+. Loss of free Ag^+ ions led to slower virus inactivation kinetics. Yet, Cu binding to NOM resulted in production of ROS and faster virus inactivation kinetics.
- In water containing low NOM (2 mg C/ L), the highest inactivation of MS2 and PhiX 174 was observed by Cu and Ag combined (a similar $K_{obs} = 3.2 \pm 0.1\ h^{-1}$).
- In water containing high NOM (20 mg C/ L), the highest inactivation kinetics of PhiX 174 (av. $K_{obs} = 3.5\ h^{-1}$) was observed by Cu and Ag combination while for MS2 ($K_{obs} = 4.8\ h^{-1}$) was observed using Cu alone.
- Synergy of Cu and Ag was observed for PhiX 174 when water contained low or high NOM, while for MS2 when water contained low or no NOM.
- Cu and Ag are promising virus disinfectants, especially in treatment options allowing for multiple hours of residence time such as safe water storage tanks.

CRediT authorship contribution statement

Mona Y.M. Soliman: Conceptualization, Investigation, Formal analysis, Methodology, Writing - Original Draft.

Gertjan Medema: Conceptualization, Supervision, Reviewing and Editing.

Doris van Halem: Conceptualization, Supervision, Reviewing and Editing, Funding acquisition.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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