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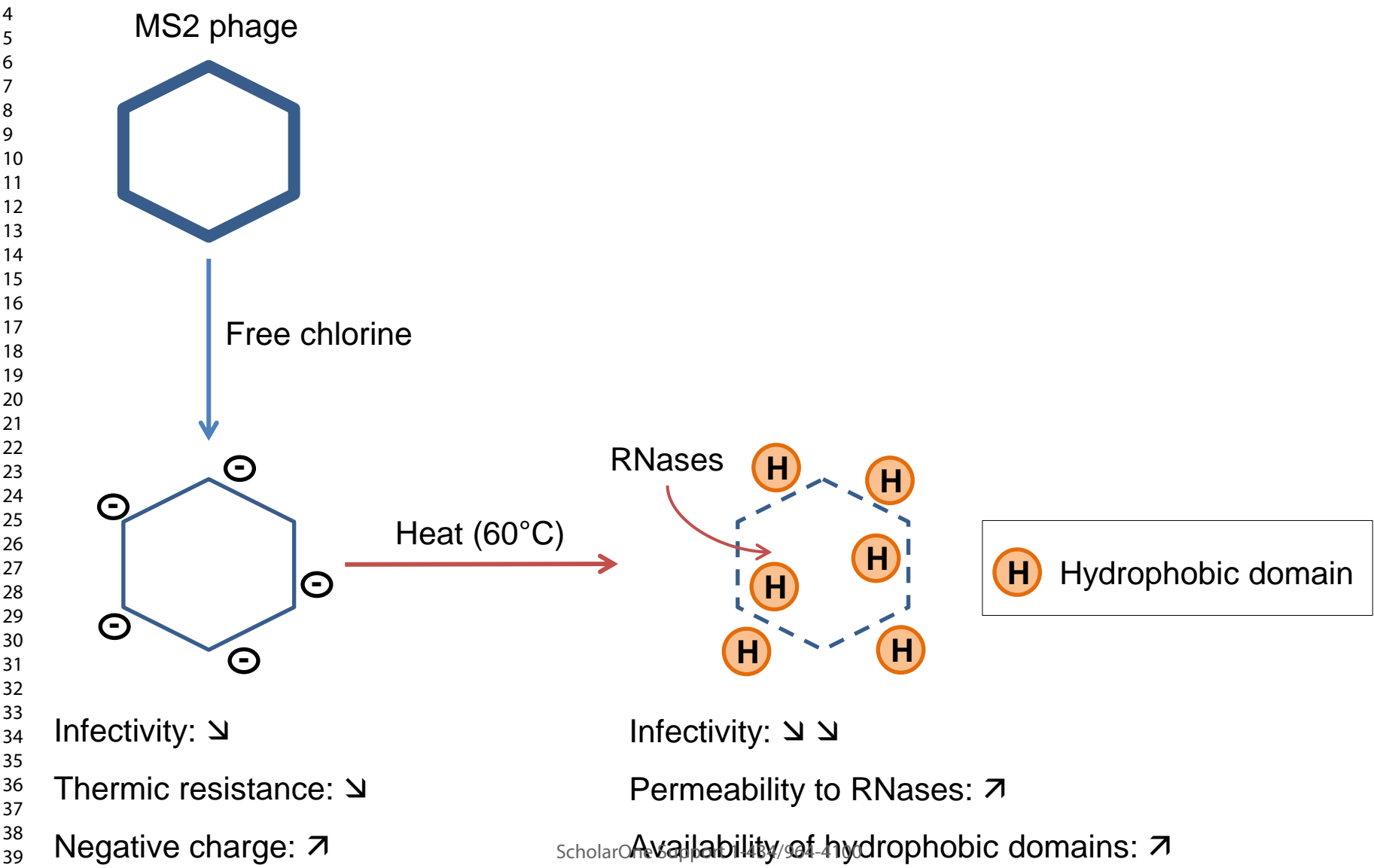
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1 **Graphical abstract**



The impact of chlorine and heat on the infectivity and physicochemical properties of bacteriophage MS2

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One-sentence summary: Exposure of MS2 phage to free chlorine followed by heat leads to higher inactivation, permeability to RNases, and availability of hydrophobic domains within the capsid than does chlorine applied alone.

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ABSTRACT

Enteric viruses and bacteriophages are exposed to various inactivating factors outside their host, and among them chlorine and heat are the most commonly used sanitizer in water industry and **treatment** in the food industry, respectively. Using MS2 phages as models for enteric viruses, we investigated the impact of free chlorine and heat on their physicochemical properties. Free chlorine was first evaluated alone. No increase in either capsid permeability or hydrophobicity was observed. The negative surface charge slightly increased suggesting molecular changes in the capsid. However, a weakening of the capsid by chlorine was suggested by differential scanning fluorimetry. This phenomenon was confirmed when chlorination was followed by a heat treatment. Indeed, an increase in the inactivation of MS2 phages and the permeability of their capsids to RNases was observed. More interestingly, an increase in the expression of hydrophobic domains at the phage surface was observed, but only for phages remaining infectious. The chlorine-caused weakening of the capsid suggested that, for an optimal use, the oxidant should be followed by heat. The increased permeability to RNases and the expression of hydrophobic domains may contribute to the development or improvement of molecular methods specific for infectious enteric viruses.

KEYWORDS: free chlorine, heat, MS2 bacteriophage, surface properties, hydrophobicity, charge

INTRODUCTION

Enteric viruses and phages are exposed to various inactivating factors in water, in foodstuffs, and on surfaces. Among them, heat and chlorine are one of the most cost-effective and easy-to-apply treatments and sanitizers in the food and water industries. The level of inactivation of enteric viruses by heat has been widely monitored in food and environmental matrices in which it is considered as one of the main inactivating factors (Hirneisen *et al.* 2010; Bertrand *et al.* 2012). Chlorine is a major disinfection treatment of water and food for its broad-spectrum antimicrobial activity (Hirneisen *et al.* 2010; Boudaud *et al.* 2012; Girard *et al.* 2016; Meireles *et al.* 2016). Although virus inactivation by heat or chlorine has been evaluated in numerous studies, the mechanisms leading to non-infectious virus particles still remain partially understood. Comparison of the impact of different inactivating treatments on virus functions (i.e. host cell binding, genome injection and replication), using MS2 phage as model of pathogenic enteric viruses, has revealed differences in the mechanisms of inactivation between physical treatments such as heat and chemical oxidants such as free chlorine, but also within treatment groups (Wigginton *et al.* 2012).

Heat induces structural and conformational changes of the capsid proteins leading to at least inactivation through alteration of the binding capacity to host cells, modification of surface properties, and increase in permeability to enzymes and intercalating dyes. These findings have been reported for poliovirus, hepatitis A virus, feline calicivirus, and MS2 phage (Breindl 1971; Nuanualsuwan and Cliver 2003; Pecson, Martin and Kohn 2009; Wigginton *et al.* 2012; Brié *et al.* 2016). Heat has been shown to have, if any, only a very low impact on the virus genome (Wigginton *et al.* 2012). Hydrophobicity seems to be the surface property the most affected by heat treatment. Indeed, the expression of hydrophobic domains has been reported for MS2 phages exposed to heat but for remaining infectious particles only (Brié *et al.* 2016). An increase in hydrophobicity has also been observed for poliovirus exposed to 50°C, which has been linked to the externalization of the N-terminal region of viral protein VP1 (Fricks and Hogle 1990; Curry *et al.*

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1996). Heat-induced modification of the electrostatic charge is less obvious. Indeed, a decrease in the isoelectric point (IEP) has been observed for poliovirus heated at 56°C and linked to the loss of viral RNA genome and capsid protein VP4 (O'Brien and Newman 1979; Breindl 1971). Only a slight decrease in the global negative charge has been reported for MS2 phages after exposure to 60°C (Bri  *et al.* 2016). The structural changes in the capsid proteins caused by heat seem to alter the structure specifically involved in host cell binding, and to be linked to modification of hydrophobic domain expression.

Free chlorine has been shown to alter both genome- and protein-mediated functions of MS2 phage, more specifically genome injection and replication, but not its binding capacity to host cells (Wigginton *et al.* 2012). Free chlorine has also been shown to affect proteins of pathogenic enteric viruses such as picornaviruses (Nuanualsuwan and Cliver 2003) and adenovirus (Page, Shisler and Mari as 2010). Moreover, the loss of genome replication has been shown to coincide with extensive genome damage (Wigginton *et al.* 2012). The reaction of free chlorine with amino acids constituting the capsid proteins leads to the formation of numerous products such as carbonyl groups resulting from the oxidization of lysine, arginine, threonine, and proline. A relationship between the number of carbonyl groups and the rate of free chlorine-induced inactivation has been reported for enteric viruses belonging to *Astroviridae*, *Reoviridae*, *Caliciviridae*, and *Picornaviridae* (Sano *et al.* 2010; Tojo *et al.* 2013; Sano *et al.* 2015). The oxidization of amino acids by chlorine could also result in modifications of the electrostatic charge, which, together with hydrophobicity, is the major component of the surface properties of virus particles. To our knowledge, the electrostatic charge has not previously been explored for enteric viruses exposed to free chlorine. Concerning the hydrophobicity of chlorine-exposed viruses, a recent study has shown that exposure to sodium hypochlorite modified the profile of the hydrophobic probe SYPRO Orange in Q  phage belonging to F-specific RNA phage genogroup III. Indeed, an increase in hydrophobicity was observed at the beginning of the experiment; this meant that sodium

hypochlorite revealed some hydrophobic parts of the capsid or increased the permeability of the capsid allowing the probe to reach inside hydrophobic regions (Loison *et al.* 2016).

The study of the impact of heat and free chlorine on enteric viruses requires methods suited for the specific detection of infectious viral particles, which are the only ones that pose a risk to human health. Indeed, studies performing a simultaneous and quantitative comparison between infectivity loss and genome degradation have showed a higher persistence of viral genome after heat treatment (Bertrand *et al.* 2012) and chlorination (Pelleieux *et al.* 2016). Thus, the detection of viral genomes by molecular methods used alone does not provide information about infectivity (Girones *et al.* 2010). Human noroviruses (NoV) and hepatitis A virus (HAV) are considered as the main pathogenic enteric viruses. However, the detection of their infectious particles remains challenging although cell culture methods have been very recently proposed for noroviruses (Jones *et al.* 2015; Ettayebi *et al.* 2016). Various methods which could be combined with molecular approaches have been evaluated in order to favor the detection of only infectious viruses. Most of them are based on the increased permeability of the capsid to enzymes or intercalating dyes such as RNases, ethidium monoazide (EMA), or propidium monoazide (PMA) (Prevost *et al.* 2016; Randazzo *et al.* 2016), or on the specific detection of oxidative damage (Tojo *et al.* 2013; Sano *et al.* 2015). However, their optimal conditions of use such as the type of treatment (i.e. physical and/or chemical) and the matrices (i.e. food and/or environmental matrices) still need to be defined.

F-specific RNA phages (FRNAPH) belong to the family *Leviviridae* and are classified in two genera, *Levivirus* and *Allovivirus*, each divided into two genogroups. These phages exhibit numerous similarities with NoV and HAV in terms of size (20 - 30 nm), genome (positive-sense single-stranded RNA), and overall capsid structure (Valegård *et al.* 1990; King *et al.* 2012). These phages infect bacteria with F pili, such as *Escherichia coli*, in the gut of mammals and are released in environments exposed to fecal contamination similarly to enteric viruses. Their physicochemical and biological characteristics and the easy-to-apply methods available for the quantification of their

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3 121 infectious particles and genome have led to their use as models to assess pathogenic enteric virus
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5 122 behavior in water (Boudaud *et al.* 2012), in food (Deboosere *et al.* 2012; Bozkurt *et al.* 2015), and
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7 123 on surfaces (Morin *et al.* 2015; Pelleieux *et al.* 2016) during treatment. Due to their structural
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9 124 similarity with the main waterborne pathogenic viruses they have also been proposed as relevant
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11 125 indicators of viral contamination in water (Cole, Long and Sobsey 2003; Ogorzaly *et al.* 2009;
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13 126 Vergara *et al.* 2015) and shellfish (Hartard *et al.* 2017) using molecular and/or cell culture methods.
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15 127 MS2 phage belonging to *Levivirus* genogroup I is the most studied of these FRNAPH, particularly
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17 128 its physicochemical characteristics (Valegård *et al.* 1990; Langlet *et al.* 2008a; Dika *et al.* 2013a)
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19 129 and the mechanisms of its inactivation (Pecson, Martin and Kohn 2009; Wigginton *et al.* 2012;
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21 130 Sigstam *et al.* 2013). Thus, MS2 seems to be a relevant model for pathogenic enteric viruses,
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23 131 especially for studying the mechanisms of inactivation by different factors, and it can be produced
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25 132 in high concentrations, an essential prerequisite for some experimental methods focusing on
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27 133 physicochemical characteristics such as electron microscopy.
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31 134 Thus, it appears that even treatments commonly used on enteric viruses such as free chlorine
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33 135 and heat need to be improved. Molecular-based methods allowing a more specific detection of
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35 136 infectious enteric viruses after such treatments are also needed. Such improvements require a better
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37 137 knowledge of the damage that occurs in enteric viruses during loss of infectivity. In such a context,
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39 138 the present work aimed to optimize the conditions of use of free chlorine and heat for the
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41 139 inactivation of enteric viruses and to evaluate their impact on the surface properties of MS2. The
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43 140 impact of free chlorine used alone was first evaluated on MS2 phages because of the poor
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45 141 knowledge about its effects on the surface properties of enteric viruses. After verification of the
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47 142 preservation of the capsid of inactivated phages, we examined in greater detail the thermal stability
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49 143 and the surface properties of MS2 phages exposed to chlorine. The weakening of the capsid
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51 144 observed in the present work when free chlorine was applied alone and the increase in
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53 145 hydrophobicity previously described for MS2 phages exposed to heat (Brié *et al.* 2016) led us to
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perform further experiments focusing on hydrophobicity, in which heat treatment was applied to MS2 phages previously exposed to chlorine. Since both free chlorine and heat have been shown to act on the capsid, it might therefore be expected that their combination would increase their impact.

MATERIALS AND METHODS

Production and purification of MS2 phage

Replication of MS2 phage (ATCC, 15597-B1) was done according to the standard procedure (ISO 10705-1 1995) (without the chloroform step) using *Escherichia coli* Hfr K12 (ATCC, 23631) as host cells.

The phages were then concentrated and purified as previously described (Brié *et al.* 2016). Briefly, the phages were centrifuged twice (8,000 g, 20 min, 4°C) and the supernatant (≈ 1 L) was filtered using a 0.22 μm filter unit (Stericup-GP Millipore Express Plus, 0.22 μm ; Merck Millipore, Merck KGaA, Darmstadt, Germany). After an ultracentrifugation step (Thermo Scientific A-621 fixed rotor, 42,500 g, 18 h, 4°C; Thermo Fisher Scientific, Waltham, MA, USA), the pellets containing the concentrated phages were resuspended in iodixanol solution (20% final, Optiprep; Axis-Shield, Dundee, Scotland) and laid on a 40% iodixanol layer in a tube (Cone-Top PA tube 14 \times 47 mm, Thermo Fisher Scientific). The density gradient was formed upon centrifugation at 160,000 g for 7 h at 15°C (Thermo Scientific TH-641 swing rotor). After centrifugation, the MS2 phages were gently removed with a syringe and dialyzed twice (one overnight, the other for 7 h) against 10 L of PBS solution with a concentration of 1 mM (1 mM Na_2HPO_4 and 14 mM NaCl), pH 7.4, using a Float-A-Lyzer G2 dialysis device (MW100 kD; Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA). These working suspensions were kept at 4°C before use. Three working suspensions were produced for this study in order to perform the experiments in triplicate on independent suspensions.

Free chlorine and heat treatments

Three independent suspensions of purified phages at a concentration of 10^{14} PFU·mL⁻¹ (250 µL in 1 mM PBS solution) (PFU: plaque-forming units) were exposed either to free chlorine alone, to heat alone or to free chlorine followed by heat. To prepare our free chlorine stock solution, concentrated sodium hypochlorite solution (5%, Acros Organics, Thermo Fisher Scientific) was diluted using ultrapure water ($> 18 \text{ M}\Omega\cdot\text{cm}^{-1}$). Determination of free chlorine concentration was performed according to the DPD (*N,N*-diethyl-*p*-phenylenediamine, Acros Organics) colorimetric method (ISO 7393-2 1985). For exposure to free chlorine alone, the working concentrations of free chlorine were 100 and 200 mg·L⁻¹ with a contact time of 10 min at 4°C. Residual free chlorine was immediately quenched by adding two moles of sodium thiosulfate equivalent to one mole of free chlorine. The heat treatments were performed at 55°C or 60°C for 10 min in pre-warmed Protein LoBind tubes (Eppendorf, Hamburg, Germany) in a water bath. Samples were immediately placed on ice after heat treatment to avoid further inactivation. When free chlorine was followed by heat, the phages were exposed to 100 mg·L⁻¹ of free chlorine for 10 min at 4°C and then to heat at 55°C or 60°C for 10 min. For each replicate, a part of the MS2 phage suspension was not exposed to free chlorine, heat or to free chlorine followed by heat and was named control phages. Thus, there were nine ($n=3\times 3$) control populations in this study.

Enumeration of infectious MS2 phages

Infectious MS2 phages were counted after treatment (I) and without treatment (I_0) by the plaque assay method using the double-agar-layer method (ISO 10705-1 1995) with *E. coli* K-12 Hfr as host cells. Three to five decimal dilutions of the MS2 phage suspensions were dropped in duplicate onto agar plates in order to have a dilution resulting in the enumeration of 10 to 100 plaques per Petri dish. Infectious concentration was expressed in PFU·mL⁻¹. The final concentration of purified MS2 phages was $\approx 10^{14}$ PFU·mL⁻¹. The loss in infectivity due to each treatment was estimated by $\log \frac{I}{I_0}$.

Viral genome extraction and quantification

The Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) method was used for the detection of potential degradation of the viral genome by free chlorine and/or heat. Thus, the genome of MS2 phage was quantified after each treatment (C) and without treatment (C₀). The genome degradation by the treatment was estimated by $\log \frac{C}{C_0}$. Prior to the RT-qPCR assays, extractions of viral RNA (50 µL) were performed using the QIAamp viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The extracts (100 µL) were immediately stored at -80°C before quantification. Quantification of the phage genome was performed by specific a RT-qPCR assay targeting a genome fragment of ≈ 150 nucleotides as previously described (Ogorzaly and Gantzer 2006). Two negative controls and one positive control (i.e. known quantity of MS2 genome) were added to each RT-qPCR analysis. RT-qPCR efficiency ranged from 80% to 95%. Determination of MS2 genome copies per mL (gc·mL⁻¹) used for the standard curve was performed as previously described (Bri  *et al.* 2016). The final concentration of purified MS2 phages was ≈ 10¹⁵ gc·mL⁻¹.

Transmission electron microscopy

Transmission electron microscopy (TEM) was used to visualize virus disruption or major structural modifications. The samples were adsorbed onto grids and negatively stained for 1 min with 2% phosphotungstic acid at pH 7.4 previously filtered with a 0.22 µm membrane filter. The examination was carried out using a CM200 transmission electron microscope (Philips, Andover, MA, USA) operating at 200 kV accelerating voltage and with a ×38,000 magnification.

Evaluation of capsid integrity using an RNase assay

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3 220 An RNase assay was used to evaluate if the capsid had maintained its integrity and was still able to
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5 221 protect the virus genome (Nuanualsuwan and Cliver 2003). Suspensions of MS2 phage, with a final
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7 222 concentration of 10^7 gc·mL⁻¹, were treated with RNase A at a final concentration of 1 µg·mL⁻¹
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9 223 (AppliChem GmbH, Darmstadt, Germany) at 37°C for 60 min as previously described (Brié *et al.*
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11 224 2016).

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16 226 **Binding of MS2 phages to *E. coli* host cells**

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18 227 The impact of free chlorine on the binding capacity of MS2 phage to its host cell *E. coli* Hfr K12
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20 228 was evaluated as previously described (Wigginton *et al.* 2012; Brié *et al.* 2016). The binding
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22 229 capacity of MS2 phage to *E. coli* was estimated by $\log \frac{Q}{Q_0}$ where Q is the viral RNA found on the
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25 230 bacteria after free chlorine treatment and Q_0 without treatment.

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29 232 **Size and electrophoretic mobility measurements**

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31 233 Size and electrophoretic mobility (µ) measurements were performed in order to evaluate the impact
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33 234 of free chlorine on the overall structure and charge density of MS2 phage, respectively. The
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35 235 experiments were achieved in disposable capillary cells (Malvern Instruments, Worcestershire, UK)
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37 236 at 23°C ± 0.1°C and pH 7.0 using a Zetasizer Nano ZS instrument (Malvern Instruments, He-Ne
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39 237 red laser, wavelength 633 nm) driven by Dispersion Technology Software (Malvern Instruments) as
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41 238 previously described (Langlet *et al.* 2008a). The slope of the electrophoretic mobility as a function
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43 239 of the logarithm of ionic strength (1.95, 19.5 and 195 mM) reflects the ratio between the density of
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45 240 electric charges located within the particle and that of charges distributed at the outer virus
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47 241 periphery. A decrease in the slope value shows a decrease in the charge density of the virus (Langlet
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49 242 *et al.* 2008a). The experimental conditions were identical to those previously described (Brié *et al.*
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51 243 2016). For each condition, measurements were obtained by means of three independent experiments
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53 244 with triplicate measures in each to ensure repeatability of the measurements.

Binding of MS2 phages to hydrophobic or positively charged beads

These assays were performed to study the variation in hydrophobicity or negative charge at the surface of MS2 phages. Magnetic beads were used in these experiments: hydrophobic beads were coated with polystyrene (PolySciences Europe GmbH, Hirschberg, Germany) and positively charged beads were BcMag PSA (Bioclone, San Diego, CA, USA). As recommended by the manufacturers, the beads were first washed twice in the buffer to be used in the assay which was either 10 mM PBS (10 mM Na₂HPO₄ and 150 mM NaCl) at pH 7 for hydrophobic beads or 50 mM PBS (50 mM Na₂HPO₄ and 20 mM NaCl) at different pH values (2.5, 3.0, 4.0, and 7.0) for ion exchange beads. The positively charged beads exhibited a pKa of ≈ 10 .

The binding assays were carried out as previously described (Bri  *et al.* 2016). The binding capacity of MS2 phage to beads was estimated by $\log \frac{C_X}{C_0}$ where C_X is the concentration of either infectious phages or viral RNA after the binding assay in suspension and C_0 the initial concentration of either infectious phages or viral RNA in suspension.

Differential scanning fluorimetry

Differential scanning fluorimetry (DSF) was performed using the hydrophobic probe SYPRO Orange. This probe has a high affinity for hydrophobic domains (Niesen, Berglund and Vedadi 2007), which are unmasked when proteins unfold. DSF was used to compare the thermal stability of MS2 phage, as measured by the denaturation temperature (T_m). Thermal melting curves of MS2 phage were obtained in 96-well plates using StepOne Plus qPCR System (Life Technologies, Thermo Fisher Scientific) and the fluorescent dye SYPRO Orange, 5,000 \times concentrate, supplied in DMSO (Invitrogen, Thermo Fisher Scientific). Reactions were performed in a 25 μ L final volume containing 5 μ L of buffer (150 mM Tris-HCl, pH 8), 2 μ L of SYPRO Orange solution 62.5 \times (final concentration = 5 \times), 16 μ L of ultrapure water ($> 18 \text{ M}\Omega \cdot \text{cm}^{-1}$), and 2 μ L of MS2 suspension from

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3 270 stock or after treatment (5.10^{14} - 10^{15} gc·mL⁻¹). The temperature was increased for all replicates from
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5 271 20°C to 95°C on a 1% stepwise gradient corresponding to $\approx 1^{\circ}\text{C}\cdot\text{min}^{-1}$ with fluorescence
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7 272 measurements. The sum of the fluorescence intensities obtained with all the filters was plotted
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9 273 against temperature (Excel software) to obtain the maximum fluorescence intensity of SYPRO
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11 274 Orange. The T_m was determined by plotting the first derivative of the previous curve; it appeared
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13 275 then as a peak (Loison *et al.* 2016). This method is also known to allow the expression of
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15 276 hydrophobic parts of the capsid and/or the permeability of the capsid to be investigated by the
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17 277 fluorescence intensity of SYPRO Orange at the beginning of the DSF experiment (Loison *et al.*
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19 278 2016).
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24 280 **Data analysis**

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26 281 The analysis was performed using the values obtained for the three independent suspensions of
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28 282 phages. Since the experimental values were not normally distributed, non-parametric tests were
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30 283 performed using StatEL 2.7 (Ad Science). The explanatory variables were the exposure to free
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32 284 chlorine alone, heat alone, and free chlorine followed by heat. For free chlorine alone, Mann-
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34 285 Whitney tests were performed to evaluate its effect on the infectivity (i.e. loss of infectivity, binding
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36 286 capacity to host bacteria), structure (i.e. genome degradation, capsid permeability to RNase, thermal
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38 287 stability), and hydrophobicity (i.e. initial adhesion of hydrophobic probe SYPRO Orange, binding
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40 288 to hydrophobic probes), and the adhesion to positively charged beads by comparison between
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42 289 control and chlorinated MS2 phages. A Kruskal-Wallis test was performed to compare the
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44 290 electrophoretic mobility between control and chlorinated MS2 phages. The effect of free chlorine
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46 291 followed by heat on the loss of infectivity, structure (i.e. genome degradation, capsid permeability
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48 292 to RNase) and hydrophobicity (i.e. initial adhesion of hydrophobic probe SYPRO Orange, binding
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50 293 to hydrophobic beads) was evaluated by comparison with free chlorine alone and heat alone using
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52 294 Kruskal-Wallis tests. For all these tests, a *p* value of <0.01 was considered as significant.
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RESULTS

Impact of free chlorine applied alone or followed by heat on the infectivity and structure of MS2 phage

The effects of free chlorine applied alone on MS2 phage were evaluated for 100 and 200 mg.L⁻¹ (Table 1). Infectivity decreased by $-1.2 \pm 0.3 \log_{10}$ for 100 mg.L⁻¹ ($p < 0.01$, Mann-Whitney test) and by $-3.5 \pm 0.9 \log_{10}$ for 200 mg.L⁻¹ ($p < 0.01$, Mann-Whitney test). The binding capacity of MS2 phage to *E. coli* K12 Hfr was not altered after exposure to 100 mg.L⁻¹ ($-0.1 \pm 0.1 \log_{10}$) ($p > 0.01$, Mann-Whitney test), whereas it decreased by $-0.8 \pm 0.1 \log_{10}$ after exposure to 200 mg.L⁻¹ ($p < 0.01$, Mann-Whitney test). The free chlorine treatment at 100 mg.L⁻¹ did not affect the detection of the genome fragment of ≈ 150 nucleotides targeted with our RT-qPCR assay since the RT-qPCR signal was not modified ($p > 0.01$, Mann-Whitney test). However, the genome detection decreased by $-0.5 \pm 0.2 \log_{10}$ for 200 mg.L⁻¹ ($p < 0.01$, Mann-Whitney test). No modification of the shape of MS2 phage was observed by TEM for these two concentrations of free chlorine (Fig. 1). The mean size of chlorinated MS2 phage was 20 ± 2 nm for 100 mg.L⁻¹ ($n=3 \times 3$) and 21 ± 1 nm for 200 mg.L⁻¹ ($n=3 \times 3$) which was similar to control phages ($p > 0.01$, Mann-Whitney test). Moreover, the limited effect of RNase treatment on RT-qPCR signal which decreased by less than $-0.3 \pm 0.1 \log_{10}$ with the two concentrations of free chlorine ($p > 0.01$, Mann-Whitney test) confirmed that the integrity of the capsid was preserved under our experimental conditions. DSF was then used to explore conformational modifications of the capsid that could not be revealed by TEM and to compare the T_m (i.e. denaturation temperature) value of MS2 phages before and after exposure to free chlorine. We observed only one T_m peak of $66.0 \pm 0.1^\circ\text{C}$ ($n=3$) before exposure to chlorine (Fig. 2). After exposure to free chlorine for 10 min, a decrease in the T_m to $62.8 \pm 0.1^\circ\text{C}$ ($n=3$) for 100 mg.L⁻¹ ($p < 0.01$, Mann-Whitney test) and $60.9 \pm 0.7^\circ\text{C}$ ($n=3$) for 200 mg.L⁻¹ ($p < 0.01$, Mann-Whitney test) was noticed. This reduction in the denaturation temperature suggested a weakening of the capsid of

MS2 phage by the oxidant. Although there was a decrease in the thermal stability of the capsid we did not find any changes in shape, size, and binding capacity, nor did the permeability to RNase increase thus suggesting that the capsid of MS2 phage maintained its integrity after exposure to free chlorine alone.

The decrease in the thermal stability after exposure to chlorine led us to expose MS2 phage to chlorine (100 mg.L⁻¹) and then to heat (55°C or 60°C). The infectivity decreased by $-1.9 \pm 0.3 \log_{10}$ for chlorine followed by heat treatment at 55°C and by $-3.2 \pm 0.6 \log_{10}$ for chlorine followed by heat treatment at 60°C. For each treatment applied separately, the loss in infectivity reached $-1.2 \pm 0.3 \log_{10}$ after exposure to free chlorine (Table 1), $-0.7 \pm 0.1 \log_{10}$ after heat treatment at 55°C (data not shown) and $-1.1 \pm 0.1 \log_{10}$ after heat treatment at 60°C (Brié *et al.* 2016). The highest loss in infectivity was obtained when free chlorine was followed by heat treatment at 60°C ($p < 0.01$, Kruskal-Wallis test). This loss in infectivity was also higher than the sum of the decrease values obtained with free chlorine and heat treatment at 60°C applied separately ($p < 0.01$, Kruskal-Wallis test). This suggested that free chlorine improved the inactivation of MS2 phage by heat treatment at 60°C. The RT-qPCR signal decreased only by $-0.1 \pm 0.4 \log_{10}$ for chlorine followed by heat treatment at 55°C and by $-0.3 \pm 0.4 \log_{10}$ for chlorine followed by heat treatment at 60°C. The concentrations in genome observed after these treatments were similar to those observed for MS2 phage exposed to 100 mg.L⁻¹ free chlorine alone and also for control MS2 phage ($p > 0.01$, Kruskal-Wallis test). Thus, the detection of the MS2 genome was not affected when free chlorine was followed by heat. As observed for free chlorine alone, no modification of the shape of MS2 phage was noted by TEM after exposure to free chlorine followed by heat (Fig. 1). The RNase treatment applied after exposure to free chlorine (100 mg.L⁻¹) followed by heat decreased the RT-qPCR signal by $-1.1 \pm 0.2 \log_{10}$ for 55°C and $-1.7 \pm 0.4 \log_{10}$ for 60°C. The log removal of genome copies by RNase treatment reached $-0.3 \pm 0.1 \log_{10}$ (Table 1) for free chlorine alone, $-0.1 \pm 0.1 \log_{10}$ (data not shown) for 55°C heat treatment alone and $-0.8 \pm 0.2 \log_{10}$ (Brié *et al.* 2016) for 60°C heat

treatment alone. The reduction in genome after chlorine followed by heat (55°C and 60°C) was higher than the sum of the log removal obtained with free chlorine and heat applied separately ($p < 0.01$, Kruskal-Wallis test). Moreover, the highest log removal of genome copies by RNase treatment was observed for MS2 phage exposed to free chlorine followed by heat treatment at 60°C ($p < 0.01$, Kruskal-Wallis test). Overall, the increased effect of free chlorine followed by heat treatment at 60°C on the loss in infectivity and on the capsid permeability to RNase was in agreement with the free chlorine-induced weakening of the capsid suggested by DSF experiments.

Impact of free chlorine on the charge of MS2 phage particles

The electrophoretic mobility (μ) was represented as a function of the logarithm of ionic strength (1.95, 19.5 and 195 mM) at pH 7.0 before and after exposure to free chlorine (100 and 200 mg.L⁻¹) in order to estimate the impact of free chlorine on the charge density of MS2 phage (Fig. 3A). A decrease in the electrophoretic mobility was observed after exposure to free chlorine at ionic strengths of 1.95 and 19.5 mM ($p < 0.01$, Kruskal-Wallis test). On the other hand, no modification of the electrophoretic mobility was observed after exposure to chlorine at an ionic strength of 195 mM ($p > 0.01$, Kruskal-Wallis test). Therefore, a lower slope value was observed for chlorinated phages than for control phages suggesting a decrease in the negative charge density of MS2 phage exposed to free chlorine. The negative surface charge of total particles (i.e. RT-qPCR results) was then estimated by binding to positively charged beads at different pH (i.e. 7.0, 4.0, 3.0, and 2.5) for control and chlorinated phages (Fig. 3B). The lowest adhesion capacity of both control and chlorinated phages on positively charged beads was obtained at pH 2.5 and 3.0 which are below the IEP of MS2 phage. Conversely, the highest adhesion capacity was obtained at pH above the IEP (i.e. 4.0 and 7.0). Thus, the IEP of MS2 phage was shown to be between 3 and 4. Interestingly, an increase in the adhesion capacity to positively charged beads was observed for MS2 phages exposed to the two concentrations of free chlorine in comparison with control phages ($p < 0.01$, Mann-

Whitney test). Thus, the increase in the negative charge density of MS2 phage after exposure to free chlorine seemed to be mainly linked to an increase in the external negative charge.

Impact of free chlorine applied alone or followed by heat on the hydrophobicity of MS2 phage particles

The effect of free chlorine on the hydrophobicity of MS2 phage was then evaluated by DSF and by its adhesion capacity to hydrophobic beads. The DSF profile (Fig. 2) showed the same fluorescence intensity of SYPRO Orange at the beginning of the experiment for control and chlorinated phages, meaning that free chlorine did not modify the overall expression of hydrophobic domains of the capsid proteins. The expression of hydrophobic domains on the surface of MS2 particles was compared between control and chlorinated phages using the adhesion ability to hydrophobic beads. The experiments were performed at pH 7.0 and high ionic strength (10 mM Na₂HPO₄ and 150 mM NaCl) in order to minimize the influence of the global charge. No adhesion to hydrophobic beads was observed for control phages and those exposed to chlorine (Fig. 4). Thus, the results obtained using these two methods suggested that free chlorine did not change the expression of hydrophobic domains of the MS2 capsid proteins or the permeability of the capsid to the probe.

Since we have previously described an increased hydrophobicity of MS2 phage after heat treatment (Bri  *et al.* 2016), DSF experiments were carried out after exposure to free chlorine (100 mg.L⁻¹) followed by heat (55 C or 60 C) and were compared to 55 C and 60 C heat treatments applied alone (Fig. 5). Similar DSF profiles with low initial fluorescence were obtained for both control MS2 phages and those exposed to the 55 C heat treatment alone. The initial fluorescence increased for MS2 phages exposed to the 60 C heat treatment alone and for those exposed to free chlorine followed by heat treatment at 55 C and 60 C. The highest initial fluorescence value was observed using 100 mg.L⁻¹ free chlorine followed by 60 C heat treatment (*p* <0.01, Kruskal-Wallis test). The decrease in the fluorescence of the DSF profiles observed between 20 C and 60 C could

have been due to probe quenching by water (Cimmperman and Matulis 2011; Loison *et al.* 2016). The increase in the initial fluorescence showed that free chlorine followed by heat led to the expression of hydrophobic domains and/or increased the permeability of the capsid allowing the probe to reach inside hydrophobic regions. The adhesion level on hydrophobic beads increased after exposure to free chlorine followed by heat treatment at 55°C or 60°C (Fig. 4); thus showing an increase in hydrophobic domains on the surface of MS2 particles. The highest adhesion level on hydrophobic beads was observed after free chlorine followed by heat treatment at 60°C (-2.4 ± 0.5 , Fig. 4) ($p < 0.01$, Kruskal-Wallis test). Remarkably, this increase in the adhesion capacity to hydrophobic beads appeared to be specific to viral particles remaining infectious.

DISCUSSION

The purpose of this study was to explore the impact of free chlorine alone or followed by heat on the loss of infectivity and the modification of the physicochemical properties of MS2 phage used as model of the main pathogenic enteric viruses. The impact of free chlorine on capsid proteins has been previously described in a few studies focusing on their altered functions. However, modification of the overall structure of viral particles may lead to modification of their surface properties which govern their behavior in the environment. Moreover, the study of surface properties may contribute to the understanding of the mechanisms involved in the inactivation process and may also help in the development of detection methods specific for infectious viruses.

MS2 phage was first exposed to free chlorine alone. The loss of infectivity of MS2 phage observed in our experiments after exposure to 100 and 200 mg.L⁻¹ of free chlorine was similar to that reported by Loison *et al.* (2016) for a Q β phage suspension. Indeed, by applying sodium hypochlorite at 1 mM (i.e. 74.4 mg.L⁻¹) for 10 min at room temperature on a Q β phage suspension at an initial concentration of $\approx 10^{12}$ PFU·mL⁻¹, these authors observed a $-1.7 \log_{10}$ reduction in infectivity. In the present work, we observed that the binding to host bacteria was altered after

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3 420 exposure to 200 mg.L⁻¹ of free chlorine only. Thus, the loss of infectivity was not due to the
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5 421 alteration of the binding capacity after exposure to 100 mg.L⁻¹ of free chlorine and was slightly due
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7 422 to the alteration of this function after exposure to 200 mg.L⁻¹ of free chlorine. Our observation of a
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9 423 high concentration of free chlorine needed to observe an impact on binding to host cell was in
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11 424 agreement with a previous study published by Wigginton *et al.* (2012). Indeed, these authors
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13 425 applied a concentration of 2-3 mg.L⁻¹ of free chlorine to an MS2 phage suspension of 10¹¹ PFU.L⁻¹
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15 426 and observed alteration of the genome injection and extensive degradation of the maturation protein
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17 427 (MP). This protein, which is present as a single copy in the capsid of MS2 phage, is clearly
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19 428 involved in the binding of MS2 phage to the F-pilus of host bacteria and probably in the injection of
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21 429 the genome into host cells. Dent *et al.* (2013) have proposed that the natural retraction of the F-pilus
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23 430 could pull the MP from its location in the attached capsid, leaving a pore through which the phage
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25 431 genome could exit into the cell. Thus, alteration of the MP by free chlorine may impact these two
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27 432 functions depending on its intensity. The sensitivity of the capsid to oxidants has also been observed
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29 433 for Q β phage with the loss of its infectivity due either to capsid destabilization or to the
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31 434 modification of capsid proteins especially the MP and the readthrough protein (Loison *et al.* 2016).
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33 435 Considering the capsid of FRNAPH, the MP appears as one of the targets of free chlorine. Thus,
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35 436 free chlorine weakens the capsid of MS2 phage and may alter the role of the MP in the genome
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37 437 injection into host cells even at low concentrations. High concentrations of free chlorine seem to
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39 438 further alter the MP with a reduced ability to bind to host cells.
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44 439 In our experiments, the genome of MS2 phage was quantified using an RT-qPCR assay which
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46 440 targets a fragment of \approx 150 nucleotides and the RT-qPCR signal was found to be affected only after
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48 441 exposure to 200 mg.L⁻¹ of free chlorine. A few studies have shown that some regions of the viral
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50 442 genome could be more sensitive than others to damage caused by free chlorine with significant
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52 443 differences depending on the virus type (Li *et al.* 2002; Xue *et al.* 2013). Free chlorine was shown
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54 444 to induce genome damage on MS2 phage at levels comparable to singlet oxygen and UV. However,
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the loss of replication function played a lesser role in the overall inactivation than in the other two inactivation treatments (Wigginton *et al.* 2012). For these authors, this suggested that the virucidal effect due to genome damage was linked to the type of modification rather than its location.

No modification of the shape of MS2 phage was observed by TEM after exposure to free chlorine. The mean size of MS2 phage before and after chlorination was similar to that previously described (Valegård *et al.* 1990; Dika *et al.* 2013a). Q β phage particles exposed to chlorine under similar conditions were also demonstrated by TEM observations to maintain their shape (Loison *et al.* 2016). Disruption of MS2 phage occurring after exposure to 72°C for 10 min has previously been shown to be related to a significant increase in RNase sensitivity (Brié *et al.* 2016). In our case, the limited effect of RNase treatment on RT-qPCR signal confirmed that the integrity of the capsid was preserved after exposure to free chlorine under our experimental conditions. DSF was then used to explore conformational modifications of the capsid that could not be revealed by TEM. We observed a similar DSF profile to that obtained in our previous study (Brié *et al.* 2016) with only one T_m peak (66°C) before exposure to chlorine. The decrease in the T_m value after exposure to free chlorine corresponded to a reduction in the denaturation temperature and suggested a weakening of the capsid of MS2 phage by the oxidant by comparison to a previous study performed on poliovirus (Robinson, Jesudhasan and Pfeiffer 2014). The weakening of the capsid might be linked to the site-specific cleavage showed on the capsid protein of MS2 phage following exposure to chlorine (Wigginton *et al.* 2012).

The reactions of hypochlorous acid with amino acids result in a wide variety of products such as sulfinic/sulfonic acids, methionine sulfone, and *N*-chlorinated species (Pattison, Davies and Hawkins 2012) which may modify the electrostatic charge of viral particles. In our study, the charge density was evaluated by measuring the electrophoretic mobility as a function of ionic strength which suggested a decrease in the negative charge density of MS2 phage exposed to free chlorine. On a qualitative level, it meant that free chlorine could change the negative charge density

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3 470 of MS2 phage particles by increasing their external negative charge and/or by decreasing their
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5 471 internal negative charge (Langlet *et al.* 2008a). The binding to positively and negatively charged
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7 472 beads at different pH showed that the IEP of MS2 phage was between 3 and 4, which was in full
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9 473 agreement with literature data in the field (Langlet *et al.* 2008b; Dika *et al.*, 2011). MS2 phage
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11 474 exposure to chlorine led to an increase in its adhesion capacity to positively charged beads at pH
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13 475 above the IEP. Thus, the increase in the negative charge density of MS2 phage observed after
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15 476 exposure to free chlorine resulted mainly from an increase in the external negative charge.

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18 477 Hydrophobicity was the other surface property monitored after exposure to free chlorine. The
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20 478 lack of adhesion to hydrophobic beads before exposure to free chlorine was in agreement with
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22 479 previous studies performed with MS2 phage (Dika *et al.* 2013b; Brié *et al.* 2016). No adhesion to
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24 480 hydrophobic beads was also observed after exposure to free chlorine. The DSF profile showed the
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26 481 same fluorescence intensity of SYPRO Orange at the beginning of the experiment for control and
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28 482 chlorinated phages. Thus, the results obtained with these two methods suggest that few hydrophobic
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30 483 domains are naturally expressed on the surface of MS2 particles and that free chlorine does not
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32 484 change the expression of hydrophobic domains of the MS2 capsid proteins or the permeability of
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34 485 the capsid to the probe. An increase in hydrophobicity by chlorine has previously been described for
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36 486 Q β phage (Loison *et al.* 2016) and for specific proteins such as α 2 chaperone (Wyatt *et al.* 2014)
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38 487 and Bovine Serum Albumin (Ashrafi Kooshk *et al.* 2012). This suggests that the expression of
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40 488 hydrophobic domains induced by chlorine depends on the composition and environment of the
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46 490 Overall, the most significant effect we observed on MS2 phages exposed to free chlorine
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48 491 alone was the decrease in the T_m in the DSF profile suggesting the weakening of the capsid. This
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50 492 led us to explore the impact of free chlorine followed by heat on MS2 phages. Indeed, we had
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52 493 previously shown that heat significantly impacts the structure of the capsid with disruption of MS2

phage particles at high temperatures (72°C) and transient expression of hydrophobic domains at lower temperatures (60°C) (Brié *et al.* 2016).

An **increased** effect on the inactivation of MS2 phage and on the permeability of its capsid to RNase was observed when the 60°C heat treatment was applied after chlorination. To our knowledge, the virucidal effect of the combination of free chlorine and heat on enteric viruses and phages has not been studied before. Indeed, chlorine-induced inactivation of enteric viruses and phages in suspension in water has only been studied for temperatures below 30°C. The combined effect of temperature and chlorination in foodstuffs has been more closely studied on bacteria, showing an increase in inactivation compared to chlorine applied alone (Klaiber *et al.* 2005; Choi *et al.* 2016). Moreover, these authors have shown that warm water (50°C) or dry heat (80°C) and chlorine can be combined without altering the organoleptic quality or the viability of vegetables or seeds.

Since hydrophobicity is known to be the main surface property affected by exposure of MS2 phages to heat (60°C) (Brié *et al.* 2016), a significant change in this parameter was expected when free chlorine was followed by heat. As previously described by Brié *et al.* (2016) for MS2 phages exposed to the 60°C heat treatment alone, the initial fluorescence of the DSF profile increased after exposure to free chlorine followed by heat. The highest initial fluorescence value was observed using free chlorine followed by a 60°C heat treatment. The increase in the initial fluorescence showed that chlorine followed by heat led to the expression of hydrophobic domains and/or increased the permeability of the capsid allowing the probe to reach inside hydrophobic regions. The adhesion test to hydrophobic beads showed an increase in the expression of hydrophobic domains at the surface of MS2 phages exposed to free chlorine followed by 60°C heat treatment. Remarkably, this increase in the adhesion capacity to hydrophobic beads appeared to be specific to viral particles remaining infectious as previously observed for MS2 phage exposed to heat alone (Brié *et al.* 2016). After exposure to heat and free chlorine, infectious MS2 phages accounted for a

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3 519 small part of the total MS2 phage particles. Thus, our results showed that the permeability of the
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5 520 capsid to the probe increased for both infectious and inactivated phages, whereas the increase in the
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7 521 expression of hydrophobic domains at the surface was specific to phages remaining infectious after
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9 522 treatment.

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11 523 Overall, our results showed that free chlorine weakens the capsid of MS2 phage and alters the
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13 524 functions of the MP at least for the highest concentration tested. The combination of heat and
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15 525 chlorine has not been evaluated before for enteric phages or pathogenic viruses, but it has been
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17 526 previously reported to be interesting for bacteria in foodstuffs. We observed that free chlorine
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19 527 followed by heat increased the inactivation of MS2 phage and the structural modifications of the
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21 528 capsid by comparison with these treatments applied separately. Our results suggested that the
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23 529 oxidant should be applied before heat to have an optimal effect. Concerning the specific detection
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25 530 of infectious viruses, the increase in permeability to enzymes such as RNases for inactivated phages
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27 531 could offer new opportunities for such methods. In our opinion, the most important result
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29 532 concerning free chlorine followed by heat is the specific increase in the expression of hydrophobic
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31 533 domains at the surface of infectious MS2 phages. Indeed, this result suggested that hydrophobic
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33 534 surfaces could be used to capture specifically infectious viruses before their detection using
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35 535 molecular methods. Finally, the modifications observed on the capsid with our treatments underline
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37 536 the need for further studies focusing on the structural modifications that occur during the viral life
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39 537 cycle and also during inactivation treatments in order to better understand the mechanisms of virus
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41 538 inactivation resulting from capsid damage.
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For Peer Review

The impact of chlorine and heat on the infectivity and physicochemical properties of bacteriophage MS2

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One-sentence summary: Exposure of MS2 phage to free chlorine followed by heat leads to higher inactivation, permeability to RNases, and availability of hydrophobic domains within the capsid than does chlorine applied alone.

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ABSTRACT

Enteric viruses and bacteriophages are exposed to various inactivating factors outside their host, and among them chlorine and heat are the most commonly used sanitizer in water industry and treatment in the food industry, respectively. Using MS2 phages as models for enteric viruses, we investigated the impact of free chlorine and heat on their physicochemical properties. Free chlorine was first evaluated alone. No increase in either capsid permeability or hydrophobicity was observed. The negative surface charge slightly increased suggesting molecular changes in the capsid. However, a weakening of the capsid by chlorine was suggested by differential scanning fluorimetry. This phenomenon was confirmed when chlorination was followed by a heat treatment. Indeed, an increase in the inactivation of MS2 phages and the permeability of their capsids to RNases was observed. More interestingly, an increase in the expression of hydrophobic domains at the phage surface was observed, but only for phages remaining infectious. The chlorine-caused weakening of the capsid suggested that, for an optimal use, the oxidant should be followed by heat. The increased permeability to RNases and the expression of hydrophobic domains may contribute to the development or improvement of molecular methods specific for infectious enteric viruses.

KEYWORDS: free chlorine, heat, MS2 bacteriophage, surface properties, hydrophobicity, charge

INTRODUCTION

Enteric viruses and phages are exposed to various inactivating factors in water, in foodstuffs, and on surfaces. Among them, heat and chlorine are one of the most cost-effective and easy-to-apply treatments and sanitizers in the food and water industries. The level of inactivation of enteric viruses by heat has been widely monitored in food and environmental matrices in which it is considered as one of the main inactivating factors (Hirneisen *et al.* 2010; Bertrand *et al.* 2012). Chlorine is a major disinfection treatment of water and food for its broad-spectrum antimicrobial activity (Hirneisen *et al.* 2010; Boudaud *et al.* 2012; Girard *et al.* 2016; Meireles *et al.* 2016). Although virus inactivation by heat or chlorine has been evaluated in numerous studies, the mechanisms leading to non-infectious virus particles still remain partially understood. Comparison of the impact of different inactivating treatments on virus functions (i.e. host cell binding, genome injection and replication), using MS2 phage as model of pathogenic enteric viruses, has revealed differences in the mechanisms of inactivation between physical treatments such as heat and chemical oxidants such as free chlorine, but also within treatment groups (Wigginton *et al.* 2012).

Heat induces structural and conformational changes of the capsid proteins leading to at least inactivation through alteration of the binding capacity to host cells, modification of surface properties, and increase in permeability to enzymes and intercalating dyes. These findings have been reported for poliovirus, hepatitis A virus, feline calicivirus, and MS2 phage (Breindl 1971; Nuanualsuwan and Cliver 2003; Pecson, Martin and Kohn 2009; Wigginton *et al.* 2012; Brié *et al.* 2016). Heat has been shown to have, if any, only a very low impact on the virus genome (Wigginton *et al.* 2012). Hydrophobicity seems to be the surface property the most affected by heat treatment. Indeed, the expression of hydrophobic domains has been reported for MS2 phages exposed to heat but for remaining infectious particles only (Brié *et al.* 2016). An increase in hydrophobicity has also been observed for poliovirus exposed to 50°C, which has been linked to the externalization of the N-terminal region of viral protein VP1 (Fricks and Hogle 1990; Curry *et al.*

1996). Heat-induced modification of the electrostatic charge is less obvious. Indeed, a decrease in the isoelectric point (IEP) has been observed for poliovirus heated at 56°C and linked to the loss of viral RNA genome and capsid protein VP4 (O'Brien and Newman 1979; Breindl 1971). Only a slight decrease in the global negative charge has been reported for MS2 phages after exposure to 60°C (Bri  *et al.* 2016). The structural changes in the capsid proteins caused by heat seem to alter the structure specifically involved in host cell binding, and to be linked to modification of hydrophobic domain expression.

Free chlorine has been shown to alter both genome- and protein-mediated functions of MS2 phage, more specifically genome injection and replication, but not its binding capacity to host cells (Wigginton *et al.* 2012). Free chlorine has also been shown to affect proteins of pathogenic enteric viruses such as picornaviruses (Nuanualsuwan and Cliver 2003) and adenovirus (Page, Shisler and Mari as 2010). Moreover, the loss of genome replication has been shown to coincide with extensive genome damage (Wigginton *et al.* 2012). The reaction of free chlorine with amino acids constituting the capsid proteins leads to the formation of numerous products such as carbonyl groups resulting from the oxidization of lysine, arginine, threonine, and proline. A relationship between the number of carbonyl groups and the rate of free chlorine-induced inactivation has been reported for enteric viruses belonging to *Astroviridae*, *Reoviridae*, *Caliciviridae*, and *Picornaviridae* (Sano *et al.* 2010; Tojo *et al.* 2013; Sano *et al.* 2015). The oxidization of amino acids by chlorine could also result in modifications of the electrostatic charge, which, together with hydrophobicity, is the major component of the surface properties of virus particles. To our knowledge, the electrostatic charge has not previously been explored for enteric viruses exposed to free chlorine. Concerning the hydrophobicity of chlorine-exposed viruses, a recent study has shown that exposure to sodium hypochlorite modified the profile of the hydrophobic probe SYPRO Orange in Q  phage belonging to F-specific RNA phage genogroup III. Indeed, an increase in hydrophobicity was observed at the beginning of the experiment; this meant that sodium

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hypochlorite revealed some hydrophobic parts of the capsid or increased the permeability of the capsid allowing the probe to reach inside hydrophobic regions (Loison *et al.* 2016).

The study of the impact of heat and free chlorine on enteric viruses requires methods suited for the specific detection of infectious viral particles, which are the only ones that pose a risk to human health. Indeed, studies performing a simultaneous and quantitative comparison between infectivity loss and genome degradation have showed a higher persistence of viral genome after heat treatment (Bertrand *et al.* 2012) and chlorination (Pelleieux *et al.* 2016). Thus, the detection of viral genomes by molecular methods used alone does not provide information about infectivity (Girones *et al.* 2010). Human noroviruses (NoV) and hepatitis A virus (HAV) are considered as the main pathogenic enteric viruses. However, the detection of their infectious particles remains challenging although cell culture methods have been very recently proposed for noroviruses (Jones *et al.* 2015; Ettayebi *et al.* 2016). Various methods which could be combined with molecular approaches have been evaluated in order to favor the detection of only infectious viruses. Most of them are based on the increased permeability of the capsid to enzymes or intercalating dyes such as RNases, ethidium monoazide (EMA), or propidium monoazide (PMA) (Prevost *et al.* 2016; Randazzo *et al.* 2016), or on the specific detection of oxidative damage (Tojo *et al.* 2013; Sano *et al.* 2015). However, their optimal conditions of use such as the type of treatment (i.e. physical and/or chemical) and the matrices (i.e. food and/or environmental matrices) still need to be defined.

F-specific RNA phages (FRNAPH) belong to the family *Leviviridae* and are classified in two genera, *Levivirus* and *Allovivirus*, each divided into two genogroups. These phages exhibit numerous similarities with NoV and HAV in terms of size (20 - 30 nm), genome (positive-sense single-stranded RNA), and overall capsid structure (Valegård *et al.* 1990; King *et al.* 2012). These phages infect bacteria with F pili, such as *Escherichia coli*, in the gut of mammals and are released in environments exposed to fecal contamination similarly to enteric viruses. Their physicochemical and biological characteristics and the easy-to-apply methods available for the quantification of their

infectious particles and genome have led to their use as models to assess pathogenic enteric virus behavior in water (Boudaud *et al.* 2012), in food (Deboosere *et al.* 2012; Bozkurt *et al.* 2015), and on surfaces (Morin *et al.* 2015; Pelleieux *et al.* 2016) during treatment. Due to their structural similarity with the main waterborne pathogenic viruses they have also been proposed as relevant indicators of viral contamination in water (Cole, Long and Sobsey 2003; Ogorzaly *et al.* 2009; Vergara *et al.* 2015) and shellfish (Hartard *et al.* 2017) using molecular and/or cell culture methods. MS2 phage belonging to *Levivirus* genogroup I is the most studied of these FRNAPH, particularly its physicochemical characteristics (Valegård *et al.* 1990; Langlet *et al.* 2008a; Dika *et al.* 2013a) and the mechanisms of its inactivation (Pecson, Martin and Kohn 2009; Wigginton *et al.* 2012; Sigstam *et al.* 2013). Thus, MS2 seems to be a relevant model for pathogenic enteric viruses, especially for studying the mechanisms of inactivation by different factors, and it can be produced in high concentrations, an essential prerequisite for some experimental methods focusing on physicochemical characteristics such as electron microscopy.

Thus, it appears that even treatments commonly used on enteric viruses such as free chlorine and heat need to be improved. Molecular-based methods allowing a more specific detection of infectious enteric viruses after such treatments are also needed. Such improvements require a better knowledge of the damage that occurs in enteric viruses during loss of infectivity. In such a context, the present work aimed to optimize the conditions of use of free chlorine and heat for the inactivation of enteric viruses and to evaluate their impact on the surface properties of MS2. The impact of free chlorine used alone was first evaluated on MS2 phages because of the poor knowledge about its effects on the surface properties of enteric viruses. After verification of the preservation of the capsid of inactivated phages, we examined in greater detail the thermal stability and the surface properties of MS2 phages exposed to chlorine. The weakening of the capsid observed in the present work when free chlorine was applied alone and the increase in hydrophobicity previously described for MS2 phages exposed to heat (Brié *et al.* 2016) led us to

perform further experiments focusing on hydrophobicity, in which heat treatment was applied to MS2 phages previously exposed to chlorine. Since both free chlorine and heat have been shown to act on the capsid, it might therefore be expected that their combination would increase their impact.

MATERIALS AND METHODS

Production and purification of MS2 phage

Replication of MS2 phage (ATCC, 15597-B1) was done according to the standard procedure (ISO 10705-1 1995) (without the chloroform step) using *Escherichia coli* Hfr K12 (ATCC, 23631) as host cells.

The phages were then concentrated and purified as previously described (Brié *et al.* 2016). Briefly, the phages were centrifuged twice (8,000 g, 20 min, 4°C) and the supernatant (≈ 1 L) was filtered using a 0.22 µm filter unit (Stericup-GP Millipore Express Plus, 0.22 µm; Merck Millipore, Merck KGaA, Darmstadt, Germany). After an ultracentrifugation step (Thermo Scientific A-621 fixed rotor, 42,500 g, 18 h, 4°C; Thermo Fisher Scientific, Waltham, MA, USA), the pellets containing the concentrated phages were resuspended in iodixanol solution (20% final, Optiprep; Axis-Shield, Dundee, Scotland) and laid on a 40% iodixanol layer in a tube (Cone-Top PA tube 14 × 47 mm, Thermo Fisher Scientific). The density gradient was formed upon centrifugation at 160,000 g for 7 h at 15°C (Thermo Scientific TH-641 swing rotor). After centrifugation, the MS2 phages were gently removed with a syringe and dialyzed twice (one overnight, the other for 7 h) against 10 L of PBS solution with a concentration of 1 mM (1 mM Na₂HPO₄ and 14 mM NaCl), pH 7.4, using a Float-A-Lyzer G2 dialysis device (MW100 kD; Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA). These working suspensions were kept at 4°C before use. Three working suspensions were produced for this study in order to perform the experiments in triplicate on independent suspensions.

Free chlorine and heat treatments

Three independent suspensions of purified phages at a concentration of 10^{14} PFU·mL⁻¹ (250 µL in 1 mM PBS solution) (PFU: plaque-forming units) were exposed either to free chlorine alone, to heat alone or to free chlorine followed by heat. To prepare our free chlorine stock solution, concentrated sodium hypochlorite solution (5%, Acros Organics, Thermo Fisher Scientific) was diluted using ultrapure water ($> 18 \text{ M}\Omega\cdot\text{cm}^{-1}$). Determination of free chlorine concentration was performed according to the DPD (*N,N*-diethyl-*p*-phenylenediamine, Acros Organics) colorimetric method (ISO 7393-2 1985). For exposure to free chlorine alone, the working concentrations of free chlorine were 100 and 200 mg.L⁻¹ with a contact time of 10 min at 4°C. Residual free chlorine was immediately quenched by adding two moles of sodium thiosulfate equivalent to one mole of free chlorine. The heat treatments were performed at 55°C or 60°C for 10 min in pre-warmed Protein LoBind tubes (Eppendorf, Hamburg, Germany) in a water bath. Samples were immediately placed on ice after heat treatment to avoid further inactivation. When free chlorine was followed by heat, the phages were exposed to 100 mg.L⁻¹ of free chlorine for 10 min at 4°C and then to heat at 55°C or 60°C for 10 min. For each replicate, a part of the MS2 phage suspension was not exposed to free chlorine, heat or to free chlorine followed by heat and was named control phages. Thus, there were nine ($n=3\times 3$) control populations in this study.

Enumeration of infectious MS2 phages

Infectious MS2 phages were counted after treatment (*I*) and without treatment (*I*₀) by the plaque assay method using the double-agar-layer method (ISO 10705-1 1995) with *E. coli* K-12 Hfr as host cells. Three to five decimal dilutions of the MS2 phage suspensions were dropped in duplicate onto agar plates in order to have a dilution resulting in the enumeration of 10 to 100 plaques per Petri dish. Infectious concentration was expressed in PFU·mL⁻¹. The final concentration of purified MS2 phages was $\approx 10^{14}$ PFU·mL⁻¹. The loss in infectivity due to each treatment was estimated by $\log \frac{I}{I_0}$.

Viral genome extraction and quantification

The Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) method was used for the detection of potential degradation of the viral genome by free chlorine and/or heat. Thus, the genome of MS2 phage was quantified after each treatment (C) and without treatment (C₀). The genome degradation by the treatment was estimated by $\log \frac{C}{C_0}$. Prior to the RT-qPCR assays, extractions of viral RNA (50 µL) were performed using the QIAamp viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The extracts (100 µL) were immediately stored at -80°C before quantification. Quantification of the phage genome was performed by specific a RT-qPCR assay targeting a genome fragment of ≈ 150 nucleotides as previously described (Ogorzaly and Gantzer 2006). Two negative controls and one positive control (i.e. known quantity of MS2 genome) were added to each RT-qPCR analysis. RT-qPCR efficiency ranged from 80% to 95%. Determination of MS2 genome copies per mL (gc·mL⁻¹) used for the standard curve was performed as previously described (Bri  *et al.* 2016). The final concentration of purified MS2 phages was ≈ 10¹⁵ gc·mL⁻¹.

Transmission electron microscopy

Transmission electron microscopy (TEM) was used to visualize virus disruption or major structural modifications. The samples were adsorbed onto grids and negatively stained for 1 min with 2% phosphotungstic acid at pH 7.4 previously filtered with a 0.22 µm membrane filter. The examination was carried out using a CM200 transmission electron microscope (Philips, Andover, MA, USA) operating at 200 kV accelerating voltage and with a ×38,000 magnification.

Evaluation of capsid integrity using an RNase assay

An RNase assay was used to evaluate if the capsid had maintained its integrity and was still able to protect the virus genome (Nuanualsuwan and Cliver 2003). Suspensions of MS2 phage, with a final concentration of 10^7 gc·mL⁻¹, were treated with RNase A at a final concentration of 1 µg·mL⁻¹ (AppliChem GmbH, Darmstadt, Germany) at 37°C for 60 min as previously described (Brié *et al.* 2016).

Binding of MS2 phages to *E. coli* host cells

The impact of free chlorine on the binding capacity of MS2 phage to its host cell *E. coli* Hfr K12 was evaluated as previously described (Wigginton *et al.* 2012; Brié *et al.* 2016). The binding capacity of MS2 phage to *E. coli* was estimated by $\log \frac{Q}{Q_0}$ where Q is the viral RNA found on the bacteria after free chlorine treatment and Q_0 without treatment.

Size and electrophoretic mobility measurements

Size and electrophoretic mobility (µ) measurements were performed in order to evaluate the impact of free chlorine on the overall structure and charge density of MS2 phage, respectively. The experiments were achieved in disposable capillary cells (Malvern Instruments, Worcestershire, UK) at 23°C ± 0.1°C and pH 7.0 using a Zetasizer Nano ZS instrument (Malvern Instruments, He-Ne red laser, wavelength 633 nm) driven by Dispersion Technology Software (Malvern Instruments) as previously described (Langlet *et al.* 2008a). The slope of the electrophoretic mobility as a function of the logarithm of ionic strength (1.95, 19.5 and 195 mM) reflects the ratio between the density of electric charges located within the particle and that of charges distributed at the outer virus periphery. A decrease in the slope value shows a decrease in the charge density of the virus (Langlet *et al.* 2008a). The experimental conditions were identical to those previously described (Brié *et al.* 2016). For each condition, measurements were obtained by means of three independent experiments with triplicate measures in each to ensure repeatability of the measurements.

Binding of MS2 phages to hydrophobic or positively charged beads

These assays were performed to study the variation in hydrophobicity or negative charge at the surface of MS2 phages. Magnetic beads were used in these experiments: hydrophobic beads were coated with polystyrene (PolySciences Europe GmbH, Hirschberg, Germany) and positively charged beads were BcMag PSA (Bioclone, San Diego, CA, USA). As recommended by the manufacturers, the beads were first washed twice in the buffer to be used in the assay which was either 10 mM PBS (10 mM Na₂HPO₄ and 150 mM NaCl) at pH 7 for hydrophobic beads or 50 mM PBS (50 mM Na₂HPO₄ and 20 mM NaCl) at different pH values (2.5, 3.0, 4.0, and 7.0) for ion exchange beads. The positively charged beads exhibited a pKa of ≈ 10 .

The binding assays were carried out as previously described (Brié *et al.* 2016). The binding capacity of MS2 phage to beads was estimated by $\log \frac{C_x}{C_0}$ where C_x is the concentration of either infectious phages or viral RNA after the binding assay in suspension and C_0 the initial concentration of either infectious phages or viral RNA in suspension.

Differential scanning fluorimetry

Differential scanning fluorimetry (DSF) was performed using the hydrophobic probe SYPRO Orange. This probe has a high affinity for hydrophobic domains (Niesen, Berglund and Vedadi 2007), which are unmasked when proteins unfold. DSF was used to compare the thermal stability of MS2 phage, as measured by the denaturation temperature (T_m). Thermal melting curves of MS2 phage were obtained in 96-well plates using StepOne Plus qPCR System (Life Technologies, Thermo Fisher Scientific) and the fluorescent dye SYPRO Orange, 5,000 \times concentrate, supplied in DMSO (Invitrogen, Thermo Fisher Scientific). Reactions were performed in a 25 μ L final volume containing 5 μ L of buffer (150 mM Tris-HCl, pH 8), 2 μ L of SYPRO Orange solution 62.5 \times (final concentration = 5 \times), 16 μ L of ultrapure water ($> 18 \text{ M}\Omega\cdot\text{cm}^{-1}$), and 2 μ L of MS2 suspension from

stock or after treatment (5.10^{14} - 10^{15} gc·mL⁻¹). The temperature was increased for all replicates from 20°C to 95°C on a 1% stepwise gradient corresponding to $\approx 1^{\circ}\text{C}\cdot\text{min}^{-1}$ with fluorescence measurements. The sum of the fluorescence intensities obtained with all the filters was plotted against temperature (Excel software) to obtain the maximum fluorescence intensity of SYPRO Orange. The T_m was determined by plotting the first derivative of the previous curve; it appeared then as a peak (Loison *et al.* 2016). This method is also known to allow the expression of hydrophobic parts of the capsid and/or the permeability of the capsid to be investigated by the fluorescence intensity of SYPRO Orange at the beginning of the DSF experiment (Loison *et al.* 2016).

Data analysis

The analysis was performed using the values obtained for the three independent suspensions of phages. Since the experimental values were not normally distributed, non-parametric tests were performed using StatEL 2.7 (Ad Science). The explanatory variables were the exposure to free chlorine alone, heat alone, and free chlorine followed by heat. For free chlorine alone, Mann-Whitney tests were performed to evaluate its effect on the infectivity (i.e. loss of infectivity, binding capacity to host bacteria), structure (i.e. genome degradation, capsid permeability to RNase, thermal stability), and hydrophobicity (i.e. initial adhesion of hydrophobic probe SYPRO Orange, binding to hydrophobic probes), and the adhesion to positively charged beads by comparison between control and chlorinated MS2 phages. A Kruskal-Wallis test was performed to compare the electrophoretic mobility between control and chlorinated MS2 phages. The effect of free chlorine followed by heat on the loss of infectivity, structure (i.e. genome degradation, capsid permeability to RNase) and hydrophobicity (i.e. initial adhesion of hydrophobic probe SYPRO Orange, binding to hydrophobic beads) was evaluated by comparison with free chlorine alone and heat alone using Kruskal-Wallis tests. For all these tests, a p value of <0.01 was considered as significant.

295
296 **RESULTS**

297 **Impact of free chlorine applied alone or followed by heat on the infectivity and structure of**
298 **MS2 phage**

299 The effects of free chlorine applied alone on MS2 phage were evaluated for 100 and 200 mg.L⁻¹
300 (Table 1). Infectivity decreased by $-1.2 \pm 0.3 \log_{10}$ for 100 mg.L⁻¹ ($p < 0.01$, Mann-Whitney test)
301 and by $-3.5 \pm 0.9 \log_{10}$ for 200 mg.L⁻¹ ($p < 0.01$, Mann-Whitney test). The binding capacity of MS2
302 phage to *E. coli* K12 Hfr was not altered after exposure to 100 mg.L⁻¹ ($-0.1 \pm 0.1 \log_{10}$) ($p > 0.01$,
303 Mann-Whitney test), whereas it decreased by $-0.8 \pm 0.1 \log_{10}$ after exposure to 200 mg.L⁻¹ ($p < 0.01$,
304 Mann-Whitney test). The free chlorine treatment at 100 mg.L⁻¹ did not affect the detection of the
305 genome fragment of ≈ 150 nucleotides targeted with our RT-qPCR assay since the RT-qPCR signal
306 was not modified ($p > 0.01$, Mann-Whitney test). However, the genome detection decreased by $-$
307 $0.5 \pm 0.2 \log_{10}$ for 200 mg.L⁻¹ ($p < 0.01$, Mann-Whitney test). No modification of the shape of MS2
308 phage was observed by TEM for these two concentrations of free chlorine (Fig. 1). The mean size
309 of chlorinated MS2 phage was 20 ± 2 nm for 100 mg.L⁻¹ ($n=3 \times 3$) and 21 ± 1 nm for 200 mg.L⁻¹
310 ($n=3 \times 3$) which was similar to control phages ($p > 0.01$, Mann-Whitney test). Moreover, the limited
311 effect of RNase treatment on RT-qPCR signal which decreased by less than $-0.3 \pm 0.1 \log_{10}$ with
312 the two concentrations of free chlorine ($p > 0.01$, Mann-Whitney test) confirmed that the integrity of
313 the capsid was preserved under our experimental conditions. DSF was then used to explore
314 conformational modifications of the capsid that could not be revealed by TEM and to compare the
315 T_m (i.e. denaturation temperature) value of MS2 phages before and after exposure to free chlorine.
316 We observed only one T_m peak of $66.0 \pm 0.1^\circ\text{C}$ ($n=3$) before exposure to chlorine (Fig. 2). After
317 exposure to free chlorine for 10 min, a decrease in the T_m to $62.8 \pm 0.1^\circ\text{C}$ ($n=3$) for 100 mg.L⁻¹ (p
318 < 0.01 , Mann-Whitney test) and $60.9 \pm 0.7^\circ\text{C}$ ($n=3$) for 200 mg.L⁻¹ ($p < 0.01$, Mann-Whitney test)
319 was noticed. This reduction in the denaturation temperature suggested a weakening of the capsid of

MS2 phage by the oxidant. Although there was a decrease in the thermal stability of the capsid we did not find any changes in shape, size, and binding capacity, nor did the permeability to RNase increase thus suggesting that the capsid of MS2 phage maintained its integrity after exposure to free chlorine alone.

The decrease in the thermal stability after exposure to chlorine led us to expose MS2 phage to chlorine (100 mg.L⁻¹) and then to heat (55°C or 60°C). The infectivity decreased by $-1.9 \pm 0.3 \log_{10}$ for chlorine followed by heat treatment at 55°C and by $-3.2 \pm 0.6 \log_{10}$ for chlorine followed by heat treatment at 60°C. For each treatment applied separately, the loss in infectivity reached $-1.2 \pm 0.3 \log_{10}$ after exposure to free chlorine (Table 1), $-0.7 \pm 0.1 \log_{10}$ after heat treatment at 55°C (data not shown) and $-1.1 \pm 0.1 \log_{10}$ after heat treatment at 60°C (Brié *et al.* 2016). The highest loss in infectivity was obtained when free chlorine was followed by heat treatment at 60°C ($p < 0.01$, Kruskal-Wallis test). This loss in infectivity was also higher than the sum of the decrease values obtained with free chlorine and heat treatment at 60°C applied separately ($p < 0.01$, Kruskal-Wallis test). This suggested that free chlorine improved the inactivation of MS2 phage by heat treatment at 60°C. The RT-qPCR signal decreased only by $-0.1 \pm 0.4 \log_{10}$ for chlorine followed by heat treatment at 55°C and by $-0.3 \pm 0.4 \log_{10}$ for chlorine followed by heat treatment at 60°C. The concentrations in genome observed after these treatments were similar to those observed for MS2 phage exposed to 100 mg.L⁻¹ free chlorine alone and also for control MS2 phage ($p > 0.01$, Kruskal-Wallis test). Thus, the detection of the MS2 genome was not affected when free chlorine was followed by heat. As observed for free chlorine alone, no modification of the shape of MS2 phage was noted by TEM after exposure to free chlorine followed by heat (Fig. 1). The RNase treatment applied after exposure to free chlorine (100 mg.L⁻¹) followed by heat decreased the RT-qPCR signal by $-1.1 \pm 0.2 \log_{10}$ for 55°C and $-1.7 \pm 0.4 \log_{10}$ for 60°C. The log removal of genome copies by RNase treatment reached $-0.3 \pm 0.1 \log_{10}$ (Table 1) for free chlorine alone, $-0.1 \pm 0.1 \log_{10}$ (data not shown) for 55°C heat treatment alone and $-0.8 \pm 0.2 \log_{10}$ (Brié *et al.* 2016) for 60°C heat

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3 345 treatment alone. The reduction in genome after chlorine followed by heat (55°C and 60°C) was
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5 346 higher than the sum of the log removal obtained with free chlorine and heat applied separately (p
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7 347 <0.01 , Kruskal-Wallis test). Moreover, the highest log removal of genome copies by RNase
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9 348 treatment was observed for MS2 phage exposed to free chlorine followed by heat treatment at 60°C
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11 349 ($p <0.01$, Kruskal-Wallis test). Overall, the increased effect of free chlorine followed by heat
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13 350 treatment at 60°C on the loss in infectivity and on the capsid permeability to RNase was in
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15 351 agreement with the free chlorine-induced weakening of the capsid suggested by DSF experiments.
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20 353 **Impact of free chlorine on the charge of MS2 phage particles**

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22 354 The electrophoretic mobility (μ) was represented as a function of the logarithm of ionic strength
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24 355 (1.95, 19.5 and 195 mM) at pH 7.0 before and after exposure to free chlorine (100 and 200 mg.L⁻¹)
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26 356 in order to estimate the impact of free chlorine on the charge density of MS2 phage (Fig. 3A). A
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28 357 decrease in the electrophoretic mobility was observed after exposure to free chlorine at ionic
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30 358 strengths of 1.95 and 19.5 mM ($p <0.01$, Kruskal-Wallis test). On the other hand, no modification
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32 359 of the electrophoretic mobility was observed after exposure to chlorine at an ionic strength of 195
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34 360 mM ($p >0.01$, Kruskal-Wallis test). Therefore, a lower slope value was observed for chlorinated
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36 361 phages than for control phages suggesting a decrease in the negative charge density of MS2 phage
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38 362 exposed to free chlorine. The negative surface charge of total particles (i.e. RT-qPCR results) was
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40 363 then estimated by binding to positively charged beads at different pH (i.e. 7.0, 4.0, 3.0, and 2.5) for
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42 364 control and chlorinated phages (Fig. 3B). The lowest adhesion capacity of both control and
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44 365 chlorinated phages on positively charged beads was obtained at pH 2.5 and 3.0 which are below the
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46 366 IEP of MS2 phage. Conversely, the highest adhesion capacity was obtained at pH above the IEP
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48 367 (i.e. 4.0 and 7.0). Thus, the IEP of MS2 phage was shown to be between 3 and 4. Interestingly, an
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50 368 increase in the adhesion capacity to positively charged beads was observed for MS2 phages exposed
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52 369 to the two concentrations of free chlorine in comparison with control phages ($p <0.01$, Mann-
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Whitney test). Thus, the increase in the negative charge density of MS2 phage after exposure to free chlorine seemed to be mainly linked to an increase in the external negative charge.

Impact of free chlorine applied alone or followed by heat on the hydrophobicity of MS2 phage particles

The effect of free chlorine on the hydrophobicity of MS2 phage was then evaluated by DSF and by its adhesion capacity to hydrophobic beads. The DSF profile (Fig. 2) showed the same fluorescence intensity of SYPRO Orange at the beginning of the experiment for control and chlorinated phages, meaning that free chlorine did not modify the overall expression of hydrophobic domains of the capsid proteins. The expression of hydrophobic domains on the surface of MS2 particles was compared between control and chlorinated phages using the adhesion ability to hydrophobic beads. The experiments were performed at pH 7.0 and high ionic strength (10 mM Na₂HPO₄ and 150 mM NaCl) in order to minimize the influence of the global charge. No adhesion to hydrophobic beads was observed for control phages and those exposed to chlorine (Fig. 4). Thus, the results obtained using these two methods suggested that free chlorine did not change the expression of hydrophobic domains of the MS2 capsid proteins or the permeability of the capsid to the probe.

Since we have previously described an increased hydrophobicity of MS2 phage after heat treatment (Brié *et al.* 2016), DSF experiments were carried out after exposure to free chlorine (100 mg.L⁻¹) followed by heat (55°C or 60°C) and were compared to 55°C and 60°C heat treatments applied alone (Fig. 5). Similar DSF profiles with low initial fluorescence were obtained for both control MS2 phages and those exposed to the 55°C heat treatment alone. The initial fluorescence increased for MS2 phages exposed to the 60°C heat treatment alone and for those exposed to free chlorine followed by heat treatment at 55°C and 60°C. The highest initial fluorescence value was observed using 100 mg.L⁻¹ free chlorine followed by 60°C heat treatment ($p < 0.01$, Kruskal-Wallis test). The decrease in the fluorescence of the DSF profiles observed between 20°C and 60°C could

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3 395 have been due to probe quenching by water (Cimmperman and Matulis 2011; Loison *et al.* 2016).
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5 396 The increase in the initial fluorescence showed that free chlorine followed by heat led to the
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7 397 expression of hydrophobic domains and/or increased the permeability of the capsid allowing the
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9 398 probe to reach inside hydrophobic regions. The adhesion level on hydrophobic beads increased after
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11 399 exposure to free chlorine followed by heat treatment at 55°C or 60°C (Fig. 4); thus showing an
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13 400 increase in hydrophobic domains on the surface of MS2 particles. The highest adhesion level on
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15 401 hydrophobic beads was observed after free chlorine followed by heat treatment at 60°C (-2.4 ± 0.5 ,
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17 402 Fig. 4) ($p < 0.01$, Kruskal-Wallis test). Remarkably, this increase in the adhesion capacity to
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19 403 hydrophobic beads appeared to be specific to viral particles remaining infectious.
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24 405 **DISCUSSION**

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27 406 The purpose of this study was to explore the impact of free chlorine alone or followed by heat on
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29 407 the loss of infectivity and the modification of the physicochemical properties of MS2 phage used as
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31 408 model of the main pathogenic enteric viruses. The impact of free chlorine on capsid proteins has
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33 409 been previously described in a few studies focusing on their altered functions. However,
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35 410 modification of the overall structure of viral particles may lead to modification of their surface
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37 411 properties which govern their behavior in the environment. Moreover, the study of surface
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39 412 properties may contribute to the understanding of the mechanisms involved in the inactivation
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41 413 process and may also help in the development of detection methods specific for infectious viruses.

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44 414 MS2 phage was first exposed to free chlorine alone. The loss of infectivity of MS2 phage
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46 415 observed in our experiments after exposure to 100 and 200 mg.L⁻¹ of free chlorine was similar to
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48 416 that reported by Loison *et al.* (2016) for a Q β phage suspension. Indeed, by applying sodium
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50 417 hypochlorite at 1 mM (i.e. 74.4 mg.L⁻¹) for 10 min at room temperature on a Q β phage suspension
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52 418 at an initial concentration of $\approx 10^{12}$ PFU·mL⁻¹, these authors observed a $-1.7 \log_{10}$ reduction in
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54 419 infectivity. In the present work, we observed that the binding to host bacteria was altered after
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exposure to 200 mg.L⁻¹ of free chlorine only. Thus, the loss of infectivity was not due to the alteration of the binding capacity after exposure to 100 mg.L⁻¹ of free chlorine and was slightly due to the alteration of this function after exposure to 200 mg.L⁻¹ of free chlorine. Our observation of a high concentration of free chlorine needed to observe an impact on binding to host cell was in agreement with a previous study published by Wigginton *et al.* (2012). Indeed, these authors applied a concentration of 2-3 mg.L⁻¹ of free chlorine to an MS2 phage suspension of 10¹¹ PFU.L⁻¹ and observed alteration of the genome injection and extensive degradation of the maturation protein (MP). This protein, which is present as a single copy in the capsid of MS2 phage, is clearly involved in the binding of MS2 phage to the F-pilus of host bacteria and probably in the injection of the genome into host cells. Dent *et al.* (2013) have proposed that the natural retraction of the F-pilus could pull the MP from its location in the attached capsid, leaving a pore through which the phage genome could exit into the cell. Thus, alteration of the MP by free chlorine may impact these two functions depending on its intensity. The sensitivity of the capsid to oxidants has also been observed for Q β phage with the loss of its infectivity due either to capsid destabilization or to the modification of capsid proteins especially the MP and the readthrough protein (Loison *et al.* 2016). Considering the capsid of FRNAPH, the MP appears as one of the targets of free chlorine. Thus, free chlorine weakens the capsid of MS2 phage and may alter the role of the MP in the genome injection into host cells even at low concentrations. High concentrations of free chlorine seem to further alter the MP with a reduced ability to bind to host cells.

In our experiments, the genome of MS2 phage was quantified using an RT-qPCR assay which targets a fragment of \approx 150 nucleotides and the RT-qPCR signal was found to be affected only after exposure to 200 mg.L⁻¹ of free chlorine. A few studies have shown that some regions of the viral genome could be more sensitive than others to damage caused by free chlorine with significant differences depending on the virus type (Li *et al.* 2002; Xue *et al.* 2013). Free chlorine was shown to induce genome damage on MS2 phage at levels comparable to singlet oxygen and UV. However,

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the loss of replication function played a lesser role in the overall inactivation than in the other two inactivation treatments (Wigginton *et al.* 2012). For these authors, this suggested that the virucidal effect due to genome damage was linked to the type of modification rather than its location.

No modification of the shape of MS2 phage was observed by TEM after exposure to free chlorine. The mean size of MS2 phage before and after chlorination was similar to that previously described (Valegård *et al.* 1990; Dika *et al.* 2013a). Q β phage particles exposed to chlorine under similar conditions were also demonstrated by TEM observations to maintain their shape (Loison *et al.* 2016). Disruption of MS2 phage occurring after exposure to 72°C for 10 min has previously been shown to be related to a significant increase in RNase sensitivity (Brié *et al.* 2016). In our case, the limited effect of RNase treatment on RT-qPCR signal confirmed that the integrity of the capsid was preserved after exposure to free chlorine under our experimental conditions. DSF was then used to explore conformational modifications of the capsid that could not be revealed by TEM. We observed a similar DSF profile to that obtained in our previous study (Brié *et al.* 2016) with only one T_m peak (66°C) before exposure to chlorine. The decrease in the T_m value after exposure to free chlorine corresponded to a reduction in the denaturation temperature and suggested a weakening of the capsid of MS2 phage by the oxidant by comparison to a previous study performed on poliovirus (Robinson, Jesudhasan and Pfeiffer 2014). The weakening of the capsid might be linked to the site-specific cleavage showed on the capsid protein of MS2 phage following exposure to chlorine (Wigginton *et al.* 2012).

The reactions of hypochlorous acid with amino acids result in a wide variety of products such as sulfinic/sulfonic acids, methionine sulfone, and *N*-chlorinated species (Pattison, Davies and Hawkins 2012) which may modify the electrostatic charge of viral particles. In our study, the charge density was evaluated by measuring the electrophoretic mobility as a function of ionic strength which suggested a decrease in the negative charge density of MS2 phage exposed to free chlorine. On a qualitative level, it meant that free chlorine could change the negative charge density

of MS2 phage particles by increasing their external negative charge and/or by decreasing their internal negative charge (Langlet *et al.* 2008a). The binding to positively and negatively charged beads at different pH showed that the IEP of MS2 phage was between 3 and 4, which was in full agreement with literature data in the field (Langlet *et al.* 2008b; Dika *et al.*, 2011). MS2 phage exposure to chlorine led to an increase in its adhesion capacity to positively charged beads at pH above the IEP. Thus, the increase in the negative charge density of MS2 phage observed after exposure to free chlorine resulted mainly from an increase in the external negative charge.

Hydrophobicity was the other surface property monitored after exposure to free chlorine. The lack of adhesion to hydrophobic beads before exposure to free chlorine was in agreement with previous studies performed with MS2 phage (Dika *et al.* 2013b; Brié *et al.* 2016). No adhesion to hydrophobic beads was also observed after exposure to free chlorine. The DSF profile showed the same fluorescence intensity of SYPRO Orange at the beginning of the experiment for control and chlorinated phages. Thus, the results obtained with these two methods suggest that few hydrophobic domains are naturally expressed on the surface of MS2 particles and that free chlorine does not change the expression of hydrophobic domains of the MS2 capsid proteins or the permeability of the capsid to the probe. An increase in hydrophobicity by chlorine has previously been described for Q β phage (Loison *et al.* 2016) and for specific proteins such as α 2 chaperone (Wyatt *et al.* 2014) and Bovine Serum Albumin (Ashrafi Kooshk *et al.* 2012). This suggests that the expression of hydrophobic domains induced by chlorine depends on the composition and environment of the protein.

Overall, the most significant effect we observed on MS2 phages exposed to free chlorine alone was the decrease in the T_m in the DSF profile suggesting the weakening of the capsid. This led us to explore the impact of free chlorine followed by heat on MS2 phages. Indeed, we had previously shown that heat significantly impacts the structure of the capsid with disruption of MS2

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phage particles at high temperatures (72°C) and transient expression of hydrophobic domains at lower temperatures (60°C) (Brié *et al.* 2016).

An increased effect on the inactivation of MS2 phage and on the permeability of its capsid to RNase was observed when the 60°C heat treatment was applied after chlorination. To our knowledge, the virucidal effect of the combination of free chlorine and heat on enteric viruses and phages has not been studied before. Indeed, chlorine-induced inactivation of enteric viruses and phages in suspension in water has only been studied for temperatures below 30°C. The combined effect of temperature and chlorination in foodstuffs has been more closely studied on bacteria, showing an increase in inactivation compared to chlorine applied alone (Klaiber *et al.* 2005; Choi *et al.* 2016). Moreover, these authors have shown that warm water (50°C) or dry heat (80°C) and chlorine can be combined without altering the organoleptic quality or the viability of vegetables or seeds.

Since hydrophobicity is known to be the main surface property affected by exposure of MS2 phages to heat (60°C) (Brié *et al.* 2016), a significant change in this parameter was expected when free chlorine was followed by heat. As previously described by Brié *et al.* (2016) for MS2 phages exposed to the 60°C heat treatment alone, the initial fluorescence of the DSF profile increased after exposure to free chlorine followed by heat. The highest initial fluorescence value was observed using free chlorine followed by a 60°C heat treatment. The increase in the initial fluorescence showed that chlorine followed by heat led to the expression of hydrophobic domains and/or increased the permeability of the capsid allowing the probe to reach inside hydrophobic regions. The adhesion test to hydrophobic beads showed an increase in the expression of hydrophobic domains at the surface of MS2 phages exposed to free chlorine followed by 60°C heat treatment. Remarkably, this increase in the adhesion capacity to hydrophobic beads appeared to be specific to viral particles remaining infectious as previously observed for MS2 phage exposed to heat alone (Brié *et al.* 2016). After exposure to heat and free chlorine, infectious MS2 phages accounted for a

small part of the total MS2 phage particles. Thus, our results showed that the permeability of the capsid to the probe increased for both infectious and inactivated phages, whereas the increase in the expression of hydrophobic domains at the surface was specific to phages remaining infectious after treatment.

Overall, our results showed that free chlorine weakens the capsid of MS2 phage and alters the functions of the MP at least for the highest concentration tested. The combination of heat and chlorine has not been evaluated before for enteric phages or pathogenic viruses, but it has been previously reported to be interesting for bacteria in foodstuffs. We observed that free chlorine followed by heat increased the inactivation of MS2 phage and the structural modifications of the capsid by comparison with these treatments applied separately. Our results suggested that the oxidant should be applied before heat to have an optimal effect. Concerning the specific detection of infectious viruses, the increase in permeability to enzymes such as RNases for inactivated phages could offer new opportunities for such methods. In our opinion, the most important result concerning free chlorine followed by heat is the specific increase in the expression of hydrophobic domains at the surface of infectious MS2 phages. Indeed, this result suggested that hydrophobic surfaces could be used to capture specifically infectious viruses before their detection using molecular methods. Finally, the modifications observed on the capsid with our treatments underline the need for further studies focusing on the structural modifications that occur during the viral life cycle and also during inactivation treatments in order to better understand the mechanisms of virus inactivation resulting from capsid damage.

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For Peer Review

FIGURE LEGENDS

Figure 1. TEM images of the effect of chlorine and heat on the particle state of MS2 phages.

(a) control phages; (b) free chlorine (100 mg.L⁻¹); (c) free chlorine (200 mg.L⁻¹); (d) free chlorine (100 mg.L⁻¹) followed by heat (55°C, 10 min); (e) free chlorine (100 mg.L⁻¹) followed by heat (60°C, 10 min). (bar, 50 nm).

Figure 2. SYPRO Orange signature of MS2 phages exposed to free chlorine. Effect of free chlorine treatment on the derivative fluorescent curve of SYPRO Orange. Blue is the control MS2 phages, red is the phages exposed to 100 mg.L⁻¹ for 10 min, and green is the phages exposed to 200 mg.L⁻¹ for 10 min. The curve represents one of three independent experiments. Peak value is indicated for each condition. Fluorescence intensity is given in arbitrary units (10⁶ × AU).

Figure 3. Electrostatic charge of control MS2 phages (before treatment) and those exposed to free chlorine (100 and 200 mg.L⁻¹ for 10 min). (a) Electrophoretic mobility (μ) as a function of the ionic strength (mM) at pH 7.4. (b) Binding assay to positively charged beads. Blue is the control MS2 phages, red is the phages exposed to 100 mg.L⁻¹ for 10 min, and green is the phages exposed to 200 mg.L⁻¹ for 10 min. The dotted lines are only guides to the eye. Three independent experiments were performed, each time with three measurements for electrophoretic mobility only. Error bars indicate standard deviations. For adhesion experiments on positively charged beads: C_x, concentration after binding assay; C₀, initial concentration.

Figure 4. Binding to hydrophobic, magnetic beads at pH 7 in 10 mM PBS (10 mM Na₂HPO₄ and 150 mM NaCl) for control MS2 phages (before treatment) and those exposed to free chlorine alone (100 and 200 mg.L⁻¹ for 10 min) or to free chlorine (100 mg.L⁻¹ for 10 min) followed by heat (55°C or 60°C for 10 min). Three independent experiments were performed. Error bars indicate standard deviations. C_X, concentration after binding assay; C₀, initial concentration.

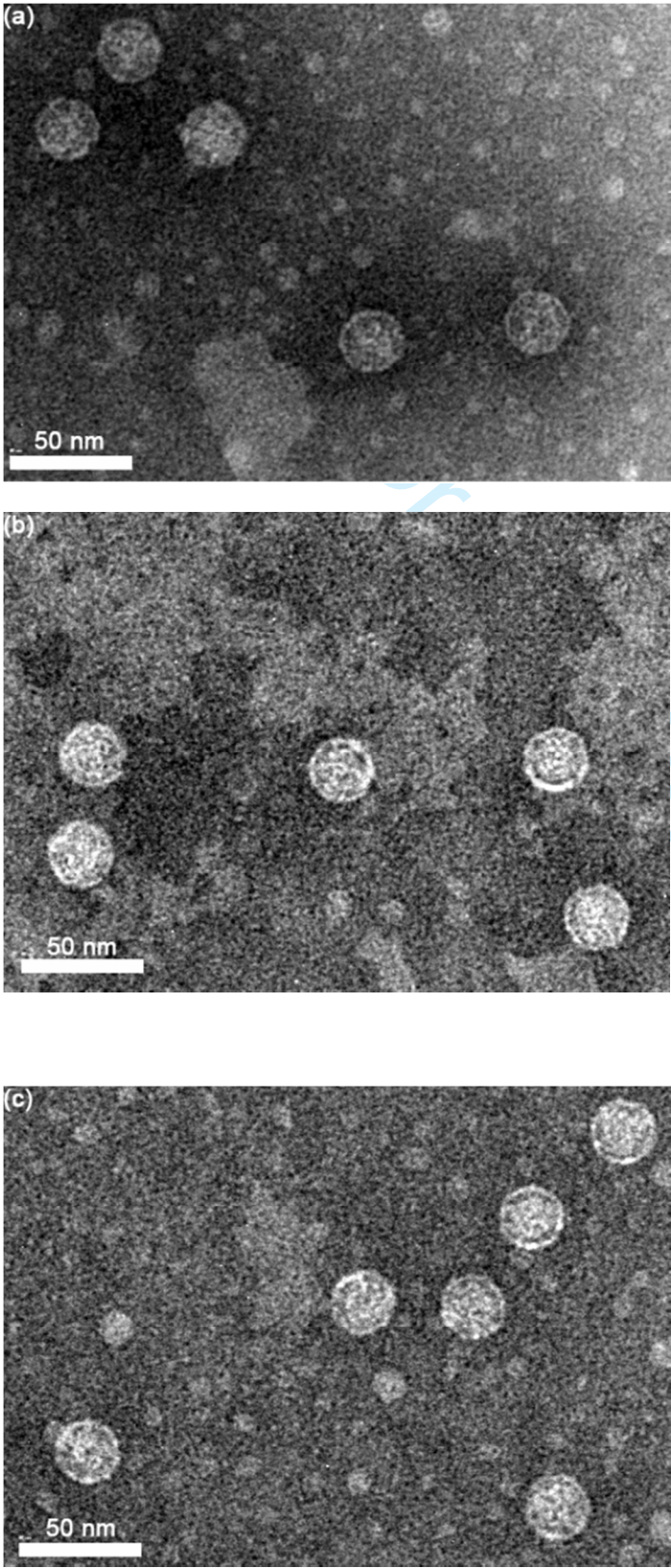
Figure 5. SYPRO Orange signature of control MS2 phages (before treatment) and those exposed to heat alone (55°C and 60°C for 10 min) or to free chlorine (100 mg.L⁻¹ for 10 min) followed by heat (55°C or 60°C for 10 min). The curve represents one of three independent experiments. Fluorescence intensity is given in arbitrary units (10⁶ × AU).

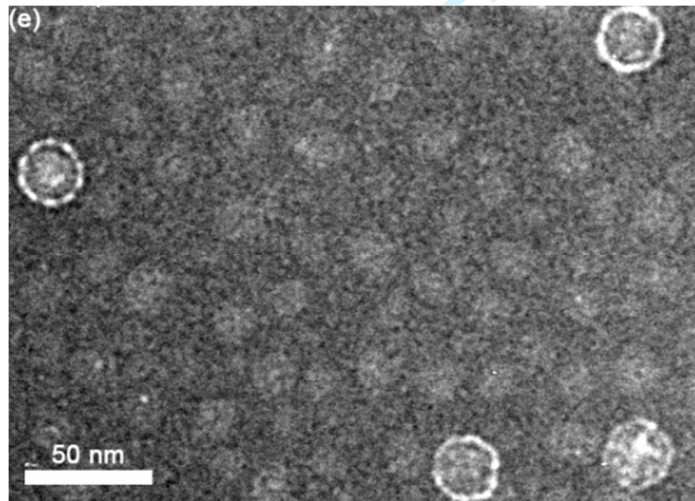
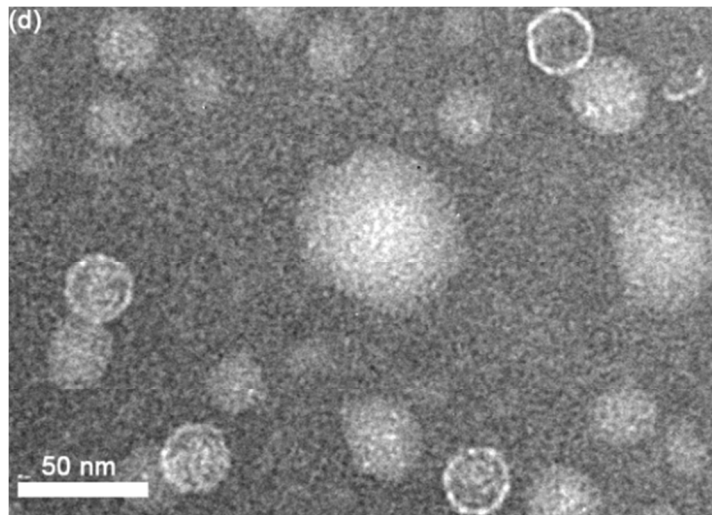
Table 1: Effect of chlorine (100 or 200 mg.L⁻¹ for 10 min at 4°C) followed or not by heat (55°C or 60°C for 10 min) on MS2 phage infectivity, genome degradation, and capsid permeability (sensitivity to RNase treatment). Experiments were performed in triplicate. SD = Standard Deviation.

Free chlorine treatment (10 min)	100 mg.L ⁻¹	200 mg.L ⁻¹	100 mg.L ⁻¹	100 mg.L ⁻¹
Heat treatment	None	None	55°C, 10 min	60°C, 10 min
Loss of infectivity (log ₁₀ ± SD)	-1.2 ± 0.3	-3.5 ± 0.9	-1.9 ± 0.3	-3.2 ± 0.6
Loss of genome (log ₁₀ ± SD)	0.0 ± 0.0	-0.5 ± 0.2	-0.1 ± 0.4	-0.3 ± 0.4
Loss of genome with RNase treatment (log ₁₀ ± SD)	-0.3 ± 0.1	-0.1 ± 0.1	-1.1 ± 0.2	-1.7 ± 0.4

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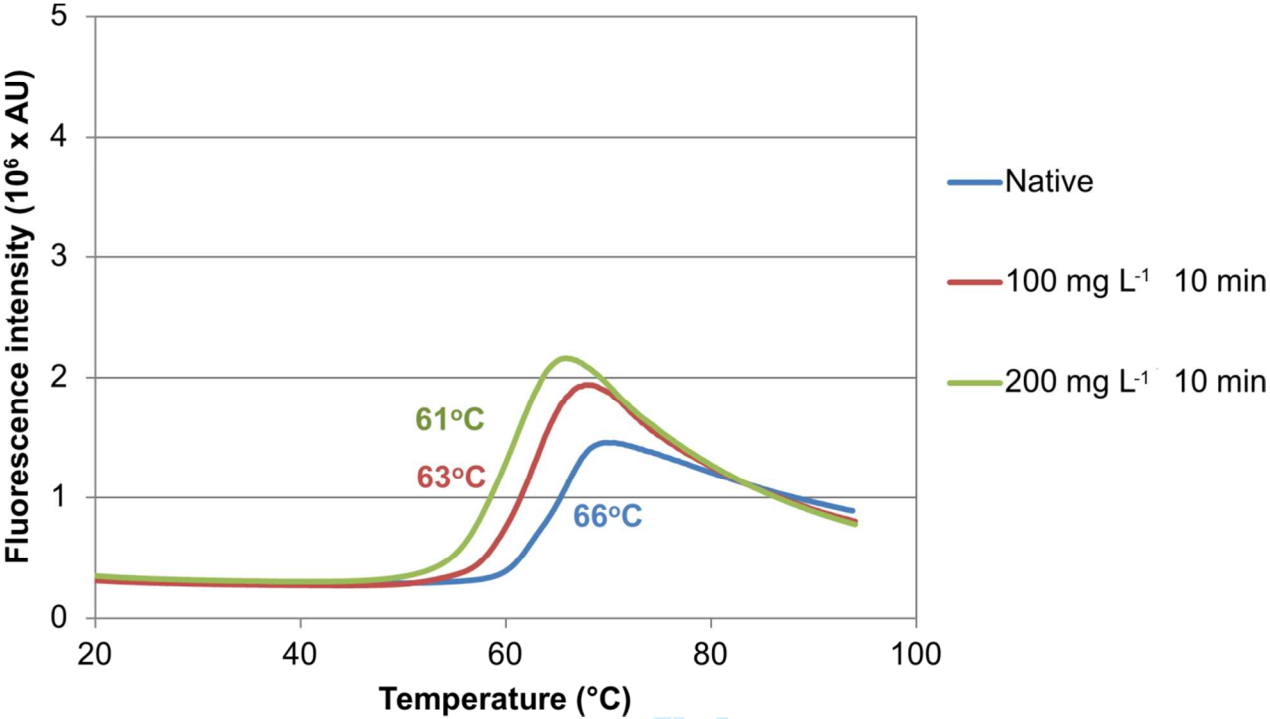
Figure 1





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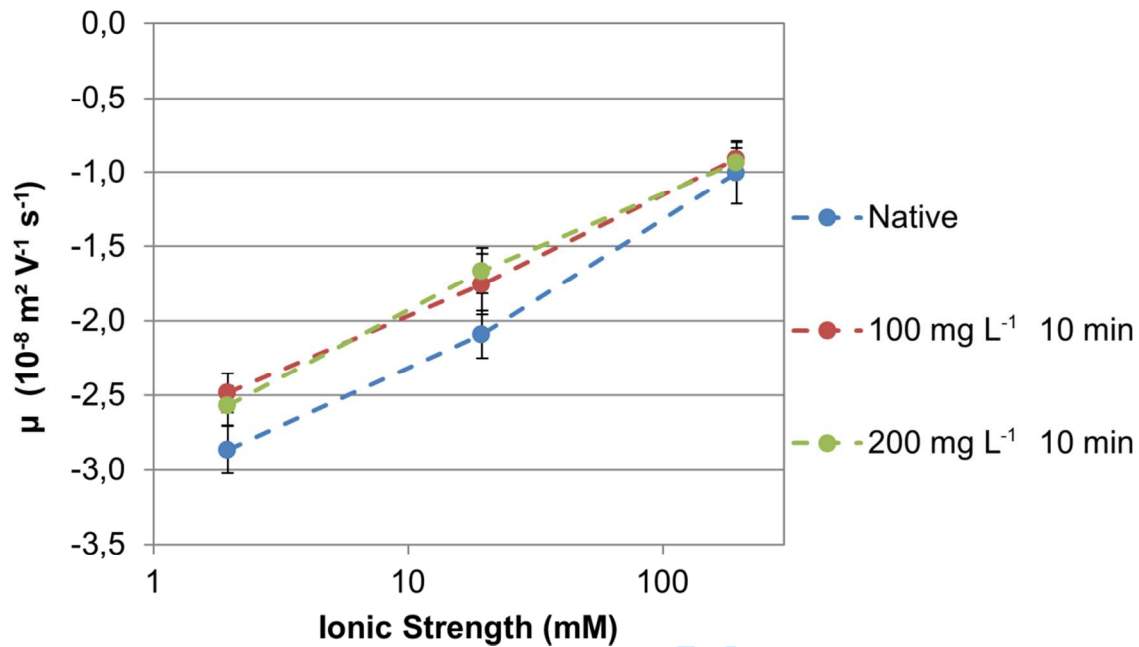
Figure 2



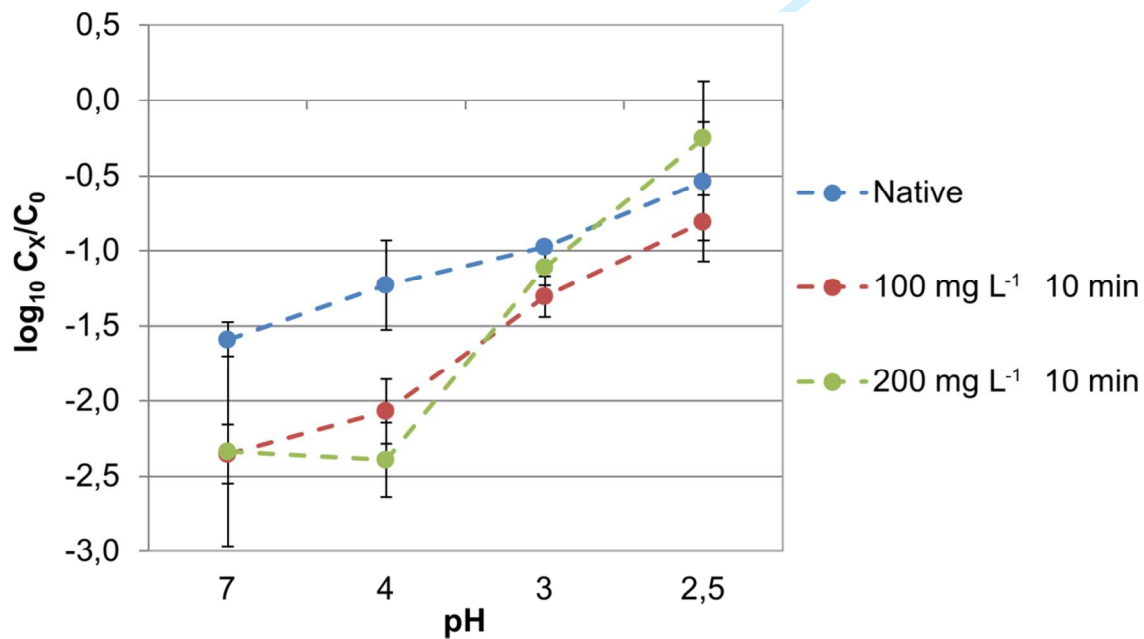
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Figure 3

(a)

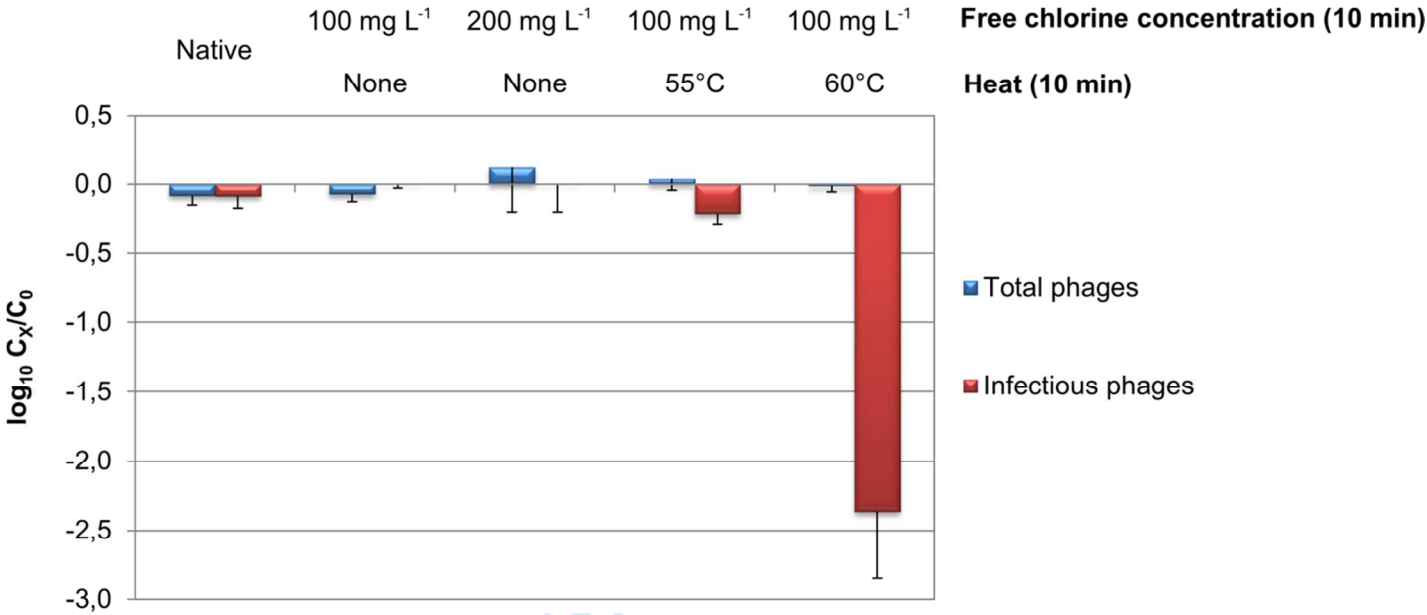


(b)



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Figure 4



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Figure 5

