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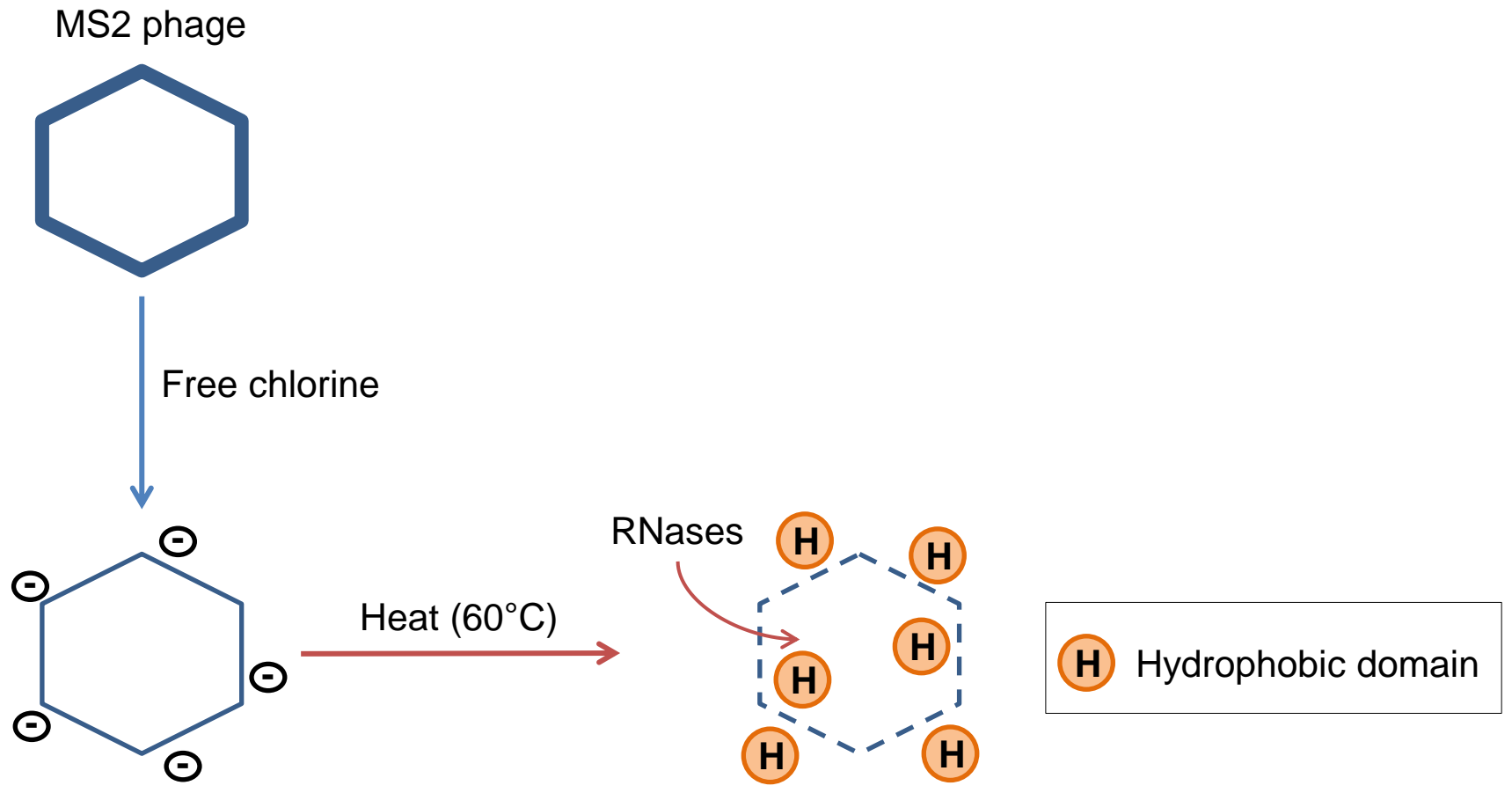
The impact of chlorine and heat on the infectivity and physicochemical properties of bacteriophage MS2

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1 **Graphical abstract**

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Infectivity: ↘

Infectivity: ↘ ↘

Thermic resistance: ↘

Permeability to RNases: ↗

Negative charge: ↗

Availability of hydrophobic domains: ↗

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3 1 **The impact of chlorine and heat on the infectivity and physicochemical properties of**
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5 2 **bacteriophage MS2**
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9 4 Adrien Brié^{1,2}, Christophe Gantzer¹, Nicolas Boudaud², and Isabelle Bertrand^{1*}
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12
13 6 ¹Université de Lorraine, CNRS, LCPME, Nancy, F-54000, France
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15 7 ²Food Safety Department, ACTALIA, Saint Lô, F-50000, France.
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24 11 **One-sentence summary:** Exposure of MS2 phage to free chlorine followed by heat leads to higher
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26 12 inactivation, permeability to RNases, and availability of hydrophobic domains within the capsid
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28 13 than does chlorine applied alone.
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47
48 22 ***Corresponding author:** Isabelle Bertrand, Laboratoire de Chimie Physique et Microbiologie pour
49
50 23 l'Environnement (LCPME), UMR 7564 CNRS Université de Lorraine, Faculté de Pharmacie, 5 rue
51
52 24 Albert Lebrun, BP 80403, 54001 Nancy, France.
53
54 25 Tel: +33-3-72-74-73-52; Fax: +33-3-83-27-54-44; E-mail: isabelle.bertrand@univ-lorraine.fr
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3 26 **ABSTRACT**
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5 27 Enteric viruses and bacteriophages are exposed to various inactivating factors outside their host, and
6
7 28 among them chlorine and heat are the most commonly used sanitizer in water industry and
8
9 29 **treatment** in the food industry, respectively. Using MS2 phages as models for enteric viruses, we
10
11 30 investigated the impact of free chlorine and heat on their physicochemical properties. Free chlorine
12
13 31 was first evaluated alone. No increase in either capsid permeability or hydrophobicity was
14
15 32 observed. The negative surface charge slightly increased suggesting molecular changes in the
16
17 33 capsid. However, a weakening of the capsid by chlorine was suggested by differential scanning
18
19 34 fluorimetry. This phenomenon was confirmed when chlorination was followed by a heat treatment.
20
21 35 Indeed, an increase in the inactivation of MS2 phages and the permeability of their capsids to
22
23 36 RNases was observed. More interestingly, an increase in the expression of hydrophobic domains at
24
25 37 the phage surface was observed, but only for phages remaining infectious. The chlorine-caused
26
27 38 weakening of the capsid suggested that, for an optimal use, the oxidant should be followed by heat.
28
29 39 The increased permeability to RNases and the expression of hydrophobic domains may contribute
30
31 40 to the development or improvement of molecular methods specific for infectious enteric viruses.
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42 **KEYWORDS:** free chlorine, heat, MS2 bacteriophage, surface properties, hydrophobicity, charge
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46 INTRODUCTION

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

60 Heat induces structural and conformational changes of the capsid proteins leading to at least
61 inactivation through alteration of the binding capacity to host cells, modification of surface
62 properties, and increase in permeability to enzymes and intercalating dyes. These findings have
63 been reported for poliovirus, hepatitis A virus, feline calicivirus, and MS2 phage (Breindl 1971;
64 Nuanualsuwan and Cliver 2003; Pecson, Martin and Kohn 2009; Wigginton *et al.* 2012; Brié *et al.*
65 2016). Heat has been shown to have, if any, only a very low impact on the virus genome
66 (Wigginton *et al.* 2012). Hydrophobicity seems to be the surface property the most affected by heat
67 treatment. Indeed, the expression of hydrophobic domains has been reported for MS2 phages
68 exposed to heat but for remaining infectious particles only (Brié *et al.* 2016). An increase in
69 hydrophobicity has also been observed for poliovirus exposed to 50°C, which has been linked to the
70 externalization of the N-terminal region of viral protein VP1 (Fricks and Hogle 1990; Curry *et al.*

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2
3 71 1996). Heat-induced modification of the electrostatic charge is less obvious. Indeed, a decrease in
4
5 72 the isoelectric point (IEP) has been observed for poliovirus heated at 56°C and linked to the loss of
6
7 73 viral RNA genome and capsid protein VP4 (O'Brien and Newman 1979; Breindl 1971). Only a
8
9 74 slight decrease in the global negative charge has been reported for MS2 phages after exposure to
10
11 75 60°C (Brié *et al.* 2016). The structural changes in the capsid proteins caused by heat seem to alter
12
13 76 the structure specifically involved in host cell binding, and to be linked to modification of
14
15 77 hydrophobic domain expression.

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18 78 Free chlorine has been shown to alter both genome- and protein-mediated functions of MS2
19
20 79 phage, more specifically genome injection and replication, but not its binding capacity to host cells
21
22 80 (Wigginton *et al.* 2012). Free chlorine has also been shown to affect proteins of pathogenic enteric
23
24 81 viruses such as picornaviruses (Nuanualsuwan and Cliver 2003) and adenovirus (Page, Shisler and
25
26 82 Mariñas 2010). Moreover, the loss of genome replication has been shown to coincide with extensive
27
28 83 genome damage (Wigginton *et al.* 2012). The reaction of free chlorine with amino acids
29
30 84 constituting the capsid proteins leads to the formation of numerous products such as carbonyl
31
32 85 groups resulting from the oxidization of lysine, arginine, threonine, and proline. A relationship
33
34 86 between the number of carbonyl groups and the rate of free chlorine-induced inactivation has been
35
36 87 reported for enteric viruses belonging to *Astroviridae*, *Reoviridae*, *Caliciviridae*, and
37
38 88 *Picornaviridae* (Sano *et al.* 2010; Tojo *et al.* 2013; Sano *et al.* 2015). The oxidization of amino
39
40 89 acids by chlorine could also result in modifications of the electrostatic charge, which, together with
41
42 90 hydrophobicity, is the major component of the surface properties of virus particles. To our
43
44 91 knowledge, the electrostatic charge has not previously been explored for enteric viruses exposed to
45
46 92 free chlorine. Concerning the hydrophobicity of chlorine-exposed viruses, a recent study has shown
47
48 93 that exposure to sodium hypochlorite modified the profile of the hydrophobic probe SYPRO
49
50 94 Orange in Q β phage belonging to F-specific RNA phage genogroup III. Indeed, an increase in
51
52 95 hydrophobicity was observed at the beginning of the experiment; this meant that sodium
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3 96 hypochlorite revealed some hydrophobic parts of the capsid or increased the permeability of the
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5 97 capsid allowing the probe to reach inside hydrophobic regions (Loison *et al.* 2016).
6

7 98 The study of the impact of heat and free chlorine on enteric viruses requires methods suited for
8
9 99 the specific detection of infectious viral particles, which are the only ones that pose a risk to human
10
11 100 health. Indeed, studies performing a simultaneous and quantitative comparison between infectivity
12
13 101 loss and genome degradation have showed a higher persistence of viral genome after heat treatment
14
15 102 (Bertrand *et al.* 2012) and chlorination (Pelleieux *et al.* 2016). Thus, the detection of viral genomes
16
17 103 by molecular methods used alone does not provide information about infectivity (Girones *et al.*
18
19 104 2010). Human noroviruses (NoV) and hepatitis A virus (HAV) are considered as the main
20
21 105 pathogenic enteric viruses. However, the detection of their infectious particles remains challenging
22
23 106 although cell culture methods have been very recently proposed for noroviruses (Jones *et al.* 2015;
24
25 107 Ettayebi *et al.* 2016). Various methods which could be combined with molecular approaches have
26
27 108 been evaluated in order to favor the detection of only infectious viruses. Most of them are based on
28
29 109 the increased permeability of the capsid to enzymes or intercalating dyes such as RNases, ethidium
30
31 110 monoazide (EMA), or propidium monoazide (PMA) (Prevost *et al.* 2016; Randazzo *et al.* 2016), or
32
33 111 on the specific detection of oxidative damage (Tojo *et al.* 2013; Sano *et al.* 2015). However, their
34
35 112 optimal conditions of use such as the type of treatment (i.e. physical and/or chemical) and the
36
37 113 matrices (i.e. food and/or environmental matrices) still need to be defined.
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41 114 F-specific RNA phages (FRNAPH) belong to the family *Leviviridae* and are classified in two
42
43 115 genera, *Levivirus* and *Allovivirus*, each divided into two genogroups. These phages exhibit
44
45 116 numerous similarities with NoV and HAV in terms of size (20 - 30 nm), genome (positive-sense
46
47 117 single-stranded RNA), and overall capsid structure (Valegård *et al.* 1990; King *et al.* 2012). These
48
49 118 phages infect bacteria with F pili, such as *Escherichia coli*, in the gut of mammals and are released
50
51 119 in environments exposed to fecal contamination similarly to enteric viruses. Their physicochemical
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53 120 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
6
7 123 on surfaces (Morin *et al.* 2015; Pelleieux *et al.* 2016) during treatment. Due to their structural
8
9 124 similarity with the main waterborne pathogenic viruses they have also been proposed as relevant
10
11 125 indicators of viral contamination in water (Cole, Long and Sobsey 2003; Ogorzaly *et al.* 2009;
12
13 126 Vergara *et al.* 2015) and shellfish (Hartard *et al.* 2017) using molecular and/or cell culture methods.
14
15 127 MS2 phage belonging to *Levivirus* genogroup I is the most studied of these FRNAPH, particularly
16
17 128 its physicochemical characteristics (Valegård *et al.* 1990; Langlet *et al.* 2008a; Dika *et al.* 2013a)
18
19 129 and the mechanisms of its inactivation (Pecson, Martin and Kohn 2009; Wigginton *et al.* 2012;
20
21 130 Sigstam *et al.* 2013). Thus, MS2 seems to be a relevant model for pathogenic enteric viruses,
22
23 131 especially for studying the mechanisms of inactivation by different factors, and it can be produced
24
25 132 in high concentrations, an essential prerequisite for some experimental methods focusing on
26
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
32
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
36
37 137 knowledge of the damage that occurs in enteric viruses during loss of infectivity. In such a context,
38
39 138 the present work aimed to optimize the conditions of use of free chlorine and heat for the
40
41 139 inactivation of enteric viruses and to evaluate their impact on the surface properties of MS2. The
42
43 140 impact of free chlorine used alone was first evaluated on MS2 phages because of the poor
44
45 141 knowledge about its effects on the surface properties of enteric viruses. After verification of the
46
47 142 preservation of the capsid of inactivated phages, we examined in greater detail the thermal stability
48
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
52
53 145 hydrophobicity previously described for MS2 phages exposed to heat (Brié *et al.* 2016) led us to
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3 146 perform further experiments focusing on hydrophobicity, in which heat treatment was applied to
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5 147 MS2 phages previously exposed to chlorine. Since both free chlorine and heat have been shown to
6
7 148 act on the capsid, it might therefore be expected that their combination would increase their impact.
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10 11 150 **MATERIALS AND METHODS**

12 13 14 151 **Production and purification of MS2 phage**

15
16 152 Replication of MS2 phage (ATCC, 15597-B1) was done according to the standard procedure (ISO
17
18 153 10705-1 1995) (without the chloroform step) using *Escherichia coli* Hfr K12 (ATCC, 23631) as
19
20 154 host cells.

21
22 155 The phages were then concentrated and purified as previously described (Brié *et al.* 2016). Briefly,
23
24 156 the phages were centrifuged twice (8,000 g, 20 min, 4°C) and the supernatant (\approx 1 L) was filtered
25
26 157 using a 0.22 μ m filter unit (Stericup-GP Millipore Express Plus, 0.22 μ m; Merck Millipore, Merck
27
28 158 KGaA, Darmstadt, Germany). After an ultracentrifugation step (Thermo Scientific A-621 fixed
29
30 159 rotor, 42,500 g, 18 h, 4°C; Thermo Fisher Scientific, Waltham, MA, USA), the pellets containing
31
32 160 the concentrated phages were resuspended in iodixanol solution (20% final, Optiprep; Axis-Shield,
33
34 161 Dundee, Scotland) and laid on a 40% iodixanol layer in a tube (Cone-Top PA tube 14 \times 47 mm,
35
36 162 Thermo Fisher Scientific). The density gradient was formed upon centrifugation at 160,000 g for
37
38 163 7 h at 15°C (Thermo Scientific TH-641 swing rotor). After centrifugation, the MS2 phages were
39
40 164 gently removed with a syringe and dialyzed twice (one overnight, the other for 7 h) against 10 L of
41
42 165 PBS solution with a concentration of 1 mM (1 mM Na₂HPO₄ and 14 mM NaCl), pH 7.4, using a
43
44 166 Float-A-Lyzer G2 dialysis device (MW100 kD; Spectrum Laboratories, Inc., Rancho Dominguez,
45
46 167 CA, USA). These working suspensions were kept at 4°C before use. **Three working suspensions**
47
48 168 **were produced for this study in order to perform the experiments in triplicate on independent**
49
50
51 169 **suspensions.**
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55 170

171 **Free chlorine and heat treatments**

172 Three independent suspensions of purified phages at a concentration of 10^{14} PFU·mL⁻¹ (250 µL in
173 1 mM PBS solution) (PFU: plaque-forming units) were exposed either to free chlorine alone, to heat
174 alone or to free chlorine followed by heat. To prepare our free chlorine stock solution, concentrated
175 sodium hypochlorite solution (5%, Acros Organics, Thermo Fisher Scientific) was diluted using
176 ultrapure water ($> 18 \text{ M}\Omega\cdot\text{cm}^{-1}$). Determination of free chlorine concentration was performed
177 according to the DPD (*N,N*-diethyl-*p*-phenylenediamine, Acros Organics) colorimetric method (ISO
178 7393-2 1985). For exposure to free chlorine alone, the working concentrations of free chlorine were
179 100 and 200 mg·L⁻¹ with a contact time of 10 min at 4°C. Residual free chlorine was immediately
180 quenched by adding two moles of sodium thiosulfate equivalent to one mole of free chlorine. The
181 heat treatments were performed at 55°C or 60°C for 10 min in pre-warmed Protein LoBind tubes
182 (Eppendorf, Hamburg, Germany) in a water bath. Samples were immediately placed on ice after
183 heat treatment to avoid further inactivation. When free chlorine was followed by heat, the phages
184 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
185 10 min. For each replicate, a part of the MS2 phage suspension was not exposed to free chlorine,
186 heat or to free chlorine followed by heat and was named control phages. Thus, there were nine
187 (n=3x3) control populations in this study.

189 **Enumeration of infectious MS2 phages**

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

196

197 **Viral genome extraction and quantification**

198 The **Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR)** method was used
199 for the detection of potential degradation of the viral genome by **free chlorine and/or heat**. Thus, the
200 **genome of MS2 phage was quantified after each treatment (C) and without treatment (C₀)**. The
201 **genome degradation by the treatment was estimated by $\log \frac{C}{C_0}$** . Prior to the RT-qPCR assays,
202 extractions of viral RNA (50 μ L) were performed using the QIAamp viral RNA kit (Qiagen,
203 Hilden, Germany) according to the manufacturer's recommendations. The extracts (100 μ L) were
204 immediately stored at -80°C before quantification. Quantification of the phage genome was
205 performed by specific a RT-qPCR assay targeting a genome fragment of ≈ 150 nucleotides as
206 previously described (Ogorzaly and Gantzer 2006). **Two negative controls and one positive control**
207 **(i.e. known quantity of MS2 genome) were added to each RT-qPCR analysis**. RT-qPCR efficiency
208 ranged from 80% to 95%. Determination of MS2 genome copies per mL ($\text{gc}\cdot\text{mL}^{-1}$) used for the
209 standard curve was performed as previously described (Brié *et al.* 2016). The final concentration of
210 purified MS2 phages was $\approx 10^{15}$ $\text{gc}\cdot\text{mL}^{-1}$.

211

212 **Transmission electron microscopy**

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

218

219 **Evaluation of capsid integrity using an RNase assay**

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2
3 220 An RNase assay was used to evaluate if the capsid had maintained its integrity and was still able to
4
5 221 protect the virus genome (Nuanualsuwan and Cliver 2003). Suspensions of MS2 phage, with a final
6
7 222 concentration of 10^7 gc·mL⁻¹, were treated with RNase A at a final concentration of 1 µg·mL⁻¹
8
9 223 (AppliChem GmbH, Darmstadt, Germany) at 37°C for 60 min as previously described (Brié *et al.*
10
11 224 2016).

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13 225

14 15 16 226 **Binding of MS2 phages to *E. coli* host cells**

17
18 227 The impact of free chlorine on the binding capacity of MS2 phage to its host cell *E. coli* Hfr K12
19
20 228 was evaluated as previously described (Wigginton *et al.* 2012; Brié *et al.* 2016). The binding
21
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
23
24
25 230 bacteria after free chlorine treatment and Q_0 without treatment.

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28 29 232 **Size and electrophoretic mobility measurements**

30
31 233 Size and electrophoretic mobility (μ) measurements were performed in order to evaluate the impact
32
33 234 of free chlorine on the overall structure and charge density of MS2 phage, respectively. The
34
35 235 experiments were achieved in disposable capillary cells (Malvern Instruments, Worcestershire, UK)
36
37 236 at 23°C ± 0.1°C and pH 7.0 using a Zetasizer Nano ZS instrument (Malvern Instruments, He-Ne
38
39 237 red laser, wavelength 633 nm) driven by Dispersion Technology Software (Malvern Instruments) as
40
41 238 previously described (Langlet *et al.* 2008a). The slope of the electrophoretic mobility as a function
42
43 239 of the logarithm of ionic strength (1.95, 19.5 and 195 mM) reflects the ratio between the density of
44
45 240 electric charges located within the particle and that of charges distributed at the outer virus
46
47 241 periphery. A decrease in the slope value shows a decrease in the charge density of the virus (Langlet
48
49 242 *et al.* 2008a). The experimental conditions were identical to those previously described (Brié *et al.*
50
51 243 2016). For each condition, measurements were obtained by means of three independent experiments
52
53 244 with triplicate measures in each to ensure repeatability of the measurements.

245

246 Binding of MS2 phages to hydrophobic or positively charged beads

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

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

259

260 Differential scanning fluorimetry

261 Differential scanning fluorimetry (DSF) was performed using the hydrophobic probe SYPRO
262 Orange. This probe has a high affinity for hydrophobic domains (Niesen, Berglund and Vedadi
263 2007), which are unmasked when proteins unfold. DSF was used to compare the thermal stability of
264 MS2 phage, as measured by the denaturation temperature (T_m). Thermal melting curves of MS2
265 phage were obtained in 96-well plates using StepOne Plus qPCR System (Life Technologies,
266 Thermo Fisher Scientific) and the fluorescent dye SYPRO Orange, 5,000 \times concentrate, supplied in
267 DMSO (Invitrogen, Thermo Fisher Scientific). Reactions were performed in a 25 μ L final volume
268 containing 5 μ L of buffer (150 mM Tris-HCl, pH 8), 2 μ L of SYPRO Orange solution 62.5 \times (final
269 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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2
3 270 stock or after treatment (5.10^{14} - 10^{15} gc·mL⁻¹). The temperature was increased for all replicates from
4
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
6
7 272 measurements. The sum of the fluorescence intensities obtained with all the filters was plotted
8
9 273 against temperature (Excel software) to obtain the maximum fluorescence intensity of SYPRO
10
11 274 Orange. The T_m was determined by plotting the first derivative of the previous curve; it appeared
12
13 275 then as a peak (Loison *et al.* 2016). This method is also known to allow the expression of
14
15 276 hydrophobic parts of the capsid and/or the permeability of the capsid to be investigated by the
16
17 277 fluorescence intensity of SYPRO Orange at the beginning of the DSF experiment (Loison *et al.*
18
19 278 2016).

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24 280 **Data analysis**

26 281 The analysis was performed using the values obtained for the three independent suspensions of
27
28 282 phages. Since the experimental values were not normally distributed, non-parametric tests were
29
30 283 performed using StatEL 2.7 (Ad Science). The explanatory variables were the exposure to free
31
32 284 chlorine alone, heat alone, and free chlorine followed by heat. For free chlorine alone, Mann-
33
34 285 Whitney tests were performed to evaluate its effect on the infectivity (i.e. loss of infectivity, binding
35
36 286 capacity to host bacteria), structure (i.e. genome degradation, capsid permeability to RNase, thermal
37
38 287 stability), and hydrophobicity (i.e. initial adhesion of hydrophobic probe SYPRO Orange, binding
39
40 288 to hydrophobic probes), and the adhesion to positively charged beads by comparison between
41
42 289 control and chlorinated MS2 phages. A Kruskal-Wallis test was performed to compare the
43
44 290 electrophoretic mobility between control and chlorinated MS2 phages. The effect of free chlorine
45
46 291 followed by heat on the loss of infectivity, structure (i.e. genome degradation, capsid permeability
47
48 292 to RNase) and hydrophobicity (i.e. initial adhesion of hydrophobic probe SYPRO Orange, binding
49
50 293 to hydrophobic beads) was evaluated by comparison with free chlorine alone and heat alone using
51
52 294 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

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3 320 MS2 phage by the oxidant. Although there was a decrease in the thermal stability of the capsid we
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5 321 did not find any changes in shape, size, and binding capacity, nor did the permeability to RNase
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7 322 increase thus suggesting that the capsid of MS2 phage maintained its integrity after exposure to free
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9 323 chlorine alone.

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11 324 The decrease in the thermal stability after exposure to chlorine led us to expose MS2 phage to
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13 325 chlorine (100 mg.L^{-1}) and then to heat (55°C or 60°C). The infectivity decreased by $-1.9 \pm 0.3 \log_{10}$
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15 326 for chlorine followed by heat treatment at 55°C and by $-3.2 \pm 0.6 \log_{10}$ for chlorine followed by
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17 327 heat treatment at 60°C . For each treatment applied separately, the loss in infectivity reached –
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19 328 $1.2 \pm 0.3 \log_{10}$ after exposure to free chlorine (Table 1), $-0.7 \pm 0.1 \log_{10}$ after heat treatment at 55°C
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21 329 (data not shown) and $-1.1 \pm 0.1 \log_{10}$ after heat treatment at 60°C (Brié *et al.* 2016). The highest
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23 330 loss in infectivity was obtained when free chlorine was followed by heat treatment at 60°C (p
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25 331 <0.01 , Kruskal-Wallis test). This loss in infectivity was also higher than the sum of the decrease
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27 332 values obtained with free chlorine and heat treatment at 60°C applied separately ($p <0.01$, Kruskal-
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29 333 Wallis test). This suggested that free chlorine improved the inactivation of MS2 phage by heat
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31 334 treatment at 60°C . The RT-qPCR signal decreased only by $-0.1 \pm 0.4 \log_{10}$ for chlorine followed by
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33 335 heat treatment at 55°C and by $-0.3 \pm 0.4 \log_{10}$ for chlorine followed by heat treatment at 60°C . The
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35 336 concentrations in genome observed after these treatments were similar to those observed for MS2
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37 337 phage exposed to 100 mg.L^{-1} free chlorine alone and also for control MS2 phage ($p >0.01$, Kruskal-
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39 338 Wallis test). Thus, the detection of the MS2 genome was not affected when free chlorine was
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41 339 followed by heat. As observed for free chlorine alone, no modification of the shape of MS2 phage
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43 340 was noted by TEM after exposure to free chlorine followed by heat (Fig. 1). The RNase treatment
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45 341 applied after exposure to free chlorine (100 mg.L^{-1}) followed by heat decreased the RT-qPCR signal
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47 342 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
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49 343 RNase treatment reached $-0.3 \pm 0.1 \log_{10}$ (Table 1) for free chlorine alone, $-0.1 \pm 0.1 \log_{10}$ (data
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51 344 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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353 **Impact of free chlorine on the charge of MS2 phage particles**

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

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3 370 Whitney test). Thus, the increase in the negative charge density of MS2 phage after exposure to free
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5 371 chlorine seemed to be mainly linked to an increase in the external negative charge.

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9 373 **Impact of free chlorine applied alone or followed by heat on the hydrophobicity of MS2 phage**
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11 374 **particles**

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13 375 The effect of free chlorine on the hydrophobicity of MS2 phage was then evaluated by DSF and by
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15 376 its adhesion capacity to hydrophobic beads. The DSF profile (Fig. 2) showed the same fluorescence
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17 377 intensity of SYPRO Orange at the beginning of the experiment for control and chlorinated phages,
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19 378 meaning that free chlorine did not modify the overall expression of hydrophobic domains of the
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21 379 capsid proteins. The expression of hydrophobic domains on the surface of MS2 particles was
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23 380 compared between control and chlorinated phages using the adhesion ability to hydrophobic beads.
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25 381 The experiments were performed at pH 7.0 and high ionic strength (10 mM Na₂HPO₄ and 150 mM
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27 382 NaCl) in order to minimize the influence of the global charge. No adhesion to hydrophobic beads
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29 383 was observed for control phages and those exposed to chlorine (Fig. 4). Thus, the results obtained
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31 384 using these two methods suggested that free chlorine did not change the expression of hydrophobic
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33 385 domains of the MS2 capsid proteins or the permeability of the capsid to the probe.

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35 386 Since we have previously described an increased hydrophobicity of MS2 phage after heat
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37 387 treatment (Brié *et al.* 2016), DSF experiments were carried out after exposure to free chlorine (100
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39 388 mg.L⁻¹) followed by heat (55°C or 60°C) and were compared to 55°C and 60°C heat treatments
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41 389 applied alone (Fig. 5). Similar DSF profiles with low initial fluorescence were obtained for both
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43 390 control MS2 phages and those exposed to the 55°C heat treatment alone. The initial fluorescence
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45 391 increased for MS2 phages exposed to the 60°C heat treatment alone and for those exposed to free
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47 392 chlorine followed by heat treatment at 55°C and 60°C. The highest initial fluorescence value was
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49 393 observed using 100 mg.L⁻¹ free chlorine followed by 60°C heat treatment ($p < 0.01$, Kruskal-Wallis
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51 394 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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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.

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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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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3 445 the loss of replication function played a lesser role in the overall inactivation than in the other two
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5 446 inactivation treatments (Wigginton *et al.* 2012). For these authors, this suggested that the virucidal
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7 447 effect due to genome damage was linked to the type of modification rather than its location.
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9 448 No modification of the shape of MS2 phage was observed by TEM after exposure to free
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11 449 chlorine. The mean size of MS2 phage before and after chlorination was similar to that previously
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13 450 described (Valegård *et al.* 1990; Dika *et al.* 2013a). Q β phage particles exposed to chlorine under
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15 451 similar conditions were also demonstrated by TEM observations to maintain their shape (Loison *et*
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17 452 *al.* 2016). Disruption of MS2 phage occurring after exposure to 72°C for 10 min has previously
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19 453 been shown to be related to a significant increase in RNase sensitivity (Brié *et al.* 2016). In our
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21 454 case, the limited effect of RNase treatment on RT-qPCR signal confirmed that the integrity of the
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23 455 capsid was preserved after exposure to free chlorine under our experimental conditions. DSF was
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25 456 then used to explore conformational modifications of the capsid that could not be revealed by TEM.
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27 457 We observed a similar DSF profile to that obtained in our previous study (Brié *et al.* 2016) with
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29 458 only one T_m peak (66°C) before exposure to chlorine. The decrease in the T_m value after exposure
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31 459 to free chlorine corresponded to a reduction in the denaturation temperature and suggested a
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33 460 weakening of the capsid of MS2 phage by the oxidant by comparison to a previous study performed
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35 461 on poliovirus (Robinson, Jesudhasan and Pfeiffer 2014). The weakening of the capsid might be
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37 462 linked to the site-specific cleavage showed on the capsid protein of MS2 phage following exposure
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39 463 to chlorine (Wigginton *et al.* 2012).
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44 464 The reactions of hypochlorous acid with amino acids result in a wide variety of products such
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46 465 as sulfinic/sulfonic acids, methionine sulfone, and *N*-chlorinated species (Pattison, Davies and
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48 466 Hawkins 2012) which may modify the electrostatic charge of viral particles. In our study, the
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50 467 charge density was evaluated by measuring the electrophoretic mobility as a function of ionic
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52 468 strength which suggested a decrease in the negative charge density of MS2 phage exposed to free
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54 469 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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42 489 protein.

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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

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3 494 phage particles at high temperatures (72°C) and transient expression of hydrophobic domains at
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5 495 lower temperatures (60°C) (Brié *et al.* 2016).

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7 496 An **increased** effect on the inactivation of MS2 phage and on the permeability of its capsid to
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9 497 RNase was observed when the 60°C heat treatment was applied after chlorination. To our
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11 498 knowledge, the virucidal effect of the combination of free chlorine and heat on enteric viruses and
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13 499 phages has not been studied before. Indeed, chlorine-induced inactivation of enteric viruses and
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15 500 phages in suspension in water has only been studied for temperatures below 30°C. The combined
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17 501 effect of temperature and chlorination in foodstuffs has been more closely studied on bacteria,
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19 502 showing an increase in inactivation compared to chlorine applied alone (Klaiber *et al.* 2005; Choi *et*
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21 503 *al.* 2016). Moreover, these authors have shown that warm water (50°C) or dry heat (80°C) and
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23 504 chlorine can be combined without altering the organoleptic quality or the viability of vegetables or
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25 505 seeds.

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28 506 Since hydrophobicity is known to be the main surface property affected by exposure of MS2
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30 507 phages to heat (60°C) (Brié *et al.* 2016), a significant change in this parameter was expected when
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32 508 free chlorine was followed by heat. As previously described by Brié *et al.* (2016) for MS2 phages
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34 509 exposed to the 60°C heat treatment alone, the initial fluorescence of the DSF profile increased after
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36 510 exposure to free chlorine followed by heat. The highest initial fluorescence value was observed
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38 511 using free chlorine followed by a 60°C heat treatment. The increase in the initial fluorescence
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40 512 showed that chlorine followed by heat led to the expression of hydrophobic domains and/or
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42 513 increased the permeability of the capsid allowing the probe to reach inside hydrophobic regions.
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44 514 The adhesion test to hydrophobic beads showed an increase in the expression of hydrophobic
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46 515 domains at the surface of MS2 phages exposed to free chlorine followed by 60°C heat treatment.
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48 516 Remarkably, this increase in the adhesion capacity to hydrophobic beads appeared to be specific to
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50 517 viral particles remaining infectious as previously observed for MS2 phage exposed to heat alone
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52 518 (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

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3 1 **The impact of chlorine and heat on the infectivity and physicochemical properties of**
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5 2 **bacteriophage MS2**

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9 4 Adrien Brié^{1,2}, Christophe Gantzer¹, Nicolas Boudaud², and Isabelle Bertrand^{1*}
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13 6 ¹Université de Lorraine, CNRS, LCPME, Nancy, F-54000, France

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15 7 ²Food Safety Department, ACTALIA, Saint Lô, F-50000, France.
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24 11 **One-sentence summary:** Exposure of MS2 phage to free chlorine followed by heat leads to higher
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26 12 inactivation, permeability to RNases, and availability of hydrophobic domains within the capsid
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28 13 than does chlorine applied alone.
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48 22 ***Corresponding author:** Isabelle Bertrand, Laboratoire de Chimie Physique et Microbiologie pour
49
50 23 l'Environnement (LCPME), UMR 7564 CNRS Université de Lorraine, Faculté de Pharmacie, 5 rue
51
52 24 Albert Lebrun, BP 80403, 54001 Nancy, France.
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54 25 Tel: +33-3-72-74-73-52; Fax: +33-3-83-27-54-44; E-mail: isabelle.bertrand@univ-lorraine.fr
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3 26 **ABSTRACT**
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5 27 Enteric viruses and bacteriophages are exposed to various inactivating factors outside their host, and
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7 28 among them chlorine and heat are the most commonly used sanitizer in water industry and
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9 29 treatment in the food industry, respectively. Using MS2 phages as models for enteric viruses, we
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11 30 investigated the impact of free chlorine and heat on their physicochemical properties. Free chlorine
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13 31 was first evaluated alone. No increase in either capsid permeability or hydrophobicity was
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15 32 observed. The negative surface charge slightly increased suggesting molecular changes in the
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17 33 capsid. However, a weakening of the capsid by chlorine was suggested by differential scanning
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19 34 fluorimetry. This phenomenon was confirmed when chlorination was followed by a heat treatment.
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21 35 Indeed, an increase in the inactivation of MS2 phages and the permeability of their capsids to
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23 36 RNases was observed. More interestingly, an increase in the expression of hydrophobic domains at
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25 37 the phage surface was observed, but only for phages remaining infectious. The chlorine-caused
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27 38 weakening of the capsid suggested that, for an optimal use, the oxidant should be followed by heat.
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29 39 The increased permeability to RNases and the expression of hydrophobic domains may contribute
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31 40 to the development or improvement of molecular methods specific for infectious enteric viruses.
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42 44 **KEYWORDS:** free chlorine, heat, MS2 bacteriophage, surface properties, hydrophobicity, charge
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46 INTRODUCTION

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

60 Heat induces structural and conformational changes of the capsid proteins leading to at least
61 inactivation through alteration of the binding capacity to host cells, modification of surface
62 properties, and increase in permeability to enzymes and intercalating dyes. These findings have
63 been reported for poliovirus, hepatitis A virus, feline calicivirus, and MS2 phage (Breindl 1971;
64 Nuanualsuwan and Cliver 2003; Pecson, Martin and Kohn 2009; Wigginton *et al.* 2012; Brié *et al.*
65 2016). Heat has been shown to have, if any, only a very low impact on the virus genome
66 (Wigginton *et al.* 2012). Hydrophobicity seems to be the surface property the most affected by heat
67 treatment. Indeed, the expression of hydrophobic domains has been reported for MS2 phages
68 exposed to heat but for remaining infectious particles only (Brié *et al.* 2016). An increase in
69 hydrophobicity has also been observed for poliovirus exposed to 50°C, which has been linked to the
70 externalization of the N-terminal region of viral protein VP1 (Fricks and Hogle 1990; Curry *et al.*

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3 71 1996). Heat-induced modification of the electrostatic charge is less obvious. Indeed, a decrease in
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5 72 the isoelectric point (IEP) has been observed for poliovirus heated at 56°C and linked to the loss of
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7 73 viral RNA genome and capsid protein VP4 (O'Brien and Newman 1979; Breindl 1971). Only a
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9 74 slight decrease in the global negative charge has been reported for MS2 phages after exposure to
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11 75 60°C (Brié *et al.* 2016). The structural changes in the capsid proteins caused by heat seem to alter
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13 76 the structure specifically involved in host cell binding, and to be linked to modification of
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16 77 hydrophobic domain expression.

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18 78 Free chlorine has been shown to alter both genome- and protein-mediated functions of MS2
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20 79 phage, more specifically genome injection and replication, but not its binding capacity to host cells
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22 80 (Wigginton *et al.* 2012). Free chlorine has also been shown to affect proteins of pathogenic enteric
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24 81 viruses such as picornaviruses (Nuanualsuwan and Cliver 2003) and adenovirus (Page, Shisler and
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26 82 Mariñas 2010). Moreover, the loss of genome replication has been shown to coincide with extensive
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28 83 genome damage (Wigginton *et al.* 2012). The reaction of free chlorine with amino acids
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30 84 constituting the capsid proteins leads to the formation of numerous products such as carbonyl
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32 85 groups resulting from the oxidization of lysine, arginine, threonine, and proline. A relationship
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34 86 between the number of carbonyl groups and the rate of free chlorine-induced inactivation has been
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36 87 reported for enteric viruses belonging to *Astroviridae*, *Reoviridae*, *Caliciviridae*, and
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38 88 *Picornaviridae* (Sano *et al.* 2010; Tojo *et al.* 2013; Sano *et al.* 2015). The oxidization of amino
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40 89 acids by chlorine could also result in modifications of the electrostatic charge, which, together with
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42 90 hydrophobicity, is the major component of the surface properties of virus particles. To our
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44 91 knowledge, the electrostatic charge has not previously been explored for enteric viruses exposed to
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46 92 free chlorine. Concerning the hydrophobicity of chlorine-exposed viruses, a recent study has shown
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48 93 that exposure to sodium hypochlorite modified the profile of the hydrophobic probe SYPRO
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50 94 Orange in Q β phage belonging to F-specific RNA phage genogroup III. Indeed, an increase in
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52 95 hydrophobicity was observed at the beginning of the experiment; this meant that sodium
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3 96 hypochlorite revealed some hydrophobic parts of the capsid or increased the permeability of the
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5 97 capsid allowing the probe to reach inside hydrophobic regions (Loison *et al.* 2016).
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7 98 The study of the impact of heat and free chlorine on enteric viruses requires methods suited for
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9 99 the specific detection of infectious viral particles, which are the only ones that pose a risk to human
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11 100 health. Indeed, studies performing a simultaneous and quantitative comparison between infectivity
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13 101 loss and genome degradation have showed a higher persistence of viral genome after heat treatment
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15 102 (Bertrand *et al.* 2012) and chlorination (Pelleieux *et al.* 2016). Thus, the detection of viral genomes
16
17 103 by molecular methods used alone does not provide information about infectivity (Girones *et al.*
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19 104 2010). Human noroviruses (NoV) and hepatitis A virus (HAV) are considered as the main
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21 105 pathogenic enteric viruses. However, the detection of their infectious particles remains challenging
22
23 106 although cell culture methods have been very recently proposed for noroviruses (Jones *et al.* 2015;
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25 107 Ettayebi *et al.* 2016). Various methods which could be combined with molecular approaches have
26
27 108 been evaluated in order to favor the detection of only infectious viruses. Most of them are based on
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29 109 the increased permeability of the capsid to enzymes or intercalating dyes such as RNases, ethidium
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31 110 monoazide (EMA), or propidium monoazide (PMA) (Prevost *et al.* 2016; Randazzo *et al.* 2016), or
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33 111 on the specific detection of oxidative damage (Tojo *et al.* 2013; Sano *et al.* 2015). However, their
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35 112 optimal conditions of use such as the type of treatment (i.e. physical and/or chemical) and the
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37 113 matrices (i.e. food and/or environmental matrices) still need to be defined.
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41 114 F-specific RNA phages (FRNAPH) belong to the family *Leviviridae* and are classified in two
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43 115 genera, *Levivirus* and *Allovivirus*, each divided into two genogroups. These phages exhibit
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45 116 numerous similarities with NoV and HAV in terms of size (20 - 30 nm), genome (positive-sense
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47 117 single-stranded RNA), and overall capsid structure (Valegård *et al.* 1990; King *et al.* 2012). These
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49 118 phages infect bacteria with F pili, such as *Escherichia coli*, in the gut of mammals and are released
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51 119 in environments exposed to fecal contamination similarly to enteric viruses. Their physicochemical
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53 120 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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3 146 perform further experiments focusing on hydrophobicity, in which heat treatment was applied to
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5 147 MS2 phages previously exposed to chlorine. Since both free chlorine and heat have been shown to
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7 148 act on the capsid, it might therefore be expected that their combination would increase their impact.
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11 150 **MATERIALS AND METHODS**

14 151 **Production and purification of MS2 phage**

16 152 Replication of MS2 phage (ATCC, 15597-B1) was done according to the standard procedure (ISO
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18 153 10705-1 1995) (without the chloroform step) using *Escherichia coli* Hfr K12 (ATCC, 23631) as
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20 154 host cells.

22 155 The phages were then concentrated and purified as previously described (Brié *et al.* 2016). Briefly,
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24 156 the phages were centrifuged twice (8,000 g, 20 min, 4°C) and the supernatant (\approx 1 L) was filtered
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26 157 using a 0.22 μ m filter unit (Stericup-GP Millipore Express Plus, 0.22 μ m; Merck Millipore, Merck
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28 158 KGaA, Darmstadt, Germany). After an ultracentrifugation step (Thermo Scientific A-621 fixed
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30 159 rotor, 42,500 g, 18 h, 4°C; Thermo Fisher Scientific, Waltham, MA, USA), the pellets containing
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32 160 the concentrated phages were resuspended in iodixanol solution (20% final, Optiprep; Axis-Shield,
33
34 161 Dundee, Scotland) and laid on a 40% iodixanol layer in a tube (Cone-Top PA tube 14 \times 47 mm,
35
36 162 Thermo Fisher Scientific). The density gradient was formed upon centrifugation at 160,000 g for
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38 163 7 h at 15°C (Thermo Scientific TH-641 swing rotor). After centrifugation, the MS2 phages were
39
40 164 gently removed with a syringe and dialyzed twice (one overnight, the other for 7 h) against 10 L of
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42 165 PBS solution with a concentration of 1 mM (1 mM Na₂HPO₄ and 14 mM NaCl), pH 7.4, using a
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44 166 Float-A-Lyzer G2 dialysis device (MW100 kD; Spectrum Laboratories, Inc., Rancho Dominguez,
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46 167 CA, USA). These working suspensions were kept at 4°C before use. Three working suspensions
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48 168 were produced for this study in order to perform the experiments in triplicate on independent
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50 169 suspensions.
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171 **Free chlorine and heat treatments**

172 Three independent suspensions of purified phages at a concentration of 10^{14} PFU·mL⁻¹ (250 µL in
173 1 mM PBS solution) (PFU: plaque-forming units) were exposed either to free chlorine alone, to heat
174 alone or to free chlorine followed by heat. To prepare our free chlorine stock solution, concentrated
175 sodium hypochlorite solution (5%, Acros Organics, Thermo Fisher Scientific) was diluted using
176 ultrapure water ($> 18 \text{ M}\Omega\cdot\text{cm}^{-1}$). Determination of free chlorine concentration was performed
177 according to the DPD (*N,N*-diethyl-*p*-phenylenediamine, Acros Organics) colorimetric method (ISO
178 7393-2 1985). For exposure to free chlorine alone, the working concentrations of free chlorine were
179 100 and 200 mg·L⁻¹ with a contact time of 10 min at 4°C. Residual free chlorine was immediately
180 quenched by adding two moles of sodium thiosulfate equivalent to one mole of free chlorine. The
181 heat treatments were performed at 55°C or 60°C for 10 min in pre-warmed Protein LoBind tubes
182 (Eppendorf, Hamburg, Germany) in a water bath. Samples were immediately placed on ice after
183 heat treatment to avoid further inactivation. When free chlorine was followed by heat, the phages
184 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
185 10 min. For each replicate, a part of the MS2 phage suspension was not exposed to free chlorine,
186 heat or to free chlorine followed by heat and was named control phages. Thus, there were nine
187 ($n=3\times 3$) control populations in this study.

189 **Enumeration of infectious MS2 phages**

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

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197 Viral genome extraction and quantification

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

211

212 Transmission electron microscopy

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

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219 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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14 225

15 16 226 **Binding of MS2 phages to *E. coli* host cells**

17
18 227 The impact of free chlorine on the binding capacity of MS2 phage to its host cell *E. coli* Hfr K12
19
20 228 was evaluated as previously described (Wigginton *et al.* 2012; Brié *et al.* 2016). The binding
21
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
23
24
25 230 bacteria after free chlorine treatment and Q_0 without treatment.

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27 231

28 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
32
33 234 of free chlorine on the overall structure and charge density of MS2 phage, respectively. The
34
35 235 experiments were achieved in disposable capillary cells (Malvern Instruments, Worcestershire, UK)
36
37 236 at 23°C ± 0.1°C and pH 7.0 using a Zetasizer Nano ZS instrument (Malvern Instruments, He-Ne
38
39 237 red laser, wavelength 633 nm) driven by Dispersion Technology Software (Malvern Instruments) as
40
41 238 previously described (Langlet *et al.* 2008a). The slope of the electrophoretic mobility as a function
42
43 239 of the logarithm of ionic strength (1.95, 19.5 and 195 mM) reflects the ratio between the density of
44
45 240 electric charges located within the particle and that of charges distributed at the outer virus
46
47 241 periphery. A decrease in the slope value shows a decrease in the charge density of the virus (Langlet
48
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.

245

246 Binding of MS2 phages to hydrophobic or positively charged beads

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

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

259

260 Differential scanning fluorimetry

261 Differential scanning fluorimetry (DSF) was performed using the hydrophobic probe SYPRO
262 Orange. This probe has a high affinity for hydrophobic domains (Niesen, Berglund and Vedadi
263 2007), which are unmasked when proteins unfold. DSF was used to compare the thermal stability of
264 MS2 phage, as measured by the denaturation temperature (T_m). Thermal melting curves of MS2
265 phage were obtained in 96-well plates using StepOne Plus qPCR System (Life Technologies,
266 Thermo Fisher Scientific) and the fluorescent dye SYPRO Orange, 5,000 \times concentrate, supplied in
267 DMSO (Invitrogen, Thermo Fisher Scientific). Reactions were performed in a 25 μ L final volume
268 containing 5 μ L of buffer (150 mM Tris-HCl, pH 8), 2 μ L of SYPRO Orange solution 62.5 \times (final
269 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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2
3 270 stock or after treatment ($5 \cdot 10^{14}$ - 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
6
7 272 measurements. The sum of the fluorescence intensities obtained with all the filters was plotted
8
9 273 against temperature (Excel software) to obtain the maximum fluorescence intensity of SYPRO
10
11 274 Orange. The T_m was determined by plotting the first derivative of the previous curve; it appeared
12
13 275 then as a peak (Loison *et al.* 2016). This method is also known to allow the expression of
14
15 276 hydrophobic parts of the capsid and/or the permeability of the capsid to be investigated by the
16
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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23 24 280 **Data analysis**

25
26 281 The analysis was performed using the values obtained for the three independent suspensions of
27
28 282 phages. Since the experimental values were not normally distributed, non-parametric tests were
29
30 283 performed using StatEL 2.7 (Ad Science). The explanatory variables were the exposure to free
31
32 284 chlorine alone, heat alone, and free chlorine followed by heat. For free chlorine alone, Mann-
33
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
37
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
41
42 289 control and chlorinated MS2 phages. A Kruskal-Wallis test was performed to compare the
43
44 290 electrophoretic mobility between control and chlorinated MS2 phages. The effect of free chlorine
45
46 291 followed by heat on the loss of infectivity, structure (i.e. genome degradation, capsid permeability
47
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.

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

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2
3 320 MS2 phage by the oxidant. Although there was a decrease in the thermal stability of the capsid we
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5 321 did not find any changes in shape, size, and binding capacity, nor did the permeability to RNase
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7 322 increase thus suggesting that the capsid of MS2 phage maintained its integrity after exposure to free
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9 323 chlorine alone.

10
11 324 The decrease in the thermal stability after exposure to chlorine led us to expose MS2 phage to
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13 325 chlorine (100 mg.L⁻¹) and then to heat (55°C or 60°C). The infectivity decreased by $-1.9 \pm 0.3 \log_{10}$
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15 326 for chlorine followed by heat treatment at 55°C and by $-3.2 \pm 0.6 \log_{10}$ for chlorine followed by
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17 327 heat treatment at 60°C. For each treatment applied separately, the loss in infectivity reached –
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19 328 $1.2 \pm 0.3 \log_{10}$ after exposure to free chlorine (Table 1), $-0.7 \pm 0.1 \log_{10}$ after heat treatment at 55°C
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21 329 (data not shown) and $-1.1 \pm 0.1 \log_{10}$ after heat treatment at 60°C (Brié *et al.* 2016). The highest
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23 330 loss in infectivity was obtained when free chlorine was followed by heat treatment at 60°C (p
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25 331 <0.01 , Kruskal-Wallis test). This loss in infectivity was also higher than the sum of the decrease
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27 332 values obtained with free chlorine and heat treatment at 60°C applied separately ($p <0.01$, Kruskal-
28
29 333 Wallis test). This suggested that free chlorine improved the inactivation of MS2 phage by heat
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31 334 treatment at 60°C. The RT-qPCR signal decreased only by $-0.1 \pm 0.4 \log_{10}$ for chlorine followed by
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33 335 heat treatment at 55°C and by $-0.3 \pm 0.4 \log_{10}$ for chlorine followed by heat treatment at 60°C. The
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35 336 concentrations in genome observed after these treatments were similar to those observed for MS2
36
37 337 phage exposed to 100 mg.L⁻¹ free chlorine alone and also for control MS2 phage ($p >0.01$, Kruskal-
38
39 338 Wallis test). Thus, the detection of the MS2 genome was not affected when free chlorine was
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41 339 followed by heat. As observed for free chlorine alone, no modification of the shape of MS2 phage
42
43 340 was noted by TEM after exposure to free chlorine followed by heat (Fig. 1). The RNase treatment
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45 341 applied after exposure to free chlorine (100 mg.L⁻¹) followed by heat decreased the RT-qPCR signal
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47 342 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
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49 343 RNase treatment reached $-0.3 \pm 0.1 \log_{10}$ (Table 1) for free chlorine alone, $-0.1 \pm 0.1 \log_{10}$ (data
50
51 344 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
10
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
29
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
37
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
45
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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3 370 Whitney test). Thus, the increase in the negative charge density of MS2 phage after exposure to free
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5 371 chlorine seemed to be mainly linked to an increase in the external negative charge.

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9 373 **Impact of free chlorine applied alone or followed by heat on the hydrophobicity of MS2 phage**
10
11 374 **particles**

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13 375 The effect of free chlorine on the hydrophobicity of MS2 phage was then evaluated by DSF and by
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15 376 its adhesion capacity to hydrophobic beads. The DSF profile (Fig. 2) showed the same fluorescence
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17 377 intensity of SYPRO Orange at the beginning of the experiment for control and chlorinated phages,
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19 378 meaning that free chlorine did not modify the overall expression of hydrophobic domains of the
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21 379 capsid proteins. The expression of hydrophobic domains on the surface of MS2 particles was
22
23 380 compared between control and chlorinated phages using the adhesion ability to hydrophobic beads.
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25 381 The experiments were performed at pH 7.0 and high ionic strength (10 mM Na₂HPO₄ and 150 mM
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27 382 NaCl) in order to minimize the influence of the global charge. No adhesion to hydrophobic beads
28
29 383 was observed for control phages and those exposed to chlorine (Fig. 4). Thus, the results obtained
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31 384 using these two methods suggested that free chlorine did not change the expression of hydrophobic
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33 385 domains of the MS2 capsid proteins or the permeability of the capsid to the probe.

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37 386 Since we have previously described an increased hydrophobicity of MS2 phage after heat
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39 387 treatment (Brié *et al.* 2016), DSF experiments were carried out after exposure to free chlorine (100
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41 388 mg.L⁻¹) followed by heat (55°C or 60°C) and were compared to 55°C and 60°C heat treatments
42
43 389 applied alone (Fig. 5). Similar DSF profiles with low initial fluorescence were obtained for both
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45 390 control MS2 phages and those exposed to the 55°C heat treatment alone. The initial fluorescence
46
47 391 increased for MS2 phages exposed to the 60°C heat treatment alone and for those exposed to free
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49 392 chlorine followed by heat treatment at 55°C and 60°C. The highest initial fluorescence value was
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51 393 observed using 100 mg.L⁻¹ free chlorine followed by 60°C heat treatment ($p < 0.01$, Kruskal-Wallis
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53 394 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 ,
16
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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23 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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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
30
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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3 445 the loss of replication function played a lesser role in the overall inactivation than in the other two
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5 446 inactivation treatments (Wigginton *et al.* 2012). For these authors, this suggested that the virucidal
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7 447 effect due to genome damage was linked to the type of modification rather than its location.
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9 448 No modification of the shape of MS2 phage was observed by TEM after exposure to free
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11 449 chlorine. The mean size of MS2 phage before and after chlorination was similar to that previously
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13 450 described (Valegård *et al.* 1990; Dika *et al.* 2013a). Q β phage particles exposed to chlorine under
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15 451 similar conditions were also demonstrated by TEM observations to maintain their shape (Loison *et*
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17 452 *al.* 2016). Disruption of MS2 phage occurring after exposure to 72°C for 10 min has previously
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19 453 been shown to be related to a significant increase in RNase sensitivity (Brié *et al.* 2016). In our
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21 454 case, the limited effect of RNase treatment on RT-qPCR signal confirmed that the integrity of the
22
23 455 capsid was preserved after exposure to free chlorine under our experimental conditions. DSF was
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25 456 then used to explore conformational modifications of the capsid that could not be revealed by TEM.
26
27 457 We observed a similar DSF profile to that obtained in our previous study (Brié *et al.* 2016) with
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29 458 only one T_m peak (66°C) before exposure to chlorine. The decrease in the T_m value after exposure
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31 459 to free chlorine corresponded to a reduction in the denaturation temperature and suggested a
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33 460 weakening of the capsid of MS2 phage by the oxidant by comparison to a previous study performed
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35 461 on poliovirus (Robinson, Jesudhasan and Pfeiffer 2014). The weakening of the capsid might be
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37 462 linked to the site-specific cleavage showed on the capsid protein of MS2 phage following exposure
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39 463 to chlorine (Wigginton *et al.* 2012).
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44 464 The reactions of hypochlorous acid with amino acids result in a wide variety of products such
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46 465 as sulfinic/sulfonic acids, methionine sulfone, and *N*-chlorinated species (Pattison, Davies and
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48 466 Hawkins 2012) which may modify the electrostatic charge of viral particles. In our study, the
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50 467 charge density was evaluated by measuring the electrophoretic mobility as a function of ionic
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52 468 strength which suggested a decrease in the negative charge density of MS2 phage exposed to free
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54 469 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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42 489 protein.

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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

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3 494 phage particles at high temperatures (72°C) and transient expression of hydrophobic domains at
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5 495 lower temperatures (60°C) (Brié *et al.* 2016).

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7 496 An increased effect on the inactivation of MS2 phage and on the permeability of its capsid to
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9 497 RNase was observed when the 60°C heat treatment was applied after chlorination. To our
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11 498 knowledge, the virucidal effect of the combination of free chlorine and heat on enteric viruses and
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13 499 phages has not been studied before. Indeed, chlorine-induced inactivation of enteric viruses and
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15 500 phages in suspension in water has only been studied for temperatures below 30°C. The combined
16
17 501 effect of temperature and chlorination in foodstuffs has been more closely studied on bacteria,
18
19 502 showing an increase in inactivation compared to chlorine applied alone (Klaiber *et al.* 2005; Choi *et*
20
21 503 *al.* 2016). Moreover, these authors have shown that warm water (50°C) or dry heat (80°C) and
22
23 504 chlorine can be combined without altering the organoleptic quality or the viability of vegetables or
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25 505 seeds.

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27
28 506 Since hydrophobicity is known to be the main surface property affected by exposure of MS2
29
30 507 phages to heat (60°C) (Brié *et al.* 2016), a significant change in this parameter was expected when
31
32 508 free chlorine was followed by heat. As previously described by Brié *et al.* (2016) for MS2 phages
33
34 509 exposed to the 60°C heat treatment alone, the initial fluorescence of the DSF profile increased after
35
36 510 exposure to free chlorine followed by heat. The highest initial fluorescence value was observed
37
38 511 using free chlorine followed by a 60°C heat treatment. The increase in the initial fluorescence
39
40 512 showed that chlorine followed by heat led to the expression of hydrophobic domains and/or
41
42 513 increased the permeability of the capsid allowing the probe to reach inside hydrophobic regions.
43
44 514 The adhesion test to hydrophobic beads showed an increase in the expression of hydrophobic
45
46 515 domains at the surface of MS2 phages exposed to free chlorine followed by 60°C heat treatment.
47
48 516 Remarkably, this increase in the adhesion capacity to hydrophobic beads appeared to be specific to
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50 517 viral particles remaining infectious as previously observed for MS2 phage exposed to heat alone
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52 518 (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
4
5 520 capsid to the probe increased for both infectious and inactivated phages, whereas the increase in the
6
7 521 expression of hydrophobic domains at the surface was specific to phages remaining infectious after
8
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
14
15 525 chlorine has not been evaluated before for enteric phages or pathogenic viruses, but it has been
16
17 526 previously reported to be interesting for bacteria in foodstuffs. We observed that free chlorine
18
19 527 followed by heat increased the inactivation of MS2 phage and the structural modifications of the
20
21 528 capsid by comparison with these treatments applied separately. Our results suggested that the
22
23 529 oxidant should be applied before heat to have an optimal effect. Concerning the specific detection
24
25 530 of infectious viruses, the increase in permeability to enzymes such as RNases for inactivated phages
26
27 531 could offer new opportunities for such methods. In our opinion, the most important result
28
29 532 concerning free chlorine followed by heat is the specific increase in the expression of hydrophobic
30
31 533 domains at the surface of infectious MS2 phages. Indeed, this result suggested that hydrophobic
32
33 534 surfaces could be used to capture specifically infectious viruses before their detection using
34
35 535 molecular methods. Finally, the modifications observed on the capsid with our treatments underline
36
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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2
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4
5 545 experiments.

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8
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21 553 Crudettes, and Rosée des Champs).
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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.

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5 Figure 4. Binding to hydrophobic, magnetic beads at pH 7 in 10 mM PBS (10 mM Na₂HPO₄
6 and 150 mM NaCl) for control MS2 phages (before treatment) and those exposed to free
7 chlorine alone (100 and 200 mg.L⁻¹ for 10 min) or to free chlorine (100 mg.L⁻¹ for 10 min)
8 followed by heat (55°C or 60°C for 10 min). Three independent experiments were performed.
9 Error bars indicate standard deviations. C_X, concentration after binding assay; C₀, initial
10 concentration.
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22 Figure 5. SYPRO Orange signature of control MS2 phages (before treatment) and those
23 exposed to heat alone (55°C and 60°C for 10 min) or to free chlorine (100 mg.L⁻¹ for 10 min)
24 followed by heat (55°C or 60°C for 10 min). The curve represents one of three independent
25 experiments. Fluorescence intensity is given in arbitrary units (10⁶ × AU).
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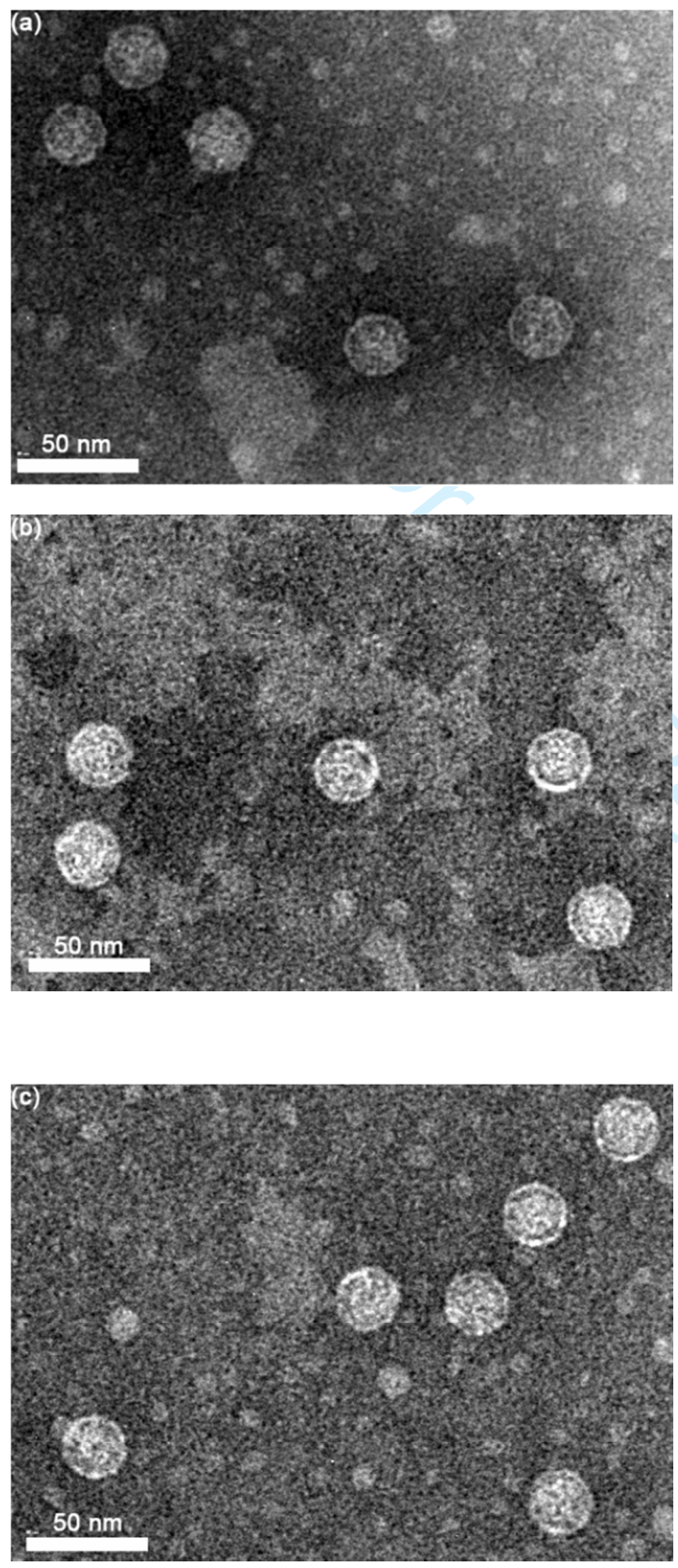
Table 1: Effect of chlorine (100 or 200 mg.L⁻¹ for 10 min at 4°C) followed or not by heat (55°C or 60°C for 10 min) on MS2 phage infectivity, genome degradation, and capsid permeability (sensitivity to RNase treatment). Experiments were performed in triplicate. SD = Standard Deviation.

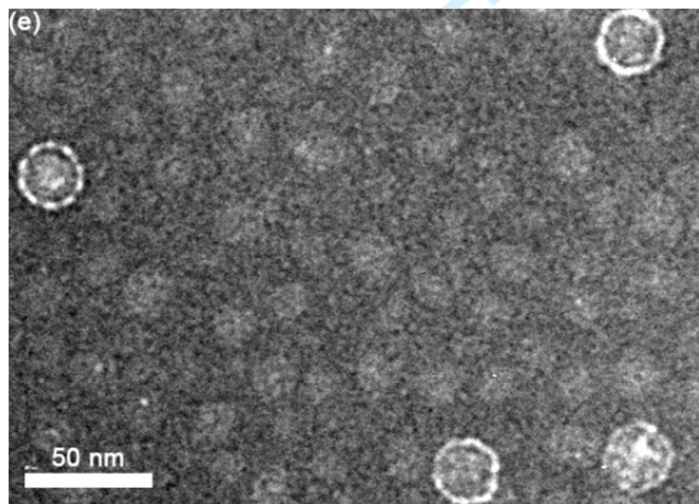
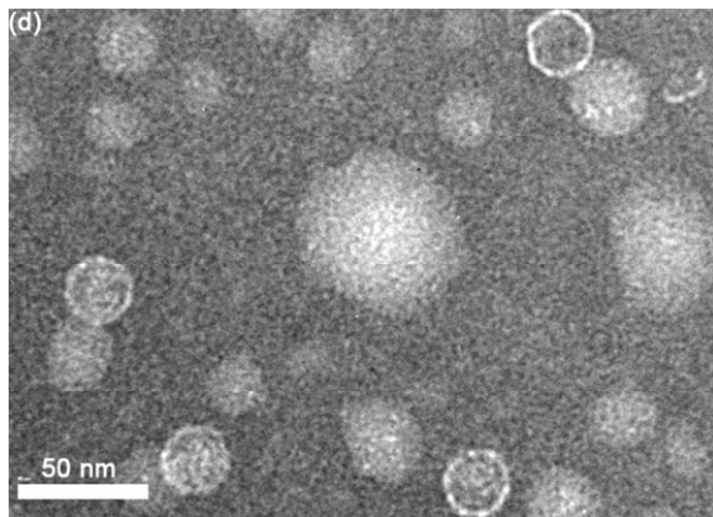
Free chlorine treatment (10 min)	100 mg.L ⁻¹	200 mg.L ⁻¹	100 mg.L ⁻¹	100 mg.L ⁻¹
Heat treatment	None	None	55°C, 10 min	60°C, 10 min
Loss of infectivity (log ₁₀ ± SD)	-1.2 ± 0.3	-3.5 ± 0.9	-1.9 ± 0.3	-3.2 ± 0.6
Loss of genome (log ₁₀ ± SD)	0.0 ± 0.0	-0.5 ± 0.2	-0.1 ± 0.4	-0.3 ± 0.4
Loss of genome with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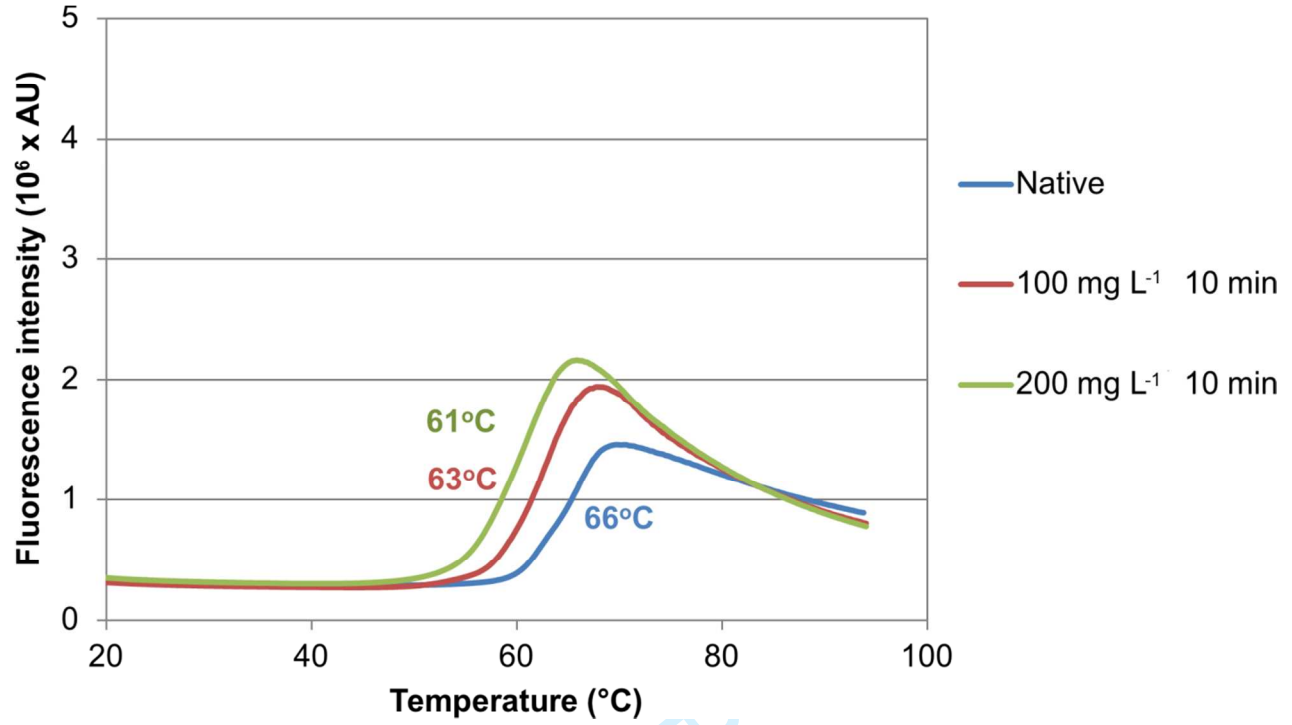
Figure 1





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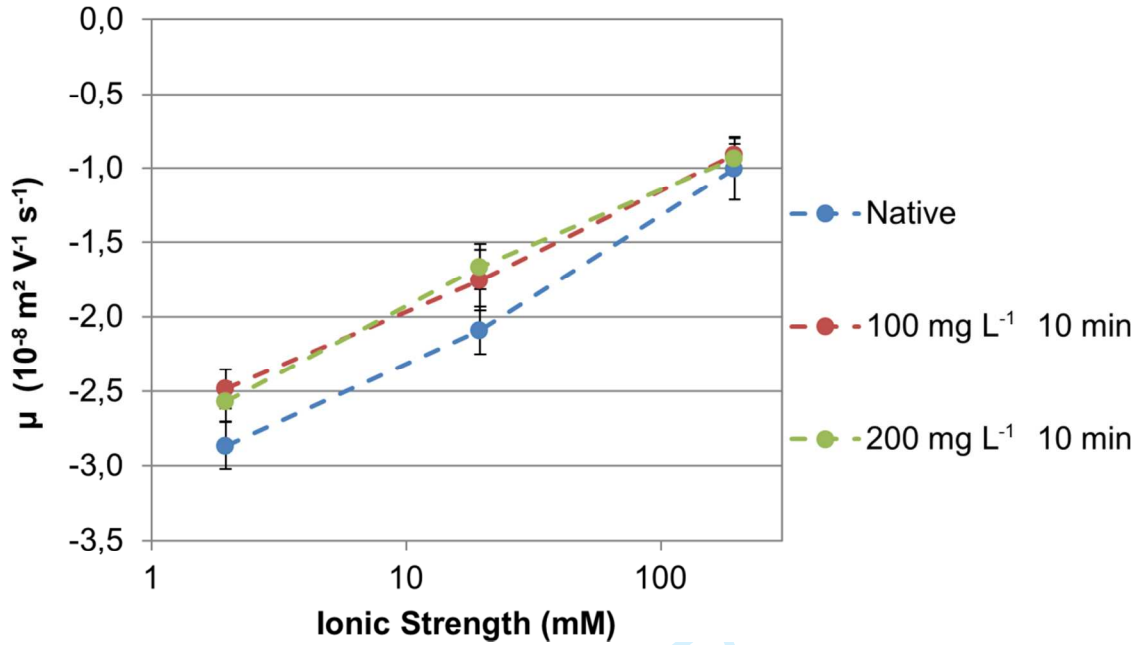
Figure 2



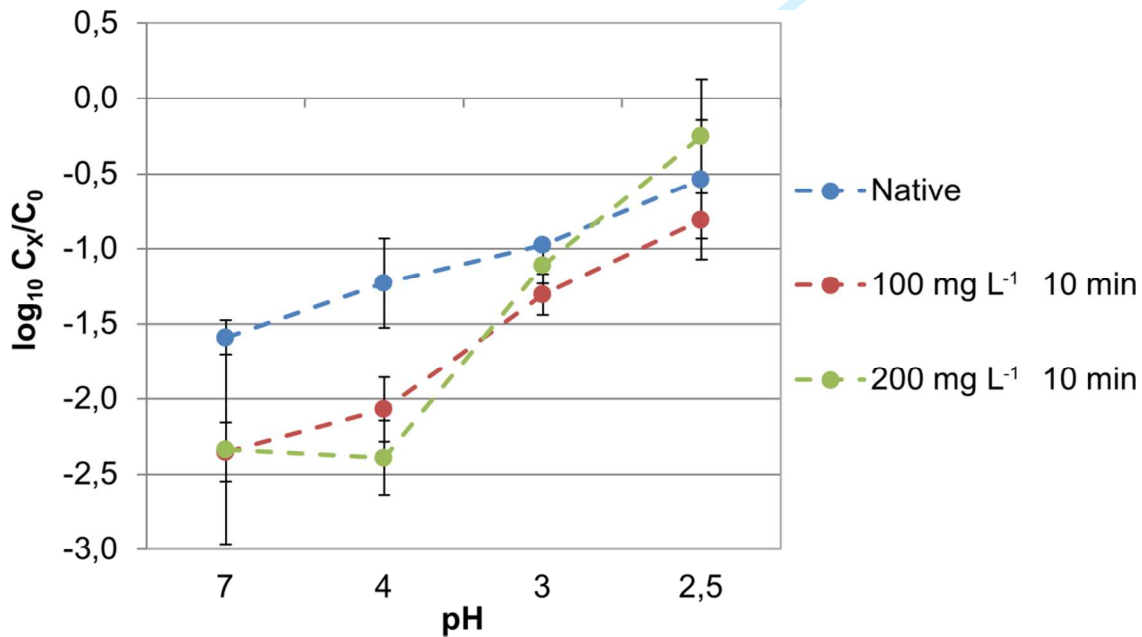
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Figure 3

(a)

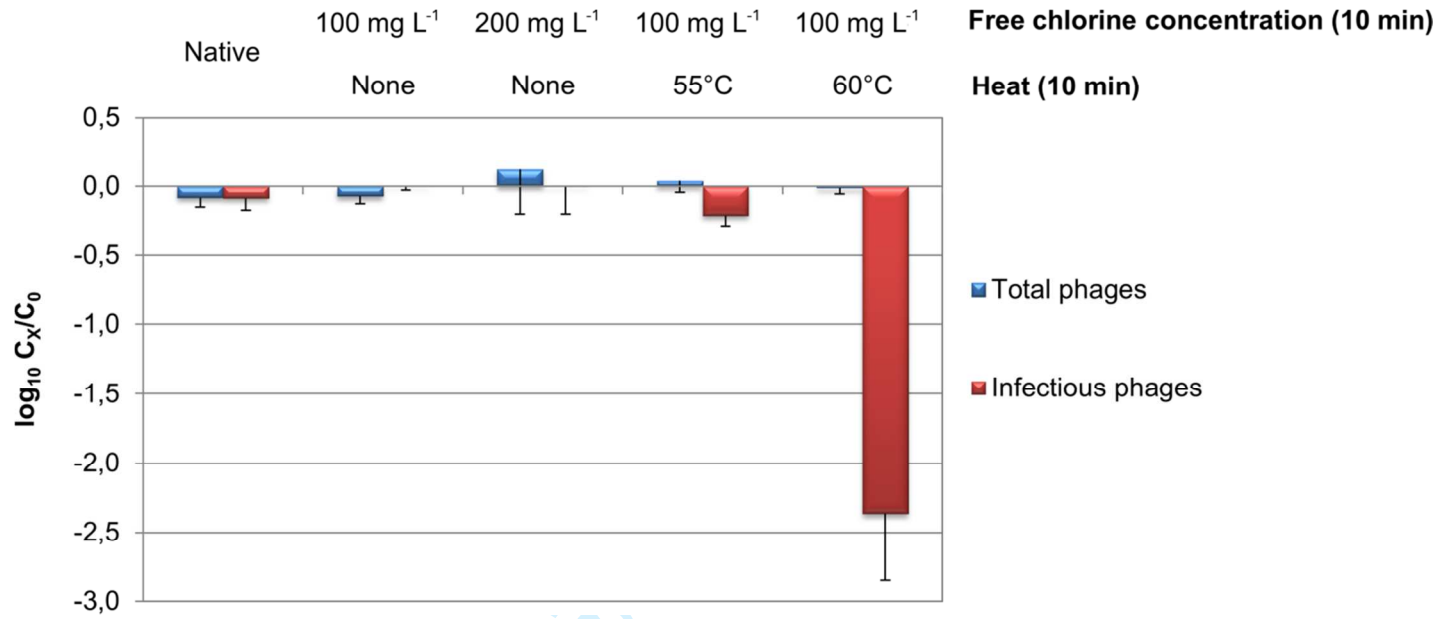


(b)



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Figure 4



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4 **Figure 5**
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