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1 **When are bacteria dead? A step towards interpreting flow cytometry profiles after**
2 **chlorine disinfection and membrane integrity staining**

3

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18 survival

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19 A B S T R A C T

20

21 Encouraged by the successful use of flow cytometry in water research, this versatile technology
22 is increasingly employed by drinking water providers. In addition to quantifying concentrations
23 of total bacteria, the use of a fluorescent viability stain allows the distinction between intact and
24 membrane-damaged cells, which makes it ideally suited for assessment of disinfection
25 efficiency. In contrast to plate counting, the technology allows the visualization of the gradual
26 loss of membrane integrity. Although this sensitivity *per se* is very positive, it creates the
27 problem of how to compare this detailed viability information with binary plate counts where
28 a colony is either formed or not. Guidelines are therefore needed to facilitate interpretation of
29 flow cytometry results and to determine a degree of membrane damage where bacteria can be
30 considered 'dead'. In this study we have tackled this problem by subjecting *E. coli* and
31 environmental micro-organisms in real water to a gradient of increasing chlorine
32 concentrations. Resulting flow cytometric patterns after membrane integrity staining were
33 compared with culturability and in part with redox activity. For laboratory-grown bacteria,
34 culturability was lost at lower disinfectant concentrations than membrane integrity making the
35 latter a conservative viability parameter. No recovery from chlorine was observed for four days.
36 For real water, loss of membrane integrity had to be much more substantial to completely
37 suppress colony formation, probably due to the heterogenic composition of the natural
38 microbial community with different members having different susceptibilities to the
39 disinfectant.

40 **Introduction**

41 Flow cytometry as a rapid method for quantifying concentrations of total and intact bacterial
42 cells is becoming an important tool for assessing microbiological water quality. Robust
43 protocols have been developed [1,2,3]. For the assessment of total cell concentrations,
44 validation in ring trials led to the recommendation of the technology in the Swiss Food
45 Compendium for assessing microbiological fresh water quality [4]. The importance of flow
46 cytometry will be greatly strengthened in the future with online measurements, which are
47 already being practiced in academic research [5,6] and which will become more common with
48 the availability of commercial instruments allowing high frequency assessments.

49 Applications of flow cytometry includes quantification of bacterial concentrations in
50 raw water [7,8], monitoring changes along water treatment processes [9,10] and evaluating
51 disinfection efficiency [11,12]. Of particular importance for disinfection is understanding and
52 assessing bacterial viability so that water treatment practitioners can distinguish between
53 bacterial populations with different viability statuses. A common staining technique that is used
54 to achieve this involves two fluorescent dyes, SYBR Green I (or alternatively SYBR Green II)
55 and propidium iodide. While the green SYBR dye enters all cells, independent of their
56 membrane integrity, the red 'viability dye' propidium iodide selectively enters cells with
57 compromised cell membranes [13]. This double staining was applied in this study to all samples
58 to assess cell envelope integrity. The two dye approach (using SYTO™ 9 and propidium iodide)
59 also forms the basis of the well-known LIVE/DEAD® BacLight™ bacterial viability kit applied
60 in microscopy and other assays.

61 One of the most critical hurdles for flow cytometry to evolve from a research tool to a
62 diagnostic method that can realistically be applied in the water industry, is the use of fixed
63 operational and gating settings. In the flow cytometric vocabulary, gates are areas on the scatter

64 plot that are defined by the user and determine the signals that are deemed relevant. Gates that
65 work well with the majority of water samples have been developed for proprietary flow
66 cytometers [3,13,14]. In combination with cell integrity staining, bacteria that are located in
67 such a gate are seen to have intact cell membranes. However, despite the benefit of such fixed
68 gates, the question remains as to the extent to which cells that are detected within the gated
69 areas (after cell integrity staining) are viable in the traditional sense meaning whether they grow
70 on nutrient agar and therefore fulfil the classical criterion of being 'viable'. When cells are
71 subject to detrimental or even lethal conditions (with an effect on membrane integrity), a
72 transition in the fluorescence pattern from the position of cells being defined as intact to the
73 position of cells being defined as irreversibly damaged has been described [12,13]. The shape
74 of the transition is characteristic and, depending on instrumentation, signal compensation and
75 other settings, often resembles a 'crescent moon'. The movement of signals reflects the amount
76 of propidium iodide entering the cells and thus the range of different integrity states between
77 two extremes of being completely intact/live and heavily damaged/dead. It occurs because there
78 is a shift in cells having a strong green and weak red signal intensity to an increased red and
79 weaker green intensity [13]. The question that arises from this distinctive transitory shift is at
80 which point in the transition the signals can be attributed to cells still being alive and where
81 cells should be considered as dead?

82 To make a step towards a better interpretation of flow cytometric patterns, we subjected
83 *E. coli* (in two different cell concentrations in 0.2 µm filtered mineral water) to a chlorine
84 gradient of increasing strength. We compared the resulting flow cytometry patterns after cell
85 integrity staining with the culturability of the cells and their redox activity. For the *E. coli*
86 suspension in a concentration of 10^5 cells mL^{-1}, we additionally addressed whether the bacteria
87 (after chlorine disinfection and neutralization of the disinfectant) can recover and potentially
88 regain culturability after repair of damage, while being stored in water. To see whether results

89 obtained from pure bacterial suspensions hold true also for natural water, pre-chlorine contact
90 tank samples from a water treatment plant were subjected to a chlorine gradient and FCM
91 patterns were compared with heterotrophic plate counts.

92 **Materials and methods**

93

94 ***Bacteria and growth conditions***

95 *Escherichia coli* (*E. coli*; ATCC 25922) were grown in 10% tryprone soy broth (TSB CM0129,
96 Oxoid, Basingstoke, Hampshire, UK) for 15 hours at room temperature (approx. 20°C) at 290
97 rpm. The optical density (OD_{600}) of the culture was adjusted with 10% broth to 1.0 using a
98 spectrophotometer (Jenway 6310, Essex, Dunmow, CM6 3LB). This optical density
99 corresponds to a bacterial concentration of 10^9 *E. coli* cells mL^{-1}. Aliquots of 1 mL were
100 subsequently harvested by centrifugation (5,000 g, 5 min) and the supernatants carefully
101 removed. The resulting bacterial pellets were washed three times by resuspension in 0.2 µm
102 filtered mineral water (Evian, France) with a pH of 7.2. Bacterial suspensions were either used
103 undiluted (for high cell density experiments) or diluted 100-fold by adding 100 µL cell aliquots
104 to 10 mL of filtered mineral water to obtain a concentration of 10^7 cells mL^{-1}. This first dilution
105 was followed by another 50- fold dilution resulting in a suspension of 2 x 10^5 cells mL^{-1}.

106

107 ***Preparation of chlorine demand-free glassware***

108 All experiments were performed using chlorine demand free glassware to minimize interference
109 of substances exerting chlorine demand with the effect of the disinfectant on bacteria. Organic
110 contaminants were removed following the method described by Charnock and Kjønnø [15].
111 Borosilicate glass beakers were initially machine washed with detergent, rinsed first with
112 normal tap water and subsequently rinsed three times with ultrapure water. Beakers were filled
113 with 0.2N hydrochloric acid (HCl), covered with aluminium foil and left overnight to hydrolyze
114 organic compounds. Acid was discarded the next morning and glassware was rinsed three times
115 with ultrapure water, air-dried and capped with aluminium foil. Removal of residual carbon

was achieved by heating to 550°C for at least six hour in a Muffle furnace (Muffle Furnace 1400, Pave Testing Ltd, Hertfordshire, UK). Glassware was stored in a dry place until use.

Chlorine disinfection

The chlorine solution was made in chlorine demand-free glassware by diluting the chlorine stock solution (11.15%, Sigma Aldrich, St. Louis, MO, USA) to the desired final concentration using ultrapure water (Ultra GE MK2 Purelab, Elga, High Wycombe, Buckinghamshire, UK). Chlorine was applied to three systems:

Chlorine exposure of E. coli at a high cell concentration. *E. coli* cells resuspended in mineral water and with an OD_{600} of 1.0 were aliquoted (1 ml each) into chlorine-demand free glass tubes. Chlorine solutions (200 µl each) with concentrations of 6, 15, 30, 60, 120 and 240 mg L^{-1} were added to the cell suspensions to obtain final chlorine concentrations of 1, 2.5, 5, 10, 20 and 40 mg L^{-1} followed by thorough mixing. Chlorine exposure was stopped after 10 min by addition of 50 µl of 0.1 N sodium thiosulphate (Acros Organics, Geel, Belgium). Cells were washed twice with filtered mineral water to remove residual chlorine following the procedure described earlier and eventually resuspended in 1 ml of filtered mineral water.

Chlorine exposure of E. coli at a low cell concentration. For experiments using an *E. coli* concentration of 10^5 cells mL^{-1}, the chlorine stock was diluted with ultrapure water to a concentration of 200 mg L^{-1}. This solution was further diluted with filtered mineral water (to ensure osmotic balance) in seven conical flasks to obtain volumes of 49.5 ml with the following chlorine concentration gradient: 0, 0.005, 0.01, 0.025, 0.05, 0.1 and 0.25 mg L^{-1}. Then 0.5 mL of the 10^7 cells mL^{-1} bacterial solution was added to the conical flasks to a final concentration of 10^5 cells mL^{-1}. The flasks were shaken at 290 rpm on an orbital shaker for 30 minutes. The chlorine was immediately quenched by adding 0.5 mL of thiosulfate (0.1 N; Acros organics by thermos Fischer Scientific, Geel, Belgium).

141 *Disinfection of real water samples.* A water sample was collected post rapid gravity filtration
142 from a water treatment works (WTW) in Scotland, UK. In brief, the process stream was (A)
143 raw water inlet, (B) coagulation, (C) dissolved air flotation, (D) rapid gravity filtration, (E)
144 chlorination, (F) storage and distribution. Disinfection experiments were performed by adding
145 aliquots of chlorine solution to 100 mL of water in AOC-free conical flasks. Final chlorine
146 concentrations were 0.01, 0.025, 0.05, 0.10, 0.25, 0.50, 0.75 and 1.00 mg L^{-1}. Samples without
147 chlorine served as controls. The flasks were shaken for 30 minutes to allow for sufficient mixing
148 and chlorine was subsequently quenched by addition of 1 mL of 0.1 N of thiosulfate (Acros
149 Organics, Geel, Belgium).
150

151 ***Analysis by cultivation***

152 For high cell density experiments, 100 µl aliquots of cell suspensions were pipetted into the top
153 row of sterile 96-well microtiter plates (Porvair Sciences Ltd., Wales, UK). Using a
154 multichannel pipette, these suspensions were successively diluted by a factor of 10 by mixing
155 10 µl of cells with 90 µl of 0.2 µm filtered mineral water pre-aliquoted in the lower rows. All
156 dilutions (1 µl each) were spotted onto square Petri dishes (manufacturer) filled with Membrane
157 Lactose Glucuronide Agar (MLGA CM1031, Oxoid, Basingstoke, Hampshire, UK). After brief
158 drying, plates were incubated at 35°C for approximately 20 hours.

159 When working with low cell densities and real water, samples were filtered after
160 chlorine disinfection and chlorine quenching by applying 1 mL or 10 mL aliquots onto 0.45 µm
161 filters (Millipore S-PAK® 47 mm, Watford, UK) placed on a vacuum manifold (Combisart,
162 Sartorius, UK). To facilitate even filtration of 1 mL aliquots, 5 mL of filtered mineral water
163 was added to each funnel before the addition of the sample. Filters were placed on 55 mm petri-
164 dishes containing MLGA (for *E. coli*) or R2A (for real water samples). The petri-dishes were

165 subsequently incubated for 24 hours at a temperature of 35°C (to allow growth of *E. coli*) or for
166 5 days at 22°C (to allow growth of heterotrophic bacteria from real water).

167 Pictures of plates were made on a ProXima C16 Phi+imaging system (Isogen Life
168 Science, Netherlands) using the following grayscale settings: exposure 40 ms, zoom 3.0, iris
169 3.1, focus 84, no filter.

170

171 ***Measurement of redox activity***

172 To measure redox activity, 17 mg of WST-8 (GenScript, Piscataway, USA) and 13.8 mg of
173 menadione (2-methyl-1,4-naphthoquinone; ACROS Organics, Geel, Belgium) were dissolved
174 in 2.43 ml nanopure water and 10 ml DMSO, respectively, to obtain concentrations of 10 mM
175 and 8 mM. Stock solutions were stored at -20°C. A detection reagent was prepared by mixing
176 WST-8, menadione and water at ratios of 9:1:10. 20 µl of this solution was aliquoted into the
177 wells of a 96 well plate, followed by addition of 100 µl of TSB. The reaction was started from
178 addition of 80 µl of cell suspension using a multichannel pipette. After thorough mixing by
179 pipetting up and down several times, plates were immediately transferred to a TECAN
180 M200 plate reader (TECAN, Austria). Signals were measured at 460 nm at time 0 and after 1,
181 6, 12 and 24 hours. Prior to each measurement, plates were shaken for 5 sec (linear shaking
182 using an amplitude of 3).

183

184 ***Flow cytometric analysis***

185 Flow cytometric analysis was carried out following the protocol developed by Hammes et al.
186 (2008) with a few amendments. A 10,000× stock of SYBR Green I (SG; cat. S-7567; Life
187 Technologies Ltd., Paisley, UK) was diluted with dimethyl sulphoxide (DMSO) (Fisher
188 Scientific, Fair Law, NJ) to a working 100× concentration stock. This working stock solution
189 was mixed with propidium iodide (PI) (1 mg mL^{-1}, corresponding to 1.5 mM; cat. P3566; Life

190 Technologies Ltd, Paisley, UK) at a ratio (v/v) of 5:1 (v/v, SG:PI). 2.4 µL of dye solution was
191 aliquoted into the wells of a 96-well plate prior to addition of 200 µL of cell suspension using
192 a multichannel pipette (final dye concentrations: 1xSG, 3 µM PI). For real water, total cell
193 concentrations were determined accordingly by staining with 2 µL of SYBR Green I, but
194 omitting PI. Mixtures were incubated at 37°C for 13 min (SLMB 2012) in a Grant-bio PHMP
195 thermo-shaker (Grant Instruments Ltd, Cambridgeshire, UK) at 600 rpm, followed by analysis
196 on a BD Accuri C6 cytometer (Becton Dickinson UK Ltd., Oxford, UK) equipped with a 588
197 nm laser. The settings were as follows: 25 µL sample volume and fast flow rate (66 µL/min).
198 To eliminate background noise, the trigger was set on FL-1 with a threshold of 2,000 units for
199 pure culture samples and to 600 for real water samples. Signals were recorded on a FL-3 (red
200 fluorescence, 670 nm) vs FL-1 (green fluorescence, 533 nm) density plot, using the gate
201 settings described by Gatza et al. [14].

202 **Results**

203 *E. coli*, used here as a prominent indicator organism, and bacteria in a real water were subjected
204 to increasing chlorine concentrations followed by assessment of culturability and indirect
205 viability parameters. *E. coli* suspensions were used both at high cell concentrations (10^9 cells
206 mL^{-1}, allowing for activity and membrane integrity measurements on a fluorescence plate
207 reader) and at low cell concentrations (10^5 cells mL^{-1}). For the latter, the sustainability of the
208 chlorine effect was studied over 72 hours.

209

210 ***Relationship between culturability, activity and membrane integrity for high cell densities***

211 *E. coli*, grown overnight and washed in mineral water, was subjected to chlorine concentrations
212 up to 40 mg L^{-1} for 10 min. Following chlorine exposure and quenching, samples were serially
213 diluted and aliquots of the dilution series spotted on MLGA plates to test for viability (Figure
214 1A). A suspension that was not exposed to chlorine served as a control, growth was obtained
215 up to a dilution of 10^5-fold. Compared to the control, increasing chlorine concentrations up to
216 5 mg L^{-1} resulted in an incremental loss of culturability. No growth was observed after exposing
217 cells to chlorine concentrations exceeding 10 mg L^{-1}. This loss in culturability was reflected in
218 an increasing loss in redox activity as measured by the reduction of the water soluble
219 tetrazolium dye WST-8 on a plate reader (Figure1B). Development of reduction activity was
220 measured for up to 24 hours. Activity was measured fastest for the control that had not been
221 exposed to chlorine. As is typical for this type of assay, signal intensity declined after reaching
222 peak absorbance. For samples exposed to chlorine concentrations of 1, 2.5 and 5 mg L^{-1}, WST-
223 8 reduction was increasingly delayed in a concentration-dependent manner. At 5 mg L^{-1}
224 chlorine, activity was only measured after a long delay (12-24 hours) suggesting that bacteria
225 either needed time for cellular recovery or that the proportion of active cells was low. Redox

226 activity was eventually eliminated after exposure to 10 mg L^{-1} chlorine or higher concentrations,
227 in line with the complete loss of culturability at this disinfectant concentration.

228 The status of membrane integrity is reflected in the flow cytometric patterns (Figure1C).
229 Here, chlorine exposure led to the distinctive migration of signals from the red gate to a zone
230 outside of the gated area confined by a dotted line. Under the chosen instrument settings the
231 migration can be referred to as 'crescent moon'. At low chlorine concentrations (1-2.5 mg L^{-1}),
232 this signal migration was visible in its very early state and was more prominent with increasing
233 chlorine concentration at 5 mg L^{-1} and 10 mg L^{-1}. At higher chlorine concentrations all signals
234 had disappeared from the gated area indicating heavy membrane damage.

235

236 ***Relationship between culturability and membrane integrity for low cell densities***

237 A similar experiment was performed with an *E. coli* suspension at a low cell concentration of
238 10^5 cells ml^{-1}. Compared to the high cell density experiment, substantially lower disinfectant
239 concentrations were required due to the lower chlorine demand exerted by the bacterial
240 biomass.

241 Cells were challenged with increasing chlorine concentrations, inducing both an
242 increasing loss of membrane integrity and culturability (Figure 2A). Strong growth of undiluted
243 suspensions on filters was obtained for chlorine concentrations up to 0.025 mg L^{-1}, coinciding
244 with the majority of flow cytometric signals being located in the gated area. At a chlorine
245 concentration of 0.05 mg L^{-1}, a strong signal migration was evident with cells migrating into an
246 interim zone defined by a second gate. Culturability for this sample was limited to a few
247 colonies on the filter. Higher chlorine concentrations resulted in FCM signals clearly located in
248 the zone associated with heavy membrane damage, in agreement with the complete loss of
249 culturability observed.

250 Dilution of cell suspensions allowed for quantification of colony forming units. Fig. 2B
251 shows culturable cell numbers both directly after disinfection (and quenching) and their change
252 over 72 hours to address the question whether the effect of chlorine was sustainable. No
253 significant difference in culturability was obtained between samples without chlorine exposure
254 or after challenge with 0.005 or 0.01 mg L^{-1} chlorine. Concentrations of culturable *E. coli*
255 remained similar to time point zero for the following 72 hours. A disinfectant concentration of
256 0.025 mg L^{-1} resulted in a modest loss of culturability at time point zero followed by a small
257 further decline over the studied time course. At 0.05 mg L^{-1} chlorine a substantial drop in CFU
258 (by nearly 4 log units) was obtained directly after disinfection reflecting the visibly strong
259 reduction seen when filtering undiluted cell suspensions. Concentrations of culturable cells
260 remained thereafter comparable to time point zero. The culture data was in agreement with
261 cultivation-independent data. Flow cytometric patterns are shown for samples subjected to
262 0.025 and 0.05 mg L^{-1} chlorine (Figure 2C). Initially the bacterial population comprised the *E.*
263 *coli* population that was used for the experiment. These specific *E. coli* populations remained
264 static in the FCM plots over the 72 hours of the experiment suggesting that the cells that form
265 those clusters did not recover and restore membrane integrity. *E. coli* FCM signals were,
266 however, increasingly supplemented by other signals originating from unidentified bacteria that
267 grew under the given non-selective conditions. This was supported by the appearance of reddish
268 colonies on the filters on MLGA agar (*E. coli* cells should appear green on this media). The
269 bacteria giving rise to the red colonies were not added deliberately, but probably represent a
270 'contaminating' bacterial population that entered the vials after
271 chlorine quenching and during sample processing. This population was obtained reproducibly.
272 The fact that these bacteria could grow suggests that conditions were suitable for cell replication
273 and probably also repair of damage. They were therefore seen as a valuable "internal growth
274 control'. Lack of *E. coli* regrowth was not due to insufficient chlorine neutralization, lack of

275 nutrients or otherwise adverse conditions. For this reason, the terminology 'viable-but not
276 culturable' (VBNC) is therefore avoided for the bacteria with transitory fluorescent signals.
277 The presence of VBNC cells can of course not be excluded and would need further experiments.

278

279 ***Relationship between culturability and membrane integrity for real water samples***

280 Pre-contact tank water from a drinking water works was subjected to a chlorine gradient from
281 0.025 to 1 mg L^{-1} for 30 min each. Not considering chlorine demand, this range corresponds to
282 Ct values between 0.75 to 30 mg min L^{-1}, comparable with experiment 2. The pH of the water
283 was 6.25 and the chlorine demand within 30 min was measured to be approximately 0.11 mg
284 L^{-1}. The effect of chlorine was measured both by counting of colonies supported by R2A (5
285 days at 22°C) and flow cytometry. Both techniques showed that an increasing concentration of
286 chlorine had an increasing impact on the bacterial community naturally contained in this water.
287 Despite variation between different samples in regard to overall cell numbers and
288 susceptibilities to chlorine, the overall observation was that culturability did not drop faster than
289 the intact cell signals in flow cytometry. Whereas the culturability of *E. coli* was shown to be
290 impacted at lower chlorine concentrations than flow cytometric signals (meaning culturability
291 was affected stronger than membrane integrity), the trend was in part reversed with real water.
292 A representative example is shown in Figure 3A. In other repeats, culturability was not lost
293 completely even with highest chlorine concentrations, meaning that colony formation of some
294 bacterial survivors persisted even when severe membrane damage was inflicted on the majority
295 of the bacterial population. For the example shown in Figure 3A, data is quantitatively
296 expressed in Figure 3B demonstrating that log removal of intact cells was faster than the log
297 removal of heterotrophic plate counts when using chlorine. The decrease in intact cell
298 concentrations was accompanied also by a decrease in total cell concentrations, although less

299 dramatically (Figure 3C). This can be explained by the fact that chlorine at higher doses does
300 not only damage the bacterial cell envelope, but also their nucleic acids. This damage can result
301 in weaker dye binding and fluorescent staining [16].

302 Interestingly, colonies arising on R2A along the chlorine gradient displayed different
303 colours. Whereas beige/cream colours dominated in the unchlorinated sample, their relative
304 proportion dropped with increasing chlorine concentrations to 0.25 mg L^{-1} (Figure 3D). Pink
305 colonies on the other hand tended to increase along the disinfectant gradient and yellowish
306 colonies were visible up to 0.1 mg L^{-1} chlorine. Overall the colony composition of samples
307 subjected to 0.25 and 5 mg L^{-1} chlorine was clearly distinct from the one in the non-chlorinated
308 sample. The observation suggested that different groups of microorganisms contained in real
309 water have very different susceptibilities to chlorine.

Discussion

Fluorescent staining using a combination of a membrane-permeant and a membrane-impermeant dye is one of the most commonly used cultivation-independent methods in microbiology research to assess microbial viability. Although it is based on only one viability criterion and therefore rather distinguishes between ‘intact’ and ‘damaged’ cells than ‘live’ and ‘dead’ cells, this procedure has beyond doubt added a vast new layer of knowledge to viability research compared to exclusively cultivation-based approaches. With the disclaimer that “no staining technique can give a guaranteed answer about a bacterial cell’s reproductive viability” [17], analysis of membrane damage has been suggested to be well suited for viability assessment when severe physico-chemical cellular damage is expected, including disinfection by oxidants [18,19]. The attractiveness for combining this staining approach with flow cytometry lies in the ability of the method to visualize intermediate integrity states as they occur for example after cell injury at low disinfection intensity. With the wider use of the method in routine water laboratories whose traditional focus is on culturability to comply with regulations, the interpretations of the resulting fluorescent patterns in relationship to classical culture data (that will for the foreseeable future remain the gold standard) is becoming more important.

Viability of laboratory-grown E. coli along transition from intact to damaged

A nice example of the ability of flow cytometry to reflect the transition from intact to damaged was demonstrated by Berney et al. [13] when studying the effect of artificial UV-A light. Increasing irradiation resulted in the curve-shaped transition that was later also described for other treatments like heat [20] or chlorine dioxide [12]. The goal of our research was to produce such transitions for chlorine exposure. Adequate disinfection conditions were chosen to obtain a gradient with distinct flow cytometric patterns. Data suggests that cells

335 whose fluorescence signals are located in the transitory staining zone had either just lost
336 viability or were in the process of losing it. *E. coli* subjected at high cell concentrations to 5 or
337 10 mg L^{-1} chlorine show greatly reduced growth or had just lost culturability, respectively
338 (Figure 1). Flow cytometric signals for both samples were in transition. The same result was
339 obtained with *E. coli* at lower cell concentration (Figure 2). As soon as flow cytometric
340 bacterial signals began their transition from intact to damaged in the characteristic crescent
341 moon fashion, culturability was impacted. The impact of chlorine on viability was also clearly
342 visible, on the other hand, when measuring redox activity (Figure 1). To overcome the lack of
343 sensitivity of this plate reader-based assay, the assay time however had to be extended to 24
344 hours to allow the treated (and probably injured) cells to measurably reduce the added dye
345 WST-8. Redox activity within the assay time was still measureable after exposure to 5 mg L^{-1}
346 disinfectant, although with a substantial delay compared to sample without chlorine. *E. coli*
347 exposed to higher chlorine concentrations did not display measurable activity. Flow
348 cytometric signals of these cells were either in the process of leaving the gated area or were
349 located outside of it. The observation is relevant as for heat inactivation actively respiring
350 cells were associated previously with a higher rate of recovery [17].

351 Overall the interim state visualized by flow cytometry by the migration of fluorescent
352 signals can be seen as a critical transition between live (in the classical meaning of culturable)
353 and dead. Culturability was hereby impacted earlier than membrane integrity, which matches
354 the outcome of other studies [19,21,22]. When measuring different viability parameters of
355 chlorine-exposed *E. coli* O157:H7, Lisle et al. reported that the physiological indices were
356 affected in the order: viable plate counts > substrate responsiveness > membrane potential >
357 respiratory activity > membrane integrity [19]. The reason probably lies in the fact that the
358 action of chlorine is not selective and oxidative damage is inflicted not only to the cell
359 envelope, but also to other cellular components like key enzymes and nucleic acids [23]. In

360 the case of other treatments, the relationship can be different. When assessing the effect of
361 different stresses on different individual viability parameters of *Listeria monocytogenes*, it
362 was observed that the order they were lost strongly depended on the treatment that was
363 applied [24]. In the case of detergent application (in form of quaternary ammonium
364 compounds), loss of culturability and membrane integrity was better correlated than in the
365 case of treatment with oxidants. This suggest that the data of the present study only apply to
366 chlorine, whereas flow cytometric signals have to be newly matched with culturability and
367 other viability parameters for other biocidal treatments.
368

369 ***Effect of chlorine on bacteria in real water***

370 Interestingly the relationship between membrane damage and culturability was different for
371 the bacterial population naturally contained in water than for laboratory-grown *E. coli* when
372 exposed to chlorine. With real waters, colony formation was partly still obtained even when
373 the majority of flow cytometric signals had disappeared from the gated area. The
374 microbiological diversity contained in real water might be the most self-evident explanation.
375 It is, for example, known that gram-positive bacteria are more resistant to chlorine than gram-
376 negative ones [25]. Apart from different susceptibilities of bacterial species to chlorine,
377 environmental bacteria tend to be generally more resistant than laboratory grown cultures.
378 Factors that can contribute to increased chlorine resistance of environmental bacteria include
379 bacterial attachment to particles and aggregation and nutrient effects [26-28] and general
380 greater diversity in physiological traits [12]. For *E. coli,* the development of a slimy coat has
381 been reported after successive chlorinations accompanied with a 10-fold increase in resistance
382 (29). Any relationship between plate counts and flow cytometric results might in part also be
383 attributed to the growth medium used for plating. It is important to reflect in this context that
384 the number of obtained colonies is not fixed, but depends on the choice of media and the

385 given growth conditions [30,31]. Further research will be necessary to substantiate the effect
386 of growth conditions (including temperature and recovery periods) and to compare the
387 behaviour of micro-organisms in more types of environmental water. As the bacterial
388 populations in different water types have different compositions, they show different
389 percentages of culturability and have different growth requirements. As culturability by itself
390 is limited to a small fraction of the total bacterial population, it is prone to variation. Another
391 question that remains to be answered is whether the chlorine effect with real water is
392 sustained over several days, similar to the outcome seen with laboratory grown *E. coli*. More
393 recovery assays in presence of different nutrient concentrations and recovery supplement will
394 be necessary in future studies as done previously with heat-injured cells [17].
395 Last but not least the observation that colours of colonies obtained at different chlorine
396 concentrations were subject to a trend (with pink colonies gaining relative abundance)
397 supported the selective effect of chlorine on different bacterial species. Differential resistance
398 of waterborne bacteria has been reported before for both chlorine-treated wastewater effluent
399 [32] and drinking water with chloramine residual [33]. For the latter, pyrosequencing in
400 combination with viability PCR showed a clear effect of increasing Ct values of
401 monochloramine exposure on the composition of the intact fraction of the bacterial
402 community. A similar outcome can be expected for chlorine.

403 *Conclusions.*

404 This study addressed the relationship between bacterial culturability and flow cytometric
405 signals after staining with SYBR Green I and propidium iodide. Laboratory-cultured bacteria
406 were seen to lose culturability when the membrane was not fully compromised. Without
407 applying resuscitation measures or adding recovery supplement, the effect of chlorine
408 appeared sustainable over several days supporting the view that bacteria identified with strong
409 membrane damage might be seen as dead in a relevant time frame for water treatment and
410 supply systems. Environmental bacteria, on the other hand, required a complete migration
411 from the applied 'intact cell envelope' gate to ensure loss of colony formation. The most
412 probable reason for the higher chlorine resistance of bacteria in the latter is in the diversity of
413 the bacterial flora contained in real waters. Different colours of colonies arising after
414 treatment with different chlorine concentrations support the view of different bacterial
415 chlorine susceptibilities. Future research involving cell sorting might be able to attribute their
416 positions in the flow cytometric patterns.

417 **Figure Legend**

418

419 Figure 1. Measurement of the effect of chlorine on high cell densities of *E. coli* (10^9 cells mL^-
420 ^{1}) based on assessment of culturability (A), redox activity (B), and membrane integrity (C).
421 Culturability was determined by growth on MLGA plates and redox activity by measurement
422 of WST-8 reduction on a plate reader platform. Cells were allowed to develop redox activity
423 for 1, 6, 12 or 24 hours after neutralization of the chlorine and addition of WST-8. Membrane
424 integrity was determined using a flow cytometer after staining samples with SYTO9 and
425 propidium iodide. Error bars show standard deviations from three independent repeats.
426 Representative pictures are shown for culturability and flow cytometry.

427

428 Figure 2. Effect of chlorine on viability of *E. coli* at a cell density of 10^5 cells mL^{-1} measured
429 by culturability and flow cytometry. Cell suspensions were exposed to chlorine for 30 min
430 followed by chlorine quenching. (A) Flow cytometric profiles of *E. coli* directly after
431 disinfection in comparison with growth of 1 mL cell suspensions on filters. (B) Change in
432 concentrations of culturable *E. coli* directly after disinfection and after 24, 48 and 72 hours of
433 storage in filtered mineral water. (C) Change in flow cytometric profiles of *E. coli* within 72h
434 after exposure to 0.025 and 0.05 mg L^{-1} chlorine and subsequent neutralization. Error bars show
435 standard deviations from three independent repeats. Representative pictures are shown for
436 culturability and flow cytometry.

437

438 Figure 3. Effect of chlorine on viability of bacteria in natural water. (A) Environmental bacteria
439 were enumerated following chlorine disinfection (exposure time of 30 min and subsequent
440 quenching) using R2A medium. Complimentary flow cytometry dot plots are shown for the
441 intact cell counts (B) Loss of viability shown as log removals relative to the average counts at

442 time zero for both FCM and HPC analysis. (C) Concentrations of total and intact cells after
443 exposure to different chlorine concentrations as derived by flow cytometry. (D) Changes in
444 colony compositions on R2A agar along the applied chlorine gradient. All values represent
445 averages from three independent repeats and error bars represent standard deviation of the
446 analysis. Representative pictures are shown for culturability and flow cytometry.

447

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449

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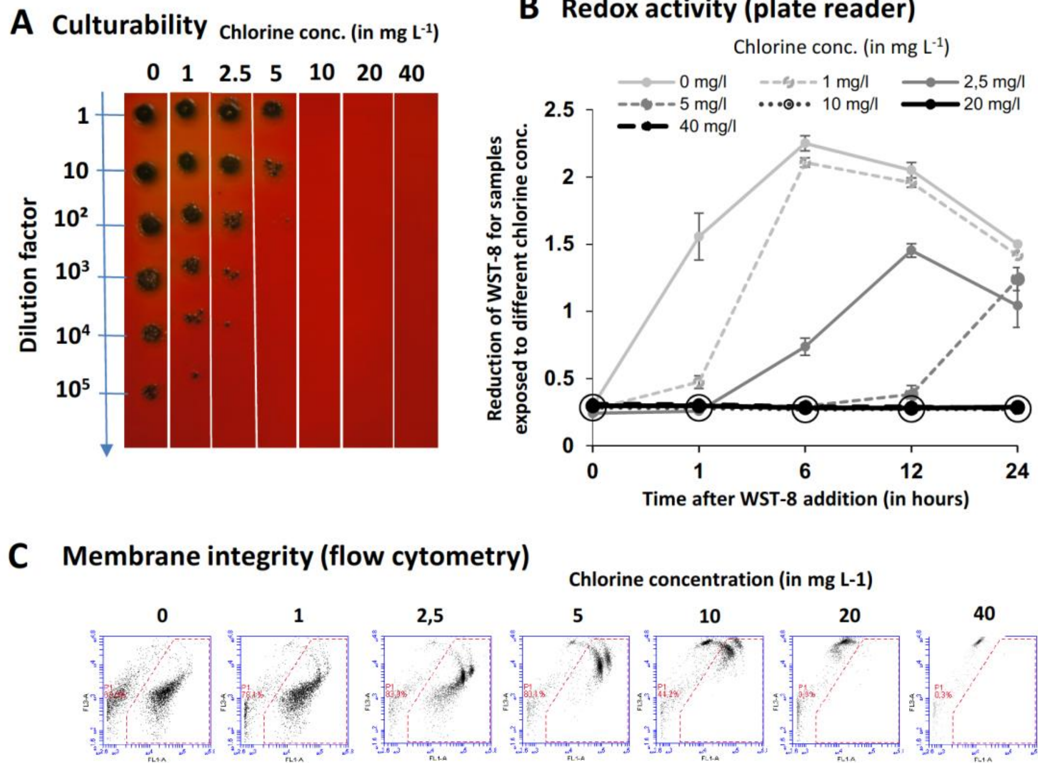

Figure 1. Measurement of the effect of chlorine on high cell densities of *E. coli* (10^9 cells mL^{-1}) based on assessment of culturability, metabolic activity, and membrane integrity. Culturability was determined by growth on MLGA plates and metabolic activity by measurement of WST-8 reduction on a plate reader platform. Cells were allowed to develop metabolic activity for 1, 6, 12 or 24 hours (after neutralization of the chlorine). Membrane integrity was determined both on a plate reader and a flow cytometer. Error bars show standard deviations from three independent repeats. Representative pictures are shown for culturability and flow cytometry.

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Fig. 2

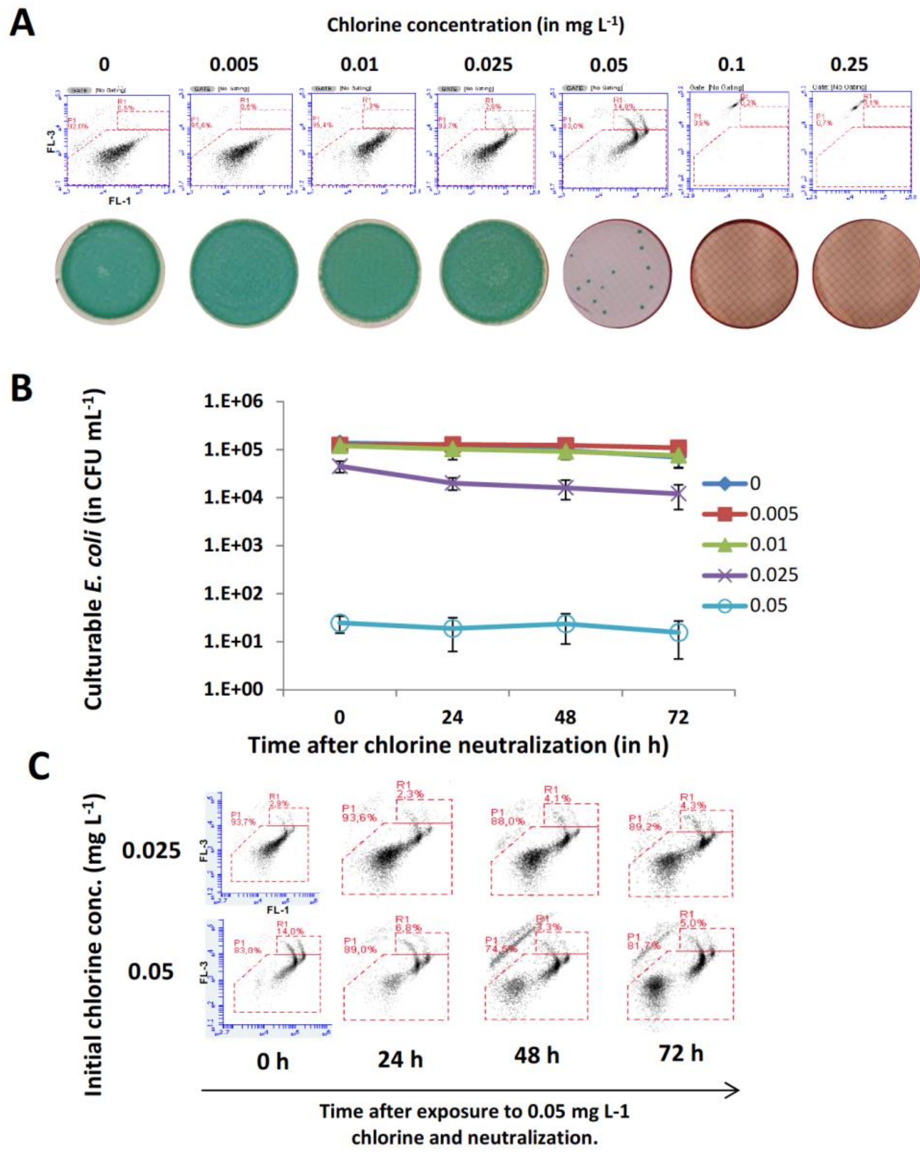

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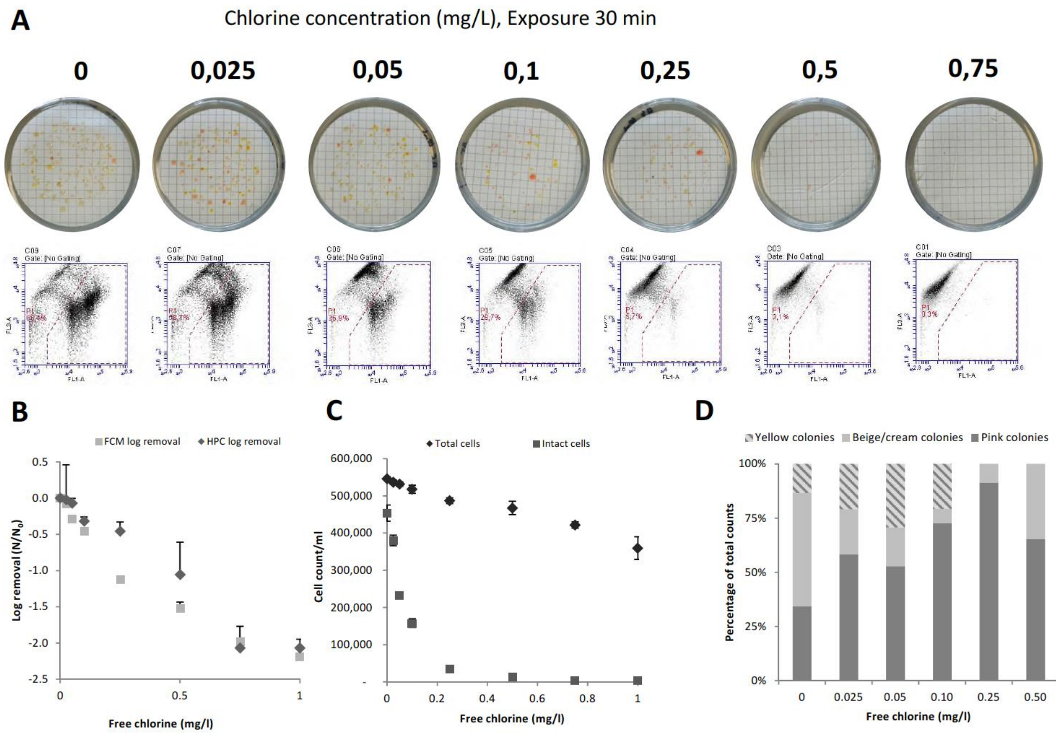
Fig. 3



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