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

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A B S T R A C T

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

Introduction

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

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

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

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

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

Materials and methods

Bacteria and growth conditions

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

Preparation of chlorine demand-free glassware

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

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

Analysis by cultivation

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

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

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

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

Measurement of redox activity

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

Flow cytometric analysis

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

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

Results

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

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

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

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

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

Relationship between culturability and membrane integrity for low cell densities

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

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

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

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

Relationship between culturability and membrane integrity for real water samples

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

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

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

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

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

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

Effect of chlorine on bacteria in real water

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

given growth conditions [30,31]. Further research will be necessary to substantiate the effect of growth conditions (including temperature and recovery periods) and to compare the behaviour of micro-organisms in more types of environmental water. As the bacterial populations in different water types have different compositions, they show different percentages of culturability and have different growth requirements. As culturability by itself is limited to a small fraction of the total bacterial population, it is prone to variation. Another question that remains to be answered is whether the chlorine effect with real water is sustained over several days, similar to the outcome seen with laboratory grown *E. coli*. More recovery assays in presence of different nutrient concentrations and recovery supplement will be necessary in future studies as done previously with heat-injured cells [17].

Last but not least the observation that colours of colonies obtained at different chlorine concentrations were subject to a trend (with pink colonies gaining relative abundance) supported the selective effect of chlorine on different bacterial species. Differential resistance of waterborne bacteria has been reported before for both chlorine-treated wastewater effluent [32] and drinking water with chloramine residual [33]. For the latter, pyrosequencing in combination with viability PCR showed a clear effect of increasing Ct values of monochloramine exposure on the composition of the intact fraction of the bacterial community. A similar outcome can be expected for chlorine.

Conclusions.

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

Figure Legend

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 (A), redox activity (B), and membrane integrity (C). Culturability was determined by growth on MLGA plates and redox activity by measurement of WST-8 reduction on a plate reader platform. Cells were allowed to develop redox activity for 1, 6, 12 or 24 hours after neutralization of the chlorine and addition of WST-8. Membrane integrity was determined using a flow cytometer after staining samples with SYTO9 and propidium iodide. Error bars show standard deviations from three independent repeats. Representative pictures are shown for culturability and flow cytometry.

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

Figure 3. Effect of chlorine on viability of bacteria in natural water. (A) Environmental bacteria were enumerated following chlorine disinfection (exposure time of 30 min and subsequent quenching) using R2A medium. Complimentary flow cytometry dot plots are shown for the 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.

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547

Fig. 1

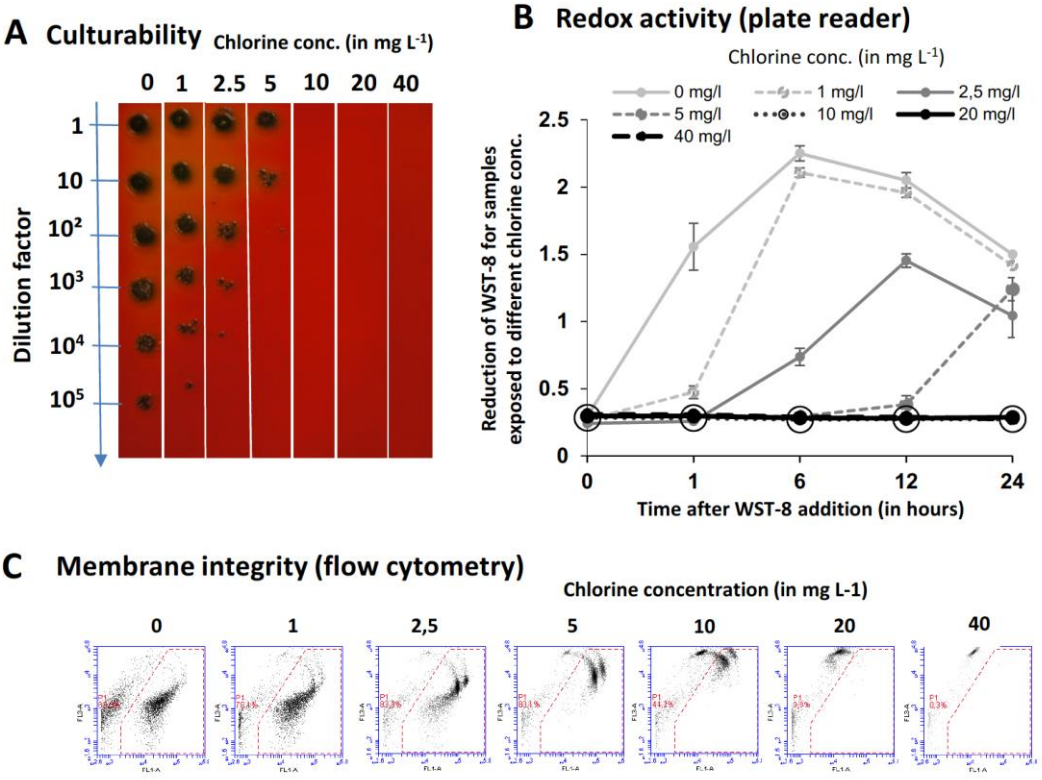

Figure 1. Measurement of the effect of chlorine on high cell densities of *E. coli* (10^9 cells mL^{-1}) based on assessment of culturability, metabolic activity, and membrane integrity. Culturability was determined by growth on MLGA plates and metabolic activity by measurement of WST-8 reduction on 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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549

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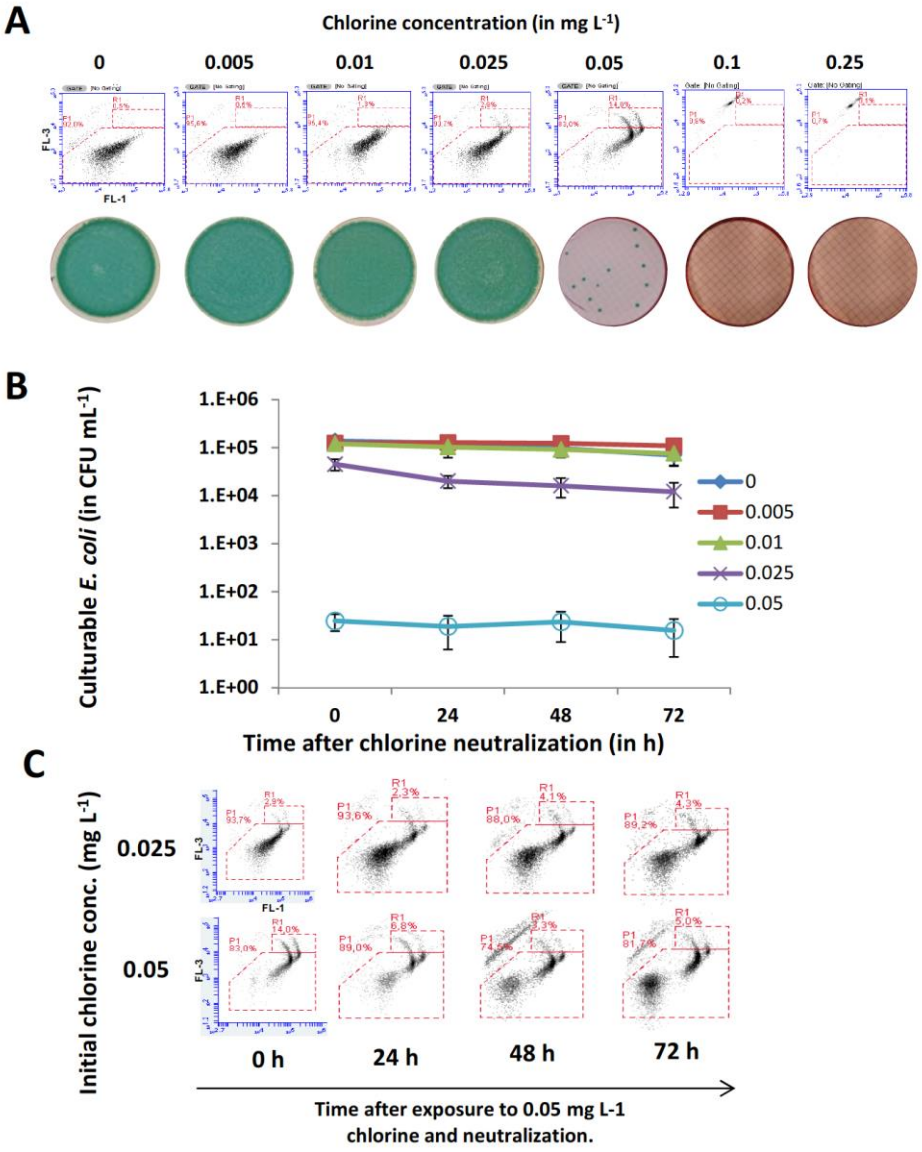
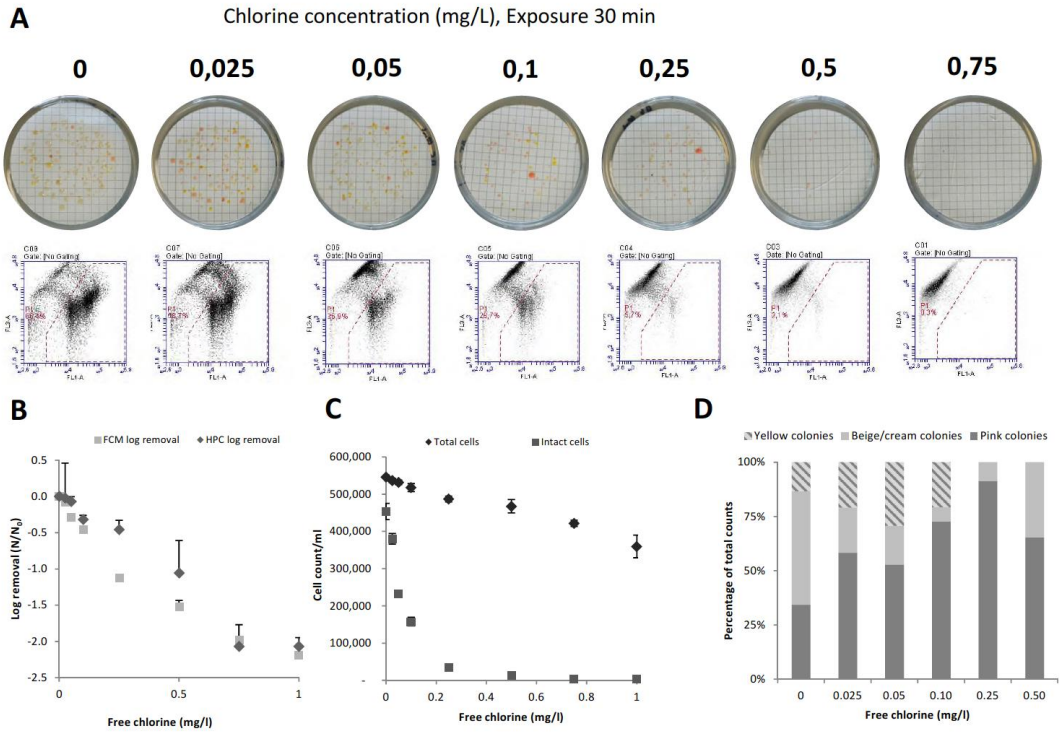
Fig. 2


A
Chlorine concentration (in mg L-1)
0
0.005
0.01
0.025
0.05
0.1
0.25
FL-3
FL-1
B
Culturable E. coli (in CFU mL-1)
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
0
24
48
72
Time after chlorine neutralization (in h)
0
0.005
0.01
0.025
0.05
C
Initial chlorine conc. (mg L-1)
0.025
0.05
0 h
24 h
48 h
72 h
Time after exposure to 0.05 mg L-1
chlorine and neutralization.

551

552

Fig. 3


A
Chlorine concentration (mg/L), Exposure 30 min
0
0,025
0,05
0,1
0,25
0,5
0,75
B
FCM log removal
HPC log removal
Log removal (N/N0)
Free chlorine (mg/l)
C
Total cells
Intact cells
Cell count/ml
Free chlorine (mg/l)
D
Yellow colonies
Beige/cream colonies
Pink colonies
Percentage of total counts
Free chlorine (mg/l)

553

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

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