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Flow-cytometric quantification of microbial cells on sand from water biofilters

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ABSTRACT

Rapid quantification of absolute microbial cell abundances is important for a comprehensive interpretation of microbiome surveys and crucial to support theoretical modelling and the design of engineered systems. In this paper, we propose a protocol specifically optimised for the quantification of microbial abundances in water biofilters using flow cytometry (FCM). We optimised cell detachment from sand biofilter particles for FCM quantification through the evaluation of five chemical dispersants (NaCl, Triton-X100, $CaCl_2$, sodium pyrophosphate (PP), Tween 80 combined with PP), different mechanical pretreatments (low and high energy sonication and shaking) and two fixation methods (glutaraldehyde and ethanol). The developed protocol was cross-compared using other established and commonly employed methods for biomass quantification in water filter samples (adenosine triphosphate (ATP) quantification, real-time quantitative PCR (qPCR) and volatile solids (VS)). The highest microbial count was obtained by detaching the biofilm from biofilter grains and dispersing clusters into singles cells using Tween 80 and sodium pyrophosphate combined with four steps of high energy sonication (27W, for 80 s each step); glutaraldehyde was shown to be the best fixative solution. The developed protocol was reliable and highly reproducible and produced results that are comparable to data from alternative quantification methods. Indeed, high correlations were found with trends obtained through ATP and qPCR ($\rho = 0.98$ and $\rho = 0.91$) measurements. The VS content was confirmed as an inaccurate method to express biomass in sand samples since it correlated poorly with all the other three methods ($\rho = 0.005$ with FCM, 0.002 with ATP and 0.177 with qPCR). FCM and ATP showed the strongest agreement between absolute counts with a slope of the correlation equal to 0.7, while qPCR seemed to overestimate cell counts by a factor of ten. The rapidity and reproducibility of the method developed make its application ideal for routine quantification of microbial cell abundances on sand from water biofilters and thus useful in revealing the ecological patterns and quantifying the metabolic kinetics involved in such systems.

1. Introduction

Water filtration, with sand or granular activated carbon (GAC), is a conventional treatment process widely used in traditional drinking water treatment plants, whereby raw water is passed through a porous bed of filter medium in order to remove fine particles and soluble organic matter. In addition to removing particles from the water stream, sand and GAC particles are an excellent support for the development of biofilms; in fact, highly diverse microbial communities have been found populating these systems (Pinto et al., 2012). Water biofilter functionality and performance, under different conditions, have been investigated and reported in several studies (LeChevallier et al., 1992; Liu et al., 2001; Moll et al., 1999). More recently, research has focused on understanding the composition of the complex microbial communities inhabiting water filters by employing molecular biology tools (Bai et al., 2013; Haig et al., 2014, 2015; Palomo et al., 2016; Pinto et al., 2015; White et al., 2012). These studies have collected a considerable amount of phylogenetic and potential physiological

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Abbreviations

ATP	Adenosine Triphosphate
BOT	Bottom
DWTP	Drinking Water Treatment Plants
EPS	Extracellular polymeric substances
EtOH	Ethanol
FCM	Flow cytometer
GAC	Granular Activated Carbon
GLUT	Glutaraldehyde
HES	High Energy Sonication
ICC	Intact Cell Concentration
LES	Low Energy Sonication
MID	Middle
PBS	Phosphate Buffer
PP	Sodium Pyrophosphate
qPCR	Quantitative Polymerase Chain Reaction
RGF	Rapid Gravity Filter
SSF	Slow Sand Filter
TCC	Total Cell Concentration
TEC	Total Extracted Cells

information, contributing to a better understanding of some of the ecological functions of these communities in drinking water systems (Gülay et al., 2016) and their impact on the water in the distribution system (Pinto et al., 2012).

Studying microbial diversity in water filters is an exciting new research direction, but the accurate quantification of bacteria in these filters is equally important. It is an often under-appreciated, complementary element of microbial ecology, which is essential for determining bacterial growth rates and substrate utilisation kinetics, for theoretical modelling (Meynet et al., 2014, 2012), mass balances (Vignola et al., 2018) and for comprehensive interpretation of microbiome surveys (Props et al., 2017). Therefore, the search for rapid and reliable techniques to estimate microbial cell numbers in diverse environments, and in filter media specifically, has become a scientific priority (Davis, 2014).

Microbial quantification has traditionally been carried out using plating techniques, which are characterised by long processing time and strong biases due to the fact that the majority of prokaryotes fail to grow on culture media (Staley and Konopka, 1985). Epifluorescence microscopy (EFM) was developed as a standardised method allowing direct microscopic counts of microbial cells stained with fluorescence dyes. This method was successfully employed to count bacteria in freshwater (Jones, 1979), marine water (Daley, 1979), soil (Barra Caracciolo et al., 2005; Elazhari-Ali et al., 2013; Riis et al., 1998) and sediments samples (Amalfitano and Fazi, 2008). The main factors hampering the wider application of these methods for biomass quantification include the laborious and time-consuming nature of the analytical procedure, especially when working with solid matrices.

Alternative approaches which allow for a quick and rapid direct estimation of biomass on biofilter media particles include i) adenosine triphosphate (ATP) quantification, as proposed by (Velten et al., 2007); and ii) real-time quantitative PCR (qPCR) (Gülay et al., 2016).

One of the most promising alternatives to traditional quantification methods to assess drinking water treatment plant (DWTP) performance, which so far has mainly been applied to water samples, consists of counting cells by flow cytometry (FCM) in combination with sensitive nucleic acid-specific dyes. FCM has become the method of choice for quantifying microbial cells in aquatic samples, combining high sample throughput with speed and accuracy (Hammes et al., 2008; Van Nevel et al., 2017). Moreover, FCM has proved to be a useful tool not only for assessing cell abundance and viability but also for microbial community profiling (Berney et al., 2008; Prest et al., 2014, 2013; Ramseier et al., 2011).

While FCM is widely used for water, its application on samples such as sand particles, sediment or soil is still limited due to difficulties and complications linked to working with biofilms attached to a solid matrix. The presence of detritus, minerals and extracellular polymeric substances (EPS) can produce a high fluorescence background and interferes with cell counting (Kuwae and Hosokawa, 1999). In such environments, cells are attached to particle surfaces and to each other through the EPS. Cells need to be detached from the solid matrix, transferred into a liquid matrix and separated from the EPS structure into single cells in order to accurately quantify them using FCM.

A study from Magic-Knezev and van der Kooij (2004) developed a biofilm detachment protocol for filter media samples (sand and GAC), quantifying the dispersed cells using ATP measurements. In the development of a biofilm detachment method for FCM applications, it is not only crucial to obtain high yields of detached cells but also to ensure dispersal of flocs/clusters into single cells while avoiding cells lysis. Cells are counted on the FCM only when they maintain structural integrity with either partially damaged or intact membranes (Berney et al., 2007), while the presence of flocs can lead to underestimations (cells grouped together are counted as one single event) and pose a risk of blockage to the instrument. A study from Lavergne et al. (2014) proposed a detachment protocol specifically optimised for its use in combination with FCM for coastal marine sediment samples. To our knowledge, no simple and standardised method has yet been developed for water filter sand samples. Thus, this paper aims to optimise and critically describe a rapid FCM protocol to enumerate microbial cells present in biofilms attached to water filter media and to evaluate the protocol against other culture-independent quantification approaches typically used for such samples, namely ATP, qPCR and volatile solids (VS).

2. Methods

2.1. Samples collection

Samples were collected from two slow sand filters (SSF) and two rapid gravity filters (RGF) belonging to three different DWTPs (Table 1, Table SI1) operated by Northumbrian Water Ltd. Filters were fully drained overnight before sampling. Sand cores were extracted by means of a metal sediment corer, allowing the sampling of the top (TOP), middle (MID) and bottom (BOT) layers of the filter bed. Aliquots of sand were transferred into sterile 500 ml plastic containers, transported to the lab at ~4 °C and stored in the

Table 1
Drinking Water Treatment Plants (DWTP) details, type of filter and position of the sample.

Sample ID	DWTP	Filter	Process Configuration	Position
DWTP1-1	DWTP1	RGF[a]	Dissolved air Flotation, RGF and SSF	TOP[a][b]
DWTP1-2	DWTP1	RGF	Dissolved air Flotation, RGF and SSF	TOPb
DWTP1-3	DWTP1	RGF	Dissolved air Flotation, RGF and SSF	MID[c]
DWTP1-4	DWTP1	RGF	Dissolved air Flotation, RGF and SSF	BOT[d]
DWTP1-5	DWTP1	SSF[e]	Dissolved air Flotation , RGF and SSF	TOP
DWTP1-6	DWTP1	SSF	Dissolved air Flotation, RGF and SSF	MID
DWTP2-1	DWTP2	RGF	Coagulation/flocculation, RGF	TOP
DWTP2-2	DWTP2	RGF	Coagulation/flocculation, RGF	MID
DWTP3-1	DWTP3	SSF	RGF, SSF	TOP
DWTP3-2	DWTP3	SSF	RGF, SSF	MID

[a]Rapid Gravity Filter; [b]Top layer; [c]Middle layer; [d]Bottom layer; [e]Slow Sand Filter.

dark at 4 °C until use; sample storage differed between the different optimisation experiments, however in any cross-comparison experiment each sample was analysed, for all the four counting methods, the same day in order to avoid discrepancies that may result from different storage times. All the method optimisation experiments were performed using only one type of sand collected from the top layer of the SSFs in DWTP1 (Table 1).

2.2. Optimisation of biofilm detachment

The method proposed by Magic-Knezev and van der Kooij (2004) was used as a starting point and optimised for its application with FCM analysis in order to improve the enumeration of microbial cells in sand samples. The optimisation was performed through three main steps:

1. The identification of a dispersant solution capable of enhancing the biofilm detachment and its dispersal into single cells;
2. The evaluation of different mechanical treatments (low and high energy sonication, shaking) to be combined with the identified dispersant;
3. The identification of a suitable fixative method to protect cells from lysis during the developed detachment treatment.

Cells were extracted from 2 g of wet sand (sample DWTP1-5, sampled as described in 2.2) submerged in 50 ml of an extraction solution and subjected to one cycle of low energy sonication (LES) using a sonicating water bath (Decon FS200b; 120W; 40 KHz) for 3 min, followed by different cycles (according to the optimisation experiment) of high energy sonication (HES) using an ultrasonic probe (Cole Parmer Instrument, Ultrasonic processor), with a power input of 27W, for 80 s at each step. Fresh extraction solution (different solutions were tested according to the experiment) was added at each treatment step, while the sonicated solution was transferred into autoclaved 500 ml Duran bottles. 2 ml aliquots of the extraction solution, at each treatment step, were collected along with aliquots of the final cumulative solution; transferred into sterile Falcon tubes and fixed with a solution of glutaraldehyde 1% v/v at a ratio of 1:1 v/v. After fixation samples were stored in the dark at 4 °C and analysed within 2 days with FCM to obtain total and intact cell concentrations (see below). The total number of cells extracted from 2 g wet weight (WW) of sand was expressed as the sum of the cells recovered at each step. Replicate extractions were performed, and each extraction was analysed in duplicate with FCM.

2.2.1. Optimisation of pre-treatments for biofilm detachment

2.2.1.1. Chemical treatment: assessment of different dispersants versus tap water. Five dispersants were tested for enhanced biofilm detachment from sand particles: two salt solutions, sodium chloride (NaCl) and calcium chloride ($CaCl_2$) dissolved in deionised water (DI) 0.3 M and 0.21 M respectively; the non-ionic surfactant TritonX-100 (TRITONX-100) at a concentration of 1000 mg/L in DI water; the ionic dispersant sodium pyrophosphate (PP) 10 mM in DI water; and a combination of polyoxyethylene-sorbitan monooleate (Tween 80, Sigma) 5% v/v in a solution of PP 10 mM (TWEEN-PP). All the dispersants were autoclaved prior to use, with the exception of the TritonX-100 and the Tween 80.

The extraction was carried out as described in section 2.2 with one cycle of LES and two cycles of HES. Autoclaved tap water (120 °C, 15 min) (TAP) (Magic-Knezev and van der Kooij, 2004) was used as a control solution against which the recovery of cells from biofilm was expressed. The cumulative recovery of cells, at each treatment step, was expressed as the number of cells extracted with the specific extraction solution compared to the total amount of cells extracted in the control samples (extracted with tap water) by the whole treatment.

$$Recovery\ compared\ to\ TAP\ (\%)_{(STEP)} = \frac{cumulative\ n\ of\ cells\ extracted_{(DISPERSANT)}}{cumulative\ final\ n\ of\ cells\ extracted_{(TAP)}} * 100$$

2.2.1.2. Physical treatment: low and high energy sonication, shaking. The effect of mechanical treatment, in combination with chemical treatment, on cell detachment was tested by subjecting sand samples, immersed in the chosen dispersant, to one cycle of LES and seven cycles of HES (section 2.1). A control with autoclaved tap water was extracted in the same way.

The effect of mechanical shaking compared to sonication was also tested using an orbital shaker. Wet sand (2 g) (sample DWTP1-5, Table 1) was subjected to four cycles of shaking on an orbital shaker (250 rpm for 30 min). Fresh extraction solution was added at each treatment step, while the exhausted solution was transferred to sterile containers. Aliquots of the extraction solution were withdrawn at each treatment step, straight after the solution addition (T_0) and after each shaking cycle (T_1 to T_4); after collection, samples were fixed and stored for FCM analyses as previously described. During the same experiment, 2 g WW of the sand from the same sample (DWTP1-5) was subjected to four cycles of HES. Samples of the extraction solutions were collected at each cycle of sonication, treated and analysed as previously described in order to compare the extraction efficiency of the two mechanical methods (sonication versus shaking) at each step. For both methods, a dispersant was used as an extraction solution, and autoclaved tap water was used as a control.

2.2.1.3. Fixative optimisation. Two fixative solutions were tested: (1) a solution of glutaraldehyde 1% v/v in DI water (GLUT-fixed) and (2) a solution of absolute ethanol in autoclaved phosphate buffer saline (PBS) 50% v/v (EtOH-fixed); a solution of autoclaved PBS was employed as a fixative free control (PBS-control). Glutaraldehyde has previously been suggested as an effective fixative for FCM analyses (Kamiya et al., 2007) and the measurement of viable cells using the live/dead stain (Hu et al., 2017). A 2 ml aliquot of each fixative solution was added to 2 g WW of sand (DWTP1-5, Table 1). Samples were incubated with the fixative for 30 min at room temperature before extraction. Cells were extracted via three cycles of HES using TWEEN-PP as an extraction solution and autoclaved tap water as a control. Samples were collected and analysed as described in 2.1.

2.3. Cell abundance in sand filters: comparison of FCM with quantitative PCR (qPCR), volatile solids (VS) and ATP

To further evaluate the protocol, the method for FCM analysis was applied to 10 different sand samples collected from two slow sand filters (SSF) and two rapid gravity filters (RGF) belonging to three different DWTPs (Table 1). Each sand sample was extracted in triplicate, and the extraction was analysed in duplicate by FCM. Cell counts obtained with the FCM method were compared with three other biomass quantification methods: qPCR, ATP quantification and VS content. Samples were collected as described in section 2.1.

ATP: ATP was measured as described in Velten et al. (2007), with a few minor modifications. Briefly, 200 mg WW of sand particles were transferred into sterile 2 ml sterile reaction tubes together with 100 µl of phosphate buffer, where ATP was previously deactivated as described in (Velten et al., 2007), and heated for at least

10 min in a heating block (30 °C). Simultaneously 300 μL of BacTiter-Glo™ (Promega, Madison, WI, USA) was transferred to a separate sterile reaction tube and heated at 30 °C for 3 min in a heating block. No rinsing of the sand samples was performed in order to obtain comparable results with the other methods tested. After 3 min incubation, the BacTiter-Glo™ reagent was added to the sand sample, the mixture was vortexed for 5 s and placed for a further 1.5 min in the heating block to complete the reaction. After the reaction, 200 μL of the supernatant was transferred into the well of a 96-well plate (Greiner Bio-One Ltd., UK)) and the relative light units (RLU) were measured with a microplate reader (SpectraMax M3, Molecular Devices, CA) after exactly 30 s. Results were converted to ATP concentrations using a calibration curve established with pure ATP standards (Promega, Madison, WI, USA) over a concentration range of 1 to 0.05 μM of ATP. All samples were analysed in triplicate. A conversion factor of 8.9×10^{-17} g ATP per cell (Hammes et al., 2010) was used to convert ATP concentrations into cell abundances.

qPCR: DNA was extracted from 0.5 g WW of sand using the FastDNA® SPIN Kit for Soil (MP-Biomedicals, Santa Ana, USA) following the manufacturer's protocol, but adding three more cycles of homogenisation in the FastPrep® instrument and incubating (55 °C for 5 min) the elute before final centrifugation. The 338F (Bakke et al., 2011) and 1046R (Huber et al., 2009) primers were used to target the 16S rRNA gene, assuming that each organism in our microbial community contained one copy of the gene. qPCR assays were performed on a CFX96 real-time PCR detection system (Bio-Rad, Hemel Hempstead, United Kingdom), using the following temperature profile: 98 °C for 3 min for 1 cycle; and 98 °C for 5 s, followed by 60 °C for 5 s, for 39 cycles.

Each amplification reaction was run in triplicate and contained 3 μL of template DNA, to assure a concentration of between 10 and 100 ng/ml of genomic DNA (or molecular-grade water for blanks), 0.5 μL of forward and reverse primer (10 pmol/μL), 5 μL of SsoFast EvaGreen Supermix (Bio-Rad, Hemel Hempstead, United Kingdom), and 1 μL of molecular-grade water. Standards, in concentrations ranging between 10^2 and 10^8 fragment copies per μL, were obtained from circular plasmids containing the target fragment of DNA (16S rRNA gene fragment, 1515 bp, inserted into the plasmid of the *E.coli* clones using pA-pH primers), and a calibration curve was generated in every qPCR run. Melt-curve analysis (between 65 and 95 °C) was performed at the end of each qPCR run to assess the specificity of the products.

VS: Sand samples were dried overnight at 105 °C and then combusted in a muffle furnace (1 h at 450 °C) to determine the total dry mass and VS content (U.S. EPA, 2001).

2.4. Flow-cytometric measurements, FCM

Total and intact cell concentration (TCC, ICC) measurements on the extraction solution samples were performed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with a 15 mW 488 nm air-cooled argon-ion laser. Two stock solutions of SYBR® Green I (SGI) alone and combined with propidium iodide (SGI-PI) were prepared as follows: to prepare the SGI solution SYBR® Green I (10,000 x in DMSO, Sigma Aldrich, Darmstadt, Germany) was diluted 1:100 in EDTA[1] (1 mM); to prepare the SGI-PI solution PI (1.6 mM) was mixed with SYBR® Green I (10,000 x in DMSO) and diluted with EDTA (1 mM) for a final concentration in the stock solution of 0.6 mM (for PI) and 100 x respectively (for SYBR). The microbial cells in the sample aliquots were stained separately with 10 μL/ml SGI or 10 μL/ml SGI + PI and incubated in the dark for 13 min before measurement. Where necessary, samples were diluted in filtered (0.22 μm; polyethersulfone membrane, Merck Millipore, Kenilworth, NJ) DI water so that the events per second detected by the instrument were always less than 400. Readings were collected in logarithmic mode and analysed with Flowing Software 2.0. Electronic gating was used to separate selected signals (prokaryotic cells) from the background (inorganic and organic particles) (Berney et al., 2007; Hammes et al., 2008; Prest et al., 2013). Negative controls, consisting of the extraction solutions without sand addition, which were treated following the sample extraction procedures, were analysed for each FCM run and the values were subtracted from the final FCM measurements.

2.5. Statistical analyses

All statistical analyses were performed using Minitab and R software. The effect of the different dispersants and the different mechanical treatments on biofilm detachment was tested using paired t-tests with the controls. Effects of fixative conditions were tested with a 2-way analysis of variance (ANOVA) with Tukey's pairwise comparisons. Linear models were used to detect significant differences among the protocol developed (FCM) and the other three methods tested (ATP, qPCR, VS). A Pearson product-moment correlation coefficient (ρ) was calculated to determine the relationship between FCM counts with either ATP or qPCR counts or VS content.

3. Results

3.1. Chemical treatment: dispersants

The highest recovery was obtained with the solution of TWEEN-PP, where after one cycle of low energy sonication (LES) and two cycles of high energy sonication (HES) 24 ± 1% more cells were extracted compared to TAP (paired *t*-test, $p < 0.05$) (Fig. 1a). No statistical difference was observed among the recoveries of the $NaCl_2$, $CaCl_2$ and PP solutions. The number of cells extracted with Triton-X100 was significantly lower (paired *t*-test, $p < 0.05$) than the controls (52 ± 8% of the control). Three main extraction patterns were observed. TWEEN-PP triggered the release of the majority of cells immediately after its addition to the samples, with a second increase after the first HES treatment (Fig. 1b). Autoclaved TAP water seemed to release cells in two distinct phases: half of the cells were released in the first two steps (53% of cells released during addition and LES), 40% were released in the HES1. The rest of the dispersants showed a constant linear release throughout the four treatment steps (average 25% removal at each step), with no treatment showing more extraction efficiency than the others.

3.2. Mechanical treatment: sonication steps and shaking

Having established that the solution of TWEEN-PP was the most effective dispersant, an experiment was performed in order to find the minimum number of sonication treatments able to ensure a satisfactory removal of biomass from sand particles. Samples treated with TWEEN-PP displayed a significantly higher number of total extracted cells (TEC) compared to autoclaved tap water. The highest amount of cells was extracted, in both sets of samples, after the addition of the extraction solution (T_0) and in the first HES treatment (Fig. 2).

The treatment effectiveness attenuated after the HES1 in both the tap water and dispersant-treated samples (Fig. 2). In TWEEN-PP treated samples, no statistically significant difference was observed between the total amount of cells extracted at the end of the whole treatment and the one measured at HES2 (one-way *t*-test,

[1] Ethylenediaminetetraacetic acid.

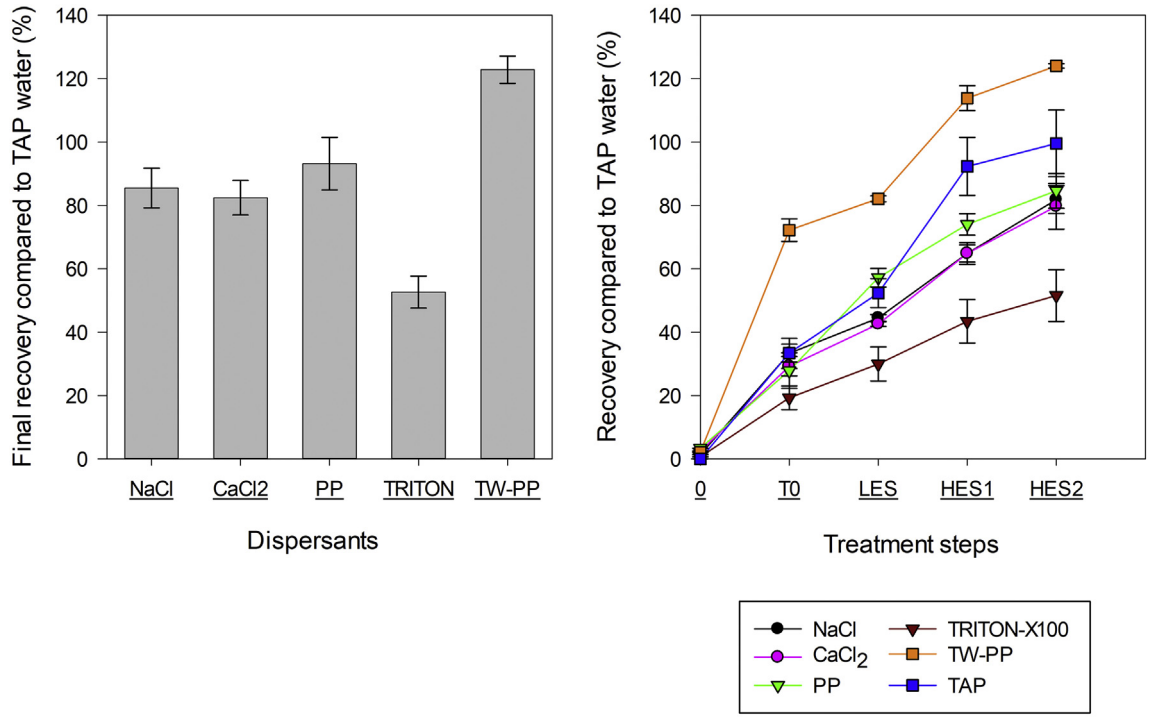

Fig. 1. Effect of the five dispersant solutions NaCl, Triton-X100 (TRITON), $CaCl_2$, sodium pyrophosphate (PP), and Tween 80 combined with PP (TW-PP) on cell recovery compared to (a) control samples (TAP) at the end of the treatment and, (b) at each treatment step: after dispersant addition (T_0), after the step of Low Energy Sonication (LES) and after the two steps of High Energy Sonication (HES).

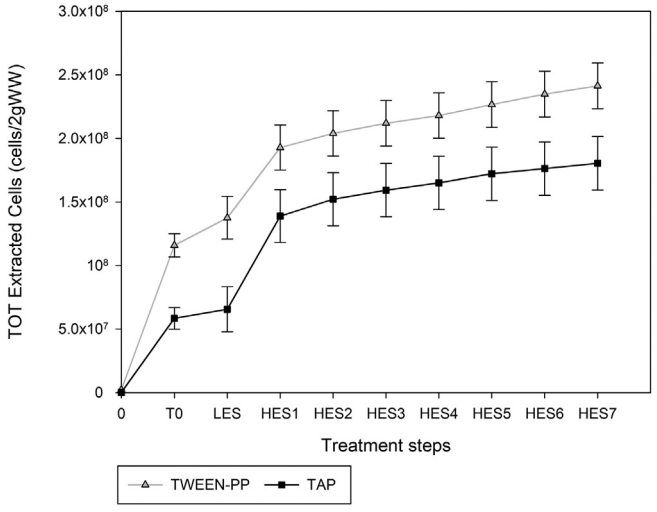

Fig. 2. Effect of biofilm detachment and floc dispersal after dispersant addition (T0), after low energy sonication (LES) and seven high energy sonication steps (HES1; HES7) on biofilm detachment and floc dispersal from 2 g WW of sand.

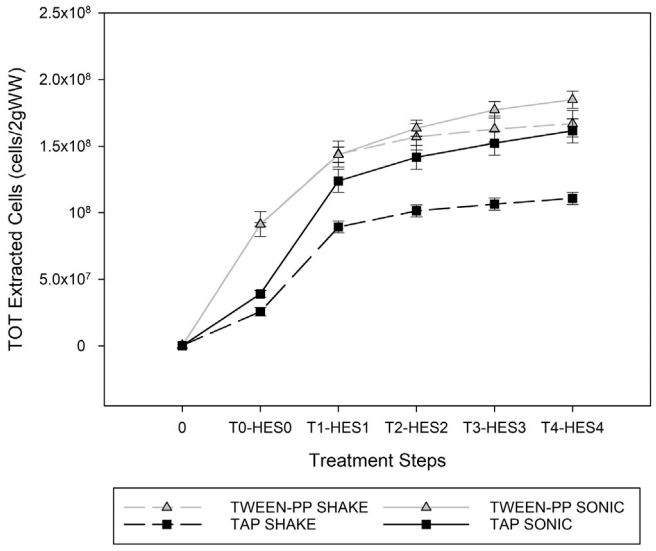

Fig. 3. Effect of high energy sonication on biofilm detachment and floc dispersal (continue line) at each sonication step (HES0; HES4) compared to shaking (dashed line) at each shaking step (T_0; T_4).

$p = 0.057$). In samples extracted with tap water, the plateau seemed to be reached later: the increment given by each consecutive HES step was statistically insignificant after HES3 (one-way *t*-test, $p = 0.08$).

The effect of shaking was also tested and compared to sonication (Fig. 3). The combination of TWEEN-PP with HES was the most effective method among the four tested. The amount of cells extracted using TWEEN-PP with sonication was significantly higher than those extracted by the other three methods (ANOVA, $p < 0.05$); the worst performing treatment was the tap water combined with shaking, while the difference between TWEEN-PP with shaking and tap water with sonication was statistically insignificant (*t*-test, $p = 0.22$). As previously observed, the highest amount of cells was recovered at HES0 (after the dispersant addition) and after the first cycle of HES and the recovery efficiency of the treatment also attenuated after the first cycle of HES (Fig. 3).

The number of cells extracted in shaken samples was significantly higher for the TWEEN-PP solution than for tap water (paired *t*-test, $p < 0.01$). The highest number of cells was detached in the first two steps of treatment (T_0 and T_1); the removal efficiency dropped drastically after the first two steps in both solutions.

3.3. Fixative optimisation

The TEC from glutaraldehyde-fixed samples (GLUT-fixed) was significantly higher than the TEC from ethanol-fixed (EtOH-fixed) samples and from the PBS-control (ANOVA, $p < 0.001$) when TWEEN-PP was employed as an extraction solution (Fig. 4). No statistically significant difference was observed between the EtOH-fixed samples and the PBS-control (ANOVA, $p > 0.05$).

The same pattern was observed when autoclaved tap water was employed as the extraction solution: the TEC from the GLUT-fixed samples was significantly higher than the amount extracted from the EtOH-fixed samples and the PBS-control (ANOVA, $p < 0.01$). No statistically significant difference was observed between the EtOH-fixed and the control samples (ANOVA, $p > 0.05$).

The TEC with TWEEN-PP solution was significantly higher than the number of cells extracted by autoclaved TAP water in all the samples (GLUT- and EtOH-fixed and PBS-control), confirming that TWEEN-PP is more effective as an extraction solution (*t*-test, $p < 0.05$) (Fig. 4a).

The number of intact cells was also measured during this experiment. Contrary to what was observed with the total cells, the number of intact cells extracted by using autoclaved tap water was, for all the samples (EtOH-fixed, GLUT-fixed, and PBS-control), significantly higher than the amount extracted when using TWEEN-PP (*t*-test, $p < 0.05$) (Fig. 4b).

Among the samples extracted with TWEEN-PP, no statistically significant difference was observed between the intact cells measured in GLUT-fixed samples and the PBS-control (ANOVA, $p > 0.05$). On the other hand, the number of intact cells extracted from the EtOH-fixed samples was two orders of magnitude lower than that extracted from the other two sets of samples (Fig. 4b).

3.4. Protocol cross-comparison with other biomass quantification methods

All the assessments above resulted in a final protocol that seemed to detach biofilm formed on the surface of and between sand grains satisfactorily and effectively, and dispersed the biofilm (Fig. SI1) from such samples allowing microbial cells to be counted using FCM (Fig. 5). However, the biofilm still attached on sand grains was not quantified in this study. Ten sand samples, collected from several water filters, were tested by quantifying cell counts with the developed protocol, which were compared with those of other commonly used techniques for cell biomass quantification (see Fig. 6).

1. Aliquots of sand samples (2g in duplicate) were fixed with a glutaraldehyde solution (1% v/v) for 30 min at room temperature (1:1 ml of fixative solution to gWW of sand). Straight after fixation, cells were extracted via four steps of HES performed with a sonication probe (80 s, 27 W). A combination of [sodiumpyrophosphate (10 mM) + Tween 80 (5%)] was employed as an extraction solution; fresh solution (50 ml) was added to sand after each treatment step, while the exhausted solution was transferred into a sterile glass container.
2. Aliquots of the cumulative solutions were collected, fixed in a solution of glutaraldehyde (1% v/v) (1:1 v/v fixative solution/sample)) stored in the dark at 4 °C and analysed by FCM within two days.
3. Controls of dried (105 °C, overnight) and incinerated (550 °C, 1h) sand were extracted, for each sample tested, following the same protocol and analysed by FCM in order to distinguish background noise created by organic and inorganic particles. Indeed, each sand sample showed unique background fluorescence signals created by particle autofluorescence, controls were vital to design tailored settings of the electronic gate.

In order to get comparable results among the four quantification methods, each sample was analysed, for every single method, within the same day.

FCM estimates of cell abundances were always lower than those given by ATP and qPCR, by an average factor of 0.70 ± 0.05 and 0.09 ± 0.01 respectively. However, despite differences in absolute numbers, cells abundances assessed by FCM were significantly correlated with those obtained with ATP ($p < 0.01$, Pearson's $\rho = 0.98$) and qPCR ($p < 0.01$, $\rho = 0.911$). Highly significant

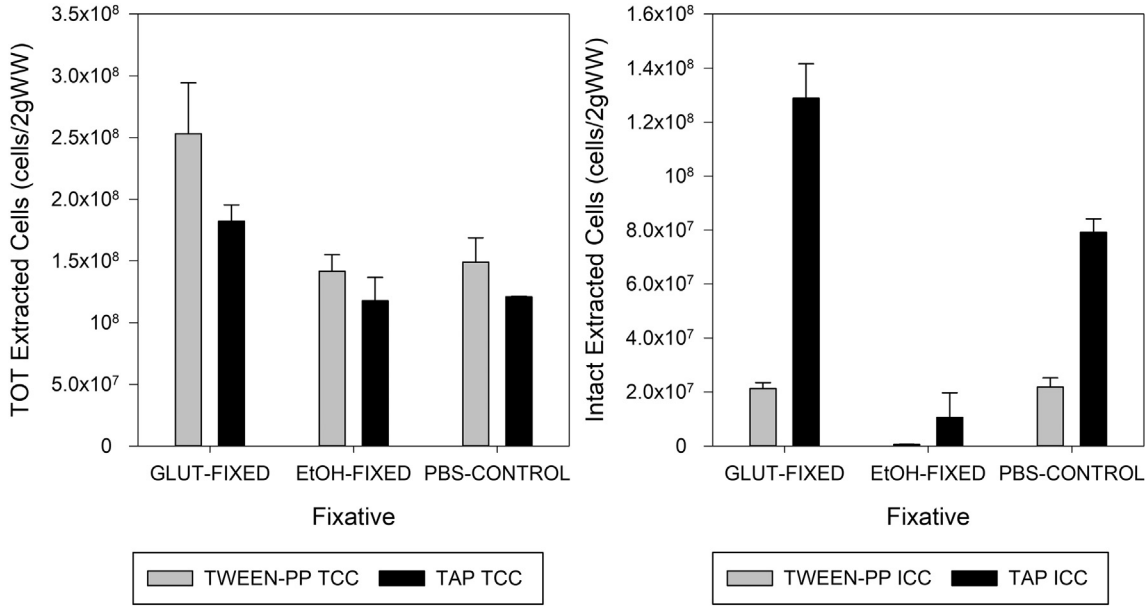

Fig. 4. (a) Total cells extracted with TWEEN-PP (orange) and TAP (blue) from samples fixed with glutaraldehyde, ethanol:PBS and from the PBS-control; (b) Intact cells extracted with TWEEN-PP (orange) and TAP (blue) from samples fixed with glutaraldehyde, ethanol:PBS and from the PBS control.

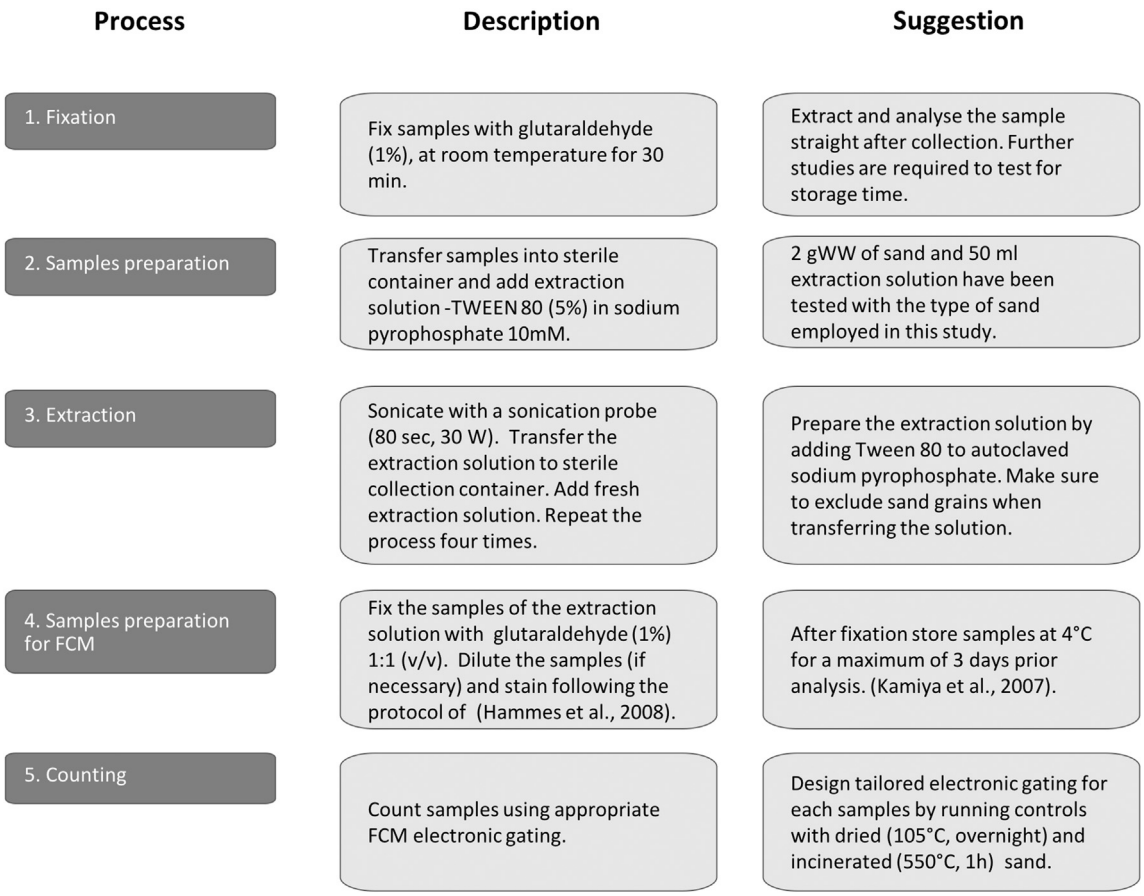

Fig. 5. Diagram describing the final optimised protocol steps for the extraction of cells from water filter sand grains.

correlations were also found between ATP and qPCR abundances ($p < 0.01$, $\rho = 0.93$) (Table 2). Very low correlations were found between all three methods and quantification of biomass using volatile solids (VS).

4. Discussion

In this study, we present a flow-cytometric method specifically optimised for enumerating cells present on the surface of sand particles used in water filters. This method constitutes a considerable improvement on previously developed protocols for biomass quantification on sand samples from water biofilters (Magic-Knezev and van der Kooij, 2004). The protocol developed allows direct cell quantification free from potential biases introduced by assumptions such as equal average ATP content per cell (Frossard et al., 2016) and overcomes the limitations associated with qPCR quantification methods such as DNA extraction efficiency and yield (Bremen et al., 1999; Feinstein et al., 2009), and primer specificity. We optimised cell detachment for FCM quantification using a combination of chemical surfactants and ionic dispersants, together with low and high-energy sonication as a mechanical pre-treatment, and different fixative methods. Furthermore, we cross-compared the developed protocol against other established and commonly employed methods for biomass quantification for water filter samples.

4.1. Optimisation of pre-treatments for biofilm detachment

Chemical treatment: The combination of a surfactant (Tween-80) with an ionic dispersant (sodium pyrophosphate) created the best conditions for the detachment of biofilm from particle surfaces and their dispersal into single cells, allowing the highest counts in flow-cytometric analyses. The surfactant Triton-X100 detached significantly fewer cells than all the other solutions tested, in contrast with previous studies (Chen and Stewart, 2000). Different dispersants have previously been studied to enhance the detachment of cell agglomerates and biofilms from surfaces. In an experiment conducted by Chen and Stewart (2000), solutions of NaCl, $CaCl_2$ and Triton-x100 performed best in removing biofilm from stainless-steel slides. Magic-Knezev and van der Kooij (2004) successfully employed autoclaved tap water, in combination with several sonication steps, for the detachment of biofilm from sand and GAC particles as evaluated by ATP quantification, while Brown et al. (2015) and Lavergne et al. (2014) showed that the combination of a non-ionic surfactant (Tween 80) and an ionic dispersant was the best performing solution to disperse activated sludge flocs and detach biofilm from coastal sediments. We observed no significant differences in the number of total extractable cells from sand samples treated with autoclaved tap water, NaCl, $CaCl_2$ and PP solutions.

The five dispersants used clustered according to three different extraction patterns similar to those previously observed by Ugolini et al., (2013), where a Tween mix (1% Tween 80 in 0.1 M EDTA and 0.1M TSPP[2]) was tested, among other dispersants, to detach biofilm from freshwater sediment columns. The solution released the majority of cells shortly after application, followed by drastic, rapid attenuation of its extraction efficiency. Ugolini et al., 2013 also

[2] Tetrasodium Pyrophosphate decahydrate.

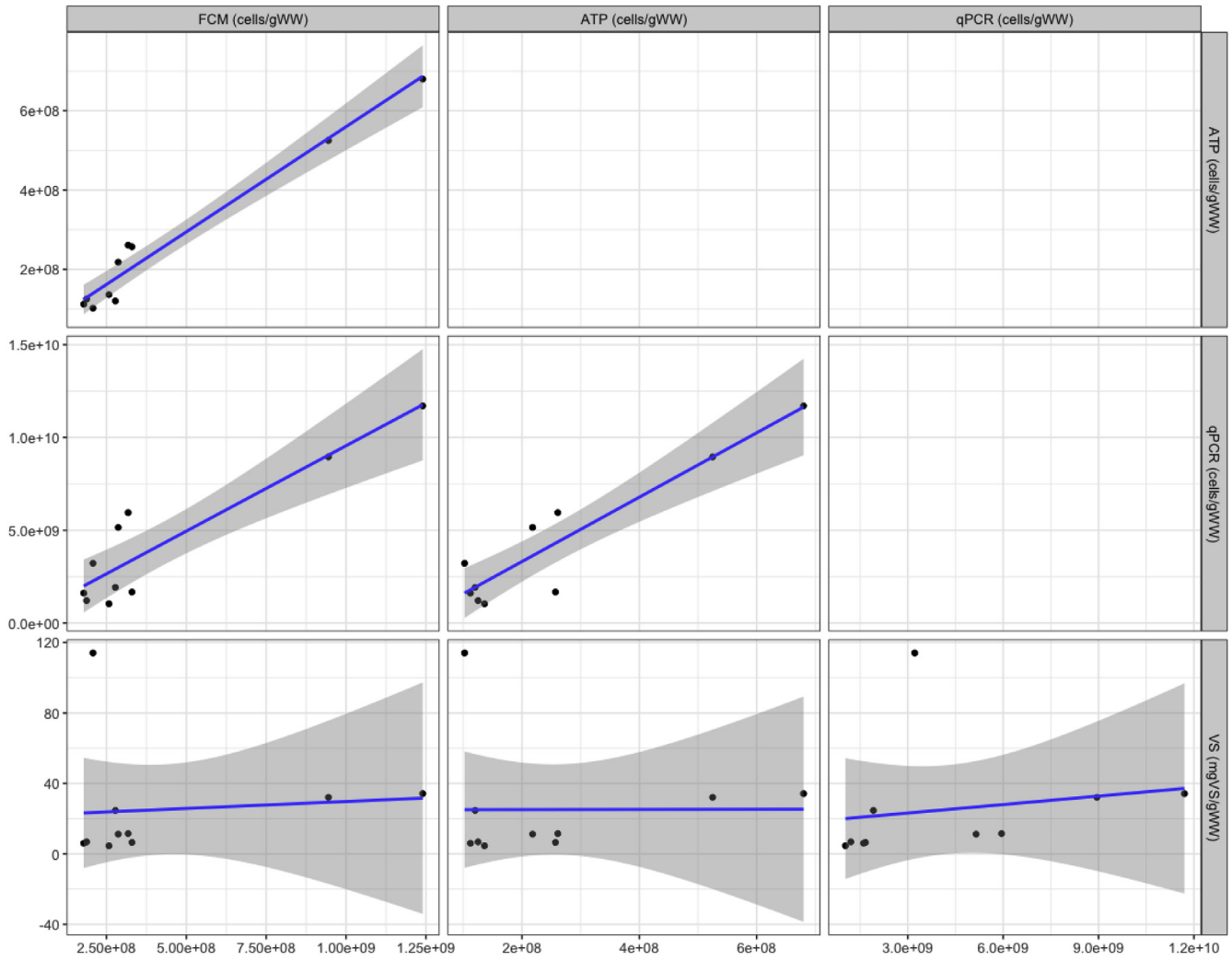

Fig. 6. Correlation between biomass quantifications methods; FCM on cells extracted with the protocol developed in this article (concentration expressed as cells/gWW), ATP quantified as described in (Velten et al., 2007) (concentration expressed as cells/gWW, qPCR performed as described in section 2.3 (concentration expressed as cells/gWW) and VS quantified as described in section 2.3 (concentration expressed as mgVS/gWW).

observed that solutions of NaCl and $CaCl_2$ showed a slower and more prolonged response similar to that described in this paper (Ugolini et al., 2013).

Each of the solutions employed in this study promotes biofilm detachment and dispersal through different mechanisms. Non-ionic surfactants, such as Tween-80 and Triton X-100 act by disrupting hydrophobic interactions involved in cross-linking the biofilm matrix. This triggers the solubilisation of hydrophobic molecules that constitute the biofilms' extracellular polymeric substances (EPS), such as extracellular lipids, which play an important role in biofilm adhesion (Chen and Stewart, 2000). Such an effect would explain the high numbers of cells extracted at T_0 by the TWEEN-PP dispersant. However, it is still not clear why in this experiment, a completely different behaviour, and a significantly lower extraction efficiency, was observed for Triton X-100. The increased extraction efficiency of TWEEN-PP might be explained by the fact that when the two dispersants are combined they may act through two different mechanisms: TWEEN dissolves hydrophobic molecules (as described above) and PP acts as a chelating agent, scavenging bivalent cations such as Ca^{2+} and Mg^{2+} that crosslink the negatively charged groups on the surface of EPS constituents (Ugolini et al., 2013; Velji and Albright, 1984).

Table 2
Correlation coefficient (Pearson), significance levels (p-values), slopes and intercepts of regressions of cell abundances per g of wet weight.

	ρ	*p*	*Slope*	*Intercept*
FCM vs. ATP	0.983	<0.001	0.70 ± 0.05	3.62E+07
FCM vs. qPCR	0.911	<0.001	0.09 ± 0.01	3.98E+07
ATP vs. qPCR	0.930	<0.001	0.12 ± 0.01	1.09E+08
VS vs. FCM	0.049	<0.001		
VS vs. ATP	0.002	<0.001		
VS vs. qPCR	0.177	<0.001		

Mechanical treatment: We found that four cycles of high-energy sonication in a TWEEN-PP solution are the most effective treatment for optimal cell counts among all the treatments tested. Sonication is a mechanical treatment often proposed for the removal of biofilm from a plastic material (Proctor et al., 2016), sand and GAC particles (Li et al., 2010; Magic-Knezev and van der Kooij, 2004), and marine sediments (Danovaro et al., 2001). Low energy sonication has been shown to be less effective than high energy sonication in detaching biofilm from GAC and sand particles, a finding that has been confirmed by our results (Magic-Knezev and van der Kooij, 2004). In our experiment, we subjected sand particles to one cycle of LES, followed by seven cycles of HES. We

observed no significant difference between the number of cells extracted after the addition of the extraction solution (TWEEN-PP or TAP) and after one cycle of LES, in both sets of samples. The cumulative ATP yield of the biomass that Magic-Knezev and van der Kooij, 2004 obtained in their experiment using LES treatment (on sand samples) was 50% of the biomass obtained with HES at power input comparable to the one employed in our experiment (27 W).

HES treatment showed a quick and drastic decrease in its extraction efficiency, reaching a plateau already after one HES cycle (for TWEEN-PP) or after two HES (for TAP), confirming a trend already observed by Magic-Knezev and van der Kooij (2004). However, in order to ensure maximum reproducibility and to obtain comparable results, we suggest a minimum of four HES cycles run for up to 80 s at a power input of 27 W.

Shaking was also evaluated as a mechanical pre-treatment of sand particles, as an alternative to sonication. Results suggested that the combination of chemical dispersant TWEEN-PP and HES was still the treatment ensuring the highest number of cells extracted. However, the combination of TWEEN-PP with four cycles of shaking allowed the final recovery of the same amount of cells obtained by the combination of autoclaved tap water and HES; this kind of treatment might be useful when HES cannot be applied.

Fixative optimisation: Two common fixation solutions were evaluated for their ability to preserve and protect microbial cells during the extraction protocol proposed (glutaraldehyde and a solution of ethanol: PBS 50%). The results strongly suggested that the fixation methods could affect the extraction performance and the number of both intact and total microbial cells recovered by the treatment.

The number of total cells extracted from GLUT-fixed samples was significantly greater than the amount extracted from EtOH-fixed and PBS-control samples for both extraction solutions used (TWEEN-PP and TAP water) (Section 3.3).

Solutions of detergents such as Tween-20 or Triton-x100 are often used to allow the permeabilisation of cell membranes in order to gain access to intracellular areas; they act by breaking lipid-lipid and lipid-protein interactions, causing the solubilisation of lipids forming the cell membrane.

Two main fixation methods can be identified in the literature to protect cells: additive and denaturing fixations (Chao and Zhang, 2011; St-Laurent et al., 2006). Glutaraldehyde, along with other aldehydes such as formaldehyde and paraformaldehyde, belongs to the group of additive fixative solutions; these solutions fix cells through the formation of covalent chemical bonds between the proteins of the cellular membrane. The effect is the creation of an outer cell cross-link structure. This might explain the significantly higher amount of cells detected in the GLUT-fixed samples extracted with TWEEN-PP compared to the controls. The detergent enhances the biofilm breakage through the dissolution of the EPS lipids, but at the same time, dissolves lipids of the cellular membrane. This induces cells to burst unless they are protected by the outer cross-link structure between membrane proteins created by the additive fixative solutions. Moreover, the outer cross-linked structure created by glutaraldehyde bonds proved to be an effective strategy for protecting cells from the shear forces created by sonication, preventing both their burst and their damage. In fact, the amount of both total and intact cells extracted from GLUT-fixed samples was significantly higher than that recovered in the control samples when autoclaved tap water was used as an extraction solution.

Glutaraldehyde, however, was not able to protect cells from damage due to the dissolution of the lipid membrane. The number of intact cells measured in GLUT-fixed samples extracted with TWEEN-PP was one order of magnitude lower than the amount measured in the same set of samples extracted with autoclaved tap water. This suggests that, while the outer structure of cells remains intact (allowing the count of total cells using FCM), pores created in the cell membrane by lipid dissolution caused the double-positively charged molecule of the propidium iodide (PI) stain to penetrate and stain the cells with a stronger red fluorescence. In this way, the PI-positive cells with damaged membranes can clearly be distinguished from intact cells during FCM analyses (Ramseier et al., 2011).

Ethanol proved to be an ineffective fixative; failing to protect cells against burst or damage induced by chemical dissolution of the membrane or by shear forces created by sonication. In fact, no statistical difference was observed between the number of total cells measured in the EtOH-fixed samples and controls extracted with both solutions. Moreover, we observed that ethanol dramatically damaged the cellular membrane: the number of intact cells measured in EtOH-fixed samples was significantly lower than the controls, for both extraction solutions. Ethanol belongs to the denaturating fixative solutions group. These solutions act by denaturing proteins through the reduction of their solubility and/or disruption of hydrophobic interactions. Their application could induce the dissolution of cell membrane lipids with the formation of large pores in its structure, therefore explaining the low counts obtained.

4.2. Cross-comparison with other methods

The protocol proposed appeared to be a consistent and reliable method for measuring microbial cells in sand samples from water filters using FCM. Despite differences in the absolute numbers, the cell abundances measured with the protocol correlated highly with the values obtained with other classic methods employed for biomass quantification for these environments: qPCR and ATP analysis. The absolute counts obtained with the FCM method were typically lower than the corresponding counts obtained with the ATP assay and with qPCR as suggested by the slope of the linear correlations: 0.7 and 0.09 respectively.

A previous study, carried out on samples collected from several environments (sediments, soils, and sludge), directly compared cell abundances measured via the ATP assay and FCM on suspensions obtained after cell detachment via three cycles of sonication in a fixative solution (Frossard et al., 2016). The study showed that estimates based on the ATP assay yielded significantly higher average microbial abundances than the FCM method; the slope of the correlation between FCM and ATP was on average 0.36. In that study, researchers attributed the discrepancy to the different storage times (4 weeks for FCM against the 24h for ATP) and preservation methods (paraformaldehyde for FCM and phosphate buffer for ATP) employed by the two protocols. The much higher slope observed in our study (0.7 rather than 0.36) might be explained by the fact that the four different methods were performed, on every single sample, on the same day of collection; overcoming, in this way, biases induced by different preservation strategies and time. The FCM estimated slightly lower cell abundances than the ATP assay. This discrepancy could be attributed to the measurement of extracellular ATP or additional non-microbial ATP belonging to other microorganisms, such as fungi, or micro-invertebrates as well as fine roots and small plant residues that are not quantified in the FCM method. Other potential causes are an underestimation of the average ATP content per cell, and uncertainty in the determination of the slope from the data.

On the other hand, the high discrepancy between qPCR and FCM absolute numbers could result from problems of non-specificity of the qPCR primers used, or from the presence of free DNA from decayed cells, causing qPCR to overestimate the number of cells present in a sample.

Alternatively, another reason for the high discrepancies in counts observed between the method proposed and the other two analysed, could be an incomplete removal of biofilm from the sand grain surfaces; a qualitative analysis of the sand grains before and after the detachment protocol, using optical microscopy (Fig. 1 SI), showed effective detachment of biofilm. However, while the method seems to effectively detach most of the biofilm developed on the surfaces and between sand grains, where we expect most of the cells to be located, an unknown percentage of undetached biofilm or individual cells might still be present in crevices within sand particles.

Finally, VS had much weaker correlations (Pearson correlation ranging from 0.001 to 0.19) as compared with all other measures of biomass concentration. This is not surprising, as VS includes organic matter other than cells, such as the organic matter naturally present in water and coagulated during the water treatment process.

It is important to underline that the method here proposed has been optimised using only one type of sand. Factors such as different filter media (GAC versus sand), filter media characteristics (grain dimension and roughness), biofilm age and composition might affect the extraction efficiency. While the protocol shows good reproducibility comparable with other common methods for the quantification of cell abundances in sand samples, we suggest that additional experiments should be performed to check the reproducibility of the protocol and its efficacy on samples with characteristics different from those investigated in this study.

5. Conclusions

- The results show that the optimised protocol presented is a reliable and highly reproducible method for enumerating total cells on sand particles;
- The protocol proposed using FCM for cell quantification produces cell abundances that correlate well with alternative quantification methods: high correlations were found with counts obtained with ATP and qPCR;
- The VS content was confirmed as an inaccurate method to express biomass in sand samples;
- Despite a good correlation between the trends of cell abundances, qPCR seems to overestimate absolute cell counts while FCM and ATP produce similar values.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2018.05.053.

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