



# A flow cytometry method for bacterial quantification and biomass estimates in activated sludge

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## ABSTRACT

Absolute bacterial quantification receives little serious attention in the literature compared to sequencing, conceivably because it is considered unimportant and facile, or because existing methods are tedious, laborious and/or biased in nature. This is particularly true in engineered systems, including activated sludge, where such information underpins their design and operation. To overcome these limitations we built upon existing work and optimised and comprehensively validated, through comparison with epifluorescence microscopy (EFM), a rapid and precise flow cytometric protocol to enumerate total bacterial numbers in activated sludge. Insights into potential biases were evaluated using appropriate statistical analyses on this comparison, which spanned four orders of magnitude, as well as comparing volatile suspended solid (VSS) concentrations. The results suggest flow cytometry (FCM) is a rapid, reproducible and economical technique for quantifying total bacterial numbers and biomass concentrations in activated sludge, despite within order of magnitude discrepancies with EFM counts, which had inherent and evidently greater errors and biases than FCM. The use of FCM for routine monitoring over both EFM and VSS should help further understanding of the microbial ecology in, and the operation of, engineered systems.

## 1. Introduction

Accurate quantification is a fundamental and invaluable prerequisite for comprehending the ecological role of microorganisms in natural and engineered ecosystems. Quantification underpins parameters important in predicting microbial behaviour, from empirical models of bacterial growth and kinetics (Monod, 1949) to ecological theories of microbial assembly and dynamics (MacArthur and Wilson, 1967; Tilman, 1977; Sloan et al., 2006). Yet absolute quantification of microbes, particularly bacteria and individuals of specific bacterial taxa, receives relatively little attention; relative taxa abundances, garnered from sequencing technologies, dominate contemporary microbial ecology (Props et al., 2017). However, proportional abundances have inherent biases (Angly et al., 2014; Widder et al., 2016) and disregard inter-sample differences in cell density, both of which can lead to differing biological interpretations (see Angly et al. (2014) and Props et al. (2017) for further discussion). Thus absolute abundance measurements, applicable at the community level, have recently been called for in microbial ecology (Widder et al., 2016).

Quantification is particularly important in engineered biological

wastewater treatment systems such as activated sludge (AS), a global economically important biotechnology. Monitoring key functional organisms, as well as total biomass, is essential for anticipating and obviating plant failure (Baptista et al., 2014), as well as facilitating rational improvements in system design. The measurement of total bacterial abundance, combined with high throughput sequencing, in principle, should allow such absolute taxon abundances to be calculated (Props et al., 2017). Nevertheless total bacterial numbers, in AS and other complex environments, are infrequently determined, perhaps owing to the tedious, laborious and/or biased nature of available methods (Frossard et al., 2016).

Traditional, culture based methods (Pike et al., 1972; Banks and Walker, 1977) detect only a small fraction of the total population (Wagner et al., 1993), whilst enumeration by epifluorescence microscopy (EFM) (Kepner and Pratt, 1994), often considered the “gold standard” of total bacterial quantification (Seo et al., 2010), involves considerable observer bias and is time consuming (Frossard et al., 2016). More recently quantitative real-time PCR (qPCR) targeting the bacterial 16S rRNA gene has greatly simplified total bacterial quantification (Dionisi et al., 2003), due to its high sample throughput, high

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precision (Klein, 2002) and sensitivity across an extensive linear range (Suzuki et al., 2000; Klein, 2002). Yet, like its predecessors, it has its limitations, suffering from many of the biases associated with PCR. These include only measuring gene copy number (not cell numbers) and its dependence on factors other than the amplification reaction (e.g. sample preparation, DNA extraction, standard quality, choice of target gene and amplification primers and probes) (Suzuki et al., 2000; Klein, 2002; Yu et al., 2005).

As a consequence practitioners and many research laboratories still rely heavily on the conventional gravimetric measurement of volatile suspended solids (VSS) as a proxy for total bacterial abundance/active biomass. Gravimetric methods, which date from the 19th century, are inaccurate, imprecise and time-consuming (Ziglio et al., 2002). As such there is a need, both in academia and industry, for a rapid, cheap and reliable technique for quantifying total bacterial numbers in AS.

One of the most promising solutions is flow cytometric quantification following nucleic acid staining with the same fluorescent dyes used in EFM analysis, for example 4', 6-diamidino-2-phenylindole (DAPI) and SYBR Green I. Flow cytometry (FCM) overcomes many of the above mentioned disadvantages of both EFM and qPCR respectively and thus has been used to enumerate bacteria in a diverse range of environments, including drinking water (Hammes et al., 2008), natural and agricultural soils (Bressan et al., 2015; Frossard et al., 2016), stream, lake and ocean sediments (Danovaro et al., 2002; Duhamel and Jacquet, 2006; Frossard et al., 2016) and sand filters (Frossard et al., 2016; Vignola et al., 2018). Although in principle bacterial quantification in AS is problematic, due to the aggregated nature of bacterial growth in suspended biofilms/multispecies microcolonies (flocs), in practice FCM has been used to quantify bacteria, cell viability and bacterial biomass (Ziglio et al., 2002; Falcioni et al., 2006; Foladori et al., 2007; Foladori et al., 2010; Ma et al., 2013; Abzazou et al., 2015) following floc disaggregation by mechanical homogenisation and/or sonication.

However, significant improvements are possible. Chemical dispersants may be more effective at disaggregating cells than the use of physical methods alone (Brown et al., 2015). Furthermore, previous protocols used in AS were neither comprehensively optimised nor validated against a more widely accepted, “gold standard” quantification method, rarely used comprehensive statistical analysis and were confined to narrow cell concentration ranges that are not environmentally relevant (Ziglio et al., 2002; Falcioni et al., 2006; Foladori et al., 2007; Foladori et al., 2010; Ma et al., 2013; Abzazou et al., 2015). In this paper we describe the optimisation and validation (by comparison with EFM) of a rapid, cheap and precise FCM protocol to enumerate total bacterial numbers in AS. We anticipate that a simple and rapid quantification protocol will be of benefit to researchers and practitioners alike. In particular such a method will encourage the use of total bacterial numbers in conjunction with high throughput sequencing data.

## 2. Methods

### 2.1. Protocol optimisation

#### 2.1.1. Samples

AS samples were collected from the aeration basin (3600 m<sup>3</sup>) of a full scale, nitrifying AS plant situated in North East England, United Kingdom (FS 1). Samples were collected in 50 mL polypropylene containers and immediately fixed with absolute ethanol at a ratio of 1:1 v/v before being transported at ~4 °C, aliquoted into 1 mL sub-samples and stored at -20 °C for up to 6 months. Sub-samples were mixed via manual shaking for 10 s prior to pre-treatment (Table 1, described in Section 2.1.2). Once established optimal pre-treatments were used in subsequent experiments.

#### 2.1.2. Pre-treatments for floc disruption

**2.1.2.1. Chemical treatment.** The surfactant polyoxyethylene-sorbitan monooleate (Tween 80, Sigma) and the ionic dispersant sodium

**Table 1**

Treatments to which AS samples were subjected in order to optimise FCM enumeration of bacterial cells.

Treatment	Levels
Chemical Extraction	5% Tween 80 and 10 mM SP
Physical Extraction	Mechanical mixing, sonication for 1, 2, 3, 4, 5 and 8 min
Dye Type	SYBR Green I, SYBR Green II, SYBR Gold and SYTO 9
Dye Dilution	1:30, 1:100 and 1:200
Incubation Temperature	RT, 44, 60 and 80 °C

RT = Room temperature.

pyrophosphate (SP, Sigma) were tested in combination, 5% and 10 mM respectively, as a sample pre-treatment for AS floc disruption, as used previously for viral quantification (Brown et al., 2015). Dispersants were combined, added to sub-samples and mixed via manual shaking for 30 s followed by incubation for 15 min in the dark at room temperature. The treatment was performed in triplicate, with a paired control (dispersant free sub-sample) per replicate. SP was autoclaved prior to use whilst Tween 80 was not.

**2.1.2.2. Physical treatment.** The effect of mechanical mixing, in combination with chemical treatment, on floc disruption was tested using a magnetic stirrer and mixing bar, with sub-samples being mixed at 200 rpm during the 15 min incubation period described in 2.1.2.1. The effect of ultrasound treatment was also evaluated using a sonicating water bath (USC 300 T; 200 W; 45 KHz), with sub-samples being run for 1, 2, 3, 4, 5 and 8 min. Sonication was interrupted for 30 s every minute, during which time the samples were shaken manually (Danovaro et al., 2001). Each treatment was analysed in triplicate, with a paired control (sub-samples without mechanical mixing or sonication) per replicate.

#### 2.1.3. Staining optimisation

The nucleic acid dyes SYBR Green I, SYBR Green II, SYBR Gold and SYTO 9, which preferentially bind to double stranded DNA (dsDNA); single stranded DNA (ssDNA), RNA and dsDNA; and ssDNA and RNA respectively (SYBR Gold and SYTO 9), were tested separately, at varying dilutions (1:30, 1:100 and 1:200 v/v of each dye's stock solution and dimethyl sulfoxide (DMSO), chosen in accordance with Duhamel and Jacquet (2006) and Foladori et al. (2010)). Those that target both DNA and RNA, and/or have better extinction coefficients and quantum yields at 488 nm, may be expected to result in a greater signal to noise ratio and therefore achieve higher counts (Table S1). All dyes were tested simultaneously at each dilution using triplicate sub samples, with triplicate controls (the same sub samples stained as described in 2.1.5) for each dilution. Incubation at different temperatures, 44, 60 and 80 °C respectively, was also investigated, since heat treatment can increase cell permeability, denature nucleic acids and thus improve staining efficiency (Lebaron et al., 1998; Brussaard, 2004). All temperatures, chosen in accordance with those used previously for viral quantification (Brussaard, 2004), were tested concurrently using triplicate sub samples and controls (the same sub samples incubated as described in 2.1.5).

#### 2.1.4. Dilution optimisation

The optimum number of events per second for FCM bacterial counting was assessed by running triplicate sub-samples at varying dilutions (1:250, 1:500, 1:750, 1:1000, 1:1250, 1:1500, 1:1750 and 1:2000). Once established dilutions falling in the optimal events per second range were analysed (for consistency typically 4 dilutions, see Section 2.1.5).

#### 2.1.5. Fluorescent staining

To reduce coincidence, two or more bacteria and/or particles being

simultaneously within the sensing zone, pre-treated sub-samples were diluted with autoclaved 0.2 µm filtered TE-buffer (10 mM Tris-HCl 1 mM EDTA; pH 8.0, Sigma), as recommended for FCM viral quantification (Brussaard, 2004). Although not specifically tested, TE-buffer is thought to permeabilise cell membranes (facilitating dye uptake) and further release bacteria bound to extracellular polymeric substances (EPS, EDTA destroys cation links between EPS polymers) (Zhang et al., 2000; Carreira et al., 2015), increasing fluorescent signals and counts respectively (Brussaard, 2004). Four 1 mL dilutions, 1:200, 1:300, 1:400 and 1:500, were prepared per replicate for all treatments, unless otherwise stated (Section 2.1.4 and analysis thereafter). Diluted sub-samples were stained using either 10 µL of SYBR Green I (1:30 v/v dilution of commercial stock solution with DMSO) for 15 min in the dark at room temperature (as described by Foladori et al. (2010)), or a modification of this using other dyes (Section 2.1.3). Note 10 µL of dye and a 15 min incubation period was used regardless of stain type, stain dilution factor, or staining temperature. The differentiation and quantification of both live and dead cells, using propidium Iodide, was purposely omitted, since contemporary high throughput sequencing typically incorporates both live and dead cells (although it is possible to exclude the latter, see Albertsen et al. (2015)).

## 2.2. Flow cytometry analysis

Sample dilutions were analysed in duplicate using a FACScan flow cytometer (Becton Dickinson, California) equipped with a 15-mW air-cooled argon-ion laser emitting light at 488-nm. Green fluorescence (GFL) was measured at 530 nm (FL1 channel) and the flow cytometer was set up as follows: gain FL1 = 540, gain side scatter (SSC) = 540, flow rate = low. Readings were collected in logarithmic mode (at least 5000 events per sample) and triggered on GFL (threshold = 230, determined using 0.2 µm filtered AS samples), CountBright™ absolute counting beads (ThermoFisher, UK) were used as a volumetric standard. Data was analysed using FCS Express (v.6.04.0015, De Novo Software, California), with electronic gates, defined based on previous mixed and pure culture experience (data not shown) and 0.2 µm filtered AS samples, used to quantify the desired events. Presentation of the data as FL1/SSC density plots (Fig. S1 A) enabled the best distinction between stained bacteria and other microbial cells and/or background noise, thus filtration to remove such particles was avoided. To facilitate the correction of bacterial counts for noise, TE-buffer blanks were pre-treated and analysed identically to sample dilutions.

## 2.3. Protocol validation

### 2.3.1. Samples

AS samples were collected from both full-scale and lab-scale systems to obtain counts spanning several orders of magnitude, an important consideration when validating agreement between two quantification methods (Baptista et al., 2014). Samples were collected from, i) ten full-scale AS plants (see Table 2; including three samples taken at different times from FS 1, a local wastewater treatment plant (WWTP)), making 12 samples in total, ii) eight lab-scale AS sequence batch reactors (SBRs) run at varying solids retention times (SRTs, 2–14 days), with one sample each taken at SRTs of 2, 4, 6, 8, 10, 11, 12 and 14 days. The SBRs had a working volume of 1 L, were seeded and fed with AS and settled sewage collected from FS 1 and had a hydraulic retention time (HRT) of 2.86 days. All samples were collected, fixed and stored as previously described. Samples were mixed via manual shaking for 10 s and pre-treated following the optimal protocol before being split into two 500 µL aliquots for FCM and EFM total bacterial quantification respectively. Prior to ethanol fixation the suspended solids (SS) and VSS concentration of each sample was also determined according to Standard Methods (APHA, 1998).

### 2.3.2. Cytometry versus microscopy

FCM aliquots were diluted and stained following the optimal protocol and then analysed as described in 2.2. EFM aliquots were similarly diluted using autoclaved 0.2 µm filtered TE-buffer (10 mM Tris-HCl 1 mM EDTA; pH 8.0, Sigma) for reasons discussed in 2.1.5, with four 1 mL dilutions (1:50, 1:100, 1:500 and 1:1000) prepared in duplicate. Dilutions were stained with 50 µL of SYBR Green I (1:50 v/v dilution of commercial stock solution with DMSO) and incubated for 15 min in the dark at room temperature. Dilutions were then filtered onto 0.2 µm-pore-size black polycarbonate filters (Millipore, MA) before being washed twice with 1 mL of autoclaved 0.2 µm filtered TE-buffer (10 mM Tris-HCl 1 mM EDTA; pH 8.0, Sigma) and mounted onto glass microscope slides using Citifluor AF1 (Citifluor Ltd., Hatfield, US) antifadent and glass cover slips secured using nail varnish. Counts were obtained at x 1000 magnification using a Nikon Eclipse Ci-L microscope (CFI Plan Apo Lambda x 100 objective, NA = 1.45, WD = 0.13) equipped with a pE-300<sup>white</sup> illumination unit (CoolLED Ltd., Andover, UK), set to blue excitation, with appropriate filter sets. Dilutions resulting in > 30 cells per field of view, captured in JPEG format using a Retiga 6000 charge-coupled-device camera (QImaging, Surrey, Canada), were used to calculate total bacterial counts as described by Kepner and Pratt (1994), with 20 random fields of view counted per filter ( $n = 20$ ).

## 2.4. Statistical analysis

All statistical analysis was undertaken in RStudio (v. 1.0.143) using R version 3.4.0 (R Core Team, 2017). The Shapiro-Wilk Test ( $P > 0.05$ , shapiro.test, “stats” v. 3.4.0, R Core Team (2017)) and the Bartlett Test ( $P > 0.05$ , bartlett.test, “stats” v. 3.4.0, R Core Team (2017)) were utilised to test normality and homogeneity of variance respectively, unless stated otherwise.

### 2.4.1. Protocol optimisation

Bacterial counts (and fluorescence intensities, section 1.1.3) were compared and analysed for significant differences using ANOVA with Tukey's pairwise comparisons (TukeyHSD, “stats” v. 3.4.0, R Core Team (2017)). Prior to analysis data was checked for normality and homogeneity of variance as in 2.4.

### 2.4.2. Fluorescent microscopy cell distribution and count transformation

Counts obtained by EFM were evaluated as described previously (Davenport and Curtis, 2004). For each replicate normality (as in 2.4), skewness (skewness, “timeDate” v. 3012.100, Rmetrics Core Team (2015)), kurtosis (kurtosis, “timeDate” v. 3012.100, Rmetrics Core Team (2015)) and the standardised index of dispersion (Eq.S1) were calculated across all fields of view, the latter being compared to a tabulated critical value of the  $\chi^2$  distribution for the appropriate degrees of freedom ( $n - 1$ ) at a 0.05 significance level (Rohlf and Sokal, 1995). Homogeneity of variance across all replicates was also checked (as in 2.4). Where necessary field of view counts were transformed based on the most common Box-Cox transformation, calculated per replicate (BoxCox.lambda, “forecast” v. 8.0, Hyndman and Khandakar (2008)), and then back transformed to assess agreement with FCM counts (section 2.4.3).

The influence of bacterial abundance and VSS on cell dispersion was assessed by calculation of Spearman's rank correlation coefficients (cor.test, “stats” v. 3.4.0, R Core Team (2017)), all variables were  $\log_{10}$  transformed prior to analysis. Its use, over Pearson correlation, was justified since all variable combinations were not bivariate normal ( $P < 0.05$ , roystonTest, “MVN” v. 4.0.2, Korkmaz et al. (2014)).

### 2.4.3. Agreement between cytometry and fluorescence microscopy counts

The statistical analysis recommended for comparing FCM and EFM counts, as performed in this section, is summarised in the supplementary information (Fig. S3).

**Table 2**  
FCM and EFM TBN's and solids data for each AS sample.

Sample	FCM TBN's (cells mL <sup>-1</sup> )	EFM TBN's (cells mL <sup>-1</sup> )	SS (g L <sup>-1</sup> )	VSS (g L <sup>-1</sup> )
R SRT 2	1.90 ± 0.009 × 10 <sup>7</sup>	3.29 ± 0.076 × 10 <sup>7</sup>	0.13	0.11
R SRT 4	1.08 ± 0.008 × 10 <sup>8</sup>	1.62 ± 0.001 × 10 <sup>8</sup>	0.26	0.23 <sup>†</sup>
R SRT 6	1.81 ± 0.029 × 10 <sup>8</sup>	2.83 ± 0.184 × 10 <sup>8</sup>	0.3	0.23
R SRT 8	2.04 ± 0.018 × 10 <sup>8</sup>	3.05 ± 0.057 × 10 <sup>8</sup>	0.48	0.48
R SRT 10	6.68 ± 0.136 × 10 <sup>8</sup>	1.32 ± 0.185 × 10 <sup>9</sup>	0.83	0.57
R SRT 11	9.22 ± 0.021 × 10 <sup>8</sup>	2.21 ± 0.068 × 10 <sup>9</sup>	0.57	0.42 <sup>ψ</sup>
R SRT 12	3.56 ± 0.004 × 10 <sup>8</sup>	7.43 ± 0.014 × 10 <sup>8</sup>	0.74	0.64
R SRT 14	5.18 ± 0.005 × 10 <sup>8</sup>	9.31 ± 0.742 × 10 <sup>8</sup>	0.6	0.49
FS 1 A	1.31 ± 0.058 × 10 <sup>9</sup>	4.08 ± 0.231 × 10 <sup>9</sup>	1.95	1.44
FS 1 B	1.16 ± 0.007 × 10 <sup>9</sup>	3.44 ± 0.139 × 10 <sup>9</sup>	2.95	2.13 <sup>†</sup>
FS 1 C	1.09 ± 0.050 × 10 <sup>9</sup>	2.92 ± 1.029 × 10 <sup>9</sup>	4.02	2.92 <sup>†</sup>
FS 2	1.67 ± 0.028 × 10 <sup>9</sup>	4.53 ± 0.358 × 10 <sup>9</sup>	1.19	0.97 <sup>ψ</sup>
FS 3	2.02 ± 0.002 × 10 <sup>9</sup>	5.18 ± 0.289 × 10 <sup>9</sup>	1.83	1.48
FS 4	3.31 ± 0.069 × 10 <sup>9</sup>	9.26 ± 0.965 × 10 <sup>9</sup>	2.22	1.87
FS 5	2.62 ± 0.074 × 10 <sup>9</sup>	6.27 ± 0.007 × 10 <sup>9</sup>	2.15	1.73
FS 6	2.31 ± 0.005 × 10 <sup>9</sup>	4.97 ± 0.312 × 10 <sup>9</sup>	1.92	1.52
FS 7	1.06 ± 0.015 × 10 <sup>9</sup>	2.57 ± 0.362 × 10 <sup>9</sup>	2.57	2.04 <sup>†</sup>
FS 8	3.95 ± 0.012 × 10 <sup>9</sup>	1.25 ± 0.134 × 10 <sup>10</sup>	3.22	2.67
FS 9	3.06 ± 0.006 × 10 <sup>9</sup>	6.26 ± 0.419 × 10 <sup>9</sup>	3.93	2.87
FS 10	3.94 ± 0.007 × 10 <sup>9</sup>	1.11 ± 0.116 × 10 <sup>10</sup>	4.63	3.67

R = Reactor sample, FS = Full scale sample. TBN, total bacterial numbers. ± denotes standard deviation across duplicate samples. Samples VSS advocates greater (†) and fewer (ψ) bacterial cells than those quantified.

**2.4.3.1. Difference between abundance estimates.** Bacterial counts obtained by FCM and EFM were initially compared and analysed for significant differences using the Kruskal-Wallis test ( $P > 0.05$ , kruskal.test, “stats” v. 3.4.0, R Core Team (2017)), since some counts were non-normal and had heterogeneous variance.

**2.4.3.2. Relatedness, agreement and bias.** The association, or relatedness (Giavarina, 2015), between the two methods was further assessed using linear regression (.lm, “stats” v. 3.4.0, R Core Team (2017)), whilst Bland Altman analysis, used extensively in clinical sciences (bland.altman.stats, “BlandAltmanLeh” v. 0.3.1, Lehnert (2015)), was used to assess their agreement and to identify the nature and magnitude of any bias (Giavarina, 2015). The influence of VSS concentrations, as alluded to previously (Frossard et al., 2016), and bacterial abundance on FCM and EFM agreement was also assessed using linear regression analysis, with differences in FCM and EFM counts, identified during Bland Altman analysis, being regressed on VSS concentrations and EFM bacterial abundances.

All linear models were checked visually for homoscedasticity, linearity and residual autocorrelation, whilst model residuals were checked for normality as in 2.4 (Norman and Streiner, 2008; Zuur et al., 2010; Ghasemi and Zahediasl, 2012). For Bland Altman analysis the calculated differences between FCM and EFM bacterial counts were checked for normality as described in 2.4 so that 95% limits of agreement could be estimated. To meet these assumptions FCM and EFM counts, as well as their differences, were log<sub>10</sub> transformed for both types of analysis, as were VSS concentrations.

**2.4.3.3. Precision, reliability and repeatability.** The precision, reliability and repeatability of both methods were assessed by one way repeat measures ANOVA (aov, “stats” v. 3.4.0, R Core Team (2017)), calculation of intraclass correlation coefficients (ICCs, Eq.S2) and repeatability coefficients respectively (RCs, Eq. S3). ICCs were calculated as the index of contribution of random measurement errors to the total variance (Eq.S2). Separate ANOVA models were produced for each method, with dependent variables (each set of replicates) checked for normality as in 2.4. As only two repeat measures were undertaken the variance in replicates was also assessed as in 2.4. In this instance, to meet the normality assumption of ANOVA, replicate FCM and EFM counts were square root transformed.

#### 2.4.4. Association between abundance estimates and VSS

FCM and EFM counts ( $X$ ) were initially converted into bacterial biomass estimates ( $M$ ) using Eq. (1) and assuming a bacteria biovolume ( $V$ ) of 0.25 μm<sup>3</sup> cell<sup>-1</sup> (Frølund et al., 1996), a carbon content per unit of cell volume ( $C_s$ ) of 310 fg C μm<sup>-3</sup> (Fry, 1990) and considering only ~53% of a cell's dry weight in activated sludge constitutes carbon (Foladori et al., 2010).

$$M \text{ (mg VSS)} = X \times \left( \frac{V \times C_s \times 10^{-12}}{0.53} \right) \quad (1)$$

The association between VSS and both FCM and EFM total bacterial counts and biomass estimates was then assessed using linear regression and Bland Altman analysis, performed and checked as in 2.4.3.2 using log<sub>10</sub> transformed counts, biomass estimates and VSS concentrations.

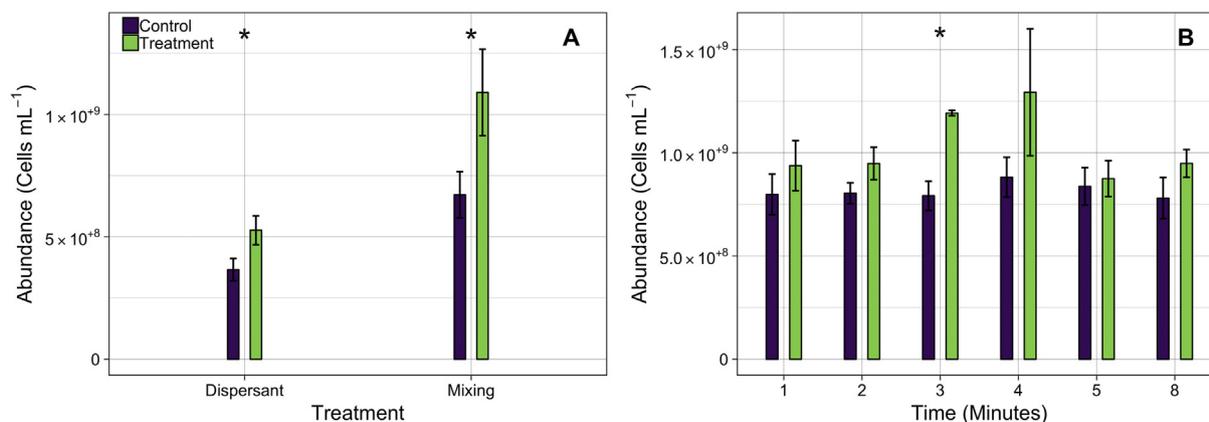
### 3. Results

#### 3.1. Optimisation of protocol for AS total bacterial enumeration by flow cytometry

AS samples incubated with Tween 80 and SP displayed significantly higher counts than untreated samples, increasing by 45 ± 22% from 3.66 ± 0.46 × 10<sup>8</sup> cells mL<sup>-1</sup> in the controls to 5.27 ± 0.59 × 10<sup>8</sup> cells mL<sup>-1</sup> in the treated samples (Fig. 1 A, ANOVA:  $P < 0.05$ ). Mechanical mixing further increased counts by 61 ± 10%, with 6.72 ± 0.94 × 10<sup>8</sup> and 1.09 ± 0.18 × 10<sup>9</sup> cells mL<sup>-1</sup> recorded in the controls and treated samples respectively (Fig. 1 A, ANOVA:  $P < 0.05$ ).

Sonication for 3 min also had a statistically significant effect on counts when compared with unsonicated controls (ANOVA:  $P < 0.05$ ), increasing by 51 ± 14% (7.92 ± 0.71 × 10<sup>8</sup> cells mL<sup>-1</sup> and 1.19 ± 0.01 × 10<sup>9</sup> cells mL<sup>-1</sup> respectively, Fig. 1 B). All other sonication times, 1, 2, 4, 5 and 8 min respectively, had no statistically significant effect (ANOVA:  $P > 0.05$ , Fig. 1 B).

The highest absolute counts were achieved using SYTO 9 at a dilution of 1:30, with a 22 ± 1.6% increase observed when compared to the SYBR Green I controls (1.57 ± 0.419 × 10<sup>9</sup> and 1.29 ± 0.32 × 10<sup>9</sup> cells mL<sup>-1</sup> respectively, Fig. 2 A). This increase was not significant, nor was it significantly higher than those obtained using SYBR Green II and SYBR Gold at a 1:30 dilution (ANOVA:  $P > 0.05$  for all comparisons). Counts obtained with dyes at 1:100 and 1:200 dilutions were also non-significantly different from the SYBR



**Fig. 1.** Effect of dispersants (Tween 80 and SP), mechanical mixing (A) and sonication time (B) on FCM TBNs. Main bars indicate mean TBN's across triplicates, whilst error bars indicate standard deviation across triplicates. TBN, total bacterial numbers. \*Treatment is significantly different from the control, ANOVA:  $P < 0.05$ .

Green I controls, with small increases and decreases observed (ANOVA:  $P > 0.05$ , Fig. 2). The greatest fluorescence intensity was achieved using SYBR Green II at a 1:200 dilution ( $345.67 \pm 9.03$  a.u., Fig. 2 B); a significant increase when compared to the controls (ANOVA:  $P < 0.05$ ). Likewise at a 1:100 dilution SYBR Green II achieved the greatest fluorescence intensity ( $330.57 \pm 33.37$ , Fig. 2 B), yet at a 1:30 dilution the fluorescence intensity of SYTO 9 was the highest and significantly so when compared to the controls ( $298.74 \pm 26.21$  a.u., ANOVA:  $P < 0.05$ , Fig. 2 B).

An incubation temperature of  $60^\circ\text{C}$  gave the highest counts ( $2.48 \pm 0.42 \times 10^9$  cells  $\text{mL}^{-1}$ ) when compared to the room temperature controls; however this difference was non-significant (ANOVA:  $P \geq 0.05$ , Fig. 3 A).

Finally a 1:1250 dilution, or an event rate of  $233 \pm 24$  events  $\text{s}^{-1}$ , gave the highest count of  $1.56 \pm 0.18 \times 10^9$  cells  $\text{mL}^{-1}$ , although this was not significantly different from those obtained at other dilutions (ANOVA:  $P \geq 0.05$ , Fig. 3 B). However; dilutions achieving an event rate between  $167 \pm 22$  and  $366 \pm 43$  events  $\text{s}^{-1}$  (1:750–1:1750) generally gave higher counts than those with event rates outside of this range (1:250, 1:500 and 1:2000 dilutions,  $931 \pm 122$ ,  $538 \pm 53$  and  $134 \pm 25$  events  $\text{s}^{-1}$  respectively).

### 3.2. Final protocol

Following optimisation a final protocol was produced (Fig. 4). Cells were detached and homogenised in AS samples using a combination of chemical (addition of Tween 80 and SP) and physical extraction (mechanical mixing and sonication) methods. Samples were then diluted,

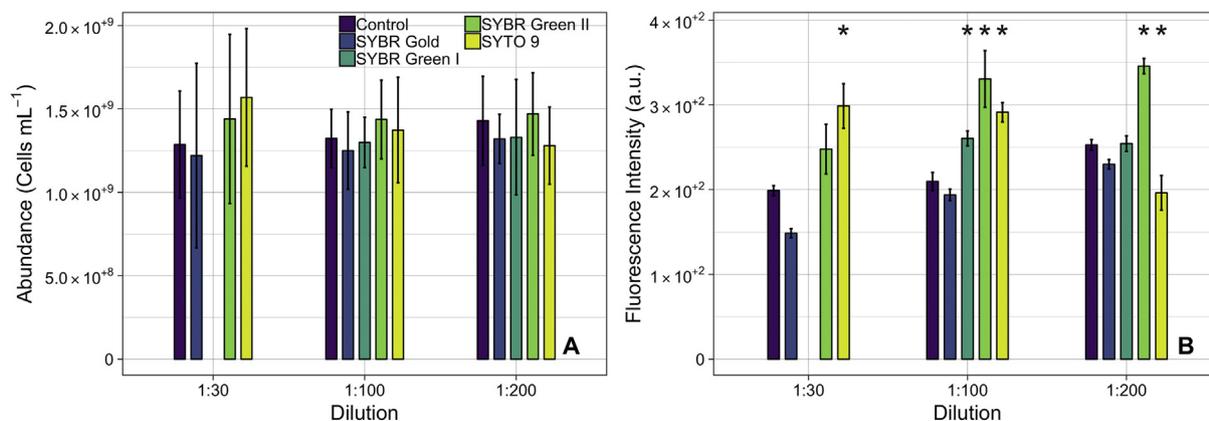
stained with SYTO 9 (1:30 dilution of stock solution) and incubated at  $60^\circ\text{C}$ . All steps included in the protocol provided statistically significant increases in counts and/or fluorescent signal when compared to respective controls. The only exception being incubation at temperature, its inclusion was based purely on the highest count being achieved at  $60^\circ\text{C}$ .

### 3.3. Microscopy cell dispersion and count correction

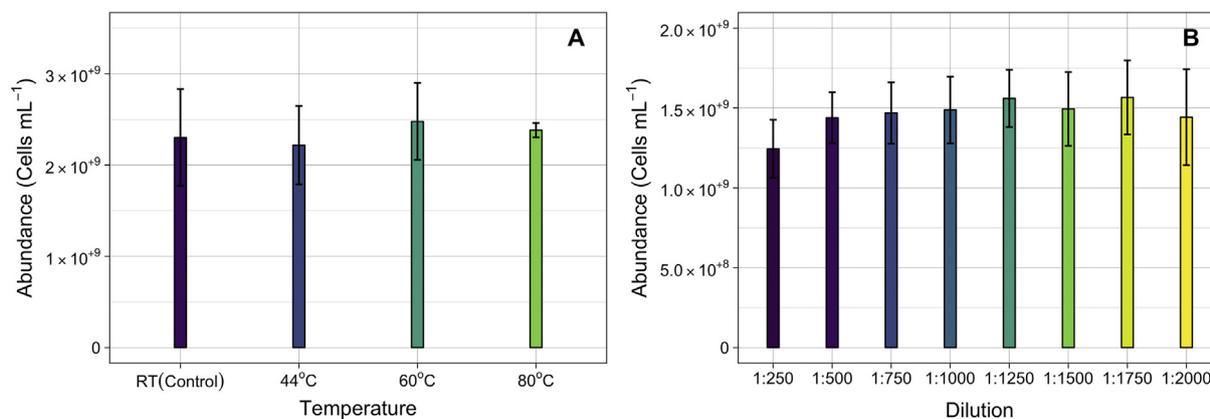
Bacterial cells were contagiously dispersed, or followed a negative binomial distribution, on polycarbonate filters. Whilst the majority of replicates conformed to a normal distribution there was evidence of both positive and negative skewness and kurtosis (Table S2). Consequently field of view counts were squared before total bacterial abundance determination and then back transformed. Calculated per replicate index of dispersions were positively associated with both bacterial abundance (Spearman  $\rho = 0.64$ ,  $P < 0.001$ ) and VSS concentrations (Spearman  $\rho = 0.66$ ,  $P < 0.001$ ).

### 3.4. Agreement between cytometric and microscopy counts

The estimated counts from both methods correlated but did not agree. Although appearing similar across multiple samples (Table 2) FCM and EFM counts were significantly different (kruskal.test  $P < 0.05$ ,  $n = 20$ ). The best-fit linear regression model for  $\log_{10}$  FCM and  $\log_{10}$  EFM counts yielded a highly significant, positive correlation coefficient and high coefficient of determination ( $P < 0.001$ ,  $R^2 = 0.99$ , Fig. 5 A). The slope coefficient and intercept were also



**Fig. 2.** Effect of dye type and dilution on FCM TBN's (A) and corresponding fluorescence intensities (B). Main bars indicate mean TBN's (A) and fluorescence intensity (B) across triplicates, whilst error bars indicate standard deviation across triplicates. Control is SYBR Green I at a 1:30 dilution. a.u., arbitrary units. TBN, total bacterial numbers. \* Treatment is significantly different from the control, ANOVA:  $P < 0.05$ .



**Fig. 3.** Effect of incubation temperature (A) and sample dilution (B) on FCM TBN's. Main bars indicate mean TBN's across triplicates, whilst error bars indicate standard deviation across triplicates. RT, room temperature. TBN, total bacterial numbers.

highly significant ( $P < 0.001$ ,  $n = 20$ ), however both were significantly different from 1 and 0 respectively implying inconsistencies between the two methods (Table 3).

Bland Altman analysis of counts also identified inconsistencies. FCM consistently underestimated total cell numbers compared to the “gold standard” EFM reference method by a mean bias estimate of  $-0.354 \log_{10} \text{ cells mL}^{-1}$  across the measurement range, suggesting FCM counts were, on average,  $\sim 44\%$  of EFM counts (Fig. 5 B). Further analysis highlighted inconsistency in this bias estimate. When full-scale and lab-scale reactor samples were analysed separately the bias estimated shifted to  $-0.416$  and  $-0.26 \log_{10} \text{ cells mL}^{-1}$  respectively,  $\sim 38\%$  and  $\sim 55\%$  of EFM counts (Fig. 5 C). Indeed the bias estimate was inconsistent proportionally across the cell abundances evaluated (Fig. 5. D), thus FCM/EFM counts were significantly similar at low abundance (reactor samples,  $\text{kruskal.test } P > 0.05$ ,  $n = 8$ ) yet significantly different at high abundance (full-scale samples,  $\text{kruskal.test } P < 0.001$ ,

$n = 12$ ). A best-fit linear regression model of FCM/EFM count differences against EFM counts confirmed that as cell abundance increases so does the difference between FCM and EFM counts ( $P < 0.001$ ,  $R^2 = 0.65$ , Table 2 and Fig. 5 B). Similarly a highly significant, positive linear relationship was found between VSS concentrations and a decrease in the agreement between FCM and EFM counts ( $P < 0.001$ ,  $R^2 = 0.56$ , Table 3, Fig. 6 B).

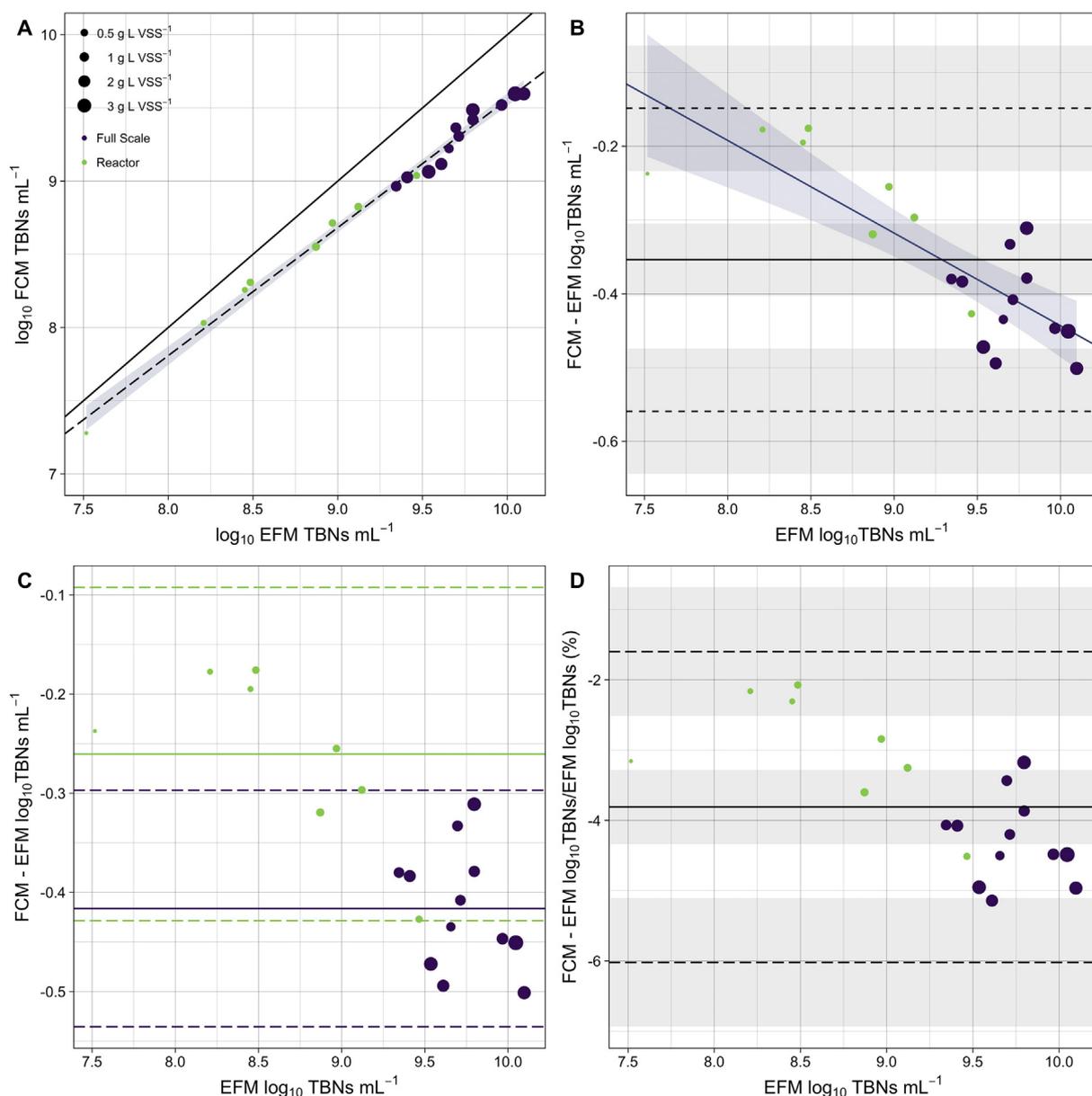
All samples however, whether analysed together or as individual sources (Fig. 5 B, C and D), fell within the 95% limits of agreement determined by Bland Altman analysis, indicating that FCM counts are accurate to within half an order of magnitude relative to EFM counts.

### 3.5. Precision of cytometric and microscopy counts

The precision and reliability of both methods was high. Decomposition of the variation in each method by source identified that

Process	Method	Notes
1. Fixation and Storage	Fix sample with ethanol at a ratio of 1:1 v/v, store at $-20^\circ\text{C}$ .	Fix in the field and analyse as soon as possible.
2. Physicochemical Extraction	Add Tween 80 (5 %) and SP(10mM) to well mixed sample, mix at 200 rpm for 15 mins in the dark.	Heat surfactant solution to $\sim 60^\circ\text{C}$ to decrease viscosity. Spinvane stirring bars (Sigma-Aldrich, UK) enable mixing of small volumes.
3. Physical Extraction	Sonicate for $3 \times 1$ minute bursts in sonicating waterbath, rest for 1 minute between bursts.	Floating tube racks facilitate this process (Sigma-Aldrich, UK).
4. Sample Preparation	Dilute sample in EDTA, stain with SYTO 9 and incubate at $60^\circ\text{C}$ for 15 mins.	Run numerous dilutions and allow them to cool for 5 mins prior to analysis.
5. Enumeration	Count sample dilutions for 1 min at a flow rate between $25 - 50 \mu\text{L min}^{-1}$ .	Event rate should be $> 150$ but $< 400 \text{ second}^{-1}$ for optimal TBN's.
6. Data Analysis	Gate bacteria population and subtract blank values.	Gates should be consistent.

**Fig. 4.** Optimised protocol suggested on the basis of our findings, including processes, methodology and critical notes. TBN, total bacterial numbers.



**Fig. 5.** Statistical comparisons of FCM and EFM TBN's. FCM  $\log_{10}$  TBN's  $\approx$  EFM  $\log_{10}$  TBN's (A), point size is related to VSS concentration and the solid and dashed lines, and associated shaded area, represent the theoretical slope of a 1:1 relationship and the best-fit regression line and its 95% CI's respectively. Bland Altman analysis of FCM and EFM TBN's using all samples (B,  $n = 20$ , Shapiro-Wilk Test  $P = 0.22$ ), full scale and reactor samples separately (C, full scale  $n = 12$ , Shapiro-Wilk Test  $P = 0.75$  and reactors (C,  $n = 8$ , Shapiro-Wilk Test  $P = 0.32$ ) and all samples expressed as a percentage of EFM TBN's (D). Point size is related to VSS concentration, whilst solid and dashed lines, and associated shaded areas (B and D), represent mean differences, 95% limits or agreement, and 95% CI's respectively. Blue solid line and associated shaded area (B) represent the best-fit regression line and 95% CI's for FCM/EFM  $\log_{10}$  TBN differences  $\approx$  EFM  $\log_{10}$  TBN's. CI, confidence intervals. TBN, total bacterial numbers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

variance between sample replicates (within-sample) and measurement error (residual variance) was very small in comparison to that between samples (Table 4), producing ICC's of 1 and 0.89 for FCM and EFM counts respectively. The variance in EFM counts, particularly residual variance associated with extraneous factors, was however greater than that found for FCM.

The repeatability of FCM was also greater than that of EFM, with RC's of 1091 and 9835 respectively, indicating that on 95% of occasions the absolute difference between replicates was estimated to be no  $> 0.12$  and  $9.76 \times 10^7$  cells  $\text{mL}^{-1}$  for FCM and EFM respectively. This represents  $\sim 0.1$  and 2.4% of mean FCM and EFM bacterial counts respectively.

### 3.6. Relationship between VSS and bacterial counts

VSS concentrations appeared to increase with increasing FCM and EFM counts, particularly at the lower end of the measurement range (reactor samples, Fig. 6 A). The best-fit linear regression models,  $\log_{10}$  FCM counts  $\approx \log_{10}$  VSS and  $\log_{10}$  EFM counts  $\approx \log_{10}$  VSS, support this linear association, yielding highly significant, positive correlation coefficients and high coefficients of determination ( $P < 0.001$ ,  $R^2 = 0.81$ ,  $P < 0.001$ ,  $R^2 = 0.83$ , respectively, Table 3). While having similar intercepts the slope coefficient of  $\log_{10}$  FCM counts  $\approx \log_{10}$  VSS encompassed 1, suggesting VSS concentrations appear more consistent with FCM counts across the measurement range.

Regression and Bland Altman analysis of FCM and EFM biomass

**Table 3**  
Linear regression analysis of FCM and EFM TBN's and VSS.

Coefficient	Estimate	SE	t statistic	P-value		95% CI (min, max)
$\log_{10}$ FCM $\approx \log_{10}$ EFM <sup>a</sup>						
Intercept	0.812	0.194	4.19	$5.5 \times 10^{-4}$	***	0.405, 1.220
EFM TBN's	0.875	0.021	42.0	$< 2.0 \times 10^{-16}$	***	0.831, 0.918
$\log_{10}$ FCM /EFM Differences $\approx \log_{10}$ EFM <sup>b</sup>						
Intercept	0.821	0.194	4.91	$5.5 \times 10^{-4}$	**	0.405, 1.219
EFM TBN's	-0.126	0.021	-6.03	$1.1 \times 10^{-5}$	***	-0.169, -0.082
$\log_{10}$ FCM /EFM Differences $\approx \log_{10}$ VSS <sup>c</sup>						
Intercept	-0.203	0.112	-1.81	0.09		-0.438, 0.033
VSS	0.186	0.037	5.02	$8.9 \times 10^{-5}$	***	0.108, 0.264
$\log_{10}$ FCM TBN's $\approx \log_{10}$ VSS <sup>d</sup>						
Intercept	5.192	0.441	11.78	$6.77 \times 10^{-10}$	***	4.266, 6.117
VSS	1.250	0.146	8.58	$9.0 \times 10^{-8}$	***	0.942, 1.556
$\log_{10}$ EFM TBN's $\approx \log_{10}$ VSS <sup>e</sup>						
Intercept	4.989	0.481	10.37	$5.1 \times 10^{-9}$	***	3.980, 5.945
VSS	1.436	0.159	9.03	$4.2 \times 10^{-8}$	***	1.120, 1.770
$\log_{10}$ FCM Biomass $\approx \log_{10}$ VSS <sup>f</sup>						
Intercept	-1.644	0.441	-3.73	$1.53 \times 10^{-3}$	**	-2.569, -0.718
VSS	1.250	0.146	8.58	$8.96 \times 10^{-8}$	***	0.944, 1.556
$\log_{10}$ EFM Biomass $\approx \log_{10}$ VSS <sup>g</sup>						
Intercept	-1.846	0.481	-3.84	$1.20 \times 10^{-3}$	**	-2.857, -0.836
VSS	1.436	0.159	9.03	$4.22 \times 10^{-8}$	***	1.102, 1.771

<sup>a</sup>  $n = 20$ . Multiple  $R^2 = 0.99$  and adjusted  $R^2 = 0.99$ . Shapiro-Wilk Test  $P = 0.71$ .

<sup>b</sup>  $n = 20$ . Multiple  $R^2 = 0.67$  and adjusted  $R^2 = 0.65$ . Shapiro-Wilk Test  $P = 0.71$ .

<sup>c</sup>  $n = 20$ . Multiple  $R^2 = 0.58$  and adjusted  $R^2 = 0.56$ . Shapiro-Wilk Test  $P = 0.95$ .

<sup>d</sup>  $n = 20$ . Multiple  $R^2 = 0.80$  and adjusted  $R^2 = 0.79$ . Shapiro-Wilk Test  $P = 0.58$ .

<sup>e</sup>  $n = 20$ . Multiple  $R^2 = 0.82$  and adjusted  $R^2 = 0.81$ . Shapiro-Wilk Test  $P = 0.34$ .

<sup>f</sup>  $n = 20$ . Multiple  $R^2 = 0.80$  and adjusted  $R^2 = 0.79$ . Shapiro-Wilk Test  $P = 0.58$ .

<sup>g</sup>  $n = 20$ . Multiple  $R^2 = 0.82$  and adjusted  $R^2 = 0.81$ . Shapiro-Wilk Test  $P = 0.34$ . SE = Standard Error, CI = Confidence Intervals. TBN, total bacterial numbers.

\*\*  $P < 0.01$ ,

\*\*\*  $P < 0.001$ .

estimates (i.e.  $\text{mg L}^{-1}$ ),  $\log_{10}$  FCM biomass  $\approx \log_{10}$  VSS and  $\log_{10}$  EFM biomass  $\approx \log_{10}$  VSS, highlighted FCM systematically underestimated VSS concentrations by about an order of magnitude (mean bias estimate of  $-0.89 \pm 0.29 \log_{10} \text{mg L}^{-1}$ , Fig. 6 C and E). This systematic error bias is a consistent  $\sim 13\%$  of VSS measurements, ranging from 3 to 38%. In contrast, EFM biomass estimates had a non-systematic bias of  $-0.54 \pm 0.35 \log_{10} \text{mg L}^{-1}$  with VSS measurements, equivalent to  $\sim 29\%$  of VSS. In this case, bias was over an order of magnitude at low VSS concentrations ( $\sim 4\%$  of VSS) and less than half an order of magnitude at higher concentrations ( $\sim 102\%$  of VSS); and increased non-systematically in absolute value as VSS concentrations increased (Fig. 6 D and F).

#### 4. Discussion

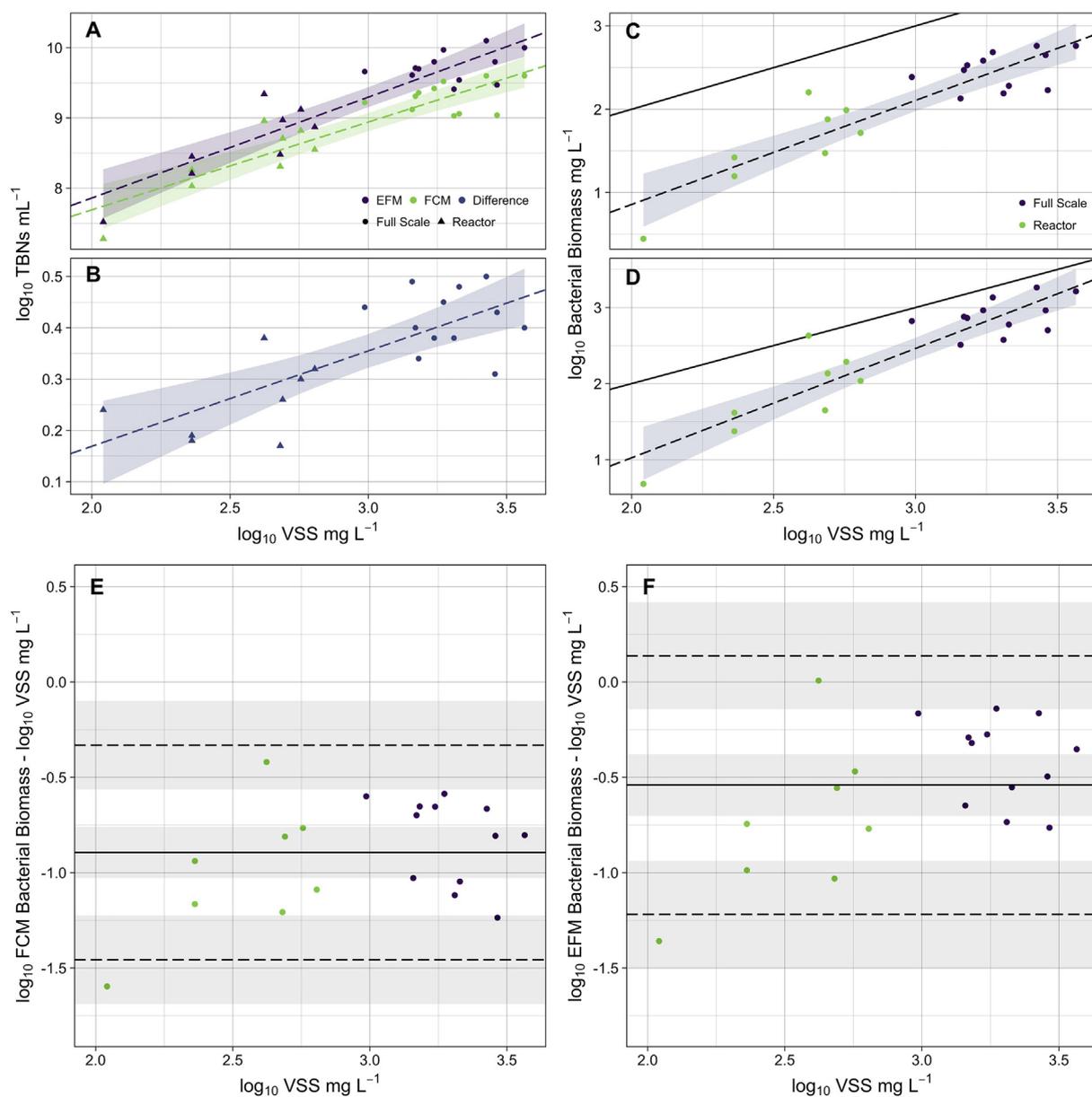
The optimised FCM protocol presented here reliably detected changes in AS total bacterial numbers, which were accurate to within half an order of magnitude of EFM counts. Yet linear regression and Bland Altman analysis highlighted that agreement between the two methods decreased with increasing counts. We posit that greater bacterial numbers and/or biomass is driving the observed differences across the measurement range. The precision of FCM counts was superior to those obtained by EFM, implying the latter may not be a reliable reference method. Furthermore, there was evidence that FCM could be a useful substitution for VSS measurements in AS.

Robust statistical evaluations of FCM bacterial counts are rare in AS and other complex environments. An analogous study by Abzazou et al. (2015) found good agreement between AS FCM and EFM total bacterial numbers, with the former constituting  $\sim 75\%$  of those garnered from the latter, as we observed at lower abundances. Unfortunately the previous comparison was made across too small a range ( $\sim 1.5\text{--}4.5 \times 10^8 \text{ cells mL}^{-1}$ ) to be relevant in full-scale systems (Abzazou et al., 2015). Other studies (Falcioni et al., 2006; Foladori et al., 2010; Ma et al., 2013) omitted validation of FCM bacterial counts

or regrettably drew limited, and possibly misleading conclusions, about their precision and accuracy by using simple EFM pairwise comparisons of relatively few samples (Ziglio et al., 2002; Abzazou et al., 2015). These statistical oversights may explain why the divergence between FCM and EFM bacterial counts has not been observed previously in AS, but is ubiquitous in other complex environments where robust statistics were employed, including stream, lake (Duhamel and Jacquet, 2006; Frossard et al., 2016) and coastal sediments (Lavergne et al., 2014), natural and agricultural soils and slow sand filters (Frossard et al., 2016). By contrast, direct and statistically robust FCM/EFM comparisons from less complex environments, including freshwater (del Giorgio et al., 1996; Felip et al., 2007) and seawater (Gasol et al., 1999; Troussellier et al., 1999; Jochem, 2001), typically yield a 1:1 relationship, though there are exceptions (e.g. Lebaron et al. (1998) and Santic et al. (2007)).

This between-study variation in the relationship between FCM and EFM bacterial counts could be attributable to the use of different flow cytometers and/or the subjectivity of manual gating. Indeed both have been shown to influence FCM counts (Maecker et al., 2005; Bashashati and Brinkman, 2009; G erikas Ribeiro et al., 2016) and certainly warrant more attention in the literature. Differences in organic matter content between complex and less complex environments could equally account for this between-study variation, as well as the breakdown in agreement between FCM and EFM counts at higher VSS concentrations observed here and elsewhere (Frossard et al., 2016).

In EFM uneven filter pore clogging (Moran et al., 1999) and consequential differences in sample volume passing individual pores could increase at higher organic matter concentrations, amplifying heterogeneous cell distribution (contagious dispersion), as we observed, and thus the sensitivity of counts (Seo et al., 2010). Virus particles and extracellular DNA, particularly if bound to organic matter and/or mineral particles, could also complicate EFM counts at higher organic matter concentrations, producing false positives and inflating abundances (Frossard et al., 2016). However, similar biases would also



**Fig. 6.** Statistical comparisons of FCM and EFM bacterial counts and biomass estimates with VSS. FCM log<sub>10</sub> TBN's, EFM log<sub>10</sub> TBN's (A) and FCM - EFM log<sub>10</sub> TBN's  $\approx$  VSS (B), dashed lines, and associated shaded area, represent the best-fit regression line and its 95% CI's respectively. FCM log<sub>10</sub> (C) and EFM log<sub>10</sub> (D) biomass estimates  $\approx$  VSS, solid and dashed lines, and associated shaded area, represent the theoretical slope of a 1:1 relationship and the best-fit regression line and its 95% CI's respectively. Bland Altman analysis of FCM (E,  $n = 20$ , Shapiro-Wilk Test  $P = 0.45$ ) and EFM (F,  $n = 20$ , Shapiro-Wilk Test  $P = 0.65$ ) biomass estimates and VSS. Solid and dashed lines, and associated shaded areas represent mean differences, 95% limits of agreement and 95% CI's respectively. CI, confidence intervals. TBN, total bacterial numbers.

**Table 4**

Decomposition of variances and intraclass correlation coefficients.

Method	Mean Square for between-sample variance	Mean Square for within-sample variance	Residual Mean Square for within-sample variance	ICC	RC
FCM <sup>a</sup>	636,572,609	31,353	123,797	1	1091
EFM <sup>b</sup>	1,924,975,343	351,906	12,237,594	0.89	9835

<sup>a</sup>  $n = 20$ .  $P$ -Value = 0.621. F-Value = 0.253. Shapiro-Wilk Test  $P = 0.58$  (replicate A) and 0.63 (replicate B).

<sup>b</sup>  $n = 20$ .  $P$ -Value = 0.867. F-Value = 0.029. Shapiro-Wilk Test  $P = 0.60$  (replicate A) and 0.65 (replicate B). ICC, intraclass correlation coefficient. RC, repeatability coefficient.

complicate FCM analysis. Coincidence of fluorescently labelled cells with mineral pieces and/or organic particles could block cell excitation and detection, whilst increasing organic matter content could reduce floc disaggregation and increase coincidence through cell clumping. In this study however, all samples were optimally diluted to avoid

coincidence and intact flocs were seldom observed in EFM fields of view, suggesting neither were likely sources of FCM bias.

It is interesting to note FCM biomass estimates showed a strong, positive and systematic linear disagreement with VSS, the estimates implying bacterial cells consistently constitute  $\sim 13\%$  of VSS

concentrations in AS. This value is in agreement with the 10–15% previously observed (Frølund et al., 1996) and is intuitively reasonable given VSS measures all organic matter in AS, which includes EPS as well as live and dead microbial cells. In contrast EFM biomass estimates indicate a VSS cell contribution of ~29%. Although rational this increased non-systematically as VSS concentrations increased. Indeed those observed at high cell/VSS concentrations are intuitively illogical, such as a ~102% cell contribution to VSS, and imply EFM overestimates bacterial abundance in AS at high cell/VSS concentrations.

The presented FCM protocol generates highly reproducible and more accurate bacterial counts than EFM, is amenable to automation and high throughput (Van Nevel et al., 2013), and is thus ~100 and ~20 times quicker and cheaper than EFM respectively (Fig. S2, Tables S3 and S4). These factors make FCM an ideal standard for bacterial quantification in research. Moreover, FCM correlates well with VSS, providing important cell number data without the confounding complications of organic matter, which could mask true cell numbers and thus operational issues when VSS is used (examples highlighted in Table 1). Although more costly than VSS analysis, FCM's amenability to automation and retrospective analysis of appropriately stored samples makes it an ideal replacement method of VSS for practitioners (Fig. S2, Table S3 and S4). Cell numbers could help operators balance costs against the risk of failure more rationally (e.g. increase/decrease aeration at low/high cell numbers), as well as provide a more informed line of enquiry when investigating failure. The use of FCM community fingerprinting techniques alongside the presented protocol would also aid in this endeavour (De Roy et al., 2012; Kinet et al., 2016).

In summary, we have shown that FCM is a good and arguably superior substitute for direct microscopic counts and an adequate substitute for VSS. We believe its use will enable better use of amplicon sequencing in wastewater research. Additionally, using FCM *in lieu* of VSS could enable the industry to reduce the time of biomass monitoring and thus increase the frequency of its measurement, thereby aiding the operation and control of activated sludge plants. Extension and further development of the method to quantify specific functional groups would certainly be valuable in this regard.

## Conflict of interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mimet.2019.03.022>.

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