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Development and validation of an alternative parameter for quantification of signals emitted by fluorescently labelled bacteria in microscopic images

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A B S T R A C T

In this study, an alternative parameter for quantifying the signals of fluorescently labelled bacteria (e.g. propidium iodide, Cyanine 3, etc.) in microscopic images was investigated. Three common parameters (mean grey value (MGV), mean grey value which is corrected for the background (MGVcwB) and the signal to background ratio (SBR) per bacterial cell) are used as reference parameters. As an alternative, the coefficient of variation (CV) is defined as the ratio of the logarithm of the standard deviation and the logarithm of the mean grey value of a bacterial cell in a microscopic image. The actual fluorescence value was safeguarded by measuring commercially available fluorescence latex microspheres at regular time intervals within our study. The precision and the correlation of the respective values of MGV, MGVcwB, SBR and CV taken from identical images were measured and subsequently normalized in order to enhance the inter-parameter comparability. The average precision of CV was the highest (89% ± 14) with decreasing numbers for MGVcwB, SBR, and MGV (78% ± 25, 71% ± 32, and, 52% ± 22, respectively). Changes in operational parameters, e.g., microscope settings, protocol steps, etc., yielded good results for the CV but less precise results for MGV, MGVcwB, and SBR in the analyses of identical images.

In conclusion, using the alternative parameter CV, changes in the composition of microbial ecosystems may thus be investigated at the highest precision level.

1. Introduction

Several physical and biological characteristics of bacterial cells can nowadays be detected and quantified using analyses of microscopic images. The samples can either be directly analysed or in combination with staining methods for the detection of specific biomolecules or structures inside the cell. Examples of these well-known methods are Fluorescence In Situ Hybridisation (FISH) or direct staining of DNA/RNA using fluorescent labels like propidium iodide, acridine orange, Cyanine 3, or, Fluorescein isothiocyanate (Hoshino et al., 2008; Langendijk et al., 1995; Poulsen et al., 1993; Seo et al., 2010; Tamminga et al., 2016; Waters and Swedlow, 2007).

Fluorescent labels may be useful to get insight into the cellular DNA/RNA-concentrations of individual bacterial cells (Amann and Fuchs, 2008; Wagner et al., 2003). Since cellular DNA/RNA-concentrations may be indicative of metabolic functioning, such fluorescence labelling provides direct information on the metabolic state of the bacterial cell (Blazewicz et al., 2013). Also, fluorescence measurements may be useful to interpret the DNA/RNA-binding effects of the matrix. Finally, in optimization studies of the different experimental procedures, the effect of the buffer composition, used for dissolving the dye, may be studied (Bouvier and del Giorgio, 2003; Langendijk et al., 1995; Ootsubo et al., 2003).

In many studies, cellular fluorescence signals are expressed as grey values and presented as relative fluorescence units (RFU) (Akram et al., 2015; Hoshino et al., 2008; Lebaron et al., 1997; Stiefel et al., 2015). Cellular fluorescent signals may also be expressed using well-known parameters such as: mean grey value per bacterial cell (MGV) (Poulsen et al., 1995, 1993), mean grey value per bacterial cell corrected for the background (MGVcwB) (Strack et al., 2013), or signal to background ratio (SBR) (Fuchizawa et al., 2008; Waters and Swedlow, 2007). If only the mean grey value is measured, it should be realized that this parameter consists of both the actual fluorescence signal and the background fluorescence signal (which may consist of photon counting (Poisson) noise and various forms of illumination and detector noise) (Pang et al., 2012; Waters, 2009; Waters and Swedlow, 2007). This dependency makes MGV vulnerable to inter-sample differences in background fluorescence signals. The parameters MGVcwB and SBR are also dependent on the fluorescence signal of the image background. The increased fluorescent signal of the image background may be caused by non-specific binding of the fluorescent label to molecules in the matrix, incorrect protocol implementation, or improper equipment handling

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(Bouvier and del Giorgio, 2003; Webb and Brown, 2013). These fluctuations in MGVcwB and SBR measurements will directly affect the comparability of the fluorescent signals of bacterial cells between, or within experimental studies (Daims and Wagner, 2007; Pang et al., 2012; Waters, 2009; Waters and Swedlow, 2007; Zhou et al., 2007). These fluctuations may restrict the use of fluorescence quantitative microscopic imaging in studies in which quantification of intensities is of paramount importance e.g. measurement of metabolic activity and physiological conditions (Bouvier and del Giorgio, 2003).

Since MGV, MGVcwB, and SBR are vulnerable to the influences mentioned here above, an alternative parameter, which uses the distribution of the fluorescence signal of each bacterial cell instead of the average fluorescence per bacterial cell in the calculations of MGV, MGVcwB, and SBR, will be investigated. A possible parameter describing the distribution of the fluorescence signal of an individual bacterial cell is the coefficient of variation. Since coefficients of variation (CV) are dimensionless, they are especially suitable to compare fluorescence signals which originate from different samples. To the best of our knowledge, this parameter has not been used to compare the intensities of different samples of bacterial cells before.

In this study, the reproducibility of each of the reference parameters (MGV, MGVcwB, SBR) was tested and compared to the reproducibility of the CV. These tests were performed by the analysis of a series of microscopic fluorescent images obtained at different conditions. *Escherichia coli* was used in combination with general DNA-staining and fluorescence in situ hybridization. Subsequently, the parameters MGV, MGVcwB, SBR, and CV were calculated from the fluorescence intensities of all bacterial cells present in a microscopic image. After the reproducibility-values of each of the four parameters were established, the influence of several environmental factors was explored. The experimental focus was on the comparison of a) different solvents for the general DNA-stain, b) different washing buffers used in the FISH procedure, and c) the light intensity and magnification at which the image was recorded.

This study presents a promising alternative parameter for quantifying and evaluating fluorescence signals of bacterial cells in microscopic images among different samples.

2. Materials and methods

2.1. Bacterial strain and growth conditions

In all experiments, *Escherichia coli* (DSMZ 301) was used. Bacteria were cultivated in 5 mL TSB (Oxoid, CM0129) during 20 h at 37 °C and 100 µL of this culture was subcultured in 5 mL TSB during 18 h at 37 °C until stationary phase was reached before using the cells in the experiments.

2.2. Staining protocols

2.2.1. DNA and RNA staining of bacterial cells on microscopic glass slides

2.2.1.1. Sample preparation. For the fixation of the bacterial cells, one part culture, one part formaldehyde (37% *v*/v) and eight parts sodium chloride solution (0.9% (*w*/*v*) NaCl) were mixed and incubated during 30 min at room temperature. After fixation, the bacteria were spotted on glass slides (diagnostic slides, 8 wells, 6 mm diameter) and air-dried during 30 min. Dehydration (96% (*v*/v) ethanol) was applied to ensure total water removal and to allow easy transport of the dye or probe into the bacterial cells.

2.2.1.2. DNA staining. After the staining solution was added to the samples, they were incubated for 10 min in the dark at room temperature. The staining solution consists of 0.03 mg/mL propidium iodide (Fluka, Sigma-Aldrich ecno. 2470810). Six different solvents were used in this investigation: 1) Milli-Q water, 2) 70% (*v*/v) ethanol, 3) 96% (v/v) ethanol, 4) 2× SCC (0.6 M NaCl, 30 mM sodium citrate, pH 7.0), 5) phosphate buffer (PBS) (75.6 mL, 0.1 M Na_2HPO_4 and 24.4 mL, 0.1 M NaH_2PO_4, pH 7.0) and 6) the reference solvent staining buffer (100 mM Tris pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$). The slides were washed using Milli-Q for 15 min in the dark at room temperature.

2.2.1.3. Fluorescence in situ hybridisation. After the staining solution was added, the samples were incubated for 60 min in the dark at 50 °C. The staining solution consists of 5'-Cy3 labelled EUB338 probe (Amann et al., 1990) (12 ng/µL) (sequence; 5′- GCT GCC TCC CGT AGG AGT −3′), hybridisation buffer (0.9 M NaCl, 20 mM Tris-HCl pH 7.5, 0.01% (*w*/*v*) SDS and 20% (v/v) formamide, 0.5% (v/v) Triton X-100). The slides were washed using one of the 5 different washing buffers: 1) the reference washing buffer (0.9 M NaCl, 20 mM Tris-HCl pH 7.5 and 5 mM EDTA pH 8.0), 2) 2× SCC, 3) PBS, 4) Milli-Q or 5) 0.1 M sodium citrate buffer pH 6.6 for 15 min in the dark at room temperature.

2.2.1.4. Sample preservation. Slides were air-dried in the dark, and each spot was covered with approx. 5 µL mounting medium (Invitrogen, I7224 component H) to prevent the degradation of the fluorochrome. Directly were the slides photographed.

2.3. Experimental design

2.3.1. Verification of the fluorescence signals

Within each experiment fluorescence latex microspheres (InSpeck™ Red (580/605) Microscope Image Intensity Calibration Kit (diameter 2,5 µm), Invitrogen catalogue number: I7224) were spotted on glass slides (diagnostic slides, 8 wells, 6 mm diameter) and air-dried during 30 min. Each spot was covered with approx. 5 µL mounting medium (Invitrogen, I7224 component H) to prevent the degradation of the fluorochrome. The slides were photographed directly thereafter and MGV, MGVcwB, SBR and CV values were calculated from each microscopic digital image. This dataset was used as a reference to verify the accuracy of the fluorescence measurements and to monitor the analytical stability of the fluorescence microscope image processing system.

2.3.2. Testing the reproducibility

The reproducibility of the staining technique using each parameter (MGV, MGVcwB, SBR, and CV) was tested with 8 different bacterial cultures. The reproducibility of the fluorescence microscope image processing system was tested using ten aliquots of each culture. These were collected and processed according to the DNA staining protocol described above. After staining, each sample was photographed at 4 different locations on each microscopic slide, using the following exposure times: 80 ms, 400 ms, 800 ms, and 1200 ms.

2.3.3. Evaluating the effect of different solvents on fluorescence signals produced by DNA staining

Five different solvents were tested: Milli-Q, 70% ethanol, 96% ethanol, 2× SCC, and PBS. The data were compared to the reference solvent staining buffer to examine the fluorescence intensity signals as expressed by the four different parameters (MGV, MGVcwB, SBR, and CV). All tests were performed using the same bacterial culture. The samples were processed according to the DNA staining protocol described above. After staining, each sample was photographed at 4 different locations on the microscopic slides. An exposure time of 500 ms was used.

2.3.4. Evaluating the effect of different wash buffers on fluorescence signals produced by Fluorescence in situ Hybridisation

One bacterial culture was processed according to the FISH protocol described earlier. 2× SCC, PBS, Milli-Q, and sodium citrate buffer were tested in comparison to the reference washing buffer. After staining, each sample was photographed at 4 different locations on the microscopic slides. An exposure time of 500 ms was used.

2.4. Data acquisition

For all experiments, an Olympus (BH2-RFL) fluorescence microscope equipped with an Olympus mercury (BH2-RFL-T3) lamp, exciter filter BP545 in combination with barrier filter r-610 from Olympus was used to get fluorescence signals and a Lumenera USB 2.0 camera type LM135 (image sensor: Sony ICX205 (mono), sensitivity: 2.5 DN/(nJ/cm^2) at 8-bit and gain 1, read Noise: 8e- and Dark Current Noise: 2e- at 25 °C) was used. The image resolution was 1392 × 1040, and the image type was 8-bit greyscale LUT (lookup tables for displaying each of the 256 possible pixel values). Images were taken with the software Lucam capture v5.0.1 and stored in Tiff format. A magnification of 200× (Olympus lens: UVFL 20×, NA: 0.65, Din 160 and coverslip thickness 0.17) was used to photograph the bacterial cells within the different samples. Therefore, the pixel resolution is 4.0 pixels/μm. Calibration of the optical system was performed using a microscale measuring staff (Carl Zeiss, Jena, Germany) with intervals of 10 μm. The exposure time varied and is specified per experiment. For every image, the gain was set to 1.0, and the saturation was set to 1.0.

2.5. Data processing

The segmentation algorithm of Tamminga et al. was used (Tamminga et al., 2016). Biologically significant binarization of the acquired images was done with ImageJ-software version 1.47 m (Abràmoff et al., 2004). Objects that were too small to represent a bacterium (Palumbo et al., 1984) or had a lower intensity than the mean background of the image were omitted. This binarized image was subsequently projected over the original fluorescent image to determine different parameters such as mean grey value, standard deviation (SD) and the area of the identified bacterial cell, as well as the average background of the corresponding image.

2.6. Data interpretation

2.6.1. The four parameters

MGV, the first parameter, is the raw average intensity value of a bacterial cell. The MGV is the sum of the grey values of all pixels which make up a bacterial cell, divided by the number of those pixels. The MGV may be indicative of the overall amount of DNA/RNA within a bacterial cell, hence its metabolic state.

The second parameter, MGVcwB, is the raw average intensity value of a bacterial cell corrected for the mean background intensity value of the corresponding fluorescent image. Calculating the mean background can be performed by deleting the bacterial cells from the image. Subsequently, the mean background is the sum of the grey values of all pixels in the corrected image divided by the number of those pixels.

The third parameter, SBR, is the ratio between the mean grey value of the bacterial cell and the mean background of the corresponding image.

The fourth parameter, CV, is the ratio between the logarithm of the standard deviation and the logarithm of the mean grey value of the pixels which make up the bacterial cell. The direct use of the MGV and SD leads to problems such as identical CVs at different values of the MGV and their SD per bacterial cell in the same image. An example of such an identical CV is that a bacterial cell with an MGV of 35 and an SD of 1.6 yields the same CV as a second bacterial cell with an MGV of 70 and an SD of 3.2. As mentioned previously, MGV gives an indication of the overall amount of DNA/RNA within a bacterial cell. Therefore the first bacterial cell in this example should at least have a lower CV than the second bacterial cell. To accomplish this, the MGV and their respective SD values per bacterial cell will first be transformed logarithmically before calculating the CV. As a result, the CV for the first bacterial cell in the previously mentioned example will be lower than the CV of the second bacterial cell, respectively 0.13 and 0.27.

2.6.2. Data normalization

The parameters MGV and MGVcwB of bacterial cells vary between 0 and 255. The parameter SBR of bacterial cells varies between 1 and 13. The parameter CV of bacterial cells varies between −0.4 and 1. Inter-parameter comparison was done after normalization of these four parameters. The following equations were used to normalize the four parameters:

$$\textit{Normalized MGV or Normalized MGVcwB} = \frac{((\text{MGV or MGVcwB value}) - 0)}{(255 - 0)}$$

$$\textit{Normalized SBR} = \frac{(\text{SBR value} - 1)}{(13 - 0)}$$

$$\textit{Normalized CV} = \frac{(\text{CV value} - -0.4)}{(1 - -0.4)}$$

2.7. Statistical analysis

Since the number of data points may rise to high numbers, the normalized data were divided into 20 classes, each with a class-width of 0.05.

2.7.1. Testing the reproducibility

Reproducibility is evaluated using the chi-squared test ($p = .05$). The chi-square test makes use of expected and observed data. In this case, expected data is the average of the normalized parameter scores (MGV, MGVcwB, SBR, and CV) per class of the 10 aliquots. Furthermore, the observed data comprise of the individual normalized parameter scores. As a result, reproducibility of 50% means that 5 out of 10 aliquots were statistically indistinguishable from the average.

2.7.2. Evaluating the effect of different solvents on fluorescence signals produced by DNA staining

A reference dataset was used to test the effect of dye-solvents on bacterial cell intensity (MGV, MGVcwB, SBR, and CV). The different datasets were tested using the chi-square test ($p = .05$) compared to the reference dataset. In this case, the reference dataset was obtained using staining buffer as the golden standard.

2.7.3. Evaluating the effect of different wash buffers on fluorescence signals produced by fluorescence in situ hybridisation

A reference dataset was used to test the effect of washing buffers on bacterial cell intensity (MGV, MGVcwB, SBR, and CV). The different washing buffer datasets were tested using the chi-squared test ($p = .05$) compared to the reference dataset. In this case, the reference dataset was obtained using wash buffer as the golden standard.

3. Results

In Fig. 1, a photomicrograph of *E. coli* stained with propidium iodide is depicted (A) and its corresponding binary projection (B).

The results of the reproducibility tests are summarized in Table 1. The average reproducibility of the eight bacterial cultures when measuring the MGV is approximately 52% ± 22. If the same data is corrected with the corresponding image background (MGVcwB), the average reproducibility becomes approximately 78% ± 25. This background correction increased the reproducibility of the results by almost 25%. When calculating the SBR and CV of the same data, an average reproducibility is achieved of approximately 71% ± 32 and of 89% ± 14, respectively. When the data are arranged into subcategories of exposure times (400, 800 and 1200 ms), the highest average reproducibility is achieved at 1200 ms for all four parameters (MGV: 50% ± 25, MGVcwB: 80% ± 19, SBR: 62% ± 41 and CV: 92% ± 13). The data in Table 1 were obtained from the analysis of

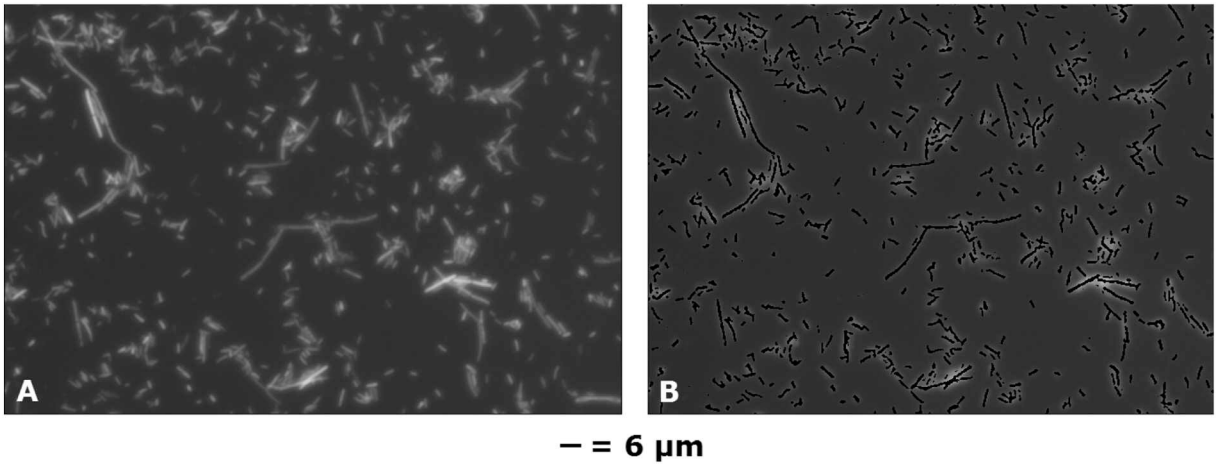

Fig. 1. An example of DNA stained bacterial cells. The raw image (A) and the binary projection (B). These images belong to sample 15. Exposure time was 800 ms and the magnification factor 200×.

Table 1
Reproducibility values of normalized intensity data per parameter and per sample and categorised for exposure time.

Bacterial culture Number	MGV[a] [%]	MGVcwB[a] [%]	SBR[a] [%]	CV[a] [%]	Exposure Time [ms]
1	70	80	100	90	80
2	80	100	90	100	400
3	20	30	70	70	400
4	20	50	10	70	400
5	50	100	100	100	400
6	60	90	100	90	400
7	50	100	80	100	400
Mean ± SD (n = 6)	47 ± 23	78 ± 31	75 ± 34	88 ± 15	
8	60	30	50	90	500
9	40	50	60	80	500
10	90	100	90	100	800
11	20	60	80	80	800
12	30	60	10	50	800
13	60	100	100	100	800
14	70	100	90	100	800
15	60	100	70	100	800
Mean ± SD (n = 6)	55 ± 26	87 ± 21	73 ± 33	88 ± 20	
16	30	70	80	100	1200
17	30	70	0	70	1200
18	40	60	90	100	1200
19	90	100	100	100	1200
20	60	100	40	100	1200
Mean ± SD (n = 5)	50 ± 25	80 ± 19	62 ± 41	92 ± 13	
Overall Mean ± SD (n = 20)	52 ± 22	78 ± 25	71 ± 32	89 ± 14	Variable

[a] Value consisted of 10 individual measurements.

250–12,000 cells from 1 of 10 individual aliquots (n) using 4 microscopic images each, of different cultures (1 − 20) (Table 2).

In Fig. 2 the parameter scores obtained from the fluorescence signal of cells stained with propidium iodide dissolved in different solvents are shown. In the calculation of the MGV, the dilution buffer 2× SCC does not result in significant differences when compared to the reference buffer (staining buffer). The MGVcwB obtained with all dilution buffers result in values that significantly differ from the reference buffer. The calculated SBR values, from the experiments with dilution buffers PBS, 2× SCC, and Milli-Q do not differ from the result of the reference buffer. When calculating the CV of the fluorescence signal obtained from cells stained with propidium iodide dissolved in each of the dilution buffers result in similar values except for 96% ethanol.

In Fig. 3 the parameter scores obtained from the fluorescence signal from cells hybridized with EUB338 and washed with different buffers are shown. When calculating the MGV, the washing buffers Milli-Q and sodium citrate buffer do not result in significant differences when compared to the reference washing buffer. The MGVcwB obtained with the washing buffers Milli-Q and PBS do not result in significant differences when compared to the reference washing buffer. The SBR, however, was significantly different from the reference buffer in all experiments with each of the different washing buffers. Finally, the CV, from experiments with all washing buffers seems to give results that do not differ significantly from the reference washing buffer.

4. Discussion

Fluorescence signals of stained bacterial cells are usually measured using mean grey value of objects (MGV) (Poulsen et al., 1995; Poulsen et al., 1993), mean grey value of objects corrected with the background (MGVcwB) (Strack et al., 2013) or the signal to background ratio (SBR) (Fuchizawa et al., 2008; Waters and Swedlow, 2007). Since these parameters are known to depend considerably on the conditions at which the experiments are performed (Webb and Brown, 2013) and on the background fluorescence signal (Waters, 2009; Waters and Swedlow, 2007), another parameter, the coefficient of variation (CV), was investigated for its robustness and suitability in the analysis of fluorescent signals obtained from bacterial cells in microscopic images.

This study resulted in three primary conclusions:

1) The mean reproducibility of the CV-values obtained in this study amount up to 90% or higher, while the reproducibility of the other parameters (MGV, MGVcwB, and SBR) does not exceed 80%.
2) The precision of said reproducibility values (expressed as relative standard deviation values) ranges from 14% to 32%, which renders the precision of the CV estimation superior to the precision of the other three parameters.
3) Finally, the latter also indicates that the CV is likely to be less dependent on environmental factors e.g. microscope settings, protocol steps, etc.

With regard to the first conclusion, it is stated that the background signal indeed has a detrimental effect on the reproducibility values of

Table 2
The number of bacterial cells per sample (4 images) used for the analyses of MGV, MGVcwB, SBR and CV.

Bacterial culture	Bacterial cell number per sample (4 images)										Exposure Time
Number	1 (n)	2 (n)	3 (n)	4 (n)	5 (n)	6 (n)	7 (n)	8 (n)	9 (n)	10 (n)	[ms]
1	1521	1248	1120	1937	1089	1382	1865	1602	1664	1105	80
2	1645	1371	1191	1727	1201	1489	2005	1558	1752	1180	400
3	3462	6420	4603	5064	4222	2382	2742	2858	1983	3000	400
4	2280	2231	2256	2876	2108	2926	2113	1521	1232	1459	400
5	3636	3868	2706	2574	3574	2437	2865	2953	2876	3333	400
6	4937	4211	4739	4680	5462	4419	5394	4165	3022	3633	400
7	10,495	10,575	11,938	12,108	9993	10,049	10,053	10,241	9745	9616	400
8	440	455	356	559	463	351	268	548	461	528	500
9	452	488	306	456	370	475	508	440	355	632	500
10	1563	1291	1128	1640	1183	1465	1866	1486	1655	1112	800
11	3344	3233	4421	4993	4056	3213	2765	2835	1971	3072	800
12	2242	2206	2210	2772	2052	2816	2088	1485	1212	1434	800
13	4108	3881	2745	2650	3754	3472	2972	3074	3050	3594	800
14	5614	5260	5274	5255	6097	4786	6020	4704	3375	4089	800
15	9897	9895	11,140	11,388	9352	9503	9480	9538	9281	9118	800
16	3326	3129	4342	4886	4000	3180	2732	2839	1966	3145	1200
17	2292	2231	2224	2759	2064	2824	2097	1505	1241	1471	1200
18	4291	4172	4691	4713	3936	3547	3092	4704	3213	3582	1200
19	6082	5676	5652	5523	6505	5074	6447	5066	3597	4423	1200
20	9607	10,480	10,730	11,399	9008	9148	9292	9021	9415	8875	1200

the reference parameters which depend on i.e. MGV, MGVcwB, and SBR. Therefore, it is advisable to use a background-independent parameter i.e. CV in measuring intensity signals in fluorescently labelled bacterial cells in microscopic images. Also remarkable is the fact that the background-independent parameter, CV, is estimated more precisely. The increased precision directly results in a higher resolution of the CV, rendering it the parameter of choice for measuring ($p = .05$) small differences in fluorescence signals of bacterial cells in microscopic images. As far as the exposure time is concerned, it should be realized that increasing the exposure time results in a stronger fluorescence signal of the bacterial cell and simultaneously in a stronger fluorescence signal of the background (Pang et al., 2012). Given the fixed minimum and maximum values of the detector, the realistic range of detection will be diminished (Frigault et al., 2009), thereby hampering a correct calculation of the MGV, MGVcwB, and SBR due to signal levels below or above the detector limits. Since the parameter CV does not rely on this phenomenon, the lesser dependence of the CV on the exposure time may be explained.

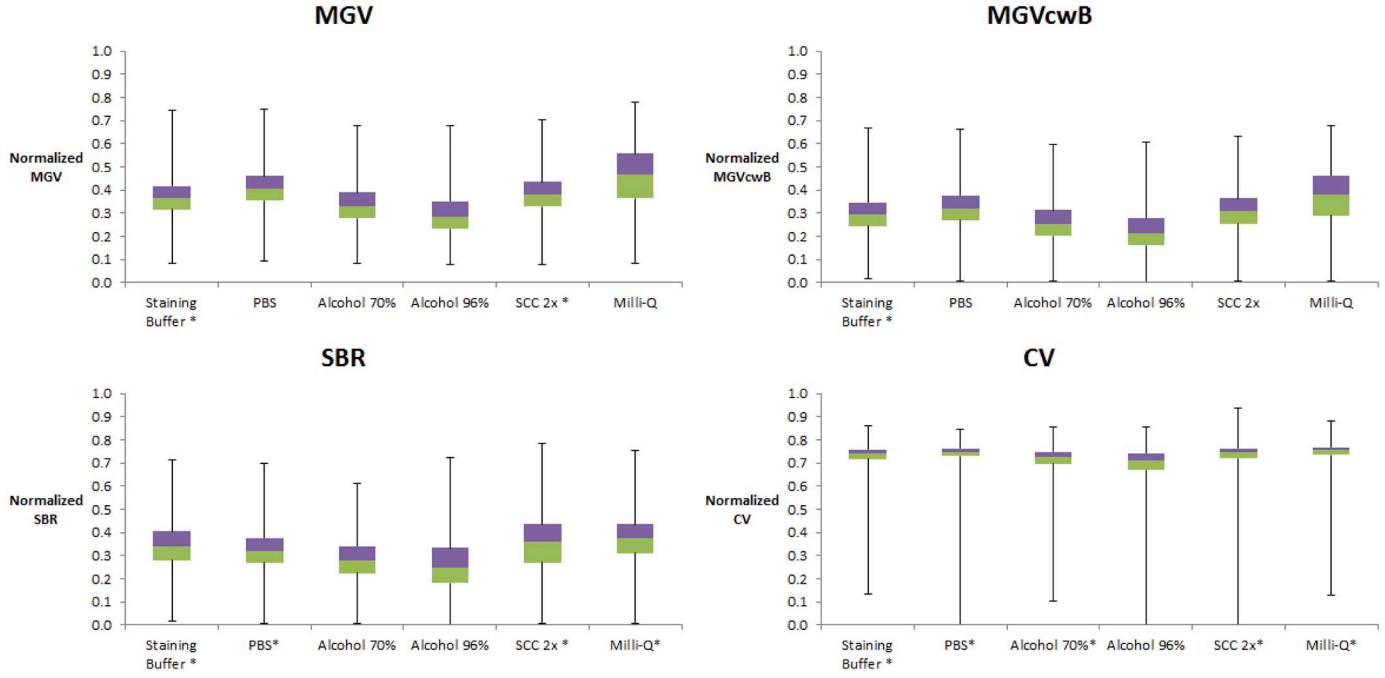

Fig. 2. Assessment of the analysis of the fluorescence signal from cells stained with propidium iodide dissolved in different solvents. Four different parameters were measured and calculated (MGV, MGVcwB, SBR, and CV). The x-axis presents different solvents. The total bacterial cell numbers per buffer are for Staining Buffer: 1469; PBS: 2540; Alcohol 70%: 2054; Alcohol 96%: 2798; and 2× SCC: 1853. The data from the dye solvents with an asterisk are statistically similar to the Staining Buffer (chi-squared test, $p < .05$).

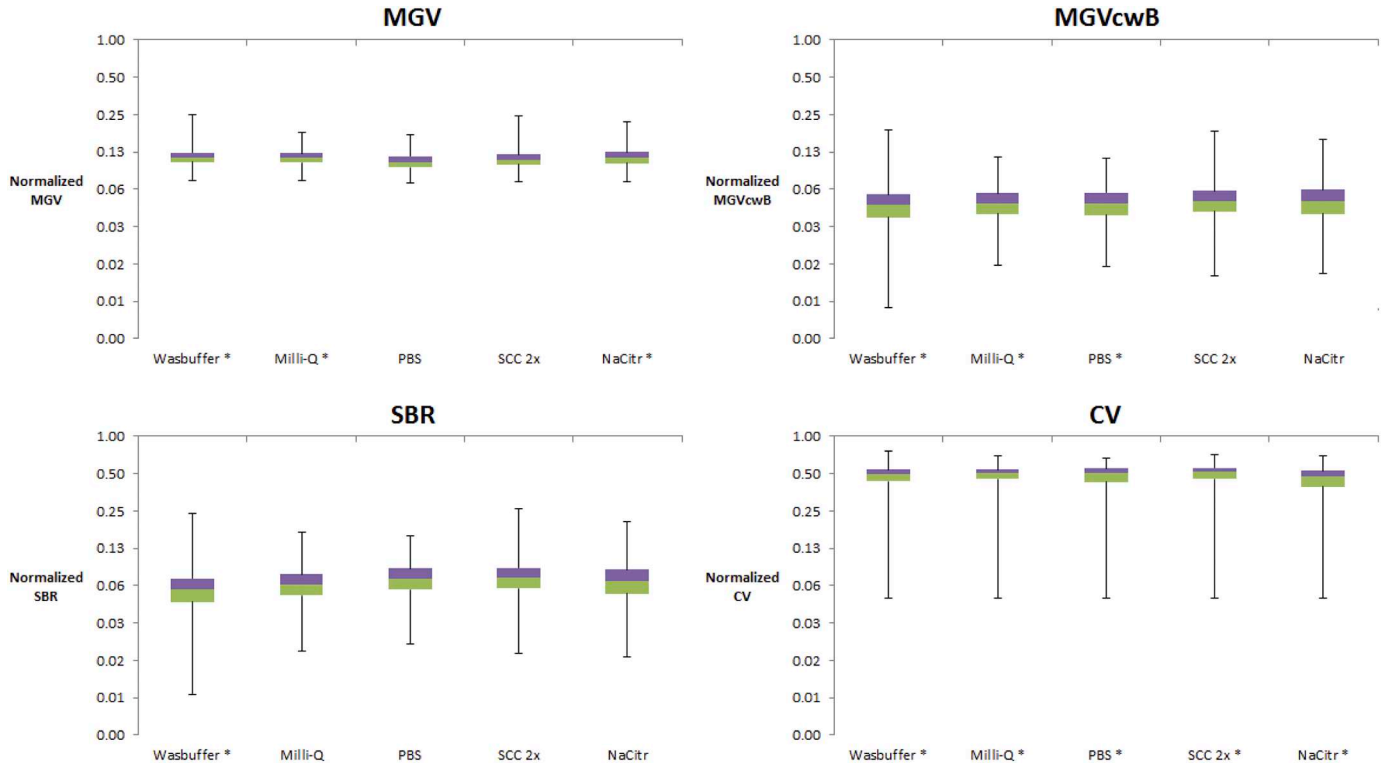

Fig. 3. Assessment of washing buffers after hybridisation with probe EUB338. Four different parameters were measured and calculated (MGV, MGVcwB, SBR, and CV). The x-axis presents different washing buffers. The total bacterial cell numbers per buffer are for Wash buffer: 13668; Milli-Q: 6048; PBS: 3528; 2× SCC: 7721; and sodium citrate buffer (NaCitr): 7974. The data from the washing buffers with an asterisk are statistically similar to the Staining Buffer (chi-squared test, $p < .05$).

Two practical trials were performed to gain insight into the influence of environmental factors on the behaviour of the four parameters. The first practical trial was focused on the effect of the solvent of propidium iodide, the nucleic acid dye. The second practical trial was focused on the effect of differences in the composition of washing buffers when the cells were hybridized with the EUB338 probe.

The interpretation of the data of the two practical trials confirms the fact that environmental factors influence measurements of the fluorescence signals (Figs. 2 and 3). Since the data (MGV, MGVcwB, SBR, and CV) originate from identical images, the influence of procedural factors (Bouvier and del Giorgio, 2003) on the observed differences are excluded. In addition, the definition of the parameters (MGV, MGVcwB, and SBR) includes some influence of the background signal (Waters, 2009; Waters and Swedlow, 2007) which is reflected by variability in the values of the reference parameters. The CV parameter is independent of the background signal and will consequently be less vulnerable to environmentally induced differences in background signals. The background-independency directly results in significantly more precise calculations of fluorescence intensities of fluorescence-labelled bacterial cells in microscopic images.

In conclusion, the proposed alternative parameter CV shows the best performance for measuring the signal intensity of fluorescence-labelled bacterial cells in microscopic images. Since this parameter may be indicative of the distribution of DNA/RNA at different locations within a bacterial cell (Blazewicz et al., 2013), for example, ribosomes, messenger-RNA or plasmids, it may well be applicable in studies concerned with measuring metabolic activity or physiological conditions of fluorescent bacterial cells in microscopic images. Because of its methodological independence, the parameter CV may be used in images produced by different imaging systems such as imaging flow cytometers (Headland et al., 2015) and fluorescence microscopy systems. Using the alternative parameter CV, changes in the composition of microbial ecosystems may thus be investigated at the highest precision level.

Declaration of Competing Interest

None.

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