



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Two-colour fluorescence fluorimetric analysis for direct quantification of bacteria and its application in monitoring bacteria growth in cellulose degradation systems

Citation for published version:

Duedu, K & French, C 2017, 'Two-colour fluorescence fluorimetric analysis for direct quantification of bacteria and its application in monitoring bacteria growth in cellulose degradation systems', *Journal of microbiological methods*, vol. 135, pp. 85-92. <https://doi.org/10.1016/j.mimet.2017.02.006>

Digital Object Identifier (DOI):

[10.1016/j.mimet.2017.02.006](https://doi.org/10.1016/j.mimet.2017.02.006)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Journal of microbiological methods

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



1 **Two-colour fluorescence fluorimetric analysis for direct quantification of bacteria and**
2 **its application in monitoring bacteria growth in cellulose degradation systems**

3 Kwabena O. Duedu^{1,2*} and Christopher E. French¹

4 *¹Institute of Quantitative Biology, Biochemistry and Biotechnology, School of Biological Sciences,*
5 *University of Edinburgh, Edinburgh EH9 3FF, UK*

6 *²Department of Biomedical Sciences, School of Basic & Biomedical Sciences, University of Health &*
7 *Allied Sciences, Ho, Ghana*

8 *Corresponding author

9

10 Email addresses:

11 kduedu@uhas.edu.gh (K.O.Duedu)

12 c.french@ed.ac.uk (C.E.French)

13

14

15 **Highlights**

- 16 • An assay for quantification of bacteria in mixtures containing insoluble substrates
- 17 • The assay uses SYBR Green I and propidium iodide dual staining with fluorimetry
- 18 • DNA standards were used to define an equivalent fluorescence DNA (EFD) unit
- 19 • Inter-lab/instrument comparisons are possible when the DNA standards are used

20

21

22 **Abstract**

23 Monitoring bacteria growth is an important technique required for many applications such as
24 testing bacteria against compounds (eg. drugs), evaluating bacteria composition in the
25 environment (eg. sewage and wastewater or food suspensions) and testing engineered
26 bacteria for various functions (eg. cellulose degradation). Traditionally, rapid estimation of
27 bacterial growth is performed using spectrophotometric measurement at 600 nm (OD600) but
28 this estimation does not differentiate live and dead cells or other debris. Colony counting
29 enumerates live cells but the process is laborious and not suitable for a large number of
30 samples. Enumeration of live bacteria by flow cytometry is a more suitable rapid method with
31 the use of dual staining with SYBR I Green nucleic acid gel stain and Propidium Iodide (SYBR-
32 I/PI). Flow cytometry equipment and maintenance costs however is relatively high and
33 unavailable in many laboratories that may require a rapid method for evaluating bacteria
34 growth. We therefore sought to adapt and evaluate the SYBR-I/PI technique of enumerating
35 live bacteria cells for cheaper platform, a fluorimeter. The fluorimetry adapted SYBR-I/PI
36 enumeration of bacteria in turbid growth media had direct correlations with OD600 ($p > 0.001$).
37 To enable comparison of fluorescence results across labs and instruments, a fluorescence
38 intensity standard unit, the equivalent fluorescent DNA (EFD) was proposed, evaluated and
39 found useful. The technique was further evaluated for its usefulness in enumerating bacteria
40 in turbid media containing insoluble particles. Reproducible results were obtained which
41 OD600 could not give. An alternative method based on the assessment of total protein using
42 the Pierce Coomassie Plus (Bradford) Assay was also evaluated and compared. In all, the
43 SYBR-I/PI method was found to be the quickest and most reliable. The protocol is potentially
44 useful for high-throughput applications such as monitoring of growth of live bacteria cells in
45 96-well microplates and in assessing *in vivo* activity of cellulose degrading enzyme systems.

46
47 **Keywords**

48 Quantification of bacteria; Fluorimetry; SYBR Green; Propidium iodide; Cell density;
49 Cellulose

50 **1. Introduction**

51 Monitoring bacterial growth is essential for assessing many microbial applications.
52 Determination of bacterial cell numbers can be done by direct and indirect methods. The
53 commonest and oldest methods for this are turbidimetric measurements (optical density at
54 600 nm, OD600) and viable (plate/colony) counts (Breed and Dotterrer, 1916, Koch, 1970).
55 Turbidimetric measurements are indirect methods which are fast and usually preferred when
56 a large number of cultures are to be counted. The readings obtained from these
57 measurements are a representation of the cell numbers (Koch, 1970). However to obtain
58 definitive numbers, the readings must be correlated initially with cell number determined by
59 other means (eg. plate counts). Plate counts on the other hand gives a direct measure of
60 viable cells within the sample. Direct enumeration by microscopy using Petroff-Hausser
61 counting chambers can also be performed (Treuer and Haydel, 2011). The aforementioned
62 methods are however not universally applicable due to various limitations. Turbidimetric
63 methods are unreliable for direct enumeration of bacteria cells in media containing insoluble
64 substances such as food and environmental samples. Unavailability of suitable culture media
65 and low concentrations of viable bacteria are major limitations to plate counting. To get around
66 these challenges, different approaches based on the use of fluorochromes have been devised
67 to investigate microbial viability and density (Barbesti et al., 2000, Caron and Badley, 1995,
68 Diaper et al., 1992, Foladori et al., 2010, Kaprelyants and Kell, 1992, Porter et al., 1996,
69 Tamburini et al., 2014).

70

71 Fluorochromes used in staining and enumerating bacteria cells by flow cytometry are based
72 on membrane integrity, DNA binding and energy transfer between the fluorochromes
73 (Barbesti, Citterio, Labra, Baroni, Neri and Sgorbati, 2000, Gregori et al., 2001, Humphreys et
74 al., 1994, Sgorbati et al., 1996). Barbesti et al (2000) demonstrated that when DNA is
75 simultaneously stained by SYBR-I (membrane permeant) and PI (non-membrane permeant)
76 there is a decrease in the fluorescence of SYBR-I and an increase in the fluorescence of PI.
77 This is due to a strong energy transfer between the two fluorochromes which facilitates

78 discrimination between living and dead bacteria. This transfer is due to the extremely high
79 quantum yield of DNA bound SYBR-I complex (~0.8, Molecular Probes Inc., USA) and the
80 overlapping of its emission spectrum (Fig. 1) with the absorption spectrum of PI. As a result,
81 the fluorescence of SYBR-I is 'quenched' by PI when stained with both. 'Dead cells' are
82 regarded as cells with compromised membranes. The compromised membrane integrity
83 allows both PI and SYBR-I to permeate the cells at which point such cells will fluoresce red
84 (PI) when excited. 'Live cells' on the other hand allows only SYBR-I to permeate and when
85 excited, fluoresce green.

86

87 Figure 1. Excitation and emission spectra of SYBR green I and propidium iodide from
88 Fluorescence SpectraViewer, Life Technologies

89

90 Fluorescence has been used in quantitation for a long time. Although the technique has
91 improved over time, a major challenge it faces is standardization and references for
92 fluorescent measurements. A special issue on "Quantitative Fluorescence Cytometry: An
93 Emerging Consensus" published by the journal *Cytometry* identified some of these challenges
94 (Lenkei et al., 1998). Among them were **(1)** inter-laboratory comparisons (Purvis and Stelzer,
95 1998, Waxdal et al., 1998, Zenger et al., 1998), **(2)** instrumentation (Purvis and Stelzer, 1998,
96 Wood, 1998, Wood and Hoffman, 1998), and **(3)** reagent and calibration standards (Gratama
97 et al., 1998, Lenkei et al., 1998, Purvis and Stelzer, 1998, Schwartz et al., 1998, Shapiro et
98 al., 1998, Wood and Hoffman, 1998, Zhang et al., 1998). A common response to these
99 challenges has been the development of a fluorescence intensity standard (FIS), the MESF
100 (molecules of equivalent soluble fluorochrome) for use in flow cytometry (Gaigalas et al., 2001,
101 Schwartz et al., 2004, Schwartz et al., 2002, Wang et al., 2002). The MESF is based on a
102 comparison between the number of fluorophores in two solutions, where one solution is a
103 standard with known values. The standard is often a suspension of labelled microbeads.
104 [Although all these standardizations contribute to making the use of flow cytometry a better](#)
105 [platform for enumerating live bacteria than OD600 and colony counting, there are some](#)

106 challenges that do not favour its wide use. First, the flow cytometers cost relatively high and
107 not available in many laboratories monitoring and enumerating bacteria growth. Secondly, the
108 use and interpretation of flow cytometric data require special training and expertise.
109 Furthermore, flow cytometers require regular servicing which is not available particularly in
110 most developing regions such as sub-Saharan Africa. There is therefore a need to adapt the
111 SYBR-I/PI principle of differentiating and enumerating bacteria for a simple and cheaper
112 platform such as fluorimetry. As mentioned above, a major challenge for fluorescence
113 measurement is the inability to make comparable fluorescence intensity measurements
114 across laboratories and between different instruments. In response to this, a FIS based on
115 DNA stained with SYBR-I and PI was also developed. This standard, like the MESF used in
116 flow cytometry, is based on equivalency between the intensity of fluorophores in two solutions,
117 one standard (known concentration(s) of DNA) and the other the unknown sample. An
118 alternative method for semi-quantification of bacteria using the total protein content of the
119 sample was also evaluated and compared with the two-colour fluorescence method.

120

121 As the world's fossil fuel reserves deplete, there is a growing need to develop sustainable
122 fuels (Creutzig et al., 2015, Fulton et al., 2015, Nuffield Council on Bioethics, 2011). Solar
123 electric, hydropower (hydroelectric, tidal and ocean thermal power) and geothermal appear
124 are safe sustainable sources of energy. However, these sources are not practically useful in
125 the long-distance transport sector, thus emphasizing the need for liquid fuels. The only
126 sustainable source of liquid fuels appears to be plant derived biofuels. The United States (US)
127 and the European Union (EU) have individually set targets to expand the production and use
128 of biofuels. The Energy Independence and Security Act (EISA) of 2007 establishes that
129 blending of renewable fuels (Biomass diesel and cellulosic biofuels) into transportation fuels
130 in the US should increase from 9 billion gallons in 2008 to 36 billion gallons by 2022 (Van Dyk
131 and Pletschke, 2012). Similarly, the EU has also projected biofuel supply in transportation
132 fuels to reach 25% by 2030 (Himmel *et al.*, 2007; US EPA, 2013). Despite the potential of
133 plant derived biofuels to meet these targets, large scale applications are currently problematic

134 due to the difficulties in converting plant biomass (mostly made of cellulose, hemicellulose,
135 lignin, pectin, and protein) into bioalcoholic derivatives and biodiesel. Naturally occurring
136 cellulose degrading microbes use a battery of multiple catalytic enzymes to hydrolyse
137 cellulose. We have described applications of synthetic biology that expand the technical
138 capabilities of engineering efficient cellulose degrading enzyme systems (Duedu and French,
139 2016, French et al., 2015, Lakhundi et al., 2016) making it potentially easier to develop an
140 ideal biofuel producing microorganism (IBPM) (French, 2009). Characterization and fine
141 tuning of microbial cellulose degrading systems requires reliable methods for monitoring
142 growth of cells on cellulose as a main carbon source. Substrates used for such experiments
143 (eg. avicel, paper and pre-treated plant materials) are generally insoluble. This makes the use
144 of turbidimetric methods (eg. OD600) which are fast and suitable for screening large numbers
145 of samples unreliable. Colony counting on the other hand is an arduous process. Dual staining
146 of samples with SYBR-I and PI has been demonstrated as a useful method for quantifying
147 bacteria in environmental and food samples using flow cytometry. The application of this
148 method for fluorimetry has not been reported. Here, SYBR-I/PI dual staining was used to
149 evaluate the cell content of cultures containing microcrystalline cellulose (avicel).

150

151 **2. Materials and Methods**

152 **2.1 Reagents, equipment and sample preparation**

153 The SYBR-I/PI protocol was evaluated using DNA and cell suspensions of *Escherichia coli*
154 strains JM109 and DH5 α and *Citrobacter freundii* strains ATCC8090 and SBS197. The
155 SBS197 strain was obtained from the Biology Teaching Laboratory of the School of Biological
156 Sciences (SBS), University of Edinburgh. *E. coli* and *C. freundii* were chosen because they
157 are the hosts we use in our biomass degradation experiments (Lakhundi, Duedu, Cain, Nagy,
158 Krakowiak and French, 2016). *E. coli* and *C. freundii* were grown in 100 ml Luria Broth (LB)
159 overnight at 37°C, 200 RPM. The cells were centrifuged at 5000 x g for 10 minutes, the
160 supernatant discarded and the pellet resuspended in 5 ml 1 x PBS. Human genomic DNA

161 (200 ng μl^{-1}) was obtained from Bioline, London, UK and used as a calibration standard.
162 Additionally, plasmid DNA was extracted from *E. coli* JM109 harbouring the BioBrick plasmid,
163 pSB1A2-BBa_K523025 using the QIAGEN plasmid midi kit (QIAGEN GmbH, Hilden,
164 Germany) according to the manufacturer's instructions. Dilutions of the DNA were made in
165 nuclease free water and stained with the fluorochromes. Propidium iodide (1mg ml^{-1} stock
166 solution) and SYBR-I (10,000 X concentrate) were obtained from ThermoScientific, Eugene,
167 OR, USA. SYBR-I dilutions were made using Pierce™ Dimethylsulfoxide (DMSO)
168 (ThermoScientific, Rockford, IL, USA). Fluorescence measurements were taken using the
169 Modulus™ Single Tube multimode reader (P/N 998-9203, Turner BioSystems, Sunnyvale,
170 CA, USA).

171

172 **2.2 Fluorescence staining of cells and DNA**

173 One ml of diluted DNA or cell suspension was stained with 10 μl each of SYBR-I (1:30 dilution
174 of commercial stock) and PI in a cuvette (FisherBrand FB55147, Fisher Scientific,
175 Loughborough, UK). The samples were covered with parafilm, mixed by inverting about 6
176 times and incubated in the dark for 15 minutes at room temperature.

177

178 **2.3 Fluorescence measurement and energy transfer**

179 Fluorescence was measured using the blue (P/N 9200-040, $\lambda_{\text{ex}} = 460 \text{ nm}$, $\lambda_{\text{em}} = 515 - 570 \text{ nm}$)
180 or green (P/N 9200-042, $\lambda_{\text{ex}} = 525 \text{ nm}$, $\lambda_{\text{em}} = 580 - 640$) Modulus™ fluorescence kits (Turner
181 BioSystems, Sunnyvale, CA, USA). Measurements obtained from the instrument were given
182 in fluorescence units (FSU). To convert the FSU to a FIS which can be compared between
183 instruments and across labs, a standard was generated using dilutions of DNA stained with
184 either SYBR-I, PI or both. For each standard 10 ml was prepared. Aliquots of 1 ml were
185 transferred to labelled cuvettes in triplicates for SYBR-I, PI and SYBR-I/PI staining. All
186 samples were measured individually in both the red and green fluorescence channels. Energy
187 transfer from SYBR-I to PI was evaluated.

188

189 **2.4 Discrimination and semi-quantification of cells**

190 To assess the discriminatory capacity of the protocol, various degrees of damage were
191 induced in the *E. coli* and *C. freundii* cells. To induce damage, 500 µl of cell suspension was
192 ultrasonicated at 10 µm (amplitude) for various intervals. The MSE Soniprep 150 Plus
193 Ultrasonic Disintegrator (MSE (UK) Ltd, London, UK) with the exponential probe was used to
194 sonicate cells. Cell damage was assessed by performing total protein quantification on 100 µl
195 of each sample using the Pierce Coomassie plus (Bradford) assay kit (ThermoScientific,
196 Rockford, IL, USA). The ultrasonicated samples were then stained with SYBR-I, PI or both.
197 Measurements were taken for all samples in both the green and red fluorescence channels.

198

199 **2.5 Determination of how presence of cellulose particles affects fluorescence**

200 To assess the effects of addition of cellulose on fluorescence, 20 mg of cellulose powder (20
201 µm microcrystalline powder, Sigma Aldrich, Irvine, UK) was added to cell suspensions. Two
202 sets of cell suspensions were prepared at different cell densities. The turbidity of cells was
203 determined by measuring the absorbance at 600 nm using the absorbance module (Model
204 E6076, GLOMAX MultiJR, Promega, Southampton, UK) with the Modulus™ reader. To one
205 set of cells, cellulose powder was added and the turbidity re-measured. Both sets of samples
206 were stained in parallel with SYBR-I/PI and the fluorescence determined as described above.

207

208 **2.6 Total protein assay**

209 The Pierce Coomassie plus (Bradford) assay was used to determine the total protein
210 concentration in cells. Reactions were set up in 1 ml volumes containing 100 µl of sample and
211 900 µl of Bradford reagent and mixed. To determine release of soluble protein from sonicated
212 cell suspensions, cells were removed by centrifugation (10,000 g, 10 min) and 100 microliters
213 of supernatant was assayed. The reaction mixture was incubated at room temperature for 10
214 minutes according to the manufacturer's instructions after which absorbance was measured
215 at 600 nm using the Modulus™ reader. To measure total protein content of intact cells, 100 µl

216 of the cell suspension was added to 900 μ l of Bradford reagent, mixed and incubated at 65°C
217 for one hour to lyse the cells. The samples were allowed to cool for one hour at room
218 temperature during which time cellulose particles settle at the bottom that so they do not
219 interfere with the absorbance measurements. Absorbance values were converted to protein
220 concentration by comparing with a standard curve prepared with dilutions of the 2 mg ml⁻¹
221 bovine serum albumin (BSA) included in the kit.

222

223 **2.7 Statistical analysis**

224 Microsoft Excel was used for data entry, organization and generation of graph plots. Statistical
225 tests were conducted in IMB SPSS version 21. Correlations and tests for linearity between
226 fluorescence and DNA concentration or cell density were analysed using Pearson's product-
227 moment correlation tests. To determine whether fluorescence depended on cell density or
228 degree of damage, Kendall's rank correlation (tau b) was used. All experiments were
229 performed in at least three replicates and the means plotted with standard error values.

230

231 **3. Results**

232 **3.1 Staining characteristics and energy transfer**

233 Both stains fluoresced in either channel due to the overlap between their absorption and
234 emission spectra. Furthermore, fluorescence values recorded for SYBR-I were higher than
235 those of PI for the same sample. SYBR-I fluorescence in the green channel was 10.06 % (SD
236 = \pm 2.16) that of the fluorescence recorded from the red channel. Fluorescence of propidium
237 iodide in the green channel was 0.34 % (SD = \pm 0.25) of that in the red channel (supplementary
238 information figure 1). Background fluorescence of PI is not likely to affect fluorescence of
239 SYBR-I in the green channel but that of SYBR-I will potentially affect the fluorescence of PI in
240 the red channel.

241

242 Energy transfer was assessed after compensation for SYBR-I and PI emission in the red and
243 green channels respectively as described by Barbesti et al (2000). The energy transfer from
244 SYBR-I to PI was evaluated as a decrease in green SYBR-I fluorescence and increase in PI
245 fluorescence of DNA samples stained with both fluorochromes as compared to the same
246 samples stained with only one of the two fluorochromes. Reduction of SYBR-I fluorescence
247 was 98.6 % (SD = ± 0.18) whereas increase in PI was 120.2 % (SD = ± 15.5) indicating a strong
248 discriminatory capability (table 1 and figure 2).

249

250 *Table 1: Energy transfer from SYBR-I to PI*

251 *Figure 2: Differences between single and dual staining of DNA*

252

253 **3.2 Definition of an equivalent fluorescent DNA (EFD) unit**

254 A fluorescence intensity standard was developed based on the fluorescence of DNA stained
255 with either of the two fluorochromes to enable comparison of fluorescence across laboratories
256 and instruments. An equivalent fluorescent DNA (EFD) unit was defined as the amount of
257 fluorescence obtained from staining 1 ng μl^{-1} of DNA for 15 minutes at room temperature
258 (25°C). A best fit line obtained for each stain was used to determine the EFD unit for that
259 particular stain after compensating for fluorescence of SYBR-I and PI in the red and green
260 channels (figure 3a).

261

262 **3.3 Assessment of single fluorescent staining of bacteria cultures and their** 263 **discriminatory capability**

264 Quantification of bacterial cells was assessed first by staining dilutions of *E. coli* suspensions
265 with single fluorochromes. There was no correlation between cell density (OD600) and the
266 fluorescence obtained when PI alone was used to stain cells (figure 3b). On the other hand, a
267 strong correlation ($R^2 = 0.988$) between cell density and fluorescence was obtained with

268 SYBR-I alone. Although SYBR green I is a green fluorescent stain, red fluorescence was
269 detected from cultures stained with SYBR green I. This fluorescence was however 3 to 6 fold
270 lower than the green fluorescence detected.

271

272 To investigate discrimination of live and dead cells, we needed a system where specific
273 degrees of cell damage could be attained. Ultrasonication of cell suspensions for increasing
274 duration of time gave increasing extracellular protein concentrations indicating release of
275 protein from cells whereas the cell density measured by absorbance at 600 nm slightly
276 decreased (figure 3c). Complete lysis of all cells by incubating at 65 °C for one hour post-
277 sonication resulted in protein concentrations levels that were similar to each other irrespective
278 of the duration of sonication. (supporting information figure 2). To confirm that this damage
279 also resulted in release of varying amounts of DNA, suspensions were stained with both
280 SYBR-I and PI. Fluorescence increased with staining by both stains confirming that varying
281 degrees of cell damaged had been achieved (Figure 3d).

282

283 *Figure 3: DNA standard preparation and estimation of the EFD of damage-induced and non-*
284 *damaged cells stained with single fluorochromes*

285

286 **3.4 Discriminating between live and dead cells by dual staining**

287 To demonstrate that simultaneous staining of cells with both SYBR-I and PI can allow
288 quantification of live and dead cells using fluorimetry, cell suspensions of *E. coli* JM109 and
289 *C. freundii* NCIMB11409 were sonicated to induce various degrees of cell damage and stained
290 with both SYBR-I and PI. The population of live cells as determined by dual staining decreased
291 with increasing sonication duration whilst the dead cells increased (Figure 4a and 4b). There
292 was a strong direct correlation between duration of sonication and the dead cell population for
293 both *E. coli* ($R^2 = 0.99$; figure 5-8c) and *C. freundii* ($R^2 = 0.99$ for both *E. coli* and *C. freundii*).
294 There was an inverse correlation between the duration of sonication and the live cell

295 population, ($R^2 = 0.87$, *E. coli*; $R^2 = 0.76$, *C. freundii*). Furthermore there were strong
296 correlations between the dead cell measurements and the extracellular protein released as a
297 result of sonication for both *E. coli* ($R^2=0.98$) and *C. freundii* ($R^2=0.99$) (supporting information
298 figure 3).

299

300 *Figure 4: Dual staining with SYBR-I and PI to discriminate live and dead E. coli and C. freundii*
301 *cells*

302

303 **3.5 Effects of the presence of insoluble cellulose on turbidity of cell suspensions**

304 To determine what effects addition of an insoluble substance will have on the cell density
305 measurement (OD600), cellulose powder was added to cell suspensions after initial OD600
306 had been measured. As expected, the addition of cellulose resulted in high OD600 values
307 (supporting information figure 4). The values however did not correlate with the values
308 obtained before the addition of cellulose indicating presence of insoluble substances will lead
309 to inaccurate OD600 values. To investigate whether leaving cuvettes to stand for some time
310 to allow avicel to settle would improve the correlation between OD600 without and with avicel,
311 OD600 measurements were taken at 5 and 15 minutes after addition of avicel. The OD600
312 did not significantly change with longer standing time. There was still no correlation with the
313 original OD due to cells alone. The values for 15 minutes wait were however lower than the
314 values for 5 minutes as a result of settling of particles (supporting information figure 5). The
315 rate of decrease was similar for all the samples tested.

316

317 **3.6 Detecting live and dead cells in the presence of cellulose**

318 To investigate the ability of the SYBR-I and PI dual staining to estimate the amount of cells
319 present in suspensions with cellulose, correlations between OD600 and fluorescence were
320 determined from *E. coli* and *C. freundii* cell suspensions with or without cellulose. The addition
321 of avicel did not significantly affect the fluorescence measurements (Figure 5 (a)). Pearson's

322 correlation was used to test whether there was any correlation between OD600 before addition
323 of avicel and the live cells (green fluorescence) before and after addition of avicel. Strong
324 correlations were obtained for all cells whether avicel was present or not (Table 2). To
325 determine whether the fluorescence measured depended on the OD600, Kendall tau
326 correlation test was performed (Table 2). The results showed that irrespective of the type of
327 bacteria used or whether there was avicel or not, fluorescence depended on the OD600 before
328 addition of avicel.

329 *Table 2: Significance of correlations between OD600 and fluorescence in the presence or*
330 *absence of cellulose*

331

332 **3.7 Assessing growth in the presence of cellulose by total protein estimation**

333 Total protein was determined using the coomassie assay on samples following incubation at
334 65°C for one hour as described above. There was no significant difference between total
335 protein for cells with or without avicel ($p = 0.117$ (*E. coli*) and 0.600 (*C. freundii*); figure 5 (b)).
336 Significant correlations were observed between OD600 and the total protein of *E. coli* without
337 cellulose ($p = 0.001$, $R^2 = 0.960$) and with cellulose ($p = 0.003$, $R^2 = 0.923$) as well as *C.*
338 *freundii* without cellulose ($p < 0.001$, $R^2 = 0.990$) and with cellulose ($p < 0.001$, $R^2 = 0.984$).
339 Total protein was statistically dependent on OD600 of cells ($\tau_b = 1$, $p \leq 0.011$).

340

341 **4. Discussion**

342 There have been tremendous advances in fluorescence cytometry (instruments, fluorophores
343 and methods) over the years which enable direct analysis and quantification of bacteria and
344 other cells in different environments (Foladori, Bruni, Tamburini and Ziglio, 2010, Gregori,
345 Citterio, Ghiani, Labra, Sgorbati, Brown and Denis, 2001, Lebaron et al., 1998, Lenkei, Mandy,
346 Marti and Vogt, 1998, Melamed et al., 1972, Nunez et al., 2004, Tamburini, Foladori,
347 Ferrentino, Spilimbergo and Jousson, 2014). [In this study, it has been demonstrated that a](#)
348 [simple two colour fluorescence fluorimetric technique can effectively be used to monitor](#)

349 growth of bacteria in turbid growth cultures including those containing insoluble (eg. cellulosic)
350 substrates. This is essential for assessing *in vivo* activity of cellulose degrading enzyme
351 systems for biofuel production (Duedu and French, 2016, Lakhundi, Duedu, Cain, Nagy,
352 Krakowiak and French, 2016). The total protein assay evaluated in this study was also found
353 to be suitable for this purpose. The fluorescence technique however has some advantages
354 over the total protein technique. Whilst the fluorescence method can be completed in about
355 20 minutes, the total protein will be completed in not less than two hours. Additionally, the
356 stains used in the fluorescence technique are not regarded as harmful by European Union
357 regulations whereas the protein reagent is harmful. Another advantage of using the SYBR-I/PI
358 method over the total protein is that it allows direct estimation of live and dead cells making it
359 suitable for experiments where cell lysis is suspected. With an appropriate standard curve (eg.
360 plate count or flow cytometry), the green fluorescence values can be converted directly to cell
361 numbers.

362

363 Barbesti et al (2000) demonstrated that there is energy transfer from SYBR-I to PI in bacterial
364 cells stained with both fluorochromes. This study has demonstrated that the energy transfer
365 also occurs with DNA in solution but at a slightly higher percentage. This is expected as the
366 fluorochromes readily bind DNA and do not need to cross barriers (eg. membranes) as occurs
367 for cells (Lebaron, Parthuisot and Catala, 1998, Melamed, Adams, Zimring, Murnick and
368 Mayer, 1972). The strong energy transfer facilitates discrimination of live and dead bacteria
369 and it has been shown that it is not affected by the metabolic state (eg. stationary or
370 exponential) or the type of bacteria (gram positive or negative) (Barbesti, Citterio, Labra,
371 Baroni, Neri and Sgorbati, 2000). The discriminatory properties of the SYBR-I/PI dual staining
372 have been shown to be very effective, with strong correlations demonstrated between dead
373 cells (red fluorescence) and the degree of induced damage (sonication). This also means that
374 the fluorimetric application of the SYBR-I/PI method can account not only for cells with
375 compromised cell membranes as happens in flow cytometry but also for completely lysed
376 cells. Flow cytometry will count cells meaning completely lysed cells will not be accounted for.

377 This makes the application of two colour fluorescence staining with fluorimetry applicable in a
378 wider context.

379

380 Obtaining conditions necessary for the ideal measurement of fluorescence intensity is in
381 practice very difficult (Gaigalas, Li, Marti, Henderson, Vogt and Barr, 2001). The development
382 of the MESF unit is a practical approach that seeks to take the application of fluorescence
383 cytometry from just enumeration to actual quantitation. With this unit, quantitative fluorescence
384 data are no longer dependent on the instrument or the environment within which cells are
385 present (eg. media) but are standardized and comparable with others (Schwartz, Gaigalas,
386 Wang, Marti, Vogt and Fernandez-Repollet, 2004). Although the unit has not been evaluated
387 on other fluorescence platforms such as fluorimeters, there are some foreseeable challenges.
388 Fluorimeters do not count cells but rather give a value for the total fluorescence obtained. The
389 fluorescence obtained is also dependent on the amount of DNA present which these stains
390 (i.e. SYBR-I and PI) bind to. Thus, a recombinant organism harbouring a large piece of foreign
391 DNA will likely produce higher fluorescence than the wild type. The equivalent fluorescent
392 DNA (EFD) unit developed in this study leverages the principles of the MESF and the
393 foreseeable challenges of its application in fluorimetry to best serve its purpose. The EFD
394 value obtained for a sample can be directly compared to another sample of the same
395 constitution provided the DNA standard and samples were measured on the same instrument.
396 Rather than using a known quantity of standard beads, the EFD can be converted to a
397 quantitation standard when cell quantities are standardized with plate counts or another
398 appropriate measure.

399

400 Despite the advantages highlighted, the method does have some limitations. On its own, dual
401 staining followed by fluorescence measurement does not give absolute quantification of cells.
402 A standard curve must be generated, for example from plate counts or other suitable method,
403 and used to estimate the number of cells from the fluorescence reading. Furthermore, the
404 method is not suitable for directly comparing quantitation obtained from different bacteria, due

405 to its dependence on the amount of DNA present and inability to separate brightly and dimly
406 fluorescing cells.

407

408 **5. Conclusions**

409 Analysis of bacterial cells stained with SYBR-I and PI using a fluorimeter has been evaluated.
410 The application of this dual fluorescence staining technique in fluorimetry is simple, fast and
411 can be easily adopted for automation or large screening applications [such as with 96-well](#)
412 [plates](#). With fluorimetry, dual staining of samples with SYBR-I and PI will detect not only cells
413 with compromised membranes but completely lysed cells as well. This property can be an
414 advantage or disadvantage depending on the application. The use of the EFD as a unit for
415 comparing fluorescence intensity in fluorimetry across laboratories and instrument platforms
416 has also been demonstrated. This standard is cheap and can easily be made, yet is suitable
417 for comparison with other samples or applications.

418

419

420 **6. References**

421 Barbesti, S., Citterio, S., Labra, M., Baroni, M.D., Neri, M.G., Sgorbati, S., 2000. Two and
422 three-color fluorescence flow cytometric analysis of immunoidentified viable bacteria.
423 *Cytometry*. 40, 214-218.

424 Breed, R.S., Dotterrer, W.D., 1916. The number of colonies allowable on satisfactory agar
425 plates. *Journal of bacteriology*. 1, 321-331.

426 Caron, G.N.-V., Badley, R.A., 1995. Viability assessment of bacteria in mixed populations
427 using flow cytometry. *Journal of Microscopy*. 179, 55-66.

428 Creutzig, F., Ravindranath, N.H., Berndes, G., Bolwig, S., Bright, R., Cherubini, F., Chum, H.,
429 Corbera, E., Delucchi, M., Faaij, A., Fargione, J., Haberl, H., Heath, G., Lucon, O., Plevin, R.,
430 Popp, A., Robledo-Abad, C., Rose, S., Smith, P., Stromman, A., Suh, S., Masera, O., 2015.
431 *Bioenergy and climate change mitigation: an assessment*. *GCB Bioenergy*. 7, 916-944.

432 Diaper, J.P., Tither, K., Edwards, C., 1992. Rapid assessment of bacterial viability by flow
433 cytometry. *Applied Microbiology and Biotechnology*. 38, 268-272.

434 Duedu, K.O., French, C.E., 2016. Characterization of a *Cellulomonas fimi*
435 exoglucanase/xylanase-endoglucanase gene fusion which improves microbial degradation of
436 cellulosic biomass. *Enzyme and Microbial Technology*. 93–94, 113-121.

437 Foladori, P., Bruni, L., Tamburini, S., Ziglio, G., 2010. Direct quantification of bacterial biomass
438 in influent, effluent and activated sludge of wastewater treatment plants by using flow
439 cytometry. *Water Research*. 44, 3807-3818.

440 French, C.E., 2009. Synthetic biology and biomass conversion: a match made in heaven? *J*
441 *R Soc Interface*. 6 Suppl 4, S547-558.

442 French, C.E., Horsfall, L., Barnard, D.K., Duedu, K., Fletcher, E., Joshi, N., Kane, S.D.,
443 Lakhundi, S.S., Liu, C.-K., Oltmanns, J., Radford, D., Salinas, A., White, J., Elfick, A., 2015.
444 Beyond Genetic Engineering: Technical Capabilities in the Application Fields of Biocatalysis
445 and Biosensors. In: B. Giese, C. Pade, H. Wigger, A. von Gleich (Eds.), *Synthetic Biology*,
446 Springer International Publishing, pp. 113-137.

447 Fulton, L.M., Lynd, L.R., Körner, A., Greene, N., Tonachel, L.R., 2015. The need for biofuels
448 as part of a low carbon energy future. *Biofuels, Bioproducts and Biorefining*. 9, 476-483.

449 Gaigalas, A.K., Li, L., Marti, G., Henderson, O., Vogt, R., Barr, J., 2001. The development of
450 fluorescence intensity standards. *J Res Natl Inst Stand Technol*. 106, 381-389.

451 Gratama, J.W., D'Hautcourt, J.-L., Mandy, F., Rothe, G., Barnett, D., Janossy, G., Papa, S.,
452 Schmitz, G., Lenkei, R., The European Working Group on Clinical Cell, A., 1998. Flow
453 cytometric quantitation of immunofluorescence intensity: Problems and perspectives.
454 *Cytometry*. 33, 166-178.

455 Gregori, G., Citterio, S., Ghiani, A., Labra, M., Sgorbati, S., Brown, S., Denis, M., 2001.
456 Resolution of viable and membrane-compromised bacteria in freshwater and marine waters
457 based on analytical flow cytometry and nucleic acid double staining. *Applied and*
458 *environmental microbiology*. 67, 4662-4670.

459 Humphreys, M.J., Allman, R., Lloyd, D., 1994. Determination of the viability of trichomonas
460 vaginalis using flow cytometry. *Cytometry*. 15, 343-348.

461 Kaprelyants, A.S., Kell, D.B., 1992. Rapid assessment of bacterial viability and vitality by
462 rhodamine 123 and flow cytometry. *Journal of Applied Bacteriology*. 72, 410-422.

463 Koch, A.L., 1970. Turbidity measurements of bacterial cultures in some available commercial
464 instruments. *Analytical Biochemistry*. 38, 252-259.

465 Lakhundi, S.S., Duedu, K.O., Cain, N., Nagy, R., Krakowiak, J., French, C.E., 2016.
466 *Citrobacter freundii* as a test platform for recombinant cellulose degradation systems. *Letters*
467 *in Applied Microbiology*. DOI: 10.1111/lam.12668. (Accepted article available online).

468 Lebaron, P., Parthuisot, N., Catala, P., 1998. Comparison of blue nucleic acid dyes for flow
469 cytometric enumeration of bacteria in aquatic systems. *Applied and environmental*
470 *microbiology*. 64, 1725-1730.

471 Lenkei, R., Gratama, J.W., Rothe, G., Schmitz, G., D'Hautcourt, J.L., Årekrans, A., Mandy, F.,
472 Marti, G., 1998. Performance of calibration standards for antigen quantitation with flow
473 cytometry. *Cytometry*. 33, 188-196.

474 Lenkei, R., Mandy, F., Marti, G., Vogt, R., 1998. Quantitative fluorescence cytometry: an
475 emerging consensus, *Cytometry*, John Wiley & Sons, Inc.

476 Melamed, M.R., Adams, L.R., Zimring, A., Murnick, J.G., Mayer, K., 1972. Preliminary
477 evaluation of acridine orange as a vital stain for automatic differential leukocyte counts. *Am J*
478 *Clin Pathol*. 57, 95-102.

479 Nuffield Council on Bioethics, 2011. *Biofuels: ethical issues*, Nuffield Council on Bioethics,
480 Abingdon.

481 Nunez, R., Garay, N., Villafane, C., Bruno, A., Lindgren, V., 2004. Description of a flow
482 cytometry approach based on SYBR-14 staining for the assessment of DNA content, cell cycle
483 analysis, and sorting of living normal and neoplastic cells. *Exp Mol Pathol*. 76, 29-36.

484 Porter, J., Deere, D., Pickup, R., Edwards, C., 1996. Fluorescent probes and flow cytometry:
485 new insights into environmental bacteriology. *Cytometry*. 23, 91-96.

486 Purvis, N., Stelzer, G., 1998. Multi-platform, multi-site instrumentation and reagent
487 standardization. *Cytometry*. 33, 156-165.

488 Schwartz, A., Gaigalas, A.K., Wang, L., Marti, G.E., Vogt, R.F., Fernandez-Repollet, E., 2004.
489 Formalization of the MESF unit of fluorescence intensity. *Cytometry Part B: Clinical Cytometry*.
490 57B, 1-6.

491 Schwartz, A., Marti, G.E., Poon, R., Gratama, J.W., Fernández-Repollet, E., 1998.
492 Standardizing flow cytometry: A classification system of fluorescence standards used for flow
493 cytometry. *Cytometry*. 33, 106-114.

494 Schwartz, A., Wang, L., Early, E.A., Gaigalas, A.K., Zhang, A., Marti, G.E., Vogt, R.F., 2002.
495 Quantitating Fluorescence Intensity From Fluorophore: The Definition of MESF Assignment.
496 *J Res Natl Inst Stand Technol*. 107, 83-91.

497 Sgorbati, S., Barbesti, S., Citterio, S., Bestetti, G., De Veechi, R., 1996. Characterization of
498 number, DNA content, viability and cell size of bacteria from natural environments using
499 DAPI/PI dual staining and flow cytometry. *Minerva Biotech*. 8, 9-15.

500 Shapiro, H.M., Perlmutter, N.G., Stein, P.G., 1998. A flow cytometer designed for fluorescence
501 calibration. *Cytometry*. 33, 280-287.

502 Tamburini, S., Foladori, P., Ferrentino, G., Spilimbergo, S., Jousson, O., 2014. Accurate flow
503 cytometric monitoring of *Escherichia coli* subpopulations on solid food treated with high
504 pressure carbon dioxide. *J Appl Microbiol*. 117, 440-450.

505 Treuer, R., Haydel, S.E., 2011. Acid-fast staining and Petroff-Hausser chamber counting of
506 mycobacterial cells in liquid suspension. *Curr Protoc Microbiol*. Chapter 10, Unit 10A 16.

507 Wang, L.L., Gaigalas, A.K., Schwartz, A., Abbasi, F., Vogt, R.F., 2002. Quantitating
508 fluorescence intensity from fluorophores: Practical use of MESF values. *J Res Natl Inst Stand*
509 *Technol*. 107, 339-353.

510 Waxdal, M.J., Monical, M.C., Palini, A.G., 1998. Inter-laboratory relative fluorescence intensity
511 measurements using FlowCal 575 calibration beads: A baseline study. *Cytometry*. 33, 213-
512 218.

513 Wood, J.C.S., 1998. Fundamental flow cytometer properties governing sensitivity and
514 resolution. *Cytometry*. 33, 260-266.

515 Wood, J.C.S., Hoffman, R.A., 1998. Evaluating fluorescence sensitivity on flow cytometers:
516 An overview. *Cytometry*. 33, 256-259.

517 Zenger, V.E., Vogt, R., Mandy, F., Schwartz, A., Marti, G.E., 1998. Quantitative flow cytometry:
518 Inter-laboratory variation. *Cytometry*. 33, 138-145.

519 Zhang, Y.-z., Kemper, C., Bakke, A., Haugland, R.P., 1998. Novel flow cytometry
520 compensation standards: Internally stained fluorescent microspheres with matched emission
521 spectra and long-term stability. *Cytometry*. 33, 244-248.

522

523 **Figures**

524 ***Figure 1. Excitation and emission spectra of SYBR green I and propidium iodide from***
525 ***Fluorescence SpectraViewer, Life Technologies***

526

527 ***Figure 2: Differences between single and dual staining of DNA***

528 DNA was serially diluted and stained with either SYBR-I or PI alone and fluorescence
529 determined. Fluorescence determination was performed in the red fluorescence channel for
530 PI alone (a) and in the green fluorescence channel for SYBR-I alone (b). Dual stained DNA
531 was measured in both the red and green fluorescence channels. Six replicates were
532 performed.

533

534 ***Figure 3: DNA standard preparation and estimation of the EFD of damage-induced and***
535 ***non-damaged cells stained with single fluorochromes***

536 A. Standard curves for DNA stained with each of the fluorochromes were made to develop
537 the equivalent fluorescent DNA unit. DNA was serially diluted and the concentrations
538 determined using the nanodrop 2000 (ThermoScientific, Wilmington, DE, USA).
539 Diluted DNA was stained with either SYBR-I or PI and the fluorescence measured in
540 the green or red fluorescence channels respectively. Plots represent six replicates of
541 each dilution that was prepared and measured.

542 B. Cell suspensions were tested and compared with the DNA standard curves to
543 determine the EFD. Cell suspensions were prepared by resuspending pellets from
544 overnight cell cultures. The OD600 was predetermined and the pellets were serially
545 diluted and stained with either SYBR-I or PI. Two biological replicates (each made up
546 of three technical repeats) were performed.

547 C. Cell damage was confirmed by determining the total protein concentration of the cell
548 suspensions. Cell suspensions were ultrasonicated to induce various degrees of cell
549 damage. Suspensions were centrifuged to remove debris and non-lysed cells and the

550 supernatant collected and tested. The total protein was determined using the Bradford
551 assay.

552 D. Fluorescence and total protein were determined for cell suspensions sonicated to
553 induce various degrees of membrane damage. Fluorescence of PI as a result of cell
554 damage determined by total protein was compared.

555

556 ***Figure 4: Simultaneous staining with SYBR-I and PI to discriminate live and dead E. coli***
557 ***and C. freundii cells***

558 Discrimination of live and dead cells was achieved by simultaneously staining cell suspensions
559 of *E. coli* and *C. freundii* that have been damaged to various degrees. Live cells were obtained
560 as the fluorescence in the green channel whereas dead cells were obtained as the
561 fluorescence in the red channel. Three biological repeats were performed.

562

563

564 ***Figure 5:***

565 ***(a) Simultaneous staining is sufficient to discriminate and quantify live and dead cells***
566 ***in the presence of insoluble cellulose***

567 Cell suspensions of different densities were used. Fluorescence measurements were obtained
568 for cells prior to and after addition of avicel. Cell suspensions of *E. coli* (a and b) and *C. freundii*
569 (c and d) were used. Three biological repeats were performed.

570 ***(b) Total protein estimation to estimate growth in the presence of cellulose***

571 Total protein were obtained for cell suspensions prior to and after addition of avicel. The plots
572 show the original OD600 (i.e. before addition of avicel) versus the total protein with and without
573 avicel. Three biological replicates were performed.

574 **Tables**575 **Table 1: Energy transfer from SYBR-I to PI**

Amount of DNA (ng μl^{-1})	SYBR-I decrease (%)	Standard Deviation (\pm)	PI increase (%)	Standard Deviation (\pm)
10	-99.0	0.27	146.7	23.31
15	-99.8	0.07	121.9	17.84
20	-99.4	0.14	98.4	31.13
25	-99.7	0.11	115.6	31.60
30	-99.4	0.13	118.6	37.61

576 Values are means of three measurements. SYBR-I fluorescence was almost quenched when DNA was
 577 stained with both stains.

578

579 **Table 2: Significance of correlations between OD600 and fluorescence in the presence or**
 580 **absence of cellulose**

		Pearson Correlation	Kendall's tau_b Correlation
JM109	Correlation Co-efficient	0.995**	1.000**
	Sig. (2-tailed)	0.000	0.000
JM109+avicel	Correlation Co-efficient	0.990**	1.000**
	Sig. (2-tailed)	0.000	0.000
NCIMB	Correlation Co-efficient	0.978**	1.000**
	Sig. (2-tailed)	0.000	0.000
NCIMB+avicel	Correlation Co-efficient	0.942**	1.000**
	Sig. (2-tailed)	0.002	0.000

581

582 Correlations were tested for linearity (Pearson) and dependence (Kendall's tau) between the
 583 OD600 values and the fluorescence measurements. **Correlation is significant at the 0.01
 584 level (2-tailed).





