

New methodology for viability testing in environmental samples

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Abstract

Environmental samples can be complex and are comprised of microorganisms and a matrix of decaying organic matter as well as an inorganic phase such as sand or precipitated material (waste water, sludge, soils, etc.). Nucleic acid dyes have recently been developed to address the growing need for environmental analyses (cell staining, counting, viability testing and specific organism identification). However, certain dyes may not be ideally suited for testing of environmental samples, because they readily adhere to the substrate material as well as their target molecule, resulting in increased non-specific binding and background fluorescence. The aim of this study was to address the limitations of the widely used and commercially available Live/Dead BacLight™ Bacterial Viability kit (Molecular Probes, Eugene, OR). A new combination of nucleic acid dyes, i.e. SYTO® 13 and SYTOX® Orange (Molecular Probes, Eugene, OR), was proposed as an alternative. The dyes were carefully chosen for their spectral separation and increase of fluorescence quantum yield. A protocol for this combination was first designed and optimized and the two staining assays were compared against suspensions of live and dead *E. coli*, mixed in different proportions and it was shown that both protocols performed equally on pure cultures. However, when testing activated sludge samples, the commercial kit showed greater background fluorescence and non-specific binding than the alternate combination. Therefore, the proposed dye combination and its corresponding protocol are deemed more suitable for use on complex environmental samples than the Live/Dead BacLight™ Bacterial Viability kit.

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1. Introduction

In environmental studies, growth-based assays are traditionally used to determine bacterial inventories. However, such plate counts only estimate the numbers of culturable, active bacteria that are able to initiate cell division at a sufficient rate to form colonies [1]. This excludes a large proportion of the bacteria encountered in environmental samples that might be viable but not culturable on conventional substrates. Dyes such as acridine orange (AO) or 4,6 diamino-2-phenylindole (DAPI) have been used to estimate bacterial numbers in diverse environmental samples; these dyes function by staining any DNA-containing cells. They eliminate unspecific staining of dead cells that do not contain a significant amount of DNA, but include inactive bacteria in the count. Direct optical detection

methods such as epifluorescent microscopy (EPM), which utilize stains specific to biological molecules (e.g. DNA stains), are increasingly being employed for microbial characterization due to the awareness of problems associated with culture-based assays [2]. Other methods, such as universal probe hybridisation have been used to assay all cells containing a threshold amount of rRNA [3–4]. While the presence of rRNA is not direct proof of metabolic activity, it indicates, at least, the potential viability of the cell. Moreover, numerous stains (e.g. the redox dye CTC, lipophilic cationic dyes and propidium iodide) have been developed for the assessment of cell function including reproductive ability, metabolic activity, membrane activity, and membrane potential. These methods have been employed to distinguish viable, but non-culturable, microorganisms that would be discounted in traditional, growth-based assays. Furthermore, accurately assessing the viability of bacteria involved in engineered biological treatments (e.g. wastewater treatment) is essential in order to estimate the system productivity, biomass and substrate uptakes [5,6]. The Live/Dead BacLight™ Bacterial Viability kit is a widely used method to measure viability and differentiate between living

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and dead cells by detecting if their membrane system is intact [7]. This kit is comprised by two nucleic acid probes, green-fluorescent SYTO[®]9 and red-fluorescent Propidium Iodide (PI). These stains differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. The Live/Dead BacLight[™] Bacterial Viability kit has been proven to accurately quantitate a broad range of bacterial species. However, autofluorescence and non-specific binding issues can be encountered when testing more complex samples such as wastewater, due to the relatively low increase of fluorescence quantum yield of the 'dead' stain PI, upon binding to nucleic acids.

The objective of this study was to evaluate whether an alternate viability assay to the available commercial kit would be more suitable to complex environmental samples, by reducing background fluorescence and non-specific binding issues, previously mentioned. SYTO[®]13 and SYTOX[®] Orange (Molecular Probes, Eugene, OR) were selected for their spectral and DNA/RNA binding characteristics and have been previously used in viability testing in cell suspensions. LeBaron *et al.* used SYTO[®]13 for assessment of viability of individual bacterial cells in seawater samples [8]. Comas-Riu and Vives-Rego used both SYTO[®]13 and PI for viability testing and cell sorting of endospore-forming *P. polymixa* cultures [9], while Rodriguez *et al.* successfully detected viable and damaged cells in *Salmonella typhirium* and *Stapilococcus aureus* suspensions using the same dye combination [10]. Burnett *et al.* showed that SYTOX[®] Orange stained dead *E. coli* cells on apple cuticles, more effectively than PI and provided an intensity fluorescent label, which could be easily distinguished from the background fluorescence [11]. This new combination of dyes was compared to the Live/Dead BacLight[™] Bacterial Viability kit (detailed information on these dyes is provided later in the text). Comparison was carried out against pure *Escherichia coli* (*E. coli*) cultures and municipal activated sludge samples. Live and dead *E. coli* cells were mixed in different proportions, stained with both probe combinations and stained samples were scanned with a confocal laser-scanning microscope (CLSM). Image-Pro[®] Plus image analysis software (Media Cybernetics Inc., Silver Spring, MD) was used to quantitate the relative amounts of stained (live and dead) cells to assess suitability of the proposed dye combination for viability testing. A similar procedure was performed against activated sludge samples in order to evaluate background fluorescence and non-specific binding issues. Quantitative results from the image analysis were compared to reveal the most appropriate assay for this type of samples.

2. Materials and methods

2.1. Bacterial samples

2.1.1. *E. coli* cultures

Escherichia coli (ATCC 23724) was grown overnight in 20 mL of nutrient broth at 37 °C. After centrifugation at 2000 rpm for 15 min, the pellets were resuspended in sterile deionized water for live bacteria and in 70% isopropyl alcohol for dead bacteria (contact time for total mortality: 30 min).

Following direct counting with Crystal Violet, suspensions were diluted up to 10 million per milliliter. Suspensions were centrifuged at 2000 rpm for 15 min and resuspended in Tris buffer. Live (L) and dead (D) bacteria were mixed together in different proportions (100L:0D, 75L:25D, 50L:50D, 25L:75D, and 0L:100D) in one milliliter aliquots for staining.

2.1.2. Activated sludge samples

Wastewater samples from aerated activated sludge treatment train were obtained from a local wastewater treatment plant (Public Water Works Department, City of Oak Ridge, TN). In order to produce dead samples from live samples for viability testing, incubation in 70% isopropyl alcohol was initially chosen. However, it appeared to impact the structural integrity of the bacterial aggregates, making it very difficult to compare images from live and dead samples, respectively. Therefore, autoclaving (121 °C, 30 min) of the samples was used instead.

Live and autoclaved wastewater samples were stained at their original concentration.

2.2. Viability staining

2.2.1. Live/dead[®] BacLight[™] bacterial viability kit

The BacLight[™] kit was used according to protocol issued by Molecular Probes [12].

2.2.2. SYTO[®]13 and SYTOX[®] Orange

In order to compare combination of SYTO[®]13 and SYTOX[®] Orange dyes (Invitrogen Molecular Probes, Eugene, OR) for comparison with commercial Live/Dead[®] BacLight[™] bacterial viability kit (Invitrogen Molecular Probes, Eugene, OR), it was necessary to test various concentrations of each dye on pure *E. coli* cultures. The following concentration series, prepared in Phosphate-Buffered Saline solution (PBS 1X, Mediatech Inc., Herndon, VA), were evaluated for desired fluorescence, for each dye, respectively: (1.25, 2.5, 5, 10, 20 μM) and (0.0125, 0.025, 0.05, 0.10, 1, 2.5, 5 μM). One milliliter bacterial suspensions were stained with the above concentrations of dyes, and incubated at room temperature (approx. 25 °C), in the dark, for fifteen minutes. After brief vortexing, 10 μL of stained suspension was spotted onto a microscope slide, air-dried for 5 min, heat fixed for approximately 1–2 s, and sealed for microscopic analysis. Based upon desired level of fluorescence, optimum concentrations of the two dyes were established to be 10 μM for SYTO[®]13 and 2.5 μM for SYTOX[®] Orange.

2.3. Confocal laser-scanning microscopy (CLSM) and image analysis

Stained bacterial suspensions were viewed using a Leica DMIRBE CLSM with a 100× objective (1000× total magnification) (viable cells were observed with a 488 nm excitation, 535 nm bandpass emission filter and dead bacteria were seen with a 488 nm excitation, 580 nm emission longpass filter). Five random fields, in stacks of six, were chosen and

captured using the Leica CLSM. Images were then quantitated using Image-Pro[®] Plus image analysis software (Media Cybernetics Inc., Silver Spring, MD). Composite images of stacks were obtained for each channel and converted into intensity levels per pixel that was compiled and analyzed with Microsoft[®] Office Excel (Microsoft Corp., Redmond, WA).

2.4. Quality controls

In order to insure that the collected data was not biased by autofluorescence intensity, unstained samples of *E. coli* and wastewater were tested using the previously described protocol. The autofluorescence background was established and removed from the actual samples by using gain and offset functions of the Leica CLSM.

3. Results and discussion

3.1. Relevance of dye combinations for viability testing

To provide further insight on the two dye combinations to be compared and justify the choice of the two alternative dyes, detailed information is presented here. The Live/Dead BacLight[™] Bacterial Viability kit is comprised of SYTO[®]9 and PI, while the proposed alternate dye combination is comprised of SYTO[®]13 and SYTOX[®] Orange. The SYTO[®] family of dyes (which includes SYTO[®]9), are essentially non-fluorescent until they bind to nucleic acids, whereupon their fluorescence quantum yield may increase by 1000-fold or more [13]. These dyes are freely permeant to most cells and their affinity for nucleic acids is moderate. They can therefore be displaced by higher-affinity nucleic acid stains such as PI. Because the membrane of intact cells offers a barrier to entry of these higher-affinity nucleic acid stains, the combination of a green-fluorescent SYTO[®] dye with a red-fluorescent, high-affinity nucleic acid stain such as PI allows for simultaneous

staining of the live- and dead-cell populations. Although the green-fluorescent SYTO[®] dye will still bind to nucleic acids in dead cells, it will be displaced or its fluorescence quenched by the red-fluorescent dye, resulting in a yellow-, orange- or red-fluorescent dead-cell population [13]. The excitation/emission maxima for these dyes are at 480/500 and 537/620 nm for SYTO[®]9 and PI, respectively. Thus, with an appropriate mixture of the SYTO[®]9 and PI stains, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. PI is a high-affinity nucleic acid stain that increases its fluorescence quantum yield by only 20–30 fold [14] upon binding to nucleic acids, leading to potentially high background fluorescence when the dye non-specifically stains non-biological material, e.g. organic and inorganic matter.

SYTO[®]9 was not commercially available as individual dye at the time of this study, therefore SYTO[®]13 was chosen as alternative to SYTO[®]9, as it is also one of the SYTO[®] family of dyes (previously described) and exerts similar excitation and emission spectra as SYTO[®]9 (5 nm difference). As with PI, SYTOX[®] Orange is a high-affinity nucleic acid stain, impermeant to live cells but easily penetrates cells with compromised plasma membrane. However, SYTOX[®] Orange exerts an increase of its fluorescence quantum yield by 500 fold upon binding to nucleic acids [15], resulting in a much larger difference in fluorescence between specific and non-specifically bound stain compared to PI. The quenching of SYTO[®]13 fluorescence in the presence of SYTOX[®] Orange is also expected for staining of metabolically inactive cells. The excitation/emission maxima for the alternate dye combination are at 489/505 and 548/571 nm for SYTO[®]13 and SYTOX[®] Orange, respectively. Thus, with an appropriate mixture of the SYTO[®]13 and SYTOX[®] Orange stains, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent orange-red.

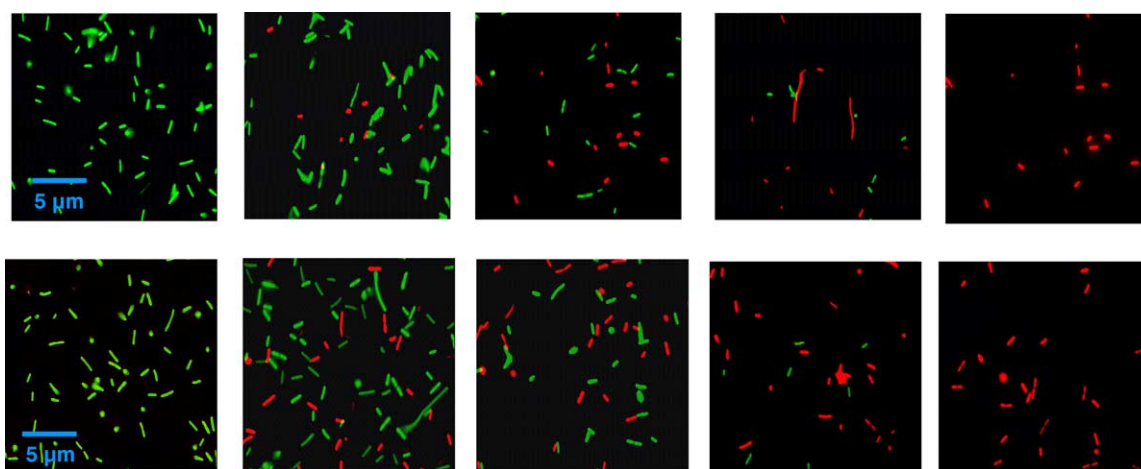


Fig. 1. Image series for comparison of dye combinations in mixed *E. coli* suspensions. Metabolically active cells are stained green and dead cells are stained red. Live/Dead BacLight[™] Bacterial Viability Kit (Molecular probes, Eugene, OR), top; SYTO[®]13/SYTOX[®] Orange (Molecular probes, Eugene, OR), bottom. From left to right, the target live/dead ratios were 100, 75, 50, 25 and 0%, respectively. At 1000 \times magnification using a Leica DMIRBE CLSM, viable cells were observed with a 488 nm excitation, 535 nm bandpass emission filter and dead bacteria were seen with a 488 nm excitation, 580 nm emission longpass filter and imaged with Image Pro Plus software.

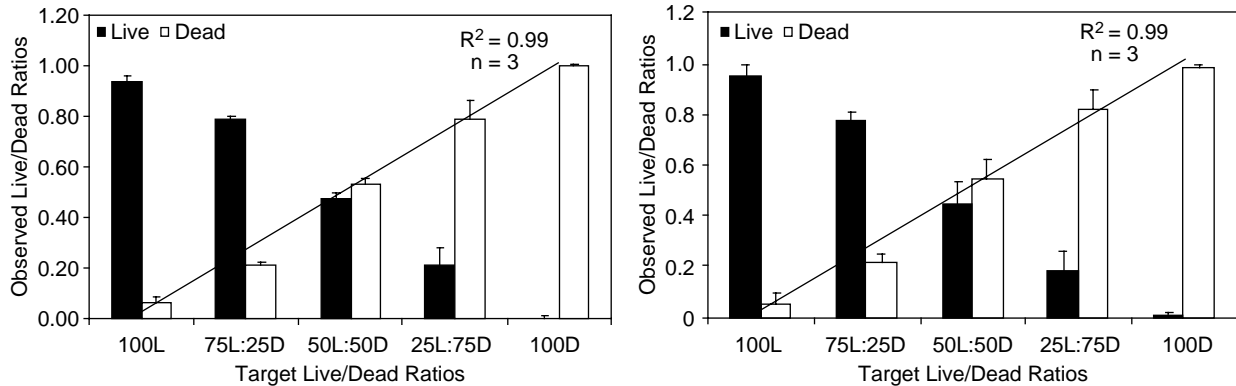


Fig. 2. Observed vs target live/dead ratios—staining of mixed live and dead suspensions of *E. coli* with: left: Live/Dead BacLight™ bacterial viability kit (Molecular probes, Eugene, OR); Right: SYTO® 13/SYTOX® Orange (molecular probes, Eugene, OR) dye combination. One sample was tested for each mixed live/dead *E. coli* suspension (target live/dead ratios of 100, 75, 50, 25, and 0%, respectively). Three fields of view ($n=3$) were randomly chosen and imaged for each sample, for both channels (Green/Live and Red/Dead). Results from each channel were compiled and averaged over the fields of view and relative proportions (observed) of green/live (black bars) and red/dead (white bars) fluorescence (with corresponding standard deviations) were calculated for each sample and plotted against target live/dead ratios. A linear regression was performed between observed and target live/dead ratios and exerted $R^2=0.99$.

3.2. Comparison on pure *E. coli* suspensions

The response from the two kits was analyzed and compared for mixed sample with different proportions of live and dead bacteria, i.e. *E. coli*. Fig. 1 shows the processed images obtained from these suspensions, for both dye combinations and Fig. 2a and b show the live/dead ratios derived from these images, which demonstrates that there is very slight difference between the two assays. Further more, the full paired data (number of pixels for each intensity level of the grayscale) from both sets was compiled and a standard Student *t*-test ($\alpha=0.05$, $n=1020$) was performed to compare the mean intensity from both dye combinations and no significant difference was detected, i.e. $p\text{-value} = 0.50 > \alpha$.

3.3. Comparison on wastewater samples

Images in Fig. 3 illustrate the stark contrast between the two viability kits. Live and dead wastewater samples were stained with both dye combinations. Images A and C show the live (green) and dead (red) Channels with SYTO® 13/SYTOX® Orange simultaneous staining, for live and dead wastewater, respectively. Images B and D show the live (green) and dead (red) Channels with Live/Dead BacLight™ Bacterial Viability Kit, for live and dead wastewater, respectively. Images obtained with the SYTO® 13/SYTOX® Orange combination are exempt of background fluorescence on both channels, while some background fluorescence from PI (i.e. red channel) is present on image B. To confirm this trend, ratios of Red/Green channel fluorescence, i.e. dead/all cells ratio in field of view, were evaluated by staining fresh wastewater with SYTOX® Orange/SYTO® 13 and PI/ SYTO® 9. The number of pixels per intensity level was averaged over the grayscale for each series ($n=6$) and each fluorescent dye in both combinations. The results were expressed as the mean (\pm SD; $n=6$) ratio between the red and green fluorescence channel for both assays. SYTOX® Orange stained $24 \pm 14\%$ of the total cells, whereas

PI showed considerably higher staining with $62 \pm 25\%$. Images obtained from stained live and dead wastewater were converted into pixel per intensity data for each dye pair, in order to assess their sensitivity for low intensity levels, i.e. background intensity. An intensity threshold of 75 was empirically chosen for assessment of the background fluorescence. Z-test for means with unequal variances was performed with Data Analysis Tool Pak (Microsoft® Excel) to assess mean difference in background fluorescence between two samples

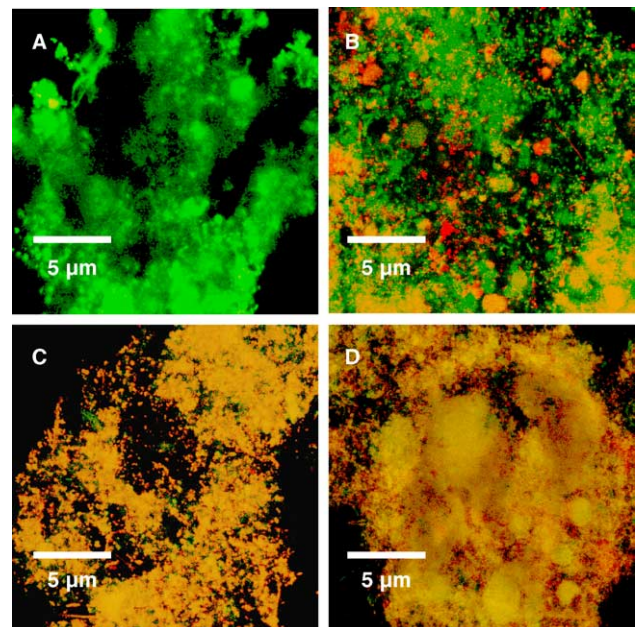


Fig. 3. Comparison of the dye combinations for wastewater sample. Images A and C are live-green and dead-red channels for SYTO® 13/SYTOX® Orange. Images B and D are Live-Green and Dead-Red Channels for Live/Dead BacLight™ Bacterial Viability Kit. 1000 \times on Leica DMIRBE CLSM, viable cells were observed with a 488 nm excitation, 535 nm bandpass emission filter and dead bacteria were seen with a 488 nm excitation, 580 nm emission longpass filter and imaged with Image Pro Plus software.

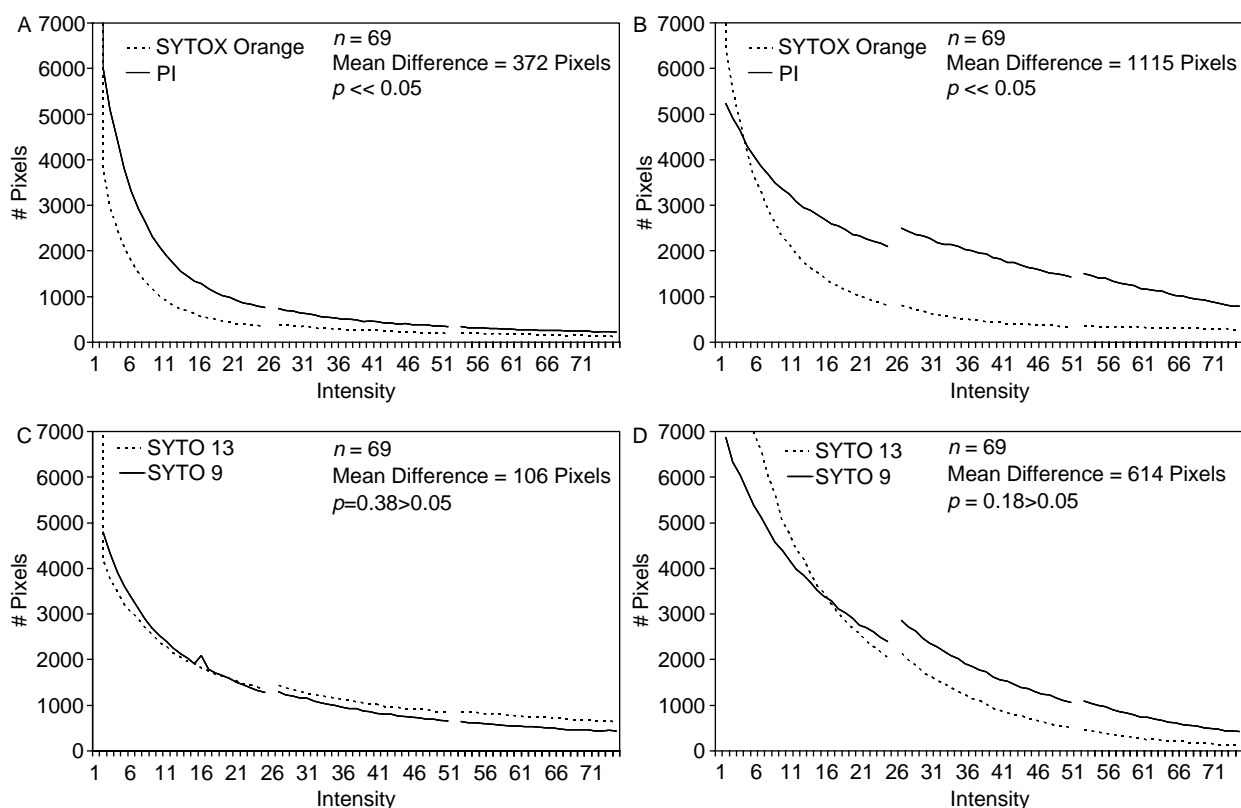


Fig. 4. Number of Pixels as a function of Intensity—comparison of dyes for background fluorescence (intensity below threshold of 75). Images were taken from six fields of view for each slide of spotted wastewater sample, respectively stained with each dye being compared. Number of pixels for each intensity level was obtained and averaged over the field of views. All data points with intensity below the arbitrarily chosen intensity threshold of 75 ($n=69$) were plotted. Z-test for means with unequal variances was performed with Data Analysis Tool Pak (Microsoft[®] Excel). A: Stained live wastewater—comparison of SYTOX[®] Orange and PI. SYTOX[®] Orange stained live wastewater exerts significantly less fluorescence ($p \ll 0.05$, mean difference of 372 pixels per intensity level across the grayscale) than PI stained live wastewater. B: stained dead wastewater—comparison of SYTOX[®] Orange and PI. SYTOX[®] Orange stained dead wastewater exerts significantly less fluorescence ($p \ll 0.05$, mean difference of 1115 pixels per intensity level across the grayscale) than PI stained dead wastewater. C: stained live wastewater—comparison of SYTO[®]13 and SYTO[®]9. No significant difference was observed ($p=0.38 > 0.05$, mean difference of 106 pixels per intensity level across the grayscale) between SYTO[®]13 stained live wastewater and PI stained live wastewater. D: stained dead wastewater—comparison of SYTO[®]13 and SYTO[®]9. No significant difference was observed ($p=0.18 > 0.05$, mean difference of 614 pixels per intensity level across the grayscale) between SYTO[®]13 stained dead wastewater and PI stained dead wastewater.

stained with different dyes. Fig. 4a and b present a comparison of ‘dead’ stains, SYTOX[®] Orange and PI, for live and dead wastewater, respectively. On Fig. 4a, the curve for SYTOX[®] Orange was consistently lower than the curve for PI across the grayscale, i.e. the intensity axis (below the threshold). The mean difference was significant (372 pixels per intensity level across the grayscale, $p < 0.05$, $n=69$), and showed that SYTOX[®] Orange exhibited less background fluorescence than PI on live wastewater. SYTOX[®] Orange also proved to exert less background fluorescence in dead wastewater, with curve significantly lower than PI (1115 pixels per intensity level across the grayscale, $p \ll 0.05$, $n=69$, Fig. 4b). Therefore, SYTOX[®] Orange appears to significantly reduce background fluorescence in both instances. Fig. 4c and d present a comparison of ‘all cells’ stains, SYTO[®]13 and SYTO[®]9, for live and dead wastewater, respectively. Similar background fluorescence was observed for both dyes in presence of metabolically active wastewater ($p=0.38$, $n=69$, Fig. 4c) and dead wastewater ($p=0.18$, $n=69$, Fig. 4d).

4. Conclusions

The results presented above demonstrated that both dye combination, i.e. SYTO[®]13/SYTOX[®] Orange and SYTO[®]9/PI performed similarly on a pure single bacterial suspension (*E. coli*). However, when tested against a more complex matrix, comprised of inorganic and organic compounds (activated sludge), the ‘dead cell’ dye (PI) of the Live/Dead BacLight[™] bacterial viability kit yielded significantly greater non-specific binding to the matrix and greater background fluorescence (especially in presence of dead bacteria) than the proposed alternative SYTOX[®] Orange. No significant difference between the two ‘all cell’ dyes SYTO[®]9 and SYTO[®]13 was found, in terms of background fluorescence. Selecting both dyes for the proposed combination, based on their spectral separation and quantum yield increase upon binding to nucleic acids, proved beneficial in enhancing reduction of background fluorescence and non-specific binding, in the case of aerated activated sludge.

The SYTO[®]13/SYTOX[®] Orange combination and its corresponding protocol are therefore considered to be more suitable than the Live/Dead BacLight[™] bacterial viability, for assessing viability in more complex biological matrices.

Further investigation is needed in order to test other matrices, but the results presented in this paper clearly showed that the proposed combination outperformed the commercial kit for assessing viability in activated sludge samples.

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