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Enumeration of water-borne bacteria using viability assays and flow cytometry: a comparison to culture-based techniques

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Abstract

Maintaining optimal conditions in catchments or distribution systems relies heavily on water authorities having access to rapid and accurate water quality data, including an indication of bacteriological quality. In this study, the BacLight™ bacterial viability kit and carboxyfluorescein diacetate (CFDA) were coupled with flow cytometry (FCM) for rapid detection of physiologically active bacteria from raw and potable waters taken from various locations around South Australia. Results were compared to the direct viable count (DVC) and quantitative DVC (qDVC), in addition to the culture-based methods of the heterotrophic plate count (HPC) and a commercial SimPlate™ technique. Raw and potable water analysis revealed that DVC and culture-based techniques reported significantly fewer viable bacteria compared to the number of physiologically active bacteria detected using the rapid FCM assays, where this difference appeared to be nonlinear across different samples. Inconclusive results were obtained using qDVC as a viability assay. In particular, HPC results were 2–4 log orders of magnitude below that reported by the FCM assays for raw waters. Few bacteria in potable waters examined were culturable by HPC, even though FCM assays reported between 5.56×10^2 and 3.94×10^4 active bacteria ml^{-1}. These differences may be attributed to the presence of nonheterotrophic bacteria, sublethal injury or the adoption of an active but nonculturable (ABNC) state.

Keywords: ABNC; BacLight kit; CFDA; Flow cytometry; Heterotrophic plate count; qDVC

1. Introduction

Water-testing laboratories commonly evaluate bacteriological water quality using the heterotrophic plate count (HPC), which assesses the number of bacteria in water samples that are able to form visible colonies on a solid medium, under specified test conditions (e.g. medium nutrients, incubation time, incubation temperature, etc.) (Australian Drinking Water Guidelines, 1996). However, this technique only reveals the presence of culturable heterotrophic bacteria.

Microorganisms in the environment are exposed to conditions that cause survival stress. To counter this,

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some bacteria are capable of maintaining metabolic activity whilst developing a recalcitrance to culture. Such bacteria are often defined as 'viable but non-culturable' (VBNC) (McDougald et al., 1998). However, this term is not accurate by its strictest definition, unless subsequent resuscitation of VBNC bacteria, leading to extended cellular division, is demonstrated. As outlined by Kell et al. (1998), a more accurate term is 'active but nonculturable' (ABNC) as it describes bacteria which exhibit measurable traits of physiological activity but fail to grow to a detectable level. Also, chlorine used in many potable waters for disinfection may cause sublethal injury of some bacteria (McFeters et al., 1986; du Preez et al., 1995), thereby rendering them nonculturable. These factors question whether the use of laborious and time-consuming culture methods, such as HPC which takes a minimum of two days to return a result, truly provide the best indication of the bacteriological quality of water or the effectiveness of disinfection procedures.

Flow cytometry (FCM), coupled with advancements in fluorescent dye technology (Porter et al., 1996), has become a valuable tool for the detection of bacteria in aquatic environments (Vives-Rego et al., 2000). FCM can perform detailed light scattering and fluorescent signal measurements on single bacterial cells at a rate in excess of 1000 cells s^{-1} (Vives-Rego et al., 2000). Hence, bacterial data from a water sample can be acquired and processed rapidly and, if necessary, corrective action can be taken sooner when compared with samples processed using conventional culture-based techniques.

There is a range of fluorescent dyes that can be chosen for use in conjunction with FCM for characterising the metabolic status of bacterial cells. The LIVE/DEAD® BacLight™ bacterial viability kit, developed by Molecular Probes (Eugene, OR, USA), has been widely used, in conjunction with FCM, to assess the active bacterial process of maintaining membrane integrity. The kit consists of SYTO-9, a green fluorescent nucleic acid stain which is membrane permeable and labels bacteria with both intact and damaged membranes, and propidium iodide (PI), an orange/red fluorescent nucleic acid stain that is membrane impermeable and so can only stain bacteria with compromised membranes (Haugland, 1999). When the stains are used simultaneously and illuminated with blue light, those bacteria with intact membranes appear fluorescent green; however, bacteria with damaged membranes, permeable to PI, have a red fluorescence as a result of an energy transfer between the two fluorophores (Vives-Rego et al., 2000). The BacLight™ kit has had applications in various areas of bacteriological research (Couto and Hogg, 1999; Ericsson et al., 2000; Auty et al., 2001) but has been used specifically for the direct enumeration of physiologically active bacteria from drinking (Boulos et al., 1999) and bottled mineral water (Ramalho et al., 2001).

FCM can also be used for the assessment of physiologically active bacteria based on measurements of intracellular esterase activity. A variety of cell permeant, esterified fluorogenic substrates are available that are colourless until cleaved by active intracellular enzymes and the fluorescent product is accumulated within the cell. Some of these include ChemChrome B (Reynolds et al., 1997), fluorescein diacetate (FDA) and calcein acetoxymethyl ester (Calcein-AM) (Diaper and Edwards, 1994). Carboxyfluorescein diacetate (CFDA), a derivative of FDA with enhanced retention characteristics (Haugland, 1999), has also been applied for the rapid enumeration of esterase active bacteria in water used in the manufacturing process of pharmaceutical products (Kawai et al., 1999) and in fresh water environments (Porter et al., 1995).

The aim of this study was to comprehensively evaluate a variety of techniques for the comparison between numbers of active and culturable bacteria in raw and potable waters, collected from various locations around South Australia. FCM was used for rapid enumeration of physiologically active bacteria by staining samples with the BacLight™ kit and CFDA. The direct viable count (DVC) (Joux and LeBaron, 1997), a recent adaptation to the DVC procedure known as quantitative DVC (qDVC) (Yokomaku et al., 2000) and SimPlate™ (Jackson et al., 2000) were also tested. Results were compared against the standard HPC technique. This study revealed a huge disparity between viability as defined by culture compared to assays that assess more specific indicators of physiological activity alone. Results also demonstrate the disparity between numbers of active and culturable bacteria is nonlinear between samples. The most feasible explanation for this nonlinearity is different proportions of bacteria adopting an ABNC state, in addition to other factors such as sublethal

injury or the presence of nonheterotrophic bacteria. The significance of these findings is discussed in relation to the on going use of HPC, and the potential for rapid FCM assays to give overall catchment or distribution system information with regard to bacteriological quality.

2. Materials and methods

2.1. Bacterial strains

Cultures used in this study included *Aeromonas hydrophila* (ATCC 7966), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 11775), *Pseudomonas aeruginosa* (ATCC 10145) and *Staphylococcus epidermidis* (ATCC 12228).

2.2. FCM

All experiments were performed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an air-cooled 15 mW argon ion laser, emitting at a fixed wavelength of 488 nm. Fluorescent filters and detectors were all standard with green fluorescence collected in the FL1 channel (530 ± 30 nm), orange fluorescence collected in the FL2 channel (585 ± 42 nm) and red fluorescence collected in the FL3 channel (>670 nm). All parameters were collected as logarithmic signals. Data were analysed using CellQuest™ software (Becton Dickinson). The addition of fluorescent microspheres (Fluoresbrite™ 0.95 μm diameter yellow-green fluorescent microspheres, Polysciences, Warrington, PA) to each sample was performed as a means of normalising cell fluorescence. For bacterial enumeration, where quantification of sample volume analysed was required, the outer sample injection port envelope was removed and samples were placed in 12×75 mm plastic tubes and weighed before and after analysis. This method was shown to be more accurate than the bead ratio method (data not shown).

2.3. Total count

Total numbers of bacteria (both active and inactive) in raw and potable waters were enumerated by staining with SYTO-9 (2.0 μM final concentration) for 15 min at room temperature in the dark followed by FCM detection.

2.4. BacLight™ kit

Cultured bacteria were used to assess the ability of FCM, combined with the BacLight™ kit, to distinguish between physiologically active cells in a mixture containing high numbers of inactive cells. *A. hydrophila*, *B. subtilis*, *E. coli*, *P. aeruginosa* and *S. epidermidis* were grown to exponential phase in Luria-Bertani (LB) broth (1% (w/v) tryptone; Oxoid, 0.5% (w/v) yeast extract; Oxoid, 0.5% NaCl, pH 7.0), each separated into two equal suspensions and centrifuged at $10{,}000 \times g$ (Sorvall® RC-5B Refrigerated Superspeed Centrifuge) for 15 min at 4 °C. Supernatant was aspirated and one pellet was resuspended in Milli-Q water (Millipore Ultra-Pure Water System) (this tube labelled 'live'). The other was resuspended in 70% ethanol and heat treated at 60 °C for 30 min (this tube labelled 'dead'). Dead cells, which were assessed for culturability by spread plating 0.1 ml onto R2A medium (Oxoid Australia, Heidelberg, Australia) and incubating at 35 °C for 72 h, showed no growth. Both suspensions were then centrifuged at $10{,}000 \times g$ for 15 min at 4 °C followed by resuspension in Milli-Q water. Bacterial concentrations of 'live' and 'dead' cells were adjusted to OD_{670} 0.03 in MilliQ water followed by a further 100-fold dilution. For each bacterial type, volumes of 'live' and 'dead' cell suspensions were mixed to achieve active concentrations ranging from 0 to 100% (all mixtures were 1 ml total). Staining was performed by combining equal volumes of SYTO-9 (3.34 mM) and PI (20 mM), dissolved in dimethyl sulfoxide (DMSO), prior to use and adding 1.0 μl of this mixture directly to 1.0 ml of the appropriate bacterial suspension. Incubation was performed for the predetermined optimal staining time of 15 min (data not shown) in the dark at room temperature.

Subsequent BacLight™ kit staining of raw and potable waters was performed on 1 ml samples as described above.

2.5. CFDA

Stock CFDA (Molecular Probes) was prepared at a concentration of 10 mM in anhydrous DMSO. The

optimal staining conditions for CFDA described by Hoefel et al. (2003) were used herein.

For enumeration of esterase active bacteria in raw and potable waters, 900 μl of sample was supplemented with 90 μl of sterile 1.0 M phosphate buffer (pH 8.0) and 10 μl of 50 mM EDTA. Staining was carried out by the addition of CFDA to the sample at a final concentration of 10 μM followed by incubation at 35 °C in the dark for 10 min. After this time, bacteria were enumerated directly by FCM and the dilution accounted for in the final calculation.

2.6. DVC

DVC was carried out as described by Joux and LeBaron (1997), with minor modifications, for enumeration of bacteria responsive to nutrient addition by assessing cell elongation. Optimisation of yeast extract concentration and incubation time was performed on two raw waters (A and B). Both raw waters were enriched with yeast extract (Oxoid) at concentrations of 50, 100, 500 and 1000 μg ml^{-1}. All samples were incubated statically at 35 °C in the presence of a cocktail of antibiotics containing nalidixic acid (20 μg ml^{-1}; ICN Biomedicals), piromidic acid (10 μg ml^{-1}; ICN Biomedicals), pipemidic acid (10 μg ml^{-1}; ICN Biomedicals), cephalexin (10 μg ml^{-1}; ICN Biomedicals) and ciprofloxacin (0.5 μg ml^{-1};ICN Biomedicals) for 0, 18 and 24 h in the dark. To test bacterial regrowth, both samples were also supplemented with yeast extract (50 μg ml^{-1}) but no antibiotics. Following incubation after the times mentioned above, cells were stained with 0.05% (v/v) SYBR Green II (Molecular Probes) for 15 min in the dark at room temperature. At each time interval, a total cell count and an elongated cell count (DVC-positive) was performed by EFM.

For subsequent environmental enumeration, samples were enriched with 100 μg ml^{-1} yeast extract, the cocktail of antibiotics (as above) and incubated at 35 °C for 24 h.

2.7. qDVC

The qDVC method was carried out as described by Yokomaku et al. (2000), with minor modifications. All samples were enriched with yeast extract (100 μg ml^{-1}) and a cocktail of antibiotics containing nalidixic acid (20 μg ml^{-1}), piromidic acid (10 μg ml^{-1}), pipemidic acid (10 μg ml^{-1}), cephalexin (10 μg ml^{-1}) and ciprofloxacin (0.5 μg ml^{-1}). In addition, glycine (2% [w/v]) was also added and samples were incubated at 35 °C for 24 h in the dark. During incubation, glycine interfered with cell wall peptidoglyan synthesis causing substrate responsive cells to form unstable spheroplasts that were lysed by freezing in liquid nitrogen for 1 min followed by thawing at room temperature. Remaining cells, unresponsive to substrate addition, were enumerated by staining with SYTO-9 (2.0 μM final concentration) in the dark at room temperature for 15 min followed by FCM enumeration. The number of physiologically active cells estimated by the qDVC assay was calculated using the equation below:

$$\mathrm{qDVC} = \mathrm{qDVC_o} - \mathrm{qDVC}_t$$

where $\mathrm{qDVC_o}$ represents the number of cells before incubation and qDVC_t represents the number of cells remaining after incubation and freeze thaw treatment. For the above equation to be applied, qDVC_t must be significantly less than $\mathrm{qDVC_o}$. To test this statistically, all data was log-transformed and the *F*-test applied to assess variance equivalence between the two sets of data. The one tailed *t*-test was then applied to determine if significantly fewer cells remained at qDVC_t. If this was true, the above calculation was performed. However, if qDVC_t was not significantly less than $\mathrm{qDVC_o}$, the result was deemed zero.

2.8. HPC

HPC were performed in accordance with the Australian Standard (Australian Standard, 1995) with minor modifications. Two media were used, R2A (Oxoid) and tryptone soya agar (TSA; Oxoid). Both media were shown to give superior recoveries to plate count agar and nutrient agar (data not shown). Dilutions, when necessary, were performed in maximum recovery buffer (0.1% (w/v) neutralised bacteriological peptone, 0.85% (w/v) NaCl, pH 7.0). Incubation was performed using standard conditions of 35 °C for 48 h, 20 °C for 72 h or 20 °C for 72 h followed by a subsequent 35 °C for 48 h. Spread plates, performed

Table 1
Sample types and chlorine residuals[a]

Sample	Sample type	Chlorine residual (mg l^{-1})
Raw 1	reservoir	NA
Raw 2	reservoir	NA
Raw 3	reservoir	NA
Raw 4	lake	NA
Raw 5	lake	NA
Raw 6	marine	NA
Raw 7	WFP raw water	NA
Potable 1	WFP treated water	3.8
Potable 2	WFP treated water	ND
Potable 3	WFP treated water	3.1
Potable 4	town supply	0.6
Potable 5	town supply	0.8
Potable 6	tap water	0.9
Potable 7	tap water	0.5

WFP, water filtration plant; NA, not applicable; ND, not determined.
[a] No samples are from the same location.

on R2A medium using 0.1 ml of sample (or appropriate dilution) spread over the surface of the solid medium and incubated at 35 °C for 48 h, showed no significant difference to the associated HPC using R2A (data not shown).

2.9. SimPlate™

SimPlate™ for HPC (IDEXX Laboratories, Westbrook, ME) was purchased in unit-dose form. Samples were processed in accordance with the manufacturer's specifications. Briefly, 10 ml of sample (or appropriate dilution) was added to a dehydrated medium tube and dissolved by shaking. The contents were poured onto the centre of the plate base and the plate covered. To distribute the sample into the wells, the plate was swirled gently and then tapped lightly to dislodge any air bubbles. Excess sample was drained into the absorbent pad by tipping the plate 90–120° and the plate inverted for incubation at 35 °C for 48 h. After that time, the number of positive wells was determined by fluorescence emitted upon illumination with long wavelength UV light (365 nm), indicating that microbial enzymes had hydrolysed the substrate medium causing the release of 4-methylumbelliferone.

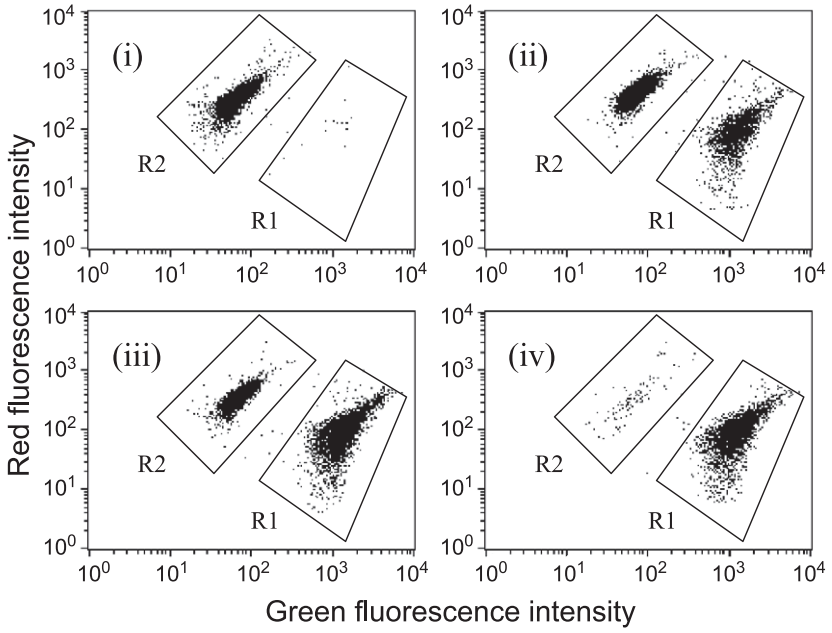

Fig. 1. FCM dot plot profiles for mixtures of *E. coli* cells at (i) 0%, (ii) 30%, (iii) 60% and (iv) 100% viability after staining with the BacLight™ kit. R1 defines the active region and R2 defines the inactive region.

The most probable number (MPN) was obtained using the MPN table provided.

2.10. Raw and potable waters

A total of seven raw waters and seven potable (treated) waters were collected from various locations around South Australia and are listed in Table 1. Residual chlorine in potable waters was neutralised by the addition of 100 mg l^{-1} sodium thiosulphate upon sampling (Australian Standard, 2001) and samples stored at 4 °C until analysis.

2.11. Statistical analysis

When required to determine whether or not there was a significant difference between series of data, *t*-test analyses were performed on logarithmically transformed data.

3. Results

3.1. BacLight™ kit

The BacLight™ kit was assessed for detection of active cells in the presence of inactive cells by FCM (e.g. *E. coli*; Fig. 1). For all bacteria tested, there was clear separation and strong association between the percentage of active cells added and the percentage measured by FCM (*A. hydrophila*: $y = 0.91x + 5.8$, $R^2 = 0.98$; *B. subtilis*: $y = 0.94x + 1.23$, $R^2 = 0.99$; *E. coli*: $y = 0.96x + 2.47$, $R^2 = 0.99$; *P. aeruginosa*: $y = 0.91x + 4.37$, $R^2 = 0.99$; *S. epidermidis*: $y = 0.95x + 2.67$, $R^2 = 0.99$). In some instances, a small percentage of the cells in the supposed 100% physiologically active suspension showed permeability to PI although this did not significantly affect correlation.

Separation between active and inactive bacteria in raw and potable waters was not as pronounced (for an example, see Fig. 2). In some instances, there was a range of cells with an intermediate capacity for PI uptake.

3.2. DVC

Optimisation of yeast extract concentration and cell incubation time was performed on two raw waters.

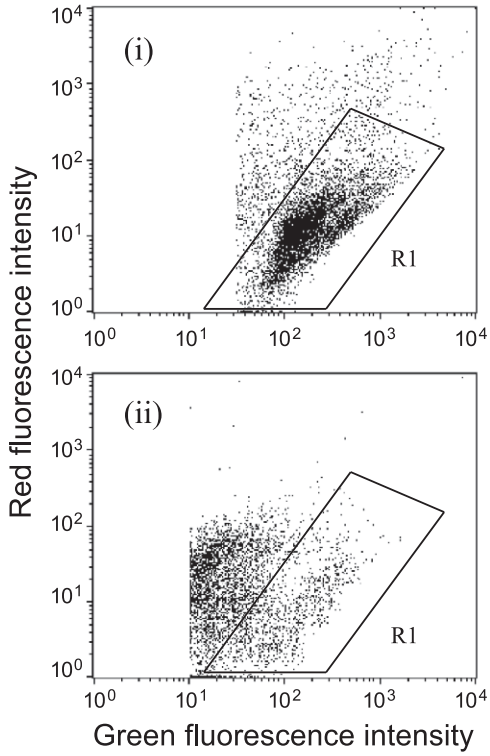

Fig. 2. FCM analysis of environmental samples: (i) Raw 7 and (ii) Potable 1 after being stained with the BacLight™ kit. R1 defines the active region.

Sample A, in the presence of antibiotics, had no significant increase in cell number after 24 h ($P > 0.05$; Fig. 3). However, significant growth was detected in the non-antibiotic control after 18 h ($P < 0.002$). There were significantly more elongated cells (DVC-positive) detected after the 24 h incubation compared with 18 h incubation for all concentrations of yeast extract ($P < 0.05$), although no significant difference between the number of DVC-positive cells for the different yeast extract concentrations tested existed.

Sample B returned similar results to sample A with no growth detected in the samples after 24 h in the presence of the antibiotics, whereas significant growth was detected in the non-antibiotic control after this time ($P < 0.002$; Fig. 3). There were significantly more DVC-positive cells after 24 h compared with 18 h for yeast extract concentrations of 50 and 100 μg ml^{-1} ($P < 0.05$); however, there was

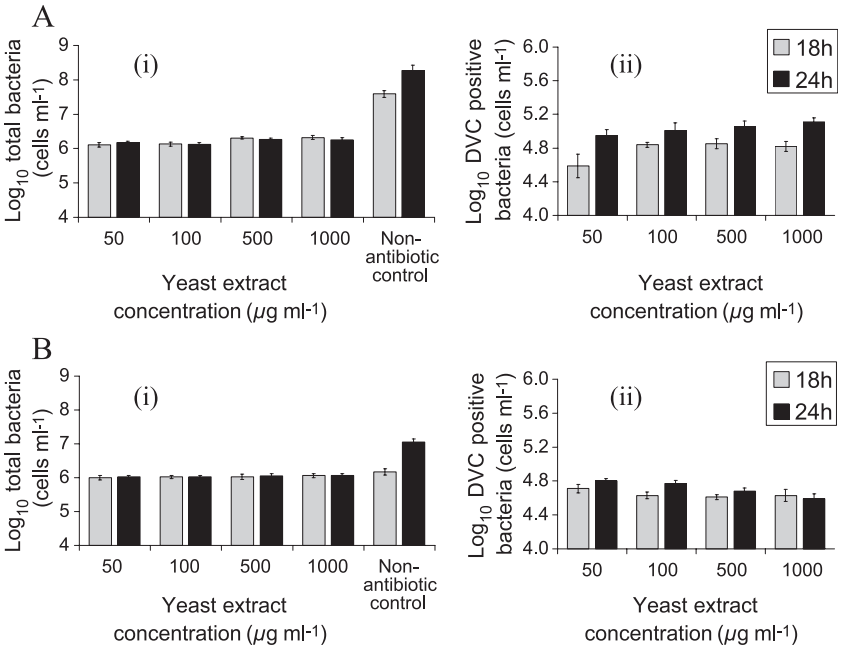

Fig. 3. Response of two raw waters (A and B) to the addition of yeast extract on (i) total bacterial counts, and (ii) DVC-positive counts, after 18 and 24 h. Initial $\log_{10}$ total counts were 6.09 and 5.97 for samples A and B respectively. Data are averages of five replicates. Error bars represent standard deviations.

no significant difference for yeast extract concentrations of 500 and 1000 μg ml^{-1} ($P > 0.05$). Based on this information, a yeast extract concentration of 100 μg ml^{-1} and an incubation time of 24 h at 35 °C were deemed optimal. These parameters were used also for qDVC.

3.3. Bacterial enumeration from raw and potable waters

A range of water samples were analysed for numbers of total bacteria (SYTO-9 staining and FCM analysis), those maintaining membrane integrity (BacLight™ kit staining and FCM analysis), active intracellular esterase activity (CFDA staining and FCM analysis), response to substrate addition (DVC with EFM analysis and qDVC with FCM analysis), and culturable on solid media (R2A and TSA pour plates at various temperatures and SimPlate™). Results of these analyses are shown in Figs. 4 and 5.

For the raw waters tested, both the BacLight™ kit and the CFDA assay gave similar results (with four out of the seven samples correlating statistically, $P > 0.05$). A larger variation between the two tests was observed with the potable samples ($P < 0.05$). The number of physiologically active bacteria enumerated from the raw waters by these two techniques was relatively high compared to the total count (BacLight™ kit 63.5–94.6% and CFDA 36.4–92.9%). For potable waters, due to the water treatment process, there were significantly fewer numbers of active bacteria compared to the total count (BacLight™ kit 0.08–5.0% and CFDA 0.2–3.6%).

Assessing substrate responsiveness by DVC for raw waters gave significantly fewer numbers of active counts compared to the BacLight™ kit and the CFDA assay ($P < 0.002$), generally in the order of 1 log. However, DVC counts, for raw waters, were significantly higher compared to HPCs ($P < 0.002$). No DVC-positive bacteria were detected in any potable waters, whereas the BacLight™ kit and CFDA assays indicated positive results ranging from anywhere between 5.56×10^2 and 3.94×10^4 active bacteria ml^{-1}.

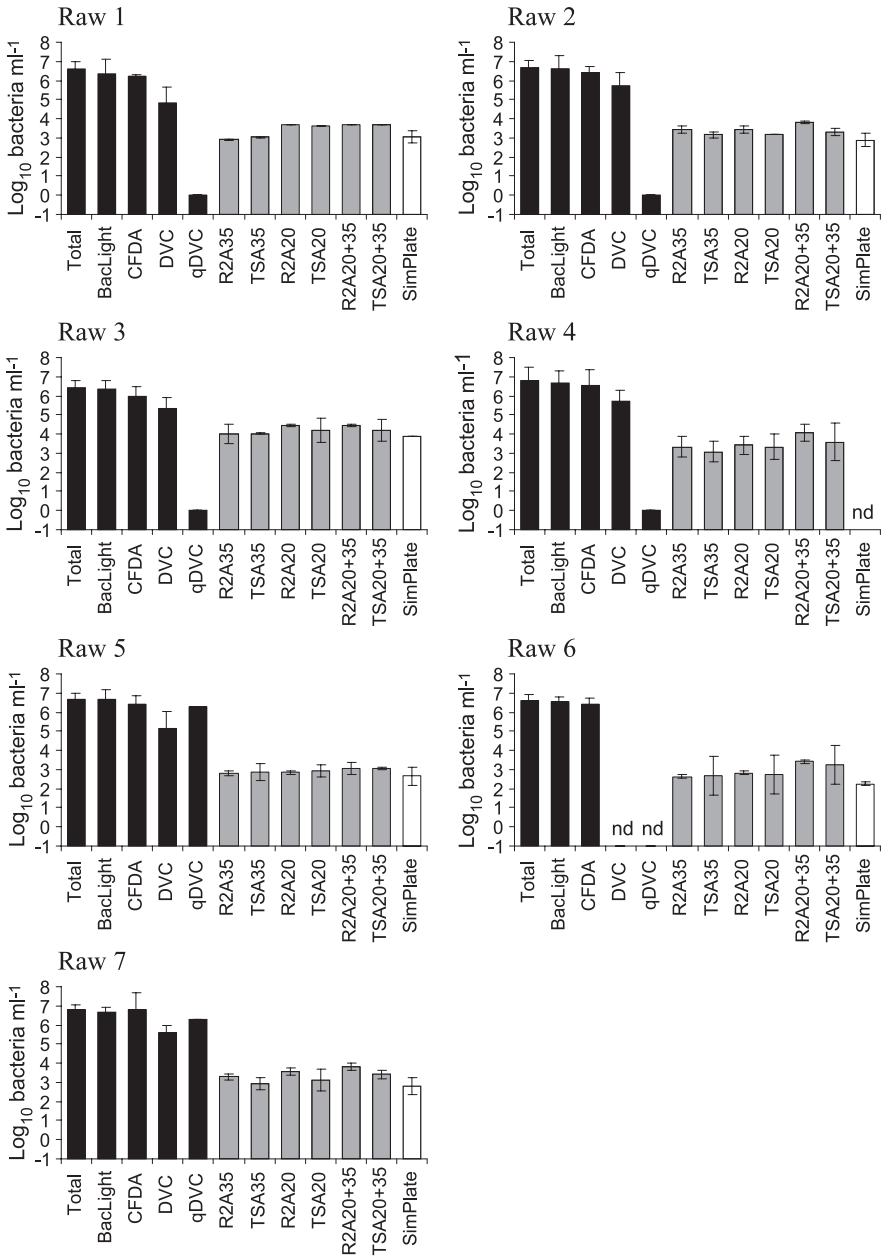

Fig. 4. Graphical representation of bacterial analysis in raw waters. Results are reported as $\log_{10}$ bacteria ml^{-1}. Black columns represent physiological activity assays. Grey columns represent culture assays. White columns represent SimPlate MPN technique. Error bars represent standard deviations of four replicates for activity assays and three replicates for plate counts. ND, not determined.

In raw waters, when there were statistically fewer bacteria remaining after qDVC incubation and freeze thawing ($qDVC_t$) compared to before ($qDVC_o$), qDVC counts were higher than those reported by the DVC procedure ($P < 0.01$), but for most samples, a zero result was reported for qDVC. Potable water

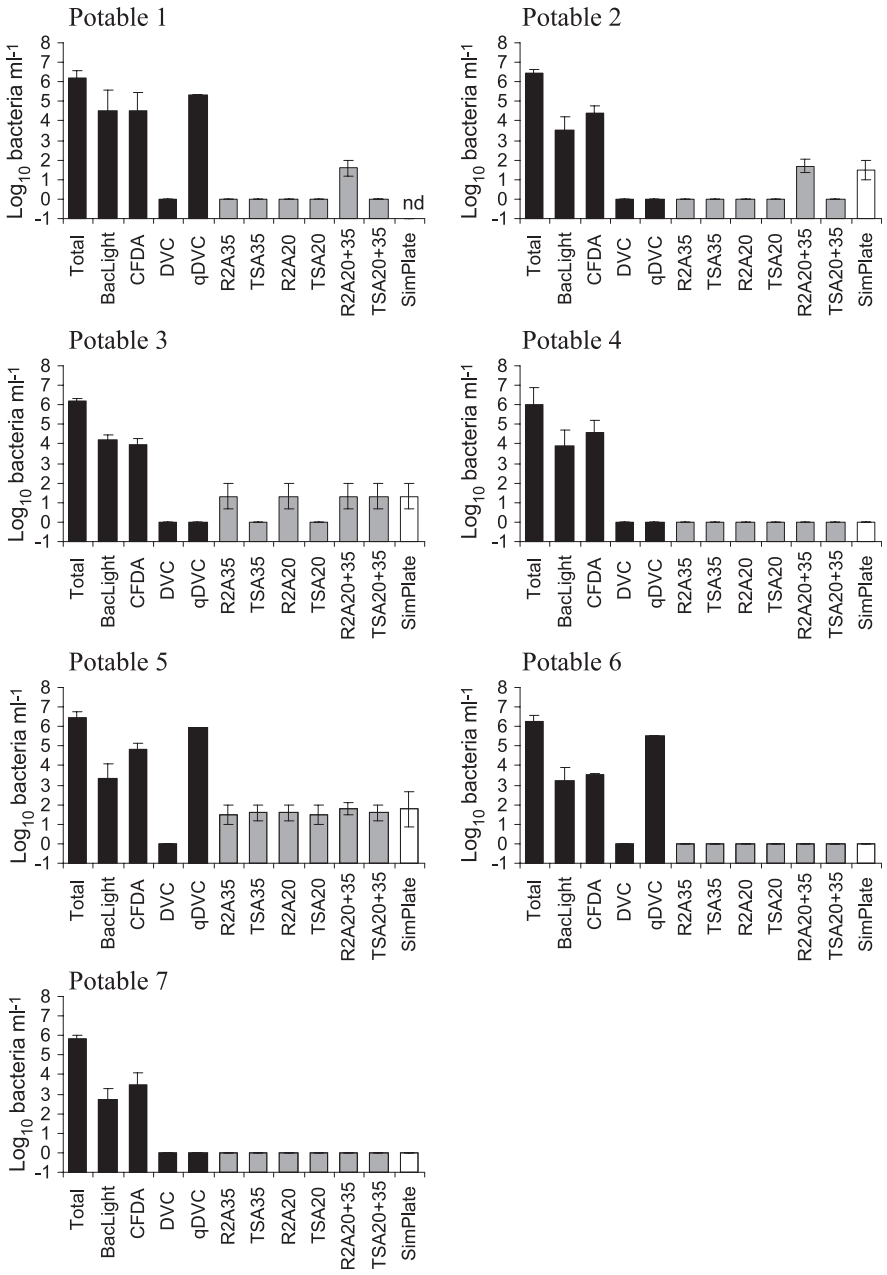

Fig. 5. Graphical representation of bacterial analysis in potable waters. Results are reported as $\log_{10}$ bacteria ml^{-1}. Black columns represent physiological activity assays. Grey columns represent culture assays. White columns represent SimPlate MPN technique. Error bars represent standard deviations of four replicates for activity assays and three replicates for plate counts. ND, not determined.

analysis revealed qDVC results of either zero or significantly greater than BacLight™ or CFDA assays (to be discussed).

HPCs for raw waters never exceeded values above 0.40%, 1.15% and 1.20% of the total bacteria present for incubation conditions of 35 °C for 48 h, 20 °C for

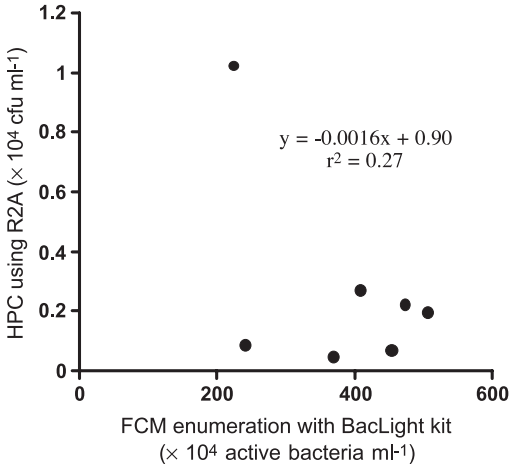

Fig. 6. Graphical representation of the nonlinearity between the number of physiologically active bacteria, assessed using the BacLight™ kit, and the number of culturable bacteria, using R2A medium incubated at 35 °C for 48 h, in raw waters.

72 h or 20 °C for 72 h followed by subsequent 35 °C for 48 h, respectively. In addition, HPCs were generally between 2 and 4 log fold less than the active numbers reported by the BacLight™ kit and CFDA assays. This difference between numbers of physiologically active and culturable bacteria appeared to be nonlinear across different samples, as illustrated in Fig. 6.

For potable waters, HPCs for all incubation conditions used never exceeded 0.002% of the total bacterial number present, where in most cases no colonies were observed on plates of undiluted samples. This was observed even though BacLight™ kit and CFDA assay reported between 5.56×10^2 and 3.94×10^4 active bacteria ml^{-1}.

Evaluation of the SimPlate™ method against R2A medium incubated at 35 °C for 48 h using potable waters showed that all six samples tested with SimPlate™ were not significantly different to R2A results ($P > 0.05$). For raw waters, four out of six samples tested by SimPlate™ had no significant difference to R2A results ($P > 0.05$).

4. Discussion

Changes in microbiological water quality have direct impacts on the decisions made by water authorities striving to maintain safe conditions in catchments or distribution systems and correct decision making relies heavily on having access to rapid and accurate bacteriological data. Technological advances in FCM and fluorescent probes now offer a realistic approach for direct bacterial activity assessment in waters (Porter et al., 1997; Vives-Rego et al., 2000) returning bacteriological data rapidly and with high accuracy. Here we report a comprehensive study of raw and potable waters, comparing various techniques used to evaluate the physiological activity of bacteria. From these data, we have demonstrated the absence of a consistent proportionality or linear relationship between numbers of active and culturable bacteria in these water types.

Assessing the active process of maintaining membrane integrity or the presence of active intracellular esterases was comparable for determining numbers of physiologically active bacteria in raw and potable waters overall. As a percentage of the total bacteria present, there were significantly fewer of these physiologically active bacteria in potable waters compared to raw waters. This is most likely due to the effects of chlorination on the water supply as highlighted in disinfection studies by Lisle et al. (1999) and Boulos et al. (1999) who report decreases in active counts of *E. coli* O157:H7 and *Citrobacter freundii*, respectively, as measured with the BacLight™ kit.

For raw and potable waters tested, DVC counts were significantly less than FCM active counts, where no DVC-positive cells were detected in any potable waters. However, active cells were detected by the BacLight™ kit and CFDA assays. This suggests that although chlorination of a water supply may not totally diminish membrane integrity or intracellular esterase activity, it appears to be successful at disrupting the mechanisms required for uptake and utilisation of substrates leading to cell division. However, evidence to the contrary exists because several colonies were seen on the plate counts of those potable water samples that returned a zero result for DVC. This indicates that the DVC method did not account for some substrate responsive cells, most probably due to limitations in the number of cells a microscope operator can count compared to the number of bacteria present in 1 ml of cell suspension used for HPC.

For raw waters in general, DVC counts were significantly greater than associated HPCs. This ob-

servation is consistent with findings by Joux and LeBaron (1997) and may be expected, as bacteria previously exposed to environmental stresses may only have the ability to divide a limited number of times, giving a positive DVC result, but are unable to produce visible colonies on solid media. However, contradictory results have been published with DVC counts less than plate counts in raw waters (Yokomaku et al., 2000). This discrepancy may be due to difficulties in microscopic assessment of elongation associated with a positive DVC cell and may be overcome using automated image analysis techniques with pre-programmed cell size measurements. Alternatively, a new method developed by Yokomaku et al. (2000), known as qDVC may be used, eliminating the need for detection of cell elongation associated with the DVC assay. In this study, when there were significantly fewer bacteria after qDVC incubation and freeze thawing compared to before, qDVC resulted in higher active counts than the DVC procedure, in agreement with Yokomaku et al. (2000). However, in many instances, there was no statistical difference between the number of bacterial cells remaining after freeze thaw treatment compared to before. This is expected if the result is truly zero, but occurred in many raw waters when DVC-positive results were in excess of 10^4 cells ml^{-1}. As Yokomaku et al. (2000) suggest, different ecosystems have different bacteria present and it may be that the addition of glycine for these particular indigenous environmental bacteria was not successful in interfering with peptidoglycan synthesis for cell wall formation. For potable waters, when there was a statistical difference between cell numbers after freeze thawing compared to before, qDVC counts were significantly higher than BacLight™ kit and the CFDA assay. This occurred even when DVC results and plate counts returned a zero result. These results suggest that the qDVC assay is not suited for potable waters. We hypothesise that cell lysis may occur, in some samples, upon freeze thawing due to the detrimental effect that extended exposure to chlorine has on bacterial cell membranes rather than due to the formation of unstable spheroplasts by glycine, hence giving false-positive results.

Previous reports have shown the ratio of colony forming units, measured by HPC, to total bacteria, represent less than 2% in river waters (Yamaguchi and Nasu, 1997) and less than 1% in seawaters (Bernard et al., 2001). Results of this study agree with these observations. In addition, we found that HPC counts were generally 2–4 log fold less than the numbers of active bacteria reported by the BacLight™ kit and CFDA assays. This level of difference has also been seen previously in raw waters comparing the CFDA assay and HPC (Porter et al., 1995; Yamaguchi and Nasu, 1997).

Due to the effects of disinfection on potable waters, generally no culturable bacteria were detected by HPC, even though physiologically active bacteria were still reported by BacLight™ and CFDA assays. A similar study by Reynolds et al. (1997), who used the esterase substrate ChemChrome B (as measured using a ChemScan) for bacterial enumeration on potable waters, reported that the assay gave in the order of 100 fold greater counts of active bacteria compared to the number culturable on R2A medium. As Reynolds et al. (1997) state, it is unclear whether the observed difference is due to bacteria that have adopted an ABNC state or simply an intrinsic inability to grow on the solid medium under the specific incubation conditions being used. Induction of an ABNC state may be due to environmental stresses, such as starvation and temperature extremes, expected in raw waters. However, in addition to these factors, bacteria in potable waters are exposed to chlorine, which may also induce an ABNC state, or result in sublethal injury thereby rendering the bacteria nonculturable.

In general, the performance of SimPlate™ was equal to that of HPC, using R2A agar with an incubation period of 35 °C for 48 h as a reference. Results here suggest that SimPlate™ may potentially be a suitable replacement for the HPC in raw and potable waters. SimPlate™ has advantages over HPCs in that medium preparation or sterilisation is not required. In addition, counting of positive fluorescent wells is simple and less time-consuming than colony counting on standard HPC media (Jackson et al., 2000). Although it may be a suitable replacement, SimPlate™ has similar problems to HPC in that a time-consuming incubation period is required, compared with the rapid assays used in this study, and bacteria that require more complex techniques for culture are unable to be detected. Such bacteria include those in the beta-subclass *Proteobacteria* that

are able to oxidise ammonia (Rotthauwe et al., 1997), causing a loss of disinfection residual if established in chloraminated water systems. Detection of such bacteria requires complex laboratory techniques and a minimum of three weeks incubation (Matulewich et al., 1975). In contrast, use of rapid fluorescent assays would allow the identification of significant numbers of these microorganisms quickly so that remedial action can be performed in a timely fashion.

Even though HPC results in this study reported significantly fewer culturable bacteria compared to the rapid assays that assess for physiological activity (generally between 2 and 4 log fold), the difference appeared to be nonlinear across samples (Fig. 6). If a linear (proportional) correlation existed, regression analysis would identify the data as having an r^2 value not significantly different to 1. However, the slope of the trend line would be significantly less than 1 as HPC consistently reported fewer numbers than the rapid assays. If it had been shown that HPC was linearly consistent in reporting fewer culturable bacteria compared to the rapid assays, a culturable count could have been correlated to the number of physiologically active bacteria by estimation via a standard curve against the rapid assay results. However, a linear correlation did not exist and may be due to different waters containing populations of bacteria varying in their ability to be detected by HPC. Reasons for this may include differences in the percentage of heterotrophic versus nonheterotrophic bacteria between samples, in addition to different proportions of sublethally injured or ABNC bacteria. Therefore, even though HPC (or SimPlate™) may be a suitable tool for monitoring changes in bacterial water quality over time for a particular catchment or distribution system, the results do not linearly correlate with the number of physiologically active bacteria present. If these data are required, rapid assays, such as the BacLight™ kit or CFDA with FCM detection, are better suited.

5. Conclusion

This study has comprehensively analysed bacteria in both raw and potable waters using various assays that target traits of physiological activity. HPC reported significantly fewer viable bacteria (as assessed by culturability) when compared to the number of active cells in these waters. It was also revealed that between samples there was no consistent proportionality comparing numbers of active and culturable bacteria, meaning water utilities cannot directly equate the number of active bacteria detected to the number that may be cultured by HPC. Rapid assays used here have the potential to replace time-consuming culture-based techniques for the bacteriological assessment of water especially reporting active counts of nuisance microorganisms that are difficult to culture, e.g. ammonia-oxidising bacteria. Such rapid assessment would allow appropriate management practices to be implemented sooner, resulting in operational cost savings.

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