Comparison of enumeration techniques for the investigation of bacterial pollution in the Berg River, Western Cape, South Africa

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Abstract

The study was aimed at assessing techniques, which would provide an accurate indication of the planktonic bacterial pollution load in the Berg River, Western Cape, South Africa. Sampling of sites started in June 2004 and continued for a period of 1 year until June 2005. The most probable number (MPN) technique was used to determine the level of faecal coliforms and *E. coli*, while the heterotrophic plate count method was used to determine the amount of culturable micro-organisms in planktonic samples. The flow cytometry (FCM) and direct acridine orange count (DAOC) (epifluorescence microscopy) techniques were employed to evaluate total bacterial counts in planktonic (water) samples. The highest MPN and heterotrophic plate counts were recorded in Week 37 at site B2 at 1.7 x 10^7 micro-organisms/100 mℓ and 1.04 x 10^6 micro-organisms/mℓ, respectively. In comparison, the viable FCM counts, were significantly higher ($p < 0.05$) for that period at 1.7 x 10^7 micro-organisms/mℓ. The highest total FCM count of 3.7 x 10^7 micro-organisms/mℓ was recorded in Week 41 at Site B2. In comparison the highest DAOC of 8.3 x 10^6 micro-organisms/mℓ was obtained in Week 29 at Site B2. Results showed that on average the heterotrophic plate count represented a fraction (< 3.65%) of the total FCM counts. The total DAOC count also represented a fraction (< 43.08%) of the total FCM count for most of the sampling period. Results therefore showed that the FCM proved to be more effective in evaluating microbial pollution in water samples.

Keywords: bacterial pollution, direct acridine orange count, flow cytometry, heterotrophic cell counts, planktonic organisms, river water

Introduction

Water scarcity is becoming a major problem in South Africa, as dams serving communities with drinking water and water for daily household use, have been less than 30% full in recent years (Department of Water Affairs and Forestry (DWAF), 2005). River water, in combination with groundwater, is considered a suitable alternative as a utilisable and potable water source. However, in South Africa, rivers are steadily becoming more contaminated and in some cases even toxic, due in large part, to urbanisation. Communities located in rural and some semi-urban areas lack adequate domestic water supplies and wastewater treatment facilities. In many instances sullage and excreta from these informal settlements are discharged into stormwater drainage pipes, which directly flow into nearby rivers. The low rainfall and increasingly high temperatures then leads to the proliferation of micro-organisms which in turn leads to a significant increase in waterborne diseases (Gerba, 1996; WHO-UNICEF, 1999). The contaminated river water also affects the farms downstream from the pollution source that utilises the water as a means of irrigation.

The Plankenbrug River (Stellenbosch) and Berg River (Paarl) are two rivers in the Cape Metropolitan-Boland area that are regarded as highly polluted. Informal settlements inhabit their banks, and stormwater drainage pipes from these settlements flow directly into these rivers (Barnes, 2003). Previous studies have recorded an *E. coli* count as high as 2.44 x 10^9 CFU per 100 mℓ water in the Berg River at an Mbekweni stormwater drainage pipe during the 2003 summer season (Barnes, 2003). Raw sewage spills from sewer pump stations in Wellington (near Paarl), overstressed sewer mains in the Paarl area. and stormwater effluent from informal settlements in the Paarl and Wellington areas were identified as possible sources of the pollution. These microbial counts significantly ($p < 0.05$) exceed the stipulated water quality guidelines indicating that these rivers, which serve as recreational and irrigational water sources, need to be monitored on a regular basis.

The most probable number (MPN) technique is routinely used to determine levels of all gas-producing contaminants in river water, which include the faecal coliforms and *E. coli*. However, this technique does not determine or indicate the level of other culturable micro-organisms that might be present in the water. For this purpose a conventional heterotrophic plate-count technique is performed. There are, however, two fundamental problems which inhibit the effectiveness of this quantitative culturing technique, i.e. the culturability of the samples on which the technique relies, as well as the selectivity of the medium on which bacteria are cultivated (Ward et al., 1992; Amann et al., 1995). Therefore, to base viability solely on the plate count method would not be sufficient proof of the total cell count. Furthermore, certain organisms such as *Vibrio cholerae* and *E. coli* have the ability to enter a non-culturable state in response to adverse environmental conditions (Xu et al., 1982; Colwell et al., 1985).

Flow cytometry and epifluorescence microscopy are techniques widely used not only to determine total cell counts, but also the ratios of live (viable) cells to permeabilised (injured) and dead cells (Hiraoka and Kimbara, 2002). Epifluorescence microscopy, in conjunction with specific fluorochromes, has

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become one of the standard techniques that are currently used to estimate the abundance, biomass, biovolume, size and physiological activity of bacteria obtained from aquatic bodies (Hobbie et al., 1977; Kepner and Pratt, 1994; Lisle et al., 1999; McFeters et al., 1999). Even though this enumeration technique is fairly simple and easy to perform, care should be taken as certain factors such as the presence of nonbacterial biomass or debris, if not eliminated or minimised, may influence cell counts and hence affect the reliability of resulting data.

Flow cytometry could be regarded as a more reliable approach for the enumeration of microorganisms. LIVE/DEAD BacLight™ Bacterial Kits (Molecular Probes, 1995) are used in flow cytometric analysis to investigate the microbiological effects of the two fluorochromes, SYTO 9 and propidium iodide. These two fluorochromes are nucleic acid-binding stains and can be applied to estimate both viable and total bacterial counts in water samples (Boulos et al., 1999). An appropriate mixture of the two fluorochromes will stain bacterial cells with intact membranes green (SYTO 9) and those with damaged membranes a fluorescent red (propidium iodide) (Braga et al., 2003).

The aim of this study was to assess enumeration techniques, which would provide an accurate indication or estimation of the planktonic bacterial pollution load in the Berg River, Western Cape, South Africa. The MPN technique was used to determine the level of faecal contamination in river water. The conventional plate count technique was used to determine heterotrophic counts in the water samples. In addition, all water samples were subjected to flow cytometric analysis and the direct acridine orange count technique using epifluorescence microscopy in order to obtain total cell counts, i.e. the culturable and non-culturable population.

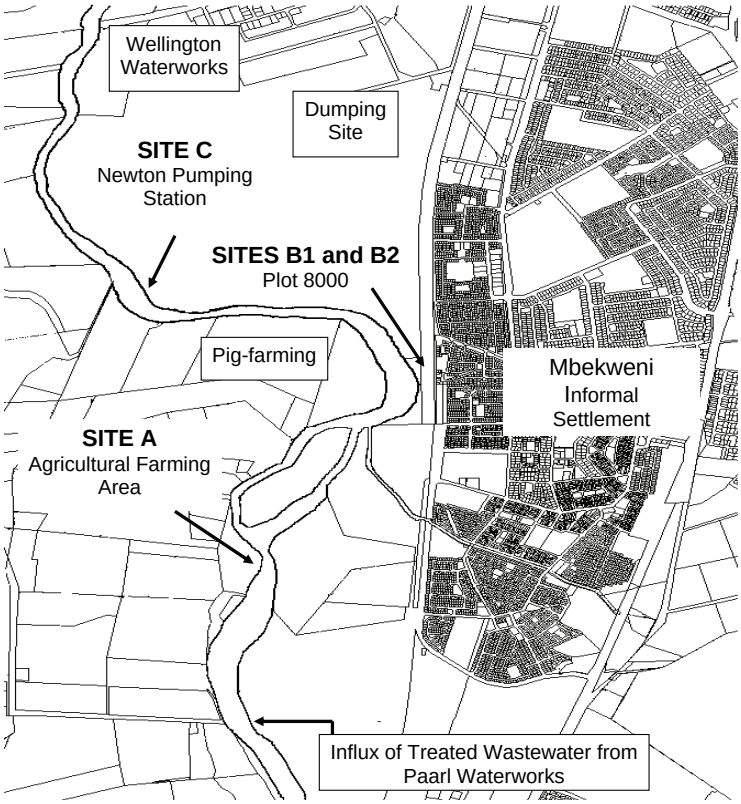

Figure 1
Map of the Berg River indicating the different sampling points: Site A - agricultural farming area; Sites B1 and B2 (Plot 8000) - close to the informal settlement of Mbekweni and Site C - the Newton pumping station.

Materials and methods

Sampling sites

Sampling sites at the Berg River location are indicated in Fig. 1 and included Site A (agricultural farming area); Site B (informal settlement of Mbekweni - sites B1 and B2) and, Site C (Newton pumping station). Site B2 is known as Plot 8000 and is the site where stormwater drainage pipes from the informal settlement flow directly into the river. The Newton pumping station services the residential area of Newton as well as certain sections of Mbekweni. Sampling of sites started in June 2004 and continued for a period of 1 year until June 2005. The temperature and pH of the river water were measured using a hand-held mercury thermometer and portable pH meter (Hanna Instruments), respectively. Water samples were collected in 1 ℓ sterile Nalgene-polypropylene bottles and stored on ice to maintain a low temperature.

Most probable number (MPN) and heterotrophic plate counts

The MPN technique was modified by Barnes (2003) and involved the inoculation of sample water into lauryl tryptose tubes containing Durham tubes, followed by incubation for 48 h at 37ºC (indicating all gas-producing organisms). All tubes indicating gas formation were regarded as positive presumptive tests and the presumptive coliform count was read off De Mans tables (American Public Health Association, 1992; 1995). These positive tubes were re-inoculated into brilliant green bile broth and tryptose water tubes respectively, according to the guidelines set out by the South African Bureau of Standards (SABS, 1984). These guidelines also incorporate the standard methods set out by the American Public Health Association, American Water Works Association and the Water Environment Federation (American Society for Microbiology, 1997). Positive tubes were incubated in a 44.5ºC water-bath for 24 h. Gas production in the brilliant green tubes (indicating faecal coliforms) was compared to growth in the tryptose water tubes (indicating *E. coli*). The presence of *E. coli* was confirmed with a colour change from clear to pink or red after the addition of Ehrlich's reagent into the tryptose water tubes.

Total heterotrophic counts were done in triplicate on nutrient agar (NA) (Merck, Biolab Diagnostics) plates after serial dilutions (10^{-1} to 10^{-7}) of sample water were performed. Plates were incubated for 3 to 4 d at 37ºC. Thereafter, the number of visible cells [colony-forming units (CFUs)] were counted and recorded.

Flow cytometry (FCM)

For the flow cytometry-based assay, individual samples were subjected to a Becton Dickinson FACSCalibur flow cytometer for analysis. The Becton Dickinson FACSCalibur flow cytometer has a 15 mW, 488 nm argon-ion laser. A doublet discrimination module, which uses pulse width and area to eliminate cell clumping (doublets and triplets), in conjunction with a LIVE/

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DEAD™ bacterial stain, allows for the differentiation between bacterial cells and debris. Flow cytometry therefore, employs the principles of light scattering, light excitation and emission of fluorochrome molecules to generate data from particles or cells in the size range of 0.5 µm to 40 µm in diameter (Current Protocols in Cytometry, 2005). The addition of fluorescent beads enables the calculation of absolute or total cell counts in samples. The absolute number (cells/µℓ) of positive cells in a sample can be determined by comparing cellular events to the bead events measured by the flow cytometer. For this study, the bacterial population was identified and gated on a forward scatter (FSC) vs. a side scatter (SSC) dot plot and a SSC vs. fluorescence channel 2 (FL-2) at 585/42 nm dot plot. The bead count was identified and gated on a SSC vs. fluorescence channel (FL-1) dot plot. All parameters were measured using a logarithmic amplification scale. A threshold of 52 FSC channels was set to remove sample debris. Only bacterial cells satisfying both gates were collected for subsequent analysis.

Depending on the amount of debris present, certain samples were filtered through a 0.45 µm filter before analysis. The staining procedure was performed by combining equal volumes of PI (propidium iodide) (4 µℓ) and SYTO 9 (4 µℓ) in BacLight™, dissolved in 1 mℓ sterile distilled H_2O. The stained samples (1 mℓ sample stained with 200 µℓ BacLight™) were kept in the dark for 15 min , after which 50 µℓ liquid counting beads (BD™ Cell Viability Kit, BD™ Liquid Counting Beads) were added. The samples were then subjected to the flow cytometer for analysis and the concentrations of total cell populations were determined (Eq. (1)). In order to avoid excessive compensation of fluorescence overlap, SYTO 9 green emittance fluorescence was measured in fluorescence Channel 1 (FL-1) at 530/30 nm and the PI was measured in fluorescence Channel 3 (FL-3) at 670/LP nm. As previously mentioned, the addition of beads allows for the calculation of total cell counts (i.e. viable plus dead cells) in samples. After optimisation, each water sample was subjected to the flow cytometer until a total of 250 counting bead events were detected. An *E. coli* laboratory strain was used as control.

$$\frac{\text{Number of events in cell region}}{\text{Number of events in bead region}} \times \frac{\text{Number of beads/test}}{\text{test volume}} \times \text{dilution factor} \quad (1)$$

[Bead concentration recorded at 988/µℓ (value found on the vial of BD liquid counting beads obtained from BD™)]

Direct acridine orange count (DAOC)

The total number of micro-organisms in the water samples were measured by means of epifluorescence microscopy, with acridine orange (Sigma) as the fluorochrome. Samples (2 mℓ) were filtered through Millipore membrane filters with a pore size of 0.22 µm. Cells captured on the filter were stained with 2 mℓ acridine orange (160 mg/ℓ) for 5 min. Total cell counts were obtained using a Zeiss Epifluorescent microscope (100x magnification). A minimum of 5 different fields was enumerated for all the water samples, for each respective sampling time.

Statistical analysis

Repeated Measures Anovas (RMAs) were performed on all data obtained as outlined in Dunn and Clark (1987), using Statistica™. In each RMA the residuals were analysed to determine if they were normally distributed. In all hypothesis tests a significance level of 5% was used as standard.

Note: Unit clarification

The MPN results are expressed in micro-organisms per 100 mℓ, whereas the CFU, FCM and DAOC results are expressed in micro-organisms per mℓ.

Results and discussion

Most probable number (MPN)

The levels of planktonic organisms associated with faecal pollution were evaluated by performing the MPN method (Fig. 2). The MPN method distinguishes between the total number of possible gas-producing organisms, which includes faecal coliforms [(FC) indicates all indicator organisms] and *E. coli*-organisms within water samples (Oblinger and Koburger, 1975). On average the total MPN counts ranged from 1.6 x 10^3 micro-organisms/100 mℓ recorded at site A in the first week of sampling, to 3.5 x 10^7 micro-organisms/100 mℓ observed at

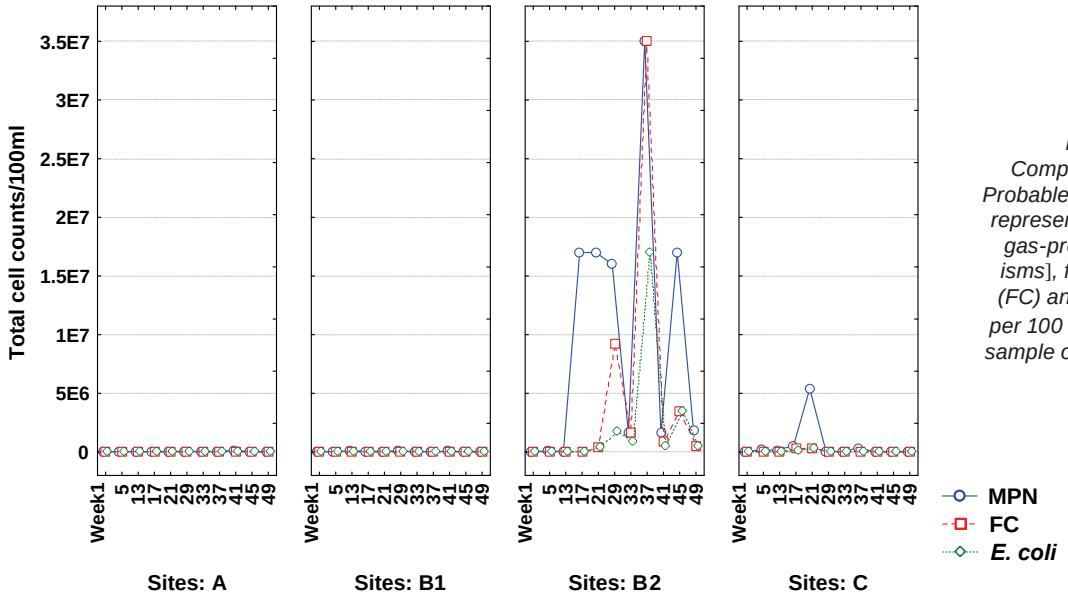

Figure 2
Comparison of Most Probable Number [(MPN), representing all possible gas-producing organisms], faecal coliforms (FC) and E. coli *counts per 100 mℓ of river water sample over the sampling period*

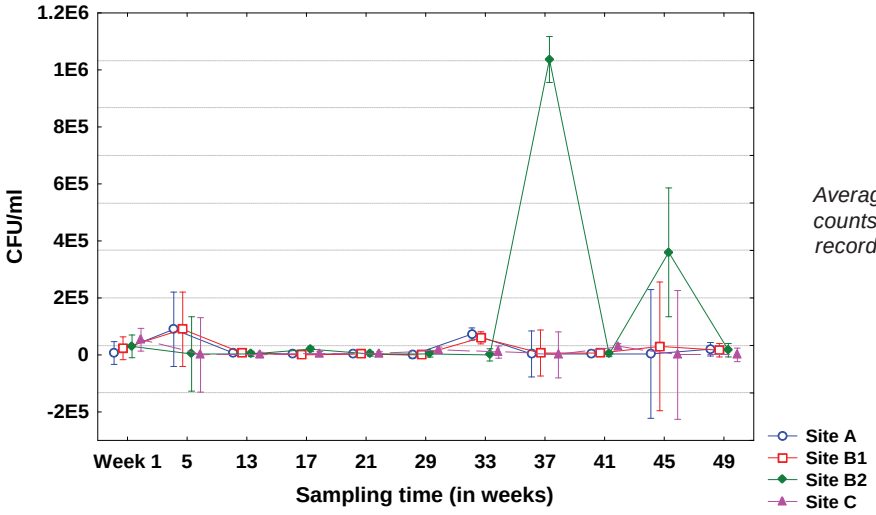

Figure 3
Average heterotrophic plate counts for all sites analysed recorded over the sampling period.

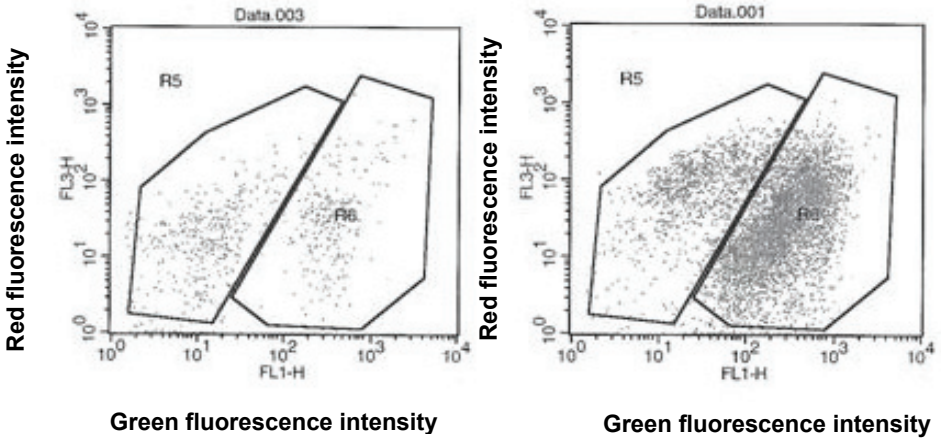

Figure 4
Flow cytometric analyses of river water samples: (a) total cell counts from a planktonic sample at site B1 in week 1 of sampling; (b) E.coli used as control, after staining with the BacLight™ probe. R5 and R6 indicate the dead and live populations, respectively.

Site B2 in Week 37 of the sampling period. In comparison, the lowest faecal coliform count of 1.7 x 10^2 micro-organisms/100 mℓ was recorded in Week 17 at Site A, whereas the highest FC count of 3.5 x 10^7 micro-organisms/100 mℓ water was observed at site B2 in Week 37. Corresponding *E. coli* counts ranged from 0.36 x 10^2 micro-organisms/100 mℓ in Week 1, to 1.7 x 10^7 micro-organisms/100 mℓ in Week 37, both recorded at Site B2. These results are significantly ($p < 0.05$) higher than the maximum limit of 2 000 organisms/100 mℓ set for planktonic organisms in river water by the SABS Guidelines (1984). Furthermore, during the one-year sampling period, the *E. coli* counts fell within the accepted range less than 13 times for all samples (i.e. A, B1, B2 and C) analysed. It should also be noted that in two distinct cases during the sampling period, namely Week 1 at Sites A and B1, no *E. coli* counts were detected. Results clearly show that increases in microbial activity was experienced at Site B2 for most of the sampling period, with the highest MPN, FC and *E. coli* counts measured at this site.

Total heterotrophic plate counts

Results obtained for the average heterotrophic plate counts for the planktonic samples analysed at the respective sites in the Berg River are depicted in Fig. 3. On average the heterotrophic plate counts ranged from 3 x 10^3 micro-organisms/mℓ recorded at various sites throughout the sampling period to 1.04 x 10^6 micro-organisms/mℓ recorded at Site B2 in Week 37. Even though the plate counts remained constantly low at all the sites for most of the sampling period, significant ($p < 0.05$) increases were observed in Weeks 37 and 45 at Site B2 where counts of 1.04 x 10^6 micro-organisms/mℓ and 3.6 x 10^5 micro-organisms/mℓ were recorded, respectively. High MPN counts (Fig. 2) were also observed at Site B2 for Weeks 37 and 45. The high microbial input at site B2 could be ascribed to the fact that stormwater drainage pipes from the informal settlement flow directly into the river at this site.

Flow cytometric analyses

Live/Dead ratios of planktonic populations were obtained using flow cytometer analyses, in conjunction with the Live/Dead BacLight™ probe and liquid beads (BD™). Two distinct populations of live and dead cells (R5 and R6, respectively) were observed by distinguishing between their fluorescence intensities (Fig. 4), i.e. either red or green fluorescence.

The total cell counts obtained are presented in Fig. 5. The lowest planktonic counts of 1.5 x 10^6 and 1.6 x 10^6 micro-organisms/mℓ, were recorded at sites B1 and B2 in Weeks 5 and 49, respectively. In comparison, the highest total cell counts of 3.1 x 10^7, 3.7 x 10^7 and 3.5 x 10^7 micro-organisms/mℓ were observed in Weeks 1, 41 and 45 respectively, all at Site B2.

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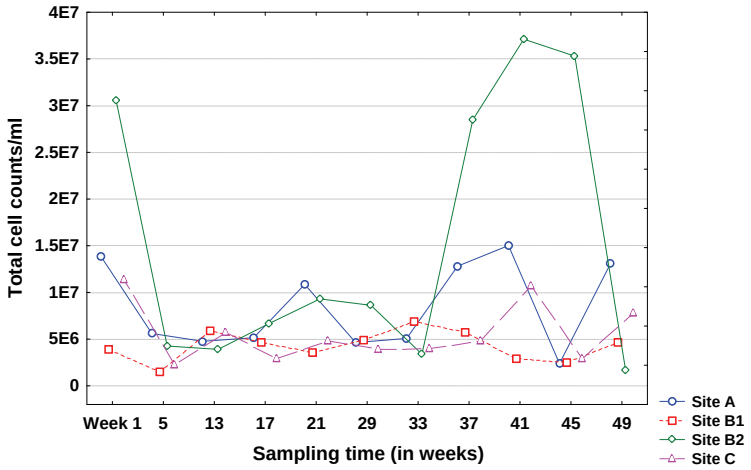

Figure 5
Enumeration of total bacteria by means of flow cytometric analysis (FCM) recorded over the sampling period.

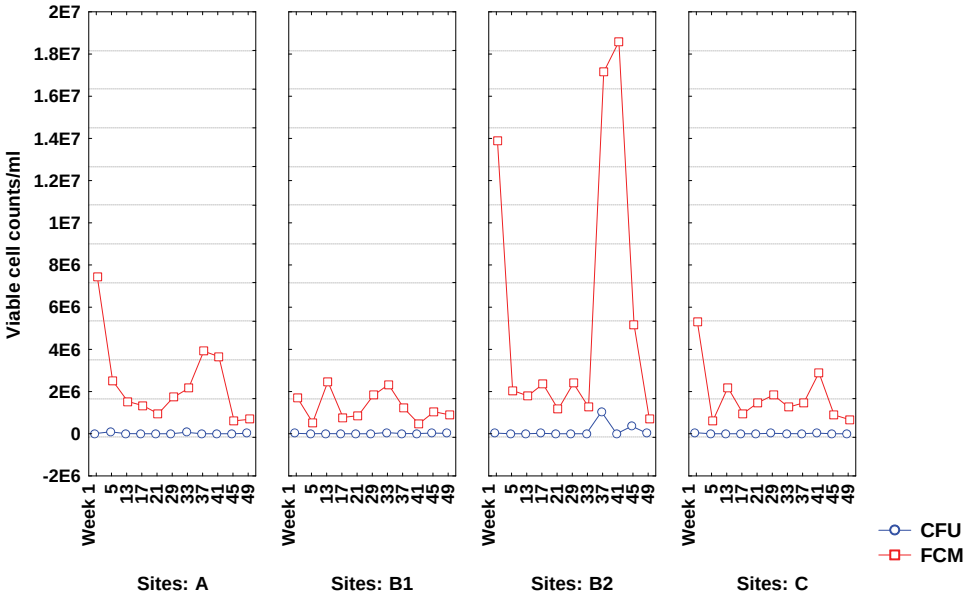

Figure 6
Comparison of colony forming units by heterotrophic plate counts to viable cell counts by means of flow cytometric analysis

Comparisons of heterotrophic plate counts vs. the viable cell counts obtained by flow cytometry are shown in Figure 6. It was observed that when compared to the CFU counts obtained by the heterotrophic plate count technique, flow cytometric (FCM) analysis yielded significantly ($p < 0.05$) higher viable counts in the planktonic samples.

The highest CFU count of 1.04 x 10^6 micro-organisms/mℓ (Figure 3) was recorded in Week 37 at site B2. A corresponding FCM viable count of 1.72 x 10^7 micro-organisms/mℓ was recorded for the same sampling time. In addition, for Weeks 1 and 41, FCM counts of 1.4 x 10^7 and 1.9 x 10^7 micro-organisms/mℓ, for viable micro-organisms, respectively were recorded at Site B2, compared to the CFU counts of 3 x 10^4 and 3 x 10^3 micro-organisms/mℓ, recorded for the same week. As shown in Figs. 2 and 3 significant increases in MPN and CFU counts were observed in Weeks 37 and 45. The FCM results showed a significant increase in total cell counts in both these weeks, as well as in Week 41. The temperatures recorded in Weeks 37, 41 and 45 ranged from 19 to 25°C. However, irrespective of the high water temperature of 23.4°C measured in Week 41, a low heterotrophic plate count was recorded at this site. Correspondingly, low MPN, FC and *E. coli* counts were also observed at this site for the same sampling time.

The relative values (by means of percentage ratios) of the heterotrophic plate count to the total FCM count, viable FCM count to total FCM count and heterotrophic plate count to viable FCM count are presented in Tables 1 to 3, respectively. Results showed that on average the heterotrophic plate count represented only a fraction [3.65% (Week 37, Site B2)] of the total biomass obtained by FCM analysis. In comparison, the viable FCM count accounted for 60.18% of the total FCM count for Site B2 in the same week of sampling (Table 2). In addition, the heterotrophic plate count represented only a fraction (6.06%) of the viable FCM count for the same sampling period at Site B2. The highest heterotrophic plate count represented 6.96% of the viable FCM count (Week 45 at Site B2) and only a fraction of 1.02% of the total FCM count (Table 1) for the same sampling site and period. Results clearly show that the FCM technique is the more reliable enumeration technique for microbial populations obtained from environmental samples. The higher FCM results could also be ascribed to the fact that this technique is able to detect certain populations in the environment, which enter a viable but non-culturable state when exposed to stressful conditions. It is thus evident that the traditional plate-count method only provides an indication of the viable-culturable cells present in the river water, and thereby only accounts for a fraction of

TABLE 1
The average percentage ratio of heterotrophic plate counts to total cell numbers based on flow cytometric analysis for all samples analysed over the sampling period

Parameter	1	5	13	17	21	29	33	37	41	45	49
A	0.07	1.61	0.21	0.06	0.03	0.00	1.38	0.02	0.02	0.13	0.15
B1	0.51	0.20	0.17	0.00	0.08	0.00	0.87	0.17	0.35	1.19	0.43
B2	0.10	0.00	0.08	0.30	0.03	0.03	0.00	3.65	0.01	1.02	1.23
C	0.44	0.00	0.00	0.10	0.06	0.51	0.25	0.00	0.28	0.00	0.00

TABLE 2
The average percentage ratio of viable cell numbers to total cell counts based on flow cytometric analysis for all samples analysed over the sampling period

Parameter	1	5	13	17	21	29	33	37	41	45	49
A	53.48	44.87	32.11	25.87	8.77	37.31	42.46	30.54	24.37	25.66	5.25
B1	43.15	36.10	41.68	16.68	24.11	36.94	33.80	21.28	16.29	41.48	19.31
B2	45.51	48.15	45.94	35.39	12.44	27.78	37.38	60.18	50.09	14.64	44.20
C	46.48	26.64	38.02	32.56	31.02	47.04	31.70	30.52	26.99	30.84	8.64

TABLE 3
The average percentage ratio of heterotrophic plate counts to viable cell numbers based on flow cytometric analysis for all samples analysed over the sampling period

Parameter	1	5	13	17	21	29	33	37	41	45	49
A	0.13	3.58	0.65	0.23	0.31	0.00	3.25	0.08	0.08	0.49	2.91
B1	1.18	0.57	0.40	0.00	0.35	0.00	2.58	0.82	2.13	2.88	2.22
B2	0.22	0.00	0.17	0.85	0.26	0.13	0.00	6.06	0.02	6.96	2.79
C	0.95	0.00	0.00	0.31	0.20	1.09	0.79	0.00	1.04	0.00	0.00

the total viable population within samples, which could also explain the low MPN and CFU counts observed in Week 41. In order to determine the exact level of pollution in the river water, it is thus essential that accurate methods of measuring total microbial activity for planktonic micro-organisms be employed.

Even though the flow cytometry technique provides accurate relative quantification (using fluorescent liquid beads to determine the percentage abundance) of cells in various sample types, technical limitations do exist. The essential reagents or components required for the FCM technique are costly and the limited applicability as a result of the type of flow cytometer, adds to the restrictions of applying this technique for routine analysis (Montes et al., 2006).

Direct acridine orange count (DAOC)

The average total cell counts of planktonic samples as obtained by DAOC analysis are represented in Fig. 7. The total cell counts obtained from the DAOC method were lower in all the water samples, when compared to total counts obtained by flow cytometry analyses (Fig. 8). The highest DAOC count of 8.3 x 10^6 micro-organisms/mℓ for planktonic samples was recorded in Week 29 at Site B2. The FCM analysis for Week 29 yielded comparable total counts of 8.62 x 10^6 micro-organisms/mℓ. The lowest planktonic DAOC count of 4 x 10^4 micro-organisms/mℓ was recorded at Site B1 in Week 49, with a corresponding FCM count of 4.7 x 10^6 micro-organisms/mℓ. Even though the FCM counts fluctuated throughout the sampling period, results clearly

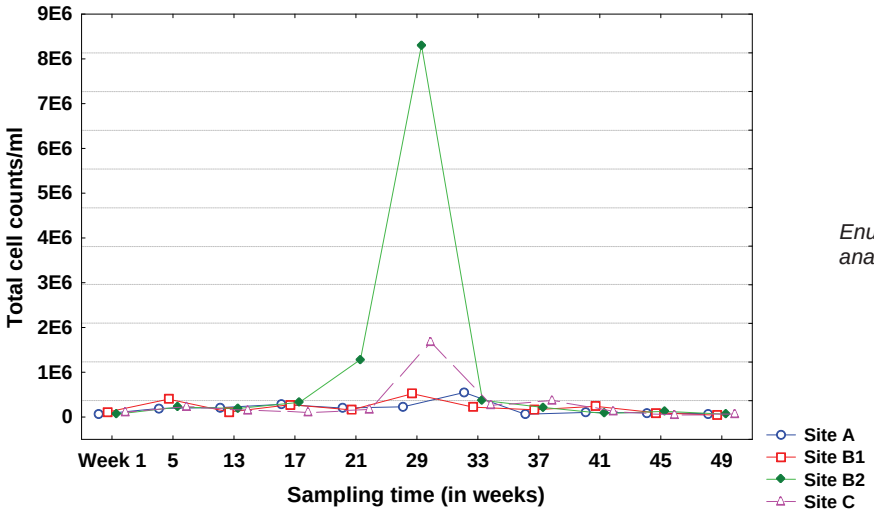

Figure 7
Enumeration of total bacteria for all sites analysed by means of the direct acridine orange count (DAOC)

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TABLE 4
The average percentage ratio of heterotrophic plate counts to total cell numbers based on direct acridine orange count analysis for all samples analysed over the sampling period

Parameter	1	5	13	17	21	29	33	37	41	45	49
A	16.96	46.74	4.77	1.07	1.50	0.00	12.78	4.87	2.97	3.23	26.78
B1	18.39	0.76	8.88	0.00	1.91	0.00	27.43	6.21	4.17	32.25	49.25
B2	46.74	0.00	1.56	6.11	0.23	0.04	0.00	490.06	3.88	286.26	31.81
C	44.38	0.00	0.00	3.05	1.91	1.19	3.87	0.00	24.11	0.00	0.00

TABLE 5
The average percentage ratio of total cell numbers based on direct acridine orange count compared to total cell numbers based on flow cytometric analysis for all samples analysed over the sampling period

Parameter	1	5	13	17	21	29	33	37	41	45	49
A	0.43	3.44	4.40	5.47	1.85	4.92	10.79	0.48	0.67	3.90	0.57
B1	2.76	27.08	1.90	5.94	4.42	10.56	3.18	2.81	8.32	3.70	0.87
B2	0.21	5.13	4.93	4.91	13.78	96.35	10.99	0.74	0.21	0.36	3.87
C	0.99	10.20	2.66	3.35	3.29	43.08	6.47	7.65	1.16	1.86	0.93

show that the FCM method yields more accurate data for total cell counts than the DAOC method.

The DAOC also displayed questionable results when compared to the heterotrophic plate count data (Fig. 3). The highest CFU counts of 1.04 x 10^6 micro-organisms/mℓ and 3.6 x 10^5 micro-organisms/mℓ were recorded in Weeks 37 and 45, respectively at Site B2. In comparison, the DAOC counts measured for the same sampling time were lower than the CFU counts, at 2.1 x 10^5 micro-organisms/mℓ and 1.3 x 10^5 micro-organisms/mℓ, respectively. Corresponding FCM viable counts of 1.72 x 10^7 and 5.2 x 10^6 micro-organisms/mℓ were measured at this site for the two weeks, respectively.

Percentage ratios of the heterotrophic plate count to total DAOC count as well as the total DAOC to total FCM counts are represented in Tables 4 and 5, respectively. Results showed that on average the heterotrophic plate count represented < 49.25% of the total DAOC count (Site B1 in Week 49). In comparison, the percentage ratio of the heterotrophic plate count to the total FCM count (Table 1) was recorded at 0.43% at Site B1 for the same sampling period with the total DAOC count representing only 0.87% of the total FCM count. Discrepancies in the percentage ratio of the heterotrophic plate count and total DAOC counts were observed in Week 37 (490.06%) and Week 45 (286.26%) (Table 4) respectively. Generally, the total DAOC count should represent all culturable and non-culturable micro-organisms, while the heterotrophic plate count (culturable organisms) should thus only account for a fraction of the total DAOC count. In general, the total DAOC count on average represented < 43.08 % of the total FCM count (Table 5) for most of the sampling period. However, in Week 29 at Site B2 the total DAOC count could be compared to the total FCM count as the DAOC count represented 96.35 % of the total FCM count.

A significant limitation to be considered when using membrane filtration in retaining and concentrating bacteria is the lack of a sufficient number of cells to be counted on the filter's surface. An increased microbial population usually provides a level of acceptable reliability to the resulting data (Fry, 1990). Care should also be taken where increased volumes of water are filtered through a single membrane, as nonbacterial biomass and debris tend to clog filters routinely used in total-direct-count methods (Lisle et al., 2004). A factor, which could also influence results, is that the DAOC technique involves physical counting of the micro-organisms in conjunction with epifluorescence microscopy, whereas the FCM method analyses total cell counts by means of computer software.

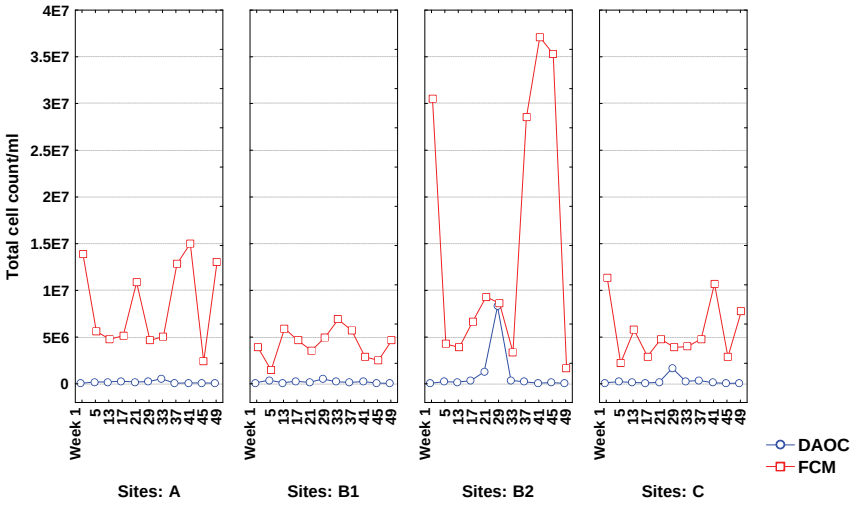

Figure 8
Comparison of total counts obtained by means of flow cytometric analysis to direct acridine orange count using epifluorescence microscopy

Conclusions

The major conclusions of the study are as follows:

- On average, the MPN counts notably exceeded the maximum limit of 2 000 micro-organisms/100 mℓ (South African Bureau of Standards, 1984) for river water
- Only 2% of the total MPN, 23% of faecal coliforms and 30% of *E. coli* counts fell into the accepted maximum limit range
- The highest overall counts for MPN (faecal coliforms, *E. coli*), heterotrophic counts, DAOC and FCM were observed at Site B2 which is the site where stormwater drainage pipes from the informal settlement discharge into the river
- Overall higher viable cell counts were obtained from FCM analysis when compared to cell counts obtained by means of the heterotrophic plate count technique, which could be ascribed to the fact that the heterotrophic plate-count technique only accounts for viable culturable micro-organisms whereas FCM analysis detects viable-culturable micro-organisms as well as those in a viable-but-non-culturable state
- The heterotrophic plate count thereby represented only a fraction < 3.65% of the total FCM count and < 6.06% of the viable FCM count (Site B2 in Week 37) of the sampling period
- The heterotrophic plate counts represented < 49.25% of the total DAOC count with exceptions in Weeks 37 and 45 where higher heterotrophic plate counts with percentage ratios of 490.06% and 286.26% respectively, for heterotrophic plate counts vs. DAOC counts were recorded. This indicates that inconsistencies could be experienced with the DAOC technique.
- In addition, the FCM technique indicated significantly ($p < 0.05$) higher total counts than those observed by the DAOC technique. The only comparable DAOC to FCM count was observed in Week 29 at Site B2 where a 96.35% percentage ratio was recorded. The FCM technique therefore proves to be a more effective technique to routinely compare and evaluate the presence of most if not all, populations in the river water samples.

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