Enumeration of soil bacteria with the green fluorescent nucleic acid dye Sytox green in the presence of soil particles

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Received 1 June 2004; received in revised form 2 July 2004; accepted 2 July 2004
Available online 13 August 2004

Abstract

Total counts in soils are usually determined using fluorescent dyes, such as DAPI or Sybr green, due to fluorescence enhancement if they are bound to nucleic acids. Unfortunately, these commonly used dyes stain soil particles as well. Therefore, besides fluorescence enhancement, sufficient spectral differentiation is also required. We present a new procedure that overcomes the problems of visualising bacteria on surfaces in soil and avoids the separation of soil particles to a large extent. Spectral differentiation between bacteria and soil matrix is achieved by using Sytox green and a suboptimal excitation wavelength. Bacteria exhibit a bright green fluorescence, while soil particles fluoresce blue or red. Slight homogenisation and sedimentation of the sand and coarse silt that were too big for microscopic investigations were the only separation steps required. We compared the proposed Sytox green staining with Sybr green staining. The recovery of Sybr green-stained cells amounted to 38%, whereas in samples stained by Sytox green 81% of the spiked cells were counted. Sytox green can also be combined with fluorescence in situ hybridisation (FISH) using deep red dyes such as Cy5.

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Keywords: Sytox green; Total counts; Digital image analysis

1. Introduction

Fluorescent dyes and epifluorescent microscopy have been used for more than 25 years for the rapid and sensitive enumeration of total counts of bacteria in soils and sediments (Hobbie et al., 1977; Porter and Feig, 1980; Zweifel and Hagström, 1995). Fluorescent dyes optimally suited for total counts should stain all bacteria independent of their physiological state and metabolic activity. Therefore, fluorescent dyes should preferably stain cellular DNA to mark DNA-containing particles as total counts (Zweifel and Hagström, 1995).

The presence of autofluorescent particles and debris stained by unspecific binding of the fluorescent dyes in samples from soils and sediments limits the use of the most common dyes such as DAPI and
Acridine orange (Kepner and Pratt, 1994). Novel nucleic acid dyes are promising (Lebaron et al., 1998a; Weinbauer et al., 1998) because they offer a better signal-to-background ratio by an increase in fluorescence during DNA binding. Sybr green I (Sybr green), Sytox green, Pico green and Syto dyes are a new generation of nucleic acid dyes and have been tested by several researchers for the enumeration of bacteria in aquatic systems (Veldhuis, 1997; Lebaron et al., 1998a; Suzuki et al., 1998). Only Sybr green has been described in detail so far for use in soil and sediment samples (Weinbauer et al., 1998). In contrast to Sybr green, Sytox green does not stain live cells and the common use of Sytox green is restricted in terms of distinguishing dead from live cells (Roth et al., 1997; Lebaron et al., 1998b; Thevissen et al., 1999; Green et al., 2000; Chang et al., 2001; Burnett and Beuchat, 2002; Theis et al., 2003) and for counterstaining cell tissues (http://www.probes.com).

In fact, in order to use Sytox green as a dye for total counts, the membranes of cells first have to be permeabilised by detergents. Wobus et al. (2003) mentioned Sytox green for total counts instead of DAPI, but they did not permeabilise the cells before staining.

Increasing signal strengths of DNA-bound dyes alone cannot cope with the problems of unspecific binding. The number of unspecific binding sides for the fluorescent dyes in environmental samples, e.g., on clay particle surfaces, may be significant in comparison to the number of DNA binding sites. Non-DNA particles may exhibit higher fluorescent intensities than microorganisms. In image analysis it has proved difficult to morphologically distinguish bacteria from nonbacterial features because these features are often of a similar shape and size to the bacteria (Nunan et al., 2001). Another source of errors is the fact that bacterial fluorescent signals can be masked by an underlying unspecific fluorescent signal, because in soils most of the bacteria are attached to particles in contrast to free-living bacteria in an aquatic environment (Kuwae and Hosokawa, 1999). Thus, a direct measurement of the abundance and biovolume of soil bacteria by epifluorescence microscopy has up to now only been possible when bacteria can be detached or segregated from aggregates including mineral particles and detritus.

Several authors (Kepner and Pratt, 1994; Riis et al., 1998; Kuwae and Hosokawa, 1999; Gough and Stahl, 2003) tried to reduce the presence of particles by separating them before the counting procedure. They found that a satisfactory separation of microorganisms from clay or silt was not possible; therefore, a compromise between removing the soil particles and quantifying the microorganisms is necessary. Removing all soil particles results in the removal of most bacteria. On the other hand, maintaining the soil particles results in unspecific staining of the latter.

We present a new procedure overcoming the problems of visualising bacteria on soil surfaces and avoiding the separation of soil particles to a significant extent. Slight homogenisation and sedimentation of the sand and coarse silt that are too big for microscopic investigations were the only separation steps. Fluorescence enhancement of DNA-bound dye and sufficient spectral differentiation between bacteria and the soil matrix enables total counting of bacteria. Spectral differentiation between bacteria and the soil matrix is achieved by using Sytox green and a suboptimal excitation wavelength. Bacteria exhibit a bright green fluorescence, while soil particles fluoresce blue or red. This colour split allows digital image analysis to be performed on the basis of the Red–Green–Blue (RGB) colour space.

To the best of our knowledge, the fluorescent dye Sytox green has not so far been used successfully for epifluorescent counts of soil and sediment bacteria. We investigated whether this fluorescent dye would improve the visualisation of bacteria in clay, sand, organoclay suspensions and soils.

2. Materials and methods

2.1. Soils and matrices

Soil samples were collected in Stolberg and Kaldenkirchen (North Rhine-Westphalia, Germany). The soil from Stolberg was a silty loam (orthic luvisol) soil with 1.7% Corg, whereas the soil from Kaldenkirchen was a loamy sand (gleyic cambisol) soil with 1.2% Corg. Soil samples were collected at depths of 0–0.3 m by spading. The soils were transported on ice and stored at 4 °C before being preserved (1–2 days). The soils were sieved (<2 mm) before use. Ca-bentonite was purchased from Süd-Chemie (Munich, Germany) and cleaned to mont-
morillonite as described elsewhere (Witthuhn et al., in press), Quartz sand (Tecosil -50+100) was purchased from Frechener Quarzwerke (Cologne, Germany). Both clay and sand had an organic content of <0.1%. 2C18-montmorillonite organoclay was prepared as described elsewhere (Witthuhn et al., in press), the C<sub>org</sub> content was 14.6%.

2.2. Organisms and growth conditions

The following bacterial strains were used: Pseudomonas putida (German-type culture collection DSMZ) DSMZ no. 8368, Bacillus subtilis DSMZ no. 347, Pseudomonas fluorescens DSMZ no. 50108 and Ralstonia eutropha JMP134-1, provided by Dietmar Pieper from the German Research Centre for Biotechnology (GBF, Braunschweig, Germany). All the organisms were grown in medium 461 (according to DSMZ protocol). Aliquots of cell suspensions were taken at the end of the log phase and in the late stationary phase.

2.3. Comparison of Sytox green total counts with Sybr green and DAPI total counts in aquatic suspensions

Bacterial cell densities were determined by using an Elzone counter (Particle Data Elzone 280 PC, Luxembourg) as described previously (Wilhelm et al., 1998) and by fluorescence microscopy techniques using DAPI (Kepner and Pratt, 1994), Sybr green (Weinbauer et al., 1998) and Sytox green. Cells from the late log phase were used. The cell counts determined by fluorescence microscopy were related to those by obtained by the Elzone counter.

2.4. Spiking of cells into matrices

Cells of P. fluorescens from the log and late stationary phase were spiked into the diluted soil and the other matrices to test whether these cells could be accurately counted. The suspension concentration of the soil and matrices ranged from 1 to 10 g/l. For example, 1 g of soil or matrix was suspended in 99 ml of 0.01 M sterile sodium pyrophosphate solution in an Erlenmeyer flask and shaken for 30 min at 200 rpm. After 10 s of sedimentation, an aliquot of 0.5 ml was taken from the centre of the liquid at half the depth with a sterile syringe (1 ml, B. Braun, Melsungen, Germany). 1.5 ml of a 4% (w/v) paraformaldehyde solution in PBS buffer (pH 7) was added to the sample and incubation was performed in an Eppendorf tube on a shaker (Eppendorf Thermomixer 5437, Germany) for 1 h at 200 rpm and room temperature. We tested the need to fixate the soil sample before preparing the suspension according to Weinbauer et al. (1998) and found no difference in cell counts. Therefore, we suspended the soil sample before fixation to save preparation steps.

2.5. Staining procedure

The staining procedure for Sytox green differs from Sybr green because staining cells with Sytox green requires permeabilisation of membranes.

For Sybr green staining, an aliquot of the sample was sonicated on ice for 1 min before incubation with the dye, according to Weinbauer et al. (1998). The sample was pipetted into a FinStar (Poschen et al., 2002) staining reactor containing 4 ml of phosphate buffered saline (PBS; pH 7) as described previously (Amann et al., 1990). For filtration, an Anodisc filter (Whatman, 0.2 μm, 25-mm diameter) was used according to Weinbauer et al. (1998). The liquid sample was sucked off with 130 mbar underpressure and the retained particles were washed twice with 4 ml PBS buffer (pH 7). After washing, the filter was coated with 1.5 ml of 5 μM Sybr green solution in PBS buffer (pH 7) and incubated for 15 min. Two washing steps were performed subsequently as described above. The filter was mounted into a drop of immersion oil on a microscope slide after drying and a drop of immersion oil was put on the surface. A cover slide was used and an additional drop of immersion oil was put on the cover slide. The sample was viewed under blue excitation light (Nikon B-2A, excitation 450–490 nm, dichroic mirror 505 nm, longpass>520 nm) at 600-fold magnification.

For Sytox green staining, an aliquot of the sample was pipetted into 4 ml of hybridisation buffer (Tris 2.4 g/l; SDS 2.0 g/l; Na<sub>3</sub>EDTA 2.0 g/l; MgCl<sub>2</sub> 0.2 g/l; NaCl 52.6 g/l; pH 7, according to Amann et al. (1990) to permeabilise the cell membrane. The hybridisation buffer was put inside a FinStar-staining reactor as described elsewhere (Poschen et al., 2002). Millipore filters (GPIB, 0.2 μm, 25-mm diameter) were used for filtration. Incubation took 1 h at 46°C.
This step was necessary to enable the dye to enter the cell, because Sytox green cannot enter the cells of live bacteria with an intact membrane (Lebaron et al., 1998b). The liquid sample was sucked off with 130 mbar underpressure and the retained particles were washed four times with 4 ml PBS buffer (pH 7). The FinStar was cooled down to room temperature to avoid weak staining and afterwards the filter was coated with 1.5 ml of 25 μM Sytox green solution in PBS buffer (pH 7) and incubated for 5 min. Four washing steps were subsequently performed as described above. The wet filter was placed on a microscope slide onto a 30 μl drop of PBS buffer (pH 7), and a drop was put on the filter surface. A cover slide and a drop of immersion oil were then applied. The sample was viewed under UV excitation at 600-fold magnification with a filter set for DAPI (Nikon UV-2A, excitation 330–380 nm, dichroic mirror 400 nm, longpass>420 nm).

2.6. Microscope procedure and data analysis

The microscope used was a PC-supported Nikon ECLIPSE E 1000 with a motorised and PC-controlled three-axis crosstable from Merzenhäuser (Germany). The images were captured with a CCD camera, Sony DXC-9100 P, and a Matrox Corona Framegrabber. The crosstable, microscope and image acquisition were controlled by the LUCIA 32 4.11 software (Nikon, Germany). The images were stored as 8-bit bitmaps. For every sample, 30 pictures were acquired randomly to avoid a subjective selection of “good” images. For the random selection of closeups, the “random search” function in the Lucia software was used to control the crosstable.

For a reproducible and user-independent analysis of the fluorescent images, the digital image analysis software ZEISS KS 400 V 3.0 (Carl Zeiss, Germany) was used. The image processing program was modified according to Bloem et al. (1995) and is described below. The colour channel of interest (green) was first extracted from the RGB image and processed in order to get a mask image. Shading correction was implemented without a background image. The grey tone image of the green channel was filtered 25 times by a lowpass filter of 25×25 matrix resulting in a reference image. The reference image was processed with the grey tone by the following equation, resulting in a shadow-corrected image.

\[
\text{Greyvalue}(x,y)_{\text{corrected}} = \frac{\text{Greyvalue}(x,y)_{\text{original}}}{\text{Greyvalue}(x,y)_{\text{referenz}}} X_{\text{referenz}}
\]

For image information (bacterial cells) enhancement, an edge detection operator was used on the shadow-corrected image. A highpass filter of 3×3 matrix was applied 10 times resulting in a highpass-filtered image. The dynamics of the grey tones was reduced in a subsequent step to simplify the following thresholding step. The highpass-filtered image was delineated with a threshold of 1 and a matrix size of 5 resulting in a delineated image.

For image information extraction, thresholding was used on the delineated image. This was the only user-interactive intervention to prevent background noise from being counted. After thresholding of the delineated image, a mask image was generated. Two binary operators binfill and binscrap were then applied. Binfill fills holes in objects, sometimes resulting from the highpass filter, and binscrap removes noise up to the size of two pixels that can be defined as electronic noise (Inoué and Spring, 1997). The processed binary image was labelled with the original image in an overlay procedure, whereas the cells were marked and counted automatically in the next step. Comparisons were made between manual counting and automatic counting. To check the comparability of Sytox green with other dyes, e.g.,

Fig. 1. Absorption spectrum of Sytox green in PBS buffer (pH 7).
attached dyes in fluorescence in situ hybridisation (FISH) probes, emission intensities were recorded at several excitation/emission wavelengths.

2.7. Spectrophotometric measurements

Emission spectra of matrix suspensions stained with Sytox green were recorded with a Perkin-Elmer LS-50 B fluorescence spectrophotometer. Slurries and solutions were suspended in PBS buffer (pH 7). The absorption spectrum of Sytox green solution (25 μM) was recorded on a Beckman coulter DU 800 (Germany) spectrophotometer (Fig. 1).

3. Results

Total counts by microscopy techniques resulted in recovery values between 75% and 89% related to cell counts of the Elzone counter (Table 1). DAPI, Sybr green and Sytox green-staining procedures gave the same cell counts in aquatic systems, whereas the DAPI counts served as a standard. The counting accuracy for Sybr green and Sytox green was first examined in the presence of pure clay (montmorillonite), sand and organoclay (2C18-montmorillonite) particles to avoid the interference of autochthonous microorganisms. Soils were then spiked with bacteria to test recovery under real environmental conditions.

3.1. Recovery of spiked cells in the presence of soil components

Total counts and recovery of Sybr green- and Sytox green-stained cells in the presence of clay and sand were similar (Table 2). In contrast, Sybr green and Sytox green recovery values differed markedly in the presence of organoclay, recovery values being 50–90% higher with Sytox green staining. Cells on montmorillonite or sand surfaces were detected due to their intensive green colour. However, cells on organoclay surfaces could hardly be detected using Sybr green staining. The unspecific fluorescence of Sybr green bound to organoclay is shown in Fig. 2, and for Sytox green in Fig. 3. The emission peak of Sybr green bound to organoclay ranged from green to red (Fig. 2), indicating a yellow fluorescence as observed in the images. The unspecific fluorescence signal of Sytox green is split into a blue and a green emission peak (Fig. 3). The signal of the blue peak is twice that of the signal of the green peak resulting in

Table 1

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>DAPI (% to counter)</th>
<th>Sybr green (% to counter)</th>
<th>Sytox green (% to counter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas fluorescens</td>
<td>81±31</td>
<td>84±22</td>
<td>85±27</td>
</tr>
<tr>
<td>Pseudomonas putida</td>
<td>83±26</td>
<td>80±25</td>
<td>87±21</td>
</tr>
<tr>
<td>Ralstonia eutropha</td>
<td>75±34</td>
<td>85±29</td>
<td>89±12</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>78±32</td>
<td>82±36</td>
<td>80.5±33</td>
</tr>
</tbody>
</table>

No matrix was added. All staining experiments were done in 12 replicates. Variability in cell counts between images refers to 30 images.

Table 2

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Physiological status of P. fluorescens cells</th>
<th>Recovery by Sybr green staining (%)</th>
<th>Recovery by Sytox green staining (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clay</td>
<td>Log-phase cells</td>
<td>80±27</td>
<td>82±25</td>
</tr>
<tr>
<td></td>
<td>Late stationary cells</td>
<td>85±32</td>
<td>84±23</td>
</tr>
<tr>
<td>Sand</td>
<td>Log-phase cells</td>
<td>79±26</td>
<td>81±31</td>
</tr>
<tr>
<td></td>
<td>Late stationary cells</td>
<td>76±32</td>
<td>84±28</td>
</tr>
<tr>
<td>Organoclay</td>
<td>Log-phase cells</td>
<td>55±31</td>
<td>74±32</td>
</tr>
<tr>
<td></td>
<td>Late stationary cells</td>
<td>42±23</td>
<td>79±25</td>
</tr>
</tbody>
</table>

All experiments were done in five replicates. One-hundred percent recovery is defined as the cell density of the sample measured by an Elzone counter. Variability in cell counts between images refers to 30 images.
easy visualisation of green cells on blue surfaces. No red peak appeared when Sytox green was bound to organoclay, but red organoclay particles were observed in the images. This colour effect can be explained by surface interactions with dye–dye complexes resulting in spectral shifts to longer wavelengths as was found for acridine orange bound to clay surfaces (Cohen and Yariv, 1984). The physiological status of the cells (log or late stationary phase) had no significant influence on recovery (Table 2).

3.2. Recovery of spiked cells in the presence of soils

Diluted soil samples (1 g/l) of Stolberg soil and Kaldenkirchen soil were stained with either Sytox green or Sybr green according to the procedures described in Materials and methods. The cell count of bacteria naturally present (autochthonous) determined with Sytox green staining amounted 9.24×10^8±1.82×10^8 g^{-1} soil for the Stolberg soil and 5.55×10^8±2.22×10^8 g^{-1} for the Kaldenkirchen soil. With Sybr green staining these amounts were 3.22×10^8±2.58×10^8 and 1.99×10^8±1.34×10^8, respectively. The comparison of Sybr green and Sytox green staining in soils showed a worse recovery of Sybr green staining like in the presence of organoclay. In Figs. 4 and 5, images of Sytox green- and Sybr green-stained soil samples are shown. Using Sybr green, no cells were detected on the surfaces of soil particles due to the intensive yellow fluorescence (Fig. 4). In contrast, cells were easily differentiated from the underlying surface by Sytox green staining due to the colour split and the weak unspecific fluorescence of the dye bound to soil particles (Fig. 5). The colour split could only be induced using suboptimal UV excitation.

Theoretically, the amount of autochthonous bacteria could be nearly twice as high as measured by the staining procedure, because half of the number of the bacteria could have been masked by soil particles, when most of them are attached to soil particles. To verify this assumption, the two diluted soils were spiked with varying amounts of P. fluorescens cells from the late log phase and stained in situ with either Sybr green or Sytox green. For Sybr green staining, no correlation between spiked and recovered cells was found in the presence of soil particles (Table 3). However, the recovery of spiked cells increased with increasing amounts of spiked cells for Sytox green staining. Recovery values in both soils reached those obtained from pure aquatic cell suspensions when the amount of spiked cells exceeded the amount of autochthonous cells by a factor of >2; the amount of autochthonous cells have been previously subtracted (Fig. 6).

Manual counting of cells compared to digital image analysis resulted in 5% higher cell counts, whereas the time needed for manual counting was much higher. Thirty images were analysed by the computer in 1–2 min, while manual counting took 0.5–1 h. The image
Fig. 4. Sybr green-stained soil sample from Stolberg soil.

Fig. 5. Sytox green-stained soil sample from Stolberg soil.
processing program developed for Sytox green-stained samples proved suitable for replacing manual counting by digital image analysis for environmental samples.

### 3.3. Crosstalk of Sytox green with other dyes

For the combination of Sytox green total count staining with other applications like FISH, the crosstalk (interference of unspecific fluorescence signals excited at suboptimal wavelengths) of Sytox green has to be investigated to avoid false results by unspecific emission. Sytox green shows a bright absorption spectrum (Fig. 1) and emits fluorescence light both by UV and by blue light excitation. We found that Sytox green had a red fluorescence emission even when excited by green light. Table 4 shows the compatibility with common FISH dyes and the percentage of crosstalk at suboptimal excitation. The percentages were normalized to maximal fluorescence emission of Sytox green at optimal excitation wavelength (488 nm). Cy5 appeared to be a suitable dye.

### 4. Discussion

The use of Sytox green for total counts was reported by Wobus et al. (2003). The authors favoured the significantly lower background fluorescence over the widely used DAPI stain. Unfortunately, neither proof of validity nor a picture was shown in the article. In fact, because of the lack of the permeabilisation step it is doubtful whether all the cells were stained. To achieve permeabilisation, we used the permeabilisation procedures as described by Amann et al. (1990) for fluorescence in situ hybridisation.

<table>
<thead>
<tr>
<th>Amount of spiked cells</th>
<th>Recovery Sybr green Kaldenkirchen (%)</th>
<th>Recovery Sytox green Kaldenkirchen (%)</th>
<th>Recovery Sybr green Stolberg (%)</th>
<th>Recovery Sytox green Stolberg (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.89 x 10^8</td>
<td>33.9 ± 118.5</td>
<td>29 ± 98.7</td>
<td>37.9 ± 60.5</td>
<td>52.7 ± 43.7</td>
</tr>
<tr>
<td>1.92 x 10^8</td>
<td>5.3 ± 203.3</td>
<td>59.8 ± 39</td>
<td>26.7 ± 50.4</td>
<td>75.5 ± 52.1</td>
</tr>
<tr>
<td>2.76 x 10^8</td>
<td>22 ± 108.3</td>
<td>82.4 ± 32.1</td>
<td>5.2 ± 588.2</td>
<td>81.5 ± 38.7</td>
</tr>
</tbody>
</table>

The percentage recovery is the number of total cell count, corrected for the autochthonous cell count, determined by Sytox green staining, and divided by the number of spiked cells. Variability in cell counts between images refers to 30 images.

### Table 4

Fluorescence intensity of Sytox green-stained cells of _P. fluorescens_ by different excitation wavelengths

<table>
<thead>
<tr>
<th>Excitation wavelength (nm)</th>
<th>Emission window (nm)</th>
<th>Filter set for signal intensity, normalised to emission intensity of Sytox green at 520 nm (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>488</td>
<td>510–530</td>
<td>Sytox green</td>
</tr>
<tr>
<td>543</td>
<td>570–700</td>
<td>Cy3</td>
</tr>
<tr>
<td>594</td>
<td>615–700</td>
<td>Texas Red</td>
</tr>
<tr>
<td>647</td>
<td>670–700</td>
<td>Cy5</td>
</tr>
<tr>
<td>676</td>
<td>695–730</td>
<td>Cy5.5</td>
</tr>
</tbody>
</table>

Emission intensity was measured by using common fluorescent filters for commonly used dyes. The percentages were normalized to maximal fluorescence emission of Sytox green at optimal excitation wavelength (488 nm).
(FISH) and by Poschen et al. (2002). We found that these protocols were compatible with Sytox green staining. Neu et al. (2002) recently reported that Sytox green can be excited at suboptimal wavelengths. As shown in Fig. 1, Sytox green has absorption maxima at 360 and 490 nm. The use of suboptimal excitation wavelengths causes a split in emission wavelengths. DNA-containing bacteria exhibited a bright green fluorescence, while soil particles fluoresced blue or red. This so-called colour split enables the counting of particle-associated bacteria. The use of Sytox green has the clear disadvantage of passing through more steps in the staining procedure. Aquatic samples and suspensions of pure clay and sand Sybr green staining may be preferred, because Sybr green and Sytox green have similar spectral properties and give the same recovery values. However, matrices like organoclay suspensions and soils staining with Sytox green were shown to be superior to staining with Sybr green. The comparison in the presence of organoclay showed markedly lower recovery values using Sybr green staining since particle-associated green fluorescent bacterial cells are masked by the bright yellow soil particles. The yellow fluorescence signal consists of green and red fluorescence signals, green cells could therefore not be visualised on bright yellow surfaces. One reason for the limitation of the use of Sybr green staining in soil can be the unspecific binding of the dye to organic-coated minerals. Staining of bacterial cells with Sytox green in the presence of organoclay particles showed green cells on blue or red organoclay particles. The blue fluorescence of organoclay particles could be explained by the spectroscopic measurements of Sytox green bound to organoclay (Fig. 3). One reason for the red fluorescence of organoclay particles may be the dye–dye interaction on the surface of the surfactant-modified silica layer since fluorescing red particles were visible in soil suspension too. Masking of bacteria even became significant in the presence of soil because of the higher suspension concentration. Sybr green showed no correlation of spiked to recovered cells, while there was a linear increase in recovered cells stained with Sytox green.

Recovery values of up to 85% were found when the amount of spiked cells exceeded the amount of autochthonous cells by a factor of 2 to 3. The lower recovery values obtained with less bacteria spiked could be explained by the fact that soil particles covered underlying bacteria. Recovery values would be higher using a lower soil particle concentration, as shown with the organoclay suspensions.

Removing a significant amount of soil particles before staining would probably lead to higher counts of free suspended bacteria, but the particle-associated bacteria would be removed too. As a consequence, the remaining nonparticle-associated bacteria would be distributed more homogeneously and standard deviations would be much lower; however, the lost of particle-associated bacteria could lead to an underestimation of the total counts. This may be the reason for the lower standard deviations found in literature for total counts (Kepner and Pratt, 1994; Riis et al., 1998; Kuwae and Hosokawa, 1999; Gough and Stahl, 2003).

The sample preparation and staining procedure proposed in this paper leads to the removal of those particles which have to be excluded anyway. After 10 s of sedimentation, only particles >0.1 mm are excluded. Nevertheless, this step is necessary because particles bigger than 0.1 mm could not be observed by an epifluorescence microscope because the distance from filter surface to objective lens is only 0.2 mm including the cover slide. It may be possible to overcome these limitations by using a confocal laser-scanning microscope without cover slide and water objective.

Overall, standard deviations were quite high, but it was realistic considering the procedure used. Thirty images were acquired randomly from the whole filter surface, not influenced by a subjective choice of “good” and “bad” closeups by an experimenter. Due to the nonhomogeneous distribution of the soil sample on the 2.5-cm filter surface, the cell density varied rather strongly. Manual selection of closeups instead of random selection by the crosstable software resulted in lower standard deviations. Results were not reproducible when images were acquired manually by different experimenters.

We showed that the colour split of Sytox green-stained samples resulting in green bacteria and red or blue fluorescing particles is very useful for video acquisition based on the RGB colour space. Downstream image analysis systems with automatic detection of cells and user-reduced interaction enables a high throughput of samples, with almost the same efficiency.
(5% lower counts) as manual counting. In addition, Sytox green would be an ideal counterstain for environmental FISH staining, when dyes with emission wavelengths greater than 670 nm are used for FISH.

Acknowledgements

We thank C.J. Soeder and H. Vereecken (ICG-IV) for their continuous support.

References


