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Quantification of aquatic sediment prokaryotes—A multiple-steps optimization testing sands from pristine and contaminated aquifers

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ABSTRACT

The total number of prokaryotic cells in aquatic sediments is a crucial measure tightly linked to energetic constraints, microbial productivity, and ecosystem status. While counting cells in water samples is routine, reliable procedures for the determination of prokaryotic cells associated to aquatic sediments are especially needed. A protocol for the direct quantification of prokaryotic cell abundance in aquatic sediments was developed and tested with sandy material from pristine and contaminated aquifers. Individual steps required in sample preparation, including preservation, cell dislodgement, density gradient centrifugation providing sample purification, and staining, were critically evaluated and optimized. Quantification of cells is conducted by flow cytometry promoting a high-throughput sample processing. Although tested for only two different types of aquifer sediments, researchers may easily adapt the protocol for their individual samples and purposes, thus conducting a brief efficiency re-evaluation of individual steps highlighted in this study.

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1. Introduction

Most prokaryotic cells (Eubacteria and Archaea) in aquatic sediments are associated with sediment surfaces, forming microcolonies and biofilms. Only a small fraction (0.01–10%) is generally found suspended in the pore water (e.g. Alfreider et al., 1997; Duhamel and Jacquet, 2006; Griebler et al., 2002). Consequently, the analysis of prokaryotes in groundwater ecosystems requires reliable techniques for the preservation and quantification of cells attached to sediment grains.

A first step in quantification of sediment prokaryotes is an adequate preservation of fresh samples. Most commonly, water and sediment samples dedicated to direct cell counts are fixed with paraformaldehyde, formaldehyde, glutardialdehyde or a combination of metal ions and sodium azide (Günther et al., 2008). Following preservation, cells need to be efficiently dislodged from particles keeping cell destruction small to get rid of organic and inorganic particles others than cells. Detachment of cells is mainly accomplished by homogenization and sonication (e.g. Danovaro and Fabiano, 1995), while removal of unspecific particles is mainly achieved via sample dilution (Gough and Stahl, 2003) and density gradient centrifugation (DGC) (e.g. Amalfitano et al., 2009). Equally important is the use of appropriate fluorochromes for cell staining. Dislodgement and cell-separation treatments not only release prokaryotic cells from sediment surfaces but often result in a substantial yield of bacteria-sized organic and inorganic particles that interfere with counting. There is independent evidence that cells stained by green fluorescent dyes are easier to distinguish from non-specifically stained particles than cells stained with dyes such as DAPI and Acridine Orange (Griebler et al., 2001; Gruden et al., 2003).

Quantification of prokaryotes is routinely based on direct counting, either via epifluorescence microscopy (e.g. Epstein and Rossel, 1995) or flow cytometry (e.g. Amalfitano and Fazi, 2008). For statistical relevance a high number of cells per sample needs to be counted (Kirchman et al., 1982) which makes the microscopic approach time-intensive and limits the number of samples processed in a study. In contrast, flow cytometry enables high throughput measurements. Both techniques are applied routinely with water samples, but it is still challenging to quantify prokaryotes initially attached to sediment surfaces.

Here we report on the optimization of critical steps in the quantification of prokaryotic cells from aquatic sediments including sample preservation, cell dislodgement, density gradient centrifugation, and staining. A standard protocol for fast and direct quantification of by means of flow cytometry is provided.

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Exemplarily applied for two selected types of sediment, i.e. sands from a pristine and an organically contaminated aquifer, the protocol can easily be adapted to for other kinds of sediments.

2. Materials and methods

2.1. Sediment samples

Most experiments, during development of the standard protocol for the quantification of aquifer sediment prokaryotes, were conducted with natural unsorted fluvio-glacial quaternary carbonate sandy aquifer sediment (grain diameter 0.063–4 mm) sampled from a local gravel pit, which was, for the duration of the study, continuously percolated with groundwater from the on-site quaternary aquifer. In the following this sediment is termed "test sediment". At some points, sterile quartz sand (grain diameter: 200–300 μm, sterilized for 2 h at 450 °C) was used as additional control sediment (see below). At a later stage of the study, fresh sediment samples from a sandy porous aquifer at a former gas works site in Düsseldorf-Flingern (Germany) was collected by means of drilling ("contaminated sediment"). Here, along a vertical cross section through a contaminant plume of aromatic hydrocarbons, sediment subsamples originating from 6.0 to 10.4 m below land surface (bls) were collected and analyzed. The medium sand fluvial deposits of the Rhine river mainly consisted of quartz (80–90%) with only a small silt and clay content (10%). A detailed description of this sampling site including sediment collection is given elsewhere in Anneser et al. (2008).

2.2. Bacterial cultures

For the purpose of standardization and evaluation of cell recovery, cultures of *Pseudomonas putida* F1 were grown in an oxic carbonate-buffered freshwater mineral medium, respectively (Widdel and Bak, 1992). Cells were harvested at the end of their exponential growth phase by centrifugation. Cell pellets were resolved in PBS buffer (Dulbecco, Biochrom AG), fixed in 2 mL 2.5% glutardialdehyde and stored at +4 °C for further use. *P. putida* is a fast growing soil bacterium degrading organic solvents and, hence, similar to microorganisms at the sampling site in Düsseldorf.

2.3. Sediment preservation tests

Different fixatives in various concentrations were tested for sediment samples with respect to cell quantification and quality of staining. Aliquots of 0.5 cm^3 (corresponding to 0.26 g dry weight) of fresh "test sediment" were fixed for a minimum of 15 min in (1) 0.5 mL of 37% formaldehyde, (2) 1.5 mL of 4% formaldehyde, or (3) 1.5 mL of 2.5% glutardialdehyde, and if not processed immediately, stored at 4 °C in the dark (e.g. Böckelmann et al., 2003). Additionally, different sediment aliquots were fixed in 1.5 mL of 4% paraformaldehyde, incubated for three days at 4 °C in the dark, then washed twice with 1 mL of PBS and spun down at 4000 × *g* for 30 min. Subsequently these sediment samples were resolved and stored in a mixture of 0.75 mL PBS buffer and 0.75 mL ethanol (absolute) (Merck) at 4 °C in the dark.

2.4. Detachment, loss and damage of cells

Before the dislodgment of prokaryotic cells from sediment, the supernatant containing the fixative was removed after centrifugation (12,000 × *g* for 2 min) and 1.5 mL particle free PBS buffer (0.2 μm filtrated) was added. Dislodgement of the cells was tested comparatively (1) in an ultrasonic bath (Branson digital sonifier 450) at 20% amplitude from 20 to 360 s with pulsed and continuous sonication, (2) in a swing mill (MM 200, Retsch) at 10, 20 and 30 Hz and (3) using a test tube shaker (Thermomixer comfort, Eppendorf) at 1400 rpm. These treatments were also tested with culture bacteria amended to sterile quartz sand to evaluate cell damage and recovery. Sample aliquots (in triplicates) received the addition of the surface active detergent sodium pyrophosphate (PPI, 100 mM), prior to the mechanical treatment (Griebler et al., 2001). Different settings (intensity and duration) of ultrasonication were further tested to properly disaggregate cell pellets of culture bacteria after centrifugation steps to avoid pronounced cell damage or loss. Cell aggregation and possible damage was counterchecked microscopically. To estimate the efficiency of cell release from sediment samples treated in the swing mill, the supernatant was collected and replaced by new PBS buffer. The remaining sediment sample was then homogenized again in the swing mill. This procedure was repeated three times and the subsequently collected supernatants were further processed for DGC as described below.

2.5. Density gradient centrifugation (DGC)

Since high background of unspecifically stained particles constituted the most serious problem with quantification of sediment prokaryotes, reduction of these small inorganic bacteria like particles via DGC was a crucial step. 5 mL of a 4 °C-cold Nycodenz solution (Progen Biotechnik GmbH) at a final concentration of 1.3 g mL^{-1} and pH 8 (Lindahl and Bakken, 1995) was transferred to a 10-mL-centrifuge tube. Thereafter, the supernatant (1.5 mL at room temperature) from the treated sediment sample containing the dislodged prokaryotic cells was placed on top of the cold Nycodenz solution. Density gradient centrifugation was performed in a Centricon ultra centrifuge (T-2190, TST 41.14 swing rotor) and particles were spun down at 15,500 × *g* for 1 h at 4 °C. After centrifugation subfractions of 1 mL were collected by pipetting from top to bottom.

To qualitatively and quantitatively verify the centrifugation step, total cell numbers of triplicate samples were determined microscopically (see below) in all sample fractions. As a first test, cells of a *P. putida* F1 culture were diluted in 1.5 mL PBS aliquots to a final concentration of ca. 10^7 cells mL^{-1}. The aliquots were applied on 5 mL of cold Nycodenz solution and centrifuged as described above. Total recovery from the different sample fractions separated after DGC was determined in comparison to the total microscopic counts of non-centrifuged control samples. For comparison, a test was run with quartz sand samples spiked with *P. putida* cells to estimate the amount of bacterial cells spun down together with particles. The pellet in the centrifugation tubes were exemplarily resolved in 1.5 mL PBS buffer, treated in the swing mill and applied once again on cold Nycodenz for repeated DGC. Finally, real sediment samples ("test sediment" and "contaminated sediment") were treated in the swing mill and the supernatant was placed onto the Nycodenz solution. Total cells were quantified by flow cytometry.

2.6. Staining of prokaryotes

For staining, a number of fluorescent dyes were tested with epifluorescence microscopy at various concentrations; 4′,6-diaminido-2-phenylindole (DAPI, Diagnostica Merck) at 0.01 to 0.5 mg mL^{-1}, CYTO 59 (5 mM, Molecular Probes) at 0.1 μL mL^{-1} and 0.2 μL mL^{-1}, acridine orange (Sigma) at 4 μg mL^{-1}, and SYBR Green I (stock 10,000× concentrated, Invitrogen; working solution 1:10 with PBS buffer) at 0.5 to 20 μL mL^{-1} final concentration. Based on the results from microscopic test, only SYBR Green I (3 μL mL^{-1} and 6 μL mL^{-1}) and CYTO 59 (0.6 μL mL^{-1}) were further evaluated with flow cytometry.

In addition, 0.5 cm^3 aliquots of sterile sediment diluted in 1.5 mL PBS buffer were stained with SYBR Green I (3 μL mL^{-1} working

solution) after treatment in the swing mill (20 Hz, 3 min). Some of these sample aliquots were centrifuged at 10,000 × *g* for 10 min, then washed with PBS after staining, and compared to sterile sediment aliquots treated the same way but without staining. These samples were examined by flow cytometry to check for unspecific staining and de novo generation of small particles due to the detachment procedure.

2.7. Microscopic counts

Subsamples after DGC (0.5 to 2 mL) were diluted in particle free PBS buffer to a final volume of 5 mL, stained with 25 μL working solution of SYBR Green I and incubated for 10 min in the dark. The samples were briefly mixed and filtered onto 0.2 μm black polycarbonate filters (Millipore). Subsequently filters were washed once with 5 mL PBS buffer, removed from the filtration funnel and embedded in anti-fading fluorescent oil on a microscope slide. Cells were counted with a Zeiss Axiolab microscope at 1000×. A minimum of 200–400 cells and not less then 10 observation fields were examined (Kirchman et al., 1982). The total number of prokaryotic cells was calculated following the equation in Kepner and Pratt (1994).

2.8. Flow cytometry

For flow cytometry analysis, sample aliquots (mostly 100 μL) were stained with SYBR Green I (3 μL mL^{-1} working solution) and incubated for 10 min in the dark. As an internal standard, Trucount beads (Becton Dickinson) dissolved in fresh 0.2 μm-filtrated PBS buffer were used. For practical reasons and economic consideration, one Trucount tube was amended with 4 mL of PBS buffer and then split into four aliquots. Sample analysis was performed with a LSR II flow cytometer (488 nm and 633 nm laser, Becton Dickinson). The instrument settings were as follows: forward scatter (FSC) 350 mV, side scatter (SSC) 300–370 mV, bandpass filter 530 nm (B530) 500–580 mV, and bandpass filter 610 nm (B610) 650 mV adjusted based on a good separation of *P. putida* cells and Trucount beads. The counting gate for prokaryotic cells was set at the beginning of analysis and then repeatedly checked with a *P. putida* culture comparing green (B530) and red (B610) fluorescence and further adjusted to separate the cell populations from background particles. To minimize background noise, the signal threshold was adjusted to 200 mV. All analyses were performed at a minimum flow rate (about 10 μL min^{-1}). Samples were measured in triplicates and the BD FACSDiva software package (Becton Dickinson) was used for data analysis. In order to compare the results obtained by flow cytometry with results from microscopy, aliquots of the same samples were counted in parallel.

Calculation of the total number of cells per cm^3 sediment (N_{bac}) was derived through the experiments according to:

$$N_{bac} = \frac{cells_{counted} \times beads_{total} \times V_{fractions}}{beads_{counted} \times V_{sample} \times V_{sediment}} \times 1.43 \times 1.28 \times 1.1$$

where $cells_{counted}$ = number of events in the gate for the prokaryotic cells, $beads_{counted}$ = number of events in the gate for the beads, $beads_{total}$ = total number of beads in the sample, $V_{fractions}$ = volume of the measured sample fractions [mL], V_{sample} = volume of the sample taken for the flow cytometer [mL], $V_{sediment}$ = volume of sediment treated [cm^3], 1.43 = cell dislodgement efficiency factor (see Section 3), 1.28 = corrections factor for the loss of prokaryotic cells during the DGC not found in the target fractions 2 & 3 (see Section 3), and 1.1 = factor which corrects for sediment pore water not transferred after dislodgment.

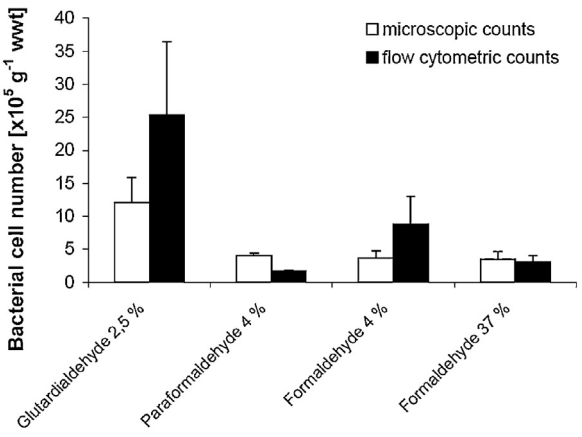


Fig. 1. Total direct counts of prokaryotic cells subsequent to different fixation treatments and comparatively determined by flow cytometry and epifluorescence microscopy. Values are triplicates + SD.

2.9. Statistics

For statistical evaluation of the data, the statistic package in SIGMA Plot 11 and the software R (version 2.9.1) were used. In SIGMA Plot, student's *t*-test or Mann–Whitney Rank test was applied for comparison of two groups of values. To compare multiple groups, one-way ANOVA was used. In R, two-sample *t*-test with unequal variances to test statistical significance was applied. Differences were considered to be significant at a confidence level of $p < 0.05$ in all tests.

3. Results and discussion

A multitude of studies has shown a close link between the total number of bacteria (prokaryotes) and sediment grain size, organic matter content, microbial respiration and grazing, as well as individual biogeochemical processes (e.g. Bott and Kaplan, 1985; Dale, 1974; Deflaun and Mayer, 1983; Griebler et al., 2001; Lucchesi and Santangelo, 1997). Similarly, a tight relation between the total number of prokaryotic cells and the degree of contamination and biodegradation activity in polluted aquifers have been shown (Haack and Bekins, 2000; Anneser et al., 2010; Meckenstock et al., 2010). The total number of prokaryotic cells in sediments is thus indicative for hot spots and a crucial parameter to understand the ecosystem status and functioning. In consequence, fast and reliable methods for sample preservation, preparation and quantification of cells are needed.

3.1. Evaluation of fixatives

The comparison of different fixatives and dyes at varying concentrations showed highest cell numbers, best fluorescence yield and minimum background staining of particles when fixed with 2.5% glutardialdehyde and stained with SYBR Green I. This was evident for sediment samples but also for water samples (data not shown). Fig. 1 shows the difference in total counts of SYBR Green I stained cells subsequent to different fixation treatments. Glutardialdehyde fixed samples revealed significantly higher cell numbers for microscopic (One Way ANOVA, $p = < 0.001$ to 0.002) and flow cytometric counts (One Way ANOVA, $p = 0.001$ to 0.005).

It was shown, e.g. for marine water samples, that fluorescence was best with cells not fixed at all when compared to fixation with formaldehyde (Lebaron et al., 1998). This approach, however, has serious limitations. First, samples without any fixation need to be cooled and processed within a few hours of collection (Ziglio et al., 2002), while fixation allows storage of samples for several

weeks to months (Davey and Kell, 1996). Even storage at 4 °C may lead to significant cell loss, as it was shown for sediment samples (Duhamel and Jacquet, 2006). Even more important with sediment samples, fixation with aldehydes leads to denaturation of proteins and gives the cells robustness which is advantageous during the process of dislodgement (homogenization and sonication) from sediment particles (Griebler et al., 2001).

With water, sample preservation with formaldehyde or paraformaldehyde is the most common approach (Joachimsthal et al., 2003). However, a better performance of glutardialdehyde fixation when compared with formaldehyde and paraformaldehyde has been reported by Lunau et al. (2005) and Martens-Habbena and Sass (2006), respectively. Our study supports these findings with respect to cells from a shallow sandy Quaternary aquifer. However, these observations are in contradiction to findings of Duhamel & Jacquet who obtained higher bacterial counts from sediment samples when fixed with formaldehyde (Duhamel and Jacquet, 2006).

3.2. Comparison of different staining solutions

Several independent studies showed that cells stained by green fluorescent dyes are easier to distinguish from non-specifically stained particles than cells stained with dyes such as DAPI and Acridine Orange (Griebler et al., 2001; Gruden et al., 2003). This is confirmed by our study. With our microscopic observations, the best results were obtained for SYBR Green I stained samples at a final dye concentration of 0.8–1 $\mu L\,mL^{-1}$. With the flow cytometry analysis, where only SYBR Green I and CYTO 59 have been tested, no significant difference between the various SYBR Green I concentrations tested could be found. Consequently a final concentration of 0.3 $\mu l\,mL^{-1}$ was applied.

3.3. Efficiency of cell dislodgement and recovery

Homogenization (e.g. Dale, 1974), ultrasonication (e.g. Epstein and Rossel, 1995), and the combination of both (Velji and Albright, 1993; Weinbauer et al., 1998) have been found most suitable in the past for the detachment of cells from particles. Homogenization separates cells from sediment particles by mechanical forces. Ultrasonication causes separation of cells and sediment particles as well as disaggregation of cells by vibration. Both techniques may also be applied to destroy or digest cells (Lindahl, 1996). Consequently, the intensity and duration of these treatments are crucial. Here, homogenization was used to dislodge cells from sediment particles and brief ultrasonication was applied to disaggregate cells after DGC (see below). Treatment of sediment samples in an ultrasonic bath was not further considered for cell detachment as in preliminary experiments it was found to produce a huge amount of small inorganic particles (data not shown).

To test the destructive force and cell recovery efficiency related to homogenization by means of a mini shaker and a swing mill at different settings, sterile quartz sediment was spiked with a known number of cells from a *P. putida* culture. Processing sediment aliquots in the swing mill at 20 Hz for 3 min resulted in least cell loss and thus in best recovery, while mixing on a shaker at 1400 Hz and 10 Hz for 3 min each produced significantly lower cell numbers (t-test, $p < 0.01$). Similarly, the swing mill treatment at higher intensity (30 Hz) revealed significant lower cell counts (t-test, $p < 0.05$), indicating increased cell damage (Fig. 2).

Testing the efficiency of cell removal our standard protocol (see later) was repeatedly applied to various types of sediments (spiked quartz sand, the test sediment, and the contaminated sediment). It was found that only 70 ± 5% of the prokaryotes were released and recovered in the first treatment round, while the following repetitions disclosed significantly lower numbers of cells; i.e. 17 ± 2% in the second, 8 ± 1% in the third and 5 ± 2% in the fourth run. A higher cell release efficiency of up to 90% has been reported for different types of sediments applying a comparable detachment protocol (Griebler et al., 2001). A much lower cell recovery of only 25% and less have been reported by Lindahl (1996).

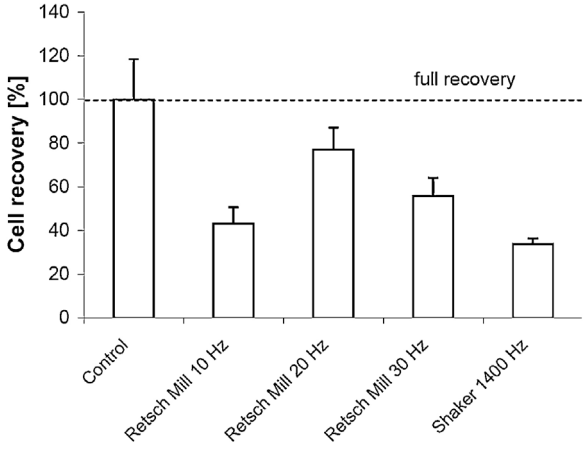


Fig. 2. Recovery of culture bacteria (*P. putida*) spiked to sterile quartz sand subsequent to different homogenization treatments, i.e. in a swing mill at different intensities or in a mini shaker. The number of cells spiked served as the control. Values are triplicates + SD.

A large number of publications compare procedures for detachment of prokaryotic cells from sediment particles. Buesing and Gessner (2002), for example, systematically compared ultrasonic bath, ultrasonic probe, homogenizer and blender for varying times of treatment of sediment and found the mechanical removal to be more efficient than ultrasonication, which is clearly supported by our findings. Mermillod-Blondin et al. (2001) evaluated ultrasonic bath against ultrasonic probe, and the latter was found to be more effective and less damaging to cells, a finding which is supported by earlier results of Epstein and Rossel (1995). Even minor differences (e.g. instrument settings, power) in detachment procedures with the same basic approach and equipment can lead to significantly different results (Buesing and Gessner, 2002; Epstein and Rossel, 1995). Besides the time and intensity of treatment, sample size may play an important role (Epstein and Rossel, 1995). Moreover, the addition of surface active detergents like PPI, Tween80 or methanol was shown to potentially improve the removal efficiency (Bakken and Lindahl, 1995; Epstein and Rossel, 1995), but may at the same time interfere with cell counting (Griebler et al., 2001; Velji and Albright, 1993). Supplementary application of enzymatic treatment for the degradation of extracellular polymeric substances was used by Kallmeyer et al. (2008). None of the latter two approaches have been applied in this study. We conclude that there is obviously not the one method of choice. Rather, a proper determination of detachment efficiency is important.

The success in detachment may also vary with species and strains of prokaryotic organisms (Lindahl and Bakken, 1995). One explanation for this may be the growth stage of the bacterial cells. Slowly growing cells have been shown to develop stronger hydrogen bonding to sediment particles than fast growing cells (Priemé et al., 1996). The distinct behavior of different species and fast growing versus slowly growing cells may potentially influence the cell removal efficiency in natural sediment samples, an aspect which awaits further investigation.

Without doubt, to obtain an optimized detachment protocol a compromise has to be found between detachment efficiency and cell damage. Based on the different removal efficiencies, various correction factors have been established (Ellery and Schleyer, 1984; Griebler et al., 2001). Based on our findings, we set a correction

Table 1
Quantitative distribution of prokaryotic cells from selected sediment samples in the Nycodenz-gradient after DGC as determined by flow cytometry ($n = 3$).

Fraction	mL	*P. putida* + sterile "test sediment" (%)	*P. putida* (%)	*P. putida* + quartz sand (%)	"Contaminated sediment" (%)
1	0–1	0.5 ± 1	1 ± 1	3.5 ± 6.5	1 ± 0.5
2	1–2	74 ± 4	81.5 ± 9.5	68 ± 15.5	66 ± 10
3	2–3	9 ± 5	9 ± 7.5	12 ± 7.2	12 ± 6.5
4	3–4	3.5 ± 2	1.5 ± 1	8.5 ± 4	5.5 ± 4
5	4–5	6 ± 2	1.5 ± 1	3.5 ± 0.2	6 ± 2
6	5–6.5	7 ± 0.5	5.5 ± 8	4 ± 2.2	9.5 ± 8

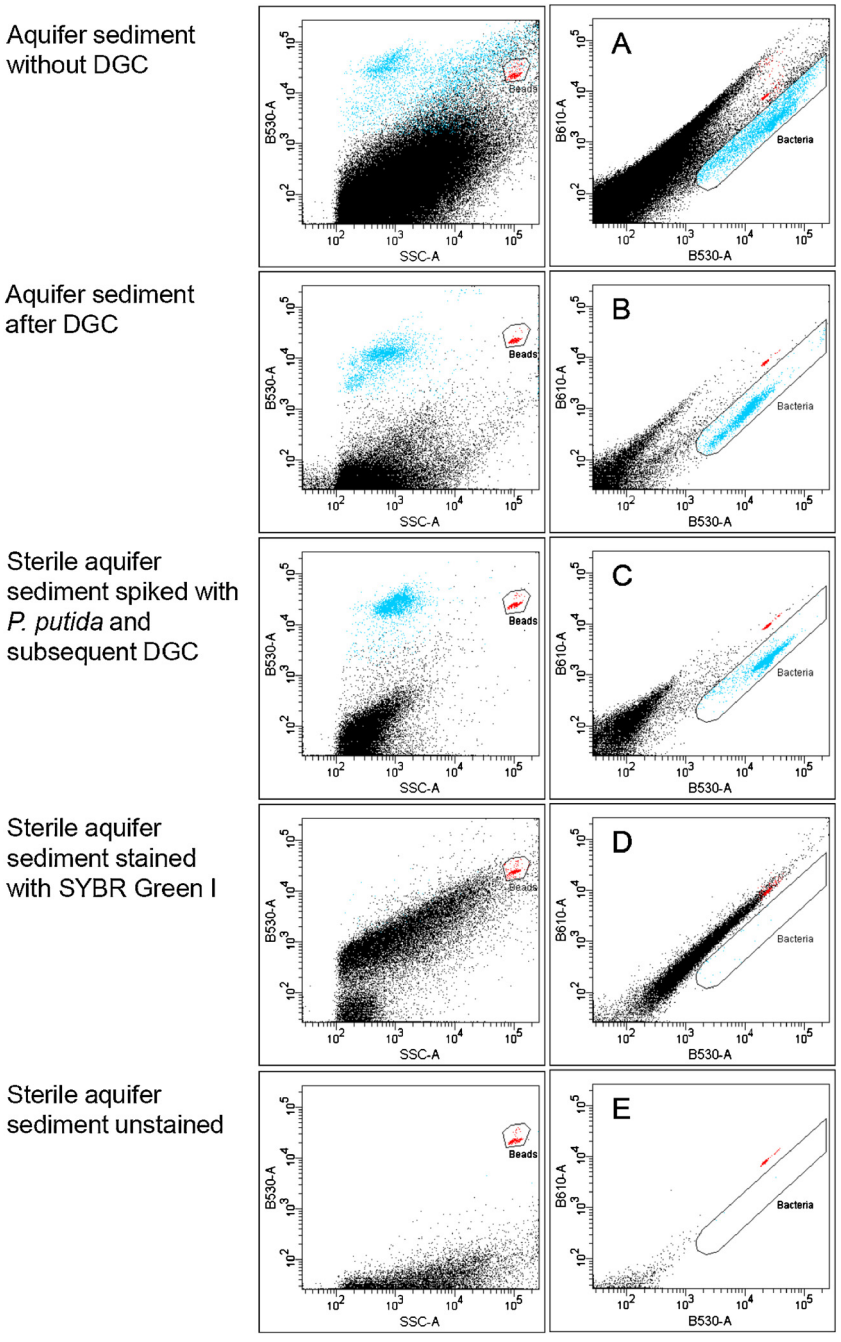


Fig. 3. Flow cytometry results for (A) "test sediment" processed without DGC, (B) "test sediment" treated following our standard protocol, (C) sterile "test sediment" spiked with *P. putida* and subsequent DGC, (D) sterile "test sediment" stained with SYBR Green I, and (E) unstained sterile "test sediment". All samples contained the same amount of fluorescent beads used as internal standard.

factor of 1.43 in accordance to the 70% removal efficiency during a one step dislodgement treatment in the swing mill with the two different sediments tested. Approaches alternative to a cell dislodgment are to simply correct for potential masking by sediment particles, as applied for soft sediments (Schallenberg et al., 1989), or to work with highly diluted samples to reduce the load of inorganic particles (Gough and Stahl, 2003). Boenigk (2004) tried to reduce the number of interfering particles in sediments containing clay and fine silt by partial dissolution with hydrofluoric acid, which on the other hand led to an increase of background fluorescence after staining.

3.4. Density gradient separation of cells and inorganic particles

Density gradient centrifugation of water samples containing prokaryotic cells dislodged from different sandy aquifer sediments or sterile sediment spiked with *P. putida* cells revealed a similar pattern. The bulk of cells after centrifugation were always found in fraction 2 and 3 of the centrifuge tube, with the major portion of cells contained in fraction 2. Details of the distribution of cells along the Nycodenz density gradient are provided in Table 1.

In numbers, 75% to 85% of all cells accumulated in the fractions 2 and 3. For our standard protocol, we set a correction factor of 1.28 to account for the loss of cells to other fractions of the Nycodenz gradient. Similarly, Bakken and Lindahl (1995) found more than 95% of a pure culture of bacteria and about 89% of bacteria extracted from soil in the cushion (comparable to fraction 2 in our approach) of the Nycodenz after DGC. Up to 30% of the cells after DGC have been found in the pellet (Kallmeyer et al., 2008), which contained cells that remained sorbed to particles that were spun down during centrifugation. In our experiments, actually only 4–9.5% of the cells were found in the bottom fraction of the centrifugation vial which contained the pellet. In addition to counting the cells via flow cytometry following our standard protocol (see below), we examined the pellets microscopically and this revealed similar results (data not shown).

The qualitative improvement of sample preparation, i.e. the reliable separation of non-prokaryotic "particles" from cells, is highlighted in Fig. 3. From Charts 3A and 3B the tremendous reduction of the huge particle load (noise) is obvious. Moreover, spiking sterile "test sediment" with *P. putida* cells confirmed the reliable separation of bacterial cells from the background noise (Fig. 3C). Fig. 3D and E underline the effect of unspecific staining, when adding the fluorescence dye to sterile, organic carbon free (muffled) "test sediment".

The non-specific staining of sediment particles is a well-known phenomenon which happens with nearly all kinds of DNA-specific dyes (Epstein and Rossel, 1995). Klauth et al. (2004) compared different green fluorescent nucleic acid dyes focusing on non-specific staining with sediments. In general, SYTOX Green exhibited a significantly lower background staining effect to sediments than other dyes. However, the results varied broadly with different types of soils tested (Klauth et al., 2004).

With all samples tested, a sufficient and reproducible separation of prokaryotic cells from other particles could be obtained when plotting the green (B530) versus the red fluorescence (B610) of SYBR Green I stained samples (Fig. 3). A similar separation and quantification strategy was already followed for water samples by Hammes and Egli (2005).

3.5. Microscopy vs. flow cytometry

When staining samples subsequent to DGC with SYBR Green I, microscopic observations showed bright greenish cells besides many light green and yellow particles with merely moderate fluorescence. Only the green and bright fluorescing objects with a regular round shape (coccoides, rods) and size of prokaryotes were considered prokaryotic cells, leading to cell numbers slightly lower, but not statistically different from that obtained by flow cytometry. A similar result was obtained when comparing both methods with samples from a *P. putida* culture (Table 2), corresponding to the results of Gruden et al. (2003).

Table 2
Total cell counts from flow cytometry and direct microscopic observation of samples from a *P. putida* culture and replicate samples from treated "test sediment" subsequent to treatment according to our evaluated 'standard protocol'.

Counting method	*P. putida* [mL^{-1}]	Sediment cells [cm^{-3}]
Microscopy	$1.26 \times 10^8 \pm 2.25 \times 10^7$	$1.20 \times 10^6 \pm 2.25 \times 10^5$
Flow cytometry	$1.92 \times 10^8 \pm 3.45 \times 10^7$	$1.98 \times 10^6 \pm 2.74 \times 10^5$

An advantage of microscopic counts is the direct observation, so particles can be assessed by taking into account their size, shape and staining behavior. However, the assessment of fluorescing objects based on their fluorescence intensity relies on the counter's subjective perception, which was shown to vary considerably among researchers (Joachimsthal et al., 2003).

With flow cytometry fluorescing particles are only lighted once when they pass the laser beam so fading is no problem. The flow cytometer is able to exactly measure fluorescence intensities. Therefore, it can be clearly defined which particles are considered as prokaryotes based on their fluorescence and/or scattering properties. On the other hand, there is a certain range of error in data analysis, i.e. for instance when setting the gates in the plots (see Fig. 3). The decision what to include in the gate relies at least partly on human judgment, since calibration is difficult. Fluorescent beads and cells from cultures are useful but may have a different behavior than individual populations in natural sediment prokaryotic communities (Fig. 3A and C). Without doubt, flow cytometry allows an unprecedented high sample throughput.

With water samples flow cytometry developed to a routine technique for quantification of cells as well as for the characterization of cell size, cell structure, nucleic acid content and viability (Berney et al., 2008; Hammes et al., 2010). Especially, the distinction of high and low nucleic acid cells gained momentum recently for studies of the dynamic physiology of prokaryotes in the environment (Bouvier et al., 2007; Wang et al., 2009). This approach awaits application with sediment samples in the near future.

3.6. Standard protocol for flow cytometry analysis

Based on the optimization of the various critical steps in the quantification of sediment prokaryotes, the following standard protocol is proposed (Fig. 4): 0.5 cm^3 of fresh sandy sediment is fixed with 1.5 mL of 2.5% glutardialdehyde and stored at 4 °C in the dark until further processing. After brief centrifugation the glutardialdehyde containing supernatant is replaced by 1.5 mL PBS. The sample is then treated in a swing mill for 3 min at 20 Hz. Thereafter, the turbid supernatant is collected and layered on 5 mL 4 °C-cold Nycodenz solution in a 10-mL-centrifuge tube. DGC follows as described above. Fractions 2 and 3, containing the bulk of cells, are collected and combined. Subsequently, an aliquot volume is subsampled and diluted in particle free PBS containing fluorescence beads at a ratio of 1:10 or 1:100, depending on the expected cell number in the sample. Finally, samples are stained with SYBR Green I (3 $\mu L\,mL^{-1}$ of diluted working solution) and analyzed by flow cytometry (for details see Section 2).

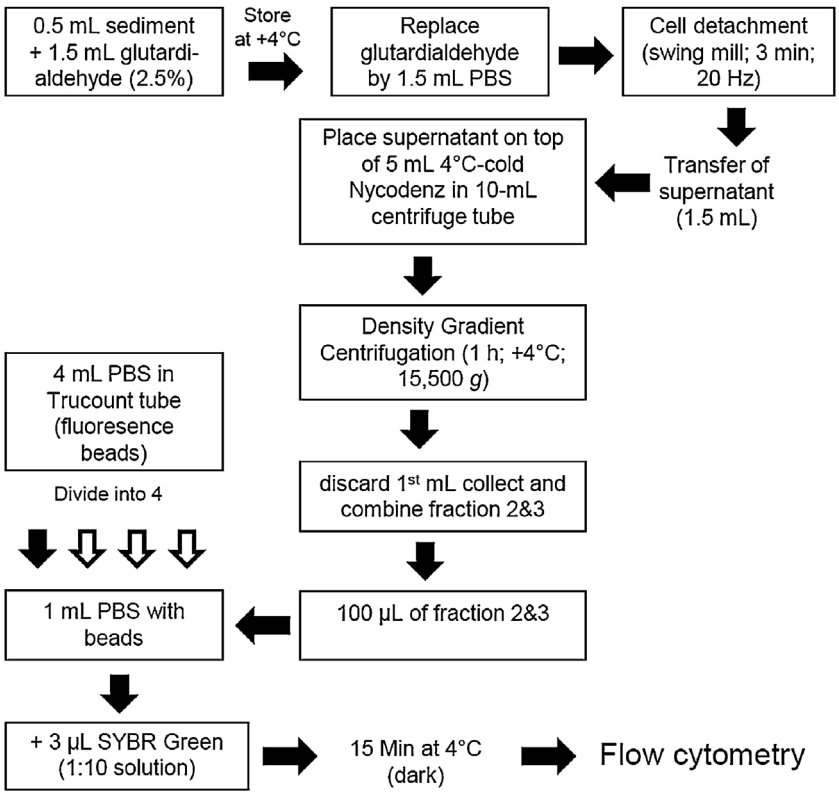


Fig. 4. Flow diagram highlighting the different steps of the 'standard protocol' for the quantification of sediment prokaryotes.

4. Conclusions

A robust and reliable protocol for the direct quantification of prokaryotic cells in sandy sediments is introduced. Critical steps in sample preservation as well as preparation prior to counting including cell detachment, density gradient centrifugation, and staining, were re-examined and improved in a concerted way. As the final step, flow cytometry is applied for cell counting allowing high-throughput sample processing. Without doubt, the efficiency of a single quantification protocol strongly depends on the specific properties of the sediment sample. The stepwise evaluation and in-depth discussion of the various critical steps in sample preparation provided in this manuscript allow the reader an individual systematic and easy modification of our protocol for other types of sediment.

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