



A disintegration method for direct counting of bacteria in clay-dominated sediments: dissolving silicates and subsequent fluorescent staining of bacteria

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Abstract

The masking of bacteria by abundant microparticles of the clay and silt fraction and cell losses due to sonication hampered direct enumeration of bacteria in sediments dominated by fine sediments. These problems can be circumvented by dissolving silicate fine particles using hydrofluoric acid and subsequent staining of bacteria by DTAF. The developed disintegration method partly replaces mechanical separation of bacteria from sediment particles by chemical disintegration of the silicates. Recovery efficiency ranged from 90% to 111% for different clays and clay-dominated sediments. Especially for the analysis of fine sediments and clays, this method circumvents both strong dilution of the sediment sample and harsh sonication. The method can also therefore be used in sediments where particle abundance is several orders of magnitude higher than bacterial abundance and simple dilution would not suffice in reliably counting bacteria.

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1. Introduction

Enumeration of microorganisms in sediments is generally limited by separating organisms from sediment particles. Especially in clays and fine sediments, where particles are much more abundant than bacteria, simple dilution often does not suffice for reliable counts. Solutions for counting bacteria in such fine sediments are controversially discussed. Soil and sediment bacteria are usually enumerated

by applying fluorescent stains after strong dilution of the sediment suspension by epifluorescence microscopy or confocal laser scanning microscopy (Bloem et al., 1995; Sunamura et al., 2003; Gough and Stahl, 2003; Weinbauer et al., 1998) rather than using indirect methods such as the most probable number (MPN) methods. Whereas MPN methods depend on culturability of the bacteria, direct counting is limited especially in fine sediments due to limits in the detachment and separation of bacteria from sediment particles. Quantitative detachment of bacteria is generally preferable for enumeration of sediment bacteria (Fry, 1988). Different detachment methods have been suggested for separating bacteria from sediment par-

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ticles including strong sonication and shaking (Buesing and Gessner, 2002). Cell disruption is one of the risks of sonication and therefore a protocol avoiding harsh sonication is preferable.

Separation of bacteria from fine sediment particles is also limited. Differential filtration methods have been applied towards the separation of larger protists from sediment particles (Griffiths and Ritz, 1988), but such methods are not suitable for separating bacteria from fine sediment particles, i.e. clay particles. Strong dilution is the usual procedure for reducing the particle abundance. This procedure, however, has its limits when the abundance of fine sediment particles is several orders of magnitude higher than bacterial abundance, as is the case in clays. Even less than 1 mg of clay particles completely covers the area of a 25-mm filter and therefore may mask bacteria. Different staining protocols have been suggested for differential staining of bacteria (Kuwaie and Hosokawa, 1999; Noble and Fuhrman, 1998; Griebler et al., 2001; Weinbauer et al., 1998). Commonly applied dyes such as 4',6-diamidino-2-phenylindole (DAPI) and even the novel high-affinity nucleic acid dyes such as SYBR Green I and SYTO 13 (Molecular probes) tend to bind at least to some clay minerals (Weinbauer et al., 1998; own observation). Even though these dyes are specific enough for routine investigations in many silt- and sand-dominated soils, their applicability is limited when nonspecific binding to clay minerals occurs. The reduction of sediment particles prior to analysis of the sample is therefore desirable to reduce both the particles that may mask bacteria, as well as nontarget binding sites for fluorescence dyes. The applicability of a modified protocol for solving silicates using hydrofluoric acid (HF) in combination with fluorescent staining of bacteria for subsequent direct enumeration of sediment bacteria was tested. In geosciences, hydrofluoric acid is used for disintegration and subsequent element analysis in silicates. The harsh conditions that are usually applied destroy bacterial cells. I therefore optimised the acid treatment for microbiological purposes. This procedure significantly reduces the amount of sediment particles without destroying bacterial cells and allows for low intensity sonication as bacteria are released from sediment surfaces chemically rather than mechanically.

2. Methods

2.1. Media used for the maintenance of microbes

Nutrient broth soytone yeast extract (NSY) medium was used for the maintenance of bacterial strains (Hahn et al., 2003). For the growth of bacterial strains, the inorganic basal medium was supplemented with 3 g l⁻¹ of nutrient broth, soytone, peptone, and yeast extract (all obtained from Difco). For the initial washing of the bacterial cultures and for the maintenance of axenic *Poteroiochromonas* sp. strain JBM10 cultures, the inorganic basal medium (see above) was used.

2.2. Microbial strains

Tests were performed using the ultramicrobacterial strains (<0.1 µm³) MWH-Mo1 [Actinobacteria, closest known relative *Clavibacter michiganensis* (Microbacteriaceae); Hahn et al., 2003], MWH-MoNR1 (Betaproteobacteria; next relative *Polynucleobacter necessarius*; Hahn, in press), the bacterial strain *Listonella pelagia* CB5 (99.6% similarity; genbank synonym *Vibrio pelagius*; Hahn, 1997; Hahn and Höfle, 1998), and a natural bacterial community from Lake Mondsee sampled from 1-m depth. The bacterial strains are representatives of typical free-living bacteria including gram-positive as well as gram-negative strains. In addition, the axenic chrysoomonad flagellate *Poteroiochromonas* sp. strain JBM10 isolated from an artificial pond in Mondsee (Boenigk et al., in preparation) was used. As bacteria-free controls, 0.2-µm poresize-filtered cell suspensions were used.

2.3. Influence of acid treatment and staining protocol on the detectability of bacteria

Bacterial strains were grown in organic NSY medium. Aliquots were taken from the exponential and stationary growth phases. Cells were collected by centrifugation (5000 × g for 5 min) and resuspended in inorganic NSY medium. Cell suspensions were diluted to a cell concentration of 10⁷ cells/ml. The natural bacterial community was directly taken from Lake Mondsee and immediately processed without further concentration steps. Cells were preserved with formalin (final concentration 2%) and

glutaraldehyde (final concentration 1%) 30 min prior to the experiments.

Bacterial cells were exposed to 1 M HCl and 22 M HF, respectively, for 5 and 30 min. Either 2 M HCl or 44 M HF were added to the bacterial suspension to the above final concentrations. After 5 and 30 min, the acid cell suspension was neutralised using a 2 M KOH/0.1 M K_2HPO_4 solution. The HCl-treated cell suspensions were, in addition, directly filtered onto black nucleopore filters and washed with 2 ml of 1 M K_2HPO_4 and subsequently with 1 ml aqua dest. Bacteria were then stained in suspension or on the filter by applying the seven below staining protocols. In a second treatment, bacteria were stained prior to the addition of acids. Acid treatment was additionally applied after bacterial suspension had been collected onto filters. The filters were exposed to the acids and washed subsequently in 1 M K_2HPO_4 , 1 M $CaCl_2$, and aqua dest after 5 and 30 min.

To determine the suitability of different stains, staining was applied prior to and after exposure of the bacterial cells to acids. After staining, the bacteria were collected onto black polycarbonate filters (Millipore) backed by 0.45- μ m poresize cellulose nitrate membrane filters (Millipore) by vacuum filtration (usually 20 kPa). Filters were inspected using a Zeiss Axioplan 40 equipped with a HBO 103 mercury lamp for fluorescence. Image analysis was carried out using a Zeiss Axioplan equipped with a HBO 103 mercury lamp and the LUCIA image analysis software. The following staining protocols were tested:

4',6-Diamidino-2-phenylindole (DAPI): Staining procedure followed the protocol by [Porter and Feig \(1980\)](#). DAPI stock solution (100 mg l^{-1}) was added to the cell suspension providing a final concentration of 20 mg l^{-1} . After 5 and 30 min of incubation, an aliquot was filtered onto black nucleopore filters and checked under the epifluorescence microscope using filter set 01 for UV excitation (Zeiss no. 488001-0000).

5-([4,6-Dichlorotriazin-2-yl]amino)fluorescein (DTAF): 2 M K_2HPO_4 solution was added to the bacterial suspension to a final concentration of 0.1, 0.5, and 1 M K_2HPO_4 . DTAF concentrations were adjusted to 0.2, 0.1, and 0.033 mg ml^{-1} and the cell suspension was incubated for 60, 75, and 90 min at 65°C . Cells were then collected onto black nucleopore filters (20 and 45 kPa) and inspected under an epifluorescence microscope using filter set 09 for blue excitation (Zeiss no. 488009-0000).

The green fluorescent dyes SYBR Green I [$10,000 \times$ in dimethyl sulfoxide (DMSO); Molecular Probes, chemical no. S-7567], Pico-Green (dsDNA quantitation kit; Molecular Probes, chemical no. P-7581), and SYTO 13 (Molecular Probes, chemical no. S-7575): The bacterial suspensions were stained for 30 min with a 2000- to 10,000-fold diluted stock solution of the stains and subsequently filtered onto black nucleopore filters using filter set 09 for blue excitation.

Primulin: a stock solution containing 0.1 M TRIS, 250 mg l^{-1} Primulin, and 3% formalin was prepared and 5 ml of cell suspension was mixed with 10 ml of the stock solution. Cells were incubated for 20 min and subsequently filtered onto black nucleopore filters. Filters were inspected under an epifluorescence microscope using a filter set 09 for blue excitation.

2.4. Minimizing of unspecific staining: optimisation of neutralisation and washing procedure

Different neutralisation procedures using distinctive concentrations and combinations of Na_2CO_3 , K_2HPO_4 , KOH, and NaOH were tested. Background fluorescence and staining of nontarget objects, i.e. sediment particles, as well as the filter, was always problematic. To minimize these effects, different washing procedures were tested. These tests were only performed for staining protocols which proved to stain bacterial cells after acid treatment (see below). After staining and filtration, the filters still mounted onto the filtration funnel were washed with (1) 1 ml (2 ml) aqua dest, (2) 1 ml (2 and 5 ml) 1 M K_2HPO_4 solution, (3) 1 ml (5 and 10 ml) 1 M HCl, (4) 1 ml (2, 4, 6, 8, and 10 ml) 2 M NaOH, (5) 1 ml 2 M KOH, or (6) 1 ml (2 ml) 0.5 M KOH. In addition, combinations of washing steps using the above solutions were tested. Washing was tested using vacuum filtration at 20, 40, and 50 kPa.

2.5. Acid resistance of bacterial cells

Cell losses due to acid treatment were determined. Cells were exposed to 22 M HF for 20, 40, 60, 120, 240, and 360 min at 0, 25, 30, 60, and 80°C . Afterwards, cell suspensions were neutralised and stained using the proposed protocol and counted

under the epifluorescence microscope. Controls were counted using the original cell suspension stained with DAPI for 30 min. In addition, hydrofluoric acid concentrations of 36.5%, 24.5%, 12%, and 6% were applied for different time intervals at 0, 30, and 60 °C.

2.6. Enumeration of soil and sediment bacteria

Clay- and loam-dominated soils, as well as different clays were used: a kaolinite-dominated clay (Heide, 1955) and a kaolinite/montmorillonite-dominated clay

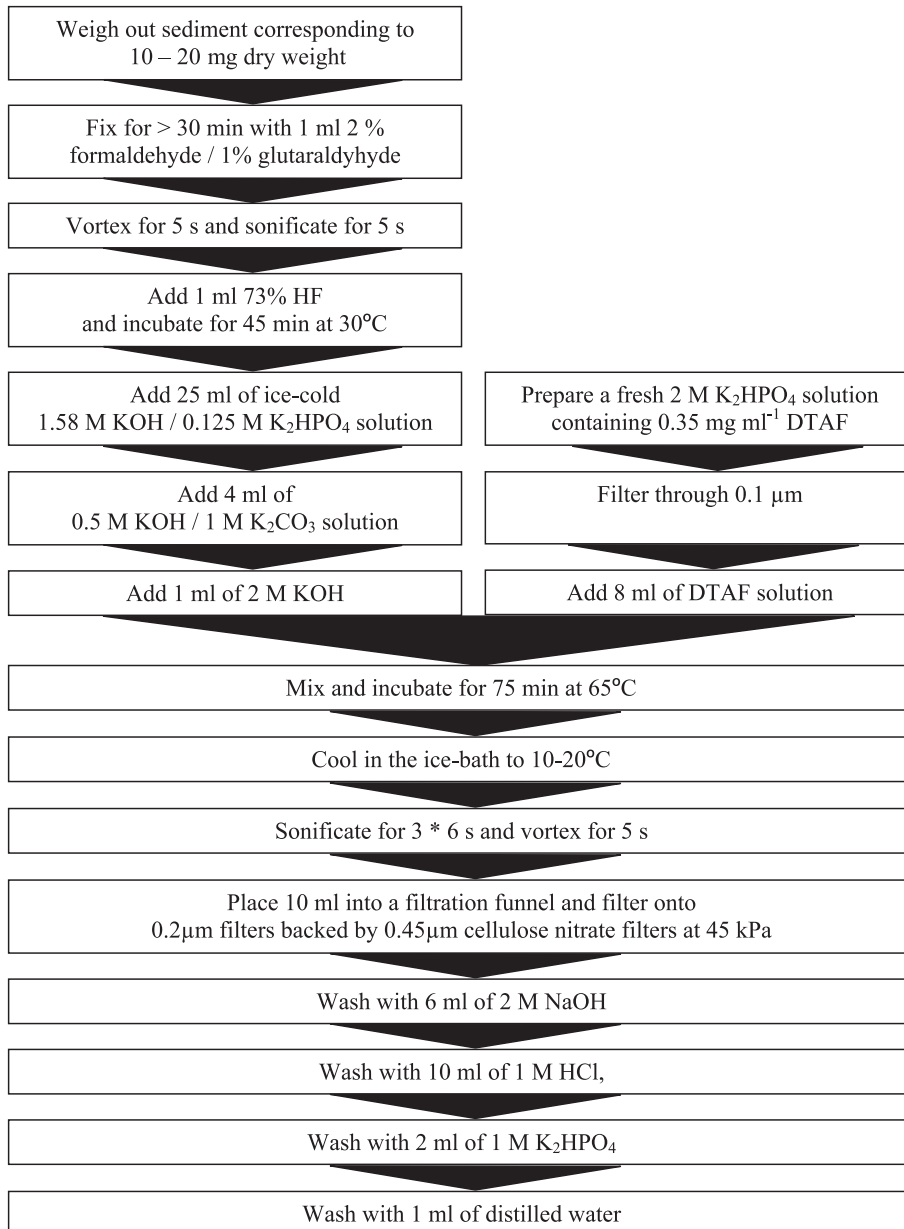


Fig. 1. Scheme of the proposed disintegration method for preparing filters for counting bacteria in sediment samples using epifluorescence microscopy. For explanations, see text.

(Villwock, 1959) from clay pit Kärlich in the Eifel further referred to as “Kärlich 1” and “Kärlich 2”, a commercial bentonite (IKOMONT WB 90, IKO Minerals, Mannheim, Germany), and a commercial kaolinite (Edelkaolin, Fa. Kamig, Schwertberg, Austria).

Natural soils were taken from different locations near Mondsee. All sediments were dominated by particles of the clay and fine silt fraction.

Water content of the sediments was determined by drying the sediments at 105 °C for 24 h. For the

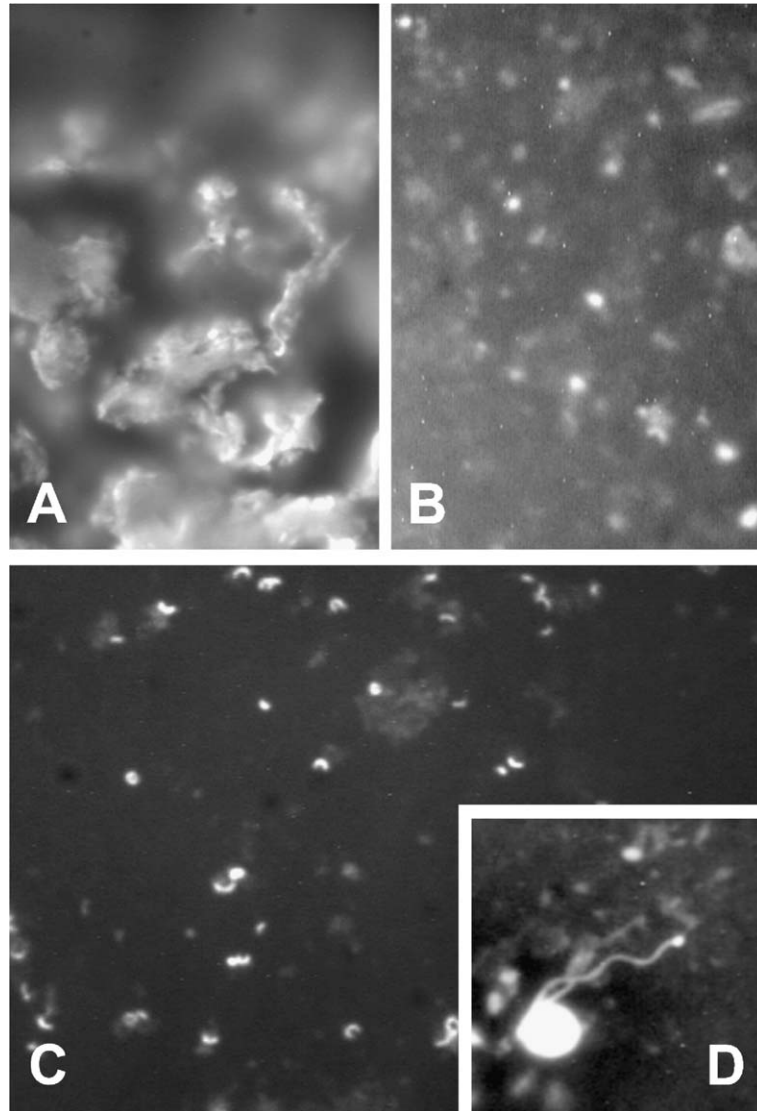


Fig. 2. Epifluorescence images of the clay “Kärlich 2” spiked with the ultramicrobacterium MWH-MoNR1 and the chrysoomonad JBM10. (A) Two milligrams of original spiked clay after DAPI staining filtered onto a 25-mm polycarbonate filter and inspected at 100 × magnification. (B) One milligram of spiked clay after staining with SYBR Green I following a previously published protocol (Weinbauer et al., 1998). Clay covers the entire filter area and most bacteria are covered by clay particles. Background fluorescence due to stained clay particles is high. (C) Two milligrams of spiked clay after applying the disintegration method. The bacterial strain shows the curved morphology which is typical for this strain. (D) Two milligrams of clay spiked with the chrysoomonad strain JBM10 after applying the disintegration method. The flagellate cell and the long and the short flagellum are clearly visible.

analyses, sediment subsamples corresponding to 10–20 mg of dry sediment was preserved with 1 ml of 2% formaldehyde/1% glutaraldehyde solution and stored at 4 °C. To test the recovery efficiency of cells, samples were spiked at a concentration of 8×10^9 cells g^{-1} soil with the bacterial strain MWH-MoNR1 and the chryomonad strain JBM10, respectively. Cells were counted after applying the proposed protocol (see below) in spiked and unspiked samples.

For comparison purposes, filters were additionally prepared following a slightly modified protocol proposed by Weinbauer et al. (1998) using DAPI and SYBR green I for direct enumeration of bacteria in a sediment subsample of 0.1 mg. For the comparison of methods, 0.2- μ m Anodisc filters (Whatman) as proposed by Weinbauer et al. (1998) as well as nucleopore filters were tested. At least 400 cells from at least 10 eye fields were counted per filter (Kirchman, 1993).

2.7. Proposed disintegration and staining protocol

A scheme of the proposed protocol is shown in Fig. 1. Immediately prior to the acid treatment, the fixed samples were vortexed for 5 s and subsequently sonicated for 5 s in an ultrasonic bath (Bandelin Sonnex RK100). Samples were augmented with 1 ml 73% HF and incubated for 45 min at 30 °C. The cell suspension was then neutralised by adding 25 ml of ice-cold 1.58 M KOH/0.125 M K_2HPO_4 solution, subsequently 4 ml of 0.5 M KOH/1 M K_2CO_3 solution, and finally 1 ml of 2 M KOH.

A 2 M K_2HPO_4 solution containing 0.35 mg ml^{-1} DTAF was freshly filtered using 0.1- μ m single-use syringe filters (Sartorius no. 16553). Eight milliliters of this suspension were added to the neutralised cell suspension and incubated for 75 min at 65 °C. The suspension was then cooled in the ice bath to 10–20 °C, sonicated for 3×6 s (Bandelin Sonnex RK100) and vortexed for a few seconds. Ten milliliters of the suspension was then placed into a filtration funnel and filtered onto 0.2- μ m Anodisc filters (Whatman) backed by 0.45- μ m poresize cellulose nitrate membrane filters (Millipore) by vacuum filtration at 45 kPa. Filters were then subsequently washed with 6 ml of 2 M NaOH, 10 ml of 1 M HCl, 2 ml of 1 M K_2HPO_4 , and 1 ml of distilled water without reducing the vacuum pressure. Filters were then inspected under

an epifluorescence microscope (Fig. 2) using filter set 09 for blue excitation (Zeiss no. 488009-0000).

3. Results and discussion

Methods that quickly assess microbial density in soil and sediments are needed in environmental microbiology (Resina-Pelfort et al., 2003; Yu et al., 1995; Gough and Stahl, 2003). Direct counting of bacteria in sediments is limited due to masking of bacteria by sediment particles. Masking can be reduced but not excluded by diluting the sample. For accurate counting, however, an average number of around 10 bacteria per microscope ocular field has been suggested (Gough and Stahl, 2003). In fine sediments and clays, the abundance of sediment particles is some orders of magnitude higher than bacterial abundance and simple dilution is not sufficient for separating bacteria from sediment particles.

3.1. Comparison of staining procedures

The use of either Anodisc filters or nucleopore filters had no effect on staining of bacteria and clay particles. Due to the perfect plain surface of the Anodisc filters, these were, however, superior to nucleopore filters. Staining in separate vessels generally provided superior results to staining in the filter funnel, as background fluorescence was reduced. All DNA/RNA stains including DAPI and the green fluorescent dyes faded away during acid treatment, and acid-treated cells did not respond to the staining anymore. Only DAPI-stained cells persisted the HCl treatment, but the remaining signal was weak and completely faded away within a few hours. DTAF and Primulin did persist HCl and HF treatment, but background fluorescence was always high and crystals were visible on the filter. Only DTAF and Primulin, when applied after acid treatment, stained bacteria satisfactorily. Background fluorescence was, however, high and washing of the filters to remove unspecifically bound dyes was necessary. In the case of Primulin, distinction of bacteria from other particles was hardly possible. DTAF staining after acid treatment is therefore recommended. Washing with HCl removed phosphate and carbonate precipitation and provided satisfactory results as long as no sediment

particles were present. In the presence of sediment particles, however, strong precipitation masked the bacterial cells. Additional washing with strong bases was necessary to remove surplus DTAF. Washing with NaOH and subsequent washing with HCl removed surplus DTAF effectively without any bleaching of bacteria, whereas KOH bleached bacterial cells. It was essential to remove the DTAF solution quickly once placed onto the filter, thus requiring a relatively high filtration pressure (40–50 kPa) and immediate washing of the filters without delay. The applied filtration pressure had no significant impact on bacterial numbers (*t*-test, $p \gg 0.05$). Washing solutions have to be added immediately after the filter drops have dried. This was observed to be essential for adding the NaOH and HCl solution. A delay in adding NaOH solution resulted in high background fluorescence, and a delay in adding HCl solution resulted occasionally in the bleaching of bacterial cells resulting in a weak fluorescence yield.

Increased temperature generally increased the disintegration of silicates, as well as bacteria. The best results were obtained for temperatures between 30 and 60 °C for less than 1 h of incubation. In the 0 °C treatment, silicates hardly disintegrated at all even after 24 h, whereas in the 80 °C treatment, a fraction

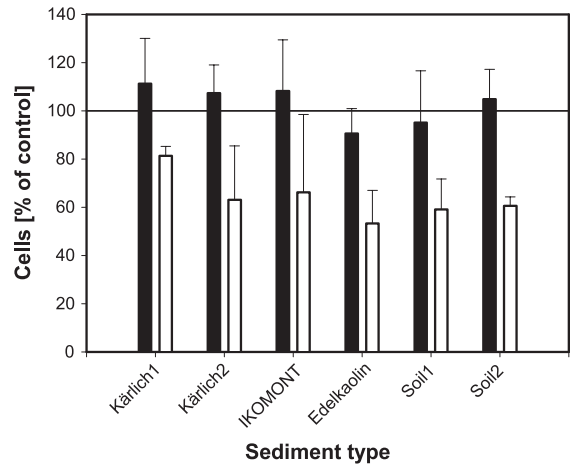


Fig. 4. Recovery efficiency (mean \pm SD) of the bacterial strain MWH-MoNR1 (filled bars) and the chryomonad strain JBM10 (open bars) spiked to different sediment samples. Cell counts are presented as percentage of cell abundance in the original cell suspension (mean \pm SD). Control counts were performed using the original bacterial and flagellate cell suspension.

of the bacterial cells disintegrated even after short incubation times. The time course experiments showed no significant cell disruption due to acid treatment at 30 °C for short-term exposure. Losses

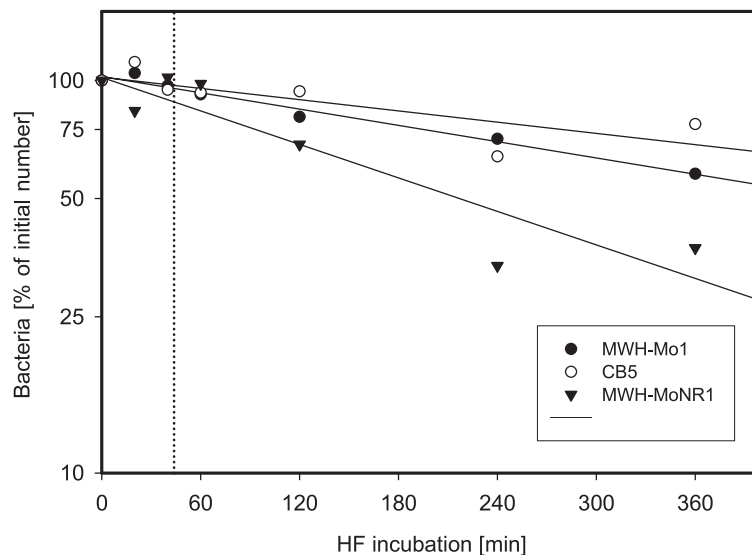


Fig. 3. Changes in the abundance of bacterial test strains after incubation with 36.5% hydrofluoric acid (HF) at 30 °C. Relative abundance of bacteria is plotted against incubation time. Note that even the ultramicrobacterial strains MWH-Mo1 and MWH-MoNR1 show no significant cell losses (see text) for incubation times of less than 1 h. The proposed method includes HF treatment for 45 min only (dotted line).

in bacterial abundance were not significant for treatments below 1 h of HF incubation for all tested strains (Fig. 3) and for the natural bacterial community (*t*-test, $p \gg 0.05$). Only when exposed to hydrofluoric acid for several hours were bacterial losses considerable.

3.2. Cell counts in fine sediments and recovery efficiency of spiked cells

Cell counts in the spiked samples showed that even ultramicrobacteria quantitatively persisted hydrofluoric acid incubation and could be enumerated in sediment samples (Fig. 4). The efficiency of recovery for the MWH-MoNR1 strain averaged 102.5% (range 90–111%). This indicated that the enumeration of cells was not obscured and emphasises that cells are not destroyed by the treatment even though this strain belongs to the ultramicrobacteria ($< 0.1 \mu\text{m}^3$) and was the least resistant to hydrofluoric acid of the tested strains. In addition, no significant losses occurred in the natural bacterial population (*t*-test, $p > 0.05$). It is therefore concluded that natural bacteria also persist the treatment quantitatively. In contrast to the bacteria, efficiency of recovery for flagellates was only around 60% (Fig. 4). Even though flagellates seem to persist acidic conditions during the treatment, the above protocol cannot be recommended as a standard protocol for counting flagellates.

Direct staining of sediments using SYBR Green I or DAPI (Weinbauer et al., 1998) could not be used for the enumeration of bacteria as bacterial cells were masked by clay particles (Fig. 2; Table 1) even in subsamples of less than 0.1-mg sediment. Background

fluorescence of dyes, which were bound to sediment particles, was always high for DAPI and partly high for SYBR Green I (Fig. 2). An exception was the soil sample 2 as the abundance of fine sediment particles was not as high as in the other sediments. In this sample, bacterial counts using the proposed disintegration protocol ($1.96 \times 10^8 \pm 0.31 \times 10^7$ bacteria gram^{-1}) were significantly higher (*t*-test, $p = 0.036$) than using dilution and staining only ($1.16 \times 10^8 \pm 0.21 \times 10^7$ bacteria gram^{-1}).

4. Conclusions

This study indicates that using conventional methods, a fraction of bacteria is masked by sediment particles or is even disrupted by the mechanical treatment. In contrast, hydrofluoric acid can be used for disintegrating silicates in soils and sediments without damaging bacterial cells (Fig. 2). Even though the fluorescent stain DTAF tends to bind to all kinds of particles, the washing procedure reduces background fluorescence significantly, thus allowing for proper differentiation of bacterial cells from remaining sediment particles. In contrast to methods using mechanical force to detach bacteria from sediment particles, i.e. sonication and shaking, the proposed method uses chemical disintegration of the substratum and allows therefore for gentle sonication procedures which are desirable to prevent cell disruption (Buesing and Gessner, 2002). The suitability of the proposed method and its advantage to conventional methods for enumeration of bacteria in clay-dominated sediments is demonstrated for clay-dominated sediments from natural deposits as well as for fine sediment-dominated soil samples.

The most frequent problems hampering the enumeration of cells in direct counting of sediment samples, i.e. masking of bacteria by sediment particles and staining of nontarget particles, could be circumvented with the proposed method. Disintegration of silicates proved to significantly reduce sediment particles in clay-dominated sediments without significant losses in bacteria. Overall, the use of the proposed disintegration method allows for gentle mechanical treatment of sediment samples and opens a new methodological approach for the enumeration of sediment bacteria.

Table 1

Cell counts in soil and clay samples using the proposed disintegration method and a direct counting method using the specific fluorochrome SYBR Green I

| | Bacterial abundance [bacteria g^{-1} sediment] | |
|------------------|---|---|
| | SYBR Green I | Proposed disintegration method |
| Soil 1 | unspecific staining of clay particles too high | $1.10 \times 10^8 \pm 0.73 \times 10^7$ |
| Soil 2 | $1.17 \times 10^8 \pm 0.08 \times 10^8$ | $1.96 \times 10^8 \pm 0.31 \times 10^8$ |
| Clay | unspecific staining of clay particles too high | $7.43 \times 10^6 \pm 3.22 \times 10^6$ |
| Clay “Kärlich 1” | unspecific staining of clay particles too high | |
| Clay “Kärlich 2” | unspecific staining of clay particles too high | $1.17 \times 10^7 \pm 0.97 \times 10^7$ |

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