Limnologica 56 (2016) 6–13

Contents lists available at ScienceDirect

Limnologica

journal homepage: www.elsevier.com/locate/limno

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

Anne Bayer[1], Regina Drexel, Nina Weber, Christian Griebler*

Institute of Groundwater Ecology, Helmholtz Zentrum München, 85764 Neuherberg, Germany

ARTICLE INFO

Article history:
Received 10 March 2015
Received in revised form
11 September 2015
Accepted 2 November 2015
Available online 23 November 2015

Keywords:
Aquifer sediments
Direct cell counts
Sediment bacteria
Detachment
Density gradient centrifugation
Flow cytometry
Method

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.

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.

* Corresponding author. Tel.: +49 0 89 3187 2564; fax: +49 0 89 3187 3361.
E-mail address: griebler@helmholtz-muenchen.de (C. Griebler).
[1] Present address: Bavarian Environment Agency, Demollstr. 31, 82407 Wielenbach, Germany.

http://dx.doi.org/10.1016/j.limno.2015.11.003
0075-9511/

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