Journal of Microbiological Methods 126 (2016) 42–47

Contents lists available at ScienceDirect

Journal of Microbiological Methods

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

The precision of bacterial quantification techniques on different kinds of environmental samples and the effect of ultrasonic treatment

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ARTICLE INFO

Article history:
Received 22 March 2016
Received in revised form 11 May 2016
Accepted 11 May 2016
Available online 13 May 2016

Keywords:
Fluorescence microscopy
Gelix One®
Most probable number
PCR
Sediment
Variability

ABSTRACT

The precision of cell number quantification in environmental samples depends on the complexity of the sample and on the applied technique. We compared fluorescence microscopy after filtration, quantification of gene copies and the cultivation based most probable number technique for their precision. We further analyzed the effect of increasing complexity of the sample material on the precision of the different methods by using pure cultures of *Pseudomonas aeruginosa*, fresh water samples and sediment slurries with and without ultrasonic treatment for analyses. Microscopy reached the highest precision, which was similar between pure cultures and water samples, but lower for sediment samples due to a higher percentage of cells in clusters and flocks. The PCR based quantification was most precise for pure cultures. Water and sediment samples were similar but less precise, which might be caused by the applied DNA extraction techniques. MPN measurements were equally precise for pure cultures and water samples. For sediment slurries the precision was slightly lower. The applied ultrasonic treatment of the slurries dispersed the cell clusters and flocks, increased the precision of microscopical and MPN measurements and also increased the number of potential colony forming units. However, the culturable cell number decreased by half. For MPN quantification of viable cells in samples with a high proportion of clustered cells we therefore recommend an optimization of ultrasonic treatment and a confirmation by microscopy and cultivation to reach highest possible dispersion of the cells with a minimum of inactivation. As a result of these observations we suggest a correction factor for MPN measurements to consider the effect of sonication on complex samples. The results are most likely applicable to other complex samples such as soil or biofilms.

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1. Introduction

The quantification of bacterial cells is an important tool to characterize environmental systems and to estimate or explain in situ substrate turn over processes. Besides molecular based techniques, microscopical counting and cultivation based methods such as plate count or most probable number (MPN) are still important and commonly used (Bjornsdottir-Butler et al., 2011).

The precision, defined as the reproducibility of results at unchanged conditions (Hospodsky et al., 2010), has to be taken into account when analyzing the spatial or temporal variability of bacterial cell numbers by different methods. Several studies revealed that plate counting, PCR-based methods or microscopical measurements reach higher precision compared to MPN approaches (Barbosa et al., 1995; Ben-David and Davidson, 2014; Chae et al., 2008), which are very imprecise (Blodgett, 2010; Noble et al., 2003) and large effort is needed to obtain statistically reliable results (Corry et al., 2012). However, it is barely discussed, whether the theoretical precision such as the confidence interval for MPN-values or the precisions obtained by calibrations with pure cultures (Barbosa et al., 1995; Bjornsdottir-Butler et al., 2011) or known amounts of DNA are applicable for complex environmental samples such as water and especially sediment samples.

The precision might differ from ideal conditions and different methods might be affected to a different degree by the complexity of the sample. Preliminary observations let us assume that the statistical variability of cultivation based and microscopical techniques will increase with increasing sample complexity, while molecular methods should not be affected. To validate this assumption we compared different enumeration techniques: the direct counting by fluorescence microscopy, the cultivation based method MPN and a PCR based semi-quantitative method on samples of different complexity: pure culture samples, fresh water samples, sonicated and untreated sediment samples. Since the applied molecular technique, the quantification of PCR products after electrophoresis, is not commonly used, we will give some comments on their suitability.

Microscopical counting and cultivation based methods such as plate count or most probable number (MPN) require evenly distributed single cells. However, in complex systems, bacteria are not necessarily single celled and evenly distributed either by the formation of any kind of

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http://dx.doi.org/10.1016/j.mimet.2016.05.006
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aggregates (compact clusters, loose flocks) or attached to particles or bound in biofilms. Different methods for desorption and separation of cells such as mechanical agitation, sonication, homogenization or the use of detergents are available (Both et al., 1990; Degrange and Bardin, 1995). Various effects of ultrasonic treatments on the total bacterial cell number or the number of colony forming units have been reported. Some observed a decrease (Lindahl and Bakken, 1995) due to damage of the cells (Gao et al., 2014), an inhibiting or negligible effect on bacterial growth (Vázquez et al., 2015) or an increase (Curtis et al., 1975), caused by a better dispersion. Other studies applied ultrasonic treatment without reporting or confirming the effect on the sample (Ferris and Hirsch, 1991; McNamara et al., 2002; Straub and Buchholz-Cleven, 1998).

To analyze the effect of ultrasonic treatment on complex samples we compared the culturable most probable number and the precision of this method before and after ultrasonic treatment of sediment slurries. We also documented the effect by microscopical counts of the total cell number and potential colony forming units and as a consequence recommend a correction factor for MPN results to consider these effects.

2. Material and methods

2.1. Total cell counting

The total amount of bacterial cells from samples of increasing complexity (increasing amount of cell aggregates, taxonomic diversity and dead organic matter for cell attachment): pure cultures of *Pseudomonas aeruginosa* (ATCC 27853) and *Pseudomonas aureofaciens* (DSMZ 6698), fresh water samples and organic rich sediment from the lake Scharmützelsee at the sampling site Rietz (Grüneberg et al., 2011; Nixdorf et al., 2008), were counted by fluorescence microscopy (Nikon Eclipse LV 100 with NIS Elements BR) at 200 and 400 fold magnification (Fig 1). In addition, the number of microscopical or potential colony forming units were counted. We defined these as cellular units, either single cells or aggregates of several cells, which can be microscopically characterized as one unit (Fig. 1) and which would have the potential to form a colony on agar or to give a positive result in a MPN tube. This parameter is essential to measure the success of the ultrasonic treatment and to estimate errors of MPN measurements caused by the amount of cells in clusters compared to the total cell number. The amount of cells in dense clusters was estimated regarding the size and brightness of the cluster and the mean size of single cells. 1 mL of a fresh water or an appropriate diluted sediment or pure culture sample (usually from the 10^{-3} dilution step of the MPN dilution series) were incubated with a Syto 9-Propidium iodide mixture (LIVE/DEAD® BacLight™, Invitrogen Deutschland according to manufactures instructions) and filtered through a counting filter (Isopore Membrane Filters, 0.2 μm GTBP, Merck, Ireland) using a filtration unit (Sartorius, Göttingen, Deutschland) according to Hobbie et al. (1977). Samples were analyzed with 3 to 5 replicate filters. At least 5 pictures per filter were taken and at least 4 areas of 50 × 50 μm or 100 × 100 μm per picture were examined to meet the requirements recommended by (Chae et al., 2008; Lisle et al., 2004).

2.2. Molecular approach for microbial enumeration

DNA was extracted from 1 mL of pure cultures of *P. aeruginosa* (ATCC 27853) and *P. aureofaciens* (DSMZ 6698) and from 1.5 mL of a 1:100 diluted sediment sample. For fresh water samples, 50 mL were filtered as described in Section 2.1. Filters and native sediment samples were stored at −20 °C until extraction. Cells were resuspended in 1 mL of PBS extraction buffer, spinned down at 1200 g and the pellet was used for DNA extraction by several application steps of extraction buffer/chloroform and isopropanol according to Rathsack et al. (2014). The DNA samples were resuspended in 100 μL of purified water and stored at −80 °C. As a control for a constant extraction and PCR efficiency, DNA of a known cell number of *P. aeruginosa* (ATCC 27853) was extracted and amplified parallel.

The 16S rDNA was amplified using the universal bacterial primer set 27f (5′-AgAgTTTgATC(A/C)TggCTCA-3′) and 1525r (5′-AggAggTgATCCAgCC-3′) (Lane, 1991). Agarose gels were stained with ethidium bromide (1%) and the amount of the PCR-products were quantified from pictures with the software Gelix One® G230 (biostep®, Jahnsdorf, Germany, Fig. 2) in comparison with a quantification marker (MassRuler DNA Ladder Mix, Thermo Scientific, Schwerte, Germany). The number of cells was calculated with a calibration curve (Fig. 3), which was established with tree independent dilution series of known cell numbers of *P. aeruginosa* (ATCC 27853) and *P. aureofaciens* (DSMZ 6698) (see Section 2.1). The calibration curve was fitted onto the data with Origin Pro 8.5 (Origin Lab Coorperation) using log-transformed cell numbers.

2.3. Most probable number

The number of culturable cells in pure cultures, fresh water and sediment samples was measured by turbidity as Most Probable Number (MPN) according to Michels et al. (2008) from one tenfold dilution series per sample with three tubes per dilution step with 3 to 5 replicates in Nutrient Broth II (Sifin, Berlin, Germany) with 1.5 mg L^{-1} KNO_3. MPN-values were taken from Blodgett (2010) or calculated with the BAM MPN calculator program (http://www.unc.edu/courses/2008fall/envr/431/001/BAM-MPN.xls). Test tubes were examined for bacterial growth.

2.4. Ultrasonic treatment

To separate cell clusters in the sediment slurries a maximum of 2 mL of a tenfold diluted sediment sample was transferred in to a glass tube

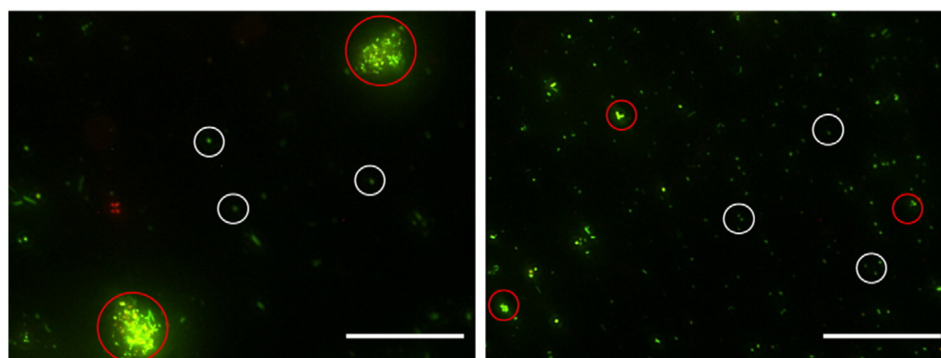

Fig. 1. Examples of microscopical potential colony forming units, either single cells (white circles) or cell aggregates (red circles) before (left) and after 30 s of ultrasonic treatment (right), bar = 50 μm).

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