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Use of silica microspheres having refractive index similar to bacteria for conversion of flow cytometric forward light scatter into biovolume

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ABSTRACT

This research describes an alternative approach for the rapid conversion of flow cytometric Forward Angle Light Scattering (FALS) into bacterial biovolume. The Rayleigh–Gans theory was considered for explaining the main parameters affecting FALS intensity: sensitivity analysis of the model was carried out, taking into account the parameters characteristic of bacterial cells and characteristics of the flow cytometer. For particles with size in the typical range of bacteria, the FALS intensity is affected mainly by volume and refractive index of bacterial cells and is approximately independent of the shape of the cells. The proposed conversion from FALS intensity into bacterial biovolume is based on a calibration curve determined by using silica microspheres having relative refractive index as far as possible similar to that of bacteria. The approach was validated for two different flow cytometers (the first equipped with an arc lamp and the second with a laser) by comparing the biovolume distribution obtained from FALS conversion with the biovolume measured conventionally under epifluorescence microscopy. The specific case of bacteria taken from a WWTP was addressed. Compared to the time-consuming conventional microscopic approach, the application of FALS for sizing bacterial biovolume could be a very promising tool being completed in few minutes, simultaneously to the enumeration of bacteria during the flow cytometric analysis.

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1. Introduction

In biotechnological processes and in environmental samples, viable bacterial biomass is an important issue involved in mass balance and in evaluating bacteria dynamics (growth or decay). Cell concentration, biovolume and the specific carbon content or the dry weight of cells need to be known for the calculation of bacterial biomass (Fry, 1990). In environmental samples, cell sizing with epifluorescence or confocal microscope is the conventional approach for biovolume determination. Alternatively, the flow cytometry technique

offers a great potential, thanks to the possibility of measuring simultaneously viable bacterial concentration and their forward angle light scattering which is correlated to the cellular size (or biovolume). Flow cytometry is a single-cell analysis and is showing increasing interest in environmental microbiology for its rapidity in quantifying microorganisms. This technique takes only a few minutes for the analysis of several hundred or thousand cells in bacterial suspensions with accuracy and high precision in the enumeration (Porter et al., 1997; Steen, 2000). In the case of environmental samples, flow cytometric analysis requires firstly cell staining with

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fluorescent probes in order to discriminate microorganisms from other non-fluorescent non-biotic particles. For example, the staining of cellular components by fluorescent dyes allows the total bacteria number to be identified and their viability, death or metabolic activity to be discriminated. Then total, viable or dead bacteria can be rapidly and automatically enumerated by flow cytometry on the basis of their fluorescence emission (Nebe-von-Caron et al., 2000; Ziglio et al., 2002). A flow cytometer is usually equipped for the simultaneous acquisition of two or more fluorescent signals and two light scattering signals for each cell passing through the focus. In particular, the light scattering acquired by the flow cytometer in the forward direction – that is for angles of about $1\text{--}2^\circ$ and indicated as FALS (Forward Angle Light Scattering) – depends on bacteria size (Salzman et al., 1990).

Empirical equations have often been sought by several authors for the conversion of FALS signal into bacteria size (Bouvier et al., 2001; Julià et al., 2000; Davey and Kell, 1996). For microorganisms, most authors agree that FALS increases monotonically with cell size and is a non-linear function of the volume or the diameter of the bacteria (Davey and Kell, 1996; Julià et al., 2000). In some cases the non-linear curve was fitted using a second-order polynomial curve (Davey and Kell, 1996; Julià et al., 2000).

A rational approach for the conversion of FALS into biovolume based on the Rayleigh–Gans theory was proposed by Koch et al. (1996). On the basis of this theory, the FALS intensity is proportional to the sixth power of the equivalent sphere radius (or to the second power of particle volume).

Several authors mention the possibility of evaluating the bacteria size by comparing FALS signal produced by the cells to that given by microspheres (beads) of known diameter (*inter alia* Koch et al., 1996). In this procedure, as underlined by Koch et al. (1996), the refractive index of the series of particles must be the same. Synthetic beads for flow cytometry applications are usually made of polystyrene or latex. FALS signal produced by bacteria generally has a lower intensity than the one produced by synthetic beads of the same size or volume as the bacterium, due to the much higher refractive index of polystyrene or latex than cells. This can induce an underestimation of the actual biovolume of bacteria, and therefore the use of latex particles as size standards for biological cells may be problematic (Davey et al., 1993; Robertson et al., 1998).

This aspect focuses on the importance of comparing FALS of particles having the same optical characteristics, especially for the relative refractive index. FALS intensity produced by cells in starved strains or in natural communities can differ from exponentially growing cells, as a consequence of the different refractive indexes due to the different metabolic status of the cells grown with excess of substrate or under limiting conditions (Bouvier et al., 2001). Refractive index of bacteria may vary from 1.36–1.40 for bacterial cells growing in minimal medium or in environments with limited substrate (Valkenburg and Woldringh, 1984; Robertson et al., 1998) to 1.40–1.41 for bacilli in cultures (Ross, 1957). Considering marine bacteria and phytoplankton cells growing in natural environments where nutrients are less abundant, refractive indices have been estimated to be in the range 1.39–1.40 (Jonasz et al., 1997; Morel and Ahn, 1990) and in the range 1.39–1.45,

respectively (Twardowski et al., 2001; Stramski et al., 2001). Refractive index of marine microorganisms such as *Synechococcus* and eukaryotes is 1.41–1.43 on average (Green et al., 2003). The purified organic material from a typical phytoplankton cell has an average refractive index of about 1.53 (Twardowski et al., 2001), so that it is not the organic material but the large proportion of water that gives bacteria low refractive indices. We are not aware of any directly measured value for the refractive index of bacteria present in wastewater and activated sludge in the literature. It was estimated in this research on the basis of the agreement between the FALS converted into biovolume and the microscopic sizing of bacteria.

The present research describes a new and alternative approach to rapidly convert FALS intensity measured by flow cytometry into bacterial biovolume by using silica microspheres, having optical characteristics – especially the relative refractive index – as far as possible similar to those of bacteria.

The Rayleigh–Gans theory was considered for understanding and supporting the conversion from FALS intensity into bacterial biovolume, according to the model of Koch et al. (1996). The Rayleigh–Gans law is applicable only if the phase shift between the waves scattered from different points of the target is low. This condition is satisfied if: (a) the size of bacteria is not too large with respect to the incident light wavelength and (b) the refractive index of bacteria is similar to that of the surrounding medium: an index of refraction only 3–6% higher than that of the surrounding medium minimizes phase change and allows the use of Rayleigh–Gans theory (Robertson et al., 1998; Koch et al., 1996). Both these hypotheses are usually satisfied for bacteria in environmental samples and in WWTPs. A sensitivity analysis was applied to evaluate how the flow cytometer configuration and the physical properties of bacteria, both involved in the Rayleigh–Gans model, affect FALS intensity.

The specific case of bacteria taken from a WWTP was addressed. The approach was applied to bacteria in wastewater and activated sludge and was validated by comparing the biovolume distribution obtained from FALS conversion with the biovolume measured using a conventional approach based on microscopy.

By means of the proposed calibration procedure, a rapid conversion of the FALS signal (measured usually in arbitrary units) into biovolume (measured in μm^3) is possible, independent of the geometry and set-up of the specific cytometer.

2. Materials and methods

2.1. Synthetic beads

Non-fluorescent silica microspheres (produced by MicroParticles GmbH, Germany) with different diameters were used to assess the calibration curve of FALS intensity. The selected silica beads have a refractive index of 1.42, which is close to the typical values of environmental bacteria. Six types of microspheres with diameter between 0.5 and $1\ \mu\text{m}$ were chosen. Also fluorescent polystyrene beads (produced by

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