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Approbation of Microbially Available Phosphorus (MAP) Determination Method by Flow Cytometry

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Abstract. Phosphorus (P) is among the most important nutrients required for bacterial growth. It has a great influence on microbial activity even at very small concentrations. Existing chemical methods are not able to determine P at low enough concentrations and to quantify biologically available phosphorus fractions. Therefore, a method of microbially available phosphorus (MAP) determination is used to quantify the amount of P at concentrations below 20 µg/l. Additionally, this method determines the amount of P that can be directly used by microorganisms.

Originally it was determined by inoculating sample by Pseudomonas fluorescens (now Ps. brenneri) P17 strain and spread-plated on R2A agar for enumeration. Further, a more rapid method was developed by replacing heterotrophic plate count (HPC) by flow cytometry (FCM).

In this paper the use of FCM for MAP determination is validated and compared with HPC method. The results of calibration are presented. The original pure P17 strain was used as inoculum and standards with different PO_4-P concentrations were inoculated at 30°C. The gained yield factor by FCM was $1.59x10^8$. FCM results showed strong correlation (R^2=0.99) with HPC results, as pure culture was used. Therefore, flow cytometry is a rapid alternative to heterotrophic plate count method for microbially available phosphorus determination.

Keywords: microbially available phosphorus, flow cytometry, drinking water.

I. INTRODUCTION

Phosphorus (P) compounds are among the most important microbial nutrients. The lack of P in the water to be transported or raw water may hinder the growth of microorganisms, thus preventing the formation of biofilm. P is considered to be the limiting nutrient in waters with high humic content or in boreal regions, e.g., northern Europe, Russia, and North America [1], as natural waters in these regions have high organic carbon content. Previous research has shown that by decreasing P concentrations water may become more microbiologically stable, as it limits bacterial growth [2], [3], [4].

Original MAP determination assay for drinking water relies on the growth potential of *Pseudomonas fluorescens* P17 strain [5] by means of heterotrophic plate count (HPC) method. However, in its original form it is regarded as time-consuming and labour-intensive enumeration. To overcome such problems, modifications have been made with natural microbial consortia and rapid enumeration tools, such as flow cytometer [6]. Here we used an originally suggested *Pseudomonas fluorescens* P17 (now *Ps.brenneri)* strain and approbated flow cytometer (FCM) as an alternative to classical cell cultivation. To ensure result compatibility, a thorough method comparison and calibration was performed. The approbation on artificially recharged groundwater station`s effluent was implemented.

II. MATERIALS AND METHODS

The method was adapted according to the one described by Lehtola et.al in 1999 [5]. Similar bacterial culture and stock solutions were used. The measurements were performed by means of flow cytometry, implementing optimized MAP determination method as recently described by Wen et al. in 2016 [6].

A. Preparation of glassware

All glassware and plastic caps were washed in the dishwasher with phosphate-free detergents, additionally rinsed several times with deionized water and left to dry. Then everything was sealed with aluminium folium. Glassware was placed into the oven for three hours at 500°C. Bottles` plastic caps were autoclaved at 121°C for 20min.

B. Inoculum

Freshly cultivated pure microbial culture *Pseudomonas brenneri* P17 (ATCC 49642) was used, as suggested by Lehtola et al., as this strain has phosphatase activity [5]. At first cells were grown in liquid R2A medium. Afterwards they were washed

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and inoculated into 0.1μm filtered Evian (Danone, France) water with added CH_3COONa as a carbon source to achieve the concentration of 1mg C l^{-1}. Incubation was performed at 30°C using orbital shaker at 150RPM for 24hours.

C. Preparation of standards and samples for MAP determination

Salts and acetate stock solutions were added to standards and samples in excess to ensure that P would be the only limiting nutrient. Salts stock consisted of NH_4NO_3, $MgSO_4$ x $7H_2O$, $CaCl_2$ x $2H_2O$, KCl and NaCl suspension in deionized water, resulting in final concentrations of 250μg N l^{-1}, 10μg Mg l^{-1}, 27μg Ca l^{-1}, 53μg K l^{-1} and 40μg Na l^{-1} in samples and 15,000μg N l^{-1}, 600μg Mg l^{-1}, 1,600μg Ca l^{-1}, 3,200μg K l^{-1} and 2,400μg Na l^{-1} in standards. As additional carbon source, acetate (CH_3COONa) stock solution was added to reach a concentration of 2,000μg C l^{-1} in the standard or sample.

Disodium hydrogen phosphate (Na_2HPO_4) was added to standard in different concentrations as phosphorus reference with values in the range 0–12.6μg P l^{-1}.

Afterwards water samples were pasteurized in a heated water bath at 60°C for 50min. After cooling till the room temperature, *Ps.brenneri* cells were added to achieve the concentration of 10^3TCC ml^{-1}. Prepared samples were kept in a shaker-incubator at 30°C and 150RPM until steady state of bacterial growth was reached. All samples were prepared in triplicates to ensure greater precision.

D. Measurements

Total cell count was measured using flow cytometer PartecCyFlow® SL (Partec, Germany) by method described previously in recent studies [7], [8]. Briefly, 1ml of sample was heated for at least 3min at 35°C. Further, 10μl of SYBR Green I (Invitrogen, Switzerland) and 10μl of EDTA was added and left to stain for 10min at 35°C in the dark before measurement. Prior to measurements samples were optionally diluted in 0.1μm filtered Evian in order not to exceed instrumental detection limit. PartecCyFlow® SL has a blue 25mW solid state laser emitting light at a fixed wavelength of 488nm. Green fluorescence was collected at 520±10nm, red fluorescence above 630nm, and high angle sideward scatter (SSC) at 488nm. The instrument was set on parameters described by Nescerecka et al. [9], i.e., the trigger was set on the green fluorescence channel and data were acquired on two-parameter density plots while no compensation was used for any of the measurements. Results were expressed as total cell count (TCC) in microbial cells per ml of water sample.

For classical cultivation, heterotrophic plate count method was used by spread-plating sample on R2A agar medium [10] and incubated for 2 days at 30°C in the dark before enumeration. Results were expressed in colony forming units (CFU) per ml of water sample.

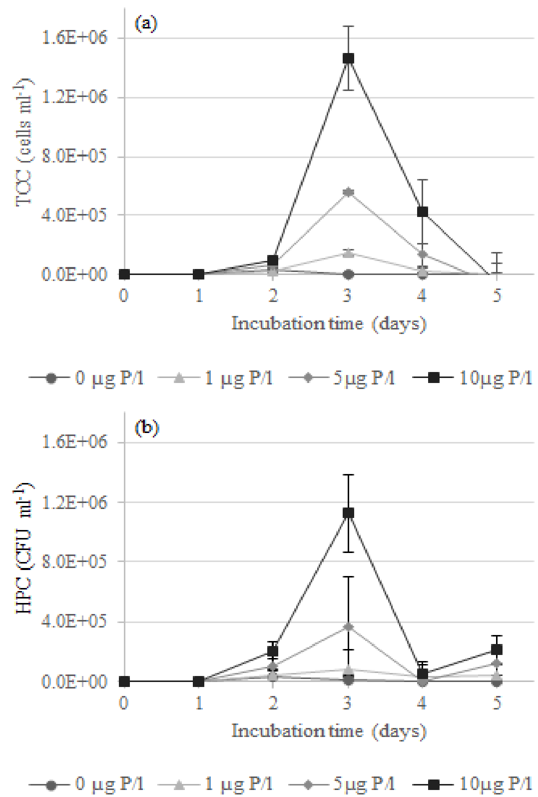

Fig. 1 Comparison between maximum growth of *Ps.brenneri* cells by FCM (a) and HPC method (b) in standards with different phosphorus concentrations

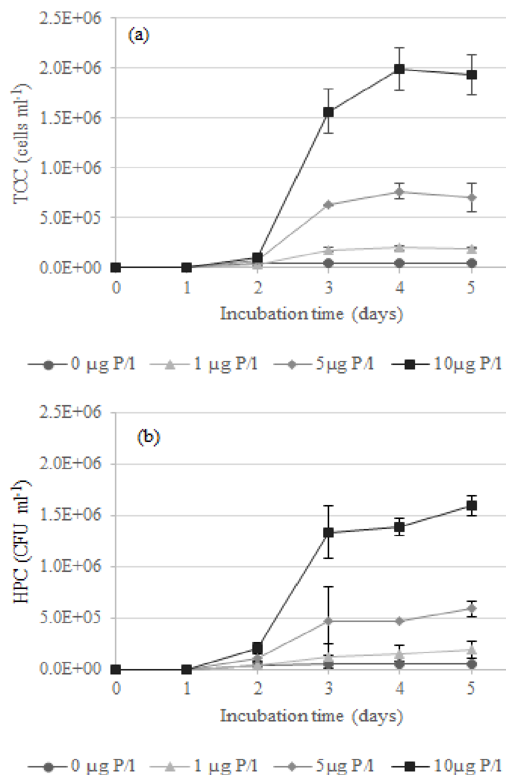

Fig. 2 Comparison between cumulative growth of *Ps.brenneri* cells by FCM (a) and HPC method (b) in standards with different phosphorus concentrations

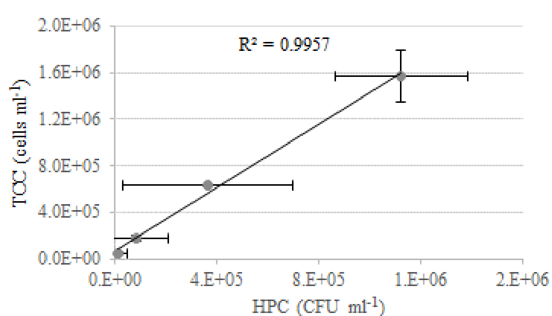

Fig. 3 Relationship between total cell count (TCC) on the day of steady state reached, measured by FCM, and colony forming units (CFU) on the day of maximum cell growth, measured by HPC method

III. RESULTS AND DISCUSSION

A. Method comparison between MAP determination by flow cytometry and heterotrophic plate count methods

The comparison was made for four phosphorus concentrations in the range of 0–10μg P l^{-1}. To compare the methods, the day of maximum growth (Fig. 1) and the day of steady state reached (Fig. 2) was determined. Both, cultivation and flow cytometry enumeration methods, showed that the maximum growth of *Ps.brenneri* in standard sample was reached on the third day and steady state was reached on the third or fourth day, depending on the sample. A very strong correlation was achieved between FCM and HPC (Fig. 3) results, displaying R^2 of 0.99.

In case of HPC, the day of maximum growth of bacteria in a water sample is used for conversion into MAP. For Lehtola et al. [5] it was day four to six. The time required to reach the maximum growth was longer due to a lower temperature, as they incubated samples at 15°C in contrary to 30°C used in this study.

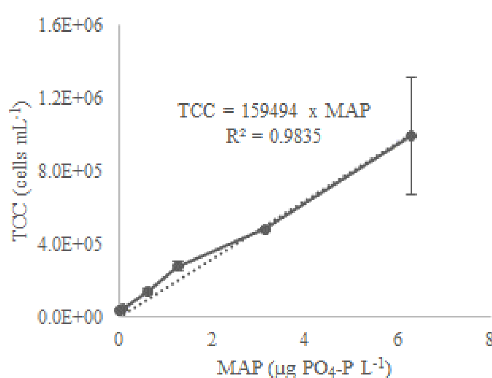

Fig. 4 Relation between the growth of *Pseudomonas brenneri* cells, measured with FCM, and phosphorus concentration in MAP standardization

B. Calibration curve

The MAP concentration for calibration was measured at the day, when steady state of bacterial growth was reached, i.e., when the maximum amount of TCC was registered, on contrary to HPC method, when the day of maximum growth was used, i.e., the day, when the greatest increase of CFU was registred. That is due to different appearance of result curves directly gained by each method. Additionally, at the point of steady state the maximum possible bacterial growth would be reached and no further growth would be possible due to P limitation. In this study the fourth day was used for further determination of yield factor.

The calibration was made for seven phosphorus concentrations in the range of 0–12.6μg P l^{-1}. The point of 12.6μg l^{-1} was not used in calibration as it was more away from linear correlation. That corresponds to observations by Lehtola et al. [5], who also amended standards with 2,000μg C l^{-1} in form of CH_3COONa and found no linear correlation with P concentrations above 10μg l^{-1}. Therefore, this range might be applied as MAP detection limit within this study.

Nevertheless, Wen et al. [6] found that linear range was 0–5μg l^{-1}, 0–10μg l^{-1} and 0–20μg l^{-1} if the amount of acetate spiked was 0.5mg l^{-1}, 1mg l^{-1} and 2mg l^{-1}, respectively. However, in this study the amount of carbon spiked to reach final concentration of 2mg l^{-1} did not show linear correlation for the point of 12.6 μg l^{-1}.

The calibration curve (Fig. 4) displayed strong linear correlation with R^2=0.98. The transformation of TCC into MAP was made using following equation (1):

$$TCC = 159494 \times MAP. \qquad (1)$$

It gives a yield factor of 1.59×10^8, which means that 1μg of PO_4-P corresponds to 1.59×10^8 cells of *Ps.brenneri*.

Lehtola et al. gained a yield factor of 3.7×10^8 [5], Polanska et al.– 3.2×10^8 [3], Jiang et al.– 1.1×10^9 [11] by spread-plating method and using *Pseudomonas fluorescens* as inoculum.

Wen et al. [6] compared P17 strain and natural microbial consortium of Evian water as inoculum and gained slopes of 1.8×10^8 and 9.4×10^8, respectively, using flow cytometry. They explained lower yield factor as a result of lower phosphatase activity, when comparing pure culture with natural microbial consortium. The value gained in this study is comparable to the value gained by Wen et al. by FCM [6]. Although, there is a lower phosphotase activity of P17 if compared to natural microbial consortium, the method is still applicable for MAP determination.

IV. APPROBATION IN LATVIAN CONDITIONS

The gained equation (1) was applied to determine the amount of MAP in artificially recharged groundwater effluent from the station (Riga, Latvia). A three weeks long study with three days per week sampling gave an average MAP concentration of 18.2±2.9μg P l^{-1} for groundwater samples. Such

concentration is out of calibration range and is nearly twice as high as data obtained in 2007 at the same station by HPC method (10.2±1.9µg P l^{-1}) [12] and the difference is even greater if compared to the amount presented in 2004 (3.4±2.4µg P l^{-1}) [2]. That might be attributed to increased pollution level or seasonal variations. To note, the consumption of drinking water in Riga has decreased almost threefold since 1995 [13] and in the last years infiltration basins for artificial recharge are used only to insure their serviceability.

Furthermore, as calibration limit is out of range, an increased acetate concentration needs to be applied, as suggested by Wen et al. [6]. However, a precise amount of carbon dose needs to be specified to fit the necessary conditions.

V.CONCLUSIONS

Flow cytometry is more advantageous method for MAP determination if compared to heterotrophic plate count due to its rapidness, ease of operation and safer sample measurement backup, as any doubtful cases can be detected immediately and remeasured.

The gained yield of this study by using FCM is 1.59×10^8 cells µg of PO_4-P^{-1}. At last, it is useful to provide wider MAP determination range by increased sodium acetate concentration.

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