Novel method to quantify intracellular accumulation of polyphosphate in EBPR systems

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Abstract: A new method for intracellular storage polyphosphate (poly-P) identification and quantification in enhanced biological phosphorus removal (EBPR) systems is proposed based on image analysis. In EBPR systems, 4',6-diamidino-2-phenylindole (DAPI) is usually combined with fluororescent *in situ* hybridization (FISH) to evaluate the microbial community. The proposed technique is based on an image analysis procedure specifically developed for determining poly-P inclusions within biomass suspension using solely DAPI by epifluorescence microscopy. Due to contradictory literature DAPI concentrations used for poly-P detection, the present work assessed the optimal DAPI concentration for samples acquired at the end of the EBPR aerobic stage when the accumulation is performed. Digital images were then acquired and processed by means of image processing and analysis. Regarding image analysis results and considering the current operational conditions, a promising correlation could be found between average poly-P intensity values and the analytical determination, although presenting a correlation coefficient somewhat far from the ideal. The proposed methodology can be seen as a promising alternative procedure to quantify intracellular poly-P accumulation in a faster and less labor intensive way.

Keywords: Enhanced biological phosphorus removal (EBPR); polyphosphate (poly-P); image analysis.

INTRODUCTION

Phosphorus (P) plays an essential role in cellular functions and its importance to biochemistry, biology and ecology has long been documented. In biological systems, P is present mainly as free phosphate ions, inorganic polymeric phosphates, or organic phosphorus (Majed et al., 2012). Polyphosphate (poly-P), a linear polymer of orthophosphate abundant in the environment and a key component in wastewater treatment (Diaz and Ingall, 2010), is of particular interest due to its cellular functions, which include phosphate and energy storage, pH buffering, cell envelope formation and function, stress response and survival and stationary phase adaptation (Madjed et al., 2012). In wastewater treatment, enhanced biological phosphorus removal (EBPR) has been considered a well-established process to achieve low P levels, preventing eutrophication of waterways. EBPR is based on the activity of polyphosphate-accumulating organisms (PAOs) able to take up a large amount of P and store it in the form of intracellular (poly-P) (Oehmen et al., 2007). Poly-P, particularly in this biological process, is a high energy storage molecule that, upon hydrolysis, can supply ample energy for biochemical reactions within the cell.

The oldest and most extensively used methods to document the presence of poly-P are cell staining with toluidine blue, neutral red, methylene blue (Kulaev & Vagabov, 1983) or Neisser. However, these traditional staining techniques may be problematic since they require practice, accuracy and precision in the discoloration step, and may be of limited application in large and dense flocs that do not decolorize correctly. A

fluorescent dye 4',6-diamidino-2-phenylindole (DAPI) is commonly combined with fluorescent *in situ* hibridization (FISH) for the detection of nucleic acids in epifluorescence microscopy. However, DAPI is also widely used to visualize poly-P granules where the staining depends on a polyphosphate-mediated metachromatic reaction, causing a shift in the emitted fluorescence from blue to a bright yellow-green (Günther et al., 2009). Nevertheless, a literature review shows that the used DAPI concentration can vary from 5 to 50 µg mL^{-1} depending on the excitation wavelengths. In consequence, the present study aimed first at identifying the best DAPI concentration for staining intracellular poly-P, in an EBPR system. Simultaneously, a novel method to quantify intracellular poly-P, based on quantitative image analysis, is proposed. For this purpose, 20 samples from an EBPR system were analyzed and 6 DAPI concentrations were studied.

MATERIALS AND METHODS

Experimental section: Experimental results were obtained in a lab-scale sequencing batch reactor (SBR) with 4 L. A synthetic wastewater was used containing acetate and propionate as the main carbon sources, and orthophosphate addition, obtaining a COD/P ratio of 10 mg COD mg $P\text{-}PO_4^{-1}$ to provide selective advantages to PAOs according to the findings of Oehmen et al. (2006). The system was operated with a cycle time of 6 h consisting of 120 min anaerobic including 5 min feed, 180 min aerobic, and 60 min settling/wasting. Two on/off control valves were used, bubbling nitrogen or compressed air into the reactor during anaerobic and aerobic periods, respectively. The pH was maintained around 7.5 by a pH controller.

Analytical procedures: The bulk P concentration was determined by segmented flow analysis (Skalar Analytical, The Netherlands), as described by Freitas et al. (2005). Total P concentration was determined in duplicate using segmented flow analysis (Skalar Analytical, The Netherlands), in mixed liquor samples, after digestion with potassium persulphate in 0.03 M H_2SO_4 according to Freitas et al. (2005). The intracellular poly-P (Int. poly-P) was determined subtracting the bulk P to the total P.

Samples fixation: Fresh sludge samples were collected at the end of the aerobic stage and fixed in paraformaldehyde, then washed with phosphate buffer saline solution (PBS), and stored in a PBS/ethanol solution at -20 ºC prior to further analysis.

Staining procedure: Intracellular poly-P granules were observed using DAPI staining. A DAPI "working solution" of 100 µg mL^{-1} was previously prepared. This solution was protected from light to avoid photodegradation of the reagent. To study the best DAPI concentration to survey poly-P inclusions, under the current conditions, the proposed methodology was performed as follows. For each sample, 1 mL of fixed cell suspension was first centrifuged at 4500 rpm for 5 min. Then, the pellet cells were washed, suspended in deionised water and incubated with DAPI at room temperature in the dark for 10 min, for final concentrations of 5, 10, 15, 20, 25, and 50 µg mL^{-1}. Samples were next centrifuged at 4500 rpm for 5 min. The pellet cells were finally washed and suspended in deionised water. To guarantee good quality images for the implementation of quantitative image analysis, a tissue grinder was used to disrupt the granules formed during the staining procedure.

Image acquisition procedure: To monitor the intracellular poly-P of the sludge, staining and epifluorescence microscopy were performed for 20 samples. Images were acquired in the upper, middle and bottom of the slide, resulting in a total of 150 images (3 × 50 images per slide). Samples were examined by means of an

epifluorescence microscope Olympus BX51 (Olympus, Tokyo, Japan) at 400× total magnification using a constant exposure time of 100 ms for intensity measurements. A long pass filter was used with an excitation bandpass of 365-370 nm and emission cut off at 421 nm, with the stained poly-P inclusions presenting an emission spectra around 525-575 nm. Images were acquired at 1360 × 1024 pixels, and 24-bit RGB format through the commercial software Cell^B (Olympus, Tokyo, Japan).

Image analysis procedure: The image processing and analysis was based on the identification and quantification of both individual poly-P granules and poly-P regions. The fundamental parameters that the program determined for these regions were areas, area percentages and total and average intensities. Images accounting for the main image analysis procedure steps are presented in Figure 1.

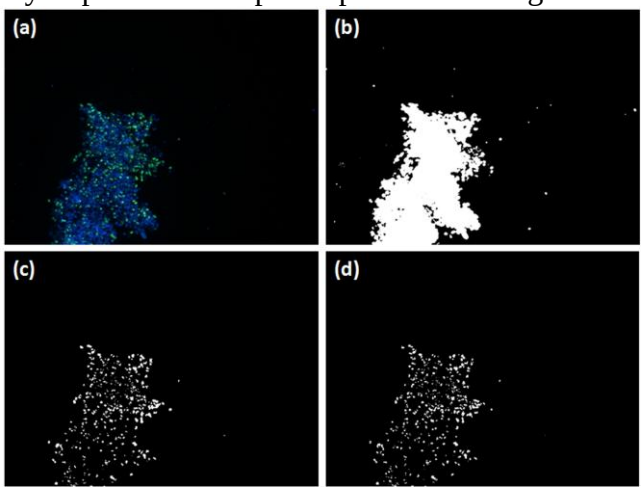

Figure 1 (a) DAPI original image, (b) segmented floc, (c) poly-P regions, (d) poly-P granules.

RESULTS AND DISCUSSION

The analysis on the effect of DAPI concentration on the poly-P intensity of the acquired images is presented in Figure 2a. The normalized values of the average intensity of intracellular poly-P represent the values for each studied DAPI concentration, normalized by the value at the lowest DAPI concentration (5 μg mL^{-1}). It is clear that, up until a DAPI concentration of 25 μg mL^{-1} the dependency on the DAPI concentration can be well modeled. In fact, for the average intensity, a logarithmic trend (Figure 3a) with a regression coefficient of 0.999 was found. Regarding the results obtained considering also 50 μg mL^{-1} of DAPI, a lower regression coefficient was attained, leading to the conclusion that at this DAPI concentration the above dependencies were not maintained anymore. Regarding the overall result, it is apparent that the best DAPI concentration was around 25 μg mL^{-1}. From then on, the increase in DAPI concentration no longer affects the acquired image poly-P intensities in a predictable manner. Therefore, for further analysis, 25 μg mL^{-1} was selected as the best DAPI concentration to quantify intracellular poly-P storage. Regarding the linear regression obtained for the Poly-P average intensity with Int. poly-P (mg L^{-1}) (Figure 2b) and excluding only 3 outliers, a satisfactory regression coefficient of 0.77 was achieved for the average poly-P intensity until 200 mg of poly-P L^{-1}. It was also clear that the average poly-P intensity could be a suitable parameter to estimate the real concentration of intracellular storage poly-P concentration. Although the obtained correlation factor is still somewhat distant from 1, these results can be seen as promising to effectively quantify intracellular accumulation of poly-P in EBPR systems.

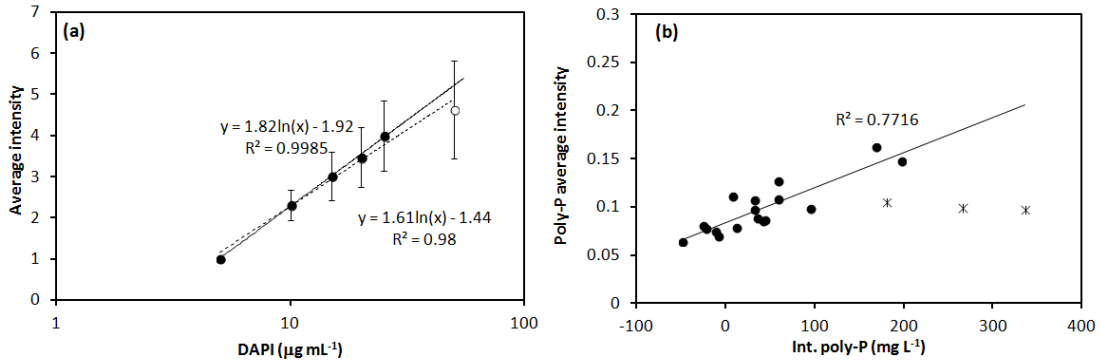

Figure 2 (a) Intensity ratio for the average poly-P and (b) Poly-P average intensity related to Int. poly-P (mg L^{-1}) analytical determination.

CONCLUSIONS

The ability to quantify intracellular accumulation of poly-P in EBPR systems using epifluorescence microscopy combined with image analysis was studied in this work. The preliminary results showed that the dependency of the image analysis parameters on the DAPI concentration can be well modeled up to 25 µg mL^{-1} of DAPI, and that at higher concentrations this dependency model was not maintained anymore. The proposed methodology can be seen as a promising alternative procedure to quantify intracellular poly-P, a significant energy source for biochemical reactions within cells.

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