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Original Research

Analysis of Methods for Determining DPAO Fraction in Phosphorus Accumulating Organisms

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Abstract

We attempt to answer the question whether it is possible to directly compare the results of phosphorus uptake batch tests (PUBT) conducted by researchers who use different methods of determining the DPAOs-to-PAOs ratio. This issue is raised because personally obtained results clearly show that DPAO fractions calculated with various methods proposed in the literature differ significantly. In the article the factors influencing the results of PUBT are discussed in detail. The study demonstrated that errors in determined values of DPAO fraction may be caused by the invitation of inappropriate value of reduction coefficient for anoxic conditions (η). The conclusion is that the η value should be accurately estimated in each test, independently for every active sludge.

Keywords: nitrogen and phosphorus removal, denitrifying dephosphatation, phosphorus uptake batch test

Introduction

When bacteria capable of accumulating orthophosphates not only under aerobic but also under anoxic conditions (DPAO) were discovered in the 1980s, a new method for removal of phosphorus from wastewater opened up before chemical process engineers. This new method was cost-effective as it allowed simultaneous removal of phosphorus and nitrogen at a significantly lower total demand for organic carbon. Potential financial benefits resulting from lower demand for organic substrates, shorter aeration times (oxygen is used only for nitrification and accumulation of orthophosphates remaining after anoxic phase) and lower sludge yields due to higher proportion of anoxic to aerobic conditions led to research on the practical application of denitrifying *P* removal in full-scale wastewater treatment plants [1].

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A mandatory condition for the occurrence of denitrifying phosphorus removal is the presence of an appropriate mass of bacteria capable of taking up orthophosphates under anoxic conditions (DPAO) in activated sludge. Fulfillment of this condition does not always secure the occurence of anoxic ortho-P uptake. But as the operational conditions required for removal of orthophosphates under anoxic conditions are practically the same as those required for the growth of DPAO, maintaining environmental conditions promoting growth of DPAO should result in an increase of denitrifying phosphorus removal rates.

Undoubtedly, the precise quantification of the number of DPAO must be preceded by their identification, which is not an easy task. It is reported in the relevant literature that attempts to identify denitrifying *P* removal bacteria involve either its isolation from activated sludge [2-4] or the use of DNA identification technology [5-9]. In any case, highly specialized personnel or specialized equipment are required.

Other useful tools for tracking changes in the quantity of denitrifying phosphorus accumulating organisms

(DPAO) are commonly used phosphorus uptake batch tests [10-15]. These tests are based on the idea by Kerrn-Jespersen and Henze [16] that all phosphorus accumulating organisms (PAO) can be divided into 2 subgroups: those for which the only possible electron acceptor is oxygen, and those which apart from oxygen can also use nitrates (DPAO). The phosphorus uptake test is run in parallel under anaerobic-aerobic and anaerobic-anoxic conditions, which allows for the estimation of the concentration of DPAO by determining their percent share in the total amount of PAO in the system. This is possible through a comparison of phosphorus uptakes under anoxic and aerobic conditions, as in the first case, the process can be carried out only by DPAO, while under aerobic conditions, all PAO are capable of accumulating the ortho-phosphates.

In the literature, two methods of interpreting the results of phosphorus uptake batch tests are presented. Hu et al. [19] identified the fraction of DPAO in PAO as the ratio of the total amount of phosphorus taken up by the biomass under anoxic and aerobic conditions. On the other hand Wachtmeister et al. [17] stressed that it is the ratios of initial *P* uptake rates that should be considered. During a calculation of DPAO/PAO ratio with the second method, Meinhold et al. [18] suggested that as the denitrifying activity of the bacteria is about 80% of their aerobic activity, a correction factor η=0.8 for anoxic conditions should be introduced.

The question arises whether it is possible to directly compare the results of phosphorus uptake batch tests conducted by researchers who use different methods of determining the DPAO/PAO ratio.

The article attempts to answer this question by presenting personally measured results of the phosphorus uptake batch tests and discussing the values of DPAO/PAO ratios identified according to the methodology of Wachtmeister et al. [17], Meinhold et al. [18], and Hu et al. [19]. Additionally, a detailed analysis of the factors influencing the results obtainable by various methods is also presented.

Experimental Procedures and Methodology of the Determination of DPAO/PAO Ratio

The phosphorus uptake batch tests have been performed on the activated sludge collected from a laboratory-scale 28 L sequencing batch reactor (SBR) model operating at 3 cycles per day and treating synthetic wastewater mimicking the composition of average municipal wastewater. In a search for the best operating conditions for the reactor, which will lead to a synergic removal of nitrogen and phosphorus, changes in the phase arrangement in the cycle of the reactor have been periodically introduced. A detailed description of operating conditions of SBR can be found at [20] and [21]. A set of phosphorus uptake batch tests was performed within 6 months on the activated sludge system operating with a different phase arrangement in a cycle.

PUBT methodology assumes a 2-stage experimental process – initially under strictly anaerobic conditions and then in two parallel reactors under aerobic and anoxic conditions (Fig. 1). Sludge for analysis was withdrawn from the SBR in idle phase. It was then washed with deaerated water in order to remove all organic compounds, ammoniacal nitrogen and nitrates present in the liquid phase. Anaerobic phase was carried out in a 2.0 L test reactor mixed with a magnetic stirrer and with simultaneous introduction of nitrogen gas above the surface of the liquid to avoid diffusion of oxygen from air into the liquid phase. Concentration of mixed liquor suspended solids in the test reactor was chosen to be approximately 3 gSS/L. Food source for the microorganisms was a solution of acetic acid whose pH had been corrected to the value of about 7.0, with 5% NaOH. During first stage of the measurement, i.e. the anaerobic process, the test reactor was sampled every 15 minutes. The samples were vacuum filtered on 0.45 µm membrane filters and concentration of P-PO_4^{3-} was measured in the filtrate. Anaerobic phase was maintained in the test reactor until a steady-state constant concentration of ortho-P was attained in the liquid phase.

Before proceeding to the 2nd stage of the test, the sludge was again washed with water in order to remove from the liquid all organic compounds remaining after the anaerobic phase. Next, the biomass with PAO and internally accumulated PHA was divided into two test reactors: aerobic and anoxic. Initial concentrations of orthophosphates in the treated liquid was chosen to equal approximately 80 mg P-PO_4^{3-}/L. Aerobic conditions in the aerobic reactors were maintained by sparging air through the bulk liquid using aquarium air stones. The resulting DO concentration was approximately 5 mgO_2/L. Anoxic conditions in the second reactor were attained by dosing a nitrate-rich solution and constant introduction of gaseous nitrogen above the surface of the liquid. Both reactors were sampled every 15 minutes. The samples were immediately filtered through 0.45 µm membrane filters and the concentration of P-PO_4^{3-} was measured in the filtrate. This test was carried out until no further changes in the concentration of P-PO_4^{3-} in both reactors could be observed. This indicated an end of orthophosphate uptake by PAO.

The tests were carried out at a constant temperature of 17ºC.

A description of these batch test results confined itself to a determination of the rate and efficiency of phosphorus

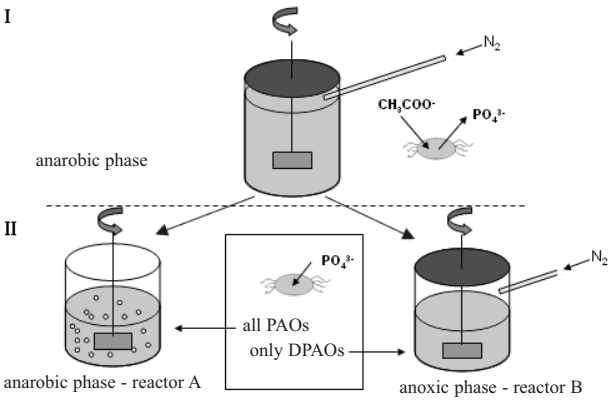

Fig. 1. The phosphorus uptake batch test procedure.

Table 1. Summary of the efficiencies and rates of initial phosphorus uptake under anoxic and aerobic conditions in tests 1 and 2.

Test No.	MLSS [g MLSS/L]	Anoxic conditions			Aerobic conditions		
		Δ P upt. [mg P/g MLSS]	Direction parameter	P uptake rate [mg P/g MLSS·min]	Δ P upt. [mg P/g MLSS]	Direction parameter	P uptake rate [mg P/g MLSS·min]
TEST 1	3.56	12.55	-0.1547	0.0435	16.99	-0.7742	0.2175
TEST 2	3.19	8.21	-0.0501	0.0157	20.07	-0.3888	0.1217

Table 2. DPAO/PAO fraction in tests 1 and 2 calculated with the methods proposed in literature.

Test No.	Percent share of DPAO in PAO calculated as:		
	$\Delta P_{anox}/\Delta P_{aerobic}$	$v_{anox}/v_{aerobic}$	$(\Delta P_{anox}/0.8)/\Delta P_{aerobic}$
TEST 1	73.90%	20.00%	25.00%
TEST 2	40.90%	12.90%	16.10%

uptake under aerobic and anoxic conditions and determination of the relationships between these values. DPAO/PAO ratio was calculated with two methods proposed in the literature: as a ratio of the total phosphorus uptake under anoxic and aerobic conditions (% DPAO I) and as a ratio of the initial *P* uptake rates under these conditions. Calculations with the second method had two variants – η values of η=1 (% DPAO IIa) and η=0.8 (% DPAO IIb) have been assumed respectively, as suggested by Meinhold et al. [13].

The percent share of DPAO in PAO was calculated using the following formulas:

$$\%\,DPAO\,\mathrm{I} = \frac{\Delta P_{anox}}{\Delta P_{aerobic}} \qquad (1)$$

$$\%\,DPAO\,\mathrm{IIa} = \frac{v_{anox}}{v_{aerobic}} \qquad (2)$$

$$\%\,DPAO\,\mathrm{IIb} = \frac{v_{anox}}{v_{aerobic} \cdot 0.8} \qquad (3)$$

...where:

ΔP_{anox} – phosphorus uptake efficiency under anoxic conditions [mg P-PO_4^{3-}/g MLSS]

$\Delta P_{aerobic}$ – phosphorus uptake efficiency under aerobic conditions [mg P-PO_4^{3-}/g MLSS]

v_{anox} – phosphorus uptake rate under anoxic conditions [mg P-PO_4^{3-}/g MLSS·min]

$v_{aerobic}$ – phosphorus uptake rate under aerobic conditions [mg P-PO_4^{3-}/g MLSS·min]

Results

During the period of SBR operation, 10 batch tests were carried out, often in considerable intervals and with the SBR working in different operating conditions and thus at virtually completely different sludges. Figs. 2 and 3 illustrate the changes in orthophosphate concentrations under anoxic and aerobic conditions in the two sample batch tests (1 and 2).

The data that allowed us to assess DPAO/PAO fraction with the methods used in tests 1 and 2 are shown in Table 1, the final results of the tests are presented in Table 2.

In more than half-year period of research, 8 more batch tests were carried out in an identical manner as batch tests 1 and 2. Fig. 4 shows the values of a DPAO/PAO ratio calculated for all 10 tests according to the methods under consideration.

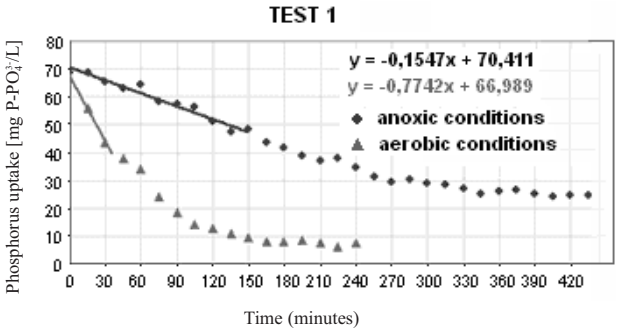

Fig. 2. Changes in orthophosphate concentrations under aerobic and anoxic conditions in the test No. 1.

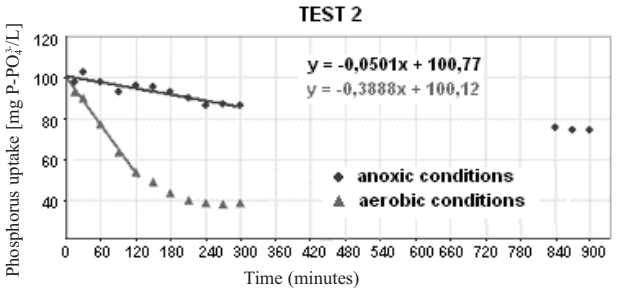

Fig. 3. Changes in orthophosphate concentrations under aerobic and anoxic conditions in the test No. 2.

Discussion of Results

The two methods of interpreting results of phosphorus uptake batch tests summarized briefly in this article have been widely reported in the literature. To date, researchers investigating a denitrifying *P* removal process essentially chose one of these methods and used it for the assessment of the DPAO/PAO ratio. Meinhold et al. [18] made a careful analysis of the various methods for obtaining the characteristics of the PAO population and obtained similar results. However, these authors were not engaged in experimental research but used computer simulations of processes with chosen mathematical models. Nevertheless, as is clear from Fig. 4, the results from all tests calculated with various methods differ significantly. Moreover, for each activated sludge sample the calculated DPAO/PAO ratio was consistently much higher according to the first method (% DPAO I), which computes the share of DPAO in PAO as the ratio of *P* uptake rates under anoxic and aerobic conditions. In test 9 such a calculated DPAO share was only about 40% higher from the one calculated by the method of initial uptake rate ratios, while in tests 1, 2, 6, 7, and 10, this difference was more than three-fold. An introduction of the correction coefficient η=0.8 for the anoxic conditions to the method based on initial uptake rate ratios brings the results obtained by the two methods closer, but only very slightly. For example, in test 1, the share of DPAO calculated with the method based on uptake efficiencies is about 74% and introduction of a coefficient η=0.8 in the method of initial uptake rates method increased the DPAO/PAO value only from 20 to 25%. For both methods to give similar results, the η coefficients in tests 1 and 2 would need to be approximately 0.3. In other tests this coefficient would have to vary from 0.2 to 0.7. However, nowhere in the literature on the process of denitrifying *P* removal were the reported efficiency coefficients of nitrate respiration in relation to aerobic respiration so low. All models give the value of 0.8 and Kuba et al. [22] proved that η might even be equal to 1.

All this leads to the assumption that the methods presented in the literature for estimating a DPAO/PAO ratio do not produce equivalent results. Before reaching such a conclusion, however, it is necessary to assess if the methodology of all the tests has been closely followed and to analyze all the factors that influence the results produced from these methods.

Determination of phosphorus uptake efficiency is relatively simple. In addition to preventing diffusion of oxygen under anoxic conditions and assuring accurate measurements of orthophosphate concentrations, it is only required to allow a sufficiently long duration time for both phases to ensure that the biomass is no longer capable of any further storage of phosphorus. To fulfill this requirement, in this article the aerobic-anoxic phases were 15 hours long. However, the uptake of phosphorus under aerobic conditions is found to cease earlier than under anoxic conditions. A premature termination of the test could possibly lead to underestimation of phosphorus uptake under anoxic conditions. As a consequence, in the calculation of *P* uptake efficiency ratio under anoxic and aerobic conditions, the nominator would have been smaller than the actual value. Elimination of this error could therefore only lead to an even larger difference between the results obtained from the two DPAO/PAO ratio calculation methods.

Some controversy may be raised by the method of determining the initial *P* uptake rate in aerobic and anoxic conditions. Since individual measurements are always associated with some errors and in all the tests it has been observed that changes in orthophosphate concentration both in anoxic and aerobic conditions are initially approximately linear, the initial *P* uptake rates were determined from the slope of the initial sections of curves considered linear. The errors committed here seem to be negligible. The determination coefficient R^2 values for the sections of the curves considered linear under aerobic conditions leave no doubt as to the selection of the trend line – in both tests the R^2 values are larger than 0.99 The linearity of ortho-P concentration changes under anoxic conditions in tests 1 and 2 is somewhat less certain as the R^2 values in this instance were respectively 0.95 and 0.88. For the results from both methods in test 1 (already with a correction factor η=0.8) to be exactly the same, the uptake rate under anoxic conditions would have to be threefold greater than the calculated one and to be equal 0.129 mg P/g MLSS·min instead of 0.0435 mg P/g MLSS·min. Line 2 in Fig. 5 shows how the initial changes in orthophosphate concentrations would look under anoxic conditions in test 1.

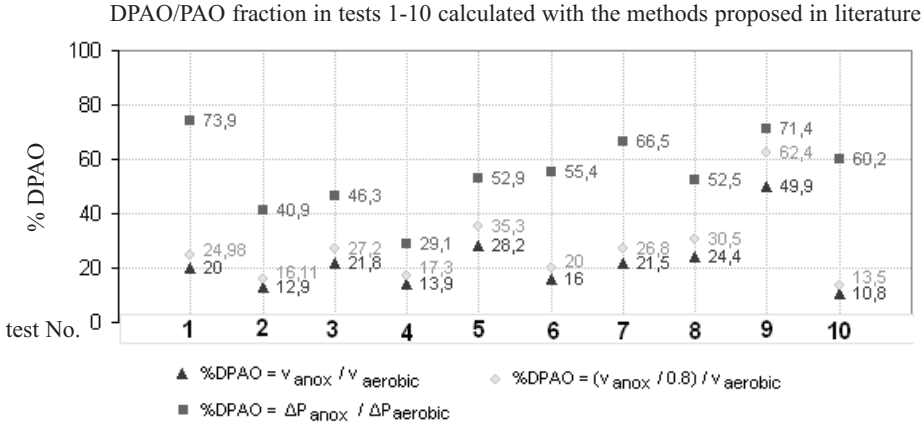

Fig. 4. Percent share of DPAO in PAO calculated with the literature published methods using 10 phosphorus uptake batch tests.

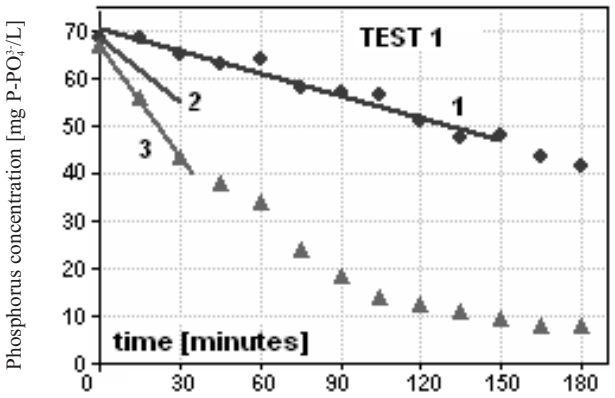

Fig. 5 Graphical representation of the measured phosphorus uptake rate under anoxic conditions (1) and the theoretical rate (2), at which the two discussed DPAO fraction estimations would have produced the same results. Curve 3 corresponds to orthophosphates uptake under aerobic conditions.

It is clear that all the experimentally determined data points are situated above the theoretically calculated curve for the rate of *P* uptake under anoxic conditions (2). This also clearly indicates that the inaccuracy of determining the initial *P* uptake rate under anoxic conditions is not the reason for discrepancies in the results obtained by the two methods for estimating DPAO/PAO fraction. The above findings suggest that the sources of discrepancies in the results obtained from both methods should be sought in the manner of interpretation rather than in the actual methods of determining the rate and efficiency of phosphorus uptake.

The essence of phosphorus uptake batch tests is based on the assumption that the differences in the rates and efficiencies of phosphorus uptake under anoxic and aerobic conditions only arise from the differences in the quantity of bacteria capable of carrying out the process under both circumstances. Since under anoxic conditions the oxidation of PHA is catalyzed by the respiratory enzymes present only in DPAO and under aerobic conditions, this process is carried out by all PAO bacteria, the ratio of the initial speed (after an introduction of the appropriate value of the η coefficient) can be identified with the share of DPAO in all PAO. In order for these dependencies to be met, it is required that the changes in the rate of *P* uptake become independent from all the factors other than the activity of the bacterial respiratory enzymes catalyzing the processes in anoxic and aerobic conditions. It is therefore important to provide exactly the same conditions in both batch reactors (anoxic and aerobic). Particularly important here is the equality of biomass concentrations and of those external factors that may affect the phosphorus uptake rate. The rates of all chemical reactions are dependent on temperature and the concentrations of the substrates. The temperature and the biomass concentrations in both batch reactors were the same at all times. According to the kinetics described with the Michaelis-Menten equation [23], at substrate concentrations above the concentration limits, the rates of biochemical reactions are constant and depend solely on the amount of enzyme catalyzing the reaction (Fig. 6).

In order for the ratio of initial *P* uptake rates to be synonymous with a DPAO/PAO ratio, it is therefore necessary to meet the condition requiring that all reaction substrates in the early stages of the anoxic and the entire aerobic phase are in large excess (their concentrations in every case should be higher than Sx), and therefore have no effect on the measurements of the phosphorus uptake rate. The substrates for these reactions are: oxygen, nitrates, orthophosphates, and PHA, which is internally stored inside the cells of PAO. DO concentration in the aerobic phases was always around 5 mgO_2/l and was definitely not limiting the uptake of phosphorus. Nitrates and phosphates were also added in large excess (for extra safety, starting from test 2, the amount of phosphates per 1g MLSS at the beginning of both phases was further increased). The only substrate whose concentration could not be manually manipulated and increased in the early stages of aerobic and anoxic phases was PHA, which is internally stored by PAO and DPAO during the earlier anaerobic phases. The anaerobic phases in each time, however, lasted until a complete cessation of the orthophosphate release and the accompanying accumulation of PHA. It also seems that the way the tests have been performed provided sufficient amounts of PHA in the early stages of the anoxic and aerobic phases for the initial rate of phosphorus uptake to be independent of the quantity of this substrate. However, even in the situation where the initial rate *P* uptake was limited by PHA, following the assumption accepted in the literature about the equality of reaction stoichiometry in all PAO under anaerobic conditions, the rate of *P* uptake under anoxic and aerobic conditions would decrease in the same proportion to the maximum rate (v_{max} in Fig. 6), which would otherwise be noted in the absence of any limiting substrates. Therefore, the limiting effect of the internally stored PHA in PAO during initial stages of anoxic and aerobic phases should not have any effect on the measured ratio of initial *P* uptake rates. The fact that *P* uptake rates depended solely on the fixed quantity of respiratory enzymes catalyzing the oxidation of PHA in a given phase is supported by the linear character of the initial sections of the curves (Figs. 2 and 3). It seems therefore, that all the assumptions have been met. In order for the method of calculating the DPAO/PAO fraction based on the ratio of initial *P* uptake rates to give the

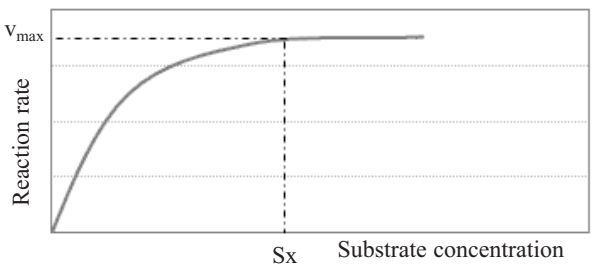

Fig. 6. Schematic of Michaelis-Menten enzymatic reaction kinetics [23].

correct results, it is only required to substitute into equation 2 an appropriate value of coefficient η, which describes the difference in the rate of *P* uptake by DPAO under anoxic and aerobic conditions.

The second method of assessing the DPAO/PAO fraction is in turn based on the assumption that the values of phosphorus uptake efficiency depend solely on the amounts of PAO possessing the ability to accumulate phosphorus in a given phase. As under anoxic conditions, phosphorus is taken up from wastewater only by DPAO, and under aerobic conditions all PAO are capable of accumulating phosphorus, the ratio of *P* uptake efficiency under anoxic and aerobic conditions should be in a 1:1 ratio with a DPAO/PAO ratio. The measurements of *P* uptake efficiency in contrast to the *P* uptake rate do not depend on the amount and activity of the enzymes catalyzing the Bio-P processes. At reduced amounts of active enzymes, the processes may run at a slower pace, but will not lead to lower uptake efficiencies. The efficiency of a Bio-P process, i.e. the amount of phosphorus accumulated by a unit of biomass is limited by the availability of substrates for the reaction, uptake of phosphorus stops upon exhaustion of at least one of them. In accordance with the above points, the electron acceptors and phosphates in the early stages of anoxic and aerobic phases were added in large excess and clearly did not limit the process. Therefore, it seems that phosphorus uptake ceased at the time of the exhaustion of the pool of PHA stored inside the bacterial cells. Since it is assumed that the amount of stored PHA after the anaerobic phase is equal in all the PAO, it can be assumed that the measured values of *P* uptake efficiencies are proportional to the mass of PAO involved in a given phase of the process. However, one additional condition must be met: the stoichiometric relationship between the amount of phosphorus taken up and the amount of PHA being oxidized must be equal for all PAO. In the meantime, it is known that phosphorus uptake is associated with the accumulation of energy, and this energy is generated in lesser amounts during anoxic respiration if compared to aerobic respiration. Perhaps in order to make the calculations of the DPAO/PAO ratio with this method a bit more credible, another correction factor η' should also be introduced into this calculation method.

Conclusions

The analysis carried out in this article demonstrated that it is not appropriate to compare the results of phosphorus uptake batch tests when different methods of determining the DPAO/PAO fraction have been used. Failure to introduce a correction coefficient for the anoxic conditions during the calculations of DPAO/PAO ratio with any of the methods proposed in the literature, or the use of its improper value, may result in errors in the calculated value of the DPAO/PAO ratio. Erroneous values obtained from one or both methods lead to differences in the calculated values of DPAO/PAO, which was indeed observed in this study. Since, as shown in the article, the ratio of *P* uptake rates by DPAO under aerobic and anoxic conditions η may not be equal to the ratio of the *P* uptake efficiencies produced by these bacteria (η'), it appears that the values of the correction coefficients that should be taken into account when calculating the share of DPAO with both methods may not be identical. Furthermore, it is possible that the values of these coefficients are different for DPAO obtained from different sludges. For accurate estimation of the share of DPAO in PAO it is necessary to precisely determine for each test the value of coefficient η or η' depending on the adopted calculation method. Perhaps coefficient η may be equal to 0.8 on many occasions as suggested Meinhold et al. [18], but it does not seem that this parameter value will be correct for each activated sludge sample. Similarly, the coefficient η' may be equal to 1 only in special cases. All of this indicates that it is advisable to conduct further research and analysis, which in future will allow for the development of a methodology for determining the coefficient values η and η' characteristic to each sludge sample.

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