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Metabolic modelling of full-scale enhanced biological phosphorus removal sludge

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ABSTRACT

This study investigates, for the first time, the application of metabolic models incorporating polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) towards describing the biochemical transformations of full-scale enhanced biological phosphorus removal (EBPR) activated sludge from wastewater treatment plants (WWTPs). For this purpose, it was required to modify previous metabolic models applied to lab-scale systems by incorporating the anaerobic utilisation of the TCA cycle and the aerobic maintenance processes based on sequential utilisation of polyhydroxyalkanoates, followed by glycogen and polyphosphate. The abundance of the PAO and GAO populations quantified by fluorescence *in situ* hybridisation served as the initial conditions of each biomass fraction, whereby the models were able to describe accurately the experimental data. The kinetic rates were found to change among the four different WWTPs studied or even in the same plant during different seasons, either suggesting the presence of additional PAO or GAO organisms, or varying microbial activities for the same organisms. Nevertheless, these variations in kinetic rates were largely found to be proportional to the difference in acetate uptake rate, suggesting a viable means of calibrating the metabolic model. The application of the metabolic model to full-scale sludge also revealed that different *Accumulibacter* clades likely possess different acetate uptake mechanisms, as a correlation was observed between the energetic requirement for acetate transport across the cell membrane with the diversity of *Accumulibacter* present. Using the model as a predictive tool, it was shown that lower acetate concentrations in the feed as well as longer aerobic retention times favour the dominance of the TCA metabolism over glycolysis, which could explain why the anaerobic TCA pathway seems to be more relevant in full-scale WWTPs than in lab-scale systems.

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1. Introduction

The biological removal of phosphate (also known as enhanced biological phosphorus removal or EBPR) has been incorporated into several wastewater treatment plant (WWTP) configurations and provides a more economical and sustainable alternative to chemical precipitation methods of P removal (Mino et al., 1998; Oehmen et al., 2007).

For more than 20 years, an effort has been made to develop and apply activated sludge models (ASM) to describe and predict the activated sludge processes, which are a useful tool for plant design and optimisation (Henze et al., 2000). While ASM models use a grey-box approach and focus on macroscopic processes, a different modelling approach, relying on metabolic and biochemical pathways, describes the energy, redox and mass balances of the cell processes (Smolders et al., 1994a). When comparing the two strategies, ASM models require a plant-tailored calibration procedure that can affect a higher number of variables, whereas metabolic models have been reported to require a simpler calibration procedure, since all of the equations for the microbial processes are interdependant (Seviour et al., 2010). Both approaches have been combined in the Technical University of Delft model (TUDP) and successfully applied for full-scale WWTPs, describing anaerobic, anoxic and aerobic processes of polyphosphate accumulating organisms (PAOs) (Van Veldhuizen et al., 1999; Brdjanovic et al., 2000; Meijer et al., 2001).

EBPR is carried out by PAOs using three different internal storage compounds that generate energy and/or reducing power, i.e., polyphosphate (poly-P), glycogen and polyhydroxyalkanoate (PHA). Anaerobically, volatile fatty acids (VFAs) like acetate and propionate are converted into PHA by consuming poly-P and glycogen, while PHA is then consumed for P uptake and glycogen production under anoxic and aerobic conditions. Additionally, PAOs have to withstand competition from glycogen accumulating organisms (GAOs), for which external parameters, such as temperature, pH, COD:P ratio and carbon source, play a significant role (Oehmen et al., 2007). Hence, the initial metabolic models developed for PAOs (Smolders et al., 1994a, 1995; Kuba et al., 1996; Murnleitner et al., 1997), were expanded to include the metabolic pathways of two main GAOs, i.e., *Competibacter* and *Defluviicoccus vanus*-related organisms, as well as the effects of temperature, carbon source and pH on their metabolism (Lopez-Vazquez et al., 2009). Moreover, the denitrification capacities of *Accumulibacter*, the main PAO known, and *Competibacter*- and *Defluviicoccus*-GAOs were further incorporated in the model by Oehmen et al. (2010b).

However, these new additions have only been validated in lab-scale systems containing enrichments of PAOs and GAOs and have not previously been tested on full-scale sludge. While simplified metabolic model calibration strategies have been proposed based on lab-scale results (Oehmen et al., 2010b), it is necessary to test these theories using full-scale sludge in order to evaluate their applicability to more complex situations. It is noteworthy to mention that when modelling the performance of full-scale systems, there is an added complexity, since not only could there be unknown PAOs and/or GAOs whose contribution to the phosphorus removal process is still unknown, but known PAOs such as *Tetrasphaera* could be active, whose metabolism related to EBPR is still largely unclear. Adding to this complexity is the fact that wastewater influents contain a wider diversity of organic carbon sources that are subject to much variability, and PAOs and GAOs make up a much smaller fraction of the total microbial community in full-scale sludge as compared to lab-scale systems.

Although PAOs have been typically modelled as using the glycolysis pathway as their sole source of anaerobic reducing power generation, it has been suggested that the role of the anaerobic TCA cycle in real WWTPs might be greater than expected as compared to lab-scale results (Pijuan et al., 2008; Lanham et al., 2013). In fact, Zhou et al. (2009) have shown that the TCA might have a particularly prominent role when PAOs face conditions of glycogen shortage. Since WWTPs deal with variable influent compositions and often with limited carbon substrate availability, this might be the reason for a greater reliance on the TCA cycle in WWTPs as opposed to lab-scale reactors (Lanham et al., 2013). Therefore, in order to improve the applicability of metabolic models, particularly with respect to full-scale situations, the relevance of incorporating the TCA cycle activity into the model should be assessed.

Furthermore, in previous metabolic models the aerobic maintenance processes predict cell decay at low PHA levels, which is not consistent with experimental findings. Experiments on the endogenous metabolism of PAOs (Lopez et al., 2006; Lu et al., 2007; Vargas et al., 2013) observed that the aerobic maintenance processes were dependant on glycogen and polyphosphate degradation following PHA depletion, with minimal cell decay. This is a particularly relevant factor to include when applying the model to full-scale systems, where the level of polymers stored by the sludge is much lower as compared to lab-scale systems.

In this study, a simplified version of the metabolic models previously developed by Lopez-Vazquez et al. (2009) and Oehmen et al. (2010b) was adapted in order to incorporate the anaerobic TCA utilisation of PAOs, in addition to the previously implemented glycolytic pathway. The resulting model was tested by describing the anaerobic/aerobic chemical transformations observed in activated sludge batch tests fed with acetate as carbon source. The tests were carried out with sludge from four different EBPR WWTPs with differing microbial compositions (including different fractions of *Accumulibacter*, *Competibacter* and *Defluviicoccus*) and metabolisms, as shown in Lanham et al. (2013). Special attention was paid to the required calibration procedure necessary in order to describe the activity of each biomass, and where possible, simplified calibration procedures that could be applicable to the modelling industry were evaluated. In addition, theoretical simulation studies were conducted between PAOs using solely the TCA cycle anaerobically (PAO_TCA) and PAOs using glycolysis (PAO_Glyc) in order to better understand the conditions which may lead to the use of one metabolic pathway over the other. Thus, this study is also relevant to improve our knowledge about factors that influence the microbial metabolism in EBPR systems, which is necessary in order to better understand and optimise the performance of the process.

2. Materials and methods

2.1. Experimental results

Four WWTPs were modelled in order to describe the anaerobic and aerobic chemical transformations observed in activated sludge tested in lab-scale batch tests, fed with acetate, at neutral pH (7) and at 20 °C. The WWTPs studied had either an A2/O configuration (Portuguese WWTPs: PT_1 and PT_2) or a Biodenitro configuration coupled to a return sludge side-stream hydrolysis process (RSS) (Danish WWTPs: DK_1 and DK_2). Experiments were carried out in winter and summer for the Portuguese WWTPs and only in winter for the Danish WWTPs (Lanham et al., 2013). Two of the WWTPs (PT_1 and DK_2) only had *Accumulibacter*-PAOs (approximately 4% as determined by quantitative fluorescence *in situ* hybridisation (qFISH)), whereas PT_2 had significant amounts of *Defluviicoccus*- and *Competibacter*-GAOs (4–8%). DK_1 presented one time point with almost 1% of *Competibacter*. Also, Type I and Type II *Accumulibacter* were quantified using specific oligonucleotide probes, Acc-I-444 and Acc-II-444 targeting type-I and type-II *Accumulibacter*-PAOs (Flowers et al., 2009). The biovolume of these *Accumulibacter* types was determined as described in Lanham et al., 2013. A complete account of the WWTPs characterisation, the batch test results and the microbial population quantification can be seen in Lanham et al. (2013).

2.2. Model description

The model developed in this study was based on a simplified version of previous metabolic models defined by Lopez-Vazquez et al. (2009) and Oehmen et al. (2010b) and was compiled using AQUASIM software (v. 2.1, Reichert (1994)).

The present model focused only on acetate as the sole external carbon source, converted into polyhydroxyalkanoate (PHA). It does not address pH nor temperature dependencies (since these were controlled at 7 and 20 °C in all batch tests) and aims at describing the anaerobic and aerobic transformations of *Accumulibacter*-PAOs (abbreviated to ACC) and *Competibacter* and *Defluviicoccus*-GAOs (abbreviated to GB and DEF).

The model describes the acetate (S_{HAc}) and the phosphate (S_{PO_4}) concentrations observed in the medium, the concentration of PHA (X_{PHA}), glycogen (X_{GLY}) and polyphosphate (X_{PP}) inside the bacterial cells, as well as the concentration of PAOs and GAOs (X_{ACC}, X_{GB} or X_{DEF}). The initial values for these parameters in the model were based on the experimental results. The initial concentrations of the PAO and GAO biomass fractions were based on the active biomass concentrations (as given by the volatile suspended solids (VSS) minus the organic storage polymers, PHA and glycogen, cf., Smolders et al. (1994b), converted to CmM via the biomass formula of $CH_{1.84}O_{0.5}N_{0.19}$ (Zeng et al., 2003)), and multiplied by the fraction of *Accumulibacter* (X_{ACC}), *Competibacter* (X_{GB})and *Defluviicoccus* (X_{DEF}) detected by qFISH (Oehmen et al., 2010b). In systems containing PAOs and GAOs simultaneously, their initial fraction of glycogen and PHA was estimated based on the specific anaerobic yields for each compound (cf., Table 1) in each type of organism as exemplified in Eq. (1) for the initial PHA fraction of *Competibacter* ($X^{GB}_{PHA,ini}$).

$$X^{GB}_{PHA,ini} = X_{PHA,ini} \times \frac{f_{GB} \times Y^{GB}_{PHA,HAc}}{f_{GB} \times Y^{GB}_{PHA,HAc} + f_{ACC} \times Y^{ACC}_{PHA,HAc} + f_{DEF} \times Y^{DEF}_{PHA,HAc}} \quad (1)$$

where f_{GB}, f_{ACC} and f_{DEF} are the fraction of each of these organisms as exemplified in Eq. (2) for *Competibacter*.

$$f_{GB} = \frac{X_{GB}}{X_{GB} + X_{ACC} + X_{DEF}} \quad (2)$$

The anaerobic stoichiometry of PAOs was based on acetate uptake coupled with polyphosphate hydrolysis and phosphate release, glycogen degradation and PHA production. Anaerobic maintenance processes were modelled as polyphosphate hydrolysis and subsequent phosphate release, which was followed by glycogen degradation, if low polyphosphate levels were attained. A complete account of the anaerobic reactions and kinetics in the model is given in Appendix A–I.

The anaerobic yields were defined based on the utilisation of the anaerobic TCA cycle or the glycolysis pathway, as determined experimentally by the anaerobic glycogen per acetate yield obtained in the activated sludge batch tests (Lanham et al., 2013). The overall reactions for the TCA cycle or the glycolysis stoichiometry were based on the Comeau-Wentzel (Comeau et al., 1986; Wentzel et al., 1986) and Mino (Mino et al., 1987) models, respectively, as presented by Smolders et al. (1994a) and shown in Eqs. (3) and (4), respectively. Greater explanation of the incorporation of the TCA cycle stoichiometry is detailed in Section 3.1.1.

Table 1 – Anaerobic stoichiometric parameters for *Accumulibacter* (ACC), *Competibacter* (GB) and *Defluviicoccus* (DEF) at pH 7, 20 °C and with acetate as the carbon source. The yields for the two possible metabolisms for *Accumulibacter*, with glycolysis (ACC_Gly) and with the anaerobic TCA cycle (ACC_TCA) are indicated.

			ACC_Gly	ACC_TCA	GB	DEF
Phosphate released per acetate yield	$Y_{PO_4,HAc}$	P-mol/C-mol	0.5	0.75	0	0
Glycogen consumed per acetate yield	$Y_{GLY,HAc}$	C-mol/C-mol	0.5	0	1.12	1.12
PHA produced per acetate yield	$Y_{PHA,HAc}$	C-mol/C-mol	1.33	0.89	1.86	1.86
Reference			(Smolders et al., 1994a)	(Smolders et al., 1994a)	(Filipe et al., 2001a; Zeng et al., 2002)	(Filipe et al., 2001a; Zeng et al., 2002)

The overall acetate uptake reaction where the TCA cycle is incorporated is shown below (C-mol basis):

$$\text{Acetate} + (0.5 + \alpha_{ACC})HPO_3 + \left(\frac{1}{3} - 0.5 + \alpha_{ACC}\right)H_2O \rightarrow 0.89PHA + 0.11CO_2 + (0.5 + \alpha_{ACC})H_3PO_4 \quad (3)$$

The overall acetate uptake reaction where glycolysis is incorporated is shown below (conversion of acetate, glycogen and polyphosphate into PHB, CO_2 and phosphate):

$$\text{Acetate} + 0.5\text{Glycogen} + (0.25 + \alpha_{ACC})HPO_3 \rightarrow 1.33PHA + 0.17CO_2 + (0.25 + \alpha_{ACC})H_3PO_4 + \left(\frac{5}{12} - (0.25 + \alpha_{ACC})\right)H_2O \quad (4)$$

where α_{ACC} is the energy of transport of one C-mol of acetate across the cell membrane.

GAOs were modelled in the same way as PAOs, except with a different stoichiometry (see Table 1) and excluding the processes dependant on polyphosphate or phosphate. The overall anaerobic acetate uptake of GAOs is shown in Eq. (5) (Filipe et al., 2001a; Zeng et al., 2002):

$$\text{Acetate} + (1 + 2\alpha_{GAO})\text{Glycogen} \rightarrow \left(1.75 + \frac{5}{3}\alpha_{GAO}\right)PHA + \left(0.25 + \frac{1}{3}\alpha_{GAO}\right)CO_2 \quad (5)$$

The aerobic stoichiometry and kinetics of the PAO model are based on the method proposed by Murnleitner et al. (1997), where PHA is degraded to contribute to phosphate uptake, polyphosphate formation and glycogen replenishment. Growth is determined in the model from the difference between the degraded PHA and the glycogen and polyphosphate produced. The aerobic stoichiometry depends on the ATP production yield per NADH oxidised with oxygen through oxidative phosphorylation, which is known as the P/O ratio or δ. The same processes are applied to the GAO models with the exception of the phosphate uptake and the polyphosphate formation processes. As explained in more detail in Section 3.1.3, the aerobic maintenance processes rely sequentially on PHA, glycogen and polyphosphate as energy sources. The kinetic processes and parameters utilised in this model, summarised in Appendix A-H, were mostly consistent with those presented in Lopez-Vazquez et al. (2009) and Oehmen et al. (2010b), except for the differences specified in Section 3.1.

2.3. Model calibration and validation strategy

The PAO model was first calibrated using the averaged results from two replicate batch tests of plant PT_1, in winter time, at standard conditions (acetate as the carbon source, pH = 7 and 20 °C). The calibration procedure was performed on the key kinetic parameters, namely the maximum anaerobic acetate uptake rate (q_{HAc}^{max}), the aerobic polyphosphate formation rate (q_{PP}), the aerobic PHA consumption rate (q_{PHA}) and the aerobic glycogen production rate (q_{GLY}). The calibrated values were determined based on a deviation between the experimental values and the modelled results of less than 10%, calculated by the normalised root mean squared deviation (NRMSD) (cf. 2.4).

The resulting calibrated model was then used to describe the experimental results obtained for the other experiments at different time-points or in different WWTPs. In order to achieve this, the strategy detailed in Oehmen et al. (2010b) was used, where q_{HAc}^{max} was first recalibrated, if needed, followed by adjusting all other aerobic kinetic rates proportionally. During this validation procedure, the initial concentrations of acetate, phosphate, PHA, glycogen and polyphosphate were set to the initial experimental values.

The *Competibacter* model and the *Defluviicoccus* model were first calibrated in one batch test of plant DK_1 and two averaged replicate batch tests of plant PT_2 (summer), respectively. The same procedure as for PAOs was applied to adjust the models to the experimental results of plant PT_2 (winter).

2.4. Error and sensitivity analysis

The deviation for n data points of the values predicted by the model (x_i^{model} at time point i) and the experimental values (x_i^{exp} at time point i) was determined by calculating the normalised root mean squared deviation (NRMSD) as stated in Eq. (6):

$$NRMSD = \frac{\sqrt{\frac{\sum_{i=1}^{n}\left(x_i^{exp} - x_i^{model}\right)^2}{n}}}{x_{max}^{exp} - x_{min}^{exp}} \quad (6)$$

where x_{max}^{exp} and x_{min}^{exp} are the maximal and minimal experimental values measured for that parameter.

Sensitivity analyses were performed to assess the impact of the aerobic PHA degradation rate and of the initial polyphosphate concentration by varying the value of these parameters by ±50% and then determining the effect on growth, levels of storage compounds (PHA, glycogen and polyphosphate) and on phosphorus removal in long-term simulations of 40 d.

2.5. Simulation studies

Long-term simulations were performed by defining a sequencing batch reactor (SBR) in AQUASIM as a mixed reactor compartment (maximum volume = 8 L), with a sludge retention time (SRT) of 10 d, a hydraulic retention time of approximately 0.6 d, and a 7-h cycle. The simulations were executed for 40 d to ensure that repeatable profiles were achieved (4 × SRT), similar to what is described in Oehmen et al. (2010b).

The simulations were performed either for sensitivity analysis purposes (see 2.4) or for microbial competition estimates. In the latter, simulations were performed to determine the theoretical competition between the two anaerobic metabolisms that are at the focus of this work. Two theoretical distinct PAO organisms were defined, one using only the anaerobic TCA metabolism and the other only using the glycolysis metabolism. While it is recognised that PAOs can potentially perform both processes simultaneously, this approach allowed examination of the factors that influence the competition between different metabolic pathways. Due to hypotheses suggested by Lanham et al. (2013), the competition between the two metabolisms was assessed at different acetate concentrations in the feed, from 0.25 to 5 C-mM, and at different aerobic phase durations, from 3 to 12 h. Simulations

that varied the acetate concentration were performed at a constant aerobic phase duration of 4 h and simulations that varied the duration of the aerobic phase were performed at a constant acetate concentration in the feed of 2 C-mM.

3. Results and discussion

3.1. Model development

The goal of this study was primarily to adapt metabolic models developed for lab-scale systems to describe the processes occurring in full-scale sludge. Although lab-scale systems are essential to achieve a deeper understanding about key metabolic transformations, the higher complexity of full-scale WWTPs might require adjustments to the models developed upon lab-scale data, regarding the diversity of influent characteristics, configurations and operating conditions existing in WWTPs. In particular for EBPR systems, the diversity of PAOs and GAOs and of their metabolic activities in real plants might be much higher than that obtained in lab-scale reactors. Moreover, the enrichments obtained in the latter can contain an abundance of these specific populations that are amplified as compared to full-scale systems, thus diluting possible competition and synergetic interactions of flanking populations. In this study, some parameters and concepts had to be adjusted from the models developed by Lopez-Vazquez et al. (2009) and Oehmen et al. (2010b), as detailed below.

3.1.1. Incorporating the TCA cycle stoichiometry

Both Pijuan et al. (2008) and Lanham et al. (2013), two EBPR studies concerning full-scale activated sludge, have observed the partial utilisation of the anaerobic TCA cycle to different extents, as shown by the stoichiometric yields, which, as reviewed by Oehmen et al. (2010a), can indicate that different biochemical pathways are being employed. Smolders et al. (1994a) proposed two metabolic models for the anaerobic metabolism of PAOs, one using the TCA cycle and the other employing glycolysis. The anaerobic TCA cycle utilisation is associated with a higher phosphorus release to acetate uptake yield ($Y^{ACC}_{PO_4,HAc}$) and a lower glycogen consumption and PHA production per acetate uptake ($Y^{ACC}_{GLY,HAc}$ and $Y^{ACC}_{PHA,HAc}$, respectively) than what is observed when using the glycolytic pathway (Smolders et al., 1994a; cf., Table 1). Lanham et al. (2013) observed different degrees of TCA cycle utilisation in different plants, and even in the same plant at different periods in time, which were proportional to the glycogen consumption to acetate uptake yield. Furthermore, this study verified correlations of the glycogen consumption yield coefficients with the PHA production and P release yields that supported the glycolysis and TCA models. Therefore, the incorporation of the TCA cycle metabolism was performed by adjusting the $Y^{ACC}_{PO_4,HAc}$ and the $Y^{ACC}_{PHA,HAc}$ yields such that they are dependent on the $Y^{ACC}_{GLY,HAc}$ yields observed in the batch tests.

Another important aspect is that, within the cases where the anaerobic TCA cycle was relevant, a discrepancy was observed between some of the $Y^{ACC}_{PO_4,HAc}$ values obtained experimentally and those predicted by the TCA model. The reason for this could likely stem from the fact that $Y^{ACC}_{PO_4,HAc}$ is dependent on the energetic requirement for acetate uptake across the cell membrane (α_{ACC}), which may vary depending on whether PAOs employ the TCA cycle or glycolysis. Thus, the α_{ACC} value that Smolders et al. (1994a) found using the glycolysis pathway (via a lab-scale culture) is not necessarily transferable to the TCA cycle model. This energetic requirement (α_{ACC}), is known to be dependent on pH (Smolders et al., 1994a; Filipe et al., 2001b) but also on the VFA uptake mechanism of the cell (Oehmen et al., 2010a). Since pH was controlled at 7 in all experiments, we hypothesised that the reason for this occasional discrepancy was due to the α_{ACC} parameter changing as a function of the PAO population, with different *Accumulibacter* sub-groups possessing different VFA uptake mechanisms. Supporting this hypothesis is the fact that a correlation was observed in this study between the α_{ACC} parameter and the abundance of *Accumulibacter* Type I and Type II vs. total *Accumulibacter* (Table 2). In cases where total *Accumulibacter* correlates closely (between 75 and 100%) with the sum of Type I plus Type II, a higher α_{ACC}, and consequently a higher $Y^{ACC}_{PO_4,HAc}$ was observed. When Type I plus Type II accounted for between 25 and 75% of the total *Accumulibacter* population, the α_{ACC} agrees well with that predicted by the glycolysis model. Interestingly, Zhou et al. (2009) also found a higher $Y^{ACC}_{PO_4,HAc}$ value than the one predicted by Smolders et al.

Table 2 – Abundance of total Type I and Type II *Accumulibacter* organisms as well as the value of $f_{ACCI,ACCII}$ used to model each experiment, the resulting α_{ACC} and the corresponding NRSMD indicating the agreement of the modelled phosphate values to the experimental results.

		Accumulibacter (% PAO mix)	Type I *Accumulibacter* (% Acc-I-444)	Type II *Accumulibacter* (% Acc-II-444)	Ratio type I + II vs. Total *Accumulibacter*	$f_{ACCI,ACCII}$	NRSMD (%)
PT_1 summer	Exp. 1	2.9	0.6	0.7	0.5	0	0.9
PT_1 winter	Exp. 1	3.1	1.4	1.4	0.9	1	1.7
	Exp. 2	3.4	1.6	1.6	0.9	1	
PT_2 summer	Exp. 1	2.8	0.5	0.8	0.5	0	3.6
	Exp. 2	3.3	0.3	1.4	0.5	0	
PT_2 winter	Exp. 1	3.3	2.7	2.8	1.7	1	2.0
	Exp. 2	4.7	2.6	2.8	1.1	1	
DK_1 winter	Exp. 1	3.7	1.6	2.1	1.0	1	1.4
	Exp. 2	3.1	1.9	1.9	1.3	1	2.4
	Exp. 3	3.1	1.7	1.5	1.0	1	1.5
DK_2 winter	Exp. 1	6.8	2.3	1.6	0.6	0	0.6
	Exp. 2	6.9	1.3	1.3	0.4	0	

(1994a) when the TCA cycle was active, corroborating the results of this study. Therefore, two new terms were introduced into the model to describe the value of $Y^{ACC}_{PO_4,HAc}$ in the case of the glycolysis and TCA cycle pathways (Eqs. (7) and (8)). The first term, defined in Eq. (7), accounts for a higher $Y^{ACC}_{PO_4,HAc}$ value when the TCA cycle is active instead of glycolysis, due to the necessity to increase the hydrolysis of polyphosphate to accommodate for the ATP production that is no longer being generated by glycolysis. This is dependent on the observed glycogen per acetate yield ($Y^{ACC}_{GLY,HAc}$) and the model predicted yields via glycolysis and the TCA cycle shown in Table 1. The second term impacts the α_{ACC} parameter, as shown in Eq. (8). This term is expressed as a function of $f_{ACCI,ACCII}$, which describes the composition of the *Accumulibacter* population (i.e., whether the total *Accumulibacter* population assessed through the PAOMIX probes was mainly comprised of Type I plus Type II as assessed through the FISH probes, or of other clades). Furthermore, it is important to note that this increase in ATP requirement for acetate transport was not observed when the glycolysis pathway was active, but only when the TCA cycle was used and the total *Accumulibacter* population was well described by the sum of Type I and Type II, as reflected in equation (8) through dependence on the $Y^{ACC}_{GLY,HAc}$ term. This suggests that different *Accumulibacter* sub-populations possess different acetate uptake mechanisms. Additionally, when comparing the VFA uptake energetic requirements of PAOs and GAOs (Table 3), it can be observed that α appears to be dependent on the level of glycolysis activity of the cell, in addition to the VFA uptake mechanism. For instance, PAOs and GAOs are capable of using additional VFA uptake mechanisms besides the direct use of ATP (see Oehmen et al. (2010a) for a review). These mechanisms become more prevalent when higher quantities of glycogen are used by the cell, and thus, they are more significant in GAOs than PAOs. Furthermore, *Defluviicoccus* GAOs have been found to have two simultaneous extra proton motive force (pmf) generation capacities (i.e., through fumarate reductase and methylmalonyl-CoA decarboxylase), while *Competibacter* GAOs only have one (through fumarate reductase) (Saunders et al., 2007; Burow et al., 2008). This correlates very well with the ATP requirement for transport found in these organisms through metabolic models (Filipe et al., 2001a; Oehmen et al., 2006).

$$Y^{ACC}_{PO_4,HAc} = \frac{1}{4} + \left(Y^{ACC\_Gly}_{GLY,HAc} - Y^{ACC\_TCA}_{GLY,HAc}\right) \times \left(1 - \frac{Y^{ACC}_{GLY,HAc}}{Y^{ACC\_Gly}_{GLY,HAc}}\right) + \alpha^{ACC}_{HAc} \quad (7)$$

$$\alpha^{ACC}_{HAc} = -1.1 + 0.19 \times pH + \frac{1}{4}\left(1 - \frac{Y^{ACC}_{GLY,HAc}}{Y^{ACC\_Gly}_{GLY,HAc}}\right) \times f_{ACCI,ACCII} \quad (8)$$

where $f_{ACCI,ACCII}$ is 1 for a higher coverage of total *Accumulibacter* by Types I and II (75–100% of total *Accumulibacter*) and 0 for a lower coverage (25–75% of total *Accumulibacter*). As previously reported by Smolders et al. (1994a), the range of α is constrained between 0 and 0.5 mol ATP/C-mol acetate uptake. The values of the yields for the two *Accumulibacter* metabolisms (ACC_TCA and ACC_Gly) are stated in Table 1.

$Y^{ACC}_{PHA,HAc}$ was defined using a similar strategy as for $Y^{ACC}_{PO_4,HAc}$ (Eq. (9)), where more PHA is produced via the glycolysis pathway per mole of acetate uptake as compared to the TCA cycle.

$$Y^{ACC}_{PHA,HAc} = Y^{ACC\_TCA}_{PHA,HAc} + \left(Y^{ACC\_Gly}_{PHA,HAc} - Y^{ACC\_TCA}_{PHA,HAc}\right) \times \frac{Y^{ACC}_{GLY,HAc}}{Y^{ACC\_Gly}_{GLY,HAc}} \quad (9)$$

3.1.2. *Anaerobic maintenance processes*

The anaerobic maintenance coefficient (m^{ANA}_{ACC}) was determined in a blank test without the addition of acetate, and its value was determined by calculating the rate of ATP production from the rate of P release observed during this test. The batch tests conducted in WWTPs that carried out a chemical phosphate precipitation polishing step, namely DK_1 and DK_2, presented a relatively high anaerobic P release, which could be partially due to anaerobic dissolution of iron-phosphate precipitates caused by iron-reducing bacteria (Nielsen, 1996). Since it was not possible to distinguish between the P release attributed to anaerobic maintenance and chemical dissolution in the tests performed, it was opted to model this additional P-release as an increased anaerobic maintenance coefficient, instead of adding a new chemical P release process (Table 4). Therefore, the anaerobic maintenance coefficient (m^{ANA}_{ACC}) should be considered as a lumped parameter for the Danish WWTPs.

3.1.3. *Aerobic maintenance processes*

In previous models, the aerobic maintenance was modelled as a function of cell decay, which is indirectly related to PHA degradation (Murnleitner et al., 1997). For this reason, at low PHA concentrations, the model predicted cell decay, which contrasts with studies where the endogenous mechanisms of PAOs and GAOs were characterised (Lopez et al., 2006; Lu et al., 2007; Vargas et al., 2013). Based on the results of Brdjanovic et al. (1998), Lopez et al. (2006), Lu et al. (2007) and Vargas et al. (2013), the aerobic maintenance process was expanded

Table 3 – Summary of the energy requirement for acetate transport (α) in PAOs and GAOs.

	$f_{ACCI,ACCII}$	α	Additional active pmf generating mechanisms	References
ACC_TCA	1	0.5	none	This study Zhou et al. (2009)
ACC_TCA	0	0.25	Fumarate reductase or other	This study García-Martín et al. (2006) Flowers et al., 2013 Smolders et al. (1994a)
ACC_Gly	–	0.25	Fumarate reductase or other	Smolders et al. (1994a) García-Martín et al. (2006) Flowers et al., 2013
Competi	–	0.06	Fumarate reductase	Filipe et al. (2001a) Saunders et al. (2007)
Defluvi	–	0	Fumarate reductase and Methylmalonyl-CoA decarboxylase	Oehmen et al. (2006) Burow et al. (2008)

Table 4 – Parameters adjusted during calibration.

		PAO model (Portuguese experiments)	PAO model (Danish experiments)
m_{ANA}^{ACC}	mol-ATP/C-mol/h	2.35[a]	40
$K_{S,PHA}$	C-mol/C-mol	1	1
$f_{GLY,max}$	C-mol/C-mol	0.8	0.8

[a] According to Smolders et al. (1994a).

to include a sequential dependence on PHA, glycogen and then polyphosphate. These studies have shown that PAOs tend to rely on energy generation from their storage polymers prior to cell decay, an effect that is of high importance particularly in the substrate-limited conditions that normally exist in full-scale WWTPs, unlike lab-scale systems. The kinetic equations of the three sequential aerobic maintenance processes are defined in Eqs. (10)–(12):

$$m_{ACC,PHA}^{AER} = m_{PHA}^{ACC} \times X_{ACC} \times \frac{X_{PHA}^{ACC}}{X_{PHA}^{ACC} + K_{PHA}^{ACC}} \quad (10)$$

$$m_{ACC,GLY}^{AER} = m_{GLY}^{ACC} \times X_{ACC} \times \left(1 - \frac{X_{PHA}^{ACC}}{X_{PHA}^{ACC} + K_{PHA}^{ACC}}\right) \times \frac{X_{GLY}^{ACC}}{X_{GLY}^{ACC} + K_{GLY}^{ACC}} \quad (11)$$

$$m_{ACC,PP}^{AER} = m_{PP}^{ACC} \times X_{ACC} \times \left(1 - \frac{X_{PHA}^{ACC}}{X_{PHA}^{ACC} + K_{PHA}^{ACC}}\right) \times \left(1 - \frac{X_{GLY}^{ACC}}{X_{GLY}^{ACC} + K_{GLY}^{ACC}}\right) \times \frac{X_{PP}^{ACC}}{X_{PP}^{ACC} + K_{PP}^{ACC}} \quad (12)$$

where, m_{PHA}^{AER}, m_{GLY}^{AER} and m_{PP}^{AER} are the aerobic maintenance coefficients based on PHA, glycogen and polyphosphate respectively. m_{PHA}^{AER} was defined according to Lopez-Vazquez et al. (2009) (Eq. (15)), while m_{GLY}^{AER} and m_{PP}^{AER} are defined in this study. From Eqs. (13) and (14), the degradation of one C-mol of glycogen or of one P-mol of polyphosphate produces $1 + \delta/2$ or 1 ATP-mol, respectively. These coefficients are proportional to how much glycogen or polyphosphate would be needed to produce the ATP requirements for aerobic maintenance, defined as the constant variable m_{ATP}^{AER} (Smolders et al., 1994b), hence obtaining the expressions presented in Eqs. (16) and (17).

Glycogen degradation to produce ATP (C-mol basis):

$$\text{Glycogen} + \frac{1}{4}O_2 \rightarrow \frac{2}{3}\text{PHA} + \frac{1}{3}CO_2 + \frac{1}{3}H_2O + \frac{1+\delta}{2}\text{ATP} \quad (13)$$

Polyphosphate degradation to produce ATP (Smolders et al., 1994a):

$$HPO_3 + H_2O \rightarrow \text{ATP} + H_2PO_4 \quad (14)$$

$$m_{PHA}^{AER} = \frac{12 \times m_{ATP}^{AER}}{6 + 27\delta} \quad (15)$$

$$m_{GLY}^{AER} = \frac{2 \times m_{ATP}^{AER}}{1 + \delta} \quad (16)$$

$$m_{PP}^{AER} = m_{ATP}^{AER} \quad (17)$$

Table 5 – Stoichiometric matrix for the aerobic maintenance coefficients. For a definition of the different yield coefficients, cf. Appendix A–H.

Components	Processes		
	$m_{HAc,PHA}^{ACC}$ (Lopez-Vazquez et al., 2009)	$m_{HAc,GLY}^{ACC}$ This study	$m_{HAc,PP}^{ACC}$ This study
S_{O_2}	$\frac{Y_{HAc,X}^{ACC}}{Y_{O,X}^{ACC}} - 1$	$-\frac{1}{4}$	
S_{PO_4}	$i_{BM,P} \times Y_{HAc,X}^{ACC}$		1
S_{CO_2}	$1 - 0.37 \times Y_{S,X}^{ACC}$	$\frac{1}{3}$	
X_{ACC}	$-Y_{HAc,X}^{ACC}$		
X_{PHA}^{ACC}		$\frac{2}{3}$	
X_{GLY}^{ACC}		-1	
X_{PP}^{ACC}			-1

The stoichiometric matrix for these maintenance coefficients is given in Table 5.

It was assumed that the aerobic maintenance processes would be similar in GAOs, as in PAOs, i.e., with the sequential utilisation of PHA and glycogen for aerobic maintenance, excluding of course the process dependent on polyphosphate. This is consistent with the results found by Vargas et al. (2013), where GAOs aerobic maintenance processes relied on glycogen degradation following PHA depletion.

3.2. Model calibration and validation in the different WWTPs

The PAO model was first calibrated using results from PT_1 during the winter (Fig. 1). The strategy used in this study was to input the abundance of *Accumulibacter*, as determined by quantitative FISH, multiplied by the active biomass concentration (Oehmen et al., 2010b). This allowed to account for the microbial community covered by the model, which in the case of a WWTP sludge is only a minor fraction of the total community (in this study, usually approximately 5% of the total population, and never surpassing 12%). This step alone yielded a fairly correct description of the chemical transformations, with some adjustment required with respect to the kinetic parameters.

Similarly to lab-scale studies, the calibration procedure involved the adjustment of the q_{HAc}^{max}, the q_{PHA}, the q_{PP} and the q_{GLY} parameters (Table 6). In general, the rates were slightly lower than the values obtained by Lopez-Vazquez et al. (2009), except for glycogen, which had a slightly faster rate than found in lab-scale systems (Table 6). After the calibration procedure, the model fitted very well with the experimental results, with error values below 5% for PHA, glycogen and P, as determined by the NRMSD (Table 8). The strong fitting of the model to the data further supports the anaerobic TCA cycle activity in some sludges (e.g., Fig. 1a). It should be noted that both the glycolysis and TCA models are based on a steady-state assumption for intermediates including NADH, ATP and acetyl-CoA. Some studies have found that the levels of some intermediates in cells (e.g., NADH) can fluctuate over time and space in WWTPs (Wos and Pollard, 2009). Nevertheless, if NADH were to be consumed anaerobically in PAOs, without generation through the TCA cycle (or glycolysis), then

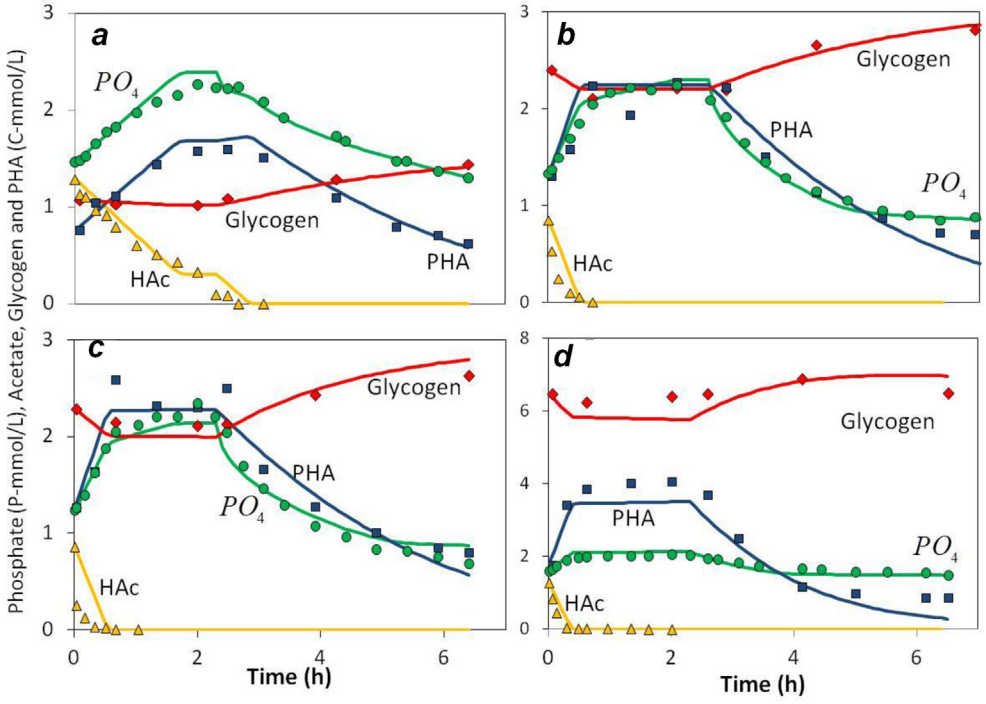

Fig. 1 – Comparison of model predictions and experimental data for a) PAO model in PT_1 (winter), b) PAO model in DK_1 (winter_1), c) *Competibacter* and PAO model in DK_1 (winter_3), d) *Defluviicoccus*, *Competibacter* and PAO model in PT_2 (summer). Experimental data shown for phosphate (circles), PHA (squares), acetate (triangles) and glycogen (diamonds).

acetate would be completely converted to PHA at a ratio of 1 C-mol PHA/C-mol HAc (since some acetyl-CoA must be oxidised to CO_2 to generate reducing equivalents via the TCA cycle), leading to an underestimation of anaerobic PHA production by the model. Such a discrepancy was not observed in this study, which validates the hypothesis that the TCA cycle and/or glycolysis were responsible for anaerobic NADH generation.

Other minor modifications from the lab-scale models are shown in Table 4, notably of which was the need to increase the maximal glycogen fraction (f_{GLY}^{max}), which was found to exceed the previously estimated threshold (0.27 C-mol/C-mol as used in Lopez-Vazquez et al. (2009)) when measured in the activated sludge. This parameter was introduced in the model to prevent the prediction of an unrealistically high level of glycogen accumulation (Meijer et al., 2002). However, considering the low quantity of PAOs and GAOs in full-scale sludge, the measured glycogen value might be more highly influenced by the presence of other hydrolysable sugar-polymers resulting, for example, from exopolymeric substances, from the presence of other EBPR bacteria (e.g., *Tetrasphaera* or unidentified populations) or other populations present in the sludge.

It is noticeable that despite the fact that glycogen levels are approximately 1 C-mmol/L in sludge from PT_1, the microorganisms hardly consume glycogen anaerobically (Fig. 1), which could suggest a depletion of their internal storage reserves and that the remaining glycogen quantified resulted from other glucose sources.

The calibration of the GAO models necessitated the inclusion of PAOs as well, since in the set of WWTPs modelled, there was no situation where GAOs, either *Competibacter* or *Defluviicoccus*, were present without PAOs. The DK_1 winter_3 experiment, with a population of *Accumulibacter* and some *Competibacter*, was used for calibrating the *Competibacter* model, whereas PT_2 summer, with a population containing *Accumulibacter*, *Competibacter* and *Defluviicoccus* was used to calibrate the *Defluviicoccus* model. Despite this challenge, the phosphate profile was effectively described by introducing, as initial concentrations, the GAO and *Accumulibacter* abundance in the proportions determined by qFISH, confirming once more the success of this methodology (Fig. 1 and Table 8). The stoichiometry of glycogen and PHA was also generally well described. It should be noted that the calibrated anaerobic and

Table 6 – Kinetic constants for the PAO model applied to different tests, given as a function of the initially calibrated q_{HAc}^{max}, q_{PP}, q_{PHA} and q_{GLY} for PT_1 (winter).

C-mol/C-mol/h or P-mol/C-mol/h	PT_1 (winter)	PT_1 (summer)	DK_1 (winter-1)	DK_1 (winter-2)	PT_2 (winter)	PT_2 (summer)	DK_2 (winter)	(Lopez-Vazquez et al., 2009)
q_{HAc}^{max}	0.15	$3q_{HAc,ACC}^{max}$	$3q_{HAc,ACC}^{max}$	$3q_{HAc,ACC}^{max}$	$1q_{HAc,ACC}^{max}$	$3q_{HAc,ACC}^{max}$	$1q_{HAc,ACC}^{max}$	0.2
q_{PP}	0.010	$3q_{PP}^{ACC}$	$3q_{PP}^{ACC}$	$3q_{PP}^{ACC}$	$1q_{PP}^{ACC}$	$3q_{PP}^{ACC}$	$1q_{PP}^{ACC}$	0.02
q_{PHA}	0.20	$1q_{PHA}^{ACC}$	$1q_{PHA}^{ACC}$	$1q_{PHA}^{ACC}$	$2q_{PHA}^{ACC}$	$1q_{PHA}^{ACC}$	$1q_{PHA}^{ACC}$	0.8
q_{GLY}	0.020	$3q_{GLY}^{ACC}$	$3q_{GLY}^{ACC}$	$3q_{GLY}^{ACC}$	$1q_{GLY}^{ACC}$	$3q_{GLY}^{ACC}$	$1q_{GLY}^{ACC}$	0.015

Table 7 – Kinetic constants for the GAO models in applied to different experiments, given as a function of the initially calibrated rates in DK_1 winter_3 (*Competibacter*) and PT_2 summer (*Defluviicoccus*).

C-mol/C-mol/h	Calibration		Validation	
	Competi	*Defluvi*	*Competi*	*Defluvi*
	DK_1 (winter-3)	PT_2 (summer)	PT_2 (winter)	
q_{HAc}^{max}	0.30	0.20	$0.5q_{HAc,GB}^{max}$	$0.4q_{HAc,DEF}^{max}$
q_{PHA}	0.30	0.20	$1q_{PHA}^{GB}$	$0.8q_{PHA}^{DEF}$
q_{GLY}	0.20	0.13	$0.5q_{GLY}^{GB}$	$0.4q_{GLY}^{DEF}$

aerobic kinetic parameters of *Competibacter* were consistently higher than those of *Defluviicoccus* (Table 7), which is consistent with the results of Lopez-Vazquez et al. (2009) and Oehmen et al. (2010b). Indeed, these studies showed that it was necessary to model each group of GAOs independently, primarily due to their different carbon source preferences (*Competibacter* were able to take up acetate at a much faster rate than propionate, while *Defluviicoccus* preferred propionate uptake).

The next step was to apply the calibrated models to other experiments. Once more, the strategy of inputting the PAO and GAO abundance as determined by FISH proved very effective in determining the overall cycling of the target parameters (Fig. 2 and Table 8). However, in some situations the model required some adjustments in order to correctly describe the experimental data. When applying the model to the Danish plants, the anaerobic maintenance coefficient had to be adjusted in order to also account for a higher phosphorus release due to the presence of chemical precipitants (see 3.1.2 and Table 4). Additionally, the acetate consumption rate had to be adjusted for most experiments. The aerobic kinetic parameters were also adjusted but in the same proportion as for the acetate uptake rate as shown in Tables 6 and 7. This corroborates the findings from lab-scale studies presented in (Oehmen et al., 2010b). Interestingly, different kinetics were observed in Portuguese plants when comparing summertime (faster) and wintertime (slower) experiments. In Denmark, despite the fact that the experiments were all performed in winter-like conditions, DK_1 revealed the same kinetics as the summertime Portuguese experiments, whereas DK_2 agreed with the Portuguese wintertime kinetics (Tables 6 and 7), suggesting this effect might not entirely be related to seasonal effects, but could reflect either different levels of activity of PAO and GAO cells, or the presence of other PAOs and GAOs besides *Accumulibacter*, *Competibacter* and *Defluviicoccus*.

One exception to the proportionality between parameters was the aerobic PHA degradation rate, which remained constant in most experiments (except in DK_2, Table 6) and therefore was independent of the overall activity factor discussed above. Since PHA is not an exclusive polymer of PAOs and GAOs, it could be produced or consumed by other organisms present in the sludge, thus influencing the rates

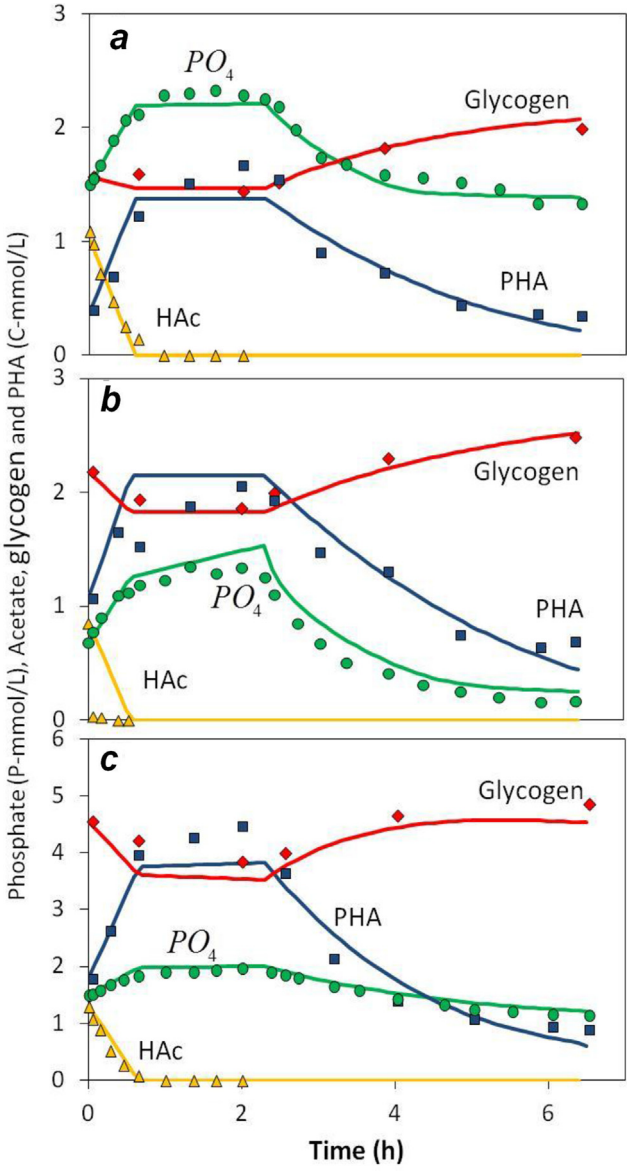

Fig. 2 – Comparison of model predictions and experimental data for a) and b) PAO model in PT_1 summer and DK_1 winter_2, respectively, c) PAO and GAO model in PT_2 winter, with experimental data for phosphate (circles), PHA (squares), acetate (triangles) and glycogen (diamonds).

Table 8 – The normalised mean root squared deviation (NRMSD) between the experimental results and model predictions in the different experiments.

	NRMSD (%)		
	Glycogen	PHA	PO4
PAO model			
PT_1 (winter)	2.4	2.4	1.7
PT_1 (summer)	4.5	3.8	1.7
DK_1, exp. 1	3.7	4.1	1.4
DK_1, exp. 2	5.8	5.4	2.4
DK_2	7.0	3.2	0.6
PAO and GAO model			
DK_1, exp. 3	8.5	3.2	1.5
PT_2 (summer)	28.3	3.8	3.6
PT_2 (winter)	13.3	2.9	2.0

observed. Nevertheless, Meijer et al. (2002) already discussed that the model was more sensitive to stoichiometry than to kinetics, in steady state conditions. In fact, when varying the aerobic PHA degradation rate by ±50%, a difference of less than 10% was observed for the PAO biomass concentration and <1% for the concentration of soluble phosphate after 40 d (results shown in Supplementary Material – Appendix J). Considering the finding that the q_{PHA} rarely changed, and when it did change an appreciable effect on the prediction of P removal or growth was not observed, it is suggested that this kinetic parameter can be assumed to be constant while q_{HAc}^{max}, q_{PP} and q_{GLY} can be assumed to vary by an identical factor. This could be a useful strategy during future full-scale metabolic model calibration endeavours, thereby avoiding the direct measurement of PHA and glycogen, which are usually not feasible to quantify at full-scale facilities.

Finally, in some experiments the initial conditions of poly-P content were adjusted in order to adequately predict the aerobic phosphate uptake trend. It seemed that in some cases aerobic phosphate uptake seemed to be influenced by a factor other than PHA limitation, since a deceleration was observed even when PHA was still available. In fact, in most experiments, PHA levels seemed to not decrease beneath a value of approximately 0.5 C-mmol/L (Figs. 1 and 2). This suggests that either there could be other PHA sources from PHA accumulating organisms other than PAOs and GAOs (also suggested by Meijer et al., 2002) or that the deceleration in phosphate uptake may have been due to the level of poly-P accumulation within PAOs, which is known to affect the P uptake rate (Smolders et al., 1995). The second option was evaluated by performing a sensitivity analysis on the initial poly-P concentration (usually set as $0.75^{*}f_{PP,max}$) in long term simulations (40 d) (results in Supplementary material - Appendix J). The results showed that even when varying the initial concentration of polyphosphate between $0.50^{*}f_{PP,max}$ and $1.00^{*}f_{PP,max}$, the models converged to the same steady-state conditions, and therefore this effect is only relevant to accurately describe the start-up conditions.

Overall, experimental results for the various measured parameters were well described with NRSMD values below 10% (Fig. 2 and Table 8). Glycogen was the only parameter shown to be more difficult to describe in some tests with error values higher than 10% (Table 8), which is consistent with literature findings (Lopez-Vazquez et al., 2009).

3.3. *Competition between PAOs using glycolysis vs. TCA*

The additional elements incorporated in this study to the metabolic models developed previously (Lopez-Vazquez et al., 2009; Oehmen et al., 2010b) were successful in describing the overall transformations observed in activated sludge performing enhanced biological phosphorus removal with acetate as the carbon source. Thus, the model was used as a tool to understand the mechanisms that could lead to an advantage of the TCA metabolism (ACC_TCA) over the glycolysis metabolism (ACC_Gly). It is desirable to understand the factors that lead to the observation of the TCA metabolism in WWTPs, and explain why it is observed at a lesser extent in lab-scale SBRs.

In Lanham et al. (2013), it was hypothesised that low carbon substrate levels in the influent, as well as long aeration periods, could lead to a lower availability of glycogen, and hence to the use of the TCA cycle over the glycolysis pathway. In order to verify this hypothesis, simulations were carried out in this study with different acetate concentrations in the influent and also in SBR cycles with different aeration periods (Fig. 3). Although higher acetate concentrations clearly led to higher overall growth as compared to lower acetate concentrations, the relative proportion of ACC_TCA and ACC_Gly was found to vary. The results reveal that at higher substrate concentrations, e.g., at 5 C-mmol/L of acetate in the feed, a situation similar to the conditions used in most lab-scale reactors (e.g., 3.4 C-mmol/L in Acevedo et al. (2012), 4.7 C-mmol/L in Zhou et al. (2009) and 6.25 C-mmol/L in Lopez et al. (2006)), a predominance of the glycolytic metabolism is observed, whereas for lower substrate concentrations, i.e., lower than 2 C-mmol/L, which is closer to the acetate or total volatile fatty acid (VFA) concentration available in WWTPs (Zeng et al., 2006), the TCA metabolism gains an advantage. As seen by the simulations, this advantage likely derives from the fact

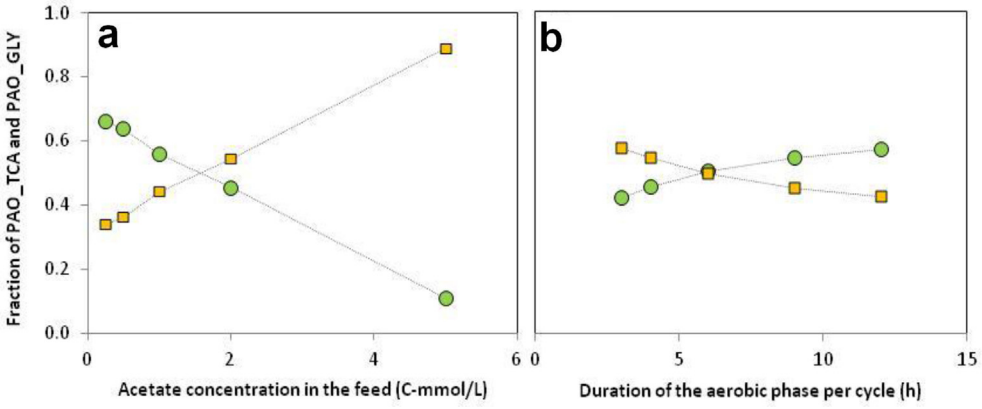

Fig. 3 – Effect of acetate feed concentration (a) and duration of the aerobic phase (b) on the competition between the two metabolisms: TCA cycle (circles) vs. glycolysis (squares). Simulations were run for 40 d using the PT_1 (summer) calibrated model. Simulations in (a) were conducted with an aeration period of 4 h and simulations in (b) were performed at an initial acetate concentration of 2 C-mM.

that the aerobic replenishment of storage polymer is prioritised as compared to growth (Murnleitner et al., 1997), therefore the ACC_Gly spends more PHA on restoring the consumed glycogen in aerobic conditions than the ACC_TCA. Thus, at lower acetate concentrations, the ACC_TCA metabolism gains an advantage, since less PHA is used for glycogen production, and therefore, more PHA is available for growth in comparison with ACC_Gly. Additionally, at lower acetate concentrations, PAOs become limited in glycogen resources. In this situation, the ACC_TCA metabolism still assures the anaerobic acetate uptake, since this process is not dependent on glycogen, when the glycolytic metabolism is no longer able to support acetate uptake. The reason why the glycolysis pathway dominates in lab-scale systems, which are commonly operated with higher carbon loading, is likely because ACC_Gly metabolism (1.33 C-mol/C-mol HAc) is able to produce more PHA anaerobically than ACC_TCA (0.89 C-mol/C-mol HAc). At non-limiting acetate concentrations, this higher PHA content leads to a higher level of biomass growth through ACC_Gly than ACC_TCA.

The effect of the aeration phase duration is less pronounced on the selection of one metabolism over the other. However, for aeration periods lasting 3–4 h, which constitute the most common conditions in lab-scale reactors (e.g., 2.7 h–3.5 h in Lopez et al. (2006), Zhou et al. (2009) and Acevedo et al. (2012)) the glycolytic metabolism is prevalent, whereas for longer aeration periods, such as higher than 6–7 h, which were similar to the aerobic retention times in the four WWTPs modelled here (see Lanham et al. (2013) for more details on plant operation) the TCA metabolism appears more favourable. This effect likely results from glycogen limitation as well, but this time due to the aerobic maintenance processes, which in this model rely on glycogen and then on polyphosphate after PHA depletion, as suggested by Lopez et al. (2006) and Lu and Keller (2007). As mentioned above, the previous models, which based aerobic maintenance indirectly on PHA through modelling a decrease in the biomass concentration (Murnleitner et al., 1997; Lopez-Vazquez et al., 2009; Oehmen et al., 2010b), predict aerobic cell decay when PHA levels are depleted, observable even in cycles of 6 h (typical of lab-scale SBRs). This does not describe what is known for PAO and GAO aerobic endogenous metabolism, where cell decay was minimal as compared to glycogen and polyphosphate degradation (Lopez et al., 2006; Lu and Keller, 2007; Vargas et al., 2013). Therefore, the model proposed in this study predicts a consumption of glycogen, and eventually, if glycogen levels are depleted, a release of phosphate, which agrees better with what was observed in previous studies (Lopez et al., 2006; Lu and Keller, 2007; Vargas et al., 2013).

4. Conclusions

In this study, a metabolic model was applied to describe the activity of one biomass group of PAOs and two biomass groups of GAOs in activated sludge collected from four WWTPs. The model incorporates the anaerobic TCA cycle activity of PAOs in order to describe the observed biochemical transformations, as well as modification of previously proposed anaerobic and aerobic maintenance processes. The model was able to predict accurately the experimental results, by defining the initial abundance of PAOs and GAOs with the fraction quantified by FISH. The changes in anaerobic acetate uptake rate amongst the different sludges were proportional to changes in aerobic P uptake rate and glycogen production rate, while the aerobic PHA kinetics remained mostly stable in different plants and for different seasons. This suggests a simplified model calibration procedure that avoids the need for PHA and glycogen measurements. Additionally, it was suggested that the acetate uptake mechanism of *Accumulibacter* varies according to the different clades, with differences observed in the energetic requirements for acetate uptake depending on the *Accumulibacter* diversity. Long-term simulations using the model showed that low carbon substrate concentrations in the feed and long aerobic phases lead to an advantage of the anaerobic TCA metabolism over the glycolysis metabolism, which would explain the higher importance of this metabolism in WWTPs as opposed to what is most commonly observed in lab-scale reactors.

This study illustrated how metabolic modelling could be used as a research tool to test hypotheses and to determine new metabolic properties of *Accumulibacter*, particularly with respect to its acetate uptake mechanism. It was found to be a robust means of describing and predicting with relative ease the EBPR metabolism observed in full-scale WWTPs. Further research should be conducted to integrate this improved version of the metabolic model with ASM models in order to describe the operation of biological nutrient removal plants in steady-state or dynamic conditions.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2014.08.036.

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