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4 **Denitrification Activity of Polyphosphate Accumulating Organisms**  
5 **(PAOs) in Full-Scale Wastewater Treatment Plants**  
6

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16

17 **ABSTRACT**

18 A comprehensive assessment of full-scale EBPR plants (5 plants, 19 independent tests) was undertaken  
19 to determine their effectiveness in terms of aerobic and anoxic P removal. By comparing parallel P  
20 uptake tests under only aerobic or under anoxic-aerobic conditions, results revealed that introducing  
21 an anoxic stage led to an overall P removal of on average 90% of the P removed under only aerobic  
22 conditions. This was achieved with negligible higher PHA and glycogen requirements, 30% lower  
23 overall oxygen consumption and with the simultaneous removal of nitrate, reducing up to an estimate  
24 of 70% of carbon requirements for simultaneous N and P removal. Varying fractions of Denitrifying  
25 Polyphosphate Accumulating Organisms (DPAOs), from an average of 25% to 84%, were found in  
26 different plants. No correlation was found between the DPAO fractions and EBPR configuration, season  
27 nor the concentration of any of the microbial groups measured via quantitative fluorescence *in situ*  
28 hybridisation. These included Type I and Type II *Ca. Accumulibacter* and Glycogen Accumulating  
29 Organisms, suggesting that chemical batch tests are the best methodology for quantifying the  
30 potential of anoxic P removal in full-scale WWTPs.

31 **KEYWORDS**

32 Denitrification, DPAOs, EBPR, Enhanced Biological Phosphorus Removal, Full-scale, Nutrient removal  
33

## 34 INTRODUCTION

35 Enhanced Biological Phosphorus Removal (EBPR) is a widely adopted technology for treating  
36 wastewater including the efficient removal of phosphorus. It addresses the growing concern of the  
37 environmental impacts caused by excessive nutrients in water bodies, known as eutrophication. This  
38 technology is based on alternating anaerobic and aerobic conditions to encourage the uptake of  
39 “luxury” phosphorus (P) by organisms who thrive under these conditions: Polyphosphate Accumulating  
40 Organisms (PAOs). There are many possible variations of the conventional configuration  
41 (Anaerobic/Aerobic or A/O), including the addition of one or multiple anoxic stages to achieve  
42 denitrification with the simultaneous removal of P (Sedlak 1991). Denitrification is achieved through  
43 the action of multiple groups of heterotrophic microorganisms, including PAOs (DPAOs), who can use  
44 nitrogen compounds (nitrate and/or nitrite) as electron acceptors. Theoretically and as shown in lab-  
45 scale studies, anoxic phosphorus removal is advantageous since it allows simultaneous P and nitrate  
46 removal on the same carbon source thus reducing oxygen requirements, as well as sludge production  
47 (Kuba et al. 1996a). However, whilst the process is well-established, very limited stoichiometric, kinetic  
48 and microbiological data is available on the capacity of full-scale EBPR plants to achieve simultaneous  
49 denitrification and phosphorus removal. Considering the recent advances provided by many studies  
50 on the identification of potential DPAOs (e.g., Carvalho et al. 2007; Flowers et al. 2009) and on their  
51 observed kinetic and stoichiometric parameters in enriched populations at lab-scale (e.g., Lanham et  
52 al. 2011; Rubio-Rincón et al. 2017; Ribera-Guardia et al. 2016; Saad et al. 2016), it is timely to  
53 investigate how this knowledge translates into the characterisation of full-scale communities.

54 PAOs and their known competitors, Glycogen Accumulating Organisms (GAOs), often also present in  
55 EBPR systems, have both been shown to denitrify. Within these organism groups, evidence has shown  
56 diversity within PAOs and GAOs in their denitrification pathways, from full denitrification from nitrate  
57 and/or nitrite to no denitrification. Early studies with limited microbial characterisation suggested two  
58 fractions of PAOs: DPAOs, able to remove phosphate in anoxic conditions (including the use of nitrate  
59 and nitrite), and non-DPAOs, only able to remove phosphate using oxygen as electron acceptor (Kern-  
60 Jespersen et al. 1993). Further studies, in particular of organisms belonging to the genus *Ca.*  
61 *Accumulibacter*, the most well-characterised PAO, showed different results regarding the  
62 denitrification capabilities of PAOs with nitrate and nitrite. Authors highlighted potential  
63 morphological (Carvalho et al. 2007), taxonomic (Flowers et al. 2009) and genetic (García-Martín et al.  
64 2006) differences that may explain different phenotypes. Fundamentally there are still two viable  
65 hypotheses: i) the existence of separate phenotypes including non-DPAOs, nitrite-DPAOs (mostly  
66 correlated with *Accumulibacter* clade II), and nitrate-DPAOs (mostly correlated with *Accumulibacter*  
67 clade I) (Flowers et al. 2009, Lanham et al. 2011); ii) only one phenotype exists, nitrite-DPAOs, and the  
68 observed conversion of nitrate to nitrite in different studies is provided via flanking populations (e.g.,  
69 Saad et al. 2016; Rubio-Rincón et al. 2017). *Tetrasphaera*-PAOs have been less studied. However,  
70 based on data of four isolates, they were shown to possess the genes for the reduction of nitrate to  
71 nitrite and some isolates (*T. japonica* and *T. elongata*) were shown to use nitrate and nitrite as electron  
72 acceptors (Kristiansen et al. 2012). Nevertheless, the amount of anoxic P uptake determined by a  
73 denitrifying *Tetrasphaera*-PAO enrichment has been found to be low (Marques et al. 2018). Regarding  
74 GAOs, as with *Accumulibacter*-PAOs, results indicate that taxonomic differences could result in  
75 different capacities to utilise nitrate and nitrite in both major studied organisms: *Ca. Competibacter*  
76 and *Defluviicoccus vanus*-related GAOs. For example, some *Competibacter* sub-groups could not  
77 denitrify, whilst others could use nitrate but not nitrite and some could use both (Kong et al. 2006). As

78 for *D.vanus*-related GAOs, Cluster 1 was shown to use nitrate whereas Cluster 2 could not and none  
79 could use nitrite (Wang et al. 2008; Burow et al. 2007). Due to the different findings as to the  
80 denitrification capacities of organisms involved in EBPR, a quantification of DPAOs could potentially be  
81 correlated with the abundance of Accumulibacter Type I PAOs or with the presence of GAOs.

82 Over time, different methods have been suggested to determine the fraction of DPAOs in mixed  
83 systems by quantification of the chemical transformations of phosphate and nitrogen. The assumption  
84 would be that under anoxic conditions, only PAOs able to use nitrate and/or nitrite (i.e. DPAOs) would  
85 remove phosphate, whereas under oxic conditions all PAOs would be able to remove phosphate. Thus,  
86 the rates or yields of P removal under anoxic conditions vs. aerobic conditions would be proportional  
87 to the ratio of DPAOs vs. PAOs. Initially, the ratio of the rate of P removal in parallel anoxic and aerobic  
88 batch tests was suggested (Wachtmeister et al. 1997). Subsequent improvements were proposed, first  
89 by Meinhold et al. (1999) and then by Oehmen et al. (2010), to use the ratio of phosphate removal to  
90 avoid kinetic biases. Based on these methods, sporadic estimations of DPAOs vs. non-DPAOs have been  
91 carried out in lab-scale (Oehmen et al. 2010; Wachtmeister et al. 1997), pilot scale (Meinhold et al.  
92 1999; García-Usach et al. 2010) and full-scale (Wachtmeister et al. 1997; Bai et al. 2011) studies.  
93 However, there has not been a systematic assessment of the efficiency of anoxic P removal vs. aerobic  
94 P removal in full-scale systems using nitrate, coupled with a microbiological analysis that could attempt  
95 to link DPAO fractions to the presence of Accumulibacter Type I and Type II or GAOs.

96 Therefore, this study investigated the activity of DPAOs in five full-scale EBPR plants with different  
97 configurations and over a period of time. The data collected was able to provide an important insight  
98 into the variability of the kinetics and the microbial composition of simultaneous denitrification and P  
99 removal within different full-scale plants, as well as shed some light on some of the factors that might  
100 influence DPAO selection.

## 101 **METHODS**

### 102 **Sample collection**

103 Five full-scale EBPR plants, labelled PT1-PT2 and DK1-DK3 respectively, were included in this study:  
104 three conventional anaerobic/anoxic/aerobic systems (A<sub>2</sub>O) (Beirolas WWTP, SIMTEJO, Lisbon,  
105 Portugal; Setubal WWTP, Águas do Sado, Setúbal, Portugal; Hjørring WWTP, Hjørring, Denmark) and  
106 two adapted Biondenitro configurations (Aalborg West and Aalborg East WWTP, Aalborg, Denmark). All  
107 plants were sampled at the end of the aerobic phase during winter conditions and the Portuguese  
108 plants were also sampled in summer conditions as detailed in Lanham et al. (2013).

### 109 **Offline Batch Tests and Chemical Analysis**

110 The activity of the PAO community in each sampled WWTP was tested using offline batch tests  
111 supplied with synthetic medium containing acetate as the main substrate. Nineteen tests were run as  
112 a 2-h anaerobic phase with the addition of an acetate (10-15 mg-C/L) and phosphate (30-40 mg-P/L)  
113 pulse. This slight variation is due to adjustments in the carbon and P concentration after the initial tests  
114 to ensure that a reasonable anaerobic carbon profile was determined and that P would always be in  
115 excess during the aerobic and anoxic phases. At the end of the anaerobic phase, the mixed liquor was  
116 split into two separate reactors, one aerobic (oxygen levels close to saturation) and one anoxic (pulse  
117 of 25 mg-N/L of nitrate) and run for approximately 4-6 h until PHA reserves were consumed and P  
118 removal stabilised, as proposed in Oehmen et al. (2010). In aerobic tests, the oxygen uptake rate (OUR)  
119 was measured by stopping the recirculation of mixed liquor into a respirometric side chamber and

120 measuring the decrease in dissolved oxygen (DO). In thirteen out of the nineteen experiments, a  
121 further aerobic phase was supplied after the anoxic phase to observe aerobic uptake of phosphate. pH  
122 and temperature were controlled at  $7.0 \pm 0.2$  and  $20 \pm 1$  °C respectively and dissolved oxygen was kept  
123 below 0.1 mg/L in anaerobic and anoxic conditions. Full details of sampling and operation are provided  
124 in Lanham et al. (2013).

125 The chemical transformations in the reactors were monitored by periodic sampling (typically every  
126 5-20 min) for measurements of acetate, phosphate and ammonia in the supernatant and of PHA,  
127 glycogen and total phosphorus in the biomass. For each phase, initial and final samples of glycogen  
128 were measured in triplicate. Acetate was determined by high pressure liquid chromatography,  
129 phosphate was measured by the ascorbic acid colorimetric method and ammonia was measured by a  
130 gas sensing combination electrode. Lyophilised biomass samples were digested and used for the  
131 quantification of PHA and glycogen via gas and liquid chromatography respectively. Volatile suspended  
132 solids (VSS) were determined by the gravimetric method according to APHA (1995). Full details of the  
133 analytical methods are described in Lanham et al. (2013). Nitrate and nitrite were monitored in the  
134 supernatant in each of the reactors, with greater detail in the anoxic phase. For Portuguese tests and  
135 Danish Plant DK3, the analysis was done using ion chromatography according to Lanham et al. (2011)  
136 and for DK1 and DK2 using colorimetric quantitative test strips for nitrate and nitrite (Merck,  
137 Germany). The chemical concentrations are given in C-mol of active biomass (X) by subtracting the  
138 amount of glycogen and PHA from the VSS (biomass formula  $CH_{1.84}O_{0.5}N_{0.19}$  (Zeng et al. 2003)).

#### 139 **Quantitative fluorescence *in situ* hybridisation (qFISH)**

140 qFISH was performed on all mixed liquor samples using an Axioskop epifluorescence microscope (Carl  
141 Zeiss, Oberkochen, Germany) to quantify target organisms by their biovolume as detailed in Nielsen  
142 (2009) and Mielczarek et al. (2012). The organisms targeted are detailed in Lanham et al. (2013). In  
143 addition to this, *Accumulibacter*-PAO Type I and II were targeted using probes Acc-I-444 and Acc-II-444  
144 by (Flowers et al. 2009a).

#### 145 **Estimation of carbon requirements**

146 The carbon requirements were estimated in three different configurations: anoxic EBPR (A2O, this  
147 study), conventional nitrification-denitrification, and EBPR (A/O) with additional denitrification. These  
148 were calculated based on the level of nitrate reduction achieved in this study, i.e., removal of a  
149 minimum of 25 ppm-N of nitrate. An anoxic EBPR system, as presented in this study, would consume  
150 the COD provided in this study (i.e., 10-15 C-ppm of acetate equivalent to 27-40 ppm COD) and in  
151 addition to nitrate removal, could remove a minimum of 9 ppm-P. A nitrification-denitrification  
152 system, would require 72 ppm-COD for the removal of the same quantity of nitrate (assuming 2.86  
153 mg-COD/mg  $NO_3-N$  as per ASM1 model (Henze et al. 1987)). Finally, adding the two values above, the  
154 combination of an A/O EBPR with a separate nitrification-denitrification would require a total of 99-  
155 112 ppm-COD (COD for EBPR + COD for denitrification).

156

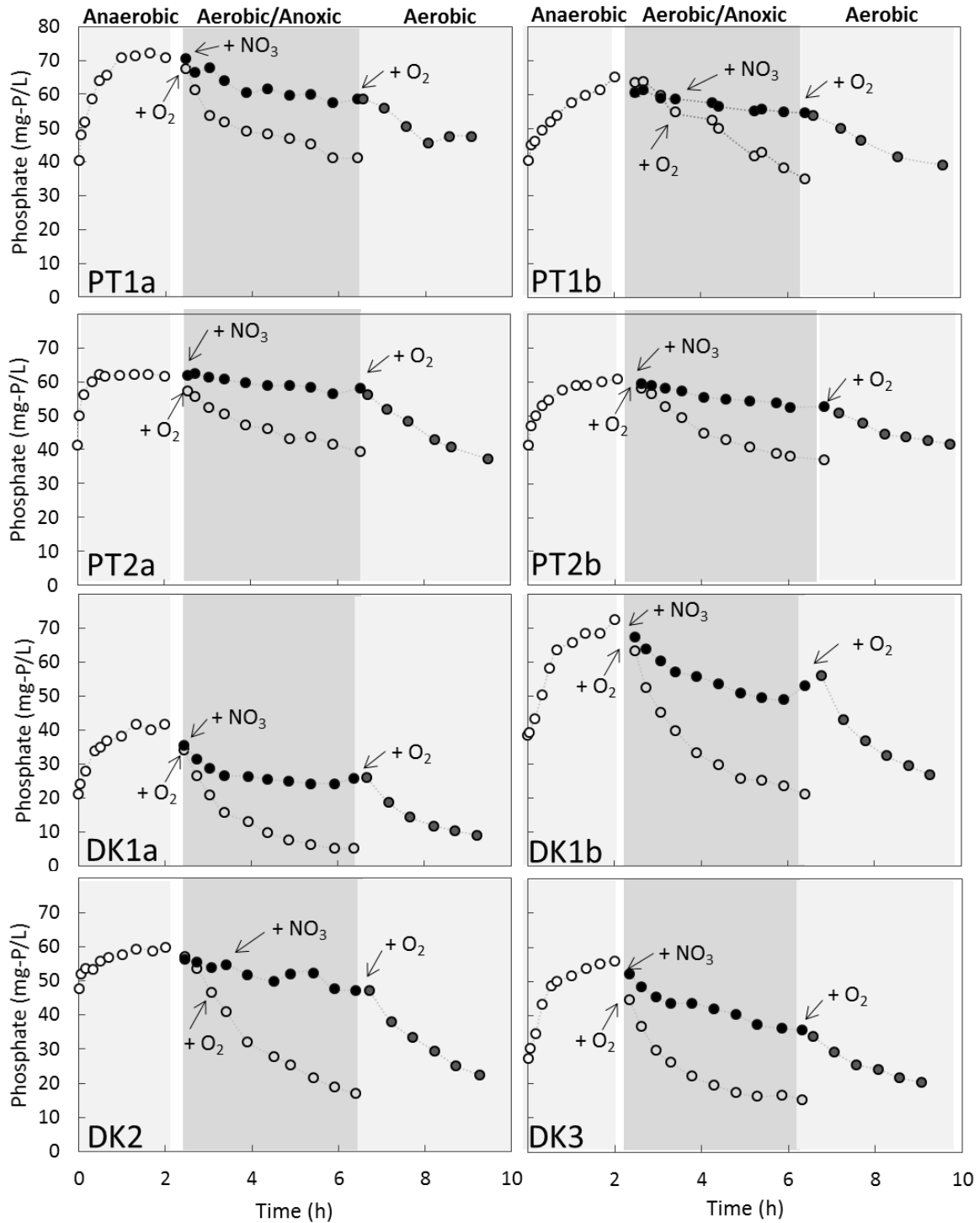
## 157 **RESULTS AND DISCUSSION**

### 158 **Aerobic and Anoxic P removal capacity in different full-scale WWTPs**

159 The first objective was to compare the anoxic and aerobic phosphate uptake in different full-scale EBPR  
160 plants using offline batch tests with acetate. After one single anaerobic phase, the mixed liquor was  
161 split into parallel aerobic and anoxic batch tests (addition of a nitrate spike). Examples of the  
162 phosphate cycling in the different experiments are given in **Error! Reference source not found.** All

163 plants included EBPR configurations with anoxic conditions, hence it is not surprising that all mixed  
164 liquors were able to remove phosphate both aerobically and anoxically. On average, under anoxic  
165 conditions, DPAOs were only able to remove 35% of the P removed under aerobic conditions. These  
166 values varied from plant to plant, proportionally to the different DPAO fractions (Figure 3), with PT2 in  
167 the summer presenting the lowest anoxic P removal (13%) and DK1 the highest (46%). However, when  
168 supplied with an additional aerobic phase, the total P removed is  $90 \pm 3\%$  of the P removed under only  
169 aerobic conditions (**Error! Reference source not found.**). This came at the expense of a marginal PHA  
170 and glycogen increase ( $109 \pm 7\%$  and  $106 \pm 7\%$ ) (**Error! Reference source not found.**). In terms of  
171 oxygen requirements, these depended on the fraction of DPAOs available and how much P was  
172 removed anoxically, but on average this configuration required only 70% of the oxygen needed for a  
173 full aerobic removal, as also observed by Kuba et al. (1996a) in a lab-scale system, with the added  
174 benefit of also removing approximately up to 25 ppm-N. This means simultaneously removing N and P  
175 at the expense of only 27-40 ppm COD instead of 99-112 ppm COD (c.f. methods section), i.e. a  
176 reduction in carbon requirements of approximately up to 70%, an estimate that is moderately higher  
177 to what was obtained by Kuba et al. (1996a) with lab-scale reactors (50%).

178

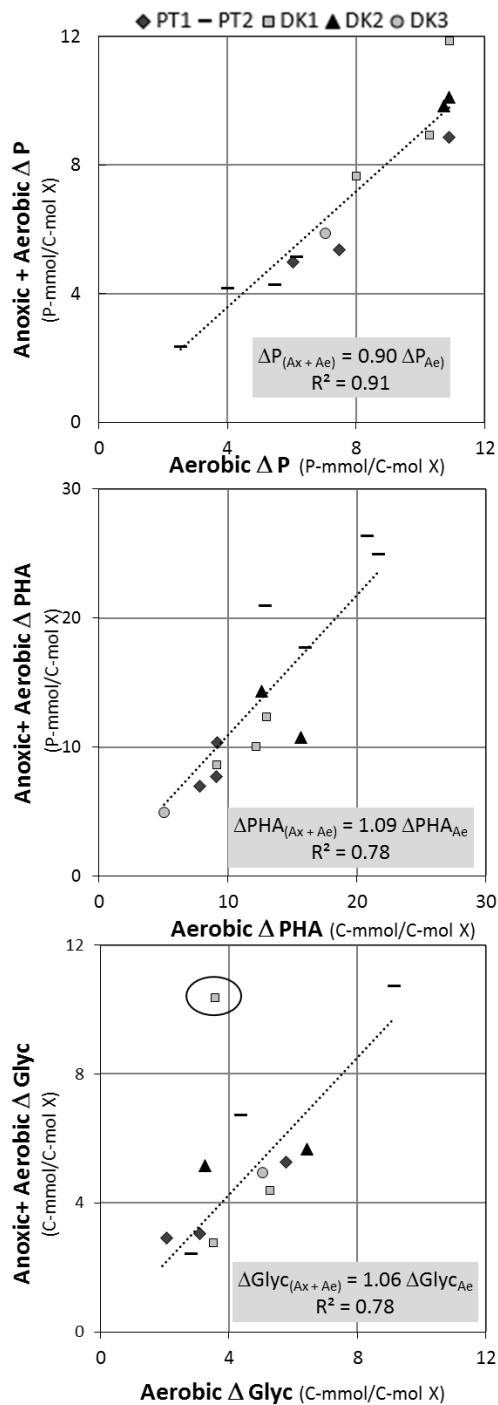


179 *Figure 1: Phosphate profiles in a sample of off-line batch tests across the 5 EBPR plants. The different data points were*  
 180 *obtained under anaerobic conditions (white circles) and then the sludge was partitioned into 2 parallel tests, one under*  
 181 *anoxic conditions (black circles) and one under aerobic conditions (light grey circles), and finally the anoxic sludge was*  
 182 *subjected to an aerobic phase (dark grey circles). Samples were run in triplicate and standard deviation was below 5%.*  
 183 *Anaerobic and aerobic profiles of other compounds are shown in Lanham et al. 2013.*

184

185

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187

188 *Figure 2: Correlation between phosphate (top), PHA (middle) and glycogen (bottom) uptake under aerobic conditions vs.*  
 189 *anoxic + aerobic conditions in the different plants (PT1 – diamond, PT2 – dash, DK1 – square, DK2 – triangle, DK3 – circle).*  
 190 *The circled result has been excluded from the linear regression, due to an extremely high measurement of glycogen in the*  
 191 *anoxic phase that could suggest an experimental error.*  
 192

193 In all batch tests, the rate of anoxic P removal (rP) was on average a third of the rate of aerobic P  
 194 removal (Table 1). This is similar to results obtained by Bai et al. (2011), also with full-scale sludge.  
 195 Once oxygen is introduced at the end of the anoxic phase, P uptake re-starts at a higher rate, but lower  
 196 than the one observed when only an aerobic phase is supplied, as also observed by Kerrn-Jespersen  
 197 and Henze (1993). Both anoxic and aerobic P-uptake are accompanied by the intracellular use of PHA



198 and glycogen replenishment. This suggests that PAOs are active under anoxic conditions with nitrate,  
199 either because they can directly denitrify or because flanking populations are converting nitrate to  
200 nitrite as proposed by Rubio-Rincón et al. (2017)), and this would be the rate limiting step as no nitrite  
201 accumulation was observed (typically below detection limit of 1 ppm-N).

202 Under anoxic conditions, excluding the results from PT2, where a high number of GAOs was detected,  
203 the yields of glycogen replenishment on intracellular PHA (Glyc/PHA) were within the ranges proposed  
204 in previous metabolic models (Table 2). However, the yield of phosphate removal at the expense of  
205 PHA (P/PHA) seemed more variable and higher than expected (Table 2). This could suggest the  
206 presence of other PAOs not or less reliant on PHA and glycogen, such as for example *Tetrasphaera*-  
207 PAOs, that would be able to contribute to some P removal (Marques et al. 2017). However, even if  
208 *Tetrasphaera* has been detected in significant quantities in all samples in this study (*cf.* Lanham et al.  
209 (2013)), they are the most abundant in plants DK1, DK2 and then PT1, which does not seem to align  
210 with the calculated P/PHA yields, with the highest being in DK3 followed by PT1.

211 In terms of the ratios of P removed per electron acceptor (P/O<sub>2</sub> for oxygen and P/N for nitrate) (Table  
212 1), the values are similar to the ones reported by Bai et al (2011) for full-scale experiments but  
213 generally lower than previous values reported in literature for lab-scale enriched cultures both with  
214 nitrate and nitrite as electron acceptor (Table 1). This implies a lower overall efficiency of full-scale  
215 sludge to remove P in function of their electron acceptor. This could be a result of the lower  
216 concentration of PAO organisms in the mixed liquor coupled with a residual maintenance activity of  
217 other microbial communities relying on these electron acceptors. This emphasizes the importance of  
218 measuring the aerobic and anoxic P removal in full-scale systems in addition to the fundamental  
219 studies in lab-scale enriched communities.

220

221

222 *Table 1: Stoichiometric and kinetic values related to P uptake under aerobic and anoxic conditions. Results shown are averages*  
 223 *for all sampling points (n = number of tests), standard deviation and range of values to illustrate the spread of data.*

		<b>Aerobic</b>		<b>Anoxic</b>		<b>Aerobic (after Anoxic)</b>	
		P/O <sub>2</sub>	rP	P/N	rP	P/O <sub>2</sub>	rP
		P-mol/O <sub>2</sub> -mol	mg-P/gVSS.h	P-mol/N-mol	mg-P/gVSS.h	P-mol/O <sub>2</sub> -mol	mg-P/gVSS.h
PT1 summer		0.38 ± 0.03	8 ± 3	0.25 ± 0.08	2.9 ± 0.8	0.15 ± 0.04	2.9 ± 0.8
n <sub>AE</sub> or n <sub>AX</sub> = 4, n <sub>AX+AE</sub> = 2		(0.35 – 0.42)	(3.7 – 11.4)	(0.14 – 0.32)	(1.7 – 3.6)	(0.12 – 0.18)	(2.3 – 3.8)
PT1 winter		0.40 ± 0.06	3 ± 1	0.17 ± 0.02	1.0 ± 0.5	ND	2.0
n <sub>AE</sub> or n <sub>AX</sub> = 2, n <sub>AX+AE</sub> = 1		(0.35 – 0.44)	(2.5 – 4.0)	(0.15 – 0.18)	(0.7 – 1.4)		(n=1, 2.0)
PT2 summer		0.20 ± 0.08	2.0 ± 0.2	0.05 ± 0.01	0.6 ± 0.3	0.2 ± 0.1	1.9 ± 0.2
n <sub>AE</sub> or n <sub>AX</sub> = 2, n <sub>AX+AE</sub> = 2		(0.14 – 0.25)	(1.9 – 2.1)	(0.04 – 0.06)	(0.4 – 0.9)	(0.13 – 0.29)	(1.7 – 2.0)
PT2 winter		0.25 ± 0.04	2.5 ± 0.2	0.16 ± 0.02	0.8 ± 0.1	0.21 ± 0.04	1.8 ± 0.1
n <sub>AE</sub> or n <sub>AX</sub> = 2, n <sub>AX+AE</sub> = 2		(0.22 – 0.27)	(2.4 – 2.7)	(0.15 – 0.17)	(0.7 – 0.8)	(0.18 – 0.23)	(1.7 – 1.9)
DK1 winter		0.40 ± 0.07	7.2 ± 0.7	ND	3 ± 2	0.3 ± 0.1	4 ± 1
n <sub>AE</sub> or n <sub>AX</sub> = 4, n <sub>AX+AE</sub> = 3		(0.29 – 0.45)	(6.4 – 8.0)		(1.6 – 5.6)	(0.27 – 0.45)	(2.9 – 5.5)
DK2 winter		0.40 ± 0.08	5 ± 1	ND	1.2 ± 0.2	0.43 ± 0.0	4.2 ± 0.9
n <sub>AE</sub> or n <sub>AX</sub> = 3, n <sub>AX+AE</sub> = 2		(0.31 – 0.47)	(4.1 – 6.0)		(1.0 – 1.4)	(0.42 – 0.43)	(3.6 – 4.9)
DK3 winter		0.29 ± 0.08	6.1 ± 0.0	0.26 ± 0.08	2.0	0.24	2.0
n <sub>AE</sub> or n <sub>AX</sub> = 2, n <sub>AX+AE</sub> = 1		(0.24 – 0.35)	(6.1 – 6.1)	(0.20 – 0.31)	(n=1, 2.0)	(n=1, 0.24)	(n=1, 2.0)
Full-scale, HAC, NO <sup>3-</sup>	Kuba et al. (1997) (n=1)	-	13	-	6	-	-
	Bai et al. (2011) (n=3)	-	2.00 – 3.85	0.05 – 0.3	0.54 – 2.22	-	-
Lab scale, HAC, NO <sup>3-</sup>	Kuba et al. (1993)	0.91	29 - 46 <sup>a</sup>	0.94	29 - 46 <sup>a</sup>	-	-
	Carvalho et al. (2007)	-	-	0.6 <sup>b</sup>	0.6 – 8.4	-	-
Lab scale, HPr, NO <sup>3-</sup>	Carvalho et al. (2007)	-	-	0.82 <sup>b</sup>	3.1 – 19.5	-	-
	Guisasola et al. (2009)	-	-	1.6 <sup>b</sup>	0.6 - 2.2	-	-
Lab scale, HAC, NO <sup>2-</sup>	Saad et al. (2016)	1.38	-	0.70	17.1	-	-
Lab scale, HPr, NO <sup>2-</sup>	Saad et al. (2016)	1.28	-	0.46	12.4	-	-
	Guisasola et al. (2009)	-	-	0.29 <sup>b</sup>	3.7 - 7.13	-	-

<sup>a</sup>calculated by converting gSS to gVSS using 0.65-0.70 gVSS/gSS

<sup>b</sup>calculated from anoxic rates of P-uptake/N-uptake

224  
225

226 Table 2: Experimental results for offline anoxic batch tests (n=19) followed by an aerobic phase (n= 13). Results shown are  
 227 averages rounded to one decimal (n = number of tests) with a standard deviation and the range of values to illustrate the  
 228 spread of the data.

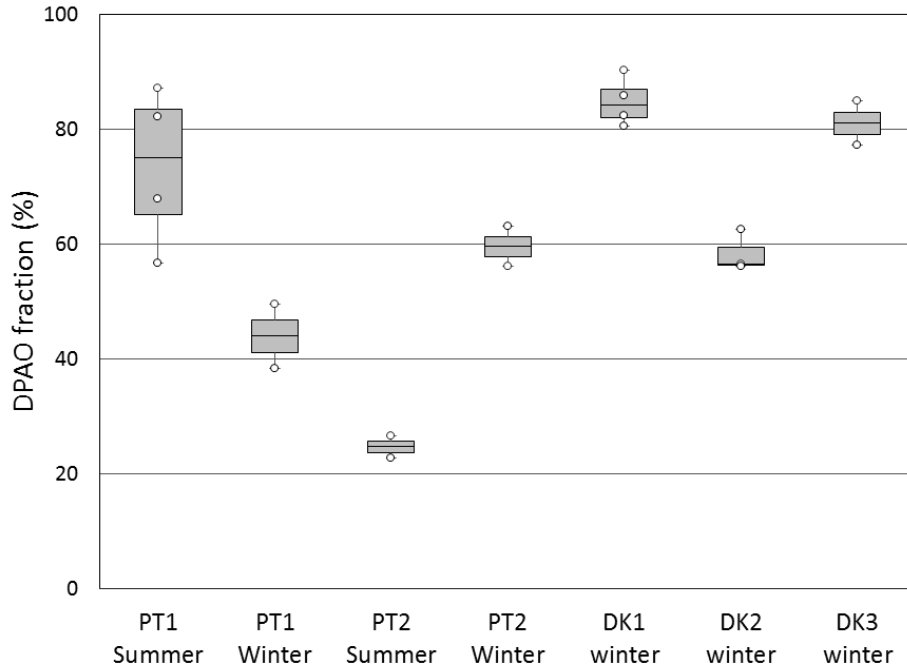
	Anoxic + Aerobic Experimental Results			
	Anoxic Phase		Aerobic Phase	
	P/PHA P-mol/C-mol	Glyc/PHA C-mol/C-mol	P/PHA P-mol/C-mol	Glyc/PHA C-mol/C-mol
PT1 summer (n <sub>AX</sub> = 4, n <sub>AX+AE</sub> = 2)	0.7 ± 0.2 (0.53 – 0.83)	0.7 ± 0.5 (0.31 – 1.26)	1.7 ± 0.7 (1.20 – 2.17)	0.9 ± 0.2 (0.77 – 1.06)
PT1 winter (n <sub>AX</sub> = 2, n <sub>AX+AE</sub> = 1)	0.7 ± 0.5 (0.26 – 1.04)	0.3 ± 0.1 (0.28 – 0.40)	0.9 (n=1, 0.86)	0.3 (n=1, 0.28)
PT2 summer (n <sub>AX</sub> = 2, n <sub>AX+AE</sub> = 2)	0.0 ± 0.0 (0.03 – 0.05)	0.1 (n=1, 0.13)	0.3 ± 0.0 (0.31 – 0.32)	0.1 (n=1, 0.10)
PT2 winter (n <sub>AX</sub> = 2, n <sub>AX+AE</sub> = 2)	0.2 ± 0.0 (0.19 – 0.25)	0.1 ± 0.0 (0.05 – 0.10)	0.2 ± 0.0 (0.15 – 0.19)	0.5 ± 0.1 (0.38 – 0.57)
DK1 winter (n <sub>AX</sub> = 4, n <sub>AX+AE</sub> = 3)	0.7 ± 0.2 (0.59 – 1.00)	0.5 ± 0.6 (0.02 – 1.36)	1.3 ± 0.2 (1.05 – 1.42)	0.7 ± 0.2 (0.52 – 0.90)
DK2 winter (n <sub>AX</sub> = 3, n <sub>AX+AE</sub> = 2)	0.7 ± 0.3 (0.50 – 1.05)	0.3 ± 0.1 (0.24 – 0.39)	0.9 ± 0.1 (0.81 – 0.90)	0.5 ± 0.2 (0.36 – 0.59)
DK3 winter (n <sub>AX</sub> = 2, n <sub>AX+AE</sub> = 1)	1.0 ± 0.1 (0.92 – 1.07)	0.5 ± 0.6 (0.06 – 0.88)	1.4 (n=1, 1.38)	1.0 (n=1, 1.04)
PAO model	0.30 <sup>b</sup>	0.41 <sup>b</sup>	0.41 <sup>a</sup>	0.42 <sup>a</sup>

<sup>a</sup> According to Smolders et al. (1995), <sup>b</sup> According to Kuba et al. (1996b)

229

230 **Calculation of DPAO fraction**

231 The experimental results obtained from the offline batch tests provided a means to determine the  
 232 fraction of DPAOs ( $f_{DPAO}$ ) and non-DPAOs ( $f_{n-DPAO}$ ) over total Accumulibacter-PAO in each of the  
 233 sampling points (Figure 3). For this purpose, a modification of the method proposed in Oehmen et al.  
 234 (2010) was used (Equations (1)(2), where  $f_{DPAO}$  is assumed to be correlated with the quantity of P  
 235 removed under anoxic conditions with nitrate as the electron acceptor vs. the quantity of P removed  
 236 under aerobic conditions, in parallel batch tests containing the same sludge. The modification accounts  
 237 for nitrate having a lower energetic efficiency than oxygen as an electron acceptor due to a lower ATP  
 238 production during the oxidative phosphorylation mechanism (Kuba et al. 1996b; Murnleitner et al.  
 239 1997). The phosphorus uptake in anoxic conditions was corrected using the P/O ratios ( $\delta_{aer}$ , and  
 240  $\delta_{anox}$ ), i.e. the amount of ATP produced per oxidised NADH<sub>2</sub> during phosphorus uptake under aerobic  
 241 and anoxic conditions respectively. Smolders et al. (1994) proposed that  $\delta_{aer}$  is 1.85 and Kuba et al.  
 242 (1996b) proposed that  $\delta_{anox}$  is 1.



243

244 Figure 3: Boxplot representing DPAO fractions (white circles) in each plant and sampling period (winter and summer).  
 245

246 The highest denitrification activity was found in DK1, with a median of 84% of the PAO population  
 247 being DPAOs, and the lowest (25%) was found in PT2 during the summer sampling. In the plants with  
 248 seasonal sampling, there were quite significant differences in DPAO activity, suggesting that this  
 249 capacity can fluctuate quite significantly (from medians 75% to 44% in PT1 and 25% to 60% in PT2 for  
 250 summer and winter samplings respectively).

$$f_{DPAO} + f_{n-DPAO} = 1 \quad (1)$$

$$f_{DPAO} = \frac{\Delta P_{anox}}{\Delta P_{aer}} \times \frac{\delta_{aer}}{\delta_{anox}} \quad (2)$$

251

252 **Correlation between microbial composition and DPAO activity**

253 All samples contained 2-7% of *Ca. Accumulibacter*. Total concentrations of putative *Tetrasphaera*-  
 254 PAOs were higher, ranging from 15-30%, however considering most are not likely to consume acetate,  
 255 their activity should not be expressed in the experimental results. Finally, *Competibacter* and  
 256 *Defluviicoccus*-related GAO concentrations were far more variable ranging from 1 to 6%. 7 out of the  
 257 19 samples contained either only *Competibacter*-GAOs (n=1), only *Defluviicoccus*-related GAOs (n=4)  
 258 or both (n=2) in concentrations above 0.3%. These results are detailed in Lanham et al. (2013).

259 The ability of PAOs to denitrify from nitrate has been suggested to be linked with diversity within  
 260 different types of *Accumulibacter* (Flowers et al. 2009), with some evidence suggesting that Type I  
 261 would be able to denitrify from nitrate, whereas Type II only from nitrite, although Saad et al. (2016)  
 262 has also reported a population of *Accumulibacter* clade IC not able to denitrify from nitrate. However  
 263 in this study, there does not seem to be any correlation between the biovolume of Type I

264 Accumulibacter nor its fraction over the total PAO biovolume and the capacity to remove P under  
265 anoxic conditions in each of the plants (n = 19, R<sup>2</sup> is 0.27 and 0.06 respectively). Similarly, no correlation  
266 was found for the biovolume of Accumulibacter Type II nor its fraction over total PAO biovolume (n =  
267 19, R<sup>2</sup> are both 0.00).

268 Average results for the q-FISH quantification of Type I and Type II Accumulibacter biovolumes in the  
269 different samples across the five tested WWTPs are shown in Table 3. It is important to note that these  
270 two probes do not necessarily cover all the diversity within Accumulibacter-PAOs (Flowers et al. 2009)  
271 and there might even be some overlap, as can be seen by calculating the ratio of the sum of Type I and  
272 Type II over total PAO determined by the PAOmix FISH probe (Table 3). In fact, for many of the samples,  
273 these two probes only covered approximately 50% of the organisms targeted by the probe PAOmix. In  
274 some others, their value was higher than PAOmix, which could indicate some Accumulibacter over-  
275 estimation using the PAOmix probe due to the inclusion of *Propionivibrio aalborgensis*, a putative GAO,  
276 as recently presented by Albertsen et al. (2016).

277 It has also been suggested that PAOs would only be able to denitrify from nitrite and that they would  
278 rely on flanking populations to produce nitrite from nitrate (Rubio-Rincón et al. 2017) and potentially  
279 on GAO-related populations. However, from the samples collected, there was also no observable  
280 correlation between the presence of GAOs and any increased phosphate removal under denitrification  
281 conditions. This applies for Competibacter-GAOs (n=19, R<sup>2</sup> is 0.00, 0.00 and 0.05 for the relationship  
282 between DPAO fraction and Competibacter biovolume, fraction of Competibacter over PAO biovolume  
283 and over total GAO biovolume, respectively) and for *Defluviicoccus*-related GAOs (n=19, R<sup>2</sup> is 0.20 and  
284 0.30 for the relationship between DPAO fraction and *Defluviicoccus* biovolume and fraction of  
285 *Defluviicoccus* over total PAO biovolume, respectively). In fact, the samples with the highest  
286 concentration of GAOs, observed in plant PT2, resulted in the lowest P removal in anoxic conditions.

287 In conclusion, even if a wide range of DPAO activity has been found, varying seasonally and from plant  
288 to plant, this could not be explained by the microbiological results in terms of the presence of  
289 Accumulibacter Type I or II, Competibacter or any other detected GAOs. Therefore, chemical batch  
290 tests are the best methodology to assess a plant's potential of simultaneous P and N removal.

291

292 *Table 3: Microbial composition of sampling points in each WWTP in terms of Accumulibacter type I and type II. Values given*  
 293 *are averages with standard deviations, plus the range of experimental values.*

	Type I Accumulibacter (% biovolume Acc-I-444)	Type II Accumulibacter (% biovolume Acc-II-444)	Ratio Type I + Type II vs. Total Accumuli.
PT1 summer (n = 4)	0.5 ± 0.1 (0.39 – 0.66)	0.7 ± 0.0 (0.65 – 0.71)	0.4 ± 0.1 (0.30 – 0.53)
PT1 winter (n = 2)	1.5 ± 0.1 (1.41 – 1.59)	1.5 ± 0.1 (1.41 – 1.59)	0.9 ± 0.0 (0.90 – 0.94)
PT2 summer (n = 2)	0.4 ± 0.1 (0.31 – 0.49)	1.1 ± 0.4 (0.84 – 1.36)	0.5 ± 0.0 (0.47 – 0.51)
PT2 winter (n = 2)	2.7 ± 0.0 (2.62 – 2.68)	2.8 ± 0.0 (2.80 – 2.84)	1.4 ± 0.4 (1.14 – 1.65)
DK1 (n = 4)	1.6 ± 0.2 (1.41 – 1.88)	1.7 ± 0.4 (1.22 – 2.08)	1.0 ± 0.2 (0.79 – 1.25)
DK2 (n = 3)	1.9 ± 0.5 (1.26 – 2.28)	1.7 ± 0.4 (1.29 – 2.09)	0.5 ± 0.2 (0.37 – 0.68)
DK3 (n = 2)	1.5 ± 0.1 (1.39 – 1.55)	1.3 ± 0.1 (1.18 – 1.37)	0.6 ± 0.0 (0.59 – 0.62)

294

295

## 296 CONCLUSIONS

297 This work systematically assessed the capacity of five full-scale EBPR plants to remove phosphorus  
 298 under anoxic and aerobic conditions, as well as the fraction of DPAO organisms in each of the sampling  
 299 points. The results of a total of 19 independent tests showed that all plants were able to remove  
 300 phosphate anoxically, even if only partially (35%) and at lower rates than in aerobic conditions.  
 301 However, in a configuration with anoxic followed by aerobic conditions, approximately the same  
 302 phosphate removal was achieved (90%), with only 70% of the oxygen requirements, and given the  
 303 simultaneous nitrate uptake, reducing up to 70% of the carbon requirements for N and P removal.  
 304 DPAO fractions varied across plants and in the same plant over time, from 25% (PT2) to 84% (DK1).  
 305 There was no correlation found between the microbial composition of the samples, especially in terms  
 306 of Accumulibacter Type I or Competibacter as suggested in the literature, with the abundance of  
 307 DPAOs through anoxic and aerobic batch tests. These results suggest that chemical tests are still the  
 308 best method to determine the performance of a full-scale EBPR system under anoxic conditions and  
 309 that further work still has to ascertain which microbial groups are responsible for anoxic P removal  
 310 from nitrate.

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