Factors affecting *Accumulibacter* population structure in full- and laboratory-scale biological reactors with nutrients removal

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ABSTRACT

The structure of *Accumulibacter* lineage was examined over a three-year period in six full-scale wastewater treatment plants and compared to the population in a laboratory-scale reactor. The *Accumulibacter* lineage reached 69% of all bacteria in the laboratory-scale reactor and contained clades IA and IIA,C,D only. In full-scale plants, *Accumulibacter* constituted up to 12%, correlated with sludge loading with BOD, COD, N and P. Clade IA was more abundant after periods with low temperatures, whereas clades IIA,C,D presented opposite variations. The fraction, unrevealed by clade-specific probes, constituted 31–62% of the *Accumulibacter* lineage in all but one full-scale plant – the population in the plant with significant industrial contribution in the influent resembled the low diversity in the laboratory-scale reactor. Selection of specific clades in the laboratory-scale reactor was associated with its different performance, despite stable operational conditions being maintained through the study. It implies that high relative abundance of *Accumulibacter* in bacterial community is not enough for efficient P removal and the effectiveness may also be associated with the presence of specific clades. A considerable fraction of *Accumulibacter* in full-scale plants, which is not targeted by clade-specific probes, should be further investigated to better characterize clades that may affect effectiveness of phosphorus removal.

Key words | activated sludge, EBPR, ecological selection, FISH, PAO, *ppk1*

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INTRODUCTION

Enhanced biological phosphorus removal (EBPR) is a widely used method to eliminate phosphorus from wastewater in activated sludge systems. The process is mediated by polyphosphate accumulating organisms (PAOs), which are enriched in EBPR systems under alternating anaerobic/aerobic cycles. '*Candidatus* Accumulibacter phosphatis' (hereafter referred to as *Accumulibacter*) is a model PAO, which has been found in both full-scale wastewater treatment plants (WWTPs) and laboratory-scale reactors (Oehmen *et al.* 2007). Based on the analysis of polyphosphate kinase genes (*ppk1*), *Accumulibacter* has been divided into two ecotypes – Type I and II, consisting of 5 clades (IA-E) and 9 clades (IIA-II_I), respectively (He *et al.* 2007; Peterson *et al.* 2008; Mao *et al.* 2015). These clades differ in their ecophysiology, including variety of pathways in carbon metabolism, abilities to reduce nitrate and nitrite, or substrate affinities (Flowers *et al.* 2013; Skennerton *et al.* 2015; Camejo *et al.* 2016). It is hypothesized that the effectiveness of the EBPR process may depend to some extent on the structure and function of the *Accumulibacter* population (Slater *et al.* 2010; Gonzales-Gil & Holliger 2011). However, most WWTPs configurations were designed in an empirical manner, without full knowledge about the main PAOs, such as *Accumulibacter* or *Tetrasphaera* (Oehmen *et al.* 2007; Muszyński *et al.* 2013). The EBPR process is often examined in laboratory-scale reactors, fed with synthetic wastewater containing a single source of carbon. In such artificially controlled operational conditions bacteria experience selective pressures, which are markedly different to those encountered in full-scale systems. The aim of this study was to analyse the structure and seasonal variations of *Accumulibacter* lineage over a three-year period in six full-scale WWTPs, and to find connections between abundance of *Accumulibacter* lineage and

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operational parameters. WWTPs differed in the configuration of reactors and main technological parameters of treatment process. Chemical and operational data were analysed to assess their influence on the abundance and structure of *Accumulibacter* lineage. Furthermore, diversity of *Accumulibacter* lineage in full-scale WWTPs was compared with its diversity in a laboratory-scale sequencing batch reactor (SBR), which was seeded with activated sludge from one of the examined full-scale WWTPs.

MATERIALS AND METHODS

WWTPs and laboratory-scale reactor data

Full-scale WWTPs. Six full-scale municipal WWTPs, located in the central part of Poland and designated I-VI, were selected for the three-year research which was carried out in the period September 2011 to March 2014. WWTPs treated typical domestic wastewater, except WWTPs II and IV, where industrial contribution (expressed as BOD_5) in influents ranged 20–25% (slaughterhouse, dairy) and 30–50% (fruit and vegetables processing, sugar refining), respectively. The main operational parameters of the plants are listed in Table 1 (all the data were provided by the plant operators). All the WWTPs had nitrification and denitrification tanks for biological N removal and four of them (except WWTPs II and VI) also anaerobic tanks to favour EBPR. Activated sludge samples were collected twice a year, in early spring (at the beginning of March) and in early autumn (at the end of September). The samples were taken from the aerobic process tanks and kept on ice during transportation to the laboratory.

Laboratory-scale SBR. A laboratory-scale reactor with a working volume of 6.9 l was seeded with sludge from WWTP V and operated for over 100 days for *Accumulibacter* enrichment as described previously by Muszyński & Miłobędzka (2015). Briefly, the reactor cycle consisted of an anoxic/anaerobic period of 120 min (including 10 min of filling), an aerobic period of 190 min, a settling period of 40 min and a decantation period of 10 min. This resulted in a 6 h cycle and a hydraulic retention time (HRT) of 12 h. At the end of the aerobic period, the excess sludge was withdrawn once a day as mixed liquor to maintain a solids retention time (SRT) of 8–18 days and mixed liquor suspended solids (MLSS) of 3–4 g/l. The reactor was fed with a synthetic medium containing (mg per litre): 770 CH_3COONa, 1.5 peptone, 1.5 yeast, 153 NH_4Cl, 180 $MgSO_4{\cdot}7H_2O$, 21.5 $CaCl_2$, 0.9 $FeCl_3{\cdot}6H_2O$, 0.09 H_3BO_3, 0.018 $CuSO_4{\cdot}5H_2O$, 0.108 KI, 0.072 $MnCl_2{\cdot}4H_2O$, 0.036 $Na_2MoO_4{\cdot}2H_2O$, 0.072 $ZnSO_4{\cdot}7H_2O$, 0.09 $CoCl_2{\cdot}6H_2O$, 6 EDTA, 112 K_2HPO_4 and 88 KH_2PO_4, corresponding to an influent concentration of 600 mg COD/L and COD/P ratio of 15:1. After 80 days of operation, stable reactor performance was achieved, resulting in efficient COD and P removal (>92% and >98%, respectively) and low concentrations in the effluent (<50 mg COD/L and <0.8 mg P/L).

Molecular analysis

Quantitative fluorescence in situ hybridization (qFISH). The microbial abundance was examined using qFISH as described previously (Nielsen *et al.* 2009). 6-Fam labelled EUBmix oligonucleotide probes (equimolar mixture of EUB338, EUB338II, and EUB338III) were used to target the entire bacterial community. The abundance of the whole *Accumulibacter* lineage, Type I *Accumulibacter* (clade IA and others) and Type II *Accumulibacter* (clades IIA, IIC and IID) was determined directly by qFISH using PAOmix (equimolar mixture of PAO462, PAO651 and PAO846), Acc-I-444 and Acc-II-444. The abundance of other *Accumulibacter* clades, not targeted by either of the clade-specific probes, was calculated by subtracting the abundance of Type I and II *Accumulibacter* from the total abundance of the whole lineage. Detailed information about the probes used in the study is given in probeBase (Greuter *et al.* 2016), except for Acc-I-444 and Acc-II-444, which are described by Flowers *et al.* (2009). Similar quantification procedures were performed to those described in Muszyński *et al.* (2015). Briefly, 20 separate images for each probe were captured with a Nikon Eclipse 50i microscope (60× objective) and analysed using ImageJ software (Collins 2007). The microbial abundance (biovolume, expressed as % of EUBmix), which was relative to the pixel area of cells positive for the specific probe, was then quantified as a percentage of the pixel area for all bacteria positive for the EUBmix (a mean of 20 separate measurements). Standard error was calculated as a standard deviation of the percentage abundance of specific bacteria divided by a square root of 20 measurements.

Polyphosphate kinase genes (ppk1). Microdiversity of *Accumulibacter* lineage was investigated using polymerase chain reaction (PCR) and clade-specific *ppk1* primers. Genomic DNA was isolated with a PowerSoil® DNA Isolation Kit (MO BIO), and the obtained DNA was stored at −80 °C until analyses. Five sets of primers were used for clades of Type I and clades IIA, IIB, IIC and IID (He *et al.* 2007). The PCR was carried out using a Mastercycler

Table 1 | Characteristics of influents to biological reactors, effluents and operational parameters in WWTPs investigated in this survey. mean values (used for statistical analyses) and ranges (in parentheses) are listed for each parameter

Parameter	WWTP I	WWTP II	WWTP III	WWTP IV	WWTP V	WWTP VI
Size designed/actual (PE)	55,400/73,400	83,000/99,000	163,500/110,000	53,040/76,000	7,500/6,000	27,000/18,500
Reactor type	A2O	ANOX/AERO	AN tank + OD	UCT	A2O	Carrousel
Predenitrification	Yes	No	No	No	Yes	No
Presettling	Yes	Yes	Yes	Yes	No	No
Fermenter	Yes	No	No	No	No	No
Aeration	Diffusers	Surface (vertical)	Surface (horizontal) (ORP controller)	Diffusers	Diffusers	Surface (vertical) + diffusers
P-precipitation	Occasionally (PIX)	Continuous (PIX)	Occasionally (PIX)	Occasionally (PIX)	NO	Occasionally (PIX)
Industrial wastewater (% of influent BOD_5)	<10%	20–25%	<10%	30–50%	<2%	<2%
Sludge loading [g BOD_5/gMLSS/d]	0.03 (0.02–0.07)	0.06 (0.02–0.26)	0.03 (0.01–0.08)	0.06 (0.02–0.25)	0.06 (0.01–0.30)	0.05 (0.02–0.12)
Sludge loading [g COD/gMLSS/d]	0.09 (0.02–0.20)	0.14 (0.04–1.27)	0.09 (0.02–0.34)	0.09 (0.02–1.28)	0.22 (0.01–0.73)	0.12 (0.04–0.32)
SVI [mL/g]	225 (108–389)	178 (93–262)	206 (152–240)	89 (35–213)	152 (56–395)	127 (91–199)
SRT [d]	24 (10–59)	38 (1–218)	37 (9–397)	33 (9–238)	17 (3–111)	29 (25–33)
Influent to biological reactor						
BOD_5 [mg/L]	176 (79–392)	285 (77–673)	350 (144–793)	844 (160–4,900)	411 (35–921)	457 (179–613)
COD [mg/L]	452 (98–1,334)	640 (168–6,241)	988 (280–2,455)	1,402 (157–29,146)	1,477 (284–4,728)	1,021 (334–1,568)
N_{total} [mg N/L]	68 (24–149)	67 (13–139)	82 (8–151)	74 (23–888)	88 (18–153)	85 (52–132)
P_{total} [mg P/L]	10 (3.4–46)	10 (3.6–51)	17 (6.4–46)	18 (3.0–320)	22 (3.5–63)	14 (5.3–47)
BOD_5/N [g BOD_5/g N]	2.7 (1.3–6.3)	4.5 (1.1–19)	5.3 (1.6–46)	11 (2.5–70)	4.9 (0.3–20)	5.5 (3.5–11)
COD/N [g COD/g N]	6.8 (2.4–14)	10 (5.0–74)	14 (3.9–115)	17 (4.8–109)	17 (3.8–81)	12 (6.5–16)
BOD_5/P [g BZT_5/g P]	20 (5.7–39)	31 (4.3–101)	24 (6.8–96)	68 (7.5–295)	22 (1.4–115)	39 (13–62)
COD/P [g ChZT/g P]	50 (16–121)	69 (8.0–655)	69 (7.8–375)	90 (13–607)	73 (12–328)	85 (25–122)
pH	7.6 (7.2–7.9)	7.6 (5.5–8.2)	7.6 (7.4–8.1)	7.4 (5.9–9.2)	8.3 (6.7–8.8)	7.5 (7.2–7.8)
effluent						
BOD_5 [mg O_2/L]	60 (3.0–13)	15 (4.3–34)	5.5 (1.9–12)	8.2 (1.0–42)	3.5 (0–30)	9.3 (0–75)
COD [mg O_2/L]	59 (25–114)	45 (21–154)	25 (10–47)	72 (7.2–227)	33 (12–87)	43 (15–149)
N_{total} [mg N/L]	22 (8.1–85)	18 (0.4–91)	5.0 (2.1–12)	11 (0.2–53)	14 (1.5–48)	13 (2.2–41)
P_{total} [mg P/L]	0.9 (0.1–2.0)	1.1 (0.1–42)	0.4 (0.1–0.7)	0.8 (0.02–20)	0.3 (0.01–2.1)	0.6 (0.1–11)
pH	7.5 (6.5–7.9)	7.6 (7.0–7.9)	7.8 (7.6–8.0)	7.9 (7.2–9.0)	NA	7.2 (6.7–7.8)

A2O, anaerobic–anoxic–aerobic; AERO, aerobic; AN, anaerobic; ANOX, anoxic; BOD, biological oxygen demand; COD, chemical oxygen demand; MLSS, mixed liquor suspended solids; NA, not available; OD, anoxic/aerobic oxidation ditch with oxidation-reduction potential controller; PE, population equivalent; PIX, iron based coagulants; SVI, sludge volume index; SRT, sludge retention time; UCT, University of Cape Town.

proS (Eppendorf) in a 50 µl reaction volume with 1 µM of primers and 0.05 U/µl of DNA Maxima HotStart Taq polymerase (Thermo Scientific). The PCR program consisted of an initial 4-min hot-start at 95 °C, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing for 45 s (61 °C for clade I, IIA, IIB, and 66 °C and 63 °C for clades IIC and IID, respectively) and extension at 72 °C for 30 s with final elongation at 72 °C for 5 min. The presence of PCR amplicons was visualized by 2% agarose gel electrophoresis. Some randomly chosen bands were cut out of the gel and sequenced to confirm their identity (Laboratory of DNA Sequencing and Oligonucleotide Synthesis, IBB PAS).

Chemical analyses

Soluble orthophosphate and chemical oxygen demand (COD) were determined by the use of standard LCK vial test kits (HACH-Lange). Mixed liquor suspended solids (MLSS) were determined at the end of the aerobic periods in accordance with Standard Methods (Clescerl *et al.* 1999).

Statistical measures and methods

In order to find the strength of the relationship between the quantified bacteria populations and the operational parameters, correlation analyses (with Pearson's product moment correlation coefficient and Spearman's rank correlation coefficient) were performed. Seasonal variations of bacterial abundance were searched by analysis of variance (ANOVA) with significance level 0.05.

RESULTS AND DISCUSSION

Abundance of *Accumulibacter* in WWTPs and in laboratory-scale SBR

Accumulibacter abundance in the laboratory-scale SBR, determined by qFISH with the PAOmix probe, reached 69 ± 3% of all bacteria after 51 days and remained stable until the end of the study (Figure S1, available with the online version of this paper). By using two clade-specific probes, it was revealed that the *Accumulibacter* community contained clades IA and IIA,C,D only (89% and 11% of the total content, respectively – Figure 2; Figures S2 and S3, available online). The *Accumulibacter* population in full-scale WWTPs constituted up to 12% of all bacteria depending on the plant (Figure 1(a)). The range of the abundance (1–12%) was comparable to those reported by Wong *et al.* (2005), López-Vázquez *et al.* (2008), Gu *et al.* (2008), and Mielczarek *et al.* (2013) for Japanese (4–18%), Dutch (6–16%), American (5–15%) and Danish (2–8%) plants, respectively, but slightly lower than the abundance observed by He *et al.* (2008) in full-scale municipal WWTPs (9–24%). Kong *et al.* (2005) showed that *Accumulibacter* were dominant PAOs mainly in domestic plants (9–17%), but hardly present (3%) in most industrial WWTPs receiving food processing wastewater. In WWTPs investigated in this study, the abundance of clades IA and IIA,C,D was on a similar level (0–6% and 0–4% of all bacteria, respectively) and the average biovolumes (2.2% and 1.3% for IA and IIA,C,D, respectively) were slightly larger than the abundance observed by Mielczarek *et al.* (2013) in 28 Danish WWTPs (1.3% and 0.9%, respectively). However, in contrast to the

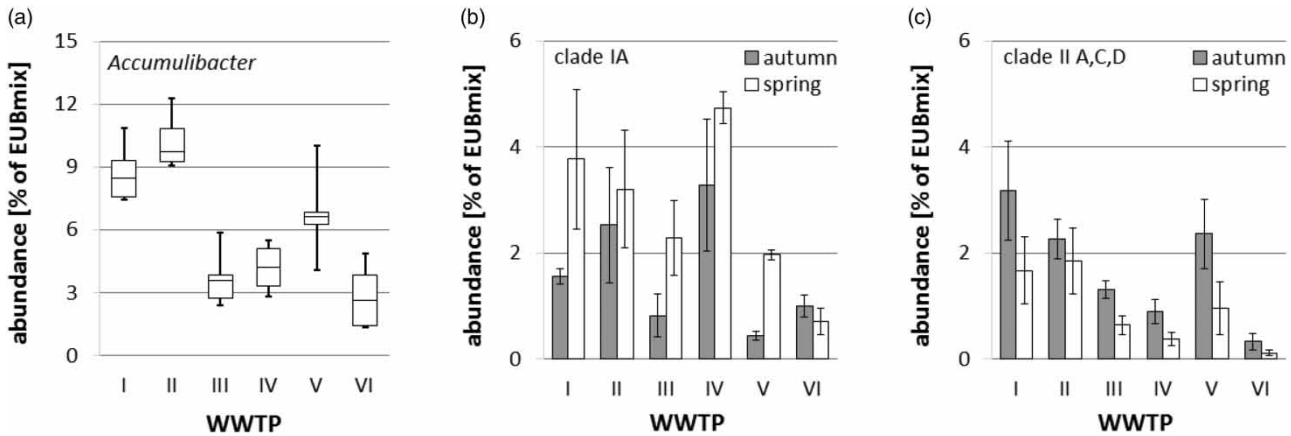

Figure 1 | Abundance of total *Accumulibacter* lineage (a) and clade-level seasonal variations (b and c) in full-scale WWTPs (I-VI) over a three-year study determined by qFISH. The bottom and top of each box (a) are the first and third quartiles, the band inside the box is the median, the whiskers represent the minimum and maximum values of each data set. The error bars (b and c) illustrate calculated standard errors of average values.

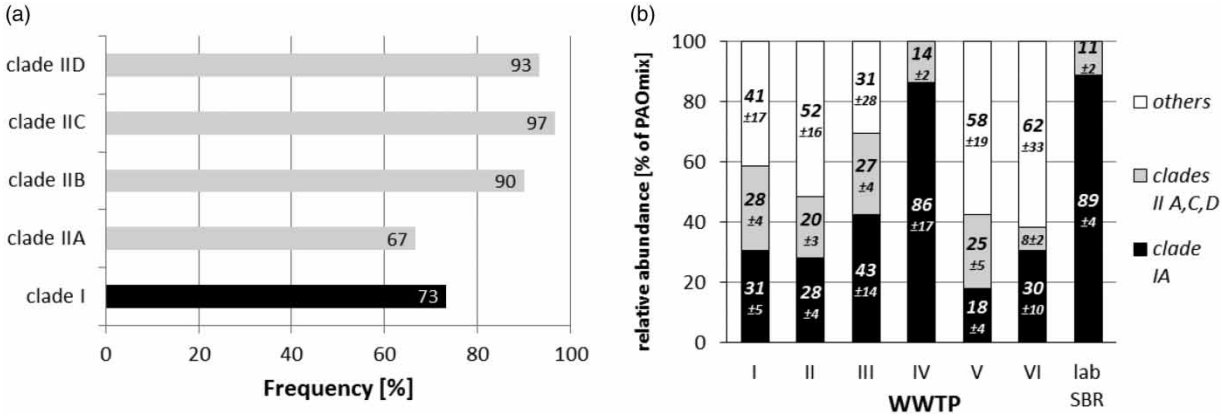

Figure 2 | Frequency of occurrence of *Accumulibacter* clades (percentage of samples in which the clade was detected) based on *ppk1* gene (a) and relative abundance (average values ± standard errors) of clade IA and clades IIA, C, D within *Accumulibacter* lineage based on qFISH (b) in full-scale WWTPs (I-VI) and laboratory-scale SBR over a three-year period and 100 days, respectively.

laboratory-scale reactor, the fraction of *Accumulibacter*, which was not targeted by two clade-specific probes, constituted 31–62% of the whole lineage in all plants except WWTP IV (Figure 2). It was a remarkably higher level than the respective 20–30% observed in Danish WWTPs (Mielczarek *et al.* 2013). The presence of cells not targeted by two clade-specific probes is not surprising, as the probes are recommended for use mainly in well-characterized laboratory-scale bioreactors (Flowers *et al.* 2009). The probe Acc-I-444 (used to detect Type I *Accumulibacter*) targets clade IA and some but not all other Type I clades, and the probe Acc-II-444 (used to detect Type II *Accumulibacter*) actually targets clade IIA, some IIC and some IID. Consequently, in full-scale systems, which are usually characterized by higher biodiversity than laboratory-scale reactors, there are some cells not targeted by any of the clade-specific probes. Thus the smaller the fraction targeted by the PAOmix probe, but uncovered with the two clade-specific probes, the potentially smaller the diversity of *Accumulibacter* lineage is. Interestingly, the *Accumulibacter* population in WWTP IV consisted mainly of clades targeted by Acc-I-444 and Acc-II-444 probes (relative abundance 86% and 14%, respectively) and resembled the low diversity of the laboratory-scale community (Figure 2), despite the SBR being seeded with sludge from WWTP V. Therefore, the abundance of the remaining fraction of *Accumulibacter* (not targeted by the clade specific probes) could be negligible. There was 52% contribution of industrial wastewater in the influent to WWTP IV, which could have a potential effect on the relative abundance of *Accumulibacter* clades. On the other hand, a fraction of the *Accumulibacter* lineage, untargeted by clade-specific FISH probes, was relatively high (52%) in WWTP II, which was the other plant with a significant contribution of industrial wastewater in the influent. These results may suggest that specific substrates contained in the influent rather than simply the percentage contribution of industrial wastewater may be the selective factors for *Accumulibacter* clades selection in activated sludge. Confirmation of this observation, however, requires additional detailed research.

Mao *et al.* (2015) implied that in addition to the composition of wastewater, operation parameters may also induce proliferation of different *Accumulibacter* clades. WWTP IV had the UCT configuration, which was different from the other systems investigated in this study. In all the examined plants (except WWTP IV), the return sludge (which contains nitrate) was recycled from the final clarifier to the first chamber, which was usually the anaerobic tank. As a consequence, the first part of this tank is operated in anoxic conditions instead of in anaerobic conditions, and easily biodegradable low molecular organic substrates are utilized by denitrifiers first, before they are available to *Accumulibacter*. To overcome this detrimental effect of nitrate on EBPR, in WWTP IV (with UCT configuration) the return sludge is pumped first into the anoxic rather than the anaerobic tank and mixed liquor is recycled from the anoxic tank to the anaerobic tank. Its principal advantage over the other systems examined in our study is that there is an opportunity for nitrate in the return sludge to be denitrified in the anoxic tank before entering the anaerobic tank, so *Accumulibacter* is favoured in competition with denitrifiers for easily biodegradable substrates in influent wastewater. Theoretically, conditions for PAOs in UCT systems are more favourable than in other plants examined during this study, because VFA are fully available for PAOs and they can be converted into PHAs. However, the effect of this configuration on

selection of *Accumulibacter* clades needs to be verified in a study with more WWTPs tested.

Slater *et al.* (2010) suggested that clade IA has low substrate affinity and therefore it is commonly the dominant clade of *Accumulibacter* in most laboratory-scale reactors, which are operated in fed-batch mode (similarly to the SBR in this study), selecting for bacteria with high substrate uptake rate. Full-scale systems are usually operated in continuous feed of wastewater, therefore they select for bacteria with high substrate affinity, like clade IIC. In this study, members of clade IA were predominant in the laboratory-scale SBR and WWTP IV. However, WWTP IV did not differ in terms of sludge loading with BOD_5 or COD from other WWTPs and no correlations were found between the abundance of clade IA and sludge loading with BOD_5 or COD (as discussed later). Bacteria require specific organic compounds to grow, probably therefore such 'lumped' parameters, like BOD_5 or COD, were not helpful in examining decisive factors that determined the *Accumulibacter* population structure within this study. However, due to its limited scope, this observation requires additional detailed research.

Seasonal variations and correlations between *Accumulibacter* and operational parameters

Only few statistically significant ($p < 0.05$), strong and medium ($|r| > 0.4$) correlations were found between influent or operational parameters and *Accumulibacter* in full-scale WWTPs (Table 2). *Accumulibacter* abundance increased with sludge loading with N (similarly to the findings of López-Vázquez *et al.* (2008)) and P, but it was negatively correlated with BOD, COD, BOD/N, COD/N, BOD/P and COD/P ratios in influent, in contrast to the results of Mielczarek *et al.* (2013). There was a strong positive correlation between abundance of clades IIA,C,D and the whole lineage of *Accumulibacter*. Similarly to the total *Accumulibacter* content, clades IIA,C,D correlated with sludge loading with N and P, as also seen by Mao *et al.* (2015) for clade IID, which was dominant in one in 18 WWTPs from 6 countries. Negative medium correlations of clades IIA,C,D were found for BOD, BOD/P and COD/P ratios. Type I (clades IA and others) did not correlate with any examined operational parameters, but interestingly it was more abundant in spring (after a long period with low temperatures),

Table 2 | Correlations between abundance of *Accumulibacter* lineage and clades and influent and operational parameters (only those with statistically significant values) in full-scale WWTPs, tested by Pearson's product-moment correlation coefficient and Spearman's rank correlation coefficient. The strength of statistically significant ($p < 0.05$) positive and negative correlations is illustrated by saturation of green and red colours, respectively. The full colour version of this figure is available in the online version of this paper, at http://dx.doi.org/10.2166/wst.2018.267

Parameter		Pearson coefficient			Spearman coefficient		
		Accumulibacter lineage	*Accumulibacter* clade I	*Accumulibacter* clade IIA, C, D	*Accumulibacter* lineage	*Accumulibacter* clade I	*Accumulibacter* clade IIA, C, D
Sludge loading with N [g N/gMLSS/d]	r	0.665	-0.053	0.566	0.671	-0.113	0.617
	p	*0.000*	*0.760*	*0.000*	*0.000*	*0.211*	*0.000*
Sludge loading with P [g P/gMLSS/d]	r	0.323	-0.237	0.447	0.396	-0.227	0.477
	p	*0.054*	*0.164*	*0.006*	*0.017*	*0.184*	*0.003*
BOD_5 [mg/L]	p	-0.494	0.262	-0.461	-0.580	-0.038	-0.543
	p	*0.002*	*0.123*	*0.005*	*0.000*	*0.824*	*0.001*
COD [mg/L]	r	-0.430	-0.126	-0.383	-0.492	-0.229	-0.389
	p	*0.009*	*0.464*	*0.022*	*0.002*	*0.178*	*0.019*
BOD_5/N [g BOD_5/g N]	r	-0.441	0.207	-0.369	-0.519	-0.020	-0.397
	p	*0.007*	*0.226*	*0.027*	*0.001*	*0.909*	*0.016*
COD/N [g COD/g N]	r	-0.408	-0.142	-0.295	-0.433	-0.214	-0.267
	p	*0.014*	*0.407*	*0.081*	*0.008*	*0.209*	*0.116*
BOD_5/P [g BOD_5/g P]	r	-0.344	0.416	-0.382	-0.372	0.145	-0.434
	p	*0.040*	*0.012*	*0.021*	*0.026*	*0.398*	*0.008*
COD/P [g COD/g N]	r	-0.409	0.085	-0.414	-0.391	0.005	-0.418
	p	*0.013*	*0.623*	*0.012*	*0.018*	*0.976*	*0.011*
Accumulibacter lineage	r		0.251	0.609		0.340	0.614
	p		0.140	0.000		*0.042*	*0.000*

r coefficient value	correlation strength
-1,0 ÷ -0,9	very strong
-0,9 ÷ -0,8	
-0,8 ÷ -0,7	strong
-0,7 ÷ -0,6	
-0,6 ÷ -0,5	medium
-0,5 ÷ -0,4	
-0,4 ÷ -0,3	weak
-0,3 ÷ -0,2	
-0,2 ÷ -0,1	none
-1,0 ÷ 0	
0 ÷ 0,1	
0,1 ÷ 0,2	
0,2 ÷ 0,3	weak
0,3 ÷ 0,4	
0,4 ÷ 0,5	medium
0,5 ÷ 0,6	
0,6 ÷ 0,7	strong
0,7 ÷ 0,8	
0,8 ÷ 0,9	very strong
0,9 ÷ 1,0	

whereas clades IIA, C, D presented opposite seasonal variations, confirmed by ANOVA (Figure 1(b) and 1(c)). This resulted in a lack of clear seasonal trend for the whole *Accumulibacter* lineage (not shown). Seasonal patterns similar to those observed in the present study were also reported by Flowers *et al.* (2013), who investigated bacterial community dynamics over a two-year period in two different treatment trains of a full-scale WWTP. Strong positive correlation between abundance of clade IIA and temperature was revealed, whereas clade IA was more abundant during winter. Surprisingly, no statistically significant correlations were found between both clades in the present study, implying lack of functional divergence between them. Furthermore, abundance of individual clades in each WWTP varied within wide ranges, resulting in high values of coefficient of variation (cv = 40 ÷ 96%), whereas the total *Accumulibacter* community was relatively stable (cv = 13 ÷ 55%).

The obtained results show that despite rather stable abundance of the whole *Accumulibacter* lineage (determined by the PAOmix probe) throughout a year, the abundance of individual clades presented seasonal changes in all the examined WWTPs. This may reflect the adaptation of *Accumulibacter* clades to low or higher temperatures regardless of the reactor configuration or influent or operational parameters. Type I (clades IA and others) and Type II (clades IIA,C,D) occupy different temperature niches and therefore studies on *Accumulibacter* metabolism, which are carried out in different specific temperatures, may not cover the entire *Accumulibacter* community but only specific temperature-dependent clades. Considering significant differences in the metabolism of individual clades (e.g. denitrification capabilities), this can have a crucial impact on the conclusions. Depending on the temperature, the EBPR performance of reactors with similar abundance of *Accumulibacter* lineage may be different due to the selection of different clades. Successful EBPR is usually observed at low temperatures, whereas in warm climates (>20 °C), deterioration of the EBPR process is frequently reported, mainly because PAOs at higher temperatures are less competitive than GAOs, which become dominant (Oehmen *et al.* 2007). However, Ong *et al.* (2014) showed that at 32 °C particular *Accumulibacter* clades can coexist with GAOs without compromising EBPR activity. The smaller *Accumulibacter* population and the larger population of GAOs did not deteriorate the EBPR performance. That proved that, at high temperatures, the EBPR process does not solely depend on the size of the *Accumulibacter* population but rather on the presence and ecophysiology of particular clades.

Distribution of different clades of *Accumulibacter*

The *ppk1* gene encodes the polyphosphate kinase, which is responsible for polyphosphate synthesis by *Accumulibacter*. The *ppk1* gene evolves faster than 16S rRNA genes and provides better resolution to observe microdiversity within *Accumulibacter* lineage (He *et al.* 2007; Mao *et al.* 2015). Therefore *ppk1*-PCR was used in this study to better resolve the *Accumulibacter* communities in full-scale WWTPs and in the laboratory-scale SBR. *Accumulibacter* structure in the laboratory-scale SBR varied significantly over time despite stable operational conditions being maintained through the whole study. Four detected clades (I, IIB, C and D) were present in the original seed (Table S1, Supplementary Material, available online). However, after 50 days of the SBR operation there was a marked change in the *Accumulibacter* population – clade IIA was selected instead of clade IIB. Selection of 2 clades only (I and IID) was observed after 101 days in the SBR. These changes were associated with different EBPR performance of the reactor, which substantially improved after 80 days, despite the abundance of the whole *Accumulibacter* lineage (determined by qFISH) being stable starting from the 51st day of the study. This shows that the mere relative abundance of *Accumulibacter* is not enough for efficient P removal, but *Accumulibacter* clades determine the effectiveness of EBPR. Slater *et al.* (2010) showed that in four laboratory-scale SBRs, clades IA and IIC were associated with good and poor EBPR performance, respectively.

It is hypothesized that varying reactor operational conditions may select for different *Accumulibacter* clades due to differences in their metabolism (Flowers *et al.* 2009; Slater *et al.* 2010; Acevedo *et al.* 2012). However, transient shifts between different dominant clades in laboratory-scale SBRs were also observed by Gonzales-Gil & Holliger (2011) and Camejo *et al.* (2016), although no specific changes in operational conditions were identified. Another probable explanation could be that laboratory-scale systems are particularly susceptible to perturbations due to unique conditions and small volumes (Slater *et al.* 2010). Also a phage pressure cannot be ruled out as a factor triggering population changes in the laboratory-scale SBR, because lytic-bacteriophage events are proposed as selective forces for *Accumulibacter* clades selection (Skennerton *et al.* 2015; Camejo *et al.* 2016).

Different clades seem to be associated with different habitats, and only few clades are usually identified as dominant clades in laboratory-scale SBRs. The *Accumulibacter* lineage was far more diverse in the full-scale WWTPs, but similarly to the survey of Mielczarek *et al.* (2013) the distribution of clades among WWTPs showed no obvious pattern

nor seasonal variations (Table S1, Supplementary Material). On average, 4 clades were present in each plant; among them, clades IIC, D and B were detected most frequently (in more than 90% of the samples). However, *Accumulibacter* of clade IA (and others of Type I) were usually the most abundant as shown by qFISH, despite being detected by *ppk1*-PCR in 'only' 73% of the samples (Figure 2). A higher microdiversity of *Accumulibacter* clades in full-scale plants was also reported by He *et al.* (2007), who showed that *ppk1* genes from laboratory-scale reactors were affiliated only with clades I and IIA, while *Accumulibacter* in full-scale WWTPs were represented by at least three clades. This reflects higher complexity and fluctuations in operational parameters and wastewater composition, which create more niches available to *Accumulibacter* clades with different ecophysiology, when compared to the unique conditions in laboratory-scale systems.

The FISH analysis is based on relatively broad probes, which do not reveal the microdiversity of the *Accumulibacter* lineage. However, the *ppk1* gene analysis, which is a higher resolution biomarker, still does not provide enough accuracy either, unless primers for the remaining clades are applied. Mao *et al.* (2015) revealed that abundance of total *Accumulibacter* lineage, determined by qPCR with the same primers as used in the present study, accounted for less than half of that population as determined by 16S rRNA genes. Novel primers for newly discovered clades are being designed; however, the unclassified *Accumulibacter* abundance in some samples still remains as high as 64.4% (Zhang *et al.* 2016). Existing primers are being improved to avoid cross-hybridization (Camejo *et al.* 2016), but primer design works proceed slowly. Furthermore, bias related to DNA extraction and different copy numbers of genes make results of this investigation difficult to compare with qFISH. Albertsen *et al.* (2012) demonstrated significant discrepancies between qFISH counts and metagenomic read numbers, therefore the reasonable strategy seems to be not relying on a single approach but using more than one method in parallel whenever possible.

CONCLUSIONS

Accumulibacter clades distribution in full-scale WWTPs is governed by season, but the frequency of their occurrence do not implicate the abundance. Higher microdiversity than in laboratory-scale reactors reflects complexity and temporal fluctuations of wastewater. Sludge loading with BOD, COD, N and P correlate with total *Accumulibacter* abundance in full-scale WWTPs, whereas specific substrates present in industrial wastewater may be key factors in the clades selection. However, further work is required to confirm this observation, given the changes in *Accumulibacter* population structure in the laboratory-scale reactor while maintaining stable operational conditions and a constant wastewater composition throughout the entire experiment. A probable explanation could be the unique conditions and relatively small volume of the laboratory-scale reactor, which define specific ecological niches and select for particular clades. The considerable fraction of *Accumulibacter* in full-scale plants, which is not targeted by any clade-specific probe yet, depicts the need for further research, especially when the probes used to cover *Accumulibacter* lineage also target a closely related GAO – *Propionivibrio aalborgensis* (Albertsen *et al.* 2016).

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