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"*Candidatus* Accumulibacter delftensis": A clade IC novel polyphosphate-accumulating organism without denitrifying activity on nitrate

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ABSTRACT

Populations of "*Candidatus* Accumulibacter", a known polyphosphate-accumulating organism, within clade IC have been proposed to perform anoxic P-uptake activity in enhanced biological phosphorus removal (EBPR) systems using nitrate as electron acceptor. However, no consensus has been reached on the ability of "*Ca.* Accumulibacter" members of clade IC to reduce nitrate to nitrite. Discrepancies might relate to the diverse operational conditions which could trigger the expression of the Nap and/or Nar enzyme and/or to the accuracy in clade classification. This study aimed to assess whether and how certain operational conditions could lead to the enrichment and enhance the denitrification capacity of "*Ca.* Accumulibacter" within clade IC. To study the potential induction of the denitrifying enzyme, an EBPR culture was enriched under anaerobic–anoxic–oxic (A2O) conditions that, based on fluorescence *in situ* hybridization and *ppk* gene sequencing, was composed of around 97% (on a biovolume basis) of affiliates of "*Ca.* Accumulibacter" clade IC. The influence of the medium composition, sludge retention time (SRT), polyphosphate content of the biomass (poly-P), nitrate dosing approach, and minimal aerobic SRT on potential nitrate reduction were studied. Despite the different studied conditions applied, only a negligible anoxic P-uptake rate was observed, equivalent to maximum 13% of the aerobic P-uptake rate. An increase in the anoxic SRT at the expenses of the aerobic SRT resulted in deterioration of P-removal with limited aerobic P-uptake and insufficient acetate uptake in the anaerobic phase. A near-complete genome (completeness = 100%, contamination = 0.187%) was extracted from the metagenome of the EBPR biomass for the here-proposed "*Ca.* Accumulibacter delftensis" clade IC. According to full-genome-based phylogenetic analysis, this lineage was distant from the canonical "*Ca.* Accumulibacter phosphatis", with closest neighbor "*Ca.* Accumulibacter sp. UW-LDO-IC" within clade IC. This was cross-validated with taxonomic classification of the *ppk1* gene sequences. The genome-centric metagenomic analysis highlighted the presence of genes for assimilatory nitrate reductase (*nas*) and periplasmic nitrate reductase (*nap*) but no gene for respiratory nitrate reductases (*nar*). This suggests that "*Ca.* Accumulibacter delftensis" clade IC was not capable to generate the required energy (ATP) from nitrate under strict anaerobic-anoxic conditions to support an anoxic EBPR metabolism. Definitely, this study stresses the incongruence in denitrification abilities of "*Ca.* Accumulibacter" clades and reflects the true intra-clade diversity, which requires a thorough investigation within this lineage.

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1. Introduction

Enhanced biological phosphorus removal (EBPR) is a process applied worldwide to remove phosphorus in wastewater treatment plants (WWTP) (Henze et al., 2008). EBPR is carried out by microorganisms that are capable of storing phosphorus beyond their growth requirements as polyphosphate (poly-P), known as polyphosphate-accumulating organisms (PAOs) (Comeau et al., 1986). The PAO guild comprises multiple genera (Stokholm-Bjerregaard et al., 2017), with "*Candidatus* Accumulibacter" as well-described primary population. The relative abundance of "*Ca.* Accumulibacter" (hereafter referred to as PAO) has been correlated with good EBPR in WWTPs with different configurations worldwide (Kong et al., 2002; Zilles et al., 2002; Saunders et al., 2003; He et al., 2005; Wong et al., 2005). "*Ca.* Accumulibacter" performs different metabolic processes depending on the availability of terminal electron acceptors. Under anaerobic conditions (*i.e.*, no electron acceptor available according to environmental engineering lexicon), this PAO stores carbon (*e.g.*, acetate, propionate) as poly-β-hydroxyalkanoates (PHAs) at the expense of polyphosphate (poly-P) hydrolysis and glycogen degradation. Thereafter, when an electron acceptor is available such as dissolved oxygen under aerobic conditions, and nitrite and presumably nitrate under anoxic conditions, the organism consumes the stored PHA to replenish its poly-P and glycogen storage pools, for biomass synthesis and cellular maintenance (Comeau et al., 1986; Wentzel et al., 1986; Smolders et al., 1994a, 1994b; Kuba et al., 1996b).

Previous studies have suggested that members of the PAO guild have different affinities and potentials to use nitrate or nitrite as electron acceptors for anoxic P-uptake (Kerrn-Jespersen and Henze, 1993; Kuba et al., 1993, 1996a, 1997; Ahn et al., 2001a, 2001b). Kerrn-Jespersen and Henze (1993) have postulated the existence of two types of PAOs: one denitrifying-PAO type able to use nitrate and oxygen as electron acceptors (herein identified as or DPAO) and another PAO type capable of using only oxygen. Through the long-term operation of two sequencing batch reactors (SBRs) operated under anaerobic-anoxic (A_2) and anaerobic-oxic conditions (A/O), Kuba et al. (1993) have observed that DPAO could exhibit an anoxic EBPR activity similar to that of PAO on oxygen.

Carvalho et al. (2007) observed that rod-shaped PAOs exhibited satisfactory anoxic P removal on nitrate while coccus-shaped PAO showed poor anoxic EBPR activity. Later on, based on fine-scale differences in the genetic sequences of the *ppk1* gene, Peterson et al. (2008) identified the existence of two "*Ca.* Accumulibacter" clades I and II, with several subclades (IA-ID and IIA-IIG). Oligonucleotide probes and primers set were designed on this molecular basis for rapid detection by fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR). Based on these findings and correlations with process performances, Oehmen et al. (2010a,b) re-assessed the different PAO phenotypes observed by Carvalho et al. (2007), and suggested that clade I was responsible for the anoxic P-uptake activity observed in EBPR systems. In agreement, Flowers et al. (2009) observed that a culture enriched in clade I (*ca.* 70 ± 11% based on FISH bio-volume) was able to denitrify without requiring any acclimatization step, while a culture dominated by clade II (approx. 55 ± 7% clade II and 32 ± 0.5% clade I per bio-volume) could not. Nevertheless, after a 24-h acclimatization period to the presence of nitrate, both EBPR systems performed a simultaneous denitrification and P-uptake activity. The anoxic P-uptake activity observed in the latter clade II culture was associated to the presence of the clade I fraction in the system (Flowers et al., 2009).

Lanham et al. (2011) enriched a clade I culture (approx. 90% bio-volume) under anaerobic-anoxic-oxic (A_2O) conditions. Their enrichment was capable to take-up about 12 mg PO_4–P g^{-1}VSS h^{-1} using nitrate as electron acceptor. Contrary to the strict A_2 operating conditions of Kuba et al. (1993), the authors reported that maintaining an oxic stage seemed to have been a key condition to secure the reactor stability and obtain a highly enriched clade I culture. On the other hand, through the execution of short-term studies, Saad et al. (2016) and Rubio-Rincón et al. (2017a) reported that clade I (further identified via *ppk* gene sequence analysis as populations of "*Ca.* Accumulibacter" clade IC) was unable to use nitrate as electron acceptor as efficient as oxygen, or nitrite. They suggested that when anoxic P-uptake takes place, the underlying members of clade IC may use the nitrite generated from the reduction of nitrate by side populations within the microbial community, possibly glycogen-accumulating organisms (GAOs) or ordinary heterotrophic organisms (OHOs). Nevertheless, both studies were based on the conduction of short-term (hours) batch activity tests with a culture of clade IC affiliates enriched under A/O conditions. In contrast, recent studies performed by Camejo et al. (2016) have suggested that some populations of clade IC can efficiently use nitrate as electron acceptor for the oxidation of PHA and P-uptake.

From a molecular and microbial perspective, early genome-centric metagenome analyses of populations of "*Ca.* Accumulibacter" clades IA and IC have not been able to detect the respiratory nitrate reductase gene (*nar*) required for nitrate respiration (Flowers et al., 2013; Skennerton et al., 2014), similar to clade IIA and IIF. Recently, Camejo et al. (2018) have identified one "*Ca.* Accumulibacter" population of clade IC harbouring a *nar* gene. The denitrification pathway of the aforementioned clades, only includes the presence of the periplasmic nitrate reductase gene (*nap*) and the required genes to denitrify from nitrite onwards (García Martín et al., 2006; Flowers et al., 2013; Skennerton et al., 2014). According to Moreno-Vivián et al. (1999) the main difference among these genes is the potential of their expressed enzyme (Nap, Nar) to generate energy as ATP. While both enzymes can reduce nitrate to nitrite only the Nar enzyme has been correlated with enough generation of energy as ATP to sustain bacterial growth and other microbial processes (Moreno-Vivián et al., 1999). As such and in view of the contradictory findings previously described, the gene expression mechanisms could be responsible for the different anoxic P-uptake activities of clade IC phylotypes in EBPR systems reported in literature, as first suggested by Skennerton et al. (2014).

It remains unclear which fraction of "*Ca.* Accumulibacter" clade IC species harbor the *nap* or *nar* genes, and, if these genes are present in their genome, which operational factors could induce their enzymatic expression. We aimed to assess the influence of different operating conditions on a clade IC enriched culture (Rubio-Rincón et al., 2017a). The main factors studied were: (*i*) a high P/COD ratio (0.06 g P g^{-1} COD); (*ii*) a long sludge retention time (SRT) of 15 d; (*iii*) a low P/COD ratio of 0.03 g P g^{-1} COD; (*iv*) a short aerobic SRT of 1.5 d; and, (*v*) pulse and continuous nitrate dosing modes. Combining mixed-culture stoichiometry and kinetics investigation with molecular biology and genome-centric metagenomics analysis of the here proposed "*Ca.* Accumulibacter delftensis" clade IC, this study contributes to assess conditions that may stimulate and enhance the use of nitrate and elucidate their potential role in EBPR systems. It further stresses the need to reappraise metabolic functionalities inside and across clades of the "*Ca.* Accumulibacter" lineage – and microbial lineages in general – beyond correlations observed between relative abundances of clades and biochemical conversions monitored.

2. Materials and methods

2.1. Reactor operation

A culture of a "*Ca.* Accumulibacter" clade IC population was enriched in a 3-L, double-jacket, stirred-tank reactor (Applikon, Delft, The Netherlands) with a working volume of 2.5 L. A volume of 500 mL of activated sludge from the EBPR WWTP Nieuwe Waterweg (Hoek van Holland, The Netherlands) was used as inoculum. Prior to the start of the study, the reactor was operated under anaerobic-oxic (A/O) conditions for more than a year (experimental period A; Rubio-Rincón et al., 2017a). Thereafter, the operational conditions were changed to anaerobic-anoxic-oxic (A_2O) (experimental period B; Table 1). Once the system was operated under A_2O in a pseudo steady state (experimental period B), the medium composition was changed from the one used by Smolders et al. (1994a,b) to the one used by Kuba et al. (1993) (experimental period C) with low and high potassium concentration, respectively. In order to give the opportunity to the (assumed slow-growing) DPAOs to proliferate, the SRT was extended from 8 to 15 d in the experimental period D. Due to the potential role of GAOs in the denitrification activities observed in EBPR systems (Rubio-Rincón et al., 2017a), the feeding P/COD ratio was decreased from 0.06 to 0.03 g P g^{-1} COD (experimental period E and F, respectively). In the experimental period G, to address if DPAOs could be *r*- or *k*-strategists, the nitrate dosing mode was changed from a pulse feeding carried out in 1 min to a longer feeding period of 30 min at a 1 mL/min flowrate (as studied by Kuba et al., 1993; Tu and Schuler, 2013), keeping the same nitrate concentration dosed (±10 mg NO_3–N L^{-1}). Finally, to wash the aerobic PAOs out and select for DPAOs, the length of the aerobic phase was gradually decreased, and consequently the aerobic SRT was shortened from 2.2 days to 0.9 days (in experimental period G) and further to 0.4 days (in experimental period H), which is lower than the minimum aerobic SRT estimated by Brdjanovic et al. (1998) (of about 1.25 d) for A/O-enriched PAO.

The hydraulic retention time (HRT) was 12 h. The pH was controlled at 7.6 ± 0.1 with the addition of 0.4 mol L^{-1} NaOH and 0.1 mol L^{-1} HCl. Temperature was controlled at 20 ± 1 °C. In order to create and maintain the anaerobic conditions, dinitrogen gas was sparged at the bottom of the reactor during the first 30 min of the anaerobic phase and a water lock was installed at the off-gas outlet. Nitrate was fed either as pulse or continuously for 30 min (in accordance to the corresponding experimental period) from a bottle containing a 1 g NO_3–N L^{-1} stock solution. The dissolved oxygen (DO) concentration was controlled at 20% of the saturation level by sparging on/off compressed air and dinitrogen gas. Both gases were controlled at 10 L h^{-1}. DO and pH levels were monitored continuously. Ortho-phosphate (PO_4–P), mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentrations were measured twice per week. When no significant changes in these parameters were observed for more than 3 SRTs, it was assumed that the system had reached pseudo steady-state conditions.

2.2. Nitrate-based DPAO batch activity tests

In order to assess if the biomass-specific organic load (food-to-microorganisms, F/M ratio), affecting the anaerobic PHA storage and therefore the intracellular carbon availability and its potential impact on the anoxic phosphorus uptake, two batch tests were carried out *ex situ* with half (batch 1D) and twice (batch 2D) the F/M ratio (37 and 148 mg COD g^{-1} VSS, respectively) applied in the regular operation of the parent reactor. The medium composition was the same like in the operation of the parent reactor. Each batch test was performed with 200 mL of MLSS (collected and transferred during experimental period D, Table 1) in a double jacketed reactor operated at 20 °C with a 400 mL working volume. The cycle of the batch tests was composed of 1 h of anaerobic and 4 h of anoxic phases. In each batch test, nitrate was fed as a pulse reaching a concentration of around 45 mg NO_3–N L^{-1}. Dinitrogen gas was continuously sparged at the bottom of the reactor at 10 L h^{-1} in order to maintain the anaerobic conditions. pH was kept at 7.6 ± 0.1 with the automatic addition of 0.4 mol L^{-1} HCl and 0.4 mol L^{-1} NaOH.

2.3. Cultivation medium

The cultivation medium were prepared in two separate bottles of 10 L (carbon and mineral solutions), and concentrated 10 times. The influent medium fed contained per liter: 400 mg COD (composed by acetate and propionate supplied in a 3:1 COD ratio), 4 mg Ca^{+}, 36 mg SO_4^{2-}, 9 mg Mg^{2+}, 1 mg yeast extract, 20 mg N-allylthiourea (ATU) and 300 μL of trace element solution prepared according to Smolders et al. (1994a,b). In addition, the influent medium for (i) the experimental periods A and B contained per liter: 36 mg NH_4–N, 25 mg PO_4–P, 19 mg K^{+}, and 18 mg Na^{+}; (ii) for periods C, D and E the medium composition was change to increase the concentration of potassium as reported by Kuba et al. (1993) to: 83 mg NH_4–N, 25 mg PO_4–P, 50 mg K^{+} and 0 mg Na^{+}; and, (iii) for F, G and H: 83 mg NH_4–N, 15 mg PO_4–P, 38 mg K^{+}, and 0 mg Na^{+} per liter.

2.4. Chemical analyses

Ortho-phosphate (PO_4–P), nitrite (NO_2–N), MLSS, and MLVSS were analytically determined as described in APHA (2005). Nitrate (NO_3–N) was measured according to ISO 7890/1 (1986). Acetate and propionate were measured using a Varian 430-GC Gas

Table 1
Main different operational conditions applied during the experimental periods.

Exp. Period	Days on operation	Operation A_2O (anaerobic-anoxic-oxic)	mg P/mg COD (influent)	Dosed nitrate concentration mg NO_3–N/L (feeding mode)	Sludge retention time		
					Total SRT (d)	Anoxic SRT (d)	Aerobic SRT (d)
A	N.A.	2h - 0h - 2.5h	0.06[a]	N.A.	8	N.A.	3.0
B	0–40	0.6h - 2h - 1.8h	0.06[a]	20 mg/L (pulse)	8	2.6	2.2
C	40–102	0.6h - 2h - 1.8h	0.06[b]	14 mg/L (pulse)	8	2.6	2.2
D	102–167	0.6h - 2h - 1.8h	0.06[b]	13 mg/L (pulse)	15	5.0	4.1
E	167–212	0.6h - 2h - 1.8h	0.06[b]	11 mg/L (pulse)	8	2.6	2.2
F	212–218	0.6h - 2h - 1.8h	0.03[b]	11 mg/L (pulse)	8	2.6	2.2
G	218–265	0.6h - 3h - 0.8h	0.03[b]	30 min at 1 mL/min (prolonged)	8	4.0	0.9
H	265–276	0.6h - 3.5h - 0.3h	0.03[b]	Between 30 and 80 min at 1 mL/min (prolonged)	8	4.4	0.4

[a] Phosphate was added as sodium phosphate.
[b] Phosphate was added as potassium phosphate.

Chromatograph (GC) equipped with a split injector (200 °C) and a WCOT Fused Silica column (105 °C) coupled to a FID detector (300 °C). Helium was used as carrier gas and 50 μL of butyric acid as internal standard.

2.5. Characterization of microbial community compositions

In order to estimate the relative abundance of the microbial communities along the different experimental periods, FISH analyses were performed as described by Amann (1995). Since *Propionivibrio* was not observed with 16S rRNA gene-based amplicon sequencing, and because the PAO 651 FISH probe suggested by Albertsen et al. (2016) has a coverage of 71% of the species from the genus "*Ca.* Accumulibacter", the mix probe of PAO 651, PAO 462, and PAO 846 suggested by Crocetti et al. (2000) was used to target the "*Ca.* Accumulibacter" genus (with a 89% coverage). To differentiate among the different PAO clades, the probes Acc-1-444 (PAO I) and Acc-2-444 (PAO II) were used (Flowers et al., 2009). Glycogen-accumulating organisms (GAOs) were identified with the GB probe according to Kong et al. (2002). *Defluvicoccus* clades 1 and 2 were identified with TFO-DF215, TFO-DF618, DF988, and DF1020 probes (Wong et al., 2004; Meyer et al., 2006). Vectashield with DAPI was used to avoid the fading of staining and stain all living organisms (Nielsen et al., 2009). FISH quantification of each probe was performed by image analysis of 25 random pictures taken with an Olympus BX5i microscope and analyzed with the software Cell Dimensions 1.5. The standard error of the mean was calculated as the standard deviation divided by the square root of the number of pictures.

2.6. Analyses of bacterial community compositions by V4–V6 16S rRNA gene-based amplicon sequencing

Genomic DNA (gDNA) was extracted using the Ultraclean Microbial DNA extraction kit supplied by MOBIO laboratories Inc. (CA, USA) according to the manufacturer's protocol except that the bead-beating was substituted by a combination of 5 min heating at 65 °C and 5 min beat-beating to ensure maximum yields. To check for quality and quantity, the gDNA extracts were loaded onto a 1% agarose gel in 1x TAE running buffer. Analysis of the extracted gDNA showed a large high molecular weight fraction and well visible DNA yields in comparison to the Smart ladder (Eurogentech Nederland b.v.).

The extracted gDNA was subsequently used for a two-step PCR reaction targeting the 16S rRNA gene of most bacteria and archaea, using the primers U515F (5′-GTGYCAGCMGCCGCGGTA-3′) and U1071R (5′-GARCTGRCGRCRRCCATGCA-3′) following Wang and Qian (2009). The first amplification step was performed to enrich for 16S rRNA genes, via quantitative PCR (qPCR). The qPCR reaction comprised 2x iQ™ SYBR® Green Supermix (Bio-rad, CA, USA), 500 nmol L^{-1} primers each, and 1–50 ng gDNA template added per well (final volume of 20 μL by adding MiliQ water). The qPCR program went along a first denaturation at 95 °C for 5 min followed by 20 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 40 s and elongation at 72 °C for 40 s, prior to final elongation at 72 °C for 7 min. During the second step, 454-adapters (Roche) and MID tags at the U515F primer, were added to the products of step one. This protocol was similar to the ones previously described, but only Taq PCR Master Mix (Qiagen Inc, CA, USA) was used. The program was run for 15 cycles, the template, product from step one was used as template DNA and diluted ten times. After the second amplification, 12 PCR products were pooled in equimolar ratio and purified over an agarose gel using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific, The Netherlands). The resulting library was send for 454 sequencing and run in 1/8 lane with titanium chemistry by Macrogen Inc. (Seoul, Korea).

After sequencing, the reads library was imported into the CLC genomics workbench v7.5.1 (CLC Bio, Aarhus, DK) and (quality, limit = 0.05 and max. two ambiguities allowed) trimmed to a minimum of 200 bp and average of 284 bp. After trimming, the datasets were de-multiplexed resulting in 12 samples with an average of 7800 reads per sample. A build-it SILVA 123.1 SSURef Nr99 taxonomic database was used for BLASTn analysis on the reads under default conditions. To identify chimeric sequences we used the online tool DECIPHER (Wright et al., 2012). A very small portion of reads had a non-significant match and were discarded. The chimera check was done, but eventually not implemented as they were in minor amounts present and of non-significant importance to the final results. Sequences were only included if the E-value was sufficient low ($<E^{-50}$). The top result was imported into an excel spreadsheet and used to determine taxonomic affiliation and species abundance.

2.7. Molecular analysis of "Ca. Accumulibacter" clades by PCR amplification and sequencing of the ppk1 functional gene

A direct PCR was performed to identify the "*Ca.* Accumulibacter" clade enriched in the biosystem based on the polyphosphate kinase (*ppk1*) functional gene as described by McMahon et al. (2007). The PCR amplicons were produced using ACCppk1-254F (5′-TCAC CACC GACG GCAA GAC-3′) and ACCppk1-1376R (5′-TCGA TCAT CAGC ATCT TGGC-3′) primers, and (Sanger) sequenced by BaseClear, Leiden, the Netherlands. Both strands were quality checked and found non-ambiguous. Subsequently both were aligned to yield a high quality, near complete, ppk1 gene. The phylogenetic tree was constructed using the neighbor joining method implemented in the CLC genomics workbench package, as described by Saad et al. (2016). In total 332 amino-acid positions were used for calculations.

2.8. Genome-centric metagenomic analysis of the "Ca. Accumulibacter" and flanking lineages present in the reactor

Two biological samples collected during the anaerobic-aerobic and anaerobic-anoxic-aerobic operations were selected for metagenome sequencing analysis. Wet-lab treatments of aliquots of the EBPR biomass were performed to suppress their background contribution of accompanying populations to the metagenomes and to eventually isolate the "*Ca.* Accumulibacter" genome bin by differential coverage. Synthetic shifts in relative abundances of microbial populations were achieved by contacting the biomass to different substrate, temperature, and dissolved oxygen conditions in shake flasks.

gDNA was extracted from these samples using the Fast DNA Spin Kit for Soils (MP Biomedicals, USA) by 4 series of 40 s of bead beating. The bacterial community compositions were characterized beforehand by V1–V3 16S rRNA gene-based amplicon sequencing analysis (primer pair 8F/518R) according to the MiDAS field guide of activated sludge (McIlroy et al., 2015).

The gDNA extracts were purified and prepared for sequencing of the metagenomes using the Nextera XT DNA Sample Preparation Kit (Illumina, USA) according to manufacturer's instructions. All purifications steps were performed using the Agencourt AMPure XP clean-up system (Beckman Coulter, USA). The dual-multiplexed pools of tagmented and indexed DNA fragments were sequenced on a MiSeq benchtop sequencer (Illumina, USA) at a sequencing depth of 1 Gbp per sample with paired-end reads of 300 × 300 bp. Quality controls were performed using DNA Analysis D1000 ScreenTape assays on a 2200 TapeStation instrument (AgilentTechnologies, USA). gDNA concentrations were accurately measured using Quant-iT dsDNA Assay Kits (ThermoFischer

Scientific, USA) on an Infinite M1000 PRO plate reader (Tecan, Switzerland).

The metagenomics sequencing raw datasets were processed using the CLC Genomics Workbench (Qiagen Bioinformatics, Denmark). The resulting *de novo* assembly and coverage files of the metagenomes of the EBPR sludge and background community treatment were loaded into mmgenome (Karst et al., 2016) in R for dual coverage binning of the near-complete genome of the "*Ca.* Accumulibacter" and flanking lineages present in the bioreactor following the method initially developed by Albertsen et al. (2013).

The single-lineage genome assemblies were uploaded into a KBase narrative (Arkin et al., 2016) for phylogenetic classification and functional gene annotation using the RAST toolkit (Brettin et al., 2015) after quality control using checkM (Parks et al., 2015), BLASTn, ARB Silva, RNAmmer, tRNAscan-SE, and QUAST. The obtained assembly of the near-complete genome of the "*Ca.* Accumulibacter" population selected in the system was taxonomically classified at high resolution against the 28 draft genome assemblies available for this lineage in on-line databases such as NIH GenBank (Benson et al., 2005), NCBI RefSeq (O'Leary et al., 2016), and JGI IMG (Chen et al., 2017), and imported manually in KBase. Reference genome assemblies that were deprived of gene and protein annotations of coding sequences were annotated in KBase prior to phylogenetic tree reconstruction.

The genome-centric phylogenetic tree was computed in KBase from reference alignments based on 49 highly conserved clusters of orthologous groups of proteins and using the FastTree2 approximate maximum likelihood method. The FastANI algorithm was used to discriminate between unique genomes (<95%, new population) or to highlight overlapping genomes (>99%) by fast computation of pairwise average nucleotide identity (FastANI) values. The genome-based phylogenetic tree was cross-validated with the *ppk1*-gene-based taxonomic classification across the "*Ca.* Accumulibacter" lineage: the *ppk1* gene sequence was retrieved from the genome assembly and matched against sequences of *ppk1* gene PCR amplicons obtained from the EBPR biomass. Based on the uniqueness of the near-complete genomes obtained, we here proposed the provisional novel species names "*Ca.* Accumulibacter delftensis sp. nov.", "*Ca.* Nocardioides delftensis sp. nov.", and "*Ca.* Thermomonas delftensis sp. nov.". Since no isolate and pure culture is available for these organisms, the provisional names were proposed according to recommendations of Murray and Stackebrandt and principles of the International Code of Nomenclature of Prokaryotes. A digital protologue table was developed for the description of the provisional taxa, following identical procedure adopted lately by Andersen et al.. The genome assemblies and annotations are deposited in KBase, and will be made publicly available in GenBank/European Nucleotide Archive (in progress). Output milestones of the genome-centric metagenomics analysis are available in Supporting Information.

2.9. Stoichiometric and kinetic parameters of interest

The ratio of phosphorus released to VFA uptake (P/VFA) under anaerobic conditions was calculated based on the observed net phosphorus released at the end of the anaerobic period per VFA consumed. The phosphorus content in the biomass was calculated based on a mass balance performed using data from the pseudo steady-state conditions, as described by Kuba et al. (1993). The anaerobic metabolic activity of the sludge was characterized using the following anaerobic biomass-specific rates of interest:

i) $q^{MAX}_{PO_4,AN}$ Maximum observed anaerobic phosphorus release rate, in mg PO_4–P g^{-1} VSS h^{-1}.

ii) $m_{PO_4,AN}$ Anaerobic endogenous phosphorus release rate observed once VFA were taken up, in mg PO_4–P g^{-1} VSS h^{-1}.

iii) $q_{PO_4,VFA}$ Anaerobic phosphorus release rate due to VFA uptake, calculated according to:

$$q_{PO_4,VFA} = q^{MAX}_{PO_4,AN} - m_{PO_4,AN} \quad (1)$$

iv) q^{MAX}_{VFA} Maximum observed anaerobic VFA uptake rate observed, in mg COD g^{-1} VSS h^{-1}.

Oxygen uptake rate (OUR) profiles were determined based on DO consumption over time. In order to measure the DO consumption, during the oxic stages the EBPR sludge was recirculated from the parent reactor through a separate 10 mL biological oxygen monitor (BOM) unit for 2–3 min. Once the DO measurements were stable, the sludge recirculation was stopped and the DO concentration profiles were recorded. The DO concentrations were kept above a set point of 2 mg O_2 L^{-1} by periodically re-starting the sludge recirculation. This procedure was repeated along the oxic phases. The BOM unit was equipped with a WTW OXi 340i DO probe (Germany). The anoxic and aerobic biomass-specific rates of interest were:

i) $q_{NO_3,Ax}$ Nitrate uptake rate, in mg NO_3–N g^{-1} VSS h^{-1}.

ii) $q_{PO_4,Ax}$ Anoxic phosphorus uptake rate, in mg PO_4–P g^{-1} VSS h^{-1}.

iii) $q_{PO_4,Ox}$ Aerobic phosphorus uptake rate, in mg PO_4–P g^{-1} VSS h^{-1}.

All rates were calculated by linear regression based on the observed profiles as described in Smolders et al. (1995).

3. Results

3.1. Operation of the reactor under anaerobic-oxic (A/O) conditions

The EBPR reactor was operated for more than a year under A/O conditions showing a pseudo steady-state performance (Fig. 1A). All VFAs were anaerobically consumed during the first 15 min of reaction at a maximum biomass specific rate of 269 mg COD g^{-1} VSS h^{-1} (q^{MAX}_{VFA}), with a phosphorus release of 199 mg PO_4–P g^{-1} VSS h^{-1} ($q^{MAX}_{PO_4,AN}$). Once all VFA were taken up, a residual P-release rate of 2.5 mg PO_4–P g^{-1} VSS h^{-1} ($m_{PO_4,AN}$) was observed and assumed to correspond to the anaerobic endogenous P-release (*i.e.*, for cellular maintenance). Under the presence of oxygen, phosphorus was taken up at a rate of 58 mg PO_4–P g^{-1} VSS h^{-1} ($q_{PO_4,Ox}$). The observed ratio of phosphorus taken up per total oxygen consumed was 1.63 mg P mg^{-1} O_2 (equivalent to 0.42 mol P mol^{-1} e^- transferred during respiration).

3.2. Long-term operation under anaerobic-anoxic-oxic (A_2O) conditions

As observed in Fig. 1 B-G, in the anaerobic stage of the A_2O system, all VFAs were taken up and phosphorus was released at a maximum anaerobic rate ($q^{MAX}_{PO_4,AN}$) of between 164 and 254 mg PO_4–P g^{-1} VSS h^{-1}. Once the VFAs were consumed, the phosphorus released due to maintenance activities ($m_{PO_4,AN}$) was considerably higher in the experimental periods B, C, and E (12.0 ± 0.8 mg PO_4–P g^{-1} VSS h^{-1}) than in D, F, and G (1.9 ± 1.7 mg PO_4–P g^{-1} VSS h^{-1}). The P/VFA ratios were rather stable in the experimental periods B, C, D, and E (0.66 ± 0.06 mg PO_4–P mg^{-1} COD), and higher than in F and G (0.50 ± 0.06 mg PO_4–P mg^{-1} COD).

Under anoxic conditions, accumulation of nitrite was never

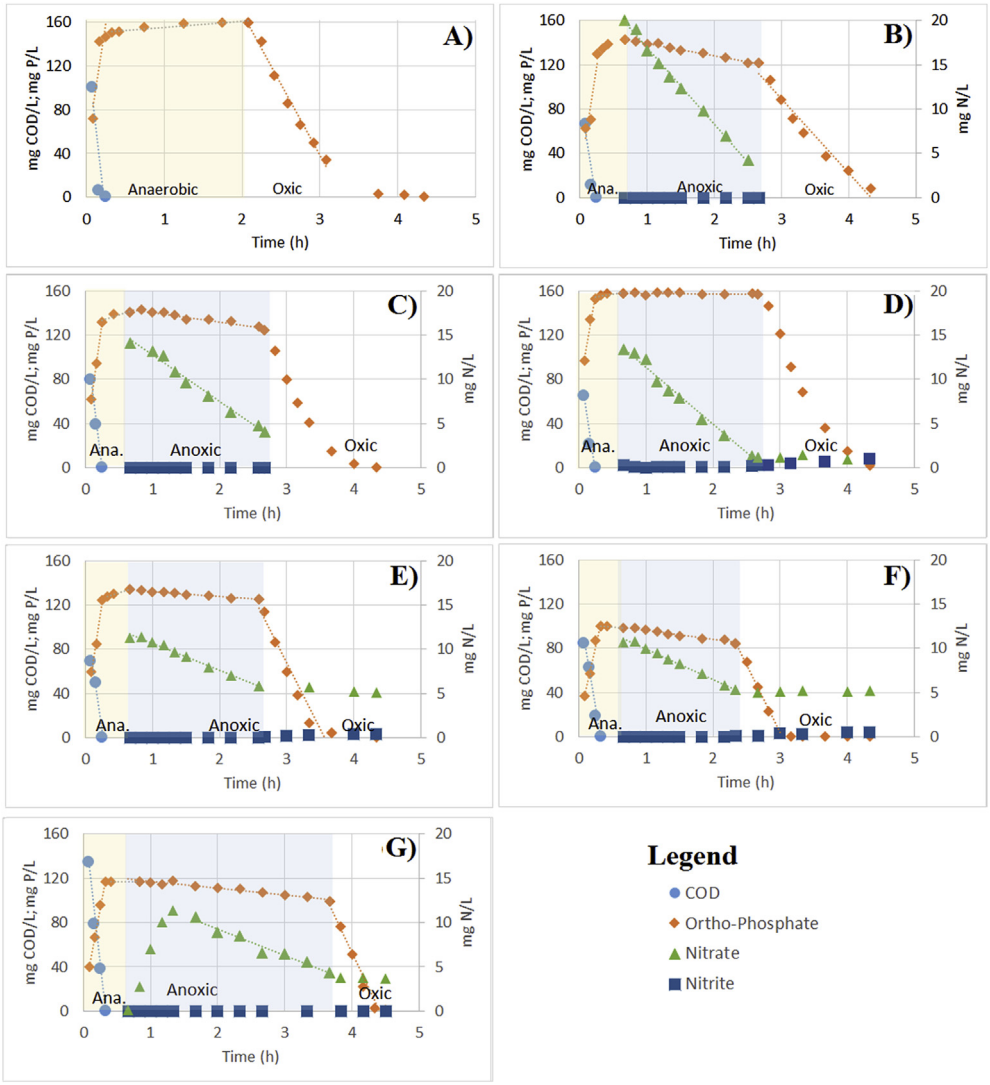

Fig. 1. Concentration profiles of ortho-phosphate (diamond), VFA as COD (circle), nitrate (triangle), and nitrite (square) observed during the SBR cycles of the different A/O (A) and A_2O (B–G) experiments conducted under different combinations of anaerobic (ana.), anoxic, and oxic phase lengths to potentially enhance the denitrification capacity of DPAOs susceptible to metabolize nitrate.

detected, in none of the experiments (Fig. 1B–G). A slightly higher denitrification activity of 3.9 mg NO_3–N g^{-1} VSS h^{-1} was observed in the first experimental period (B) after switching to the A_2O conditions. In the rest of the experimental periods, the denitrification rates did not increase considerably and remained around 2.2 ± 0.5 mg NO_3–N g^{-1} VSS h^{-1}. Table 2 shows the A_2O rates and stoichiometry parameters calculated in each experimental period. Compared to the aerobic P-uptake rates (of up to 79 mg PO_4–P g^{-1} VSS h^{-1}), a relatively low anoxic P-uptake rate ($q_{PO_4,Ax}$) was observed in periods B, C, and F (5.0 ± 0.3 mg PO_4–P g^{-1} VSS h^{-1}), which even decreased to 3.7 mg PO_4–P g^{-1} VSS h^{-1} in period G, and became negligible in period D. As much, the relative anoxic phosphorus uptake rates reached 13% of the aerobic phosphorus uptake rates (Table 2). In all cases, phosphorus was fully removed during the oxic phase (Fig. 1).

3.3. Minimal aerobic SRT

In the experimental phase H, as an attempt to favor the growth of a DPAO capable of using nitrate over strict aerobic PAOs, the oxic SRT was reduced below the minimum required for aerobic PAOs to grow as described by Brdjanovic et al. (1998). After two days of operation with an oxic SRT of approximately 0.4 d, the VFAs started to leak into the anoxic phase (74 mg COD L^{-1} at day 2 of operation). Likewise, the anaerobic phosphorus release decreased from 117 mg PO_4–P L^{-1} to 32 mg PO_4–P L^{-1} on the 3rd day of operation. The nitrate dose was increased daily to prevent nitrate limitation, while avoiding to exceed a concentration of more than 3 mg NO_3–N L^{-1} in the oxic phase, which may leak into the anaerobic stage. Despite these measures, no DPAO activity was observed and no phosphorus was removed in neither the anoxic nor the oxic phases. Thus, up to 27 mg PO_4–P L^{-1} were observed at the end of the oxic phase (day 2 of operation).

3.4. Assessment of the effects of the F/M ratio on the anoxic phosphorus uptake activity

In the batch activity test 1D (conducted *ex situ* with half of the F/M ratio applied to the parent reactor), the acetate uptake rate ($q_{PO_4,VFA}$) and the maximum phosphorus release rate ($q^{MAX}_{PO_4,AN}$) were 75.2 mg COD g^{-1} VSS h^{-1} and 72.2 mg PO_4–P g^{-1} VSS h^{-1}, respectively. These rates were considerably slower than the ones

Table 2
Anaerobic, anoxic and aerobic kinetics and stoichiometry of the PAO metabolisms calculated along the different experiments.

Exp. Period	q_{VFA}^{MAX}	$q_{PO_4,AN}^{MAX}$	$m_{PO_4,AN}$	q_{PO_4,NO_3}	q_{PO_4,O_x}	q_{NO_3}	P/NO_3	P/O_2	VSS/TSS[a]
	mgCOD/gVSS.h	$mgPO_4$-P/gVSS.h				$mgNO_3$-N/gVSS.h	mol P/mol e^-		g/g
A	269	199	2.5	N.A [d]	58	N.A [d]	N.A [d]	0.42	0.59
B	181	185	13.3	5.0	39	3.9	0.11	0.46	0.62
C	271	240	11.7	4.8	64	3.0	0.13	0.47	0.58
D	150	130	3.8	0.2	50	2.5	0.01[b]	0.53	0.56
E	272	254	12.0	2.9	79	2.0	0.13	0.50	0.61
F	210	164	1.8	5.4	77	2.1	0.24	N.C.[d]	0.77
G	331	193	0.3	3.7	79	1.8	0.22	0.43	0.77
Activity Test									
1D	75	72	N.O.[d]	−2.7[c]	N.A [d]	0.8	N.A [d]	N.A [d]	0.60
2D	265	235	8.3	0.7	N.A [d]	1.95	0.03	N.A [d]	0.60

[a] Calculated at the start of each test.
[b] Value below 0.01 P-mol/e^-.
[c] Phosphorus was released instead of taken up.
[d] N.O. not observed; N.A. not applicable; N.C. not calculated.

observed in the batch test 2D, executed with twice the F/M ratio fed to the parent reactor: 265 mg COD g^{-1} VSS h^{-1} and 235 mg PO_4–P g^{-1} VSS h^{-1}, respectively. Despite these differences, the observed P/VFA ratios were not considerably different between the two batch tests (0.60 and 0.66 at the batch tests 1D and 2D, respectively).

The anoxic phosphorus uptake and denitrification rates were rather different in the two batch tests (Fig. 2). In the 1D batch test, a nitrate reduction rate of 0.8 mg NO_3–N g^{-1} VSS h^{-1} was observed, while phosphorus was released at a rate of 2.7 mg PO_4–P g^{-1} VSS h^{-1} (Fig. 2A). On the contrary, in the 2D batch test a faster nitrate reduction rate of 1.95 mg NO_3–N g^{-1} VSS h^{-1} was observed together with a marginal phosphorus uptake rate of 0.7 mg PO_4–P g^{-1} VSS h^{-1}.

3.5. Identification of predominant bacterial populations along the experimental periods

FISH analyses were performed to identify the predominant microorganisms present in the systems and potentially involved in EBPR over the different experimental periods. Fig. 3 shows a representative image of the microbial composition at the start and end of this research (experimental periods A and G, respectively). The relative abundance of PAOs related to the genus "*Ca.* Accumulibacter" (PAOmix probe set) compared to all organisms (stained with DAPI) decreased from 98%, 95%, 76%–52% along the experimental periods A, B, D and G, respectively. Despite these differences, the fraction of the "*Ca.* Accumulibacter" clade I (Acc-1-444 probe) within PAOs did not change along the experiments (97 ± 4%; Fig. 3). Across experimental periods, GAOs were never abundant: the genera "*Ca.* Competibacter" and *Defluviicoccus* composed less than 5% of the total microbial populations, according to both FISH (Fig. 3) and V3–V4 16S rRNA gene amplicon sequencing (Table 3).

The amplicon sequencing profiles displayed a decrease in the relative abundance of the genus "*Ca.* Accumulibacter" from 53% to 33% from experimental periods A to G, respectively (Table 3).

Finer-scale characterization of the "*Ca.* Accumulibacter" clades based on PCR and sequencing analyses of the *ppk1* gene showed that the system was mainly composed of members of the clade PAO IC at the start and end of the experiments (Fig. 4).

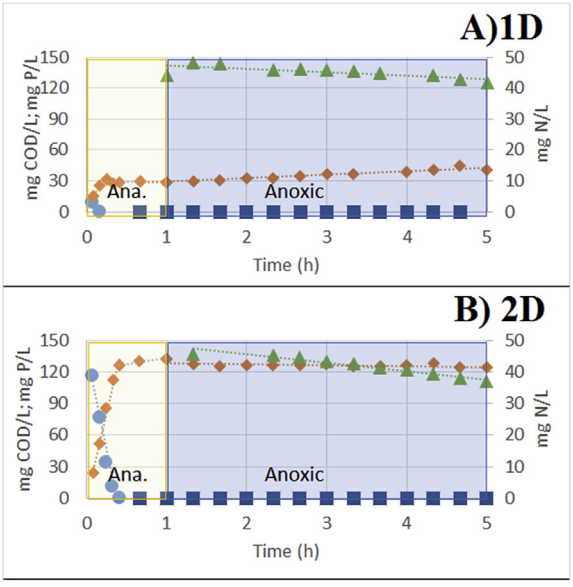

Fig. 2. Concentrations profiles of COD as VFA (circle), ortho-phosphate (diamond), nitrate (triangle) and nitrite (square) observed in the anoxic batch activity tests conducted *ex situ* with A) half F/M (37 mg COD g^{-1} VSS) and B) twice as high F/M (148 mg COD g^{-1} VSS) ratio applied in the parent reactor.

3.6. Near-complete genomes of "Ca. Accumulibacter delftensis" clade IC and two flanking populations were retrieved from the metagenome of the EBPR biomass and enabled genome-centric analysis

Three near-complete genomes were retrieved by differential coverage binning (Fig. 5A) for sub-lineages of the main gammaproteobacterial-betaproteobacteriales genus "*Ca.* Accumulibacter" (genome size of 5.3 Mbp, completeness of 100%, contamination of 0.2%, GC content of 62.2%) and of the two accompanying actinobacterial genus *Nocardioides* (4.7 Mbp, 99.2%, 0.7%, 68.6%) and gammaproteobacterial genus *Thermomonas* (2.9 Mbp, 99.5%, 0.4%, 68.4%) (Table 5) from the metagenome of the EBPR biomass exposed to A/O and subsequently A_2O conditions. Note that the class of *Betaproteobacteria* has recently been reclassified as order *Betaproteobacteriales* in the class of *Gammaproteobacteria*. Detailed features of the genome bins are provided in Fig. S1 and Table S1 of the Supplementary Information. The genome assemblies were submitted to GenBank/European Nucleotide Archive (in progress).

Phylogenetic classification of the high-quality genome assemblies against reference genomes of these lineages available in online databases allowed to delineate key populations involved in the biosystem, from alignments based on 49 highly conserved

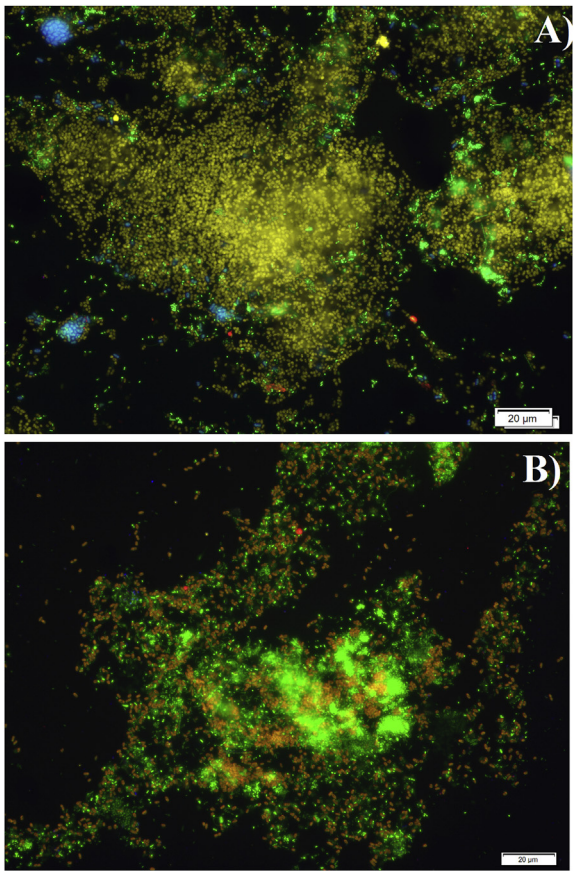

Fig. 3. Characterization of microbial populations by FISH during A) A/O conditions and 0.06 g P/g^{-1} COD fed (Exp. period A) and B) during A_2O conditions and 0.03 g P g^{-1} COD fed (Exp. period G): in green all living organisms which are not PAO nor GAO (DAPI), in red PAO which are not PAOI (Cy3; PAO Mix), in yellow PAOI (FAM; Acc-1-444), in blue GAO (Cy5; GB). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

clusters of orthologous groups of proteins. A new population of the here-proposed "*Ca.* Accumulibacter delftensis sp. nov." clade IC was identified by fine-scale differentiation out of the about 30 (draft) genomes of "*Ca.* Accumulibacter" publicly available. The recently characterized "*Ca.* Accumulibacter sp. UW-LDO-IC" was the closest neighbor but formed a different phylotype inside clade IC (Fig. 5B). Computation of pairwise average nucleotide identity (ANI) scores stated the uniqueness of the candidate species "*Ca.* Accumulibacter delftensis" (pairwise ANI < 95%). The two main accompanying populations of the here-proposed "*Ca.* Nocardioides delftensis sp. nov." and "*Ca.* Thermomonas delftensis sp. nov." affiliated with closest neighbours of *Nocardioides jensenii* and *Thermomonas fusca*, respectively, but also formed new candidate species according to pairwise ANI scores (<95%). Extraction of the full-length 16S rRNA genes (Table S4) from the three genomic datasets and mapping against NCBI and ARB Silva databases provided additional verification. The digital protologue Table 5 provides the main characteristics of the near-complete genomes of these proposed, yet not-isolated, new candidate species. The ANI analysis further highlighted that several genome assemblies that have been previously deposited in databases under different reference numbers for the "*Ca.* Accumulibacter" lineage are overlapping (pairwise ANI > 99%). This has notably arisen from re-use of deposited sequencing datasets in bioinformatics studies. The pangenome of ANI-filtered unique single-lineage genomes of "*Ca.* Accumulibacter" populations displayed diverse non-core genetic features in the assemblies (Fig. S2).

3.7. Matching of genome-based and ppk1-based phylogenetic characterizations

The phylogenetic output of the high-resolution analysis conducted on the near-complete genome of "*Ca.* Accumulibacter delftensis" clade IC was cross-validated to the sequencing of *ppk1*-gene based PCR amplicons. The sequence of the *ppk1* gene retrieved from the genome assembly of "*Ca.* Accumulibacter delftensis" clade IC (Table S5) displayed a perfect match to the sequence of the *ppk1* gene amplified from the DPAO biomass. In the functional-gene-

Table 3
Relative abundances of the ten predominant phylotypes detected at the genus level over the experimental periods A (A/O), B (A_2O), D (A_2O; extended overall SRT) and G (A_2O; Short aerobic SRT) by 16S rRNA gene-based amplicon sequencing analysis.2

Closest bacterial genus	Relative abundances (% sequencing read counts)			
	EXP. A	Exp. B	Exp. D	Exp. G
"*Candidatus* Accumulibacter"	52.6	35.4	34.6	32.9
Thermomonas	1.4	11.3	13.3	13.4
Flavobacterium	0.0	0.0	13.2	10.3
Chryseobacterium	7.8	6.5	4.5	7.7
Terrimonas	0.0	6.0	3.2	6.5
Thiobacillus	3.9	2.8	8.9	3.5
SJA 28	1.7	4.0	0.0	0.0
Thaurea	4.9	2.3	3.3	0.0
Competibacter	0.0	0.1	0.8	1.3
Niabella	0.0	3.2	0.0	0.0

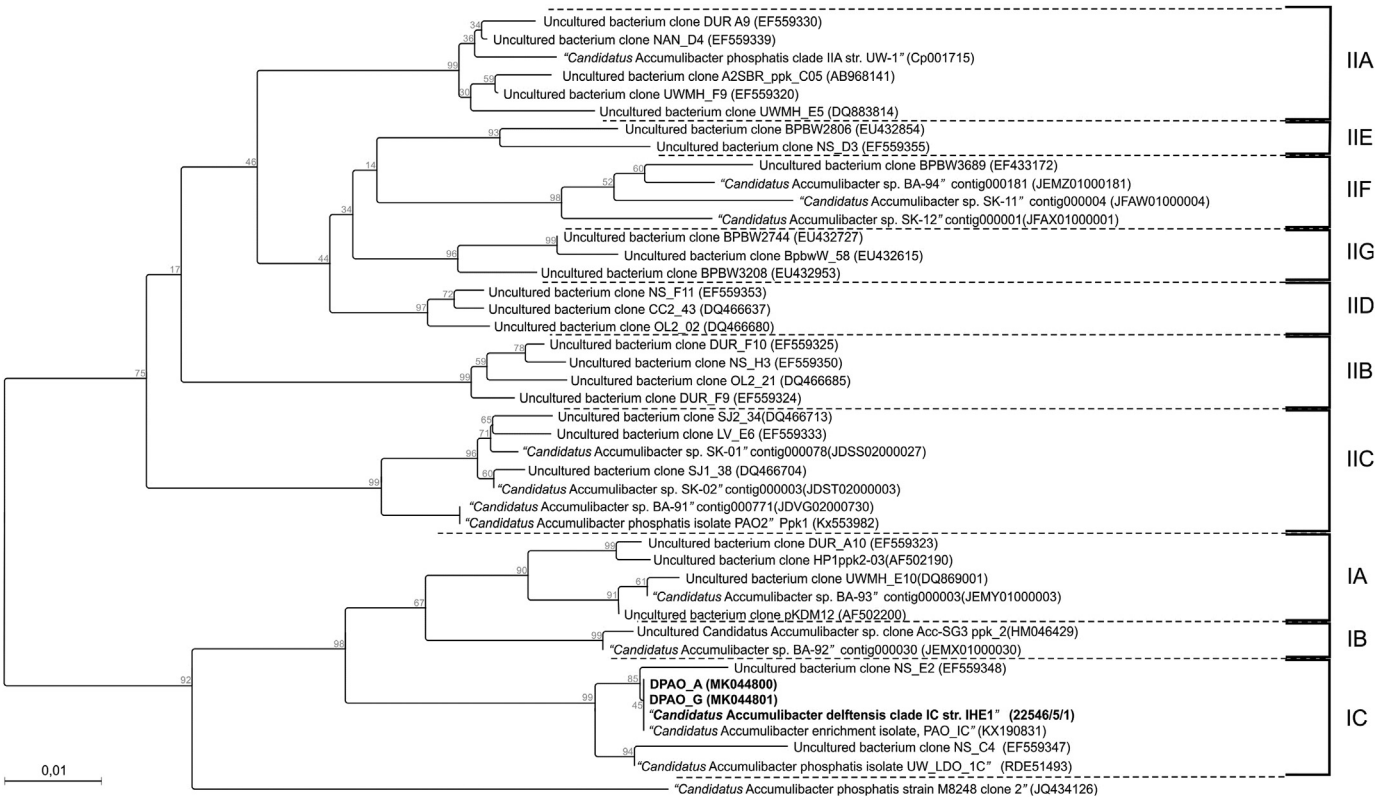

Fig. 4. Phylogenetic tree used to highlight the clade of "*Ca.* Accumulibacter phosphatis" detected within the referenced Types IA-C and IIA-F based on PCR amplification and sequencing of the *ppk1* marker gene in the samples collected during experimental period A (DPAO_A) and during experimental period G (DPAO_G). The *ppk1* gene from the metagenome ("*Candidatus* Accumulibacter delftensis" clade IC) was also included as a reference.

based phylogenetic tree, its *ppk1* nucleotide composition was closely related to reference sequences of clade IC. It formed a separate population than "*Ca.* Accumulibacter sp. UW-LDO-IC" after comparison with the *ppk1* sequence retrieved from the deposited assembly of the latter lineage. Thereafter, the genome-based and *ppk1*-based classification approaches matched, resulting in phylogenetic trees of analogous structures and robust molecular differentiation of the lineages.

3.8. Genome-centric investigation of denitrification marker genes of "Ca. Accumulibacter delftensis" clade IC, "Ca. Nocardioides delftensis", and "Ca. Thermomonas delftensis"

The underlying coding sequences of the 3 retrieved near-complete genomes of "*Ca.* Accumulibacter delftensis", "*Ca.* Nocardioides delftensis", and "*Ca.* Thermomonas delftensis" were annotated and assigned to 30 functional gene categories (Table S2), including the nitrogen metabolism (Table 4 and Table S3). Functional gene annotations of the genome assemblies highlighted a putative complementary interaction between these populations along the nitrogen cycle (Table 4, and Table S3 in Supplementary Information). The "*Ca.* Accumulibacter delftensis" genome assembly comprised genes coding for nitrate/nitrite transporter, assimilatory nitrate reductase (*nas* gene), periplasmic nitrate reductase precursor, and periplasmic nitrate reductases (*nap*), but no genes coding for respiratory nitrate reductases (*nar*). It further harbored a whole set of nitrite (*nir*), nitric oxide (*nor*), and nitrous oxide (*nos*) reductases. The "*Ca.* Nocardioides delftensis" genome assembly contained assimilatory nitrate reductase, nitrate/nitrite transporter, nitrite reductase [NAD(P)H] large subunit, and respiratory nitrate reductases, but no further denitrification genes. Interestingly, the "*Ca.* Thermomonas delftensis" genome assembly mainly harbored respiratory nitrate reductases (alpha, beta, delta, and gamma chains), and some denitrification gene homologues (*nir*, *nor*).

4. Discussion

4.1. Effect of different operating conditions on anoxic phosphorus uptake activity

The anoxic phosphorus uptake rates observed in the experimental periods conducted under different operating conditions were considerably lower than the oxic P-uptake rates (Table 2). The anoxic P-uptake rate reached at most 13% of the oxic P-uptake rate. Thus, even though there could be some denitrification associated with an anoxic P uptake, this seems not be significant when compared with the oxic P uptake. This is in agreement with the study of Lanham et al. (2011) who observed a faster oxic than anoxic P-uptake rate in an A_2O reactor. Alternatively, Kuba et al. (1993) and Lee and Yun (2014) reported that nitrate could be used as electron acceptor as efficient as oxygen by PAO for P-uptake. However, Kuba et al. (1993) and Lee and Yun (2014) have applied an A_2 configuration in contrast to this study where an A_2O configuration was used. This suggests that the oxic phase in each cycle may have hampered the selection for a PAO and/or a microbial population which could enhance the use nitrate as efficient as oxygen for P-uptake. The aim of this study was to evaluate whether various operational conditions could enhance the denitrification capacities of a "*Ca.* Accumulibacter" clade I culture. Operational changes were applied for a relatively short time, allowing for the enzymatic induction but minimizing any shifts in the microbial community composition.

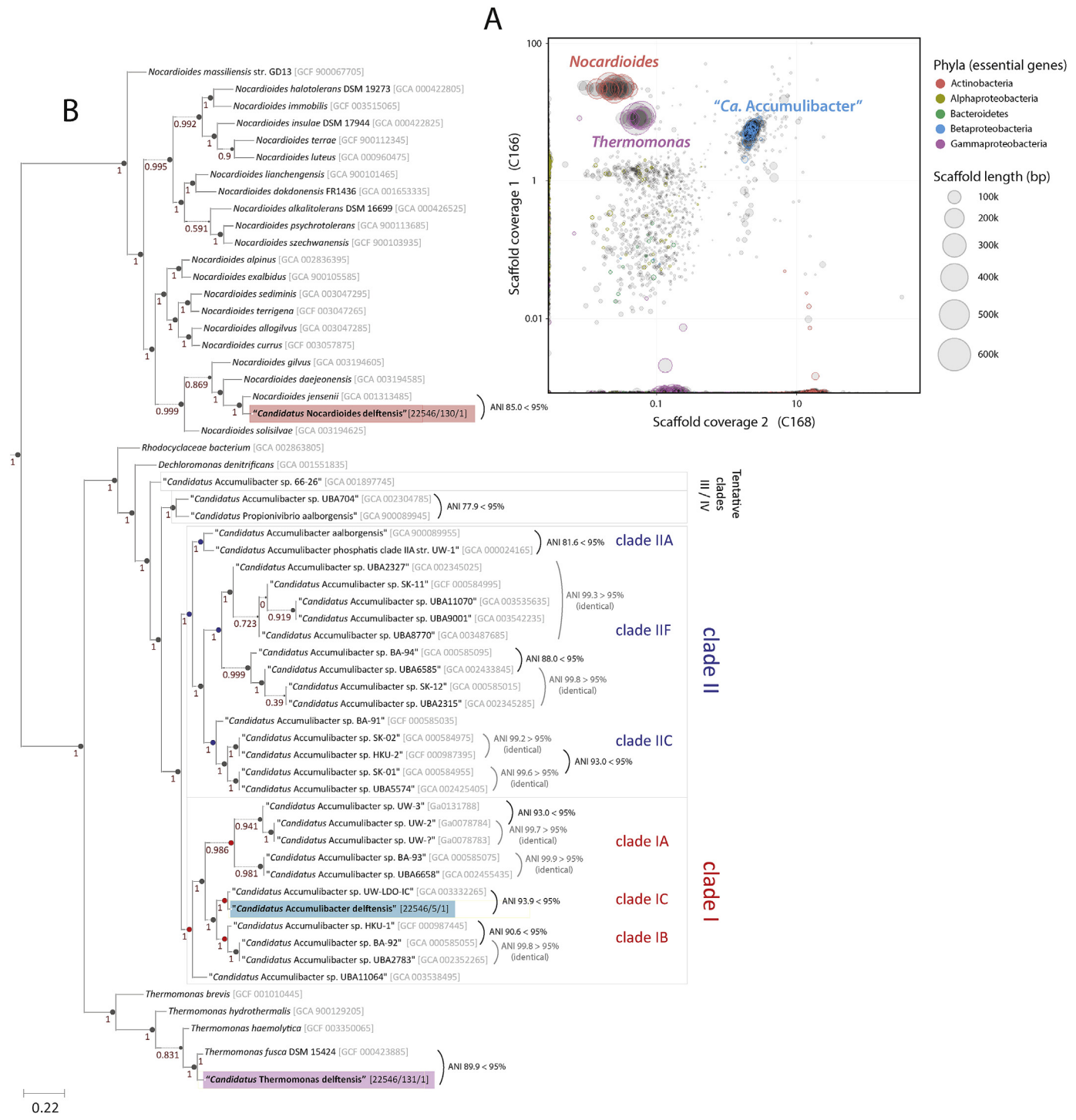

Fig. 5. Extraction and phylogenetic analysis of the near-complete genome of "*Ca.* Accumulibacter delftensis clade IC" out of the metagenome of the EBPR biomass. **(A)** Differential coverage plot used to extract the single-lineage genomes of "*Ca*. Accumulibacter delftensis" (*blue scaffolds*),"*Ca.* Nocardioides delftensis" (*orange scaffolds*), and "*Ca.* Thermomonas delftensis" (*pink scaffolds*) populations via the mmgenome workflow. Genome scaffolds are displayed by circles scaled by nucleotide length (bp) and colored after taxonomic classification of essential genes at phylum level. Two sequencing datasets were used here to generate the differential coverage, namely from the biomass sample directly collected from the denitrifying EBPR reactor stage (*scaffold coverage 1, C166*) and the wet-lab treatment of the biomass used to generate a synthetic shift in predominant populations (*scaffold coverage 1, C168*). Each set of isolated and colored scaffolds represent a genome. **(B)** Phylogenetic analysis of the near-complete genome of "*Ca.* Accumulibacter delftensis" clade IC (*highlighted in yellow*) against all single-lineage genomes of "*Ca.* Accumulibacter" populations recovered from the 28 assemblies available in on-line public databases such as NIH GenBank (*GCA accession numbers*), NCBI RefSeq (*GCF accession numbers*), and JGI MGM (*Ga accession numbers*). The "*Ca.* Accumulibacter" lineage is composed of two primary clades I (subgroups A-E) and II (subgroups A-I) (see Fig. 4). "*Ca.* Accumulibacter delftensis" clade IC was classified within "*Ca.* Accumulibacter" clade IC together with the UW-LDO-IC population characterized by Camejo et al. (2018) but remains a different phylotype, underlying the difference in denitrification patterns. The two other single-lineage near-complete genomes of "*Ca.* Nocardioides delftensis" and "Ca. Thermomonas delftensis" recovered from the metagenome of the EBPR biomass are also displayed on this tree (*highlighted in yellow*); the phylogenetic identification of these near-complete genomes is available in Fig. S2 of the supplementary information. The phylogenetic tree was computed in KBase from reference alignments based on 49 highly conserved clusters of orthologous groups of proteins and using the FastTree2 approximate maximum likelihood method. Scale bar: number of nucleotide substitutions per site. The pairwise average nucleotide identity (ANI) scores discriminates between unique genomes (ANI > 99%) and overlapping genomes (ANI < 95%). Several assemblies present in on-line databases under different registry numbers code for identical sub-lineages. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

As the anoxic growth yield can be up to 70% of the aerobic growth yield (Kuba et al., 1993), the SRT was extended from 8 to 15 d in the experimental period D as suggested by Ahn et al. (2002). However, this exerted an adverse effect by decreasing the anoxic activity from 3.9 to 2.5 mg NO_3–N g^{-1} VSS h^{-1} (experimental phase B, and D, respectively). In contrast to the studies of Kuba et al. (1993) and Ahn et al. (2002), a clear anoxic P-uptake was never observed. It may be concluded that either the denitrification activity observed in this study was not carried out by the "*Ca.* Accumulibacter" population selected in this system, or the culture was not capable to restore its poly-P storage pools under the applied conditions. Further activity tests with the biomass of experimental period D showed that under a low F/M ratio, phosphate was surprisingly released under anoxic conditions at a rate of 2.7 mg PO_4–P g^{-1} VSS h^{-1}. Despite that nitrate was present in the activity test (Fig. 2A), the release of phosphate strongly suggests that the "*Ca.* Accumulibacter" affiliate of clade IC obtained in this enrichment culture could not use nitrate as electron acceptor to generate enough energy from its putative reduction. Thus, as suggested by Rubio-Rincón et al. (2017a) and Ribera-Guardia et al. (2016), this PAO may prefer to use nitrite (generated by side populations, among them possibly GAOs, via the reduction of nitrate to nitrite) as electron acceptor for the oxidation of PHA. The nitrite produced by side populations would be lower according to a decrease in the organic load per biomass (F/M), resulting in energy limitation by the oxidation of PHA by PAOs and a subsequent anoxic P-release as observed in Fig. 2A.

To further assess if a side population was responsible for the denitrification observed in the system, the P/COD ratio in the influent was decreased with the aim to increase the GAO fraction from experimental period F onwards. The population of "*Ca.* Accumulibacter" clade IC remained the main PAO detected (approx. 97 ± 4%) but decreased from 98% to 52% from experimental periods A to G (Fig. 3), similar to the study of Carvalho et al. (2007). However, GAOs did not proliferate as expected (up to 1% in the experimental period G; Fig. 3). Likely, the relatively high pH of 7.6 limited the proliferation of GAOs (Smolders et al., 1995). At the same time, after the switch from A/O to A_2O conditions, an increase in the fraction of potentially denitrifying *Thermomonas* and *Chryseobacterium* genera was observed (13% and 8%, respectively in experimental period G; Table 3). Despite that *Thermomonas* and *Chryseobacterium* are capable to reduce both nitrate and nitrite (Mergaert et al., 2003; Kundu et al., 2014), the biomass-specific denitrification activity did not increase together with their enrichment (2.1 and 1.8 mg NO_3–N g^{-1} VSS h^{-1} in experimental periods F and G, respectively). The non-PAO-based denitrification activity measured in the system remained uncertain.

Additional analyses of mRNA-based functional gene expression or protein translation within the nitrogen cycle either via reverse transcription and qPCR or higher-throughput molecular analyses like metatranscriptomics and metaproteomics, respectively, would have helped to identify the biochemical pathways activated together with the bacterial populations involved. The role of the side populations within the microbial community (*e.g.*, *Thermomonas, Flavobacterium, Chryseobacterium, Terrimonas, Nocardioides*) remains also unclear. Under the operating conditions applied here, they did not significantly contribute to the denitrification activity. This suggests that other factors or microorganisms interact and can (*e.g.*, GAOs were suggested by Rubio-Rincón et al. (2017a)) enhance the anoxic P-uptake activity over nitrite in EBPR cultures.

Kuba et al. (1993) enriched a DPAO culture under A_2 conditions, dosing nitrate continuously during the anoxic stage. This dosing mode could benefit k-strategist DPAOs. Therefore, in period G, nitrate was dosed continuously during the anoxic phase as described earlier. Certain increase in the anoxic P-uptake per electron available was observed from 0.13 to 0.24 mol P mol^{-1} e^- (between periods F and G, respectively). The anoxic range from 0.13 to 0.24 mol P mol^{-1} e^- ratio observed along this study was comparable to the one of 0.19 mol P mol^{-1} e^- reported by Kuba et al. (1993). However, the anoxic P-uptake rate was slower than the reported by Kuba et al. (1993) (up to 5.4 mg PO_4–P g^{-1} VSS h^{-1} compared to 30–46 mg PO_4–P g^{-1} VSS h^{-1}, respectively). This indicated that the dosing mode did not play a role to enhance the anoxic phosphorus uptake activity of the sludge.

As an attempt to favor the growth of DPAOs over strict aerobic PAOs, the operational conditions were gradually modified from anaerobic-anoxic-oxic to anaerobic-anoxic (experimental periods G and H). However, once the aerobic SRT was reduced below the minimal required, acetate started to leak into the anoxic phase (after just 2 days of operation). Despite that nitrate was not limiting, likely PHAs were not completely oxidized during the anoxic and oxic phases. Thus, PAOs could not take up sufficient phosphorus or produce enough glycogen for anaerobic VFA uptake. This is in agreement with Lanham et al. (2011) who concluded that an oxic phase was essential to maintain a good removal of phosphorus, where most of the phosphorus uptake occurred. Therefore, it is suggested that the culture of "*Ca*. Accumulibacter delftensis" clade IC enriched in this study cannot solely rely on the use of nitrate for energy generation for metabolic processes (*e.g.*, via PHA oxidation).

Recently, in contrast with these findings and those of Saad et al. (2016), where no significant anoxic phosphorus uptake was observed, Camejo et al. (2016) reported the anoxic P-uptake activity on nitrate (11 ± 1.7 mgPO_4-P g^{-1} VSS h^{-1}) of a "*Ca.* Accumulibacter" clade IC culture. However, functional diversity in sub-lineages can happen, making that the *ppk* analysis cannot be used to anticipate denitrification capabilities. Hence, as discussed in the following sections, the correlative trends between *ppk1*-based taxonomic classification and denitrification activities monitored in EBPR systems is no longer valid. The sole detection of microbial clades is not sufficient to explain biochemical conversions.

The genome-centric metagenomics analysis of the near-complete genomes of the "*Ca.* Accumulibacter delftensis" clade IC and "*Ca*. Thermomonas delftensis" lineages of the EBPR biomass subjected to A/O and A_2O conditions interestingly highlighted a putative interaction potential between the two populations from nitrate reduction to nitrite by *Thermomonas* (harbors genes coding for respiratory nitrate reductases, *nar* but genes for nitrite reduction were not found in the assembly) prior to respiration of nitrite by "*Ca.* Accumulibacter" along with P-removal. The "*Ca.* Nocardioides delftensis" lineage harbored functional genes for both nitrate and nitrite respiration. The involvement of this population that can display filamentous phenotypes is supposed to primarily occur under disturbed conditions like those where VFA leak into the anoxic stage. These functional potentials underlie hypothetical interactions between "*Ca.* Accumulibacter" and these accompanying populations in the nitrogen cycle within the ecosystem of lab-scale denitrifying EBPR biomass. However, the previously proposed reduction mechanism from nitrate to nitrite performed by *Thermomonas* and/or *Nocardioides* might be conducted by other organisms in full-scale wastewater treatment plants. Further research and analytical methods are needed to elucidate microbial interactions between "*Ca.* Accumulibacter" and other genera (*e.g.*, "*Ca.* Competibacter", *Thermomonas, Nocardioides,* "*Ca.* Microthrix") on the nitrogen cycle.

4.2. Possible metabolic pathway for nitrate reduction observed in this study

Only a marginal nitrate consumption of up to 3.9 mg NO_3–N g^{-1}

Table 4
Functional genetic signatures coding for enzymes catalyzing pathways of nitrogen metabolism within the near-complete genomes of "Ca. Accumulibacter delftensis clade IC str. IHE1" (size of 5.3 Mbp, completeness of 100%, contamination of 0.2%), Nocardioides delftensis str. IHE1 (4.7 Mbp, 99.2%, 0.7%), and Thermomonas delftensis str. IHE1 (2.9 Mbp, 99.5%, 0.4%) lineages recovered from the metagenomes of the biomass subjected to A/O prior to A2O conditions. These genomic signatures highlight a putative complementary interaction between Thermomonas and "Ca. Accumulibacter" along nitrate and nitrite reductions, respectively. Annotations of the genome bin were performed using the RAST toolkit in KBase. Zero-values mean not detected in the genome bins. The heatmap highlights the presence (blue) and absence (red) of functional genes relating to the nitrogen cycle in the near-complete genomes. The names of the functional genes are highlighted in blue or red according to presence/absence in the near-complete genome of "Ca. Accumulibacter delftensis clade IC str. IHE1".3

Functional signatures of the nitrogen cycle detected in single-lineage near-complete genomes
(# instances)

"Ca. Accumulibacter delftensis clade IC str. IHE1"	*Nocardioides delftensis* str. IHE1	*Thermomonas delftensis* str. IHE1		"Ca. Accumulibacter delftensis clade IC str. IHE1"	*Nocardioides delftensis* str. IHE1	*Thermomonas delftensis* str. IHE1	
73	35	16	**Functional categories in nitrogen metabolism** *(57 / 170 entries)* [a]				
[b] 2	0	0	**Nitrogen fixation** *(2 / 43 entries)* [d]	3	2	0	**Allantoin utilization** *(2 / 11 entries)*
[c] 1	0	0	4Fe-4S ferredoxin, nitrogenase-associated	2	1	0	2-hydroxy-3-oxopropionate reductase
1	0	0	NifX-associated protein	1	1	0	Glycerate kinase
1	8	0	**Nitric oxide synthase** *(1 / 3 entries)*	3	2	1	**Cyanate hydrolysis** *(1 / 8 entries)*
1	8	0	putative cytochrome P450 hydroxylase	3	2	1	Carbonic anhydrase
18	12	6	**Ammonia assimilation** *(12 / 24 entries)*	13	11	4	**Nitrate and nitrite ammonification** *(15 / 37 entries)*
2	1	0	Ammonium transporter	1	2	0	Assimilatory nitrate reductase large subunit
1	0	0	Ferredoxin-dependent glutamate synthase	1	0	0	Cytochrome c-type protein NapC
3	2	1	Glutamate synthase [NADPH] large chain	1	0	0	Ferredoxin-type protein NapF (periplasmic nitrate reductase)
0	1	0	Glutamate synthase [NADPH] putative GlxC chain	2	0	0	Ferredoxin-type protein NapG (periplasmic nitrate reductase)
2	1	1	Glutamate synthase [NADPH] small chain	1	0	0	Nitrate reductase cytochrome c550-type subunit
1	1	1	Glutamate-ammonia-ligase adenylyltransferase	1	1	0	Nitrate/nitrite transporter
0	1	0	Glutamine amidotransferase protein GlxB	1	3	0	Nitrite reductase [NAD(P)H] large subunit
1	0	0	Glutamine amidotransferase, class-II	1	0	0	Nitrite reductase [NAD(P)H] small subunit
1	2	1	Glutamine synthetase type I	1	0	0	Nitrite reductase probable [NAD(P)H] subunit
1	0	0	Nitrogen regulation protein NR(I)	1	0	0	Periplasmic nitrate reductase precursor
4	1	1	Nitrogen regulatory protein P-II	2	0	0	Polyferredoxin NapH (periplasmic nitrate reductase)
2	1	1	[Protein-PII] uridylyltransferase	0	1	1	Respiratory nitrate reductase alpha chain
				0	2	1	Respiratory nitrate reductase beta chain
7	0	2	**Nitrosative stress** *(3 / 11 entries)*	0	1	1	Respiratory nitrate reductase delta chain
2	0	1	Nitric-oxide reductase, quinol-dependent	0	1	1	Respiratory nitrate reductase gamma chain
2	0	0	Nitrite-sensitive transcriptional repressor NsrR				
3	0	1	NnrS protein involved in response to NO	16	0	3	**Denitrification** *(11 / 21 entries)*
				0	0	1	Copper-containing nitrite reductase
10	0	0	**Dissimilatory nitrite reductase** *(10 / 11 entries)*	3	0	0	Cytochrome cd1 nitrite reductase
1	0	0	Cytochrome c55X precursor NirC	1	0	0	Nitric oxide responding transcriptional regulator Dnr (Crp/Fnr family)
3	0	0	Cytochrome cd1 nitrite reductase	2	0	1	Nitric oxide reductase, quinol-dependent
1	0	0	Heme d1 biosynthesis protein NirF	2	0	0	Nitrous oxide reductase maturation protein NosD
1	0	0	Heme d1 biosynthesis protein NirG	1	0	0	Nitrous oxide reductase maturation protein NosF (ATPase)
1	0	0	Heme d1 biosynthesis protein NirH	1	0	0	Nitrous oxide reductase maturation protein, outer-membrane lipoprotein NosL
1	0	0	Heme d1 biosynthesis protein NirJ	1	0	0	Nitrous oxide reductase maturation transmembrane protein NosY
1	0	0	Nitrite reductase associated c-type cytochorome NirN	1	0	0	Nitrous-oxide reductase
1	0	0	Uroporphyrinogen-III methyltransferase	3	0	1	NnrS protein involved in response to nitric oxide
1	0	0	Nitrite reductase associated c-type cytochorome NirN	1	0	0	NnrU family protein, required for expression of nitric oxide and nitrite reductases
1	0	0	Uroporphyrinogen-III methyltransferase				
				0	0	0	**Amidase clustered with urea and nitrile hydratase functions** *(0 / 1 entry)*

[a] Number of entries reported across functional categories of the nitrogen metabolism: 57 different genes detected over a maximum possible total of 170
[b] Total of instances (sum of gene copies) detected per functional category
[c] Number of instances (gene copies) detected for each functional gene entry (line)
[d] Number of single entries detected among the total number of single entries per functional category

VSS h^{-1} was observed in the enriched "*Ca.* Accumulibacter" IC culture (experimental period B). Alternatively to the role of side-populations, the consumption of nitrate can be associated to growth (nitrate assimilation; Nas), generation of metabolic energy (nitrate respiration; Nar) and/or dissipation of the excess of reducing power (nitrate dissimilation; Nap) (Moreno-Vivián et al., 1999). According to previous studies (Skennerton et al., 2014) different clades of "*Ca.* Accumulibacter" contain either the nitrate respiration gene (*nar;* clade IIC) or the periplasmatic nitrate dissimilation gene (*nap*; clades IC, IA, IIA, IIF). Only the *nap* gene has been previously identified in the metagenomes of enriched EBPR cultures composed of the clades IA and IC (Flowers et al., 2013;

Table 5
Digital protologue table for "*Candidatus* Accumulibacter delftensis sp. nov.", "*Candidatus* Nocardioides delftensis sp. nov.", and "*Candidatus* Thermomonas delftensis sp. nov. ".

Taxonumber	(in progress)	(in progress)	(in progress)
Species name	Accumulibacter delftensis	Nocardioides delftensis	Thermomonas delftensis
Genus name	Accumulibacter	Nocardioides	Themomonas
Specific epithet	delftensis	delftensis	delftensis
Species etymology	delftensis (delf.ten'sis. N.L. fem. adj. delftensis pertaining to the city of Delft, The Netherlands, home of IHE Delft where the organism was enriched. Delft originates from from the Dutch word *delf, i.e.*, 'ditch', name of city's main canal		
Species status	sp. nov.	sp. nov.	sp. nov.
LCA tax. Silva	K: *Bacteria* P: *Proteobacteria* C: *Gammaproteobacteria* [a] O: *Betaproteobacteriales* [a] F: *Rhodocyclaceae* G: "*Ca.* Accumulibacter"	K: *Bacteria* P: *Actinobacteria* C: *Actinobacteria* O: *Propionibacteriales* F: *Nocardiodaceae* G: *Nocardioides*	K: *Bacteria* P: *Proteobacteria* C: *Gammaproteobacteria* O: *Xanthomonadales* F: *Xanthomonadaceae* G: *Thermomonas*
Relationship to O_2	Facultative aerobe	Facultative anaerobe	Facultative aerobe
Energy metabolism	Chemoorganotroph	Chemoorganotroph	Chemoorganotroph
16S rRNA gene accession number	(in progress)	(in progress)	(in progress)
MAG/SAG accession number	(in progress)	(in progress)	(in progress)
Genome status	draft	draft	draft
Genome completeness	100.0%	99.223%	99.483%
Genome size	5′278′942 bp	4′727′224 bp	2′911′105 bp
Mean GC mol %	62.19%	68.57%	68.43%
# Contigs	361	32	26
N50	36′145	242′893	255′668
L50	47	7	4
Genome contamination	0.187%	0.691%	0.431%
Predicted tRNA genes	54	47	46
rRNA operons	1	1	1
Predicted genes	4′913	4′434	2′422
Coding genes (CDS)	5′329	4′641	2′666
Coding density (%)	87.2%	91.4%	91.4%
Annotated genes	3′955	3′756	2′334
Annotated domains	16′726	14′606	14′487
Country of origin	Netherlands		
Region of origin	Delft, Zuid Holland		
Source of sample	Activated sludge enrichment culture		
Sampling date	September 2015		
Geographic location	IHE Delft Institute for Water Education		
Latitude	52.0090° N		
Longitude	4.3569° E		
Assembly	Replicate different samples		
Sequencing technology	Illumina MiSeq		
Binning software	mmgenome through R		
Assembly software	CLC workbench		
Authors	Rubio-Rincón F. J.[§], Weissbrodt D. G.[§], Lopez-Vazquez C. M., Welles L, Abbas B., Albertsen M., Nielsen P. H., van Loosdrecht M. C. M., Brdjanovic D.		
Title	"*Candidatus* Accumulibacter delftensis sp. nov.": a clade IC novel polyphosphate-accumulating organism without denitrifying activity on nitrate		
Journal	Water Research		
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Submitter; e-mail	David G. Weissbrodt d.g.weissbrodt@tudelft.nl		

[a] The class of *Betaproteobacteria* has recently been reclassified as order *Betaproteobacteriales* within the class of *Gammaproteobacteria* (https://doi.org/10.1038/nbt.4229).

Skennerton et al., 2014). Consistently, in the present study, the genome assembly of "*Ca.* Accumulibacter delftensis" clade IC harbored *nas* and *nap* genes but not the *nar* gene (Table 4), which could explain the low anoxic P-uptake activity measured in the bioreactor. Camejo et al. (2016) have suggested that an enriched clade-IC population of "*Ca.* Accumulibacter sp. UW-LDO-IC" contained all the enzymes necessary to perform a full denitrification, which has been confirmed by their subsequent integrated 'omic' analysis (Camejo et al., 2018). However, the actual operating conditions required to enhance the presence and activation of the required enzymes remain unclear.

From the nitrogen cycle perspective, the two most characterized populations of this interesting clade IC, namely "*Ca.* Accumulibacter delftensis" clade IC and "*Ca.* Accumulibacter sp. UW-LDO-IC", display fine-scale differentiation in genes coding for nitrate reduction (antagonist presence/absence of *nap* and *nar* genes). IHE1 primarily harbors periplasmic (*nap*) and lacks respiratory (*nar*) nitrate reductases, which is exactly the opposite by UW-LDO-IC.

As described by Moreno-Vivián et al. (1999), the Nap enzyme is not involved in the anaerobic respiration as the enzyme is independent of the energy conserving *cytochrome bc1* complex. Instead, the electron required for the reduction of nitrate could be obtained via NADH, which passes through proton translocating NADH dehydrogenases (Bedzyk et al., 1999). Interestingly, the subunit *napD,* which seems to be an important precursor of the subunit *nap*A (Moreno-Vivián et al., 1999) was not detected in the genome assembly of "*Ca.* Accumulibacter delftensis" clade IC (Table S3 in Supporting Information). This is notable since *napA* that is responsible for the reduction of nitrate to nitrite can likely not be promoted in absence of *napD*. Thus, based on such genetic impotent, it is unlikely that the PAO population in our system was capable to utilize nitrate. Moreover, the existence and activation of the Nar enzyme appears to be required for a considerable anoxic phosphorus uptake using nitrate by "*Ca.* Accumulibacter". The

absence of *nar* gene in the genome assembly underlines that this functionality cannot be achieved by the selected "*Ca.* Accumulibacter delftensis" clade IC.

Regarding the current molecular techniques applied, based on fine-scale genetic analyses of the *ppk1* gene, it is possible to differentiate among the "*Ca.* Accumulibacter" lineages (McMahon et al., 2007). Despite the general correlative assumptions that these clades seem to have the same encoding for denitrification, they exhibit different anoxic P-uptake capabilities on nitrate (*e.g.*, anoxic activities of clades IC and IIA) (García Martín et al., 2006; Flowers et al., 2008, 2013, Oehmen et al., 2010b, 2010c; Skennerton et al., 2014; Camejo et al., 2016, 2018; Ribera-Guardia et al., 2016; and this study). In view of these observations, the traditionally accepted engineering classification of the denitrifying populations of "*Ca.* Accumulibacter" grouped based on the *ppk* gene as either clade I ("with full denitrification capacity from nitrate onwards") or clade II ("from nitrite onwards") as modeled by Oehmen et al. (2010c) does not seem to be valid and supported as a stand-alone approach. Further studies should include detection and expression of the key denitrifying genes (*e.g.*, *nar*) or proteins (*e.g.*, Nar), in addition to the clade differentiation using the *ppk* marker gene.

It should be thoroughly investigated how different operating conditions can enhance the selection of DPAOs, the activation of their genetic signatures, and their catabolic regulation depending on terminal electron acceptors available. The influence of environmental and operational conditions on the gene regulation (Skennerton et al., 2014) and their interaction with the different metabolic pathways and energy consumption deserve special attention.

Definitely, microbial ecology have suggested that many functional traits are not necessarily conserved between lineages (Martiny et al., 2015). Ancestral genome reconstructions have shown that denitrification genes are part of the flexible genome, and that numerous other important genes of EBPR metabolisms have been acquired by horizontal transfer (Oyserman et al., 2016). Hence, the incongruency between studies on denitrification traits of "*Ca.* Accumulibacter" clades does reflect the true intra-clade diversity, which requires thorough investigation within the "*Ca.* Accumulibacter" lineage. This statement can be broadened over the complexity of clades of heterotrophic denitrifiers, whose genetic pools may result from a rather random acquisition process. Certainly, the concepts and justification of clades need strong reappraisal across microbial tree of life (Parks et al., 2017) toward a more accurate representation of actual metabolisms, together with consideration of horizontal transfer of catabolic genes in activated sludge microbiomes.

4.3. Possible sources of carbon for denitrification by side populations

If ultimately the selected DPAOs are not able to denitrify from nitrate at relevant and applicable rates, it remains possible that the denitrification observed in EBPR systems is carried out partially (from nitrate to nitrite) by other microbial populations. However, such potential heterotrophic denitrifiers should be provided with electron donors. This adds up to the former questions regarding the factors (*e.g.*, organic substrate availability and substrate storage under anaerobic conditions) and underlying the presence of a diversity of potential denitrifiers in the bacterial community of EBPR processes (Weissbrodt et al., 2014). Previous research on EBPR have reported the leakage of certain residual concentrations of (non-VFA) organic compounds as a function of the HRT (Ichihashi et al., 2006). The residual organic compounds present in the anoxic stage could be potentially used by ordinary heterotrophic organisms (OHOs) as carbon source for denitrification purposes. Kuba et al. (1993) have observed around 5 mg TOC L^{-1} leaking into an anoxic phase, which have led to up to 3.8 mg NO_3–N L^{-1} reduction (if nitrate was reduced to nitrite). Moreover, nitrate (from anoxic zones) can also intrude into anaerobic stages (via internal recirculation flowrates or due to the residual concentrations of these components observed in the end of the alternating aerobic or anoxic phases), independently of the experimental periods. Thus, OHOs could grow in the system on the VFAs fed and nitrate available contributing to the direct denitrification from nitrate to nitrite and even in the subsequent anoxic phase triggered by the endogenous processes. In some cases, the presence of nitrate in anaerobic stages have nevertheless been reported to favor the growth of DPAOs in EBPR systems (Ahn et al., 2001b).

Another possibility is the potential presence of other microorganisms capable to store VFAs under anaerobic conditions and then to oxidize them using nitrate. Tu and Schuler (2013) proposed that under carbon limiting conditions PAOs would outcompete other PHA-accumulating organisms such as GAOs. This could be the case in the activity test 1 D performed here with half the organic loading per biomass (Fig. 2A), where a denitrification rate of 0.8 mg NO_3–N g^{-1} VSS h^{-1} was observed together with an anoxic P-release rate of 2.7 mg PO_4–P g^{-1} VSS h^{-1}. On the contrary, in the activity test 2D conducted with double the organic loading per biomass, a denitrification rate of 1.95 mg NO_3–N g^{-1} VSS h^{-1} with an anoxic phosphorus uptake rate of 0.7 mg PO_4–P g^{-1} VSS h^{-1} were observed. Thus, it is suggested that the anoxic P-uptake observed in this study was carried out over the nitrite generated by other PHA-accumulating organisms that might have benefitted from the relatively higher availability of electron donors.

Ekama and Wentzel (1999) pointed out that alternating anoxic-oxic conditions stimulated the presence of filamentous bacteria. The appearance of filamentous bacteria has also been reported in the studies of Kerrn-Jespersen and Henze (1993) and comprised up to 10% of the biomass in the studies of Lanham et al. (2011). Early studies of Kuba et al. (1993) also reported considerable amounts of sludge washed out through the effluent due to settling problems, suggesting the presence of filamentous bacteria. These filamentous bacteria (as the frequently observed *Thiothrix* (type 021N) or/and "*Ca.* Microthrix" in wastewater treatment plants) can reduce nitrate into nitrite (Williams and Unz, 1985; Nielsen et al., 2000; Hesselsoe et al., 2005; McIlroy et al., 2013) and have also been identified as one of the most abundant side populations present in EBPR lab-systems (Garcia-Martin et al., 2006). Interestingly, recently some of these (*e.g.*, *Thiothrix*) have been reported to also contribute to EBPR (Rubio-Rincón et al., 2017b). Further characterization of the metabolisms of the side populations is needed to elucidate their potential involvement in the anoxic P-uptake activities of EBPR cultures.

5. Conclusions

The "*Ca.* Accumulibacter delftensis" clade IC culture enriched and genetically characterized in this study did not exhibit a considerable anoxic P-uptake on nitrate: it was as high as 13% of the phosphorus uptake observed under aerobic conditions. A decrease in the organic load fed per biomass resulted in the anoxic release of phosphorus, indicating that this specific population of the clade IC was unable to use nitrate as electron acceptor. Eventually, the system collapsed when the aerobic SRT decreased below the minimum required, reinforcing the idea that our clade IC cannot rely solely on the use of nitrate as terminal electron acceptor. The putative complementary interaction potential between side populations of the genera *Thermomonas* and *Nocardioides* (nitrate respiration) and the "*Ca.* Accumulibacter" population (nitrite respiration) was highlighted from single-lineage genome

assemblies extracted from the biomass subjected to A/O and A_2O conditions. We further showed that genome-based and *ppk1*-gene-based phylogenies were congruent for the classification of known populations within the "*Ca.* Accumulibacter" lineage, with genome-centric metagenomics approach providing definite advantage to capture functional potential of the phylotypes. Next research should target functional expression and ecophysiological analyses to validate actual biochemical pathways involved by targeted populations, together with microbial interactions in denitrifying EBPR systems. We here stressed that correlating denitrifying activities to "*Ca.* Accumulibacter" clade differentiation by *ppk* gene is not valid. Deep reconsideration of the true intra-clade diversity in functional traits, that results from evolutionary and horizontal gene transfer processes, is paramount.

6. Nucleotide sequence accession numbers

The ppk1 gene sequences obtained in this study were deposited in the GenBank database under accession numbers MK044800-MK044801-MH899086. The 16S-rRNA gene amplicon libraries have been deposited as a SRA archive under project PRJNA495757. The genome assemblies are deposited on KBase, and are submitted to GenBank/ENA (in progress). The sequences of the full-length 16S rRNA gene and of the *ppk1* retrieved from the genomes are submitted to GenBank/ENA (in progress).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.03.053.

7. Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Ahn, J., Daidou, T., Tsuneda, S., Hirata, a, 2001a. Metabolic behavior of denitrifying phosphate-accumulating organisms under nitrate and nitrite electron acceptor conditions. J. Biosci. Bioeng. 92 (5), 442–446.

Ahn, J., Daidou, T., Tsuneda, S., Hirata, A., 2001b. Selection and dominance mechanisms of denitrifying phosphate-accumulating organisms in biological phosphate removal process. Biotechnol. Lett. 2005–2008.

Ahn, J., Daidou, T., Tsuneda, S., Hirata, A., 2002. Characterization of denitrifying phosphate-accumulating organisms cultivated under different electron acceptor conditions using polymerase chain reaction-denaturing gradient gel electrophoresis assay. Water Res. 36 (2), 403–412.

Albertsen, M., Hugenholtz, P., Skarshewski, A., Nielsen, K.L., Tyson, G.W., Nielsen, P.H., 2013. Genome sequences of rare, uncultured bacteria obtained by differential coverage binning of multiple metagenomes. Nat. Biotechnol. 31 (6), 533–538.

Albertsen, M., McIlroy, S.J., Stokholm-Bjerregaard, M., Karst, S.M., Nielsen, P.H., 2016. "Candidatus Propionivibrio aalborgensis": a novel glycogen accumulating organism abundant in full-scale enhanced biological phosphorus removal plants. Front. Microbiol. 7 (JUL), 1–17.

Amann, R.I., 1995. In situ identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In: Akkermans, A.D.L., Van Elsas, J.D., De Bruijn, F.J. (Eds.), Molecular Microbial Ecology Manual. Springer Netherlands, Dordrecht.

APHA, AWWA, WEF, 2005. Standard Methods for the Examination of Water and Wastewater, 22th ed. American Water Works Assn.

Arkin, A.P., Stevens, R.L., Cottingham, R.W., Maslov, S., Henry, C.S., Dehal, P., Ware, D., Perez, F., Harris, N.L., Canon, S., Sneddon, M.W., Henderson, M.L., Riehl, W.J., Gunter, D., Murphy-Olson, D., Chan, S., Kamimura, R.T., Brettin, T.S., Meyer, F., Chivian, D., Weston, D.J., Glass, E.M., Davison, B.H., Kumari, S., Allen, B.H., Baumohl, J., Best, A.A., Bowen, B., Brenner, S.E., Bun, C.C., Chandonia, J.-M., Chia, J.-M., Colasanti, R., Conrad, N., Davis, J.J., DeJongh, M., Devoid, S., Dietrich, E., Drake, M.M., Dubchak, I., Edirisinghe, J.N., Fang, G., Faria, J.P., Frybarger, P.M., Gerlach, W., Gerstein, M., Gurtowski, J., Haun, H.L., He, F., Jain, R., Joachimiak, M.P., Keegan, K.P., Kondo, S., Kumar, V., Land, M.L., Mills, M., Novichkov, P., Oh, T., Olsen, G.J., Olson, B., Parrello, B., Pasternak, S., Pearson, E., Poon, S.S., Price, G., Ramakrishnan, S., Ranjan, P., Ronald, P.C., Schatz, M.C., Seaver, S.M.D., Shukla, M., Sutormin, R.A., Syed, M.H., Thomason, J., Tintle, N.L., Wang, D., Xia, F., Yoo, H., Yoo, S., 2016. The DOE Systems Biology Knowledgebase (KBase), 096354. Org.

Bedzyk, L., Wang, T., Ye, R.W., 1999. The periplasmic nitrate reductase in Pseudomonas sp. strain G-179 catalyzes the first step of denitrification. J. Bacteriol. 181 (9), 2802–2806.

Benson, D.A., Karsch-Mizrachi, L., Lipman, D.J., Ostell, J., Wheeler, D.L., 2005. GenBank. Nucleic Acids Res. 33, D34–D38. Database issue.

Brdjanovic, D., Van Loosdrecht, M.C.M., Hooijmans, C.M., Alaerts, G.J., Heijnen, J.J., 1998. Minimal aerobic sludge retention time in biological phosphorus removal systems. Biotechnol. Bioeng. 60 (3), 326–332.

Brettin, T., Davis, J.J., Disz, T., Edwards, R.A., Gerdes, S., Olsen, G.J., Olson, R., Overbeek, R., Parrello, B., Pusch, G.D., Shukla, M., Thomason, J.A., Stevens, R., Vonstein, V., Wattam, A.R., Xia, F., 2015. RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. Sci. Rep. 5, 8365.

Camejo, P.Y., Owen, B.R., Martirano, J., Ma, J., Kapoor, V., Santodomingo, J., McMahon, K.D., Noguera, D.R., 2016. Candidatus Accumulibacter phosphatis clades enriched under cyclic anaerobic and microaerobic conditions simultaneously use different electron acceptors. Water Res. 102, 125–137.

Camejo, P.Y., Oyserman, B.O., McMahon, K.D., Noguera, D.R., 2018. Integrated 'omic' Analyses Provide Evidence that a Ca. Accumulibacter Phosphatis 2 Strain Performs Denitrification under Micro-aerobic Conditions (bioRxiv).

Carvalho, G., Lemos, P.C., Oehmen, A., Reis, M.A., 2007. Denitrifying phosphorus removal: linking the process performance with the microbial community structure. Water Res. 41 (19), 4383–4396.

Chen, I.-M.A., Markowitz, V.M., Chu, K., Palaniappan, K., Szeto, E., Pillay, M., Ratner, A., Huang, J., Andersen, E., Huntemann, M., Varghese, N., Hadjithomas, M., Tennessen, K., Nielsen, T., Ivanova, N.N., Kyrpides, N.C., 2017. IMG/M: integrated genome and metagenome comparative data analysis system. Nucleic Acids Res. 45 (D1), D507–D516.

Comeau, Y., Hall, K., Hancock, R., Oldham, W., 1986. Biochemical model for enhanced biological phosphorus removal. Water Res. 20 (12), 1511–1521.

Crocetti, G.R., Hugenholtz, P., Bond, P.L., Schuler, a, Keller, J., Jenkins, D., Blackall, L.L., 2000. Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. Appl. Environ. Microbiol. 66 (3), 1175–1182.

Ekama, G.A., Wentzel, M.C., 1999. Difficulties and developments in biological nutrient removal technology and modelling. Water Sci. Technol. 39 (6), 1–11.

Flowers, J.J., He, S., Carvalho, G., Peterson, S.B., Lopez, C., Yilmaz, S., Zilles, J.L., Morgenroth, E., Lemos, P.C., M, M. a, Crespo, M.T.B., Noguera, D.R., Mcmahon, K.D., 2008. Ecological differentiation of accumulibacter in EBPR reactors. Environment 2008 (17), 31–42.

Flowers, J.J., He, S., Malfatti, S., del Rio, T.G., Tringe, S.G., Hugenholtz, P., McMahon, K.D., 2013. Comparative genomics of two "Candidatus Accumulibacter" clades performing biological phosphorus removal. ISME J. 7 (12), 2301–2314.

Flowers, J.J., He, S., Yilmaz, S., Noguera, D.R., McMahon, K.D., 2009. Denitrification capabilities of two biological phosphorus removal sludges dominated by different "Candidatus Accumulibacter" clades. Environmental microbiology reports 1 (6), 583–588.

García Martín, H., Ivanova, N., Kunin, V., Warnecke, F., Barry, K.W., McHardy, A.C., Yeates, C., He, S., Salamov, A. a, Szeto, E., Dalin, E., Putnam, N.H., Shapiro, H.J., Pangilinan, J.L., Rigoutsos, I., Kyrpides, N.C., Blackall, L.L., McMahon, K.D., Hugenholtz, P., 2006. Metagenomic analysis of two enhanced biological phosphorus removal (EBPR) sludge communities. Nat. Biotechnol. 24 (10), 1263–1269.

He, S., Gu, A.Z., McMahon, K.D., 2005. The role of Rhodocyclus-like organisms in biological phosphorus removal: factors influencing population structure and activity. Proceedings of the Water Environment Federation 14, 1999–2011, 2005.

Henze, M., van Loosdrecht, M.C.M., Ekama, G.A., Brdjanovic, D., 2008. Biological Wastewater Treatment-Principles, Modelling and Design, first ed. IWA publishing.

Hesselsoe, M., Nielsen, J.L., Roslev, P., Nielsen, P.H., 2005. Isotope labeling and microautoradiography of active heterotrophic bacteria on the basis of assimilation of 14CO(2). Appl. Environ. Microbiol. 71 (2), 646–655.

Ichihashi, O., Satoh, H., Mino, T., 2006. Effect of soluble microbial products on microbial metabolisms related to nutrient removal. Water Res. 40 (8), 1627–1633.

Karst, S.M., Kirkegaard, R.H., Albertsen, M., 2016. Mmgenome: a Toolbox for Reproducible Genome Extraction from Metagenomes, 059121 bioRxiv.

Kerrn-Jespersen, J.P., Henze, M., 1993. Biological phosphorus uptake under anoxic and aerobic conditions. Water Res. 27 (4), 617–624.
Kong, Y., Ong, S., Ng, W., Liu, W., 2002. Diversity and distribution of a deeply branched novel proteobacterial group found in anaerobic–aerobic activated sludge processes. Environ. Microbiol. 4 (11), 753–757.
Kuba, T., Loosdrecht, M. Van, Heijnen, J., 1996a. Phosphorus and nitrogen removal with minimal COD requirement by integration of denitrifying dephosphatation and nitrification in a two-sludge system. Water Res. 1354 (96), 1702–1710.
Kuba, T., Van Loosdrecht, M.C.M., Brandse, F. a., Heijnen, J.J., 1997. Occurrence of denitrifying phosphorus removing bacteria in modified UCT-type wastewater treatment plants. Water Res. 31 (4), 777–786.
Kuba, T., Murnleitner, E., van Loosdrecht, M.C., Heijnen, J.J., 1996b. A metabolic model for biological phosphorus removal by denitrifying organisms. Biotechnol. Bioeng. 52 (6), 685–695.
Kuba, T., Smolders, G., van Loosdrecht, M.C.M., Heijnen, J.J., 1993. Biological phosphorus removal from wastewater by anaerobic-anoxic sequencing batch reactor. Water Sci. Technol. 27 (5), 241–252.
Kundu, P., Pramanik, A., Dasgupta, A., Mukherjee, S., Mukherjee, J., 2014. Simultaneous heterotrophic nitrification and aerobic denitrification by Chryseobacterium sp. R31 isolated from abattoir wastewater. BioMed Res. Int. 2014.
Lanham, A., Moita, R., Lemos, P., Reis, M., 2011. Long-term operation of a reactor enriched in Accumulibacter clade I DPAOs: performance with nitrate, nitrite and oxygen. Water Science & 352–359.
Lee, H., Yun, Z., 2014. Comparison of biochemical characteristics between PAO and DPAO sludges. J. Environ. Sci. 26 (6), 1340–1347.
Martiny, J.B.H., Jones, S.E., Lennon, J.T., Martiny, A.C., 2015. Microbiomes in light of traits: a phylogenetic perspective. Science 350 (6261) aac9323-aac9323.
McIlroy, S.J., Kristiansen, R., Albertsen, M., Karst, S.M., Rossetti, S., Nielsen, J.L., Tandoi, V., Seviour, R.J., Nielsen, P.H., 2013. Metabolic model for the filamentous 'Candidatus Microthrix parvicella' based on genomic and metagenomic analyses. ISME J. 7 (6), 1161–1172.
McIlroy, S.J., Saunders, A.M., Albertsen, M., Nierychlo, M., McIlroy, B., Hansen, A.A., Karst, S.M., Nielsen, J.L., Nielsen, P.H., 2015. MiDAS: the field guide to the microbes of activated sludge. Database 2015 (2), 1–8.
McMahon, K.D., Yilmaz, S., He, S., Gall, D.L., Jenkins, D., Keasling, J.D., 2007. Polyphosphate kinase genes from full-scale activated sludge plants. Appl. Microbiol. Biotechnol. 77 (1), 167–173.
Mergaert, J., Cnockaert, M.C., Swings, J., 2003. Thermomonas fusca sp. nov. and Thermomonas brevis sp. nov., two mesophilic species isolated from a denitrification reactor with poly(E-caprolactone) plastic granules as fixed bed, and emended description of the genus Thermomonas. Int. J. Syst. Evol. Microbiol. 53 (6), 1961–1966.
Meyer, R.L., Saunders, A.M., Blackall, L.L., 2006. Putative glycogen-accumulating organisms belonging to the Alphaproteobacteria identified through rRNA-based stable isotope probing. Microbiology 152 (2), 419–429.
Moreno-Vivián, C., Cabello, P., Blasco, R., Castillo, F., Cabello, N., Marti, M., Moreno-vivia, C., 1999. Prokaryotic nitrate Reduction : molecular properties and functional distinction among bacterial nitrate reductases. J. Bacteriol. 181 (21), 6573–6584.
Nielsen, P.H., Daims, H., Lemmer, H., Arslan-Alaton, I., Olmez-Hanci, T., 2009. FISH Handbook for Biological Wastewater Treatment. IWA Publishing.
Nielsen, P.H., Muro, M.A. de, Nielsen, J.L., 2000. Studies on the *in situ* physiology of *Thiothrix* spp. present in activated sludge. Environ. Microbiol. 2 (4), 389–398.
O'Leary, N.A., Wright, M.W., Brister, J.R., Ciufo, S., Haddad, D., McVeigh, R., Rajput, B., Robbertse, B., Smith-White, B., Ako-Adjei, D., Astashyn, A., Badretdin, A., Bao, Y., Blinkova, O., Brover, V., Chetvernin, V., Choi, J., Cox, E., Ermolaeva, O., Farrell, C.M., Goldfarb, T., Gupta, T., Haft, D., Hatcher, E., Hlavina, W., Joardar, V.S., Kodali, V.K., Li, W., Maglott, D., Masterson, P., McGarvey, K.M., Murphy, M.R., O'Neill, K., Pujar, S., Rangwala, S.H., Rausch, D., Riddick, L.D., Schoch, C., Shkeda, A., Storz, S.S., Sun, H., Thibaud-Nissen, F., Tolstoy, I., Tully, R.E., Vatsan, A.R., Wallin, C., Webb, D., Wu, W., Landrum, M.J., Kimchi, A., Tatusova, T., DiCuccio, M., Kitts, P., Murphy, T.D., Pruitt, K.D., 2016. Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. Nucleic Acids Res. 44 (D1), D733–D745.
Oehmen, A., Carvalho, G., Freitas, F., Reis, M.A., 2010a. Assessing the abundance and activity of denitrifying polyphosphate accumulating organisms through molecular and chemical techniques. Water Sci. Technol. 61 (8), 2061.
Oehmen, A., Carvalho, G., Lopez-Vazquez, C., van Loosdrecht, M.C., Reis, M.A.M., 2010b. Incorporating microbial ecology into the metabolic modelling of polyphosphate accumulating organisms and glycogen accumulating organisms. Water Res. 44 (17), 4992–5004.
Oehmen, A., Lopez-Vazquez, C.M., Carvalho, G., Reis, M.A.M., van Loosdrecht, M.C.M., 2010c. Modelling the population dynamics and metabolic diversity of organisms relevant in anaerobic/anoxic/aerobic enhanced biological phosphorus removal processes. Water Res. 44 (15), 4473–4486.
Oyserman, B.O., Moya, F., Lawson, C.E., Garcia, A.L., Vogt, M., Heffernen, M., Noguera, D.R., McMahon, K.D., 2016. Ancestral genome reconstruction identifies the evolutionary basis for trait acquisition in polyphosphate accumulating bacteria. ISME J. 10 (12), 2931–2945.
Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., Tyson, G.W., 2015. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res. 25 (7), 1043–1055.
Parks, D.H., Rinke, C., Chuvochina, M., Chaumeil, P.-A., Woodcroft, B.J., Evans, P.N., Hugenholtz, P., Tyson, G.W., 2017. Recovery of nearly 8,000 metagenome-assembled genomes substantially expands the tree of life. Nature Microbiology 2 (11), 1533–1542.
Peterson, S.B., Warnecke, F., Madejska, J., McMahon, K.D., Hugenholtz, P., 2008. Environmental distribution and population biology of Candidatus Accumulibacter, a primary agent of biological phosphorus removal. Environ. Microbiol. 10 (10), 2692–2703.
Ribera-Guardia, A., Marques, R., Arangio, C., Carvalheira, M., Oehmen, A., Pijuan, M., 2016. Distinctive denitrifying capabilities lead to differences in N2O production by denitrifying polyphosphate accumulating organisms and denitrifying glycogen accumulating organisms. Bioresour. Technol. 219, 106–113.
Rubio-Rincón, F.J., Lopez-Vazquez, C.M., Welles, L., van Loosdrecht, M.C.M., Brdjanovic, D., 2017a. Cooperation between Candidatus Competibacter and Candidatus Accumulibacter clade I, in denitrification and phosphate removal processes. Water Res. 120, 156–164.
Rubio-Rincón, F.J., Welles, L., Lopez-Vazquez, C.M., Nierychlo, M., Abbas, B., Geleijnse, M., Nielsen, P.H., van Loosdrecht, M.C.M., Brdjanovic, D., 2017b. Long-term effects of sulphide on the enhanced biological removal of phosphorus: the symbiotic role of Thiothrix caldifontis. Water Res. 116, 53–64.
Saad, S.A., Welles, L., Abbas, B., Lopez-vazquez, C.M., Loosdrecht, M.C.M., Brdjanovic, D., 2016. Denitrification of nitrate and nitrite by 'Candidatus Accumulibacter phosphatis' clade IC. Water Res. 105, 97–109.
Saunders, a M., Oehmen, a, Blackall, L.L., Yuan, Z., Keller, J., 2003. The effect of GAOs (glycogen accumulating organisms) on anaerobic carbon requirements in full-scale Australian EBPR (enhanced biological phosphorus removal) plants. Water Sci. Technol.: a journal of the International Association on Water Pollution Research 47 (11), 37–43.
Skennerton, C.T., Barr, J.J., Slater, F.R., Bond, P.L., Tyson, G.W., 2014. Expanding our view of genomic diversity in Candidatus Accumulibacter clades. Environ. Microbiol. 17, 1574–1585.
Smolders, G.J., van der Meij, J., van Loosdrecht, M.C., Heijnen, J.J., 1994a. Stoichiometric model of the aerobic metabolism of the biological phosphorus removal process. Biotechnol. Bioeng. 44, 837–848.
Smolders, G.J.F., van der Meij, J., van Loosdrecht, M.C.M., Heijnen, J.J., 1995. A structured metabolic model for anaerobic and aerobic stoichiometry and kinetics of the biological phosphorus removal process. Biotechnol. Bioeng. 47 (3), 277–287.
Smolders, G.J.F., Van Der Meij, J., Van Loosdrecht, M.C.M., Heijnen, J.J., 1994b. Model of the anaerobic metabolism of the biological phosphorus removal process: stoichiometry and pH influence. Biotechnol. Bioeng. 43, 461–470.
Stokholm-Bjerregaard, M., McIlroy, S.J., Nierychlo, M., Karst, S.M., Albertsen, M., Nielsen, P.H., 2017. A critical assessment of the microorganisms proposed to be important to enhanced biological phosphorus removal in full-scale wastewater treatment systems. Front. Microbiol. 8 (APR), 1–18.
Tu, Y., Schuler, A.J., 2013. Low acetate concentrations favor polyphosphate-accumulating organisms over glycogen-accumulating organisms in enhanced biological phosphorus removal from wastewater. Enviromental Science & Technology 47, 3816–3824.
Wang, Y., Qian, P.Y., 2009. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. PLoS One 4 (10).
Weissbrodt, D.G., Shani, N., Holliger, C., 2014. Linking Bacterial Population Dynamics and Nutrient Removal in the Granular Sludge Biofilm Ecosystem Engineered for Wastewater Treatment 1–17.
Wentzel, M., Lötter, L., Loewenthal, R., Marais, G., 1986. Metabolic behaviour of Acinetobacter spp. in enhanced biological phosphorus removal- a biochemical model. WaterSA 12 (4), 7700.
Williams, T.M., Unz, R.F., 1985. Filamentous sulfur bacteria of activated sludge: characterization of Thiothrix, Beggiatoa, and Eikelboom type 021N strains. Appl. Environ. Microbiol. 49 (4), 887–898.
Wright, E.S., et al., 2012. DECIPHER, a search-based approach to chimera identification for 16S rRNA sequences. Appl. Environ. Microbiol. https://doi.org/10.1128/AEM.06516-11.
Wong, M.T., Mino, T., Seviour, R.J., Onuki, M., Liu, W.T., 2005. In situ identification and characterization of the microbial community structure of full-scale enhanced biological phosphorous removal plants in Japan. Water Res. 39 (13), 2901–2914.
Wong, M.T., Tan, F.M., Ng, W.J., Liu, W.T., 2004. Identification and occurrence of tetrad-forming Alphaproteobacteria in anaerobic-aerobic activated sludge processes. Microbiology 150 (11), 3741–3748.
Zilles, J.L., Peccia, J., Kim, M., Hung, C., Noguera, D.R., 2002. Involvement of rhodocyclus -related organisms in phosphorus removal in full-scale wastewater treatment plants involvement of rhodocyclus -related organisms in phosphorus removal in full-scale wastewater treatment plants. Appl. Environ. Microbiol. 68 (6), 2763–2769.