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“*Candidatus* *Dechloromonas* phosphoritropha” and “*Ca.* *D.* phosphorivorans”, novel polyphosphate accumulating organisms abundant in wastewater treatment systems

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Members of the genus *Dechloromonas* are often abundant in enhanced biological phosphorus removal (EBPR) systems and are recognized putative polyphosphate accumulating organisms (PAOs), but their role in phosphate removal is still unclear. Here, we used 16S rRNA gene sequencing and fluorescence in situ hybridization (FISH) to investigate the abundance and distribution of *Dechloromonas* spp. in Danish and global wastewater treatment plants. The two most abundant species worldwide revealed in situ dynamics of important intracellular storage polymers, measured by FISH-Raman in activated sludge from four full-scale EBPR plants and from a lab-scale reactor fed with different substrates. Moreover, seven distinct *Dechloromonas* species were determined from a set of ten high-quality metagenome-assembled genomes (MAGs) from Danish EBPR plants, each encoding the potential for polyphosphate (poly-P), glycogen, and polyhydroxyalkanoates (PHA) accumulation. The two species exhibited an in situ phenotype in complete accordance with the metabolic information retrieved by the MAGs, with dynamic levels of poly-P, glycogen, and PHA during feast-famine anaerobic-aerobic cycling, legitimately placing these microorganisms among the important PAOs. They are potentially involved in denitrification showing niche partitioning within the genus and with other important PAOs. As no isolates are available for the two species, we propose the names *Candidatus* *Dechloromonas* phosphoritropha and *Candidatus* *Dechloromonas* phosphorivorans.

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INTRODUCTION

The enhanced biological phosphorus removal (EBPR) process is becoming globally popular in wastewater treatment. In EBPR, phosphorus (P) removal can be achieved without chemical addition and the P-enriched biomass can be used for P recovery or as agricultural fertilizer [1]. The EBPR process depends on the ability of polyphosphate accumulating organisms (PAOs) to store polyphosphate (poly-P) as an intracellular storage compound when exposed to alternating carbon-rich anaerobic (feast) and carbon-deficient aerobic (famine) conditions. The metabolism of well-described PAOs, such as *Ca.* *Accumulibacter* and *Tetrasphaera*, has been extensively investigated in lab-scale enrichments [2–6] and recently verified in full-scale EBPR plants [7]. While *Ca.* *Accumulibacter* cycles poly-P during dynamic feast-famine conditions with PHA and glycogen storage, *Tetrasphaera* spp. exhibit a different behavior, as no PHA or glycogen have been identified in situ [7].

Several putative PAOs have been identified through the detection of intracellular poly-P [8–11], including the genus *Dechloromonas*, first described in EBPR plants by Göel et al. [12]. Over recent years, *Dechloromonas* has been consistently found in high abundances in many EBPR plants in Japan [13], China [14], Denmark [15], and worldwide [1]. *Dechloromonas* spp. were originally isolated as aromatic compound-degrading bacteria and facultative anaerobic nitrate-reducing bacteria [16, 17]. Since

then, several *Dechloromonas* populations have been shown to accumulate poly-P and PHA in situ and/or in axenic cultures [11, 13, 18], and several studies have also suggested their potential role as PAOs [18–21]. Furthermore, analysis of the *Dechloromonas aromatica* strain RCB genome revealed the genes required for poly-P accumulation, including polyphosphate kinase, and exopolyphosphatase [22]. *Dechloromonas* spp. likely also have important nitrogen cycling roles in full-scale EBPR plants, as uncultured *Dechloromonas* has the ability to respire with NO_3^- and/or NO_2^- in situ [11, 23, 24], and are suggested to be denitrifying PAOs [20, 21]. Other uncultured members of the genus *Dechloromonas* have also shown the potential for a glycogen accumulating organism (GAO) phenotype [23, 25], long considered competitors of PAOs. The genus encompasses a broad range of metabolisms integral to the wastewater treatment ecosystem, exposing the need for a deeper characterization of these organisms.

Recent advances in high-throughput sequencing methods have enabled the creation of comprehensive ecosystem-specific full-length 16S rRNA gene databases [26]. These databases can be used for detailed phylogenetic analyses, facilitating the design of specific fluorescence in situ hybridization (FISH) probes and re-evaluation of existing ones for the identification and characterization of key species [27]. Moreover, it is now possible to assemble high-quality, near-complete genomes from deeply sequenced

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metagenomes, revealing the metabolic potential of these organisms [27–29]. However, in situ validation of the genomic potential is critical and required to confirm their predicted role in the system. Recently, Raman microspectroscopy combined with FISH was used to identify and quantify intracellular storage compounds in *Ca. Accumulibacter* and *Tetrasphaera* [7]. While these lineages accounted for 24–70% of total P removal [7], undescribed PAOs likely remove a large portion of P. Identifying these novel PAOs and obtaining more insights into their physiology is essential to improve the management and efficacy of resource recovery in EBPR treatment plants.

In this study, we present and characterize novel uncultured species belonging to the genus *Dechloromonas* and show that they are actively involved in P removal in full-scale EBPR plants. We confirmed the presence and dynamics of intracellular poly-P and other storage compounds in the novel species using the new Raman-based approach on activated sludge from full-scale WWTPs. The effect of several individual substrates on P-release was tested using sludge from a lab-scale sequencing batch reactor (SBR). Moreover, genes suggesting the potential for PAO metabolism were identified in ten high-quality *Dechloromonas* metagenome-assembled genomes (MAGs) from Danish EBPR plants. The combination of genome-based investigations and in situ analyses provides the first detailed insight into the ecophysiology of these abundant, widespread, and novel PAOs.

MATERIALS AND METHODS

Full-scale activated sludge batch experiments for P cycling

Batch experiments were conducted on fresh activated sludge to analyze the poly-P-content per cell of FISH-identified *Dechloromonas* cells under anaerobic and aerobic conditions. Fresh samples were collected from four large full-scale Danish WWTPs (Lynetten, Ejby Mølle, Viby, and Aalborg West) and aerated for 30 min to exhaust most intracellular carbon source reserves. After aeration, sludge was transferred to serum bottles and sealed with a butyl septum and aluminum cap. A substrate solution containing acetate, glucose, and casamino acids was added, with a final concentration of the three components of 500, 250, and 250 mg L⁻¹, respectively. Ultrapure nitrogen was used to flush the headspace in each bottle to ensure anaerobic conditions. The serum bottles were kept at room temperature (~22 °C) with shaking for 3 h. Samples for ortho-P analysis were taken every 20 min for the first hour of the experiment, and every 30 min during the remaining 2 h. Initial samples (0 h) and at the end of the experiment (3 h) were fixed for FISH and Raman analyses (see below).

P cycling experiments in SBR reactor

A sequencing batch reactor (SBR) with 5 L working volume was operated with 8 h cycles based on Marques et al. [2]. Each cycle included a 4 h anaerobic phase, a 1 h settling/decant phase, and a 3 h aerobic phase. The SBR was operated with a hydraulic retention time of 13 h, a solid retention time of 15 d, organic loading rate of 0.6 g COD L⁻¹ d⁻¹ and phosphate loading rate of 0.2 g P L⁻¹ d⁻¹. To maintain anaerobic conditions, nitrogen was bubbled continuously at a flowrate of 4 L min⁻¹. The aerobic phase was controlled at oxygen saturation point (~9 mg L⁻¹) by bubbling compressed air at a flowrate of 4 L min⁻¹. Temperature was controlled at 19 ± 1 °C. pH was controlled at 7.5 by dosing HCl (0.5 M) or NaOH (0.4 M). During aerobic and anaerobic phase, the SBR was completely stirred using an overhead stirrer set at 400 rpm. The SBR was fed with a synthetic medium containing casein hydrolysate (4.4 g L⁻¹; 5.5 g COD L⁻¹) (Sigma-Aldrich, USA) during the anaerobic phase in six pulses every 30 min (30 mL/pulse). After the decanting phase, during the first 5 min of the aerobic phase, the SBR was fed (3.1 L) with mineral medium containing per litre: 317 mg K₂HPO₄, 190 mg KH₂PO₄, 123 mg NH₄Cl, 198 mg MgSO₄·7H₂O, 92 mg CaCl₂·2H₂O, 1.7 mg N-Allylthiourea, 6.7 mg ethylenediaminetetraacetic, and 0.7 mL of micronutrient solution. The micronutrient solution was based on Smolders et al. [30] and contained per litre: 1.5 g FeCl₃·6H₂O, 0.15 g H₃BO₃, 0.03 g CuSO₄·5H₂O, 0.18 g KI, 0.12 g MnCl₂·4H₂O, 0.06 g Na₂MoO₄·2H₂O, 0.12 g ZnSO₄·7H₂O, and 0.15 g CoCl₂·6H₂O.

Batch tests were performed to evaluate the phosphate release from different carbon sources. Biomass from the SBR was harvested after the aerobic phase (i.e., after phosphate uptake) and directly used for the batch

test without amendments. 25 mL of biomass was dispensed in 30 mL serum bottles and sealed with butyl septa and aluminum caps. The serum bottles were flushed with nitrogen (4 L min⁻¹) for 15 min to ensure anaerobic conditions. Five carbon sources (casein hydrolysate, acetate, glutamate, glycine, or glucose) were tested individually. The cycle started with the addition of 200 µL of stock solution to an initial cycle concentration of 200 mg COD L⁻¹. The anaerobic cycle lasted for 4 h. The bottles were mixed by manual swirling before each sampling event. Blank controls without carbon source addition were used to evaluate the endogenous phosphate release. All the experiments were performed in duplicate. Samples from the start (0 h) and the end of the experiment (4 h) were fixed for FISH and Raman analyses.

Chemical analyses

The ortho-P release into the liquid phase was analyzed in accordance with ISO 6878:2004 using the ammonium molybdate-based colorimetric method. Samples for total P measurement were taken at the start of the P-release experiment. Sludge collected from the bottles was homogenized and stored at -20 °C until further analysis. 67% nitric acid was used to dissolve 0.5 mL of each sludge sample and the samples were microwave heated according to U.S. EPA (2007). The total amount of P in the samples was analyzed by Inductively Coupled Plasma Optical Emission Spectrometry in accordance with Jørgensen et al. [31].

Sampling and fixation

Biomass samples from SBR batch reactors and full-scale activated sludge were either stored at -80 °C for sequencing workflows or fixed for FISH with 96% ethanol or 4% PFA, as previously described [32] and stored at -20 °C until analysis.

DNA extraction

DNA extraction of activated sludge samples from the MiDAS collection [33] was performed as described by Stokholm-Bjerregaard et al. [15]. Briefly, DNA was extracted using the FastDNA spin kit for soil (MP Biomedicals), following the manufacturer's indications, but with an increase of the bead beating to 6 m/s for 4 × 40 s, using a FastPrep FP120 (MP Biomedicals).

Community profiling using 16S rRNA gene amplicon sequencing

Amplicon sequence variant (ASV) analysis are described in Dueholm et al. [26] and Nierychlo et al. [34]. Community profiling of Danish WWTPs was performed using 16S rRNA gene amplicon sequencing data collected from 2006 to 2018 from 27 different Danish WWTPs, as part of the MiDAS project [33]. Data were analyzed using R (version 3.5.2) [35], RStudio software [36] and visualized using ampvis2 [37] and ggplot2 [38].

Phylogenetic analysis and FISH probes evaluation

Phylogenetic analysis of 16S rRNA gene sequences and evaluation of existing FISH probes for the genus *Dechloromonas* were performed using the ARB software v.6.0.6 [39]. A phylogenetic tree was calculated based on the full-length 16S rRNA gene sequences retrieved from the MiDAS 3.7 database [26] and Silva 138 SSURef Nr99 [40], using the maximum likelihood method and a 1000-replicate bootstrap analysis. Similarity of the full-length 16S rRNA gene sequences retrieved from the MAGs (see below) and already published sequences was evaluated using blastn [41]. Coverage and specificity of the existing probes designed in our previous studies Bet135 [11] and Dech443 [23] were re-evaluated and validated in silico with the MathFISH software for hybridization efficiencies of target and potentially weak non-target matches [42]. All probes were purchased from Sigma-Aldrich (Denmark) or Biomers (Germany), labeled with 6-carboxyfluorescein (6-Fam), indocarbocyanine (Cy3), or indodicarbocyanine (Cy5) fluorochromes.

Fluorescence in situ hybridization (FISH) and quantitative FISH (qFISH)

FISH was performed on full-scale activated sludge samples as well as sludge from SBR reactor as described by Daims et al. [43]. Optimal formamide concentration and use of competitors or helper probes was applied as recommended by the authors for each probe [11, 23]. Quantitative FISH (qFISH) biovolume fractions of individual *Dechloromonas* spp. were calculated as a percentage area of the total biovolume, hybridizing the EUBmix probes,

that also hybridizes with the specific probe. qFISH analyses were based on 30 fields of view taken at 630× magnification using the Daime image analysis software [44]. Microscopic analysis was performed with a white light laser confocal microscope (Leica TCS SP8 X).

Raman microspectroscopy and absolute quantification of intracellular storage polymers

Raman microspectroscopy was applied in combination with FISH as previously described [7]. Briefly, FISH was conducted on optically polished CaF₂ Raman slides (Crystran, UK). *Dechloromonas*-specific (Cy3) probes [11, 23] were used to locate the target cells for Raman analysis. After bleaching, 100 spectra from single-cells were obtained using a Horiba LabRam HR 800 Evolution (Jobin Yvon—France) equipped with a Torus MPC 3000 (UK) 532 nm 341 mW solid-state semiconductor laser. The Raman spectrometer was calibrated prior to obtaining all measurements to the first-order Raman signal of Silicon, occurring at 520.7 cm⁻¹. The incident laser power density on the sample was attenuated down to 2.1 mW/μm² using a set of neutral density filters. The Raman system is equipped with an in-built Olympus (model BX-41) fluorescence microscope. A 50X, 0.75 numerical aperture dry objective (Olympus M Plan Achromat- Japan), with a working distance of 0.38 mm, was used throughout the work. A diffraction grating of 600 mm/groove was used and the Raman spectra collected spanned the wavenumber region of 200–1800 cm⁻¹. The slit width of the Raman spectrometer and the confocal pinhole diameter were set respectively to 100 μm and 72 μm. Raman spectrometer operation and subsequent processing of spectra were conducted using LabSpec version 6.4 software (Horiba Scientific, France). All spectra were baseline corrected using a 6th order polynomial fit. Absolute quantification of intracellular storage polymers was carried out as described by Fernando et al. [7]. Briefly, the method assumes that the intensity of the Raman signal is directly dependent on the amount of the analyte in a determined area. The average amount of storage polymers per cell was calculated as a factor of a constant determined during calibration [7], the average charge-coupled device counts determined during the experiment with the previous settings, and the average area of cells measured by image analysis.

Identification, annotation, and metabolic reconstruction of *Dechloromonas* spp

A set of 1083 high-quality MAGs were searched for *Dechloromonas* spp. based on taxonomic classification using GTDB-Tk [45] v1.0.2 [27]. Distinct species were determined using dRep [46] v2.3.2 and 95% average nucleotide identity (ANI) genome clustering. ANI based on blast (ANiB) was conducted on the MAGs and genomes using pyani v0.2.10 and the arguments “average_nucleotide_identity.py -m ANiB” [47]. The resulting ANiB_percentage_identity.tab and ANiB_alignment_coverage.tab tables were processed in R v4.0.3 [35] to make heatmaps using the libraries gplots, RColorBrewer and reshape2. The MAGs were annotated with KEGG orthology [48] numbers using EnrichM (<https://github.com/geronimp/enrichM>) v0.5.0 against the uniref100 database, with pathways identified based on genes and modules defined by KEGG. Pathways were considered complete if 100% of the genes in the KEGG module were identified. For *Ca. D. phosphorivorans*, the species was presented as encoding the pathway if at least one of the three MAGs encoded the full pathway, see STable1 and SDataFile1 for details. The 16S, 23S, 5S rRNA, and tRNA genes were identified using Prokka [49] v1.14 and Infernal [50] v1.1.2. Fxtract (<https://github.com/ctSkennerton/fxtract>) v2.3 extracted the 16S rRNA gene sequences for placement in the phylogenetic 16S rRNA gene tree. The phylogenetic genome tree of *Dechloromonas* was created using GTDB-Tk v1.0.2 and the concatenated alignment of 120 single copy proteins. The concatenated protein alignments created by GTDB-Tk were used as input into IQ-TREE v2.0 [51] using the WAG + G model and 100 bootstrap iterations, with 5 *Ca. Accumulibacter* genomes used as an outgroup.

RESULTS AND DISCUSSION

Diversity and distribution of *Dechloromonas* spp. in full-scale wastewater treatment plants

Phylogenetic analysis based on full-length high quality 16S rRNA gene sequences retrieved from 24 Danish WWTPs [26] revealed the presence of 12 *Dechloromonas* species (Fig. 1). Some of them were well-known from axenic cultures, such as *Dechloromonas denitrificans* [17], *Dechloromonas hortensis* [52],

and *Dechloromonas agitata* [16], while the majority were novel and undescribed.

A 12 year survey of 20 Danish WWTPs using data from the MiDAS project [34] showed that *Dechloromonas* was the second most abundant genus among well-recognized and putative PAOs (Fig. 2A). This high abundance is in accordance with our recent study of PAOs and putative PAOs across the world in 101 EBPR plants [1]. The genus constituted on average 2.6% of the total reads across all Danish plants with abundances reaching 20% in some samples. The two most abundant species (Fig. 2B), for which we propose the names *Candidatus Dechloromonas phosphoritropha* and *Candidatus Dechloromonas phosphorivorans*, were targeted by the existing FISH probes Bet135 [11] and Dech443 [23], respectively, with good specificity and coverage (Fig. 1). ASV85 was classified to *Ca. D. phosphorivorans* with the aid of 16S rRNA genes retrieved from the MAGs (see below), indicating its potentially higher abundance in some WWTPs. The 16S rRNA gene sequences retrieved from the MAGs were also used for comparison with full-length 16S rRNA gene sequences published in previous studies [11, 13, 18, 53] and associated to the genus *Dechloromonas*. In all the cited studies, ten 16S rRNA gene sequences (AF450470, AF450475, AF447801, EU850366, EU850385, LC145227, LC145228, LC145284, DQ640664, DQ640700) had a similarity >95% to the full-length 16S rRNA gene sequences of the two novel species, and therefore most likely belonging to the same genus. Application of the probes to fixed activated sludge biomass showed rod-shaped cells (1.4 × 1.2 μm and 1.2 × 0.8 μm, respectively), often arranged in spherical microcolonies (Fig. 3). Quantitative FISH analyses confirmed the relative abundances obtained by 16S rRNA gene amplicon sequencing of selected samples (Table 1), showing little differences, most likely due to the presence of several 16S rRNA gene copies in some of the species [22].

Ca. D. phosphorivorans and *Ca. D. phosphoritropha* were also abundant according to our recent MiDAS global WWTP survey [54] of 480 plants (Suppl. Fig. 1). As expected, the highest abundance was observed in WWTP performing the EBPR process (Suppl. Fig. 1A), but the two species were also present in plants performing only nitrification and denitrification, confirming their potential involvement in nitrogen removal in full-scale plants. *Ca. D. phosphorivorans* was dominant in the global EBPR plants (Suppl. Fig. 1B), reaching the highest average read abundances in Switzerland (6.5%), United Kingdom (4.6%), and Czech Republic (4.2%). When present, *Ca. D. phosphoritropha* was generally observed at lower abundances, with the highest mean read abundance in Denmark and Poland (0.3%).

In situ quantification of storage polymers in *Dechloromonas* spp. in full-scale EBPR plants

In order to identify, quantify, and explore the dynamics of intracellular storage polymers, such as poly-P, PHA, and glycogen, in the *Dechloromonas* spp., we performed anaerobic-aerobic P-cycling experiments with fresh activated sludge from four full-scale EBPR plants. Since the specific carbon preferences for *Dechloromonas* were not known, a mixture of acetate, glucose, and casamino acids was used as carbon source during the anaerobic phase to accommodate a large range of potential requirements. Pure culture and in situ studies of *Dechloromonas* species have shown they can grow on volatile fatty acids and casamino acids [17], which is very similar to the well-known PAO *Ca. Accumulibacter* as substrates for P release under anaerobic conditions, but partly different from the abundant PAO *Tetrasphaera*, which can use sugars and amino acids [2, 55, 56]. The addition of substrate stimulated an anaerobic release of ortho-P during the 3 h anaerobic phase (Suppl. Fig. 2), as is typically seen for activated sludge from EBPR plants.

In situ identification and quantification of intracellular storage products using FISH-Raman microspectroscopy was performed on

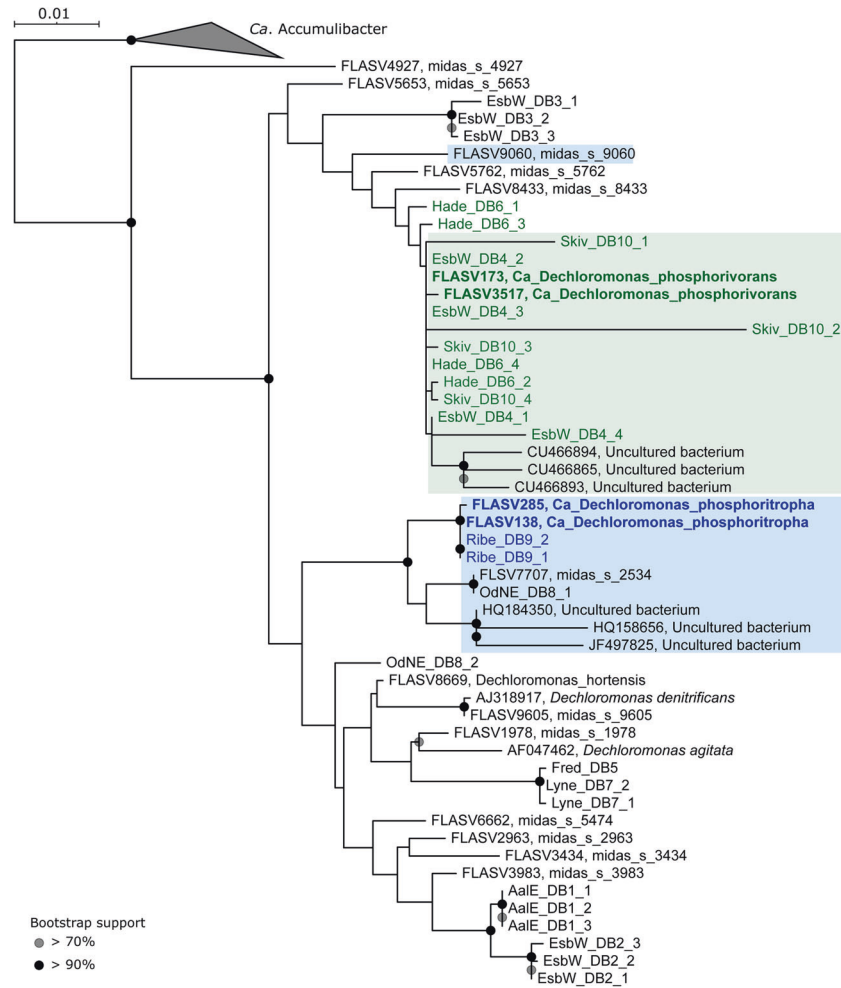


Fig. 1 Phylogenetic tree of the genus *Dechloromonas*. Maximum-likelihood (PhyML) 16S rRNA gene phylogenetic tree of the genus *Dechloromonas* including full-length sequences from MiDAS3 database (FLASVxxx), MAG (xxxx_DBx_x) and Silva138 SSURef Nr99 (indicated by sequence accession numbers). Colors indicate the two most abundant *Dechloromonas* species in activated sludge (*Ca. D. phosphorivorans* in green, and *Ca. D. phosphoritropha* in blue, shown in bold) and their related MAG 16S rRNA gene sequences. Coverage of FISH probes Dech443 and BET135 is shown as green- and blue-shaded area, respectively. Bootstrap values from 1000 re-samplings are indicated for branches with >70% (gray circle), and >90% (black) support. The scale bar represents substitutions per nucleotide base.

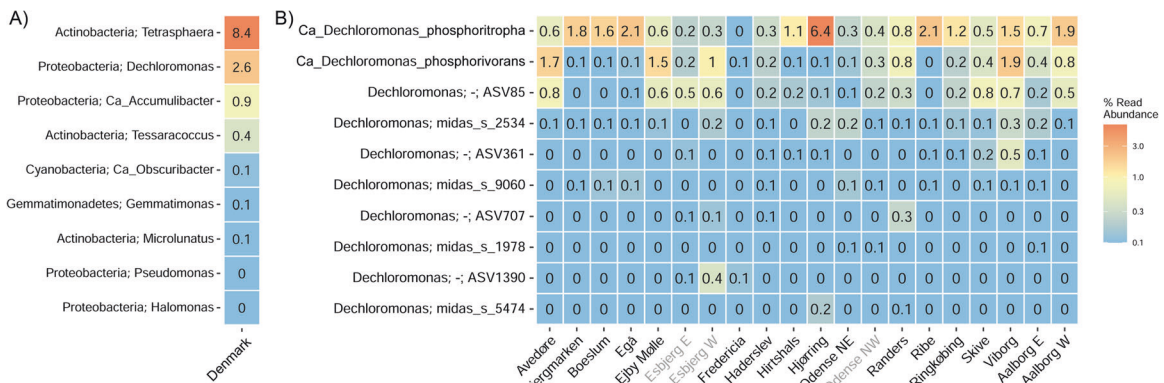


Fig. 2 Abundance and distribution of *Dechloromonas* spp. in Danish full-scale WWTPs. **A** Abundance of known or putative PAOs in Denmark (phylum and genus name is given). **B** Abundance of *Dechloromonas* species in Danish WWTPs. ASV numbers represent taxa that could not be confidently classified at species level, as indicated by "-". ASV85 corresponds to *Ca. D. phosphorivorans*. Names of non-EBPR plants are shown in gray.

Ca. D. phosphoritropha in all four WWTPs. For *Ca. D. phosphorivorans*, lower abundances in the other three WWTPs limited FISH-Raman quantification to Viby WWTP. In all cases both species contained all three storage polymers (poly-P, glycogen, and PHA),

and they both exhibited dynamic cycling of the polymers. In accordance with the accepted metabolic model for conventional PAOs as exemplified by *Ca. Accumulibacter* [55], the amount of intracellular poly-P was lowest after the anaerobic phase and

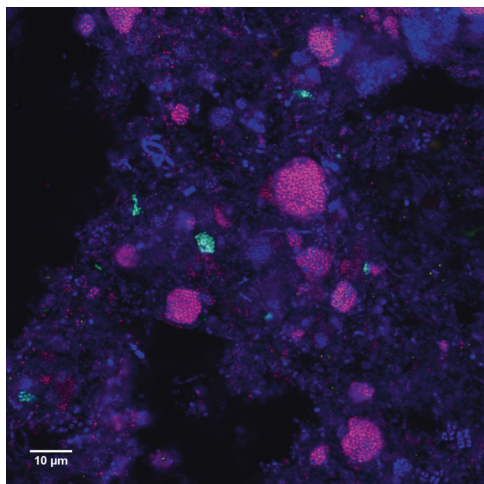


Fig. 3 Composite FISH micrographs of the two most abundant *Dechloromonas* species in Danish WWTPs. Specific probes Bet135 (Cy3-label, red) and Dech443 (FLUOS, green) are targeting the *Ca. D. phosphoritropha* and *Ca. D. phosphorivorans*, respectively. All bacteria are targeted by EUBmix probe (Cy5-label, blue) and overlap appears in magenta (*Ca. D. phosphoritropha* - Bet135) or cyan (*Ca. D. phosphorivorans* - Dech443).

Table 1. Abundance estimation: 16S rRNA amplicon sequencing and qFISH (percentage of total) of full-scale activated sludge samples.

WWTP	Sample date	Abundance (%)	
		Sequencing ^a	qFISH
Candidatus <i>Dechloromonas phosphoritropha</i>			
Aalborg West	August 2013	9.9	6.6 ± 2
Damhusaaen	August 2015	2.6	2.4 ± 1.7
Egaa	November 2011	7.4	6.5 ± 2.4
Hjørring	November 2009	17.4	10.9 ± 3.9
Ribe	August 2014	8.1	5.8 ± 2.1
Candidatus <i>Dechloromonas phosphorivorans</i>			
Aalborg East	February 2006	3.7	<1
Damhusaaen	February 2015	0.4	<1
Hjørring	August 2013	2.2	<1

^aTaken from the 16S rRNA gene amplicon sequencing MiDAS survey of Danish WWTPs [34].

highest after the aerobic phase, with some variations between plants (Fig. 4A, Table 2). The highest values measured in situ for *Ca. D. phosphoritropha* was around 6.5×10^{-14} g P cell⁻¹ while *Ca. D. phosphorivorans* contained 6.3×10^{-14} g P cell⁻¹ (Table 2). These values are slightly lower than for *Ca. Accumulibacter* ($5\text{--}10 \times 10^{-14}$ g P cell⁻¹) but higher than for *Tetrasphaera* ($1\text{--}2 \times 10^{-14}$ g P cell⁻¹), where their cell size seemed to correlate with poly-P content, with average *Dechloromonas* biovolume ($2.35 \mu\text{m}^3$) lower than *Ca. Accumulibacter* ($3.14 \mu\text{m}^3$), but higher than *Tetrasphaera* ($0.45 \mu\text{m}^3$) [57].

The PHA content was also dynamic, with a build up during the anaerobic phase, reaching a similar level in all plants of approx. 21.2×10^{-14} g C cell⁻¹ (Fig. 4B, Table 2). Similarly, the glycogen level was reduced from $\sim 7.2 \times 10^{-14}$ in the aerobic state to $\sim 1.3 \times 10^{-14}$ g C cell⁻¹ in the end of anaerobic phase (Fig. 4C, Table 2). These conversions likely reflect the hydrolysis of glycogen used as an energy source for PHA formation under anaerobic conditions and its replenishment in the aerobic phase. The levels of PHA and

glycogen were slightly lower than values recorded for *Ca. Accumulibacter* ($30\text{--}39 \times 10^{-14}$ g C cell⁻¹ and $31\text{--}43 \times 10^{-14}$ g C cell⁻¹ for PHA and glycogen, respectively) most likely due to its smaller size [57]. In both *Dechloromonas* species, the C/P and C/C molar ratio were within the range of 0.3–0.4 that are reported for PAOs in previous studies [58, 59]. Our findings show that both *Dechloromonas* species express a phenotype very similar to the canonical PAO metabolism as described by *Ca. Accumulibacter*, with dynamic levels of poly-P, PHA, and glycogen under feast-famine conditions.

Dynamics of storage compounds in *Dechloromonas* spp. fed with different substrates

To further investigate the physiology and the in situ dynamics of intracellular storage compounds in probe-defined *Ca. D. phosphoritropha* and *Ca. D. phosphorivorans* cells, feast-famine cycling experiments were carried out with biomass from a lab-scale SBR reactor. The reactor was conducted to enrich for novel PAOs (anaerobic/aerobic cycles with casein hydrolysate organic substrate added during the anaerobic phase) and contained 1–2% *Dechloromonas* spp. as quantified by qFISH. Different carbon sources (casein hydrolysate, acetate, glutamate, glycine, or glucose) were tested to investigate the potential to induce anaerobic poly-P hydrolysis and P release, indicating uptake of that particular substrate and conversion to PHA (Table 2). Addition of various substrates under anaerobic conditions was followed by a release of P and a subsequent uptake of P during aerobic conditions (SFigures 3–4). Both *Dechloromonas* species had in the end of the aerobic phase intracellular poly-P content in the range of $6.7\text{--}7.0 \times 10^{-14}$ g P cell⁻¹ (Fig. 4D, Table 2), which is the same range as observed in the four full-scale EBPR plants tested. All substrates induced anaerobic degradation of intracellular poly-P with casein hydrolysate and acetate giving the highest release for both *Dechloromonas* species, and the two specific amino acids glycine and glutamate the lowest (Fig. 4D, Table 2 and Suppl. Fig. 3). Glucose induced P-release from the biomass and also intracellular dynamics of poly-P, PHA, and glycogen. However, glucose uptake is not observed in *Dechloromonas* pure cultures [17] and is also not encoded by MAGs representing the two species (see below). Thus, the observed glucose pattern may be due to uptake and metabolism by other members of the community, which then provide substrates that can be used by the *Dechloromonas* species. Intracellular cycling of PHA and glycogen occurred in accordance with the results from the full-scale biomass investigation (Fig. 4E, F, Table 2), and with similar C/P and C/C ratios of 0.3–0.4.

Interestingly, these substrate uptake patterns of *Dechloromonas* species, also observed by Qiu et al. [56], indicate a niche partially overlapping with the two other common PAOs, *Ca. Accumulibacter* with acetate as primary substrate [55, 60, 61], and *Tetrasphaera* with amino acids [2, 62], although some *Ca. Accumulibacter* may also use amino acids [55, 60, 61].

Metabolic potential of uncultivated *Dechloromonas* spp. in EBPR systems

In order to look further into the potential physiology of the *Dechloromonas* genus in activated sludge, ten *Dechloromonas* MAGs obtained by Singleton et al. [27] from Danish EBPR plants were annotated and the potential for particular pathways important for the PAO metabolism were identified (Figs. 5, 6). The MAGs represented 7 distinct species at the 95% ANI cutoff [63]. *Ca. D. phosphoritropha* was represented by Ribe_DB9, and clustered at the species level (98% ANI; Suppl. Fig. 5) with the MAG *Dechloromonas* sp002396525, which is a lower quality MAG lacking rRNA genes assembled from a Danish WWTP short-read metagenome (NCBI accession ERX656399) [29]. *Ca. D. phosphorivorans* was represented by the MAGs Skiv_DB10, EsbW_DB4 and Hade_DB6, of which Skiv_DB10 was the highest quality and

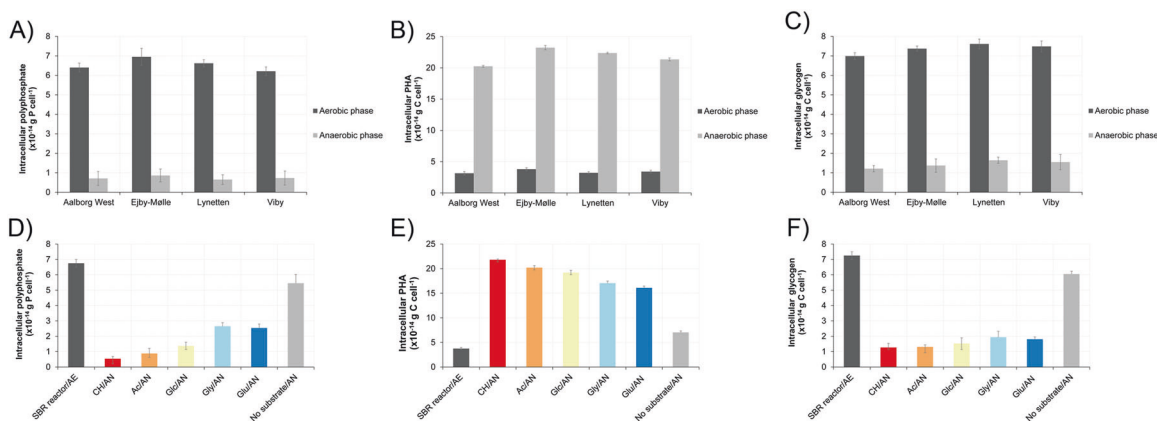


Fig. 4 Dynamics of storage polymers in *Ca. D. phosphoritropha*. The content was measured by Raman microspectroscopy in FISH-defined cells by end of aerobic and anaerobic phases. **A** Poly-P levels in *Ca. D. phosphoritropha* in different full-scale EBPR plants. **B** PHA dynamics in *Ca. D. phosphoritropha* in different full-scale EBPR plants. **C** Glycogen dynamics in *Ca. D. phosphoritropha* in different full-scale EBPR plants. **D** Poly-P dynamics in *Ca. D. phosphoritropha* in SBR reactor fed with different substrates. **E** PHA dynamics in *Ca. D. phosphoritropha* in SBR reactor fed with different substrates. **F** Glycogen dynamics in *Ca. D. phosphoritropha* in SBR reactor fed with different substrates. SBR reactor is the initial sample collected at the end of the aerobic phase. CH = casein hydrolysate, Ac = acetate, Glc = glucose, Gly = glycine, Glu = glutamate. The sample indicated as “no substrate” has not been amended with additional substrate during anaerobic phase (control). AE = aerobic. AN = anaerobic.

Table 2. Summary of all storage compounds investigated in FISH-defined *Dechloromonas* cells in SBR reactor and EBPR plants.

Sample type	Aerobic phase			Anaerobic phase		
	Poly-P ($\times 10^{-14}$ gP cell $^{-1}$)	PHA ($\times 10^{-14}$ gC cell $^{-1}$)	Glycogen ($\times 10^{-14}$ gC cell $^{-1}$)	Poly-P ($\times 10^{-14}$ gP cell $^{-1}$)	PHA ($\times 10^{-14}$ gC cell $^{-1}$)	Glycogen ($\times 10^{-14}$ gC cell $^{-1}$)
<i>Candidatus Dechloromonas phosphoritropha</i>						
EBPR plant Aalborg West ^a	6.40 ± 0.23	3.14 ± 0.28	6.99 ± 0.17	0.71 ± 0.36	20.2 ± 0.14	1.21 ± 0.16
EBPR plant Ejby-Mølle ^a	6.95 ± 0.44	3.79 ± 0.24	7.37 ± 0.13	0.86 ± 0.33	23.2 ± 0.36	1.37 ± 0.34
EBPR plant Lynetten ^a	6.52 ± 0.18	3.22 ± 0.17	7.61 ± 0.25	0.65 ± 0.25	22.3 ± 0.13	1.64 ± 0.16
EBPR plant Viby ^a	6.21 ± 0.22	3.41 ± 0.23	7.48 ± 0.28	0.73 ± 0.36	21.3 ± 0.23	1.55 ± 0.39
SBR reactor/casein hydrolysate	6.75 ± 0.25	3.74 ± 0.36	7.25 ± 0.13	0.54 ± 0.14	21.8 ± 0.17	1.27 ± 0.27
SBR reactor/acetate				0.88 ± 0.33	20.2 ± 0.41	1.31 ± 0.13
SBR reactor/glucose				1.37 ± 0.25	19.2 ± 0.47	1.53 ± 0.37
SBR reactor/glycine				2.65 ± 0.23	17.0 ± 0.43	1.94 ± 0.39
SBR reactor/glutamate				2.54 ± 0.26	16.1 ± 0.36	1.81 ± 0.15
SBR reactor/no substrates				5.45 ± 0.58	7.03 ± 0.32	6.05 ± 0.18
<i>Candidatus Dechloromonas phosphorivorans</i>						
EBPR plant Viby ^a	6.30 ± 0.27	3.52 ± 0.15	7.01 ± 0.38	0.99 ± 0.38	21.2 ± 0.28	1.33 ± 0.36
SBR reactor/casein hydrolysate	6.61 ± 0.23	3.63 ± 0.24	7.16 ± 0.24	0.61 ± 0.14	20.1 ± 0.42	1.07 ± 0.22
SBR reactor/acetate				0.97 ± 0.36	21.1 ± 0.15	1.23 ± 0.17
SBR reactor/glucose				1.46 ± 0.29	19.3 ± 0.16	1.45 ± 0.34
SBR reactor/glycine				2.22 ± 0.13	17.9 ± 0.25	1.87 ± 0.17
SBR reactor/glutamate				2.15 ± 0.22	18.0 ± 0.11	1.81 ± 0.24
SBR reactor/no substrates				5.25 ± 0.56	7.38 ± 0.33	5.92 ± 0.13

^aEBPR biomass was fed with a mixture of acetate, glucose, and casamino acids.

selected as the species representative (Figs. 4, 5). None of these MAGs possessed >95% average nucleotide identity to the isolates (SUPPL. Fig. 5), so they all represent novel uncultured species.

Metabolic reconstruction of the *Dechloromonas* species MAGs confirmed the presence of central carbon pathways, including the glycolysis, TCA cycle, and glyoxylate pathways, along with genes

essential for PHA and glycogen accumulation (Figs. 5, 6 and Suppl. Table 1). We confirmed the genomic potential for uptake of two carbon sources used in the P-release experiments by identifying the transporters for acetate and glutamate (Fig. 6). However, glycine (*cycA*) and glucose transporter genes could not be detected in the genomes, suggesting that other members of the

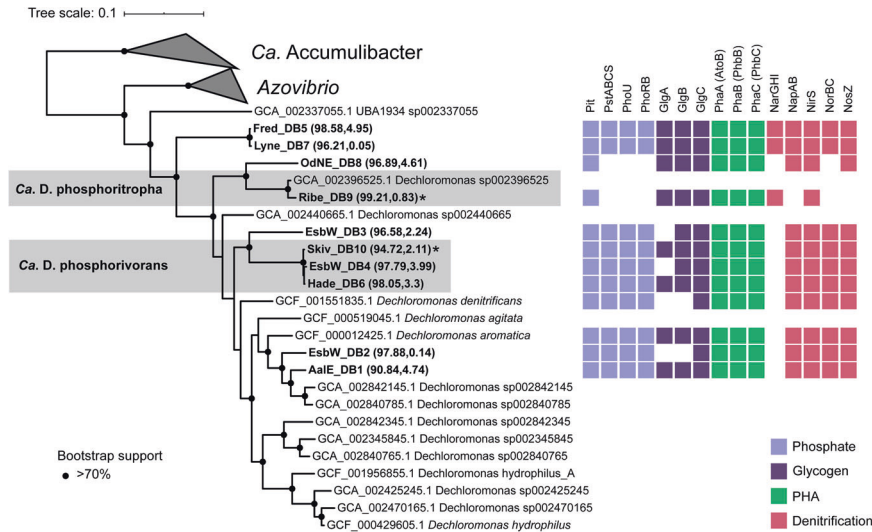


Fig. 5 Phylogenetic tree and metabolic potential of *Dechloromonas* spp. Maximum likelihood tree created using the WAG + GAMMA model in IQ-TREE and the alignment of 120 concatenated proteins created by GTDB-Tk with GTDB release89. Five *Ca. Accumulibacter* genomes were used as the outgroup. Genome completeness and contamination % estimates for the MAGs are indicated within the parentheses. Genomes in bold indicate the MAGs recovered from the Danish WWTPs. The gray boxes denote the *Ca. D. phosphoritropha* and *Ca. D. phosphorivorans* species clusters, and the species representatives are represented by the MAGs Ribe_DB9 and Skiv_DB10 respectively, indicated by asterisks. Bootstrap support >70% is indicated by the solid black circles. NosZ was not identified in the *D. denitrificans* genome, however, it is present in NCBI under accession number KT592356.1.

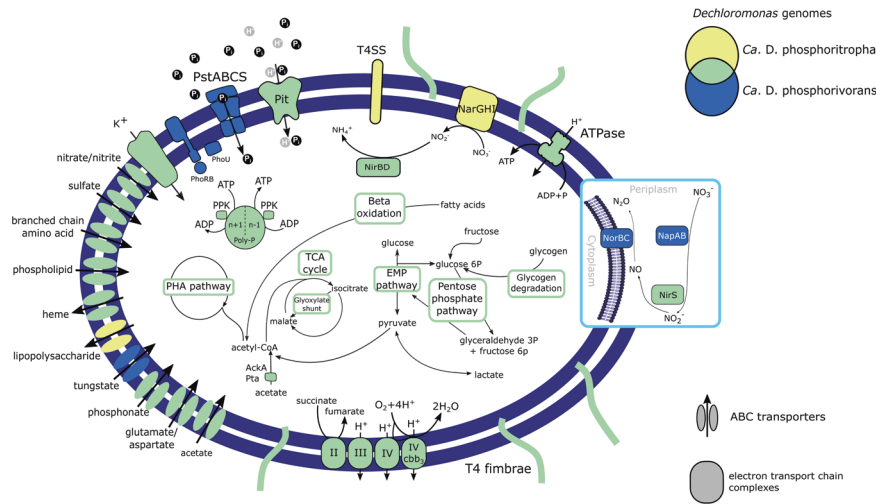


Fig. 6 Metabolic model of the two most abundant *Dechloromonas* species. Colors indicate the species or combination of species (Venn diagram) that encode the potential for the enzyme or cycle. TCA = tricarboxylic acid cycle, EMP = Embden-Meyerhof-Parnas pathway (glycolysis), PHA = polyhydroxyalkanoate pathway, I = complex I NADH dehydrogenase, II = complex II succinate dehydrogenase, III = complex III cytochrome bc1, IV = cytochrome c oxidase, IV cbb3 = complex IV cytochrome cbb3 oxidase, Pit = inorganic phosphate transporter family, PstSCAB = inorganic phosphate ABC transporter, PhoRB = two component system for phosphate regulation, PhoU = phosphate transport system accessory protein, Poly-P = polyphosphate, Ppk = polyphosphate kinase, T4SS = type IV secretion system, NapAB = periplasmatic nitrate reductase, NarGHI = membrane-bound nitrate reductase, NirS = nitrite reductase, NorBC = nitric oxide reductase, AckA = acetate kinase, Pta = phosphotransacetylase.

community process these substrates into components that could be used by the *Dechloromonas* species in the SBR experiment. The identification of both glycine hydroxymethyltransferase (*glyA*) and glutamate dehydrogenase (*gdhA*) in the MAGs suggests that the *Dechloromonas* species process these amino acids to pyruvate and 2-oxoglutarate, respectively, for integration into central carbon metabolism. Interestingly, all MAGs encoded the complete pathway for PHA accumulation, also confirmed in the genomes of the two closely related pure cultures, *Dechloromonas agitata* and *Dechloromonas denitrificans*. *Ca. D. phosphoritropha*, and *Ca. D. phosphorivorans* encoded the genes for glycogen accumulation,

supporting the experimental results. Two of the other MAGs, representing different *Dechloromonas* spp. (EsbW_DB3 and EsbW_DB2), lacked the genes for glycogen synthase (*glgA*) and 1,4-alpha-glucan branching enzyme (*glgB*) for the glycogen accumulation pathway (Figs. 5, 6 and Suppl. Table 1). These genes were also absent in the isolate *D. denitrificans*. Other carbon metabolisms encoded by the MAGs included the potential for beta-oxidation of fatty acids, and fructose degradation (Suppl. Table 1 and Suppl. Data File 1). Lactate (both isomers for *Ca. D. phosphoritropha*, D-lactate only for others) may be used as an additional electron donor and carbon source, as in *D. denitrificans*

[17], or could be produced as a product of fermentation of pyruvate to lactate, depending on the prevailing environmental conditions.

Although the potential for polyphosphate metabolism was present in all the MAGs and in *D. denitrificans* and *D. aromatica*, a key difference was found in the phosphate transporters. While the majority of the MAGs possessed the high-affinity PstSCAB transporter system, the most abundant *Ca. D. phosphoritropha* (Ribe_DB9) and its close relative OdNE_DB8 possessed only the low-affinity Pit transporters (Fig. 5). This supports the proposal that Pit is the phosphate transporter vital to the PAO phenotype [64]. The Pit system is essential as it generates a proton motive force by the export of metal-phosphates in symport with a proton under anaerobic conditions, which seems to drive volatile fatty acids uptake in *Ca. Accumulibacter* [64, 65]. Moreover, the high-affinity PstSCAB transporter system may not be essential in a P-rich environment such as activated sludge. Further analysis, for example with the aid of transcriptomics, may be useful to determine the relevance and usage of these transporters in different conditions. A potential GAO phenotype in situ was previously suggested for probe-defined *Dechloromonas* (likely *Ca. D. phosphorivorans*) [23], but GAOs typically encode only the high affinity PstSCAB uptake system [64]. The presence of genes encoding the Pit system, supported by the experimental poly-P accumulation evidence, is in accordance with a conventional PAO metabolism. It is possible that *Dechloromonas* species, as previously shown for *Ca. Accumulibacter* [66, 67], can exhibit different metabolisms, spanning from polyphosphate- to glycogen-based phenotypes depending on the environmental conditions, but additional analysis is needed to verify this potential metabolic versatility.

Nitrogen metabolism also varied between the different MAGs. All the MAGs presented the potential for dissimilatory nitrate reduction and some of them (EsbW_DB2, Fred_DB5, and Lyne_DB7) possessed the genes for nitrogen fixation (Figs. 5, 6 and Suppl. Table1). The majority of MAGs (8/10) encoded the potential for complete denitrification, while *Ca. D. phosphoritropha* and OdNE_DB8 only encoded reduction of nitrate to nitric oxide (Figs. 5, 6). For OdNE_DB8, the absence of the nitric oxide reductase (NorBC) may be due to incompleteness, as this genome contained all other components of the denitrification pathway, including the nitrous oxide reductase (NosZ). The *Ca. D. phosphoritropha* genome did not encode the periplasmic nitrate reductase NapAB, present in all other recovered genomes, but the respiratory nitrate reductase NarGHI (which is also present in combination with NapAB in Fred_DB5 and Lyne_DB7). This could indicate a difference in their ecological niche, as the Nap enzyme is usually not involved in anaerobic respiration [68], while the presence of a respiratory nitrate reductase may allow *Ca. D. phosphoritropha* to dominate under anoxic conditions. Previous studies on *Ca. Accumulibacter* species [68–70] showed that the Nar enzyme is essential for anoxic phosphorus uptake using nitrate, and its absence in the other MAGs may indicate an inability to use this metabolic function. The subsequent steps of the denitrification pathway also varied between the different MAGs. All of them encoded a dissimilatory nitrite reductase (NirS), while nitric oxide reductase (NosZ) was found in all the MAGs, excluding *Ca. D. phosphoritropha*. As this MAG lacked other genes of the denitrification pathway (Figs. 5, 6, Suppl. Table1), important for the reduction of toxic intermediates [71], this organism most likely lost the metabolic capability to use nitric oxide and nitrous oxide as terminal electron acceptors. Additional in situ studies are needed to verify this metabolic trait and its possible effects on niche partitioning within the genus and with other important PAOs.

Taxonomic proposal

As no axenic cultures representing the novel species are available, we propose the names *Candidatus Dechloromonas phosphoritropha* sp. nov. [phos.pho.ri.tro'pha. from N. L. masc. N. *phosphorus*,

phosphorus; Gr. Masc. n. *trophos*, one who feeds; N. L. fem. adj. *phosphoritropha*, indicating the ability to accumulate poly-phosphate] and *Candidatus Dechloromonas phosphorivorans* [phos.pho.ri.vo'rans, from N.L. masc. n. *phosphorus*, phosphorus; L. pres. part. *vorans*, devouring; N.L. part adj. *phosphorivorans*, indicating the ability to accumulate poly-phosphate] for the two species, based on the recommendations by Murray and Stackebrandt [72]. The complete protologues for the formal proposal of the new species is given in Suppl. Table 2.

Concluding remarks and future perspectives

Here, we have provided the first experimental and genomic insights into the physiology of uncultivated *Dechloromonas* spp. We have shown that these lineages are PAOs that are often abundant and actively involved in P removal in full-scale EBPR plants worldwide, and they should be regarded as important PAOs along with *Ca. Accumulibacter* and *Tetrasphaera*. They exhibit a physiology very similar to *Ca. Accumulibacter*, but seem more diverse in their substrate utilization profile and share some traits with *Tetrasphaera* too. They may also be important for nitrogen removal with most species possessing full denitrifying capabilities. The co-occurrence of these three genera in most EBPR plants suggests niche differentiation and functional redundancy, which may be even more pronounced by the presence of other PAOs. Some putative PAOs can occasionally be abundant in specific plants [15] and our recent discovery of a new putative PAO, *Ca. Methylophosphatis* [27], strongly suggests there are undescribed PAOs. This has important implications for the study of EBPR communities, as they are critical to nutrient cycling in many wastewater treatment plants and warrant specific attention as societies transition to improve resource recovery from these systems.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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