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Population dynamics of bacteria involved in enhanced biological phosphorus removal in Danish wastewater treatment plants

*Artur Tomasz Mielczarek, Hien Thi Thu Nguyen, Jeppe Lund Nielsen, Per Halkjær Nielsen**

Department of Biotechnology, Chemistry, and Environmental Engineering, Aalborg University, Sohngaardsholmsvej 49, DK-9000 Aalborg, Denmark

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ABSTRACT

The enhanced biological phosphorus removal (EBPR) process is increasingly popular as a sustainable method for removal of phosphorus (P) from wastewater. This study consisted of a comprehensive three-year investigation of the identity and population dynamics of polyphosphate-accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs) in 28 Danish municipal wastewater treatment plants with nutrient removal. Fluorescence *in situ* hybridization was applied to quantify ten probe-defined populations of PAO and GAO that in total constituted a large fraction (30% on average) of the entire microbial community targeted by the EUBmix probes. Two PAO genera, *Accumulibacter* and *Tetrasphaera*, were very abundant in all EBPR plants (average of 3.7% and 27% of all bacteria, respectively), and their abundance was relatively stable in the Danish full-scale plants without clear temporal variations. GAOs were occasionally present in some plants (*Competibacter* in 11 plants, *Defluviicoccus* in 6 plants) and were consistent in only a few plants. This shows that these were not core species in the EBPR communities. The total GAO abundance was always lower than that of *Accumulibacter*. In plants without EBPR design, the abundance of PAO and GAO was significantly lower. *Competibacter* correlated in general with high fraction of industrial wastewater. In specific plants *Accumulibacter* correlated with high C/P ratio of the wastewater and *Tetrasphaera* with high organic loading. Interestingly, the relative microbial composition of the PAO/GAO species was unique to each plant over time, which gives a characteristic plant-specific "fingerprint".

1. Introduction

The enhanced biological phosphorus removal (EBPR) process is a relatively inexpensive and sustainable method for the removal of phosphorus (P) from wastewater. The EBPR process is based on the ability of polyphosphate-accumulating organisms (PAOs) to take up P and accumulate it intracellularly as polyphosphate when exposed to alternating anaerobic and aerobic conditions. These bacteria can be removed with surplus sludge for further processing of the P, e.g. for P reuse. The EBPR process was discovered almost 60 years ago, but only in the past 10–15 years have we gained more detailed knowledge of the microbiology involved (reviewed by Seviour et al., 2003; Oehmen et al., 2007; McMahon et al., 2010).

The key PAO organism is believed to be the uncultured betaproteobacterial '*Candidatus* Accumulibacter phosphatis' (Crocetti et al., 2000; He and McMahon, 2011a; Hesselmann et al., 1999; Zilles et al., 2002), hereafter called *Accumulibacter*.

* *Corresponding author*. Tel.: +45 9940 8503; fax: +45 9814 1808.
E-mail address: phn@bio.aau.dk (P.H. Nielsen).
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The bacterial *ppk1* gene, which encodes for the enzyme polyphosphate kinase responsible for polyP synthesis in many bacteria, can be used for a high-resolution phylogenetic analysis of PAOs. McMahon et al. (2007) conducted an analysis of *ppk1* genes from ten EBPR plants and observed two major types of *Accumulibacter* (Type I and Type II). He et al. (2007) also retrieved fragments of 16S rRNA and *ppk1* genes from lab-scale and several full-scale EBPR systems. Phylogenies reconstructed from 16S rRNA genes and *ppk1* were largely congruent with *ppk1*, providing higher phylogenetic resolution and at least five subgroups (clades) emerging under the two major types. These two types of *Accumulibacter* commonly exist in treatment plants (He et al., 2007; McMahon et al., 2002; Peterson et al., 2008) and show distinct morphotypes, coccibacilli, and cocci, respectively (Carvalho et al., 2007; Flowers et al., 2008). Many studies of their physiology have been carried out, primarily in enriched lab-scale reactors. These have sought to elucidate important biochemical pathways (Mino et al., 1998; He et al., 2010; Wexler et al., 2009), substrate preference (Kong et al., 2004; Oehmen et al., 2005b), denitrification capability (Kong et al., 2004; Flowers et al., 2009), and operational conditions affecting their growth, such as temperature and pH (Oehmen et al., 2007; van loosdrecht et al., 1997; Zilles et al., 2002).

Putative PAOs are also found within the *Actinobacteria* (Eschenhagen et al., 2003; Kong et al., 2005; Beer et al., 2006; Nguyen et al., 2011). In full-scale plants, two morphologically distinct cells (cocci and rods) from the genus *Tetrasphaera* were found to be involved in P removal (Kong et al., 2005). Nguyen et al. (2011) showed a higher diversity of the *Tetrasphaera* genus and designed oligonucleotide probes for FISH detection of three clades (1–3). *Tetrasphaera* can constitute up to 30–35% of the total biovolume of bacteria in some full-scale plants (Nguyen et al., 2011; Nielsen et al., 2010); this number is much higher, compared to *Accumulibacter*, which constitute around 3–10% of the total population (e.g., Gu et al., 2008; He et al., 2008). The physiology of the genus *Tetrasphaera* is poorly described, although the species seem to be more diverse in terms of substrate uptake than *Accumulibacter* and can ferment glucose under anaerobic conditions (Kong et al., 2008; Nguyen et al., 2011). They seem to occupy a slightly different ecological niche, compared to *Accumulibacter*, possibly contributing to ensure the stability of the EBPR process.

The glycogen-accumulating organisms (GAO) are often present in EBPR plants, where they compete with PAOs for uptake of organic substrate in the anaerobic tanks. Unfortunately, GAOs do not accumulate P, so they are unwanted members of the microbial community in both lab-scale reactors and full-scale plants (Crocetti et al., 2002; Kong et al., 2002; Nielsen et al., 2010). Two main groups of GAOs are known today, the alphaproteobacterial *Defluviicoccus vanus*-related bacteria and the gammaproteobacterial '*Candidatus* Competibacter phosphatis' (hereafter called *Competibacter*) (Crocetti et al., 2002; Meyer et al., 2006; Wong et al., 2004). They have been observed competing with PAOs in both full-scale and lab-scale reactors (Burow et al., 2007; Kong et al., 2006; Nielsen et al., 2010; Oehmen et al., 2005b; Thomas et al., 2003). Several subgroups of *Competibacter* have been identified in lab-scale reactors (Kong et al., 2002), and some of them are also present in full-scale plants (Kong et al., 2006). The potential to denitrify has been observed for a minority of the *Competibacter* sub-populations (Kong et al., 2006; Zeng et al., 2003), but not for members of the *D. vanus* – related GAOs (Burow et al., 2007; Wang et al., 2008).

A number of factors are known to affect the growth of PAOs and GAOs and their competition. However, most of this understanding comes from studies in highly enriched lab-scale reactors, where the growth conditions rarely resemble full-scale plants, and little information is presently available about key factors controlling the populations in full-scale EBPR plants. It is known that certain operational parameters, such as pH, temperature, wastewater type, and COD/P ratio, concentration of oxygen or nitrite as well as specific plant configurations can have an effect on the stability and diversity of these populations, but the reasons for this are not always clear (Oehmen et al., 2007; Pijuan et al., 2005, 2006; Van Loosdrecht et al., 1997). Furthermore, we do not know much about the temporal stability of these populations in full-scale systems, a matter of great importance for the operation of these plants.

Recently, we have published a conceptual ecosystem model of the EBPR process (Nielsen et al., 2010). We presented the average microbial composition of 25 Danish EBPR plants and found the presence of a core population common to all plants. Several PAO species were among these. The main aim of the study presented here was to conduct detailed investigations into the abundance and temporal variations of the key PAO and GAO populations associated with the EBPR process in these full-scale plants. Furthermore, an attempt was made to find correlations between abundances of different species, as well as between species and plant design and operational parameters.

2. Material and methods

2.1. Sampling and plant data

Data from 28 full-scale biological nutrient removal plants were gathered over a three-year period from 2009 to 2011 (see list of plants in Table 1A) and included in the "Microbial database for Danish wastewater treatment plants" (Nielsen et al., 2010), now called MiDas-Dk (www.midas-dk.dk). Data was obtained from a core 25 wastewater treatment plants over the three year period, with an additional three plants investigated in the first year. Extensive information has been collected on process design, operation, influent and effluent concentrations of COD, N and P fractions, industrial load, suspended solid concentrations in activated sludge, diluted sludge volume index as well as information on major problems encountered, such as bio-P process removal disruptions or foaming/bulking problems. All plants experienced only minor operational problems during the period and consistently complied to the effluent limits of 8 mg/L total N, 0.5 or 1 mg/L total P and 70 mg/L total COD. Seven plants were sampled four times a year, the remaining plants twice a year. In addition, Aalborg East and Hjørring WWTP were sampled twice a week, from November 2008 to January 2009, in order to monitor short-term changes in the PAO/GAO bacterial community. Activated sludge samples were taken from the

Table 1 – (A). Details on the Danish nutrient removal plants investigated 2007–2011. All plants carry out nitrification, denitrification and chemical or biological P removal. (B) Details on the Danish nutrient removal plants investigated 2007–2011.

Type	WWTP name	Samplings per year	Size designed (PE)	Size actual (PE)	Alternating operation	Recirculation	Sidestream hydrolysis	Presettling	Dosage of PAC	External carbon source
EBPR WWTP	Bjergmarken	4	125,000	83,000	+	–	–	+	+	None
	Egå		120,000	84,000	–	+	+	–	–	None
	Ejby Mølle		410,000	236,000	+	–	–	+	+	None
	Hjørring		160,000	100,000	–	+	–	+	+	None
	Skive		123,000	39,590	–	+	+	–	–	None
	Aalborg West		330,000	195,000	+	–	+	+	+	Molasses
	Aalborg East		100,000	45,000	+	–	+	–	–	Molasses
	Boeslum	2	26,000	10,000	–	+	–	+	+	None
	Fornæs		60,000	45,000	–	+	–	–	–	None
	Fredericia			420,000	–	+	–	–	–	None
	Haderslev		100,000	48,460	+	–	–	–	–	None
	Kerteminde		25,000	16,000	–	+	–	–		None
	Kolding		125,000	80,000	+	–	–	+	+	None
	Lundtofte		135,000	110,000	+	–	–	+	+	Propylene/ Ethylene glycol
	Mørke		14,000	8300	+	–	–	+	+	None
	Odense Nordøst		37,000	26,000	+	–	–	–	–	None
	Randers		130,000	75,000	–	+	+	+	+	Acetic acid
	Ringkøbing		42,500	20,000	+	–	+	+	+	None
	Søholt		100,000	65,000	+	–	+	+	+	None
	Viby		100,000	41,200	–	+	+	–	–	None
	Åby		93,000	71,000	+	–	+	+	+	None
Non EBPR WWTP	Avedøre		345,000	330,000	+	–	–	–	–	None
	Hirtshals			133,000	+	–	–	–	–	None
	Horsens		140,000	140,000	–	+	–	+	+	Molasses
	Marselisborg		220,000	157,000	+	–	–	+	+	None
	Odense Nordvest		75,000	51,000	+	–	–	–	–	None
	Viborg		80,000	68,000	+	–	–	+	+	Methanol
	Aars		105,000	60,000	+	–	–	–	–	None

WWTP name	Industrial contribution (% of COD)	Industry type	Sludge age (range summer) (d)	Sludge age (range winter) (d)	DSVI range (ml/g)	Temperature (Winter–Summer) C	C/P average (gCOD/gP)	P (in and out) (mg/L)	Ortho P release after 120 min (mgP/gSS)
Bjergmarken	20	Slaughterhouse, diary, enzyme production	25–30	30–35	85–152	13–16	98	8 – 0.44	13.1 ± 0.6
Egå	40	Hospital, Silk factory, incineration plant	20–25	25–0	90–150	10–18	66	8.8 – 0.18	13.5 ± 0.5
Ejby Mølle	55	Paper factory, food, incineration plant, hospital	20–25	25–30	104–200	13–18	71	–0.68	13.0 ± 0.6
Hjørring	30	Food	20–25	25–30	60–100	9–17	–	10.3 – 0.48	12.5 ± 0.4
Skive	20	Mixed	20–25	25–30	85–118	8–18	–	5.7 – 0.89	14.5 ± 0.8
Aalborg West	30	Diary	20–25	25–30	107–157	10–18	65	4.9 – 0.27	9.3 ± 0.5

(*continued on next page*)

Table 1 – (*continued*)

WWTP name	Industrial contribution (% of COD)	Industry type	Sludge age (range summer) (d)	Sludge age (range winter) (d)	DSVI range (ml/g)	Temperature (Winter–Summer) C	C/P average (gCOD/gP)	P (in and out) (mg/L)	Ortho P release after 120 min (mgP/gSS)
Aalborg East	10	Slaughterhouse, chemical	20–25	25–30	62–122	8–18	78	8.6 – 0.38	11.9 ± 1.1
Boeslum	5	Mixed	25–30	30–45	66–92	10–15	77	12.9 – 0.22	14.0 ± 0.6
Fornæs	75	Chemical	20–25	25–30		10–20	61	4.7 – 0.32	
Fredericia	40	–	20–25	25–30	51–166	–	85	14.3 – 0.83	13.5 ± 0.6
Haderslev	5	Mixed	20–25	25–30	76–132	–	–	8.3 – 0.38	
Kerteminde	10	Food, metallurgy	20–25	25–30	78–130	12–16	–	8.1 – 0.10	
Kolding	14	Food, metallurgy	20–25	25–30	90–135	9–15	51	7.1 – 0.62	10.9 ± 0.7
Lundtofte	5	Chemical	20–25	25–30	121–127	10–18	–		13.5 ± 0.8
Mørke	5	Mixed	20–25	25–30	68–87	–	71	10.1 – 0.35	11.4 ± 0.4
Odense Nordøst	21	Food, mixed	20–25	25–30	87–185	10–15	115	–0.23	13.0 ± 0.3
Randers	5	Diary, food	25–30	30–35	79–117	9–18	89	5.6 – 0.39	13.3 ± 0.9
Ringkøbing	10	–	25–30	30–35	124–149	9–13	70	4.6 – 0.16	9.3 ± 0.5
Søholt	35	Textile, food	20–25	25–30	89–105	9–17	59	17.0 – 0.12	10.9 ± 0.5
Viby	5	–	20–25	25–30	97–118	8–17	40	6.7 – 0.22	8.8 ± 1.4
Åby	20	Mixed	20–25	25–30	96–171	12–20	42	8.6 – 0.28	9.8 ± 0.4
Avedøre	20	Pharmaceutical, food	20–25	25–30	136–260	12–18	74	9.8 – 0.71	
Hirtshals	70	Fish, food, mixed	20–25	25–30	91–112	–	54	15.2 – 0.17	11.4 ± 0.5
Horsens	60	Slaughterhouse, mixed	20–25	25–30	76–129	12–18	70	3.6 – 0.10	12.5 ± 0.3
Marselisborg	45	Slaughterhouse, food	20–25	25–30	72–107	–	47	5.2 – 0.27	11.6 ± 0.5
Odense Nordvest	18	Food, mixed	20–25	25–30	110–198	11–18	83	–0.04	13.1 ± 0.6
Viborg	10	Hospital	25–30	30–35	76–155	11–15	69	6.1 – 0.35	13.5 ± 0.5
Aars	85	Slaughterhouse	25–30	30–35	39–133	9–17	59	18.0 – 0.22	13.0 ± 0.6

Description.
PE – Population Equivalent.
– no/absence.
+ yes/presence.

aerobic process tank and kept below 4 °C, and a fraction was fixed for FISH analysis (Nielsen, 2009). Anaerobic release tests of orthophosphate by addition of acetate were conducted once for all plants (in May/June 2012) in order to test the biological phosphorus removal potential. Acetate (150 mg/l final concentration) was added to duplicate stirred activated sludge samples, and the anaerobic P released after 3 h at 20 °C was recorded and normalised to SS (Table 1B).

2.2. FISH identification

A range of oligonucleotide probes targeting different bacterial species were applied (Table 2). The coverage of *Tetrasphaera* probes is shown in Figure S1. Detailed information about most of these probes is given in probeBase (Loy et al., 2007). The oligonucleotide probes were labelled with 5(6)-carboxy-fluorescein-N-hydroxy-succinimide ester (FLUOS) or with the sulfoindocyanine dyes (Cy3 and Cy5) (Biomers, Ulm/Donau, Germany).

The procedure was performed according to the guidelines as detailed by Nielsen (2009) and visualized with an Axioskop epifluorescence microscope (Carl Zeiss). Quantification of probe-defined populations (qFISH) was carried out according to Morgan-Sagastume et al. (2008). In short, each sample was diluted and homogenized before a very thin layer was applied to glass microscope slides. After FISH, twenty random fields were chosen and images acquired with a specific probe-defined population (Cy3) and the general EUBmix (FLUOS). The area of probe-defined populations, relative to the total biomass area, was determined by image analysis software (ImageJ, http://rsbweb.nih.gov/ij/), and custom-made macros were used for post-processing and data acquisition of all images. The total biomass of bacteria in all plants was determined by the EUBmix and by DNA staining with 4′6-diamidino-2-phenylindole, DAPI (Kapuscinski, 1995). Standard deviation for all values and probes was between 16 and 20% of the average value indicated. The standard deviation was calculated from three replicate sludge samples. The standard error of the mean between the samples was between 16 and 20%, depending on the probes applied, and thus was assumed as valid for all the samples. Only averages are shown in the figures.

2.3. Polyphosphate kinase (ppk) gene

PCR amplification of *ppk* fragments using five clade-specific *ppk1* primers was performed as follows: Total genomic DNA was extracted from frozen sludge samples by using the PowerSoilTM DNA Isolation Kit (MO BIO). Five clade-specific *ppk1* primer sets (I, IIA, IIB, IIC, and IID) (He et al., 2007) were used. PCR conditions were optimized for each primer set. The PCR was carried out in a 50-μl thermal cycler (MJ Research, USA) with 1 μM of primers and 0.1 unit of HotStart Taq polymerase (Invitrogen, USA), using a cycling regime of 95 °C for 3 min (1 cycle); 94 °C for 30 s, an optimized annealing temperature for 45 s (61 °C for *ppk1* clade I, IIA, IIB, and 63 °C, 66 °C for *ppk1* clade IIC and IID, respectively), and 72 °C for 30 s (40 cycles); and 72 °C for 5 min (1 cycle). The presence or absence of a PCR product was visualized by agarose gel electrophoresis. If present, some randomly chosen bands were cut out of the gel and sequenced to confirm their identity.

2.4. Statistical measures and methods

All values used for the calculations, charts and statistical analyses were from qFISH analyses together with plant-specific data related to the different sampling times and other plant specific data. Standard statistical comparisons and graphing were performed in Microsoft Excel and Prism. Correlation and cluster analyses were performed in SPSS 19 package and Excel-XLSTAT (Brace et al., 2006). These analyses

Table 2 – Overview of specificity of oligonucleotide probes applied for quantitative FISH analyses.

Affiliation	Probe name	Coverage	Reference
Phylum	EUB338 Most *Bacteria*	EUB338 Most *Bacteria*	Amann et al. (1990)
	EUB338-II	*Planctomycetales*	Daims et al. (1999)
	EUB338-III	*Verrucomicrobiales*	Daims et al. (1999)
Proteobacteria	PAOmix (PAO462, PAO651and PAO846)	Most *Accumulibacter*	Crocetti et al. (2000)
	Acc-I-444	*Accumulibacter* clade IA and others	Flowers et al. (2008)
	Acc-II-II-444	*Accumulibacter* clade IIA, some clade IIC and IID	Flowers et al. (2008)
	DF1 mix (TFO_DF218 and TFO_DF618)	*Defluviicoccus* spp. (Type 1)	Wong et al. (2004)
	DF2 mix (DF988 and DF1020)	*Defluviicoccus* spp. (Type 2)	Meyer et al. (2006)
	Gbmix (GAOQ989 and GB_G2)	Most *Competibacter*	Kong et al. (2002)
Actinobacteria	Actino-221	*Tetrasphaera* – Type 1	Kong et al. (2005)
	Actino-658	*Tetrasphaera* – Type 2	Kong et al. (2005)
	Tet1-266	*Tetrasphaera* – Clade 1	Nguyen et al. (2011)
	Tet2-892	*Tetrasphaera* – Clade 2A	Nguyen et al. (2011)
	Tet2-174	*Tetrasphaera* – Clade 2B	Nguyen et al. (2011)
	Tet3-654	*Tetrasphaera* – Clade 3	Nguyen et al. (2011)

included multivariate analysis of variance (MANOVA) (Foster et al., 2005), with significance level alpha = 0.05 and the Wilks' lambda and Mann–Whitney *U* tests. Unscrambler 11.01 and Excel-XLSTAT were used for principal component analysis (PCA). The correlation analysis aimed to find weak ($|r| \leq 0.4$), medium ($0.4 < |r| \leq 0.6$), strong ($0.6 < |r| \leq 0.8$) or very strong ($0.8 < |r| \leq 1$), correlations between given parameters and abundances of bacteria. The PCA was used in order to find groups and relationships between the given parameters and bacterial abundance in the multidimensional space. The PCA correlation circles were used to determine the relationships between the given parameters in between principal components. The circles show a projection of the initial variables in the factors space. When two variables are far from the centre and close to each other, they are significantly positively correlated (*r* close to 1); if they are far from the centre and orthogonal, they are not correlated (*r* close to 0).

3. Results

3.1. Wastewater treatment plants

The 28 WWTPs included in the survey all had biological N-removal (nitrification and denitrification), and 21 were also configured for EBPR (Table 1). The plants ranged in size between 8300 and 420,000 population equivalents (PE), and their process configurations were either alternating or recirculating (Henze et al., 2001). The fraction of industrial contribution to the organic matter in the influent varied between 0 and 70%, thus representing a relatively broad range of plant types and operational configurations. The yearly temperature range was between 7 and 20 °C. In the plants with EBPR, 12 plants had normal anaerobic tanks on the main stream for mixing return sludge and incoming wastewater, whereas 9 plants had side stream hydrolysis (SSH) instead of an anaerobic tank on the main stream (Vollertsen et al., 2006). Typically, 20–30% of the return activated sludge entered the SSH tank, whereas the other part was returned to the denitrification tank. The residence times in the anaerobic tanks were 12–48 h. All EBPR plants had an active EBPR process as shown by anaerobic ortho-P release upon acetate addition. A screening of all plants showed a release capacity of 8–15 mgP/gSS (Table 1B), which is slightly higher than many other full-scale plants (Zhang et al., 2011). All plants received small doses of iron compounds (iron chloride or iron sulphate) for improving precipitation of phosphorus and enhancing flocculation. Effluent concentrations of total P were usually around 0.5 mgP/L in most plants, and always below the limit of 1.0 mgP/L.

3.2. Morphology of PAOs and GAOs

During the FISH analyses of activated sludge samples, the morphologies of abundant PAOs and GAOs were observed and compared in the 21 different EBPR plants.

3.3. PAOs

Accumulibacter targeted by the PAOmix usually consisted of relatively large rod-shaped cells and were mainly found as microcolonies. Two distinct morphotypes representing two *Accumulibacter* clades targeted by probe Acc-I-444 (clade IA and others) and probe Acc-II-444 (most of clade IIA, IIC and IID) were present, consistent with the findings of Flowers et al. (2008). In some plants, many *Accumulibacter* cells, giving a positive signal for the PAOmix probe set, were not targeted by the two clade-specific probes (Fig. 1).

Four probes were applied to describe three distinct clades of *Tetrasphaera*. Each probe covered several morphotypes, as also described by Nguyen et al. (2011); namely cocci in clusters of tetrads, small cocci, branched rods, short rods in clumps and filamentous bacteria with varied in widths (Fig. 2). The two probes Actino-221 and Actino-658 (designated Type 1 and Type 2 by Kong et al. (2005)) only covered part of the three *Tetrasphaera* clades, with their morphologies being primarily small cocci in tetrads (Actino-221) and short rods, fairly small in size and almost always in colonies (Actino-658). The coverage of these probes is shown in Fig. S1.

3.4. GAOs

Competibacter was targeted by the GAOmix and showed large coccoidal cells as previously described (Crocetti et al., 2002;

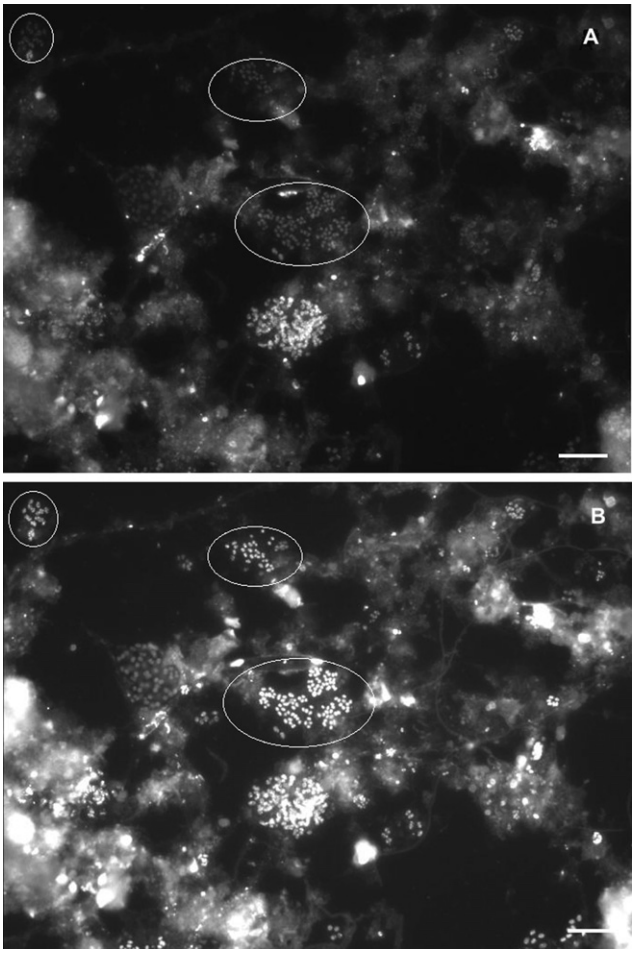

Fig. 1 – *Accumulibacter* not targeted by probes for *Accumulibacter* clade I or II (probes Acc-I-444 and Acc-II-444) (a), but they were targeted by PAOmix (b).

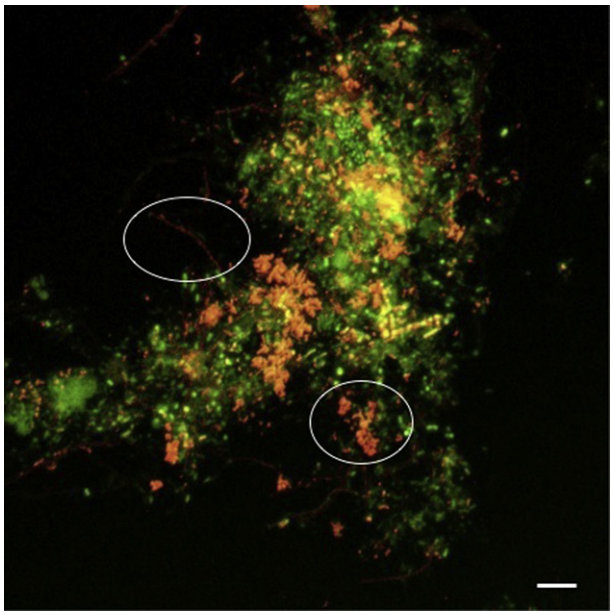

Fig. 2 – Example of activated sludge from Aalborg East WWTP with high abundance of *Tetrasphaera* with different morphologies (clades 1, 2A, 2B and C, red/yellow, all labelled with Cy3). EUBmix shows all bacteria (green, FLUOS). Scale bar 10 μm.

Kong et al., 2002; Levantesi et al., 2002). The bacteria were present both as single cells and as large microcolonies, in some cases grouped in tetrads. The morphology of *Defluviicoccus* (targeted by probes DF1Mix and DF2Mix) was relatively diverse, and positive cells were cocci-shaped cells present in small microcolonies, as previously described (Meyer et al., 2006; Wong et al., 2005). DF2Mix also gave a positive signal with relatively wide, but short filaments, as described by Nittami et al. (2009). However, this is likely due to the mis-hybridisation of the DF2mix probe set to members of the filamentous Cluster III *Defluviicoccus*, as has been previously demonstrated (Nittami et al., 2009).

3.5. *Abundance of PAOs and GAOs in Danish treatment plants in 2009–2011*

In all EBPR plants examined, both *Tetrasphaera* and *Accumulibacter* were abundant PAOs (Figs. 3 and 4). Most abundant was *Tetrasphaera* clade 3, ranging from 0.5% to 18% of the total biovolume, and *Tetrasphaera* clade 2A (0.5–9%) (Fig. 3). The total abundance of *Tetrasphaera* was typically 20–25%, and in a few cases up to 25–30% of all bacteria with an average of 21% for all plants. This very high abundance was supported by Gram staining. *Accumulibacter* was less abundant (3.9%) on average, with a range of 2–8% of all *Bacteria* (Fig. 3). Both clades of *Accumulibacter* were present in relatively constant amounts in all plants, although clade 1, which is assumed to be able to denitrify, was more often present in higher abundance. The fraction targeted by the PAOmix, but not with the two specific clade probes, each constituted 0.25–1.2% in the plants (or 20–30% of all *Accumulibacter*, Fig. 4a, b). In all treatment plants investigated, the total biovolume of *Tetrasphaera* was significantly higher than that of the *Accumulibacter*.

The two types of GAOs, *Competibacter* and *Defluviicoccus*, were only found in significant amounts in some of the plants (Fig. 3). *Competibacter* was consistently present in only two plants over the three years (Ejby Mølle and Fredericia) in abundances up to 7.2%, but nearly always in abundances slightly lower than *Accumulibacter* in the same plant. They were occasionally present in nine plants. *Defluviicoccus* (Type 1 and 2) were only occasionally present in seven plants, and consistently in two (Bjergmarken and Hjørring), constituting up to 4.7% of the total biovolume. In these seven plants, *Defluviicoccus* was present both as cocci and filaments; mainly as the latter morphotype in the Bjergmarken plant. In two plants, *Competibacter* and *Defluviicoccus* coexisted most of the time (Ejby Mølle, Hjørring), but only occasionally in Bjergmarken.

In the seven non-EBPR plants with chemical P removal, the PAO and GAO populations were less abundant, compared to the EBPR plants analysed in this study. All clades of *Tetrasphaera* were detected with an average abundance of 4.7%, 5.8%, 4.4% and 5.9% for clades 1, 2A, 2B, and 3, respectively, compared to 3.8% determined for the *Accumulibacter* (see Fig. S2). Some EBPR activity potentially took place, even though the plants were not designed for it. As in the EBPR plants, *Tetrasphaera* were always more abundant than *Accumulibacter*. Interestingly, *Competibacter* and *Defluviicoccus* were recorded occasionally in these plants in relatively high numbers (up to 6.7%). They coexisted in one non-EBPR plant (Horsens) throughout the 3-year study period.

3.6. *Population dynamics of PAOs and GAOs*

Variations in PAO and GAO populations were investigated over 3 years in the full-scale EBPR plants (Fig. 4). When the averages of the populations for all EBPR plants were compared, no seasonal variations could be detected. Most of the individual plants showed some temporal variations, but no clear difference between winter/summer could be found for any of the probe-defined PAO and GAO populations. The three clades in *Tetrasphaera* showed the greatest temporal variations, whereas *Accumulibacter* appeared more stable. Analysis of the mutual dependency of PAO clades with Pearson's correlation did not show any strong correlation between the variation in abundance in the three *Tetrasphaera* clades (also their cumulative sum) and the two *Accumulibacter* clades (and their cumulative sum) (Table 3). This indicates a lack of direct connection between these two genera. *Competibacter* showed some temporal variation in the few plants where it was present, but did not follow the trends of either of the PAO group populations.

The general level of PAOs and GAOs was different from plant to plant, although some variation was found in the individual plants over time. It was possible to identify plants with a very stable, relatively high or low content of one or more groups of the detected PAO/GAO (e.g., Aalborg East, PAOs; Ejby Mølle, *Competibacter*), but also some plants with a less stable and more changing microbial population (e.g., Egå) (see Fig. 4b). The temporal variations of *Accumulibacter* were determined in greater detail in two treatment plants

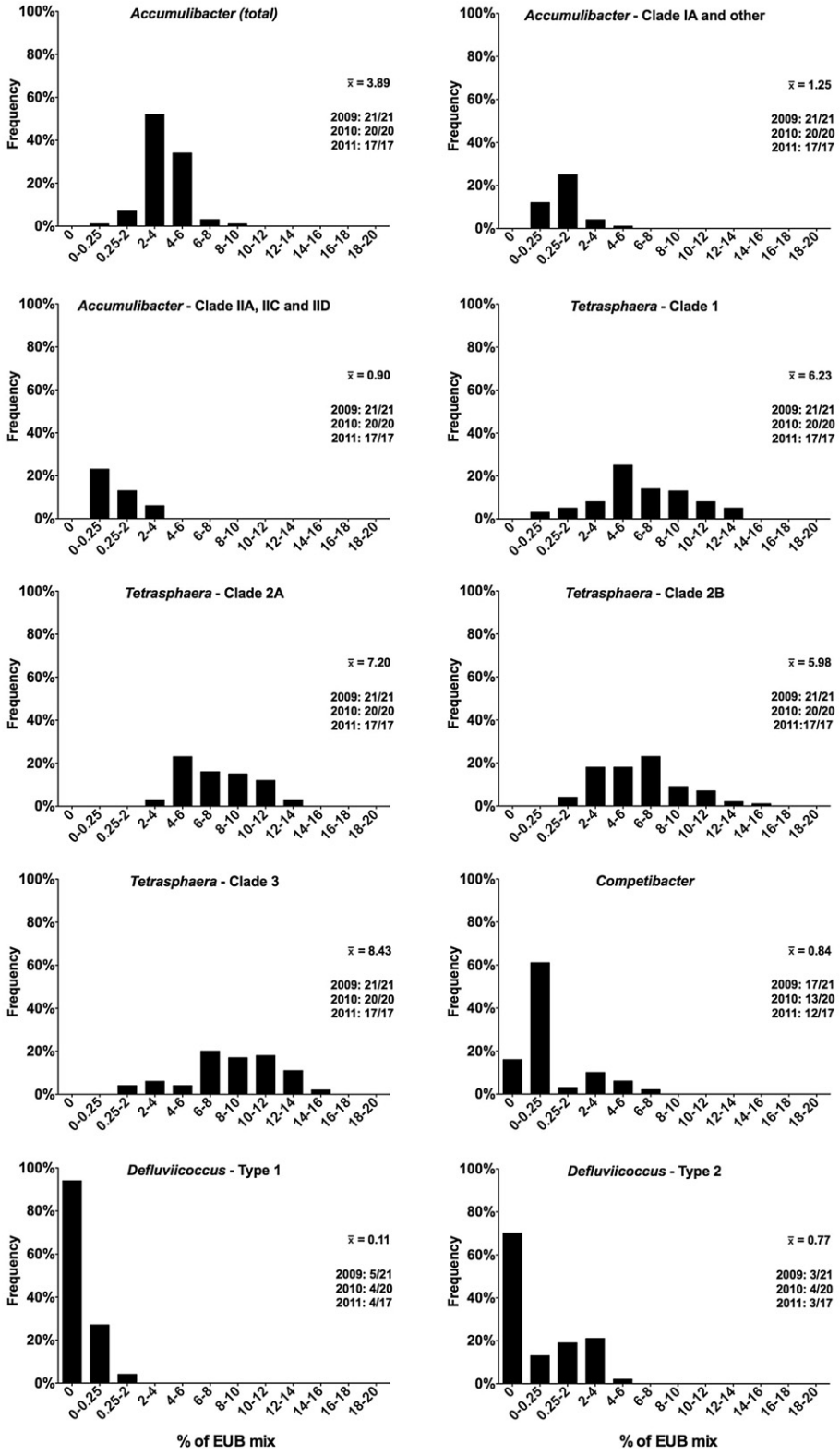

Fig. 3 – Histograms representing percentage distribution (qFISH data) of PAO and GAO in all tested Danish EBPR wastewater treatment plants in 2009–2011. represents averages of all plants (in % of EUBmix). The number of plants with an abundance > 0.25% is indicated for 2009, 2010, and 2011. The Y-axis shows the frequency distribution and the X-axis the percentage of bacteria enumerated by qFISH.

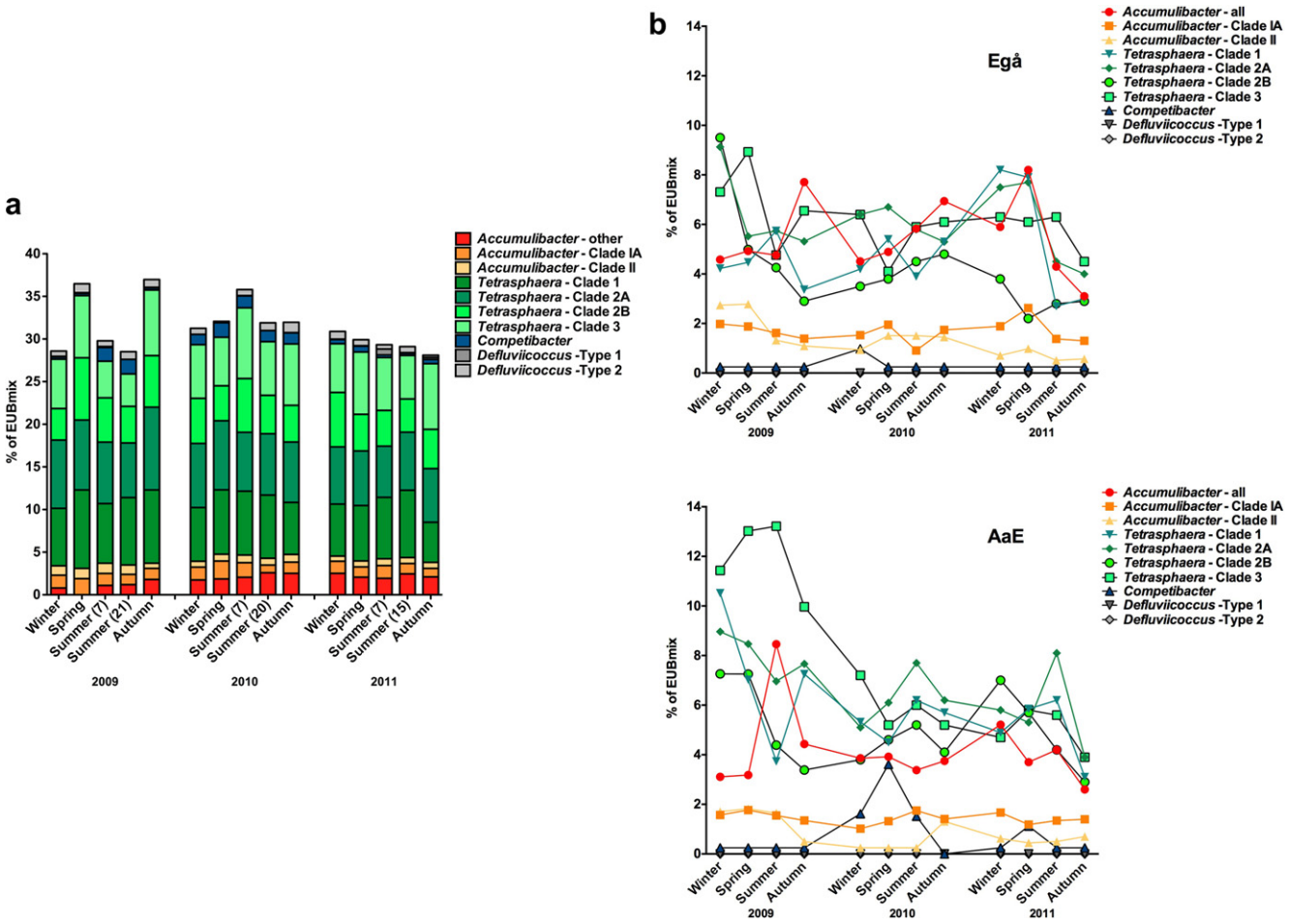

Fig. 4 – a). Relative average abundance of PAO and GAO during the period 2009-2011 (winter (February), spring (May), summer (August) and autumn (November)) in Danish EBPR wastewater treatment plants. (b). Temporal variations in PAO and GAO populations at Egå and Aalborg East WWTPs.

(Aalborg East and Hjørring), with sampling twice a week over a three-month period, in order to see whether there were variations not seen by the 2-4 yearly samples from each plant (Fig. S3). In both plants, only minor temporal variations, marginally outside the normal standard error on the mean (16–20% of the average), were detected for either the total *Accumulibacter* population or the individual clades (I and II). Many *Accumulibacter* not detected by the two clade probes (approx. 30% of total *Accumulibacter*) were present in both plants (Fig. S2). *Competibacter* was below 0.25% in these plants during this period.

3.7. *Distribution of different clades of* Accumulibacter

In order to investigate the diversity of *Accumulibacter* in the different treatment plants, PCR and clade-specific *Accumulibacter ppk1* primer sets were applied (Table 3). Each of the five *Accumulibacter* clades were found in at least one plant and with clades I and IIC present in nearly all. Clades IIA, and particularly clade IID, were found in very few plants. Most plants had 2-3 different clades present, but none had all. Three plants had only one. The distribution of clades showed no obvious pattern, see below for detailed statistical analyses. The *ppk1* gene analysis generally agreed with the FISH results from same plants. In only one plant (Horsens) did we observe that FISH analysis indicated the presence of clades I and II, but *ppk1* analysis only detected one.

3.8. *Statistical analyses of population structure and treatment plant characteristics*

Different statistical analyses were applied to find possible correlations between i) the different populations in the plants and ii) specific probe-defined populations and various treatment plant characteristics such as design type, loading, etc.

3.8.1. *Mutual correlations between PAO and GAO*

Simple mutual correlation analyses were performed on abundances of all probe-defined PAOs and GAOs. Only a few relatively strong correlations were found. The two groups of *Tetrasphaera* as detected by the probes designed by Kong et al. (2005) had a positive correlation, indicating that the same factors promote their presence in treatment plants. *Tetrasphaera* clade 1 had a medium strength correlation with clades

Table 3 – Distribution of different clades of *Accumulibacter* (based on *ppk1* gene) compared with quantitative FISH analyses in 27 Danish wastewater treatment plants with and without EBPR.

WWTP name	Type of WWTP	Clade I	FISH Clade I	Clade IIA	Clade IIB	Clade IIC	Clade IID	FISH Clade II
Bjergmarken	EBPR	–	+	+	+	+	–	+
Egå		+	+	–	+	+	–	+
Ejby Mølle		+	+	+	+	+	–	+
Hjørring		–	–	+	+	–	+	+
Skive		+	+	–	–	+	–	+
Aalborg West		+	+	–	–	+	–	+
Aalborg East		+	+	+	+	+	–	+
Boeslum		+	+	–	–	+	–	+
Fornæs		–	–	–	–	+	–	+
Fredericia		+	+	–	–	–	–	–
Haderslev		–	–	–	–	+	+	+
Kerteminde		+	+	–	–	+	–	+
Kolding		+	+	–	–	+	–	+
Mørke		+	+	–	–	–	–	–
Odense NE		+	+	–	–	+	–	+
Randers		–	–	–	+	+	–	+
Ringkøbing		+	+	–	–	+	–	+
Søholt		+	+	–	–	+	+	+
Viby		+	+	–	–	–	–	–
Åby		+	+	–	–	+	–	+
Avedøre	Non EBPR	+	+	–	+	+	–	+
Hirtshals		+	+	–	–	+	–	+
Horsens		–	+	–	–	+	–	+
Marselisborg		+	+	–	–	+	–	+
Odense NW		–	–	–	–	+	–	+
Viborg		–	–	+	+	+	–	+
Aars		–	–	+	+	+	–	+

2A and 3 (as defined by Nguyen et al., 2011) (Fig. 5a). Other correlations were either weak or not significant. The fraction of *Accumulibacter* not detected by the two clade-specific probes (total minus clade I + II) and the total amount of *Tetrasphaera* was also used in the analyses, but did not indicate any strong correlations (data not shown).

3.8.2. *Correlations between PAO/GAO populations and treatment plant design and process parameters*

Two types of EBPR plant design were included in the correlation analysis: alternating operation and recirculation. Furthermore, they were either with or without side stream hydrolysis (Table 1A). The system configurations were indicated as binary values (1 for yes and −1 for no). This correlation analysis (Fig. 5b) showed no significant correlations, meaning there does not appear to be any direct pattern or affinity between the tested plant configurations and bacterial group abundances.

The abundance of the PAO and GAO groups was also tested in order to find correlations with processes recorded in the plant parameters (Fig. 5b). Only one significant strong correlation was found, the sludge loading correlated with *Tetrasphaera* Type 1 (nomenclature after Kong et al., 2005). This was not confirmed for the different *Tetrasphaera* clades. Other medium strength correlation was also found between *Tetrasphaera* Types 1 and 2, *Competibacter*, and percentage of industrial wastewater.

Plant by plant correlation analyses were also performed. When data, (both process parameters and qFISH data) from each of the seven plants sampled 4 times a year over 3 years was used, a medium correlation was found between C/P ratio of influent wastewater and *Accumulibacter* in nearly all plants. Using the same dataset, abundance of *Tetrasphaera* Types 1 and 2 were positively correlated with sludge load and total COD influx (results not shown).

3.8.3. *Principal component analysis*

Principal component analysis was conducted in order to find any correlations not only in one, but in the multivariate planes between different parameters from the microbial database. The bacterial populations, treatment design, and process parameters were used as parameters in the analysis. PCA did not identify any correlations, hence the PCA's correlation circle could not be used for detailed description. Moreover, the two highest factors (1 and 2) explained only 46% of the variables (data not shown).

Attempts to differentiate the observations (samples) in the multivariate space in terms of seasonality, plant design, and process parameters were not successful. For all sets tested there were no patterns, and up to eight principal components were necessary to explain the environments. No specific patterns for PAO and GAO in separate samples from 2009, 2010, and 2011 were found.

3.8.4. *Hierarchical cluster analysis*

Hierarchical cluster analysis was performed in order to find hidden (unclear correlations in 2D graphs) relationships between groups of PAO and GAO. The results depicted in Fig. S4 shows that *Tetrasphaera* clades 1, 2A, and 3 group together, whereas *Tetrasphaera* clade 2B did not affiliate with other clades. The total amount of *Accumulibacter* and its two clades group together. However, as with the *Tetrasphaera* 2B

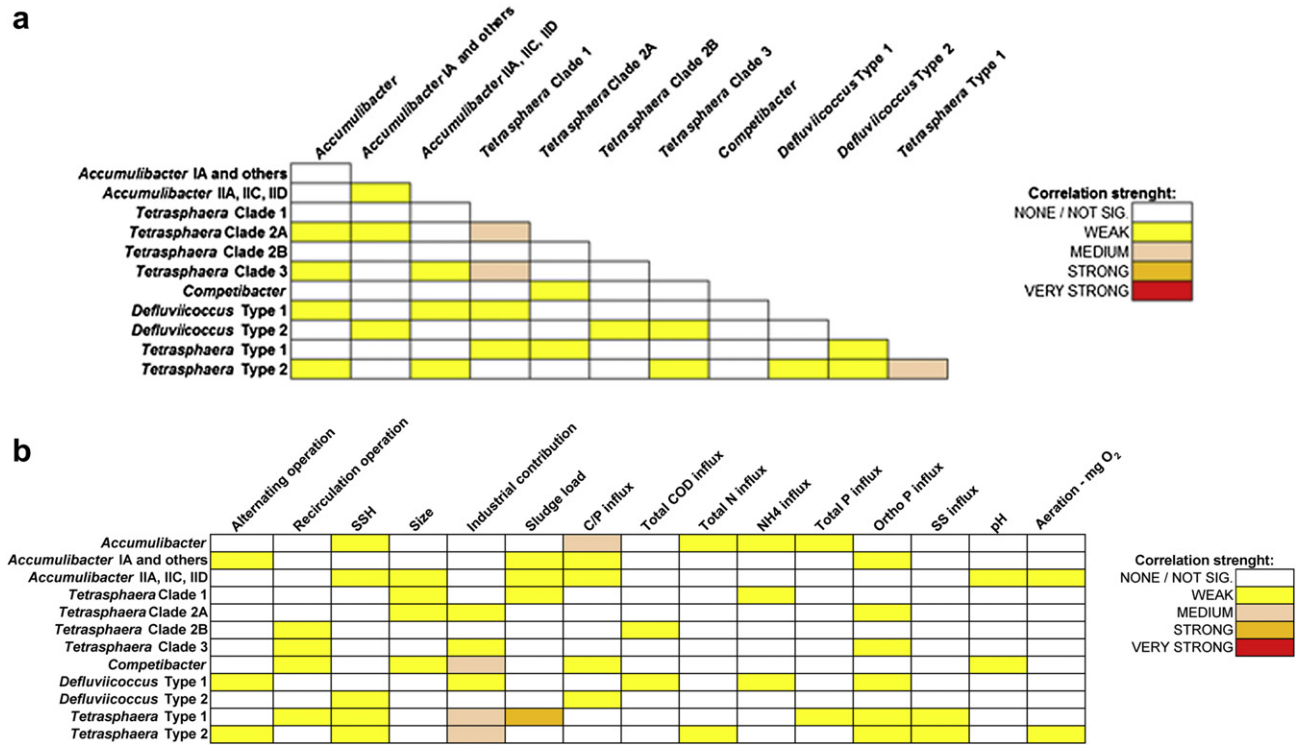

Fig. 5 – a). Correlation analysis showing strength of mutual relationships between single PAO/GAO species. (b). Correlation analysis showing strength of relationships between single PAO/GAO species and process parameters.

clade, *Competibacter* is also clustering completely separately. Fig. S4 shows no direct clustering of PAO and GAO and plant design.

3.8.5. MANOVA

FISH analyses of the 28 treatment plants showed the presence of core PAO/GAO bacteria in all plants. Since the abundances of these were relatively similar among the plants and periods, the general impression was that the composition was relatively stable in each plant over the three years, at least according to the FISH quantification. In order to determine whether each plant consistently had a unique, but rather stable PAO/GAO population, the FISH-quantified populations were considered as a multivariate response with the treatment plant as an explanatory variable. MANOVA multivariate tests were carried out on all samples for all time periods. The test was based on Wilkis' lambda, which differentiates between similarity and diversity among plants. The value of 0.018 of Wilkis' lambda (F (150,220) = 1.03; $p < 0.001$) indicated large statistical difference between the individual plants. Thus, all plants consistently had a unique composition of PAO/GAO throughout the three years. This result is also supported by the distribution of PAO and GAO in the seven plants as seen on the Redundancy analysis plot (Fig. S5), which shows large dispersion of the samples.

4. Discussion

This is the first study in which a large number of full-scale EBPR plants have been investigated in detail with molecular methods for the major putative PAO and GAO groups and their temporal dynamics described over a three-year period. The present very comprehensive set of data from 21 EBPR and 7 non-EBPR plants has made it possible to gain a better understanding of the abundances of the populations, their temporal variations, and, to some extent, which factors might determine the presence and stability of the different populations. It was clearly shown that the different FISH-defined groups of PAO were present in all EBPR plants and are thus members of the core community, whereas the GAOs were only present in few plants. The most surprising and important result is the discovery of distinctly different population compositions, or 'fingerprints', in the different EBPR plants. The common core of PAOs with different relative abundances, and the presence of GAOs in only some plants, resulted in unique plant-specific microbial fingerprints, as shown by MANOVA. This study also shows that bacteria related to the genus *Tetrasphaera* were very abundant in all plants and they can thus be assumed to be important for processes in full-scale EBPR systems.

4.1. *Population composition and dynamics*

The abundance of PAOs was relatively high and typically constituted approx. 15–20% of the entire population. All probe-defined populations of *Accumulibacter* and *Tetrasphaera* clades were present in all 21 EBPR plants, although with diverse abundances. Our data show co-existence between these two groups, as also reported in previous studies by Beer et al. (2006), Kong et al. (2005), Nguyen et al. (2011) and recently in MBR-reactors by Silva et al. (2012). Presumably, these are able to coexist since they have differences in physiology and therefore occupy different niches. This also shows a high

functional redundancy among PAOs and that they all belong to the core community in the Danish EBPR plants. *Tetrasphaera* was the dominant genus (among PAO) in all plants, both EBPR and non-EBPR. However, it is important to mention that not all bacteria covered by *Tetrasphaera* probes always behave as PAO. Most non-filamentous probe-defined *Tetrasphaera* detected in the plants investigated are assumed to be true PAO as they contain polyphosphate and can actively take up radiolabelled orthophosphate under aerobic conditions, after they have accumulated organic substrate in previous anaerobic phases (Nguyen et al., 2011). However, *Tetrasphaera* species are also able to denitrify and ferment and seem to be physiologically very versatile and active in most phases in EBPR plants, not only as PAOs (Kristiansen et al., 2012).

Accumulibacter, which is the model PAO organism, was typically present with 2–8% of the biovolume. This is a lower level than the 4–22% observed in many other full-scale studies (Chua et al., 2006; Gu et al., 2008; He et al., 2008; Kong et al., 2004; Lopez-Vazquez et al., 2008; Saunders et al., 2003; Zhang et al., 2011). Whether this shows actual differences in abundances in full-scale plants or whether methodological variations in conducting qFISH in different studies is not known. Both *Accumulibacter* clades were present in all plants as well as other *Accumulibacter* not targeted by the two clade-specific probes. A high microdiversity of *Accumulibacter* clades in full-scale plants was reported by He et al. (2007), and besides the fact that gene probes targeted all *ppk*-defined clades (Flowers et al., 2008), there were still some cells not targeted by clade-specific probes. However, this is not so surprising, since Flowers et al. (2008) has emphasized that these probes should be used in lab-scale reactors. This thus, also shows the higher diversity of *Accumulibacter* in full-scale systems and depicts the necessity of investigating the remaining unknown part. Interestingly, in some plants, there were many clades (based on *ppk1* genes), whereas, in others, there were few. This clade diversity was also found in the metagenome of Aalborg East treatment plant (Albertsen et al., 2012), and such diversity is assumed to be important for the stability of treatment plants (He and McMahon, 2011b). Potentially, the different clades have different sensitivities to phage attack, one of the factors that has been suggested to influence their population composition (Kunin et al., 2008). Such microdiversity may also have a stabilizing effect on the performance of the EBPR process.

The three clades of *Tetrasphaera* also showed very high phenotypic microdiversity, with each clade probe set giving a positive hybridisation signal with cocci, rods, and filamentous morphotypes. It is still not possible to resolve this diversity with available FISH probes. The two probes Actino-221 and Actino-658 (Kong et al., 2005) only covered a part of the three *Tetrasphaera* clades described by Nguyen et al. (2011). Probe Actino-221 covers largely the same sequences as Tet2-892, but does not target the sequences covered by Tet2-174. In clade 3, probe Tet3-654 targets almost the same sequences as Actino-658, but microscopy shows that probe Tet3-654 targets more morphologies and more cells (Nguyen et al., 2011). Thus, the recommendation for future FISH studies is use of Tet1-266 for clade 1, Tet2-174 and Tet2-892 for clade 2, and Tet3-654 for clade 3.

Both GAO species investigated, *Competibacter* and *Defluviicoccus*, were only present in significant numbers (>0.25%) in less than half of all EBPR plants (11 and 6 plants, respectively). Other studies (i.e., Burow et al., 2007; Lopez-Vazquez et al., 2009b; Wong et al., 2004) also describe relatively similar abundance for both *Competibacter* and *Defluviicoccus* not exceeding 3–4%. These studies all took place in the relatively temperate part of Europe, which may explain the abundance being lower than observed in warm climates such as Australia (Burow et al., 2007).

In this study, we only applied the broad GAOmix probes to quantify *Competibacter* and did not investigate any potential microdiversity for the GAOs. It is known from previous studies that *Competibacter* has a significant microdiversity in Danish plants, with several probe-defined subgroups (Kong et al., 2006). It was interesting that a few plants (Ejby Mølle, Bjergmarken, Hjørring) had GAOs constantly during the three-year investigation period. This indicates that some plant-specific factors (wastewater, design, operation) determined the presence of these bacteria in the plants. The three plants with the consistent presence of GAO all had a relatively high content of industrial contribution, which may explain their presence, either due to surplus organic substrate in wastewater or due to the presence of specific substrates that may promote the GAOs. This is also in agreement with the observed correlation for *Competibacter* abundance with the amount of industrial contribution and the sludge loading (see below).

Filamentous *Defluviicoccus* can cause bulking problems if present in large numbers in EBPR plants (Nittami et al., 2009), but they were only found in a few plants and in very low numbers (see details in Mielczarek et al., 2012).

All non-EBPR plants also had PAO, and three of these plants also had GAOs in amounts higher than 0.25%. *Tetrasphaera* and *Accumulibacter* were detected in lower abundances than in EBPR plants, but were present in all periods and all plants. Their relatively high abundance might be due to presence of occasional anaerobic periods in the plants with alternating operation, providing suitable growth conditions, although the systems were not designed for biological P removal. The presence of *Accumulibacter* and *Tetrasphaera* has also been shown in MBR reactors without a defined anaerobic zone (Silva et al. 2012). The extent of anaerobic periods to ensure presence of *Accumulibacter* has been discussed, and there are reports of EBPR systems without anaerobic phases where the *Accumulibacter* are present and potentially responsible for observed P-removal (Ahn et al., 2007). It might also be possible that *Accumulibacter* is adjusting to the conditions by switching to alternative metabolic pathways as proposed by Ahn et al. (2009) and Barat et al. (2006). The presence of GAO in two non-EBPR plants has also been observed in different types of plants worldwide (McIlroy and Seviour, 2009; Pisco et al., 2009) showing that GAO can be present in treatment plants without constant and stable anaerobic periods. Since *Competibacter*, *Accumulibacter*, and *Tetrasphaera* contain denitrifying strains, this capability may also explain their wide distribution in non-EPBR systems.

Most probe-defined PAO populations showed a high degree of temporal stability. The two plants with very frequent sampling (twice per week, Hjørring and Aalborg East) showed marginal short-term fluctuations. All seven plants investigated 12 times over three years showed only limited variation, indicating presence of very stable communities. Only two

Tetrasphaera clades (clade 1 and 2A) showed some temporal variation. This may indicate that the communities have reached a level of equilibrium and that the plants were running consistently with relatively small variations in wastewater characteristics and plant operation (i.e., stable COD level – data not shown). This is in agreement with a previous study of the filamentous bacteria in the same plants (Mielczarek et al., 2012). The GAO populations showed temporal variations in many plants, but were also constant in a few plants as described above.

Only a few other studies have investigated population stability of microbial communities in full-scale plants (Ofiteru et al., 2010; Wang et al., 2009, 2010a, 2010b, 2010c, 2011). The majority have observed a higher level of temporal variations and also a larger process instability compared to our study. However, the population dynamics were analysed with the use of T-RFLP, functional genes analyses, and DGGE, which can be difficult to compare to qFISH as FISH probes often cover several closely related strains/species. Other observations from pilot-scale or lab-scale reactors typically show much greater and more significant changes in bacterial community structure, particularly among heterotrophic bacteria (Kaewpipat and Grady, 2002; Nadarajah et al., 2007). As discussed elsewhere (Mielczarek et al., 2012), the relatively high stability in the Danish plants may be due to the fact that all plants are large nutrient removing plants with long-term operational stability (experienced plant operators with excellent supervision), alternating aerobic/nitrifying and anaerobic/denitrifying conditions, long sludge age (25–35 days), moderate annual temperature range (7–20 °C), stable pH, and a rather complex wastewater composition. These are all factors that might have a great impact on the temporal stability of the entire community.

The method applied to describe the diversity may to some extent explain the different results in population stability in the different studies. The FISH-based approach relies on relatively broad probes, which do not reveal changes in abundances in very closely related strains. Use of DGGE or TRFLP finger-printing methods may reveal, at least in qualitative terms, fine-scale changes in the populations (e.g., Ofiteru et al., 2010). However, bias related to extraction of nucleic acids and/or PCR, as well as varying copy numbers of the 16S rRNA gene in different species, makes this type of investigation difficult to compare with the quantitative FISH analysis (Albertsen et al., 2012). The FISH method, together with the more novel amplicon sequencing, remains the methodological approach of choice for a quantitative analysis of these microbial communities. Furthermore, FISH provides an important visualization of the spatial arrangement of bacteria in the aggregates.

4.2. *Correlations between PAO/GAO, plants and operation*

Several factors related to the wastewater characteristics, plant design and operation are believed to affect the presence of PAO and GAO. Only a few full-scale studies exist, and they have shown that PAOs are primarily affected by pH, temperature, COD/P ratio, presence of pre-settling, and sludge digestion, and more (He et al., 2007, 2010; Lopez-Vazquez et al., 2008, 2009a, 2009b; van Loosdrecht et al., 1997; Whang and Park, 2006). Although we included all these factors in our global analyses of the full-scale plants, only a few (weaker) correlations were found for the amount of industrial contribution and the sludge loading. They both promoted *Tetrasphaera* and *Competibacter*. The plant-specific investigations also showed a positive correlation to high C/P ratio and loading, so it seems this study supports the general observation that PAOs and GAOs are most abundant in plants with a high level of organics in the wastewater. It was interesting that the use of an alternating or recirculating plant design did not affect the PAO/GAO populations. Also, we were not able to see correlation of either GAO/PAO with total nitrogen, as seen by Zhang et al. (2011).

Correlation and clustering analyses did not reveal any strong affiliation with any of the bacterial species and plant design and operation. However, FISH numbers showed that more plants with SSH were free of *Competibacter*, compared to plants without, in specific years. More studies into the direct influence of this system configuration on bacterial populations in treatment plants are necessary.

It was not possible to check either temperature or pH effect on GAO populations, as was suggested by other studies (Lopez-Vazquez et al., 2009b; Oehmen et al., 2005a, 2007), mainly because Danish treatment plants do not operate at high temperatures (>20 °C) and have very constant pH values.

When we narrowed the analyses to single plants over time we found a few medium strength correlations between process parameters and microbial populations. The C/P ratio and *Accumulibacter* (in nearly all plants) correlated, and *Tetrasphaera* types 1 and 2 were positively correlated with medium strength with sludge load and total COD influx. Similar types of correlations were found regarding filamentous microorganisms in the same treatment plants (Mielczarek et al., 2012). Another explanation could be that some of the probes applied were relatively broad (PAOmix and GAOmix), so changes or variations in specific populations were not possible to observe.

The high similarity of the Danish EBPR plants in terms of plant design, operation and wastewater composition, with only small variations of PAO-GAO communities, made it difficult to discover global correlations. This was also stressed by the fact that the correlations became weaker the more data we included, with few strong correlations left when several years were included. Since the individual plants seem to be relatively stable in community composition and operation, a better way is to study the individual plants in more detail over time to discover factors of importance for the specific populations. Major changes in wastewater characteristics, plant design, or operation may reveal more specific changes in the PAO/GAO populations.

5. Conclusions

- Both *Accumulibacter* and *Tetrasphaera* were abundant PAOs, and their numbers were relatively stable throughout a three-year observation period in 28 Danish full-scale activated sludge treatment plants with N and P removal. In the EBPR plants, these PAOs constituted on average of 30% of the bacteria detected by the EUBmix probes.

- *Tetrasphaera* was the most abundant PAO (average 27%) and in all EBPR plants significantly outnumbered *Accumulibacter* (average 4%).
- The two GAOs, *Competibacter* and *Defluviicoccus*, were present in only a few plants and then in lower abundances than *Accumulibacter*. Their presence did not correlate with poor plant P-removal performance.
- The abundance of PAOs and GAOs was lower in plants without EBPR design.
- It was possible to distinguish population compositions in the different EBPR plants. The relative compositions of PAO and GAO in the individual plants over three years were unique in each plant, as shown by MANOVA.
- The abundance of *Competibacter* correlated with high industrial contribution to the wastewater. In specific plants *Accumulibacter* correlated with high C/P and *Tetrasphaera* with high loading.
- Few global correlations were found between PAO/GAO population abundances and plant design, operation, and wastewater characteristics.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2012.12.003.

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