

POLYPHOSPHATE ACCUMULATING ORGANISMS IN TREATMENT PLANTS WITH DIFFERENT WASTEWATER COMPOSITION

Adam MUSZYŃSKI ^{a*}, Monika ZAŁĘSKA-RADZIWIŁŁ ^b

^a Dr.; Faculty of Environmental Engineering, Department of Biology, Warsaw University of Technology, Nowowiejska 20, 00-653 Warsaw, Poland

*E-mail address: adam.muszynski@is.pw.edu.pl

^b Associate Prof.; Faculty of Environmental Engineering, Department of Biology, Warsaw University of Technology, Nowowiejska 20, 00-653 Warsaw, Poland

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Abstract

Fluorescence *in situ* hybridization was applied to investigate the community structure of polyphosphate accumulating organisms (PAOs) in activated sludge of four full-scale wastewater treatment plants (WWTPs) with different configurations of bioreactors and wastewater composition. PAOs constituted 18-36% of the entire bacterial biovolume and they were more abundant in autumn than in spring. More than half of the probe-defined PAOs community were *Tetrasphaera*, which were particularly abundant in WWTPs with significant contribution of industrial wastewater or sewage from septic tanker trucks. *Accumulibacter*-PAOs accounted for up to 12% of all bacteria and were more abundant in the WWTP receiving typical domestic wastewater. *Halomonas*-PAOs did not generally exceed 1% of total biovolume and constituted a minor fraction of the PAOs community in all the tested WWTPs. Significant contribution of industrial wastewater and sewage from septic tanker trucks were the decisive factors in determining the PAOs community structure.

Streszczenie

Zbadano strukturę populacji bakterii akumulujących fosfor (PAO) w osadzie czynnym czterech oczyszczalni różniących się układem technicznym i składem dopływających ścieków. Badania prowadzono metodą fluorescencyjnej hybrydyzacji *in-situ*. Ogólna liczebność PAO w osadzie czynnym była wyższa na jesieni niż na wiosnę i zawierała się w granicach 18-36% ogółu bakterii. Ponad połowa PAO w badanych układach należała do rodzaju *Tetrasphaera*, które były szczególnie liczne w osadzie czynnym układów z dużym udziałem ścieków dowożonych transportem asenizacyjnym oraz pochodzących z przemysłu spożywczego. Zawartość *Accumulibacter* nie przekraczała 12%, osiągając najwyższe wartości w oczyszczalni, do której doprowadzono typowe ścieki bytowe. Liczebność *Halomonas* nie przekraczała na ogół 1% i bakterie te stanowiły mniejszość w populacji PAO we wszystkich badanych układach. Istotnym czynnikiem, wpływającym na strukturę PAO, był rodzaj dopływających ścieków, a w szczególności udział ścieków przemysłowych i dowożonych transportem asenizacyjnym.

Keywords: Activated Sludge; Nutrients Removal; *Accumulibacter*; *Tetrasphaera*; *Halomonas*.

1. INTRODUCTION

Enhanced biological phosphorus removal (EBPR) is based on circulation of activated sludge through anaerobic and aerobic conditions to enrich polyphosphate-accumulating organisms (PAOs). In anaerobic conditions PAOs take up readily degradable organic substrates and store them as intracellular polymers -

polyhydroxyalkanoates (PHAs). The energy for this comes from hydrolysis of intracellular polyphosphate (poly-P) and orthophosphate is released from PAOs cells. In aerobic conditions, PAOs use the stored PHAs as the endogenous source of carbon and energy for biomass synthesis. The surplus energy is used for the excessive orthophosphate uptake and stored in

poly-P chains. Phosphorus is eliminated from wastewater by removal of excess activated sludge with high poly-P content.

Majority of studies on abundance of PAOs in activated sludge are carried out in laboratory-scale reactors or pilot plant systems [1, 2]. Lab-scale reactors, which are fed with synthetic sewage containing usually only acetate as a single energy source, are dominated by “*Candidatus Accumulibacter phosphatis*” (hereafter referred to as *Accumulibacter*), which can account for up to 90% of all bacteria [3]. These PAOs can play an important role also in full-scale systems where they constitute 5-22% of the community biomass, as demonstrated by Kong et al. [4]. Zilles et al. [5] showed that *Accumulibacter* represented 13-18% of the total bacterial population in two American full-scale WWTPs. In seven Dutch municipal WWTPs, *Accumulibacter* comprised in average around 9% of total biomass [6].

Other putative PAOs, which are frequently detected mostly in full-scale systems, are members of

Tetrasphaera-genus. These PAOs were detected in high numbers (up to 25 and 35% of the bacterial biomass) in full-scale EBPR plants studied in Australia and Denmark, respectively [7-9]. *Tetrasphaera*-related PAOs were particularly abundant in industrial WWTPs, although they were present in significant numbers also in plants treating mainly domestic wastewater [8].

A potentially important role of other PAOs in the EBPR process was suggested by Nguyen et al [10]. The research, carried out in 30 full-scale Danish WWTPs, revealed that “*Candidatus Halomonas phosphatis*” (hereafter referred to as *Halomonas*) made up 0.5-5.7% of all bacteria and were often in higher abundance than *Accumulibacter*.

The aim of the study was to investigate the abundance of potential PAOs in full-scale WWTPs in Poland. Quantitative fluorescence in situ hybridization (qFISH) with the recently evaluated oligoprobes was used to analyse the PAOs community structure (including *Accumulibacter*, *Halomonas* and

Table 1.
Operational data and influent characteristics of the WWTPs investigated in this survey

Parameter	WWTP I	WWTP II	WWTP III	WWTP IV
size (PE)	73 400	99 000	76 000	18 500
reactor type	A2O	AO (anoxic+aerobic)	UCT	Carrousel (anoxic+aerobic)
predenitrification	Yes	No	No	No
presettling	Yes	Yes	Yes	No
fermenter	Yes	No	No	No
selector	No	No	No	Yes
aeration	fine bubble diffusers	surface (vertical type)	fine bubble diffusers	fine bubble diffusers surface (vertical type)
supplementary chemical P-precipitation	Yes	Yes	Yes	Yes
wastewater type (% of overall BOD ₅)	domestic	domestic industrial (20-25%): <i>slaughterhouse</i> <i>diary</i>	domestic industrial (30-50%): <i>fruit & vegetables</i> <i>processing</i> , <i>sugar refining</i>	domestic septic tanker trucks (10-25%)
COD [mg/L]	622-3645	303-4099	508-3259	512-1760
BOD ₅ [mg/L]	220-1300	260-2576	380-1900	320-790
N total [mg/L]	51-133	41-113	50-129	54-190
P total [mg/L]	18-124	7-32	11-64	5-21
pH	7.5-7.9	7.1-8.2	7.0-8.4	7.1-7.8
comments	sludge bulking	sludge overloadings shock loadings unstable N removal	foaming (winter) industry campaigns: – seasonal sludge overloadings – shock loadings	septic tanker trucks: – shock loadings – toxic wastewater

Tetrasphaera), in four WWTPs with different configuration and wastewater. The microbial population was examined twice – in spring and in autumn, after periods of stable low and high temperatures, respectively. The obtained results show richness of PAOs community in activated sludge of full scale WWTPs, with composition of the inflowing wastewater as a key factor in the PAOs selection.

2. MATERIALS AND METHODS

2.1. Sampling and WWTPs data

Four full-scale WWTPs, located near Warsaw (Poland), and differing in configurations and treated wastewater, were selected. The main operational parameters of the plants are listed in Table 1 (all data provided by the plants operators). The plants ranged in size from 18,500 to 99,000 population equivalents (PE). The fraction of industrial contribution (from food industry mainly) to the organic matter in the influent, depending on the plant, was 0-50%. All the WWTPs had biological N-removal (nitrification and denitrification) and two of them (WWTP I and WWTP III) also the well-defined enhanced biological phosphorus removal (EBPR) step. The plants experienced only minor operational problems (deterioration of nitrification and sludge settling properties in winter), however, biological phosphorus removal was insufficient. Iron-based coagulants (PIX) were dosed occasionally in all plants to improve phosphorus elimination when P concentration in effluent exceeded the respective maximum permissible values. In WWTP I the EBPR process was intensified by producing in a fermenter volatile organic compounds from primary and secondary sludge. Effluent from the fermenter, rich in easily degradable substrates, was pumped into the predenitrification and anaerobic tanks.

The research was carried out within one year. Activated sludge samples were collected twice, in early spring (at the beginning of March) and in early autumn (at the end of September), on the assumption that the microbial community structure was then stable after long periods of low and high temperatures, respectively. The samples were kept on ice during transportation to the laboratory and then fixed properly for FISH analyses in paraformaldehyde or in ethanol for Gram-negative and Gram-positive bacteria, respectively, as detailed by Nielsen et al. [11].

2.2. qFISH analyses

FISH procedures were performed according to Nielsen et al. [11]. When required, permeabilisation of the cells was applied to improve the penetrability of oligonucleotide probes, using lysozyme (360 000 U/ml), achromopeptidase (60 U/ml) and 0.1M HCl solutions. A 6-Fam labelled EUBmix oligoprobe (equimolar mixture of EUB338, EUB338II, and EUB338III) was used to target the entire bacterial community.

Oligoprobes PAOmix (equimolar concentration of PAO462, PAO651 and PAO846), Hal180 and Tet1-266, Tet2-174, Tet2-892, Tet3-654, targeting most *Accumulibacter*-, *Halomonas*- and *Tetrasphaera*-related PAOs, respectively, were labelled with Cy3. The more broadly specific Pse136 probe (labelled with Cy3) was applied in a hierarchical approach to decrease the possibility of false positives for probe Hal180. When necessary, the probes were used in combination with their corresponding unlabelled competitors and/or helpers. Detailed information about the probes used in the study is given in probeBase [12].

Similar quantification procedures were performed to those previously described [2]. Twenty separate images for each probe were captured with a Nikon Eclipse 50i microscope. ImageJ software [13] was used to determine the biovolume of bacteria, which is relative to the pixel area of the cells positive with the specific probe. The microbial abundance (expressed as a percentage of the EUBmix probe) was then quantified as a percentage of the pixel area for all bacteria positive with the EUBmix probe and was calculated as a mean of 20 separate measurements. SE was calculated as a standard deviation of the percentage abundance of the specific bacteria divided by a square root of 20 measurements.

3. RESULTS AND DISCUSSION

The community structure of PAOs in activated sludge was determined by qFISH using oligoprobes targeting three different genera: *Accumulibacter*, *Tetrasphaera* and *Halomonas*. Bacteria hybridizing with these probes constituted 18-36% of the biovolume determined by the EUBmix probe. This is consistent with the results obtained by Mielczarek et al. [14] for 28 Danish full-scale WWTPs, where PAOs on average accounted for 30% of all bacteria. Seasonal variations in the abundance of entire PAOs community was observed in each WWTP – more bacteria were detected in autumn than

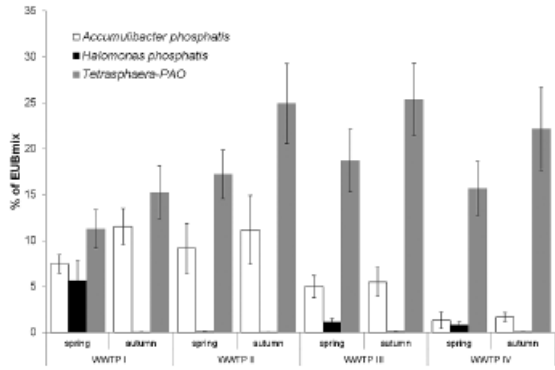


Figure 1. The abundance of putative PAOs in activated sludge of the investigated WWTPs, determined by qFISH using oligoprobes PAOmix, Hal180 and Tet1-266+Tet2-892+Tet2-174+Tet3-654 for detection of *Accumulibacter phosphatis*, *Halomonas phosphatis* and *Tetrasphaera*-related PAOs (sum of clades 1, 2A, 2B and 3), respectively. The error bars illustrate the calculated standard error

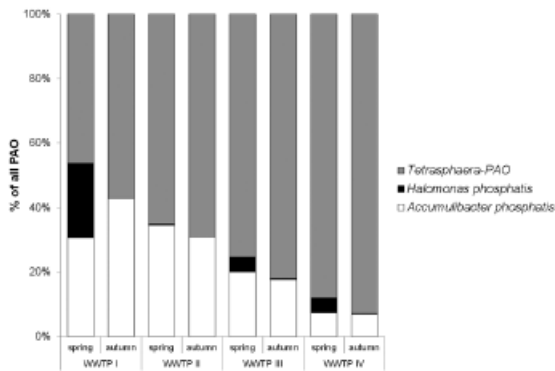


Figure 2. PAOs community structure in activated sludge of the investigated WWTPs, determined by qFISH. For description of the respective oligoprobes see Fig. 1

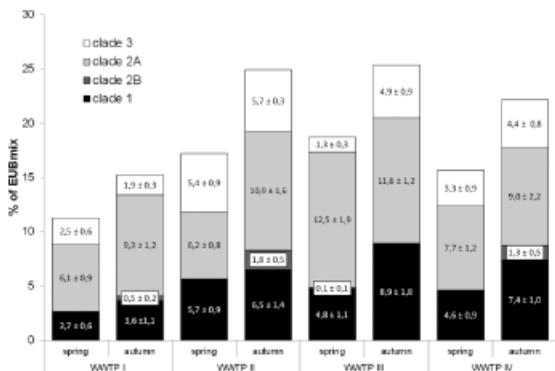


Figure 3. The composition of *Tetrasphaera*-related PAOs population in the investigated WWTPs, determined by qFISH. Percentages of individual populations and standard errors are shown. For description of the respective oligoprobes see Fig. 1.

in spring (Fig. 1). Interestingly, the percentage of each tested probe-defined population varied in different WWTPs, but usually more than half of the probe-defined PAOs population were *Tetrasphaera*-PAOs (Fig. 2). *Tetrasphaera*-PAOs were particularly abundant in plants with significant contribution of industrial wastewater (WWTP II and III) or sewage from septic tanker trucks (WWTP IV). Three clades of *Tetrasphaera*-PAOs targeted by 4 probes constituted 11-25% of the total bacterial biomass present in 4 tested WWTPs. This is comparable to results (18-30%) obtained by Nguyen et al. [9] in 5 Danish WWTP with recirculating (A2O) and BioDenipho (an anaerobic chamber ahead of a tank with alternating denitrifying and nitrifying conditions) configurations. Beer et al. [15] and Kong et al. [8] demonstrated also that *Actinobacteria* are present in large numbers in full-scale WWTPs and may play an important role in EBPR processes. Most plants examined by Beer et al. [15] had 3-stage Bardenpho, UCT and Carrousel configurations, similar to WWTPs I, III and IV, respectively, whereas Kong et al. [8] tested a Biotenipho WWTP. Mielczarek et al. [14] showed that *Tetrasphaera* in 28 Danish WWTPs comprised typically 20-25% of all bacteria and these PAOs significantly outnumbered *Accumulibacter* (2-8%), regardless the bioreactor configuration. However, in specific plants *Tetrasphaera* correlated with high organic loading. Our research revealed also that *Tetrasphaera*-PAOs were particularly abundant (up to 25% of total bacterial biomass) in WWTPs II and III with high BOD₅ of wastewater.

In the tested WWTPs the most abundant *Tetrasphaera*-PAOs were branched rods, short rods in clumps and clusters of tetrads from clades 2A and 1, targeted by Tet2-892 and Tet1-266 (6-13% and 3-9% of all bacteria, respectively) (Fig. 3). Less numerous (1-6% of EUBmix) were short and branched rods and small cocci from clade 3 (targeted by Tet3-654). These results were close to those obtained by Nguyen et al. [9] and Mielczarek et al. [14]. However, in this survey clade 2B of *Tetrasphaera*-PAOs (targeted by Tet2-174) was detected in lower abundance (< 2%), only in autumn in WWTPs I, II and IV (filaments from this *Tetrasphaera* group were not quantified as they do not take up phosphate aerobically).

The range of *Accumulibacter* abundance (1-12%) in this study was comparable to those reported by Nielsen et al. [16], López-Vázquez et al. [6], Wong et al. [17] and Gu et al. [18] (1-11%, 6-16%, 4-18% and 5-15%, respectively), but slightly lower than those observed by He et al. [19] in full-scale municipal WWTPs (9-24%). However, despite this distinct dif-

ference in bacterial abundance there were no clear correlations between plant design and *Accumulibacter* percentages in activated sludge – majority of the WWTPs examined by the cited authors, as well as in the present study, had similar configurations (UCT, modified UCT, A2O and AO). Furthermore, the abundance of *Accumulibacter* in Danish plants with Biotrid configuration, which were examined by Nielsen et al. [16] and Mielczarek et al. [14], was almost the same as in the plants with recirculating design (A2O, AO or UCT) or Carrousel bioreactors, tested by the same or other authors [6, 14, 16, 17, 18]. It seems that use of an alternating or recirculating plant design did not affect the *Accumulibacter* populations and a type of wastewater and concentration of different substrates in influents were more decisive factors in determining the PAOs community structure.

The relative abundance of *Accumulibacter* in the whole PAO population reached the highest values in WWTP I (Fig. 2), which received typical domestic wastewater. Kong et al. [8] showed also that *Accumulibacter* were dominant PAOs mainly in the domestic plants (9-17%), but hardly present (3%) in most industrial WWTPs receiving food processing wastewater. *Halomonas*-PAOs did not generally exceed 1% of total biovolume in the surveyed WWTPs in this study (except WWTP I in spring when these bacteria accounted for nearly 6%), and these bacteria constituted a minor fraction of the PAOs community in all tested WWTPs. However, the survey conducted by Nguyen et al. [10] on the occurrence of *Halomonas* in 23 full-scale EBPR plants revealed that they constituted up to 6% of all bacteria, and they may be more abundant than *Accumulibacter*.

Muszyński et al. [20] investigated long-term dynamics of microbial population in activated sludge of a full-scale wastewater treatment plant over a two-year-period and concluded that variations in microbial community structure reflected differences in bacterial ecophysiology and affinities to different substrates. *Tetrasphaera*-PAOs are more diverse in substrate uptake than *Accumulibacter*. Most of them take up amino acids and glucose preferably under both anaerobic and aerobic conditions [8]. Some *Tetrasphaera* members are even more versatile and, apart from glucose or amino acids, they can also utilize glutamate and acetate in anaerobic phase [9]. Furthermore, most *Tetrasphaera* ecotypes excrete extracellular amylases and are specialized degraders of starch and other polysaccharides [16]. Therefore *Tetrasphaera* are highly abundant in WWTPs receiv-

ing industrial wastewater from food processing, as it was in the case of WWTPs II and III. As these PAOs are able also to ferment glucose and grow under anaerobic conditions, they occupy different ecological niches in comparison to *Accumulibacter*, which can take up primarily volatile fatty acids (acetate and propionate) and, to less extent, amino acids [21]. On the other hand, *Halomonas*-PAOs were shown to take up a narrower selection of substrates (only acetate, propionate and ethanol) compared with *Accumulibacter*, which suggests that only relatively few low molecular-weight substances present in wastewater can be utilized by these microorganisms [10]. It can be concluded that decisive factors in deciding which of the PAO populations outcompete the other in activated sludge of EBPR systems, appear to be availability of acetate (or other volatile fatty acids), glucose, amino acids and ethanol, as well as long residence time in anaerobic tanks, where the ability to ferment is substantial for growth.

The PAOs community structure in the investigated full-scale WWTPs was different from those observed in lab-scale reactors fed with acetate, which are usually dominated by *Accumulibacter* clades [22, 23]. However, lab-scale systems are usually operated as fill-and draw reactors under artificially controlled conditions. Therefore bacteria in such conditions experience selective pressures which are markedly different from those encountered in full-scale continuous-flow systems. Well-defined operational parameters in lab reactors reduce the number of ecological niches available to bacteria [2]. One of the most important factors affecting PAOs community is C/P ratio in influent. It was shown that that low COD/P ratio promotes growth of PAOs in both flocculent and granular sludge in lab-scale reactors [23, 24]. PAO community in granular sludge can be almost completely replaced by GAOs when dosage of phosphate is stopped [25]. Under extended phosphorus limitation in wastewater, PAOs no longer synthesize poly-P and behave as GAOs [23, 26, 27]. On the other hand Mielczarek et al. [9] showed that *Accumulibacter* correlated with high C/P ratio of wastewater in full-scale Danish WWTPs. However, Nielsen et al. [16] concluded that the concentration of different substrates in the inflowing wastewater rather than BOD₅, COD or sludge loading, seems to be the more decisive factor in determining the microbial community structure. This study showed that PAOs community structure was affected by a contribution of industrial wastewater and/or sewage from septic tanker trucks.

4. CONCLUSIONS

In the tested full-scale WWTPs with nutrients removal:

- composition of the inflowing wastewater seem to be key factors in the PAOs selection; significant contribution of industrial wastewater and sewage from septic tanker trucks affected PAOs community structure to a large extent,
- *Tetrasphaera*-related bacteria seem to be the major PAOs, *Accumulibacter* were less abundant,
- *Halomonas*-PAOs constituted a minor fraction of the PAOs community.

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