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Comparative analysis of biological phosphate removal (BPR) and non-BPR activated sludge bacterial communities with particular reference to *Acinetobacter*

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The bacterial community of a biological phosphate removal (BPR) activated sludge process was studied and compared to that of a non-BPR process treating the same municipal waste water. Bacterial isolates from the BPR process, as characterized by whole cell fatty acids, belonged to more than twenty genera, with *Micrococcus, Staphylococcus* and *Acidovorax* scoring highest. *Acinetobacter* spp represented 4% of cultured bacteria, ≤3% as estimated by fluorescence *in situ* hybridization, and well under 10% on the basis of the proportion of ubiquinone *Q9* in the sludge. The mole proportions of ubiquinones, *Q8* : *Q10* : *Q9* in the sludge were maintained fairly stable at approximately 9:4:1. The spectra of the isolated strains and the proportions of ubiquinones in the processes (BPR *vs* non-BPR) were otherwise similar, but a significant number of isolates related to actinomycetes were obtained from the BPR sludge only. The BPR process did not enrich *Acinetobacter*. Pure cultures of *Acinetobacter* isolated from the sludge stained for polyphosphate, but *Acinetobacter* cells responding to the ACA probe in native sludge from the BPR process did not. Instead, the bulk of the polyphosphate in the BPR sludge was located in a distinct morphotype of large, coccoid, highly clustered cells.

Keywords: activated sludge; biological phosphate removal; polyphosphate; *Acinetobacter*

Introduction

Bacterial communities in activated sludge have been studied by cultivation [13], by using respiratory quinones [11] or polyamines [2] as chemical biomarkers, by cloning and sequencing 16S rDNA [3,22,23], and by using fluorescence *in situ* hybridization [16,26]. Biological phosphate removal (BPR) in activated sludge is assumed to rely on bacteria accumulating phosphorus in excess as polyphosphate (polyP) [4,8]. Although there are many successful applications and several process configurations for BPR, the roles of different bacteria are obscure [15,21,25]. *Acinetobacter* spp are the best known polyP-accumulating bacteria isolated from activated sludge [17,19], but their importance in BPR *in situ* is not clear. *Acinetobacter* numbers detected in BPR sludge by fluorescence *in situ* hybridization were less than 10% of all bacteria [26], and *Acinetobacter* numbers detected by immunofluorescence did not correlate with the rate of phosphorus removal [5]. In addition to *Acinetobacter*, several other bacteria capable of polyP accumulation have been isolated from BPR sludge [18,24].

We studied the bacterial communities in two processes treating the same municipal waste water in southern Finland, a BPR and a non-BPR process. Both processes incorporated biological nitrogen removal. Aerobic bacteria were cultured from activated sludge and characterized by whole cell fatty acid compositions. The communities were also characterized by using ubiquinones as chemical biomarkers. *Acinetobacter* numbers were estimated and their polyP accumulation was studied in culture as well as *in situ* under process conditions. In addition, the stability of the phosphorus accumulated by isolated bacteria in culture was studied. In the process studied, BPR was shown to rely on a morphologically distinct polyphosphate-accumulating organism, not belonging to the bacterial genus *Acinetobacter*. Isolated *Acinetobacter* strains were unable to retain their accumulated polyphosphate under stationary phase conditions prevailing in activated sludge.

Materials and methods

Sludge samples

Sludge was taken from two activated sludge processes at Suomenoja municipal waste water treatment plant (235 000 population equivalents) purifying residential waste containing 8% industrial waste water. A pilot scale (42 m^3) mixed reactor biological P and N removal process (UCT-type, Figure 1), and a full-scale plug flow biological N removal line (2500 m^3) with simultaneous chemical phosphate precipitation ($FeSO_4 \cdot 7H_2O$, 60 mg L^{-1}) were studied. The latter (recirculation DN-process) incorporated successive anoxic (denitrification) and aerobic (nitrification) compartments. Average sludge ages were 17.5 and 21 days for the BPR and the non-BPR process, respectively. In the BPR process, having a flow rate of 5.5 m^3 h^{-1}, the mean hydraulic retention times were 1.3, 2.7 and 3.6 h, for the anaerobic, anoxic and aerobic compartments, respectively. In the non-BPR process, having a flow rate of 210 m^3 h^{-1}, the mean hydraulic retention time was 5.9 h, for both the anoxic and

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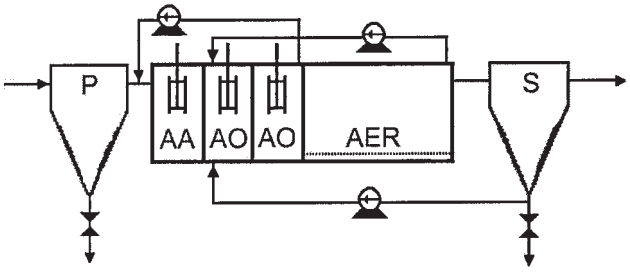


Figure 1 Diagram of the pilot scale BPR process: anaerobic (AA) and anoxic (AO) compartments with sludge propellers, and aerobic (AER) compartment with bottom aeration. Hydraulic retention times were 1.3, 2.7 and 3.6 h in the AA, AO and AER compartments, respectively. (P) primary clarifier, (S) secondary clarifier.

aerobic compartments. The untreated influent waste water contained on the average (mg L^{-1}): total P 7.4, total N 53, and BOD_7 195. Grab samples of mixed liquor were taken from the anaerobic and aerobic compartments of the pilot process and from the anoxic compartment of the full-scale process.

Isolation and identification

Serial dilutions of homogenized sludge samples were plated on Plate Count Agar (Difco, Detroit, MI, USA; diluted 1/5 or full strength) and incubated at 20°C for at least 10 days. All colonies were picked for purification by successive platings from those plates containing the order of 30 colonies. For the isolation of *Acinetobacter*, activated sludge extract agar supplemented with acetate [8] was used. These plates were incubated at 20°C for 2 days. For identification, isolates were grown for 24 h at 28°C on Trypticase Soy Broth (BBL, Cockeysville, MD, USA) agar plates; slow growers for 6 days. Isolates were identified by whole cell fatty acids as described in [27] using the Microbial Identification System (MIDI Inc, Newark, DE, USA) with the aerobic TSBA library Version 3.8.

Microscopy

Bright field microscopy was performed with an Olympus BH-2 microscope with 100× and 10× objectives. Fluorescent microscopy was performed with an Olympus AX70 Provis microscope fitted with UV and U-MNG filters and a 60× objective. Slides on Fujichrome 100 were digitized with a Polaroid SprintScan scanner, and the Adobe PhotoShop 3.0 program was used to overlay the fluorescent oligonucleotide probe and polyP signals. PolyP in the cells was visualized with toluidine blue [7] for bright field, and with 4′,6-diamidino-2-phenylindole (DAPI, 50 μg ml^{-1}) [24] for fluorescence microscopy. Toluidine blue-stained samples were destained for 1–2 min, with 1% H_2SO_4.

Oligonucleotide probing and quinone analysis

Tetramethyl rhodamine-labeled 18 mer oligonucleotide probe ACA (5′-ATCCTCTCCCATACTCTA-3′) for *Acinetobacter* was obtained from MWG Biotech (Ebersberg, Germany). The probe, hybridization, and sample preservation were as described in [26]. The number of ACA positive bacteria on the microscope slides was recorded from field areas representing approximately 2000 counts of total bacteria (after hybridization stained for 5 min with DAPI, 0.3 μg ml^{-1}) [14]. Sludge ubiquinones were extracted and purified as described in [10], but acetone was used for sludge extraction [6]. Samples were prepared and run on HPLC (Waters, Milford, MA, USA) fitted with a C18 reverse phase column (0.3 m × 3.9 mm, 3 μm, Nova-pak) using a 3:1 mixture of methanol and di-isopropyl ether as eluent [20].

^{33}P-phosphate release

Release of accumulated phosphorus was studied in stationary phase cultures in 1/5 diluted Brain Heart Infusion Broth (Difco) at 28° C with shaking (150 rpm). ^{33}P-orthophosphate (110 TBq $mmol^{-1}$; Amersham, Bucks, UK), 30 000 dpm in 100 μl of 10 mM sodium phosphate pH 7.0, was added to the 5-ml cultures containing 3.5 mM sodium phosphate. ^{33}P was measured from centrifuged culture supernatants by liquid scintillation counting after 4 days of growth to calculate P taken up and again after 9 days of growth to calculate the percentage of P released back in 5 days.

Results

Activated sludge bacterial communities were studied in a biologically P- and N-removing pilot scale process (Figure 1) and in a full-scale process line with biological N removal and chemical simultaneous phosphate precipitation. Average P, N and BOD_7 reductions were 72, 74 and 91% for the pilot process and 95, 61 and 98% for the full-scale process line. Both processes received the same waste water having a temperature of about 10°C during winter and about 20°C during summer (average 15°C). Any difference in their bacterial communities therefore should reflect differences in the processes, ie a BPR process *vs* a non-BPR process.

Bacterial isolates from the BPR process characterized by whole cell fatty acids were placed into more than twenty genera (Table 1), with *Micrococcus* (24%), *Staphylococcus* (14%), and *Acidovorax* (13%) scoring highest. *Micrococcus* and *Acidovorax* were also the most frequent isolates in the non-BPR process. The only clear difference between the spectra of the isolates from the two processes was the occurrence (16%) of the Gram-positive, irregular, actinomycete-related organisms *Aureobacterium*, *Cellulomonas*, *Corynebacterium*, *Nocardia*, *Rhodococcus*, *Streptoverticillium* and non-identified tuberculostearic acid-containing actinomycetes among the BPR isolates, and their absence among the non-BPR isolates. In addition, *Moraxella* and *Stenotrophomonas* were found only in the BPR samples. The relative proportion of *Acinetobacter* seemed no higher in the BPR process. The low number of isolates does not allow conclusive judgments, but with the exception of the actinomycete-related organisms, the spectrum of genera found appears much the same in the two processes. However, more than one third of all primary colonies ceased to grow when purified, and many poorly growing isolates were lost before characterization because of takeover of the cultures by *Bacillus cereus* and *B. thuringiensis*. Both of these bacteria seemed to be indigenous to the BPR as well as the non-BPR sludge (not included in Table 1).

Microscopic study of toluidine blue-stained sludges

Table 1 Bacteria isolated from the non-BPR and BPR activated sludge

Genus[a]	Number of isolates		Average match[a], 1000–0 (highest/lowest)
	non-BPR	BPR	
Acidovorax	2	13	430 (796/44)
Acinetobacter	1	4	520 (724/254)
Aeromonas	1	3	660 (841/567)
Aureobacterium		1	660
Bacillus[b]	1	5	610 (837/32)
Cellulomonas		1	600
Clavibacter	1	3	490 (865/94)
Comamonas	1	1	360 (649/69)
Corynebacterium		1	210
Flavobacterium	1		300
Hydrogenophaga		1	370
Janthinobacterium		1	410
Listeria		1	430
Micrococcus	4	24	660 (904/287)
Moraxella		2	640 (698/579)
Neisseria	1	3	630 (715/527)
Nocardia		3	330 (437/73)
Paenibacillus		1	160
Pseudomonas		1	760
Rhodococcus		2	360 (387/339)
Staphylococcus	1	14	480 (796/117)
Stenotrophomonas		2	490 (964/17)
Streptoverticillium		1	330
Actinomycete[c], NI[d]		7	
NI	3	5	
Total	17	100	

[a]Suggestion of the whole cell fatty acid identification system used (library version 3.8).
[b]Excluding *B. cereus* and *B. thuringiensis*.
[c]On the basis of tuberculostearic acid.
[d]NI, not identified.

revealed that the polyP-containing microbial cells in the BPR sludge were unevenly distributed and formed discrete patches (Figure 2), in contrast to the non-BPR sludge (with simultaneous chemical phosphate precipitation), where very small or no polyP-containing microcolonies were seen (Figure 3). The bulk of the toluidine blue positive matter (polyP) in the BPR sludge concentrated in one morphotype of cells: bigger than average, intensely staining coccoid cells, occurring in large strikingly homogenous clusters (Figure 4). Stained with DAPI (50 μg ml^{-1}) these polyP-containing clusters showed bright fluorescence (Figure 5).

Acinetobacter cells were counted after hybridization with the tetramethyl rhodamine-labeled ACA probe (Figure 6) and numbered in all samples $\leq$ 3%. Samples were taken during 25.7.95–6.11.95 at 7 days from the aerobic and the anaerobic compartments of the BPR process and the denitrification compartment of the non-BPR processes, respectively. The *in situ* function of *Acinetobacter* as a polyP-accumulating bacterium under BPR process conditions was assessed microscopically. When the spatial distribution of polyP revealed by DAPI staining (Figure 5) was compared with the spatial distribution of *Acinetobacter* cells revealed by the ACA probe (Figure 6), it was found that the clustered cells containing the bulk of the polyP were not *Acinetobacter* cells, and that the phylogenetically stainable *Acinetobacter* cells did not contain significant amounts of DAPI stainable polyP under process conditions (Figures 5–7).

Sixteen isolates identified as *A. calcoaceticus*, *A. lwoffii*, *A. johnsonii* and *A. radioresistens* were isolated from the BPR process and their ability to accumulate polyP was studied in the laboratory at 20°C in shaken cultures containing (g L^{-1}) tryptone 1, yeast extract 0.5 and glucose 0.2. The soluble PO_4-P content of the medium was $\leq$9 mg L^{-1}. Samples after different times of growth were stained with DAPI and inspected microscopically. PolyP was detected (but only in a fraction of the cells) in 11 of the 16 cultures after 3 days, after 5 days only in one culture, and after 7 days in none of the cultures. When the strains that were positive after 3 days were transferred to new culture and grown for one day, more than 50% of the cells in all cultures showed the presence of polyP. The results showed that polyP accumulation in *Acinetobacter* was a characteristic of young, actively growing cells, and that *Acinetobacter* cells in advanced stationary phase contained less polyP than cells in early stationary phase.

The capacity of activated sludge bacteria to retain the accumulated phosphorus was studied by measuring phosphorus release under aerobic conditions in stationary phase cultures from selected isolates grown in the presence of ^{33}P-labeled phosphate. There were considerable differences in the capacity of the sludge bacteria to retain their accumulated phosphorus (Table 2). Whereas the *Neisseria* isolates studied released in 5 days 97% of the previously accumulated phosphorus, the *Nocardia* and *Rhodococcus* isolates released $\leq$5%. The other isolates studied were intermediate between these extremes. Thus, the inability to retain under advanced stationary phase conditions the phosphorus accumulated during the preceding growth phase seems not to be solely a characteristic of *Acinetobacter*, as shown above, but a more common trait among activated sludge bacteria.

The bacterial communities of the processes were monitored over time using ubiquinones as biomarkers. Figure 8 shows the time profile of the mole% distribution of ubiquinones and temperature in the BPR-process. Ubiquinone

Figure 2 The uneven distribution and formation of microcolonial patches by polyP-containing cells in the BPR sludge. Sample from the aerobic compartment of the pilot process. PolyP staining with toluidine blue (dark areas). Bar = 100 μm.

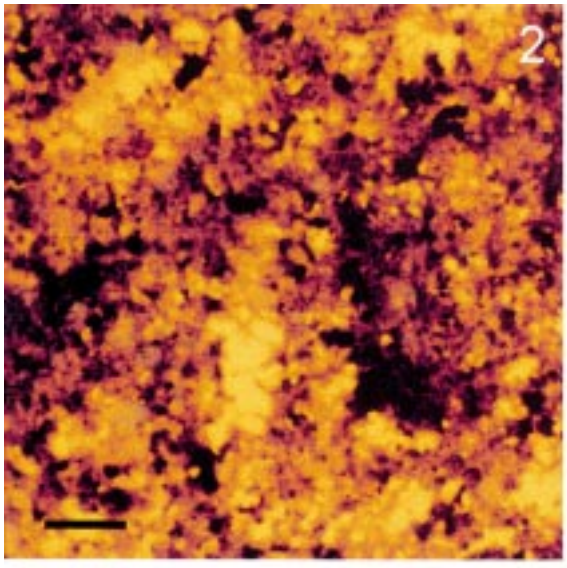
Figure 3 Only very small or no polyP-containing microcolonies were found in non-BPR sludge with simultaneous chemical phosphate precipitation. In this case the sample was taken from a full-scale line without N removal (all other data given for the non-BPR process, relate to a full-scale line with denitrification-nitrification). Toluidine blue staining. Bar = 100 μm.

Figure 4 The bulk of polyP in the BPR sludge was in homogenous clusters composed of cells having a distinct coccoid morphotype. Only little polyP was found in other kinds of cells. Aerobic stage sludge stained with toluidine blue. Bar = 10 μm.

Figure 5 Spatial distribution of polyP in BPR sludge (brightly fluorescing areas). Aerobic stage sludge stained with DAPI (50 μg ml^{-1}). Bar = 10 μm.

Figure 6 Phylogenetic staining of the BPR sludge by *in situ* hybridization with the ACA probe (most intense red fluorescence). The same sample and microscopic field as in Figure 5. Bar = 10 μm.

Figure 7 Different locations of *Acinetobacter cells* and polyP in the sludge of the BNR process. The ACA-signal spots of Figure 6 overlaid in pseudocolor with Figure 5. Bar = 10 μm.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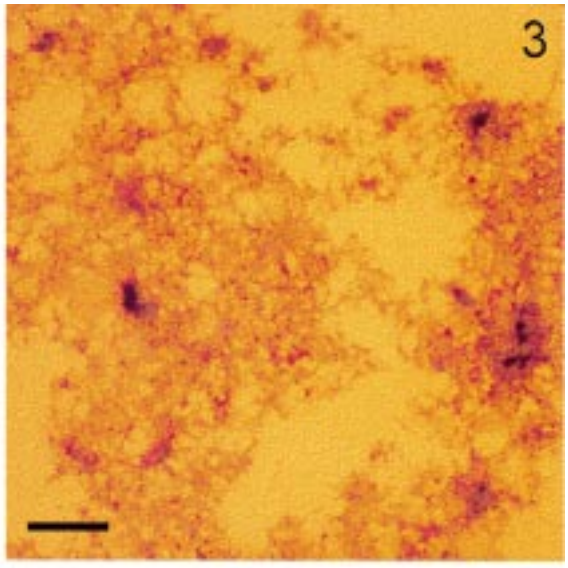
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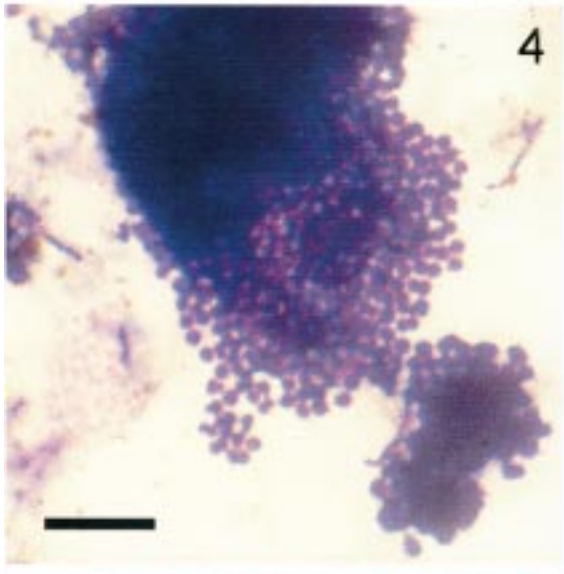
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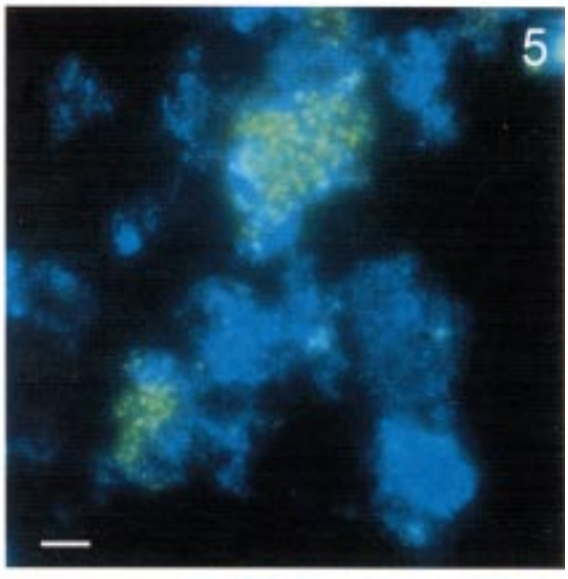
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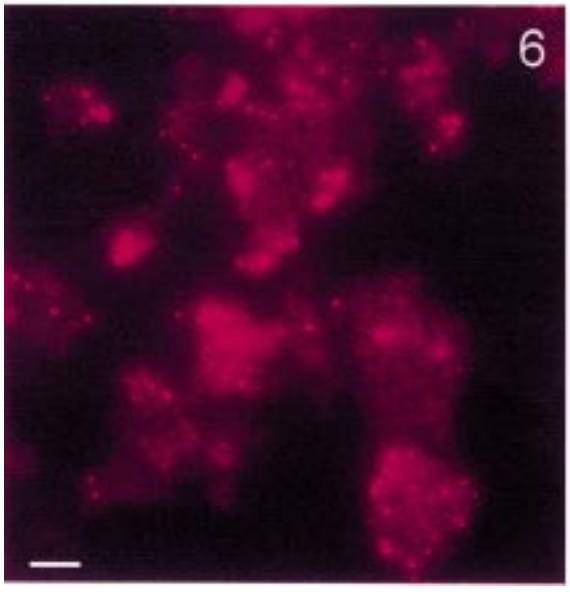
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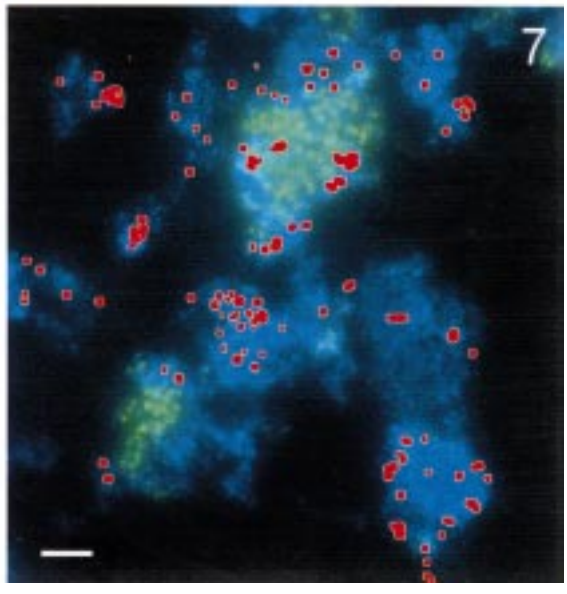
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composition showed little change over the more than 100 days of observation, indicating no major changes in the Gram-negative microbial community in the period from July to November, although the temperature of the process declined steadily from above 20°C to below 17°C during this time (25.7.95–6.11.95). Figure 8 also shows that the ubiquinone composition of the BPR sludge was similar in the anaerobic and the aerobic stages of the process. This indicates that sludge recycling through the consecutive anaerobic, anoxic and aerobic phases of the process (hydraulic retention times 1.3, 2.7 and 3.6 h, respectively) resulted in no detectable change in the biomass diversity of Gram-negative bacteria. The percentage ubiquinone composition in the anaerobic stage of the BPR process was (temporal mean ± σ): *Q8* 64 ± 3; *Q10* 29 ± 3 and *Q9* 7 ± 3, whereas the percentage composition in the denitrification stage of the non-BPR process sampled at the same times was *Q8* 56 ± 34; *Q10* 37 ± 32 and *Q9* 7 ± 8. The relative amount of ubiquinone *Q9* was the same in the BPR and the non-BPR processes, but the proportion of *Q8* was somewhat lower and the proportion of *Q10* somewhat higher in the non-BPR sludge. The *Q8*:*Q10*:*Q9* ratios were rather similar however, 9:4:1 for the BPR sludge and 8:5:1 for the non-BPR sludge. The clearest difference between the ubiquinone compositions in the two processes was the high temporal variation of the composition in the non-BPR sludge as indicated by the large standard deviations.

Discussion

Characteristics of the influent waste water and process design are the determinants of the activated sludge microbial community of a waste water purification plant. In the present work information was sought on the effect of combining BPR with a denitrification-nitrification process. This was accomplished by comparing two processes receiving the same waste water. The BPR process incorporated as its first stage an anaerobic compartment absent from the non-BPR process. On the basis of the bacterial genera isolated, there was overall similarity between the bacterial communities of the processes. This may not be so unexpected since both processes incorporated aerobic as well as anoxic (denitrification) stages, and an anaerobic stage, as in the BPR process, differs from an anoxic stage only in not containing nitrate. The isolates obtained in this study showed, however, one major difference between the bacterial communities: the occurrence of several actinomycete-related genera among the BPR isolates, and their absence among the non-BPR isolates suggests that these bacteria might play a role in biological phosphate removal.

Some sludge bacteria grow better on standard laboratory media in mixed, rather than in pure culture, and some grow only as long as their endogenous pool of growth factor(s) lasts. This was manifested in the cessation of growth during purification of more than one third of all primary colonies taken for purification. The takeover of cultures by bacilli is thought to result from the tight adherence of the particles making up the sludge flock and the occurrence of bacilli in the flocks as spores which germinate later in the course of the isolation process.

The roughly similar average ubiquinone compositions in the two processes studied were in line with the idea of a rather high overall similarity between their bacterial communities, based on the isolation of mostly the same bacterial genera from both processes. Similar ubiquinone compositions, as found in this study, have been already earlier reported by others [11] in full and laboratory scale activated sludge processes with or without BPR. The drawback of ubiquinones as chemical biomarkers is that they only reflect the Gram-negative portion of the community [6].

Ubiquinone *Q9* is the principal ubiquinone of *Acinetobacter* [10]. The relative amount of ubiquinone *Q9* found in the BPR process sets the maximum possible proportion of *Acinetobacter* at 10%. However, as *Acinetobacter* probably is not the only ubiquinone *Q9*-containing Gram-negative present in the sludge, and as a major portion of the sludge bacteria seem to be Gram-positives (two-thirds of

Table 2 Aerobic release of accumulated phosphorus in advanced stationary phase from bacteria isolated from BPR sludge

Bacterium[a]	Match[a] (1000–0)	Phosphorus released in 5 days (% from accumulated)
Neisseria flavescens	690	100
Neisseria subflava	560	94
Bacillus pumilus	840	72
Streptoverticillium reticulum	330	65
Clavibacter michiganense	860	47
Listeria ivanovi	430	39
Acidovorax avenae	510	29
Micrococcus luteus	820	27
Acidovorax avenae	410	16
Staphylococcoccus warneri	800	14
NI[b]		9
Nocardia asteroides	440	5
Rhodococcus rhodnii	390	2

[a]Suggestion of the whole cell fatty acid identification system used (library version 3.8).
[b]Not identified, did not grow on the identification medium.

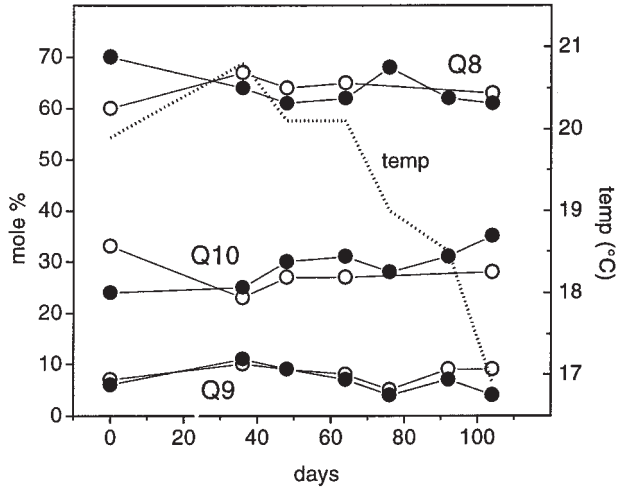

Figure 8 Time-dependent changes in the ubiquinone composition of different stages of the BPR process. Q8–Q10, ubiquinones; closed symbols, anaerobic stage; open symbols, aerobic stage. Temperature was measured from the aerobic compartment. The experiment was carried out from 25.7.95 to 6.11.95.

cultured isolates), the true proportion of *Acinetobacter* of all bacteria must be well under 10%. This is in line with the cultivation-based (4%) and *in situ* hybridization-based ($\leq$ 3%) *Acinetobacter* estimates in the BPR process. On the basis of cultivation, *in situ* hybridization and the proportion of ubiquinone *Q9*, there was no enrichment of *Acinetobacter* in the BPR process as compared to the non-BPR process.

DAPI binds to DNA when added to a microbial sample at low concentration (0.3 μg ml^{-1}). The resulting complex gives a blue emission upon UV-excitation, which is used for fluorescence microscopic determination of total bacteria [9,14]. When used at a high concentration (50 μg ml^{-1}), DAPI also binds to polyP, and this complex gives upon UV-excitation a yellow emission [1]. This has been used to detect polyP-containing bacteria in activated sludge [24]. We used both DAPI and toluidine blue [7] to stain polyP-containing cells. By both staining methods the bulk of the polyP in the BPR process was found inside clumps of cells growing in grape-like clusters. This kind of polyP-containing cells has been found in significant amounts in BPR processes with initial anoxic or anaerobic zones, and they have been thought of as acinetobacters [12]. The idea of the association of the grape-like cluster-forming polyP organisms with *Acinetobacter* has support in publications [4,8], where clustered polyP-cells in BPR sludges were described as well as the isolation of polyP-accumulating *Acinetobacter* cultures from the same sludges. However, it was not shown in these, or later publications, that the grape-like cluster-forming polyP organism observed in the sludges was indeed *Acinetobacter*.

We assessed *in situ* this question and the capacity of *Acinetobacter* to accumulate polyP under BPR process conditions. This was done by mapping the location in the sludge flock of *Acinetobacter* cells hybridized with the ACA probe and comparing that with the location of polyP stained with DAPI (Figure 7). The results clearly show that the cells in the grape-like clusters containing the bulk of the polyP under process conditions did not belong to any of the described *Acinetobacter* species. The ACA (23a) probe used is complementary to a signature present in all so far validly described species of the genus *Acinetobacter* [23]. Although most of the *Acinetobacter* strains isolated from the BPR process accumulated polyP in actively growing laboratory cultures, they did not contain clearly stainable amounts of polyP under process conditions.

BPR processes comprise successive anaerobic and aerobic phases. Phosphate is removed from the waste water mainly as polyP accumulated in aerobic microorganisms in the aerobic phase. Release of phosphate from the sludge in the anaerobic phase is thought to be a prerequisite for its subsequent aerobic accumulation in excess [21]. Under aerobic conditions phosphate should not be released from the microorganisms. We found that it was a common feature of BPR sludge bacteria, including *Acinetobacter*, to release phosphorus accumulated during active growth under aerobic conditions, when the cultures were held for a prolonged time under stationary phase conditions. There were considerable differences between the isolates in this respect, however.

In order to obtain good waste water purification results in a short hydraulic retention time, the concentration of biomass in an activated sludge process is kept high by pumping most of the settled sludge back to the process. The mean residence time of sludge microbes in nutrient-removing processes is often of the order of weeks (in this study $\geq$ 2 weeks); ie sludge microbes divide and double their biomass on the average only once in about 2 weeks. In addition, oxygen is available for the aerobic microorganisms only part of the time, in rather short pulses (in this study $\leq$ 50% of the time, in pulses of 3.6 h). As a result of these conditions, activated sludge microbes, including the polyP-accumulating organisms, are most of the time far from active, exponential growth, but instead in or near stationary phase. This must be especially true for organisms that occur in big clusters, diffusion limited for oxygen, nutrients and metabolic end products of the cells. As this kind of organism nevertheless contains the bulk of the polyP in the process, we conclude that the organism in the grape-like clusters likely has the capacity of polyP formation under stationary phase conditions.

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