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Microbial community composition and function in wastewater treatment plants

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Abstract

Biological wastewater treatment has been applied for more than a century to ameliorate anthropogenic damage to the environment. But only during the last decade the use of molecular tools allowed to accurately determine the composition, and dynamics of activated sludge and biofilm microbial communities. Novel, in many cases yet not cultured bacteria were identified to be responsible for filamentous bulking and foaming as well as phosphorus and nitrogen removal in these systems. Now, methods are developed to infer the *in situ* physiology of these bacteria. Here we provide an overview of what is currently known about the identity and physiology of some of the microbial key players in activated sludge and biofilm systems.

Abbreviations: EBPR – enhanced biological phosphorus removal; FISH – fluorescence *in situ* hybridization; GAO – glycogen-accumulating organism; PAO – polyphosphate-accumulating organism; PHA – polyhydroxyalkanoates; wwtp – wastewater treatment plant

Introduction

Wastewater treatment is one of the most important biotechnological processes which is used worldwide to treat municipal and industrial sewage. This review focuses on the microbiology of the activated sludge process. In addition, we also cover activated sludge and biofilm nutrient removal plants in which anaerobic and aerobic treatments are combined to allow for complete nitrogen and/or biologically enhanced phosphorus removal. In all plant types, prokaryotic microorganisms dominate and are responsible for the observed conversions. On the other hand, certain microorganisms cause the most frequently encountered problems in wastewater treatment like activated sludge bulking and foaming. Consequently, the efficiency and robustness of a wwtp mainly depend on the composition and activity of its microbial community. Although biological wastewater treatment has been used for more than a century, research on the microbiology of this process suffered from severe methodological limitations (Wagner 1993) until a decade ago. Only after the introduction of a set of different molecular techniques in wastewater microbiology (for example Wagner 1993, 1994; Bond 1995; Schramm 1996; Snaidr 1997; Juretschko 1998; Lee 1999; Purkhold 2000), it has become possible to determine the composition and dynamics of microbial communities in these systems and to identify the microbial key players for the different process types. It is the aim of this review to summarize these new insights and to provide some outlook on how this knowledge could be used to improve the performance of wwtps. It should be noted that the bacterial nomenclature proposed in the taxonomic outline (released 1, April 2001) of the second edition of Bergey's manual of systematic bacteriology (http://www.cme.msu.edu/bergeys/) was used throughout this review. Furthermore, anaerobic ammonium oxidizing bacteria, which were recently detected in wwtps (Schmid 2000, 2001), are not in-

cluded in this review because a separate manuscript dealing with these bacteria is included in the ISME 9 special volume of Antonie van Leeuwenhoek (Strous 2002).

Microbial diversity of wastewater treatment plants

The species richness of the microbial communities of five laboratory-scale reactors and three wwtps has been analyzed by 16S rRNA gene phylogenetic inventories established by using bacterial or universal primers (Bond 1995; Snaidr 1997; Christensson 1998; Dabert 2001; Daims 2001; Liu 2001; Juretschko 2002). Results of these surveys are summarized in Table 1. It is obvious that the number of plants analyzed by the 16S rRNA approach is too low to infer ultimate conclusions. However, already in the available studies members of 13 bacterial divisions, of the 36 divisions currently identified for the bacterial domain (Hugenholtz 1998), were detected, indicating considerable microbial diversity in wwtps. Consistent with previous quantitative FISH experiments using group-specific rRNA-targeted oligonucleotide probes (Wagner 1993, 1994; Manz 1994; Kämpfer 1996; Manz 1996; Neef 1996; Bond 1999; Liu 2001), *Proteobacteria* are abundant in each library and represent more than 50% of the clones in five of the eight surveys. With one exception (Christensson 1998), *Betaproteobacteria* are the most frequently retrieved members of this division. Apart from the *Proteobacteria*, molecular isolates affiliated to the *Bacteroidetes*, the *Chloroflexi* and the *Planctomycetes* were retrieved in significant numbers in several of the libraries. One library of a reactor designed for enhanced biological phosphorus removal in a continuous flow system is dominated by high-G+C Gram positive bacteria (*Actinobacteria*), consistent with the proposed importance of these bacteria for phosphorus removal (see below) (Wagner 1994).

However, the relatively low coverage values (Giovannoni 1995; Singleton 2001, Juretschko 2002) of some of these libraries demonstrate that the number of clones analyzed were too low in these studies to adequately represent the diversity in the established libraries (Table 1). Furthermore, the 16S rRNA approach suffers from numerous biases introduced in the DNA extraction, PCR amplification, and cloning procedures (Meyerhans 1990; Suzuki & Giovannoni 1996; Juretschko 1998; Suzuki 1998). Therefore quantitative data on the microbial community composition can only be obtained if the 16S rRNA approach is combined with quantitative dot blot or *in situ* hybridization techniques.

So far, the microbial community structures of activated sludges from only two wwtps have been investigated using the full-cycle rRNA approach which includes the establishment of a 16S rRNA gene clone library, the design of a set of clone-specific oligonucleotide probes for FISH, and the determination of the abundance of the respective bacterial populations by quantitative FISH. Snaidr et al. analyzed a high-load aeration basin of a large municipal wwtp (Amann 1996; Snaidr 1997) while Juretschko et al. studied an intermittently aerated industrial wwtp containing a nitrifiying and denitrifying microbial community (Juretschko 1998; Juretschko 2002). The results from the respective 16S rRNA gene clone libraries are shown in Table 1. Snaidr and colleagues designed probes for a few selected clones and found a high microdiversity of bacteria of the beta1 group of *Betaproteobacteria* in the municipal activated sludge. Furthermore, 3 and 4% of all microbial cells in this system could be assigned to *Sphingomonas*- and *Arcobacter*-related populations, respectively.

The composition of the microbial community in the industrial plant was investigated in more detail using semi-automatic quantitative FISH (Juretschko 2002). Hybridization with group-specific probes demonstrated that the *Betaproteobacteria* made up almost half of the total biovolume of those bacteria detectable with the bacterial probe set. Other *in situ* important groups were the *Alphaproteobacteria*, the *Nitrospira*-phylum, the *Planctomycetes*, and the *Chloroflexi*. The composition of the *Betaproteobacteria* within this system was further analyzed using clone-specific probes for quantitative FISH. Bacteria related to *Zoogloea ramigera* and *Azoarcus sensu lato* were the most abundant members of this class *in situ*, and accounted for 36 and 34% of the biovolume of the *Betaproteobacteria*. In addition, significant numbers of the ammonia-oxidizer *Nitrosococcus mobilis* (which was not present in the clone library), *Alcaligenes latus*-, and *Brachymonas denitrificans*-related microorganisms were recorded. In total only 2% of the *Betaproteobacteria* detectable *in situ* could not be assigned to a specific genus.

Table 1. Summary of 16S rRNA gene-based diversity surveys of wastewater treatment plants and reactors. SBR = sequencing batch reactor. SBBR = sequencing biofilm batch reactor. Clones were assigned to different OTUs (operational taxonomic units) if they shared less than 97% 16S rRNA gene sequence similarity with each other (adapted from A. Loy, 2002)

wwtps and lab-reactors	No. of		Coverage	Bacterial phyla[9] (No. of OTUs in respective phylum)																		
	Clones sequenced	OTUs	C[10] [%]	*Proteobacteria*[8]																		
				α	*β*	*γ*	*δ*	*ϵ*	*Bacteroidetes*	*Acidobacteria*	*Firmicutes*	*Actinobacteria*	*Nitrospira*	*Verrucomicrobia*	*Planctomycetes*	*Chlorobi*	*Chloroflexi*	*Fibrobacteres*	*Fusobacteria*	OP11	Unaffiliated
High-load aeration basin of a full-scale municipal plant[1]	62	25	77	3 (1)	52 (9)	18 (7)		15 (1)			10 (5)			2 (1)			2 (1)					
Nitrifying/ denitrifying industrial plant[2]	94	53	64	26 (15)	31 (9)		2 (2)			5 (3)	1 (1)	1 (1)	2 (1)	3 (2)	12 (10)	1 (1)	16 (8)					
Nitrifying SBBR1[3]	96	33	78	5 (4)	51 (11)	22 (6)	4 (4)	1 (1)	2 (2)	5 (2)		1 (1)	8 (2)									
EBPR lab-scale SBR1 + sodium acetate[4]	97	69	48	13 (8)	33 (20)	8 (4)	3 (3)	2 (2)	5 (5)	3 (2)	1 (1)	4 (3)	3 (3)	1 (1)	13 (9)		4 (3)		3 (2)		3 (3)	
EBPR lab-scale SBR2 unsupplemented[4]	92	75	28	17 (15)	25 (13)	10 (9)	1 (1)	7 (5)	13 (9)	7 (5)		2 (2)	2 (2)	1 (1)	9 (8)		3 (3)		2 (2)			
EBPR lab-scale continuous flow reactor[5]	51	30	51	16 (8)	8 (2)	8 (3)	4 (2)	4 (2)	6 (1)	2 (1)		37 (3)			8 (4)	2 (1)	6 (3)					
EBPR lab-scale SBR[6]	92	50	46	4 (3)	17 (8)	5 (2)	3 (3)		39 (17)	9 (6)		4 (2)	3 (1)	4 (3)	3 (2)	3 (2)				1 (1)		
EBPR lab-scale reactor[7]	150	16	93	5 (1)	14 (1)	7 (2)	1 (1)		50 (7)		5 (1)	9 (2)						9 (1)				

[1] (Snaidr 1997); [2] (Juretschko 2002); [3] (Daims 2001a); [4] (Bond 1995); [5] (Christensson 1998); [6] (Dabert 2001); [7] (Liu 2001); [8] *Proteobacteria* are present at the class level due to the extensive representation of this phylum; [9] Relative incidence of phylum-level representatives in respective studies, [10] coverage was calculated according to the formula $C = [1 - (n1 \times N^{-1})] \times 100\%$, $n1$ = number of OTUs consisting of only one sequence, N = number of all sequences in the 16S rRNA gene clone library.

The filamentous bacteria

The efficiency of the activated sludge process is strongly influenced by the settleability of the sludge flocs in the succeeding sedimentation tanks in which the biomass is separated from the treated sewage. Filamentous bacteria can dramatically decrease the settleability of activated sludge flocs (sludge bulking) or cause floating and foam formation of the biomass (sludge foaming). Despite the inclusion of selectors in the plant design, which helps in some cases to control the overgrowth of filamentous bacteria, sludge bulking and sludge foaming are still a major problem in many primarily industrial wwtps. Therefore, there is considerable interest to identify the filamentous bacteria in wwtps and to characterize their physiological properties in order to develop specific control strategies to suppress their growth.

Traditionally, filamentous bacteria were analyzed in activated sludge by using standard light microscopy. For provisional identification of these microorganisms, keys were developed using: (i) the reaction of the filaments to Gram- and Neisser-staining, and (ii) the morphological characteristics of the filaments (Eikelboom 1975; Jenkins 1993) and type numbers were assigned to the different filaments (e.g. Eikelboom Type 021N). Since then a considerable number of filament types were enriched (mainly by micromanipulation) and their 16S rRNA gene sequences were determined (for example Blackall 1996; Bradford 1996; Seviour 1997; Howarth 1999; Kanagawa 2000; Snaidr 2001). The phylogenetic tree shown in Figure 1 presents an overview on the phylogenetic affiliation of those filaments. Several filament types, for example Eikelboom type 1863 or '*Nostocoida limicola*', harbor phylogenetically unrelated species. Today, many of the filaments can specifically and rapidly be detected in activated sludge using rRNA-targeted oligonucleotide probes (Wagner 1994; Erhart 1997; de los Reyes 1998; Kanagawa 2000). *In situ* probing demonstrated polymorphism of several filaments within activated sludge (Wagner 1994), a fact which further complicates morphology-based identification. Furthermore, application of the full-cycle rRNA approach revealed that especially industrial wwtps harbor a variety of filamentous bacteria which can not be found in the traditional identification keys (e.g. Juretschko 2002).

The availability of FISH probes for many of the filaments now allows to investigate their *in situ* physiology within activated sludge systems (Nielsen 1998). *Mircothrix parvicella* was shown to be a specialized lipid consumer being able to take up long chain fatty acids (but no short chain fatty acids or glucose) under anaerobic conditions and subsequently use the storage material for growth when nitrate or oxygen is available as electron acceptor (Andreasen & Nielsen 1997; Nielsen 2001). However, *M. parvicella* could not take up phosphorus under aerobic conditions excluding its importance for enhanced biological phosphorus removal (see below). In addition, *Thiothrix* sp. filaments in industrial wwtps were shown to be physiologically very versatile since they incorporated radioactively labeled acetate and/or bicarbonate under heterotrophic, mixotrophic and chemolithoautotrophic conditions. The *Thiothrix* filaments were active under anaerobic conditions (with or without nitrate) in which intracellular sulphur globules were formed from thiosulphate and acetate was taken up (Nielsen 2000).

Microorganisms responsible for enhanced biological phosphorus removal and nitrogen removal

The identification and characterization of those bacteria responsible for phosphorus and nitrogen removal in wwtps is complicated by the fact that 16S rRNA sequence-based identification of a microorganism does generally not allow to infer its functional properties. Phylogenetically closely related microorganisms may possess different metabolic potentials while on the other hand several physiological traits like the ability to denitrify are found dispersed in many different phylogenetic lineages. Therefore, the full-cycle rRNA-approach has to be supplemented with other techniques which allow a functional assignment of the detected bacteria to identify the members of the most important physiological groups in wwtps. In detail, the use of: (i) lab-scale reactors, inoculated with activated sludge, which select for the respective functional microbial group (Burrell 1998; Hesselmann 1999; Strous 1999; Crocetti 2000), (ii) functional genes coding for key enzymes of certain metabolic pathways as phylogenetic and physiological markers for the respective guild (Juretschko 1998; Purkhold 2000), (iii) the combination of FISH and microelectrodes (Schramm 1996; Okabe 1999; Schramm 1999, 2000), (iv) the recently developed combination of FISH and microautoradiography (Lee 1999; Daims 2001b) allowed to identify the bacteria catalyzing important transformations of biological nutrient removal.

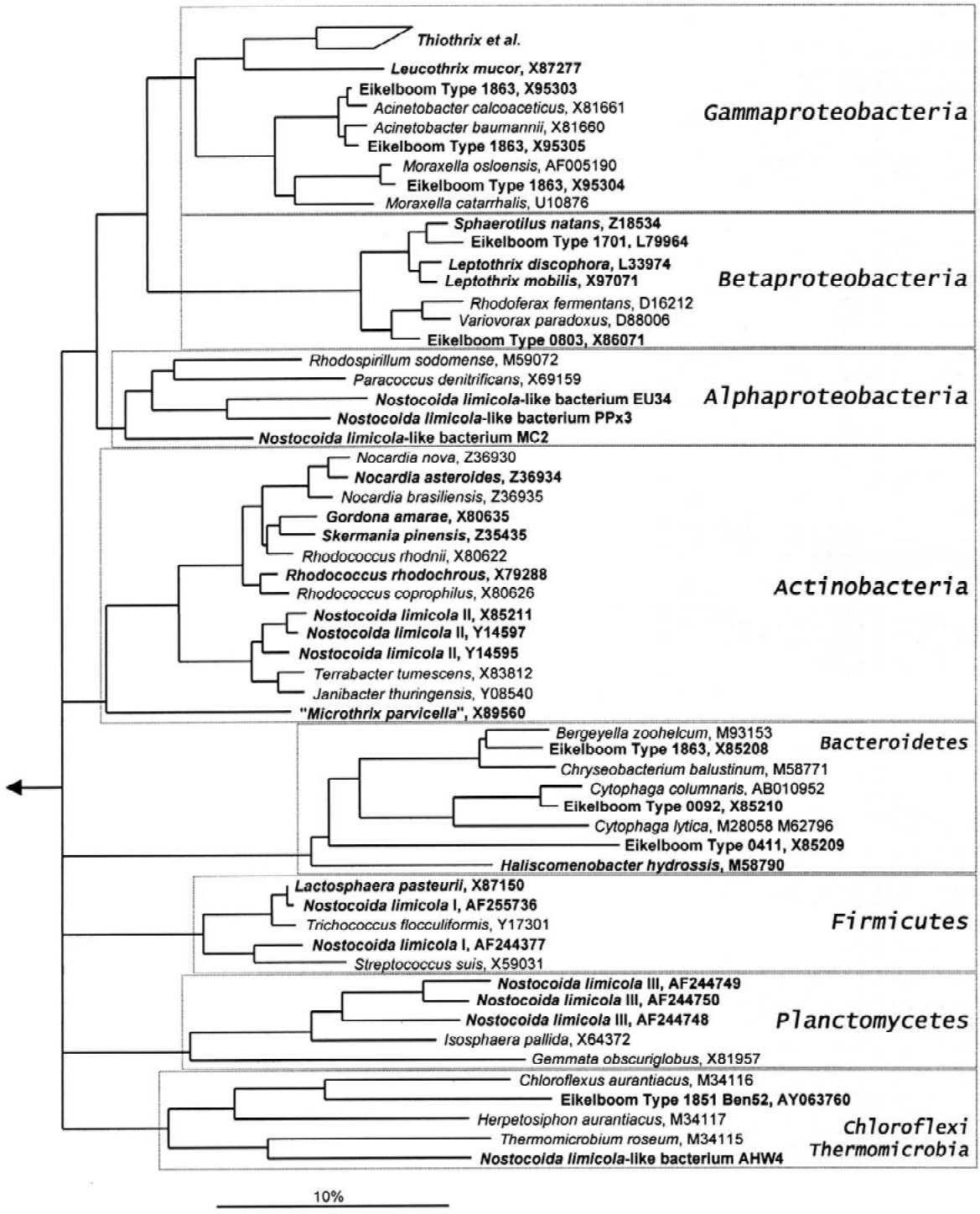


Figure 1. 16S rRNA-based tree showing the phylogenetic affiliation of filamentous bacteria occurring in activated sludge (labeled in bold). It should be noted that the *Thiothrix* et al. group contains also at least three phylogenetically distinct lineages including filaments of the Eikelboom Type 021N (Kanagawa 2000). The tree was calculated using the neighbor-joining method with a 50% bacterial conservation filter. Multifurcations connect branches for which a relative order could not be unambiguously determined by applying different treeing methods. The bar corresponds to 10% estimated sequence divergence.

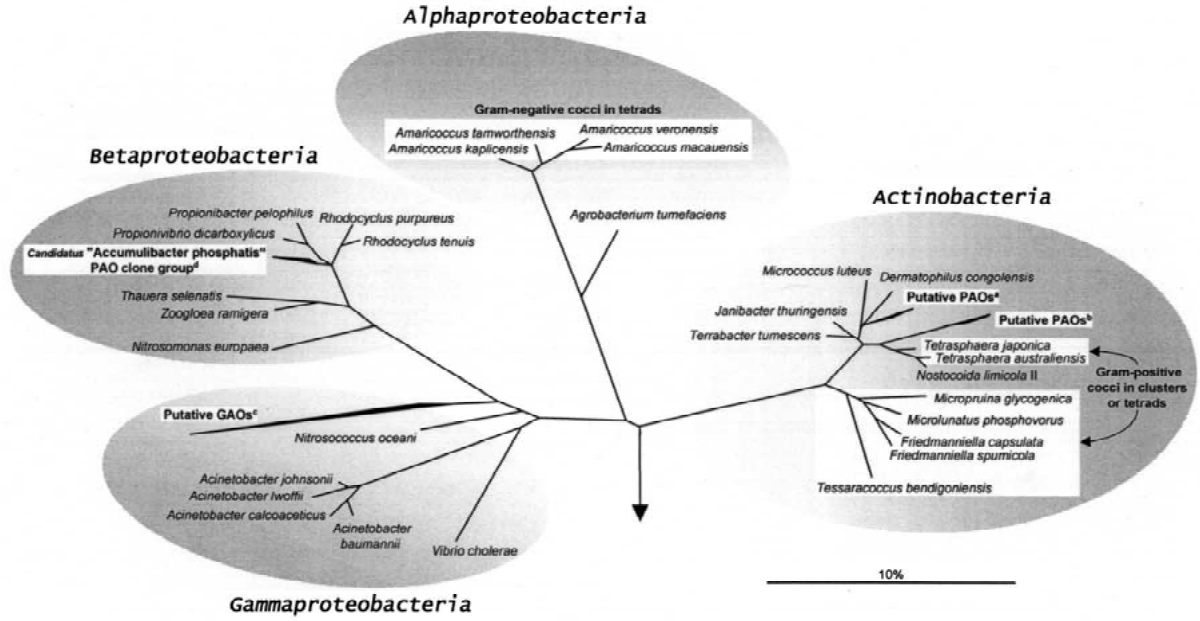


Figure 2. 16S rRNA-based tree showing the phylogenetic affiliation of putative polyphosphate accumulating organisms (PAOs), glycogen accumulating organisms (GAOs) and G-bacteria. The tree was calculated using the maximum-likelihood method with a 50% bacterial conservation filter. The bar corresponds to 10% estimated sequence divergence. [a] 16S rRNA gene clones: AF109792, AF109793, AF124650, AF124655 (Liu 2000). [b] 16S rRNA gene clones: AJ225335, AJ225341, AJ225342, AJ225348, AJ225351, AJ225353, AJ225355, AJ225356, AJ225360, AJ225364, AJ225370, AJ225376-AJ225379, Y15796, AF255628, AF255629 (Christensson 1998; Liu 2001).[c] 16S rRNA gene clones: AF093777, AF093778, AF093780, AF093781, AF314424, AF124652, AF124656 (Nielsen 1999; Liu 2000; Dabert 2001). [d] 16S rRNA gene clones: AJ224947, AF255631, AF255641, AF204244, AF204245, AF204247, AF204248, AF314417, AJ225350, AJ225358, X84620 (Bond 1995; Christensson 1998; Hesselmann 1999; Crocetti 2000; Dabert 2001; Liu 2001).

Microorganisms of importance for enhanced biological phosphorus removal

Phosphorus removal from wastewater is important to prevent eutrophication and is therefore an integral part of modern municipal and industrial nutrient removal wwtps. Phosphorus can be precipitated by the addition of iron or aluminum salts and subsequently be removed with the excess sludge. Chemical precipitation is a very reliable method for phosphorus removal but increases significantly the sludge production and thus creates additional costs. Furthermore, the use of chemical precipitants may introduce heavy metal contamination into the sewage and increases the salt concentration of the effluent. Alternatively, phosphorus removal can be achieved by microbiological mechanisms in a process called EBPR, for reviews see (Jenkins & Tandoi 1991; van Loosdrecht 1997; Mino 1998, 2000). This process is characterized by cycling the activated sludge through alternating anaerobic and aerobic conditions. In the anaerobic stage the bacteria responsible for EBPR are supposed to gain energy from polyphosphate hydrolysis accompanied by subsequent P_i release for uptake of short-chain fatty acids and their storage in form of PHA. Two different models were postulated for the production of the reducing equivalents for this anaerobic metabolism (Comeau 1986; Mino 1987) In the subsequent aerobic stage, the PAOs possess a selective advantage compared to other microorganisms which were not able to take up fatty acids under the preceding anaerobic conditions by utilizing the stored PHA in an otherwise carbon-poor environment. In parallel, PAOs restore their polyphosphate pools by aerobic uptake of available phosphate from the wastewater. After sedimentation in the secondary clarifier, a part of the biomass is recycled to the anerobic stage and mixed with new wastewater while the excess sludge containing the intracellular polyphosphates is removed from the system.

In contrast to chemical precipitation, EBPR plants have been frequently reported to fail. This raised interest in the microbiology of the process. Traditionally, based on cultivation experiments *Gammaproteobacteria* of the genus *Acinetobacter* were believed to be the only PAOs (Fuhs & Chen 1975; Lötter & Murphy 1985; Bayly 1989). However, today it has become

apparent that *Acinetobacter* can accumulate polyphosphate but does not possess the above described PAO metabolism (e.g. van Loosdrecht 1997). Furthermore, cultivation-independent methods like fluorescent antibody staining (Cloete & Steyn 1987), respiratory quinone profiles (Hiraishi 1989), and FISH with a genus-specific probe (Wagner 1994; Kämpfer 1996) demonstrated that the relative abundance of *Acinetobacter* in EBPR systems was dramatically overestimated due to cultivation biases further confirming that *Acinetobacter* is not of importance for EBPR.

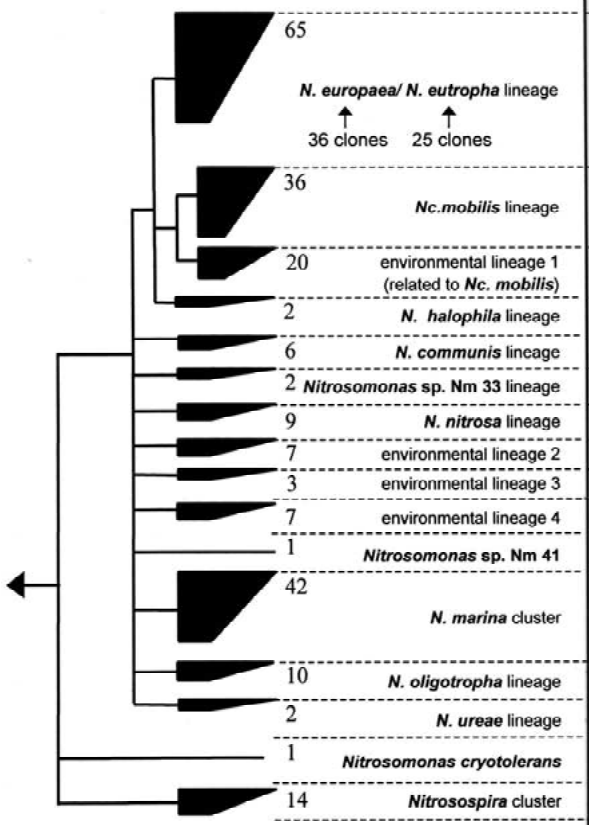
Several other bacteria isolated from EBPR reactors have been suggested as PAO candidates. *Microlunatus phosphovorus*, a high-G+C Gram-positive bacterium accumulates aerobically polyphosphate and consumes it under anaerobic conditions but fails to take up acetate or accumulate PHA under anaerobic conditions (Nakamura 1991, 1995). FISH with a probe specific for *Microlunatus phosphovorus* demonstrated the presence of this organism in an EBPR plant (2.7% of the total bacteria) (Kawaharasaki 1998) but no direct indications for the importance of this bacterium for EBPR are available Furthermore, *Lampropedia* spp. were shown to possess the basic metabolic features of a PAO but their acetate-uptake-phosphate-release ratio was much lower that EBPR models predict, and no additional data regarding the abundance or activity of these morphologically conspicuous bacteria in EBPR systems have been published.

Compared to these cultivation-based attempts, the hunt for PAOs was more successful using molecular tools for analyses of EBPR systems. *Betaproteobacteria* and high-G+C Gram-positive bacteria (*Actinobacteria*) increased in number after addition of acetate to the raw sewage of a EBPR-full-scale wwtp suggesting that these groups benefit from the enhanced availability of short chain fatty acids in the anaerobic basin and thus represent candidates for PAO (Wagner 1994). Additional support for the importance of both groups for EBPR stems from FISH experiments in an efficient EBPR laboratory-scale sequencing batch reactor (Bond 1999) and respiratory quinone profiling in a laboratory-scale EBPR system (Liu 2000). Recently, *Actinobacteria* related to the suborder *Micrococcineae* were reported to be abundant in EBPR systems and thus might be important for EBPR (Christensson 1998; Crocetti 2000; Liu 2001) (Figure 2). While the function of *Actinobacteria* as PAOs still has to be proven, evidence is available that *Betaproteobacteria* of the family '*Rhodocyclaceae*' (Figure 2) are important PAOs in the so far investigated EBPR systems. These bacteria, for which the name *Candidatus* 'Accumulibacter phosphatis' was suggested (Hesselmann 1999) were present in significant numbers in EBPR systems (Bond 1995; Hesselmann 1999; Crocetti 2000; Dabert 2001; Lee 2001). Furthermore, phosphorus accumulation by these bacteria in the aerobic phase was demonstrated by sequential FISH and polyphosphate staining (Crocetti 2000; Liu 2001). In addition, acetate uptake in the anaerobic phase and phosphorus uptake under aerobic conditions could be demonstrated for *Candidatus* 'Accumulibacter phosphatis' using FISH and microautoradiography (Lee 2001).

A potential reason for the failure of EBBR plants is the presence of bacteria which use previously stored compounds such as glycogen (also referred to as GAOs) to compete with the PAOs for substrate uptake under anaerobic conditions (Satoh 1992; Cech & Hartman 1993; Liu 1997). Cech and Hartmann (Cech & Hartman 1993) described Gram-negative cocci in clumps and packages of tetrads in activated sludge and called these morphotypes G-bacteria, since their numbers increased after glucose addition. Mino et al. (1998) suggested that bacteria with a similar morphology are GAOs. However, recent molecular and physiological analyses of various G-bacteria isolated from wwtps revealed a high phylogenetic diversity of this morphotype and provided no support for their role as GAOs (Seviour 2000) (Figure 2). These results suggest that GAOs should be defined by their phenotype and not by their cell morphology. Recently, molecular community analyses of deteriorated EBPR reactors revealed the predominance of a novel bacterial group within the *Gammaproteobacteria* (Figure 2; Nielsen 1999), which are good GAO candidates since they probably accumulate PHA, and store little or no polyphosphates (Nielsen 1999; Liu 2001; Crocetti 2001).

Nitrifying bacteria

The nitrifiers encompass two groups of microorganisms, the ammonia- and the nitrite-oxidizing bacteria, which catalyze the oxidation of ammonia to nitrite and of nitrite to nitrate, respectively. Since most of the nitrogen in the influent of a wwtp is present either in form of urea (which is hydrolyzed to ammonia) or ammonium/ammonia, the nitrifying bacteria play a central role in nitrogen removal in wwtps. It is important to lower the ammonia concentrations in the effluent of wwtps since this compound is toxic to aquatic life and promotes eutrophication in the receiving wa-



No. of isolates /described species	Isolates obtained from	Number of wwtp clones	No. of wwtps in which the respective clones were detected
4	Wwtp, soil	61	10
4	Wwtp, seawater	32	5
0	-	20	6
2	Seawater, alkaline lake	0	0
1	Soil	5	3
1	Soil	1	1
1	Wwtp	8	1
0	-	7	4
0	-	3	1
0	-	7	4
1	Soil	0	0
3	Sandstone, seawater, brackish water	39	6
1	Soil	9	4
1	Soil	1	1
1	Arctic seawater	0	0
8	Soil	6	2
28		199	

Figure 3. Diversity of *amoA* clones retrieved from different nitrifying wwtps. A schematic AmoA–based phylogenetic classification of the beta-proteobacterial ammonia oxidizing bacteria is shown. Multifurcations connect branches for which a relative order could not be unambiguously determined by applying different treeing methods. The cluster designations were adopted from those of Purkhold et al. (2000). The height of each tetragon indicates the number of sequences in the cluster. Gammaproteobacterial ammonia-oxidizers are not included since they currently have not been detected by FISH nor by *amoA*-analyses in nitrifying wwtps (adapted from A. Loy, 2002).

ters. Nitrifying bacteria are extremely slow-growing microorganisms and are recalcitrant to cultivation attempts. Due to the sensitivity of nitrifying bacteria to disturbances like pH- and temperature shifts, breakdown of the nitrification process is frequently reported from municipal and especially industrial wwtps.

According to textbooks the model ammonia-oxidizer is *Nitrosomonas europaea*. However, FISH analyses in nitrifying activated sludge and biofilms showed that other ammonia-oxidizers are more important. In an industrial nitrifying/denitrifying plant the dominant ammonia-oxidizer was *Nitrosococcus mobilis*, a bacterium which was previously considered to occur in brackish water only (Juretschko 1998). Subsequently, *N. mobilis* was also detected in significant numbers in a nitrifying sequencing batch biofilm reactor (Daims 2001a). In contrast, *Nitrosospira*-related ammonia-oxidizers were found to be dominant *in situ* in a laboratory scale fluidized bed reactor (Schramm 1998). Although *Nitrosospira* was also reported in a PCR-based study as important ammonia-oxidizer genus in wwtps (Hiorns 1995), this finding could not be confirmed by FISH analyses of various wwtps and by a large *amoA*-based ammonia-oxidizer diversity survey in wwtps (Purkhold 2000). Today it is generally accepted that nitrosomonads (including *Nitrosococcus mobilis*) and not nitrosospiras (encompassing the genera *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio*) are important for ammonia oxidation in wwtps. This perception is also reflected in Figure 3 which shows the affiliation of 199 *amoA* clones retrieved from various nitrifying wwtps. Only

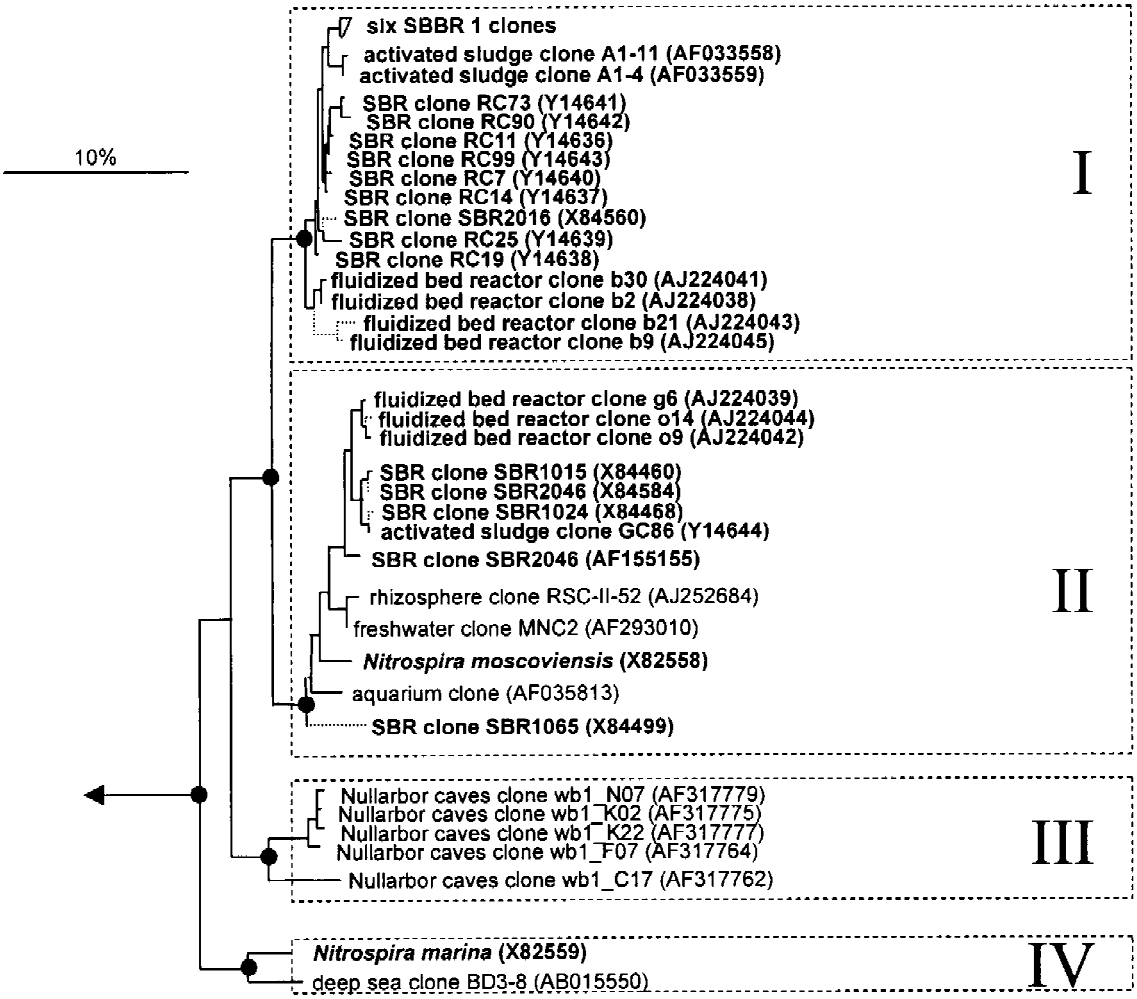


Figure 4. Phylogenetic tree of the genus *Nitrospira* based on comparative analysis of 16S rRNA sequences. The basic tree topology was determined by maximum likelihood analysis of all sequences longer than 1300 nucleotides. Shorter sequences were successively added without changing the overall tree topology. Branches leading to sequences shorter than 1000 nucleotides are dotted to point out that the exact affiliation of these sequences cannot be determined. Black spots on tree nodes symbolize high parsimony bootstrap support above 90% based on 100 iterations. The scale bar indicates 0.1 estimated changes per nucleotide. Clones from wwtps and reactors as well as sequences that belong to isolated strains are depicted in bold. The four sublineages of the genus *Nitrospira* are boxed and marked by the numbers I to IV.

6 *amoA* clones from these systems cluster with the nitrosospiras while the remaining 193 clones are affiliated with the nitrosomonads. Figure 3 also shows that almost all recognized lineages of betaproteobacterial ammonia-oxidizers can be found in wwtps. Numerically, the *Nitrosomonas europaea/Nitrosomonas eutropha*-lineage, the *Nitrosococcus mobilis*-lineage, and the *Nitrosomonas marina* cluster are most frequently detected.

In conclusion, wwtps harbor a diversity of ammonia-oxidizers of the *Betaproteobacteria*, which was enormously underestimated previously. Most of these ammonia-oxidizers are, based on comparative *amoA* sequence analyses, relatively close relatives of described ammonia-oxidizer species. Interestingly, quantitative FISH results indicate that some nitrifying wwtps are dominated by a single ammonia-oxidizer species (Juretschko 1998) while other plants harbor at least five different co-existing ammonia-oxidizer populations which are present in significant numbers (Daims 2001a).

Traditionally, *Nitrobacter* was considered as the most important nitrite-oxidizer in wwtps (Henze 1997). Therefore, the finding that *Nitrobacter* could

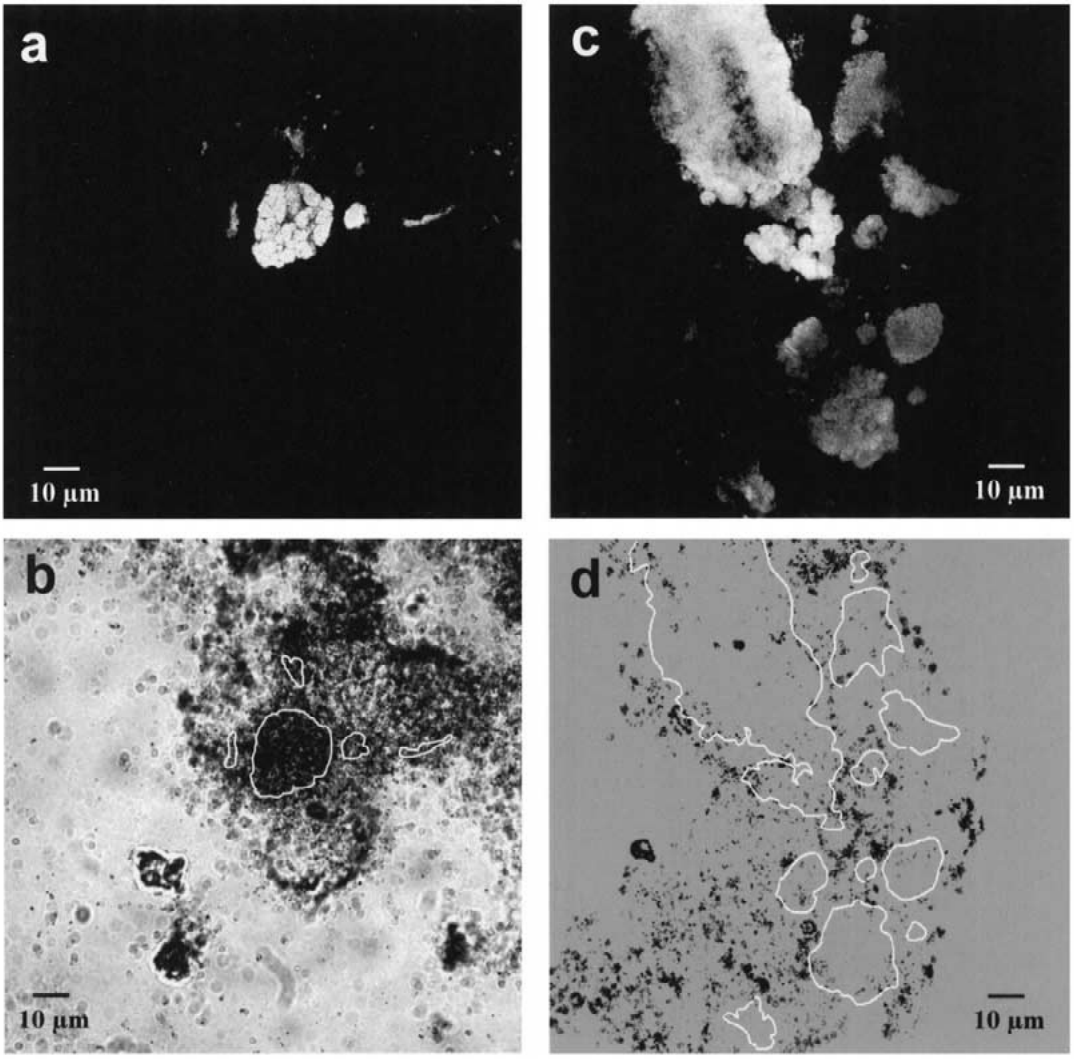


Figure 5. Analyses of the *in situ* physiology of *Nitrospira*–like bacteria. Uptake of radioactive bicarbonate by *Nitrospira*-like bacteria in a biofilm from a sequencing batch reactor under aerobic incubation conditions. (a) shows *Nitrospira* cells stained by a specific probe (b) shows only the radiographic film to visualize the microautoradiographic signal at the position of the *Nitrospira* cells (indicated by white lines). Other microautoradiographic signals were caused by CO_2-fixing bacteria, which were not detected by the *Nitrospira*-specific probe. (c, d) No uptake of acetate by *Nitrospira*-like bacteria in the same biofilm under aerobic incubation conditions. (c) shows *Nitrospira* cells stained with the specific probe. (d) shows the micrograph of the radiographic film at the same position. The localization of the *Nitrospira* microcolonies is indicated by white borderlines.

not be detected by FISH with specific 16S rRNA-targeted probes in various nitrifying wwtps came as a surprise (Wagner 1996). Using the full cycle rRNA approach the occurrence of novel, yet uncultured *Nitrospira*-like nitrite-oxidizing bacteria in nitrifying wwtps could be demonstrated (Juretschko 1998; Okabe 1999; Daims 2000, 2001b; Gieseke 2001). The importance of these microorganisms for nitrite-oxidation in wwtps was also confirmed by reactor enrichment studies (Burrell 1998). Today, four different phylogenetic lineages, two of them containing 16S rRNA gene clones of wwtps, within the genus *Nitrospira* have been recognized (Figure 4; Daims 2001b) and phylum- as well as genus-specific probes suitable for FISH are available (Daims 2001b). Combination of FISH and microautoradiography showed that the *Nitrospira*-like nitrite-oxidizers in activated sludge fix CO_2 and can also grow mixotrophically using pyruvate but not acetate, butyrate, and propionate (Figure 5; Daims 2001b).

It has been postulated that the predominance of *Nitrospira*-like bacteria over *Nitrobacter* in most wwtps is a reflection of their different survival strategies. While *Nitrospira*-like nitrite-oxidizers are, according to data extracted from microelectrode-FISH analyses, *K*-strategists and thus may possess a low μ_{max} but are well-adapted to low nitrite and oxygen concentrations, *Nitrobacter* is postulated to be a relatively fast-growing *r*-strategist with low affinities to nitrite and oxygen (Schramm 1999). Since nitrite-concentrations in most reactors from wwtps are low, *Nitrospiras* will outcompete *Nitrobacter* in these systems. In plants with temporally or spatially elevated nitrite concentrations, for example in nitrifying sequencing batch reactors, both nitrite-oxidizers should be able to co-exist. Consistent with this hypothesis co-occurrence of *Nitrobacter* and *Nitrospira*-like bacteria has been observed by FISH in a nitrifying sequencing batch biofilm reactor (Daims 2001a). We recently started to investigate the competition between *Nitrospira* and *Nitrobacter* in controlled chemostat experiments. Two chemostats were inoculated with the same nitrifying biofilm containing *Nitrospira*-like nitrite-oxidizers and operated under identical conditions (oxygen, temperature, pH, and liquid retention time). After addition of *Nitrobacter* sp. to the chemostats, the nitrite concentration in the influent of one of the reactors was increased such that nitrite peaks (up to 80 mg l^{-1}) in the effluent of this reactor were detectable. Consistent with the above mentioned *K*/*r*-hypothesis, this perturbation stimulated the growth of *Nitrobacter* while in the undisturbed control reactor *Nitrospira* dominated (Figure 6; R. Nogueira, unpubl.). Interestingly, the dominance of *Nitrobacter* over *Nitrospira* caused by the elevated nitrite concentrations could not be reverted by lowering the available nitrite concentration to the original level. One possible explanation for this result is that *Nitrobacter* if present at a certain cell density is able to inhibit the growth of *Nitrospira*-like nitrite oxidizers.

Denitrifying bacteria

Denitrification is used in wastewater treatment to convert the product(s) of nitrification into gaseous nitrogen compounds (mainly dinitrogen) and thus to remove them from the sewage. Most attempts to identify and enumerate denitrifiers in activated sludge are based on cultivation-dependent approaches. Members of the genera *Alcaligenes*, *Pseudomonas*, *Methylobacterium*, *Bacillus*, *Paracoccus* and *Hyphomicrobium* were isolated as part of the denitrifying microbial flora from wwtps (Sperl & Hoare 1971; Attwood & Harder 1972; Knowles 1982; Schmider & Ottow 1986; Vedenina & Govorukhina 1988). The latter genus was also detected microscopically by its typical cell morphology in denitrifying activated sludge (Timmermans & van Haute 1983; Nyberg 1992). However, little is known about whether the above listed bacterial genera are representative for the *in situ* active denitrifiers in wwtps. Neef et al. using specific FISH probes detected significant numbers of *Paracoccus* spp. and *Hyphomicrobium* spp. in a denitrifying sand filter supplemented with methanol as reduced carbon compound for nitrate reduction. But both genera were present in numbers below 0.1% of the total cell counts in a non-denitrifying sand filter run in parallel without addition of methanol, indirectly suggesting an active participation of both genera in the denitrification process (Neef 1996). Molecular studies of the community composition of denitrifying bacteria are difficult to perform since the denitrifying phenotype can not be inferred from the phylogeny of a microorganism. However, the combination of FISH and microautoradiography (Lee 1999) allows identification of denitrifiers *in situ* by performing two types of experiments in parallel. In the first experiment the wwtp sample is incubated under anaerobic condition in absence of nitrate or nitrite with radioactively labeled substrates which are typically used as electron donors for denitrification. In the second experiment the sample is incubated with the same labeled substrates under anaerobic conditions but in the presence of nitrate or nitrite. Bacterial species, identified by FISH, which take up substrate under anaerobic conditions exclusively in the presence of nitrate or nitrite are most likely denitrifiers. The use of this technique in combination with the full-cycle rRNA approach revealed that novel, still uncultured *Azoarcus*-related bacteria are important denitrifiers in an industrial nitrifying/denitrifying wwtp (Juretschko 2000).

Conclusions and future perspectives

During the last decade, the application of molecular tools in wastewater microbiology has revolutionized our view on the microbial ecology of these systems. Different groups of still not culturable bacteria are identified and shown to be responsible for sludge bulking, enhanced biological phosphorus removal, nitrite

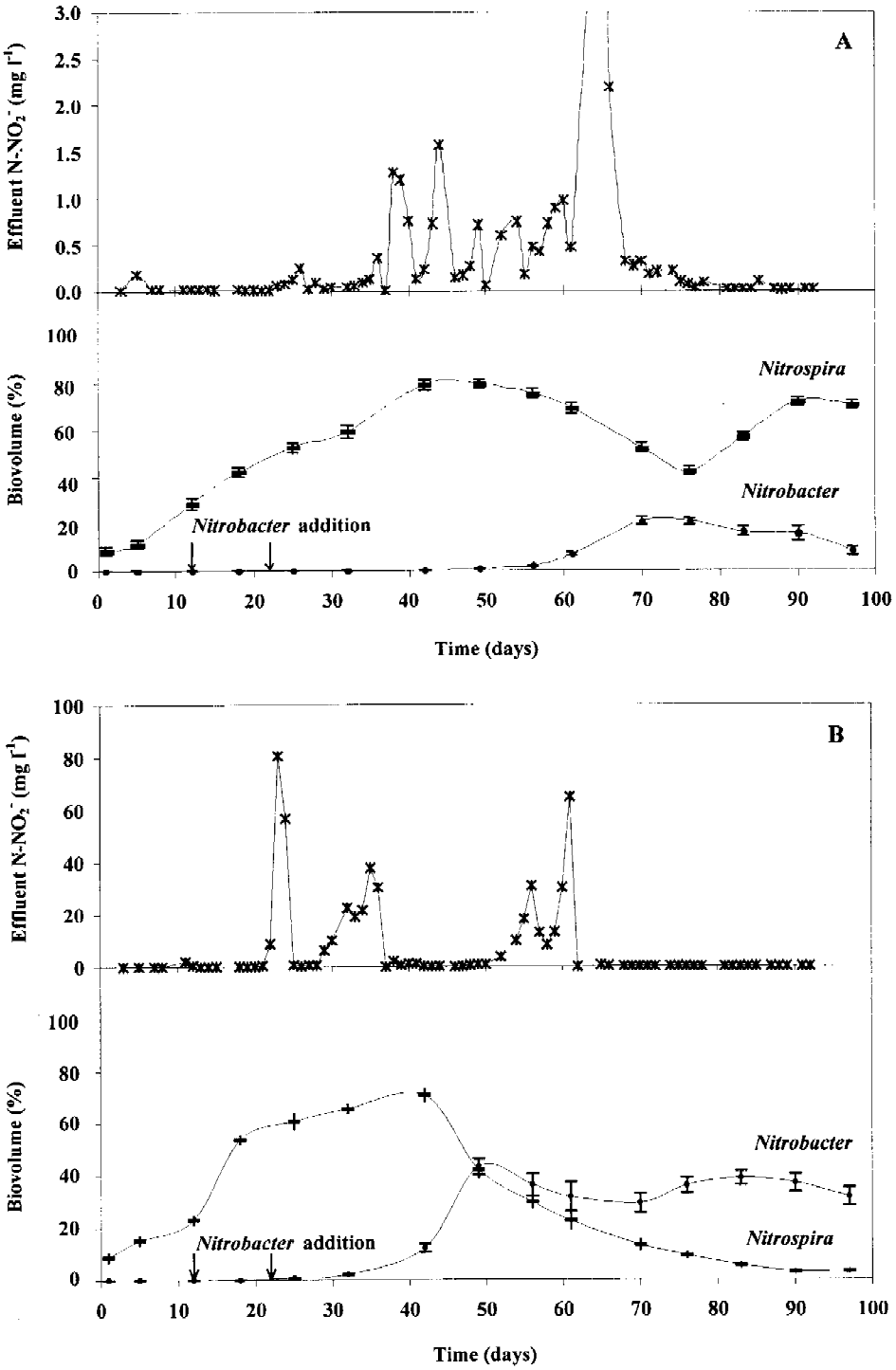


Figure 6. Competition between *Nitrospira* and *Nitrobacter* in chemostats. Two parallel chemostats were inoculated with nitrifying activated sludge containing *Nitrospira*-like bacteria. Subsequently, *Nitrobacter* was added (arrows). In one chemostat nitrite peaks in the effluent (up to 80 mg l^{-1}) were induced by temporarily elevating the nitrite concentrations in the influent (B; upper panel). The other chemostat served as control reactor which always had nitrite effluent concentrations of below 5 mg l^{-1}(A, upper panel). The relative biovolume of *Nitrospira* and *Nitrobacter* (to the biovolume of all cells detectable by FISH) was determined by quantitative FISH in the chemostats for a period of 98 days (A, B; lower panels).

oxidation, and denitrification. Surprisingly, the model organisms described in text books for these processes and for ammonia oxidation are shown to be generally not of importance for wastewater treatment. It is important to note that significant diversity exists in each of these functional groups of bacteria. In the next research phase in wastewater microbiology, more detailed knowledge on the biology of the above mentioned non-cultured bacteria needs to be gained. Therefore, an increased effort on the development of suitable cultivation strategies for these bacteria is needed. In parallel, the use of techniques referred to as 'environmental genomics' should allow to investigate the genome composition of these bacteria without the need of cultivation (Schleper 1998).

Regarding application, the most obvious benefit of the progress described is that it provides a basis for a more knowledge-driven treatment of wwtp failures. One strategy to improve a particular aspect of process performance in a wwtp, for example during start-up or after its breakdown, is the addition of specialized microorganisms or activated sludge from another wwtp (Rittmann & Whiteman 1994). This operational tool which is called bioaugmentation does however frequently fail (e.g. Bouchez 2000 and references therein). Such failure is typically caused by addition of the 'wrong' microorganisms, for example the model organisms for nitrification, which can not compete successfully with the autochthonous bacteria in the plant and are thus eliminated or washed-out. Therefore, it was (and still is) important to identify those microorganisms responsible for nitrogen or phosphorus removal in a functioning wwtp. If problems arise these results can be used as guidance to select the appropriate bacterial additive (for culturable microorganisms) or a well-suited activated sludge from a neighboring wwtp containing a comparable microbial flora. Once the appropriate bacteria have been selected they need to be protected for example by polymer embedding from grazing by protozoa (Bouchez 2000).

Compared to curing failure of a certain process in a wwtp by bioaugmentation, protecting the plant from process deterioration is a more sustainable strategy. For this purpose we need to understand the links between the diversity of a functionally important bacterial group and the stability of the catalyzed process. Preliminary observations indicate that plants with low functional redundancy due to the presence of a low diversity of bacteria of a certain functional group are more sensitive to failure of the respective process than plants harboring a high diversity of the same bacterial group. If increase in diversity can indeed be proven to cause process stabilization then it will be important to learn how plant design and process parameter control can be optimized to increase the diversity of functionally important bacterial groups. To answer these ecologically and economically important questions it will be necessary to determine the microbial community composition of a large number of different samples obtained from tightly controlled reactor studies as well as full scale wwtps. Due to the tediousness of many of the established molecular methods this kind of research will greatly benefit from the implementation of modern high throughput techniques like DNA microarrays for measuring microbial community composition in complex samples (Guschin 1997).

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