



***Microbial Community Analysis of two
Full-scale
Wastewater Treatment Plants:
Oerlinghausen & Bad Salzulfen***

**Final Report
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Table of Contents

1. Aim and methodology	3
Project aim.....	3
Partners and Period.....	3
Sample description	3
Methods	3
2. Results	5
Overall bacterial groups.....	5
Detailed studies - Oerlinghausen Wastewater Treatment Plant	10
Denitrifiers.....	11
Putative PAOs and GAOs.....	12
Filamentous Bacteria	13
Other bacteria	16
Detailed studies – Bad Sadsalzulfen Wastewater Treatment Plant	17
Denitrifiers.....	18
Putative PAOs and GAOs.....	19
Filamentous Bacteria	20
Other bacteria	23
General Discussion.....	24
General characteristics and comparison of populations	24
Differences in the microbial composition in activated sludge and effluent from clarifiers.....	24
Corellations.....	25
Appendix 1	28
Appendix 2	29
Appendix 3	30
Appendix 4 Light microscopy investigation	31
Oerlinghausen.....	31
Bad Salzulfen.....	32
Appendix 5. Filament index, SVI and total filamentous bacteria	33
Oerlinghausen.....	33
Bad Salzulfen.....	34

1. Aim and methodology

Project aim

The aim of the project is to:

1. Analyse the microbial population of activated sludge from two full-scale German wastewater treatment plants with the use of molecular methods. Besides community analysis detailed population dynamics – shifts in populations - was determined.
2. Detect potential quantitative differences of microbial populations in activated sludge (AS) reactor and effluent from clarifier.

Partners and Period

The project has been running as a collaboration between LimnoTec Abwasseranlagen GmbH and Aalborg University (AAU), Section of Biotechnology, in the period August 2008-June 2010.

Sample description

Activated sludge samples were sent from two German full-scale N/DN WWTPs: Oerlinghausen (OE) and Bad Salzulfen (BS) 17 times in the period 10.09.2008 – 30.03.2010. Two samples came from each plant, one from mixed activated sludge (BB3) and one from the clarifier (NKS).

Upon arrival, light microscopically examination of fresh activated sludge was performed. All samples were fixed in formamid (FA) or ethanol (EtOH) prior and frozen down for later community analysis by quantitative Fluorescence in situ Hybridization (q-FISH) analysis. An overview of the samples received is shown in Appendix 2.

Methods

Light microscopy was used to determine overall activated sludge characteristic and presence of filaments (filament index, FI) in most of the samples. The microbial composition of activated sludge was conducted by epifluorescence microscopy (q-FISH) analysis.

A range of specific gene probes was used for q-FISH in order to determine the abundance of key bacterial groups i.e. nitrifiers, denitrifiers, potential PAO (Polyphosphate Accumulating Organisms), GAO (Glycogen Accumulating Organisms), protein hydrolysers, fermenters and filamentous bacteria. All the specific probes were labelled with Cy3 (red-fluorochrome) while the general probe targeting all bacteria (EUBmix) was labelled with FLUOS (green). Table 1 in Appendix summarizes all probes and their target populations.

The number of specific gene probes used in the first survey (described in Report - December 2009) was rather high and later reduced as many of these did not target any bacteria. Instead broader gene probes were applied to target broader groups of bacteria to give a better overview of the microbial populations.

Note: All results presented are given as a percentage out of all bacteria analysed with the gene probe EUBmix. This number is an average and the standard deviation is 15-

20% of the average. The notation 0.2% is used when the bacteria are present but in a percentage lower than our quantification limit of 0.5%.

2. Results

The very first FISH analyses described in the first report (Report– December 2009) were carried on the basis of previous studies of Danish treatment plants and results published in other international publications. The selection of gene probes was based on results from the “Microbial Database for Danish Treatments plant”, where we have monitored the population of microbial communities in more than 30 full-scale nutrient removing plants for four years. In these, it was possible to identify 60 to 80% of all bacteria in the activated sludge systems from nutrient removal systems. Results from these studies are used in this report as “Danish Standard Microbial Composition”. This composition can be found in Appendix 3.

Overall bacterial groups

Only about 30% of the microbial populations in the two German treatment plants could be identified with specific gene probes in the preliminary analysis in December 2009. Therefore, we have also applied broader probes and also some new specific probes based on these results. This set of probes has been used to determine the general compositions as shown in Table 1 and 2.

Oerlinghausen. The main groups were Alphaproteobacteria, Chloroflexi and Actinobacteria. This is to some extent different from Danish plants where Betaproteobacteria are most common. Chloroflexi and Actinobacteria are also common, but Alphaproteobacteria are not. Among both Alphaproteobacteria and Chloroflexi there were many filamentous bacteria.

Bad Salzufen. The main groups were Alphaproteobacteria, Chloroflexi and Actinobacteria. As mentioned above this is to some extent different from Danish plants where Betaproteobacteria are most common. Chloroflexi and Actinobacteria are also common, but Alphaproteobacteria are not. Among both Alphaproteobacteria, Actinobacteria and Chloroflexi there were many filamentous bacteria.

Overall bacterial profile.

Table 1. Oerlinghausen - microbial composition

AS reactor	Alpha - proteobacteria	Beta - proteobacteria	Gamma - proteobacteria	Actinobacteria	Firmicutes	Chloroflexi	TOTAL
10-sep-08							
1-okt-08	8.0	0.3	2.5	3.6	0.9	44.6	59.7
22-okt-08	10.2	1.0	3.1	4.3	1.9	37.1	57.6
3-dec-08	13.3	3.1	3.2	12.5	0.9	32.4	65.5
17-dec-08	11.7	1.2	2.8	5.7	0.8	27.5	49.7
17-feb-09	1.3	3.1	5.5	10.4	0.9	10.8	32.0
11-mar-09							
2-jun-09	10.3	5.2	2.2	3.8	0.7	16.5	38.7
1-jul-09	23.7	4.8	6.1	9.4	0.3	29.0	73.3
29-jul-09	20.4	3.2	7.1	9.8	0.5	12.4	53.4
26-aug-09	14.3	1.1	13.6	5.8	0.9	24.7	60.4
22-sep-09							
18-nov-09	3.5	2.2	12.4	8.8	0.5	8.0	35.3
16-dec-09	7.6	3.3	4.9	10.7	0.2	16.7	43.5
2-feb-10	5.7	2.2	6.2	6.5	0.2	2.8	23.6
2-mar-10	8.0	3.2	1.5	8.9	0.2	3.2	25.0
30-mar-10	8.4	2.7	1.4	10.3	0.2	4.3	27.3

Clarifier	Alpha - proteobacteria	Beta - proteobacteria	Gamma - proteobacteria	Actinobacteria	Firmicutes	Chloroflexi	TOTAL
10-sep-08							
1-okt-08	10.4	0.8	3.6	14.7	0.2	17.6	47.4
22-okt-08	12.8	0.7	5.0	10.2	0.2	10.5	39.5
3-dec-08	9.5	2.2	7.5	7.1	1.0	10.6	37.9
17-dec-08	14.2	0.7	6.3	7.7	0.2	30.8	60.0
17-feb-09	13.4	1.5	4.9	5.9	1.3	12.7	39.7
11-mar-09							
2-jun-09							
1-jul-09	11.3	0.3	4.4	10.1	0.2	10.9	37.1
29-jul-09	14.5	2.4	4.3	11.5	0.7	17.4	50.8
26-aug-09	7.8	0.3	4.8	9.2	1.4	24.6	48.1
22-sep-09							
18-nov-09	23.6	2.9	7.1	7.2	1.4	35.0	77.2
16-dec-09	13.8	2.7	5.8	11.3	0.9	23.1	57.7
2-feb-10	7.4	6.8	2.6	12.2	0.2	2.5	31.7
2-mar-10	21.4	3.3	3.0	14.5	0.2	3.1	45.5
30-mar-10	24.5	4.7	3.9	11.7	0.2	5.8	50.8

Table 2. Bad Salzulfen. Overall microbial composition.

AS reactor	Alpha proteobacteria -	Beta proteobacteria -	Gamma proteobacteria -	Actinobacteria	Firmicutes	Chloroflexi	TOTAL
10-sep-08	7.9	0.3	1.5	1.1	2.2	12.0	25.0
1-okt-08	6.0	0.3	1.6	3.3	5.9	13.5	30.5
22-okt-08	8.5	6.3	2.4	4.5	3.8	8.5	33.8
3-dec-08	7.4	5.2	3.1	2.2	3.9	7.1	28.9
17-dec-08	7.7	3.8	1.6	2.5	5.5	10.2	31.4
17-feb-09	6.9	1.3	2.4	5.1	1.7	5.4	22.9
11-mar-09	8.5	2.1	3.6	6.7	1.1	4.9	27.0
2-jun-09	6.8	1.7	2.0	7.6	2.2	3.2	23.5
1-jul-09	7.9	1.6	1.0	6.5	1.1	5.1	23.2
29-jul-09	9.3	1.2	0.7	3.1	3.1	11.3	28.8
26-aug-09	8.5	0.3	2.2	2.3	2.2	6.3	21.7
22-sep-09							
18-nov-09	10.4	3.4	0.7	3.7	3.8	10.4	32.6
16-dec-09	14.5	3.7	0.6	6.3	4.0	7.1	36.2
2-feb-10	9.8	4.3	2.5	9.3	5.4	8.5	39.9
2-mar-10	9.5	4.6	3.4	7.9	1.7	5.4	32.5
30-mar-10	11.7	4.0	3.9	8.1	3.4	6.7	37.8

Clarifier	Alpha proteobacteria -	Beta proteobacteria -	Gamma proteobacteria -	Actinobacteria	Firmicutes	Chloroflexi	TOTAL
10-sep-08	5.5	1.7	1.3	3.5	3.6	8.1	23.8
1-okt-08	6.5	1.4	1.5	1.1	5.7	12.4	28.6
22-okt-08	13.4	7.3	2.1	4.0	7.4	24.8	59.0
3-dec-08	9.8	6.1	2.9	0.7	3.9	12.0	35.5
17-dec-08	8.9	7.2	2.0	2.4	2.7	8.2	31.4
17-feb-09							
11-mar-09	12.6	4.1	1.5	5.0	1.0	5.3	29.7
2-jun-09	8.6	1.3	0.7	7.8	1.4	8.3	28.1
1-jul-09	0.9	3.2	0.6	4.0	1.6	7.9	18.2
29-jul-09	10.1	0.3	0.5	0.3	0.2	9.1	20.4
26-aug-09	10.3	0.3	0.7	4.4	1.3	11.2	28.2
22-sep-09							
18-nov-09	9.4	4.3	0.4	3.2	2.4	8.4	28.2
16-dec-09	10.6	3.9	1.3	7.3	1.2	9.4	33.7
2-feb-10	11.3	5.8	1.8	12.1	3.5	12.9	47.4
2-mar-10	14.5	6.4	2.9	8.9	5.2	5.0	43.0
30-mar-10	13.5	5.3	3.4	10.1	3.9	7.2	43.4

Figure 1 and 2 show minimum and maximum coverage in both examined plants. During the period we were able to identify from 24 to 73% of the population (bacteria coverage) in Oerlinghausen. In Bad Salzuflen we could only determine 24-40% by using the broad probes. The results depict that the microbial composition in both wastewater treatment plants is indeed different than Danish treatment plants, what supports the previous initial observations. Furthermore, a significant fraction remain unidentified. This is very surprising since we have applied broad probes that should target the majority of bacteria normally present in wastewater treatment plants

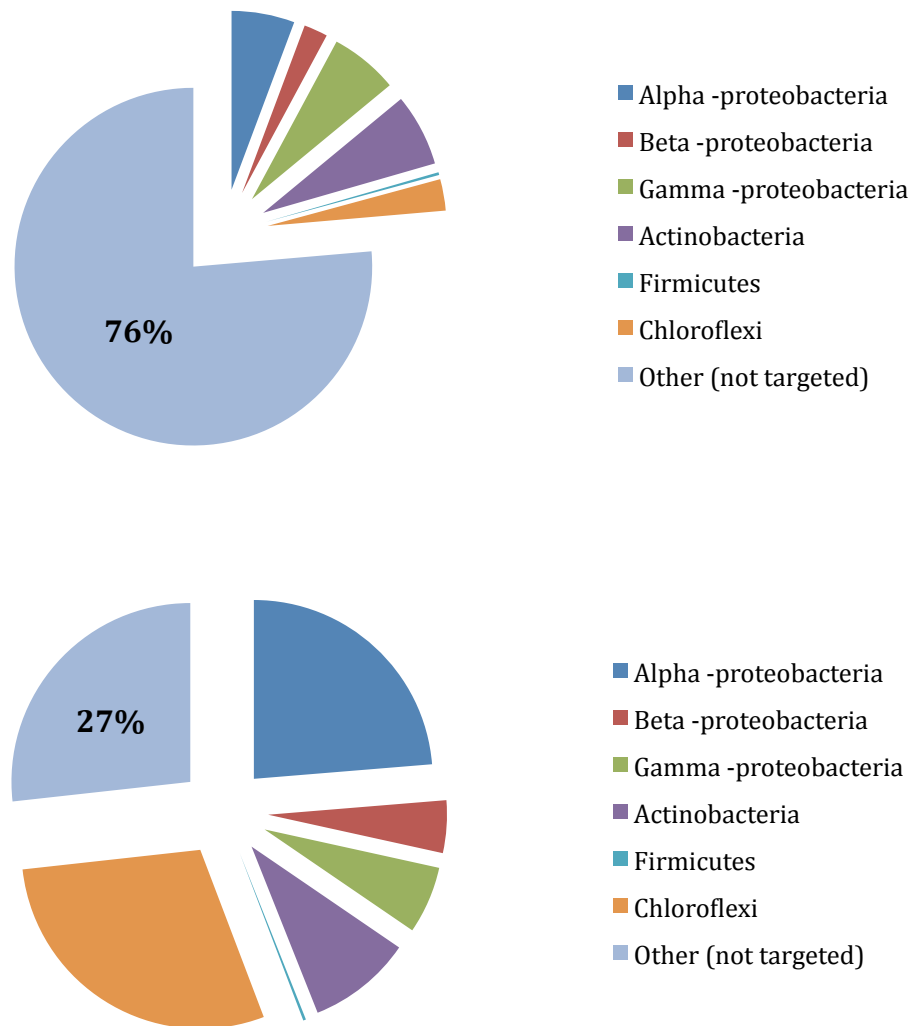


Figure 1. Oerlinghausen - Minimum and maximum coverage (AS reactor)

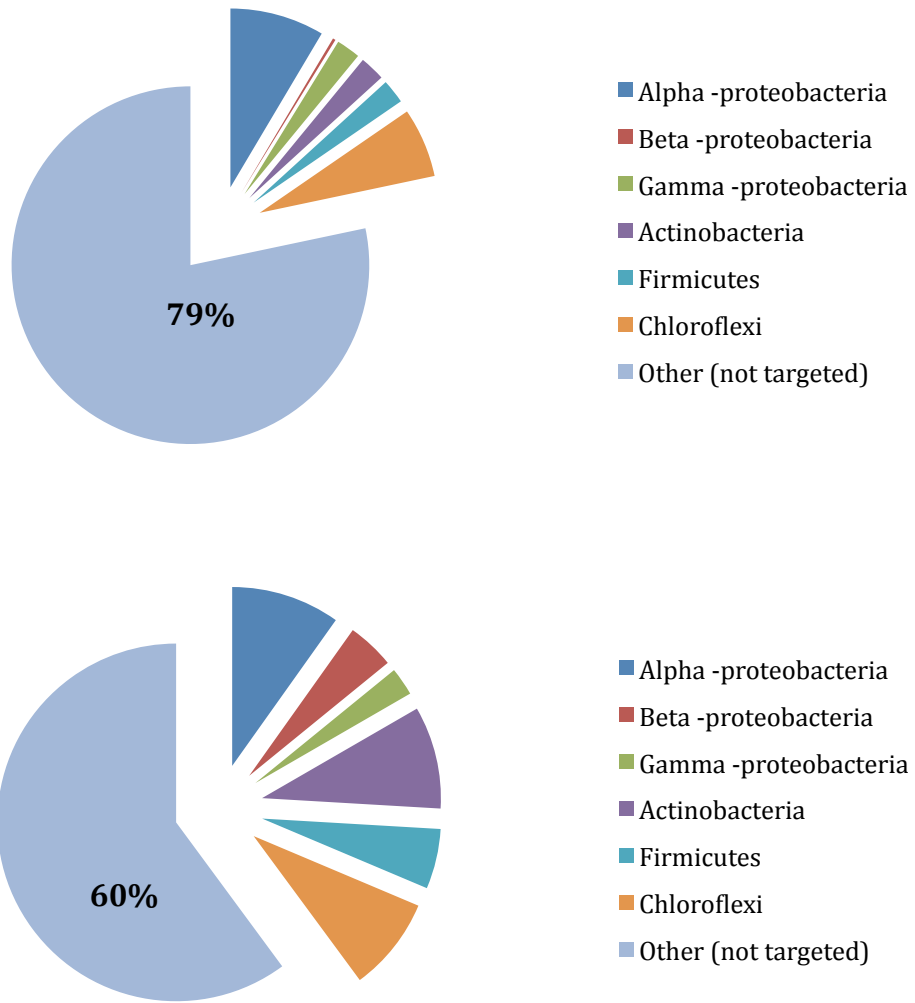


Figure 2. Bad Salzulfen - minimum and maximum coverage (AS reactor)

Detailed studies - Oerlinghausen Wastewater Treatment Plant

Nitrifiers

Ammonium oxidizers (AOB). In the activated sludge samples, the total amount of AOBs was low most of the time ranging from 0.25 to 3% of the total population. The amount was highest summer-fall and lowest winter-spring. In some cases we could not detect any at all. We were not able to detect which species were present and it was not the most common ones present in Danish plants (Nitrosomonas and Nitrospira). Typical values in Danish plants are in average 3-4% for AOB throughout the entire year. The results indicate that the oxidation of ammonium to nitrite only has worked in some periods and that it most of the time has been poor.

Nitrite Oxidizers (NOB): Surprisingly, we were not able to detect any NOBs in any of the samples. That means that the oxidation of nitrite to nitrate has most likely not taken place in the plant. Normal range in Danish plants is 3-4% with Nitrospira as dominant species.

Comparing the numbers in activated sludge and the clarifier/effluent, we could not see a clear relationship although there was a tendency of higher number of AOB in the clarifiers when there were many in the activated sludge.

The lack of NOB and the varying and general low number of AOB show that the nitrification had serious problems in the treatment plant. Likely reasons can be presence of toxic compounds in influent or too short aerobic sludge age. In both cases the NOB are most sensitive and will react (disappear) first. Even if the conditions improve, it takes long time to re-establish the populations due to their slow growth rate.

Table 3. Oerlinghausen. Nitrifiers

	Total AOB	AOB	AOB	NOB	NOB
AS reactor		Nitrospira	Nitrosomonas	Nitrobacter	Nitrospira
10-sep-08					
1-okt-08	0.2	0.2	0.2	0.2	0.2
22-okt-08	0.2	0.2	0.2	0.2	0.2
3-dec-08	1.3	0.2	0.2	0.2	0.2
17-dec-08	0.2	0.2	0.2	0.2	0.2
17-feb-09	1.3	0.2	0.2	0.2	0.2
11-mar-09					
2-jun-09	2.3	0.2	0.2	0.2	0.2
1-jul-09	2.9	0.2	0.2	0.2	0.2
29-jul-09	1.1	0.2	0.2	0.2	0.2
26-aug-09	1.5	0.2	0.2	0.2	0.2
22-sep-09					
18-nov-09	3.0	0.2	0.2	0.2	0.2
16-dec-09	2.3	0.2	0.2	0.2	0.2
2-feb-10	0.2	0.2	0.2	0.2	0.2
2-mar-10	0.2	0.2	0.2	0.2	0.2
30-mar-10	3.4	0.2	0.2	0.2	0.2

	Total AOB
Clarifier	
10-sep-08	
1-okt-08	0.2
22-okt-08	1.2
3-dec-08	2.5
17-dec-08	0.2
17-feb-09	1.6
11-mar-09	
2-jun-09	
1-jul-09	0.2
29-jul-09	0.2
26-aug-09	1.4
22-sep-09	
18-nov-09	1.5
16-dec-09	2.7
2-feb-10	1.3
2-mar-10	1.9
30-mar-10	1.2

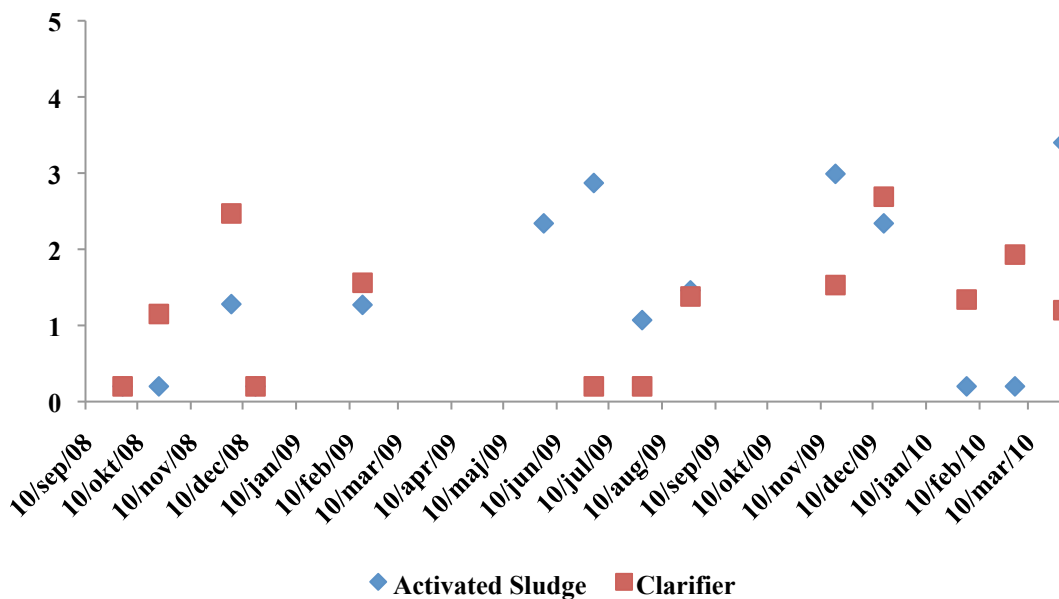


Figure 3. Total AOB in Oerlinghausen

Denitrifiers

Initially we tested six specific probes, but only two of them gave some significant numbers (Azoarcus and Rhodobacter). The other probes tested can be seen in the Appendix 1. The other probes, not used this time, target abundant populations in Danish plants: Curvibacter, Zoogloea and Thauera.

Rhodobacter was detected occasionally in Oerlinghausen, but generally only in low amounts. Azoarcus was only sporadically found in even lower amounts.

We were not able to find the species that are commonly associated with denitrification process in the DN plants in Denmark. One possible explanation is that the number of dedicated denitrifiers is low in the plant due to the poor performance of nitrification. However, most likely some Chloroflexi (described below as filamentous bacteria) are denitrifiers and they may carry out a significant part of the denitrification in periods. However, there are a significant number of non-identified bacteria in the plant and some of these may be unknown denitrifiers.

Table 4. Denitrifiers. Oerlinghausen

	AS reactor		Clarifier	
	Azoarcus	Rhodobacter	Azoarcus	Rhodobacter
10-sep-08				
1-okt-08	0.2	0.2	0.2	0.9
22-okt-08	0.2	0.2	0.2	0.2
3-dec-08	0.2	0.2	0.2	0.2
17-dec-08	0.2	0.2	0.2	0.2
17-feb-09	0.2	0.7	0.2	0.2
11-mar-09				
2-jun-09	0.2	0.2		
1-jul-09	0.2	0.9	0.2	0.2
29-jul-09	0.2	0.2	0.2	0.2
26-aug-09	0.9	0.2	0.2	0.2
22-sep-09				
18-nov-09	0.2	1.3	0.2	0.2
16-dec-09	0.2	0.9	0.2	0.2
2-feb-10	0.2	0.7	0.2	0.9
2-mar-10	0.2	0.8	0.2	3.2
30-mar-10	0.2	0.2	0.2	0.2

Putative PAOs and GAOs

Since the treatment plant does not carry out biological P-removal it was not expected to find any PAOs or GAOs in the plant. The best known PAO, *Accumulibacter* or the best known GAO, *Competibacter* were not present.

The only representatives of putative PAO were two types of *Tetrasphaera*. They can also be involved in other processes such as hydrolysis and fermentation, so their presence does not mean that biological P-removal took place, but a certain removal is possible. Their abundance was substantial, but the stability of the population was relatively low and they varied from 0.25 – 7.5%. In some periods the abundance of *Tetrasphaera* was below the detection limit.

The relative amount of *Tetrasphaera* was generally highest in activated sludge. However, from 18.11.2009 we were able to identify them also in larger fractions in clarifier. *Tetrasphaera* type 2 consists mainly of filamentous bacteria, most probably *Nostocoida limicola*.

Table 5. Oerlinghausen. PAO

	AS reactor		Clarifier	
	Tetrasphaera type 1	Tetrasphaera type 2	Tetrasphaera type 1	Tetrasphaera type 2
10-sep-08				
1-okt-08	5.3	3.2	1.4	0.2
22-okt-08	7.5	1.7	1.3	0.2
3-dec-08	6.6	3.5	0.7	0.2
17-dec-08	3.2	1.0	0.8	3.2
17-feb-09	1.1	0.9	1.5	3.7
11-mar-09				
2-jun-09	1.7	0.2		
1-jul-09	1.1	0.2	0.2	0.2
29-jul-09	1.3	2.2	0.2	2.9
26-aug-09	1.7	1.0	1.6	0.2
22-sep-09	3.1			
18-nov-09	3.1	3.2	6.3	6.9
16-dec-09	4.6	2.2	4.3	3.3
2-feb-10	3.3	0.2	1.8	1.7
2-mar-10	0.2	0.2	0.2	1.3
30-mar-10	3.2	2.1	1.7	1.5

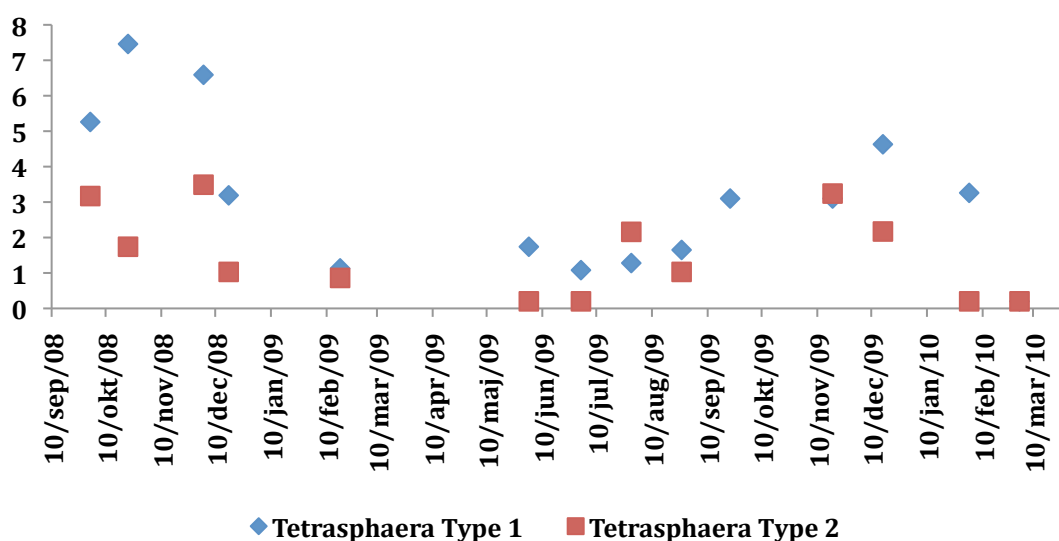


Figure 4. Tetrasphaera in Oerlinghausen WWTP

Filamentous Bacteria

The filamentous bacteria constituted the biggest part of the microbial population in this system occasionally even up to 50% of the biomass. The very high content of filaments is higher than typical Danish plants where they rarely exceed 30%. Fewer filaments were present in the last year (summer 2009-2010), see Table 6 and 7.

Filaments belonging to Chloroflexi and Alphaproteobacteria were most dominant, constituting up to approx. 90% of all filaments detected. Thiothrix/021N were also present, but in a lower percentage. These are unusual in BNR plants and are usually

present in plants without proper denitrification and with a high level of industrial wastewater. Microthrix were not present which is unusual for BNR plants.

Figure 5 shows the distribution and changes of the filamentous bacteria in time. In the first sampling period (2008), we could observe a very high abundance of Chloroflexi followed by a decline. Later on, several filaments were coexisting.

There was no clear difference in relative number of filamentous bacteria in the clarifier and activated sludge reactor.

Table 6. Filamentous bacteria. Oerlinghausen

AS reactor	Chloroflexi	Microthrix	H. hydrossis	Alpha – filaments	T-021N Thiothrix
10-sep-08					
1-okt-08	44.6	0.2	0.2	5.8	1.5
22-okt-08	37.1	0.2	0.2	8.0	1.5
3-dec-08	32.4	0.2	1.5	6.3	3.4
17-dec-08	27.5	0.2	0.2	7.9	1.9
17-feb-09	10.8	0.2	1.4	0.2	0.9
11-mar-09					
2-jun-09	16.5	0.2	2.2	5.3	3.2
1-jul-09	29.0	0.2	2.8	5.9	3.7
29-jul-09	12.4	0.2	2.7	4.9	6.6
26-aug-09	24.7	1.3	3.8	6.9	9.4
22-sep-09			1.4	0.2	4.3
18-nov-09	8.0	0.2	1.4	0.2	4.3
16-dec-09	16.7	0.2	1.0	0.2	1.2
2-feb-10	2.8	0.2	0.2	0.2	0.2
2-mar-10	3.2	0.2	0.2	1.5	0.2
30-mar-10	4.3	0.2	0.2	1.3	0.2

Clarifier	Chloroflexi	Microthrix	H. hydrossis	Alpha – filaments	T-021N Thiothrix
10-sep-08					
1-okt-08	17.6	0.2	0.2	0.2	1.4
22-okt-08	10.5	1.3	0.2	2.5	1.3
3-dec-08	10.6	0.2	0.7	3.8	4.4
17-dec-08	30.8	0.2	0.2	4.3	1.2
17-feb-09	12.7	0.2	0.8	0.2	0.2
11-mar-09					
2-jun-09					
1-jul-09	10.9	0.2	0.2	0.2	10.2
29-jul-09	17.4	0.2	0.2	0.2	1.2
26-aug-09	24.6	0.2	0.2	3.3	4.1
22-sep-09					
18-nov-09	35.0	0.2	0.2	4.7	0.8
16-dec-09	23.1	0.2	0.2	0.2	0.6
2-feb-10	2.5	0.2	0.2	0.2	0.2
2-mar-10	3.1	0.2	0.2	0.2	0.2
30-mar-10	5.8	0.2	0.2	3.2	0.2

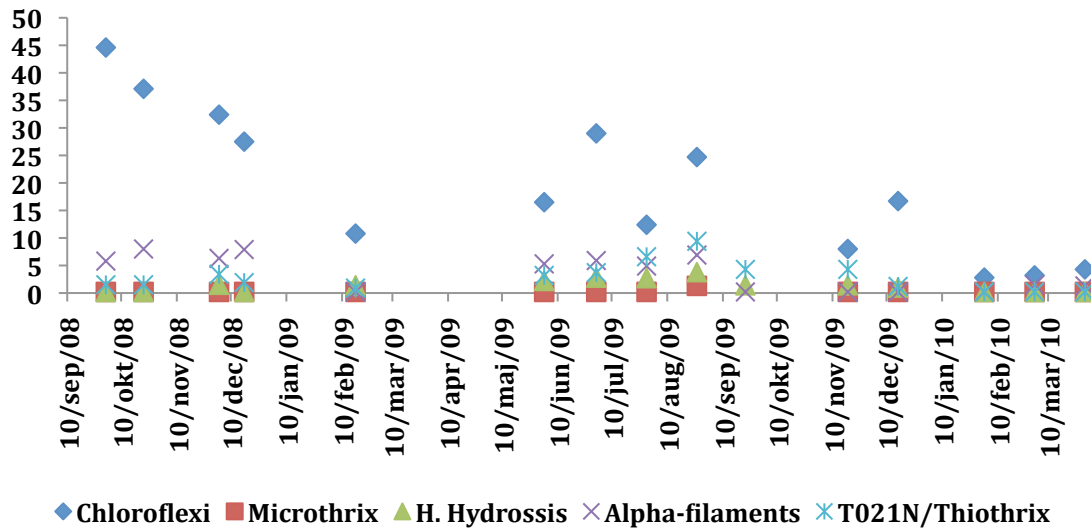


Figure 5. Filamentous bacteria - Oerlinghausen

The number of filamentous bacteria was very large in some periods, and although many of them are growing inside the flocs, they may strongly affect the settling properties. This can be assessed by looking at the filament index as shown in Table 7 and Appendix 4. Generally, the FI is 1.5-3 (scale 0-5) indicating acceptable number of filaments protruding from the flocs in most cases. An FI of 3 may be critical. The very high FI in clarifier samples August-December 2009 indicate an enrichment of these filaments in the effluent part.

Table 7. Oerlinghausen – filament index

	Oerlinghausen (OE)	
	AS REACTOR	CLARIFIER
10-sep-08		
1-okt-08		
22-okt-08		
3-dec-08		
17-dec-08	3.0	1.5
17-feb-09		
11-mar-09		
2-jun-09	1.5	1.0
1-jul-09	2	0
29-jul-09		
26-aug-09	2.0	4.0
22-sep-09		
18-nov-09	2.5	4.0
16-dec-09	3	4.0
2-feb-10	1.5	2.0
2-mar-10	1.5	1.5
30-mar-10	1.0	1.5

We have also performed a more detailed analysis of the Chloroflexi species (Table 8). We have applied four specific gene probes to detect different types of these filamentous bacteria in the most abundant sample (from 1st October 2008).

Table 8. Oerlinghausen. Species of Chloroflexi (October 1, 2008)

AS reactor	
CFX1851	17.2
CFX 678	15.3
CFX 654	3.2
CFX 0092	0.2
Unidentified fraction	8.9

The most abundant species in that sample was Chloroflexi type 1851 and 678. Type 654 was less abundant, whereas 0092 was practically not present. With specific probes we were able to identify 75% of the filaments from the Chloroflexi phylum. The presence of high amounts of Type 1851 is unusual. These have often epiphytic bacteria. We do not know much about their function, but they are most likely involved in extracellular degradation of protein and complex polysaccharides.

Other bacteria

Saprospiraceae include several different bacteria and many of them are associated with protein hydrolysis. They were detected mainly as epiphytic bacteria attached on some filamentous bacteria, probably mainly to certain Chloroflexi types such as type 0041 and type 1851. The amount of the Saprospiraceae of 1-5% (see Table 9) is approx. half of that in Danish treatment plants.

Saprospiraceae was generally more abundant in the activated sludge reactor compared to the secondary clarifier.

Table 9. Oerlinghausen. Protein hydrolysers (Saprospiraceae)

	AS reactor	Clarifier
10-sep-08		
1-okt-08	0.3	0.8
22-okt-08	1.0	0.7
3-dec-08	3.1	2.2
17-dec-08	1.2	0.7
17-feb-09	3.1	1.5
11-mar-09		
2-jun-09	5.2	
1-jul-09	4.8	0.3
29-jul-09	3.2	2.4
26-aug-09	1.1	0.3
22-sep-09	2.2	
18-nov-09	2.2	2.9
16-dec-09	3.3	2.7
2-feb-10	2.2	6.8
2-mar-10	3.2	3.3
30-mar-10	3.5	3.2

Detailed studies – Bad Sadsalzulfen Wastewater Treatment Plant

Nitrifiers

AOB: The total level in activated sludge tank was rather constant around 2.5% (interval 1.5-3.5%) which is a little lower than typical Danish plants, but probably sufficient to ensure a stable ammonium oxidation most of the time. We were not able to specify any AOB species.

NOB: We were not able to detect any NOB in the samples. Thus, the nitrification process seems also not to work well in this plant.

Table 10. Bad Salzulfen. Nitrifiers

AS reactor	Total AOB	AOB Nitrosospira	AOB Nitrosomonas	NOB Nitrobacter	NOB Nitrospira
10-sep-08	0.2	0.2	0.2	0.2	0.2
1-okt-08	1.5	0.2	0.2	0.2	0.2
22-okt-08	2.2	0.2	0.2	0.2	0.2
3-dec-08	2.6	0.2	0.2	0.2	0.2
17-dec-08	2.6	0.2	0.2	0.2	0.2
17-feb-09	2.5	0.2	0.2	0.2	0.2
11-mar-09	2.4	0.2	0.2	0.2	0.2
2-jun-09	2.1	0.2	0.2	0.2	0.2
1-jul-09	2.4	0.2	0.2	0.2	0.2
29-jul-09	1.9	0.2	0.2	0.2	0.2
26-aug-09	2.6	0.2	0.2	0.2	0.2
22-sep-09					
18-nov-09	3.2	0.2	0.2	0.2	0.2
16-dec-09	1.4	0.2	0.2	0.2	0.2
2-feb-10	1.9	0.2	0.2	0.2	0.2
2-mar-10	1.8	0.2	0.2	0.2	0.2
30-mar-10	3.4	0.2	0.2	0.2	0.2

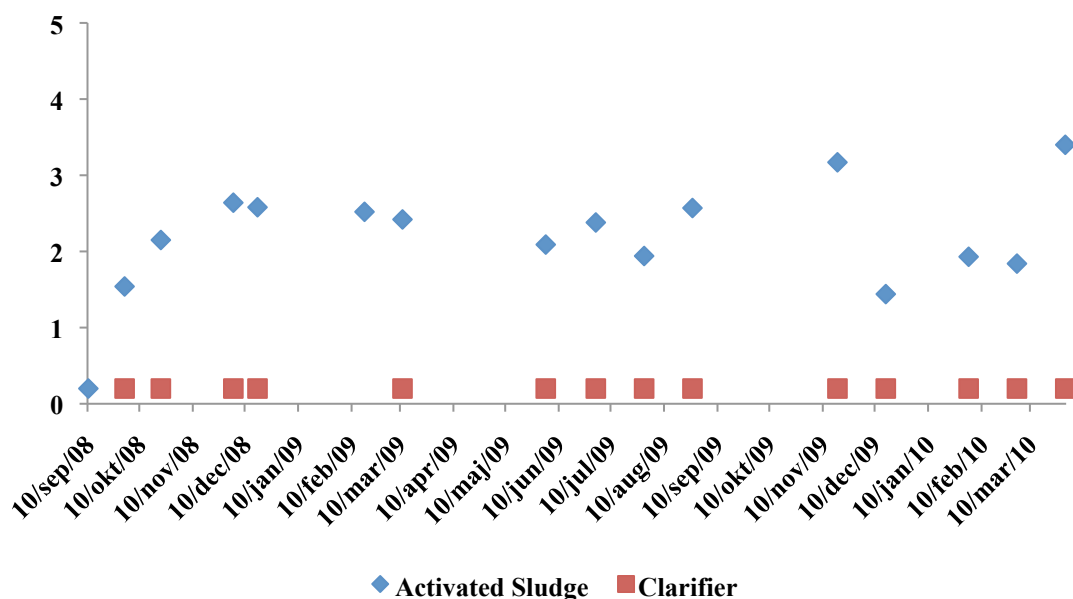


Figure 6. Total AOB in Bad Salzulfen

The clarifier did not show presence of any AOB (Fig. 6), which must be due to an undiscovered error in the analyses.

Denitrifiers

We tested six specific probes but only two of them gave significant numbers (Azoarcus and Rhodobacter). The other probes tested can be seen in Appendix 1.

Most target abundant populations in Danish plants: Curvibacter, Zoogloea and Thauera.

Azoarcus was found in amounts up to 2% but it varied over time. Rhodobacter was detected occasionally, but only in low amounts.

We were not able to find the species that are commonly associated with denitrification in the DN plants in Denmark. One possible explanation is that the number of dedicated denitrifiers is low in the plant due to the poor performance of nitrification. As also mentioned above, most likely some Chloroflexi (described below as filamentous bacteria) are denitrifiers and they may carry out a significant part of the denitrification in periods. However, there are a significant number of non-identified bacteria in the plant and some of these may be denitrifiers.

Table 11. Bad Salzulfen Denitrifiers.

	AS reactor		Clarifier	
	Azoarcus	Rhodobacter	Azoarcus	Rhodobacter
10-sep-08	0.2	0.2	0.2	0.2
1-okt-08	0.2	0.2	0.2	0.2
22-okt-08	1.6	1.0	0.2	0.2
3-dec-08	1.5	0.2	1.1	1.0
17-dec-08	1.9	0.2	0.2	0.8
17-feb-09	0.9	0.2		
11-mar-09	0.7	0.2	0.2	0.9
2-jun-09	0.9	0.2	0.2	0.2
1-jul-09	0.6	0.2	0.2	0.2
29-jul-09	0.2	0.2	0.2	0.2
26-aug-09	0.2	0.2	0.2	0.2
22-sep-09				
18-nov-09	0.2	0.2	0.6	0.2
16-dec-09	0.7	0.2	0.8	0.2
2-feb-10	0.9	0.6	0.8	0.2
2-mar-10	0.2	0.2	1.8	0.2
30-mar-10	0.2	0.2	0.2	0.2

Putative PAOs and GAOs

Since the treatment plant does not carry out biological P-removal it was not expected to find any PAOs or GAOs in the plant. The best known PAO, *Accumulibacter* or the the best known GAO, *Competibacter* were not present. The only representatives of putative PAO were two types of *Tetrasphaera*. They can also be involved in other processes such as hydrolysis and fermentation, so their presence does not mean that biological P-removal took place, but a certain removal is possible. Their abundance was substantial, but the stability of the population was relatively low and they varied from 0.2 – 8.5%, thus indicating very unstable system. In some periods the abundance of *Tetrasphaera* was below the detection limit.

The relative amount of *Tetrasphaera* was generally highest in activated sludge compared to the clarifier.

Tetrasphaera type 2 was mainly filamentous bacteria, most probably *Nostocoida limicola*.

Table 12. Bad Salzulfen. Putative PAOs

	AS reactor		Clarifier	
	Tetrasphaera type 1	Tetrasphaera type 2	Tetrasphaera type 1	Tetrasphaera type 2
10-sep-08	0.2	0.2	1.1	0.2
1-okt-08	0.2	0.2	0.6	0.6
22-okt-08	0.2	0.2	1.0	1.1
3-dec-08	1.4	0.2	0.8	1.4
17-dec-08	1.6	3.9	3.6	3.8
17-feb-09	3.4	8.3		
11-mar-09	4.7	1.9	2.7	5.8
2-jun-09	1.3	1.4	2.6	2.4
1-jul-09	1.1	1.7	1.1	1.8
29-jul-09	0.5	2.1	0.7	0.9
26-aug-09	0.8	0.2	0.2	0.6
22-sep-09				
18-nov-09	0.8	1.4	0.2	2.0
16-dec-09	3.8	0.8	0.2	1.8
2-feb-10	2.9	1.3	1.6	3.9
2-mar-10	1.4	3.5	3.5	4.5
30-mar-10	2.7	3.6	2.8	2.9

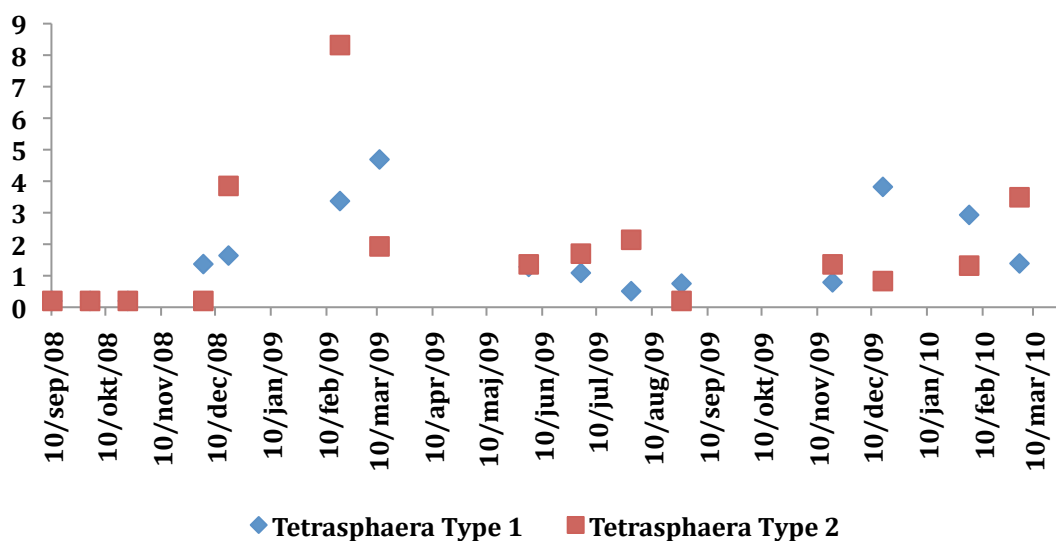


Figure 7. Tetrasphaera in Bad Salzulfen WWTP

Filamentous Bacteria

The filamentous bacteria constituted a big part of the microbial population in this system occasionally up to 25% of the biomass. This is similar to typical Danish NBR plants.

Filaments belonging to Chloroflexi and Microthrix were dominant and it is also normal for Danish BNR plants. There were only few samples where both species were equally present. There was no clear seasonal pattern of their presence. Microthrix is usually most dominant during winter periods in European plants.

There were generally more filaments detected in the clarifier than in the activated sludge reactor. Chloroflexi was mostly dominant in the reactor, whereas Microthrix was more in clarifier in the last sampling period.

Figure 8 shows the changes of filamentous bacteria in time in Bad Salzulfen WWTP. The abundance of Chloroflexi was decreasing in 2008, whereas Microthrix was increasing. Microthrix was dominant filamentous bacteria in nearly whole 2009. In the end of 2009 Microthrix population decreased and from that point co-dominant with Chloroflexi.

Table 13. Bad Salzulfen Filamentous bacteria.

AS reactor	Chloroflexi	Microthrix	H. hydrossis	Alpha – filaments	T-021N Thiothrix
10-sep-08	12.0	2.7	0.2	0.2	0.2
1-okt-08	13.5	6.1	0.2	0.2	0.2
22-okt-08	8.5	4.3	0.2	0.2	0.2
3-dec-08	7.1	5.4	0.2	0.2	0.2
17-dec-08	10.2	0.2	0.9	0.2	0.2
17-feb-09	5.4	11.2	0.9	0.2	0.2
11-mar-09	4.9	13.8	1.2	0.2	0.2
2-jun-09	3.2	22.5	1.4	0.2	0.2
1-jul-09	5.1	18.1	0.7	0.2	0.2
29-jul-09	11.3	12.7	0.2	0.2	0.2
26-aug-09	6.3	4.3	0.2	0.2	0.2
22-sep-09					
18-nov-09	10.4	6.4	0.2	0.2	0.2
16-dec-09	7.1	7.3	0.2	0.2	0.2
2-feb-10	8.5	7.1	1.5	0.2	0.2
2-mar-10	5.4	3.9	0.2	0.2	0.2
30-mar-10	6.7	4.2	0.2	0.2	0.2

Clarifier	Chloroflexi	Microthrix	H. hydrossis	Alpha – filaments	T-021N Thiothrix
10-sep-08	8.1	8.7	0.2	0.2	0.2
1-okt-08	12.4	5.4	0.2	0.2	0.2
22-okt-08	24.8	3.6	0.2	0.2	0.2
3-dec-08	12.0	7.4	0.9	0.2	0.2
17-dec-08	8.2	9.6	0.2	0.2	0.2
17-feb-09					0.2
11-mar-09	5.3	7.5	0.2	0.2	0.2
2-jun-09	8.3	26.0	0.2	0.2	0.2
1-jul-09	7.9	20.4	0.7	0.2	0.2
29-jul-09	9.1	12.0	0.2	0.2	0.2
26-aug-09	11.2	7.2	0.2	0.2	0.2
22-sep-09					
18-nov-09	8.4	6.8	0.2	0.2	0.2
16-dec-09	9.4	12.9	0.2	0.2	0.2
2-feb-10	12.9	14.0	1.2	0.2	0.2
2-mar-10	5.0	17.7	1.3	0.2	0.2
30-mar-10	7.2	6.4	3.1	0.2	0.1

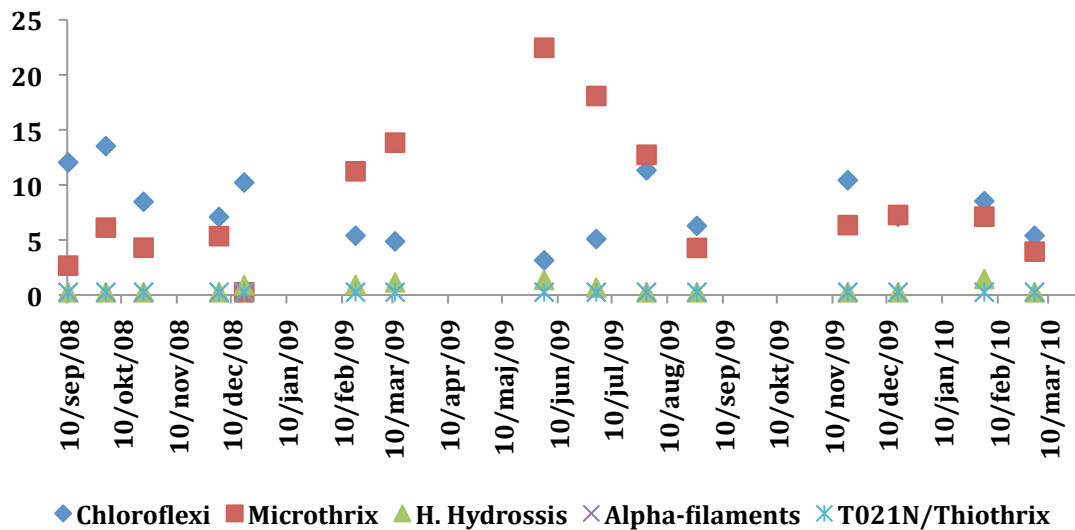


Figure 8. Bad Salzulfen - filamentous bacteria

The number of filamentous bacteria can seem large in some periods, but most of these are in fact growing inside the flocs, so they may not affect the settling properties very much. This can be assessed by looking at the filament index as shown in Table 14. Generally, the FI is below 3 (scale 0-5) indicating low or acceptable number of filaments presumably not causing serious settling properties.

Table 14. Bad Salzulfen – filament index (FI)

	Bad Salzulfen (BS)	
	AS REACTOR	CLARIFIER
10-sep-08		
1-okt-08		
22-okt-08		
3-dec-08		
17-dec-08	2.5	1.0
17-feb-09		
11-mar-09		
2-jun-09	1.5	1.0
1-jul-09	1.0	0.5
29-jul-09		
26-aug-09	1.5	0
22-sep-09		
18-nov-09	2.0	1.5
16-dec-09	2.5	1.0
2-feb-10	2.5	1.5
2-mar-10	1.0	2.5
30-mar-10	1.5	1.0

We have also performed detailed analyses and identification of Chloroflexi species (Table 15). We have applied four specific gene probes to detect different types of these filamentous bacteria in the most abundant sample (from October 22, 2008).

Table 15. Bad Salzsulfen. Species of Chloroflexi (October 22, 2008)

AS reactor	
CFX1851	0.2
CFX 678	0.2
CFX 654	0.2
CFX 0092	1.3
Unidentified fraction	23.5

Surprisingly we were only able to detect one type present (type 0092) and only in very low abundance. The other types were not present so most of the Chloroflexi types remain unknown.

Other bacteria

Saprosiraceae include several different bacteria and many of them are associated with protein hydrolysis. They were detected mainly as epiphytic bacteria attached on some filamentous bacteria, probably mainly to certain Chloroflexi types such as type 0041 and type 1851. The amount of the Saprosiraceae is rather similar to that in Danish treatment plants but it varied very much here in contrast to Danish plants.

Saprosiraceae was generally more abundant in the secondary clarifier compared to the activated sludge reactor.

Table 16. Bad Salzsulfen. Protein Hydrolysers

	AS reactor	Clarifier
10-sep-08	0.2	1.7
1-okt-08	0.2	1.4
22-okt-08	6.3	7.3
3-dec-08	5.2	6.1
17-dec-08	3.8	7.2
17-feb-09	1.3	
11-mar-09	2.1	4.1
2-jun-09	1.7	1.3
1-jul-09	1.6	3.2
29-jul-09	1.2	0.2
26-aug-09	0.2	0.2
22-sep-09		
18-nov-09	3.4	4.3
16-dec-09	3.7	3.9
2-feb-10	4.3	5.8
2-mar-10	4.6	6.4
30-mar-10	3.7	5.3

General Discussion

General characteristics and comparison of populations

Both treatment plants differ in their microbial populations from the “Danish standard” wastewater plant populations. We were not able to detect the major groups that usually constitute the largest part of Danish microbial communities, such as denitrifiers and most nitrifiers. Most of these bacteria usually belong to the Betaproteobacteria, but they were only present in very low number. The significant unidentified fraction is very surprising, since we have applied broad probes that should target the majority of bacteria normally present in wastewater treatment plants.

The dominant group was filamentous bacteria, which constituted up to 30-50% of the total biomass. This is very much and did also influence the settling properties seriously in periods. It is, however, important to remember that a large fraction of the filamentous bacteria were hidden inside the flocs so they did not always cause settling problems.

We have also been able to see the differences in both activated sludge samples from Oerlinghausen and Bad Salzulfen. Whereas nearly all the groups have similar percentage and species composition, the filamentous part was the most dissimilar. In Oerlinghausen case Chloroflexi type was dominant, but also *H. hydrossis*, Alphaproteobacteria and Thiothrix were present. This is typical for a nonBNR plant. In contrast, in Bad Salzulfen we were able to detect both Microthrix and Chloroflexi which is typical for Danish BNR plants.

We were also trying to follow the patterns and dynamics of all the groups, but no clear pattern was detected. The general stability of both treatment plants was low and there were no direct correlations with neither abundance of coherent species nor season of the year.

Differences in the microbial composition in activated sludge and effluent from clarifiers

One important idea of this study was that it should be possible to carry out a direct selective surplus sludge removal of an activated sludge fraction (effluent from clarifier) that is characterized by a higher concentration of flocs with poor settling characteristics. In other words: In the surplus sludge there should be a higher concentration of activated sludge flocs with worse mean settling characteristics than the mean settling characteristics of the total activated sludge.

The microbial composition was quantified in both the AS and the effluent in the two plants. It gave, however, not clear-cut results in terms of different ratios. We need to get more info about the performance of this selective removal before we can analyse in more details the results. A few attempts to find correlations have been made. Figure 9 shows the abundance of Chloroflexi in AS and effluent in the two plants. If it was the same we should expect a straight line with the slope of 1. It seems not to be the case, but the tendency is not systematic. Many point are on the “slope=1” line and the ones outside may be due to special Chloroflexi species that were enriched. This was not further investigated.

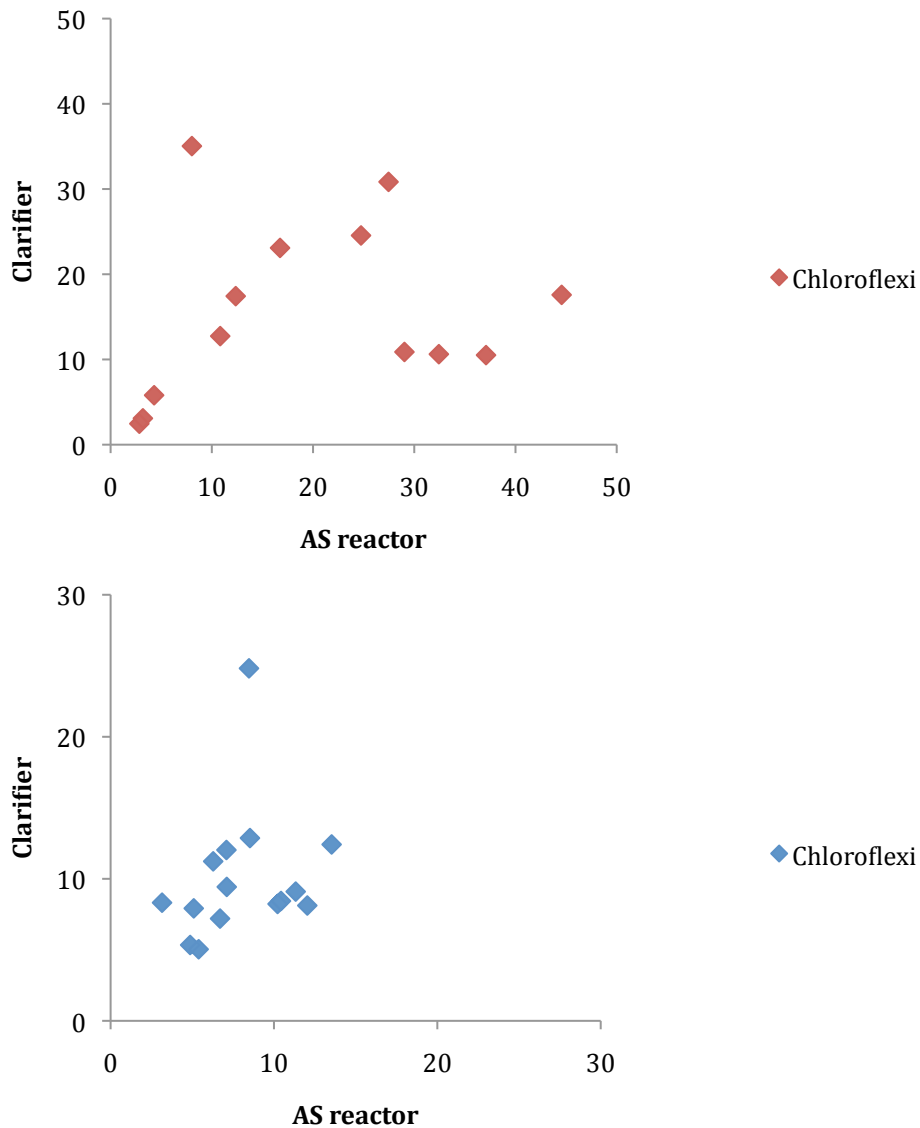


Figure 9. Comparison of the microbial communities in AS and effluent in the two treatment plants. The upper one is Oerlinghausen (red) and the lower one Bad Salzlufen (blue).

Corellations

We have tested whether it is possible to see some correlation between filamentous bacteria and some operational parameter, e.g. sludge volume index (SVI). Figure 10 and 11 show the population dynamics of the filament's population together with SVI measured on site.

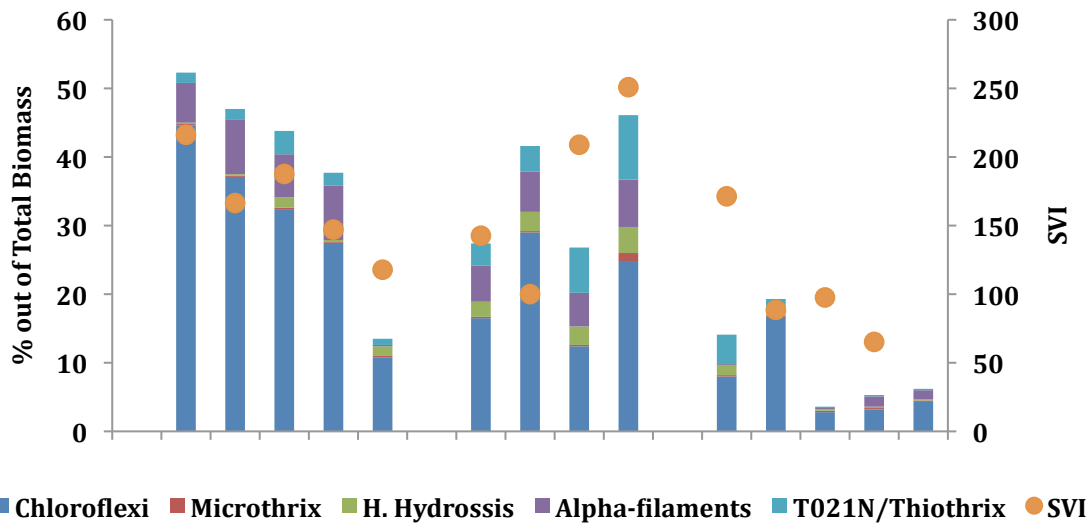


Figure 10. SVI and filamentous bacteria – Oerlinghausen – Activated sludge reactor

The combination of both cumulative “filamentous bars” and SVI points could show if the SVI is influenced by the presence of the filaments. This is the case only in Oerlinghausen where Chloroflexi dominated, and SVI varied with the abundance of the filament population. This was also to some extent the case for the filament index (FI), which indicates no to vey many (0-5 scale), see Appendix 4. To study in more details the relations, we applied more sensitive statistical methods described below.

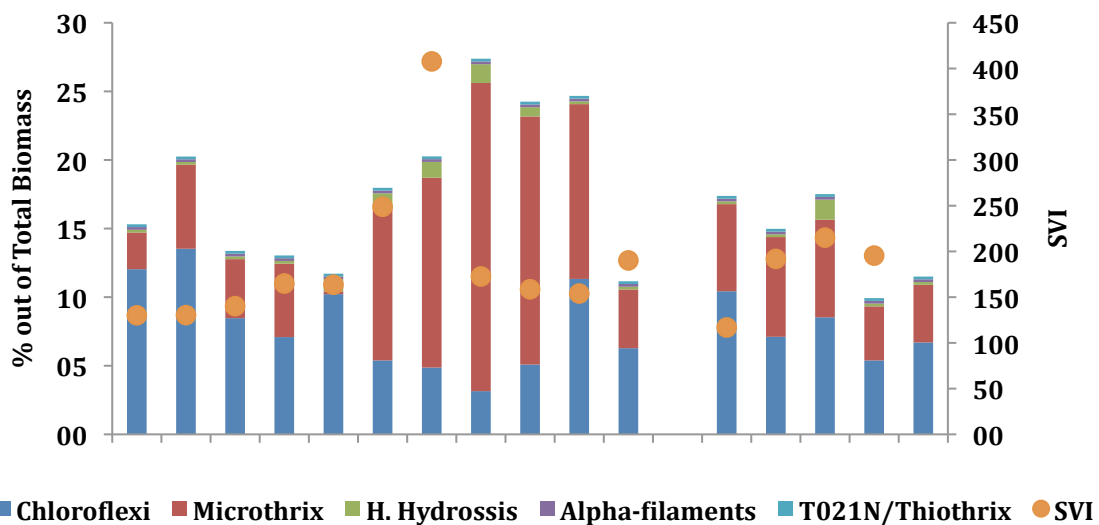


Figure 11. SVI and filamentous bacteria - Bad Salzulfen – Activated sludge reactor

In order to go deeper into possible correlation among the microbial populations, we have applied some statistical analyses. One of the most common methods to discover the relationship between bacteria is the correlation analysis. The result of this calculation is a correlation coefficient - r - that determines the dependence between e.g. to bacteria species. The coefficient varies from -1 to +1 and that value determines the strength of the correlation, as indicated in the following table.

Table 8. Correlation coefficient and its strength

r	Correlation strength	Labeling
0.00 - 0.19	None	N
0.20 - 0.39	Weak	W
0.40 - 0.69	Medium	M
0.70 - 0.89	Strong	S
0.90 - 1.00	Very Strong	VS

In our analysis we are looking for mainly strong and very strong correlations.

First we have checked, as mentioned before, the correlation between filaments and SVI. The correlation matrix in Table 9 shows that there were no direct relation between neither of filamentous species nor sum of filaments and SVI.

Table 9. Correlation matrix - Filamentous bacteria vs SVI

	<i>Chloroflexi</i>	<i>Microthrix</i>	<i>H. hydrossis</i>	<i>Alpha-filaments</i>	<i>T021N/Thiothrix</i>	<i>Sum of filaments</i>
SVI	N	W	N	N	N	N

The next relation tested was the mutual relation between each specific bacterial species. (Table 10). There was only one strong correlation among the all tested, namely the correlation between Alpha-filaments and Chloroflexi. It means that whenever there was more Chloroflexi in the sample we can also expect a higher presence of the Alpha-filaments.

Table 10. Correlation matrix - Mutual relations among different bacteria species

	<i>Chloroflexi</i>	<i>Microthrix</i>	<i>H. hydrossis</i>	<i>Alpha-filaments</i>	<i>T021N/Thiothrix</i>	<i>Tetrasphaera t1</i>	<i>Tetrasphaera t2</i>	<i>Total AOB</i>	<i>Nitrosospira</i>	<i>Azoarcus</i>	<i>Rhodobacter</i>	<i>Saprosiraceae</i>
<i>Chloroflexi</i>	n/a											
<i>Microthrix</i>	M	n/a										
<i>H. hydrossis</i>	N	N	n/a									
<i>Alpha-filaments</i>	S	M	W	n/a								
<i>T021N/Thiothrix</i>	W	W	M	M	n/a							
<i>Tetrasphaera t1</i>	W	N	N	W	N	n/a						
<i>Tetrasphaera t2</i>	N	N	N	N	N	M	n/a					
<i>Total AOB</i>	N	N	N	N	N	N	N	n/a				
<i>Nitrosospira</i>	N	N	N	N	N	N	N	N	n/a			
<i>Azoarcus</i>	W	W	N	N	N	N	N	N	N	n/a		
<i>Rhodobacter</i>	W	W	N	N	N	N	N	N	N	N	n/a	
<i>Saprosiraceae</i>	N	N	N	N	W	N	W	N	N	W	W	n/a

Appendix 1

Overview of gene probes tested and applied for q-FISH. Probes in **Bold** were applied for quantification in most samples.

targeted group	probe name	%FA	target
Nitrifiers	Nso190	55	Betaproteobacterial ammonia-oxidizing bacteria (AOB)
	Nsv443	30	Nitrosospira spp. (AOB)
	Nsm156	5	Nitrosomonas spp., Nitrosococcus mobilis(AOB)
	Nit3	25	Nitrobacter (NOB)
	Ntspa662	35	Genus Nitrospira(NOB)
Denitrifiers	Curvi997	35	Curvibacter (Aquaspirillum delicatum ATCC14667; Pseudomonas lanceolata AB021390 + few more)
	ZRA	35	Zoogloea lineage, not Z. resiniphila
	Azo644	20	Azoarcus cluster
	Thau-646	45	Thauera
	PAR651	40	Paracoccus
	G Rb	30	Rhodobacter, Roseobacter
PAO	PAOmix	35	"Candidatus" Accumulibacter phosphatis
	Acc-I-444	35	Accumulibacter-clade IA and others
	Acc-II-444	35	Accumulibacter clade IIA, IIC, IID
	Actino-221	30	Tetrasphaera related PAO
	Actino-658	40	Tetrasphaera related PAO
	Bet135	45	related to Rhodocyclaceae clones - putative PAO
GAO	Gbmix	35	All Competibacter GAO's
Protein hydrolysers	SAP-309	25	Saprospiraceae
	Bac111	25	Skagen clones in Saprospiraceae
Fermenters	Str (Strept)	30	Streptococcus spp.
Filamentous bacteria	CXF1223	35	phylum Chloroflexi, together with GNSB941
	CFX 0092	35	Chloroflexi type 0092
	CFX 1851	35	Chloroflexi type 1851
	CFX 678	35	Chloroflexi type 678 (unpublished)
	CFX 654	35	Chloroflexi type 654 (unpublished)
	Mpa-all-1410	20	all Microthrix
	HHY-all-654	35	Haliscomenobacter hydrossis
	Nos 174	30	Nostocoida limicola
	TM7-905	20	Phylum candidate division TM7
	ALF968	35	Alphaproteobacteria (filaments)
	G123T	40	all type 021N/Thiothrix
General bacterial groups	ALF968	35	Alphaproteobacteria
	BET42a	35	Betaproteobacteria
	GAM42a	35	Gammaproteobacteria
	HGC 69a	25	Actinobacteria
	LGCmix	35	Firmicutes

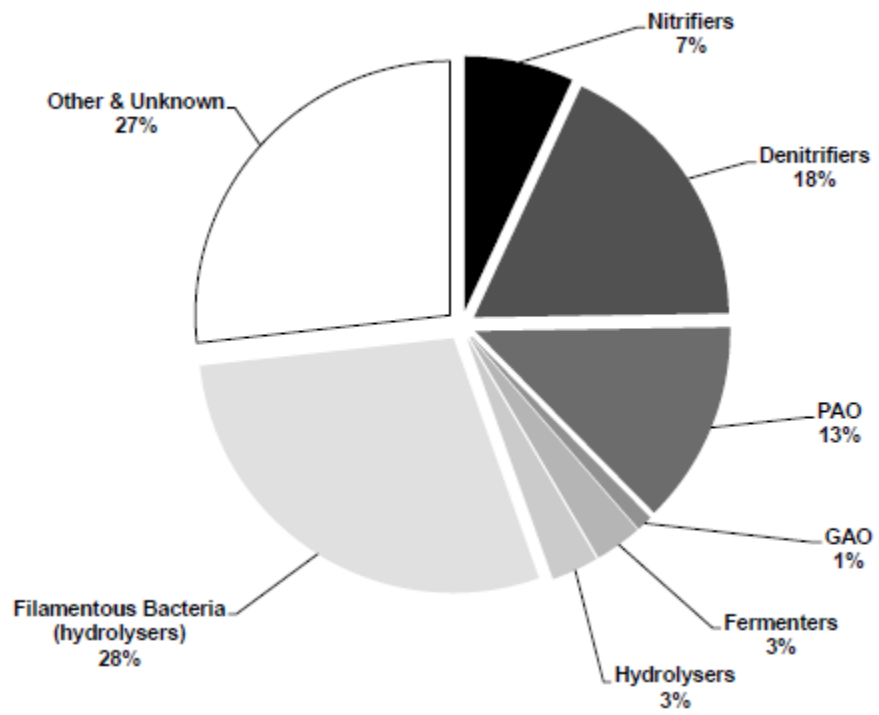
Appendix 2

Detailed sample description

	Oerlinghausen (OE)			Bad Salzuflen (BS)			Unified label
	Sampling	AS REACTOR	CLARIFIER	Sampling	AS REACTOR	CLARIFIER	
1				10.09.08	+	+	10.09.08
2	01.10.08	+	+	01.10.08	+	+	01.10.08
3	21.10.08	+	+	22.10.08	+	+	22.10.08
4	02.12.08	+	+	03.12.08	+	+	03.12.08
5	16.12.08	+	+	17.12.08	+	+	17.12.08
6	17.02.09	+	+	18.02.09	+	-	17.02.09
7				11.03.09	+	+	11.03.09
8	03.06.09	+	-	03.06.09	+	+	02.06.09
9	30.06.09	+	+	30.06.09	+	+	01.07.09
10	29.07.09	+	+	29.07.09	+	+	29.07.09
11	26.08.09	+	+	26.08.09	+	+	26.08.09
12	23.09.09	+	+	23.09.09	+	+	22.09.09
13	18.11.09	+	+	18.11.09	+	+	18.11.09
14	16.12.09	+	+	16.12.09	+	+	16.12.09
15	02.02.10	+	+	02.02.10	+	+	02.02.10
16	02.03.10	+	+	02.03.10	+	+	02.03.10
17	30.03.10	+	+	30.03.10	+	+	30.03.10

Appendix 3

“Standard” Danish Activated Sludge composition from a typical EBPR plant.



Appendix 4 Light microscopy investigation

(Filament Index and floc properties)

Oerlinghausen

AS reactor	FI	Floc size	Floc form		
10-sep-08					
1-okt-08					
22-okt-08					
3-dec-08					
17-dec-08	3.0	>0.25 mm	Robust	Round	Compact
17-feb-09					
11-mar-09					
2-jun-09	1.5	>0.25 mm	Robust	Irregular	Compact
1-jul-09	2	>0.25 mm	Robust	Irregular	
29-jul-09					
26-aug-09	2.0	>0.25 mm	Robust	Round	Compact
22-sep-09					
18-nov-09	2.5	>0.25 mm	Robust	Irregular	Compact
16-dec-09	3	>0.25 mm	Robust	Round	Compact
2-feb-10	1.5	0.025-0.25 mm	Robust	Irregular	Compact
2-mar-10	1.5	0.025-0.25 mm	Robust	Round	Compact
30-mar-10	1.0	0.025-0.25 mm	Robust	Irregular	Compact

Clarifier	FI	Floc size	Floc form		
10-sep-08					
1-okt-08					
22-okt-08					
3-dec-08					
17-dec-08	1.5	>0.25 mm	Robust	Irregular	Compact
17-feb-09					
11-mar-09					
2-jun-09	1.0	0.025-0.25 mm	Robust	Irregular	Compact
1-jul-09	0	<0.25 mm			
29-jul-09					
26-aug-09	4.0	>0.25 mm	Robust	Irregular	Compact
22-sep-09					
18-nov-09	4.0	>0.25 mm	Robust	Irregular	Compact
16-dec-09	4.0	>0.25 mm	Robust	Irregular	Compact
2-feb-10	2.0	>0.25 mm	Robust	Irregular	Compact
2-mar-10	1.5	0.025-0.25 mm	Robust	Irregular	Compact
30-mar-10	1.5	0.025-0.25 mm	Robust	Irregular	Compact

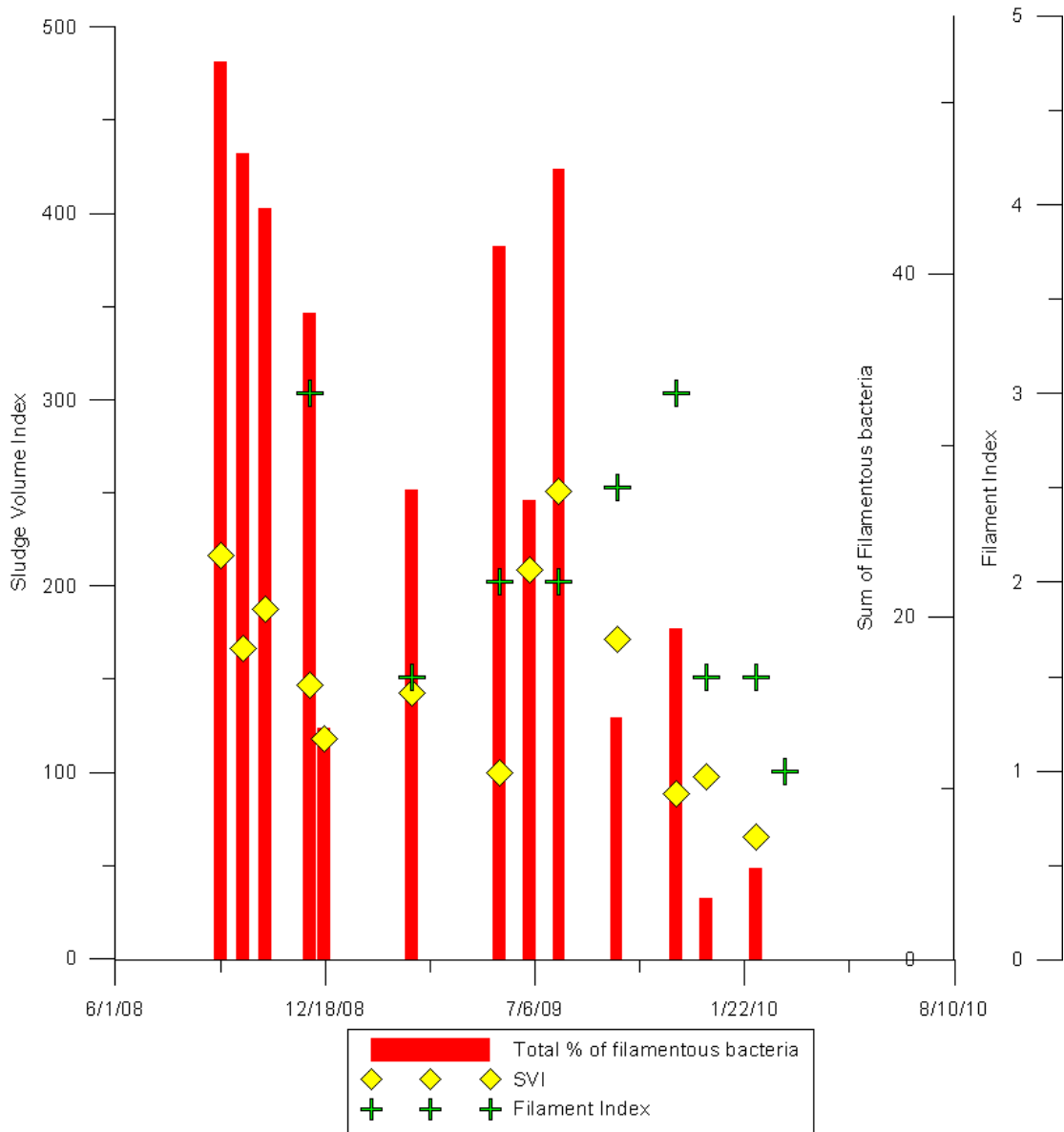
Bad Salzfén

AS reactor	FI	Floc size	Floc form		
10-sep-08					
1-okt-08					
22-okt-08					
3-dec-08					
17-dec-08	2.5	>0.25 mm	Robust	Round	Compact
17-feb-09					
11-mar-09					
2-jun-09	1.5	0.025-0.25 mm	Robust	Irregular	Compact
1-jul-09	1.0	0.025-0.25 mm	Robust	Round	
29-jul-09					
26-aug-09	1.5	0.025-0.25 mm	Robust	Irregular	Compact
22-sep-09					
18-nov-09	2.0	0.025-0.25 mm	Robust	Round	Compact
16-dec-09	2.5	>0.25 mm	Robust	Round	Compact
2-feb-10	2.5	0.025-0.25 mm	Robust	Irregular	Compact
2-mar-10	1.0	0.025-0.25 mm	Robust	Round	Compact
30-mar-10	1.5	0.025-0.25 mm	Robust	Irregular	Compact

Clarifier	FI	Floc size	Floc form		
10-sep-08					
1-okt-08					
22-okt-08					
3-dec-08					
17-dec-08	1.0	0.025-0.25 mm	Robust	Round	Difuse
17-feb-09					
11-mar-09					
2-jun-09	1.0	0.025-0.25 mm	Robust	Irregular	Compact
1-jul-09	0.5	<0.25 mm	Robust	Irregular	
29-jul-09					
26-aug-09	0	<0.25 mm			
22-sep-09					
18-nov-09	1.5	0.025-0.25 mm	Robust	Irregular	Difuse
16-dec-09	1.0	0.025-0.25 mm	Robust	Round	Compact
2-feb-10	1.5	0.025-0.25 mm	Robust	Irregular	Compact
2-mar-10	2.5	0.025-0.25 mm	Robust	Round	Difuse
30-mar-10	1.0	0.025-0.25 mm	Robust	Irregular	Compact

Appendix 5. Filament index, SVI and total filamentous bacteria

Oerlinghausen



Bad Salzulfen

