

Article

Low-Temperature Adapted Nitrifying Microbial Communities of Finnish Wastewater Treatment Systems

Antonina Kruglova ^{1,*}, Jenni Kesulahti ¹, Khoi Minh Le ¹, Alejandro Gonzalez-Martinez ², Anna Mikola ¹ and Riku Vahala ¹

¹ Department of Built Environment, Aalto University, FI-00076 AALTO, Tietotie 1E, P. O. Box 15200, 02150 Espoo, Finland; jenni.kesulahti@gmail.com (J.K.); lminhkhoi@gmail.com (K.M.L.); anna.mikola@aalto.fi (A.M.); riku.vahala@aalto.fi (R.V.)

² Institute of Water Research, University of Granada, 18071 Granada, Spain; agon@ugr.es

* Correspondence: antonina.kruglova@aalto.fi

Received: 30 June 2020; Accepted: 26 August 2020; Published: 31 August 2020



Abstract: In this study, the microbial community of nitrifying activated sludge adapted to Finnish climate conditions was studied to clarify the microbial populations involved in low-temperature nitrification. Microbial community analysis of five full-scale wastewater treatment plants (WWTPs) showed several differences compared to WWTPs from other countries with a similar climate. In particular, very low abundance of ammonium oxidizing bacteria (AOBs) (altogether < 0.25% of total community) as well as typical NOBs (<0.35%) and a high abundance of orders *Cytophagales* and *Micrococcales* was observed in all Finnish WWTPs. To shed light on the importance of autotrophic and heterotrophic nitrifying processes, laboratory studies of activated sludge were carried out with a presence of and a lack of organic carbon in wastewater at 10 ± 1 °C. Two different sludge retention times (SRTs) were compared to determine the effect of this operational parameter on low-temperature nitrogen removal. The important role of previously reported *Candidatus Nitrotogaarctica* for nitrite oxidizing in cold climate conditions was confirmed in both full-scale and laboratory scale results. Additionally, potential participation of *Dokdonella* sp. and *Flexibacter* sp. in nitrogen removal at low-temperatures is proposed. Operation at SRT of 100 days demonstrated more stable and efficient nitrogen removal after a sharp temperature decrease compared to 14 days SRT.

Keywords: nitrogen removal; activated sludge; ammonium oxidizing bacteria; nitrogen oxidizing bacteria; low temperature

1. Introduction

Today, the main responsibility of conventional municipal wastewater treatment systems is to remove organic nutrients such as nitrogen and phosphorus from wastewaters in order to prevent environmental pollution due to human activities. Conventional biological nitrogen removal, based on nitrification and denitrification, is an underlying process in the majority of wastewater treatment plants (WWTPs). The stable and high efficiency of these WWTPs depends on the microbial composition and diversity of functional microbial groups in activated sludge [1]. Consequently, design and operational control of existing WWTPs are based on the gained knowledge of the optimal parameters for the functional microbial communities. A successful nitrification (the oxidation of ammonia to nitrite, and further to nitrate) is traditionally related to sequential activity of ammonia-oxidizing and nitrite-oxidizing bacteria (AOB and NOB, respectively) [2]. Temperature is considered to be one of the key factors affecting growth of the most-described AOBs and NOBs with an optimum value being around 28 °C, however cold-adapted AOB and NOB species have been reported in several studies [3,4].

In Finland, average the temperature of wastewater is in the range of 5–12 °C during most of the year and 14–21 °C in the shorter and warmest period, whilst stable and efficient nitrification can be reached at down to 8 °C [5,6]. There are only a few studies on activated sludge microbial communities operated at this temperature range. According to Gonzalez-Martinez et al. [7], the diversity of bacteria and archaea in WWTPs decreases with the decrease of operational temperature. Decrease of AOB and ammonium oxidizing archaea (AOA) diversities due to a low temperature effect was also reported by Urakawa et al. [8]. Accordingly, low concentrations of conventional nitrifiers have been detected in activated sludge of WWTPs in northern Finland as well as in pilot WWTPs operated at 10 ± 2 °C [7,9]. Unknown temperature-tolerant AOA and heterotrophic nitrifiers were proposed as possible contributors [9,10]. However, at the moment there is no available data on the key nitrifying bacteria and archaea of low-temperature adapted municipal-activated sludge and thus operational parameters of WWTPs are not optimized for cold regions. At the same time, Kruglova et al. [5,6] reported high sensitivity of nitrifying processes to temperature fluctuation at typical operation conditions while operation at longer sludge retention time (SRT) increased the resistance of the nitrifying community to low-temperature stress accompanied by microbial population shifts.

The objectives of this research were to study the microbial community structure of activated sludge adapted to Finnish climate conditions. Clarifying the groups of microorganisms, which are important for low-temperature nitrification, can help to improve day-to-day operation and troubleshooting of the activated sludge processes at WWTPs. To shed light on the importance of autotrophic and heterotrophic nitrifying processes, laboratory studies were carried out with the presence and lack of organic carbon in wastewater. In parallel, experiments with long SRT were performed in order to evaluate whether optimizing this operational parameter could stabilize efficient nitrogen removal in WWTPs in cold regions.

2. Material and Methods

2.1. Sampling

The full-scale activated sludge samples were obtained from five large highly nitrifying WWTPs of Finland. The laboratory low-temperature-adapted activated sludge was obtained from four pilot reactors operated in water laboratory of Aalto University, Espoo. The sampling locations are presented in Figure 1.



Figure 1. Sampling locations. 1–5—full-scale wastewater treatment plants (WWTPs), 6–9 pilot reactors.

The main characteristics of WWTPs are presented in Table 1.

Table 1. General characteristics of studied wastewater treatment plants (WWTP1-5).

Sample	Name	Location	WWTP1 Helsinki	WWTP2 Porvoo	WWTP3 Hyvinkää	WWTP4 Turku	WWTP5 Tampere
Influent characteristics	T _{month.ave} (°C)	Spring	11.5 ± 1	6.9 ± 0.5	8.6 ± 0.5	11 ± 0.5	15 ± 0.5
		Summer	17.1 ± 0.5	17 ± 0.5	14.5 ± 0.5	17.9 ± 0.5	20.5 ± 0.5
	Q _{ave} (m ³ /day)	Spring	280,000	16,000	112,000	180,000	85,000
		Summer	280,000	10,000	9500	60,000	75,000
	pH	Spring	6.1	7.1	7.3	7.4	7.5
		Summer	6.2	7.1	7.4	7.3	7.4
	BOD _{7ATU} (mg/L)	Spring	350	370	190	220	100
		Summer	320	270	200	360	310
	COD _{Cr} (mg/L)	Spring	570	780	450	550	810
		Summer	660	580	650	750	540
	N _{tot} (mg/L)	Spring	51	46	48	43	61
		Summer	60	55	52	76	54
	NH ₄ -N (mg/L)	Spring	35	27	45	26	28
		Summer	35	44	47	57	37
	P _{tot} (mg/L)	Spring	6.9	8.8	6.3	6	3.7
		Summer	8.9	6.6	7.4	8.3	8.5
SS (mg/L)	Spring	300	710	180	430	60	
	Summer	380	260	270	240	570	
Operational parameters	T _{month.ave} (°C)	Spring	14	8	10	13	16
		Summer	19	17	17	19	21
	SRT (day)	Spring	12	14	14	16	6
		Summer	12	8	16	14	10
	DO (mg/L)	Spring	1.9 ± 1.7	2.6 ± 0.1	1.7 ± 0.4	1.6 ± 1.1	3.1 ± 0.5
		Summer	1.9 ± 1.7	3.5 ± 1	1.9 ± 0.3	2.2 ± 1.3	2.1 ± 0.5
	MLSS (mg/L)	Spring	3300	4800	6800	4600	4700
		Summer	2300	2800	4500	4100	5300
WWTP performance	BOD _{7ATU} , removal (%)		97.9	98.8	98.8	98.7	98.5
	COD _{Cr} , removal (%)		92.5	95	95.8	94.1	94
	N _{tot} , removal (%)		91.5	75.7	82.9	80.3	28.3
	P _{tot} , removal (%)		96.5	97	97.7	97.6	97.4
	Nitrification efficiency (%)		98.2	93.3	99.6	97	97.8
	SS, removal (%)		98	99	98.8	99.1	98.4

Full-scale sampling campaigns were organized over the spring and summer periods. Laboratory studies were carried out over autumn–winter periods. One litre of full-scale activated sludge for each sample was delivered to the Water Laboratory of Aalto University 24 h after sampling. From pilot reactors, 0.5 litre of activated sludge were collected for each sample. After collection and delivery to the laboratory, the samples were well mixed, and each sample was divided into two replicates which were immediately centrifuged for 10 min at 5000 rpm. Supernatant was removed and pellets were frozen at −20 °C for further processing.

2.1.1. Full-Scale Study

Full-scale samples were collected twice: in March–May (spring samples) when wastewater temperature was rising from −7 to −12 °C (15 °C in one location) and in the July–August period (summer samples), when the wastewater temperature was between −17 and −22 °C.

2.1.2. Pilot Study

Four pilot reactors (two equal Sequencing batch reactors (SBRs) and two equal Membrane Bioreactors (MBRs)) were operated at 10 ± 1 °C with synthetic municipal-like wastewater. The details of pilot reactor design are presented elsewhere [5,9]. The main operational characteristics during the experimental period are presented in Table 2. Initial synthetic wastewater at the beginning of the experiment contained 130.8 mg L⁻¹ of CH₃COOH* 3 H₂O, 209.7 mg L⁻¹ of yeast extract

and 184.68 mg L⁻¹ of peptone as a carbon source. Other components included NH₄Cl, KH₂PO₄, CaCl₂·2H₂O, MgSO₄·7H₂O, trace nutrients and NaHCO₃ [5]. To remove the source of organic carbon from wastewater, yeast extract and peptone were replaced with acetate in two steps. First, yeast extract and peptone were removed while the concentration of CH₃COOH* 3 H₂O was increased to 392 mg L⁻¹. Next, all three components were totally removed, and the amount of molecular carbon was compensated by NaHCO₃.

Samples from pilot reactors were taken first after the activated sludge from WWTP 1 was adapted to the laboratory conditions. The second sampling was performed after the organic carbon source was removed from the wastewater and the process performance was showing stable results. The main characteristics of the pilot-scale reactors and synthetic wastewater quality on the sampling days are presented in Table 2.

The quality of the influent, effluent and activated sludge was monitored regularly in accordance with Finnish Standards (SFS). The analyses and Standard Methods affiliations are listed in Table S1 of Supplementary materials.

2.2. Samples Processing

Frozen samples were sent to the Granada University in Spain for DNA extraction. DNA isolation was done using the FastDNA SPIN Kit for Soil. Extraction was made according to the manufacturer's instructions (MP Biomedicals, Solon, Ohio, United States). The primer pair 28F (5'-GAGTTTGATCNTGGCTCAG-3')-519R (5'-GTNTTACNGCGGCKGCTG-3') was employed to amplify the V1-V2-V3 hypervariable regions of the 16S rRNA gene of the domain bacteria. For the samples from pilot studies, in addition to the primer pair, Arch519wF (5'-CAGCM GCCGCGGTAA-3')-Arch1017R (5'-GGCCATGCACCWCCTCTC-3') was used to amplify archaeal hypervariable regions V4-V6 of 16S rRNA [11]. The PCR steps were as follows: for bacteria 94 °C for 5 min, followed by 32 cycles at 94 °C for 30 s, 60 °C for 40 s and 72 °C for 60 s and a final elongation step at 72 °C for 5 min; for archaea preheating during 5 m at 95 °C; 40 cycles of 30 s at 95 °C, 40 s at 57 °C, 90 s at 72 °C, and a final elongation step for 25 s at 80 °C [12].

DNA samples were sent for analysis to the United States at the Lubbock, Texas, at the Research and Testing Laboratory (hereinafter RTL). Next generation sequencing (Illumina Miseq apparatus, Illumina, San Diego, CA, USA) was applied to describe bacterial communities and diversity of nitrifying bacteria in activated sludge samples. The sequencing target was variant regions V1, V2, and V3 of the bacterial 16S rRNA gene. Illumina MiSeq Reagent Kit v3 reagent kit (Illumina, San Diego, USA) was used for sequencing. The primers used were 28F-519R pairs of primers.

The sequence data obtained after Illumina sequencing were processed and quality filtered in RTL. Subsequently, those sequences length was less than half of the expected sequence length with the primers used deleted. In addition, sequences that did not have the intact barcode or the separable bar code were removed [13].

The remaining sequences were clustered into operational taxonomic units (OTUs) in RTL. Clustering was done using the UPARSE algorithm and Edgar's methodology [14]. Central OTU has been used for taxonomic classification. The taxonomic classification for OTUs was obtained using the USEARCH global search algorithm. Taxonomic information has been retrieved from RTL's own database. The database used is based on the National Center for the Biotechnology Information (NCBI) database. In the taxonomic assay, the percentages of sequences were 97% for bacterial species and 95% for archaea.

Results from RTL were analysed by both Excel 2016 software and QIIME (Quantitative Insights Into Microbial Ecology, [15] with 1.9.1 according to RTL's methodology [13].

Table 2. The main operational parameters of the pilot-scale reactors.

Laboratory Reactor	Type of Wastewater (Experimental Stage)	Sequencing Batch Reactors		Membrane Bioreactors		
		1	2	1	2	
Synthetic influent wastewater characteristics	T (°C)			10 ± 1		
	V (L)		12	15		
	Q (L/day)	6			15	
	pH			8 ± 0.4		
	BOD ₇ ATU (mg/L)	Municipal-like			360	
		50% lower organic carbon			101	
		No organic carbon source			22.5	
	COD _{Cr} (mg/L)	Municipal-like			525	
		50% lower organic carbon			300	
		No organic carbon source			85	
	N _{tot} (mg/L)	Municipal-like			53	
		50% lower organic carbon			52	
		No organic carbon source			53	
	NH ₄ -N (mg/L)	Municipal-like			15	
50% lower organic carbon				34		
No organic carbon source				52		
P _{tot} (mg/L)	Municipal-like			12		
	50% lower organic carbon			10		
	No organic carbon source			9		
Operational	T (°C)			10 ± 1		
	SRT (d)	14			100	
	DO (mg/l)	6			8	
	MLSS (mg/L)	Municipal-like	2.3	2.3	5	4.8
		50% lower organic carbon	2.2	2.4	5.3	4.9
		No organic carbon source	1.1	1.2	4.5	4.4
	MF membrane		–			+

Table 2. Cont.

Laboratory Reactor		Type of Wastewater (Experimental Stage)	Sequencing Batch Reactors		Membrane Bioreactors	
			1	2	1	2
Process performance	COD _{Cr} , removal (%)	Municipal-like	86	87	87	90
		50% lower organic carbon	62	70	47	60
		No organic carbon source	75	63	93	64
	NH ₄ ⁺ , removal (%)	Municipal-like	74	90	99	97
		50% lower organic carbon	95	99	99	99
		No organic carbon source	97	99	99	99
	SS, removal (%)	Municipal-like	99.5	99.5	99.9	99.9
		50% lower organic carbon	99.5	99	99.9	99.9
		No organic carbon source	99.5	98.7	99.9	99.9

3. Results and Discussion

3.1. Microbial Communities of Finnish WWTPs

Altogether, 228 species from 285 genera of 125 families, 66 orders and 32 classes belonging to 17 bacterial phyla were identified in full-scale samples. On the phylum level, the studied full-scale bacterial communities of activated sludge had a typical structure. Proteobacteria were the most dominant phylum found in all the samples. The other abundant phyla were actinobacteria (in most of the samples) or acidobacteria (in WWTP2), followed by *Bacteroidetes* and *Firmicutes* (Figure 2).

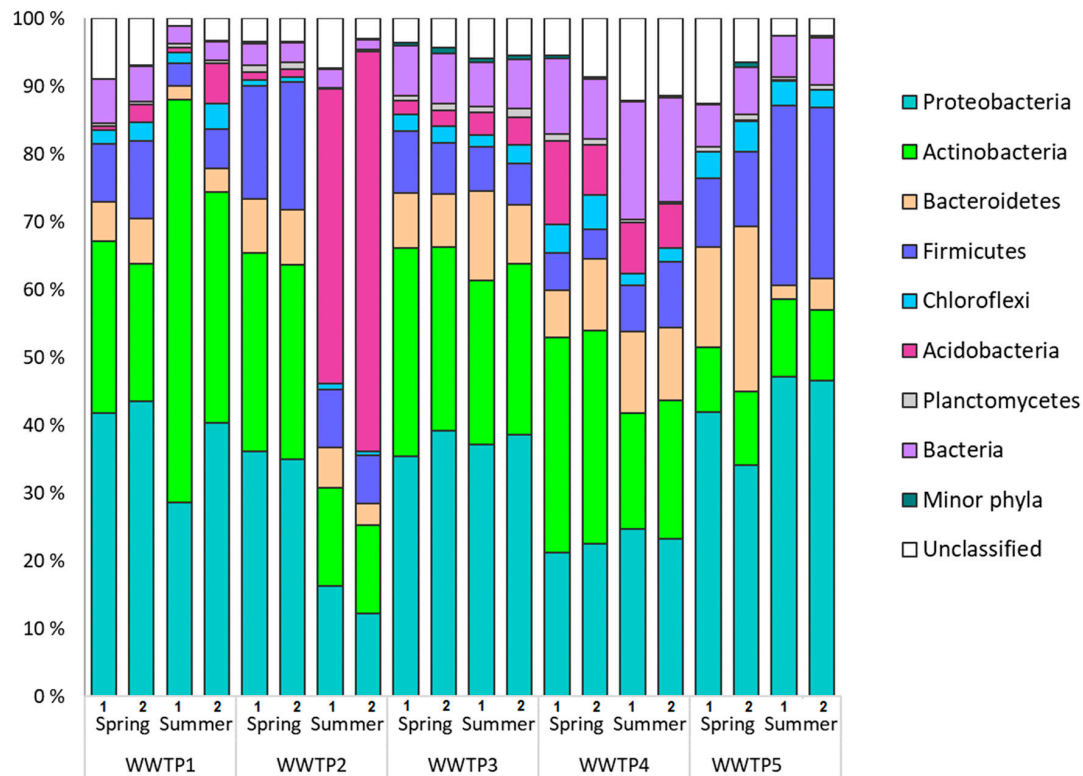


Figure 2. Relative abundance of bacterial phyla in Finnish wastewater treatment plants (WWTPs 1–5) during the spring and summer season.

Proteobacteria are commonly reported as the most abundant bacterial phyla of WWTP-activated sludge as well as *Acidobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicutes*, which are reported as the next most dominant phyla [16–18].

Clear clustering of the samples from each WWTP, despite the small differences between the seasons, was observed using principle coordinates analyses (PCoA) based on Bray–Curtis distances (Figure S1 of Supplementary material). On the class level, most of the *Proteobacteria* in the samples were represented by alpha- and betaproteobacteria (from 30% to 88% of total community together). The dominance of these two classes in the active sludge has also been reported in other studies of WWTP microbial communities [17,19,20].

Spring and summer microbial communities on order level are presented in Tables S2 and S3 of Supplementary Materials, respectively. The most abundant bacteria (>1% of microbial community) in both spring and summer samples of all WWTPs belonged to the orders *Cytophagales*, *Sphingobacteriales*, *Lactobacillales*, *Clostridiales*, *Rhizobiales*, *Rhodobacteriales*, *Burkholderiales* and *Rhodocyclales*. In addition, in summer samples, high abundance of *Micrococcales* in all WWTPs was observed.

Orders *Sphingobacteriales*, *Clostridiales*, *Rhizobiales*, *Burkholderiales* and *Rhodocyclales* were also reported previously in literature as core orders of WWTP-activated sludge [7,17,21]. *Lactobacillales*

and *Rhodobacterales* were found in the study of seven WWTPs from northern Finland, where a high abundance of these bacteria was also reported in all studied influents [7]. In addition, both orders were reported in high abundance in Danish WWTPs with similar yearly temperatures to Finland, ranging from +7 to +20 °C [16,22].

Therefore, the main differences between the structures of the studied microbial communities compared to other reported WWTP data include high abundance of orders *Cytophagales* and *Micrococcales*.

Five bacterial genera presented in the highest abundance in most of the samples are shown in Figure 3. Four of them, including *Candidatus Microthrix*, *Trichococcus*, *Rhodobacter* and *Hyphomicrobium*, were previously reported as the most abundant activated sludge bacteria found in the study of 20 WWTPs in Denmark [22]. In addition, high abundance of denitrifying acidobacterium *Geothrix* was observed in three WWTPs with a sharp increase of up to 58% of the community in summer samples of WWTP2.

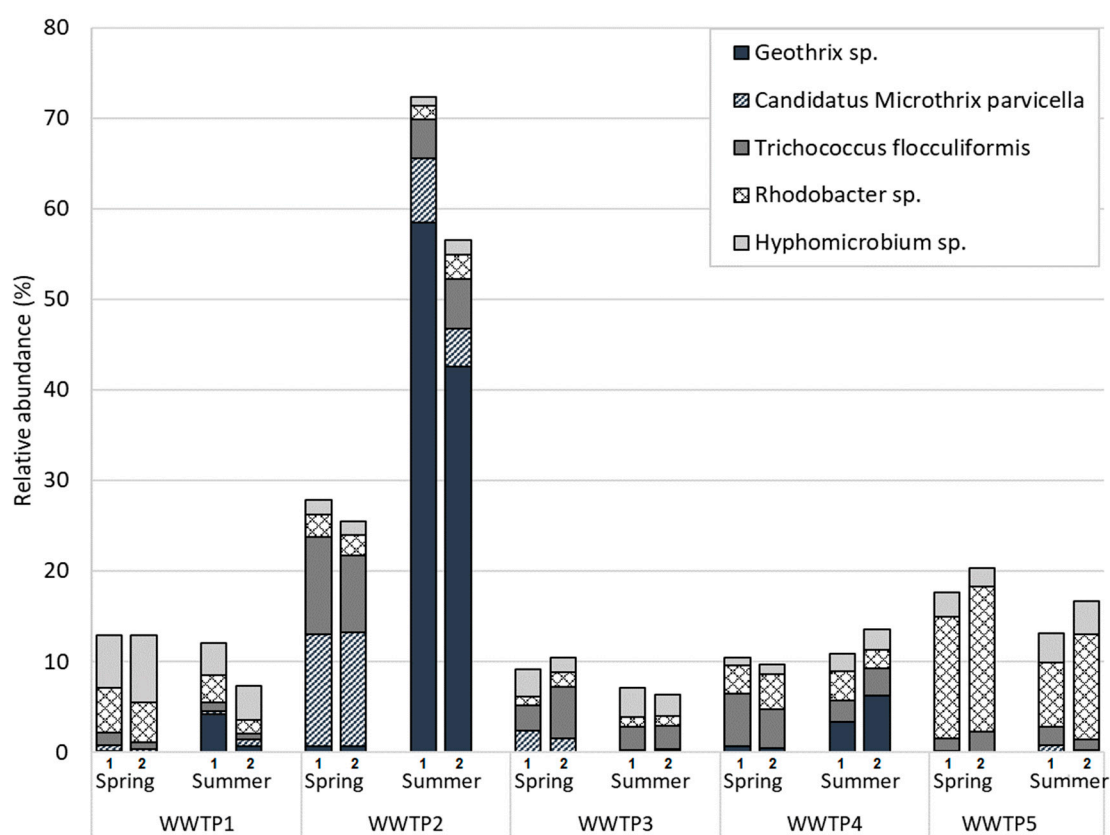


Figure 3. Relative abundance of the dominant genera in activated sludge of Finnish wastewater treatment plants (WWTPs) during the spring and summer season.

Despite the fact that *Geothrix* sp. is strictly anaerobic Fe (III)-reducing bacteria, it was also reported in dominant abundance, representing most of the acidobacteria in Danish WWTP-activated sludge as well as in northern Finnish WWTPs and seems to be the typical genus for activated sludge of WWTPs in Nordic countries [7,19]. The contribution of *Geothrix* in biological removal processes is unclear but all the studied WWTPs are using ferrous or ferric sulphate coagulants for phosphorus precipitation. The dosing points for coagulants are in sand removal and in the beginning of secondary clarifiers. Additionally, in WWTP2, polyaluminum chloride was dosed to primary clarification during the summer months, which could affect bacterial community formation. Finally, *Geothrix* sp. was recently reported among dominating species in activated sludge, facing the selective pressure of

several antibiotics due to its wide antibiotic resistance [23]. Further studies are needed to clarify the engineering value of this bacterial genus.

Nitrifying bacteria were represented by ammonium oxidizers *Nitrosomonas*, *Nitrosovibrio* and *Nitrospira* and nitrite oxidizers *Candidatus Nitrotoga arctica* and *Nitrospira*. In general, typical nitrifiers presented in unexpectedly low abundance in most of the WWTP samples. The overall abundance of AOBs was below 0.25% in all the bacterial communities and less than 0.35% of NOB bacteria altogether were identified in all the samples except WWTP5 spring samples, where Ca. *Nitrotoga arctica* presented in above 4% of total community (Figure 4).

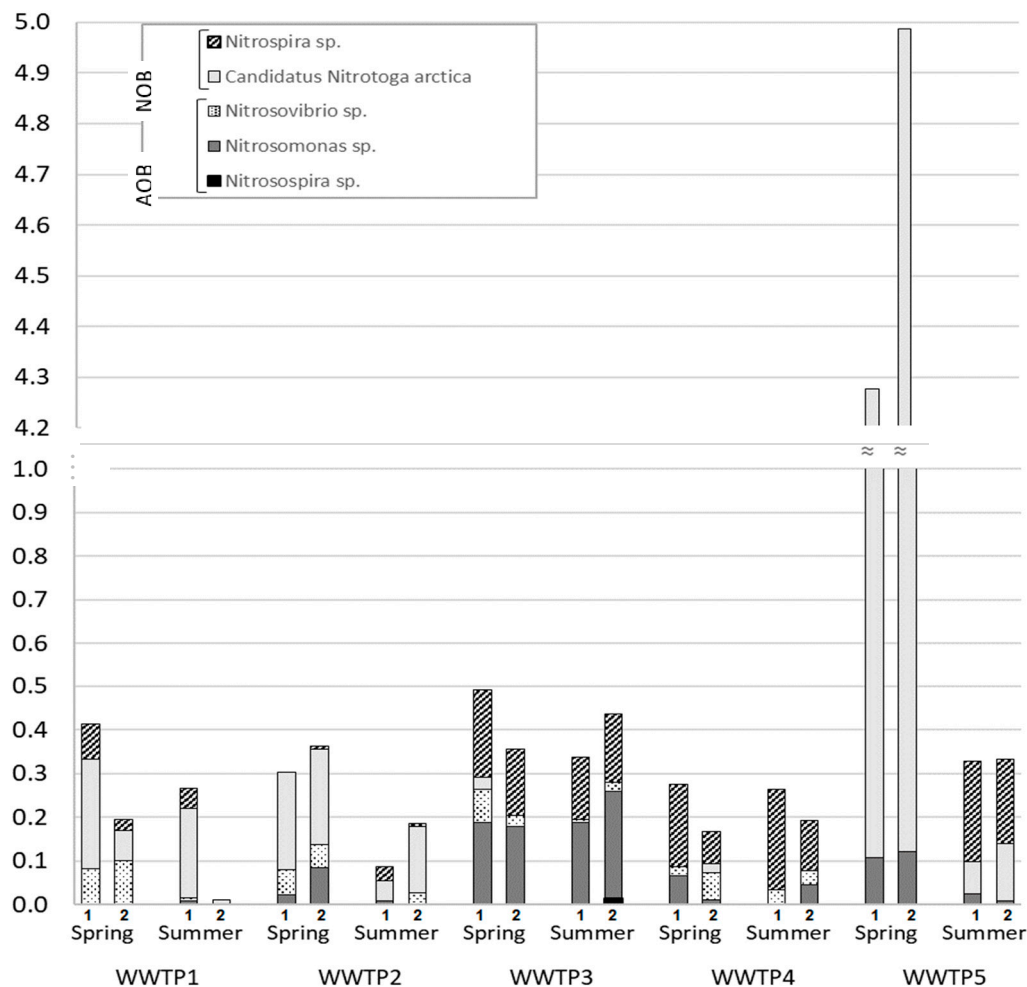


Figure 4. Relative abundance of nitrifying bacteria in studied WWTPs.

No increase in nitrifying bacteria was observed with increasing temperature. Furthermore, in three WWTPs, the amount of AOB and NOB noticeably decreased during summer compared to the spring season. At the same time, nitrification efficiency remained stable for all the experimental period with over 97% in four WWTPs and 93% in WWTP2 of ammonia transformation to nitrate, showing that the identified autotrophic nitrifying bacteria had little or no effect on low-temperature nitrification (Table 2). In the study of 50 Danish WWTPs [16], despite the seasonal variations, the abundance of total AOBs were reported above 2%, as well as the abundance of *Nitrospira* being above 2%, however no other typical NOBs were found in activated sludge [16].

Previous studies of AOBs have shown that the most often reported factors decreasing the diversity of the AOB community are the temperature, ammonium concentration and the presence of industrial wastewaters [1]. For instance, ammonia-low wastewater may explain the absence of *N. europaea* and *Nitrosococcus mobilis*. *Nitrosomonas* and *Nitrospira* are typically the most abundant nitrifying bacteria of

activated sludge from Nordic countries [22]. However, lower abundance of these genera and presence of *Ca. Nitrotoga arctica* in all WWTPs may indicate the effect of low temperatures on the overall nitrifying communities, since the growth optimum for *Ca. Nitrotoga arctica* is 10 °C with the possible growth range 4–22 °C [3]. This also may explain the lower abundances of this species in summer samples. Further studies with more samples from different seasons of the year must be considered to support these findings.

3.2. Acclimation of Activated Sludge to Laboratory Conditions

Activated sludge from WWTP1 was transferred to laboratory reactors to study the role of autotrophic and heterotrophic nitrifiers in a cold-temperature-adapted microbial community. The experimental period was started with an organic carbon load typical for Finnish full-scale processes (municipal-like wastewater) with a following reduction in the organic carbon source. The main operational parameters of the influent and their effect on the ammonia oxidation and nitrogen oxides formation in SBR and MBR reactors, are presented in Figure 5.

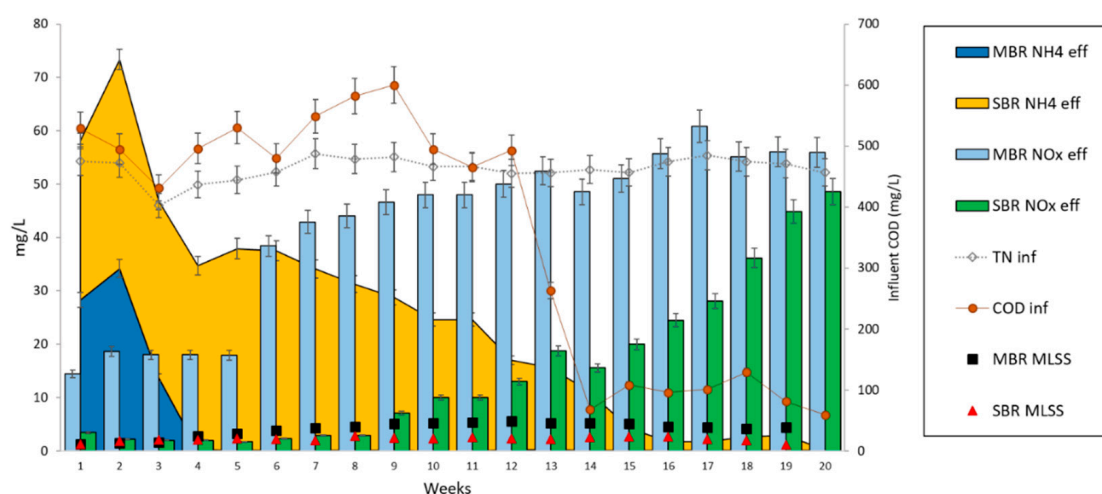


Figure 5. Ammonium oxidizing efficiency during the experimental period. The results presented as average value between the two reactors. The error bars represent standard errors. Wastewater composition is changed during Weeks 12–14.

Efficient removal of ammonia was reached in all of the reactors, however adaptation properties between MBR and SBR reactors were noticeably different. Predictably, activated sludge in MBRs showed faster adaptation to 10 °C conditions and total ammonia removal was reached after four weeks, while in SBRs, the removal of ammonia and the appearance of nitrogen oxides were limited for 11 weeks and then gradually increased over the five following weeks. These findings are in accordance with previously reported data showing that longer SRT may significantly improve the temperature adaptation process in activated sludge [5,6]. Faster development of the nitrifying community can be explained by differences in microbial composition of activated sludge of SBR and MBR as well as by possible wash-out of slow growing nitrifiers in the SBRs.

3.3. Effect of Organic Carbon Source on the Dynamics of Activated Sludge Microbial Community

Consequently, to nitrification efficiency, the schedule of microbial communities sampling was planned. First microbial community samples were collected on the fifth week of the experiment, representing the activated sludge community under the effect of the temperature decrease, while MBRs were nitrifying and no nitrification was observed in SBRs. The additional sample of SBR microbial communities was taken after the appearance of the nitrification during Week 9. The next sampling for microbial analyses was performed in Week 13 of the experiment, representing the community during

the decrease of the organic carbon load in the wastewater. The final samples were taken during Week 20, when all of reactors were nitrifying with the inorganic wastewater.

3.3.1. Bacteria

Dynamics of bacterial communities were evaluated by PCoA (Figure 6). Microbial communities of activated sludge operated under the same operational conditions (SRT, organic load) separated in clear clusters. The longest distance was observed between the communities of MBR and SBR reactors at the end of the experiment, with a reduced organic carbon source in wastewater ($SBR_{\text{low organic}}$, $MBR_{\text{low organic}}$ and $SBR_{\text{inorganic}}$, $MBR_{\text{inorganic}}$), probably due to the longest operation with different SRT under changing conditions. Additionally, there were noticeable differences between not-nitrifying microbial communities ($SBR_{\text{no nitrification}}$ in Figure 6) and nitrifying-activated sludge samples from the same reactors ($SBR_{\text{Municipal-like}}$ in Figure 6).

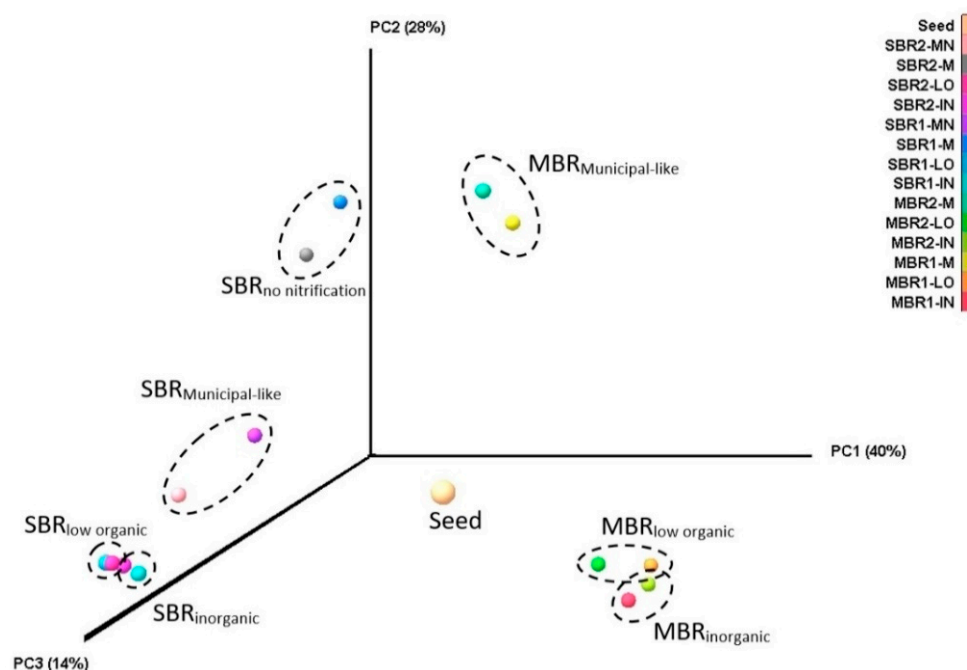


Figure 6. Bray–Curtis principle coordinates analyses (PCoA) plot showing the dynamics of microbial community of seed sludge under operation in pilot MBR and SBR reactors with different wastewater composition.

The dynamics of bacterial populations on order level are presented in Figure 7. Similar to the full-scale data, *Cytophagales* and *Micrococcales* seem to be the most important functional groups in conventional activated sludge due to their abundance in SBR samples. The increase of nitrifying efficiency also positively correlated with the abundance of *Cytophagales*, but negatively affected the abundance of *Micrococcales*. Additionally, the growth of *Xanthomonadales* and unclassified bacteria most closely related to *Proteobacteria* was observed with the increase in nitrifying efficiency and the decrease in organic carbon.

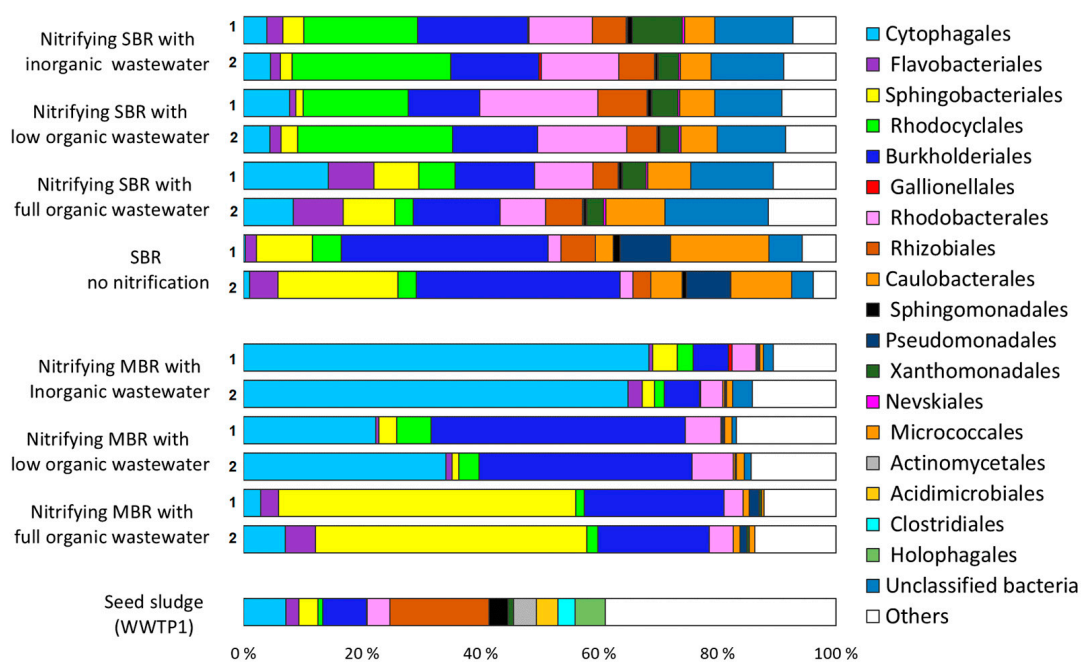


Figure 7. Relative abundance of dominant bacterial orders in pilot reactors during the experimental period.

In MBR samples, the abundance of *Micrococcales* noticeably decreased during the experiment. Conversely, *Cytophagales* dramatically increased during the experimental period, showing the positive correlation of the abundance with the decrease of the organic carbon source.

Sphingobacteriales was negatively correlated with the decrease in the organic carbon source and the increase of the nitrifying efficiency in both SBR and MBR reactors. It could be concluded that both *Micrococcales* and *Sphingobacteriales* are not the important contributors in low-temperature nitrification. However, slow-growing *Sphingobacteriales* might be important heterotrophic participants in the nitrogen-removing cycle.

The dynamics of bacterial communities on the genus level (>0.5% relative abundance at least one sample) is presented in Figure 8. The increase of *Rhodobacter* and *Sphingobacterium* during the experiment was observed in all the reactors, showing the selective advantage of these heterotrophic/mixotrophic genera from studied conditions.

Noticeable increase of *Dokdonella* sp. from order *Xanthomonadales* was observed with the increase of nitrifying activity and a decrease of the organic carbon source in SBR reactors. In MBR reactors, there was a sharp increase of *Flexibacter* sp. from order *Cytophagales* during the experimental period in MBRs and the vast majority of this species in final samples with no organic carbon in wastewater was observed. The role of these two genera in activated sludge was not discussed in previous studies of WWTP microbial compositions.

None of the cold-adapted AOB species previously reported in the literature were found in the activated sludge samples of this study. The appearance of NOB was observed with only one representing species *Ca. Nitrotoga arctica* in all the samples from Week 20 sampling (up to about 0.3% of the SBR community and 0.6% of MBR community). Since the seed sludge was taken during the autumn period, in consistency with the full-scale data, the temperature of the initial community operation was too high for this species' selective growth. After operation for 20 weeks at 10 °C *Ca. Nitrotoga arctica* could appear in the community as an active NOB.



Figure 8. Dynamics of the most abundant bacterial genera in SBR and MBR pilot reactors before and after the change of carbon source in wastewater.

3.3.2. Archaea

AOAs were expected to contribute to low temperature nitrification, since their range of adaptation temperatures is much wider compared to AOBs [24]. Recently, Gonzalez-Martinez et al. [7] showed the positive correlation between ammonium oxidation in WWTPs of northern Finland with unclassified *Euryarchaeota* genera. However, in this study, no clear correlation was observed. A study of the full-scale activated sludge sample from WWTP1 showed that unclassified *Archaea* represented 1.4% of the community and 1% was represented *Methanobrevibacter* sp. From pilot studies, archaea were identified only in one SBR sample and two MBR from the first and the last sampling dates. Since the results were not repeatable between the pilot reactors, no conclusions could be drawn from these data.

Zhang et al. [25] speculated that the lack of the data on the diversity of AOA in activated sludge could be due to the problems during the amplification. Consequently, in this study, five samples out of sixteen had low amplification rates and four had no amplification. Therefore, further studies of AOA contributions in low-temperature nitrification are needed with primary PCR protocol optimization.

4. Conclusions

A study of five full-scale Finnish WWTPs shows the typical highly nitrifying activated sludge bacterial community structure for low-temperature operated WWTPs on order level. It was observed that the most abundant bacterial genera identified in full-scale activated sludge elsewhere in Northern Europe were also typical for Finnish municipal WWTPs. However, several differences were observed, most probably caused by lower temperature conditions and specific wastewater composition and operational conditions typical for Finland. In particular, very low abundance of typical AOBs and NOBs and high abundance of orders *Cytophagales* and *Micrococcales* distinguished studied WWTPs.

The important role of previously reported *Candidatus Nitrotoga arctica* for nitrite oxidizing in cold climate conditions was confirmed in this study. Further studies on genera *Dokdonella* sp. and *Flexibacter* sp. are proposed for deeper understanding of the nitrogen removal cycle in low-temperature adapted WWTPs. It can be concluded that prolongation of sludge retention time from 14 days to 100 days is beneficial for nitrogen-removal efficiency at sharp temperature fluctuations. Further studies of AOA contribution with developed analytical methods are suggested.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4441/12/9/2450/s1>, Figure S1: Bray-Curtis PCoA plot showing the clustering of the samples from different WWTPs (clusters demonstrated by dashed lines), Table S1: Operational parameters, analysed during the study and Standard Methods, Table S2: Microbial communities of Finnish wastewater treatment plants (WWTPs) during the spring season, Table S3: Microbial communities of Finnish wastewater treatment plants (WWTPs) during the summer season.

Author Contributions: Conceptualization, A.K. and A.M.; methodology, J.K., A.G.-M. and A.K.; software, J.K., A.G.-M.; validation, A.K., J.K. and A.G.-M.; formal analysis, J.K., K.M.L.; investigation, J.K., K.M.L.; data curation, A.K., A.M.; writing—original draft preparation, A.K.; writing—review and editing, A.K.; visualization, A.K.; supervision, A.M., A.G.-M. and R.V.; project administration, A.M., R.V.; funding acquisition, A.M., R.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: We would like to acknowledge the Finnish Water Utilities Association (VVY) for the support of this study and the water utilities for providing samples and operational protocols.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Jaranowska, P.; Cydzik-Kwiatkowska, A.; Zielińska, M. Configuration of biological wastewater treatment line and influent composition as the main factors driving bacterial community structure of activated sludge. *World J. Microbiol. Biotechnol.* **2013**, *29*, 1145–1153. [[CrossRef](#)] [[PubMed](#)]
2. Awolusi, O.O.; Kumari, S.K.S.; Bux, F. Ecophysiology of nitrifying communities in membrane bioreactors. *Int. J. Environ. Sci. Technol.* **2015**, *12*, 747–762. [[CrossRef](#)]

3. Alawi, M.; Lipski, A.; Sanders, T.; Eva Maria, P.; Spieck, E. Cultivation of a novel cold-adapted nitrite oxidizing betaproteobacterium from the Siberian Arctic. *ISME J.* **2007**, *1*, 256–264. [[CrossRef](#)] [[PubMed](#)]
4. Karkman, A.; Mattila, K.; Tamminen, M.; Virta, M. Cold temperature decreases bacterial species richness in nitrogen-removing bioreactors treating inorganic mine waters. *Biotechnol. Bioeng.* **2011**, *108*, 2876–2883. [[CrossRef](#)] [[PubMed](#)]
5. Kruglova, A.; Ahlgren, P.; Korhonen, N.; Rantanen, P.; Mikola, A.; Vahala, R. Biodegradation of ibuprofen, diclofenac and carbamazepine in nitrifying activated sludge under 12 °C temperature conditions. *Sci. Total Environ.* **2014**, *499*, 394–401. [[CrossRef](#)]
6. Kruglova, A.; Kråkström, M.; Riska, M.; Mikola, A.; Rantanen, P.; Vahala, R.; Kronberg, L. Comparative study of emerging micropollutants removal by aerobic activated sludge of large laboratory-scale membrane bioreactors and sequencing batch reactors under low-temperature conditions. *Bioresour. Technol.* **2016**, *214*, 81–88. [[CrossRef](#)]
7. Gonzalez-Martinez, A.; Sihvonen, M.; Muñoz-Palazon, B.; Rodriguez-Sanchez, A.; Mikola, A.; Vahala, R. Microbial ecology of full-scale wastewater treatment systems in the Polar Arctic Circle: Archaea, Bacteria and Fungi. *Sci. Rep.* **2018**, *8*, 2208. [[CrossRef](#)]
8. Urakawa, H.; Tajima, Y.; Numata, Y.; Tsuneda, S. Low Temperature Decreases the Phylogenetic Diversity of Ammonia-Oxidizing Archaea and Bacteria in Aquarium Biofiltration Systems. *Appl. Environ. Microbiol.* **2008**, *74*, 894–900. [[CrossRef](#)]
9. Kruglova, A.; Gonzalez-Martinez, A.; Kråkström, M.; Mikola, A.; Vahala, R. Bacterial diversity and population shifts driven by spotlight wastewater micropollutants in low-temperature highly nitrifying activated sludge. *Sci. Total Environ.* **2017**, *605*, 291–299. [[CrossRef](#)]
10. Cavicchioli, R.; Siddiqui, K.S.; Andrews, D.; Sowers, K.R. Low-temperature extremophiles and their applications. *Curr. Opin. Biotechnol.* **2002**, *13*, 253–261. [[CrossRef](#)]
11. Orcutt, B.N.; Lapham, L.L.; Delaney, J.; Sarode, N.; Marshall, K.S.; Whaley-Martin, K.J.; Slater, G.; Wheat, C.G.; Girguis, P.R. Microbial response to oil enrichment in Gulf of Mexico sediment measured using a novel long-term benthic lander system. *Elem. Sci. Anth.* **2017**, *5*, 18. [[CrossRef](#)]
12. Gonzalez-Martinez, A.; Garcia-Ruiz, M.J.; Rodriguez-Sanchez, A.; Osorio, F.; Gonzalez-Lopez, J. Archaeal and bacterial community dynamics and bioprocess performance of a bench-scale two-stage anaerobic digester. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 6013–6033. [[CrossRef](#)] [[PubMed](#)]
13. RTL Genomics 2016. Data Analysis Methodology. Version 1.8. 2016. Available online: http://www.researchandtesting.com/docs/Data_Analysis_Methodology.pdf (accessed on 17 November 2016).
14. Edgar, R.C. UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* **2013**, *10*, 996–998. [[CrossRef](#)]
15. Caporaso, J.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F.; Costello, E.; Fierer, N.; Peña, A.; Goodrich, J.; Gordon, J.; et al. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **2010**, *7*, 335–336. [[CrossRef](#)] [[PubMed](#)]
16. Mielczarek, A.T.; Saunders, A.M.; Larsen, P.; Albertsen, M.; Nielsen, J.L.; Nielsen, P.H. The Microbial Database for Danish wastewater treatment plants with nutrient removal (MiDas-DK)—A tool for understanding activated sludge population dynamics and community stability. *Water. Sci. Technol.* **2013**, *67*, 2519–2526. [[CrossRef](#)] [[PubMed](#)]
17. Wang, X.; Hu, M.; Xia, Y.; Wen, X.; Ding, K. Pyrosequencing analysis of bacterial diversity in 14 wastewater treatment systems in China. *Appl. Environ. Microbiol.* **2012**, *78*, 7042–7047. [[CrossRef](#)]
18. Zhang, T.; Shao, M.; Ye, L. 454 pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants. *ISME J.* **2012**, *6*, 1137–1147. [[CrossRef](#)]
19. Fredriksson, N.J.; Hermansson, M.; Wilén, B.-M. Long-term dynamics of the bacterial community in a Swedish full-scale wastewater treatment plant. *Environ. Technol.* **2019**, *40*, 912–928. [[CrossRef](#)]
20. Hu, M.; Wang, X.; Wen, X.; Xia, Y. Microbial community structures in different wastewater treatment plants as revealed by 454-pyrosequencing analysis. *Bioresour. Technol.* **2012**, *117*, 72–79. [[CrossRef](#)]
21. Gonzalez-Martinez, A.; Rodriguez-Sanchez, A.; Lotti, T.; Garcia-Ruiz, M.; Osorio, F.; Gonzalez-Lopez, J.; van Loosdrecht, M.C.M. Comparison of bacterial communities of conventional and a-stage activated sludge systems. *Sci. Rep.* **2016**, *6*, 18786. [[CrossRef](#)]

22. McIlroy, S.J.; Saunders, A.M.; Albertsen, M.; Nierychlo, M.; McIlroy, B.; Hansen, A.A.; Karst, S.M.; Nielsen, J.L.; Nielsen, P.H. MiDAS: The field guide to the microbes of activated sludge. *Database* **2015**, *2015*, bav062. [[CrossRef](#)] [[PubMed](#)]
23. Zhao, R.; Feng, J.; Liu, J.; Fu, W.; Li, X.; Li, B. Deciphering of microbial community and antibiotic resistance genes in activated sludge reactors under high selective pressure of different antibiotics. *Water Res.* **2019**, *151*, 388–402. [[CrossRef](#)] [[PubMed](#)]
24. Yin, Z.; Bi, X.; Xu, C. Ammonia-Oxidizing Archaea (AOA) Play with Ammonia-Oxidizing Bacteria (AOB) in Nitrogen Removal from Wastewater. *Archaea* **2018**, *2018*, 8429145. [[CrossRef](#)] [[PubMed](#)]
25. Zhang, T.; Ye, L.; Tong, A.H.Y.; Shao, M.-F.; Lok, S. Ammonia-oxidizing archaea and ammonia-oxidizing bacteria in six full-scale wastewater treatment bioreactors. *Appl. Microbiol. Biotechnol.* **2011**, *91*, 1215–1225. [[CrossRef](#)] [[PubMed](#)]



© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).