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An Evolutionary Performance of Ammonia Oxidation Process in Azo Dye Containing Waste Water Treatment Plant

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Abstract

In this study, it was shown that azo dye containing effluent treatment plant's influent had the same clear distinctive communities, in terms of total and ammonia oxidizing bacteria. In studying these bacterial communities, It has been demonstrated that certain species is not seem to be numerically very abundant, can be very close active as RNA signal. Thus, when studying the activities and performance of a waste water treatment plant, an rRNA evaluation advisable, especially when focused on bacterial subgroups, i.e. ammonia oxidizing bacteria. This study thus showing the utility of the control implementation of the process a combination of nitritation study both the parameters and molecular nitritational fingerprinting of the ammonia oxidizing bacteria community. Moreover, clustering and - above all - moving window analyses are shown valuable tools to monitor community shifts effluent treatment plants.

Keywords: Ammonia oxidizing bacteria; rRNA; Azo dye; DGGE; RT-PCR

Introduction

Nitrogen discharges into the environment pose multiple threats to ecosystem health, including toxicity (NH_3), oxygen depletion (NH_4, NO_2, and organic N), and stimulation of algal blooms (NH_4, NO_2, NO_3, and organic N) [1]. To prevent these adverse impacts, wastewater treatment plants use bioreactors to oxidize ammonium to nitrate, and where nitrate removal is also required, design features for de nitrification are also included [2]. Nitrification, the key and often rate-limiting step in N removal [3], entails the two-step microbial oxidation of ammonia to nitrate *via* nitrite. The two steps are catalyzed by chemo lithotrophic ammonia-oxidizing bacteria and nitrite-oxidizing bacteria. While some heterotrophic bacteria [4] and anaerobic ammonia-oxidizing bacteria [5] oxidize ammonia, ammonia oxidizing bacterial are thought to be largely responsible for the oxidation of ammonia in WWTPs. In particular, members of the betaproteobacterial genera Nitrosomonas and Nitrosospira are thought to be the most important ammonia oxidizing bacterial in activated sludge [6]. Recently, however, it was discovered that autotrophic oxidation of ammonia is not restricted to the domain Bacteria. Gustavsson, et al. [7] isolated an Ammonia-Oxidizing Archaeon (AOA) named Nitrosopumilus maritimus from the rocky substratum of a tropical marine aquarium tank. N. maritimus is the first cultivated representative of the ubiquitous marine "group 1" Crenarchaeota and, like ammonia oxidizing bacterial, grows chemo lithoautotrophically by oxidizing ammonia to nitrite under mesophilic conditions. In addition, N. maritimus contains putative genes for all three subunits (amoA, amoB, and amoC) of ammonia monooxygenase, the key enzyme used for bacterial ammonia oxidation. Thus, a critical link connecting archaeal

Ammonia monooxygenase genes originally identified through metagenomic studies and the process of aerobic ammonia oxidation has now been established [8-10]. Jaroszynski, et al. [11]. Recently demonstrated the presence and expression of archaeal amoA genes in soil, while Kartal, et al. [12]. Demonstrated the ubiquity of AOA in marine and estuarine sediments, as well as in oxic and suboxic water columns. Considering that domestic wastewater contains 1 to 2mM ammonium (25mg N/liter) along with 1mM N as organic nitrogen (15mg N/liter) [13], it seemed possible that activated sludge bioreactors could also harbour ammonia oxidizing bacteria. The community composition of the functional bacterial group's active in WWTPs can be assessed by Denaturing Gradient Gel Electrophoresis (DGGE) analysis of 16S rRNA [14-16]. DGGE is a molecular technique that allows the separation of DNA fragments of the same length (up to 500bp) [17], but with different base-pair sequences. It was introduced into environmental microbiology to determine the genetic diversity of complex microbial populations [17]. Ever since, it has been shown to be useful for studying the bacterial communities in a variety of environmental samples, e.g. soil [16] plants [17], water [18] and activated sludge [18]. This molecular technique is also useful for studying population dynamics and stress responses [14-18]. Hence, DGGE analysis can be regarded as an elegant tool to investigate microbial populations or bacterial subpopulations when using group specific primers for the PCR reaction preceding the DGGE profiling. Possible shifts in these communities can further be analysed by clustering and moving window analysis [19]. The 16S rRNA gene pool diversity is typically the basis for analysis of the microbial community. The sample concentration of DNA is not directly linked with cell activity, because DNA is relatively stable. As a result, DNA-based fingerprints reveal the community structure of the most abundant bacteria, however, without differentiating on basis of their activity. On the contrary, with rRNA - based fingerprints the most active microorganisms can be studied, because non active cells will have limited rRNA amounts to be transcribed into cDNA

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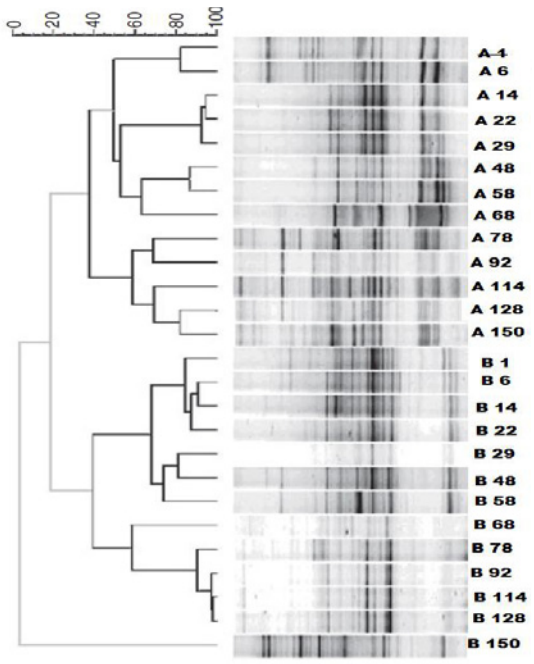

Figure 1: Cluster analysis of the denaturing gradient gel electrophoresis pattern for the bacterial community on DNA basis of wastewater treatment plants A and B.

[20]. DGGE was used to molecularly screen out of the bacterial and specifically the ammonia oxidizing bacterial communities in the activated sludge of a dye containing effluent. Shifts were investigated in nitrification performance reflected in the total number of bacteria and / or the ammonia oxidizing bacterial communities. In addition, a comparison of DNA and RNA -based DGGE analysis was performed in order to investigate the most suitable method for studying wastewater treatment plant failures.

Materials & Methods

Sample collection & analysis

Activated sludge samples were collected from aeration tank of dye containing effluent treatment plant. Some physicochemical properties of the activated sludge samples are presented in (Table 1). All samples were collected from an aeration tank at each facility in February 2006, with the 1-l sample frozen at -20°C until use. Conductivity, dissolved oxygen and pH were measured on site. Water samples were stored at 4°C until water chemistry analysis. Total ammonia cal nitrogen and NO_2 were measured by colorimetric methods using an Auto Analyzer II. Sediment and water samples were collected from the 12 ammonia transect sites on 21 October 2010 and from the five additional locations along the river on 11 November 2010. Wastewater effluent directly from the pipeline outlet, 50m above site downstream1, was also collected on 21 October 2010. Triplicate sediment samples were collected using a plastic core tube from each site. Approximately 100 to 300 ml of water was filtered onsite onto 0.22-m-pore size Sterivex filters. Both sediment samples and all filters were kept on dry ice during transportation and stored at 80°C until DNA extraction.

Analysis of nitrification process

Nitrite, nitrate and the ammonium content of the effluent was analyzed by ion chromatography after a 10-min centrifugation at 5000g and filtering through a 0.45µm filter. A DX-600 system was used which consisted of a Dionex AS50 auto sampler, a Dionex GP50 pump and a Dionex ED50 electrochemical detector. Data analysis was performed with Peak Net 6 software system version 6.10. The Ionpac columns AS9-HC, and CS12-HC were used for anion and cation separation, respectively. The mobile phase consisted of Na_2CO_3 and methane sulfonic acid to the anion and cation analysis respectively. The flow rate was 1mlmin. Kjeldahl nitrogen was analyzed by standard methods [21]. And the data were checked for the absence of a link between the nitrogen load of System and the amount of the Nkjeldahl the waste water. This way, any changes in the wastewater Kjeldahl could be due to failing ammonia oxidizers. The organic nitrogen was calculated as the difference between Kjeldahl and NH_4+-N.

Genomic DNA extraction and PCR amplification

DNA was extracted from sediment samples using Mo Bio Power Soil DNA Kit following the manufacturer's protocol. Triple samples from each location were drawn and maintained independently shaft separate biological replicates. Nucleic acids from the Sterivex filters were extracted by a protocol by Vlaeminck [22]. All recovered DNA concentrations were accurately estimated using a Nano Drop spectrophotometer ND -100 and a 2.0 qubit fluorometer with a double-stranded DNA specific dye. Extracts were then diluted to 5 to 10 ngµl^{-1} in preparation for qPCR. The qPCR amplifications were formed six genes targeted by primer sets and conditions. Mix for each QPCR reaction contained 5 l or 2 iQ SYBR Green Supermix, 0.3M of each primer, 0.3M bovine serum albumin, and 5 to 10ng DNA template in a total volume of 10µL. All amplifications were performed in duplicate on CFX96 Real-Time System. PCR products from each qPCR primers were used as standard templates. Genomic DNA from Escherichia coli was amplified to generate a bacterial 16S rRNA standard template. Plasmids with inserts or amplified DNA from an aquarium bio filter [19] served as a template for the generation of PCR amplicons or AOA 16S rRNA, AOA Amoa, and ammonia oxidizing bacterial Amoa genes for qPCR standards. Environmental tests with high abundance of anammox bacteria and possibly were amplified and assembled to generate anammox bacterial 16S rRNA any and 16S rRNA gene standard templates. Each PCR product was purified using a Min Elute Kit and quantified by Nano Drop ND -100 spectrophotometer. Ten-fold serial dilutions were formed in a range from 101 to 107 copies in order to create a standard curve for each earlier. All standard curves were linear, with an efficiency of 80 to 98% and R2 values of 0.995. The specificity of qPCR was confirmed by melting curve agarose gel electrophoresis analysis and after each run.

Analysis of denaturing gradient gel electrophoresis

DGGE patterns obtained were subsequently analyzed by using Bio Numerics software version 2.0. This software compression increases define the different levels, background subtraction, marker assisted normalization, offset - which includes intensity differences between the lanes and allocation different bands in each lane. A matrix or similarities of densiometric curves the band patterns was calculated to be based on the pearson product-moment correlation coefficient and dendrograms were created using UPGMA bond. Relevant and non-relevant clusters were

Further Cluster separated by the cut-off method. The moving

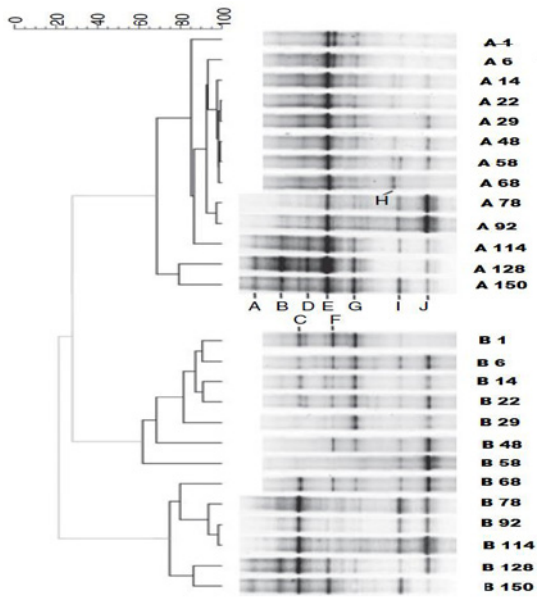

Figure 2: Denaturing gradient gel electrophoresis pattern of the ammonia oxidizing bacteria on basis of DNA analysis of wastewater treatment plants A and B.

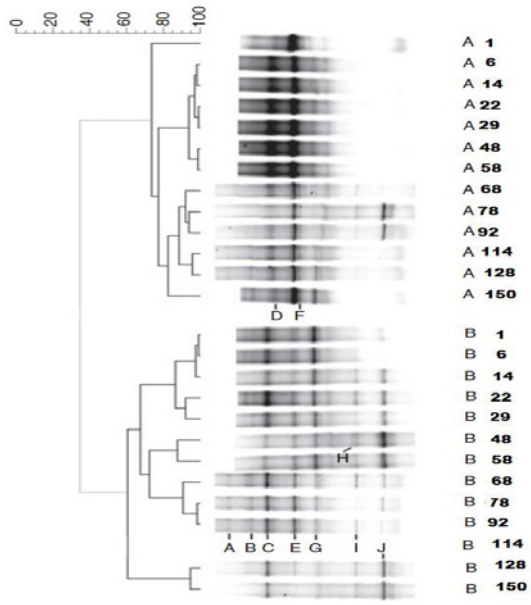

Figure 3: Denaturing gradient gel electrophoresis pattern of the ammonia oxidizing bacteria on basis of RNA analysis of wastewater treatment plants A and B.

window analysis consisted of plot evolution in time of the correlation values between two consecutive analyze dates and are useful when evaluating bacterial community stabilities [23].

RNA extraction and RT-qPCR

RNA extractions samples was performed using RNA Power Soil total RNA Isolation Kit by following the manufacturer's protocol, and Power Water Sterivex DNA isolation kit with a minor modification to the manufacturer protocol to recover extracted RNA. In the letter, the extracted Sterivex, the filters were incubated at 70°C instead of 90°C for 5 minutes. Either 1.5ml or ST4 and 1.5ml of ethanol only instead of 3ml or ST4, was optimized the concentration of salt is applied to the selective binding of RNA on the filter membrane, accordion thing to step 8 and 25 of the manual, respectively. Total RNA was eluted with nuclease-free water in place of ST7 in the last step. The amount and quality of extracted RNA was verified using 1% agarose gel electrophoresis and 2.0 qubit fluorometer, with an RNA specific dye. Reverse transcription and cDNA synthesis was formed by a previously published protocol [20]. All RT-qPCR amplifications were performed using the same primer set and conditions as described in the qPCR

Denaturing gradient gel electrophoresis & cloning

DGGE was carried out as described previously [21], using the D Code universal mutation detection system and 1.5-mm 8% polyacrylamide gels containing denaturant gradients of 40 to 60%, for separation of CTO189f-GC plus CTO654r PCR products and of 35 to 62% for analysis of 357f-GC plus 518r PCR products. The gels were stained for 20min in ethidium bromide and de stained twice for 10 min in 1x TAE buffer (48.22 g of Tris base, 2.05 g of anhydrous sodium acetate, and 1.86g of disodium EDTA. $2H_2O$ [pH 8] in 1 liter of distilled H_2O) prior to UV trans illumination. The gels were also silver stained for better visual resolution of complex community banding patterns. Three purified PCR products resulting from amplification with primers amof plus amor or PLA4 0f plus 1492r were pooled to minimize PCR drift [22,24], ligated into the pGEM T-vector system and transformed into XL1- Blue MRF Kan super competent E. coli cells as specified by the manufacturer. Transformed colonies were screened for inserts of the correct size by PCR amplification with the specific primers described above. For each PCR amplicon, 150 clones with the correct fragment the size of the inserts was subjected to nested PCR with the primer sets described above and GC 357F-518r plus the PCR products were screened by DGGE Sequence differences within the hypervariable V3 region. For β-proteobacterial ammonia oxidizing bacterial, Clone libraries were selected for full-length sequencing of the entire insert represents all recognized clones with different DGGE migration patterns. Ten repeated clones of the recognized main-proteobacterial sequences show identical was also selected migration patterns on DGGE gels for sequencing. Clones from libraries generated by the PV assay were selected at random for the full length sequencing of the entire insert. The selected clones were amplified with vector M13f and M13R primers and M13 PCR products were purified by standard preparative agarose gel electrophoresis as described above. To ensure complete full-length sequence read without ambiguities of sequences of potentially closely related β-proteobacterial ammonia oxidizing bacterial, M13 vector products was sequenced in both directions using the reading SP6 and T7 vector primers the internal 16S rDNA primer CTO654r and 518f (reverse complementary to the above-described 518r). M13 vector derivatives of PV assay was sequenced using vector primers SP6 and T7, and internal 16S rDNA primer pf1053r. For sequence analysis purposes, the desired DGGE band fragments were cut out and cloned with the pCR 2.1-TOPO_ cloning kit according to the manual instructions. DNA sequence analysis was performed using the BLAST server of the National Centre for

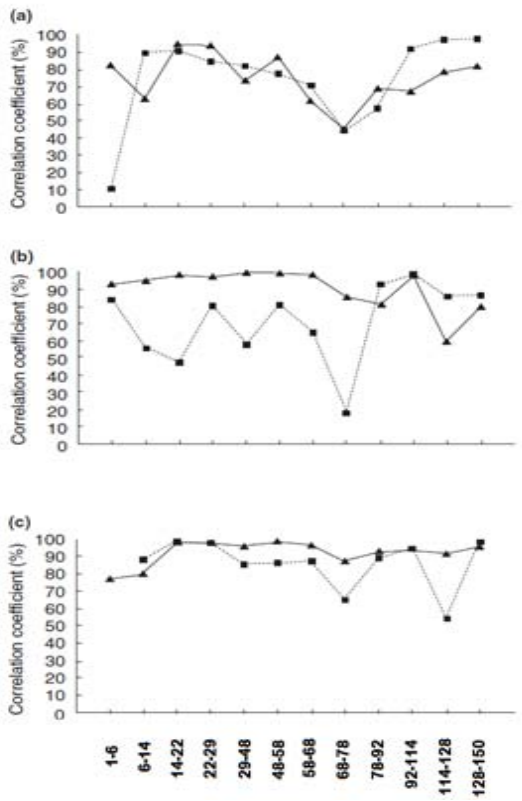

Figure 4: Moving window correlation on the DNA level of the total bacteria (a), and on the DNA (b) and RNA (c) level of the ammonia oxidizing bacteria communities. Each time both wastewater treatment plants A (full line) and B (dashed line) curves are depicted.

Table 1: Characterization of Activated Sludge Sample.

Sample	pH	TN (mg/L)	NH_4-N (mg/L)	MLSS (mg/L)	MLVSS (mg/L)
A	7.2	56.5	39	2480	1880
B	7.3	64.2	52	2260	1740
C	8	94	74	4240	3470
D	6.9	30.8	88	4860	3950
E	7	92	104	2340	1840
F	7.1	60	109	2100	1690
G	8.2	48.6	95	1800	1280
H	8.2	72.4	90	1240	860
I	7.9	66.4	78	2860	1840
J	7.7	155	72	3450	2880
K	7.5	47	66	3280	2460
L	7.9	128.4	148	3040	2210
M	7.4	139.2	138	2270	1750
N	7.3	154.8	70	3160	2430

Biotechnology Information (http://www.ncbi.nlm.nih.gov) with the BLAST algorithm and specifically with the BLASTN program.

Results

Overall sediment DNA consisted of large molecular weight DNA fragments (10 to 20 kb). Raw nucleic acid extracts showed little contamination and PCR amplification was generally successful without further purification of the extract. However results was more consistent and PCR product yields were higher after a dialysis "clean-up" step, which was better than dilution crude nucleic acid extracts. Direct PCR amplification of purified extracts with CTO189f plus CTO654r primers, the AMO161f plus AMO1301r primers and PV assay generated products for almost all sampling sites and depths (10 to 20 ngμL^{-1}). All nested PCR amplifications PCR provided strong signals amplification product with concentrations in the range of 25 to 35 ngμL^{-1}. Aliquots of nucleic acid extracts were sufficient quality to generate direct amplicons of high yield in RT-PCR amplifications without further purification. However, Only weak products or products of illegitimate fragment size was obtained using AMO1301r or 1492r as the transcription terminator primer in RT-PCR, which prevents the formation of large fragment RT-PCR products, which are suitable for cloning and detailed phylogenetic analysis. No direct or embedded DNA PCR or RTPCR The product was obtained by NOC1-45f plus NOC2-1168r primer.

Performance evaluation of nitrification process

For a period of about 5 months nitrification was monitored in azo dye containing effluent treatment plant treating the same influent. (Table 1) shows an overview of the nitrification parameters measured with periods of poor nitritation performance indicated in blue. This was based on waste water concentrations of ammonia - nitrogen > 2mg N l^{-1+}. Almost all nitrogen in the influent is present as recalcitrant nitrogen (Table 1), indicating the importance of ammonification process. Waste water treatment plant A an experienced poor Nitritation around day 1, around day 64 and from day 120 to 148. Waste water treatment plant B had poor nitritation from day 64 to 78, and from day 134 to 148. Overall, the main differences nitrogen between the two wastewater treatment plants was observed around day 78 and 120 respectively.

Denaturing gradient gel electrophoresis analysis

The results of cluster analysis of DNA is based on denaturing gradient gel electrophoresis revealed that each treatment plant had a distinct bacterial community. The separate clusters of day 1 sample wastewater treatment plant B was due to the start up of it (Figure 1). Furthermore, for each wastewater treatment plant type, smaller clusters-although not separated by statistical cluster cut-off method-indicated less society shifts over time. Over time, the bacterial communities of wastewater treatment plant A moved between Days 6 and 14, day 28 and 43, and day 64 and 78 cases of treatment plants B shifts occurred between day 22 and 29, and day 45 and 58. For both types of wastewater treatment plants shifted around Day 64 was the most significant one (Figure 1). This day 45 shifts were also clearly noted in the moving window analysis based on the DGGE profiles obtained after PCR amplification with total bacterial primers (Figure 4a). Curves for both Types of treatment plants followed a similar trend to start with a high-correlation coefficient decreased in the direction of a day 45 Low point after which both curves rose again. Furthermore, it was noted that this latest rise was higher for sewage treatment B than for wastewater treatment plant A, which also fluctuated more in the period until day 58. The low correlation coefficient for wastewater treatment plant B at the beginning of the curve was due to the start-up reasons. In addition to the DNA-based DGGE, one rRNA based DGGE were performed to compare the

Table 2: Organic nitrogen, ammonium-N, nitrite-N, nitrate-N and total nitrogen values for the different sampling points.

	Influent					Effluent A					Effluent B		
Time	N_{org} (mgL^{-1})	NH_4-N (mgL^{-1})	NO_2^-N (mgL^{-1})	NO_3^-N (mgL^{-1})	N-Total (mgL^{-1})	N_{org} (mgL^{-1})	NH_4-N (mgL^{-1})	NO_2^-N (mgL^{-1})	NO_3^-N (mgL^{-1})	N-Total (mgL^{-1})	N_{org} (mgL^{-1})	NH_4-N (mgL^{-1})	NO_2^-N (mgL^{-1})
1	104	10	0	0	124	60	6	20	48	134	22	1	0
6	286	2	0	0	288	15	2	1	35	53	12	0	0
14	72	4	0	0	76	12	0	1	28	41	19	1	0
22	120	38	0	0	158	2	0	0	16	18	24	1	0
29	70	15	0	0	85	0	0	0	8	8	17	0	0
45	94	18	0	0	112	10	0	0	14	24	11	1	0
58	118	8	0	0	126	8	24	0	24	56	14	76	2
68	270	6	0	0	276	28	2	18	36	84	9	84	1
78	298	1	0	0	299	18	1	10	42	81	6	1	0
92	75	0	0	0	75	12	8	2	20	42	12	8	0
114	97	0	0	0	97	2	28	0	12	42	24	2	1
128	112	0	0	0	112	20	5	2	4	31	7	0	0
150	128	0	0	0	128	4	2	1	2	9	2	0	0

active total bacterial communities in both treatment plants. Both rRNA-based DGGE patterns grouped in the same manner as their respective DNA based DGGEs did (data not shown).

Denaturing gradient gel electrophoresis of ammonia oxidizing bacteria

As for the analysis of the bacterial community, a denaturing electrophoretic analysis gradient gel was performed on bacterial community oxidizing ammonia. We made a comparison between the ammonia oxidizing bacteria numerically the most abundant (DNA) and ammonia most active oxidizing bacteria (RNA) present in the samples. For waste treatment plant water A, the differences in the bonds of presence and domination between DNA and electrophoresis analysis models by denaturing gradient gel based RNA was observed. In total, more than 10 different groups visible, although a number of bands were quite low. The 10 predominant bands in denaturing gradient electrophoresis gel analysis model (A through J designated in (Figure 2 & Figure 3)) were partially sequenced. B and C showed 97% similarity with Nitrosomonas europaea ATCC 19.718 (BX321856), the band E 100% similarity Nitrosomonas eutropha isolate Nm57T (AJ298739), the band F 96% similarity with Nitrosomonas oligotropha isolate Nm45T (AJ298736), and Group G 100% similarity N. europaea ATCC 19718 (BX321856). Although clonation procedure was repeated several times, no clones carrying the correct sequence was obtained for bands A, B, D and H. The two groups I and J showed 97% similarity with DGGE beta proteobacteria found unkempt band CJ2-B4 WWout (AY583658) and 97% similarity with Aqua Spirillum Serpent (AB074518). Therefore, they can be regarded as the result of non-specific primer CTO and was excluded from further statistical analysis (both move the aggregation and analysis of the window) and discussion of the oxidizing ammonia bacterial analysis.

DNA based ammonia oxidizing bacteria denaturing gradient gel electrophoresis pattern of waste water treatment plant A hardly changed in the first 3 months. Only one ammonia oxidizing bacteria ribotype (band E) dominated and one other ammonia oxidizing bacteria band was clearly present (G band, which bands I and J was not ammonia oxidizing bacteria s). DNA based ammonia oxidizing bacteria denaturing gradient gel electrophoresis analysis of the wastewater treatment plant B depicted a different situation (Figure 2). It revealed the presence of three predominant ammonia oxidizing bacteria s (C, F, and G) in the first 2 months. Over time, the number of different ammonia oxidizing bacteria s increased. From day 78 on, ammonia-oxidizing community remained more or less constant. Band C dominated the ammonia oxidizing bacteria population. In the last two sampling dates (days 128 and 150) society was more dynamic with number of different higher and the ammonia oxidizing bacteria ribotype E becomes more dominant than before. This resulted in domination of bands B, C and E (Figure 2). Moving window analysis based on DNA denaturing gradient gel electrophoresis profiles for the ammonia oxidizing bacteria s, showed that the ammonia-oxidizing bacterial communities from both treatment plants were clearly different correlation coefficient curves, but both responded with a decrease in the direction of day 48 (Figure 4b). The treatment plant a curve started with high and stable correlation coefficients were more volatile from day 58 on. It was also noted that the low point of this curve was situated one month later with respect to the low point of the bacterial communities curves (Figure 4a). The wastewater treatment plant B curve had an opposite course; it fluctuated much before the day 48, had a very deep minimum at day 48 and changed into a more stable and high baskets behind (Figure 4b). RNA based ammonia oxidizing bacteria denaturing gradient gel electrophoresis gel of the plant A produced a different pattern than the DNA-based ammonia oxidizing bacteria denaturing gradient gel electrophoresis gel (Figure 3). As DNA analysis showed dominance only a ribotype (band E), RNA study showed band that both D and E are represented as active ammonia-oxidizing ribotypes. Between day 114 and day 128, the band D temporarily less dominant, but the band E remained prevalent until the end of sampling period. RNA based ammonia oxidizing bacteria denaturing gradient gel electrophoresis gel of the waste water treatment plant B demonstrated co-dominance of the belt C and G on the at the beginning of the sampling period. Band C remained dominant all the time and were even the only dominant

ammonia oxidizing bacteria ribotype between days 92 and 114. But the band G, exchanged his dominance with band E at the end of the sampling period. Moving window analysis for the RNA-based denaturing gradient gel electrophoresis profiles of ammonia oxidizing bacteria communities both wastewater treatment plants showed different rates over time (Figure 4c). Except for the first 2 weeks and decreased around day 64, the treatment plant A curve stayed very high, suggesting a stable society. The wastewater treatment plant B curve fluctuated more and revealed a variable (up to 40% change) RNA community structure from day 48 on.

Discussion

Nitrification parameters and structure of microbial community of azo dye containing waste water treatment plants (A and B) the processing of the same influent was monitored. Based on the residual NH_4+-N data in (Table 2), with the exception of the start-up values experienced Nitritation only failures from around day 48. These parameters were changing Nitritation also reflected in the local community change of ammonia oxidizing bacterias as demonstrated the statistical treatment of ammonia oxidizing bacteria denaturing gradient gel electrophoresis fingerprint (Figure 2-4). The shifts were both detected by Cluster analysis and moving window analysis based on denaturing gradient gel electrophoresis patterns. Furthermore, molecular analysis tools have advantage to identify key microbial species involved in the failure of nitritation. This change in nitritation performance was related to the production of imidazoles from the pharmaceutical company from day 64 on, and thus the confrontation of the microbial populations of the wastewater treatment plants with chemicals used in this production process, which is present in the inlet. Imidazole production related products and used chlorinated and non chlorinated solvents are well known alternative substrates for ammonia and thereby inhibit the growth of ammonia oxidizing bacteria [22]. They affect Ammonium Mono Oxygenase (AMO) activity by three different mechanisms: (i) direct bond, and interaction with AMO, (ii) interference in the provision of reducing agent necessary for the monooxygenase activity, and (iii) oxidation of substrates to yield products which are very reactive and inactivate AMO and / or other enzymes [23,24]. To follow up possible errors in Nitritation process the remaining NH_4^+ N at the end of the treatment plant was monitored. The data were checked for the absence of a correlation between the nitrogen load of the system and the amount of the Nkjeldahl wastewater. This way, possible changes in the wastewater Nkjeldahl may be due to failing ammonia oxidizers. The measurement the quantities of organic nitrogen in the inlet and waste water, as an indication of a well performing ammonification was included, as this is a preliminary step towards Nitritation [25,26]. And incoming contained high organic nitrogen levels. This was of particular interest to this research, in displaying the presence of a preceding stage of ammonification wastewater treatment plant B, but not in wastewater treatment plant A. This prior pelvic wastewater treatment plant B was used to optimize the conversion of the high amounts of organic nitrogen to ammonia, which is easily accessible to the ammonia oxidizing bacterias. In wastewater treatment plant A this conversion the ammonia will take place in the mixed aeration tank itself, resulting in a delay in the supply of ammonia to the ammonia oxidizing bacterias. In the last decade there has been much interest in the rRNA analysis [27,28] as a correlation was found between the growth rate of bacteria and cellular rRNA concentration [29]. The data in this study supports the hypothesis that rRNA analysis does not only present, but also to the activity of bacteria. The ammonia oxidizing bacteria DGGE analysis of the waste water treatment plant a showed that 16S rRNA based analysis can actually reveal a different pattern of microbial community structure than DNA-based analysis (Figure 3 *vs* Figure 2). The fact that the band D is only slightly present at the DNA level (Figure 2), but strongly present at RNA levels (Figure 3) suggests that these bacteria are active and thus play a functional role than expected from the DNA-based DGGE. The results obtained of the total bacteria suggested that the differences between DNA and RNA based analysis are less evident. Therefore, it is appears that a 16S rRNA analysis is valuable for assessment of the relative activity of the tested bacteria, particularly when focusing on subsets of the entire bacterial communities. Another strategy to evaluate activity of ammonia oxidizing bacteria community could have been based on using primers directed against the functional gene involved in the Nitritation; The AMoA gene [30,31]. However, a previous study compared the organic AmoA approach to the more traditional 16S rRNA approach and showed a similar evolutionary the ratio of ammonia oxidizing bacterias based on these two methods [32]. A recent study showed even a more superior resolution of 16S rRNA *vs* AMoA analysis partly because AMoA region is highly conserved [33]. It is also reported that AMoA-based assays may miss several ammonia oxidizing bacterial populations than 16S rRNA-based assays [34]. Moreover, the solution of phylogeny based on 16S rDNA sequences in the genus Nitrosomonas is reported to be fairly good [35]. Therefore, because all ammonia oxidizing bacterias found in this study nitrosomonads would AMoA approach have contributed more information to our results than that obtained using the 16S rRNA primers CTO. Moving window analysis is a useful technique to follow-up stability or change in the time of microbial communities [36]. (Figure 4 a,b) yielded that although the bacterial communities in both wastewater treatment plants studied follows almost the same course, this is not the case for ammonia oxidizing bacteria subgroup. On ammonia oxidizing bacteria level, it was also noticed that the treatment plant B showed more instability throughout the complete monitoring period than waste water treatment plant A. In addition, one re stabilization (after the disturbance at day 64) of the total bacterial communities were reached by the end of the measuring period (Figure 4a), even though the stabilization was not reached ammonia oxidizing bacteria during the group kept on fluctuating (Figure 4b). Offsets have been reported to be related to a change in the functional performance of the bacterial communities. Tsushima, et al. (2007) showed that a temporary malfunction of nitrification process can result in a row coming and disappearance of certain species. Because changes in RNA is known to be more rapid and to precede alterations in the DNA after a few days, a direct comparison between DNA and RNA analysis could only be made if RNA was sampled more frequently [34-37]. Both the cluster and moving window the analysis showed clearly the usefulness of examining bacterial subgroups and perhaps indicates that the ammonia oxidizing bacterias could be a useful indicator subgroup to study treatment plants disorders. Further study on this path could lead for an accurate picture of the ammonia oxidizing bacterias is more resistant to environmental disturbances. It could eventually allow fine-tune operational wastewater treatment plant parameters

for the benefit of presence, survival and growth of this ammonia oxidizing bacteria species. As previously reported, it is important to control the non specificity using primers CTO [30-33]. For this purpose and at the same time identify the key ammonia-oxidizing bacteria the two treatment plants, sequence analysis of the most dominant denaturing gradient gel electrophoresis bands was performed. It appeared that the bands I and J was more closely related to the Serpens A., one common species of Wastewater treatment plants [37] than to the non-specific bands already found before when using CTO primers [38]. The other bands showed great similarity to Nitrosomonas spp. The fact that in the two treatment plants only Nitrosomonas spp. found - though CTO primers also potentiate other ammonia oxidizing bacterias such Nitrosococcus spp. and Nitrosospira (Rascal Keep, et al. 2003.) - Similar to a previous study, only nitrosomonads found in all but two of the 11 wastewater treatment plants analyzed [22] and with a recent study [35,36]. But this is not a general finding Nitrosococcus mobilis-like bacteria also has been reported as the dominant ammonia oxidizing bacteria activated species sludge [37-39]. In this study, it was shown that two sewage treatment plant treats the same inflowing had clearly distinctive community, in the form of everything and ammonia oxidizing bacteria. When studying these bacterial communities, it was demonstrated that certain species does not appear to be numerically very abundant, can be very asset according RNA signal. Therefore, when studying activity and performance of water treatment plant, one rRNA evaluation is advisable, especially when the focus is on bacterial sub-groups. This research therefore demonstrates the usefulness of monitoring nitritation process performance through a combination of the study of both parameters and molecular nitritational fingerprints of ammonia oxidizing bacteria community. Furthermore, clustering and in particular moving window analysis are shown to be valuable tools for monitoring changes in society wastewater treatment plants.

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