Molecular biological methods (DGGE) as a tool to investigate nitrification inhibition in wastewater treatment

N. Kreuzinger*, A. Farnleitner, G. Wandl*, R. Hornek** and R. Mach****

* Institute for Water Quality and Waste Management, Department for Chemistry and Biology of Water, Vienna University of Technology, Austria (E-mail: *norbkreu@iwag.tuwien.ac.at*)

** Institute for Chemical Engineering, Microbial Biochemistry and Gen Technology Group, Vienna University of Technology, Austria

Abstract Incomplete nitrification at an activated sludge plant for biological pre-treatment of rendering plant effluents led to a detailed investigation on the origin and solution of this problem. Preliminary studies revealed that an inhibition of ammonia oxidising microorganisms (AOM) by process waters of the rendering plant was responsible for the situation. We were able to show a correlation between the existence of specific AOM and nitrification capacity expressed as oxygen uptake rate for maximal nitrification ($OURN_{max}$). Only *Nitrosospira sp*. was found in the activated sludge of the rendering plant and another industrial wastewater treatment plant with problems in nitrification, while reference plants without nitrification problems showed *Nitrosomonas spp*. as the predominant ammonia oxidising bacteria. By accompanying engineering investigations and experiments (cross-feeding experiments, operation of a two-stage laboratory plant) with molecular biological methods (DGGE – Denaturing Gradient Gel Electrophoresis) we were able to elaborate an applicable solution for the rendering plant. Laboratory experiments with a two-stage process layout finally provided complete nitrification overcoming the inhibiting nature of process waters from the rendering plant. DGGE analysis of the second stage activated sludge from the laboratory plant showed a shift in population structure from *Nitrosospira sp*. towards *Nitrosomonas spp*. simultaneous to the increase of nitrification capacity. Nitrification capacities comparable to full-scale municipal wastewater treatment plants could be maintained for more than two months. As the design of wastewater treatment plants for nitrification is linked to the growth characteristics of *Nitrosomonas spp*., established criteria can be applied for the redesign of the full-scale plant.

Keywords 16S rDNA; activated sludge process; DGGE; nitrification inhibition; *Nitrosospira*; wastewater treatment

Introduction

Different process effluents of a rendering plant are pre-treated together on site in an industrial wastewater treatment plant based on the activated sludge process. Treated wastewater is characterised by high concentrations of nitrogen (mean value for TKN between 1997 and 2001 = 430 mg/l) and COD (mean value 6.33 g/l) especially during summer months (Nowak *et al*., 1999). The single stage activated sludge plant for biological pre-treatment of the rendering plant effluent consists of an equalisation tank (700 m^3), an anoxic denitrification tank (550 m^3), an aeration tank (1,440 m^3) divided into three cascades operated in sequence (580 + 430 + 430 m^3) and a final clarifier (150 m^3). The average temperature in the aeration tank is 28°C and the typical sludge retention time is about 30 days. Treated wastewater (~ 326 m^3/day) is discharged into the sewerage of a nearby municipality and contributes 2% to the influent of the corresponding wastewater treatment plant. Despite a high sludge retention time in the wastewater treatment plant of the rendering plant repeating breakdowns of the biological nitrification process were observed resulting in high ammonium concentrations in the effluent of the plant. Detailed investigations were undertaken to elaborate a solution for this problem. In order to get a holistic view on the origin of the problems and possibilities for solving them, different tools, for example, mass

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balances, respirometry, inhibition tests, cross-feeding experiments and molecular methods were applied (Wandl *et al*., 2001). Each of these tools provided specific information and, in combination, allowed promising engineering strategies to establish stable conditions for nitrification in the investigated wastewater treatment plant. This paper deals with the implementation of 16S rDNA based denaturing gradient gel electrophoresis (DGGE) for ammonia oxidising beta-subclass *Proteobacteria* (β-AOM) and its results into the investigations and experiments performed.

Material and methods

Investigated activated sludge samples

Table 1 gives a summary of activated sludge samples and abbreviations used for the investigation and a short description of corresponding wastewater treatment plants (WWTP).

F-A is the WWTP receiving pre-treated wastewater from the objective treatment plant F-R. Activated sludge from WWTP F-M is used for seeding of F-R in the case of total loss of nitrification. F-D is an industrial WWTP receiving process waters from chemical industry and suffering from similar problems as F-R. P-MD is a pilot plant based on membrane technology for the food processing industry, again with severe nitrification inhibition in the full-scale plant of this company. Because all industrial plants investigated here have problems in meeting legal standards for nitrogen in their effluent, no names are indicated for them. P-V indicates the second stage activated sludge of a pilot plant operated in connection with the upgrading of the main treatment plant of Vienna, Austria.

Cross feeding experiments

In order to obtain information on the inhibition of the process waters of the rendering plant, cross-feeding experiments were performed. Reaction tanks were operated as sequencing batch reactors with a sludge retention time of 14 days and addition of corresponding wastewater 2–3 times a day (Wandl *et al*., 2001). The pH was kept above 6.5 to prevent interference with nitrification. Dosage of NaOH automatically was controlled by a pH probe. Subtraction of excess sludge was done manually once a week; experiments were carried out over a period of four months. Three parallel setups were chosen (Table 2). Container A (LX-A) was operated with activated sludge from the rendering plant (F-R), fed with waste-

Table 1 Abbreviations, activated sludge samples and corresponding treatment plants used (PE = population equivalent based on BOD_{60})

Abbreviation	Treatment plant	Type	Capacity in PE_{60}	
F-R	Anonymous	Investigated rendering plant	Industrial	67,000
F-A	Ager West	Full-scale treatment plant	Municipal	60,000
F-M	Marchtrenk	Full-scale treatment plant	Municipal	12,000
F-D	Anonymous	Full-scale treatment plant	Industrial	300,000
P-MD	Anonymous	Membrane pilot plant	Industrial	40
P-V	Vienna	Two-stage pilot plant	Municipal	2,000
LS-1/21st/2nd stage sludge from lab-scale two-stage plant				
LX-A/B/CSludge from cross-feeding experiments A, B and C in lab-scale batch plants				

Table 2 Experimental setup and abbreviation for cross feeding experiments. Note: F-R is the rendering plant, P-V is the municipal plant

Setup	Abbreviation	Sludge from . . .	Wastewater from . . .
A	LX-A	F-R	F-R
B	LX-B	F-R	P-V
C	LX-C	P-V	F-R

water from the rendering plant and represented a reference comparable to the full-scale plant. Container B (LX-B) was operated with activated sludge from the rendering plant (F-R) but fed with municipal wastewater from the pilot plant Vienna (P-V). Container C (LX-C) was operated with activated sludge from the pilot plant Vienna (P-V) and received wastewater from the rendering plant (F-R).

Two-stage laboratory plant experiments

A two-stage laboratory plant was set up to investigate the reduction of inhibition from process waters of the rendering plant within the first stage, and therefore uninfluenced nitrification in the second stage (Wandl *et al.*, 2001). Both stages of the laboratory plant have their own equalisation tanks continuously supplying the corresponding 4 l aeration tanks (continuous aeration) with wastewater. Both aeration tanks were filled with sludge from the pre-treatment plant of the rendering plant (F-R). The first stage received wastewater from the rendering plant, and the second stage the settled effluent of the first stage. Excess sludge was abstracted manually once a day in both stages, realising sludge retention times of about 1.6–2 days in the first stage and 14 days in the second stage. After 4 months of operation, the first stage was bypassed and the second stage received the wastewater of the rendering plant directly.

Respirometry

For determination of specific nitrification capacity of activated sludge samples, autotrophic oxygen uptake rates ($OURN_{max}$) were determined according to Nowak *et al.* (1994). A fresh and well aerated sample of activated sludge from the investigated aeration tank or the lab-scale plant was poured into a reaction chamber ($V = 0.5$ l). One experimental setup was performed with an excess of ammonia (final concentration 15 mg/l NH_4-N) for stimulation of maximal respiration of nitrifying bacteria; a second experiment was done with the nitrification inhibitor ATU (allyl-thio-urea) to a final concentration of 10 mg/l for determination of endogenous respiration. Decrease of oxygen in the closed reaction chamber is measured online with an oxygen probe. The results are combined to obtain maximal respiration for nitrification, and afterwards were normalised to 20°C and 1 g mixed liquor suspended solids and classified into five groups for better interpretation (Table 3).

DNA extraction

Sewage sludge from the respective locations of the wastewater treatment or lab-scale experiment plant were taken in sterile 50 ml polypropylene tubes (Sterilin, Austria), immediately closed and stored at –20°C until further processed. For DNA extraction the sewage sludge was centrifuged at 15.000 rpm (4°C for 10 minutes) and the resulting pellet was thereafter washed twice with sterile saline. DNA was subsequently extracted from 0.25 g of the wet pellet using the Ultra Clean™ Soil DNA kit (MoBio Laboratories Inc., Solana Beach, CA) which includes a beat beating and a spin column purification step (Tsai and Rochelle, 2001). Successful extraction was confirmed by gel electrophoresis, and DNA was stored at –20°C until being further processed. DNA extractions from *Nitrosomonas europaea* ATCC 19718 and *Escherichia coli* type strain DSM 30083, which were used as

Table 3 Classification for oxygen uptake for maximal nitrification ($OURN_{max}$) of activated sludge (mg O_2 l^{-1} h^{-1} g^{-1} suspended solids at 20°C)

$OURN_{max}$ in mg O_2 l^{-1} h^{-1} g^{-1} (20°C)				
0 ($OURN_{max}$ < 0.5	0.5 ($OURN_{max}$ < 1.5	1.5 ($OURN_{max}$ < 5	5 ($OURN_{max}$ < 10	10 ($OURN_{max}$
–	~	±	+	++

positive and negative controls during PCR and DGGE, were performed using standard protocols (Sambrook *et al*., 1989).

PCR amplification

The analysis was based on a 16S-rDNA nested PCR approach including an initial step specific for ammonia oxidising beta-subclass *Proteobacteria* (β-AOM) and a second nested step for amplifying a region spanning the variable V3 region of the ribosomal eubacterial DNA. PCR was performed using triplicate reactions of 10^{-1}, 10^{-2}, 10^{-3} tenfold dilutions from the respective DNA templates by using a Biometra Tri-Thermoblock (Biometra, Göttingen, FRG). The initial PCR was performed as previously described (Bano and Hollibaugh, 2000) and based on primers Nit A and Nit B (Voytek and Ward, 1995; see Table 4). In addition, BSA (Boehringer, Mannheim, Germany) was used as an additive in a final concentration of 0.3 mg/ml to the reaction mixture to relieve possible PCR amplification inhibition (Kreader, 1996). The nested PCR step was based on V3 16S-rDNA specific primers PRBA 338f and PRUN 518r (see Table 4). The primer PRBA 338f bears a 5′ attached GC-rich sequence (GC-clamp, underlined sequences in Table 4) for subsequent DGGE analysis (Ovreas *et al*., 1997).

Denaturing gradient gel electrophoresis (DGGE)

For rapid screening of DNA sequence differences in the amplified 16S rDNA V3 region, a DGGE analysis was performed with the recovered amplicons containing the attached GC-clamp. The DGGE approach was performed as described in detail by Farnleitner *et al*. (2001). Selected bands on the DGGE gels were recovered, sequenced and subsequently submitted to the Basic Local Alignment Search Tool (BLAST; *http://www.ncbi.nlm.nih.gov*) in order to allocate to available 16S-rDNA sequences. Two DDGE regions representing a *Nitrosomonas sp*. (❶ in Figures 1 and 2) and a *Nitrosospira sp*. (❷ in Figures 1 and 2) cluster were identified.

Results and discussion

Preliminary investigations

As a first step for the investigation on nitrification inhibition at the wastewater pre-treatment plant of the rendering plant effluents, activated sludge from several wastewater treatment plants (Table 1) providing different nitrification performance (Table 5) were investigated by means of DGGE (Figure 1). It can be shown that the high nitrification performance of F-A, F-V and F-M (for abbreviations see Table 1) correspond well with the existence of a typical DGGE-cluster characterised as *Nitrosomonas spp*. (Figure 1 and Table 5). On the other hand, the sludge from the investigated rendering plant (F-R) with hardly any nitrification showed a DGGE-band characterised as *Nitrosospira sp*. (see Figure 1 and Table 5). DGGE gave no signal for the sludge from F-D with no nitrification at all (Figure 1).

Table 4 Primers used for β-AOM PCR and following nested PCR step with GC-clamp

Primer	Sequence
Nit A	5′- CTTAAGTGGGGAATAACGCATCG
Nit B	5′- TTACGTGTGAAGCCCTACCCA
PRBA 338f	5′- CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG ACTCCTACGGGAGGCAGCAG
PRUN 518r	5′- ATTACCGCGGCTGCTGG

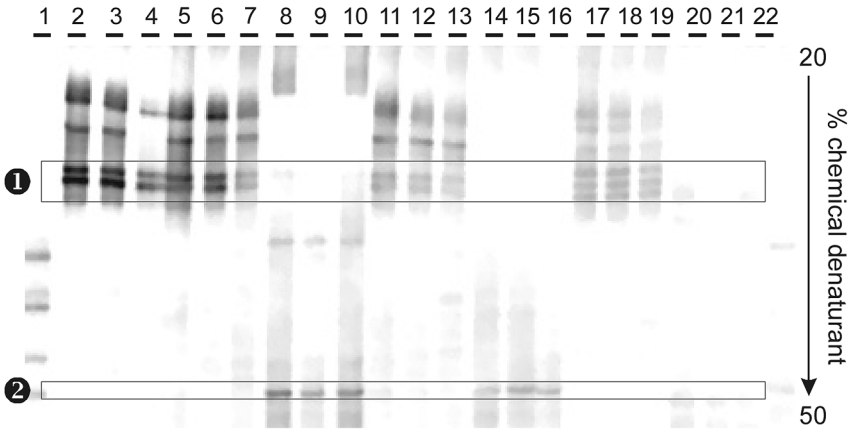

Figure 1 Negative image of 16S-rDNA DGGE amplicon profile variations based on β-AOM PCR using three different template concentrations per sample. Note: Lane 1 is DGGE marker; description of other lanes as specified in Table 5. ❶ = DGGE cluster for *Nitrosomonas spp*; ❷ = DGGE cluster for *Nitrosospira sp*.)

Table 5 Description of lanes in Figure 1 and results from investigated wastewater treatment plants. Nitrification performance according to Table 3

WWTP abbreviation	Lanes (Figure 1)	Origin of samples	Date	DGGE cluster	Nitrification performance	$OURN_{max}$ in mg O_2 l^{-1} h^{-1} g^{-1} (20°C)
F-A	2–4	Aeration tank 2	28.1.2000	❶	++	10
F-A	5–7	Aeration tank 3	28.1.2000	❶	++	10
F-R	8–10	Aeration tank 3	24.2.2000	❷	~	1.4
P-V	11–13	Aeration tank 1	24.2.2000	❶	++	14
F-R	14–16	Aeration tank 3	13.1.2000	❷	–	0.2
F-M	17–19	Aeration tank 1	22.2.2000	❶	++	11.3
F-D	20–22	Aeration tank 1	22.2.2000	–	–	0

Cross-feeding experiments

Evaluation of results from cross-feeding experiments was based on oxygen consumption for the maximal nitrification rates ($OURN_{max}$) observed. $OURN_{max}$ was determined three times a week during the four month duration of the experiment. At the start up phase of the experiment, nitrification capacities were determined and DGGE was performed for utilised activated sludges F-R and P-V (see Table 5), indicating the presence of *Nitrosospira sp*. in F-R (lanes 8–10 in Figure 1) and *Nitrosomonas spp*. in P-V (lanes 11–13 in Figure 1) as expected from preliminary investigations and nitrification performance. For the whole duration of the experiment, nitrification performance in the reference experiment LX-A (for a description see Table 2) corresponded well with the full-scale plant showing low nitrification (classification "~" according to Table 3) as expected. DGGE performed after four months showed the same picture as for the fresh sludge used for start up (compare lane 11 and 14 in Figure 2) indicating only the presence of *Nitrosospira sp*. Nitrification in experiment LX-C within two weeks decreased from "+ +" measured for the "fresh" activated sludge of P-V to the same level of "~" as observed in LX-A, indicating the inhibitory nature of the rendering plant process waters. Although *Nitrosomonas spp*. were present in the sludge at the beginning of the experiments (see lanes 11–13 in Figure 1) none could be verified after four months (see lane 10 in Figure 2) showing that wastewater from the rendering plant is inhibiting growth of *Nitrosomonas spp*. but not of *Nitrosospira sp*.

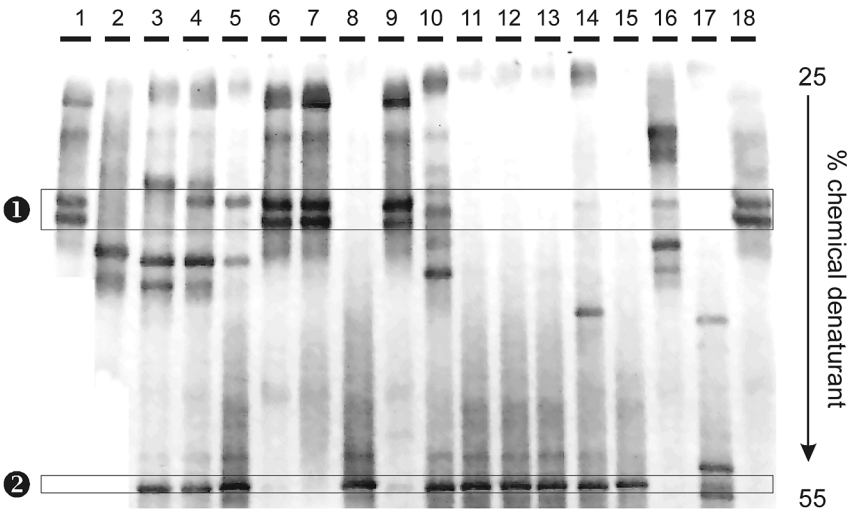

Figure 2 Negative image of 16S-rDNA DGGE amplicon profile variations based on β-AOM. Description of lanes as specified in Table 6. Lanes 1 and 7: DGGE marker (positive control F-A). Note: ❶ = DGGE cluster for *Nitrosomonas spp*; ❷ = DGGE cluster for *Nitrosospira sp*.

Table 6 Description of lanes in Figure 2 and results of investigated activated sludges from full-scale plants and laboratory experiments. Nitrification performance according to Table 3

Abbreviation	Lane (Figure 2)	Origin of samples	Date	DGGE cluster	Nitrification performance	$OURN_{max}$ in mg O_2 l^{-1} h^{-1} g^{-1} (20°C)
LS-2	2	Lab-experiments 2nd stage	16.3.2001	–	–	0
LS-1	3	Lab-experiments 1st stage	21.2.2001	❷	–	0.3
LS-2	4	Lab-experiments 2nd stage	21.2.2001	❶ ❷	++	10.5
F-R	5	Full-scale rendering plant RS	18.1.2001	❷	~	0.5
LS-2	6	Lab-experiments 2nd stage	18.1.2001	❶	++	14
LS-2	8	Lab-experiments 2nd stage	21.10.2000	❷	~	0.33
LX-B	9	Lab-experiments cross-feeding	27.6.2000	❶	++	11.2
LX-C	10	Lab-experiments cross-feeding	27.6.2000	❶❷	±	3.8
LX-A	11	Lab-experiments cross-feeding	27.6.2000	❷	~	1.5
F-R	12	Full-scale rendering plant AT3	9.10.2000	❷	±	2.4
F-R	13	Full-scale rendering plant AT3	28.6.2000	❷	±	2.8
F-R	14	Full-scale rendering plant AT3	24.2.2000	❷	~	0.5
F-R	15	Full-scale rendering plant AT3	13.1.2000	❷	–	0.3
P-MD	16	Industrial membrane pilot plant	28.11.2000	(❶)	±	2.7
F-D	17	Industrial plant; negative control	22.2.2000	(❷)	–	0
F-A	18	Municipal plant; positive control	27.1.2000	❶	++	14.4

Experiment LX-B (sludge from F-R and wastewater from P-V) started with low nitrification performance "~" measured for F-R, but within one month increased to "++" as typically for the activated sludge from P-V. DGGE at the end of the experimental period indicated a shift in population structure from *Nitrosospira sp*. towards *Nitrosomonas spp*. (compare lanes 14 and 9 in Figure 2). This shift may probably be due to seeding of *Nitrosomonas sp*. by the municipal wastewater used in experiment LX-B. In any case, *Nitrosomonas spp*. ruled out initially present *Nitrosospira sp*. by better or faster growth. *Nitrosospira sp*. could not be detected at the end of the experiments.

Two-stage laboratory plant experiments

Based on the results from cross-feeding experiments that indicate a possible development

of *Nitrosomonas sp*. out of the F-R sludge dominated by *Nitrosospira sp*., experiments with a two-stage laboratory plant were performed. The idea was to remove the causes of nitrification inhibition in the process waters of the rendering plant by a high loaded first stage in order to establish full nitrification in a low loaded second stage. Initially sludge from F-R was used for the set up of both the first and the second stage of the laboratory plant. DGGE only showed a *Nitrosospira sp*. related cluster for this sludge (lane 12 in Figure 2). For further DGGE analysis, the main emphasis was put on the sludge of the second stage, as the sludge retention time in the first stage was too short (1.6–2 days) to allow development of ammonium oxidising bacteria. Sludge retention time of the second stage was 14 days. Twelve days after the beginning of the experiment the first DGGE was performed for the second stage sludge (lane 8 in Figure 2). As expected from respirometry (lane 8 in Table 5), no *Nitrosomonas sp*. was detected at that time. After approximately one month a steady increase in the nitrification performance of the second stage could be observed and after three months a second DGGE of the sludge from the second stage indicated a shift in population from *Nitrosospira sp*. towards *Nitrosomonas spp*. (lane 6 in Figure 2). In order to exclude a possible influence from a variation in the characteristic of the applied wastewater itself (reduction of inhibition and therefore stimulation of *Nitrosomonas sp*.), sludge from the full-scale plant (F-R) was also investigated using DGGE. The results (lane 5 in Figure 2) show that still *Nitrosospira sp*. was predominant in the full-scale plant. This indicates that inhibition can be removed in the first stage and full nitrification can be established in the second stage of the laboratory plant. At the end of the experiment, the first stage was bypassed and the wastewater from the rendering plant was fed directly to the second stage without changing corresponding sludge retention time. Within two weeks *Nitrosomonas sp*. vanished from the system (lane 2 in Figure 2), again indicating the inhibitory effect of the rendering plant wastewater to growth of *Nitrosomonas sp*.

Conclusions

Community analysis of β-AOMs by DGGE was shown to be a suitable and sensitive tool to support established engineering tools in the investigation of wastewater treatment processes as respirometry or mass balances. Results from DGGE were conclusive, useful for the interpretation of other experiments and assisted engineers to find strategies in order to solve nitrification problems in wastewater treatment. This work showed a fruitful collaboration between engineers and scientists revealing new possibilities for understanding and optimising applied biological processes for technical applications.

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