Monitoring of population shifts in an enriched nitrifying system under gradually increased cadmium loading

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ABSTRACT

The changes in nitrifying bacterial population under cadmium loading were monitored and evaluated in a laboratory scale continuous-flow enriched nitrification system. For this purpose, the following molecular microbiological methods were used: slot–blot hybridization, denaturing gradient gel electrophoresis (DGGE), real-time PCR followed by melting curve analysis, cloning and sequence analysis. The initial cadmium concentration was incrementally increased from 1 to 10 mg/l which led to a drop in ammonia removal efficiency from 99 to 10%. Inhibition was recovered when cadmium loading was stopped. During the second application of cadmium, nitrifying population became more tolerant. Even at 15 mg/l Cd, only a minor inhibition was observed. To investigate the variations in ammonia and nitrite oxidizing bacteria populations in a period of 483 days, ammonia monooxygenase (*amoA*) and 16S rRNA genes-based molecular techniques were used. An obvious shift was experienced in the diversity of ammonia oxidizers after the first application of 10 mg/l Cd. Metal-tolerant ammonia oxidizing species became dominant and the microbial diversity sharply shifted from *Nitrosomonas* and *Nitrosococcus* sp. to *Nitrosospira* sp. which were observed to tolerate higher cadmium loadings. This result indicated that the extent of nitrification inhibition was not only related to the metal concentration and quantity of microorganisms but also depended on the type of species.

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1. Introduction

In biological treatment systems, nitrification is the key process for the removal of nitrogen from wastewater. As known it is a two-step process consisting of the oxidation of ammonia to nitrite by ammonia-oxidizing bacteria (AOB) such as *Nitrosomonas* and *Nitrososopira*. Then, nitrite is converted to nitrate by nitrite-oxidizing bacteria (NOB) such as *Nitrobacter* and *Nitrospira*. Ammonia oxidation is the rate-limiting step in most nitrogen removal processes because of the slow growth rate and high sensitivity of these organisms to toxic compounds [1,2]. The presence of various organic and inorganic compounds and the DO, temperature and metal levels can affect the specific growth rate of ammonia oxidizing bacteria in various environments [3–6]. The nitrification process is often susceptible to metal toxicity which has received special attention in recent years [5–7].

Metal uptake is a physicochemical process rather than biological, because sorption plays an important role and uptake does not depend on cell viability and bacterial growth [5,8–10]. In bacterial cells, free heavy metal ions form unspecific complex compounds, which lead to toxic effects [9]. These inhibitory effects on microbial growth have been investigated by many researchers. Most of the research on the toxicity of inhibitors addresses the maximum concentration which can be tolerated by microorganisms [11,12].

Metals have several effects on microbial growth, either as trace elements or inhibitors. There are few studies on nitrifying populations in which the toxic effects of heavy metals, their threshold levels and the degree of nitrification inhibition are studied [5–7,13–15]. Some microorganisms can tolerate toxicity of heavy metals, while others cannot. Recent studies indicated that acclimation can reduce the negative effects of toxicity and lead to the dominance of new tolerant species [6,16,17]. However, it has not been clarified yet what kind of acclimated microorganisms can tolerate heavy metal toxicity. Some researchers examined the effects of heavy metals on nitrifying systems using pure cultures of *Nitrosomonas* species and studied the threshold levels [18,19]. However, it is well known that there is a high diversity of ammonia-oxidizing

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bacteria in the environment. In general, members of the *Nitrosomonas europaea* lineage could out-compete *Nitrosospira* sp. in habitats containing high substrate concentrations because of their higher maximum growth rates. On the other hand, *Nitrosospira* sp. were found to be better competitors under low substrate conditions and in environments contaminated with toxic metals [3,20].

The results of pure culture studies are valid only under laboratory conditions and cannot be extended to more complex environments where various microorganisms can exist. Complex environments may permit the generation of new species tolerating higher toxic levels. In literature, the toxic range of copper extends from 5 mg/l [14] to 150 mg/l [21] which shows that data are very scattered. In former studies on heavy metal inhibition, the bacterial mass or concentrations were taken into consideration and the community structures of nitrifying species were not monitored [22]. However, in a natural environment, diverse species are available. Although much is known about the basic metabolism of toxicity and threshold levels, little is known about the bacterial structure, diversity and function in mixed cultures.

The objective of this study was to assess whether any changes occurred in the nitrifying population in the presence of high Cd or the existing bacteria became acclimated to toxic levels of Cd. Therefore, the variation in nitrifying bacterial community was monitored and evaluated using *amoA* and 16S rRNA gene based molecular methods. Ammonia oxidizing bacterial population was monitored using denaturing gradient gel electrophoresis (DGGE) and real-time PCR followed by melting curve analysis. Microbial species were identified by cloning and DNA sequencing analysis. Nitrite-oxidizing bacterial species were detected by slot–blot hybridization analysis.

2. Materials and methods

2.1. The continuous-flow nitrification system

The lab-scale continuous-flow reactor used in this experiment was fed with synthetic wastewater. The seed sludge was taken from the Pasakoy Advanced Wastewater Treatment Plant (Istanbul, Turkey) and was enriched in a batch reactor before being transferred to the continuous-flow system [23]. The sludge was enriched in terms of nitrifiers by feeding only ammonia-nitrogen and necessary minerals. The effective volume of the reactor was 20 l. The reactor was operated at 20 days of sludge retention time and 1 day of hydraulic retention time [24]. A temporized peristaltic pump was used to recycle the settled sludge at a flow rate of 10 l/day

which was equal to the influent flow rate. Temperature and pH were continuously controlled by a submerged heater and a pH controlling system, respectively. The dissolved oxygen in the tank was monitored using a dissolved oxygen meter and probe. Cadmium and ammonia nitrogen were simultaneously added to the influent of the continuous-flow reactor to investigate the inhibitory effect (Table 1). Both free cadmium (Cd^{2+}) and total cadmium concentrations were measured by voltammetry (VA 797 Computrace, Metrohm Inc.). To monitor the extent of nitrification, nitrogen in the form of ammonia, nitrite, nitrate, and also biomass concentrations were daily measured according to the Standard Methods [25]. For investigation of the microbial community in the nitrification tank, samples were taken from the mixed liquor at days 47, 104, 113, 137, 153, 203, 232, 305, 328, 342, 356, 426, 468, and 480 and stored frozen (−20 °C) before DNA isolation.

2.2. DNA extraction and PCR amplification

Nucleic acid extraction was performed using FastDNA SPIN kit (Q-BIOgene) according to the manufacturer's instructions with a few modifications [26]. The amount and purity of DNA was estimated spectrophotometrically by measuring the optical density at 260 and 280 nm. DNA was diluted to obtain suitable PCR amplicons, as templates for subsequent PCRs. Full-length bacterial 16S rDNA fragments were amplified for slot–blot hybridization with general bacterial primers, 27 forward (AGA GTT TGA TCC TGG CTC AG) and 1510 reverse (GGT TAC CTT GTT ACG ACT T) [26]. For cloning experiments, *amoA* gene fragments (491 bp) were amplified as described previously [26] with *amoA*-1F (GGG GTT TCT ACT GGT GGT) and *amoA*-2R (CCC CTC KGS AAA GCC TTC TTC) primer sets. For DGGE analysis, the PCR approach was used to add a 33 base GC-clamp to the amplicon, in order to improve the melting behavior of fragments, as described by Nicolaisen and Ramsing [27]. The PCR products were subsequently cooled to 4 °C.

2.3. Slot–blot hybridization

DIG-labeled oligonucleotide probes targeting *Nitrospira* (GGA ATT CCG CGC TCC TCT) and *Nitrobacter* (CCT GTG CTC CAT GCT CCG) were used to investigate the nitrite oxidizing population in an enriched nitrification system as described by Mertoglu et al. [26]. For slot–blot analysis, DIG-labeled oligonucleotide probes were hybridized using DIG Easy Hybridization buffer (Boehringer Mannheim GmbH) and washed with DIG Wash and Block Buffer Set (Boehringer Mannheim GmbH). Afterwards, the anti-DIG alkaline

Table 1
Influent ammonia and cadmium metals, application periods and sampling days

Application period (day)	Average influent ammonia-nitrogen (mg/l)	Influent cadmium (mg/l)	Samples
1–16	50	–	–
17–48	200	–	–
49–68	250	–	Day 47 (C1)
69–89	50–60	–	Day 104 (C2)
90–116	206	–	Day 113 (C3)
117–134	230	1.0	Day 137 (C4)
135–159	224	2.0	Day 153 (C5)
160–192	221	Recovery period	
193–223	215	2.5	Day 203 (C6)
224–250	223	Recovery period	Day 232 (C7)
251–278	224	4.5	
279–306	214	6.0	Day 305 (C8)
307–327	213	8.0	Day 328 (C9)
328–347	225	10.0	Day 342 (C10)
348–426	218	Recovery period	Day 356 (C11)
			Day 426 (C12)
427–468	212	10.0	Day 468 (C13)
469–491	224	15.0	Day 480 (C14)

phosphatase conjugate was applied to visualize the hybridization signals [26].

2.4. Denaturing gradient gel electrophoresis

DGGE was performed with 8% (w/v) polyacrylamide containing a linear chemical gradient ranging from 20 to 50% denaturant. The denaturing gradient gels were run at 100 V and 60 °C for 15 h with the Dcode Universal Mutation System (Bio-Rad). Silver-staining and development of the gels were performed as described elsewhere [28].

2.5. Cloning and sequencing analysis

The phylogenetic analysis of ammonia oxidizing bacteria was started with the purification of PCR products of *amoA* genes using the QIAquick PCR purification kit (Qiagen) prior to cloning. Then, the purified amplicons were ligated into the pTZ57R/T vector (Fermentas) and were cloned in competent *E. coli* JM109 cells using the InsT/Aclone™ PCR Product Cloning Kit (Fermentas) with ampicillin selection and blue/white screening, following the manufacturer's protocol. White colonies were picked up from each cloned sample and reamplified. Subsequently, PCR amplicons of clone inserts were screened by denaturing gradient gel electrophoresis to determine the representative clones for sequencing. The reamplification product was also evaluated by DGGE to verify the purity and correct the mobility of the reamplified product. The reamplified product was compared with the raw samples; it should give one band and match the raw sample.

Before DNA sequencing, the plasmids of selected transformants were purified using the Wizard Plus SV Miniprep DNA purification kit (Promega). DNA sequences were analyzed in SeqLab Sequence Laboratories (Göttingen, Germany). Afterwards, a similarity search, in the GenBank database, with the derived partial *amoA* sequences from the clones, was performed using the BLAST search program available on the internet (National Center for Biotechnology Information sequence search service).

2.6. Real-time PCR and melting curve analysis

Ammonia oxidizing bacteria (AOB) were analyzed by real-time PCR using primers that targeted the *amoA* gene [26] with a LightCycler device (Roche, Mannheim, Germany). The standard SYBR Green detection was carried out using the LightCycler FastStart DNA Master SYBR Green I kit (Roche) following the manufacturer's protocol. Reactions were performed in a volume of 20 μl. The reaction mixture contained 2 μl, 1× Mastermix (Roche); 2 μ1, 25 mM MgCl$_2$; 1.25 μM concentrations of each primer, PCR grade distilled water and 2 μl diluted template DNA. Real-time PCR was started with an initial denaturation at 95 °C for 10 min. Subsequently, the cycling program was followed by 40 cycles of 5 s of denaturation at 95 °C, 20 s of annealing at 57 °C, and 45 s of elongation at 72 °C. Fluorescence was detected after each cycle at 84 °C to avoid detection of primer dimmers [29]. In all applications, negative controls without template DNA were subjected to the same procedure to detect any possible contamination.

The specificity of amplified PCR products were assessed by performing a melting curve analysis, gradual denaturation with a temperature transition rate of 0.1 °C s^{-1} from 65 to 95 °C with a continuous monitoring of fluorescence. The melting temperature (T_m) of PCR products is the temperature at which 50% of the strands of the target gene have dissociated, and was derived from the inflection point of the fluorescence (F) versus temperature (T) curves, or the peak value of the $-dF/dT$ versus T curves [30]. An external standard curve showing the relationship between *amoA* copy numbers

and $C(t)$ values was constructed using serial dilutions of a known copy number of *amoA* gene. The R^2 values were greater than 0.99 for all standard curves. The standard copy number of *amoA* gene was prepared from PCR products of previously cloned *Nitrosomonas eutropha*. The concentration of amplified DNA was determined by measuring absorbance at 260 nm with the Shimadzu UV2450 spectrophotometer (Shimadzu Co., Kyoto, Japan).

3. Results

3.1. Reactor performances

The continuous-flow nitrifying reactor was operated for 483 days at various ammonia and cadmium loadings to investigate the inhibitory effects of cadmium on nitrifying bacteria [23,24]. In the first period from 0 to 116 days, the reactor was operated at various ammonia loadings without any metal addition. Three sludge samples were taken in this no-Cd load period (samples C1–C3). Nitrification efficiencies were usually above 90%. After the continuous dosing of 1 mg/l of cadmium in the influent, no decrease was observed in ammonia removal rates. Moreover, no further increase in inhibition was observed when the influent Cd concentration was stepwise increased up to 10 mg/l (samples C4–C9). The ammonia removal efficiency decreased almost to zero when 15 days had passed after the first application of 10 mg/l Cd (sample C10). Cadmium addition was interrupted to allow the recovery of microbial activity because along with a decrease in ammonia removal also a drop was observed in biomass concentration. Then, during this period, only ammonia and the necessary minerals were dosed into the reactor (samples C11–C12). The amount of nitrifying bacteria restored back within 2 months. In the second application inhibition reached nearly 60% although the influent Cd concentration was set again as 10 mg/l (sample C13). In the next step, where the cadmium concentration was further elevated to 15 mg/l, ammonia removal efficiencies were unexpectedly high at 50% (sample C14). Fig. 1 depicts ammonia loading rates and corresponding reactor performances. Table 1 summarizes the different periods of operation showing the influent ammonia and cadmium.

3.2. Slot–blot hybridization

For the detection of the nitrite oxidizing bacterial community group-specific oligonucleotide probes were applied. Hybridization results indicated that *Nitrospira* and *Nitrobacter* were the major nitrite oxidizers existing in the reactor (Fig. 2).

3.3. DGGE and sequence analysis

The variations in ammonia oxidizing community were monitored for more than 450 days using 14 sludge samples (C1–C14). A low diversity was observed in the case of ammonia-oxidizing bacteria in each individual lane (Fig. 3). Representative bands from different DGGE lanes were compared to the cloned *amoA* plasmids. Each individual band was sequenced after matching with the DGGE lanes. The DGGE fingerprints of the amplified *amoA* populations showed no difference between the samples C1–C2, C3–C5, C7–C9, C10–C11 and C12–C14. As no significant differences were observed in population structure, only the samples C1, C3, C6, C8, C10, and C12 in Fig. 3 were cloned and analyzed for phylogenetic affiliation of retrieved sequences. All retrieved sequences were related to previously published *amoA* sequences (Fig. 3).

In sample C1, all amoA clones were categorized under *Nitrosomonas* species, uncultured *Nitrosomonas* sp. clone Y34, uncultured *Nitrosomonas* sp. clone Y35, and *Nitrosomonas* sp. GH22 (Fig. 4). When Cd addition started, *Nitrosococcus mobilis* species
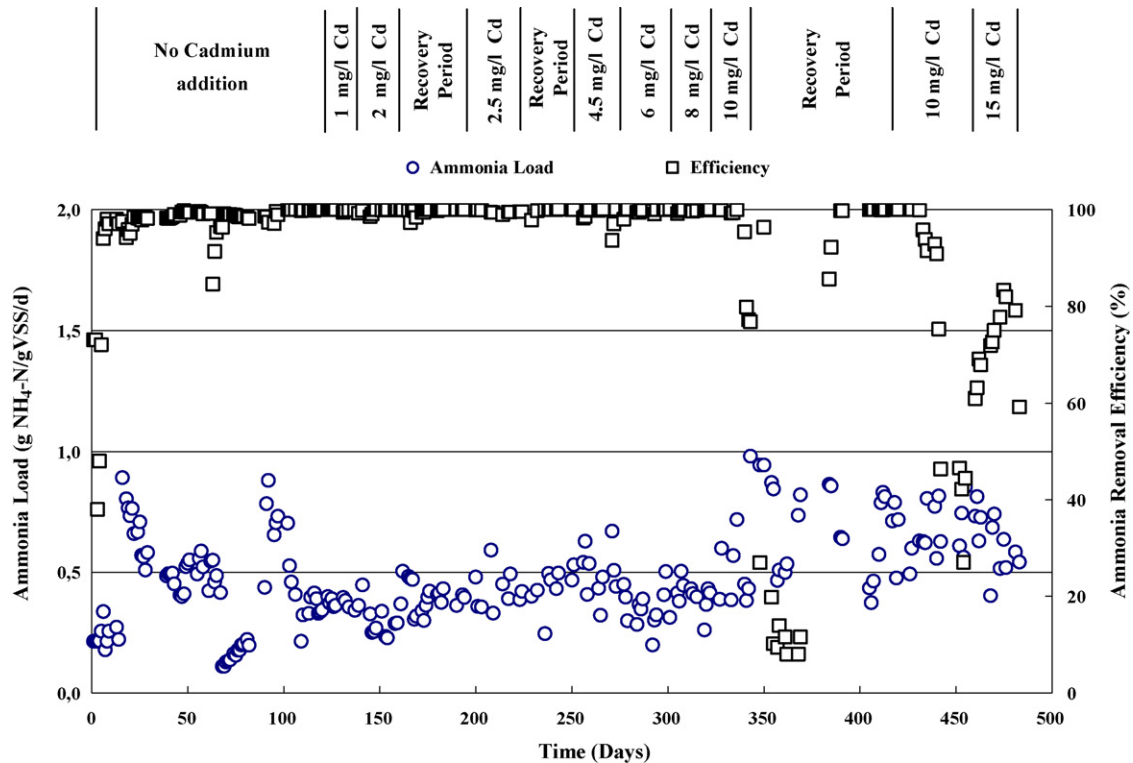
Fig. 1. Applied ammonia loading rates and reactor performance under gradually increased cadmium loading.

began to appear while uncultured *Nitrosomonas* sp. clone Y35 disappeared (C3–C5). In the DGGE fingerprint of sample C1, the uncultured *Nitrosomonas* sp. clone Y34 disappeared while *N. mobilis* became predominant. Uncultured *Nitrosomonas* sp. clone Y34 was also detected in sample C10 and C11. Apparently, the nitrifier community did not change significantly between sample C1 and C11. After the first inhibitory impact of cadmium, the microbial community underwent a considerable change and *Nitrosomonas* and *Nitrosococcus* shifted to *Nitrosospira* sp. NpAV (C12–C14). A similar banding pattern was observed between samples C12 and C14.

3.4. Real-time PCR and melting curve

The quantitative real-time PCR method was applied to quantify the numbers of *amoA* gene copies. However, no noticeable change was detected throughout the sampling period. This is thought to arise due to the constant numbers of *amoA* gene copies in the

enriched nitrifier population per gram of suspended solids (data not shown). Following PCR amplification, the melting curve analysis was immediately applied to 14 diluted DNA samples and revealed major amplification peaks between 80 and 95 °C, indicating identical melting peaks (Fig. 5). When double-stranded DNA molecules or PCR products are subjected to gradual heating, they melt in a series of steps and different species exhibit different identical melting peaks as a result of different GC contents. When the melting curve analysis is completed, individual melting peaks represent different species.

In our study, the melting curve analysis was used to monitor microbial changes during the operation. This method can be applicable only if the system harbors few numbers of nitrifying bacteria. Identical melting peaks were clearly distinguishable and a microbial shift was observed similar to the DGGE and cloning experiments (Fig. 5). Only one identical melting peak was identified from sample C1 to C5 and this belonged to the uncultured *Nitrosomonas*
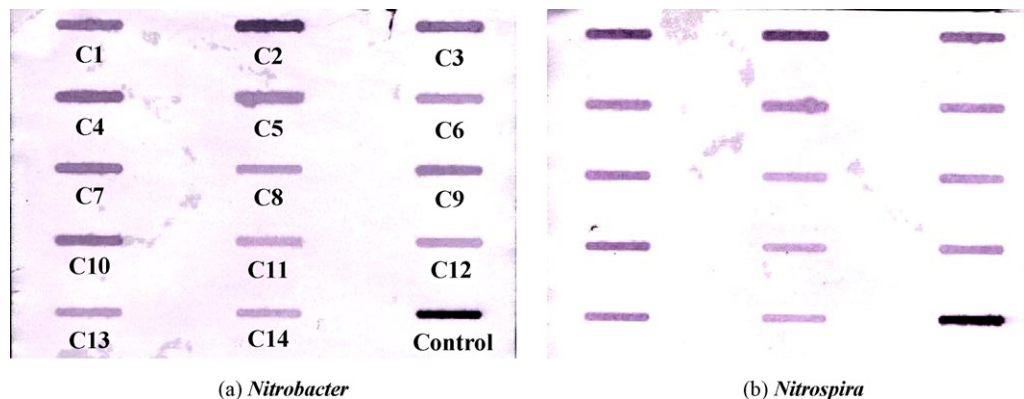

(a) *Nitrobacter*

(b) *Nitrospira*

Fig. 2. Nitrite oxidizing bacteria, *Nitrobacter* (a) and *Nitrospira* (b) changes during the operation of nitrifying reactor. Thickness of the bands is directly proportional to the amount of target DNA. For efficient comparison DIG labeled 1 ng pBR328 control DNA (linearized with BamHI) was applied to each membrane.
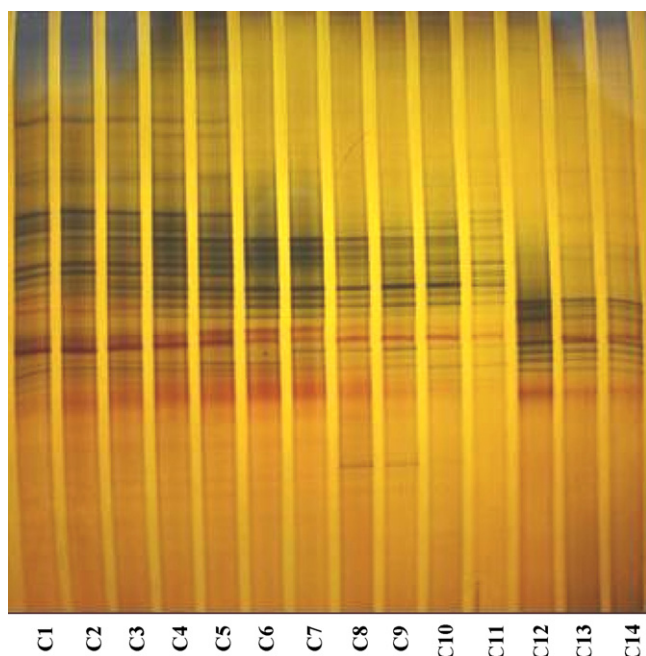
Fig. 3. DGGE profiles of PCR-amplified *amoA* gene fragments retrieved from 14 sludge samples.
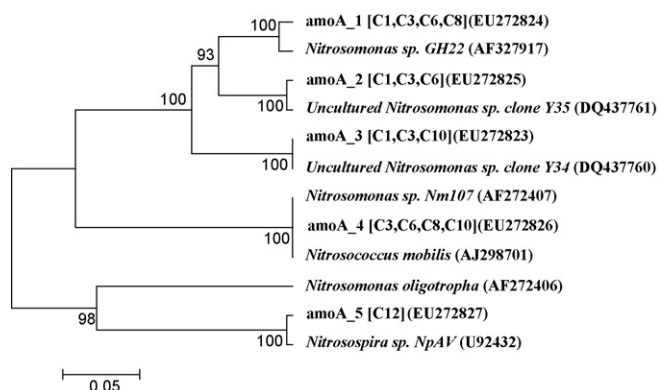

Fig. 4. A neighbor-joining trees of *amoA* clones from nitrifying reactor. The significance of each branch is indicated by bootstrap values. The scale bar represents 0.05 inferred substitutions per nucleotide position.
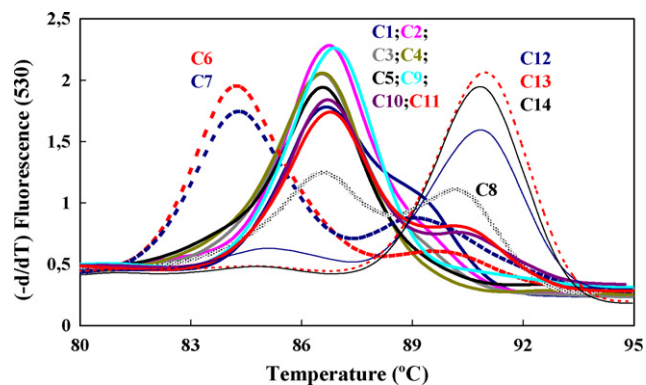

Fig. 5. Melting curve analysis was carried out for 14 samples directly after real-time PCR amplification. Whole melting curve peaks were placed at the same axis.

sp. *clone Y34* species. In the sample C6, the peak resembled to the *N. mobilis* species and the melting temperature (T_m) differed from the C5 sample. Two melting peaks were observed in sample C7: uncultured *Nitrosomonas* sp. *clone Y34* and *Nitrosospira* sp. *NpAV* with apparent T_m of 86.7 and 89.6 °C, respectively. *Nitrosospira* sp. *NpAV* appeared in sample C8 and disappeared immediately in sample C9. The great community changes observed in sample C12 were also shown in the DGGE analysis. The melting peaks shifted from 87.0 °C (C11) to 91.2 °C (C12); from uncultured *Nitrosomonas* sp. *clone Y34* to *Nitrosospira* sp. *NpAV* species, respectively. The differences in the melting peaks of samples C11 and C12 are shown in Fig. 5. Three main identical melting peaks were detected in this experiment. The variation in T_m was calculated 0.5 °C when the same DNA template was amplified in four independent reactions. In an additional series of experiments with DNA from eight different dilutions, the T_m variation was 0.3 °C. These results suggest that melting peak data are highly reproducible under fixed assay conditions.

4. Discussions

In the continuous-flow nitrifying system; ammonia removal was not significantly hindered when the system was monitored using ammonia removal without or with Cd at levels less than 10 mg/l (Fig. 1). The nitrification efficiency was monitored using conventional parameters and no adverse effect of cadmium was assessed during this period. However, the application of molecular tools (DGGE and real-time PCR analyses) clearly showed that microbial population changed although ammonia removal was almost not affected. When the cadmium concentration in the influent to the reactor was elevated to 10 mg/l, ammonia removal decreased sharply. Our microbial analysis based on two important hypotheses: either microorganisms had acclimated their specific genes to the toxic environment or a microbial shift occurred from sensitive to tolerant microorganisms. In our case, all results indicated that microbial acclimation was not observed. Otherwise, the same type of species should be detected after Cd addition and should be not lost in the reactor. Our molecular results clearly demonstrated that cadmium resistant bacteria became dominant following the first inhibition by Cd and microbial community shifted from *Nitrosomonas* and *Nitrosococcus* to *Nitrosospira* sp. Thus, these *Nitrosospira* species which were exceptionally tolerant to Cd were gradually becoming dominant. These aspects indicate why molecular identification of nitrifiers is crucial in inhibition studies along with conventional monitoring.

Toxic effects of heavy metals on microbial growth have been investigated by many researchers. Most of the researchers focused on the maximum concentration that could be tolerated by pure or mixed cultures and have studied the degree of inhibition and recovery mechanisms [4,7,13,14,16]. However, a long-term monitoring of tolerant or sensitive microorganisms was not done and microbial changes have not been studied in detail. Particularly, the information is very limited in the case of nitrifying populations exposed to metals. Lee et al. [14] reported that when copper concentration reached 5 mg/l, nitrification was partially suppressed, while Tomlinson et al. [21] reported significantly different copper toxicity levels such as 150 mg/l for nitrifying populations. In all reported studies the characterization of biomass was based on VSS measurements and determination of biomass activity while bacterial composition and community structures were disregarded [22]. Braam and Klapwijk [31] showed that the inhibitory effect of copper on nitrification depended on the concentration of volatile suspended solids and pH. However, microbial diversity studies and population monitoring give a more accurate idea about the inhibitory

effects of heavy metals than physical or chemical measurements alone.

The general belief is that ammonia oxidizers are more susceptible to heavy metal toxicity than nitrite oxidizers in nutrient removal systems. Lee et al. [14] investigated the effects of copper and nickel on *Nitrosomonas* and *Nitrobacter* species in a suspended growth and an attached-suspended growth system. They observed that *Nitrosomonas* sp. were more sensitive to copper and nickel than *Nitrobacter* sp. and ammonia oxidation was the rate limiting step under metal-stressed conditions. In literature, *Nitrosomonas* species were generally taken as representatives of ammonia oxidizers in pure culture nutrient removal studies [14,18,19]. However, there is a high diversity of ammonia oxidizing bacteria in full-scale nutrient removal systems. In particular, members of *Nitrosospira* sp. were found to be predominant in habitats exposed to low-substrate or high toxic metal concentrations because of their low substrate affinities [3,20]. This is an important factor and the wide variation in the threshold levels reported in laboratory- and full-scale studies can be attributed to this factor. There is even a difference between the inhibitory effect of metals in batch and continuous-flow systems [23,24]. In the work of Semerci [24] the inhibitory effect of cadmium was modeled in the continuous-flow system and the importance of cadmium speciation in the sludge and bulk phases was discussed. In our work, which demonstrates a through microbial characterization of this continuous-flow study in great detail, microbial shifts were clearly observed from *Nitrosomonas* sp. to *Nitrosospira* sp. in an enriched nitrifying system. Obviously, *Nitrosospira* sp. tolerated higher cadmium concentrations compared to *Nitrosomonas* sp. Sakano and Kerkhof [32] also reported a microbial shift from groups dominated by organisms containing *Nitrosomonas*-like and *Nitrosospira*-like *amoA* genes to groups containing only *Nitrosospira*-like *amoA* genes in a laboratory-scale ammonia biofilter. Experiments using pure *Nitrosomonas* cultures may never represent mixed culture studies and would be not suitable for the determination of threshold levels or toxicity. Juliastuti et al. [15] determined the maximum specific growth rate of autotrophic biomass under inhibitory conditions. They summarized that literature threshold value of inhibition significantly higher than the experimental limits of their research. However, the microbial composition of nitrifying bacteria was also not investigated in that study.

Cadmium is widely known to be a very hazardous pollutant having toxic effects in various ecosystems [33]. On the molecular level, cadmium uptake is barely understood [13]. According to Tsai et al. [7,16], the toxic effect of cadmium in nitrification occurs at a relatively low level of 5 mg/l. They also implied that 2 mg/l cadmium initially affected the biological phosphate removal in an A_2O pilot plant. Nitrification substantially dropped at 5 mg/l cadmium in their study. They also found that *Nitrosomonas communis* was the predominant microorganism in the class *Betaproteobacteria* before inhibition had occurred [16]. Tomlinson et al. [21] pointed out that cadmium caused nitrification upset in activated sludge and inhibited pure cultures of AOB. The mechanism of this inhibition is unknown.

Although *Nitrospira* sp. were reported as the main nitrite oxidizers generally present in environmental samples [20,26], our study revealed that the nitrifying reactor both harbored *Nitrospira* and *Nitrobacter* species. Kelly et al. [34] emphasized that cadmium shock affects ammonia oxidation but does not significantly impact nitrite oxidation. This theory was confirmed with our slot–blot experiment. In our study, the same nitrite oxidizing species *Nitrobacter* and *Nitrospira* were detected at varying cadmium concentrations (Fig. 2), despite changes in the ammonia oxidizing bacteria in the sludge (Fig. 5).

The comparison of the community changes in ammonia oxidizers was performed using DGGE and real-time PCR melting curve analysis based on *amoA* gene of all the sludge samples (Figs. 3 and 5). A microbial shift was clearly observed with these two methods. Interestingly, the number of identical melting peaks and DGGE banding patterns were not equally observed. DGGE analysis always harbored higher diversity because of the great sensitivity of the sequence variation detection. The melting curve analysis is a fast, simple and specific method to detect microbial community changes if the system comprises a non-intensive bacterial community. However, the melting curve analysis is less sensitive compared to the DGGE analysis. We believe that the melting curve mutation detection analysis will be used for environmental studies in near future as an alternative to the DGGE. The experimental procedure which includes DNA extraction, real-time PCR and melting curve analysis lasted less than 4 h. The melting curve analysis was also used in the selection of clones for DNA sequencing in order to proceed rapidly and to eliminate the need for gel electrophoresis. This application was also cross-checked using the DGGE analysis. In literature, there are only few studies about the melting curve analysis and up to date none of them were used for monitoring of microbial shifts. Randegger and Hächler [35] concluded that the melting curve mutation works perfectly with small or single-copy DNA templates and is a powerful tool for important epidemiological studies. This makes the method a serious candidate for implementation into routine diagnostics.

5. Conclusion

The microbial shift from *Nitrosomonas* and *Nitrosococcus* sp. to *Nitrosospira* sp. under gradually increased cadmium loading was monitored in a laboratory scale nitrification system using different molecular methods; slot–blot hybridization, denaturing gradient gel electrophoresis, real-time PCR, cloning and sequence analysis. The results showed that the inhibitory effect and quantity of microorganisms depended on metal concentration and the type of microbial species present in the sludge. In the sludge, metal-tolerant ammonia oxidizing *Nitrosospira* species became dominant after the first application of cadmium. Then, the sludge tolerated higher cadmium concentrations. The melting curve analysis was performed to monitor population shifts and selection of clones for sequencing. The study demonstrated the superiority of the melting curve approach in environmental biotechnology.

Acknowledgements

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