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DNA microarray detection of nitrifying bacterial 16S rRNA in wastewater treatment plant samples

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Abstract

A small scale DNA microarray containing a set of oligonucleotide probes targeting the 16S rRNAs of several groups of nitrifying bacteria was developed for the monitoring of wastewater treatment plant samples. The microarray was tested using reference rRNAs from pure cultures of nitrifying bacteria. Characterization of samples collected from an industrial wastewater treatment facility demonstrated that nitrifying bacteria could be detected directly by microarray hybridization without the need for PCR amplification. Specifically, the microarray detected *Nitrosomonas* spp. but did not detect *Nitrobacter*. The specificity and sensitivity of direct detection was evaluated using on-chip dissociation analysis, and by two independent analyses—an established membrane hybridization format and terminal restriction fragment length polymorphism fingerprinting (T-RFLP). The latter two analyses also revealed *Nitrospira* and *Nitrobacter* to be contributing populations in the treatment plant samples. The application of DNA microarrays to wastewater treatment systems, which has been demonstrated in the current work, should offer improved monitoring capabilities and process control for treatment systems, which are susceptible to periodic failures.

Keywords: Microarray; Nitrification; Nitrifiers; Wastewater treatment

1. Introduction

Nitrification, the conversion of ammonia to nitrate via nitrite, is a crucial part of the global nitrogen cycle and is also a critical step in many wastewater treatment schemes. The maintenance of stable nitrifying bacterial communities in these treatment systems is critical to their proper functioning, but can be difficult because nitrifying bacteria are sensitive to shifts in pH, temperature, and a number of inhibitors (Rittmann and McCarty, 2001). Nitrifying bacteria have proved

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particularly difficult to study by conventional cultivation techniques because of their long generation times and low growth rates (Purkhold et al., 2000), which can result in underestimations of their numbers (Konuma et al., 2001). Therefore, a rapid, culture-independent detection technique for nitrifiers would be useful for the management of wastewater treatment systems.

Molecular techniques are increasingly used to detect specific groups of microorganisms without cultivation. DNA probes complementary to the 16S rRNAs are now commonly used for the study of microbial populations in complex systems (Sahm et al., 1999). However, membrane-based (Sahm et al., 1999) and in situ (Okabe et al., 1999) hybridization techniques severely limit the number of probes that can be applied simultaneously. The high probe capacity of DNA microarrays provides a format for simultaneous hybridization of much larger numbers of nucleic acids. This technology is increasingly used to measure changes is gene expression (e.g. Schena et al., 1995), and has recently been applied to the detection of bacterial genes in environmental samples (Wu et al., 2004, 2001; Rhee et al., 2004). Relatively few studies have demonstrated the utility of DNA microarrays incorporating 16S rRNA-targeted oligonucleotide probes for characterizing environmental populations of bacteria (El Fantroussi et al., 2003; Loy et al., 2002).

We are developing a microarray format in which oligonucleotide probes targeting the 16S rRNAs are individually immobilized within polyacrylamide gel pads bound to the surface of a glass slide (Guschin et al., 1997; Liu et al., 2001). RNA isolated from pure cultures or environmental samples serves as the target for hybridization to the immobilized probes. Since the rRNAs are naturally amplified, often present in thousands of copies per cell, they can often be detectable directly, eliminating the need for PCR amplification. Avoiding PCR is a significant advantage, as PCR can fail or lead to biases that confound ecological studies (Becker et al., 2000).

The objective of the current study was to determine if DNA microarrays could be useful for the detection of nitrifying bacteria in wastewater treatment systems. The study was designed specifically to determine if nitrifier rRNA could be detected directly by microarray hybridization and if melting profile analysis could be used to achieve a high level of specificity. A small-scale DNA microarray containing a set of eight oligonucleotide probes targeting both ammonia-oxidizing (AOB) and nitrite-oxidizing bacteria (NOB) was fabricated and tested using reference rRNAs from pure cultures of nitrifying bacteria. This was then used to assess the presence of nitrifying bacterial populations in an industrial wastewater treatment plant that included two sequential aeration tanks. This demonstrated the direct detection of specific nitrifying bacteria in a wastewater treatment plant without amplification, as confirmed by two widely-used techniques, membrane hybridization and terminal restriction fragment length polymorphism (T-RFLP) analysis.

2. Materials and methods

2.1. Reference RNAs

Nitrosomonas eutropha strain C91 and *Nitrosospira briensis* strain C128 were used as reference organisms for DNA microarray testing and for membrane hybridization. *Nitrobacter winogradskyi* (ATCC 14123) and *Nitrospira marina* strain Nb-295 were also used as reference organisms for membrane hybridization. Due to the long generation times and low growth rates of these organisms, reference RNAs were produced by in vitro transcription. For each organism, DNA was extracted from a frozen cell pellet by bead beating (Kuske et al., 1998) using the FP120 Cell Disrupter (Qbiogene, Inc., Carlsbad, CA). The 16S rRNA genes were amplified by PCR using primers 11F (5′-GTTTGATCCTGGCTCAG-3′) and 1512AR (5′-ACGGYTACCTTGTTACGACTT-3′) and cloned using TOPO Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA). The identity of the clones was confirmed by sequencing using a MegaBACE 1000 DNA Sequencer (Amersham Pharmacia Biotech, Piscataway, NJ) and the following primers: M13R and T7 (Invitrogen, Carlsbad, CA) and 700F and 700R (Urbach et al., 2001). Plasmids containing the 16S rRNA gene sequences were isolated using Ultra Clean Mini Plasmid Prep Kit (MoBio Laboratories, Solana Beach, CA), linearized by restriction digestion, and used as templates for in vitro transcription using a commercial RNA transcription kit (New England Bio Labs Inc., Beverly, MA). The number of mismatches between the probes and the reference RNA sequences (Table 1) was determined using the HP Calculator (available at http://stahl.ce.washington.edu.).

2.2. Wastewater treatment plant samples

On 31 January 2001, samples were collected from the first- and second-stage aeration tanks (AT1 and AT2) of the Borden Chemicals and Plastics (BCP) activated sludge treatment facility in Illiopolis, IL. BCP produces polyvinyl chloride (PVC) resin with ammonium as a by-product, resulting in a wastewater with an ammonium content typically about 60 mg/L NH_4^+-N. This facility achieves stable nitrification (i.e. conversion of essentially 100% of the ammonium to nitrate). Multiple grab samples of mixed liquor were collected from AT1 and AT2 in 2-ml cryovials containing 0.5 g baked zirconium beads (0.1 mm diameter; BioSpec Products, Bartlesville,

Table 1
Oligonucleotide probes included on microarray

Probe name	Full name[a]	Probe sequence (5′-3′)	Target organism(s)	Probe source	Mismatches to reference organisms	
					N. briensis	*N. eutropha*
UNIV907	S-*-Univ-0907-a-A-22	cccgtcaattcctttgagttt	All life	Amann et al. (1992)	0	0
UNIV1390	S-*-Univ-1390-a-A-18	gacgggcggtgtgtacaa	All life	Zheng et al. (1996)	0	0
NSO1225	S-G-Nso-1225-a-A-20	cgccattgtattacgtgtga	Most β-proteobacterial AOB[b,c]	Mobarry et al. (1996)	0	0
NSO190	S-G-Nso-0190-a-A-19	cgatcccctgctttttctcc	Many β-proteobacterial AOB[b,c]	Mobarry et al. (1996)	0	1
NEU	S-G-Nsom-0653-a-A-18	ccctctgctgcactcta	Most halophilic and halotolerant *Nitrosomonas* spp[b]	Wagner et al. (1995)	1	0
NSOM156	S-G-Nsom-0156-a-A-19	tattagcacatctttcgat	*Nitrosomonas* spp.	Mobarry et al. (1996)	2	0
NSV443	S-G-Nso-0443-a-A-19	ccgtgaccgtttcgttccg	*Nitrosospira* cluster 1-3[b]	Mobarry et al. (1996)	0	5
NBAC1000	S-G-Nbac-1000-a-A-15	tgcgaccggtcatgg	*Nitrobacter* spp.	Mobarry et al. (1996)	6	6

[a]Full names have been standardized by Alm et al. (1996).
[b]Koops et al. (2003).
[c]AOB = ammonia oxidizing bacteria.

OK). Sample tubes were transported on dry ice and stored at −80 °C. Total RNA was extracted from sludge samples by bead-beating (2 × 30 s) using the FP120 Cell Disrupter (Qbiogene) as previously described (Stahl et al., 1988). RNAs were treated with RNase-free DNase (Amersham Pharmacia Biotech, Piscataway, NJ) as described previously (Moran et al., 1993). The RNA concentration was determined by spectrophotometric analysis at 260 nm. Three replicate RNA extractions for each tank were combined in order to have enough RNA for analysis.

2.3. Microarray analysis

DNA microarrays including the probes listed in Table 1 were fabricated as previously described (Urakawa et al., 2002). Each gel pad contained 3 pmol of probe and each probe was immobilized within two gel pads on each microarray. RNAs from wastewater samples and reference samples were fragmented and labeled by the hydroxyl radical method (Kelly et al., 2002) using the following reactant concentrations: 5 mM o-phenanthroline hydrochloride monohydrate, 500 μM $CuSO_4$, 1 mM lissamine-rhodamine B ethylenediamine (Molecular Probes, Inc., Eugene, OR), 4 mM H_2O_2, 20 mM sodium phosphate, and 20 mM $NaCNBH_3$. After fragmentation and labeling the RNA concentration was determined by spectrophotometric analysis at 260 nm. Microarray hybridization was carried out with 5–10 μg-labeled RNA as previously described (El Fantroussi et al., 2003). After 12–16 h hybridization in the dark at room temperature (20 °C), microarrays were washed twice with room-temperature washing buffer (20 mM Tris-HCl [pH 8.0], 5 mM EDTA, 4 mM NaCl). Analyses of images and thermal dissociation curves were carried out as previously described (Urakawa et al., 2002). Signal intensities were compared by ANOVA using SAS 6.11 (SAS Institute Inc., Cary, NC).

2.4. Membrane hybridization

RNA samples from AT1 and AT2 were analyzed by membrane hybridization using the following probes: S-*-Univ-0907-a-A-22 (UNIV907), S-G-Nso-1225-a-A-20 (NSO1225), S-G-Nsom-0653-a-A-18 (NEU), S-G-Nso-0443-a-A-19 (NSV443), and S-G-Nbac-1000-a-A-15 (NBAC1000), as well as two additional probes targeting nitrite oxidizers: S-G-Nit-1035-a-A-18 (NIT3) which targets the *Nitrobacter* genus (Wagner et al., 1996) and S-G-Ntspa-0662-a-A-18 (NTSPA662) which targets the *Nitrospira* genus (Daims et al., 2001). UNIV907 was used as the universal probe for the membrane hybridization instead of the more commonly used S-*-Univ-1390-a-A-18 (UNIV1390) because previous microarray analysis (data not shown) suggested that there was some degradation of the UNIV1390 target in the

environmental samples. RNAs were applied to nylon membranes (Magna Charge nylon membrane, Micron Separation Inc., Westboro, MS), and hybridization with radiolabeled probes was performed as described previously (Zheng et al., 1996). The retained 32P-labeled probe was quantified using the Cyclone Storage Phosphor System (Packard Instrument Co., Meriden, CT) and analyzed with the OptiQuant software package (Packard Instrument Co.).

2.5. T-RFLP Analysis

DNA was isolated from sludge samples with the UltraClean Soil DNA Kit (MoBio Laboratories, Inc.). A nested PCR strategy was used for amplification of 16S rRNA genes from AOB and NOB (Regan et al., 2002). The initial PCR was performed using the universal primers 11F (5′-GTTTGATCCTGGCT-3′) and 1492R (5′-TACCTTGTTACGACTT-3′), followed by specific amplification of AOB and NOB 16S rRNA genes using EUB338 (5′-GCTGCCTCCCGTAGGAGT-3′) as a forward primer and the following reverse primers: NSO1225R (specific for β-proteobacterial AOB; Mobarry et al., 1996), NIT3R (specific for *Nitrobacter*; Wagner et al., 1996), and NTSPA685R (specific for *Nitrospira*; Hovanec et al., 1998). All primers were synthesized by Qiagen, Inc. (Valencia, CA), and EUB338 included 6-FAM attached to the 5′ end. The products of each PCR reaction were purified by Qiaquick PCR purification kit (Qiagen) and digested with *MspI* restriction enzyme (New England Biolabs Inc.) according to the manufacturer's protocol. Restriction digests were analyzed using a 3100 Capillary DNA Sequencer/Genotyper (Applied Biosystems), and fragment sizes were determined using GeneScan software version 3.5.2. The fragment sizes were compared to expected fragments derived from TAP T-RFLP analysis (Ribosomal Database Project version 8.0; Cole et al., 2003).

3. Results

The reproducibility of microarray hybridization was assessed using triplicate hybridizations of in vitro-transcribed RNA from *N. briensis* and *N. eutropha*. ANOVA analysis indicated that there were no significant differences in signal intensities at room temperature between replicate hybridizations, replicate microarrays, or for identical probes immobilized at different locations on the same microarray.

When the microarrays were hybridized with in vitro-transcribed RNA from *N. briensis* and *N. eutropha* and then washed and imaged at room temperature, probe-target duplexes with 2 or more mismatches showed average signal intensities 78% lower than signal intensities for perfect match duplexes (significant at $p<0.001$). However, for this set of probes, average signal intensities for probe-target duplexes with a single mismatch showed no significant difference from perfect match probe-target duplexes. For example, after room temperature hybridization and washing, *N. briensis* and *N. eutropha* showed significantly different signals for NSV443 (0 and 5 mismatches, respectively), but there were no significant differences in signals for S-G-Nso-0190-a-A-19 (NSO190) (0 and 1 mismatches) or for NEU (1 and 0 mismatches) (Fig. 1). However, as previously documented (Urakawa et al., 2002, 2003) the determination of dissociation kinetics for each probe-duplex provides additional data for the duplex structures retained on each array element. Thus, in order to resolve single and double mismatch duplex structures, we examined the dissociation of target from all probes on the microarray using in vitro-transcribed RNA from *N. eutropha* and *N. briensis*. For individual 16S rRNA target sequences, the melting profiles and experimentally determined T_ds were highly reproducible, showing very low standard deviations (Fig. 2a and Table 2). When *N. eutropha* and *N. briensis* rRNAs were compared, UNIV1390, which is a perfect match for both rRNA targets, resulted in identical melting profiles (data not shown), as was also reflected by indistinguishable mean T_d values (Table 2). Melting profiles for NEU hybridized to *N. eutropha* (0 mismatches) and to *N. briensis* (1 mismatch) were well resolved (Fig. 2b), having significantly different T_ds (mean difference of 4.8 °C, $p<0.001$) (Table 2). These data confirmed previously

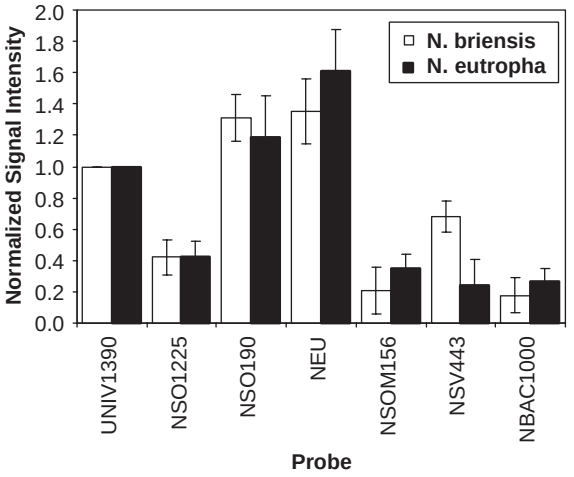

Fig. 1. Signal intensities at room temperature for hybridizations of DNA microarray with in vitro-transcribed RNA from *Nitrosospira briensis* and *Nitrosomonas eutropha*. Probe signals were normalized by dividing by the signal of UNIV1390. Data represent mean values ($n=6$), and error bars reflect the standard deviations for each mean.

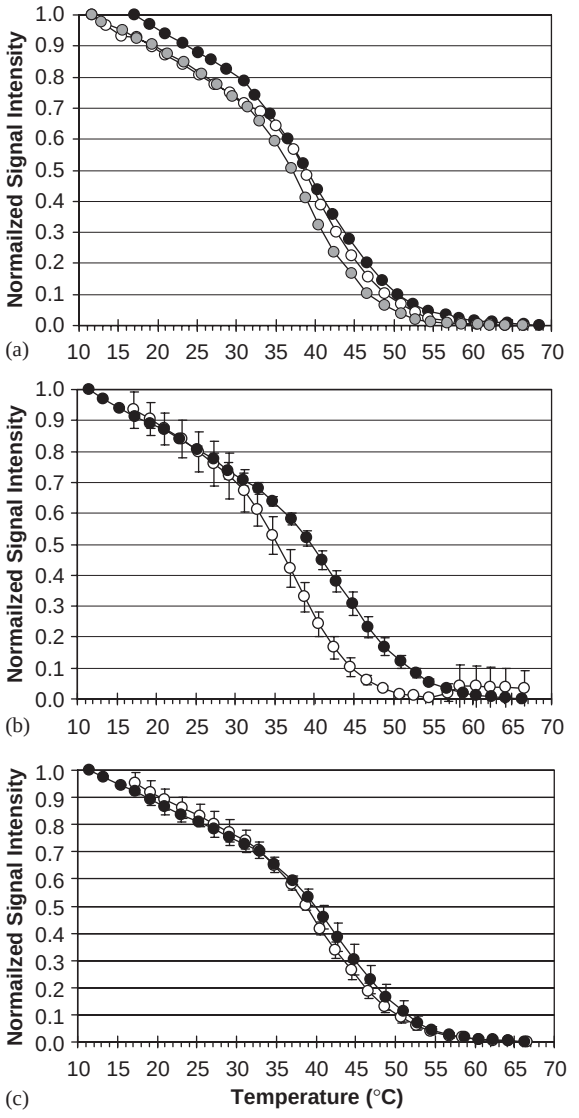

Fig. 2. Microarray melting profiles for probe NSO190 hybridized with in vitro-transcribed RNA from *Nitrosospira briensis* (a); Microarray melting profiles for probes NEU (b), and NSO190 (c) hybridized with in vitro-transcribed RNA from *Nitrosomonas eutropha* (●) and *Nitrosospira briensis* (○). Data points represent mean values ($n = 3$), and error bars reflect the standard deviations for each mean.

published model studies showing that a single base mismatch could be discriminated on the microarray (Urakawa et al., 2003). However, melting profiles and T_d values showed no significant differences for NSO190 when hybridized to *N. briensis* (0 mismatches) and *N. eutropha* (1 mismatch) (Fig. 2c and Table 2). Thus, in the case of NSO190, a single base mismatch was not discriminated on the microarray. Although NEU and NSO190 have similar lengths (18 and 19 bases, respectively) and G+C contents (61% and 58%, respectively), the position and type of mismatch differ. While the mismatch between NEU and *N. briensis* is a U/G (target/probe) mismatch located 8 bases from the 3′ terminus of the probe, the mismatch between NSO190 and *N. eutropha* is a G/T (target/probe) mismatch located 5 bases from the 3′ terminus of the probe.

Operating data for the activated sludge plant are summarized in Table 3. For the 30 days prior to sampling, the average chemical oxygen demand (COD) removal efficiency for the plant was 95%, while the average ammonium-removal efficiency was 99.7%. For AT1, the average dissolved oxygen (DO) for the 30 days prior to sampling was 3.5 mg/L, and the average pH was 8.2. For AT2 during this period the average DO was 3.3 mg/L and the average pH was 8.2. Thus, the activated sludge plant had effective ammonium removal, and the pH and DO values for both tanks were within the range for growth of nitrifying bacteria (Rittmann and McCarty, 2001).

After room temperature hybridization and washing, RNA from AT1 and AT2 showed signals for NEU (Fig. 3), which targets halophilic and halotolerant *Nitrosomonas* (Wagner et al., 1995). Melting-profile analysis was used to validate the signal observed for NEU in the activated sludge samples. Melting profiles for NEU for AT1 and AT2 matched the curve for *N. eutropha* (0 mismatches) (Fig. 4) with no significant difference in experimentally determined T_ds among AT1, AT2, and *N. eutropha* (40.4, 40.1, and 40.8 °C, respectively, $p > 0.05$). This result indicated that samples collected from AT1 and AT2 contained RNA that was a perfect match for NEU. RNA from tanks AT1 and AT2 also showed hybridization to NSO190 (Fig. 3), which targets many β-proteobacterial AOB (Mobarry et al., 1996). Dissociation analysis demonstrated that the melting curves for NSO190 for AT1 and AT2 matched those of *N. eutropha* and *N. briensis* (data not shown), with no significant difference in experimentally determined T_ds among AT1, AT2, *N. eutropha*, and *N. briensis* (41.8, 40.9, 41.1, and 39.7 °C, respectively, $p > 0.05$). As was discussed above, melting profile analysis was not able to achieve single base mismatch discrimination when NSO190 was hybridized with reference RNAs, so this analysis alone could not ensure that any of the rRNA recovered from AT1 and AT2 contained a perfect match with probe NSO190.

The microarray analysis of AT1 and AT2 samples was compared to two independent analyses—membrane hybridization and terminal restriction fragment length polymorphism fingerprinting (T-RFLP). Membrane hybridization showed signals for AOB (NSO1225, NEU, NSV443) and NOB (NIT3 and NTSPOA 662), but no signal for NBAC100 (Fig. 5). Membrane hybridization indicated that NEU targets represented approximately 2% of the total microbial communities in

Table 2
Experimentally determined T_ds (°C) for three replicate hybridizations of in vitro-transcribed 16S rRNA from *Nitrosospira briensis* and *Nitrosomonas eutropha*

Probe	*Nitrosospira briensis*				
	A	B	C	Mean	Std Dev
NSO190	39.4	39.7	40.1	39.7	0.4
NSV443	40.6	39.9	35.2	38.6	2.9
NEU	36.5	35.6	35.7	36.0	0.5
UNIV1390	40.0	39.9	38.4	39.5	0.9
	Nitrosomonas eutropha				
NSO190	40.8	42.3	40.3	41.1	1.1
NSV443	nd*	nd	nd	nd	nd
NEU	40.1	40.8	41.4	40.8	0.7
UNIV1390	41.0	37.5	41.1	39.8	2.1

**N. eutropha* contains 5 mismatches for probe NSO443. There was not a significant hybridization signal for this probe/target, so a T_d value was not determined.

Table 3
Operating data for the biological treatment stages of the BCP wastewater treatment plant. Each data point represents the average of daily measurements taken over the 30 days immediately prior to sample collection

Total flow (m^3/day)	1238
Solids retention time (days)	41
Mixed liquor volatile suspended solids (mg/L)	6000
T1 dissolved oxygen (mg/L)	3.3
T2 dissolved oxygen (mg/L)	3.5
Mixed liquor pH	8.2
Sludge recycle ratio (%)	220
Influent COD (mg/L)	1700
Effluent COD (mg/L)	84.5
Influent ammonium (mg-N/L)	58.1
Effluent ammonium (mg-N/L)	0.2

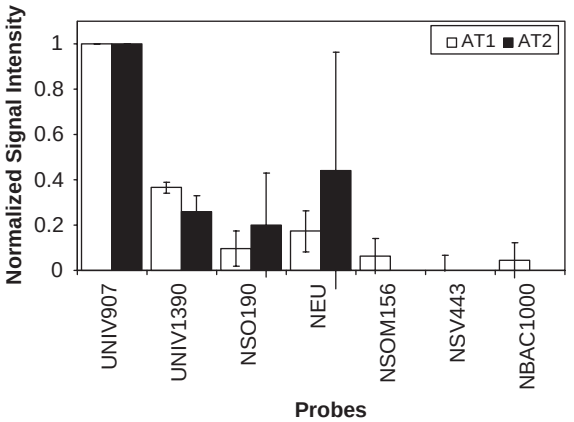

Fig. 3. Signal intensities for microarray hybridizations of RNA extracted from samples collected from the first-stage aeration tank (AT1) and the second-stage aeration tank (AT2) of the BCP activated sludge plant. The signal for each probe was normalized by dividing by the signal of probe UNIV907. The microarray used in this experiment did not include probe NSO1225. Data represent mean values ($n = 6$), and error bars reflect the standard deviations for each mean.

AT1 and AT2 (Fig. 5). A terminal digestion fragment of 166 bp (Fig. 6a and b), corresponding to a fragment common to the *Nitrosomonas europaea* and *Nitrosomonas marina* lineages (Groups 1 and 4 as defined by Koops and Pommerening-Röser, 2001), was identified in both AT1 and AT2 reactors using the NSO1225 probe as a primer for T-RFLP analysis. Both reactor systems yielded a peak at 141 bp (Fig. 6c and d) using the *Nitrobacter*-specific primer NIT3, and a 277 bp fragment (Fig. 6e and f) common to several *Nitrospira*-like sequences was identified using NTSPA685 as the reverse primer.

4. Discussion

Testing of this DNA microarray demonstrated that single base-pair mismatch discrimination could be achieved for NEU and full-length rRNA targets by inclusion of a dissociation analysis to characterize the duplex structure(s) formed on each array element. These results confirmed our group's earlier work using short (38 nucleotide) synthetic RNA targets to demonstrate single base mismatch discrimination for NSOM653 (identical to NEU) (Urakawa et al., 2002, 2003). Although this method of analysis should improve interpretation of microarray data, as compared to analyses that rely on one set of hybridization and wash conditions for an entire array of probes, we note that

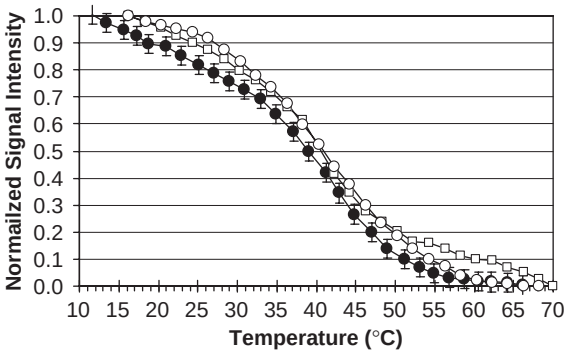

Fig. 4. Melting profiles for probe NEU hybridized with in vitro-transcribed RNA from *Nitrosomonas eutropha* (●) and native RNA from tank AT1 (○) and AT2 (□).

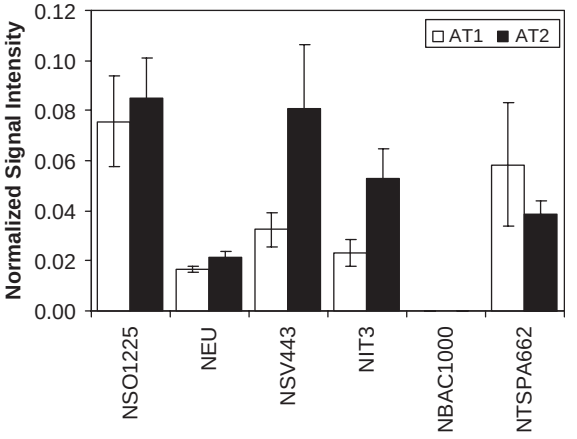

Fig. 5. Signal intensities for membrane hybridization of RNA extracted from samples collected from the first-stage aeration tank (AT1) and the second-stage aeration tank (AT2) of the BCP activated sludge plant. The signal for each probe is expressed as the fraction of target 16S rRNA relative to total rRNA quantified using the UNIV907 probe. Data represent mean values ($n = 3$), and error bars reflect the standard deviations for each mean.

there are instances in which single base pair differences are not resolved by comparative dissociation analysis. For example, this study could not discriminate a single base mismatch with the NSO190 probe. The extensive dissociation dataset developed for different mismatch composition duplex structures developed by Urakawa et al. (2003) offers general guidelines for predicting the relative destabilization by single mismatches within short duplexes. In particular, near terminal mismatches contributed to the greatest loss of discrimination. Thus, the localization of the mismatched nucleotide relative to the nearest terminus for the NEU and NSO190 probes (5 versus 8 nucleotides, respectively), each having similar lengths and G + C content (18 and 19 bases, and 61% and 58%, respectively) may have contributed to differing discrimination. However, mismatch composition also contributes to relative stability, and the U/G (target/probe) mismatch between NEU and *N. briensis* rRNA may be more destabilizing than the G/T mismatch for *N. eutropha*. We anticipate that as a more complete understanding of mismatch composition and position contributions to destabilization is achieved, probes will be redesigned to provide greater discrimination.

Hybridization of the wastewater treatment plant samples demonstrated that RNA from nitrifying bacteria could be detected in activated sludge samples by extraction and direct hybridization to a DNA microarray without the need for PCR amplification (Fig. 3). The microarray showed significant hybridization signals for NEU (most halophilic and halotolerant *Nitrosomonas* spp.) which represented approximately 2% of the total microbial communities in AT1 and AT2 (Fig. 5). The specificity of detection of NEU targets in the activated sludge samples was confirmed by melting profile analysis on the microarray. The microarray detection of *Nitrosomonas* in AT1 and AT2 was also confirmed by membrane hybridization with NEU (Fig. 5) and by T-RFLP analysis with AOB-specific primers (Fig. 6a and b). The detection of *Nitrosomonas* in the activated sludge samples agrees with previous studies which have detected *Nitrosomonas* in sewage treatment plants using NEU (Wagner et al., 1995, 1996).

Although our microarray detected hybridization to the NEU and NSO190 probes, it did not detect NOB with the NBAC1000 probe (Fig. 3), which targets *Nitrobacter* spp. (Mobarry et al., 1996). This agrees with previous studies that also failed to detect *Nitrobacter* in wastewater treatment systems using 16S rRNA targeted probes (Juretschko et al., 1998; Wagner et al., 1996). Although the NBAC1000 target could not be detected by membrane hybridization, hybridization was observed for two probes for which signal was below detection using the microarray format, the *Nitrospira*- and *Nitrobacter*-specific probes NTSPA662 and NIT3 (Fig. 5). These populations were also detected by T-RFLP analysis using NOB-specific primers (Fig. 6).

The detection of *Nitrospira* in this system is consistent with recent results showing that *Nitrospira* spp. are commonly abundant in activated sludge (Juretschko et al., 1998). However, the detection of *Nitrobacter* with NIT3 was surprising because other researchers have not detected *Nitrobacter* with NIT3 in wastewater treatments systems, despite the fact that it was possible to culture *Nitrobacter* from these systems (Juretschko et al., 1998; Wagner et al., 1996). In addition to possible system-specific differences, variation between our results and the more common failure to detect *Nitrobacter* spp.

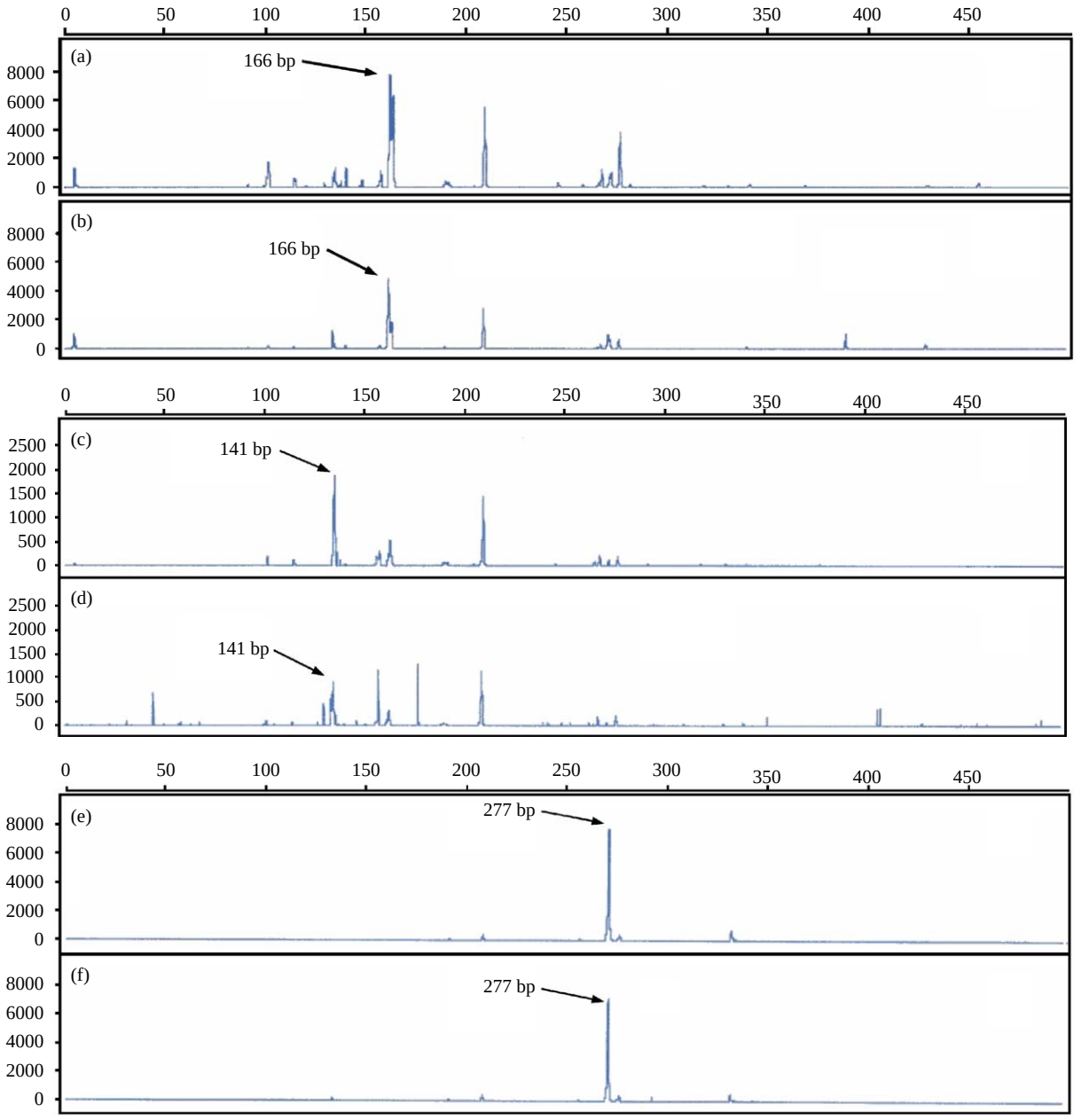

Fig. 6. T-RFLP profiles for AT1 (a, c, and e) and AT2 (b, d, f) run with primers 338F and the following reverse primers: NSO1225 (a and b), NIT3 (c and d), and NTSPA685 (e and f). Sizes of significant peaks are indicated.

may be a result of using membrane hybridization and T-RFLP, rather than the more commonly employed fluorescent in situ hybridization (FISH) method.

5. Conclusions

These analyses have demonstrated the utility of this DNA microarray format for detecting nitrifying bacteria in wastewater treatment systems without target amplification, as is commonly accomplished using PCR amplification. In addition, the inclusion of a dissociation analysis should serve for improved discrimination between target and non-target hybridization events. Although dissociation analysis using this microarray system in its current form of implementation does not discriminate between all single mismatch variants, we anticipate that discrimination can be improved via

optimization of probe design to bias it towards more general discrimination. These features, together with the well recognized advantages of high probe capacity, suggest that DNA microarrays should become useful tools for determining presence/absence of specific clades of nitrifiers in wastewater treatment systems. The linking of this type of microarray data to long term operational data should help to improve our understanding of niche differentiation among the nitrifier clades. The necessary next steps include an increase in the scale of the microarray (i.e. increase in number of probes) as well as longer term sampling.

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