Journal of Experimental Microbiology and Immunology (JEMI) Vol. 4:66-73

Comparisons and Analyses of Probe Temperature Elution Profiles of *Nitrospira*

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It was recently discovered in sequencing batch reactors (SBR) that *Nitrospira*, which is the dominant nitrifying bacteria in bioreactors, cannot be detected in cultures where the activity of nitrite oxidation is high. In order to investigate this problem, RNA was isolated from the SBR samples collected on different dates. The isolated RNA was immobilized onto a nylon membrane by slot blotting, and then radioactively labelled with Ntspa 454 and Ntspa 685 probes. The probe elution profiles of each of the samples were characterized by washing the individual bands at a series of increasing wash temperatures. It was found that the dissociation temperature (T_d) for the SBR samples were lower than the pure culture *Nitrospira moscoviensis* RNA. This difference in T_d explains why *Nitrospira* could not be detected despite the high nitrifying activities in the SBR. The probes were washed at the theoretical T_d, a higher temperature than the actual T_d of the bioreactor samples, which causes most of the probes to elute off, leaving only trace amounts of probe bound on the membrane. The minimal amounts of bound probe left on the membrane create the misconception that *Nitrospira* is not present. Comparing the autoradiogram and the elution profiles of the two probes, it is suspected that the dominant organism present in the bioreactor is *Nitrospira marina*. However, it is also possible that the probes are binding to novel, modified strains of *Nitrospira*. Further research must be performed to determine the dominant bacteria present in the bioreactors.

Lithoautotrophic nitrification is the main aerobic process in microbial nitrogen cycling (15) that involves a two-step nitrification process. The process begins with the conversion of ammonia (NH_4^+) to nitrite (NO_2^-) (ammonia oxidation), and is followed by the conversion of nitrite (NO_2^-) to nitrate (NO_3^-) (nitrite oxidation) (5, 15). It was discovered that the rate of nitrite oxidation is as fast as or faster than the rate of ammonia oxidation (15).

Many autotrophic and heterotrophic bacteria are involved in this nitrification process. The four known types of nitrite-oxidizing bacteria are *Nitrobacter*, *Nitrococcus*, *Nitrospina*, and *Nitrospira* (3,8). In wastewater systems, *Nitrosomonas* is the primary organism responsible for ammonia oxidation (2), and *Nitrospira* is the primary organism responsible for nitrite oxidation (2,6,12,15). *Nitrospira* uses nitrite as sole energy source, and grows well in about 0.35mM of nitrite (4). Even though *Nitrospira* are the primary nitrifying organisms, they have a low affinity for substrates and they grow at a slow rate, doubling in numbers every 12 to 32 hours (4). Two cultivated species of *Nitrospira* are *Nitrospira marina*, a strain isolated from ocean water, and *Nitrospira moscoviensis*, a strain isolated from an iron pipe of a heating system in Moscow, Russia (4).

Previous studies have shown that *Nitrospira* cannot be detected by the oligonucleotide probes Ntspa 454 and Ntspa 685 in sequencing batch reactor (SBR) cultures that are rapidly converting nitrite to nitrate (16). The nitrifying activities measured in the bioreactors do not correspond to the presence of *Nitrospira*. The nitrifying activity can be detected by measuring the amount of nitrate produced and the presence of *Nitrospira* bacteria can be detected by probing their 16S RNA with specific oligonucleotide probes. Since the probes are specific for the bacteria, they should bind if the bacteria are present. The detection of nitrite conversion activity indicates the presence of nitrifying bacteria. However, it is possible that the bacteria could not be detected because of DNA contamination (13) or because the probes were not binding properly to the RNA. The cause for such an observation could possibly be mismatches between the DNA probe and the RNA sequences due to nucleotide sequence differences. If the nucleotide sequence does not completely match the probe, the probe-RNA hybrid will dissociate at a lower temperature.

Such mismatches may be due to the fact that *Nitrospira* in bioreactors behave differently from the pure *Nitrospira* cultures; therefore, the dissociation temperature at which 50% of the bound probe is released from the hybrid (10) would be different. The difference could be the cause of the low detection of the presence of *Nitrospira* in the bioreactor. Other causes for mismatches could be the presence of other dominant nitrifying organisms, or novel, modified forms of *Nitrospira*.

This study attempts to investigate the problem by indirect radioactive labelling of the RNA which was isolated from the SBR culture samples collected on different dates. The radioactive probe bound membranes were then washed at different temperatures in order to characterize a probe elution profile and to determine the temperature of dissociation (T_d) for the various samples.

MATERIALS AND METHODS

Nucleic acid extraction and quantification. RNA was extracted from frozen bacterial samples collected from the sequencing batch reactor 3 (SBR3) from the UBC Civil Engineering Department. Samples were taken on March 15, March 24, April 18, April 26 and May 27 during the year of 2003 and were stored at -80°C. Pure *Nitrospira moscoviensi*s culture was used as a positive control. Bacterial cells of approximate volume of 2.5 mL were thawed on ice, and 1 mL of Trizol™ reagent (GIBCO BRL) was added to it to disrupt the cells and protect the released RNA from degradation. For every milliliter of Trizol, 0.2mL of chloroform was added to the cell pellet for phase separation, and 0.1 mm diameter glass beads (SIGMA) were added to the vial until it was full. The cells were lysed by mechanically disrupting the cells using a Beadbeater (Minibeadbeater, Biospec Products) at 4800rpm for a total of 3 minutes, cooling the mixture on ice after every minute. The mechanical disruption was then followed by a 10 minute cooling at room temperature, and centrifugation at 14000 rpm for 15 minutes.

After centrifugation, two layers formed: a clear aqueous top layer, and a pink organic lower layer. The aqueous layer, which contains the RNA, was removed and precipitated with isopropanol. The mixture was incubated at room temperature for 10 minutes, and the RNA was pelleted by centrifugation. The RNA pellet was washed with 75% ethanol, and then resuspended and dissolved in 100μL of diethylpyrocarbonate (DEPC) treated water to minimize the amount of contaminating ribonuclease. RNA was then quantified by measuring the absorbance at 260nm and 280nm (*A*260 and *A*280) using the relationship [RNA]=0.063(*A*260)-0.036(*A*280) (11). The isolated RNA was then stored at -80°C until it was used.

The purity and integrity of the RNA samples were verified by gel electrophoresis. The apparatus was DEPC treated to ensure that it was ribonuclease free. The samples were run on a 1.2% agarose gel at 100V for 1 hour.

Slot Blotting and Probe Hybridization. The isolated RNA was immobilized onto a nylon membrane (Zeta-probe, Bio-Rad) by slot blotting (7, 17). One microgram of RNA was added to each of the slots in the slot blot apparatus (Bio-Dot SF Microfiltration apparatus, Bio-Rad). After blotting the RNA onto the membrane according to the manufacturer's protocol, the membrane was baked at 80°C for 30 minutes and stored between two filter papers in a plastic bag until it was used.

The probes used for this experiment were prepared by the UBC Nucleic Acid and Protein Synthesizing Unit (NAPS). Two probes were chosen for hybridization with the isolated RNA: S-G-Ntspa-0685-a-A-22 and S-*-Ntspa-0454-a-A-19. As shown in Table 1, Ntspa 685 probe targets the aquarium clone 710-9, *N. marina* and *N. moscoviensis*, while the Ntspa 454 is more specific and targets the aquarium clone 710-9 and *N. moscoviensis* only (6). The probes were labelled at the 5' end with γ-^{32}P-ATP (10mCi/mL) using the KinaseMax™ kit (Ambion) according to the manufacturer's protocol.

Prior to the hybridization, the nylon membranes with immobilized RNA were placed in hybridization tubes and pre-warmed in the hybridization buffer (0.9 M NaCl, 50 mM NaPO4, 5mM EDTA, 10X Denhardt Solution, 0.5 mg/mL poly(A)) at 40°C for 2 hours (9). A total of 20,000,000 counts of γ-^{32}P-ATP labelled probes were then added to the tubes for the hybridization. The nylon membranes were hybridized overnight with the radioactive probes at 40°C in hybridization buffer. After hybridization, the membranes were washed twice at 40°C for 30 minutes with wash buffer (1x SSC (0.15M NaCl, 0.015M sodium citrate) with 1% sodium dodecyl sulfate (SDS)), and exposed to x-ray film overnight (16).

Table 1. Oligonucleotide probe sequences and positions for *Nitrospira*

Probe	Position	Target (5' to 3')	T_d (°C)	Wash T (°C)	Specificity	Reference
S-G-Ntspa-0685-a-A-22	664 – 454	CAC CGG GAA TTC CGC GCT CCT C	63.0	60.0	*N. moscoviensis*, *N. marina*, and 710-9 clone	1, 6
S-*-Ntspa-0454-a-A-19	435 – 454	TCC ATC TTC CCT CCC GAA AA	58.5	56.0	*N. moscoviensis* and 710-9 clone	6

Determination of Dissociation Temperature

The T_d of the oligonucleotide probes were then determined by measuring the amounts of probe eluted over a series of increasing wash temperatures ranging from 40°C to 80°C (7). Individual bands identified from the autoradiogram corresponding to the slots in the slot blot apparatus were cut out from the membrane and placed in 0.6 mL Eppendorf tubes. To each of the bands, 500μL of wash buffer was added. The tubes were then placed in a thermal cycler and heated at the wash temperature for 35 minutes. After the incubation period, the wash solutions were removed from the Eppendorf tubes and placed in scintillation vials. New pre-warmed buffer was added and the tubes were incubated at the new temperature. These steps were repeated until the final temperature in the series was reached. The Cherenkov radioactivity in the wash buffers was then measured by the scintillation counter.

RESULTS

Gel Electrophoresis. Two distinct bands corresponding to the 23S and 16S prokaryotic rRNA were detected by gel electrophoresis (data not shown). The overall quality of the RNA was good and the degree of RNA degradation was too small to considerably affect the results.

Autoradiogram. The x-ray films were exposed to the radioactive probes that were bound to the RNA immobilized on the nylon membranes. The intensities of the bands correspond to the amount of probe bound to the RNA. Both Ntspa 454 and Ntspa 685 were well bound to the pure culture *N. moscoviensis* RNA. It was found that there were more Ntspa 685 probes bound to the samples than there were Ntspa 454 probes (Fig. 1). When probed with Ntspa 454, the intensities of the bands for the samples were low, even though the intensity for the pure culture was high. The intensities of the bands for each of the replicates were consistent, meaning that the same amounts of probes were bound onto the membrane.

There appears to be a trend in the intensity of the bands when the samples were probed against Ntspa 685 as seen in Fig. 2b. The intensity of the bands increased from March 15 to April 18, decreased on April 26, and then increased again where the intensity was highest on May 27. This suggests that the amount of *Nitrospira* increases at first, then slightly decreases, and then increases to its peak. This trend could not be confirmed with the Ntspa 454 probe because fewer probes were bound to the RNA immobilized on the membrane. When probed against Ntspa 454, the bands (row 2, Fig.1a) corresponding to the March 24 sample, were more intense than the other bands. This suggests that the amount of *Nitrospira* was perhaps highest on March 24.

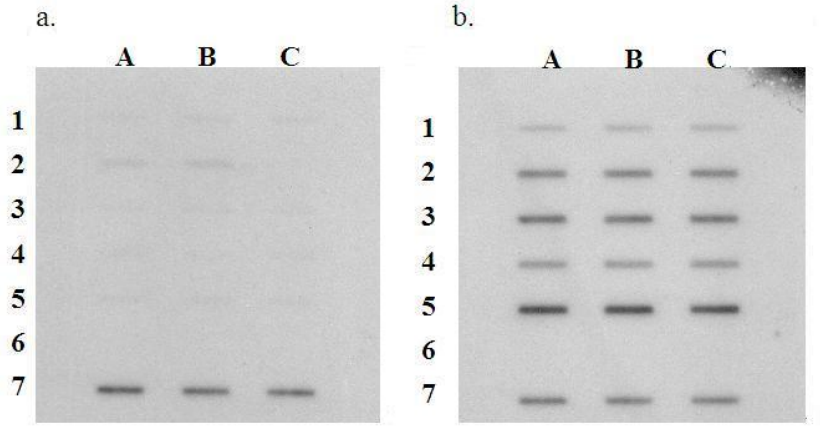

Figure 1. Autoradiogram of hybridization with (a) Ntspa 454 probe and (b) Ntspa 685 probe. For both autoradiograms, row 1 to 5 corresponds to the March 15, March 24, April 18, April 26 and May 27 bioreactor RNA samples. Row 6 corresponds to the blank and row 7 corresponds to the pure *Nitrospira* sample. Columns A to C are replicates of each of the samples.

Comparison of Temperature Curves. The probe elution curves for the Ntspa 685 and Ntspa 454 probes were generated based on the Cherenkov counts of the samples since the wash buffers were first counted without the addition of scintillation fluid. Since some of the counts were quite close to the background counts, scintillation fluid was added to the first set of samples (March 15) to determine if the addition of the scintillation fluid would significantly improve the results. Fig. 2 compares the temperature wash curves of the March 15 sample with and without scintillation fluid added. The shapes of the two temperature wash curves are very similar, even though the actual amount released varies slightly. Therefore, Cherenkov counts were used for the rest of the analysis.

A background count of 19.8 counts per minute (cpm) was subtracted from each of the individual counts. For both the Ntspa 685 and the Ntspa 454 probes, the probe release curves of the SBR samples behaved somewhat differently than those of the pure *Nitrospira moscoviensis* culture. The Cherenkov counts and the scintillation counts between replicates were similar. The standard deviations of the data are shown by the error bars in the temperature wash curves (Fig. 3).

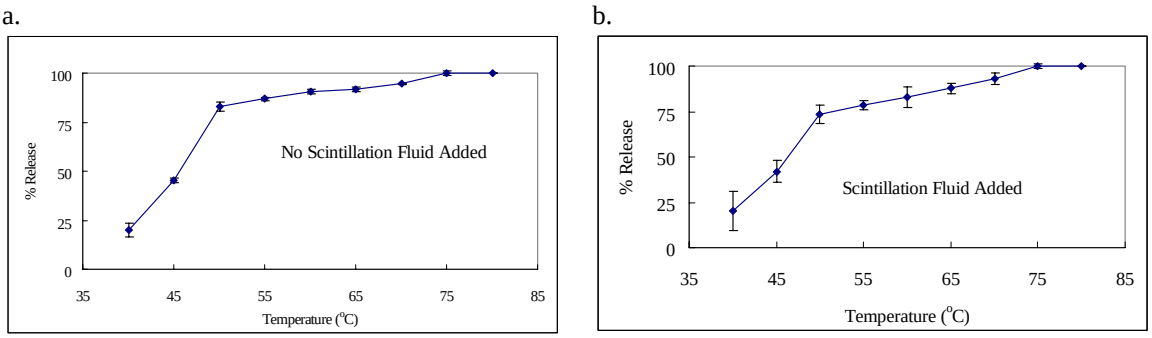

Figure 2. Temperature wash curves of the March 15 Sample (a) without scintillation fluid and (b) with scintillation fluid for the Ntspa 685 probe.

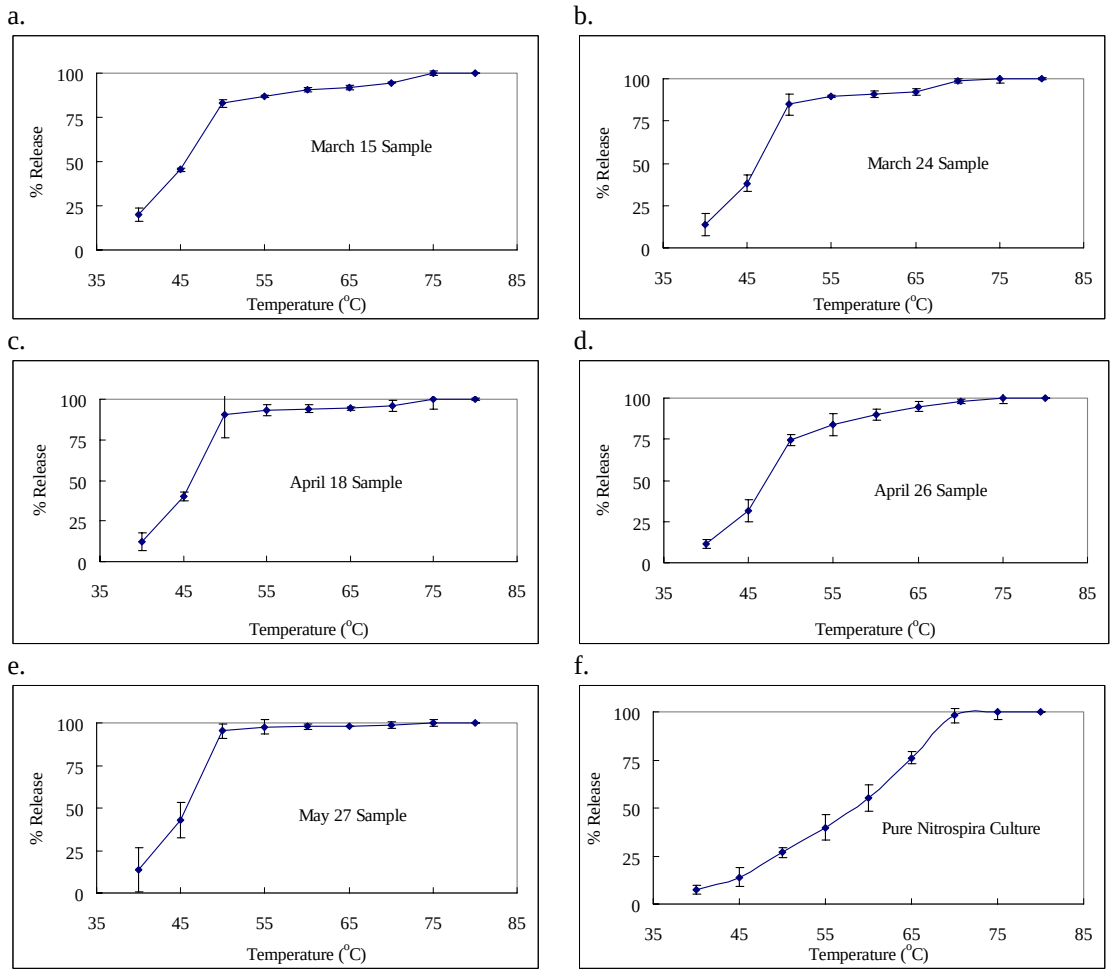

Figure 3. Temperature wash curves for the Ntspa 685 probe. (a) March 15 Sample (b) March 24 Sample (c) April 18 Sample (d) April 26 Sample (e) May 27 Sample (f) pure *Nitrospira* culture.
The error bars in the curves correspond to the standard deviations of the Cherenkov counts of the replicates.

For the Ntspa 685 probe, all of the release curves for the SBR samples look similar to each other. Most of the probes were washed off at temperatures between 40°C to 50°C, as shown by the steep slopes in Fig. 3. The release curve for the pure *Nitrospira* agrees with literature (6); however, the T_d of the experimental results appears to be lower than that described by Hovanec *et al.* as shown in Table 2. Furthermore, the T_d obtained for the SBR samples were lower than the pure *N. moscoviensis* RNA in the experiment. Theoretically, the release curve should start off flat, then rises with an increasing slope until it plateaus off at approximately 70°C (6). The T_d of Ntspa 685 with the pure *Nitrospira* culture release curve should be approximately 63°C. The pure *Nitrospira* curve obtained from the experiment was very close to what was expected. The probe started washing off gradually at 40°C, and the probe release continued to increase until 70°C and then levelled off. However, the elution curve of the pure *Nitrospira* culture appears to be more smooth and sigmoidal than the curve published by Hovanec *et al.* (6).

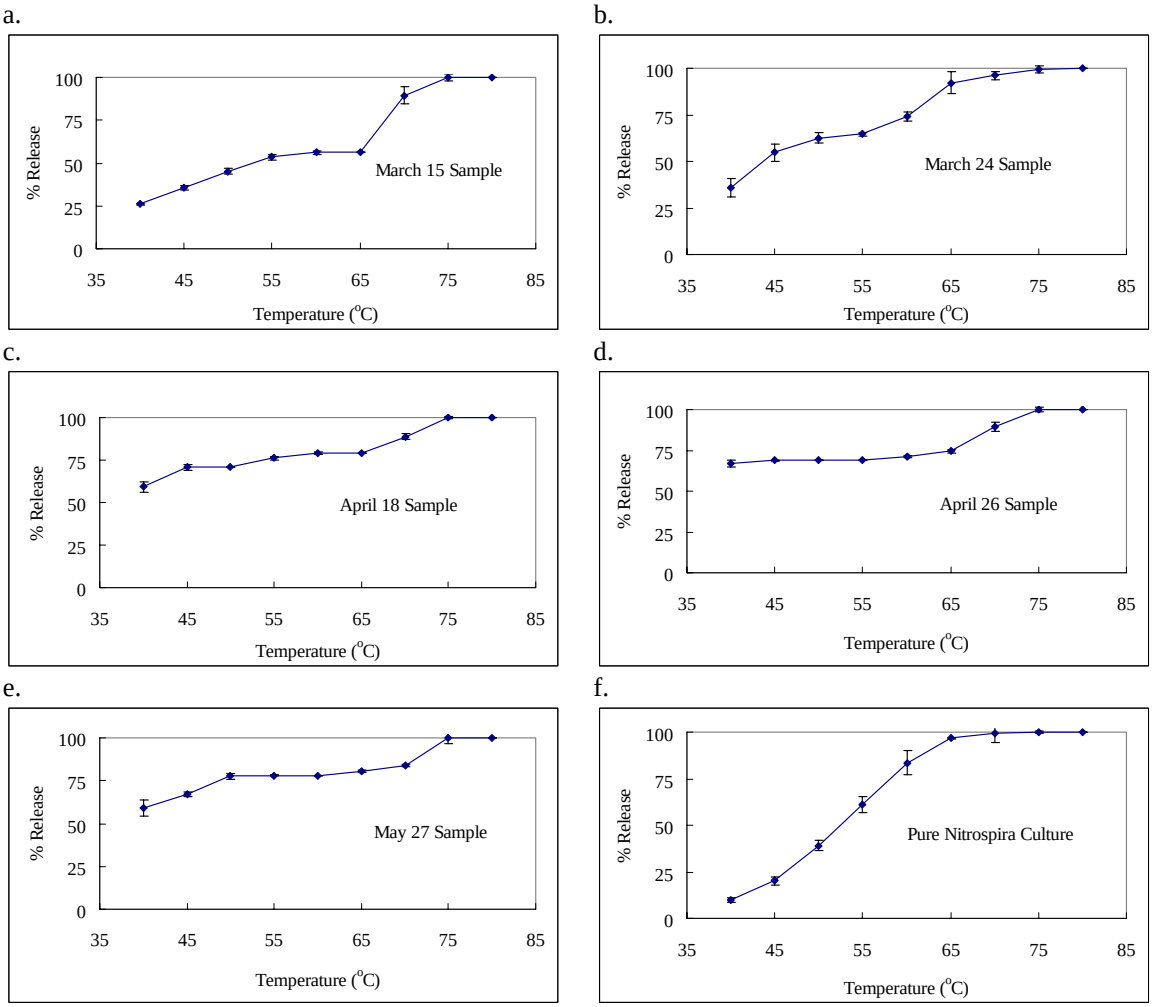

Figure 4. Temperature wash curves for the Ntspa 454 probe. (a) March 15 Sample (b) March 24 Sample (c) April 18 Sample (d) April 26 Sample (e) May 27 Sample (f) pure *Nitrospira* culture. The error bars in the curves correspond to the standard deviations of the scintillation counts of the replicates.

For the Ntspa 454 probe, the release curves for each of the environmental samples were slightly different. It appears that there are two sigmoidal curves present. For the March 15 sample, the probes washed off gradually over a range of 40°C to 55°C, remained on the sample from 55°C to 65°C, and then the remaining probes were washed off from 65°C to 75°C as shown in Fig. 4. The behaviour of the March 24 sample was similar to the March 15 sample. There appears to be two curves in this sample: one going from 40°C to 55°C and one from 55°C to 75°C.

However, the curve appears to be smoother for the March 15 sample. In both of these samples, the beginning flat region of the first sigmoidal curve cannot be observed. The April 18, April 26, and May 27 samples appear to have two curves, resembling the March 15 and March 24 curves in nature. However, more than 50% of the probes were already washed off before the beginning of the temperature wash at 40°C.

Table 2. T_d of Ntspa 454 and Ntspa 685 probes with SBR RNA samples and pure *Nitrospira* RNA samples

	Ntspa 454	**Ntspa 685**
March 15	52°C	45.5°C
March 24	44°C	46°C
April 18	---[a]	46°C
April 26	---[a]	46°C
May 27	---[a]	45.5°C
Pure *Nitrospira* culture	52.5°C	58.5°C
Literature Results[b]	58.5°C	63°C

[a] T_d cannot be determined for these samples because more than 50% of the probe has been released by 40°C.
[b] Based on the results of Hovanec *et al* (6)

The probe elution curve for the pure *Nitrospira* RNA appears to agree with the literature (6); however, the T_d of this curve is lower than that reported by Hovanec et al. as shown in table 2. Theoretically, the release curve should be sigmoidal. It should start off relatively flat, then rises with an increasing slope until it plateaus off at approximately 63°C -65°C (6). The T_d of Ntspa 454 with the pure *Nitrospira* culture release curve should be approximately 58.5°C. In the experimental result, the pure *Nitrospira* behaved similar to the expected results. However, the flat region at the beginning of the curve was not observed. The probe had already started washing off at 40°C and the probe release continued to increase until 65°C and then levelled off.

DISCUSSION

Since the same amounts of RNA were loaded onto the slot blot apparatus, the intensity of the bands from the autoradiogram in Fig. 1b should be consistent between samples. However, as shown in Fig. 1b, the intensities of the bands in rows 1-5 are not the same. This is because the RNA sample used for slot blotting is the total RNA, and not specifically *Nitrospira* RNA. Therefore, the amount of probe bound to the membrane varies according to the amount of *Nitrospira* RNA bound on the membrane.

From the autoradiogram (Fig. 1b), it is evident that different amounts of Ntspa 685 probe bind to the different SBR samples collected on different dates. This variability in probe binding was not observed for the Ntspa 454 probe because the amount of probe bound to the membrane was inadequate to allow one to observe a significant difference. The variation in band intensities indicates that the population of bacteria in the bioreactor was changing. As the ammonia oxidizing bacteria convert ammonia to nitrite, *Nitrospira* and other nitrite oxidizers increase in numbers. However, the *Nitrospira* concentration in the SBR appears to have decreased on April 26, and increased again on May 27. It is uncertain as to why *Nitrospira* would behave in this manner, and further research must be done to better understand the behaviour of *Nitrospira* in bioreactors.

Although the same amount of RNA sample was added to each of the wells for slot blotting, the amount of Ntspa 454 probes and Ntspa 685 probes bound to the same samples are different. Ntspa 454 did not bind as well to the RNA samples as shown by the faint bands in Fig. 1a. This problem is not associated with the Ntspa 454 probe itself because the probe bound well to the pure culture *Nitrospira moscoviensis* RNA.

A possible explanation for this observation is that the major nitrite oxidizing organism present in the sequencing batch reactor is the *Nitrospira marina.* As previously mentioned, Ntspa 454 binds to *N. moscoviensis* and the aquarium clone 710-9; however, Ntspa 685 binds to *N. moscoviensis*, clone 710-9, and *N. marina* (6). The amount of Ntspa 454 probe bound to the membrane is low compared to the amount of Ntspa 685 bound, which suggests that the Ntspa 685 is binding to the *N. marina* instead of *N. moscoviensis* or clone 710-9. This observation corresponds to the previous findings that the concentration of *N. moscoviensis* is relatively low in cultures that are converting nitrite to nitrate (15).

Based on the autoradiogram and the probe elution profile for the Ntspa 685 and Ntspa 454 probes (Figs. 3 & 4), it appears that the dominant organism present in the sequencing batch reactor is *Nitrospira marina*. The shape of the elution curves resembled what was expected; however, the T_d was lower than expected based on Hovanec's findings (6) using pure culture *N. marina* RNA.

The Ntspa 454 probe was able to bind well to the pure culture *N. moscoviensis* RNA. Since this probe only recognizes *N. moscoviensis* and clone 710-9 RNA, the faint bands observed for the SBR samples in the autoradiogram suggests that these two strains are present in small amounts. Since the concentrations of *N. moscoviensis* and clone 710-9 are low, this implies that the binding of Ntspa 685 to *N. moscoviensis* and clone 710-9 RNA do not have significant contributions to the intense bands observed for the Ntspa 685 probe binding activity in Fig. 1b. Therefore, the Ntspa 685 probe must be binding to *N. marina* or a novel strain of *Nitrospira* that has not yet been discovered since not all the variants of *Nitrospira* are known. Since Ntspa 685 was not probed against pure *N. marina* RNA, it cannot be proven in this study that most of the Ntspa 685 probes were binding to *N. marina*.

According to the elution curves in Fig. 4, the T_d of the Ntspa 454 probe appears to have decreased at a later bioreactor sample collection date (Table 2). This suggests that the *Nitrospira* population might have shifted. For Ntspa 454, the T_d of the March 15 sample matches the T_d of the pure *N. moscoviensis* sample (Table 3). This could mean that *N. moscoviensis* was present on March 15; however, it is also possible that the T_d coincidentally matched. The T_d of the samples after the March 15 sample tend to decrease. The April 18, 26 and May 27 had already eluted more than 50% of the probe before 40^{o}C, which is the starting point of the characterization of the elution profile. Therefore, this observation suggests that the amount of *N. moscoviensis* was slowly decreasing, and the probe was detecting another organism that poorly binds to Ntspa 454, which could possibly be *N. marina*.

According to the elution curves in Fig. 3, the T_d of the Ntspa 685 probe appears to be constant, and the shapes of the elution curves are similar. This suggests that the population is consistent. Since *N. marina* is the dominant bacteria in this situation, it is most likely that the consistent population observed was due to *N. marina*.

For Ntspa 685, the T_d of the SBR samples did not match the T_d of the pure *N. moscoviensis* sample (Table 3). It is questionable as to whether the organism Ntspa 685 detected is actually *N. marina* because the T_d does not agree with the T_d of the *N. marina* that Hovanec *et al.* found (6). Other experimental conditions were used by Hovanec (6), such that Hovanec's findings cannot be directly compared to the experimental results presented in this paper. Different organisms could be present in the cultures used for the experiment. Such differences would be affect the rRNA present, and thus would affect the ability of the probes to bind to the immobilized RNA. Further research must be performed in order to determine which organism the probes are binding to.

Nitrifying bacteria are not detected by Ntspa 454 and Ntspa 685 in cultures that are rapidly converting nitrite to nitrate because bioreactor samples behave somewhat differently from pure cultures. The T_d of the bioreactor samples are lower than the T_d of pure culture samples because the probes can bind to other organisms that have a similar sequence in the bioreactor samples. A probable reason for why *Nitrospira* cannot be detected despite the high nitrifying activity is because the probes were washed at the theoretical wash temperature based on the T_d determined for pure culture samples. As shown through this experiment, the T_d for bioreactor samples are different from the T_d of the pure culture samples. Therefore, as the probes are washed at the theoretical T_d, most of the probes would be washed off. As a result, the amount of probe bound to *Nitrospira* would be too little to be detected and would create the misconception that the nitrifying bacteria were not present.

FUTURE EXPERIMENT

The present study should be repeated with a few modifications to the experimental design. For the slot blotting procedure, 3μg of RNA should be applied to the nylon membrane. This is to ensure that there is more target RNA for the Ntspa 454 probe in order to identify a trend in band intensities such as observed for Ntspa 685. In addition, pure cultures of both *N. marina* and *N. moscoviensis* should be used as positive controls for comparison with the probe elution curve and T_d values generated from the SBR samples. The temperature for the washes should range from 30^{o}C to 80^{o}C in order to characterize the entire probe elution profile.

This experiment can be repeated using other probes in order to test the diversity of the *Nitrospira* present in the bioreactor. Less specific probes can be used in addition to Ntspa 454 and Ntspa 685 (table 3). By using the new probes, the presence of other strains of *Nitrospira* or *Nitrospira*-related species can be detected, and perhaps a new dominant species can be found. After repeating the experiment with the modifications, cloning could be performed to determine whether modified strains or novel nitrifying bacteria are present in the bacterial population.

Table 3. Suggestions of less specific *Nitrospira* probes

Probe	Specificity	Target Sequence (5' to 3')	Reference
NSR 826	*Nitrospira* spp.	GTAACCCGCCGACACTTA	14
NSR 1156	*Nitrospira* spp.	CCCGTTCTCCTGGGCAGT	14
NSR 447	*Nitrospira spp.*	GGTTTCCCGTTCCATCTT	14
S-G-Ntspa-0662-a-A-18	*Nitrospira* phylum	GGAATTCCGCGCTCCTCT	3
S-*-Ntspa-0712-a-A-21	*Nitrospira* and *Nitrospira* related species	CGCCTTCGCCACCGGTGTTCC	3
S-*-Ntspa-1026-a-A-18	*N. moscoviensis* and clone A-4 and A-11	AGCACGCTGGTATTGCTA	8

ACKNOWLEDGEMENTS

The helpful support of Dr. William Ramey and Gaye Sweet with the experimental design and troubleshooting are much appreciated.

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