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An Evaluation of the Relationship between the Most Probable Number (MPN) Assay and 16S rRNA Hybridization Technique in Characterizing and Quantifying Nitrosomonas Species in Sludge Wastewater

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Nitrosomonas species are the main organisms involved in the oxidation of ammonium to nitrite in nature. The traditional method for enumerating *Nitrosomonas* species is via the Most Probable Number (MPN) Technique. However, this is a slow and labourious method due to the slow growth rate of nitrifiers. Also, the method does not distinguish different species of nitrifiers. Oligonucleotide probes are now used as a method for identifying particular species of nitrifying bacteria. However, the correlation between these methods is unclear. Sludge wastewater sample from a bioreactor was used to study the potential correlation between the MPN technique and the oligonucleotide probe technique. In order to investigate this relationship, multiple replicate dilutions of the sludge wastewater were made and each dilution was grown and processed using the MPN technique. After a period of growth, a record was made of the presence or absence of ammonia oxidation. RNA was extracted from these growth samples were probed with 16S rRNA specific probes Nso 190 and Nsm 156. It was found that the original sludge sample had 1.4 x 10^6 viable cells/ 100mL with 95% confidence lower limit of 6.1 x 10^5 and an upper limit of 2.8 x 10^6. However, the poor integrity of the isolated RNA prevented any identification of *Nitrosomonas* species in the MPN sample.

The biogeochemical cycles describe the cyclic nature of the five essential nutrients of life. One of which is nitrogen. Nitrogen is an essential component in amino acids, which are the building blocks of proteins, and in nucleic acids, which form the genetic material of the cell. Nitrogen gas makes up about 78% of the earth's atmosphere, but the triple bonds in this molecule makes the gas inert. Nitrogen fixation in bacteria converts N_2 to forms of fixed nitrogen compounds that are more available to the biosphere. To complete the cycle, the denitrification process returns nitrogen gas (N_2) back to the atmosphere. In nature, nitrogen is found in a variety of valence states from -3 in NH3 to +5 in NO3- (3, 8). The energy released by changes in the redox potential of the element is harnessed by organisms to maintain life (7).

Nitrification is the process by which ammonium is oxidized to nitrate. This process is carried out by two distinct groups of bacteria: the ammonium oxidizing bacteria (AOB), which oxidize ammonium to nitrite, and the nitrite oxidizing bacteria (NOB), which oxidize nitrite to nitrate (11). *Nitrosomonas europaea*, an obligate lithoautotrophic ammonia-oxidizing bacterium, is the most studied of the ammonia-oxidizing bacteria that are participants in the biogeochemical nitrogen cycle. These bacteria also are important players in the treatment of industrial and sewage waste in the first step of oxidizing ammonia to nitrate. *N.europaea* is also capable of degrading a variety of halogenated organic compounds (6). The ability of nitrifying organisms to degrade some pollutants may make these organisms attractive for controlled bioremediation in nitrifying soils and waters.

N. europaea is a gram negative chemolithotroph that oxidizes ammonia to nitrite and can be found in places such as soil, sewage, freshwater, and building surfaces in polluted areas where the air contains high levels of nitrogen compounds (3). *N. europaea* grows at an optimum pH of 6.0-9.0, temperature range of 20°C to 30°C and under aerobic metabolism conditions. In addition to the previously mentioned conditions, a special selective medium that is inorganic and includes ammonia as an electron donor, bicarbonate as the sole carbon source for the isolation of *Nitrosomonas*. Because of the large amounts of ammonia this bacterium needs to consume for energy to divide, cell division can take up to several days. Due to their slow growth rates and undefined growth requirements, lithotrophic nitrifiers are difficult to isolate (2).

The standard method to enumerate nitrifying bacteria is by the most probable number technique (MPN) which indicates numbers of viable nitrifying bacteria. The MPN assay is performed by setting up multiple replicate dilutions of samples and recording the presence or absence of nitrifying activity. Unfortunately, the MPN assay is inefficient method for the enumeration of nitrifying bacteria due to their slow growth rate and moreover, nitrifiers are small organisms and growth cannot be determined using optical density measurements. With the development of

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molecular techniques, researchers have applied methods such as PCR and probe hybridization in the identification of nitrifying bacteria. In a RNA blot, Ribonuclease (RNA) from nitrifying bacteria is immobilized on a nylon membrane by slot blotting. Then the blot is hybridized with nucleotide probes with specific affinity for 16S rRNA of isolated species of nitrifiers. The detection of probes bound to the membrane would indicate the presence of a particular strain of nitrifying bacteria in the original sample. However, previous studies have detected a consistent discrepancy between the nitrification activity in bioreactors and the binding of 16sRNA probes of known nitrifiers (1, 11). For example, the level of ammonium converted to nitrite measured remains high when the amount of organisms detected by the Nso190 probe (specific to all β-proteobacteria ammonia-oxidizers) is decreased by 50% (12, 13). This observation could be caused by mismatches between the RNA 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. Other causes for mismatches could be the presence of other nitrifying organisms, or other species of *Nitrosomonas*.

This study attempts to investigate the relationship between the MPN assay and the results of the probe assays. In addition, this study will also determine if the MPN enriches for a diversity of AOB. MPN assay was performed on a sludge sample was obtained from a bioreactor to determine the number of *Nitrosomonas* cells in the initial sludge sample. The samples from the MPN assay was then assayed by DNA probes specific to the 16sRNA of Ammonia-oxidizing β-*Proteobacteria* and species of *Nitrosomonas* to determine the identity of AOB enriched by the MPN assay (1).

MATERIALS AND METHODS

MPN growth tubes and media. The Most Probable Number Assay for determining the number of Nitrosomonas cells in a sludge sample was set up as follows. A sludge sample (Methanol Bio-P Sludge- October 7, 2004) was obtained from UBC Department of Civil Engineering. Sludge sample was centrifuged at 4200g for 10 minutes at 4°C. The MPN assay was conducted in 50mL test tubes with an inverted Durham tube to trap any gas produced as a result of fermentation. The fermentation tubes filled with *Nitrosomonas europaea* medium (ATCC medium 2265. Details can be found at http://www.atcc.org/mediapdfs/2265.pdf). Seven series of fermentation tubes were set up, ranging from 10^{-1} to 10^{-7}. Quadruplicate fermentation tubes were set up for series 10^{-1} and 10^{-2}. Series 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} were set up in replicates of ten. Final volume of media in fermentation tube was 30mL. The sludge pellet was inoculated into series 10^{-1} and subsequent serial dilutions were made. The fermentation tubes were incubated in the dark at 30°C for 5 weeks.

Nitrogen assay. The nitrate reduction test was used to monitor the growth of Nitrosomonas. At week 3 and 5, the fermentation tubes were examined for pH change, the presence of gas and the production nitrite ions in the medium. A pH change would be indicated by a change in media color as the pH indicator Phenol red (14) was added to the media. The presence of nitrogen gas would be indicated by gas bubbles trapped in the Durham tube. The production of nitrite ions were detected by the addition of sulfanilic acid and N, N-dimethyl-1-naphthylamine to culture (7).

RNA extraction protocol. The Nitrosomonas cells from the MPN tubes were harvested by vacuum filtration through a 0.22um (Millipore GVWP). Cells were washed off from the membrane and resuspended in 1ml of fresh media. The cells were then microfuged at 14000 rpm for 15 minutes.

The cell pellets from the MPN tubes and a frozen pellet from a pure Nitrosomas culture were suspended in 1 mL of Trizol™ reagent (Invitrogen) was added to disrupt the cells. The Trizol® reagent is a monophasic solution of phenol and guanidine isothiocyanate and it disrupts cells while inactivating RNase activity. 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. A second extraction was performed on the remaining organic layer by adding again Trizol reagent and chloroform and repeating the beating and centrifugation procedures as mentioned above. The clear aqueous top layer was removed and combined with the aqueous layer from the first extraction 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 (A260 and A280) using the relationship [RNA]=0.063(A260)-0.036(A280). 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 (RNase) free.

Determination of the Quality of RNA using Gel Electrophoresis. The integrity of the extracted RNA was characterized by gel electrophoresis in 1X TAE buffer (40 mM Tris pH 8.0, 1.14 ml of glacial acetic acid, 1 mM EDTA) with 1.2% agarose gel at 100V for 1 hour. The gels were stained with ethidium bromide and run at 70 V for about 1 hour. The gels were visualized and photographed under a UV transilluminator. Note that due to the lack of RNA from the MPN cultures, only the quality of the RNA extracted pure Nitrosomonas sample was assessed by gel electrophoresis.

Slot Blotting. RNA samples were immobilized onto BioRad transblot nitrocellulose membrane (6.45μm) as per the Alkaline RNA Denaturation and Fixation protocol in the Bio-Dot SF Microfiltration Apparatus Instruction Manual. The RNA samples were denatured with 5 volumes of ice cold 10mM NaOH, 1mM EDTA immediately before blotting onto membrane. The samples are then applied onto a pre-wet membrane. Then sample wells were washed with cold 10mM NaOH, 1mM EDTA. Vacuum was applied to pull liquid through the membrane after each application. After blotting, the membrane was air-dried and then baked at 80ºC for 30 min before tube hybridization.

Primer Design. Oligonucleotide probes were synthesized and purified at the Nucleic Acid Protein Service Unit (NAPS) at University of British Columbia using Applied Biosystems automated synthesizers. The probes were biotin-labeled for detection with streptavidin-peroxidase (POD) and the chemiluminescent substrate luminol.

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TABLE 1. Names, target positions, sequences, and specificities of the probes used in this study.

Probe	*E. coli* 16S rRNA position	Sequence	Probe dissociation temperature (°C)	Targeted groups
NSO 190	190-208	5'-CGATCCCCTGCTTTTCTCC-3'	62	Ammonia-oxidizing β-*Proteobacteria* (11)
Nsm 156	156-174	5'-TATTAGCACATCTTTCGAT-3'	46	*Nitrosomonas* C-56, *Nitrosomonas europaea*, *Nitrosomonas eutropha*, and *Nitrosococcus mobilis*(11)

Hybridization. BM Chemiluninescence Blotting Kit (Biotin/Streptavidin) from Roche Applied Science was used. The processing of the membrane and detection of nucleic acid probes was performed as per manufacturer's protocol. Due to the limited RNA samples, the membrane was divided into two parts. Membrane 1 was first probed with Nso 190 and membrane 2 probed with Nsm 156. After initial hybridization, the probes were stripped off and probed with the other oligonucleotide probe. Prior to hybridization, the nylon membranes with the immobilized RNA were placed in hybridization tubes and pre-warmed in hybridization buffer (5x SSC, 2% blocking reagent, 0.1% N-laurylsarcosine, 0.02% SDS, 50% formamide) at 40°C for 3 hours. Probes were then added to the hybridization tubes at 800 ng/ml. The nylon membranes and the probes were left overnight to hybridize at 40°C. After hybridization, the membranes were washed at twice in 2x washing solution (2x SSC, 0.1% SDS) for 5 minutes per wash at room temperature. Followed then by two washes in 0.1x washing solution (0.1x SSC, 0.1% SDS) for 15 minutes per wash at 65°C for Nso190 probe and 46°C for Nsm 156 probe. After washing, the membranes were blocked with 1% blocking solution for 30 minutes at room temperature followed by incubation of the membranes in Streptavidin-POD diluted in 1% blocking solution at 10 mU/ml for 30 minutes. The membranes are then washed with a solution of maelic acid and 0.3% Tween 20 for 15 minutes and subsequently with just maelic acid.

Chemiluminescence detection. Detection reagent was prepared by combining substrate solution A and starting solution B included in the chemiluminescence kit in a ratio of 100:1. After draining off excess maleic acid, the detection reagent was poured onto the membranes allowing the horseradish peroxidase (POD) to catalyze the oxidation of luminol in the presence of hydrogen peroxide. This reaction forms an activated intermediate product which emits light energy in its conversion back to ground state. The POD was allowed to react with the bound probes for 1 minute and exposed to autoradiography film for intervals of 5, 10, and 30 minutes.

Stripping and reprobing. The bound probes to the membrane were stripped by incubating the membrane twice with 50% dimethylformamide, 1% SDS, 50 mM Tris-HCl at 68°C for 30 minutes. The membranes were then rinsed with 2x SSC and reprobed with the alternate probe in the same manner as described above.

RESULTS

Most Probable Number Assay. Bacterial density can be estimated by the Poisson formula or from the table using the number of positive tubes in multiple dilutions. MPN tables are based on the assumption of a Poisson distribution (random dispersion). The "most probable number" for bacteria density in the original sludge sample is calculated by using Table 2. Using results from 10^{-5}, 10^{-6}, and 10^{-7}, the MPN of viable cells in the original sludge sample is 1.4 x 10^6 cells/ 100mL with 95% confidence lower limit of 6.1 x 10^5 and an upper limit of 2.8 x 10^6.

TABLE 2. Summary of results of the MPN assay.

Dilution of series	Number of positive tubes	Total replicates
10^{-1}	3	4
10^{-2}	4	4
10^{-3}	10	10
10^{-4}	10	10
10^{-5}	10	10
10^{-6}	7	10
10^{-7}	2	10

RNA preparation and assessment of RNA integrity. The RNA preparation from frozen *Nitrosomonas* pellet and sludge sample yielded two bands corresponding to the 23S and 16S rRNA via detection by gel electrophoresis (data not shown). The overall integrity of the RNA was determined to be adequate by the marginally acceptable ratio of A_{260}/A_{280} spectrophotometric methods but the presence of bands showed that the degree of RNA degradation was small. However, the RNA extractions from MPN assay samples were determined to be low yield and low quality via spectrophotometric methods and gel electrophoresis was not able to make out any distinct 16S rRNA bands (Table 3).

Hybridization autoradiogram. As indicated in Table 1, Nso 190 probe should be able to identify all β-proteobacteria and Nsm 156 probe is specific to a couple species of Nitrosomonas. By using differential probing by Nso 190 and Nsm 156, this study hoped to identify Nitrosomonas and distinguish them from other AOB that may be enriched by the MPN method. The autoradiograms were exposed to the chemiluminescent probes that were in turn bound to the immobilized RNA on the nylon membranes. Therefore, the intensities of the bands not only allude to the presence of the 16S rRNA of that the particular probe is specific to, but also to the amount of RNA that is present on the nylon membrane. A standard of Nitrosomonas RNA titrating from 5μg to 0.5μg so that any bands resulting from the MPN assay samples can be used to extrapolate

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TABLE 3. Summary of RNA integrity analysis via spectrophotometry.

Sample	Dilution of sludge sample	A_{260}	A_{270}	A_{280}	Ratio	Concentration (µg/ml)	Total RNA (µg)
Nitrosomonas	1	0.484	0.423	0.297	1.63	495.00	44.55
Sludge	1	0.265	0.212	0.162	1.64	271.58	24.44
1	10^{-5}	0.797	1.540	0.699	1.14	50.09	3.01
2	10^{-5}	0.886	1.704	0.771	1.15	56.12	3.37
3	10^{-5}	0.478	1.002	0.457	1.05	27.32	1.64
4	10^{-5}	0.436	0.897	0.428	1.02	24.12	1.45
5	10^{-5}	0.456	0.863	0.435	1.05	26.14	1.57
6	10^{-5}	0.634	1.233	0.570	1.11	38.84	2.33
7	10^{-5}	0.593	1.179	0.553	1.07	34.90	2.09
8	10^{-5}	1.344	2.414	1.081	1.24	91.51	5.49
9	10^{-5}	0.872	1.638	0.742	1.18	56.45	3.39
10	10^{-5}	0.621	1.216	0.561	1.11	37.85	2.27
11	10^{-6}	0.483	1.002	0.470	1.03	27.02	1.62
12	10^{-6}	0.551	1.115	0.516	1.07	32.27	1.94
13	10^{-6}	0.653	1.130	0.643	1.02	35.98	2.16
14	10^{-6}	0.345	0.651	0.353	0.98	18.05	1.08
15	10^{-6}	0.772	1.264	0.654	1.18	50.18	3.01
16	10^{-6}	0.603	1.218	0.560	1.08	35.66	2.14
17	10^{-6}	0.603	1.209	0.553	1.09	36.16	2.17
18	10^{-6}	1.213	2.227	1.014	1.20	79.83	4.79
19	10^{-6}	0.402	0.854	0.393	1.02	22.36	1.34
20	10^{-6}	0.316	0.710	0.335	0.94	15.70	0.94

TABLE 4. Schematic detailing layouts of the slot blot membrane. The amount and identity of the RNA samples are indicated. The membrane was cut into two with part (a) becoming membrane 1 and part (b) becoming membrane 2.

(a) (b)

	A	B	C	D	E	F
1	*Nitrosomonas* 5µg	sludge 5µg	sample 32	*Nitrosomonas* 5µg	sludge 5µg	sample 42
2	*Nitrosomonas* 4µg	sludge 4µg	sample 33	*Nitrosomonas* 4µg	sludge 4µg	sample 43
3	*Nitrosomonas* 3µg	sludge 3µg	sample 34	*Nitrosomonas* 3µg	sludge 3µg	sample 44
4	*Nitrosomonas* 2µg	sample 27	sample 35	*Nitrosomonas* 2µg	sample 37	sample 45
5	*Nitrosomonas* 1µg	sample 28	sample 36	*Nitrosomonas* 1µg	sample 38	sample 46
6	*Nitrosomonas* 0.5µg	sample 29	blank	*Nitrosomonas* 0.5µg	sample 39	blank
7	blank	sample 30	blank	blank	sample 40	blank
8	blank	sample 31	blank	blank	sample 41	blank

the amount of RNA resulting from that sample. The probe Nso 190 bound very little to the Nitrosomonas RNA on membrane 1 with only a very faint band at the 5µg as seen in figure 2. Compared to the probing of membrane 2 with Nsm 156, in which the autoradiogram produced a distinct band even at 0.5µg of Nitrosomonas RNA (Fig. 1).

Figure 1 shows detection of Nitrosomonas 16S rRNA in the RNA extracted from sludge wastewater sample using Nsm 156 probe on membrane 2. All 3 concentrations (5µg, 4µg, and 3µg) were detected and the intensity of the bands decreased with decreased amounts of RNA immobilized on the nylon membrane.

After stripping and reprobing with the alternate probe, similar results were seen. Nsm 156 was able to detect a gradient of Nitrosomonas RNA on membrane 1 (Fig. 4) whereas Nso 190 did not reveal any distinct bands to the Nitrosomonas RNA on membrane 1 (data not shown).

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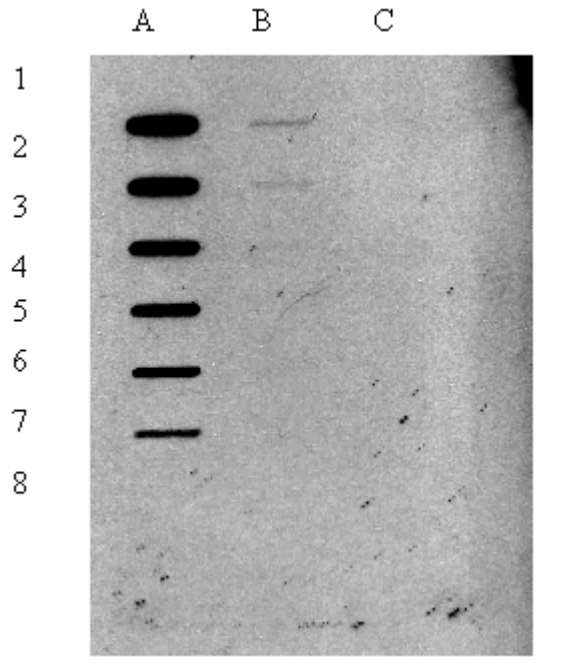

FIG. 1 Autoradiogram of hybridization of Nsm 156 onto membrane 2 (10 minute exposure). Refer to Table 4 for identity of samples.

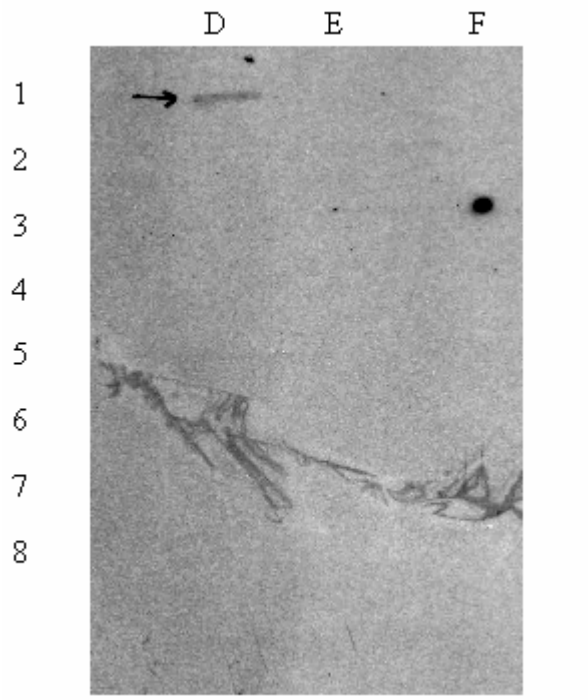

FIG. 2 Autoradiogram of hybridization of Nso 190 onto membrane 1 (10 minute exposure). Refer to figure 1 for identity of samples. The streak in the middle was a shadow produced by the wrinkles in the plastic wrap in the exposure process.

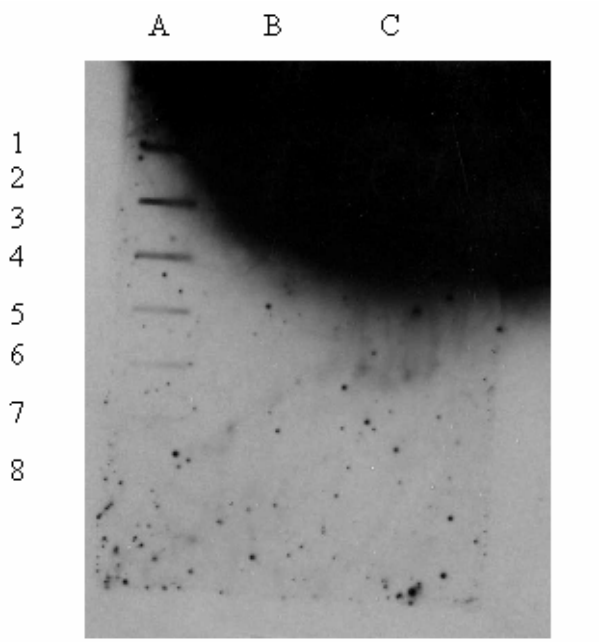

FIG. 3 Autoradiogram of hybridization of Nsm 156 onto membrane 1 (30 minute exposure). Refer to figure 1 for identity of samples. The large black area that obscures the top right corner of the autoradiogram was caused by inadequate sealing of the film cassette during film exposure. Therefore light leaked into the cassette and bleached out that area of the autoradiograph film.

DISCUSSION

MPN assay enumerates the amount of viable organisms in a sample. According to the MPN assay, there are 1.4 x 10^6 viable growth units (GU) in the original sludge sample. Although MPN cannot distinguish between the different organisms that may be present in the sludge wastewater (9), certain parameters of the MPN assay allude to the identity of the organism that was enriched in the MPN assay. First, the media used in the fermentation tubes only consisted of inorganic materials such as ammonia so that only AOB are able to grow. Also other parameters such as temperature and lack of light were optimal for AOB such as *Nitrosomonas* to flourish (2). Second, the nitrate reductase test of the supernatant detected nitrite. Since there was no nitrite in the original media, the nitrate must be the product of the nitrification. It would be possible to have nitrite oxidizing bacteria (NOB) since such organisms oxidize nitrite to nitrate in their metabolic growth, so NOB can flourish using the products of AOB metabolism. However, the nitrate reductase test detected no nitrate in any of the sample supernatants which suggests that NOB are present in the samples. Also, the absence of nitrogen gas production further signifies the lack of NOB in the sludge. Therefore, the MPN assay can conclude that

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there are AOB present in the original sludge wastewater sample and all the growth units can be attributed only to the presence of AOB in the sludge wastewater sample. Even though *Nitrosomonas europaea* is the primary AOB found in wastewater, other species of *Nitrosomonas* could be found in wastewater such as *Nitrosomonas halophila*, *Nitrosomonas communis*, *Nitrosomonas ureae*, *Nitrosomonas marina*, *Nitrosomonas aestuarii*, *Nitrosomonas oligotropha*, *Nitrosomonas cryotolerans*, and *Nitrosomonas nitrosa* (11, 12). Therefore, the 16s rRNA assay was performed to identify the particular *Nitrosomonas* species present in the sludge wastewater.

From the autoradiogram, it is evident that the two probes Nso 190 and Nsm 156 have a very different binding affinity to the *Nitrosomonas* RNA in the blot analysis. The binding of oligonucleotide probes is dependent on many factors such as temperature, NaCl concentration and formamide concentration of the wash buffer (7). One possible explanation for the binding of Nsm 156 to the pure *Nitrosomonas* RNA sample but the Nso 190 probe's greatly reduced affinity to the RNA is that the washing temperature of the Nso 190 probe was set too high. The washing temperature used in this study was 68°C but according to Wong (15), the Nso 190 probe dissociation temperature ranges from 44.1°C to 51.9°C (14), which is much lower than the melting temperature described by Mobarry et al at 68°C (11). The more than 15°C difference in washing temperature might be enough to cause total dissociation of bound probes off the nylon membrane. Nsm 156 has an optimum NaCl concentration of 56 mM (5). Compared to Nso 190, the optimum NaCl concentration is 20 mM (4). This detail is important because higher NaCl concentration will favour the nonspecific binding and may be misleading. The concentration of NaCl in 0.1x washing solution is 15mM. The NaCl concentration in the washing solution is much lower than optimum for Nsm 156 which may have allowed the probe to bind more efficiently than Nso 190 because the comparatively lower NaCl concentration might have allowed for more mismatching. Formamide concentration should not have played a part in probe binding because no formamide was used in the wash buffer. However, it can be seen from the autoradiogram that the Nso 190 probe is sensitive to *Nitrosomonas* RNA at 5µg while the Nsm156 is sensitive to amounts of RNA less than 0.5µg.

If there were slight mismatching of the nucleotides to the RNA coupled to too high of a washing temperature, significant amounts of probes that were supposed to be bound to the RNA would be washed off (14). Although the sequence of Nso 190 is specific to 16s rRNA of β-proteobacteria which include *Nitrosomonas*, there are still many species of *Nitrosomonas* that are not yet isolated or sequenced. The particular strain of *Nitrosomonas* present on the nylon membrane may have sequences corresponding to the Nsm 156 probe but not the Nso 190 oligonucleotide probe. Although it should be noted that this hypothesis is highly unlikely since 16s rRNA is highly conserved.

The probe Nso 190 is designed to detect all ammonia oxidizers of the β-subclass of proteobacteria. However, according to Purkhold et al. (12) Nso 190 probe does not react with species of *Nitrosomonas* such as *N. communis*, *N nitrosa*, *N. oligotropha*, and *N. ureae*. The probe Nsm 156 targets species of *Nitrosomonas* such as *N. europaea*, *N. eutropha*, *N. C56* and *Nitrosococcus mobilis* (11). A possible explanation of the differential sensitivity to the *Nitrosomonas* RNA is that the species of organism is one that Nsm 156 is specific to, but not Nso 190 such as *Nitrosococcus mobilis*, which is a AOB in the γ-subdivision of the Proteobacteria class. Since Nso 190 is specific to β-proteobacteria, it would not be able to detect *N. mobilis* RNA while Nsm 156 can.

A study by Behrens et al (4) investigated the hybridization of rRNA-targeted oligonucleotides. They hypothesized that if probes were targeted to the surface of the ribosomal subunit, then the increase accessibility of the probe to RNA binding site would increase sensitivity of probe assays. However, the results showed little correlation between probe hybridization efficiency and the proximity of the probe target region to the surface of the three-dimensional model of the 30S ribosomal subunit. Moreover, according the predicted secondary structures of *E. coli* 16s rRNA model, both Nso 190 and Nsm 156 probes are located at exposed portions of the secondary structure of the ribosomal subunit. Therefore, the secondary structure of the RNA is not believed to have any pronounced effect on the binding of the oligonucleotide probes.

None of the RNA samples from MPN assay were detected by either Nso 190 or Nsm 156 as seen in figures 1, 2, and 3. The integrity of the RNA extracted from the MPN assay was very poor as assessed by spectrophotometric method of ratio A_{260}/A_{280}. Due to the small amounts of RNA extracted, RNA integrity analysis by gel electrophoresis was not performed in order to converse RNA for slot blotting. It is strongly hypothesized that the RNA degradation by RNase was very great so that the probes would not be able to bind efficiently to the RNA samples immobilized on the nylon membrane. Therefore, even if *Nitrosomonas* RNA was extracted from the MPN assay, the RNase degradation would have prevented the 16s rRNA probes from identifying the presence of *Nitrosomonas* RNA.

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FUTURE EXPERIMENTS

Since the present study did not yield any conclusive results as to whether the MPN assay enriched for *Nitrosomonas* species, the present study should be repeated. The lack of good integrity RNA extracted from the MPN assay is the main barrier in this study. Therefore, it is suggested that this study be repeated using extra anti-RNase precautions to prevent RNase degradation. To obtain higher yield from RNA extraction, the MPN assay can be performed in larger vessels to obtain more cells. Future experiments can also include the use of other 16s rRNA probes such as Nsc 128 to detect γ-proteobacteria (11) such as *Nitrosococcus mobilis* that are also AOB that may be enriched by the *Nitrosomonas* media in the MPN assay.

Other aspects of the enrichment of MPN assays can be studied such as the temporal enrichment of the MPN assay. Perhaps different nitrifiers are enriched at different times during an MPN assay. Also, by varying the growth conditions of the MPN assay, one can study the effect of growth condition on the expression of rRNA in nitrifying bacteria.

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