A new simple method for the enumeration of nitrifying bacteria in different environments

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ABSTRACT

In this work, a simple, safe and rapid method for enumerating nitrifying bacteria was used as an alternative to traditional harmful chemical methods. The enumeration of nitrifying bacteria was based upon the change of color of the growth media containing pH indicators in response to acid production during nitrification. The oxidation of ammonia to strong acids by nitrifiers leads to pH decrease, which can be detected by pH indicators such as methyl orange (MO), bromocresol green (BCG), methyl red (MR), bromothymol blue (BTB), and phenol red (PhR) using the Most Probable Number (MPN) technique. The use of these pH indicators revealed a higher estimate than the classical chemical methods in all tested samples. Ammonium oxidizer counts always exceeded those of nitrite oxidizers in the surveyed environments. The time required for the detection of growth (positive tubes in MPN) was descending in the following order: MO, BCG, MR, BTB and PhR. The time to detection was shorter for ammonium oxidizers than for nitrite oxidizers. Generally, nitrifier counts were very low in soils compared with farmyard manure or sewage effluent. Incubation periods for both organisms differed from 4 to 8 weeks depended upon the indicator used. Finally, it could be concluded that the use of pH indicators, especially phenol red, as proposed in this study was accurate, sensitive and successfully applicable for the enumeration of nitrifiers in different environments.

Keywords: ammonium oxidation; nitrite oxidation; Most Probable Number; pH indicators; environmental samples

Nitrifying bacteria are widespread in soil and are responsible for the maintenance of soil fertility and a part of the nitrogen cycle (Troeh and Thompson 1993). Nitrifiers are difficult to cultivate, enumerate or isolate because of their very specific growth requirements, and are very sensitive to any environmental changes as well as many organic and inorganic substances. The enumeration of nitrifying bacteria is typically carried out using a Most Probable Number (MPN) technique (Alexander and Clark 1965, Finstein 1968, Ghiorse and Alexander 1978, Li et al. 2006) based on the detection of the production of HNO_2 and HNO_3 or the disappearance of NH_4^+ or NO_2^- from the medium. The detection of HNO_2, HNO_3 and NH_4^+ is usually done using chemical methods. The standard chemical methods employed to detect the presence of HNO_2, HNO_3 and NH_4^+ rely on the use of hazardous chemicals such as α-naphthylamine and sulfanilic acid in the case of HNO_2 or diphenylamine in the case of HNO_3. Other disadvantages of chemical tests are those that are relatively laborious, expensive, and time consuming (Sarathchandra 1979, Baikun and Shannon 2007). Membrane filter techniques for the enumeration of nitrifying bacteria were developed to avoid some of these problems, and the counts obtained by this method were in good agreement with the results obtained by using the MPN method (Finstein 1979, Li et al. 2006). More recently PCR-based techniques were developed for the detection and counting of nitrifier population *in situ* (Degrande and Bardin 1995, Jang et al. 2005, Pollard 2006, Yapsakli et al. 2011). Although membrane- and PCR-based methods avoid the use of hazardous chemicals, they are still relatively labor-intensive. This work aimed at developing a simple and inexpensive method for the enumeration of nitrifying bacteria based upon the change of color of growth media containing pH indicators in response to acid production during nitrification.

MATERIAL AND METHODS

Nitrifier enrichment medium. Nitrifiers were enriched on Nakos and Wolcott (1979) medium containing (g/L): 0.3 $(NH_4)_2SO_4$; 0.136 $CaCl_2$; 0.175 $MgSO_4 \cdot 7\ H_2O$; 0.5 $NaHCO_3$; 13.5 Na_2HPO_4; 0.7 KH_2PO_4; 0.005 $FeSO_4 \cdot 7\ H_2O$ and 0.00375 $NaMoO_4$. The pH of the medium was adjusted to 8.2 and autoclaved at 121°C for 20 min.

Original and modified media for the enumeration of nitrifiers. Two different media by Alexander and Clark (1965) were used for cultivating and enumerating nitrifying bacteria. The first medium was used for the cultivation and enumeration of all nitrifiers (ammonium and nitrite oxidizers). The chemical composition (g/L) of this medium was: 0.5 $(NH_4)_2SO_4$; 1.0 K_2HPO_4; 0.03 $FeSO_4 \cdot 7\ H_20$; 0.3 NaCl; 0.3 $MgSO_4 \cdot 7\ H_2O$; 7.5 $CaCO_3$. The second medium (0.006 $NaNO_2$; 1.0 K_2HPO_4; 0.3 NaCl; 0.1 $MgSO_4 \cdot 7\ H_2O$; 0.03 $FeSO_4 \cdot 7\ H_2O$; 0.3 $CaCl_2$ and 1.0 $CaCO_3$) was used for the cultivation and enumeration of nitrite oxidizers only. These two media were modified to be appropriate for counting the nitrifying bacteria (ammonium and nitrite oxidizers) depending upon the change in pH of the growth media containing pH indicators. The modification was principally to avoid the buffering effect of $CaCO_3$ present in the original media by replacing it with $CaCl_2$ to keep the calcium concentration at the same level. K_2HPO_4 was replaced by K_2SO_4 and $Ca_3(PO_4)_2$ to keep phosphorus and potassium at appropriate levels. In addition, the $NaNO_2$ concentration was increased to 0.01 g/L in the nitrite oxidizer medium. The pH indicators (MO, BCG, MR, BTB and PhR) were individually added to the enumeration media before sterilization. The initial pH of the media was adjusted to 7 with 0.01 mol/L NaOH and 5 mL portions were distributed in tubes that were plugged and autoclaved at 121°C for 20 min. After sterilization the pH of the media was increased to 8.2 by the addition of sterile NaOH.

Sample materials and nitrifier stock culture preparation. Different samples, including different soil types, farmyard manure, aerated sewage effluent and enriched nitrifiers stock culture, were tested.

Three types of soils differing in their texture class, i.e. sand, sandy loam and clay loam, were air dried, sieved to pass a grade 2 mesh sieve and fortified with 200 ppm ammonical nitrogen as $(NH_4)_2SO_4$. The moisture of the soils was adjusted to 60% of their water holding capacity (WHC), which was kept constant through the daily compensation of evaporation losses.

Farmyard manure (organic fertilizer) obtained from the Faculty of Agriculture Farm at the Fayoum University, Fayoum, Egypt, contained about 0.3% nitrogen and 10% organic matter. Aerated sewage effluent samples were obtained from the sewage treatment station in Fayoum.

For the preparation of nitrifier stock culture, Nakos and Wolcott (1972) medium was inoculated with 5% of activated sewage effluent containing a high level of NH_4-N (> 1%) to produce an enriched culture of nitrifying bacteria (ammonium and nitrite oxidizers). To obtain the final stock culture, the original Alexander and Clark (1965) medium was inoculated with the previously enriched culture and incubated at 30°C for 24 h, then subcultured daily into new fresh medium.

Enumeration of nitrifying bacteria. Nitrifying bacteria were enumerated by the MPN technique using both modified media of Alexander and Clark (1965). Tenfold dilution series of soils, manure, sewage effluent, and stock culture were prepared in physiological solution. Each sample was inoculated in 25 tubes for 5 appropriate successive dilutions. All assays were performed in triplicate and all tubes were incubated for 5–8 weeks at 30°C. For the detection of positive samples, the tubes were visually scored at two-day intervals for the production of sufficient acid to decrease the pH and thus change the color of the indicator added as an indication of nitrifier growth (ammonium and nitrite oxidizers). As a control, nitrite and nitrate were assayed using the standard chemical method (Griess-Ilosvay reagent) after 8 weeks (Bremner et al. 1965, Charlot 1965). In the case of the enumeration of nitrite oxidizers, only the positive tubes (negative for nitrite detection) were chemically distinguished using the α-naphthylamine sulfanilic acid sodium acetate method (Charlot 1965). The pH of the inoculated tubes was also measured with a pH meter at the end of the incubation period (8 weeks) to compare with the pH indicators used. A Most Probable Number (MPN) table (Cochran 1950) was used to determine numbers of nitrifying bacteria. In the case of using the first modified medium, combined counts of ammonium and nitrite oxidizers were obtained. In the case of the second modified medium only nitrite oxidizers were counted. Counts of ammonium oxidizers were determined by subtracting the nitrite oxidizer count from the combined nitrifier count obtained with the first medium.

RESULTS AND DISCUSSION

Initially a preliminary trial was applied in order to determine the most appropriate concentration of each indicator that can easily be visually distinguished without ambiguity using diluted HCl as acidifier in different media. A diphenylamine sulfuric acid assay for the detection of both nitrite and nitrate was carried out for the purpose of comparison (Table 1). An assay for nitrite only using α-naphthylamine sulfanilic acid was also performed.

Table 1 lists the results obtained with different pH indicators and their respective pH ranges. As methyl orange has a useful pH range of 3.1 to 4.4, more acid production is required to effectuate a change of color than for the other indicators, while phenol red, with a pH range of 6 to 7.6, requires the least production of acid for a positive result. The concentrations of the indicators were kept as low as possible to avoid the toxic effects of high concentration of the indicators on the autotrophic nitrifiers, while still allowing the color change to be easily observed. The appropriate concentrations of the indicators employed in this work were 5, 4, 3, 10 and 5 mL/L for MO, BCG, MR, BTB and PhR, respectively. While Sarathchandra (1979) used phenol red at a concentration of 7.5 mg/L for counting nitrifiers, in our work only 0.2 mg/L were used to avoid the toxic effects of phenol red.

Table 2 shows the numbers of ammonium oxidizers determined by the classical diphenylamine method compared with the suggested method (pH indicators) using a stock culture (10^9 cells/mL) in modified Alexander and Clark (1965) medium. Phenol red indicator scored the highest ammonium oxidizer count (1.48×10^9 cells/mL, 1667% of the result obtained with diphenylamine), which was very close to the cell density in the original inoculum (stock culture). MO gave the lowest count (2.05×10^7 cell/mL, 23.1% of the diphenylamine count). This result was expected as the acidity needed for phenol red to respond is very low compared to that needed in the case of MO. The count determined using the classical diphenylamine method was 8.88×10^7 cells/mL. BCG, MR and BTB recorded 7.03, 11.08 and 77.03×10^7 cells/mL, respectively. Phenol red needed the shortest incubation time (4–5 weeks) while methyl orange needed an incubation period of 7–8 weeks until a change of color was observed.

The same trends of ammonium oxidizer counts were observed in the different environmental samples tested (soils, farmyard manure, and sewage effluent). In the case of sandy loam and clay soil, the ammonium oxidizer counts with phenol red always exceeded those of methyl orange by 7 to 10-fold and gave similar counts as those determined using the diphenylamine method. Using methyl orange as an indicator recorded 0.2, 0.9 and 10.1×10^5 cells/g in sand, sandy loam and clay loam soils, respectively, while in the case of PhR the corresponding counts were 11.1, 93.7 and 972.1×10^5 cells/g, respectively. In addition, the detection of positive tubes was possible after only 4–5 weeks in the case of phenol red as indicator, and only after 8 weeks in the case of methyl orange. The other three indicators occupied intermediate positions between these two indicators. In soils, diphenylamine showed counts very similar to those of MR and BTB. In farmyard manure, the same trends for ammonium oxidizer counts were observed as in soil samples, but counts were higher than those found in soils. Counts using MO were

Table 1. Some characteristics and preparation of indicators used

Indicator	pH range	Preparation	Concentration (mL/L)
Methyl orange	3.1–4.4 red–yellow	0.1 g salt/L water	5
Bromocresol green	3.8–5.4 yellow–blue	0.1 g + 14.3 mL of 0.01 mol/L NaOH/250 mL water	4
Methyl red	4.2–6.3 red–yellow	0.1 g + 300 mL of ethanol + 200 mL of water	3
Bromothymol blue	6.0–7.6 yellow–blue	0.1 g + 16 mL 0.01 mol/L NaOH/250 mL water	10
Phenol red	6.8–8.4 yellow–red	0.1 g + 28.2 mL 0.01 mol/L NaOH/250 mL water	5

Table 2. Ammonium oxidizer and nitrite oxidizer counts using pH indicators compared to the traditional chemical method

Indicator	Stock culture		Sandy soil		Sandy loam		Clay loam		Farmyard manure		Sewage effluent	
	Ammonium oxidizer											
	No. $\times 10^6$	*% Di.ph	No. $\times 10^5$	*% Di.ph	No. $\times 10^5$	*% Di.ph	No. $\times 10^5$	*% Di.ph	No. $\times 10^5$	*% Di.ph	No. $\times 10^5$	*% Di.ph
Methyl orange	20.5	23.1	0.2	1.8	0.9	3.19	10.1	10.6	60.0	6	182.3	12.31
Bromocresol green	70.3	79.17	0.8	7.27	11.01	39.04	19.6	19.52	221.1	22.16	634.7	42.87
Methyl red	110.8	124.77	7.0	63.63	13.1	46.45	93.8	93.43	783.3	78.31	988.1	66.74
Bromothymol blue	770.3	867.45	9.0	81.81	37.2	131.91	124.2	123.7	1001.7	100	1600.2	108.1
Phenol red	1480.0	1666.67	11.1	100.1	93.7	332.27	792.1	788.94	1600.2	1597	8600.3	580.87
Diphenylamine	88.8	100	11.0	100	28.2	100	100.4	100	1000.2	100	1480.6	100
	Nitrite oxidizers											
Indicator	No. $\times 10^5$	*% Di.ph	No. $\times 10^4$	*% Di.ph	No. $\times 10^4$	*% Di.ph	No. $\times 10^4$	*% Di.ph	No. $\times 10^4$	*% Di.ph	No. $\times 10^4$	*% Di.ph
Methyl orange	18.41	200.77	0.43	33.1	1.01	14.13	1.09	5.95	12.41	12.13	11.33	6.36
Bromocresol green	73.91	806	1.22	93.85	15.33	215	33.14	181	212.13	207.4	191.17	107.3
Methyl red	113.88	1241.88	11.32	870.7	22.15	309.8	128.17	700	813.22	794.9	711.23	399.2
Bromothymol blue	830.33	9054.85	16.81	1293	42.13	589	190.37	1039.7	1123.8	1098.5	1017.18	570.9
Phenol red	1212.3	13220.28	1.70	130.7	101.12	1414.3	933.13	5096.3	1701.12	1662.9	1703.30	955.9
Sulfanilic acid and α-naphthylamine	10.12	110.36	3.70	284.62	8.12	113.57	21.11	115.3	109.18	106.7	188.16	105.6
Diphenylamine	9.17	100	1.30	100	7.15	100	18.31	100	102.30	100	178.18	100

$$*\text{Diphenylamine (\%)} = \frac{\text{Count by pH indicator} \times 100}{\text{Count by Diphenylamine}}$$

60×10^5 cells/g (6% of diphenylamine count) in farmyard manure after 7 weeks of incubation and were 1600×10^5 and 1000×10^5 cells/g with PhR and diphenylamine, respectively. Diphenylamine counts for ammonium oxidizers were very close to those with BTB. Active sewage effluent revealed higher counts, with 182, 8600 and 1480×10^5 cells/g, respectively, but positive tubes were visually recognizable after only 4–5 weeks of incubation. Using a pH meter at the end of incubation periods showed that there were no great differences between the pH values determined by the indicators and those determined using the pH meter. It was noticed that in the case of some samples (soils, organic matter) lower dilutions (10^{-1}–10^{-2}) often lead to high turbidity in tubes, making it difficult to observe the color change. Therefore, if the count is low and samples are turbid, the pH indicator test is not as reliable and the chemical test should be carried out. In practice, the abundance of nitrifiers in turbid samples such as soil or manure is usually high enough to require higher dilution factors, and turbidity does therefore not pose a problem.

Table 2 illustrates the nitrite oxidizer counts (HNO_2 to HNO_3) obtained from different samples using the different pH and chemical indicators in the modified medium of Alexander and Clark (1965). The total nitrifier count (ammonium and nitrite oxidizers) was determined in medium containing $(NH_4)_2SO_4$, and nitrite oxidizers alone were counted using medium containing $NaNO_2$. The number of nitrite oxidizers was then subtracted from the total nitrifier count to give the ammonium oxidizer counts presented in Table 2. It was clear from the results illustrated in Table 2 that the same trends were observed for both ammonium and nitrite oxidizers. Phenol red as pH indicator scored the highest counts in all examined samples, but counts of nitrite oxidizers were always less than those of ammonium oxidizers with all indicators used. Many authors reported that in nature, i.e. soils, sewage, water and balanced environment (without addition of nitrite), counts of ammonium oxidizers always exceeded those of nitrite oxidizers by an order of magnitude (Charlot 1965, Ghiorse and Alexander 1978, El-Shahawy

and Al-Mashhady 1984). In addition, it was noticed that nitrite oxidizers needed a relatively long incubation period (8 weeks) to produce sufficient nitric acid from nitrite to allow detection. This was expected because nitrite oxidizers such as *Nitrobacter* are well known to be more sensitive to environmental factors than ammonium oxidizers (e.g. *Nitrosomonas*). Similar to the chemical methods, the method presented here still requires lengthy incubation periods (weeks) due to the slow growth of nitrifying bacteria. Unlike the chemical methods, however, the acidification of the medium can be easily followed continuously by simple visual inspection, rather than through labor-intensive and potentially hazardous chemical assays. The use of pH indicators in weakly buffered medium offers the further advantage that the color change upon acidification past the pH threshold of the indicator is unambiguous; as soon as the critical pH is reached the color change is complete. Using diphenylamine for the detection of both nitrite and nitrate and sulfanilic acid α-naphthylamine for the detection of a decrease of nitrite in the counting of nitrite oxidizers yielded very similar results, confirming the validity of the methods (Table 2). Finally, it could be concluded that the use of pH indicators, especially phenol red, as proposed in this study was an accurate, sensitive and successfully applicable method for the enumeration of nitrifiers in different environments.

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REFERENCES

Alexander M., Clark F. E. (1965): Nitrifying Bacteria. In: Black C.A., Evans D.D., White J.L., Ensminger L.E., Clark F.E. (eds): Methods of Soil Analysis. Part 2. American Society of Agronomy, Madison, 1477–1483.

Baikun L., Shannon I. (2007): The comparison of alkalinity on ORP as indicators for nitrification and denitrification in a sequencing batch reactor (SBR). Biochemical Engineering Journal, *34*: 248–255.

Bremner J.M. (1965): Inorganic forms of nitrogen. In: Black C.A. (ed): Methods of Soil Analysis – Part 2: Chemical and Microbiological Properties, Agronomy No. 9, American Society of Agronomy Inc., Madison.

Charlot G. (1965): Colorimetric Determination of Elements. Principles and Methods. Elsevier Publishing Co., New York.

Cochran W.G. (1950): Estimation of bacterial densities by means of the 'most probable number'. Biometrics, *6*: 105–116.

Degrande V., Bardin R. (1995): Detection and counting of *Nitrobacter* populations in soil by PCR. Applied and Environmental Microbiology, *61*: 2093–2098.

El-Shahawy R.M., Al-Mashhady A.S. (1984): Nitrification of ammonium sulphate and urea fertilizers under saline condition. Zentralblatt für Mikrobiologie, *139*: 343–347.

Finstein M.S. (1968): Enumeration of autotrophic ammonium-oxidizing bacteria in marine waters by a direct method. Applied Microbiology, *16*: 1646–1649.

Ghiorse W.C., Alexander M. (1978): Nitrifying populations and the destruction of nitrogen dioxide in soil. Microbiology Ecology, *4*: 233–240.

Jang A., Okabe S., Watanabe Y., Kim In S., Bishop L. (2005): Measurement of growth rate ammonia oxidizing bacteria in partially submerged rotating biological contactor by fluorescent in situ hybridization (FISH). Journal of Environmental Engineering and Science, *4*: 413–420.

Li H., Yang M., Zhang Y., Yu T., Kamagata Y. (2006): Nitrification performance and microbial community dynamics in a submerged membrane bioreactor with complete sludge retention. Journal of Biotechnology, *123*: 60–70.

Nakos G.G., Wolcott A.R. (1972): Bacteriostatic effect of ammonium on *Nitrobacter agilis* in mixed culture with *Nitrosomonas europaea*. Plant and Soil, *36*: 521–527.

Pollard P.C. (2006): A quantitative measure of nitrifying bacterial growth. Water Research, *40*: 1569–1576.

Sarathchandra S.U. (1979): A simplified method for estimating ammonium oxidising bacteria. Plant and Soil, *52*: 305–309.

Troeh R.F., Thompson L.M. (1993): Soil and Soil Fertility. Oxford University Press, New York, 193–213.

Yapsakli K., Aliyazicioglu C., Mertoglu B. (2011): Identification and quantitative evaluation of nitrogen-converting organisms in a full-scale leachate treatment plant. Journal of Environmental Management, *92*: 714–723.

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