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FEMS Microbiology Ecology 32 (2000) 167–175

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Quantitative analysis of ammonia oxidising bacteria using competitive PCR

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Received 13 August 1999; received in revised form 18 February 2000; accepted 17 March 2000

Abstract

Culture-based methods for enumeration, such as most probable number (MPN) methodologies, have proved inefficient due to difficulties in the isolation and cultivation of ammonia oxidising bacteria in the laboratory. Biases are associated with the isolation of bacteria in selective media and organisms cultivated in the laboratory may not be truly representative of those in the environment. In this study, we developed a competitive PCR (cPCR)-based method based on the amplification of 16S rRNA genes specific for the β-subgroup proteobacterial ammonia oxidising bacteria for enumeration of these organisms. Populations in both agricultural soils and estuarine sediments were quantified by traditional MPN and by cPCR. The numbers of ammonia oxidisers for both sample types were significantly underestimated by conventional MPN and were 1–3 orders of magnitude lower than those obtained by cPCR. Higher numbers of ammonia oxidisers found in fertilised plots in agricultural soils by the cPCR technique were not observed in MPN estimates. It was necessary to construct a separate standard curve for each sample type as differences in DNA extraction, quantity and purity had a significant bearing on the ease of PCR of both competitor and target DNA.

Keywords: Competitive PCR; Ammonia oxidiser; Most probable number; Nitrification; Soil; Sediment

1. Introduction

Autotrophic ammonia oxidising bacteria play a central role in the cycling of nitrogen in natural environments, oxidising ammonia, produced by decomposition of organic material, to nitrite which is subsequently oxidised to nitrate [1]. Ecological studies of this important microbial group have been severely limited by the lack of reliable and convenient techniques for estimating cell or biomass concentrations. The most common approach to enumeration employs the most probable number (MPN) method, incubating inocula from serial dilutions of environmental samples in liquid inorganic medium containing ammonium [2,3]. Growth is assessed after a period of incubation, typically 4 weeks, by acid production, production of nitrite and/or nitrate and/or removal of ammonium. This provides estimates of viable cell concentrations but is limited by the well-accepted disadvantages associated with techniques based on laboratory cultivation [4,5]. These include the requirement for extraction of attached cells, selectivity of culture media and laboratory growth conditions, the inability to detect non-culturable cells and competition between indigenous populations, and inhibition by non-ammonia oxidisers in growth media. The long incubation periods required reduce the practical value of the technique and variation in dormancy and lag periods result in continued appearance of positive cultures after several months [6]. Traditional viable cell enumeration on solid media has been attempted [7], with detection of acid production by an overlay of $CaCO_3$, but it shares many of the limitations of MPN enumeration. More recently, a microcolony method has been employed [8]. This greatly reduces incubation periods and may detect viable cells that do not grow for prolonged periods. Estimation of total cell concentrations of ammonia oxidisers has been carried out using immunofluorescent antibody techniques [9,10]. This approach requires initial cultivation of cells to generate antibodies, does not distinguish live and dead cells and is difficult when cells are attached to particulate material or present at low concentrations.

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0168-6496/00/$20.00
PII: S0168-6496(00)00028-3

The advent of molecular techniques for the analysis of autotrophic ammonia oxidising bacteria in environmental samples [11–15] has provided evidence of previously uncharacterised diversity and has increased concerns that enumeration techniques based on laboratory cultivation may misrepresent the size of natural populations. Molecular techniques provide alternative approaches for enumeration, through quantification of DNA sequences specific to particular groups of organisms. These techniques are based on PCR amplification of target DNA sequences from DNA extracted from the environment, and are independent of laboratory cultivation of target organisms. Primers may be designed for amplification of 16S rRNA genes or functional genes, enabling detection of different microbial groups, while sensitivity can be increased by nested PCR [15,16].

Two approaches have been used for quantification of PCR amplification products from environmental DNA, MPN-PCR and competitive PCR (cPCR). The former involves serial dilution of extracted DNA with detection in replicate samples at each dilution using PCR amplification [17,18]. Numbers of target sequences are calculated from published tables, as for MPN cell counts. cPCR involves co-amplification of the target DNA and an internal standard, or competitor DNA, which is similar to but distinguishable from the target [19]. Estimation of the number of target sequences is achieved by comparison of ratios between target and competitor sequences with those of a standard curve generated by the amplification of competitor DNA with a range of target DNA concentrations.

The aim of this study was to develop competitor sequences for cPCR enumeration of ammonia oxidising bacteria and to compare cPCR and MPN enumeration approaches in terrestrial and aquatic environments. The method employed 16S rDNA primers developed in previous studies characterising natural populations of ammonia oxidising bacteria belonging to the β-subgroup of the proteobacteria [13,20]. These are believed to be the dominant group of ammonia oxidising bacteria in natural environments.

2. Materials and methods

2.1. Bacterial strain, culture conditions and DNA extraction

The competitor DNA sequences COMP1 and COMP2 were constructed from DNA isolated from *Nitrosomonas europaea*, grown in modified Skinner and Walker (1961) medium [21] and incubated in the dark at room temperature. Acid produced during ammonia oxidation was neutralised by addition of 5% (w/v) sodium carbonate until ammonia was fully converted and cells were harvested by centrifugation at $10\,000 \times g$ for 15 min at room temperature. A known volume of Chelex 100 (50–100 μl) was added to the cell pellet and cells were lysed by boiling for 10 min. Cell debris was removed by centrifugation in a micro-centrifuge for 10 min at room temperature. Total cell concentration was determined by microscopic counts using a Thoma slide.

2.2. PCR amplification

PCR amplification was carried out with a standard PCR master mix [15] in a 50-μl reaction. Single stage PCR amplifications were carried out using the forward and reverse primers CTO189f and CTO654r [20], which amplify all known β-proteobacterial ammonia oxidiser sequences. For a nested PCR, primary amplification was carried out using the forward and reverse primers βAMOf [13] and CTO654r, respectively. Products of primary amplification (1 μl) were then amplified with the primers CTO189f and CTO654r. Initial denaturation was carried out at 95°C for 5 min, followed by 20 or 25 cycles of 94°C for 40 s; 55°C for 30 s and 72°C for 2 min; and 72°C for 5 min. For single stage PCR, amplification was carried out over 30 rather than 25 cycles. Known concentrations of the competitors COMP1 or COMP2 were added to the PCR reaction tubes after addition of template and master mix.

2.3. Construction of competitor and verification of amplification efficiency

Internal standards COMP1 and COMP2 were prepared by deletion of a portion of the *N. europaea* 16S rRNA gene using a standard PCR reaction with a modified reverse primer CTO654r/INT1 (CTAGCYTTGTAGTTTCAAACGCCTTTACGCCCAGTAATTCCG (CTO654r underlined)) as described by [22] (Fig. 1). PCR of *N. europaea* DNA with this primer and either of the forward primers βAMOf or CTO189f yields a product 60 bases shorter than that generated by PCR with the reverse primer CTO654r. These products can be differentiated on a 2% (w/v) agarose gel. COMP1 and COMP2 were prepared by amplification of *N. europaea* DNA with CTO654r/INT1 and either CTO189f or βAMOf, respectively. COMP1 had primer recognition sites CTO189f and CTO654r while COMP2 had primer recognition sites βAMOf, CTO189f and CTO654r enabling amplification using nested PCR. The COMP1 and COMP2 amplification products were gel-purified and cloned into *Escherichia coli* using the TA cloning kit (Invitrogen, Groningen, The Netherlands). Plasmids were purified from transformed cells using the wizard *SV* plasmid purification kit (Promega, Southampton, UK).

2.4. Verification of competitor amplification efficiency

Amplification efficiencies of competitor DNA and *N. eu-*

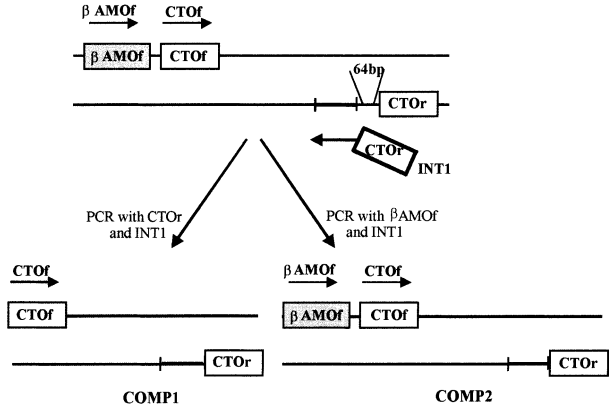

Fig. 1. Schematic representation illustrating the construction of the cPCR competitors COMP1 and COMP2. Competitors were constructed by deletion of a 64-bp region of the 16S rRNA gene of *N. europaea*. The upper region shows recognition sites for the primers βAMOf, CTO189f and CTO654r. Primer recognition site INT1, 64 bp upstream of the CTO654r binding site, is also shown in bold. PCR was carried out with either of the forward primers and a modified CTO654r primer, INT1, which has the CTO654r primer attached to its 3′ end. The products of the PCR are the competitors COMP1 and COMP2.

ropaea DNA were compared using a PCR reaction with the primers CTO189f and CTO654r over 15–40 cycles. Amplification products were quantified by image analysis of 5–10 μl of PCR products separated on 2% (w/v) agarose gels for 45 min at 100 V and post-stained for 30 min in 10 μg ml^{-1} ethidium bromide in 1×TAE buffer and destained for 10 min in 1×TAE buffer. A mass ladder (Life Technologies, Paisley, UK) was included on each gel and densitometry was carried out using either Molecular Analyst software (Bio-Rad, Hertfordshire, UK) or Phoretix 1D software (Phoretix Ltd., UK). Band intensities were normalised using the mass ladder as controls. To ensure that the amplification products of a nested PCR gave the same ratio of COMP2 to target DNA after the primary and secondary amplifications, a dilution series of *N. europaea* DNA (corresponding to 10^0–10^7 cells per reaction) was amplified with 40 pg of COMP2. Ratios of the competitor and target products were quantified as above.

2.5. Optimisation and calibration

Estimation of PCR products from target DNA requires optimisation of amplification cycle number to ensure that the PCR is in the exponential phase. DNA was extracted from 10^7 cells of *N. europaea* by boiling in Chelex 100, as described above, and then diluted 100- and 10 000-fold in sterile milli-Q water. PCR amplification was carried out for 15, 20, 25, 30, 35 and 40 cycles on all three DNA samples using the primer set βAMOf and CTO654r, and products were run on a 2% agarose gel. Nested PCR, using the primer set CTO189f and CTO654r, was then carried out on 1 μl of PCR products from amplification products of each DNA dilution employing 20 and 25 cycles. The products of both the primary and secondary reactions were run on 2% agarose gels, stained, destained and quantified as described above.

The concentrations of competitor and target DNA in each sample must be closely matched to reduce the effects of competition for primers and nucleotides particularly when carrying out nested PCR. A dilution series of competitor DNA (4 fg–40 ng per 50-μl reaction) was amplified with a constant amount of target DNA in a nested PCR reaction and 5–10 μl of product visualised and quantified as above. Calibration curves for enumeration of pure cultures of *N. europaea* were determined by extraction of DNA from serial, 10-fold dilutions of cells from a fully-grown culture and PCR amplification of the total supernatant following DNA purification. Calibration curves for enumeration in environmental samples were prepared by addition of a 10-fold dilution series of *N. europaea* cells to either soil or sediment to give final cell concentrations in the range 10^0–10^6 cells g^{-1}. DNA extracted from each dilution was amplified in the presence of a known amount of competitor DNA. Soils had been γ-irradiated but sediment samples were not sterilised to maintain their structure and water content. A sediment sample to which no cells had been added served as a blank and the ratio of COMP:target obtained for this sample was subtracted from that of other samples.

DNA was extracted from 5 g of soil [23] and humic contaminants were removed by gel electrophoresis in a 1% (w/v) low melting agarose gel (Gibco BRL, Gaithersburg, MD, USA) (40 V for 4 h). The DNA was excised from the gel, removed by β-agarase treatment (Boehringer Mannheim Corp., Indianapolis, IN, USA) and concentrated through a Microcon 100 filtration unit (Amicon Inc., Beverly, MA, USA). DNA quantity and purity were determined by measurement of absorbance at 260 nm and 280 nm. DNA was extracted from 1-g samples of sediment using a modified method of [11]. Approximately 0.5 g of sediment was added to a sterile 2-ml Eppendorf tube containing 0.5 g of glass beads (Hybaid Ltd., Middlesex, UK), 300 μl of 120 mM phosphate buffer (pH 8.0), 200 μl of Tris–HCl (pH 8.0) and 250 μl of Tris-buffered phenol. The mixture was agitated twice for 5 s in a Ribolyser (Hybaid Ltd.) before centrifugation at 11 600×*g* for 10 min. The supernatant was transferred to a fresh 1.5-ml Eppendorf tube, 500 μl of phenol was added and mixed gently and the two phases separated by centrifugation at 11 600×*g* for 10 min. The aqueous layer was transferred to a fresh Eppendorf tube and 500 μl of phenol:chloroform:isoamyl alcohol (25:24:1) was added, mixed briefly and the aqueous phases separated by centrifugation. The aqueous phase was removed and washed four times by addition of an equal volume of chloroform:isoamyl alcohol (24:1) and centrifugation at 3000×*g* for 15 min in a Microcon 100 filtration unit. DNA was resuspended in 50 μl of sterile distilled water and purified by gel electrophoresis.

2.6. cPCR of environmental samples

Ammonia oxidiser cell concentrations were estimated in Michigan soils and Ythan estuary sediment samples by cPCR using COMP1 (single stage PCR) and COMP2 (nested PCR), respectively. Soil samples were collected in October 1996 from cultivated and successional sites at the Long Term Ecological Research Site (LTER) at the Kellogg Biological Station, Southwest Michigan, USA (http://www.kbs.msu.edu/lter). Ten composite samples (5-cm depth) were taken from each of three replicate plots of each treatment. Treatments were chosen to assess the effects of fertilisation and tillage and cultivation on ammonia oxidiser numbers. The composite samples were pooled and passed through a 1-cm sieve to remove large stones, twigs and plant material. Soil for DNA extraction was aliquoted and stored frozen at −20°C. Sediment samples were collected at low tide from four stations of the Ythan estuary (Aberdeen, UK) in October 1997. The Ythan estuary is heavily influenced by agricultural run off and has been designated a nitrate vulnerable zone (European Council Directive (91/676/EEC)) due to increasing eutrophication. Station A was the seaward station at the mouth of the estuary while Stations B and C were within the River Ythan and were brackish and freshwater, respectively. Triplicate cores (11-cm diameter × 0.5-cm depth) were collected from each station and the top 0.5 cm was stored at 4°C prior to analysis. Sediment from Station A was sieved through a 2-mm mesh sieve to remove macrofauna. The water content of soil and sediment samples was determined by oven drying.

2.7. Enumeration of ammonia oxidising bacteria by MPN and cPCR

MPNs were determined in microtitre plates by the method of [3]. Soil samples (10 g) were blended with 190 ml of 100 mM phosphate buffer (pH 7) in a Waring blender for 1 min and 150 μl of the soil suspension was added to the eight wells of the first column of a 96-well microtitre plate; each well contained 150 μl of ammonia oxidiser growth medium with 50 μg NH_4^+-N ml^{-1}. A 150-μl sample was then taken from the first column and added to the next column of eight wells and the procedure repeated to give eight replicates of a series of 18, 2-fold dilutions. Sediment samples were removed with 1-ml syringes with the luer end removed and placed in 20 ml of phosphate buffer containing 20, 3-mm diameter sterile glass beads and shaken vigorously for 15 min to dislodge bacteria from sediment particles. After settling for 1 min, 150 μl of sediment suspension was removed and added to each of the eight wells in the first column of a microtitre plate which contained 150 μl of modified Skinner and Walker medium or Skinner and Walker medium in artificial seawater in each of the 96 wells. The plates were placed on top of a pad of water-saturated tissue and wrapped in saran wrap to avoid evaporation during incubation. After incubation for 4 weeks in the dark at room temperature, growth was determined by a colour change from pink to yellow due to acid production. The presence of nitrate and nitrite was checked as an indicator of ammonia oxidation by the addition of diphenylamine reagent (0.2 g in 100 ml concentrated sulfuric acid). MPN values were calculated according to the tables of [3].

3. Results

3.1. Optimisation of PCR conditions

For accurate determination of the amount of target in the environmental samples, it is essential that the competitor and target DNA amplify with the same efficiency. This was confirmed by PCR amplification of both COMP2 and *N. europaea* DNA over a range of PCR cycles. No significant difference in the volumes of PCR products was observed (Fig. 2); COMP2 and *N. europaea* amplification was in the linear phase between 15 and 30 cycles.

As COMP2 can be used in a nested PCR, optimum PCR cycling conditions which would allow the amplification of a range of DNA concentrations were determined. DNA extracted from 10^7 *N. europaea* cells was diluted 100- and 10 000-fold, to give concentrations of 0.4 ng, 4 pg and 40 fg μl^{-1}. PCR was carried out using CTO primers with 1 μl of each sample over 15–40 cycles. From the amplification products from 20 and 25 cycles, 1 μl was taken and amplified over a further 15–30 cycles. Image analysis of the products from both primary and secondary amplifications showed that linearity of PCR amplification was achieved when using 20 cycles followed by 20 cycles when the starting DNA concentration was in the range 40 fg–4 pg μl^{-1} (Fig. 3). Higher starting DNA concentrations showed that the PCR reached a plateau by 20 cycles in the primary amplification. The products from the secondary amplifications were at saturation by 15 cycles.

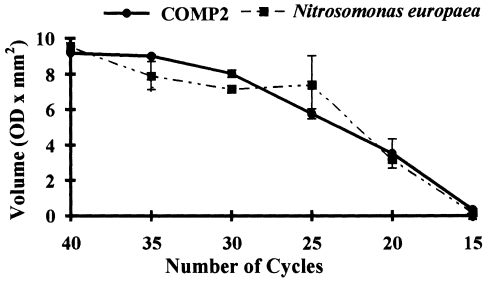

Fig. 2. Efficiency of amplification of competitor, COMP1, and *N. europaea* DNA amplified over a range of PCR cycles (15–40) with the CTO primer pair. Products of triplicate PCR reactions were run on a 2% (w/v) agarose gel and the image was quantified using Molecular Analyst software (Bio-Rad). Bands were normalised to the Gibco mass ladder and the density plotted against the cycle number.

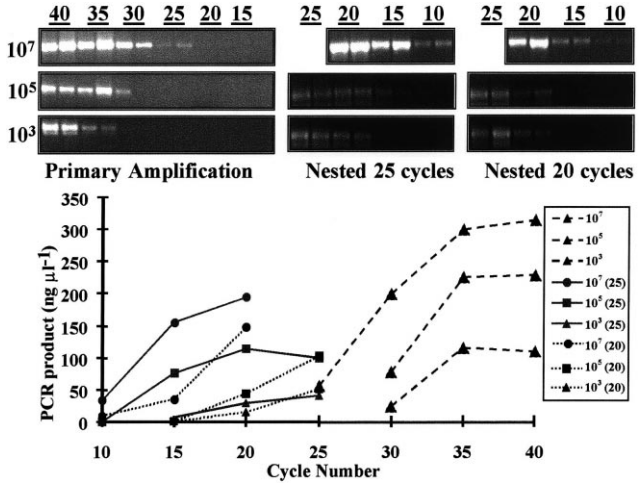

Fig. 3. Estimation of optimum PCR conditions for nested PCR. DNA extracted from 10^7 *N. europaea* cells was diluted 100- and 10 000-fold and the three samples amplified over a series of PCR cycles (15–40) with the βAMOf and CTO654r primers. Subsamples (1 µl) from the products of the 20- and 25-cycle reactions were amplified for a further 10–25 cycles in a nested PCR with the CTO189f and CTO654r primers. Aliquots of the products (5 µl) of both primary and secondary amplifications were run on 2% agarose gels and the images quantified using Phoretix 1D image analysis software with normalisation of the Gibco mass ladder. The three plates show the gel images of the products from the primary, secondary (25 cycles) and secondary (20 cycles) amplifications, respectively. The graph shows the quantification of the products plotted against the cycle number.

The competitor COMP1 was evaluated in agricultural soils from Michigan, USA. In these soils, the presence of β-proteobacterial ammonia oxidisers was shown by the positive amplification in a primary PCR with CTO primers, indicating a relatively high abundance of these bacteria in the soils. Using this primary amplification protocol, the COMP1 concentration at the crossover point was 7 pg when comparing the COMP1 dilution with soils amended with *N. europaea* cells.

Detection of ammonia oxidiser rDNA in sediments from the Ythan estuary required use of a nested PCR for which COMP2 competitor was used at a concentration for cPCR 1000-fold lower than for primary amplifications with COMP1. Fig. 4, bottom right, demonstrates that, even with a competitor concentration (COMP2) as low as 40 fg, a band could be detected after secondary amplification. This concentration was at the limit of detection for this system and, at this low concentration, no band was visible from the target DNA (Fig. 5, lower gel), consisting of *N. europaea* DNA and sediment DNA extracted from site A.

It was hoped to overcome the inconvenience of using different concentrations of competitor for each environment, and hence separate standard curves, by preparing a calibration curve using pure culture DNA. As seen from Fig. 4, top left, with *N. europaea* DNA alone, even a primary amplification was possible and the crossover point for the competitor was at 400 pg. This demonstrates the inhibitory nature of environmental DNA and the lack of suitability of calibration curves produced with pure culture DNA.

3.2. Calibration curves

Calibration curves were prepared for competitors, COMP1 and COMP2, in soils and sediment samples. The calibration for COMP1 was prepared with DNA extracted from 1-g aliquots of γ-irradiated Michigan soil previously inoculated with a dilution series of between 10^7 and 10^0 *N. europaea* cells g^{-1}. DNA was only visible on ethidium bromide-stained gels from soil samples inoculated with 10^7 cells. The DNA extracted from soils inoculated with *N. europaea* (10^7 cells g^{-1} soil) was serially diluted 10-fold and then amplified in a PCR reaction with 7 pg of COMP1 DNA and the ratio of template to COMP1 was quantified by image analysis of gels using Molecular Analyst software (Bio-Rad, Hertfordshire, UK). The ratio of COMP1:target DNA was directly proportional to the number of cells ($r^2 = 0.992$) (Fig. 5A).

Calibration curves for COMP2 were prepared using riverine sediment from the Ythan estuary. Uninoculated sediment was included as a control and the ratio of COMP2: target for the control was subtracted from that of the sediments inoculated with *N. europaea*. Calibrations were prepared using two methods. In the first, *N. europaea* cells were added to eight, 1-g subsamples of riverine sediment to give final cell counts between 0 and 10^7 cells g^{-1}. In the second, DNA from the sediment containing 10^7 *N. europaea* cells g^{-1} was serially diluted to give eight samples of

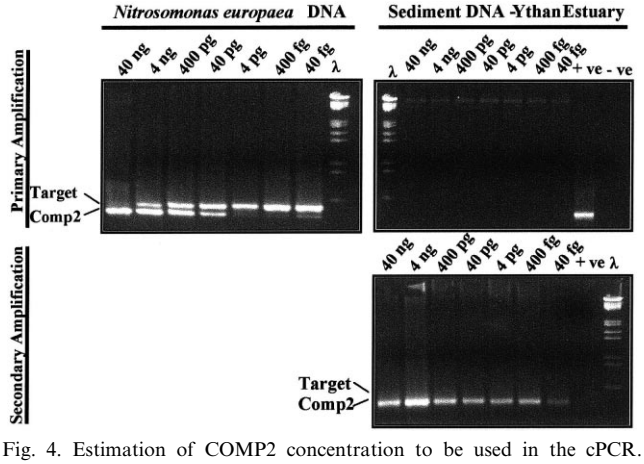

Fig. 4. Estimation of COMP2 concentration to be used in the cPCR. Sediment samples from the Ythan estuary, UK, were amplified initially using the primer set βAMOf/CTO654r. The top left figure shows a dilution series of COMP2 DNA (between 40 ng and 40 fg) amplified with 100 ng of DNA from a fully-grown culture of *N. europaea*. The top right figure shows the same dilution series with *N. europaea* DNA but also amplified in the presence of 100 ng of Ythan estuary sediment DNA from Station C. The bottom right figure shows the secondary amplification products of the products from the top right figure with CTO189f/CTO654r primer sets.

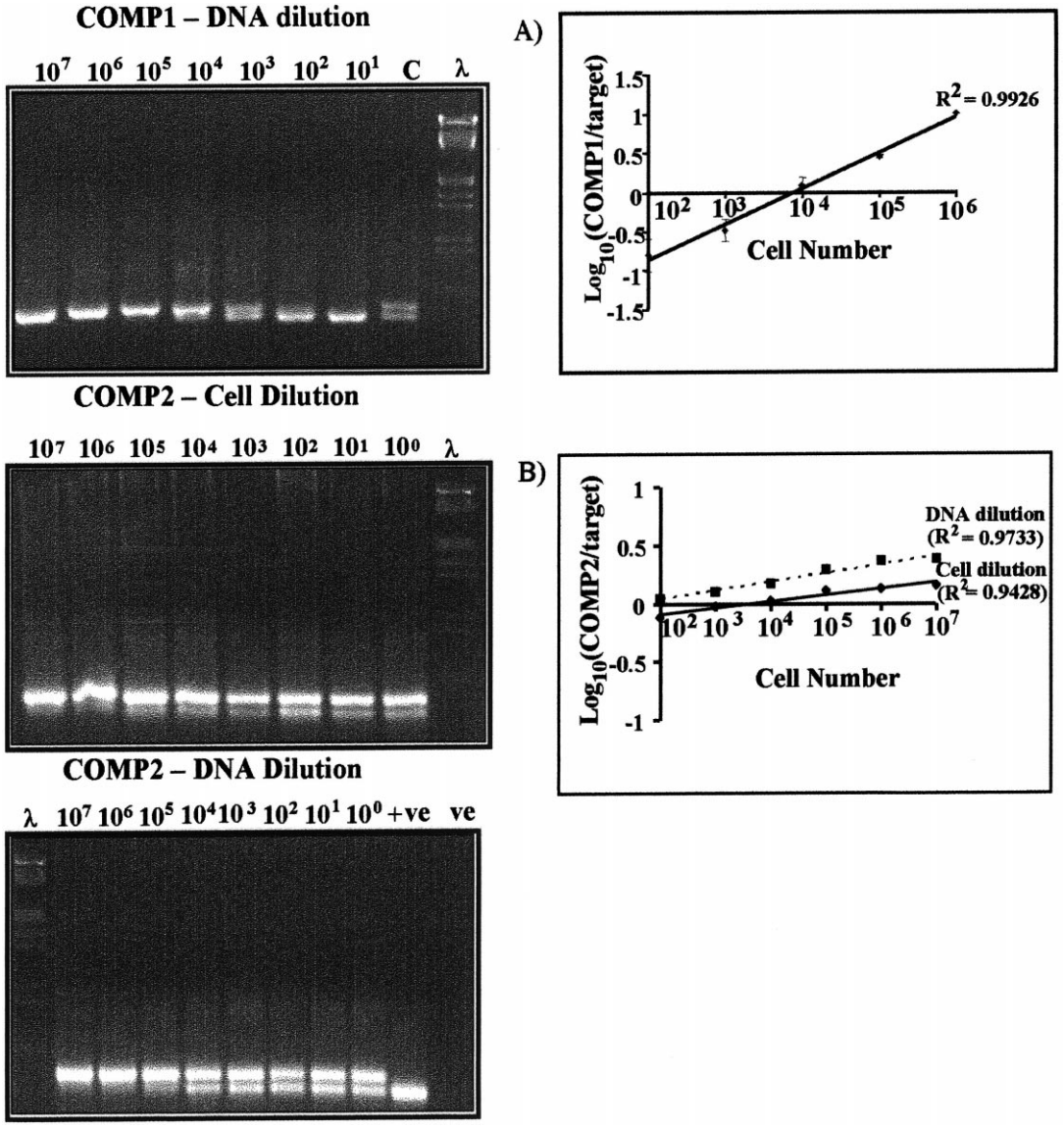

Fig. 5. Calibration curves of COMP1 and COMP2 for agricultural soils and sediments from the Ythan estuary, respectively. The gels show amplification products for soils and sediments inoculated with *N. europaea* DNA and amplified in the presence of 7 pg of COMP1 (upper plate) and 40 fg of COMP2 (lower plates): primary amplification products with CTO189f and CTO654r primers, for soil samples, and secondary amplification products (βAMOf/CTO654r followed by CTO189f/CTO654r), for sediment samples. The two upper plates show a dilution series of DNA extracted from both soil and sediment to which *N. europaea* cells (10^7) were added. For the bottom image, a dilution series of cells between zero and 10^7 was added to the sediment prior to DNA extraction. The band intensities were quantified and the ratio of COMP2:target plotted against cell number. The upper graph (A) shows the calibration of COMP1 in soil for the upper gel picture. The lower graph (B) shows the calibration curves for COMP2 in sediment for cell dilution and DNA dilution.

a 10-fold dilution range. In each case, the DNA was amplified with 40 fg of COMP2 in a nested PCR (Fig. 5B). The calibration curves were prepared as for COMP1 by plotting the ratio of COMP2:target against the cell number. In the second method, it was assumed that the DNA content of cells was linear between 10^0 and 10^7 cells. PCR products of both methods gave the same results, with a band from COMP2 becoming visible from the samples with 10^5 added *N. europaea* cells, but analysis of digitised images indicated that the curves were statistically different ($P = 0.014$) (Fig. 5B).

3.3. Numbers of ammonia oxidising bacteria in environmental samples

Numbers of ammonia oxidising bacteria were calculated for Michigan soils by both traditional MPN and cPCR (Fig. 6). In general, no treatment effects were seen in the soils using MPN data ($P = 0.094$ and 0.361 for fertilised (F) and tilled (Tr2 and Tr7T) treatments, respectively). Numbers of ammonia oxidisers calculated by cPCR were 1–2 orders of magnitude higher than those calculated by MPN ($P = 0.022$). Numbers ranged between 10^4 and 10^6

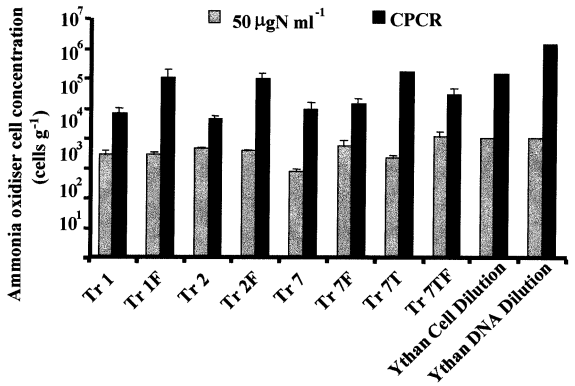

Fig. 6. Ammonia oxidiser cell concentrations in LTER soils and Ythan estuary sediments estimated by the MPN method using medium containing 50 μg NH_4^+-N ml^{-1} and by cPCR. Counts were determined for triplicate samples in October 1996 for LTER soils and in November 1997 for Ythan sediments. Results are shown as mean of triplicate samples ± S.E.M. Tr1 and Tr2 are cultivated soils while Tr7 is a successional grassland soil. The suffix F refers to a fertilised treatment while Tr2 and the suffix T refers to soils which have been tilled.

cells g^{-1} and, in contrast to the MPN data, there was a noticeable treatment effect in the cultivated treatments, Tr1 and Tr2, where fertilised treatments had higher ammonia oxidiser numbers (cPCR: $P = 0.014$, MPN: $P = 0.361$).

In the Ythan sediments, numbers of ammonia oxidisers calculated by cPCR were again 2–3 orders of magnitude higher than those calculated by MPN (1.1×10^4 cells g^{-1}) (Fig. 6). However, the ratio of COMP2:target DNA for the marine and brackish sediments did not fall on the calibration curve prepared in riverine sediments, preventing enumeration. In addition, the numbers of ammonia oxidisers calculated for the riverine sediments varied by an order of magnitude depending on the calibration curve used. In this case, the cell dilution method gave higher numbers (1.4×10^7 cells g^{-1}) than those calculated by the DNA dilution method (1.5×10^6 cells g^{-1}).

4. Discussion

Estimation of the abundance of ammonia oxidisers is traditionally achieved by MPN enumeration, which is generally considered to underestimate population size. Molecular techniques provide an alternative approach to quantification, and between 10- and 1000-fold differences have been reported between MPN and PCR quantitative methods of other microbial groups [24]. In this study, a cPCR method for estimating the abundance of ammonia oxidising bacteria in soil and sediment samples was developed and evaluated. Our results show that cPCR is superior to MPN-PCR. MPN-PCR gave inconsistent results, was cumbersome, expensive and prone to contamination due to high numbers of PCR reactions required for each sample. The inconsistency of the technique is thought to result from the variety of PCR inhibitors (humic acids) that are co-extracted with the DNA from environmental samples. Dilution of the sample usually overcomes the inhibition by diluting out the contaminants but where the concentration of the target is already low, as in the case of ammonia oxidisers, dilution can result in the extinction of target DNA [25].

Optimisation of the PCR conditions and preparation of accurate calibration curves were labour intensive [26]. When comparing agricultural soil samples from the same location, one calibration curve was sufficient, as had been shown by van Elsas et al. [27] and Hallier-Soulier et al. [28]. The soil samples were from the same soil type, which would have been relatively homogeneous due to cultivation. However, all sediment samples could not be evaluated on the same calibration curve, as sediment from the three sample locations, marine, brackish and riverine, contained different levels of humic contaminants. In aqueous environments, which are less complex than soils and sediments, calibrations carried out in a range of samples showed no statistical difference [29] and separate calibration curves for water and sediment were necessary in a study of marine microcosms inoculated with a *Pseudomonas* strain [27]. To overcome the problem of separate calibration curves for each environment, Möller and Jansson [30] added the competitor after cell lysis and co-extracted it along with the DNA. Our approach differed, in that competitor and target DNA were added after extraction, as we experienced differences in extraction efficiencies for different sample types. For the sediment, calibration curves prepared by addition of known concentrations of *N. europaea* cells and by dilution of DNA from the highest concentration were compared. This method has been used in some studies [24,31] but data from the Ythan estuary showed that calibration curves generated by this method gave lower numbers of ammonia oxidisers than by the extraction of DNA from soils with the addition of a dilution series of cells. The difference between the two curves probably arises from dilution of inhibitory substances as well as target DNA resulting in different slopes. This has been shown to give 10-fold differences in the numbers calculated for some samples.

Nested procedures using either two PCR steps or a PCR step followed by a probing step are commonly used to increase the sensitivity of cPCR [29,30]. Our study demonstrated that, by using two 25-cycle rounds of PCR, the products were in the linear phase of PCR for initial DNA concentrations between 40 fg and 4 pg μl^{-1}. Both soil and sediment samples had the same order of magnitude of ammonia oxidisers present when the numbers were calculated using conventional MPN counts, yet only the sediment samples required a nested PCR. This was attributed to higher levels of inhibitory substances in the sediment DNA, as amplification along with the DNA of a pure culture strain of *N. europaea* and COMP2 DNA only gave products after the second round of PCR. Due to

the different levels of inhibitory compounds between soil and sediment, distinct concentrations of competitor DNA were required for each and it was impossible to use a standard calibration made from pure cultures of ammonia oxidiser cells.

A 1–2 orders of magnitude increase in the numbers of ammonia oxidisers calculated by traditional MPNs was found for both the agricultural soil and sediment, agreeing with the suggestion that less than 1% of the bacterial community is culturable [33]. Amplification of bacterial sequences may provide a better representation of the bacteria present than culture-based methods [5,33,34]. Despite the possible biases of DNA extraction (inefficient or preferential cell lysis), PCR biases (including primer selectivity and the presence of inhibitory substances), it is considered more accurate than culture-based methods and is the only method available for bacterial groups with no cultured representatives [35].

Other studies have targeted the *amoA* gene for cPCR analysis of ammonia oxidiser communities [36,37] but the lack of information on *amoA* gene copy number and relative lack of sequence information for the design of suitable primers reduce its reliability. In addition, other groups of organisms, including the methanotrophic bacteria, have been shown to possess genes homologues of *amoA* genes that could prevent accurate quantification of ammonia oxidiser numbers.

We have shown that the numbers of ammonia oxidisers calculated by a nested cPCR are up to 1000 times higher than those calculated by traditional MPNs, suggesting that these bacteria constitute a greater proportion of the bacterial community than previously thought. Numbers determined by cPCR showed treatment effects masked by the culture dependent quantification method of MPN. cPCR has been shown to be a very useful tool but preparation of a suitable calibration curve for accurate determination of numbers is labour intensive. In addition, we have shown the use of a nested PCR process for cPCR, which is crucial for the assessment of groups of organisms not present in high abundance in environmental samples, for example the nitrifying bacteria, but are nevertheless performing a vital function in the community. Techniques such as these will prove essential in the quantification of some groups of bacteria for which no culture-based method exists.

Acknowledgements

This project was funded by NSF grants to the Center for Microbial Ecology (DEB912006) and to the LTER (DEB8702332).

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