The development and use of real-time PCR for the quantification of nitrifiers in activated sludge

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Abstract Chemical analytical data has long been used to monitor the performance of activated sludge plants even though the process relies on the performance of microorganisms. It is now evident that a rapid and reliable quantitative method is required, to be able to monitor the organisms responsible for nutrient transformation and their activities, allowing avenues for more efficient nutrient removal. The development of real-time or quantitative polymerase chain reaction (PCR) also known as TaqMan® or 5'-nuclease assay has allowed the rapid, quantitative analysis of DNA templates, eliminating some of the variability traditionally associated with other quantitative techniques. In this study analysis of Nitrospira spp., one of the key organisms in nitrite oxidation in wastewater treatment, was used to validate real-time PCR for the their quantification in activated sludge. A probe and primer set, targeting the 16S rRNA gene of Nitrospira spp. was designed according to the constraints of the TaqMan® specifications. Samples used to evaluate the method included DNA from the sludge from full-scale wastewater treatment plants and laboratory scale systems. The reproducibility, quantitative efficiency and specificity were assessed in the evaluation. It was concluded that the method is sensitive and reproducible but has some constraints on the quantitative efficiency. A survey of full-scale systems for Nitrospira spp. was carried out and the results are presented here.

Keywords Activated sludge; BNR; microbial ecology; Nitrospira; quantification; real-time PCR

Introduction

The removal of nutrients such as nitrogen and phosphorus from wastewaters is important to prevent eutrophication. Although biological nutrient removal (BNR) relies on microbial activity, its monitoring relies on chemical and physical parameters rather than microbiological data (Seviour et al., 1999). The application of biological quantification and in situ identification techniques for the monitoring and understanding of these microorganisms might result in more efficient nutrient removal. BNR plants are designed and modelled, and accurate quantitation of the responsible microorganisms would improve these models. Immunological (Sandén et al., 1994), blot hybridisation (Rittman et al., 1999) and fluorescence in situ hybridisation (FISH) (e.g. Schramm et al., 1999) methods exploit molecular biological approaches to quantify BNR organisms.

The development of real-time PCR or TaqMan® has eliminated the variability traditionally associated with PCR allowing routine and reliable quantification of PCR amplicons. It is a single-step quantification technique where the accumulation of the amplicons is directly monitored either during or after PCR (Becker et al., 2000). Real-time PCR involves the attachment of a probe, labelled with both a fluorochrome and a quencher, to a target sequence. When the fluorochrome and quencher are in close proximity, no fluorescence is produced. However, during PCR the exocatalytic activity of the DNA polymerase cleaves the probe from the target sequence, separating the fluorochrome from the quencher, resulting in a fluorescence signal. This signal is then detected by a spectrophotometer like the PE Applied Biosystems 7700 sequence detection system resulting in a curve (Figure 1). The cycle threshold ($C_T$) is calculated as the cycle number at which the reaction begins to become exponential (PE Biosystems, 1999). The cycle threshold ($C_T$) of each sample is
then compared to a standard curve and the result is a numerical value of the number of target sequences in the sample.

Real-time PCR has largely been limited to pathogen diagnosis, but recent research has seen its more widespread application for Archaea and Bacteria such as for specific marine organisms (Suzuki et al., 2000) and ammonia-oxidisers in arable soil (Hermansson and Lindgren, 2001).

In this study we investigated real-time PCR as a quantification method for Nitrospira spp., implicated as one group of organisms responsible for nitrite oxidation in wastewater treatment (Burrell et al., 1998; Juretschko et al., 1998).

**Materials and methods**

**TaqMan® primer and probe design**

A probe and primer set targeting the 16S rRNA gene of Nitrospira spp. was designed according to TaqMan® specifications. Clone clade 1 of Nitrospira spp. (Figure 2) was targeted as these sequences were obtained from sludges with operating conditions similar to full-scale wastewater treatment plants (WWTP). Primer Express (PE Applied Biosystems, USA) and the ARB database were used to design the primers while the basic local alignment search tool was employed to check the specificity.

**Standards**

Plasmid DNA was extracted (ABI Prism Miniprep kit, PE Applied Biosystems, USA) from an overnight Luria Burtani agar culture of the Escherichia coli harbouring clone SBR2046 (Figure 2), grown at 37°C. DNA concentration was determined spectrophotometrically using GeneQuant (Pharmacia LKB Biochrom Limited, Cambridge, UK). The number of plasmid copies within the extracted sample was calculated from this concentration using the known weight of one plasmid. A ten fold dilution series of the plasmid from $10^9$ to $10^0$ copies were used as standards for the method.

**Controls and sample preparation**

Positive controls for Nitrospira spp. were clones SBR1024, SBR1015 and SBR2046 (Clone clade 1, Figure 2). Negative controls were clones SBR2016, RC7 (non-target...
Nitrospira spp. from Clone clade 2, Figure 2), GCP112 (a polyphosphate accumulating organism, Crocetti et al., 2000) and genomic DNA from E. coli. The DNA of both positive and negative control clones was extracted as described above and from E. coli and sludge samples by the FastDNA® SPIN Kit (Bio101, USA) method. The latter technique was evaluated along with other methods and found the most reliable and efficient at DNA extraction.

Real-time PCR assays
Reactions were carried out using the protocol outlined in the TaqMan® Universal mastermix manual (PE Applied Biosystems, USA). Probe and primers were used at a final concentration of 200 nM. The fluorescent signal emitted during the PCR was detected using the ABI prism 7700 sequence detection system (PE Applied Biosystems, USA). The cycling regime was as follows: hold at 50°C for 2 min; hold at 95°C for 10 min; 45 cycles of 95°C for 15 sec and 60°C for 1 min; hold at 25°C for 2 min.

Full scale activated sludge plant survey
Mixed liquor samples were taken from the aerobic zones of five Queensland WWTPs. Mixed liquor volatile suspended solids concentration in mg/L (MLVSS) was measured on fresh samples according to APHA (1995). For TaqMan® assays, DNA was isolated from 10 mg of fresh sludge by the FastDNA® SPIN Kit (Bio101, USA) method and stored until analysed at 20°C. Nitrospira rRNA operon numbers were determined by TaqMan®.

Results and discussion
The Nitrospira spp.-specific TaqMan® primers and probe are described in Table 1. The reproducibility of the real-time PCR was tested by comparing the curves and cycle thresholds of randomly chosen DNA samples. Two examples of the reproducibility are shown in Figure 3. SPR was DNA from a nitrifying lab-scale sequencing batch reactor (SBR) from the University of Queensland. Three analyses of this sample gave cycle
thresholds of 23.97, 24.27, and 23.06 with a standard deviation of ± 0.63. Noosa WWTP, had cycle thresholds of 27.35, 27.74, and 27.38 with a standard deviation of ± 0.22. The reproducibility was tested by comparing the Nitrospira spp. template numbers determined by real-time PCR from ten replicates of one sample. Figure 4 shows the reproducibility result from Brendale WWTP.

The specificity of the Nitrospira spp. real-time PCR assay was assessed by firstly consulting the public database GenBank to demonstrate that the sequences of the probe and primers (Table 1) were specific to clone clade 1 (Figure 2) sequences. DNA from clones SBR2016 and RC7 (both from clone clade 2, Figure 2), and from clone GCP112 (Crocetti et al., 2000) and the genome of E. coli was used to assess the experimental specificity of the Nitrospira spp. real-time PCR assay. Unexpectedly, a high signal intensity was observed for clone SBR2016 (clone clade 2, Figure 5). Resequencing of the 16S rDNA insert in clone SBR2016 demonstrated high similarity with sequences in clone clade 1 and likely mislabelling of the clone.

The quantitative efficiency of the Nitrospira spp. real-time PCR was assessed by diluting DNA extracted from Noosa WWTP sludge and clone SBR1015, carrying out real-time PCR and then calculating the Nitrospira spp. templates in the undiluted DNA samples. The results for SBR1015 demonstrate approximately the same number of templates in all dilutions (Figure 6). For Noosa WWTP, the Nitrospira spp. 16S rDNA values are comparable from the undiluted sample to the 1 in 40 dilution. The 1 in 80 dilution gives a much higher

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number of *Nitrospira* spp. 16S rDNAs and a number of theories could explain this. Sample dilution could reduce the template number below the detection level and therefore the value determined is that from background amplification. If an inhibitory component were present, the 1 in 80 dilution could be below the PCR inhibition giving the correct result for template numbers. Sample dilution was further investigated and results in Figure 7 for two WWTPs (Noosa – A and B; and Gibson Island), also demonstrate that sample dilution gives higher results for real-time PCR of *Nitrospira* spp. than undiluted samples.

A preliminary survey of *Nitrospira* spp. rRNA operon numbers in full-scale installations was conducted on five Queensland WWTPs using the real-time PCR assay (Table 2). We have expressed the final results as *Nitrospira* spp. rRNA operons/mg MLVSS so that comparison between plants could be facilitated. We presume that the rRNA operon numbers are correlated with *Nitrospira* organism numbers but currently the number of rRNA operons per *Nitrospira* cell is not known and we are not prepared to speculate on this point. The ammonia in the influent for all plants ranged from 21 to 42 mg/L (Table 2) and for the majority, the nitrification was nearly complete. Only Oxley WWTP had very poor nitrification with 48.1% ammonia removal. This plant had the lowest number of *Nitrospira* spp. rRNA operons/mg MLVSS (4.4 × 10⁸) while, Noosa, Gibson Island and Wacol WWTPs had approximately 10⁷–10¹⁰ *Nitrospira* spp. rRNA operons/mg MLVSS and demonstrated 98.8–99.8% nitrification. On the surface it appears that for these four plants nitrification performance is correlated with *Nitrospira* cell numbers. At Luggage Point the correlation appears to be not upheld because there are only 6.0 × 10⁸ *Nitrospira* spp. operons/mg MLVSS but nitrification is 99.7% complete. At Luggage Point, it could be that organisms other than *Nitrospira* are responsible for nitrite oxidation or that the NO₂ formed during ammonia oxidation is being denitrified, thus not requiring nitrite oxidising bacteria.

**Conclusions**

In this study of *Nitrospira* spp. real-time PCR, the specific, sensitive and reproducible quantification of *Nitrospira* was investigated. Clone clade 1 (Figure 2) represents the target sequences, as these clones were obtained from the biomass of SBRs operating at nitrite concentrations like those found in typical domestic WWTPs. Studies by many groups have demonstrated the importance of *Nitrospira* to nitrite oxidation (Burrell *et al*., 1998; Juretschko *et al*., 1998; Schramm *et al*., 1998).

Real-Time PCR has definite potential for quantification of BNR organisms. Significant

![Figure 7](https://iwaponline.com/wst/article-pdf/46/1-2/267/476898/267.pdf)
insight to the community composition could be achieved through quantification of key members. The population sizes of both phosphorus and nitrogen removing organisms could be incorporated into models along with chemical and process data to optimise the process. However, certain aspects of the method need to be addressed in order to obtain reliable, accurate results. The bias of DNA extraction should not be overlooked and while the reproducibility and specificity are reliable and accurate, the quantitative efficiency needs to be researched further. The approach as described by Hermansson and Lindgren (2001) could be incorporated where correction for sample inhibition was included. In conclusion, real-time PCR should be exploited to further enhance current microbial enumeration methods.

Acknowledgements

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References


