Microbial quantification in activated sludge: the hits and misses

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Abstract Since the implementation of the activated sludge process for treating wastewater, there has been a reliance on chemical and physical parameters to monitor the system. However, in biological nutrient removal (BNR) processes, the microorganisms responsible for some of the transformations should be used to monitor the processes with the overall goal to achieve better treatment performance. The development of in situ identification and rapid quantification techniques for key microorganisms involved in BNR are required to achieve this goal. This study explored the quantification of Nitrospira, a key organism in the oxidation of nitrite to nitrate in BNR. Two molecular genetic microbial quantification techniques were evaluated: real-time polymerase chain reaction (PCR) and fluorescence in situ hybridisation (FISH) followed by digital image analysis. A correlation between the Nitrospira quantitative data and the nitrate production rate, determined in batch tests, was attempted. The disadvantages and advantages of both methods will be discussed.

Keywords Activated sludge; fluorescence in situ hybridisation (FISH); microbial quantification; Nitrospira; real-time PCR

Introduction

The removal of nutrients such as nitrogen and phosphorus from wastewater is important to prevent eutrophication. Although biological nutrient removal (BNR) relies on microbial activity, process monitoring focuses mainly on chemical and physical parameters rather than microbiological data (Seviour et al., 1999). However, the monitoring by molecular genetic methods of the key microorganisms might permit better prediction of process performance and improved process efficiency.

Several molecular biological techniques have been developed for microbial quantification. However, there are various hurdles in the development of rapid and reliable methods. Methods used for the quantification of nitrifiers in activated sludge include competitive ELISA assays using monoclonal antibodies (Aamand et al., 1996) and quantitative competitive PCR (Dionisi et al., 2002) but neither is routinely employed. Two molecular genetic methods, real-time PCR and fluorescence in situ hybridisation (FISH) followed by digital image analysis, were developed in this study for quantification of nitrite oxidising bacteria (NOB) in activated sludge. Real-time PCR quantifies the number of target DNA sequences in a sample, while FISH quantifies a particular microbial group as a percentage of total bacteria.

Nitrospira sp. in activated sludge was examined because these bacteria are key nitrite oxidisers in this environment (Burrell et al., 1998; Hovanec et al., 1998; Juretschko et al., 1998). Their study is complicated because they are slow growing and present in quite low abundance.

Methods

Sample collection and preparation

Mixed liquor (500 mL) was collected from the aeration zones of Wacol and Oxley...
wastewater treatment plants (WWTP) in South-East Queensland at the same time of the day each Monday and Thursday from the 20th September to the 22nd October 2001. Samples were stored at 4°C until processing which was typically 1 hr after collection. The mixed liquor volatile suspended solids (MLVSS) was measured on the collection day (APHA et al., 1992) and an aliquot of mixed liquor was fixed in paraformaldehyde and stored at −20°C.

**Determination of nitrate production rates**
The nitrate production rate was determined in 500 mL batch reactors in which 200 mL of sludge was augmented with nitrite to a final concentration of 15 mg/L NO₂⁻N. The batch reactor was sampled every 15 min for 2 hr, samples were filtered (0.22 µm mixed cellulose esters Millipore filters), diluted 1:2 with milliQ water and analysed for NOₓ-N and NO₂-N using flow injection analysis (FIA). The nitrate production rate was determined in mg-NO₃-N/L/hr.

**Extraction of DNA and real-time PCR**
Genomic DNA was extracted from 250 µL of mixed liquor on collection day using the FastDNA spin kit (QBiogene, USA) and stored at −20°C until real-time PCR was carried out (Hall et al., 2002). Results were expressed as number *Nitrospira* cells per mg MLVSS with the assumption that each *Nitrospira* cell has one copy of the rrn operon.

**FISH and digital image analysis**
Samples were prepared (Daims et al., 2001b) and FISH probed (Manz et al., 1992) with EUBALL, specific for the domain bacteria (Daims et al., 1999) labelled with Cy5 which is excited by red light and emits in the infrared and Ntspa662 (Daims et al., 2001a) for *Nitrospira* sp. labelled with Cy3 which is excited by green light and emits in the red. After FISH, samples were observed and images collected using a BioRad Radiance 2000 confocal laser scanning microscope (CLSM) (Björnsson et al., 2002). The recording of images and set-up of microscope followed previously reported procedures (Daims et al., 2001b). For each quantification, 30 microscope fields at random positions and focal planes were recorded using a 40×, oil immersion 1.3 NA SFluor Nikon lens collecting 8-bit, 512 × 512 pixel images with an approximate 1 µm z-depth. Images were stored as RGB TIFF images and imported into the software Image Pro Plus 4 (Media Cybernetics). Several images were examined to determine the optimum threshold values for all channels and these values were then used to determine the area ratio for all 30 images in the sample. The countable pixel area of the specific Cy3 signal (Ntspa662) was then expressed as a mean percentage of the pixel area count from the Cy5 signal (EUBALL) for the 30 images.

**Results and discussion**
Wacol WWTP is an extended aeration system with an anoxic zone in a recirculation configuration designed for the removal of nitrogen and carbon, and operating with a sludge age of approximately 30 d. Oxley WWTP is a highly-loaded, single aeration tank configuration to achieve carbon removal only, with a sludge age of approximately 3–5 d. Wacol WWTP had an average MLVSS value of 1966 mg/L whereas Oxley WWTP had an average MLVSS of 658 mg/L, over the time period of sampling. Table 1 shows the average influent concentrations of NH₃-N and total Kjeldahl nitrogen (TKN) and effluent concentrations of NH₄-N, NO₂⁻N, NO₃⁻N and TKN. The nitrate production rates measured for both Wacol and Oxley WWTP are shown in subsequent figures in combination with data generated by the molecular methods (Figures 2, 3 and 4).
Real-time PCR involves the use of conventional PCR primers, nucleotides and thermostable DNA polymerase for the PCR. In addition there is an oligonucleotide with an attached fluorescent reporter and quencher whose close proximity obviates the capacity of the fluorochrome to emit light if excited. The fluorescently-labelled oligonucleotide hybridises to the target DNA between the PCR primer sites. During the PCR, the exocatalytic activity of the DNA polymerase cleaves the probe from the target sequence, separating the quencher from the fluorochrome, allowing the fluorochrome to be excited and to emit light. The emitted light is detected by a spectrophotometer, like the 7700-sequence detection system (PE Applied Biosystems, USA). The result is a curve, similar to that presented in Figure 1. The cycle threshold is then compared to a standard curve and the result is a numerical value of the number of target sequences in the sample.

Clones of *Nitrospira* 16S rDNA (Burrell et al., 1998) were used to standardise and optimise the *Nitrospira* real-time PCR (Hall et al., 2002) where data for 16S rDNA operons/mg MLVSS were generated and converted to numbers of *Nitrospira* cells/mg MLVSS assuming each *Nitrospira* cell has one *rrn* operon. Figure 2 shows the results for samples collected between the 20th September and 11th October, 2001. Several orders of magnitude variation in *Nitrospira* cells/mg MLVSS were observed from week to week in both plants. While such large swings in the microbial populations in that timeframe are unlikely they might be possible. However, there appears no correlation between the number of *Nitrospira* cells/mg MLVSS and the nitrate production rate (Figure 2). The latter data was generated using batch tests with nitrite addition and reflect the “potential nitrate production rate” of the sludge or more simply, the maximal nitrite oxidation activity of the biomass. This is different from the nitrite oxidation performance occurring at the plant during its operation, but directly reflects the combined measure of nitrite oxidising bacteria multiplied by their specific activity. Lack of accord between the numbers of *Nitrospira* in the

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**Table 1** Representative values for typical plant performance of Wacol WWTP and Oxley WWTP

<table>
<thead>
<tr>
<th>Influent (mg/L)</th>
<th>Effluent (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₃-N</td>
<td>TKN*</td>
</tr>
<tr>
<td>Wacol</td>
<td>42</td>
</tr>
<tr>
<td>Oxley</td>
<td>27</td>
</tr>
</tbody>
</table>

* TKN = total Kjeldahl nitrogen

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**Figure 1** Model of single amplification plot X axis – Cycle number $C_T$ – cycle threshold i.e. the point at which the amplification begins to be exponential. Y-axis – fluorescent signal
sludge and their activity may be due to varying activity of *Nitrospira* cells. Alternatively, the data from the real-time PCR may not be able to accurately quantify *Nitrospira* or organisms other than *Nitrospira* could be responsible for nitrite oxidation. Real-time PCR for other nitrite oxidisers was not developed.

The advantages of real-time PCR include its ability to detect a single copy of DNA (high sensitivity), no post-PCR analysis and specificity (Hall *et al.*, 2002). However, preliminary results suggest DNA extraction provides a major bias and that a single method for accurate reproducible DNA extraction from activated sludge is not yet possible (Hall *et al.*, 2002).

In FISH, fluorescently tagged oligonucleotide probes enter fixed bacterial cells, and, if the complementary nucleotide sequence to the probe is present inside the bacteria, the probe will be irreversibly trapped there. If no target is present, the probe is washed from the cell. The number of *Nitrospira* sp. as a percentage of the total bacteria was determined. However, it is difficult to convert this biovolume data to numbers of *Nitrospira*/mg MLVSS and therefore a direct comparison with the real-time PCR data cannot be made. Figures 3 (Wacol WWTP) and 4 (Oxley WWTP) show the FISH results superimposed with nitrite oxidation activity from the batch test (mg nitrate produced/L/hr).

The results from the Wacol WWTP (Figure 3) show good correlation between the abundance of *Nitrospira* and nitrite oxidation activity. Possible errors in the chemical analysis may account for a decrease in the rate, yet no corresponding drop in the abundance of *Nitrospira* (sample date 18/10). Nevertheless, the correlation between *Nitrospira* and nitrite oxidation is better when FISH was used for *Nitrospira* determination than when real-time PCR was used.

Figure 4 shows the combination of the results obtained from Oxley WWTP. Samples taken on dates from the 8th October to the 22nd October presented as zero percent indicate no *Nitrospira* cells were observed. Whilst no correlation can be made on three data points we can conclude that the Oxley WWTP has no significant population of *Nitrospira* and that the nitrite oxidation activity is significantly lower than at the Wacol WWTP.

FISH using rRNA-targeted oligonucleotides is frequently applied to a diverse range of samples. We showed that over a 4 week period we were able to monitor the population and the results correlated quite well with chemical analysis. An advantage of this analysis is, unlike PCR and other DNA based methods, FISH is not subject to method bias or inconsistencies such as were found in DNA extraction (Hall *et al.*, 2002) and it is very rare that inhibitors affect the result. The other advantage of FISH is that the method can be applied to
a wide range of microorganisms and samples. The use of CSLM, however, is essential in getting representative results from densely agglomerated microbial populations, such as the activated sludge flocs studied in this work.

Conclusions

In this study of microbial quantification in activated sludge we chose to look at the quantification of *Nitrospira* as a model to test two popular molecular methods, Real-Time PCR and FISH followed by digital image analysis. Studies by many groups have demonstrated the importance of *Nitrospira* to nitrite oxidation (Burrell et al., 1998; Hovanec et al., 1998; Juretschko et al., 1998) and significant insight to the community composition could be achieved through quantification of key members.

Real-Time PCR, while proving to be a highly sensitive and specific molecular method, is reliant on a reliable and accurate DNA extraction method. FISH and image analysis on the other hand is not dependent on such a method and has proven to be a more reliable method for monitoring NOB in activated sludge systems. But the next question is what are we trying to quantify? There are numerous microbial quantification methods and each will...
give us a different part of the story. We believe that the methods complement each other and should be used in combination to give operators and researchers a broader assessment of the activated sludge community.

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References