Use of P-17 and NOX specific primer sets for assimilable organic carbon (AOC) measurements

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Abstract In this study, two primer sets were at first designed specific to P-17 and NOX, the two bacterial species used for measurements of assimilable organic carbon (AOC) in water, based on their respective 16S rDNA sequences. These two primer sets were then used to measure the AOC content in 41 water samples by quantitative real time polymerase chain reaction (qRT-PCR). Results showed that P-17 and NOX cell numbers quantified by qRT-PCR using the designed primer sets were consistently higher (about 40.8% for P-17 and 54.8% for NOX measurements) than those by the traditional but time-consuming plate culture method. This proposed method not only saves the 5 days needed for plate culture, but also may detect AOC at concentrations as low as 0.3 μg-acetate-C/l and 0.6 μg-oxalate-C/l, which are lower than those measured by the plate culture and other methods. Using this new method, the water from the tap and a local reservoir were found containing 122 μg/l and 194 μg/l AOC, respectively, accounting for 5.7% and 7.8% of TOC.

Keywords AOC; assimilable organic carbon; molecular technique; NOX; P-17; qRT-PCR

Introduction

The growth potential of heterotrophic bacteria in the oligotrophic water distribution systems depends mainly on the availability of trace organic matters, the content of which is commonly expressed as assimilable organic carbon (AOC). Bacteria may grow in water with AOC as low as 2–5 μg/l (Van der Kooij, 1982). Instead of measuring the carbon content directly, AOC is measured indirectly from the growth of model bacterial species, as proposed by Van der Kooij (1982) and subsequently modified by others (Kemmy *et al.*, 1989; LeChevallier *et al.*, 1993). According to the *Standard Methods* (APHA, 1996), AOC of a water sample is calculated from the concentration increases of two model bacteria, i.e. *Pseudomonas fluorescens* strain P-17 and *Spirillum* strain NOX, using established calibration curves after 7–9 days of incubation followed by 5 additional days of plate culture. The culturing process is, however, not only time-consuming but also laborious (Kemmy *et al.*, 1989; LeChevallier *et al.*, 1993; Haddix *et al.*, 2004).

Numerous attempts have been made in the past two decades to simplify such an AOC measurement protocol. Instead of using the plate culture method, cell yields of P-17 and NOX may be quantified from the increases of ATP (adenosine triphosphate) concentration (LeChevallier *et al.*, 1993) or the bioluminescent intensity (CheckLight Ltd., Qiryat-Tiv'on., Israel., 2006). Although these modified methods are more time-saving, they are not applicable, due their limited sensitivity, for drinking water which may have AOC below 30 μg/l. In addition, these modified methods cannot distinguish P-17 and NOX cells, and thus may to cause erroneous AOC measurements (Van der Kooij *et al.*, 1982; Van der Kooij and Hijnen, 1984; Kaplan *et al.*, 1993; LeChevallier *et al.*, 1993; Frias *et al.*, 1994; Haddix *et al.*, 2004).

Quantitative real-time polymerase chain reaction (qRT-PCR) is a rapidly emerging method for the enumeration of microbes (Grüntzig *et al.*, 2001). Using this method, a

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selected DNA fragment is extracted and then multiplied by polymerase. Concentration of the PCR product is monitored throughout the amplification cycles using fluorescent reagents targeting either the double-stranded DNA or the specific DNA fragment. The fluorescent intensity, which reflects the amplicon concentration in real time, increases with the number of PCR cycle. The threshold cycle, C_t, at which the amplified target DNA concentration reaches the threshold detection level, is inversely proportional to the log value of the initial concentration of the target DNA. Based on a pre-calibrated standard curve, the initial concentration of the target DNA can be estimated from the C_t measurement. This method has so far been applied mostly to the quantification of pure cultures, such as toxigenic *Escherichia coli* (Oberst *et al.*, 1998) and phytopathogenic *Ralstonia solanacearum* (Weller *et al.*, 2000). However, its application for the quantification of uncultured bacteria, as many of those present in environmental samples, has still been very limited (Zhang and Fang, 2006).

This study was conducted to demonstrate the application of qRT-PCR to enumerate P-17 and NOX cells in the AOC measurement of water samples. After sequencing the DNA of P-17 and NOX, two specific primer sets specific respectively to these two species were designed. The AOC of a water sample can then be measured from the C_t values of P-17 and NOX using the pre-calibrated standard curves. This proposed method not only saves time but also are more accurate in cell counting, as compared to the traditional plate culture method. A total of 41 water samples were measured for AOC by both methods for comparison.

Materials and methods

Pseudomonas fluorescens P-17 and Spirillum strain NOX

Pure cultures of *Pseudomonas fluorescens* P-17 and *Spirillum* strain NOX purchased from Kiwa Water Research (The Netherlands) were stored at −70 °C in an aqueous solution containing 20% glycerol and 2% peptone (LeChevallier *et al.*, 1993; Haddix *et al.*, 2004). The respective stock solutions of P-17 and NOX were prepared following the *Standard Methods* (APHA, 1996). Cell concentrations of P-17 and NOX in individual stock solutions were determined by microscopic counting after 4′-6-diamidino-2-phenylindole (DAPI) staining.

Primer design and evaluation

The stock solutions were used as the templates for whole-cell PCR, using the *Eubacteria*-specific primer set of 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and the prokaryote universal primer 1490R (5′-GGTTACCTTGTTACGACTT-3′), to obtain full 16S rDNA sequences of P-17 and NOX. After purifying using the Promega kit (Madison, WI, USA), the 16S rDNA fragments were sequenced using an auto-sequencer (ABI model 377A, Perkin-Elmer Ltd., Foster City, CA) and the dRhodamine Terminator Cycle Sequencing FS Ready Reaction kit (Perkin-Elmer., Foster City, CA) using the primer sets of EUB8F, 1490R and 1055R (5′-CACGAGCTGACGACAGCCAT-3′).

The 16S rDNA sequences of P-17 and NOX were then compared with the closely related sequences in the GenBank by BLAST (http://www.ncbi.nlm.nih.gov/) and aligned them using the BioEdit (Hall, 1999). Two primers were designed targeting the 16S rDNA fragments specific to P-17 and NOX individually. The P-17 primer set includes a forward primer P17-F (5′-TAACGTTCGGAAACGGACGC-3′, T_m 62.0 °C and GC content 55.0%) and a reverse primer P17-R1 (5′-GCCACTAAGAGCTCAAGGCT-3′, T_m 62.0 °C and GC content = 55.0%). The NOX primer set includes a forward primer NOX-F (5′-GTCGGGACTTAATTGTCTTGGT-3′, T_m 58.5 °C and GC content 45.5%) and NOX-R (5′-CACCCTCTAATCTCTCAGAGG-3′, T_m 58.5 °C and GC content 52.4%).

PCR were then performed using iQ SYBR Green Supermix kit (Bio-Rad) using the designed primer set for either P-17 or NOX, and for the corresponding cell stock solution template following the Supermix Reaction Set Up protocols (Bio-Rad). iQ SYBR Green Supermix contains the non-specific fluorescence dye SYBR Green which has a high affinity for double-stranded DNA. Amplification began with an initial hold at 95 °C for 3 min, followed by 35 cycles of polymerase chain reactions, and an final extension at 72 °C for 1 min. Each cycle of polymerase chain reactions consisted of 95 °C for 30 s of denaturation, 30 s of annealing and 72 °C 30 s of extension. The optimal annealing temperature, judging from PCR efficiency, was identified as 54.4 °C for both primer sets after comparing PCR efficacies at six annealing temperatures ranging from 53.3 to 59.2 °C. The C_t values determined by the iCycler iQ PCR Detection System (Bio-Rad, USA) were then plotted against the Log_{10} values of starting cell numbers to establish the respective standard curves for P-17 and NOX over a concentration range of 32 folds.

AOC measurements based on population growth of P-17 and NOX

Water samples collected from the tap of local water supply and the Pokfulam reservoir were measured for their AOC concentrations for comparison by both the traditional plate culture and the proposed qRT-PCR methods. To avoid the interference from organics in the containers, all glassware used in this study for AOC measurements were heated to 180 °C to remove volatile organics (APHA, 1996; Charnock and Kjønnø, 2000). Residual chlorine in the tap water samples was removed by adding 1 ml 10% (w/v) sodium thiosulfate solution to each litre of water. The depletion of residual chlorine was subsequently confirmed by a portable spectrophotometer (Hach, DR2400, USA).

All water samples were first pasteurized at 70 °C for 30 min. Each sample (40 ml) was inoculated with 500 cells/ml each of P-17 and NOX in ten separate vials (45 ml each), nine of which used for AOC measurements and the remaining one served as blank control, and incubated following the *Standard Methods* (APHA, 1996). Samples (0.2 ml each) were taken from each vial on days 7, 8 and 9 of incubation and measured in parallel for cell concentrations by the plate culture and the qRT-PCR methods.

Comparison of quantifications of P-17 and NOX by plate culture and by qRT-PCR

A total of 41 sets of AOC measurement were conducted for water samples collected from the tap of local water supply system and from the Pokfulam Reservoir. Each set consisted of two AOC measurements, one by the plate culture method and the other by the qRT-PCR method using the designed primer sets, for comparison.

For each AOC measurement using the qRT-PCR method, P-17 and NOX cells in vial were quantified in triplicate to obtain an average C_t value. For each measurement using the plate culture method, water in each vial were diluted to three approximate cell concentrations (ca. 10, 10^2, 10^3 cells/ml) and incubated in duplicate at room temperature on plate of R2A medium. P-17 colonies (3–4 mm in diameter with diffuse yellow pigmentation) became visible after 3 days, while NOX colonies (1–2 mm in white dots) after 5 days. The colony numbers of P-17 and NOX were counted separately (APHA, 1996).

Measurements of AOC and total organic carbon

AOC concentration of a given water sample was calculated from the cell increases of P-17 and NOX, based on the *Standard Methods*, using the established cell yield values of 4.1×10^6 cells/μg-acetate-C for P-17 and 2.9×10^6 cells/μg-oxalate-C for NOX. The total organic carbon (TOC) concentrations of water samples were measured by a TOC Analyzer (Shimadzu TOC-5000, Japan) with a detection limit of 0.02 mg/l (Cinar, 2004).

Accession numbers

Nucleotide sequence data reported in this paper have been submitted to the GenBank, EMBL and DDBJ databases and assigned the following accession numbers: EF552157 (P-17) and EF552158 (NOX).

Results and discussions

Optimal annealing temperature and primer specificity

Six annealing temperatures ranging from 53.3 to 59.2 °C were tested using the gradient function of qRT-PCR machine for the two primer sets. For each annealing temperature, a standard curve was established for 5 concentrations over a range of 32 folds. Results showed that the highest PCR efficiencies (71% for P-17, and 84.4% for NOX) were achieved at the annealing temperature of 54.4 °C, which was used in the subsequent study.

In order to check if there was any possible interference of NOX on the quantification of P-17, a test was conducted by adding the P-17 primer set to the NOX template for PCR amplification. The negative result confirmed the specificity of the P-17 primer set, as evidenced by the lack of NOX amplification by PCR. Similarly, the specificity of the NOX primer set was also confirmed by another test using NOX primer set in P-17 template amplification. These results show that the co-presence of NOX and P-17 in a water sample does not interfere with the quantification of these two individual species by the qRT-PCR method.

PCR efficiency and detection limit

A set of standard curves plotting C_t against the Log_{10} value of initial cell numbers for P-17 and NOX has to be established for each set of measurements using qRT-PCR. The standard curve should be linear, as illustrated in Figure 1, and the slope of which represents the PCR efficiency (Zhang and Fang, 2006). A total of 12 sets standard curves, similar to Figure 1, were plotted. Results show a high degree of linearity for both P-17 ($R^2 = 0.986–0.997$) and NOX ($R^2 = 0.994–0.999$). The PCR efficiencies were 71.0–79.0% (averaging 75.1%) for P-17 and 80.3–89.8% (averaging 84.4%) for NOX.

Using the cell yield coefficients given by *Standard Methods* (APHA, 1996), i.e. 4.1×10^6 cells/μg-acetate-C for P-17 and 2.9×10^6 cells/μg-oxalate-C for NOX, the AOC concentrations were estimated from the C_t values of P-17 and NOX using the standard curves. As shown in Figure 1, qRT-PCR may detect AOC at concentrations as low as 0.3 μg-acetate-C/l and 0.6 μg-oxalate-C/l, which are lower than the 1 μg-AOC/l measured by the conventional plate culture method (APHA, 2005), the 30 μg-acetate-C/l

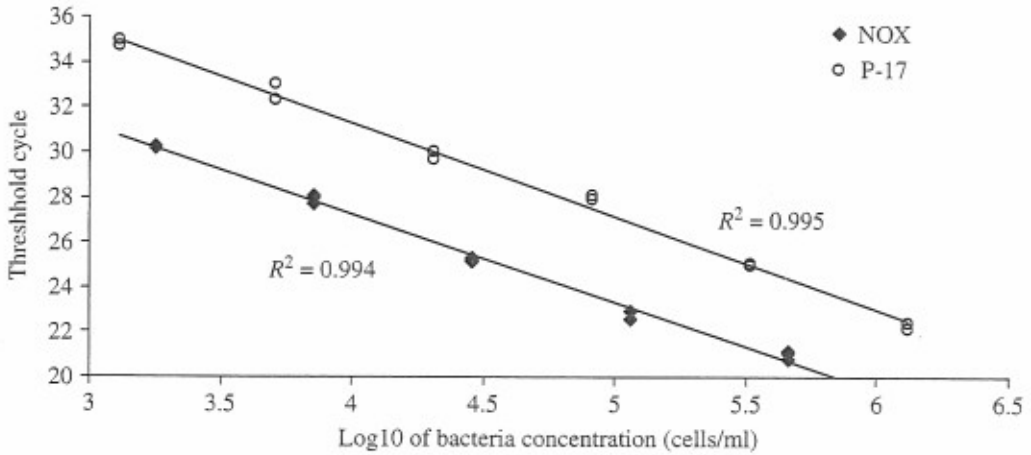

Figure 1 Typical standard curves for P-17 and NOX

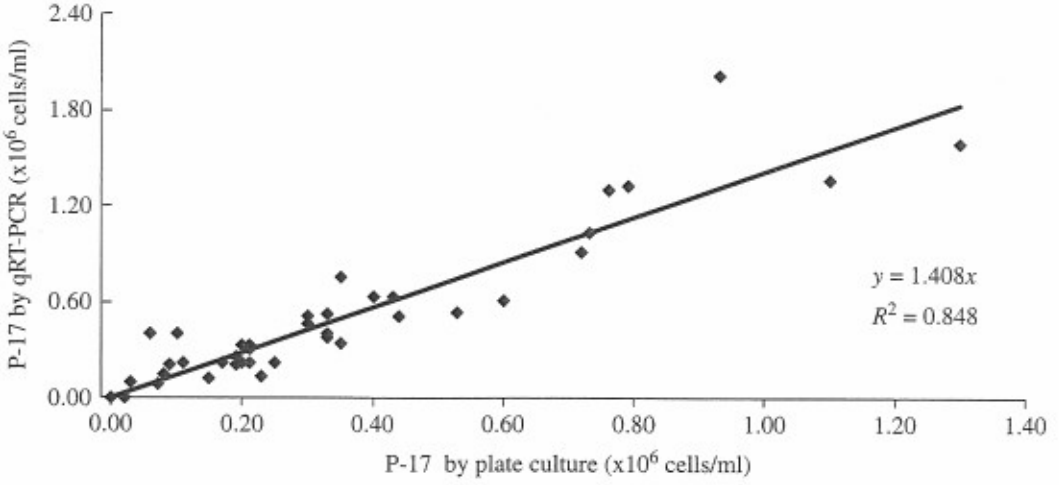

Figure 2 Comparison of P-17 cell concentrations measured by qRT-PCR and plate culture methods ($N = 41$)

reported by bioluminescent intensity (Haddix *et al.*, 2004), and the 1.5 μg-acetate-C/l and 4 μg-oxalate-C/l reported by ATP (LeChevallier *et al.*, 1993).

Since the default threshold fluorescent intensity is automatically set by the qRT-PCR instrument, the C_t values may vary slightly for the same solution if measured days apart. This may result in a slight shift of the standard curve. It is thus advised to update the standard curves periodically for AOC measurements.

Comparison of qRT-PCR and plate culture methods

A total of 41 water samples were measured for their NOX and P-17 concentrations after incubation, as required in AOC measurements, by both the qRT-PCR using the designed primer sets and the conventional plate culture method. Results are compared in Figures 2 and 3 for P-17 and NOX measurements.

Figures 2 and 3 show that in general cell measurements based on qRT-PCR were linearly correlated with those based on plate culture (with R^2 of 0.848 and 0.873 for P-17 and NOX, respectively). Based on the slopes in Figures 2 and 3, measurements based on qRT-PCR were on average 40.8% higher than those measured by plate culture for P17 and 54.8% higher for NOX. This is due to the underestimation of total cell numbers by the plate culture method, which counts viable cells only, but not the non-viable ones

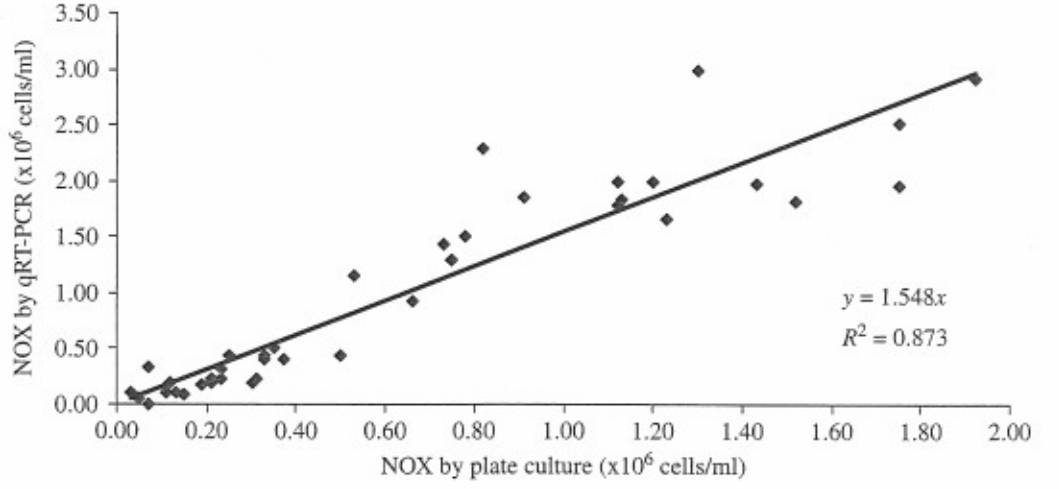

Figure 3 Comparison of NOX cell concentrations measured by qRT-PCR and plate culture methods ($N = 41$)

Table 1 AOC measurements of water samples from the tap and Pokfulam Reservoir ($N = 3$)

Water source	Method	AOC (μg/l)			TOC (mg/l)	AOC/TOC
		AOC_{P-17}	AOC_{NOX}	AOC_{Total}		
Tap	qRT-PCR	65 ± 17	57 ± 16	122 ± 23	2.15 ± 0.06	5.7%
	Plate culture	49 ± 17	38 ± 18	87 ± 27	2.15 ± 0.06	4.0%
Reservoir	qRT-PCR	130 ± 30	64 ± 14	194 ± 18	3.01 ± 0.09	6.4%
	Plate culture	100 ± 70	39 ± 10	139 ± 75	3.01 ± 0.09	4.6%

(LeChevallier *et al.*, 1993). On the other hand, qRT-PCR counts both viable and non-viable cells.

Table 1 summarizes AOC measurements of water samples collected from the tap of local water supply and the Pokfulam Reservoir by both qRT-PCR and plate culture methods, as well as the TOC measurement for comparison. It shows that AOC measurements by qRT-PCR were about 40% higher those by the traditional plate culture method. According to the qRT-PCR results, the tap water in Hong Kong contained 122 μg/l AOC, which accounts for 5.7% of the 2.15 mg/l of TOC. Water in the Pokfulam Reservoir contained 194 μg/l AOC, which accounts for 6.4% of the 3.01 mg/l of TOC. For comparison, AOC concentrations in tap and river water were reported ranging from 50 μg/l to 250 μg/l and TOC concentrations from 2 to 4 mg/l (Liu *et al.*, 2002; Museus and Khan, 2006).

Conclusion

This study successfully demonstrated the use of newly designed primer sets of P-17 and NOX for AOC measurements by qRT-PCR. P-17 and NOX cell concentrations quantified by qRT-PCR were consistently higher (about 40.8% for P-17 and 54.8% for NOX measurements) than those by the traditional but time-consuming plate culture method. Using this new method, the water from the tap and a local reservoir were found containing 122 μg/l and 194 μg/l AOC, respectively, accounting for 5.7% and 6.4% of TOC. Compared to the plate culture and other methods, the qRT-PCR method not only saves time, but also may detect AOC at lower concentrations, i.e. as low as 0.3 μg-acetate-C/l and 0.6 μg-oxalate-C/l.

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