Appl Microbiol Biotechnol (2006) 70: 281–289
DOI 10.1007/s00253-006-0333-6

MINI-REVIEW

Tong Zhang · Herbert H. P. Fang

Applications of real-time polymerase chain reaction for quantification of microorganisms in environmental samples

Received: 1 December 2005 / Revised: 9 January 2006 / Accepted: 10 January 2006 / Published online: 10 February 2006
© Springer-Verlag 2006

Abstract Due to the advanced development of fluorogenic chemistry, quantitative real-time polymerase chain reaction (qRT-PCR) has become an emerging technique for the detection and quantification of microorganisms in the environment. Compared with the conventional hybridization- and PCR-based techniques, qRT-PCR not only has better sensitivity and reproducibility, but it is also quicker to perform and has a minimum risk of amplicon carryover contamination. This article reviews the principle of this emerging technique, its detection reagents, target DNAs, quantification procedures, and affecting factors. The applications of qRT-PCR for the quantification of microorganisms in the environment are also summarized.

Introduction

Analytical techniques targeting 16S rDNA or functional genes were widely used for microbial quantification. These include hybridization-based techniques, such as membrane hybridization (Raskin et al. 1995) and fluorescence in situ hybridization (FISH) (Okabe et al. 1999) as well as polymerase chain reaction (PCR)-based techniques, such as denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1993) and cloning-sequencing (Zhang and Fang 2001; Zhang et al. 2003a). Hybridization methods, which have detection limits in the order of 10^5 DNA/RNA copies or greater, are in general less sensitive. They can thus only be used for environmental samples of relatively high microbial concentrations. PCR-based methods, on the other hand, are capable of detecting DNA/RNA at low concentrations. However, the precision of PCR-based methods may be compromised due to a number of factors. These may include reagent depletion, competition of amplicons with primers, and the loss of polymerase activity as the number of amplification cycle increases (Schneegurt and Kulpa 1998). To overcome such deficiency, a new technique known as quantitative real-time PCR (qRT-PCR) has emerged for the detection and quantification of microorganisms at low concentrations.

The qRT-PCR method monitors the amount of PCR products of DNA as they are amplified in real time (Higuchi et al. 1993; Heid et al. 1996; Gibson et al. 1996). From the change of PCR product concentration throughout the amplification cycles, the initial concentration of the target DNA/RNA can be estimated. The first qRT-PCR instrument was commercialized in 1997 (DeFrancesco 2003) soon after the concept was demonstrated in 1993 (Higuchi et al. 1993). Since then, qRT-PCR was widely applied in medical research (Klein 2002; Ginzinger 2002). This article reviews the principle of qRT-PCR, fluorescent reagents and quantification procedures, and its applications for the quantification of microorganisms in environmental samples.

Principle

During PCR, the target DNA sequence is amplified over a number of denaturation–annealing–extension cycles. In a conventional PCR, only the final concentration of the amplicon may be monitored using a DNA-binding fluorescent dye. However, in the qRT-PCR, the concentration of the amplicon is monitored throughout the amplification cycles using a group of new fluorescent reagents. These reagents bind with the amplicon without causing damage at the end of each cycle so that amplification may continue to proceed. The fluorescence intensity emitted during this process reflects the amplicon concentration in real time.

Figure 1a are the plots of fluorescence intensity throughout the amplification cycles in whole cell PCR, using a series of five standard solutions as examples. These solutions contained *Microcystis aeruginosa* PCC 7820 at

T. Zhang · H. H. P. Fang (✉)
Environmental Biotechnology Laboratory,
Department of Civil Engineering,
The University of Hong Kong,
Pokfulam Road,
Hong Kong, China
e-mail: hrechef@hkucc.hku.hk
Tel.: +86-852-28592660
Fax: +86-852-25595337

varied concentrations from 4.6×10^3 to 4.6×10^7 cells/ml. The threshold cycle C_t is defined as the cycle at which the fluorescence intensity crosses over a level where the amplification enters a logarithmic growth phase. C_t is inversely proportional to the log value of the initial concentration of the target, as illustrated in Fig. 1b for *M. aeruginosa* PCC 7820. Based on the standard curve like Fig. 1b, concentration of the target DNA in an environmental sample can be estimated from its C_t measurement in qRT-PCR.

Figure 1a also shows that as the number of PCR cycle further increases, the amplification efficiency reduces gradually. This is due to a number of factors including the depletion of reagents, the inhibition caused by the increased product concentration, and the gradual loss of polymerase activity. Thus, amplicon concentration and the corresponding fluorescence intensity beyond a certain number of amplification cycles cannot be used to estimate the initial concentration of the target DNA (Schneegurt and Kulpa 1998). Figure 1a shows that the fluorescent intensities of the five standard solutions after 50 cycles were nearly the same despite the differences of their initial concentrations.

Detection reagents

Fluorescent reagents used for qRT-PCR can be classified into three categories: (1) dyes that bind with double-stranded DNA (dsDNA) (Hernández et al. 2004); (2) DNA-sequence specific probes, including TaqMan probe (Haugland et al. 1999), molecular beacon (Briones and Raskin 2003), and dual hybridization probe (Glazer and Mathies 1997; Reina et al. 2005); and (3) DNA sequence-specific primers including Amplifluor primer (Hernández et al. 2004), scorpion primer (Taveau et al. 2002), Light Upon eXtension (LUX) primer (Donia and Pana 2005), and universal template (Zhang et al. 2003b). Among these fluorescent reagents, SYBR Green I, TaqMan probe, and molecular beacon, are the most widely used in qRT-PCR applications of environmental samples.

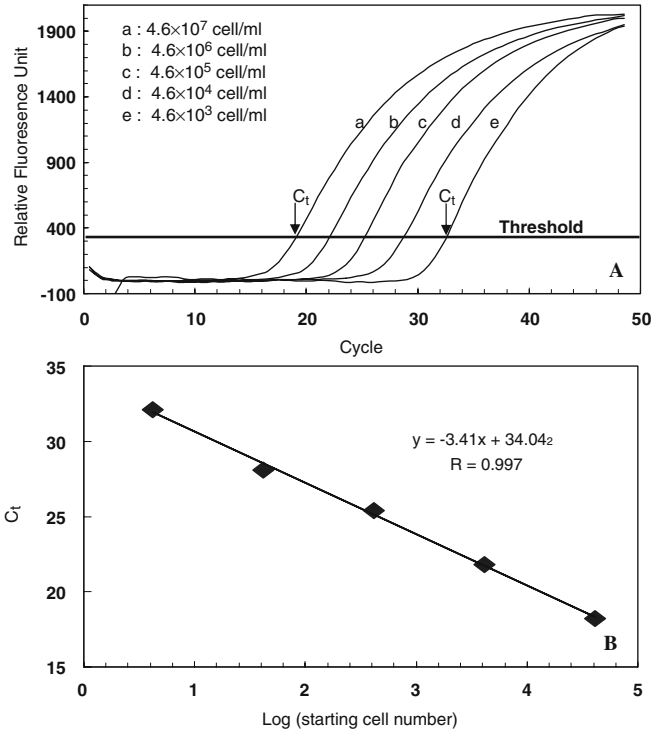

Fig. 1 Quantification of *Microcystis aeruginosa* PCC 7820 using qRT-PCR. **a** Relative fluorescence intensity of five standard solutions of PCC 7820 throughout amplification cycles where C_t represents the threshold cycle number. **b** The standard curve for qRT-PCR measurement of PCC 7820

dsDNA-binding dyes

During the extension phase in the PCR cycle, dsDNA are synthesized from the denatured single-stranded DNA. The fluorescence intensity of a dsDNA-binding dye increases as a result when the dye binds with the increased amount of amplified dsDNA. Such an increase can be monitored in real time (Morrison et al. 1998). For qRT-PCR applications, the fluorescence intensity of the dye has to be substantially increased when it binds with the dsDNA from its autofluorescence intensity. For example, the fluorescent intensities of ethidium bromide, i.e., the first dsDNA-binding dye used for qRT-PCR (Higuchi et al. 1993), increase 25 times after binding with dsDNA; whereas the intensity of SYBR Green I, i.e., the most commonly used dsDNA-binding dye (Nadkarni et al. 2002; Stubner 2002; López-Gutiérrez et al. 2004), increases 200 times. Other dsDNA-binding dyes used include BEBO (Bengtsson et al. 2003) and Thiazole Orange (Benveniste et al. 1996).

There are three most notable advantages of the dsDNA-binding dye: (1) it is simple to use without the complicated requirement for the design of a probe; (2) it can be used in conjunction with any PCR primer set; and (3) it is less costly compared to hybridization probes, such as TaqMan probe and molecular beacon. This technique, on the other hand, has its limitations (Sharkey et al. 2004) resulting from: (1) its inability to discriminate different dsDNA segments because the dye binds with all dsDNA indiscriminately; (2) the formation of primer dimmers, which may affect its detection sensitivity; and (3) the mistaken amplification of nontarget DNA segments. Thus, an accurate primer design and the optimization of the PCR conditions are crucial when DNA-binding dyes are used.

Table 1 contains the list of primers used in SYBR Green I qRT-PCR for environmental samples. It should be noted that most primer pairs might produce unspecific products when applied to complex environmental samples of high microbial diversity. This may partly invalidate applications of qRT-PCR using SYBR Green I as detection reagent. Thus, an additional melting curve analysis is often required to ensure the quality of the amplified product (Zhang and Fang 2005; Bischoff et al. 2005).

Table 1 Primers and probes used in qRT-PCR related to environmental microbiology

Target	Forward Primer / Reverse Primer	TaqMan Probe[a]	Reference
Prokaryote 16S rDNA	Uni340F / Uni806R	Uni516F	Takai and Horikoshi 2000
Eubacteria 16S rDNA	519f / 907r		Stubner and Meuser 2000
	1055f / 1392r	16STaq1115	Dionisi et al. 2003
	Bac349F / Bac806R	Bac516F	Takai and Horikoshi 2000
	BACT1369F / PROK1541R (or 1492R)	TM1389F	Suzuki et al. 2000
Archaea 16S rDNA			
Archaea	Arch349F / Arch806R	Arch516F	Takai and Horikoshi 2000
Archaea	ARCHMIX1369F / PROK1541R	TM1389F	Suzuki et al. 2000
Marine *Archaea* Group I	ARCHGI334F / ARCHGI554R	TM519AR	Suzuki et al. 2000
Marine *Archaea* Group II	ARCHGI333F / ARCHGI554R	TM519AR	Suzuki et al. 2000
Methanobacterium	Mbac-0398f / Mbac-0578r	Taq-Mbac-0526	Sawayama et al. 2006
Methanothermobacter	Mbac-0398f / Mthb-0589r	Taq-Mthb-0549	Sawayama et al. 2006
Methanosarcina	Msar-0450f / Msar-0589r	Taq-Msar-0540	Sawayama et al. 2006
Methanosaeta	Msae-0387f / Msae-0573r	Taq-Msae-0540	Sawayama et al. 2006
Methanotrophs *pmo*A gene			
Methylobacter/Methylosarcina	A189F / Mb601R		Kolb et al. 2003
Methylococcus	A189F / Mc468R		Kolb et al. 2003
Methylosinus	II223F / II646R		Kolb et al. 2003
Methylocapsa	A189F / Mcap630		Kolb et al. 2003
Nitrifying bacteria			
N oligotropha like *amo*A	*amo*No550D2f / *amo*No754r	*amo*NoTaq729	Harms et al. 2003; Okano et al. 2004
AOB 16S rDNA	(CTO 189fA/B and CTO 189fC) / RT1r	TMP1	Hermansson and Lindgren 2001
Nitrospira 16S rDNA	NSR1113f / NSR1264r	Taq-NSR1143	Harms et al. 2003; Dionisi et al. 2002
Denitrifying bacteria (nitrate reductase gene)			
Gene *nar*G	*nar*G 1960m2f / *nar*G 2050m2r		López-Gutiérrez et al. 2004
Gene *nir*K	*nir*K876 / *nir*K1040		Sonia et al. 2004
Sulfate-reducing bacteria 16S rDNA			
Desulfovibrionaceae	9/27f / DSVIB679r		Stubner 2004
Desulfobacteraceae	DSBAC355f / 519r		Scheid and Stubner 2001
Desulfobulbus	DSBb279f / 519r		Stubner 2004
Desulfomicrobium	DSV-IV-214f / 519r		Manz et al. 1998
Desulfobacter	519f / DSB985r		Manz et al. 1998
Desulfobacterium	DSB219f / 907r		Devereux et al. 1992
Desulforhabdus/Synthrophobacter	519f / DSRhab1037r		Stubner 2004
Desulfotomaculum lineage 1	DEM116f / DEM1164r		Stubner 2002
Desulfotomaculum clones	519f / DEM-RC1015r		Stubner 2002
Desulfuromonas acetoxidans	519f / D-acet1027r		Stubner 2002
Dissimilatory sulfite reductase gene	DSR3FRExt / DSR1Fext		Tang et al. 2004
Cyanobacteria			
Cyanobacteria 16s rDNA	CYAN108F / CYAN 377R	Taq-CYAN328R*	Rinta-Kanto et al. 2005
Microcystis 16S rDNA	MICR 184F / MICR 431R	Taq-MICR228F*	Rinta-Kanto et al. 2005
Toxin synthase gene *mcy*B	*mcy*B 2959F / *mcy*B 3278R		Nonneman and Zimba 2002
Toxin synthase gene *mcy*D	*mcy*D F2 / *mcy*D R2	Taq-mcyD F2*	Rinta-Kanto et al. 2005
Pollutant degraders			
MTBE-degrader 16S rDNA	963f / 1076r	Taq-1030	Hristova et al. 2001
hydrocarbon-degrader *bss*A gene	*bss*Af / *bss*Ar	Taq-*bss*A	Beller et al. 2002
Trichloroethylene-degrader *mmo*C gene	SF-1 / SR-3	C821	Kikuchi et al. 2002

[a]All TaqMan probes were labeled with FAM at the 5′ end. All TaqMan probes were labeled with TAMRA at the 3′ end except for those marked with asterisk (*), which were labeled with BHQ. Those not using TaqMan probe used SYBR Green I

TaqMan probe

The TaqMan probe is a linear oligonucleotide with a 5′ end fluorophore (called reporter) and a 3′ end quencher. The fluorescence emitted by the reporter is greatly dimmed by the nearby quencher, which disperses energy as heat rather than fluorescence via a fluorescence resonance energy transfer (FRET) mechanism. Figure 2 illustrates the role of TaqMan probe in a qRT-PCR amplification cycle. After DNA denaturation, TaqMan probe is hybridized with the target DNA segment prior to annealing. During the subsequent extension phase, *Taq* DNA polymerase hydrolyzes the TaqMan probe bound on the target DNA strand, cleaving nucleotides from the probe and releasing the reporter into the solution. This results in a drastic increase of reporter's fluorescence intensity due to its disconnection with the quencher on the probe. As PCR proceeds, the fluorescence intensity increases proportionally with the amount of the amplicons (Holland et al. 1991; Heid et al. 1996).

Reporter fluorophores commonly used include FAM (6-carboxyfluoroscein), TET (tetrachloro-6-carboxyfluorescein), JOE (2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein), VIC, Cy3, Cy5, Texas Red, and HEX (hexacholoro-6-carboxyfluorescein). There are a number of quenchers available, the most common of which being TAMRA (6-carboxytetramethylrhodamine). Two new quenchers were recently introduced to replace TAMRA: minor groove binding (MGB) quencher and black hole quencher (BHQ). They have lower fluorescence background, higher sensitivity, higher hybridization stability, better resolution of fluorescence spectrum, longer storage life, plus higher reproducibility and specificity (Wilson et al. 2005). However, it should be noted that TaqMan technique, as compared to SYBR Green I, is more costly, more difficult in probe design, and limited to the detection of shorter PCR product, normally less than 150 bps.

Molecular beacon

Like the TaqMan probe, molecular beacon is also a fluorescent oligonucleotide with a 5′ end reporter and a 3′ end quencher. However, unlike TaqMan probe, molecular beacon has a special "hairpin" structure as illustrated in Fig. 3. As a result, the fluorescence emitted by the reporter is greatly suppressed by the quencher due to their close proximity (Tyagi and Kramer 1996). Upon hybridization with the target denatured DNA segment during annealing, the reporter's fluorescence intensity increases as its distance from the quencher increases. The fluorescence intensity thus becomes the indicator of the target DNA concentration. As temperature increases during the subsequent extension, molecular beacon detaches from the DNA segment, retaining its hairpin structure, and can rebind to the target DNA segment in the next cycle.

In addition, molecular beacon has a very high specificity, i.e., it requires a perfect hybridization with the target due to the high thermal stability of its hairpin structure. It is able to discriminate DNA sequences that differ by a single nucleotide substitution. It is thus suitable for mutation analysis and for single nucleotide polymorphism detection. On the other hand, design of a molecular beacon and its detection conditions are very demanding.

Molecular beacon has been used for gene expression (Taveau et al. 2002), single-nucleotide polymorphism detection (Mhlanga and Malmberg 2001), and pathogen detection (Belanger et al. 2003). Its application to environmental sample analysis is still very limited (Harms et al. 2003). Similar to TaqMan probe, molecular beacon is costly and often used for the detection of short PCR product. However, due to its complex configuration and required binding property, molecular beacon is more difficult to design than the TaqMan probe.

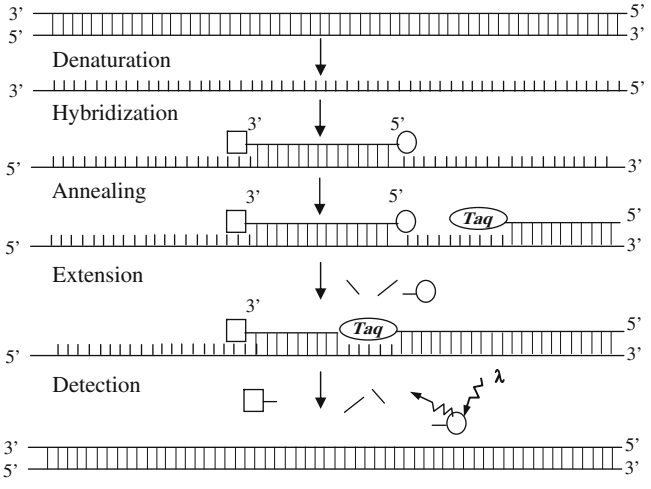

Fig. 2 The role of TaqMan probe in a qRT-PCR amplification cycle (reporter ○, quencher □)

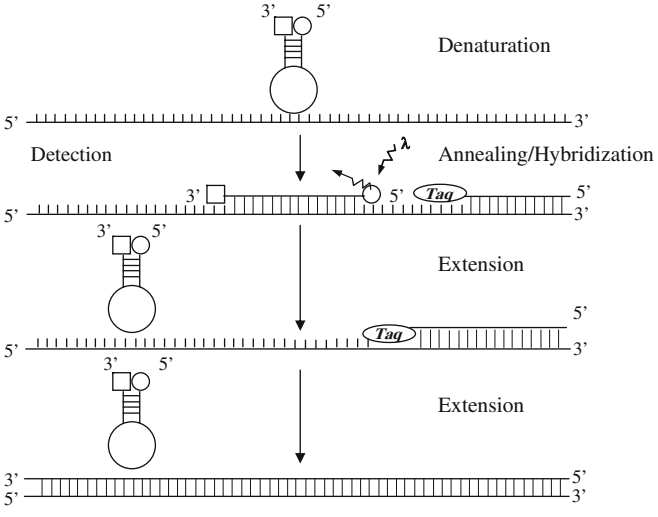

Fig. 3 The role of molecular beacon in a qRT-PCR amplification cycle (reporter ○, quencher □)

Target DNA sequences

16S rDNA and functional genes are the most common target DNA sequences for microbial quantification using qRT-PCR. Although other DNA sequences, such as 5S rDNA, 23S rDNA, and 16S-23S rDNA interspacer region can also be used as targets, their applications are very limited so far.

16S rDNA

16S rDNA contains conserved and variable regions according to their genetic stability. Based on variations of DNA sequences in these regions, organisms can be classified into different taxonomy levels. Organisms within a domain often share the same DNA sequence in the most conserved region, whereas species of the same genus may be discriminated from DNA sequence in the variable regions (Woese et al. 1990; Skovhus et al. 2004). qRT-PCR probes and primers may be designed either basing on the more than 150,000 16S rDNA sequences available in the genetic databases, such as GenBank, RDP, and ARB, or by modifying the 1,200 primers and FISH probes available in the probeBase (http://www.microbial-ecology.net/probebase/).

Functional genes

Functional genes (Wagner and Loy 2002) shared by microorganisms of similar physiological characteristics can also be used as target in qRT-PCR. Currently, more than 14,000 DNA sequences are known for more than 100 functional genes. There are many functional genes that may be used for environmental applications. These include those of nitrification/denitrification (Purkhold et al. 2000; López-Gutiérrez et al. 2004; Okano et al. 2004), nitrogen fixation (Gruntzig et al. 2001; Qiu et al. 2004), carbon dioxide fixation (Wu et al. 2001), polymer degradation (Wu et al. 2001), dissimilatory sulfate reduction (Wagner et al. 1998), methane oxidation (Maria et al. 2002), methane production (Hales et al. 1996), organic contaminant degradation (Rhee et al. 2004), metals reduction (Wu et al. 2001), and algal toxin production (Foulds et al. 2002; Rinta-Kanto et al. 2005).

Quantification

Standard curve

For a qRT-PCR analysis, a standard curve like Fig. 1b is needed for each target 16S rDNA or gene. Such a curve may be constructed from serial dilutions of a given source, which could either be cells, which are extracted genomic DNA of a pure culture or plasmids with the target DNA insert, or PCR-amplified DNA segments. Cells in the serial standard solutions can be quantified by plate counting or microscopic counting. Genomic DNA, plasmids, and PCR product may be quantified by spectrophotometry at 260 nm or by kits using dsDNA-binding dyes such as PicoGreen.

The precision of microbial quantification using qRT-PCR relies on the assumption that the environmental sample and the standard solutions share the same PCR efficiency. It is thus crucial to check the PCR efficiency in the analysis of both standard solutions and environmental samples (Devers et al. 2004). For a PCR process,

$$N_n = N_o \times \left(1 + \eta/100\%\right)^n$$

where N_n is number of amplified target at the end of nth cycle of amplification, N_o the initial number of target, and η the PCR efficiency (Abell and Bowman 2005). At 100% efficiency, two DNA segments are produced from a target segment in each PCR cycle. The value of threshold cycle C_t can then be expressed as:

$$C_t = (\log N_t - \log N_o)/\log\left(1 + \eta/100\%\right)$$

where N_t is the number of amplified target after the threshold cycle C_t. The slope (S) of a standard curve of C_t vs log N_o, as illustrated in Fig. 1b, equals to $-1/\log\left(1 + \eta/100\%\right)$. The slope of an ideal PCR amplification is thus equal to −3.32 (i.e., −1/log 2). In practice, a reliable standard curve should have a R^2 value of more than 0.95 and a slope between −3.0 and −3.9 corresponding to PCR efficiencies of 80–115%. The standard cure in Fig. 1b shows a slope of −3.41 corresponding to an efficiency of 96% and a R^2 of 0.997.

Detection limit, dynamic range, and factors affecting quantification

The dynamic range refers to the range in which the target quantity is linear with C_t in the standard curve (van der Velden et al. 2003). Probes, primers, and PCR conditions should be optimized not just for a low detection limit but also for a broad dynamic range (Panicker et al. 2004; Ponchel et al. 2003). Under the most optimum conditions, SYBR Green I and TaqMan probe may have a detection limit as low as two cells per PCR sample and a dynamic range of 10^7 (Bassler et al. 1995: Chen et al. 1997).

The precision of qRT-PCR analysis may be affected by PCR inhibitors present in the environmental sample. Ideally, each sample should be serially diluted and tested to determine PCR efficiency to see if there are any PCR inhibitors present (Stubner 2002; Harms et al. 2003). The effect of the PCR inhibitor may be reduced by conducting the test at higher degrees of dilution (Stubner 2002; Harms et al. 2003). Careful handling and storage of sample, primers, probes, and enzymes are crucial to the accuracy of microbial quantification. In addition, the DNA extraction efficiency, which may vary from batch to batch, has a significant effect on the reliability of qRT-PCR (Dionisi et al. 2003).

Applications in environmental samples analysis

Early qRT-PCR applications were mainly for the detection and quantification of pathogens, such as *Salmonella* spp. (Chen et al. 1997), toxigenic *Escherichia coli* (Oberst et al. 1998), *Stachybotrys chartarum* (Haugland et al. 1999), *Ehrlichia* spp. (Leutenegger et al. 1999), *Listeria monocytogenes* (Bassler et al. 1995; Nogva et al. 2000), and *Vibrio cholerae* (Lyon 2001). More recently, applications of qRT-PCR were extended to environmental samples' analysis as summarized in Table 1.

Eubacteria and *Archaea*

As shown in Table 1, a universal PCR primer set and a universal probe were designed for prokaryote, i.e., *Eubacteria* and *Archaea* (Takai and Horikoshi 2000). In addition, four probes/primers were designed for *Eubacteria* (Stubner and Meuser 2000; Dionisi et al. 2003; Takai and Horikoshi 2000; Suzuki et al. 2000) and two for *Archaea* (Takai and Horikoshi 2000; Suzuki et al. 2000). Nadkarni et al. (2002) showed that the microbial quantity in an anaerobic sample measured by qRT-PCR using a universal probe was 40-fold greater than that measured by the conventional culture methods.

Subgroups in *Archaea*

In addition to the qRT-PCR primer/probe set for the whole domain of *Archaea*, several primer/probe sets were developed, which are specific for different *Archaea* subgroups (Table 1). Using these sets, Sawayama et al. (2006) showed that *Methanosarcina* spp. and *Methanobacterium* spp. immobilized in the polyurethane foam in a reactor were 7.6×10^{9} and 2.6×10^{8} DNA-segment/ml, respectively, about 1,000 times higher than those in the original anaerobically digested sludge.

Methanotrophic bacteria

Methane oxidation in the environment is mostly accomplished by methanotrophic bacteria. As shown in Table 1, primer sets were developed to target the *pmo*A gene, which encodes the α subunit of particulate methane monooxygenase of four methanotroph groups: *Methylococcus*, *Methylobacter/Methylosarcina*, *Methylosinus*, and *Methylocapsa* (Kolb et al. 2003). Detection limits were between 10 and 100 cells per PCR sample. qRT-PCR analysis of soil samples spiked with these four bacteria recovered only 20% of *Methylosinus*, but almost all the added cells of the other three groups.

Nitrifying bacteria

Ammonium oxidation by autotrophic ammonia-oxidizing bacteria (AOB) is a key process in agricultural/natural ecosystems and wastewater treatment (Jordan et al. 2005). Using qRT-PCR based on 16S rDNA, the AOB concentration in a fertilized soil was found as 6.2×10^{7} cells/g, three times higher than that in the unfertilized soil (Hermansson and Lindgren 2001). AOB population in soil may also be quantified by qRT-PCR targeting the ammonia-monooxygenase gene (*amo*A) (Okano et al. 2004) with a detection limit of 1.3×10^{5} cells/g.

qRT-PCR was also used to monitor the nitrifying bacteria population dynamics in wastewater treatment plants (Hall et al. 2002; Harms et al. 2003; Limpiyakorna et al. 2005). In 12 activated sludge samples collected over a year, the mean concentrations were $4.3\pm2.0\times10^{11}$ cells/l for *Eubacteria*, $3.7\pm3.2\times10^{10}$ cells/l for *Nitrospira*, $1.2\pm0.9\times10^{10}$ cells/l for all AOB, and $7.5\pm6.0\times10^{9}$ cells/l for *N. oligotropha*-like AOB (Harms et al. 2003). Ammonia-oxidizing rates were estimated as $7.7–12.4\times10^{-12}$ mol/(h·cell), comparable to the $0.5–25.0\times10^{-12}$ mol/(h·cell) found in soil (Okano et al. 2004). The qRT-PCR standard curves for *Nitrospira* 16S rDNA, AOB 16S rDNA, and *N. oligotropha*-like *amo*A were linear over six orders of magnitude and that of *Eubacteria* 16S rDNA was linear over four orders of magnitude.

Denitrifying bacteria

Denitrification is performed by phylogenetically diverse groups of bacteria. It is thus difficult to design a universal primer/probe set for denitrifiers based on 16S rDNA. Recently, qRT-PCR was developed to quantify denitrifier based on functional genes, such as gene *nar*G encoding the α subunit of the membrane-bound nitrate reductase (López-Gutiérrez et al. 2004) and gene *nir*K encoding nitrite reductase (Sonia et al. 2004). The qRT-PCR standard curves were linear over seven orders of magnitude and with a sensitivity of 100 DNA target segments per PCR sample. The results also showed that *nar*G quantity ranged between 5.08×10^{8} and 1.12×10^{11} DNA-segment/g-soil (López-Gutiérrez et al. 2004), far higher than the *nir*K quantity of 9.7×10^{4} to 3.9×10^{6} DNA-segment/g-soil (Sonia et al. 2004).

Sulfate-reducing bacteria

Sulfate-reducing bacteria (SRB) is a group of polyphyletic microorganisms. Due to the well-established phylogenetic database of 16S rDNA, many qRT-PCR primer/probe sets specific for different classification levels (down to species) are available for SRB, as shown in Table 1. The qRT-PCR analysis for gram-positive SRB *Desulfotomaculum* lineage 1 had the detection limit of 100 DNA target segment per PCR sample, equivalent to 10^{6} DNA-segments/g-soil (Stubner 2002), while the detection limits for gram-

negative SRB were between 2×10^5 and 4×10^3 DNA-segments/g-soil (Stubner 2004). In addition, qRT-PCR primer set for dissimilatory sulfite reductase gene was also developed (Tang et al. 2004).

Cyanobacteria

Microcystis is one of the most common cyanobacteria that may cause serious algae bloom. As shown in Table 1, quantification of *Microcystis* was accomplished by the qRT-PCR utilizing primer/probe sets specific for *Microcystis* 16S rDNA, and microcystin synthetase genes of *mcy*B (Nonneman and Zimba 2002) and *mcy*D (Foulds et al. 2002; Rinta-Kanto et al. 2005). Results showed that *Microcystis* concentration in the bloom area varied from 2×10^3 to 4×10^8 cells/l (Rinta-Kanto et al. 2005). The quantification result was valuable in the identification of the blooming sources (Rinta-Kanto et al. 2005).

Pollutant degrader

A methyl *tert*-butyl ether (MTBE) degrading bacteria PM1 was detected using the TaqMan qRT-PCR. The detection limit was 2,000 cells/l in pure culture or 1.80×10^5 cells/l in a mixture of PM1 and *Escherichia coli*. The quantification results indicated that increases in PM1 concentration corresponded to the rate of MTBE removal (Hristova et al. 2001). qRT-PCR analyses for hydrocarbon-degrader *bss*A gene, encoding benzylsuccinate synthase (Beller et al. 2002), and trichloroethylene-degrader *mmo*C gene, encoding a soluble methane monooxygenase (Kikuchi et al. 2002), had the same detection limit of five DNA target segments per PCR sample. The dynamic ranges of qRT-PCR analysis were over seven orders of magnitude for the former gene and five for the latter.

Acknowledgement The authors wish to thank the Hong Kong Research Grants Council for the financial support of this study (HKU 7106/04E).

References

Abell GCJ, Bowman JP (2005) Colonization and community dynamics of class *Flavobacteria* on diatom detritus in experimental mesocosms based on Southern Ocean seawater. FEMS Microbiol Ecol 53:379–391

Bassler HA, Flood SJA, Livak KJ, Marmaro J, Knorr R, Batt CA (1995) Use of a fluorogenic probe in a PCR-based assay for the detection of *Listeria monocytogenes*. Appl Environ Microbiol 61:3724–3728

Belanger SD, Boissinot M, Clairoux N, Picard FJ, Bergeron MG (2003) Rapid detection of *Clostridium difficile* in feces by real-time PCR. J Clin Microbiol 41:730–734

Beller HR, Kane SR, Legler TC, Alvarez PJJ (2002) A real-time polymerase chain reaction method for monitoring anaerobic, hydrocarbon-degrading bacteria based on a catabolic gene. Environ Sci Technol 36:3977–3984

Bengtsson M, Karlsson HJ, Westman G, Kubista M (2003) A new minor groove binding asymmetric cyanine reporter dye for real-time PCR. Nucleic Acids Res 31:e45

Benveniste O, Vaslin B, Villinger F, Grand RL, Ansari AA, Dormont D (1996) Cytokine mRNA levels in unmanipulated (ex vivo) and in vitro stimulated monkey PBMCs using a semi-quantitative RT-PCR and high sensitivity fluorescence-based detection strategy. Cytokine 8:32–41

Bischoff C, Lüthy J, Altwegg M, Baggi F (2005) Rapid detection of diarrheagenic *E. coli* by real-time PCR. J Microbiol Methods 61:335–341

Briones A, Raskin L (2003) Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. Curr Opin Biotechnol 14:270–276

Chen S, Yee A, Griffiths M, Larkin C, Yamashiro CT, Behari R, Paszko-Kolva C, Grandis SAD (1997) The evaluation of a fluorogenic polymerase chain reaction assay for the detection of *Salmonella* species in food commodities. Int J Food Microbiol 35:239–250

DeFrancesco L (2003) Real-time PCR takes center stage. Anal Chem 75(7):175A–179A

Devereux R, Kane MD, Winfrey J, Stahl DA (1992) Genus- and group-specific hybridization probes for determinative and environmental studies of sulfate-reducing bacteria. Syst Appl Microbiol 15:601–609

Devers M, Soulas G, Martin-Laurent F (2004) Real-time PCR reverse transcription PCR analysis of expression of atrazine catabolism genes in two bacterial strains isolated from soil. J Microbiol Methods 56:3–15

Dionisi HM, Layton AC, Harms G, Gregory IR, Robinson KG, Sayler GS (2002) Quantification of *Nitrosomonas oligotropha*-like ammonia-oxidizing bacteria and *Nitrospira* spp from full-scale wastewater treatment plants by competitive PCR. Appl Environ Microbiol 68:245–253

Dionisi HM, Harms G, Layton AC, Gregory IR, Parker J, Hawkins SA, Robinson KG, Sayler GS (2003) Power analysis for real-time PCR quantification of genes in activated sludge and analysis of the variability introduced by DNA extraction. Appl Environ Microbiol 69:6597–6604

Donia MD, Pana A (2005) Use of armored RNA as a standard to construct a calibration curve for real-time RT-PCR. J Virol Methods 126:157–163

Foulds IV, Granacki A, Xiao C, Krull UJ, Castle A, Horgen PA (2002) Quantification of microcystin-producing cyanobacteria and *E. coli* in water by 5′ nuclease PCR. J Appl Microbiol 93:825–834

Gibson UE, Heid CA, Williams PM (1996) A novel method for real time quantitative RT-PCR. Genome Res 6:995–1001

Ginzinger DG (2002) Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Exp Hematol 30:503–512

Glazer AN, Mathies RA (1997) Energy-transfer fluorescent reagents for DNA analyses. Curr Opin Biotechnol 8:94–102

Gruntzig V, Nold SC, Zhou J, Tiedje JM (2001) *Pseudomonas stutzeri* nitrite reductase gene abundance in environmental samples measured by real-time PCR. Appl Environ Microbiol 67:760–768

Hales BA, Edwards C, Ritchie DA, Hall G, Pickup RW, Saunders JR (1996) Isolation and identification of methanogen-specific DNA from blanket bog peat by PCR amplification and sequence analysis. Appl Environ Microbiol 62:668–675

Hall SJ, Hugenholtz P, Siyambalapitiya N, Keller J, Blackall LL (2002) The development and use of real-time PCR for the quantification of nitrifiers in activated sludge. Water Sci Technol 46:267–272

Harms G, Layton AC, Dionisi HM, Gregory IM, Garrett VM, Hawkins SA, Robinson KG, Sayler GS (2003) Real-time PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant. Environ Sci Technol 37:343–351

Haugland RA, Vesper SJ, Wymer LJ (1999) Quantitative measurement of *Stachybotrys chartarum* conidia using real time detection of PCR products with TaqMan fluorogenic probe system. Mol Cell Probes 13:329–340

Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. Genome Res 6:986–994

Hermansson A, Lindgren PE (2001) Quantification of ammonia oxidizing bacteria in arable soil by real-time PCR. Appl Environ Microbiol 67:972–976

Hernández M, Estev Te, Prat S, Pla M (2004) Development of real-time PCR systems based on SYBR Green I, Amplifluor and TaqMan technologies for specific quantitative detection of the transgenic maize event GA21. J Cereal Sci 39:99–107

Higuchi R, Fockler C, Dollinger G, Watson R (1993) Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology (N Y) 11:1026–1030

Holland PM, Abramson RD, Watson R, Gelfand DH (1991) Detection of specific polymerase chain reaction product by utilizing the 5′-3′ exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc National Acad Sci U S A 88:7276–7280

Hristova KR, Lutenegger CM, Scow KM (2001) Detection and quantification of MTBE-degrading strain PM1 by real-time TaqMan PCR. Appl Environ Microbiol 67:5154–5160

Jordan FL, Cantera JJL, Fenn ME, Stein LY (2005) Autotrophic ammonia-oxidizing bacteria contribute minimally to nitrification in a nitrogen-impacted forested ecosystem. Appl Environ Microbiol 71:197–206

Kikuchi T, Iwasaki K, Nishihara H, Takamura Y, Yagi O (2002) Quantitative and rapid detection of the trichloroethylene-degrading bacterium *Methylocyctis* sp. M in groundwater by real-time PCR. Appl Microbiol Biotechnol 59:731–736

Klein D (2002) Quantification using real-time PCR technology: applications and limitations. Trends Mol Med 8:257–260

Kolb S, Knief C, Stubner S, Conrad R (2003) Quantitative detection of methanotrophs in soil by novel pmoA-targeted real-time PCR assays. Appl Environ Microbiol 69:2423–2429

Leutenegger CM, Pusterla N, Mislin CN, Weber R, Lutz H (1999) Molecular evidence of ticks coinfected with Borrelia burgdorferi sensu lato and the human granulocytic ehrlichiosis agent in Switzerland. J Clin Microbiol 37:3390–3391

Limpiyakorna T, Shinoharab Y, Kurisub F, Yagib O (2005) Communities of ammonia-oxidizing bacteria in activated sludge of various sewage treatment plants in Tokyo. FEMS Microbiol Ecol 54:205–217

Lopez-Gutierrez JC, Henry S, Hallet S, Martin-Laurent F, Catroux G, Philippot L (2004) Quantification of a novel group of nitrate-reducing bacteria in the environment by real-time PCR. J Microbiol Methods 57:399–407

Lyon WJ (2001) TaqMan PCR for detection of *Vibrio cholerae* O1, O139, non-O1, and non-O139 in pure cultures, raw oysters, and synthetic seawater. Appl Environ Microbiol 67:4685–4693

Manz W, Eisenbrecher M, Neu TR, Szewzyk U (1998) Abundance and spatial organization of gram-negative sulfate-reducing bacteria in activated sludge investigated by in situ probing with specific 16S rRNA targeted oligonucleotides. FEMS Microbiol Ecol 25:43–61

Maria PO, McDonald IR, Groleau D, Murrell JC, Miguez CB (2002) Detection of methanotrophs with highly divergent *pmoA* genes from Arctic soils. FEMS Microbiol Lett 209:313–319

Mhlanga MM, Malmberg L (2001) Using molecular beacons to detect single-nucleotide polymorphisms with real-time PCR. Methods 25:463–471

Morrison TM, Weiss JJ, Wittwer CT (1998) Quantification of low-copy transcripts by continuous SYBR green I monitoring during amplification. Biotechniques 24:954–962

Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial population by DGGE analysis of polymerase chain reaction amplified genes encoding for 16S rRNA. Appl Environ Microbiol 62:2676–2680

Nadkarni MA, Martin FE, Jacques NA, Hunter N (2002) Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiol 148:257–266

Nogva HK, Rudi K, Naterstad K, Holck A, LilleHaug D (2000) Application of 5′-nuclease PCR for quantitative detection of *Listeria monocytogenes* in pure cultures, water, skim milk, and pasteurized whole milk. Appl Environ Microbiol 66:4266–4271

Nonneman D, Zimba PA (2002) PCR-based test to assess the potential for microcystin occurrence and channel catfish production ponds. J Phycol 38:230–233

Oberst RD, Hays MP, Bohra LK, Phebus RK, Yamashiro CT, Paszko-Kolva C, Flood SJA, Sargeant JM, Gillespie JR (1998) PCR-based DNA amplification and presumptive detection of *Escherichia coli* O157:H7 with an internal fluorogenic probe and the 5′ nuclease TaqMan assay. Appl Environ Microbiol 64:3389–3396

Okabe S, Satoh H, Watanabe Y (1999) In situ analysis of nitrifying biofilms as determined by the in situ hybridisation and the use of microeletrodes. Appl Environ Microbiol 65:3182–3191

Okano Y, Hristova KR, Leutenegger CM, Jackson LE, Denison RF, Gebreyesus B, Lebauer D, Scow KM (2004) Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. Appl Environ Microbiol 70:1008–1016

Panicker G, Myers ML, Bej AK (2004) Rapid detection of *Vibrio vulnificus* in shellfish and Gulf of Mexico water by real-time PCR. Appl Environ Microbiol 70:498–507

Ponchel F, Toomes C, Bransfield K, Leong FT, Douglas SH, Field SL, Bell SM, Combaret V, Puisieux A, Mighell AJ, Robinson PA, Inglehearn CF, Isaacs JD, Markham AF (2003) Real-time PCR based on SYBR Green I fluorescence: an alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions. BMC Biotechnol 13:18

Purkhold U, Pommerening-Roser A, Juretschko S, Schmid MC, Koops HP, Wagner M (2000) Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: implications for molecular diversity surveys. Appl Environ Microbiol 66:5368–5382

Qiu XY, Hurt RA, Wu LY, Chen CH, Tiedje JM, Zhou JZ (2004) Detection and quantification of copper-denitrifying bacteria by quantitative competitive PCR. J Microbiol Methods 59:199–210

Raskin L, Amann RI, Poulsen LK, Rittmann BE, Stahl DA (1995) Use of ribosomal RNA-based molecular probes for characterization of complex microbial communities in anaerobic biofilms. Water Sci Technol 31:261–272

Reina EI, Kurokawa K, Fujioka A, Sharma A, Mayer BJ, Matsuda M (2005) A FRET-based probe for epidermal growth factor receptor bound non-covalently to a pair of synthetic amphipathic helixes. Exp Cell Res 307:142–152

Rhee SK, Liu XD, Wu LY, Chong SC, Wan XF, Zhou JZ (2004) Detection of genes involved in biodegradation and biotransformation in microbial communities by using 50-mer oligonucleotide microarrays. Appl Environ Microbiol 70:4303–4317

Rinta-Kanto JM, Ouellette AJA, Boyer GL, Twiss MR, Bridgeman TB, Wilhelm SW (2005) Quantification of toxic *Microcystis* spp during the 2003 and 2004 blooms in western lake Erie using quantitative real-time PCR. Environ Sci Technol 39:4198–4205

Sawayama S, Tsukahara K, Yagishita T (2006) Phylogenetic description of immobilized methanogenic community using real-time PCR in a fixed-bed anaerobic digester. Bioresour Technol 97:69–76

Scheid D, Stubner S (2001) Structure and diversity of gram-negative sulfate-reducing bacteria on rice roots. FEMS Microbiol Ecol 36:175–183

Schneegurt MA, Kulpa CF (1998) The application of molecular techniques in environmental biotechnology for monitoring microbial systems. Biotechnol Appl Biochem 27:73–79

Sharkey FH, Banat IM, Marchant R (2004) Detection and quantification of gene expression in environmental bacteriology. Appl Environ Microbiol 70:3795–3806

Skovhus TL, Ramsing NB, Holmstrom C, Kjelleberg S, Dahllof I (2004) Real-time quantitative PCR for assessment of abundance of *Pseudoalteromonas* species in marine samples. Appl Environ Microbiol 70:2373–2382

Sonia H, Baudoin E, López-Gutiérrez JC, Martin-Laurent F, Brauman A, Philippot L (2004) Quantification of denitrifying bacteria in soils by *nirK* gene targeted real-time PCR. J Microbiol Methods 59:327–335

Stubner S (2002) Enumeration of 16S rDNA of *Desulfotomaculum* lineage 1 in rice field soil by real-time PCR with SybrGreen detection. J Microbiol Methods 50:155–164

Stubner S (2004) Quantification of gram-negative sulphate-reducing bacteria in rice field soil by 16S rRNA gene-targeted real-time PCR. J Microbiol Methods 57:219–230

Stubner S, Meuser K (2000) Detection of *Desulfotomaculum* in an Italian rice paddy soil by 16S ribosomal nucleic acid analyses. FEMS Microbiol Ecol 34:73–80

Suzuki MT, Taylor LT, DeLong EF (2000) Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′-nuclease assays. Appl Environ Microbiol 66:4605–4614

Takai K, Horikoshi K (2000) Rapid detection and quantification of members of the archaeal community quantitative PCR using fluorogenic probes. Appl Environ Microbiol 66:5066–5072

Tang Y, Shigematsu T, Morimura IS, Kida K (2004) The effects of micro-aeration on the phylogenetic diversity of microorganisms in a thermophilic anaerobic municipal solid-waste digester. Water Res 38:2537–2550

Taveau M, Stockholm D, Spencer M, Richard I (2002) Quantification of splice variants using molecular beacon or scorpion primers. Anal Biochem 305:227–235

Tyagi S, Kramer FR (1996) Molecular beacons: probes that fluoresce upon hybridization. Nat Biotechnol 14:303–308

van der Velden VH, Hochhaus A, Cazzaniga G, Szczepanski T, Gabert J, van Dongen JJ (2003) Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. Leukemia 17:1013–1034

Wagner M, Loy A (2002) Bacterial community composition and function in sewage treatment systems. Curr Opin Biotechnol 13:218–227

Wagner M, Roger AJ, Flax JL, Brusseau GA, Stahl DA (1998) Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. J Bacteriol 180:2975–2982

Wilson PE, Kazadi W, Kamwendo DD, Mwapasa V, Purfield A, Meshnick SR (2005) Prevalence of pfcrt mutations in Congolese and Malawian *Plasmodium falciparum* isolates as determined by a new Taqman assay. Acta Trop 93:97–106

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains *Archaea, Bacteria*, and *Eucarya.* Proc National Acad Sci U S A 87: 4576–4579

Wu LY, Thompson DK, Li GS, Hurt RA, Tiedje JM, Zhou JZ (2001) Development and evaluation of functional gene arrays for detection of selected genes in the environment. Appl Environ Microbiol 67:5780–5790

Zhang T, Fang HHP (2001) Phylogenetic diversity of a SRB-rich marine biofilm. Appl Microbiol Biotechnol 57:437–440

Zhang T, Fang HHP (2005) 16S rDNA clone library screening of environmental samples using melting curve analysis. J Chin Inst Chem Eng 28:1153–1155

Zhang T, Fang HHP, Ko BCB (2003a) Methane producing bacteria in the corrosion biofilm on mild steel. Appl Microbiol Biotechnol 63:101–106

Zhang Y, Zhang D, Li W, Chen J, Peng Y, Cao W (2003b) A novel real-time quantitative PCR method using attached universal template probe. Nucleic Acids Res 31:e123