MINIREVIEW

Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology

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Abstract

Quantitative PCR (Q-PCR or real-time PCR) approaches are now widely applied in microbial ecology to quantify the abundance and expression of taxonomic and functional gene markers within the environment. Q-PCR-based analyses combine 'traditional' end-point detection PCR with fluorescent detection technologies to record the accumulation of amplicons in 'real time' during each cycle of the PCR amplification. By detection of amplicons during the early exponential phase of the PCR, this enables the quantification of gene (or transcript) numbers when these are proportional to the starting template concentration. When Q-PCR is coupled with a preceding reverse transcription reaction, it can be used to quantify gene expression (RT-Q-PCR). This review firstly addresses the theoretical and practical implementation of Q-PCR and RT-Q-PCR protocols in microbial ecology, highlighting key experimental considerations. Secondly, we review the applications of (RT)-Q-PCR analyses in environmental microbiology and evaluate the contribution and advances gained from such approaches. Finally, we conclude by offering future perspectives on the application of (RT)-Q-PCR in furthering understanding in microbial ecology, in particular, when coupled with other molecular approaches and more traditional investigations of environmental systems.

Introduction

The application of PCR in combination with the extraction of nucleic acids (DNA and RNA) from environmental matrices has been central to the development of culture-independent approaches in microbial ecology. These methods, which have been applied since the early 1990s (e.g. Giovannoni *et al.*, 1990), enabling the analysis of the total microbial communities present within environmental systems, have revolutionized our understanding of microbial community structure and diversity within the environment. Coupling environmental nucleic acid isolation to subsequent PCR amplification of both taxonomic (i.e. rRNA) and functional gene markers and in combination with DNA fingerprinting- and sequencing-based analyses has enabled description of the hitherto uncharacterized majority of environmental microorganisms (Head *et al.*, 1998) driving the discovery of new microbial lineages and enabling the description of genetic diversity in a wealth of functional gene markers (Larkin *et al.*, 2005). Although recently developed ultra-high-throughput sequencing technologies such as pyrosequencing (Margulies *et al.*, 2005; Edwards *et al.*, 2006) now dwarf PCR-based sequence studies in terms of sequence coverage, the ability of the PCR to specifically target particular taxonomic or functional markers from domain – down to strain – or phylotype levels means that PCR will remain an invaluable method in the molecular microbial ecologist's toolbox. Nevertheless, PCR has inherent limitations (von Wintzingerode *et al.*, 1997), particularly those that result in biases in the template to product ratios of target sequences amplified during PCR from environmental DNA (Suzuki & Giovannoni, 1996; Polz & Cavanaugh, 1998), with such amplification biases found to increase with increasing numbers of PCR cycles. These limitations presented a significant challenge to microbial ecologists who were interested in determining the abundance of individual genes present in environmental samples. To circumvent such challenges, an adaptation of the PCR method developed by Holland *et al.* (1991) utilizing the so-called '5′ nuclease assay' was applied to quantify target 16S rRNA genes amplified from environmental DNA by PCR (Becker *et al.*, 2000; Suzuki *et al.*, 2000; Takai & Horikoshi, 2000). This

development had been facilitated by the earlier combination of the 5′ nuclease assay developed by Holland *et al.* (1991) with fluorescence detection following cleavage of an internal (*Taq*Man™) DNA probe (Livak *et al.*, 1995), enabling the accumulation of amplicons to be monitored after each cycle (in real-time) and hence facilitating quantitative determination of the initial template gene (or transcript) numbers.

Quantitative-PCR or Q-PCR (often referred to as real-time PCR) is now widely used in microbial ecology to determine gene and/or transcript numbers present within environmental samples. The target specificity of any Q-PCR assay is determined by the design of the primers (and in some cases an internal probe), allowing quantification of taxonomic or functional gene markers present within a mixed community from the domain level down to the quantification of individual species or phylotypes. Q-PCR has been shown to be a robust, highly reproducible and sensitive method to quantitatively track phylogenetic and functional gene changes across temporal and spatial scales under varying environmental or experimental conditions. Moreover, the quantitative data generated can be used to relate variation in gene abundances and/or levels of gene expression (in terms of transcript numbers) in comparison with variation in abiotic or biotic factors and/or biological activities and process rates. The provision of Q-PCR data sets that describe the abundance of specific bacteria or genes to complement other quantitative environmental data sets is of increasing importance in microbial ecology as it furthers understanding of the roles and contributions of particular microbial and functional groups within ecosystem functioning. Furthermore, reverse transcription (RT) analyses are now increasingly combined with Q-PCR methods in RT-Q-PCR assays, offering a powerful tool for quantifying gene expression (in terms of numbers of rRNA and mRNA transcripts) and relating biological activity to ecological function.

In this review, we firstly discuss the mechanistic aspects of Q-PCR and RT-Q-PCR methodologies, hereafter defined collectively as (RT)-Q-PCR, and highlight the key experimental considerations in the design and implementation of (RT)-Q-PCR protocols and the analysis of resultant data sets. Secondly, we explore the application of (RT)-Q-PCR approaches in microbial ecology, and finally we discuss how these methods can be applied together with other molecular and also conventional approaches to provide an increased understanding of microorganisms within environmental systems.

Advantages of Q-PCR over traditional end-point PCR

Q-PCR approaches combine the detection of target template with quantification by recording the amplification of a PCR product via a corresponding increase in the fluorescent signal associated with product formation during each cycle in the PCR. Quantification of gene (or transcript) numbers is determined during the exponential phase of the PCR amplification when the numbers of amplicons detected are directly proportional to the initial numbers of target sequences present within the environment (discussed in more detail in Target quantification). Quantification of the target gene during exponential amplification avoids problems that are associated with so-called 'end-point' PCR (in which amplicons are only analysed after completion of the final PCR cycle). In end-point PCR, the proportions of numerically dominant amplicons do not necessarily reflect the actual abundances of sequences present within the environment due to the inherent biases of PCR that are associated with amplification of targets from mixed template community DNA (Reysenbach *et al.*, 1992; Suzuki & Giovannoni, 1996; Polz & Cavanaugh, 1998). Moreover, Q-PCR that uses fluorescence-based detection offers greater sensitivity and enables discrimination of gene numbers across a wider dynamic range than is found with end-point PCR; for example twofold changes in target concentration can be discriminated using Q-PCR. Before the development of fluorescence-based Q-PCR-based methods, two alternative PCR-based methods for gene number quantification had been developed, namely competitive PCR (Diviacco *et al.*, 1992) and limiting dilutions or most probable number (MPN)-PCR (Skyes *et al.*, 1992). However, these methods are time- and resource-consuming, requiring post-PCR analysis, and have now largely been replaced by fluorescence-based Q-PCR methods.

Fluorescence detection chemistries used to detect template amplification during Q-PCR

Quantitative real-time PCR works in essentially the same manner as end-point PCR, i.e. multiple amplification cycles in which template DNA is initially denatured, followed by annealing of oligonucleotide primers targeting specific sequences, followed by subsequent extension of a complementary strand from each annealed primer by a thermostable DNA polymerase, resulting in an exponential increase in amplicon numbers during the PCR. However, in contrast to end-point PCR, the increase in amplicon numbers is recorded in 'real-time' during the PCR via detection of a fluorescent reporter that indicates amplicon accumulation during every cycle. Two reporter systems are commonly used, namely, the intercalating SYBR green assay (Wittwer *et al.*, 1997) and the *Taq*Man probe system (Holland *et al.*, 1991; Livak *et al.*, 1995).

SYBR green binds to all double-stranded DNA via intercalation between adjacent base pairs. When bound to DNA,

a fluorescent signal is emitted following light excitation (Fig. 1a). As amplicon numbers accumulate after each PCR cycle, there is a corresponding increase in fluorescence. Because SYBR green binds to all double-stranded DNA, it is essential to use primer pairs that are highly specific to their target sequence to avoid generation of nonspecific products that would contribute to the fluorescent signal, resulting in an overestimation of the target. Extensive optimization of primer concentrations used in SYBR green Q-PCR assays may be required to ensure that only the targeted product is formed. Primer pairs that exhibit self-complementarity should also be avoided to prevent primer–dimer formation. A post-PCR dissociation (melting) curve analysis should be carried out to confirm that the fluorescence signal is generated only from target templates and not from the formation of nonspecific PCR products. During a dissociation curve, the double-stranded template is heated over a temperature gradient and fluorescence levels are measured at each discrete temperature point. As the double-stranded template is heated, it denatures, resulting in a corresponding decline in fluorescence due to SYBR green dissociation from the double-stranded product (Giglio *et al.*, 2003; Gonzalez-Escalona *et al.*, 2006). The temperature at which 50% of the double-stranded template is denatured can be used to confirm that the template being targeted is present, along with the presence of other nonspecific template and primer dimers in much the same way as agarose gel electrophoresis of an end-point PCR product is used.

The *Taq*Man probe method utilizes a fluorescently labelled probe that hybridizes to an additional conserved region that lies within the target amplicon sequence. The *Taq*Man probe is fluorescently labelled at the 5′ end and contains a quencher molecule at the 3′ end (Livak *et al.*, 1995). The close proximity on the probe of the quencher molecule to the fluorophore prevents it from fluorescing due to fluorescent resonance energy transfer. During the annealing step of each cycle of the PCR, primers and the intact probe bind to their target sequences. During subsequent template extension, the 5′ exonuclease activity of the *Taq* polymerase enzyme cleaves the fluorophore from the *Taq*Man probe and a fluorescent signal is detected as the fluorophore is no longer in close proximity to the quencher (Fig. 1b). Amplification of the template is thus measured by the release and accumulation of the fluorophore during the extension stage of each PCR cycle. The additional specificity afforded by the presence of the *Taq*Man probe ensures that the fluorescent signal generated during Q-PCR is derived only from amplification of the target sequence. Multiple *Taq*Man probes and primer sets can be used in different Q-PCR assays to differentiate between closely related sequences (Smith *et al.*, 2007), or alternatively, probes can be labelled with different fluorophores, facilitating the development of multiplex Q-PCR protocols whereby different targets can be coamplified and quantified within a single reaction (Neretin *et al.*, 2003; Baldwin *et al.*, 2003, 2008). For example, Baldwin *et al.* (2003) developed a multiplex Q-PCR assay targeting a number of different aromatic oxygenase genes using bacterial strains and then subsequently applied the assay to simultaneously quantify aromatic oxygenase genes in contaminated groundwater (Baldwin *et al.*, 2008). *Taq*Man probes are, however, a more expensive option than using SYBR green chemistry and the former requires the presence of an additional conserved site within the short amplicon sequence to be present. Identification of three conserved regions within a short region (typically *c.* < 100 bp) may not

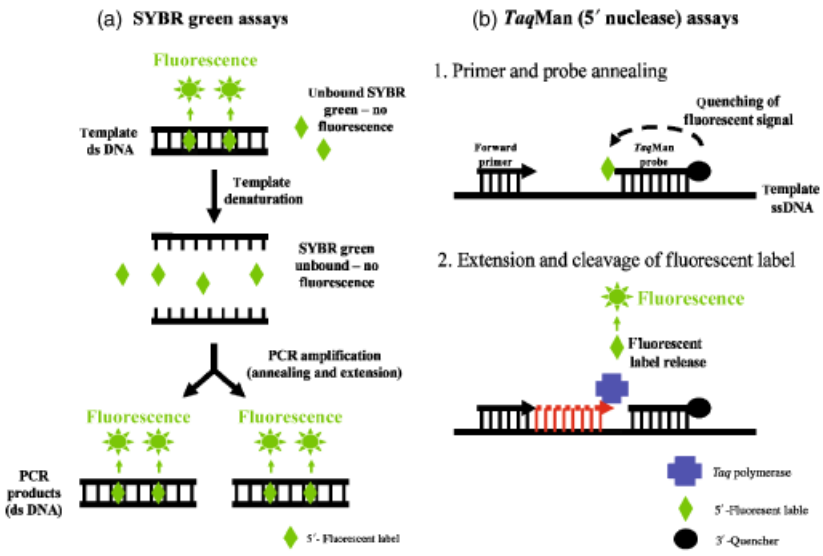

Fig. 1. Real-time PCR chemistries: (a) SYBR green detection. SYBR green binds to all double-stranded DNA and emits a fluorescent signal. In its unbound state, SYBR green does not fluoresce. Template amplification is therefore measured in each cycle by the corresponding increase in fluorescence. (b) *Taq*Man (5′ nuclease) assay using *Taq*Man® probes. During annealing, the *Taq*Man probe and primers bind to the template. When the *Taq*Man probe is intact, energy is transferred between the quencher and the reporter; as a result, no fluorescent signal is detected. As the new strand is synthesized by *Taq* polymerase, the 5′ exonuclease activity of the enzyme cleaves the labelled 5′ nucleotide of the probe, releasing the reporter from the probe. Once it is no longer in close proximity, the fluorescent signal from the probe is detected and template amplification is recorded by the corresponding increase in fluorescence.

always be possible, especially when primer/probe combinations are being designed to target divergent gene sequences. More recent advances in *Taq*Man probe technology have involved the introduction of the minor groove binder (MGB) probe (Kutyavin *et al.*, 2000). The MGB molecule is attached to the 3′ end of the probe and essentially folds back onto the probe. This not only increases the stability of the probe, but allows the design of shorter probes (13–20 bp) than are required for traditional *Taq*Man probes (20–40 bp), while at the same time, maintaining the required hybridization annealing temperature.

Target quantification using the cycle threshold (C_t) method

Irrespective of the fluorescence chemistry used, quantification of the target template DNA is carried out in essentially the same manner. There are a number of different commercially available instruments to carry out Q-PCR, each with its own associated software. Currently, there is considerable debate as to which algorithms are the best used to analyse Q-PCR data (reviewed in Rebrikov & Trofimov, 2006). All the Q-PCR platforms collect fluorescent data from every amplification cycle and the increase in fluorescence is plotted against the cycle number, resulting in the typical amplification curve shown in Fig. 2. The Q-PCR amplification curve can be subdivided into four stages, namely background noise, where the background fluorescence still exceeds that derived from initial exponential template accumulation, exponential amplification, linear amplification and a plateau stage. During the exponential phase of the amplification, the amount of target amplified is proportional to the starting template and it is during these cycles that gene numbers are quantified using the C_t method. The C_t is reached when the accumulation of fluorescence (template) is significantly greater than the background level

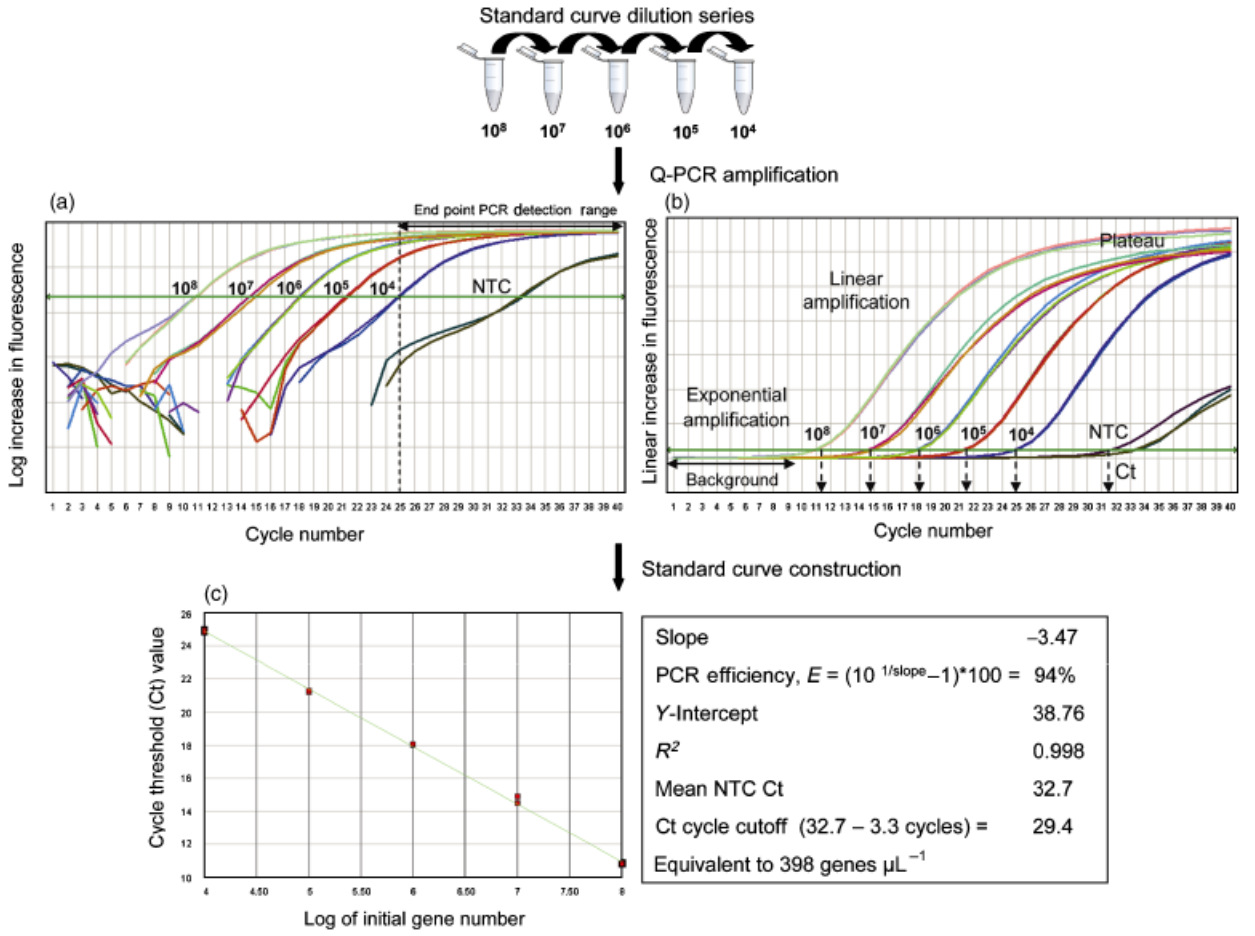

Fig. 2. Q-PCR amplification from known concentrations of template DNA to construct standard curves for quantification of unknown environmental samples. (a) Log plot of the increase in fluorescence vs. cycle number of DNA standards ranging from 1×10^4 to 1×10^8 16S rRNA gene amplicons μL^{-1}. (b) Linear plot indicating the three phases of a PCR amplification, the corresponding C_t values for each of the amplified standards and for the NTC. (c) Simple linear regression of the C_t values (from b) vs. log of the initial rRNA gene number. Q-PCR descriptors are shown (boxed).

(Heid *et al.*, 1996). During the initial cycles, the fluorescence signal due to background noise is greater than that derived from the amplification of the target template. Once the C_t value is exceeded, the exponential accumulation of product can be measured. When the initial concentration of the target template is higher, the C_t will be reached at an earlier amplification cycle.

Quantification of the initial target sequences of an unknown concentration is determined from the C_t values and can be described either in relative or in absolute terms. In relative quantification, changes in the unknown target are expressed relative to a coamplified steady state (typically a housekeeping) gene. Any variation in the presence (or expression) of the housekeeping gene can potentially mask real changes or indicate artificial changes in the abundance of the gene of interest. While this approach is commonly applied for studying eukaryotic gene expression (reviewed in Bustin, 2002), it is more difficult to apply this method for studying prokaryotic genes where the identification of a valid steady-state reference gene is problematic. Burgmann *et al.* (2007) nevertheless successfully utilized such an approach when confirming microarray-based determination of the transcriptional responses of *Silicibacter pomeroyi* to dimethylsulphoniopropionate additions. From microarray experiments, they identified a gene whose expression was not altered by experimental conditions and used the expression of this gene to normalize levels of expression of the target genes of interest in RT-Q-PCR assays. In a number of other studies, gene and transcript numbers of the target gene of interest have been normalized to the numbers of 16S rRNA gene or transcripts (Neretin *et al.*, 2003; Treusch *et al.*, 2005; Kandeler *et al.*, 2006). For example, Treusch *et al.* (2005) normalized the number of *amoA* transcripts to numbers of 16S rRNA gene transcripts in RNA extracted from ammonia-amended or unamended soils. They reported a statistically significant increase in *amoA* transcript numbers in the ammonia-amended soils. However, although 16S rRNA genes and transcripts are now commonly used in this manner, the application of such an approach is controversial, especially when studying genes/transcripts amplified from nucleic acids extracted from complex environmental samples. This is, in particular, because 16S rRNA gene copy and transcript numbers are highly variable, with the number of 16S rRNA genes per operon varying dramatically between species (1–15 copies) while 16S rRNA gene transcription rates are regulated primarily by resource availability (Klappenbach *et al.*, 2000). The 16S rRNA genes and transcripts cannot therefore be considered as a steady-state (housekeeping) gene, especially when studying genes/transcripts in environmental samples.

In absolute quantification protocols, the numbers of a target gene or transcript are determined from a standard curve generated from amplification of the target gene present at a range of initial template concentrations, and then the C_t values for each template concentration are determined. Subsequently, a simple linear regression of these C_t values is plotted against the log of the initial copy number (Fig. 2). It should be ensured that the C_t value of the most diluted template DNA used to construct the standard curve is at least a log fold lower (3.3 cycles) than the C_t value of the no template control (NTC). Quantification of the unknown target template is determined by comparison of the C_t values of the target template against the standard curve. However, in reality, this 'absolute' quantification of the target gene represents quantification of the target in comparison against a constructed standard curve, rather than as an absolute measurement of the number of target genes present within an environmental sample. Any number of factors involved in the construction of the standard curve including the initial quantification of the standard curve template, serial dilution of the template and the algorithmic determination of the C_t value (Love *et al.*, 2006) contribute to the final quantification of the environmental sample. As a consequence, it is recommended that the following descriptors of the standard curve are reported for each Q-PCR amplification: amplification efficiency (E), the linear regression coefficient (r^2) and especially the y-intercept value, which uniquely describes the standard curve and indicates the sensitivity of the reaction (Smith *et al.*, 2006; Fig. 2). Furthermore, the C_t value of the NTC and its equivalent value in terms of gene numbers should be reported. Moreover, we have previously demonstrated that even highly reproducible standard curves may result in statistically significant differences in gene numbers for the same template (with equivalent C_t values) when gene numbers are quantified within different Q-PCR assays (Smith *et al.*, 2006) due to the log nature of the curve, whereby minor differences in C_t values and standard curves result in large differences in gene copy numbers.

Biological and methodological factors affecting quantification of genes and transcripts from environmental samples

Q-PCR-based quantification of gene/transcript numbers amplified from nucleic acids isolated from environmental samples is further influenced by a number of other compounding factors. Firstly, the choice of method used for nucleic acid extraction will be a major determinant on the final quantification. Nucleic acid extraction efficiencies vary considerably between different methods and the final nucleic acid yield is dependent on both the method used and the type of environmental sample being studied (Martin-Laurent *et al.*, 2001). Moreover, many different extraction protocols are used for different environmental

samples and within different laboratories, making direct comparison of absolute gene numbers between studies extremely problematic. Hence, in order to compare gene/transcript numbers from different environmental samples, it must first be ensured that the same extraction procedure is used for each sample. Furthermore, while the presence of PCR inhibitors in nucleic acids extracted from environmental samples and their subsequent effect on Q-PCR is well established (Stults *et al.*, 2001), the concentration at which inhibitors no longer affect the Q-PCR for any sample is not known *a priori* and must be determined empirically (Stults *et al.*, 2001) to ensure that the environmental template and the standard curve target gene have equivalent amplification efficiencies.

The sensitivity of Q-PCR allows quantification of very low numbers of target genes, with detection limits as low as two copies of a gene in a Q-PCR (Fey *et al.*, 2004) reported in the literature. However, statements pertaining to the sensitivity and lower detection limits of Q-PCR should be qualified by providing information on the amplification signal detected, if any, within the NTC. This is because quantification of low numbers of the target gene may be artificially increased by the presence of an amplification signal within the reaction that is equivalent to that quantified within the NTC. While details of amplification signals in the NTC are sometimes reported in the literature (Suzuki *et al.*, 2000; Gruntzig *et al.*, 2001; Baldwin *et al.*, 2003; Smith *et al.*, 2006; McKew *et al.*, 2007), many studies do not provide such details (Panicker *et al.*, 2004; Kandeler *et al.*, 2006; Coolen *et al.*, 2008). It is, however, recommended that details of the C_t values of the NTC and their equivalent gene numbers should be reported for all Q-PCR assays in order to determine the lower limits of detection for the reaction. To ensure that the NTC (if detected) does not contribute to the fluorescence signal of either the standard curve or the target sequence in the environmental DNA sample, it is recommended that the C_t value of both the most dilute DNA standard and of the unknown target gene should have C_t values of 3.3 cycles (a log value) fewer than that of the NTC C_t value (Smith *et al.*, 2006).

The determination of gene and transcript numbers amplified from environmental samples generated by different research groups will entail any number of the aforementioned variables in the Q-PCR protocol and may be affected by the initial extraction of nucleic acids, the preparation, quantification and amplification of the standard curve template (Love *et al.*, 2006), variations in the efficiencies of the subsequent Q-PCR, differences in the Q-PCR platform, associated software and reagents that are used, as well as variations due to different researchers and laboratories. Therefore, the generation of 'absolute' gene numbers can only be considered as being meaningful for the individual study in question and even then, such a direct comparison should be used only for gene numbers determined within a single Q-PCR assay and using the same standard curve (Smith *et al.*, 2006).

Quantifying gene expression by RT-Q-PCR

Combining Q-PCR with an initial RT reaction facilitates the quantification of RNA transcripts (rRNA or mRNA), enabling quantitative estimates of the activity of specific taxa or functional guilds within a microbial community. Isolation of total RNA directly from complex environmental samples is typically problematic, as RNA is a labile molecule with a potentially short half-life (Grunberg-Manago, 1999). As with DNA quantification, the first step towards accurate RNA quantification lies in the preparation of a high-quality template, free from inhibitors (Stults *et al.*, 2001). However, simple dilution to reduce the concentration of inhibitors present in the RNA template may be problematic as the kinetics of the RT reaction can be affected adversely by low RNA template concentrations (Chandler *et al.*, 1998). This effect has been demonstrated in environmental samples using a dilution series of environmental RNA within the RT reaction, which resulted in at least log fold differences in the number of transcripts (transcripts per gram sediment) quantified from different dilutions of RNA (Smith *et al.*, 2006). Moreover, due to the sensitivity of (fluorescence-based) Q-PCR methods, it is particularly important that the RNA template is free from contaminating DNA that could contribute to the final amplification signal. Absolute numbers of RNA transcripts should be determined from standard curves constructed from cDNA (i.e. reverse transcribed RNA) and not from a double-stranded DNA template. Furthermore, the efficiency of the initial RT step is critical for sensitive and accurate quantification as the amount of cDNA produced must accurately reflect the starting RNA concentration.

RT-Q-PCR amplifications can be conducted using either a one-step or a two-step reaction. In a one-step RT-Q-PCR, both the RT reaction and the Q-PCR are carried out consecutively in a single tube. RNA is first reverse transcribed, with all resultant cDNA serving as templates in the subsequent Q-PCR amplification. In addition to the reduced risk of contamination and the convenience of setting up only a single reaction, a further advantage of this method is that all the resulting cDNA produced is used to quantify the target RNA sequence. However, for the study of eukaryotes, one-step RT-Q-PCR reactions have been reported to have reduced sensitivity (Bustin, 2002) as reaction conditions are compromised to accommodate the two different enzymes required within a single reaction. In a two-step RT-Q-PCR protocol, the RT reaction and the subsequent Q-PCR are carried out separately. Firstly, cDNA is generated in an independent RT reaction and subsequently an aliquot of this cDNA is used as a template for

the Q-PCR. An advantage of this method is that the RT reaction can be optimized to increase cDNA yield as can the subsequent Q-PCR amplification. Furthermore, cDNA generated in the RT reaction can be used as a template for a number of different Q-PCR reactions. If random primers are used in the initial RT reaction, then any number of subsequent gene-specific Q-PCR amplifications using the randomly primed cDNA can be conducted, making a two-step reaction a more economically viable option for RT-Q-PCR than a one-step reaction. While random primers can maximize the number of different cDNA templates generated, gene-specific reverse primers can increase the sensitivity and specificity of the cDNA created and at the same time reduce the amount of unspecific background cDNA. However, this may be dependent on the gene-specific primer. For example, Nícolaisen *et al.* (2008), in a study of *tfdA* gene expression in soil, showed that use of random primers in the RT reaction as opposed to using gene-specific primers was optimal for RT-Q-PCR of *tfdA* transcripts. Clearly, the choice of the protocol, enzyme, primer and reaction conditions will influence the quantification of the RNA template from the environment.

Practicalities of (RT)-Q-PCR protocols

Although the physical set-up of (RT)-Q-PCR amplifications to quantify gene or transcript numbers from environmental nucleic acids is straightforward, the quantitative data generated from these reactions can be affected by many compounding factors. Consequently, such factors as discussed earlier in this review need to be carefully considered when designing, developing and implementing (RT)-Q-PCR protocols. Details of some additional key considerations and recommendations for the use of (RT)-Q-PCR are given below.

(RT)-Q-PCR amplicons should be short, ideally between 50 and 150 bp in length. While, the GC content of the primers can range between 20% and 80% (although paired primers should have similar melting temperatures; T_m), a high GC content will increase the specificity of the reaction, which is of particular importance for SYBR green assays. When designing a *Taq*Man probe, the probe should be situated as close as possible to the forward primer without overlapping. The probe should not have a guanine nucleotide at the 5′ end or have more guanines than cytosines as guanine residues are natural quenchers. The T_m of the probe should be 8–10 °C above the T_m of the primers. When designing primers and probes, it may be difficult to meet all the above criteria. However, satisfying as many of these as possible within the constraints of the assay design will maximize the likelihood of successful quantification. As outlined earlier (see Fluorescence detection chemistry section), SYBR green primer sets may require extensive optimization to ensure a single specific amplicon and that primer dimers are not produced; this must also be confirmed by dissociation curve analysis. Highly reproducible DNA and RNA standard curves can be created by dilution of known concentrations of standards (see Smith *et al.*, 2006 for details). Care should be taken to avoid repeated freeze–thawing of templates used to construct standard curves. As quantification of genes or transcripts from an environmental samples is calculated from the standard curve, a full description of the standard curve (r^2, slope, efficiency and *y*-intercept value) should be given when reporting gene and/or transcript numbers. Biological (not just technical) replication (at least $n = 3$) is essential for (RT)-Q-PCR to enable statistical investigation of differences in gene or transcript numbers between samples or treatments. Finally, as there are numerous compounding factors that can affect quantification, we recommend that comparisons between absolute gene or transcript numbers generated from different Q-PCR assays (or indeed studies) should not be made.

Application of Q-PCR for investigating the microbial genetic potential within the environment

The first applications of Q-PCR in microbial ecology were reported in three papers published in November 2000, which used *Taq*Man-based assays to target 16S rRNA genes (Becker *et al.*, 2000; Suzuki *et al.*, 2000; Takai & Horikoshi, 2000). Becker *et al.* (2000) demonstrated the ability of *Taq*Man probes to determine the abundance of a specific ecotype of *Synechococcus* sp. BO 8807 against a mixed background of phylogenetically related bacteria using artificial mixed communities. Suzuki *et al.* (2000) exploited the specificity and the sensitivity of *Taq*Man Q-PCR assays to determine spatial and temporal quantitative differences in the distributions of *Synechococcus*, *Prochlorococcus* and archaea in marine waters, while Takai & Horikoshi (2000) quantified archaeal 16S rRNA gene numbers within samples from a deep sea hydrothermal vent effluent, hot spring water and from hot spring and freshwater sediments. By targeting highly conserved regions of the 16S rRNA gene, Q-PCR assays have been designed to quantify 'total' bacterial (and/or archaeal) numbers while targeting of taxa-specific sequences within hypervariable regions within the gene enables quantification of sequences from phylum to species levels, provided that there are sequence data available that enable the design of primers and probes. A caveat of this approach must be stressed; 16S rRNA gene numbers from environmental samples cannot be converted to cell numbers as the exact number of copies of the 16S rRNA gene in any given bacterial species varies (Klappenbach *et al.*, 2000). Table 1 details commonly used rRNA Q-PCR primer and probe sets.

Table 1. Quantitative PCR primer and probe sets targeting small subunit ribosomal RNA genes of bacteria, archaea and fungi

Target	Detection chemistry	Primer/probe	Sequence (5′–3′)	Amplicon length (bp)	Temp. (°C)	References
Prokaryote 16S rRNA gene	TM	Uni 340F	CCT ACG GGR BGC ASC AG	466	57	Takai & Horikoshi (2000)
		Uni 806R	GGA CTA CNN GGG TAT CTA AT			
		TM 516F	TGY CAG CMG CCG CGG TAA HAC VNR S			
Bacterial 16S rRNA gene	TM	BACT1369F	CGG TGA ATA CGT TCY CGG	123	56	Suzuki *et al*. (2000)
		PROK1492R	GGW TAC CTT GTT ACG ACT T			
		Probe TM 1389F	CTT GTA CAC ACC GCC CG			
Bacterial 16S rRNA gene	TM	331F	TTC TAC GGG AGG CAG CAG	466	60	Nadkarni *et al*. (2002)
		797R	GGA CTA CCA GGG TAT CTA ATC CTG TT			
		Probe Bac*Taq*	CGT ATT ACC GCG GCT GCT GGC AC			
Archaeal 16S rRNA gene	TM	Arch 349F	GYG CAS CAG KCG MGA AW	457	59	Takai & Horikoshi (2000)
		Arch 806R	GGA CTA CVS GGG TAT CTA AT			
		TM Arch 516F	TGY CAG CCG CCG CGG TAA HAC CVG C			
Archaeal 16S rRNA gene	SG	Ar109f	ACK GCT CAG TAA CAC GT	806	52	Lueders & Friedrich (2003)
		Ar915r	GTG CTC CCC CGC CAA TTC CT			
Fungal 18S rRNA gene	SG	EUK 345F	AAG GAA GGC AGC AGG CG	149	60	Zhu *et al*. (2005)
		EUK 499R	CAC CAG ACT TGC CCT CYA AT			
Fungal 18S rRNA gene	SG	Fung5f	GTAAAAGTCCTGGTTCCCC	550	48	Smit *et al*. (1999)
		FF390r	CGA TAA CGA ACG AGA CCT			Vainio & Hantula (2000)
						Lueders *et al*. (2004)

TM, *Taq*Man probe; SG, SYBR green; Temp., annealing temperature.

Quantification of eukaryotes within environmental samples by Q-PCR can be carried out by targeting the 18S rRNA gene (Lueders *et al*., 2004; Zhu *et al*., 2005) or the internal transcribed spacer (ITS) region (Landeweert *et al*., 2003; Kennedy *et al*., 2007). The ITS region is often targeted for the design of taxon-specific Q-PCR assays as it provides a greater degree of sequence differentiation between species and lower within-species variability (Kennedy *et al*., 2007) than is seen for the 18S rRNA gene. As with quantification of 16S rRNA gene numbers, Q-PCR-derived ITS region and 18S rRNA gene numbers cannot be directly equated to cell numbers. However, numbers of fungal rRNA gene or ITS numbers per volume of sample can be used to compare the relative numbers of fungi between different environmental samples (Guidot *et al*., 2002).

In addition to quantitative data on taxonomic markers, Q-PCR has also been applied to quantify functional genes within the environment. By targeting functional genes that encode enzymes in key metabolic or catabolic pathways, the (genetic) potential for a particular microbial function within a particular environment can be assessed. To understand microbial functioning in the environment at a molecular level, it is essential not only to know what genes are present and the diversity of these genes but also to determine their abundance and distribution within the environment. To this end, Q-PCR assays have been designed to target microbially mediated biogeochemical processes in the environment. Quantification of functional genes involved in ammonia oxidation (Hermansson & Lindgren, 2001; Okano *et al*., 2004; Treusch *et al*., 2005; Leininger *et al*., 2006; Mincer *et al*., 2007), nitrate reduction and denitrification (Lopez-Gutiérrez *et al*., 2004; Henry *et al*., 2006; Smith *et al*., 2007), sulphate reduction (Leloup *et al*., 2007), methanogenesis (Denman *et al*., 2007) and methane oxidation (Kolb *et al*., 2003) have been investigated (see Table 2 for details of nitrogen cycle Q-PCR analyses). In a particularly striking example of the value of such functional gene Q-PCR assays, the relative contributions of ammonia-oxidizing archaea and bacteria to the first step of nitrification (ammonia oxidation) have been investigated both in soils (Leininger *et al*., 2006; He *et al*., 2007b) and in seawater (Mincer *et al*., 2007) by determination of the abundance of archaeal- and bacterial-related *amoA* genes. These studies have suggested that archaea and not bacteria are the numerically dominant ammonia oxidizers in both environments. The results of such studies are therefore encouraging a re-evaluation of our basic understanding of nitrogen cycling and the relative importance of bacteria and archaea (or specific taxa or functional guilds within the domains) within key environmental processes. While these studies have greatly enhanced our understanding of gene numbers in the environment, the next step to further our understanding is to link variation in genetic potential (i.e. gene numbers) within a system in relation to variation in rates and activity of the biologically driven environmental processes in question, and hence enabling improved understanding of the underpinning factors that influence microbial functioning within the environment. As Q-PCR is a sensitive and specific method to track changes in the abundance (and expression) of specific target functional genes, it lends itself to experiments

Table 2. Q-PCR primers and probes targeting genes encoding enzymes involved in nitrogen cycling

Functional group	Target gene	Comment/environment	References
Nitrogen fixation	*nifH*	Suite of *Taq*Man probes and primers designed to quantify *nifH* transcripts from seawater	Church *et al*. (2005)
		SYBR green primers targeting the *nifH* gene of *Synechococcus* sp. OS-B' isolate and used to quantify *nifH* transcripts from a hot spring microbial mat	Steunou *et al*. (2006)
Ammonia oxidation	*amoA*	*Taq*man probe and primers targeting known bacterial ammonia oxidizers. Used to quantify genes from soil	Okano *et al*. (2004)
		*Taq*Man probe and primers designed from the alignment of environmental mRNA and DNA clones from soil samples. Used to quantify transcripts from a soil microcosm	Treusch *et al*. (2005)
Nitrate reduction	*narG*	SYBR green primers designed from environmental soil clone libraries. Used to quantify *narG* from a range of soil types	Lopez-Gutiérrez *et al*. (2004)
		Suite of *Taq*Man primers and probes designed from environmental clone library. Used to target *narG* genes and transcripts from estuarine sediments	Smith *et al*. (2007)
		SYBR green primer set designed from all available *narG* sequences in the public database and used to quantify genes from river sediment, range of soils, water and biofilms	Bru *et al*. (2007)
	napA	Suite of *Taq*Man primers and probes used to target *napA* genes and transcripts from estuarine sediments	Smith *et al*. (2007)
		SYBR green primer set used to quantify genes from river sediment, soils, water and biofilms	Bru *et al*. (2007)
Nitrite reduction	*nirS*	*Taq*Man probes and primers targeting *Pseudomonas stutzeri*-related *nirS* genes. Used to quantify *nirS* genes from soil and contaminated groundwater	Gruntzig *et al*. (2001)
		Suite of *Taq*Man primers and probes designed from *nirS* mRNA clone library. Used to quantify genes and transcripts from estuarine sediments	Smith *et al*. (2007)
	nirK	SYBR green primer set designed from all *nirK* sequences available at the time. Used to quantify genes from a range of soil types	Henry *et al*. (2006)
Nitric oxide reduction	*norB*	Two SYBR green primer sets targeting the cytochrome c electron donor (cNOR) form of the enzyme; designed from cultured soil isolates. Used to quantify c*norB* from soil microcosms	Dandie *et al*. (2007)
Nitrous oxide reduction	*nosZ*	Two SYBR green primer sets designed from diverse *nosZ* sequences. Used to quantify *nosZ* genes from a range of soil types	Henry *et al*. (2006)
Nitrate ammonification	*nrfA*	*Taq*Man primers and probe targeting *nrfA* in estuarine sediments	Smith *et al*. (2007)

that further investigate the environmental controls/effects on the numbers of the target gene (and hence the organisms carrying these genes) and subsequently on the environmental process that these genes (and organisms) encode. A recent study by Dandie *et al*. (2007) has adopted such an approach by quantifying the response of denitrifying populations within soil microcosms amended with varying concentrations of glucose (as an electron donor) designed to induce different rates of denitrification. Denitrifier population numbers were assessed using the nitric oxide reductase (*cnorB*) gene as a proxy for denitrifier numbers targeting two populations ($cnorB_P$: *Pseudomonas* and $cnorB_B$: *Bosea*, *Bradyrhizobium*, *Ensifer*). These mesocosm experiments indicated that denitrification rates and microbial respiration increased significantly with increasing addition of glucose and that this was accompanied by increases in $cnorB_P$, but not $cnorB_B$ populations, revealing population-specific responses to carbon amendment.

Functional genes encoding key reactions in biodegradation pathways of environmental pollutants have also been targeted by Q-PCR analysis (Baldwin *et al*., 2003, 2008; Devers *et al*., 2004; Gonod *et al*., 2006; McKew *et al*., 2007; see Table 3 for details of Q-PCR primer and/or probe sets). The accurate quantification of key genes such as those encoding mono-oxygenase and diooxygenase enzymes involved in the catabolic conversions of environmental pollutants *in situ* will greatly enhance our understanding and importantly improve our knowledge of the biotic potential within an environment for successful bioremediation, and further how indigenous or augmented microorganisms respond to biostimulation protocols. For example, the effects of biostimulation and bioaugmentation remediation strategies on the activity of hydrocarbon degrading bacteria in seawater microcosms containing crude oil were investigated over a 30-day period by targeting alkane hydroxylase and aromatic ring hydroxylating dioxygenase genes by

Table 3. Q-PCR primers and probes targeting genes involved in biodegradation

Target chemical	Functional group	Target gene	Comment/environment	References
Herbicide atrazine	Atrazine degrading bacteria	*atzA*, *B*, *C*, *E* and *F*	SYBR green primers targeting *atz* catabolic gene expression in two atrazine-degrading bacteria	Devers *et al*. (2004)
Herbicide MCPA	MCPA (4-chloro-2-methylphenoxy-acetic acid) and 2,4-dichlorophenoxyacetic degrading bacteria	*tfdA*	SYBR green primers targeting *tfdA* gene in soil. Primers described originally by Vallaeys and colleagues, and later adapted by Gonod and colleagues, for Q-PCR	Vallaeys *et al*. (1996) (primers) Gonod *et al*. (2006) (Q-PCR assay conditions)
			SYBR green Q-PCR primer set designed from the alignment of 23 known *tfdA* genes and used to track quantitative changes in *tfdA* gene numbers in soil during degradation of MCPA	Bælum *et al*. (2006)
Trichloroethene and *cis*-dichloroethene (*cis*-DCE)	Bacteria involved in reductive dechlorination of TCE 2 and oxidation of *cis*-DCE	16S rRNA gene	Suite of group specific primers for SYBR green Q-PCR targeting of CFB, *Alphaproteobacteria* and *Burkholderiales* in hydrocarbon, trichloroethene and *cis*-DCE contaminated groundwater	Miller *et al*. (2007)
Chlorinated ethenes	Anaerobic reductive dehalogenases (RDase)	*tceA*, *vcrA* and *bvcA*	*Taq*man primer and probe sets targeting the *tceA, vcrA* and *bvcA* genes of *Dehalococcoides* spp. in groundwater	Lee *et al*. (2008)
Halogenated compounds	Reductive dehalogenating bacteria	16S rRNA and *rdh* genes	SYBR green primer sets targeting the 16S rRNA gene of two known dehalogenating bacteria and a SYBR green primer set targeting a *rdh* gene designed from sequences retrieved from marine sediments amended with 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (TeCDD)	Ahn *et al*. (2007)
Methyl *tert*-butyl ether (MTBE)	MTBE-degrading bacterial strain PM1	16S rRNA gene	*Taq*man primer and probe set targeting the MTBE degrading bacterial strain PM1 in groundwater and sediments	Hristova *et al*. (2001)
Hydrocarbons (aliphatic and aromatic)	Hydrocarbonoclastic bacteria	*alkB2*, *alkB*, *phnA*	SYBR green primers targeting the alkane hydroxylase in *Alcanivorax borkumensis* and *Thakassolituus oleivorans* and the aromatic ring-hydroxylating dioxygenase gene from *Cycloclasticus* spp.	McKew *et al*. (2007)
Hydrocarbons (aromatic)	Toluene- and xylene-degrading bacteria	*bssA*	*Taq*man probe and primer set targeting *bssA* gene in a variety of toluene-degrading denitrifying bacteria	Beller *et al*. (2002)
Hydrocarbons (aromatic)	Aromatic compound degrading bacteria	Entire subfamilies of related oxygenase genes rather than species-specific genes	Paper outlines the development of a suite of SYBR green primer sets targeting biphenyl dioxygenase, naphthalene dioxygenase, toluene dioxygenase, toluene/xylene monooxygenases, phenol monooxygenase and ring-hydroxylating toluene monooxygenase genes	Baldwin *et al*. (2003)

Q-PCR while simultaneously measuring the degradation of the crude oil (McKew *et al*., 2007). This study revealed that specific taxa within these hydrocarbon-degrading bacterial communities were directly influenced by application of different biostimulation approaches involving addition of nutrients and/or bioemulsifiers. Q-PCR is a valuable tool for investigating the potential within the environment for biodegradation of other pollutants, such as herbicides. For example, Q-PCR has been used to study the potential for biodegradation of the herbicide 4-chloro-2-methylphenoxyacetic acid (MCPA) in different soil types (Bælum *et al*., 2006) by targeting and quantifying the *tfdA* gene involved in the initial degradation step of the compound. This study showed a five- and threefold log increase in *tfdA* gene

numbers over time in soil microcosms amended with either a high (20 mg kg^{-1}) or a low (2.3 mg kg^{-1}) dose of MCPA, respectively, with increases in *tfdA* genes inversely proportional to MCPA degradation. Moreover, this study also demonstrated the diagnostic potential of using SYBR green dissociation curve analysis of Q-PCR products to identify shifts in the dominant *tfdA* populations over time and during degradation. Subsequent clone library analysis showed that class III *tfdA* genes were responsible for MCPA degradation and not class I *tfdA* genes, which were dominant before the degradation process was initiated.

Quantifying gene expression in environmental samples using RT-Q-PCR: a step closer to determining the functioning of target genes in the environment

RT-Q-PCR can be used to detect and quantify mRNA transcripts of interest in complex environmental samples both in a sensitive and a specific manner. However, RT-Q-PCR to investigate gene expression (rRNA or mRNA) within environmental samples has been far less widely applied than Q-PCR-based assessment of gene numbers (i.e. from DNA) in the environment. This is primarily due to the difficulties of extracting intact RNA, and particularly intact mRNA, from environmental samples. While the quantification of both rRNA genes and/or functional genes from the environment can be used as an indicator of the genetic potential within an environment and is suggestive of potential functional activity within a community, molecular investigation of biological activity should preferably determine changes in gene expression and ideally of mRNA transcripts encoded by specific functional genes. A limited number of studies have indeed shown the successful quantification by RT-Q-PCR of a number of functional gene transcripts from a range of environments including aquatic ecosystems (Holtzendorff *et al.*, 2002; Wawrik *et al.*, 2002; Fey *et al.*, 2004; Church *et al.*, 2005; Gonzalez-Escalona *et al.*, 2006; Lee *et al.*, 2008) but also in estuarine sediments (Smith *et al.*, 2007), soil (Treusch *et al.*, 2005; Nícolaisen *et al.*, 2008), hot spring microbial mats (Steunou *et al.*, 2006) and blood and faecal samples (Matsuda *et al.*, 2007). In a particularly elegant application of RT-Q-PCR, Steunou *et al.* (2006) investigated changes in expression of *Synechococcus* spp. *nif* (*nifH*, *nifD*, *nifK*) genes in a hot spring microbial mat over a 12-h period. Transcripts in the mat were only detected and quantified at the end of the day, when the mat became anoxic. They further quantified expression of key genes involved in photosynthesis, respiration and fermentation processes within the hot spring microbial mat to build an overview of the energy-generating processes that may drive N_2 fixation. Lee *et al.* (2008) used RT-Q-PCR to quantify expression of reductive dehalogenase (*vcrA*, *bvcA* and *tceA*) genes as biomarkers of *Dehalococcoides* spp. activity and to distinguish the roles of different strains of *Dehalococcoides* during bioremediation and bioaugmentation of groundwater contaminated with trichloroethene. RT-Q-PCR indicated that *vcrA* and *bvcA* gene transcripts were highly expressed in all samples, whereas the *tceA* transcripts were inconsistently quantified and were at lower levels, indicating that *Dehalococcoides* spp. carrying *vcrA* and *bvcA* genes played a more important role in trichloroethene *in situ* bioremediation. These two examples highlight how the application of RT-Q-PCR in the environment will undoubtedly further our understanding of the many important processes that are mediated by microorganisms.

Combining (RT)-Q-PCR with other approaches to provide greater insight into community function and dynamics

Linking the structure and composition of microbial communities with the biological function that individual species or functional guilds convey is a key objective within microbial ecology. Stable isotope probing (SIP) (Radajewski *et al.*, 2000; Manefield *et al.*, 2002) can be used to directly link distinct taxa within a mixed microbial community to specific metabolic processes, particularly carbon utilization/degradation, using labelled substrates such as ^{13}C. During microbial growth, these substrates are incorporated into the nucleic acids (DNA or RNA) from members of the community that are directly (or indirectly) utilizing the labelled substrate, and the 'heavy' labelled nucleic acid can be separated using density gradient ultracentrifugation from the (unlabelled) nucleic acids representative of other members of the community that do not utilize the substrate. Lueders *et al.* (2004) combined SIP with (RT)-Q-PCR for quantitative measurements of a domain-specific template distribution through the differentially labelled fractions of DNA and RNA extracted from soil microcosms following SIP incubation. This enabled the community dynamics of methanotrophs in rice field soils to be tracked over time. Bacterial, archaeal and eukaryote rRNA genes were quantified in the heavy labelled fractions, indicating not only the presence of a dynamic methanotroph community that was enriched over time but also direct or indirect incorporation of the labelled ^{13}C into eukaryotes (fungi and protozoa).

Microarrays are now being increasingly used to simultaneously screen microbial communities within diverse environments for the presence (and, in principle, the abundance) of specific ribo- or phylotypes (taxa) and/or functional genes. Microarray platforms such as the PhyloChip (Brodie *et al.*, 2007) and GeoChip (He *et al.*, 2007a) are now affording a previously unparalleled opportunity to undertake targeted phylogenetic marker-based and functional

gene surveys of environments. Nevertheless, the potential for providing quantitative assessments of gene abundance from microarrays as applied to nucleic acids extracted from environmental samples is often compromised by the requirement for an initial amplification step from the environmental DNA (or RNA), often via PCR amplification (Brodie *et al.*, 2007), but alternatively via rolling linear amplification (He *et al.*, 2007a), before microarray hybridization. Consequently, and especially in those microarray studies requiring an intermediate PCR amplification, any quantitative interpretation of such data sets should be treated with caution, as these results will be susceptible to the same biases that are associated with any end-point PCR protocol (Reysenbach *et al.*, 1992). Nevertheless, microarray experiments can be used to identify potentially interesting quantitative changes in taxon- or gene-specific abundance between environmental samples that can then be validated by Q-PCR-based approaches, and hence Q-PCR can be recommended as a fast, target-specific method for validation of the (semi-) quantitative results generated from the increasing number of environmental microarrays (Rhee *et al.*, 2004; Brodie *et al.*, 2006, 2007; Burgmann *et al.*, 2007; He *et al.*, 2007a).

One major disadvantage of Q-PCR-based approaches is the requirement for prior sequence data of the specific target gene of interest. Consequently, Q-PCR can only be used for targeting of known genes. Historically, and until recently, sequence information has primarily been derived from genome or gene fragment sequences from cultured organisms and/or from clone libraries generated by PCR using primers that are themselves based on current sequence knowledge. Hence, accessing the 'unknown' using Q-PCR or indeed any PCR-based methods is inevitably limited to the analysis of sequences related to those that have already been characterized. Because molecular analysis of environmental microorganisms has repeatedly shown that the majority of microorganisms (and their genes) in the environment are highly divergent from those of most cultured organisms, this represents a Catch 22 situation for the development of new PCR-based assays. In recent years, this problem has, however, been circumvented by the introduction of metagenomic approaches that provide a PCR-independent assessment of microbial diversity. Two main strategies have been utilized, namely clone library-based metagenomes (Vergin *et al.*, 1998; Beja *et al.*, 2000; Venter *et al.*, 2004) and more recently ultra-high-throughput sequencing approaches such as pyrosequencing (Edwards *et al.*, 2006; Dinsdale *et al.*, 2008). The latter, particularly, offers considerable benefits both in terms of providing much larger data sets than can be generated via library-based approaches, and as importantly, by avoiding potential sequence-specific cloning biases. Moreover, where pyrosequencing is used to target-specific genes (e.g. rRNA genes; Sogin *et al.*, 2006), such data sets provide only semi-quantitative assessments of the diversity and/or the abundance of particular phylotypes that can again be validated by (RT)-Q-PCR-based approaches.

Conclusions

In conclusion, (RT)-Q-PCR-based approaches represent fast, effective methods enabling the quantification of gene and/or transcript numbers within environmental samples, providing unparalleled specificity and sensitivity to target sequences present within a mixed community background. As with all methodologies, the validity of the resulting data sets should be considered against the specificity and experimental variability associated with the method. In particular, for Q-PCR-based assays, the value of such data sets should be considered in relation to the specificity of the primer (and probes) used in the amplification and with respect to instrument, user and most importantly experimental variability associated with the method. Moreover, in order to maximize the value of (RT)-Q-PCR-based approaches for furthering biological understanding in microbial ecology, their value is greatest when used in combination with other (and often process-based) assessments of ecosystem function.

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