Quantitative Real-time Polymerase Chain Reaction for Tracking Microbial Gene Expression in Complex Environmental Matrices

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Abstract
Environmental matrices are highly diverse in their composition and range from simple (e.g. water) to highly complex (e.g. organic soils/biosolids). Analysis of microbial gene expression from such substrates is done for variety of purposes which could range from bio-surveillance to elucidation of biological function of a target microbe. Quantitative real-time PCR (RT-qPCR) has become a technique of choice for studying such bio-processes, due to its unique ability to both detect and quantify a target transcript in real-time. Challenges in extracting inhibitor-free, structurally intact RNA, amenable for a sensitive technique like RT-qPCR, has however proved to be a major impediment in our ability to rigorously implement this highly versatile technology. Despite these 'substrate defined' limitations, many attempts have been made to implement the RT-qPCR technology. Efforts like these have given us invaluable insight into the expression status of a particular transcript and hence, the biological functioning of the microbe, specifically under natural *in situ* conditions. As a result, it has enhanced our understanding of the role and diversity of many microbial populations which, previously was not possible using conventional molecular approaches. In this article, we have sought to summarize such technical problems faced by molecular environmental microbiologist and solutions developed to mitigate those challenges.

Introduction
The key to studying gene expression quantitatively in environmental samples lies in one's ability to effectively and most importantly, specifically detect messenger RNA (mRNA), corresponding to the activity of interest. In this regard, the use of reverse transcription (RT) in conjunction with quantitative real-time polymerase chain reaction (qPCR) has become a method of choice for quantifying specific mRNA's (Filion, 2012).

The unique ability of the RT-qPCR based method to analyse large number of samples rapidly, with high degree of sensitivity and specificity, makes it a logical choice over other forms of RNA detection methodologies for example Northern blotting, *in situ* hybridization, RNase protection assays and semi-quantitative RT-PCR analyses amongst others. Some of the aforementioned non-qPCR methods are highly laborious and time consuming to perform, making them unsuitable for high-throughput experimental designs. Moreover, some of them, for example RNase protection assay and Northern blotting, have a lower threshold for detection compared with RT-qPCR (Sharkey *et al.*, 2004). In summary, RT-qPCR is a technique of choice to detect target mRNA, if high degree of specificity, sensitivity and rapidity is desired. This article focuses on the methods, variants and technical limitation in quantifying mRNA's from complex environmental matrices.

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Microbial gene expression in environmental matrices
Detection and monitoring of microbial gene expression from an environmental sample has become an integral part of microbial ecology, bioremediation, biothreat analysis and diversity monitoring, amongst others (Sharkey *et al.*, 2004). While there are several molecular tools available to analyse microbial gene expression *per se* (Fleming and Sayler, 1995), the introduction of the RT-qPCR technology adds a very different dimension, not afforded by any of the earlier techniques (Sharkey *et al.*, 2004). With RT-qPCR technology, we have the unique ability to perform two specific functions simultaneously – monitor and quantify transcripts from target microbes, all under real-time conditions. The ease by which these two functions can be implemented by RT-qPCR, has significantly enhanced our ability to understand the microbial processes occurring under natural conditions at such fine scale and resolution, not previously afforded by any of the known conventional gene targeting techniques.

Analysing microbial gene expression in an environmental matrix involves multifaceted handling and processing steps. The complexity of these initial steps is heavily dependent on the type of matrix in question. While there are simple matrices generated through 'controlled' experimental conditions, there are myriad of matrices one has to contend with, especially naturally sourced, starting from simple (e.g. water) to highly diverse and complex types (e.g. soil, raw sewage/biosolids and food). Since each of the aforementioned matrices possess different levels of compositional complexity which, in most cases is poorly defined, one's ability to reliably implement a sensitive technology like RT-qPCR, has hinged on one major factor – 'pre-processing' of target matrices. This, in other words, implies extraction of target components from the matrix in such forms that, it becomes amenable for RT-qPCR analysis. In practical terms, this involves the use of specific RNA extraction protocols which, in principle are not only optimized for an efficient recovery of nucleic acids (RNA in the present context), but are also capable of mitigating co-extraction of endogenous confounding substances. These 'confounding' substances or inhibitors could be broadly defined as chemical moieties which, directly/indirectly interact with the components of the RT-qPCR reaction, leading to dampening of the final fluorescence signal. Such undesirable interactions invariably lead to an aberrant,

and in many instances, an irreproducible gene expression quantification pattern (Suslov and Steindler, 2005).

Based on the wide diversity and hence the compositional complexity of environmental matrices *per se*, the array of such chemical inhibitors, documented till date, have been extremely vast (Wilson, 1997). These inhibitors, whose list is continually increasing, has an amazing range of diversity, from simple forms of heavy metals, commonly found in food/water-based matrices (Kreader, 1996; Ijzerman *et al.*, 1997), to the highly amorphous 'brown contaminating' substances, endemic to organic/sedimentary soils (Watson and Blackwell, 2000). Developing a viable RNA extraction protocol, capable of mitigating the effects of these endogenous inhibitory substances from each type of environmental matrix documented till date, is one major challenge faced by microbiologists working in this area of research. This is in sharp contrast to the wide scale application of RT-qPCR technology in other areas of microbial research, where inhibitor mitigation is easily achieved through the use of highly controlled/defined microbial cultivation substrates (e.g. artificial growth media) and a well-optimized RNA extraction protocol. Since it is impossible to 'dictate' the composition of an environmental matrix, especially those sourced naturally, the only option till date has been to manipulate/optimize the nucleic acid extraction protocols, so that a high quality, inhibitor-free RNA, compatible to the sensitive requirements of RT-qPCR, is obtained. This, however, has proven to be a major challenge, since developing highly optimized protocols for each type of matrix, or class of matrices, is not only technically impossible, but also logistically impractical. In summary, this 'substrate defined' technical limitation, is a major bottleneck in our ability to rigorously implement the highly versatile RT-qPCR technology in environmental microbiology. Achieving a minimal 'quality benchmark' for this first and highly critical pre-processing step, has been a major and a continual challenge for environmental microbiologist.

In the subsequent sections, we aim to describe the technical challenges faced by many molecular environmental microbiologists interested in analysing microbial gene expression from environmental matrices, especially complex ones. Some of the areas covered include different type of RNA extraction methodologies, the invariant problem of endogenous inhibitors (which are co-extracted with RNA), common strategies employed to mitigate the effects of the same, finally followed by representative examples of specific environmental matrices namely soil, food/water and sludge/biosolids where RT-qPCR has successfully been implemented to quantify microbial gene expression.

Problems in extracting RNA from environmental matrices and quality issues

The first step towards analysing gene expression in any system is the invariant process of extracting RNA using an extraction protocol, optimized to yield high-quality, intact RNA. Low-quality RNA may compromise the derived expression results as previously observed (Raeymarkers, 1993; Imbeaud *et al.*, 2005). Developing an optimized RNA extraction protocol, suitable for a wide-range or even a subset of environmental matrices with a common compositional makeup, has been a major challenge for researchers. Notwithstanding the availability of 'off the shelf' commercial kits capable of extracting high quality total RNA from environmental samples, the end user still has to empirically test a particular kit's suitability on his/her target matrix. This in many cases is extremely cumbersome and especially difficult if the sample amounts are exceedingly limiting. Moreover, commercial kits are only customized to work optimally for certain matrix type(s) and thus, may not be suitable for a particular user-defined matrix. Also, if the experimental set-up demands a high throughput RNA extraction, the overall cost of nucleic acid preparation can be a major deciding factor for a user to take into consideration.

The inclusion of a robust internal/external extraction control(s), is one way to circumvent the issue of variable extraction efficiencies between different extraction protocols, as previously reported (Hoorfar *et al.*, 2003, 2004; Nolte 2004; van Doorn *et al.*, 2009). However, the unavailability of an universal external control(s), specifically designed for environmental matrices, at least until recently (González-Escalona *et al.*, 2009; Deer *et al.*, 2010; DeCoste *et al.*, 2011), and lack of unanimity amongst researchers on the choice of an optimal internal control, are some of the technical bottlenecks which have prevented researchers to implement testing RNA extraction protocols, either user developed or commercially available kits, in order to test their suitability for a particular matrix or class of matrices.

RNA extraction protocols

Although many protocols for extracting genomic DNA from environmental samples have been established till date (Bakken and Lindahl, 1995; Bakken and Frostegård, 2006), developing protocols capable of yielding high-quality, intact mRNA from the same substrate, remains a major obstacle. This is primarily due to the extreme labiality of the mRNA *per se*, especially of the prokaryotic origin which has a very short (in minutes) half-life (Takayama and Kjelleberg, 2000). Of the limited number of RNA extraction protocols developed till date for environmental matrices, one is able to always infer a well defined logical work flow. This work flow could be at best described into two steps: (a) cell lysis and release of RNA into the aqueous solution and (b) selective elution/precipitation of RNA. Any improvements to the current extraction protocols or development of new ones have always focused on modifying substeps within these two main steps.

The first step in extracting any RNA target is its release, preferably complete from the living cells. An inefficient or even an incomplete lysis could result in low or unrepresentative RNA amounts which, if submitted to RT-qPCR analysis would give either an aberrant or irreproducible gene expression pattern (Smith *et al.*, 2006). Cell lysis is easily achieved in simple matrices (e.g. water) however; this has proven to be extremely difficult for highly complex environmental matrices like sludge, natural soil and unprocessed food. This difficulty is most probably attributed to the components of the complex matrix which bind/adsorb the target components hence, making it difficult to access the cells for a complete or at least a representative lysis (Hahn *et al.*, 1990). Cell lysis is easily achieved through the use of chaotropic agents capable of permeabilizing the cells in simple matrices such as food (e.g. yoghurt) and water. Some of the commonly used agents include detergents such as sodium lauryl sulfate (SLS), lauryl sarcosyl and Triton X-100, EDTA and lysozyme, amongst others (reviewed

by Bakken and Frostegård, 2006). Since the major goal is to isolate RNA, these disrupting agents are used in combination with chemicals which inhibit the activity of the ubiquitously present RNase's which are released post cell lysis into the extraction solution. Some of the most commonly used agents include: lithium chloride (LiCl), cetyl-trimethyl ammonium bromide (CTAB), guanidinium thiocyanate (GdnHCl) and guanidine isothocyanate (GTC) (Sambrook and Russel, 2001). Complex matrices like soil and biosolids require a harsh physical disruption to release the microbial RNA contents into the extraction media (reviewed by Bakken and Frostegård, 2006). Some of the commonly employed methods to achieve this include, but are not limited to, mortar grinding with/without liquid nitrogen (Zhou *et al.*, 1996; Frostegård *et al.*, 1999; Hurt *et al.*, 2001) and bead beating (Bürgmann *et al.*, 2001). The physical disruption is usually carried out in the presence of the aforementioned chaotropic agents so that, post cell disruption, the released nucleic acids (total RNA) is not subject to RNase mediated degradation. Addition of diethyl pyrocarbonate (DEPC), a potent inhibitor of RNases, directly in the extraction buffer, has also been described for matrices which are known to contain high amounts of endogenous RNases (Bleve *et al.*, 2003). While there are many 'home brew' recipes for RNA extraction from environmental matrices (Ogram *et al.*, 1995; Bürgmann *et al.*, 2001; Hurt *et al.*, 2001), many commercial kits are now increasingly becoming available which work on a pre-defined environmental matrix; for example, the Mo Bio water and soil RNA extraction systems from Mo Bio Laboratories (Carlsbad, CA, USA) or the ZR soil/faecal RNA extraction kit from Zymo Research Corp (Irvine, CA, USA), amongst few others. Owing to proprietary and intellectual concerns, the exact description of the composition of the solutions used in most commercial kits is not always available.

Quality of RNA

The RNA obtained following extraction is highly unstable and susceptible to degradation by endogenous RNase's (Bustin, 2002, 2005), especially mRNAs which are several kilobases long. Apart from this, degradation could also occur due to handling errors and improper storage of the sample under suboptimal temperature conditions (Schoor *et al.*, 2003). Owing to the inherent chemical instability and its ability to get degraded due to base- or enzyme-catalysed hydrolysis, assessment of integrity of the extracted RNA is the first step in ensuring that the RNA submitted to RT-qPCR analysis would result in a reproducible data. For that, the general criteria for RNA integrity, irrespective from which environmental matrix it has been extracted from, could be summarized as:

- $A_{260\,nm}/A_{280\,nm}$ spectrophotometric absorbance value > 1.8;
- free of or extremely low genomic DNA contamination;
- free of enzymatic inhibitors of the reverse transcriptase enzyme and other enzymatic components of the RT-qPCR reaction;
- free of divalent ions and other co-factors which affect PCR reaction;
- low amounts/free of endogenous nucleases.

Conventional methods for RNA assessment are often not sensitive enough and also not specific enough for single-stranded RNA (Fleige and Pfaffl, 2006). This makes it difficult to assess the quality of extracted RNA from a wide range of environmental matrices. The interference from other non-target components during spectrophotometric assessment which include genomic DNA, proteins and other chemical moieties, also confound the true quality of the RNA. The conventional methods to assess RNA integrity can be done by classical denaturing agarose gel assessment, spectrophotometric assessment at multiple UV/VIS range wavelengths using a conventional spectrophotometer or a NanoDrop™ instrument, to a more modern lab-on-chip microfluidic platforms like the Bioanalyzer™ 2100 (Agilent Technologies, USA) and Experion™ (Bio-Rad Laboratories, USA).

Owing to the invariable low yields in extracting RNA from environmental samples, quantification and assessment of nucleic acids are more pre-disposed towards protocols which consume fewer amounts of the sample. This makes logical sense as there are always inherent procedural difficulties in extracting high quality RNA from environmental samples, forcing the end user to judiciously make use of the nucleic acid sample. Of the methods available for RNA assessment, the spectrophotometric methods using UV/VIS are the most popular and are performed at multiple wavelengths to assess truly the quality of the extracted RNA. Being non-discriminative, spectrophotometric quantification is done at 240 nm (background absorption and possible contamination), 260 nm (specific for nucleic acids), 280 nm (specific for proteins) and 320 nm (background absorption and possible contamination). On the basis of absorption at 260 nm, the quantity, and by the ratio at other wavelength values, namely $A_{260\,nm}/A_{280\,nm}$, $A_{260\,nm}/A_{240\,nm}$ and $A_{260\,nm}/A_{320\,nm}$, the purity can be assessed (Fleige and Pfaffl, 2006). $A_{260\,nm}/A_{280\,nm}$ ratio > 1.8 is considered an acceptable indicator of good RNA yield, suitable for downstream RT-qPCR analysis. However, it cannot determine the presence of contaminating inhibitors, which has to be empirically determined. This method however, has one major drawback- its inability to discern between RNA and carryover genomic DNA which, also contributes to the absorption value at 260 nm. This contamination could lead to serious overestimation (less than 50% accuracy) of the final RNA amounts in samples containing carryover DNA, bringing into question the reliability of this widely used estimation parameter (Bustin and Nolan, 2004). A more modern approach to correct this anomaly has been the use of advanced instrumentation in conjunction with RNA specific dyes. The NanoDrop ND-3300 instrument from NanoDrop Technologies, is an example of this approach and is increasingly becoming popular amongst researchers. RNA specific dyes like the RiboGreen™ dye (Molecular Probes, USA) can be used to detect RNA in conjunction with the NanoDrop quantification instrument. The main advantage of this quantification approach being the low requirement of sample (1–2 µl) and the ability of the instrument to read spectra over a wide-range (240–700 nm) on a single run.

The aforementioned spectrophotometric assessment methods, both conventional and modern, have one major drawback- the difficulty in assessing the integrity of the RNA preparation in the sample. Assessment by resolving the RNA sample on a denaturation agarose gel, to visually

inspect the integrity of the 18S/28S or 16S/25S ribosomal RNA, is one possible approach. This however is extremely impractical if the experimental set-up is high throughput or if the sample amount is limiting. Both these are woefully unsuitable for assessing RNA extracted from environmental samples which generally are high-throughput and precious.

The modern lab-on-chip technologies holds much promise and is able to alleviate all the aforementioned drawbacks inherent to conventional quantification methodologies. The Agilent 2100 Bioanalyzer and the Experion system are part of this new generation of technologies which allows the end user to assess the RNA sample's quality, quantity and most importantly, its integrity (18S/28S or 16S/25S rRNA). All the quantifications are performed on a micro-fabricated chip which requires extremely small amounts of starting sample. The development of a RNA assessment quality parameter called the RNA integrity number (RIN) for the Agilent 2100 Bioanalyzer (Agilent Technologies, 2004; Schroeder *et al.*, 2006), is a step in the right direction to develop parameter(s) which, could be reliably used to compare the quality of starting samples. Since it is easy to directly compare samples which are submitted to RT-qPCR analysis, such universal parameters serve another important function-make it simpler for laboratories to cross collaborate and compare results. These quantification platforms although extremely accurate and promising, are however expensive. This makes it difficult to implement them in experimental situations which require high throughput quantification of the starting sample or for laboratories operating on a modest research budgets.

Inhibitors from environmental matrices: diversity and mitigation

Notwithstanding the difficulties in extracting high quality intact RNA from environmental matrices, one invariant factor an environmental microbiologist has to always contend with is the presence of substrate derived chemical inhibitors (reviewed by de Franchis *et al.*, 1988; Wilson 1997; Filion *et al.*, 2003; Sutlovic *et al.*, 2005). These chemical compounds are natural components of the environmental matrices and are invariably co-extracted with RNA preparation. The passive presence of these chemical moieties in the final RNA preparation has one unintended consequence-inhibition of the downstream RT-qPCR enzymatic reaction, including the reverse transcription reaction which is required for generating the cDNA template (Johnson *et al.*, 2005). Practically speaking, inhibition *per se* during the whole RT-qPCR work flow could also be the end consequence of other factors which are of 'non-sample' origin, for example poorly controlled reaction conditions, contaminants in reagents, user derived introduction of contamination or some complex interactions that, at best are difficult to describe or distinguish (Wilson, 1997). In the present context, we have sought to focus on the substrate (i.e. environmental matrix) derived inhibitors and their effect on downstream enzymatic reaction.

Till date, a wide range of these endogenous chemical inhibitors have been identified from different environmental matrices (reviewed by Wilson, 1997). The list of known inhibitors presented in this review, however, does not include any process/protocol introduced chemical moieties like phenol, EDTA, CTAB etc., which are also inhibitory to the enzymatic components of RT-qPCR (Demeke and Jenkins, 2010). Empirical extraction of nucleic acids from various environmental substrates carried out till date has made it fairly possible to predict, assuming the elimination is not total, the nature of inhibitor(s) which, may be present in the final RNA preparation. For example, one can expect the presence of 'brown contaminating' and humic/fulvic acids-based substances in RNA preparations originating from organic soil and sediments (Watson and Blackwell, 2000). Similarly, polyphenols and tannins based inhibitors are endemic to wines and fermented beverages (Zoecklein *et al.*, 1999; Phister and Mills, 2003; Delaherche *et al.*, 2004; Martorell *et al.*, 2005) and therefore, expected to get co-extracted during the RNA extraction protocol. As a result, an intuitive decision making process goes into selecting an optimal RNA extraction protocol, for a particular target environmental matrix. If an optimized protocol is unavailable, a specific RNA extraction protocol has to be 'tailored' for the target matrix, so that an efficient mitigation of the expected inhibitory factor(s) known to be prevalent within the target matrices could be achieved.

Mitigating the effects of endogenous inhibitors on RT-qPCR, co-extracted during RNA extraction, is one of the major challenges faced by researchers. Since it is difficult to describe the quantitative levels of a particular inhibitor(s) present within a target matrix sample, especially those sourced naturally, a two pronged inhibitor mitigation strategy is generally employed by most researchers. As a first part of this strategy, and the most logical, is the development of RNA extraction protocol(s) which, not only are capable of extracting high-quality, intact RNA, but are also able to efficiently sequester any matrix originating inhibitors (Bürgmann *et al.*, 2003; Monnet *et al.*, 2008; Ulve *et al.*, 2008). Considering the wide range and complexities amongst environmental matrices *per se*, tailoring any extraction protocol to yield high quality RNA, which is not only intact, but also inhibitor-free, has proven to be an extremely challenging undertaking.

Based on literature evidence, the most common strategy has been to use specific chemicals/additives in the RNA extraction protocol itself which, bind/adsorb to the inhibitor(s). In this way, a large load of inhibitors are removed during the extraction process itself, making the final RNA preparations virtually free of or having a very minimum amounts of chemical inhibitors. This 'pre-emptive' strategy is widely employed and some of the common examples of the chemicals which aid in reducing/eliminating the endogenous load of inhibitors are: acid phenol–chloroform–isoamyl alcohol (25:24:1) to remove inhibitors from sediments and soils (Tsai and Olson, 1991), $AlNH_4(SO_4)_2$ to reduce co-purification of PCR inhibitors (Braid *et al.*, 2003), a combination of 5% polyethylene glycol 8000 (PEG), polyvinylpolypyrrolidone (PVPP) and Sepharose 4B-polyvinylpolypyrrolidone spin columns (Arbeli and Fuentes, 2008), polymeric adsorbent Superlite DAX-8 (Schriewer *et al.*, 2011), flotation of substrate (Jacobson *et al.*, 2009), commercial glass milk, Ionex and membrane-based spin columns (Tsai and Olson, 1991; Wiedbrauk *et al.*, 1995; Braid *et al.*, 2003). These approaches have been found to be optimal for those environmental matrices where the level of target transcript(s) is relatively high. This being important as the use of these inhibitor adsorption chemicals requires one to use multiple phase extraction/elution steps. Such multistep recovery processes, result in a considerable

loss of nucleic acids, making it difficult to detect moderate to rare transcripts.

The aforementioned mitigation strategies, although successful in most of the cases, work especially well when the target matrices have a simpler composition, for example water, or if the extraction matrices originate from highly controlled in vitro experimental set-ups. When highly complex environmental matrices like natural organic soils; organic sediments/sludge are used, which are known to carry a very heavy load of endogenous inhibitors, this mitigation approach is generally found to be inadequate. As a result, additional steps are deemed necessary to completely alleviate the effects of the inhibitor(s) which, do get co-extracted in the final RNA preparation. A very common strategy to achieve this goal is through the use of additives which bind/chelate the inhibitors in the RT-qPCR reaction itself. However for this strategy to work, the additive should be compatible with the enzymatic components of the RT-qPCR reaction itself. Identifying such novel additives, which are not only highly efficient in quenching the actions of an inhibitor, but compatible with the other enzymatic components of the RT-qPCR reaction, has been a major challenge. Some of the amplification facilitators/additives identified till date, which are able to satisfy these stringent requirements include the use of 'inhibitor tolerating' DNA polymerases (Al-Soud and Rådström, 1998), like bovine serum albumin (BSA) and single-stranded DNA binding T4 gene 32 protein (Kreader, 1996), bovine lacto transfer technique optimizer (BLOTTO) and spermidine, amongst others (Wang *et al.*, 2007). Irrespective of which one of these aforementioned strategies is employed; one of the most basic approach has always been to serially dilute the RNA sample to overcome the inhibitory effects of the endogenous inhibitor (Tsai and Olson, 1992; Queipo-Ortuno *et al.*, 2008). This simple approach, in conjunction to the aforementioned approaches, works well in most of the cases, however, this strategy is feasible only when the target transcript is present in high copy numbers. Excess dilution can lead to false negatives when targets are diluted below their detection limits or when the inhibitor(s) cannot be diluted to levels below their interference threshold (Queipo-Ortuno *et al.*, 2008).

Of the plethora of inhibitory moieties identified during RNA extraction from environmental matrices, very little is known about the actual mechanism through which this inhibition takes place. It is generally assumed that an inhibitor generally acts at multiple steps of the whole RT-qPCR analysis process. These steps could broadly be divided into three categories: (1) the nucleic acid extraction step, where it prevents complete cell lysis leading to an unrepresentative sampling; (2) the capture step, where RNA is recovered leading to an inefficient recovery or degradation; and, finally, (3) at the individual enzymatic step(s) of the RT-qPCR protocol which results in signal dampening. Since it is sometimes difficult to define an inhibition phenomenon at both the lysis and capture phases, the most logical target to characterize an inhibitor(s), has been to assay for any changes (usually negative), in the fidelity of the RT-qPCR enzymes, including the reverse transcriptase (RT) enzyme used for generating the cDNA.

Based on the multistep enzymatic cascade of RT-qPCR, the interaction of the inhibitor could itself be elucidated into three potential steps: (1) binding of the inhibitor to the enzymes of RT-qPCR namely: reverse transcriptase and DNA polymerase; (2) interaction of the inhibitor with the DNA; and finally (3) the interaction with the polymerase during primer extension cycle. This categorization is by no means absolute, since an inhibitor may act in more than one way and relationships between chemical, enzymatic, and physical factors often cannot be distinguished (Wilson, 1997). It is also probable that many inhibitors act through various physical and chemical means by interfering with the interaction between DNA and polymerase, for example 1 ng of humic acid is known to inhibit Taq DNA polymerase and reverse transcriptase (Opel *et al.*, 2010). Other factors which directly affect the components of the RT-qPCR are nucleases which hydrolyse DNA, divalent ions which bind to the co-factors required for the functioning of the polymerase, polyamines which prevent the DNA polymerase to access the template, amongst others (reviewed by Wilson, 1997; Hugget *et al.*, 2008).

Examples of common environmental matrices

A quick meta-analysis of those studies where qPCR and RT-qPCR techniques have been implemented to quantify microorganisms from environmental matrices, reveal a striking disparity- a much larger number of studies used DNA as a target instead of mRNA. This large scale bias in the use of DNA rather than RNA based targets could be attributed to the innate difficulties in extracting inhibitor-free RNA, as described in great detail in the earlier sections. Specifically, the compounded inefficiencies usually associated with RNA extraction from environmental matrices, the problem of accessing the cells enmeshed within the matrix component (for lysis to release of nucleic acids), high prevalence of endogenous RNase's, the extreme labiality of RNA species and co-extraction of inhibitors, could be some of the technical difficulties which in our opinion tip a researcher to favour DNA based targets. Despite these 'substrate defined' technical bottlenecks, it's highly satisfying to note that many attempts have indeed been made till date, in analysing the *in situ* microbial gene expression from various environmental matrices.

Amongst the host of RNA based gene transcripts as a target for RT-qPCR analysis, the choice of ribosomal RNA (rRNA) to infer the physiological status of a microorganism has certainly been very controversial (Smith *et al.*, 2006). Though, it is difficult for us to justify or debunk the validity of any study carried out till date where, modulation of a particular rRNA transcript(s) has successfully been used to describe the physiological functioning of the target microorganism(s), arguments certainly compelling against this approach, cannot be ignored. One of primary argument being the well known fact that the copy number of rRNA operons varies widely within a genome, therefore measuring the rRNA transcript numbers is not an accurate 'reflection of the functional processes' carried out by the target organism (Smith *et al.*, 2006). Also, the transcription rates of the rRNA is regulated by resource availability (Klappenbach *et al.*, 2000), for example starved cells tend to keep their ribosomal machinery active for a longer time (Wagner *et al.*, 2003), thereby potentially skewing the actual gene expression results. To circumvent such technical problems, functional genes are now being seen as more logical targets in other areas of microbiology, including environmental microbiology (Bælum *et al.*, 2008; Nicolaisen *et al.*,

2008). Keeping these caveats in mind and avoiding any unnecessary complications, we have in the future sections, only considered those studies where mRNA transcripts have been used as targets to analyse endogenous microbial gene expression from environmental matrices using RT-qPCR. Some of these common environmental matrices are described below.

Soil

Soil is home to highly diverse, complex microbial communities, which provide immense ecological services to mankind and its functioning, both direct and indirect, deeply impact many biological and bio-geochemical processes (Gutknecht *et al.*, 2006). Because of this importance, amongst all the known environmental matrices where the RT-qPCR technology has been implemented, it either pertains to soil or matrices derived from it. This is despite severe technical handicaps usually associated with working with this matrix, for example heavy compositional complexity and presence of chemical inhibitors, amongst others. Since only 1% of the endogenous soil microbial population is cultivable (Davis *et al.*, 2005), the acute gap in our information pertaining to the exact genetic makeup of the target microorganism(s), makes it even more challenging to design specific RT-qPCR primers/probes when soil is the substrate in question.

With specific reference to soils, one overarching question molecular microbiologists have constantly been interested in is the possibility of developing a quantitative understanding of both the abilities and diversity of complex natural soil microbial communities, preferably under *in situ* conditions (Bælum *et al.*, 2006; Bælum and Jacobsen, 2009). While some headway has been made in addressing this question, the introduction of RT-qPCR, a molecular tool with an undeniable ability to analyse target microorganism(s) with an extreme high specificity, sensitivity and rapidity, has given a tremendous boost to one's ability to address this seemingly simple, yet technically intractable question. The latter being primarily attributed to the high compositional complexity and intrinsic diversity amongst natural soil matrices *per se*. Notwithstanding this technical challenge, such understanding is extremely important if one needs to define the precise 'structure–function' relationship amongst soil based microbial communities. Of all the studies carried out till date, two distinct experimental approaches could be seen: (a) *in situ* gene expression experiments, where the expression of the target gene(s) is analysed directly from the natural soils and (b) seeding experiments, where the natural soil samples are seeded with a specific test microbe and its expression studied under defined test conditions.

An *in situ* analysis of microbial gene expression is seen as the 'holy grail' by many interested in understanding the endogenous microbial structure–function relationship (Bælum *et al.*, 2006). Owing to low efficiency of RNA extraction, high prevalence of endogenous inhibitors and rapid degradation of mRNA, especially when working with natural field soils, realization of this goal has not been possible as one would have expected (Bælum *et al.*, 2006). As a result, most gene expression studies have tended to use soil matrices with low concentrations of co-extractable enzymatic inhibitors, organic radicals or nucleases (Holmes 2004; Steunou *et al.*, 2006; Smith *et al.*, 2007). Despite this, researchers still have attempted, and in some cases succeeded, in analysing soils matrices, e.g. peat soil for microbial gene expression which, earlier were thought to be intractable due to their harsh endogenous constituents (Freitag and Prosser, 2009).

Understanding the *in situ* temporal expression of a gene has a special significance in certain area of soil microbiology, for example bio-remediation where such understanding is important to implicate the role of a gene in the actual mineralization/degradation process. An illustrative example could be the work of Holmes *et al.* (2004), who showed that it's possible to monitor the *in situ* changes in the expression pattern of the *nifD* gene in Geobacteraceae present in aquifer sediment in response to fluctuating sources of nitrogen. When these *in situ* results were compared with the highly controlled laboratory experimental set-ups, the *in situ* expression rate of the *nifD* gene was close to 100-fold higher. The authors suggested that there are 'unknown factors' operating in natural systems which, cannot be mimicked under controlled conditions. In other words, if an effective bio-remediation strategy has to be formulated using Geobacteraceae; one needs to understand the expression pattern of the *nifD* gene preferably under *in situ* conditions. This study underscores the utility of *in situ* systems to understand the exact metabolic state of the target microorganism. Similar studies where transcription dynamics of microbial genes have been assessed *in situ* using RT-PCR targeted *atzA* and *atzD* genes expression involved in the bio-degradation of the pesticide atrazine in the soil drilosphere, which essentially is a soil influenced by earthworm secretions (Monard *et al.*, 2010), *alkB* (alkane monooxygenase) involved in the microbial degradation of hydrocarbons in Antarctic soils (Powell *et al.*, 2006) and the *mcrA* (methyl coenzyme M reductase) and *pmoA* (methane monooxygenase) genes involved in the methanogenesis process in peat soils (Freitag and Prosser, 2009; Freitag *et al.*, 2010) amongst others (Bælum *et al.*, 2008; Chin *et al.*, 2008; O'Neil *et al.*, 2008; Mårtensson *et al.*, 2009; Mouser *et al.*, 2009; DiDonato *et al.*, 2010; Liu *et al.*, 2010a,b; N'Guessan *et al.*, 2010; Paulin *et al.*, 2010; Yuan *et al.*, 2011). The methanogenesis study in peat soils (Freitag and Prosser, 2009; Freitag *et al.*, 2010) is especially notable, considering the extreme difficulties in working with peat-based substrates. Components of peat soil are detrimental for RT-qPCR analysis since it is difficult to extract high-quality nucleic acids, owing to its high acidity (pH 3.8), high water content (80–90%), high concentrations of co-extractable organic matter, high capacity of complexing nucleic acids (Crecchio and Stotzky, 1998) and high concentrations of stable free radicals (Shinozuka *et al.*, 2001) and phenolics (Vaughan and Ord, 1982).

Limited numbers of spiking experiments have also been reported whereby; natural soil is first seeded with a test microorganism (containing the gene(s) of interest) and then, transcriptional modulation of the(se) gene(s) monitored using RT-qPCR. Subsequent work where such type of approach has been taken include quantification of *invA* mRNA in *Salmonella* spp. seeded in soil and chicken manure (Jacobsen and Holben, 2007; García *et al.*, 2010); herbicide degradation genes in *Cupriavidus necator* (Nicolaisen *et al.*, 2008), subsurface sediment (Holmes *et al.*, 2004; Elifantz *et al.*, 2010), *bphAa* gene involved in the degradation of polychlorinated biphenyl (PCB) in *Rhodococcus* sp. strain RHA1 (Wang *et al.*, 2008), *tfdC* and C230 genes involved in the cleavage of the of the

pollutant 2,4-D (Lillis *et al.*, 2010), and more recently, the expression of two antibiotic encoding biosynthetic genes in the bacterium *Pseudomonas* sp. LBUM300 of biocontrol interest (DeCoste *et al.*, 2011; Novinscak and Filion, 2011).

Food and other edible matrices

Microbial gene expression using RT-PCR in food and water based matrices (e.g. beef, chicken, carbonated beverages, fresh produce, wine, drinking water) is mainly performed for biosafety and surveillance purposes (Fratamico, 2001; Straub and Chandler, 2003; Rowan, 2011); as part of mandates usually delegated to governmental regulatory authorities (USFDA, 2001; WHO, 2002). In the interest of public safety at large and the societal importance of food and water, microbiological testing of these matrices is not only expected to be very rapid, but also extremely accurate (Jaykus, 2003). With the ability to acquire data in real-time, coupled with no post-sample processing, the RT-qPCR technology is an ideal technique to further this goal. Despite some initial cost concerns *viz à viz* the traditional protocols which are generally slower and cumbersome to perform, the RT-qPCR technology is now a widely accepted analytical tool in determining food microbial biosafety (Jaykus, 2003; Hanna *et al.*, 2005). The implementation of the RT-qPCR technology, specifically addresses the accuracy factor also interpreted as the 'zero tolerance' policy in the food microbial biosafety industry. Departure from such excruciatingly high verification standards could result in an erosion of consumer confidence, leading to potential severe economic consequences to both- producer and the end consumer.

Viability status of the target microorganism is extremely critical to accurately determine the biosafety status of a food sample (Keer and Birch, 2003). The conventional cultivation protocol even though highly cumbersome, specifically addresses this aspect, and therefore continues to be a mainstay technique in food microbiology (Harrigan, 1998). The relatively non-specific and time consuming aspect of this approach affects the decision making process in determining the biosafety status of food samples. As a result, alternatives are constantly being sought, which do not suffer from some of the logistical drawbacks. The advent of PCR changed this and offered a viable alternative to these cultivation based protocols (Krascsenicsova *et al.*, 2008). Initially, the PCR based protocols made extensive use of DNA based targets, including ribosomal (rDNA) in microbial food biosafety sciences (Vaitilingom *et al.*, 1998; Fortin *et al.*, 2001; Rijpens and Herman, 2002; Yaron and Matthews, 2002; Rodriguez-Lazaro *et al.*, 2003; Malorny *et al.*, 2007). Though able to detect the presence/absence of the target microorganism, this DNA based approach falls short in determining the viability status of the target microorganism. As viability *per se* is critical in accurately determining the biosafety status of food/water, decisions which have solely relied on DNA-based data, have attracted a great deal of controversy (Drahovska *et al.*, 2001) and continues to do so (Fey *et al.*, 2004). One major criticism against this approach is the well known fact that DNA is able to persist in dead cells, and therefore any positive PCR signal could potentially be contaminated, or at worst originate wholly from these non-viable, dead cells (Gonzalez-Escalona *et al.*, 2009). To avoid such confounding situations, non-DNA targets have been proposed and for that matter, less stable mRNA offers a much more attractive alternative (Sheridan *et al.*, 1998).

As mentioned earlier, expression analysis of functional gene transcripts allows one not only to detect the presence/absence of the target microorganism within a food sample, but also to infer its viability (Fey *et al.*, 2004). In food-based matrices, for mRNA *per se* to be a reliable target for RT-qPCR and an accurate indicator of cell metabolic status, it should meet certain criteria. These criteria could be summarized as:

1 For specificity purposes, the target mRNA transcription should be species or strain specific.
2 The target mRNA transcription should have a sufficiently brief half-life, so as to allow its detection.
3 The target mRNA transcription should ideally be expressed at sufficiently high copy numbers.
4 Its expression pattern should have some correlation with well-defined physiological parameters (typically spoilage).

Identifying candidate transcripts which are able to satisfy all the aforementioned criteria's, has proven to be an extremely difficult undertaking for food microbiologists. Thus, it is not surprising to note that there aren't many studies in the literature where RT-qPCR has been used to assess microbial gene expression in food/water based matrices. This difficulty in identifying a candidate mRNA could also be one reason where researchers have been forced to use the rRNA targets for determining the biosafety status of food (Hierro *et al.*, 2006; Wan *et al.*, 2006), despite the well known fact that rRNA expression is not too tightly linked to the physiological status of an organism (Ruimy *et al.*, 1994; Gourse *et al.*, 1996) and hence, not an ideal reliable indicator of cell viability status (Fey *et al.*, 2004, Gonzalez-Escalona *et al.*, 2009). Most, if not all the studies which have sought to assess the biosafety status of a particular food sample using mRNA based targets, have invariably made use of the 'endpoint reporting method' i.e. conventional agarose gel electrophoresis (Vaitilingom *et al.*, 1998; Fortin *et al.*, 2001; Yaron and Matthews, 2002). Though highly invaluable from a scientific stand point, these reverse transcription-PCR (RT-PCR) studies would have spurred, as one might expect, a rapid adaptation of the RT-qPCR technology, to further enhance the dynamics and accuracy of detecting the target microorganism(s), in the area of food microbial biosafety. However, looking at the number of studies in the literature, this 'natural progression' leading to a wide-scale adaptation of the RT-qPCR technology, has not yet happened. The main reasons cited behind this application gap, is the high set up and implementation costs of the RT-qPCR platform (Jaykus, 2003; Hanna *et al.*, 2005) and the exceptionally low tolerance for false-positive results in the area of food biosafety which is not totally difficult to rule out, considering the highly sensitive nature of this fluorescence-based detection technique.

Bleve *et al*. (2003), for the first time implemented the RT-qPCR technique along with the conventional end-point RT-PCR technique to quantify yeast and mould contaminants in yogurts and other pasteurized fruit-derived products (fruit juices and preserves). Yeast and mould based microorganisms have been heavily implicated in food spoilage and contamination. Using the fungal specific actin (*act*) transcripts as target, they were able to show that

it is possible to detect viable and viable but not culturable (VBNC) cells from food samples. No signal was however detected from dead cells. The dynamics of this detection was very interesting and depended on the nature of the detection system employed. Specifically, the RT-PCR was able to detect 10 CFU/ml and 10^2–10^3 CFU/ml of the fungi in pure culture and artificially contaminated food products, respectively. However, when the RT-qPCR system was used, the detection limit was raised 10- to 1000-fold higher, for the same target and extraction matrices. The authors noted that the extremely large dynamic range of detection, typical for RT-qPCR, is the key towards offsetting some of the limitations of the RT reaction, which is inherently vulnerable to inhibition by some of the matrix components (e.g. nucleases, fats, low pH, and chelating Mg^{2+} ions), used in this particular study. Other examples of RT-qPCR-based assessment of endogenous microbial gene expression in processed foods include: quantification of the *sea* and *sed* gene transcripts encoding enterotoxins A and D to quantify *Staphylococcus aureus* in cheese and milk (Duquenne, 2010), meat (Dolan *et al.*, 2010), pork (Techathuvanan *et al.*, 2010) and finally, *Salmonella* spp. in chicken and eggs (Wang and Mustapha, 2010).

In the realm of unprocessed foods/water-based matrices, there are few examples to be found where RT-qPCR has been used to determine the endogenous microbial gene expression. Excellent examples of unprocessed matrices are fresh vegetable products (e.g. raw salads) and drinking water. While fresh products are consumed raw with minimal pre-treatment, water is subject to microbial contamination either at source or during distribution to end consumers. In other words, it is an important vehicle for transmission of a large number of microbial borne diseases and therefore a subject of constant surveillance by regulatory authorities (Straub and Chandler, 2003; Howard *et al.*, 2006; Fong *et al.*, 2007). Fey *et al.* (2004) showed for the first time that it was possible to detect *Salmonella enterica* serovar Typhimurium using RT-qPCR in environmental water samples, using two RNA targets: the mRNA encoding for the *invA* (invasion protein A) gene and 16S rRNA. Notwithstanding the successful demonstration by these authors of RT-qPCR to detect microbial gene expression in water, there have been no further reported studies on similar lines, except for the detection of water based RNA viruses (Baert *et al.*, 2008; Parshionikar *et al.*, 2010). One major reason for this could be the technical difficulties in concentrating the target microorganisms from very large volumes of water (typically 100 ml to 1 litre), so as to get enough biomass for a representative and reproducible extraction of RNA (Rutjes *et al.*, 2005). Other examples of RT-qPCR in water based matrices are for the presence of yeast in wine (Hierro *et al.*, 2006) and juice based products (Connor *et al.*, 2005; Wan *et al.*, 2006). Both these studies made use of rRNA as targets to detect the viability status of the targeted microorganisms. Similarly, RT-qPCR has been used for detection of various serovars of *S. enterica* in raw vegetables and fresh products, specifically spinach (Gonzalez-Escalona *et al.*, 2009) and peppers (Miller *et al.*, 2010), targeting the *invA* gene transcripts.

Environmental biosolids/sludge

Analysis of microbial gene expression in biosolids/faecal samples is done primarily as public biosafety measures to detect the active presence of microbes which are known to affect human health. Most of the studies done till date on these environmental matrices have been qualitative, i.e. detect the presence/absence of the target microbial pathogen (Novinscak *et al.*, 2008; Karpowicz *et al.*, 2009). Recovery of RNA from biosolids/sludge is difficult due to endemic presence of array of inhibitors like humic acids, fulvic acids, fats, and proteins (Rosen *et al.*, 1992; Tsai and Olson, 1992; Whitehouse and Hottel, 2007). Environmental biosolids, especially urban sludge, is known to contain a heavy load of these substances, in addition to numerous organic/inorganic compounds, such as polyphenols and heavy metals (USEPA, 2000). These compounds have been found to get concentrated with microbial activity, resulting in its co-extraction during RNA extraction (Rock *et al.*, 2010). Owing to these reasons, analysis of microbial gene expression in biosolids using RT-qPCR is extremely difficult and predictably very few studies on this substrate can be found in literature (Dunaev *et al.*, 2008; Matsuda *et al.*, 2009; Kubota *et al.*, 2010; Rock *et al.*, 2010).

Conclusions

It is beyond doubt that there has been resurgence in the application of nucleic acid targeting tools in every aspect of biology, owing to reduced technology costs, constant development of effective nucleic acids recovery protocols, which also include commercial options, and last but not the least, the ever increasing access to post-analysis processing systems, i.e. software, online databases. These aforementioned advancements have however failed to provide the necessary traction for researchers working in the area of environmental microbiology, owing to certain difficulties endemic to this area of research. Of several, two major issues which have made it difficult to rapidly introduce advanced molecular tools in this area: (a) limited genetic information of environmental microbes due to extremely low cultivability and (b) practical difficulties in extracting high quality nucleic acids from environmental matrices, suitable for sensitive application like RT-qPCR. Despite these 'cultivation-defined' and 'substrate-dependent' technical bottlenecks, DNA/RNA targeting molecular tools, mainly end-point PCR-based, have extensively been used to analyse the expression status of targeted microbial transcripts. Though qualitative, these preliminary studies have given us invaluable insights into the functioning of the target microbe under defined experimental conditions.

To overcome some of the well known technical limitations of end-point PCR, detection platforms like RT-qPCR have proven to be an ideal solution. Using this highly versatile technology, it is now possible to simultaneously detect and quantify in real-time, the expression pattern of a microbial transcripts isolated from environmental substrates. Practical difficulties in working with environmental matrices have however impeded our ability to robustly apply this versatile technology. Technical issues such as extraction of inhibitor-free, structurally intact, total RNA from environmental substrates which is suited for a sensitive technique like RT-qPCR, continues to be a major impediment. It is however satisfying to note that despite these severe 'substrate defined' limitations, several attempts have indeed been made to implement the RT-qPCR technology in environmental microbiology. Efforts like these have given us invaluable insights into the

expression status of a particular transcript and hence, the biological functioning of microbes, specifically under natural *in situ* conditions. In summary, a better understanding of the technical difficulties faced by microbiologist in working with environmental matrices, especially those sourced naturally, should go a long way in developing efficient mitigation strategies (Filion, 2012).

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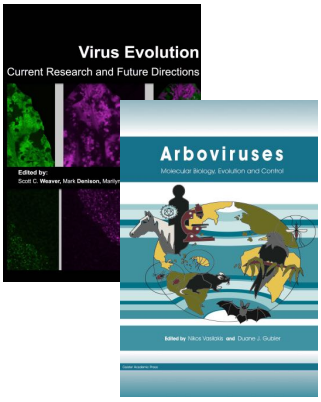

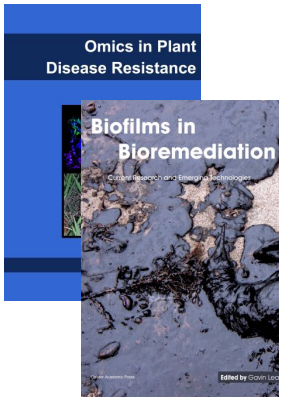

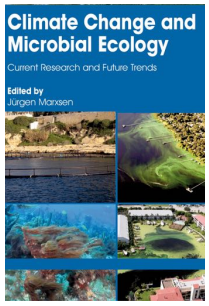

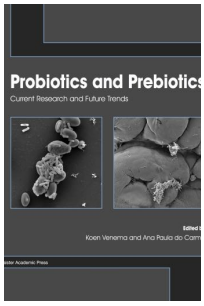

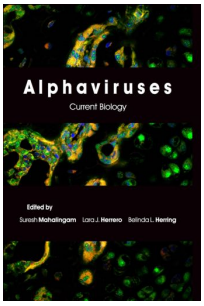

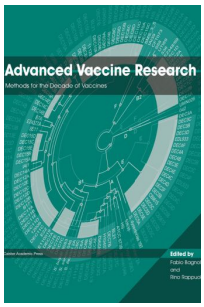

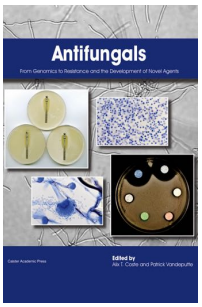

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