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Redefining relativity: quantitative PCR at low template concentrations for industrial and environmental microbiology

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The application of PCR techniques in environmental and industrial microbiology is complicated by innumerable organic and inorganic contaminants and enzyme inhibitors that copurify with nucleic acids. These complications are compounded in quantitative PCR (qPCR) methods, which are predicated upon subtle yet significant assumptions of amplification efficiency and the representativeness of the sample with respect to the environment or industrial process from which it was obtained. In low-biomass and/or low-template situations, additional concerns related to target gene spatial heterogeneity in the sample, differential DNA (or RNA) extraction efficiency, molecular sampling error, attenuation of PCR inhibitors and amplification bias can quickly undermine fundamental assumptions of conventional competitive PCR (cPCR) and most-probable-number PCR (MPN-PCR) formats. A critical evaluation of cPCR and MPN-PCR assumptions is therefore presented within the context of environmental microbiology and low-template enumerations. Fundamental conclusions from the analysis of qPCR assumptions are that: (a) environmental qPCR enumerations are invariably estimates, not absolute enumerations, which are *relative* to the PCR standard; (b) traditional cPCR assays are ill-suited for environmental applications, especially in low-biomass situations; and (c) both cPCR and traditional MPN-PCR practices insufficiently account for field-scale, process-level or experimental variations that arise and become amplified in PCR enumerations. Thus, sample representativeness and errors related to sample replication are frequently more important than errors related to the qPCR assay itself. Based upon this critique of qPCR assumptions, an alternate qPCR method for routine environmental application is described which is based upon replicative limiting dilution analysis and the pragmatic tradeoffs between analytical sensitivity and practical utility.

Keywords: quantitative PCR; competitive PCR; MPN-PCR; low template; bias; polymerase chain reaction; environmental microbiology

Introduction

The advent of nucleic acid technology has initiated a new era in environmental and industrial microbiology by providing specific, sensitive detection of (uncultured or unculturable) microorganisms in complex chemical and biological backgrounds. As Sayler [57] surmised, the ultimate application of nucleic acid technology is to provide knowledge of the absolute composition and structure of microbial communities and the dynamics of individual populations or genes within that community. At the forefront of nucleic acid technology is the polymerase chain reaction (PCR), universally employed for its ability to exponentially amplify a target gene of interest for facile detection. A recent summary of PCR technology for environmental applications has been published, with a cautionary preface and introductory chapter alerting the reader to inherent difficulties and nuances associated with PCR amplifications from environmental materials [61]. To be sure, the PCR is a powerful technique that has found the widest application in clinical or laboratory settings where routine samples consist of abundant, highly purified DNA. A 'routine' environmental sample, however, also contains innumerable organic contaminants, metals, chelators, humic acids or other inhibitory compounds that can copurify with nucleic acids and complicate the amplification process (recently reviewed in [69]). To realize the full potential of the PCR in environmental situations, then, one should be aware of its limitations at levels of chemical and genetic complexity not normally encountered in traditional molecular biology laboratories.

While standard PCR techniques can be problematic, their complexity pales in comparison to quantitative PCR (qPCR) methods in an environmental context. Numerous articles, reviews and methods manuals have been written describing quantitative PCR methods in laboratory settings [eg 2–4,16,18,22,24,25,28–30,32,33,35,37,38,46,51,54,60, 65,68,71,73], but development and application of these techniques for environmental samples has been slow [7,11,31,36,39,41,43,49,50,55,64]. The relative absence of quantitative PCR methodologies for environmental applications might be due to the chemical complexity of environmental samples, but may also result from the array of methods described in the nucleic acid literature and uncertainty regarding the underlying assumptions (Table 1) involved with the assay. Like prior environmental experience with standard PCR, the wholesale adoption of clinical qPCR methods without careful consideration of the assumptions, strengths and weaknesses of the various techniques is wrought with difficulty. The intent of this article is *not* to provide an exhaustive review of quantitative PCR

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Table 1 Summary of quantitative PCR methodology and data output

	cPCR	MPN-PCR	RLD-PCR
Format	Coamplified internal standard	Dilution to extinction	Dilution to extinction, with true replication
Enumeration relative to	Internal standard	No standard	External standard
Assumptions	Many	Few	Very few
Validation requirements	Extensive	Few	Few
Sensitivity	Excellent, to single-copy	Excellent, to single-copy	Excellent, to single-copy
Precision	Excellent	Very good, within limits of dilution	Very good, within limits of dilution
Accuracy relative to assumptions	Variable	Good	Excellent
Accuracy relative to the sample	Variable, highly dependent on satisfying all assumptions and representativeness of single sampling event	Variable, dependent on representativeness of single sampling event	Good, accounts for variation in PCR and sampling events

cPCR = competitive PCR formats; MPN-PCR = most-probable-number PCR including MPN statistics; RLD-PCR = replicative limiting dilution PCR with statistics described in the text. Similar considerations apply to quantitative RT-PCR methods but are not discussed here.

theory, as this can be found in previously cited references. Rather, the purpose of this article is to identify some key theoretical and practical limitations of conventional qPCR techniques as they apply to environmental samples and low-biomass (or low copy number) situations, and introduce a generalized qPCR approach for environmental samples that attempts to account for and minimize these limitations.

Quantitative competitive PCR

Theoretical considerations

Quantitative, competitive PCR (cPCR) techniques are generally classified as those where a known amount of internal standard is coamplified with the target gene of interest (Figure 1a). During the exponential phase of the PCR reaction, the amount of PCR product accumulated after *n* cycles (T_n and S_n) is described by the equations:

$$T_n = T_o\,(1 + E^{\mathrm{T}})^n \tag{1}$$

$$S_n = S_o\,(1 + E^{\mathrm{S}})^n \tag{2}$$

where T_o and S_o are the initial amounts of target (T) and standard (S), respectively, and E^{T} and E^{S} are the mean amplification efficiencies of the target and standard for all cycles. From these two equations, a general formula for experimentally determining T_o becomes:

$$\log(T_n/S_n) = \log T_o - \log S_o + n \times \log[(1 + E^{\mathrm{T}})/(1 + E^{\mathrm{S}})] \tag{3}$$

A basic assumption for absolute quantitation by competitive PCR techniques is that the efficiency of amplification be the same for both the target (T) and standard (S), in which case Equation (3) simplifies to:

$$\log(T_n/S_n) = \log T_o - \log S_o + n \tag{4}$$

According to Equations (3) and (4), a graph relating $\log(T_n/S_n)$ to $\log S_o$ should form a straight line *having a slope of –1* provided the ratio $(1 + E^{\mathrm{T}})/(1 + E^{\mathrm{S}})$ remains constant. Indeed, much attention has been given to the property of linearity, but little notice is ever given to the basic mathematical prediction and requirement that the slope = –1 independent of assumptions of equal amplification efficiency (below). Raeymaekers [51] provided an excellent mathematical treatment of this obvious and continued oversight of basic theory. Even if $E^{\mathrm{T}} \neq E^{\mathrm{S}}$, the main effect on the log-log plot is a line-shift in parallel compared to the plot when the efficiencies of amplification of target and standard are equal. In practice, however, it is impossible to detect a parallel shift because there is no reference point for the standard. Raeymaekers further pointed out that there is no theoretical ground to choose the point where $\log(T_n/S_n) = 0$, the point of equivalent product accumulation, for calculations of T_o if the slope of the graph is –1 as required. From these fundamental theoretical considerations, a quantitative, competitive PCR assay yielding a graph relating $\log(T_n/S_n)$ to $\log S_o$ which is nonlinear *or* does not have a slope of –1 cannot be used for absolute [4,5,31,39,41,71] *nor* relative quantification of T_o. If the amplification efficiency of target and standard remains constant through all PCR cycles, even for $E^{\mathrm{T}} \neq E^{\mathrm{S}}$, a *relative* quantitation of T_o is still possible [51].

Assumptions of equal amplification efficiency

From Equations (1) and (2), it is obvious that small differences in amplification efficiency between target and standard templates will generate very different amounts of final PCR product. The competitive PCR concepts involving a coamplified internal standard were developed to improve the reliability of the quantitative results by providing a means to correct for variations in PCR efficiency [20,73]. Internal controls also help control for variations in thermal cycler performance and reagent formulations that might otherwise lead to unequal amplification efficiencies. Since both the target and standard compete for amplification in the same tube, any variable affecting amplification efficiency is therefore assumed to have the same effect on both templates [31,73]. Thus, considerable attention is focused upon accurate determination of final PCR product yield, rather than the basic assumption of equal amplification efficiency.

The amplification of a target gene and competitive template in a single reaction tube is directly analogous to the coamplification of specific alleles in genetic analyses or multiple 16S rRNA genes from an environmental nucleic

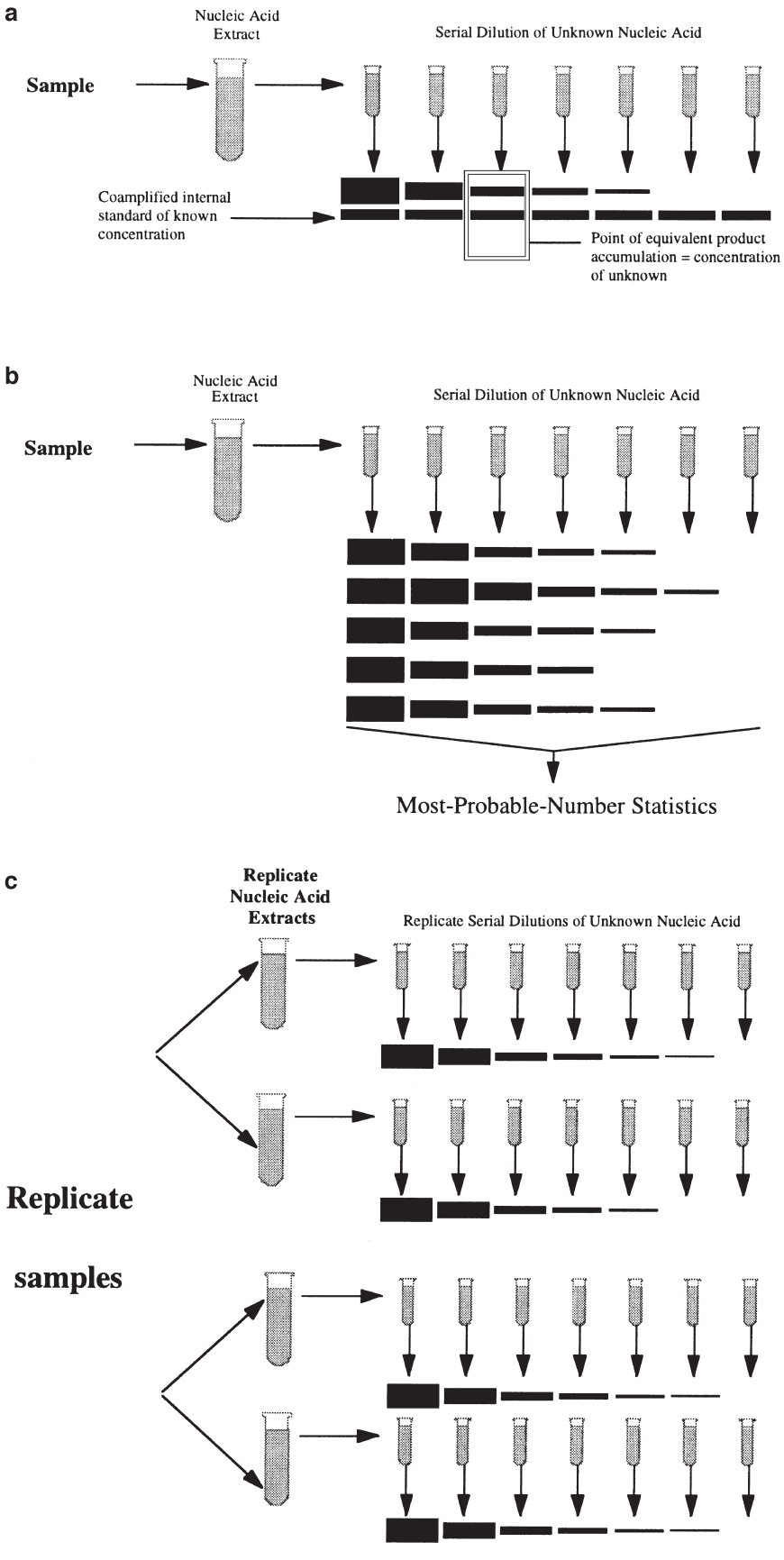

Figure 1 Graphic representation of quantitative PCR methods. Panel (a) illustrates the competitive PCR format; panel (b) represents the standard MPN-PCR format; panel (c) shows an example of replicate limiting dilution analysis presented herein.

acid extract. In both cases, there is ample evidence that preferential amplification of one template over another sequence can and does occur [45,53,56,58,67,70]. Preferential amplification can occur: (a) due to differential denaturation of templates; (b) from differential annealing of primers (eg amplification of a heterologous internal standard); (c) as a result of different target length; (d) if the target DNA is degraded or impure; or (e) when the initial number of sampled genomes (or targets) is very small. Indeed, a 6-bp difference was enough to consistently bias the amplification of a trinucleotide repeat region in favor of the smaller amplicon, with damaged template, low template quantities or addition of monovalent salts also contributing to PCR bias for the smaller target [45]. Even though the mechanism of PCR inhibition by humic acids and other copurified environmental constituents is not understood, it is clear that the added chemical complexities of and PCR inhibitors in environmental samples will exacerbate the effects and cause(s) of preferential amplification.

Even if care is taken to measure the overall amplification efficiency of nucleic acids isolated from a sample relative to a standard template under identical chemical conditions [39,43], the resulting quantitation still cannot be considered 'absolute' unless there is a reference point from which to evaluate amplification efficiency of the standard. Thus, a competitive PCR assay will always be *relative* to the standard. Admittedly, there are many situations where the 'relative' enumeration is virtually indistinguishable from the 'absolute' number of target molecules in the sample [20], especially at higher template concentrations (eg $\geq 10^7$ copies). In these situations, the inhibitory effects of environmental contaminants can be mitigated by sample dilution prior to the PCR, and complications due to preferential amplification are minimized when the number of target molecules is high. Möller and Jansson [43] have also introduced the concept of competitor co-extraction to account for both nucleic acid extraction and amplification efficiencies, a methodological advance which significantly improves the accuracy of cPCR quantitation. At low template concentrations, however, the complicating chemical factors of environmental samples, coupled with previously described idiosyncrasies of the PCR, can combine to introduce error in a quantitative, competitive PCR format due to significant differences in (and possibly changing) amplification efficiencies (E^T and E^S) during the early rounds of the amplification process [20].

Low template concentrations

As pointed out by others [20], an exhaustive evaluation of all qPCR assumptions is frequently unnecessary for a relatively accurate cPCR enumeration. This assertion is equally true for many environmental or industrial applications of qPCR techniques. At low template concentrations, however, additional considerations apply. For example, the initial solution phase polymerase:primer:template hybridization and complex is a random occurrence due to the phenomenon of molecular sampling error [20,45,67]. For highly purified DNA samples, molecular sampling error has been observed at target copy number ranging from 20–78 copies [45,67]; in a highly multiplexed situation, such as the concurrent amplification of 16S rRNA genes from total microbial community DNA, there is some evidence that molecular sampling error occurs at target densities orders of magnitude higher than in highly purified DNA samples with limited target gene sequence complexity [12]. These additional random and nonuniform processes in the early phase of the PCR can alter E^T and/or E^S, which will result in PCR enumeration error. In most qPCR situations, then, there will be a lower limit of (relative) quantitative detection. For environmental or industrial samples, the complicating factors of variable chemical backgrounds suggest that the lower limit of *reproducible* detection and enumeration is likely to be higher than otherwise expected. Therefore, the limits of detection and enumeration should be defined for each target of interest and each environmental sample under investigation.

The interaction between environmental contaminants and the PCR process is not understood, nor is the interaction between humic acids, metals, residual detergents, proteins, chelators and nucleic acids present in an environmental extract. Since many inhibitors of the PCR copurify with nucleic acids from environmental samples, nucleic acids are either extensively purified, or the environmental sample is simply diluted to a point where the inhibitory effect is no longer noticeable [13,26,36,41]. The net result in both cases is to reduce the target concentration proportionately, sometimes by several orders of magnitude, which increases the probability that T_o is within the range where molecular sampling error prevails. It is therefore imperative that the standard curve be generated in the expected target concentration range and satisfy the basic mathematical predictions set forth above. In addition, extensive purification or environmental sample dilution does not guarantee that the inhibitors are no longer present and affecting the PCR, a complication likely to surface at low template concentrations.

There is a qualitative (albeit not yet quantified) difference between isolating nucleic acids from environmental samples amended with log-phase cells [36,43,50,62–64] and environmental samples containing low native concentrations of target or overall biomass (eg, the deep subsurface [10,12,21,34,47]). Native microorganisms are frequently starved, metabolically inactive, dormant (eg spores) and/or physically damaged, lifestyle differences that may significantly affect the availability of nucleic acids for extraction and analysis. In the former case, excellent extraction efficiencies and qPCR detection limits have been achieved, whereas little work has been performed under the latter conditions. In low-biomass samples (ie $\leq 10^6$ cells g^{-1}), it is clear that overall nucleic acid extraction efficiency is extremely low [47]. The practical consequence of low biomass samples and concomitant inefficiencies in extraction and recovery of nucleic acids is that the effective T_o concentration required for detection increases and enumeration is less accurate [eg 50,52]. Due to poor nucleic acid recovery from low biomass samples and the added concerns of molecular sampling error and preferential amplification, competitive PCR techniques appear to be less suited for low-biomass or otherwise low-copy environmental situations than in highly purified, high-biomass samples containing high or low quantities of target.

Most-probable-number PCR

Theoretical considerations

Most-probable-number PCR (MPN-PCR) techniques are offshoots of the traditional microbiological enumeration procedure that combines dilution to extinction with enrichment culture [17]. It comes as no suprise, then, that the MPN-PCR techniques have been the preferred qPCR method in environmental microbiology [1,19,49,50,52,64] with only infrequent application in general molecular biology ('Limiting Dilution' [59,60]). MPN theory is based upon the assumptions that: (a) the elements to be enumerated are in solution; (b) they must be randomly distributed throughout the solution; and (c) a single copy of target gives rise to a positive signal. The emphasis for PCR applications is that the endpoint is all-or-none, rather than a quantifiable endpoint as described above for competitive PCR methods. The assay consists of a serial dilution of the sample, and replicate PCRs for each point in the dilution series (Figure 1b). Around the limit of dilution, an individual tube contains either no targets and gives a negative result, or contains one or more targets which give rise to a positive result. The distribution of targets within each reaction tube is assumed to conform to Poisson statistics, such that the mean number of targets per reaction tube is given by:

$$m = -1\mathrm{n}p_o \tag{5}$$

where m = the mean target density and p_o is the proportion of negative reactions at each dilution point.

The MPN technique is a procedure for obtaining *estimates* and does not provide absolute quantitation. The precision of an MPN assay is directly related to the number of replicate tubes assayed at each dilution of the sample, with more tubes providing smaller coefficients of variation around the true target density. With respect to the dilution ratio, however, the average precision is practically identical for any dilution ratio between 2 and 10, provided the total number of tubes in the assay is identical, although the coefficient of variation is more stable and tends to be slightly lower for a 2-fold dilution series [17].

Assumptions

Unlike competitive PCR techniques, there are relatively few assumptions for MPN-PCR that can be violated. Quantitation by MPN does not require the use of an added internal standard and the endpoint is a simple all-or-none determination. Because the endpoint is based upon the terminal plateau phase of the PCR, the technique is relatively robust and able to handle relatively wide variations in amplification efficiency without affecting the estimation of DNA target numbers [59].

As discussed above, however, environmental contaminants can interfere with the PCR, and the assumption of single-copy detection applies *to the sample*, not to the PCR or the positive control templates. As with cPCR methods, the effects of environmental contaminants will be minimized or unnoticeable at relatively high template concentrations, but may have significant effects on MPN-PCR enumerations at low target densities or low overall biomass. Therefore, controls must be included with each MPN enumeration to ensure that single-copy detection occurs in the same chemical matrix as the target. In addition, it is extremely important to ensure that negative results are due to the absence of target, rather than inefficient amplification (false negatives). Practical approaches to deal with these issues are described below.

The assumption of random template distribution is critical to MPN theory. MPN tables tend to overestimate the accuracy of the MPN method, since they are derived on the assumption that the mathematical analysis corresponds exactly to the practical situation [17]. In reality, the distribution of targets in solution does *not* conform to a Poisson distribution [6,23], an error which is propagated throughout the serial dilution [15] resulting in confidence limits around the estimate which can span two orders of magnitude [42]. While a plateau-phase detection point will accommodate day-to-day variations in amplification efficiency, pipetting and dilution errors manifest themselves as altered extinction points in a dilution series and contribute to enumeration error above and beyond statistical theory. At low overall concentrations of DNA, nucleic acid aggregation [8] during repeated sample freezing and thawing may also alter the distribution of target within the sample extract, further eroding the primary assumption of the MPN approach.

One dilution series is not enough

An implicit and often unstated assumption is that the sample under investigation is *representative* of the environment or industrial process from which it was taken. It is well-known that microorganisms are distributed non-randomly in nature, and their tendency to aggregate, clump or adhere to surfaces also affects their distribution in environmental and industrial processes. The distribution of microorganisms in mixed-phase environments, then, are probably very different from their distribution within the experimental sample represented as a liquid nucleic acid extract. Differential and/or incomplete DNA extraction efficiencies [11,27,40,44,47,62,63,66] can also affect target density in the final nucleic acid extract. In combination, these sources of error could be more important than the large standard deviation of the MPN estimate itself [17]. Indeed, whether the assay format is competitive- or MPN-PCR, *sample* replication and sample *representativeness* are important considerations that are not explicitly addressed in many qPCR analyses of environmental systems.

Precision *vs* practical utility

The foregoing discussion emphasized that quantitation of nucleic acids by cPCR or MPN-PCR is complicated by a number of technical considerations. It is also clear that the vagaries of microbial spatial heterogeneity, environmental sample chemistry, PCR inhibitors and low template concentrations only serve to undermine pre-analysis assumptions and increase the requirements for method validation and quality control. The question then becomes, is high analytical precision necessary?

As noted for clinical samples, high analytical precision does not necessarily translate into adequate assay performance, defined as the ability to detect a target sequence at a given level of sensitivity or analytical precision [48]. Assay

performance depends on sample preparation to ensure the presence of an adequate number of targets, absence of enzymatic inhibitors, and appropriate sampling time (stage of disease; before, during, or after therapy). In addition, the volume of sample tested has a direct bearing on assay performance for low-copy targets. For example, an assay with an analytical sensitivity of one target molecule will only give a positive result in $\leq$70% of all reactions performed if there is an average of only one target copy in the reaction volume. In order to ensure that 99% of all samples will generate a positive post-amplification, single-copy signal, the sample would need to contain an average of 10 target molecules irrespective of enzyme inhibitors. As discussed previously, the effects of inhibitors are themselves thought to be more prevalent at low copy numbers of target. Precision in practical terms also depends on the number of target molecules of interest. If thousands of targets are present per sample, a relatively insensitive and imprecise amplification method will provide adequate assay performance. Therefore, it is important to differentiate between high analytical precision and functional performance and practicality, since the amplification system may not always provide adequate overall performance despite high analytical precision [48].

Similar, practical concerns have been voiced for the application of nucleic acid technology in applied bioremediation situations [9]. In order for nucleic acid methods, including qPCR techniques, to contribute important and timely information concerning microbiological processes, they must provide timely and cost-effective information that cannot be obtained by other analyses. In addition, basic or applied qPCR studies of environmental or certain industrial processes need to consider the larger ecological or functional properties of the system: microbial spatial variability is an obvious, inherent and frequently overlooked factor which can significantly skew and distort conclusions based on nucleic acid (and other) analyses [9]. Other variables of practical importance include (but are not limited to): the extraction efficiency of *in situ* nucleic acids (as opposed to estimates based on log-phase, spiked cells); extraction and amplification bias; expected (absolute) target density and chemical/genetic complexity in which the target exists; total biomass; lower detection limits which also account for PCR inhibition; and consistent, reliable, replication of PCR data which can be compared across spatially or temporally related samples. Assumptions related to sample selection and processing in an environmental or industrial context, then, may ultimately be more important than the assumptions, practice and limitations of the qPCR techniques themselves. The joint considerations of practical utility and the properties of environmental samples therefore call into question the need for high analytical precision of routine qPCR techniques.

A practical compromise

qPCR by replicate limiting dilution analysis

Given the multitude of PCR and environmental considerations, the frequent requirement for low-copy detection in low-biomass environments, and a need to develop new PCR assays for varying genes and mRNAs on a regular basis, we have developed an alternative qPCR technique which is sufficiently precise for many ecological and applied purposes, yet simple enough for rapid development and practical application. Our approach, which is usually linked to a broader ecological question or process, comes from several conclusions related to and derived from the previous discussion. These conclusions include: (a) cPCR assumptions are rarely satisfied in an environmental context; (b) cPCR techniques are not reliable in a low-biomass, low copy-number context; (c) the quality control requirements for cPCR methods are more difficult to address than and outweigh the added effort required to perform multiple PCRs per sample (as in MPN-PCR); (d) insufficient attention is given to the *relative* amplification of the standard under idealized and actual PCR conditions; and (e) both cPCR and traditional MPN-PCR practices insufficiently account for field-scale, process-level or experimental variations that arise and become amplified in PCR enumerations conducted on a *single* sample.

Given these conclusions and outlook on qPCR methods, the generalized but distinguishing features of our assay are that: (a) we acknowledge forthrightly that all enumerations are *relative* to the standard, such that every enumeration is only an *estimate*; (b) the assay has a known lower detection limit, but not necessarily single-copy sensitivity; (c) we use and prefer the dilution-to-extinction *concept* but do not use MPN statistics; (d) the dilution series is constructed in keeping with the ecological context and tolerable variation in the estimate or target density; (e) we make extensive use of spiked controls to estimate the extent of PCR inhibition and minimum detection limits *in the sample*; (f) we make extensive use of external standards to calibrate the enumeration through comparative analysis of product yields; and, most importantly, (g) we perform replicate nucleic acid extractions from the sample, with replicate serial dilutions prepared from each nucleic acid extract prior to the PCR. The experimental setup of our qPCR assay relative to competitive- or MPN-PCR procedures is presented graphically in Figure 1, with a comparative representation of underlying features described in Table 1.

In critiquing the method and comparing it to more established assay formats, it is important to keep in mind the tradeoffs in analytical precision that come with practical utility, and that increased precision can be obtained by using two-fold base dilutions rather than 10-fold dilutions, performing multiple nucleic acid extractions and replicate dilutions, or validating all of the assumptions involved in a cPCR assay. The basic experimental design for each sample, however, consists of two nucleic acid extractions, with two dilution series from each extract, with a single PCR for each dilution point (Figure 1c). In this sense, it is a 'non-quantitative' or 'relative' MPN experiment as defined by Sykes [59,60]. Nevertheless, the net result is a 2×2 matrix, yielding four replicate dilution series. The total number of PCR reactions per sample depends on the extent of sample dilution. A typical enumeration in our lab involves four replicate 10-fold dilution series spanning five orders of magnitude, or 20 total PCR reactions (as shown in Figure 1c). A geometric mean value is calculated from each of the endpoint enumerations from each of the four dilution series, which can be compared to estimates between samples through analysis of variance [72].

Estimating target densities

A first-generation example of our limiting dilution analysis, including the rationale for sacrificing analytical sensitivity for practical utility in a field-scale bioremediation application, has been previously described [11]. Since then, we have routinely incorporated replicate sample extractions and replicate dilution series in our assays. Figure 2, for example, shows limiting dilution PCR results used for the enumeration of ammonia-oxidizing bacteria in several soil types receiving a nitrogen amendment and incubated for 3 weeks. The purpose of this experiment was to compare nitrifier populations based upon a culturable MPN assay and qPCR enumeration. Nucleic acids were isolated from replicate 4-g aliquots of each sediment using a direct lysis and purification protocol described elsewhere [13]. Replicate 10-fold dilutions of each sediment extract were prepared, and PCR was performed on one 1-μl aliquot from each tube of the 10-fold dilution series using 16S rDNA primers targeting terrestrial ammonia oxidizing bacteria and a cycling protocol described elsewhere [13]. The detection limit with this set of primers and PCR conditions was reproducibly 0.1–1 pg *Nitrosomonas europaea* genomic DNA, corresponding to 20–200 cell equivalents of DNA assuming 5 fg DNA cell^{-1}. Estimates of nitrifier abundance per gram of sediment were calculated by:

(PCR detection limit) × (Extinction point) × (Conversion factor(s))

The PCR detection limit is a *best-case* estimate derived from the positive control for the assay. Hence, a ≥ symbol is always used to indicate that the enumeration is a conservative estimate bounded by the (unknown) extent of PCR inhibition in the sample and the extinction point indicated by the dilution series. Thus, if the PCR detection limit is 20 cell equivalents, the last positive signal occurs at the 10^3 dilution, and the dilution series is initiated with 10 mg equivalent of soil extract (Figure 2; Agropyron + N, lanes 27 and 32), then the estimated biomass of ammonia-oxidizing bacteria is $\geq 2 \times 10^6$ cells g^{-1}. The actual target density lies somewhere between 2×10^6 and 2×10^7 cells g^{-1}; if there were more than 2×10^7 cells g^{-1} in the sample, a positive PCR product would be observed at the 10^4 dilution. Limiting dilution estimates for the other soil samples are given in Table 2.

The accuracy of each enumeration can undoubtedly be increased by introducing conversion factors for nucleic acid extraction efficiency and recovery [11,39], DNA content per cell, and target copy number per cell when known (or estimated) [39], or by analyzing a 2- or 3-fold dilution series rather than a 10-fold dilution series. Unless these combined influences represent a 10-fold (90%) decrease in DNA or target yield, however, the order of magnitude of the estimate will be unchanged.

Due to the extraction and quantification uncertainties described above, differences of less than one order of magnitude between samples or treatments are of little practical or ecological consequence. In this particular example, the accuracy of enumeration and inferences regarding nitrification capacity in the test soils are not necessarily synonymous, because the range of microorganisms capable of

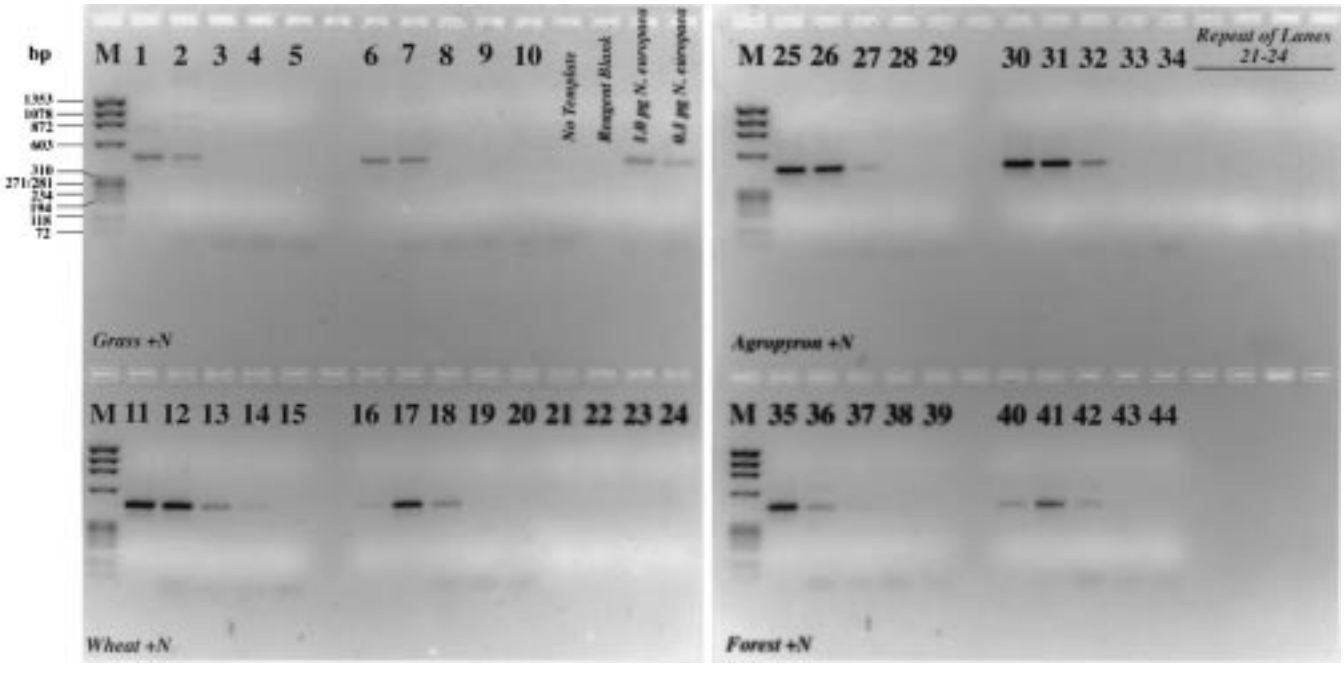

Figure 2 Replicate limiting dilution analysis of terrestrial ammonia oxidizer 16S rDNAs from $t = 0$ soils immediately after amendment with nitrogen fertilizer. Results from only one (of two) replicate soil extract(s) are shown. Two-fifths of each PCR were analyzed on 2% agarose gels in 1× tris-acetate-EDTA running buffer as described in [13]. Lane assignments are: M = Ø × 174 × *Hae*III molecular weight marker, with fragment sizes indicated in base pairs (bp); 1–5 and 6–10 are replicate 10-fold dilution series (from 10^{-1} to 10^{-5}) of soil nucleic acids recovered under temperate bluebunch grass; 11–15 and 16–20 are the same as 1–5 except from soil underneath arid wheatgrass; 21–24 are undiluted grass, wheat, agropyron and forest samples; 25–29 and 30–34 are the same as 1–5 except underneath agropyron cryptogamic crust; 35–39 and 40–44 are the same as 1–5 except from a temperate forest soil.

Table 2 Replicate limiting dilution estimates of nitrifier cell density in soils amended with nitrogen (N) fertilizer and incubated for 3 weeks

Soil	Treatment	$t = 0$ weeks	GM	$t = 3$ weeks	GM
Grass	+N	10^5, 10^5, 10^6, 10^6	($10^{5.5}$)	10^6, 10^6, 10^6, 10^7	($10^{6.2}$)
Grass	–N			10^6, 10^6, 10^6, 10^6	($10^{6.0}$)
Wheat	+N	10^7, 10^6, 10^6, 10^7	($10^{6.5}$)	10^7, 10^7, 10^6, 10^7	($10^{6.7}$)
Wheat	–N			10^6, 10^7, 10^7, 10^7	($10^{6.7}$)
Forest	+N	10^6, 10^6, 10^7, 10^8	($10^{6.7}$)	10^7, 10^7, 10^7, 10^7	($10^{7.0}$)
Forest	–N			10^7, 10^7, 10^7, 10^8	($10^{7.2}$)
Agropyron	+N	10^6, 10^6, 10^6, 10^6	($10^{6.0}$)	10^7, 10^7, 10^8, 10^8	($10^{7.5}$)
Agropyron	–N			10^6, 10^6, 10^7, 10^7	($10^{6.5}$)

Estimates were derived from dilutions as described in the text, omitting the correction factor for genome size (*ca* 5 fg cell^{-1}). Geometric mean (GM) values are shown within parentheses.

nitrification is both broad and indeterminate. Consequently, the variability of the data from the two different sediment extractions and each of the two dilution series generated from each sediment extract is of more practical importance than a single extraction and enumeration with no estimate of variability *in the soil sample*. This distinction is especially evident for the forest soil amended with nitrogen at the $t = 0$ timepoint (Table 2); the enumeration varies over two orders of magnitude with a geometric mean value of $10^{6.7}$ (according to [72]). If a traditional MPN-PCR assay were performed on the *one* extract and dilution series where the estimate of nitrifier abundance was $\geq 2 \times 10^8$ cells g^{-1}, the result would normally be interpreted as a very precise estimate of target density that was very unrepresentative of the actual target density of the soil sample as a whole. With the replicative limiting dilution analysis presented here, however, a more practical and accurate estimate of nitrifier population densities *in the sample*, and data from which to calculate a standard deviation of the estimate specific to the sample, were obtained.

The spiked control

For the example given in Figure 2, we observed a positive PCR signal for each soil and dilution series, a common result for relatively high biomass, near-surface environments and industrial processes but unusual for low biomass/template samples. From lanes 16, 21–24, and 40, however, it is evident that some level of polymerase inhibition occurred in the 10^0 and 10^1 template dilutions. When performing a quantitative PCR assay of any type, it is important to distinguish between the absence of target and PCR inhibition or inefficient amplification. Discriminating between a true negative and false negative can only be accomplished with 'spiked' controls, in which a known amount of the positive control template is added into PCR reactions that also contain the sample nucleic acid extract (and dilutions thereof). The 'spiked' control is fundamentally different from the positive control (or standard curve) for the PCR assay, in that the latter is purified template added to pure water which can overestimate the PCR detection limit *in the sample extract*. Despite their obvious importance, spiked controls are used only occasionally [11–13,55,64], perhaps due to the anecdotal observation that most ecological and industrial assays are performed on high-biomass samples with significant target gene density.

For low-biomass and low copy-number applications, significant concerns regarding a spiked control relate to the actual detection limit of the PCR, the actual target density in the sample, and the spacing of the dilution series. That is, the quantity of control template added to the test sample(s) should not be so high as to increase the target concentration (T_o) above detectable levels by a copy number effect alone. In this respect, the quantity of spiked template should be of the same or lower order of magnitude as the expected target density in the sample, and confine the endpoint signal to a single step in the dilution series. For example, given a hypothetical detection limit of 2000 copies and a 5-fold dilution series, the quantity of spiked control in each reaction should be $<$8000 copies to avoid extending the actual (and idealized) assay extinction point to the next highest dilution in the series. Likewise, for a detection limit of 20 copies and a 10-fold dilution series, the idealized spiked control should add $<$180 copies of control template. In both cases, the preferred option is to add as little spiked template as possible. A practical application of the spiked control is presented in Figure 3, in which a qPCR assay was developed for detecting specific biodegradative genes in soils contaminated with the jet fuel JP-5 [11]. Ten-fold dilutions of soil nucleic acid extract were prepared and run in parallel with a spiked control containing *ca* 3650 copies of catechol 2,3 dioxygenase (*cdo*), 365 copies of naphthalene dioxygenase (*nahAc*) and 230 copies of alkane hydroxylase (*alkB*) genes as indicated in the figure legend. PCR inhibition is clearly evident in the 1:10 template dilution (lanes 2, 7, 11, 16, 20 and 25) but is unnoticeable in the 1:100 dilution. While the spiked control was not necessary to enumerate *cdo* or *nahAc* genes in this sediment, it was of obvious importance for estimating an upper limit of *alkB* density in the sample. Since there was no product obtained in the dilution series but PCR inhibition was removed at the 1:100 template dilution, we can back-calculate to a *minimum target density* required to observe a positive PCR signal under the conditions and sensitivity of the assay and as revealed by the unspiked positive controls. In this case, the minimum detectable *alkB* gene density was $\sim 2.3 \times 10^5$ copies g^{-1} ((PCR detection limit of 230 copies) $\times$ (10^2 dilution at which inhibition is overcome) $\times$ (correction factors to normalize for 1 g sediment)). Therefore, gene density in the sample was $\leq$ 2.3×10^5 copies g^{-1} sediment. Similar estimates and calcu-

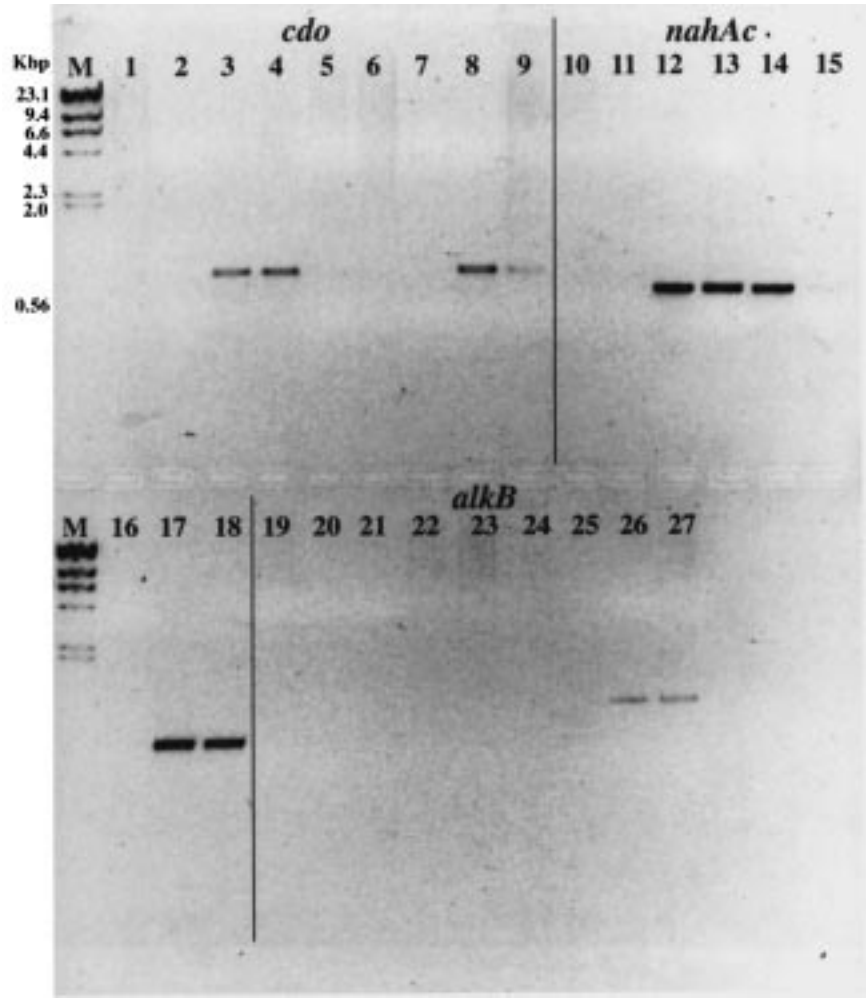

Figure 3 Detection of specific biodegradative genes in a JP-5-contaminated soil. Nucleic acid extraction and PCR conditions for each gene are described in [11]. One tenth of each reaction was analyzed on 1% agarose gels in 1 × tris-acetate-EDTA buffer. Lane assignments are: M = λ × *Hin*dIII, with fragment sizes indicated in kilobases (kbp); 1, 10 and 19 are no template controls for *cdo*, *nahAc*, and *alkB* genes, respectively; 2–6, 11–15, and 20–24 are 10-fold serial dilutions (from 10^{-1} to 10^{-5}) of nucleic acid extract; 7, 16 and 25 are 10^{-1} dilutions of soil nucleic acid extract spiked with 3650, 365 and 230 (plasmid) copies of *cdo*, *nahAc*, and *alkB* genes, respectively; lanes 8, 17, and 26 are the same as 7, 16 and 25 except using 10^{-2} dilutions of soil nucleic acid extract; and lanes 9, 18, and 27 are PCR-positive controls of 3650, 365 and 230 target copies of *cdo*, *nahAc*, and *alkB* genes, respectively.

lations for minimum detection limits at this site before and after active bioventing are reported elsewhere [11].

As described above and illustrated in Figure 3, the PCR detection limit *in the sample* may be significantly higher than that of the positive control reactions (which contain no environmental extract). Figure 4, for example, shows that the extent of PCR inhibition can be template concentration-dependent and highly variable at low template concentrations. In this example, PCR sensitivity was 12.8 fg genomic DNA (*ca* 3 cell equivalents), but reproducible measures of PCR inhibition were not obtained even in the presence of 250 fg added standard template (*ca* 50 cell equivalents). That is, not only was there variability in endpoint detection between replicate dilution series at each concentration of template, but there was also no obvious titration effect of the inhibitor even at the highest template concentration (Figure 4; compare lanes 9 to 8, and 18 to 17). In this case, reproducible measures of PCR inhibition were only obtained with 1.0–2.5 pg standard template (200–1250 cell equivalents) even though the actual PCR detection limit was 2.56–12.8 fg genomic DNA. These results suggest that the effects of molecular sampling error and PCR inhibitors at very low template concentrations also affect the spiked control. Consequently, there is a balance between the effects of a spiked control on T_o and stochastic processes as they occur in the PCR during the amplification of nucleic acids extracted from the environment. In practice, then, the optimal concentration of spiked standard needs to be determined empirically for each primer:template pair under investigation.

Estimating the extent of PCR inhibition

In the replicate-limiting dilution qPCR format presented here, the PCR conditions are always such that the reactions enter the plateau phase of amplification and estimates of target density *in the sample* are based upon an all or none

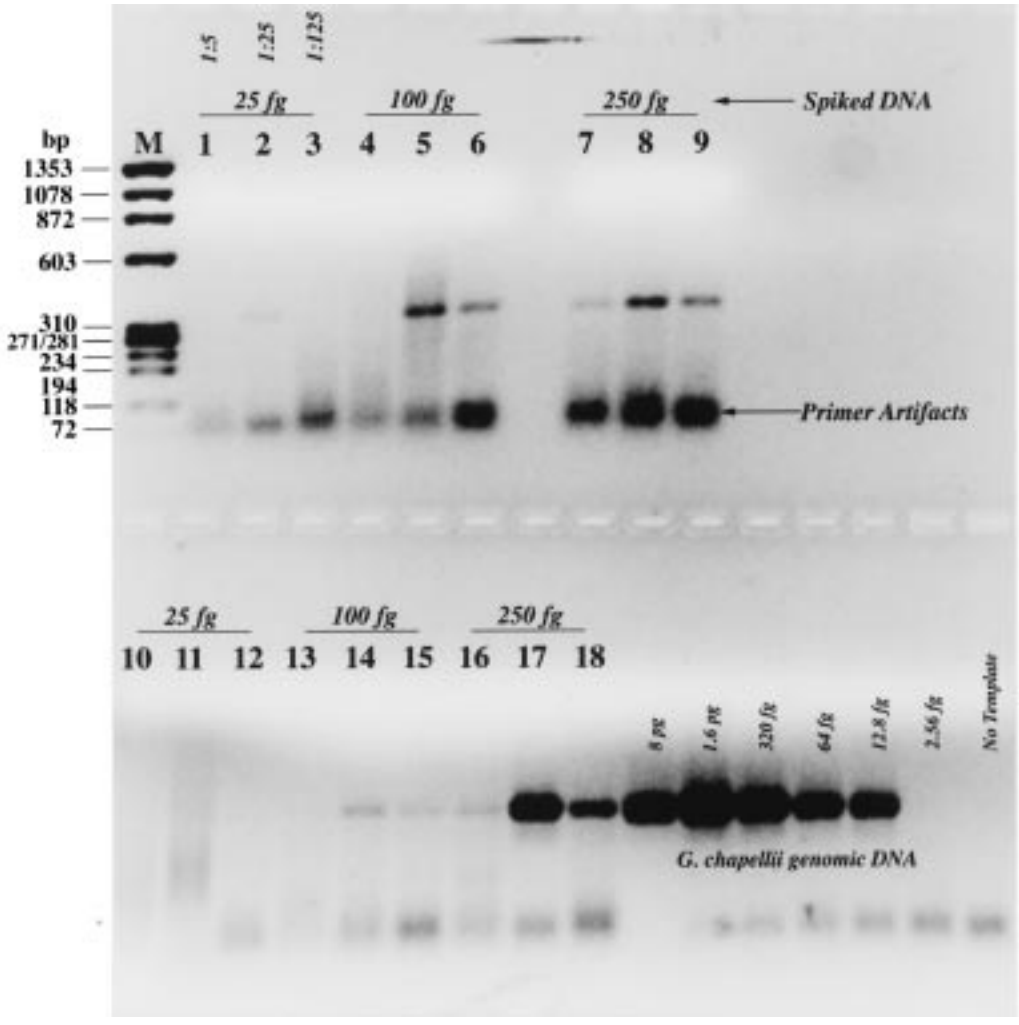

Figure 4 Estimating the extent of PCR inhibition after mock purification of genomic DNA with a biotinylated universal 16S rDNA primer and streptavidin-coated paramagnetic beads according to the manufacturer's protocol (Promega, Madison, WI, USA). Two microliters from each mock hybridization eluant were used to initiate replicate 5-fold dilution series of mock 'template'. *Geobacter chapellii* genomic DNA was used as the spiked control and reference standard at the levels indicated in the figure. *G. chapellii*-specific 16S rDNA primers of sequence 401f: 5′ AASCCTGACGCAGCRACGCC and 683r: 5′ TCTACGGATTTCACTCCTACAC were used to amplify a 280-bp fragment with a hot start cycling regime. Final PCR conditions were 2 μl mock eluant (and dilutions thereof), 10 mM Tris pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 μM each primer, 200 μM each dNTP and 1.25 units LD-*Taq* polymerase (Perkin-Elmer, Foster City, CA, USA) in 25 μl total volume using 0.2-ml thin-walled tubes and a 9600 thermal cycler (Perkin-Elmer). Cycling conditions were for 5 cycles of 94°C for 40s, 60°C for 10 s, 72°C for 75 s followed by 40 cycles of 94°C for 12 s, 65°C for 10 s, 72°C for 80 s with a 2-s extension per cycle, and a final 20-min extension at 72°C. The total contents of each reaction were analyzed on 2% agarose gels in 1 × tris-acetate-EDTA running buffer. Lane assignments are: M = Ø× 174 × *Hae*III, with fragment sizes indicated in base pairs (bp); 1–3 are 5-, 25- and 125-fold dilutions of mock eluant spiked with 25 fg *G. chapellii* genomic DNA; 4–6 are the same as 1–3 except spiked with 100 fg genomic DNA; 7–9 are the same as 1–3 except spiked with 250 fg; lanes 10–18 are the same as 1–9 except using 2 μl of the second (of four) replicate dilution series as 'template'. Amplification products from control PCRs using a 5-fold dilution series of *G. chapellii* standard are shown in the lower panel.

determination. However, the dilution series of reference standard (S_o), which is used to calibrate the relative enumeration, represents the maximum PCR product accumulation at each concentration of input template for each PCR run. In this respect, PCR product accumulation is qualitatively different from measures of PCR product accumulation during competitive PCR analyses, in that cPCR methods (usually) compare product accumulation within the linear portion of the exponential amplification curve. Consequently, the S_o limiting dilution series can be used to estimate: (a) the extent of PCR variability between and within replicate PCR runs; and (b) the extent of PCR inhibition, by comparing the PCR product yields between the S_o dilution series and the spiked controls. In Figure 4, for example, densitometry was performed on individual bands and corrected for background. Comparing pixel densities between the second and third 5-fold dilutions within the 250-fg-spiked controls (lane 8 *vs* 9; lane 17 *vs* 18) indicates that the third dilution in both replicates amplified with only 74–84% efficiency relative to the second dilution in the series, and that the 250-fg-spiked controls in replicate 1 (top of gel) amplified with only 43–49% efficiency relative to the same controls in replicate 2 (bottom of gel; lane 8 *vs* 17 and 9 *vs* 18). Further, the 250-fg-spiked controls in replicate 1 only amplified with 33–44% efficiency relative to the 64-fg standard; the relative efficiencies for replicate 2 ranged from 77–91%. Variations in amplification efficiency within a dilution series and between replicates disappeared

with 1 pg spiked controls (not shown), which suggests that the observed variability with ≤ 250-fg-spiked control resulted from the combined influences of experimental variations during sample preparation prior to the PCR, pipetting errors between replicate dilution series, PCR inhibitors, and molecular sampling error at *ca* 50 cell equivalents of genomic DNA (*ca* 50–500 copies of 16S rRNA assuming 1–10 copies cell^{-1}). This example also stresses the importance of *replicate controls* for obtaining estimates of PCR inhibition or PCR augmentation [14], and how both the PCR enumeration and estimates of PCR inhibition are *relative* to the standard (S_o) controls.

In practice, our spiked controls typically require 500 fg–2.5 pg genomic DNA to avoid the type of uncontrollable variation described above (Figure 4), a level of DNA input representing 100–500 cell equivalents of genomic DNA assuming 5 fg DNA cell^{-1}. From a copy number perspective, this level of spiked genomic DNA could also represent upwards of 5000 targets assuming 10 copies per genome. It is interesting to note that reproducible amplification of our spiked controls requires 10^2–10^3 copies of (genomic DNA) target, even though the PCR detection limit for unspiked genomic DNA is 1–100 copies of DNA. Because the spiked controls and standards are generated from the same template stock solution, it is difficult to rationalize the discrepancy between amplification of a spiked control and standard templates due to template effects alone. Rather, these observations suggest that environmental (and other) PCR inhibitors attenuate PCR amplification (or molecular sampling error) at template concentrations below 10^3–10^4 (genomic) copies, a copy-number effect and hypothesis previously put forth within the context of generating 16S rDNA clone libraries from low biomass (10^4–10^6 cells g^{-1}), deep subsurface sediments [12].

Conclusions

The intent of this review is to identify specific theoretical and practical assumptions that are required for quantitative PCR (and RT-PCR) analyses, and elucidate how the assumptions and conditions of qPCR can be easily violated within an environmental context. In light of the unique aspects of environmental samples and their relationship to qPCR or experimental assumptions, it becomes clear that qPCR enumerations are *relative* to the standard template. Further, the practical PCR detection limits in environmental samples can be orders of magnitude higher than in the positive control standards used to calibrate the enumeration. Relativity does not necessarily imply inaccuracy, however; in high biomass systems, imprecise enumerations can be relatively accurate. Nevertheless, the rigorous fulfillment of the many assumptions required for high analytical precision can give way to more practical qPCR approaches and compromises without invalidating the utility or meaning of qPCR analyses in many environmental and industrial applications. In this respect, the concepts of *sample* replication and sample *representativeness* are (re)emphasized, pointing to potentially significant oversights which are not explicitly addressed in many qPCR studies. Assumptions related to sample processing in an environmental or industrial context, then, are likely to be of more practical importance than the assumptions, practice and limitations of the qPCR techniques themselves. Special consideration was also given to qPCR assays performed on low-biomass and/or low-template samples, situations that predominate nucleic acid analyses of deep subsurface environments, arid ecosystems and many other practical gene-based detection problems.

Given the special considerations of low-biomass, environmental samples and conclusions related to qPCR methods and assumptions, a generalized qPCR method based upon replicative limiting dilution analysis was suggested as a compromise between analytical precision, accuracy and practical utility. The distinguishing features of the replicate limiting dilution qPCR assay are: that all enumerations are estimates which are relative to the standard; a known lower detection limit, but not necessarily single-copy sensitivity; that preference is given to the limiting dilution concept, but without MPN statistics; the dilution series is constructed in a manner consistent with the ecological context and tolerable variations in qPCR estimates of target density; extensive use of spiked controls to evaluate the PCR as it occurs in the sample and the relative extent of PCR inhibition arising from the environmental sample; external standards used to calibrate the enumeration; and, most importantly, replicate nucleic acid extractions from the sample, with replicate serial dilutions prepared from each nucleic acid extract. By developing a simplified qPCR format which relies on relatively few PCR assumptions and takes into account the unique aspects of environmental samples, it is hoped that adaptations of this method will make qPCR analyses more straightforward and practical for environmental and industrial microbiology applications.

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