Evaluating real-time PCR for the quantification of distinct pathogens and indicator organisms in environmental samples

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Abstract We evaluated quantitative real-time PCR (qPCR) and RTqPCR (for RNA species) for their ability to quantify microorganisms and viruses in problematic environmental samples such as cattle manure, digester material, wastewater and soil. Important developments included a standard spiking approach which compensated for methodological bias and allowed sample-to-sample comparison and reliable quantification. Programme CeTe was developed to calculate endogenous concentrations of target organisms (nucleic acid copies) for each sample separately from the generated standard curves. The approach also permitted assessment of the detection limit of the complete method, including extraction. It varied from sample to sample, due to different extraction efficiencies and variable co-extraction of PCR inhibitors. False negative results were thereby avoided. By using this approach we were able to optimise a DNA extraction protocol from the different tested sample types. Protocols for the extraction of RNA species from environmental samples were also optimised. DNA was (almost) not degraded after lethal shock (autoclaving) in the sterile environment. In contrast, the parallel selective cultivation and qPCR results for various microbial parameters from an anaerobic digester chain suggested that DNA from decaying organisms was readily recycled in metabolically active environments. It may, therefore, be used to determine viable organisms in samples exhibiting substantial metabolic turnover. It is proposed that our standard spiking approach, including data evaluation by the program CeTe, should be considered in future standardisation and norms for the quantification of nucleic acid containing organisms in environmental and product samples.

Keywords Environmental samples method detection limit; quantitative real-time PCR; standard spiking

Introduction

Quantitative real-time polymerase chain reaction (qPCR) is a relatively new molecular biology technique (Holland *et al*., 1991; Heid *et al*., 1996). This technology enables quantification of distinct operational phylogenetic or taxonomic units of any nucleic acid (DNA or RNA) containing organisms in a given sample. It combines high specificity with high sensitivity and high sample processing capacity and speed, provided that primers and probes (if used) are adequately designed and extraction and measurement thoroughly optimised (Lebuhn *et al*., 2003). If no pre-extraction enrichment step is included, it avoids cultivation-based bias. Generated results, hence, quantitatively reflect the organic relations in the original sample if all steps in the experimental procedure have been adequately addressed.

Several variants of qPCR have been developed which, for example, rely on unspecific nucleic acid staining (SYBR Green), hybridisation probes, amplifluors, molecular beacons, (PNA based) light-up probes, (duplex) scorpion primers, lux primers, or 5′ nuclease activity-dependent hydrolysis (TaqMan) probes. These use different detection stategies that may be applied alternatively for specific purposes. Several reports suggest (e.g. Jebbink *et al*., 2003) that the hydrolysis probe approach, which we currently use, is among the most specific and sensitive of the qPCR methods. For the quantitative detection

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of RNA species, a reverse transcription (RT) step is inserted upstream of the PCR program (RTqPCR). Numerous reports have shown that the quantification range of (RT)qPCR is linear over at least 6-log units. Although the qPCR approach appears to be highly suited for various applications, e.g. routine monitoring for specific (pathogenic) organisms or organism groups (in particular for those which are difficult or impossible to cultivate, e.g. *Cryptosporidium*, Norwalk virus), several points of criticism have been raised which need to be addressed before qPCR may be accepted by standardisation authorities (e.g. DIN, ISO, CEN) and substituted for conventional cultivation based techniques.

1. A major bottleneck has been that real-time PCR cyclers were expensive – meanwhile, affordable apparatus is available.

2. Specificity of the detection system (primers, probes) was frequently questioned. Suitable design of primers and probes requires skilled know-how, and specificity must be demonstrated *in silico* and *in vitro*. Databank updates must be checked regularly and the systems eventually accommodated. Meanwhile, there is broad acceptance that specificity of properly designed PCR is superior to selective cultivation that frequently requires tedious additional biochemical verification steps.

3. Method detection limits for environmental and tissue samples, particularly in association with possibly co-extracted PCR-inhibitors, are not adequately addressed. This is a valid point, and the criticism also applies for other methods, including quantification by (selective) cultivation. Endogenous or exogenous standards, which may consist of unrelated sequences, are frequently included in the PCR mixture or compared separately for quantification (Chung *et al.*, 1999; Fontaine and Guillot, 2002). However, such approaches do not account for potential losses during extraction, nucleic acid shearing and PCR inhibition by co-extracted inhibitors, particularly at low template concentrations, and may cause substantial underestimation for environmental samples. In addition, unrelated internal standards may cause quantification bias due to preferential amplification in the PCR step.

4. The reliability of molecular biology, and specifically PCR-based methods, to quantify the fraction of active and infectious cells is called into question. Selective cultivation is based on the assumption that a single cell in the sample can be grown and determined specifically. However, it has repeatedly been shown that (even potentially pathogenic) active or viable but not cultivable cells (A/VBNC) may be present, which are only detected by molecular biology tools (Lleò *et al.*, 2001), and can cause underestimation by cultivation. RTqPCR targeting messenger-RNA (mRNA) is among the most powerful approaches to assess viability (Keer and Birch, 2003) but presents potential pitfalls (Freeman *et al.*, 1999). Quantitative extraction of (particularly bacterial) mRNA from environmental samples is currently being optimised, but the question is open, in how far an mRNA pool in a sample (in relation to DNA) can be taken to quantify the active fraction of an investigated organism in an environmental or tissue sample. The generation of suitable standards is a challenging task, because the level of transcription is dependent on various environmental factors.

In the present paper we report on a standard spiking approach by which the method detection limit and the investigated organism in an environmental sample can be reliably assessed. This approach also compensated for methodological bias potentially occurring between extraction and result, thereby invalidating major points of criticism associated with the use of (RT)qPCR for the specific quantification of organisms in environmental samples. For the quantification of the viable or active fraction, we evaluated the use of qPCR (target DNA) for (environmental) samples with substantial physiological activity, assuming high nucleic acid turnover. The rationale was that DNA was more robust and less prone to degradation than mRNA, and that by using DNA as a target, at least no potentially

fatal underestimation would occur. An optimised protocol for the quantitative extraction of DNA from environmental samples has recently been developed (Lebuhn *et al*., 2003). Examples for the application to fresh, autoclaved and UV-treated cattle manure, digester material and wastewater are given and the results discussed in relation to respective data from selective cultivation.

Materials and methods

Environmental samples, strains, selective cultivation and (RT)qPCR systems

Fresh cattle manure and material from full-scale anaerobic digesters (mesophilic–thermophilic–mesophilic) connected in series (Effenberger *et al*., 2002) were obtained from a farm situated near Rosenheim (Germany). The first mesophilic digester (Meso1, 50 m^3) was operated at 35–37°C, the second (tubular, longitudinally mixed) digester at 48–51°C (Thermo, 46 m^3) and the third at 35–37°C (Meso2, 150 m^3). Autoclaving was at 121°C for 15 min and UV treatment at 254 nm for 15 min. Wastewater was from the Garching municipal wastewater treatment plant. Manure and digester samples were homogenised (Ultra-Turrax, Vortex). Wastewater samples were concentrated by centrifugation (for bacteria, cryptosporidia) or ultracentrifugation (for viruses) and resuspension in 0.85% KCl or PBS. Samples were aliquoted and processed immediately or stored at –20°C (only for DNA analyses).

Coliform and faecal coliform bacteria, Enterobacteriaceae and *Escherichia coli*, intestinal enterococci, *Enterococcus faecium* and *E. faecalis* were determined as reported previously (Lebuhn *et al*., 2003). *Bacillus cereus* was determined by plating on PEMBA agar (Oxoid) and a specific qPCR hydrolysis probe system was developed for bacteria belonging to the *B. cereus* group. *Clostridium perfringens* was determined on TSCF agar (Merck) and by using a newly developed specific qPCR system. *Yersinia enterocolitica* and human health-relevant cryptosporidia were determined by newly developed qPCR systems and enteroviruses (ss-RNA) by a newly developed specific RTqPCR system. Development and synthesis of primers and probes was as reported previously (Lebuhn *et al*., 2003).

Standard spiking, nucleic acid extraction and (RT)qPCR

Standards for spiking were quantified by plating serial dilutions and colony counting (bacteria) or microscopically in Abbe-Zeiss counting cell chambers (*Cryptosporidium parvum* strain Iowa). Inactivated human poliovirus 1 (Sabin), quantified by autoradiography with excess labelled probe of known concentration (Dr J.M. Lopez-Pila, Umweltbundesamt Berlin, pers. comm.) was kindly provided by Dr S. Ihle (Umweltbundesamt, Berlin). For standard spiking, washed and diluted (in sterile 0.85% KCl) aliquots of bacteria were stored at –20°C, of virus at –80°C and of *Cryptosporidium* at 4°C. Aliquots of a sample (40–50 µL) were spiked with several low (1–100 cells) and high (10^3–10^6 cells) amounts of the target standards in order to assess the lower method detection limit and the slope of the standard curve individually for each sample. Sterile water was spiked to determine endogenous concentrations of target organisms.

DNA extraction, using our optimised protocol (the Bio101 FastDNA® SPIN Kit for soil, and qPCR, using Qiagen thermoactivated HotStarTaq DNA Polymerase, specifically adjusted stringent combined primer annealing/extension) were performed as described previously (Lebuhn *et al*., 2003). In qPCR we used 6 mM MgCl$_2$, 600 nM primers, 300 nM probe, 1 µM ROX and 200 µM dNTPs found to work optimally for cattle manure DNA extracts (not shown). Different protocols were compared to optimise extraction of viral RNA (vRNA): Bio101 FastRNA™ Kit-RED (BFKR), Bio101 RNaid Plus SPIN Kit (BRPS), Qiagen RNeasy® Plant Mini Kit (QRP), QIAamp Viral RNA Mini Kit (QVR) and QIAamp MinElute Virus Spin Kit using 1 µg and 5.6 µg carrier RNA (QMV_1, QMV_5.6).

Optionally, an upstream bead-beating step using Bio101 Lysing Matrix E tubes (30 s, speed 5.5; optimal for soil) was included. In the two-tube reaction, extracted RNA was reverse transcribed (57.5°C, 30 min) using Invitrogen ThermoScript™ RNase H⁻ Reverse Transcriptase, and cDNA was purifed using either the Qiagen DNeasy® Plant Mini Kit (QDP) or the Promega Wizard® SV Gel and PCR Clean-Up System (PWS), and qPCR was as described above. Alternatively, RNA was transcribed and amplified in a one-tube reaction using the Qiagen QuantiTect Probe RT–PCR Kit (RT: 52.5°C, 30 min; qPCR conditions: see above).

Data evaluation, method detection limit and quantification by program CeTe

In cultivation-based approaches, colony-forming units (CFU) or the most probable number (MPN) per analysed volume were determined. For the quantification of endogenous target organism concentrations in a sample by qPCR, we used the standard curve generated for the differently spiked sample aliquots including the non-spiked aliquot. Measurements usually consisted of two to three replicates. The method detection limit, being particularly dependent on extraction losses and the influence of co-extracted PCR inhibitors, was defined for each sample individually as the lowest quantity of spiked target organism that produced a positive signal to noise ratio in the analysed volume (Figure 1) multiplied by the fraction of extracted per analysed volume. It described the sensitivity of the complete determination system for the target organism in a specific sample and extracted volume. If a non-spiked sample produced signals (positive endogenous contents), the Ct value *vs* spike plot (semi-logarithmic) for the sample was analysed by program CeTe (Lebuhn and Majewski, in preparation) that used an iteration algorithm to calculate the endogenous content and the standard deviation from the standard curve which included the data for the non-spiked aliquot. Results were, therefore, independent of (normalised for) eventual extraction and measurement bias, with sample-to sample comparison being possible. Extraction efficiencies and impacts of methodological variation were calculated by comparison of the corresponding Ct value *vs* spike curves in the linear region at a defined Ct value (e.g. Figure 1). The concentration of *E. coli* DNA (number of target operons) in the genetically engineered DNA-polymerase was determined by qPCR and subtracted as background from the results for *E. coli* and Enterobacteriaceae.

Results and discussion

Method detection limit and influencing factors of qPCR and RTqPCR

For our optimised standard spiking qPCR approach (target DNA) on cattle manure and concentrated (waste)water, typical recovery rates ranged between 66.2% and 13.4%. If all (or 2.5 μL) of the extract (100 μL) was analysed, the method detection limit ranged between 1.5 and 7.5 (or 60.4–298.5) target gene copies per extracted sample volume (typically 40 mg for samples with high contents of phenolic, humic compounds). These were very good values, considering the high contents of these PCR inhibitors in such samples and the short and simple applied extraction procedure. Typically 1–2 DNA copies calculated to be present as a spike in the PCR reaction volume were detected. However, a bottleneck of this miniprep-approach for samples that are difficult to concentrate was the low quantity that could be extracted. For example, for 1g cattle manure or soil, 37.8–186.6 for 100 μL extract (or 1,510.6–7,462.7 for 2.5 μL) target genes should have been present to generate a positive signal if a single extraction was performed. The development of large-scale preparation systems may alleviate this shortcoming. For samples that could be concentrated, e.g. by centrifugation such as (waste)water samples, this problem was of minor importance because large sample volumes could be processed.

Figure 1 shows an example for a poor extraction of DNA from cattle manure (qPCR for

Y. enterocolitica) using a suboptimal protocol (Lebuhn *et al.*, 2003) where humic compounds were co-extracted (yellow-brown colour) and extract purification resulting in DNA losses (Lebuhn *et al.*, 2003) was necessary. The extraction efficiency was consequently low (4.3%) and the method detection limit was high (4.5×10^4 DNA copies for a single qPCR measurement). Co-eluted PCR inhibitors and/or DNA shearing by the bead-beating, which had an effect particularly at low template concentrations (Figure 1), may have contributed. Figure 1 also shows that quantification in an environmental extract from a separately prepared standard curve (e.g. culture suspension) was not reliable. A single spike of calibrator DNA into the PCR reaction was also error-prone and may cause substantial systematic underestimation or even false negative results. This may have been due to (partial) PCR inhibition at low template concentrations and potential nucleic acid losses during extraction (Figure 1).

We are currently evaluating and optimising extraction methods for viral RNA for the above-mentioned problematic environmental samples and subsequent RTqPCR analysis. The two-tube RTqPCR approach was more sensitive by a factor of 5.1 than the one-tube RTqPCR for a purified enterovirus suspension. For a spiked and extracted (QVR) wastewater sample, the two-tube RTqPCR was 4,052-fold more sensitive, probably mainly due to the dilution of PCR inhibitors in the two-tube system. Further developments will focus on the two-tube systems, although these were slightly more labour intensive.

When the different tested RNA extraction systems were compared for enterovirus-spiked wastewater samples (two-tube RTqPCR, calculated 230 genomes in qPCR reaction), signals were only obtained for the extracts from BRPS and QRP systems, which will be further optimised. RNA was apparently sheared by introducing the bead-beating (optimised for bacteria in soil, cattle manure) as an initial step for pure enterovirus suspensions; no or only minor detrimental influences were obtained for RNA from spiked concentrated wastewater and spiked material from the thermophilic digester (not shown). How far reduced bead-beating intensity may be helpful needs to be tested.

Purification of cDNA before qPCR was found to be helpful in eliminating PCR inhibitors, with the QDP system being 3.6-fold more effective than the PWS system. We optimised the combinations of BRPS/QDP and QRP/QDP in two-tube systems and achieved recovery rates between 1.0 and 16.2%, with method detection limits of about 1.6×10^3 genomes/50 μL cattle manure, digester material or concentrated wastewater for a
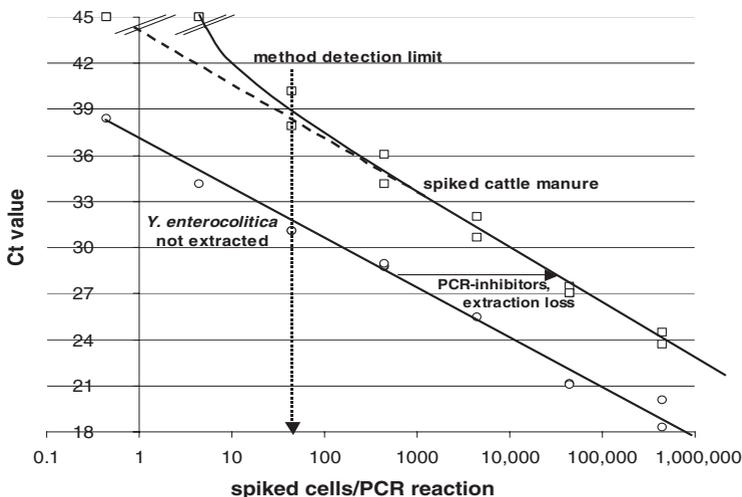

Figure 1 Suboptimal DNA extraction; method detection limit and influencing factors

single 2.5 µL analysis. Since these values were still relatively low, we are comparing the combined systems to a magnetic-beads-based extraction approach.

Bacteria in cattle manure, digester samples and sterilised samples – comparison of quantification by cultivation and qPCR

In Table 1, data from quantification of Enterobacteriaceae by qPCR and coliforms using Fluorocult in fresh and differently sterilised cattle manure are presented.

Table 1 shows that, as expected, no regrowth occurred after sterilisation during the storage time. DNA was only slightly reduced by the lethal shock autoclaving but substantially after 2× autoclaving with UV treatment. Widmer *et al.* (1999) similarly found almost no decrease in the level of various nucleic acids in *C. parvum* oocyst samples at conditions allowing (almost) no biological turnover activity, whereas degradation (also of DNA and rRNA, but particularly of mRNA) occurred at conditions allowing metabolic activity in the axenic samples. Figure 2 shows results for *E. coli* and faecal coliforms from the anaerobic digester chain.

Concomitantly with the decrease in viable faecal coliform numbers, we obtained a clear reduction in the concentration of *E. coli* DNA. The slight final increase indicated that regrowth had occurred in the digest storage tank (Figure 2). Data for Enterobacteriaceae, coliforms, (intestinal) enterococci, *Enterococcus faecium* and *E. faecalis* showed the same behaviour (not shown). With prolonged incubation in/on the cultivation media, typically higher values were obtained for (faecal) coliforms and enterococci, particularly in the thermophilic reactor, as well as in soil samples (not shown), indicating transition of bacteria to

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Table 1 Enterobacteriaceae and coliforms in fresh and differently sterilised cattle manure

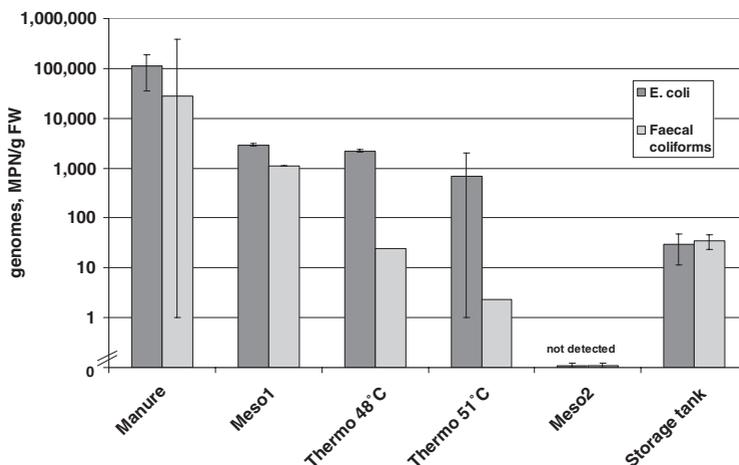|	Fresh	Cattle manure		
		Autoclaved	Autoclaved, 15min UV	Autoclaved x2 15min UV
Time between treatment and extraction (d)	1–7	13	3	2
Enterobacteriaceae (Mean ± SD genomes/mL)	$2.34 \pm 2.24 \times 10^5$	$3.79 \pm 0.17 \times 10^4$	$6.22 \pm 4.45 \times 10^4$	44.6 ± 26.9
Coliforms (Mean ± SD MPN/mL)	$0.28 \pm 3.53 \times 10^5$	<1	<1	<1

Figure 2 Quantification of *Escherichia coli* and faecal coliforms in compartments of the anaerobic digestion chain

the ABNC state. The presence of ABNC cells could also explain the systematic differences observed for viable bacteria and corresponding DNA (typically higher qPCR values), as exemplified by faecal coliforms and *E. coli* DNA in Figure 2. No reduction was obtained for *C. perfringens* using both selective cultivation and qPCR assays with almost no reduction of *B. cereus* (selective cultivation) and *B. cereus* group bacilli (qPCR). A preliminary experiment indicated substantial reduction of *B. cereus* group bacteria at 55°C – the envisaged optimum temperature of the thermophilic digester. *Y. enterocolitica* and *C. parvum* were below the method detection limit.

We concluded from the results that DNA released from decayed bacteria was degraded in the functioning digester ecosystems. DNA may thus be used as a valid parameter to quantify viable organisms in environments with substantial metabolic turnover activity. Subsequent experiments will address turnover rates and the usefulness of DNA for environments with lower metabolic activity, such as freshwater and bulk soil. Our results suggested that (RT)qPCR could be substituted for corresponding selective cultivation approaches, e.g. to determine the hygienic safety of environments and products, if the standard spiking approach and data evaluation using our program CeTe was included, in order to avoid potential biases. We propose that this approach should be considered in future standardisation and norms for the quantification of nucleic-acid-containing organisms in environmental and product samples.

Conclusions

1. If properly designed, quantitative (reverse transcription) Real-Time PCR ((RT)qPCR) was a highly suitable, convenient and versatile tool to quantify nucleic acid-containing organisms specifically in diverse (environmental) samples. It provided many striking advantages over traditional (selective) cultivation (Lebuhn *et al*., 2003).

2. If the highest possible sensitivity was required, e.g. for the quantification of pathogens in environmental or food samples, optimised extraction procedures specific for the type of nucleic acid investigated, such as those that we have developed and proposed, need to be applied in order to guarantee maximum extraction yield and purity.

3. Reliable quantification was achieved by our standard spiking procedure, including data evaluation by the program that we developed: CeTe. This approach compensated for potential methodological bias and allowed sample-to-sample comparison, provided the opportunity to assess the methodological detection limit, and avoided potential false negative results.

4. If our standard spiking approach was applied, major points of criticism towards the application of the (RT)qPCR methodology, e.g. for hygiene monitoring of environmental samples, could be rejected. We propose, therefore, that this approach should be considered in future standardisation and norms for the quantification of nucleic-acid-containing organisms in environmental and product samples.

5. We focused on DNA as the predominant parameter for routine analyses. The rationale was that DNA was more robust and less prone to degradation than mRNA, and that by using DNA as a target, underestimation could be avoided.

6. A major question to be addressed by future research is: how DNA may be used to quantify explicitly viable and potentially infectious agents. Our results indicated that DNA could be used for samples with high intrinsic metabolic turnover. Subsequent experiments will show whether DNA may also be used for environments with low turnover rates, or whether the responsiveness of more labile mRNA may then be a more suitable parameter.

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