Using quantitative real-time PCR to determine the hygienic status of cattle manure

M. Lebuhn*, M. Effenberger**, A. Gronauer**, P.A. Wilderer* and S. Wuertz¹

* Lehrstuhl und Versuchsanstalt für Wassergüte- und Abfallwirtschaft, Am Coulombwall, 85748 Garching, Germany (E-mail: m.lebuhn@bv.tum.de)
** Bayerische Landesanstalt für Landtechnik, Am Staudengarten 3, 85354 Freising, Germany
¹ Current affiliation: Department of Civil and Environmental Engineering, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA

Abstract We developed a suitable system of DNA extraction and real-time quantitative polymerase chain reaction (qPCR) for the specific and sensitive quantification of pathogens and other relevant (indicator) organisms in recalcitrant material such as cattle manure. PCR inhibition by coextraction of humic compounds was minimized in this system, resulting in detection sensitivity of one target DNA copy per reaction well. Data from qPCR analysis for Escherichia coli agreed with cultivation based results, but orders of magnitude more fecal enterococci, Enterobacteriaceae and Campylobacter jejuni, were determined by qPCR than by cultivation. These bacteria may have been in a potentially hazardous active but non-cultivable state. The qPCR system is much less time consuming than conventional cultivation, highly specific, can detect non-cultivable organisms, provides high measurement throughput, and is cost attractive. It should be considered as an alternative in various application areas for (prescribed routine) cultivation based assays, e.g. for biosafety and hygiene monitoring.

Keywords Anaerobic digestion; DNA extraction; hygienization; pathogens; PCR inhibitors; quantitative real-time PCR

Introduction

Anaerobic digestion (AD) of organic wastes is being increasingly used in agricultural and waste(water) treatment practices to produce biogas fuel and organic fertilizer. It is aimed at recycling nutrients and turns out to be a profitable alternative to conventional combustion or aerobic treatment. Moreover, AD can contribute to minimize dissipation of fossil energy resources and greenhouse gas emissions.

Various AD techniques have been developed to optimize energy yield, minimize digest volume, and avoid sanitary risks if the digest is to be reused e.g. in agriculture as fertilizer (van Lier et al., 2001). Several studies (e.g. Oropeza et al., 2001) have indicated that efficient hygienization during AD may only be achieved if a thermophilic (50–60°C) step with guaranteed retention time at this temperature is included in the process.

The aspect of hygienization efficiency of AD has gained major interest in Germany. Water protection areas have been enlarged in the recent past, and in certain areas farmers with animal husbandry are confronted with the problem of increasing animal waste production but at the same time diminishing land area to apply the manure. This is particularly relevant for former water protection zone III areas that were, or will be, given the status water protection zone II, in which storage and application of manure is generally prohibited (BStMLU, 1994; DVGW, 1995; Anon., 1996). However, exceptions from these restrictions may be made if the material to be applied has undergone a treatment that guarantees sufficient destruction or inactivation of relevant pathogens.

According to German ordinances on drinking water, recreational (bathing) water and biological wastes, quantification of pathogens relies on cultivation techniques. Indicator
organisms such as (fecal) coliforms and fecal enterococci are typically examined to prove fecal contamination and conclusions about health risks. Actually, local authorities may occasionally prescribe more specific additional examination of (drinking) water for *Salmonella* sp., *Pseudomonas aeruginosa*, *Legionella* sp., *Campylobacter* sp., EHEC, *Cryptosporidium parvum*, *Giardia intestinalis* (*G. lamblia*), coliphages or enteropathogenic viruses, in addition to *Clostridium perfringens*.

However, several major drawbacks are connected with these culture-based techniques. Most importantly, the determinations require one to several days to obtain results. This may be critical if decisions on eventual measures must be made rapidly. In addition, cultivation techniques frequently lack sufficient specificity. Active/viable but not cultivable (A/VBNC) and eventually pathogenic cells may not grow on the (artificial) media but may become virulent in their natural host (McDougalld *et al.*, 1998; Lleò *et al.*, 2001; Thomas *et al.*, 2002). The above mentioned protozoan parasites and the Norwalk virus cannot be cultivated, and thermophilic campylobacters are difficult to cultivate under laboratory conditions. These organisms, however, are among the leading causes of human enteric diseases worldwide. Just like spore-forming bacteria they escape the indicator-based surveillance concept due to their resistance against most practised sanitizing measures (e.g. Fayer *et al.*, 2000). Forthcoming EU directives will increasingly consider distinct pathogens to be determined by state-of-the-art methods.

One of the most promising molecular biology tools to quantify organisms is the real-time quantitative polymerase chain reaction (qPCR) (Holland *et al.*, 1991; Heid *et al.*, 1996). This technique offers excellent specificity, high sample throughput and very short analysis time, and is able to quantify even non-cultivable organisms in a sample. A major bottleneck, however, is the lack of a reliable and convenient nucleic acid (DNA, RNA) extraction protocol from environmental samples that contain PCR-inhibitors such as humic acids. If sufficiently present in the extract, they can generate false negative results (Tebbe and Vahjen, 1994).

The scope of a joint project of Bavarian research institutions and local water suppliers is to evaluate the potential of a three-stage AD process (Effenberger *et al.*, 2002) for the environmentally sustainable and economical control of pathogens. In the frame of this project, we are developing and applying the qPCR technique in parallel with conventional cultivation to quantify indicator organisms and distinct pathogens of public concern in cattle manure before, during and after digestion. In this paper, we report on recent progress in the extraction of DNA from cattle manure which is extremely rich in humic acids and therefore problematic for the PCR process. Preliminary qPCR data for *Escherichia coli*, *Enterococcus faecalis*, *E. faecium*, and *Campylobacter jejuni* are presented and discussed along with results from cultivation assays.

**Methods**

**Cattle manure**

Semi-liquid cattle manure was obtained from local farmers and processed immediately. Experiments were also performed with stored (4°C), autoclaved (15 min, 121°C) and UV (254 nm) irradiated samples.

**Cultivation based quantification of bacterial groups**

For quantification of bacteria by classical cultivation, 1 mL cattle manure aliquots were serially diluted in phosphate buffered saline (PBS). Most probable numbers were obtained for coliform and fecal coliform bacteria using the Fluorocult® laurylsulfate system (Schindler, 1991). Fecal enterococci were determined according to ISO/DIS 7899-2 using membrane filtration (0.45 µm cellulosenitrate filters, equipment from Sartorius), and
cultivation on Slanetz–Bartley and Esulin bile agars (Oxoid). Data for thermophilic campylobacters were obtained by plating serial dilutions on modified Karmali selective agar (Oxoid) and incubating microaerobically at 37°C for 48 h in anaerobic jars (Merck).

**DNA extraction, primers and probes**

Prior to DNA extraction, 40 µL (standard spiked, see below) cattle manure aliquots were diluted, homogenized, and washed serially in PBS and 0.85% KCl, or were not washed. We tested the influence of polyvinylpyrrolidone (PVP, 0.85%, 1.7%, 3.4%) and cetyltrimethylammonium bromide (CTAB, 5%, 10%, 20%, in 1 M NaCl) for their ability to remove PCR inhibitors and support polymerase activity.

Different extraction kits (Qiagen QIAamp® Stool Mini Kit, QIA; MoBio Ultra Clean™ Soil DNA Isolation Kit, MoBio; Bio 101 FastDNA® SPIN Kit for Soil, Bio101) were tested (Huber, 2002) along with conventional phenol-chloroform-isoamylalcohol-extraction using our primer/probe set developed for *E. coli* (see below). If amplification was not satisfactory, DNA extracts were further purified using Promega Wizard® PCR Prep or Qiagen DNEnasy DNA purification systems. DNA extracts were analyzed immediately or after short-term storage at 4°C. Long-term storage was at –20°C.

Primers and fluorogenic hydrolysis probes for qPCR were designed for *Enterobacteriaceae*, *E. coli*, *E. faecium*, *E. faecalis* and *C. jejuni* (patents pending), and tested *in silico* using biocomputing tools provided by HUSAR ([http://genome.dkfz-heidelberg.de/](http://genome.dkfz-heidelberg.de/)) and Genomatix ([http://www.genomatix.de/](http://www.genomatix.de/)). Primers and probes were synthesized by Thermo Hybaid. Specificity was confirmed *in vitro* using whole cell qPCR analysis of lysates of our strain collection comprising ca. 250 different bacterial species and different strains per species (not shown).

**Quantitative real-time PCR (qPCR)**

For qPCR of *C. jejuni*, a single copy gene fragment was cloned in *E. coli* using the Invitrogen TOPO TA Cloning® kit for sequencing. The insert copy number in *E. coli* was determined by counting clone colony numbers on Luria Bertani agar and calculating the relation between qPCR signals obtained for the cloned insert and a single copy housekeeping gene of *E. coli*.

For qPCR, reagents and material from Applied Biosystems or Qiagen, TIB MolBiol and Eppendorf were used. Fluorescence signals were monitored simultaneously in a 96 well format using the Applied Biosystems GeneAmp® 5700 sequence detection system. In this system, fluorescence is measured online and normalized for experimental variation monitored by the intrinsic passive reference dye ROX. Data for exponential specific amplification are expressed as ΔRn values. The cycles where amplification exceeds the threshold ΔRn values are defined as the Ct (threshold cycle) values. The Ct values are a function of the target DNA template concentration, and are usually used to calculate the target starting copy number by interpolation of data from a standard curve (Lie and Petropoulos, 1998).

Quantification of bacteria (signature DNA sequences) in the samples (“endogenous contents”) was done using the programme CeTe (patent pending) and calibration curves from prequantified internal positive controls that were spiked into sample aliquots, in order to normalize for extraction losses and eventually reduced amplification by coextracted PCR inhibitors. Since extraction efficiencies can be variable, we followed this strategy in order to achieve reliable quantification. This also allowed method detection limits to be determined for given sample extracts.
Results and discussion

DNA extraction

PCR amplifiable DNA was obtained consistently only when not more than ca. 40 µL of cattle manure was applied to the utilized MiniPrep extraction kits. The theoretical method detection limit using these kits was 250 organisms per 1 mL of cattle manure when only 1 sample of 40 µL was extracted, and 10 µL of 100 µL extract were applied in one single qPCR analysis. This statistical threshold is reduced to ca. 42 organisms per mL cattle manure when 2 extractions and 3 measurement repetitions are performed, as we usually did. This limitation of the MiniPrep extraction systems may be overcome by using more expensive MaxiPrep extraction kits and/or increasing extraction and measurement repetitions. Less problematic in this respect are water samples since they can be concentrated and typically do not contain similarly high concentrations of PCR inhibitors. The method detection limit is hence much lower. We were able to detect the presence of 1 salmonella in 10 mL of collective basin or process water of composting plants using our qPCR technique (Burtscher and Lebuhn, 2002).

Phenol-chloroform-isoamylalcohol-extraction of cattle manure did not yield PCR amplifiable DNA (data not shown). The best results (lowest Ct values) were obtained for the Bio101 and MoBio extraction kits (Huber, 2002), with the Bio101 system being more efficient for suboptimally washed samples (Figure 1). This was probably due to a better removal of PCR inhibitory compounds, as evidenced by the presence of yellow–brown colour in some (non-amplifiable) extracts.

With increasing addition of PVP to cattle manure, Ct values became higher (not shown), indicating that the extraction became less efficient, probably because PVP precipitated or occluded the DNA. There was no significant effect of CTAB addition to cattle manure on Ct values (not shown). Neither addition of PVP nor of CTAB was helpful in improving DNA extraction from cattle manure.

In contrast, repeated washing of cattle manure in PBS/KCl prior to extraction greatly improved the quality of the DNA extract (Figure 1), probably by removing humic compounds which are inhibitory to the PCR process.

Post-extraction DNA purification using Promega Wizard® or, more effectively, Qiagen DNeasy systems rendered some non-amplifiable extracts amplifiable (not shown). However, since data indicated that a portion of the extracted DNA was lost during the

![Figure 1](https://iwaponline.com/wst/article-pdf/48/4/97/423441/97.pdf)

**Figure 1** Effect of washing on the efficiency of DNA extraction from cattle manure using two different extraction kits.
puriﬁcation procedures (not shown), we applied these steps only if absolutely required, and then for all extracts of a coherent determination.

Particularly for the quantiﬁcation of pathogens in environmental samples, it is highly desirable to obtain the lowest possible detection limit in order to detect low numbers of the target organisms and avoid false negative results. Washing of the sample should hence be generally applied or considered as a means if no ampliﬁcation is achieved. Additional post-extraction puriﬁcation steps may be helpful only if signiﬁcant amounts of inhibitors were coextracted.

Quantiﬁcation of bacteria in cattle manure

The time necessary to perform the complete analyses was 6–8 h for the qPCR approaches (including DNA extraction) and 24–72 h for the cultivation-based systems. The essentially lower analysis time provided by qPCR can accelerate urgent decision times, e.g. if contamination of drinking water is suspected. High sample throughput (96 measurements simultaneously) and relatively low analysis costs (Huber, 2002) qualify qPCR as a potentially ideal routine screening system.

Quantitative data for bacteria and bacterial groups in fresh cattle manure obtained by culture based systems and qPCR technique are compiled in Table 1.

According to the compilation in Table 1, results for fecal coliform bacteria and Escherichia coli were equivalent, suggesting that the qPCR system may substitute the cultivation-based analysis. However, for the other bacteria/bacterial groups, 10–1,000 fold lower values were obtained by the applied cultivation approaches than by the corresponding qPCR analyses (Table 1). Probable explanations are differences in speciﬁcity between the compared systems. In addition, many target bacteria in the cattle manure may have been in a dormant, non-cultivable state. This is typical e.g. for enterococci and campylobacters at suboptimal conditions (McDougald et al., 1998; Lleò et al., 2001; Thomas et al., 2002).

Experiments using reverse transcription of messenger RNA (mRNA) before qPCR (RTqPCR) may prove the presence of and quantify such ABNC cells.

When samples were stored at 4°C for 3 weeks, values for coliform bacteria as well as for fecal coliform bacteria dropped to 9.3 × 10^4 – 1.1 × 10^5 cfu per 10 mL cattle manure. Approximately 3% of these bacteria apparently became non-cultivable or died during the storage period. In contrast, qPCR values for E. coli remained unchanged after storage of 2 weeks at 4°C, suggesting that DNA of the non-viable or dead cells was not signiﬁcantly degraded. No cfu was detected after autoclaving, whereas qPCR values for E. coli and

<table>
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<th>Table 1</th>
<th>Cultivation-based and qPCR analyses of bacteria and bacterial groups in fresh cattle manure</th>
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<tr>
<td>Bacterium/bacterial group</td>
<td>Culture-based technique (cfu per 10 mL cattle manure)</td>
</tr>
<tr>
<td>Coliform bacteria</td>
<td>4.6 × 10^6</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>7.0 × 10^6 – 1.8 × 10^8</td>
</tr>
<tr>
<td>Fecal coliform bacteria</td>
<td>3.0 × 10^6 – 4.6 × 10^6</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>1.3 × 10^6 – 4.0 × 10^6</td>
</tr>
<tr>
<td>Fecal enterococci</td>
<td>2.0 × 10^5 – 2.7 × 10^5</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>1.1 × 10^7</td>
</tr>
<tr>
<td>Enterococcus faecium</td>
<td>3.5 × 10^7</td>
</tr>
<tr>
<td>Thermophilic campylobacters</td>
<td>n.d.                                           ¶</td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td>2.6 × 10^5 §</td>
</tr>
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If several experiments were performed, the range between minimum and maximum values are shown

Cfu, colony forming units. n.d., not detected

| E. coli DNA traces present in the Taq-polymerase were subtracted as background
| Unspeciﬁc microﬂora background present
| At the detection limit, extrapolated value
Enterobacteriaceae dropped only by ca. 3% (not shown). After repeated autoclaving and UV illumination (254 nm, 15 min) no E. coli and Enterobacteriaceae DNA was detected. Although this points to persistence of DNA after lethal stress, released DNA may be readily degraded in functioning ecosystems. Experiments on stability of DNA in natural environments under typical conditions are required to indicate in which cases DNA data may be used to predict (potentially) active organisms. For this purpose, the potentially powerful RTqPCR approach using mRNA as an indicator of viability still presents pitfalls (Freeman et al., 1999), particularly with respect to the instability of bacterial mRNA in environmental samples. The use of rRNA, the typical target of fluorescent in-situ hybridization (FISH) is no alternative to the (RT)qPCR approach, because rRNA is as stable and resistant to degradation as DNA (Widmer et al., 1999).

Conclusions
Real-time quantitative PCR (qPCR) is a highly valuable tool to quantify specific organisms in environmental samples. The major bottlenecks in this approach are the presence of PCR inhibitors such as humic acids and the relatively low nucleic acid quantity that can be extracted using miniprep systems from problematic matrices such as cattle manure. However, using our optimized DNA extraction protocol for this material, we were able to amplify even one target DNA molecule in the qPCR analysis well. The qPCR approach is an attractive alternative to the culture-based systems for quantification of (indicator) organisms, since results are generated much faster, distinct organisms (e.g. pathogens) can be determined specifically, non-cultivable but active and potentially infectious agents can be quantified, and the system provides a high throughput and is cost effective. The qPCR system appears to be highly suited to determine the fate of specific organisms or pathogens, e.g. during anaerobic digestion. It should also be considered as a convenient means to monitor the hygienic status of other (environmental) samples of public concern, e.g. food, drinking or recreational water.

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