Hygienization by anaerobic digestion: comparison between evaluation by cultivation and quantitative real-time PCR

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Abstract In order to assess hygienization by anaerobic digestion, a comparison between evaluation by cultivation and quantitative real-time PCR (qPCR) including optimized DNA extraction and quantification was carried out for samples from a full-scale fermenter cascade (F1, mesophilic; F2, thermophilic; F3, mesophilic). The system was highly effective in inactivating (pathogenic) viable microorganisms, except for spore-formers. Conventionally performed cultivation underestimated viable organisms particularly in F2 and F3 by a factor of at least 10 as shown by data from extended incubation times, probably due to the rise of sublethally injured (active but not cultivable) cells. Incubation should hence be extended adequately in incubation-based hygiene monitoring of stressed samples, in order to minimize contamination risks. Although results from qPCR and cultivation agreed for the equilibrated compartments, considerably higher qPCR values were obtained for the fermenters. The difference probably corresponded to DNA copies from decayed cells that had not yet been degraded by the residual microbial activity. An extrapolation from qPCR determination to the quantity of viable organisms is hence not justified for samples that had been exposed to lethal stress.

Keywords Active but not cultivable cells; anaerobic digestion; hygienization; overestimation by qPCR; qPCR; underestimation by cultivation

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Introduction

There is increasing concern about livestock farms being potential sources of water- and foodborne illness (Bicudo *et al.*, 2000). Livestock breeding and animal agriculture are considered responsible for the transmission of pathogens and undesired compounds in the environment. Accordingly, there is discussion (not only) in the European Union as to whether or not future legislation should demand hygienization of animal waste before application to land.

Besides generation of a versatile renewable energy source (biogas), anaerobic digestion (AD) offers other benefits such as recycling of nutrients, reduction of odor, improvement of fertilizing effects and inactivation of various pathogens present in animal manure. At mesophilic temperatures, however, AD may not provide sufficient decay of pathogenic organisms within practicable treatment times (24 h or less), whereas thermophilic conditions (about 55 °C) can reduce the numbers of several pathogenic bacteria, viruses and parasites in liquid animal wastes by several orders of magnitude within hours, with temperature being the dominant inactivating factor (e.g. Oechsner and Doll, 2000; Oropeza *et al.*, 2001).

Conventionally, hygienization is evaluated by cultivation techniques using indicator bacteria such as (fecal) coliforms and intestinal enterococci (fecal streptococci). However, it has repeatedly been shown that various bacteria can enter an ABNC (active but not

cultivable) state particularly under unfavourable conditions (Lleò *et al.*, 2001; Thomas *et al.*, 2002). This can result in an underestimation of potential pathogens and give rise to health risks if people or animals come into contact with the contaminated charge. Additionally several pathogens such as *Cryptosporidium parvum* or Norwalk (like) viruses are difficult to cultivate or cannot be cultivated, and must be determined by molecular biology approaches such as PCR.

Quantitative real-time PCR (qPCR) is a powerful new molecular biology technique to quantify distinct organisms (i.e. specific gene copy numbers) in a sample (Holland *et al.*, 1991; Heid *et al.*, 1996). We have recently developed an optimized protocol for the extraction of PCR-amplifiable DNA from problematic substrates such as cattle manure and digester contents, and proven that (pathogenic) organisms in such samples can be quantified specifically and sensitively by qPCR (Lebuhn *et al.*, 2003). However, PCR based approaches rely on the presence of DNA, and DNA can be present in samples after a lethal shock although cultivation results indicate that the target organisms are not viable anymore (Lebuhn *et al.*, 2004). This may result in overestimation of (pathogenic) organisms particularly in samples that have undergone a germ-reducing treatment.

Objectives of this study were to: (i) evaluate the potential of a full-scale anaerobic digester chain to sanitize cattle manure, and (ii) to assess the significance of results from hygiene monitoring by parallel cultivation and DNA based qPCR approaches.

Methods

Biogas plant, fermenter and cattle manure samples

The biogas plant, situated on a farm near Rosenheim (Germany), consisted of three anaerobic digesters (F1: mesophilic; F2: thermophilic; F3: mesophilic) connected in series. The first mesophilic digester (F1, 50 m^3) was operated at 35–38 °C, the second (tubular, mixed with baffles) digester at 48–51 °C or at 55°C (F2, 46 m^3), and the third at 35–38 °C (F3, 150 m^3). Technical details of the reactor chain have been presented by Effenberger *et al.*, 2003. Preliminary results on various parameters concerning the performance of the single reactors and the reactor chain, e.g. biogas and methane production, process stability and efficiency, as well as substrate degradation were presented by Effenberger *et al.* (2004). Various microbial parameters were monitored in parallel using selective cultivation and qPCR for the 5 compartments: manure input from healthy cattle, the 3 digesters, and the terminal storage tank. Two different strategies for the evaluation of germ and specific genome reduction were applied: (i) random sampling and (ii) charge tracing (follow-up of a specific charge) by probing the compartments after the respective calculated hydraulic retention times.

Samples (ca. 100 mL) were taken from the compartments and immediately processed in the laboratory. After homogenization with an UltraTurrax for 1 min at medium speed, serial dilutions were made in phosphate buffered saline (PBS) for the cultivation approaches. For qPCR, samples were washed 3 times (14,000 g for 3 min) for PCR inhibitor removal, subsequently twice in PBS and then once in 0.85% KCl, as specified recently (Lebuhn *et al.*, 2003).

Quantification by cultivation

Coliforms and fecal coliforms, *Enterobacteriaceae* and *Escherichia coli*, total and intestinal enterococci, *Enterococcus faecium* and *E. faecalis* were determined by cultivation and specific qPCR as reported previously (Lebuhn *et al.*, 2003). Incubation of coliforms and fecal coliforms in Fluorocult medium was for 24 h, 48 h and for 84 h in order to detect ABNC or sublethally injured cells.

Bacillus cereus was determined by plating on PEMBA as specified by the manufacturer (Oxoid), and by qPCR targeting the *B. cereus* group. *Clostridium perfringens* was determined in TSCF agar as specified by the manufacturer (Merck) and by specific qPCR as described previously (Lebuhn *et al.*, 2004).

Thermophilic campylobacters and *Yersinia enterocolitica* were determined after pre-enrichment on mCCDA and CIN-agar, respectively, as specified by the manufacturer (Oxoid), by most probable number (MPN) and specific MPN-qPCR analysis. Thermophilic campylobacters were pre-enriched in Preston bouillon supplemented with CCDA and slaked horse blood (all products from Oxoid) at 44 °C for 2 d at microaerobic conditions, and *Y. enterocolitica* was pre-enriched in Ossmer bouillon (Merck) at 30 °C for 1 d. Developed colonies were tested by specific qPCR (3 repetitions for each dilution), and the results were evaluated by MPN statistics.

Quantification by real-time PCR (qPCR)

Specific primers and probes for 5′-nuclease assays were designed using the programme 'Signature' (Lebuhn and Majewski, in preparation) running on CLUSTAL alignments of sequences showing significant similarity to the target DNA and suitable outgroups. The chosen primer/probe combinations were tested positively for specificity *in silico* using programmes MatInspector, BLASTN and FASTA and *in vitro* against our strain collection (ca. 500 different strains) comprising phylogenetically close relatives of the respective target species.

After standard-spiking of the washed samples (Lebuhn *et al.*, 2003; Lebuhn *et al.*, 2004), DNA was extracted using the Bio 101 FastDNA® SPIN Kit for Soil following the manufacturer's suggestions. Optimized qPCR was performed as described recently (Lebuhn *et al.*, 2003; Lebuhn *et al.*, 2004) using either thermoactivated HotStarTaq DNA Polymerase (Qiagen) or Platinum Taq DNA Polymerase (Invitrogen). For data evaluation and quantification of endogenous contents in the environmental samples, programme CeTe was used as reported previously (Lebuhn *et al.*, 2003).

Results and discussion

Random sampling

For the random sampling approach, selective cultivation and specific qPCR analyses both showed a strong reduction of fecal coliforms (4.5–5.5 log units) and *E. coli* genomes (2.5 log units) in the digester cascade, although the thermophilic reactor was run only at suboptimal temperatures (48–51 °C) (Figure 1). Re-germination was observed in the terminal storage tank, resulting in a reduction of 3.0–3.5 log units over the total sampling chain for fecal coliforms and of 2.5 log units for *E. coli* genomes (Figure 1).

The parallel results from selective cultivation and qPCR agreed quantitatively only for the compartments with equilibrated conditions, in particular for fresh manure and (less so) for the terminal storage tank, whereas consistently higher qPCR values were obtained for the digesters (Figures 1 and 2). A very good agreement between the qPCR and the cultivation-based results was also obtained for the other monitored parameters. However, for *Enterobacteriaceae* vs. coliforms and *Enterococcus faecium* and *E. faecalis* vs. total and intestinal enterococci, this was only true for the compartments with equilibrated conditions and high microbial (degradation) activity (not shown). For these parameters, less reduction than for fecal coliforms/*E. coli* was obtained in the three fermenters. Almost no reduction was obtained for the spore-forming *Bacillus cereus* group bacteria, and no reduction for the highly heat-resistant *Clostridium perfringens* spore-formers. Thermophilic campylobacters were detected at very low concentrations only when the enrichment step was introduced in fresh manure (FM), in F1 and in the terminal storage tank (TST),

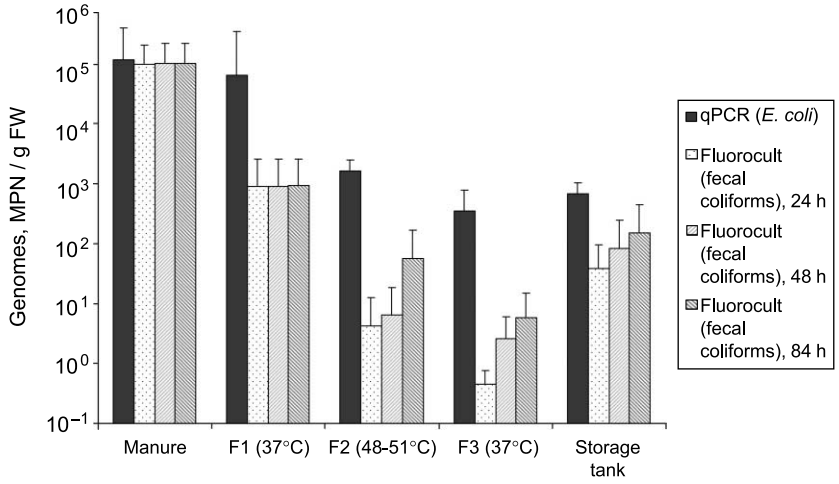

Figure 1 Reduction of *E. coli* genomes and of fecal coliforms at different incubation times in Fluorocult medium in the anaerobic fermenter cascade, random sampling

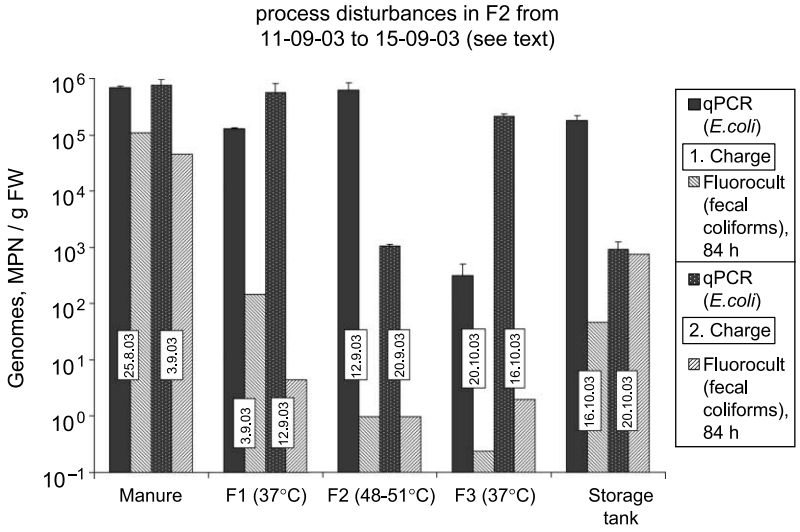

Figure 2 Reduction of *E. coli* genomes and fecal coliforms in the anaerobic fermenter cascade, charge tracing experiments

with FM > F1 > TST. *Yersinia enterocolitica* was detected only at very low concentrations in FM and in F1.

This indicated that: (i) strong reduction of indicator and potentially pathogenic bacteria (except for spore-formers) was obtained in the reactor chain (particularly in F1 and F2), and that; (ii) specific qPCR may substitute for cultivation based determinations, but only for equilibrated substrates which had not been exposed to lethal stress recently.

Strong evidence for the generation of ABNC cells in the fermenters came from higher counts for fecal coliforms (Figure 1), coliforms, and (intestinal) enterococci (not shown), when the samples were incubated for a longer time (48 h, 84 h) than conventionally applied. After incubation for 3.5 days, viable counts for (fecal) coliforms did not increase anymore. Apparently both decay and transition of bacteria to an ABNC state had occurred in the digesters. Our data indicated, that at least ten times more fecal coliforms

(and coliforms, not shown) were present as ABNC cells in the fermenters F2 and F3, and at least five times more in the terminal storage tank (Figure 1), suggesting that these parameters may be largely underestimated if the conventional incubation for 24 h is used for disturbed samples.

Figure 1 also shows that the qPCR values for *E. coli* genomes in the fermenters were consistently higher by a factor of 50–100 than the corresponding values for fecal coliforms after 84 h incubation. We believe that this difference corresponds to DNA copies from decayed *E. coli* cells that had not yet been degraded by the residual microbial activity in the fermenters. This indicates that qPCR may largely overestimate viable organisms in samples that had been exposed to lethal stress.

Charge tracing

In the course of the charge-tracing experiments, failures in the combined heat and power unit (CHPU) occurred on 11-09-03 and on the following few days, resulting in temperature drops (to 48 °C) in the thermophilic fermenter F2, reduced stirring, and transient feeding stops. These accidents had (almost) no influence on the results from cultivation as well as on most of the monitored qPCR parameters. However, qPCR data for *E. coli* and *Enterobacteriaceae* were decoupled from those of fecal coliforms and coliforms, with in some cases unusually high qPCR values in the fermenters F2 and F3 (Figure 2).

Reasons may have been: (i) local over-exposure to high temperatures in the thermophilic F2 resulting in high decay of *E. coli* and susceptible *Enterobacteriaceae* due to the prolonged hydraulic retention time and the reduced stirring; and (ii) charge dislocation (mix-up with non-target material) due to interrupted feeding. Moreover, we observed a sink body in F2 which may have been mobilized by the irregular feeding and stirring. The sink body most likely contained high concentrations of cell debris and DNA, because: (i) it was exposed for a long time to the hot fermenter wall, resulting in increased microbial decay; and (ii) because the high temperature impeded DNA degradation and reuse (cf. Figure 1, Lebuhn *et al.*, 2004).

Consequences of the CHPU accident on microbial parameters were detected by the qPCR data but not by the cultivation results. However, interpretation of the data with respect to hygienization was only possible on the basis of the results from cultivation.

Our results indicate that established cultivation routines may underestimate potential pathogens particularly in disbalanced (non-equilibrated) environments due to the transition of germs to the ABNC state. ABNC cells are included in the results generated by qPCR, but their specific quantification separately from dead cells is still problematic. The application of RTqPCR targeting specific messenger RNA may be helpful in resolving this problem.

Although DNA targeting techniques are obviously prone to overestimation of viable organisms in samples that had been exposed to lethal stress and were not re-equilibrated (reuse of released DNA by the surviving populations), the particular advantages of qPCR (Lebuhn *et al.*, 2003) are still standing. In combination with reliable extraction routines (Lebuhn *et al.*, 2003, 2004) qPCR is an ideal rapid, specific, sensitive and high-throughput tool for monitoring environmental samples. Even if qPCR can result in overestimation, this is not associated with health risks. However, underestimation (i.e. of ABNC cells, cf. Figure 1) by established cultivation routines brings about the danger of contamination and infection. Moreover, PCR–based methods are until now the approaches of choice to determine organisms and viruses which are difficult to cultivate or cannot be cultivated in environmental and product samples.

The routine screening of environmental (and e.g. product such as food) samples by qPCR and, in the case of positive qPCR results, the subsequent very sensitive traditional

cultivation with prolonged incubation times appears to be an optimal solution to guarantee hygienic safety. If qPCR results, which are obtained already 6 hr after sampling, show unusually high (i.e. potentially hazardous) levels of indicators or pathogens, production or utilization can be stopped transiently until verification by cultivation provides a solid basis for further decisions. It is predicted that by applying this combined approach in hygiene monitoring, it is possible to greatly reduce the danger of epidemies, outbreaks and toxicoses by microbes or microbial products.

Conclusions

The applied system of optimized nucleic acid extraction, standard spiking, qPCR and data evaluation proved to be highly reliable, fast, specific and sensitive to quantify the number of genes (genomes) of distinct (pathogenic) organisms in environmental samples.

The anaerobic full-scale fermenter cascade (mesophilic - thermophilic - mesophilic) was highly effective in inactivating (pathogenic) viable microorganisms, except for spore-formers.

Since conventionally performed cultivation was shown to underestimate viable organisms particularly in sublethally stressed systems (ABNC cells) by a factor of at least 10, incubation times should be adequately increased for such samples to minimize hygienic risks.

The extrapolation from qPCR results to the number of viable and potentially infectious (micro)organisms is only possible for equilibrated but not for stressed samples, since qPCR detects the number of genes or genomes of viable organisms and of the decayed fraction of which the DNA has not yet been degraded by the survivors. The history of a sample must be considered if such extrapolations are intended.

We propose an upstream qPCR monitoring for distinct (pathogenic) organisms in (hygiene) monitoring routines optionally followed by verification by cultivation to: (i) reduce analysis time and manpower, (ii) warrant hygienic safety by monitoring specific (non-cultivable) pathogens; and (iii) allow for prompt action in cases of positive results.

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