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Comparison of DNA extraction kits and modification of DNA elution procedure for the quantitation of subdominant bacteria from piggery effluents with real-time PCR

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Abstract

Four commercial DNA extraction kits and a minor modification in the DNA elution procedure were evaluated for the quantitation of bacteria in pig manure samples. The PowerSoil®, PowerFecal®, NucleoSpin® Soil kits and QIAamp® DNA Stool Mini kit were tested on raw manure samples and on lagoon effluents for their ability to quantify total bacteria and a subdominant bacteria specific of pig manure contamination: *Lactobacillus amylovorus*. The NucleoSpin® Soil kit (NS kit), and to a lesser extent the PowerFecal® kit were the most efficient methods. Regardless of the kit utilized, the modified elution procedure increased DNA yield in the lagoon effluent by a factor of 1.4 to 1.8. When tested on 10 piggery effluent samples, compared to the QIAamp kit, the NS kit combined with the modified elution step, increased by a factor up to $1.7 \log_{10}$ the values of the concentration of *L. amylovorus*. Regardless of the type of manure, the best DNA quality and the highest concentrations of bacteria were obtained using the NS kit combined with the modification of the elution procedure. The method recommended here significantly improved quantitation of subdominant bacteria in manure.

Introduction

Pig manure hosts a complex community of microorganisms that may vary during storage and during aerobic or anaerobic digestion. Two microbial aspects of manure treatment are the most frequently studied: (1) the health risks associated with pathogenic bacteria and (2) the role of the microorganisms in the transformation of the organic matter. Cultivation methods are usually used to evaluate the impact of manure treatment on pathogens and on indicator bacteria (fecal coliforms, enterococci) whereas molecular methods are more appropriate to identify the dominant microbial groups and shifts in the community composition (Leung and Topp 2001; Peu et al. 2006; Spence et al. 2008; Liu et al. 2009; Barret et al. 2012) and to detect or quantify specific markers of pig manure contamination (Ufnar et al. 2007; Marti et al. 2009, 2010; Mieszkin et al. 2009; Arthurson 2011). While methods based on culture are limited by the cultivability of bacteria, molecular methods may be biased by differential extraction of DNA from manure. Indeed, manure contains different substances including proteins,

fulvic acids, and humic acids (Moller et al. 2004; Plaza et al. 2006), which can inhibit polymerase chain reaction (PCR). Moreover, aerobic and anaerobic digestion of pig manure produces effluents with different organic composition which can affect the efficiency of PCR. Most of the protocols for DNA extraction from manure samples are designed to isolate DNA from feces (Marti et al. 2009; Barkovskii et al. 2012) or from soil (Leung and Topp 2001; Ufnar et al. 2007; Spence et al. 2008; Mieszkin et al. 2009; Barkovskii et al. 2012). These DNA extraction procedures are essentially based either on mechanical and chemical lysis by chaotropic agents, detergents, and denaturing agents, or by a combination of heat, detergent, and mechanical action. Their efficiency in extracting DNA, which has been tested on feces (Tang et al. 2008), on sludge (Vanysacker et al. 2010) or on soil samples (Whitehouse and Hottel 2007; Dineen et al. 2010; Knauth et al. 2013), varies depending on the matrix analyzed. However, the efficiency of commercial kits for piggery effluents has not been fully explored. Furthermore, the quantitation bias is a major concern when PCR applications focus on the concentration of specific markers during the treatment process, as a low DNA extraction yield may lead to misinterpretation of the impact of the treatment. For such studies, highly efficient extraction of DNA from different type of manure is thus required.

The aims of this study were to compare the ability of four commercial DNA extraction kits to quantify total bacteria and, a specific pig manure marker, *Lactobacillus amylovorus*. In addition to the efficiency of the lysis the protocol used for DNA elution from the purification columns can contribute to differences in the recovery of DNA – we also compared procedures for DNA elution from the columns. The manure samples were selected for their different composition and for the treatment they underwent: raw manure stored in a tank, and treated manure stored in a lagoon after biological treatment and settling. The quantity and quality of the extracted DNA and quantitation of total bacteria and of *L. amylovorus* were compared to assess the performance of each kit.

Materials and Methods

Collection of manure samples

The efficiency of the DNA extraction kits and of the elution procedures were compared on two types of effluents collected from a piggery located in Brittany (France). Raw manure stored in a tank was centrifuged and further treated by aerobic digestion. Sludge was then separated from the liquid phase in a settling tank. The supernatant (liquid above the sludge) was removed and sent to a lagoon. Samples of raw manure and of lagoon effluent

were collected. The characteristics of the raw manure and of the lagoon effluent were, respectively: dry matter, 43 and 7.6 $g \cdot L^{-1}$; organic matter, 27.4 and 2.0 $g \cdot L^{-1}$; total Kjeldahl nitrogen, 4.5 and 0.2 $g \cdot L^{-1}$; a pH of 7.8 and 8.6. Additional samples (eight raw manure samples and two lagoon effluents) were collected in eight other piggeries in order to compare the performance of the kits for the quantitation of bacteria. Samples were collected in a 1 L flask and stored at 4°C until analysis.

Kit selection and DNA extraction

DNA extraction was performed using four commercial kits following the manufacturer's instructions. The kits, which used silica spin filter technology, were chosen for their easy and fast extraction methods. The following kits were evaluated: PowerSoil® DNA isolation and PowerFecal® kits (Mo Bio Laboratories Inc., Carlsbad, CA), QIAamp® DNA stool mini kit (Qiagen Inc., Valencia, CA), and Nucleo-Spin® Soil (Macherey-Nagel, Düren, Germany). For NucleoSpin® Soil, sample lysis was performed with the optional enhancer SX solution, as recommended by the manufacturer. In the rest of this article, the four kits are referred as PS, PF, QI, and NS, respectively.

To enable direct comparison of the kits, the same amount of starting material was used. DNA was extracted either from 250 mg of raw manure or from 250 mg of pellet after centrifugation of 90 mL of lagoon effluent at 16,000g for 30 min.

For each kit, two procedures were tested.

Procedure 1. DNA was eluted from the spin column with 100 μL of the elution buffer following the manufacturer's instructions.

Procedure 2. The elution procedure was similar to that described for procedure 1, except that to optimize DNA yield, the eluent was split into four successive elutions, each performed with 25 μL of elution buffer. The volume obtained after each centrifugation was pooled in one tube.

The kits performed with procedure 2 are further referred to as: PS_M, PF_M, QI_M NS_M.

The samples of manure and lagoon effluent were extracted in triplicate using each kit. Extracted DNA was stored at −20°C.

DNA yield and quality

The quality of the DNA extracts was estimated by running 3 μL of purified DNA on a 1.5% agarose gel using 1× Tris-borate-Ethylenediaminetetraacetic acid (EDTA) buffer and a Lambda DNA HindIII marker (Promega, Madison, WI). Gels were visualized under UV light after

gel staining with gel red (Interchim, Montluçon, France). The quantity (ng) and quality of extracted DNA were determined using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

PCR

The presence of PCR inhibitors was assessed using a quantitative PCR assay targeting the bacterial 16S rDNA gene. DNA was amplified with universal eubacterial primers W18 and W02 (Table 1).

Each PCR mix was prepared in a single tube. PCR reactions contained 1 μL of template DNA undiluted or diluted 10 or 100-fold in water, 0.6 μmol/L (each) forward and reverse primers, 2.5 U of Taq DNA polymerase (Sigma Aldrich, Saint Quentin Fallavier, France), 1× Taq DNA polymerase buffer 5× (Sigma Aldrich), deoxynucleoside triphosphates (Sigma Aldrich) at 2 mmol/L, 1.5 mmol/L $MgCl_2$ (Sigma Aldrich). The reaction mixture was brought to a final volume of 25 μL with water. PCR cycling was carried out using MJ Mini (Biorad, Paris, France). Bacterial 16S rDNA fragments were amplified in the following conditions: one cycle at 95°C for 2 min; 30 cycles of 94°C for 1 min, a denaturation step at 50°C for 1 min, one cycle at 72°C for 1 min; followed by a final extension at 72°C for 10 min. For each PCR reaction, a positive control (DNA from pure culture of *Listeria monocytogenes*) and a negative control (DNA free water) were used. The sizes of the amplification products were confirmed by agarose gel electrophoresis (1× Tris-borate-EDTA 1.5% [wt/vol] agarose) with 1.5 kb DNA BenchTop ladder (Promega). The PCR products were visualized under UV light after gel staining with gel red (Interchim). A PCR product with 1500 bp was considered a positive result.

Quantitative PCR for *L. amylovorus* and total bacteria

L. amylovorus and total bacteria were quantified using primer pair OTU171_RDA_F/OTU171_RDA_R and 1055F/1392r (targeting 440pb of the 16S rDNA region), respectively (Table 1). The reaction mixture consisted of 12.5 μL of IQ SYBR Green Supermix (Bio-Rad), a 200 nmol/L concentration of each primer, 2 μL of 1/10 diluted DNA, and 9.5 μL of water to reach a final volume of 25 μL. The PCR program for *L. amylovorus* is described in detail in Konstantinov et al. (2005). The PCR program for total bacteria included one cycle at 95°C for 10 min, 45 cycles at 95°C for 30 sec, one cycle at 60°C for 50 sec, and one at 72°C for 30 sec. PCR reaction was prepared using the automated pipetting system ep*Motion*® (Eppendorf, Le Pecq, France). PCR amplification was performed using a Bio-Rad CFX96 real-time PCR machine with Bio-Rad CFX Manager software, version 1.1 (Bio-Rad). All qPCR were performed in triplicate. Standard curves for *L. amylovorus* were constructed as described by Marti et al. (2010). A standard curve for total bacteria was generated as follows: The 16S rDNA fragments were cloned with StrataClone PCR Cloning Kit following the manufacturer's instruction (Agilent Technologies, Massy, France). Then plasmid extraction was performed with Qiagen plasmid mini prep (Qiagen) and purified with Wizard PCR clean Up (Promega, Wilmington, DE). The concentration of the initial plasmid solution was determined using Nanodrop. The standard curves were generated using 10-fold dilution of the plasmid solution containing 10^{10}–10^2 DNA copies·μL^{-1}. For each run, melting curve, and positive and negative controls were used.

Statistical analysis

The amounts of extracted DNA and bacterial concentrations were analyzed using a repeated-measures one-way ANOVA followed by a Student–Newman–Keuls test in an all pairwise fashion. Bacterial concentrations were logarithmically transformed prior to analysis. All statistical tests were performed with XLSTAT 2010.4 (Addinsoft SARL, Paris, France).

Results

The extraction efficiency of the four commercial kits, used according the manufacturer's instructions, was compared to that of the same kits with a modification of the final DNA elution step. ANOVA (Table 2) revealed a significant effect of the DNA extraction method on the amount

Table 1. Sequence of the primers used in this study.

Name	Sequence	TM (°C)	Size amplicon (pb)	Reference
W18	5-' GAGTTTGATCMTGGCTCAG-3'	50	1500	Godon et al. (1997)
W02	5-GNTACCTTGTTACGACTT-3'			Weisburg et al. (1991)
OTU171_RDA_F	5'TTCTGCCTTTTTGGGATCAA-3'	60	320	Konstantinov et al. (2005)
OTU171_RDA_R	5'CCTTGTTTATTCAAGTGGGTGA-3'			
1055F	5'-ATGGCTGTCGTCAGCT-3'	60	440	Ferris et al. (1996)
1392r	5'-ACGGGCGGTGTGTAC-3'			Amann et al. (1995)

of DNA and on bacterial densities regardless of the type of manure used (raw manure or lagoon effluent).

Total DNA yield

All four kits successfully evaluated DNA from manure and lagoon effluent but with varying efficiency (Fig. 1). The NS_M kit produced two times more DNA than the Qi kit regardless of the matrix. DNA yields extracted from raw manure with the NS kit significantly differed from yields extracted with the Qi, PS and PF kits. The average concentrations of DNA extracted with the NS kit and the Qi kit were 29.7 ± 3.5 ng·μL^{-1} and 15.4 ± 1.5 ng·μL^{-1}, respectively. It is noteworthy that with the Qi kit, the same amount of DNA was recovered after a second elution performed with 100 μL of elution buffer on the column. With the other kits, the amount of DNA recovered in the second elution was very low and not significant compared to the amount obtained in the first elution (data not shown). In the case of the lagoon effluent, the difference in DNA yields between the NS kit and the others was less marked. However, the highest concentration of DNA in this matrix was also obtained with the NS kit (87.5 ± 0.8 ng·μL^{-1}). The modification of the elution procedure improved the yield of DNA extracted. The amounts of DNA extracted from manure were slightly higher using procedure 2, whereas the average DNA yield ratios from the lagoon effluent differed significantly with procedure 2 and procedure 1 and ranged from 1.4 to 1.8. The QI and QI_M kits showed low reproducibility for the lagoon effluent, and the Qi_M kit showed low reproducibility for manure, as indicated by the high variability of extraction efficiency between replicates (Fig. 1).

DNA purity

The level of DNA purity was determined by the $A_{260/280}$ and $A_{260/230}$ ratios. The Qi kit provided the lowest level of DNA purity (Table 3). Regardless of the matrix, the $A_{260/280}$ ratios of DNA extracted with procedures 1 and 2

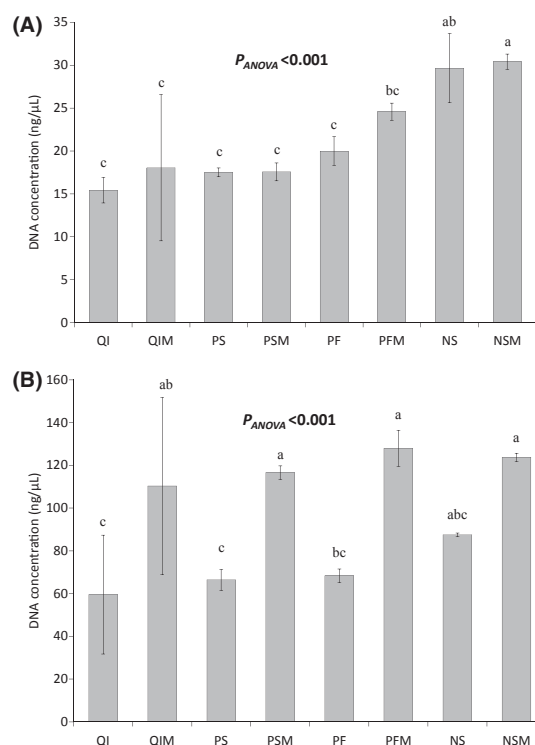**Figure 1.** Concentration of DNA (ng·μL^{-1}) extracted by each kit per elution in manure (A) and lagoon effluent (B). The ANOVA P value is indicated in the figure. Letters above the bars indicate statistical grouping (Newman–Keuls test). When letters are shared among groups, they are not statistically different ($P < 0.05$).

were comparable, ranging between 1.72 and 1.84 and between 1.70 and 1.96, respectively (Table 3), indicating that protein contamination was negligible. The $A_{260/230}$ ratios with procedure 1 were low. They ranged from 0.87 to 1.21 for manure and from 1.42 to 1.77 for lagoon effluent. However, they were systematically increased with procedure 2. The extractions performed with the PF_M and NS_M kits had the highest $A_{260/230}$ ratios, independently of the matrix (1.74 and 1.56 for manure, and 1.82 and 1.93 for lagoon effluent, respectively). The integrity of DNA extract was analyzed by agarose gel electrophoresis (Table 3). Six of eight extractions produced undegraded DNA. The lowest yield was obtained when DNA was extracted from manure with the Qi kit. DNA extracted with the PS and PS_M kits produced a smear, indicating that DNA was probably degraded and thus unsuitable for bacterial quantitation. It is noteworthy that in preliminary test, a Qiagen protocol consisting of two elutions of 100 μL was tested. However, this protocol did not enhance the yield and the purity of the DNA compared to the one step elution commonly recommended by the manufacturer (data not shown). To determine the relative

Table 2. ANOVA results for DNA amount and bacterial concentrations as dependent variables and DNA kit extraction as predictor variables.

Variables	Matrix	Degrees of freedom	F-value	P
DNA	Manure	7	8.7	0.0002
	Lagoon	7	6.7	0.001
Total bacteria	Manure	7	205.1	<0.0001
	Lagoon	7	77.2	<0.0001
Lactobacillus amylovorus	Manure	7	7.9	0.0003
	Lagoon	7	54.8	<0.0001

Table 3. Comparison of DNA extraction kits on pig manure (Man) and lagoon effluent (Lag).

Kit	DNA purity				DNA integrity[1]		Presence of PCR inhibitors[2]	
	$A_{260/280}$		$A_{260/230}$					
	Man	Lag	Man	Lag	Man	Lag	Man	Lag
QI	1.72 ± 0.20	1.81 ± 0.07	0.87 ± 0.30	1.42 ± 0.30	±	**++**	Yes[3]	Yes[3]
QI$_M$	1.79 ± 0.03	1.92 ± 0.03	1.40 ± 0.11	1.79 ± 0.16	++	**++**	No	No
PS	1.79 ± 0.11	1.87 ± 0.01	0.99 ± 0.26	1.80 ± 0.46	S	S	No	No
PS$_M$	1.96 ± 0.27	1.88 ± 0.02	1.27 ± 0.28	1.83 ± 0.16	S	S	No	No
PF	1.83 ± 0.18	1.82 ± 0.02	1.17 ± 0.19	1.63 ± 0.04	++	**++**	No	No
PF$_M$	1.70 ± 0.18	1.81 ± 0.06	1.74 ± 0.36	1.82 ± 0.12	++	**++**	No	No
NS	1.83 ± 0.15	1.84 ± 0.05	1.21 ± 0.52	1.77 ± 0.17	++	**++**	No	No
NS$_M$	1.80 ± 0.06	1.87 ± 0.02	1.56 ± 0.28	1.93 ± 0.13	++	**+**	No	No

NS, NucleoSpin® Soil kit; PS, PowerSoil®, PF, PowerFecal®; QI, QIAamp®.
[1] ++, bright band; +, faint band; ±, very faint band; S, smear.
[2] Observed using bacterial universal primers in the 1:10 dilution.
[3] Presence of PCR inhibitor was not observed in the 1:100 dilution.

sensitivity of the extraction kits, a dilution endpoint experiment using bacterial universal primers, was carried out. Extracts from samples were serially diluted and analyzed by PCR (Table 3). Samples of manure and lagoon effluent consistently yielded amplification products in 1:10 dilutions except with the Qi kit. The optimum DNA yield – in terms of DNA quantity and quality – was obtained with the PF$_M$ and NS$_M$ kits.

Quantitation of total bacteria and *L. amylovorus*

A real-time PCR assay targeting the 16S rDNA or a genomic fragment of DNA for quantitation of total bacteria and *L. amylovorus*, respectively, was used to compare the efficiency of the four extraction kits and the two elution procedures from manure and lagoon effluent. Each kit was successful in quantifying *L. amylovorus* (Table 4).

However, regardless of the matrix, there was a significant difference in the amount of total bacteria and *L. amylovorus* measured. Depending on the kit, the amount of *L. amylovorus* ranged between 3.3×10^5 and 2.6×10^6 CFU eq·mL^{-1} in manure and between 1.7×10^2 and 3.8×10^3 CFU eq·mL^{-1} in lagoon effluent. In agreement with the extracted DNA yield, the highest amounts were obtained with the NS$_M$ kit and the lowest with the PS and QI$_M$ kits. The low quantitation of total bacteria and *L. amylovorus* with the PS kit is probably due to degraded DNA, underlining the importance of checking DNA integrity. Although the difference in the amount was not systematically significant, except with the Qi kit, the concentrations of total bacteria and *L. amylovorus* were always higher with procedure 2 than with procedure 1. To check their efficiency, the four kits were tested on supplementary samples of raw manure and lagoon effluents from eight other piggeries. For each kit,

Table 4. Mean and standard deviation for concentrations of total bacteria and *Lactobacillus amylovorus* in manure and lagoon effluent after DNA extraction using four kits and two procedures of elution.

Kit	Total bacteria (gene copies mL^{-1})		*L. amylovorus* (CFU eq·mL^{-1})	
	Manure	Lagoon	Manure	Lagoon
QI	6.2×10^9 (8×10^8)**C**	6.8×10^7 (1.3×10^7)**E**	6.9×10^5 (4×10^4)**BC**	8.9×10^2 (8×10^1)**D**
QI$_M$	5.7×10^9 (2.2×10^8)**C**	3.3×10^7 (1.3×10^7)**F**	4.2×10^5 (1.9×10^5)**BC**	1.4×10^3 (1.4×10^2)**BC**
PS	1.4×10^9 (6.8×10^7)**E**	1.4×10^7 (6.1×10^4)**G**	3.3×10^5 (6.4×10^4)**C**	1.7×10^2 (5.1×10^1)**E**
PS$_M$	2.0×10^9 (3.2×10^8)**D**	4.9×10^7 (1.2×10^7)**E**	3.8×10^5 (1.1×10^5)**BC**	1.2×10^3 (1.2×10^2)**CD**
PF	6.5×10^9 (6.1×10^8)**C**	9.3×10^7 (3.8×10^6)**CD**	9.3×10^5 (4.8×10^4)**BC**	1.7×10^3 (1.4×10^2)**BC**
PF$_M$	9.0×10^9 (1.1×10^9)**B**	1.8×10^8 (1.1×10^7)**B**	1.3×10^6 (1.1×10^5)**AB**	2.2×10^3 (4.6×10^2)**B**
NS	1.2×10^{10} (7.2×10^8)**A**	1.1×10^8 (1.0×10^7)**C**	2.3×10^6 (2.1×10^4)**A**	1.9×10^3 (8×10^2)**BC**
NS$_M$	1.3×10^{10} (6.1×10^8)**A**	4.6×10^8 (7.2×10^7)**A**	2.6×10^6 (4.8×10^4)**A**	3.8×10^3 (2.5×10^2)**A**

Letters in bold indicate statistical groupings (Newman–Keuls test). When letters are identical among groups, they are not statistically different ($P < 0.05$); NS, NucleoSpin® Soil kit; PS, PowerSoil®, PF, PowerFecal®; QI, QIAamp®.

only the procedure corresponding to the highest concentrations of bacteria was chosen (procedure 1 for the Qi kit and procedure 2 for the three other kits). The concentrations of total bacteria and *L. amylovorus* obtained with the NS_M kit were systematically higher than those obtained with the QI and PS_M kits. They were similar to that obtained with the PF_M kit only in one manure sample and were higher for the other samples (data not shown). The average differences in calculated bacterial concentrations between the NS_M kit and each other kit ranged from 0.2 to 1.1 logarithmic units (Table 5). As observed on the manure and the lagoon effluent tested in the first experiments, the lowest differences in concentrations were observed between the NS_M kit and the PF_M kit.

Discussion

DNA extraction is a critical step because it can affect both the quality and quantity of DNA extracted. Furthermore, an increase in DNA yield is important to ensure that the DNA sample is representative of the sample gene pool (Burgmann et al. 2001). The extraction yield mainly depends on the physical properties and chemical composition of the matrix (Zhou et al. 1996; Lakay et al. 2007; Yankson and Steck 2009). Indeed, extraction kits that are effective for feces or environmental matrices – such as activated sludge and soil – may not be appropriate for piggery effluents. The objective of the study was thus to compare the efficiency of commercial extraction kits for

Table 5. Comparison of the efficiency of NS_M kit versus three kits in quantifying total bacteria and *Lactobacillus amylovorus* in pig manure samples and lagoon effluents.

Target (units)	Kit compared to NS_M	Differences of bacterial concentrations[1]	
		Manure (8)[2] Mean [min–max]	Lagoon (2) Mean [min–max]
Total bacteria (gene copies·mL^{-1})	QI	0.7 [0.5–1.3]	1.1 [0.7–1.5]
	PS_M	0.7 [0.4–1.3]	0.4 [0.1–0.7]
	PF_M	0.2 [0.0–0.6]	0.1 [0.1–0.1]
L. amylovorus (CFU eq·mL^{-1})	QI	0.7 [0.1–1.7]	–[0.8 to >1.0[3]]
	PS_M	0.6 [0.1–1.3]	0.5 [0.2–0.7]
	PF_M	0.5 [0.0–1.2]	0.3 [0.2–0.5]

Comparison was determined by subtracting the concentration of bacteria (expressed as log_{10}) obtained with the NS_M kit from that obtained with each other kit. NS, NucleoSpin® Soil kit; PS, Power-Soil®, PF, PowerFecal®; QI, QIAamp®.
[1]Calculated as follows: log_{10} (concentration with the NS_M kit)- log_{10} (concentration with the Qi, PS_M or PF_M kit).
[2]Number of samples.
[3]*L. amylovorus* was not quantified with the QI kit.

DNA quantitation of piggery effluents using real-time PCR. Because the chemical and physical properties of these types of effluents can vary depending on their management (storage or biological treatment), we evaluated two types of manure – raw manure and lagoon effluent, a by-product of the biological treatment – with different levels of dry matter (43 and 7.6 g·L^{-1}). A modification of the elution step was tested to improve the extraction efficiency of the kits. The kits were selected on the basis of their widespread use for fecal and environmental samples (McGarvey et al. 2004; Grewal et al. 2006; Heuer and Smalla 2007; Ufnar et al. 2007; Ravva et al. 2011; Dalkmann et al. 2012; Lins et al. 2012), because they do not require special equipment and because they extract DNA rapidly (60–90 min).

Significant differences in DNA yields were observed among the kits. The Qi kit showed poor reproducibility and provided a lower yield than the other kits assessed in this study. This low yield may be due to incomplete cell lysis, as the Qi kit uses a chemical lysis procedure whereas the PS, PF and NS kits combine a chemical and a mechanical lysis by bead beating. Indeed, bead beating has been shown to improve DNA extraction efficiency of archae in samples of human feces (Salonen et al. 2010). The Qi kit, which appeared to be the least appropriate kit regardless of the level of organic matter of the effluent, was shown to be the most effective method for the extraction of DNA from human feces compared to four other commercial kits (McOrist et al. 2002). However, in agreement with our results, (Whitehouse and Hottel 2007) showed that this kit was less efficient for the extraction of DNA from pig feces.

The results of this study showed that the NS kit yielded the higher concentrations of DNA. This is in agreement with the study of Knauth et al. (2013), who compared three commercial kits using paddy soils and found that the NS kit provided the highest DNA extraction efficiency. The NS kit combines a specific buffer and a column to remove inhibitors which may be more efficient than the buffer used in the PS and PF kits.

The use of four successive elutions (procedure 2) improved the efficiency of DNA extraction, especially in the case of the lagoon effluent, leading to an increase of up to a factor 2 in DNA yield. The weaker improvement of procedure 2 on the DNA yield observed for raw manure may be explained by the amount of organic matter in the two matrices at the beginning of the extraction step. Centrifugation of the lagoon effluent produced a pellet containing around 25 times more organic matter than that in the manure analyzed. Procedure 2, which slows down the elution time, may be more efficient in eluting DNA in presence of higher concentrations of contaminants than procedure 1.

In addition to the amount of DNA extracted, the purity of the DNA, estimated by the ratios $A_{260/280}$ and $A_{260/230}$ and the integrity of the extracts checked by agarose gel electrophoresis, are also important parameters for gene amplification by PCR (von Wintzingerode et al. 1997). While the protein purity appeared to be satisfactory, the DNA extraction from the samples led to low $A_{260/230}$ ratios, which reflected the co-extraction of contaminants absorbing at 230 nm such as residual phenol from nucleic acid extraction and humic and fulvic acids (Whitehouse and Hottel 2007; Techer et al. 2010). Interestingly, procedure 2 systematically led to an increase in $A_{260/230}$ independently of the matrix, suggesting that the split of the elution volume improved the removal of contaminants from DNA.

Regardless of the extraction procedure and of the matrix, the PS kit showed a smear on agarose gel. This was not observed with the PF kit, which differed from the PS kit by an additional heat lysis step. To improve the quality of DNA extracted from biosolids, Taskin et al. (2011) modified the procedure recommended with the PS kit (1) by increasing the incubation time of the two buffers used for the cell lysis from 5 to 10 min, and (2) by heating the elution buffer to 60°C and then incubating it for 5 min at room temperature. These modifications were tested on the manure and the lagoon effluent in this study but did not improve the DNA yield (data not shown).

Given the high level of organic matter in piggery effluents, the extraction of DNA may lead to the co-extraction of humic acids and other contaminants that inhibit Taq polymerase activity (von Wintzingerode et al. 1997; Iacovacci et al. 2003). Only the Qi kit required a 100-fold dilution to be able to remove PCR inhibitors. The reagent used for removal of enzyme inhibitors contained in the Qi kit appeared to be less efficient than the specific buffers used by the three other kits and the column in the NS kit. Once again, procedure 2 reduced the PCR inhibitors with the Qi kit, confirming the importance of this extraction step not only in the DNA yield but also for the removal of the PCR inhibitors. Although QI has been used for environmental samples, it was initially developed for DNA extraction from stool. In contrast, NS and PS were developed for soil samples and PF is a modification of PS kit, adapted for feces. However, the inhibitors of PCR are mainly represented by humic acids in soil (von Wintzingerode et al. 1997) and by polysaccharides in stool (Agusti et al. 2010; Hall et al. 2013). The presence of humic and fulvic acids in manure may explain the lower efficiency of the QI compared to the three other kits. The efficiency of the kits was tested for the quantitation of total bacteria and of a marker of pig manure contamination, *L. amylovorus* whose levels, using the Qi kit, were close to or below the detection limit of qPCR in lagoon effluents (Marti et al. 2010). It clearly appears that

except for the Qi kit, the modification in the elution procedure led to an increase in bacterial amounts. Furthermore, in accordance with the results of the DNA extraction, a higher quantitation of bacteria was obtained using the NS_M kit. When tested on 10 additional samples, compared with the results of the NS_M kit, the underestimation of the amount of *L. amylovorus* was substantial and reached up to 1.7 log_{10}. It is noteworthy that the Qi kit which was previously used to extract DNA from manure and feces of cattle or pig (Lier et al. 2008; Tang et al. 2008; Liu et al. 2009; Marti et al. 2010; Alexander et al. 2011; Grewal et al. 2006), gave the lowest amount of bacteria and failed to detect *L. amylovorus* in one lagoon effluent, suggesting that the occurrence of subdominant bacteria in piggery effluents may be underestimated with this kit. In conclusion, our results highlighted the fact that increasing the DNA extraction yield makes it possible to quantify subdominant bacteria in manure and lagoon effluents. The PS kit led to a degraded DNA and the Qi kit did not remove all the PCR inhibitors which led to significant underestimation of the concentrations of bacteria. By contrast, the NS kit and, to a lesser extent, the PF kit appeared to be more appropriate for these matrices. The modification of the final elution step increased the yield of DNA extracted and improved the quantitation of bacteria regardless of the target (total bacteria or less abundant specific bacteria). Therefore, to quantify bacteria in piggery effluents, we recommend the NS kit and the minor modification of the final elution step as described in "Materials and Methods" for commercial extraction kits using silica spin filter technology.

Acknowledgments

Conflict of Interest

None declared.

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