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Comparison of strategies for the isolation of PCR-compatible, genomic DNA from a municipal biogas plants

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Abstract

The goal of the project was the extraction of PCR-compatible genomic DNA representative of the entire microbial community from municipal biogas plant samples (mash, bioreactor content, process water, liquid fertilizer). For the initial isolation of representative DNA from the respective lysates, methods were used that employed adsorption, extraction, or precipitation to specifically enrich the DNA. Since no dedicated method for biogas plant samples was available, preference was given to kits/methods suited to samples that resembled either the bioreactor feed, e.g. foodstuffs, or those intended for environmental samples including wastewater. None of the methods succeeded in preparing DNA that was directly PCR-compatible. Instead the DNA was found to still contain considerable amounts of difficult-to-remove enzyme inhibitors (presumably humic acids) that hindered the PCR reaction. Based on the isolation method that gave the highest yield/purity for all sample types, subsequent purification was attempted by agarose gel electrophoresis followed by electroelution, spermine precipitation, or dialysis through nitrocellulose membrane. A combination of phenol/chloroform extraction followed by purification via dialysis constituted the most efficient sample treatment. When such DNA preparations were diluted 1:100 they did no longer inhibit PCR reactions, while they still contained sufficient genomic DNA to allow specific amplification of specific target sequences.

Keywords: Biogas plant; Genomic DNA; Humic acids; PCR

1. Introduction

Within the last decade, biogas plants have consolidated their position in the recycling of organic wastes to energy. The complex biotransformations taking place within the reactor are carried out by a diverse consortium of strictly anaerobic bacteria and archaea, which evolves over the operating time of the plant, i.e. often years to decades. In order to better understand and optimise the day-to-day operation of such biogas reactors, an identification of the resident microbial consortium would be of value. While it is not always possible to propagate all members of the consortium characteristic of an established biogas plant under laboratory conditions, the microorganisms can at least be identified by molecular biological methods such as PCR of specific sequences of microbial DNA. However, the availability of effective extraction methods yielding high quality DNA is an essential prerequisite for such approaches.

To our knowledge, no suitable method for the isolation of total microbial community DNA from samples representative for biogas plants has been published. However, protocols have been proposed for the isolation of genomic DNA from diverse environmental sources. Such environmental samples were expected to share some of the challenges posed by our technical ones, as they are similar in complexity. In our study, we therefore considered in particular kits/methods that were either intended for samples similar to the feed of the biogas plant, i.e. foodstuffs, or kits for the analysis of environmental samples including soil and wastewater. A screening of methods reported in the pertinent literature for the isolation of genomic DNA from complex environmental sources, such as soil [1,2], compost [3,4], activated sludge [5,6], sediment [7], or waste water [8] gives evidence that such methods tend to co-extract substances, especially humic acids [9], which are inhibitory to key enzymatic processes in molecular biology and which most likely will also be present in samples drawn from the biogas plant. Tsai and

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Olson [10] state that as little as 10 ng pure humic acids can inhibit a PCR reaction. Although some authors [11] were able to overcome this problem by adding extra polymerase to their PCR mix, the removal of the humic acids from the DNA is generally considered crucial for successful PCR. Such a separation is quite challenging, as the class of humic substances resembles nucleic acids in regard to negative charge density. Some methods have been described in the literature that presumably achieve the removal of humic acids from DNA-containing samples, including, but not restricted to, hydroxyapatite chromatography [12], polyacrylamide gel and Sephadex columns [10], polyvinylpyrrolidone and polyvinylpolypyrrolidone spin columns [13], electrophoresis in a low-melting point agarose gel [14], flocculation with aluminium sulphate [15], selective precipitation of the DNA with spermine [16], removal of DNA by magnetic capture-hybridisation [17], or dialysis [18]. The application of bovine serum albumin has also been reported to significantly reduce the PCR inhibition by haemin and other substances [19]. Dimethyl sulfoxide and glycerol are known to enhance the performance by destruction of complex structures and binding of the enzyme to the target. Eppendorf GmbH offers a ready to use MasterTaq® Kit containing a PCR enhancer for 'difficult' assays. Some authors report good results when using this approach for PCR with DNA isolated from soil contaminated with humic acids and heavy metals [20,21]. Similarly, dilution (e.g. 1:100) of the genomic DNA (and concomitantly putative inhibitors) prior to PCR may render a contaminated sample more suitable to PCR. However, in general little is known about the applicability of these methods to a given sample preparation.

The aim of this study was the identification of a robust and reliable method for the isolation and purification of high molecular weight genomic DNA representative of an established, thermophilic biogas plant. Attention was paid to time and effort required by the different methods. The method proposed by us should be of general value to the investigation of microbial consortia responsible for biotransformations in complex environments.

2. Experimental

2.1. Samples

Samples were from the biogas plant Biokraftwerke Fürstenwalde GmbH, Fürstenwalde/Spree, Germany. The plant has been continuously operated since 1998. The biological fraction of municipal solid waste, waste from agriculture, free flowing commercial waste, liquid manure, as well as 'difficult' bio-wastes requiring sanitation such as waste from grease separators and canteen kitchens are processed in this plant under thermophilic conditions (55 °C). Samples from the mash entering the bioreactor, the reactor content itself, as well as two of the bioprocess' products, namely 'liquid fertiliser' and the process water, were drawn at the plant and instantly stored at −20 °C for transport. Upon arrival, samples were allowed to thaw to 4 °C, aliquoted and kept frozen at −20 °C until further processing. Aliquoting directly was not an option.

2.2. Isolation of genomic DNA

For DNA isolation, 2 ml sample aliquots were thawed. The solids and microorganisms were collected by centrifugation (16,060 × *g*, 4 °C, 15 min), the supernatants were discarded. The pellets were subjected to the selected DNA isolation method. Six commercial kits were selected for the subsequent DNA isolation based on their dedicated area of application, i.e. the selection was restricted to kits either to be used with samples similar to the substrates processed in the biogas plant (i.e. kits intended for foodstuffs) or kits intended for environmental samples including wastewater. Kits and experimental procedures are listed below. Unless indicated otherwise, kits were used according to the manufacturer's instructions. This includes protocols for sample handling and lysis. Buffers and reagents were used as recommended. The exact composition of many of these reagents is proprietary.

2.2.1. Ultra Clean™ Soil DNA Isolation Kit (MO BIO Laboratories, Inc., CA, USA)

This kit was used with two lysis protocols. In particular, the cells were either lysed by heat (70 °C, 10 min) or by shearing with beads (vigorous shaking in a horizontal position for 10 min).

2.2.2. NucleoSpin® Food, Genomic DNA from Food (Macherey-Nagel GmbH & Co. KG, Germany)

For this kit DNA isolation was performed either with or without adding 200 μg ribonuclease A (Sigma–Aldrich, Germany) per sample.

2.2.3. Quantum Prep® AquaPure Genomic DNA Isolation Kit and InstaGene™ Matrix (both: Bio-Rad Laboratories GmbH, Germany)

These two kits yield solutions containing two fractions: the dissolved genomic DNA as well as cell debris bound to a proprietary matrix. The suspensions were centrifuged prior to further use.

2.2.4. Genomic DNA Purification Kit (Fermentas GmbH, Germany)

Contrary to the other kits evaluated in this study, this kit includes a chloroform extraction step and a precipitation of the DNA with ethanol.

In addition to the direct application of the commercially available kits, the combination of the QIAamp® DNA Stool Mini Kit (Qiagen GmbH, Germany) and the QIAamp® DNA Blood Midi Kit (Qiagen GmbH) was investigated, as suggested by Zoll et al. [22] for the isolation of bacterial DNA from soil. Briefly, the samples were incubated at 95 °C for 10 min, and centrifuged at 1930 × *g* for 5 min. The supernatant was transferred into a fresh tube. Ten millilitres of the ASL-solution was added (proprietary composition, solution provided with the kit), and the solution was shaken for 1 min. After adding one tablet InhibitEX, the mixture was shaken for 1 min, and centrifuged at 3990 × *g* for 5 min. The supernatant was transferred into a fresh tube, and 10 ml each of absolute ethanol (Merck KGaA, Germany)

and AL-buffer were added and blended by inverting the tube. Aliquots of 4 ml of this solution were loaded onto the column and centrifuged (3990 × *g*, 1 min). The eluate was discarded, and this step was repeated for the remaining solution. Once the entire volume had been processed, the column was washed with 1 ml AW1-buffer, and then with 1 ml AW2-buffer (composition of both buffers proprietary, buffers were used as provided with the kit). The column was transferred into a new tube and dried by centrifugation (1930 × *g*, 15 min). For elution, 300 μl sterile water was loaded onto the column, and incubated for 5 min at ambient temperature. The DNA was eluted by centrifugation (3990 × *g*, 5 min). The eluate was re-loaded onto the column and the elution-step was repeated. Then the DNA-solution was transferred into a sterile tube for storage.

Finally, a standard method ('miniprep') for the preparation of genomic DNA from pure bacterial cultures as published by Wilson [23] was used in modified form. Briefly, 14.4 mg lysozyme (Merck KGaA) was dissolved in 4.8 ml TE-buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The pellets obtained from centrifuging the samples were resuspended in 567 μl of this lysozyme-solution. Thirty microliters of 10% (w/v) SDS-solution and 3 μl proteinase K-solution (20 mg/ml) were added, and blended by inverting the tubes. After incubation for 1 h at 37 °C, 150 μl 5 M NaCl-solution and 80 μl CTAB/NaCl-solution (10%, 0.7 M) at a temperature of 65 °C were added. The samples were blended by inversion, and incubated for 10 min at 65 °C. When the suspensions had reached room temperature, 50 μl ribonuclease A-solution (3 mg/ml TE-buffer) were added, the samples were blended and incubated for 1 h at 37 °C. The first extraction step was carried out with 800 μl phenol:chloroform:isoamyl alcohol (25:24:1) (Carl Roth GmbH & Co. KG, Germany) by blending and centrifuging (16,060 × *g*, 4 °C, 5 min). The aqueous phase was transferred into a fresh tube and 800 μl chloroform:isoamyl alcohol (1:1) (Carl Roth GmbH & Co. KG) were added for the successive extraction. The sample was blended and centrifuged (16,060 × *g*, 4 °C, 5 min). The aqueous phase was again transferred to a fresh tube and this step was repeated twice. Finally, the DNA was precipitated from the aqueous phase by adding 600 μl isopropanol (Merck KGaA) and incubated for 30 min at −20 °C. The precipitate was pelleted by centrifugation (16,060 × *g*, 4 °C, 15 min), the supernatant was discarded, and the pellet was washed with 1 ml 70% (v/v) ethanol (−20 °C). The dried pellet was re-suspended in 100 μl TE-buffer and stored at 4 °C.

2.3. Further purification of genomic DNA

All further purification procedures were carried out with DNA isolated by the miniprep method described above. For DNA purification by electrophoresis, 25 μl of DNA-solution (equivalent to approximately 32 μg DNA) were separated in a 1% (w/v) agarose gel in 1× TAE-buffer (40 mM Tris, 1 mM Na_2EDTA, pH 8.0) at 150 V for 1 h. The gel was stained with ethidium bromide, and the DNA was visualised under UV light. The parts of the gel containing the DNA were excised with a scalpel. For electroelution, approximately 10 cm of Spektra/Por dialysis membrane tube 3 with a MWCO of 3500 (Spectrum Laboratories, Inc., CA, USA) were soaked in deionised water. Prior to use, the water was drained and the dialysis tube closed at one end. Two millilitres of 1× TAE-buffer was pipetted into the tube and the gel clipping was transferred into the tube. Air bubbles were removed, the tube was closed at the second end and placed into the electrophoresis chamber such that the gel clipping was oriented the same way as before. Electrophoresis was carried out using the same parameters as before for 30 min. Afterwards, the progress of elution was checked under UV light every 10 min. Once no fluorescence was detected anymore in the gel, the membrane was inserted in the inverse direction, and electrophoresed for 2 min to free DNA possibly sticking to the membrane. The buffer containing the DNA was transferred into a sterile Falcon tube, and the DNA was precipitated with 200 μl 3 M potassium acetate-solution (pH 5.0) und 17.8 ml absolute ethanol. After incubation at −20 °C for 2 h, the DNA was collected by centrifugation (3990 × *g*, 4 °C, 15 min). The pellet was washed twice with 70% (v/v) ethanol (−20 °C). The air-dried pellet was re-suspended in 50 μl TE-buffer.

For DNA purification via spermine-precipitation a method originally developed by Hoopes and McClure [24] was applied in the version published by Reineke et al. [16]. Briefly, a 25 μl DNA-solution aliquot (equivalent to approximately 32 μg DNA) was blended with 1 μl spermine-solution (50 mM spermine tetrahydrochloride (Sigma–Aldrich) in sterile water). The tube was incubated for 15 min on ice and centrifuged (16,060 × *g*, 4 °C, 12 min). The supernatant was discarded, and the pellet was washed twice with 70% (v/v) ethanol (−20 °C). In order to remove the spermine, 100 μl of the following freshly prepared buffer (1 volume 0.3 M sodium acetate, 10 mM $MgCl_2$, and 3 volumes ethanol) were added, the solution was blended and incubated on ice for 1 h. The DNA was collected by centrifugation (16,060 × *g*, 4 °C, 10 min) and the supernatant was discarded. The pellet was washed twice with 70% (v/v) ethanol (−20 °C). The air-dried pellet was re-suspended in 25 μl TE-buffer.

For DNA purification via dialysis the procedure described by Kiesslich et al. [18] was applied with slight modification. A sterile beaker containing 1 l buffer (1 mM EDTA, 1 mM NaCl, 10 mM Tris/HCl, pH 8.0) was prepared. Using sterile forceps, a sterile standard MF-Millipore filter membrane with a pore size of 50 nm (Millipore GmbH, Germany) was placed on the surface, the glossy side facing upwards. Fifty microliters of DNA-solution (equivalent to approximately 64 μg DNA) was applied to the membrane in tiny drops, and the beaker was covered with sterile aluminium foil. The dialysis was carried out over night (approximately 12 h) at ambient temperature. The DNA-solution (approximately 100 μl) was transferred into a sterile tube and the membrane was rinsed with 100 μl sterile water. This water was pooled with the DNA-solution, and 20 μl 3 M potassium acetate-solution as well as 1780 μl absolute ethanol were added. The DNA was precipitated by incubation at −20 °C for 2 h and centrifuged (16,060 × *g*, 4 °C, 15 min). The supernatant was discarded and the pellet was washed twice with 70% (v/v) ethanol (−20 °C). The air-dried pellet was re-suspended in 50 μl TE-buffer.

Table 1
DNA yields obtained when treating 2 ml liquid fertiliser samples according to the different isolation methods

Method[a]	Yield[b] (μg)	Concentration[c] (μg/ml)	$A_{260/280}$	$A_{260/230}$	Hands-on-time[d] (min)
Ultra Clean™					
Mechanical lysis	2.76	110.4	1.17	0.54	30–40
Heat lysis	1.58	63.1	1.17	0.39	30–40
NucleoSpin®					
With RNase	4.54	90.9	1.21	0.70	30–40
Without RNase	3.80	76.1	1.28	0.73	30–40
QIAamp®	13.08	87.2	1.52	0.92	60
Quantum Prep®	n.a.	n.a.	1.18	0.65	30–40
InstaGene™	n.a.	n.a.	1.20	0.58	15
Genomic DNA	n.a.	n.a.	1.19	0.69	30–40
Miniprep	63.90	1278.3	1.31	0.72	30–40

For spectrophotometry samples were diluted 1:50 in Millipore water. n.a.: not applicable, solutions were very turbid and/or brown.
[a] Ultra Clean™: Ultra Clean™ Soil DNA Isolation Kit; NucleoSpin®: NucleoSpin® Food, Genomic DNA from Food; QIAamp®: QIAamp® DNA Stool Mini Kit and QIAamp® DNA Blood Midi Kit; Quantum Prep®: Quantum Prep® AquaPure Genomic DNA Isolation Kit; InstaGene™: InstaGene™ Matrix; Genomic DNA: Genomic DNA Purification Kit; miniprep: miniprep of bacterial genomic DNA.
[b] Microgram DNA obtained per milliliter of original sample.
[c] In the obtained isolate.
[d] Approximated, time, e.g. required for incubation steps not included.

2.4. Quantitation of DNA

The DNA-yields of the diverse isolation methods were roughly evaluated via spectrophotometry (BioPhotometer, Eppendorf AG, Germany) at 260 nm assuming that an absorbance of 1.0 units corresponds to a DNA concentration of 50 μg/ml [25]. In addition, DNA purities were evaluated via the absorbance ratios $A_{260/280}$ and $A_{260/230}$.

As second means of DNA-quantitation agarose gel-electrophoresis (Febikon Labortechnik GmbH, Germany) with gels containing 1% (w/v) agarose in 0.5× TAE-buffer (20 mM Tris, 0.5 mM Na_2EDTA, pH 8.0) was performed. The gels were stained with ethidium bromide (1 μg/ml) and the DNA was visualised under UV light (Transilluminator, Fisherbrand, Fisher Scientific GmbH, Schwerte, Germany). Pictures of the gels were taken with a digital camera (DC290 Zoom, Kodak, Stuttgart, Germany). This method was also applied to the PCR products. The DNA concentration was estimated visually in comparison to the quantitative markers HyperLadder I and II (Bioline GmbH, Germany) as well as the MassRuler™ DNA Ladder Mix (Fermentas GmbH, Germany). Although a distinct band could be seen in the high molecular weight range of the gels, a quantitation of this DNA by scanning was not possible due to a high noise level produced by the smears present in many of the gels.

2.5. Determination of the residual inhibitory potential of the purified DNA

DNA extracts prior and after purification were assessed for their potential to inhibit polymerase by spiking an established PCR-assay. Briefly, a PCR targeting the green fluorescent protein (GFP) sequence with the primers GFP-fw (5′-TCC CCC GGG GGA GC ATG GCT AGC AAA GGA GAA GAA CTT TTC ACT-3′) and GFP-rv (5′-TCC CCC GGG GGA TTA TTT GTA GAG CTC ATC CAT GCC ATG TGT AAT-3′) was carried out (product size 0.8 kb). Each 25 μl assay contained PCR mixture [1× ThermoPol buffer, 5% (v/v) dimethyl sulfoxide, 5% (v/v) glycerol, 0.25 mM of each dNTP and 1 U *Taq* polymerase (New England Biolabs GmbH, Germany)], 50 nmol of each primer, and 0.5 μg template pGLO plasmid containing the GFP sequence (Bio-Rad Laboratories GmbH, Germany). This PCR mix was spiked with the DNA-containing solutions (1 μl) obtained by the various isolation procedures as well as with the DNA-solutions produced by the three investigated purification methods. All solutions were investigated undiluted (for concentrations see Tables 1 and 2) as well as diluted 1:10 and 1:100 in sterile water. A PCR assay with sterile water instead of biogas plant DNA served as positive control. The PCR temperature programme consisted of one denaturation step at 95 °C for 5 min, 35

Table 2
DNA yields obtained when DNA isolated from the liquid fertiliser according to the miniprep method (dubbed ‘unpurified isolate’ in the table below) was further purified with different methods

Method	DNA concentration[a] (μg/ml)	Yield[b] (%)	$A_{260/280}$	$A_{260/230}$
Unpurified isolate	1278.3	100	1.31	0.72
Electrophoresis and electroelution	94.7	7.4	1.14	0.45
Spermine precipitation	1102.9	86.3	1.33	0.87
Dialysis	432.9	33.9	1.50	0.90

For spectrophotometry samples were diluted 1:50 in Millipore water.
[a] In the obtained solution.
[b] Compared to the unpurified isolated.

cycles of 95, 60 and 72 °C for 1 min each, and a final extension step at 72 °C for 10 min.

2.6. PCR of bacterial 16S rRNA

To evaluate the suitability of the DNA-solutions as target for specific bacterial sequences, PCR for bacterial 16S rRNA was conducted with the primers pA/pH [26]. Each 25 μl assay contained the following PCR mixture [1× ThermoPol buffer, 0.25 mM of each dNTP, 5% (v/v) dimethyl sulfoxide, 5% (v/v) glycerol, 5 μg BSA and 1 U *Taq* polymerase (New England Biolabs GmbH)], as well as 20 nmol of each primer and 1 μl DNA-solution gained with the methods described above (undiluted, 1:10, and 1:100 diluted). Sterile water was used as negative control, 1 μl DNA (concentration 30 μg/ml) from *E. coli* as positive control. The temperature programme consisted of one denaturation step at 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, and a final extension step at 72 °C for 5 min.

3. Results and discussion

3.1. Isolation of DNA

Protocols for the isolation of genomic DNA can be divided in direct and indirect methods according to the procedure employed for bacterial lysis. While the direct methods lyse the cells in the matrix and then separate the DNA from the matrix and cell debris, the indirect methods separate the cells from the matrix prior to lysis. The former methods generally yield more DNA, so they are usually applied for gene bank constructions, accepting the co-extraction of eukaryotic DNA [27]. As we intended to obtain genomic DNA representative of the whole microbiota, rather than selectively lyse specific bacteria or archaea, we had to assure release of maximal amounts of genomic DNA, i.e. a high initial DNA yield. We therefore evaluated only direct lysis protocols. An initial centrifugation step was introduced to concentrate the sample, pelleting the matrix as well as the microorganisms. Four sample types representative for crucial stages in the biogas production plant were identified and used in the experiments: the mash entering the reactor, the reactor content itself, as well as the 'liquid fertiliser' and the process water leaving the reactor. Of these, the liquid fertiliser was considered the most challenging, because of its high content of inhibitory substances. Protocols were therefore first evaluated for the liquid fertiliser. In a second step, their applicability to the other biogas plant samples was verified.

The chosen DNA isolation protocols covered various typical approaches to DNA capture including adsorption, extraction and precipitation. The results obtained with the investigated methods in case of the liquid fertilizer sample are compiled in Table 1. When the time and effort necessary for DNA preparation per sample were compared, see Table 1, the InstaGene™ Matrix Kit performed best, while the combination of the QIAamp® kits clearly required the longest hands-on-time time. The yields (A_{260}) and purities ($A_{260/280}$ and $A_{260/230}$ ratios) of the DNA-solutions were assessed spectrophotometrically. While the $A_{260/280}$ ratio is a measure for the contamination with proteins, the $A_{260/230}$ ratio is a general measure for co-extracted contaminants containing peptide bonds and/or aromatic residues, in our case presumably mostly contaminating humic acids. For 'pure' DNA preparations $A_{260/280}$ ratios should range between 1.8 and 2.2 and $A_{260/230}$ ratios between 1.5 and 1.8 [25]. It is unlikely, however, that DNA prepared from a biogas plant (or from some other complex environmental source) will reach such values. Due to the likely residual contamination, the spectrophotometric quantitation of the DNA can only be considered as a very rough estimate. In certain cases, when the solutions were very turbid and/or brown (see below), we did not convert the optical density into a concentration (yield). Taking this *caveat* into consideration, DNA yields ranged from 1.58 μg/ml sample (Ultra Clean™ Soil DNA Isolation Kit in combination with heat lysis) to 63.90 μg/ml sample (miniprep method). As evidenced by agarose gel electrophoresis, all methods yielded relatively high molecular weight DNA with an average fragment size above the size limit of our system (10 kb). In spite of the sometimes harsh lysis conditions, apparently little destruction by shearing took place. An exact size determination of the isolated DNA was not carried out, as we intended to follow with PCR and not with direct cloning. Finally the methods were used to process the other sample types, i.e. mash, reactor content, and process water. The methods gave similar values for the yields and purities for these samples as for the liquid fertilizer used during method development. The only exception was the combination of the QIAamp® kits, which gave a rather low yield in case of the reactor content sample.

The kits involving adsorption of the DNA to a column material gave clear solutions, while DNA-solutions obtained by the other kits/methods were brown and turbid, making the continued presence of humic substances likely. Of the three column-based methods, the combination of the QIAamp® kits gave the best results in terms of yield and purity with a recovery of 13 μg DNA prepared from 1 ml of sample and $A_{260/280}$ and $A_{260/230}$ ratios of 1.53 and 0.92, respectively. However, when the quantities (concentrations) obtained were qualitatively assessed via gel electrophoresis, values were significantly lower than those obtained via spectrophotometric quantification, i.e. approximately 20.0 μg DNA/ml versus 87.2 μg DNA/ml. This indicates that despite the misleading clarity of the DNA-solution, optically active contaminants must have adulterated the photometric readings. This together with the fact that very low yields (<10 μg DNA/ml sample) were found spectrophotometrically when the kit was used with samples drawn directly from the biogas reactor, led us to abandon the method.

The least DNA was isolated with the Ultra Clean™ Soil DNA Isolation Kit following heat lysis, while mechanical lysis in this case resulted in almost twice the yield. Electrophoresis showed no differences in the DNA size between these two lysis methods. This kit was reported to give PCR-compatible DNA from soil samples, and our yields and DNA sizes are comparable to those published [14,28]. The NucleoSpin® Genomic DNA from Food Kit performed only slightly better concerning yield and the purity. Due to the low yields, these methods were also abandoned. The Quantum Prep® AquaPure Genomic DNA Iso-

lation Kit, the InstaGene™ Matrix Kit, and the Genomic DNA Purification Kit gave very turbid and brown solutions. We therefore refrained from calculating yields for these extractions based on spectrophotometric readings. This result is not surprising, as these kits/protocols are intended for samples low in solids, e.g. the InstaGene™ Matrix Kit for the isolation of bacterial DNA from wastewater [29]. In our case they were clearly unsuitable.

Of all investigated method, the miniprep adapted by us from a protocol originally intended for pure bacterial cultures resulted in the highest yield (63.90 μg DNA/ml sample, see Table 1) combined with high purity (only the QIAamp® combination yielded higher purities, albeit at significantly lower yield). Presumably this is the result of the several extraction steps with organic solvents that are part of this protocol. A similar observation has been made by others in the case of DNA extracted from soil [30]. Of the evaluated isolation procedures, this method thus met the demand for a high DNA yield in combination with high purity best.

It was subsequently shown, see Section 3.3, that none of the extracted DNA samples was directly suitable for PCR, even in the presence of additives, after dilution, or when the MasterTaq® Kit was used. Thus, the DNA-solutions had to be purified further. Since the miniprep method resulted in the highest yields together with good purity, this method was selected as basis for these subsequent experiments.

3.2. Purification of DNA

Additional purification of the DNA with the aim of rendering it suitable for PCR was attempted by three methods, namely agarose gel electrophoresis followed by electroelution from the gel, spermine precipitation of the DNA, and dialysis through nitrocellulose membranes. The methods were selected on account of their perceived efficiency, ease of handling, and costs. In this context it should be mentioned that on account of the high molecular weight of the DNA isolated in this study, a purification with column-based DNA clean-up kits [31,32], such as the Wizard® SV Gel and PCR Clean-Up System (Promega GmbH, Germany), was not an option, since it was not possible to elute the DNA from such clean-up columns (proven by staining the column, data not shown).

The results obtained with the three purification methods are compiled in Table 2. The data for the miniprep sample (dubbed 'untreated sample') are given for comparison. For the purification by electroelution, an aliquot of the DNA-solution obtained by the miniprep method was subjected to agarose gel electrophoresis. The fraction above 10 kb was cut from the gel with a scalpel and treated as detailed in Section 2. The precipitated DNA pellet was of white colour, indicating that some purification had taken place. However, the recovery yield of this procedure was only 7.4%, and the purity ratios were lower than for the unpurified DNA-solution. To some extent the low recovery yield as determined by a comparison of the spectrophotometric readings at 260 nm may be caused by an overestimation of the DNA content of the original miniprep solution due to the presence of optically active contaminants. However, since visually the DNA fraction excised from the agarose gel corresponded to the main fraction of the DNA preparation, the low recovery yield of this method is most likely also caused by insufficient electroelution and subsequent further losses during DNA precipitation. Spermine precipitation resulted in a slight increase in the $A_{260/230}$ ratio from 0.72 to 0.87 of the DNA preparation, see Table 2, signalling that some of the humic acid contaminants had been removed, although the effect was not strong enough to result in a visible difference. The recovery yield of this method, on the other hand, was more than 85%.

The most significant purification effect in our case was observed when the DNA obtained via miniprep protocol was subjected to dialysis through nitrocellulose membranes. After dialysis the originally darkish brown droplets had gained volume and were translucent. The $A_{260/280}$ ratio was found to increase from 1.31 to 1.50 and the $A_{260/230}$ ratio from 0.72 to 0.90. Approximately one-third of the DNA was recovered with this method. The purity of the DNA isolated with miniprep and purified with dialysis is thus comparable to that of DNA isolated with the combination of the QIAamp® kits ($A_{260/280}$ ratio 1.50 versus 1.52; $A_{260/230}$ 0.90 versus 0.92), while the yields are at least by a factor of five higher. Moreover, the miniprep/dialysis method was found also fully applicable to the other types of sample to be analysed from the biogas reactor, as shown in Table 3. For all samples the $A_{260/280}$ ratio increased with dialysis, with the mash displaying the lowest (1.27–1.47) and the reactor content the highest (1.37–1.66) values. In case of the $A_{260/230}$ ratios the value was higher after dialysis in case of the reactor content, the liquid fertiliser and the process water, while the value decreased after purification in case of the mash. The recovery rates based on the photometric readings were roughly 30% for all sample types except the mash, where approximately 5% were recovered. When the relative DNA contents were evaluated by agarose gel electrophoresis, no corresponding

Table 3
DNA yields of samples from mash, reactor contents, liquid fertiliser and process water obtained with the miniprep method ('isolation') followed by dialysis ('purification')

Sample	After isolation			After purification		
	$A_{260/280}$	$A_{260/230}$	DNA content (μg/ml)	$A_{260/280}$	$A_{260/230}$	Yield[a] (%)
Mash	1.27	0.80	2702.7	1.47	0.67	4.6
Biogas reactor	1.37	0.88	1416.0	1.66	1.19	30.6
Liquid fertiliser	1.31	0.72	1278.3	1.50	0.90	33.9
Process water	1.33	0.81	1143.0	1.61	1.07	29.3

Concentrations were obtained spectrophotometrically, samples were diluted 1:50 in Millipore water prior to analysis.
[a] Compared to the same sample after purification.

decrease of the DNA concentration before and after dialysis was observed.

We are aware of the fact that the qualities (purities) of the DNA-solutions obtained in this study are not comparable to the ones of DNA from pure bacterial cultures. However, this is most likely the case for the majority of DNA-solutions from environmental samples. Gabor et al. [27], e.g. found similar magnitudes of DNA yield for activated sludge samples.

3.3. Behaviour of the DNA in the PCR assay

Two types of PCR assay were performed, one to evaluate the inhibitory effect of the contaminants in the prepared microbial DNA-solutions, the other to estimated the suitability of the prepared DNA to act as template for PCR. The first PCR assay (evaluation of the inhibitory potential) was based on an established PCR reaction targeted the Green Fluorescent Protein (GFP) sequence in a mixture containing 5 μg of a template plasmid (pGLO) containing this sequence. This PCR mix was spiked with the original (obtained after miniprep) as well as the various purified (electroelution, spermine precipitation, dialysis) DNA-containing solutions prepared in this study. All solutions were added in the concentration as obtained (see Table 2) as well as diluted 1:10 and 1:100. The results for one of the sample types, namely the liquid fertiliser, are shown in Fig. 1. According to these results, only the 1:100 diluted DNA from the miniprep method gave a band (lane 3, Fig. 1). This confirms that as expected, e.g. from the darkish colour, the DNA-solution as originally obtained by the miniprep method did indeed still contain considerably inhibitory activity. Moreover, in spite of their misleading 'transparency', the DNA-solutions obtained after electroelution from the agarose gel also inhibited the PCR reaction when added to the mix in undiluted form. A faint band was obtained for the 1:10 dilution (lane 5, Fig. 1), while the band obtained in the presence of the 1:100 dilution (lane 6, Fig. 1) was of at least equal intensity as that of the control sample containing just the PCR mix (lane C, Fig. 1). The result was less clear for the spermine precipitation product, where we found that PCR was inhibited by the solution itself as well as the 1:100 dilution, while a faint PCR product band was present for the 1:10 dilution. The PCR was repeated several times always yielding the same result. Finally, very good results were obtained for the DNA purified by dialysis, where the band of the PCR product obtained in the presence of the 1:100 dilution was of at least equal intensity as that of the control sample, while a faint band could be obtained in case of the 1:10 dilution. From these results, it would seem that either agarose gel followed by electroelution or dialysis and to some extent even the miniprep method results in a DNA preparation that in diluted form does not inhibit the enzymes of the PCR overmuch.

The suitability of the prepared biogas plant derived DNA to serve itself as templates for PCR amplification was evaluated using the bacterial 16S rRNA PCR assay. DNA from *E. coli* was used as positive control in the assay. All DNA isolates prepared by us were included in the investigation (undiluted and in diluted form) together with all purified DNA preparations (undiluted and in diluted form). According to these experiments, none of the crude DNA preparation obtained by any of the seven isolation procedures gave, either undiluted or in diluted form, the expected specific 1.5 kb product (data not shown, miniprep data included in Fig. 2), although a very faint band was perhaps observed in case of the miniprep DNA diluted 1:100, Fig. 2. Similar results, i.e. no evidence of the amplified target sequence in the gel, were obtained for the DNA isolate (miniprep protocol) further purified by electroelution or spermine precipitation, Fig. 2. In case of the spermine precipitation this can simply be explained by the presence of inhibitory substances, as this mixture also had given low amplification in case of the PCR targeting the GFP-sequence as discussed above. However, in case of the DNA purified by electroelution, at least the 1:100 dilution had been shown to not inhibit the PCR reaction in general (see above). The failure to produce a specific PCR product here, is thus most likely not

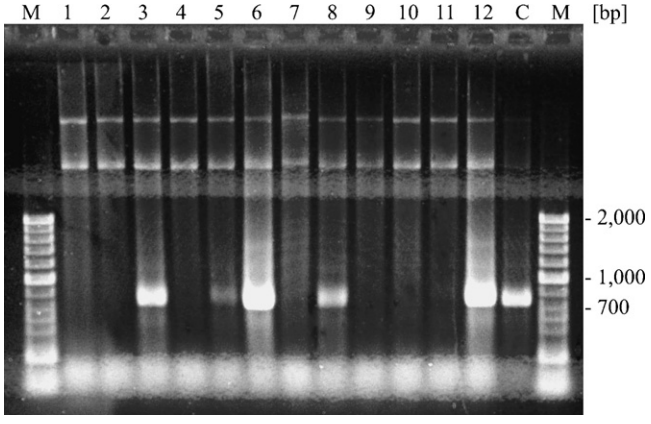

Fig. 1. Results of the PCR assay for GFP (product size 0.8 kb) spiked with DNA-solutions obtained with the miniprep method (1–3), as well as purified DNA-solutions with electroelution (4–6), spermine precipitation (7–9) and dialysis (10–12). For each set, the first reaction was spiked with undiluted DNA, the second 1:10, and the third with 1:100 diluted DNA. C: positive control (sterile water was added instead of DNA-solution), M: Marker (HyperLadder II).

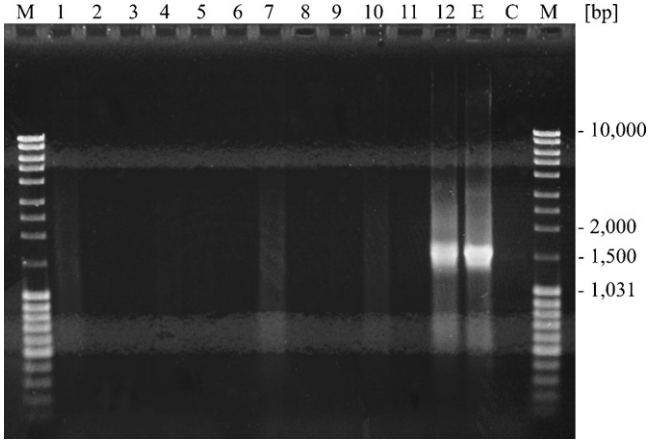

Fig. 2. Results of the PCR assay for bacterial 16S rRNA (product size 1.5 kb) with DNA-solutions obtained with the miniprep method (1–3), as well as purified DNA-solutions with electroelution (4–6), spermine precipitation (7–9) and dialysis (10–12). For each set, the first reaction is based on the undiluted template DNA (for concentrations see, Table 2), while the template DNA was diluted 1:10 in the second and 1:100 in the third reaction. E: positive control, DNA from *E. coli*, concentration: 30 μg/ml, C: negative control (sterile water), M: marker (MassRuler^{TM} DNA Ladder Mix).

due to inhibition, but rather to the low quantity of target DNA available in the sample after 1:100 dilution, note that this method had given the lowest DNA yields of the investigated ones. The PCR was repeated several times, always giving the same results.

The only successful specific amplification of the target sequence was observed in case of the DNA isolated by the miniprep method, further purified by dialysis, and diluted 1:100, i.e. the second type of DNA preparation found compatible to PCR in general in the GFP amplification experiments discussed above. In this case analysis of the PCR product by agarose gel electrophoresis (lane 12 in Fig. 2) yielded a product band of similar intensity as the positive control.

4. Conclusions

The extraction of high quality DNA representative of the entire microbial consortium remains a problem in the case of complex environments. As a rule, no standardised kits are available. In such case, we recommend the use of a robust general DNA extraction protocol, taken from the range of methods used in bacterial molecular biology, combined with an efficient means to remove contaminants that inhibit enzymes crucial for the subsequent (PCR) steps. The first step should be governed by DNA yield rather than purity, as DNA that is not extracted at this stage cannot be available later. Inhibitory compounds are better removed during the second step. Once a representative sample has been prepared, further dilution to reduce the residual inhibitory power should be no problem given the amplification potential of PCR. While such an approach gave good results in case of the biogas plant samples, a similar comparison of methods would also be valuable for samples from complex natural environments.

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