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Assessing the bias linked to DNA recovery from biofiltration woodchips for microbial community investigation by fingerprinting

Léa Cabrol · Luc Malhautier · Franck Poly ·
Anne-Sophie Lepeuple · Jean-Louis Fanlo

Abstract In this study, we explored methodological aspects of nucleic acid recovery from microbial communities involved in a gas biofilter filled with pine bark woodchips. DNA was recovered indirectly in two steps, comparing different methods: cell dispersion (crushing, shaking, and sonication) and DNA extraction (three commercial kits and a laboratory protocol). The objectives were (a) to optimize cell desorption from the packing material and (b) to compare the 12 combinations of desorption and extraction methods, according to three relevant criteria: DNA yield, DNA purity, and community structure representation by denaturing gradient gel electrophoresis (DGGE). Cell dispersion was not influenced by the operational parameters tested for shaking and blending, while it increased with time for sonication. DNA extraction by the laboratory protocol provided the highest DNA yields, whereas the best DNA purity was obtained by a commercial kit designed for DNA extraction from soil. After successful PCR amplification, the 12 methods did not generate the same bias in microbial community representation. Eight combinations led to high

diversity estimation, independently of the experimental procedure. Among them, six provided highly similar DGGE profiles. Two protocols generated a significantly dissimilar community profile, with less diversity. This study highlighted the crucial importance of DNA recovery bias evaluation.

Keywords DNA extraction · Cell dispersion · DGGE · Biofiltration · Diversity · Microbial community structure

Introduction

Biofiltration is an efficient technology for the purification of waste gases loaded with volatile organic compounds and other malodorous substances. Waste gas continuously goes through a reactor packed with an organic or inorganic material, which acts as a carrier for biofilm development. Compost, peat, or woodchips are generally used as organic material (Devinny et al. 1999). The purification relies on the oxidation of contaminants by microorganisms. The microbial community established in a biofilter is complex and uncharacterized, adapted to local conditions (pH, moisture, and concentrations) and grows as attached biomass embedded in an organic matrix mainly constituted of exopolysaccharides.

Since the last decade, molecular tools such as nucleic acid fingerprinting, quantitative PCR, or clone libraries have been applied to biofiltration to gain insight into the diversity and the structure of the microbial communities, thus providing a better understanding of microbial implication in biofiltration (e.g., Steele et al. 2005; Ying et al. 2008).

By granting access to uncultivable microorganisms, these molecular tools eliminated the cultivation bias (Amann et al. 1995). However, methodological bias still occurs at several steps, beginning with the recovery of

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genetic material from a complex matrix. DNA recovery can be biased by lack of exhaustivity, lack of representativity, and lack of purity, which can bother its subsequent applications (Tebbe and Vahjen 1993). The organic nature of the packing material even increases the difficulty to recover representative and clean DNA extracts. Thus an essential preliminary task for molecular investigation of microbial communities is to implement and optimize a methodology for the recovery of high yield and high purity nucleic acids. To get samples as representative as possible of the initial diversity, this methodology has to be the least selective as possible.

In other ecosystems, such as soil, a lot of work has been carried out to compare and implement DNA recovery methods that exhibit an unbiased sampling of the investigated community (Robe et al. 2003). Within biofiltration context, very little attention was paid to the methodological aspects of nucleic acids recovery, despite their crucial importance in final result significance (Khammar et al. 2004). DNA recovery methods are hugely dependent on the packing material specificities (size, organic/inorganic nature, density, porosity, and nutrient availability); maybe that is why they have neither been standardized nor optimized to date.

In this study, we explored methodological aspects of nucleic acid recovery from microbial communities involved in a laboratory biofilter filled with pine bark woodchips. DNA was recovered thanks to indirect methods carried out in two successive steps: cell desorption and DNA extraction. Three different methods were tested for cell desorption (crushing, shaking, and sonication). For DNA extraction, three commercial kits were assayed (two of them being specific for soil), as well as a laboratory protocol. The objectives of the present work were (a) to optimize cell desorption from the packing material and (b) to compare the combinations of desorption and extraction methods, according to three relevant criteria: DNA yield, DNA purity, and representation of microbial community structure.

Materials and methods

Sampling of biofilter packing material

Experiments were conducted on a lab-scale biofilter (1 m height, 125 mm in diameter) treating an H₂S stream (10 mg H₂S/m³; 500 m/h). The packing material was kept at a constant moisture content of 68% by regularly spraying a salt mineral nutrient solution (KH₂PO₄ 3.3 mM, Na₂HPO₄ 3.3 mM, MgCl₂ 0.2 mM, CaCl₂ 0.1 mM, FeCl₂ 9 μM, H₃BO₃ 46.2 μM, MnCl₂ 9.1 μM, CuCl₂ 0.016 μM, Zn 0.015 μM, Na₂MoO₄ 0.01 μM, and CoCl₂ 0.017 μM). The biofilter was run at ambient temperature. The organic packing material was constituted of pine bark wood chips

screened between 6 and 16 mm and characterized by a bulk density of 330 kg/m³, a void fraction of 58%, and a pH of 5.8.

At the time of sampling, the biofilter had reached a steady state, with complete H₂S removal, which indicated an effective microbial colonization. Woodchips were sampled from the biofilter at the same time and same location (0.5 m height) and splitted into 44 samples of 5 g each, suspended in 15 mL of sterile physiological saline solution (NaCl 9 g/L) so as to avoid osmotic shocks and maintain cellular integrity.

Cell dispersion methods

Three detachment methods were investigated: blending, shaking, and sonication (Fig. 1). Blending was performed by an Ultra-Turrax T25 basic (Ika, Freiburg, Germany) at maximal revolution speed of 24,000 rpm. Shaking was performed by a Vibro-Shaker MM200 (Retsch, Haan, Germany), at maximal rotating frequency of 30 Hz. Sonication was performed by an ultrasonic bath (Branson, Danbury, CT, USA) at maximal energy output of 70 W.

After dispersion in saline solution, woodchips were discarded, and the liquid phase, containing the suspended microorganisms, was recovered. One milliliter aliquots of the liquid suspension was collected, fixed with 3.7% formaldehyde, and stored at 4°C until enumeration.

Operating conditions for dispersion optimization

For each desorption method, the influence of treatment duration was assessed (Fig. 1). Four durations were tested for sonication (from 5 to 60 min) and three for blending (from 0.5 to 2 min). For shaking desorption, the influence of glass beads was also assessed. Three amounts of sterile glass beads (1 mm diameter) were assayed: 0, 5, and 10 g. For the two-factor optimization, the experimental design required seven assays according to a Doehlert matrix, as shown in Fig. 1.

For the three dispersion methods, each treatment condition was repeated on triplicate samples.

Microscopic direct counts

Total bacteria were enumerated by fluorescence microscopy after staining with 4'-6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St Louis, MO, USA). Whole experiment was done in sterile conditions. After serial dilutions with physiological serum, samples were stained with DAPI at a final concentration of 20 μg/mL during 30 min in the dark under shaking. Stained bacteria were collected on 0.2 μm polycarbonate membrane filters (Millipore, Billerica, MA, USA) by vacuum microfiltration. Filters were observed with an epifluorescence microscope (Leica, Wetzlar, Germany)

Fig. 1 Experimental design for the optimization of dispersion methods

Initial sample S		S ₁ Shaking		Duration (min)	Glass beads (g)	Samples
S ₂ Sonication		S ₃ Blending		1	5	3 S ₁₁
Duration (min)	Samples	Duration (min)	Samples	4.5	0	3 S ₁₂
5	3 S ₂₁	0.5	3 S ₃₁	4.5	10	3 S ₁₃
15	3 S ₂₂	1	3 S ₃₂	8	5	5 S ₁₄
30	3 S ₂₃	2	3 S ₃₃	11.5	0	3 S ₁₅
60	3 S ₂₄			11.5	10	3 S ₁₆
				15	5	3 S ₁₇

equipped with a blue excitation filter (BP 340–380 nm) and a barrier filter LP 425. Thirty microscopic fields per slide were enumerated.

DNA extraction methods

The experimental procedure is presented in Fig. 2. Each sample was duplicated and constituted of 5 g of woodchips suspended in 15 mL of sterile physiological serum (NaCl 0.9%). After cell detachment, the woodchips were discarded, and the liquid phase containing suspended cells was centrifuged at 10,000 rpm for 10 min. The cell pellet was recovered, and DNA was extracted from a pellet fraction using one of the four following methods:

- PowerSoil DNA Kit (MoBio, Carlsbad, CA, USA)
- FastDNA® SPIN Kit for Soil (MP Biomedicals, Irvine, CA, USA)
- NucleoSpin® Tissue Kit (Macherey-Nagel, Duren, Germany)
- A laboratory protocol adapted from Godon et al. (1997)

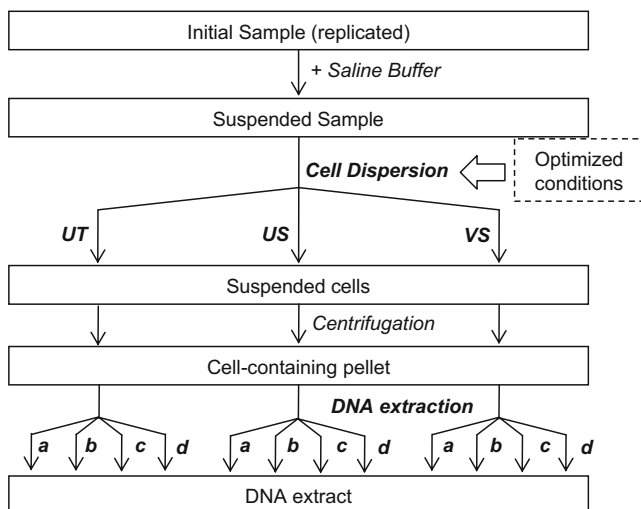


Fig. 2 Experimental design to test 12 combinations of desorption and extraction methods. *UT* blending by Ultra-Turrax, *US* ultrasonication, *VS* shaking with Vibro-Shaker. *a, b* Two commercial kits designed for DNA extraction from soil, *c* universal commercial kit for DNA extraction, *d* extraction method according to a laboratory protocol

Extraction by commercial kits was performed according to the manufacturers' instructions. For comparison purpose, the amount of cell pellet used for extraction and the elution volume for final DNA recovery were standardized in all four cases (450 mg and 50 µL, respectively).

Procedures *a* and *b* involved chemical and mechanical means (bead beating) for cell lysis, whereas procedure *c* relied on chemical and enzymatic lysis (proteinase K). Sodium dodecyl sulfate was included for cell disruption in methods *a* and *c*, while guanidine thiocyanate and guanidine hydrochloride were used for purification in kits *b* and *c*, respectively. The three kits performed DNA purification by selective DNA binding on silica spin columns and ethanol washing.

The fourth method was adapted from the one described by Godon et al. (1997). Briefly, the cell pellet was resuspended in 385 µL of 4 M guanidine thiocyanate–0.1 M Tris (pH 7.5) and 115 µL of 10% *N*-lauroyl sarcosine. After the addition of 500 µL of 5% *N*-lauroyl sarcosine–0.1 M in phosphate buffer (pH 8.0), the sample was incubated at 70°C for 1 h. One volume (500 µL) of 0.1-mm-diameter sterile zirconium beads (Sigma-Aldrich) was added, and the sample was shaken at maximum speed (30 Hz) for 10 min in a Vibro-Shaker MM200 (Retsch, Haan, Germany). Then DNA was isolated and separated from contaminants according to Godon's protocol (Godon et al. 1997) with proteinase K, polyvinylpyrrolidone, and RNase and further purified with phenol–chloroform–isoamyl alcohol.

Evaluation of DNA yield and purity

Extracted DNA (5 µL) was visualized by gel electrophoresis on 1% agarose gel. DNA amount was further determined by absorbance at 260 nm using a UV spectrophotometer (Eppendorf, Hamburg, Germany). DNA purity was determined by the ratio of absorbance at 260 nm and absorbance at 280 nm, considering that the absorbance at 280 nm was mainly due to protein contamination. It is generally assumed that DNA is free from protein contamination when this ratio is higher than 1.7 (e.g., Stach et al. 2001).

PCR amplification

The V3 region (196 bp, corresponding to the *Escherichia coli* position F337-R533) of the eubacterial 16S rRNA gene was PCR amplified using primers V3F (5'-ACTCCTACGG GAGGCAGCAG-3') and V3R (5'-ATTACCGCG GCTGCTGGCAC-3'), both slightly modified from Muyzer et al. (1993). A 40-base GC clamp was attached to the 5' end of the forward primer. Amplification was performed on ~100 ng DNA template (approx. 1–2 μ L) in a final volume of 50 μ L containing 0.6 μ M of each primer (Sigma-Aldrich), 0.8 mM of dNTP mix (0.2 mM each dNTP, Abcys, Paris, France), 10 μ L of 5 \times Phusion HF Reaction Buffer (Finnzymes, Espoo, Finland), and 1 U of Phusion Hot Start High fidelity DNA Polymerase (Finnzymes, Espoo, Finland). PCR was achieved by Tpersonal thermocycler (Biometra, Goettingen, Germany) using the following program: initial denaturation at 98°C for 4 min, 30 cycles consisting of 30 s denaturation at 98°C, 30 s annealing at 68°C and 30 s extension at 72°C, and final extension at 72°C for 10 min. Negative controls were included to verify the absence of contamination. PCR products were visualized by electrophoresis on 2% agarose gel and quantified by absorbance at 535 nm after PicoGreen staining (Quant-iT ds DNA HS reagent, Invitrogen, OR, USA).

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was performed with Ingeny phor U-2 system (Goes, The Netherlands) according to the protocol of Muyzer et al. (1993), using 8% polyacrylamide gels prepared with denaturing gradient ranging from 43% to 63% (where 100% denaturant contained 7 M urea and 40% (v/v) formamide). Samples of approx. 500 ng of PCR product were loaded on the gel, along with reference standards for further pattern alignment. Electrophoresis was run at 60°C and 100 V in 1 \times TAE buffer, for 16 h. Gels were stained with Sybr green I (Sigma-Aldrich), washed, and photographed at 520 nm. Gels images were analyzed with Bionumerics software (Applied Maths, Gent, Belgium).

Statistical analysis

After dispersion and enumeration, mean cell numbers and standard deviations were calculated on triplicate samples. When a single parameter was varied (duration), results were statistically analyzed by ANOVA: Variance homoscedasticity was verified with a Hartley test, and means were compared with a Fisher–Snedecor test at a risk level of 0.05. When two parameters varied (duration and glass bead amount), the response surface was analyzed according to a Doehlert matrix.

For DNA yields and DNA purity, means and standard deviations were evaluated on duplicate samples. The significance of the results was checked by ANOVA (Fisher–Snedecor test at a risk level of 0.05).

The DGGE gels were analyzed in two ways. First the diversity was measured by the Shannon index H' , which took into account both the number of bands and their relative intensity: $H' = -\sum_i p_i \log(p_i)$ where p_i is the relative abundance of the i th band of the profile.

The variability of H among the samples was compared to the variability within eight independent replicates (obtained independently by one of the methods) by ANOVA with a Fisher–Snedecor test. Secondly, the DGGE profiles were further compared by similarity analysis and clusterization. The pair-wise similarity index $d_{i,j}$ between profiles i and j was calculated by Bray–Curtis coefficient, on the basis of both band positions and their relative abundance:

$$d_{i,j} = 1 - \sum_{k=1}^S \frac{|p_{i,k} - p_{j,k}|}{p_{i,k} + p_{j,k}}$$
 where $p_{i,k}$ is the relative abundance of the k th band in the profile i , and $p_{j,k}$ is the relative abundance of the k th band in the profile j .

The initial data matrix (relative intensities according to position) was reduced (division by variance) and transformed by square root to enhance the weight of minor bands. The samples were hierarchically clustered according to their similarity level, using unweighted pair group method with the arithmetic averaging (UPGMA) linking, with Matlab® software (The Mathworks, Natick, MA, USA).

Results

Optimization of cell dispersion

Bacteria counts after dispersion from biofiltration wood-chips are presented in Table 1.

The influence of blending duration on cell dispersion efficiency was assessed. Between 0.5 and 2 min, blending duration did not significantly influence cell recovery. The mean recovery yield was $2.0 \times 10^9 \pm 5.1 \times 10^7$ cells/g DW, which represented a standard deviation of 3% upon the three durations assayed (Table 1). However there was an influence of blending duration on standard deviation: from 2% for 0.5 min up to 18% for 2 min (Table 1). Indeed the longer the blending, the more organic particles were suspended, which seriously increased microscopic counting errors.

The influence of sonication duration on cell dispersion efficiency was assessed. ANOVA revealed that cell recovery improved significantly from $1.3 (\pm 0.1) \times 10^9$ to $2.3 (\pm 0.08) \times 10^9$ cells/g DW when sonication duration increased from 5 to 60 min (Table 1).

Table 1 Influence of treatment duration and glass bead amount on microbial cell number recovered per gram of dry material after three dispersion methods (shaking, blending, and sonication)

N° of cells x10 ⁹ /g DW (SD)							
Shaking with glass beads				Sonication		Blending	
	0 g beads	5 g beads	10 g beads				
1 min	nd	3.1 (0.4)	nd	5 min	1.3 (0.1)	0.5 min	2.0 (0.04)
4.5 min	2.8 (0.06)	nd	2.8 (0.3)	15 min	1.7 (0.2)	1 min	1.9 (0.2)
8 min	nd	2.4 (0.5)	nd	30 min	1.7 (0.08)	2 min	2.0 (0.4)
11.5 min	2.5 (0.2)	nd	2.1 (0.3)	60 min	2.3 (0.08)		
15 min	nd	3.2 (0.2)	nd				

Cells were enumerated by microscopic counts after DAPI staining. Standard deviations calculated on triplicates are shown in parentheses. For shaking procedure, the experimental design was built according to a Doehlert matrix (evaluation of two-factor influence by response surface)

For shaking desorption, the influence of treatment duration and glass bead amount were evaluated according to a Doehlert experimental design (Fig. 1). Cell recovery (Y) was assessed as a function of treatment duration (X_1) and glass bead amount (X_2) according to the following second-order model:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2$$

The model coefficients were determined with NemrodWTM software. Given our experimental enumerations (Table 1), the only significant coefficient was the constant b_0 ($p < 0.01$). Hence cell detachment efficiency was influenced neither by shaking duration nor by glass bead addition. The mean recovery yield was $2.7 \times 10^9 \pm 4.0 \times 10^8$ cells/g DW upon the seven conditions tested (Table 1).

Comparison of dispersion methods

The cell yields recovered by shaking (averaged upon the seven conditions tested), blending (averaged upon the three conditions tested), and sonication (maximum after 60-min treatment) were compared by ANOVA (Fisher–Snedecor test, risk level 0.05). The mean desorption yield obtained by the three methods was $2.3 \times 10^9 \pm 3.4 \times 10^8$ cells/g DW (Table 1), and under the operating conditions tested in this study, there was no significant difference between blending, shaking, and sonication in terms of cell recovery. As a result, all three methods had to be further compared on the basis of more appropriated criteria, relative to DNA recovery. For subsequent experiments, optimal (or easiest) conditions for cell dispersion were chosen as follows: 1 min for blending, 60 min for sonication, and 10 min for shaking, without glass beads.

Evaluating recovery efficiency by DNA yield

Three dispersion treatments (shaking, sonication, and blending) and four extraction methods (two commercial kits

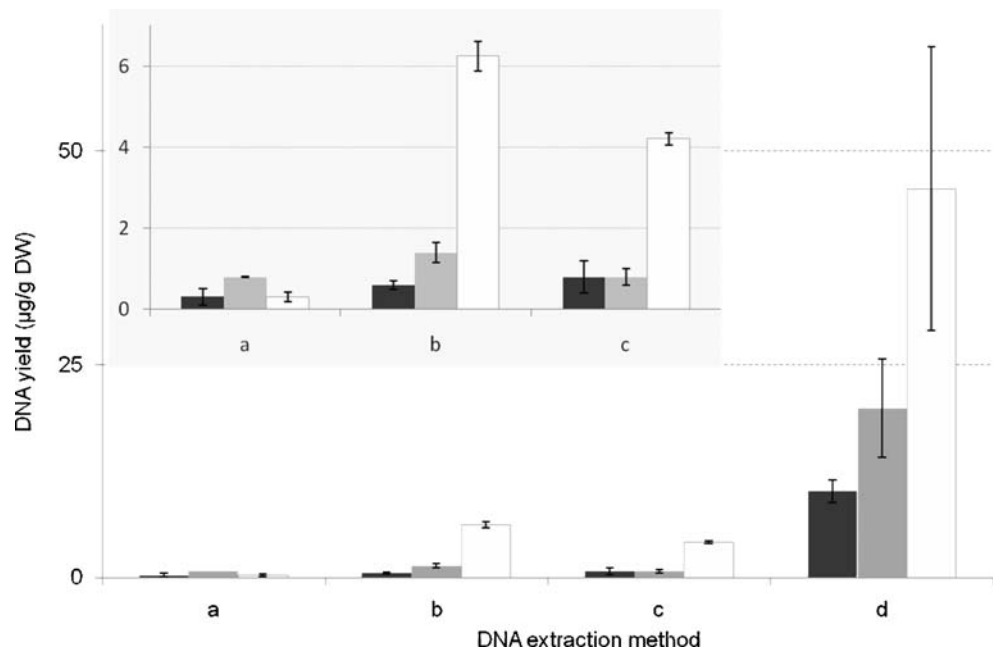
specific for DNA extraction from soil a and b , one universal commercial extraction kit c , and a laboratory extraction protocol d) were combined on duplicated samples. All combinations successfully extracted DNA, which was visible on agarose gels (data not shown). The 12 combinations were first compared on the basis of DNA recovery yield (Fig. 3), separating the effect of dispersion treatment from the effect of extraction procedure on DNA yield.

As a preliminary remark, it is important to note that, given the important variations of cell counts (microscopic counts were made difficult by contamination from the packing material itself), the cell yield was not a statistically sufficient criterion to detect any effect of the desorption method on cell detachment, whereas DNA yield was a finer indicator which enabled to distinguish statistically between dispersion methods.

First the dispersion method had a significant effect on DNA recovery yield, as revealed by ANOVA. Whatever the extraction method was, the lowest DNA yields were always obtained after blending dispersion. They were below 1 $\mu\text{g/g}$ DW when blending was combined to the three commercial kits a , b , and c (0.6 ± 0.3 $\mu\text{g/g}$ DW) and even did not exceed 10.2 ± 1.3 $\mu\text{g/g}$ DW with the reference protocol d (Fig. 3). Blending led to a homogenized suspension where the relative proportion of cells was low compared to the crushed woodchips (dilution effect). Whatever the extraction method was, the highest DNA yields were always obtained when the dispersion step was performed by shaking. The mean DNA amounts reached 4.7 ± 0.9 and 46 ± 16 $\mu\text{g/g}$ DW when shaking was combined with kit extraction and laboratory protocol, respectively (Fig. 3). Vigorous shaking detached efficiently the microorganisms from the carrier, avoiding too much dilution with wood particles. Cell removal by sonication resulted in intermediate DNA yields.

Secondly, the extraction method had also a significant effect on DNA recovery yield, as indicated by ANOVA. The

Fig. 3 DNA yield ($\mu\text{g/g DW}$) obtained by different combinations of desorption and extraction methods, evaluated by A_{260} . Cell dispersion was performed by blending (in *black*), sonication (in *gray*), and shaking (in *white*). DNA extraction was performed by three commercial kits (*a* and *b* were specific for DNA extraction from soil, and *c* was a universal extraction kit) and a laboratory protocol (*d*). Mean concentrations and standard deviations were calculated on duplicates. The *inset* displays magnified data for *a*, *b*, and *c* methods



laboratory protocol *d* adapted from Godon et al. (1997) extracted on average 17 times more DNA than the three commercial kits did, which confirmed their lack of exhaustivity (Fig. 3). The laboratory protocol, in combination with blending, sonication, and shaking, allowed to recover, respectively, 10.2 ± 1.3 , 19.9 ± 5.8 , and 45.6 ± 16.6 μg of DNA per gram of dry material, which represented, respectively, 18, 20, and 13 times more DNA than the kit-extract yields. The commercial kits exhibited different extraction efficiencies. DNA extraction by kit *a* was the least efficient (0.5 ± 0.2 $\mu\text{g DNA/g DW}$ on average), while kits *b* and *c* provided satisfactory DNA amounts when associated to shaking desorption (6.3 ± 0.4 and 4.2 ± 0.2 $\mu\text{g/g DW}$, respectively).

Evaluating recovery efficiency by DNA purity

The effect of desorption method on DNA purity was evaluated. As shown in Table 2, DNA extracted after blending desorption was the most contaminated with proteins, with a mean ratio A_{260}/A_{280} of 1.45 ± 0.18 , probably because of the huge amount of organic material released during blending. DNA purity (averaged upon the four extraction methods) was similar when desorption was performed by shaking or by sonication (ratio A_{260}/A_{280} of 1.61 ± 0.09 and 1.63 ± 0.15 , respectively).

The effect of extraction method on DNA purity was evaluated. As shown in Table 2, the universal kit *c*,

Table 2 DNA purity evaluated by A_{260}/A_{280} ratios, after different combinations of cell dispersion methods (blending, sonication, and shaking) and extraction methods

DNA purity estimated by A_{260}/A_{280} ratio				
	Blending	Sonication	Shaking	Average 3 dispersions
DNA extraction method a	1.66 (0.02)	1.70 (0)	1.59 (0)	1.65 (0.06)
DNA extraction method b	1.51 (0.01)	1.76 (0.02)	1.75 (0.01)	1.67 (0.14)
DNA extraction method c	1.23 (0.05)	1.42 (0.01)	1.59 (0.01)	1.41 (0.18)
DNA extraction method d	1.42 (0.01)	1.65 (0.01)	1.52 (0.1)	1.53 (0.11)
Average a,b,c,d	1.45 (0.18)	1.63 (0.15)	1.61 (0.1)	

Mean values and standard deviation (in parentheses) were calculated on duplicates. Data in bold are the ratios higher than 1.75

a, *b* two commercial kits designed for DNA extraction from soil, *c* universal commercial kit for DNA extraction, *d* extraction method according to a laboratory protocol

immediately followed by the laboratory protocol *d*, was always the least efficient for DNA purification, whatever the dispersion method was (ratio A_{260}/A_{280} of 1.41 ± 0.18 and 1.53 ± 0.11 , respectively, averaged upon the three desorption methods). The commercial kits specifically intended for DNA extraction and purification from soil (*a* and *b*) removed more successfully the protein contamination (ratio A_{260}/A_{280} of 1.65 ± 0.06 and 1.67 ± 0.14 , respectively, averaged upon the three desorption methods). The protocol *b* was particularly convenient as it was the only one to provide a ratio A_{260}/A_{280} exceeding 1.75 when associated to sonication and shaking dispersion.

Effect of DNA recovery method on microbial diversity

The 12 DNA samples corresponding to different desorption and extraction combinations were PCR amplified, targeting the V3 region of 16S rDNA, and further analyzed by DGGE (Fig. 5). To a first approximation, it is commonly assumed that each DGGE band of a profile corresponds to a single operational taxonomic unit (OTU) of the microbial community, while its relative intensity stands for the relative abundance of this population in the total community. This assumes that we are neglecting the possible PCR and DGGE biases, such as co-migration, artificial bands, and preferential amplification (Fromin et al. 2002; Sipos et al. 2007).

The diversity, estimated by the Shannon index, is presented in Fig. 4. The rather high index indicated that the biofilter community was diverse and evenly distributed among the different species (absence of dominant OTU). When considering the whole set of 12 samples, ANOVA revealed a significant effect of the experimental procedure

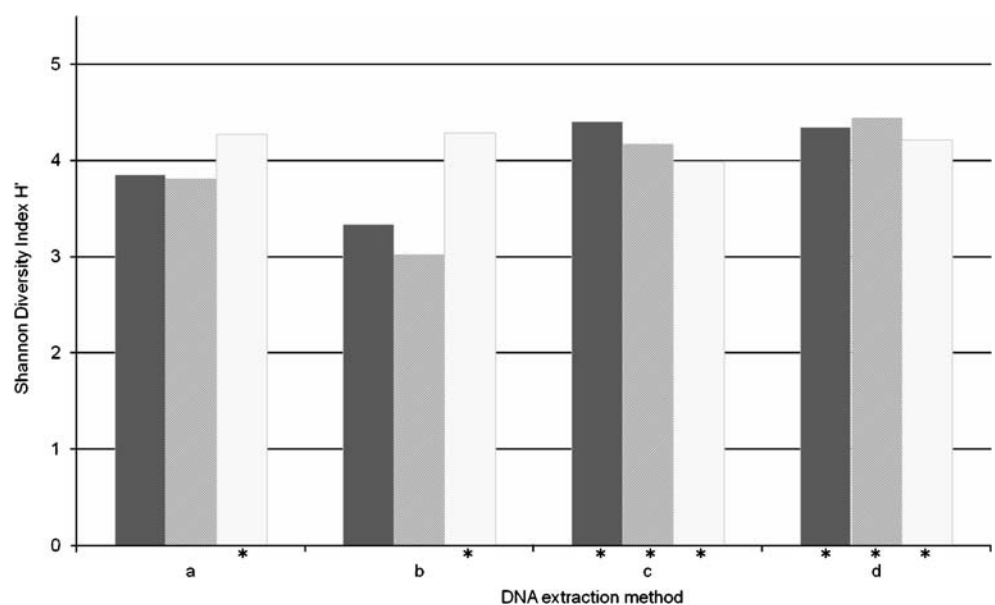
on the estimation of diversity ($p < 0.1\%$), when compared to the replicate pool.

A closer look at the diversity results enabled to separate two groups of methods (Fig. 4). A homogeneous subset of eight samples emerged from statistical analysis, characterized by high diversity and low variability ($H = 4.3 \pm 0.1$). This subset was called high diversity (HD). ANOVA revealed that there was no significant effect of the desorption/extraction method on the measure of diversity for this subset ($p < 0.05\%$). The eight HD samples (marked by an asterisk in Fig. 4) were obtained by extraction kits *a* and *b* associated with shaking desorption, as well as extraction methods *c* and *d* associated with all desorption methods (Fig. 4). The four remaining methods (namely extraction kits *a* and *b* associated to sonication and blending) led to a significantly lower representation of microbial diversity ($H = 3.5 \pm 0.4$): This group was called LD for low diversity.

Effect of DNA recovery method on microbial community structure representation

The DGGE profiles of DNA obtained after the 12 combinations of dispersion and extraction procedures were further compared by multivariate analysis based on similarity analysis and clusterization, and the resulting dendrogram is presented in Fig. 5. In order to validate the methodology, we evaluated the reproducibility of the whole experiment (from sampling until DGGE): The similarity index within the eight-replicate pool reached $81\% \pm 7$ (Bray–Curtis index) or $85\% \pm 8$ (Dice index). This reproducibility was in the same range as previous studies where

Fig. 4 Microbial diversity as a function of both extraction and desorption methods employed to recover DNA. Diversity was evaluated by Shannon index on the basis of DGGE profiles. Cell dispersion was performed by blending (*dark gray*), sonication (*gray*), and shaking (*white*). DNA extraction was performed by three commercial kits (*a* and *b* were specific for DNA extraction from soil, and *c* was a universal extraction kit) and a laboratory protocol (*d*). The samples marked with an asterisk displayed a statistically equivalent diversity index, independent of the desorption/extraction method ($p < 0.05$)



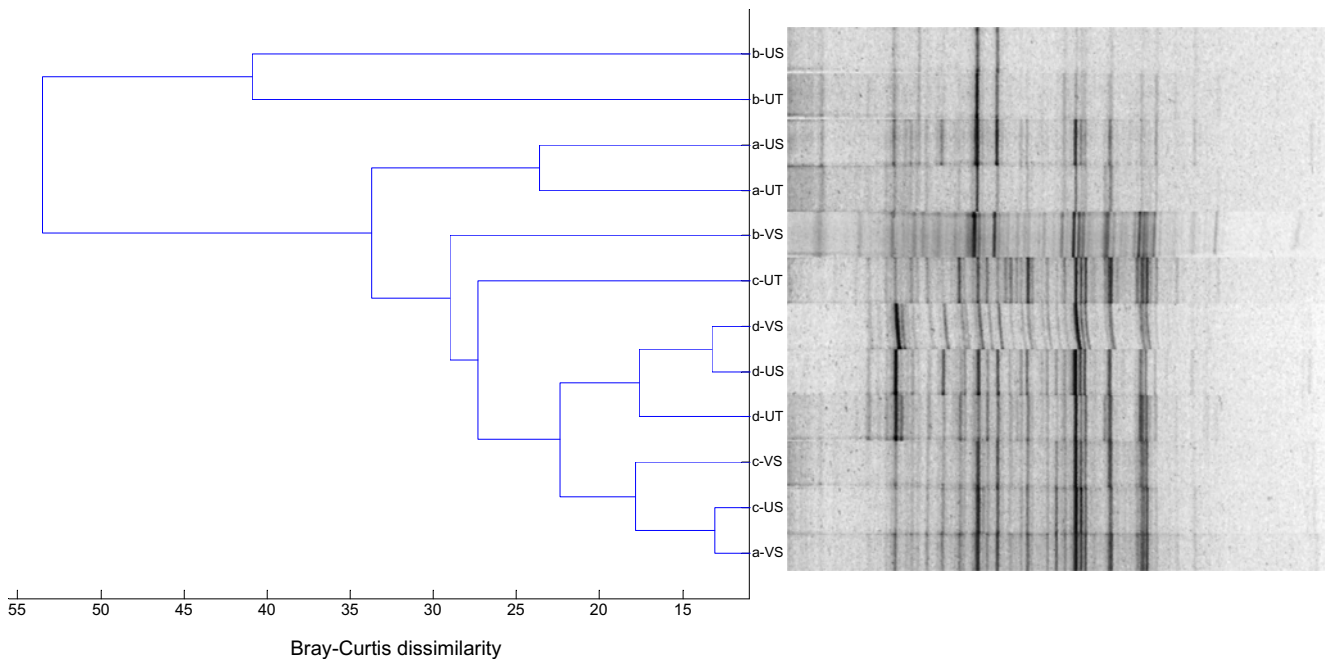


Fig. 5 UPGMA dendrogram constructed from similarity matrix (Bray–Curtis coefficient) representing dissimilarity of DGGE profiles generated from DNA samples obtained by different desorption and extraction methods. Cell dispersion was performed by blending (*UT*),

sonication (*US*), and shaking (*VS*). DNA extraction was performed by three commercial kits (*a* and *b* were specific for DNA extraction from soil, and *c* was a universal extraction kit) and a laboratory protocol (*d*).

duplicate profiles were characterized by high similarity (>85% according to Jaccard coefficient for Carrigg et al. 2007 and even >90% according to Dice coefficient for Kozdrój and van Elsas 2000).

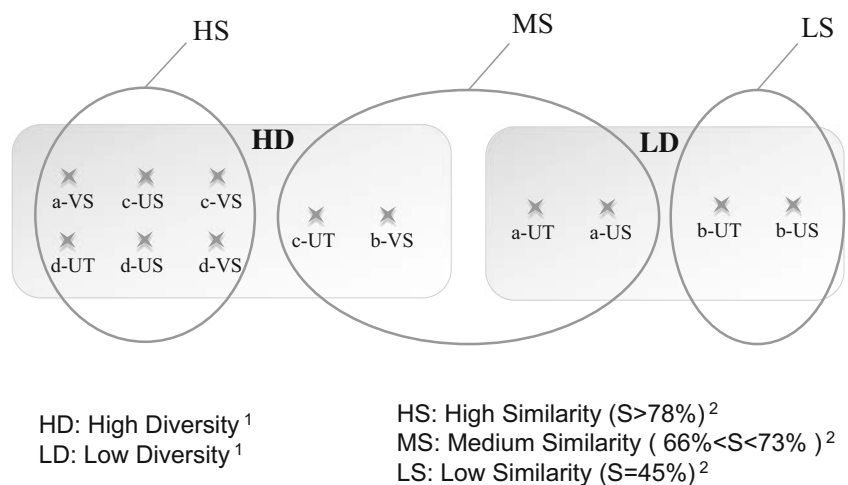
As seen in Fig. 5, the 12 samples could be clustered in three major groups.

A first group of six samples ($\{a\text{-VS}, c\text{-US}, c\text{-VS}\}$ and $\{d\text{-UT}, d\text{-US}, d\text{-VS}\}$, each clustering with more than 82% similarity) constituted a highly similar (78%) group (called HS for high similarity), influenced very little by the desorption/extraction procedure. Within this group, the desorption/

extraction procedure had no more effect on the microbial structure than the inter-replicates variability. It is interesting to note that all samples obtained with the laboratory extraction protocol (highest DNA yields) clustered in this HS group, as summarized in Fig. 6.

A four-sample group (*c-UT*, *b-VS*, *a-UT*, and *a-US*), called MS for medium similarity, was separated from the previous HS group, with similarity values ranging from 66% up to 73%, highlighting a very slight effect of these four treatments on microbial pattern representation, which did not affect dramatically the microbial structure depicted.

Fig. 6 Partition of DNA samples obtained by different dispersion and extraction methods, according to community representation: 1 Diversity was estimated by Shannon index from DGGE profiles of V3 sequence. 2 Similarity was estimated by Bray–Curtis coefficient from DGGE profiles of V3 sequence. The dispersion methods were symbolized by *UT* (blending), *US* (sonication), and *VS* (shaking). The extraction methods were two commercial kits specific for soil (*a* and *b*), one universal commercial kit (*c*), and a laboratory protocol (*d*)



Eventually, two samples (b-UT and b-US) clearly differentiated from the others with only 45% similarity (LS group for low similarity).

The pair-wise similarity approach was more sensitive and revealed deeper insight in community analysis than the sole diversity index, useful for global information. An equivalent diversity was necessary but not sufficient for concluding to high similarity (Fig. 6): Whole HS samples were identified as HD members as well, but the reverse was not true. Despite their equivalent high diversity, two HD samples (b-VS and c-UT) dissociated from the previous ones by slight dissimilarities in band positions and relative intensities: The diversity estimation is not sufficient to assess the impact of extraction procedure on community structure representation (Fig. 6). The least similar samples (b-UT and b-US) were also characterized by the lowest diversity (LD). However, in two cases, the low diversity was not correlated to a strong difference in community structure profile (a-UT and a-US clustered with the MS group), so we can presume that the univariate approach had overestimated the differences between profiles, probably due to minor populations.

Discussion

Cell dispersion

Operating conditions for cell dispersion by blending, shaking, and sonication of microbial communities growing on wood chips in a gas biofilter were optimized. Blending efficiency was not influenced by treatment duration, as already reported (Courtois et al. 2001; Khammar et al. 2004). The positive influence of treatment duration on cell dispersion by sonication was in accordance with a previous work on peat and activated carbon where more bacteria were released after 60-min sonication than after 0.5 min (Khammar et al. 2004). Eventually, in the same study, glass beads and treatment duration had no significant effect on cell detachment by shaking (Khammar et al. 2004), as demonstrated again in the present work, despite the difference of material and the difference of target population (CFU Vs total bacteria).

Cell dispersion efficiency was compared after blending, shaking, and sonication. The three methods released statistically the same amount of microorganisms from the woodchips, which was in accordance with previous results on a sandy soil (Ramsay 1984). Shaking and sonication were also equivalent for biomass removal from peat and activated carbon biofilters (Khammar et al. 2004). Blending was not more efficient for cell release in the present case, unlike a common result in environmental samples (Gabor et al. 2003; Khammar et al. 2004; Lindahl and Bakken 1995).

The mean cell yield obtained from the biofiltration woodchips used in this study was comparable to desorption yields reported in the literature for other organic matrix with various detachment methods, ranging from 3.5×10^7 cells/g DW (Bertrand et al. 2005, on soil samples; Gabor et al. 2003, on marine sludge) to 1.0×10^{11} cells/g DW (Böckelmann et al. 2003, on soil samples). However, these cell counts should not be considered as exhaustive, as cell desorption has been reported to miss a large proportion of microorganisms (usually more than 50%, assessed for example by Gabor et al. 2003), which remain attached to organic particles and trapped inside microaggregates (Stefan et al. 1988).

In the context of gas biofiltration, apart from the study of Khammar et al. (2004) who evaluated cell desorption efficiency by CFU counts only, very few studies assessed the efficiency of the desorption protocol. In rare cases, cell suspension was verified microscopically: Friedrich et al. (1999) and von Keitz et al. (1999) obtained between 4.9×10^9 and 7.8×10^{10} cells/g DW from crushed tree root biofilters.

Evaluation of DNA recovery efficiency

The effect of both dispersion and extraction procedures on DNA yield obtained after different combinations of dispersion and extraction methods was evaluated. First, blending dispersion was clearly not suitable for high yield DNA recovery, while shaking dispersion proved to be more appropriated to the pine bark woodchips assayed in this study. Main studies referred to bead-beating as the most efficient lysis technique (Lakay et al. 2007; Moré et al. 1994), but the present work revealed that this mechanical step was not sufficient to achieve high DNA yields.

Secondly, we highlighted the better efficiency of laboratory protocols compared to commercial kits, as reported by several studies (from two to 16 times higher DNA yields) on different kinds of samples: soils of various composition (Carrigg et al. 2007; Martin-Laurent et al. 2001; Stach et al. 2001), food (Abriouel et al. 2006), sediment, and activated sludge (Roh et al. 2006). It was not verified however on samples with very low biomass content, such as of deep sub-seafloor biosphere (Webster et al. 2003). The lower DNA yields obtained with commercial kits could be explained by poor lysis efficiency, DNA loss during purification, or DNA degradation by remaining nuclease activity. In addition, the higher DNA yields obtained with the laboratory protocol could be attributed to the combination of several lysis techniques (mechanical, chemical, and enzymatic means), which allowed to target larger microbial spectra. Indeed, various studies attributed the DNA extraction success to the lysis step and highlighted that the combination of different successive disruption techniques increased the DNA recovery, compared

to a sole technique (Carrigg et al. 2007; Frostegård et al. 1999; Krsek and Wellington 1999).

Comparing commercial kits and laboratory protocol was not so obvious when considering DNA contamination by proteins. Our observations (see “Results”) confirmed that, according to the samples and to the procedures carried out, commercial kits can be more efficient for DNA purification than laboratory protocols (Martin-Laurent et al. 2001; Stach et al. 2001; Webster et al. 2003), or less efficient (Jacobsen and Rasmussen 1992; Krsek and Wellington 1999; Schneegurt et al. 2003).

The DNA yields reported in the literature are extremely variable. As it has been extensively described, they depend on the extraction method (Martin-Laurent et al. 2001) as well as on the environmental matrix: its physicochemical properties (Carrigg et al. 2007; de Liphay et al. 2004), its organic content (Zhou et al. 1996), and the microbial community which colonized it. The yields obtained in the present study were globally in accordance with previous findings on environmental samples, which for example were ranging from 0.2 to 21 μg DNA/g soil (Kuske et al. 1998), inferior to 2 μg DNA/g soil (Martin-Laurent et al. 2001), from 3 to 50 μg DNA/g soil (Stach et al. 2001), or even ranging from 30 to 90 μg DNA/g DW for compost samples of high organic content (LaMontagne et al. 2002). In the context of gas biofiltration, none optimization study was carried out to date, to our knowledge, and a sole study reported DNA extraction yields from 20 to 60 ng of DNA per gram of inorganic material (i.e., perlite; Sakano and Kerkhof 1998).

Evaluation of DNA quality

The DNA extracts obtained by different combinations of dispersion and extraction procedures were also compared on the basis of quality criteria. First, DNA extracts observed on agarose gels displayed smear indicating DNA shearing. Secondly, DNA extracts were probably contaminated by humic substances, as suggested by A_{260}/A_{230} ratios lower than 2. Despite these two low-quality indicators, the PCR amplification was successfully achieved for all samples (always yielding more than 12.5 ng of V3 amplicon per nanogram of template DNA). Several authors verified that DNA shearing did not prevent PCR amplification, especially when a short template was targeted (Bürgmann et al. 2001; Kuske et al. 1998; Miller et al. 1999; Picard et al. 1992). On soil DNA extracts, the PCR success or failure seemed to be more influenced by protein contamination than by humic contamination (Krsek and Wellington 1999). PCR amplification could be successfully achieved even though DNA exhibited low purity ratios (LaMontagne et al. 2002), and conversely some enzymatic activities were inhibited

despite high A_{260}/A_{280} and A_{260}/A_{230} ratios (LaMontagne et al. 2002; Steffan et al. 1988).

Impact of extraction procedure on community structure representation

The DNA extracts obtained by different combinations of dispersion and extraction procedures were compared after 16S amplification and DGGE, on the basis of band positions and relative abundances. A summary of results is presented in Fig. 6. From the univariate analysis of Shannon index, two sample groups were separated, according to the dispersion/extraction procedure implemented to generate the DGGE profile: a highly diverse group of eight samples (HD) and a low diversity group of four samples (LD). The effect of extraction procedure on diversity was ambiguous: It existed when considering the whole set of 12 samples, but it disappeared when focusing on the HD subset. The influence of DNA extraction procedure on diversity measurement had been reported by others on various environmental samples (Carrigg et al. 2007; de Liphay et al. 2004; Gabor et al. 2003), with bead-beating methods often resolving higher diversity (Carrigg et al. 2007; de Liphay et al. 2004). On the contrary, previous studies also highlighted that there was no influence of extraction procedure on diversity (Abriouel et al. 2006; LaMontagne et al. 2002).

The low diversity was correlated with poor extraction efficiency, as the four LD samples had among the worst DNA yields (Figs. 3 and 4). There was no link between low diversity and DNA purity (Table 2 and Fig. 4), in contrast to a previous study on compost samples (LaMontagne et al. 2002). As we could assume, the methods providing the highest DNA yields (i.e., all combinations with laboratory protocol *d*, see Fig. 3) were part of the HD group. However surprisingly they did not provide a significantly higher diversity than the rest of the HD group, characterized by lower DNA yields (Figs. 3 and 4). In other words, several methods poorly efficient in terms of DNA yield (e.g., a-VS, c-UT,...) gave rise all the same to the largest diversity, as frequently reported (de Liphay et al. 2004; Gabor et al. 2003; Kozdrój and van Elsas 2000; LaMontagne et al. 2002; Stach et al. 2001). This could be attributed to a DNA yield threshold: below it, the extraction was not exhaustive enough to resolve all the diversity; above it, the diversity ceased to increase with DNA yield because (a) no new OTU representant was extracted or (b) the DGGE sensitivity limit was reached (Fromin et al. 2002).

The multivariate analysis of similarity index distinguished three sample groups, according to the dispersion/extraction procedure: a highly similar group of six samples (HS), a medium similar group of four samples (MS), and a low similarity group of two samples (LS).

The similarity of community profiles depicted by different extraction methods had been reported by others (Courtois et al. 2001; LaMontagne et al. 2002). For Kozdrój and van Elsas (2000), the similarity of DGGE profiles obtained after four different extraction methods reached 82% according to Dice index. On the contrary, previous studies also reported that the choice of DNA extraction protocol gave rise to significantly distinct representations of microbial structure, affecting the presence and absence of bands as well as their relative abundance (Bertrand et al. 2005; Bürgmann et al. 2001; de Liphay et al. 2004; Krsek and Wellington 1999; Peng et al. 2007). The impact of DNA recovery method on the outcome of ecological representation was emphasized by low similarity values of DGGE profiles generated after different extraction procedures, from 65% (Carrigg et al. 2007, according to Jaccard coefficient) to 20% (Gabor et al. 2003, according to Dice coefficient).

One should be careful when investigating the impact of DNA extraction on diversity and community structure representation from literature data because it is hugely dependent on the sample types (e.g., Abriouel et al. 2006), and also on the observation level (genus vs species, whole community vs specific population) for the generation of community patterns (Martin-Laurent et al. 2001; Stach et al. 2001). The profiles are or are not affected according to the level considered.

How to choose the most appropriated combination of dispersion and extraction methods

The objective of this study was to implement a reliable method for the recovery of DNA from complex samples (i.e., woodchips involved in gas biofiltration and colonized with uncharacterized biomass) in order to use this genetic material for further molecular analyses with as few bias as possible. An experimental strategy was proposed to compare different combinations of cell desorption and DNA-extraction methods on the basis of several criteria: DNA yield, DNA purity, subsequent PCR feasibility, and diversity representation.

This study did not aim to provide a single general protocol for optimal DNA recovery from biofiltration packing material, but presented a systemic guideline to compare desorption and extraction methods, assessing their impact on quantity, quality, and representativity of DNA samples. Cell dispersion by shaking seemed the most appropriated to our pine bark woodchips. Except the laboratory protocol (which is too time consuming for routine analysis of numerous samples), the combination of shaking desorption and DNA extraction with kit *b* provided the best efficiency in terms of both DNA yield and purity, as well as high diversity. This is interesting because in

many cases, when the purity is improved, the yield decreases, and vice versa, so a compromise shall be found between quantity and quality exigencies. The DGGE pattern generated after the combination of shaking and kit *b* was rather similar to most of the other patterns.

This study showed that DNA extraction from a complex sample could not grant an exhaustive view of the microbial community. It highlighted the crucial importance which must be given to the evaluation of bias linked to DNA extraction when microbial ecology concerns are questioned in biofiltration ecosystems. This integrated comparative approach should be applied to each new sample, as DNA recovery is hugely influenced by the matrix specificities. The extraction step is only one of the numerous possible bias occurring during molecular analysis, from the sampling itself up to the PCR amplification or the fingerprinting analysis (Fromin et al. 2002; Sipos et al. 2007).

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