Development of a reliable extraction method for the recovery of total genomic DNA from woodchip colonizing biofilm involved in gas biofiltration

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ABSTRACT

This preliminary study focused on a critical step for the characterization of microbial ecosystem involved in biofiltration. Two aspects of nucleic acid recovery were explored: (i) cell dispersion (three methods tested) and (ii) total DNA extraction (four methods tested). The objective is to select the optimal combination of desorption/extraction methods, allowing subsequent molecular investigations to be reliable. Three relevant criteria are used to assess extraction efficiency: DNA amount and purity, and subsequent amplification feasibility.

1 INTRODUCTION

During the past two decades, most studies concerning biofiltration concentrated on two main objectives: (i) assessment of operating parameter impact (e.g. packing material impact, pH effect: Kim *et al*., 2000; Prado *et al*., 2006); and (ii) definition of the system limits under different loading conditions (Aizpuru *et al*., 2001; Vergara-Fernandez *et al*., 2007). Both problematic were based on elimination performance evaluation, without regard for intrinsic biological phenomena. It was noticeable that a

number of studies tended to consider the system according to a «black-box» approach, while in a biofilter the pollutant removal is mainly due to the microbial component, whose structure and activity still remain unclear or even unknown.

Microbial ecology aims to characterize microbial communities by their structure (i.e. their diversity, stability, spatial and temporal dynamics, occurrence of specific groups), as well as their interactions with the environment. Along the last decade, staggering progress in molecular biotechnologies offered powerful tools which made possible the fine characterization of microbial communities, granting access to uncultivable microorganisms. Among these molecular tools are quantitative PCR, fingerprintings, clone libraries sequencing, and, more recently, metagenome shotgun sequencing. They have been applied in various ecosystems, such as soil (Patra *et al*., 2006), sea sediments (Venter *et al*., 2004), anaerobic sludge (Godon *et al*., 1997), wastewater treatment biofilters (Ahn *et al*., 2004). More and more studies are carried out to elucidate community structures in gas biofilters. To date, several molecular tools have been used to gain insight into the dynamic diversity of bacterial communities in biofilters: ARISA (Steele *et al*., 2005), SSCP (Khammar *et al*., 2005), RFLP (Khammar *et al*., 2005), *amoA* gene PCR, cloning and sequencing (Sakano and Kerkhof, 1998), DGGE on 16S rDNA (Sercu *et al*., 2005; Cai *et al*., 2006; Li and Moe, 2004; Shim *et al*., 2006; Chung, 2007), DGGE on 16S rRNA (Sercu *et al*., 2006), FISH (Friedrich *et al*., 1999; Friedrich *et al*., 2003). Adopting a microbial ecology approach is of prime interest to reach a better understanding of biological mechanisms occurring within a biofilter. This better understanding may help to control, stabilize and optimize the biological process.

Biofilms involved in biofilters are constituted of a complex and uncharacterized microflora attached to the packing material. Thus an essential preliminary task for the investigation of microbial communities with molecular tools is to implement and optimize a methodology for the recovery of 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 ecosystem studies, such as soil, a lot of work was carried out to compare and implement DNA recovery methods that exhibit an unbiased sampling of the investigated community (Robe *et al*., 2003). However, within biofiltration context, very little attention was paid to the methodological aspects of nucleic acids recovery, despite their crucial importance in final results significance (Khammar *et al*., 2004; Li and Moe, 2004). DNA recovery methods are very heterogeneous and have neither been standardized nor optimized to date. Indeed, they are hugely dependent on the packing material specificities (size, organic/inorganic nature, density, hardness, porosity).

In this preliminary study we explored methodological aspects of the nucleic acids recovery from microbial communities involved in a laboratory scale biofilter

filled with pine bark woodchips. Considering the packing material used in this work, direct DNA extraction could not be applied. Hence two successive steps had to be performed: cell desorption (crushing, shaking, sonication) and DNA extraction (three commercial kits –two of which being specific for soil– and a reference protocol). The objectives of the present work were (i) to optimize cell desorption from the packing material, and (ii) to select the optimal combination of desorption and extraction methods. To assess DNA recovery efficiency, importance was attached to three relevant criteria: extracted DNA amount and purity, as well as subsequent amplification yield (this latter data will only be presented orally).

2 MATERIALS AND METHODS

2.1 First step: Optimization of cell desorption

2.1.1 Biofilter setup

Experiments were conducted on the biomass which colonized a lab-scale biofilter (1 m height, 125 mm diameter) filled with pine bark woodchips (initial porosity of 37%) and treating an H_2S stream (10 mg_{H2S}/m^3; 500 m/h). The packing material was kept at constant humidity by regularly spraying a salt mineral nutrient solution, whose composition was previously described (Lalanne *et al*., 2007), at a rate of 150 mL every six hours. The biofilter was run at ambient temperature.

2.1.2 Desorption methods and operating conditions for optimization

Three commonly used detachment methods were investigated for microorganism removal from woodchip support: blending (performed by an Ultra-Turrax -T25 basic, Ika); shaking (performed by a Vibro-Shaker –Retsch MM200); sonication (performed by an ultrasonic bath -Branson sonifier bath, Energy). One or two parameters were retained as potentially influent to define optimal conditions for each method, as shown in Fig.1. Other parameters (revolution speed, rotating frequency) were maximal.

Initial sample *(44 *5 g)*

S_1 Shaking *(23 * 5 g)*

S_2 Sonication *(12*5g)*

Duration (min)	Samples
5	3 S_{21}
15	3 S_{22}
30	3 S_{23}
60	3 S_{24}

S_3 Blending *(9*5g)*

Duration (min)	Samples
0.5	3 S_{31}
1	3 S_{32}
2	3 S_{33}

Duration (min)	Glass beads (g)	Samples
1	5	3 S_{11}
4.5	0	3 S_{12}
4.5	10	3 S_{13}
8	5	5 S_{14}
11.5	0	3 S_{15}
11.5	10	3 S_{16}
15	5	3 S_{17}

Figure 1. Desorption methods and optimization conditions.

Sonication duration range was chosen from previous results (Khammar *et al*., 2004), which demonstrated that ultrasonic treatment needed higher duration to suspend microorganisms. On the contrary, blending treatment did not need more than two minutes for a complete homogenization of the suspension.

2.1.3 Sampling scheme

At the time of sampling, the biofilter had reached a steady state, with complete H_2S removal. Each treatment was carried out in triplicate, on woodchips extracted from the biofilter at the same time and at the same location (about 0.5 m high). Sampling procedure is shown in Fig. 1. Each sample was constituted of 5 g of woodchips, suspended in 15 mL of sterile physiological serum (NaCl, 9 g/L). Aliquots of 1 mL of the liquid suspension were collected and enumerated.

2.1.4 Microscopic direct counts

Total bacteria were enumerated by fluorescence microscopy using DAPI staining (Sigma, USA). Whole experiment is done in sterile conditions. After serial dilutions, samples were stained with DAPI at a final concentration of 20 µg/mL during 30 minutes in the dark in a shaker (200 rpm). Stained bacteria were collected on 0.2 µm polycarbonate membrane filters (Millipore GTBP, Ireland) by vacuum microfiltration. Filters were mounted on microscope slides in Mounting Medium (Sigma, USA) and observed with an epifluorescence microscope (Leica DMLB) equipped with a blue excitation filter (BP 340-380 nm) and a barrier filter LP 425. Thirty microscopic fields per slide were enumerated.

2.2 Second step: combination of cell desorption and DNA extraction

2.2.1 Biofilter setup

Experiments were conducted on the same pilot-scale unit, but the biofilter was treating a VOC mixture made of acetaldehyde, acetone, butanal, MEK, DMDS, butanoic acid, isovaleric acid. At the time of biomass sampling, operating conditions were as follows: 10 mg/m^3 for each compound; gas velocity at 100 m/h.

2.2.2 Experimental procedure

The experiment involved 12 samples, each constituted of 5 g of woodchips suspended in 15 mL of sterile physiological serum (NaCl, 9 g/L). Each sample was repeated twice. As shown in Figure 2, each sample was submitted to one of the three desorption methods, under previously optimized conditions (described in section 3.1.). After centrifugation of liquid phase at 10 000 rpm for 10 minutes, the pellet was subjected to DNA extraction, using one of the four following methods: I. PowerSoil DNA Kit, MoBio (Ozyme, France); II. FastDNA® SPIN Kit for Soil, Qbiogene (MP Biomedicals, France); III. NucleoSpin® Tissue Kit (Macherey –Nagel, France); IV. An extraction protocol adapted from Godon *et al*. (1997).

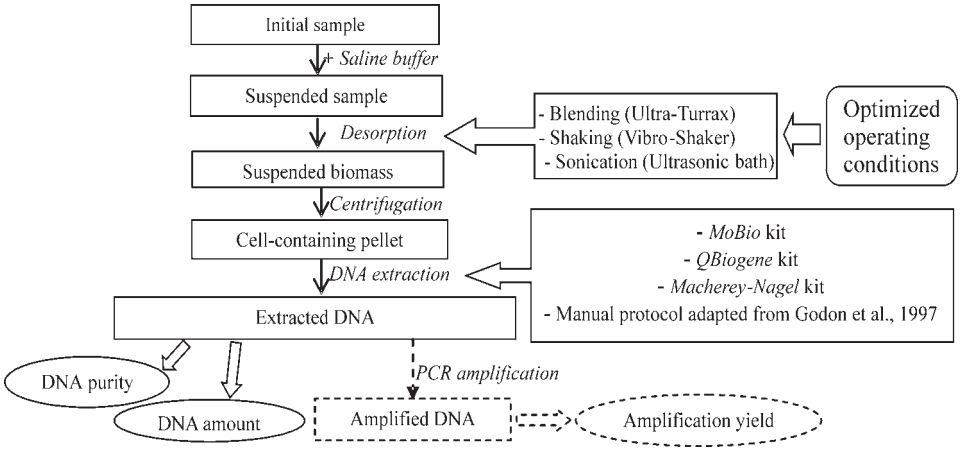

Figure 2. Schematic procedure to assess the optimal combination of desorption and extraction methods (in dotted lines: experiments in prospect).

2.2.3 Extraction and purification of total genomic DNA

Extraction by commercial kits was performed according to the manufacturers' instructions, using a Vibro Shaker Retsch for cell disruption. In all cases, elution volumes were 50 µL.

The fourth method was slightly modified from the one described by Godon *et al*. (1997), as follows, to ensure the largest sample size. The microbial cell fraction-containing pellet obtained after desorption and centrifugation was resuspended in 385 µL of 4M guanidine thiocyanate-0.1 M Tris (pH 7.5) and 115 µL of 10%-N-lauroyl sarcosine. Samples were stocked at -20°C. After the addition of 500 µL of 5% -N-lauroyl sarcosine-0.1 M 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) was added and the sample was shaken at maximum speed (30 Hz) for 10 min in a Vibro Shaker (Retsch MM200). Polyvinylpolypyrrolindone (PVPP, 15 mg) was added. The sample was vortexed and centrifuged for 3 min at 12 000 rpm. The supernatant was recovered. The pellet was washed with 500 µL of TENP (50 mM Tris [pH 8.0], 20 mM EDTA [pH 8.0], 100 mM NaCl, 1 % PVPP) and centrifuged for 3 min at 12 000 rpm. The new supernatant was pooled with the first one. The washing step was repeated three times. The pooled supernatants were centrifuged for 3 min at 12 000 rpm to remove particles, and then split into two 2-mL tubes.

Samples were incubated for 1h30 at 56 °C with 30 µL of proteinase K (20 mg/mL), and then incubated 1 h more at 37°C with 20 µL of RNase A (10 mg/mL). Samples were split into 500 µL subsamples and crude DNA was purified by addition of 1 mL of phenol-chloroforme-isoamyl alcohol (25:24:1). After centrifugation for 5

min at 10 000 rpm, the upper phase was recovered. Nucleic acids were precipitated by the addition of 50 µL of sodium acetate 3M and 1 mL of cold absolute ethanol. Samples were incubated for 15 min at -80°C and 30 min at -20°C. After centrifugation for 30 min at 14 000 rpm, supernatant was discarded and DNA pellet was washed with about 1 mL of cold 70% ethanol, dried for 10 min at room temperature and resuspended in 50 µL of Tris-EDTA 0.1X.

2.2.3 Evaluation of DNA recovery efficiency

Sizing and quantification of extracted DNA were assessed by electrophoresis. 5 µL of extraction product were loaded in 1% agarose gel. Migration was performed at 85 V, for 45 min, in 1X TAE buffer and gel was stained with ethydium bromide. DNA amount was further determined by absorbance at 260 nm using an UV spectrophotometer (Biophotometer, Eppendorf). DNA concentration was calculated considering that 1 UDO corresponds to a double-strand DNA concentration of 50 µg/mL, in 1 cm cuvettes. DNA purity was determined by the ratio of absorbance at 260 nm and absorbance at 280 nm (Biophotometer, Eppendorf), considering that the absorbance at 280 nm is mainly due to protein contamination.

3 RESULTS AND DISCUSSION

3.1 Optimization of cell desorption

Bacterial counts after desorption are presented in Figure 3. When a single parameter was variable (treatment duration), results were statistically analyzed by ANOVA (analysis of variance). After verifying variance homoscedasticity with a Hartley test, the significance of differences between means was established by the Fisher-Snedecor test with a risk level of 0.05. It appeared that for blending desorption (Figure 3.A), treatment duration between 0.5 and 2 minutes did not influence the amount of recovered cells.

Nevertheless, increasing blending duration led to more deviation. Indeed the longer was the blending, the more organic particles were suspended, which seriously hampered microscopic counting, thus leading to higher experimental errors. On the contrary, cell counts obtained after different sonication durations were not statistically equal (Fig. 3.B): cell removal was significantly improved by increasing sonication duration up to 60 minutes. Concerning shaking desorption, a Doehlert matrix was built. In the model provided by NemrodW analysis, the most significant coefficient is the constant one, i.e. the coefficient linked to none experimental factor (data not shown). Hence it can be concluded that neither shaking duration nor shearing force increase (by adding glass beads) improved shaking efficiency. These results are in accordance with those of Khammar *et al*. (2004), which detected no significant effect of glass

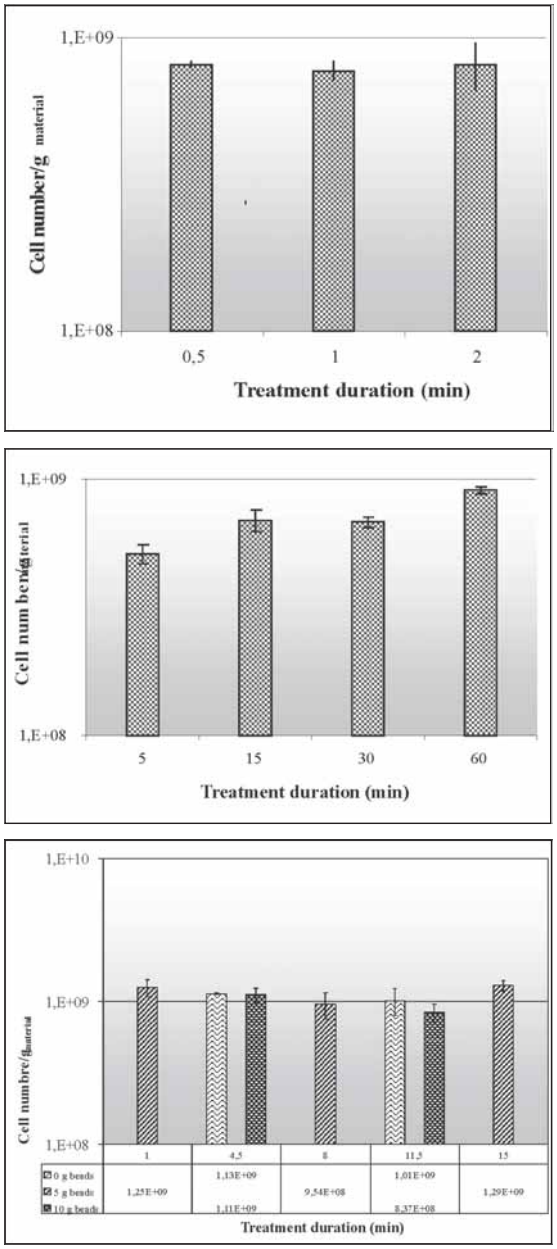

Figure 3. Influence of treatment duration and glass bead amount on total microbial cells recovered after desorption and enumerated by DAPI (*A: blending by Ultra-Turrax; B: Sonication; C: Vibro-Shaking*). Graphs are in logarithmic scale and error bars represent standard deviation calculated on triplicates.

beads and treatment duration on cell detachment from peat and activated carbon by blending and shaking and highlighted treatment duration effect for sonication.

As a result, optimal conditions for desorption treatments are chosen as follows: 1 min for blending; 60 min for sonication; 10 min without glass beads for shaking.

Blending could have been thought to be the most efficient detachment method, as it allowed recovery and homogenization of the initial material in its entirety (no biomass was lost), while sonication and shaking only suspended microorganisms (a fraction of biomass left on the support may be lost). This was observed by Khammar *et al*. (2004). Nevertheless, in this previous study, biomass detachment was only evaluated by the number of viable and cultivable microorganisms. As enumeration results were in the same range whatever was the desorption method, whole three methods had to be further compared, on the basis of more accurate criteria.

3.2. Optimal combination of cell desorption and DNA extraction methods

Electrophoresis results are shown in Fig. 4. Only one sample is presented for each duplicate (except for *MoBio* extraction method, where results were not reproducible).

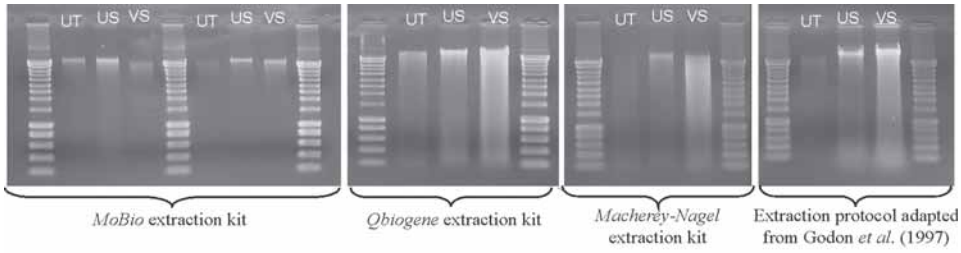

Figure 4. Electrophoresis of DNA extracted by four different methods, after three different desorption treatments *(UT: UltraTurrax; US: UltraSonication; VS: VibroShaking).*

Conclusions drawn from band intensity observations were confirmed and completed by absorbance measures, as shown in Fig. 5. It is important to note the bad reproducibility of the results between duplicates. This would be explained by the random aspect of bacterial colonization on the initial 5g-sampling.

It is obvious that Godon-adapted extraction protocol led to significantly higher DNA amounts, when compared to commercial kit extraction (about 15 times higher on average). This is observed independently of the previous desorption methods used. But this protocol being highly time-consuming, it is not realistic to envisage its use for routine DNA extraction of numerous samples. It is thus considered as a reference protocol.

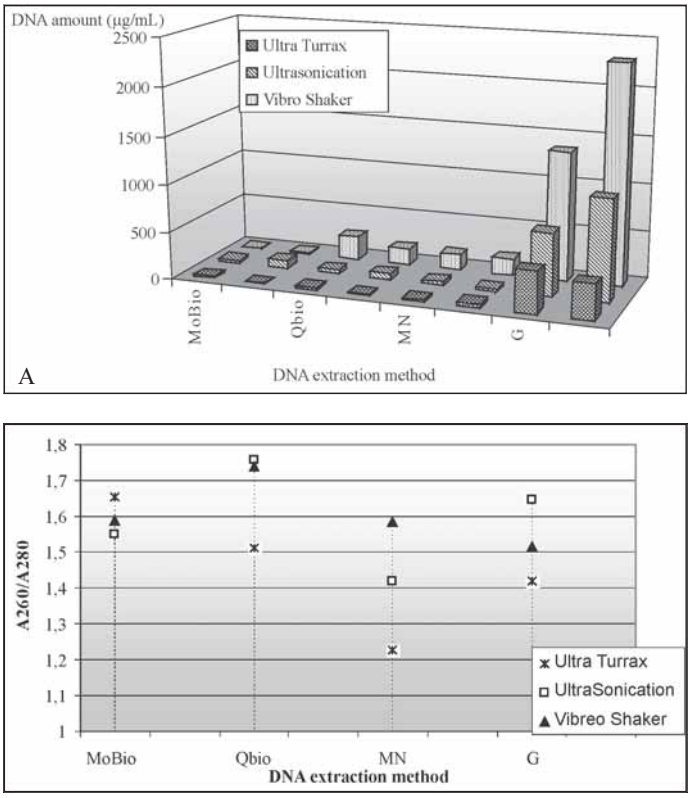

Figure 5. DNA concentration and purity after different methods of desorption and extraction, evaluated by A_{260} and ratio A_{260}/A_{280}, respectively (concentrations shown for both duplicates).

- It should be noted that, whatever the extraction method was, desorption treatment by *Ultra Turrax* was clearly not suitable for high DNA extraction yield. DNA concentration after kit extraction was about 20 µg/mL and did not exceed 400 µg/mL with reference extraction protocol. Indeed, *Ultra Turrax* treatment led to a single-phase suspension where the whole initial material was homogenized. Therefore, in the pellet obtained after centrifugation, the relative proportion of cells was very low compared to the proportion of crushed wooden material. Moreover, blending detachment gave the worst results in terms of DNA purity (Fig. 5.B), probably because of the high amount of organic material in blended samples.
  In all cases, DNA recovery was higher when previous desorption was performed by *Vibro Shaker*. After shaking detachment, DNA concentration was about 130 µg/mL when kit-extracted and even reached 2000 µg/mL

with reference protocol. Cell removal by sonication gave intermediate results in terms of DNA recovery.

- DNA extraction by *MoBio* kit was the least efficient for DNA recovery (<30 µg/mL). In contrast, DNA extraction by both *Qbiogene* and *Macherey-Nagel* kits provided satisfactory DNA amounts, especially after biomass removal by shaking (about 200 µg/mL).
- Considering DNA purity, the worst results were obtained with the universal kit (Macherey-Nagel), followed by reference extraction protocol. Better DNA purity was gained with the two commercial kits specifically designed for DNA extraction and purification from soil samples. These kits (MoBio and Qbiogene) aimed to remove DNA contaminating organic substances, which seemed to result in improved DNA purity (Fig. 5.B). DNA extraction by *Qbiogene* kit was even more interesting as it was the only case where the ratio A_{260}/A_{280} exceeded 1.75.

To conclude, the experimental strategy implemented in this study allowed comparison and selection of a reliable combination of cell-desorption and DNA-extraction methods, considering both DNA amount and DNA purity as decisive criteria (Fig. 6). As a result, the most appropriate methodology seems to be a desorption step with Vibro-Shaking, followed by an extraction step with Qbiogene kit.

	MoBio kit	Qbiogene kit	Macherey-Nagel kit	Reference protocol
Blending (Ultra-Turrax)	- ; ✶	- ; -	- ; -	✚ ; -
Sonication	- ; -	- ; ✶ ✶	- ; -	✚✚ ; ✶
Shaking (Vibro-Shaker)	- ; -	✚ ; ✶ ✶	✚ ; -	✚✚ ; -

Figure 6. Summary of different combinations efficiency, basing on recovered DNA quantity (symbolized by ✚) and purity (designed by ✶).

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