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Comparison of Total DNA Extraction Methods for Microbial Community form Polluted Soil

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Abstract

DNA isolation represents the basic and probably the most important step in molecular biology for microbial strains, and even more, for microbial community analyses. Despite the development of molecular protocols for DNA microbial community isolation, there are still many drawbacks dependent of samples composition, and even the commercially available genomic isolation kits have significant limitations in recovering high genomic DNA amounts, especially from soil samples. Our study is aiming to compare and optimize a total microbial community DNA isolation protocol from polluted soil samples, estimating the amount and the purity of genomic DNA per g of soil, versus time requirements for each protocol, taking under consideration that our soil samples have a high content of humic acids. We checked several protocols for total DNA extraction, CTAB based, including a specific Kit for Soil DNA Isolation Kit NorgenBiotek. We estimated the time needed for each protocol, the amount of the DNA per gram of polluted soil, proteins and RNA contamination grade, by spectrophotometric analysis, but also the grade of PCR amplification inhibition. The most efficient method for our soil samples with high content of humic acid, suitable for further molecular analyses was the total DNA microbial community sample retrieved from Sagova et al. (2008) based protocol, with several adjustments. This protocol will be valuable for molecular analysis on microbial community profiling from environmental samples, especially from polluted soils.

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Keywords: DNA isolation; microbial community; soil; pollution

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1. Introduction

Soil microorganisms represent an important part of the Earth's biodiversity and have a critical role in biogeochemistry cycles as well as ecosystem functioning (Petric et al., 2011; Fornasier et al., 2014**).** Moreover, many human activities, such as industrial development, agriculture, pollution, and the use of chemical compounds, affect soil microbial communities (Maron et al., 2011**)**. Therefore, assessing and understanding soil microbial diversity and function is fundamental for environmental management and for evaluating soil quality. In this regard it is very important to apply appropriate molecular techniques protocols for studying soil microbial communities. Majority of these molecular techniques start with extraction and purification of nucleic acids methods, as a key point of most microbial ecology studies (Lakay et al., 2007).

DNA isolation from soil represents a challenging procedure because many contaminants, especially humic acids, co-precipitate and interfere with nucleic acid extraction (Zhou et al., 1996; Lakay et al., 2011; Kasu and Shires, 2015). Many studies tried to establish the most suitable total soil DNA extraction protocol, but the diversity of soil type and various step of protocols applied, make very difficult to integrate all the metagenomics data (Philippot et al, 2010; Plassart et al., 2012). Several scientists also initiated an ISO/CD 11063 standard, in order to reunite and to give a direction in soil microbial diversity studies (Philippot et al., 2010), but even this protocol had to be improved (Plassart et al., 2012). The amount, the PCR efficiency and necessary time for these protocols, correlated with soil characteristics, are the most important items in choosing a specific method.

The majority of these studies are focused on various unpolluted type of soils, but few of them are directed to polluted ones. Most of the biodegradation and bioremediation studies on microbial communities from polluted environments they pay less attention to the DNA isolation step. In this study we aimed to compare and evaluate a number of procedures recommended by other studies of soil DNA isolation protocols, for a specific type of kerosene-polluted soil, in order to use such extracts in further molecular analyses regarding microbial communities from a constructed microcosm.

2. Materials and Methods

2.1. Soil Samples

Soil representing garden-soil was collected from Botanical Garden University of Bucharest, Romania, being part of a microcosm intentionally polluted with a concentration of 58,8g kerosene/kg soil. Replicate soil cores from the microcosm were then bulked to obtain a composite sample and stored at -70^{o}C, prior to DNA extraction.

2.2. DNA Extraction Procedures

Five methods of DNA extraction (Table 1) were tested in order to choose the most suitable for specific soil samples. DNA was extracted from three technical replicates for each method, after several procedures of homogenizing, starting from the same 500mg of soil and resuspending the DNA pellets in 50µl water endonuclease free, for an easier evaluation procedure. The most important step in these procedures is represented by the cell lysis that can be obtained with various treatments of soil samples, e.g. with liquid nitrogen, microwaves or different size of glass or ceramic beads. We used two sizes of glass beads (Table 1) in order to detach the cells from soil matrix and in the same time to obtain the cell lysis.

Soil (0.5g) was homogenized on a vortex mixer (S8A Stuart) at 2200rpm for 5min with 600µl of extraction buffer (50mM Na-phosphate buffer [pH 8], 50mM NaCl, 500mM Tris-HCl [pH 8], and 5% SDS) and 300µl of phenol-chloroform–isoamylic alcohol (25:24:1 v/v) and 0.5g sterile glass beads 0.5-mm diameter, for **S** and **SP** protocol.

Resulted lysate was centrifuged at 16000g for 2 minutes. The supernatant was mixed with the same volume of phenol-chloroform–isoamylic alcohol (25:24:1 v/v) and centrifuged at 6,000g for 5min. The upper layer was transferred and mixed with an equal volume of chloroform-isoamylic alcohol (24:1 v/v) and centrifuged at 16,000g for 5 min for S protocol, and prior to this step at SP protocol, we added a 100µl proteinase K stock solution, and incubated for 30min at 37 ^{o}C. The supernatant was then precipitated with 1 volume of ice-cold isopropanol, and

incubated at -20 °C for 20min. In S-CTAB protocol the proteinase step was replaced by a 30 min incubation time at 65°C after adding 100 µl CTAB/NaCl solution.

ISOm procedure is a modified version by Plassart et al. (2012) named GnS-GII. Soil 0.5 g of was added to a tube containing 0.5 g of glass beads of 0.5 mm and, respectively, 0.1mm diameter. Soil sample was first mixed with 800µl of extraction buffer of 100mM Tris (pH 8.0), 100mM EDTA (pH 8.0), 100 mM NaCl and 2% (w/v) sodium dodecyl sulfate. Tubes were then shaken horizontal for 5 minutes at 2200 rpm on a vortex mixer (S8A Stuart) and incubated for 30 min at 65°C, before centrifugation at 14,000 g for 1 min. The supernatant was removed carefully and treated with 1/10 volume of 3M potassium acetate for proteins precipitation, followed by an incubation at -20 °C for 15 min. The precipitated proteins were removed by centrifugation at 14,000g for 5min at 4°C. Finally, nucleic acids were precipitated by adding 1 volume of ice-cold isopropanol. The DNA pellets obtained after centrifugation 14,000 g for 5min at 4°C were resuspended in the same volume for all methods, respectively 50 µl of endonuclease-free water. Sample aliquots of 0.5 g soil were added directly to Powerbead tubes of Soil DNA Isolation Kit Norgen Biotek along with 60 µL of Lysis Solution and isolated DNA was further purified according to the manufacturer's instructions.

Visualization of DNA amount was made in 1% agarose gel with TBE1X (Tris/Borate/EDTA) buffer and then stained with ethidium bromide. The visual estimations from the gel were then correlated to the spectrophotometric measurements at 260nm made by the NanoVuePlus HG Healthcare Life Science spectrophotometer. Soil DNA extraction was carried out in triplicate (n = 3) and DNA quantification for each extract was carried out in triplicates and DNA yields were estimated as average value with standard deviation (g DNA/g soil). Absorption ratios A_{260}/A_{280}, A_{260}/A_{230} were calculated in order to characterize the DNA extracts, and presented also as mean value (Table 2).

Table 1. Experimental design for comparison of soil DNA extraction methods

Method	Soil pretreatment	Estimated time	DNA extraction
S	0.5 g glass beads 0.5-mm diameter	4h	Modified by Sagova et al. (2008) from Miller et al. (1999)
SP	0.5 g glass beads 0.5-mm diameter	5h	Modified of Sagova et al. (2008)
S-CTAB	0.5 g glass beads 0.5-mm diameter and 0.5g glass beads of 0.1mm diameter	5h	Modified of Sagova et al.(2008)
GnS-GII	0.5 g glass beads 0.5-mm diameter and 0.5gglass beads of 0.1mm diameter	4h	Modified of Plassart et al. (2012)
N	Glass beads provided in the isolation kit	2h	Soil DNA Isolation Kit NorgenBiotech

2.3. PCR assays

The quality of DNA isolates was estimated by quantifying amplification of 16S rRNA genes using the universal primers specific for Eubacteria: GM3F (5'-AGAGTTTGATCMTGGC-3') and GM4R reverse (5'-TACCTTGTTACGACTT-3') according to Muyzer et al. (1995), using a 50°C alignment temperature on a Mini Cycler MJ Research. PCR assays were carried out in a 30µl reaction volume containing PCR Master Mix 2X (ThermoScientific), with and without 3mg/ml Bovine Serum Albumin (BSA) and 50µM of each primer, including a *no template* control and a positive control represented by chromosomal DNA from *P. aeruginosa* PAO1. Template DNA concentration was determined and we used 25ng of crude DNA extract for each PCR reaction. Amplification products from 10 reactions for each procedure were checked for correct size (1500bp) by 1% agarose gel electrophoresis.

3. Results and Discussions

3.1 Soil Samples

Graving determination by sieving revealed that studied soil is a brown soil with 29% clay, 35% silt and 36% sand. The physico-chemical analysis showed a high concentration of humic acids 60%, a medium nitrogen and total carbon content and a pH of 6.3, having characteristics for a garden soil (data not shown).

3.2. DNA Extraction Procedures

Total DNA was successfully isolated from the soil samples using each of the methods of DNA extraction protocol. We underline that we started the protocols from the same soil sample, using the same amount of soil (500mg), and the final resuspension of the DNA was made in the same 50µl volume. The gel electrophoresis was run with the same DNA samples volume. We did not perform any additional purification steps, like DNA Purification Kit. All the PCR reactions were performed after normalizing the DNA concentration of each sample.

The spectrophotometrical data suggest that the S-CTAB and S protocol yielded the most suitable DNA, showing similar ratio A_{260}/A_{280} and A_{260}/A_{230} (Table 2), but with a two times higher concentration for S protocol of 49.38± 9.82 $\mu g \cdot g^{-1}$ (Figure 1 and Table 2) in a shorter time. The ratio A_{260}/A_{280} and A_{260}/A_{230} values obtained in this study, for all DNA extraction methods, were similar and some times better than those reported in the scientific literature (Table 2).

Table 2. DNA concentration and purity assessed by spectrophotometrical determinations.

Technical protocol	DNA concentration $\mu g \cdot g^{-1}$soil	A_{260}/A_{280}	A_{260}/A_{230}	References
SK	40.00	nd.	nd.	Sagova et al. 2008
S	49.38±9,8	1.523 ± 0.02	0.697 ± 0.02	This study
SP	75.70±9.4	0.742 ± 0.02	0.387 ± 0.08	This study
SDS based method	40.00	1.29 ± 0.0001	0.27 ± 0.0004	Fornasier et al.2014
SDS-chloroform	53 ± 8.0	nd.	nd.	Miller et al.1999
S-CTAB	25.58±8.62	1.562 ± 0.02	0.622 ± 0.02	This study
CTAB	17.5 ±1.2	1.35 ± 0.04	0.91 ± 0.03	Zhou et al. 1996
GnS-GII	40±6.16	1.552 ± 0.05	0.567 ± 0.05	This study
GnS-GII	26.26±2.22	nd.	nd.	Plassart et al.2012
PowerSoil DNA isolation Kit MoBio	1.01 ± 0.28	nd.	nd.	Terrat et.al 2012
Soil DNA Isolation Kit	1.08± 0.18	2.3176 ± 0.17	0.296 ± 0.12	This study

nd = not determined

Also, the amounts of DNA recovered after each extraction method was varying from 1 to 75µg DNA/g soil were in the same range of magnitude or even higher than previously reported (Table 2) (Zhou et al.,1996; Miller et al., 1999; Sagova et al., 2008; Plassart et al., 2012; Zhao and Xu, 2012). As we expected and other studies also confirmed (Terrat et al., 2012; Plassart et al., 2012; Zhao and Xu, 2012), specific soil DNA isolation kits recover only a small part of total microbial community DNA. In our case study, the highest DNA concentration was achieved using a Sagova et al. (2008) protocol followed by a proteinase K treatment (Figure 2).

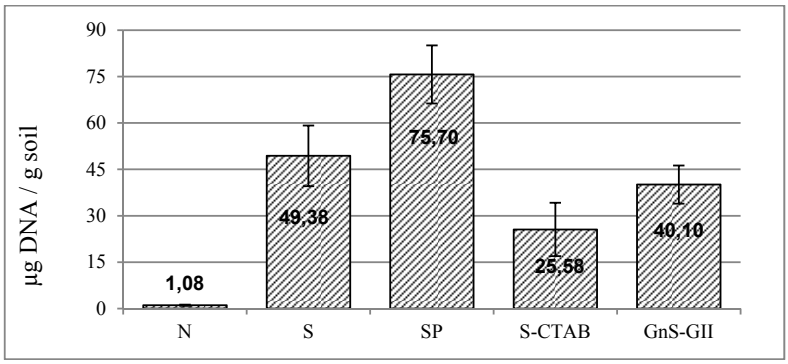

Fig. 1. DNA yield averages $\mu g \cdot g^{-1}$ from studied soil by each protocol.

The gel electrophoresis (Figure 2) shows the differences between the quality and quantity of the DNA applied, the DNA profiles revealed the contamination with proteins for S, SP, S-CTAB and GnS-GII protocols, but a much higher DNA concentration than N protocol. The fragmentation of DNA in all protocols, exempt N was similar due to mechanic treatments of soil samples with glass beads.

Several studies (Sagova et al., 2008) tried to estimate correlations (as regression functions) between DNA yield and soil type, pH, organic matter, factors that can influence development of specific microbial groups with aggregates properties, so the amount of DNA obtained is not always an indicator of DNA extraction efficiency. In this case, working on the same soil sample, general characteristics are the same, but is possible to be influenced by some micro-aggregates from soil structure that cannot be quantified by general characteristics of soil type. It was expected that a CTAB base protocol applied to high humic acid soil, to yield a higher DNA concentration because it has a good performance in reducing humic acid contamination from nucleic acid extracts (Zhou et al., 1996), but it has a lower concentration in our case (Figure 2), in a longer time procedure.

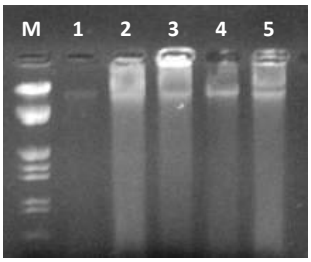

Fig. 2 Agarose gel electrophoresis of genomic DNA extracted from kerosene-polluted soil.
M - DNA Ladder λ/ *Eco*RI and *Hind*III; 1. Soil DNA Isolation Kit Norgen Biotek; 2.S; 3. SP; 4.S-CTAB; 5.GnS-GII.

ISO base protocol GnS-GII retrieved a better DNA extract than in Plassart et al. (2012) study. We obtained a much higher concentration; yet, we did not apply a Clean-up step, as purification procedures usually lead to DNA loss.

For S protocol the DNA amount and absorbance ratio, indicated that this protocol is the best suited for the studied soil with a high humic acid content, generating a good DNA concentration in less time, approximately 4h, comparing with the other procedures tested in this study.

3.3. PCR assay

PCR amplification of the 16S rRNA genes was successful with all DNA extracts, except SP protocol, but resulted in different relative intensity of PCR products (38 successful PCRs out of 50). The intensity of the amplification was higher when 3mg/ml BSA was added to PCR reactions (Figure 3).

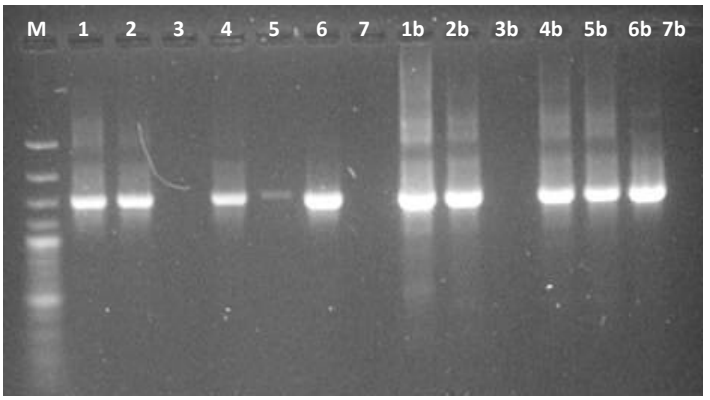

Fig. 3. Agarose gel of 16S rDNA amplicon obtained using various procedures from soil samples (*b* lanes = +3mg/ml BSA): DNA Ladder λ/ *Eco*RI and *Hin*dIII (M); Soil DNA Isolation Kit NorgenBiotek (1, 1b); S (2, 2b); SP(3, 3b); S-CTAB (4, 4b); GnS-GII (5, 5b); *P. aeruginosa* PAO1 (6,6b); No DNA control (7, 7b).

No PCR product of the negative control was detected and the intensity of PCR product for *P. aeruginosa* PAO1 was similar with and without BSA. Previous studies reported a higher PCR efficiency but DNA amount used for PCR reactions was between 1ng (Plassart et al. 2012) to 50-100ng (Sagova et al., 2008; Zhao et al., 2012). There was no amplification for the SP protocol, with or without adding BSA for 10 PCR reactions. We presume that, because we performed no additional purification in this SP protocol, part of proteinase K remained trapped in residual humic acids or other contaminants from soil, having an inhibitory effect on *Taq* polymerase activity.

4. Conclusions

In this study we have compared 5 methods of DNA extraction for a rich humic acid and clay content soil polluted with kerosene, in order to evaluate the most suitable and fastest one. The DNA extraction procedure tested in this study are fast, simple and retrieved a good nucleic acid concentration and purity, compared to other studies. Our results showed that adjusted S protocol is efficient for extracting high molecular weight DNA from the tested soil, and the extracted DNA can be further used for PCR-based molecular ecology studies. From our knowledge there has been no other similar studies on such high concentration of humic acid and clay soil type. Thus, our study succeeded in establishing a protocol for soil DNA extraction that can be applied to soil and sediments that are very rich in humic acid content, having a wide range of applications from agriculture and pollution monitoring to biodiversity and forensics profiling.

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References

Fornasier F., Ascher J., Ceccherini M.T., Tomat E., Pietramellara G.A., 2014. A simplified rapid, low-cost and versatile DNA-based assessment of soil microbial biomass. Ecological Indicators 45: 75–82.

Kasu M., Shires K., 2015. The validation of forensic DNA extraction systems to utilize soil contaminated biological evidence. Legal Medicine, 17(4): 232–238.

Lakay F.M., Botha A., Prior B.A., 2007. Comparative analysis of environmental DNA extraction and purification methods from different humic acid-rich soils. Journal of Applied Microbiology, 102(1): 265-273.

Maron P.A., Mougel C., Ranjard L., 2011. Soil microbial diversity: Methodological strategy, spatial overview and functional interest. Comptes Rendus Biologies, 334: 403–411.

Miller D.N., Bryant J.E., Madsen E.L., Ghiorse W.C., 1999. Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. Applied and Environmental Microbiology, 65(11): 4715-4724.

Muyzer G., Teske A., Wirsen C.O., Jannasch H.W., 1995. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Archives of Microbiology, 164(3): 165-172.

Petric I., Philippot L., Abbate C., Bispo A., Chesnot T., Hallin S., Laval K., Lebeau T., Lemanceau P., Leyval C., Lindström K., Pandard P., Romero E., Sarr A., Schloter M., Simonet P., Smalla K., Wilke B.M., Martin-Laurent F., 2011. Inter-laboratory evaluation of the ISO standard 11063 "Soil quality — Method to directly extract DNA from soil samples". Journal of Microbiological Methods 84: 454–460.

Philippot L., Abbate C., Bispo A., Chesnot T., Hallin S., Lemanceau P., Lindström K., Pandard P., Romero E., Schloter M., Simonet P., Smalla K., Wilke B.M., Petric I., Martin-Laurent F., 2010. Soil microbial diversity: an ISO standard for soil DNA extraction. Journal of Soils and Sediments, 10(7): 1344-1345.

Plassart P., Terrat S., Thomson B., Griffiths R., Dequiedt S., Lelievre M., Regnier T., Nowak V, Bailey M., Lemanceau P., Bispo A., Chabbi A., Maron P.A., Mougel C., Ranjard L., 2012. Evaluation of the ISO Standard 11063 DNA extraction procedure for assessing soil microbial abundance and community structure. PloS One, 7(9): e44279.

Sagova-Mareckova M., Cermak L., Novotna J., Plhackova K., Forstova J., Kopecky J., 2008. Innovative methods for soil DNA purification tested in soils with widely differing characteristics. Applied and Environmental Microbiology, 74(9): 2902-2907.

Terrat S., Christen R., Dequiedt S., Lelièvre M., Nowak V., Regnier T.,Bachar D., Plassart P., Wincker P., Jolivet C., Bispo A., Lemanceau P., Maron P.A., Mougel C., Ranjard L., 2012. Molecular biomass and MetaTaxogenomic assessment of soil microbial communities as influenced by soil DNA extraction procedure. Microbial Biotechnology, 5(1): 135-141.

Zhou J., Bruns M.A., Tiedje J.M., 1996. DNA recovery from soils of diverse composition. Applied and Environmental Microbiology, 62(2): 316-322.

Zhao F., Xu K., 2012. Efficiency of DNA extraction methods on the evaluation of soil microeukaryotic diversity. Acta Ecologica Sinica, 32(4): 209-214.