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First characterization of activated sludge bacterial bioma by biomolecular (PCR) method

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Abstract:
The water treatment carried out with biological processes artificially reproduces the natural process of the biogeochemical cycle of the detritus present in aquatic environments and partly in the soil. The growing biomass in the plant is called "activated sludge"; usually it is mainly composed by bacteria (95%) and by Protozoa and Metazoans (5%). Microorganisms composing activated sludge have always been of great interest to microbiologists. The sludge biological composition is e a good indicator of its state of health, and can show the need for process or management changes, the treatment potential, the possible presence in the sewage of substances harmful to the process, the sludge pathologies and the best approach to further treatment of the mud. This work is part of a full-scale experimentation project of the AS Diffusion system for the abatement of osmogenic compounds emitted by biological treatment processes (TOASD project). The task of the project concerns the verification of any effects of selective pressure on the composition of the biological sludge induced by the AS diffusion system, applied to a biological line, in comparison with a parallel line on which the system is not applied. An initial characterization of the microorganisms present in the activated sludge of the plant chosen for the project is carried out before starting the project. The characterization is carried out through the extraction of DNA, with its quantification and determination of quality. A PCR analysis follows with the identification of the main Kingdoms and phylum of bacteria, for a first screening in order to continue the investigations to identify the species present. A performance check was carried out on the preparatory and DNA extraction procedures from the biological sludge matrix and an initial characterization of the microorganisms present before the project was started, with the quantification and determination of the quality of the extracted DNA. The NucleoSpin® Soil kit from MACHEREY-NAGEL demonstrated improved qualitative and quantitative efficacy. A DNA extraction procedure from samples stored by freezing in 150 ml aliquots and gradually thawed was found to be qualitatively and quantitatively better. All sequencing confirmed the amplification of the sequence sought and produced by the PCR. DNA of generic bacteria and archaea are always present in the 16S region. The Phylum Bacteroidetes, Termotogae (Fervidobacteria sp), Chlorobi, Spirochetes are absent in the present case. Furthermore, the Deltaprotebacteria class is absent. On the confirmation of bacterial presence, the subsequent research work by species and subsequently quantification with realtime PCR will be developed.

Key Word*: biological water treatment plant, as diffution, sludge ecology, biomolecular characterizzation*

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I. Introduction

The water treatment carried out with biological processes artificially reproduces the natural process of the biogeochemical cycle of the detritus present in aquatic environments and partly in the soil. In fact, purification plants with a predominantly biological component can be considered as artificial ecosystems in extreme conditions.

The natural phenomenon reproduced is a microbial mixed biotransformation, followed by a share of predation on the bacteria by other superior microorganisms.

In the municipal wastewater treatment plants the dissolved substrate to be removed consists of a very heterogeneous mixture of organic and inorganic compounds; the microorganisms involved in the process are mainly mixed colonies of heterotrophic bacteria, breakers of organic matter of prevalent fecal origin, and coming from the anthropic food cycle. The growing biomass in the plant is called "activated sludge"; usually it is mainly composed by bacteria (95%) and by Protozoa and Metazoans (5%).

The role of activated sludge in the treatment process is twofold: metabolizing the organic substance and forming mud flocs that can be separated from the treated water [1]. In the metabolization of organic matter, a fundamental role is played by bacteria, while other species help to contain the growth of bacteria by limiting their excessive development and thus determining the maintenance of the ecosystem balance. The biochemical reactions that take place depend on the steps in the process. The aerobic phase is prevalent. In this phase, the

oxidation of the organic substances and of the reduced inorganic compounds present occurs, using atmospheric or pure oxygen (aerobic respiratory heterotrophic metabolism); oxidized forms of the elements that constitute the original substances are released (mineralization).

Often there is also an anoxic phase, where mainly the oxidized forms of nitrogen are denitrified to molecular nitrogen (anoxic respiratory heterotrophic metabolism). A respiratory degradation is thus achieved, based on bacterial development that uses compounds, considered pollutants, as a growth substrate. These bacteria grow aggregated with the organic particles they feed on, forming sludge floks, which can be separated, by gravity or filtration, from the purified water in specific process stages, allowing the clarification of the sewage.

A part of the removed substrate is mineralized with the formation of gaseous products of bacterial catabolism released into the atmosphere; a second part constitutes a solid-liquid residue, the highly putrescible sludge, that must still be treated and reused or disposed in a hygienically and ecologically correct way [2]. Also for sludge management a biological treatments can be employed, with the purpose of reducing its volume, putrescibility, odors and enterobacterial load and prepare it for reuse or disposal.

Microorganisms composing activated sludge have always been of great interest to microbiologists. The sludge biological composition is e a good indicator of its state of health, and can show the need for process or management changes, the treatment potential, the possible presence in the sewage of substances harmful to the process, the sludge pathologies and the best approach to further treatment of the mud. Numerous laboratory cultivation studies have been conducted to isolate and identify bacteria [3,4,5], which have provided interesting insights into microbial diversity. However, plating or numerical probability techniques are always selective and therefore cannot produce sufficient documentation of the true structure of the community. As a result, several attempts have been made over the past decade to analyze the structure of the activated sludge bacterial community by direct methods. In recent years, group and gender specific oligonucleotide probes have been used to directly analyze the community structure of activated sludge by in situ hybridization [6,7,8].

This work is part of a full-scale experimentation project of the AS Diffusion system for the abatement of osmogenic compounds emitted by biological treatment processes (TOASD project). The task of the project concerns the verification of any effects of selective pressure on the composition of the biological sludge induced by the AS diffusion system, applied to a biological line, in comparison with a parallel line on which the system is not applied.

In this study, an initial characterization of the microorganisms present in the activated sludge of the plant chosen for the project is carried out before starting the project. The characterization is carried out through the extraction of DNA, with its quantification and determination of quality. A PCR analysis follows with the identification of the main Kingdoms and phylum of bacteria, for a first screening in order to continue the investigations to identify the species present.

II. Material And Methods

Study area

The treatment plant of the Municipality of Turi is recently built. It has a capacity of 10,000 equivalent inhabitants; is developed on two separate treatment lines, including a complete process scheme of equalization, primary sedimentation, pre-denitrification, oxidation, secondary sedimentation, filtration, sanitation and aerobic treatment of the sludge, with subsequent dehydration. The phases of screening, sieving and emergency clariflocculation are single. The plant shows excellent functional characteristics of the water and sludge line, and is among the best and most modern in the province of Bari.

The two lines and common sections are also equipped with covers and suction systems and air treatment with activated carbon.

Sampling

Photo no. 1:on the left line A and on the right line B of the oxidation tank of the Turi treatment plant.

The sampling of the aerated mixture was carried out at the Turi treatment plant, a plant identified for carrying out the experimentation.

The purpose of this activity is to set up the procedures for the characterization of the sludge of the biological oxidation lines.

The sampling took place at the two oxidation tanks present at the plant indicated respectively as line A and line B (Photo no 1).

The sample was collected through a container drawn directly from the oxidation tank; the sampling point and system coincide with the sampling point usually used for routine monitoring activities carried out by the plant operator.

Sampling was performed at a depth of few decimeters below the free surface of the tanks, in order to avoid the collection of any foam, in accordance with the guidelines of the sampling procedures. Before pouring the sample into the transport containers (rinsed with the same aerated mixture), the sample was manually stirred in order to avoid the decantation of the solid fraction which would negatively affect its representativeness.

The sample was collected in sterile 5-liter tanks, leaving enough headspace to prevent sludge anoxia during the transportation.

Two aliquots were taken for each line, for a total of 10 l of sample each. The sample was then labeled and placed in portable refrigerators to keep them at a constant temperature during transportation.

Sample preparation activity in the laboratory

Different preparatory procedures were applied to the sample matrix to determine their possible impact on the quantity and quality of extractable DNA, listed below:

1) Use of the fresh sample as it is, with filtering of three volume aliquots of 10 ml each on 0.25 µm NC filters and subsequent extraction.
2) Use of the fresh sample as it is, with filtering of three volume aliquots of 10 ml each on 0.25 µm NC filters and subsequent freezing of the 3 filters at -20 ° before the DNA extraction steps
3) Freezing of 10 ml aliquots of sample as it is at T -20 °, for subsequent phases of thawing, filtration and DNA extraction
4) Freezing of 150 ml aliquots of sample as it is at T -20 °, for subsequent steps of thawing, filtration and DNA extraction

DNA extraction was carried out with the following deadlines:

• Extraction 1) The fresh sample was processed immediately after filtering;

• Extraction 2) The frozen filters were processed directly after 72 hours (without waiting for defrosting);

• Extraction 3) The 10 ml aliquot was thawed after 72 hours, filtered using 0.25 µm filters and processed;

• Extraction 4) The 150 ml aliquot was returned to 4 ° C after 72 hours, for gradual thawing, and after 24 hours a volume of 10 ml was taken, filtered using 0.25 µm filters and processed.

DNA extraction

DNA extraction was initially performed by comparing two different commercial kits, namely NucleoSpin® Soil (MACHEREY-NAGEL, Germany) and DNeasy PorwerSoil Kit from QUIAGEN, applying the standard protocol in both cases.

PCR conditions

Table no 1 lists the PCR primers and the thermal cycles conditions used in this study. PCR amplifications were performed using 50 µl total volume mixture obtained adding 4 µl HOTFIREPOOL (5x), 2 µl forward primer (10 pmol), 2µl reverse primer (10 pmol); 5 µl DNA (20 ng/ µl) in 37 µl of water.

Amplification of PCR products was confirmed by electrophoresis through "E-Gel™ Power Snap Electrophoresis System". Using 1.2% E-Gel™ agarose gel pre-stained with SYBR™.

Table no1: *Primer*

Target Group	Primer	Primer Sequence (5'-3')	Amplicon Length (bp)	T. Annealing (°C)	Ref.
Region 16S	16SF	AGA GTT TGA TCA TGG CTC AG	1500	60	[9]
	16SR	TAC GGC TAC CTT GTT ACG ACT T			
All Bacteria	Eub338F	ACT CCT ACG GGA GGC AGC AG	200	60	[10]
	Eub518R	ATT ACC GCG GCT GCT GG			
Archea	Arch16F	CTG GTT GAT CCT GCC AG	300	58	[11,12]
	Arch344R	TTC GCG CCT GST GCR CCC CG			
Alphaproteobacteria	Alf28f	ARC GAA CGC TGG CGG CA	750	58	[13]
	Alf684r	TAC GAA TTT YAC CTC TAC A			
Betaproteobacteria	Beta359f	GGG GAA TTT TGG ACA ATG GG	450	58	[13]
	Beta682	ACG CAT TTC ACT GCT ACA CG			
Gammaproteobacteria	Gamma395f	CMA TGC CGC GTG TGT GAA	600	57	[13]
	Gamma871r	ACT CCC CAG GCG GTC DAC TTA			
Nitrospira	NSR 1113f	CCT GCT TTC AGT TGC TAC CG	151	60	[14]
	NSR 1264r	GTT TGC AGC GCT TTG TAC CG			
Bacteroidetes	798cfbF	CRA ACA GGA TTA GAT ACC CT	240	61.5	[15]
	cfb967R	GGT AAG GTT CCT CGC GTA T			
FIRMICUTES	Firm934F	TGA AAC TYA AAG GAA TTG ACG	126	60	[16]
	Firm1060R	ACC ATG CAC CAC CTG TC			
ACTINOBACTERI	AcT920F3	TAC GGC CGC AAG GCT A	280	59	[16]
	Act1200R	TCR TCC CCA CCT TCC TCC G			
CYANOBACTERIA	CYA106F	CGG ACG GGT GAG TAA CGC GTG A	675	60	[17]
	CYA781R	GAC TAC AGG GGT ATC TAA TCC CTT T			
PLANCTOMICETES	Pla46f	GGA TTA GGC ATG CAA GTC	1500	60	[18]
	1542r	AAG GAG GTG ATC CAG CCG CA			
CHLOROFLEXI	UNIV530F	GTG CCA GCM GCC GCG G	400	60	[19]
	GNSB941R	AAA CCA CAC GCT CCG CT			
ACIDOBACTERIUM	31f	GAT CCT GGC TCA GAA TC	1461	53	[20,21]
	1492r	GGT TAC CTT GTT ACG ACT T			
TERMOTOGAE (FERVIDOBACTERIUM)	FerF	TGA AAC TYA AAG GAA TTG ACG	710	60	In this study
	FerR	ACC ATG CAC CAC CTG TC			
CHLOROBI	AF34	GCT CAG GAC GAA CGT TG	1471	60	[22]
	AF35	TTA GCC CCA GTC ACT AAG			
SPIROCHETES	SpiroF	CCA GAC TCC TAC GGG AGG CAG	500	60	[23]
	SpiroR	CTC GTG CCG TGA GGT GTT GGG			

Nucleotide sequence analysis

All the PCR products were sequenced by Mycrosint Lab (GERMANY). The sequencing analyses were conducted using the BLAST program [61] in the GenBank.

III. Result and discussion

DNA extraction

The NucleoSpin® Soil kit by MACHEREY-NAGEL has shown a better efficacy in the extraction by producing a much greater quantity of DNA than the DNeasy PorwerSoil Kit by QUIAGEN and a quality of the extracted DNA that has a very low percentage of residual impurities downstream of the extraction (photo 2 and photo 3). In fact, a concentration of extracted DNA equal to 15-20 ng / µl was obtained using the Quiagen kit and a concentration of 95-100 ng / µl with the MACHEREY-NAGEL kit. Moreover the DNA extracted with the Quiagen kit has a smir due to residual impurities or degraded DNA; instead, with the extraction using the MACHEREY-NAGEL kit the smir is completely absent. (photo no 2 and photo no 3).

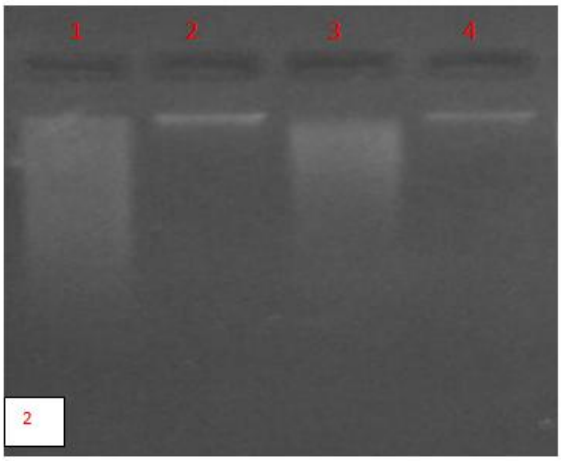

Photo no 2: *results with DNeasy PorwerSoil kit QUIAGEN: 1 - sludge Line A; 2 - 0.25µm filter of 10 ml sample sludge Line A; 3 - sludge Line B; 4 - 0.25 µm filterof 10 ml sample sludge Line B.*

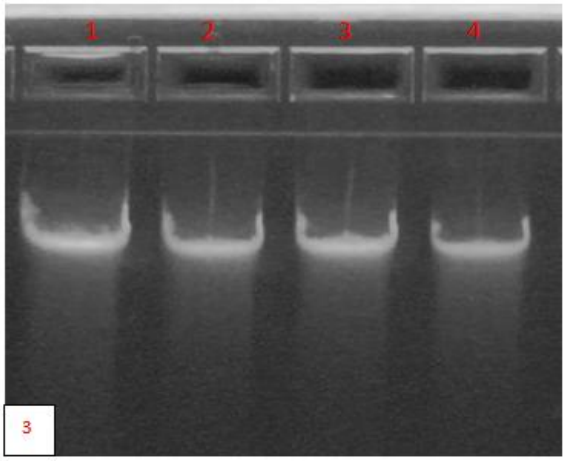

Photo no 3.*results with NucleoSpin® Soil MACHEREY-NAGEL kit: 1 - sluidge Line A; 2 - 0.25 µm filter of 10 ml sample sludge Line A; 3 - sludge Line B; 4 - 0.25 µm filter of 10 ml sample sludge Line B.*

DNA analysis, extracted from samples subjected to the four preparatory procedures previously described and with the use of the NucleoSpin® Soil Kit by MACHEREY-NAGEL, did not reveal significant qualitative differences (photo no 4 and photono 5).

The quantity of extracted DNA (table no2) is always greater by adopting the "Extraction 4" procedure, ie from frozen samples in 150 ml aliquots and gradually thawed. This result appears to be of interest for subsequent evaluations.

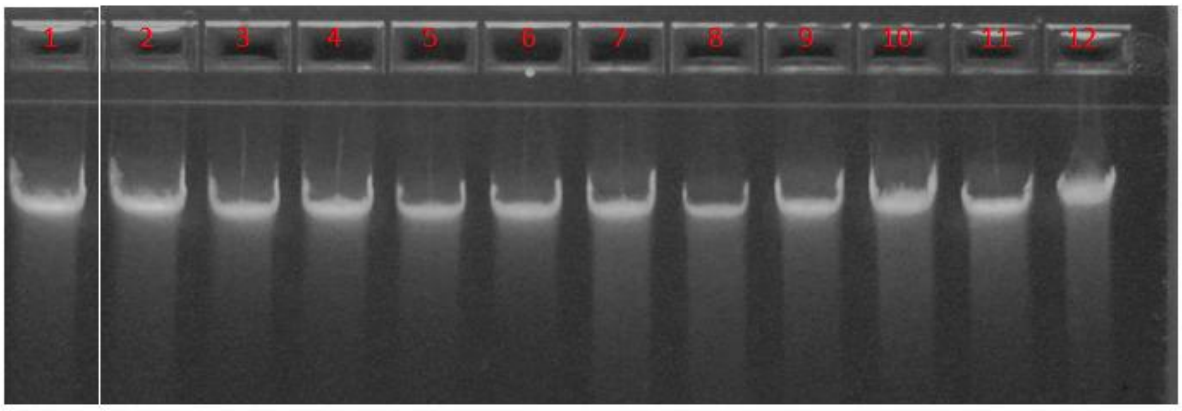

Photo no 4: *electrophoretic run DNA extracted with NucleoSpin® Soil MACHEREY-NAGEL kit:* ***Wells 1 to 3*** *- "Extraction 1" line A;* ***Wells 4 to 6*** *- "Extraction 1" line B;* ***Wells 7 to 9*** *- "Extraction 2" line A****; Wells from 10 to 12*** *- "Extraction 2" line B*

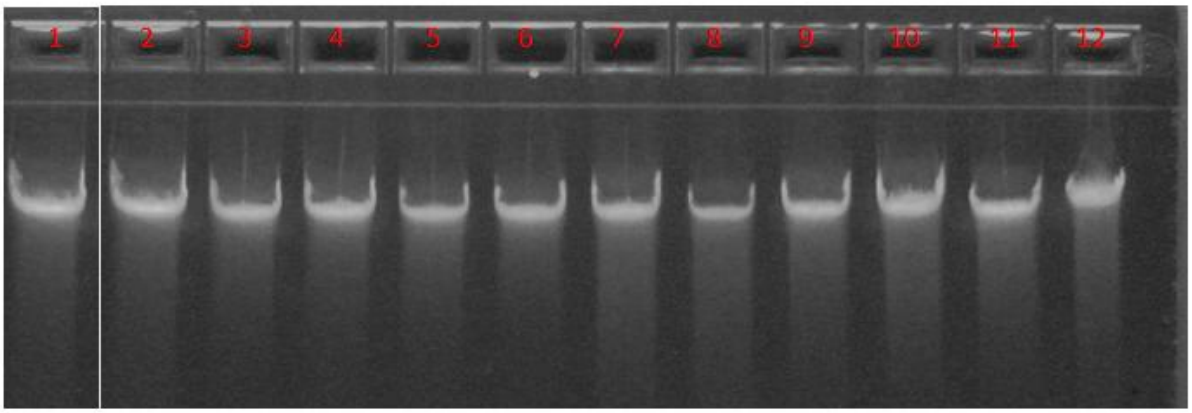

***Photo no 5:**. electrophoretic run DNA extracted with NucleoSpin® Soil MACHEREY-NAGEL kit: Wells 1 to 3 - "Extraction 3" line A; Wells 4 to 6 - "Extraction 3" line B; Wells 7 to 9 - "Extraction 4" line A; Wells from 10 to 12 - "Extraction 4" line B*

Table no2:*amount of DNA extracted from the samples in triplicate*

DESCRIPTION	SLUDGE FILTERED (ml)	DNA (ng/µl)
"Extraction 1" line A	10	93,20
"Extraction 1" line A	10	87,40
"Extraction 1" line A	10	112,00
"Extraction 1" line B	10	100,00
"Extraction 1" line B	10	75,00
"Extraction 1" line B	10	74,60
"Extraction 2" line A	10	102,00
"Extraction 2" line A	10	62,40
"Extraction 2" line A	10	81,40
"Extraction 2" line B	10	97,80
"Extraction 2" line B	10	84,80
"Extraction 2" line B	10	94,20
"Extraction 3" line A	10	104,00
"Extraction 3" line A	10	122,40
"Extraction 3" line A	10	74,00
"Extraction 3" line B	10	106,00
"Extraction 3" line B	10	102,00
"Extraction 3" line B	10	112,00
"Extraction 4" line A	10	141,20
"Extraction 4" line A	10	134,00
"Extraction 4" line A	10	134,40
"Extraction 4" line B	10	107,20
"Extraction 4" line B	10	120,80
"Extraction 4" line B	10	130,00

Biomolecular Analysis

All the previously indicated protocols were applied, through a verification and fine-tuning of the amplification procedures and selecting the optimal PCRs. All the amplified products were sent for sequencing at the MICROSYNTH SEQLAB center (Germany) for the verification of amplification of the searched fragment. All sequencing confirmed the amplification of the sequence sought and produced by PCR. The analysis was carried out on the 4 types of preparatory procedure adopted for the samples and previously described

- Extraction 1) The fresh sample was processed immediately after filtering;
- Extraction 2) The frozen filters were processed directly after 72 hours (without waiting for defrosting);
- Extraction 3) The 10 ml aliquot was thawed after 72 hours, filtered using 0.25 µm filters and processed;
- Extraction 4) The 150 ml aliquot was returned to 4 ° C after 72 hours, for gradual thawing, and after 24 hours a volume of 10 ml was taken, filtered using 0.25 µm filters and processed.

The results are shown in the Table no 3.

Of particular interest was the difference in abundance, carried out with a visual method using an electrophoretic run on 1.2% agarose gel. There are significant differences between the investigations carried out on the fresh sample and those carried out on previously frozen samples on which amplification was carried out following thawing after 72 hours. In particular: Nitrospira, Cyanobacteria, Planctomycetes and Cloroflexy are present in all the samples but much more abundant in the "Extraction 4" samples. Acidobacteria is absent in the

filters of fresh samples, while a slight band can be seen in the "Extraction 4" samples.

This observation allows us to deduce that the freezing and thawing of the sample, with subsequent filtration of the aliquot to be analyzed, significantly affects both the quantity of extractable DNA and its quality. Probably the physical action that the specific matrix undergoes from freezing allows the extraction of DNA from very resistant bacteria due to the structural characteristics of the cell wall and membrane, probably not very degradable by the lysis action of the kit enzymes alone.

Table no 3:*result of biomolecular analysis*

PCR	EXTRACTION 1		EXTRACTION 2		EXTRACTION 3		EXTRACTION 4	
	LINE A	LINE B	LINE A	LINE B	LINE A	LINE B	LINE A	LIN B
REGION 16S	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT
KINGDOM								
GRNERIC BACTERIA	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT
ARCHEA	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT
PHYLUM								
NITROSPIRA	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT
BACTEROIDETES	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT
FIRMICUTES	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT
ACTINOBACTERIA	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT
CYANOBACTERIA	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT
PLANCTOMYCETES	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT
CLOROFLEXY	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT
ACIDOBACTERIA	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	LIGHT BAND	LIGHT BAND
TERMOTOGAE (FERVIDOBACTERIA SP)	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT
CHLOROBI	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT
SPIROCHETES	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT
CLASSE								
ALPHAPROTEBACTERIA	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT
BETAPROTEBACTERIA	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT
GAMMAPROTEBACTERIA	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT
DELTAPROTEBACTERIA	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT

IV. Conclusion

The comparison on the use of the main commercial kits used for the extraction of DNA from biological sludge samples, the NucleoSpin® Soil kit by MACHEREY-NAGEL has shown a better efficacy, producing a much greater quantity of DNA (95-100 ng / µl of DNA) compared to the QUIAGEN DNeasy PorwerSoil Kit (15-20 ng / µl of DNA). Furthermore, the "NucleoSpin® Soil" kit produced an extracted DNA quality with a very low percentage of residual impurities. In fact, the DNA extracted with the QUIAGEN kit has a smir due to residual impurities or degraded DNA, completely absent, however, with the extraction using the MACHEREY-NAGEL kit.

The 4 different storage procedures of the samples before DNA extraction resulted in significant qualitative and quantitative differences in the extracted DNA. The "Extraction 4" procedure is better, ie from frozen samples in 150 ml aliquots and thawed gradually.

This observation allows us to deduce that the freezing and thawing of the sample, with subsequent filtration of the aliquot to be analyzed, significantly affects both the quantity of extractable DNA and its quality. Probably the physical action that the specific matrix undergoes from freezing allows the extraction of DNA from very resistant bacteria due to the structural characteristics of the cell wall and membrane, probably not very degradable by the lysis action of the kit enzymes alone.

All sequencing confirmed the amplification of the sequence sought and produced by PCR.

The DNA of generic bacteria and archaea are always present in the 16S region. The Philum Bacteroidetes, Termotogae (Fervidobacteria Sp), Chlorobi, Spirochetes are absent in the present case. Furthermore, the Deltaprotebacteria class is absent. On the confirmation of bacterial presence, the following research work will be developed by species and subsequently quantification with Pcr realtime.

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