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Earlier Observation of Applicability of Biomolecular and Chemical Analysis to Soil and Shallow Groundwater in Nitrogen Biogeochemical Local Cycle Evaluation

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Abstract:
The presence of nitrate in groundwater has long been considered as one of the main issues on a global scale. The identification of various contamination sources is then particularly relevant, along with the assessment of the negative impact of agricultural activities. This work presents the first attempt to apply the developed methodologies to phenomena of groundwater contamination from nitrates, with the purpose of distinguishing the type of contamination when related to particular microbiomes environments of soil and shallow groundwater. Analyses of the main chemical parameters and of the microbiome involved in the nitrogen biogeochemical cycle were carried out through specific definition of metagenomics techniques. The analytical procedure adopted, implemented with further quantitative real-time to estimate specific microbial communities in different matrices, showed to be suitable for the identification of factors responsible for nitrate contamination. In addition, it provides useful information on the relationship and possible interaction between soil and groundwater. Interesting differences emerge between the possible chemical and microbiological exchange between soil and groundwater, and the quality of soil itself, due to different agronomic practices a soil use. Future application of the Real Time and the quantification of population abundance per species will allow more detailed information about these phenomena.

Key Word*: Nitrogen biogeochemical cycle, soil, shallow groundwater, biomolecular technique, nitrate pollution.*

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I. Introduction

Groundwater is one of the main Earth's freshwater resources for industrial processes, agricultural activities and human life. Nitrogen compounds are the major chemicals artificially released into the environment as a consequence of the growing need for food and energy production [1] [2].

Several studies have highlighted how the excessive use of fertilizers generates a surplus of fertilizer compared to the farms real need. In these cases, the nitrates present in fertilizers can be easily washed off by rainfall and irrigation, with possible consequent formation of high nitrate concentrated polluted plumes in groundwater, following hydrodynamic dispersion [3][4][5][6].

Another anthropic source of nitrates is the sewage, mainly from small towns or isolated houses, in the absence of a collection grid [8]. Livestock is another leading contributing factor [8].

For all the above mentioned reasons, nitrates are the form of nitrogen most frequently exceeding the maximum admissible concentrations in groundwater set at 50 mg/lby the Directive 91/676/CEE.

Today is well known that the whole subsoil system is colonized by microorganisms, mainly present in microbial communities, constituted of bacteria and Archaea, but also of protozoa and fungi, carrying out relevant roles in biogeochemical processes [9].

The groundwater ecosystems are affected by the presence of environmental limiting factors (lack of light, limited nutrient availability, low temperatures, etc.) conditioning the existence of peculiar microbial communities [10].

Microbial communities in the aquifers are constituted of Heterotrophic bacteria well adapted to underground environment [11] [12] [13] and they characterize the hydrological, chemical and geological heterogeneity of the groundwater [11] [12].

The presence of different biotic and abiotic factors may directly or indirectly influence the microbial diversity of ecosystems, determining three different dynamics of transformation of micro biotic communities: quantitative variation of certain bacteria strains already present therein; appearance of new strains of alien bacteria; disappearance of some bacteria strains. Moreover, depending on the type of alteration (from diffuse

source or not) and on the class of pollutants, there are different impacts on microbial communities of groundwater.

All previous works, had the principal purpose of evaluating groundwater and soil status, classifying them on a chemical basis in homogeneous area, but without identifying the type of potential sources and a direct correlation between the anthropogenic activity and the trend of nitrates concentration. Moreover, a microbial characterization (through the identification of the development of different microbial species), that may reinforce the evidence of nitrate contamination and discriminate the type of sources, is still lacking.

The present paper shows a first combined chemical-physical and microbiological characterization of water and soil matrices of two sample areas of Apulian region, taking advantage of specific sampling protocols and biomolecular analysis of organisms of nitrogen cycle. It defines the first observations on the potential interactions between the soil and shallow groundwater as regards such parameters. Moreover, it provides first considerations on the potential effects of different agricultural practices on the chemical and biological properties of soil and shallow groundwater in those areas.

In our view, these results provide a starting point for future studies that could include the identification of potential nitrates sources as regards natural background values.

II. Material And Methods

Study area

Puglia is located at the south-eastern of Italy and extends for 19,350 km2 with a perimeter of 1,260 km and an overall coastal development of 784 km, the largest in mainland Italy.

For the scope of this work, two areas of the Puglia Region have been examined: the first is the Ionic arc (called Area A) the second is located between the municipality of Nardò area and the municipality of Gallipoli (called Area B).

On the basis of the lithological and hydrogeological assessments, the sampling sites with the relative sampling depths were identified. For each sampling site, in addition to the surface groundwater sample, two soil samples were taken upstream and downstream relative to the groundwater flow. Each sample was identified using the following nomenclature: area of origin (Point A.), point number (1.), identification of the matrix: if it is a water sample (W) and in the case of the soil if upstream (UW) or downstream (DW) of the groundwater flow.

In Area A, a total of 6 sampling sites were identified positioned on two lines parallel to the coast. The sampling points have been named for the water samples: Point A.1.W, Point A.2.W, Point A.3.W, Point A.4.W, Point A.5.W, Point A.6.W; the soil samples for each site: Point A.1.U.W, Point A.1.D.W, Point A.2.U.W, Point A.2.D.W, Point A.3.U.W, Point A.3.D.W, Point A.4.U.W, Point A.4.D.W, Point A.5.U.W, Point A.5.D.W, Point A.6.U.W, Point A.6.D.W.

In Area B, a total of 4 sampling sites have been identified, positioned at point 1 in the municipality of Nardò which persists on the upper groundwater of the Salento and the other 3 in the municipality of Gallipoli which persist on the central groundwater of the Salento. The sampling points have been named for the water samples: Point B.1.W, Point B.2.W, Point B.3.W, Point B.4.W, the soil samples for each site: Point B.1.U.W, Point B.1.D.W, Point B.2.U.W, Point B.2.D.W, Point B.3.U.W, Point B.3.D.W, Point B.4.U.W, Point B.4.D.W. A particular point is the Point B.1.white sampling in a biological land.

Sampling

The sampling sites, with the relative sampling depths, were identified. Two soils samples, placed upstream and downstream of the groundwater flow, were taken for each site, in addition to the shallow groundwater sample.

The soil matrix sample was carried out in according to the "Methods of Soil Chemical Analysis" issued by the Ministry of Agricultural and Forestry Politics, approved with the Ministerial Decree of the 13th September 1999 (Uff. Journal Suppl. Ordin. n° 248, 21/10/1999).

The basic aim of this sampling procedure is to obtain a truly representative sample of the soil under investigation.

Samples were collected from the soil at a depth between 10-15 cm using a sterile spatula and excluding the first two centimeters presenting grass. They were then put inside sterile envelopes and stored at 10°C.

Water matrix samples were taken according to the APAT-IRSA Methods[14]. A pre-sampling purging was carried out in order to remove stagnant water from the well and to sample ground water (purging equal to the volume of the water column so as to sample ground water in proximity to the well).

Two samples were taken from each well, for biomolecular analyses, using a sterile PVC container and collecting a volume of 5 liters per sample.

Chemical analysis

The soil samples were collected in order to make a set of analyses according to the "Official Methods of Soil Chemical Analysis (MUACS), as stated by Ministerial Decree of the 13th September 1999, Ministry of Agricultural and Forestry Politics".

The sample preparation is such that the smallest weighing should be representative of the entire sample collected in the field. In particular, the following parameters were analyzed: temperature, organic carbon (Walkey-Black Method) (Method VII.3), organic matter, total nitrogen, organic nitrogen, nitrate, nitrite and ammonia.

The water samples collected underwent a set of analyses according to the "Water Analytical Methods – Manual and Guidelines 29/2003- APAT/IRA-CNR" (APHA-AWWA-WEF, 1998) [14]. In particular, the following parameters were analyzed: pH, Temperature, total nitrogen, organic nitrogen, nitrate, nitrite, ammonia and Chemical Oxygen Demand (COD).

DNA extraction

The NucleoSpinSoil kit (MACHEREY-NAGEL) was employed for the extraction of bacterial DNA from soil samples. It is designed for DNA molecules with high molecular weight of microorganisms such as positive and negative gram, archaea, fungi and algae present in soil, mud and sediment samples.

Bacterial DNA extraction from water samples was carried out through filtration, with a 0.22 µm filter, until reaching a volume of filtered water of 1 liter per filter.

The filter was then used for DNA extraction of microorganism present in water sample.

Filters were processed with NucleoSpinSoil (MACHEREY-NAGEL) using the same procedures for soil matrix, previously described. It is advisable that quantification and quality control of the extracted DNA is carried out through agarose gel electrophoresis.

Bacterial DNA was quantified using the Qubit™ 4 Fluorometer, while DNA quality was verified through electrophoretic run using the E-Gel™ Power Snap Electrophoresis System".

PCR conditions

Table1 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 1.5% E-Gel™ agarose gel pre-stained with SYBR™.

Table 1. 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	[15]
	16SR	TAC GGC TAC CTT GTT ACG ACTT			
All Bacteria	Eub338F	ACT CCT ACG GGA GGC AGC AG	200	60	[16]
	Eub518R	ATT ACC GCG GCT GCT GG			
Archea	Arch16F	CTG GTT GAT CCT GCC AG	300	58	[17],[18]
	Arch344R	TTC GCG CCT GST GCR CCC CG			
Alphaproteobacteria	Alf28f	ARC GAA CGC TGG CGG CA	750	58	[19]
	Alf684r	TAC GAA TTT YAC CTC TAC A			
Betaproteobacteria	Beta359f	GGG GAA TTT TGG ACA ATG GG	450	58	[19]
	Beta682	ACG CAT TTC ACT GCT ACA CG			
Gammaproteobacteria	Gamma395f	CMA TGC CGC GTG TGT GAA	600	57	[19]
	Gamma871r	ACT CCC CAG GCG GTC DAC TTA			
Nitrospira	NSR 1113f	CCT GCT TTC AGT TGC TAC CG	151	60	[20]
	NSR 1264r	GTT TGC AGC GCT TTG TAC CG			
Bacteroidetes	798cfbF	CRA ACA GGA TTA GAT ACC CT	240	61.5	[21]
	cfb967R	GGT AAG GTT CCT CGC GTA T			

Species	Nitrifying Bacteria				
	Primer	Primer Sequence (5'-3')	Amplicon Length (bp)	T. Annealing (°C)	Ref.
Nitrosomonas spp.	NsomoF	GTG GGG AAT TTT GGA CAA TG	900	60	in this study
	NsomoR	TTA CGT GTG AAG CCC TAC CC			

Species	Nitrifying Bacteria				
	Primer	Primer Sequence (5'-3')	Amplicon Length (bp)	T. Annealing (°C)	Ref.
Nitrosovibrio sp.	NvibrioF	GTG GGG AGC AAA CAG GAT TA	400	60	in this study
	NvibrioR	GCG CCA TTG TAT TAC GTG TG			
Nitrococcus spp.	NcoccusF	GGT CTG AGA GGA CGA TCA GC	400	60	in this study
	NcoccusR	CTA CGC ATT TCA CCG CTA CA			
Nitrobacter spp.	NitroF	TCA CTA GTG GCG CAC GTA AC	400	56	in this study
	NitroR	CTA CAA TGG CGG TGA CAA TG			
Nitrospiraceae sp.	NspiracF	ACC GGA TAT GGT GAT TTG GA	850	60	in this study
	NspiracR	TGC ATG TCA AAC CCA GGT AA			

Species	Denitrificant Bacteria				
	Primer	Primer Sequence (5'-3')	Amplicon Length (bp)	T. Annealing (°C)	Ref.
Hydrogenophilus sp.	HydroF	TGG GCT CAA CCT AGG AAT TG	600	60	in this study
	HydroR	ATG ACG TGT GAA GCC CTA CC			
Hyphomicrobium sp.	HyphoF	TGA TGA AGG CCT TAG GGT TG	800	58	in this study
	HyphoR	CAT TGT CAC CGC CAT TGT AG			
Rhodopseudomonas sp.	RhodoF	GCG GGA AGA TAA TGA CGG TA	400	60	in this study
	RhodoR	CAT TGT CAC CGC CAT TGT AG			
Pseudomonas spp.	PsF	TTA GCT CCA CCT CGC GGC	600	58	[22]
	PsR	GGT CTG AGA GGA TGA TCA GT			
Xanthomonas sp.	XantF	TGG GGA GCA AAC AGG ATT AG	500	62	in this study
	XantR	AGC CCT CTG TCC CTA CCA TT			
Kingella sp. .	KinF	CCA ATC CGA AAG ATT GGC TA	550	60	in this study
	KinR	ACG CAT TTC ACT GCT ACA CG			
Halomonas sp.	HaloF	AGA GGA TGA TCA GCC ACA CC	950	60	in this study
	HaloR	GCG ATA TTG CAA CCC TTT GT			

Species	Nitrifying / Denitrificant Bacteria				
	Primer	Primer Sequence (5'-3')	Amplicon Length (bp)	T. Annealing (°C)	Ref.
Paracoccus spp.	ParaF	TAA TAC CGT ATG CGC CCT TC	900	60	in this study
	ParaR	AAC TTC ATG GGG TCG AGT TG			
Alcaligenes sp	AlcaF	AAG GCT CAC CAA GGC AAC TA	900	60	in this study
	AlcaR	GTA CAA GAC CCG GGA ACG TA			

Nucleotide sequence analysis

All the PCR products were sequenced by Mycrosint Lab (GERMANY). The sequencing analyses were conducted using the BLAST program [23] in the GenBank.

III. Results

Chemical Analysis

Nitrate concentrations resulted lower than the law's limits (50 mg N/l) in all the water samples (figure 1) within the range 1 mg N /l - 32.60 mg N /l; in soil samples resulted in the range 10 mg N/kg - dry soil and 56 mg N/kg - dry soil, excepted Point A.3.U.W and Point A.3.D.W (1 mg N/kg - dry soil) and Point B.1.white (0.1 mg N/kg - dry soil) (figure 2). On the basis of these results, it can therefore be stated that the probability that nitrate concentration levels will exceed the threshold limits in the shallow groundwater, only due to percolation and infiltration of water through soil, is low.

Nitrites and ammonia concentrations were below or near detection limits in all water samples. In soil samples resulted less than 1 mg N/kg - dry soil, with the exception in Point B.1.white (1.09 mg N/kg - dry soil for nitrites concentration and 18.49 mg N/kg - dry soil for ammonia concentration) and in Point A.5.D.W and

Point A.5.U.W (ammonia concentrations: 9.54 mg N/kg - dry soil and 7.67 mg N/kg - dry soil respectively) (fig 1 and fig. 2).

The organic nitrogen concentrations in water samples resulted in the range 1.98 mg N/l - 51.95 mg N/l (figure 1). The ratio nitrates/organic nitrogen at each sampling point could be due to a different kinetic of nitrification. The organic nitrogen concentrations in soil samples widely ranged from 100 mg N/kg - dry soil to 700 mg N/kg - dry soil with an anomalous very low concentration (96.30 mg N/kg - dry soil) in the Point B.1.white (figure 2).

No correlation was found between organic nitrogen and COD in water samples; this, therefore, implies the presence of different composition in oxidisable matter present in the groundwater (figure 3).

The organic matter in soil presents a wide range of concentrations (figure 4), with very high values (8.2% Point B.1.U.W and 8.7% Point B.3.D.W) and some cases of very low concentrations (0.6% Point B.1.white and 0.2% Point A.3.D.W), with a correlation index (0.72) (figure 5) between organic matter and organic nitrogen, suggesting the presence in the soil of more homogenous organic compounds than in groundwater.

The nitrification rate,at the moment of sampling, was defined by the evaluation of nitrates and total nitrogen concentrations. In some cases, almost the complete nitrification of the total existing nitrogen is detected.It suggests an inconstant and/or not recent provision of organic substance in groundwater. In all the other cases a nitrification levels lower than 50% was detected; it would mean a constant and/or recent provision of organic substance in groundwater.

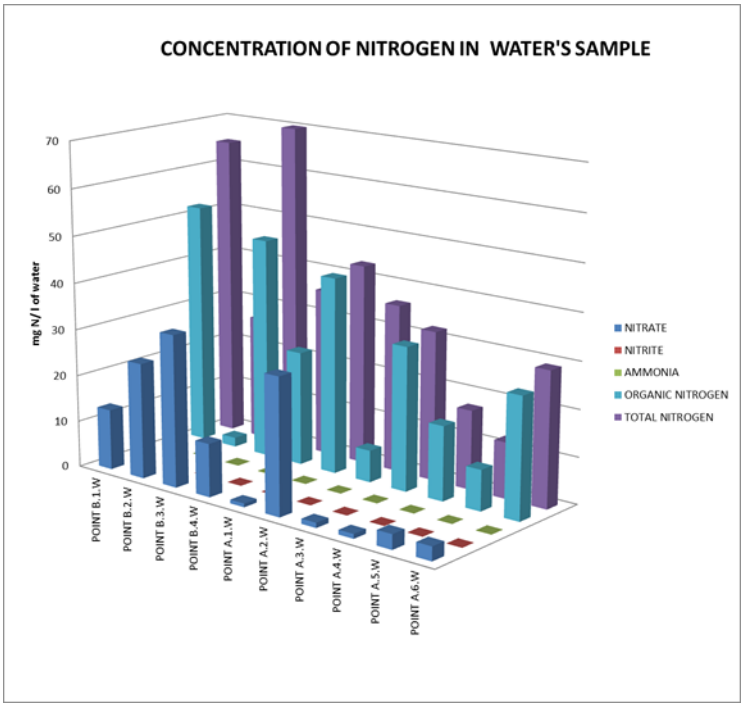

Figure 1. Concentration of nitrogen compounds in water's sample.

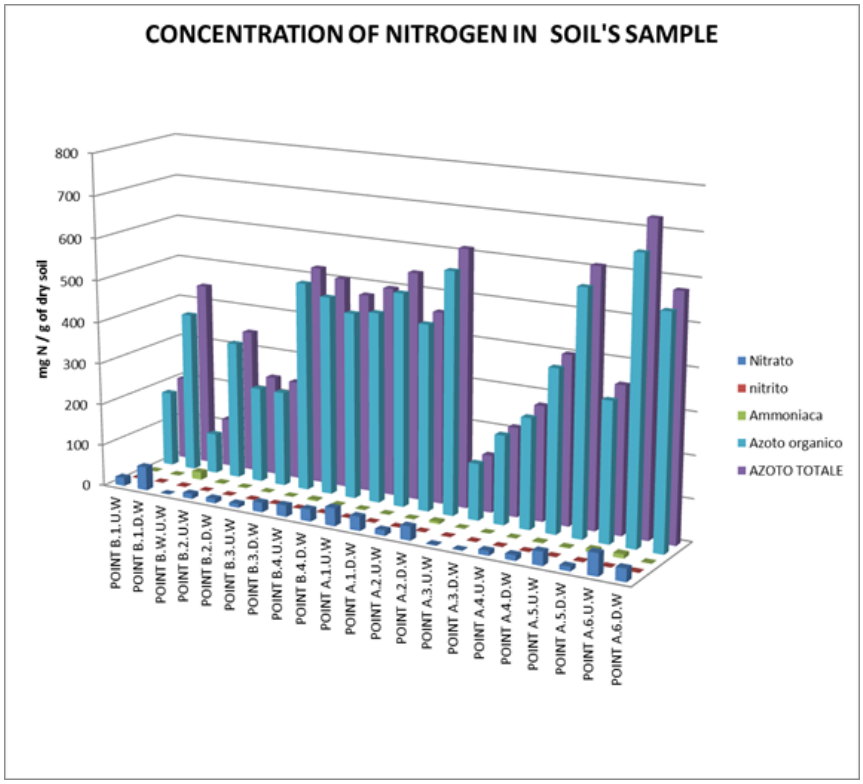

Figure2. Concentration of nitrogen compounds in soil's sample.

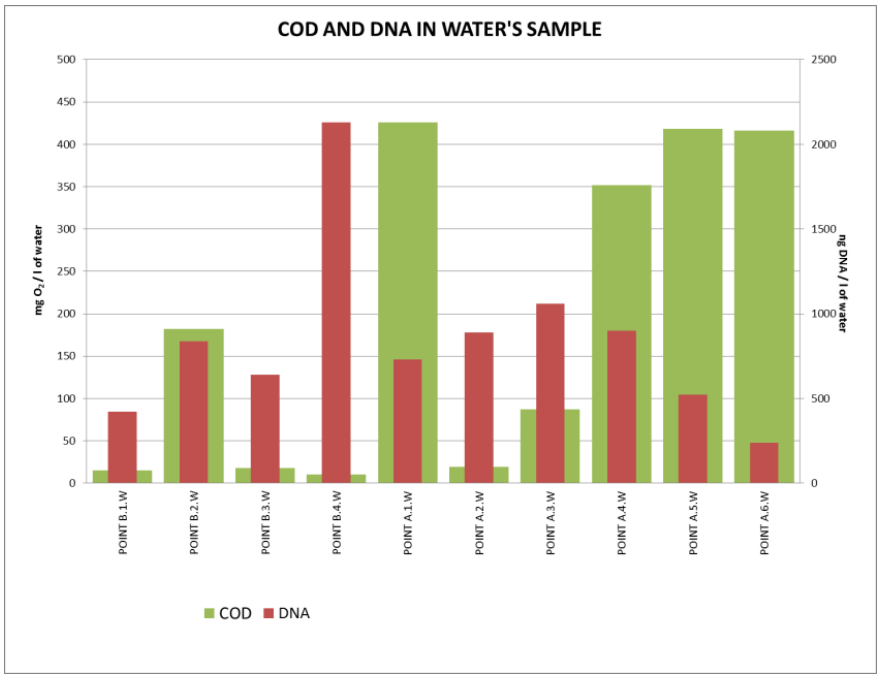

Figure3. Concentration of COD and DNA in water's sample.

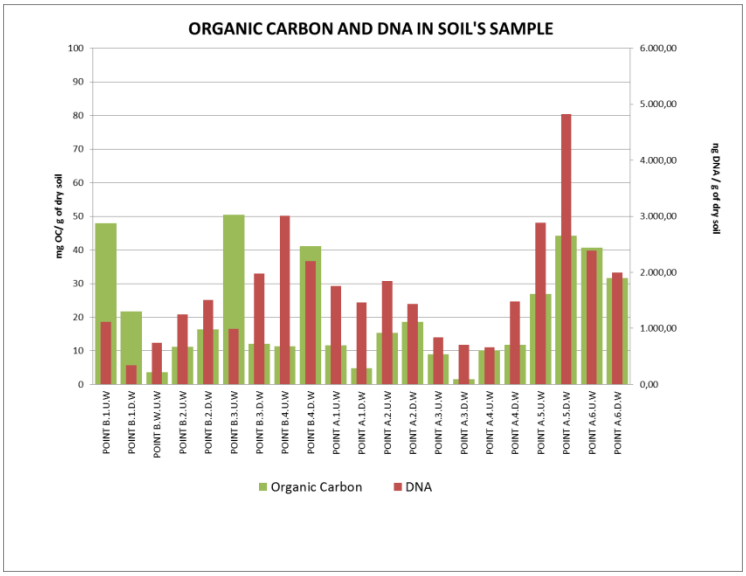

Figure 4. Concentration of Organic Carbon and DNA in soil's sample.

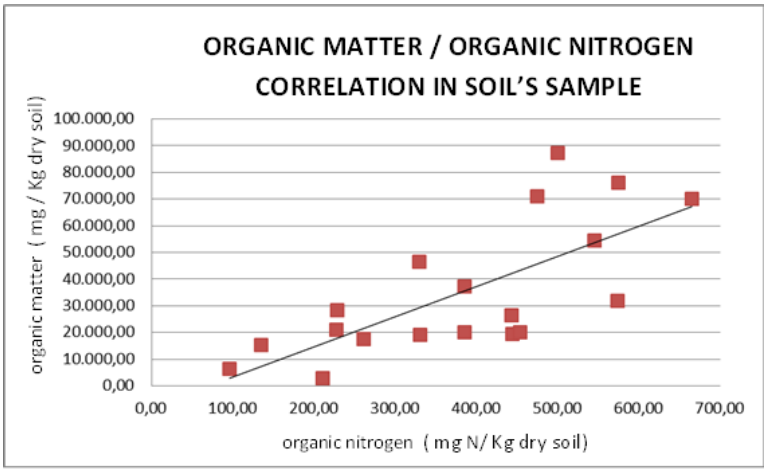

Figure5. Correlation Organic Matter/organic nitrogen in soil sample.

Biomolecular Analysis

DNA concentration in water sample resulted in the range 500 ng DNA/l - 1000 ng DNA/l, except Point A.6.W (236.8 ng DNA/l) and Point B.4.W (2128.47 ng DNA/l) (figure 3). In the soil samples the DNA extract resulted in the range (658.88 ng DNA/g - dry soil - 3012.67 ng DNA/g - dry soil), except Point B.1.D.W (338.59 ng DNA/g - dry soil) and Point A.5.D.W (4819.07 ng DNA/g - dry soil) (figure 4). The analysis of the DNA concentration had identified distinguishable range of DNA concentration in relation to the different use of soil: orchards present 1400 ng DNA/g - dry soil - 1900 ng DNA/g - dry soil; uncultivated field 1100 ng DNA/g - dry soil - 2900 ng DNA/g - dry soil; olive groves 1700 ng DNA/g - dry soil - 3000 ng DNA/g- dry soil; vegetable gardens 900 ng DNA/g - dry soil - 1500 ng DNA/g - dry soil; grasslands 300 ng DNA/g - dry soil - 600 ng DNA/g - dry soil ; arable lands 700 ng DNA/g - dry soil - 850 ng DNA/g - dry soil; vineyard 600 ng DNA/g - dry soil - 1500 ng DNA/g - dry soil. A high DNA concentration was present in the biodynamic vineyard (4800 ng DNA/g - dry soil) and in the field with a biological farming practice (2000 ng DNA/g - dry soil). Particularly relevant the results regarding the vegetable garden with a very low DNA concentration (740 ng DNA/g - dry soil) (figure 6).

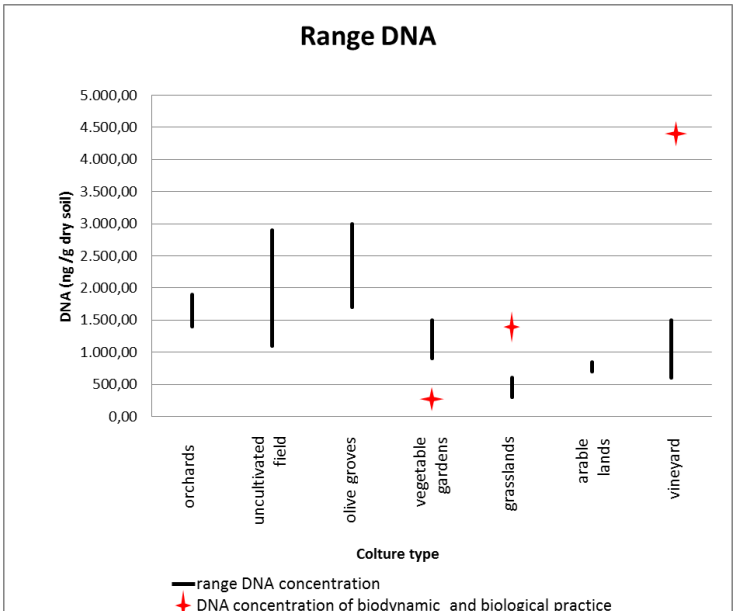

Figure 6. Range of DNA concentration in soil for different cultivation type.

Case of biodynamic and biological agriculture practice.

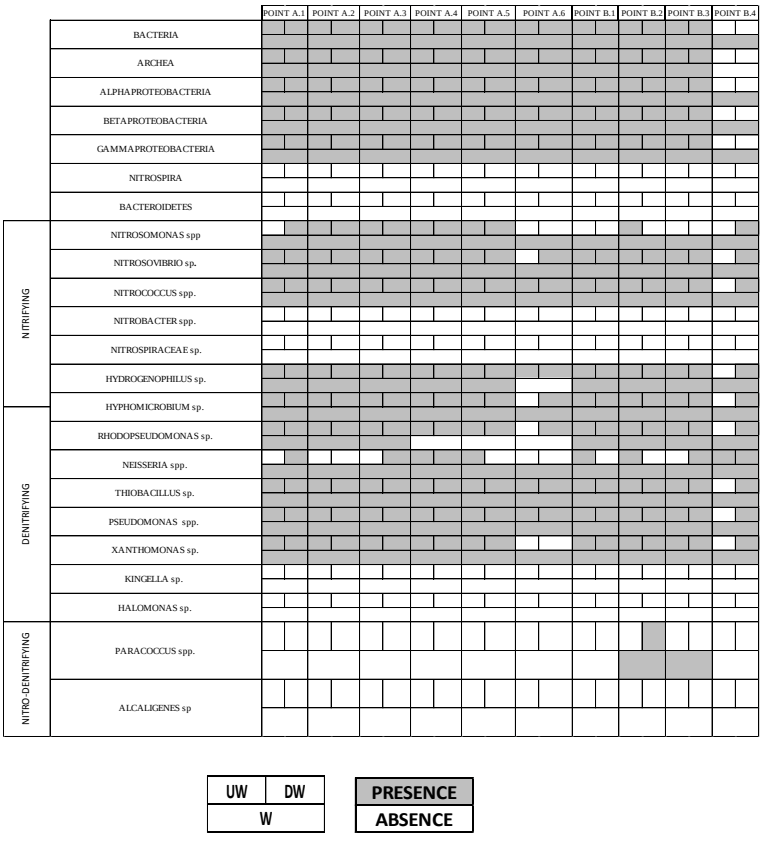

Figure7. PCR schematic result of presence and absence of DNA amplification. SU: Soil sample upstream; SD: Soil sample downstream; W: water sample

The PCR analysis (figure 7) determined the presence of generic bacterial DNA in all samples and of Archaea DNA except in Point B.4.W for water samples and in Points B.3.D.W and Point B.4.U.W for soil sample. In all samples the presence of Alfaproteobacteria, Betaprotobacteria and Gammaproteobacteria was detected, except soil samples Points B.3.D.W and Point B.4.U.W.

The absence of Nitrospira and Bacteroidetes DNA has been shown in all samples.

The analyses of nitrifying species demonstrate the absence in all samples of Nitrobacter sp. DNA; the presence of Nitrosovibrio sp. and Nitrococcus spp. DNA, except for the soil sample Point B.4.U.W; the presence of Nitrosomonas spp. and Nitrococcus spp. DNA in all water samples and absent in soil samples Point B.2.D.W, Point B.3.U.W, Point B.3.D.W, Point B.4.U.W, Point B.1.U.W, Point B.1.D.W, Point B.1.white and Point A.6.U.W and Point A.6.D.W.

The DNA of Nitrospiraceae sp. where always absent in all samples, thus confirming the observations Nitrospira class carried out and previously described.

The analyses of the denitrifying species demonstrate: the presence of Rhodopseudomonas sp. DNA in all samples except in Point A.4.W and Point A.6.W of the water sample, and in Point B.4.U.W and Point A.6.U.W of soil sample; the presence of Hyphomicrobium sp. in all samples except for the soil samples Point B.4.U.W and Point A.6.U.W; the presence in all sample of the Thiobacillus sp. DNA and Pseudomonas spp. DNA, except for soil sample Point B.4.U.W; the absence in all sample of Kingella sp. DNA and Halomonas sp. DNA, the presence of Neisseria spp. DNA in all water sample and in soil sample only in Points B.2.U.W, Points B.3.D.W, Point B.4.U.W, Point B.1.U.W and Point A.1.D.W, Point A.4.U.W, Point A.4.D.W and Point A.5.U.W; the presence of Xanthomonas sp. DNA in all samples except for Points B.4.U.W, Point A.6.U.W and Point A.6.D.W; the presence of Hydrogenophilus sp. DNA is observed in all water samples, except for Point A.6.W and of soil, except for Point B.4.U.W and Point A.6.U.W.

The analyses of the nitro-denitrifying species showed: the presence of Paracoccus spp. DNA only in water sample Point B.2.W and Point B.3.W and in Point B.3.D.W of soil sample; completely absent is the Alcaligenes sp. DNA. A critical situation is represented by the Point.B.4 were in the soil Point B.4.U.Wonly Neisseria spp. was found; not even one other bacteria of nitrogen cycle.

IV. Discussion

First Observations On The Possible Correlation Phenomena Between Soil Matrix And Water Matrix.

The nitrate concentration in soil was 60% of the cases (Point B.4, Point B.1, Point A.1, Point A.4, Point A.5, Point A.6) higher than in both groundwater samples taken downstream and upstream of the hydraulic flow. In only 20% of cases (Point B.2 and Point B.3) the nitrates concentration was higher in groundwater than in the soil. The following Points represent two particular cases: Point A.3 with a nitrate concentration in groundwater higher than in the soil taken downstream of the hydraulic flow but lower compared to the soil taken upstream of the hydraulic flow; Point A.2 with a nitrate concentration in groundwater higher than in the soil taken upstream of the hydraulic flow but lower compared to soil taken downstream of the hydraulic flow.

The nitrites, ammonium and organic nitrogen, in 100% of cases presented a concentration higher in the soil, both in hydraulic upstream and in hydraulic downstream samples, than in groundwater.

In the 30% of cases the concentration of organic nitrogen in soil exceeded by about 2 orders when compared to groundwater.

The DNA concentration between soil samples and groundwater samples determined two prevalent trends.

The main case is represented by DNA concentration in the soil taken upstream of the hydraulic flow. Actually it was considerably higher (greater than 40%) than DNA concentration in groundwater and downstream. This situation is recorded in 50% of analyzed sites, in which there is always a different soil use.

The second case shows a DNA concentration soil sample taken upstream of the hydraulic flow considerably higher than the groundwater sample, but lower than the DNA concentration in soil sample taken downstream of the hydraulic flow from the same site. This situation is recorded in 30% of cases (Point B.2, Point B.3, and Point A.5) where a different use of soil is observed except for Point B.2 with the same use of soil upstream and downstream.

The following points represented two particular cases: Point A.4 in which the DNA concentration in groundwater is considerably higher (greater than 36%) than the DNA concentration in soil sample taken upstream of the hydraulic flow, but lower than that recorded in soil sample taken downstream of the hydraulic flow; Point A.3 in which the DNA concentration in groundwater is greater than the soil sample with the same type of use of soil.

The qualitative microbial correlation of species between the soil samples in upstream of the hydraulic flow and the water sample were in the range of 75% - 94%.

In particular, there is a correspondence of 94% in species of six sites (Point B.1, Point B.2, Point A.3, Point A.4, Point A.5), 85% in Point A.1, 82% in Point B.3, 75% in Point A.6; an extreme case is Point B.4 with a correspondence lower than 44%.

The qualitative microbial correlation of species between the soil samples taken downstream of the hydraulic flow with the water sample were in a range of 85%- 100%. In particular, in the Point B.4, Point A.1, Point A.3 was find a correspondence of 100%; in the Point B.3, Point A.2 and Point A.4 was find a correspondence of 94%; in the Point B.1 and Point A.5 was find a correspondence of 85%; in the Point B.2 was find a correspondence of 82% and in the Point A.6 was find a lower correspondence equal to 69%.

Considering the soil samples of the same site, without distinguishing between samples in upstream and downstream of hydraulic flow, it is observed a more considerable correspondence of microbiological species with the equivalent water sample. In this case the correspondence was in the range 94% - 100%, except for Point B.1 with a lower correspondence (81%).

Moreover, particular cases some species are punctually present or in groundwater or in soil upstream and/or downstream, specifically, the presence of the following species was detected only in water samples: Nitrosomonas spp in point B.1 and Point B.3, in Point B.2 of Paracoccus spp., Point A.2 of Neisseria spp.; in Point A.6 of Nitrosomonas spp., Neisseria spp. and Xanthomonas sp.; only soil samples showed the presence of Rhodopseudomonas sp in Point A.4 and A.5; the presence of Hydrogenophilus sp. and of Rhodopseudomonas sp was dectected in soil samples taken upstream of the hydraulic of Point A.6.

Significant Evidence Related To Specific Agronomic Practices

The chemical analyses of vineyard with biodynamic agricultural practice determined organic matter concentration higher than in vineyards characterized by traditional agricultural practices, as well as for the organic carbon which is four times higher in the biodynamic vineyard. This situation, instead, is not recorded in the biodynamic olive grove whose concentration values of organic matter and organic carbon are within the standard range of the other olive groves analyzed.

Otherwise, the organic nitrogen concentration is substantially higher in the biodynamic olive grove than in those characterized by traditional agricultural practices, while for such parameter the biodynamic olive grove is within the range.

Nitrites and ammonia concentrations, resulted higher both in the biodynamic vineyard and in the biodynamic olive grove, if compared to the sites subjected to the same use of soil adopting traditional agricultural practices. Conversely, nitrates concentration is higher in the biodynamic olive grove, while it is within the range in the biodynamic vineyard.

As regards the sites with agronomic practice of biological type, an increase of organic matter and of organic nitrogen can be detected in the biological grassland as well as a decrease in the nitrate, nitrites and ammonia concentration compared to other sites with the same use of soil. With regard to the biological vegetable garden, an abnormal situation is detected, that is, a low organic matter and organic nitrogen concentration as well as a considerable increase of nitrite and ammonia concentration.

The analyses of DNA concentrations suggest that the biodynamic vineyard presents a high DNA concentration equal to 4800 ng DNA/g - dry soil, the grassland under biological farming practice presents a concentration equal to 2000 ng DNA/g - dry soil, while the biodynamic olive grove is within the range of the other olive groves (from 1700 ng DNA/g - dry soil to 3000 ng DNA/g - dry soil). An exceptional case is the biological vegetable garden with a very low DNA concentration equal to 740 ng DNA/g - dry soil.

As regards the analyses of microbiological species identified there is an almost complete homogeneity between the biodynamic vineyard and those subjected to a traditional agronomic activity, except for Neisseria spp which is absent in the biodynamic vineyard. No discernible characteristics appear, with respect to the microbiome, in the biodynamic olive grove, in the biological grassland and in the biological vegetable garden.

V. Conclusion

The present study is part of a research program concentrated on the verification, and further improvement, of metagenomics analyses procedures of environmental matrices with final aim to adopt such methods in the study of biogeochemical phenomena, with particular focus on the possible detection of the alteration sources of the ecological balance of anthropic origin.

This work presents the first attempt to apply the developed methodologies to phenomena of groundwater contamination from nitrates, with the purpose of distinguishing the type of contamination when related to particular microbiomics environments of soil and shallow groundwater.

Samples of surface soil were taken upstream and downstream of the groundwater collection point. Analyses of the main chemical parameters and of the microbiome involved in the nitrogen biogeochemical cycle were carried out through a specific definition metagenomics technique. The analytical procedure adopted, implemented with further quantitative real-time to estimate specific microbial communities in different matrices,

showed to be suitable for the identification of factors responsible for nitrate contamination. In addition, it provides useful information on the relationship and possible interaction between soil and groundwater. However, this is attainable only with a tight monitoring of all parameters throughout the time, with a number of points (samples) sufficiently representative, and accompanied by any useful information about possible changes of farming methods and agronomic techniques.

The threshold level for nitrates in groundwater has not been reached in all cases.

Differences emerge between the types of organic molecules containing or not containing nitrogen in groundwater. This result will be useful to further studies in identifying the structure of these molecules and, therefore, determine whether they originate from soil natural composition or anthropogenic pressure. Moreover, it could represent a factor of selective pressure imparted on different microbial consortia with different proportional rates between denitrifying and nitrifying populations.

A constant proportion between nitrogen compounds concentration in soil and in groundwater is not evident. A concentration of nitrite, ammonia and organic nitrogen is recorded higher in soil than in groundwater.

The soil of biodynamic vineyard shows The highest DNA concentration equal to more than double the second greatest value found in the grassland soil under stringent biological activity.

The presence of bacterial species in the nitrogen cycle in the two matrices shows a highly articulated framework, with some species always absent and others always present in all the matrices.

There is a correlation between the microbiological species present in soil and groundwater, ranging from 94% to 100%. This finding could become particularly relevant with the future application of Real-Time and the quantification of population abundance per species.

As regards the distinctive characteristics of different use of soil and agronomic practices, there is an evident distinction of chemical parameters (as the organic matter) in the biodynamic vineyard cultivation. Conversely, other differences appear less marked at this study phase.

A critical situation is represented by the Point.B.4 were in the soil Point B.4.U.W only Neisseria spp. was found; not even one other bacteria of nitrogen cycle.

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