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Use of Molecular Techniques for Identification of Nitrate Contamination Sources in Groundwater

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Abstract

The groundwater ecosystems are regulated by the limiting environmental factors (absence of light, availability of nourishing, low temperatures, etc.) that select microbial communities well-fitting conditions of “extreme” life. The microbial communities in the groundwater are mainly constituted by well adapted heterotrophy and they are characterized by the state of hydrological chemistry and geologic heterogeneity of the stratum. The microbial communities of the groundwater develop according to the typology of contamination (point or diffused) and the category of contaminants as well. The "classical methods" procedures of microbial community analysis provide for the identification of microorganisms from pure culture isolation. These analyzes are not sufficient to identify most species of microorganisms and are limited to cultivable species which represent a very small percentage of all species present in nature. Research on environmental microbiology has shown that microbial communities play a functional role in controlling ecosystems, which cannot be attributed to individual species. New methodologies have been developed that enable to analyze the structure of microbial communities independently of the isolation phase. These work illustrated a pilot action carried out to identify potentially contaminating sources of nitrate in the ground on the whole Apulian territory (livestock effluents, urban wastewater, sewage sludge), by the correlation between certain sources of contamination and specific bacterial species.

Keywords: Bacteria Investigation, Biomolecular Technique, Groundwater, Nitrate Contamination.

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Introduction

The groundwater ecosystems are limited by the presence of environmental factors (absence of light, limited availability of nourishing, low temperatures, etc.) that condition the existence of peculiar microbial community, sometime in conditions of life so called "extreme."

Today it is known that the whole system of the subsoil is colonized by the microorganisms, mainly present in microbial community, constituted by bacteria and Archaea, but also from protozoa and fungi, that develop remarkable roles in the trials biogeochemical.

The microbial communities in the groundwater are mainly constituted by well adapted heterotrophy to the underground environment and they are characterizing by hydrological, chemistry and geologic of the stratum. The presence of different biotic and abiotic factors can influence in direct or indirect way the microbial difference in the ecosystems; it is generally characterized by a typical stable bacterial community that is directly influenced by the chemical-physical parameters. It is influenced from the spatial heterogeneity of the geologic unity, from the pressures anthropic on the same one, from the nature of the layer of impending ground the stratum, from the biogeography and from the use of the superficial soil.

The stability of the bacterial communities is interrupted if there are spatial variations of the groundwater chemical-physical parameters, caused by a superficial contamination. Such change can determine three different dynamics of transformation of the micro biotic communities present: (i) quantitative variation of already determined present bacterial fetterless in them; (ii) inclusion of new alien bacterial fetterless; (iii) disappearance of some bacterial fetterless - cause the presence of toxic substances - with possible occupation of the ecological site of a new alien microorganism. Besides, in base to the typology of contamination (point or diffused) and the class of contaminants different impacts are had on the microbial communities of the groundwater.

The "standard" procedures of analysis of the microbial communities, or "classical methods", they foresee the identification of the microorganisms through the isolation of pure crops, followed by test that they analyze some morph-physiological and biochemical characteristics. These analyses are not enough for the identification of the greatest part of the microorganisms and they are limited to the cultivable species that represent a minimum percentage of all the species present in nature. These tests, introduce besides the serious limit to ask for a notable waste of time, and they economically result often onerous. During the last decades, the searches in the field of the environmental microbiology have shown that microbial communities develop a functional role of control of the ecosystems that is not referable to single species. For these reasons, new methodologies have been developed. One of the most important technic is the biomolecular analysis that is based on the individualization of a specific genomics region of the microorganism DNA. With the development of the DNA analysis it has been reached levels of sensibility ever gotten with the traditional other techniques of survey. These methods are usually rapid and they can be applied for the search both of specific pathogens that of groups of microorganisms. The identification of the present species in the environmental matrix and of the relative

DNA allows a direct correlation of the possible contamination with the sources to it connected, also supporting studies of molecular epidemiology and phylogenetic analysis.

Molecular diagnosis is generally more sensitive and more specific to traditional culture methods and requires shorter time for identification. The gene sequencing of amplified fragments also allows the identification of the present bacterial species. With specific reference to the definition of the source of nitrate contamination, the identification of the microbiological species present in the groundwater allows to identify the source of contamination. Several studies have shown that certain sources of contamination are related to well-defined bacterial species, in particular anthropogenic contamination can be identified by identification of BIFIDOBACTERIUM (Barrett et al., 2002) and by ENTEROCOCCUS FAECALIS AND FACIUM (Boccia et al., 2002; Eigner et al., 2008), or contamination due to incorrect spillage or manure accumulation can be detected by identifying BACTEROIDES-PREVOTELLA, ENTEROCOCCUS AVIUM, ENTEROCOCCUS CASSELIFLAVUS, ENTEROCOCCUS DURANS, ENTEROCOCCUS GALLINARUM, ENTEROCOCCUS HIRAE, ENTEROCOCCUS SACCHAROLYTICUS (Savichtcheva and Okabe, 2006). For this purpose, a pilot action has been carried out to identify potentially contaminating sources of nitrate in the groundwater on the whole Apulian territory, distinguishing the origin of nitrate from livestock effluents, urban wastewater, sewage sludge. Consequently, the priority objective of this action was to identify with greater certainty the causes of nitrate contamination by distinguishing the agricultural, livestock or civil source. With specific reference to the definition of the source of nitrate contamination, the identification of the species present in the environmental matrix (water) of its nucleotide sequences allows to identify the certain source of contamination. Through the recognition bacterial species with their genetic kit, it is possible to determine whether contamination comes from a strictly anthropogenic source (such as untreated septic tanks or sewage sludge) or it comes from an inadequate spread of animal flywheels.

In the present work, the biomolecular technical has been used for conducting an investigation metagenomica within an pilot action realized for the Apulian Region and finalized to the individualization of the potential sources of contamination by nitrates in a number selected of portions of aquifer through wells located on the Apulian territory. In the specific one, an analysis has been conducted contemplated for the identification of bacterial kind selected that they are indicative of the source of origin of the nitrates in water samples. The principal objective of the job has been that to identify exactly the origin of the in relief nitrates in the investigated waters samples, with the purpose to individualize the causes of contamination (zootechnology outflowing, urban waste, muds of purification) in the resulted wells polluted in comparison to natural values leading.

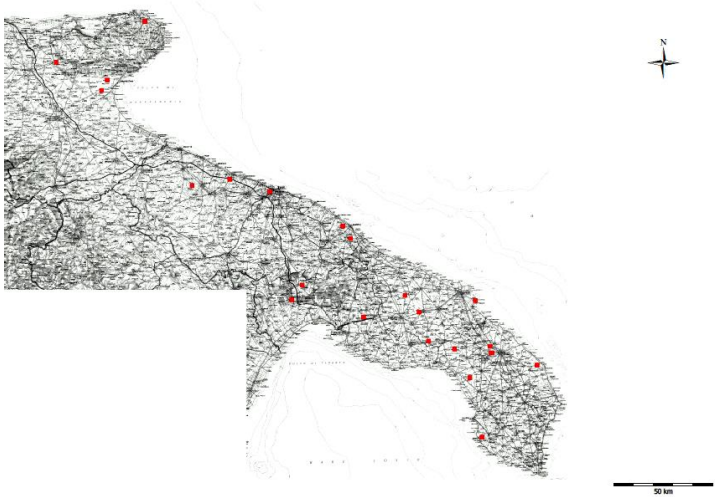
Materials and Methods

Sampling Activities

Within the present activity it has been planned and realized a specific country of sampling that has interested 22 wells displaced on the territories of the five provinces of the Apulian Region. Such wells have been selected on the base of the results gotten in precedents activity of monitoring of the regional water bodies that have underlined the presence of contamination from nitrates (superior concentration to the 50 mg/l NO_3).

For the present activity it has been applied in all the wells a sampling "instant" type dynamic or in flow according to the formalities brought in the D.lgs. 31/2001. Overall there has been investigated: 5 wells in the province of Foggia, 4 in the province of Bari, 4 in the province of Brindisi, 3 in the province of Taranto and 6 in the province of Lecce (Figure 1).

Figure 1. *Identification of the Wells for the Study*



Chemical Analysis

On every sample of water, the analytical determinations of a set of chemical parameters, has been done. It has been conducted (Table 1) with the purpose to be able to verify the actual state of the contamination of the previously identification. The analysis of the parameters is finalized to the evaluation of the typology of contamination in the champions of water. Elevated values of ammonia and nitrate can be correlated to anthropic contaminations or to imputable contaminations to the presence of breeding of livestock; besides, an excessive content of phosphates and sulfates can be correlated to intensive agricultural activity. Finally, the analyses of the metals (lead, cadmium, nickel and zinc), have been conducted for underlining possible imputable contaminations to industrial activity or of illegitimate disposal of solid and liquid refusals.

Table 1. *Chemical Analysis*

Parametro	**Unità di misura**
pH	
Electrical conductivity	mS/cm
Salinity	%NaCl
Total Dissolved Solid	mg/L
Nitrate	mg/L di NO_3
Ammonium	mg/L di NH_4
Nitrite	mg/L di NO_2
Total Nitrogen	mg/L di N
Chemical Oxigen Demand	mg/L O_2
Phosphate	mg/L di PO_3
Sulphate	mg/L di SO_4
Copper	mg/L di Cu
Cadmium	mg/L di Cd
Piombo	mg/l di Pb
Nichel	mg/l di Ni
Zinc	mg/l di Zn

Biomolecular Analysis

On every of the sample of water has been realized a genetic investigation to the identification of a number of indicative bacterial species of the source of origin of the nitrates.

For the molecular analyses, between 150 and 250mL of water (depending on the amount of suspended particulate matter (SPM)) were filtered through 0.22 mm nitrocellulose filters (Durapore, 45mm diameter) in triplicates and the filters were frozen (20 1C) until the DNA extraction. DNA was extracted from the filters by a bead-beating method with the FastDNA spin Kit for soil (Bio 101, Lajolla, CA, USA) according to the manufacturer's instructions. The DNA extracts were then stored at -20°C. The DNA was visualized by UV transillumination (GelDoc 200, BioRad). Digital images of the gels were obtained with a CCD camera controlled by the software Quantity one (BioRad).

On the base of previous studies and of the bibliographical indications, it was created a genomic investigation to identifying the bacterial species brought in Tab II, through the individualization of specific genic sequences of the region 16S rDNAs. These analysis was been done using the Polymerase Chain Reaction (PCR). It allows the amplification of the genetic sequences after opportune extraction of the bacterial DNA.

For the analysis of 16S rDNA it was used generic primers 16Sf/16Sr (5'-AGA GTT TGA TCA TGG CTC AG-3'/5'-TAC GGC TAC CTT GTT ACG ACT T-3') that amplified a sequence of 1.500 bp. The reaction was carried out with a solution of 50 μL containing: 20 ng of DNA, each deoxynucleoside triphosphate at a concentration of 200 to 400 mM, 1.5 mM $MgCl_2$, 10 mM NaCl, 0.01 mM EDTA, 0.1 mM dithiothreitol, 5 mM Tris HCl (pH 8.0), 2% dimethyl sulfoxide, 5% glycerol,

0.1% Triton X-100, and 2.5 U of Taq polymerase. The concentration of each primer was equal to 0.15 mM. The reaction step was been: incubation 94°C for 2 minutes, 30 cycles with incubation 94°C for 1 minute, annealing 45°C for 1 minute and elongation 72°C for 1 minute, final elongation 72°C for 2 minutes.

The identification of the BIFIDOBACTERIUM - present bacterium exclusively in human feces (Layton et al., 2006; Anne et al., 2000; Kimura et al., 1997; Kreader, 1995; Resnick and Levin, 1981) – it has been conducted using probes site-specific for the region 16S rDNAs. For the analysis of 16S rDNA it was used generic primers Bif164F / Bif601R (5'- GGG TGG TAA TGC CGG ATG -3'/5'- TAA GCG ATG GAC TTT CAC ACC -3') that amplified a sequence of 313 bp. The reaction was carried out with a final solution 50 μl:10 ng of DNA, for each deoxynucleoside triphosphate a concentration of 200 to 400 mM, 1.5 mM of MgCl2, 10 mM of NaCl, 0.01 mM of EDTA, 5 mM of Tris HCl (pH 8.0) and 2, 5 U of Taq polymerase. The concentration of each primer was 0.15 mM. The reaction step was been: incubation 94°C for 5 min, 35 cycles: incubation 94°C for 30 sec, annealing 53°C for 1 min, elongation 72°C for 1 minute, final elongation 72°C for 6 min.

The identification of the BACTEROIDES-PREVOTELLA, present bacterium exclusively in bovine feces (Layton et al., 2006; Anne et al., 2000; Fiksdal et al., 1985), it has been realized using probes site-specific for the region 16S rDNAs For the analysis of 16S rDNA it was used specific primers GM5F/ DS907-reverseb (5'-CCTA CGG GAG GCA GCA G-3'/5'-CCC CGT CAA TTC CTT TGA GTT T-3') that amplified a sequence 453bp and 222bp. The reaction was carried out with a final solution 50 μl:20 ng of DNA, for each deoxynucleoside triphosphate a concentration of 200 to 400 mM, 1.5 mM of MgCl2, 10 mM of NaCl, 0.01 mM of EDTA, 5 mM of Tris HCl (pH 8.0) and 2, 5 U of Taq polymerase. The concentration of each primer was 0.25 mM. The reaction step was been: incubation 95°C for 5 min, 35 cycles: incubation 95°C for 1 min, annealing 65°C for 1 min, elongation 72°C for 3 minutes, final elongation 72°C for 10 min.

The identification of ENTEROCOCCUS FECALIS, present in human and feces (Jackson et al., 2004; Baele et al., 2000; Deasy et al., 2000; Ke et al., 1999) it has been realized using probes site-specific for the region 16S rDNAs For the analysis of 16S rDNA it was used specific primers FL1/ FL2 (5'- ACT TAT GTG ACT AAC TTA ACC -3'/5'- TAA TGG TGA ATC TTG GTT TGG -3') that amplified a sequence of 450 bp. The reaction was carried out with a final solution 50 μl: 10 ng of DNA, for each deoxynucleoside triphosphate a concentration of 200 to 400 mM, 1.0 mM MgCl2, 10 mM NaCl, 0.01 mM EDTA, 5 mM Tris HCl (pH 8.0) and 2.5 U of Taq polymerase. The concentration of each primer was equal to 0.15 mM. The reaction step was been: incubation 95°C for 10 min, 35 cycles : incubation 95°C for 30 sec, annealing 50°C for 1 min, elongation 72°C for 1 minute, final elongation 72°C for 5 min.

The identification of ENTEROCOCCUS FECIUM, present in human feces (Boccia et al., 2002; Eigner et al., 2008) it has been realized using probes site-specific for the region 16S rDNAs For the analysis of 16S rDNA it was used specific primers FM1B / FM2B (5'- ACA ATA GAA GAA TTA TTA TCT G -3'/5'- CGG CTG CTT TTT TGA ATT CTT CT -3') that amplified a sequence of 350 bp. The reaction was carried out with a final solution 50 μl:10 ng of DNA, for each deoxynucleoside

triphosphate a concentration of 200 to 400 mM, 1.0 mM of MgCl2, 10 mM of NaCl, 0.01 mM of EDTA, 5 mM of Tris HCl (pH 8.0) and 2, 5 U of Taq polymerase. The concentration of each primer was 0.15 mM. The reaction step was been: incubation 95°C for 10 min, 35 cycles: incubation 95°C for 30 sec, annealing 50°C for 1 min, elongation 72°C for 1 minute, final elongation 72°C for 5 min.

The identification of the NITROBACTER - present bacterium exclusively agriculture fertilizers (Degrange and Bardin, 1995; Cébron and Garnier, 2005; Chartrain et al., 1983; Bock et al., 1990) – it has been conducted using probes site-specific for the region 16S rDNAs. For the analysis of 16S rDNA it was used specific primers FGPS1269/ FGPS872 (5'- TTT TTT GAG ATT TGC TAG -3'/5'- CTA AAA CTC AAA GGA ATT GA -3') that amplified a sequence of 220 bp. The reaction was carried out with final solution 50 µl: 20 ng of DNA, for each deoxynucleoside triphosphate a concentration of 200 to 400 mM, 1.5 mM MgCl2, 10 mM NaCl, 0.01 mM EDTA, 0.1 mM dithiothreitol, 5 mM Tris HCl (pH 8.0), 2% dimethyl sulfoxide, 5% glycerol, 0.1% Triton X-100 and 2.5 U of Taq polymerase. The concentration of each primer was equal to 0.15 mM.

The reaction step was been: incubation 95°C for 10 min, 35 cycles: incubation 95°C for 1 min, annealing 50°C for 1 min, elongation 72°C for 1 minute, final elongation 72°C for 7 min.

The identification of the NITROSPIRA - present bacterium exclusively agriculture fertilizers (Dionisi et al., 2002; Cébron and Garnier, 2005; Bock and Koops, 1992; Burrell et al., 1998; Juretschko et al., 1998; Gieseke et al., 2001) – it has been conducted using probes site-specific for the region 16S rDNAs. For the analysis of 16S rDNA it was used specific primers 16F27N /16R1525 (5'-CCA GAG TTT GAT CMT GGC TCA G-3'/5'- TTC TGC AGT CTA GAA GGA GGT GWT CCA GCC-3') that amplified a sequence of 397 bp. The reaction was carried out with a final solution 50 µl:20 ng of DNA, for each deoxynucleoside triphosphate a concentration of 50 mM, 1.5 mM of $MgCl_2$, 10 mM of NaCl, 0.01 mM of EDTA, 5 mM of Tris HCl (pH 8.0) and 2.5 U of Taq polymerase. The concentration of each primer was 0.20 mM. The reaction step was been: incubation 94°C for 5 min, 35 cycles: incubation 94°C for 2 min, annealing 55°C for 1 min, elongation 72°C for 1 minute, final elongation 72°C for 15 min. The identification of ENTEROCOCCUS, present in animal feces (Savichtcheva and Okabe, 2006) it has been realized using probes site-specific for the region 16S rDNAs For the analysis of 16S rDNA it was used specific primers 27f/1525R (5'-CCA GAG TTT GAT CCT GGC TCA G-3'/5'-TTC TGC AGT CTA GAA GGA GGT GGT CCA GCC-3') that amplified a series of sequence from 220 bp to 500 bp. The reaction was carried out with a final solution 50 µl:20 ng of DNA, for each deoxynucleoside triphosphate a concentration of 200 mM, 3.75 mM of MgCl2, 10 mM of NaCl, 0.01 mM of EDTA, 5 mM of Tris HCl (pH 8.0) and 5 U of Taq polymerase. The concentration of each primer was 0.20 mM. The reaction step was been: incubation 94°C for 3 min, 35 cycles: incubation 94°C for 30 sec, annealing 63°C for 30 sec, elongation 72°C for 2 minutes, final elongation 72°C for 10 min.

PCR amplifications were carried out in a total volume of 50 mL in 0.2 mL tubes using a DNA-microcycler (BioRad), according to the thermal profiles described in Table 3. Negative (sterile milliQ water) and positive control tubes were

simultaneously performed for all PCR reactions to avoid any false positive or false negative results. Aliquots of amplification products were analyzed by gel electrophoresis on 2.0 % agarose gels (Euroclone).

The biomolecular information has been elaborate through bioinformatics technic with the purpose to be able to identify with precision the referable bacterial species to specific sources of contamination. The bioinformatics study has allowed, besides, the identification of the present bacterial community in the sampled wells. The Bacterial species that have been used in the study are shown in Table 2.

Table 2. *Bacterial Species used in the Study*

Bacterial species	**Kind of contamination**
Bifidobacterium	Anthropic contamination (Barrett and Howard, 2002)
E. Faecalis	Present in wastewater (Boccia et al., 2002; Eigner et al., 2008)
E. Faecium	Present in wastewater (Boccia et al., 2002; Eigner et al., 2008)
Bacteroides-Prevotella	Present in animal feces (Savichtcheva and Okabe, 2006)
E.Avium	
E. Casseliflavus	
E. Durans	
E. Gallinarum	
E. Hirae	
E. Saccharolyticus	
Nitrobacter	Intensive agriculture (Cébron and Garnier, 2005)
Nitrospira	Intensive agriculture (Cébron and Garnier, 2005)

Results

Chemical analyzes revealed that of 22 wells investigated, nitrate contamination (exceeding the law limit of 50 mg/l of NO_3) was recorded in 7 wells: one in the province of Foggia, one in the province of Bari, four in the province of Brindisi and one in the province of Lecce.

The biomolecular analyses had demonstrated that the Bacteroides-Prevotella bacterial strain, typically seen in bovine stool (Layton et al., 2006; Anne et al., 2000; Fiksdal et al., 1985), was found exclusively in two of the twenty-two water samples investigated whose chemical analyzes have highlighted limitations of the regulatory limits for the concentration of nitrates: one taken in the province of Foggia and the other taken in the province of Brindisi. The Enterococcus Faecalis was found: in two samples taken in the province of Foggia, in one sample taken in the province of Taranto and in four of the five samples taken in the province of Brindisi, for three of them the analytical determinations of the nitrates have highlighted exceedances of the normative threshold concentration. Therefore, in such wells the contamination is due to anthropic sources because the species found is typically present in human feces (Jackson et al., 2004; Baele et al., 2000;

Deasy et al., 2000; Ke et al., 1999). This bacterial species has also been found in two samples of Lecce. The Nitrobacter was found in all samples of water taken in the province of Bari, in a sample taken in the province of Taranto and in three samples taken in the province of Lecce. Of these, a value of nitrates above the regulatory limits was observed exclusively for the 7BA sample. Therefore the identification of this bacterial strain, present in the areas in which there is a particular use of specific agricultural fertilizers (Cébron and Garnier., 2005; Chartrain et al., 1983; Bock et al., 1990; Degrange and Bardin, 1995) is indicative of contamination from nitrates from agricultural sources. The Bifidobacterium was found: in four water samples taken in the province of Foggia, in one sample of the province of Taranto, in four samples taken in the province of Brindisi and in two samples of the province of Lecce, the presence of this bacterial species has not been found in any sample of water of the province of Bari. The Enterococcus Faecium was found: in two samples of the province of Foggia, in one sample taken in the province of Taranto and in two samples taken in the province of Brindisi.

Regarding Enterococcus avium, Enterococcus casseliflavus, Enterococcus durans, Enterococcus gallinarum, Enterococcus hirae and Enterococcu saccharolyticus did not produce any amplified in any of the 22 samples analyzed.

Discussion

The integrated analysis of the results obtained with the chemical and metagenomic investigation activities makes it possible to respond to the priority objectives foreseen by the present action and it is therefore possible to define that of 22 wells investigated, nitrate contamination (exceeding the law limit of 50 mg / L NO3) was recorded in seven wells: one in the province of Foggia, one in the province of Bari, four in the province of Brindisi, one in the province of Lecce. The biomolecular analyses have demonstrated in particular the presence of same species with particular correlation to the contamination present in the wells. In particular in eleven well, four of the province of Foggia, one of the province of Taranto, four of the province of Brindisi and two of province of Lecce, it was found the typical bacterial correlated to a contamination caused by human feces the Bifidobacterium, Enterococchi Fecium and Enterococchi Faecalis; in two wells, one of the province of Foggia and one of the province of Brindisi, it was found the typical bacterial correlated to a contamination caused by cattle feces the Bacteroides – Prevotella; in eight wells, four of province of Bari, one of province of Taranto and three of province of Lecce, it was found the typically bacterial presence correlated to a contamination of nitrate caused by an intensive agriculture the Nitrobacter e Nitrospira; only one well of province of Taranto not find nobody of the bacterial examined. In all well it is not found Enterococcus avium, Enterococcus casseliflavus, Enterococcus durans, Enterococcus gallinarum, Enterococcus hirae and Enterococcu saccharolyticus and for these reason it is possible to exclude all the contamination correlated to these microbiological indicator.

Conclusions

This pilot action was aimed to identify potential sources of nitrate contamination classifying the origin of nitrate from mineral fertilizers, zootechnical effluents, urban wastewater, sewage sludge, etc. through a biomolecular approach. The survey activities were conducted on 22 wells - identified on the regional territory on the basis of the results obtained with nitrate concentration monitoring - conducted in the period 2008-2014. In these wells, the regulatory limits had been exceeded (50 mg / L, D.Lgs. 152/06).

The integrated analysis of the results obtained with the chemical and metagenomic investigation activities makes it possible to respond to the priority objectives foreseen by the present action and it is therefore possible to define that:

A. Of a total of 22 wells investigated, nitrate contamination (exceeding the law limit of 50 mg/L) was recorded in 7 wells: one in the province of Foggia, one in the province of Bari, four in the province of Brindisi, one in the province of Lecce. For each of these wells the source of nitrate origin was identified and reported in the Table 3. In addition, the results have made it possible to identify the wells to be "monitored" in the monitoring activities of the water bodies to be planned, since they have concentrations of nitrates in the range 40-50 mg/L, and identify their source Table 4.

Table 3. *Contaminated Wells and their Origin*

Wells	NO_3 (mg/L)	Source of contamination
Foggia	69.5	Cattle breeding
Bari	72.2	Agriculture
Brindisi	70.4	Cattle breeding
Brindisi	163.02	Urban Wastewater
Brindisi	161.7	Urban Wastewater
Brindisi	91.7	Urban Wastewater
Lecce	53.1	Pig breeding

Table 4. *Wells Monitored and Possible Source of Contamination*

Wells	NO_3 (mg/L)	Source of contamination
Foggia	42.08	Urban Wastewater
Bari	49.17	Agriculture
Lecce	48.28	Agriculture
Lecce	47.84	Agriculture

B. The type of pollution has been uniquely identified for each contaminated well.
C. The identification of the sources of contamination produced results consistent with the information obtained with the inspection operations of the areas of location of the wells examined.
D. The results obtained, according to which the main sources of nitrate pollution in the samples of water examined are livestock (cattle and pigs) and urban wastewater related to sewage treatment plants (sewers) not adequately collected, it is possible to hypothesize some mitigation actions. Specifically,

with regard to the causes of agricultural breeding, it is suggested to define measures that, in the Vulnerable Areas of Nitrates of Agricultural Origin (ZVN), are aimed at: (i) intensify the controls by the competent Authorities, (ii) ensure the compilation and delivery of the required documentation (Company Register, Complete/Simplified Communication, PUA) by the farmers, iii) planning appropriate dissemination activities of the Regional Nitrate Action Plan in force in order to more effectively implement prevention, control and assistance to the agricultural world in the area concerned. These technical assistance activities could be aimed at supporting the actors involved in tackling business issues such as: the adaptation of storage containers for manure and sewage, the design of suitable plants for the treatment of farmed wastes in order to reduce the nitrogen charge before any spreading of them for soil fertilization, the rationalization of the use of synthetic chemical fertilizers in agricultural crops of relevance for the Apulian region.

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