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Nitrate and Ammonia Contamination in Groundwater and their Effect on Microbial Community in Apulia Region

Angelantonio Calabrese[1*], Mariavirginia Campanale[2], Fabiola Iurino[1]

[1] Water Research Institute- National Research Council, Bari 70132, Italy

[2] Engeo soc.cop. a.r.l., Bari 70122, Italy

* Corresponding author's e-mail: angelantonio.calabrese@ba.irsa.cnr.it

ABSTRACT

Due to its widespread presence in groundwater, nitrate contamination has become a major global concern. Identifying the different sources of this contamination, particularly those related to agricultural practices, is therefore crucial to assess its negative impacts. The European Nitrates Directive (91/676/EEC) requires the protection of all natural freshwater resources with a maximum nitrate concentration threshold of 50 mg$NO_3^+L^{-1}$, applicable to all groundwater, regardless of its intended use. Many studies have used a chemical approach to identify these contaminations, but one of the possible approaches to identify contamination and the source of the contamination is a microbiological approach. An aquifer's unique groundwater fingerprint: its hydrology, chemistry, and geology are shaped by the communities of heterotrophic bacteria that thrive in this underground environment. The present study carries out an evaluation of the impact of nitrate and ammonia on the bacterial community of groundwater, in particular by studying the correlations between the two chemical forms analyzed and some of the native species most present in nitrifying and denitrifying groundwater. These evaluations make it possible to identify the microbial species subject to the variation of ammonia and nitrate concentrations and to evaluate the extent of variation in the natural environment, providing useful information on the variation of the chemical compound, validating the innovative thesis of being used as a natural tracer for the identification of potential direct or indirect contamination.

Keywords: nitrate, ammonia, nitrifying and denitrifying microrganism, microrganism dynamics.

INTRODUCTION

Nitrate (NO_3^+) has emerged as a critical global threat, with regions worldwide grappling with polluted groundwater [Lal et al. 2020; Haller et al., 2013; Wang et al., 2016; Stuart and Lapworth,2016]. Health concerns surrounding nitrate contamination have been debated for years, with potential links to increased risks of methemoglobinemia and certain cancers [World Heath Organization (WHO), 2004]. Nitrate pollution also has a significant environmental impact, contributing to eutrophication, or excessive nutrient enrichment, of surface waters [Amoatey and Baawain, 2019]. This problem is largely attributed to diffuse agricultural pollution, which has intensified since the mid-20th century with the rise of intensive farming practices [Foster and Bjerre., 2023]. Estimates suggest that agriculture is the source of 70–80% of nitrate groundwaters contamination [Department For Environment, Food And Rural Affairs (DEFRA), 2002]. However, it's important to recognize that agricultural activities are not the sole culprit. High nitrate levels in water can also arise from discharges from septic systems and leaky sewage infrastructure, atmospheric deposition of nitrogen compounds and application of sewage sludge and manure to land [Gutiérrez et al., 2018].

Similar to the World Health Organization (WHO), the European Union (EU) has established a maximum nitrate level of 50 milligrams per liter (mg/L) for drinking water, expressed as nitrate ions (NO_3^-). This limit is a key component of the Nitrates Directive (91/676/EEC), which focuses on protecting freshwater sources.

However, the EU takes a broader approach to nitrate contamination. Recent research suggests that even lower levels, between 1 and 2 mg/L of nitrate-nitrogen (NO_3-N), can contribute to excessive nutrient enrichment (eutrophication) in pristine lakes and rivers. This highlights the need for stricter regulations in certain cases, particularly for unpolluted waters. In contrast, for already nutrient-rich waters, phosphorus might be a more critical factor [Swe et al., 2021].

The Water Framework Directive (2000/60/EC) complements the Nitrates Directive by aiming to achieve "good ecological status" for all groundwater resources by 2015. This includes maintaining nitrate concentrations within legal limits.

Data from the European Environment Agency (EEA) reveals concerning levels of nitrate contamination in European groundwater. In 2003, a significant portion of groundwater bodies exceeded a benchmark of 25 mg/L of nitrate ions (NO_3^-). Countries like Spain, the UK, Germany, France, and Italy faced particularly high levels, while Scandinavian and Baltic states had a lower percentage exceeding the limit.

This issue is not limited to Europe. Elevated nitrate concentrations have also been documented in other parts of the world, including Australia [Australian State of the Environment Committee (ASEC), 2001] and North America[Hudak, 2018; Liang et al., 2020].

Addressing nitrate contamination presents a significant challenge due to its long-term, widespread, and ongoing nature [Zhang and Hiscock, 2016; Chen et al., 2019]. Current mitigation strategies primarily focus on two approaches: continued implementation of land-use controls, this involves establishing protection zones around vulnerable water sources. These zones aim to reduce nitrate infiltration into the subsurface through various management practices [Johnson et al., 2023] and relying on natural attenuation processes, this strategy leverages natural mechanisms in the environment that can break down or transform nitrate before it reaches groundwater sources.

Groundwater ecosystems stand out from surface soil and aquatic environments due to the absence of photosynthesis and limited fresh, readily available organic carbon. These factors shape the microbial communities within aquifers, dominated by heterotrophs adapted to thrive in the nutrient-poor (oligotrophic) conditions.

Recently, scientists discovered that lithoautotrophs, microbes that use inorganic chemicals for energy and fix carbon dioxide, are also essential for these ecosystems.

Groundwater habitats are a mosaic of hydrological, chemical, and geological variations. While the vertical layering of strata within aquifers can be highly complex and unique, conditions within specific zones can be remarkably stable. Due to the absence of sunlight, scarcity of organic carbon and nutrients, and consistently cool temperatures, near-surface aquifers can be considered harsh environments for many life forms [Zagmajster et al., 2018].

Despite the constant and seemingly harsh conditions, groundwater microbes have adapted remarkably well. In fact, for them, sudden environmental changes pose a greater threat than the stable state often perceived as extreme. Groundwater ecosystems exhibit a remarkable diversity in size and complexity. They range from small alluvial aquifers flanking rivers, stretching just a few kilometers, to vast regional aquifers spanning hundreds of kilometers. These ecosystems can be as small as a cave pool or encompass entire karst systems with intricate networks snaking through mountains. Notably, many of these subsurface environments are interconnected, forming a vast network of ecosystems [Amanambu et al., 2020]. The boundaries (ecotones) between soils, vadose zones (unsaturated zones above groundwater), aquatic sediments and saturated groundwater layers are considered hotspots for microbial diversity and activity. Microbial communities within groundwater ecosystems act as facilitators, influencing the movement of nutrients, particles, organisms, and energy across various zones. However, transfer rates can vary significantly and may be slow enough, especially in deep subsurface habitats, to allow for allopatric evolution (evolution in isolation) even among microorganisms [Thullner and Regnier, 2019].

Groundwater ecosystems harbor a vast number of bacteria, ranging from 10^2 to 10^6 cells per cubic centimeter in the water itself and 10^4 to 10^8 cells per cubic centimeter in the sediment [Thullner and Regnier., 2019]. Estimates suggest that between 6% and 40% of all prokaryotes (bacteria and archaea) on Earth might reside within the subsurface. This hidden world encompasses bacteria, archaea, protozoa, yeasts, and other fungi [Ittner et al., 2018].

Interestingly, the distribution of some microorganisms, particularly micro-eukaryotes (organisms with complex cells), appears limited to shallow, near-surface groundwater [Ittner et al.,

2018]. Most prokaryotic life in aquifers is found attached to sediment particles, rock surfaces, and organic debris, forming microcolonies or biofilms [Hofmann et al., 2020]. This attachment strategy offers advantages in environments with limited carbon and nutrients. Additionally, sediment surfaces provide greater geochemical diversity and more ecological niches compared to the open water within the aquifer. The proportion of microbes floating freely in groundwater (suspended) compared to those clinging to sediment particles (attached) is heavily influenced by three factors: Availability of dissolved organic carbon (DOC) and nutrients encourage the growth of free-floating microbes; finer-grained sediments offer more surface area for microbes to attach to, promoting a higher attached population and mineralogy of the sediments: The ratio of attached to free-living microbes can vary significantly across different groundwater environments, potentially spanning several orders of magnitude (from 0.2 to 1000 or more) [Chang et al., 2018; Retter et al., 2021]. While early studies using isolated microbes hinted at significant differences between free-floating and attached communities, relatively few studies have directly compared their microbial diversity. Capturing a complete picture of microbial diversity in groundwater requires well-designed sampling strategies that account for both spatial and temporal variations. A crucial consideration is the relationship between the effective habitat size needed by a microbe and the sampling resolution [Shafi et al., 2017; Shoemaker et al., 2017].

Surprisingly, the diversity of prokaryotes found in just a 100 cm^3 sediment sample can rival the regional diversity of animals [Hermans et al., 2022] highlight how sample size can significantly bias our understanding of microbial patterns in subsurface environments. Most studies suggest that at least a portion of the microbes in aquifers are actively alive. However, pointed out, knowing the percentage of active cells isn't enough [Kieft et al., 2018]. Ideally, we'd understand the «in situ» activity of these microbes. Unfortunately, this remains a challenge even a decade later. Most methods for measuring microbial activity rely on incubating freshly collected samples in the lab, which can overestimate actual activity levels.

Studies using radioisotopes to track bacterial growth in groundwater sediments at depths of 200–450 meters suggest slow doubling times. These estimates varied depending on the tracer used, with 14C-labeled substrates indicating times between 1 and 320 days, while 3H-labeled substrates suggested much longer times, potentially thousands of days.

However, models based on groundwater chemistry and balancing the amount of material entering and leaving the system imply even slower growth rates, potentially in the range of centuries. Overall, microbial activity in the subsurface appears to be significantly slower than in surface environments, potentially by a factor of 10 billion (10 orders of magnitude). New methods, such as stable isotope tracing and activity-based molecular techniques, offer promise for more precise measurements of microbial activity in groundwater directly within its natural environment (in situ) [Vargas-García et al., 2023].

The present work analyzes the presence of denitrifying and nitrifying microorganisms in groundwater through biomolecular analysis. A key step is the analysis of the correlation between the chemical compounds detected and the presence of the nitrifying and denitrifying microorganisms and how the correlation varies over time. In order to have a complete picture of the microbial activity, biomolecular studies and comparisons with chemical elements have been carried out through the main bacterial species identified in groundwater [Calabrese et al., 2020]. The principal aim is to determined a new sistem to identify the microbial species subject to the variation of ammonia and nitrate concentrations and to evaluate the extent of variation in the natural environment, providing useful information on the variation of the chemical compound, validating the innovative thesis of being used as a natural tracer for the identification of potential direct or indirect contamination

MATERIALS AND METHODS

Monitoring area

Following analysis of existing databases, six areas in Puglia were identified with similar lithology (rock composition) and soil type. Within each of these areas, 24 sub-areas were chosen based on a consistent land use, excluding urbanized zones. This resulted in a total of 144 sub-areas for further monitoring. Six wells capturing water from the surface aquifer were identified within each monitoring area. This selection process yielded a total of 864 wells. Two rounds of sampling were conducted at each well, once in 2021 and again in 2022.

Chemical analysis

Ammoniacal nitrogen

The determination of the ammonia nitrogen concentration was carried out as per Method C-Spectrophotometric determination described in chap. 4030 pp. 519-523 of "Analytical Methods for Water – Manual and Guidelines 29/2003 – APAT/IRSA-CNR

Nitric nitrogen

The determination of the nitric nitrogen concentration was carried out as described in chap. 4050 pp. 533-536 of "Analytical Methods for Water – Manual and Guidelines 29/2003– APAT/IRSA-CNR".

Biomolecular analysis

DNA extraction and sequencing

Standard bead-beating protocols from MACHEREY-NAGEL (Germany) were employed for DNA extraction from the samples. The Environmental Microbiology and Molecular Biology laboratory at CNR-IRSA Rome quantified the extracted DNA using a Qubit™ 4 Fluorometer (Invitrogen, Thermo Fisher Scientific). Next-generation sequencing of the DNA was performed on an Illumina MiSeq platform using GENE AMPLICON SEQUENCING (MiSeq, Illumina). The resulting sequences were then compared to those available in the GenBank database via a BlastN search (https://blast.ncbi.nlm.nih.gov/).

Primer design, qPCR conditions and data analysis

Primers for bacterial amplification were designed using the Clone Manager Suite version 6 software (Sci-Ed, Cary, NC). To ensure specificity, these primer pairs were then evaluated for sequence similarity against known entries in the GenBank database via a BlastN 2.9 search. The designed primer pairs were specific to each target 16S rRNA gene species and aimed to amplify a fragment of approximately 100 to 250 base pairs (bp) in length (Table 1).

A StepOne Plus™ Real-Time PCR System (Applied Biosystems) was used for quantitative analysis. The thermal profile employed the following conditions: UDG activation – 50°C for 2 minutes, denaturation – 94°C for 2 minutes; amplification (40 cycles): denaturation – 94°C for 15 seconds; annealing – 65°C for 30 seconds; elongation – 72°C for 30 seconds (fluorescence data acquisition); melting curve analysis (for product specificity).

Each 20 µL reaction contained: 10 µL PowerUp™ SYBR™ Green Master Mix (Thermo Fischer Scientific); 10 pmol of forward and reverse primers; 1 µL of template DNA (40 ng/µL).

All reactions were run in triplicate and analyzed using StepOne Plus™ software (Applied Biosystems). PCR efficiency was calculated using the formula: $Efficiency = -1 + 10^{\wedge(-1/slope)}$.

Following next-generation sequencing (NGS) of the samples, specific bacterial groups were chosen for further analysis based on their role in nitrogen cycling: Nitrification – *Nitrosomonas* spp., *Nitrosovibrio* sp., and *Nitrobacter* spp.;

Table 1. Primers used for real time PCR in this study

Aim	Target gene	Primers	Sequence 5′–3′	References
Bacteria	16S rRNA	1055F	ATG GCTGTC GTCAGCT	[Ferris et. al, 1996]
		1392R	ACG GGC GGTGTGTAC	
Ammonia oxidisers	Amo	amoA-1-F	GGG GTT TCTACTGGTGGT	[Li et al., 2012]
		amoA-2R	CCC CTCKGSAAAGCC TTC TTC	
Nitrite oxidisers	Nxr	nxrA-RT-F	GTG GTC ATG CGC GTT GAG CA	[Gerbl et al., 2014]
		nxrA-RT-R	TCG GGA GCG CCA TCA TCC AT	
All known Planctomycetes	Hzo	hzoCl1f1	TGYAAGACYTGYCAYTGG	[Kim et al., 2014]
		hzoCl1r2	ACTCCAGAT RTG CTGACC	
Denitrifiers	NirS	nirS 1f	TAC CAC CCSGARCCG CGCGT	[Ziembińska-Buczyńska et al., 2019]
		nirS 3r	GCC GCC GTC RTGVAGGAA	
	NirK	nirK876	ATYGGC GGVCAYGGC GA	
		nirK1040	GCC TCGATCAGRTTRTGGTT	

denitrification – *Thiobacillus* sp., *Pseudomonas* spp., and *Xanthomonas* sp.

Unique regions within the 16S rRNA gene sequences obtained from NGS were identified and used to design specific primers for real-time PCR. This design process utilized Clone Manager Suite version 6 software (Sci-Ed, Cary, NC). The designed primer pairs were verified for specificity against known sequences in the GenBank database using a BlastN search. Each primer pair targeted a specific fragment size between 100 and 250 base pairs (bp) for the targeted species (Table 2).

RESULTS AND DISCUSSION

Chemical analysis of the surface groundwater showed a high variability of nitrate and ammonia concentrations with values ranging from 0 to 386.92 mgNO_3^- L^{-1} and 0 to 4.85 mg$NH_4^+L^{-1}$, respectively.

Real-time PCR analyses were performed on all samples to quantify the abundance of nitrifying and denitrifying bacteria. Specific target genes were used to identify these functional groups.In particular, the quantification analyses, doing with real time PCR, revealed the presence of nitrifying microorganisms with a range from 0% to 30,58% of presence and the presence of denitrifying microorganisms with a range from 0% to 29,18% of presence.

The analyses carried out on the samples showed a high correlation between the presence of nitrate$^-$ and denitrifying bacteria and the presence of NH_4^+ and nitrifying bacteria. Relative to the presence of nitrates, there is an R_2 correlation equal to 0.97 (Fig. 1a). as far as the presence of ammonia is concerned, also in this case the correlation with the presence of nitrifying bacteria is very high with R^2 equal to 0.9792 (Fig. 1b)

Correlation analyses were carried out for the two individual sampling campaigns carried out in 2021 and 2022. There is a high correlation

Table 2. Primers used for real time PCR in this study

Aim	Primers	Sequence 5′–3′	Fragment length (bp)	Annealing (°C)
Nitrosomonas spp.	NitrosomonasF	CGC ATC GAA AGA TGT GCT AA	178	59
	NitrosomonasR	CGT GTC TCA GTC CCA GTG TG		
Nitrosovibrio sp.	NitrosovibrioF	GTG GGG AGC AAA CAG GAT TA	182	60
	NitrosovibrioR	CAC ATA ATC CAC CGC TTG TG		
Nitrobacter spp.	NitrobacterF	GGG CGT AGC AAT ACG TCA GT	192	59
	NitrobacterR	CTA CTG ATC GTC GCC TTG GT		
Thiobacillus sp	ThiobacillusF	GTG GGG AAT ATT GGA CAA TG	192	59
	ThiobacillusR	CTT GCA CCC TCC GTA TTA CC		
Pseudomonas spp.	PseudomonasF	GGT CTG AGA GGA TGA TCA GT	215	59
	PseudomonasR	CCG GTG CTT ATT CTG TTG GT		
Xanthomonas sp	XanthomonasF	TGG GGA GCA AAC AGG ATT AG	201	60
	XanthomonasR	CGT TGC ATC GAA TTA AAC CA		

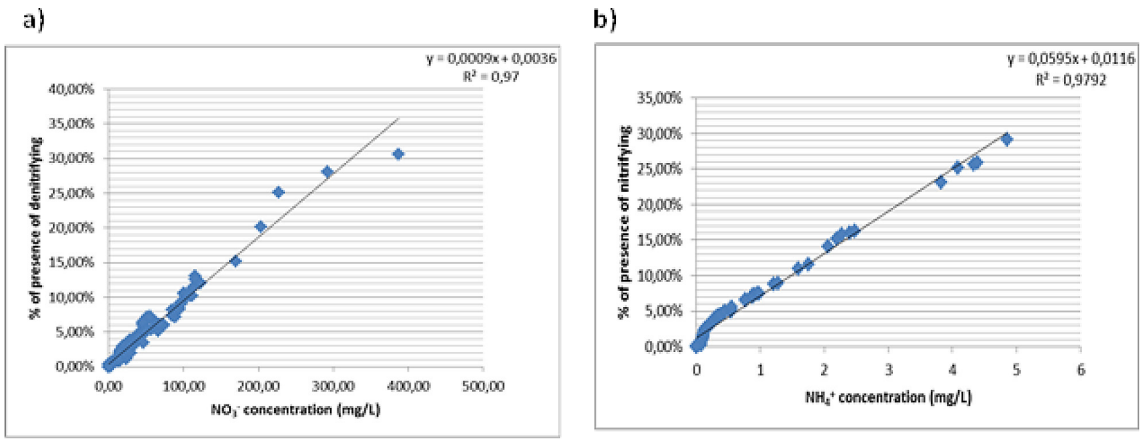

Figure 1. a) Correlation between nitrate concentration in groundwater and % of denitrifyng bacteria; b) correlation between ammonia concentration in groundwater and % of denitrifyng bacteria

between nitrate concentrations and the presence of denitrifying bacteria for both annalualities (R^2 2021 equal to a 0.9647 R^2 2022 equal to a 0.9811) (Fig 2a and Fig 2b). The same high correlation is found in the correlation between ammonia and the percentage of presence of nitrifying bacteria (R^2 2021 equal to a 0.9765 R^2 2022 equal to a 0.9808) (Fig. 2c and Fig. 2d).

Furthermore, by carrying out a temporal analysis through the algebraic difference of the values of the chemical and microbiological analysis obtained in the two campaigns, it is possible to see how the high correlation value also remains between the variations in nitrate concentration with the changes in the percentage of presence of denitrifying bacteria and the variations in ammonia with the variations in the percentage of presence of nitrifying bacteria (R^2 2021 equal to a 0.9687, R^2 2022 equal to a 0.9931) (Fig. 2e and Fig. 2f).

Classification of nitrifying and denitrifying agents

A sequencing of the DNA extracted was carried out and the analysis of the species present will

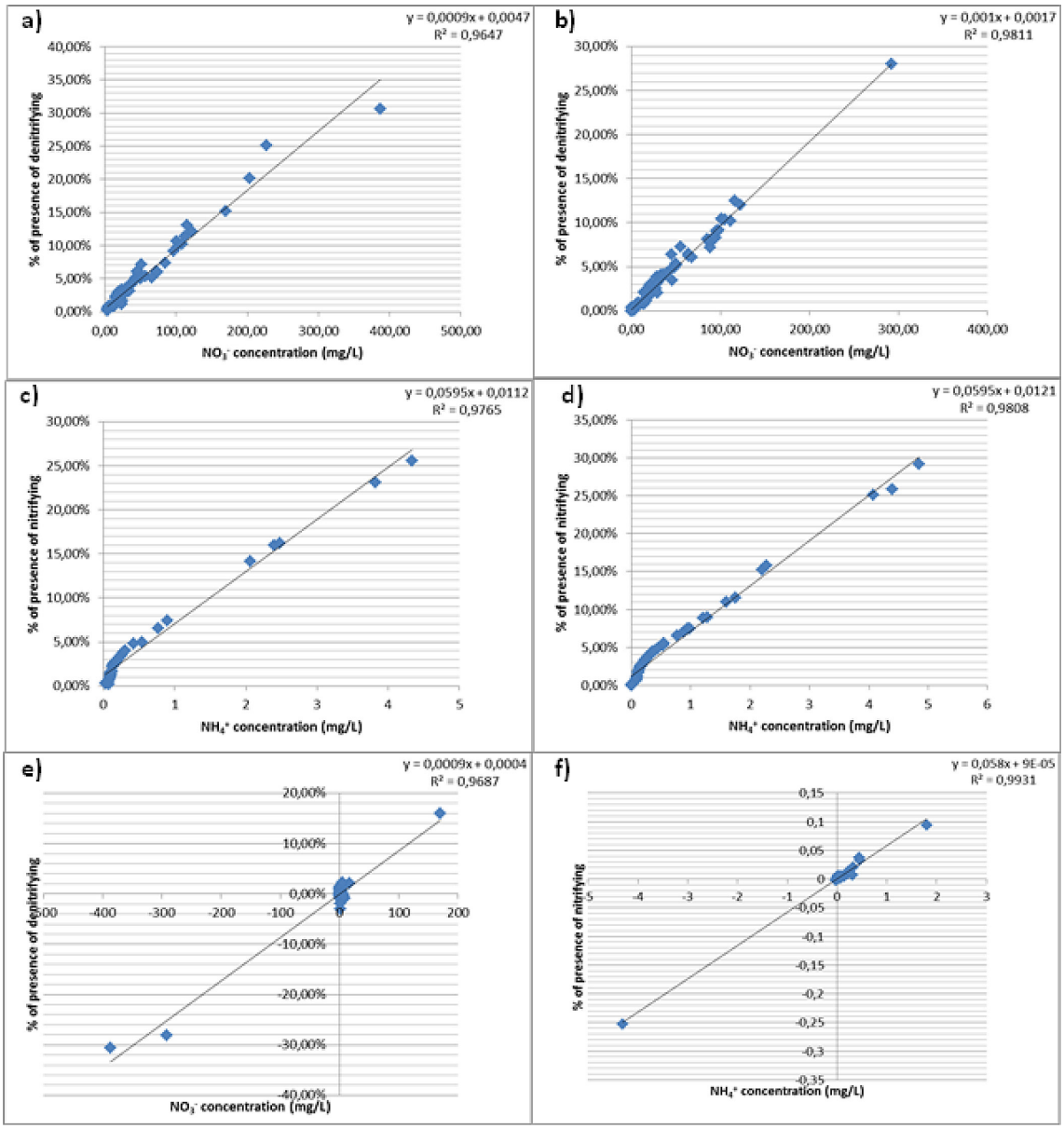

Figure 2. a) Correlation between nitrate concentration and % of denitrifyng bacteria in 2021; b) correlation between nitrate concentration and % of denitrifyng bacteria in 2022; c) correlation between ammonia concentration and % of nitrifyng bacteria in 2021; d) correlation between ammonia concentration and % of nitrifyng bacteria in 2022; e) correlation between variation of nitrate concentration and variation of % of denitrifyng bacteria; f) correlation between variation of ammonia concentration and variation of % of nitrifyng bacteria

be done. With regard to denitrifying species, the sequencing of DNA extracted from the samples highlighted in 2021 (Fig. 3a) a prevalent presence of *Pseudomonas* sp. equal to 50.50%, *Thiobacillus* sp. 8.45%, *Xanthomonas* sp. 36.31% and other species equal to 4.74%. With regard to the year 2022 (Fig. 3a), there is a prevalence of the presence of *Pseudomonas* sp. equal to 48.78%, *Thiobacillus* sp. 6.15%, *Xanthomonas* sp. 31.56% and other species equal to 13.51%.

With regard to nitrifying species in 2021 (Fig. 3b), the main presence of *Nitrobacter* sp. was found with a percentage of 49.55%, 31.25% of *Nitrosovibrio* sp., 11.20% of *Nitrosomonas* sp. and 8.00% of other nitrifying species. For the year 2022 (Fig. 3b), a homologous result was obtained with a percentage of presence of 41.84% *Nitrobacter* sp., 30.60% of *Nitrosovibrio* sp., 7.10% of *Nitrosomonas* sp. and 20.46% of other nitrifying species.

Real-time analysis

Through the real time it was possible to obtain the individual concentrations of the major species found in the samples in order to be able to carry out a correlative analysis with the respective concentrations of the nitrogenous forms found. With regard to *Pseudomonas* sp. there was a high correlation with the presence of nitrate for 2021 (Fig. 4a) and 2022 (Fig. 4b) with an R^2 of 0.9905 and 0.9888 respectively. This high correlation with the chemical compound is also found with regard to the variation of nitrate and the variation in the presence of *Pseudomonas* sp. (Fig. 4c) with an R^2 equal to 0.9752.

Thiobacillus sp. presents a high correlation in 2021 (Fig. 5a) and in 2022 (Fig. 5b) with the presence of nitrate with an R^2 of 0.9861 and 0.9793 respectively. This high correlation with the chemical compound is also found with regard to the variation of nitrate and the variation in the presence of *Thiobacillus* sp. (Fig. 5c) with an R^2 equal to 0.9721.

Xanthomonas sp. presents a high correlation in 2021 (Fig. 6a) and in 2022 (Fig. 6b) with the presence of nitrate with an R^2 of 0.9802 and 0.986 respectively. This high correlation with the chemical compound is also found with regard to the variation of nitrate and the variation in the presence of *Xanthomonas* sp. (Fig. 6c) with an R^2 equal to 0.9721.

Nitrobacter sp., for 2021 (Fig. 7a) and 2022 (Fig. 7b), precents a high correlation with the presence of ammonia with an R^2 of 0.9753 and 0.9805 respectively. This high correlation with the chemical compound is also found with regard to the variation of ammonia and the variation in the presence of *Nitrobacter* sp. (Fig. 7c) with an R^2 equal to 0.9707.

Nitrosomonas sp. species, in 2021 (Fig. 8a) and in 2022 (Fig. 8b) presents a high correlation with ammonia with an R^2 of 0.9784 and 0.9716 respectively. This high correlation with the chemical compound is also found with regard to the variation of ammonia and the variation in the presence of *Nitrosomonas* sp. (Fig. 8c) with an R^2 equal to 0.9747.

Nitrosovibrio sp., in 2021 (Fig. 9a) and in 2022 (Fig. 9b), presents a high correlation with ammonia with an R^2 of 0.9769 and 0.9724 respectively. This high correlation with the chemical compound is also found with regard to the variation of ammonia and the variation in the presence of *Nitrosovibrio* sp. (Fig. 9c) with an R^2 equal to 0.9711.

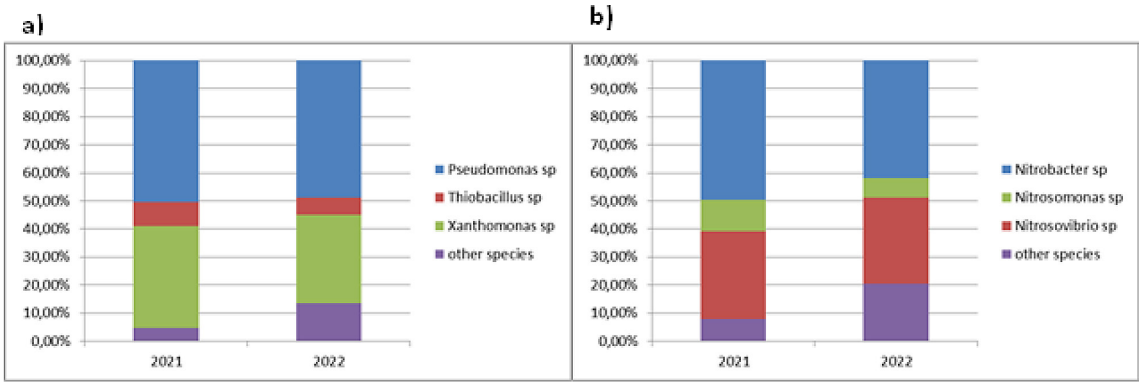

Figure 3. a) Distribution of denitrifying species in the year 2021 and in the year 2022; b) distribution of nitrifying species in the year 2021 and in the year 2022

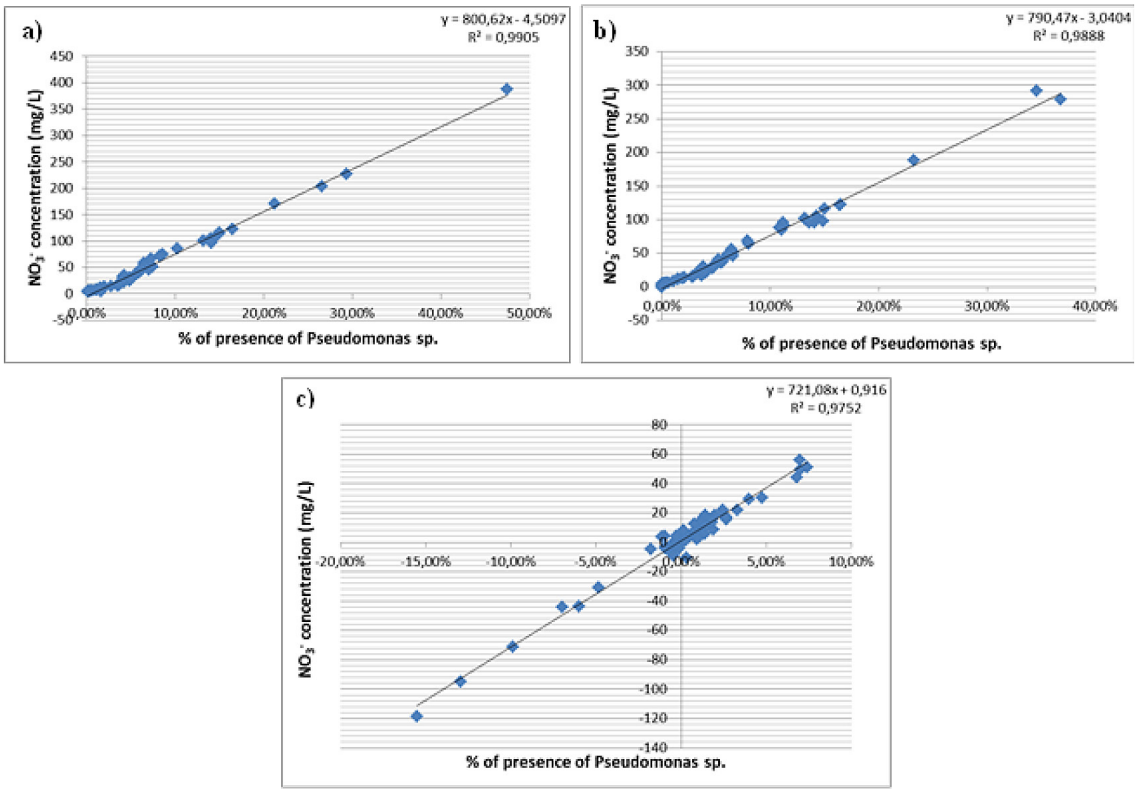

Figure 4. a) Correlation between nitrate concentration and *Pseudomonas* sp. in 2021; b) correlation between nitrate concentration and *Pseudomonas* sp. in 2022; c) correlation between change in nitrate concentration and change in the presence of *Pseudomonas* sp.

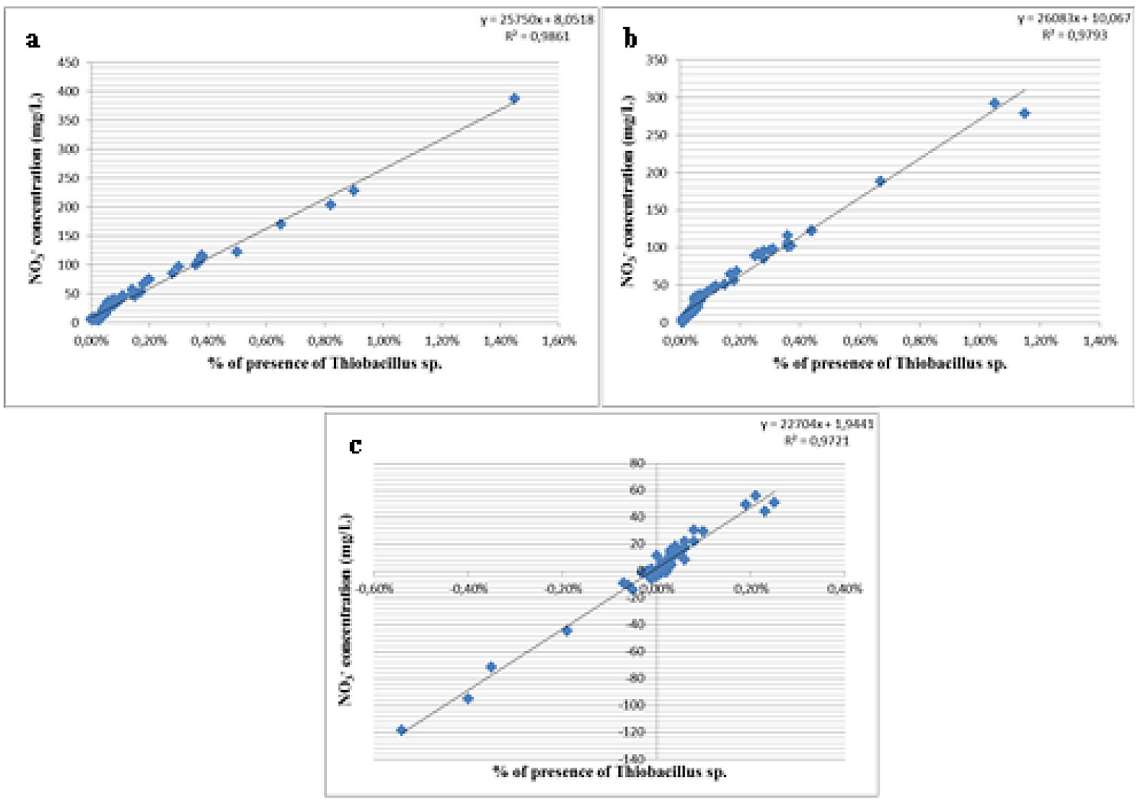

Figure 5. a) Correlation between nitrate concentration and *Thiobacillus* sp. in 2021.; b) correlation between nitrate concentration and *Thiobacillus* sp. in 2022; c) correlation between change in nitrate concentration and change in the presence of *Thiobacillus* sp.

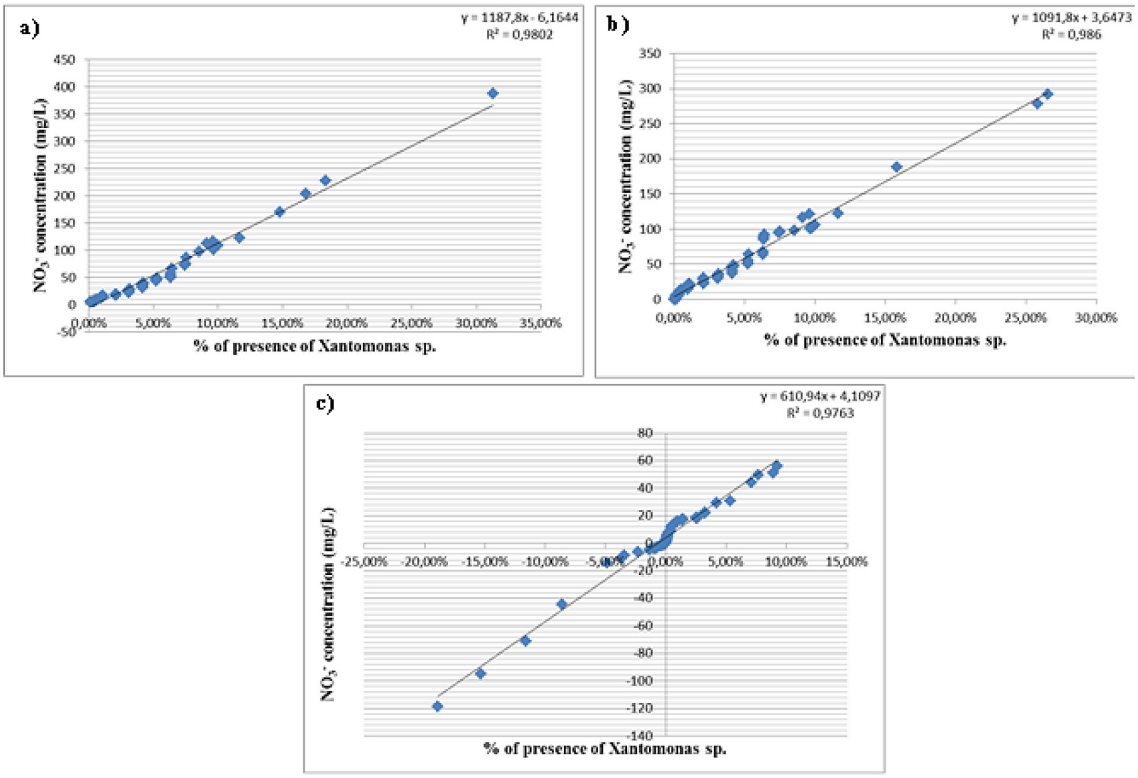

Figure 6. a) Correlation between nitrate concentration and *Xanthomonas* sp. in 2021; b) correlation between nitrate concentration and *Xanthomonas* sp. in 2022; c) correlation between change in nitrate concentration and change in the presence of *Xanthomonas* sp.

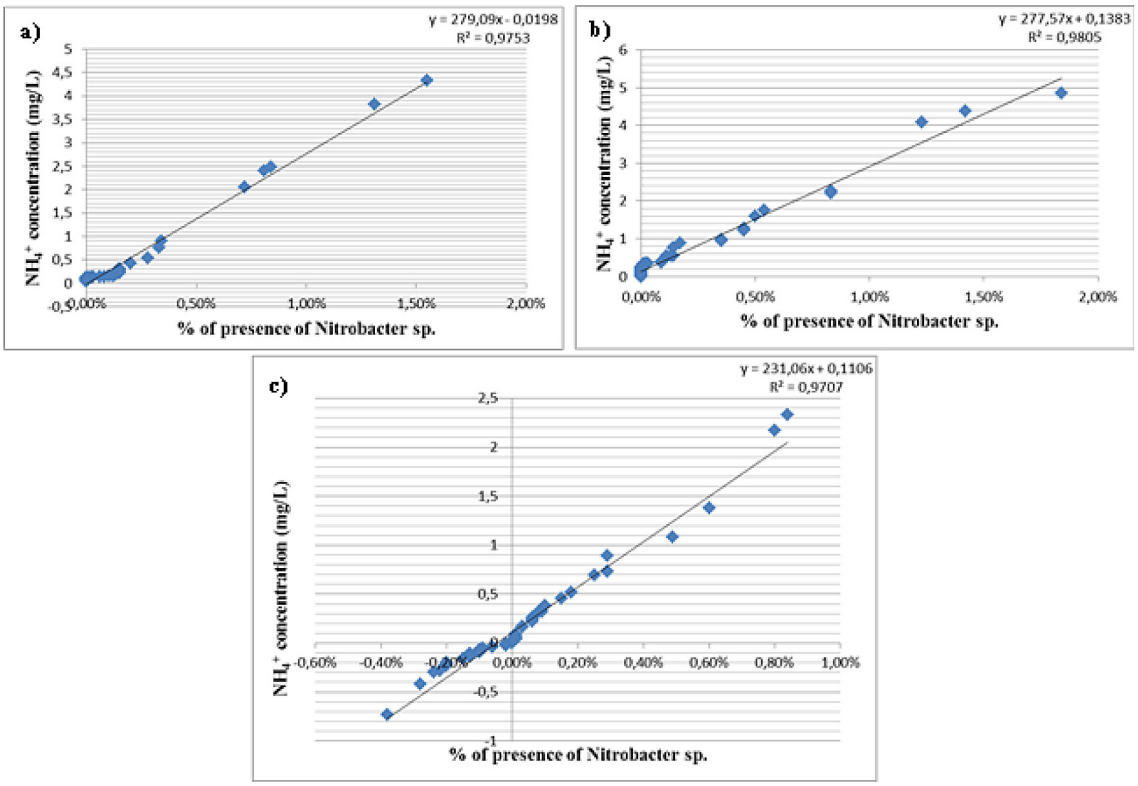

Figure 7. a) Correlation between ammonia concentration and *Nitrobacter* sp. in 2021; b) correlation between ammonia concentration and *Nitrobacter* sp. in 2022; c): correlation between change in ammonia concentration and change in the presence of *Nitrobacter* sp.

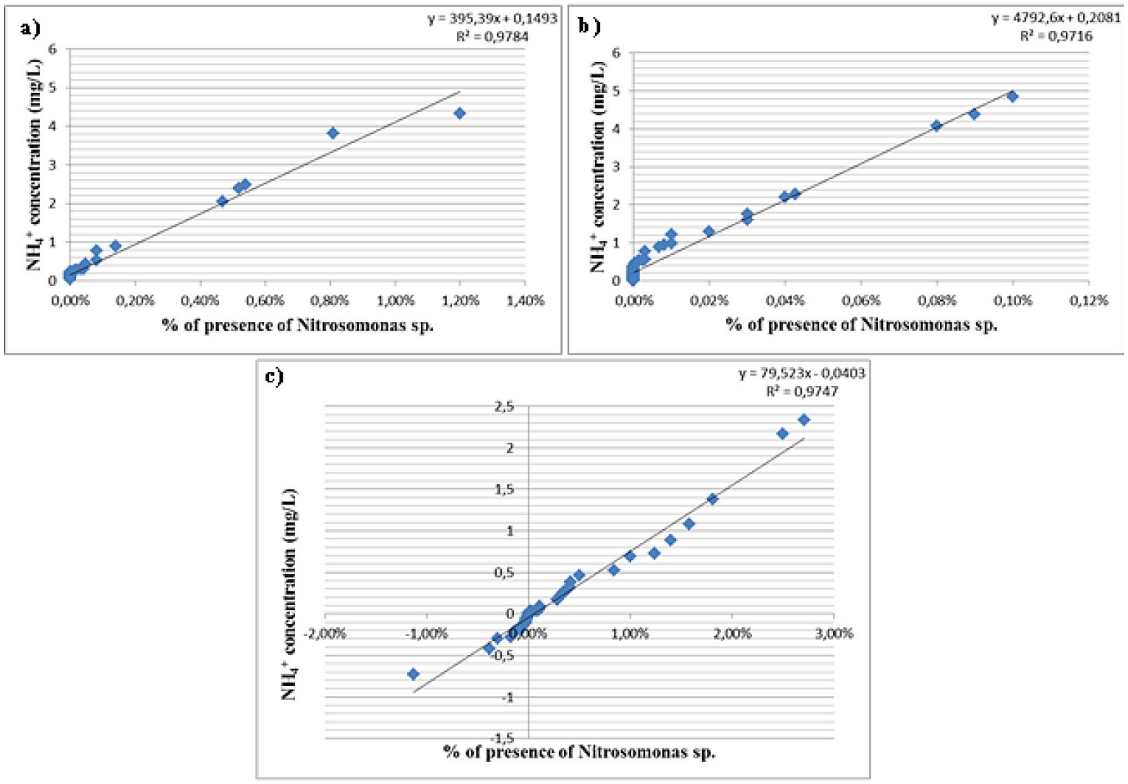

Figure 8. a) Correlation between ammonia concentration and *Nitrosomonas* sp. in 2021; b) correlation between ammonia concentration and *Nitrosomonas* sp. in 2022; c) correlation between variation in ammonia concentration and change in the presence of *Nitrosomonas* sp.

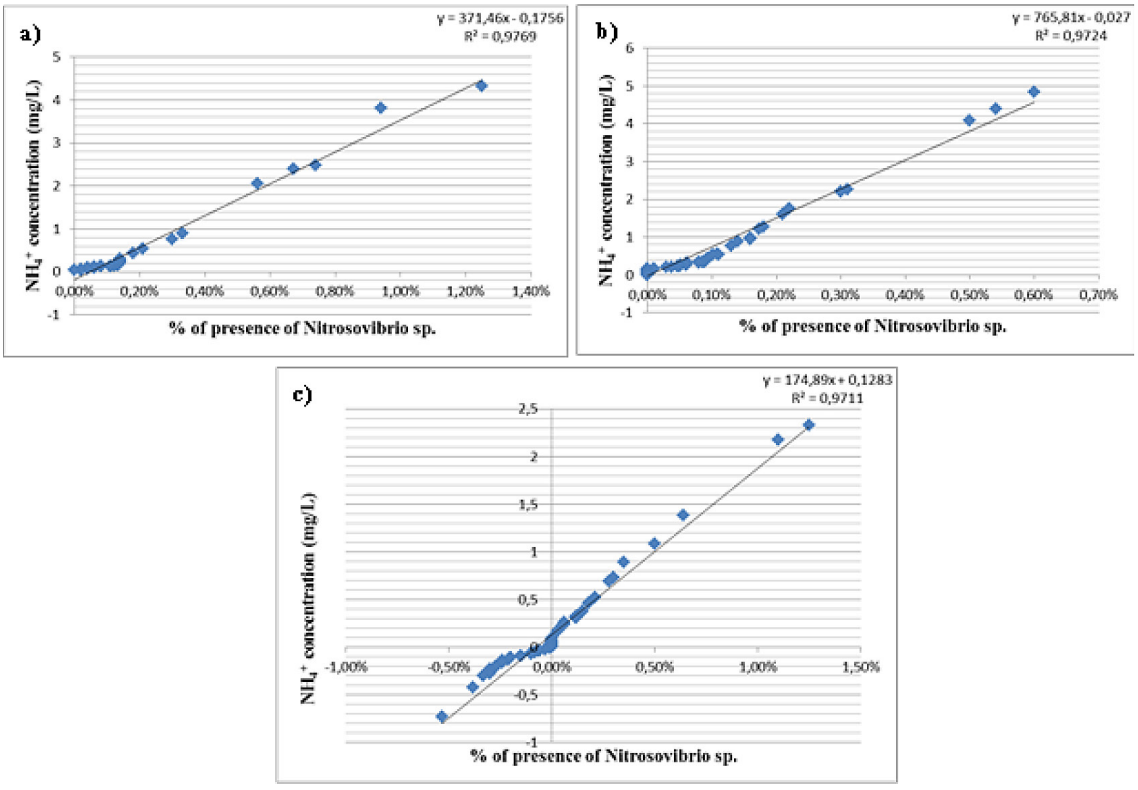

Figure 9. a) Correlation between ammonia concentration and *Nitrosovibrio* sp. in 2021; b) correlation between ammonia concentration and *Nitrosovibrio* sp. in 2022; c) correlation between variation in ammonia concentration and change in the presence of *Nitrosovibrio* sp.

CONCLUSIONS

The results obtained from the research showed that in all the points analyzed there is a high correlation between nitrifying microbial species and nitrate concentrations and denitrifying microbial species and ammonia concentrations. The analysis of the most abundant species identified through the sequencing of the extracted DNA, have highlighted how some species such as *Pseudomonas* sp., *Thiobacillus* sp. and *Xanthomonas* sp., in relation to denitrifying species, and *Nitrobacteer* sp., *Nitrosomonas* sp. and *Nitrosovibrio* sp., in relation to nitrifying species, are closely related to the concentration of the growth substrate, i.e. nitrate for denitrifying species and ammonia for nitrifying species. This correlation is also perfectly preserved in the variation of the substrate, demonstrating how it is the first factor in the selection of the microbial species present, causing a variation in the presence of individual species.

The analyses carried out have highlighted how the microbial species analyzed can be considered as species indicating the presence and variation in groundwater of nitrate and ammonia. For these reason they can be considered as possible natural tracers respectively for a potential nitrate contamination or a potential ammonia contamination bearing in mind that with regard to nitrates the NO_3^+ threshold of 50 mgl coincides with a concentration of *Pseudomonas* sp. equal to 7.32%, Thiobacillus sp equal to 0.32% and *Xanthomonas* sp. equal to 6.27%. With regard to ammonia, the species identified and their quantification allow us to understand and analyze that if there is a percentage of presence greater than 0.5%, it would be possible to trace a concentration of ammonia such as to determine contamination due to a direct introduction not related to soil leaching.

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