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Nitrate Contamination: The Use of Nitrosovibrio Sp. And Pseudomonas Sp. To Identify the Source of Contamination

Angelantonio Calabrese[1], Emanuele Barca[1] And Mariavirginia Campanale[2]
[1] *Water Research Institute- National Research Council, Bari 70132, Italy.*
[2] *Engeo soc.cop. a.r.l., Bari 70122, Italy*

Abstract:
Nitrogen compounds are among the major chemical agents artificially released into the environment as a consequence of the growing need for food and energy production. Several studies have highlighted how the excessive use of fertilizers generates a surplus that overcomes 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 plumes in groundwater, due to hydrodynamic dispersion. Another anthropic source of nitrates is the sewage. For all these reasons, nitrates are the form of nitrogen that more frequently exceeds the maximum admissible concentrations in groundwater. 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. The activities describe in these work analysed the correlation between the NH_4^+, NO_3^- and two microorganism (Nitrosovibrio sp. and Pseudomonas sp.) to create a matrix for identifying sources of nitrate contamination.

Key Word*: Nitrate, Nitrosovibrio sp., Pseudomonas sp., range of applicability*

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I. Introduction

One of the main forms of groundwater contamination is linked to the presence of nitrogenous compounds. The growing needs of nitrogen by the human population for the production of food and energy have led to an increase in the production of nitrogen in reactive form (i.e. all forms except the molecular state N_2)[1,2]. It is estimated that the input of reactive nitrogen from human activities, primarily industrial processes and the use of fertilizers, grew by 225% from 1970 to 2000[3,4] and that this represents 45% of the total nitrogen fixed on Earth[5]. The amount of nitrogen that is not removed and/or immobilized from the soil can leach[6,7,8,9] until it reaches groundwater and surface water bodies, causing various negative effects both in the environment and in the man. However, not all forms undergo the leaching action in the soil, the only highly leachable form is nitric nitrogen More abundant, more mobile and therefore absorbed in greater quantities by plants, unlike ammonia nitrogen which is very volatile and is not subject to leaching also because it is absorbed very quickly by plants and mainly used for the synthesis of amino acids[10].

The natural sources of nitrates are formed by the biological processes of mineralization and fixation of atmospheric nitrogen. The nitrates thus produced can then leach, conveyed by rain or irrigation water, reach the groundwater and then migrate into it thanks to the phenomena of advection and hydrodynamic dispersion. Further natural sources of nitrates can be calcareous deposits or encrustations, igneous rocks (in the form of ammonium compounds) and lake sediments[11]. On average, it can be assumed that the concentrations in the groundwater due to natural phenomena are modest, in the order of 3-10 mg/l[12]. Natural denitrification helps to keep nitrate concentrations relatively low in surface and groundwater. Higher concentrations of nitrates can be attributed to sources of anthropic origin, mainly linked to the use in agriculture of nitrogen fertilizers, both natural organic (manure) and inorganic and/or chemical organic[13].

Simple nitrogen-based fertilizers can contain nitrogen in nitric, ammonia, nitric-ammonia and organic forms. Fertilizers with nitrate nitrogen are directly and readily absorbed by plants, while those with ammonia nitrogen, although having the same physiological value, are absorbed directly more slowly, but are mostly used indirectly undergoing transformation into nitrogen in the soil nitrate by nitrifying bacteria[14]. Fertilizers based on organic nitrogen exert their action very slowly, having to undergo successive transformations into ammonia and then nitric nitrogen in the soil. The main nitric nitrogen fertilizers are sodium nitrate ($NaNO_3$) and calcium ($Ca(NO_3)_2$). The latter also acts as a corrective for soils lacking or poor in calcium. Among the nitrogen fertilizers are ammonium sulfate ($(NH_4)_2SO_4$) and ammonium carbonate ($(NH_4)_2CO_3$). A double-acting nitrate, containing equal parts of both nitric and ammonia nitrogen, is ammonium nitrate (NH_4NO_3). It is the most widely used compound in almost all fertilizer producing countries, but has the drawback of a high

hygroscopicity which makes storage difficult. Furthermore, ammonium nitrate is susceptible to detonation due to its sensitivity to heat and shock, so it is diluted with inert substances such as calcium carbonate, diatomaceous earth or clay[15].

Organic nitrogen fertilizers are mainly urea and calcium cyanimide. Urea, CO(NH□)□, is the solid fertilizer with the highest nitrogen title, about 46%; its physiological-agricultural action is comparable to that of ammonia nitrogen, given that it is easily hydrolyzed in the soil, turning into ammonium carbonate[15].

The use of chemical fertilizers was created to meet the need for plant nutrients for their development; on the other hand, however, poor management in the use of fertilizers has been the cause of massive nitrate pollution in groundwater. Several studies have shown that fertilizers are often administered in a single solution, thus exceeding the absorption capacity of plants. The plants, on the other hand, require multiple administrations during the different seasons. But above all it has been found that the fertilizers are supplied in excessive quantities with respect to the real needs of the crops[16]. The Report on the State of the Environment presented by the Ministry of the Environment to Parliament in 2001, refers to a use of more than 4,600,000 t of fertilizers containing N, P and K which correspond to about 890,000 t of nitrogen, with an average around 53 kg/ha of nitrogen. The geographical distribution of these inputs of nutrients is very diversified, reaching in certain provinces inputs such as to generally have a surplus of more than 200 kg/ha. When such excess fertilization occurs, the nitrates present in the fertilizers are easily subject to leaching by rainfall and irrigation. In this way, in the underlying groundwater, the formation of polluted plumes with high concentrations of nitrates can occur[17].

Another anthropic source of nitrate contamination is made up of waste produced by civil settlements, above all relating to small towns or isolated houses, where wastewater treatment is often entrusted to disposal systems such as septic tanks. Such plants can generate an effluent with a nitrogen concentration ranging between 30 and 100 mg NO_3-NL^{-1} [18] .The wastewater produced by larger settlements is usually directed to biological purification plants. If there are no industrial inputs, generally the wastewater entering a purification plant is characterized by an ammonia nitrogen concentration of 12÷45 mg NH_4-NL^{-1} [19] .

Another significant contribution is due to animal husbandry; a study by the University of Nebraska estimated that the waste produced by these activities contain about 100÷400 g N/Kg of animal weight, resulting in a concentration in wastewater of 150÷500 mg NL^{-1} [20] .

Given the different inputs of the chemical form in the groundwater, it becomes extremely difficult to identify the source exactly. Another limiting factor for identifying the source of nitrates is the microbial activity present in groundwater.

Today it is well known that the entire subsoil system is colonized by microorganisms, mainly present in microbial communities, consisting of bacteria and Archaea, but also by protozoa and fungi, which play relevant roles in biogeochemical processes [20] .

All the previous works carried out had the main purpose of evaluating the state of groundwater and soils, classifying them on a chemical basis by homogeneous area, without however allowing the identification of the type of potential sources and a direct correlation between anthropic activity and the trend of nitrate concentration . Furthermore, a microbial characterization (through the identification of the development of different microbial species) that can strengthen the evidence of nitrate contamination and discriminate the type of sources is still lacking.

The denitrification process constitutes the final phase of the natural nitrogen cycle; it takes place by exploiting the ability of bacterial populations to reduce the nitrate ion into molecular nitrogen through the oxidation of organic or inorganic species used as electron donors [21] .

Denitrifying bacteria are facultative bacteria that carry out the process in the absence of free oxygen in the environment, using that contained in the nitrate (anoxic conditions).

Among the processes for the removal of nitrates from groundwater, those that exploit heterotrophic de-nitrification are the most studied and have found wider application in the open field[22] .

The heterotrophic process of denitrification is the most studied in open field installations[22] . The electrons necessary for denitrification originate from the oxidation of the organic substance by the biomass. The bacterial strains capable of operating the denitrification process are ubiquitous and multiple, of which the most abundant belong to the Pseudomonas family.

The presence of heterotrophic bacteria in nature never limits the denitrification process; on the contrary, the availability of organic carbon is identified as the major factor limiting the process kinetics[23] .

Ammonium (NH_4^+) is present in groundwater naturally as a result of anaerobic degradation of organic matter and artificially as a result of organic waste disposal. Anthropogenic NH_4^+ is one of the main dissolved components in some types of peak groundwater contaminants. NH_4^+ concentrations of the order of 1-10 $mmolL^{-1}$ have been observed in groundwater contaminated by landfill leachate and wastewater disposal practices[24] . Rarely, agricultural systems and practices can be impacted by a high presence of NH_4^+ in aquifers which can cause degradation of the quality and usability of groundwater, with substantial effects on water-rock

interactions, and can be a substantial cause of N in surface waters receiving water discharge[25] . The movement of ammonium is very slow and can be retarded by chemical processes in the soil such as uptake or biological processes such as microbiologically induced transformations, depending on the geochemistry of the aquifer and the nature of the groundwater flow[26] . Furthermore, since ammonia nitrogen is extremely volatile, it is difficult for it to leach into the soil and be transported to the groundwater, creating a consequential contamination [27], therefore the various studies carried out have shown that the main source of ammonia nitrogen contamination in groundwater is wastewater from purifiers or leachate leaching from landfills[28] .

Nitrification is a globally relevant process of the nitrogen cycle mediated by a certain group of microorganisms[29] . The discovery of complete ammonia oxidizers (comammox bacteria, CMX) has challenged the paradigm of a strict division of labour in the nitrification process[30] While the oxidation of ammonia is carried out by oxidizing bacteria (AOB) and archaea (AOA)[28] and oxidation of nitrites by nitrite oxidizing bacteria (NOB)[29], CMX of the genus Nitrosovibrio sp. can perform the complete oxidation of ammonia (NH3) to nitrate (NO3-) at one time[31,32] . CMXs have been frequently reported from engineered environments such as wastewater treatment plants[33] and from natural environments such as soil[34, 35] , surface freshwater[36,37] and groundwater[38,39] . However, our knowledge of the role and relevance of CMX for nitrification activity in natural environments is still poor.

The present work has the main objective of identifying, through laboratory tests and elaborations of a mathematical algorithm, the main groundwater dynamics linked to the presence of nitrate of two microbial species, Nitrosovibrio sp. and Pseudomonas sp., identified as the main species of forms of nitrification and denitrification, which can be used as indicators of the impacting activities and by modelling the trend of the species to create a method for identifying the sources of contamination linked to nitrate.

II. Material And Methods

Organisms and cultural media

For the creation of solutions with microorganisms, pure cultures produced by specific laboratories at known concentrations were used: (1% per ml of solution) relative to Nitrosovibrio sp. and Pseudomonas sp.

The cultures were analysed and DNA extraction tests were performed using the kit. Then NGS were performed relative to the DNA extracted from the individual cultures and BLAST alignments were performed with the NCBI database to verify the presence of the species. Subsequently, primers and protocols were created for the amplification of specific sites of both cultures.

For Nitrosovibrio sp. the reaction amplification mix contained 10 µl of PowerUp™ SYBR™ Green Master Mix 2x mix contains SYBR Green dye, Dual-Lock Taq DNA Polymerase, dNTP with dUTP/dTTP mix, thermolabile UDG, passive reference ROX dyes and buffer components optimized; 1 µl Primer Forward (NitrosovibrioF: 5' – GTG GGG AGC AAA CAG GAT TA – 3', T. M. 59.93°C); 1 µl Primer Reverse (NitrosovibrioR: 5' – CAC ATA ATC CAC CGC TTG TG – 3', T.M. 59.99°C); 1 µl of DNA (about 40 ng/µl) and water for an amplification solution equal to 25 µl. The real time thermal cycle consisted of 3 phases: - UDG activation: 50°C x 2'; Denaturation: 95°C x 2'; 40 cycles: Denaturation 95°C x 15", annealing 60°C x 40", elongation 72°C x 1'. The Real Time product was a specific fragment with a length of 182 bp (Fig.1).

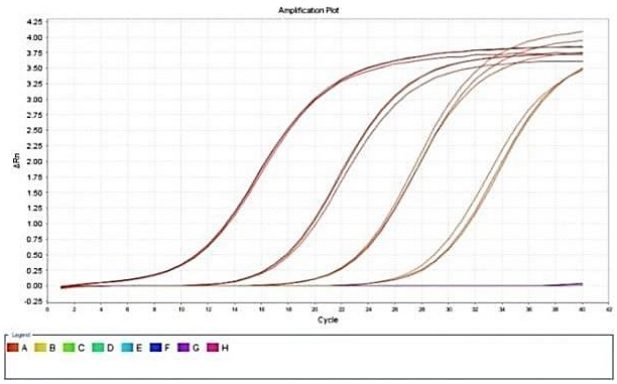

Figure no 1. Amplification of real time PCR Nitrosovibrio sp. cultural media with different dilution.

For the Pseudomonas sp. species, the reaction amplification mix contained 10 µl of PowerUp™ SYBR™ Green Master Mix 2x mix contains SYBR Green dye, Dual-Lock Taq DNA Polymerase, dNTP with dUTP/dTTP mix, thermolabile UDG, passive reference ROX dyes and buffer components optimized; 1 µl Primer Forward (PseudomonasF: 5' – GGT CTG AGA GGA TGA TCA GT – 3', T.M. 60.02°C); 1 µl Primer Reverse (Pseudomonas R: 5' – CCG GTG CTT ATT CTG TTG GT – 3', T.M. 59.99°C); 1 µl of DNA (about 40 ng/µl) and water for an amplification solution equal to 25 µl. The real time thermal cycle consisted of 3 phases:

- UDG activation: 50°C x 2'; Denaturation: 95°C x 2'; 40 cycles: Denaturation 95°C x 15", annealing 60°C x 40", elongation 72°C x 1'. The Real Time product was a specific fragment with a length of 215 bp (Fig. 2).

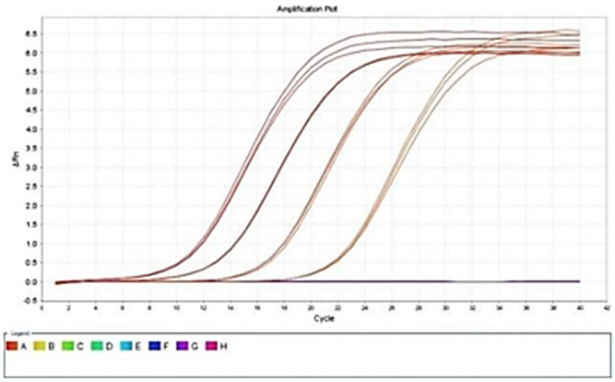

Figure no 2. Amplification of real time PCR Pseudomonas sp. cultural media with different dilution.

Batch experiments

For the purpose of monitoring the trend of NO_3^- and of the bacterial species, two different series of tests were set up, in the first the production of NO_3^- from the metabolism of Nitrosovibrio sp. with NH_4^+as substrate, while in the second test the production of NH_4^+ from the metabolism of Pseudomonas sp. with NO_3^- as substrate.

Test 1

A solution containing NH_4^+ at different initial concentrations (10 mg $NH_4^+L^{-1}$; 40 mg $NH_4^+L^{-1}$; 180 mg $NH_4^+L^{-1}$ and 300 mg $NH_4^+L^{-1}$) was inserted into a 500ml batch. For each experimental line, a verification triplicate and a blank line containing only ultrapure water were created. For each experimental line, a solution containing 5% of the presence of Nitrosovibrio sp. was added to each batch.

All trials were placed at room temperature in a rotary shaker at 120 rpm.

Samples were taken from each line at regular time intervals on which chemical analyses were carried out in relation to NO_3^- and NH_4^+ and analysis of the percentage of presence of Nitrosovibrio sp. through analysis with Real time PCR.

Test 2

A solution containing NO_3^- at different initial concentrations (10 mg $NO_3^-L^{-1}$; 40 mg $NO_3^-L^{-1}$; 180 mg $NO_3^-L^{-1}$ and 300 mg $NO_3^-L^{-1}$) was inserted into a 500ml batch. For each experimental line, a verification triplicate and a blank line containing only ultrapure water were created. For each experimental line, a solution containing 5% of the presence of Pseudomonas sp. was added to each batch.

All trials were placed at room temperature in a rotary shaker at 120 rpm.

Samples were taken from each line at regular time intervals on which chemical analyses were carried out in relation to NO_3^- and NH_4^+ and analysis of the percentage of presence of Nitrosovibrio sp. through analysis with Real time PCR.

III. Result

With regard to test 1 carried out using the Nitrosovibrio sp., daily measurements were carried out for an overall period of 50 days. In all tests, a direct correlation between the decrease in the NH_4^+ used by the species and the increase in the concentration of NO_3^- can be seen. In the test in which the concentration of 10 mg $NH_4^+L^{-1}$ was entered, there was an increase in Nistrosomonas sp. with the achievement of the maximum % presence on day 15 (75%). This peak is reached in conjunction with the equalization of the concentration between the two chemical forms NO_3^- and NH_4^+. Subsequently, due to the decrease in the growth substrate, a decrease in the presence of the bacterial species is noted. Particular is the indication of the trend of Nitrate with a maximum concentration reached on day 25 followed by a decrease also due to the disappearance of the producing species (Fig. 3).

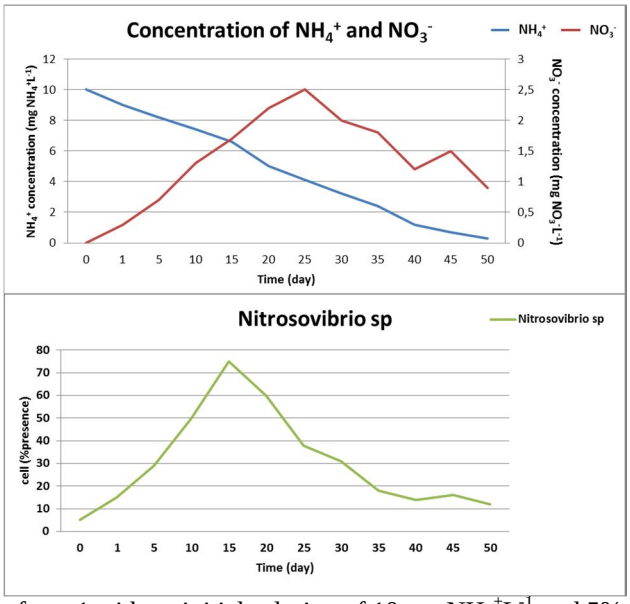

Figure no 3.Result of test 1 with an initial solution of 10 mg $NH_4^+L^{-1}$ and 5% of Nitrosovibrio sp.

In the test in which the concentration of 40 $NH_4^+L^{-1}$ was entered, there was an increase in Nistrosomonas sp. with the achievement of the maximum % presence on day 15 (75%). This peak is reached in conjunction with the equalization of the concentration between the two chemical forms NO_3^- and NH_4^+. Subsequently, due to the decrease in the growth substrate, a decrease in the presence of the bacterial species is noted. Particular is the indication of the trend of Nitrate with a maximum concentration reached on day 25 followed by a decrease also due to the disappearance of the producing species (Fig. 4).

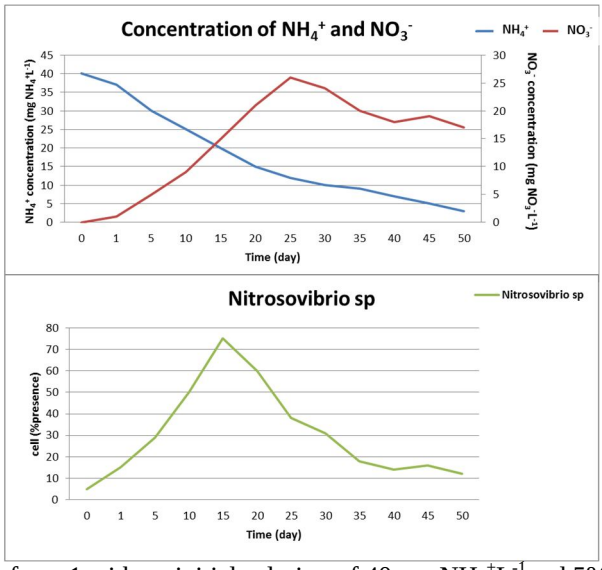

Figure no 3. Result of test 1 with an initial solution of 40 mg $NH_4^+L^{-1}$and 5% of Nitrosovibrio sp.

In the test in which the concentration of 180 mg $NH_4^+L^{-1}$ re was entered, there was an increase in Nitrosovibrio sp. with the achievement of the maximum % presence on day 20 (80%). This peak is reached in a delayed manner relative to the equalization of the concentration between the two chemical forms NO_3^- and NH_4^+. It denotes a stasis of the presence of Nitrosovibrio sp. which begins from the moment in which the two chemical compounds are equal in concentration until shortly after the NO_3^- peak (20mg NO_3^- L^{-1}) where a constant decrease begins both in the bacterial species and in the produced compound (Fig. 5).

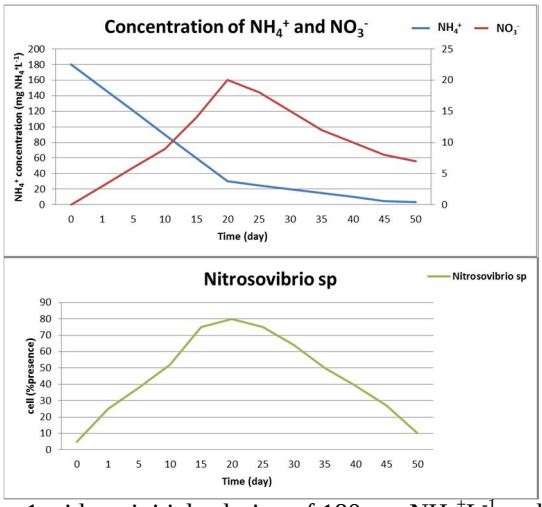

Figure no 5.Result of test 1 with an initial solution of 180 mg $NH_4^+L^{-1}$ and 5% of Nitrosovibrio sp.

In the test in which the concentration of 300 mg $NH_4^+L^{-1}$ was entered, there was an increase in Nitrosovibrio sp. with the achievement of the maximum % presence on day 20 (91%). This peak is reached in a delayed manner relative to the equalization of the concentration between the two chemical forms NO_3^- and NH_4^+. It denotes a stasis of the presence of Nitrosovibrio sp. which begins from the moment in which the two chemical compounds are equal in concentration until shortly after the NO_3^- peak (equal to 50mg $NO_3^-L^{-1}$) where a constant decrease of both the bacterial species and the produced compound(Fig. 6).

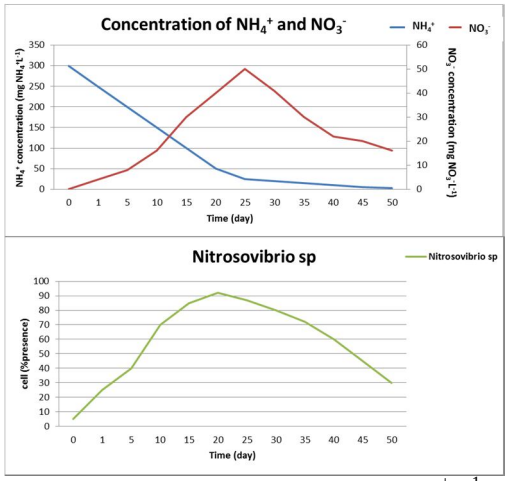

Figure no 6.Result of test 1 with an initial solution of 300 mg $NH_4^+L^{-1}$ and 5% of Nitrosovibrio sp.

With regard to test 2 carried out using the Pseudomonas sp., daily measurements were carried out for an overall period of 50 days. In all tests, a direct correlation between the decrease in NO_3^- used by the species as a growth substrate and the increase in the concentration of NH_4^+ can be seen. In particular, in the test in which the concentration of 10 mg NO_3^- L^{-1} was entered, there was an increase in Pseudomonas sp. with the achievement of the maximum % presence on day 20 (29%). This peak is reached gradually, slightly offset from the NH_4^+ peak (2.2 mg NH_4^+ L^{-1}). Subsequently, due to the decrease in the growth substrate, a decrease in the presence of the bacterial species is noted(Fig.7).

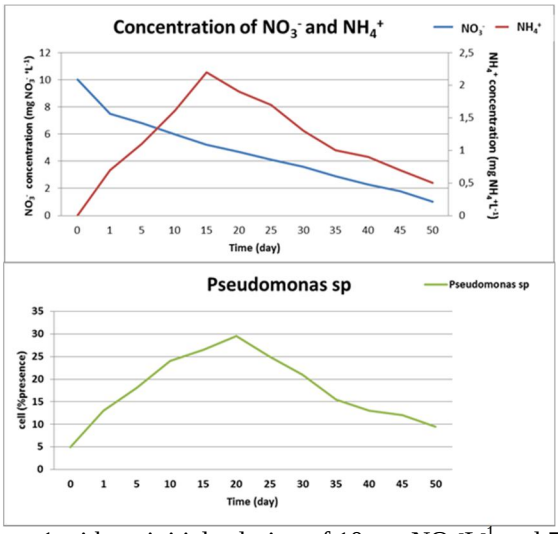

Figure no 7.Result of test 1 with an initial solution of 10 mg $NO_3^-L^{-1}$ and 5% of Pseudomonas sp.

In the test in which the concentration of 40 mg $NO_3^-L^{-1}$was entered, there was an increase in Pseudomonas sp with the achievement of the maximum % presence on day 15 (30%). This peak is reached after equalization of the concentration between the two chemical forms NO_3^- and NH_4^+. Subsequently, due to the decrease in the growth substrate, a decrease in the presence of the bacterial species is noted. Particular is the indication of the trend of ammonia nitrogen with a maximum concentration reached on day 20 (27 mg NH_4^+ L^{-1}) the peak is reached during the plato phase of the species followed by a decrease also due to the disappearance of the producer species (Fig.8).

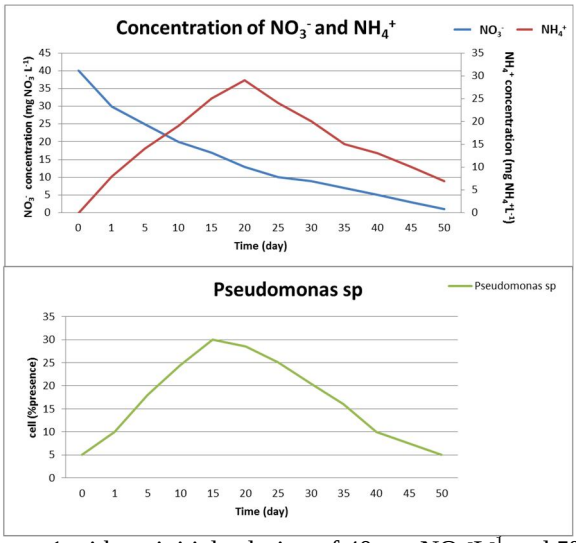

Figure no 8.Result of test 1 with an initial solution of 40 mg $NO_3^-L^{-1}$ and 5% of Pseudomonas sp.

In the test in which the concentration of 180 mg $NO_3^-L^{-1}$ was entered, there was an increase in Pseudomonas sp. with the achievement of the maximum % presence on day 20 (43%). This peak is reached in a delayed manner relative to the equalization of the concentration between the two chemical forms NO_3^- and NH_4^+. It denotes a stagnation of the presence of Pseudomonas sp. which begins from the moment in which the two chemical compounds are equal in concentration encloses the peak of NH_4+ (equal to 35 mg NH_4^+ L^{-1}) where a constant decrease of both the bacterial species and the chemical compounds begins (Fig. 9).

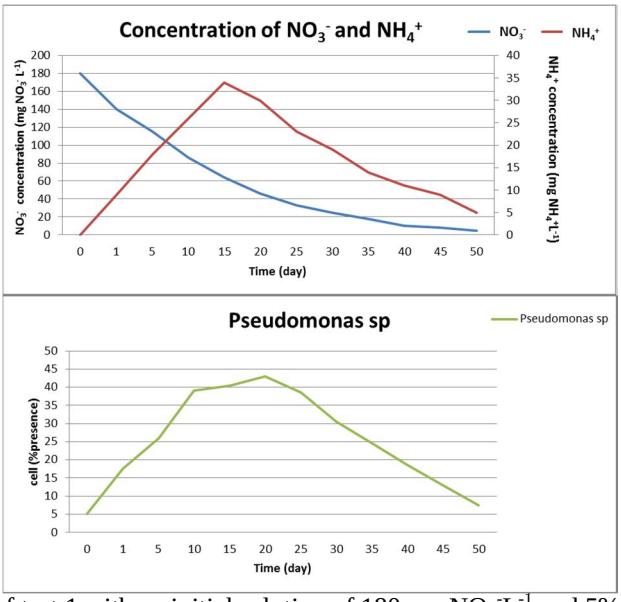

Figure no 9.Result of test 1 with an initial solution of 180 mg $NO_3^-L^{-1}$ and 5% of Pseudomonas sp.

In the test in which the concentration of 300 mg $NO_3^-L^{-1}$ was entered, there was an increase in Pseudomonas sp. with the achievement of the maximum % presence on day 20 (86%). This peak is reached in a delayed manner relative to the equalization of the concentration between the two chemical forms NO_3^- and NH_4^+. It denotes a stagnation of the presence of Pseudomonas sp. which begins from the moment in which the two chemical compounds are equal in concentration encloses the peak of NH_4^+ (53 mg NH_4^+ L^{-1}) where a constant decrease of both the bacterial species and the chemical compounds begins (Fig. 10).

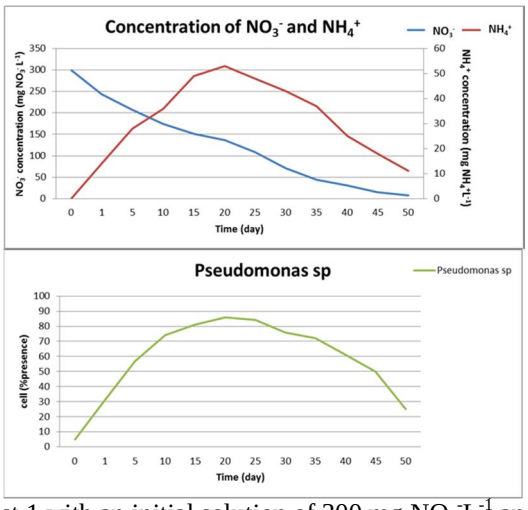

Figure no 10.Result of test 1 with an initial solution of 300 mg $NO_3^-L^{-1}$ and 5% of Pseudomonas sp.

Experimentation shows a trend with a well-defined correlation between growth substrates, microbial species and bacterial products. In particular, in the first test it is possible to define, on the basis of the results and the curves, a ratio between the NH_4^+ as a growth substrate, the Nitrosovibrio sp. and the NO_3^- produced, in the second test it is possible to define a correlation between NO_3^- as a growth substrate, the Pseudomonas sp. and the NH_4^+ produced.

Functional relationshp NH4+ Nitrosovibrio sp. and the NO3-

Nitrovibrio sp., in a controlled environment, has a different reactivity to the concentrations of NH4+ available which is used as a growth substrate. This direct relationship occurred in all four types of experimentation and in all of them it was demonstrated that the species releases NO3- in different quantities precisely on the basis of the concentrations of substrate available. By means of the machine learning methods (the earth function of the package earth in R) it is possible to identify the underlying mathematical structure. These relationships are expressed by the following mathematical equations:

1) initial concentration of NH_4^+ = 10 mg $NH_4^+L^{-1}$

$$NO_3^- = 2{,}5346030 - 0{,}4022424 * h(4{,}1 - NH_4^+) - 0{,}4262388 * h(CNitrovibrio\ -4{,}1) \quad (1)$$

RSq=0,9532636

Were:
$h(4{,}1 - NH_4^+) = max(0, 4{,}1 - NH_4^+) = \{4{,}1 - NH_4^+, \text{ if } 4{,}1 - NH_4^+ > 0; \text{ and } 0, \text{ if } 4{,}1 - NH_4^+ \leq 0\}$
And
$h(CNitrovibrio - 4{,}1) = max(0, CNitrovibrio\ -4{,}1) = \{ CNitrovibrio\ -4{,}1, \text{ if } CNitrovibrio\ -4{,}1 > 0; \text{ and } 0, \text{ if } CNitrovibrio\ -4{,}1 \leq 0\}$

2) initial concentration of NH_4^+ = 40 mg $NH_4^+L^{-1}$

$$NO_3^+ = 23{,}5231355 - 0{,}7595329 * h(12 - NH_4^+) - 0.9213660 * h(CNitrovibrio\ -12) \quad (2)$$

RSq= 0,9617057

Were:
$h(12 - NH_4^+) = max(0, 12 - NH_4^+) = \{12 - NH_4^+, \text{ if } 12 - NH_4^+ > 0; \text{ and } 0, \text{ if } 12 - NH_4^+ \leq 0\}$
And:
$h(CNitrovibrio - 12) = max(0, CNitrovibrio\ -12) = \{ CNitrovibrio\ -12, \text{ if } CNitrovibrio\ -12 > 0; \text{ and } 0, \text{ if } CNitrovibrio\ -12 \leq 0\}$

3) initial concentration of NH_4^+ = 180 mg $NH_4^+L^{-1}$

$$NO_3^- = 18.2714170 - 0.5320438 * h(25 - NH_4^+) - 0.1228122 * h(CNitrovibrio\ -25) \quad (3)$$

RSq= 0.9745508

Were:
$h(25 - NH_4^+) = max(0, 25 - NH_4^+) = \{25 - NH_4^+, \text{ if } 25 - NH_4^+ > 0; \text{ and } 0, \text{ if } 25 - NH_4^+ \leq 0\}$
And:
$h(CNitrovibrio\ -25) = max(0, CNitrovibrio\ -25) = \{ CNitrovibrio\ -25, \text{ if } CNitrovibrio\ -25 > 0; \text{ and } 0, \text{ if } CNitrovibrio\ -25 \leq 0\}$

4) initial concentration of NH_4^+ = 300 mg $NH_4^+L^{-1}$

$$NO_3^- = 20.9222754 - 0.0723706 * NH_4^+ + 0.8537040 * h(CNitrovibrio\ -60) \quad (4)$$

RSq= 0.9381285

Were:
$h(CNitrovibrio\ -60) = max(0, CNitrovibrio\ -60) = \{ CNitrovibrio\ -60, \text{ if } CNitrovibrio\ -60 > 0; \text{ and } 0, \text{ if } CNitrovibrio\ -60 \leq 0\}$

Functional relationship NO_3^- Pseudomonas sp. and the NH_4^+

Pseudomonas sp., in a controlled environment, has a different reactivity to the concentrations of NO_3^- available which is used as a growth substrate. This direct correlation occurred in all four types of experimentation and in all of them it was demonstrated that the species releases NH_4^+ in different quantities precisely on the basis of the concentrations of substrate available. These correlations are expressed by the following mathematical equations:

1) initial concentration of NO_3^- = 10 mg NO_3^- L^{-1}

$$NH_4^+ = 0.9922114 - 0.4489849 * h(3.1 - NO_3^-) + 0.3861165 * h(CPseudomonas\text{-}3.1) \quad (5)$$

RSq=0.9526371

Were:
$h(3.1- NO_3^-) = max(0, 3.1- NO_3^-) = \{ 3.1- NO_3^-, if\ 3.1- NO_3^- > 0;\ and\ 0, if\ 3.1- NO_3^- \leq 0\}$
And:
$h(CPseudomonas\ -3.1) = max(0, CPseudomonas\ -3.1) = \{2, CPseudomonas-3.1, if\ CPseudomonas-3.1>0; and\ 0, if\ CPseudomonas-3.1\leq 0\}$

2) initial concentration of NO_3^- = 40 mg NO_3^- L^{-1}

$$NH_4^+ = 17.4721968 - 0.2036135 * NO_3^- - 0.4074921*h(32-CPseudomonas) + 0.4516922 *h(CPseudomonas\ -32) \quad (6)$$

RSq=0.9478251

Were:
$h(32- CPseudomonas) = max(0, 32- CPseudomonas) = \{ 32- CPseudomonas, if\ 32- CPseudomonas > 0;\ and\ 0, if\ 32- CPseudomonas \leq 0\}$
And:
$h(CPseudomonas\ -32) = max(0, CPseudomonas\ -32) = \{ 2, CPseudomonas\ -32, if\ CPseudomonas\ -32>0;\ and\ 0, if\ CPseudomonas\ -32\leq 0\}$

3) initial concentration of NO_3^-= 180 mg NO_3^- L^{-1}

$$NH_4^+ = 14.8405833 - 0.3315147 * h(49- NO_3^-) + 0.4280695 * h(CPseudomonas\ -49) \quad (7)$$

RSq=0.9748552

Were:
$h(49- NO_3^-) = max(0, 49- NO_3^-) = \{ 49- NO_3^-, if\ 49- NO_3^- > 0;\ and\ 0, if\ 49- NO_3^- \leq 0\}$
And:
$h(CPseudomonas\ -49) = max(0, CPseudomonas\ -49) = \{ 2, CPseudomonas\ -49, if\ CPseudomonas\ -49>0;\ and\ 0, if\ CPseudomonas\ -49\ -32\leq 0\}$

4) initial concentration of NO_3^- = 300 mg NO_3^- L^{-1}

$$NH_4^+ = 25.996262 - 0.445326 * h(61- NO_3^-) + 1.037893 * h(CPseudomonas\ -61) \quad (8)$$

RSq=0.98321

Were:
$h(61- NO_3^-) = max(0, 61- NO_3^-) = \{ 61- NO_3^-, if\ 61- NO_3^- > 0;\ and\ 0, if\ 61- NO_3^- \leq 0\}$
And:
$h(CPseudomonas\ -61) = max(0, CPseudomonas\ -61) = \{ 2, CPseudomonas\ -61, if\ CPseudomonas\ -61>0;\ and\ 0, if\ CPseudomonas\ -61\leq 0\}$

IV. Conclusion

The tests carried out have demonstrated a high correlation between the growth substrates, the relative species and the product of the metabolism. Furthermore, the experimentation showed that there is a strong correlation which highlights both the production of NO3+ and its use.

On the basis of the results obtained it was therefore possible to create ranges for the definition of the increase of nitric nitrogen and therefore to define through the microbial activity whether there is a direct contamination caused by leaching of the compound or an indirect contamination due to the introduction in groundwater of compounds from which nitrate derives, mainly compounds containing ammonia nitrogen such as wastewater.

These ranges therefore define the derivation of the possible contribution on the basis of a complex matrix.
Here are the matrices:

a) Definition of the origin of the Nitrate from sources other than agricultural activities:

ΔNO_3^+ concentration	**ΔNitrovibrio sp and ΔNH_4^+ concentration**
$0.3\leq\Delta NO_3^-\leq 0.6$	$1\%\leq\Delta$Nitrovibrio sp$\leq 2.5\%$ & $-1\leq\Delta NH_4^+\leq -0.8$
$0.6<\Delta NO_3^-\leq 1$	$2.5\%<\Delta$Nitrovibrio sp $\leq 10\%$ & $-3\leq\Delta NH_4^+<-1$
$1<\Delta NO_3^-\leq 3$	$10\%<\Delta$Nitrovibrio sp $\leq 15\%$ & $-5\leq\Delta NH_4^+<-3$

$3<\Delta NO_3^-\leq 5$	$15\%<\Delta$Nitrovibrio sp $\leq 25\%$ & $-15\leq\Delta NH_4^+<-3$
$5<\Delta NO_3^-\leq 6$	$25\%<\Delta$Nitrovibrio sp $\leq 30\%$ & $-30\leq\Delta NH_4^+<-15$
$6<\Delta NO_3^-$	$30\%<\Delta$Nitrovibrio sp & $-30<\Delta NH_4^+$

b) Definition of the origin of the nitrate from leaching caused by agricultural activities:

ΔNO_3^+ concentration	**ΔPseudomonas sp and ΔNH_4^+ concentration**
$0.8\leq\Delta$NO3-≤ 1	$0.3\%\leq\Delta$Pseudomonas sp $\leq 1.6\%$ & $0.4\leq\Delta$NH4+≤ 0.7
$1<\Delta$NO3-≤ 3	$1.6\%<\Delta$Pseudomonas sp $\leq 10\%$ & $0.7<\Delta$NH4+≤ 2.5
$3<\Delta$NO3-≤ 10	$10\%<\Delta$Nitrovibrio sp $\leq 16\%$ & $2.5<\Delta$NH4+≤ -5
$10<\Delta$NO3-≤ 18	$16\%<\Delta$Nitrovibrio sp $\leq 20\%$ & $5<\Delta$NH4+≤ 7
$18<\Delta$NO3-≤ 40	$20\%<\Delta$Nitrovibrio sp $\leq 26\%$ & $7<\Delta$NH4+≤ 9
$40<\Delta$NO3-≤ 56	$26\%<\Delta$Nitrovibrio sp $\leq 30\%$ & $9<\Delta$NH4+≤ 14
$56<\Delta$NO3-	$30\%<\Delta$Nitrovibrio sp & $14<\Delta$NH4+

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