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ISOTOPIC STUDIES OF NITRATES – A SHORT REVIEW

Beata Gebus, Stanisław Hałas

Mass Spectrometry Laboratory, Institute of Physics, UMCS, pl. Marii Curie-Skłodowskiej 1, 20-031 Lublin, Poland; e-mail: beata.gebus@poczta.umcs.lublin.pl

ABSTRACT

Nitrogen is an essential element for life. One of its available forms are nitrates – the compounds playing a key role in the biogeochemical N cycle. However, excessive amounts of nitrates may be harmful to organisms and the environment, therefore in recent years the emphasis is on continuous monitoring of quality of consumed water. Nitrates from different sources have wide, but different ranges $\delta^{15}N$ and $\delta^{18}O$. Also researchers observed typical changes in both delta values induced by biological processes and in the case of mixing water with anthropogenic nitrates. This is a reason why the isotopic analysis are often used to identify the source of contamination in a reservoir or to quantitatively describe the processes occurring in an ecosystem.

In this review article, we present a model of the global nitrogen cycle, along with the latest data on the disturbances caused by human activity. We describe the processes occurring in the N cycle and biogeochemical mechanisms, which modify the nitrogen isotopic composition in their compounds. We also present a short description of analytical techniques utilized for studying isotopic compositions of nitrates. In addition, we discussed the methods for extraction and preparation of nitrates from freshwater and ocean water, by determining the $\delta^{15}N$, $\delta^{18}O$ and $\delta^{17}O$ (or $\Delta^{17}O$) values. The final part is a description of applications of developed techniques for environmental research.

Keywords: nitrogen, nitrate, isotopic analysis, $\delta^{15}N$ value, $\delta^{18}O$ value

INTRODUCTION

The aim of this review paper is to outline the biogeochemical transformations of nitrate occurring in nature. This topic has recently become important to us because since we started the study of nitrogen cycle using the isotope approach.

Nitrogen, as an element forming nucleic acids, amino acids or proteins, is essential for the correct functioning of all living organisms. The ability of N_2 fixation from the atmosphere, the largest reservoir of this element, is due only by a small group of bacteria known as diazotrophs, that which use in this process 16 adenosine triphosphates (ATP) per 1 molecule of N_2 (Young, 1992). However, the other organisms do not have a nitrogenase enzyme required for this process, therefore for most plants and animals nitrogen must be supplied in different forms. These include nitrates, essentially in appropriate amounts, in excess they become harmful.

Nitrates are ubiquitous in our lives and they are a source of assimilable nitrogen for plants and animals. They are compounds with low toxicity and they are not a direct threat to human health or life, being relatively rapidly removed from the body. However, in reducing conditions they are converted to nitrite, which cause a methemoglobinemia (O'Neill, 1993; Price, 1998; Nascimento et al., 2008) or form carcinogenic nitrosamines (Richard, 1980; Bruning-Fann & Kaneene, 1993; Jakszyn & González, 2006). Excessive amounts of nitrates in waters also favors the anthropogenic eutrophication. Its result is not only a visible degradation of water quality, but often irreversible changes in a whole ecosystem. One of the useful tools is isotopic composition studies of nitrate, complemented with concentration measurements of the N compounds in analyzed waters. This makes it possible to determine, whether the main source of NO_3^- is manure or fertilizers, municipal and industrial sewage or NO_3^- derived from nitrification in soil or atmospheric nitrate deposition (Mayer, 2005; Leśniak, 2006).

The nitrates represent a large group of chemical compounds, which includes both inorganic and organic compounds. A planar structure of NO_3^- ions have a sp^2 hybridizations type, in which a central nitrogen atom is surrounded by three identically bonded oxygen atoms. Around each oxygen atom two non-binding electron pairs are located thus, they could easy create new compounds by binding with any cations. In nature they are present in small amounts as three mineral salts. Due to the industrial demand, about a hundred years ago the production of synthetic nitrogen fertilizers started using the Haber-Bosch technology to fix atmospheric nitrogen (Joo et al., 2013).

Almost all inorganic nitrate salts are highly soluble in water; the NO_3^- ions are a source of assimilable nitrogen for plants. Nitrates have a variety of uses, including the preservation of food, production of medicine or explosive materials. Widely present in industry, they penetrate into surface waters, thereby jeopardizing humans and ecosystems. Due to the harmful effects of excessive nitrates on living organisms, for almost 20 years researchers have paid a special attention to a continuous monitoring of water quality and applicability of biological processes

for wastewater treatment. In this case the isotopic methods are very useful, they can help to determine the origin of analyzed nitrate and give information about processes occurring in nature.

GLOBAL NITROGEN CYCLE

Nitrogen is one of the elements widespread in nature. It may occur in several oxidation states from -3 to +5. In free state it exists as a diatomic molecule, N_2, which comprises somewhat over 78% volume of the atmosphere. It belongs to essential nutrients and is often a limiting factor for biological productivity of aquatic and terrestrial organisms (Vitoušek & Howarth, 1991). Among other elements necessary for life, nitrogen is characterized by chemical passivity during most of the biogeochemical processes and by the fact that its primary and the largest reservoir is the atmosphere. The remaining nitrogen reservoirs are depleted by orders of several magnitude.

The nitrogen cycle can be divided into two subcycles:

- small one includes the biosphere and lithosphere; its interior (also called heterotrophic) circulation represents the changes that take place in the lithosphere, and its external (autotrophic) circulation with participation of plants;
- large one carried out in atmosphere, biosphere, hydrosphere and lithosphere.

By biological and chemical reduction of atmospheric N_2 (and scarce oxidation), the nitrogen can be moved from the large to the small subcycle. Released into the atmosphere, the gaseous products of nitrogen transformation and nitrogen leaching from pedosphere to hydrosphere enter small and large cycles (Staszewski, 2012).

By decades scientists have been trying to develop a detailed model of nitrogen cycle in both, the pre-industrial period and after the anthropogenic modification (i.e. Galloway et al., 2004, Gruber, 2008). In 2013, a research group: Joo – Li – Lerman presented a model of balanced nitrogen cycle (Fig. 1), with sizes of reservoirs and mass fluxes between them. For calculations they used the latest version of TOTEM I and II (Terrestrial Ecosystem Model of Atmosphere Ocean; Ver et al., 1999; Lerman et al., 2004), which is a model based on global biogeochemical cycles coupling with carbon, nitrogen, phosphorus and sulfur on the Earth. The natural balance in the nitrogen cycle was disrupted by human activity. The Industrial Revolution, besides the growth of human living standards, also disturbed the biogeochemical cycles of most important systems on the Earth.

According to Lerman et al. (2004), five anthropogenic impacts to nitrogen cycle include:

- emissions of NO_x into the atmosphere, associated with the combustion of fossil fuels, a development of industry and transport; the lifetime of NO_x in atmosphere is rather short, but it is converted to nitric acid and deposited in terrestrial and marine reservoirs;

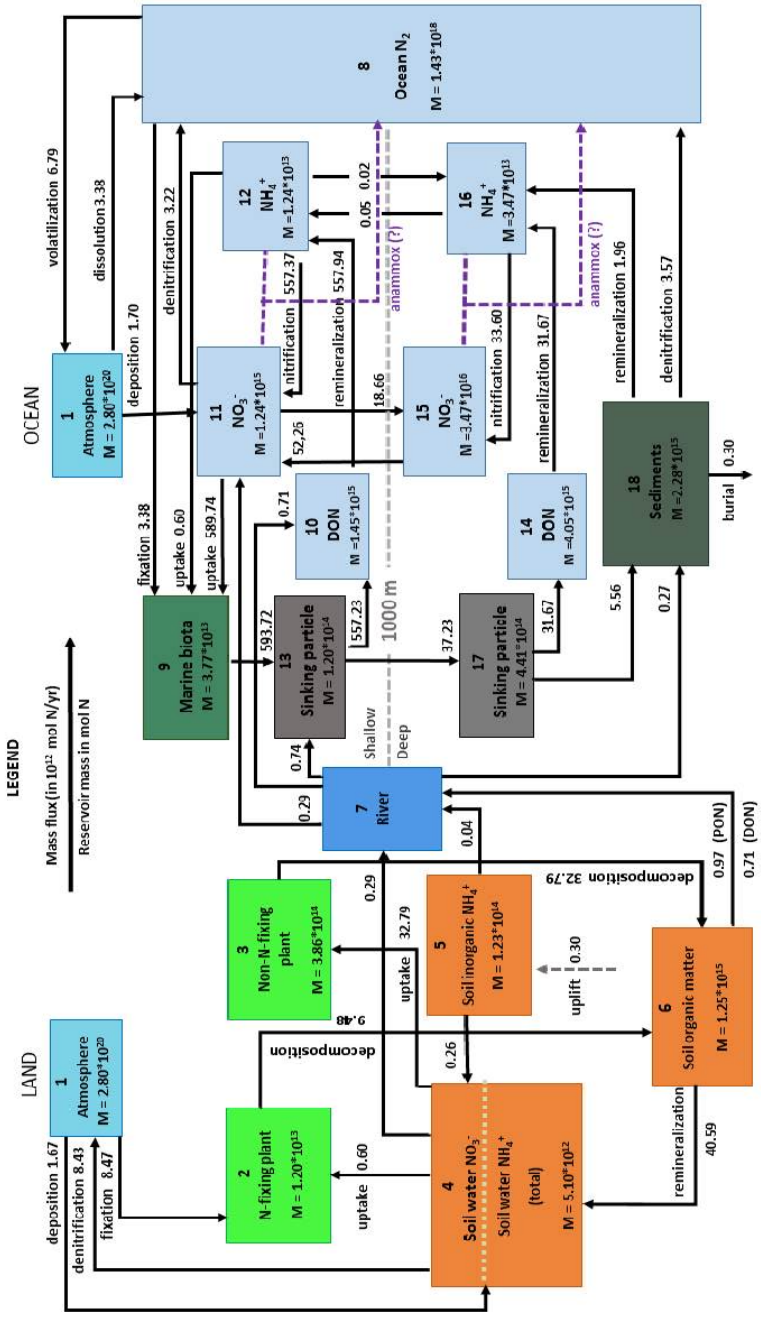

Figure 1: The global nitrogen cycle (after Joo et al., 2013)

- changes related to a land development; the effect of these changes was the transfer of nitrogen from soils to oceans, as a consequence of soil erosion, dissolving of minerals and surface water runoff;
- the use of N-containing fertilizers. Smil reported (1991), that only 45% of applied nitrogen is converted to biomass. The remainder contributes to disturbance of nitrogen cycle – this portion can be transported to coastal areas, stored in soil, leached into groundwater or emission into the atmosphere;
- city sewage whose participation in nitrogen cycle disturbances grows with increasing of population in the world;
- temperature and biological uptake by carbon coupled with the Redfield ratio[1].

In 1999, Ver et al. reported the biggest disturbances in the nitrogen cycle were caused by changes in land use resulting from the use of nitrogen fertilizers. These changes do not introduce a new source of nitrogen, but they are responsible for its transport from pedosphere to hydrosphere. Adding a new reactive N to soil is offset by nitrogen losses associated with changes in land use.

ISOTOPIC COMPOSITION OF DIFFERENT NITROGEN COMPOUNDS

Nitrogen has only two stable isotopes: ^{14}N (99.635%), and ^{15}N (0.365%). For this reason, the ^{15}N abundance in the air is constant, $^{15}N/^{14}N = 0.0036765$ (Joo et al., 2013). Usually the isotopic element composition is given in delta notation, indicating the relative deviation of the sample isotope ratio to the standard one, expressed in permill. For nitrogen (eq. 1):

$$\delta^{15}N\,[‰] = \left(\frac{R_{sample}}{R_{standard}} - 1\right) * 1000 = \left(\frac{\frac{^{15}N}{^{14}N}_{sample}}{\frac{^{15}N}{^{14}N}_{standard}} - 1\right) * 1000 \qquad [1]$$

where R_{sample}, $R_{standard}$ is a ratio of heavy isotope to light isotope, in the examined sample and the standard one, respectively. A positive δ value means enrichment, and negative δ – depletion of the sample in the heavy isotope of a given element. A suitable value in isotopic studies is isotopic fractionation, $\varepsilon_{\rho/s}$, expressed by eq. 2:

$$\varepsilon_{\rho/s}\,[‰] = \left(\frac{\delta^{15}N_{product} - \delta^{15}N_{substrate}}{\delta^{15}N_{substrate} + 1000}\right) * 1000 \approx \delta^{15}N_{product} - \delta^{15}N_{substrate} \qquad [2]$$

[1] Redfield ratio is the atomic ratio of carbon, nitrogen and phosphorus found in phytoplankton and throughout the deep oceans. This empirically developed stoichiometric ratio is found to be C:N:P = 106:16:1.

If $\varepsilon_{p/s} > 0$, then the product is enriched in ^{15}N, if $\varepsilon_{p/s} < 0$ – depleted; between factors $\varepsilon_{p/s}$ and $\varepsilon_{s/p}$ is relationship: $\varepsilon_{p/s} = -\varepsilon_{s/p}$, where *s* and *p* refer to substrate and product, respectively.

This value is useful to describe quantitative changes in biogeochemical processes and theoretical modeling of nitrogen cycle in different catchments.

Oxygen has three stable isotopes, ^{16}O (99.757%), ^{17}O (0.038%) and ^{18}O (0.205%) (Rosman & Taylor, 1999). The isotopic composition of oxygen is usually given in relation to international standard, SMOW (*Standard Mean Ocean Water*). In case of oxygen, usually $\delta^{18}O$ is measured, but now, with new isotope techniques, we can measure also $\delta^{17}O$, which requires much more precise measurements. Both delta values are defined by eq. 3 below.

$$\delta^{18}O\ [‰] = \left(\frac{\frac{^{18}O}{^{16}O}_{sample}}{\frac{^{18}O}{^{16}O}_{standard}} - 1 \right) * 1000 \ ; \ \delta^{17}O\ [‰] = \left(\frac{\frac{^{17}O}{^{16}O}_{sample}}{\frac{^{17}O}{^{16}O}_{standard}} - 1 \right) * 1000 \quad [3]$$

Based on the statistical-mechanical theory developed by Urey (1947), Bigeleisen & Mayer (1947) and Bigeleisen & Wolfsberg (1958), stated that all the conventional thermodynamic and kinetic isotope effects follow the mass-dependent relationship: $\delta^{17}O = 0.52 * \delta^{18}O$. This is because $\delta^{17}O$ is sensitive to the $^{17}O - ^{16}O$ mass difference (1 amu) in the fractionation process and $\delta^{18}O$ is sensitive to the $^{18}O - ^{16}O$ mass difference (2 amu); a slope of ~0.5 is observed in a plot of $\delta^{17}O$ *versus* $\delta^{18}O$. At higher isotopic measurement precision, the slope is observed to vary between 0.500 and 0.526 for different systems. Recently, Hoffman & Pack (2010) have precisely determined the slope of terrestrial fractionation line as 0.5252 ± 0.0008 on the basis of both delta determinations for 161 rocks and mineral samples.

The term $\Delta^{17}O$ called "isotopic anomaly", or oxygen-17 excess, is commonly used to express the deviation from a normal mass-dependent fractionation process:

$$\Delta^{17}O = \delta^{17}O - 0.52 * \delta^{18}O \quad [3a]$$

A mass-independent process is thus any that produces a positive or negative value of $\Delta^{17}O$.

The isotopic composition of nitrogen in its compounds varies within a wide range (Russell et al., 1998; Mayer, 2005; Leśniak, 2006; Joo et al., 2013). Each of these pools is characterized by a typical range of $\delta^{15}N$ values, but part of them in some cases may overlap. Microorganisms prefer lighter isotopes, and therefore the biological reservoirs are normally depleted in heavy isotopes, the ^{15}N or ^{18}O. On the other hand, the remaining reservoirs, including organic and inorganic nitrogen, are enriched in ^{15}N, which suggests, that they are residues after removal of ^{14}N preferred by microorganisms (Joo et al., 2013). The process, in which lighter isotopes are preferred, is also a volatilization of ammonia from a surface layers of soil to the atmosphere. Especially significant changes in isotopic composition of nitrogen in remaining soil ammonium ions are observed in alkaline soil with a high pH (Chmura, 2005). The other processes, which modify the isotopic composition of

nitrogen compounds without participation of microorganisms, are sorption and desorption. The anion-exchange centers prefer the lighter isotopes in NO_3^- ions, both nitrogen and oxygen (Delwiche & Stein, 1970), whilst the cation-exchange centers prefer the heavier isotope, the ^{15}N, from adsorbed fraction of NH_4^+.

Table 1. shows the isotopic composition of nitrogen in various chemical compounds (after Leśniak, 2006), whereas Table 2 presents values of isotopic fractionation, ε, in different microbial process (after Casciotii, 2009).

TABLE 1: Isotopic composition of nitrogen and oxygen in various nitrogen compounds (after Leśniak, 2006).

Reservoir	**Nitrogen compound**	**Isotopic composition [‰]**
Atmosphere	N_2, NH_3, NH_4^+, NO_3^-, N_2O	$-15 < \delta^{15}N < 15$ $20 < \delta^{18}O_{NO_3^-} < 70$
Pedosphere	N_{org}	$\delta^{15}N < 0$
	NH_4^+	$-10 < \delta^{15}N < 5$
	NO_2^-	$-20 < \delta^{15}N < 5$
	NO_3^-	$-5 < \delta^{15}N < 15$ $-5 < \delta^{18}O_{NO_3^-} < 15$

TABLE 2: The values of isotopic fractionation, $\varepsilon_{s/\rho}$, in different microbial process (after Casciotii, 2009 and references therein).

Process	**Reaction**	**$\varepsilon_{s/\rho}$ [‰]**
N_2 fixation	$N_2 \rightarrow N_{org}$	-2 to +2 ‰
NH_4^+ assimilation	$NH_4^+ \rightarrow N_{org}$	+14 to +27 ‰
Nitrification	$NH_4^+ \rightarrow NO_2^-$ $NO_2^- \rightarrow NO_3^-$	+14 to 38 ‰ -12.8 ‰
Denitrification	$NO_3^- \rightarrow NO_2^-$ $NO_2^- \rightarrow NO$ $N_2O \rightarrow N_2$	+13 to +30 ‰ +5 to +25 ‰ +4 to +13 ‰
Nitrate assimilation	$NO_3^- \rightarrow NO_2^-$	+5 to +10 ‰

BIOGEOCHEMICAL MECHANISMS OF MODIFYING A NITROGEN ISOTOPIC COMPOSITION

The nitrogen cycle in nature is dominated by reactions involving microorganisms. Nitrates are a source of assimilable nitrogen, wherein crucial is nitrate to nitrite reduction, taking place via nitrate reductase enzyme. Used by both prokaryotes and eukaryotic cells, this plays a key role in the biogeochemical nitrogen cycle, being substrates or products in a number of transformations, see Fig. 2. The knowledge of microbial nitrogen metabolism is very important, because it has industrial applications ranging from wastewater treatment to bioremediation and a potential future use in biocatalysis for chemical production (Rick & Stuart, 2001).

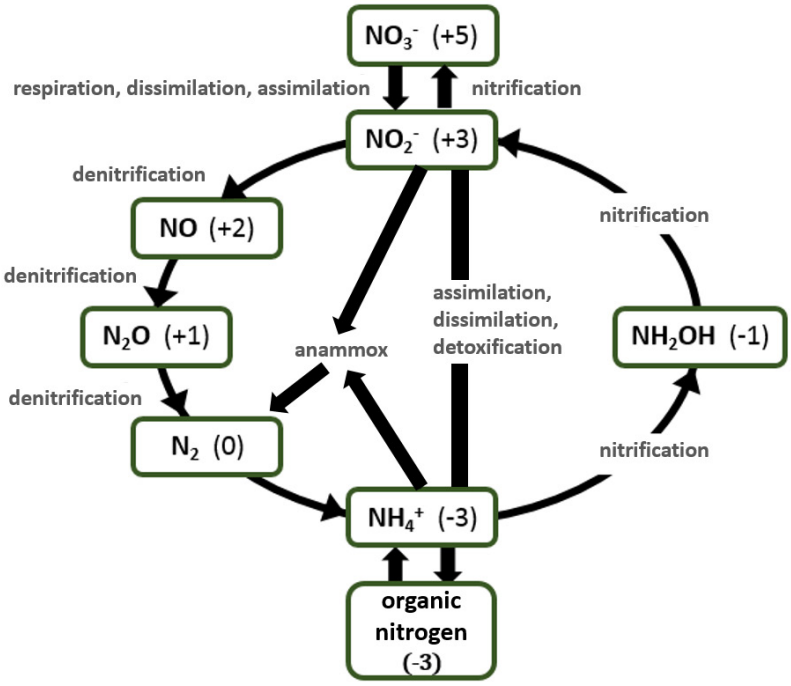

Figure 2: Schematic view of nitrogen biological cycle (based on Richardson, 2001, with added anammox process). In parentheses the oxidation degrees of nitrogen in its compounds are given.

Below various processes of biological nitrogen cycle are shortly described.

Nitrogen fixation is a conversion of atmospheric N_2 to ammonia, which can be metabolized in cells of living organisms. Dinitrogen-fixing bacteria are both aerobic and anaerobic. This process may occur in the presence of appropriate enzyme, called nitrogenase. The nitrogenase is very sensitive to a presence of oxygen, therefore bacteria have developed mechanisms to protect them from destruction by O_2

(O'Neill, 1993). The schematic equation of biological N_2 fixation can be summarized as follows:

$$N_2 + 16ATP + 8e^- + 8H^+ \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i \quad [4]$$

Nitrogen fixation is carried out by bacteria of different physiological groups: free-living in soil and water reservoir (*Azotobacter, Clostridium, Nostoc*), which could stay related with plants roots (*Azospirillum*), or living in a close symbiotic systems of plants (*Rhizobium* – legumes, *Frankie* – alder) (Martyniuk, 2008).

Assimilation is the process of ingestion by plants simple inorganic compounds containing nitrogen, such as NO_3^-, NO_2^- and NH_4^+. In the presence of a suitable reductase, nitrite or nitrate ions are reduced to ammonium ions, which are converted to organic matter. The resulting products are depleted in heavy isotope (both nitrogen and oxygen).

The research reveals that plants grown on a medium supplemented with SO_4^- accumulate much more nitrates than plants in the control group (Buczek & Marciniak, 1990). Studies by the other group of researchers (Rożek et al., 2004) showed, that increased accumulation of nitrate at high sulfate content in the soil occurs due to the antagonism between NO_3^- and SO_4^{2-} during the absorption step, the antagonism between collection of SO_4^{2-} and Mo^{2+} ions, and the competitiveness of the reduction processes of nitrate and sulfate in plants.

Dissimilation is called the use by bacteria in anaerobic conditions oxidized nitrogen form (nitrate or nitrite), as an alternative of electrons acceptor to the free-oxygen (Kotowska & Włodarczyk, 2005). Nitrate reduction leads in this case to producing nitrite or ammonia. This process is generally referred to as nitrate respiration. When the main product of nitrate reduction process is ammonium, we are talking about the fermentation nitrate reduction (Fenchel & Blackburn, 1979) whereas in the case of nitrate reduction to N_2O or N_2 we are talking about denitrification. Dissimilatory reduction to NH_4^+ takes place under the same conditions as appropriate denitrification and then competes with the use of nitrate ions (Tiedje, 1981).

Mineralization (ammonification) is decomposition of complex organic compounds containing nitrogen into NH_3 or NH_4^+. Mineralization takes place, e.g. after the biological death of animal or plant. The products of this process may be used by other organisms. One example of such a mineralization may be urea hydrolysis (eq. 5):

$$(NH_2)_2CO + H_2O \rightarrow 2NH_3 + CO_2 \quad [5]$$

A small isotopic fractionation is related to mineralization process. Some researchers expand this concept to a multi-stage nitrate production from organic matter; then fractionation is much higher (see Table 2).

Nitrification is a two-step process of ammonium oxidation to nitrate, carried out by three microbial groups: (1) autotrophic ammonia oxidizers, (2) autotrophic nitrite oxidizers, and (3) heterotrophic nitrifiers (Prosser, 2005). Because they are primarily

autotrophic, nitrification provides a unique link between the carbon and nitrogen cycles (Casciotti et al., 2011).

The first step, the ammonia oxididation to nitrite (eq. 6, by Prosser, 2005), is carried out by ammonia oxidizing bacteria (AOB). The AOB are primarily a chemolitotrophs, although some of them might consume organic substances (for example acetate or pyruvate) to mixotrophic growth. Included in this groups of bacteria are: *Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosovibrio, Nitrosolobus*.

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O$$
$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \quad [6]$$

Generated H^+ ions lead to environmental acidification, limiting a sustained growth. The second step, oxidation of NO_2^- to NO_3^-, is provided by nitrite oxidizing bacteria (NOB), which are relative autotrophs. Examples of such bacteria are: *Nitrobacter, Nitrospiran* and *Nitrococcus*. Eq. 7 shows a nitratation scheme (Prosser, 2005):

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \quad [7]$$

In favorable conditions for both reactions, the second stage occurs soon after the first, hence excludes the possibility of nitrites accumulation. Soil microorganisms may adapt to a different conditions, but nitrification is generally low at: low pH, low O_2 concentration, low soil moisture, and high C/N ratio (Kotowska & Włodarczyk, 2005). According to Park et al. (2007), optimal pH conditions for *Nitrosomonas* growth is 8.2 ± 0.3, and for *Nitrobacter*: 7.9 ± 0.4; the concentration of dissolved oxygen should be in the range of 1–1.5 mg O_2/L. The presence of ammonia and nitric acid (III) may cause inhibition of nitrification (Anthosien, 1976).

Another type of nitrification is heterotrophic nitrification. This process (the oxidation of inorganic and organic reduced forms of N to nitrate) is carried out by certain fungus types and heterotrophic bacteria. In some organisms, the mechanism is similar to aerobic denitrification, with the participation of fungi (fungal nitrification). This type of nitrification may be important in acid soils or where C:N ratios and heterotroph biomass are high (Prosser, 2005).

Denitrification is a multi-stage reduction of nitrate to gaseous end products, N_2 or N_2O. Denitrification occurs with a participation of heterotrophic bacteria (*Pseudomonas, Micrococus, Alcaligenes, Flavobacterium, Bacillus, Achromobacter*) or autotrophic ones *Thiobacillus denitrificans*. Heterotrophic bacteria obtain energy from oxidation of organic compounds, for example (Niżyńska, 2003):

$$5CH_2O + 4NO_3^- + 4H^+ \rightarrow 2N_2 + 5CO_2 + 7H_2O \quad [8]$$

and autotrophic bacteria from oxidation of inorganic compounds (Hiscock et al., 1991):

$$5FeS_2 + 14NO_3^- + 4H^+ \rightarrow 7N_2 + 10SO_4^{2-} + 5Fe^{2+} + 2H_2O \quad [9]$$

As reported by Knowles (1982), nearly all denitrifiers reduce NO_3^- to N_2, but a small group of these bacteria do not have the adequate reductase (e.g. some species of *Chromobacterium, Micrococcus* and *Bacillus*), then reduction is finished at N_2O. There are also strains, such as *Achromobacter*, which reduce NO_2^-, but they couldn't reduce NO_3^-. Each step of reduction is catalyzed by different enzyme.

Denitrification is an effective method of removing nitrate, therefore this process is used in some wastewater treatment plants. Denitrification is progressing smoothly in favourable conditions, otherwise it is necessary to add organic compound – for example methanol or acetic acid. Denitrification is a process, in which we can see a large isotopic fractionation of nitrogen and oxygen (Table 2). It has a distinctive influence on the $\delta^{15}N$ values in nitrates: with decreasing nitrate concentrations, the $\delta^{15}N$ value grows exponentially (Chmura, 2005).

Anammox (anaerobic ammonium oxidation) is an autotrophic biological process running under anaerobic conditions, in which a complete conversion of NH_4^+ to N_2 (without supplying an external organic carbon source; in this reaction, NO_2^- ions are electron acceptors in a process of biological oxidation to N_2) takes place. Microorganisms responsible for an Anammox process belong to three groups of bacteria: Brocadia (*B. anammoxidans and B. fulgida*), Kuenenia (*K. Stuttgartiensis*) and Scalindua (*S. wagneri, S. brodae, S. sorokinii*). They are characterized by the same metabolism, a similar ultrastructure and low growth (0.1–0.15/day). Activity of Anammox bacteria is 25 times higher than oxygen nitrozobacteria (in denitrification) and over 7 times lower than that of nitrozobacteria under an aerobic conditions (Błaszczyk, 2007).

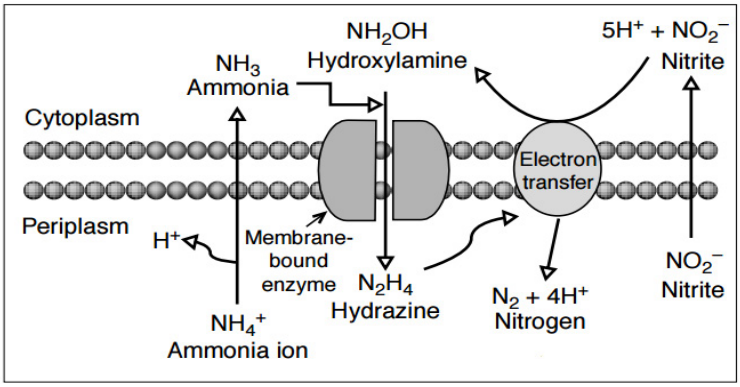

Figure 3: A scheme of anaerobic ammonium oxidation by *Planctomycetales* (Rick & Stuart, 2001). Anammox is coupled with nitrite reduction. Ammonia and hydroxylamine are converted to hydrazine by a membrane-bound enzyme, which is then oxidized in the periplasm. Jetten et al. (1997) propose two potential pathways of electron transfer for nitrite reduction: one system involves a single enzyme that is responsible for hydrazine oxidation and nitrite reduction, and the other involves a nitrite-reducing enzyme that mediates formation of hydroxylamine while an electron transport chain enzyme supplies the electrons.

The combination of partial nitrification and anammox process can be used to remove nitrogen from wastewater with a high content of ammonia nitrogen generated during dewatering of the digested sludge or landfill leachate. It can also be used in purification of industrial wastewater, eg. in the food sector (Kettler, 1997) and wastewater from animal husbandry (Egli et al., 2007). Its advantage is that microorganisms which carried out this process do not require the addition of any organic compounds, which are needed for denitrification bacteria. It also does not cause a large increase of biomass. The most important advantage of Anammox is removal of nitrogen without dissolved oxygen, which greatly reduce operating costs. Nowadays, this process is implemented at a full scale in several wastewater treatment plants, e.g. in Hattingen (Germany) and Dutch Lichteenvorde and Odburger.

METHODS OF NITRATE DETECTION

Nitrates can be detected in several ways:

- in the so-called "ring test", consisting in the reduction of nitrate to nitrogen oxide (II) using a solution of iron sulfate (II) and concentrated sulfuric acid (VI); resulting $Fe(NO)SO_4$ creates a brown "ring" on the border solutions;
- in Devarda's test (de Groot, 2009), which consists in reducing the nitrates to ammonia – gas with a characteristic odor (to NO_3^- solution is adding a few milliliters of NaOH and a small amount of Devarda melt, next solution is heated);
- on the basis of oxidizing influence of NO_3^- on certain organic compounds, for example diphenylamine, which in a solution of concentrated sulfuric acid is colorless (in its reduced form), and becomes dark-blue when passing into oxidized form.

The concentration of NO_3^- ions dissolved in water can be measured colorimetrically using the Griess reagent (e.g. Ellis et al., 1998; Xu et al., 2000), by high-performance liquid chromatography (e.g. Gierak & Leboda, 1999; Torrento et al., 2010) or using micro ion selective electrodes (e.g. Badea et al., 2001, Gebus & Hałas, 2012). In the case, when concentration of nitrite in analyzed water is sufficient, the first method seems to be inappropriate.

METHODS OF NITRATE ISOTOPIC ANALYSES

The first study of nitrogen isotopic composition in nitrate started in the 50s of the twentieth century. Bremner & Show (1955) described a method for nitrogen analysis in nitrate and ammonia extracted from soil. In this method NH_4^+ and NO_3^- are extracted at a pH of 1.0–1.5 with a mixture of potassium sulphate and sulphuric

acid. The ammonia is determined by distillation with magnesium oxide at room temperature in a modified Conway microdiffusion unit. Ammonia plus nitrate is determined on a separate sample of the same extract; nitrate is reduced to ammonia by using titanous hydroxide and subsequent distillation with magnesium oxide. Both reactions, reduction and distillation, are carried out in a modified microdiffusion unit at 25°C. As authors said, their method is applicable to colored extracts from soils and could be useful for determination of ammonia and nitrate in plant materials.

In the same year, Hoering (1955) described a method for nitrate reduction by iron in dilute sulphuric acid. The N_2 end-product was flushed by a He carrier through a liquid nitrogen and Cu-CuO silica glass furnace at 700°C for purification, respectively. Purified and separated N_2 is trapped on a charcoal at liquid nitrogen temperature, then its isotopic composition was analyzed (de Groot, 2009).

Widely used is a method that converts nitrate to ammonium by a Kjeldahl reaction (Bremner, 1965; Bremner & Edwards, 1965). This in turn is converted in the next step to N_2 gas by one of several methods (Silva et al. 2000):

(1) combustion of a dried ammonium salt (Kendall & Grim, 1990, described below),
(2) steam distillation of ammonium followed by oxidation with a hypobromite solution, and purification of N_2 in a Cu/CuO furnace (Bremner, 1965; Bremner & Edwards, 1965),
(3) distillation followed by ammonium collection on a suitable zeolite and combustion to N_2 (Velinsky et al., 1989),
(4) slowly ammonium diffused into an acid solution or onto acidified filter paper, and combusted/reacted to N_2 (MacKown et al., 1987; Sigman et al., 1997, described below).

Adapted from Kline & Caplan (1975) "ammonia diffusion" method, was described by Sigman et al. (1997) and it involves:

- sample concentration by boiling or evaporation,
- conversion of nitrate to ammonia using Devarda's alloy,
- the gas-phase diffusion of ammonia onto an acidified glass fiber disk which is sandwiched between two porous Teflon membranes.

The authors described the conditions necessary to effect complete ammonia recovery from natural seawater samples and the use of Devarda's alloy under these conditions (Fig. 4 and Tab. 3). While the samples have an incubation time of 4 days or longer, they find out that the diffusion method allows for higher throughput than the distillation method because samples can be run conveniently in large batches.

This nitrate extraction method gives highly reproducible, complete recovery of nitrate and a standard deviation for isotopic analysis of <0.2‰ down to 5 pM nitrate (or lower). The Devarda's alloy used by the authors results in a blank of -0.4 nmol N per 100 ml of seawater, for this blank the authors made an isotopic correction.

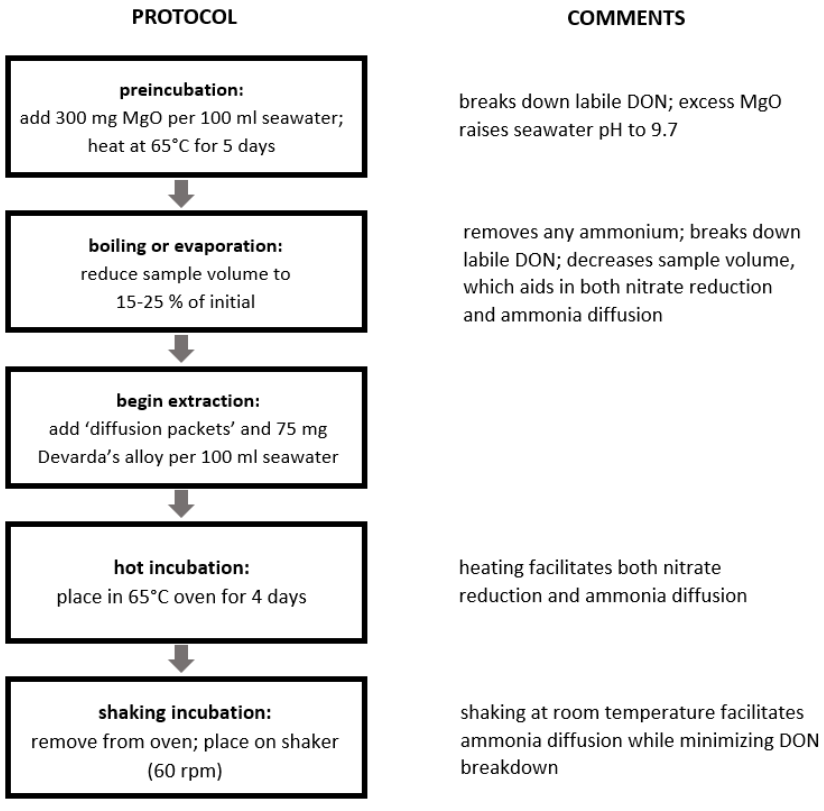

Figure 4: A protocol summary chart for the nitrate extraction method (Sigman et al. 1997).

TABLE 3: Conditions for nitrate extraction incubations (Sigman et al. 1997).

Sample nitrate (µM)	Initial sample volume (ml)	Final sample volume (ml)	Devarda's alloy (mg)	Incubation time at 65°C (days)	Total incubation time [a] (days)
20	100	15-20	75 (50-60)[b]	4 (2)[c]	6 (4)
10	200	30-40	150 (100-120)	4 (2)	8 (6)
5	400	60-80	300 (200-240)	4	12
2.5	800	120-160	600 (400-480)	4	16

[a] The difference between total incubation time and incubation time at 65°C is time that samples are to be incubated on a shaker.

[b] The values given in the table are recommended amounts. The values in parentheses are minimum amounts that have been shown to give reproducible isotopic data.

[c] The values given in the table are recommended times. The values in parentheses are minimum times that have been shown to give reproducible isotopic data, if this information exists.

The next technique adapted from Kline & Caplan (1975) for application to low-level nitrate samples was described by Tanaka & Saino (2002).

Another method to determine isotopic ratio of nitrogen in NO_3^- ions, from soil and water samples, is described by Russow (1999). In this method nitrate was injected (about 10 mL) into reaction vessel containing a mixture of 15% $TiCl_3$ solution and concentrated H_2SO_4, where the reaction takes place according to the following equation:

$$NO_3^- + 3Ti^{3+} + 4H^+ \rightarrow NO + 2H_2O + 3Ti^{4+} \quad [10]$$

Reaction gases were carried away from the reaction vessel by a He flow, NO was purified in a traps system and then measured on a quadrupole mass spectrometer (Fig. 5). With the same reaction line nitrites could by analyzed too. In this case the reaction vessel should contain a mixture of NaI (or KI) and H_3PO_4.

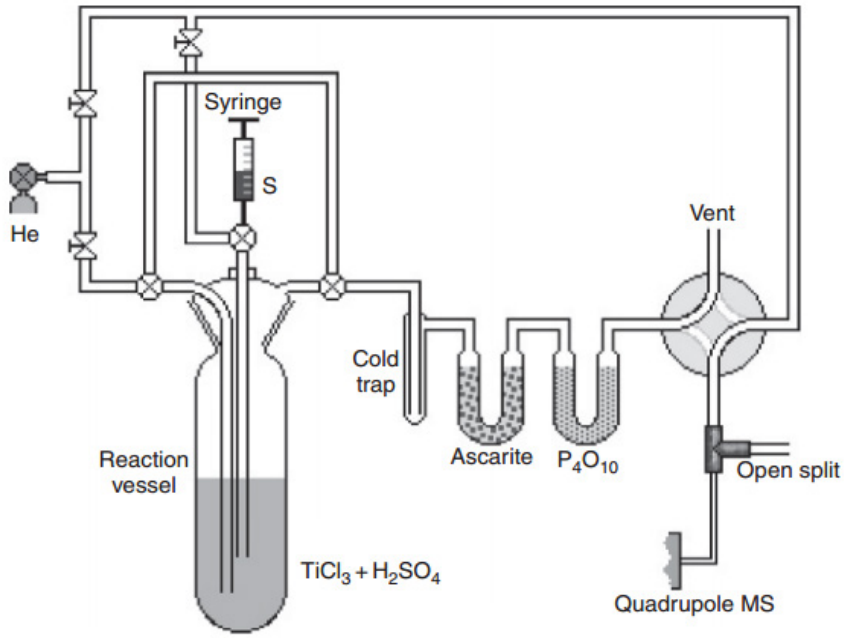

Figure 5: Nitrate reaction device coupled to a quadrupole mass spectrometer for determination of $\delta^{15}N$ values (after Russow, 1999). S = sample

Since the 80s of the twentieth century the "combustion" method has become popular, enabling analysis of $\delta^{18}O$ in nitrates. Originally, it was the mercury cyanide off-line combustion method described in 1987 by Amberger & Schmidt. Nitrate, in the KNO_3 form, and mercury cyanide, in molar ratio 3:4, were mixed and homogenized in an agate mortar. Then aliquots containing 100 µmole of KNO_3 were loaded into 9-mm glass tubes, evacuated overnight at 100°C, and sealed. The tubes were baked at 560°C for 6 h and then cooled to room temperature. Gases from the

combustion tubes were separated cryogenically and then CO_2 gas was analyzed for the $\delta^{18}O$ value (Révész & Böhlke, 2002). In view of the toxicity of cyanide and mercury compounds, this has been gradually displaced by the more safety analytical method.

The "on-line" carbon combustion method was described by Kornexl et al. (1999). KNO_3 samples were dried in a 90°C oven overnight to remove a moisture contamination. Aliquots containing about 3 μmole of nitrate were loaded into silver capsules and combusted in a ThermoQuest/Finnigan TC/EA unit. The samples were placed in a reaction tube held at 1450°C, and a continuous He flow of 70 mL/min transported the product to a GC column (5 Å molecular sieve, 80–100 mesh, 2 feet) held at 70°C for separation of CO from other gases. The purified reaction products, mainly N_2 and CO, were transferred to a Finnigan Delta Plus mass spectrometer and analyzed for oxygen isotopic composition (Révész & Böhlke, 2002).

In 1997 Révész et al. described a low temperature graphite off-line combustion method. The mixture of nitrate sample (as in the previous case, the authors used KNO_3) and catalyzed C, containing up to 1% by weight Pd or Ni (in molar ratio 1:4) were mixed and homogenized in an agate mortar. Next, aliquots of mixture were loaded into 9-mm glass tube with a small slab of 0.0025 mm thick gold foil. The tubes were evacuated overnight at 100°C, sealed, baked at 520°C for 24 h by the following reaction:

$$4KNO_3 + 5C \rightarrow 2K_2CO_3 + 3CO_2 + 2N_2 \tag{11}$$

and then cooled slowly. End-products gases from combustion tubes were separated cryogenically; the CO_2 gas was transferred by cryogenic distillation into a sample tube for $\delta^{18}O$ analysis. The CO traces from N_2 fraction were removed by "re-combustion" with Cu_2O + CaO. Because in tubes remained K_2CO_3, it was reacted with phosphoric acid at 25°C to release CO_2. To obtain the $\delta^{18}O$ value of the original KNO_3, the authors combined the results for the CO_2 and K_2CO_3 by equation:

$$\delta^{18}O_{KNO_3} = 0.9967\ \delta^{18}O_{CO_2} - 3.29 \tag{12}$$

As the authors described, these combustion techniques are associated with a small oxygen isotope exchange between a glass and a sample, which depends on glass type and should be added as a correction to the true $\delta^{18}O$ value. This method could be used to nitrate extraction for natural-abundance level of nitrogen isotopic measurement of oceanic nitrate.

Kendall & Grim (1990) described a combustion method to analyze nitrogen isotope ratio from organic and inorganic materials. In this case authors used aliquots of samples required to produce 6–60 pmoles of N_2. The samples with reagents: 10–500 mg CaO, 3 g of cupric oxide wire, 4 g of cupric oxide and 5 g of copper granules (amounts of copper and copper oxide could probably be reduced by 50% as the authors indicated) were put in 23 cm x 9 mm Vycor tubes. The tubes were evacuated and sealed to a length of about 18 cm. Next, they were shaken and vibrated to mix the sample with the reagents and then combusted at 850°C. After 2

hours, the tubes were slowly cooling to a room temperature. CaO was used for the quantitative removal of CO_2 and water. In the next step tubes were loaded into a tube cracker mounted to the inlet system and evacuated. The tubes were frozen in liquid nitrogen for 15 min before loading, cracked and purified. Then N_2 was expanded into the mass spectrometer for analysis.

In 1983 Unger & Heumann described a new calibration method for nitrate determination in water samples. In this method production of negative nitrogen dioxide thermal ions was used to measure NO_2 in a mass spectrometer after conversion into nitrate. In the article the authors discussed the optimum conditions for the ionization process, such as filament material, filament temperature and sample compounds (sodium nitrate, lead nitrate and nitron nitrate). By this method the nitrate traces could be analyzed down to the ng/g-level. According to the authors, this technique could be applied for the standardization of other analytical methods. The relative external standard deviation of the isotope ratio measurement is in the range of 0.2‰. The method can be used to determine NO_x, nitrite and nitrate.

Very popular nowadays nitrate isotope analysis method was described by Silva et al. (2000). In this method nitrate is pre-filtered and then collected on anion exchanging resin columns in the field, then the columns are subsequently transported to the laboratory where the sample is adequately prepared. According to the authors, nitrate is eluted from the anion exchange resins, AG1-X8 with 3M HCl. Next, the nitrate-bearing acid eluant is neutralized with Ag_2O and filtered to remove a precipitate of silver chloride. Then the solution is freeze-dried to obtain solid $AgNO_3$, which in the next step is combusted to N_2 in sealed quartz tubes for $\delta^{15}N$ analysis. The tubes are loaded with combustion reagents (CaO, CuO, and Cu wire), evacuated and combusted according to the method of Kendall & Grim (1990) described above. During evacuation, the part of tubes with $AgNO_3$ are covered by paper or foil jackets to prevent photodegradation of silver nitrate. As the authors describe, the 1σ analytical precision in this case is ±0.05‰.

Prior to the $\delta^{18}O$ analysis, the silver nitrate solution should be further removed by non-nitrate oxygen-bearing anions and dissolved organic matter. Sulfates and phosphates are stripped by adding 1M $BaCl_2$, the solution is then filtered and passed through a cation exchange column to remove excess Ba^{2+} ions. In the next step the solution is re-neutralized with Ag_2O, filtered, agitated with activated carbon to remove dissolved organic matter and freeze-dried. The resulting silver nitrate crystals are combusted with graphite in a closed quartz tube to produce CO_2, which is cryogenically purified and analyzed for its oxygen isotope composition. The 1σ analytical precision in this case is 0.5‰. The scheme of extraction method proposed by Silva et al. (2000) is shown in Fig. 6.

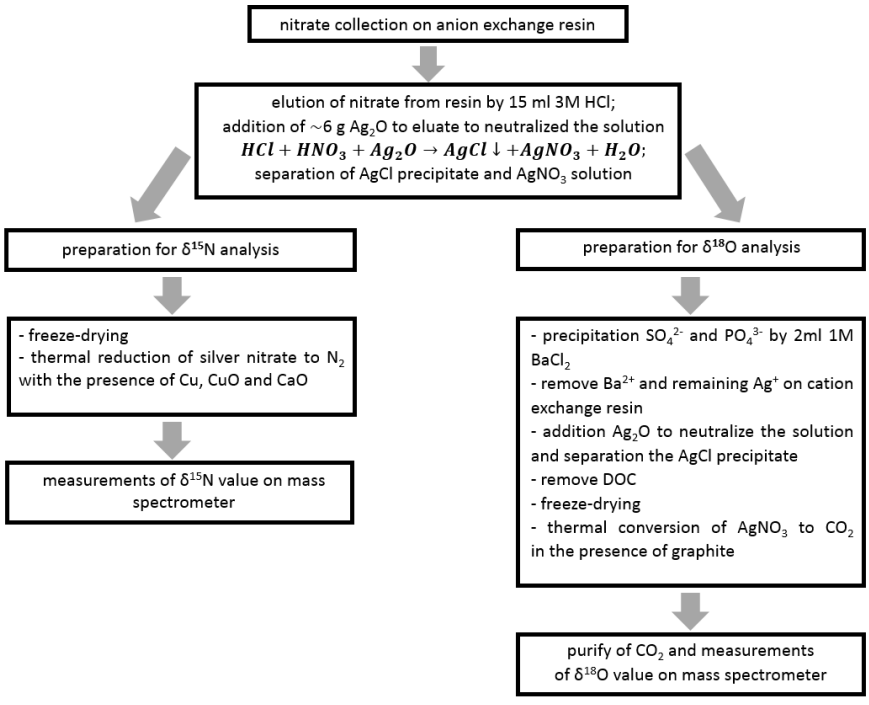

Figure 6: A protocol summary chart for the nitrate extraction method described by Silva et al. (2000).

As the authors said, the method is convenient, economic, has excellent precision for $\delta^{15}N$ and produces higher yields of CO_2 for $\delta^{18}O$ measurements. For the others benefits they include is elimination of the need to transport large volumes of water to a laboratory, elimination of the need for hazardous preservatives and ability to concentrate nitrate from fresh waters.

Another, already popular, method for measuring the isotopic composition of oxygen and nitrogen from nitrates is a denitrifying method. The method is described by the authors in two parts (Sigman et al. 2001 for nitrogen isotopes, Casciotti et al. 2002 for oxygen isotopes). This method is based on isotopic analysis of N_2O generated from nitrates by denitrification bacteria, which don't have a nitrous oxide reductase. In the first article (Sigman et al. 2001) the authors described the protocol for nitrogen isotopic analysis. For the tested method, researchers used two well-known denitrifier bacteria strains: *Pseudomonas chlororaphis* and *Pseudomonas aureofaciens.* As the authors said, both strains have similar characteristics with regard to blanks and reaction times, but only *P. aureofaciens* could be useful to determine $\delta^{18}O\text{-}NO_3^-$. Both strains cultivated at room temperature on tryptic soy agar containing the same nitrate and ammonium amendments as the liquid medium (10 mM potassium nitrate and 1 mM ammonium sulfate). The cultures are then grown for min 6 days - this time is required to complete O_2 consumption from the

headspace and the amended nitrate. To ensure an anaerobic conditions and remove N_2O produced by original nitrate, each sealed vial is purged at 10–20 mL/min for 2 hours with N_2 gas. After sample addition, the vials are incubated overnight to complete nitrate conversion to N_2O. Next, 0.1–0.2 ml of 10 N sodium hydroxide is injected into each headspace vial to a pH >12, to stop the reaction. In the last step N_2O is stripped from each sample vial by using a helium carrier gas, purified, and analyzed for its isotopic composition using an isotope ratio mass spectrometer.

The precision in this case is better than 0.2‰ (1σ) for nitrate concentrations <1 μM. For samples with ≥1 μM NO_3^- the blank of the method is less than 10% of the signal size and could be more reduced, as authors said. The adventage of the method also includes: small sample size and time required to analysis, a reproducible isotopic analysis of samples with very low nitrate concentration and an ability to analyze the oxygen isotope composition of seawater nitrate.

In the case of oxygen isotopic analysis the same authors used strains *Pseudomonas aureofaciens* and *Corynebacterium nephridii*. In this case working cultures grow in 130-ml batches of tryptic soy broth amended with 10 mM KNO_3, 7.5 mM NH_4Cl and 36 mM KH_2PO_4. The cultures are inoculated with 0.5 ml of starter culture and grow in sealed bottles (160-ml capacity) on shaker at room temperature. Sample preparation follows the method of Sigman et al. 2001, described above. Working cultures grown for 6–10 days, than the vials are crimp-sealed with teflon-backed silicone septa and purged for 3 hours with N_2. In the next step, aliquots of dissolved nitrate (10–20 nmol) are added to the sample vials and are incubated overnight to allow for complete nitrate conversion to N_2O. Then 0.1 mL of 10 N NaOH to stop bacterial activity and scavenge CO_2. The oxygen isotopic composition is measured on a Finnigan DELTA ^{plus} isotope ratio mass spectrometer in a continuous-flow mode. As the authors said, the results from the denitrifier method and other techniques used to compare the $\delta^{18}O$ values agree well for many groundwater, precipitate, and salt samples. The denitrifier method achieves a very high level of sensitivity, providing similar precision for $\delta^{18}O$ with 2–3 orders of magnitude less nitrate required per analysis. This allows analysis of samples with low nitrate concentrations (≤1 μM) and limited volumes (10 mL for 1 μM NO_3^-).

In 2011 McIlvin & Casciotti described a few novel approach of denitrifier method, which yielded increased precision and throughput of NO_3^- isotopic analysis.

Böhlke et al. (2003) described the isotope-ratio analysis of N and O from nitrate samples. The nitrogen isotopic composition is analyzed by off-line reduction with copper. Dried samples of KNO_3 or $NaNO_3$ (approximately 80 μmol/aliquot) were loaded into quartz glass tubes with 1.5 g mixture of $Cu+Cu_2O$ and 0.3 g of pre-baked CaO for high-temperature conversion to N_2 gas. The tubes with reactants were evacuated and sealed, then baked at 850°C for 2 h to produce N_2 and next, slowly cooled to room temperature to remove traces of H, C, and S gases by reaction with CaO. Baked tubes were broken manually in a tube cracker under vacuum and the purified N_2 gas was analyzed by a Finnigan MAT 251 dual inlet isotope-ratio mass spectrometer.

The $\delta^{18}O$ value is determined by on-line reduction with carbon. For O isotope-ratio analyses, aliquots of the same nitrate salts (approximately 4.5 μmol/aliquot)

were loaded into 3.5 x 5 mm Ag foil capsules, which were folded shut, placed in an autosampler on a Thermoquest-Finnigan thermo-chemical elemental analyzer and flushed with high purity He (modified from Kornexl et al. 1999 and Revesz & Böhlke 2002). Samples were dropped sequentially into a granular carbon crucible held within a vitreous carbon reaction oven held at 1325°C within an alumina cylinder that also was flushed with He. The obtained CO gas was carried in a He stream through a gas chromatograph to the inlet of a Finnigan Delta XP continuous-flow isotope-ratio mass spectrometer and analyzed against a tank of CO reference.

McIlvin et al. (2005) proposed a new method for both $\delta^{15}N$ and $\delta^{18}O$ analyses from freshwater and seawater, through chemical conversion of nitrates into nitrous oxide. The method is based on the reduction of nitrates to nitrites using spongy cadmium and then reduced to nitrous oxide by sodium azide in an acetic acid buffer. For separate nitrite analysis, the cadmium reduction step is simply bypassed. The nitrous oxide obtained is then analyzed in a CF IRMS for N and O isotopic values. Nitrogen and oxygen isotopic fractionation and oxygen atom exchange were consistent within each batch of analysis, these effects were included in the determination of a true value of both deltas in tested samples.

Undoubtedly the great advantage of this method is the ability to analyze nitrogen and oxygen isotope ratios separately in NO_2^- and NO_3^-, in the presence of nitrates. The researchers analyzed the nitrate samples from 0.5 to 40 μM concentration range. As the authors said, 1σ analytical precision is less than 0.2‰ for nitrogen and 0.5‰ for oxygen (better in the case of nitrite analyses). They claim also, that this method may prove to be simpler, faster, and obtain isotopic information for lower concentrations of nitrate and nitrite than other methods.

In 2011 Huber et al. presented a new simple method for the isolation of nitrate, which is based on the different solubilities of inorganic salts in an acetone/hexane/water mixture. In this mixture all major nitrate salts are soluble but other oxygen-bearing compounds are not (most inorganic carbonates, sulfates, and phosphates). In first step nitrate was concentrated by freeze-drying, dissolved in the ternary solvent and separated from insoluble compounds by centrifugation. The used barium iodide in solution was precipitated with anhydrous barium nitrate.

For the $\delta^{18}O$ analysis, dried $Ba(NO_3)_2$ by conversion into CO gas via pyrolysis with a high-temperature conversion elemental analyzer is used. Dry silver capsules reacted with a glassy carbon at 1450°C in a continuous flow of ultra-pure helium gas. Before pyrolysis, the products, mainly dinitrogen and carbon monoxide, were transfered to the IRMS; they passed through H_2O and CO_2 absorption traps and were separated on a column with a molecular sieve (5 Å) held at 50°C.

For the $\delta^{15}N$ analysis, samples are combusted in an elemental analyzer (EA) coupled to an IRMS system. The method was tested down to 20 μmol NO_3^- with a reproducibility (1σ) of 0.1‰ for nitrogen and 0.2–0.4‰ for oxygen isotopes. As the authors said, in this method a little isotopic fractionation (^{15}N enrichment of +0.2‰) was observed, which is associated with an incomplete precipitation process. For oxygen a correction for the incorporation of water in the precipitated $Ba(NO_3)_2$ has to be applied. Despite these drawbacks, this method is highly efficient and cost-effective.

Noting the lack of "off-line" method, where during one preparation could be obtained gases to measure the two delta values ($\delta^{15}N$, $\delta^{18}O$), we attempted to fill this gap. Our method is based on the simultaneous conversion of the thermal decomposition products of $AgNO_3$ to N_2 and CO_2, whose isotopic compositions ($\delta^{15}N$, $\delta^{18}O$) are then measured by a 3-collector mass spectrometer. The reaction is carried out at 850°C for 2 hours in a vacuum system ($p<10^{-2}$ mbar) to decompose $AgNO_3$ to Ag, NO_2 and O_2. Then, NO_2 and O_2 are reduced by spectrally pure graphite in the presence of Pt-Ir catalyst (Gebus et al., 2012). In the method homogenized mixture of 12 mg $AgNO_3$ and 3 mg C is used. At this temperature we observed also small amounts of CO and NO_2. This fact does not significantly affect the $\delta^{18}O$ value, but makes impossible a reliable determination of $\delta^{15}N$. Therefore, the traces of CO and NO_2 were removed in "Cu/CuO furnace". We are currently working on achieving the best precision in this preparation (Gebus et al., in progress).

In recent years there are also developed techniques to measure $\delta^{17}O$ in nitrate samples. According to Kendal et al. (2007), the analysis of $\Delta^{17}O$ in nitrates could be a very useful tool to describe the atmospheric processes and in ecosystem studies because it is an unambiguous tracer of atmospheric NO_3^- (e.g. Michalski et al. 2003, Michalski et al. 2004, Leis et al. 2015).

Michalski et al. (2002) described a method for $\Delta^{17}O$ in nitrates, dedicated to collected atmospheric nitrate aerosols analysis. Nitrate was isolated and converted to $AgNO_3$ by the method described by Silva et al. (2000). The oxygen isotopic composition of $AgNO_3$ was determined by its partial conversion to O_2 and analysis on a Finnigan-Mat 251 isotope ratio mass spectrometer. According to these authors, the analytical precision of complete experimental method is ±0.2‰ for 5 mmol NO_3^- samples.

Schauer et al. (2009) presented a method used for measurement of nitrate $\Delta^{17}O$ by silver salt pyrolysis. Silver nitrate samples are dropped into a quartz column held at 550ºC. The O_2 obtained during pyrolysis was purified away from other products using a molecular sieve trap held at liquid nitrogen temperature. The O_2 sample is then passed through a capillary GC and into an IRMS for 32, 33, 34 mass/charge measurement. This method requires about 200 nmol O_2. As the authors presented, the precision $\Delta^{17}O$ is ±1.0‰ and the accuracy is ±0.4‰.

In the next years this group described a method of silver nitrate analysis (Schauer et al. 2012, Geng et al. 2013) at sub-micromole levels using the pyrolysis method. Schematic view of apparatus used in this technique is shown in Fig 7.

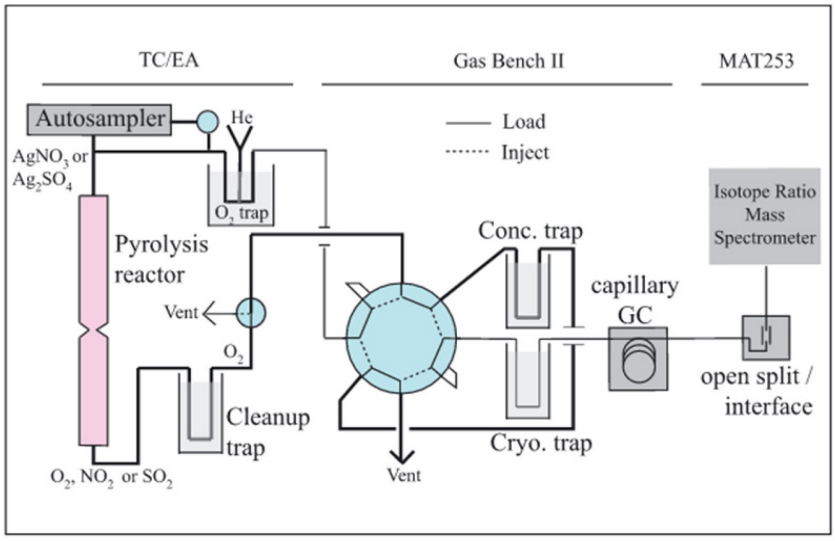

Figure 7: Flow path diagram of the TC/EA-GB-IRMS system (Geng et al. 2013).

First a sample was preprocessed to dissolve ions in aqueous solution and pre-concentrate the solution by evaporation. Next step was a separation using ion chromatography and then conversion of nitrate into $AgNO_3$. Samples were dried to produce solid silver nitrate, used in the next step. The silver nitrate was thermally decomposed in quartz and silver sample containers to O_2 and by-products in a TC/EA (mainly NO_2) were subsequently removed from the system. Next step was trapping and extraction of the O_2 sample with a Gas Bench interface and subsequent determination of the evolved O_2 isotope ratios ($^{17}O/^{16}O$ and $^{18}O/^{16}O$) using an isotope ratio mass spectrometer. Analytical precision (1σ) in this case is better than ±0.7‰ (Geng et al., 2013).

THE ISOTOPIC COMPOSITION OF NITRATE ($\delta^{15}N$, $\delta^{18}O$) AS A TOOL TO IDENTIFY SOURCES OF WATER POLLUTION

The investigations of light stable isotopes have many applications, including hydrology and environmental studies. From the observation that the isotopes do not participate equally in the natural processes but undergo fractionation, i.e. in a given reaction are preferred lighter or heavier isotopes of the element, a slight modification of products isotopic composition. Above we presented data on variation ranges of the isotopic composition of nitrogen compounds and the mechanisms affecting their modification. Although the $\delta^{15}N$ values in some cases substantially overlap, the researchers managed to isolate the variation ranges of nitrates isotopic compositions derived from different sources. Thereby, a few decades ago $\delta^{15}N$ values made it

possible to distinguish nitrate from various sources (e.g. Mariotti et al., 1981), where the primary reservoirs do not mix with each other.

Development of agriculture and industry has contributed to new nitrates sources to the nitrogen cycle. Identification of the source of contamination can be difficult if the effects of different processes are overlapped. Currently, however, there are adequate tools for identification of anthropogenic pollutions in water reservoirs. Now, thanks to development of isotope techniques, we can measure both delta values ($\delta^{15}N$ and $\delta^{18}O$) in NO_3^- ions. Thereby, isotopic studies supplemented by analysis of concentration changes of nitrogen compounds in seasonal cycles could determine the origin of contamination in analyzed waters (Vitoušek et al., 1997; Kendall et al., 1998; Mayer, 2005; Hosono et al., 2013). Such studies can determine if nitrates in the water are derived from atmospheric deposition, fertilizers or manure used in nearby agricultural areas or formed during the soil nitrification process (Fig. 8).

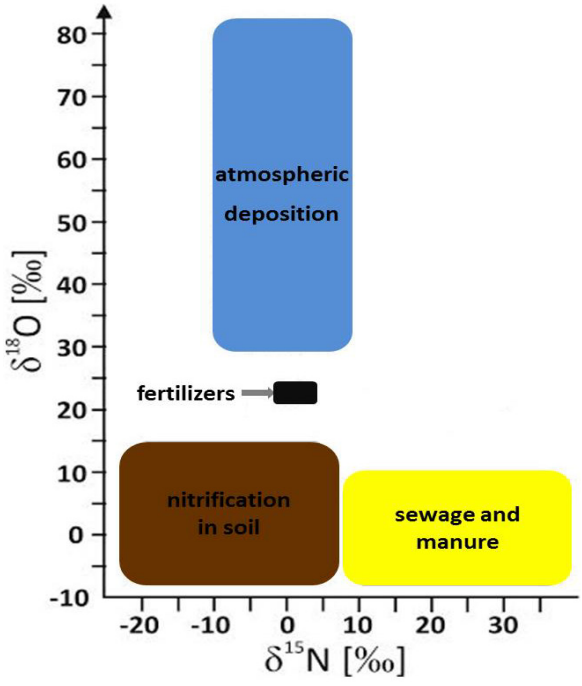

Figure 8: Ranges of isotopic compositions for major nitrate sources (based on Mayer et al., 2002; Mayer 2005).

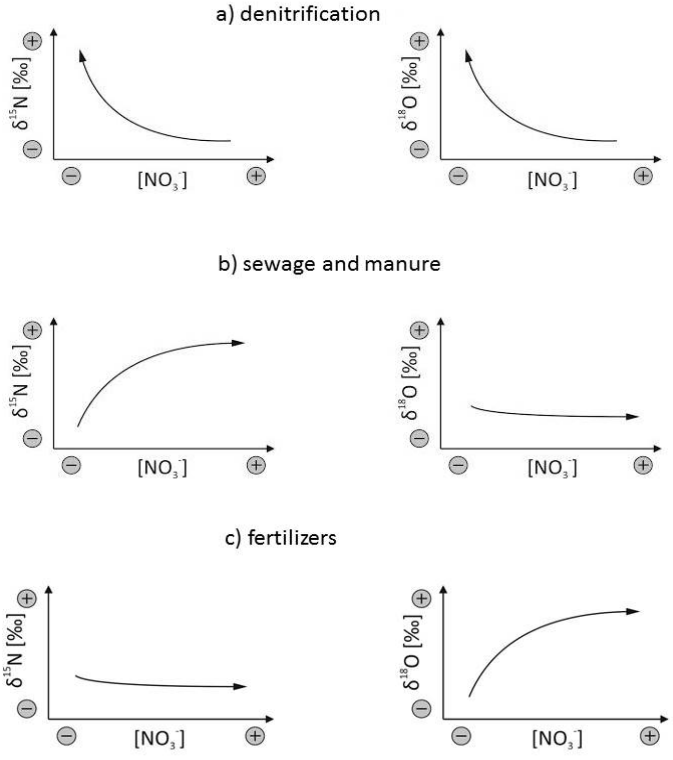

Figure 8 a-c): Trends in the $\delta^{15}N$ and $\delta^{18}O$ value:
- during denitrification (a)
- mixing of waters with sewage and manure (b)
- mixing of waters with fertilizers (c)

As Mayer (2005) pointed, nitrate in surface water or groundwater rarely has the same isotopic composition as nitrate in fertilizers or atmospheric deposition. Usually it is assumed that low $\delta^{18}O$ values (<15‰) of aqueous nitrate indicate nitrate from atmospheric deposition ($\delta^{18}O$ nitrate > +30‰) and nitrate from fertilizers ($\delta^{18}O$ nitrate ~ 23‰) does not behave conservatively in the unsaturated water zone, but rather undergoes an intense immobilization–mineralization cycle in the soils (e.g. Mengis et al., 2001).

Researchers also observed typical changes of nitrate concentrations and $\delta^{15}N$-NO_3^- values for an admixture of nitrate from an anthropogenic source (Fig. 8 b, c). Using a combined chemical and isotopic techniques, they identified nitrate derived from sewage (Aravena et al., 1993) or manure (Rock & Mayer, 2004) in surface waters and groundwater. By studying the isotopic composition of nitrogen and oxygen in NO_3^- ions we can also follow the processes in nitrogen biogeochemical cycle (Delwiche, 1981; Jaffe, 1992; Casciotti, 2009; Casciotti et al. 2013), both in the qualitative and quantitative manner.

CONCLUSIONS

In this article we have reviewed the biogeochemical transformations of nitrate occurring in nature. In the recognition of these transformations steps, a particular role is played by the isotopic techniques of nitrate determinations. The studies of nitrate isotopic composition ($\delta^{15}N$, $\delta^{18}O$) allow us to distinguish natural and anthropogenic nitrates and identify the sources of water contaminations. On the other hand, the analysis of $\Delta^{17}O$ value in nitrates samples is used to study the atmospheric processes and their influence on ecosystem.

All the described techniques are also helpful to better understand the mechanisms of processes occurring in nitrogen cycle. The development of technology makes it possible to obtain more precise results of isotopic analyses while reducing the amount of sample required for analysis. We do hope that this trend will continue and that the study of stable isotopes will gain followers in other areas of knowledge.

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