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Nitrate, nitrite and microbial denitrification in drinking water from Ozun village (Covasna County, Romania) and the association between changes during water storage

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SUMMARY. Severe groundwater contamination affects drinking water quality in many vulnerable zones across Romania. Ingestion of high levels of nitrate and nitrite can lead to the emergence of compounds with major toxicological effects. The present study was aimed to assess nitrate and nitrite concentrations in parallel with the activity of denitrifying bacteria, in different groundwater sources from Ozun village, Covasna County. Natural denitrification potential based on local microbiota was investigated during water storage for one month. Results indicate that tap water supplied from the public system complies with drinking water criteria, but most domestic wells are not safe. Nitrate concentrations exceeded the maximum limit allowed for drinking water in most of the samples collected from dug wells, while nitrite ions occurred within the mandatory limit. Denitrifying bacteria were detected in all groundwater samples, with the exception of one well. Regrowth of denitrifying bacteria was observed during water storage, but significant reduction of nitrate, nitrite or their sum of ratio did not occurred as a general rule. The association between percentage changes in bacterial counts, nitrate and nitrite concentrations was not statistically significant. In conclusion, enhancing the bioremediation potential of local microbiota by groundwater storage at household level is not an efficient strategy for nitrate/ nitrite removal.

Keywords: denitrifying bacteria, groundwater, nitrate, nitrite, water storage.

Introduction

Water, together with air, nutrients, light and climate represent important natural resources, sustainability of life on Earth becoming questionable as a result of their continuous deterioration and depletion. Over the last decades, local and global consequences of

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environmental degradation and pollution became more obvious (Hill, 2010), impacting the ecological balance and the human health (Myers *et al.*, 2013; Suk *et al.*, 2016). Pollution occurs as a result of accidental or deliberate discharge of harmful substances (fertilizers, pesticides, organic contaminants, metal compounds, etc.), after which the environment suffers significant changes and the biogeochemical cycling is altered. The most common contaminants affecting water and soil are nitrates, phosphates and organic matters. These substances are harmless and even necessary at certain levels, but are harmful at higher concentrations (Butiuc-Keul, 2014).

Many thresholds for human and ecosystem health have been exceeded due to pollution with nitrogen compounds (Erisman *et al.*, 2013).Especially in agriculture, soil fertilization is required for the high productivity of crops. Excessive application of chemical fertilizers, natural compost, or mixtures, often leads to over-fertilization, soil becoming saturated in reactive nitrogen species (Fields, 2004). The soil and vegetation cannot retain and use them and therefore, depending on their solubility, such contaminants end up leaching into the vadose zone and groundwater (Dahan *et al.*, 2014). During the nitrogen cycle, nitrogen compounds are affected by the ammonifying, nitrifying and denitrifying bacteria. Ammonium ions ($NH4^+$) resulting from mineralization of organic matter are oxidized to nitrite (NO_2^-) and then to nitrate (NO_3^-) by nitrogen-fixing bacteria in a process known as nitrification. Denitrifying bacteria, also known as nitrate reducers, belong to the category of chemotrophic microorganisms that oxidize hydrogen to release energy, having the ability to reproduce the natural circuit of nitrogen. Denitrification occurs by reduction of inorganic nitrogen compounds, nitrate being reduced to nitrite and then to nitrous oxide (N_2O) and to molecular nitrogen (N_2), under anaerobic conditions (Muntean, 2009).

Although nitrate concentrations have slightly decreased over the past decades in some European rivers, overall, nitrate levels in groundwater have remained constant (Grizetti *et al.*, 2011). Across Romania, many aquifers are highly contaminated, in different regions of the country, from the karst systems into the mountains (Epure and Borda, 2014) to the seaside (Vulpaşu and Racoviţeanu, 2016). Nitrate and nitrite have been frequently detected in drinking water drawn from underground sources (Burcă *et al.*, 2015; Mureșan *et al.,* 2011; Pele *et al.*, 2010; Roşu *et al.,* 2014; Török-Oance *et al.*, 2013; Vasilache *et al.*, 2012), in concentrations exceeding the maximum limit of 50 mg/L for nitrate and 0.5 mg/L for nitrite, respectively (Council Directive 98/83/EC; Law 458/2002).

Nitrate removal methods include biological denitrification. The possibility of enhancing natural denitrification is currently receiving attention in relation to the problem of nitrate in groundwater (WHO, 2011).

The purpose of this study is to investigate the well water quality in Ozun village, Covasna County, an area vulnerable to nitrate contamination. Also, given the population habits to store drinking water in households, an experiment was conducted in order to assess the changes of water properties over one month. Fresh water samples from private wells and from the public network were analyzed for nitrate, nitrite and denitrifying bacteria. After 30 days of storage, chemical and microbiological analysis was repeated.

Materials and methods

Ozun is the largest commune of Romania, located in the southern part of the Covasna County, Romania (Fig. 1). Seven villages belong to Ozun commune: Ozun, Sântionlunca, Lisnău, Bicfalău, Lunca Ozunului, Măgheruș, Lisnău-Vale. The commune has a population of 4599 inhabitants (Ráduly, 2011).

Figure 1. Location of the study area. Image taken from GoogleEarth

The main source of drinking water is groundwater. The old centralized system, supplied through 3 drilling boreholes of medium depth (50-80 m), is currently under expansion. Measurements on the quality of drinking water are carried out regularly by the Water Management System Sfântu Gheorghe. Previous data indicate an excess of iron, manganese and nitrate. As a result, the area is included in the nitrate vulnerable zone. The households not connected to the public network are using private hand-dug wells. Well water quality is not regularly monitored to ensure compliance with drinking water standards. Domestic wells are highly exposed to nitrate and other types of pollution, mainly due to leakage from manure storage, mineral fertilizers, sewage and detergents from private homes, household waste deposits and those from authorized and unauthorized industrial activities (Ráduly, 2011).

Location of sampling points

For the investigations and experiments performed in the present study, a number of 10 samples have been collected from domestic wells and from the public system (Fig. 2), in April 2015:

- Eight samples were collected from private wells with daily use for drinking, household needs and irrigation (W1-W8);
- Two samples were collected from the public water supply, one from the reservoir before treatment (R) and one from the tap (T).

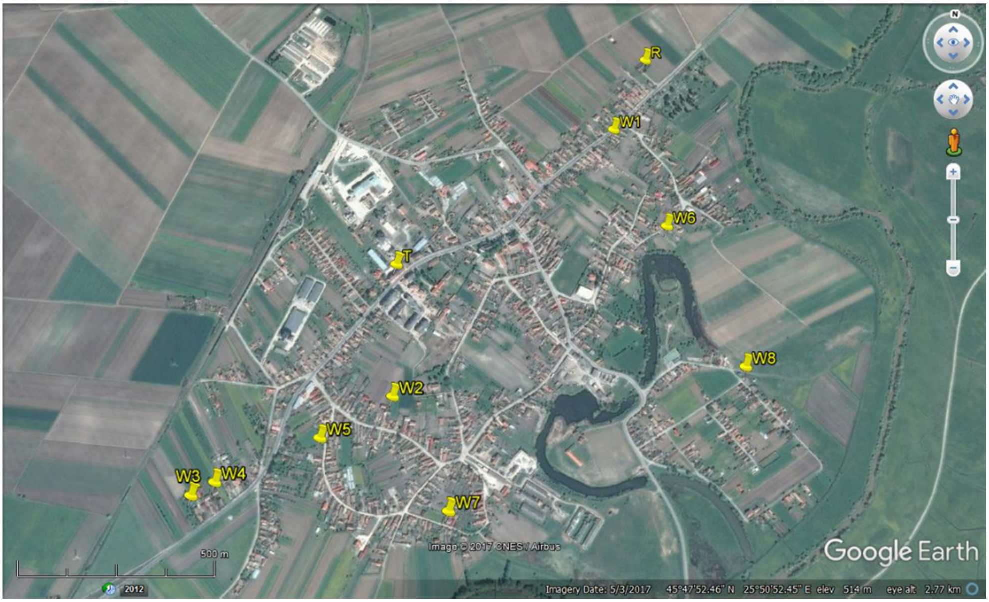

Figure 2. Location of sampling points across Ozun village. W1 - W8: domestic wells; R: reservoir of the public drinking water system; T: tap water from public network. Image taken from GoogleEarth

Water storage and analysis

Samples were analysed for nitrate, nitrite and denitrifying bacteria within 12 hours after collection (day 1). Then, considering the common practices of household water storage, the samples were kept in closed polypropylene bottles for one month at room temperature, and the analysis was repeated (day 30). All the procedures for sampling, sample processing and execution of microbiological analysis were carried out in sterile conditions. The analyzes were performed in laboratories of Faculty of Biology and Geology, Babeș-Bolyai University, Cluj-Napoca, Romania.

Determination of nitrate and nitrite

The measurement of nitrate was carried out using the spectrophotometric method, according to the international standard (SR ISO 7890-1/1986). It is based on the formation of yellow 4-nitro-2,6-dimethylphenol, when nitrate reacts with 2,6-dimethylphenol, under acidic conditions. Nitrite levels were measured by spectrophotometric reading, a pink diazonium salt resulting from nitrite reaction with Griess reagent, sulphanilic acid (4-aminobenzosulfoacid) and 1-naphtylamine (SR EN ISO 26777/2002).

Assessment of microbial denitrification

The most probable number of denitrifying bacteria was estimated by the method of multiple tubes (Farkas, 2015). Each water sample and at least three subsequent dilutions were inoculated in Allen broth, a culture medium rich in nitrate (KNO_3 1g/L, KH_2PO_4 0.4 g/L, K_2HPO_4 0.5g/L, $CaCl_2$ 0.2 g/L, $MgSO_4$x $7H_2O$ 0.25 g/L, $FeCl_3$ 0.001 g/L). The inoculated tubes were incubated at 25 °C for 14 days. Denitrification was evident at the end of the incubation period through the accumulation of gas (nitrogen or nitrous oxide) in the Durham tubes.

Risk assessment of nitrate and nitrite in drinking water

Because of the possibility of the cumulative hazard due to simultaneous occurrence of nitrate and nitrite in drinking water, the sum of the ratios (R) of the concentration (C) of each to its guideline value (GV) was assessed for each sampling point, in freshwater and stored water:

$$\frac{C_{nitrate}}{GV_{nitrate}} + \frac{C_{nitrite}}{GV_{nitrite}} \leq 1$$

The GV of 50 for nitrate and GV of 3 for nitrite were considered (Council Directive 98/83/EC; Law 458/2002). Based on epidemiological evidence for methaemoglobinaemia in infants, the sum of ratios should not exceed 1 (WHO, 2011).

Statistical analysis

In exploratory analyses, the results were statistically assessed using the analysis of variance (Kruskal-Wallis non-parametric test) to find out any significant differences between the parameter levels in fresh water and also after 30 days of storage. The change in nitrite or nitrate concentration was compared with the variation of denitrifying bacteria after storage, by using the association between percentage changes in a regression framework. Statistical analyses were performed using the Real Statistics Resource Pack software for Microsoft Excel (Zaiontz 2015), with a significance level of $p < 0.05$.

Results and discussion

Occurrence of nitrate and nitrite in drinking water

At day 1, nitrate concentrations varied between 9.94 and 150.16 mg/L, exceeding the maximum limit allowed (50 mg/L) in 6 groundwater samples. Only four samples, including two wells and the public drinking water system were found to correspond with drinking water criteria. After one month of storage, recorded nitrate levels ranged between 2.40 and 151.81 mg/L. The changes were not uniform, in some of the samples (W1 and W2) a nitrate raise have been recorded, while in other samples (W3-W8, R and T) a nitrate reduction have been observed (Fig.3).

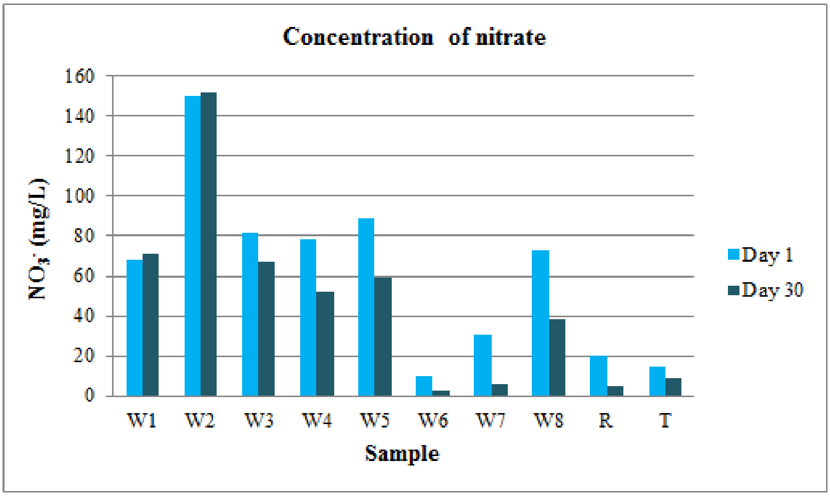

Figure 3. Nitrate concentrations in groundwater and tap water. W1-W8=well water, R=reservoir, T=tap water from the public network

A percentage of 88% groundwater samples from dug wells, in Ozun village, contained high levels of nitrate (above 10 mg/L), 75% being non-compliant with drinking water standards. Other studies investigating drinking water from domestic wells across Romania also warn on nitrate contamination. In Cluj County, 46,4% of wells and springs were found contain more than 10 mg NO_3^-/L (Mureșan *et al.*, 2011), while all groundwater samples from the most urbanized village near Cluj-Napoca exceeded 10 mg NO_3^-/L, 56% of the wells being non-compliant with drinking water criteria (Roşu *et al.*, 2014). Unfortunately, in Romania there are still many areas in which these values are significantly exceeded (Fleşeriu and Oroian, 2010). Extreme values, over 400 mgNO_3^-/L were found in Matca, Galaţi County, Săhăteni, Buzău County (Pele *et al.*, 2010) and Ştefăneşti, Botoşani County (Vasilache *et al.*, 2012), while more than 500 mgNO_3^-/L have been detected in Brăneşti, Ilfov County (Pele *et al*., 2010; Tociu *et al*., 2016). Factors such as the complex biogeochemical cycle of nitrogen in soils, nitrate leaching from the agricultural system and dispersion in the groundwater contribute to extended nitrate contamination (Marinov and Marinov, 2014; Wick *et al.*, 2012). Severe contamination leads to nitrate concentration in shallow groundwater.

At day 1, nitrite was detected in concentrations up to 0.309 mg/L (Fig. 4), the maximum limit allowed for drinking purposes (0.5 mg/L) being not exceeded. After 30 days, nitrite levels were observed to increase in three water samples (W1, W2 and W6), while in five samples a nitrite reduction have been noticed (W3, W4, W5, R and T). In samples W7 and W8 nitrite has not been detected neither at the time of water collection, nor after one month of storage.

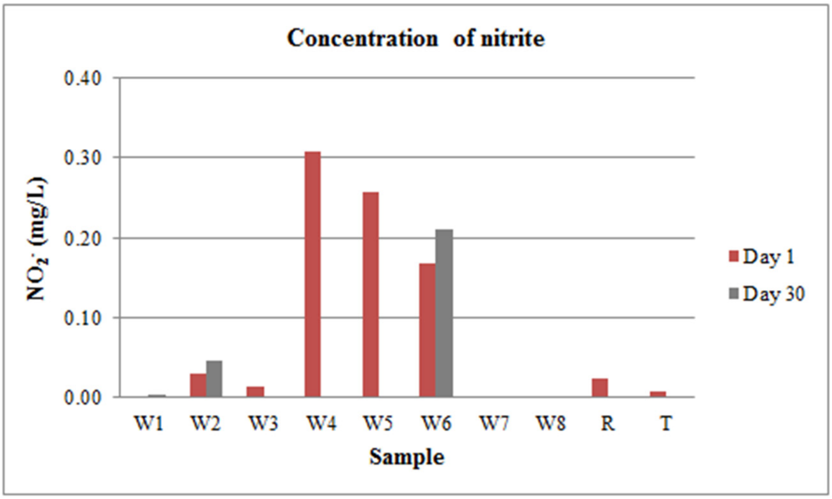

Figure 4. Nitrite concentrations in groundwater and tap water. W1-W8=well water, R=reservoir, T =tap water from the public network

Both the groundwater from private wells and drinking water from the public system of Ozun village contained low nitrite levels. Previous studies investigating drinking water from domestic wells across Romania frequently indicate compliance with drinking water limit (Mureșan *et al*., 2011; Roşu *et al*., 2014; Tociu *et al*., 2016), but extreme nitrite values, over 1.1 mg NO_2^-/L were found in Matca, Galaţi County (Pele *et al*., 2010), 1.4 mg NO_2^-/L in Gătaia, Timiş County (Török-Oance et al., 2013), 1.5 mg NO_2^-/L in Sadoveni, Botoşani County (Vasilache *et al*., 2012) and over 1.6 mg NO_2^-/L in Feleacu, Cluj County (Burcă *et al*., 2015).

Activity of denitrifying bacteria

In all fresh water samples, bacteria able of denitrification were present excepting one well (W7), where a maximum of 3.1 cells/mL (W1) have been reached. A general increase in the number of denitrifying bacteria was achieved during water storage, but not for W1. At day 30, the highest values were obtained for wells W5 (92 cells/mL), W8 (54 cells/mL) and for the public reservoir (35 cells/mL) (Fig. 5).

Previous studies proved that in drinking water systems denitrifying bacteria are generally present in low number. Denitrification occurs mostly in biofilms, the ecophysiological group of denitrifying organisms being usually overwhelmed by the ammonifyiers (Farkas *et al.*, 2013). Biological nitrate reduction occurs in the presence of organic material, under anaerobic conditions, leading to the production of nitrite which is then broken down further to harmless elemental nitrogen (WHO, 2011).

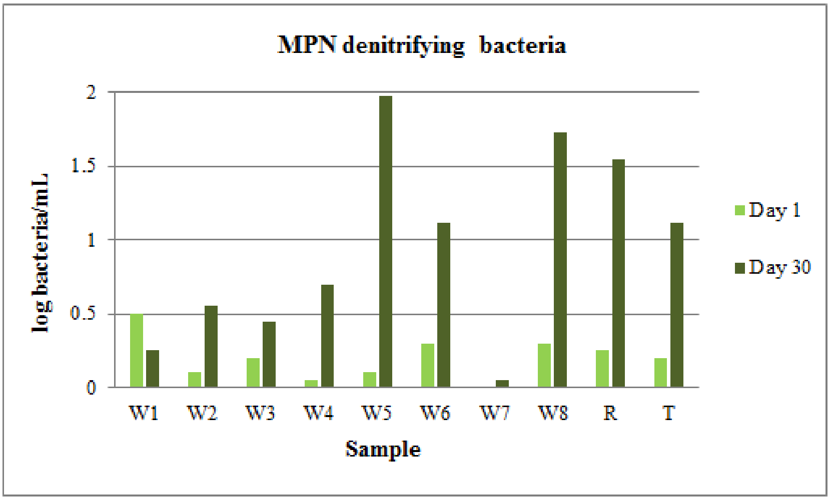

Figure 5. Changes in concentration of denitrifying bacteria in groundwater and tap water. MPN=most probable number, W1-W8=wells, R=reservoir source for public network, T=tap

Nitrate/nitrite risk for human health and water quality changes during storage

The sum of ratios for nitrate and nitrite, signifying the cumulative hazard due to their simultaneous occurrence in drinking water, was greater than 1 in six out of eight wells, reaching the maximum value of 3.01 in W2. After one month of storage, a slight decrease in the sum of ratios for nitrite and nitrate was observed in most of the samples, except for W1 and W2. At day 30, in five groundwater samples the ratio was greater than 1, with a maximum value of 3.05 in W2 (Table 1). Both samples collected from the public system were compliant with drinking water guidelines that recommend a ratio below 1 (WHO, 2011). Comparing to domestic wells, a lower ratio was obtained for the groundwater sampled from the deep reservoir supplying the public network. Well depth, type, age and condition are important variables influencing the water quality. Nitrate contamination is often associated with shallow wells (Fewtrell, 2004; Tociu *et al.*, 2016), but drilling at higher depths does not guarantee the higher quality water in terms of nitrate concentration (Vulpaşu and Racoviţeanu, 2016). In such cases, groundwater nitrate may rather occur from geological sources, than as a consequence of anthropic activities. Nitrogen release form bedrock has a potentially significant impact on localized nitrogen cycles (Holloway and Dahlagren, 2002).

Therefore, water from dug wells should be used with caution for human consumption, especially by vulnerable categories such as infants, toddlers and pregnant women. It is well known that nitrate itself and in small quantities is relatively harmless, but its reaction products, e.g. nitrite or nitrosamines, can affect human health (Kross, 2002). Ingestion of water contaminated with nitrate and nitrite leads to the disease called methaemoglobinaemia (Fewtrell, 2004). This intoxication is a serious hazard for Romanian infants, especially in areas where drinking water is used from polluted wells (Zeman *et al.*, 2002; Iacob *et al.*, 2012). The association between high groundwater nitrate levels and elevated methaemoglobin levels in infants fed with drinking water–diluted formulas has been demonstrated by epidemiological studies. However, more recent investigations suggest other sources of nitrogenous substance exposures in infants, including protein-based formulas and foods, or the production of nitrate precursors by bacterial action in the infant gut in response to inflammation and infection (Richard *et al.*, 2014). Other health effects associated with drinking water nitrate and nitrite are cancer, inflammatory bowel disease, adverse reproductive outcomes, thyroid hypertrophy, diabetes, increased blood pressure and acute respiratory tract infections (Ward *et al.*, 2005).

Table 1.

Nitrite, nitrite, denitrifying bacteria and R in fresh water and stored water.
R = sum of ratios for nitrate and nitrite.

Sample	Nitrate (mg/L)		Nitrite (mg/L)		R		Denitrifying bacteria (cells/mL)	
	Day 1	Day 30	Day 1	Day 30	Day 1	Day 30	Day 1	Day 30
W1	68.23	71.18	0.000	0.004	3.1	1.4	1.36	1.42
W2	150.16	151.81	0.030	0.047	0.4	3.4	3.01	3.05
W3	81.32	67.56	0.014	0.000	0.7	2.2	1.63	1.35
W4	78.72	51.65	0.309	0.000	0.2	4.9	1.68	1.03
W5	89.06	59.43	0.258	0.000	0.4	92.0	1.87	1.19
W6	9.94	2.40	0.168	0.210	1.7	13.0	0.25	0.12
W7	30.77	5.68	0.000	0.000	0.0	0.2	0.62	0.11
W8	72.50	38.22	0.000	0.000	1.3	54.0	1.45	0.76
R	19.92	4.80	0.024	0.000	1.0	35.0	0.41	0.09
T	14.79	8.81	0.007	0.000	0.8	13.0	0.30	0.18
Kruskal-Wallis test	$p = 0.15$		$p = 0.08$		$p = 0.15$		$p = 0.003$	

The multiplication of denitrifying bacteria was observed during water storage, but nitrate and nitrite reduction did not occurred as a general rule. In some samples, an increase of nitrate and/or nitrite content occurred at day 30, comparing to day 1. The difference between the number of denitrifying bacteria at day 1 and at day 30 was statistically significant (p-value = 0.003), while no significant differences occurred in nitrate, nitrite concentrations and their sum of ratios within one month of storage (Table 1). No significant associations were observed in the percentage changes of denitrifying bacteria and nitrate (r = 0.01; p-value = 0.97) or nitrite (r = 0.39; p-value = 27), neither between percentage changes of nitrate and nitrite (r = 0.28; p-value = 0.44). Therefore, the complex microbial activity of heterogenic consortia present individually in each well hinders generic predictions on drinking water quality during storage. Despite the enhancing denitrification due to microbial regrowth, groundwater storage at household level is not efficient for nitrate/nitrite removal. Optimized bioremediation strategies are needed in order to ensure drinking water safety.

Conclusions

According to analyses carried out in this study, water from the public network of Ozun village complies with drinking water regulations, but domestic wells are not safe. Nitrate concentrations exceeded the maximum limit allowed for drinking water in most of the samples collected from dug wells, while nitrite ions occurred within the mandatory limit. Denitrifying bacteria were detected in all groundwater samples, with the exception of one well.

The multiplication of denitrifying bacteria was observed during water storage, but a significant reduction of nitrate, nitrite or their sum of ratio did not occurred as a general rule. In conclusion, enhancing the bioremediation potential of local microbiota by groundwater storage at household level is not an efficient strategy for nitrate/nitrite removal.

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