DENITRIFICATION DITCHES AS A BIOREMEDIATION TOOL FOR THE REMOVAL OF THE NITROGEN POLLUTION AND PROTECTION OF GROUND WATER IN RURAL AREA

BARIERY DENITRYFIKACYJNE JAKO NARZĘDZIE W BIOREMEDIACJI DLA USUWANIA ZANIECZYSZCZEŃ AZOTOWYCH I OCHRONY WÓD GRUNTOWYCH W OBSZARACH ROLNICZYCH

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1. ABSTRACT

The production of food and energy for growing human population the early twenty-first century had contributed more than 10 times the anthropogenic emissions of nitrogen compounds in comparison with the end of the nineteenth century. The application of methods based on denitrification process, which advances in the last decade has created an opportunity to increase the efficiency of ground water protection in the ecosystem scale. Depending on the specificity of a nitrogen pollution source, different biotechnology can be applied in the field. In a catchment of intensive farming or pasture, around the point source, e.g. storage manure, or near the coastline, denitrification ditches will be the most appropriate solution. Denitrification in the catchment can be aided by increasing the organic carbon content in the soil. The aim of the study was to build an underground denitrification ditches around the manure storing place and monitor its effectiveness and also the selection of genetic markers for tracking the complete process of denitrification and the optimization of genetic analyses involving PCR. It was constructed by mixes brown coal and calcium coal with soil perpendicular to groundwater flow. Preliminary results showed average 65 % reduction of nitrate nitrogen. Considering the low construction costs, high efficiency and lack of landscape intrusion, denitrification ditches seem to be a good solution to reduce nitrate contamination, especially for small farms.

Key words: point-source nitrogen pollutants, bioremediation, denitrification process, denitrifying bacteria.

1. ABSTRAKT

Produkcja żywności i energii dla wzrastającej liczby ludności na początku XXI wieku przyczyniła się ponad 10-krotnego wzrostu emisji związków azotu w porównaniu do IXX wieku. Zastosowanie metod wykorzystujących proces denitryfikacji znacząco rozwinęło się w ostatnim dziesięcioleciu, co daję ogromne możliwości efektywnej ochrony wód gruntowych w skali ekosystemu. W zależności od specyfiki źródła zanieczyszczeń różne biotechnologie mogą być stosowane w terenie. W zlewniach intensywnie użytkowanych rolniczo, pastwiskowo, wokół punktowych źródeł zanieczyszczeń azotanami, jak składowiska nawozów organicznych bezpośrednio na gruncie oraz w pobliżu linii brzegowych wód powierzchniowych bariery denitryfikacyjne wydają się być najbardziej korzystnym rozwiązaniem problemu zanieczyszczeń azotanami. Denitryfikacja w zlewni jest warunkowana dostępnością węgla organicznego w glebie. Celem badań była budowa oraz monitoring efektywności pracy podziemnej bariery denitryfikacyjnej wokół miejsca nieprawidłowego składowania nawozów organicznych. Ponadto, podjęto się selekcji właściwych markerów genetyczne i optymalizacji metody PCR w celu śledzenia obecności bakterii denitryfikacyjnych odpowiedzialnych za proces redukcji azotanów. Do konstrukcji bariery wykorzystano węgiel brunatny i węgiel wapienny wymieszany z ziemią z rowu, przez

który prostopadle przepływają zanieczyszczone azotanami wody ze składowiska obornika. Wstępne wyniki wskazują na średnią 65 % redukcję azotanów w wodach przepływających przez barierę. Biorąc pod uwagę niskie koszty konstrukcji, wysoką efektywność oczyszczania, brak ingerencji w krajobraz bariery denitryfikacyjne wydają się być dobrym rozwiązaniem dla redukcji zanieczyszczeń azotanowych, zwłaszcza w małych gospodarstwach.

2. INTRODUCTION

The production of food and energy for the growing human population of the early twenty-first century has contributed to more than 10 times the anthropogenic emissions of nitrogen compounds than those being produced at the end of the nineteenth century. Agriculture has changed the natural flow of nitrogen (N) and led to a number of changes, such as an increase in the amount of ozone in the troposphere, a more acute greenhouse effect due to N_2O emission, greater acidification of soils and surface waters, reduction of biodiversity, increased eutrophication of aquatic ecosystems and more widespread adverse health effects resulting from the accumulation of nitrates in groundwater used as drinking water. As nitrate (NO_3^-) is the most mobile form of nitrogen in soil, it is the most dangerous pollutant of water, causing many diseases, such as methemoglobinemia, as well as carcinogenic and mutagenic changes. Nitrogen, like phosphorus, contributes to the eutrophication of freshwater ecosystems, which in turn results in the intensive development of toxic algal blooms and the deterioration of drinking water sources.

The elaboration and application of methods based on the denitrification process over the last decade have created an opportunity to increase the efficiency of ground water protection on the ecosystem scale. Depending on the specificity of the source of nitrogen pollution, different solutions can be applied in the field. In a catchment of intensive farming or pasture, around point sources such as storage manure, or near the coastline, denitrification ditches will be the most appropriate solution.

Denitrification takes place in soil and in the subsurface groundwater [1,2]. In soils, particularly in rural areas, the process of denitrification, on the one hand, leads to loss of nitrate as a nutrient for plants, but on the other hand, it prevents the migration of unused nitrates into groundwater, reducing their pollution. In general, the absence of oxygen and the presence of organic carbon, reduced sulfur or iron facilitate the occurrence of denitrification. In soils, the denitrification process can be carbon limited, especially at greater depths, significantly reducing the likelihood of the soil solution being fully denitrified before it becomes drainage water [3]. The availability of organic carbon seems to be one of the most important factors that affects denitrifying processes in the soil which can be regulated by man. Increasing the amount of the external carbon source to reduce high levels of ambient nitrate is considered an efficient and low-cost tool to prevent eutrophication, especially in areas where human activity places greater nitrogen loads on the catchment. These external carbon sources, which supply greater quantities of carbon than those found naturally, can be straw, mulch, sawdust or woodchips [4, 5, 6] or even forms that are not usually found in the natural environment, like methanol, ethanol and acetate. Preliminary results of our field study suggest that NO_3^- removal is most effective when denitrification ditches are installed around and under a point source if pollution perpendicularly intercepts the flow path of NO_3^- .The schematic for the use of denitrification ditches for NO_3^- removal from a point nitrogen pollution source (e.g. storage manure) are shown in Fig. 1A and 1B.

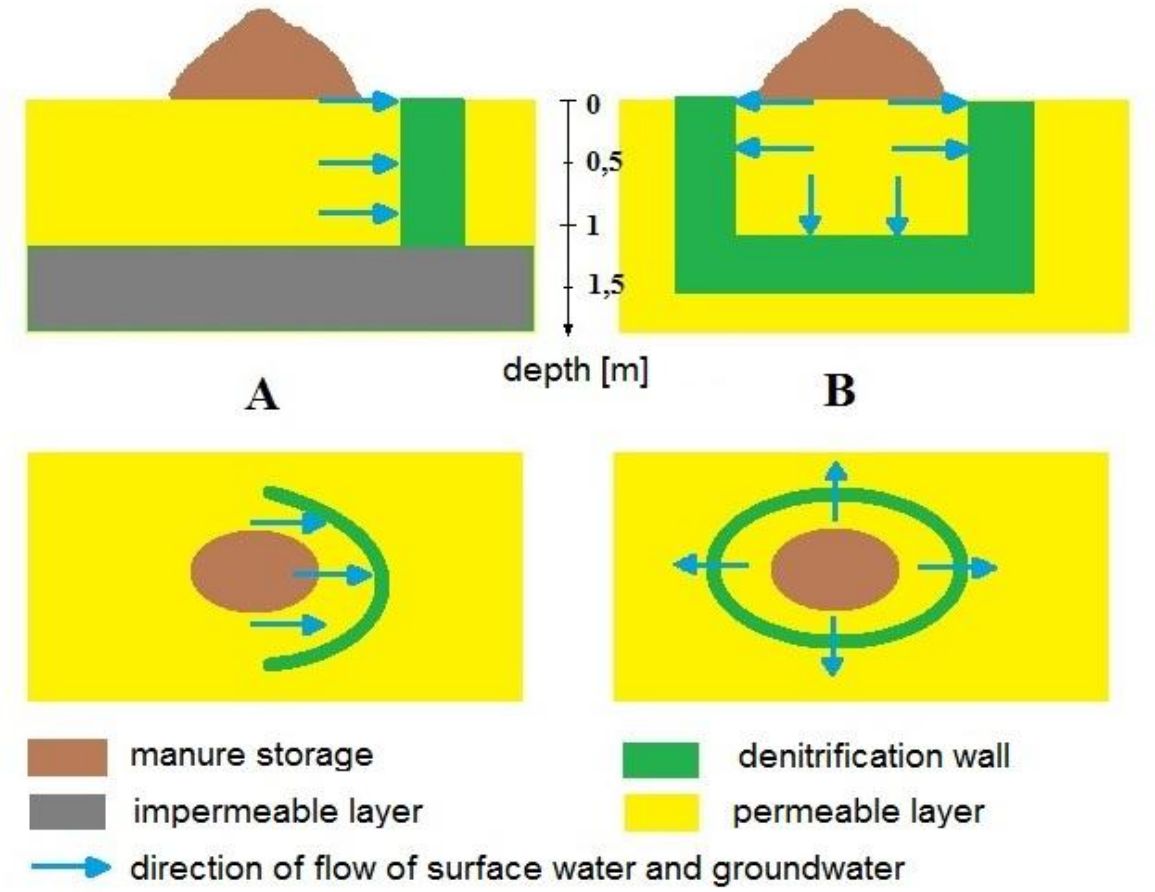

Fig. 1.Different schematics of the use of denitrification wall/ditches for NO_3 removal from a point nitrogen pollution source [5]

A. in the case of a shallow impervious layer
B: in the case without an impervious layer

3. MATERIALS AND METHODS

The source of pollution in this study was an manure heap from a fattening farm which had lain unprotected for 12 years (Phot.1a). The manure was produced by ca. 20 cows. Manure is removed from the heap twice a year. Since it is unprotected, nitrogen compounds are leached by water run-off. Prior to deciding on the placement of denitrification ditches, groundwater levels and contamination levels were monitored for three months. The average concentration of NO_3^- was estimated to be above 300 mgl^{-1}, with the highest values being above 2000 mgl^{-1}

The dump was constructed using a CAT excavator: the ditch was 1-1.5 m deep and 1 m wide, dug perpendicular to the slope/direction of groundwater flow. A mixture of brown coal and calcium coal was used as the source of organic carbon, constituting about 30% of the wall volume (Phot.1b, c). Brown coal and calcium coal were carefully mixed by the excavator with soil dug out from the trench during construction. On decomposition, sawdust added to the coal mixture provides a source of organic carbon for the denitrifying bacteria, thus converting the nitrates present in the drainage flowing through the ditches to their gaseous form. Ground waters were at the level 1 – 1.3 m deep.

Piezometers were used to take samples of groundwater were taken once a month to analyze nitrate nitrogen (NO_3^-) and ammonium ion (NH_4^+) content using ionic chromatography with DIONEX (mg l^{-1}).

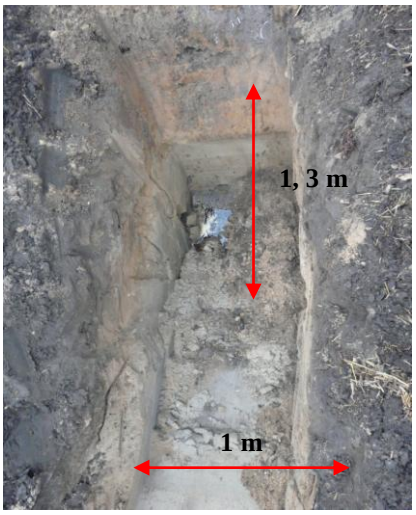

Photo 2. Example from the demonstration site, restoration of a point source of nitrogen – manure storage site in the village of Jervonice, central Poland [5]

A – Manure storing site before constructing the ditches
B- mixed material, brown and calcium coal
C – underground constructed ditches

The aim of the first phase of the study was the selection of genetic markers for tracking the complete process of denitrification, and the optimization of genetic analyses involving PCR. The three soil samples from the inside of the denitrification ditch were collected in March 2012, 2013, and December 2013. The DNA was isolated from soil using a FastDNA® SPIN Kit for Soil (MP Biomedicals, Ohio, USA) or a Gene MATRIX Soil DNA Purification Kit (EurX Ltd., Gdańsk, Poland). The amplification of selected functional reductase genes was prepared according to Braker et al. [7] for *nirK*, Braker et al. [8] for *qnorB* and Rich et al. [9] for *nosZ* (Tab. 1). However the exact quantities of DNA and PCR annealing temperatures were modified.

4. RESULTS

The ditches were constructed to achieve nitrate reduction. Nitrates are the most mobile forms of nitrogen in soil and hence, they are most dangerous for the pollution of waters. After the first two years of the operation of the walls, it was observe 65% average reduction of nitrate ions in the groundwater around the ditches (Fig. 2).

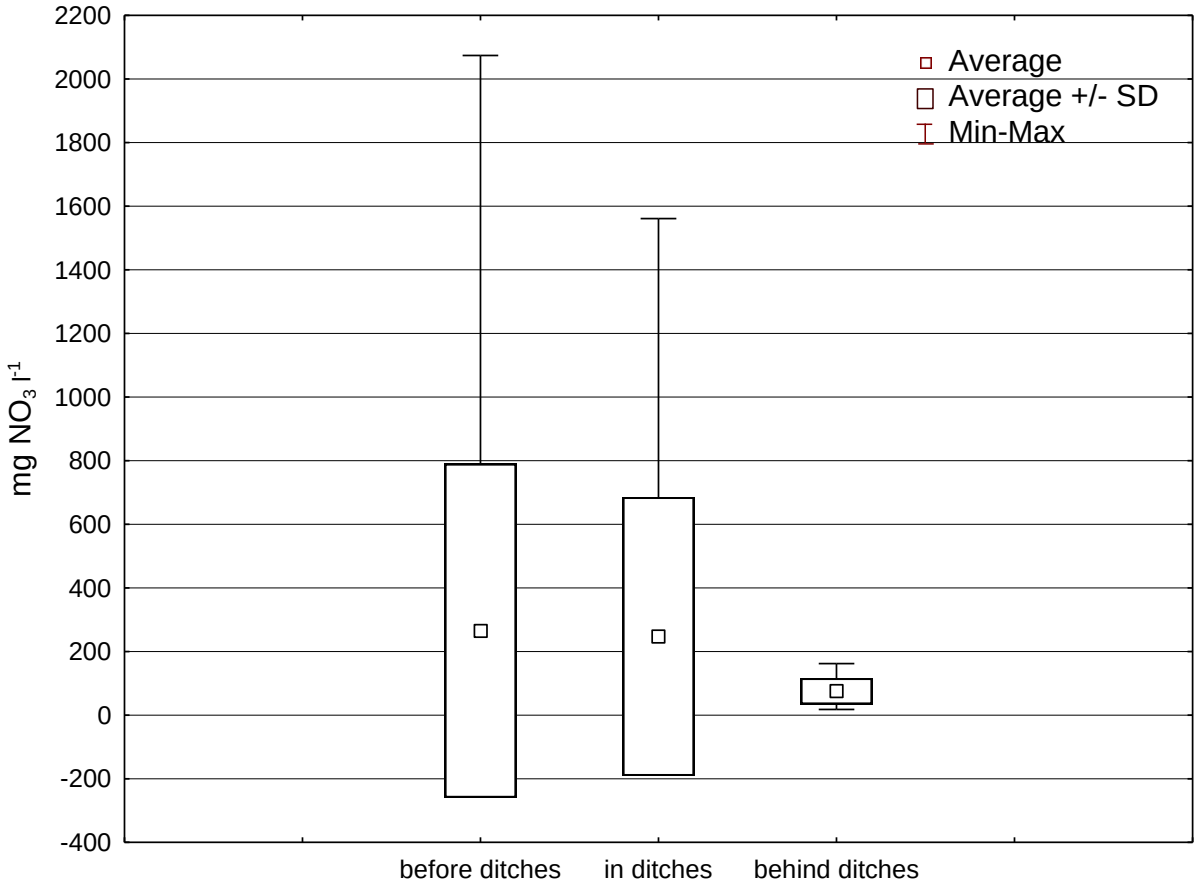

Fig. 2. Average (and +/- DS and Min/Max value) nitrate concentration in ground water befor, in and behind ditches in Jervonice demonstration site.

In our pilot study, bacteria with denitrifying potential were identified directly in the studied bioreactor, a denitrification ditch filled with brown coal, based on amplification of fragments of selected genes for NO_2^- reductase (*nirK*), NO reductase (*qnorB*) and N_2O reductase (*nosZ*) (Tab. 1).

Table1. Genes and their primers used for detection of denitrifying bacteria

Gene function	Gene name	Amplified size	Primers	Primer sequences (5' - 3')	References
NO_2^- reductase	*nirK*	514 bp	nirK1F	GGMATGGTKCCSTGGCA	[7]
			nirK5R	GCCTCGATCAGRTTRTGG	
NO reductase	*qnorB*	262 bp	qnorB2F	GGNCAYCARGGNTAYGA	[8]
			qnorB5R	ACCCANAGRTGNACNACCCACCA	
N_2O reductase	*nosZ*	700 bp	nosZ-F-1181	CGCTGTTCITCGACAGYCAG	[9]
			nosZ-R-1880	ATGTGCAKIGCRTGGCAGAA	

I – Inosine; M→A+C; K→G+T; S→C+G; R→A+G; Y→C+T; N→A+C+G+T.

The genes *nirK*, *qnorB* and *nosZ* were amplified in all three samples (Tab 2). The bacteria capable of complete denitrification were present in two samples taken in March and one sample taken in December

Table 2 .The results of amplification of genes participating in the denitrification pathway isolated from bacteria in soil from the denitrification ditch filled with brown coal and calcium coal

Sampling data	Functional genes of reductases		
	nirK $NO_2 \rightarrow NO$	***qnorB*** $NO \rightarrow N_20$	***nosZ*** $N_2O \rightarrow N_2$
March 2012	+	+	+
December 2012	+	+	+
March 2013	+	+	+

Additionally, the fragment of the *nosZ* gene from the sample collected on December was sequenced. The homology searches were performed using the National Center for Biotechnology Information microbial and nucleotide Basic Local Alignment Search Tool (BLAST) network service (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence of *nosZ* was 82% homologous with that of the genus *Pseudomonas*, particularly strain *P. stutzeri* TR2 (GeneBank: AB764137.1).

5. DISCUSSION

Denitrification ditches are as yet a new solution, and more studies are needed to fully realize their potential, but the results of previous studies have focussed attention on them. These solutions are effective in removing nitrate pollution from water bodies [10, 11, 12, 6], and above all, they are economically competitive compared to other solutions. These studies indicate the need for more research to obtain more detailed information on the operation of denitrification ditches and new possibilities for their applications and activation.

Previously, our studies have shown that denitrifying bioreactors installed in the field need time to achieve optimum reduction of nitrates. Moreover, the denitrifying ditches described in the previous section, were designed, and operated, without a precise knowledge of the microorganisms that inhabit them. Therefore, one aim of our present study on the optimization of denitrifying ditches is to identify the microbial community within the ditch and their mode of activity. Bacterial denitrification plays an important role in global nitrogen cycles. Oxidized nitrogen compounds including nitrate (NO_3^-), nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O) are used by denitrifying bacteria as an alternative electron acceptors for energy production under anaerobic or oxygen-limited conditions [13]. During complete denitrification, NO_3^- and NO_2^- are reduced to gaseous compounds, NO and N_2O, and

eventually to nitrogen gas (N_2). Bacterial denitrification is dependent on the specific activity of reductases synthesized by a number of genes: *napA* and *narG* (NO_3^- reductase) [14], *nirK* and *nirS* (NO_2^- reductase) [7], *cnorB* and *qnorB* (NO reductase) [8] and the *nosZ* gene (N_2O reductase) [9]. However, not all denitrifying bacteria synthesize a complete set of enzymes needed for the full denitrification process [15]. In this case, incomplete denitrification can lead to the emission of nitrous oxide, a potent greenhouse gas implicated in destruction of the ozone layer. As complete denitrification depends not only of the soil denitrifier community, but also on the environmental conditions which influence the condition of the bacteria, both of these aspects need to be monitored simultaneously [14, 16]. The construction of the bioreactor, and the level of the organic substrate within it, are important factors in the accumulation of intermediate denitrification products. The factors limiting denitrification are carbon and nitrate availability, dissolved oxygen, temperature, pH, and the concentrations of nitrate, nitrite and ammonia.
Since denitrification is not specific to any one phylogenetic group of bacteria in the environment [15, 17, 18], to identify the diversity of bacteria in the denitrification pathway, it is necessary to analyze the functional reductase genes given above. In addition, this kind of analysis is more appropriate for identifying the denitrifying population of bacteria than taking a phylogenetic approach, as the functional genes are more specific to the process.

6. CONCLUSIONS

The protection and restoration of an ecosystem should employ methods that use ecosystem properties, which can contribute to its resilience and are able to react flexibly to anthropopressure [19, 20]. These treatments aim to restore the biogeochemical cycles of evolutionarily-shaped properties and increase the resilience of the environment to anthropopressure [19]. The application of denitrification as ecosystem biotechnology, is one of the basin-level activities which are required by the Water Framework Directive to achieve a *good ecological status* for water by 2015 [19, 22, 20]. In the case of Poland, national legislation does not cover all sources of pollution by nitrogen compounds. The obligation to have manure slabs applies only to large farms and pig farms. The farms must have tanks for liquid livestock manure: both manure and slurry. Smaller farms with a small number of poultry or pigs and other species are ignored. When these installations are not present, nitrogen leach through the soil and penetrate the water resources [23].
Despite adopting the regulations concerning the disposal of nitrates given in the Nitrates Directive [24], Poland has still not defined a sufficient number of zones as being threatened by pollution, and much less, taken action to reduce the amount of nitrate pollution in these areas. In the light of this, and the fact that Poland was taken to court by the European commission on 24 January 2013 in connection with nitrate pollution of water supplies (Reference: IP/13/48), this proposal to employ the natural capacities of the environment, by using the activity of denitrifying bacteria together with appropriate carbon sources, appears to be both an appropriate and a most necessary solution. The obtained results suggest that the construction of denitrification ditches and addition of locally available, organic carbon is an efficient (average 65 % reduction) tool for controlling point sources of nitrate pollution of both surface and groundwater in our climate zone. Considering the low construction costs, low labor inputs, high efficiency and lack of landscape intrusion, denitrification ditches seem to be a good solution to reduce nitrate contamination, especially for small farms.

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