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Nitrates removal from wastewater using bacteria *Thiobacillus denitrificans*

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Abstract

The work is focused on the removal of nitrates from model wastewaters using autotrophic denitrification by *Thiobacillus denitrificans*. Elemental sulphur was used as the electron donor. The main goal of theoretical part is focused on the bacteria *Thiobacillus denitrificans* and the mechanisms of its action in the autotrophic treatment of nitrate-containing waters. The experiments were performed dealing with the systematic study of nitrate removal by autotrophic denitrification from laboratory prepared wastewater samples, which were poor in the content of organic substances and at the same time in which the concentration of nitrate ions does not exceed 100 $mg.L^{-1}$. The experiments were carried out in a batch arrangement, where limestone was added to the reaction mixtures to control the pH of denitrification processes. The influence of a number of factors, such as temperature, the size of the feed fraction used, mixing and the addition of phosphorus and ferric iron to the reaction mixture was studied. The second part of the experiments was focused on a long-term study of autotrophic denitrification in a continuous arrangement, i.e. flow column, where only elemental sulphur was used as a fixed bed. The results of the experiments show that the reduction process can be characterized with sufficient accuracy as a pseudo-zero order reaction.

Abstrakt

Disertační práce je zaměřena na odstraňování dusičnanů z odpadních vod metodou autotrofní denitrifikace bakterií *Thiobacillus denitrificans*, kde je jako elektronový donor použita elementární síra. Teoretická část shrnuje a popisuje metody odstraňování dusičnanů z vod, přičemž stěžejní část je zaměřena na bakteriální kmen *Thiobacillus denitrificans* a mechanismy jeho působení při autotrofním čištění vod obsahujících dusičnany. Experimentální část je zaměřena na systematické studium odstraňování dusičnanů autotrofní denitrifikací z uměle připravovaných odpadních vod, které jsou chudé na obsah organických látek a zároveň v nich koncentrace dusičnanových iontů nepřesahuje hodnoty 100 $mg.l^{-1}$. První část experimentů se zabývala vsádkovým uspořádáním, kde byl k regulaci pH denitrifikačních procesů přidáván do reakčních směsí vápenec a kde byla pozornost věnována studiu vlivu řady faktorů, jako je teplota, velikost použité frakce náplně, míchání a přídavek fosforu a trojmocného železa do reakční směsi. Druhá část experimentů byla zaměřena na dlouhodobé studium autotrofní denitrifikace v průtočné koloně, kde byla jako nehybná náplň použita pouze elementární síra. Výsledky experimentů ukazují, že proces redukce lze s dostatečnou přesností charakterizovat jako reakci pseudo nultého řádu.

Keywords:

Autotrophic Denitrification, Nitrates Removal, *Thiobacillus denitrificans*, Sulphur.

Klíčová slova:

Autotrofní denitrifikace, odstraňování dusičnanů, *Thiobacillus denitrificans*, síra.

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1 Introduction

Nitrate concentration in wastewater, groundwater and source of drinking water has been increasing in recent years. It is a result of the intensive application of nitrogen fertilizers and increasing population growth and its standard of living. Nitrates ions are soluble in water and are not retained in the soil. It can lead to their easy migration and thus to pollution of all parts of the aquatic ecosystem. Nitrate is commonly regarded as a contaminant due to its environmental and public health impacts [1].

1.1 Methods of nitrate removal from water

There are several effective methods of nitrate (or nitrite) ions removal from contaminated water. These methods can be divided into groups according to the mechanism of removal: physicochemical, chemical and biological methods.

Physicochemical methods of nitrate removal include for example ion exchange, distillation and membrane separation processes including electrodialysis. These methods can remove nitrates from water with relatively high efficiency. Unfortunately, disadvantage of these methods is usually in the form of highly concentrated salt solution. It usually requires their additional processing [2].

Chemical methods of removing nitrates from water include reduction. There are a variety of nitrate reducing agents such as aluminium, iron, ammonia and some organic substances. The product of reduction reactions is a diverse mixture of nitrogen, nitrite, ammonia and other substances, many of which have a negative impact on the environment [2].

In general, biological methods for removing nitrate ions from water are based on the metabolic enzymatically catalysed conversion of nitrates to nitrites. Nitrites are further reduced to nitrogen oxides and the final product of denitrification is nitrogen gas molecules - equation (1) [3]. Each of the reactions is enzymatically catalysed by a specific enzyme.

$$NO_3^- \xrightarrow{Nar} NO_2^- \xrightarrow{Nir} NO_{(g)} \xrightarrow{Nor} N_2O_{(g)} \xrightarrow{Nos} N_{2\,(g)} \quad (1)$$

A key factor in using these methods is the reduction of nitrate ions to nitrite ions, which can accumulate in the system and are more toxic than nitrates.

Many organisms, including fungi and protozoa, have denitrification capabilities, but most of this is a variety of bacteria. Not all denitrifying bacteria form all three intermediates during denitrification, some may form only one, two or even none of the intermediates. Therefore, denitrification is considered as complex community process where a number of organisms work together to complete the process [3].

Bacteria genera that can use nitrates as an electron acceptor and produce nitrogen gas under anoxic conditions include, for example, *Pseudomonas, Bacillus, Escherichia, Alcaligenes* and *Thiobacillus* [2,3].

In an aerobic environment, oxygen molecules become terminal electron acceptors in the metabolic processes of denitrifying organisms. Under anoxic conditions, these microorganisms can use chemically bound oxygen, as is the case with the nitrate ion,

which then play a role as an electron acceptor instead of oxygen molecules. This is called denitrification. Thus, denitrification processes can only take place in an environment without free molecular oxygen (dissolved oxygen concentration up to 0.5 $mg.L^{-1}$) [4].

The enzymatic apparatus using free oxygen is activated at dissolved oxygen concentrations above 1.0 $mg.L^{-1}$ - free molecular oxygen is as an inhibitor of denitrification processes [1]. At the same concentration of dissolved oxygen, denitrifying enzymes are also deactivated.

Both inorganic (autotrophic denitrification) and organic substances (heterotrophic denitrification) are used as electron donors in metabolic processes.

1.2 Autotrophic denitrification

The autotrophic denitrification is based on the removal of nitrate ions by bacteria using inorganic substances for their metabolic activity. These processes take place spontaneously in soil and groundwater. On the other hand, they are practically absent in surface waters, where we can find much higher concentrations of organic substances. Nitrates are removed here mainly by heterotrophic denitrification.

In contrast to heterotrophic denitrification, autotrophic denitrification does not require the supply of a more expensive organic substrate [3]. Furthermore, the growth properties of the cells of autotrophic denitrifying bacteria are much lower than those of heterotrophs, leading to the production of less bacterial sludge. Thus, autotrophic denitrification may be a suitable solution mainly for the removal of nitrates from wastewater with low organic load [2].

As a substrate, resp. electron donors, autotrophically denitrifying bacteria use inorganic substances - especially hydrogen, elemental sulphur and some of its compounds (sulphane, pyrite, thiosulphate, etc.) [2].

The oxidation of elemental sulphur in autotrophic denitrification is shown in equation (3) [5]:

$$5S + 2H_2O + 6NO_3^- \rightarrow 5SO_4^{2-} + 4H^+ + 3N_2 \quad (3)$$

During the process, a considerable amount of H^+ ions is formed, while the environment is acidified. The optimal pH for autotrophic denitrification is in the range of 6.5 - 7.5. If the pH is outside the range of 5.5 to 9, denitrification is completely stopped, although bacterial cells can survive [6].

Sodium bicarbonate is one of the best known and most commonly used substances for this purpose. An alternative to sodium bicarbonate can be cheaper limestone (calcium carbonate). The following equations (4) - (6) show the corresponding chemical reactions [7]:

$$CaCO_3(s) + H^+ \rightarrow HCO_3^- + Ca^{2+} \quad (4)$$

$$HCO_3^- + H^+ \rightarrow H_2CO_3 \quad (5)$$

$$H_2CO_3 \rightarrow H_2O + CO_2 \quad (6)$$

The calcium ions then react with the formed sulphate ions to form insoluble calcium sulphate. The reactions further release carbon dioxide, which can also serve as

a carbon source for chemolithoautotrophic bacteria. The disadvantage of using limestone as a pH regulator in autotrophic denitrification processes is the increase of water hardness and the total content of dispersed solids. Another disadvantage is the insufficient dissolution rate of limestone in combination with the high concentration of nitrates in the purified water. In addition, the sulphates formed can passivate the surface of the limestone. In published works, the ratio of sulphur to limestone is often reported as 3: 1. However, from the point of view of minimizing the reactor volume, the authors give an optimal volume ratio of sulphur and limestone fed to the autotrophic denitrification reactor of approximately 1:1 [7].

The optimum temperature for autotrophic denitrification processes is in the range from 33 °C to 35°C. At temperatures above 40°C, these processes are completely stopped [6].

It is also necessary to monitor the concentration of nitrite ions. Inhibition of autotrophic denitrification by nitrite ions occurs in the presence of concentrations above 50 $mg.L^{-1}$ $N\text{-}NO_2^-$ (i.e. approximately 164 $mg.L^{-1}$ NO_2^-) [6]. One possible explanation for the accumulation of nitrite ions in the reaction mixture may be too high concentration of dissolved oxygen. Of the four denitrifying enzymes, the enzyme nitrate reductase is the least susceptible to the presence of oxygen. Thus, there may be a situation where the oxygen concentration in the system still allows the action of nitrate reductase and the production of nitrites, but the presence of oxygen prevents further reductions mediated by the other three enzymes [8].

Autotrophic denitrification processes can also be adversely affected by high concentrations of sulphate formation, at sulphate concentrations higher than 2 $g.L^{-1}$ [6].

The best known bacterial species that have denitrifying properties include *Thiobacillus denitrificans* and *Thiomicrospira denitrificans* [7].

1.3 Bacteria *Thiobacillus denitrificans*

T. denitrificans is widely found mainly in soil, mud, freshwater and marine sediments, domestic and industrial wastewater treatment plants and septic tanks. It is not pathogenic or toxic organism. *T. denitrificans* can move with the help of a polar flagellum [9].

T. denitrificans is strictly chemolithoautotrophic (uses only inorganic substances), facultative anaerobic and able to denitrify. *T. denitrificans* is able to oxidize (use as an energy source) sulphite (SO_3^{2-}), thiosulphate ($S_2O_3^{2-}$), elemental sulphur (S^0), sulphides (S^{2-}, especially in the form of FeS_2) or tetrathionate ($S_4O_6^{2-}$) and thiocyanate (SCN^-). These substrates can utilize bacterial cells under both aerobic and anaerobic conditions, oxidizing them to sulphate ions (SO_4^{2-}). During oxidation reactions, electrons are released which react either with free oxygen molecules (aerobic environment) or with oxygen bound in the form of nitrates, nitrites and nitrogen oxides (anoxic environment) [9].

The energy generated by the processes in the oxidation of sulphur and selected *thio* compounds can then be used to assimilate carbon dioxide or sodium bicarbonate. These substances can also be used as a carbon source.

T. denitrificans bacterial cells also contain bacterial enzymes that reduce nitrate and nitrite ions, the concentration and activity of which are related to the concentration of dissolved oxygen in the bacterial environment. Due to their enzymatic equipment, *T. denitrificans* bacteria are able to live in both aerobic and anaerobic environments and at the same time always adapt to the change of environment from aerobic to anaerobic and vice versa without permanent loss of denitrifying properties [10].

The optimum temperature for the growth and prosperity of these bacteria is in the range of 28 to 32 °C, while the optimum pH values of the environment are in the range of 6.8 to 7.4. Values of pH decrease significantly during cultivation [9].

Under aerobic conditions, the reduced growth factor is reduced inorganic sulphur compounds, while under anaerobic conditions, nitrates and nitrites are [10].

1.4 Nitrates removal by bacteria *Thiobacillus denitrificans*

Either the *T. denitrificans* strain itself or a consortium of autotrophic organisms was used to test the technology of nitrate removal from water using autotrophic denitrification, of which *T. denitrificans* was part of. A variable parameter in the use of this method is also the use of various electron donors. Elemental sulphur has the advantage that its particles can form the filler of the reactor and thus directly become a carrier for bacterial cells. In some cases, limestone is also added to the reactor along with the sulphur to adjust the pH. Most often, sulphur and limestone are fed to the reactors in a ratio of 1:1 [7].

Autotrophic denitrification by *T. denitrificans* was tested in terms of technical arrangement in both batch and continuous flow regimens. The application of autotrophic denitrification technology by *T. denitrificans* was tested with different types of water (underground, surface, industrial, municipal wastewater and drinking water) [7].

2 Aims of the thesis

The aim of the experimental study will be to test and set technological limits for the removal of nitrates from model-prepared wastewater poor in organic matter with nitrate content up to 100 $mg.L^{-1}$, by the process of autotrophic denitrification by *T. denitrificans* bacteria.

The first part of the experiments consists of batch experiments, where elemental sulphur mixed with limestone in a weight ratio of 1:1 will be used as a carrier for bacterial cells and an electron donor. Limestone will be used to regulate the pH. The course of denitrification processes and their influence by factors such as temperature, size of used sulphur and limestone fraction, mixing and addition of phosphorus and ferric iron in various concentrations to the reaction mixture will be described. The acquired knowledge will be further applied in the following flow experiments.

In the second part of the experimental work, attention will be focused on experiments in a flow column, where only elemental sulphur will be used as the packing. The results will be critically evaluated, including the possibility of use for real waters polluted with nitrates.

3 Experimental part

3.1 Cultivation of microorganism

The collection strain *Thiobacillus denitrificans* DSM 12475 (Leibniz Institute DSMZ, Germany) was used for all experiments in this work. The strain was then cultured according to the recommended conditions [11].

3.2 Batch experiments

Clear borosilicate glass bottles were used as reaction vessels. Sulphur was used as a carrier of bacterial cells and as an electron donor for the processes of *T. denitrificans* in reactors. In the first part of the batch experiments, a particle size fraction of 2.5 - 5.0 mm (mean particle size 3.54 mm) was used and the weight was 50, 100 or 200 g of sulphur in the reaction vessel (Table 1). In the second part of the batch experiments in the reaction vessels, crushed sulphur was used to a powder with a fraction size of 140 - 200 μm (mean particle size 167 μm). In these vessels, the weight was determined so that the surface of the sulphur particles corresponded to the surface of the particles in the case of sulphur vessels with a larger fraction size (Table 2).

Table 1 – Content of reaction mixtures with a fraction of used sulphur with a mean particle size of 3.54 mm and limestone with a mean particle size of 3.46 mm

Label	S (g)	$CaCO_3$ (g)	P ($mg.L^{-1}$)	Fe ($mg.L^{-1}$)
K1a	50	50	-	-
K1b	50	50	-	-
K2a	100	100	-	-
K2b	100	100	-	-
K3	200	200	-	-
P1	50	50	0.5	-
P2	50	50	1.0	-
P3	50	50	1.5	-
P4	50	50	2.0	-
P5	50	50	3.0	-
P6	100	100	0.5	-
P7	100	100	1.0	-
P8	100	100	1.5	-
P9	200	200	1.0	-
Fe1	100	100	-	0.05
Fe2	100	100	-	0.1
Fe3	100	100	-	0.5
Fe4	100	100	-	1.0

Limestone was also added to the reaction vessels to adjust the pH. The weight ratio of sulphur and limestone dosed to all experimental vessels was always 1:1. Where

a larger particle size sulphur fraction was used in the reaction vessel, limestone with dimensions of 3.0 - 4.0 mm (mean particle size 3.46 mm) was dosed. In other experiments with a smaller sulphur fraction size, limestone with a particle size of 2 μm was used. Each reaction vessel further contained 1 L of sterile sodium nitrate solution with a concentration of 100 $mg.L^{-1}$ NO_3^- dissolved in either demineralized or drinking tap water and 1 mL of bacterial suspension of *T. denitrificans* in liquid medium S6 with an optical density of 0.17 McFarland scale. Other components added to the reaction vessels were NaH_2PO_4 and $FeCl_3$ (sterile solutions autoclaved for 15 min at 121 °C).

Table 2 – Content of reaction mixtures with a fraction of used sulphur with a mean particle size of 167 μm and limestone with a mean particle size of 2 μm. Here K = drinking water, D = demineralized water.

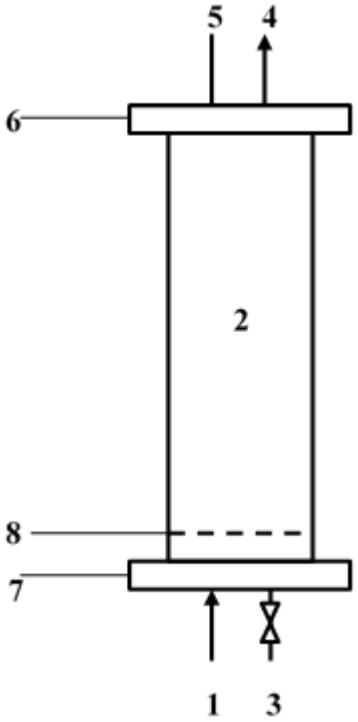
Label	S [g]	$CaCO_3$ [g]	Water	Stirred [150rpm]	T [°C]
K4	100	100	K	yes	22
K5	2.23	2.23	D	yes	22
K6	4.46	4.46	D	yes	22
K7	8.92	8.92	D	yes	22
K8	2.23	2.23	K	-	22
K9	4.46	4.46	K	-	22
K10	8.92	8.92	K	-	22
K11	2.23	2.23	K	yes	22
K12	4.46	4.46	K	yes	22
K13	8.92	8.92	K	yes	22
K14	2.23	2.23	K	-	33
K15	4.46	4.46	K	-	33
K16	8.92	8.92	K	-	33
K17	2.23	2.23	K	yes	33
K18	4.46	4.46	K	yes	33
K19	8.92	8.92	K	yes	33

At the beginning and throughout all experiments (usually every 7 days), samples of the liquid reaction mixture were taken. The content of nitrate, nitrite and in some cases also phosphate and sulphate ions was subsequently determined in the samples taken. Furthermore, pH, ORP and dissolved oxygen concentrations were determined. The experiments were monitored until the concentrations of nitrates and nitrites in the determined samples were reduced to values close to zero.

3.3 Flow column experiments

A column made of Plexiglas was used for flow-through experiments. It was a tube with a diameter of 150/140 mm, provided with flanges, with an active column length of 670 mm and a free volume of 10.3 L. On the lower flange, inside the column, a sieve with 4 mm meshes was placed on short legs (height 20 mm), which served as a

granulate carrier. The dosing into the column was from the bottom, the overflow from the top. The technological scheme of the flow column is shown in Figure 1.



1 - Supply of nutrient solution from the reservoir
2 - Column containing solid bacterial support
3 - Drain
4 - Overflow of the solution passed through the column
5 - Venting
6 - Upper flange
7 - Bottom flange
8 - Sieve

Figure 1 – Technological scheme of the flow column

The column was packed with sulphur (fraction size 5.0 to 8.0 mm) and *T. denitrificans* bacteria up to 582 mm (solids volume was 9 L).

Table 3 – Concentration of the components of the working solution and the rate of its dosing into the flow column

Days	cNO^{3-} [$mg.l^{-1}$]	Flow [$ml.min^{-1}$]	$P\text{-}PO_4^{3-}$ [$mg.l^{-1}$]	$cNaHCO_3$ [$mg.l^{-1}$]	$cKHCO_3$ [$mg.l^{-1}$]
0 - 20	50	23.6	1.0	50	-
21 - 97	25	19.3	1.0	50	-
98 - 150	15	7.0	1.0	50	-
151 - 255	25	8.5	1.0	50	-
256 - 428	30	9.9	1.0	-	50

The column experiments were run for a total of 428 days, with a working solution containing $NaNO_3$, Na_2HPO_4 (concentration 1 $mg.L^{-1}$ $P\text{-}PO_4^{3-}$), $NaHCO_3$ and $KHCO_3$ being metered into the column. The concentration of the working solution components and the rate of its dosing into the column are shown in Table 3. For the first 100 days of the experiment, the working solution was prepared from demineralized water. Subsequently, (until the end of the column operation) the working solution was prepared from drinking water from the water supply system of the University of Pardubice.

The content of nitrates and phosphorus was regularly determined in the input samples of the working solution (twice a week). The content of nitrates, nitrites, sulphates, phosphorus, dissolved oxygen, pH and redox potential (ORP) was determined in the samples at the outlet of the column at the same intervals.

3.4 Analytical methods

When culturing *T. denitrificans* in liquid medium, the turbidity of the suspension was continuously measured in a DEN-1B densitometer (BioSan, Latvia) at a wavelength of 565 nm with the reported results in McFarland turbidity scale units.

Molecular biological methods were used to confirm the presence of *T. denitrificans*. The measurements were performed at Tomas Bata University in Zlín using molecular biological methods.

The concentration of nitrate ions in the reaction mixture was determined according to the standard ČSN ISO 7890-3 (75 7453) from 1994. The standardized method ČSN EN 26777 (75 7452) was used to determine the content of nitrite ions. Determination of phosphate ion content was also performed using the standardized method ČSN EN ISO 6878 (75 7465).

Ion chromatography with a modular chromatograph based on Shimadzu parts was also used for determination of nitrates, sulphates and phosphorus concentrations.

Values of pH were measured potentiometrically using a combination electrode connected to a portable HQ 30d multimeter (Hach, Germany). ORP values were also measured on the same multifunction device, using the appropriate ORP probe (Hach, Germany). Dissolved oxygen values were measured electrochemically using a Cyberscan DO 300 membrane probe (Eutech Instruments, USA).

4 Results and discussion

4.1 Batch experiments

From the measured data, the effects of a number of process factors on the course of autotrophic denitrification were evaluated. These were the effect of temperature (22 and 33 °C), the size of the fraction used, the stirring of the reaction mixture (unmixed vessels versus mixtures which were stirred on a shaker at 150 rpm throughout the experiment) and the effect of phosphorus and ferric iron at various concentrations.

4.1.1 Control mixtures

First, the processes taking place in batch reaction vessels labelled K1a,b, K2a,b and K3, which are hereinafter referred to as control reaction mixtures, to which no additives were added, were described and evaluated (Table 1). Figure 2 shows the nitrate content of reaction vessels K1a, K2a and K3 during the experiment. In the first start-up phase of the experiment, a gradual decrease in the concentration of nitrate ions in all reaction mixtures is evident. This can be caused by two factors. The first of these is

called the lag phase, when the cells of microorganisms adapt to the new environment. The second factor is the presence of molecular oxygen in the reaction mixture. At the beginning of all experiments, a dissolved oxygen concentration of approximately 5.9 $mg.L^{-1}$ was measured in the reaction mixtures. However, once the oxygen from the reaction mixture is depleted and the condition of the anoxic environment is met (dissolved oxygen concentration below 0.5 $mg.L^{-1}$) and at the same time the bacterial cells are adapted to the new environment, there is a more significant reduction of nitrates, which continues to virtually zero values.

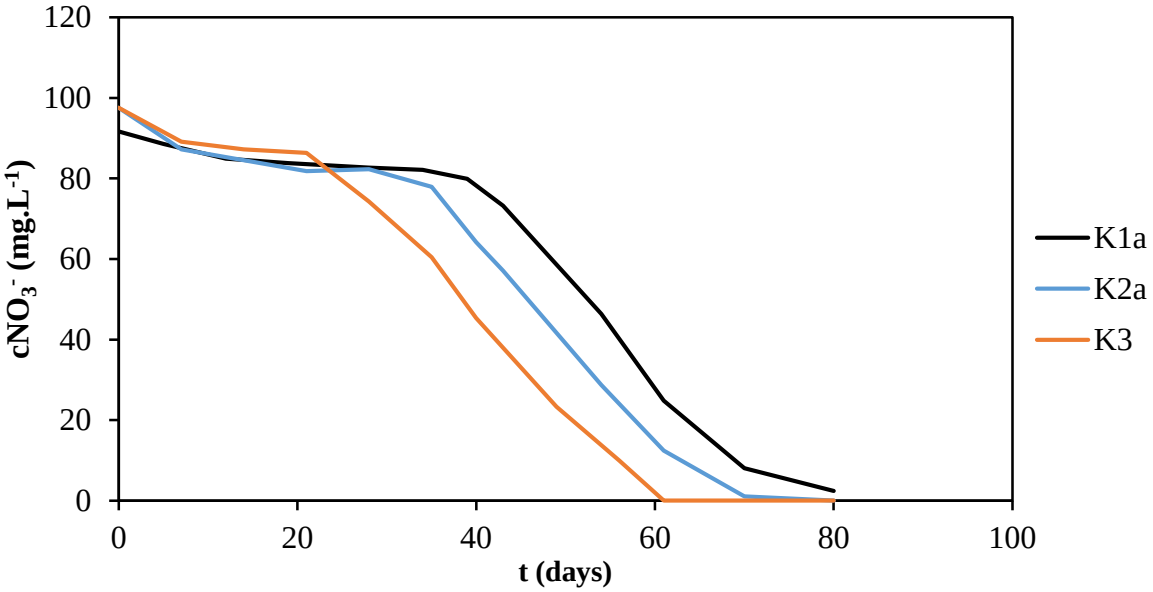


Figure 2 – Nitrate content in control reaction vessels K1a (50 g S), K2a (100 g S) and K3 (200 g S)

The measured data show that the adaptation time of bacterial cells can also be affected by the amount of the charge used, i.e. for a charge with the same mean particle size, mediated by the size of the reaction surface. The concentration of nitrates in the reaction systems decreased by 15 to 22 % during the start-up phase. The onset time was 34 to 51 % of the total experiment time. The degradation time of the remaining nitrate ions after the lag phase was close to 40 days. The almost linear course of the decrease part of the measured curve (Figure 2) indicates that the reaction rate does not depend, with the exception of extremely low concentrations, on the concentration of nitrate ions, which is typical for pseudo-zero order reactions. The values of the reaction constants obtained by regression of the experimental data were in the reaction vessel K1a -1.88 $mg.L^{-1}day^{-1}$, in K2a -2.05 $mg.L^{-1}day^{-1}$ and in K3 -2.15 $mg.L^{-1}day^{-1}$.

To verify the reproducibility of the measurements, the experiments in reaction vessels K1 and K2 were prepared in duplicate (i.e., two reactors designated K1a and K1b, respectively K2a and K2b with the same initial composition). The course of the experiments shows a very good reproducibility of the measurements in the reaction vessels.

A typical course of nitrate reduction is demonstrated in more detail in Figure 3 for system K1a. Total nitrogen values were calculated as the sum of nitrate and nitrite

nitrogen concentrations. It can be seen from (Figure 3) that nitrite ions begin to appear in the reaction mixture as an intermediate of the decomposition reaction in connection with the onset of the degradation process. The subsequent reduction of nitrites is slower than the primary reduction of nitrates, and thus their undesired accumulation in the reaction system occurs. However, the concentration of nitrite ions was about two orders of magnitude lower than the concentration of nitrate ions throughout the process.

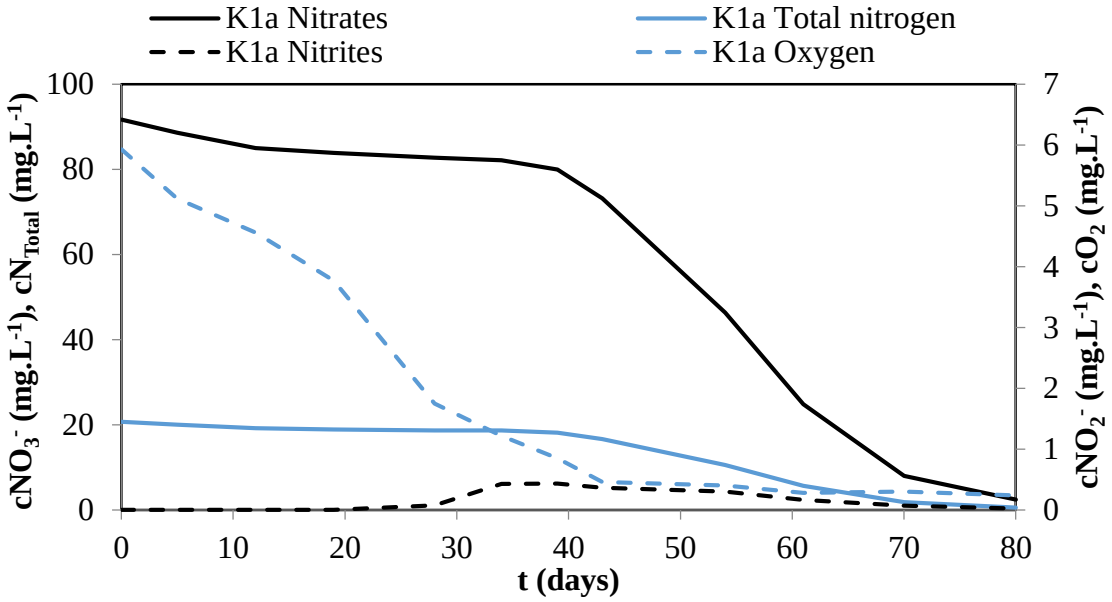


Figure 3 – Concentrations of nitrates, nitrites, total nitrogen and dissolved oxygen in reaction vessel K1a (50 g S)

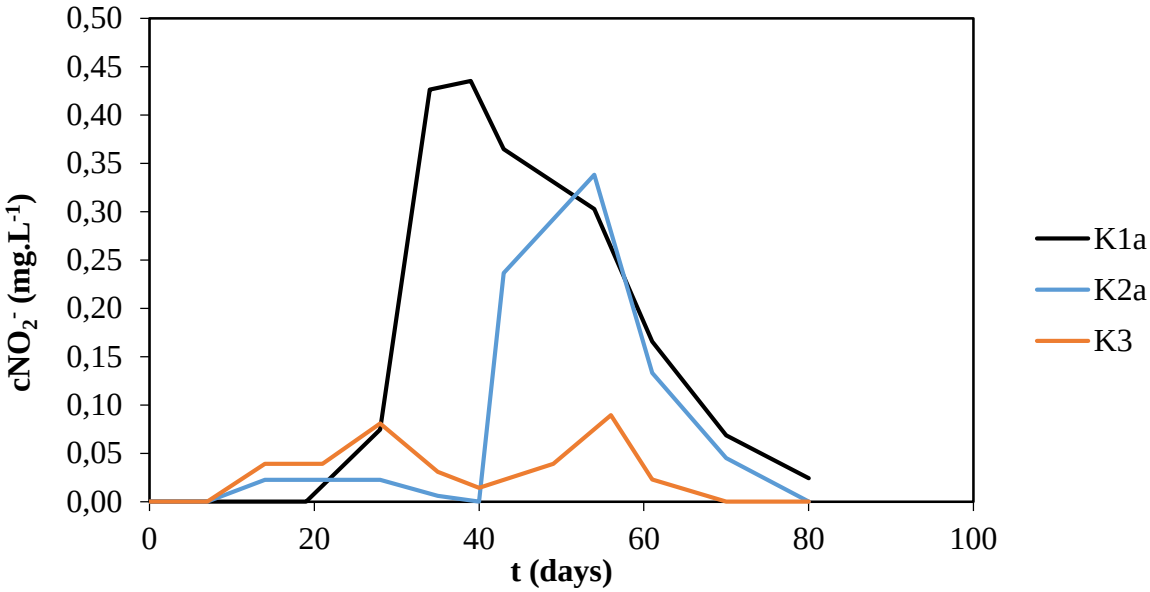


Figure 4 – Changes in nitrite concentration in control reaction vessels K1a (50 g S), K2a (100 g S) and K3 (200 g S)

The concentration of nitrite ions in the individual reaction systems throughout the experiment is shown in Figure 4. The concentrations of measured nitrites in these three systems did not exceed values 0.45 mg.L^{-1} NO_2^-.

Changes in total nitrogen concentration in experiments in control batch reaction vessels are shown in Figure 5. It is clear that the concentrations of the excess intermediate are very low and hardly affect the overall nitrogen balance. On the other hand, with regard to the higher potential toxicity of nitrite ions, even these low concentrations may limit the possible industrial use of the studied process.

Experiments have further shown that the addition of a neutralizing agent - limestone in a ratio of 1:1 to the amount of sulphur is sufficient. Values of pH ranged from 7.0 to 7.7 during all experiments.

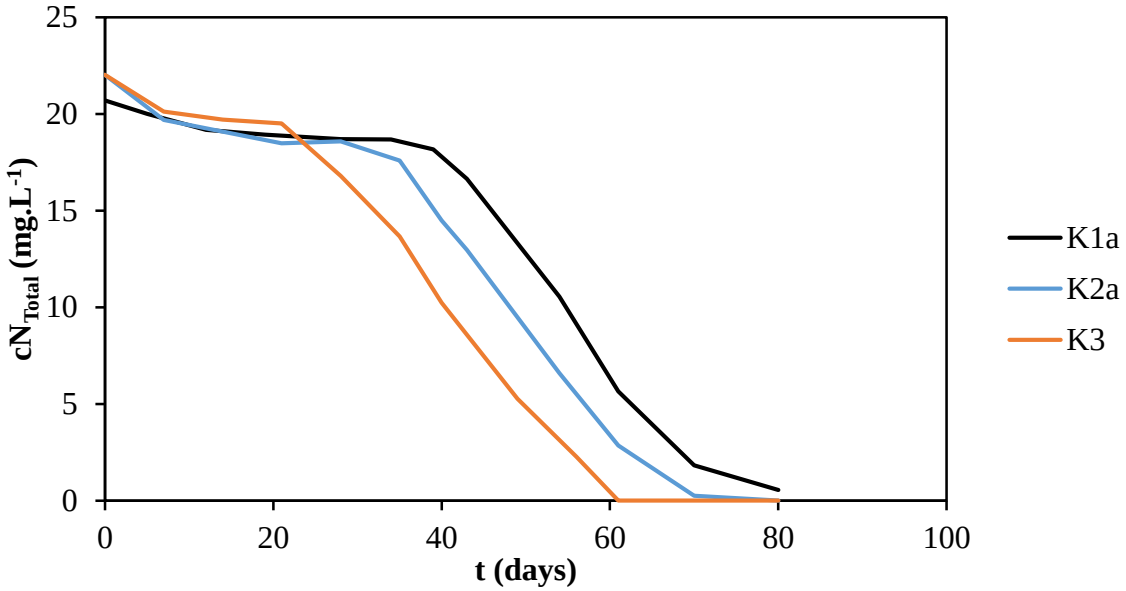


Figure 5 – Changes in total nitrogen content in control reaction vessels K1a (50 g S), K2a (100 g S), and K3 (200 g S)

4.1.2 Influence of phosphorus on the course of autotrophic denitrification

Phosphorus is one of the main biogenic elements and in recent years, phosphorus concentrations have increased significantly in almost all types of water that could be treated by autotrophic denitrification. Therefore, the effect of phosphorus additions (in the form of NaH_2PO_4) on the course of the autotrophic denitrification reaction was experimentally studied.

For this set of experiments, a total of nine reaction mixtures labelled P1 to P9 with different initial concentrations of NaH_2PO_4 with concentrations corresponding to phosphorus amounts ranging from 0.5 to 3.0 $mg.L^{-1}$ were prepared (Table 1).

No interruption of the regulatory role of limestone was observed during all the experiments performed, and the possible formation of insoluble phosphates on the surface of the limestone can therefore be neglected. Some of the free calcium ions usually precipitate as calcium sulphate. Phosphate, which is practically insoluble in water as calcium phosphate, only begins to form at pH 9.5, i.e. under conditions outside the scope of the experiments performed [12].

The length of the onset phase in P1 to P5 systems was 24 to 54 % of the experiment time and 6 to 23 % of nitrates were degraded. However, the measured data (Figure 6) do not show a clear effect of the content of added phosphorus on the length of this onset phase. It turns out that the random effects due to the initial oxygen content in the reaction mixture and the adaptability of the microorganisms to the new environment are more pronounced than the effect of the amount of phosphorus added to the reaction mixture.

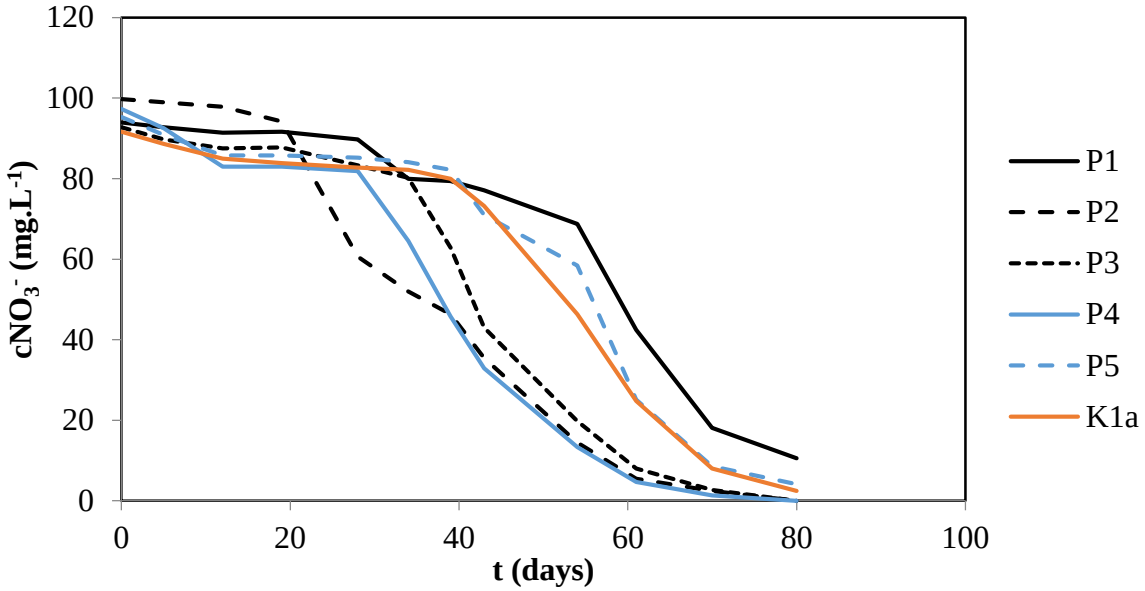


Figure 6 – Influence of dosed phosphorus on changes in nitrate concentration during autotrophic denitrification (systems with 50 g S). Here P1 (0.5 mg.L^{-1} P), P2 (1.0 mg.L^{-1} P), P3 (1.5 mg .L^{-1} P), P4 (2.0 mg.L^{-1} P), P5 (3.0 mg.L^{-1} P) and K1a (0 mg.L^{-1} P)

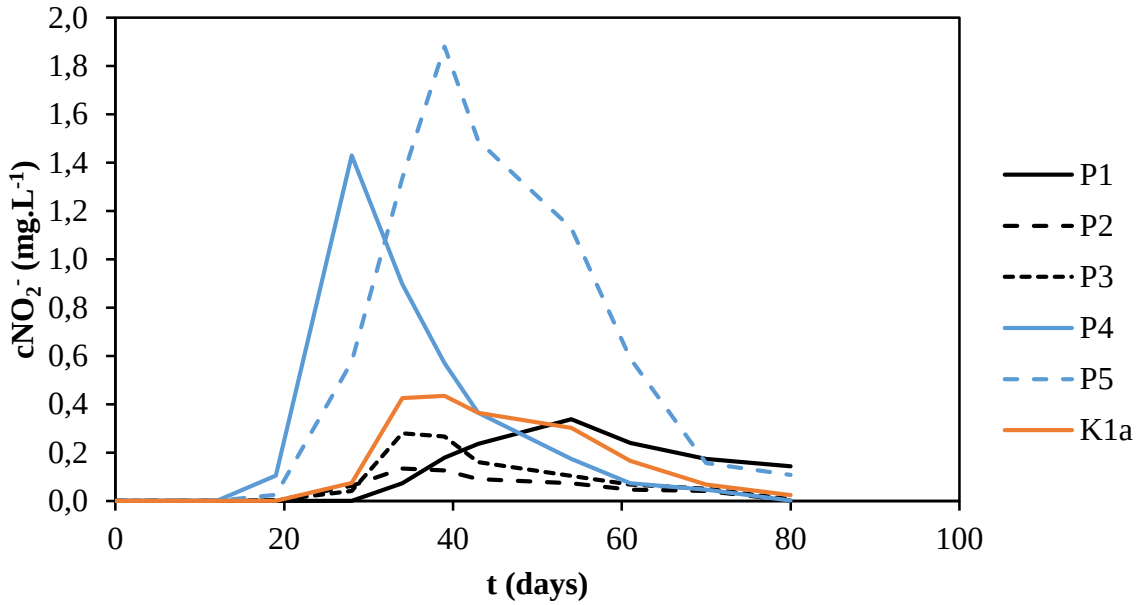


Figure 7 – Time changes of nitrite concentration in reaction vessels P1 (0.5 mg.L^{-1} P), P2 (1.0 mg.L^{-1} P), P3 (1.5 mg.L^{-1} P), P4 (2,0 mg.L^{-1} P), P5 (3.0 mg.L^{-1} P) and K1a (0 mg.L^{-1} P)

Rate constants for nitrate degradation in reaction vessels were determined (P1 -1.54 $mg.L^{-1}day^{-1}$, P2 -1.65 $mg.L^{-1}day^{-1}$, P3 -1.86 $mg.L^{-1}day^{-1}$, P4 -1.82 $mg.L^{-1}day^{-1}$ and P5 -1.71 $mg.L^{-1}day^{-1}$). It is obvious that the course of autotrophic denitrification characterized by the rate constant *k* is not systematically influenced by the amount of phosphorus in the reaction mixture.

The effect of phosphorus addition on changes in nitrite ion concentration during experiments in systems P1 to P5 is shown in Figure 7. High concentrations of undesired nitrites were recorded in reaction mixtures P4 and P5 (2.0 and 3.0 $mg.L^{-1}$ of phosphorus, when nitrite concentrations exceed 1.4 $mg.L^{-1}$).

Another set of experiments (P6 to P9) was focused on studying the effect of phosphorus addition to systems with higher amounts of sulphur in the reaction mixture (100 and 200 g S) with the concentration of added phosphorus ranging from 0.5 $mg.L^{-1}$ to 1.5 mg $.L^{-1}$ (Table 1). From the evaluated reaction constants of nitrate degradation (P6 -2.29 $mg.L^{-1}day^{-1}$, P7 -2.29 $mg.L^{-1}day^{-1}$, P8 -1.95 $mg.L^{-1}day^{-1}$ and P9 -2,31 $mg.L^{-1}day$) it is evident that even the reduction of nitrates is not affected by the concentration of phosphorus, in all tested systems with different sulphur weights.

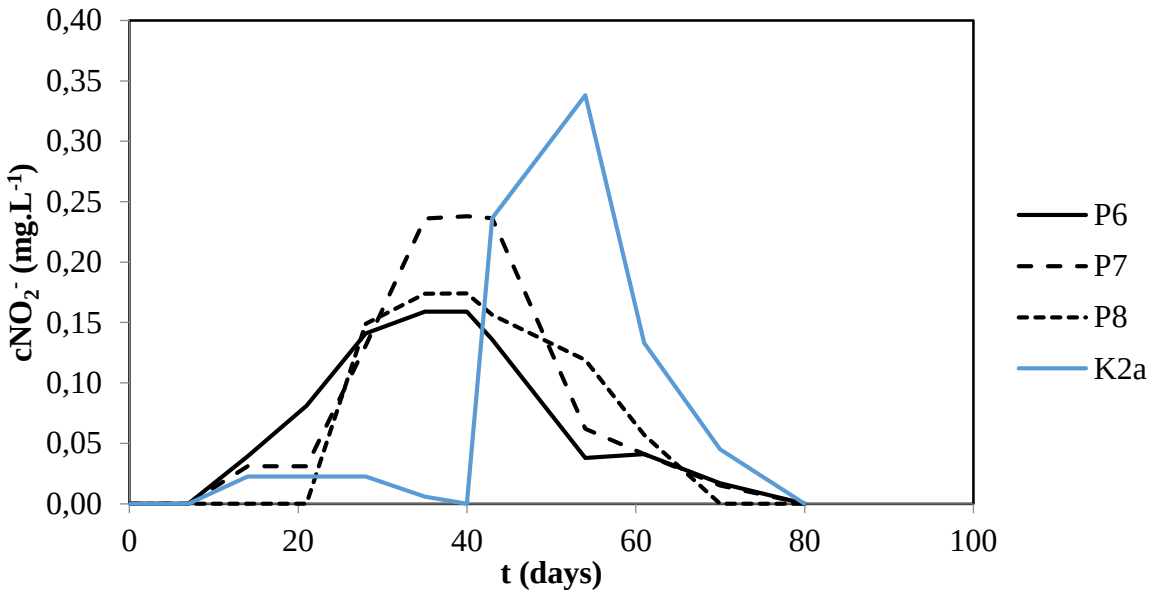


Figure 8 – Time changes of nitrite concentration in reaction vessels P6 (0.5 $mg.L^{-1}$ P), P7 (1.0 $mg.L^{-1}$ P), P8 (1.5 $mg.L^{-1}$ P) and K2a (0 mg $.L^{-1}$ P)

In contrast, the effect of added phosphorus on the formation and subsequent reduction of nitrite ions is evident (Figure 8). In the reaction vessels marked P6 and P8, the nitrite concentrations did not exceed 0.179 $mg.L^{-1}$. In vessel P7 (1.0 $mg.L^{-1}$ of phosphorus at the beginning of the experiment), the highest measured value of nitrite concentration was 0.248 $mg.L^{-1}$.

Figure 9 shows a comparison of the nitrate content in reaction vessels K1a, K2a, K3, P2, P7 and P9 and Figure 10 shows a comparison of changes in nitrite content in the same reaction vessels. These are systems that differ in the sulphur content with limestone (50, 100 or 200 g each) and at the same time the containers marked with the letter P have an additional phosphorus content of 1.0 $mg.L^{-1}$ (Table 1). As can be seen

(Figures 9 and 10), the higher sulphur content did not significantly affect the course of the reduction of nitrates or nitrites.

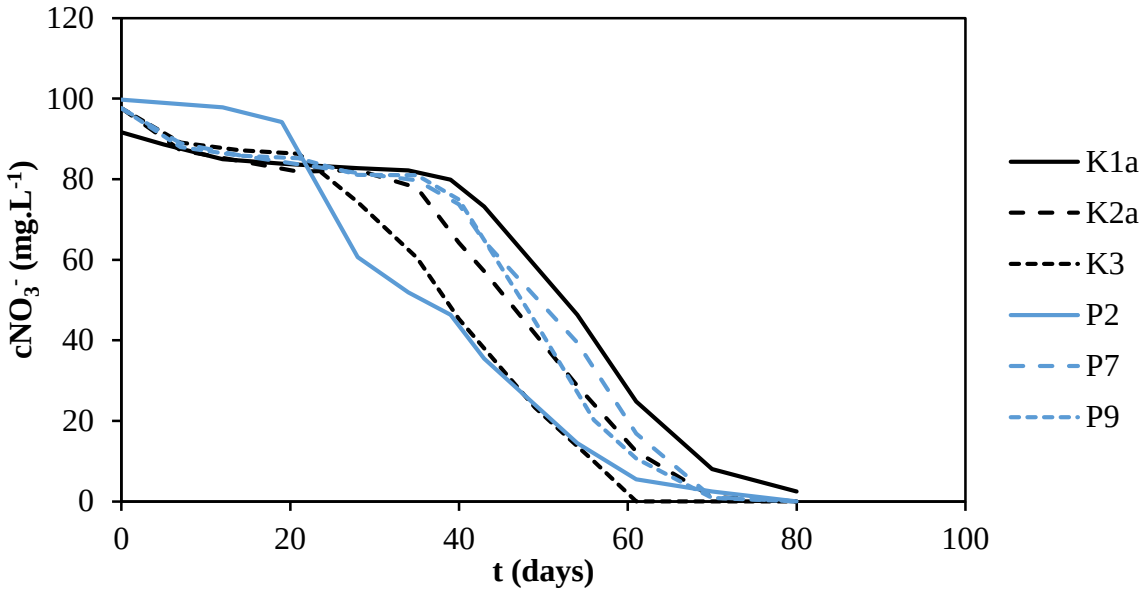


Figure 9 – Influence of dosed phosphorus on changes in nitrate concentration during autotrophic denitrification in systems with different concentration S and with the addition of P 1.0 mg.L^{-1}. Here K1a (50 g S), K2a (100 g S), K3 (200 g S), P2 (50 g S and 1.0 mg.L^{-1} P), P7 (100 g S and 1.0 mg. L^{-1} P) and P9 (200 g S and 1.0 mg.L^{-1} P)

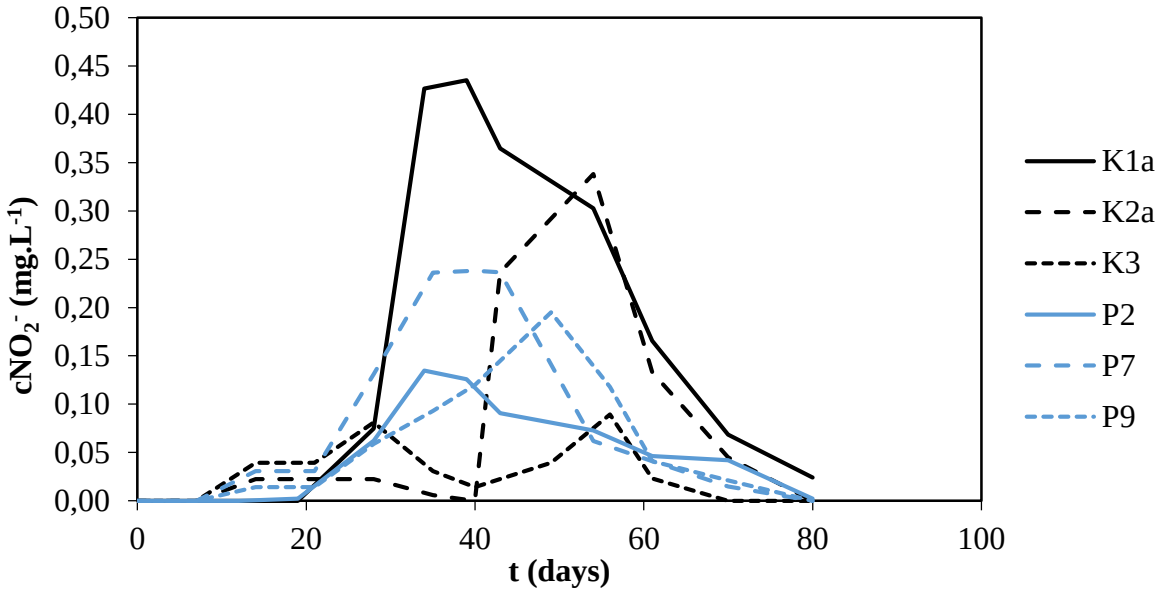


Figure 10 – Time changes of nitrite concentration in reaction vessels K1a (50 g S), K2a (100 g S), K3 (200 g S), P2 (50 g S and 1.0 mg.L^{-1} P), P7 (100 g S and 1.0 mg.L^{-1} P) and P9 (200 g S and 1.0 mg.L^{-1} P)

4.1.3 Influence of iron ions on the course of autotrophic denitrification

Iron is one of the trace biogenic elements. In the form of $FeSO_4.7H_2O$, iron contains nutrient media for the cultivation of the genus *Thiobacillus* and in the form of $FeCl_3$ media for the growth of specifically *T. denitrificans*. Dissolved iron is present in

acidic and neutral waters rich in oxygen as a trivalent cation Fe^{3+}. In addition to higher concentrations of nitrate ions, wastewater may also contain increased concentrations of Fe^{3+} ions, so this part of the experiments is focused on the study of the effect of iron additions in the form of $FeCl_3$ on the course of autotrophic denitrification. It was studied in four prepared reaction mixtures designated Fe1 to Fe4 (initial iron concentration ranging from 0.05 to 1.0 $mg.L^{-1}$, Table 1).

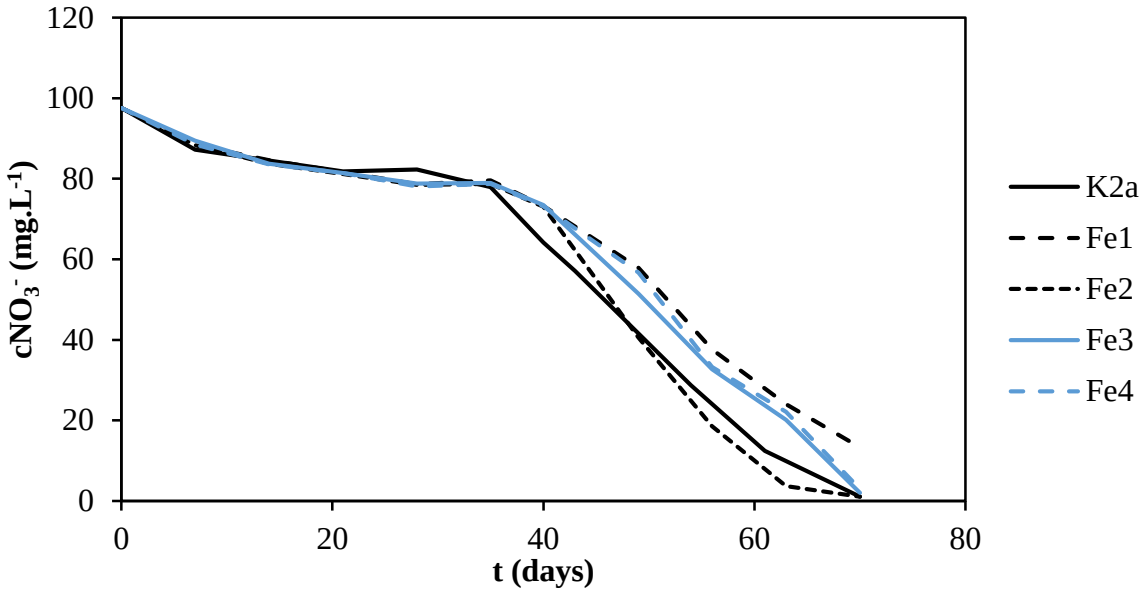


Figure 11 - Influence of dosed iron on changes in nitrate concentration during autotrophic denitrification in systems with added Fe^{3+}. Here Fe1 (0.05 $mg.L^{-1}$ Fe^{3+}), Fe2 (0.1 $mg.L^{-1}$ Fe^{3+}), Fe3 (0.5 $mg.L^{-1}$ Fe^{3+}), Fe4 (1.0 $mg.L^{-1}$ Fe^{3+}) and K2a (0 $mg.L^{-1}$ Fe^{3+})

In Figure 11 is not seen the direct effect of the concentration of iron ions on the length of the onset phase (a period of 35 days during which approximately 19 % of the originally dosed nitrates were degraded). It turns out that, as with phosphorus, the random effects of the initial oxygen content of the reaction mixture and the adaptability of the microorganisms to the new environment are more pronounced than the effect of the amount of Fe^{3+} ions fed to the reaction mixture.

Nitrate degradation rate constants were evaluated (Fe1 -1.90 $mg.L^{-1}day^{-1}$, Fe2 -2.26 $mg.L^{-1}day^{-1}$, Fe3 -2.19 $mg.L^{-1}day^{-1}$ and Fe4 -2.19 $mg.L^{-1}day^{-1}$). Compared to the K2a system, the absolute value of the reaction constant increases by 12 to 16 %. This could indicate a positive effect of the addition of iron ions on the first step of denitrification - the reduction of nitrates.

The Figure 12 shows the increased accumulation of nitrite ions in the presence of iron ions. The highest concentration of nitrite ions was measured in the Fe2 system, i.e. in the case with the highest rate of nitrate ion degradation (Figure 11). It is evident that the presence of Fe^{3+} mainly affects the first step of denitrification. This means that the reduction of nitrates to nitrite is accelerated. However, the degradation of the nitrites formed is most likely not affected by the addition of Fe^{3+} ions, so that this system of subsequent reactions results in the above-mentioned increase in the accumulation of nitrite ions in the reaction mixture.

Even in this case, the total nitrogen content (as the sum of nitrate and nitrite nitrogen) was calculated from the measured data of nitrate and nitrite content. And despite the accumulation of nitrite in the tested systems in relatively high concentrations, the values of total nitrogen throughout the experiment were declining.

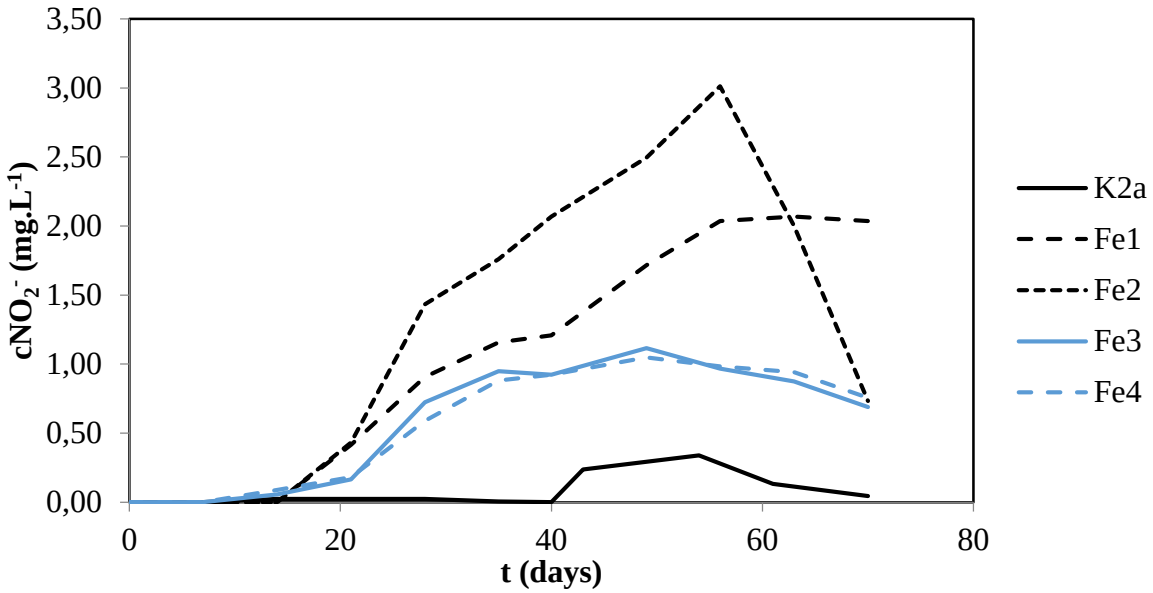


Figure 12 - Time changes of nitrite concentration in reaction vessels with the addition of Fe^{3+}. Here Fe1 (0.05 $mg.L^{-1}$ Fe^{3+}), Fe2 (0.1 $mg.L^{-1}$ Fe^{3+}), Fe3 (0.5 $mg.L^{-1}$ Fe^{3+}), Fe4 (1.0 $mg.L^{-1}$ Fe^{3+}) and K2a (0 $mg.L^{-1}$ Fe^{3+})

4.1.4 Influence of particle size and amount of sulphur on the course of autotrophic denitrification

System K4 contained sulphur and limestone with larger particle size than in system K12 (Tables 1 and 2). The sulphur content in the K12 system (4.46 g) was determined so that the total surface area of the sulphur particles in the K12 reaction system was the same as in the K4 system.

In the reaction vessel with the larger sulphur particle size fraction, the onset phase lasted approximately 55 % of the total experiment time (Figure 13). In a reaction mixture with a smaller sulphur particle size, the onset region can be divided into two distinct parts. In the first part (for about 32 days) no nitrates were reduced, nor were nitrites formed. Then, in the second part, there is an increase in the nitrite concentration, reaching its flat maximum (42 to 79 days) and a subsequent decrease in the nitrite concentration to very low values (93 days). This whole period of time is accompanied by a slight linear decrease in the nitrate concentration. From day 93, the decrease in the nitrate concentration in the reaction mixture is very steep and within 9 days 81 % of the original amount of nitrate was broken down.

From the comparison of rate constants to the pseudo-zero order, it is clear that a higher absolute value was determined for the final denitrification in the K12 system (-0.84 and -10.5 $mg.L^{-1}day^{-1}$, respectively) compared to the K4 system (-2.40 $mg.L^{-1}day^{-1}$). This may be due to the fact that smaller particles were suspended in the entire volume of the sodium nitrate solution when the reaction vessels were stirred,

and the effect of external diffusion was also included when larger particles were used. In addition, the use of a sulphur fraction with a smaller particle size, while maintaining the same surface area of the particles, can be advantageous in terms of lower consumption of sulphur and limestone and thus their cost.

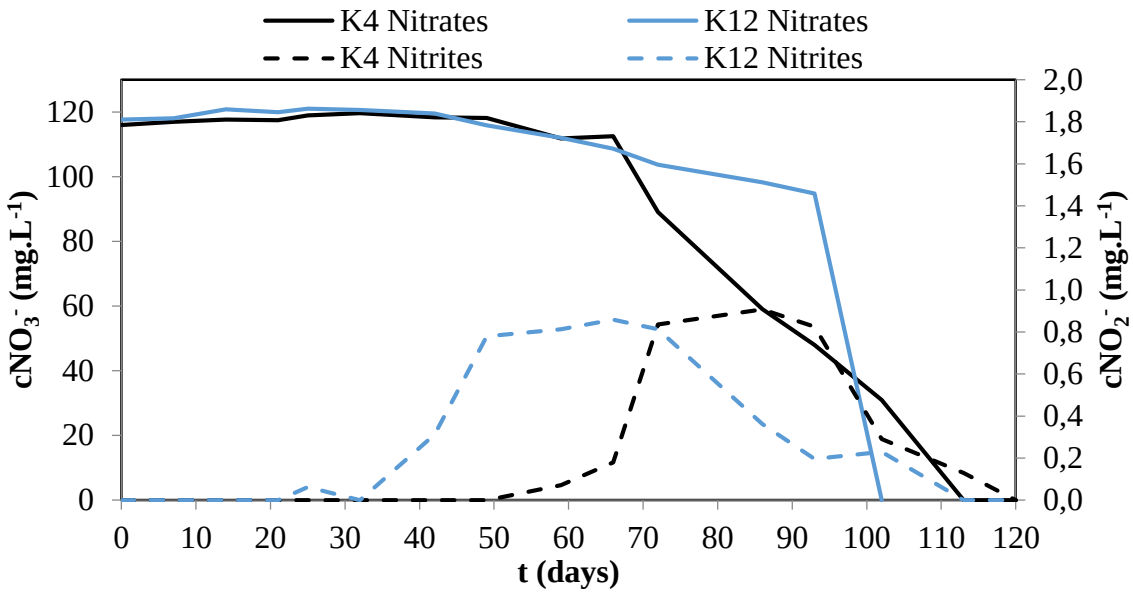


Figure 13 - Nitrate content and time changes of nitrite concentration in reaction systems K4 (S with a mean particle size of 3.54 mm) and K12 (S with a mean particle size of 167 μm)

Figure 13 shows that even when a finer sulphur fraction is used, undesired accumulation of nitrite ions occurs in the reaction mixture. In both systems, the values of measured concentrations of nitrite ions did not exceed 1 $mg.L^{-1}$. Compared to the comparative experiments from the first series of measurements, the use of smaller sulphur particles shows a higher rate of nitrate degradation in the final phase and at the same time a higher concentration of nitrite accumulated in the reaction mixture.

The influence of drinking tap water on the course of degradation was tested in a set of experiments designated as K5 to K7 and K11 to K13 (Table 2). From the measured data it is evident that the effect of preparation of the mixture from demineralized water or tap water is not statistically significant. At the same time, a higher concentration of nitrite was observed in the reaction mixture (the maximum value approximately 1.1 $mg.L^{-1}$). This phenomenon may be due to the use of the smaller particle size packing fraction discussed above and at the same time to the intensive stirring of the reaction mixture throughout the experiment.

4.1.5 Influence of stirring the reaction mixture on the course of autotrophic denitrification

Another monitored parameter of autotrophic denitrification was the effect of stirring the reaction mixtures on the course of the process. For this purpose, systems K8 to K13 were prepared (Table 2).

The length of the onset phase was almost the same in all six systems and was 55 to 60 % of the experiment time (Figure 14). During this phase, about 8 mg of nitrates were degraded. The rate constants for nitrate degradation in the K8 system were -2.38 $mg.L^{-1}day^{-1}$, K9 -2.73 $mg.L^{-1}day^{-1}$, K10 -2.77 $mg.L^{-1}day^{-1}$, K11 -3.09 $mg.L^{-1}day^{-1}$, K12 -3.03 $mg.L^{-1}day^{-1}$ and K13 -3.08 $mg.L^{-1}day^{-1}$. It can be seen that the stirring of the reaction mixture has a positive effect on the rate of nitrate reduction. This phenomenon is most likely due to the better availability of nitrate ions for the cells of microorganisms during intensive stirring of the reaction mixture

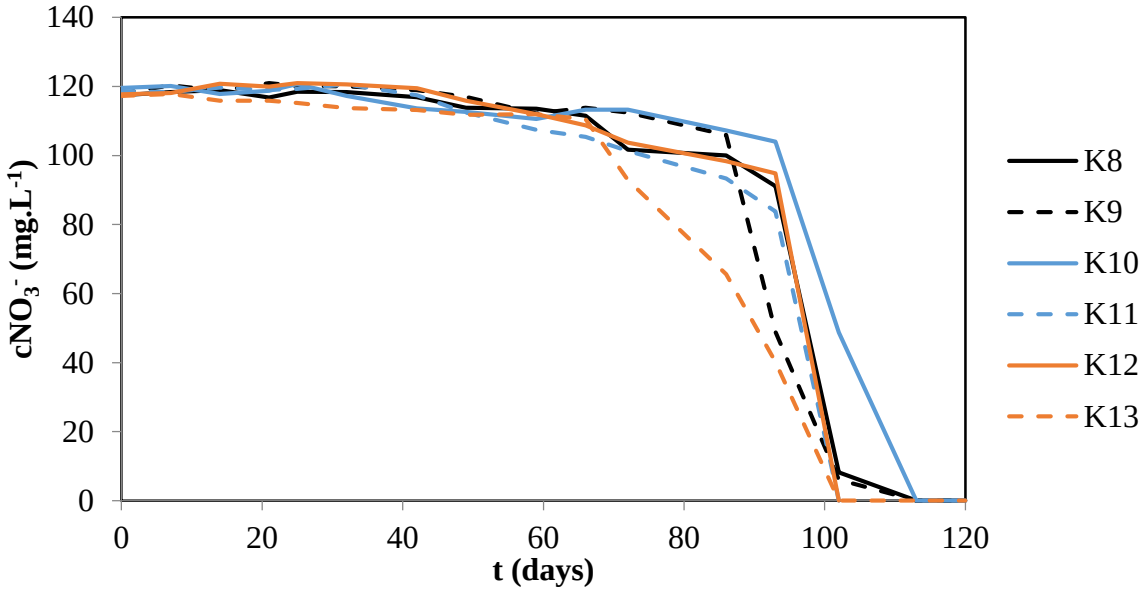


Figure 14 - Nitrate content in stirred and unmixed reaction mixtures K8 to K13. Here K8 (unmixed, 2.23 g S), K9 (unmixed, 4.46 g S), K10 (unmixed, 8.92 g S), K11 (stirred, 2.23 g S), K12 (stirred, 4.46 g S) and K13 (stirred, 8.92 g S)

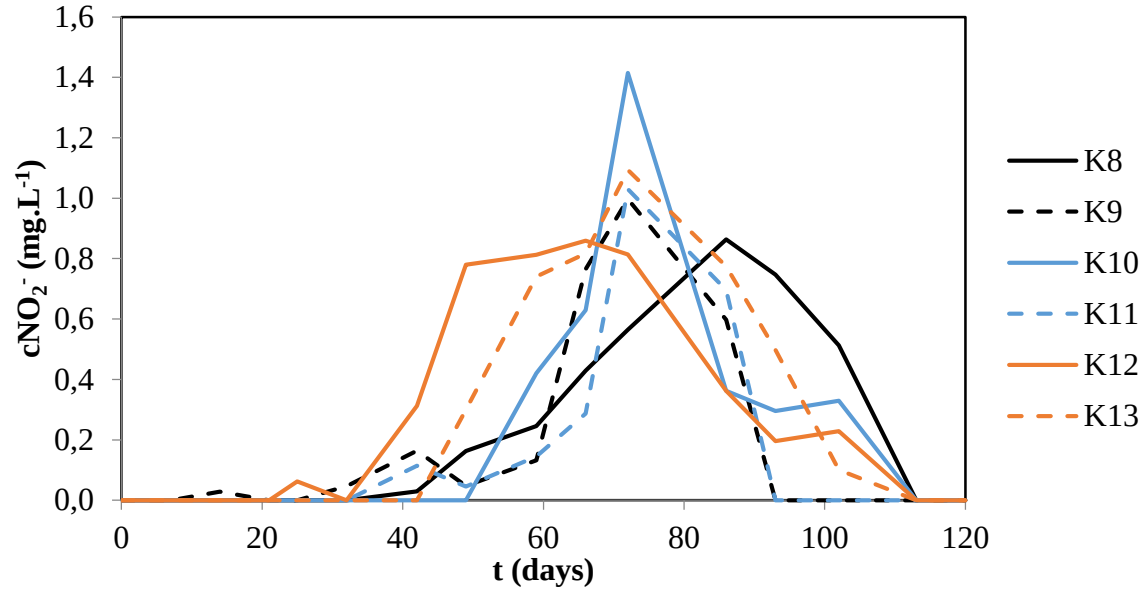


Figure 15 - Time changes of nitrite concentration in stirred and unmixed mixtures K8 to K13. Here K8 (unmixed, 2.23 g S), K9 (unmixed, 4.46 g S), K10 (unmixed, 8.92 g S), K11 (stirred, 2.23 g S), K12 (stirred, 4.46 g S) and K13 (stirred, 8.92 g S).

Undesirable accumulation of nitrite with maxima from 0.9 to 1.4 mg.l^{-1} was detected in all six monitored mixtures (Figure 15). The reason may be the previously discussed increase in the rate of nitrate degradation and thus the faster formation of nitrite as a consequence of the use of a filler fraction with a smaller particle size.

4.1.6 Influence of temperature on the course of autotrophic denitrification

To study the effect of temperature on autotrophic denitrification, systems labelled K8 to K10 and K14 to K16 were prepared (Table 2).

The onset time was shorter in the higher temperature systems (Figure 16) – 49 % of the total experiment length compared to 60 % in the room temperature systems.

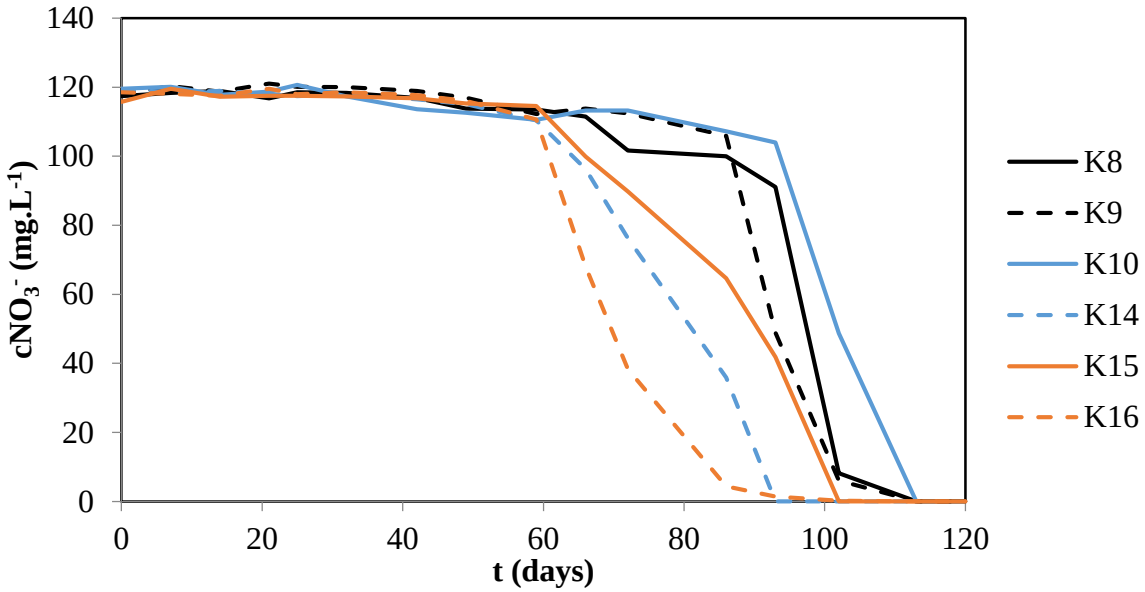


Figure 16 - Nitrate content in systems with different temperatures during autotrophic denitrification. Here K8 (22 °C, 2.23 g S), K9 (22 °C, 4.46 g S), K10 (22 °C, 8.92 g S), K14 (33 °C, 2.23 g S), K15 (33 °C, 4.46 g S) and K16 (33 °C, 8.92 g S).

The rate of nitrate degradation was also higher in higher temperature systems. The values of rate constants for nitrate degradation were in the system K8 -2.38 mg.L^{-1}day^{-1}, K9 -2.73 mg.L^{-1}day^{-1}, K10 -2.77 mg.L^{-1}day^{-1}, K14 -3.24 mg.L^{-1}day^{-1}, K15 -2.74 mg.L^{-1}day^{-1} and K16 -3.26 mg.L^{-1}day^{-1}. The higher rate of nitrate degradation probably had an effect on the higher accumulated nitrite content during the experiment, as the rate of nitrite degradation was probably not affected by temperature. In the monitored mixtures, the highest nitrite concentrations reached from 0.9 to 1.4 mg.L^{-1} (Figure 17).

Finally, three reaction mixtures, designated K17 to K19, were prepared and tested at a higher temperature (33 °C) and stirred at the same time (Table 2).

The shortest onset phase (Figure 18) was observed in all three tested mixtures compared to all batch experiments in the second series (49 days, i.e. 41 % of the experiment time). At the end of the onset phase, nitrates were degraded at the highest

observed rate from all batch experiments performed. The average value of the rate constant *k* was -6.73 $mg.L^{-1}day^{-1}$, i.e. almost twice than the values evaluated so far in the previously described experiments.

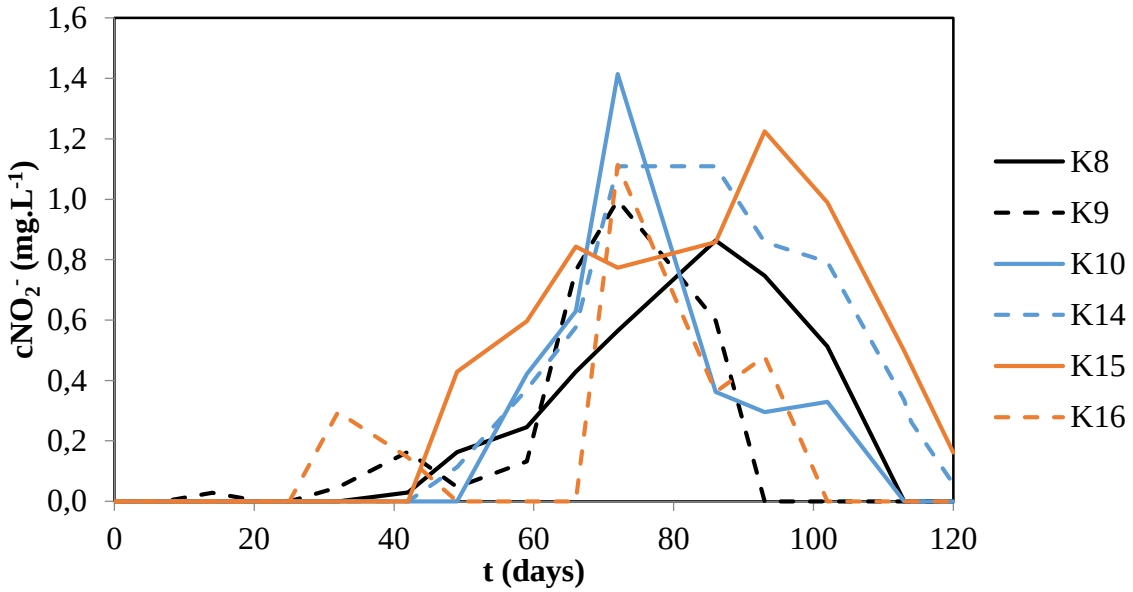


Figure 17 - Time changes of nitrite concentration in systems tested at different temperatures. Here K8 (22 °C, 2.23 g S), K9 (22 °C, 4.46 g S), K10 (22 °C, 8.92 g S), K14 (33 °C, 2.23 g S), K15 (33 °C, 4.46 g S) and K16 (33 °C, 8.92 g S)

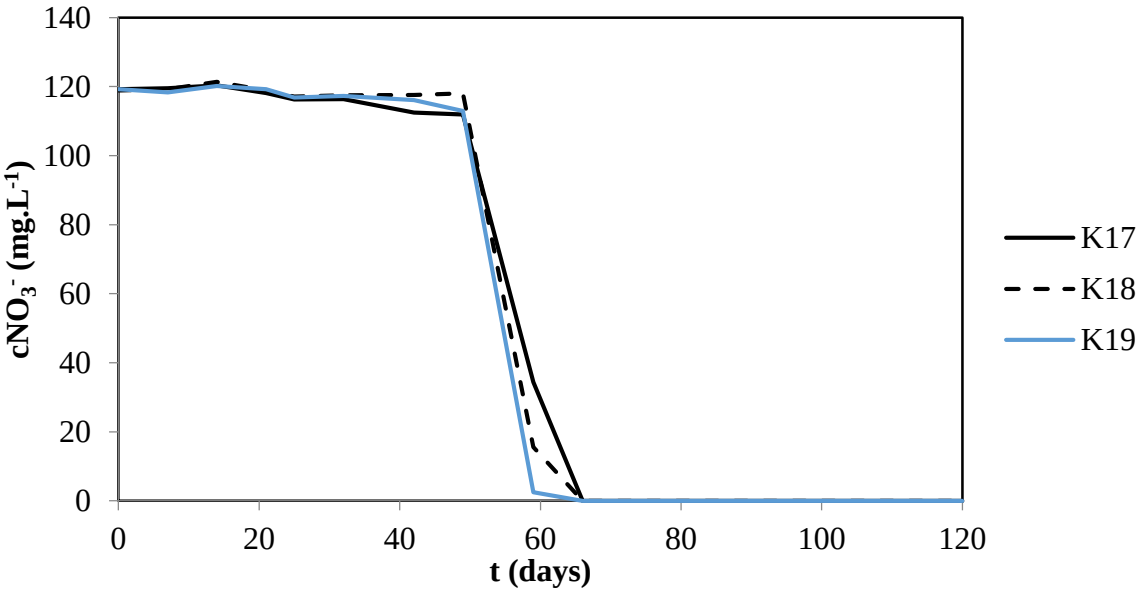


Figure 18 - Nitrate content during autotrophic denitrification in stirred systems at 33 °C. Here K17 (2.23 g S), K18 (4.46 g S) and K19 (8.92 g S)

A desirable effect of the combination of higher temperature and stirring was also the faster decomposition of nitrite, as their concentration in the reaction mixture was not as high as in the other cases measured in the second series of experiments. The values of nitrite concentration in the tested mixtures K17 to K19 (Fig. 19) did not exceed the value of 0.5 $mg.L^{-1}$.

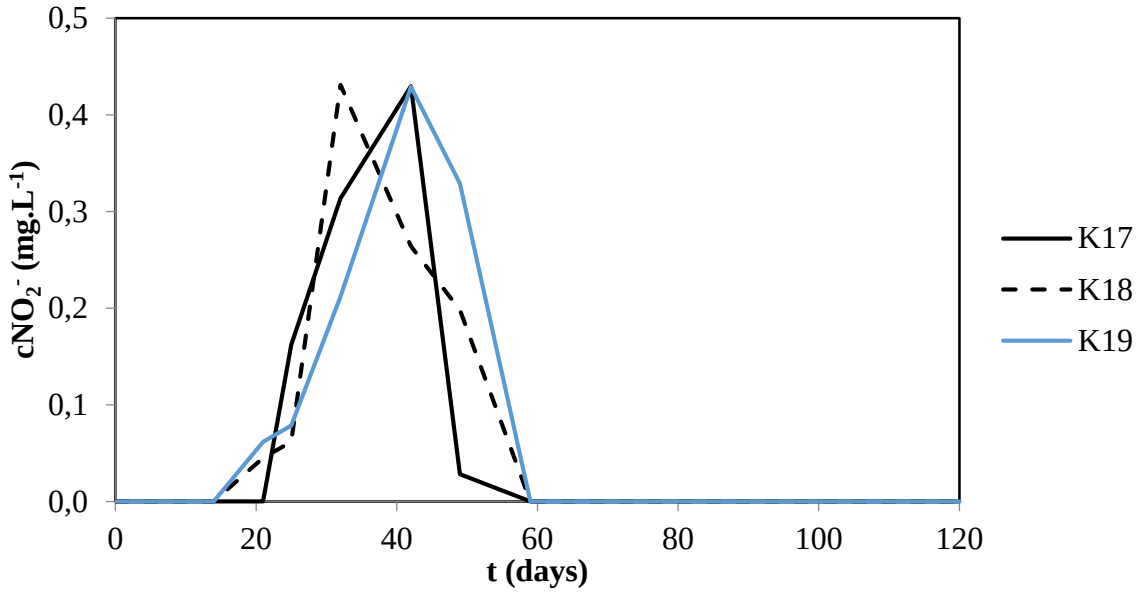


Figure 19 - Time changes of nitrite concentration in stirred systems at 33 °C. Here K17 (2.23 g S), K18 (4.46 g S) and K19 (8.92 g S)

4.2 Denitrification processes in a flow column

The experiments in the flow column lasted a total of 428 days. The concentrations of the individual components of the working solution and the rate of its dosing into the column are given in Table 3. Due to their long-term nature, the experiments were performed at room temperature (22 °C) and a larger particle fraction was used to ensure adequate hydraulic resistance. Thus, the optimal conditions determined in previous batch experiments were not used directly. The course of denitrification processes in the monitored flow column is shown in Figure 20.

In the first twenty days of the experiment, 70.8 mg of $NO_3.h^{-1}$ passed through the column (flow rate 23.6 $mL.min^{-1}$, concentration 50 $mg.L^{-1}$ NO_3^-). During these twenty days, the nitrate concentration at the outlet of the column gradually decreased. The lowest output value of the nitrate concentration was reached at the end of the monitored time interval, namely 23.1 $mg.L^{-1}$, i.e. 46 % of the input value of the nitrate concentration, and thus approximately 38.1 mg $NO_3^-.h^{-1}$ was removed. On the other hand, the values of the nitrite content gradually increased and reached a value close to 10 $mg.L^{-1}$.

After 20 days, the ORP values dropped from initial value 142.4 mV to 100 mV and the dissolved oxygen content from initial 7.9 to 7.0 $mg.L^{-1}$. Nevertheless, the determined values of dissolved oxygen concentration for autotrophic denitrification were too high. From the 21st day of the experiments in the flow column, the input content of nitrates was reduced to 25 $mg.L^{-1}$ until the 97th day of the experiment. At the same time, the flow of the working solution into the column was also reduced to 19.3 $mL.min^{-1}$ (dosing 29.0 mg $NO_3^-.h^{-1}$). The required increase in the efficiency of nitrate removal from the working solution did not occur, but there was a reduction in the nitrite content in the outlet (concentrations just above 1 $mg.L^{-1}$). At the same time, the

measured values of ORP at the outlet (about 80 mV) and the dissolved oxygen content (below 4 mg.L^{-1}) also decreased slightly.

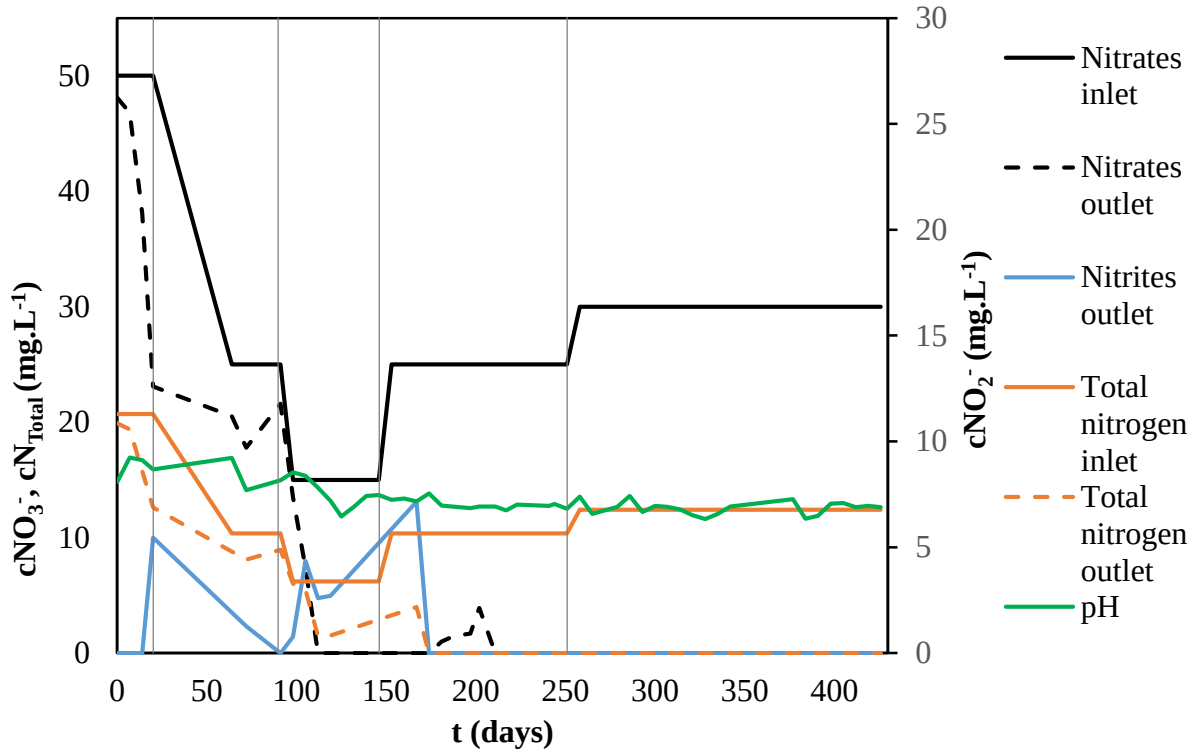


Figure 21 - Time course of basic parameters of autotrophic denitrification process in a flow column.

For further measurements in the flow column (98th to 150th day of the experiment) the input content of nitrates was again reduced (to 15 mg.L^{-1}) and at the same time the flow through the column to 7 mL.min^{-1} (dosing 6.3 mg NO_3^-.h^{-1}). In this case, there was a complete decomposition of nitrate ions to virtually zero values at the outlet. However, the values of the concentration of nitrite ions at the outlet were still high - they averaged around 4 mg.L^{-1} and at the end of this period they reached values of almost 10 mg.L^{-1}. However, the ORP values at the outlet of the column fell below 40 mV. At the same time, the dissolved oxygen values dropped below 3 mg.L^{-1}.

In the following test period (151st to 255th day of the experiment), 25 mg.L^{-1} of nitrate was metered into the column and the working solution was pumped at a flow rate of 8.5 mL.min^{-1}.

For about the first 170 days of the experiment, the operation of the column and the processes of microorganisms taking place in it stabilized (including their adaptation and multiplication). After this time, virtually complete removal of dosed nitrates has been achieved. At the same time, nitrites were not formed and their output concentration was below the limit of measurement detection.

The pH of the input (working) solution was determined to be 8.07. At the outlet, the pH values ranged from 6.33 to 9.23.

In the fifth and final stage of testing, which began on day 256 and lasted until day 428, the concentration of nitrate ions in the working (inlet) solution was increased to 30 $mg.L^{-1}$ and the flow rate was increased to 9.9 $mL.min^{-1}$. Under these conditions, no residual concentrations of nitrates or nitrites were detected in the column effluent (off-layer water flow rate 0.643 $mm.min^{-1}$, 17.8 mg of $NO_3^-.h^{-1}$ were treated).

At the outlet of the column, the highest measured concentration of sulphates at the outlet was 237.7 $mg.L^{-1}$.

During the whole experiment, a sample of immobile filling with microorganisms was taken several times, which was handed over to the laboratory of the Department of Environmental Protection Engineering of Tomas Bata University in Zlín, where the presence of *T. denitrificans* bacteria was confirmed by molecular biological methods.

Thus, with the help of the operated column and its tested setting, it was possible to remove up to 17.8 mg of $NO_3^-.h^{-1}$, i.e. approximately 4 mg of $N\text{-}NO_3^-.L^{-1}.h^{-1}$.

5 Conclusions

The aim of this experimental work was a systematic study of nitrate removal from prepared wastewater, which is poor in organic matter content and at the same time the concentration of nitrate ions does not exceed 100 $mg.L^{-1}$, by autotrophic denitrification using *T. denitrificans* bacteria.

In the first part of the study of autotrophic denitrification by *T. denitrificans*, attention was paid to batch experiments, which were used to describe the course of partial processes related to autotrophic denitrification. A total of 34 batch reaction vessels were prepared, the contents of which, and thus also the carrier of bacterial cells and the electron donor, consisted of lump sulphur with different particle sizes. Crushed limestone was also added to the reaction vessels to control pH values. Experiments have shown that the addition of a neutralizing agent - limestone in a weight ratio of 1:1 to the amount of sulphur is sufficient to maintain the pH in the range of 7.0 to 7.7.

In all tested reaction mixtures, the so-called lag phase was first observed in the initial stage of the process, during which there was only a slight loss of nitrates due to the adaptation of bacterial cells to the new environment and the presence of molecular oxygen in the mixture. The lag phase time ranged from 34 % to 51 % of the experiment time and 15 % to 22 % of the dosed nitrates were degraded. This was followed by an intensive reduction of nitrates to virtually zero values.

The absolute value of the rate constant evaluated for individual experiments ranged from 1.54 to 10.5 $mg.L^{-1}.day^{-1}$. The highest values, i.e. the highest rate of nitrate degradation, were determined for systems with intensive mixing combined with larger particles and a temperature of 33 °C, or smaller particles at a lower temperature of 25 °C.

It has been observed experimentally that the subsequent reduction of the nitrites formed is usually slower than the primary reduction of the nitrates, and thus their undesired accumulation in the reaction system occurs. Depending on the studied system, the maximum concentration of nitrite anion accumulation in the reaction mixture ranged from 0.09 to 3 $mg.L^{-1}$. The results of the experiments showed that at phosphorus

concentrations in the reaction mixture higher than 2 $mg.L^{-1}$, the values of the measured nitrite concentrations in the reaction mixture were higher than 1 $mg.L^{-1}$. The presence of iron ions in the reaction mixture had an even more significant effect, where their concentration of 0.05 $mg.L^{-1}$ already contributed to the formation of nitrite anions with a concentration of 1 $mg.L^{-1}$ and 3 $mg.L^{-1}$, respectively, at a concentration of iron ions of 1 $mg.L^{-1}$. The obtained findings show that the presence of Fe^{3+} accelerates the reduction of nitrates (reaction constant approx. -2.14 $mg.L^{-1}.day^{-1}$) to the formation of nitrites. However, the degradation of the formed nitrites is most likely not affected by the addition of Fe^{3+} ions. The result is an increase in the accumulation of nitrite ions in the reaction mixture.

The second part of the experiments was focused on long-term laboratory verification of the operation of a flow-through autotrophic denitrification column. Elemental sulphur was used as the filler, the particles of which were the carrier and electron donor for the bacteria *T. denitrificans*. The column was operated for a total of 428 days and a model working solution containing from 15 to 50 $mg.L^{-1}$ of nitrates was pumped into it. During the first 170 days of the experiments, the column stabilized (adaptation of bacterial cells to the new environment, reduction of nitrate input concentration, reduction of input solution flow and reduction of ORP and dissolved oxygen concentration in the output solution) and after this time a removal rate of up to 4 mg $N\text{-}NO_3^-.L^{-1}.h^{-1}$.

In general, it can be stated from the experiments that it was confirmed that the studied method of removing nitrates from water using autotrophic bacteria *T. denitrificans* is slower than the alternative use of heterotrophic organisms. However, when removing low concentrations of nitrates from water, the choice of autotrophic denitrification method could be more advantageous, as it is possible to remove nitrates down to zero concentrations without releasing more toxic nitrites. At the same time, an inorganic substrate (here sulphur) is cheaper than an organic substrate that needs to be supplied to heterotrophic organisms.

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7 List of published works and conference contributions

Patent

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Published works and conference contributions without connection to the topic of dissertation thesis

Type of published work and conference contributions	**Quantity**
Co-author of published articles	4
Co-author of poster presentation at international conferences	8
Co-author of poster presentation at national conferences	10