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NUMBERS OF SELECTED PHYSIOLOGICAL GROUPS OF BACTERIA IN DOMESTIC SEWAGE AFTER VARIOUS STAGES OF TREATMENT IN MULTI-STAGE CONSTRUCTED WETLAND[1]

Krzysztof Jóźwiakowski[*], Elżbieta Wielgosz[**]

[*]Water and Sewages Analytics Laboratory, Department of Melioration and Agricultural Construction
[**]Department of Agricultural Microbiology
University of Life Sciences in Lublin, Leszczyńskiego str. 7, 20-069 Lublin
*krzysztof.jozwiakowski@up.lublin.pl, **elzbieta-wielgosz@wp.pl

Summary. The paper presents an analysis of the numbers of selected physiological groups of bacteria in domestic sewage after various stages of treatment in a multi-stage constructed wetland with vertical and horizontal flow (VF-HF), with reed and willow. The study was performed in 2009. The greatest numbers of ammonifying and denitrifying bacteria were recorded in the raw wastewater. At the successive stages of treatment a reduction in the numbers of those bacteria was found, which indicates correct operation of the constructed wetland under analysis. The highest numbers of phase I and II nitrifying bacteria, and an increase in the concentration of $N\text{-}NO_3^-$ and $N\text{-}NO_2^-$ were observed in sewage flowing out of bed I with reed, which indicates an effective process of nitrification in that bed. The lowest numbers of nitrifying bacteria were found in the raw sewage, which was probably due to low concentration of dissolved oxygen and a high level of $N\text{-}NH_4^+$ which could be toxic to their growth. The effective process of nitrification in bed I with reed and of denitrification in bed II with willow resulted in an almost 70% efficiency of total nitrogen removal in the constructed wetland system under study.

Key words: bacteria, nitrogen, ammonification, nitrification, denitrification, sewage, constructed wetland

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INTRODUCTION

The process of biological removal of nitrogen in sewage treatment plants proceeds with participation of three groups of bacteria: ammonifying, nitrifying and denitrifying bacteria [Gallert and Winter 2005]. The presence of all three of those microbial groups in the particular elements of multi-stage constructed wetland systems is necessary for efficient transformation of organic nitrogen compounds present in sewage into forms available for plants [Vymazal *et al.* 1998]. Each of those bacterial groups requires a different environment for optimum growth, and therefore constructed wetlands should be designed in such a way as to ensure suitable conditions for correct run of the particular processes of nitrogen removal [Platze 1999].

According to Benham and Mote [1999], the processes of nitrification and denitrification are the primary mechanisms of nitrogen removal in constructed wetlands. Vymazal [2005] claims that complete nitrification can only be achieved in vertical flow constructed wetland systems (VF-CW), while horizontal flow constructed wetlands (HF-CW) ensure better conditions for denitrification.

Initially, the constructed wetlands applied in the world had the form of single-stage soil-plant systems with horizontal or vertical flow, in which reed or willow were utilised. The systems ensured over 80% effectiveness in reducing BOD_5 and COD, while their efficiency in the removal of biogenic compounds usually did not exceed 50% [Haberl *et al.* 1995, Kowalik and Obarska-Pempkowiak 1998, Vymazal 2005].

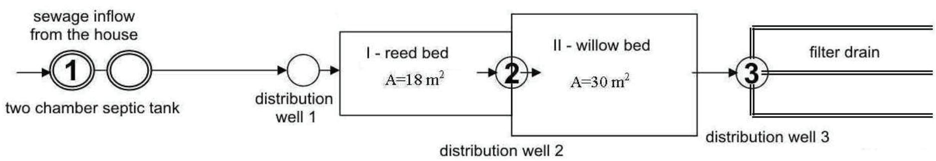
In recent years, however, it is recommended to use multi-stage constructed wetlands, so-called hybrid systems composed of two or three HF-CW and VF-CW beds that ensure better conditions for the removal of nitrogen and phosphorus from wastewaters [Luederitz *et al.* 2001, Obarska-Pempkowiak and Gajewska 2003, Arias *et al.* 2004, Krzanowski *et al.* 2005, Obarska-Pempkowiak 2005, Vymazal 2005].

The objective of this study was to analyse the numbers of ammonifying, nitrifying and denitrifying bacteria in sewage after the various stages of treatment in a multi-stage constructed wetland with vertical and horizontal flow (VF-HF). The study was undertaken as until now there is a scarcity of research in this field.

MATERIAL AND METHODS

Characterisation of the analysed object. The study was conducted in a constructed wetland located in the village of Janów near Garbów, Province of Lublin. The system is used for the neutralisation of sewage from a three-person household ($Q = 0{,}45\ m^3 \cdot d^{-1}$). In the first part of the system, sewage is purified mechanically in a two-chamber settler with a total active volume of 8.5 m^3. The second element of the constructed wetland is a system of two beds: 1 – with vertical flow and common reed *Phragmites australis* Cav. Trin. Ex Steud. (bed with an area of 18 m^2 and depth of 0.8 m), 2 – with horizontal flow and common osier *Salix viminalis* L. (bed with an area of 30 m^2 and depth of 1.2 m) (Fig. 1).

The slope of the bottom of the beds is 1% in the outflow direction. The material used in the beds is gravel (d = 5.0–10.0 mm) and coarse sand (d = 1.0–2.0 mm). The beds are isolated from the natural ground by means of hydroinsulating geomembrane PEHD with thickness of 1 mm. Sewage is supplied to the beds periodically through the use of a submerged pump located in the second camber of the preliminary settler. This permits pulsed dosage of sewage to the reed bed, at the rate of ca. 0.225 m^3, with frequency of twice a day. The hydraulic load on the reed bed is 25 $dm^3 \cdot m^{-2} \cdot d^{-1}$, i.e. 25 $mm \cdot m^{-2} \cdot d^{-1}$. Treated sewage is drained off to the ground by means of filtering drainage overgrown with *Miscanthus giganteus* [Jóźwiakowski and Goral 2007].



1, 2, 3 – sampling points

Fig. 1. Technological schematic of multi-stage constructed wetland [Jóźwiakowski and Goral 2007]

Methods. Samples of raw sewage (1) and of treated sewage flowing out of the soil-plant beds with reed (2) and willow (3) were taken for analyses in February, May, August and November, 2009 (Fig. 1). The samples were used for the determination of the numbers of ammonifying bacteria, bacteria nitrifying to phase I (oxidising $N\text{-}NH_4^+$ to $N\text{-}NO_2^-$) and II (oxidising $N\text{-}NO_2^-$ to $N\text{-}NO_3^-$), and denitrifying bacteria (reducing $N\text{-}NO_3^-$ to $N\text{-}NO_2^-$), with the index method. The most probable number (MPN) of those bacteria was read from the Mc Crady tables, based on the calculus of probability. The numbers of ammonifying bacteria were determined with the test tube method acc. to standard PN-75 C-04615/18, of autotrophic nitrifying bacteria – with the method of culturing in liquid media acc. to standard PN-77 C-04615/20, and of denitrifying bacteria – with the test tube method acc. to standard PN-75 C-04615/19. Other parameters determined in the sewage samples included also pH, concentration of O_2, total suspended solids, levels of BOD_5 and COD, as well as concentrations of total nitrogen and phosphorus, $N\text{-}NH_4^+$, $N\text{-}NO_3^-$, $N\text{-}NO_2^-$. Those analyses were made following the commonly applied methods [Hermanowicz *et al.* 1999]. The bacteriological analyses of the sewage samples were made at the Faculty of Agricultural Microbiology, and the physicochemical ones at the Water and Sewages Analytics Laboratory, Department of Melioration and Agricultural Construction, University of Life Sciences in Lublin.

RESULTS AND DISCUSSION

Figures 2, 3 and 4 present the mean values of indices and components of pollution in the sewage at various stages of treatment in the studied constructed wetland in 2009, as a background for analysis of the research results. Whereas, Table 1 presents the minimum, maximum and mean values of temperature, concentration of dissolved oxygen and ranges of pH in the studied sewage.

Ammonifying bacteria. Ammonification is the process of biochemical transformation of organic nitrogen into ammonia or into ammonium ions by heterotrophic bacteria. The process can proceed in aerobic as well as in anaerobic conditions. In household sewage treatment installations ammonification usually takes place in the preliminary settlers [Washington State Department of Health 2005].

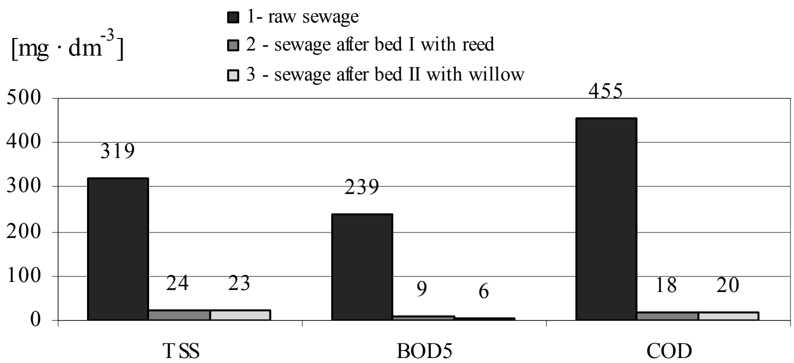


Fig. 2. Mean content of total suspended solids and levels of BOD5 and COD in the sewage at the particular stages of purification

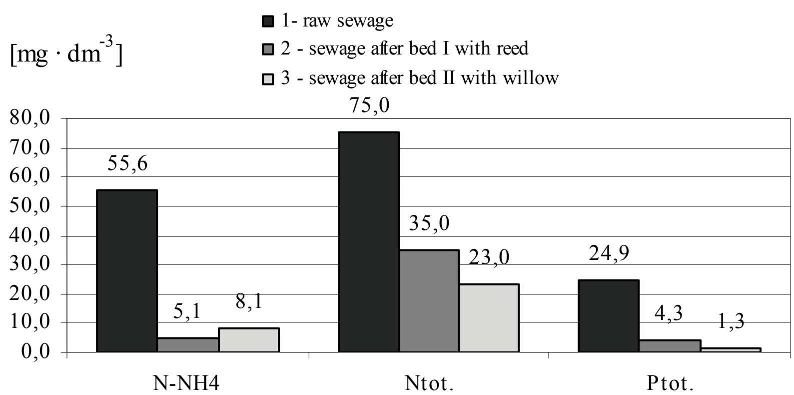


Fig. 3. Mean concentrations of $N\text{-}NH_4^+$, N_{total} and P_{total} in the sewage at the particular stages of purification

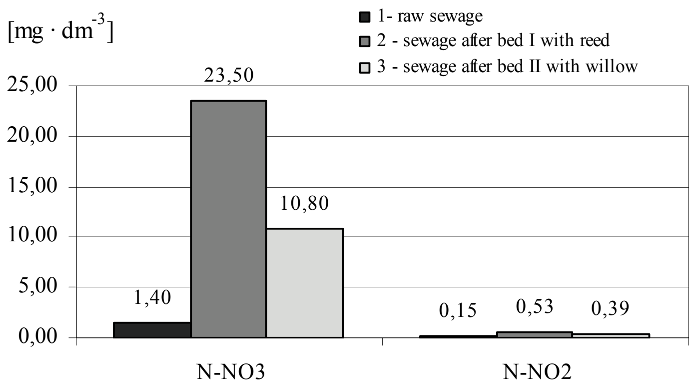


Fig. 4. Mean concentrations of $N\text{-}NO_3^-$ and $N\text{-}NO_2^-$ in the sewage at the particular stages of purification

Table 1. Temperature, concentration of dissolved oxygen and pH in the sewage at the particular stages of purification in the multi-stage constructed wetland in 2009

Kind of sewage	1 – raw sewage			2 – sewage after bed I with reed			3 – sewage after bed II with willow		
Parameters studied	min.	max.	x	min.	max.	x	min.	max.	x
Sewage temp., °C	12.8	20.6	16.0	12.2	19.9	15.5	11.1	19.6	15.0
Concentration of O_2, mg·dm^{-3}	0.33	0.59	0.42	2.15	3.28	2.82	2.20	2.99	2.55
pH	7.18	7.73	-	6.73	7.39	-	6.92	7.05	-

The great diversity of heterotrophic bacteria permits their easy adaptation to growth in a given environment, which accounts for their large numbers in raw sewage [Filipkowska and Pesta 2003]. Our study shows that also in the constructed wetland under analysis the highest numbers of ammonifying bacteria were recorded in raw sewage – varying from 2500 to 250000 MPN/1 ml, with a mean value of 98125 MPN/1 ml (Tab. 2). Also the concentration of ammonium nitrogen (55.6 mg·dm^{-3}) and those of other pollutants were the highest in the raw sewage (Fig. 2, 3, 4). The lowest numbers of ammonifying bacteria, in the range of 450–25000 MPN/1 ml (mean of 10350 MPN/1 ml), were obtained in the sewage after bed I with reed (Tab. 2). This indicates that the conditions in that bed permitted effective reduction of the numbers of those bacteria – on average by 85%. An only slightly lower reduction of ammonifying bacteria (72–81%) was observed by Filipkowska and Pesta [2003] in reed beds with vertical flow in a constructed wetland in Łęguty.

Nitrifying bacteria. Bacteria participating in phase I of the process of nitrification include *Nitrosomonas*, *Nitrosococcus*, *Nitrosolobus*, *Nitrosospira* and *Nitrosovibrio,* while phase II of the process involves the participation of *Nitrobacter*, *Nitrococcus* and *Nitrospira* [Gallert and Winter 2005]. The effectiveness

Table 2. Most probable number of ammonifying bacteria in 1 ml of sewage in 2009

Sample No.	Kind of sewage	Min.	Max.	x	σ
1	Raw sewage	2500	250000	98125	108077
2	Sewage after bed I with reed	450	25000	10350	11869
3	Sewage after bed II with willow	950	45000	14488	20679

min. – minimum value, max. – maximum value, x – mean value, σ – standard deviation

of the process of nitrification is determined primarily by such factors as temperature, pH and concentration of dissolved oxygen, as well as the load of organic pollutants, presence and concentration of toxic substances, and the concentration of nitrogen in the sewage flowing into the constructed wetland [Bernacka *et al.* 1995].

In the raw sewage entering the constructed wetland under analysis the value of pH oscillated at the level of 7.18 to 7.73 pH, and the concentration of dissolved oxygen varied from 0.33 to 0.59 $mg \cdot dm^{-3}$ (Tab. 1). For the process of nitrogen oxidation to proceed undisturbed, the minimum concentration of oxygen dissolved in the environment should be 1–2 mg $O_2 \cdot dm^{-3}$. Increased concentration of dissolved oxygen in wastewaters results in a more complete process of nitrification [Bernacka *et al.* 1995].

In this study, in the raw sewage accumulated in the preliminary settler low numbers of nitrifying bacteria of phases I and II were noted – on average 6.6 and 1.4 MPN/1 ml, respectively (Tab. 3). This was probably due to the low concentration of dissolved oxygen and to the high level of $N\text{-}NH_4^+$ (Fig. 3) that could be toxic to their growth. This is in line with observations by Bernacka *et al.* [1995] who report that high concentration of $N\text{-}NH_4^+$ in sewage may be toxic to nitrifying bacteria and inhibit their growth.

Table 3. Most probable number of nitrifying bacteria of phases I and II in 1 ml of sewage

Sample No.	Kind of sewage	Phase I				Phase II			
		min.	max.	x	σ	min.	max.	x	σ
1	Raw sewage	0.4	25	6.6	12	0.4	4	1.4	2
2	After bed I with reed	1.5	250	64.6	124	0.9	95	25.1	47
3	After bed II with willow	0.4	25	8.1	11	0.4	25	7.2	12

min. – minimum value, max – maximum value, x – mean value, σ – standard deviation

According to Brix [1990], reed plays a highly important role in soil-plant beds with vertical flow as it has the air tissue, aerenchyma, consisting of intercellular spaces through which diffusive flow of oxygen from the aboveground parts of plants to the roots and rhizomes takes place. Since they release some of the oxygen, aerobic zones are created that stimulate the decomposition of organic matter, but also stimulate the growth of nitrifying bacteria.

The study conducted in the constructed wetland under analysis showed that the highest mean number of nitrifying bacteria of phases I and II – 64.6 and 25.1 MPN/1 ml, respectively (Tab. 3) and elevated concentrations of $N\text{-}NO_3^-$

(23.5 mg·dm^{-3}) and N-NO_2^- (0.53 mg·dm^{-3}) (Fig. 4) were recorded in sewage flowing out from bed I with reed. That sewage also had a low content of N-NH_4^+ – 5.1 mg·dm^{-3} (Fig. 3), and the concentration of dissolved oxygen varied from 2.15 to 3.28 mg·dm^{-3} (Tab. 1). The results indicate that the conditions in bed I with reed permitted an effective process of nitrification, and consequently also efficient elimination of N-NH_4^+ in 90.8%. Lower effects of ammonium nitrogen elimination (75,9%) were observed by Filipkowska and Pesta [2003] in reed beds with vertical flow in a constructed wetland in Łęguty.

Denitrifying bacteria. Denitrifying bacteria play a key role in the removal of nitrogen compounds from wastewaters [Knowles 1982]. Also according to Machnicka *et al.* [2004] only denitrification can result in total elimination of nitrogen from ecosystems.

Denitrifying bacteria can be easily isolated from sediments, soil, and aquatic environments. Bacteria from the genus *Pseudomonas* are commonly believed to be the dominant microorganisms, thanks to which intensive denitrification can proceed [Lazarova *et al.* 1992, Janda *et al.* 1998]. Studies by other authors showed that also such species as *Achromobacter, Agrobacterium, Alcaligenes, Bacillus, Chromobacterium, Flavobacterium, Hyphomicrobium* and *Pseudomonas* are responsible for the process of denitrification in soil [Zumft 1992, Otlanabo 1993, Chčneby *et al.* 2000]. Therefore it appears unlikely that *Pseudomonas* is the species primarily responsible for denitrification that takes place in such unbelievably diverse microbial communities as those existing in sewage treatment plants [Lim *et al.* 2005].

According to Bernacka *et al.* [1995], the process of denitrification, apart from a suitable bacterial mass, requires the presence of nitrates in the sewage, as well as a source of energy in the form of organic matter. The optimum range of pH for the process is 6.5–7.5. The process gets rapidly inhibited when pH drops below 6 or when its value exceeds 8. Denitrification proceeds the fastest at temperature of 20°C. Further increase of temperature does not accelerate the process, and its decrease causes its inhibition only at 5°C. he presence of oxygen has an inhibiting effect on the process of denitrification, therefore oxygen concentration in the environment should not exceed 0.5 mg O_2·dm^{-3}.

The study conducted in the multi-stage constructed wetland in 2009 shows that the greatest numbers of denitrifying bacteria reducing N-NO_3^- to N-NO_2^- were noted in raw sewage accumulated in the preliminary settler (450–25000 MPN/1 ml) (Tab. 4), where there were favourable conditions for their growth – mean concentration of dissolved oxygen in raw sewage was 0.42 mg O_2·dm^{-3}, pH varied within the range of 7.18–7.73, and temperature oscillated within the range of 12.8–20.6°C (Tab. 1).

Notably lower numbers of denitrifying bacteria were recorded in sewage flowing out of bed I with reed (45–9500 MPN/1 ml) and from bed II with willow (45–2500 MPN/1 ml). Although the reaction of sewage flowing out of those beds fell within the acceptable range given by Bernacka *et al.* [1995] – it varied from

Table 4. Most probable number of denitrifying bacteria (reducing $N\text{-}NO_3^-$ to $N\text{-}NO_2^-$) in 1 ml of sewage

Sample No.	Kind of sewage	Min.	Max.	x	σ
1	Raw sewage	450	25000	7600	11756
2	After bed I with reed	45	9500	2561	4629
3	After bed II with willow	45	2500	949	1102

min. – minimum value, max – maximum value, x – mean value, σ – standard deviation

6.77 to 7.39 pH, and temperature did not drop below 11°C, but probably the oxygen conditions in beds I and II caused a reduction in the numbers of denitrifying bacteria in sewage flowing out of those beds. This may be indicated by dissolved oxygen concentrations in sewage after bed I with reed (2.15–3.28 mg $O_2 \cdot dm^{-3}$) and after bed II with willow (2.20–2.99 mg $O_2 \cdot dm^{-3}$) (Tab. 1).

Haberl *et al.* [1995] and Seo *et al.* [2010] maintain that beds of the type of HF-CW (with willow) can be successfully used in multi-stage hybrid systems as denitrifying beds for the removal of nitrates remaining after the nitrifying bed of the type of VF-CW (with reed). In the studied multi-stage constructed wetland, in sewage flowing out of bed II with willow *Salix viminalis* L. a drop in the concentration of $N\text{-}NO_3^-$ from 23.8 to 10.8 $mg \cdot dm^{-3}$ was observed (Fig. 4), which gives a mean removal efficiency at the level of 54.6%. A similar result – 58.9% was obtained by Seo *et al.* [2010] who studied the possibility of $N\text{-}NO_3^-$ removal by three willow species: *Salix gracilistyla, Salix chaenomeloides, Salix koreensis*.

The results obtained for the constructed wetland under analysis reveal (Fig. 3) that the mean efficiency of nitrogen elimination in bed II with willow is 34.3%. Mayo and Bigambo [2005] showed in a model study that in beds of the HF-CW type the efficiency of elimination of total nitrogen is 48.9%, of which the process of denitrification permits for permanent removal of total nitrogen at the level of 29.9%, another 10.2% of nitrogen is taken up by plants, and 8.2% is neutralised through the process of sedimentation.

Over the last dozen years or so the presence of the process of denitrification in sewage treatment has assumed a special significance, as $N\text{-}NO_3^-$ and $N\text{-}NO_2^-$ dumped with sewage are highly dangerous to human health [Kempster *et al.* 1997] and contribute to the eutrophisation of waters [Gray 1990]. Therefore, there is a growing preference for the construction of sewage treatment systems involving both nitrification and denitrification that permit highly efficient removal of nitrogen compounds from wastewaters.

In the multi-stage constructed wetland analysed in this study the average efficiency of elimination of total nitrogen in 2009 was 69.3%. Similar results of nitrogen removal were obtained in constructed wetlands of the VF-HF type in South Korea – 68% [Seo *et al.* 2009] and in Poland – 63–71% [Krzanowski *et al.* 2005].

CONCLUSIONS

1. The largest numbers of ammonifying bacteria and denitrifying bacteria reducing $N\text{-}NO_3^-$ to $N\text{-}NO_2^-$ were noted in raw sewage. At the particular stages of treatment a decrease was observed in the numbers of those bacteria, which indicates correct operation of the constructed wetland under analysis.

2. The highest numbers of phase I and II nitrifying bacteria and an increase in the concentration of $N\text{-}NO_3^-$ and $N\text{-}NO_2^-$ were observed in sewage flowing out of bed I with reed, which indicates effectiveness of the process nitrification in that bed. The lowest numbers of nitrifying bacteria were noted in raw sewage, which was probably due to low concentration of dissolved oxygen – below 0.5 mg $O_2 \cdot dm^{-3}$, and to high levels of $N\text{-}NH_4^+$ which could be toxic to their growth.

3. The efficient processes of nitrification (in bed I with reed) and denitrification (in bed II with willow) contributed to the nearly 70% effectiveness of removal of total nitrogen in the constructed wetland system under analysis.

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LICZEBNOŚCI WYBRANYCH GRUP FIZJOLOGICZNYCH BAKTERII W ŚCIEKACH BYTOWYCH PO RÓŻNYCH ETAPACH OCZYSZCZANIA W WIELOSTOPNIOWEJ OCZYSZCZALNI HYDROFITOWEJ

Streszczenie. W pracy przedstawiono analizę liczebności wybranych grup fizjologicznych bakterii w ściekach po różnych etapach oczyszczania w wielostopniowej hydrofitowej oczyszczalni z pionowym i poziomym przepływem ścieków (VF-HF) z trzciną i wierzbą. Badania wykonywano w 2009 r. Największą liczebność bakterii amonifikacyjnych i denitryfikacyjnych zanotowano w ściekach surowych. Na poszczególnych etapach oczyszczania stwierdzono zmniejszanie się liczby tych bakterii, co wskazuje na prawidłową pracę analizowanej oczyszczalni. Największą liczebność bakterii nitryfikacyjnych I i II fazy oraz wzrost stężenia $N\text{-}NO_3^-$ i $N\text{-}NO_2^-$ zaobserwowano w ściekach odpływających z I złoża z trzciną, co świadczy o skutecznym przebiegu procesu nitryfikacji w tym złożu. Najmniejszą liczbę bakterii nitryfikacyjnych stwierdzono w ściekach surowych, co było prawdopodobnie spowodowane przez niewielkie stężenie tlenu rozpuszczonego oraz wysoką zawartość $N\text{-}NH_4^+$, który mógł być toksyczny dla ich rozwoju. Skuteczny przebieg procesów nitryfikacji w I złożu z trzciną i denitryfikacji w II złożu z wierzbą przyczynił się do prawie 70% efektywności usuwania azotu całkowitego w badanej oczyszczalni ścieków.

Słowa kluczowe: bakterie, azot, amonifikacja, nitryfikacja, denitryfkacja, ścieki, oczyszczalnia hydrofitowa