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DEPTH DISTRIBUTION OF BACTERIAL DIVERSITY IN LAB SCALE UP FLOW CONSTRUCTED WETLANDS

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Abstract
This paper presents the results of research preformed on the biodiversity of bacteria at different depths of lab-scale constructed wetlands (CWs) systems loaded with water simulating electroplating wastewater. The bacterial biocenosis in two constructed wetland systems with different organic or mineral bed media was investigated. Samples were taken from the two columns to the metered synthetic galvanic wastewater, which included metals such as lead, nickel, zinc, copper and lactates, and cyanides. Samples were taken after 53 weeks of the experiment. The biodiversity analysis was performed on the basis of distribution of DNA fragments coding for bacterial 16S rRNA by denaturing gradient gel electrophoresis. Based on the bioinformatic analysis of the obtained fingerprints Shannon biodiversity index was calculated to estimate species diversity, and Jaccard index was determined, which allowed comparison of two communities. The study indicated that the two communities were significantly different, with a higher diversity in the column with a peat mixture

Streszczenie
W artykule przedstawiono wyniki badań dotyczących bioróżnorodności bakterii na różnych głębokościach wypełnienia złóż hydrofitowych wykorzystywanych w celu oczyszczania ścieków. Badano biocenozę bakteryjną dwóch złóż o wypełnieniu organicznym oraz mineralnym. Próbki pobrano z dwóch kolumn, do których dozowano syntetyczne ścieki galwanizerskie, w skład których wchodziły metale, takie jak: ołów, nikiel, cynk, miedź oraz mleczany, a także cyjanki. Próbki pobrano po 53 tygodniach pracy złoża.Analizę bioróżnorodności wykonano na podstawie wyników rozdziału fragmentów DNA kodujących bakteryjne 16S rRNA za pomocą elektroforezy w gradiencie czynnika denaturującego. Na podstawie analizy bioinformatycznej uzyskanego wzoru prążkowego (tzw. fingerprintu) wyznaczono współczynnik bioróżnorodności Shannona, stosowany do szacowania różnorodności gatunkowej, a także Indeks Jaccarda umożliwiający porównanie dwóch zbiorowisk bakterii. Uzyskane wyniki wykazały, że w kolumnie z wypełnieniem organicznym (torfem) bioróżnorodność bakterii jest większa w porównaniu z kolumną z wypełnienieem mineralnym.

Keywords: **Constructed wetlands; Biodiversity; DGGE.**

1. INTRODUCTION

Constructed wetlands (CWs) are specified as a halfway between aquatic and terrestrial ecosystem which exhibits some of the characteristics of each [1]. The proven water purification ability of wetlands has encouraged scientists and engineers to construct these systems to take advantage of this ability. Therefore, constructed wetlands have become commonly used as a cost-effective alternatives or useful additions to traditional wastewater treatment systems [2]. Research conducted over the years has shown that wastewater treatment in CWs can be effective for both municipal and industrial wastewater.

There are three types of CWs based on flow direction and position of water surface: systems with horizontal subsurface flow, with vertical subsurface flow and with surface flow. In constructed wetlands with vertical subsurface flow the wastewater can flow from the top to the bottom of the filter bed (system down-flow) or from the bottom to the top of the filter bed (system up-flow). Surface flow CWs are most similar to natural wetlands. These systems are characterized by the presence of water flowing over a layer of soil in which vegetation might be rooted [2].

CWs with subsurface flow are covered by marsh vegetation, and wastewater flows horizontally or vertically through a porous filter material (usually sand or gravel). Physical-chemical processes associated with the removal of contaminants such as: filtration, sedimentation, adsorption, evaporation, and the plant-induced processes are relatively well known [3]. However, the activity of the microorganisms present in the system exhibit great influence on wastewater treatment. Many important processes in wastewater treatment wetland systems are dependent on microorganisms. Although, the microbiological processes play an important role in the degradation of organic substances, transformation of nitrogen, phosphorus or sulfur compounds, little research has focused on the effect of microorganisms on the removal of pollutants in the wetland systems [4-8].

Molecular techniques are a modern tool used in the analysis of microorganisms responsible for the remediation process in wetland systems. Ibekwe et al. [9] analyzed the microbial biocenosis by denaturing gradient gel electrophoresis (DGGE) in two wetland systems used for the treatment of wastewater from dairy industry. In the study conducted by Babtiste et al. [10] fluorescence in situ hybridization (FISH) was used to assess the number of bacteria in two CWs with subsurface horizontal flow. DNA extraction and subsequent cloning was used to determine the bacteria species present in the CWs with subsurface horizontal flow. The studies demonstrated the presence of bacteria *Methylomicrobium* sp. and bacteria performing heterotrophic nitrification (*Alcaligenes*) in the outflow of the system. Most of the obtained

Figure 1.
The photo of lab-scale constructed wetlands

sequences belonged to unclassified taxa, while the second dominant group consisted of members of the phylum *Proteobacteria* [11]. In other studies, the use of cloning demonstrated the presence of bacteria such as *Desulfovibrio* and magnetic sulfate-reducing bacteria in sediments [13]. Reduction of sulphate carried by the participation of bacteria may be accompanied by abiotic reduction of metal, resulting in occurance of precipitation of the metal sulfides, which is particularly important in the case, where the wastewater containing a high concentration of metals such as iron, zinc, copper, nickel, cadmium, mercury, lead [13] is fed into the system. Also, some semi-metals can be precipitated in the bed in the form of sulfides, for example with the involvement of sulphate-reducing bacteria the reduction of arsenic (V) may occur, whereby the sulfate arsenic (As_2S_3) is precipitated [14]. Ability to grow even at high concentrations of metal, occurs in many microorganisms. This may be a result of acquired and congenital mechanisms, as well as environmental result from metal modification. Resistance may be also caused by the production of metallothioneins [15]. Despite a small demand for some elements, including the heavy metals, microorganisms in significant quantities fetch them into the cell. This phenomenon leads to the intracellular accumulation of metal cations, and is called bioaccumulation [16]. These processes can be helpful for treatment of electroplating wastewater, which contain increased amounts of metals. Summing up information the process parameters such as the type of bed, operating parameters (volume, wastewater retention time) and the type of wastewater with biodiversity and spatial distribution of microorganisms in the bed allows efficient optimization of treatment, which let obtain satisfactory effects of wastewater treatment [19].

The aim of the presented study was to get a detailed description of the microbial biocenosis et different depths in planted subsurface vertical flow to the constructed wetlands with organic or mineral bed media using molecular techniques.

2. METHODS

Experimental system

The lab-scale system of the constructed wetlands consisted of 12 columns, of which two were used for the analyses presented in the study. The lab-scale system is shown in Figure 1.

Each column of the experimental system was 80 cm high, with diameter of 20 cm, filled with mineral or organic media up to 63.5 cm, which was quartz gravel (3-8 mm) or peat (Table 1), respectively. The properties of the peat were: organic matter content of 89.9%, water capacity 746 ml/l, pH 3.5- 4.5. Both columns were fed with wastewater from the bottom of the reactor. The system was operated in the indoor conditions. After 53 weeks of operation several samples were taken from different depths of the column. Table 1 shows the depths from which samples were taken from the tested CWs.

Table 1.
Description of the samples taken from the experimental system

Column	Depth of sampling	Sample name	Bed type
1	- 10 cm	1_10	quartz gravel mixed with peat
	- 20 cm	1_20	
	- 30 cm	1_30	
	- 40 cm	1_40	
	- 50 cm	1_50	
2	- 10 cm	2_10	quartz gravel
	- 20 cm	2_20	
	- 30 cm	2_30	
	- 40 cm	2_40	
	- 50 cm	2_50	

The columns were planted with *Phragmites australis* (common reed) and *Phalaris arundinacea* (Reed canarygrass). The system was used to treat synthetic medium, emulating pretreated electroplating wastewater. The wastewater contained salts of metals: lead ($Pb(NO_3)_2$, 1 mg/l), nickel ($NiSO_4*6H_2O$, 5 mg/l), zinc ($ZnCl_2$, 5 mg/l), copper ($CuSO_4*5H_2O$, 5 mg/l) and sulfates (Na_2SO_4, 500 mg/l). Since week 44 of operation lactate was added to wastewater feeding column 2 (as 80% lactic acid, at a ratio of COD: SO_4 = 0.3) in order to increase the availability of organic carbon used by the sulfate-reducing bacteria. Since week 48 of operation the wastewater feeding the columns was enriched with cyanide (added as KCN, 2 mg/l). Lactate was added to the wastewater dispensed to the mineral-filled column in order to increase the availability of organic carbon used by the sulfate-reducing bacteria as a final electron acceptor. Because the wastewater from the electroplating plant contain cyanide, so in the final stage of the research cyanide was added to wastewater.

DNA extraction and amplification

DNA isolation was performed by mechanical method [18] using a kit Clean UP (A&A Biotechnology) for DNA purification. For 0.1 g of soil sample was used DNA extraction. Each extraction was carried out once. For amplification of the sequence of the gene encoding the bacterial 16S rRNA PCR was performed using primers 338f (5'-GCC TAC GGG AGG CAG CAG -3') and 518r (5'-ATT ACC GCG GCT GCT GG-3'). A 40-base GC clamp was attached to the 5' end of the primer 338f. DNA amplification was carried out under the following conditions: (1) initial denaturation (95°C, 10 minutes), (2) 30 cycles, each consisting of a single denaturation (95°C, 1 min), annealing (53°C, 1 minute), elongation (72°C, 2 minutes). The product was evaluated on an agarose gel (0.8% w/vol agarose, 1xTAE). Visualization of the product was made using UV light transluminator (Bio Rad Universal Hood IT).

The results of DGGE

DGGE was performed using a DCode$_{TM}$ Universal Mutation Detection System (Bio-Rad, Richmond, CA)., based on the protocol of Ziembińska et al. [18]. About 400 ng of PCR products were loaded onto 8% (w/vol) polyacrylamide (37.5:1) gel in 1×TAE. The denaturing gradients ranging from 30% to 60% (where 100% denaturant contains 7M urea and 40% formamide). The electrophoresis was run at constant conditions of 60°C and 45V for 17h. The stained gels were immediately photographed on a UV transillumination table with a Video Camera Module (Bio-Rad, USA). Bioinformatic analysis of the DGGE gel was performed using GelCompare (Applied Maths). Shannon and Jaccard indices were calculated. Shannon index was calculated on the basis of the amount and intensity of the bands on the gel. The intensity of the bands is reflected by peak heights in the densitometric curve [18]. Shannon index (H') was calculated according to following formula:

$$H' = \Sigma(n_i/N)\ln(n_i/N) \quad (1)$$

where: n_i – the height of the peak, N – the sum of all peak heights of the densitometric curve.

Jaccard index (JI) was used to determine the similarity of the communities from different samples and was calculated as follows:

$$J = 100\ (c/[a+bc]) \quad (2)$$

where: a – number of bands of sample A, b – number of bands of sample B, and c – number of bands that were present both in sample A and B.

3. RESULTS AND DISCUSSION

Using GelCompar, a software program capable of similarity/dissimilarity calculation, achieved the DGGE fingerprints of biodiversity of bacteria at different depths of lab-scale constructed wetlands (CWs) systems used for electroplating wastewater treatment. Figure 2 shows the gel image of PCR products separated by DGGE.

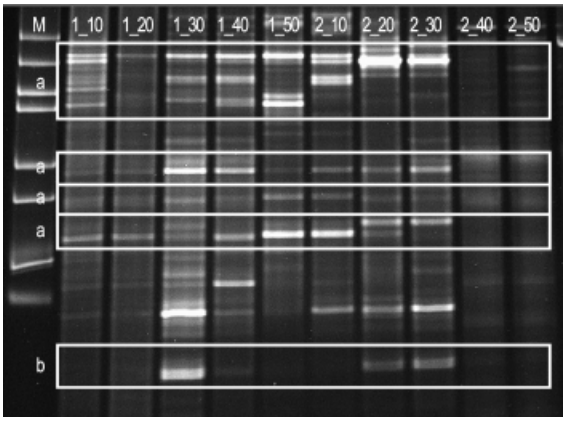

Figure 2.
DGGE profiles of PCR-amplified bacterial 16S rDNA gene in samples from two up flow wetlands composed of gravel+peat (1) or gravel (2) and five different depths (10, 20, 30, 40 and 50 centimeters from the top (a: DNA fragments with more pairs AT, b: DNA fragments with more pairs GC)

Among a number of bands visible in each fingerprint there were some bright bands observable for all or majority of samples (a), but bands present only in samples taken from the middle of the columns (depth of 20-30 cm); b) can be as well distinguished. It can by concluded that in the samples there were less fragments with more GC (three hydrogen bonds, usually located in the lower part of a gel) than fragments with more AT (two hydrogen bonds, usually located in the upper part of a gel) pairs. Fingerprints of samples taken from the bottom of column 2 (2_40 and 2_50) contain few distinct bands that may be a result of a presence of many inhibiting substances. Figure 3 presents Shannon index values obtained for samples taken from both columns. When the index has a value $H'<1$ bacterial community is characterized by low diversity, and when the index is in the range of 1-3 then distribution of individuals between species is uniform and there is no dominant species (fair biodiversity). If the index is >3 the diversity is high [19]. The H' values for column 1 (organic bed) were within the range of 2.8-3.2, which is associated with high biodiversity in the analyzed samples, while for column 2 (mineral bed) of 2.8-2.9 which means that in the diversity of bacteria was fair.

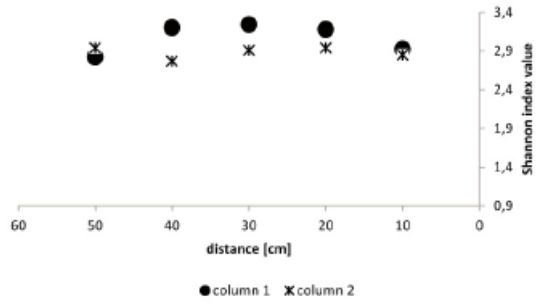

Figure 3.
Shannon index values for the samples in columns 1 and 2 (column 1: peat, column 2: gravel) collected from different layers in lab-scale constructed wetland (10, 20, 30, 40 and 50 centimeters from the top)

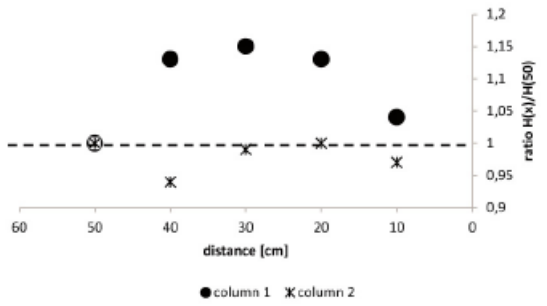

Figure 4.
Ratio of Shannon index in the selected sample (H (x)) and the sample situated closest to the inflow (H (50)) in samples collected from different depths in lab-scale constructed wetlands (10, 20, 30, 40 and 50 centimeters from the top)

Higher bacterial biodiversity was observed for organic column (column 1, filled with a mixture of peat and gravel) than in column with the mineral bed (column 2, filled with gravel). In column 1 Shannon index related to the depth of sampling presents a parabolic pattern with a maximum value at the level of 30 cm (Figure 3).

The lowest H' value was observed in the samples situated closest to the top and bottom of the column. The H' values at different levels of column 2 were similar but the highest value was observed for the level closest to the bottom of the column, thus to the wastewater inflow zone. The difference in the Shannon index values for column 1 and column 2 can result from the properties of the bed material. The bacteria in column 1 have had more available energy and carbon source throughout the experiment, However, in column 2 until the time of the experiment, feeding an additional source of carbon - lactate, which could have an effect on the bacteria in the column Moreover, probably in column with organic bed significant amounts of organic humus were present. Humic substances affect sorption ability of the wastewater constituents, including metals. It is hypothesized that close to the inflow the organic bed retain predominant proportion of wastewater contaminants in relation to the overall amount of removed contaminants. High concentration of the wastewater constituents in the lower part of the bed media may be toxic for bacteria and results in lower biodiversity. It can be assumed, that the mineral particles of the bed of column 2 did not retain the substances and the concentration gradient of contaminants along the column is smaller. Thus the biodiversity in the entire of column 2 remains at a similar level.

There was no tendency for increase of H' value with a proximity to the plants roots (top of the columns) thus no influence of plants (roots) presence was observed.

Higher similarities between certain samples were observed for column 1 (Figure 5).

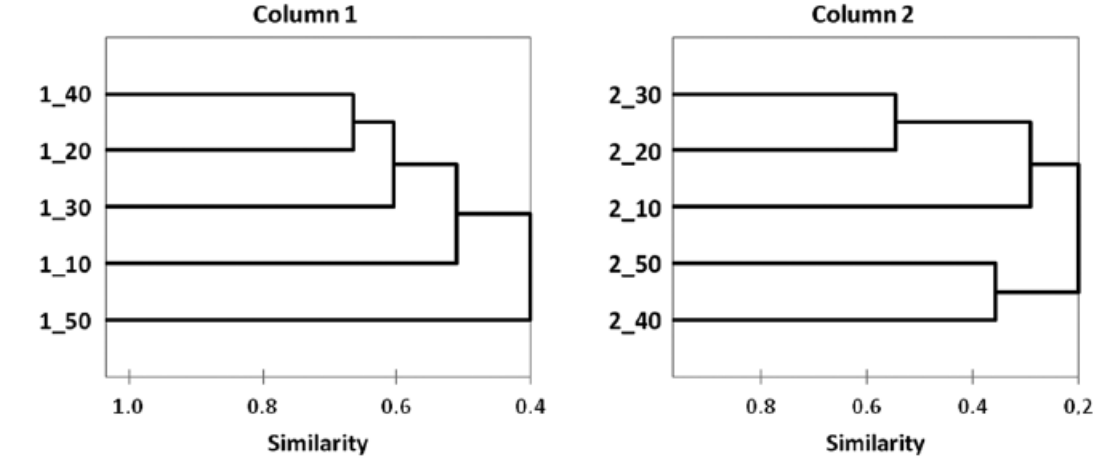

Figure 5.
Dendrograms showing similarity of bacterial 16S rDNA fingerprints for samples of column 1 (left) and column 2 (right)

The smallest similarity in column 1 occurred between samples taken from the -30 and -50 cm, while most similar bacterial communities in the samples were taken from the -20 and -40 cm In column 2, the greatest similarity was found in the samples taken from the -20 and -30 cm. The lowest value of the Jaccard's index was recoded in samples collected from the -10 and -40 cm.

Figure 6 presents the similarity of bacterial biocenosis of consecutive sample to the sample situated closest to the influent (-50 cm).

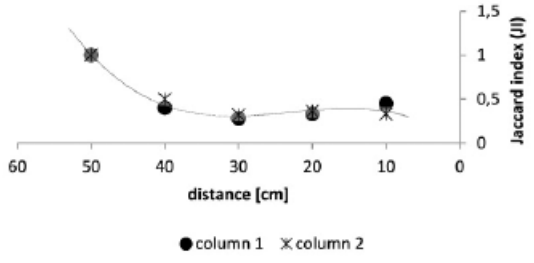

Figure 6.
Similarity of certain samples to the sample nearest the inflow made based on bioinformatic analysis in GelCompare, column 1 with organic media, and column 2 with mineral media

As shown in Figure 6 similar trend in a mineral and organic column can be observed. The JI values for the samples taken from the depths between -10 to -40 cm in comparison to the sample from depth -50 cm were within the range of 0.3-0.5. There samples were only 30-50% similar to the sample from the -50 cm. Jaccard index value indicates that 50% of bands (bacterial species) was unchangeable in the analyzed samples.

4. CONCLUSIONS

The bacterial 16S rRNA fingerprint was compared based the samples taken at different depths of two lab-scale CWs. Designated Shannon index of biodiversity indicates a greater biodiversity of bacteria at different depths in column 1 with organic filter bed. The greatest bacterial diversities were observed at the distance of 20-40 cm downward from the top of the column. In the column with mineral filter bed Shannon biodiversity index achieved similar values at different depths in lab-scale constructed wetlands. Moreover, the bacterial biocenosis located in the upper part of the columns (depth of 10-30 cm) was slightly different from the biocenosis located closer to the wastewater inlet both in column with organic and mineral filter bed.

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