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Regular paper

Qualitative variability in microbial community of constructed wetlands used for purifying wastewater contaminated with pharmaceutical substances*

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Pharmaceutical substances and their residues are increasingly present in the environment. Therefore, attempts at their removal are made by using different processes. Increasingly important among these processes are those modeled on natural phenomena which occur in wetland ecosystems, called technical scale constructed wetlands. Microbial degradation is an important process in these constructed wetlands. The biodegradation of chemicals often involves a complex series of biochemical reactions and usually varies with the microorganisms involved. The objectives of this study were to determine the impact of sulfamethoxazole and diclofenac on ammonia oxidizing bacteria and other parameters of wastewater in the microcosm of down-flow constructed wetlands. The Spearman correlation coefficient attained negative values in the case of comparison of the Shannon biodiversity index and the parameters of purified wastewater. This dependence was pronounced. In the case of pharmaceutical substances dosed with wastewater, the Spearman correlation coefficient assumed positive values. The highest value assumed by the Spearman correlation coefficient (0.9) was for the removal of diclofenac and Shannon index values for the planted columns, with a very high relationship. For unplanted columns, this value equaled 0.6. For sulfamethoxazole, the value for planted columns was 0.7, and for unplanted –0.7. The presence of plants did not have an impact on the Shannon biodiversity index.

Key words: bacterial biodiversity, constructed wetlands, pharmaceutical substances, PCR-DGGE

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INTRODUCTION

Constructed wetlands (CWs) are engineered systems, wherein the principle of operation imitates and intensifies the removal process occurring in natural wetlands. These systems are currently regarded as an alternative to more technically advanced solutions in wastewater treatment. In the early stages of development, constructed wetlands had been mainly used for domestic wastewater, which is still their most common use (Wu *et al.* 2014). The application and research on wastewater treatment in constructed wetlands has also included treatment of other types of wastewater, such as: electroplating wastewater (Sochacki *et al.* 2015), wastewater from the production of wine, from a cheese making factory, surface runoff from airports and leachate landfill (Miksch *et al.* 2015) and pharmaceutical substances. While the treatment performance in constructed wetlands is assigned to a variety of removal mechanisms, including filtration, adsorption, volatilization, sedimentation and plant uptake, it has been recognized that the removal of most pollutants in CWs is mainly due to microbial activity (Wu *et al.*, 2012; Cui *et al.*, 2013). The organic compounds are mineralized mainly by microorganisms, both under aerobic and anaerobic conditions. Nitrogen removal is assigned to microbial metabolism such as nitrification – denitrification, ammonification and anammox processes. Furthermore, the microbial community plays a vital role in sulfur transformations, and removal of heavy metals and phosphorous (Faulwetter *et al.*, 2009; Truu *et al.*, 2009; Knowles *et al.*, 2011; Saeed & Sun 2012). Ammonia oxidation is the first and rate-limiting step of nitrification that converts ammonium to nitrite in CWs (Pester *et al.*, 2012). Ammonia-oxidizing bacteria (AOB) are aerobic chemolithoautotrophic microbes. The specific molecular markers for AOB are functional genes carrying α subunit of the ammonia monooxygenase (amoA) gene and CTO region of 16S rRNA (Truu *et al.*, 2009). Based on the CTO region of the 16rRNA gene, a total of 15 different ammonia –oxidizing microbial communities can be distinguished. AOB populations belong to two phylogenetic groups: β-*Proteobacteria* (*Nitrosomonas* and *Nitrosospira* spp.) and γ-*Proteobacteria* (*Nitrosococcus oceani* and *Nitrosococcus halophilus*) (Faulwetter *et al.*, 2009). Molecular biology techniques are an effective tool in the assessment of the impact that environmental parameters have on the microbial community. PCR-DGGE, as a molecular method for rapid detection of microbial community changes or comparative analysis of environmental samples, offers more information about the distribution and composition of the microbial community. Dong & Reddy (2010) have used the PCR-DGGE technique to compare the Shannon diversity index and the richness between the influent and effluent of constructed wetlands treated with swine wastewater. These studies focused on the ammonia oxidizing bacteria due to the type of construct-

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Abbreviations: CWs, constructed wetlands; SMX, sulfamethoxazole; DCF, diclofenac; PCR-DGGE, polymerase chain reaction, denaturing gradient gel electrophoresis; AOB, ammonia oxidizing bacteria; P, planted columns; U, unplanted columns; Ph-P, planted columns fed with wastewater containing pharmaceutical substances; Ph-U, unplanted columns fed with wastewater containing pharmaceutical substances; COD, chemical oxygen demand; TKN, total Kjeldahl nitrogen; HPLC, high performance liquid chromatography

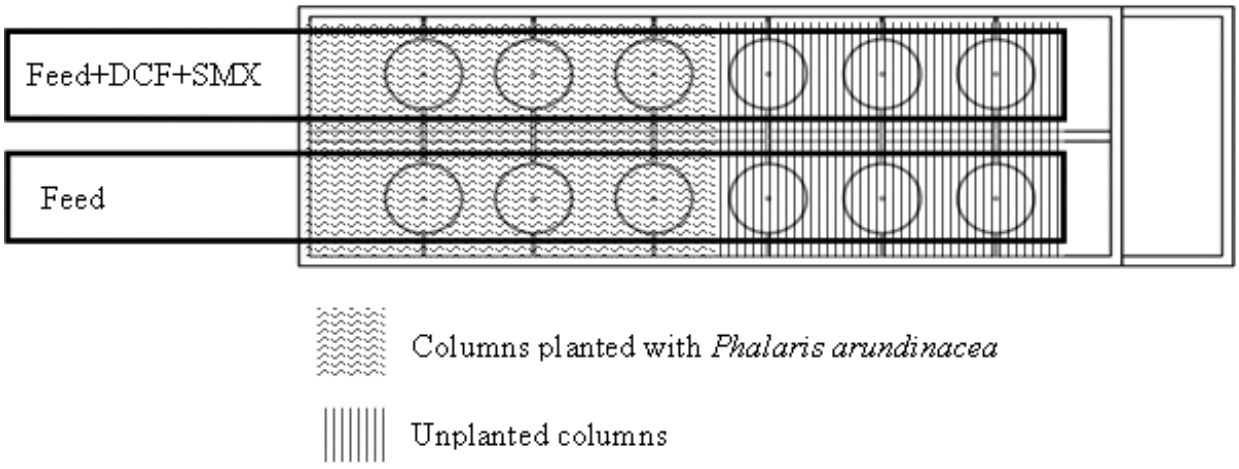

Figure 1. The scheme of the lab scale system used in the experiment

ed wetlands in this experiment (down-flow constructed wetlands), which is the most conducive system to processes of nitrification.

The aim of this research is the monitoring of the bacterial community in constructed wetlands and the assessment of the impact that pharmaceutical substances (sulfamethoxazole and diclofenac) and other parameters of wastewater have on the ammonia oxidizing bacteria (AOB).

MATERIALS AND METHODS

Sampling procedure. The experimental system used in this study and the results of physicochemical analyzes were described in Nowrotek *et al.* (2015). The samples for the analysis were taken in day 1, 27, 133, 151, 160, 182, 210, 271 of the experiment. In day 160, 182 and 210 of the experiment, wastewater containing pharmaceutical substances (sulfamethoxazole and diclofenac) were dosed into the system. The samples were taken from the top zone (about 10 cm deep) from planted and unplanted columns with *Phalaris arundinacea*. The samples were taken from 12 columns: planted columns (P, 3 replicates), unplanted columns (U, 3 replicates), planted columns fed with wastewater containing pharmaceutical substances (Ph-P, 3 replicates), unplanted columns fed with wastewater containing pharmaceutical substances (Ph-U, 3 replicates) (Fig. 1).

The constructed wetlands were fed with prepared synthetic municipal wastewater based on the protocol by Nopens *et al* (2001). The synthetic wastewater was prepared by using tap water flowing into the column that consisted of: urea (208.76 mg/L), yeast extract (264 mg/L), skim milk powder (118 mg/L), sodium acetate (510.4 mg/L), peptone (40 mg/L), KH_2PO_4 (41.37 mg/L), $KCr(SO_4)_2{\cdot}12H_2O$ (0.96 mg/L), $CuSO_4{\cdot}5H_2O$ (0.781 mg/L), $MnSO_4{\cdot}H_2O$ (0.108 mg/L), $NiSO_4{\cdot}7H_2O$ (0.359 mg/L), $PbCl_2$ (0.1 mg/L), $ZnCl_2$(0.208 mg/L), $MgSO_4{\cdot}7H_2O$ (4.408 mg/L), $FeSO_4{\cdot}7H_2O$ (11.6 mg/L). All columns were fed manually once a day with a 1 L batch of the wastewater. The wastewater was fed into the columns from Monday to Friday, on Saturday and Sunday the wastewater wasn't introduced into the columns. The experimental system was operated under laboratory conditions using high pressure sodium lighting system with various light/dark conditions changed seasonally: for summer 16 hours of light and 8 hours of dark (16/8), for fall 9/15, winter 8/16 and spring 14/10. The mean night and day temperature during the experiment was 23.1°C and 29.1°C, and the mean humidity was 59.3%. The biological samples after collection were frozen at –20°C for further procedure.

DNA isolation. DNA was isolated from samples (mixture of sand and soil) by a mechanical method. Before the isolation, 5 g of samples with 15 mL of 1PBS were shaken for 12 hours. Subsequently, 1.5 mL of extract from each sample was collected, and centrifuged for 1 min at 13000 rpm. The supernatant was decanted. Then, the sediments were washed three times with 1xPBS (Sigma), vortexed, centrifuged for 1 min at 13000 rpm, and the supernatant was decanted. For mechanical isolation, 300 mg of bead beating glass balls (Roth, Germany) and 1 mL of extraction buffer (100 mM Tris-HCl; 100 mM EDTA; 1.5 M NaCl; pH=8) were used. Then, the samples were incubated horizontally for 20 min with shaking at 1,400 rpm. Next, 200 μL of 10% SDS (Sigma) were added to the samples, and the samples were incubated for 30 min at 65°C, at 1400 rpm. After the incubation, samples were centrifuged twice for 10 min at 13000 rpm. The supernatant (0.5 mL) was placed onto silica col-

Table 1. Primers used in this study

Step	Primers			
	338f-GC/518r		CTO189f/CTO654r	
	Temperature (°C)	Time (min)	Temperature (°C)	Time (min)
Predenaturation	95	10	94	10
Denaturation x30, x34	95	1	94	1
Annealing	52	2	57	1
Elongation	72	2	72	2
Final elongation	72	12	72	12
References	Muyzer *et al.* 1993		Kowalchuk *et al.* 1997	

Table 2. PCR programs used in this study

Primer	Sequence (5′ to 3′)	Target	References
CTO189f	CCGCCGCGCGGCGGGCGGGGCGGGGGC ACGGGGGGACMAAAGYAGGGGATCG	I PCR round for 16S rRNA gene of ammonia oxidizing bacteria	Kowalchuk *et al*., 1997
CTO654R	CTAGCYTTGTAGTTTCAAACGC		
338f-GC*	TACCGGGAGGCAGCAG	I PCR round for 16S rRNA gene of ammonia oxidizing bacteria	Muyzer *et al*., 1993
518r	ATTACCGCGGCTGCTGG	16S rRNA gene for all bacteria	

* CG clamp: 5′ CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCGCC 3′

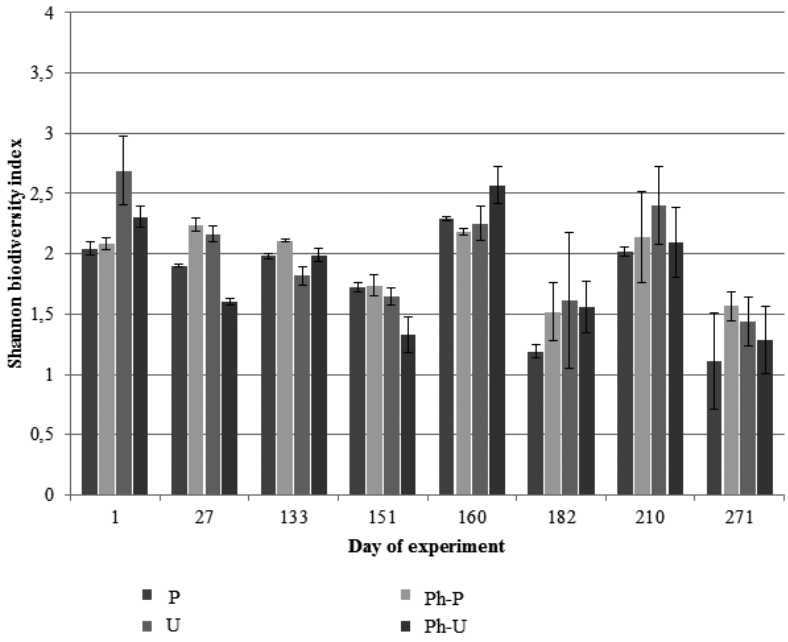

Figure 2. Shannon biodiversity index calculated on the basis of DGGE fingerprints for samples taken during experiment

umns (A&A Biotechnology), centrifuged and washed twice with A1 buffer (A&A Biotechnology). DNA was washed out from the columns with 30 μL of sterile MilliQ water and kept frozen until PCR.

PCR-DGGE analysis. The nested-PCR procedure was used in the study for AOB 16S rRNA gene amplification. In the first step, for amplification of the partial fragment of the gene encoding the bacterial 16S rRNA bacterial gene belonging to ammonia oxidizing β-proteobacteria was performed with the CTO189f/CTO654r primers. The second step was performed with the 338f-GC/518r primers. Primer sequences are presented in Table 1. Amplification programs are presented in Table 2.

The PCR mixture for first step of nested PCR contained: primers, 0.3 μL (5 pmol/μL), dNTPs 1.3 μL (20 pmol/μL, Promega), 1.2 μL $MgCl_2$ (2 mol/μL), 6 μL of 10× of reaction T*aq* buffer $MgCl_2$ free (Promega), 0.3 μL of T*aq* DNA Go Flexi polymerase (1.5 U, Promega), and 1 μL of the extracted DNA. In the mixture of the second step of nested PCR, 0.6 μL of amplicons from the first step were used. The final volume was 30 μL. PCR products were separated in 0.8% agarose (w/v, Promega) with ethidium bromide (10 mg/mL, Promega), in 1TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA; pH=8.3). For product size evaluation, 1 Kb Ladder (A&A Biotechnology) was used.

The PCR products were separated electrophoretically using DGGE *via* a Dcode™ universal mutation detection system (Bio-Rad Laboratories, USA). Samples were loaded onto 8% (w/v) polyacrylamide gels (acrylamide/*N,N*'-methylene bisacrylamide, 37.5/1, Fluka) in 30–60% gradient of urea, in 1TAE buffer (40 mM Tris, 20 mM glacial acetic acid, 1 mM EDTA; pH=8). The gels were run at 100 volts for 6 hours. After electrophoresis, the gels were stained with ethidium bromide (10 mg/mL, Promega) for 20 min, and then rinsed for 20 min in distilled water. Fingerprints were analyzed densitometrically with Quantity One 1D Software (BioRad) and the Shannon biodiversity index was calculated for all the samples.

Standard wastewater parameters. Chemical oxygen demand (COD) was determined photometrically using 1.14540.0001 and 1.14691.0001 Spectroquant COD Test purchased from Merck KGaA (Germany). Total Kjeldahl nitrogen (TKN) was determined using the distillation method in the Kjeltec 1026 system, and titration of boric acid solution containing Tashiro indicator (ISO 9001 certified). The concentrations of sulfamethoxazole (SMX) and diclofenac (DCF) were determined with high performance liquid chromatography (HPLC) in conjunction with the 3000 Ultimate system detector (Dionex Corporation, Sunnyvale, CA, USA) using a C18 Hypersil™ Gold column (250 mm × 4.6 mm; pore size: 5 μm) (Thermo Scientific, Polygen, Poland).

Statistical analysis. The Shapiro-Wilk W test was employed to test for normality of the data. To express correlation between the two variables, the Spearman correlation coefficient was calculated. Dependent groups that were of non-normally distributed data, were analyzed using the Wilcoxon matched pair test, and independent groups were analyzed using the Mann-Whitney U test. Differences were considered statistically significant if $p < 0.05$. Statistical testing was performed using the STATISTICA 10 software (StatSoft Inc., 2011).

RESULTS AND DISCUSSION

The AOB diversity of all samples was evaluated by DGGE analysis of the amplified partial 16S rRNA

Table 3. Influent and effluent concentrations (mg/L; average ± standard deviation) and removal efficiencies (%; average ± standard deviation) of the standard wastewater parameters, and SMX and DCF (L, average ± standard deviation)

Parameters		Influent	Effluent			
			Planted columns		Unplanted columns	
			Ph-P	P	Ph-U	U
COD	Concentration	840±448	47.3±16.5	46.5±17	46±23.6	43±19.5
	Removal		94.3±0.9	94.8±1.1	94.7±1.9	95±0.8
TKN	Concentration	150±13	14±4	15±5	21±2	16±3
	Removal		90.7±2.5	90.2±3.2	85.8±2.5	89.0±2.1
DCF	Concentration	5.924±2.629	2.017±0.690		2.081±0.564	
	Removal		63.47±14.28		61.63±13.22	
SMX	Concentration	6.243±2.353	3.391±7.078		3.223±0.934	
	Removal		42.01±22.24		44.64±20.32	

genes. The Shannon biodiversity index was determined on the basis of densitometry analysis. The results are presented in Fig. 2.

Analyzing the results, it can be seen that the Shannon biodiversity index decreased over time in all types of columns. The decrease of the Shannon index can by observed in planted and unplanted columns. This situation may be associated with the adaptation of the microbial community to the synthetic wastewater and laboratory conditions. The influent and effluent concentrations and removal efficiencies of the standard wastewater parameters, sulfamethoxazole and diclofenac are presented in Table 3.

The average removal of diclofenac was 63.47% and 61.63% for planted and unplanted columns. The removal of SMX was 42.01% for planted and 44.64% for unplanted columns. It was found that the presence of plants in a column had no effect on the removal of the dosed substances (Nowrotek *et al.*, 2015). A similar trend was demonstrated in the case of the Shannon index calculated for ammonia oxidizing bacteria (AOB). Neither the presence of plants in the column during the adaptation period, nor the introduction of SMX and DCF dosing could be said to affect the Shannon index value ($p > 0.05$). In order to assess the relationship between the Shannon index values and the removal of total nitrogen (TKN), chemical oxygen demand (COD), sulfamethoxazole and diclofenac Spearman correlation coefficient was calculated (Table 4).

The Shannon index value was compared with total nitrogen (TKN) because the nitrogen compounds in wastewater were in organic forms. The removal of TKN in unplanted columns without SMX and DCF was 89%, in unplanted with SMX and DCF — 85.8%, in the planted columns, the removal of TKN in the columns with SMX and DCF was 90.7%, and without SMX and DCF — 90.2%. The Spearman coefficient for comparing the removal of TKN and the Shannon index values ranged from –0.33 to –0.58 (a significant dependency). The negative value of the Spearman correlation coefficient is associated with a decrease in the value of the two compared values of the analyzed factors. Dong & Reddy (2012) also reported no significant correlation between bacterial biodiversity and the removal of nitrogen forms (NH_4^+, NO_3^-). The Spearman correlation coefficients values for COD and the Shannon index were negative, which points to a low correlation. The Spearman correlation coefficient for the Shannon index values and removal of SMX and DCF was a positive value, which is associated with a simultaneous increase in both factors being compared. The highest value (0.9), the Spearman correlation coefficient assumes for comparison of the Shannon index value and the removal of DCF in planted columns. The obtained value indicates a fairly strong relationship between the analyzed factors. For planted columns, the Spearman correlation coefficient is 0.6, which is associated with significant dependence. The Spearman correlation coefficient for removal of SMX and Shannon index value for planted columns reached 0.7, which indicates moderate correlation, and 0.6 for unplanted columns, which indicates high correlation, and large dependency.

Table 4. Spearman correlation coefficient values calculated for the selected parameters of wastewater and Shannon biodiversity index

Parameters	Type of columns			
	P	Ph-P	U	Ph-U
TKN	–0.45	–0.36	–0.33	–0.58
TOC	0.26	–0.29	–0.38	–0.14
DCF		0.9		0.6
SMX		0.7		0.6

The dendrograms constructed on the basis of the obtained fingerprints show genetic similarity among the samples. In figure 3 presents dendrograms for samples taken on day 1 (A), 151 (B) of the experiment, when wastewater without pharmaceutical substances was dosed to the system, on day 210 (C) of the experiment, when pharmaceutical substances were dosed into the system, and on day 271 (D), when again wastewater without pharmaceutical substances was dosed into the system.

Only dendrogram A presenting the relationships among AOB seems to be more coherent. Over time, the similarity of samples is changing. Observation of this trend towards changeability is difficult. It can be concluded that SMX and DCF dosed into the system with the wastewater did not modify the similarity of samples, as compared to the samples taken in the earlier stage of research. This confirms the lack of a significant effect of dosed pharmaceuticals on the bacterial community. The bacteria present in constructed wetlands adhere to the filter material or plant roots — if they are present, and on solid particles create biofilm (Watnick *et al.*, 2000). This biofilm structure is responsible for the majority of the fundamental transformations and degradation of

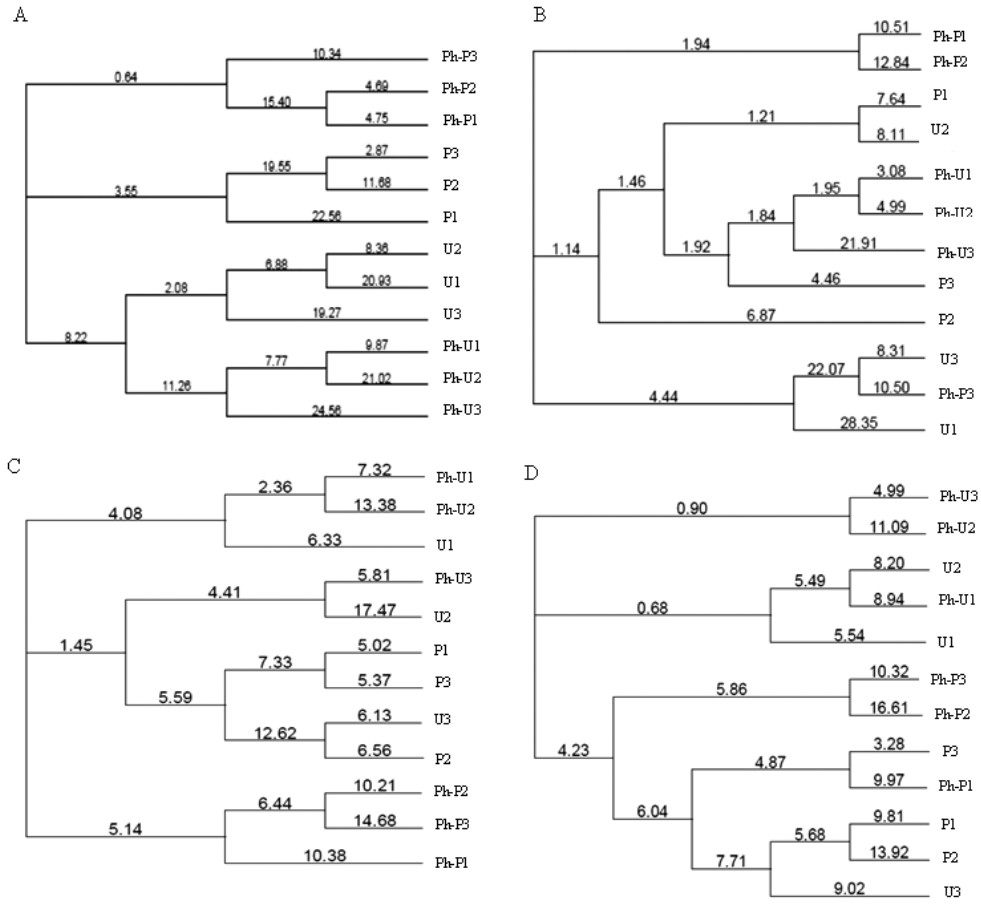

Figure 3. Dendrograms constructed using Neighbor Joining algorithm with Dice coefficient on the basis of DGGE fingerprints for AOB.
(**A**) samples taken on the first day of experiment, (**B**) samples taken on 151st day of experiment (**C**) samples taken on 210th day of experiment when to the system wastewater was dosed along with SMX and DCF, (**D**) samples taken on 272nd day of research when to the system wastewater was dosed without SMX and DCF.

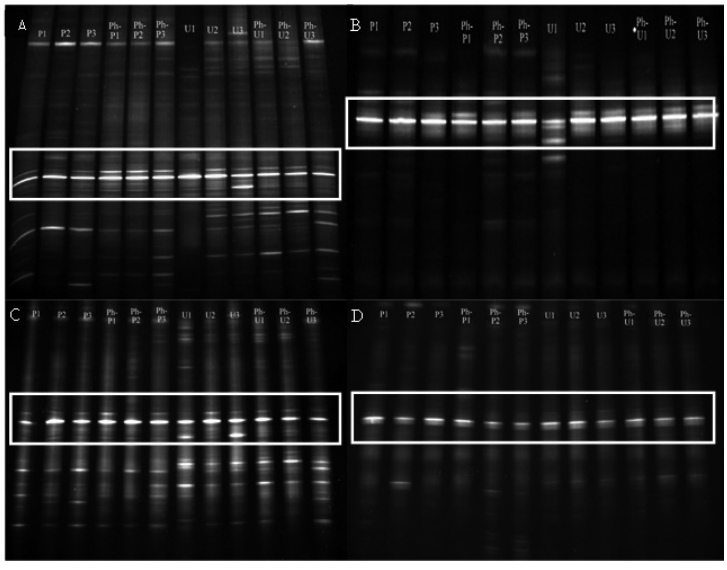

Figure 4. DGGE fingerprints obtained on the basis of 16S rRNA gene amplification for AOB
(**A**) samples taken on first day of experiment, (**B**) samples taken on 151st day of experiment (**C**) samples taken on 210th day of experiment when wastewater was dosed into the system, along with SMX and DCF, (**D**) samples taken on 272nd day of research when wastewater was dosed into the system without SMX and DCF. In the frame are marked parent genotypes during the experiment.

contaminants that are found in the wastewater (Larsen *et al* 2004; Faulwetter *et al.* 2009). It may be assumed that extracellular polymers secreted by the bacteria which create the biofilm, form a structure that protects the bacteria against the adverse effect of the dosed SMX and DCF.

The results of DGGE monitoring are presented in Fig. 4. The study showed that the ammonia oxidizing community did not change over time. In the figure 4, the dominant genotype in all types of columns can be observed with only little change in the distribution of the other bands. That also confirms that the structure of the ammonia oxidizing community did not change under the influence of SMX, DCF and other factors.

As shown, the AOB microbial community did not change during experiment. Similar results were demonstrated in sediment samples of an overland flow system treating landfill leachate containing mainly construction/industrial and household waste. In a study also based on the DGGE of CTO region of 16S rRNA gene, no significant changes were demonstrated in the structure of ammonia oxidizing bacteria community (Sundeberg *et al.* 2007a). In the case of

studies on municipal wastewater treatment plants, composition of AOB communities varied only slightly and tended to be stable in time (Dionisi *et al.* 2002; Hallin *et al.* 2005). Pell & Nyberg (1989) reported an establishment of ammonia oxidizing bacteria within 75 days. Furthermore, in vertical flow constructed wetlands treating municipal wastewater, more than 50% of the microbial biomass and bacterial activity were concentrated in the first few centimeters, and 95% in the first 10 cm of the sandy substrate filter (Tietz *et al.*, 2007). It can therefore be concluded that the microbial community in this experiment reached a stable state and the results obtained from collected samples are representative.

CONCLUSIONS

Denaturing gradient gel electrophoresis (DGGE) has been widely used to produce an overall pattern of microbial community in environmental samples. The ammonia oxidizing bacteria analyzed in this study are constituting a stable structure, insensitive to TKN and COD. The biodiversity of AOB throughout the experiment did not change significantly, and the presence of plants had no effect on the Shannon biodiversity index. Furthermore, the presence of pharmaceutical substances (SMX and DCF) in dosed wastewater also had no negative impact on the biodiversity of ammonia oxidizing bacteria. Moreover, the Spearman correlation coefficient presented a positive value and is associated with a very high sensitivity for Shannon index values and the removal of DCF. Also, for SMX these values are positive. It can be concluded, that the SMX and DCF present in dosed wastewater did not negatively affect the structure of AOB, moreover, it increased the Shannon index values.

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