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THE EFFECT OF SOME TECHNOLOGICAL PARAMETERS ON THE DIVERSITY OF IMMOBILIZED MICROORGANISMS

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Abstract: The effect of hydraulic retention time (HRT) and internal circulation capacity (q_c) on microbial diversity of immobilized biomass in the porous ceramic carrier was determined. The bioreactor, operating at HRT of 70 and 60 min, and with q_c in the range of 20–70 $dm^3 \cdot h^{-1}$, was employed for the removal of organic compounds from municipal wastewater. Microbial diversity was estimated on the basis of RISA patterns using the Shannon-Wiener index (H'). At HRT of 70 min, H' lowered from 2.48 ± 0.14 to 2.13 ± 0.23 as q_c was increased from 20 to 60 $dm^3 \cdot h^{-1}$. At HRT of 60 min, an increase in q_c from 40 to 70 $dm^3 \cdot h^{-1}$ resulted in H' drop from 2.41 ± 0.13 to 2.08 ± 0.19. At every HRT the highest efficiency of removal of organic compounds was obtained at the lowest value of q_c and the highest biomass diversity.

INTRODUCTION

In wastewater treatment technology systems with high concentrations of biomass, various reactor types are used including column or membrane reactors, fluidized beds and air-lift reactors, that are characterized by high volumetric reaction rate and long solids retention time. These reactors are considered as ecosystems of high process stability [8] and provide a larger biodiversity in comparison to reactors with suspended biomass [4].

Ecosystem stability is the outcome of functional redundancy, which is ensured by the presence of many species able to perform the same ecological function [3]. The presence of many microorganisms that are able to conduct a specific process heightens the probability that a sudden change of environmental conditions will not worsen the effectiveness of wastewater treatment, because one or more of the species will manage to adapt and assure maintenance of the specific metabolic pathway [13].

Genetic techniques offer promising opportunities for quickly analyzing the community diversity, because many samples can be analyzed relatively rapidly [12]. Recently, several molecular techniques have been developed in order to study a number and di-

versity of natural samples [6]. One of the methods is based on Ribosomal Intergenic Spacer (RIS) region analysis. RIS is located between the 16S and 23S rDNA genes in the ribosomal operon. Research showed that this region is extremely variable both in size and sequence even within closely related taxonomic groups [9]. The patterns obtained can be used to characterize different communities with respect of their species diversity [10, 17].

Microbial diversity in a bioreactor depends on the scale of process being conducted and on its operating parameters. In our column reactor with biomass immobilized in a porous carrier, internal circulation is of high importance, preventing the carrier fouling. Zielińska [23] claimed that lowering the internal circulation capacity from 60 to 20 $dm^3 \cdot h^{-1}$ resulted in an increase in sludge yield by 20% on average.

From a technological point of view, it is important to evaluate the impact of operating parameters on microbial diversity in a bioreactor. The aim of the experiment was to estimate the effect of hydraulic retention time and internal circulation capacity on microbial diversity of biomass immobilized in the ceramic carrier. The results obtained may help in selecting optimal values of the parameters at which the microbial diversity is maximized, and thus positively influence the wastewater treatment efficiency.

MATERIALS AND METHODS

Characteristics of the bioreactor

Activated sludge was immobilized inside a porous carrier formed as an 8-channeled cylinder. The carrier external diameter was 25 mm, length 1178 mm, total volume 0.6 dm^3. The carrier volume was the total volume with the volume of eight channels. The carrier producer (TAMI Industries, Germany) provided the following carrier characteristics: hydraulic diameter 6 mm, internal surface 0.2 m^2, pore diameters from 4 to 6 μm, and the material porosity of 35–40%. The carrier was made from the mixture of aluminum oxide (Al_2O_3), titanium oxide (TiO_2) and zirconium oxide (ZrO_2) and was called a ceramic carrier. From this powdered mixture a paste was made and formed in the shape of multichanneled tube. The tube was then sintered at > 1000°C.

Activated sludge, derived from a full scale wastewater treatment plant with nitrification, was the source of inoculum. The value of the Shannon-Wiener index for activated sludge used as inoculum was 2.31 ± 0.28. First, activated sludge was thickened to the concentration of about 23 g TSS·dm^{-3}. Then, the immobilization was made by circulating the activated sludge in the reactor for 24 h. The circulation was conducted in such a way that allowed for biomass flow through internal channels of the carrier and not through the space outside the carrier. As a result, biomass was immobilized both inside the pores and on the internal surfaces of the carrier. The initial carrier loading reached 18.2 g TSS·dm^{-3}. The carrier loading was calculated from the total volume of a carrier.

The carrier with immobilized biomass comprised the stationary filling of the reactor with internal circulation. The reactor scheme and its dimensions in mm (in italics) are shown in Figure 1. The carrier was fixed into the bioreactor using O-rings. Two spaces were created: the internal channels and the external space. Raw wastewater flux and the circulating stream were mixed before they flowed into the reactor. The influent was divided into two streams flowing parallel through the external space and internal channels. This allowed the pressure on the internal and external surfaces to be kept equal.

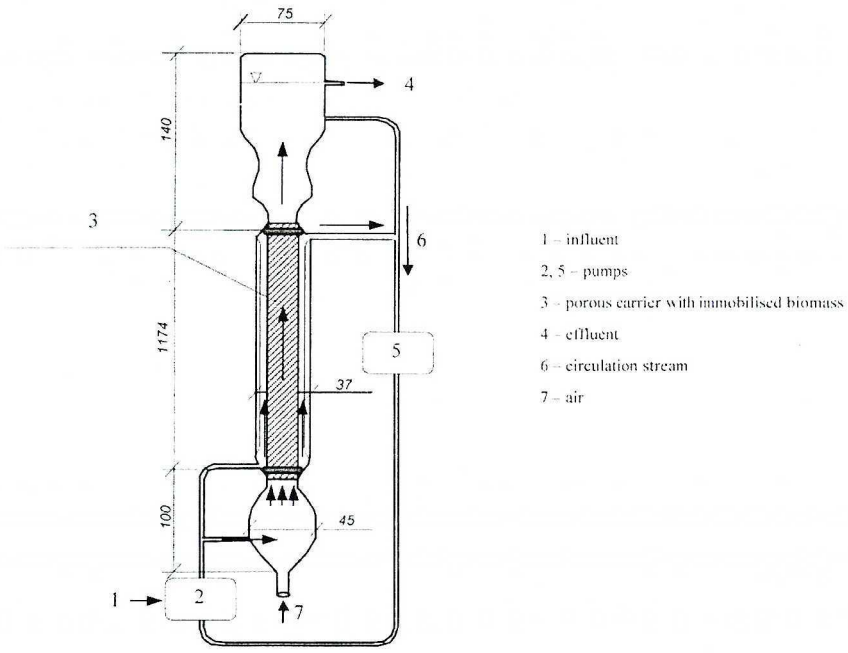

Fig. 1. Scheme of the reactor

Operational conditions

The reactor worked under aerobic conditions. In order to maintain the oxygen concentration in the reactor at the level of 2 mg $O_2 \cdot dm^{-3}$, it was necessary to supply about 120 $dm^3 \cdot h^{-1}$ of air to the reactor. DO was measured in the upper part of the reactor in the effluent. The experiment was carried out at the temperature of 20°C.

During the experimental period, municipal wastewater was taken each day directly from the inspection chamber of a sewer pipe. Typical contents of organic compounds, nitrogen compounds and total suspended solids were as follows: 274 ± 49.7 mg $COD \cdot dm^{-3}$, 47 ± 7.6 mg $TKN \cdot dm^{-3}$, 25 ± 4.0 mg $N_{NH4} \cdot dm^{-3}$, 150 ± 45.9 mg $TSS \cdot dm^{-3}$. Chemical analyses of wastewater were performed according to Polish Standards [16]. We analyzed 130 samples of wastewater.

The experiment was organized as presented in Table 1. Firstly, the study was carried out for a hydraulic retention time (HRT) of 70 min, and volumetric organic loading rate (VLR) of 5.4 kg $COD \cdot m^{-3} \cdot d^{-1}$. The experiment was repeated for a HRT of 60 min, and a VLR of 5.8 kg $COD \cdot m^{-3} \cdot d^{-1}$. HRT was altered by changing the wastewater feed rate. The volumetric loading rate was calculated per total volume of the carrier according to German ATV directions concerning dimensioning of biological beds. For both HRTs the internal circulation (q_C) was changed as shown in Table 1.

Table 1. Experimental set-up

Series	1	2	3	4	5	6
HRT [min]	70			60		
VLR [kg $COD \cdot m^{-3} \cdot d^{-1}$]	5.4			5.8		
q_C [$dm^3 \cdot h^{-1}$]	60	40	20	70	60	40

All experimental series were carried out consecutively in the same reactor in the following order: 1, 2, 3, 5, 6, 4. Before the start of the first experimental series, inoculum was immobilized in the carrier and used throughout the whole experiment. The adaptation period before every series lasted about 30 days and was considered complete when the range of changes of particular parameters of the effluent (COD, TKN, N_{NH4}) within 7 days did not exceed 5–10%. At each hydraulic retention time, after biomass adaptation for the experimental conditions, the research was carried out for about 2 weeks. During the experiment, samples were collected twice a day.

DNA isolation and RISA

The samples of biomass were taken from the effluent at the end of each experimental series and stored at -20°C prior to molecular analysis. DNA was extracted from approximately 0.5 g of immobilized biomass using a commercial DNA isolation kit (Fast DNA® SPIN® Kit for Soil, Q-BIOgene, USA) according to manufacturer's instructions. The quality and quantity of isolated DNA were measured spectrophotometrically using a Biotech Photomer (WPA, UK).

The bacterial RIS was amplified in duplicate with primers 1 and 2 described previously by Dolzani *et al.* [7]. The amplified fragment contained RIS plus approximately 380 bp, corresponding to flanking regions of genes coding for 16S and 23S rRNA. PCR was performed in an Eppendorf® Mastercycler Gradient (Eppendorf, Germany). The PCR mixture contained 50 ng of extracted total DNA, 0.5 μM of each primer, 100 μM of deoxynucleoside triphosphate (Promega, USA), 1 U of Taq DNA polymerase (Sigma, USA), $3 \cdot 10^{-6}$ dm^3 of reaction buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin, pH 8.3 at 25°C), and sterile water to a final volume of $30 \cdot 10^{-6}$ dm^3. The PCR amplification was carried out using the following program: 95°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 43°C for 30 s, extension at 72°C for 1 min, and a final elongation at 72°C for 5 min. The presence of PCR products was confirmed by analyzing $5 \cdot 10^{-6}$ dm^3 of the product on a 1.2% agarose gel stained with ethidium bromide.

After the successful DNA amplification, $10 \cdot 10^{-6}$ dm^3 of PCR products were applied directly to 6% polyacrylamide gel (29 : 1 acrylamide : bisacrylamide). Electrophoresis was carried out at 60 V for 110 min in 1x TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA; pH 8.0). After electrophoresis, the gel was stained with SYBRgold (Molecular Probes, USA) at 10 000x dilution in 1x TAE buffer for 30 min.

Stained gel was viewed with an ultraviolet transilluminator and recorded with a CCD camera (Gel Logic 200, Eastman Kodak Company, USA). Bands were detected automatically from digital images of the gel using KODAK 1D 3.6 Image Analysis Software (Eastman Kodak Company, USA). The size of PCR products was estimated using 1 kb DNA Ladder (Promega, USA). Both poliacrylamide gels were used for the determination of the Shannon-Wiener index.

Calculation methods

The structural diversity of the microbial community was examined by the Shannon-Wiener index of general diversity H' [18, 20]. H' was calculated on the basis of the bands on the gel tracks, using the densitometric curves. The intensity of the bands was reflected as peak heights in the densitometric curve. The Shannon-Wiener index was calculated according to the equation:

$$H = - \Sigma(n_i/N) \ln(n_i/N)$$

where: n_i was the height of the peak,

N was the sum of all peak heights in the densitometric curve.

The relationships between microbial diversity and technological parameters of the process were determined by correlation analyses. The dependence between the number of bands in RISA patterns and internal circulation was assessed with Pearson's correlation (R_p). Since a normal distribution could not be assumed, the correlation between H' values and internal circulation intensity was performed as Spearman's rank correlations (R_S). A statistical significance of the differences between the TSS values in a particular series was determined using the Kruskall-Wallis test. All statistical analyses were performed at significance level of 0.05 using the program STATISTICA 6.0 (StatSoft, USA).

On the basis of RISA patterns, distance matrix analyses were performed according to the method of Nei Li [14] using the program DGGEstat 1.0 (van Hannen, the Netherlands Institute for Ecological Research, NIOO-KNAW, the Netherlands). The samples were clustered using the unweighted pair group method of arithmetic averages (UPGMA), bootstrapping was conducted with 1000 replicates.

The liquid superficial velocity was determined according to Zaiat *et al.* [22].

The calculated mean values are followed by standard deviations.

RESULTS AND DISCUSSION

In the experiment, the impact of hydraulic retention time and internal circulation capacity on the microbial diversity changes in biomass immobilized in the porous ceramic carrier was examined. Both at HRT 70 and 60 min, at variable q_C, the efficiency of organic compounds (COD) removal remained at the level of about 80% (Tab. 2). However, it could be noticed that, at every HRT, the highest efficiency of organic compounds removal (E_C) was obtained at the lowest value of internal circulation capacity.

Table 2. Mean values of parameters in the effluent of the bioreactor with immobilized biomass

Series	1	2	3	4	5	6
HRT [min]	70			60		
q_C [$dm^3 \cdot h^{-1}$]	60	40	20	70	60	40
TSS in the effluent [$mg \cdot dm^{-3}$]	64 ± 58.6	65 ± 39.7	30 ± 24.8	113 ± 109.0	54 ± 62.0	57 ± 35.0
E_C [%]	76.8 ± 15.0	81.8 ± 15.2	84.6 ± 12.7	82.6 ± 16.3	80.6 ± 15.7	87.1 ± 12.0

In order to investigate the microbial diversity of biomass immobilized in the reactor, molecular analyses were carried out. Electrophoretic separation of PCR-amplified RIS region from immobilized biomass, sampled at the end of each experimental series, resulted in distinct banding patterns (Fig. 2).

The Shannon-Wiener index (H') is more and more widely used for evaluation of microbial diversity both in natural and technical biocoenoses [2, 5, 19]. This index can be successfully used for estimating the diversity of communities, however it is difficult to interpret the results obtained, and so further investigations into the dependence between technological parameters and Shannon-Wiener index values are needed [11]. In our research no statistically significant dependence between HRT and Shannon-Wiener

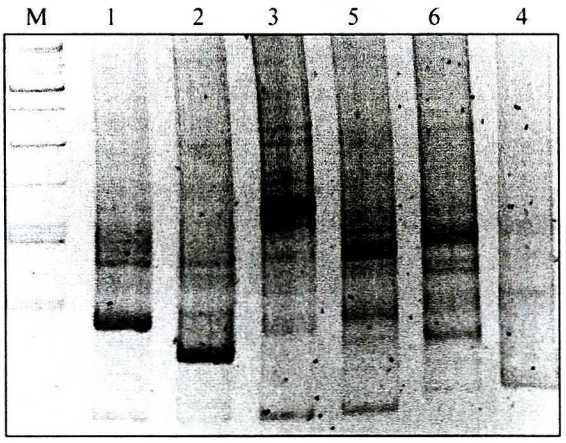

Fig. 2. Ribosomal Intergenic Spacer (RIS) region analysis of bacterial community immobilized in the porous carrier; lane labels along the top show the number of experimental series; lane M: 1 kb DNA Ladder (Promega, USA); the gel was stained with SYBRgold (Molecular Probes, USA)

index values was proved. The obtained results are in agreement with the research carried out by Nogueira *et al.* [15]. The authors did not show any effect of HRT ranging from 0.7 h to 5.0 h on the composition of nitrifiers in biofilm. In the presented experiment, however, a statistically significant correlation between internal circulation capacity (q_C) and H' values, both at HRT 70 min and 60 min was noted ($R_S = -0.85$, $p < 0.05$) (Fig. 3A). These data indicated that an increase in the internal circulation capacity resulted in a decrease in microbial diversity of biomass immobilized in the porous carrier. At a HRT of 70 min an increase of q_C from 20 to 60 $dm^3 \cdot h^{-1}$ caused a decrease of H' from 2.48 ± 0.14 to 2.13 ± 0.23, whereas at a HRT of 60 min an increase of q_C from 40 to 70 $dm^3 \cdot h^{-1}$ reduced H' from 2.41 ± 0.13 to 2.08 ± 0.19. Parallelly, a statistically significant negative correlation between the number of bands, representing different bacterial species, and internal circulation capacity (q_C) was observed ($R_P = -0.92$, $p < 0.05$) (Fig. 3B). A decline of immobilized biomass diversity did not result from the biomass washout, because no statistically significant differences between TSS values in the effluent during experimental series were observed (Tab. 2). The calculated values of the liquid superficial velocity in the reactor (from 1.55 to 5.3 $cm \cdot s^{-1}$) indicated that the flow speed of the stream through the bioreactors could have not caused the biomass washout. In general, the increase of the internal circulation capacity results in the larger total contaminants load in the wastewater stream introduced to the bioreactor. According to Atlas [1], at high nutrient loading a less diverse community develops. Our research results are in agreement with this opinion. In the reactors with bacterial communities immobilized in the carriers, part of the biomass is attached to the carrier; the other part is suspended [21]. It seems that intensive wastewater flow due to increased internal circulation capacity mainly favors the growth of bacterial species with greater ability to attach, whereas the contribution of suspended biomass is minor.

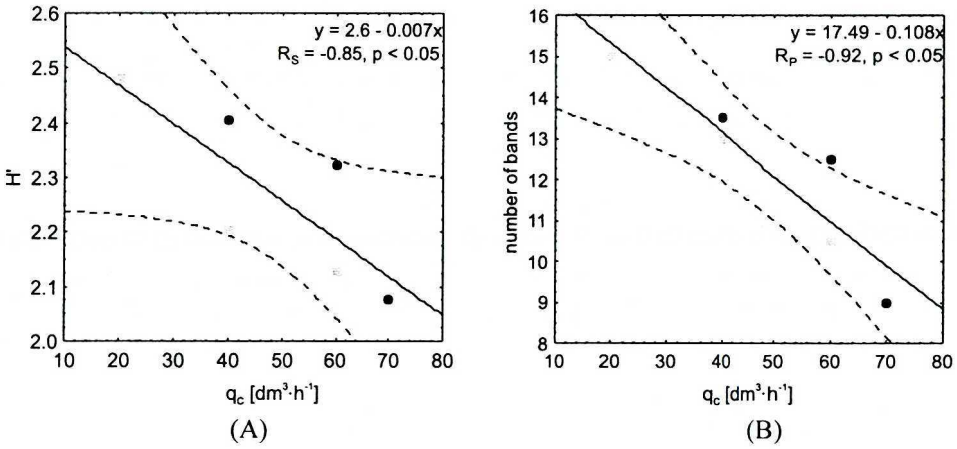

Fig. 3. Correlations between: (A) internal circulation intensity and Shannon-Wiener index values (Spearman's rank correlation), (B) internal circulation intensity and the number of bands in the RISA patterns (Pearson's correlation); black circles represent values obtained for HRT 60 min, whereas grey squares represent values obtained for HRT 70 min.

A comparison of RISA patterns obtained in different experimental series was conducted in order to determine the similarities between microbial communities in the reac-

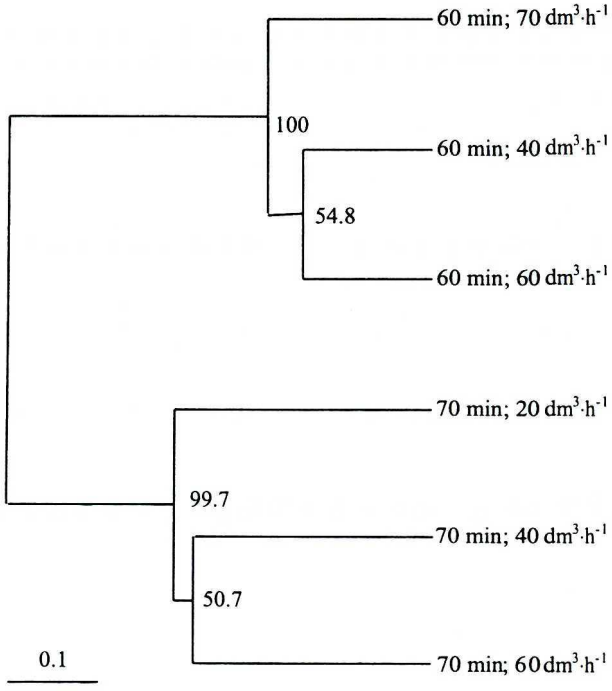

Fig. 4. Dendrogram of RISA patterns similarities among different experimental series calculated on the basis of distance matrix according to the method of Nei Li with the clustering algorithm of UPGMA; numbers adjacent to branch points are bootstrap percentages (n = 1000 replicates); in the description of branches the first number refers to HRT, the second number to the q_c value

tor in relation to technological parameters. The cluster analysis divided the RISA patterns of the microbial community immobilized in the porous carrier into two clusters (Fig. 4). A clear distinction between microorganisms communities at HRT of 70 and 60 min was found. The first cluster embraced RISA patterns obtained in the series 4–6 at HRT of 60 min, while the second cluster included patterns from the series 1–3 at HRT of 70 min. It is worth mentioning, however, that RISA similarity patterns indicated in the dendrogram corresponded to the order of series, in which the experiment was conducted. As the reactor was inoculated once only, at the beginning of the experiment, it can be assumed that biocoenoses shaped in every series fluently passed one into another. For this reason, the effect of HRT on similarities between bacterial communities immobilized in the carrier cannot be stated unequivocally.

CONCLUSIONS

1. At a given HRT, the microbial diversity depends on the internal circulation capacity. Along with an increase of wastewater flow, the Shannon-Wiener index values and the number of different bacterial species decrease.
2. At any given HRT, the highest efficiency of organic compounds removal (E_C) can be obtained at the lowest value of internal circulation capacity and the highest biomass diversity.

SYMBOLS USED

DO	– dissolved oxygen,
COD	– chemical oxygen demand,
E_C	– effectiveness of organic compounds removal,
HRT	– hydraulic retention time,
H'	– Shannon-Wiener index,
p	– significance level,
q_C	– internal circulation capacity,
RISA	– Ribosomal Intergenic Spacer Analysis,
R_P	– Pearson's correlation,
R_S	– Spearman's rank correlation,
TKN	– total Kjeldahl nitrogen,
TSS	– total suspended solids,
VLR	– volumetric loading rate.

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WPŁYW PARAMETRÓW TECHNOLOGICZNYCH NA RÓŻNORODNOŚĆ UNIERUCHOMIONYCH MIKROORGANIZMÓW

Celem pracy było określenie wpływu hydraulicznego czasu zatrzymania (HRT) i wydajności cyrkulacji wewnętrznej (q_c) na różnorodność mikroorganizmów w biomasie unieruchomionej w porowatym nośniku ceramicznym. Bioreaktor, wykorzystywany do usuwania związków organicznych ze ścieków komunalnych, był eksploatowany przy HRT 70 i 60 min oraz q_c w zakresie 20–70 $dm^3 \cdot h^{-1}$. Różnorodność mikroorganizmów była określana na podstawie wzorów RISA przy użyciu indeksu Shannona-Wienera (H'). Przy HRT równym 70 min, H' obniżył się z 2,48 ± 0,14 do 2,13 ± 0,23 ze wzrostem q_c z 20 do 60 $dm^3 \cdot h^{-1}$. Przy HRT 60 min, zwiększenie q_c z 40 do 70 $dm^3 \cdot h^{-1}$ spowodowało spadek H' z 2,41 ± 0,13 do 2,08 ± 0,19. Przy każdej wartości HRT, najwyższą efektywność usuwania związków organicznych uzyskano przy najniższej wartości q_c i najwyższej bioróżnorodności.