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# INVESTIGATION OF FUNCTIONAL DIVERSITY AND ACTIVATED SLUDGE CONDITION USING BIOLOG® SYSTEM

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Abstract
**Activated sludge method dominates among the currently used biological wastewater treatment systems. The dynamic development of this technology implies need for research, most of which are carried out in laboratory conditions. Biolog system seems to be an effective tool that allows a quick assessment of microbial activity of activated sludge. The aim of this study was to verify Biolog® EcoPlates' usage for comparing the functional and metabolic diversity of activated and excess sludge microbial communities, collected from WWTP "Klimzowiec" in Chorzów with the excess sludge subjected to the purification process, and then long-term adaptation in laboratory conditions. Changes in the functional diversity of activated sludge during the adaptation in the lab-scale were verified. It was found that the acclimatization process plays a crucial role in sludge adaptation to the laboratory environment. In addition, it has been shown that subjecting the excess sludge to purification may lead to changes in functional diversity of this community.**

Streszczenie
**Wśród współcześnie stosowanych systemów biologicznego oczyszczania ścieków dominuje metoda osadu czynnego. Dynamiczny rozwój tej technologii implikuje konieczność prowadzenia badań naukowych, które w większości wykonywane są w warunkach laboratoryjnych. System BIOLOG wydaje się być skutecznym narzędziem, pozwalającym na szybką ocenę stanu aktywności mikroorganizmów osadu czynnego. Przedmiotem badań było zastosowanie płytek Biolog® EcoPlate do porównania funkcjonalnej różnorodności i aktywności metabolicznej zespołów mikroorganizmów, występujących w osadzie nadmiernym i osadzie czynnym, pobranym z Oczyszczalni Ścieków „Klimzowiec" w Chorzowie oraz w osadzie nadmiernym, poddanym procesowi oczyszczania, a następnie 7-tygodniowej adaptacji w warunkach laboratoryjnych. Sprawdzono także zmiany różnorodności funkcjonalnej mikroorganizmów osadu czynnego pobranego z oczyszczalni ścieków podczas hodowli w warunkach laboratoryjnych. Stwierdzono, iż proces aklimatyzacji odgrywa ważną rolę w adaptowaniu osadu czynnego do warunków laboratoryjnych. Wykazano również, że poddanie osadu nadmiernego procesowi oczyszczania może prowadzić do zmian różnorodności funkcjonalnej tego środowiska.**

Keywords: **Activated sludge; Functional diversity indices; Biolog® Ecoplate; Excess sludge; Wastewater Treatment Plant.**

## 1. INTRODUCTION

Intensive industrialization and fast urbanization affect the variable quantitative and qualitative composition of wastewater introduced into the sewage treatment plants, wherefore the wastewater treatment constantly undergoes numerous modifications. Application of the systems based on intensification of the naturally occurring organic compounds biomineralization processes in Waste Water Treatment Plants (WWTPs) is not only an economic and ecological acceptable solution, but most of all the removal of more than 90% wastewater organic substances takes place [1]. Nowadays, for both large and small WWTPs, the Activated Sludge Process (ASP) allows very effective treatment of industrial and municipal sewage, representing the most commonly used technology for biological wastewater treatment. In order to facilitate the optimization of this process in WWTPs it is necessary to monitor the activated sludge quality in aeration tanks. However, the majority of research focused on improvement of the ASP is conducted in laboratory conditions. It was reported that the lab-scale bioreactors may not be possible to mimic all aspects of WWTPs due to the more complex microbiology of sewage treatment plant environment, where selective pressures likely differ from those in lab-scale systems [2]. Only a few studies have focused on the functional diversity of activated sludge microbial communities in large-scale WWTPs [3, 4]. After transferring the activated sludge into a different environment, microbial community undergoes a number of strong alterations needed for maintenance dynamic equilibrium and acclimatization to these new operational conditions [5]. The microbial communities of the activated sludge are complex assemblages of microorganisms that respond to changes in the environment by modification of their structure via shifts in species richness and rank abundance or by changes in community functions. Van der Gast et al. [6] showed the correlation between organic loading rate and temporal variability of microbial richness in laboratory-scale systems. Since microorganisms within activated sludge communities are interdependent, the minor change in the cultivation conditions, even if it affects directly only a limited number of populations, may lead to restructuration of activated sludge composition [7]. However, in WWTPs, sludge communities may consist of taxa exhibiting minimal temporal variability and subjected to more pronounced fluctuations in abundance over time [8]. Although the bacterial taxa comprise approximately 95% of the activated sludge and play a significant role in the sewage purification, information on microbial ecology and community structure in biological wastewater treatment system is still quite limited [4, 9].

The Biolog system, originally applied in soil biodiversity studies, starts to be useful tool for sewage sludge functional diversity investigation in full-scale WWTPs [3, 4, 7, 10]. Found out for heterotrophic microbial communities metabolic activity in Biolog system is based on the premise that microorganisms vary in pattern and rate at which they utilize natural carbon sources [11,12]. Hence, this method may have an application for monitoring and assessment of activated sludge condition and for prediction in advance the effects of negative factors in WWTPs such as discharge of highly-loaded wastewaters, low oxygen concentration or even environmental disasters. On the other hand this method like many others has some pros and cons. Preston-Mafham et al. [13] concluded that Biolog is more useful for comparision of communities rather than community characterization.

The primary objective of this study was to compare the functional diversity and metabolic activity of the microbial communities found in excess and activated sludge, obtained from the WWTP "Klimzowiec" in Chorzów, with the sludge that underwent long-term cultivation in lab-scale system. The second one was to investigate the changes of microbial activity patterns during the cultivation of activated sludge obtained directly from aeration tank in laboratory-scale system. The research was also aimed to determine the effect of excess sludge purification process on its functional diversity.

## 2. MATERIALS & METHODS

### 2.1. Collection and preparation of sludge samples

Sludge samples were obtained directly from the aeration tank and secondary sedimentation tank of the WWTP "Klimzowiec", Chorzów, Polska and analyzed immediately after collection.

The activated sludge (AS) from the aeration tank was transferred to the laboratory-scale activated sludge system (LSASS) and analyzed after 7 ($AS_{t7d}$) and 21 days ($AS_{t21}$) of cultivation. The excess sludge (ES) has undergone purification process as follows: 48 hours of aeration, one hour of sedimentation, supernatant replacement by tap water sitting out for 24 hours. All steps were repeated three times in order to remove all these compounds adsorbed on/in flocs which potentially could serve as the additional source

of carbon and energy for microorganisms. After purification the excess sludge (ESP) was analyzed and transferred to the LSASS for 6 ($ASL_{t6w}$) and 7 weeks ($ASL_{t7w}$) of synthetic wastewater feeding.

LSASS operated under stable temperature conditions (23°C) and was fed with synthetic wastewater of composition as described in [14], dissolved in tap water after sitting out for 24 hours. During cultivation period the mixed liquor suspended solids (MLSS) content was kept over 3 mg $dm^{-3}$ and dissolved oxygen (DO) level was maintained at range of 2 to 3 g $dm^{-3}$.

### 2.2. Measurement of sludge volume index, mixed liquor suspended solids concentration and dissolved oxygen level

The sludge volume index (SVI) was measured after 30 min sedimentation in 1-liter cylinder. MLSS concentration was determined by weighting of sludge sample that was filtered and dried to constant weight at 105°C for 2 hours. The dissolved oxygen (DO) amount in LSASS was monitored by Elmetron COG-1 oxygen electrode [15].

### 2.3. Microbial extraction and measurement of community substrate utilization profiles

EcoPlates were inoculated by 120 µl aliquot of the activated and secondary sludge suspensions prepared in the following way: (1) 20 ml aliquots of the sludge were placed into glass conical flasks and shaken at 23°C, 130 rpm, for 10 min; (2) sample homogenisation on ice for 10 sec at 11.000 rpm using a sterile IKA's Ultra-Turrax T-25 digital homogenizer; (3) 10 ml of aliquots were diluted with 90 ml 0.85% sterile saline solution and shaken again at 23°C, 130 rpm, for 10 min; (4) after 5 min settling of samples, the supernatant was adjusted to 0.05-0.01 absorbance units (at 590 nm) with 0.85% sterile saline solution before inoculation into EcoPlates for standardization the inoculum size at the level of 2-3 × $10^8$ cells $ml^{-1}$ to investigate every time the metabolic response of the same number of microorganisms.

The microplates were incubated at 23°C and absorbance of each well was measured at 590 nm on Biolog MicrostationTM on the day 0, after 24 hours and then every 12 hours for 96 hours.

Activated sludge microbial activity was expressed as the average well color development (AWCD) and calculated as the arithmetic average of the sum of positive carbon responses corrected by subtracting the water control wells values of EcoPlates [11]. To eliminate false positive responses in wells the threshold was set at 0.25 abs. units [16]. Optical density values obtained in 72 hours point of reading was used for the assessment of microbial functional diversity expressed as the richness (S; the number of utilized substrates), Shannon-Weaver (H'; substrate diversity) and evenness (I'; the equitability of activities across all utilized substrates) indices [11].

### 2.4. Determination of microorganisms concentration in activated sludge samples

Concentration of heterotrophic microorganisms in sludge samples was indirectly assessed by heterogeneous plate count method analysis (HPC) on agar medium [17]. Plates were incubated at 23°C for 7 days.

### 2.5. Statistical analysis

All data presented in this paper were statistically investigated by analysis of variance (ANOVA, post hoc test) at confidence intervals of 95% ($p < 0.05$) with Statistica 10. All data are expressed as mean and standard deviation.

## 3. RESULTS & DISCUSSION

The functionally stable and efficient operation of WWTP relies upon the abundance and activity of microbial populations included in the activated sludge assemblages. Hence, the way the activated sludge microbial communities respond to perturbations can affect the performance of wastewater treatment plant and long-term ecosystem health [18]. As the most studies on activated sludge are performed in laboratory conditions, the adaptation of inoculation sludge seems to be a crucial process to avoid mistakes in the experimental results caused by acclimatization phenomena [19]. Biolog Ecoplate method was used for the qualitative evaluation of community level physiological profiles (CLPP) of microorganisms in sludges obtained from WWTP and sludges cultivated in laboratory-scale system. As a measure of total sludge microbial metabolic activity, AWCD, followed similar patterns with incubation period in all compared sludge samples (Fig. 1). The AWCD of the AS adaptation to laboratory conditions showed that utilization of substrates by microbial communities during the acclimatization period varied significantly in line of the order: $AS > AS_{t21d} > AS_{t7d}$. The lowest of AWCD values were obtained for $AS_{t7d}$ and EPS what

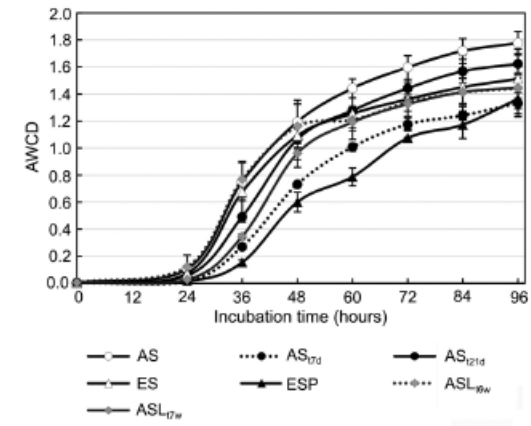


**Figure 1.**
**Comparison of average well color development (AWCD) of metabolized substrates by investigated sludge samples in Biolog EcoPlates during 96 hours of incubation**

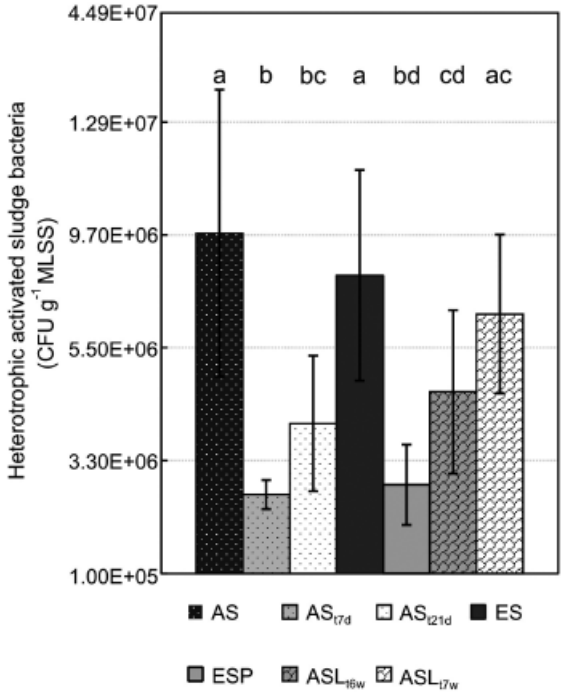


**Figure 2.**
**Comparison of the number of heterotrophic plate count activated sludge bacteria (HPC) in investigated sludge samples. Values of HPC marked with different letters were statistically significant (ANOVA, post hoc test, n=3, p<0.05)**

may suggest the reduction in number and/or species diversity of the sewage sludge community. AWCD curves obtained for $ASL_{6w}$ and $ASL_{7w}$ were similar to that appointed for ES indicating microorganisms ability to adapt to laboratory environment. Increase of metabolic capacity of sludge under stable laboratory conditions may be the result of its functional diversity recovery or enrichment of the remained microbial taxa. Regardless of sludge origin, microorganisms started to be active between 12 and 24 hour of incubation (Fig. 1). The elongation of *lag* phase after transferring AS to a laboratory environment results from the time needed for new enzymes induction during acclimatization process [5, 20].

Similarly, starvation of sludge accompanying the purification process creates conditions unfavorable for microbial community and forces them to change their functions or structure, e.g. it may lead to a sharp depression of enzymatic activity [21]. In all samples apart from ESP, microorganisms achieved stationary phase after 84 h of incubation what confirms the lowest metabolic activity of EPS. The stationary phase is determined by both, the initial inoculum density and growth rate of the species capable of utilizing substrates [13]. As the EcoPlates were incubated with the sludge suspensions of the same cell density, so the differences in lag time must result from various growth rates of the microbial communities.

HPC test has been used for the analysis of microorganisms survival in activated sludge. The higher number of culturable bacteria was determined for AS and ES (Fig. 2). During the 7 days, after AS transferring into LSASS, the number of heterotrophic microorganisms decreased rapidly, as the result of shock caused by introduction to a new environment. For ESP 3-fold reduction of microorganisms number was observed. Lack of external carbon sources and nutrients may lead to cell lysis or even death of bacteria in sludge communities [22]. However, during ESP long term cultivation in LSASS, the microbial communities were able to recover their functional capacity. The number of microorganisms showed a tendency to increase after 7 weeks of cultivation and it was similar to that obtained for ES.

Differences in microbial functional diversity among sludge samples were compared using mean Shannon-Weaver diversity (H'), richness (S) and evenness (I') indices. Significant differences in I' index for ES and ESP samples were observed (Fig. 3C). In addition, after seven days of AS adaptation H' and S indices decrease significantly (Fig. 3A, B). On the other hand, after 21 days of cultivation, H' indices were similar to those obtained for AS before introduction into LSASS. The period of 3 weeks sludge adaptation was probably sufficient to achieve the stable conditions. On the other hand, Mendrycka and Stawarz [23] pointed out the adaptation time of 9 weeks. Hence, the time and adaptation effectiveness may

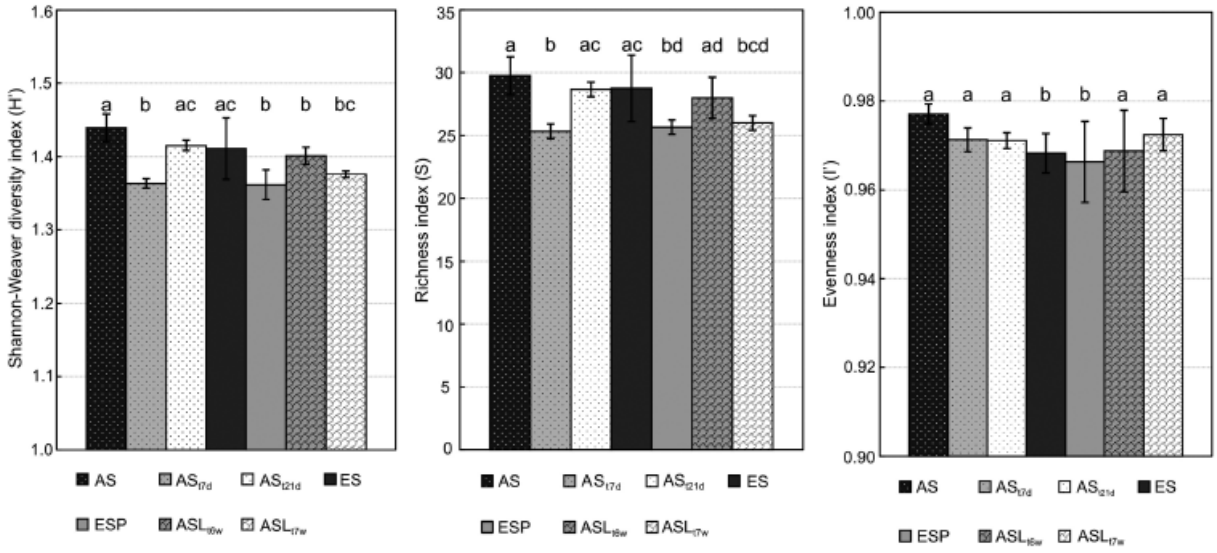


**Figure 3.**
**Model of the room made with the use of ESP-r program**

depend on many factors, e.g. primary state of activated sludge, temperature, pH or wastewater composition [24].

The values of H' and S indices for microbial communities differed because of the type of the sludge used as inoculum into LSASS (Fig. 3A, B). After purification of ES all functional diversity indices decreased, implying that some groups of microorganisms may not survive this process. If activated sludge is aerated without any additional carbon and nutrient source, only phosphate-removing bacteria (PRB) are thought to survive the aerobic starvation process, due to the presence of polyphosphate accumulating inclusions that could be utilized as a source of energy [25]. On the other hand, microorganisms surviving in the lab-scale system without any additional supplementation not necessarily may be identified as PRB but as bacteria that could accumulate and store only polysaccharides [22]. There were no significant variations in microbial functional diversity during long-term cultivation of sludge in lab-scale (Fig. 3). Similarly Kaiser et al. [10] based on the Biolog results, pointed out that metabolic capacity of activated sludge did not change over 16 weeks operation time in laboratory system.

In order to determine the degree of carbon sources utilization by the sludge bacterial communities, the Biolog EcoPlate substrates were divided into the six following guilds: (1) phenolic acids, (2) amines, (3) amino acids, (4) carbohydrates, (5) carboxylic acids

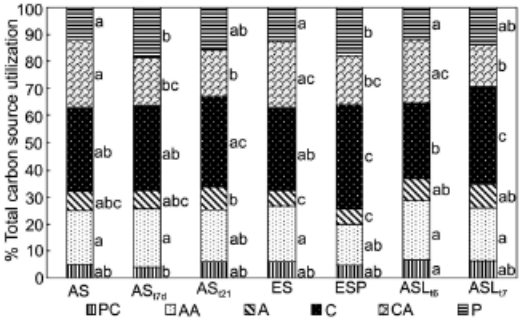


**Figure 4.**
**Comparison of substrate utilization efficiency by investigated sludge samples expressed as percentage of total carbon utilization, where (P) – phenolic compounds, (AA) – amino acids, (A) – amines (A), (C) carbohydrates, (CA) – carboxylic acids and (P) – polymers. Data are displayed as means (n=3). Values of substrates utilization marked with letters were statistically significant (ANOVA, post hoc test, n=3, $p<0.05$)**

and (6) polymers according to Insam and Rangger [12]. The only D-galactonic acid γ-lactone originally grouped as carbohydrate [12] was included in the carboxylic acids category according to [26]. All analyzed microbial communities except for ES were able to use all of 31 carbon sources, to varying degrees. Regardless of the treatment, carbohydrates were the most utilized carbon sources by investigated sludges (Fig. 4). The most intensive growth was observed while β-methyl-D-glucoside, D-xylose or D-mannitol were the energy source. Both, glucose-1-phosphate

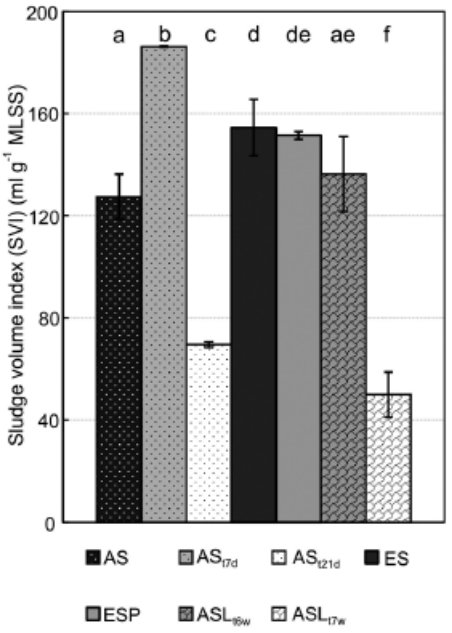


**Figure 5.**
**Comparison of the sludge biomass settling ability expressed as sludge volume index (SVI). Values of SVI marked with different letters were statistically significant (ANOVA, post hoc test, n=3, p<0.05)**

and D, L-α-glycerol phosphate served as the combined sources of phosphorus, carbon and energy for microorganisms growth. Utilization pattern of glucose-1-phosphate was similar for all sludge samples. Whereas ESP, $AS_{7d}$ and $AS_{21d}$ exhibit higher utilization of D, L-α-glycerol phosphate and glycogen than other samples. It can be suggested that under unfavorable conditions microorganisms needed more energy to carry out the modifications required for achieving the equilibrium state. Wilson et al. [27] claimed that glycogen may provide energy for microorganisms maintenance under non-growing conditions. The lowest microbial growth was observed in the presence of phenolic compounds, especially in $AS_{t7d}$ and EPS samples, that may suggest that under stress conditions microorganisms utilize preferentially the elementary carbon sources, and aromatic substrates are metabolized subsequently. From among EcoPlates substrates, 2-hydroxybenzoic acid was the single carbon source of aromatic structure not utilized by ES. In addition, microbial communities from lab-scale system metabolized this compound to a greater extent than these ones from WWTP.

Sludge volume index (SVI) was used to determine sludge physiological condition (Fig. 5). In wastewater treatment process SVI values may vary from 30 to 400 ml $g^{-1}$ MLSS [9]. The optimal SVI values of well-worked sludge vary between 70 to 150 ml $g^{-1}$. Palm and Jenkins [28] pointed out that SVI value over 150 ml $g^{-1}$ MLSS may classify the sludge as bulking one and SVI value below 70 ml $g^{-1}$ shows that the sludge settles quickly. SVI of investigated sludges revealed significantly variability and were in the range from 50 to 190 ml g-1 MLSS. The higher SVI value of 186 ml $g^{-1}$ MLSS for the $AS_{t7d}$ may be the result of changes in sludge flock structure accompanying adaptation. Whereas, decrease of SVI values for $AS_{t21d}$ to 70 ml $g^{-1}$ MLSS referred a well-adapted and well-settling sludge. The reason of SVI value reduction for $ASL_{t7w}$ to 50 ml $g^{-1}$ MLSS may be weakly structured and small flocks.

## 4. CONCLUSION

Results of this research imply that adaptation is the crucial process after sludge transferring to the laboratory conditions. It has been shown that laboratory experiment can be carried out for a long time without altering the metabolic response of microbial community. But it must be emphasized that purification process may cause a long-term changes in sludge functional diversity. It was confirmed that Biolog Ecoplate system provides information about both short-term and long-term bacterial community stability and it indicates the level of community similarity to its original state. This system can help to predict microorganisms' response to disturbances occurring in WWTPs and, hence, it may be the useful method to alleviate or prevent variations in microbial communities.

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