Environmental Engineering and Management Journal *July 2021, Vol.20, No. 7, 1099-1109*
http://www.eemj.icpm.tuiasi.ro/; http://www.eemj.eu

"Gheorghe Asachi" Technical University of Iasi, Romania

WHAT IS HIDDEN BEHIND ACTIVATED SLUDGE SUPERNATANT? FLUORESCENT STAINING AND LASER GRANULOMETRY INVESTIGATION SUPPORTED BY MACHINE LEARNING

Magdalena Domańska*, Magdalena Kuśnierz, Justyna Stańczyk

Wroclaw University of Environmental and Life Sciences, Institute of Environmental Engineering, pl. Grunwaldzki 24, 50-375 Wrocław, Poland

Abstract

Studies on the biological composition of activated sludge flocs in the operating conditions of wastewater treatment plants are generally limited to the estimation of respiratory activity or to the analysis of images made with optical microscopy. The results of these studies indirectly provide information on the microbiological composition. To date, molecular methods, although very promising, have not found a wider application in operational monitoring of wastewater treatment plants. In this paper, the supernatant was under discussion as a potential source of sampling for analyzing microbial quality changes. The results of 270 activated sludge and treated wastewater samples showed that the smallest flocs leaching to the outflow constitute a group of microorganisms that is most numerous. The studies carried out using the fluorescence *in situ* hybridization method have shown that the microorganisms responsible for the nitrification processes occur both in activated sludge and supernatant. Image analysis of microorganisms from activated sludge and supernatant stained with Live/DEAD reagent indicate that microflocs and bacteria in the outer flocs, which are relatively loosely attached to the flocculent matrix are more exposed to external factors. The results suggest that it is advisable to find information about the condition of the whole community especially for group of particles in the supernatant. In addition, the authors recommend using machine learning methods to evaluate predicting anomalies in biological composition of activated sludge flocs.

Keywords: activated sludge, fluorescence in situ hybridization method, machine learning, particle size distribution, wastewater treatment

Received: July, 2020; Revised final: November, 2020; Accepted: December, 2020; Published in final edited form: July, 2021

1. Introduction

The activated sludge process is the most commonly used one in municipal wastewater treatment (Fiałkowska and Pajdak-Stós, 2018; Seviour and Nielsen, 2010). Analytical parameters such as oxygen uptake rate (OUR), ammonium uptake rate (AUR), nitrate uptake rate (NUR), phosphate release rate (PRR) and phosphate uptake rate (PUR) are used to monitor the biochemical activity of activated sludge (Mąkinia, 2010). However, they do not provide sufficient information about the microbiological composition of the sediment. Currently, polymerase chain reaction (PCR), gene sequences and fluorescence in situ hybridization (FISH) are the most frequently used molecular methods (Domańska et al., 2014, Ferrera and Sánchez, 2016).

The FISH method, based on fluorochrome-labeled oligonucleotide probes, appears to be more accessible (Amann et al., 1995; Delong et al., 1989; Nielsen et al., 2009). This revolutionary approach allowed scientists to identify microorganisms in environmental samples (Zeng et al., 2016). Numerous FISH applications in the field of wastewater treatment

*Author to whom all correspondence should be addressed: e-mail: magdalena.domanska@upwr.edu.pl; Phone: +48 713205553; Fax: +48 71320 5579

have been implemented towards the study of microorganisms involved in the elimination of biological nitrogen and phosphorus (Wagner and Haider, 2012). Molecular techniques have contributed to a better understanding of polyphosphate and accumulating glycogen organisms (PAOs, GAOs) (Lu et al., 2017), mechanisms related to metabolic function of microorganisms, the formation process and flocs structure as well as activated granular sludge (Winkler et al., 2012).

Advanced microscopic techniques are required to understand the mechanism of sludge flocs formation and the interaction between them, but despite the great development of molecular methods and rich data on microbial communities, it is still difficult to translate lab findings into implementable solutions at full-scale wastewater treatment plants (WWTPs).

Research results have not been practically applied to monitor changes in the population of microorganisms in activated sludge yet (Cydzik-Kwiatkowska and Zielińska, 2016). Studies confirm the influence of modification in the composition of sewage flowing into the bioreactor on changes in the composition of microorganisms present in activated sludge (Stalder et al., 2013). Because nitrifiers are highly sensitive to inhibitory compounds, changes in ammonia oxidizing bacteria community could be a consequence of e.g. increase of toxic substances in raw wastewater. The presence of low concentrations of some compounds, such as active ingredients of drugs, antibiotics or estrogens, also exerts unfavorable ecological effects (Kim and Aga, 2007; Song et al., 2018). This is due to the fact that pharmaceuticals are common and incompletely removed by microorganisms during wastewater treatment. Currently, microplastics are being intensively studied mainly because they are not retained entirely at WWTPs and are present in the outflow (Koelmans et al., 2019). So far, its ecotoxicological effects on aquatic organisms is poorly understood (de Sá et al., 2018).

At present, there is a need to search for tools that will inform about the loss of microorganisms responsible for the nitrification/denitrification processes in order to reduce the risk of rapid efficiency collapse and leakage of non-treated sewage into the outflow. The increasingly greater possibilities of digital data storage have resulted in the development of analytical data mining techniques and mathematical modelling, which can be used, among other things, to support the consulting processes in the field of WWTPs exploitation (Guglielmi et al., 2020). These solutions are not commonly used to monitor processes occurring at WWTPs, but they are desirable from the point of view of extracting valuable information about their quality. Modern machine learning techniques (decision and regression trees) were used at the research stage to model and predict the functionality of WWTPs, e.g. by Atanasova and Kompare (2002). For this purpose, quantitative and qualitative data originating, among others sources, from microbiological analyses were used, and the obtained results indicate that this type of analysis makes it possible to predict abnormal operating conditions of WWTPs, such as activated sludge swelling. In the past, artificial intelligence (AI) was also used to assess the quality of wastewater treatment based on activated sludge systems, including Kohonen Self Organizing Maps (KSOM), backpropagation artificial neural networks (BPANN) and adaptive-network-based fuzzy inference system (ANFIS) (Rustum, 2009). Moreover, the results presented by Harrou et al. (2018) show the capability of the developed strategy integrating a deep belief networks (DBN) model and a one-class support vector machine (OCSVM) to monitor the WWTP, suggesting that it can raise an early alert to abnormal conditions. In Poland, data mining techniques are not used to assess the functionality of sewage treatment plants. The approach presented by Szeląg et al. (2018) demonstrates the usefulness of cascade neural network, support vector machines and boosted trees to forecast the mixed liquor suspended solids (MLSS) and food-to-mass ratio (F/M) of the activated sludge in the bioreactor. In literature on the subject, the most common application of advanced data mining techniques is the detection of sludge swelling (Amaral et al., 2013; Deepnarain et al., 2019; Han et al., 2018), while there is no mention of classification tree method used to support the image analysis of Live/DEAD-stained microorganisms. Mesquita et al. (2016) proposed Partial Least Squares (PLS) method, one of the regression techniques in machine learning, to correlate quantitative image analysis information and the key parameters.

The aim of the experiments was to check particle size distributions (PSD) in activated sludge and treated wastewater (supernatant), then to confirm with the FISH method that the bacteria responsible for the nitrification/denitrification process are leached from the activated sludge into the supernatant. The next stage was intended to verify whether more convenient methods for staining microorganisms such as Live/DEAD supported by machine learning methods (bagging decision trees) could be a source of valuable information about the condition of the microorganisms. Studies on PSD in treated wastewater and activated sludge from six mechanical-biological WWTPs located in Poland (Lower Silesia), were carried out from 2006 to 2010. The FISH method and Live/DEAD staining using activated sludge samples form Janówek WWTP (Poland) were carried out in March 2016 and June 2019 respectively.

2. Material and methods

2.1. Scope of the research

The tests were carried out on samples of activated sludge and treated wastewater derived from six mechanical-biological WWTPs located in Lower Silesia, Poland. The WWTPs were marked with symbols A to F. Selected WWTPs were characterized

by different time of operation and technological solutions. Two analyzed objects, marked A and D, worked in the flow reactor system, whereas objects E and F in the Sequencing Batch Reactor system (SBR). Such a varied selection of WWTPs allowed for a wider assessment of the activated sludge structure, PSD, microbiological recognition and drawing individual conclusions.

Activated sludge samples were taken from nitrification chambers and from SBR reactors during the nitrification phase. Treated wastewater samples from the flow system were collected at the outflow of secondary settling tanks and in the decantation phase from SBR reactors. Samples from each WWTP were collected in plastic containers in the morning, transported to the laboratory and prepared for further analysis. Studies on PSD in treated wastewater and activated sludge were carried out from 2006 to 2010 and a total of 270 samples was analyzed. The obtained results of the particle number distribution (PND) tests in activated sludge and in treated wastewater contributed to conducting microbiological tests using the FISH method in activated sludge samples and in the supernatant. These tests were carried out using 9 activated sludge samples collected from Janówek WWTP (Wroclaw, Poland).

Training and validation model of the machine learning used data from the Live/DEAD staining of fresh activated sludge taken from Janówek WWTP in June 2019. For testing the model, data of activated sludge and supernatant after 24 hours (in 4ºC), as well as with additional sodium hypochlorite after 1 and 24 hours of reaction, were considered.

2.2. Laser granulometry – sample preparation and measurements

Research on the activated sludge structure and PSD was carried out using the Mastersizer 2000 laser granulometry with a particle size range of 0.01 to 2000 μm, from Malvern Instruments Ltd. The method has been successfully used in studies on environmental pollution as a classic grain size analysis (Dąbrowska et al., 2016) and in studies on suspended solids as well as sediments in sewage and waters (Bawiec et al., 2017).

Total sample volume of treated wastewater for one measurement was about 700-800 mL. Activated sludge samples with high mass concentration had to be diluted to obtain proper values of laser light obscuration (10% 20%). After that particles were well dispersed to avoid multiple reflections in the measurement cell of the laser granulometric (Bizi and Baudet, 2006; Bushell, 2005). The laser light scattering analysis enabled to determine distributions in the form of volume function *f(vi)* and the particle number function $f(n_i)$ (the final data was based on several replications), as well as the values of mean diameters D (1.0) and D (3.2). In addition, analyses using laser granulometric were carried out in the area of activated sludge structure by assessing the fractal dimensions *(DF)*. The *DF* were calculated from the raw light scattering data and estimated using linear regression, and the student's *t* distribution was used to determine the confidence intervals of the *DF* estimator according to the method described by Guan et al. (1998). The received *DF* estimation errors for all activated sludge samples did not exceed ± 0.05.

2.3. FISH - sample preparation and analysis

The samples of activated sludge and supernatant were analyzed with the FISH method. To obtain the supernatant from the sludge, sludge samples were subjected to 30 minutes of sedimentation. Next the supernatant was centrifuged 3 times at 5000×g for 5 minutes at room temperature to obtain the right volume of sludge (pellet).

After this time the pellets from supernatant and activated sludge were washed in phosphate buffered saline (1×PBS) and fixed in 4% paraformaldehyde. The FISH procedure was carried out according to Amann et al. (1995). Well suspended sludge was placed on slides and air dried. The following probes for hybridization were applied: EUB338 (universal oligo probe which covers 90% Bacteria from Bacteria domain), NSO1225 (beta-proteobacterial ammonia-oxidizing bacteria) which targets ammonia oxidizers (beta-AOB), as well as NIT3 (*Nitrobacter* spp.) used to detect nitrite oxidizers (NOB) (Nielsen et al. 2009). Probe NSO1225 was labelled with the 6-Carboxyfluorescein dye (6-FAM) and NIT3 was labelled with carboxy-X-rhodamine (ROX). EUB338 was labelled with 6-FAM or ROX depending on the specific probe. Hybridization was performed with different stringency at 35%, 40%, respectively for NSO1225, NIT3 probes and 35-40% for EUB338 probe. The hybridization solution contained 35% or 40% formamide, 0.9 mM NaCl, 20 mM Tris-HCl (pH 7.4), and 0.01 % sodium dodecyl sulfate. Fluorescence labelled probes were mixed with the hybridization solution. Approximately 10 μL of that mix was added to each sample well and incubated in a hybridization chamber at 46°C for at least 3 hours in the dark. The chamber included saturated filter paper with excess hybridization buffer. After the hybridization step all slides were gently rinsed with pre-warmed washing buffer in the dark at 48°C for 30 min. For total cell staining DAPI was added in a final concentration of 1 μg/mL. Slides were examined using confocal microscopy (Nikon Eclipse Ni-E C2, Japan) equipped with 5-megapixel color digital camera (DS-Fi1c). The following light filter sets were used: UV-2A for DAPI (excitation 330-380, dichroic mirror 400, barrier filters 420), B-2A for 6-FAM (excitation 450-490, dichroic mirror 505, barrier filters 520) and G-2A for ROX (excitation 510-560, dichroic mirror 575, barrier filters 590). The analysis was conducted using the CFI Plan Apo 60×oil objective and pictures were taken with Nis-Elements AR 4.30 software.

2.4. Live/DEAD–staining

Two types of activated sludge samples with no

additives (100 mL) and activated sludge with 100 mL sodium hypochlorite (0.8 mg/L of free chlorine) were prepared. After 1 and 24 hours, sample of sediment and supernatant were collected. The samples of activated sludge and supernatant were analyzed using the Live/DEAD reagent of Thermo Fisher (Nr L7007) on a Nikon Eclipse Ni-E C2 microscope. A mixture of propidium iodide and SYTO 9 was added to 1 mL of each of the samples and left for 15 minutes. Then 5 µL of the solution was used for the study. To prevent the sample from fading, BacLight TM mounting oil (from Life Technologies) was added on the dried sample. The analysis was conducted using confocal microscopy as described in Section 2.3. A number of 15 photographs were taken for each sample. Data on the green and red frequency of fluorescence occurrence were obtained using the Nis-Elements AR 4.30 software.

2.5. Machine learning - bagging decision trees

The classifier learning process was based on the input data: frequency of red fluorescence, frequency of green fluorescence and quotient of frequency of red/green fluorescence. The grouping factor was the sample collection site, therefore the activated sludge from the activated sludge chamber was tested as the first classification class, and supernatant water as the second-class classification. MATLAB 2019 a software with the Classification Learner library was used for calculations.

The training data set comprised the results of analyses of 70% of photographs taken with a confocal microscope and the corresponding data in a digital version, which include the frequency of green and red fluorescence for fresh samples. The validation data set included the results of analyses of 30% of fresh sample photographs, while the testing process was carried out on data obtained during additional experiments. They were based on a sample image analysis conducted 24 hours after sampling, 1 hour after adding chlorine to the fresh sample and 24 hours after adding chlorine to the fresh sample. The experimental part was intended to show whether the emergence of abnormal conditions in the form of increasing the age of the sediment and adding chlorine will cause the classifier to be able to recognize the condition of microorganisms as abnormal.

The accuracy of the classifier in the two groups-activated sludge chamber and supernatant water – was determined on the basis of percentage accuracy and indicators accompanying the confusion matrix. These include values of true positive rate for individual real and predictive classes, known in the literature under the acronym TPR, the percentage of false negative rate (FNR), the positive predictive value (PPV) and the percentage of false discovery rate (FDR). In addition, receiver operating characteristic (ROC) curves and the associated areas under the graphs, so-called area under the ROC curve (AUC), were used.

3. Results and discussion

3.1. Particle size distributions

This research has shown that the PSD presented in the form of the volume function $f(v_i)$ differ between individual WWTPs. Table 1 presents the differences between the obtained values of mean diameters, taking into account both the particle number (D (1.0)) and the particle volume (D (4.3)). Due to the large amount of data, only the average values calculated for individual WWTPs were presented.

Due to the spatial structure of the activated sludge flocs, different *DF* values were observed for each WWTP. It was noticed that for all WWTPs the activated sludge in the biological reactors had a more compact structure (*DF* = 1.99-2.31) and a high degree of compaction, unlike treated wastewater from the settling tanks (*DF* = 1.41-2.21). The average *DF* values obtained for all samples from a given WWTP are shown in Table 1. The size of analysed WWTP was compared using the population equivalent (PE) parameter which indicates the multiple of the load of pollutants contained in the wastewater in relation to the unit load of pollutants in household wastewater discharged per capita per day.More compact structure of activated sludge flocs from bioreactors adversely affected the analysis and hindered the procedure of microorganisms identification. The flocs from the outflow had lower density, which can simplify the procedure for identifying bacteria by using lower dilution of the sample.

Table 1. Set of mean diameters and fractal dimensions determined on the basis of distributions of volume function $f(v_i)$ for treated wastewater and activated sludge samples (PE - population equivalent), D (1.0) - particle number, D (3.2) - particle volume, DF - fractal dimensions)

		Activated Sludge			***Treated Wastewater***		
WWTP	***PE***	***D(1.0),*** µm	***D(3.2),*** µm	***DF***	***D(1.0),*** µm	***D(3.2),*** µm	***DF***
A	14800	0.945	21.738	2.27	0.999	20.450	2.05
B	1972	4.645	51.153	2.18	3.006	45.287	2.02
C	6500	0.745	12.413	2.23	0.588	7.034	1.94
D	7700	5.084	81.888	2.08	2.413	46.382	1.88
E	9829	3.282	43.814	2.15	1.857	26.829	1.77
F	2748	4.800	65.476	2.16	1.243	12.405	1.96

Significant differences were also noted in the structure of suspensions comparing the $f(v_i)$ and $f(n_i)$ distributions. A few-micrometer microflocs constituted numerous groups on the $f(v_i)$ distributions. Large diameter particles determined percentage values, while the small ones are usually ignored. As a result of transforming the $f(v_i)$ into the $f(n_i)$ distribution, flocs between 2 and 10 μm constituted the biggest part in particle number of activated sludge and treated wastewater. The smallest identified particles of single bacterial cell size were in the range of 0.36 to 3.56 μm in activated sludge and 0.25 to 3 μm in treated wastewater. Differences in the particles organization on the *f(vi)* and *f(ni)* distribution are particularly visible while comparing the particle size of activated sludge flocs from 1 to 10 μm and from 10 to 1000 μm. In Fig. 1, the dashed lines indicate sizes from 1 to 10 μm and from 10 to 1000 μm, along with information on the particles percentage and particles volume. For the presented case, 2% of volume constituted 94% of the particles in the size from 1 to10 μm. It is advisable to find information about the condition of the whole community especially for this group of particles. The majority of particles forming the activated sludge flocs were really fine and constituted a small mass but occurred in large quantity. It was also recognized that the analysis of treated wastewater in terms of PSD is adequate for the analysis of activated sludge distributions (Burszta-Adamiak et al., 2010; Kuśnierz and Wiercik, 2016), but it takes place without disturbances from flocs with a compact structure. No changes in the fine PND were noticed, which provides a rationale for the implementation of the analysis procedure using supernatant.

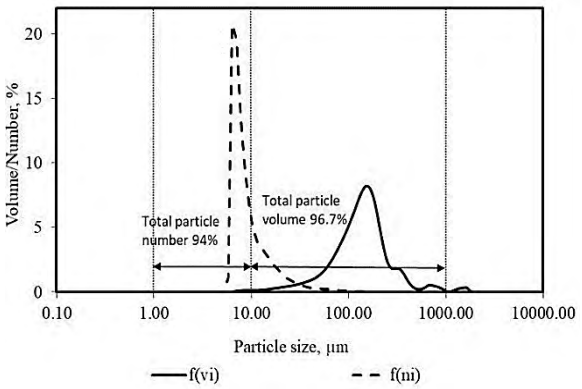


Fig. 1. Particles volume and number of d_i diameters in the total volume/number of activated sludges collected from the E WWTP

3.2. FISH analysis

The results obtained with the laser granulometric tests allowed to develop the argument for the presence of microorganisms responsible for the wastewater treatment process in the supernatant. The FISH method was used to confirm this argument. A particular advantage of the method is the direct visualization of microorganisms under the microscope. The need to target specific microorganisms and the lack of probes capable of directing all bacteria are the main disadvantages of the method. Figs. 2-3 present the results of the NSO1225 probe used to identify ammonia-oxidizing bacteria from the beta-AOB group and the NIT3 probe identifying bacteria from the *Nitrobacter* spp.

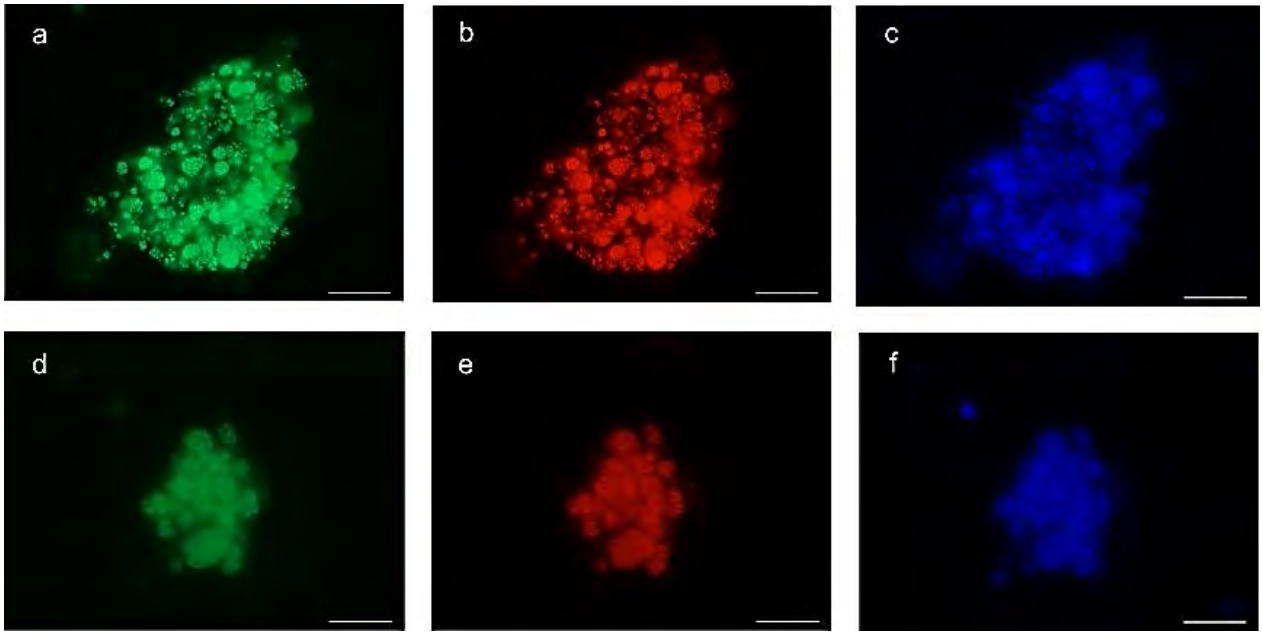


Fig. 2. FISH analysis for determination of beta-AOB in the biomass of activated sludge (a-c) and supernatant (d-e) (7.01.2016); a) and d) hybridizations with the specific oligo probe NSO1225; b) and e) hybridizations with the universal oligo probe EUB388; c) and f) DAPI staining. Scale bar 20 μm for a-c and 10 μm for d-f

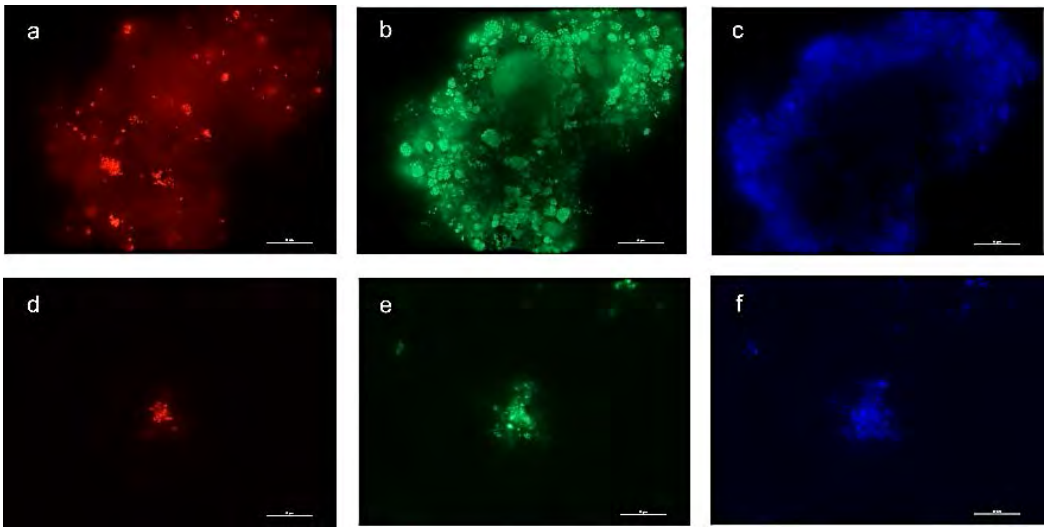


Fig. 3. FISH analysis for determination of *Nitrobacter* spp. in the biomass of activated sludge (a-c) and supernatant (d-f) (7.01.2016); a) and d) hybridizations with the specific oligo probe NIT3; b) and e) hybridizations with the universal oligo probe EUB388; c) and f) DAPI staining. Scale bar 20 μm for a-c and 10 μm for d-f

The research has shown that the beta-AOB and *Nitrobacter* spp. occur both in activated sludge and supernatant. This confirmed that the bacteria responsible for the nitrification/denitrification process are leached from the activated sludge into the supernatant. In recent years, there has been a lot of interest in the viability and cultivability of bacteria. The scientific research indicates that some bacteria are viable, but there are no tools to grow them, some bacteria lose their ability to reproduce for various reasons, and some under certain conditions remain dormant (Kell et al., 1998). This confirms that viable bacteria do not always carry out the wastewater treatment process effectively. Frølund et al. (1996) presented that activated sludge consisted mainly of protein (46-52% of dry matter), humid compounds (18-23% of dry matter) and carbohydrates (17% of dry matter). The proportions of individual components varied depending on the procedure or analytical tools, however, the mass of bacterial cells generally did not exceed 10-15% of the organic fractions of the sediment. Figs. 2-3 show a larger amount of extracellular polymeric substances (EPS) in the flocs taken directly from the activated sludge chambers than microflocs from supernatant obtained after sedimentation. The bacteria separated from the floc are likely to be a group of microorganisms that is most vulnerable to the quality of sewage inflow, which often contains pharmaceuticals or other toxic substances. As a consequence of difficulties associated with the survival of bacteria outside the flocs, the identification of microorganisms in the supernatant allows to draw conclusions about the condition of bacteria in the activated sludge chambers. Their presence or absence on the outflow as well as some transformations could indicate the quality of wastewater treatment process and shorten the time of reaction before total nitrification collapse.

A couple years ago, monitoring at water treatment plants was improved with the method of continuous monitoring of the microbiological composition (Højris et al., 2016). The device is able to measure total number of bacteria within a few minutes based on 3D images. This sensor uses databases of physical particle parameters and is able to recognize with a high probability whether a given particle is a bacterium or not. In the case of WWTPs, it is desirable to know the condition of bacteria responsible for the nitrification/denitrification processes. Despite the high complexity of reactions occurring in the reactor, the efforts are made to explain these phenomena (Fig. 4).

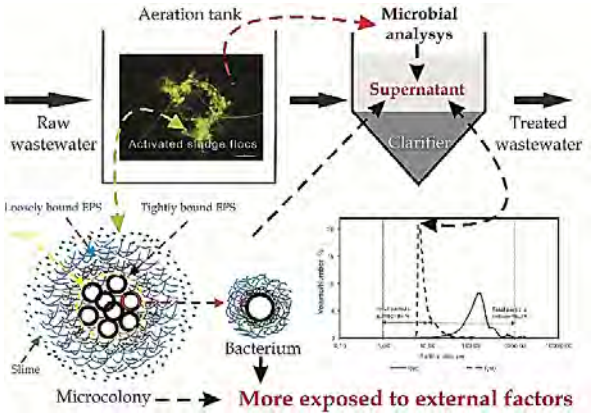


Fig. 4. The approach for the microorganisms identification in the supernatant

Micro flocs and bacteria in the outer flocs are relatively loosely attached to the flocculent matrix and more exposed to external factors. The level of activity of the microorganism in aeration tank is related to the fluctuation of dissolved oxygen (DO), pH and temperature (Cho et al., 2014; Gnida et al., 2016; Harja et al., 2016). Although reaction, temperature and oxidation might be partly controlled at WWTPs, there are many crucial factors concerning incoming sewage that cannot be managed, especially at small WWTPs. Surfactants can inhibit microorganism activity, causing fragmentation of flocs and lysis of protozoa

cells (Dereszewska et al., 2015). The increasing salinity (above 1–2%) also inhibits the activity of sludge microorganism, destroys enzymatic activity of microbes and leads to biodiversity reduction (He et al., 2017). Moussa et al. (2006) presented that *Nitrosomonas europaea* and *Nitrobacter* sp. were the only nitrifiers present at high salt concentration in sludge. What is more, increasing salinity can cause the death of salt-intolerant microorganisms (Wang et al., 2015). Microbial community can change at different COD/N ratios when treating saline wastewater (Wang et al., 2018). While describing the effect of antibiotics on the denitrification process, Roose-Amsaleg and Laverman (2016) noticed that less diverse community is more vulnerable than a diverse one. According to Schmidt et al. (2012), antibiotics are not biodegraded in artificial wastewater and, depending on the dose, nitrification was inhibited. Despite many studies in this field, there are still big gaps in the knowledge of ecotoxicological data on antibiotics (Välitalo et al., 2017).

Considering the abovementioned findings, it seems reasonable to search for answers connected with influence of undesirable factors on activated sludge population in activated sludge chambers and supernatant. For this purpose, further research was carried out using the Live/DEAD staining and machine learning.

3.3. Live/DEAD staining and machine learning

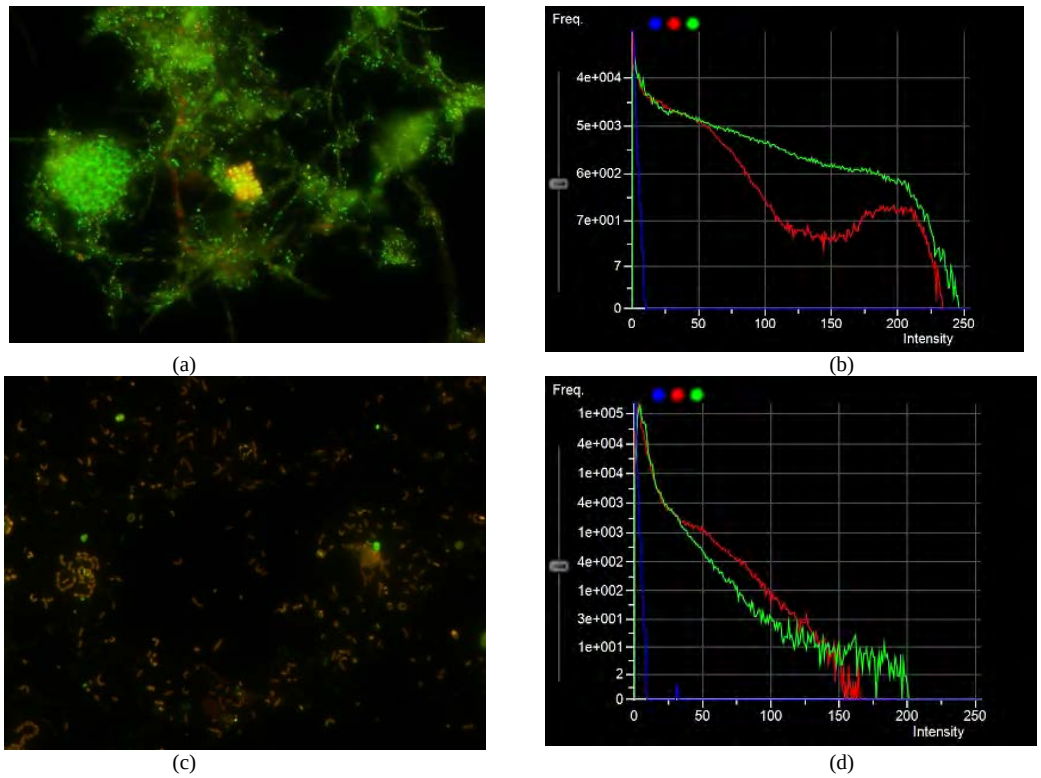
Following previous observations, the machine learning model data from the Live/DEAD staining of activated sludge and supernatant were considered. Additionally, an undesirable external factor in the form of sodium hypochlorite was introduced, which affects the condition of the activated sludge.

Fig. 5 presents the staining results (Fig. 5a,c) and the graphs of intensity and frequency of green and red fluorescence (Fig. 5b,d) obtained in the Nis-Elements AR 4.30 program. It can be seen that a greater number of dead or damaged cells (Fig. 5c) results in higher frequency of red fluorescence than green fluorescence (Fig. 5d). Fifteen photographs were taken for each sediment sample. For each photograph, 250 data on intensity, frequency and ratio of red to green fluorescence were obtained. It made it possible to collect 11 250 data for each sample. The data was then used in the machine learning process.

3.4. Training and validation dataset

The best classifier accuracy was obtained using an ensemble classifier (bagging decision trees). The overall accuracy of the learning process in correctly matching the samples to the two groups – Activated Sludge (AS) and Activated Sludge Supernatant (ASS) – was 99.2%.

The TPR and PPV ratio for the AS class was obtained at the level of 99.0% with FNR and FDR of 1%, while for ASS: TPR, PPV> 99% with FNR and FDR <1%. Subsequently, the accuracy of the classifiers was assessed using the ROC curves. The vertical axis of the ROC curve, which describes the values of the TPR match index, is called the sensitivity axis, while the horizontal axis determines the frequency of false positive rate FPR and it is called the specificity axis.



(a) (b)

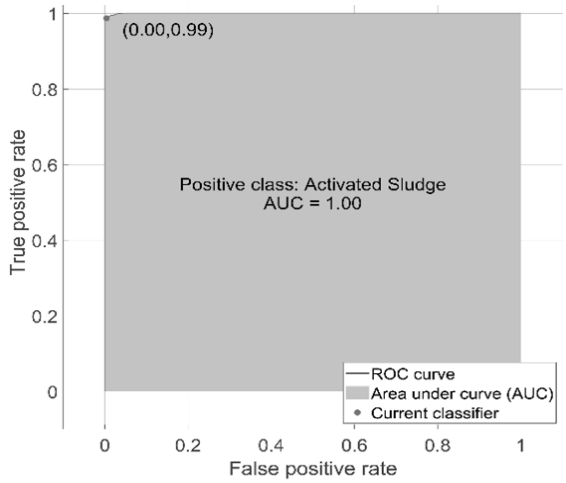
(c) (d)

Fig. 5. Microorganisms from the activated sludge (a) and supernatant (c) stained with Live/DEAD reagent and the graph of intensity vs. frequency of (red/green) fluorescence for the activated sludge (b) and supernatant (d)

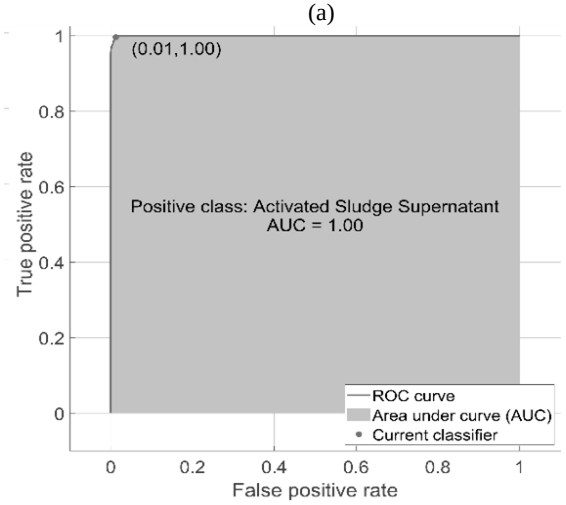
As part of the classification, it is necessary to determine the optimal cut-off point of the ROC curve, which indicates a balance between the sensitivity parameter and the specificity of the classifier. The most optimal cut-off coordinates are values equal to 0 for specificity and 1 for sensitivity-i.e. the (0,1) coordinates (Gajowniczek et al., 2014)

In order to determine the accuracy of the classifier, the area under the ROC curve graph, the so-called AUC is a very important parameter. The closer the AUC parameter is to 1, the higher the match accuracy of the classifier. It is assumed that the AUC in the range of 0.9-1.0 has a very good match of classifier, 0.8-0.9 is good, and 0.7-0.8 is satisfactory.

Fig. 6 presents the ROC curves for the two adopted classification groups: AS (Fig. 6a) and ASS (Fig. 6b). In terms of the AUC parameter, a very good classification accuracy was obtained for both classes (AUC = 1.00). It is also indicated by the cut-off point of both curves, which is close to the coordinates (0,1).



(a)



(b)

Fig. 6. Receiver Operating Characteristic curves for created classification groups: activated sludge (a) and activated sludge supernatant (b)

The classifier validation process, carried out for data obtained from the analysis of five photographs of fresh samples, showed that the classifier is to a greater extent able to recognize samples coming directly from the activated sludge chamber (99.6%), while to a lesser extent (41.9%) from the supernatant. It is caused by higher condensation of activated sludge flocs in the chamber than in the supernatant water, in which they are significantly dispersed. Although the operational condition of a WWTP with activated sludge can be more correctly diagnosed on the basis of samples obtained directly from the activated sludge chamber, it was necessary to verify the behaviour of the supernatant water samples and the activated sludge chamber samples in further experiments, which were carried out as part of the testing.

3.4. Training dataset

The results of the classifier testing process, which was based on increasing the sludge hold time (up to 24 hours) and adding sodium hypochlorite and analysing the image after 1 and 24 hours, are presented in Fig. 7. The results of Live/DEAD staining indicate that for the AS class, the accuracy gradually decreases from 99.6% for fresh sample validation to 67.3%. The addition of sodium hypochlorite resulted in a 71.6% accuracy after 1 hour and 75.7% after 24 hours. The higher accuracy of real and predictive samples results from the unambiguous destruction of the activated sludge floc structure and greater confidence of the classifier concerning grouping. This conclusion is confirmed by images obtained directly with the confocal microscope (Fig. 7). They show that in the case of sodium hypochlorite samples after 24 hours, light whose wavelength is characteristic of red light, corresponding with dead microorganisms, is clearly visible, especially on the edges of flocs. It is due to the fact that chlorine damaged bacteria on the outside of the floc and did not significantly reduce the vitality of the population inside it. In the case of tests with the addition of chlorine after 1 hour, light with green light wavelengths is visible, which is why during machine learning it is more difficult to indicate whether the condition of the activated sludge has already changed. The classifier is better at grouping unambiguous situations, which correspond with abnormal conditions – in this case, adding chlorine and waiting 24 hours.

The situation is different in the case of the ASS class, where accuracy of classifier accuracy is lower than in the case of the AS tests. The experiment based on the addition of sodium hypochlorite caused the death of microorganisms faster than in the case of tests with AS, which confirms greater sensitivity of bacteria in supernatant. In this case, due to the fact that the classifier could only use a group of dead microorganisms, which is characterized by the dominance of light whose wavelength is characteristic of red light, it was not able to carry out the classification process based on only one type of fluorescence. Nevertheless, the death of bacteria in the supernatant was observed much faster than in activated sludge after 24 hours of waiting time.

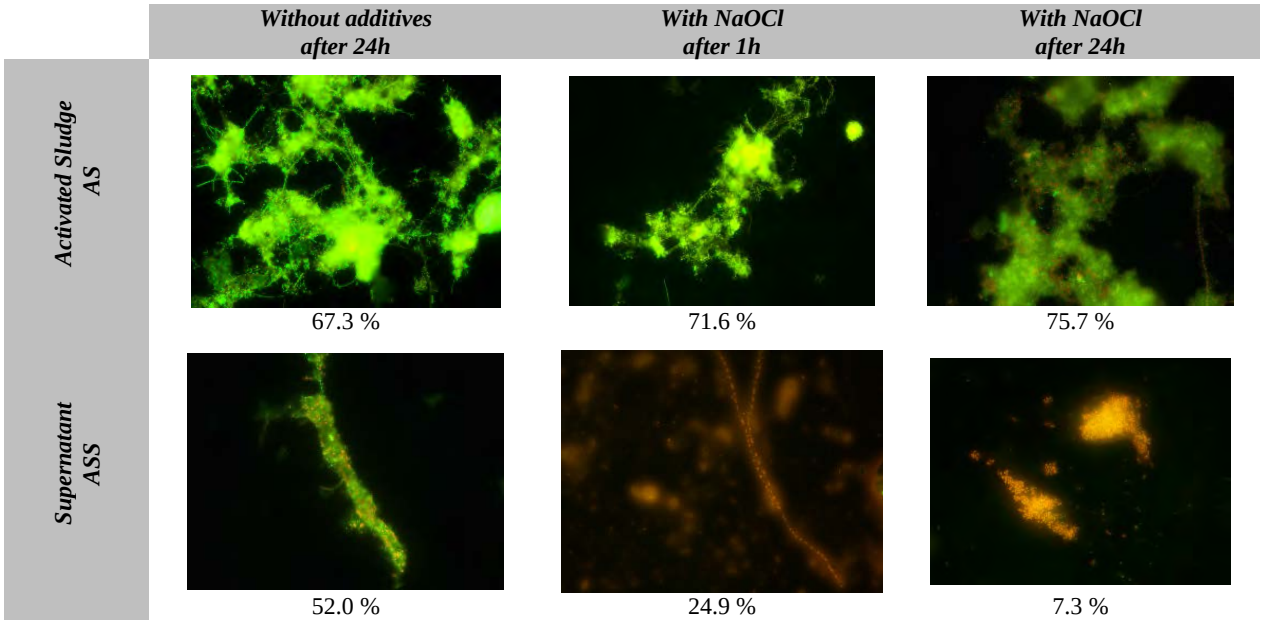


Fig. 7. Percentage accuracy of classification results for predicted group of stained bacteria from activated sludge and supernatant

The normal state of sediment flocs, dominated by living microorganisms, and it's extremely abnormal state with the dominance of dead microorganisms, results in the lowest accuracy of the classifier. The results indicate that these anomalies are identified more quickly in the supernatant (without additives after 24h) than in activated sludge (with NaOCl after 24h).

Machine learning, which in this research used the bagging decision trees, can be applied to assess the status of activated sludge based on the results of analyses carried out with a confocal microscope and Live/DEAD staining. The greatest accuracy was obtained for an intermediate state, in which the onset of the anomaly can be clearly diagnosed. These changes in the supernatant were observed faster than in activated sludge. This is due to what has been established, namely that microorganisms responsible for the wastewater treatment process could be searched in the supernatant group of very fine flocs in the range of 1-10 μm, which constitute about 90% of the total sludge particles. This indicates the usefulness of the proposed methods for detecting the condition of the activated sludge treatment plant and the ability to determine the moment at which, for some reason, its operational balance is disturbed.

4. Conclusions

The collected results confirmed the validity of conducting analyses for the determination of microorganisms in the supernatant. This is a step forward, although further research on a more suitable approach to microbiological analysis of activated sludge is needed; using machine learning is still a challenge too. The research results indicated that beta-AOB and *Nitrobacter* spp. groups occur in both the activated sludge and the supernatant. The presence or absence of the bacteria, as well as some transformation of bacteria (e.g. antibiotic-resistant bacteria) on the outflow, could indicate the quality of wastewater treatment process, demonstrate the influence of some toxic substances on the bacteria population and shorten the time of reaction before total nitrification collapse.

The data gathered for the research clearly reflect the importance of monitoring smaller size particles in the effluent. Taking into consideration that the smallest flocs leaching to the outflow constitute a group of microorganisms that is most numerous and vulnerable to external factors, it is advisable to take into consideration the samples of bacteria mostly responsible for the quality of the wastewater process in the outflow/supernatant. The presented approach requires further research in this field at various WWTPs struggling with the problem of the instability of biological nitrification.

References

Amann R.I., Ludwig W., Schleifer K.-H., (1995), Phylogenetic identification and in situ detection of individual microbial cells without cultivation, *Microbiology and Molecular Biology Reviews*, **59**, 143-169.

Amaral A.L., Mesquita D.P., Ferreira E.C., (2013), Automatic identification of activated sludge disturbances and assessment of operational parameters, *Chemosphere*, **91**, 705-710.

Atanasova N., Kompare B., (2002), *Modeling of Waste Water Treatment Plant With Regression Trees*, WIT Press, 868-879.

Bawiec A., Pawęska K., Pulikowski K., (2017), Analysis of granulometric composition of algal suspensions in wastewater treated with hydroponic method, *Water, Air and Soil Pollution*, **228**, 366.

Bizi M., Baudet G., (2006), Contribution of static light scattering to the textural characterization of large aggregates, *Journal of Colloid and Interface Science*, **300**, 200-209.

Burszta-Adamiak E., Kęszycka M., Łomotowski J., (2010), Structure and granulometric composition of suspensions in sewage sludge and activated sludge, *Environmental Engineering III*, **161**, http://doi.org/10.1201/b10566-28.

Bushell G., (2005), Forward light scattering to characterise structure of flocs composed of large particles, *Chemical Engineering Journal*, **111**, 145-149.

Cho K.H., Kim J.-O., Kang S., Park H., Kim S., Kim Y.M., (2014), Achieving enhanced nitrification in communities of nitrifying bacteria in full-scale wastewater treatment plants via optimal temperature and pH, *Separation and Purification Technology*, **132**, 697-703.

Cydzik-Kwiatkowska A., Zielińska M., (2016), Bacterial communities in full-scale wastewater treatment systems, *World Journal of Microbiology and Biotechnology*, **32**, 66, http://doi.org/10.1007/s11274-016-2012-9.

Dąbrowska J., Kaczmarek H., Markowska J., Tyszkowski S., Kempa O., Gałęza M., Kucharczak-Moryl E., Moryl A., (2016), Shore zone in protection of water quality in agricultural landscape-the Mściwojów Reservoir, southwestern Poland, *Environmental Monitoring and Assessment*, **188**, 467, http://doi.org/10.1007/s10661-016-5470-5.

Deepnarain N., Nasr M., Kumari S., Stenström T.A., Reddy P., Pillay K., Bux F., (2019), Decision tree for identification and prediction of filamentous bulking at full-scale activated sludge wastewater treatment plant, *Process Safety and Environmental Protection*, **126**, 25-34.

DeLong E.F., Wickham G.S., Pace N.R., (1989), Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells, *Science*, **243**, 1360-1363.

Dereszewska A., Cytawa S., Tomczak-Wandzel R., Medrzycka K., (2015), The effect of anionic surfactant concentration on activated sludge condition and phosphate release in biological treatment plant, *Polish Journal of Environmental Studies*, **24**, 83-91.

de Sá L.C., Oliveira M., Ribeiro F., Rocha T.L., Futter M.N., (2018), Studies of the effects of microplastics on aquatic organisms: What do we know and where should we focus our efforts in the future?, *Science of the Total Environment*, **645**, 1029-1039.

Domańska M., Kuhn R., Łomotowski J., Stańczyk E., (2014), FISH method for identification of microbes in wastewater distribution systems, *Environment Protection Engineering*, **40**, 151-160.

Ferrera I., Sánchez O., (2016), Insights into microbial diversity in wastewater treatment systems: How far have we come?, *Biotechnology Advances*, **34**, 790-802.

Fiałkowska E., Pajdak-Stós A., (2018), Temperature-dependence of predator-prey dynamics in interactions between the predatory fungus *Lecophagus* sp. and its prey L. inermis rotifers, *Microbial Ecology*, **75**, 400-406.

Frølund B., Palmgren R., Keiding K., Nielsen P.H., (1996), Extraction of extracellular polymers from activated sludge using a cation exchange resin, *Water Research*, **30**, 1749-1758.

Gajowniczek K., Ząbkowski T., Szupiluk R., (2014), Estimating the ROC curve and its significance for classification models assessment, *Quantitative Methods in Economics*, **15**, 382-391.

Gnida A., Wiszniowski J., Felis E., Sikora J., Surmacz-Górska J., Miksch K., (2016), The effect of temperature on the efficiency of industrial wastewater nitrification and its (geno) toxicity, *Archives of Environmental Protection*, **42**, 27-34.

Guan J., Waite T.D., Amal R., (1998), Rapid structure characterization of bacterial aggregates, *Environmental Science and Technology*, **32**, 3735-3742.

Guglielmi G., Coutts D., Houweling D., Peeters J. (2020), Full-scale application of MABR technology for upgrading and retrofitting an existing WWTP: performances and process modelling, *Environmental Engineering and Management Journal*, **19**, 1781-1789.

Han H.-G., Liu Z., Guo Y.-N., Qiao J.-F., (2018), An intelligent detection method for bulking sludge of wastewater treatment process, *Journal of Process Control*, **68**, 118-128.

Harja G., Nascu I., Muresan C., Nascu I., (2016), Improvements in dissolved oxygen control of an activated sludge wastewater treatment process, *Circuits, Systems, and Signal Processing*, **35**, 2259-2281.

Harrou F., Dairi A., Sun Y., Senouci M., (2018), Statistical monitoring of a wastewater treatment plant: A case study, *Journal of Environmental Management*, **223**, 807-814.

He H., Chen Y., Li X., Cheng Y., Yang C., Zeng G., (2017), Influence of salinity on microorganisms in activated sludge processes: a review, *International Biodeterioration & Biodegradation*, **119**, 520-527.

Højris B., Christensen S.C.B., Albrechtsen H.-J., Smith C., Dahlqvist M., (2016), A novel, optical, on-line bacteria sensor for monitoring drinking water quality, *Scientific Reports*, **6**, 23935, https://doi.org/10.1038/srep23935.

Kell D.B., Kaprelyants A.S., Weichart D.H., Harwood C.R., Barer M.R., (1998), Viability and activity in readily culturable bacteria: a review and discussion of the practical issues, *Antonie van Leeuwenhoek*, **73**, 169-187.

Kim S., Aga D.S., (2007), Potential ecological and human health impacts of antibiotics and antibiotic-resistant bacteria from wastewater treatment plants, *Journal of Toxicology and Environmental Health, Part B: Critical Reviews*, **10**, 559-573.

Koelmans A.A., Mohamed Nor N.H., Hermsen E., Kooi M., Mintenig S.M., De France J., (2019), Microplastics in freshwaters and drinking water: Critical review and assessment of data quality, *Water Research*, **155**, 410-422.

Kuśnierz M., Wiercik P., (2016), Analysis of particle size and fractal dimensions of suspensions contained in raw sewage, treated sewage and activated sludge, *Archives of Environmental Protection*, **42**, 67-76.

Lu Q., Qian W., Liu W., Liu H., Zhou W., Deng J., Wang Q., Sun Y., Zhou K., Ming C., (2017), A fluorescence in situ hybridization method for the measurement of denitrifying phosphorus-accumulation organisms and study on the metabolisms of activated sludge, *Journal of Cleaner Production*, **152**, 28-37.

Mąkinia J., (2010), *Mathematical Modelling and Computer Simulation of Activated Sludge Systems*, IWA Publishing, London, United Kingdom.

Mesquita D.P., Amaral A.L., Ferreira E.C., (2016), Estimation of effluent quality parameters from an activated sludge system using quantitative image analysis, *Chemical Engineering Journal*, **285**, 349-357.

Moussa M., Sumanasekera D., Ibrahim S., Lubberding H., Hooijmans C., Gijzen H., Van Loosdrecht M., (2006),

Long term effects of salt on activity, population structure and floc characteristics in enriched bacterial cultures of nitrifiers, *Water Research*, **40**, 1377-1388.

Nielsen P.H., Daims H., Lemmer H., Arslan-Alaton I., Olmez-Hanci T., (2009), *FISH Handbook for Biological Wastewater Treatment*, IWA Publishing, London, United Kingdom.

Roose-Amsaleg C., Laverman A.M., (2016), Do antibiotics have environmental side-effects? Impact of synthetic antibiotics on biogeochemical processes, *Environmental Science and Pollution Research*, **23**, 4000-4012.

Rustum R., (2009), *Modelling activated sludge wastewater treatment plants using artificial intelligence techniques (fuzzy logic and neural networks)*, PhD Thesis, Heriot-Watt University, United Kingdom.

Schmidt S., Winter J., Gallert C., (2012), Long-term effects of antibiotics on the elimination of chemical oxygen demand, nitrification, and viable bacteria in laboratory-scale wastewater treatment plants, *Archives of Environmental Contamination and Toxicology*, **63**, 354-364.

Seviour R., Nielsen P.H., (2010), *Microbial Ecology of Activated Sludge*, IWA Publishing, 1-688.

Song H.L., Huang S.B., Yang, X.L., (2018), Fate and biodegradation of estrogens in the environment and engineering systems - a review, *Environmental Engineering and Management Journal*, **17**, 977-998.

Stalder T., Alrhmoun M., Louvet J.-N., Casellas M., Maftah C., Carrion C., Pons M.-N., Pahl O., Ploy M.-C., Dagot C., (2013), Dynamic assessment of the floc morphology, bacterial diversity, and integron content of an activated sludge reactor processing hospital effluent, *Environmental Science and Technology*, **47**, 7909-7917.

Szeląg B., Barbusiński K., Studziński J., (2018), Activated sludge process modelling using selected machine learning techniques, *Desalination and Water Treatment*, **117**, 78-87.

Välitalo P., Kruglova A., Mikola A., Vahala R., (2017), Toxicological impacts of antibiotics on aquatic micro-organisms: a mini-review, *International Journal of Hygiene and Environmental Health*, **220**, 558-569.

Wagner M., Haider S., (2012), New trends in fluorescence in situ hybridization for identification and functional analyses of microbes, *Current Opinion in Biotechnology*, **23**, 96-102.

Wang Z., Gao M., She Z., Wang S., Jin C., Zhao Y., Yang S., Guo L., (2015), Effects of salinity on performance, extracellular polymeric substances and microbial community of an aerobic granular sequencing batch reactor, *Separation and Purification Technology*, **144**, 223-231.

Wang S., Gao M., Wang Z., She Z., Xin Y., Ma D., Chang Q., Li Z., Zhang J., (2018), Effect of COD/N ratio on performance of a sequencing batch reactor treating saline wastewater, *Environmental Engineering and Management Journal*, **17**, 1161-1168.

Winkler M.K., Yang J., Kleerebezem R., Plaza E., Trela J., Hultman B., van Loosdrecht M.C., (2012), Nitrate reduction by organotrophic Anammox bacteria in a nitritation/anammox granular sludge and a moving bed biofilm reactor, *Bioresource Technology*, **114**, 217-223.

Zeng T., Li D., Jiang X., Qiu W., Chen Q., Zhang J., (2016), Microbial characteristics of an ANAMMOX biofilter for sewage treatment, *Journal of Water Process Engineering*, **12**, 105-110.