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CHARACTERISTICS OF NITRIFYING BIOMASS FROM SIDESTREAM PROCESS OPERATED AT VARIOUS pH

The nitritation process, i.e., ammonia to nitrite oxidation, is effectively utilized in various methods for treating reject water. The impact of pH on characteristics of nitrifying biomass was investigated in three experiments performed at pH 6.0, 6.5, and 7.0 using real dewatering liquor from the Wrocław Wastewater Treatment Plant. A comprehensive analysis was conducted by applying both microscopic examination and digital imaging to assess the morphology of activated sludge flocs. The aim was to understand how process conditions impact the physical properties and functional performance of nitrifying biomass. The study revealed significant differences between the results of experiments, i.e., considerable changes occurred in the morphology of the activated sludge flocs, mainly their size and structure, as well as filamentous bacteria content. The most substantial changes occurred in the experiment at pH 7.0, which can be attributed to the combined effect of process conditions, i.e., the optimal pH, sludge retention time, and dissolved oxygen concentration as well as the lowest free nitrous acid and free ammonia concentration.

1. INTRODUCTION

The nitritation process, performed by ammonium oxidizing bacteria (AOB), involves the oxidation of ammonium (NH_4^+) to nitrite (NO_2^-), bypassing the full conversion to nitrate (NO_3^-), which occurs in conventional nitrification [1]. It serves as a foundational process in innovative nitrogen removal technologies such as anaerobic ammonium oxidation (Anammox) and shortcut nitrification-denitrification. When employed in wastewater treatment, particularly in sidestream treatment processes, it offers an efficient and cost-effective method for managing high nitrogen loads [2, 3]. To achieve

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stable nitritation, it is necessary to apply factors inducing inhibition of nitrite oxidizing bacteria (NOB), which include temperature, dissolved oxygen concentration, solid retention time (SRT), free ammonia (FA), and free nitrous acid (FNA) [4, 5]. The performance of the nitritation process is intimately linked to the characteristics of the nitrifying biomass. The size and structure of activated sludge flocs as well as the microbial population play a crucial role in the efficiency of the treatment process, and since the biomass is exposed to inhibitory factors in this case, the monitoring of its characteristics is essential [6].

This study investigated the effect of pH on the nitritation process and NOB inhibition stability through three experiments conducted in a sequencing batch reactor (SBR) at various pH, treating real reject water from sludge dewatering with high ammonium levels. It also aimed to explore the impact of varying pH on the characteristics of nitrifying biomass in a sidestream nitritation process, focusing on the eventual morphological changes in activated sludge flocs. Utilizing both microscopic examination and image analysis, the study provides insights into the optimal parameters for maintaining effective nitritation and stable biomass properties.

2. MATERIALS AND METHODS

Inoculum and medium characteristics. The conventional activated sludge (CAS) was used as inoculum biomass for each process start-up. The biomass was collected from a large (over 1 000 000 p.e.) municipal wastewater treatment plant (WWTP) in Poland (Wrocław) with enhanced biological nutrient removal. CAS samples were separately examined at the beginning of each experiment. The experimental reactors were fed with real reject water from the same WWTP. The composition of the influent was stable and typical of this kind of wastewater (average±SD): ammonium 840.6±100.2 mg N/dm^3, total COD 502.4±142.5 mg O_2/dm^3, soluble COD 375.8±56.3 mg O_2/dm^3, alkalinity 3740±405 mg $CaCO_3$/dm^3 and total suspended solids 92.3±42.6 mg/dm^3. More detailed information about the influent is provided in Table 1.

Table 1

Reject water characteristics

Parameter	$[NH_4^+]$	Suspended solids			Alkalinity	Total	Soluble
		Total	Volatile	Mineral	$[CaCO_3]$	COD	COD
Average	840.6	92.3	72.9	26.3	3740	502.4	375.8
Min	439	22	20	0	1985	275	198
Max	987	216	184	128	4180	1012	460
St. dev.	100.2	42.6	29.3	22.8	405	142.5	56.3
RSD*	11.9%	46.2%	40.2%	86.7%	10.8%	28.4%	15.0%
Confidence (95%)	22.2	9.7	6.8	5.6	4585	45.9	17.2
No. of samples	78	74	72	63	75	37	41

RSD – relative standard deviation, all the concentratons in mg/dm^3, ammonium related to N.

Experimental setup and operation. The nitritation process start-up was investigated at pH 6.0, 6.5, and 7.0 in a sequencing batch reactor (SBR). The reactors had a working volume of 150 dm^3 with online control of pH, dissolved oxygen concentration (DO, 1 mg O_2/dm^3), temperature (25 °C), and ammonium and nitrate concentrations. Each SBR operation cycle (240 min) consisted of an aerated reaction phase (180 min, including five feeding phases, 5 min each), sedimentation (45 min), decantation, and an idle phase (both 15 min in total). When applied, excess sludge withdrawal occurred at the end of the reaction. Operating conditions during the experiments are summarized in Table 2.

Table 2

Operating parameters

pH	DO [mg/dm^3]	*T* [°C]	SRT [day]	NLR[a] [kg N/(m^3·day)]	FNA [mg N/dm^3]	FA [mg N/dm^3]
Start-up						
7.0	0.92 ± 0.04	25.2 ± 0.4	16.0 ± 10.8	0.28 ± 0.07	0.05 ± 0.05	0.03 ± 0.04
6.5	1.26 ± 0.93	25.0 ± 0.1	12.4 ± 4.0	0.21 ± 0.09	0.13 ± 0.18	1.18 ± 2.41
6.0	1.41 ± 0.56	25.0 ± 0.2	10.8 ± 3.2	0.23 ± 0.02	0.29 ± 0.38	0.21 ± 0.08
Stable operation						
7.0	1.03 ± 1.06	26.3 ± 1.1	34.6 ± 6.7	0.33 ± 0.04	0.12 ± 0.02	0.004 ± 0.005
6.5	0.98 ± 0.26	25.0 ± 0.0	62.4 ± 24.9	0.19 ± 0.03	0.50 ± 0.06	0.19 ± 0.07
6.0	1.24 ± 0.53	25.0 ± 0.0	20.2 ± 4.6	0.23 ± 0.03	0.91 ± 0.17	0.35 ± 0.12

[a]NLR – nitrogen loading rate.

The initial part of the experiment (start-up phase) was dedicated to achieving the nitrite oxidizers suppression measured as nitrite accumulation rate (NAR) in the effluent. When the NAR reached over 90% the experiment moved into the stable operation phase. The loading rate and operating conditions were kept at a comparable level to the start-up phase, only the excess sludge withdrawal was terminated. Influent, effluent, and biomass concentrations and characteristics were determined 2–3 times a week during the whole experiment. The microscopic and molecular analyses were made at the beginning of the experiments (inoculum) and in later phases to evaluate the biomass characteristics and nitrifier population changes.

Analytical methods. When required, the wastewater samples were filtered immediately after collection using 0.45 μm fiberglass syringe filters. A Hach's (Germany) photometric cuvette tests and a DR3900 spectrophotometer were used for sample analysis: LCK303 and LCK304 (NH_4-N); LCK342 (NO_2-N); LCK340 (NO_3-N); LCK338 (total nitrogen), LCK314 and LCK514 (COD). The alkalinity was measured according to PN-EN ISO 9963-1:2001 standard. The concentrations of the total and volatile suspended solids (TSS and VSS) were measured using fiberglass filters (following PN-EN ISO 872:2007).

Biomass sampling and image acquisition. For the microscopic analysis, 3 types of slides were prepared, i.e., raw samples used for visual evaluation of sludge and samples

subjected to Gram and Neisser staining used for filamentous bacteria identification. Samples were collected directly from the reactors and immediately processed. Images were acquired by bright-field microscopy using an optical microscope Olympus CX33 and cellSens Imaging Software. For raw samples, a volume of about 0.2 cm^3 of the undiluted sample was placed on a microscope slide and covered with a 22×22 mm coverslip, while fixed smears were subjected to Gram and Neisser staining according to the method presented by van Loosdrecht et al. [7]. For the unstained samples, 10 images were taken at 40× magnification and 50 images at 100× magnification (4× and 10× objective), while for the stained samples, about 50 images were taken at 400× magnification (40× objective). One of the criteria for evaluating the condition of activated sludge was the presence of filamentous microorganisms, the amount of which was assessed using the filamentous index (*FI*) ranging from 0 (filament absent) to 5 (excessive numbers of filament) [8].

Image processing and analysis. Image analysis was carried out using DAIME (Digital Image Analysis in Microbial Ecology) software [9], which integrates image processing, image analysis, and 3-D visualization features. The analysis consisted of several steps (Fig. 1), i.e., sample and slide preparation, image acquisition, image processing, and image analysis [10]. Approximately 50 images, the quantity recommended to obtain reliable results [10, 11], of raw sludge samples were analyzed, obtaining parameters describing the count, size (e.g., total area, perimeter, the maximum and minimum diameter of flocs), and shape (circularity) of activated sludge.

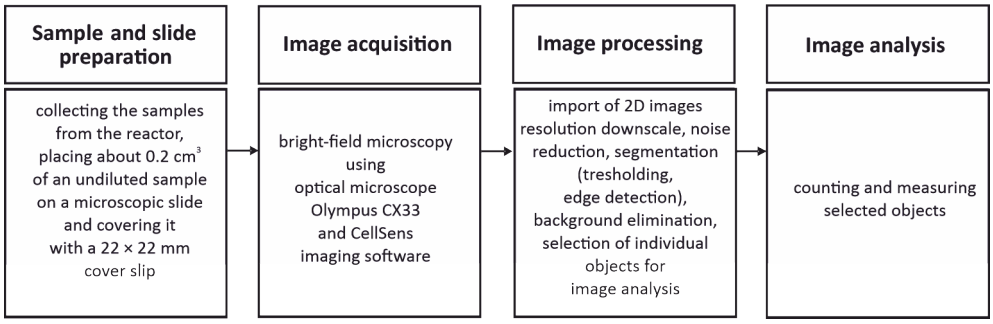

Fig. 1. Schematic representation of image acquisition and processing

3. RESULTS AND DISCUSSION

3.1. PROCESS PERFORMANCE

During all performed start-ups, the nitritation process was successfully established. The selected operating parameters ensured the occurrence of efficient suppression of the NOBs. The 90% NAR level was achieved within 26, 34, and 31 days of reactor

operation at the pH used. In the phase of stable operation, only at pH 6.0 and 6.5 the inhibition of NOB could be maintained. At pH 7.0, after two weeks of stable operation, the NOB activity was restored resulting in nitrate production and rapid decrease of the NAR level.

During stable operation, tested pH affected the substrate availability for nitritation and concentration of the compounds toxic for NOB: free ammonia (FA) and free nitrous acid (FNA). At pH 6.5 and lower, the FNA was in the range of 0.50–0.91 mg N/dm^3, while at pH 7 its concentration was much lower (0.12 mg N/dm^3) resulting in lower pressing on the NOB population. Furthermore, efficient ammonium removal at pH 7.0 resulted in very low FA in the reactor, weakening the impact of another selective factor. Compared to pH 6.0, where the ammonium oxidation rate was at the lowest level (~59%), almost complete removal of ammonium at pH 7.0 was achieved. This resulted in much lower FA concentration during SBR operation, on average 0.004 mg N/dm^3. Such a low FA level is generally considered insufficient for NOB inhibition [12]. As both, FA and FNA were considered crucial elements for sustainable NOB inhibition, the decrease of its concentration resulted in the restoration of nitratation at pH 7.0.

3.2. BIOMASS CHARACTERISTICS

3.2.1. VISUAL EVALUATION

Sludge samples obtained from CAS and start-ups were used to visually evaluate activated sludge flocs structure, strength, shape, and color, as well as protozoans and metazoans content. To obtain accurate measurements of the floc size, the samples were prepared without previous dilution [13]. A compilation of images of CAS used as inoculum and from all start-ups is presented in Fig. 2. The performed analysis revealed significant differences between the sludge samples.

When it comes to the inoculum, the samples exhibited the characteristics of a healthy activated sludge, i.e., a brown color and uniform appearance (Fig. 2a). Under the microscope, sludge flocs were visible as compact, irregularly shaped aggregates built on a skeleton of filamentous bacteria, while the samples taken from the final stage of each of the carried out experiments showed considerable differences in the morphology of the analyzed sludge. The experiment conducted at the lowest pH 6.0 remained the closest to the inoculum, as it was characterized by a predominance of small flocs while depicting smaller dispersion of the sludge (flocs were mostly separate units and did not cluster into aggregates) (Figs. 2b and 2c). In the case of the second experiment conducted at pH 6.5, much more firm and robust flocs of bigger size could be observed, with a more regular shape (Figs. 2d and 2e). The biggest change in floc morphology was observed for pH 7.0. The sludge was characterized by the largest floc size, the most compact structure, and the most regular shape among all the start-ups conducted (Figs. 2f and 2g).

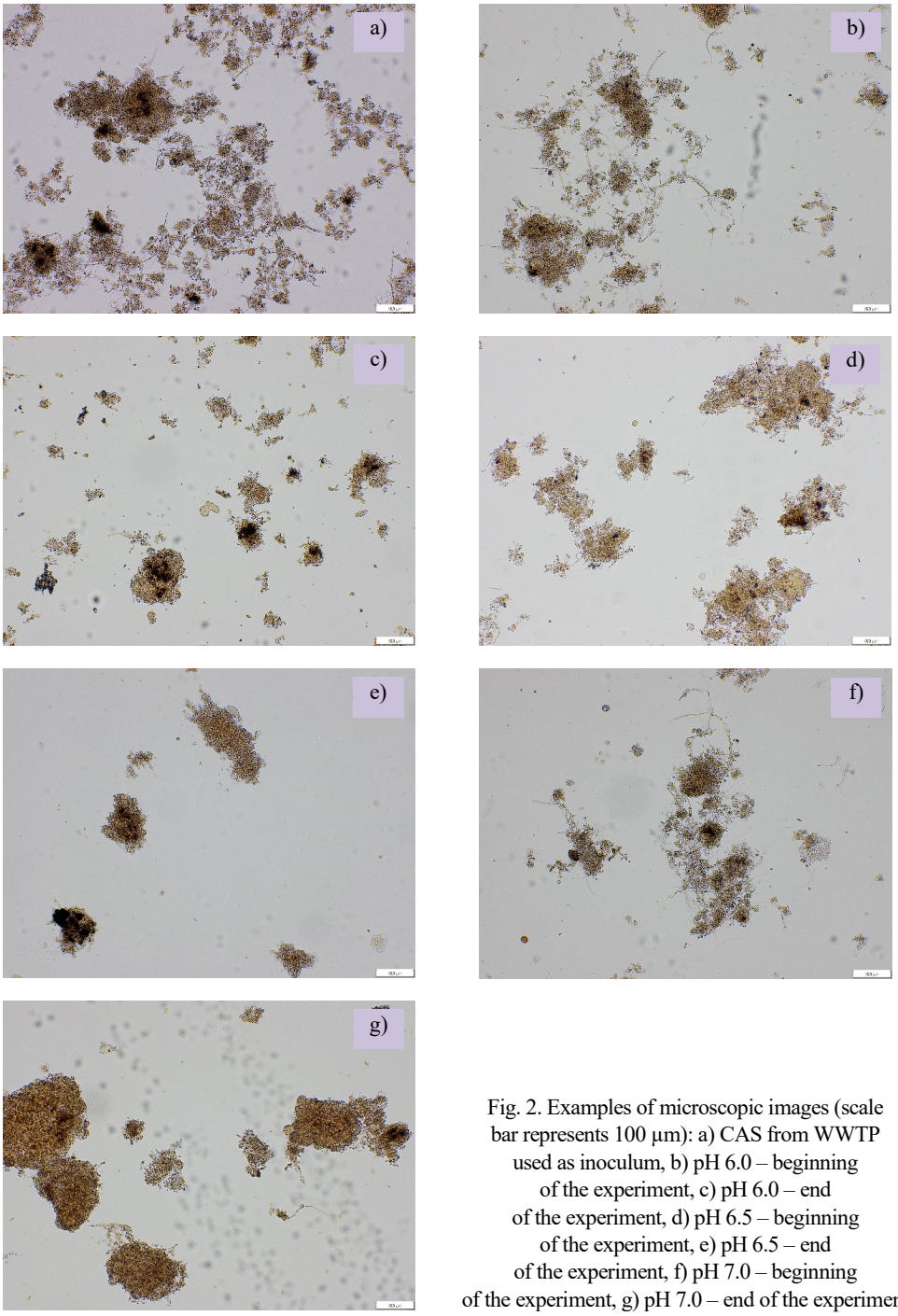

Fig. 2. Examples of microscopic images (scale bar represents 100 μm): a) CAS from WWTP used as inoculum, b) pH 6.0 – beginning of the experiment, c) pH 6.0 – end of the experiment, d) pH 6.5 – beginning of the experiment, e) pH 6.5 – end of the experiment, f) pH 7.0 – beginning of the experiment, g) pH 7.0 – end of the experiment

A number of process parameters, among which solids retention time (SRT), dissolved oxygen concentration (DO), pH, temperature, FNA and FA concentration as well as microbial community and reactor operation parameters, could have influenced the above-described changes. When it comes to the temperature and DO concentration, both of which can affect sludge morphology, all of the conducted start-ups were operated at similar values of these parameters, so they could not have caused such significant differences between the samples. The parameter that may have had a slightly greater impact on the observed changes was likely the SRT. At lower values of the SRT, more irregular flocs can be observed while at higher values they become more compact and regular. Shorter SRTs might not provide enough time for floc formation, while excessively long SRTs can lead to overgrowth of filamentous bacteria [14]. The average SRTs during a stable operation were 20, 62, and 35 days for pH 6.0, 6.5, and 7.0, respectively (Table 2).

Another parameter that could have affected the changes in activated sludge is pH. Even though for all three conducted experiments it was within the limits of the optimal pH for activated sludge (6.5–8.5) [14], this parameter had a key effect on the achieved concentration of FNA and FA in the reactor. Both these compounds display the inhibitory effect on extracellular polymeric substances (EPS) production, which can affect floc formation and strength, although it is important to note that there are few studies on EPS distribution in partial nitrification systems. Higher concentrations of these compounds can distort the structure of activated sludge flocs by interfering with EPS synthesis, which can lead to the formation of smaller and more dispersed flocs [15, 16]. On the other hand, it was found that at very low concentrations of FNA, nitrifying bacteria can produce more EPS as a defensive response [15]. These relationships are reflected in the performed microscopic examinations. At the lowest pH tested (6.0), the highest concentration of free nitrous acid was obtained (0.91±0.17 mg N/dm^3 during stable operation), which may have led to a weaker and more dispersed sludge. As for the highest pH of 7.0, the concentration was at the lowest level (0.12±0.02 mg N/dm^3 during stable operation), and the analyzed samples contained the firmest and most robust flocs of the largest size out of all three start-ups conducted, the reason for which might have been the previously mentioned defense reaction of nitrifiers.

3.2.2. MICROBIAL COMMUNITY

A healthy microbial community in activated sludge includes a variety of microorganisms such as bacteria, protozoans, and metazoans. This diversity is crucial for the breakdown of a wide range of organic pollutants [7]. At wastewater treatment plants where domestic sewage dominates, the protozoan and metazoan community is more diverse, as reflected in the inoculum from the Wroclaw WWTP's [17]. Among the observed protozoa, which are indicators of sludge health, as they help control bacterial populations and consume dispersed bacteria, aiding the clarification process [18], were Testate amoebae (*Arcella* sp.), sessile ciliates (*Epistylis* sp., *Carchesium* sp., *Vorticella* sp.), crawling ciliates (*Paramecium* sp.), free-living ciliates and occasional bacterial mono-

colonies. When it comes to metazoans, mainly nematodes and rotifiers were observed, although in small numbers. As the time of the conducted start-ups progressed, some changes in the microbial community population were observed. A disappearance of higher organisms was observed, as well as sessile ciliates and testate amoebae, while mainly free--living and crawling ciliates remained. A diverse population of microorganisms may have promoted changes in the floc morphology of the examined sludge samples, due to their feeding on various organisms and preying on the smallest flocs [19].

In addition to bacteria, organic and inorganic particles, activated sludge flocs are also formed by filamentous bacteria that form the backbone of activated sludge. However, imbalance between these microorganisms and floc-forming bacteria can lead to problems with sludge bulking or dispersed growth affecting deterioration of settling properties [20]. In the case of the conducted experiments a high number of filamentous bacteria (FI = 3.5) was found in the CAS from WWTP used as inoculum, while in the case of all the three start-ups, their amount gradually decreased and ultimately disappeared altogether reaching FI = 0, which can be observed in the images presented in Fig. 3. The population of filamentous bacteria present in activated sludge is formed by many species, the growth of which is influenced by many parameters, such as SRT, food-to-microorganisms (F/M), nutrients, DO and pH [21]. Based on the experiments, it can be concluded that the conditions under which they were conducted prevented the persistence of these microorganisms in the system.

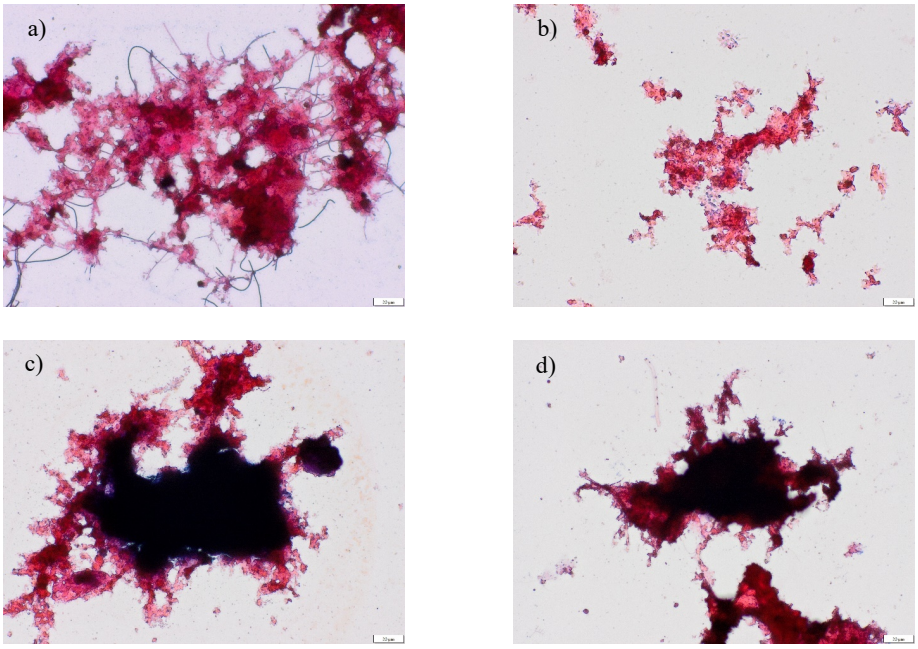

Fig. 3. Examples of microscopic images of Gram-stained samples (scale bar represents 20 μm): a) CAS from WWTP used as inoculum, b) pH 6.0, c) pH 6.5, d) pH 7.0

3.2.3. DIGITAL ANALYSIS

Among the parameters obtained in the DAIME software are the count of activated sludge flocs and their parameters such as total area (μm^2), perimeter (μm), circularity, maximum and minimum diameters (μm).

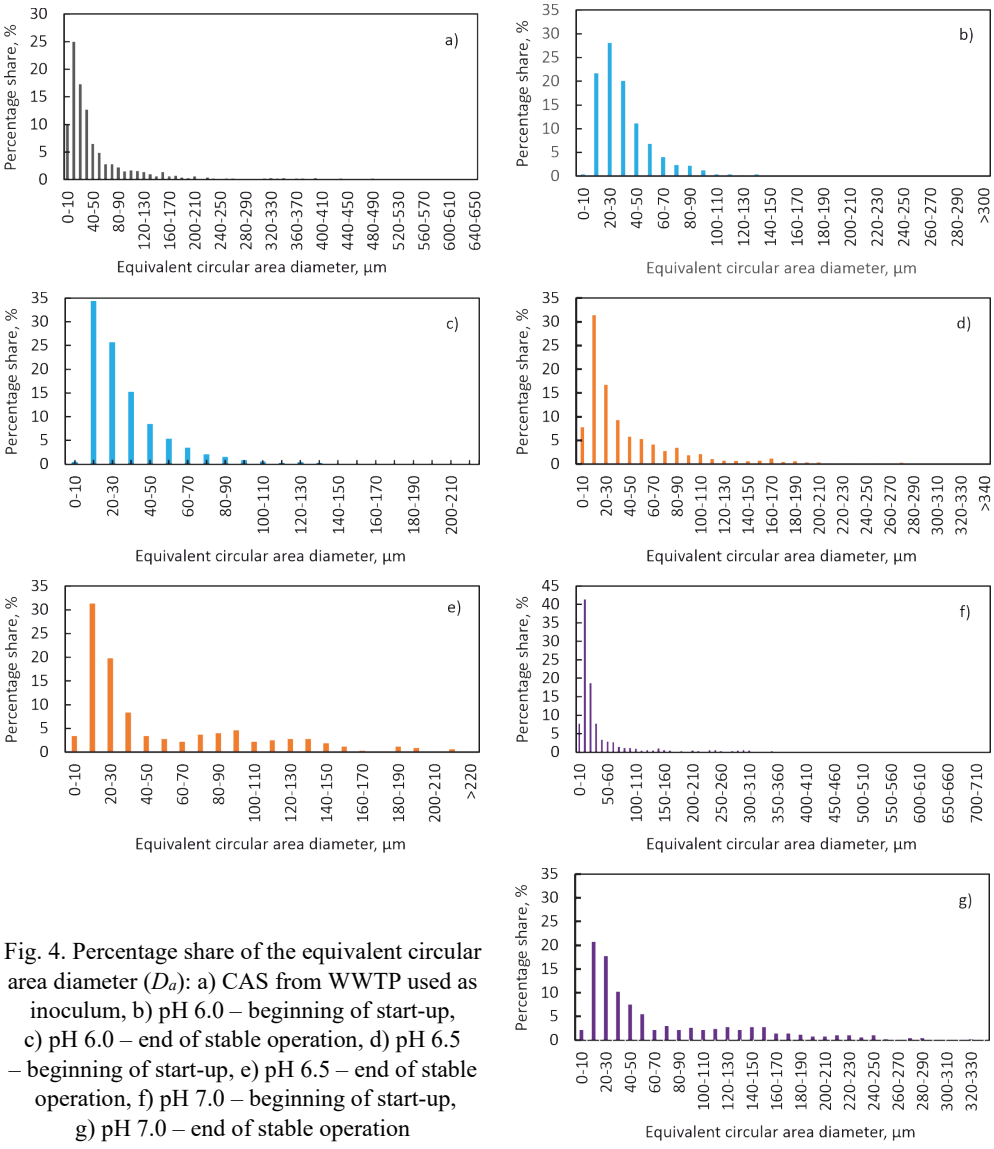

Fig. 4. Percentage share of the equivalent circular area diameter (D_a): a) CAS from WWTP used as inoculum, b) pH 6.0 – beginning of start-up, c) pH 6.0 – end of stable operation, d) pH 6.5 – beginning of start-up, e) pH 6.5 – end of stable operation, f) pH 7.0 – beginning of start-up, g) pH 7.0 – end of stable operation

However, it should be noted that computer analysis of activated sludge made up of aggregates with a rather loose structure carries a large margin of error due to the prob-

lem of determining visible boundaries between separate flocs, which may have been particularly important for the analysis of the sludge used as inoculum. To be able to compare floc size results with each other, an equivalent circular area diameter D_a was calculated (the circle of the diameter D_a has the same projected area as the particle) [22]. As can be seen from Fig. 4 showing the percentage distribution of equivalent diameter (D_a) of the inoculum and of the sludge from the beginning and end of stable operation of each conducted start-up, there were changes in the size of activated sludge flocs throughout the experiments. For the inoculum (Fig. 4a), there were individual flocs with a size as large as 650 µm, but most (71.5%) were in the 0–50 µm range. For the experiment at pH 6.0, 81.4% of flocs belonged to this range at the beginning of the experiment (Fig. 4b) and 84.5% at the end of stable operation (Fig. 4c). For pH 6.5, these values were 76.0% and 66.3% (Figs. 4d and 4e), and for pH 7.0 they were 78.8% and 58.3% (Figs. 4f and 4g), respectively. These results are confirmed by the visual analysis of the microscopic images, as it was apparent that the size of activated sludge flocs increased with the subsequent conducted experiments.

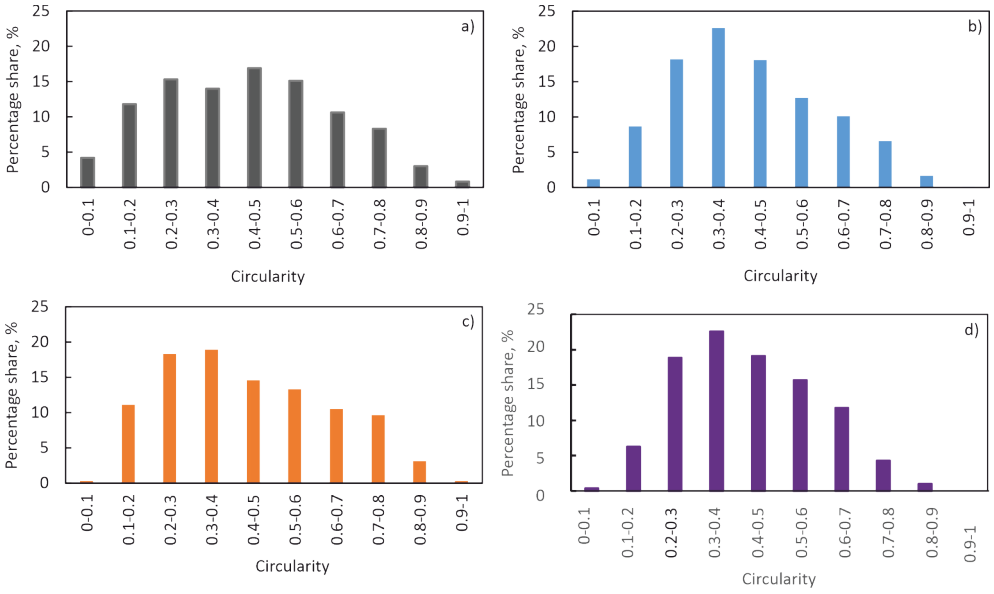

Fig. 5. Percentage share of circularity: a) CAS from WWTP used as inoculum, b) pH 6.0 – end of stable operation, c) pH 6.5 – end of stable operation, d) pH 7.0 – end of stable operation

One of the most commonly used parameters describing the shape of flocs is circularity (this parameter varies from 0 to 1 – the rounder the object the closer it is to 1) [23]. Figure 5 shows the percentage distribution of this parameter in CAS used as inoculum and in three conducted start-ups. As can be seen, all cases discussed are characterized by a similar distribution of this parameter, and most flocs take on a more irregular shape.

However, it is important to point out that their irregular shape is not an alarming factor, as, in reality, it is very rare to find activated sludge flocs with a more circular shape [8]. For a range of 0.1–0.6 of this parameter, flocs took values of 73, 80.3, 76.2, and 82.5% for CAS, pH 6.0, 6.5, and 7.0, respectively. These results show that the more regular floc shape observed in microscopic image analyses for experiments conducted at higher pH was only illusory, however, it is worth noting that measurements of this parameter for sludge with a looser structure (inoculum, pH 6.0) are subject to greater error associated with the determination of accurate floc boundaries.

4. CONCLUSIONS

Although during all performed start-ups the nitritation process was successfully established, only for pH 6.0 and 6.5 NOB inhibition could be sustained during stable operation (higher concentrations of free nitrous acid and free ammonia), while at pH 7.0 their activity was restored due to insufficient pressure caused by inhibitory factors resulting in the restoration of nitratation. The characteristics of the biomass changed significantly throughout the experiments, compared to the inoculum. Since the sludge was exposed to several factors, each of which shows a greater or lesser effect on the biomass, it can be concluded that it was the combined effect of those conditions that caused the greatest changes in the activated sludge of the experiment run at pH 7.0 (the optimal pH, SRT and DO concentration, highest temperature and lowest FNA and FA concentration) and the smallest changes for the experiment run at pH 6.0 (the lowest pH, the shortest SRT and the highest FNA and FA concentration).

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