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Development of *Arcella vulgaris* induced granule formation in an SBR

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ABSTRACT

This study reports aerobic granular sludge formation in a sequencing batch reactor (SBR) treating municipal wastewater at technical scale. An increase in temperature caused a massive increase in *Arcella vulgaris* abundance in the activated sludge. Next, the observed relative abundance of *Arcella vulgaris* gradually diminished, which may have occurred as a result of these microorganisms serving as a scaffolds for granule formation and subsequently being hidden in the granules. As a result of granule formation, biomass concentration in the SBR chamber increased from 5.9 ± 0.8 g MLSS/L to 9.1 ± 0.7 g MLSS/L; the sludge volume index (SVI) decreased from 135 ± 26 mL/g MLSS to 46 ± 16 mL/g MLSS. At the beginning of the period with mature granular sludge, crawling ciliates, attached ciliates and free-swimming ciliates co-dominated. Then a massive development of *Aspidisca cicada* resulted in crawling ciliates becoming the dominant group. The sludge biotic index (SBI) ranged from 9 to 10, which indicated first-class sludge quality.

Keywords: Aerobic granular sludge formation; Microfauna community; Municipal wastewater; Sequencing batch reactor; Testate amoebae

1. Introduction

Aerobic granular sludge is a promising technology for biological wastewater treatment, mainly due to its excellent settling ability and resistance to toxic pollutants in wastewaters [1]. This technology can shorten the time of sedimentation and simplify the separation of effluent from granular sludge. Moreover, a very high biomass concentration can be reached in treatment systems, which means that contaminants are removed at a high rate, which in turn decreases the hydraulic retention time and allows the volume of the reactors to be reduced. From a microbiological point of view, granules consist of different layers where diverse microorganisms can be present and different reactions can take place. Anaerobic and aerobic reactions can occur in the same granule, since the stratification of oxygen and substrate creates different conditions at different points in the granule. For instance, in the outer part of the granule, nitrifiers can grow, while in the inner part, denitrifiers, anammox bacteria or phosphate accumulating organisms can develop under anaerobic and anoxic conditions.

It is thought that aerobic granulation usually takes place in four stages, involving cell-to-cell contact, initial attachment to form aggregates, production of extracellular polymeric substances (EPSs) and hydrodynamic packing [2,3]. Although a number of different mechanisms for the mechanism of granulation have been proposed, the necessary factors are still a subject of discussion. Many authors have stated that the main factors favoring granulation are a high height-to-diameter ratio (H:D) of the reactor [4,5], a short settling time [6], and a high hydrodynamic shear force [2]. However, studies have not always confirmed the validity of these propositions. For example, to improve the formation of granular sludge, short settling times are usually used, ranging from 1 to 20 min, as indicated by McSwain et al. [7]. They suggested that a short settling time was necessary to create predominantly granular sludge. However, Dangcong et al. [8] cultivated aerobic granular sludge with a long settling time of 2.5 h, so the

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necessity of a short settling time is not clear. With regard to hydrodynamic shear force, Mishima, Nakamura [9] suggested that mild shear stresses on the granular sludge, a high dissolved oxygen concentration, and the growth of filamentous microorganisms may be responsible for the formation of granular sludge. In contrast, Chen et al. [10] demonstrated that regular-shaped granules were successfully cultivated with relatively high hydrodynamic shear force, and only fluffy flocs were formed with low shear force, so the importance of shear force in floc formation is also not entirely clear.

For aerobic granulation, not only technological parameters such as settling time, H:D ratio and the intensity of aeration with higher hydrodynamic shear force should be considered, but also the presence of different microfauna in the sludge, which appear to be able to serve as scaffolds for granule formation. For example, according to Chen et al. [11], granular sludge formation may be connected with the presence of filamentous bacteria which become entangled with each other, followed by flocs sticking to the tangled filaments. Weber et al. [12] stated that the cellular remnants of ciliates may serve as a skeleton for granule formation. On the other hand, Li et al. [13] indicated that granules form with the development of vorticella or rotifers. Another theory was given by Wan et al. [14]. They proposed that aerobic granules formed around inorganic cores consisting of calcium and phosphate that precipitated at alkaline pH. The mature granules comprised an inner core, a matrix layer and a rim layer with enriched microbial strains. To form the exopolysaccharide matrix, four functional bacterial strains, *Sphingomonas* sp., *Paracoccus* sp., *S. americanum* strain and *Flavobacterium* sp., grew on the cores.

The present study reports the novel observation that granular sludge formed on the shells of *Arcella vulgaris* in a technical-scale municipal wastewater plant (WWTP) working as an SBR, which has not previously been reported. In addition, the characteristics of the microfauna community during granule formation and in the mature aerobic granular sludge are presented.

2. Materials and methods

2.1. Characteristics of the WWTP

The WWTP (Olsztynek, northeast Poland) where the experiment was performed was designed for an average daily flow rate of wastewater of 4200 m^3/d and works with a mechanical-biological system. The mechanical stage contains a coarse screen (with a grid size of 2 mm) and a grit chamber, whereas the biological stage comprises 2 SBRs, working in parallel, with a total volume of 1740 m^3. The volumetric exchange rate, defined as the ratio of the volume of wastewater supplied to the reactor in a cycle to the working volume, was 0.5. The SBRs were operated with the following technological parameters: dissolved oxygen (DO) during the aeration phase of 3–4 mg/L; DO during the anoxic phase of about 0.5 mg/L, and a sludge retention time (SRT) of 22 ± 3 d. The SBRs were operated in a 12 h cycle. Each cycle consisted of the following four phases: filling/mixing (4 h), aeration (4.5 h), settling (2 h) and decantation and idle (1.5 h).

2.2. Microscopic examination

Samples for microscopic examinations were collected from the mixed liquor of the SBRs during aeration phase during a period of 360 d (from January to December). Twenty-one samples were taken for microscopic examination (grab sampling) as follows: when the reactor was working as an activated sludge reactor, 8 samples were taken one month apart; during granule formation, 1 sample was taken; and each week during operation with mature granular sludge, one sample was taken (12 samples total from this period). When mature granular sludge had been formed, protozoa and small metazoa were identified both in granules (when the granule structure allowed such these identifications to be made) and in the liquid part of the samples. During sampling of granular sludge, an enlarged pipette tip was used. Measurements of the size of flocks in activated sludge, granules and microorganisms in AS and GS were made using the Axio Vision Release 4.4 image analysis system. Protozoa and small metazoa in activated and granular sludge were observed with a Zeiss microscope Axio Imager. A1 equipped with an AxioCam MRc5 digital camera at 100–400× magnification, depending on the size of each taxon. The composition of ciliated protozoa in activated and granular sludge was determined using the keys of developed by Foissner et al. [15–18] and Curds et al. [19]. Testate amoebae were identified based on Ogden and Hedley [20]. Protozoa species were determined "in vivo" according to their morphology and movements. To improve the visibility of testate amoeba inside the granules, the Neisser staining method was used Jenkins et al. [21]. The abundance of metazoans and protozoa (without flagellates) was expressed as the arithmetic mean of the results from observations of three mixed liquor sub-samples, each with a volume of 0.025 cm^3 [22], as measured by with a gravimetrically-calibrated automatic micropipette. Finally, the abundance of individual taxa was counted in 1 cm^3 of activated and aerobic granular sludge. The density of small flagellates was measured using a counting technique described by Madoni [23]. All biological identifications were finished within 3 h of sample collection. For estimation of protist diversity, the Shannon Index (H) [24] was used. The Sludge Biotic Index (SBI) was determined using the method described by Madoni [23].

2.3. Technological analyses

In accordance with Greenberg et al. [25], the sludge volume index (SVI), mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were determined in the samples from the SBRs, and the COD (chemical oxygen demand), BOD_5 (biochemical oxygen demand), TKN (total Kjeldahl nitrogen), P_{tot} (total phosphorus) and TSS (total suspended solids) were determined in the influent and effluent.

3. Results and discussion

The raw wastewater contained relatively high concentrations of organics, expressed as COD. The content of easily biodegradable organics (BOD_5) was lower than usual in common municipal wastewater, as the ratio of BOD_5/COD was 0.5. The concentrations of total nitrogen was

rather typical for municipal wastewater, but the concentration of phosphorus was low. The characteristics of raw and treated wastewater at the WWTP in Olsztynek are presented in Table 1. Throughout the year, changes in the concentrations of pollutants in the treated wastewater were not observed.

Although the amount of sludge withdrawn from the SBR throughout the whole observation period was the same, before granulation the concentration of MLSS was 5.9 ± 0.8 g/L and that of MLVSS was 4.5 ± 0.2 g/L, and after granulation the concentration of MLSS was 9.1 ± 0.7 g/L and that of MLVSS was 7.1 ± 0.3 g/L. Despite the fact that, after 240 days, the concentration of the biomass increased substantially, the SVI decreased from 135 ± 26 mL/g MLSS to 46 ± 16 mL/g MLSS (Fig. 1), indicating good settleability. Slightly lower values of SVI were noted by Li et al. [13]. Those authors found that the mean SVI of granules in which vorticella dwelled were was 43.9 mL/g, but that of granules in which rotifers were present was even lower (33.9 mL/g). These values of SVI corresponded to settling velocities of 20.1 m/h and 38.8 m/h, respectively. Chen et al. [11] observed SVI values in granular sludge of 50–90 mL/g MLSS.

The microfauna community in the SBR showed high diversity. In this study, 23 taxa were identified altogether, including 15 species of ciliates, 3 species of testate amoebae, 2 species of naked amoebae, 2 Rotifera families, small flagellates (flagellates < 20 μm) and nematodes (Tab. 2). In activated sludge (4–245 days), the number of taxa varied from 14 to 19, in granular sludge (277–354 days), it varied from 8 to 16. In all samples (frequencies of occurrence = 100%), small flagellates, attached ciliates (*Aspidisca cicada*, *Vorticella infusionum*), carnivorous ciliates (*Holophrya discolor*), and the testate amoebae *Arcella vulgaris* were found. The attached ciliate *Carchesium polypinum* and the carnivorous ciliate *Litonotus lamella* were observed only in activated sludge. The diversity of the protozoa was significantly higher in activated sludge (H = 2.050 bits ind.$^{-1}$) than in granular sludge (H = 1.751 bits ind.$^{-1}$) (t-test, $P < 0.0001$). In all the analysed samples, the number of small flagellates counted in randomly selected passes across the slide did not exceed 9 individuals (Tab. 2).

Not counting small flagellate species, the total microfauna abundance varied from 12.960 to 37.680 ind./mL in activated sludge and from 7.680 to 31.600 ind./mL in granular sludge (Fig. 2).

Between 4 and 95 d, attached ciliates constituted the dominant group of organisms in the reactor, making up 55–66% of the community. Between 123–214 d, testate amoebae of the species *Arcella vulgaris* proliferated, which was caused by an increase in temperature. An increase in the reproduction rate of *Arcella vulgaris* with a temperature increase from 10°C to 25°C was noted by Laybourn, Whymant [26]. Similarly, Chen et al. [27] found a positive correlation between temperature increase and the abundance of the amoeba of *Arcella hemisphaerica*. The optimum temperature for the growth of most of ciliates ranges from 25 to 30°C [28].

Between 123–214 days, testate amoebae made up 46–66% of the microfauna organisms. At the end of the period with activated sludge (245th day in Fig. 2), the relative abundance of *Arcella vulgaris* gradually diminished; at the same time, granules began to form. The observed abundance of testate amoebae accounted for less than 26% of microfauna organisms in the granular sludge. This decrease in the abundance of *Arcella vulgaris* in samples taken from the SBR was probably connected with the fact that a portion of these micro-

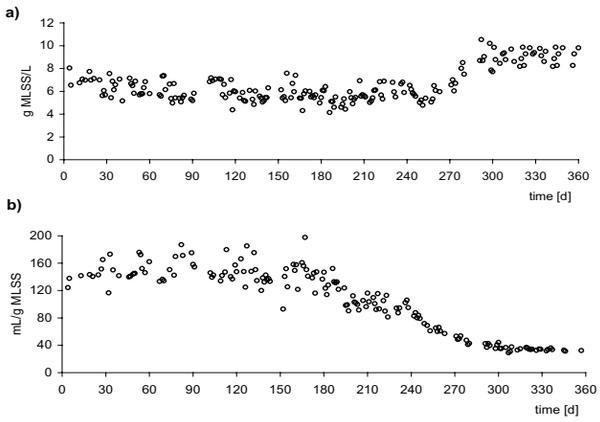

Fig. 1. Changes in biomass concentration in SBR chamber (a) and sludge volume index (SVI) (b) during the year of observations.

Table 1
Characteristics of municipal wastewater

Parameters	Unit	Raw wastewater		Treated wastewater	
		AS	GS	AS	GS
COD	mg/L	1046.0 ± 296.1	1062.0 ± 277.5	24.8 ± 7.7	22.7 ± 8.2
BOD_5	mg/L	524.0 ± 116.4	529.0 ± 113.7	3.1 ± 1.3	3.2 ± 1.2
BOD_5/COD	–	0.5	0.5	–	–
TSS	mg/L	407.6 ± 191.7	420.6 ± 184.5	4.6 ± 2.9	5.6 ± 2.6
P_{tot}	mg/L	9.7 ± 2.3	9.9 ± 2.9	0.32 ± 0.1	0.34 ± 0.2
TKN	mg/L	74.6 ± 9.5	78.5 ± 8.7	8.8 ± 5.2	9.1 ± 4.6
COD/TKN	–	14.0	13.5	–	–
BOD_5/TKN	–	7.0	6.7	–	–
COD/P_{og}	-	108.0	107.2		

Table 2
Abundance and frequency of protozoa and small metazoa in the sludge samples, AS – activated sludge (4–245 days), G – granular sludge (277–354 days)

Taxons		SBR	Abundance (ind./mL)			Frequency (%)
			Mean	Standard deviation	Min–Max	
Flagellates	Flagellates* < 20 µm	AS	4	3	1–9	100
		G	3	1	1–4	100
Crawling ciliates	*Drepanomonas revoluta*	AS	93	188	0–480	22
		G	2403	1299	0–4280	92
	Acineria uncinata	AS	156	183	0–560	67
		G	57	63	0–160	50
	Aspidisca cicada	AS	2222	1644	120–4680	100
		G	6963	9073	28680	100
	Chilodonella uncinata	AS	213	220	0–640	8
		G	10	35	0–120	78
	Euplotes affinis	AS	111	168	0–440	56
		G	17	36	0–120	25
Attached ciliates	*Vorticella infusionum*	AS	1724	1963	360–6800	100
		G	513	359	80–1280	100
	Vorticella convalaria	AS	1284	1289	240–3480	100
		G	503	768	2680	92
	Vorticella octava	AS	284	277	0–760	89
		G	30	92	0–320	17
	Epistytlis coronata	AS	1062	710	280–2240	100
		G	757	1192	0–3240	42
	Carchesium polypinum	AS	2276	1693	920–6240	100
		G	–	–	–	–
	Opercularia coarctata	AS	169	368	0–1120	33
		G	–	–	–	–
Swimming ciliates	*Enchelyomorpha vermicularis*	AS	3116	2004	1120–7640	100
		G	687	907	0–2480	75
Carnivorous ciliates	*Holophrya discolor*	AS	400	363	40–1240	100
		G	653	301	80–1000	100
	Tokophrya quadripartita	AS	44	37	0–120	78
		G	47	53	0–120	50
	Litonotus lamella	AS	36	47	0–120	33
		G	–	–	–	–
Testate amoebae	*Arcella vulgaris*	AS	8311	9195	120–21400	100
		G	1727	627	2920	100
	Cochliopodium bilimbosum	AS	342	213	80–680	100
		G	357	422	0–1320	75
	Trinema sp.	AS	27	45	0–120	34
		G	10	25	0–80	17
Naked amoebae	*Acanthamoeba* sp.	AS	142	148	0–480	89
		G	40	48	0–120	50
	Saccamoeba sp.	AS	18	35	0–80	22
		G	13	26	0–80	25
Rotifers	Lecanidae	AS	80	89	0–240	67
		G	40	78	0–240	34
	Philodinidae	AS	89	115	0–360	77
		G	23	40	0–120	34
Nematodes	Nematoda	AS	5	14	0–40	–
		G	7	23	0–80	8

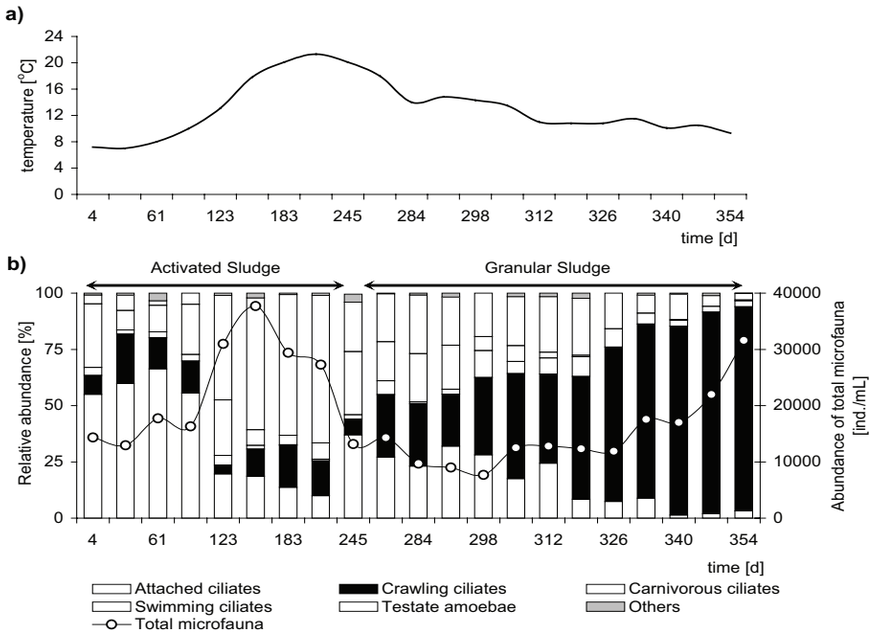

Fig. 2. Temperature changes in SBR (a) and changes in the relative abundance (%) of key groups of microfauna and in the abundance (ind./mL) of total microfauna in activated and granular sludge (b) during the year of observations.

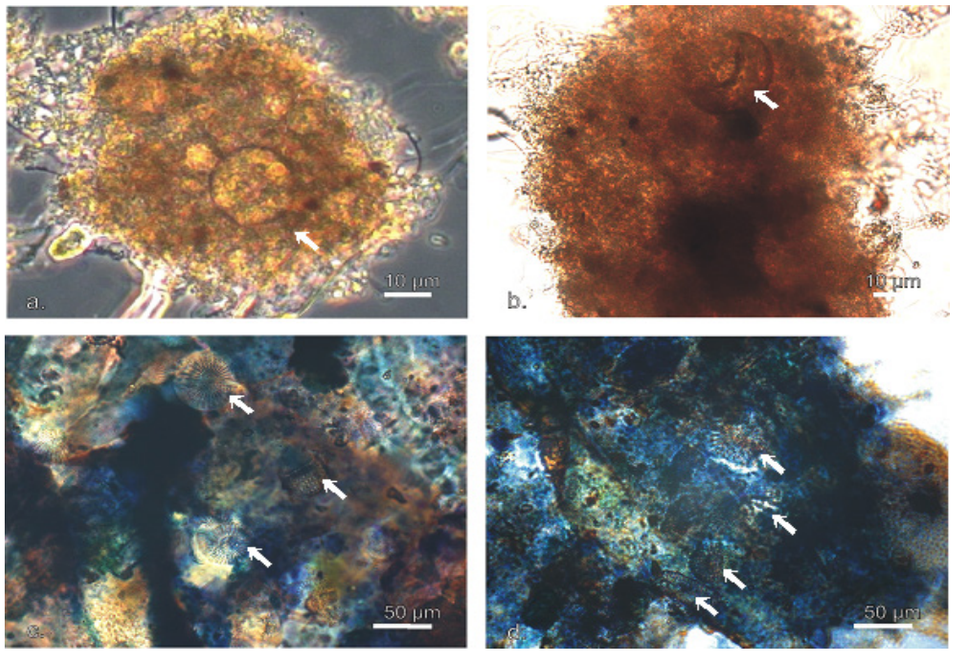

Fig. 3. Granule formation supported by testate amoebae of the species *Arcella vulgaris*, which serve as a physical support for granule formation (a-b), testate amoebae of the species *Arcella vulgaris* inside the granules (c-d); *Arcella vulgaris* has been marked with a white arrows.

fauna were hidden in the granules, as a result of which only some of them were visible and counted. As *Arcella vulgaris* were concealed in the granules, the population size of this species could easily be underestimated (this may have also taken place with the other taxa that are described later). The subsequent decrease in the abundance of testate amoebae could also be due to the decrease in temperature that took place. Due to the lower solubility of oxygen at higher temperature, the aeration intensity in the SBR was increased. This strongly influenced the hydrodynamic shear forces on

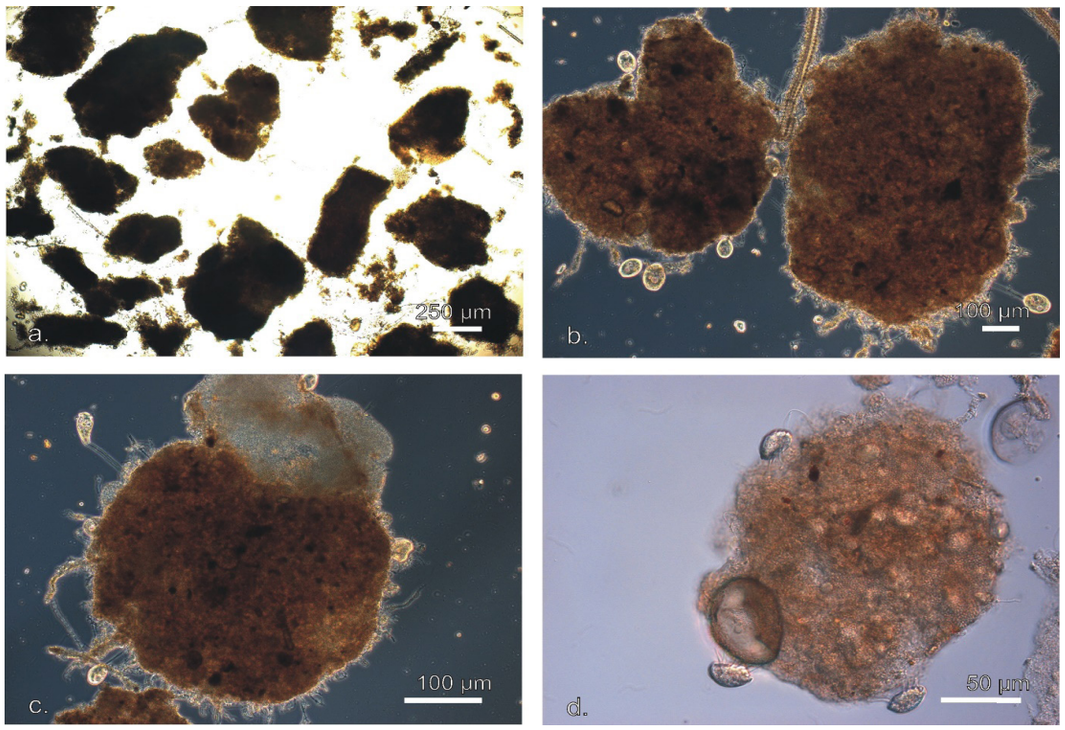

Fig. 4. Mature granular sludge from the SBR operated with municipal wastewater (a–d).

the biomass of the activated sludge. As a result of stress, the microorganisms intensively produced hydrophobic extracellular EPS with a gel structure [29]. This allowed the bacterial aggregates to adhere to the rough shells of *Arcella vulgaris*. The shells became a skeleton of spherical structures around which zoospore bacteria aggregated. The pictures of the granules with *Arcella vulgaris* inside are evidence of this phenomenon (Figs. 3, 4).

When mature granular sludge began to be present, the diameter of the granules was in the range of 200–350 µm. In this the period, crawling ciliates, attached ciliates and free-swimming ciliates (*Enchelyomorpha vermicularis* was the sole representative of the latter group), co-dominated in the microfauna community (277–291 d). Then, the abundance of attached ciliates and free-swimming ciliates decreased and a massive increase in the number of *Aspidisca cicada* resulted in crawling ciliates becoming the dominant group of organisms in the reactor, constituting 34–91% of the community (298–-354, Fig. 2). Although one cannot be certain that attached ciliates and free-swimming ciliates were not hidden in the granules, they were not visible during microscopic analysis. Taking into account the fact that the observed abundance of these taxa was lower during the later part of the period when mature granules were present than at the beginning of this period, it seems likely that the decrease in their abundance was caused by other environmental conditions. Such fluctuations in microfauna community composition are common in systems operated over long periods of time.

The granules in mature granular sludge grew to a size of up to 460–1060 µm in diameter. It is known that aerobic granules display a wide range of sizes, approximately 0.3–5.0 mm in diameter. As the diameter increases, the aerobic granule undergoes morphological and physical changes, and the diffusion of substrates is limited, which could cause problems for reactor operation. In view of the desired biological and physical properties of sludge (low SVI, effective sedimentation), a granule diameter of 1.0–3.0 mm is suggested for SBR aerobic granular sludge so that the reactor performs optimally and is economically effective [5].

During the entire period of observations, the Madoni key group with the highest score predominated (67–97%). This group included crawling, sessile ciliates and testate amoebae. This microfauna composition indicates a healthy, low-loaded sludge that produces high-quality effluent [30, 31]. The SBI, calculated on the basis of the microfauna composition, was in the range of 9–10, which indicated first-class sludge quality.

In the present study, granule formation took place in a typical SBR, and this process may have resulted from *Arcella vulgaris* serving as scaffolds for granule formation after massive development of this species. Granule formation was achieved despite the fact that the SBR chamber did not have the high height-to-diameter ratio (H:D) that is suggested for this process [4]. In the present study, the H:D ratio was 4:20 (0.2). Moreover, although some researchers have stated that one of the requirements for granule formation is a short sedimentation period lasting only a few minutes, in the present study the sedimentation period was longer (2 h, which is typical for activated sludge). This indicates that both of the above mentioned factors are not critical for granule formation. Chen et al. [11] have also stated that a high H:D ratio of the reactor and a short settling time are not essential for the formation

of aerobic granular sludge. Their reactor (conventional, continuous flow, completely mixed activated sludge system fed with synthetic municipal wastewater; settling time 2 h; dissolved oxygen concentration of 4.2 mg DO/L) was inoculated with seed sludge containing few filaments. They suggested that aerobic granules could form if provided with a sufficient number of filaments and high shear force. With sufficient shear force, the filaments become entangled with each other and flocs stick to the tangled filaments. The flocs and filaments, as well as the cavities between them are settled by Zoogloea. At the same time, the filaments strengthen the structure of the floc. With the help of the filaments and the high shear force, the flocculated sludge becomes more and more compact and smooth, and finally forms granules. According to Weber et al. [12] the cellular remnants of ciliates may act like a skeleton for granule formation. The authors found that during malt-house, brewery and artificial wastewater treatment, stalked ciliated protozoa may settle on activated sludge flocs and build tree-like colonies, that are then settled by bacteria. The ciliates become completely overgrown by the bacteria and die. Then, unstalked ciliate swarmers settle on the mature granule surfaces. Granules in which vorticella or rotifers were dwelling were also found during real domestic wastewater treatment [13]. Most vorticella anchored themselves on the granules by stalks, while rotifers attached or adhered to the surface of the granules. The vorticella and rotifers went through a process of growth, bloom and decline that was mainly caused by sludge granulation and available food from detached fine biomass particles.

4. Conclusions

Granulation took place in an SBR during municipal wastewater treatment. Granules formed despite what is considered an inappropriate H:D ratio in the SBR and a long sedimentation period. Massive development of the testate amoebae *Arcella vulgaris* took place at high temperatures in the SBR chamber (21 ± 1°C), and these amoebae may have served as scaffolds for aerobic granular sludge formation. Crawling ciliates, attached ciliates and free-swimming ciliates co-dominated at the beginning of the period with mature granular sludge. After this, crawling ciliates became the dominant group, due to massive development of *Aspidisca cicada*. A high SBI (9–10) indicated first-class sludge quality in both activated and granular sludge.

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