MIDDLE POMERANIAN SCIENTIFIC SOCIETY OF THE ENVIRONMENT PROTECTION
ŚRODKOWO-POMORSKIE TOWARZYSTWO NAUKOWE OCHRONY ŚRODOWISKA
Annual Set The Environment Protection
Rocznik Ochrona Środowiska

Tom 18.	Rok 2016	ISSN 1506-218X	733–745

Anaerobic Ciliates in Activated Sludge Communities

Roman Babko, Tatiana Kuzmina **, Volodimir Pliashechnik***, Grzegorz Łagód****, Janusz Fyda******

Schmalhausen Institute of Zoology NAS of Ukraine, Ukraine, **Sumy State University, Ukraine, ***Uzhhorod National University, Ukraine, ****Lublin University of Technology, Poland, *** Jagiellonian University, Poland*

1. Introduction

The increased volumes of wastewater have stimulated the introduction of new technologies for more efficient wastewater purification. One of the technology that was recently used at the sewage treatment plants is consists in the alternation of aerobic, anoxic and anaerobic phases (Carucci et al. 1994, Hu et al. 2005, Obaja et al. 2005, Spagni et al. 2007). Such conditions – with a high concentration of oxygen and alternating decrease of its content – influence the species composition and abundance of organisms in the activated sludge. One of the possible structural rearrangements within the activated sludge communities can be the expanded role of microaerophile and anaerobe species, among the latter especially those that are able to withstand a certain time in the oxygenated environment. Apparently, the standard (healthy) composition of the activated sludge community typical for aerobic condition changes its structure; therefore, it requires a correction of the activated sludge quality control system. The relevance of the study of plausible bioindicators in advanced treatment systems is confirmed by some recent works (Perez-Uz et al. 2010, Dubber & Gray 2011, Madoni 2011, Babko et al. 2014, Foissner 2016).

At present, there is a lot of information about the organisms inhabiting the anoxic environments or the environments with low oxygen content. Anaerobic ciliates are known to inhabit marine and freshwater bottom sediments (Fenchel 1993, Finlay et al. 1998, 1999), rice fields (Schwarz & Frenzel 2005), municipal landfills (Finlay & Fenchel 1991), as well as stomachs of ruminants (Newbold et al.1995, Tokura et al. 1999). A few of the anaerobic species can be found in the aerobic wastewater treatment plants (WWTPs) where they are an indicator of high-load activated sludge or insufficient aeration, and the poor performance of the WWTP (Foissner et al. 1995, Foissner & Berger, 1996).

Aiming to conduct a preliminary assessment of possible structural rearrangements in ciliate assemblages under aerobic-anoxic-anaerobic technologies of wastewater treatment, we have analyzed the data from treatment facilities with a complete mixing of activated sludge in aerotanks and advanced biological nutrient removal system with alternating oxygen and oxygen-absence conditions.

2. Materials and methods

The material for this work comprises the samples of activated sludge taken in two periods between 2004-2005 and 2013-2014 from 15 WWTPs in Poland. Fourteen of analyzed WWTPs are conventional plants with completely mixed aeration tanks, and 1 is an advanced biological nutrient removing wastewater treatment plant (BNR WWTP) located in Lublin, south-eastern Poland, with bioreaction chamber incorporating aerobic, anoxic and anaerobic zones, working as a modified 5-stage BARDENPHO system described in our previous papers (Babko et al. 2012, Jaromin-Gleń et al. 2013). Activated sludge from Lublin WWTP was also studied during the experiments conducted with laboratory scale sequencing batch reactors (SBRs) which simulated the aerobic/anoxic/anaerobic phases of wastewater purification process: cycle time = 720 min, number of cycles/day = 2, aeration off = 180 min, aeration on = 420 min, settle = 90 min, draw and fill = 30 min, ratio aerobic/anoxic+anaerobic time = 2.3.

A total of 235 activated sludge samples were analyzed, including 151 samples from conventional WWTPs and 84 from SBRs with changing oxygen concentration.

Total suspended solids and N-NH_3 were determined spectrophotometrically by means of DR 3600 spectrophotometer made by Hach-Lange, according to the standard methodology. In order to monitor the operating conditions, each reactor was equipped with LDO probe for dissolved oxygen measurement controlled by HACH-Lange SC1000 station, and pH as well as Redox potential electrode connected with Hach-Lange HQ 40D.

Microscopic analyzes were carried out with an Olympus CX41 microscope. Species identification was performed using the keys of Foissner et al. (1991, 1992, 1994, 1995), Foissner & Berger (1996), Jankowski (1964). The ciliates were identified with the use of phase contrast or dark-field methods, staining of nuclear apparatus with methyl green, as well as silver impregnation with protargol (Foissner, 1991). The ciliates were counted in the microsamples of 25 μm under an 18x18 mm cover glass. For each activated sludge sample 3-5 drops were analyzed, while in case of low ciliate abundance 7 drops were scanned under 100 x magnification.

Statistical data processing was performed with Past 1.57 software (Hammer et al. 2001). Additionally, the Dice's coefficient was used for the analysis of ciliate assemblage (Ludwig & Reynolds 1988).

3. Results and discussion

A total of 102 species of ciliated protozoa were found in the analyzed samples of activated sludge from the studied WWTPs. From them 82 species were found in the samples from aerotanks, while 52 species derived from aerobic/anoxic/anaerobic system (full scale WWTPs and laboratory scale SBRs). Out of this group, 10 species which are common in anaerobic habitats of natural waters were also identified (Table 1). Anaerobic ciliated protozoa and those tolerant to extremely low O_2 content were found only in 12 of the 151 samples from the aerotanks of conventional WWTPs. Among the 84 examined samples from aerobic/anaerobic/anoxic system, such species have been identified in 31 samples. In both types of wastewater treatment bioreactors anaerobic ciliates were present in low numbers: within 13.3-120.0 ind./ml in aerobic bioreactors, and 5.0-53.3 ind./ml in the devices with alterating aerobic/anaerobic/anoxic phases.

The part of anaerobic ciliates in the total ciliate abundance ranged from 0.1 to 3.8 % in the WWTP aerobic bioreactor. In the aerobic/anoxic/anaerobic conditions during normal functioning with alternate aeration and oxygen-absence phases it was about the same: 0.2 to 2.7 %, and under the violation of phases with increase the period of anoxia to 1 day, their share increased to 33.3 %. Under the aerobic/anoxic/anaerobic conditions the amount of anaerobic ciliates remains relatively stable. However, due to the decreased number of oxygen ciliate species, share of anaerobic ciliates in the total number of ciliates increases, thereby increasing their importance in communities of activated sludge.

It should be noted that the occurrence of anaerobic species in the activated sludge from aerobic/anoxic/anaerobic system has not increased in comparison with the activated sludge from the aerotanks. Moreover, some anaerobes found in aerotanks, are not registered in the activated sludge from the bioreactors with changing oxygene concentration. Instead of them, two species were found in aerobic/anaerobic/anaerobic laboratory devices with activated sludge seeded from Lublin WWTP, which were not identified in the other treatment plants – *E. vermicularis* and *A. gracilis*. The last species was characterized by a relatively high occurrence (Table 1).

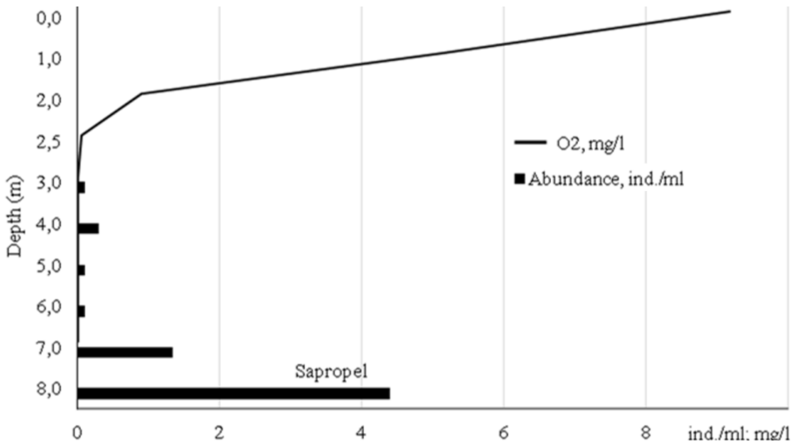
The information about the environmental preferences of this species is scarce. Kahl (1930) indicates that this species, which he calls *Holophrya gracilis* Penard, 1922 than redescribed as *A. gracilis* by Foissner et al., 1994, is an inhabitant of sapropel. According to our own data obtained from the study of lakes in the floodplain of the river Vorskla in the Ukraine (1996-2003), this species is a permanent component of the assembly of anaerobic ciliates being kept in sapropel and anoxic water column. It was recorded during all seasons; the population abundance was 0.1-4.5 ind./ml. The oxygen content and the vertical distribution of *A. gracilis* population in the floodplain lake are show in Fig. 1.

It is obvious that in natural water bodies this species chooses exclusively oxygen-absent habitats. However, it regularly appeared in the activated sludge from WWTPs with aerobic/anoxic/anaerobic conditions. It suggests that this species is able to survive in the environment with low oxygen concentration, and its appearance in the activated sludge was made possible due to the specific conditions provided by the BNR technology with changing aerobic/anoxic/anaerobic conditions.

Tabela 1. Charakterystyka ekologiczna gatunków orzęsków typowych dla warunków beztlenowych w środowisku naturalnym (zgodnie z Foissner et al. 1995, Foissner and Berger 1996, Kahl 1930*, dane własne**) oraz częstotliwość ich występowania w oczyszczalniach ścieków na terenie Polski (dane własne)
Table 1. Ecological characterization of ciliated protozoa species common in anaerobic natural habitats (according to Foissner et al. 1995, Foissner and Berger 1996, Kahl 1930*, author's own data**), and their frequency of occurrence in WWTPs in Poland (author's own data)

Species	Pref. water type	Pref. habitat	Main food	Comm.	Frequency (%)	
					Aero-tanks	ae/ano /ana
Apsiktrata gracilis	S**	Fs*	Al*, Ba**	–	0	32.1
Dexiotricha granulosa	S, F	B, A	Ba	NBE	2.0	1.2
Dexiotrichides centralis	S, F, K	Fs, B	Ba	–	0.7	0
Enchelyomorpha vermicularis	K, F, S	Fs, B	–	MET, HBE	0	1.2
Epalxella sp.	S, F	Fs	Sb	MET	0.7	0
Hexotricha caudata	S, F, K	Fs	Ba	–	1.3	0
Metopus setifer	S, F, K	Fs	Ba, Fl	MET, HBE	0.7	0
Spirostomum teres	S, F	B, P, Fs	Sb, Ba, Al, Ki	COL, HBE	1.3	0
Trimyema compressum	S, F, K	Fs	Ba	MET, COL, HBE	2.0	2.4
Uronema nigricans	F, S	B, A, P, Fs**	Ba, Fl	TRI	0.7	2.4

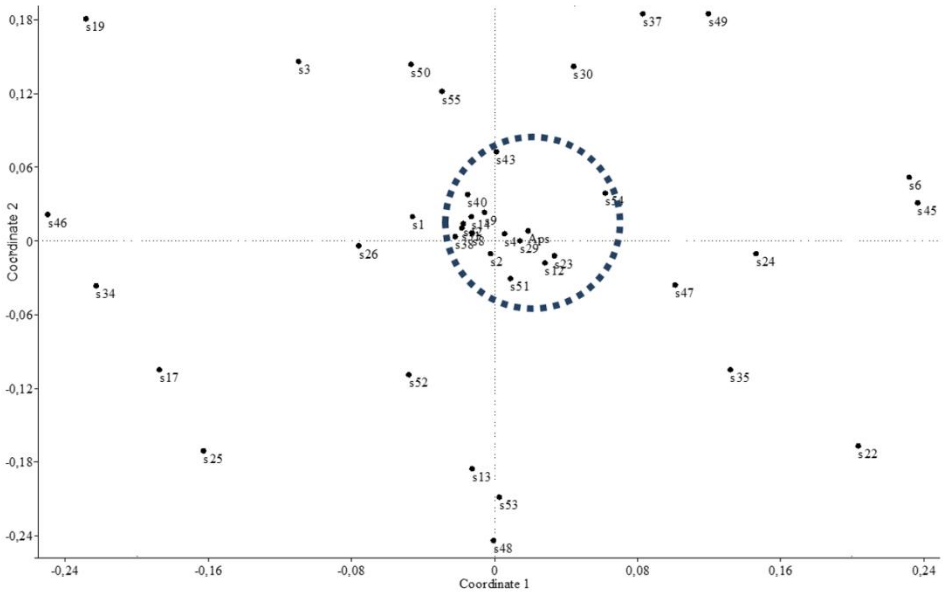
Preferred water type: F – flowing waters, K – sewage-treatment works (activated sludge plants), S – stagnant water. Preferred habitat: Fs – anaerobic mud and anaerobic zones in the pelagial, B – bottom sediments, A – Aufwuchs (periphyton), P – pelagial. Main food: Ba – bacteria, Sb – sulphur bacteria, Al – algae (except of diatoms), Fl – heterotrophic flagellates, Ki – diatoms. Community: COL – *Colpidietum colpodae*, HBE – high-load and/or oxygen deficient activated sludge, NBE – normal activated sludge, TRI – *Trithigmostometum cucullulae*.



Rys. 1. Dystrybucja obfitości *A. gracilis* oraz stężenie tlenu w zależności od głębokości jeziora zalewowego rzeki Vorskla (średnie dla m. wrz. – paźdź. 1997)
Fig. 1. Distribution of *A. gracilis* and the oxygen concentration by depth in the floodplain lake of the river Vorskla (average data for Sept. – Nov. 1997)

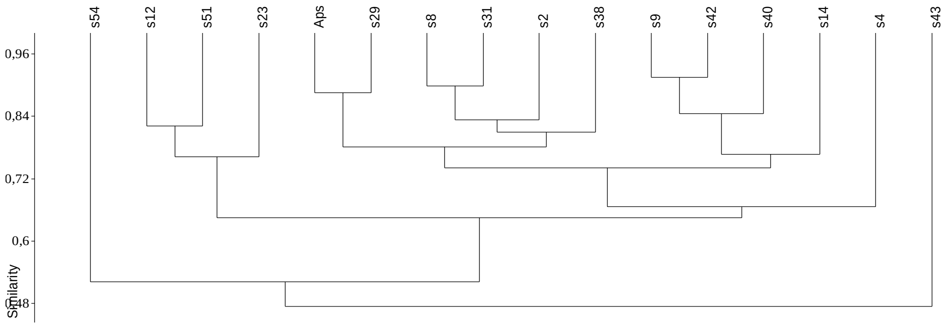
Taking into account the relatively high occurrence of *A. gracilis* in the activated sludge, an attempt to allocate a group of attendant ciliate species was made. Based on the spatial proximity on the plot, some species with the preferences similar to *A. gracilis* were marked (Fig. 2).The cluster analysis was used in order to clarify the species conjugate levels within the selected group (Fig. 3). At the level with over 60 % similarity there are two clusters. In the first cluster, three peritrich ciliates are grouped: *C. polypinum, V. aquadulcis* and *E. coronata*. 10 species form a cluster with three subclusters: *A. gracilis, H. discolor, A. uncinata*, *A. cicada*, *A. lynceus, C. margaritaceum, L. lamella, P. rouxi, P. elongata, T. potamophilus. A. gracilis* shows closest connection with *H. discolor* at the level of 90 %.

Selected species were analyzed with the use of Canonical correspondence analysis method for their preferences to 4 factors: activity of electrons also known as redox potential (Eh), activity of hydrogen ions (pH), total suspended solid (TSS) and NH_3 (Fig. 4). Among these environmental parameters, the next are dependent on oxygen: Eh, pH and NH_3 concentration. The species, which appeared closest to *A. gracilis* on factors preferences were *A. ornata*, *C. polypinum*, *C. margaritaceum*, *H. discolor*, *L. lamella*, *T. potamophilus*, *Thuricola kellicottiana*, *V. microstoma*, *P. elongata*, *A. lynceus*. Thus, using *A. gracilis* as a marker makes it possible to identify the group of ciliate species receiving similar preference in the aerobic/anoxic/anaerobic conditions.

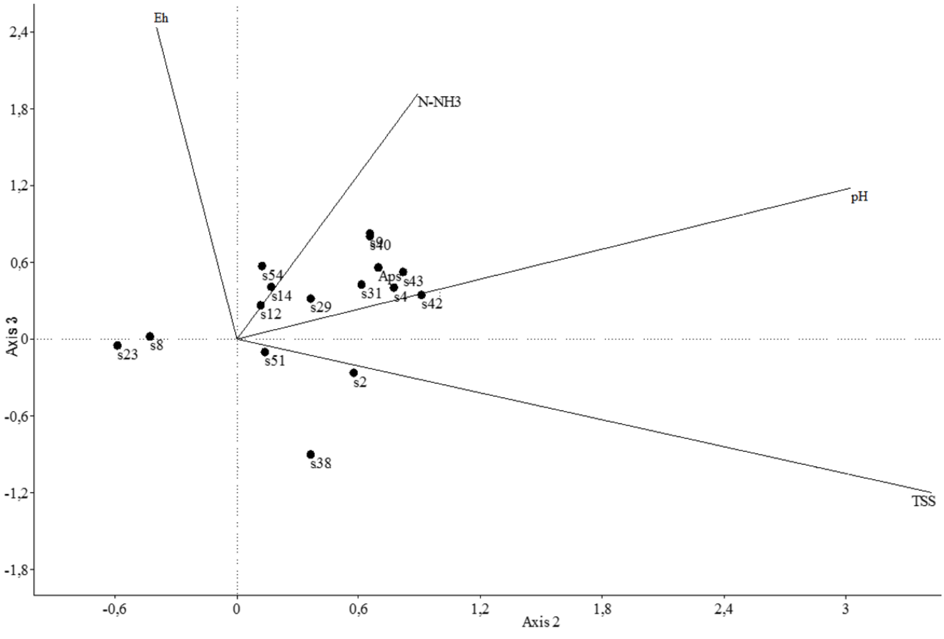


Rys. 2. Diagram ordynacji wykonany metodą skalowania wielowymiarowego 55 gatunków orzęsków w oparciu o współczynnik podobieństwa Dice'a
Fig 2. Ordination of the 55 ciliate species by non-metric multidimensional scaling with Dice coefficient similarity applied

A. gracilis, according to its frequency of occurrence (32 %), is periodically included in this group, when the conditions in aerobic/anoxic/anaerobic phases are optimized for it. At the same time, other species, which receive priority in such circumstances, form quite a stable complex (Table 2). Among selected species, the highest frequency of occurrence was achieved by *H. discolor* – more than 95 %. It enables to consider this species as a proper one. Ciliate species group "*Holophryetum discolorae*" had relatively high similarity to the ciliate species composition, present in a similar bioreactor with activated sludge from extended aeration biological nutrient removal system in the experiments with short-term anoxia (Dubber & Gray 2011) – there represented 31 % of the species selected by us.



Rys. 3. Dendrogram łączenia grup parowanych dla 16 gatunków orzęsków w oparciu o współczynnik podobieństwa Dice'a
Fig. 3. Dendrogram of paired group linkage of the 16 ciliate species using the Dice coefficient similarity



Rys. 4. Diagram ordynacyjny kanonicznej analizy zgodności 16 gatunków orzęsków z czynnikami środowiskowymi: Eh, pH, Z_{og}, N-NH_3
Fig. 4. Canonical correspondent analysis ordination diagram of 16 ciliate species with environmental variables represented by arrows. Environmental variables: Eh, pH, TSS, N-NH_3

Tabela 2.: Występowanie gatunków orzęsków z grupy "*Holophryetum discolorae*" w komorach napowietrzania oraz bioreaktorach ze zmiennym stężeniem tlenu gdzie: x – częstotliwość występowania >50%, * – częstotliwość występowania > 20% oraz ≤50%
Table 2. The occurrence of ciliate species from "*Holophryetum discolorae*" group in aerotanks and bioreactors with changing oxygen concentration; Abreviation: x – frequency of occurrence >50%, * – frequency of occurrence > 20% & ≤50%

		Aerotanks		Ae/ano/ana	
Species of *Holophryetum discolorae* group	Code	All samp.	Samp. with anaer-obes	All samp.	Samp. with an-aerobes
Acineria uncinata	s2	x	x	x	x
Acineta ornata	s4			*	x
Apsiktrata gracilis	Aps			*	x
Aspidisca cicada	s8	x	*	x	x
Aspidisca lynceus	s9	x		*	x
Carchesium polypinum	s12	x	x	x	x
Cinetochilum margaritaceum	s14		*	*	x
Epistylis coronata	s23	*	*	x	x
Euplotopsis affinis	s26	*		*	x
Holophrya discolor	s29	*	*	x	x
Litonotus lamella	s31			x	x
Plagiocampa rouxi	s38	*	*	x	x
Pseudovorticella elongata	s40			*	x
Thigmogaster potamophilus	s42				x
Vorticella aquadulcis	s51	x	x	x	x
Vorticella microstoma	s54			*	*

An even greater similarity of this assemblage was revealed with the ciliate species composition from advanced WWTPs in Spain: 63 % of species were submitted in anaerobic/anoxic/aerobic WWTP in Barcelona, 40 % – in the oxidation ditch WWTP in Seville and 50 % – in the anoxic/aerobic WWTP in Valencia (Perez-Uz et al. 2010).

Some species which were rare or not very frequent in aerotanks, showed an increase in the frequency of occurrence in aerobic/anoxic/anaerobic system, including: *Acineria incurvata, A. ornata, Epistylis longicaudatum, L. lamella, P. elongata, T. potamophilus, T. kellicottiana, Tokophrya quadripartita, V. microstoma, C. margaritaceum, H. discolor*. On the other hand, the occurrence of some species decreased, as apparent in the case of: *Chilodonella uncinata, Vorticella convallaria, V. infusionum*. This is consistent with the data presented by Dubber& Gray (2011), who noted *C. uncinata* among the species most sensitive to hypoxia. Our data are also consistent with the statement made by the same authors that the species *Opercularia microdiscum, V. microstoma, E. coronata* and *A. uncinata* which are able to successfully survive long periods in anoxic conditions. In our study the *H. discolor* and *P. rouxi* have also demonstrated a broad tolerance to anoxic conditions.

4. Conclusions

Based on the research the following conclusions were formulated:

- In the samples of activated sludge from WWTPs in Poland some species which are common in anaerobic habitats of natural waters, such as *A. gracilis, D. granulosa, D. centralis, E. vermicularis, Epalxella* sp., *H. caudata, M. setifer, S. teres, T. compressum, U. nigricans* were found.
- Anaerobic ciliates have a low incidence in aerotanks as well as aerobic/anoxic/anaerobic systems.
- Species composition of the ciliate assemblage in the changing oxygene conditions is characterized by a stable high frequency of occurrence of *H. discolor, A. uncinata, A. cicada, C. polypinum, E. coronata, L. lamella, P. rouxi, V. aquadulcis, P. elongata.*
- More frequent presence of suctoria in the ciliate assemblage under aerobic/anoxic/anaerobic conditions was noted as well. The most frequently encountered species were *A. ornata* and *T. quadripartita*.

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Orzęski anaerobowe w osadzie czynnym

Streszczenie

Występowanie wielu gatunków orzęsków beztlenowych w osadzie czynnym oczyszczalni ścieków pracujących w warunkach aerobowych było do tej pory stosunkowo rzadkie. W przypadku orzęsków beztlenowych, ich obecność traktowano jako sygnał informujący o pogorszeniu jakości osadu czynnego lub występowanie sytuacji zagrożenia dla tlenowego procesu biologicznego oczyszczania ścieków. Rozwój technologii biologicznego oczyszczania ścieków w celu zapewnienia skutecznego usuwania związków węgla, azotu oraz fosforu, doprowadził do uruchomienia nowej generacji oczyszczalni z bioreaktorami pracującymi w naprzemiennych warunkach beztlenowych, niedotlenionych i tlenowych. Wraz ze zmianą technologii powstaje pytanie co do konieczności zmian w rozumieniu i interpretacji miejsca i znaczenia orzęsków anaerobowych w zespołach osadu czynnego oraz ich roli jako indykatorów procesów oczyszczania ścieków. Regularne występowanie w osadzie czynnym, pochodzącym ze zmodyfikowanych oczyszczalni, obligatoryjnych lub fakultatywnych gatunków orzęsków anaerobowych, może być ważnym wskaźnikiem bilansu oraz zrównoważenia kolejnych faz procesu oczyszczania ścieków. W oparciu o przeprowadzone badania osadu czynnego z 15 oczyszczalni zlokalizowanych w Polsce można stwierdzić, że częstotliwość występowania orzęsków anaerobowych jest niska. W sumie zidentyfikowano 10 obligatoryjnych i fakultatywnych gatunków anaerobowych. W zmieniających się warunkach tlenowych/ anoksycznych /anaerobowych najczęściej obserwowane były *H. discolor, A. uncinata, A. cicada, C. polypinum, E. coronata, L. lamella, P. rouxi, V. aquadulcis, P. elongata*. Rozwój badań w zakresie ekologii orzęsków anaerobowych, występujących w oczyszczalniach ścieków oraz w różnych systemach laboratoryjnych, pozwoli rozszerzyć możliwości interpretacji ich roli podczas oczyszczania ścieków metodą osadu czynnego oraz określić ich potencjał jako indykatorów odnośnie występujących warunków procesowych w nowoczesnych oczyszczalniach do zintegrowanego usuwania węgla azotu i fosforu.

Słowa kluczowe: orzęski anaerobowe, osad czynny, biologiczne oczyszczanie ścieków

Keywords: anaerobic ciliates, activated sludge, biological wastewater treatment