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Diversity of Peritricha (*Ciliophora*) in Activated Sludge Depending on the Technology of Wastewater Treatment

Roman Babko[1*], Tatiana Kuzmina[2], Volodimir Pliashechnik[3], Jacek Zaburko[4], Joanna Szulżyk-Cieplak[4], Grzegorz Łagód[5*]

[1] Department Fauna and Systematics of Invertebrates, National Academy of Sciences of Ukraine, 01030 Kyiv, Ukraine

[2] Department of Ecology and Environmental Protection, Sumy State University, 40007 Sumy, Ukraine

[3] Department of Entomology and Conservation of Biodiversity, Uzhorod National University, 88000 Uzhorod, Ukraine

[4] Faculty of Mathematics and Information Technology, Lublin University of Technology, 20-618 Lublin, Poland

[5] Faculty of Environmental Engineering, Lublin University of Technology, 20-618 Lublin, Poland

* Corresponding author's e-mail: babko@izan.kiev.ua

ABSTRACT

The representation of Peritricha ciliates was studied in the activated sludge of two wastewater treatment plants (WWTP), one of which operates using a technology that includes alternating aerobic, anoxic and anaerobic conditions in bioreactors (Lublin, Poland), and the other – only aerobic conditions (Sumy, Ukraine). During the study, 14 Peritricha species were identified in the WWTP Lublin, and 13 species were identified in the WWTP Sumy. The similarity of species lists was 81.5% (Sørensen index). On the basis of on the similarity and taking into account the occurrence rate (≥ 60%), a common group of Peritricha species characteristic of the activated sludge from these two types of treatment facilities was identified, which includes 4 species of colonial Peritricha: *Carchesium polypinum*, *Epistylis coronata, Epistylis longicaudatum* and *Opercularia articulata*, and also 4 species of solitary Peritricha: *Vorticella aquadulcis*, *Vorticella convallaria*, *Vorticella infusionum* and *Vorticella microstoma*. Despite the very high similarity in species composition, statistical analysis of the population structure of Peritricha showed a clear separation of two WWTPs with different wastewater treatment technologies. If there is a significant similarity in the species composition of Peritricha, the species, based on their abundance and occurrence, are divided into two groups, focused on different technological schemes. The population structure of Peritricha responds to the changes in purification technology, showing the changes not so much in species composition as in their quantitative structure. The differences in bioreactor conditions and increased effluent treatment efficiency demonstrated by the WWTP Lublin result in differences in Peritricha species structure, which are reflected in higher Peritricha abundance in WWTP Lublin compared to WWTP Sumy.

Keywords: WWTPs, activated sludge, Attached ciliates, Peritricha.

INTRODUCTION

The process of wastewater treatment with activated sludge has been studied for more than 100 years (Ardern, Lockett, 1914), and the role of protozoa in it has been studied for almost 100 years (Agersborg, Hatfield, 1929). Protozoa eukaryotes in activated sludge, which is a unique analogue of natural biocenoses of aquatic ecosystems, are represented by a fairly diverse community including amoebas, flagellates and ciliates. More than 200 species of protozoa are known to have been found in activated sludge. Of these, about 160 are representatives of the phylum Ciliophora (Madoni, 2011). Ciliates, being represented by a large number of species with diverse ranges of tolerance to environmental conditions, are the most consistent and prominent representatives of eukaryotes in activated sludge (Madoni, 2011; Foissner, 2016). Since the beginning of research into the properties of activated sludge, it has been established that ciliates are not only important consumers of bacteria but also good indicators of the state of activated sludge (Curds, 1992; Arregui et al., 2013; Madoni, 1994; Madoni, 2011; Pauli et al., 2001; Foissner, 2016).

In the process of improving wastewater treatment technologies, the operating conditions of activated sludge are constantly changing, to which the biological system also reacts. An extremely important factor influencing the spatial distribution and functioning of aquatic communities in nature is oxygen. The effect of oxygen on ciliates was studied by Fenchel and Finlay (2008), Fenchel (2014), Babko et al. (2020). The advent of alternating aerobic and anoxic technologies has changed this critical factor in activated sludge bioreactors. Ciliates found themselves under the conditions with periodic oxygen deficiency or absence. Moreover, individual species have different tolerance to oxygen (Fenchel and Finlay, 2008). This issue is poorly developed and requires special research.

Research over the past 10-15 years shows that the activated sludge from wastewater treatment plants with advanced nutrient removal treatment exhibits different biological characteristics compared to conventional activated sludge (Dubber, Gray, 2011a b, Zhang, 2021). Such observations suggest that previous studies of conventional treatment plants cannot be directly extrapolated to newer improved systems (Pérez-Uz et al., 2010). However, even the activated sludge from conventional wastewater treatment plants cannot be considered sufficiently well-studied (Foissner, 2016). Thus, it remains relevant to constantly clarify the optimal operating parameters of activated sludge. At the same time, an important component of research is monitoring the composition and quantitative development of activated sludge organisms. It is necessary to clarify the species composition and indicator significance of individual species (Foissner, 2016).

Peritricha is an essential component of activated sludge (Madoni, 2011). These are (with few exceptions) attached colonial and solitary ciliates that feed on bacteria by filtering them from the surrounding water. Activated sludge flakes provide excellent conditions for the development of attached forms of ciliates (Peritricha), and the presence of bacteria in the liquid phase of activated sludge provides them with constant food. While bacteria perform the main work of recycling organic and inorganic substances contained in wastewater, ciliates, primarily filter-feeding ciliates, assimilate and utilize the bacterial biomass of bacteria suspended in wastewater (Curds, 1992; Arregui et al., 2013).

The purpose of this work was to compare the species composition and quantitative representation of Peritricha in the activated sludge from two WWTPs, one of which operates using a technology that includes alternating aerobic, anoxic and anaerobic conditions in bioreactors, and the other – only aerobic bioreactors.

MATERIAL AND METHOD

The representation of ciliated peritrichous protozoa (Peritricha, Ciliophora) in the activated sludge from two wastewater treatment plants was studied, one of which operates on an advanced technology involving alternating aerobic, anoxic and anaerobic conditions in bioreactors (WWTP Lublin, Poland), and the other is a conventional plant with only aerobic bioreactors (WWTP Sumy, Ukraine). Both wastewater treatment plants receive municipal wastewater. The Sumy city has a population of 256.5 thousand. The volume of wastewater entering the treatment plant is about 40,000 m^3/day. The Lublin city has a population of 348 thousand. The volume of wastewater entering the treatment plant is about 60,000 m^3/day. The indicators of treated wastewater discharged into receiving rivers are shown in Table 1. Significant differences between the conditions in the bioreactors of these wastewater treatment plants consist of different oxygen regimes. Advanced bioreactors are characterized by a wide range of dissolved oxygen content – from 0.4–4.0 mg/l (Foissner, 2016). A detailed description of the conditions in the WWTP Lublin is given in Łagod et al., (2016). In the aerobic bioreactors of the WWTP Sumy, the range of dissolved oxygen content is much smaller: 1.0–2.0 mg/l.

The samples of activated sludge from WWTP Sumy were taken twice a month from April to August 2021. The samples of activated sludge from WWTP Lublin were similarly collected twice per month between April and August 2023. After collection, the samples were transported to the

Table 1. Characteristics of treated wastewater discharged from WWTP Sumy and WWTP Lublin

Indicators	WWTP Sumy	WWTP Lublin
TSS [mg/l]	8.5–15.0	5–10
COD [mg/l]	26.2–30	18.9–27.8
$N\text{-}NH_4$ [mg/l]	3.68–11.58	0.27–4
$N\text{-}NO_3$ [mg/l]	2.54–4.88	0.669–1,1
$P\text{-}PO_4$ [mg/l]	1.48–1.9	0.13–0.28

laboratory and processed in vivo. During processing, the samples were stored in a refrigerator at a temperature of +7 ºC. The samples were processed within several hours after collection with the use of an Olympus CX41microscope. The phase contrast and dark-field methods were used when needed.

Each sample, after mixing it, the subsamples in a volume of 0.25 µl were taken with a micropipette (Madoni, 1994). Five subsamples were counted to account for all organisms, and an additional five samples were examined to determine the number of colonial peritrichs. To identify ciliate species, the following keys were used: Kahl (1930–1935); Foissner et al. (1991, 1992, 1994, 1995); Berger and Foissner (1996); Stiller (1971); Warren (1986, 1987). The ecological indices, representing the measure of biodiversity in the ciliate assemblage, were calculated: Shannon diversity index (H) and Margalef index (Magurran, 2004). In addition, a quantitative Sørensen index was calculated in this analysis. A multivariate technique was used to describe the patterns of change in the distribution of Peritricha species concerning the dependence on wastewater treatment technology. Non-metric multidimensional scaling (nMDS) was used, since it is particularly appropriate for the kinds of ecological data and provides a stress factor which indicates the stability of the ordination (Jongman et al., 1995; Beals, 2006). The best technique for nMDS is described by Shepard (1962) and Kruskal (1964). The goodness of fit of the ordination is expressed by the level of stress function and illustrated on the Shepard diagram. The nMDS technique was applied with Jaccard similarity measure to assess why the sample sites were separating. The detrended correspondence analysis (DCA) was performed to display the similarity Peritricha assemblages between the studied stations in the WWTPs of two cities. Hierarchical clustering was performed. The data were processed using PAST software, version 4.03 (Hammer et al., 2001).

RESULTS

In total, 16 species of Peritricha were found in two wastewater treatment plants; 14 species were identified in the WWTP Lublin, and 13 species in WWTP Sumy (Table 2). In turn, 11 species were observed in both treatment plants. The similarity of Peritricha species lists from these two WWTPs was 81.5% (Sørensen index). The average number of Peritricha species in samples was higher at the Lublin wastewater treatment plant (Fig. 1A). An even greater difference between Peritricha assemblages in the wastewater treatment plants studied was in population density: the average Peritricha population density was significantly higher at the Lublin wastewater treatment plant (Fig. 1b).

The average value of the Margalef index was higher in the bioreactor of WWTP Lublin, and its standard deviation had slight amplitude over the entire period of research (Fig. 2A). In the bioreactor of WWTP Sumy, the average value of the Margalef index was lower, but at the same time it had a significant fluctuation in different periods of research. The Shannon index remained fairly close in both treatment plants (Fig. 2B). Simultaneously, the Shannon index remained very close in meaning at both treatment plants. Cluster analysis was used to identify potential groups of Peritricha species based on their orientation to similar conditions. Cluster analysis showed the presence of two groups of species (Fig. 3). Changes in composition and density of Peritrich populations in two wastewater treatment plants were analyzed using the nMDS program (Jaccard index). According to the nMDS, the algorithms for changing the Peritricha composition in WWTP Sumy and WWTP Lublin were different, as can be seen in the plot. The plot shows two groups above and below the horizontal coordinate axis (Fig. 4). The next stage was to establish the positioning of Peritricha species concerning the points reflecting the Peritricha composition in monthly samples. For this purpose, the DCA method was used (Fig. 5). The percentage significance of the

Table 2. Occurrence and abundance of Peritricha found in the WWTP Sumy and WWTP Lublin

Species	Occurrence Sumy [%]	Average abundance Sumy [ind./ml]	Maximum abundance Sumy [ind./ml]	Occurrence Lublin [%]	Average abundance Lublin [ind./ml]	Maximum abundance Lublin [ind./ml]
Carchesium batorligetiense	0.0	0.0	0	80.0	151.0	375.0
Carchesium polypinum	100.0	276.0	613.3	60.0	20.0	80.0
Epistylis chrysemydis	20.0	93.3	466.7	0.0	0.0	0
Epistylis coronata	100.0	80.0	266.7	100.0	430.3	1140.0
Epistylis entzii	80.0	51.2	120.0	20.0	8.0	40.0
Epistylis longicaudatum	60.0	104.0	320.0	60.0	52.0	260.0
Epistylis plicatilis	0.0	0.0	0.0	60.0	468.0	2260.0
Opercularia articulata	60.0	210.7	680.0	80.0	24.0	100.0
Opercularia coarctata	40.0	50.7	213.3	20.0	4.0	20.0
Opercularia minima	20.0	29.3	146.7	0.0	0.0	0
Pseudovorticella elongata	40.0	24.0	80.0	80.0	65.3	186.7
Vorticella aquadulcis	60.0	50.7	200.0	60.0	376.0	1740.0
Vorticella convallaria	60.0	16.7	40.0	60.0	68.0	220.0
Vorticella infusionum	60.0	75.3	150.0	80.0	228.0	1040.0
Vorticella microstoma	80.0	102.7	413.3	100.0	184.0	300.0
Vorticella minima	0.0	0.0	0.0	60.0	99.0	280.0

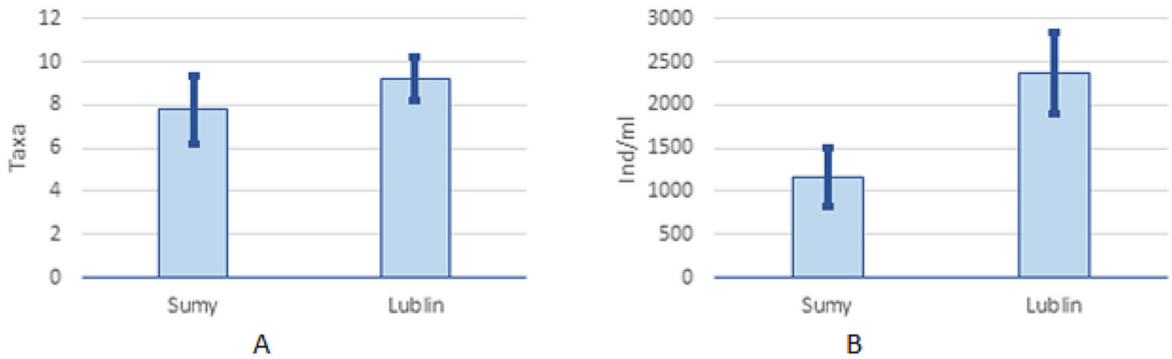

Fig. 1. Average number of species and population density observed in the WWTPs Sumy and Lublin

coordinate axes was as follows: axis 1 – 0.65; axis 2 – 0.29; axis 3 – 0.087; axis 4 – 0.027. According to the DSA analysis, all discovered species of Peritricha are divided into two groups, one of which is associated with the conditions of the WWTP Sumy, while the other group of Peritricha species was associated with the conditions of the WWTP Lublin. The analysis of the species from these two groups coincides with the composition of the groups identified by cluster analysis.

DISCUSSION

Peritricha is a permanent component of the activated sludge community form in various modifications of wastewater treatment plants in different parts of the world (Pauli et al., 2001; Liu et al., 2008; Arregui et al., 2010; Madoni, 2011; Czapluk et al., 2018; Rivas-Castillo et al., 2022).

Currently, the studies that compare the species composition and abundance of ciliate communities formed under the conditions of conventional wastewater treatment plants and improved wastewater treatment plants with nitrogen removal are ongoing (Arregui et al., 2010; Liu et al., 2008; Czapluk et al., 2018). Such studies are necessary to clarify the impact that ciliates have on the functioning of the microbial community, as well as to develop a system for indicating the state of activated sludge and communities that carry out the process of biological wastewater treatment.

The data obtained in the conducted studies showed that under the conditions of an improved

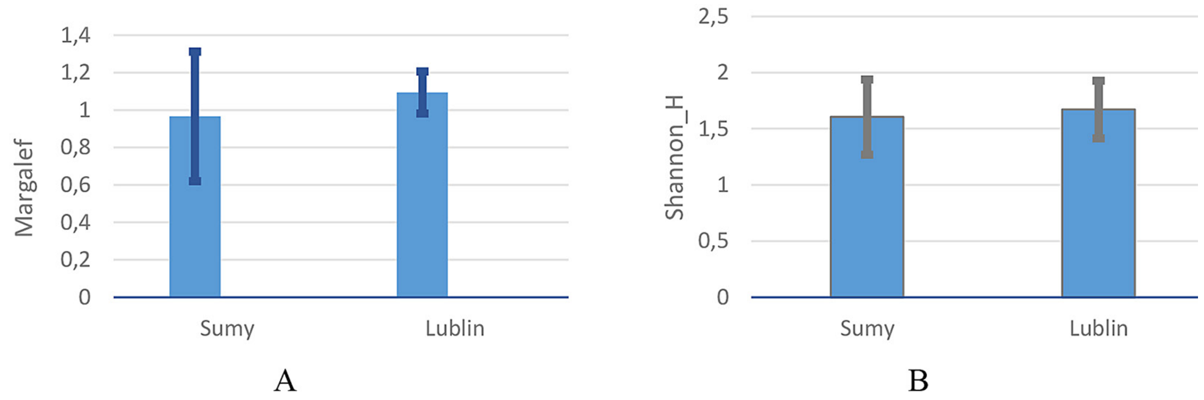

Fig. 2. Average values of the Margalef (a) and Shannon (b) indices based on the species composition and abundance of Peritricha in the WWTPs Sumy and Lublin

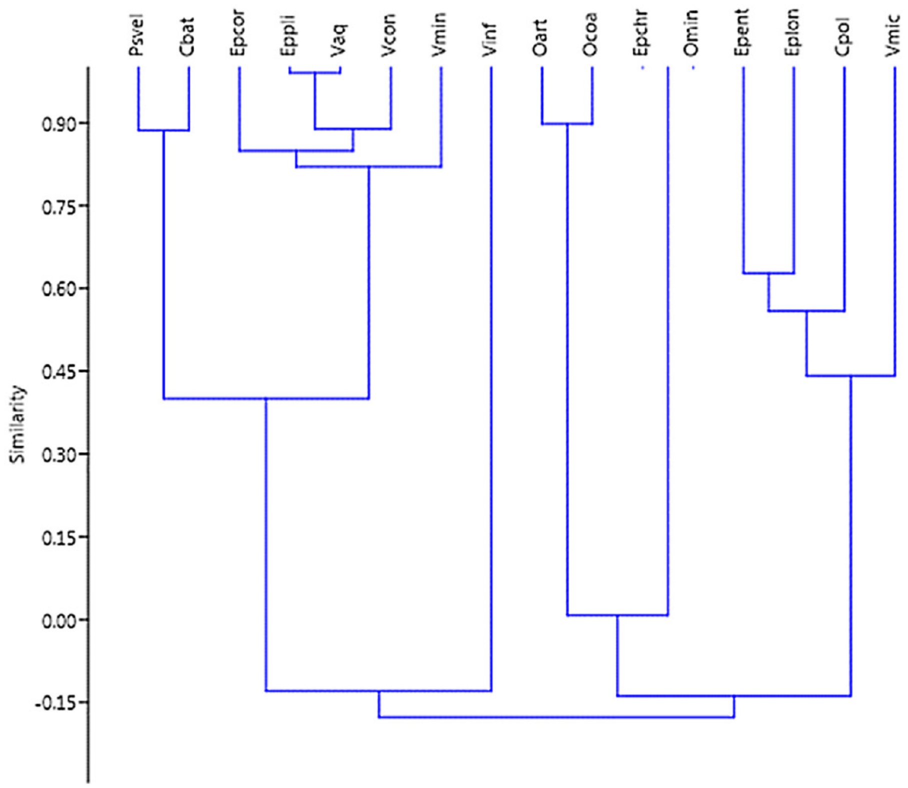

Fig. 3. Results of the similarity tree using correlation distance with paired group linkage of 16 Peritricha species, based on their population densities in activated sludge from the WWTPs Sumy and Lublin

treatment plant and a conventional treatment plant, Peritricha composition similar in species composition are formed. In the improved treatment plant, 14 species were identified, and in the conventional treatment plant, 13 species were identified, with a similarity coefficient based on the Sørensen index of 81.5%. On the basis of the occurrence rate (≥ 60%), a group of species common to two wastewater treatment plants was identified: *Carchesium polypinum*, *Epistylis coronata*, *Epistylis longicaudatum, Opercularia articulata*, *Vorticella aquadulcis*, *Vorticella convallaria*, *Vorticella infusionum* i *Vorticella microstoma*.

The number of identified Peritricha species (12–15) that is close to the obtained results is also shown by other researchers for various treatment plants (Pauli et al., 2001; Liu et al., 2008; Madoni, 2011; Czapluk et al., 2018). In some cases, the authors indicate more than 20 species, but this is the result of the discovery of rare and low-abundance species (Liu et al., 2008). At the same time, the coefficient of similarity between

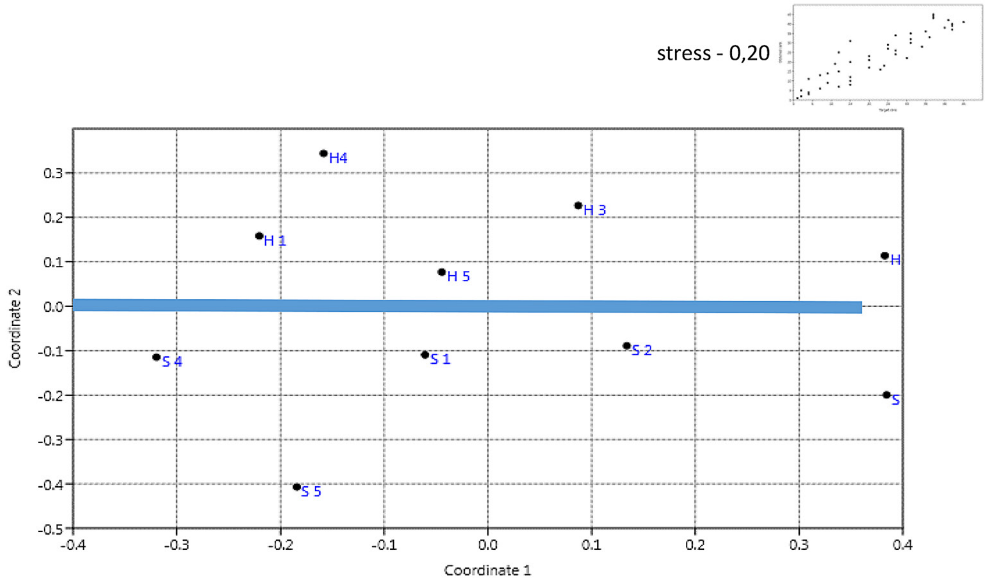

Fig. 4. The nMDS plot based on the Jaccard index. Distribution of monthly samples of activated sludge from WWTPs Sumy and Lublin based on the quantitative representation of Peritricha species. Abbreviations: station– S1-S5 Sumy WWTP and H1-H5 Lublin WWTP

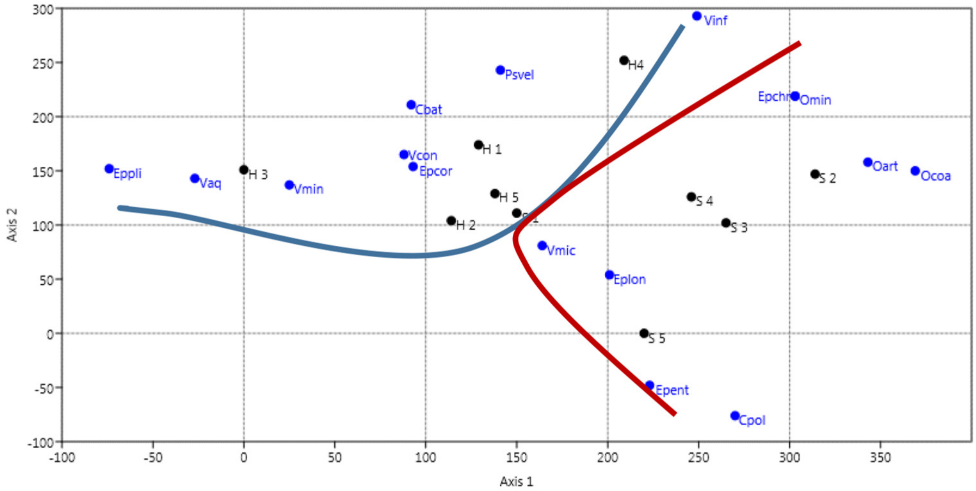

Fig. 5. DCA plot reflecting positioning of species of Peritricha relative to samples from the WWTP Sumy (station – S1-S5) and WWTP Lublin (station – H1–H5)

the obtained data and the data provided by other authors is quite high. For example, the similarity between the Peritricha from WWTP Sumy and the Peritricha from several WWTPs in Italy (Madoni, 2011) was 64%, and similarity between WWTP Lublin and WWTP Italy (Madoni, 2011) was 62%; also WWTP Sumy and WWTP Lublin were similar to the list of the Peritricha species from Wołów WWTP (Czapluk et al., 2018) 62% and 44%, respectively.

There is evidence that the structure of protozoa communities can differ significantly under the conditions of treatment plants with different modifications of the wastewater treatment process. According to some data, under the conditions of an advanced nutrient removal system "ciliate population in the oxic stage of plants with a good nitrification performance shows a very low abundance compared to other protist population" (Arregui et al., 2010). According to other data, on the contrary, ciliates, and especially Peritricha, in modern improved treatment systems reach significant abundances and surpass other groups of protozoa (Liu et al., 2008).

In the conducted studies, there was no dependence of the Peritricha species composition on the type of technological process, but the total abundance of Peritricha in the improved WWTP was, on average, almost twice higher (Fig. 1). In terms of population density in the improved WWTP the dominants were *Epistylis coronata*, *Epistylis plicatilis*, *Vorticella aquadulcis*, and *Vorticella infusionum*. The population density of these species exceeded 1000 ind/ml. In a conventional treatment plant, the populations of *Carchesium polypinum* and *Opercularia articulata* reached the highest densities, over 600 ind/ml.

Despite the high similarity in the species composition of Peritricha in the two treatment plants studied, cluster analysis showed the division of species into two groups (Fig. 3). In the first cluster, species were grouped at a level above 80%. DCA analysis showed that the species allocated to the first cluster gave preference to WWTP Lublin with an advanced nutrient removal system. Cluster 2 included the species that showed a preference for WWTP Sumy, with the relationship between species being below 60%. This indicates greater instability of the community structure under the conditions of aerobic bioreactor.

The difference in the structure of Peritricha under the conditions of the studied technological schemes was shown by nMDS analysis (Fig. 4). On the plot, species groups and sequential sampling at the two wastewater treatment plants demonstrated significant divergence in the coordinate system. This shows that representatives of Peritricha responded to differences in the conditions present in the studied bioreactors, which was manifested in changes in the abundance and occurrence of species. In the WWTP Sumy, the abundance of Peritricha, as well as diversity indices, were lower (Fig. 1 and 2). The anaerobic-anoxic-aerobic technological cycle of the advanced nutrient removal system provides greater diversity and quality of ecological niches. In turn, an increase in the abundance of filter feeder populations ensures a higher quality of wastewater treatment (Table 1).

Obviously, in addition to the indicator role of some Peritricha species (Foissner, Berger, 1996; Arregui et al., 2010; Foissner, 2016), their diversity in activated sludge is itself an important indicator. According to the performed observations, in a well-functioning treatment plant with stable activated sludge, Peritricha is usually represented by several species from 10 to 15, sometimes up to 20 species. Under the conditions with a decrease in the quality of wastewater treatment, a decrease in the number of Peritricha species below 10 is observed. Under the conditions of a decrease in the diversity of Peritricha, it is important to pay attention to the changes in the number of indicator species *Vorticella microstoma* and *Opercularia* spp., the populations of which become numerous with a decrease in the amount of dissolved oxygen and with an increase concentration of toxic substances (Madoni, 1994; Arregui et al., 2013; Foissner, 2016).

It should be noted that while diagnosing the poorly functioning activated sludge is not difficult, identifying the initial stages of deterioration in the operation of a treatment plant seems challenging. In this context, it was shown that in wastewater treatment plants operating according to different technological schemes, even with similar species composition, Peritricha shows marked changes in qualitative and quantitative structure. There is evidence that Peritricha is also a good indicator of the impact of a treatment plant on a receiving water body (Babko et al., 2022).

Along with the fact that the determination of biological indicators is an important component of monitoring the efficiency of wastewater treatment, there continue to be unsolved problems and errors associated with difficulties in determining the species composition of activated sludge organisms (Foissner, 2016). In publications, there are two types of shortcomings associated with the identification of organisms, in particular, Peritricha species: 1) insufficiently detailed identification, when recognition is carried out only to the genus; 2) overly detailed identification, where many species are identified, including synonyms, misidentifications are common, and listing of species that are not found in activated sludge. Errors in species identification significantly reduce the value of the results and, importantly, make diagnoses inaccurate.

To correctly assess the state of the activated sludge community, it is more correct to focus on species that are characterized by a high frequency of occurrence, as well as high and average abundance. There are very few such Peritricha species. Most of them are included in popular keys (Foissner, Berger, 1996); these species have a worldwide distribution.

CONCLUSIONS

The attached forms of ciliates (Peritricha) create similar species composition under conditions of an improved WWTP and a conventional

WWTP. In the improved treatment plant, 14 species were identified, and in the conventional treatment plant, 13 species were identified, with a similarity of species compositions of 81.5% according to the Sørensen index. The general group of Peritricha species for both modifications of WWTPs includes 4 colonial species: *Carchesium polypinum*, *Epistylis coronata*, *Epistylis longicaudatum* and *Opercularia articulata*, and 4 solitary species: *Vorticella aquadulcis*, *Vorticella convallaria*, *Vorticella infusionum* and *Vorticella microstoma*. Despite the high similarity in the species composition of Peritricha from the two WWTPs studied, the Peritricha populations in the advanced treatment plant (WWTP Lublin) reached greater diversity and abundance. This suggests that the Peritricha species may be indicators, sensitive to subtle differences in the conditions found in different bioreactors, or to changes in the conditions within the same type of bioreactor.

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