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Quantitative study of activated sludge population structure

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Abstract. A quantitative study of the population structure of activated sludge is an important component of biological wastewater treatment control. However, in the studying of live samples of the activated sludge, some complications arise, in particular, associated with the relatively short time of the subsample suitability. A subsample is the part of the sample that is placed on a glass slide and in which organisms are counted. The issue of optimization of counts of organisms with large amplitude of population density is considered. The results of counting ciliated protozoa in activated sludge were described. The samples were counted in 45 sub-samples of 25 μl each. An average of 10 counts was required to achieve high reliability in determining population densities with more than or equal to 1 specimen per 25 μl in sub-samples. For small population densities (less one specimen per 25 μl) of free-swimming, crawling, and sessile ciliates, 30 counts are necessary. When the density of colonial protozoan populations is established, the number of counts should be increased to 40, especially when colonies with significant differences in the number of zooids are found.

1. Introduction

Ciliated protozoa play an important role in the functioning of activated sludge [1–6] and are widely used as indicators of the wastewater treatment efficiency [4,7–11], which requires determining their species composition, diversity, and abundance [12–15]. Quantifying the population structure of activated sludge is an important step in controlling the biological wastewater treatment [16]. However, when studying live samples of activated sludge, the sample processing time is limited to a few hours to obtain adequate results. For example, a number of authors recommend a processing period not exceeding 3 hours [8,17–19]. According to other authors, the time at which the structure of the community of ciliated protozoa practically does not change is 5 hours [12] or even 8 hours [20].

For this reason, there is a need for shorter sample processing times. Therefore, when studying activated sludge, efforts tend to focus on the populations with the highest densities, leaving small



populations without proper attention. Underestimating the information on small populations can affect the results of determining environmental indices and quality of activated sludge. That said, determining the optimal parameters for the quantification process has rarely been the subject of focused research.

Different authors have used various sample volumes and number of repetitions. Many investigators use a 25 μl sub-sample volume with different numbers of replicate counts. Two replicate counts in 25 μl sub-samples were done by Puigagut et al. [21]; three 25 μL sub-samples counts were done by Martin-Cereceda et al. [22], Salvadó et al. [23], Zhou et al. [24]; at least four repeats of 25 μL sub-samples were done by Tyagi et al. [25]. Some researchers have used larger volumes of sub-samples and/or more repeat counts. For example, Chen et al. [19] used ten repeat counts in 50 μl sub-samples. Papadimitriou et al. [26] investigated 100 or 200 μl sub-samples. At the same time, most studies aimed at optimizing the number of samples in the analysis of activated sludge are focused precisely on assessing the quality of activated sludge, which allows, quite reasonably, ignoring rare species or the species with low abundance that are not essential for the purification process. In the case of studying activated sludge for its structural organization, as a community, and other ecological aspects, it is the few and rare species that become important and their identification becomes relevant. On the basis of the ecologist's needs, the experiments aimed at trying to optimize quantitative studies of activated sludge with increased attention to rare species and species with low abundance were carried out.

The research was carried out on the material taken from the urban wastewater treatment plants. The issue of optimization of counting of organisms with a large amplitude of population density was considered.

2. Material and Methods

The material involved the samples of activated sludge from wastewater treatment plants in Lublin (Poland) and Uzhgorod (Ukraine). Protozoa counting was carried out immediately after sampling and their delivery to the laboratory. The counting was carried out under a microscope at $20\times$ magnification. Sub-sample volume was 25 μl . The counts were performed by two independent researchers. For statistical analysis, the results of 45 25 μl (0.025 mL) sub-sample counts were used, which provided a large amount of data to estimate the abundance of each population. The results were divided into three groups, according to the range of population densities. The first group consisted of the populations with densities ranging from 0 to 1 specimens in 25 μl when counted, the second group 1 to 10 specimens in 25 μl , and the third group, with densities above 10 specimens in 25 μl .

The results of successive counts were averaged to obtain the best estimate of species abundance based on a given number of counts. The average of all 45 counts was taken as the "true" value of abundance. This approach was used to determine the optimal number of counts. The limits of $\pm 5\%$ of the interval around the "true" value were determined. Further, it was established on which of the consecutive counts the third hit in the limits of this interval occurred. The number of counts in which this 5% interval was reached three times was considered to be sufficient.

The data were processed using R Version 4.1.1 [27] with the tidyverse package [28]. The plots were produced using the ggplot2 [29].

3. Results

The results of population density counts in activated sludge samples were analyzed. The data obtained on the predatory sessile form such as *Tokophrya lemnae* suctorina and the free-swimming predatory ciliate *Acinertia uncinata* with densities below 0.2 specimens per 25 μl is shown in the figure (Figure 1). Obviously, the counts of sessile solitary ciliates may differ from the counts of colonial forms. For solitary sessile forms, the average number of counts required was in the range of 20 (Figure 1A). The colonial forms in this range tended to give a greater scatter of results, because of significant differences in the number of zooids in the colonies. When the colonial forms were counted, the number of repeated counts ranged from 30 to 40 times to establish a reliable average population density (Figure 1B). In both cases, for both solitary and colonial forms, the number of counts required increases with decreasing population density.

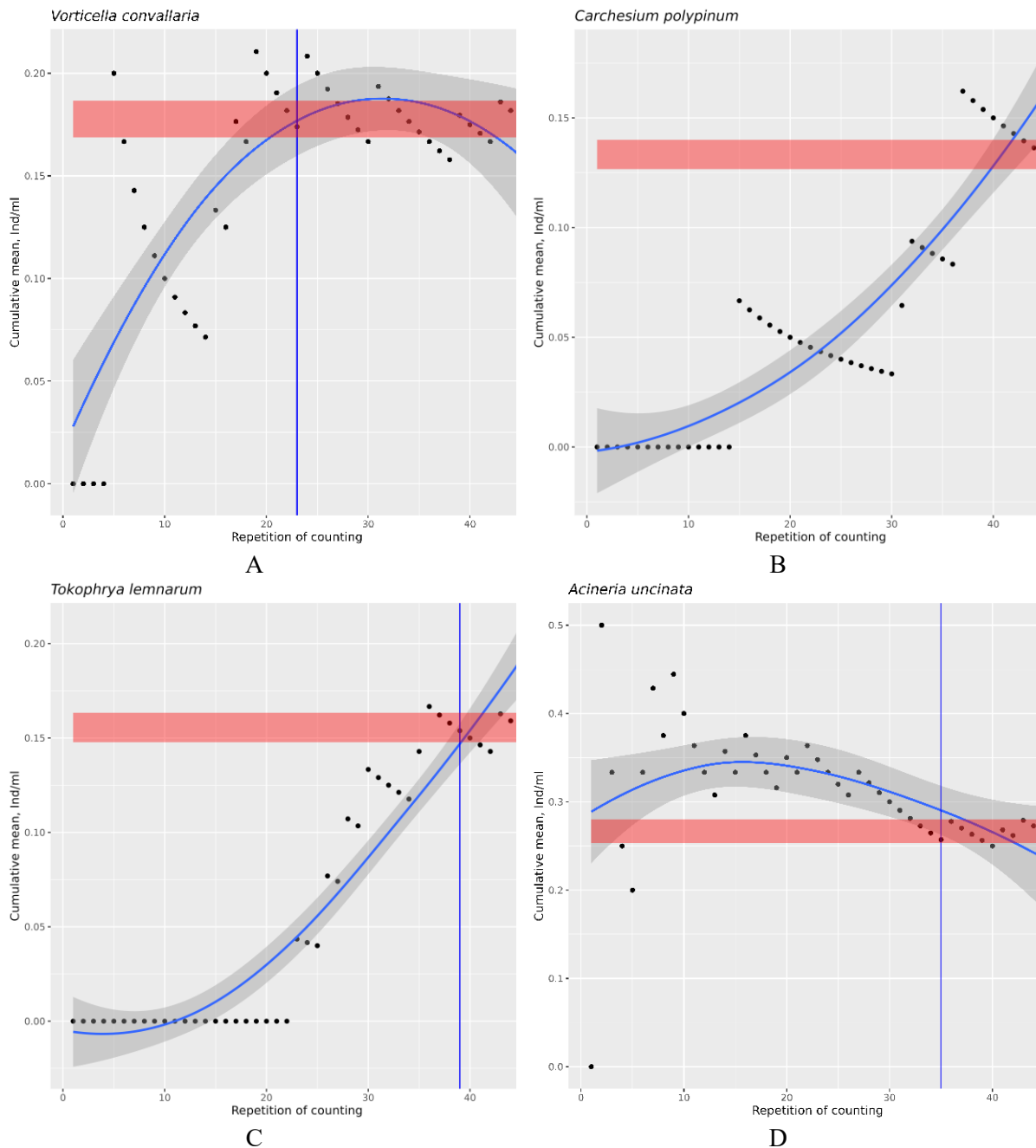


Figure 1. Cumulative averages of ciliated protozoa specimens in 25 μl sub-samples, number of specimens per sub-sample in the range 0 – 1 specimens. The red bar is the 5% interval of values around the general average. The blue line is the third hit in this interval.

Calculations of another series, in which the population density was in the range of 1 – 10 specimens in 25 μl (40 – 400 specimens per 1 milliliter), showed fairly repeatable results for both solitary and colonial forms. For example, the plots of cumulative averages for successive counts of the abundance of the solitary form, *Vorticella convallaria*, and the colonial form, *Carchesium polypinum* (Figure 2 A, B) are shown. The number of counts needed to hit the 5% interval around the general mean three times was between 9 and 11, averaging 10 counts. The number of counts for the crawling and free-swimming forms of ciliates was in the range of 9 – 12 (mean 10) (Figure 2 C, D).

High densities of prevailing populations in activated sludge are the norm, and densities counted in tens and hundreds of specimens in 25 μ l are fairly common. The results of calculations of such populations are shown in Figure 3. At population densities above 400 specimens per milliliter, the number of miscalculations at which three values fall within the required interval is also close to 10 (Figure 3 A, B).

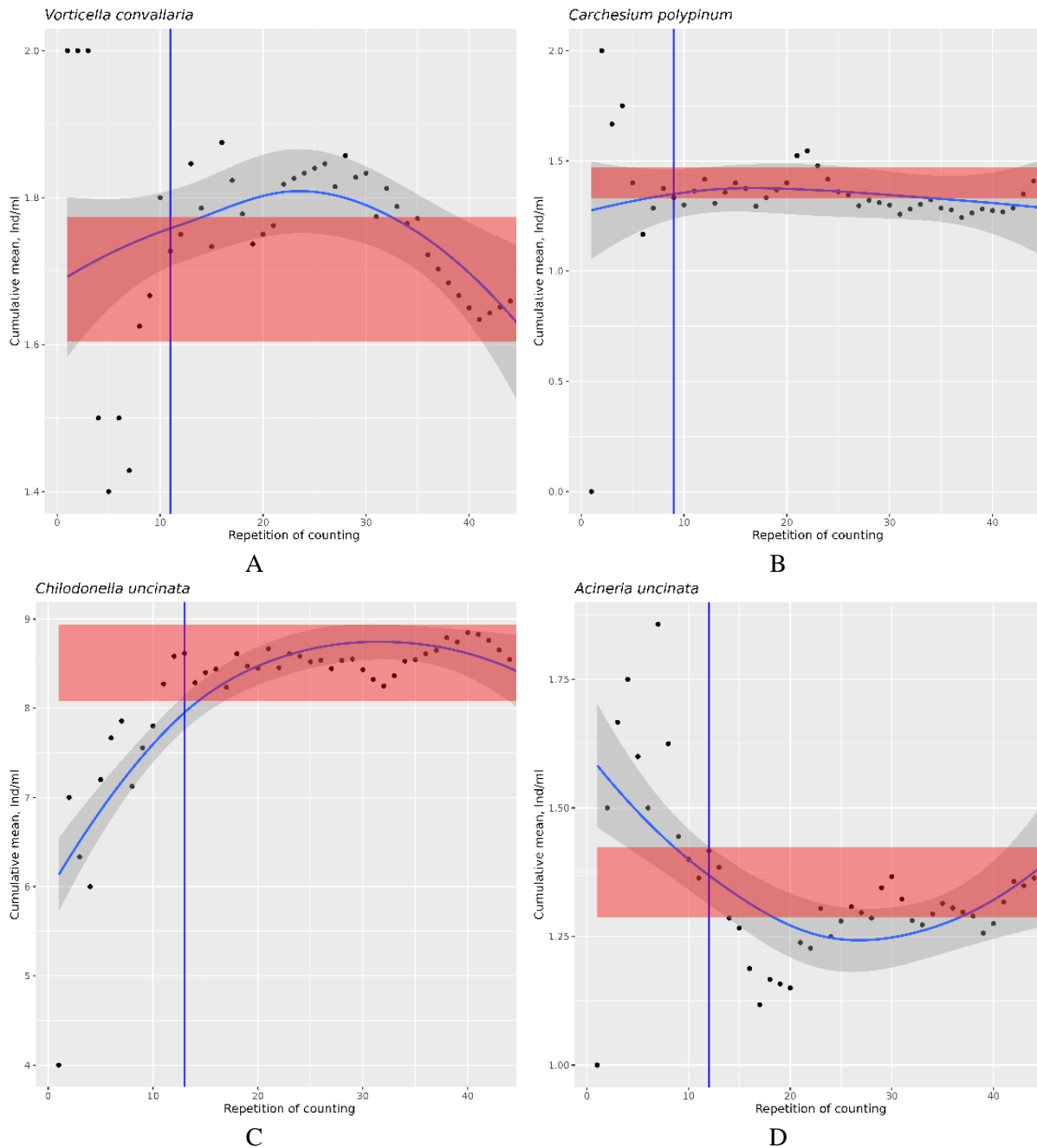


Figure 2. Cumulative averages of ciliated protozoa specimens in 25 μ l sub-samples, number of specimens per sub-sample in the range 1 – 10 specimens. The red bar is the 5% interval of values around the general average. The blue line is the third hit in this interval.

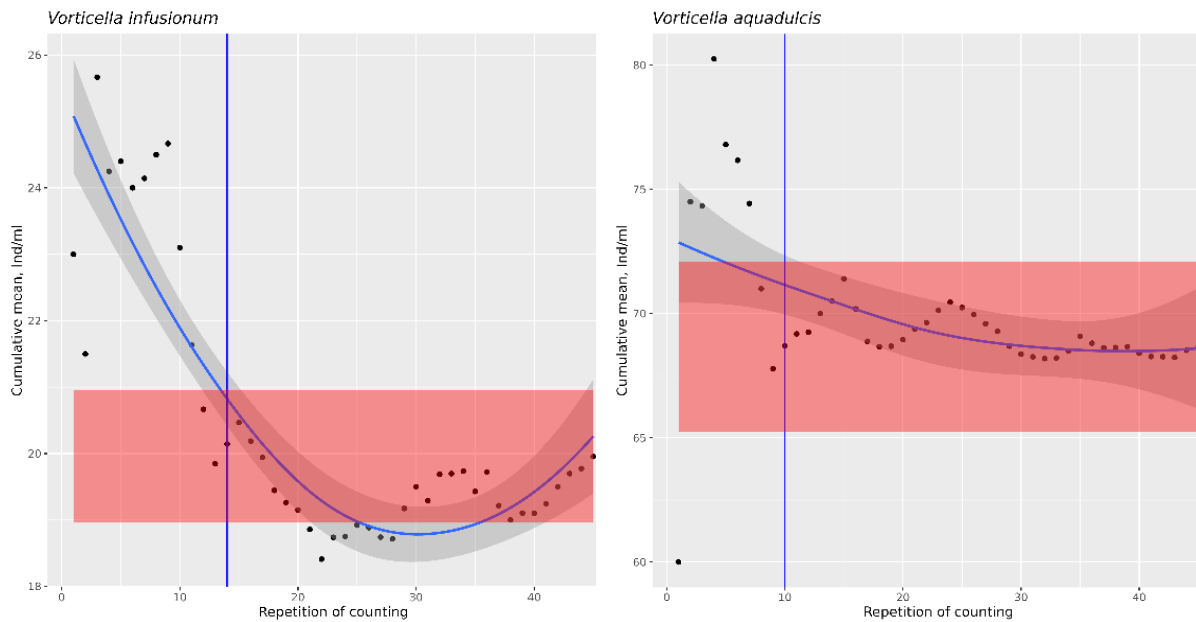


Figure 3. Cumulative averages of ciliated protozoa specimens in 25 µl sub-samples, number of specimens per sub-sample in the range greater than 10 specimens. The red bar is the 5% interval of values around the general average. The blue line is the third hit in this interval.

4. Discussion

Obviously, the probability of obtaining the most reliable information about the density of populations in the activated sludge depends not only on the number of counts, but also on a number of objective and subjective factors. The subjective factor, of course, is the experience and skills of the researcher. The objective reasons for deviations from the true value include the mobility of objects, the ability to achieve maximum homogenization (uniform distribution) of these actively moving objects before sub-sampling. For sessile forms, it is the non-uniform settlement of activated sludge flakes. All these reasons determine the scatter of the results obtained in the counts. For this reason, determining the number of counts that have a high probability of producing a result that is close to reality is a very important task. Certainly, the requirements to reliability of a result of population density calculation depend on the purpose set by the researcher. For example, Madoni believes that 1 or 2 repeated counts in sub-samples of 25 µl are sufficient to assess the quality of activated sludge using the sludge biotic index proposed by him, for the calculation of which the species composition and abundance of protozoa are estimated, [12,30]. Certainly, even with two counts, the ratio of abundant to small populations will be established quite reliably. However, there will be a high probability of unaccounted populations, with low densities, as well as obtaining a very approximate value of the density of populations with high densities. However, in the case of calculating the Madoni sludge biotic index, these inaccuracies are not important, since the main thing is the ratio of population densities, not their absolute values. At the same time, Dubber and Gray [20] determined that six repeated counts in 25 µl sub-samples provided excellent ciliate species recovery (90–95%, excluding rare species), while in two or three repeated counts, the probability of the same species recovery was 25% and 50%, respectively [20]. This is also confirmed by the results of the conducted studies. Indeed, when the population density is higher than one specimens in 25 µl (40 specimens in 1 mL), the number of counts giving a high probability of detecting most species and establishing their relatively reliable densities can be limited to 10 – 12 counts. At the same time, the number of counts should be increased up to 30 – 40 for reliable density estimation of small populations. Obviously, such a detailed study is too labor-intensive and unnecessary for practical application, for example, in the quality control of activated sludge. However, to study activated sludge in the ecological

aspect, to assess the potential diversity of protozoa, the species composition of activated sludge protozoa and others, our recommendations may be useful.

5. Conclusions

The results of determining the optimal number of repeated counts of ciliate abundance in samples of activated sludge to establish the density of their populations were presented.

It was found that using the standard technique of counting the abundance of ciliates in 25 μl sub-samples for the populations with counts equal or greater than one specimen in 25 μl , to establish a reliable average density value, the minimum number of counts is 9, and on average 10 counts are needed. For small populations of free-swimming, crawling, and colonial sessile ciliates (with a density of up to 1 specimens per 25 μl), 40 counts are necessary. For small populations of sessile solitary ciliates, 20 counts are sufficient.

Obviously, such a detailed study is too labor-intensive and unnecessary for practical application, for example, in the quality control of activated sludge. However, to study activated sludge in the ecological aspect, to assess the potential diversity of protozoa, the species composition of activated sludge protozoa and others, our recommendations may be useful.

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