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Use of *Lecane inermis* for control of sludge bulking caused by the *Haliscomenobacter* genus

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ABSTRACT

The excessive growth of filamentous bacteria that induce activated sludge bulking presents a serious problem in many wastewater treatment plants (WWTPs). In laboratory experiments, we tested the efficiency of *Lecane inermis* rotifers in reducing the abundance of *Haliscomenobacter* filaments in activated sludge samples from one municipal and one domestic WWTP. The results showed that the rotifers, by feeding on the bacterium filaments, are able to significantly reduce the quantity of *Haliscomenobacter hydrossis*-like bacteria. It was also shown that the effect of rotifers on filament abundance depends on the rotifer density, which is affected by the influent composition.

Keywords: Activated sludge; *Lecane inermis*; Wastewater treatment plants; *Haliscomenobacter hydrossis*; Sludge bulking

1. Introduction

Activated sludge technology is widely used as an effective method of wastewater treatment. Bacteria accumulated in flocs are one of the basic components of activated sludge and are involved in numerous processes that reduce the level of harmful substances in wastewater. However, protozoans and metazoans are equally important elements of activated sludge because they eliminate a substantial amount of dispersed bacteria and particles that are difficult to degrade biologically, thus helping to reduce the turbidity of treated wastewater. In addition, they are regarded as bio-indicators [1].

Filamentous organisms such as bacteria and, to a lesser extent, fungi, are components of activated sludge. According to Madoni et al. [2], these organisms create the backbone to which floc-forming bacteria adhere and grow into suitable activated sludge flocs. The balance between filamentous bacteria and floc-forming bacteria is key to effectively operating wastewater treatment plants (WWTPs) [3]. Excessive proliferation of filamentous bacteria (filamentous index (FI) exceeding 3 on the 5-point Eikelboom scale, which ranges from 0—no filaments to 5—numerous filaments) damages the desirable properties of the sludge and results in a process called "sludge bulking" [4].

Eikelboom [5] carried out fundamental studies on the identification of filamentous bacteria. He identified over 30 morphological types of filamentous bacteria in

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activated sludge and, of these, he categorized 10 that are mainly responsible for operational problems [6]. The development of culture-independent molecular methods such as 16S rRNA-based techniques [7] and fluorescent *in situ* hybridization (FISH) [8] has enabled progress and updates in the classification of filamentous bacteria. The number of known morphological types is constantly growing and, according to Guo and Zhang [9], 20 types are thought to be the agents responsible for sludge bulking.

Haliscomenobacter hydrossis is a filamentous bacterium belonging to the *Bacteroidetes* [10], found in activated sludge from both municipal and industrial WWTPs [11]. Many authors have identified *H. hydrossis* in bulking and foaming samples from Australia [12], USA [11], South Africa [13], and several European countries, such as Denmark [8] and the Czech Republic [14]. The development of *H. hydrossis* is favored by a low level of dissolved oxygen in an aeration tank, a low food-to-microorganism ratio, phosphorus deficiency, and the availability of readily biodegradable soluble substrates [6,15,16]. Although activated sludge bulking caused by the predominance of *H. hydrossis* is a rare phenomenon [10], it should be emphasized that sludge bulking is not always caused by one predominant filamentous bacterium, but by a mixture of different morphological types in a given activated sludge [17]. Elimination of this type of bulking is particularly inconvenient since the application of a specific, effective chemical compound that limits the growth of one filamentous bacterium strain often creates conditions for better growth of other strains which are insensitive to this active chemical agent [18].

Methods of *H. hydrossis* elimination that have been proposed include the addition of substrate at variable concentrations, a temporary increase of pH to 9 or hydrogen peroxide dosing [16]. The use of chemical procedures such as chlorination or hydrogen peroxide dosing is often recommended in order to reduce bulking and sludge foaming. However, these are only temporary remedies [19]. An alternative method of controlling filamentous bulking involves the addition of *Lecane* rotifers that are able to feed on filamentous bacteria and, thus, to reduce their density in activated sludge. Laboratory experiments have shown that *Lecane inermis* rotifers can significantly reduce the density of *Microthrix parvicella*, *Nostocoida limicola*-like bacteria, and Type 021N in sludge [17,20,21]. The objective of this study was to determine whether *Lecane inermis* rotifers were capable of ingesting filamentous bacteria of the *Haliscomenobacter* genus and whether this capability was influenced by the wastewater composition.

2. Material and methods

2.1. Rotifer clone

A clone (Lk3) of the *L. inermis* (Monogononta) rotifer was used in the experiments. The Lk3 strain originated from a municipal WWTP located in southern Poland. The clone was obtained from a single individual transferred with a micropipette from an activated sludge sample to a well of a tissue culture test plate containing 1 mL of mineral water. An oat grain (pre-sterilized in boiling water) was added to the well. After a few days, the content of the well was transferred to a 55-mm Petri dish. The clone was kept in a climatic chamber in the dark at 20 ± 1°C. After the density of rotifers in the Petri dish increased, the Lk3 clone was poured into other Petri dishes.

2.2. Sludge samples

The samples of activated sludge used in the experiment originated from two WWTPs located in northern Poland: a municipal WWTP in Jonkowo and a domestic one that treated wastewater from a single-family home situated in Stary Olsztyn. The Jonkowo WWTP receives up to 150 m^3 of municipal wastewater per day. Its technical system consists of a grate, a grit chamber with a degreaser, an aeration tank, and two secondary clarifiers. The effluent is discharged to a drainage ditch. The domestic WWTP in Stary Olsztyn consists of a tank with two chambers: internal (aerated) and external, which acts as a secondary clarifier. The system's capacity does not exceed 7 m^3/d. The treated wastewater flows into a drainage ditch. The activated sludge was sampled from the aeration chambers and transported to the laboratory. The containers were half-filled with sludge to prevent soluble oxygen deficiency during transport. On each occasion, the experiment was started within 2 h from the activated sludge sampling.

In each sample, filamentous bacteria were identified based on the morphological features and the results of Gram and Neisser staining according to Eikelboom's key [6]. In both samples, *H. hydrossis* was the predominant strain of filamentous bacteria. Kragelund et al. [10] have shown that for this species, nonmolecular identification techniques are often erroneous, so the term "*H. hydrossis*-like bacteria" is used in subsequent sections of this paper. The density of filamentous bacteria was expressed with the FI [6]. In the samples from both WWTPs, there were no native species of rotifers.

2.3. Experiments

Each experiment (both in Jonkowo and Stary Olsztyn) lasted one week. In the activated sludge sampled in the Jonkowo WWTP (FI = 5), a *H. hydrossis*-like bacteria and *Actinomycetes* were present in equal proportions. In the sample from the Stary Olsztyn WWTP, *H. hydrossis*-like bacteria predominated (FI = 5). From each site, 1,000 mL of activated sludge was taken and sedimented for 30 min. After sedimentation, the volume of settled sludge in the sample from Jonkowo WWTP was 970 mL; in that from the Stary Olsztyn WWTP, 980 mL. Each experiment was carried out in four beakers. These beakers contained 1,000 mL of activated sludge and were equipped with a small-bubble aeration system and placed on magnetic mixers. The *Lecane inermis* that were introduced in the famine state to two beakers were the treatment group in the experiments. At the beginning of each experiment, the density of rotifers was 600 ± 40 individuals per milliliter. Mineral water was added to two control vessels in an amount equal to the volume of culture in the experimental beakers. In the mineral water, the total amount of mineral components was 230.0 mg/L, including 131.06 mg/L of bicarbonate anions, 0.07 mg/L of fluoride anions, 5.62 mg/L of magnesium cations, 41.69 mg/L of calcium cations, and 9.65 mg/L of sodium cations. At the start of the experiments, and after four and seven days, 3 samples of 25 μL activated sludge were sampled from each beaker, for a total of 12 samples. Smears measuring 22 × 22 mm were put on microscopic slides and then stained with the Gram method. Fifteen digital images were taken of randomly selected fields of view in each smear using a Carl Zeiss AXIO Imager microscope at a total magnification of 1,000×. Each image was analyzed using the Axio Vision Release 4.4 image analysis system. The density factor ("DF") was calculated based on the average number of *H. hydrossis*-like bacteria filaments which crossed the borders of each recorded image (images were 139 × 104 μm) [22]. The density of *L. inermis* rotifers was monitored daily in each experiment. For this purpose, three 25 μL samples of sludge were collected from each experimental beaker each time. The samples were placed on a glass slides, cover slips were placed on them, and the number of rotifers that were visible under 100× total magnification was counted alive. The density of rotifers was expressed as the number of individuals per mL of activated sludge.

2.4. Statistical analyses

To test the significance of differences in the abundance of *H. hydrossis*-like bacteria, both between controls and treatments and between different days with the same treatment, the Kruskal–Wallis test and median test were used (*post hoc* multiple comparisons of mean ranks for all groups). The analyses were conducted with STATISTICA StatSoft (version 10.0) data analysis software.

3. Results and discussion

The basic characteristics of wastewater supplied to the municipal (Jonkowo) and domestic (Stary Olsztyn) WWTPs are presented in Table 1.

The effects of rotifers on *H. hydrossis*-like bacteria in sludge from different plants are shown in Figs. 1 and 2.

For the Jonkowo WWTP sludge, the Lk3 clone yielded a significant reduction in filament abundance ($p = 0.000$). Significant differences in the bacterial density between the experimental and control beakers were observed as early as on day 4 of the study ($p < 0.002$). There were significant decreases in the density of *H. hydrossis*-like bacteria in the experimental beakers from day 0 to day 4 ($p < 0.003$) and from day 4 to day 7 ($p = 0.030$) (Fig. 1(b)). The DF was not determined for *Actinomycetes* because in another study (unpublished data) we found that *Lecane inermis* rotifers do not feed on these bacteria. This is probably because *Actinomycetes* have a complex morphology with real branching that makes it difficult for the rotifers to feed on them. According to Wei et al. [23], protozoa and metazoa populations in activated sludge are limited by such morphological features as mouth and pharynx size. Thus, because *Haliscomenobacter* bacteria have a small diameter (from 0.3 to 0.5 μm) and a straight shape, and extend from the flocs like needles [16], they are easy for the rotifers to consume.

In the activated sludge from Stary Olsztyn WWTP, the DF in the beakers with rotifers decreased from the initial value of 30.5 to 0.7 on day 7 of the experiment. Although there was also a significant decrease in the density of *H. hydrossis*-like bacteria in the experimental beakers from day 0 to day 4 ($p = 0.000$), there was no significant decrease from day 4 to day 7 ($p = 0.750$) (Fig. 2(b)). The changes in the density of examined filamentous bacteria during the experiment in Stary Olsztyn are shown in Fig. 3(a)–(c).

In both experiments, the density of *H. hydrossis*-like bacteria in the control beakers remained at levels close to the initial value throughout the study (Figs. 1(a) and 2(a)).

The effectiveness of *Lecane inermis* for controlling sludge bulking is also shown by the decrease in sludge volume in samples treated with the rotifers. At

Table 1
The main characteristics of two types of wastewater (TTS—total suspended solids, COD—chemical oxygen demand, BOD_5—5 d biological oxygen demand, TN—total nitrogen, and TP—total phosphorus)

Characteristic	Unit	Jonkowo WWTP	Stary Olsztyn WWTP
TSS	mg/L	59.5	18.0
COD	mg/L	332.0	652.8
BOD_5	mg/L	192.0	215.4
TN	mg/L	76.6	62.5
TP	mg/L	6.3	10.7

the end of the experiments, after sedimentation, the volume of settled sludge in samples from Jonkowo WWTP averaged 535 ± 7.1 mL, and that in samples from Stary Olsztyn WWTP was 175 ± 21.2 mL. In samples taken from control beakers on the same day, the volume was 950 ± 14.1 mL in the sample from Jonkowo WWTP, and 940 ± 14.1 mL in the sample from Stary Olsztyn WWTP, at the same time (day 7). In the control beakers, there was a small, nonsignificant decrease in sludge volume, probably due to biomass lysis because substrate was not supplied during the 7-d experiment.

The numbers of *Lecane inermis* during both experiments are presented in Fig. 4.

In the Jonkowo sludge, rotifer abundance increased more than 6 times and the maximum number of individuals was recorded on the 6th day. In the sludge sampled in the Stary Olsztyn WWTP, the rotifer density increased over 7 times with the maximum number of individuals recorded on day 4 of the experiment. In the sludge from the Stary Olsztyn WWTP, only single *Lecane inermis* individuals were detected in the last days of the experiment, whereas on day 7, the number of rotifers exceeded 1,200 ind./mL in the sludge from the Jonkowo WWTP.

Widely applied physical and chemical methods of elimination of filamentous bacteria in activated sludge have been found to be unstable, uneconomical, and even potentially dangerous to receiving water bodies [24]. A limited number of studies have attempted to develop sustainable techniques to reduce activated sludge bulking [25]. There are reports that ciliates may help reduce the density of Type 021N bacteria [26,27]. In the case of *H. hydrossis*, Kotay et al. [24] have successfully reduced the density of bacteria under laboratory conditions by infecting them with a *Myoviridae* bacteriophage which had been previously isolated from mixed liquor from a WWTP. However, there may be a risk associated with the potential of bacteria to develop resistance to infection with a phage [25]. Therefore, the application of rotifers as a biological tool to control the filamentous bacteria density in activated sludge is promising [20]. It has been proven that *Lecane inermis* shows high efficacy in reducing the density of *M. parvicella*, *N. limicola*-like bacteria, and Type 021N bacteria in activated sludge sampled from municipal WWTPs [17,21]. No studies on filamentous bacteria in activated sludge from domestic WWTPs have yet been conducted. The characteristics of domestic wastewater are different from those typical of municipal wastewater (Table 1). This is especially true for COD, which was almost two times higher in the nontreated wastewater from Stary Olsztyn than that from Jonkowo. This may be because the residents of that household use a substantial amount of detergents with slow biodegradation [28]. This hypothesis is further confirmed by the high concentration of total phosphorus in the wastewater from the domestic WWTP.

According to Nielsen et al. [29], the members of *Bacteroidetes* are present mainly in conventional municipal WWTPs with carbon removal and nitrification but without nitrogen removal. Hence, their growth is favored by long sludge age. The facilities examined in the current study belong to this class of plants (sludge age above 20 d), which may be the reason for the predominance of *H. hydrossis*-like bacteria in the activated sludge. The initial values of the DF were 30 in Stary Olsztyn and 15 in Jonkowo. The higher density of *H. hydrossis*-like bacteria in activated sludge from the domestic water WWTP in Stary Olsztyn may have resulted from an uneven daily flow of wastewater. An irregular supply of nutrients could have induced their intensive assimilation by *Haliscomenobacter*, which may have caused the significant increase in filament density. Maintenance of a high abundance of *H. hydrossis*-like bacteria, in spite of irregular inflow, would support the thesis of Kragelund et al. [10] that they are highly resistant to famine periods.

The rotifer population grew faster and peaked sooner (day 4 as compared to day 6) (Fig. 4) in the domestic WWTP. This is probably because the initial density of *H. hydrossis*-like bacteria, as expressed by the DF, was 2 times higher in the domestic facility, despite its lower BOD_5/COD ratio (0.33, as compared to 0.58 in the municipal WWTP), perhaps due to an irregular supply of nutrients. This higher density would mean that *Lecane inermis* most likely did not have to compete so intensely for food. Although it is accepted that wastewater determines the composition of activated sludge, which in turn affects the condition of sludge organisms [6], our study suggests that other factors can influence the abundance of microorganisms in activated sludge. For example, the frequency of feeding may affect the growth of rotifers, which may well explain why the rotifer population declined so sharply when the supply of filamentous microorganisms was exhausted. Thus, it may be helpful to also consider such additional factors when designing systems to prevent sludge bulking.

The growth rates of rotifers undoubtedly had an effect on the density of *H. hydrossis*-like bacteria in the individual experiments. The results of the experiments from the Jonkowo and Stary Olsztyn WWTPs showed that *Lecane inermis* reduced the abundance of *H. hydrossis*-like bacteria in the tested samples of activated sludge (Figs. 1 and 2). The results of settling tests indicated a significant improvement of the sludge properties. On day 4 of both experiments, the rotifers had significantly reduced the density of filamentous bacteria compared to the control. In the case of activated sludge from the Stary Olsztyn WWTP, when the maximum numbers of *Lecane inermis* were recorded on day 4, the DF of filamentous bacteria in the beakers with rotifers was 9 times lower than in the control,

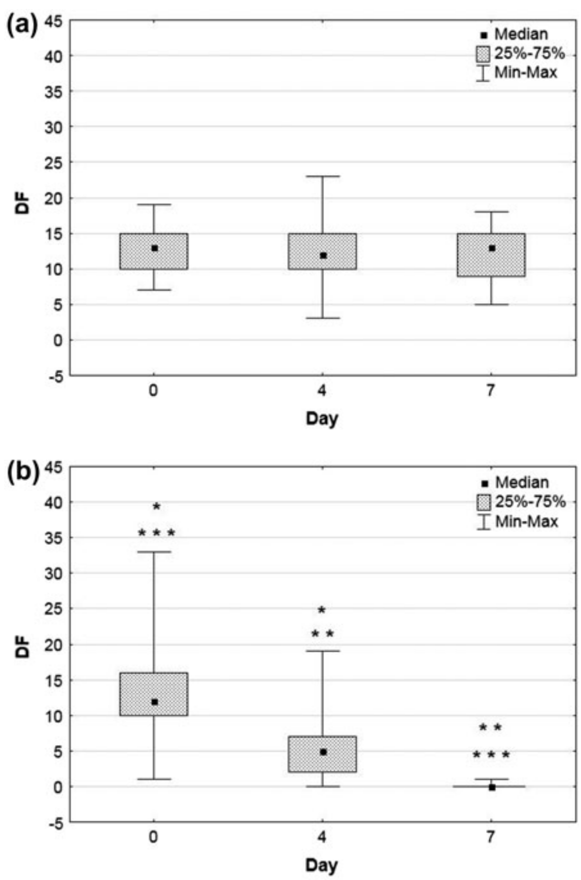

Fig. 1. Average filament DF in 25 μL sludge samples from controls (a) and treatments (b) on days 0, 4, and 7 of the Jonkowo experiment. Statistically significant differences in the DF between experimental days are indicated by *, **, and ***; significant differences were between the days that have the same number of asterisks.

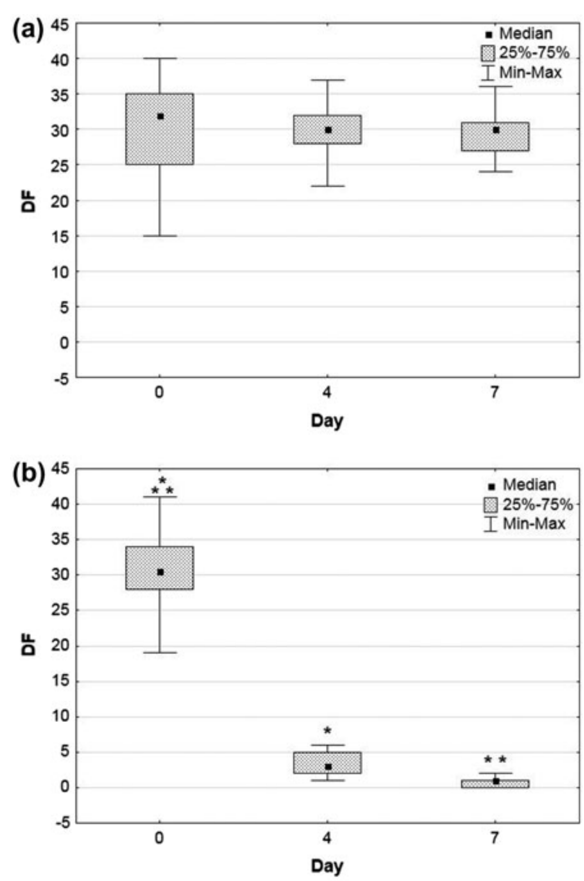

Fig. 2. Average filament DF in 25 μL sludge samples from controls (a) and treatments (b) on days 0, 4, and 7 of the Stary Olsztyn experiment. Statistically significant differences in the DF between experimental days are indicated by *, **, and ***; significant differences were between the days that have the same number of asterisks.

whereas it was only 2 times lower in the activated sludge from Jonkowo. When the rotifers in the activated sludge from Jonkowo reached their maximum density at the end of the experiment, this caused a reduction in filamentous bacteria to nearly zero. This shows that the total reduction of filamentous bacteria depends on rotifer density. Regarding the density of rotifers, after achieving maximal abundance on days 4–6 of the experiment, their abundance drastically decreased. This could have been caused by depletion of food [17].

The use of rotifers as biological tools to control filamentous bacteria growth in activated sludge seems possible and promising. Our experiments have demonstrated that *L. inermis* might be useful to control the growth of *H. hydrossis*-like bacteria, but their efficacy is influenced by the origin of the activated sludge. Further studies are thus needed to determine whether rotifers are capable of survival, multiplication, and feeding on the filamentous bacteria found in activated sludge from municipal and industrial WWTPs.

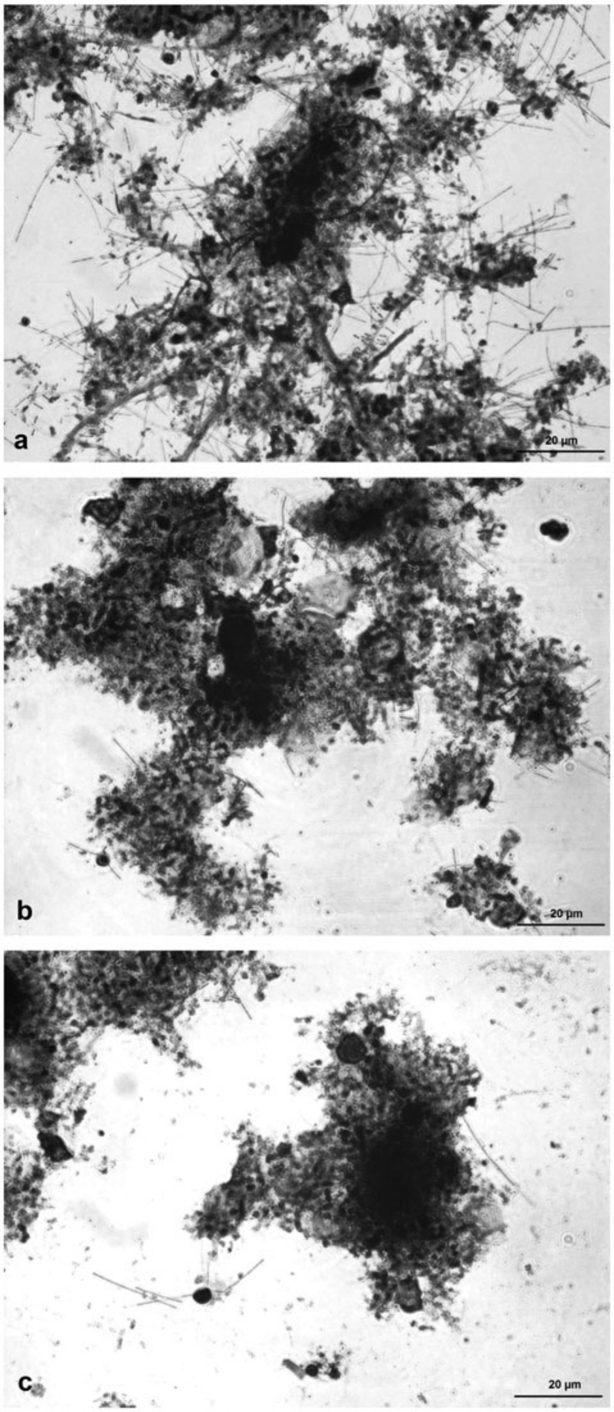

Fig. 3. Microscopic view of filamentous bacteria stained with the Gram method (sludge samples from Stary Olsztyn WWTP); starting day (a), treatment with rotifers after 4 d (b), and 7 d (c).

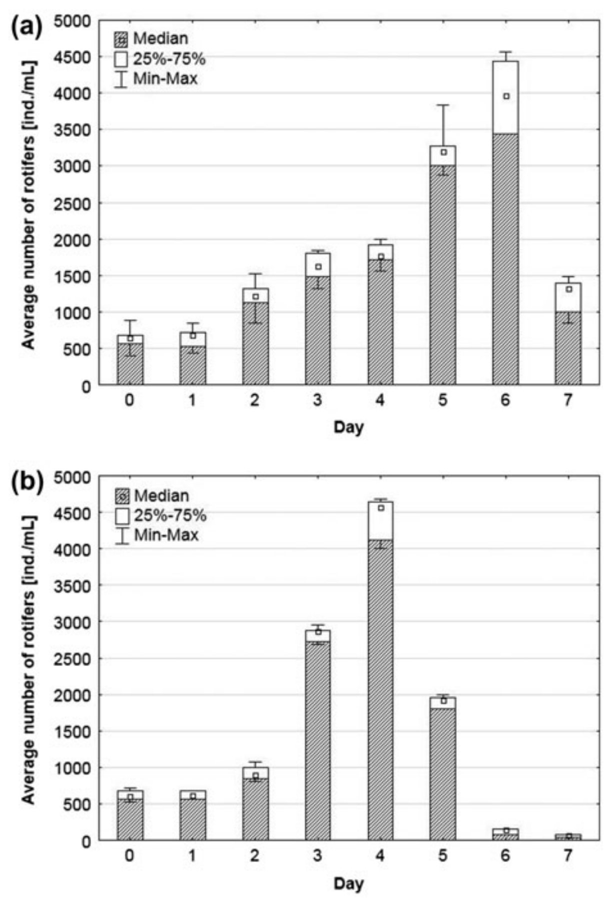

Fig. 4. Average number of rotifers in Jonkowo (a) and Stary Olsztyn (b) sludge on different days of the experiment.

4. Conclusions

The results of these studies indicate that *L. inermis* rotifers were capable of reducing the density of *H. hydrossis*-like bacteria in the activated sludge. The total reduction of filament abundance depends on the rotifer density, which is affected by the influent composition.

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