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Lecane tenuiseta (Rotifera, Monogononta) as the best biological tool candidate selected for preventing activated sludge bulking in a cold season

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ABSTRACT

Rotifers in activated sludge reduce the quantity of dispersed bacteria, ingest filamentous bacteria, enhance flocculation and limit biomass production. Growth rates of rotifers are inversely correlated with temperature; thus, their density declines drastically in wastewater treatment plants (WWTPs) during the winter. The only species already demonstrated to be able to control bulking is *Lecane inermis*. However, they cease proliferation at 8˚C. The goal of our research was to select other rotifers species whose growth rates at lower temperatures are sufficiently high to maintain a favourable density during cold seasons. We conducted selection experiments in the laboratory at temperatures reflecting the temperature distribution in the majority of municipal WWTPs in the temperate zone. In the first experiment, the general selection stage, we tested the influence of the temperatures 8, 15 and 20˚C on competition among different rotifer taxa in sludge samples originating from different WWTPs. The rotifers best adapted to lower temperatures were found among the genera *Lecane* and *Cephalodella*. The second stage, focused on *Lecane* selection, showed that the genus *Lecane* was represented most abundantly by *L. inermis* and *Lecane tenuiseta*. At 8˚C, only clones of *L. tenuiseta* exhibited positive growth rates. At 15˚C, more selected clones were identified as *L. tenuiseta* than as *L. inermis*, but the *r*-value for *L. inermis* was higher. Our results suggested that *L. tenuiseta* is the best candidate for bulking control in cold seasons.

Keywords: Activated sludge; Bulking control; Rotifers; Selection; Temperature

1. Introduction

Bulking of activated sludge reported from wastewater treatment plants (WWTPs) all over the world is most often caused by the overproliferation of filamentous bacteria. Among the extremely troublesome and frequently listed bacteria are *Microthrix parvicella*, *Nostocoida limicola* and Type 021 N [1,2]. In the temperate zone, bulking of activated sludge usually occurs during the winter and early spring seasons when the temperature in bioreactors drops below 15˚C

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due to seasonally low air temperature and/or snowmelt [1]. There are some discrepancies between the observations made in treatment plants where *M. parvicella* growth is promoted at low temperatures [1–3] and laboratory research regarding the growth kinetics of the two strains of this bacterium in pure culture, showing that the optimum temperature for its growth is above 22˚C [4]. Rossetti and co-workers [4] hypothesised that filamentous bacteria have a competitive advantage over flock-formers at lower temperatures. Some insight into why *M. parvicella* is dominant in cold seasons was also provided by Pajdak-Stós and Fiałkowska, who analysed the relationships among temperature, filament indices and indices of organisms potentially preying on filamentous forms in four Polish WWTPs with nutrient removal systems [5]. The densities of filamentous bacteria and organisms inhabiting activated sludge were estimated according to methods proposed by Eikelboom [1]. The densities of rotifers were expressed as degrees on a scale from 0 to 3 in which 0 means none and 3 means numerous individuals per slide. A similar scale from 0 to 5 was used for the estimation of filamentous bacteria abundance [1]. Temperature was shown to be negatively correlated with the *M. parvicella* index and positively correlated with an index of rotifers [5]. Previously, rotifers have been shown to be able to control *M. parvicella* density [6]. Interestingly, in activated sludge samples without rotifers, temperatures in the range of 8–20˚C did not affect the density of filamentous bacteria.

As Fiałkowska and colleagues [7] have shown, the growth rate of *Lecane inermis* drastically decreases as the temperature decreases below 10˚C [6]. At the lowest tested temperature (8˚C), only one of four clones maintained its ability to reproduce. Laboratory experiments testing the influence of different temperatures on the effectiveness of *L. inermis* to control filamentous bacteria in samples dominated by *M. parvicella* and *N. limicola* have shown that at 8˚C, the abundance of filamentous bacteria after a week of incubation with *L. inermis* was similar to the control. On the other hand, the average abundance of filamentous *M. parvicella* and *N. limicola* in the treatment (with rotifers) at 20˚C was approximately half and one-third that of the control, respectively [5].

As the positive effect of different rotifers on the properties of sludge has already been confirmed [7–12], the search for rotifers that are best adapted to function at low temperatures is needed to optimise their beneficial effects. To determine whether rotifer species already possess the capability to perform better at low temperatures, we conducted a set of selection experiments at 8, 15 and 20˚C. These temperatures reflect the temperature distribution in the majority of municipal WWTPs in the temperate zone.

The aim of the study was to find and select cold-adapted rotifer clones that can be cultured on higher scale and then used for further tests as consumers of filamentous bacteria in cold season.

2. Materials and methods

Selection of rotifer clones adapted to lower temperatures was conducted as follows:

2.1. Sampling during the winter season, when temperature did not exceed 10˚C

Sludge samples were collected during the winter from 9 different WWTPs located in southern, central and western Poland: Blachownia, Bełchatów, Chełm, Skoczów, Łęgi, Spyrkówka, Busko Zdrój and two other WWTPs encoded as BB and DOB, as requested by their operators. Each sample was divided into eight subsamples per temperature: 8, 15 and 20˚C.

2.2. Maintaining sludge samples distributed into separate wells at 8, 15 and 20˚C for two weeks

The distributed samples were incubated at different temperatures for two weeks. Thus, we checked the effect of three temperatures on competition between different rotifer taxa in activated sludge samples. The experiment was carried out in 24-well Cell Wells™ (TPP) each containing 1 mL of a well-mixed sample of activated sludge. If the rotifers proliferated in such a volume of the sludge, it allowed us to select rotifers whose initial density in sludge samples was at least 1 individual per mL. The plates with activated sludge subsamples were kept in the dark in Sanyo MLR-350 environmental test chambers and an Electrolux refrigerator, which allowed temperatures to remain constant at the two higher temperatures (15 and 20 ± 1˚C) and the lower temperature (8 ± 1˚C), respectively. For each activated sludge sample and each temperature, 8 replicates (wells) were used.

To determine which genus of rotifers developed at which temperature, after incubation, we took 2 × 25 μL of thoroughly mixed activated sludge samples from every well, covered them separately with 22 × 22 mm coverslips and counted the number of rotifers belonging to Bdelloidea, *Lecane*, *Proales* and *Cephalodella*. The composition of rotifers in the activated sludge was determined "*in vivo*" using Nomarski contrast microscopy (Olympus) at 200–400× magnification, depending on the size of the taxon's representative.

Incubation of activated sludge subsamples at 3 different temperatures was treated as a form of selection. By this way, only species proliferating fast enough at certain temperatures are capable of outcompeting other organisms and have a chance of being recovered in the 25-µL subsamples used for rotifer quantification.

2.3. Isolation of Lecane rotifer clones potentially able to ingest filamentous bacteria

To select rotifer clones potentially able to control filamentous bacteria and that grow the fastest at each temperature, we focused on genus *Lecane*, earlier reported as filamentous bacteria consumers [6]. After a consecutive week of incubation at the temperatures 8, 15 and 20˚C, to obtain separate clones, we subsampled 50 µL of activated sludge from every well, performed a dilution with Żywiec brand spring water and isolated one *Lecane* sp. individual per well. This way, for the further selection, we chose only populations sufficiently abundant to be represented in the randomly taken 50-µL subsamples. If no individuals were found in the 50-µL sample, then the well was not used for subsequent analyses. Following the subsampling, each well containing a single individual was filled with Żywiec Brand spring water and supplemented with 10 µL of the nutrition powder NOVO (denote a nutrition powder used for rotifer mass culture, patent pending procedure EPO EP 14731401.7) [13]. The plates were placed at 8, 15 and 20˚C in test chambers. After a week, all rotifers were counted, and the population growth rate (r) was calculated according to the formula: $r = 1/t(\ln(n_t) - \ln(n_o))$, where t is the day of the experiment and n is the number of rotifers. *Lecane* species were then identified. Species determination was conducted according to Segers [14]. Individuals of *Cephalodella* and *Proales* were also isolated to obtain a clonal population, but no individuals survived or reproduced.

Analysis of variance (two-way ANOVA) was used to detect significant differences in the growth rate (r) between different species (*Lecane tenuiseta* and *L. inermis*) and temperature. One-way ANOVA and *post hoc* unequal N HSD test was used for each *Lecane* species separately to detect temperature-dependent differences in growth rate. The statistical analysis was performed with Statistica 10 (StatSoft Inc. 2011).

3. Results

The method used in our experiment led to selection of rotifer clones that proliferated at a temperature of 8˚C.

3.1. Sampling during the winter season, when temperature did not exceed 10˚C

Initial selection took place in WWTPs because sampling was limited to the winter season, when temperature did not exceed 10˚C. Only six of nine sludge samples had *Lecane* rotifers present in the subsamples.

3.2. Maintaining sludge samples distributed into separate wells at 8, 15 and 20˚C for two weeks

The results of this stage of the experiment are shown in Fig. 1. The bars represent mean densities of each genus of rotifers at different temperatures. There are some differences in the composition of the rotifer communities between treatment plants. The representatives of *Lecane* and Bdelloidea were present in samples from each treatment plant, whereas *Proales* and *Cephalodella* were present in only some of the WWTPs.

Generally, bdelloid rotifers were less abundant than *Lecane*. The highest density of bdelloids was recorded in the BB sludge, in which their mean density reached up to 353 ind/mL. In contrast, *Lecane* can reach mean densities up to 4,000 ind/mL, as was observed in the sludge from Bełchatów. Bdelloids were also less sensitive to temperature. Differences in density between temperatures were less pronounced for bdelloids than for *Lecane*. In the sample from Bełchatów, there were single individuals of *Lecane* at 8˚C, whereas at 20˚C, the mean density was close to 4,000 ind/mL. On the other hand, differences in mean *Lecane* sp. density in samples from Blachownia sludge were much less pronounced, especially between 15 and 20˚C (Fig. 1).

The results obtained for *Cephalodella* sp. are the most puzzling. In some samples, these rotifers reached their highest mean density at the highest temperature (BB sludge), whereas in other cases, such as in the Busko Zdrój sludge, they reached their highest mean density at the lowest temperature.

As the main goal of this part of selection was to find rotifers at different temperatures which proliferated quickly enough to provide a promising material for further selection, the differences among the mean densities of the rotifers were not statistically analysed. Additionally, at this stage of research, the species of rotifers were not discriminated; rotifers belonging to the same genus were counted together.

3.3. Isolation of clones of Lecane rotifers potentially able to ingest filamentous bacteria

The results of this part of our research showed that *L. tenuiseta* is capable of proliferating at a temperature

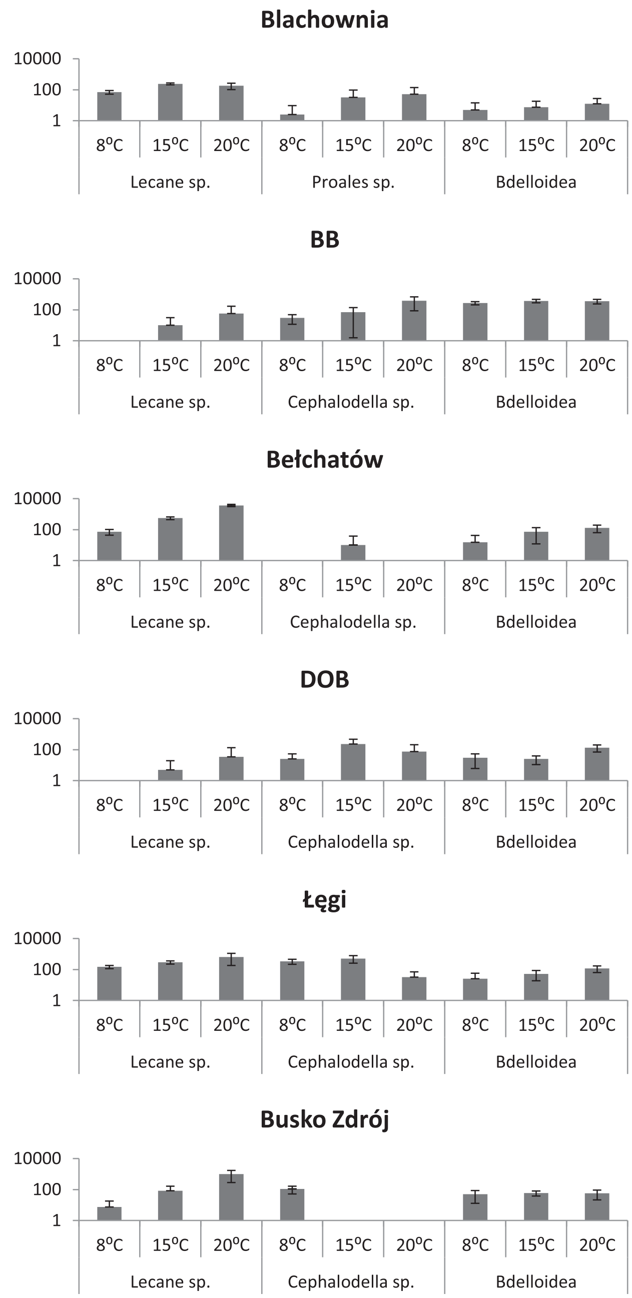

Fig. 1. The mean number of rotifers per mL (*y* logarithmic axis) at the end of the first selection experiment in activated sludge originating from the investigated WWTPs. Whiskers represent standard deviation (SD).

of 8˚C. The highest mean growth rate (*r*) reached at this temperature was 0.08 (Table 1). The highest mean growth rate of *L. inermis* at 15˚C was 0.39, whereas *L. tenuiseta* at the same temperature reached a mean growth rate $r = 0.15$. At 20˚C, *L. inermis* reached its highest mean growth rate, and in most of the clones, its value was higher than 0.50. Two-way factorial ANOVA showed significant differences in growth rate between species ($F_{1,16} = 16.10$, $p = 0.001$) and between temperatures ($F_{2,16} = 62.28$, $p < 0.001$), as well as an interaction of the two factors ($F_{2,16} = 6.36$, $p < 0.05$).

One-way ANOVA performed for 15 and 20˚C in case of *L. inermis* showed significant differences in growth rate between temperatures ($F_{1,7} = 26.43$, $p = 0.001$). For this species, 8˚C was not taken into account as there is only one clone of this species selected at this temperature. In case of *L. tenuiseta,* unequal N HSD test showed significant differences in growth rate between 8 and 20˚C ($p < 0.001$) and between 15 and 20˚C ($p < 0.01$).

The only clone of *L. inermis* isolated from different WWTPs with a distinctly lower growth rate at 20˚C was a facultatively sexual clone originating from activated sludge from Łęgi WWTP. This clone is one of only two strains of the 67 isolated that was able to produce sexual offspring. We also checked the relative proportion of *L. tenuiseta* to *L. inermis* clones maintained at different temperatures. At the lowest temperature, the proportion of *L. tenuiseta* was the highest, reaching 0.94. At the higher temperatures of 15 and 20˚C, the values were lower, at 0.78 and 0.61, respectively (Fig. 2).

4. Discussion

Our three steps selection experiments appeared to be a good way to find rotifers species which in biological bulking control could be an alternative for *L. inermis.* The growth rate of this species in winter season is not high enough to prevent the rotifers from being washed out from the WWTP system with excessive sludge. The selection method we chose, in which at certain temperature only best adapted individuals have a chance to be transferred to the next selection step, led to almost total elimination of this species at 8˚C (Fig. 2) First, selection took place in WWTPs because sampling was limited to the winter season, when the temperature did not exceed 10˚C. The presence of rotifers in 1-mL wells after incubation indicated that at least one individual of a certain species per mL was present in the original subsamples. The presence of rotifers in activated sludge during the winter season at temperatures below 10˚C reflects their ability to adapt to lower temperatures. If their growth rate at this temperature was close to 0, they would not have any chance of surviving in the WWTP with shorter sludge age. This might have been why we did not observe *Lecane* rotifers in three of nine WWTPs.

Table 1
Mean "*r*" value (±SD) of isolated *Lecane* clones at each temperature. Number of isolated clones is indicated in brackets

WWTP	Species	*r* (¯d)	Temp. 8℃	*r* (¯d)	Temp. 15℃	*r* (¯d)	Temp. 20℃
Blachownia	*L. tenuiseta*	0	(3)	0.13 ± 0.06	(8)	0.30 ± 0.04	(8)
BB	*L. tenuiseta*					0.37	(1)
	L. inermis					0.59 ± 0.01	(2)
Bełchatów	*L. tenuiseta*	0.05 ± 0.06	(8)	0.13 ± 0.12	(7)	0.23 ± 0.12	(4)
	L. inermis			0.20	(1)	0.55 ± 0.02	(2)
DOB	*L. inermis*			0.39	(1)	0.52	(2)
	L. inermis-sexual			0.30	(1)		
Łęgi	*L. tenuiseta*	0.08 ± 0.11	(2)			0.23	(1)
	L. inermis	0	(1)			0.53 ± 0.03	(4)
	L. inermis-sexual					0.43	(1)
Busko Zdrój	*L. tenuiseta*	0	(2)	0.15 ± 0.05	(3)	0.33 ± 0.11	(3)
	L. inermis			0.25 ± 0.02	(2)		

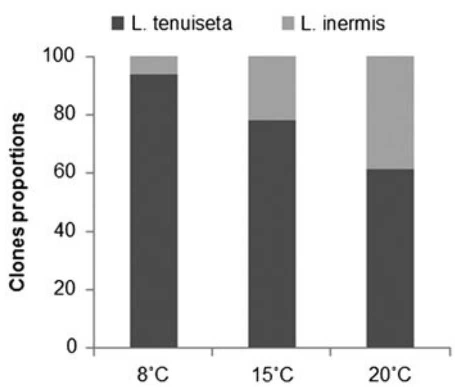

Fig. 2. Proportions of *L. tenuiseta* and *L. inermis* clones at different temperatures.

Incubation of activated sludge subsamples at 3 different temperatures was the second step of selection. Only species proliferating quickly enough at certain temperatures were able to outcompete other species and have a chance to be recovered during rotifer quantification. Representative bdelloids were present in all temperatures in 6 of the 9 investigated samples originating from different WWTPs. In the Blachownia WWTP, only solitary individuals were observed in some of the subsamples. In this sludge, the initial density of rotifers was close to one individual per millilitre, and the population of bdelloids developed only in those wells for which a solitary rotifer was randomly transferred when samples were taken. As bdelloid rotifer identification is very complicated and requires microscopic slides, all bdelloid rotifers were counted jointly without determining genus or species.

The mean densities shown in Fig. 1 suggest that activated sludge samples originating from the DOB, Łęgi and Bełchatów WWTPs were inhabited by species proliferating faster at higher temperatures, whereas in the Busko Zdrój, Blachownia and BB WWTPs, eurythermic species dominated. In the literature, there is insufficient evidence from laboratory experiments that temperature affects the growth rates of bdelloid species. Ricci [15] investigated life history traits of five strains of bdelloid species *Macrotrachela quadricornifera* originating from aquatic and moss habitats. All tested strains reacted to temperature increases within the range of 16–24℃ with increasing growth rate (*r*), but the strength of the response was strain-dependent. In our experiment, we could not exclude temperature-dependent competition between bdelloids and other constituents of the community, as different species could respond to temperature changes differently.

In natural habitats such as lakes, ponds and running waters, rotifer occurrence relative to temperature is species-dependent, as has been described in a paper analysing the temperature preferences of 225 species of rotifers from central and southern Sweden [16]. Edmondson [17] has shown that in the case of the monogonont rotifers *Keratella cochlearis*, *Kellicottia longispina* and *Polyarthra vulgaris*, the reproductive rates of all three species were strongly related to temperature. In a laboratory experiment, Ma et al. [18] showed that eight populations of *Brachionus calyciflorus* from different Chinese regions subjected to a range of mean annual temperatures differed in population intrinsic growth rate and/or in the proportion of sexual offspring produced when cultured at 18, 23 and 28℃.

Miracle and Serra [19] suggest that rotifer population growth rate increases exponentially with respect to temperature and that the slope of the response is genotype-dependent. For cold water-adapted species or clones [20,21], the slopes of the population growth rate curve are lower and the rates are globally smaller than for warm water-adapted ones. This is why, in many cases, species occurring in cold water show faster development at low temperatures but develop more slowly at high temperatures than those adapted to higher temperatures. Fontaneto et al. [22] found that species diversity of monogononts decreases at higher latitudes, whereas bdelloids are diverse at high latitudes.

All of the aforementioned results came from research on natural population of rotifers, whereas little is known regarding the extent to which the ability of rotifers to inhabit activated sludge is dependent on temperature. Pajdak-Stós and Fiałkowska [5] monitored four WWTPs for long periods and found a positive correlation between rotifer abundance and temperature within the range of 5–22˚C. No rotifers were observed in samples taken when the temperature dropped below 6˚C. This cut-off is important because conditions in WWTPs impose a constant selective pressure on the organisms inhabiting activated sludge for higher growth rates. If the organisms are not able to proliferate fast enough, they are washed out of the system with the excessive sludge. If rotifers are to be used as the remedy for the overproliferation of filamentous bacteria, selection of strains or species not only able to ingest filamentous bacteria but also characterised by a doubling time that exceeds the mean cell residence time (MCRT) at lower temperatures is necessary.

We were especially interested in the *Lecane* species as they have been shown to be able to control filamentous bacteria in activated sludge [6]. As is known from our earlier experiments, the growth rate of *L. inermis* is extremely sensitive to temperature decreases. At 8˚C, three of four examined strains completely ceased proliferation [7]. Growth rate dependence on temperature in *Lecane* sp. is clearly reflected in almost all tested WWTPs. Nevertheless, even at temperatures as low as 8˚C in samples from the Łęgi and Blachownia WWTPs, these rotifers were quite abundant (Fig. 1). However, as the next step of selection revealed, the observed differences in *Lecane* abundance were related to different species composition. In Blachownia WWTP, the only *Lecane* present was identified as *L. tenuiseta* and that was reflected in unique pattern of temperature-dependent abundance of *Lecane* genus counted in second step of selection (Fig. 1).

In the last step of selection, we isolated one *Lecane* sp. individual from every well to obtain separate clones. Strikingly, the *Lecane* genus was represented by only two species, and only 17 of 67 *Lecane* strains selected during the winter were identified to be *L. inermis* (Table 1). Such high share of *L. tenuiseta* strains suggests that this species is better adapted to lower temperatures. Only one strain of *L. inermis* survived selection at 8˚C, but was unable to proliferate. In a selection condition of 20˚C, over 40% of strains were identified as *L. inermis*. When we compared the growth rates of these two species (Table 1), the explanation is clear. At 20˚C, the growth rate of *L. inermis* is almost twice as high as that of *L. tenuiseta*. In contrast, *L. tenuiseta* showed the higher growth rate at 8˚C. Even though at 15˚C, there still were more strains of *L. tenuiseta*, the growth rates of *L. inermis* strains were higher on average (Table 1). These two morphologically similar species (Figs. 3, 4) inhabit the same niche, and coexistence of these two populations in the WWTP may help in the prevention of bulking during the winter. Based on the values of the growth rates, we conclude that among all tested populations of *L. tenuiseta*, those originating from the Łęgi and Bełchatów WWTPs appeared to be best adapted to lower temperatures. Calculations of the proportion of *L. tenuiseta* showed that this species may be a promising alternative to *L. inermis* at lower temperatures as the proportion of *L. tenuiseta* clones growing at 8˚C was close to 1.0 (Fig. 2). At this temperature, four clones of *L. tenuiseta* with positive growth rates were isolated. Those clones could be used to start mass cultures which then will be applied as a tool to prevent the overproliferation of filamentous bacteria during the winter.

Additional valuable information emerging from selection experiments confirms our earlier observations that rotifers clones maintaining ability to sexual reproduction are very rare in activated sludge [23]. In particular, the proportion of sexual strains of *Lecane* rotifers in WWTPs is very low. Our research showed that all isolated *L. tenuiseta* strains were obligatory parthenogens, and only two of the 17 *L. inermis* strains appeared to be able to reproduce sexually. Their

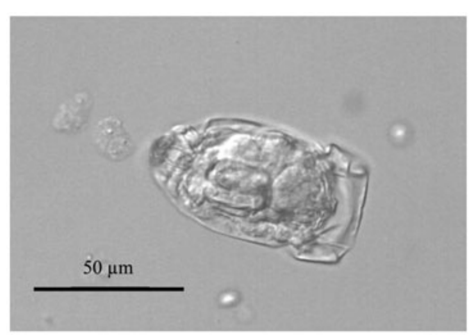

Fig. 3. Rotifer *L. inermis*.

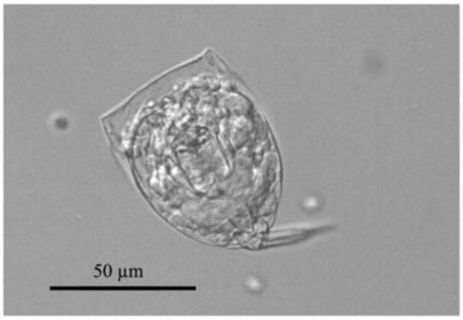

Fig. 4. Rotifer *L. tenuiseta.*

growth rate was lower in comparison with the obligatory parthenogenetic clones what is also consistent with our earlier results [23].

Year-round maintenance of *Lecane* rotifers in WWTPs is possible because of species-dependent thermal preferences. The simple method of rotifer clone selection provides a promising approach for improving the prevention and control of bulking. The clones of *L. tenuiseta* selected at the lowest temperature could be used as a component of a rotifer inoculum applied for controlling bulking in WWTPs during a cold season. Data on the growth rates of beneficial species grown at different temperatures will help the operators of WWTPs predict the fates of artificially inoculated or/and naturally occurring rotifers in thermally fluctuating activated sludge environment.

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