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THE IMPACT OF FOOD ON THE GROWTH OF CLONES OF *LECANE INERMIS*, A POTENTIAL BULKING CONTROL AGENT

Excessive growth of filamentous bacteria, inducing activated sludge bulking, presents a serious problem in many wastewater treatment plants (WWTPs). The idea of using *Lecane inermis* rotifers as a tool for controlling the filamentous bacteria density in activated sludge requires developing a method of culturing rotifers at high concentrations. The objective of this work was to determine the effect of two culture media on the growth of three *Lecane inermis* strains. The growth of rotifers from a single individual (parthenogenetic female) fed the tested culture media was observed for 10 days. The rotifers showed different individual growth, depending on the strain and type of culture medium. The results of the studies suggest that by using the tested culture media, it may be possible to select a *Lecane inermis* strain with the highest culturing capacity at high density of rotifers in order to inoculate activated sludge with these organisms in wastewater treatment plants.

1. INTRODUCTION

Activated sludge is one of the most common technologies used for wastewater treatment [1]. Activated sludge contains a diverse population. There are microorganisms including decomposers (bacteria and fungi), which take energy for their growth from dissolved organic matter in the wastewater. In addition, there are microfauna (protozoa and metazoa), which prey on the decomposers [2]. An essential part of the population in activated sludge are filamentous microorganisms. These microorganisms provide structure for the floc-forming bacteria to adhere to and grow into suitable activated

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sludge flocs. However, when filamentous bacteria are present in excessive numbers, serious operational problems arise – the bulking and foaming of activated sludge [3].

Controlling the growth of filamentous bacteria with chemical methods is only temporarily effective. Moreover, these methods have disadvantages such as high costs, increases in sludge mass and unfavourable effects on nitrification [4, 5]. An alternative method of bulking control is the use of natural enemies of filamentous bacteria as a biological tool to limit their overproliferation. It is known that *Lecane inermis* can reduce the number of filamentous bacteria in activated sludge [6–9]. These laboratory experiments showed that *Lecane inermis* rotifers can significantly reduce the density of *Microthrix parvicella*, *Nostocoida limicola*-like, type 021N and *Thiothrix* bacteria in sludge.

Although the biology of *Lecane inermis* has been quite well-described [10, 11] and it is known that this is one of the most fecund rotifers [11], with populations usually dominated by amictic females that produce an average of 20 eggs during their lifetime of approximately nine days [10], there is still a need to develop culturing methods that will provide a high density of rotifers for use on an industrial scale. It is thought that the most important factor affecting the growth and quality of rotifers is diet [12]. Therefore, we examined the effect of two culture media on the reproduction of three clones of *Lecane inermis* rotifer to investigate which food was the most effective for culturing *Lecane inermis* at laboratory scale.

2. MATERIALS AND METHODS

The rotifers culture was conducted according to Fiałkowska et al. [13] with modifications. Three clonal populations of the rotifer *Lecane inermis* (Monogononta) were used in the experiments. The rotifers were isolated in the same way from different WWTPs. The LkT clone was isolated from a WWTP located in north-eastern Poland. The other two strains, Lk3 and Lk6, were derived from two municipal WWTPs in southern Poland. All clones were obtained from single individuals that were transferred with a micropipette from an activated sludge sample to separate wells in tissue culture test plates using 1 cm^3 of mineral water as the medium. In mineral water, total amount of mineral components was 230.0 mg/dm^3, including 131.06 mg/dm^3 of bicarbonate anions, 0.07 mg/dm^3 of fluoride anions, 5.62 mg/dm^3 of magnesium cations, 41.69 mg/dm^3 of calcium cations and 9.65 mg/dm^3 of sodium cations. Oat grains (sterilized earlier in boiling water) were added to each well. The rotifers fed on the bacteria that proliferated on these grains. When the rotifers reached a density of about 100 individuals/cm^3, the clones were transferred to Petri dishes and kept in the dark in a Conviron Adaptis A 1000 climatic chamber at 20±1 °C. Further addition of oat grains caused significant turbidity in the medium; hence, we started to search for another food source.

Using two different food sources, we measured the rate at which populations of the three clones developed from single individuals, giving a total of six experimental variants. The first food source was powdered yolks of the chicken eggs (commercial industrial company) mixed with cocoa at a ratio of 3:1 (O). The second food source was yolks of chicken eggs from an eco-friendly farm that were dried at 50 °C (Z).

To investigate the reproductive capacity of individual females during their whole lifetime, the experiment lasted 10 days – one day longer than the mean lifetime of amictic females of *Lecane inermis* [10]. One rotifer was transferred to each well in a 24-well plate. This procedure was carried out separately for each clone. The females were of similar age because they were chosen from hatching eggs separated from the cultures one day previously. Each well contained 1 cm^3 mineral water and 0.001 g of media. Culture medium Z was added to twelve wells of each tissue test plate and culture medium O was added to the remaining twelve. The plates containing the rotifers were kept in Conviron Adaptis A 1000 climatic chambers at 20±1 °C. The number of living females and the eggs were counted every 24 h directly in the wells by using an inverted Nikon Diaphot microscope at a total magnification 100×. To facilitate future breeding and use of *Lecane inermis* at industrial scale, the population growth rate coefficient (r) and doubling time (t_D) were calculated for each strain as described in James et al. [14]. The dissolved oxygen concentration was not controlled in the wells during the experiment.

To investigate possible differences between experimental variants, factorial ANOVA and post hoc Scheffé tests were conducted with STATISTICA StatSoft 10.0.

3. RESULTS

All strains of *Lecane inermis* grew better on the O culture medium (powdered egg yolk mixed with cocoa) (Fig. 1). These differences were only significant for clones Lk3 and Lk6. The density of strain Lk3 was significantly higher on days 9 and 10 when fed with medium O ($p = 0.000$), with the final number of rotifers about twice as high as on medium Z. The density of strain Lk6 was significantly higher on days 7–10 when fed with medium O ($p = 0.000$); this strain showed almost no increase when fed with culture medium Z. Densities of above 80 rotifers per cm^3 were reached by strain LkT with both culture media, and by strain Lk3 only with medium O. Regardless of the culture medium, strain Lk6 proliferated much less than strains Lk3 and LkT. Of the latter two strains, Lk3 proliferated less with medium Z than with O, whereas LkT was not significantly affected by changes in medium. With all clones, the number of rotifers started to increase between day 4 and 5 of the experiment. Clone LkT with medium O had the highest density, with 90.66 individuals per cm^3 on day 10; Lk6 with medium Z had the lowest density, 4.42 per cm^3 on day 10. Clone Lk6, regardless of food source, had the lowest growth rate coefficients (Table 1).

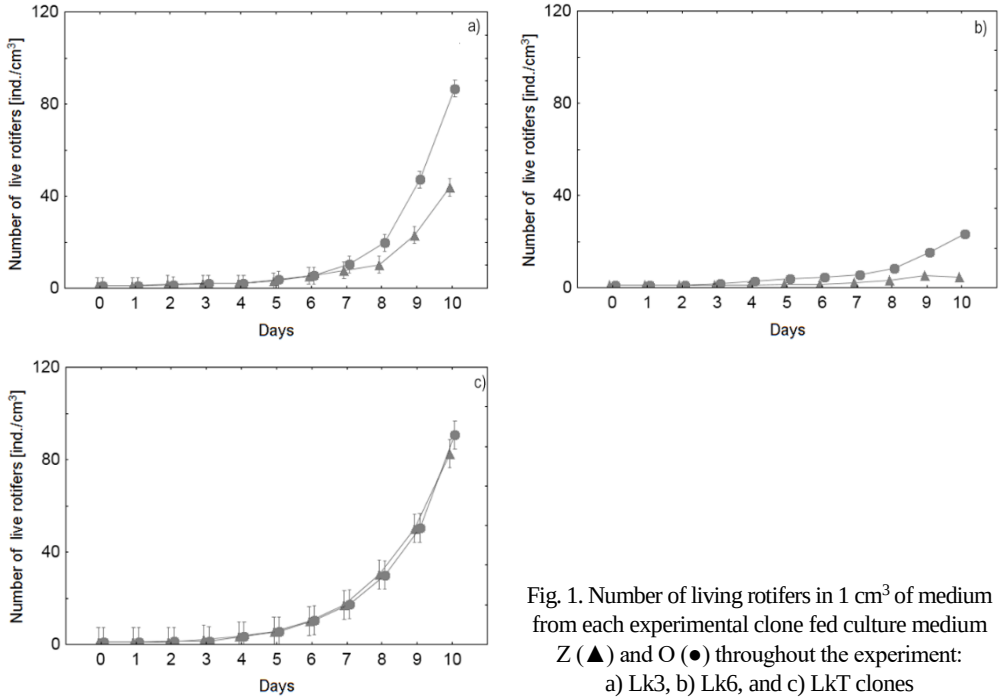

Fig. 1. Number of living rotifers in 1 cm^3 of medium from each experimental clone fed culture medium Z (▲) and O (●) throughout the experiment: a) Lk3, b) Lk6, and c) LkT clones

Table 1

Growth rate *r*-coefficients [day^{-1}] calculated for each rotifer clone between the 1st and 10th day of the experiment

Culture medium	Lk3	Lk6	LkT
Z	0.37±0.03	0.14±0.01	0.44±0.33
O	0.44±0.32	0.31±0.18	0.45±0.30

With the Lk3 and LkT clones, the growth rate coefficients were similar, with the maximum value determined for clone LkT fed with culture medium O. The doubling time (t_D) was the shortest for the clone LkT fed with culture medium O, and longest for clone Lk6 with medium Z (Table 2).

Table 2

The doubling time t_D [day] calculated for each rotifer clone between the 1st and 10th day of the experiment

Culture medium	Lk3	Lk6	LkT
Z	1.83	4.67	1.57
O	1.55	2.20	1.54

The type of food affected the number of eggs produced by the rotifers. As shown in Fig. 2, the strength of this effect depended on the strain of rotifer.

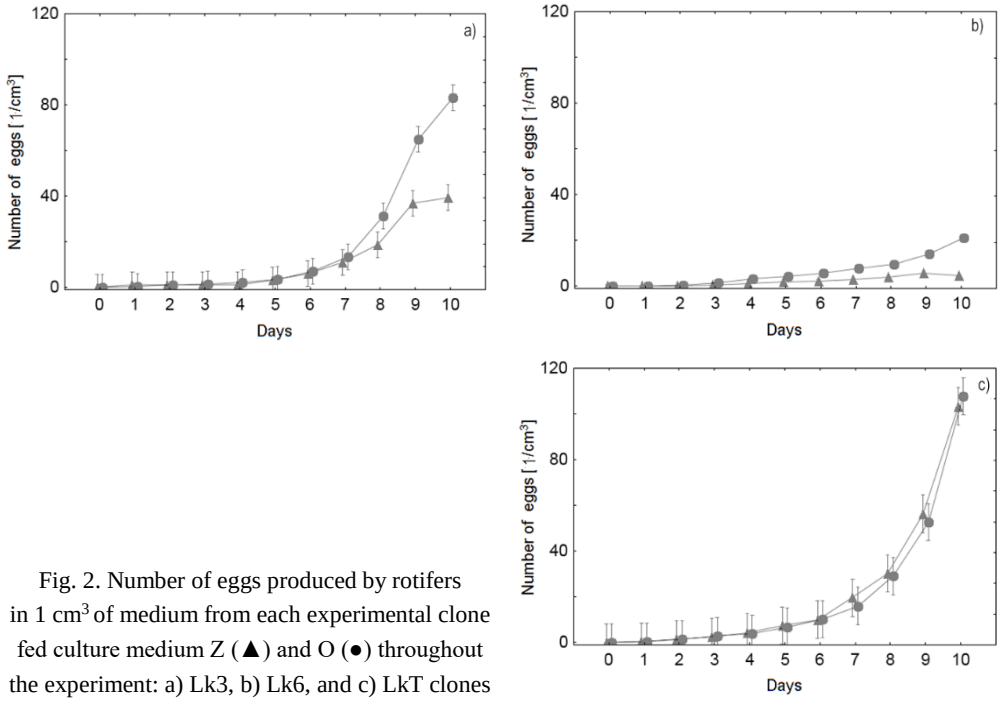

Fig. 2. Number of eggs produced by rotifers in 1 cm^3 of medium from each experimental clone fed culture medium Z (▲) and O (●) throughout the experiment: a) Lk3, b) Lk6, and c) LkT clones

All strains (except strain LkT on days 7–9) produced more eggs with culture medium O than with medium Z, although this difference was only significant for strains Lk3 and Lk6. Differences in egg production were greater with culture medium Z ($p = 0.000$) than with O. When comparing strains Lk3 and LkT, the difference in egg production was only significant with medium Z. When comparing these two strains with strain Lk6, differences in egg production were significant with both culture media.

4. DISCUSSION

A successful culture of rotifers seems to depend to a large extent on adequate data on the life history and environmental preferences of each biotype [15]. To date, many studies have been carried out on the effect of such parameters as salinity, temperature and the concentration of food on the growth of various rotifer species [16–18]. However, most of these studies have focused on laboratory trials on rotifers of *Brachionus* sp. genus, since they are important as food sources in aquaculture and as organisms used in eco-toxicological studies [19]. The using of *Lecane inermis* as a potential biological

agent to control activated sludge bulking on an industrial scale requires (similar to *Brachionus* sp.) knowledge of culture conditions to generate the maximum growth rate of these rotifers. Literature data indicate that *Lecane inermis* grow effectively on a diet consisting of oat and malted milk [10]. In the current study, oat was found out as a good medium for *Lecane inermis* development. However, at a high volume of rotifer culture and related high volume of grains, oat was decomposed which caused the increase in medium turbidity. In addition, oat grains cause the development of species that are undesirable in *Lecane inermis* culture, including fungi. In search of an alternative medium for *Lecane inermis*, we studied the results of experiments of Lubzens [20], in which nutritional values of *Brachionus calyciflorus* rotifers were enriched before using them as a food for young fish. This enrichment consisted of the addition of a suspension of egg yolk and fish oil to the culture of *Brachionus calyciflorus*. In the current study, we made attempts to introduce egg yolk as part of a diet for *Lecane inermis*.

Nhu [21] found that the production and density of rotifers depends on the availability and quality of food. Since eggs are potential offspring, their numbers may be used to explain tendencies in growth curves of zooplankton species, including rotifers [22]. Yi-Long et al. [23] proved that the type of food (various algal species, in this case) exerted a significant effect on the number of eggs produced by amictic *Brachionus calyciflorus* females. Kostopoulou and Vadstein [24] used the number of eggs produced by females of three *Brachionus plicatilis* strains to investigate the effect of food type on the reproductive efficiency of these rotifers. The effect of diet on the number of eggs produced by rotifers (*Lecane inermis*) was also found in our studies (Fig. 2). Food, as a major component of rotifer cultures, directly influences their reproduction and the subsequent population growth index [25, 26]. The presented results demonstrate that yolk is a good source of food for *Lecane inermis.* Rotifers fed on bacteria that proliferated on the yolk. In addition, we observed that they eat yolk and produce numerous eggs around yolk particles. The using of yolk from an eco-friendly farm and drying it would be too expensive in mass culturing. For this reason, in the current study, powdered yolk used for industrial purposes was investigated. This powdered yolk with cocoa (O) generated the best growth rate in all clones. The egg yolk contains valuable nutrients such as proteins, fats and carbohydrates as well as vitamins and microelements. In addition, when the culture media is mixed with cocoa, microhabitats are produced, where rotifers usually produce eggs.

The determined values of growth rate coefficient for *Lecane inermis* are comparable with those recorded for the Brachionidae family species [27]. The doubling time (Table 2) for *Lecane inermis* was 1.5 days (or more depending on the clone), which confirms the reports by Edmondson [11] on the high reproductive capacity of these organisms. *Lecane inermis* is thus classified as one of the most prolific rotifers and it has the potential to generate a high density of individuals in short-term cultures.

According to our results, the effect of food type on rotifers was strain-specific (Fig. 1). During the experiment, the number of individuals of one clone Lk6 was significantly lower for both types of culture media than the numbers of rotifers within the other clones. This proves that the reaction to the type of food may differ in individuals of the same species originating from different environments, i.e. in activated sludge from different WWTPS, as in this case. Specific reactions of different clones of the same rotifer species to environmental conditions have been discussed and reported by other authors [28, 29]. Bosque et al. [18] found that tolerance of salinity in *Brachionus plicatilis* clones depended on their origin. Hagiwara et al. [30] assumed that resistance to environmental stress in mass culture was different in individual rotifer species. Malekzadeh Viayeh et al. [15] carried out the studies on the effect of salinity and food type on four *Brachionus plicatilis* strains and two *Brachionus urceolaris* strains originating from different regions in Iran. These authors showed that the effect of analyzed environmental parameters was strain-specific and suggested that it could result from different genetic capacity of these taxa with different geographic origin and localization.

The potential using of *Lecane inermis* on a technical scale as a biological method of elimination of activated sludge bulking requires determining optimal culture conditions. Generation of rotifers with a high density in laboratory conditions will permit the application of these organisms in activated sludges with bulking. Our studies demonstrated that diet plays an important role for the growth of *Lecane inermis.* Powdered chicken egg yolk mixed with cocoa seems to be an adequate medium for *Lecane inermis.* The different level of density for individual clones which were fed with this culture medium suggests that there is a potential for experimental selection of the *Lecane inermis* strain with the highest culture capacity on an industrial scale.

5. CONCLUSIONS

Our experiment shows that industrial powdered chicken egg yolk, supplemented with cocoa, is a medium that generates better growth of the two examined *Lecane inermis* strains than with dried chicken eggs yolk from hens reared on an eco-farm. One of the clones, i.e. LkT, had a comparable density of individuals (lack of statistical differences) on both tested culture media. The results of our study indicate that the impact of food type on the rotifers was strain-specific.

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