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USE OF *LECANE* ROTIFERS FOR LIMITING *THIOTHRIX* FILAMENTOUS BACTERIA IN BULKING ACTIVATED SLUDGE IN A DAIRY WASTEWATER TREATMENT PLANT

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Abstract - Excessive growth of filamentous bacteria is a serious problem in many dairy wastewater treatment plants (WWTPs). The objective of the study was to determine whether *Lecane inermis* rotifers were able to reduce the density of *Thiothrix* bacteria in activated sludge originating from a dairy WWTP, as well as to identify the impact of rotifers on other organisms in sludge. On a laboratory scale, three experiments were conducted in which activated sludge with a predominance of *Thiothrix* was inoculated with rotifers at an initial concentration of app. 600 individuals/mL. The results showed that the rotifers, by feeding on the bacterium filaments, are able to reduce significantly the quantity of *Thiothrix*. A decline in *Thiothrix* abundance coincided with an improvement of the sedimentation properties of activated sludge. In addition, it was proven that *Lecane inermis* did not negatively affect the number of Protozoa and Metazoa in activated sludge.

Key words: Lecane inermis; activated sludge bulking; *Thiothrix;* dairy wastewater

INTRODUCTION

The volume, concentration and composition of wastewater generated by the dairy industry are strongly influenced by the production processes used in a given dairy plant (Britz et al., 2006). In general, dairy wastewater has a high biological oxygen demand (BOD) and high chemical oxygen demand (COD) due to its high content of lactose, proteins and fats (Janczukowicz et al., 2008; Lateef et al., 2013). Furthermore, wastewater generated by the dairy industry has a high concentration of total suspended solids (TSS), nitrogen, and a content of phosphorus slightly lower than nitrogen content (Kushwaha et al., 2011).

The use of activated sludge is one of the options for wastewater treatment. The method consists of using microorganisms that are capable of metabolizing lipids, proteins and lactose under aerobic conditions (Tocchi et al., 2012). Although this method is thought to be effective in the treatment of dairy wastewater, the bulking of activated sludge is a serious disadvantage (Britz et al., 2006). Bulking is most often caused by intensive growth of filamentous bacteria; this contributes to the deterioration of the settling properties of activated sludge. This deterioration causes difficulties in separating activated sludge from wastewater in secondary clarifiers, resulting in impure effluent and sludge loss.

As reported by Donkin (1997), filamentous bacteria that cause sludge bulking in industry have been associated with various operating conditions. The growth of *Thiothrix* bacteria in activated sludge is supported by uniform aeration and complete mixing in nitrification chambers (Jenkins et al., 2004), and by reduced sulfur compounds and nutrient deficiency in the influent (Fiałkowska et al., 2005; Vaiopoulou et al., 2007).

Although chemical methods, such as hydrogen peroxide dosing and chlorination, have been proposed to limit the concentration of filamentous bacteria (Fiałkowska et al., 2005), these are only effective as temporary measures (Kulkarni 2012). Taking into account the multiple disadvantages of chemical methods, the use of rotifers to control the concentration of filamentous bacteria in activated sludge seems promising (Fiałkowska, Pajdak-Stós 2008). *Lecane inermis* efficiently reduces the density of *Nostocoida limicola*-like bacteria, *Microthrix parvicella* and Type 021N in activated sludge from municipal WWTPs (Pajdak-Stós, Fiałkowska 2012; Kocerba-Soroka et al., 2013). Our previous studies have shown that *Lecane inermis* can feed on *Haliscomenobacter hydrossis*-like bacteria in activated sludge from municipal and domestic WWTPs (unpublished data). However, the use of rotifers to eliminate filamentous bacteria in activated sludge in industrial WWTPs has not yet been investigated and it has not yet been determined whether these rotifers feed on *Thiothrix*. Moreover, it is not known how *Lecane inermis* would affect other organisms in activated sludge that are necessary for sludge flocs to function properly.

The objectives of the study were: a) to determine whether *Lecane inermis* introduced to activated sludge from a dairy WWTP was capable of surviving, proliferating and reducing the concentration of *Thiothrix* bacteria, and thereby improving the settling properties of the sludge, and b) to determine how the growth of *Lecane inermis* affects other organisms present in activated sludge (Protozoa and Metazoa).

MATERIALS AND METHODS

Activated sludge samples

The samples of activated sludge used in the study originated from a WWTP at the Zakład Produkcji Mleczarskiej "Mlekpol" (ZPM "Mlekpol") dairy plant in Mrągowo (northeastern Poland). Sludge was collected from an aeration tank and transported to a laboratory. In order to prevent a deficit of dissolved oxygen during transportation, the containers were half-filled with sludge. Each time, the experiment was started no later than two hours after taking samples of activated sludge.

During sampling, abundant foam was found on the surface of the activated sludge in the aeration tank (Fig. 1). Microscopic observations showed that filamentous *Actinomycetes* bacteria were predominant in the foam.

Filamentous bacteria found in the samples of activated sludge were identified based on live observations of morphological attributes and the results of staining with Gram and Niesser methods according to the criteria specified by Eikelboom (2000) and Jenkins et al. (2004). Since only some strains of filamentous bacteria can be identified with these methods, a molecular method was also used, i.e. fluorescence *in situ* hybridization (FISH) with the following rRNA-targeted probes: EUBmix (Kragelund et al., 2007), Curvi997 (Thomsen et al., 2004), G123 T (Mielczarek et al., 2012) and TM7305 (Nielsen et al., 2009) and CFXmix (equal GNSB941 and CFX1223) (Kragelund et al., 2011). Each time, *Thiothrix* was found to be the predominant (codominant) filamentous bacterium in the tested samples of activated sludge (Fig. 2). The density of filamentous bacteria was expressed according to the filamentous index (FI) developed by Eikelboom (2000).

Characteristics of the wastewater treatment plant

The wastewater treatment system of ZPM "Mlekpol" in Mrągowo consists of a mechanical part that includes crates and a grit chamber, and a biological part in which wastewater is treated with activated sludge.

The WWTP receives, on average, 2 000 m^3 of dairy wastes per 24 h. After mechanical treatment, wastewater is directed to a 14 000 m^3 activated sludge tank where it is aerated with floating surface aerators and aerating brushes. Separation of treated wastewater from activated sludge is done in a first-degree sedimentation tank (420 m^3) and then in two second-degree sedimentation tanks (of 320 m^3 each) from which wastewater, after flowing through a measuring chamber, is directed to three oxidation ponds with a total capacity of 20 000 m^3. A water course linking Lake Piecuch and Lake Juno is the final receiver of treated wastewater. Activated sludge from the sedimentation tanks is directed to an aerobic stabilization chamber (2 000 m^3) from which, after aeration, it is recirculated to an activated sludge tank or dehydrated in a filter press.

Rotifer clone

The *Lecane inermis* rotifers (Monogononta) that were used in the study originated from a WWTP situated in southern Poland. A single *Lecane inermis* specimen, which initiated the Lk3 line, was isolated from activated sludge with a micropipette and transferred to the well of a tissue culture test plate. Medium and 1 mL of spring water were introduced into the well. The plate was kept in darkness at 20±1°C. After a few days, the content of the well, in which the number of *Lecane inermis* had reached approximately 100 ind/mL, was transferred to a 55-mm Petri dish. When the density of rotifers had sufficiently increased, part of the culture was transferred to other Petri dishes.

Experiments

On a laboratory scale, three independent one-week experiments were conducted. In experiment number 1 (E1) with activated sludge of FI = 4, *Thiothrix* was the predominant filamentous bacterium. In experiment 2 (E2) with activated sludge of FI = 3, *Thiothrix* was codominant with filamentous bacteria classified as *Chloroflexi*. In experiment 3 (E3) with FI = 4, *Thiothrix* was codominant with Type 0041.

Each experimental arrangement consisted of 4 beakers with a capacity of 2 000 mL. Each beaker was placed on a magnetic stirrer and contained 1 000 mL of activated sludge from the WWTP ZPM "Mlekpol" in Mrągowo, which was aerated with a small-bubble aeration system. The treatment consisted of two beakers into which *Lecane inermis* was introduced at 600±40 ind/mL. Control beakers were filled with water to a final volume that corresponded to that of the treatment beakers after the addition of culture.

The density of filamentous *Thiothrix* bacteria was monitored three times: at the beginning of the experiment and after its fourth and seventh days. From each beaker, 25 μL of sludge was sampled and used to prepare smears on 22 x 22 mm slides that were Gram-stained. With a Carl Zeiss AXIO Imager microscope at 1 000 x, photographs of 15 randomly selected fields on each slide were taken. The density of *Thiothrix* bacteria expressed with the DF ("density factor") index was calculated based on the average number of bacterial filaments crossing the edges of the recorded images of 139 x 104 μm in dimension.

The number of *Lecane inermis* in the experimental beakers was determined daily in each experiment and expressed by a number of individuals per mL of sludge.

On the starting day and on day 7 of the experiment, the composition of biocenosis of activated sludge (including the Protozoa and Metazoa specimens) was examined in each beaker with the keys proposed by Ricci, Melone (2000), Radwan et al. (2004) and Fiałkowska et al., (2005).

In order to determine the sedimentation properties of the activated sludge, its settling was determined at the beginning and the end (day 7) of the experiment. 1 000 mL of activated sludge was taken from each site and sedimented for 30 min.

The results of chemical analyses of influents were provided by the WWTP of ZPM "Mlekpol" in Mrągowo.

Statistical analyses

The statistical analyses were conducted with the

STATISTICA StatSoft (version 10.0) data analysis software. The normality of the distribution was confirmed by the Shapiro-Wilk test. To test the differences in the abundance of *Thiothrix* bacteria, the numbers of Protozoa and Metazoa between both controls and treatments and between treatments on different days of the experiments, ANOVA Kruskal-Wallis and median tests were used (*post hoc* multiple comparisons of mean ranks for all groups), at the significance level p <0.05.

RESULTS

A high content of organic contamination characterized the influent received by the WWTP of ZPM "Mlekpol" in Mrągowo (Table 1). The BOD_5/COD ratio in the influent was 0.42 for experiment E1, 0.49 for E2 and 0.52 for E3.

The number of *Lecane inermis* varied during the experiments (Fig. 3). In each experimental series, the rotifers introduced to the activated sludge increased to at least 3.5 times their initial number. The highest number of rotifers in experiment E1 was detected on day 3, while in experiments E2 and E3 the highest number of *Lecane inermis* was recorded on days 6 and 5, respectively.

In each of the three experiments, the DF of filamentous bacteria in activated sludge enriched with rotifers decreased from the initial values of 3.05 in E1, 2.37 in E2 and 2.70 in E3 to 0.37, 0.50 and 0.33, respectively, on day 7 of the experiment. The density of *Thiothrix* bacteria was significantly lower in the experimental beakers than in the control beakers on day 4 (p <0.05) and on day 7 (p = 0.00) of the experiment. In each of the experiments, *Thiothrix* density was significantly lower on day 4 than it was on day 0 (p <0.05), but there was no significant difference between its density on day 4 and day 7 (p >0.05). In the control beakers, the density of *Thiothrix* remained about the same as it was at the beginning of the experiments (p = 1.00). The effect of rotifers on filamentous *Thiothrix* bacteria in the individual experiments is presented in Fig. 4 and is expressed as a percentage reduction of DF.

The sedimentation of the activated sludge used in the experiments was negatively affected by the presence of filamentous bacteria. On the first day, after sedimentation the volume of activated sludge was 960 mL in E1, 890 mL in E2 and 950 mL in E3. The settling properties of the activated sludge were improved on the 7th day after addition of *Lecane inermis*. The settling of activated sludge decreased to 430±14 mL in E1, 415±21 mL in E2 and 460±14 mL in E3. On the same day, the volume of activated sludge in the control beakers after 30 min of sedimentation was 925±7 mL in E1, 805±21 mL in E2 and 915±7 mL in E3.

The number of Protozoa and Metazoa taxa in activated sludge on day 7 of the individual experiments was the same as at the beginning. The number of individual taxa on day 7 of the experiments did not significantly differ between the control beakers and the beakers with *Lecane inermis* (p = 1.00) (Table 2). In the samples of activated sludge, *Aspidisca lynceus* was most numerous. Native rotifers belonging to the Bdelloidea class were found only in the samples of sludge from E1.

DISCUSSION

To date, only a few studies have been undertaken to develop sustainable techniques for mitigating biomass bulking in activated sludge processes (Choi et al., 2011). The concept of using natural enemies of filamentous bacteria such as *Lecane inermis* rotifers may provide a basis for developing an environmentally friendly method that would prevent the bulking of activated sludge. Studies indicate that *Lecane inermis* rotifers are able to reduce the density of filamentous bacteria in activated sludge originating from WWTPs that receive municipal wastewater (Kocerba-Soroka et al., 2013). Such studies have not yet been conducted with activated sludge from an industrial WWTP. The composition of wastewater generated by industry differs substantially from municipal wastewater. The composition of industrial wastewater includes a wide spectrum of different organic compounds from varied industries and their production systems (Ibarbalz et al., 2013). In our study, activated

Fig. 1. Foam on the surface of activated sludge in the WWTP of ZPM "Mlekpol" in Mrągowo

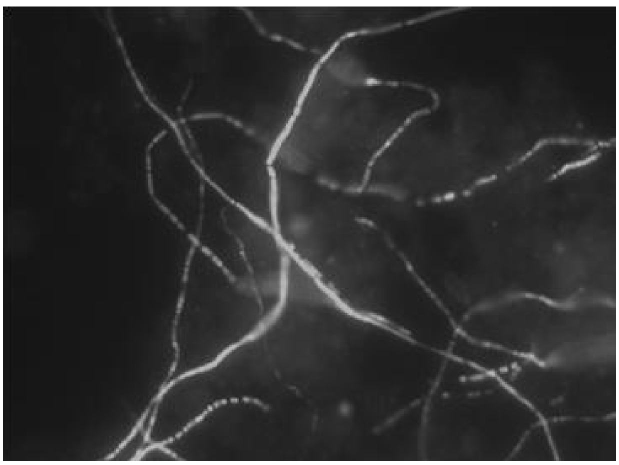

Fig. 2. *Thiothrix* in activated sludge sample after hybridization with a G123T probe

sludge originated from a dairy WWTP. Compared to reports in the literature (Donkin 1997), the composition of the influent received by the WWTP of ZPM "Mlekpol" in Mrągowo contained a high level of COD, BOD_5 and a relatively low concentration of TN and TP (Table 1). Combined with the presence of sulfates that can be reduced to sulfides, conditions in the activated sludge were favorable for the growth of filamentous *Thiothrix* bacteria.

Production processes in dairy plants use alkaline cleaning solutions to saponify lipids and remove protein substances. Disinfectants are often used that

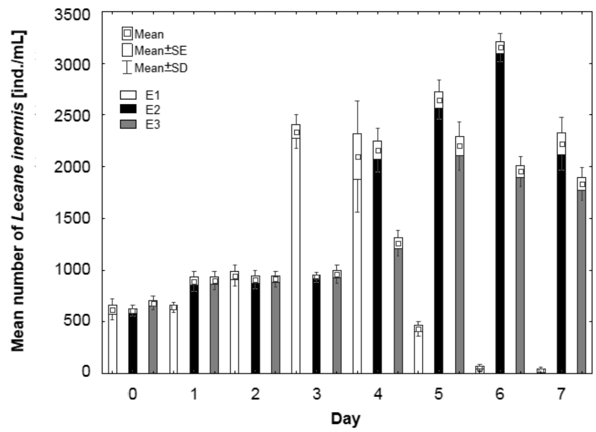

Fig. 3. Mean number of *Lecane inermis* in sludge on different days of the experiments

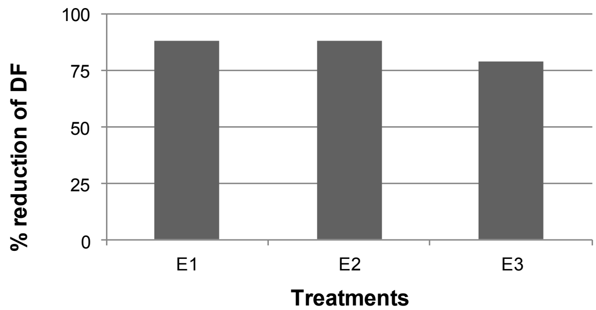

Fig. 4. Percent reduction of DF when treatment was compared to control on day 7 of the experiments

contain chlorine (e.g. sodium hypochlorite), iodine compounds or acids (Britz et al., 2006). As reported by Arvanitoyannis and Giakoundis (2006), detergents used in the dairy industry may affect the toxicity of wastewater and its susceptibility to biological treatment. The BOD_5/COD ratio is regarded as an important indicator of biodegradability of wastewater. Janczukowicz et al. (2008) reported that a value of this index lower than 0.6 indicates low susceptibility of wastewater to biological decomposition. A value lower than 0.4 can indicate the presence of toxic substances in wastewater (Arvanitoyannis and Giakoundis 2006). Rotifers, depending on their species (Pérez-Legaspi and Rico-Martínez 2003), are thought to be organisms sensitive to toxic compounds introduced to wastewater (Jenkins et al.,

Table 1. Chemical characteristics of raw wastewater at the time of sampling of activated sludge used for the experiments

Characteristic	Units	E1	E2	E3
pH	-	6.9	8.2	8.4
TSS	mg/L	675.0	738.0	615.0
BOD_5	mg O_2/L	1108.0	1260.0	1308.0
COD	mg O_2/L	2610.0	2572.0	2513.0
TN	mg N/L	115.0	120.0	120.0
$N\text{-}NH_4$	mg $N\text{-}NH_4$/L	11.3	5.8	5.9
$N\text{-}NO_3$	mg $N\text{-}NO_3$/L	23.0	44.2	46.5
TP	mg P/L	35.0	33.0	28.0
Chlorides	mg Cl^-/L	382.0	518.0	416.0
Sulfates	mg SO_4^{2-}/L	20.0	13.0	12.0

Table 2. Mean number of Protozoa and Metazoa in sludge samples from controls (C) and treatments (T) on day 7 of the experiments

Species	E1		E2		E3	
	C	T	C	T	C	T
	[ind./mL]					
Acineria uncinata (Tucolesco, 1962)	140.0	140.0	93.3	73.3	66.7	80.0
Adineta vaga (Davis, 1873)	93.3	100.0	0.0	0.0	0.0	0.0
Arcella sp.	440.0	473.3	306.7	326.7	80.0	93.3
Aspidisca lynceus (Mueller, 1773; Ehrenberg, 1830)	1273.3	1253.3	1260.0	1220.0	1386.7	1406.7
Epistylis coronata (Nusch, 1970)	93.3	73.3	66.7	60.0	66.7	73.3
Litonotus lamella (Mueller, 1773; Foissner, Berger, Blatterer, Kohmann, 1995)	220.0	240.0	220.0	220.0	73.3	100.0
Philodina roseola Ehrenberg, 1832	200.0	226.7	0.0	0.0	0.0	0.0
Paranema sp.	80.0	93.3	66.7	80.0	53.3	40.0
Vorticella convalaria (Linnaeus, 1758)	86.7	86.7	100.0	100.00	73.3	80

2004). In the present study, the BOD_5/COD ratio of the crude wastewater was 0.42-0.58 when samples were collected, indicating that the wastewater contained substances that are difficult to biodegrade. The wastewater used in E1 had a BOD_5/COD ratio of 0.42, close to that which can indicate the presence of toxic substances. Hence, there was a fear that *Lecane inermis* inoculated into the activated sludge from the dairy WWTP would not survive. However, these rotifers survived and started to proliferate, increasing to more than 3 times their initial number in each of the experiments (Fig. 3). The fact that in each experiment the maximum number of rotifers was detected on a different day may have been caused by the initial condition of *Lecane inermis*; their mobility, the activity of their corona and ability to fill of the intestine individuals changed over time. In addition, the growth of rotifers may have been determined by different compositions of the activated sludge. Even though the characteristics of the influent presented in Table 1 were similar in the individual experiments, it cannot be concluded that the conditions in the activated sludge were similar. As reported by Fiałkowska and Pajdak-Stós (2008), the chemical and biological composition of activated sludge is so complex that it is not possible to control all its characteristics.

The increase in the number of *Lecane inermis* in activated sludge undoubtedly had an impact on the density of *Thiothrix* in the individual experiments. The results of the experiments prove that *Lecane inermis* reduces the abundance of the investigated bacterium in the samples of activated sludge that originated from the dairy WWTP (Fig. 4). By day 4 of each experiment, the concentration of *Thiothrix* in the samples of sludge with rotifers was significantly lower than in the controls. Due to a decrease in filament abundance, sludge-settling properties were improved. On the last day of each experiment, the volume of sludge after sedimentation was reduced in the beakers with *Lecane inermis* compared to the controls. The lower volume of settled sludge in the control samples after 7 days compared to the volume in the initial sludge resulted from the biomass lysis, since there was no substrate supply during the 7-day experiment.

During experiments, no other organisms such as *Trithigmostoma cucullulus* or *Trochilioides recta* ciliates (which, according to the literature (Bitton 2010), may feed on filamentous bacteria) were detected in the examined samples of activated sludge (Table 2). Our own, unpublished studies with *Adineta vaga* rotifer cultures indicate that this species does not feed on filamentous bacteria.

Jenkins et al. (2004) reported that *Thiothrix* is a strain that is very sensitive to chlorination. These authors give examples of procedures for the elimination with chlorine of activated sludge bulking caused by these bacteria. Apart from the efficacy in destroying filamentous bacteria, chlorination is not neutral to other organisms in activated sludge. Kocerba-Soroka et al. (2013) reported that the minimum dose of chlorine necessary for controlling the density of filamentous bacteria Type 021N in activated sludge exerts a toxic effect on Protozoa, particularly on ciliates, leading to a loss of individuals and species. The application of incorrect chlorine doses negatively affects nitrifying bacteria, which inhibits the process of nitrification (Séka et al., 2003). The current studies demonstrated that *Lecane inermis* rotifers that reached numbers over 3 200 ind/mL in E2 did not have a negative impact on the composition of Protozoa and Metazoa in activated sludge from the dairy WWTP (Table 2). However, it is not known whether *Lecane inermis* negatively influences floc-forming bacteria. It is possible that when a source of feed (i.e., filamentous bacteria) became depleted, rotifers would start feeding on the bacteria accumulated in flocs, thereby disturbing the wastewater treatment processes. Verification of this assumption requires further studies during which rotifers would be inoculated into activated sludge in a laboratory or technical flow system with control over the composition of influents and effluents.

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