The use of rotifers for limiting filamentous bacteria
Type 021N, a bacteria causing activated sludge bulking

Wioleta Kocerba-Soroka, Edyta Fiałkowska, Agnieszka Pajdz-Stós,
Beata Klimek, Ewa Kowalska, Adam Drzewicki, Humbert Salvadó
and Janusz Fyda

ABSTRACT

The excessive growth of filamentous bacteria and the resultant bulking of activated sludge constitute a serious problem in numerous wastewater treatment plants. *Lecane inermis* rotifers were previously shown to be capable of reducing the abundance of *Microthrix parvicella* and *Nostocoida limicola* in activated sludge. In the present study, the effectiveness of four *Lecane* clones in reducing the abundance of Type 021N filamentous bacteria was investigated. Three independent experiments were carried out on activated sludge from three different treatment plants. We found that *Lecane* rotifers are efficient consumers of Type 021N filaments.

**Key words** | activated sludge, *Lecane* rotifers, municipal and industrial wastewater treatment plant, sludge bulking, Type 021N

INTRODUCTION

The activated sludge process is the most common biological wastewater treatment process in the world (Wei et al. 2003) due to its relative ease of use and efficiency. Activated sludge contains a diversity of microorganisms, primarily bacteria, ciliated protozoa and metazoa. These organisms decompose biodegradable organic constituents and remove most of the inorganic fractions, such as nitrates and phosphates, from the wastewater.

Filamentous microorganisms such as bacteria and fungi are common components of activated sludge. According to Madoni et al. (2000), they play an essential role in floc structure. Organic and inorganic particles, together with floc-forming bacteria bound to the structure, create sludge flocs often inhabited by ciliates and other microorganisms. However, when filamentous microorganisms proliferate excessively, exceeding 3 on the 5-level Eikelboom classification system, they alter floc structure and negatively influence sludge sedimentation. This phenomenon called bulking of activated sludge is a fundamental biological problem in sewage treatment plants (Madoni et al. 2000; Jenkins et al. 2004). Among the 30 primary morphological types of filamentous bacteria occurring in activated sludge in municipal treatment plants, only a few cause bulking problems. The most troublesome are *Microthrix parvicella*, commonly occurring in low-loaded municipal treatment plants, and 0092 type (Eikelboom et al. 1998; Wanner et al. 1998; Seviour & Blackall 1993; Rossetti et al. 2005). The problem is often exacerbated in industrial treatment plants where the number of filamentous bacteria types exceeds 60 and where mixtures of different types occur (Eikelboom 2006).

Type 021N usually occurs in treatment plants where industrial waste is treated alone or in combination with domestic waste (Michael et al. 1983). One factor that promotes excessive growth of Type 021N is a high fraction of brewery waste (Lacko et al. 1999). Common causes of Type 021N overproliferation include nutrient deficiency, low food to mass ratio (F/M), the presence of septic waste,
and wastes containing sulfides or having high organic acid content (Jenkins et al. 2004).

Several methods exist to limit bulking, including the application of selectors, chemical additions and process modifications; however, no broadly applicable method has yet been developed (Jenkins et al. 2004). Nevertheless, there are a few ways to control Type 021N proliferation. The most common method is chlorination, causing cell deformity, cytoplasm shrinkage and filament lysis. For filaments that lack sheaths, such as Type 021N, the sludge volume index (SVI) declines within a few days of chlorine usage. However, chlorination reduces only the symptoms of bulking. As soon as the application of chlorine is interrupted, filaments usually regrow rapidly. Séka et al. (2001) studied the effect of chlorination on sludge bulking due to 021N type and found chlorine-resistant strains of this bacterium, demonstrating that this method may be ineffective in limiting Type 021N bulking. In addition, Salvadó et al. (2000) found that the minimum dose of chlorine needed to control Type 021N would negatively affect the protist community. Haker & Jonsson (2005) tested five different chemicals (NaOCl, H₂O₂, FeCl₂, PAX-XL60 and Eco-floc) against Type 021N in a short term laboratory test. The oxidizing agents (NaOCl, H₂O₂) negatively affected not only the filaments but also the entire sludge biocenosis. Only PAX-XL60 selectively reduced filamentous bacteria without negatively affecting other microorganisms.

Recently, Lecane rotifers were found capable of consuming filamentous bacteria and effectively reducing their abundance in sludge (Fiałkowska & Pajdak-Stós 2008). However, only the effect of rotifers on Microthrix parvicella was investigated. The aim of the present study was to determine whether Lecane rotifers can also consume filamentous bacteria of Type 021N and whether this ability is clone dependent.

Sludge was sampled from aeration tanks and transported to the laboratory. Vials were filled halfway to prevent dissolved oxygen shortages during transport. Samples were refrigerated at 6°C in the laboratory and used within 24 hours.

Prior to the experiment, all sludge samples were analyzed according to Eikelboom’s method (Eikelboom 2000) to assess the density of filamentous bacteria. The filament index (FI) was evaluated by comparing microscopic images with reference manual photographs (Eikelboom 2000; Fiałkowska et al. 2005). The FI ranges from 0 to 5; however, we introduced half point intervals to increase the resolution of the scale.

**Rotifer clones**

Four different clonal populations of the monogonont rotifer Lecane inermis were cultivated in the laboratory. Strain Lk1, described by Fiałkowska & Pajdak-Stós (2008), originated from a small, domestic, wastewater treatment plant, whereas the three other clones of Lecane (Lk2, Lk3 and Lk6) originated from plants in southern Poland. All rotifer clones were obtained from single individuals transferred via micro-pipette from the sludge samples to individual wells of tissue culture test plates containing 1 ml of mineral water. An oat grain was added to each well, enabling the proliferation of different types of bacteria. After a few days, the content of the wells was transferred to 6 cm diameter Petri dishes and maintained in darkness in Sanyo MLR-350 versatile environmental test chambers at 20°C. The clones were earlier investigated in the context of growth rate dependence on temperature, medium and starvation (Fiałkowska et al. 2011; Pajdak-Stós et al. 2011).

**CR experiment**

We first evaluated the effectiveness of clones of Lk1, Lk2, and Lk3 rotifers in reducing the density of filamentous bacteria in Cell Wells™ (Corning).

According to the modified Eikelboom method, the FI of CR sludge was 3.0. Only Type 021N bacteria were observed. One millilitre of well-mixed activated sludge was added to each of 16 wells: 12 treatment and four control wells. Each well contained 100 rotifers from clones Lk1, Lk2 and Lk3 in four independent replications. The rotifers were transferred via micro-pipette into each well under stereo-microscope. Approximately 100 µl of medium was also transferred; therefore, an equal volume of mineral water was added to each of the control wells. The cell plates were left in Sanyo MLR-350 versatile environmental treatment plants were used. One plant, hereafter referred to as CR, treats brewery sewage influent (southern Poland). The remaining two plants are municipal treatment plants in Reus (Spain) and Jonkowo (northern Poland). Experiments were carried out at Jagiellonian University (CR), the University of Warmia and Mazury (Jonkowo) and the University of Barcelona (Reus).
test chambers in darkness at 20 °C. After 1 week, three 15 μl subsamples were taken from each well, mixed with 10 μl of orange acridine on a microscope slide and covered with a 22 × 22 mm cover slip. We took digital images of five randomly chosen fields of view of each slide using a Nikon Eclipse 80i fluorescence microscope equipped with a B-2A filter (excitation 450–490 nm) and using a total magnification of 1,000 × . Each image was analyzed using the Lucia image analysis system. We counted the number of acridine-colored filaments crossing the borders of 85 × 63 μm recorded images, resulting in a density factor (DF) value, as described in Fialkowska & Pajdak-Stós (2008). At the end of the experiment, 25 μl subsamples from each well were taken and fixed with Lugole solution, and the number of rotifers was counted.

Reus experiment

A similar experimental procedure was applied to the sludge from the Reus treatment plant, using the rotifer clone Lk6. During rotifer transfer, approximately 200 μl of medium was also transferred, so an equal volume of mineral water was added to each of the control wells. The cell plates were left for 1 week in a dark chamber at room temperature.

At the Reus WWTP, bacterium Type 021N dominated and reached Fl = 5. After 1 week, two 15 μl subsamples were taken from each well, mixed with 10 μl of orange acridine on a microscope slide and covered with a 20 × 20 mm cover slip. Ten images of randomly chosen fields of view were taken using a Leitz Dialux 20 fluorescence microscope at a total magnification of 400 × . The number of filaments crossing the borders of 175 × 150 μm recorded images was counted, yielding the DF value.

Jonkowo experiment

The Jonkowo experiment was carried out in six 250 ml bottles using clone Lk3. Each bottle contained 200 ml of activated sludge from Jonkowo WWTP. Type 021N and Actinomycetes were present in equal proportions in the sludge, with Fl = 5. Rotifers were added to three experimental bottles. Rotifer density at the start of the experiment reached 793 individuals per millilitre. Mineral water was added to the three remaining control bottles. Activated sludge was aerated with an aquarium pump and kept at room temperature for 1 week.

After 1 week, 25 μl subsamples from each bottle were taken, and 22 × 22 mm smears were prepared on microscope slides. Gram staining was then applied. Using a Carl Zeiss AXIO Imager microscope and a total magnification of 1,000 × , we took 15 digital images of randomly chosen fields of view for each smear. Each image was analyzed using the Axio Vision Release 4.4 image analysis system. DF was measured from 139 × 104 μm recorded images, and the mean DF was calculated for each bottle.

To obtain comparable results, the total length of filaments in each experiment was calculated following Salvadó’s (1990) equation:

\[ \text{Total length of filaments/volume} = \frac{N_i \times Ar \times V}{H \times V} \]  (1)

where: \( N_i \) = mean DF = number of intersections/number of observed fields; \( Ar \) = area occupied by the sample (cover slip area) in m²; \( H = (Le) \) (average sine) = \((Le) \times (2/\pi)\), \( Le \) = length of fields of view edges in metres; and \( V \) = volume of the sample in ml.

Statistical analyses

To test for differences in the abundance in filamentous bacteria (i.e. in DF) between controls and treatments and among rotifer clones in the CR experiment, factorial ANOVA (analysis of variance) and post-hoc Tukey HSD (honestly significant difference) tests were used. Student’s t-test was used to test whether rotifers of different clones significantly reduced the filament number. Analyses were conducted using the data analysis software system STATISTICA StatSoft, Inc. (2007), version 8.0.

RESULTS

The effects of rotifers on filamentous bacteria in sludge from different plants, expressed as the percentage reductions in the DF, are shown in Figure 1. The efficiency of rotifers in removing filamentous bacteria from activated sludge in CR varied by clone. For this sludge, filament abundance differed between the Lk2 treatments and the controls (\( p < 0.01 \)). For Lk1 and Lk3, statistical significance was less (\( p < 0.05 \) for both clones).

For the Reus sludge, the Lk6 clone yielded a strongly significant reduction in filament abundance (\( p < 0.001 \)). The average length of filaments in treatment wells was almost five times less than that of the controls (Table 1).

In Jonkowo, the average DF of the Lk3-containing bottles decreased from 19.5 to 2.9, (\( p < 0.001 \)) and the average filament length decreased from 1,222.09 to 185.22 m/ml
In the control bottles, the abundance of Type 021N remained stable throughout the experiment.

The number of live rotifers at the end of each experiment is shown in Tables 1 and 2. In the CR sludge, only solitary Lk2 rotifers remained. In the Reus sludge, rotifer abundance increased 25-fold, whereas in Jonkowo, it increased two-fold on the third day, then decreased by half of the original amount by the last day of the experiment.

**DISCUSSION**

The use of rotifers as biological tools for controlling the abundance of filamentous bacteria in activated sludge is promising (Fialkowska & Pajdak-Stós 2008). The rotifer *L. inermis* was found to be highly effective in reducing filamentous bacteria density, particularly of the problematic and common *Microthrix parvicella* and *Nostocoida limicola* (Pajdak-Stós & Fialkowska 2012). However, that study evaluated the effect of only one rotifer clone on bulkling activated sludge. On the other hand, in a study of the influence of different media on rotifer growth and reproduction, we showed that all the *L. inermis* clones investigated in the present paper survived and proliferated well after transfer to a filtrate of activated sludge (Pajdak-Stós et al. 2011). Rotifers have been observed to strongly influence the density and abundance of filamentous bacteria; however, their effectiveness depends on the

**Table 1** The initial and the final density of rotifers *L. inermis*, density factor and total length of 021N filaments during CR and Reus experiments

<table>
<thead>
<tr>
<th>CR</th>
<th>Reus</th>
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<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>Lk1</td>
<td>Lk2</td>
</tr>
<tr>
<td>Initial rotifer density [ind/ml]</td>
<td>100</td>
</tr>
<tr>
<td>FI (initial)</td>
<td>3</td>
</tr>
<tr>
<td>Rotifer density after 7 days [ind/ml]</td>
<td>0</td>
</tr>
<tr>
<td>DF (treatment) after 7 days</td>
<td>0.42</td>
</tr>
<tr>
<td>DF (control) after 7 days</td>
<td>1.02</td>
</tr>
<tr>
<td>Total length of filaments (treatment) [m/ml]</td>
<td>71.92</td>
</tr>
<tr>
<td>Total length of filaments (control) [m/ml]</td>
<td>174.66</td>
</tr>
</tbody>
</table>

**Table 2** The density of rotifers, density factor and total length of Type 021N filaments during Jonkowo experiment

<table>
<thead>
<tr>
<th>Jonkowo Lk3</th>
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<tbody>
<tr>
<td>Initial rotifer density [ind/ml]</td>
</tr>
<tr>
<td>DF initial</td>
</tr>
<tr>
<td>FI (initial)</td>
</tr>
<tr>
<td>Total length of filaments (initial) [m/ml]</td>
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<tr>
<td>Rotifer density after 3 days [ind/ml]</td>
</tr>
<tr>
<td>DF after 3 days</td>
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<tr>
<td>Rotifer density after 7 days [ind/ml]</td>
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<tr>
<td>DF (treatment) after 7 days</td>
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<td>DF (control) after 7 days</td>
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<tr>
<td>Total length of filaments (treatment) [m/ml]</td>
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<tr>
<td>Total length of filaments (control) [m/ml]</td>
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(Graph 1) The percentage of DF value reduction in treatment calculated in comparison to control on the last day of experiments.
composition of activated sludge (Fialkowska & Pajdak-Stós 2008). Therefore, it was necessary to check the effectiveness of rotifers in sludge samples from different treatment plants comprising different types of bacteria.

Our results of the CR experiment demonstrate that rotifer filament consumption is clone dependent. The observed differences in rotifer final density and effectiveness in filamentous bacteria removal likely reflect differences among Lecane rotifer clones. The growth rate of L. inermis documented by Fialkowska & Pajdak-Stós (2008) varied between 0.3 and 0.8, depending on the experimental setup.

Protozoan and metazoan populations in sewage sludge systems are primarily limited by the amount of food available and their morphological properties (size of mouth and pharynx) (Wei et al. 2003). The presence and quantities of micronutrients, such as vitamins and trace elements, also influence the condition of sewage microorganisms (Burgess et al. 1999). Rotifers are reported to feed primarily on unicellular algae and different types of bacteria; some species are predatory (King 1967; Kirk 1997). Monogonont rotifers have been reported to feed on filamentous cyanobacteria (Gulati et al. 1993; Walz 1993), although those species are planktonic. The first report of L. inermis feeding on filamentous bacteria came from Fialkowska & Pajdak-Stós (2008). The results of the present study suggest that filamentous bacteria are a sufficient food source for the survival and growth of rotifers.

The data from the Reus and Jonkowo samples reveal that rotifers can reduce the abundance of Type 021N filamentous bacteria and achieve high density. In Jonkowo, clone Lk3 reduced the filament number, and the abundance of live rotifers increased significantly during the experiment but decreased by the end. Their survival was likely the result of favorable conditions in the activated sludge, as suggested by the presence of native L. inermis. The decline in rotifer abundance at the end of the experiment might have been due to food depletion.

In the CR experiment, each Lecane clone significantly reduced filamentous bacteria density. However, at the end of the experiment, only solitary live rotifers were observed. Despite the fact that almost all the rotifers died, filament number was significantly reduced. It is possible that the bacterial filaments disintegrated due to aging and unfavorable conditions within the experimental wells; however, if so, similar results should have been observed in the control wells. An alternative possibility is that rotifers consumed some filaments early in the experiment and then died. The rotifers used in this study were isolated from municipal treatment plants and cultured under laboratory conditions for 3 years prior to the experiments. The activated sludge from the CR treatment plant contained a strong influent of brewery sewage, possibly presenting unfavorable conditions to which the rotifers were unacclimated. Food quality and quantity strongly influence rotifer growth (King 1967; Stemberger & Gilbert 1985; Kirk 1997). The effects of filamentous or/toxic bacteria on rotifers are poorly understood, with the exception of cyanobacteria: although rotifers are largely resistant to toxic cyanobacteria, some are not (for a review, see Walz (1995)). Whether filamentous bacteria in activated sludge can be toxic to rotifers requires investigation. The CR wastewater was not immediately toxic to rotifers; otherwise, the density of 021N type bacteria should have remained stable across all treatments. Some interesting observations (earlier unpublished) made in CR wastewater treatment plant showed that from February to May 2011, filamentous bacteria 021N type occurred at high abundance in activated sludge from the CR plant. In April, native L. inermis rotifers proliferated rapidly and reduced filament abundance, as shown in Figure 2. It is likely that the rotifers occurring naturally in this type of sludge are more resistant to the constituents of the influent than those bred in the laboratory. Figure 2 illustrates an example of the interaction between rotifers and filamentous bacteria in wastewater. After a rapid decrease in filament abundance, rotifer and bacterial abundance approached an equilibrium. However, if more rotifers had been introduced, the decrease in filament abundance may have been much more pronounced. The relationship between rotifer and bacterial abundance at larger scales requires further investigation.

Figure 2 | Index of filamentous bacteria 021N and rotifers (according to Eikelboom (2008)) in CR treatment plant during 3-month investigation.
An advantage of using rotifers as a biological control of activated sludge bulking is the reduced need for flocculants or chemicals such as aluminum polychlorides (PAX-es), which can have negative environmental effects and may be toxic to humans, even though they exhibit no obvious negative effects on non-target filamentous organisms in sludge (Rossetti et al. 2005). Rotifers do not pose any threat to the natural environment, as a fraction of them is potentially be modified via sludge age regulation. In the event of overproliferation and potential release into the environment, they can serve as prey for natural enemies such as insect larvae and juvenile fish. The density of rotifers in the bioreactors could also be controlled via sludge age regulation.

This study showed that clones of *L. inermis* vary in their effectiveness in reducing filamentous bacteria. An appropriate rotifer clone should be identified before using rotifers against filamentous bacteria.

**CONCLUSION**

Four clones of *Lecane inermis* are capable of limiting the proliferation of filamentous bacteria Type 021N, which is often responsible for activated sludge bulking. A significant reduction in activated sludge bulking may be achieved with this biological method, providing an alternative to chemical methods.

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