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Can a predatory fungus (*Zoophagus* sp.) endanger the rotifer populations in activated sludge?

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ABSTRACT

We investigated the influence of the predatory fungus *Zoophagus* sp. and temperature on the number of rotifers inhabiting activated sludge. When a sample of sludge was subjected to the fungus, the number of *Lecane inermis* and bdelloid rotifers was reduced at 15 °C and 20 °C, whereas at 8 °C the effect was not significant. The effect of *Zoophagus* on two *Lecane* and one bdelloid species, in conditions optimal for rotifers, confirmed the negative effect of the fungus on rotifer density. In the *L. inermis* treatment, the number of living rotifers was three times lower than in the control. These results demonstrated that the massive occurrence of predatory fungus may endanger the population of rotifers (organisms considered capable of significantly improving the quality of the effluent from wastewater treatment plants).

1. Introduction

Lecane rotifers are a promising biological tool for controlling bulking in wastewater treatment plants (WWTP). The rotifers can ingest most of the common types of filamentous bacteria that occur in activated sludge (Fiałkowska and Pajdak-Stós, 2008; Pajdak-Stós and Fiałkowska, 2012; Kocerba-Soroka et al., 2013; Drzewicki et al., 2015). Unfortunately, the factors affecting rotifer populations in wastewater treatment plants are not well understood. While monitoring rotifer/filamentous bacteria relationships in full scale WWTPs we observed a few episodes of unpredicted collapse of rotifer population. A closer look led to the discovery that the reason for rotifer mortality was not chemical, but of a biological nature. Predatory fungus armed with 'lollipop' traps attracted rotifers and killed them as described by Whisler and Travland (1974).

Predatory fungi capable of killing rotifers have previously been reported only once in activated sludge in a full-scale WWTP. Sladka and Ottova (1973) mentioned the species *Zoophagus insidians* as one of the filamentous organisms in activated sludge able to catch and digest rotifers. Although the fungus was reported in activated sludge, it is not a common species. In a thorough study of fungi in activated sludge, Cooke and Pipes (1970) did not identify any *Z. insidians* in samples from 19 different wastewater treatment plants. Sladka and Ottova (1973) suggested that this was due to the retention time. Short or long retention times are not optimal for rotifers and nematodes, which are the food source of predatory fungi.

Due to the lack of research on predatory fungi inhabiting activated sludge, their role in wastewater treatment plants is not well recognized. Our observation showed that *Zoophagus* sp. can destroy the rotifer community in activated sludge. Cooke and Ludzack (1958) described drastic impairment of laboratory activated sludge units used for nitrile removal because of the accidental development of *Z. insidians*. This growth resulted in the massive death of the rotifer *Monostyla* sp.

We performed experiments designed to test the hypothesis that the abundance of rotifers inhabiting activated sludge is affected by the presence of predatory *Zoophagus* sp. and determined whether the effect was temperature-dependent.

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2. Materials and methods

2.1. Isolation, cultivation and species determination of predatory fungus

Samples of fresh activated sludge were poured into Petri dishes and incubated at 20 °C for 1 week. Then the Petri dishes were examined using an inverted microscope. Dishes with predatory fungi attached to the bottom were rinsed with Żywiec spring water, supplied with *Lecane inermis* and incubated in Sanyo MLR-350 environmental test chambers at 20 °C for 2 weeks. After several transfers, the hyphae that were attached to the bottom of the dish were cut, and small pieces were separately transferred with a micropipette into the wells of 24 tissue test plates. To obtain a strain of the fungus, the mycelium from one well was chosen for further cultivation and the strain was named ZTk1. The fungus was identified as *Zoophagus* sp. according to its morphological features. Zoospores (conidia) were not observed.

Molecular methods were used to confirm the morphology-based identification. The DNA extraction of the mycelium samples was performed using a Genomic Mini AX Plant Kit (A&A Biotechnology). Amplification of the 18S rDNA region was performed using Pf1fw (5′-CAAGTCTGGTGCCAGCAGC-3′) and Pf2rev (5′-GACTACGACGGTATCTGATC-3′) primers (Walochnik et al., 2004). PCR conditions were optimized for a Maxima Hot Start Green PCR Master Mix (Thermo Fisher Scientific). The reaction contained 10 μg/μl DNA, 9.5 μl of nuclease-free water, 12.5 μl of Master Mix and 1 μg/10 pmol of each primer. The total volume of each reaction was 25 μl. PCR conditions included 1) an initial denaturation at 95 °C for 3 min; 2) 35 cycles consisting of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 45 s and 3) a final elongation at 72 °C for 5 min. The presence of PCR products was visualized in a 1.5% agarose gel stained with GelRed (Biotium). Isopropanol and a 0.3 M sodium acetate precipitation were used to purify the PCR products. The PCR products were sequenced using Macrogen (The Netherlands). The Pf1fw primer was used for reading sequences. Nucleotide sequences were analysed with Chromas software (www.technelysium.com.au) and compared with the published sequences in the NCBI database (www.ncbi.nlm.nih.gov) using the BLASTn algorithm.

2.2. Rotifers in activated sludge subjected to predatory fungus

Prior to the experiment, small, approximately equal pieces of *Zoophagus* hyphae were cut using a cover glass. They were transferred into 8 wells of 3 tissue culture test plates (Cell Wells™, Corning) using a Pasteur pipette. The process was performed using a stereomicroscope. A 1 ml aliquot of Żywiec spring water and 25 μl of dense (approximately 10,000 ind/ml) *L. inermis* culture were added into each well and incubated for 24 h at 20 °C. The fungus adhered to the bottom of the wells and caught some rotifers. After 24 h, the supernatant with free-swimming rotifers was removed. The remaining rotifers were dead and attached to fungus pegs. A 1 ml sample of activated sludge, obtained from a wastewater treatment plant treating brewery sewage, containing naturally occurring rotifers was added to each of the 8 wells with fungi (fungi+) and to additional 8 control wells without fungi (fungi-). The test plates were transferred into Sanyo MLR-350 environmental test chambers. The plates were stored at 8 °C, 15 °C and 20 °C, each at a different temperature, for 8 days without light. Using a micropipette, we sampled 3 × 15 μl aliquots of mixed activated sludge from each well, covered them with a 22 × 22 mm coverslip and counted the number of rotifers belonging to different taxa. The counting was performed '*in vivo*' using a Nomarski contrast microscopy (Olympus) at 200–400× magnification (depending on the size of the taxon). Species determination was conducted according to Segers (1995).

2.3. The effectiveness of predatory fungus at limiting different rotifer species in conditions optimal for rotifer growth

Three species of rotifers were used in this experiment: *L. inermis* (strain 2.B3.20), *Lecane hamata* (strain Lh1) and a bdelloid rotifer strain (Pk2). The strains originated from treatment plants located in southern Poland. All clones were obtained from single individuals transferred via a micropipette, from the sludge samples to the individual wells of tissue culture test plates. The obtained clones were cultured in Petri dishes with Żywiec spring water. NOVO powder (dry yolk supplemented with vitamins and minerals and prepared according to a confidential protocol available at the Aquatic Microbial Ecology Laboratory, Jagiellonian University) was added as the medium. All rotifer strains are maintained in the culture collection of the Institute of Environmental Sciences, Jagiellonian University, Kraków, Poland.

Experimental plates with predatory fungus were prepared according to the previously discussed method, with the exception of the initial rotifer addition. After 24 h, 100 rotifers were individually counted and transferred into each well. Eight treatment (fungi+) replicates and four control (fungi-) replicates were prepared for each rotifer species. Spring water (1 ml) and nutrition powder NOVO suspension (25 μl) were added into each well. The test plates were transferred into a Sanyo MLR-350 environmental test chamber and incubated at 20 °C for 6 d without light. The rotifers were fixed *in situ* by the addition of 25 μl of acidic Lugol (Analab, Poland) solution (Wiackowski et al., 1994) and counted using an inverted microscope. The population growth rate coefficient (r) was calculated according to formula: $r = (\ln(n_t)-\ln(n_o))/t$, where '$n_t$' is the number of rotifers at the end of experiment, 'n_0' is the initial number of rotifers and 't' is the day of experiment. Doubling time (tD) was calculated as described by James and Dias (1985).

2.4. Statistical analysis

The data were log transformed to normalise the data. Two-way factorial ANOVA with a Bonferroni correction and a *post-hoc* unequal HSD test were used to determine if the abundance of 'native' rotifers naturally inhabiting the activated sludge was significantly influenced by temperature and predatory fungi.

One-way ANOVA was used to determine if the predatory fungi significantly affected the number of artificially introduced rotifers and if the effect was species-dependent. The statistical analysis was performed using STATISTICA software, v 10.0 (StatSoft, Inc., 2011).

3. Results and discussion

As predatory fungi, especially those preying on rotifers, are one of the least known group of organisms, there are still many problems concerning their taxonomy and nomenclature (Dick, 1990; Glockling, 1997). Molecular techniques confirmed our microscopic identification. The mycelium sample was identified as *Zoophagus* sp. The 18S rDNA partial sequence of the sample demonstrated a 91% (403/433) basepair similarity to the reference sequence of *Z. insidians* deposited in the NCBI database (AB016009.2).

3.1. Rotifers in activated sludge subjected to predatory fungus

L. inermis and Bdelloidea were the only rotifers abundant enough for statistical analysis. The results of the two-way factorial ANOVA revealed that the presence of *Zoophagus* sp. in activated sludge samples significantly affected both dominant taxa of