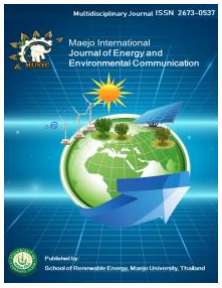
Maejo International Journal of Energy and Environmental Communication

Journal homepage: https://ph02.tci-thaijo.org/index.php/MIJEEC



ARTICLE

Predation properties of Rotifera *Lecane* isolated from an eutrophicated reservoir to toxic cyanobacteria *Microcystis*

Somsri Saenchan[1], Kazuya Shimizu[2] Norio Iwami[3], Hideaki Maseda[4], Huynh V. Vu[1], Odwa Makrwede[1], Tomoaki Itayama[1]*

[1]Graduate School of Engineering, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan
[2]Faculty of Life Sciences, Toyo University, 1-1-1 Izumino, Oura-gun Itakura, Gunma, 374-0193, Japan
[3]Graduate School of Science and Engineering, Meisei University, 2-1-1 Hodokubo, Hino, Tokyo 191- 8506, Japan
[4]Institute of Technology and Science, The University of Tokushima Graduate School, 2-1 Minamijosanjima-cho, Tokushima 770-8506, Japan.

ARTICLEINFO

Article history:
Received 19 September 2023
Received in revised form
11 October 2023
Accepted 21 October 2023

Keywords:
lake in Kashima
toxic *Microcystis* N102
L. inermis
HPLC

ABSTRACT

A rotifer was isolated from a biofilm sample collected from a eutrophic reservoir in Kashima city, Saga, Japan. After morphological and molecular phylogenetic determination using 18S rRNA gene sequencing, the isolated rotifer was identified as a *Lecane inermis*. In a mixed culture of *L. inermis* and microcystin (MC)-producing toxic *Microcystis aeruginosa* (NIES102), the number of *M. aeruginosa* cells decreased as the *L. inermis* population increased. This suggests that *L. inermis* preys on toxic *Microcystis*, resulting in a predator-prey interaction. By the 14th day of the mixed culture, 99.5% of the *Microcystis* cells were degraded. Data on the population dynamics of *L. inermis* and *M. aeruginosa* in this mixed culture were fitted to a predator-prey model, which was expressed by nonlinear differential equations. As a result, the model analysis suggested that *L. inermis* could reveal the predation properties of *M. aeruginosa* cells and may also consume bacteria as a food source. *L. inermis* is effective in promoting the degradation of MCs in the mixed culture. After 14 days of mixed culture, the degradation percentages of total MCs were 98.6% (MC-RR), 96.6% (MC-YR), and 93.9% (MC-LR).

1. Introduction

Many strains of *Microcystis* produce microcystins (MCs), a type of cyclic heptapeptide hepatotoxin (Sivonen et al., 1992). This toxic substance can be hazardous to both human and animal health, causing illness and mortality even at low concentrations in the environment (Codd et al., 2005). Therefore, to prevent the growth of toxic *Microcystis*, it is essential to develop a control method **(**Thakonget al., 2019**).** Several studies have successfully reduced cyanobacterial blooms consisting of *Microcystis* using physicochemical methods (Nakano et al., 2001). On the other hand, since predation is believed to be a significant natural loss process of *Microcystis* in eutrophicated lakes (Dryden & Wright, 1987), applying the predation ability of microfauna to toxic *Microcystis* can lead to the development of eco-friendly control methods for toxic *Microcystis* blooms.

* Corresponding author.
E-mail address: itayama@nagasaki-u.ac.jp

Nandini and Rao (1998) showed that rotifers such as *Hexarthra mira* and cladocerans (*Ceriodaphnia cornuta*) use toxic *Microcystis* cells as a food source, thereby preventing the growth of toxic *Microcystis*. Iwami et al. (1999) found that Mastigophora *Monas guttula*, Rotifera *Philodina erythrophthalma*, and Oligochaeta *Aeolosoma hemprichi*, which were isolated from biofilm samples at Lake Kasumigaura in Japan, were capable of degrading toxic *Microcystis*.

Furthermore, they were able to degrade the toxic *Microcystis* using a bioreactor containing a sponge carrier inhabited by these microfuana (Itayama et al., 2008; Iwami et al., 2000). Several other protozoa and metazoans found in lakes and reservoirs are known to prey on *Microcystis*. For example, protists testate amoebae (Nishibe et al., 2002 and *Poterioochromonas malhamensi* (Ou et al., 2005) have also been identified as predators of *Microcystis*. A recent review paper reporting on interactions between zooplankton and toxic cyanobacteria, collectively shows zooplankton preying on toxic *Microcystis* cells (Nandini & Sarma, 2023).

Of course, it is quite possible that there are many other predators other than the microfauna that can prey on toxic *Microcystis* that have been identified so far (Le-Huynh et al., 2022). Therefore, to develop effective biological control methods against toxic *Microcystis*, it is important to identify as many species that prey on toxic *Microcystis* as possible and to characterize their predation properties. This study aimed to investigate a microfauna capable of actively attacking toxic *Microcystis*, by exploring predatory rotifers commonly found in biofilms formed in eutrophic lakes and reservoirs.

2. Material and methods

2.1 Biofilm sample preparation

The biofilm sample was collected from rocks on the surface of a reservoir located in Kashima City, Saga Prefecture, Japan. The sample collection was done in November 2022, geographic coordinates 130.135483°N latitude and 33.0872220°E longitude. This reservoir experiences *Microcystis* blooms from April to December every year. The sample was then kept at 4°C in refrigerator of our laboratory.

2.2 Cultivation of toxic Microcystis

Microcystis aeruginosa NIES102 (Watanabe et al., 1995) was obtained from Microbial Culture Collection (MCC), National Institute for Environmental Studies (NIES), Japan. This *M. aeruginosa* NIES102 is a MC-producing strain and has lost its colony-forming ability. The *M. aeruginosa* cells was cultured using M11 culture medium adjusted to pH 8 (Yagi et al., 1979) under a white LED lamp (35-45 µE) at around 25°C. The *M. aeruginosa* cells were collected during centrifugation at 8000 rpm for 10 min using 50 mL centrifugation tubes, and then washed with M11 (pH 7), afterward suspended using M11 medium (pH 7). The cell suspension was stored under 4 °C until the use.

2.3 Isolation and cultivation of microorganisms

First, 1 mL of the *M. aeruginosa* cell suspension at 3.2 x 10^7 cells mL^{-1} was added to each well of a Micro24 well cell culture plate, followed by 1 mL of biofilm sample to each well. Then, the plates were incubated using the FUKUSHIMA INDUSTRIES CORP incubator under dark conditions at 23°C for 7 days. Afterward, the surviving microfauna individuals were observed using an inverted microscope (ECLIPSE TS100, Nikon Co., Japan). Then, one of the most proliferated rotifers was isolated using the glass capillary method and transferred to a petri dish. Following the isolation of the rotifer, it was washed five times with autoclave-sterilized water and cultured in a well of 24-well plates under the same conditions using the *M. aeruginosa* cell suspension. Finally, to obtain a sufficient number of clonal rotifers, the rotifers cultured in the wells were transferred to 50 mL Erlenmeyer flasks containing 15 mL of the *M. aeruginosa* cell suspension and incubated for 14 days cultivated.

2.4 18S rRNA gene-based molecular identification of rotifer

To identify the 18S rRNA gene from isolated rotifers based on molecular phylogenetic analysis, we used the direct PCR method developed by Watcharapong et al. (2019) was used. Five clonally cultured rotifer individuals were placed in Petri dishes and washed five times with autoclave-sterilized water. To remove DNA on the surface of the rotifers, 2 µL of DNase I (Takara Bio, Inc., Japan) and 7 µL of buffer solution were added to the tube containing the rotifers. The PCR tube was then incubated at 37°C for 30 min. After the incubation, 50 µL of 70% ethanol was added to the tube, which was then heated using a microwave oven (NE-EH22, Panasonic Co., Japan) at 500 W for 2 min. The tube was then subjected to ultrasonication using a Silent Sonic UT-204 (SHARP Co., Japan) with a power density of 2 W/mL for 30 min (Guo et al., 2008).

The tube was subsequently dried in an incubator at 60°C for 6 h and then frozen at -18°C. For PCR amplification, we used the TaKaRa ExTaq DNA polymerase Kit (TaKaRa Bio Inc., Japan) was used in a total volume of 50 µL with the metazoan universal eukaryotic primer set Metaz 3-F (forward primer: 5'-AACTTAAAGRAATTGACGGA-3') and Metaz5-R (reverse primer: 5'-GTGTGYACAAAGGBCAGGGAC-3') (Machida & Knowlton, 2012). The PCR amplification was performed under the following conditions: initial denaturation at 94°C for 3 min, 50 cycles of 98°C for 10 sec, 52°C for 15 sec, and 68°C for 30 sec, and a final extension step of 72°C for 7 min. After PCR amplification, a gel electrophoresis using a 2.5% agarose was performed. The gel was then visualized, and the target amplicon band was cut. The DNA was subsequently purified using the NucleoSpin® Gel and PCR clean-up kit (MACHEREY-NAGEL GmbH & Co. KG, Germany). The obtained PCR amplicon was sequenced by a company service (Fasmac Co., Japan), and the sequence was identified by BLAST search.

The multiple alignments of sequences were carried out by the Tamura-Nei method, and the phylogenetic analysis was conducted by the Neighbor-Joining method using MEGA X. The evolutionary distances were computed using the p-distance method (Nei & Kumar, 2000) and measured in units of the number of base differences per site.

2.5 Mixed culture of the isolated rotifer with M. aeruginosa

NIES102

Four replicates of 200 mL of *M. aeruginosa* NIES102 cell suspension with a concentration of 3.0×10^8 cells mL^{-1} were transferred to separate 500 mL Erlenmeyer flasks. 20 mL of culture medium from the rotifer preculture was added to three of the four flasks, whereas one flask was used as a control without adding the rotifer sample. The flasks were placed in the dark at 23°C for 12 days with reciprocal shaking at 60 rpm (MMS-1020, EYELA Japan) to culture the rotifers for 14 days. *Microcystis* cells were counted using an EOSINOPHIL counting chamber (Sunlead Glass Co., Ltd., Japan) under a microscope at 200x magnification. Simultaneously, the rotifers were also counted using 1mL counting slide glass for zooplankton (Matsunami Glass Industry Co., Ltd. Japan). The rotifer individual and *Microcystis* cell enumerations were performed in duplicate for each flask after 0 days (initial), 4 days, 6 days, 8 days, 11 days, and 14 days (final). For the control flask, *M. aeruginosa* cells were counted on the first and last day to confirm cell autolysis.

2.6 HPLC analysis of microcystins (MCs)

To achieve an acetic acid concentration of 5%, 2.6 mL of 100% analytical grade acetic acid (Fujifilm Wako Pure Chemicals, Co. Japan) was added to the first and last 50 mL samples of the mixed culture. The mixture was shaken for 30 min and then subjected to ultrasonic treatment until the cells were completely lysed (Lwin-Aye et al., 2020). The cell debris was removed by a 47 mm glass fiber filter (GA55, ADVANTECH, TOYO KAISHYA, Ltd., Japan).

InertSep RP-1 (60 mg/3mL) solid phase extraction (SPE) cartridge (GL Sciences, Inc., Japan) was washed with 5 mL of 100% methanol (HPLC grade, Fujifilm Wako pure chemicals, Co. Japan) at a flow rate of 5 mL/min, followed by twice washes with 20 mL of ultrapure water. The filtered sample was then passed through an SPE cartridge to capture MCs, then washed with 5 mL of 20% methanol and twice with 15 mL of ultrapure water. Then, MCs were eluted from the cartridge into a test tube using 5 mL of 100% methanol. The test tube was then dried by placing them in hot water at 70°C while flowing nitrogen gas at a flow rate of 100 mL/min and was completely dissolved with 0.5 mL of 75% methanol. MCs sample was subsequently filtered using a 0.22 µm pore size membrane filter (Nylon, Starlab Scientific, Germany) to obtain a purified solution ready for HPLC analysis.

A mixed standard solution of microcystin-RR (MC-RR), microcystin-LR (MC-LR) and microcystin-YR (MC-YR) of 1,000 µg L^{-1} (500 µg L^{-1} for MC-YR) were prepared from each standard chemical (Fujifilm Wako Pure Chemicals, Co. Japan). Then, standard solutions with concentrations of 1/2, 1/4, and 1/8 of the original mixed standard solution were prepared. Both the sample and the standard solutions were analyzed simultaneously using an HPLC (LC-2000 series, JASCO Co., Japan) equipped with a C-18 column (Inertsil ODS-3, 5µm, 4.6 mm×150 mm, GL Sciences, Inc., Japan). A gradient elution method was used in this system, with mobile phase A consisting of 0.05% (v/v) trifluoroacetic acid (TFA, HPLC grade, Sigma Aldrich, Co. LTD, USA) in ultrapure water, and 100% acetonitrile (HPLC grade, Fujifilm Wako pure chemicals, Co. Japan) used as mobile phase B. The gradient pattern was set as follows: it starts with 25% B (acetonitrile) and increased linearly to 45% B until 25 min, then immediately changed to 100% B and continued until 45 min, then returned to 25% B and continued until 60 min. A diode-array detector was employed to detect the signal at a wavelength of 238 nm. The concentrations of MC-RR, MC-YR, and MC-LR in each sample were calculated using the standard regression lines of MC-RR, MC-YR, and MC-LR with R^2 values of MC-RR, MC-YR, and MC-LR.

2.7 Data analysis

In all data analysis, statistical software R (version 4.2.2) was used. For nonlinear fitting of the population dynamics data on the predation prey model described by ordinary differential equations (ODEs), we used an ode solver “ode” in R package “deSolve” and the "nls.lm" in the "minpack.lm" package to apply the Levenberg-Marquardt Nonlinear Least-Squares algorithm.

3. Results and Discussion

3.1 Morphological and molecular identification of the isolated rotifer

Algal bloom (cyanobacteria bloom) has been known to occur from April to December every year in the reservoir where the biofilm samples were collected (personal information from a local resident). Therefore, the reservoir was selected as the source of isolation to find a predator for toxic *Microcystis*. In fact, we found that the microfauna present in the toxic *M. aeruginosa* cell suspension multiplied rapidly and became a potential predator for the toxic *Microcystis* cells. As a result, we were able to isolate a type of rotifer from the microfauna. It is important to note that the rotifers used in our study were clones of the same isolated rotifer.

Figure 1 Microorganism observation of *L. inermis inermis*

A typical isolated rotifer body consists of two parts: a dorsal and a ventral plate connected by lateral sulci, as shown in Figure 1 (Carl, 1929). The average dimensions of the organism were a mean length of 170 µm (std. error = 4.2 µm, n=15) and a mean breadth of 39 µm (std. error = 1.9 µm, n=15), with a flask-shaped body and flattened dorso-ventrally. Additionally, they are somewhat

cylindrical, transparent, and short, with toes that are long and slim and also being somewhat short and thick as shown in Figure 1 (Carl, 1929). According to the morphological characteristics, we identified the isolated rotifer as *Lecane inermis*, belonging to the genus of rotifers and the family of Lecanidae.We applied molecular identification method base on the 18S rRNA gene sequence, which has been used for the study of rotiferan phylogeny (Sorensen & Giribet, 2006).

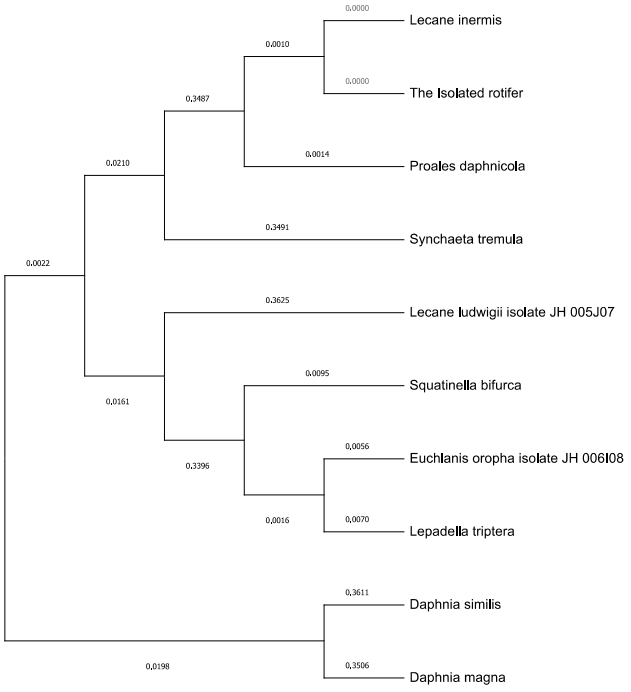


Figure 2 Phylogenetic trees of the isolated rotifer and closely related species

In our research, we used a metazoan-specific primer set (Machida & Knowlton, 2012) to obtain the 474 bp PCR amplicon. A phylogenetic tree of rotifers, which are genetically close to the isolated rotifers, using the two outer groups *Daphnia magna* and *Daphnia similis*, is shown in Figure 2. Sequencing analysis of the 18S rRNA gene of the isolated rotifer showed 100% sequence identity with *L. inermis* (Genbank accession number MT025820.1). The next closest species to the isolated rotifer on this phylogenetic tree is *Proales daphnicola* (Genbank accession number MT522677.1), with matches 99.8% identity in 18S rRNA gene sequence. However, *Proales* has different morphological characteristics from the isolated rotifers. Therefore, the isolated rotifers were identified as belonging to the order Ploima, family Lecanidae, genus *L. inermis*, and *L. inermis* based on its morphological characteristics and molecular identification based on 18S rRNA gene sequening.

3.2 Mixed culture of Microcystis NIES102 with L. inermis isolated

Isolation process as well as the preculture of *L. inermis* using toxic *M. aeruginosa cell* suspension strongly suggests that *L. inermis* has the ability to prey on toxic *Microcystis*. Additionally, we observed *L. inermis* preying on toxic *M. aeruginosa* cells using the microscope. However, it was difficult to capture clear images of the predation process. To obtain the quantitative evidence that *L. inermis* preys on toxic *Microcystis* cells, the population dynamics of isolated *L. inermis* and toxic *M. aeruginosa cells* were investigated in mixed culture for 14 days. The results of the enumeration of *M. aeruginosa* cells and *L. inermis* individuals in each flask is displayed in Figure 3. The three replicate flasks showed a similar trend in the decrease of *M. aeruginosa* cells. *M. aeruginosa cells* in flasks 1, 2, and 3 decreased slowly from day 0 (initial) until day 4, and then rapidly declined from day 4 to day 14.

The average cell density of *M. aeruginosa* in the three flasks on day 0 was 2.89 x10^6 cells mL^{-1}, decreasing to 1.43 x10^4 cells mL^{-1} on day 14. Meanwhile, *L. inermis* showed exponential growth from day 0 to day 8, but the specific growth rate from day 8 to day 14 appeared to gradually decrease. The average number density of *L. inermis* in the three flasks on day 0 was 5 individuals mL^{-1}, increasing to 242 individuals mL^{-1} on day 14. In addition, *M. aeruginosa* cell density of the control flask was 2.87 x10^6 (n=3, std. error = 3.53 x10^4) cells mL^{-1} at initial and 2.83 x10^6 (n=3, std. error = 3.53 x10^4) cells mL^{-1} at final (14 days), where the null hypothesis that there is no difference between them cannot be statistically rejected (p =0.467 >0.05, by t-test). Therefore, it was determined that there was no autolysis of *M. aeruginosa* cells in the mixed culture. Therefore, our results in mixed cultures indicate that *L. inermis* preyed on the toxic *Microcystis* cells and used them as a food source to proliferate. Over a period of 14 days, the *L. inermis* consumed the toxic *Microcystis* cells, yielding a removal rate of 99.5%.

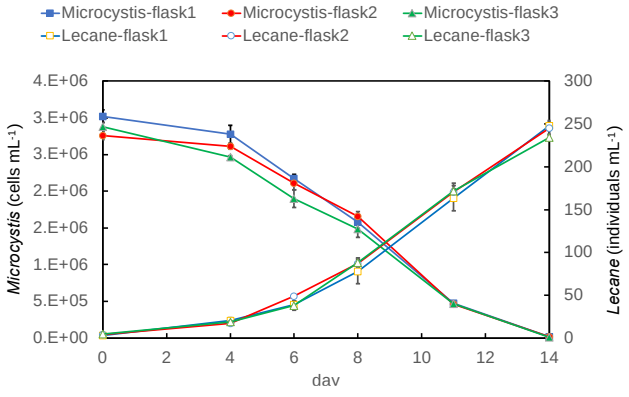


Figure 3 Changes of *L. inermis* individuals and *M. aeruginosa cells* in the mixed culture

Previous studies provide evidence of Bdelloidea *Philodina erythrophthalma* predation on toxic *Microcystis* cells (Iwami et al., 1999). On the other hand, *Brachionus calyciflorus* demonstrated grazing behavior towards both MC-producing and MC-free *Microcystis* cells. However, the growth of *Brachionus* was inhibited by the presence of *Microcystis* cells, implying that *Microcystis* cells were not a major food source for this species (Liang et al., 2017). Although the isolated rotifer *L. inermis* is widely distributed in freshwater bodies across the world (Ersch & Johann, 1827), no experiment on the predation of toxic *Microcystis* by *L. inermis* has been performed. The result of this mixed culture experiment is the first to clearly demonstrate the predation ability

of *L. inermis* to toxic *Microcystis*.

3.3 Nonlinear fitting of population dynamics data in the mixed culture

A predator-prey model with type II functional response to analyze the population dynamics data of *M. aeruginosa and L. inermis* (Turchin, 2003). The model is as follows.

$$\frac{dL}{dt} = (r(M) - \delta)\, L \quad \ldots . (1)$$

$$\frac{dM}{dt} = \left(-r(M)\frac{L}{Y}\right) \quad \ldots (2)$$

$$r(M) = \frac{r^{max} M}{K + M} \quad \ldots (3)$$

The equations include the dynamic variables of *L. inermis* population density and *M. aeruginosa* cell density, denoted by the symbols *L* and *M*, respectively. The growth of *L. inermis* is described by Equation (1). The maximum specific growth rate and half saturation constant in type II functional response *r*(*M*) are represented by *r* and *K* in equation (3) respectively. The mortality of the predator is represented by δ, and the yield when *L. inermis* consumes *M. aeruginosa* cells as a food source is represented by *Y*. Equation (2) describes the decomposition process of *M. aeruginosa* cells as prey by *L. inermis*. Equation (3) represents the predation character using a rectangular hyperbola function (Turchin, 2003) through the type II functional response *r*(*M*). Furthermore, since *M. aeruginosa* did not multiply due to the absence of light in this culture condition, equation (2) only includes the term for the decomposition of *M. aeruginosa* cells by the predation of *L. inermis*.

The nonlinear functions *L*=*L* (*t*; $\boldsymbol{p}$) and *M* =*M* (*t*; $\boldsymbol{p}$), with $\boldsymbol{p}$ representing a parameter vector (*r*, *K*, δ, Y), are defined as the solutions of model (1), (2), and (3) with the adequate initial conditions of *L* and *M*. In this regression analysis, the functions can be obtained through a numerical ODE solver (see section 2.7). The regression of the nonlinear functions *L* (*t*; $\boldsymbol{p}$) and *M* (*t*; $\boldsymbol{p}$) can be achieved by minimizing the square root of residual error $\varepsilon(\boldsymbol{p})$ using the Levenberg-Marquardt Nonlinear Least-Squares algorithm (More, 1978).

$$\varepsilon(\boldsymbol{p})^2 = \sum_{i=1}^{6}\sum_{j=1}^{6} |\boldsymbol{x}(t_i; \boldsymbol{p}) - \boldsymbol{y}_i^j|^2 \quad \ldots (4)$$

$$\boldsymbol{x}(t; \boldsymbol{p}) = \left(L(t; \boldsymbol{p}), M(t; \boldsymbol{p})\right). \quad . \quad . \quad (5)$$

$$\boldsymbol{y}_i^j = (L_i^j, M_i^j). \quad . \quad . \quad (6)$$

At different points in time t_i = (0, 4, 6, 8, 11, 14 days), data was collected for *L. inermis* and *M. aeruginosa*, denoted by L_i^j and M_i^j respectively. The index j (1...6) represents the replicates. Two replicate measurements from three replicate flasks were combined as six datasets at each time point. The three flasks showed similar population change trends for *L. inermis* and *M. aeruginosa* as shown in Figure 3, so combining the data was a sensible approach to perform nonlinear regression with sufficient accuracy. In addition, L_i^j and M_i^j were normalized by the average density of 242 individual mL^{-1} at 14 days and 2.89x10^6 cells mL^{-1} at 0 day, respectively. This normalization was necessary to evaluate the residual error $\varepsilon(\boldsymbol{p})$ equally for *L. inermis* and *M. aeruginosa.* To find a parameter $\boldsymbol{p}$ that minimizes $\varepsilon(\boldsymbol{p})^2$, we used the "nls.lm" function in the "minpack.lm" package of R. Table 1 shows the estimated parameter values, and Figure 4 displays the regression curves of *L. inermis* and *M. aeruginosa* with observed data points.

Table 1 and Figure 4 above show the estimated parameter values and regression curves of *L. inermis* and *Microcystis* at observational data points, respectively. All estimated parameters in Table 1 show statistical significance with very low p-values. The *Microcystis* and *L. inermis* regression curves in Figure 4 fit well with the data points obtained from the mixed culture experiment.

Table 1. Estimated parameters of the model (1), (2) and (3) by the nonlinear regression

	Estimate	Std. Err.	Estimate with Unit	Pr(>|t|)
r^{max}	0.869621	0.179009	0.87 day^{-1}	9.12E-05 ***
Y	0.499577	0.031773	4.19x10^{-5} L-individuals M-cells^{-1}	< 2E-16 ***
K	1.922135	0.693624	5.55x10^6 M-cells^{-1}	0.007198 **
δ	-0.097530	0.008345	-0.098 day^{-1}	< 2E-16 ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
Residual standard error: 0.01727 on 56 degrees of freedom
Note: M-cells: *Microcystis* cells, L-individuals: *Lecane* individuals

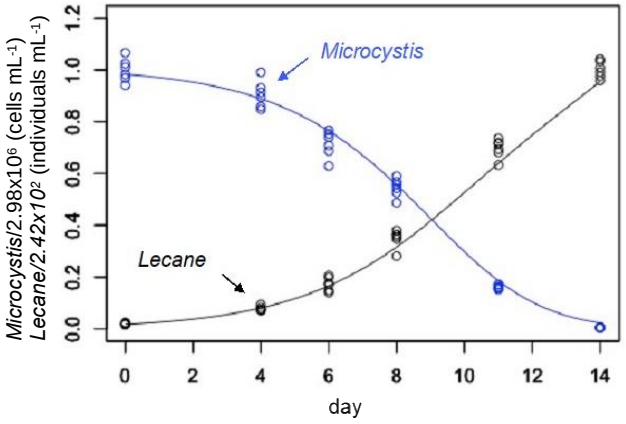
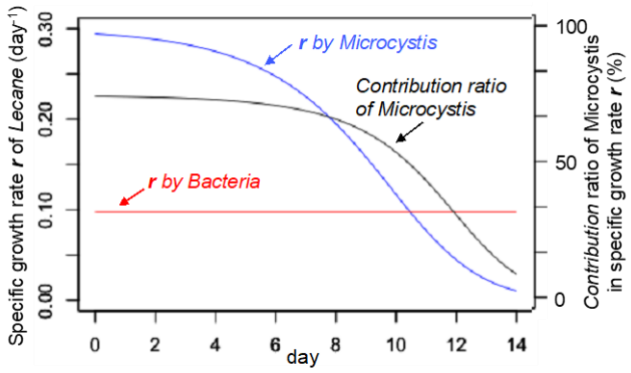


Figure 4 Regression curves using the predator-prey model for changes of *L. inermis* population and *Microcystis* cells

However, it is important to note that the estimated mortality rate of *L. inermis* was negative at -0.098 day^{-1}, indicating the growth of *L. inermis*. Additionally, this negative mortality rate suggests that the *L. inermis* grew by consuming another food source. In our experiment, *M. aeruginosa NIES*102 was cultured under axenic condition, but bacteria were introduced into the mixed culture from the isolated *L. inermis*. Bacteria could have grown in the pre-culture and the mixed culture by consuming dissolved organic matter (DOM), which may have been produced during the predation process of *M. aeruginosa cells* by *L. inermis*. Since a previous study showed that *L. inermis* consumes bacteria for growth (Agnieszka et al., 2017), this assumption is reasonable in explaining the negative mortality, indicating additional growth in

L. inermis. Under this interpretation of the negative mortality, the specific growth rates of *L. inermis* when feeding on *M. aeruginosa*



cells and when feeding on bacteria can be estimated from $r(M)$ and δ in equation (1). Figure 5 shows the results of this estimation.

Figure 5 Changes in the estimated specific growth rates of *L. inermis* when feeding on *Microcystis* and when feeding on bacteria, and the contribution ratio of *Microcystis* to the specific growth rate.

The estimated initial specific growth rate of *L. inermis* due to predation on *M. aeruginosa* cells was 0.29 day^{-1}. It was also estimated that the contribution of *M. aeruginosa* cells to the total specific growth rate was 75%. As the density of *M. aeruginosa* cells decreased, the percentage of specific growth rate of *L. inermis* due to *M. aeruginosa* cells gradually decreased. After 10 days of culture, it decreased to approximately 50%. By day 14, the specific growth rate by *M. aeruginosa* cells was 0.010 day^{-1}, and the contribution of *M. aeruginosa* cells decreased to approximately 9.6%. Equations (1) and (2) of the applied predator-prey model did not take bacterial dynamics into account. The number of bacteria might change in the mixed culture due to the proliferation consuming DOM and the predation by *L. inermis*. After 10 days of culture, the proportion of bacteria significantly increased and accounted for more than 50% of the total population. However, the regression curve of *L. inermis* seemed to go below the center of data points on days 8, 11, and 14. In order to accurately represent the obtained data, it is important to consider that the increase in bacterial numbers acts as a nutrient source, promoting further growth of *L. inermis*. In this study, since the bacterial densities in the mixed cultures were not measured, it is inappropriate to make any further assumptions or discuss this issue further. Future studies should obtain quantitative data on changes in bacterial cell density to discuss this issue in more detail.

3.4 Microcystin degradation in the mixed culture

The degradation of MCs was investigated in the mixed culture using HPLC analysis. Figure 6 shows a chromatogram of a standard solution, a 0-day sample, and a 14-sample. Table 2 shows the measured concentration values of MC-RR, MC-YR, and MC-LR. Table 3 shows the average concentrations of total MC-RR, -YR, and -LR on day 0 and their final concentrations on day 14. In Table 2, total MC means the sum of dissolved microcystin (DMC) in the medium and MC in *Microcystis* cells. Then, DMC-LR was only detected in the 14-day samples and DMC-RR and DMC-YR were not detected, as shown in Table 2 and Table 3. This result suggests that all MCs initially resided within *Microcystis* cells.

The degradation percentage after 14 days for each total MC was 98.6% (MC-RR), 96.6% (MC-YR), and 93.9% (MC-LR), respectively. Although the percentage of MC degradation was slightly lower than the cell removal rate of 99.5% for *M. aeruginosa cells*, the percentages of cell removal and MC degradation were found to be similar. It was suggested that the degradation of MCs was promoted due to the predation of *L. inermis*. This process is expected to occur because most MCs exist inside the cell bodies of *Microcystis*. When *L. inermis* preys upon the *Microcystis*, the cell bodies can be destroyed, which then exposes the internal MCs. It is speculated that MCs that leaked out of cells was degraded by microcystin (MC)-degrading bacteria (Saitou et al., 2003; Massey & Yang, 2020). In a previous study, it was found that *Microcystis*.

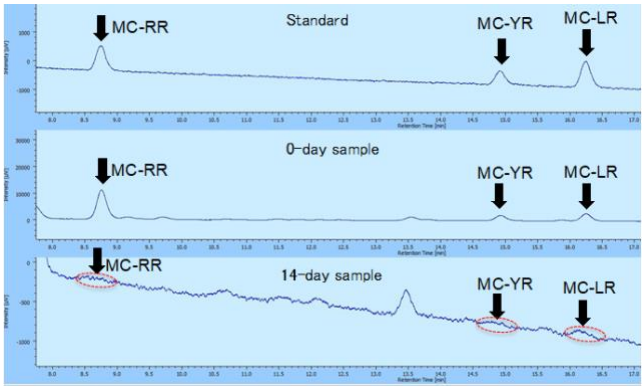


Figure 6 The three chromatograms of the mixed MC standard, day 0 samples and day 14 samples are shown below. The MC standard contained MC-RR of 500μg L^{-1}, MC-YR of 250μg L^{-1} and MC-LR of 500μg L^{-1}.

Table 2. All measured values of MCs by HPLC

Sample		MCs (μg/L)			
		MC-RR	MC-YR	MC-LR	
day 0	Initial-1	63.08	11.02	14.57	total
		ND	ND	ND	dissolved
	Initial-2	58.67	10.42	13.34	total
		ND	ND	ND	dissolved
	Mean	60.87	10.72	13.95	total
		ND	ND	ND	dissolved
	Std. Error	2.20	0.61	0.61	total
		-	-	-	dissolved
day 14	flask(1)	0.86	0.44	0.86	total
		ND	ND	0.42	dissolved
	flask(2)	0.86	0.38	0.86	total
		ND	ND	0.44	dissolved
	flask(3)	0.75	0.28	0.82	total
		ND	ND	0.42	dissolved
	Mean	0.82	0.37	0.85	total
		ND	ND	0.43	dissolved
	St. Error	0.04	0.05	0.01	total
		-	-	0.01	dissolved

Cells and MCs could be simultaneously degraded by

microfauna collected from a biofilm sample (Saito et al., 2003). The flagellate *Monas guttula* was identified as the microorganism that could decompose both *Microcystis* cells and MC-LR at the same time. The degradation of MCs by *Monas* was further studied using *M. aeruginosa* NIES102 (Fujimoto et al., 2007). Additionally, in the study two strains of MC-degrading bacteria (MG-15 and MG-22) co-existing with the flagellate *Monas* were isolated. Moreover, it was found that the degradation rate of MC-RR was faster than that of MC-LR and MC-YR by both MG-15 and MG-22.

In our study, based on the assumption of first-order kinetics of MC degradation, each specific degradation rate *k* of MC-RR, MC-YR, and MC-LR was calculated between two time points of 0 and 14 days). Under the assumption of first order kinetics, MC degradation kinetics is described as follows.

$$\frac{dM}{dt} = -kM \quad \ldots (7)$$

where M is the concentration of a MC, and the constant k is a specific degradation rate.

Since the value of k should depend on the cell density of MC-degrading bacteria, k is generally not a constant value. However, the predator-prey model well described the population dynamics of *L. inermis* under the assumption that the bacterial contribution to the specific growth rate of *L. inermis* was constant (see Figure 5), and therefore a constant MC-degrading bacterial population can be also assumed. As a result, the assumption of a constant k is acceptable for the first stage of the analysis. Therefore, the solution of the kinetic equation is simply.

$$M = M_0 \exp(-kt) \quad \ldots (8)$$

, where M_0 is the initial concentration of the MC.
In order to determine k, the logarithm transformed equation (9) is applied to achieve linear regression analysis.

$$log\ M = -kt + log\ M_0 \quad \ldots (9)$$

Table 3. Degradation of total MCs and DMCs in the mixed culture

MC-type	concentration on 0 day	concentration on 14 day	decompostion ratio	specific decompostion rate
total	mean ± std. error (μg/L)	mean ± std. error (μg/L)	percentage (%)	k ±std.error (day^{-1})
MC-RR	60.87 ± 2.20	0.82 ± 0.04	98.6	0.307 ± 0.005
MC-YR	10.72 ± 0.61	0.37 ± 0.05	96.6	0.242 ± 0.012
MC-LR	13.95 ± 0.61	0.85 ± 0.01	93.9	0.200 ± 0.003
DMC-RR	N.D.	N.D.	-	-
DMC-YR	N.D.	N.D.	-	-
DMC-LR	N.D.	0.43 ± 0.01	-	-

Regression results for MC-RR, MC-YR and MC-LR were statistically significant (p-value < 0.05). As shown in Table 3, the specific degradation rate k was highest for MC-RR, followed by MC-YR, and lowest for MC-LR. Saito et al. (2003) obtained a specific degradation rate of 1.90 h^{-1} for MC-RR, 1.25 h^{-1} for MC-YR and 0.66 h^{-1} for MC-LR by the isolated MC-degradation bacteria from Lake Kasumigaura. The fact that this order is the similar to our result can be taken as one evidence that MC-degrading bacteria contribute to MC degradation in this mixed culture experiment. Furthermore, it can be inferred that MC-degrading bacteria could immediately degrade DMC-RR and DMC-YR released during predation of *Microcystis* cells by *L. inermis*, and that DMC-RR and DMC-YR were reduced to ND levels after 14 days. On the other hand, MC-LR decomposed more slowly than the other MCs, suggesting that more DMC-LR remained in MC-LR, as shown in Table 3.

Bacterial degradation of MCs must be advantageous for microfauna living in aquatic bodies where toxic *Microcystis* proliferates. To prove that MC-degrading bacteria and predatory microorganisms work together to promote MC degradation, it is essential to detect the *mlrA* gene, which is a specific gene possessed by MC-degrading bacteria. Quantification of the *mlrA* gene during the predation process by *L. inermis* will need to be achieved in future studies.

4. Conclusion

A rotifer was found in a biofilm sample taken from a reservoir where a *Microcystis* bloom occurs every year. The morphology and 18S rRNA gene sequence analysis confirmed that the isolated rotifer belonged to the Lecanidae family, with a 100% match to *L. inermis inermis*. In the mixed culture of *L. inermis* and toxic *Microcystis* (NIES102), the number of *Microcystis* cells decreased as *L. inermis* increased, clearly indicating an interaction between predator and prey. Finally, 99.5% of *Microcystis* cells were decomposed in the mixed culture. The interaction between *L. inermis* and *Microcystis* can be described using a predator-prey model. To determine the most accurate parameters for the model, the population data of *L. inermis* and the *Microcystis* cells in the mixed culture was analyzed using a nonlinear regression method. The study found an additional growth of *L. inermis* which was needed to account for negative mortality of *L. inermis*. This suggests that *L. inermis* may consume bacteria as a food source when preying on *Microcystis* cells. By the mixed culture of *L. inermis* and toxic *Microcystis*, total MCs, which is the sum of intracellular MCs and dissolved MCs in the culture medium, were degraded to 98.7% in MC-RR, 98.5% in MC-YR, and 94.6% in MC-LR after 14 days.

Acknowledgement

This research was supported by the Project for Fostering of Proficient Researchers for the Establishment of the Research Center for Green Science (fellowship scholarship) and Grant-in-Aid for Scientific Research (B) of Japan Society for the Promotion of Science (Research No. 22H03769).

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