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Isolation and identification of two mixotrophic flagellates and their feeding on Microcystis

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Abstract

Two flagellate strains (SL1, SL2) were isolated from South Lake of Jinan University and identified as *Ochromonas* sp. and *Poterioochromonas malhamensis*, based on morphological characteristics and 18S rDNA sequencing. We explored the ability of these two strains to on *Microcystis wesenbergii*. The results showed that the growth rate of the flagellates reached 0.068 h^{-1} (*P. malhamensis*) and 0.087 h^{-1} (*Ochromonas* sp.) when feeding on *Microcystis*, and the maximum abundance of *P. malhamensis* was 1158.33×10^3 cells/ml under *Microcystis* feeding compared to 14.48 ×10^3 cells/ml under autotrophic conditions. The maximum abundance of *Ochromonas* sp. reached 1116.67×10^3 cells/ml under *Microcystis* feeding, compared to a maximum of 6.71×10^3 cells/ml under autotrophic conditions. The abundance of *M. wesenbergii* was significantly suppressed under flagellate feeding (P<0.05), with reduction rates of 38.177% (*Ochromonas* sp.) and 44.115% (*P. malhamensis*). Therefore, both flagellates were able to expand their population density by feeding on *M. wesenbergii* and showed some potential for algae removal.

Keywords

Flagellates; growth rate; nutrient patterns; Microcystis.

1. Introduction

Phytoplankton are the primary source of energy in the food web of aquatic ecosystems, but global warming and increased eutrophication of water bodies have led to the explosive growth and spread of some algae adapted to warm and high nitrogen and phosphorus water bodies, thus forming algal blooms that damage water bodies, while some cyanobacteria produce algal toxins that threaten the life and health of aquatic organisms and even humans [1]. In addition to algal toxins, cyanobacteria also secrete and produce certain chemosensitive substances that inhibit the growth of aquatic plants and animals [2]. Some cyanobacteria form colonies in the natural water column to make filter-feeding by zooplankton more difficult, while some cyanobacteria are filamentous in nature, thus hindering zooplankton feeding. Therefore, cyanobacteria, especially *Microcystis*, are a serious threat to the health of natural water bodies, and even to the safety of human water, due to their structural and toxic properties.

Protozoa are important feeders of cyanobacteria, of which flagellates feeding on *Microcystis* are by far the most studied category. As an important part of the classical food web, flagellates are widely distributed in freshwater ecosystems and are present in the habitats of all types of organisms [3]. Flagellates can be as small as 2 μm or as large as 200 μm, but most flagellates are between 2 and 20 μm in length [4].

Ochromonas is the dominant genus in the family Chrysomelidae, growing mainly in freshwater bodies such as ponds, lakes and marshes [5], and is represented by microscopic (2-10 μm) and ultramicroscopic (< 2 μm) species. The genus has mixed trophic and metabolic diversity [6, 7].

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Ochromonas grows rapidly by phagocytosis when feeding on bacteria and other small organisms [8]. However, it has been shown that heterotrophy is the main mechanism supporting the growth of *Ochromonas* and that the autotrophic mode, which relies on photosynthesis, is a survival strategy rather than a growth strategy under harsh environmental conditions [9]. *Poteriochromonas* (Chrysophyte) is a unicellular naked algae widespread in freshwater ecosystems [10] that can feed on toxic *M. aeruginosa* and biodegrade MC-LR [11]; it can also exist in a phototrophic state in food-poor environments [12, 13], which is referred to as mixotrophic.

Selective feeding by some protozoa has been reported based on prey size and surface characteristics. Among the factors influencing the foraging activity of ciliates, the size of the prey is probably the most important. In indoor experiments with ciliates fed using latex beads, starch granules and natural granules, the selectivity of ciliates for prey size was confirmed, and they were chemosensitively selective for specific algae. Grazing by the protozoan flagellate *Ochromonas* sp. can induce colony formation in *Microcystis aeruginosa*, most of which undergo aggregation to form colonies when infested with flagellates, suggesting that flagellates can induce colony formation in unicellular *Microcystis* when they feed on them [14]. These colonies can effectively prevent further feeding by the flagellates, thus increasing the survival rate of *M. aeruginosa*. Under conditions where toxins are not effective in preventing flagellate grazing [15], colony formation of *M. aeruginosa* can be considered an induced defense against flagellate grazing.

Harmful algal blooms are receiving increasing attention worldwide, and the study of *M. aeruginosa* and its toxins is one of the most representative issues. Protozoan predation on bloom-forming algae plays an important role in water purification, and a study by Zhang et al. [16] found that *Poterioochromonas* sp. could remove a high density of *M. aeruginosa* in a short time (40 h) with an inhibition rate higher than 99.9%. In addition, *Poterioochromonas* sp. was able to degrade microcystins while digesting *M.* aeruginosa cells. This study demonstrates the importance of *Poterioochromonas* sp. in aquatic ecosystems and its great potential for algae control. Yang et al. [14] isolated a strain of *Ochromonas* sp. from Yunnan Dianchi and found that the protozoan had a strong ability to swallow *M.wesenbergii*, and the removal rate of Microcystis reached more than 90% by the sixth day. The swallowing effect of *Ochromonas* sp. increased with its concentration.

In this study, two strains of flagellates were isolated and purified from South Lake of Jinan University. The two isolated flagellates were identified by morphological observation and molecular biological analysis, and their feeding ability on *Microcystis* was investigated. The results of this study are useful for understanding the role of flagellates in controlling *Microcystis*.

2. Materials and methods

2.1. Isolation and identification of flagellates

To isolate flagellates, a small drop of the concentrated lake water stock solution was pipetted onto a slide and viewed with a dissecting microscope. The tip of the micropipette, viewed through the microscope, was placed into the medium near the target flagellate. The flagellate was gently pipetted into the micropipette and transferred to a drop of sterile BG11 medium. The cells were then recaptured using a new micropipette and again transferred to a new drop of sterile medium, and the process was repeated again. Finally, all of the last drops of sterile medium containing the target cells were transferred to a flask containing sterile BG-11 medium containing glucose [17] and kept at 25 °C in the dark. Two different strains (SL1, SL2) of flagellate cells were isolated using the same method described above.

Morphological identification of the two purified flagellate cells was performed using a light microscope (Olympus, BX53; Tokyo, Japan), and then they were photographed. Prior to the

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start of the experiment, purified flagellates were cultured in BG11 medium containing glucose to maintain exponential growth. Flagellates were obtained by centrifugation collection (5000 rpm, 15 min) and identified by analysis of 18S rDNA gene sequences. According to Lara et al. [18], PCR was performed with an amplification process and specific primer design. DNA was extracted from all samples using a HiPure Plant DNA Mini Kit (Magen), and DNA concentration and purity were tested using a NanoDrop2000, while DNA extraction quality was measured by 1% agarose gel electrophoresis. PCR amplification was performed with primers F: CTATGGTCGTGTAGTATATGAAGG and R: AACACCAGCCATACGCATCC. The amplification procedure was as follows: predenaturation at 96 °C for 3 min, 30 cycles (denaturation at 96 °C for 30 s, annealing at 50 °C for 30 s, extension at 60 °C for 45 s) and a final extension at 16 °C for 10 min (PCR instrument: ABI 3730xl). The amplification system was 20 μL, which contained 4 μL 5×Fast Pfu buffer, 2 μL 2.5 mM dNTPs, 0.8 μL primers (5 μM), 0.4 μL Fast Pfu polymerase, and 10 ng DNA template. The PCR products were used for DNA sequencing (Tian yi Hui yuan, Guangzhou, China), and the DNA sequences obtained by sequencing were compared by Blast analysis with sequences found in the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/blast/). A phylogenetic analysis of two flagellate species (SL1, SL2) and other flagellate species was performed and visualized through an evolutionary tree (MEGA-X, Arizona State University, USA).

2.2. Culture of flagellates and Microcystis

The two flagellates obtained by isolation were cultured in BG11 medium and placed in a light incubator with a light-dark ratio of 12 h:12 h (L:D), a light intensity of 50 μmol m^{-2} $s^{-1,}$ and a temperature maintained at 25 °C.

To verify the feeding of both flagellates on *Microcystis*, a treatment was set up with an initial concentration of 5 × 10^3 cells/ml for flagellates and an initial abundance of 1 × 10^5 cells/ml for *Microcystis*. A flagellate autotrophic control group was also set up. Additionally, a *Microcystis* pure culture group was set up. The experiment was performed in a light incubator at 25 °C with a light-to-dark ratio of 12 h:12 h (L:D) and a light intensity of 50 μmol m^{-2} s^{-1}, and the temperature was maintained at 25 °C. The experiments were performed in 50 ml of BG11 medium, and the medium was gently stirred with a glass rod every 6 h before sampling to make it homogeneous. One milliliter of each sample was taken for cell counting (fixed with 2% Lugol reagent), each treatment was divided into 3 parallels, and the experiments lasted 72 h. The nontoxic strain of *M. wesenbergii*, FACHB-1339, was used in the experiments, with the aim of excluding the effect of algal toxins and focusing more on the feeding of flagellates on *M. wesenbergii*.

2.3. Data processing and analysis

Cell counts were performed under a light microscope (Olympus, BX51; Tokyo, Japan), and samples were dropped on a 0.1 mm^3 hemocytometer plate. The average of the number of cells in the upper and lower halves of the counting chamber of the plate was used as the cell counts. Calculation of flagellate population-specific growth rates based on the results of cell counts was performed as follows: daily specific growth rate (μ, d^{-1}): μ= $\ln N_{t+1}$-$\ln N_t$, where N_{t+1} and N_t are the abundance of flagellates on day t+1 and day t, respectively. The reduction rate of *Microcystis* by flagellates was expressed as （ϕ，%）：$\phi = \left(1 - \frac{c_t}{c_c}\right) \times 100\%$, where *Ct* is the abundance of *Microcystis* cells in the feeding group and *Cc* is the abundance of *Microcystis* cells in the control group. The clearance rate of flagellates on *Microcystis* was calculated according to the following equation: C (mL cell $^{-1}$ h^{-1}) = $\frac{\mu_{MC} - \mu_{MT}}{N_0}$, where μ_{MC} and μ_{MT} are the specific growth rates of *Microcystis* in the control and the treatment, respectively, and N_0 is the average concentration of flagellates.

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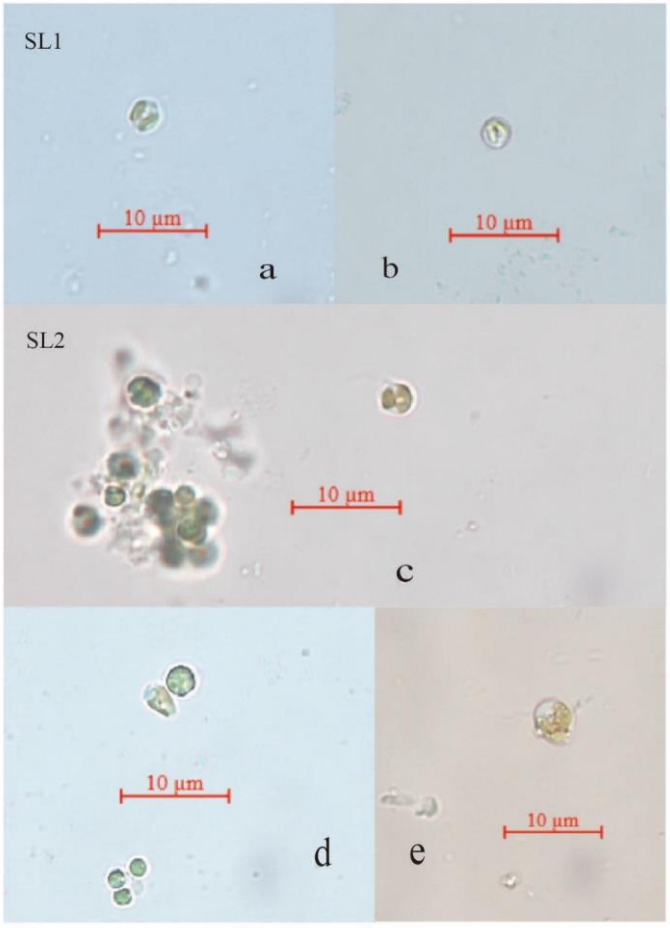
One-way ANOVA followed by Tukey's HSD test was used to analyze whether there were significant differences in the specific growth rate of flagellates under different culture modes. All statistical data were processed in SPSS18.0. Compliance with the ANOVA hypothesis was tested by normal probability plots of residuals and Levene's test for homogeneity of variances before statistical analyses were performed.

3. Results

3.1. Identification of unknown species of flagellates

Two species of flagellates (SL-1, SL-2) were isolated from the South Lake of Jinan University and cultured as monoclonal populations in the laboratory. SL-1 cells are ellipsoidal in shape, 3-8 μm in size, with two flagella of varying length. SL1 can exist as a single cell or as a group, and the single cell swims spirally with the flagellum facing forward, and the long flagellum is bent during the swimming process, while the short flagellum remains motionless, and the long flagellum is approximately 3-5 times longer than the body length. SL-1 cells have a yellow–green banded chloroplast that is unstable in shape, and the eye spot is not visible (Fig. 1a, b). SL-2 cells are spherical, poke-shaped or pear-shaped (Fig. 1d), approximately 5-10 μm in size, with two unequal flagella at the extracellular base, but the shorter flagella are difficult to observe under light microscopy.



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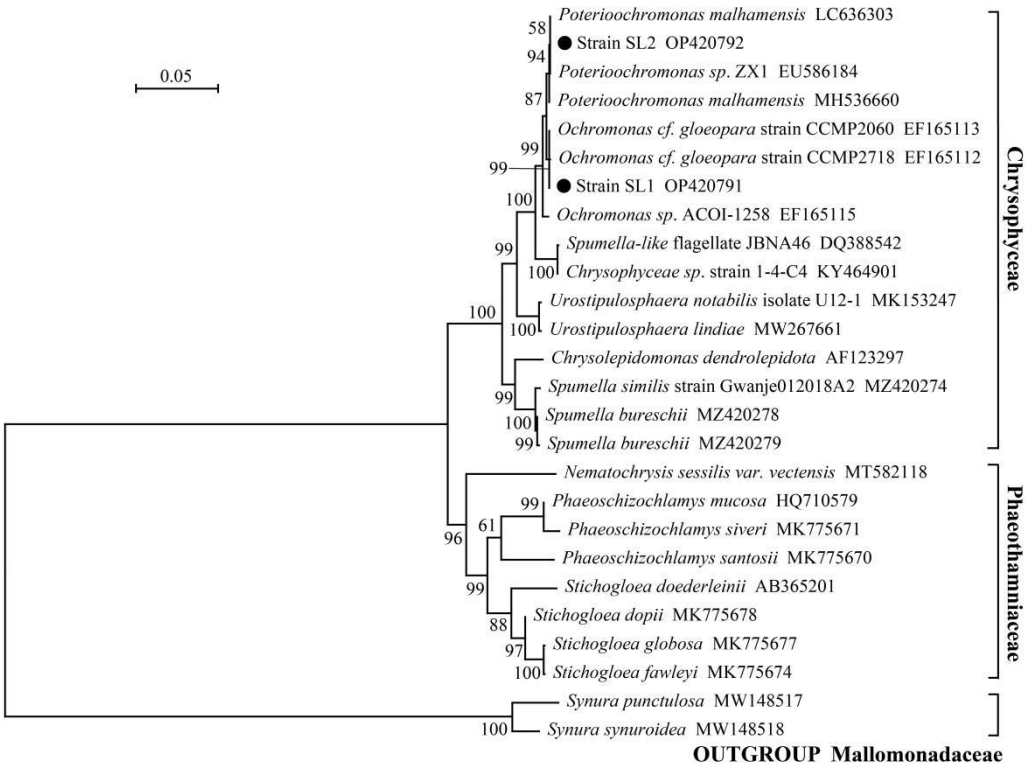

Fig. 1. Light micrographs of flagellates SL-1 and SL-2 and their NJ phylogenetic tree.

To further identify the two flagellate strains, the 18S rDNA gene sequences of SL-1 and SL-2 cells were also analyzed. According to the blast results, the phylogenetic analysis of the two flagellate strains is shown in Fig. 1. The gene sequence of strain SL-1 showed a maximum of 99% similarity to *Ochromonas* sp. strain UTEXLB 2575 (GenBank accession number: KU900228), classified as Sarcomastigophra. Chrysomonadida, Chrysomonadina, Ochromonadidea, and *Ochromonas*. The flagellate strain SL-2 showed a maximum similarity of 99.82% to *Poterioochromonas malhamensis* (GenBank accession number: MH536660) and was classified as Sarcomastigophra, Chrysomonadida, Chrysomonadina, Ochromonadidea, and *Poterioochromonas*. Based on morphology and 18S rDNA sequence alignment, strain SL-1 was identified as Sarcomastigophra, Chrysomonadida, Ochromonadidea, *Ochromonas* sp. Strain SL-2 was identified as Sarcomastigophra, Chrysomonadida, Ochromonadidea, and *Poterioochromonas malhamensis*.

3.2. Feeding on Microcystis by two flagellate strains

According to the growth of the two flagellates (SL1 and SL2) under the two *Microcystis* feeding conditions, the abundance of both flagellates increased rapidly and reached the maximum abundance at the 30th and 36th hours, respectively, and then slowly decreased to maintain at a certain level. Their growth curves showed a trend of rapid increase and then a slow decrease to maintain a stable level (Figure 2), with SL-2 decreasing more after reaching the highest point. In the experiment, the abundance of flagellates in the group without the addition of *M. wesenbergii* was always lower than that in the treatment group with the addition of *M. wesenbergii*; on the other hand, the population-specific growth rate of flagellates in the group without the addition of *M. wesenbergii* was significantly lower than that in the treatment group with the supplementation of *M. aeruginosa* (Figure 2, $P < 0.05$). The population of *M. wesenbergii* under flagellate feeding showed a trend of first decreasing and then stabilizing and finally stabilizing at approximately 60% of the initial concentration of *Microcystis*. In conclusion, both flagellates have a certain feeding effect on *Microcystis*, and both can grow rapidly in the presence of *M. aeruginosa* and eventually reach a higher population density of environmental accommodation.

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By calculating the clearance and reduction rates of the two flagellate strains, we found that the reduction rate of *M. wesenbergii* by *Ochromonas* sp. reached 38.177% at the end of the experiment, while the reduction rate of *M. wesenbergii* by *P. malhamensis* reached 44.115%. The results showed that both flagellates had a significant reduction effect on *M. wesenbergii* within 72 hours but did not completely remove *M. wesenbergii*.

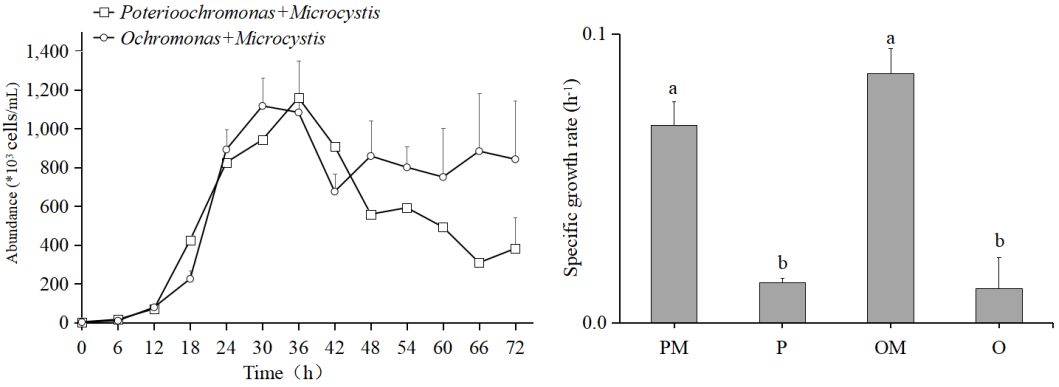

Fig. 2. Changes in the abundance of flagellate strains SL-1 (*Ochromonas* sp.) and SL-2 (*P. malhamensis*) in the group fed *Microcystis wesenbergii*. The specific growth rate (mean ± SD) of SL1 (*Ochromonas* sp.) and SL2 (*P. malhamensis*) under different nutritional conditions. PM and OM represent *P.* malhamensis +*Microcystis wesenbergii* and *Ochromonas* sp.+*Microcystis wesenbergii*, respectively, and P and O represent the group without the addition of *Microcystis wesenbergii*. Different letters above the column represent significant differences ($p < 0.05$) using one-way ANOVA followed by Tukey's HSD test.

Table 1. Growth characteristic parameters of flagellates (*Ochromonas* sp. and *P. malhamensis*)

Different flagellate treatment groups	Clearance rate of each flagellate (mL cell $^{-1}$ h^{-1})	Reduction rate of *M.wesenbergii*(%)
Ochromonas+M.wesenbergii	0.032±0.018	38.177±4.681
Poterioochromonas+M.wesenbergii	0.040±0.019	44.115±2.357

4. Discussion

4.1. Morphological characteristics and identification of flagellates

The two flagellate strains (*Ochromonas* sp. and *P. malhamensis*) isolated in this study have two unequal flagella, and the flagellar structure is not only an organ used by flagellates for locomotion but is also capable of triggering a constant-frequency water flow that pushes food particles toward the flagellate-sensitive area during feeding, thus contributing to the formation of food vesicles in the system [19]. Under the light microscope, SL2 cells were mostly spherical or ovoid and rarely pear-shaped (Fig. 1). The morphology of chloroplasts of SL2 cells also differed under different trophic modes, and their chloroplast structures were more obvious and darker under autotrophic conditions than under heterotrophic and mixotrophic modes, which is consistent with Mingyang Ma [20], who observed similar results. Based on the morphological observations, we tentatively determined that the two flagellates in the experiment, SL-1 and SL-2, belong to different genera of Sarcomastigophra, Chrysomonadida, order Chrysomonadina, Ochromonadidea: *Ochromonas* (SL1) and *Poterioochromonas* (SL2). SL2 has two unequal

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flagella at the anterior end and is a typical mixed trophic flagellate, which is very similar to SL1. The presence or absence of the capsule shell is the key to distinguishing the two, but the capsule shell structure is difficult to observe [21], and the morphology can change greatly at different times of growth when the surrounding environment and food change, so we sought further identification of the two flagellate strains starting from molecular biology.

In species identification studies of eukaryotes, because of the highly conserved nature of their gene sequences, the relationships of organisms during evolution is often inferred by comparing the variation of this sequence among different species. In this experiment, 18S rDNA sequences were used for phylogenetic tree construction. The strain of Ochromonadidea *Ochromonas cf. gloeopara* had the highest sequence similarity with the 18S rDNA sequence of SL1 in the phylogenetic tree and the strain of Ochromonadidea *Poterioochromonas malhamensis* had the highest sequence similarity with SL2. The analysis of genetic distances showed that SL1 had the lowest genetic distance from *Ochromonas cf. gloeopara* (EF165113) and SL2 had the lowest genetic distance from *Poterioochromonas malhamensis* (MH536658 and MH536660). Based on the comparison of algal cell morphology, proliferation mode and nutrient type with related literature, combined with molecular phylogenetic analysis, it was determined that the flagellates SL1 and SL2 in this study belonged to Sarcomastigophra, Chrysomonadida, order Chrysomonadina, Ochromonadidea: *Ochromonas* sp. (SL1) and *Poterioochromonas malhamensis* (SL2).

4.2. Feeding on Microcystis by two flagellate strains

The results of this experiment showed that the growth rate of flagellates was higher in the presence of added *Microcystis* than in the purely autotrophic state, suggesting that both flagellates were able to feed on *Microcystis* while growing rapidly and in large numbers to reach the environmental holding capacity. Several freshwater and marine protozoa have been reported to be able to feed on algae [22], and golden algae (*Poteriochromonas* sp. and *Ochromonas danica*) are able to feed on *M. aeruginosa* [23]. Zhang et al. [16] used light and electron microscopy to observe the uptake and digestion processes of *P. malhamensis* and *Ochromonas danica* on *M.aeruginosa* and other organic particles. The processes are as follows: (1) the plasma membrane deforms and surrounds the prey and disappears after ingestion; (2) the food vesicles are formed by the successive fusion of many uniform vesicles gathered around the prey; and (3) the prey is encapsulated in a single membrane-bound food vesicle, which is then digested. In conclusion, golden algae *Poteriochromonas* sp. are organisms that are widely found in freshwater environments and are capable of feeding on copper green microcysts. Based on these results, it is inferred that *Poteriochromonas* sp. plays an important role in the material flow of planktonic food webs and in the community structure of aquatic ecosystems.

The growth of supporting mixotrophs relies mainly on heterotrophic metabolic mechanisms, as autotrophic metabolism has limited access to material and energy and is only a survival strategy for organisms in barren environments [9], which can explain the experimental results of the present study that the maximum abundance and specific growth rates of flagellate SL1 and SL2 strains under conditions of feeding on Microcystis were greater than those of the autotrophic mode. Therefore, flagellate strains SL1 and SL2 can feed on *Microcystis* and are mixotrophic protozoans.

5. Conclusion

Two protozoa, SL1 and SL2, isolated and purified from South Lake of Jinan University, were identified by 18S rDNA molecular and morphological methods and belonged to Sarcomastigophra, Chrysomonadida, Chrysomonadina, Ochromonadidea, and *Ochromonas* sp. (SL1) and *Poterioochromonas malhamensis* (SL2). The specific growth rates of flagellates SL1 and SL2 were higher in the environment with added Microcystis than in the autotrophic

environment without added *Microcystis*, indicating that these two flagellates are heterotrophic protozoa. Both flagellates SL1 and SL2 were able to grow rapidly and abundantly by feeding on Microcystis; the autotrophic model showed much lower population growth than the treatment group fed *Microcystis*. The population growth of *M. wesenbergii* was inhibited by the feeding of flagellates.

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