Title

Grazing impact on the cyanobacterium *Microcystis aeruginosa* by the heterotrophic flagellate *Collodictyon triciliatum* in an experimental pond

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Abstract

We estimated the grazing impact of the heterotrophic flagellate *Collodictyon triciliatum* on the harmful, bloom-forming cyanobacterium *Microcystis aeruginosa* in an experimental pond during *Microcystis* bloom from summer to winter in 2010. For these experiments, we calculated the grazing rates from the *C. triciliatum* digestion rate and food vacuole content. During the study period, *M. aeruginosa* exhibited one blooming event with a maximum density of 1.1×10^5 cells ml^{-1}. The cell density of *C. triciliatum* fluctuated from below the detection limit to 291 cells ml^{-1}. The number of *M. aeruginosa* cells ingested by *C. triciliatum* food vacuoles ranged between 0.4 and 10.8 cells $flagellate^{-1}$, and the digestion rate of *C. triciliatum* at 25°C was 0.73% cell contents min^{-1}. The grazing rate of *C. triciliatum* on *M. aeruginosa* prey was 0.2–6.9 cells $flagellate^{-1}$ h^{-1}, and its grazing impact was 0.0–25.3% standing stock day^{-1}. The functional response of *C. triciliatum* to *M. aeruginosa* prey followed the Michaelis-Menten model of significance ($r^2 = 0.873$, $p < 0.001$) in our experimental systems in which the prey concentration varied from 1.0×10^4 to 2.1×10^6 cells ml^{-1}. The maximum grazing rate was 6.2 prey cells $grazer^{-1}$ h^{-1}, and the half-saturation constant was 1.2×10^5 cells ml^{-1}. We present evidence that *C. triciliatum* grazing explained the remarkable decrease in *M. aeruginosa* cell density in the pond. The present study is the

first demonstration of the high potential of protistan grazing on *M. aeruginosa* to reduce cyanobacterial blooms.

Key words: *Microcystis aeruginosa*, *Collodictyon triciliatum*, Functional response, Ingestion, Grazing impact

Introduction

The major bloom-forming cyanobacterial species *Microcystis aeruginosa* is distributed ubiquitously in eutrophic lakes worldwide. It forms toxic blooms and causes serious environmental problems due to deterioration of water quality, deoxygenation of underlying waters and their subsequent toxicity, foul odors, and an overall decrease in aesthetic value (Carmichael 1992; Park et al. 1998). Many researchers have studied the environmental factors favorable to inducing *M. aeruginosa* bloom formation (Reynolds and Walsby 1975; Dokulil and Teubner 2000; Latour et al. 2004; Gobler et al. 2007), but only limited information have been available for *M. aeruginosa* bloom depletion.

A previous study demonstrated that certain flagellate species graze on *M. aeruginosa*, including *Monas guttula* (Sugiura et al. 1992), *Poterioochromonas malhamensis* (Zhang et al. 1996), *Poterioochromonas* sp. (Zhang et al. 2009), *Collodictyon triciliatum* (Nishibe et al. 2002), *Diphylleia rotans* (Kim et al. 2006), *Ochromonas danica* (Cole and Wynne 1974), and *Ochromonas* sp. (Burkert et al. 2001; Yang et al. 2008; Baek et al. 2009; Van Donk et al. 2009; Yang et al. 2009; Wilken et al. 2010). These flagellate species retain active growth, even while feeding on toxic *M. aeruginosa*. Nishibe et al. (2002) determined the specific grazing rate of the large flagellate *C. triciliatum* on *M. aeruginosa* using a method proposed by Dolan and Šimek

(1998), and applied the rate to estimate the *in situ* grazing pressure on a cyanobacterium population in a eutrophic pond. One would expect a high impact because of the large cell size (30–50 μm in length) of the flagellate, but it in fact had a low impact on the cyanobacterium. Beyond that study, ecophysiological reports on *C. triciliatum* remain limited.

In the present study, we determined the specific grazing rate of *C. triciliatum* on *M. aeruginosa* based on the digestion rate of *M. aeruginosa* cells in the food vacuoles of *C. triciliatum* (Dolan and Šimek 1998). In addition, we examined the functional response of *C. triciliatum* to *M. aeruginosa*. Using these results, we estimated the grazing impact by the flagellate on an *M. aeruginosa* bloom that occurred in an outdoor experimental pond.

Materials and Methods

Monitoring biological abundance

We monitored a *Microcystis* bloom and *C. triciliatum* abundance in a meso-scale experimental pond (10 m × 10 m width; maximum depth, 1.7 m; water volume, ca. 70 m^3) located at the Center for Ecological Research, Kyoto University, Japan (34º 58’ 2.24’’N, 135º 57’ 38.93’’E). We artificially induced the bloom, which is

described in detail in Hodoki et al. (2011). Water samples were collected from the pond surface with a 5L plastic bucket, and poured into three sterilized polycarbonate bottles. Samples were taken at the same time of day (10:00 to 10:30 h) about once weekly from 1 July 2010 to 28 December 2010, and the water temperature was measured simultaneously with a bar thermometer. To measure the chlorophyll *a* (chl *a*) concentration, we filtered a measured portion of each water sample through a GF/F filter to retain seston, and stored the samples in a freezer at -20°C until needed for analysis. To process the samples, we placed the filter in a glass test tube along with 10 ml N, N-dimethylformamide (DMF) to extract the chl *a.* The quantity of chl *a* was determined using a spectrofluorophotometer (RF-5300 Shimadzu; Welschmeyer 1994).

Enumeration of microorganisms

To quantify the *C. triciliatum* cells and *M. aeruginosa* cells, we fixed 500 ml water samples with acidified Lugol's solution at a final concentration of 1%, and concentrated these samples using natural sedimentation. We counted the *C. triciliatum* cells in a Fuchs-Rosenthal type haemacytometer and *M. aeruginosa* in Burker-Turk type haemacytometer under a microscope at a magnification of ×400 at least three times. For enumeration of *M. aeruginosa* cell, concentrated samples were slightly sonicated at

55kw for 5 min to disperse *Microcystis* cells from colonies.

To quantify *M. aeruginosa* cells contained within the *C. triciliatum* food vacuoles, we used samples fixed with acidified Lugol's solution and the method described above. A 0.05ml aliquot of the concentrated sample was mounted on a glass slide, and at least 30 *C. triciliatum* cells were examined under a microscope at a magnification of ×400. For each sample, we averaged the numbers of *M. aeruginosa* cells ingested per flagellate.

Digestion experiment

We determined the digestion rate of *C. triciliatum* feeding on the *M. aeruginosa* population using the methods of Dolan and Šimek (1998) and Nishibe et al. (2002), with minor modifications. We isolated *C. triciliatum* from the surface water of the experimental pond in August 2010, and maintained the clonal cultures in CT medium (Watanabe and Ichimura 1997 or MCC-NIES; www.nies.go.jp/biology/mcc/home_j.htm), using *M. aeruginosa* NIES-843 as the food source for *C. triciliatum*. The microorganism cultures were maintained in 300–500 ml polycarbonate bottles at 25°C under a light intensity of 52 $\mu Em^{-2} s^{-1}$. We used *M. aeruginosa* samples that were in the exponential growth phase and *C. triciliatum* samples that were in the early stationary phase for the digestion and functional response

experiments, respectively. A 6 ml sample of the *C. triciliatum* culture was inoculated into a 100 ml polycarbonate bottle containing 12 ml *M. aeruginosa* culture (4.0×10^6 cell ml^{-1}). Following an incubation period of 12 h at 25°C in the dark, the flagellate culture was diluted with 6 ml of the mixture to 600 ml fresh CT medium in triplicate in 1 L Erlenmeyer flasks in the dark (25°C) to halt ingestion. We removed 50 ml subsamples from each flask at 30 min intervals for 150 min, and fixed these subsamples with acidified Lugol's solution at a final concentration 1%. The fixed samples were concentrated via natural sedimentation, and a 0.05ml aliquot of concentrated sample was mounted on a glass slide. *M. aeruginosa* cells as well as those found in the food vacuoles (ingested cells) were counted in 100 *C. triciliatum* cells under a microscope at a magnification of ×400.

We performed linear regression analyses and calculated the slope of the regression of ln (% initial average number of ingested *M. aeruginosa* per flagellate) versus time to yield the digestion rate. Multiplying the slope by 100 gave a digestion rate constant (% cell content min^{-1}; Dolan and Šimek 1998; Nishibe et al. 2002).

As per Nishibe et al. (2002), we corrected the digestion rate at 25°C for the *in situ* temperature in the experimental pond using $Q_{10} = 2.1$. The specific grazing rate of *C. triciliatum* (G_c, *M. aeruginosa* cells $flagellate^{-1}$ h^{-1}) was calculated by multiplying the

corrected digestion rate by the average number of ingested *M. aeruginosa* cells per flagellate for each sampling day, respectively. The clearance rate of *C. triciliatum* (nl flagellate^{-1} h^{-1}) was calculated by dividing the flagellate grazing rate by the corresponding *M. aeruginosa* cell density for each sampling day. The daily grazing impact of *C. triciliatum* on the standing stock of *M. aeruginosa* (G_i, % standing stock day^{-1}) was estimated as follows:

$$G_i = 100 \times (G_c \times N_c \times 24) / N_m,$$

where N_c (cell ml^{-1}) and N_m (cells ml^{-1}) are the cell densities of *C. triciliatum* and *M. aeruginosa* for each sampling day, respectively (Dolan and Šimek 1998; Nishibe et al. 2002).

Functional response experiment

Before conducting the functional response experiment, we diluted the *C. triciliatum* culture by adding 700 ml of the mixture to 700 ml fresh CT medium, and incubated this culture in the dark for 12 h to obtain flagellates starved for food and without residual growth, and to decrease *M. aeruginosa* abundance to a negligible level ($<1.0\times10^3$ cells ml^{-1}).

We fed the *C. triciliatum* (initial condition; 2.0×10^3 cells ml^{-1}) on *M.*

aeruginosa (initial condition; 1.0×10^4 to 2.1×10^6 cells ml^{-1}) as prey, and examined the resultant ingestion rate using the method of Jeong et al. (2005) with slight modifications. We prepared various *M. aeruginosa* cell concentrations in the culture (initial condition; 1.0×10^4 to 2.1×10^6 cells ml^{-1}) in triplicate 300 ml Erlenmeyer flasks, followed by inoculation of *C. triciliatum* at a cell density of 2.0×10^3 cells ml^{-1}. The cultures were filled with CT medium to 100 ml, and were incubated in the dark at 25°C. At 0, 15, 30 and 45 min of incubation, a 10 ml subsample was removed from each culture, transferred to a 15 ml tube, and then fixed with acidic Lugol's solution to a final concentration of 1%. We used a Burker-Turk haemacytometer to count *M. aeruginosa* cells in the sample. A 0.05ml aliquot of sample was mounted on a glass slide and count *M. aeruginosa* in the food vacuoles of *C. triciliatum*. To enumerate the *M. aeruginosa* as prey, we inspected at least 30 flagellate cells, and produced a linear regression line representing the relationship between the number of prey cells within a *C. triciliatum* cell and the incubation time. We calculated ingestion rates (prey cells $flagellate^{-1}$ h^{-1}) according to the method of Sherr et al. (1987), and fitted all derived ingestion rates to a Michaelis-Menten equation, as follows:

$$IR = \frac{I_{\max}(x)}{K_{IR} + (x)},$$

where $I_{\max}$ is the maximum ingestion rate (cells $flagellate^{-1}$ h^{-1}), and K_{IR} is the prey

concentration sustaining one-half I_{max}.

Results

Monitoring *Microcystis* bloom and *Collodictyon* abundance

The water temperature of the pond remained at about 30°C from the initial experiment day (0) to day 74, and decreased thereafter (Fig. 1A). Two relatively high chl *a* concentrations were measured on days 28 (155.3 μg L^{-1}) and 70 (138.3 μg L^{-1}; Fig. 1B). That on day 70 roughly coincided with the initial *M. aeruginosa* bloom period (day 84, 10.8×10^4 cells ml^{-1}; Fig. 1C). The abundance of *C. triciliatum* became relatively high from days 22 to 35, and from days 105 to 119, with the highest abundance (291 cells ml^{-1}) detected on day 28 (Fig. 1D). The number of *M. aeruginosa* cells ingested by each flagellate cell was high on day 84 (10.8 cells $flagellate^{-1}$), which fluctuated during the study period (Fig. 1E).

The digestion experiment showed that the average number of *M. aeruginosa* cells ingested by each flagellate cell declined over time (Fig. 2), and we determined a significant correlation between the ln (% initial average numbers of ingested *M. aeruginosa* per flagellate) and time ($r^2 = 0.909$, $p < 0.01$, n = 6). The digestion rate constants of *C. triciliatum* feeding on *M. aeruginosa* was 0.73% cell contents min^{-1},

which was close to that determined in the previous study at 25°C (0.74 ± 0.02% cell contents min^{-1}, Nishibe et al. 2002).

The *C. triciliatum* grazing rates and clearance rates were 0.2–6.9 *M. aeruginosa* cells $flagellate^{-1}\,h^{-1}$ and 2.2 to 162.2 nl $flagellate^{-1}\,h^{-1}$, respectively (Table 1). The daily grazing impact of *C. triciliatum* on *M. aeruginosa* was 0.0–25.3% of the *M. aeruginosa* standing stock (Table 1).

Functional response

The *C. triciliatum* population demonstrated a clear functional response to prey density (Fig. 3). The half-saturation constant was 1.2×10^5 cells ml^{-1}, and the maximum ingestion rate was 6.2 cells $flagellate^{-1}\,h^{-1}$.

Discussion

In this study, we demonstrated the high potential of a protistan species to reduce cyanobacterial blooms by herbivorous flagellate grazing. Our observations included temperature-independent pattern changes in *C. triciliatum* abundance (Fig. 1D; see Results section), in which *C. triciliatum* concentrations increased significantly on two occasions (from days 22 to 35, and from days 105 to 119, Fig. 1D). Hence, it is

1 likely that growth of *C. triciliatum* in the present study was independent of temperature.

2 The *M. aeruginosa* population attained its maximum concentration on day 84
3 (Fig. 1C), when the highest number of *Microcystis* cells was ingested by each *C.*
4 *triciliatum* cell (Fig. 1E). There was an abundance of *Microcystis* prey for *C. triciliatum*
5 predation during the period days 74 to 84, suggesting a high possibility for remarkable
6 *C. triciliatum* growth. However, we did not record any noteworthy growth rates for the
7 flagellate during this period (Fig. 1D). One reason for this observation is the possible
8 presence of *C. triciliatum* predators, such as large rotifers and/or
9 carnivorous/omnivorous copepods. However, lacking any evidence of *C. triciliatum*
10 predation, we can only present this possibility as speculation and recommend it as a
11 topic for future study. *C. triciliatum* attained its highest concentration on study day 28
12 (Fig. 1D), when we also recorded the highest chl *a* concentration (Fig. 1B). Previous
13 studies have reported that *C. triciliatum* is omnivorous (Mischke 1994; Nishibe et al.
14 2002). Thus, it seems likely that *C. triciliatum* could proliferate actively, with plenty of
15 food and in the absence of predators.

16 The highest number of *Microcystis* cells ingested by each *C. triciliatum* was
17 recorded on study day 84 (Fig. 1E). The abundance of *M. aeruginosa* decreased
18 significantly between days 84 and 91 (Fig. 1C). Using the specific ingestion rate of 84

day and average *C. triciliatum* number between 84 and 91 days (Heinbokel 1978), we estimated the *M. aeruginosa* loss to grazing by *C. triciliatum* between 84 and 91 days as 1.8×10^4 cells ml^{-1}. We calculated the decrease in *Microcystis* abundance during this period as 8.6×10^4 cells ml^{-1}, indicating that 21% of *Microcystis* abundance was consumed by *C. triciliatum* grazing between 84 and 91 days. According to our microscopic observations, the dominant food item of *C. triciliatum* during this period was *M. aeruginosa*. Thus, the remarkable decrease in *Microcystis* abundance recorded from days 84 to 91 was substantially due to *C. triciliatum* grazing. Grazing rates calculated from digestion rates in the present were roughly consistent with those in our previous study (Nishibe et al. 2002; Table 1). Nishibe et al. (2002) demonstrated that the grazing impact of the flagellate may be of minor importance to the decline in *M. aeruginosa* concentrations. However, in the present study, *C. triciliatum* grazing on *Microcystis* was, if only temporarily, effective for decreasing cyanobacterial abundance. The present study is thus the first to demonstrate the high potential of protistan grazing on *M. aeruginosa* to reduce the cyanobacterial bloom.

We simply calculated the grazing impact using the data on cell densities of *C. triciliatum* and *M. aeruginosa*, and in situ grazing rate of the flagellate. However, this calculation may lead to an overestimation of grazing impact. Nishibe et al. (2002)

reported that *C. triciliatum* might have preyed on small colonies (<50 cells) of *Microcystis* as well as unicellular ones. When *Microcystis* blooms attain to the stationary phase and come to senescence, colony sizes of the cyanobacteria become smaller (Ishikawa et al. 2004, Yamamoto & Nakahara 2009). During the period between 84 days to 91 days, water temperature decreased, and chlorophyll *a* concentration and *Microcystis* cell density also did (Fig. 1A, B and C). These results indicate the cyanobacterial senescence. So, it is likely that small colonies and/or unicellular *Microcysits* were abundant during the period in the present study. Considering those, we think our estimation of *Collodictyon* grazing impact on *Microcystis* abundance is valid, though there still is the possibility of overestimation.

The maximum grazing rates and half-saturation constants recorded in the present study (Table 2) are higher than those reported by Kim et al. (2006) and Wilken et al. (2010). However, these previous studies utilized small flagellate species (*Diphylleia*, ca. 25 μm and *Ochromonas*, ca. 10 μm), whereas the *C. triciliatum* cells used in the present study are comparatively large (30–50 μm). Because the specific ingestion rates of flagellates increase with increasing cell size (Jeong et al. 2005), the results of the present study are comparable to those in the previous studies on a sliding scale. Guo et al. (2008) reported that the flagellate genus *Poterioochromonas* has a

higher half-saturation constant on *M. aeruginosa* prey, and the maximum ingestion rate of the flagellate was lower than that of *C. triciliatum* (Table 2). The genus *Poterioochromonas* is a mixotroph, and some mixotrophic algae are mainly phototrophic with low phagotrophic ingestion rates (Guo et al. 2008). This may explain the high half-saturation constant of *Poterioochromonas*.

It is interesting to note that the grazing rate estimated from the digestion rate herein was close to that of the high food concentration measured in the functional response experiment (Table 1 and Fig. 3), although these methods are different. The possible reason for this similarity is that the *C. triciliatum*, in our digestion experiment, were fed under a food-replete condition, likely similar to the high food concentration used in our functional response experiment. The half-saturation constant of *C. triciliatum* in the present study (1.2×10^5 cell ml^{-1}) suggests that flagellate grazing on *Microcystis* is initiated in the early stages of the bloom (Nishibe et al. 2004; Imai et al. 2009).

As determined in the present study, and similar to previous reports, *C. triciliatum* cannot directly ingest a large colony of *Microcystis* (Nishibe et al. 2002). There may be more effective grazers of *M. aeruginosa* than flagellates, including some fish species (Miura 1990), cladoceran zooplankton (Hanazato and Yasuno 1984),

rotifers (Snell 1980; Fulton and Pearl 1987; Jarvis et al. 1987), and amoebae (Yamamoto and Suzuki 1984; Nishibe et al. 2004). In addition to grazing, other *Microcystis* consumers may include the algicidal effects of heterotrophic bacteria (Manage et al. 2000), and viruses (Yoshida et al. 2006). We have not yet collected data on *in situ* loss of *Microcystis* concentrations due to biological processes, and such loss may be synergistic with the aforementioned *Microcystis* eradicators. Additional studies are needed to understand *Microcystis* loss due to biological processes.

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Table 1. Grazing and clearance rates of *Collodictyon triciliatum* on *Microcystis aeruginosa*, and daily grazing impact of *C. triciliatum* on the standing stock of *M. aeruginosa* in an experimental pond. Min: a minimum value, Max: a maximum value, SD: standard deviation.

	Range (Min - Max)	Means ± SD
Grazing rate (*M. aeruginosa* cells flagellate $^{-1}$ h^{-1})	0.2 – 6.9	1.1 ± 1.4
Clearance rate (nl flagellate^{-1} h^{-1})	2.2 – 162.2	39.6 ± 40.0
Daily grazing impact (% standing stock day^{-1})	0.0 – 25.3	3.4 ± 7.1

Table 2. Comparison of I_{max} and K_{IR} in different predators, when fed *M. aeruginosa* or *Synechococcus*. I_{max}: maximum ingestion rate, K_{IR}: the prey concentration sustaining one-half I_{max}.

Protist	Prey	I_{max} (cells flagellate^{-1} h^{-1})	K_{IR} (10^5 cells ml^{-1})	Source
Collodictyon triciliatum	*M. aeruginosa* NIES843	6.2	1.2	Present study
Diphylleia rotans	*M. aeruginosa* NIER -10001	0.56		Kim et al. (2006)
Diphylleia rotans	*M. aeruginosa* NIES -298	0.73		Kim et al. (2006)
Diphylleia rotans	*M. aeruginosa* NIES -101 (non-toxic strain)	0.17		Kim et al. (2006)
Diphylleia rotans	*M. viridis* NIES -102	0.35		Kim et al. (2006)
Ochromonas sp.	*M. aeruginosa* PCC7806 wild type	2.21 ± 0.25	0.13 ± 0.25	Wilken et al. (2010)
Ochromonas sp.	*M. aeruginosa* PCC7807 mutant	1.68 ± 0.14	0.14 ± 0.25	Wilken et al. (2010)
Poterioochromonas sp.	*M. aeruginosa* FACHB 469	3.3	37.6	Guo et al. (2008)

Fig 1. Changes in (A) Water temperature, (B) Chlorophyll *a* concentration, abundance of (C) *M. aeruginosa* and (D) *C. triciliatum*, and (E) average number of ingested *M. aeruginosa* in the food vacuoles of *C. triciliatum*, in an experimental pond. Error bars indicate standard deviations for each mean value in (E).

Fig 2. Relationship between *M. aeruginosa* cell number in the food vacuoles of *C. triciliatum* and time. Error bars indicate ± standard error. Error bars that are not visible are hidden behind the symbols.

Fig 3. Ingestion rate (cells flagellate^{-1} h^{-1}) of *C. triciliatum* on *M. aeruginosa* as a function of the initial prey concentration (cell ml^{-1}). Each value was calculated from a linear regression curve of the number of prey cells in flagellate vacuoles over incubation time. The curve was fitted by a Michaelis-Menten equation (in Materials and methods) using all treatments in the experiment. Ingestion rate (*IR*, cell flagellate^{-1} h^{-1}) = 6.185 [x/(122280 + x)], $r^2 = 0.873$, $p < 0.001$, where x is *Microcystis* abundance.

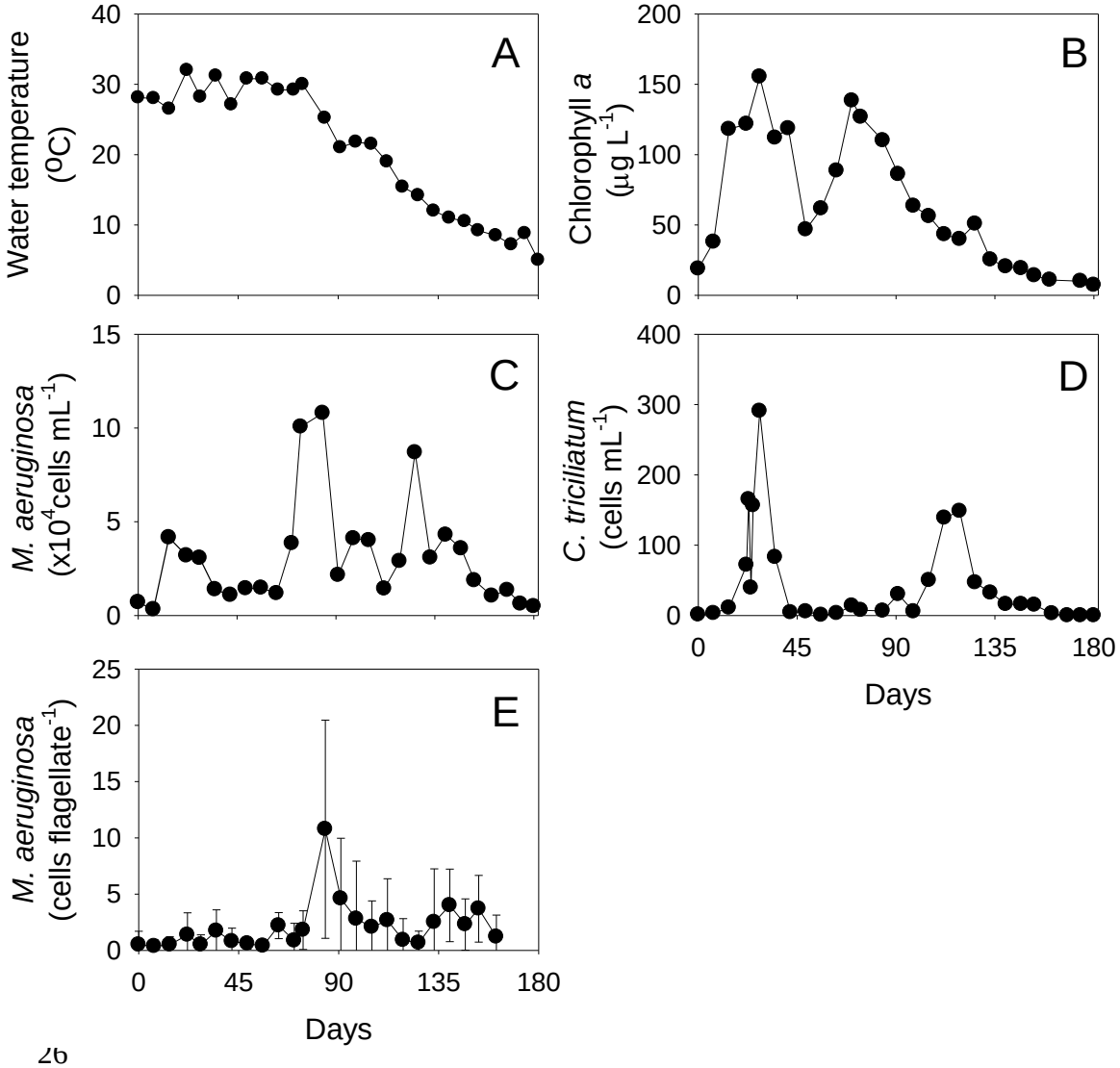

Fig. 1 Kobayashi et al.

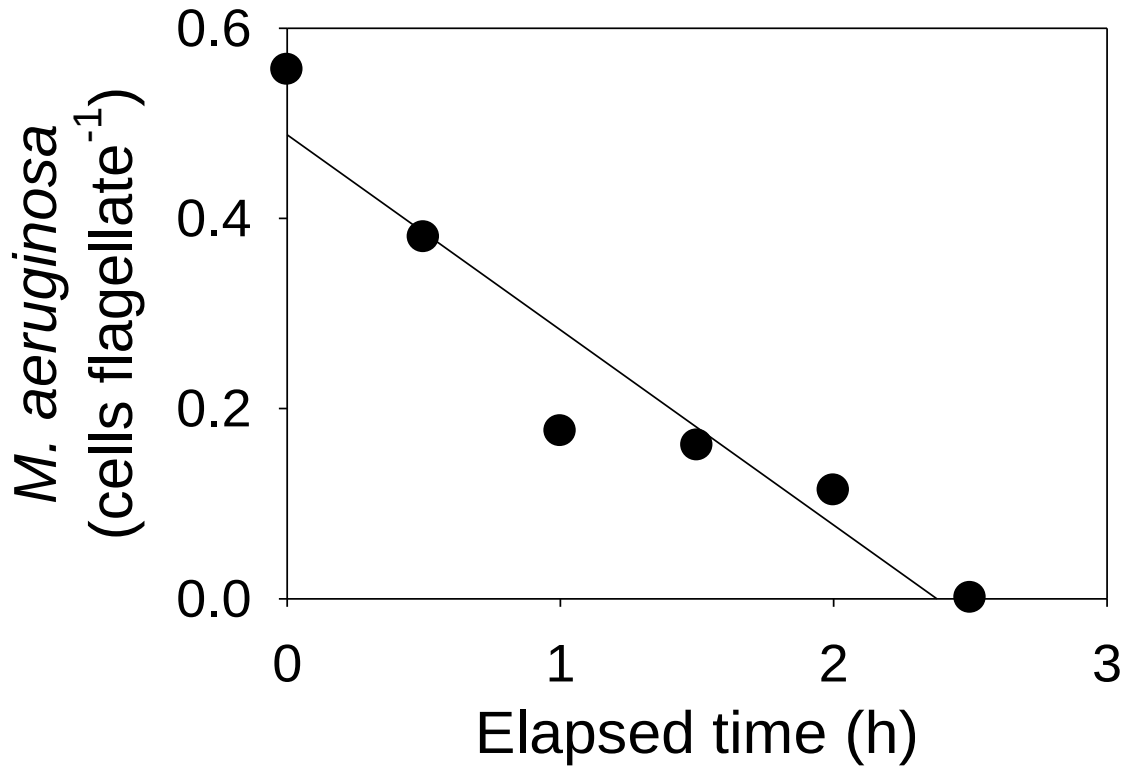

Fig. 2 Kobayashi et al.

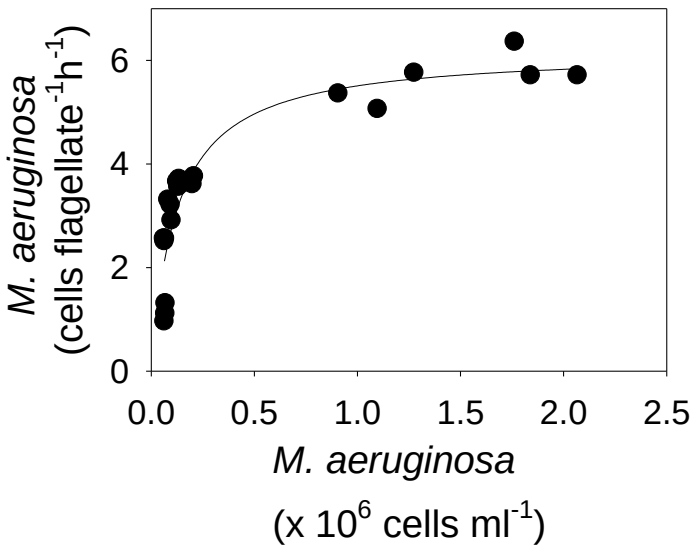

Fig. 3 Kobayashi et al.