

Ingestion of bacterial cells by the marine photosynthetic ciliate *Myrionecta rubra*

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ABSTRACT: We report the first observation of fluorescently labeled bacteria (FLB) ingestion by the cells of MR-MAL01, a temperate strain of the marine photosynthetic ciliate *Myrionecta rubra* Jankowski 1976 (= *Mesodinium rubrum* Lohmann 1908). We also investigated the time course of ingestion and digestion of bacteria as well as the ingestion rates at 3 different light intensities. In the stationary phase of growth with ambient bacterial abundance (1.4×10^6 cells ml⁻¹) under conditions of 15°C and 60 $\mu\text{E m}^{-2} \text{s}^{-1}$, the rates of FLB uptake and disappearance of *M. rubra* MR-MAL01 cells were 7.6 and 5.3 FLB grazer⁻¹ h⁻¹, respectively. The ingestion rate of *M. rubra* in cultures was calculated to be 53 bacteria grazer⁻¹ h⁻¹. The initial abundance of *M. rubra* (ca. 1.0×10^4 cells ml⁻¹) in the ingestion-digestion experiment was comparable to the natural abundance during the *M. rubra* red tide in Korean coastal waters. The bacterivory rate of *M. rubra* increased gradually as light intensity decreased from 200 to 0 $\mu\text{E m}^{-2} \text{s}^{-1}$; this might enable the photosynthetic *M. rubra* to survive under intermittently light-limiting conditions. The present study showed a novel phenomenon of the ingestion of bacterial cells by *M. rubra* and the light-affected bacterivory rates of the common red tide ciliate.

KEY WORDS: *Myrionecta rubra* · *Mesodinium rubrum* · Bacterivory · Ingestion rate · Light intensity · Mixotrophy · Red tide

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INTRODUCTION

Myrionecta rubra Jankowski 1976 (= *Mesodinium rubrum* Lohmann 1908) is a marine kleptoplastidic (=retaining plastids of donor cells) ciliate that exhibits phototrophic ability during long periods between feedings on plastid donors (Johnson & Stoecker 2005). As a functional primary producer, *M. rubra* is very common and often causes recurrent red tides in diverse marine pelagic environments, including fjords (Taylor et al. 1971, Lindholm 1985), polar water under ice, and in deep water of the adjacent seas (Putt 1990, Satoh & Watanabe 1991, Stoecker et al. 1992, 1993, Perriss et al. 1995). *M. rubra* has been considered as an unculturable species (Lindholm 1985) ever since Charles Darwin described it as 'minute animalcula darting about, and often exploding' (Darwin 1845, Taylor et al. 1971), until recently when isolates were grown as laboratory

cultures by 2 research groups (Gustafson et al. 2000, Kim 2002). Using an Antarctic strain that originated from McMurdo Sound, Gustafson et al. (2000) showed that *M. rubra* sequester organelles of ingested cryptophyte *Teleaulax acuta* cells; this enhances the photosynthetic growth of the *M. rubra* strain (Gustafson et al. 2000). The detailed feeding process and the degree of contribution of the cryptophyte ingestion to the enhanced growth of *M. rubra* were reported in another strain isolated from a temperate estuary (Yih et al. 2004). However, bacterivory by *Myrionecta/Mesodinium* species has not been seriously explored by food web researchers (Sanders et al. 1989, Sherr et al. 1991, Dolan & Marrase 1995, Perez et al. 2000), except for Sorokin & Sorokin (1996) and Sorokin et al. (1999). In the absence of any direct evidence on *M. rubra* bacterivory, the latter authors estimated considerable energy flow (ca. 100 calories m⁻² d⁻¹) from bacteria to

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'*Mesodinium rubrum*' in the ecosystem analysis of the Lena River estuary (Sorokin & Sorokin 1996) and Po River Delta (Sorokin et al. 1999).

Here, we first report novel bacterivory by a temperate strain of *Myrionecta rubra*, namely MR-MAL01 (Yih et al. 2004), and its light-affected bacterivory rates. The present study might facilitate a better understanding of the multidimensional function of *M. rubra* in various marine ecosystems.

MATERIALS AND METHODS

Clonal culture of a temperate *Myrionecta rubra* strain. Clonal cultures of experimental *M. rubra* MR-MAL01 and its prey species CR-MAL01 were established using the single cell isolation method (Guillard & Ryther 1962) with water samples collected at Gomso Bay, Korea (see Yih et al. [2004] for isolation and culturing methodology). The mean cell volume and mean equivalent spherical diameter of *M. rubra* MR-MAL01 under the culture conditions (15°C, 30 psu, and continuous illumination of 60 $\mu\text{E m}^{-2} \text{s}^{-1}$ in an f/2 culture medium) were 5996 μm^3 and 22 μm , respectively (Yih et al. 2004).

Preparation of fluorescently labeled bacteria. Ingestion and digestion rates of *Myrionecta rubra* on bacteria were measured using fluorescently labeled bacteria (FLB). Briefly, FLB were prepared as described by Sherr et al. (1987) by staining concentrated bacterial samples with 5-(4,6-dichlorotriazin-2-yl) aminofluorescein (DTAF). Bacteria (length, 1.0 to 1.9 μm ; width, 0.3 to 0.6 μm) for *M. rubra* cultures were concentrated to 200 ml by hollow fiber ultrafiltration (0.1 μm pore size) from 4 l of 3 μm prefiltered cultures. They were then centrifuged at 22 000 $\times g$ for 20 min to obtain pellets. The pellets were resuspended in 10 ml of PBS solution (0.05 M Na_2HPO_4 -0.85% NaCl, pH 9). Subsequently, 20 mg of DTAF was added, and the cell suspensions were incubated at 60°C in a water bath for 2 h. After incubation, the stained bacteria were centrifuged and washed 3 times with the PBS solution; the bacteria were then suspended in a 0.02 M tetrasodium pyrophosphate-0.85% NaCl solution. The cell suspension was sonicated using a tapered microtip (model UP-400A, Sonicor Instrument) at a 30 W power level in order to disperse large clumps. DTAF-stained bacteria were collected onto a 0.2 μm black polycarbonate filter for enumeration via epifluorescence microscopy. Aliquots (10 ml) were frozen at -20°C in 20 ml plastic vials. The FLB stocks (54 μl) were added to 100 ml samples in 500 ml polycarbonate bottles presoaked in 10% (vol./vol.) HCl and copiously rinsed with deionized water. The bacterial abundance in the *M. rubra* culture was measured by epifluorescence microscopy accord-

ing to the method described by Porter & Feig (1980). Briefly, bacterial samples were fixed with 0.2 μm filtered, borate-buffered formalin (final conc., 2%). Bacteria, which were stained with 4',6-diamidino-2-phenylindole (DAPI) and collected on a 0.2 μm black polycarbonate filter, were counted under UV excitation using an epifluorescence microscope.

Rates of FLB uptake and digestion at low light intensity. *Myrionecta rubra* cultures starved of their cryptophyte prey (Yih et al. 2004) for 20 d in the stationary phase of growth were maintained overnight under culture conditions of 15°C, 30 psu, and 60 $\mu\text{E m}^{-2} \text{s}^{-1}$ and used for the ingestion-digestion experiment. FLB were added to the *M. rubra* culture (8.0×10^3 cells ml^{-1}) until 14.3% bacterial abundance was achieved in the ciliate culture (1.4×10^6 cells ml^{-1}). The experimental design was to monitor the uptake of FLB until FLB per *M. rubra* cell stopped increasing with time, and then to effectively inhibit further uptake of FLB by a 10-fold dilution with cultured media containing the same concentration of unlabeled bacteria as used in the initial culture. For the FLB uptake experiment, 5 ml of subsamples were collected at 15 min intervals for 105 min, fixed with ice-cold glutaraldehyde (final conc., 1%), and refrigerated until microscopic examinations. The preserved subsamples were stained with DAPI and filtered onto black Nuclepore membrane filters (0.8 μm pore size), and at least 100 microscopic fields were counted. The filters were first observed under UV excitation at a magnification of $\times 1000$. When an *M. rubra* cell was located, the incident light was switched to blue, and the number of FLB contained within the *M. rubra* cell were counted. The FLB uptake rate was calculated from the changes in the number of FLB grazer $^{-1}$ with time using simple regression analysis. The per cell clearance rate (nl grazer $^{-1} \text{h}^{-1}$) was calculated by dividing the cell-specific uptake rate of FLB by the concentration of FLB per nl. The bacterivory rate was measured over the linear portion of the uptake curve. After 2 h of initial subsampling, 50 ml of the remaining culture was diluted with 450 ml of 3 μm filtered seawater containing the appropriate number of bacteria (2.17×10^6 cells ml^{-1}) to approximate the total concentration of bacteria initially present in the *M. rubra* culture. This treatment served to decrease the further uptake of FLB to a 10-fold lower rate while maintaining the same rate of bacterivory by keeping the total bacterial concentration constant. Subsamples (40 ml) were collected in 500 ml polycarbonate bottles at 15 min intervals for 105 min to monitor the decrease in FLB per *M. rubra* cell with time. The subsamples were examined for FLB per *M. rubra* cell as described earlier. A decreasing rate of FLB per *M. rubra* cell was determined via regression analysis for the linear portion of the curve for the digestion experiment.

Rates of FLB uptake at 3 different light intensities.

Aliquots of a *Myrionecta rubra* culture that was starved of its cryptophyte prey for 7 d were transferred to 3 separate bottles. Under conditions of 15°C and 30 psu conditions, each of the 3 bottles was maintained overnight at 0, 60, and 200 $\mu\text{E m}^{-2}\text{s}^{-1}$ continuous illumination. The 3 different experimental cultures of *M. rubra* (8.0×10^3 cells ml^{-1}) with initial bacterial concentration of 9.8×10^6 cells ml^{-1} and FLB concentration of 3.6×10^5 FLB ml^{-1} were used for the light intensity experiments. Subsamples (25 ml) were collected in duplicate 500 ml polycarbonate bottles at 10 min intervals for 30 min to monitor the number of FLB per *M. rubra* cell with time. The subsamples were fixed with ice-cold glutaraldehyde (final conc., 1%) and examined for FLB per *M. rubra* cell as described earlier. The initial abundance of *M. rubra* (ca. 1.0×10^4 cells ml^{-1}) and bacteria (10^6 to 10^7 cells ml^{-1}) in the above experiments is comparable to their natural abundances during the *M. rubra* red tide in Korean coastal waters (G. Myung unpubl. data).

RESULTS AND DISCUSSION

Ingestion of FLB by *Myrionecta rubra* cells

FLB were clearly observed inside most of the *Myrionecta rubra* cells (Fig. 1); this was the first direct evidence of bacterivory by *M. rubra*. However, we could not observe the actual feeding process. The FLB count per *M. rubra* cell increased almost linearly from 0 to 30 min (Figs. 2 & 3A). Although Sorokin & Sorokin (1996) and Sorokin et al. (1999) have repeatedly postulated considerable energy flow from bacteria to *M. rubra* in their ecosystem model, the possibility of *M. rubra* ingesting bacterial cells has never been experi-

mentally tested. Early observations that *M. rubra* lacks a true cytostome (Bary & Stuckey 1950, Hibberd 1977, Grain et al. 1982) led many researchers to pay little attention to the trophic modes other than the 'obligatory' phototrophism (Sieburth et al. 1978, Smith & Barber 1979, Crawford 1989) of the ciliate. However, recent reports have confirmed that phagotrophism, particularly on cryptophyte cells, as well as phototrophism is required for the sustainable growth of *M. rubra*. Gustafson et al. (2000) demonstrated the absolute requirement of the cryptophyte prey using an Antarctic isolate of *M. rubra*. Using another temperate *M. rubra* strain, Yih et al. (2004) reported a more detailed feeding process and the degree of contribution of algivory to the enhanced growth of *M. rubra*. Thus, the issue of the absence of a cytostome in *M. rubra* (Bary & Stuckey 1950) needs further thorough examination. The formation and disappearance of the temporary cytostome-like structure at the anterior tip of the ciliate body might be an aspect for further exploration (Yih et al. 2004).

Rates of FLB uptake and digestion of *Myrionecta rubra* starved for 20 d at low light intensity

At 60 $\mu\text{E m}^{-2}\text{s}^{-1}$ continuous illumination, the FLB count per *Myrionecta rubra* cell increased linearly from 0 to 60 min followed by 20 to 40 min of an 'equilibrium' period (Fig. 2). Based on the slope of the regression line for the data points between 0 and 60 min, the mean FLB uptake rate was calculated to be 7.6 FLB grazer $^{-1}\text{h}^{-1}$; this yields a mean clearance rate

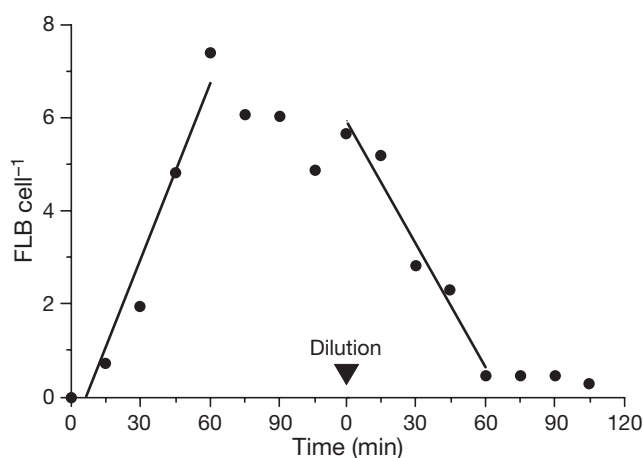


Fig. 2. *Myrionecta rubra*. Time course of ingestion and digestion of bacteria by the photosynthetic ciliate. In all cases, the correlation coefficients for the linear regression fits to the data were >0.94 . Slopes of increase and decrease in fluorescently labeled bacteria (FLB) per cell were determined via regression analysis for the linear portions of the curves for each ingestion-digestion experiment

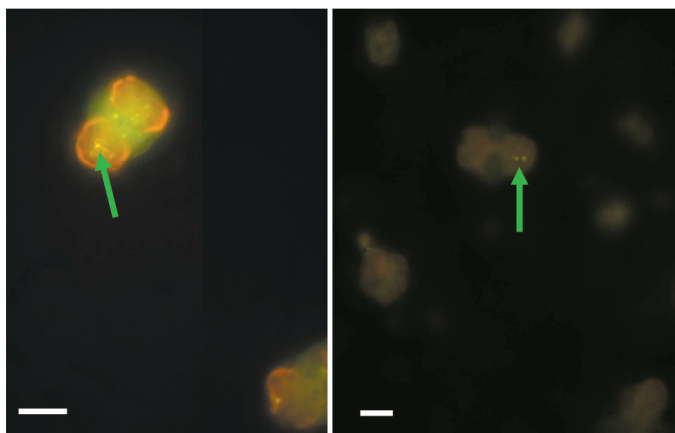


Fig. 1. *Myrionecta rubra*. Epifluorescence image of the ingested fluorescently labeled bacteria (small yellow dots indicated by arrows) inside the reddish-brown cells. Scale bars = 10 μm

of 37 nl grazer⁻¹ h⁻¹. The mean ingestion rates of *M. rubra* in the cultures was estimated to be 53 bacteria grazer⁻¹ h⁻¹. The lower ingestion rate of the experimental *M. rubra* strain than that of other bacterivorous marine ciliate species (Sherr & Sherr 1987, Sherr et al. 1989) might be associated with the mixotrophic nature of this strain.

Immediately after 10-fold dilution of the ambient FLB concentration, the FLB count per *Myrionecta rubra* cell decreased linearly for 60 min from 5.8 to 0.5 FLB grazer⁻¹ to yield a mean disappearance rate of 5.3 FLB grazer⁻¹ h⁻¹ (Fig. 2). Therefore, the time taken by *M. rubra* to digest bacteria was 1 h. This digestion time shows a good agreement with those obtained by other researchers. Fenchel (1975) reported digestion times of 30 min to 5 h for 2 bacterivorous ciliates within the temperature range of 7 to 25°C. Fok & Shockley (1985) found that the processing period in *Tetrahymena* species was 45 min at room temperature. The similar rates of FLB uptake and disappearance suggest that the plateau of the FLB uptake curve can be interpreted as the attainment of equilibrium between ingestion and digestion of FLB (Sherr et al. 1988).

Rates of FLB uptake of *Myrionecta rubra* starved for 7 d at 3 different light intensities

In all the experimental bottles, the FLB counts per *Myrionecta rubra* cell increased almost linearly from 0 to 30 min (Fig. 3A). The mean FLB uptake rates of the experimental *M. rubra* strain calculated at 0, 60, and 200 $\mu\text{E m}^{-2} \text{s}^{-1}$ were 5.9, 3.6, and 2.6 FLB grazer⁻¹ h⁻¹, respectively (Fig. 3B). The estimated mean ingestion rate of the experimental *M. rubra* strain decreased from 159 to 70 bacteria grazer⁻¹ h⁻¹ as the light intensity increased up to 200 $\mu\text{E m}^{-2} \text{s}^{-1}$. Thus, the estimated mean ingestion rate of the experimental *M. rubra* strain in the dark was ca. 2.3 times greater than that at

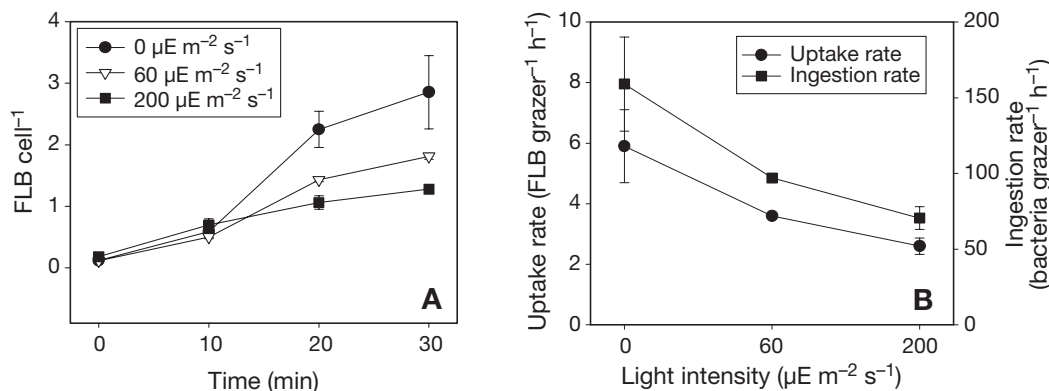


Fig. 3. *Myrionecta rubra*. Bacterivory rates of the experimental strain MR-MAL01 at 3 light intensities. (A) Time course of ingested FLB and (B) FLB uptake and ingestion rates at 0, 60, and 200 $\mu\text{E m}^{-2} \text{s}^{-1}$ continuous illumination

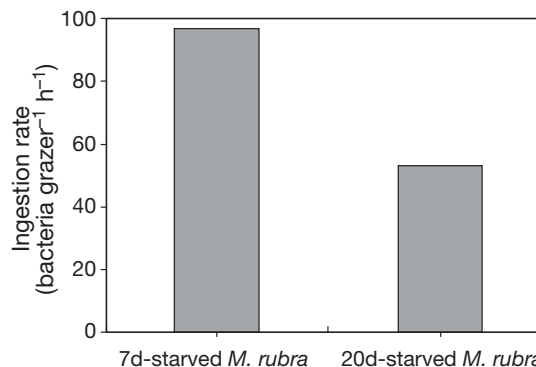


Fig. 4. *Myrionecta rubra*. Ingestion rate of bacteria by the photosynthetic ciliate decreased as the number of 'starved' days increased from 7 to 20 at 60 $\mu\text{E m}^{-2} \text{s}^{-1}$ continuous illumination

200 $\mu\text{E m}^{-2} \text{s}^{-1}$. The significant reduction in the bacterivory rate at higher light intensities implies that *M. rubra* is at the phototrophic end of the scale of nutritional modes among marine mixotrophic protists (Bird & Kalff 1987, 1989). Bacterivory could serve as a source of substitutable organic matter for the phototrophic *M. rubra* under low light conditions (Rothhaupt 1996). Bacterivory of *M. rubra* should be further studied under diverse scales of nutrient limitation, bacterial abundance, light intensity and cell density of *M. rubra* before we can extrapolate the importance of *M. rubra* bacterivory to bloom as well as non-bloom conditions in nature.

Effect of starvation period on *Myrionecta rubra* bacterivory

The estimated mean bacterivory rate of the experimental *Myrionecta rubra* strain was significantly reduced when it was starved of prey cryptophyte cells for a longer period (Fig. 4). At 60 $\mu\text{E m}^{-2} \text{s}^{-1}$, the *M.*

rubra starved for 20 d (Fig. 2) 7d (Fig. 3) ingested 53 and 97 bacteria grazer⁻¹ h⁻¹, respectively (Fig. 4). In the present study, the 'old' *M. rubra* starved for 20 d might have been photo-physiologically more retarded than the 'relatively fresh' *M. rubra* starved for 7 d. Similarly, the growth rate of the Antarctic *M. rubra* isolate gradually decreased with an increase in the 'unfed (=no addition of prey cryptophyte cells)' period (Johnson & Stoecker 2005). It can be speculated that the photosynthetic process in the *M. rubra* culture that was starved for a longer period may be weakened, thereby diminishing the physiological need for substitutable organic matter or micronutrients obtained from the ingested bacterial cells (Nygaard & Tobiesen 1993, Rothhaupt 1996).

In conclusion, the present study is the first to confirm that *Myrionecta rubra* exhibits bacterivory and may play an unrecognized role as a bacterivore, which may influence the nutrition of *M. rubra*; however, further research is required to confirm these assumptions.

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