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NOTE

# Application of a vital staining method to measure feeding rates of field ciliate assemblages on a harmful alga

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ABSTRACT: The fluorescently labeled algae (FLA) technique using a vital green fluorescent stain CMFDA (5-chloromethylfluorescein diacetate) was applied to investigate the feeding activities of a field ciliate assemblage on a harmful alga, *Heterocapsa circularisquama*. Using epifluorescence under interference blue-light excitation improved the limitations noted in the original protocol (Li et al. 1996; Aquat Microb Ecol 10: 139–147), which are due to the occurrence of green autofluorescence similar to that of CMFDA-labeled prey in natural assemblages and in food vacuoles of field protists. CMFDA-labeled *H. circularisquama* were ingested by the tintinnid ciliate *Favella taraikaensis* at the same rate as non-labeled alga, indicating that the staining with CMFDA did not influence the feeding activity of the ciliates. In an *in situ* feeding experiment, more than 6 species of heterotrophic and mixotrophic ciliates were shown to be able to feed on *H. circularisquama*, and the ingestion rates of 2 species were estimated (1.08 and 1.83 cells ind.$^{-1}$ h$^{-1}$). The FLA method with CMFDA is effective in measuring the feeding activities of ciliates and is applicable to the estimation of the grazing impact of ciliate assemblages on *H. circularisquama* populations in natural assemblages.

KEY WORDS: FLA · CMFDA · Ciliate · Feeding · Harmful alga · *Heterocapsa circularisquama*

To clarify the mechanisms by which harmful algal blooms occur, it is essential to understand the role of their grazers. In particular, herbivorous ciliates and heterotrophic dinoflagellates are important as consumers for nano-planktonic harmful algae (Caron et al. 1989, Buskey & Hyatt 1995, Nakamura et al. 1995, 1996, Kamiyama 1997). To date the feeding activities of some ciliates on harmful algae have been reported from laboratory experiments (Hansen 1989, 1995, Kamiyama 1997, Jeong et al. 1999). However, since the number of ciliate species that one can culture under laboratory conditions is limited, it is desirable to measure the feeding activities of field ciliate species on harmful algae using *in situ* experiments, but such data have scarcely been reported.

Using visually detected surrogate prey is one of the most effective methods for investigating the feeding activity of field herbivorous ciliates. In particular, the development of fluorescently labeled algae (FLA) enabled researchers to readily and accurately measure ingestion rates of marine ciliates on some algae (Rublee & Gallegos 1989) since some species of ciliates showed chemosensory selectivity for specific algae (Stoecker et al. 1981, 1986, Stoecker 1988, Taniguchi & Takeda 1988, Verity 1988). However, Putt (1991) reported that the FLA technique with heat-killed algae even caused underestimation of the ingestion rates in some herbivorous ciliates. Live, fluorescently labeled prey using HYD (hydroethidine) (Putt 1991), DAPI (2,4-diamadino-6-phenylindole) (Lessard et al. 1996) and CMFDA (5-chloromethylfluorescein diacetate) (Li et al. 1996) may be desirable alternatives to heat-killed fluorescently labeled prey. Among these vital stains, CMFDA may be the most effective dye for protists (Li et al. 1996). This is metabolized to an aldehyde-fixable, thiol-conjugated product that is trapped within cells for at least 72 h. Further, the toxicity appears to be extremely low (Li et al. 1996).

However, limitations of this technique were demonstrated in Li et al. (1996). The most serious is the existence of some green auto-fluorescence similar to that of CMFDA-labeled prey in field seawater and in the food vacuoles of field protists. They demonstrated the requirements of CMFDA control treatments. This paper reports that it is possible to improve this technique by modifying the observation method and shows

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that the modified CMFDA technique is effective in estimating the grazing impact of a field ciliate assemblage on a harmful alga.

**Materials and methods.** ***Prey alga and ciliates:*** The harmful dinoflagellate *Heterocapsa circularisquama* was used as the prey alga. Although this alga is highly toxic for bivalves specifically (Matsuyama et al. 1997), 2 species of tintinnids were found to feed on this alga and to be able to grow actively at a low concentration (<1000 cells $ml^{-1}$) (Kamiyama 1997). A strain of *H. circularisquama* (HA92-1) isolated from Ago Bay, Japan, in December 1992 was maintained in modified SWM3 medium (Chen et al. 1969, Itoh & Imai 1987). For a preliminary laboratory experiment, the tintinnid ciliate *Favella taraikaensis* isolated from Hiroshima Bay in April 1999 was used. The strain of this tintinnid was maintained in filtered seawater enriched with 0.1 ml $l^{-1}$ of f/2 iron-EDTA trace metal solution (Stoecker et al. 1988) with a mixture of *H. triquetra* and *Gyrodinium* sp. as food sources.

***Staining with CMFDA:*** The staining method with the CMFDA dye was basically followed as in Li et al. (1996). A stock solution of CMFDA (50 µM) was prepared by dissolving 1 mg of CMFDA in 0.5 ml of dimethylsulfoxide and then diluting up to an appropriate volume with filtered seawater. This solution was kept in a freezer (–20°C) until the experiments were carried out. For the experiments, part of the stock solution acclimated to room temperature was inoculated into part of *Heterocapsa circularisquama* cultures to a final concentration of 1 µM (Li et al. 1996). This culture was then incubated under dark conditions for 1 h at 20°C for a preliminary feeding experiment and 25°C for an *in situ* feeding experiment. Before the experiments, the percentage of the stained cells to the total *H. circularisquama* cells was determined. Furthermore, I checked whether fixed cells with buffered formaldehyde (final concentration: 2%; Stoecker et al. 1989) were stained or not with the CMFDA.

The stained alga was observed using an inverted microscope (Olympus IX-70) equipped with transmitted light and epifluorescence under interference blue (IB)-light excitation (Olympus U-MNIBA filter set: a 470 to 490 nm band pass filter, a DM505 dichroic splitting mirror and a BA 515-550 barrier filter).

***Preliminary feeding experiment in laboratory:*** A preliminary experiment was designed to confirm that the CMFDA-labeled alga was grazed by ciliates in the same way as the non-labeled alga. Part of the supernatant water was removed without agitation from a culture of *Favella taraikaensis* when the density of *F. taraikaensis* reached 15 ind. $ml^{-1}$. Within the supernatant water there were some algal cells carried over from the stock culture (18 cells $ml^{-1}$) but auto-fluorescence due to the food algae was hardly observed in the food vacuoles of *F. taraikaensis* (0.08 cells $ind.^{-1}$). Sixty milliliters of the supernatant water was poured into a 100 ml flask. CMFDA-labeled and non-labeled *Heterocapsa circularisquama* were added to the flasks to form a final concentration of 360 cells $ml^{-1}$, and then incubation was immediately started under conditions of 20°C and 20 µmol photon $m^{-2}$ $s^{-1}$. After 5, 10, 15, 20, 30, 40, 50 min of incubation, 2.5 ml of culture was removed from the flasks and transferred into a well of multiple-well plates (24 wells) containing 0.25 ml of 20% buffered formaldehyde for fixation of the tintinnids. Within 5 h, 23 to 27 individuals of *F. taraikaensis* for each time point were observed with the inverted microscope with transmitted light and epifluorescence, and then the average number of ingested particles was calculated. The auto-fluorescence (red) particles indicating almost all *H. circularisquama* and CMFDA-fluorescence (green) particles inside the food vacuoles were counted under blue (B)-light excitation (Olympus U-MWBV filter set: a 400 to 440 nm band pass filter, a DM500 dichroic mirror and a BA475 barrier filter) and under the IB-light excitation, respectively. Ingestion (= captured prey number per individual) by *F. taraikaensis* of non-labeled *H. circularisquama* cells was calculated by subtracting the number of green particles from the number of red particles and was compared with ingestion of the CMFDA-labeled cells for each time point.

**In situ** ***feeding experiment:*** A seawater sample was collected from a 1 m depth layer at a coastal site of western Hiroshima Bay, the Seto Inland Sea, Japan. Two liters of seawater were dispensed into two 1 l polycarbonate bottles, the CMFDA-labeled *Heterocapsa circularisquama* was added to 1 experimental bottle at a final concentration of 700 cells $ml^{-1}$ and only CMFDA solution diluted with filtered seawater (5 ml) was added to the other control bottle. Bottles were then incubated under conditions of *in situ* temperature (24.5°C) and 30 µmol photon $m^{-2}$ $s^{-1}$. After 10 and 30 min of incubation, 300 ml of the water was sampled from each incubated bottle and then divided into three 100 ml bottles containing 10 ml of 20% buffered formaldehyde for fixation of the field ciliates.

One hundred milliliters of fixed sample was settled in an Utermöhl chamber and ciliates in 3 concentrated samples were observed with the inverted epifluorescence microscope. Microscopic observations were performed with 2 serial steps. Firstly, auto-fluorescence due to chlorophyll *a* was monitored in food vacuoles of ciliates at a magnification of 150× using the inverted microscope with the transmitted light and epifluorescence under B-light excitation. Secondly, whether or not the detected particles produced green fluorescence due to the CMFDA was examined under the IB-light excitation. Ingested *Heterocapsa circularisquama*

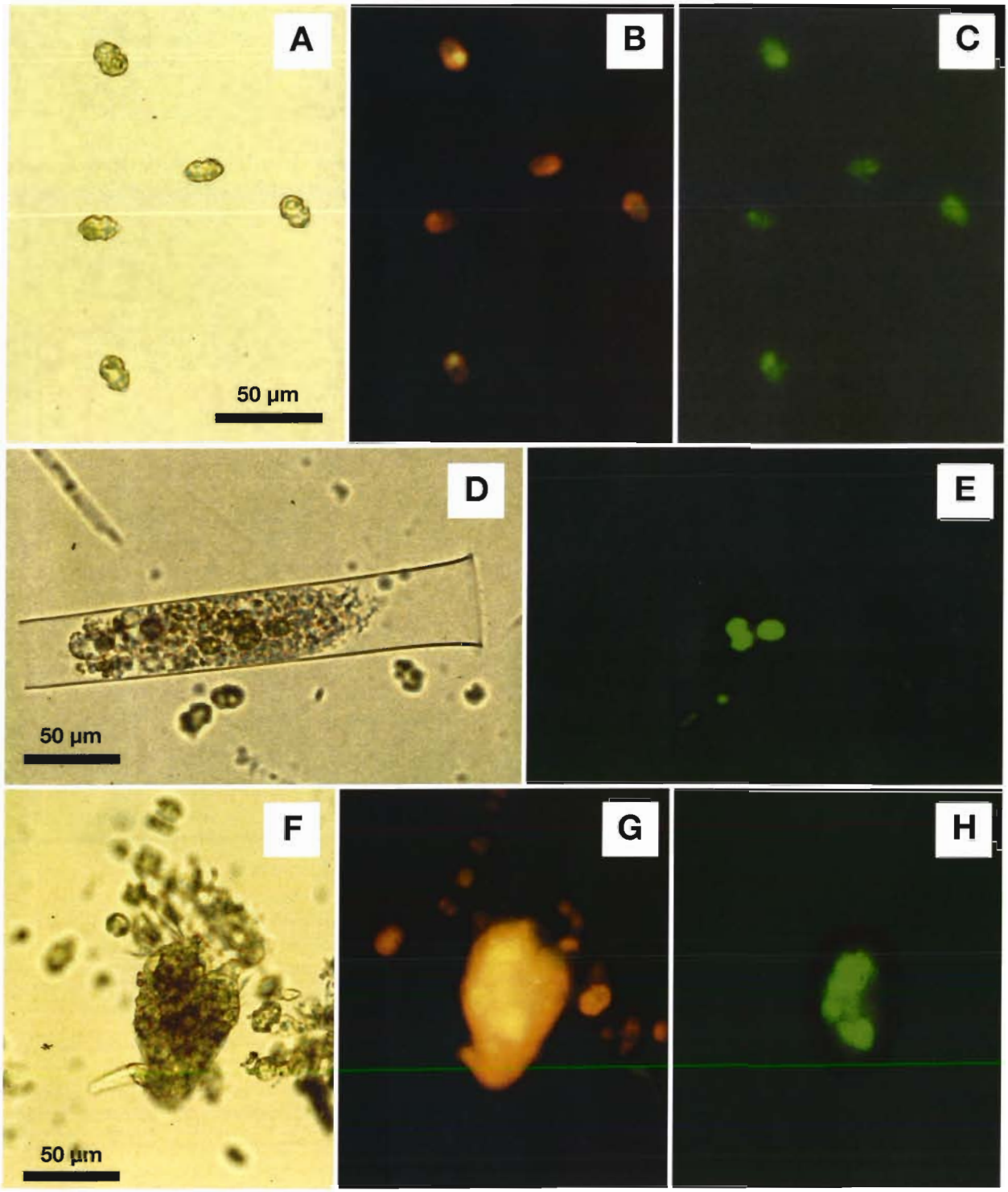


Fig. 1. Photographs of CMFDA-labeled *Heterocapsa circularisquama* observed (A) with transmitted light and (B) with epifluorescence under blue-light excitation and (C) under interference blue-light excitation; a tintinnid ciliate (*Eutintinnus lususundae*) feeding on the CMFDA-labeled *H. circularisquama* observed (D) with transmitted light and (E) with epifluorescence under the interference blue-light excitation; and an aloricate ciliate (*Tontonnia* sp.) feeding on CMFDA-labeled *H. circularisquama* observed (F) with transmitted light and (G) with epifluorescence under the blue-light excitation and (H) under the interference blue-light excitation. Note in (H) that only CMFDA fluorescence (green) within the mixotrophic ciliate can be observed under the interference blue-light excitation since red auto-fluorescence due to chlorophyll *a* is eliminated. Scale bars in (A), (D) and (F) are also applicable to (B), (C), (E), (G) and (H)

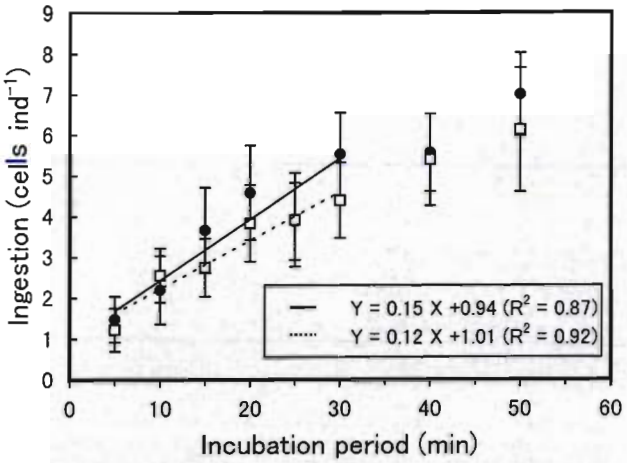


Fig. 2. Ingestion by *Favella taraikaensis* of CMFDA-labeled (●) and non-labeled (□) *Heterocapsa circularisquama* as a function of incubation period. The solid (CMFDA-labeled prey) and dotted lines (non-labeled prey) indicate linear regressions fitted to the data between 5 and 30 min of incubation. Vertical lines indicate the standard errors of the mean (n = 23 to 27)

cells should be observed under both excitations. Ingestion rates of each ciliate species feeding on *H. circularisquama* was calculated from the increase in the average number of ingested cells between 10 and 30 min of incubation. In the control bottle, there were no green particles as large as *H. circularisquama* cells within ciliates under the IB-light excitation. Hence, the ingestion rates of the field ciliate assemblage were calculated based only on the results from the experimental bottle.

**Results and discussion.** Fig. 1A to C shows photographs of *Heterocapsa circularisquama* stained with CMFDA. Green fluorescence (Fig. 1C) was not as strong as red auto-fluorescence (Fig. 1B) but it was possible to completely eliminate auto-fluorescence under the IB-light excitation (Fig. 1C).

The percentage of CMFDA-labeled cells to the total cell number (310 cells) reached 98%, suggesting that almost all cells of CMFDA-labeled *Heterocapsa circularisquama* can be effective for use as a tracer. Fixed *H. circularisquama* cells were not stained with CMFDA. This indicates that the algae in food vacuoles of protists fixed with the buffered formaldehyde are not newly stained with the CMFDA during storage periods.

The average number of CMFDA-labeled *Heterocapsa circularisquama* ingested by *Favella taraikaensis* increased linearly with increasing periods of incubation during the first 20 to 30 min (Fig. 2). This suggests that it is possible to estimate ingestion rates of field ciliates from the increase of ingested algae among more than 2 time points for the first 20 to 30 min of incubation. Similar results are reported in the feeding behavior of ciliates and other protists (e.g. McManus & Fuhrman 1988). Furthermore, ingestion by ciliates of the CMFDA-labeled *H. circularisquama* was not significantly different from ingestion of the non-labeled *H. circularisquama* at each time point, indicating that staining the alga with CMFDA does not influence the feeding activities of ciliates.

In an *in situ* feeding experiment in summer, 5 species of tintinnids and 1 species of aloricate ciliate ingested the CMFDA-labeled *Heterocapsa circularisquama*. Significant ingestion rates could be calculated for *Amphorellopsis acuta* (1.08 cells ind.$^{-1}$ h$^{-1}$) and *Laboea strobila* (1.83 cells ind.$^{-1}$ h$^{-1}$) (Table 1). Information on the feeding response of ciliates to *H. circularisquama* is limited to that of *Favella taraikaensis* and *F. azorica* (Kamiyama 1997). The result of the present study indicates that other species can also feed on *H. circularisquama* at low densities of this alga. The CMFDA-labeled *H. circularisquama* was readily observed in the food vacuoles of not only heterotrophic ciliates but also mixotrophic ciliates (Fig. 1D to H). However, except for 1 species, it was not possible to identify the species of aloricate ciliates. Hence, it was difficult to estimate the grazing impact of ciliate assemblages in this experiment. The grazing impact of the field ciliate assemblage on *H. circularisquama* and its evaluation will be addressed in another paper.

Table 1. Ingestion rates (mean ± SE) of field ciliates on CMFDA-labeled *Heterocapsa circularisquama*. nd = not determined; OD = oral diameter; ns = not significant; *$p < 0.05$

| Ciliate | 10 min of incubation | | 30 min of incubation | | Ingestion rate |
|---|---|---|---|---|---|
| | Abundance (ind. 300 ml$^{-1}$) | Ingestion (cells ind.$^{-1}$) | Abundance (ind. 300 ml$^{-1}$) | Ingestion (cells ind.$^{-1}$) | (cells ind.$^{-1}$ h$^{-1}$) |
| Tintinnids | | | | | |
| *Amphorellopsis acuta* | 253 | 0.15 ± 0.39 | 284 | 0.51 ± 0.76 | 1.08 ± 0.16* |
| *Eutintinnus lususundae* | 10 | 1.70 ± 0.48 | 6 | 2.50 ± 1.22 | 2.40 ± 1.28 ns |
| *Favella ehrenbergii* | 2 | 3.50 ± 2.12 | 0 | nd | nd |
| *Tintinnopsis cylindria* | 26 | 0.23 ± 0.43 | 22 | 0.36 ± 0.66 | 0.40 ± 0.47 ns |
| *Tintinnopsis corniger* | 10 | 0.10 ± 0.32 | 13 | 0.15 ± 0.38 | 0.16 ± 0.44 ns |
| Aloricate ciliates (OD > 30 µm) | | | | | |
| *Laboea strobila* | 35 | 0.49 ± 0.56 | 21 | 1.10 ± 1.09 | 1.83 ± 0.66* |

A characteristic of microscopical observation with the CMFDA technique is illustrated in the present study. Green fluorescent particles observed under IB-light excitation were probably more specific to CMFDA-labeled prey than that observed under the excitation with the Zeiss filter set 487709 (BP450–490 excitation filter, FT510 dichroic beam splitter and LP520 barrier filter) in Li et al. (1996). These authors pointed out the limitations of the CMFDA technique, one of which is the need for a control because there is some green auto-fluorescence observed within the protists in the addition of only CMFDA solution. Although the control treatment in the present study was different from that in Li et al. (1996), which was made from a filtrate of the algal cultures, the results demonstrate that it is not necessary to conduct a control of only CMFDA solution if IB-light excitation can be used. Furthermore, under this excitation it is possible to completely eliminate the effect of auto-fluorescence from chlorophyll *a* within the mixotrophic ciliates. Although green fluorescence due to CMFDA under this excitation is not very strong, the combination of the 2 filter sets used in the present study can be effective in readily and accurately counting particles in the food vacuoles of heterotrophic and mixotrophic ciliates, and thus enables us to estimate the grazing impact of field ciliate assemblages on stainable prey.

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