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Easy Visualization of the Protist *Oxyrrhis marina* Grazing on a Live Fluorescently Labelled Heterotrophic Nanoflagellate

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Abstract Planktonic heterotrophic flagellates are ubiquitous eukaryotic microorganisms that play a crucial role in carbon and nutrient fluxes through pelagic food webs. Here we illustrate for the first time a grazing model of planktonic dinoflagellate, *Oxyrrhis marina*, on the heterotrophic nanoflagellate *Goniomonas amphinema*, using the DNA-binding fluorescent dye Hoechst 33342. A solution of 1 μg/mL of the fluorochrome allowed viability of the prey for at least 48 hours, provided low fluorescence quenching, and labelled the flagellate without masking the cytoplasm. After 2 hours of contact between the fluorescent prey and the predator, *O. marina* population had preyed on live *G. amphinema* at an ingestion rate of 2.2 prey *Oxyrrhis*$^{-1}$ h^{-1}. Results show that this model is a time-effective and inexpensive approach for the direct observation of heterotrophic flagellate grazing. The fact that prey remain alive while predation occurs, as well as the low rate of quenching, could be of help in studying the fate of real-time trophic interactions between protists in microbial webs.

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Introduction

Heterotrophic protists play a major ecologic role in marine planktonic microbial communities because they channel a large proportion of primary carbon production to larger organisms [1]. Flagellates are the dominant component of the heterotrophic nanoplankton (2- to 20-μm size range) in terms of diversity, abundance, and biomass [2, 10, 25]. Therefore, understanding the trophic interactions between flagellates and other protists is a key question in marine ecosystem function. It has been suggested that an annual production of approximately 4,000 million tons of heterotrophic nanoflagellates (HNFs) is fed into the food chain of surface layers of the oceans and are therefore potentially available for grazing by larger protists and invertebrates [32]. In addition to introducing several other concepts, the "microbial loop" model [1] also presented the notion of top-down regulation on small heterotrophic flagellates by larger protists. Since then, ciliates and small metazoa have been shown to feed on HNFs as well as on phytoplankton prey [5, 6, 21, 29, 31, 33, 36, 37]. However, the potential grazing impact of heterotrophic dinoflagellates on HNFs has received much less attention than other facets of protist ecology.

The dinoflagellate *Oxyrrhis marina* is a widely distributed phagotrophic protist in coastal environments and common model of flagellate responses in microbial food webs, and it has been extensively reported to feed on phytoplanktonic species [7, 8,11–14, 16, 18, 24, 28, 35]. However, only two studies [19, 29] have pointed out the prospective role of heterotrophic dinoflagellates as predators of HNFs. The goal of this study was to present a protocol for the live-staining heterotrophic protists, based on the fluorochrome Hoechst 33342, showing the first evidence of the grazing by *O. marina* on *Goniomonas amphinema*, a widespread heterotrophic nanoplankton

flagellate. Hoechst 33342 is a valuable live-dye of HNFs for the following reasons:

1. It may simultaneously allow flow cytometry and fluorescence microscope analysis. Hoechst 33342 has been found to be better suited for flow cytometry than other ultraviolet light–UV dependent dyes, as diamidino phenyl indole (DAPI) because it has a higher relative fluorescence quantum yield [26].
2. It has the advantage over 488 nm excitable DNA-staining dyes (YOYO-1, YO-PRO-1, and PicoGreen), which are only to be used with aldehyde-fixed cells and must be supplemented with cofactors because they are sensitive to ionic strength [23].
3. It does not label cytoplasm significantly, thus having an advantage over other viable non–DNA-targeting dyes, which often stain the cytoplasm, thus making it difficult to distinguish the prey inside the predator.
4. It is a bright, rapid-staining, and inexpensive fluorochrome. The potential of the staining method for easily, quickly, and accurately estimating real-time ingestion of heterotrophic protists should be noted.

In addition, the significance of prey size (cell volume) selection in *O. marina* grazing is evaluated using standardised published data for both phototrophic and heterotrophic prey.

Materials and Methods

Protist Species

We tested the following cultures for the experiments: *O. marina* (CCAP 1133/4; size approximately 25 μm) and *G. amphinema* (from own culture collection; isolated from a marine planktonic sample, Wales, UK, 53.23°N, 4.164°W; size approximately 5 to 6 μm), *Metanophrys* sp. (from our own culture collection; isolated from a surface marine sample, Brighton, UK, (50.8°N, 0.1 W; size approximately 40 μm), and *Chilomonas paramecium* (CCAP 977/2a; size approximately 20 μm). All of the cultures were maintained in Plymouth Erdschreiber medium except *Chilomonas*, which was in *Chilomonas* medium (CHM) (refer to http://www.ccap.ac.uk/media/recipes.htm for recipe media). Reinoculations in fresh medium were carried out every 3 weeks. Cultures were maintained in laboratory incubators at 18°C ± 2°C in darkness.

Selection of the Fluorochrome

Three fluorescent dyes were initially tested (Fluorescein isothiocyanate [FITC], dichlorotriazinyl amino fluorescein [DTAF], and Hoechst 33342; Sigma, Saint Louis, MO). Hoechst 33342 was selected as it allowed viability and motility of the flagellates while providing stable and durable fluorescence (for 48 hours). Hoechst 33342 is a membrane-permeable fluorochrome that is specific for AT-rich regions of double-stranded DNA. The dye is excited by UV light (350 nm) and emits blue/cyan fluorescence light (460 nm). Four concentrations of Hoechst 33342 were initially tested (final concentration of 250, 33, 5, and 1 μg/mL in phosphate-buffered saline [PBS 1%]), and 1μg/mL proved to be the optimal concentration for fluorescence stability and cell viability of both the prey and the predator. The effect of Hoechst 33342 on flagellate growth was studied for 48 hours by monitoring changes in the numbers of flagellates growing in Plymouth Erdschreiber medium in both the absence (control) and the presence of the fluorochrome. Aliquots (100 μL × three replicates) were taken at elapsed times, and flagellates were enumerated using a Sedgewick-Rafter counting cell.

Grazing Experiments

O. marina log-phase cultures were centrifuged (at 2,000 rpm for 10 minutes) and acclimatised to autoclaved 0.2-μm filtered seawater (FSW) for 3 days in 50-mm diameter Petri dishes. Late–log phase cultures of *G. amphinema* were concentrated by centrifugation and resuspended in FSW. Then Hoechst 33342 (a final concentration of 1 μg/mL was added to the cultures). After 30 minutes, Hoechst-stained *Goniomonas* (fluorescent live-labelled flagellates) were centrifuged, washed thrice in FSW, and then added (at approximately 2:1 ratio prey to predator) to Petri dishes (three dishes with 10 mL volume each) containing the predator. Dishes were accommodated on orbital rotators (RotoMix at 100 rpm) and incubated in darkness at room temperature (18°C ± 2°C). At elapsed times (0, 1, 2, 24, and 48 hours), three samples were taken and fixed with a mixture of Lugol's solution (final concentration 0.1%) and formaldehyde (final concentration 2%). Samples were then deposited onto polycarbonate membrane filters (pore size 2.0 μm; Cyclopore Track Etched Membrane, Whatman R) using filtration tower equipment. Filters were then mounted on a slide between two drops of low-fluorescence immersion oil (ZEISS Immersol TM 518 F) and observed under a Zeiss upright epifluorescence microscope equipped with Axioplan 2ie filter sets for UV light. At least 30 predator cells were analysed per sample at 630 × magnification. Ingestion rate was calculated for the first 2 hours according to the following formula:

$$\text{IR (prey } \textit{Oxyrrhis}^{-1}\text{ h}^{-1}\text{)} = \text{N/t},$$

where N is the number of ingested *Goniomonas* per *Oxyrrhis* cell after a given time (t).

Prey Cell Volume Versus Ingestion Rate

To determine the relation between prey cell volume and ingestion rate by *O. marina*, we compiled a database from previously published works. Cell volume (μm^3) was calculated from equivalent spherical diameter (ESD) using the following formula [27]:

ESD = (cell volume/0.523)0.33

When ESD was not available, cell volume was calculated from provided cell measurements by assuming the geometric form of a rotational ellipsoid [29]. Ingestion rates were standardised to prey *Oxyrrhis*$^{-1}$ h^{-1}.

Results and Discussion

The growth dynamics of *O. marina* and *G. amphinema* stained with Hoechst 33342 versus that of the flagellates in the absence of the fluorochrome is shown in Fig. 1. Growth was not significantly different ($P > 0.05$) for both treatments, indicating that the fluorochrome did not affect viability of the flagellates at the concentration tested.

The grazing *O. marina* on *G. amphinema* is illustrated in Fig. 2. Ingested *Goniomonas* were clearly detected by its nucleus. The absence of cytoplasmatic staining in the predator facilitated visualization of the prey cell silhouette (Fig. 2b). Moreover, prey and predator were also easily found on the filters by phase-contrast light microscope, so cell identity was corroborated without difficulty. It must however be indicated that irrefutable demonstration of prey digestion can only be achieved by electron microscopy or other techniques that allow visualization of the interior of the cell (e.g., confocal microscopy). Despite the thorough washing of prey cells before coculture, the predator nucleus was also usually stained because of the high permeability of Hoechst 33342 (Fig. 2a–d). However, this fact did not seem to interfere with visualization of the ingested prey or with grazing because *Oxyrrhis* cells remained alive for the duration of the experiment (Fig. 1a). A maximum of three *Goniomonas* cells were detected within each *Oxyrrhis* cell (Fig. 2b). After 48 hours, Hoechst 33342–labelled *G. amphinema* cells showed well-preserved cell morphology and fluorescence that was still clearly visible (nuclear staining) (Fig. 2e). *Goniomonas* was observed feeding on background bacteria remaining in the medium (Fig. 2f and g). After 2 hours of coincubation, 30% of *Oxyrrhis* population had preyed on *Goniomonas*, and we obtained an ingestion rate of 2.2 (±0.87) prey *Oxyrrhis*$^{-1}$ h^{-1}.

The effect of prey cell volume on *O. marina* ingestion rates was calculated by plotting our data and previously published data of *Oxyrrhis* grazing on phototrophic and heterotrophic microbial prey (Fig. 3). Results indicated that

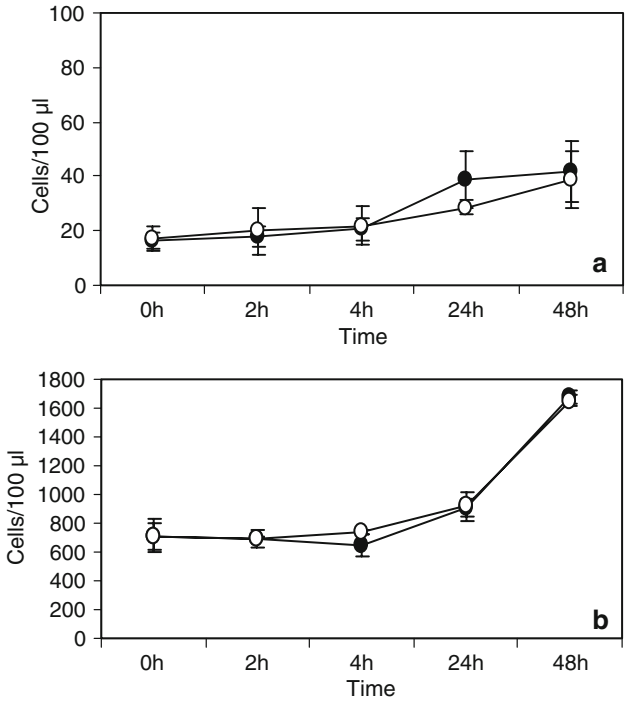

Fig. 1 Growth dynamics of *O. marina* (**a**) and *G. amphinema* (**b**) in the absence (open circles) and in the presence (closed circles) of Hoechst 33342 (1 µg/mL during the 48-hour experimental period). Data points represent average values, error bars ± SD (n = 3)

ingestion rates were moderately prey size dependent at the smallest prey cell volumes (regression coefficient, $R^2 = 0.43$, significant at $P < 0.05$). However, a large increase in prey cell volume strongly correlated with decreased ingestion rates ($R^2 = 0.98$, significant at $P < 0.05$). These results suggest that size-selective grazing of *O. marina* [14, 16] is mainly relevant for large biovolume prey. Although other factors (e.g., abundances of predator and prey, chemical properties of prey surface) should also be important for grazing parameters, it is clear that size-related preference is a chief factor for food selection in *O. marina*.

The use of live prey is essential in grazing experiments with microorganisms. Some studies show that significant differences among dead fluorescent bacteria (FLB) and living unstained bacteria exist, concluding that the number of FLB in food vacuoles may underestimate real ingestion [3]. Although it has also been reported that under food-limiting conditions, nanoflagellates display a modest preference for inert beads versus live-stained bacteria, this shifts to a strong discrimination against the beads after adding a satiating concentration of live bacteria [20]. In protist research, the use of living cells may be even more important because the fragility of their membranes and lack of rigid walls make dead organisms unsuitable as prey. Some species of protists discriminate against inert particles, including heat-killed cells [34, 35]. Live fluorescently labelled prey

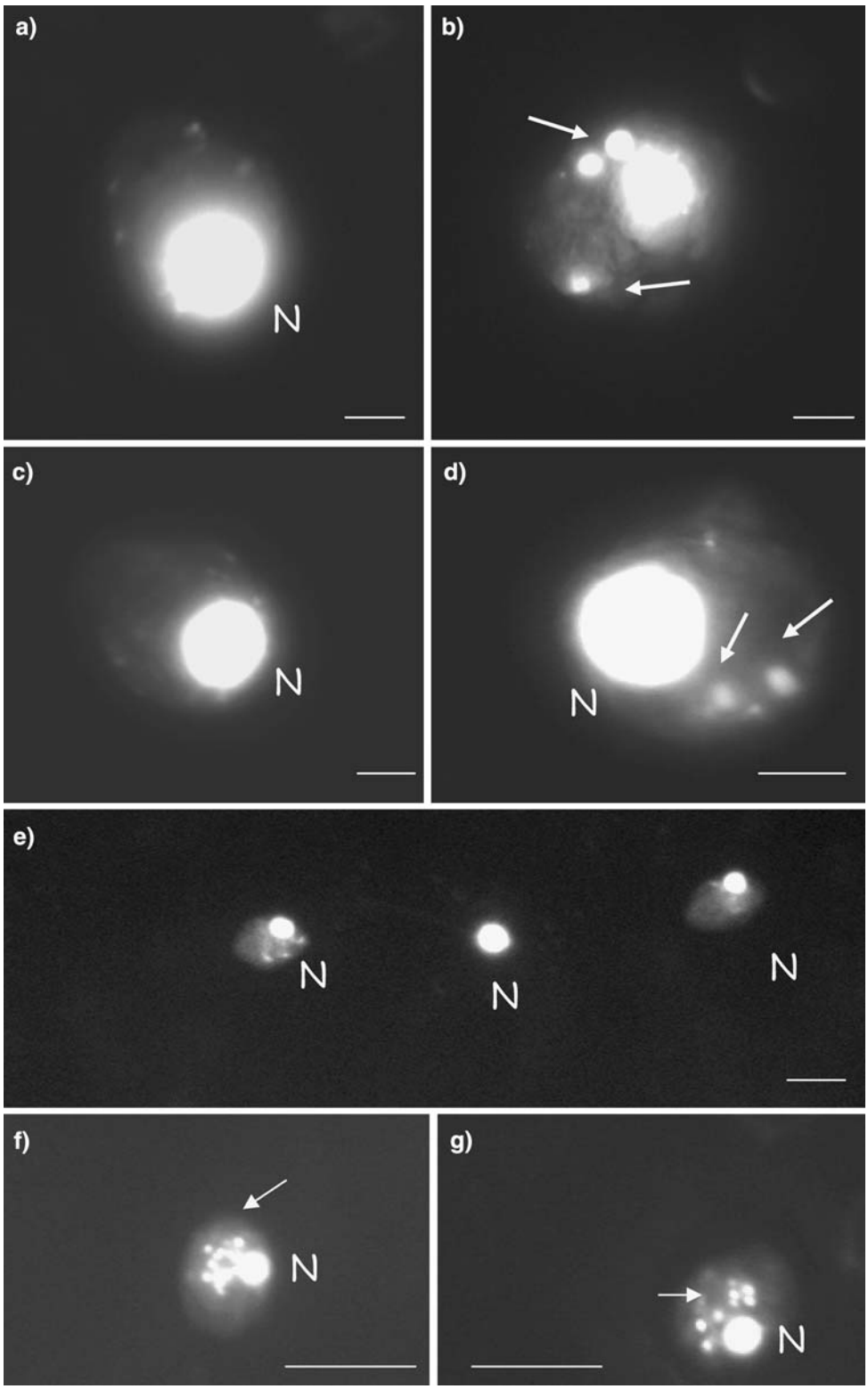

Fig. 2 Grazing of the phagotrophic dinoflagellate *O. marina* on Hoechst 33342–stained *G. amphinema*. (**a**) *Oxyrrhis* cell with no prey inside at t = 1 hour. (**b**) *Oxyrrhis* cell with prey inside (arrows) at t = 1 hour. (**c**) *Oxyrrhis* cell with no prey inside at t = 2 hours. (**d**) *Oxyrrhis* cell with prey inside (arrows) at t = 2 hours. (**e**) Hoechst 33342–stained *G. amphinema* cells after 48 hours of incubation in darkness. (**f** and **g**) Magnified *Goniomonas* cells clearly showing bacteria (arrows) in the flagellate cytoplasm (t = 48h). Bars = 5 μm. N, nucleus

offer an alternative when attempting to detect feeding. Our method allows fast and easy visualization of the heterotrophic nanoflagellate *G. amphinema* inside its predator (*O. marina*), which can facilitate the estimation of grazing rates. *O. marina* is a heterotrophic (omnivorous) prey-engulfing dinoflagellate. Diatoms, green algae, haptophytes, and cryptophytes are their major food, and only two studies [19, 29] have hitherto provided evidence of *O. marina*

feeding on HNFs. Our study provides a new record of *Oxyrrhis* predation on HNFs and supports the belief that the prey repertoire for *O. marina* is highly versatile [14]. The recognition of a top-down control of HNFs by heterotrophic dinoflagellates may result in lower predation pressure on planktonic bacteria because these are the main prey for HNFs [9, 30]. This could have important consequences for the structure and functioning of the microbial food web in pelagic ecosystems. Larger flagellates (*C. paramecium*) and ciliates (*Metanophrys* sp.) were also tested as long-term live fluorescently labelled protists. Our results showed that it was possible to live-label both organisms with the fluorochrome and that both remained stable, when labeled, for at least a 48-hour period (Fig. 4).

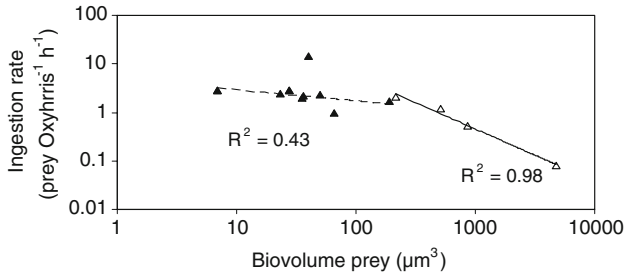

Fig. 3 Relation between prey cell volume and ingestion rates for *O. marina* [4, 7, 13, 14, 15, 17, 18, 19, 29, 35, and the current study]. The regression coefficient $R^2 = 0.43$ excludes the outlying point of the diatom *Phaeodactylum tricornuotum* [13]. Values are represented in logarithmic scale

Other investigators have previously used staining methods for monitoring grazing on HNFs. Cleven's method [6] involved indirect estimation of grazing by ciliates on HNFs using flagellates previously fed with FLB; fluorochromes (DAPI and DTAF) have also been used for direct labelling of potential flagellate prey, but no visual illustration was provided [29]. Some investigators [22] used a vital fluorescent stain chloromethyl fluorescein diacetate (CMFDA) to investigate feeding by photosynthetic dinoflagellates, but the green fluorescence of the CMFDA might be confused with the green autofluorescence of some unstained protists used as prey and has also been reported to fade quickly in presence of light [22]. The use of fluorescent macromolecules to label food vacuoles of the flagellates has also been approached [5], but success of this method could be dependent on the physiologic state of the cell.

The main characteristic of our approach is that it is straightforward and overcomes the problems associated with dead prey. The rate of fluorescence quenching is very low compared with other fluorochromes, which should make it easier to observe the fate of live-stained flagellates in field-based experiments. The fact that the cytoplasmic content of the prey can also be observed is interesting because this allows the evaluation of food channelling by HNFs (mostly of bacteria and other minute size prey) to upper trophic–level organisms. Therefore, a broad view of both top-down and bottom-up control by HNFs could be attained.

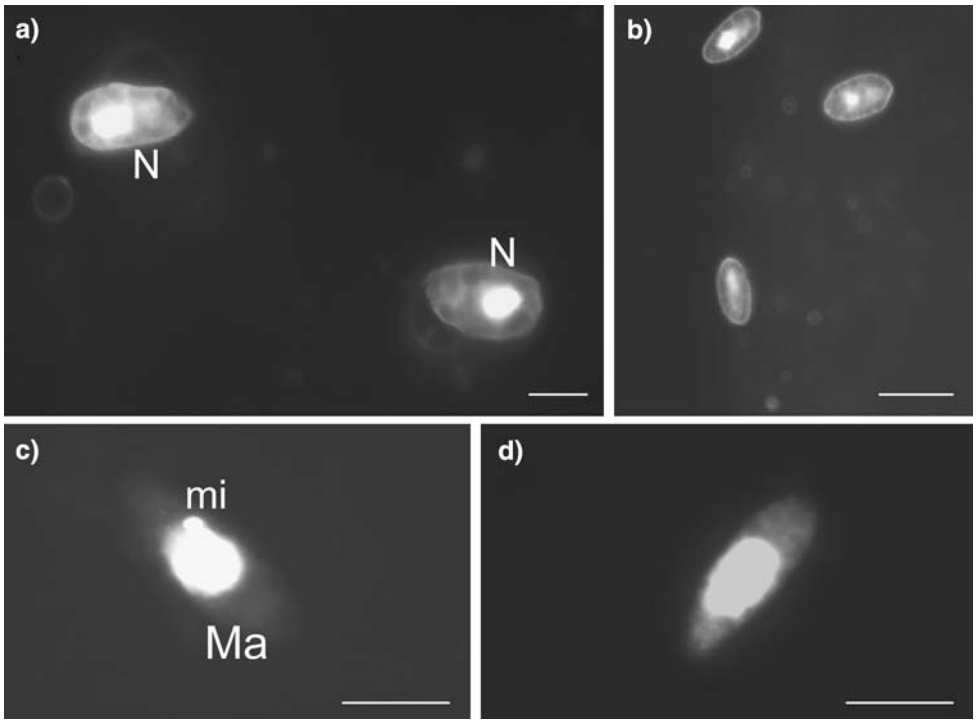

Fig. 4 Hoechst 33342–stained protists. *C. paramecium* cells at the start of the experiment (**a**) and after 48 hours of incubation in darkness (**b**). *Metanophrys* sp. cells at the start of the experiment (**c**) and after 48 hours of incubation in darkness (**d**). Bars = 20 µm in all except (a), in which bar = 10 µm. Ma: macronucleus; mi: micronucleus; N: nucleus

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References

1. Azam F, Fenchel T, Field JG, et al. (1983) The ecological role of water-column microbes in the sea. Mar Ecol Prog Ser 10:257–263
2. Berninger UG, Caron DA, Sanders RW, et al. (1991) Heterotrophic flagellates of planktonic communities, their characteristics and methods of study. In: Patterson DJ, Larsen J (eds) The biology of free-living heterotrophic flagellates. Systematics Association Special Volume No. 45. Clarendon, Oxford, UK, pp 35–56
3. Boenigk J, Arndt H, Cleven EJ (2001) The problematic nature of fluorescently labelled bacteria (FLB) in *Spumella* feeding experiments—An explanation by using video microscopy. Arch Hydrobiol 152:329–338
4. Carvalho WF, Graneli E (2006) Acidotropic probes and flow cytometry: a powerful combination for detecting phagotrophy in mixotrophic and heterotrophic protists. Aquat Microbiol Ecol 44:85–96
5. Christoffersen K, Gonzalez JM (2003) An approach to measure ciliate grazing on living heterotrophic nanoflagellates. Hydrobiologia 491:159–166
6. Cleven E (1996) Indirectly fluorescently labelled flagellates (IFLF): a tool to estimate the predation on free-living heterotrophic flagellates. J Plankton Res 18:429–442
7. Davidson K, Cunningham A, Flynn KJ (1995) A first attempt to model factors affecting the ingestion of prey by the dinoflagellate *Oxyrrhis marina*. Cytology 37:969–977
8. Davidson K, Roberts EC, Wilson AM, et al. (2005) The role of prey nutritional status in governing protozoan nitrogen regeneration efficiency. Protist 156:45–62
9. Fenchel T (1982) Ecology of heterotrophic microflagellates. IV. Quantitative occurrence and importance as bacterial consumers. Mar Ecol Prog Ser 9:35–42
10. Fenchel T (1987) Ecology of protozoa: the biology of free-living phagotrophic protists. Science Tech/Springer, Berlin, Germany
11. Flynn KJ, Davidson K (1993) Predator–prey interactions between *Isochrysis galbana* and *Oxyrrhis marina*. I. Changes in particulate delta 13C. J Plankton Res 15:455–463
12. Flynn KJ, Davidson K, Cunningham A (1996) Prey selection and rejection by a microflagellate: implications for the study and operation of microbial food webs. J Exp Mar Biol Ecol 196:357–372
13. Goldman JC, Dennet MR, Gordin H (1989) Dynamics of herbivorous grazing by the heterotrophic dinoflagellate *Oxyrrhis marina*. J Plankton Res 11:391–407
14. Hammer A, Gruttner C, Schumann R (1999) The effect of electrostatic charge of food particles on capture efficiency by *Oxyrrhis marina* Dujardin (dinoflagellate). Protist 150:375–382
15. Hammer A, Gruttner C, Schumann R (2001) New biocompatible tracer particles: use for estimation of microzooplankton grazing, digestion, and growth rates. Aquat Microb Ecol 24:153–161
16. Hansen FC, Witte HJ, Passarge J (1996) Grazing in the heterotrophic dinoflagellate *Oxyrrhis marina*: size selectivity and preference for calcified *Emiliana huxleyi* cells. Aquat Microb Ecol 10:307–313
17. Jeong HJ, Kang HJ, Shim JS, et al. (2001) Interactions among the toxic dinoflagellate *Amphidinium carterae*, the heterotrophic dinoflagellate *Oxyrrhis marina*, and the calanoid copepods *Acartia* spp. Mar Ecol Prog Ser 218:77–86
18. Jeong HJ, Kim JS, Yoo YD, et al. (2003) Feeding by the heterotrophic dinoflagellate *Oxyrrhis marina* on the red-tide raphidophyte *Heterosigma akashiwo*: a potential biological method to control red tides using mass-cultured grazers. J Eukaryot Microbiol 50:274–282
19. Jeong HJ, Song JE, Kang NS, et al. (2007) Feeding by heterotrophic dinoflagellates on the common marine heterotrophic nanoflagellate *Cafeteria* sp. Mar Ecol Prog Ser 333:151–160
20. Jürgens K, DeMott WR (1995) Behavioural flexibility in prey selection by bacterivorous nanoflagellates. Limnol Oceanogr 40:1503–1507
21. Jurgens K, Wickham SA, Rothhaupt KO, et al. (1996) Feeding rates of macro- and microzooplankton on heterotrophic nanoflagellates. Limnol Oceanogr 41:1833–1839
22. Li A, Stoecker DK, Coats DW, et al. (1996) Ingestion of fluorescently labelled and phycoerythrin-containing prey by mixotrophic dinoflagellates. Aquat Microbiol Ecol 10:139–147
23. Marie D, Vaulot D, Partensky F (1996) Application of the novel nucleic acid dyes YOYO-1, YO-PRO-1, and PicoGreen for flow cytometric analysis of marine prokaryotes. Appl Environ Microbiol 62:1649–1655
24. Martel CM (2006) Prey location, recognition and ingestion by the phagotrophic marine dinoflagellate *Oxyrrhis marina*. J Exp Mar Biol Ecol 335:210–220
25. Mills DK, Tett P, Novarino G (1994) The spring bloom in the South Western North-Sea in 1989. Netherlands J Sea Res 33:65–80
26. Monger BC, Landry MR (1993) Flow cytometric analysis of marine bacteria with Hoechst 33342. Appl Environ Microbiol 59:905–911
27. Naustvoll LJ (2000) Prey size spectra and food preferences in thecate heterotrophic dinoflagellates. Phycologia 39:187–198
28. Opik H, Flynn KJ (1989) The digestive process of the dinoflagellate *Oxyrrhis marina* Dujardin, feeding on the chlorophyte, *Dunaliella primolecta* Butcher: a combined study of ultrastructure and free amino acids. New Phytol 113:143–151
29. Premke K, Arndt H (2000) Predation on heterotrophic flagellates by protists: food selectivity determined using a live-staining technique. Arch Hydrobiol 150:17–28
30. Sanders RW, Caron DA, Berninger UG (1992) Relationships between bacteria and heterotrophic nanoplankton in marine and fresh water- an inter-ecosystem comparison. Mar Ecol Prog Ser 86:1–14
31. Sanders RW, Leeper DA, King CH, et al. (1994) Grazing by rotifers and crustacean zooplankton on nanoplanktonic protists. Hydrobiologia 288:167–181
32. Sleigh MA, Zubkov MV (1998) Methods of estimating bacterivory in protozoa. Eur J Protistol 34:273–280
33. Šolic M, Krstulovic N (1994) Role of predation in controlling bacterial and heterotrophic nanoflagellate standing stocks in the coastal Adriatic Sea: seasonal patterns. Mar Ecol Prog Ser 114:219–235
34. Stoecker D (1988) Are marine planktonic ciliates suspension feeders? J Protozool 35:252–255
35. Tillmann U, Reckermann M (2002) Dinoflagellate grazing on the raphidophyte *Fibrocapsa japonica*. Aquat Microb Ecol 26:247–257
36. Turner JT, Tester PA, Ferguson RL (1988) The marine cladoceran *Penilia avirostris* and the "microbial loop" of pelagic food webs. Limnol Oceanogr 33:245–255
37. Verity PG (1991) Measurement and simulation of prey uptake by marine planktonic ciliates fed plastidic and aplastidic nanoplankton. Limnol Oceanogr 36:729–750