Use of live, fluorescently-labeled algae for measuring microzooplankton grazing in natural communities

Rodrigo A. Martínez *, Stamatina Isari, Albert Calbet

Institut de Ciències del Mar (CSIC), Ps. Marítim de la Barceloneta 37-49, 08003 Barcelona, Spain

ARTICLE INFO

Article history:
Received 31 May 2013
Received in revised form 7 March 2014
Accepted 10 March 2014
Available online xxxx

Keywords:
IFIA
Vital stain
Fluorochrome
CellTracker
LysoSensor
Dilution experiments

ABSTRACT

Here, our goal was to develop a technique for staining live algae with vital fluorochromes and to further test whether this method may serve as a tool for examining the trophic roles and functional diversity of microzooplankton. We tested 4 fluorochromes on a total of 10 phytoplankton species, out of which only 3 proved effective, and only on some of the species tested. The fluorochrome Vybrant did not dye any algal species, CellTracker Blue successfully dyed 2 nanoflagellate species (Isochrysis galbana and Tetraselmis sp.) and one dinoflagellate (Heterocapsa sp.), and LysoSensor and LysoTracker each dyed 2 diatom species (Thalasiosira weissflogii and Skeletonema costatum). Further experiments with the 2 most successful fluorochromes (CellTracker and LysoSensor) indicated that optimum incubation times ranged from 4 to 8 h and that the percentage of stained cells was not improved at concentrations higher than 10 μM and 2 μM for CellTracker and LysoSensor, respectively. The residence times of the fluorochromes under natural light conditions were greater than 24 h (60–80% of stained algae). Labeling algae with CellTracker had no significant effect on their growth rate or C:N molar ratio. LysoSensor, however, had minor (although significant) effects on the growth rates of stained vs. unstained algae. Bottle grazing experiments showed that Oxyrrhis marina grazed on unstained nanoflagellate species at equal rates to those stained with CellTracker; however, a positive discrimination for stained cells was detected when Gyrodinium dominans was used as the grazer. We also measured microzooplankton ingestion rates in natural algal communities by combining the dilution method with the addition of live algae into a natural plankton suspension. The addition of stained algae did not significantly affect phytoplankton growth or mortality rates due to microzooplankton grazing. The low toxicity of fluorochromes and the easy visualization of labeled algae inside predators make this method a useful tool for estimating grazing rates of microzooplankton and for the quantification of different trophic interactions among protists in the microbial food web. However, given the limited number of algae species successfully stained further research is needed to obtain more universal dyes.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Microzooplankton are key components of marine food webs (Calbet and Landry, 2004; Calbet, 2008; Sherr and Sherr, 2002). Due to their high consumption rates and impacts on primary producers, these organisms may exert significant control over the size–structure of phytoplankton and the flow of matter and energy in the planktonic food web (Calbet and Landry, 2004; Schmoker et al., 2013; Vargas and González, 2004). Most knowledge of these effects has been obtained by the use of the grazing dilution technique first introduced by Landry and Hassett (1982). Among the main advantages of the technique is that it provides an estimate of the grazing impact of the entire microzooplankton community on primary producers with relatively little manipulation. However, this method is based on a black-box approach where the quantitative importance of distinct components is lost. There is a need, therefore, to approximate the function of this group in more detail (Calbet, 2008; Calbet et al., 2008).

Several methods have been suggested for investigating the foraging strategies and trophic interactions in planktonic protozoans. For instance, vacuolar content and digestion rate have been studied for specific ciliate grazers (Dolan and Coats, 1991), whereas size-fractionated incubations, which allow the segregation of grazers and prey, have been used for natural microzooplankton suspensions (e.g., Calbet, 2008; Calbet et al., 2001; Capriulo and Carpenter, 1980; Verity, 1986). Perhaps the most popular method, particularly when working under laboratory conditions with cultured organisms, has been the use of tracer particles (Archer et al., 1996; Mccmanus and Okubo, 1991; Rublee and Gallegos, 1989; Sherr et al., 1987). Various tracers have been used, including natural or artificial particles that may be radiolabeled, stained or auto-fluorescent. Tracers are incorporated into a targeted particle or cultured cell, allowing for the subsequent analysis of the vacuolar contents of the grazer. The tracing technique has been used by Sherr et al.
(1987), among others, for estimating protozoan grazing rates on dead fluorescently labeled bacteria (FLB) and was further adjusted by Rublee and Gallegos (1989) to estimate microzooplankton ingestion using heat-killed fluorescently labeled algae (FLA). However, the use of these substitutes for measuring ingestion rates may be inappropriate for some types of protists because some species may either discriminate against inert particles (Landry, 1994; Stoeker, 1988; Verity, 1991), or show atypical ingestion rates compared to the consumption of live prey (Li et al., 1996; Nygaard and Hessen, 1990; Putt, 1991). The development of new stains incorporating organic molecules allows us to label cells without loss of metabolic activity or viability (Fuller et al., 2000); this technique provides new possibilities for use as an alternative to the methods mentioned above.

In this study, we expand the use of tracers for estimating microzooplankton feeding using live, fluorescently labeled algae (FLA), which should provide a better approach for future research. FLA have been previously used to estimate the ingestion rates of copepods (Teegarden, 1999), fish larvae (Lessard et al., 1996), and protists in culture conditions (Arndt and Premke, 2000; Martín-Cereceda et al., 2008; Putt, 1991). However, to date there has been no comprehensive study assessing the effects of fluorochromes on protozoan grazers and prey, the range of applicability of this method, or optimization of a protocol for use in estimating rates in the natural environment. Our specific aim was to test different vital dyes with several species of algae, to determine different parameters (i.e., incubation time, optimal work concentration and bleaching time), and to identify the effects of dye use on both grazers and prey, thus providing a further protocol for use in future grazing experiments in the field.

2. Materials and methods

2.1. Cultures

We used 10 species of planktonic algae from several taxonomic groups to investigate the suitability of 4 fluorochromes for staining live cells (Table 1). The algae were cultured in f/2 medium (Guillard, 1975) with the addition of silicates in the case of diatoms. Cultures were maintained in 1 L sterilized glass bottles at 18 ± 1 °C under a 12:12 h light:dark cycle (100 μmol m⁻² s⁻¹), with cool white fluorescent lights. Re-inoculation of the cultures in fresh medium approximately every 2 days assured an exponential growth rate.

We used the heterotrophic dinoflagellate Oxyrhis marina (equivalent spherical diameter (ESD): 17–18 μm) and Gyrodinium dominans (ESD range: 16–20 μm) as grazers. The cultures were kept in autoclaved 0.2 μm filtered seawater (FSW) that contained trace amounts of EDTA and trace-metals (0.003 mM L⁻¹ of f/2 metal stock solution; Guillard, 1975), under the same environmental conditions described above and fed daily with the cryptophyte Rhodomonas salina. Prior to each experiment, the dinoflagellates were not fed for ~4–5 days, allowing them to graze down all R. salina cells.

2.2. Fluorochrome selection

We tested 4 fluorescent dyes, primarily characterized by their low toxicity and by the persistence of the labeling over long periods of time. The fluorochromes tested are listed in Table 1, and the specific function and range of emission of each fluorochrome are presented in Table 2. The fluorochromes Vybrant and CellTracker were tested in all algal species, whereas the other 2 were specific for staining silica frustules (LysoSensor and LysoTracker) and were tested only in diatoms, following the method described by Descies et al. (2008).

We tested 3 concentrations of Vybrant, and CellTracker (5, 10 and 20 μM) to observe the viability (motility) and labeling of the algae. To obtain these concentrations, 15, 30 and 60 μL of standard fluorochrome solution (5 mM) were inoculated into separate 15 mL of algal culture. For the diatoms, we used 2 concentrations (1 μM and 2 μM) of the fluorochromes LysoSensor and LysoTracker (standard solution: 1 mM) and the same culture volume utilized previously. All algae were incubated at 18 ± 1 °C for a period of 24 h. The viability of flagellated algae was assessed using inverted microscopy and diatom viability was assessed using epifluorescence microscopy in 1 mL aliquots taken at 3 different time intervals (2, 6, and 24 h during the incubation period). Additionally, we took 2 mL samples 2 h after the start of incubation and at the end of the incubation time period. These samples were preserved in glutaraldehyde (final concentration 1%) and further filtered onto 2 μm pore-size black polycarbonate filters. To assess whether cells had

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Species</th>
<th>ESD (μm)</th>
<th>Vybrant</th>
<th>CellTracker</th>
<th>LysoSensor</th>
<th>LysoTracker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanobacteria</td>
<td>Synechococcus sp.</td>
<td>2.5</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Flagellate</td>
<td>Nanochloropsis oculata</td>
<td>2.9</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>Micromonas pusilla</td>
<td>1.4</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>Isochrysis galbana</td>
<td>4.3</td>
<td>−</td>
<td>+</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td></td>
<td>Rhodomonas salina</td>
<td>6.8</td>
<td>−</td>
<td>−</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td></td>
<td>Tetraselmis sp.</td>
<td>8.1</td>
<td>−</td>
<td>−</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td></td>
<td>Dunaliella salina</td>
<td>5.6</td>
<td>−</td>
<td>+</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>Dinoflagellate</td>
<td>Heterocapsa sp.</td>
<td>13.6</td>
<td>−</td>
<td>+</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>Diatom</td>
<td>Skeletonema costatum</td>
<td>5.1</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Thalassiosira weisflogii</td>
<td>14.0</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Molecular weight</th>
<th>Specific action</th>
<th>Absorption (nm)</th>
<th>Emission (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vybrant</td>
<td>881.72</td>
<td>DNA-selective quantitative stain</td>
<td>506</td>
<td>534</td>
</tr>
<tr>
<td>CellTracker</td>
<td>265.03</td>
<td>Vital cytoplasmic stain</td>
<td>353</td>
<td>466</td>
</tr>
<tr>
<td>LysoSensor</td>
<td>366.42</td>
<td>Vital stain for silica synthesis</td>
<td>329</td>
<td>440</td>
</tr>
<tr>
<td>LysoTracker</td>
<td>364.40</td>
<td>Vital stain for silica synthesis</td>
<td>465</td>
<td>535</td>
</tr>
</tbody>
</table>
been stained, the latter filters were visually “scanned” under an epi-fluorescence microscope using a UV or blue light filter, depending on the fluorochrome (Table 2).

2.3. Optimal fluorochrome concentrations, staining incubation time, and residence time of the stains

Optimal concentrations and staining incubation times were established for almost all the fluorochrome-algal combinations that provided good results in our preliminary observations (i.e., CellTracker × Isochrysis galbana, Tetraselmis sp.; LysoSensor and LysoTracker × Thalassiosira weissflogii, Skeletonema costatum; Table 1). For this purpose, algal cultures growing exponentially were stained at specific concentrations (nanoflagellates: 5, 10 and 20 μM; diatoms; 1 and 2 μM) and were further incubated in triplicate in 75 mL polyethylene culture flasks on a rotating plankton wheel (0.2 rpm) for 72 h at a constant temperature (18 ± 1 °C) on a 12:12 light:dark cycle (100 μmol m⁻² s⁻¹). Aliquots of 3 mL were taken at different time intervals (2, 4, 24, 48, and 72 h); 1 mL was used to assess cell viability under an inverted microscope, and 2 mL were used to estimate the percentage of labeled cells under an epi-fluorescence microscope. The percentage of labeled cells in all groups was estimated by filtering aliquots on black polycarbonate filters, which were dyed at the previously described concentrations and incubated for a period of 2 h at a temperature of 18 ± 1 °C. After this time, we concentrated the algae by centrifugation in triplicate in 15 mL tubes for a period of 72 h at a temperature of 18 ± 1 °C. We assessed the viability of the cells and the percentage of stained cells following the protocol previously described.

To estimate the residence time inside the cells, log-phase cultures of 4 algal species (I. galbana, Tetraselmis sp., T. weissflogii and S. costatum) were dyed at the previously described concentrations and incubated in triplicate in 15 mL tubes for a period of 2 h at a temperature of 18 ± 1 °C. After this time, we concentrated the algae by centrifugation for 5 min at s rpm. The supernatant was removed, and the cell pellet was re-suspended in FSW (0.2 μM) and incubated in triplicate 75 mL polyethylene culture flasks for a period of 72 h under natural light (300 μmol m⁻² s⁻¹) and temperature conditions (26 °C). We assessed the viability of the cells and the percentage of stained cells following the protocol previously described.

2.4. Effect of staining in the cellular C:N molar ratio of algae

We assessed the influence of staining on the cellular C:N elemental ratio of the algae for one of the fluorochromes used in our study (CellTracker). Algal monocultures grown exponentially in f/2 medium (I. galbana, Tetraselmis sp., Heterocapsa sp.) were incubated at a final concentration of 5 μM of stain for 4 h at 18 °C (Teegarden, 1999). To eliminate excess stain, suspensions of stained cells were centrifuged for 10 min at 1000 rpm, the supernatant was removed, and the cells were re-suspended in FSW and left overnight to recover from the treatment. Samples of the initial stock cultures with no fluorochrome addition were processed in a similar manner (centrifuging, re-suspension in FSW, overnight recovery) and served as controls. Stained and unstained aliquots (20 mL) of known cell concentration were filtered onto pre-combusted 25 mm diameter GF/F filters (450 °C, 6 h) in duplicate and were further dried for 24 h at 60 °C. Filters were kept in a desiccator until posteriorly processed in a LECO-932 CHNS analyzer.

2.5. Effects of the fluorochromes on cultured algal growth and protozoan grazing rates

We investigated the possible effects of the experimental stains on the growth dynamics of Tetraselmis sp., I. galbana, T. weissflogii, and S. costatum. CellTracker was added to Tetraselmis sp. and I. galbana cultures at a concentration of 10 μM, whereas the fluorochrome LysoSensor was added to T. weissflogii and S. costatum at a concentration of 2 μM. Unstained algae served as controls, and both stained and unstained algae were incubated in f/2 medium, in triplicate 250 mL polyethylene culture flasks on a rotating plankton wheel for a period of 24 h. The initial cell concentrations varied between 2500 and 3800 cells mL⁻¹ for Tetraselmis sp., T. weissflogii, and S. costatum and 84,000–89,000 cells mL⁻¹ for I. galbana. Changes in the variation of cell abundances were monitored at 5 time intervals (2, 4, 8, and 24 h) using a Multisizer III particle counter.

To estimate the possible effects of the algal staining on protozoan grazing rates, we conducted 2 experiments using 2 species of heterotrophic dinoflagellates, O. marina and G. dominans, as predators. The algae Tetraselmis sp. and I. galbana were used as target prey to study the effects of CellTracker (the most successful dye) on protozoan grazing rates. Prior to the experiment, the algae were incubated in 50 mL polypropylene tubes with the fluorochrome CellTracker at a concentration of 10 μM for 24 h. Algal and dinoflagellate stock cultures were mixed in 4 L polycarbonate bottles and diluted with FSW to obtain a homogeneous mixed suspension (prey + grazer) at a concentration ca. 900 and 10,000 cells mL⁻¹, respectively. We prepared 2 types of mixed suspensions for each grazer: one with stained prey and one with unstained. Additionally, 2 single prey suspensions (10,000 cells mL⁻¹), one stained and one unstained, were used as controls for algal growth. Once the suspensions were ready, each one was divided in quadruplicate 250 mL polyethylene culture flasks, taking special care to fill them gradually in 3–4 steps, gently mixing the suspensions between fillings. One bottle of each treatment was immediately used as a sample to verify initial concentrations of prey and grazer; concentrations were quantified using a Multisizer III particle counter. The remaining flasks were placed on a plankton wheel (0.2 rpm), inside a temperature-controlled room under the temperature and light conditions described before. The experiment was terminated after ca. 24 h, and the concentrations of prey and grazers were measured as described above. To calculate grazing rates and average prey concentrations, we used Frost’s (1972) equations. Per capita values were calculated using the average concentration of grazers in each replicate (Heinbokel, 1978).

2.6. Dilution grazing experiment with natural communities

To test the viability of LFLA labeled with the selected stain for flagellated algae (CellTracker) in natural samples, we combined the staining technique with a standard dilution grazing experiment (Landry and Hassett, 1982). The aim of this experiment was twofold. First, we wanted to evaluate whether the added algae showed equivalent growth rates to those obtained for natural algae and to what degree the response of the microzooplankton community would be affected by the manipulation. Second, we wanted to identify the major grazers in the community. Surface seawater samples (from 5 m depth) were collected from Blanes Bay (41°39'N, 2°48'E), NW Mediterranean Sea on July 26, 2010 with a 15 L inner spring Teflon-coated PVC Niskin bottle. The experiment consisted of 2 sets of dilution experiments, one using non-treated natural water, and the other using water with the addition of LFLA. The LFLA were Tetraselmis sp., at a concentration of ~150 cells mL⁻¹, and I. galbana, at a final concentration of ~500 cells mL⁻¹. Both concentrations were chosen to guarantee the detection of the algae in the microscope counts, without, a priori, significantly altering the dynamics of the community. Prior to introduction into the natural sample, the algae were labeled with the fluorochrome CellTracker at a concentration of 10 μM for a period of 24 h.

A known volume of water was gravity-filtered through a Pall AcroPak 0.8/0.2 μm 500 capsule (previously flushed, including tubing, with diluted HCl and thoroughly rinsed with deionized water) to obtain a dilution series with the proportions 25, 50, 75 and 100% of the experimental water. The dilution series was prepared in 1.3 L polycarbonate bottles, which were amended with a nutrient mixture (1 μM NH₄Cl, 0.07 μM Na₂HPO₄, and 0.5 μM Na₂SiO₃) to guarantee that nutrients were not limiting in any treatment. To assess the natural growth of the algae, 2 bottles containing 100% experimental water (i.e., undiluted), without the addition of nutrients, were also prepared. The bottles were placed on a plankton wheel (0.2 rpm) to avoid sedimentation of organisms under a controlled temperature (18 ± 1 °C)
and a photoperiod of 12:12 h for a period of approximately 24 h. It should be noted that the aim of the experiments was not to accurately assess the in situ microzooplankton grazing and phytoplankton growth rates but to test the use of the fluorochromes in combination with the dilution technique. Therefore, for the sake of simplicity, we did not incubate the bottles in situ.

At the end of the incubation period, we preserved 100 mL samples in glutaraldehyde (final concentration 1%) and filtered them onto 0.2 μm black polycarbonate filters as described above. These samples were processed as above, and the presence of free LFLA and LFLA in grazer vacuoles was quantified under the epifluorescence microscope. Prey abundance was estimated by counting a minimum of 400 cells per filter. The organisms counted by epifluorescence microscopy were classified into 2 groups: <5 μm and 5–20 μm. Additionally, we used 100 to 200 mL per bottle to measure chlorophyll-a (Chlo) as an indicator of autotrophic phytoplankton biomass using the methodology employed by Strickland and Parsons (1972).

We used a Model 1 linear regression between the dilution level and the net change in Chlo concentration to obtain phytoplankton growth and mortality rates (d⁻¹). Because the addition of nutrients may cause an overestimation of phytoplankton instantaneous growth rates, unfertilized bottles (no nutrients added) were used to obtain the instantaneous in situ growth rates. The derived net growth in the unfertilized bottles was corrected for mortality by microzooplankton to obtain the instantaneous growth rate of algae (Landry and Hassett, 1982). When saturated feeding responses were observed, the instantaneous growth rate of phytoplankton was obtained by fitting linear relationships to the 3 most diluted treatments (Dolan et al., 2000; Gallegos, 1989). The microzooplankton grazing rate was calculated by the difference between the phytoplankton instantaneous growth rates (intercept) and the net growth rate in undiluted bottles enriched with nutrients.

3. Results
3.1. Fluorochrome selection

In Table 1, we present a synoptic summary of the different fluorochrome-algal tests made in our study and the staining efficiency obtained. Of the 2 fluorochromes tested on non-diatom algae, only CellTracker was relatively successful. None of the 10 algae presented a positive staining response to the Vybrant fluorochrome. In the case of the CellTracker dye, only 3 algae incorporated the fluorochrome: the di-noflagellate Heterocapsa sp., the Prymnesiophyceae I. galbana, and the Prasinophyceae Tetraselmis sp.

CellTracker-stained cells could easily be discerned from the unstained ones under an epifluorescence microscope when excited by UV light (354 nm); stained cells fluoresced bright blue-violet and presented yellow, round formations, whereas unstained cells had a reddish color (Fig. 1). Unfortunately, due to the resolution limitations of the camera, these formations are not very evident in the microphotographs presented here, although one may be able to discern them in the case of the larger alga (Heterocapsa sp.). This distinction was evident even when the prey was found inside the grazers, especially in the case of the larger prey Tetraselmis sp. (Fig. 2). For diatoms, both fluorochromes tested effectively stained the cells (Table 1); stained cells fluoresced bright yellow under blue light excitation (450–495 nm). It is important to note that in most diatom cells, regardless of fluorochrome type, only one of the valves was stained; typically the larger valve was stained, whereas the smaller looked similar to images from the unstained cells. Fig. 3 shows the staining effect of the fluorochrome LysoSensor on both species of diatom tested herein.

3.2. Optimal stain concentrations and incubation times

The percentage of stained algal cells as a function of both fluorochrome concentration and incubation time is presented in Fig. 4. Differences among concentrations were tested at each time interval using 2-way repeated measures ANOVA with Bonferroni post hoc tests (i.e., CellTracker, LysoSensor and LysoTracker trials). The fluorochrome CellTracker effectively stained the flagellates I. galbana and Tetraselmis sp., and the proportion of stained cells was highly dependent both on cell concentration and incubation time (Fig. 4A,B). The intermediate CellTracker concentration used in our study (10 μM) showed a generally greater effectiveness in staining cells of I. galbana compared with the other 2 (5 and 20 μM). This greater efficiency was particularly strong in the period between 4 and 8 h of incubation time; stained cells comprised up to 95% (at 8 h) of the total compared with the maximum number of stained cells in the low (85% at 24 h) and high (81% at 4 h) concentration treatments. In contrast, for Tetraselmis sp. the low and intermediate fluorochrome concentrations proved to be equally efficient during the first 4–8 h of incubation, staining up to 97% and 99% of the total cells after 8 h of incubation. Interestingly, in both flagellate species the highest fluorochrome concentration was generally the least effective, although the first 2–4 h may have shown similar percentages of staining to the lower concentration cultures. For all fluorochrome concentrations, the proportion of the stained cells during the first 24 h did not vary significantly with time (ANOVA, p > 0.05), however a sharp, statistically significant decrease occurred thereafter yielding a very low proportion of stained cells in both I. galbana (low: 49%, intermediate: 45%, high: 21%) and Tetraselmis sp. (low: 51%, intermediate: 63%, high: 29%) at the end of the incubation period.

Staining results in diatoms differed according to the time of incubation and the fluorochrome type and concentration used (Fig. 4C,D,E,F). Both low (1 μM) and high (2 μM) concentrations of LysoTracker stained diatoms T. weissflogii (low: 93%, high: 96%) and S. costatum (low: 91%, high: 96%) to a similar degree, with maximum values after an 8 h incubation period in all cases (ANOVA, p > 0.05). A significant decrease in the proportion of stained cells of both diatom species was recorded after 24–48 h of incubation at both concentrations; after 72 h, the proportion of stained cells was around 60% and 55% in T. weissflogii and S. costatum, respectively. Unlike LysoSensor, the fluorochrome LysoTracker seemed more effective when used at lower concentration (1 μM), and this was particularly evident in the case of the diatom T. weissflogii. We recorded the maximum percentages of stained cells for both species (T. weissflogii: 88% and S. costatum: 78%) after 4 h of incubation at the low fluorochrome concentration. However, for both diatoms, we observed a progressive drop in the percentage of stained cells after 4 h of incubation, reaching very low values at the end of the incubation period (T. weissflogii: 30–49%, S. costatum: 26–53%).

3.3. Stain duration under natural light

The residence times of fluorochromes inside the algal cells (bleaching) incubated under natural light are presented in Fig. 5. The same statistical procedure used previously was applied to testing differences among concentrations at each time interval for each of the 3 fluorochromes. In general, the effectiveness of the different concentrations of fluorochromes resembled those observed for the previous experiment. For instance, flagellated cells incubated at 5 and 10 μM concentrations of CellTracker exhibited a longer duration of staining compared with the 20 μM concentration, and the percentage of stained cells was relatively stable during the 24 h exposure to natural light (Fig. 5A,B). The intermediate concentration resulted in a relatively higher proportion of stained cells (82% in both species, 24 h) compared with the lowest concentration (I. galbana: 64%, Tetraselmis sp.: 77%, 24 h). During the second and the third day of incubation, the loss of fluorochromes became evident at all concentrations.

Diatom specific dyes presented a higher fluorescence-loss rate and seemed more sensitive to the presence of natural light than CellTracker. After a 24 h incubation period, the proportion of stained cells was higher at the 2 μM concentration for both diatom species (T. weissflogii: 53% vs. 66%; p > 0.05; S. costatum: 60% vs. 70%, p < 0.05). The fluorochrome
LysoTracker remained at a higher proportion inside the cells of T. weissflogii (52% vs. 39% p < 0.05) after 24 h when cells were stained at the lowest concentration, whereas no significant differences were observed for S. costatum (58% vs. 55%, p > 0.05).

3.4. Fluorochrome effects on algal C:N ratios, algal growth rates, and protozoan grazing rates

The influence of CellTracker on the cellular C:N ratio of 3 algal species is presented in Fig. 6. No statistically significant differences were found between stained and unstained cells in both Tetraselmis sp. (t = −0.53, df = 2, p > 0.05) and Heterocapsa sp. (t = −2.28, df = 2, p > 0.05). A significant difference was detected in the case of I. galbana (t = −4.57, df = 2, p = 0.04), although the difference was only 9%.

The results of the growth dynamics of I. galbana and Tetraselmis sp. labeled with CellTracker (at 10 μM), and S. costatum and T. weissflogii stained with LysoSensor (2 μM) versus the same, unstained, algal species are shown in Fig. 7. Comparisons between the 2 treatments were performed using a modified Michaelis–Menten adjustment model; the parameters tested are presented in Table 3. We did not find significant differences between the equation parameters (Vmax, Km; Extra sum-of-squares F test) for stained and unstained I. galbana, Tetraselmis sp. and S. costatum; therefore, we present a single model for these 3 algae. For T. weissflogii, we found small, but statistically significant, differences between labeled and unlabeled cells; therefore, both curves were treated separately.

Fig. 1. Microphotographs of A) Tetraselmis sp., B) I. galbana and C) Heterocapsa sp., taken with an epifluorescence microscope using a UV filter (354 nm) in the presence and absence of CellTracker fluorochrome (10μM concentration). Left panel: cells without the fluorochrome. Right panel: stained cells. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)
The dinoflagellate *O. marina* ingested unstained and stained cells of both prey at equal rates (*I. galbana*: $t = 0.48$, $df = 4$, $p > 0.05$; *Tetraselmis* sp.: $t = 0.10$, $df = 4$, $p > 0.05$; Fig. 8). However, *G. dominans* had slightly higher ingestion rates on labeled flagellate cells of both *I. galbana* (10% increase; $t = 7.15$, $df = 4$, $p < 0.01$) and *Tetraselmis* sp. (18% increase; $t = 6.66$, $df = 4$, $p < 0.01$; Fig. 8).

### 3.5. Dilution grazing experiments

The results of the dilution grazing experiment with and without labeled tracer cells are shown in Fig. 9. The unaltered community had a phytoplankton mortality rate of 0.70 d$^{-1}$ and instantaneous phytoplankton growth rate of 1.14 d$^{-1}$. For the treatment with the addition

![Fig. 2. Epifluorescent micrographs, taken using a UV filter (354 nm), of the dinoflagellate *Gyrodinium dominans* with ingested labeled algae A). *Tetraselmis* sp. and B) *I. galbana*. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.]

![Fig. 3. Epifluorescent microphotographs taken with a Blue light filter (450 nm) in the presence and absence of the LysoSensor fluorochrome at a concentration of 2 μM. Left panel: cells without fluorochrome and right panel: stained cells of A) *T. weissflogii* and B) *S. costatum*. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)]
of stained algae, the phytoplankton growth rate was 1.12 d\(^{-1}\), and the mortality rate was 0.59 d\(^{-1}\). Overall, the addition of algae did not affect the phytoplankton growth rate, although the microzooplankton grazing rate was reduced by 16%. However, this difference was not significant (ANCOVA test; slope: \(F = 0.72, p > 0.05\), intercept: \(F = 0.03, p > 0.05\)).

We also compared the responses of the stained algal species with their corresponding phytoplankton size-fractions (<5 \(\mu m\) for \(I. galbana\) and >5 \(\mu m\) for \(Tetraselmis\) sp.; Fig. 10). In each case, we did not find significant differences in the fitted parameters of the linear equations (ANCOVA test; \(p > 0.05\)). Finally, we used LFLA to identify the major grazers in the undiluted treatments with and without added nutrients. In Table 4, we present the results of microscopic counts and the estimated contribution of each group of predators to the total amount of microzooplankton grazing. Predator contribution was accounted for by multiplying the abundance of grazers times the proportion that presented prey inside times the average number of prey items inside each grazer. From the total impact, we calculated the contribution of each group. This approximation does not provide a real estimate of grazing because it does not consider different digestion times among groups; however, it provides a snapshot of the relevance of each group. Our data showed that the most abundant predators (nanoflagellates) had a low grazing impact, whereas aloricate ciliates and tintinnids accounted for more than 50% of the overall grazing and dinoflagellates were the second most important grazers. No significant differences were found between treatments with and without nutrients (Paired \(t\)-test, \(p > 0.5\)) for any of the predators evaluated.

**4. Discussion**

4.1. Fluorochrome selection

A variety of specific commercial stains are available, of which some either have an affinity for certain regions of the cell (e.g., DNA or cytoplasm) or may accumulate in particular organelles (McCarthy, 2007). Therefore, optimizing dye choice was a primary goal of the study. In our case, the main objective was to identify an easy to use, non-toxic fluorochrome with a fluorescence emission in a different wavelength than chlorophyll. Despite using several types of prey, i.e., from various taxa and size ranges, we were surprised by the poor results of the tested fluorochromes. Specifically, out of the 10 different algae tested here, CellTracker stained only 2 flagellates (I. galbana, Tetraselmis sp.) and one dinoflagellate (Heterocapsa sp.); whereas the fluorochrome Vybrant was not incorporated by any algae.
CellTracker is a cytoplasmic vital stain that is metabolized to a cell-impermeant fluorescent dye-thioether, allowing long-term sample storage. According to the product specifications, the dye does not interfere with the normal development of the algae and may remain within the cell for at least 72 h, allowing at least 4 cell divisions. Our results confirmed that CellTracker may be an effective fluorochrome for use with dinoflagellates; similar results were obtained for 2 other dinoflagellate species used in copepod feeding studies (Schultz and Kiorboe, 2009; Teegarden, 1999). To the best of our knowledge, there are no studies reporting results with other algal taxa. Interestingly, we found that CellTracker efficiently stained 2 of our targeted flagellates, whereas the remaining flagellate species and all other algal groups (diatoms and cyanobacteria) did not seem to incorporate the dye.

Vybrant stain is a DNA-selective dye used in live cell studies of cellular DNA content (McCarthy, 2007). It is important to note that this fluorochrome has not been used previously in marine microorganisms and the only published studies are of applications in human cells and blood (Zhao et al., 2009; Muslimovic et al., 2008). Most of the fluorochromes were developed primarily for use in mammalian cells (Fuller et al., 2000) at specific temperatures and pH; therefore, these dyes may not necessarily work in aquatic organisms or algae cells.

The low success of CellTracker vital stains could be due to their low molecular weight because many times, the brightness was determined by the number of fluorochromes per conjugate molecule (to which it binds) or by the ratio of fluorochrome molecules and ligand molecules.
The intensity of emission could have been affected by several factors such as the number of conjugation sites per molecule (target cell) or potential reductions in the solubility of the complex. In addition, some dyes increase their efficiency when bound to a particular substance or when they are in a particular environment (McCarthy, 2007). Membrane permeability also could play an important role in the penetration of vital fluorochromes into the cell. In particular, the structural barrier of the cell wall (i.e., bacterial or vegetal) may limit the penetration of the fluorochrome (Herrera et al., 2002).

The diatom species *T. weissflogii* and *S. costatum* did not incorporate any of the above-mentioned fluorochromes; however, they were stained by the diatom-specific fluorochromes tested in a present study. LysoSensor and LysoTracker fluorochromes are stains that are used to visualize silicon utilizing organisms. These stains have been previously tested in several diatom species and have been used as a tool for labeling and visualizing the synthesis of silica frustules (Desclos et al., 2008), for looking at diatom silicification in relation to environmental conditions (Quéguiner et al., 2011), and in other ecological studies of freshwater diatoms (Znachor and Nedoma, 2008).

Both fluorochromes resulted in a strong green stain of one of the shells of the diatoms (Fig. 3); this staining was well differentiated from the red-orange autofluorescence of unstained cells. Additionally, both fluorochromes had the advantage of allowing the discrimination of active cells because the fluorescence acts on biogenic silica deposited on new diatoms frustules formed during cell division. This is the reason why the staining occurs in only one of the valves. However, because only the shell was stained, the diatoms were undetectable inside grazers that feed using a pallium or tube (Hansen and Calado, 1999). Therefore, further research is necessary to identify a stable cytoplasmatic dye able to stain diatoms.

### Table 3

Parameters used to fit the model presented in Fig. 7. The model corresponds to a Michaelis–Menten model, modified by adding a constant (C) to allow intercepts different than the origin. (L): Labeled algae, (U) Unlabeled algae. We present different models for labeled and unlabeled algae only when significant differences were found between the estimated parameters (see results).

<table>
<thead>
<tr>
<th>Species</th>
<th>Vmax</th>
<th>sd</th>
<th>Km</th>
<th>sd</th>
<th>C</th>
<th>sd</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>I. galbana</em></td>
<td>2.898</td>
<td>0.01</td>
<td>2.362</td>
<td>0.03</td>
<td>3.09</td>
<td>0.13</td>
<td>0.82</td>
</tr>
<tr>
<td><em>Tetraselmis</em> sp.</td>
<td>6.714</td>
<td>0.32</td>
<td>1.035</td>
<td>0.16</td>
<td>0.32</td>
<td>0.24</td>
<td>0.99</td>
</tr>
<tr>
<td><em>S. costatum</em></td>
<td>8.429</td>
<td>0.48</td>
<td>6.445</td>
<td>0.17</td>
<td>2.16</td>
<td>0.37</td>
<td>0.81</td>
</tr>
<tr>
<td><em>T. weissflogii</em> (L)</td>
<td>8.553</td>
<td>0.42</td>
<td>4.329</td>
<td>0.22</td>
<td>0.77</td>
<td>0.63</td>
<td>0.93</td>
</tr>
<tr>
<td><em>T. weissflogii</em> (U)</td>
<td>10.496</td>
<td>0.42</td>
<td>7.746</td>
<td>0.38</td>
<td>1.37</td>
<td>1.37</td>
<td>0.93</td>
</tr>
</tbody>
</table>

Model used: \( y = \frac{V_{max}}{C + \frac{x}{K_m}} + C \).

Despite the existence of several microzooplankton works utilizing dead, labeled prey (i.e., FLA, FLB), there are few records in the literature of studies examining the fluorochrome effectiveness on algae in terms of staining time, optimal concentration and the bleaching impact of natural light in live, labeled prey. An optimal dye concentration should assure minimum negative effects on the physiology of the labeled cells coupled with good discrimination. In some cases, a lowering of the
dye/cell ratio to reduce the toxicity and the quenching effect is recommended (Rottenberg and Wu, 1998). Other studies have also reported that stains may have negative effects, thus causing a decrease in metabolic cell activity resulting in the weakness or loss of the fluorescent signal (Brussaard et al., 2001; Dorsey et al., 1989).

Contrary to what was initially expected, we found that CellTracker yielded the lowest percentages of stained cells at the highest concentration used (20 μM) compared with the 2 lower concentrations (5 and 10 μM). The low proportion of stained cells in the high concentration condition could have contributed to a shift in the physiological status and metabolic cell activity due to the toxicity of the stain. Despite being “vital stains,” most of these fluorochromes were eventually toxic to the organisms depending upon the concentration used, but an adequate dilution should not cause damage to the cells (Wenrich, 1929). During our experiment, we visually verified that many of the flagellated algae were less motile at the highest CellTracker concentration than at lower concentrations.

Bleaching of vital stains over time may be a considerable limitation when conducting long-term experiments. Our data confirmed the stability of the fluorochrome CellTracker under natural light conditions (UV-included) over a 24 h time period and the low extinction rate of fluorescence compared with other vital stains (i.e., CMFDA, Li et al., 1996). The decreased percentages of stained cells after a 24 h incubation period may also be due to cellular metabolic activity or simply attributed to cell division, which results in the loss of fluorescence through new generations of cells.

LysoSensor has several advantages over other dyes; it was easy to use for diatom mono-cultures, resulted in a high percentage of stained cells in cultures growing exponentially, and presented a relative low percentage of fluorescence extinction, although the extinction rate was not sufficient for long-term experiments (>24 h). Nevertheless, it presented lower fading rates than LysoTracker, which was bleached from both diatom species quite rapidly. This contrasts with the results of Desclés et al. (2008), who claimed a greater stability of LysoTracker over LysoSensor Blue and less background fluorescence compared with LysoSensor Yellow/Blue (used in this study).

4.3. Effects of the stains on live organisms

The use of “non-toxic” dyes in living organisms may cause a variety of responses at the organ or cellular level (Barbosa and Peters, 1971),
The results of our study showed that the estimates in the dilution experiments as suggested by Landry et al. (1995) found that the dye used (DAPI and FITC) did not affect the development of organisms in experiments over a period of 12 h. However, Putt (1991) found that the vital fluorochrome hydroethidine reduced photosynthesis in *T. galbana* by 95%, which surely affected their growth rate. The results of our study showed that the fluorochrome CellTracker did not interfere significantly with the normal growth of *Tetraselmis sp.* and *T. galbana*. Similarly, LysoSensor had no negative effects on the growth rate of *T. weissflogii*. However, the dye had a moderate effect on the growth rates of *S. costatum* after a 24 h incubation period, which calls for caution and further investigation if this fluorochrome is intended for use in physiological studies.

The effect of fluorochromes on the protozoan feeding activity seemed to differ between the 2 grazers tested in our study (*O. marina* and *G. dominans*). Previous feeding studies in protozoa using other vital dyes found modest variations in ingestion rates relative to those reported in the literature for culture experiments without added fluorochromes (e.g., Li et al., 1996; Martín-Cereceda et al., 2008). Although we found significant differences in the grazing rates between the treatments with stained and unstained algae, they could not be attributable to the changes in the elemental C:N ratio of the cell. It is likely that the effects of the dye on the movement behavior of the prey (not rigorously tested in this study) could have affected predator–prey encounter rates or capture efficiency.

### 4.4. The use of fluorochromes in dilution grazing experiments

The incorporation of labeled prey in a standard dilution grazing experiment was used to determine whether the manipulation would significantly modify the feeding behavior of the microzooplankton. Although we found no significant effects of the addition of labeled prey, we should make clear that introducing tracers at all dilution levels would not add further information to the quantification of the grazing rates. Therefore, such manipulation is not advisable, unless it is used to “refine” the grazing estimates in the dilution experiments as suggested by Landry et al. (1995). However, we recommend that the use of extra undiluted and nutrient-unamended bottles with labeled algae be added to the standard protocol as it could shed some light on the functional diversity of microzooplankton. This procedure has been successfully used in the assessment of mixotrophy to overall microzooplankton grazing impact in the Eastern Mediterranean during a mesocosm experiment (Calbet et al., 2012).

In our experiment, the lack of effects on the regression parameters of the dilution equation indicated that the concentration of labeled prey added in each experiment was appropriate. The labeled prey was sufficient to be quantifiable in the experiment and not excessive to produce changes in the grazing behavior of protozoans (e.g., feeding saturation and switching feeding responses in prey selection). We suggest the use of a concentration below 25% of the total phytoplankton standing stock for natural water samples from systems with low productivity; however, this concentration will depend exclusively on the particular system investigated. Major problems are anticipated in oligotrophic ecosystems, where small additions may modify the food web and the detection of few labeled algae may be a challenge. In these situations, and others, it may be feasible to use particle counters with imaging capability (e.g., FlowCAM, Cytobuoy, flow cytometry, etc.). Another disadvantage to be taken into consideration is the limited number of algae species successfully stained, which leaves a limited size-range of potential prey to be used in the field.

### 5. Conclusions

Advancement in trophic studies of microzooplanktonic organisms is directly related to the incorporation of new techniques and protocols, and in this case, also to the availability of new vital stains. These tools will provide greater flexibility to develop experimental designs useful in both the field and the laboratory. This research resulted in the development of a long-term fluorescent staining technique for marine nano- and dinoflagellates and diatoms that have no apparent negative effects on growth, cell viability or the grazing rates of predators. However, the low percentage of algae species stained calls for further research on new dyes with similar characteristics, but higher efficiency of staining.

Based on our experiments, we propose an optimized experimental protocol with the following steps: 1) stain cell cultures of prey in the exponential phase of growth for 4–8 h with CellTracker at a final concentration of 10 μM or with 2 μM LysoSensor (in the case of diatoms); 2) begin experiments at these concentrations and then test a range of concentrations to determine the optimal concentration for different organisms; 3) after the staining period, segregate algae from the supernatant by centrifugation (1000 rpm) for 5 min and then resuspended in FSW (0.2 μm); and 4) use stocks of stained algae for experimentation in 24 h incubations without a significant loss of fluorescence.

### Acknowledgments

This research was funded by the project PROTOS (CTM2009-08783) from the Spanish Ministry of Science and Innovation assigned to A.C., which is a contribution of the Marine Zooplankton Ecology Excellence Group from the Generalitat de Catalunya (2009SGR-1283). R.A.M. was funded by a PhD fellowship from the National Commission of Science (CONICYT), Ministry of Education, Chile. We thank M. Alcaraz for his comments on an earlier version of the manuscript.