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The strengths and weaknesses of Live Fluorescently Labelled Algae (LFLA) to estimate herbivory in protozooplankton and mixoplankton

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1. Introduction

The determination of the ecological role of marine microorganisms has been a major driver of biological oceanography research since Azam et al. (1983) coined the term “microbial loop” (Fenchel, 2008). At the time, it was difficult to quantify bacterial biomass and production rates (e.g., Hagström et al., 1979; Krambeck et al., 1981). The most successful approaches relied on radioactive isotopes (Fuhrman and Azam, 1980, 1982), but the fate of the apparently substantial bacterial production remained a mystery until Fenchel (1982) clarified the role of flagellates as important consumers of pelagic bacteria.

Logically, scientists became eager to develop methods to quantify predation rates on bacteria, and the first techniques were quickly developed (e.g., prey disappearance – Wright and Coffin, 1984; fluorescent latex beads – Borsheim, 1984; radioisotopes – Lessard and Swift, 1985; selective inhibitors – Wikner et al., 1986). Yet, the true game-changer was introduced by Sherr et al. (1987), who suggested using heat-killed fluorescently labelled bacteria (FLB).

The major advantages offered by this methodology were i) avoiding a negative selection towards inert particles like the latex beads (e.g., Epstein and Rossel, 1995; Jürgens and DeMott, 1995; Sherr et al., 1987), and ii) offering consistently-sized particles thus enabling cross-laboratory comparisons which unevenly sized beads did not allow (e.g. Chrzanowski and Simek, 1990; Monger and Landry, 1991, 1992). In fact, FLBs are still used nowadays both in marine (e.g., Avrahami and Frada, 2020) and freshwater systems (e.g., Izaguirre et al., 2021), despite the cumulative evidence that bacterivores may favour live over heat-killed bacteria (e.g., Bochdansky and Clouse, 2015; Fu et al., 2003; Bochdansky and Clouse, 2015; Wilken et al., 2019).

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Shortly after the paper by Sherr et al. (1987), Rublee and Gallegos (1989) used the same protocol (with minor differences in the centrifugation steps) to stain algae instead of bacteria (Fluorescently Labelled Algae, FLA). FLA possess similar advantages and disadvantages to FLB and, consequently, were used in more than 30 studies (up to our knowledge) across the world to quantify protistan herbivory rates. At the time of the development of the FLA technique, chemosensory selectivity had already been confirmed both in ciliates (e.g., Stoecker et al., 1981; Verity, 1988) and dinoflagellates (e.g., Hauser et al., 1974; Spero, 1985), which together would later be acknowledged as the major herbivores on a global scale (Calbet and Landry, 2004; Schmoker et al., 2013). Therefore, the question of whether dead algae would be ideal tracers was quickly raised and answered.

Unsurprisingly, the grazers preferred live algae over dead ones (e.g., Püt, 1991). The consequence was the natural displacement of research towards live fluorescent stains (e.g., Kerger et al., 1996; Lessard et al., 1996; Pfister and Arndt, 1996) and to the development of the Live Fluorescently Labelled Algae (LFLA) technique as it is mostly used nowadays (Li et al., 1996). Despite promising, the LFLA technique is not flawless since, for example, not all protists are equally stained (e.g., MacIntyre and Cullen, 2016) and the fluorescence of labelled cells fades rapidly in the light (e.g., Li et al., 1996). Nevertheless, it must be pointed out that this technique enables direct visualisation of both predator and prey simultaneously, which may be a significant advantage when attempting to study organisms like mixoplankton (single-celled organisms that have the potential to express phototrophy and phagotrophy simultaneously – Flynn et al., 2019).

Accounting for the grazing impact of mixoplankton in the field is not easy. It is difficult to ascertain when organisms are actively feeding or not (Anderson et al., 2017; Beisner et al., 2019; Flynn et al., 2019; Stoecker et al., 2017), although recent attempts have been made (e.g., Ferreira and Calbet, 2020; Ferreira et al., 2021). The fact that diel feeding rhythms are non-negligible both in protozooplankton and mixoplankton (e.g., Arias et al., 2020; Arias et al., 2021; Ferreira et al., 2021; Jakobsen and Strom, 2004; Ng et al., 2017; Strom, 2001) complicates the matter even further when conducting field studies. Indeed, a single sampling event is likely to over/underestimate the measured predation based on the organisms that are feeding the most at that time of the day.

To address the mixoplankton paradigm properly, we need a technique that can measure their grazing impact and retain its effectiveness under the different scenarios that affect grazing. This is what the LFLA technique offers, within its limitations that need to be properly discussed in light of the mixoplankton paradigm. Thus, this manuscript aimed to determine, in the laboratory, the strengths and weaknesses of the method with the description of a sound protocol for the incorporation of this methodology in situ to account for mixoplankton’s grazing in the field. For that, we used protist grazers of different taxonomic and trophic groups and conducted short-term incubations with LFLA.

## 2. Material and methods

### 2.1. Cultures

We used protozooplanktonic and mixoplanktonic predators, encompassing several taxonomic groups and feeding strategies to provide a better overview of natural populations. For a detailed description of the species used in this study and their maintenance conditions, see Table 1.

To ensure that cultures were always in exponential growth, we replenished ca. 30% of the culture with fresh medium daily. Strombidium basimorphum, Dinophysis acuminata, and their prey in the respective experiments were kept in a temperature-controlled room at 15 °C with a 14:10 L/D cycle, at a salinity of 15. All other cultures were kept in a temperature-controlled room at 19 °C with a 10:14 L/D cycle, at a salinity of 38.

### 2.2. Preparation of LFLA

I. galbana, T. chui, Heterocapsa sp., and M. rubrum were fluorescently

<table>
<thead>
<tr>
<th>Species (ESD)</th>
<th>Class (Strain ID)</th>
<th>Trophic mode</th>
<th>Feeding strategy</th>
<th>Growth medium (Prey)</th>
<th>Light (μmol photons m⁻² s⁻¹)</th>
<th>Exp.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Teledaulax amphiocoeus</em> (4.7 μm)</td>
<td>Cryptophyceae (K-1837)</td>
<td>Phytomixed</td>
<td>f/2</td>
<td>100–200</td>
<td>1 and 2</td>
<td></td>
</tr>
<tr>
<td><em>Rhodomonas salina</em> (7.5 μm)</td>
<td>Cryptophyceae (K-0294)</td>
<td>Phytomixed</td>
<td>f/2</td>
<td>100–200</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><em>Isochrysis galbana</em> (4.5 μm)</td>
<td>Prynesiophyceae (CCMP 1323)</td>
<td>Phytomixed</td>
<td>f/2</td>
<td>100–200</td>
<td>1 and 3</td>
<td></td>
</tr>
<tr>
<td><em>Tetraselmis chu'i</em> (9.2 μm)</td>
<td>Chlorodendrophyceae</td>
<td>Phytomixed</td>
<td>f/2</td>
<td>100–200</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>Heterocapsa</em> sp. (13.4 μm)</td>
<td>Dinophyceae</td>
<td>Phytomixed</td>
<td>f/2</td>
<td>100–200</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><em>Oxyrrhis marina</em> (16.5 μm)</td>
<td>Dinophyceae (ICM-ZOO-OM001)</td>
<td>Phyto</td>
<td>Direct engulfment</td>
<td>FSW (R. salina)</td>
<td>35–55</td>
<td>1</td>
</tr>
<tr>
<td><em>Gyrodinium dominans</em> (17.8 μm)</td>
<td>Dinophyceae (ICM-ZOO-GD001)</td>
<td>Pzoo</td>
<td>Direct engulfment</td>
<td>FSW (R. salina)</td>
<td>35–55</td>
<td>1</td>
</tr>
<tr>
<td><em>Lessardia elongata</em> (11.0 μm)</td>
<td>Dinophyceae (ICM-ZOO-LSP001)</td>
<td>Pzoo</td>
<td>Peduncle</td>
<td>FSW (R. salina)</td>
<td>35–55</td>
<td>3</td>
</tr>
<tr>
<td><em>Karlinodinium armiger</em> (17.8 μm)</td>
<td>Dinophyceae (ICM-ZOO-KA001)</td>
<td>Pzoo</td>
<td>Peduncle</td>
<td>FSW (R. salina)</td>
<td>35–55</td>
<td>3</td>
</tr>
<tr>
<td><em>Karlinodinium veneficum</em> (12.8 μm)</td>
<td>Dinophyceae (ICM-ZOO-KA001)</td>
<td>CM</td>
<td>Peduncle</td>
<td>FSW (T. amphioidea)</td>
<td>35–55</td>
<td>1</td>
</tr>
<tr>
<td><em>Gymnodinium litoralis</em> (19.7 μm)</td>
<td>Dinophyceae (CGA)</td>
<td>CM</td>
<td>Peduncle</td>
<td>f/2</td>
<td>100–200</td>
<td>3</td>
</tr>
<tr>
<td><em>Dinophysis acuminata</em> (29.0 μm)</td>
<td>Dinophyceae (FR101009)</td>
<td>pSNCM</td>
<td>Pzoo</td>
<td>FSW (M. rubrum)</td>
<td>35–55</td>
<td>3</td>
</tr>
<tr>
<td><em>Strombidium arenicola</em> (32.3 μm)</td>
<td>Oligotricha (ICM-ZOO-AS001)</td>
<td>pSNCM</td>
<td>Direct engulfment</td>
<td>FSW (R. salina)</td>
<td>35–55</td>
<td>1</td>
</tr>
<tr>
<td><em>Mesodinium rubrum</em> (19.9 μm)</td>
<td>Litotromatea (DK-2009)</td>
<td>pSNCM</td>
<td>Direct engulfment</td>
<td>FSW (T. amphioidea)</td>
<td>35–55</td>
<td>1</td>
</tr>
<tr>
<td><em>Strombidium basimorphum</em> (39.9 μm)</td>
<td>Oligotricha</td>
<td>GNCM</td>
<td>Direct engulfment</td>
<td>FSW (T. amphioidea)</td>
<td>35–55</td>
<td>2</td>
</tr>
</tbody>
</table>
labelled following the guidelines by Martínez et al. (2014) with slight modifications. Briefly, cells were stained overnight with the fluorescence CellTracker® Blue CMAC (7-amino-4-chloromethylcoumarin - absorption/emission maxima of 353/466 nm), a vital cytoplasmic stain, at a final concentration of 10 μM. After the staining period, the excess stain was removed from the medium by centrifugation (1000 g) for 10 min for all species except for *M. rubrum*, due to its fragility. The supernatant was discarded, and the cells were re-suspended in 0.1 μm filtered seawater. This cleaning process was repeated twice, to reduce the carryover of stain, which can enter predator cells and mask the actual ingestion of LFLA (e.g., Li et al., 1996). *M. rubrum* cells were picked individually with a drawn Pasteur pipette and transferred through five wells of 0.1 μm filtered seawater to remove the stain.

2.3. Exp. 1 – The effects of prey concentration and time on LFLA incorporation

For this experiment, we used the protozooplankton *O. marina*, *G. dominans*, and *S. arenicola*, while *K. armiger*, *K. venefericum*, and *M. rubrum* were chosen as representatives of mixoplancton. During the experiments, all predators were offered a mixture of the LFLA *I. galbana* and the respective cryptophyte prey as described before (the final percentage of LFLA was approximately 30% of the total i.e., 70% unlabelled prey – Martínez et al., 2014). For the detailed prey concentrations and proportions between LFLA and cryptophytes, see Table S1 in the Supplementary Information. The final predator concentrations were adjusted to avoid the depletion of prey at the target concentration. Additionally, we prepared a second incubation for *M. rubrum*, where we only offered the LFLA (i.e., 100% of the offered prey were labelled *I. galbana*).

Experiments were conducted in 0.1 μm filtered seawater (i.e., without added nutrients) inside 75 mL Nunc™ Non-treated flasks (Thermo Scientific™). Each experiment was carried out at two concentrations of prey, one saturating ([Prey] = High) and one non-saturating ([Prey] = Low) (see also Table S1). The experimental bottles were prepared in duplicates and filled in two to three steps using a joint suspension containing both predator and prey at the target concentrations. The suspension was gently mixed between fillings, avoiding air bubbles (Broglio et al., 2004). The bottles were incubated on a plankton wheel (0.2 rpm) at 19 °C at an irradiance of 35–55 μmol photons m⁻² s⁻¹. Each experimental bottle was sampled (9–45 mL depending on final predator concentration) every 20 min during the first 2 h of incubation, and after 3 and 5 h. The samples were fixed with cold Glutaraldehyde (4 °C, final concentration of 1%) for 2 h and then filtered with a vacuum pump onto 2 μm pore-size black polycarbonate filters, which were later mounted on microscope slides. Before filtration, these filters were placed on top of support Whatman® GF/C glass microfiber filters to ensure the homogeneity of the filtrate. The first 100 predators encountered on each slide were examined for the presence or absence of ingested prey, determined as blue-fluorescent inclusions (BFI).

2.4. Exp. 2 – The effects of diet feeding rhythms on prey incorporation and digestion

For this experiment, we used *S. basimorphum*. This species was maintained on *T. amphioxeia* before the experiment according to Maselli et al. (2020), but was allowed to decrease its prey to negligible levels before the onset of the incubations. We conducted two independent incubations with the LFLA *T. chui* in a proportion of ca. 5 prey per predator under non-saturating concentrations, one incubation during the day and one during the night. The experiments were always conducted in 0.1 μm filtered seawater.

We prepared triplicate experimental bottles, filled as described before, containing the mixture of the GNCM predator and the LFLA. We coordinated the experiment so that the initial samples for the day and night periods would be as close as possible to the lights-on/lights-off event in the temperature-controlled chamber. Both incubations lasted 5 h and were sampled hourly except for the second sampling point, which occurred within 30 min of incubation. The fixation and processing of the samples were as mentioned before. The ingested volume of LFLA stained prey (μm³ BFI⁻¹) was obtained from linear dimensions of the cells measured on photographs using the Fiji software (Schindelin et al., 2012), and its decrease rate was used as a measure of digestion (Strom, 2001). Carbon contents for *T. chui* were estimated using the equation of Menden-Deuer and Lessard (2000) for chlorophytes.

2.5. Exp. 3 – The effects of peduncle feeding on the LFLA technique

As suggested before by Archer et al. (1996), there could be issues concerning the feeding mechanism of the predator when quantifying ingestion rates based on fluorescent tracers. Accordingly, we prepared an experiment to ascertain whether the feeding mechanism could be an issue when using LFLA as tracer particles by selecting known peduncle feeders and offering them labelled prey. This experiment was designed to be qualitative instead of quantitative like the previous two experiments and, thus, the prey was always offered in a proportion of ca. 1 prey per predator.

The species chosen for this experiment were the protozooplankton *L. elongata*, and three mixoplanctonic species, *K. armiger*, *D. acuminata*, and *G. litoralis*. During the experiment, *L. elongata* and *K. armiger* were fed the LFLA *I. galbana*. *D. acuminata* was maintained as described by Nielsen et al. (2012) and Rusterholz et al. (2017) and fed fluorescently labelled *M. rubrum*. The co-existent unlabelled prey was removed before the incubation with labelled *M. rubrum* using a similar approach as used to clean the extra stain in *M. rubrum*, as previously described. Lastly, *G. litoralis* was offered the LFLA *Heterocapsa* sp. during the experiment. Food vacuoles in this species were first noticed in a monoculture that was kept under complete darkness for two days (cannibalism) and, later, in a mixed culture with *T. amphioxeia* in 0.1 μm filtered seawater (Fig. S1 in the Supplementary Information).

2.6. Calculations of clearance and grazing rates using LFLA

The average number of BFI per protist was determined using UV light by epifluorescence microscopy on samples collected at several time points. In all cases (i.e., with the different predators), the first sample was collected and fixed with glutaraldehyde within 2–3 min. Therefore, we assumed that the average BFI predator⁻¹ in this sample was due to the random overlap of predators and LFLA due to the filtration procedure (Li et al., 1996), and used it to zero the remaining samples. Plotting this information versus time (h) typically yields a linear relationship for the initial time points, levelling off as the experiment progresses due to the digestive time of algae (Rublee and Gallegos, 1989; Caron, 2001). Therefore, a bilinear model (e.g., Blackman, 1905; Jones et al., 2014) should be used to fit the experimental data points (Equation 1).

\[
LP_t = L_{P_{\text{max}}} \left(1 + \frac{t - t_{\text{sat}}}{2t_{\text{sat}}}ight)
\]

Where \(LP_t\) and \(L_{P_{\text{max}}}\) are labelled prey (BFI) predator⁻¹ at time \(t\) and at saturation, and \(t_{\text{sat}}\) is the time taken to reach \(LP_{\text{max}}\). To calculate \(t_{\text{sat}}\), we applied an objective criterion based on statistical significance (one-tailed Student’s t-test) i.e., if a given observation (BFI predator⁻¹) at time \(t\) was not significantly lower than the subsequent observation (\(P > 0.05\), \(t_{\text{sat}}\) had been found. The slope of the linear regression between \(t = 0\) and \(t = t_{\text{sat}}\) yields the ingestion of LFLA predator⁻¹ h⁻¹, which can be used to calculate \(LP_{\text{max}}\) by multiplying it by \(t_{\text{sat}}\). The ingestion rate as described before can be converted to total algae ingested per predator per hour by multiplying it by the unlabelled algae:LFLA ratio assuming that there is no discrimination for or against fluorescently labelled
tracers (e.g., Johnson et al., 2003; Kamiyama, 2000; Martínez et al., 2014). Clearance rates (both LFLA-specific and total) are determined by dividing the ingestion rates by the concentration of LFLA or by the total concentration of prey (Frost, 1972; Heinbokel, 1978). In the field, estimates of community-level herbivory are obtained by multiplying group-specific clearance rates by the abundance of each group, determining the latter with standard microscopic methods (Caron, 2001).

In the incubations where the two prey species were of different sizes (i.e., when the cryptophyte prey was R. salina), an initial and a final sample were quantified using a Beckman Coulter Multisizer III particle counter. Therefore, it was possible to determine prey-specific clearance rates based on the disappearance of individual preys. Then, we estimated the selection coefficient (Wi) and the electivity index (Ei*) according to Vanderploeg and Scavia (1979). The former is calculated according to Equation 2:

\[ Wi = \frac{F_i}{\sum F_i} \] (2)

where \( F_i \) is the clearance rate for a given food type \( i \) and \( \sum F_i \) is the sum of clearance rates on all food types. The \( E_i^* \) was calculated using Equation 3:

\[ E_i^* = \left[ \frac{Wi - \frac{1}{n}}{Wi + \frac{1}{n}} \right] \] (3)

Fig. 1. Incorporation of labelled prey (BFI predator) as a function of incubation time: a and b) protozooplanktonic dinoflagellates O. marina and G. dominans, c and d) mixoplanktonic dinoflagellates K. veneficum and K. armiger, and e) protozooplanktonic ciliate S. arenicola. All predators were incubated using two concentrations of prey, one saturating (open circles) and one non-saturating (black circles) with the exception of S. arenicola which was only followed under saturating conditions. For the detailed LP_{max} and t_{sat} parameters of the bilinear fit (Blackman, 1905; Jones et al., 2014), see Table S2. Error bars ± se.
where \( n \) is the total number of food types. Negative values imply active avoidance of prey, whereas the opposite implies selection for a given species.

3. Results

3.1. Exp. 1 – The effects of prey concentration and time on LFLA incorporation

All dinoflagellates exhibited a significantly (\( P < 0.01 \) for all species) higher \( \text{LP}_{\text{max}} \) (maximum number of labelled prey inside a predator) at high food concentrations than when the concentration of prey was low (Fig. 1). \( G. \text{dominans} \) was the predator with the largest difference between high and low concentrations, being followed by \( K. \text{armiger} \) (ca. 7.59 and 1.97x more \( \text{LP}_{\text{max}} \) at the highest prey concentration, respectively; Fig. 1b,d); \( O. \text{marina} \) increased its \( \text{LP}_{\text{max}} \) by ca. 1.84x (Fig. 1a). The species which displayed the lowest fold-increase (ca. 1.74x) in \( \text{LP}_{\text{max}} \) was \( K. \text{veneficum} \) (Fig. 1c). Only one prey concentration was studied in the case of the ciliate \( S. \text{arenicola} \) (Fig. 1e).

Overall, all species reached the plateau phase of the tracer incorporation curve within 1 h irrespective of the prey concentration. Accordingly, from the portion of the curves prior to the plateau phase, we can estimate ingestion rates on the LFLA. These values can then be converted to total ingestion and clearance rates, as summarised in Table 2. Irrespective of the predator species, higher prey concentrations resulted in higher ingestion and lower clearance rates.

As mentioned in section 2.3, we also used the pSNCM \( M. \text{rubrum} \) in the first experiment. Nevertheless, in addition to the incubation with the LFLA \( I. \text{galbana} \), i.e., provided in a mixture containing \( T. \text{amphioxæa} \) as well, we incubated it with LFLA as the sole prey. This ciliate was therefore excluded from Fig. 1 and displayed separately in Fig. 2.

When \( I. \text{galbana} \) was offered without an alternative prey (Fig. 2a,c), \( M. \text{rubrum} \) ingests it and the incorporation of BFI per predator followed the typical satiation pattern described before (with a \( t_{\text{sat}} < 1 \) h). On the other hand, if \( T. \text{amphioxæa} \) was provided in the mixture of prey (being \( I. \text{galbana} \) used only as a tracer, i.e., using the regular protocol for LFLA), the incorporation of BFI per predator is negligible (Fig. 2b). From the first experiment, we were also able to assess the selection for or against the tracer particle due to the size differences between the LFLA and the cryptophyte \( R. \text{salina} \). Accordingly, the Ei* (as calculated using Equations 2 and 3) for \( S. \text{arenicola}, O. \text{marina}, G. \text{dominans}, \) and \( K. \text{armiger} \) were summarised in Fig. 3.

Under non-saturating prey conditions (Fig. 3a), the electivity indexes for all three dinoflagellates were positive for \( R. \text{salina} \) (i.e., the cryptophyte was the preferred prey species). For both \( O. \text{marina} \) and \( K. \text{armiger} \), the Ei* value was close to 0, which suggests a negligible prey preference in these conditions for the two dinoflagellates. For \( G. \text{dominans} \) on the other hand, there was a marked preference for \( R. \text{salina} \), as seen by an Ei* value for \( I. \text{galbana} \) lower than 1.

At the highest prey concentration (Fig. 3b), \( G. \text{dominans} \) was still feeding preferentially on \( R. \text{salina} \) (i.e., Ei* > 0), although the avoidance of \( I. \text{galbana} \) was less evident (as seen by an Ei* value closer to 0). Also, \( K. \text{armiger} \) shifted from a non-selective predator at low prey concentrations to a highly selective one at saturating conditions. Contrary to \( G. \text{dominans}, K. \text{armiger} \) favoured \( I. \text{galbana} \) as prey over \( R. \text{salina} \) under saturating food conditions. The ciliate \( S. \text{arenicola} \) also shared a preference for \( I. \text{galbana} \), albeit to a smaller extent than \( K. \text{armiger} \).

3.2. Exp. 2 – The effects of diel feeding rhythms on prey incorporation and digestion

A very similar procedure was applied to follow the diel incorporation of the LFLA \( T. \text{chui} \) by the NCM \( S. \text{basimorpha} \) (Fig. 4). The \( \text{LP}_{\text{max}} \) obtained during the day incubation was ca. 2.16x higher than the one obtained during the night (\( P < 0.01 \)), but the \( t_{\text{sat}} \) were similar (ca. 2 h). Regarding volume changes in the ingested BFIs over time (Fig. 4b,d), the first 2 h induced an average reduction of 18.68 \( \mu \text{m}^3 \text{BFI}^{-1} \) during the day and 7.55 \( \mu \text{m}^3 \) during the night. Nevertheless, the parameters in both decay curves were not statistically different, likely due to the variability of the data, with the exception of the one controlling the initial volume of the BFI. Still, from the combined information (and normalized to C units) of Fig. 4a and b, one can estimate digestion rates by calculating the differences between the estimated and observed pg C per BFI. Despite having statistically insignificant decay parameters, the fact that diurnal ingestion rates were higher than the nocturnal ones resulted in significantly higher (ca. 2.88x) digestion rates between the day and the night (Fig. 4c, \( P < 0.01 \)).

3.3. Exp. 3 – The effects of peduncle feeding on the LFLA technique

To answer the question of whether the feeding mechanism could impact the conclusions drawn from an experiment with LFLA, we prepared a qualitative experiment with known tube-feeding dinoflagellates (Fig. 5). The major issue demonstrated by this experiment is the complete cytoplasm staining, noticeable irrespective of the chosen predator (i.e., an uncountable amount of prey inside), despite being particularly evident in \( D. \text{acuminata} \) (Fig. 5d). The least affected predator was \( K. \text{armiger} \) (Fig. 5a), whose cytoplasm staining was heavily dependent on the elapsed time after the beginning of the incubation. The experiment with \( G. \text{litoralis} \) (Fig. 5c) demonstrated the same issues as those mentioned before; however, as the prey offered was similarly sized to the predator, it was common to find half-eaten prey in the filter and/or more than one predator feeding on a single prey, further impairing the estimation of grazing. At last, the experiment with \( L. \text{elongata} \) (Fig. 5b) demonstrated that using LFLA to quantify grazing in peduncle feeding protozooplankton is even more difficult than in mixoplankton. It seems that the incorporation of LFLA by the former is further masked by the lack of naturally occurring pigments i.e., the result is an entirely blue staining of the predator irrespective of the feeding condition, either due to auto-fluorescence or to the complete staining of the cytoplasm due to fluorescence.
4. Discussion

The ability to deal with mixoplankton gives the LFLA technique an edge against other techniques used to estimate microbial grazing rates (e.g., the dilution technique – Landry and Hassett, 1982; quantitative protargol staining – Montagnes and Lynn, 1987). In addition, relying on microscopy (i.e., providing a direct visualisation of organisms) is advantageous as it enables a species/community-specific analysis (Caron, 2001). Indeed, it was only due to the use of microscopes that we were able to detect T. amphioxeia cells with certainty inside the dinoflagellate G. litoralis (Fig. S1b), due to the different photosynthetic pigment that each organism possesses. Feeding in this dinoflagellate had never been detected with other techniques.

Fig. 2. Incorporation of labelled prey (BFI predator$^{-1}$) by the pSNCM M. rubrum as a function of incubation time: a) I. galbana was the only prey offered – for the detailed LP$_{max}$ and t$_{sat}$ parameters of the bilinear fit (Blackman, 1905; Jones et al., 2014), see Table S2; b) the labelled I. galbana was offered as a tracer particle, i.e., T. amphioxeia was also present in the mixture of prey and c) epifluorescence pictures from the experiment without T. amphioxeia obtained under UV light excitation where BFI can be seen inside M. rubrum. Error bars ± se.

Fig. 3. Electivity indexes calculated for I. galbana and R. salina for O. marina, G. dominans, K. armiger, and S. arenicola under a) non-saturating food conditions and b) saturating food conditions. Negative values imply a negative selection and vice-versa. ND = not determined. Error bars ± se.
reported before and, thus, it can be moved from the phyto-into the mixoplankton group, namely into the CM group as it possesses its own chloroplasts (Mitra et al., 2016).

As confirmed by our first and second experiments, there are several protist predators which incorporate LFLA without major issues and under different experimental conditions (Figs. 1 and 4a). We have also confirmed the usefulness of the technique on the quantification of diel ingestion and digestion rates on an NCM species (Fig. 4b,c). We strongly believe that this is a close-to universal utility of the technique, as pigments/fluorescent tracers have been used numerous times to estimate digestion rates (e.g., Dolan and Šimek, 1998; Li et al., 2001; Nishibe et al., 2002; Setála et al., 2005). It is important to mention that the calculation of the ΔC in Fig. 4c has the implicit assumption that C scales directly with the size of the fluorescent inclusion inside the predator. However, if there is biochemical fluorescence quenching, the assumption will not hold, and the digested fraction will be over-estimated, resulting in a biased digestion rate.

In our experiments, all predators exhibited a higher LP\textsubscript{max} at higher prey concentrations than at lower prey concentrations. At high prey concentrations, the chances of encountering prey are higher (e.g., Klarboe, 2011), which results in a higher ingestion rate (Tables 2 and S2). If the LP\textsubscript{max} had been similar between the two concentrations, the explanation would likely be the internal capacity to store food; however, as this was not the case (see Fig. 1), we can rule this factor out. Digestion rates, on the other hand, may be directly responsible for the determination of t\textsubscript{sat}, which can in turn affect LP\textsubscript{max}. Higher t\textsubscript{sat} valued at higher prey concentrations (as seen in protozooplankton – G. dominans and O. marina) imply a direct correlation between ingestion and digestion, characteristic of an opportunistic predator who exploits food patches as it finds them. The opposite scenario (seen in mixoplankton – K. armiger and K. veneficum) suggests that the digestion time is always constant, irrespective of the prey food concentration. This could be explained by limited access to phagotrophic mechanisms, which would limit the digestion rate and result in quicker satiation (internal stability) of the
G. dominans displayed the largest differences in LP_{max} between saturating and non-saturating food concentrations. Additionally, this dinoflagellate exhibited a marked selectivity against labelled *I. galbana*, which was partly dependent on the concentration of prey (Fig. 3). The opposite (i.e., preference for *I. galbana*) was observed in *K. armiger* at saturating, but not at low food concentrations, whereas *O. marina*’s species preference was unaffected by the concentration of prey (Fig. 3). Despite not having been strongly emphasized in the past (but see Jürgens and DeMott, 1995 and Dolan and Šimek, 1999), this concentration-dependent selectivity could partially explain the massive dissimilarities seen in *G. dominans*’ LP_{max}. We are unable to confirm whether the results with *G. dominans* are a consequence of size or fluorochrome selectivity, although size appears to be the main driver, since Martínez et al. (2014) demonstrated that this dinoflagellate seemed to have a slight preference for fluorescent over unlabelled prey. However, the measured ingestion rates using the LFLA methodology fail to give the total ingestion of prey by *G. dominans* (see Table 2).

One of the assumptions that is crucial for the estimation of grazing rates using the LFLA technique is that the tracer is incorporated at similar rates as the remaining prey (i.e., E_{i}^{*} \approx 0 regardless of the number of prey species available). This is an issue that, as far as we know, has not been thoroughly evaluated in the past but seems to be of major importance considering our results. In fact, to illustrate the importance of considering selectivity towards or against the tracer, let us consider the scenario where *G. dominans* was offered a mix of the two prey species at the highest prey concentration. Assuming that there was no discrimination between the two prey species, we obtained a total ingestion rate of ca. 2.2 prey predator^{-1} h^{-1} (Table 2). If we consider the negative selection against the tracer (i.e., a ca. 7.4x higher clearance rate on *R. salina* than on *I. galbana*), the total ingestion rate is ca. 11.9 prey predator^{-1} h^{-1}, a value ca. 5.4x higher than the original estimation.

Similarly, the results obtained with *Karlodinium* spp. suggest that there could be problems in the extrapolation of community grazing rates...
for these species if selectivity is ignored. For instance, we know that one *K. armiger* ingests ca. 8–10 *R. salina* d⁻¹ at saturating food conditions (Arias et al., 2020; Berge et al., 2008b), a value which is ca. 7-9x higher than the one obtained for *K. veneficum* on the same prey (listed as K21 - Calbet et al., 2011). Yet, the results from the LFLA experiment at the same concentrations yielded a total ingestion rate only 1.6x higher for *K. armiger* (selectivity issues ignored, Table 2), besides estimating a higher LPmax for *K. veneficum* (Fig. 1c,d). Nevertheless, it must be noted that *K. veneficum* was offered *T. amphioxeia* instead of *R. salina* (i.e., no size differences between the LFLA and the cryptophyte during the incubations). Thus, the values displayed for *K. veneficum* in Table 2 are probably quite accurate, whereas if selectivity is considered, the total ingestion rates for *K. armiger* become only ca. 0.5 cells predator⁻¹ h⁻¹. As such, these results suggest that using a small-sized LFLA like *I. galbana* favours the estimation of grazing in *K. veneficum* over its larger congener, *K. armiger*, which is unsurprising since size preferences are typically directly correlated with one’s own size (e.g., Hansen et al., 1994).

A way to circumvent the size-selectivity issue could have been to use two different sizes of labelled prey (e.g., Calbet et al., 2012; Martínez et al., 2014). Ideally, both prey would be simultaneously labelled with fluorochromes with distinct emission spectra, which would result in a different fluorescent colour (Nelson et al., 2009; Shields and Smith, 2008). For example, *I. galbana* could be labelled with CMFDA (absorption/emission maxima of 492/517 nm i.e., green fluorescence, viable labelling - e.g., Li et al., 1996) and *T. chui* could be simultaneously labelled with CMAC (absorption/emission maxima of 353/466 nm i.e., blue fluorescence, viable labelling - e.g., Calbet et al., 2012; Martínez et al., 2014). With the assistance of two different filters fitted in the same microscope, it should be possible to obtain nearly simultaneous assessments of blue and green fluorescent inclusions within a given grazer. It should be noted, however, that fluorochromes (like our secondary prey *R. salina* and *T. amphioxeia*) remain elusive in terms of fluorochrome retention (being proflavine the only compound successfully retained by *R. salina* – Johnson et al., 2018) and are still typically detected using their own signature pigment, phycocyanin (e.g., Adolf et al., 2008; Johnson, 2015; Li et al., 2001; Li et al., 1996, Fig. S1b). For an objective analysis of species that can (or cannot) be stained with CMFDA, see Fig. 4 in MacIntyre and Cullen (2016). It is important to mention that CMFDA fades quickly in the light (Li et al., 1996), unlike CMAC (Martínez et al., 2014; this study), which could be a problem if one requires the experiment to run over more extended periods. Yet, the *t₁/₂* of the predators occurred within a short period (<1 h at 19 °C, see Figs. 1 and 2a, < 2 h at 15 °C, see Fig. 4) irrespective of prey concentration, trophic mode, and taxonomic group, which puts this problem on a secondary level. That is, of course, if prey incorporation experiments are conducted instead of prey disappearance ones (see the discussion on FLB by Caron, 2001).

Still, even if a sample is dual labelled, there may be a selection for or against the labelled prey due to the presence of the fluorochrome. In the cases where there is a positive/negative selection for the fluorochrome, an alternative could be to incorporate the fluorescent tracer on the genome of the tracer algae (for example, by fusing a green fluorescent protein, GFP, to a housekeeping gene). This is an advantage even over vital stains (Epstein and Rossel, 1995) and is getting attention from the scientific community since the GFP vector is very bright and easily detectable, besides being photo-stable (Bochdansky et al., 2015). Despite being highly promising, this approach is only publicly available for bacteria at the time of writing (e.g., Jia et al., 2002; Pu et al., 2003; Bochdansky and Clouse, 2015). Still, it is important to mention that Zhang et al. (2013) state that they have successfully inserted GFP on a strain of *Hemiselmis virens*, and detected it inside three mixoplanktonic dinoflagellates, *Prorocentrum donghaiense*, *Karenia mikimotoi*, and *Alexandrium catenella*.

Aside from size selectivity, the experiments with the pSNCM *M. rubrum* confirmed that species selectivity is a problem that cannot be circumvented. When its preferential prey was absent (see for example *Yih et al.*, 2004 and *Smith and Hansen*, 2007) the number of BFI per *M. rubrum* followed the typical satiation pattern (Fig. 2a). However, when *T. amphioxeia* was present, BFI were mostly absent inside *M. rubrum* (Fig. 2b). In the laboratory, it is possible to control both the grazer and the prey, but one cannot choose what species will be present in the field. As the functional group name suggests, *M. rubrum* is a specialised predator and retains chloroplasts mostly from a specific clade of cryptophytes, including *T. amphioxeia* among others (e.g., Hansen et al., 2012; Myung et al., 2011). Still, *M. rubrum* can ingest other flagellates as well (Ferreira and Calbet 2020; Hansen et al., 2012; Park et al., 2007; this study), and is able to retain chloroplasts from *Rhodomonas* spp. for short periods of time (Myung et al., 2011). Accordingly, our results with *I. galbana* are not surprising. Yet, in the field, the presence of *M. rubrum* is typically correlated with the presence of cryptophytes (e.g., Johnson et al., 2018), which means that it is doubtful that a field experiment will be able to capture the feeding process of this ciliate using a tracer particle that is not its own preferred prey.

Another issue with this technique that we have shown in our experiments concerns tube-feeding dinoflagellates. Indeed, as confirmed with 4 independent predator-prey combinations from distinct trophic modes, predators that feed using a peduncle tend to become entirely stained themselves instead of displaying individualized (and countable) BFI. In addition, the low grazing rates obtained with *K. armiger* (Fig. 1d) may have been due to the fact that this dinoflagellate feeds using a peduncle (Berge et al., 2008a). Based on our results with tube feeders, it is likely that some *K. armiger* did contain LFLA, but we were unable to see them because of the dispersal of the fluorochrome throughout the predator’s cytoplasm. This issue appears to become more relevant with the passing of time, as some *K. armiger* cells did, on the other hand, contain easily distinguishable and countable BFI (see Fig. 5a,c). To further complicate the peduncle feeding matter, it should be mentioned that the incorporation of ingested material through a peduncle may flow into a single (e.g., Calado and Moestrop, 1997) or several (smaller) food vacuoles (e.g., Hansen, 1991). Also, small cells like *I. galbana* (ESD ca. 4.5 μm) may be taken whole through a peduncle in some cases (e.g., Calado and Moestrop 1997), explaining why *K. veneficum* was, apparently, issue-free.

Tube feeding is a common characteristic in both naked and thecate dinoflagellates (Hansen and Calado, 1999), suggesting that this caveat of the technique may be an essential factor to consider when applying this technique in mixed assemblages in the field. One other long-known feeding mechanism in dinoflagellates (particularly in the genus *Proto- peridinium* spp.) is the deployment of a pallium, which liquefies the cytoplasmic contents of the prey extracellularly using a pseudopod, before the contents are brought into the cell (Gaines and Taylor, 1984; Jacobson and Anderson, 1986). Accordingly, pallium feeders do not transport entire prey cells into the main cell body and the presence of food vacuoles might not be a concrete measurement of ingested prey (Archer et al., 1996; Hansen and Calado, 1999). We did not experiment on pallium feeders and, as such, we can only simply hypothesise on these organisms. Still, it seems reasonable to assume that these dinoflagellates would probably appear as a very intense and uniformly bright cell, due to even incorporation of the fluorochrome. On a similar, albeit almost species-specific note, comes the feeding by toxic mucus traps. This feeding mechanism has only been reported, as far as we know, in the species *Alexandrium pseudogonyaulax* (Blossom et al., 2012, 2017) and on a *Prorocentrum* sp. (Michaela E. Larsson, MScITN Symposium, pers. comm.). To note that *Dinophysis* spp. also produces toxic mucus traps but does not carry them around the medium and, for comparison purposes, does not fall into the same category as *A. pseudogonyaulax* and *Prorocentrum* sp. (Mafra et al., 2016; Ojamäe et al., 2016; Papiol et al., 2016). *A. pseudogonyaulax* consumes whole cells through the sulcus but is unable to capture individual, motile prey cells. Therefore, it deploys a mucus trap that immobilises (but does not
kill on a short time scale) prey and, by swimming with it attached to its own body, frequently ends up entrapping several preys inside. Accordingly, these predators may affect the availability of prey without exhibiting food vacuoles or, even if showing clear and distinguishable BFIs, provide unrealistic grazing rates.

5. Conclusions

Altogether, the LFLA technique appears to be only directly applicable to organisms that feed by direct engulfment of prey. Indeed, all other feeding mechanisms (present both in protozoan- and mixotanktonic dinoflagellates) result in biased or even unmeasurable ingestion rates. For engulfment feeders, though, LFLA can be used to account for ingestion and digestion rates and, due to its short-term nature, even discriminate between day and night periods of the day. In this regard, we must stress the fact that our predators reached the plateau phase of LFLA incorporation within 1 h at 19 °C (Figs. 1 and 2a) and 2 h at 15 °C (Fig. 4a), and that digestion of prey is usually relatively fast (faster during the day than during the night – Fig. 4b,c). Therefore, to provide accurate grazing estimates and avoid finding organisms that display no food vacuoles due to digestion (e.g., Adolf et al., 2006; Matantseva and Skarlato, 2013), we recommend that all samples of a given LFLA experiment should be collected in short intervals (e.g., 10 min) and within a maximum period of 1 h (with a possible extension/shortening depending on the environmental temperature – Li et al., 2001).

At last, we have provided clear evidence that size and species-specific selectivity are hard to circumvent. As seen in the Teleaulax-Ischyrosis-Mesodinium incubation (and, on a qualitative extent, also on the Mesodinium-Dinophysys interaction), these predators exhibit species-specific selectivity, which hindered the quantification of ingested tracer prey. This conclusion is likely valid for all the specialised protist predators on Earth and implies that they may be systematically discriminant against if one decides to use LFLA in the field. Nevertheless, we proved that *M. rubrum* can be effectively labelled with CMAC, demonstrating that it is not impossible to determine grazing rates for species-specific predators if their specific prey is offered as a labelled tracer. In addition, we propose that dually labelling samples with CFMFA and CMAC should become routine when applying the LFLA technique in the field. The combined use of these two fluorochromes can either be used to distinguish different prey size ranges or, alternatively, to account for species-specific grazing within a given water column. We should keep in mind, however, that despite the proved usefulness of the LFLA technique, our conclusions are based on the assessment of a limited number of species on a limited number of prey and, therefore, further studies are needed to capture the full protistan diversity.

Author contributions

GDF, AC, and PJH conceptualized the experiments. GDF, and JP counted and photographed epifluorescence samples. SCM, AC, and PJH provided material, facilities and assistance to the counting of epifluorescence samples. SCM, AC, and PJH acquired funding for both GDF and JP. GDF and AC prepared the original draft manuscript. All authors commented and contributed to the final version of the manuscript thus justifying all authorships.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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