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Acidotropic probes and flow cytometry: a powerful combination for detecting phagotrophy in mixotrophic and heterotrophic protists

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ABSTRACT: Studies with phagotrophic organisms are hampered by a series of methodological constraints. To overcome problems related to the detection and enumeration of mixotrophic and heterotrophic cells containing food vacuoles, we combined flow cytometry and an acidotropic blue probe as an alternative method. Flow cytometry allows the analysis of thousands of cells per minute with high sensitivity to the autofluorescence of different groups of cells and to probe fluorescence. The method was first tested in a grazing experiment where the heterotrophic dinoflagellate *Oxyrrhis marina* fed on *Rhodomonas salina*. The maximum ingestion rate of *O. marina* was 1.7 prey ind.$^{-1}$ h^{-1}, and the frequency of cells with *R. salina* in the food vacuoles increased from 0 to 2.4 ± 0.5 × 10^3 cells ml^{-1} within 6 h. The blue probe stained 100% of *O. marina* cells that had *R. salina* in the food vacuoles. The acidotropic blue probe was also effective in staining food vacuoles in the mixotrophic dinoflagellate *Dinophysis norvegica*. We observed that 75% of the *D. norvegica* population in the aphotic zone possessed food vacuoles. Overall, in cells without food vacuoles, blue fluorescence was as low as in cells that were kept probe free. Blue fluorescence in *O. marina* cells with food vacuoles was 6-fold higher than in those without food vacuoles (20 ± 4 and 3 ± 0 relative blue fluorescence cell^{-1}, respectively), while in *D. norvegica* cells were 4.5-fold brighter than the ones without food vacuoles (291 ± 155 and 64 ± 23 relative blue fluorescence cell^{-1}, respectively). The use of acidotropic probes can prevent fixation artifacts such as regurgitation of food vacuoles and changes in the cellular characteristics. The combination of flow cytometry and an acidotropic probe proved to be an efficient tool in detecting phagotrophy in mixotrophic and heterotrophic marine phytoplankton species.

KEY WORDS: Mixotrophy · Phagotrophy · Acidotropic probes · Flow cytometry

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INTRODUCTION

Studies in which phagotrophy is addressed are still seriously hampered by tedious and often time-consuming methods (Riemann et al. 1995). Over the last decades, many methodologies have been developed to detect phagotrophy in heterotrophic and mixotrophic phytoplankton species. Visually detected and manually counted tracer-based techniques are the most common approaches for estimating phagotrophy in algae (Havskum & Riemann 1996, Granéli & Carlsson 1998, Legrand et al. 1998, 2001, Pålsson 2001). Surrogate prey, including dye and starch particles, fluorescent microspheres (Smalley et al. 1999), fluorescently labeled bacteria and algae have been used to investigate feeding behaviors and to measure ingestion rates of planktonic protists (Li et al. 1996).

Although the use of fluorescently labeled prey has resulted in an increased understanding of phagotrophy, advances in this field have been slow (Granéli & Carlsson 1998). Usually a large number of cells must be counted manually with the help of a microscope in order to achieve statistically significant ingestion rates, a monotonous and lengthy procedure. In addition, epifluorescence microscopy has a low resolution with regard to detection of ingested fluorescent prey, espe-

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cially in pigmented mixotrophs that exhibit high autofluorescence (Riemann et al. 1995, Havskum & Riemann 1996, Hansen 1998, Hansen & Calado 1999, Setala et al. 2005). Techniques that radioactively label the prey (Nygaard & Tobiesen 1993, Granéli et al. 1997) have the advantage of being analytically sensitive. However, these techniques have limitations, such as short-term incubations and the large amount of handling required (Smalley et al. 1999). Moreover, these approaches may have several sources of contamination: transfer of radioactivity from one organism to another without effective ingestion, labeled bacteria can be attached to the theca of mixotrophs or heterotrophs instead of being ingested, and the cell membranes can also become contaminated by the isotopes (Legrand et al. 1998).

Thus, results obtained by the use of artificial or manipulated prey coupled with microscopy can be affected by a number of errors. (1) Organisms that can actively select their prey might reject artificial or surrogate prey and phagotrophy may go undetected or be underestimated (Bockstahler & Coats 1993a,b, Havskum & Riemann 1996, Legrand et al. 1998, Smalley et al. 1999, Stoecker 1999, Hammer et al. 2001). In addition, artificial prey does not allow growth and adaptation of predators to prey (Hammer et al. 2001) and some protists can egest the particles 2 or 3 min after ingestion (Weisse 2002), leading to unrealistic ingestion rates. (2) Human counting errors (Marie et al. 2004) enhanced by the difficulties of identifying phagotrophy in algae species (Porter 1988, Legrand et al. 1996, Bouvier et al. 1998, Granéli & Carlsson 1998, Ishii et al. 2002, Rose et al. 2004), and questionable detection of phagotrophy (Stoecker 1999) also lead to under- or overestimations of the occurrence of phagotrophy among phytoplanktonic species.

The food vacuole method, mostly used to estimate grazing by heterotrophic dinoflagellates and ciliates, has also been extended to estimate phagotrophy in mixotrophs (Bernard & Rassoulzadegan 1990, Dolan & Coats 1991, Bockstahler & Coats 1993a). This method is based on observations of food vacuole content coupled to experimentally determined digestion rates (Li et al. 2001). Although the content of food vacuoles in larger protists may be identifiable, this technique is inadequate when studying small flagellates (Riemann et al. 1995). Another problem is determining whether the particle inside a cell is a prey, parasite or gamete (Granéli & Carlsson 1998, Legrand et al. 1998). Consequently, the presence of particles inside the cells does not necessarily imply phagotrophy (Porter 1988, Granéli & Carlsson 1998, Legrand et al. 1998).

An effective way of accelerating cell counting, minimizing human error and improving the sensitivity of the analysis is to use automatic particle counting (Marie et al. 2004). One of the most successful automated cell counting and analysis methods is flow cytometry, which is increasingly being used to characterize the microbial planktonic community structure in aquatic systems (del Giorgio et al. 1996, Cucci & Sieracki 2001). In the last decade, flow cytometry has been successfully applied to enumerate bacteria (del Giorgio et al. 1996, Marie et al. 1997, Troussellier et al. 1999), marine viruses (Marie et al. 1999), small ciliates (Lindström et al. 2002), and most recently to verify phagotrophy in heterotrophic cells based on acidotropic probe fluorescence (Rose et al. 2004). In contrast to the time-consuming microscopy-based methodologies (Lepesteur et al. 1993, Granéli & Carlsson 1998, Lindström et al. 2002), flow cytometry allows single-cell analysis of thousands of cells within minutes (Avery et al. 1995, Collier 2000, Marie et al. 2004), providing statistically significant data (Marie et al. 1997, 1999, Collier 2000). The precision of flow cytometric counts are on average 10 times higher than epifluorescence counts, and sample processing time can be reduced by 10- to 20-fold (del Giorgio et al. 1996).

Although flow cytometry is a versatile tool, it faces the same optical problem as microscopic analysis when analyzing heterotrophs that have fed on colorless prey or mixotrophic cells that have fed on autotrophic prey. Due to interference from the strong autofluorescence of chlorophyll and accessory pigments of the mixotrophs, it is virtually impossible to detect the ingested prey, as both have the same fluorescence. The problem is even worse for heterotrophic prey, which lack pigments.

To overcome the difficulties described above, we developed a method that combines the flexibility and speed of flow cytometry techniques with an acidotropic probe (LysoSensor™ Blue DND-167, Molecular Probes) that stains acidic organelles such as food vacuoles. The few published studies using LysoSensor™ probes can be found in medical journals (Lin et al. 2001).

Our main objective was to develop a fast and reliable method for detecting phagotrophy in phytoplanktonic species. The use of flow cytometry in combination with pH-specific probes might solve some of the methodological constraints cited above. Furthermore, our methodology allows real-time observation of the changes in the behavior of heterotrophs and mixotrophs under different environmental conditions such as nutrient sufficiency and deficiency, and different densities of co-occurring species of prey. This approach might help to more accurately assess the role of mixotrophic and heterotrophic predators in aquatic food webs.

MATERIALS AND METHODS

Flow cytometer (FCM) setup. All samples were analyzed in a FACSVantage SE FCM (Becton & Dickinson) with a 200 µm aperture nozzle, and dual excitation: 488 nm (blue laser) and 360 nm (UV laser). The cells were gated based on their size, shape and cell structural complexity using forward scatter (FSC) and side scatter (SSC) light. In addition, 3 photomultipliers measured cell autofluorescence: Green fluorescence (FL1) was collected through a 530/30 nm filter. Orange fluorescence (FL2), usually emitted by phycoerythrin (PE), was collected through a 585/42 nm filter. Red fluorescence (FL3) from chlorophyll (chl) was detected through a 675/20 nm filter. Finally, the probe's blue fluorescence (FL4) was detected in a fourth photomultiplier through a 424/44 nm filter. Flow rates and events per minute were determined by counts of reference beads (Trucount™ Tubes, Becton & Dickinson). FACS flow (Becton & Dickinson) was used as the sheath fluid for all flow cytometric analyses. This sheath fluid has lower salinity (9 psu) than the cultures (26 psu) used in the grazing experiment (see below). Note that such mismatch in salinity can cause significant distortions when using FSC to determine cell sizes <3 µm. However, the FSC of cells in the size range of 3 to 5 µm are less affected and cells >5 µm are not affected (Cucci & Sieracki 2001). In the other tests performed (see below), the difference of salinity between the sheath fluid and the natural samples (8 psu) was small.

The blue probe. LysoSensor™ probes are weak bases that are selectively concentrated in acidic organelles as a result of protonation. The protonation of the LysoSensor™ molecule results in fluorescence, and as the molecule is positively charged it will not cross membranes easily and will remain in organelles with an acidic pH (Molecular Probes information) as found in food vacuoles. The probe emits fluorescent blue light in acidic organelles when excited by UV light. Blue dyes are expected to display high signal-to-noise ratios, which are usually needed for automated flow cytometric analysis.

Test of LysoSensor™ blue probe in the heterotrophic dinoflagellate *Oxyrrhis marina*. Batch cultures: *O. marina* (Strain CCMP 604, Provasoli-Guillard National Center of Culture of Marine Phytoplankton, USA) was grown in f/50 medium (Guillard 1975) in a 2 l glass bottle and fed regularly with *Dunaliella* sp. The autotrophic prey *Rhodomonas salina* (Strain KAC 30, Kalmar University Algal Collection, University of Kalmar, Sweden) grew in f/10 medium (Guillard 1975) modified to Redfield ratio. All media were prepared with filtered (0.22 µm glass fiber filter) and autoclaved Baltic Sea surface water (7 psu). Salinity was adjusted to 26 psu by adding NaCl. The cultures were kept at 16 ± 1°C and a light intensity of 100 µE m^2 s^{-1}, with a 16:8 h light:dark cycle, till they reached high densities (4.5×10^3 and 8.5×10^5 cells ml^{-1} for *O. marina* and *R. salina*, respectively). *Dunaliella* sp. cells (previously used as food) were removed from the *O. marina* stock culture using dilution and reverse filtration (adapted from Dodson & Thomas 1964) through a 10 µm mesh-size plankton net. The volume of the culture was carefully lowered to 100 ml and diluted again up to 2 l with pure new f/50 medium. The reverse filtration process was repeated, and the medium replaced up to 1 l. To allow *O. marina* cells to recover from the filtration process and feed on the *Dunaliella* sp. cells remaining after the 'washing' process, the culture was left on standby for 24 h before beginning the grazing experiment.

The grazing experiment was performed as shown in Fig. 1. *Rhodomonas salina* was offered as prey to *Oxyrrhis marina* (8.5×10^3 and 4.5×10^3 cells ml^{-1}, respectively) in a 2:1 prey:predator ratio (mixed culture).

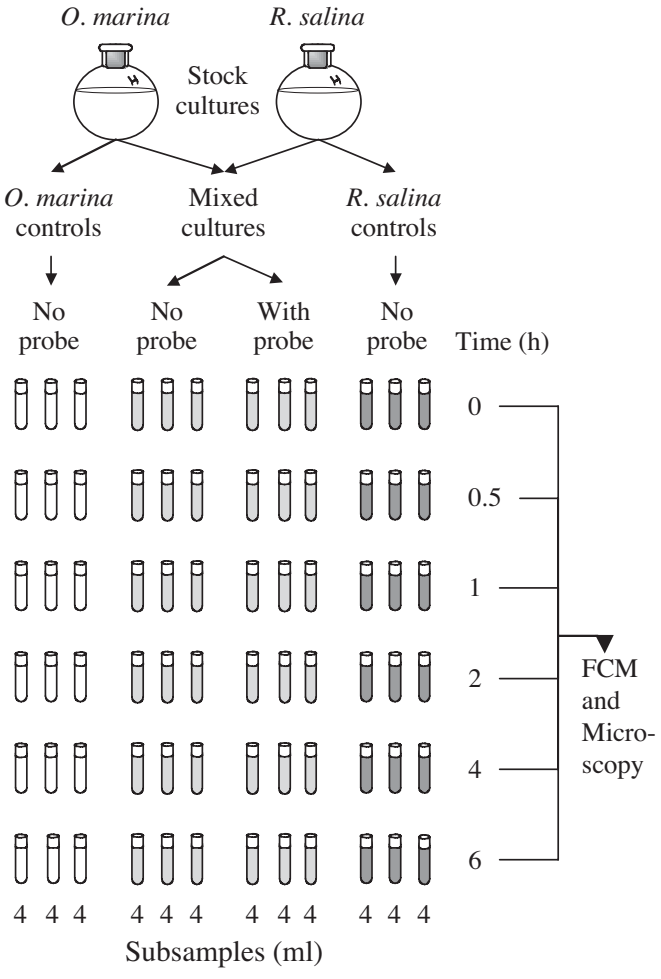


Fig. 1. Experimental setup used to compare detection of phagotrophy by *Oxyrrhis marina* cells on *Rhodomonas salina* based on LysoSensor™ blue probe and prey's autofluorescence

The controls (*O. marina* and *R. salina* monocultures) were diluted with medium to achieve the same initial cell densities as in the mixed culture. Of the cultures, 10 ml were distributed to sterile 14 ml polypropylene tubes (Falcon). Each control was composed of 18 tubes, and the mixed culture 36 tubes; the controls and half of the mixed culture tubes did not receive probe prior to flow cytometric analysis, whereas the other half of the mixed culture tubes were incubated with probe as described below. Three replicates of each control and 6 replicates of mixed culture were taken immediately after the cultures were mixed or diluted (control) and then at 0.5, 1, 2, 4, and 6 h to be analyzed. Subsamples (4 ml) were taken from every replicate to be analyzed in the FCM and observed under epifluorescence microscopy. After analysis the replicates were discarded to avoid artifacts caused by the handling process.

Probe incubation: Of LysoSensor™ Blue (DND-167, Molecular Probes), 8 µl were added to the mixed culture subsamples (4 ml, final probe concentration = 2 µM), and then incubated in darkness for 10 min at room temperature. After incubation, each subsample was analyzed in the FCM for 3 min at a flow rate of 92 µl min^{-1}, giving an average counting rate of 11 ± 2.7×10^3 cells min^{-1}. *Oxyrrhis marina* and *Rhodomonas salina* were differentiated from each other based on their relative size, shape, and pigment autofluorescence. The presence of food vacuoles in *O. marina* was detected based on probe fluorescence, which was compared with the increase in chl and PE fluorescence from the prey in the *O. marina* cells. The remaining sample was observed under UV and ordinary light in an epifluorescence microscope (Olympus BX50) in order to verify non-specific bind of the probe on cell structures other than food vacuoles.

Test of LysoSensor™ blue probe in the mixotrophic dinoflagellate *Dinophysis norvegica* from natural samples. ***Field sampling:*** Two samplings were done in the Baltic Sea (Baltic Proper region: 56° 36′ 00″ N, 16° 52′ 00″ E, and 56° 04′ 88″ N, 16° 29′ 38″ E, respectively) during the summer of 2004.

First sampling (June 29): Three vertical net (20 µm mesh-size) tows integrated cells through a 30 m deep water column. The samples were passed through a 70 µm mesh-size plankton net to remove bigger particles, such as debris and other planktonic cells. The samples containing a high density of *Dinophysis norvegica* cells (345 ± 23 cells ml^{-1}, final density) were kept in 2 polycarbonate bottles (20 l) under the same conditions as described above. LysoSensor™ Blue was tested in *D. norvegica* and other heterotrophs or mixotrophs present in the natural samples. The cells were analyzed in the FCM and observed in an epifluorescence microscope (Olympus BX50) under ordinary and UV light at 400× magnification.

Due to the high densities of *Dinophysis norvegica* in the samples from the first sampling occasion, they were used to establish incubation time and probe saturation curves for LysoSensor™ when working with a mixotrophic species.

Probe concentration: A dilution series of the probe was done in order to establish a probe saturation curve to be applied in further experiments with mixotrophic species. Different probe concentrations (0.2, 0.5, 1, 2, and 3 µl ml^{-1}) were added to aliquots of 2 ml samples (final probe concentration: 0.2, 0.5, 1, 2 and 3 µM, respectively), and incubated in darkness for 10 min at room temperature, and then counted for 1 min in the FCM according to the setup used previously.

Incubation time: Samples (2 ml) containing *Dinophysis norvegica* cells were incubated with blue probe (1 µl ml^{-1}, final probe concentration 1 µM) for 0.5, 5, 15, 30, 60, 120, and 180 min to assess the optimal incubation time to be applied in future experiments. After incubation, the samples were analyzed in the FCM for 1 min as described previously.

Second sampling (July 27): Samples were taken to quantify the percentage of *Dinophysis norvegica* cells containing food vacuoles in a vertical profile. To carry out the flow cytometric analysis, 12 l of water were pumped at 5 m intervals between the surface and 30 m depth. The samples were brought back to the lab within 2 h and kept at low room temperature (16°C). Particles >70 µm were removed from the samples as described previously. Prior to analysis, *D. norvegica* cells were concentrated and small particles removed by reverse filtration (adapted from Dodson & Thomas 1964) through a 25 µm mesh-size net. By concentrating cells, the speed of analysis increases since FCM can handle thousands of cells per second. Immediately after concentration, 2 µl of blue probe were added to 2 ml of concentrated sample (final concentration 1 µM) and incubated for 10 min at room temperature. The samples were analyzed in the FCM for 1 min according to the setup described above.

RESULTS

When excited by UV light and observed under an epifluorescence microscope, LysoSensor™ Blue was capable of staining food vacuoles in all heterotrophic and mixotrophic cells tested thus far (Fig. 2). Since LysoSensor™ is pH dependent, its fluorescence in the cells is strongly affected by number, size and digestion stage of the prey in the food vacuoles. In our test, cells containing food vacuoles had strong relative blue fluorescence, while cells without food vacuoles had fluorescence equal to the background, i.e. blue fluorescence detected in cells without probe. The blue probe

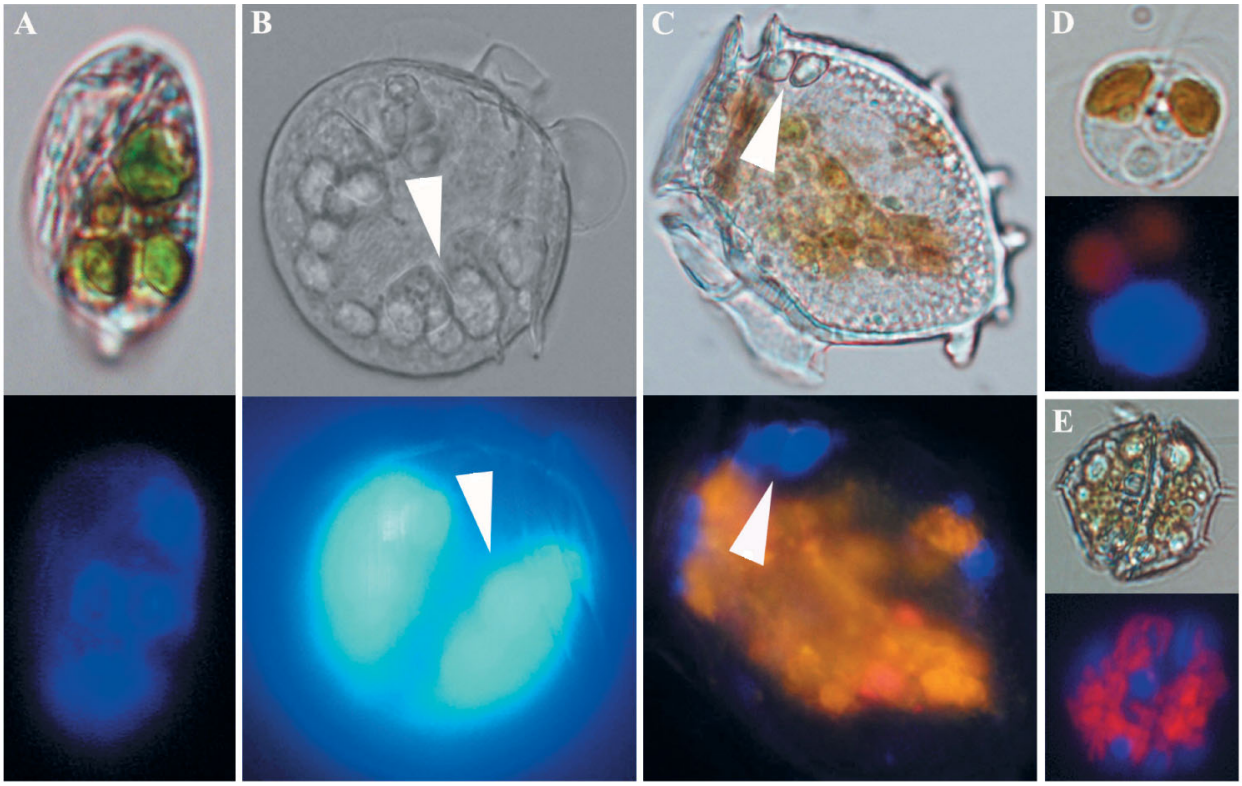


Fig. 2. Bright-field (upper panel) and epifluorescence (lower panel) micrographs of (A,B) heterotrophic and (C–E) mixotrophic species containing food vacuoles (arrowheads). Except for (A) and (D), all cells are from the Baltic Sea. (A) *Oxyrrhis marina*, (B) *Dinophysis rotundata*, (C) *Dinophysis norvegica*, (D) *Prymnesium parvum*, and (E) an unidentified peridinioid. Cells were stained with LysoSensor™ blue probe and observed under ordinary light (upper panel) and UV light (lower panel)

did not affect *Rhodomonas salina* (Cryptophyceae) and other autotrophic species such as *Dunaliella* sp. (Chlorophyceae) and *Teleaulax amphioxeia* (Cryptophyceae) in any of the preliminary observations under the microscope or FCM (data not shown).

On average, $3.8 \pm 2.7 \times 10^3$ *Oxyrrhis marina* cells ml^{-1} were analyzed per replicate of mixed culture (considering samples incubated with and without probe). The maximum ingestion rate was 1.7 *Rhodomonas salina* ind.$^{-1}$ h^{-1}, and the frequency of *O. marina* cells containing *R. salina* in the food vacuoles increased from 0 to $2.4 \pm 0.5 \times 10^3$ cells ml^{-1} within 6 h. The grazing of *O. marina* on *R. salina* was easily detected using flow cytometry and prey autofluorescence (Fig. 3). Cells of *O. marina* that had engulfed *R. salina* had PE and chl fluorescence (268 ± 42 and 295 ± 18 relative fluorescence, respectively) higher than *R. salina* cells (117 ± 10 PE and 278 ± 4 chl relative fluorescence) (Fig. 3). Conversely, *O. marina* cells that had not ingested *R. salina*, had no PE fluorescence, and chl fluorescence was very low (4 ± 0.14 chl relative fluorescence). There was no significant difference (Student's *t*-test, $t = -0.36$, $p > 0.05$) between phagotrophy detected based on prey autofluorescence and probe's blue fluorescence (Fig. 4). The cells containing *R. salina* in the food vacuoles had a strong probe signal (Fig. 5). Moreover, using the probe we were able to detect *O. marina* cells containing food vacuoles with colorless particles, i.e. food vacuoles with particles such as detritus, other *O. marina* cells (cannibalism) or partially digested food items (Figs. 3 & 4). These colorless food vacuoles also presented a high probe signal (Fig. 5), whereas in the samples without the probe (where the analysis was based only on prey autofluorescence), they were undetected (Figs. 3 & 4). In the sample with no probe, *O. marina* cells either with or without food vacuoles had very low blue autofluorescence (2 ± 0.28 and 2 ± 0.09 relative blue fluorescence, respectively).

When tested in *Dinophysis norvegica*, a mixotrophic thecate dinoflagellate (Jacobson & Andersen 1994, Gisselson et al. 2002), LysoSensor™ Blue showed similar results to those observed for the heterotrophic dinoflagellate *Oxyrrhis marina*, i.e. cells with food vacuoles had high blue fluorescence when incubated with the probe (Fig. 2), whereas cells without food vacuoles had blue fluorescence equal to background (blue autofluorescence). Using single cell analysis, 7.7 ± 0.3

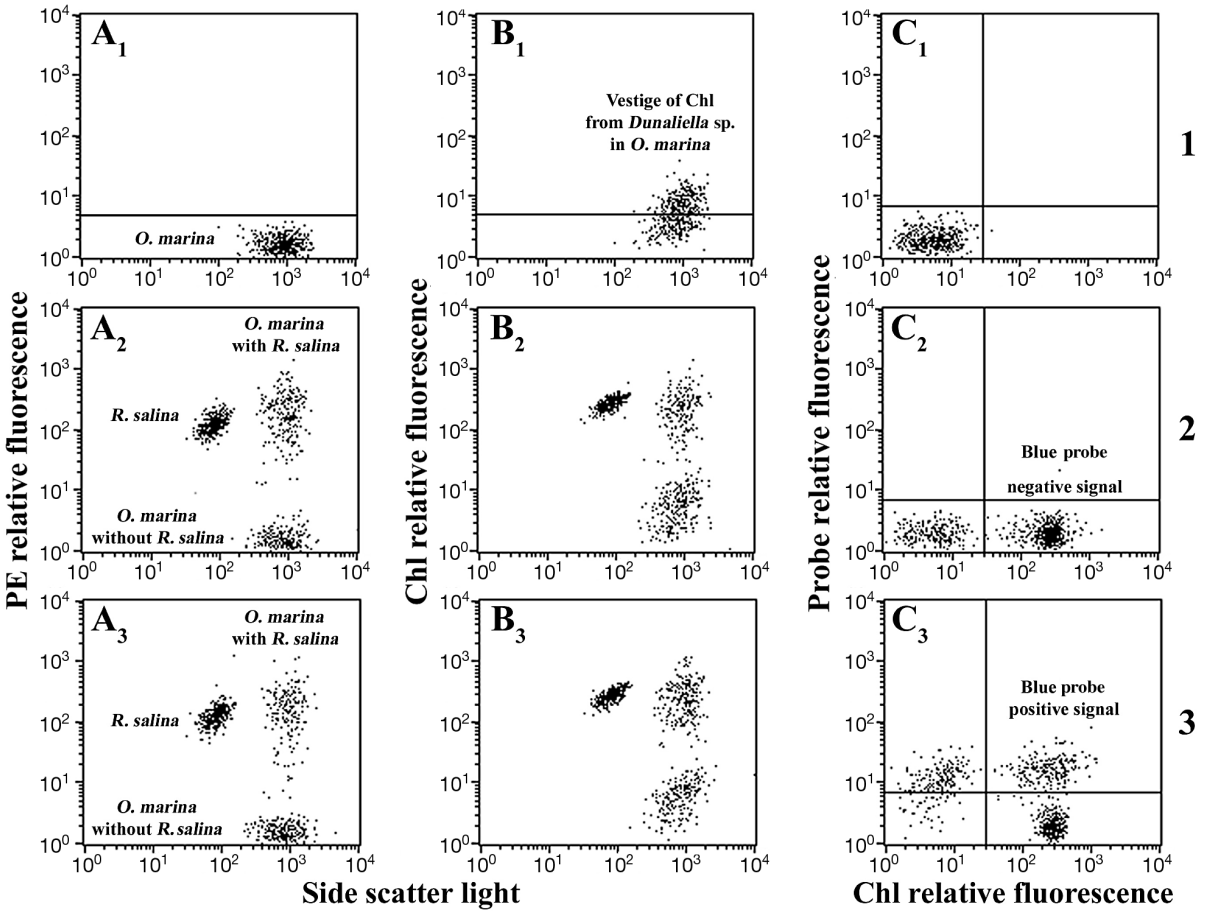


Fig. 3. Cytograms from the grazing experiment. Row 1 shows *Oxyrrhis marina* monoculture without probe; Row 2 represents the mixed cultures without probe; and Row 3 shows mixed cultures incubated with probe. The cells were analyzed based on (A) phycoerythrin (PE) orange autofluorescence and side scatter light; (B) chl red autofluorescence and side scatter light; and (C) LysoSensor™ blue probe fluorescence and chl red autofluorescence

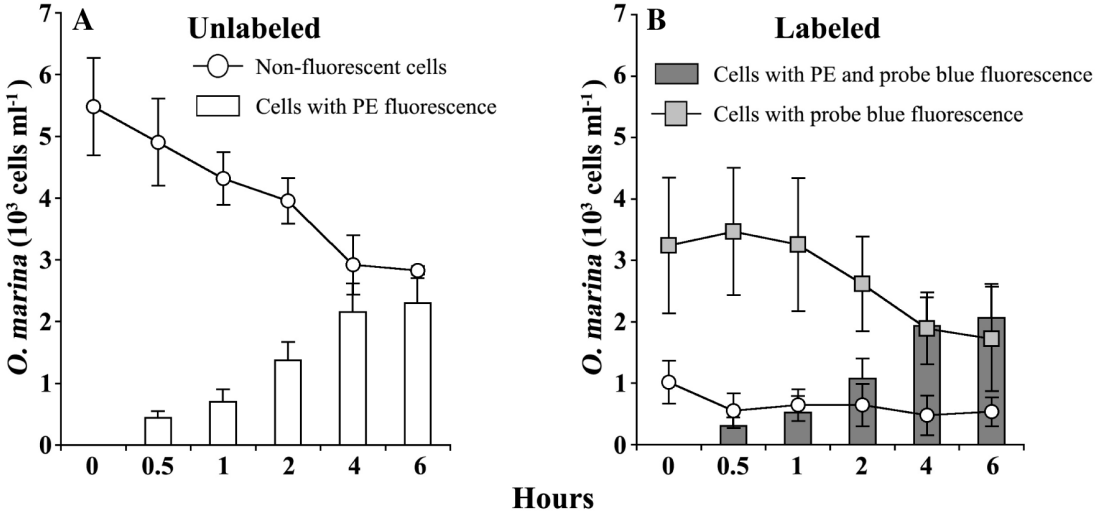


Fig. 4. Frequency of *Oxyrrhis marina* cells containing food vacuoles. Phagotrophy was detected based on (A) *Rhodomonas salina* (prey) autofluorescence, and (B) prey and probe's blue fluorescence. All food vacuoles containing *R. salina* had phycoerythrin (PE) fluorescence. When labeled, all phagotrophic cells were blue fluorescent. *O. marina* cells without food vacuoles were non-fluorescent in both labeled and unlabeled samples

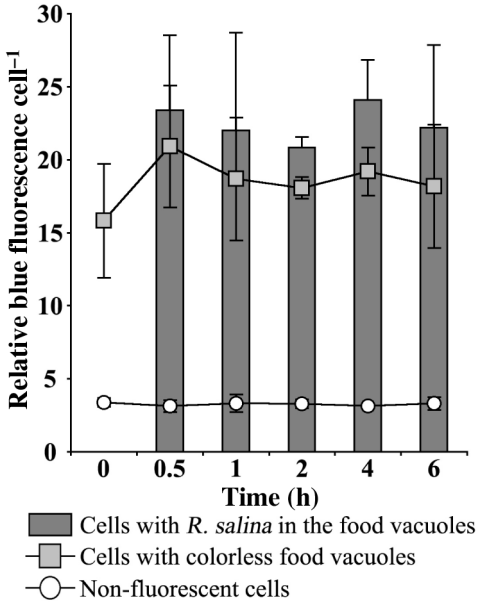


Fig. 5. Probe relative blue fluorescence per *Oxyrrhis marina* cell. For further explanations see Fig. 4

$\times 10^3$ cells were analyzed for each probe concentration tested. Probe concentrations from 0.5 to 3 µM resulted in $1.7 \pm 0.3 \times 10^3$ cells containing food vacuoles. The average blue fluorescence per cell detected in *D. norvegica* incubated with 1 µM was lower (Student's *t*-test, $p > 0.05$) than that observed in the cells incubated with 2 and 3 µM probe final concentration; however, the fluorescence per cell was highly variable in the 3 samples (Fig. 6).

The blue probe accumulated quickly in the food vacuoles. Blue fluorescence was detected between 10 and 12 s after the probe was added to the sample. This is the span between the moment that the cells were taken in the sampling tube and the moment the laser beam struck the cells. On average, $3.3 \pm 1.3 \times 10^3$ *Dinophysis norvegica* cells were analyzed for each incubation period tested. Overall, the probe showed effectiveness when incubated during periods that varied between 0.5 and 30 min, while incubations longer than 30 min led to few cells with low probe signal per cell (Fig. 7). The blue fluorescence per cell was highly variable in both the dilution series and the time series. Different digestion stages of the food items (and consequent different pH in the food vacuoles) and cells with more food vacuoles than others might have caused this high variation.

The technique of coupling flow cytometry and blue probe also proved to be efficient at detecting phagotrophy in natural populations (no adaptation time to lab conditions) of *Dinophysis norvegica*. Populations of *D. norvegica* from different depths were easily distinguished from the rest of the phytoplankton community based on their relative size (FSC), PE (FL2) and chl fluorescence (FL3). The combination of these factors in logical gates allowed us to isolate *D. norvegica* from the rest of the community and then identify cells containing food vacuoles based on probe fluorescence (Fig. 8). The percentage of *D. norvegica* containing food vacuoles increased with depth and a consequent decrease of light. The highest frequency (75%) of phagotrophic *D. norvegica* cells was observed in the

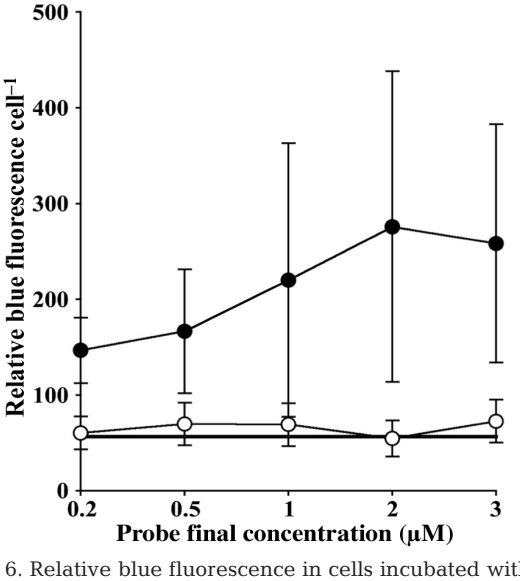


Fig. 6. Relative blue fluorescence in cells incubated with different final concentrations of LysoSensor™ blue probe: (○) cells without food vacuoles, (●) cells with food vacuoles, and (—) background, i.e. blue fluorescence in cells incubated without blue probe

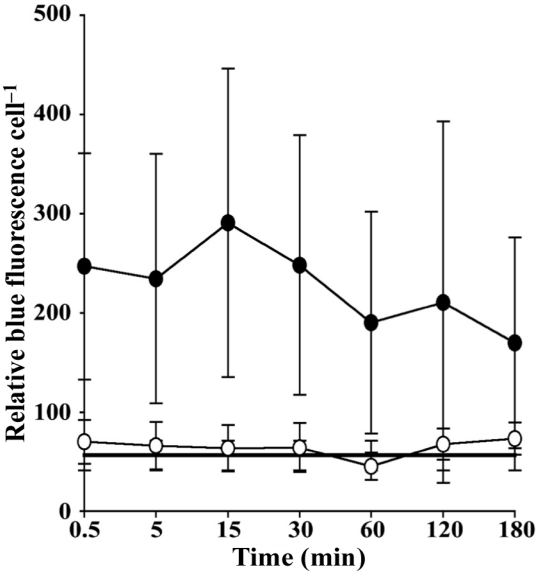
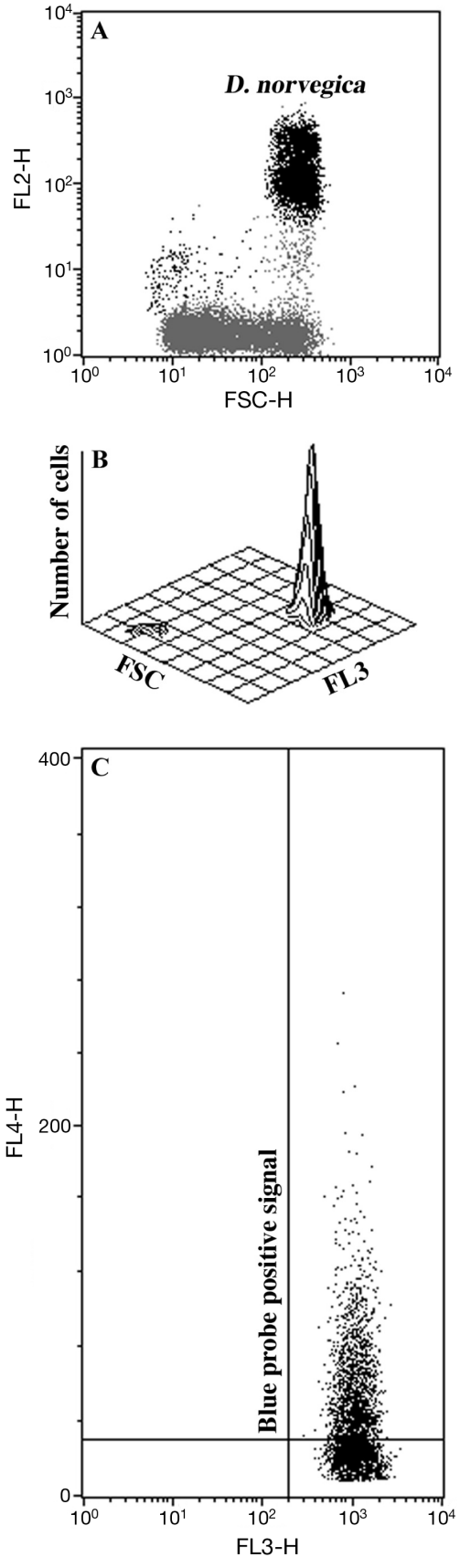


Fig. 7. Blue fluorescence in cells incubated with probe during different incubation times: (○) cells without food vacuoles, (●) cells with food vacuoles, and (—) background, i.e. blue fluorescence in cells incubated without blue probe



aphotic layer (defined as the layer where light was lower than 1% of light shining on the surface). The blue fluorescence in cells with food vacuoles was comparable to the fluorescence detected in the cells collected with net tows in the previous sampling.

DISCUSSION

It is relatively simple to identify phagotrophy by flow cytometry in a heterotrophic organism like *Oxyrrhis marina* when it has been feeding on a pigmented prey such as *Rhodomonas salina*. The detection is even simpler under controlled conditions and when the heterotroph is exposed to a single well-known species of prey. Strict heterotrophs do not have their own photosynthetic pigments (Lindström et al. 2002), permitting reliable detection of an ingested pigmented prey. Indeed, some studies have used flow cytometry to quantify protozoan grazing on photosynthetic microorganisms based on the preys' pigment fluorescence (Avery et al. 1995). However, in a natural environment mixotrophic and heterotrophic microorganisms feed on organic particles (live and non-live) from different sources, a number of which are colorless. In addition, predators might have food vacuoles in different stages of digestion (Weisse 2002). These factors make the detection and therefore quantification of phagotrophy complicated.

In the test with the heterotrophic dinoflagellate *Oxyrrhis marina*, we were able to quantify the frequency of cells containing food vacuoles based on prey autofluorescence. Prey autofluorescence in food vacuoles is affected by ingestion as well as by digestion (Weisse 2002), i.e. food vacuoles with recently engulfed prey had higher autofluorescence than those with partially digested food items. Therefore, it is possible to distinguish newly ingested prey from partially digested food items based on autofluorescence. However, if the mixotroph (or heterotroph) population was fed a colorless prey such as heterotrophic protists, bacteria or detritus, it might be difficult to distinguish the prey inside the predator. The use of the blue probe overcomes this problem because it allows the detection of the food vacuoles, whether the prey has pigments or not.

Fig. 8. Cytograms from analysis of the sample from 30 m depth (second sampling in the Baltic Sea, Summer 2004). (A) Dot plot of forward scatter (FSC) and natural phycoerythrin (PE) fluorescence (FL2). Black dots show *Dinophysis norvegica* population. Gray dots represent the remaining community. (B) Three-dimensional plot of FSC, natural chl red fluorescence (FL3), and number of cells of *D. norvegica*. (C) Dot plot of FL3 and blue fluorescence (FL4) in *D. norvegica* cells incubated with blue probe and excited by UV laser

The detection of phagotrophy in mixotrophic organisms is more complicated than in heterotrophs. Mixotrophs have pigments that may mask food vacuoles (Li et al. 1996, Hansen 1998, Hansen & Calado 1999, Stoecker 1999) and make detection of the prey by their autofluorescence virtually impossible (Riemann et al. 1995, Granéli & Carlsson 1998), even using flow cytometry. However, with the addition of the acidotropic blue probe we were able to observe a high incidence of phagotrophy in *Dinophysis norvegica* populations from natural samples.

Rose et al. (2004) successfully used flow cytometry coupled to an acidotropic green probe to detect phagotrophy in heterotrophic protists and distinguish them from autotrophic cells. Although these authors did not consider mixotrophy in their study, they verified a high probe signal in *Heterocapsa triquetra*, a recognized mixotrophic dinoflagellate (Legrand et al. 1998, Jeong et al. 2005). However, Rose et al.'s (2004) interpretation was that the probe accumulated in the plastids of *H. triquetra* and not in the food vacuoles, as we believe to have been the case. Another explanation could be a non-specific binding of the probe to membranes or other cellular structures. When testing a green acidotropic probe, we observed a low specificity for food vacuoles. The green fluorescence of the probe could be seen on cell membranes, in the cytoplasm and also in their chloroplasts (data not shown). Moreover, organic particles naturally occurring in seawater may become fluorescent when excited by blue light (Lebaron et al. 1998) and green dyes usually increase this natural background, which might be a problem in flow cytometric analysis.

In contrast to green probes, blue dyes are expected to display high signal-to-noise ratios, which are usually needed for automated flow cytometric analysis. Indeed, we did not observe blue fluorescence in autotrophic cells or mixotroph plastids during our experiments. In addition, the cells without food vacuoles that had been incubated with probe presented similar low blue fluorescence as the cells that had no contact with the probe whatsoever. In spite of the minor divergence, our results and the work presented by Rose et al. (2004) agree that the combination of flow cytometry and acidotropic probes is a useful tool in detecting phagotrophy in protists.

Although we did not see the blue probe binding to non-target structures in the cells during the microscopic observations, the flow cytometric analysis revealed large variations in the blue fluorescence in *Dinophysis norvegica* cells. We suggest that these large variations were caused by the presence of food vacuoles in different phases of digestion or different numbers of food vacuoles in the cells. *D. norvegica* uses a peduncle to suck the cytoplasm of the prey, forming numerous small food vacuoles (Schnepf & Elbrächter 1992), a phagotrophic mechanism termed myzocytosis (Jacobson & Andersen 1994). Phagotrophic mechanisms and the time of digestion can vary greatly in free-living flagellates (Gaines & Elbrächter 1987, Elbrächter 1991, Schnepf & Elbrächter 1992, Hansen 1998, Hansen & Calado 1999). As a consequence, phagotrophic cells can have food vacuoles in different stages of digestion, i.e. new food vacuoles that have been recently formed, old food vacuoles which have finished their digestion and are ready to be absorbed by the cell, and food vacuoles in a different phase of the digestion process. LysoSensor™ probes were designed to study dynamic aspects of lysosome biogenesis and function in live cells (Becton & Dickinson information). These probes are pH-dependent and their fluorescence intensity increases upon acidification. It is possible to estimate the different phases of digestion of food vacuoles based on the probe fluorescence intensity from the moment the cell engulfs the prey to the moment that the cell re-absorbs the vacuoles. However, more tests should address this matter.

Our results illustrate the effectiveness of the acidotropic probe LysoSensor™ Blue at staining food vacuoles in protists. Low probe concentration, short incubation time, and probe specificity for food vacuoles resulted in high blue fluorescence in both heterotrophic and mixotrophic cells. In addition, the blue probe caused very low background, which is desirable for flow cytometric analysis. LysoSensor™ was not harmful to species tested thus far, allowing the use of live organisms in real-time analysis, instead of fixed cells. Fixation always results in some degree of cell disruption and loss (Marie et al. 2004) and can cause changes in natural cell characteristics, such as size, shape, internal complexity, and pigment fluorescence (del Giorgio et al. 1996). Hence, FCM capabilities coupled to the LysoSensor™ Blue overcame the constraints of detecting phagotrophy in highly autofluorescent mixotrophs and heterotrophs that have fed on a colorless prey.

The combination of acidotropic blue probe and flow cytometry is an effective way of detecting food vacuoles and verifying the frequency of phagotrophy (percentage of the population containing food vacuoles in a given time) in populations of both heterotrophs and mixotrophs. However, the contents of food vacuoles cannot be directly identified based only on the probe fluorescence. Identification of the prey can be done indirectly based on autofluorescence and on other cell characteristics, a task easier to achieve in the laboratory than in field samples. To help identify the natural populations, cells can be physically sorted in the FCM and then identified using a microscope. Therefore, by performing single-cell analysis of thousands of cells of

both predator and prey it is possible to determine the percentage of the predator's population containing food vacuoles and estimating the grazing rate based on the disappearance of the prey.

The methodology we applied overcomes most of the common problems related with the detection of phagotrophy in studies of heterotrophs and mixotrophic algal species: (1) Under- or over-estimations of the frequency of phagotrophy (i.e. number of cells containing food vacuoles) caused by the use of artificial or manipulated prey (Porter 1988, Legrand et al. 1996, Bouvier et al. 1998, Granéli & Carlsson 1998, Ishii et al. 2002). (2) Transfer of radioactivity from one organism to another or contamination of the mixotrophs' cell membrane by radioactivity without effective ingestion (Legrand et al. 1998). (3) To determine whether the particle inside a mixotroph or heterotroph is a parasite or a gamete instead of prey in a food vacuole (Porter 1988, Legrand et al. 1996, Bouvier et al. 1998, Granéli & Carlsson 1998, Ishii et al. 2002). Since LysoSensor™ Blue is efficient at marking food vacuoles and its fluorescence is not masked by mixotrophs' autofluorescence, this probe might help to identify new mixotrophic species, which are presently known only as strict autotrophs.

Although the approach presented has several advantages, it also has some logistic limitations. Firstly, FCMs are relatively expensive instruments (Collier 2000, Marie et al. 2004). Secondly, machines like FACSVantage has, amongst other operational routines, manual optical alignment and requires experienced personnel to operate it (del Giorgio et al. 1996, Collier 2000, Marie et al. 2004). Finally, these 'research' grade FCMs are large and cannot be transported to oceanographic vessels, which consequently limit their use. LysoSensor™ probes are intrinsically related with the digestion process, because of this the organisms that are being studied must be alive. Therefore, the method is applicable only to field samples that can be transported quickly to the lab or to experiments with cultivable species. Standard benchtop instruments that can be transported to the field and require users with less training (del Giorgio et al. 1996) are less flexible than 'research' grade FCMs (Collier 2000). These small instruments cannot be used in combination with LysoSensor™ Blue because they are usually equipped with a single 488 nm blue laser and the probe maxima absorption is 373 nm (Molecular Probes information).

The use of the acidotropic probes can also pose some difficulties. For example, autophagy plays an essential role during cell differentiation, starvation, aging and death (Reggiori & Klionsky 2002). Therefore, the occurrence of autophagic vacuoles is not an unlikely event. Since LysoSensor™ probes are pH dependent, it is expected that they can stain autophagic vacuoles. These vacuoles are usually smaller than food vacuoles, but they can be numerous, especially when the population is under stress. Parasitophorous vacuoles, i.e. vacuoles that surround parasites (Antoine et al. 1990, Maurin et al. 1992, Stecconi-Silva et al. 2003), are other acidic vesicles that can be stained by the acidotropic probes. Since parasitism is not rare among dinoflagellates (e.g. Coats et al. 1996, Coats & Park 2002, Salomon et al. 2003a,b, Kim et al. 2004, Park et al. 2004) and other organisms, parasitophorous vacuoles can become a source of error. However, unless the frequency of cells containing parasitophorous or autophagic vacuoles is high in the population being studied, the capacity of the FCM to measure a large number of cells (Collier 2000) can diminish the possible bias caused by the occurrence of these acidic vacuoles in the cells. However, since cells are measured blindly in the FCM, to avoid or minimize bias that can be caused by a non-specific binding of the probe on non-target particles, proper controls are necessary (Marie et al. 2004). In addition, the specificity of the probe should be checked regularly by microscopic observations.

In spite of the limitations described above, the combination of acidotropic probes and flow cytometry has proved to be an excellent tool for identifying phagotrophy in both heterotrophs and mixotrophic phytoplankton species and for observing in real time the behavior of mixotrophs and heterotrophs under different environmental conditions. This prospect gives us a better understanding of the ecological role of distinct phagotrophic organisms in different aquatic environments.

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