Journal of Microbiological Methods 86 (2011) 104–107

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Note

A comparative study of fluorescence-labelled enzyme activity methods for assaying phosphatase activity in phytoplankton. A possible bias in the enzymatic pathway estimations

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ARTICLE INFO

Article history:
Received 26 December 2010
Received in revised form 18 March 2011
Accepted 18 March 2011
Available online 2 April 2011

Keywords:
Phosphatase
Ectoenzyme
Phytoplankton
ELF97-phosphate
Single cell analysis

ABSTRACT

We compared different fluorescence-labelled enzyme activity (FLEA) methods for assaying phosphatase activity in phytoplankton. Unfixed and liquid incubations are devised. We demonstrated that the presence of intracellular labelling was persistent, which could point out a source of bias in ectoenzymatic activities measurements based either on the FLEA or classical methods.

The published protocols of the enzyme-labelled fluorescence (ELF) or FLEA technique differ widely in the fixative used, the way the incubation is performed and the final support of the sample (liquid or filter). These differences have been shown to affect not only cell integrity, but also labelling efficiency (Rengefors et al., 2001; Young et al., 2010). Additionally, the presence of intracellular labelling is a problem that has not yet been solved. We compared published and new protocols for the phosphatase activity assay in natural phytoplankton communities, using the criterion that ectophosphatase (surface-bound) FLEA labelling should be maximised because these are the phosphatases playing the ecological role of catalysing reactions around the lipid boundary, where life and the surrounding environment come into contact.

Phytoplankton communities were sampled from Crous Pond (41°40′37″N, 2°35′2″E) and Redon Lake (42°38′33″N, 0°46′46″E), in Catalonia. We tested several protocols that were defined by a combination of two factors: step order and fixative (Table 1). The step order had two levels: B (Bottle) and F (Filter). B consisted in incubating the sample in a bottle, fixing it and stopping the reaction by filtration. It was based on the protocol devised by Nedoma et al.(2003). F consisted in fixing the sample, filtering it, and incubating the filters in a Petri dish. This was a modification of the protocol drawn up by Lomas et al. (2004). B or F was placed in first position in our protocol code. The fixative factor had five levels, whose initial letters were placed in the second position in our code: $HgCl_2$ (H), no fixative (X), LFT (L), ethanol + DMSO (E), and liquid N_2 (N). $HgCl_2$ 4 mM f.c. was added to samples and immediately filtered. LFT fixation was performed by adding alkaline Lugol 0.5% (vol/vol), formaldehyde 2% f.c. (pH 7) and several drops of 3% sodium thiosulfate (Sherr and Sherr, 1993). Ethanol 70% + DMSO 10% were added to the samples and left for 30 minutes before filtration (Lomas et al., 2004). In the case of the FN protocol, samples were placed in plastic vials and sunk into the liquid N_2, removed, and left to thaw prior to filtration. All the incubations were performed in the dark for 1 hour at room temperature and with a substrate concentration of 20 μM ELF97-phosphate (ELFP) (Molecular Probes, E6589). Three replicates and one negative control (without ELFP) of 30 ml each were filtered through 25 mm diameter and 2 μm pore polycarbonate filters (Millipore). Very gentle pressure (<20 KPa) was used to avoid cell disruption. Filters were left to dry on cellulose paper and stored at −20 °C. Finally, they were thawed and mounted on microscope slides using Citifluor AF1.

The composition of the phytoplankton community was determined by the Utermöhl method. Particular attention was paid to the size and shape of chloroplasts, as these characters would subsequently be used to identify taxa under the epifluorescence microscope. The different FLEA filters were analysed under a Nikon Eclipse E600 epifluorescence microscope and the percentage of ELFA-labelled cells was determined. A cell was only considered positive when a clear ELFA object was observed on or in the cell, whatever its size or intensity. Some of the experimental conditions (FH and BE in the Crous sample) resulted in non-measurable filters (Fig. 1a and b). One replicate per experimental

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doi:10.1016/j.mimet.2011.03.010

Table 1
Experimental design and codes used in this study; n = number of available replicates; unc = uncountable filters.

			Fixative				
			$HgCl_2$	No fixative	LFT	Ethanol + DMSO	Liquid N_2
Step order	Bottle	Crous	BH ($n=2$)	BX ($n=2$)	BL ($n=2$)	BE (unc)	–
	Filter		FH (unc)	FX ($n=2$)	FL ($n=2$)	FE ($n=2$)	–
	Bottle	Redon	BH ($n=3$)	BX ($n=3$)	BL ($n=2$)	–	–
	Filter		FH ($n=3$)	FX ($n=3$)	FL ($n=3$)	–	FN ($n=3$)

condition and sample was analysed with a Leica SP II spectral detection confocal microscope to determine the location of ELFA bodies. The percentage of cells with only external labelling (EXT), only internal labelling (INT), and both external and internal labelling (EXT and INT) (Fig. 1c and d, e and f) were determined by counting more than 100 cells per filter. Within each taxon, we discarded location patterns that were based on less than 5% of taxon total counts. One-way and two-way ANOVAs were performed with Statgraphics Plus 5.1 (Statistical Graphics Corp.) and STATISTICA 6.0 (Statsoft, Inc., OK, USA).

The percentage of ELFA-labelled cells in the entire phytoplankton differed significantly between protocols in both Redon (p–$v<0.0000$) and Crous (p–$v=0.0153$) samples. The step order (Crous p–$v=0.035781$ and Redon p–$v=0.001377$) was always more significant than the fixative (Crous was not significant and Redon p–$v=0.0025$) (Fig. 2, first row), and this result was consistent across most of the studied taxa. In all the cases, liquid-incubation protocols (B) significantly maximised ELFA labelling. B and F protocols differed in two factors that could explain this result: (1) the status of cells and enzymes during incubation (alive/unfixed (B) or fixed (F)), and (2) the physical support of the incubation (liquid (B) or on-filter (F)). If we consider the low labelling results of FX protocol, where cells were incubated live but on filter, as in Van Wambeke's method (Van Wambeke et al., 2008), we may conclude that apart from the expected effect of incubating fixed or unfixed samples, a physical obstruction that prevents ELFP from reaching enzymes may occur. Reasonable explanations would be either because in F protocols some phosphatases are in contact with the filter itself, or because ELFP cannot diffuse fast enough within the drop, which creates a low concentration of ELFP in the volume surrounding the cell.

The percentage of ELFA-labelled cells showed four acceptable protocols: BH, BX, BL and FH. Of these options, BH was the best in terms of the percentage of ELFA-labelled cells, but consistently performed the worst in terms of ELFA-labelling location. It was therefore an undesirable option. BX and BL had good percentage of labelled cells and good labelling location in all the cases except that BX had low EXT location in Redon sample and BL had a low percentage of labelled cells in Crous. Finally, FH had good results in both variables when the protocol worked (in Redon), but it could be unreliable as it developed huge particle aggregates in Crous. To sum up, BX and BL would be reasonably good options, BH could provide positive but ecologically uncertain results, and FH seems to be a good but unstable option.

Traditional total enzyme activity substrates (MUF-P, MFP, p-NNP, etc.) have been thought to label dissolved and/or ectoenzymes as they have physical and chemical characteristics that make it impossible for them to get into the cells by membrane transport (Chróst, 1991). The ELF substrate shares the same physical and chemical characteristics. In addition, there is evidence that ELFP and MUFP substrates react with

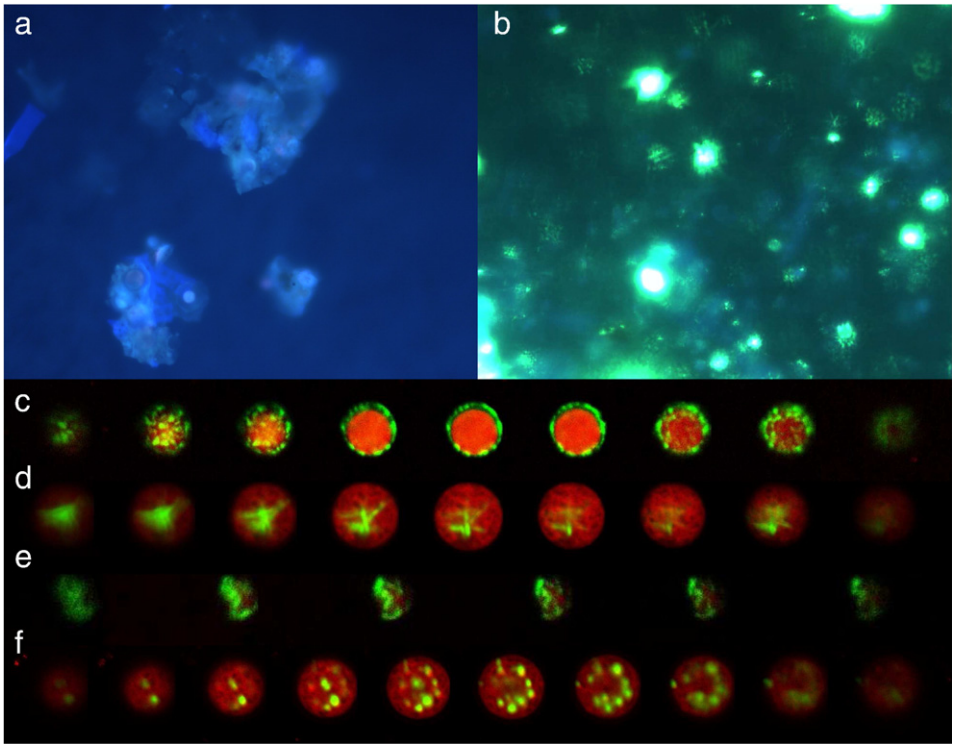

Fig. 1. Colour epifluorescent images: a and b; a) general view of protocol FH, with aggregates (Crous); b) general view of protocol BE, with extremely high unspecific ELFA labelling and non-recognisable chloroplasts (Crous). Pseudocolour optical sections at different depths through a unicellular *S. schroeteri* (c, d and f) or a small flagellated (e). Red is chloroplast and green are ELFA precipitates. c) EXT labelling; d) INT labelling; e and f) EXT & INT labelling.