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Alkaline phosphatase activity of marine bacteria studied with ELF 97 substrate: success and limits in the P-limited Mediterranean Sea

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ABSTRACT: The fluorogenic substrate Enzyme-Labeled Fluorescence 97 (ELF-P) is hydrolyzed by the P-cleaving enzyme phosphatase, producing ELF 97 alcohol (ELFA), a fluorescent-insoluble product. This reaction is used for monitoring phosphatase activity at the single-cell level. Most frequently, ELF-P has been used to determine the P-limitation status of microphytoplankton, but rarely of heterotrophic bacteria. We incubated ELF-P on filters to monitor marine bacterial cultures and oligotrophic Mediterranean Sea samples. Results were compared to classical measurements of bulk alkaline phosphatase activity using the fluorogenic substrate 4-methylumbelliferyl phosphate (MUF-P). A high percentage of the cultured cells were labeled with ELFA (the ratio of ELFA spots to total DAPI counts in P-limited cultures ranged from 26 to 100%, depending on the strain). In contrast, this ratio never exceeded 0.01% in Mediterranean samples, even when P was demonstrated to be a significant limiting factor. This protocol is useful for application on cruises and with cultures, but was not sufficiently sensitive to detect P-stressed bacterial cells in oligotrophic marine environments.

KEY WORDS: Marine bacteria · Alkaline phosphatase · Cell specific activity · Ectoenzyme · ELF97 phosphatase substrate · Phosphorus limitation

INTRODUCTION

Developing new techniques for detecting phosphorus (P)-stress in bacteria at the cell level from natural environments is a challenge in microbial ecology. Such developments are important in understanding how P-limitation can affect both the structure and the functional role of bacterial communities. The molecule 2-(5′-chloro-2′-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone (Enzyme-Labeled-Fluorescence 97 phosphatase substrate [ELF-P], Molecular Probes) is a soluble substrate, which, when cleaved by the cell's enzyme (phosphatase), produces ELF 97 alcohol (ELFA), a bright fluorescent yellow-green precipitate labeling the site of enzymatic activity (Gonzalez-Gil et al. 1998). In aquatic systems, this technique has mainly been used as a tool for identifying P-limited phytoplankton cells. To date, only 4 published studies have focused on natural bacterial communities. This includes studies on activated sludge (Van Ommen Kloeke & Geesey 1999), an acidified lake (Nedoma & Vrba 2006), and bacterial colonies and biofilms (Huang et al. 1998, Espeland & Wetzel 2001). In most of these studies, however, the abundance and activity of bacteria were fairly high.

The aims of the present study were (1) to test an easy protocol (suitable for research expeditions) on marine isolates, and (2) to test this technique in the Mediterranean Sea under P-limited conditions.

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MATERIALS AND METHODS

Two marine species were used: *Alteromonas infernus* and *Pseudomonas denitrificans.* Cells were grown in minimum requirement medium (Lyman & Fleming 1940) with a single source of carbon (C) and energy and a single source of P, plus trace amounts of iron and vitamins. The concentration of organic C (as glucose or pyruvate) was low (Table 1) in order to obtain less dense cultures. Different concentrations of inorganic P were added to produce diverse initial C/P ratios of the provided substrates (Table 1, Expts a and c). *A. infernus* was also grown in natural seawater amended with pyruvate (Table 1, Expt b). Seawater was aged in the dark for 1 mo at 4°C to remove most nutrients. This seawater was 0.2 µm filtered, and the filtrate was supplemented with excess nitrogen (0.1 mM NH_4Cl), carbon (0.33 mM C-pyruvate), and trace amounts of iron and vitamins. Phosphate (16.5 µM) was added only in the +P treatment.

The station DYFAMED (Marty et al. 2002) was studied for ELF application at the end of March 2003. Soluble reactive P (SRP) and dissolved organic P (DOP), determined as the difference between filtered samples before and after wet-oxidation, were measured according to standard colorimetric techniques (Raimbault et al. 1999).

Alkaline phosphatase (AP) activity was measured fluorometrically using 4-methylumbelliferyl phosphate (MUF-P). The linear increase in fluorescence in seawater with added MUF-P was followed over time (excitation at 365 nm and emission at 460 nm) with a Kontron SFM 23B spectrofluorometer. For *in situ* and culture samples, we used MUF-P concentrations of 1 and 50 µM, respectively, which were found to be saturating in preliminary experiments. MUF-P tracks both dissolved AP activity and particle-associated (mostly microorganisms) AP activity. Dissolved-associated AP activity was negligible in cultures (<3%) and could reach up to 36% of measured phosphatase activity at the DYFAMED station (results not shown).

Contrary to MUF-P, ELF-P substrate provides information on particle-associated AP. The Endogenous Phosphatase Detection Kit (Molecular Probes E 6601) was used. Component A (A_{kit}) was diluted 1/20 in component B (B_{kit}) of the kit in accordance with the manufacturer's instruction to generate the solution applied to the slides. Immediately after filtration of a water sample (live sample) onto a 0.2 µm pore size black polycarbonate filter, the filter was transferred onto a glass slide, and 40 µl of the diluted solution A_{kit}/B_{kit} were applied to the surface of the filter and gently spread with a plastic tip. The slide was then put into a horizontal, capped 50 ml plastic tube to which wet paper was added to prevent evaporation. Following a 1 h incubation at room temperature in the dark, the filter was removed, put briefly on a piece of absorbing paper, and then transferred into a 47 mm Petri dish containing a 47 mm absorbent pad (AP100 Millipore) soaked with a solution of phosphate buffered saline (PBS, 10 mM, pH 7.5) and 1% formaldehyde to stop the activity. The filter was transferred successively onto 3 PBS-formalin soaked pads, and left 5 min on each one. The filter was then air-dried on a piece of absorbent paper before being transferred onto a glass slide. Forty µl of a DAPI solution (25 µg ml^{-1}) were added, and the filter was incubated for 10 min in the dark. The filter was dried again on absorbent paper and mounted on a glass slide. The yellow

Table 1. *Pseudomonas denitrificans, Alteromonas infernus.* Summary of alkaline phosphatase (AP) activity in bacterial cultures, including initial conditions and the main results (bacterial numbers, ELFA spots, MUF-P based activities, and specific activities). BN: bacterial numbers (DAPI counts)

	Pseudomonas denitrificans		*Alteromonas infernus*		*Alteromonas infernus*
Code of experiment:	a		b		c
Medium:	Artificial seawater		Natural seawater		Artificial seawater
Carbon enrichment:	Glucose		Pyruvate		Pyruvate
Concentration of C (mM C)	3.3		0.33		0.55
NH_4Cl addition (mM N)	6.7		0.1		6.7
KH_2PO_4 addition (µM P)	165	8.25	16.5	0 (*in situ* 0.61)	5.8
Conditions	Excess P	Low P	Excess P	Low P	Equilibrium
Duration of the culture (d)	13	13	13	13	8
Period of max. BN (d)	8–13	7–11	5–8	7–9	3–7
Max. BN (ml^{-1})	$1.2–1.5 \times 10^8$	$3.1–4.6 \times 10^6$	$2.0–2.6 \times 10^7$	$1.2–1.7 \times 10^7$	$3.2–4.5 \times 10^7$
Period of max. AP activities (MUF-based) (d)	11–13	6–8	8–13	6–9	8
Max. MUF-based AP activities (nM h^{-1})	22–38	43–154	1400–2000	6000–9600	37 000
Period of max. specific activities (d)	0–13	6–8	2–13	2–13	8
Specific activities (amol $cell^{-1}$ h^{-1})	0.1–6.5	16–33	10–160	440–1000	1300
Period of max. ELFA spots (d)	8–12	7–11	2–13	2–13	1.2–5
Max. ELFA spots (ml^{-1})	$3–5 \times 10^4$	$3.4–7.0 \times 10^5$	$5.1 \pm 1.8 \times 10^6$	$4.8 \pm 3.0 \times 10^6$	$2–7 \times 10^7$
Ratio ELFA spots / BN (%)	0.02–0.05	9–17	30 ± 11	46 ± 28	172 ± 114

fluorescence of ELFA spots and the blue fluorescence of the DAPI stain were examined (Fig. 1) using an Olympus BH2 microscope equipped with a long pass dichroic mirror, type U (DM 400, barrier filter L420).

Variations of this protocol were tested. Following the filtration step, the filter was cut into pieces and incubated over different time periods or with different concentrations of ELF-P. In addition, a comparison was made between the abundance of ELFA spots and the rate of ELF-P hydrolysis measured in a spectrofluorometer calibrated with standard dilutions of ELFA (ref E 6578). This comparison was possible only in cultures, due to the very low sensitivity of ELFA measured spectrofluorometrically. In this attempt, 1.8 ml of *Alteromonas infernus* culture were incubated with 200 µl of a 100 mM Tris-HCl (pH 7.5) solution and 4 to 40 µl of ELF-P (ref E 6588) stock (5 mM) or diluted solutions in Milli-Q water (0.5 mM), giving final ELF-P concentrations of 2 to 100 µM. The increase in fluorescence (excitation 350 nm, emission 530 nm) was followed over time. At the end of the incubation, 500 µl were sub-sampled, fixed with 1% final concentration of formaldehyde, and filtered through 0.2 µm polycarbonate filters for epifluorescence microscopy and counting of ELFA spots.

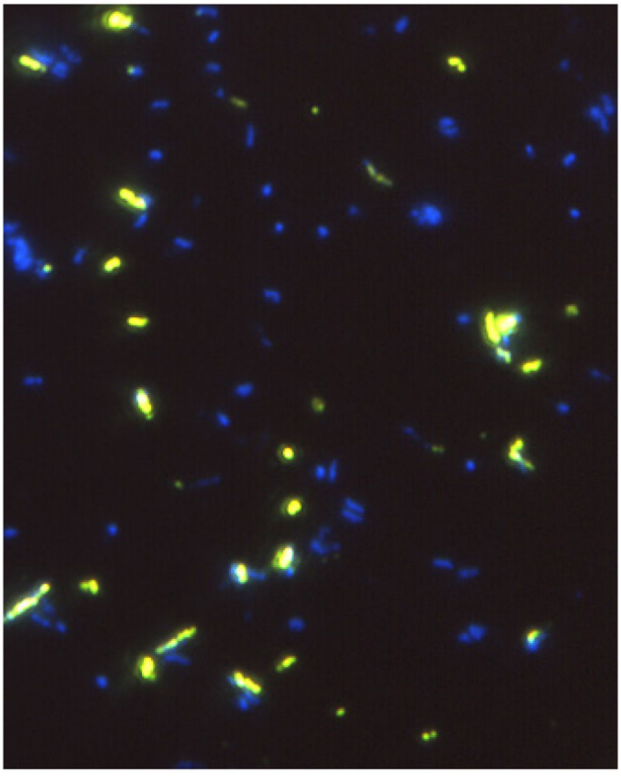

Fig. 1. *Alteromonas infernus*. ELFA labeled culture (yellow-green) counterstained with DAPI (blue) as observed with a dichroic mirror type U. Note that the ELFA signal is much more intense than DAPI

RESULTS AND DISCUSSION

Protocol

There are several important features in our protocol (see Table 2 for a comparison to previously published protocols). First, in order to work on live and unmodified samples, the ethanol pre-treatment step was omitted, since ethanol may damage cell membranes and/or induce the bleaching of pigments (Nedoma et al. 2003, Dignum et al. 2004). Second, to concentrate the cells, we used filtration because centrifugation requires high speeds and a lengthy processing time, making it less convenient when working on vessels. Third, we used the Endogenous Phosphatase Detection Kit, which is commonly used (Table 2), although only partial information regarding its composition is available (see Dignum et al. 2004, Nedoma et al. 2007). The ELF-P has been used omitting component B of the kit during incubation in the liquid phase (Nedoma et al. 2003), and ELFA precipitation was still possible (Fig. 2c,d). However, this is only feasible in cell-rich water samples with high phosphatase activity (Nedoma et al. 2003, Table 2).

Finally, we used DAPI as a counterstain because both dyes are visible (DAPI-stained bacteria in blue, ELFA spots in yellow-green) and cyanobacteria (orange) and chloroplasts (red) in natural seawater samples are easily discriminated. This also makes the technique compatible with future efforts to combine the method with fluorescent *in situ* hybridization (FISH) techniques. However, the fluorescence of the ELFA spots is much higher and fades more slowly than DAPI fluorescence (Fig. 1). Consequently, with only visual observation on the microscope, it is impossible to confirm that DAPI-stained cells are associated with an ELFA spot, since their similar size prevents seeing the DAPI stain in the same location as an ELFA spot. However, this discrimination is possible using narrow band filters and image analysis (Nedoma & Vrba 2006) or flow cytometry (Duhamel et al. 2008).

One of the first assumptions we tested was the presence of a false positive response. We showed that number of cells labeled with ELFA was higher in situations where P-limitation occurred (Table 1 [Expt a], Fig. 3c). Indeed, there was no significant labeling in C-limited cultures of marine bacteria, even at the stationary phase, suggesting that even if the substrate enters senescent cells, intracellular phosphatase cannot significantly react with ELF-P. However, P-sufficient *Alteromonas infernus* also expressed phosphatase activity (Table 1 [Expt b]), suggesting that for this strain, a fraction of phosphatase activity seemed to be constitutive. These bacteria are thus able to exert significant hydrolysis of phospho-mono-ester bonds of organic molecules even in the presence of notable sources of inorganic phosphate. In that

Table 2. Summary of ELF labeling procedures in different studies. Phytop: phytoplankton, CENT: centrifugation, ETH: treatment with 70% ethanol, PREFILT: prefiltration of the sample (followed by treatment of the filtrate), FILT: filtration of the sample (followed by treatment of the filter), WASH: rinsing procedure, FORM: formaldehyde, GLU: glutaraldehyde, PBS: phosphate buffered saline solution, EM: epifluorescence microscopy, TLM: transmitted light microscopy, CFM: flow cytometry. A_{kit}, B_{kit}: component A (ELF-P concentrate) or B (detection buffer) of the Endogenous Phosphatase Detection Kit (Molecular Probles E6601), respectively. ELP_{pure}: ELP-P 5 mM solution of Molecular Probes (E 6585). csDAPI, csPI, csAO: use of a counterstain DAPI, PI, and AO, respectively

Sample	Initial volume treated	Pre-treatment of the sample	Support for incubation	Incubation conditions	Stop reaction	Observation	Source
Marine phytop cultures	5 ml	CENT-ETH	Pellet	100 µl A_{Kit}/B_{Kit} 1/20 30 min	Washes in 10 mM PBS	CFM	Gonzalez-Gil et al. (1998)
Bacterial colonies	Colony on filter		Filter	Filter on cellulose pad saturated with A_{Kit}/B_{Kit} 1/20 30 min	Filter on pad with 1% FORM 5 min	EM - csPI	Huang et al. (1998)
Biofilm	Biofilm on a slide		Slide	Slide immersed in A_{Kit}/B_{Kit} 1/20 30 min	1% FORM cryo-embedding and sectioning	EM - csPI	
Photosynthetic biofilm	Suction of the biofilm in liquid phase	CENT	Pellet	20 µl A_{Kit}/B_{Kit} 1/20 30 min	4% GLU-FILT O.2 µm	EM-csDAPI	Espeland & Wetzel (2001)
Activated sludge flocs	1 ml		Liquid		0.1 ml drop onto a microscope slide	TLM/EM-csAO	Van Ommen Kloeke & Geesey (1999)
Eutrophic lake water-phytop	150 ml	CENT-ETH	Pellet	100 µl A_{Kit}/B_{Kit} 1/20 30 min	WASH 0.1 M PBS	TLM / EM	Rengefors et al. (2001)
Mesotrophic freshwater pond-phytop	500 ml	FILT 10 µm ETH-CENT	Pellet	100 µl A_{Kit}/B_{Kit} 1/20 30 min	WASH 0.1 M PBS	TLM/EM	Rengefors et al. (2003)
Phytop cultures (dinoflagellate)	9 ml	CENT-ETH	Pellet	100 µl A_{Kit}/B_{Kit} 1/20 30 min	CENT-WASH	TLM/EM	Dyhrman & Palenik (1999)
Field samples	1 l	FILT 0.8 µm ETH- CENT	Pellet	100 µl A_{Kit}/B_{Kit} 1/20 30 min	CENT-WASH	TLM/EM	
Trichodesmium cultures and seawater colonies	Concentrate in a 130 µm net	Isolated colonies WASH FSW	2 colonies	100 µl A_{kit}/sterile seawater 1/20 45 min	Colonies on a 5 µm filter-WASH FSW-10% FORM-transfer on filter	EM	Dyhrman et al. (2002)
Coastal - seawater phytop	1 l	FILT 0.8 µm ETH - CENT	Pellet	A_{kit}/low P-seawater 1/20 1 h	CENT-WASH	TLM/EM	Ruttenberg & Dyhrman (2005)
Oligotrophic lake phytop-bacteria	5 ml	FILT 0.4 µm in tissue culture inserts	Concentrate in inserts	200 µl A_{Kit}/B_{Kit} 1/20 1 h	FILT-RINSE	EM-csDAPI	Carlsson & Caron (2001)
Eutrophic reservoir phytop	5 ml	PREFILT 200 µm	Liquid	20 µM ELF_{pure} 1 h to 5 h	FIX 4 mM $HgCl_2$-FILT 1 µm	EM	Strojsová et al. (2003)
Lake and reservoir phytop	1 to 5 ml	PREFILT 200 µm	Liquid	20 µM ELF_{pure} 30 min to 5 h	FIX 4 mM $HgCl_2$-FILT 1 µm	EM	Nedoma et al. (2003)
Cyanobacterial cultures	14 ml	0.1% GLU or 0.01% FORM-CENT	Pellet	50 µl A_{Kit}/B_{Kit} 1/20 30 min	100 times dilution in flow cytometry sheath fluid	CFM	Dignum et al. (2004)
Marine samples	0.25 to 1 l	FILT 0.4 µm-10% DMSO- ETH	Filter	400 µl A_{Kit}/B_{Kit} 1/20 45 min	Filter back on tower RINSE FSW	EM	Lomas et al. (2004)
Lake bacterioplankton	7 ml	PREFILT 200 µm	Liquid	20 µM ELF_{pure} 0 to 40 min	2% FORM-DAPI -FILT	EM	Nedoma & Vrba (2006)
Bacterial cultures marine samples	0.5 ml (culture), 10 to 30 ml (field)	FILT 0.2 µm	Filter	30 µl A_{Kit}/B_{Kit} 1/20 30 min	Pads with 10 mM PBS + 1% FORM	EM-csDAPI	This study

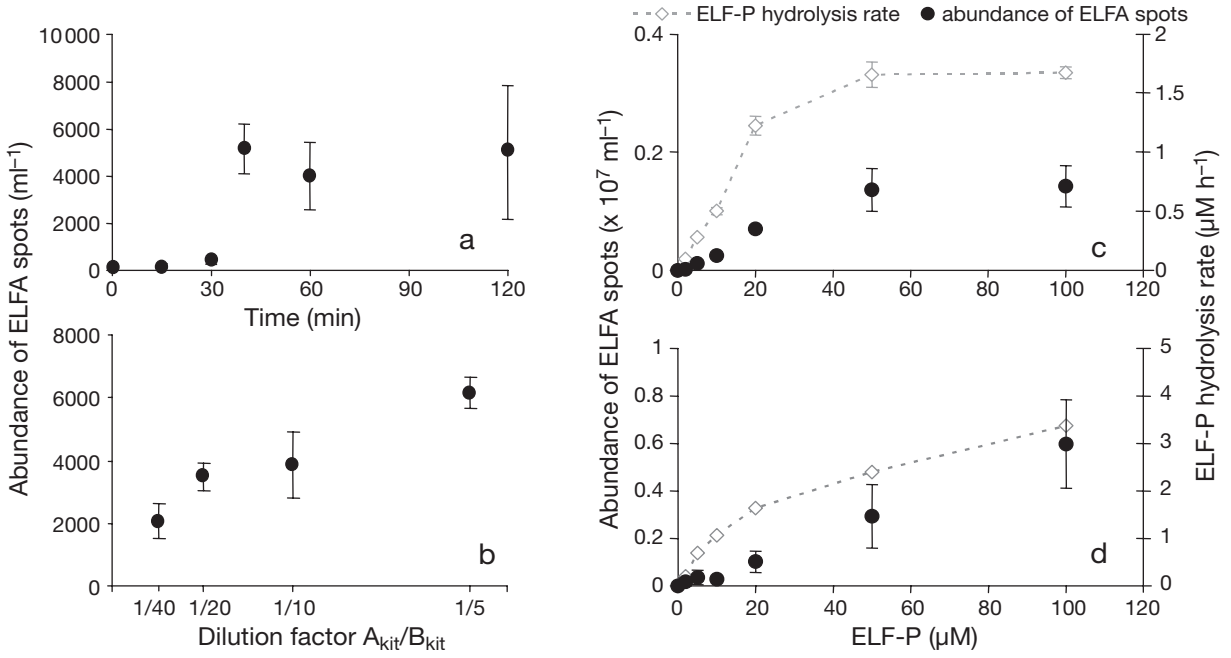

Fig. 2. Typical curves showing the effect of time of incubation (a) and concentration of ELF-P (b,c,d) on the abundance of ELFA spots and ELF-P hydrolysis rates. Data in (a) and (b) used Mediterranean Sea water samples (incubations made on filters). Data in (c) and (d) used pure cultures. ELF-P hydrolysis rate (detected by spectrofluorometry) was measurable only in P-deficient *Alteromonas infernus* cultures. Error bars are ±SE within different fields of observation or transects. A_{kit}, B_{kit}: component A (ELF-P concentrate) or B (detection buffer) of the Endogenous Phosphatase Detection Kit, respectively

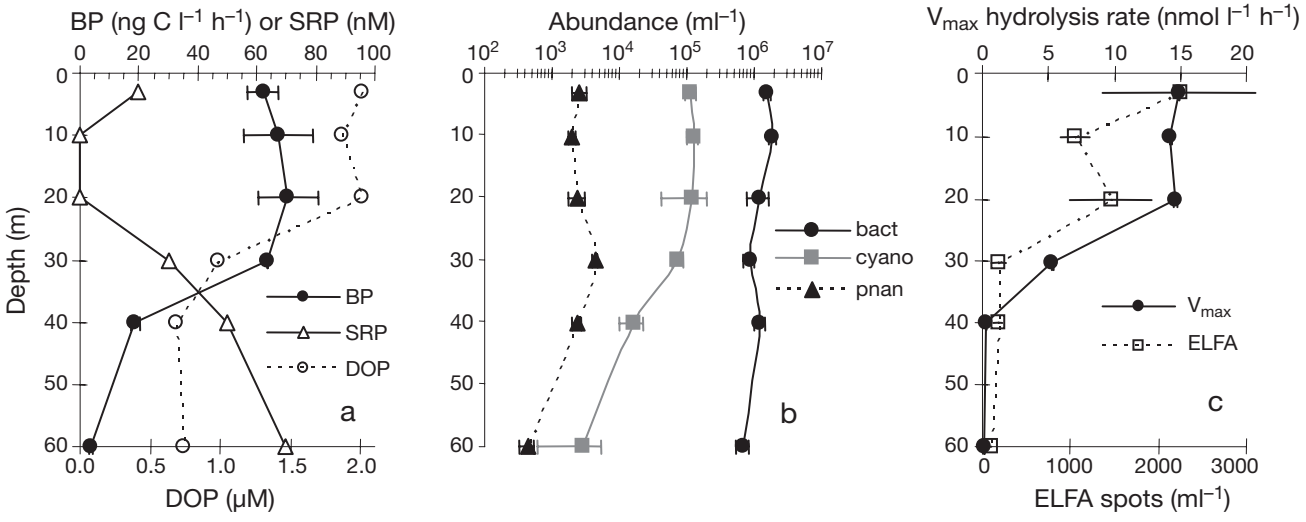

Fig. 3. Vertical profiles of bacterial production (BP), soluble reactive phosphorus (SRP) concentration and dissolved organic phosphorus (DOP) concentration (a); abundances of bacteria (bact), *Synechococcus*-like cells (cyano), and phototrophic nanoflagellates (pnan) (b); and maximum hydrolysis rate of MUF-P (V_{max} hydrolysis rate) and abundances of ELFA spots (c). All samples were collected at the DYFAMED station, NW Mediterranean Sea, sampled on 30 March 2003

case, this capacity could give access to the C or N compounds of the molecule hydrolyzed in environments deficient in labile sources of organic matter, like the deep sea (Hoppe & Ullrich 1999).

The Time 0 sample (acting as an abiotic control, because it was fixed with formalin) only exhibited a low fluorescence signal, thus confirming the absence of significant abiotic labeling with ELF-P. Development of ELFA spot abundances over time in the incubations confirmed the presence of a lag phase that was apparent in cultures as well as in natural samples (Fig. 2a). This lag time was also reported for freshwater phytoplankton (Nedoma et al. 2003, Dignum et al. 2004) and can vary with activity, temperature, and cell size (J. Nedoma unpubl. results). A

maximum concentration of label was obtained generally within 1 h in natural samples. Both the number of ELFA spots and ELF-P hydrolysis rates (measured by fluorometry) increased with the concentration of ELF-P added. The shape of these curves was not reproducible from one sample to another (Fig. 2c,d), suggesting that the range of concentrations that we tested was not always sufficient to reach maximum hydrolysis rates, or that multiphasic kinetics were present (presence of different enzymes: Hoppe 2003, Nedoma & Vrba 2006). These tests confirmed that the typical 1/20 dilution of the solutions in the kit we used (i.e. 250 µM) in the 1 h incubation is generally sufficient to optimize cell labeling.

Bacterial cultures

All tests carried out using cultures are summarized in Table 1. Maximum bacterial abundances were higher under excess-P than under low-P conditions. However, bulk activity and cell-specific activities varied depending on the strain. For *Alteromonas infernus*, differences between low-P and excess-P conditions were less pronounced than for *Pseudomonas denitrificans.* Occasionally, the average ratio of ELFA spots to DAPI counts was >1. Because ELFA spots and DAPI counts were made on 2 separate slides, it is difficult to conclude that percentages higher than 100% were really due to the formation of ELFA spots outside bacterial cells or to heterogeneity in the distribution of bacteria on the slide when abundances reached 10^7 cells ml^{-1}.

Seawater samples

Sampling was carried out during the spring phytoplankton bloom (Bourguet et al. in press) when heterotrophic bacteria (1.5×10^6 ml^{-1}) and *Synechococcus*-like cells (1×10^5 ml^{-1}) were abundant (Fig. 3b). SRP was below 20 nM or undetectable in surface layers down to 20 m depth (Fig. 3a). MUF-based AP activity was constant in the first 20 m of the profile and decreased significantly at 30 m depth (Fig. 3c). The Michaelis-Menten constant (K_m) was stable along the profile, with values ranging from 83 to 116 nM (results not presented). At most, we counted 2×10^3 ELFA spots ml^{-1} at any particular location along the profile. Being aware of the difficulty of counting rare events on filters, the vast majority of these spots were identified as unassociated spots (34 to 75% over the depth range), in contrast to spots associated with detritus (yellow DAPI particles without chlorophyll fluorescence, 21 to 55%), and only a few spots were associated with identified orange fluorescent cell (cyanobacteria, 0 to 8%) or red fluorescent cells (phototrophic nanoflagellates, 0 to 8%). The highest ratio of total ELFA spots to bacterial abundance reached was 0.01%.

A log-log plot of the distribution of ELFA spot abundance versus MUF-P based AP activity using the entire data set is shown in Fig. 4. Below a threshold of 5 to 10 nmol MUF-P hydrolyzed l^{-1} h^{-1}, a range that is frequently found *in situ*, there was no significant relationship due to the high variability. MUF-P activity was linearly related to ELFA spots over a range of 5 to 200 nmol l^{-1} h^{-1}. At MUF-P hydrolysis rates higher than 200 nmol l^{-1} h^{-1}, ELFA spot abundance leveled off.

CONCLUSIONS

Culture populations and field communities of heterotrophic bacteria exhibited different phosphatase activities as seen from the specific activities found with MUF-P substrate and the varying percentages of ELF labeling. A minimum threshold of extracellular phosphatase activity (measured by spectrofluorometry) of 10 fmol $cell^{-1}$ h^{-1} for phytoplankton (Nedoma et al. 2003) and 0.17 fmol $cell^{-1}$ h^{-1} for bacteria in acidified mountain lakes (Nedoma & Vrba 2006) is necessary to observe significant ELFA spot formation and to allow quantitative per cell estimates. Such levels were reached only in our cultures. In contrast, we were unable to detect ELFA labeling of bacteria from marine samples with the protocol developed in this study, even though SRP concentrations were undetectable in surface waters and MUF-P hydrolysis rates were relatively high. Heterotrophic bacteria were P-stressed, as shown by the stimulation of bacterial production after

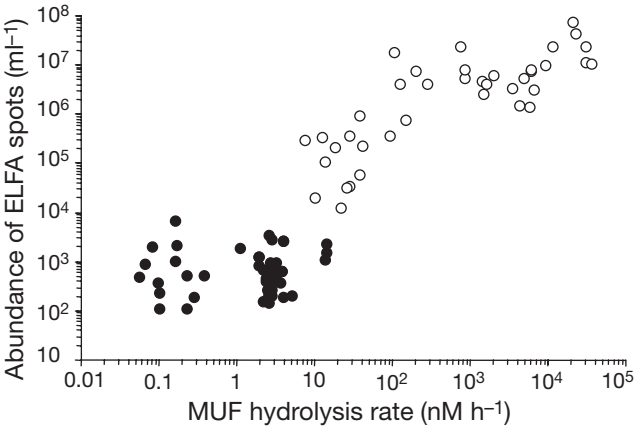

Fig. 4. Log-log plot of abundance of ELFA spots versus MUF-P alkaline phosphatase activities using the whole data set; culture data presented in Table 1, profile of March 2003 (Fig. 3), and other measurements made using the same protocol within surface waters at the DYFAMED station in October 2004 (results not presented in text). Solid symbols represent *in situ* data, open symbols are pure cultures

P amendments (results not presented). It is likely that either the concentration of ELF-P we added was different from the affinity of bacteria for this molecule, or that assay conditions were not optimal, e.g. only the most active bacteria were labeled. This problem has also been encountered with natural *Synechococcus* cells in the North Atlantic Gyre (Lomas et al. 2004). Detection of extracellular activity among small coccoids in an acidified lake was scarce, and when detectable, activity was lower than that of filaments and curved rods that systematically revealed higher activities (Nedoma & Vrba 2006). Future research should concentrate on more sensitive detection techniques such as flow cytometry, which could allow the detection of a greater number of events and the simultaneous quantification of ELF intensity per cell with a faster procedure than quantification by image analysis. This work is currently in progress.

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