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Improved methodology to measure taxon-specific phosphate uptake in live and unfiltered samples

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Abstract

Microorganisms play a major role in the marine phosphate biogeochemical cycle but the relative contribution of picoplanktonic groups is not well understood. Previous studies have shown that combining uptake measurements of radiolabeled dissolved inorganic phosphate (P_i) substrate with cell sorting by flow cytometry is a powerful tool for the assessment of P_i fluxes at the cell-specific level. Nevertheless, using ^{33}P to trace P_i uptake, we show that treatments involving fixation and filtration of the sorted groups (i.e., heterotrophic prokaryotes, *Synechococcus* and piconanophytoeukaryotes) induce leakage of radioactive P_i (up to 50% of the signal), resulting in a sizeable underestimation of the taxon-specific P_i uptake. We suggest an alternative protocol, which significantly reduces this bias. Using this optimized protocol, the samples were treated with an excess of nonradioactive P_i to stop the incubation and sorted fractions were directly collected in microtubes for radioactivity counting, avoiding signal loss due to filtration. Sorted groups were strongly and differently impacted by fixation (0.5% PFA), with the exception of *Synechococcus* cells, which showed once a 10% lower signal in samples treated with the previously used protocols compared with samples treated with ours. Based on the integrity of the live sorted cells, our improved protocol provides reproducible and accurate estimations of the taxon-specific P_i uptake (≤ 11% variation on cellular uptake rates, sd/average, $n = 69$). It was successfully applied to P-depleted oligotrophic seawater samples from the Mediterranean and will allow a comparison of taxon-specific uptake rates between sites.

Dissolved inorganic phosphate (henceforth referred to as P_i) is an essential nutrient for growth and metabolic functioning (e.g., nucleic acids, lipids, energy-transferring molecules) of living organisms, including marine microbial communities. P_i can be limiting for growth in many lakes and in some oligotrophic areas of the world ocean, where nanomolar concentrations are measured (Björkman et al. 2000; Wu et al. 2000; Moutin et al. 2002; Van Den Broeck et al. 2004). Measuring P_i uptake fluxes through different compartments of planktonic communities permits both to determine the main actors responsible for the bulk P fluxes and to assess the taxon-specific response of aquatic microorganisms to fluctuating P_i concentrations. This approach was developed primarily using size fractionation with different membrane porosities (e.g., Nausch et al. 2004; Tanaka et al. 2004; Flaten et al. 2005; Duhamel et al. 2007; Nowlin et al. 2007). However, intercomparisons between studies are challenging because of the arbitrary choice of pore sizes and membrane types, and such P_i uptake measurements suffer from overlapping in consecutive

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size-classes. This lack of specificity may lead to inaccurate estimations, particularly in oligotrophic environments where small-sized organisms dominate both autotrophic and heterotrophic compartments of the food web (Cotner and Biddanda 2002).

Flow cytometric cell sorting of radiolabeled samples is a promising tool to better characterize taxon-specific activities, like in the measurement of P_i uptake at the group level (Zubkov et al. 2007; Larsen et al. 2008; Casey et al. 2009; Michelou et al. 2011). These authors reported a non-negligible loss of radioactive signal after sample fixation, which is commonly used to stop uptake of radiolabeled compounds.

In the present study, we provide an in-depth investigation of radioactive signal losses due to fixation and introduce an alternative protocol to measure taxon-specific P_i uptake in natural samples that significantly minimizes signal loss. We also compare our revised protocol with existing ones, exposing the combined impact of fixation and filtration on the measurements. Methodological aspects that have not been highly documented thus far are developed, such as the preparation and values of blanks, and storage experiments of labeled samples.

The method presented here leads to a more accurate characterization of the taxon-specific P_i uptake, thus reducing uncertainties regarding the P_i fluxes through the microbial compartments of the surface ocean. This protocol is sensitive, reproducible, cost- and time-efficient, and finally, is easy to use on fresh samples both onboard and in the laboratory.

Materials and procedures

Sample collection

Most of the tests were conducted on coastal marine seawater samples collected during March 2008 from the oligotrophic Bay of Banyuls-sur-mer, France (NW Mediterranean Sea, Médernach et al. 2001; Grémare et al. 2003). Seawater was easily accessible and used immediately after sampling. However, additional tests were run on samples from MOLA and SOLA stations or on those acquired during the BOUM cruise in the Mediterranean Sea. In this case, sample origin is specified in the text and in the legends.

P_i analysis

Samples for dissolved inorganic phosphate analysis were filtered on combusted GF/F membrane after sampling, before being stored frozen (–20°C) in polyethylene bottles and being analyzed according to Tréguer and Le Corre (1975) on a Skalar autoanalyzer, with a detection limit of 20 nM.

P_i uptake

Fresh seawater samples (10 mL) were transferred into acid-washed and sample-rinsed polycarbonate flasks and were then spiked with 1.37 10^5 Bq of a carrier-free [^{33}P]-orthophosphoric acid solution (Perkin Elmer, equivalent to a 25 pM final P_i concentration added). Incubations were carried out at room temperature (20 ± 2°C) for 1 h, and stopped by the addition of 100 µL of a 10 mM non-radioactive KH_2PO_4 solution (i.e., cold chase, 100 µM final concentration). Four milliliters of each sample (blanks and triplicates) was stored at 4°C for a short time, in the dark, and reserved for bulk P_i uptake determination within 1 h after cold chase. Gentle filtration (< 100 mbars) was applied through a 0.2 µm porosity polycarbonate membrane (25 mm diameter) positioned on a GF/C filter that had been soaked with a saturated solution of KH_2PO_4, using a 12-position Millipore manifold. Once no liquid remained on the membranes, pressure was increased to 600 mbars for 5 s to eliminate unincorporated ^{33}P. For clarity, this filtration method will be referred to as "live filtration procedure." Five milliliters of Phase Combining System scintillation cocktail (PCS, GE Healthcare) were added to 6.5 mL polyethylene scintillation vials containing the dry filters. Radioactivity was counted using a scintillation counter (Beckman Coulter LS6500).

The leftover 6 mL samples were kept at 4°C in the dark for cell sorting analyses, which started within less than 30 min after cold chase (*see* "Cell sorting").

Sample blank

As a control for nonbiological assimilation of radiolabeled P_i, an excess of a cold KH_2PO_4 solution was added to a duplicated seawater sample at a final concentration of 100 µM, 15 min before the radioactive spiking. Replicated controls were then sorted for each group and their average blank value was systematically subtracted from the signal measured in regular samples (in dpm cell^{-1}). For the bulk activity determination, the blank value was subtracted from the whole signal (in dpm mL^{-1}).

Flow cytometric analyses

Fluorescent beads (1.002 µm; Polysciences Europe) were systematically added to the samples before analysis as an internal standard, for both sample enumeration and cell sorting. Three cytometric groups were analyzed (Fig. 1): heterotrophic prokaryotes (Hprok), *Synechococcus* (Syn), whereas picophytoeukaryotes and the few nanophytoeukaryotes were gathered as 1 group (Pic). *Prochlorococcus* cyanobacteria were absent from coastal and offshore samples at the time tests were conducted.

Enumeration of autotrophic and heterotrophic organisms

Three milliliters of the original seawater sample was fixed with formaldehyde (final concentration 2%) immediately after sampling. Samples were kept at 4°C for less than 12 h before analysis using a FACSCalibur flow cytometer (Becton Dickinson) equipped with an air-cooled argon laser (488 nm, 15 mW). Autotrophic cells were enumerated in 2-mL subsamples according to Marie et al. (2000). Syn were discriminated from other autotrophic cells (Pic) using their orange fluorescence signal (585 ± 21 nm) originating from the phycoerythrin pigment (Fig. 1A).

Hprok cell enumeration was processed on 1 mL fixed subsamples. The nucleic acid dye SYBR Green I (Invitrogen – Molecular Probes) was added at 0.025% (vol/vol) final concentration and incubated for 15 min at room temperature and in the dark before FCM analysis (Lebaron et al. 1998; Ober-

nosterer et al. 2008). Stained prokaryotic cells were excited at 488 nm, discriminated and enumerated according to their side scatter (SSC) and green fluorescence measured at 530 ± 30 nm (Fig. 1B).

The number of events per second was kept under 1000 events s^{-1} to avoid coincidence. The average volume of processed samples was 15 µL for Hprok (low flow rate, 1 min of acquisition) and 300 µL for unstained phototrophic organisms (high flow rate, 5 min of acquisition). The flow rate was calibrated every five samples on 1 mL seawater run for 5 min.

Cell sorting

From each radiolabeled sample, 2 and 4 mL were put in BD Falcon polypropylene tubes for sorting Hprok and autotrophic groups, respectively. Samples dedicated to further sorting of Hprok cells were stained with SYBR Green I as described above, for at least 30 min because the cell membranes were not permeabilized by any fixative beforehand. Sorting was performed on a FACSAria flow cytometer (Becton Dickinson) equipped with two lasers: a 488 nm (13 mW) Coherent, Sapphire Solid State, and a 633 nm (11 mW) JDS Uniphase HeNe Air-Cooled, using the same excitation wavelength and discriminating parameters as for enumeration of nonradioactive samples. The sheath fluid was 0.2 µm-filtered (stericap, Millipore) and autoclaved seawater. The instrument was controlled by a computer equipped with the FACSDiva software set on the four-way sorting 0/32/0 purity mode, which enables to sort up to 4 groups simultaneously from one sample and guarantees 99% recovery when rates are kept under 25000 events s^{-1}, according to the manufacturer.

Sorted cells were recovered in 2-mL centrifuge tubes. Cell sorting was performed at room temperature (20 ± 2°C) and, all samples included (up to 12 samples), lasted several hours (up to 7 h) due to the scarcity of organisms, in particular, nanopicophytoplankton. The tubes were centrifuged 15 s at 1000 rpm to gently bead the sorted drops, before being filled with 1.5 mL PCS scintillation cocktail and placed in the scintillation counter equipped with vented microtube holders. This method, henceforth referred to as the "live direct procedure," is compared with previously published filtration protocols for sorted samples, as detailed in the "Assessment" section (see Fig. 2).

Calculation of the bulk and taxon-specific P_i uptake rates

Samples, sample blanks, and introduced quantities of isotope were counted by liquid scintillation on the same day so as to avoid the computation of ^{33}P decay. The fraction of assimilated label per volume unit (R_t) or per group (R_{tgp}, Eq. 3) was calculated as the ratio of measured radioactivity to the introduced quantity (IQ). This IQ was measured in 10 µL subsamples of the working solution placed in scintillation vials with 5 mL of PCS cocktail.

Bulk $^{33}P_i$ uptake rate (dpm L^{-1} h^{-1}) was calculated as:

$$V = \frac{dpm_t - dpm_0}{filtered\ volume \times \Delta t} \quad (1)$$

When the in situ P_i concentration ($[P_i]$) was measurable (i.e., > 20 nM), the uptake rate (V, nM h^{-1}) was calculated as follows:

$$V = \frac{[P_i] \times R_t}{\Delta t} \quad (2)$$

Calculations for sorted groups were based on the radioactivity measured for a known number of cells (dpm $cell^{-1}$, $dpmc_t$ for the samples and $dpmc_0$ for the blanks). The introduced quantity of label was expressed per volume unit (IQ_{ml}). Thus, the fraction of assimilated label per group, R_{tgp}, was determined:

$$R_{tgp} = \frac{N_{gp} \times (dpmc_t - dpmc_0)}{IQml} \quad (3)$$

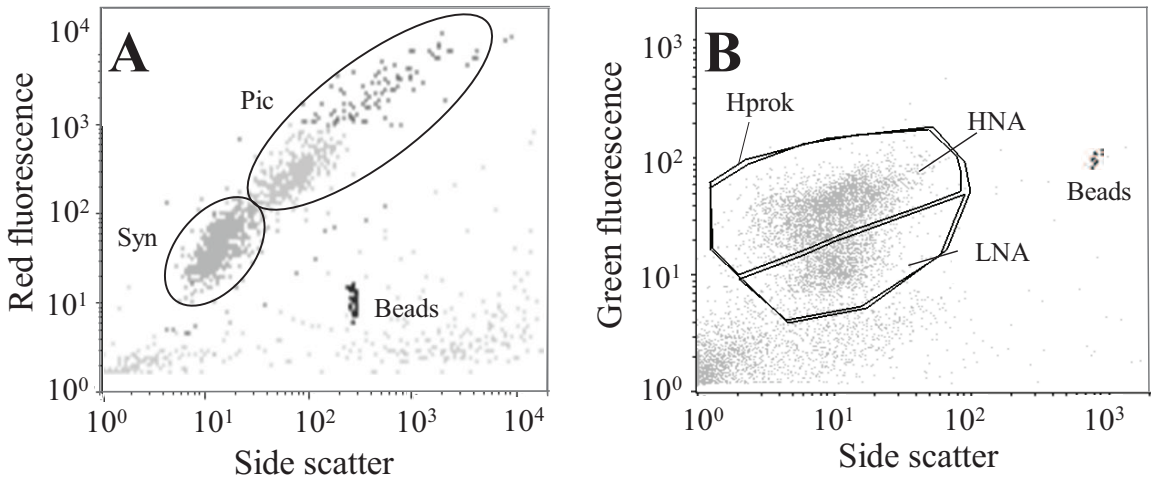

Fig. 1. FCM biparametric plot of the red (i.e., Chlorophyll) or green (i.e., SYBR Green I) fluorescence versus the side scatter (i.e., proxy for the cell structure properties) for A) autotrophic groups (*Synechococcus* = Syn, and pico/nanophytoeukaryotes = Pic) and B) SYBR Green I–stained heterotrophic groups (Hprok, including high nucleic acid content and low nucleic acid content subgroups, HNA and LNA, respectively).

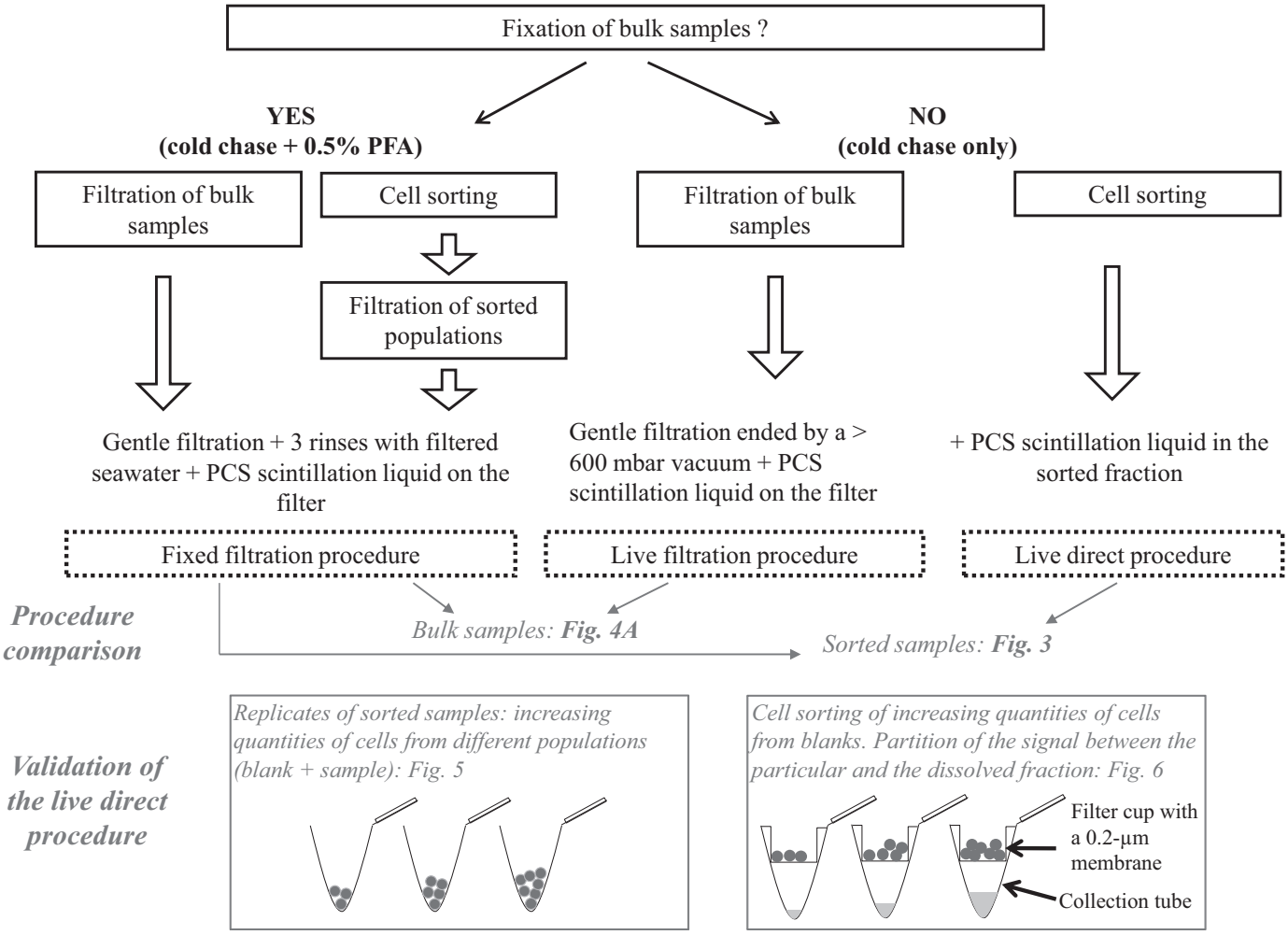

Fig. 2. Matrix of the tests presented in this study to validate the live direct procedure. All filtrations were processed on Nucleopore 0.2 µm pore-sized polycarbonate membranes (diameter of 25 mm).

Taxon-specific volumetric P_i uptake rates, V_{gp}, are estimated in nM h^{-1}, with R_{tgp} instead of R_t in Eq. 2. Using the cell abundance of a group (N_{gp}), a cell-specific uptake rate (V_{cell}, amol P $cell^{-1}$ h^{-1}) for a sorted group can be derived from the previous calculation, as follows:

$$V_{cell} = \frac{[P_i] \times R_{tgp}}{N_{gp} \times \Delta t} \quad (4)$$

Note that V_{cell} is, in fact, independent from N_{gp}, as shown by combining Eqs. 3 and 4.

Statistics

Statistical tests (parametric and nonparametric) were run using the XLStats software.

Assessment

All tests conducted to validate the protocol described in the "Materials and procedures" section are presented below and the different procedures used during the tests are schematically summarized in Fig. 2.

Live versus fixed samples

Cell loss

The effect of 0.5% PFA fixation on cell counts was tested for the three taxonomic groups of interest (Syn, Pic, and Hprok). There was no significant difference in cell counts between live and 0.5% PFA fixed samples (U = 9.000, P = 0.100, for Hprok and Syn; U = 8.500 and P = 0.200 for Pic, df = 3 for all groups). This allowed us to enumerate fixed samples while sorting live ones. Nevertheless, a better discrimination of the high and low nucleic acid content subgroups (HNA and LNA, respectively) was achieved with fixed samples, leading to higher LNA/HNA ratios in fixed samples. A higher background level was observed on the cytogram after fixation (data and cytograms not shown).

Comparison of the live direct and the fixed filtration procedures

In order to compare the most commonly used protocol (fixed filtration procedure, Fig. 2) with our new protocol (live direct procedure), we conducted experiments on seawater col-

lected at the oligotrophic SOLA station, in the Bay of Banyuls-sur-mer, France, on 3 occasions. $^{33}P_i$ incubations were processed as described in the "Material and procedures" section and were followed by cold chase. Samples were split into 2 sets before sorting: one set was fixed with 1% PFA (final concentration) while the other set was kept unfixed (live). After sorting, each series was treated with either the fixed filtration procedure or the live direct procedure, and their activities were compared (Fig. 3).

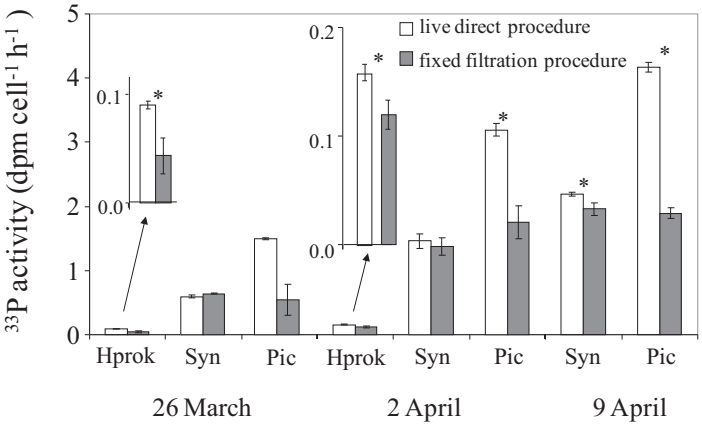

Fig. 3. ^{33}P activity per cell and per hour (mean ±SD, n = 3) of taxonomic groups sorted after incubation with $^{33}P_i$ stopped by cold chase (Syn = *Synechococcus*, Pic = piconanophytoeukaryotes, Hprok = heterotrophic prokaryotes) from 3 different experiments conducted on surface sea water samples from SOLA station. Sorted groups were treated either using the fixed filtration procedure (gray bars) or the live direct procedure (white bars). Rates have been corrected for their respective blank. An asterisk (*) indicates a significant difference between the two methods.

The loss of activity due to fixation and filtration is particularly pronounced for fragile phytoplanktonic cells such as Pic cells (31 ± 26% loss) and heterotrophic prokaryotes (39 ± 18% loss), while no or slightly significant effect was measured for *Synechococcus* cells (17 ± 23% loss). When considering bulk signals, the loss in 0.5% PFA fixed samples compared with live samples could reach up to 50%, with an average of 43 ± 6% (data not shown, n = 3 series of triplicates).

An alternative protocol

We have shown that the use of a fixative to stop the incubation of a radiolabeled substrate followed by a filtration step can cause considerable signal underestimation. Considering the well-known issue of physical damages caused by vacuum filtration (Arthur and Rigler 1967; Goldman and Dennett 1985) and the inevitable leakage of cell content (Lasker and Holmes 1957; Berman 1973) reported for some autotrophic populations, we concluded about the need for a revised methodology that would avoid those major issues.

Our protocol thus uses cold chase only instead of fixatives and does not require vacuum filtration of sorted cells, and its suitability was verified through critical tests such as sample storage after stopped incubation, reproducibility of the measurements and relevancy of the live blanks.

Sample storage

It can take a few hours to sort cells for the measurement of significant signals (i.e., those at least 3 times higher than the blank value) since autotrophic organisms are not highly abundant in oligotrophic environments such as the NW Mediterranean Sea, and since the $^{33}P_i$ has to be introduced at a trace level. Therefore, we have determined how the storage time between the end of the incubation and the cell sorting steps affects the signal, over periods of up to 48 h (Fig. 4A).

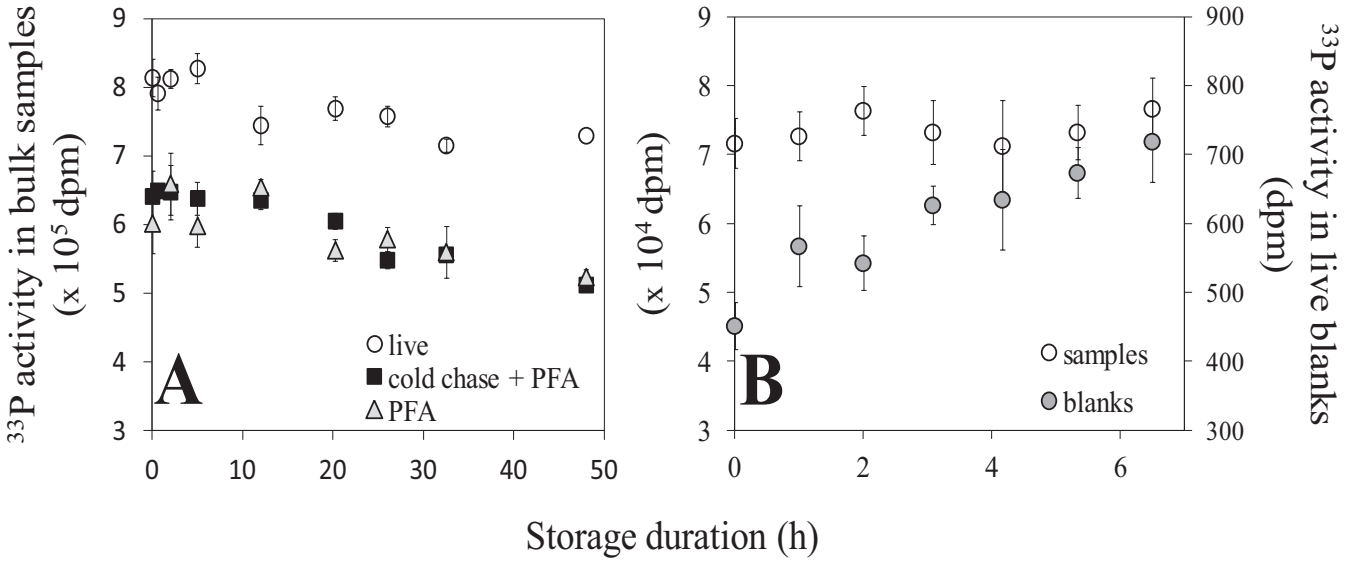

Fig. 4. Variation in bulk samples ^{33}P activity at 4°C in the dark. A: over 48 h (× 10^5 dpm) in samples stopped by cold chase only (circles, 'live'), by cold chase with PFA (squares, 'cold chase + PFA' –0.5% final concentration), or by PFA only (triangles, 'PFA only' –0.5% final concentration), mean ± SD, n = 3. B: over 7 h in samples (× 10^4 dpm, left y-axis) and blanks (dpm, right y-axis) treated by cold chase only.

The effect of storage at 4°C in the dark was tested on 30 mL samples and incubation was stopped either by cold chase only, by cold chase followed by 0.5% PFA fixation, or by a 0.5% PFA fixation only (Fig. 4A). Three milliliter subsamples were filtered at different times after cold chase. Fixed samples were subjected to the fixed filtration procedure (Fig. 2), whereas live samples were treated with the live filtration procedure. Activities in both 0.5% PFA fixation treatments (PFA only or cold chase + PFA) were not significantly different ($F = 1.167$, $\alpha = 0.01$, $df_1 = 8$, $df_2 = 8$, $P = 0.833$), therefore data were pooled ($n = 6$ at each time point) for further description and comparison with the live filtration procedure. Immediately following the end of incubation (0 h, Fig. 4A), the signal measured in fixed bulk series was significantly lower (24%, $t = 7.589$, $df = 4$, $P < 0.001$) than in the live series. This result underlines the immediate effect of PFA on signal loss. After 48 h storage, signal loss was 10% in the live series whereas it was significantly higher in the fixed samples (20% and 13% for the cold chase + PFA and the PFA series, respectively). The storage of live samples is thus more reliable in terms of signal conservation over 48 h than is the storage of fixed samples.

Considering that the use of PFA leads to high signal loss, this test was conducted again on live samples only, for a shorter duration and shorter time intervals (7 h, Fig. 4B). In the samples, radioactivity did not vary significantly ($F = 1.610$, $df = 6$, $P = 0.168$) over the 7 h following cold chase (Fig. 4B), suggesting that within this period there was no loss of $^{33}P_i$ signal. We also focused in this test on the time variability of the live blank signals. In spite of a significant signal increase over time (63%, $F = 15.92$, $df = 6$, $P < 0.10^{-4}$), blank signals remained low over 7 h storage (452 to 719 dpm, Fig. 4B), representing less than 1% of the lowest sample activity. Blank signal can thus be considered as a background signal. A delay of 7 h was the maximum storage time necessary for processing samples from the oligotrophic NW Mediterranean, and so, as long as this time is respected, the activity did not change significantly. For longer periods (up to 48 h), a loss is observed but values are still reasonable and quantifiable. Using live samples allowed optimal conservation of the signal over 7 h with no significant change and with the lowest loss over 48 h, when compared with other treatments involving a fixative.

Reproducibility

The reproducibility of P_i uptake measurement was assessed on live unfiltered sorted samples in order to check the reliability of the method and to avoid, if possible, the need for replication in further work (Table 1). Variability is compared with results obtained in fixed sorted samples after the fixed filtration procedure.

The mean coefficient of variation (CV) of the ^{33}P uptake rates was significantly higher when samples were treated with the fixed filtration procedure ($P = 0.033$, Table 1), and both methods were evaluated using comparable ranges of sorted cells and uptake rates. These results show that the live direct procedure described in this study provides more reproducible values than the fixed filtration procedure for measuring taxon-specific P_i uptake rates. We have thus applied this optimized method based on live and unfiltered sorted fractions to assess P_i uptake from natural samples.

Blank preparation

In previous methods, blanks corresponded to samples with addition of a reagent (e.g., $HgCl_2$, trichloroacetic acid, PFA) that killed cells before addition of the radioactive isotope (Duhamel et al. 2006; Tanaka et al. 2006; Larsen et al. 2008). To compare live blanks with the killed blanks cited in the literature, triplicate 10 mL samples were treated either with 4% trichloracetic acid (Tanaka et al. 2006; Larsen et al. 2008), $HgCl_2$ (20 mg L^{-1} final concentration, Kattner 1999; Moutin et al. 2005), or with 1% PFA final concentration (Zubkov et al. 2007). Only bulk unsorted samples were used in this test: they were treated either with the live filtration procedure or with the fixed filtration procedure, for live and killed blanks, respectively. Fifteen minutes after the addition of the tested reagents, samples were spiked with $^{33}P_i$. Cold chase was performed in all samples 1 h after the label spiking, and was followed by filtration. No significant difference was found between live blanks obtained with an excess of P_i and blanks treated with a fixative ($K = 8.007$, $df = 3$, $P = 0.046$, data not shown). In this experiment, the blanks obtained by the differ-

Table 1. Variability of the $^{33}P_i$ uptake rates (dpm $cell^{-1}$ h^{-1}) measured for different samples and groups treated with the live direct procedure or the fixed filtration procedure: range of the quantity of sorted cells and measured uptake rates, mean and range of coefficients of variation (CV) calculated on n triplicated values.

			N (cells mL^{-1})		^{33}P uptake rates (dpm $cell^{-1}$ h^{-1})		CV (%)		
Material	Procedure	n	Min	Max	Min	Max	Mean	Min	Max
sorted Syn	direct live	24	1500	20000	0.0024	1.137	4.9	1.03	11.33
sorted Pic	direct live	27	1500	15000	0.0022	4.173	6.1	0.87	20.83
sorted Hprok	direct live	18	50000	500000	0.0010	0.121	11.0	3.23	30.22
sorted Syn	fixed filtration	9	1000	12000	0.0138	1.046	20.8	4.94	45.29
sorted Pic	fixed filtration	9	1000	9000	0.0228	1.110	11.7	3.96	20.69
sorted Hprok	fixed filtration	7	25700	400000	0.0053	0.141	15.4	2.74	28.13

ent treatments represented less than 2% of the signal measured in the samples.

Live blanks submitted to early cold chase 15 min before the radioactive isotope addition were also used in our protocol because blank values were low and were comparable to values measured in killed blanks, and finally because the scattergrams visualized from samples and blanks were similar.

In the following tests, only the live direct procedure is involved to check the validity of our protocol.

Blank-to-signal values

Data were used to test the variability among samples of the blank-to-signal ratio (Table 2). The blank-to-signal ratio was generally lower for bulk activity than for sorted groups because of the nonincorporated radioactivity recovered in the micro tubes (see next paragraph). For Hprok sorts, blank-to-signal ratio reached up to 0.35 (Table 2).

These results obtained on samples from various environments show that blank values are variable but that they represent, on average, less than 7% and 17% of the signals measured in regular samples from autotrophic and heterotrophic sorted samples, respectively.

Due to their high variability, these results emphasize the necessity of measuring a blank value for each sorted group. As Hprok group was always the fraction collected that was accompanied by the largest volume of liquid, we further investigated the origin of the blank signal and its evolution following increasing numbers of cells sorted.

Origin of the blank signal in the live direct procedure:

In the present protocol, scintillation cocktail was added directly to the sorted fractions, which were composed of up to 300 µL of a mixture of sorted cells and liquid. We chose the PCS scintillation cocktail because of its potential to mix with up to 50% saline solutions (e.g., seawater).

Each sorted fraction is composed of drops containing the targeted cells surrounded by an undetermined volume of liquid (sample seawater and sheath fluid). Since the radioactivity in live blanks increased with the number of sorted cells (Fig. 5), the potential source(s) of nonincorporated radioactivity recovered in the sorted fractions had to be quantified.

Table 2. Range (minimum and maximum), mean and standard deviation (sd) of the blank-to-signal ratio obtained for bulk samples (using the live filtration procedure) and sorted populations (Syn, Pic, and Hprok, using the live direct procedure). N is the number of blank-to-sample ratios calculated from experiments using samples from the Bay of Banyuls, the SOLA and MOLA stations, and from the BOUM cruise.

	Blank-to-signal ratio (%)			
	Bulk	**Syn**	**Pic**	**Hprok**
N	29	28	20	21
min	0.06	1.8	0.59	2.5
max	8.7	18.5	13.6	35.0
mean ±SD	1.2 ± 1.8	6.0 ± 4.3	4.6 ± 4.2	16.4 ± 11.0

For the next experiment, Hprok was chosen because of its higher blank-to-signal values compared to other groups, and also because sorting this group leads to the recovery of the highest volumes of liquid. Increasing numbers of Hprok cells from live blanks were sorted in triplicate. To separate the particulate from the dissolved fractions, sorted cells were recovered in 2 mL micro tubes (Ultrafree MC, Millipore) containing a filter cup equipped with an inner 0.22 µm Durapore membrane (cf. experimental design, Fig. 2). After 15 to 60 s of centrifugation at 1000 rpm, the filter cup was removed and was inserted into a scintillation vial with PCS cocktail to determine the particulate fraction activity.

The volume of liquid recovered in the collection tubes was proportional to the number of sorted cells with 1.18 ± 0.06 µL (average ± SD, $n = 7$) of sample for 1000 Hprok cells. The collection tubes were filled with PCS liquid to determine the dissolved fraction of radioactivity. The radioactivity of the particulate fraction remained constant regardless of the number of sorted cells (150 ± 23 dpm, $n = 12$, Fig. 6), indicating that nonbiological uptake was negligible and that the background could be attributed to the adsorption of the radioisotope onto the Durapore membrane only. A linear relationship was found between the radioactivity in the filtrate (i.e., in the liquid surrounding sorted cells, Fig. 6) and the number of sorted cells with a slope of 0.014 dpm cell^{-1} ($r_{\_} > 0.99$, $P < 2.10^{-4}$). These results suggest that the radioactivity measured in a sorted control is mainly attributable to the sample layer surrounding single sorted cells and including some unincorporated radioactivity, and is proportional to the number of sorted cells.

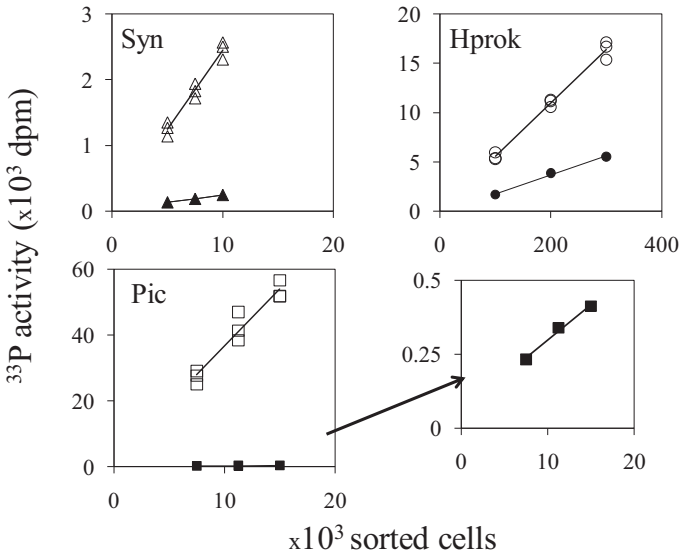

Fig. 5. Examples of the variation of the radioactive signal according to the number of sorted cells for 3 groups treated with the live direct procedure: *Synechococcus* (Syn, $r_{\_} = 0.96$), pico/nanophytoeukaryotes (Pic, $r_{\_} = 0.94$), and stained heterotrophic prokaryotes (Hprok, $r_{\_} = 0.97$). Open symbols: incubated samples; black symbols: blank samples.

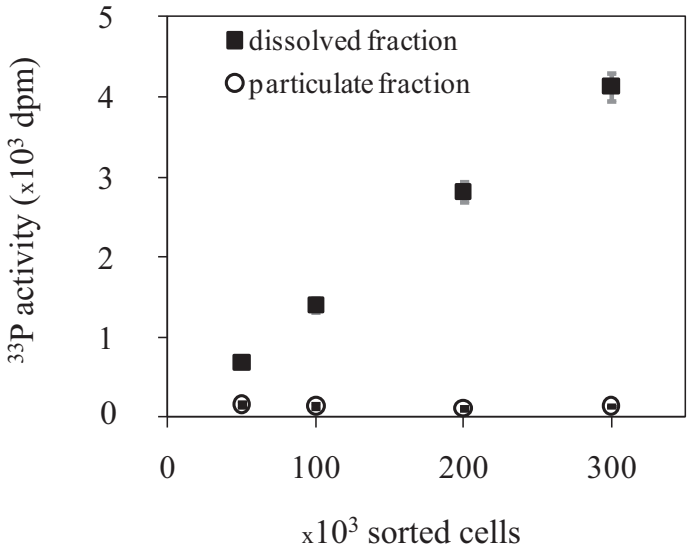

Fig. 6. Variation of the radioactive signal in a blank according to the number of Hprok cells sorted in the particulate (circles) and the dissolved phases (squares, y = 0.014x + 16.775, $r\_$ = 0.999) associated to the sorted fraction (average ± SD, *n* = 4).

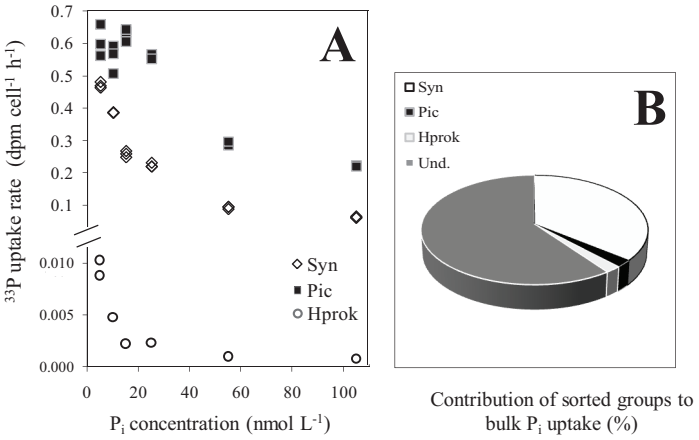

Fig. 7. P_i uptake by Synechococcus (Syn, n = 3), piconanophytoeukaryotes (Pic, *n* = 3), and heterotrophic prokaryotes (Hprok, *n* = 2). A) Taxon specific $^{33}P_i$ uptake rate(dpm cell^{-1} h^{-1}) versus P_i concentration (nmol L^{-1}), B) contribution of Syn, Pic, Hprok, and undetermined (Und.) groups to bulk P_i uptake (%) under in situ P_i concentration (5 nmol L^{-1}).

This test proves that the nonbiological activity obtained in samples prepared according to our new protocol is due solely to the dissolved fraction and is correctly measured in the live blanks.

Application

We applied our improved method on triplicate samples incubated at increasing P_i concentrations and measured taxon-specific $^{33}P_i$ uptake rate normalized to cell abundance in Syn, Pic, and Hprok groups (Fig. 7A). Seawater was sampled at the offshore MOLA station (7 May 2008, 25 m) where the in situ P_i concentration was below the 20 nM detection limit of the auto analyzer. From the samples where no cold P_i solution was added, we could estimate the contribution of the different groups to the bulk P_i uptake (Fig. 7B).

This experiment showed that cell ^{33}P uptake activity is higher for Pic cells, followed by Syn, while Hprok cells show activities about 50-fold lower (Fig. 7A). This is not surprising as cell activities are systematically related to cell size (Bjornsen et al. 1989).

Volumetric uptake rates of the 3 groups only add up to 40% of the bulk P_i uptake flux (Fig. 7B), with a high contribution of Syn (35%), and equal contributions of Pic and Hprok. The high contribution of undetermined group(s) might be partly attributable to *Prochlorococcus*, which may be found at this offshore station in May, but which we could not detect by flow cytometry. These cyanobacteria can indeed play a major role in P_i uptake fluxes in P_i-depleted waters like the North Atlantic compared to Syn and Pic (Zubkov et al. 2007). Considering an in situ concentration of 5 nM, cell P_i uptake rates (V_{cell}, Eq. 4) were of 0.89, 0.07, and 0.01 amol P cell^{-1} h^{-1} for Syn, Pic, and Hprok, respectively.

Discussion

Measuring phosphorus uptake (in both inorganic and organic forms) is essential to better understand processes controlling planktonic activities in marine environments, especially in oligotrophic and P-depleted systems. It has indeed been shown that global change may lead to increased oligotrophication of surface waters caused by intensifying stratification (Polovina et al. 2008). Taxon-specific P_i uptake data are an effective tool to refine biogeochemical models relying on heterotrophic and autotrophic compartments. Taxon-specific P_i-uptake kinetic parameters can be assessed using per cell uptake rates and may be used in mechanistic models (Mauriac et al. 2011). On the contrary, volumetric data are particularly suitable to feed global biogeochemical models (Thingstad 2005; Macias et al. 2010).

One of the most problematic steps used in previously published methods to measure taxon-specific P_i uptake lies in the use of a fixative before samples are sorted (Fu et al. 2006; Zubkov et al. 2007; Larsen et al. 2008; Casey et al. 2009). This study shows that signal loss due to a 0.5% PFA fixation ranged between 17% and 50%, for both bulk and sorted samples. This variable signal loss makes data comparison among studies difficult and is a serious drawback that should not be ignored especially when estimating biogeochemical P fluxes over large scales. Because signal loss in fixed cells is variable between groups and between samples, no empirical correction could be estimated and applied to fixed samples. Previous studies examined the effect of fixatives on cell counts because fixation is necessary when storing samples before analyses (Troussellier et al. 1995; Del Giorgio et al. 1996; Gasol and Duarte 2000; Kamiya et al. 2007). However, results did not lead to unanimous conclusions. Kamiya et al. (2007) showed that there was no significant variation in bacterial counts due to fixation (1% paraformaldehyde PFA + 0.01% glutaraldehyde GA). On the

contrary, Troussellier et al. (1995) concluded that each fixative (formaldehyde FA, PFA, and GA, 2% final concentration) has a different effect on a given group, generally inducing a decrease in bacterial counts. We found no significant cell loss in fixed samples (analyzed 1 h after fixation) as compared to live samples. Thus, cell leakage, rather than cell loss, should explain the loss of radioactive signal after sample fixation. In P_i uptake studies, sample fixation has often been controversial (Zubkov et al. 2007; Larsen et al. 2008) and our study demonstrates the reliability of an alternative protocol avoiding fixation.

By avoiding the fixation and filtration steps, the improved protocol presented here saves considerable costs due to materials (e.g., filters, vials, cocktail) and radioactivity handling. Our methodology has been tested in a Mediterranean area through coastal and offshore sampling, and it works in those P_i-depleted (< 20 nM) surface and low-biomass oligotrophic waters (comparable to the Sargasso Sea).

With the use of a single sample for the treatment of the bulk community and the sorted fractions, it is also possible to optimize time by reducing the number of replicates or increasing the number of samples to process (e.g., more depths or treatments). Taxon-specific P_i uptake rates measured in oligotrophic samples with this optimized protocol are less affected by methodological biases, and thus provided more accurate estimates.

Using this improved methodology, P_i uptake by phytoplanktonic eukaryotes might lead to higher values in environment where it may have been underestimated due to cell leakage. To assess P_i fluxes in a microbial planktonic foodweb, the next methodological limit will be the feasibility of sorting large-sized and undamaged cells (e.g., flagellates, nano-microphytoplankton cells).

Comments and recommendations

When using the presented improved protocol, nonincorporated radioisotope present in the liquid surrounding each sorted cell is recovered in the sorted fractions and is not removed as with a filtration procedure. This signal needs to be quantified in a blank and subtracted from the activity of a given group. To accurately measure taxon-specific P_i uptake rates, we recommend to systematically prepare blanks and substract its value to the corresponding group of interest.

Although by-sorting was shown to be insignificant in natural communities from the Sargasso Sea (Zubkov et al. 2007), we recommend checking the by-sorting of heterotrophs along with autotrophs when starting a series of experiments, even when using the highest purity level on the sorter. Using the lowest signals detected in sorted Hprok along our study and results from Larsen et al. (2008: autotrophic to heterotrophic by-sorting ratio from 1.51 to 4.86), we evaluated the bias introduced by by-sorting. Calculations were processed to maximize the by-sorting impact on P_i uptake measurements, and resulted in a 10% overestimation of Pic uptake rates, which is close to the inner variability of cell uptake rate measurements (Table 1). A hypothesis might be that these by-sorted heterotrophic prokaryotes are attached to pico/nanophytoeukaryotes and that they could supply autotrophic cells with freshly incorporated and/or regenerated P_i (Carrillo et al. 2008).

Our method is sufficiently sensitive to be applied to natural groups from P-depleted environments; however it should be used only when samples can be fully processed shortly after sampling. When fixation is necessary in place of an operational embarked cell sorter, the leakage of radioactive P_i due to fixation should be checked systematically for all groups and all samples.

As for traditional bulk measurements, several steps of the protocol require adjustment according to cell abundance and activity, as well as P_i turnover time, before this method can be applied to a new environment. We thus recommend users run several tests to adjust the quantity of introduced label, incubation time and incubated volume, the number of sorted cells and potential by-sorting before using the protocol described in this study with their samples. We recommend using our improved protocol to measure taxon-specific Pi uptake rates so that measurements can be compared between systems.

Finally, a future step in the evaluation of the contribution of taxonomic groups to the total P_i uptake will be the determination of biomass-specific P_i uptake rates (i.e., the P_i uptake per biomass unit instead of P_i uptake per abundance unit). To measure accurate values of P biomass obtained from the same sample as the labeled sample, a high resolution technique such as X-ray fluorescence spectroscopy is necessary. This is, so far, the most accurate method to obtain a specific P cellular content (fg P $cell^{-1}$, Tanaka et al. 2006; Løvdal et al. 2008), when there is enough particulate material samples.

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