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Specific rates of leucine incorporation by marine bacterioplantkon in the open Mediterranean Sea in summer using cell sorting

A. Talarmin[1,2,3,4], **F. Van Wambeke**[1,2], **P. Catala**[3,4], **C. Courties**[3,5], and **P. Lebaron**[3,4]

[1]Université de la Méditerranée – Aix Marseille 2, UMR 6117, Centre d'Océanologie de Marseille, Case 901, Campus de Luminy, 13288 Marseille, France
[2]CNRS/INSU, UMR 6117, LMGEM, Case 901, Campus de Luminy, 13288 Marseille, France
[3]UPMC Univ Paris 06, Laboratoire ARAGO – Observatoire Océanologique, 66651 Banyuls/mer, France
[4]CNRS, UMR 7621, LOMIC, Observatoire Océanologique, 66651 Banyuls/mer, France

[5]CNRS, UMS 2348, Observatoire Océanologique, 66651, Banyuls/mer, France

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Correspondence to: A. Talarmin (agathe.talarmin@univmed.fr)

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Abstract

Cell-specific leucine incorporation rates were determined in early summer across the open stratified Mediterranean Sea along vertical profiles from 0 to 200 m. During the period of our study, the bulk leucine incorporation rate was on average 5.0 ± 4.0 ($n = 31$) pmol leu l^{-1} h^{-1}. After ^{3}H-radiolabeled leucine incorporation and SyBR Green I staining, populations were sorted using flow cytometry. Heterotrophic prokaryotes (Hprok) were divided in several clusters according to the cytometric properties of side scatter and green fluorescence of the cells: the low nucleic acid content cells (LNA) and the high nucleic acid content cells (HNA), with high size and low size (HNA-hs and HNA-ls, respectively). LNA cells represented 45 to 63% of the Hprok abundance between surface and 200 m, and significantly contributed to the bulk activity, from 17 to 55% all along the transect. The HNA/LNA ratio of cell-specific activities was on average 2.1 ± 0.7 ($n = 31$). Among Hprok populations from surface samples (0 down to the deep chlorophyll depth, DCM), HNA-hs was mostly responsible for the leucine incorporation activity. Its cell-specific activity was up to 13.3 and 6.9-fold higher than that of HNA-ls and LNA, respectively, and it varied within a wide range of values (0.9–54.3×10^{-21} mol leu $cell^{-1}$ h^{-1}). At the opposite, ratios between the specific activities of the 3 populations tended to get closer to each other, below the DCM, implying a potentially higher homogeneity in activity of Hprok in the vicinity of nutriclines. *Prochlorococcus* cells were easily sorted near the DCM and displayed cell-specific activities equally high, sometimes higher than the HNA-hs group (2.5–55×10^{-21} mol leu $cell^{-1}$ h^{-1}). We then showed that all the sorted populations were key-players in leucine incorporation into proteins. The mixotrophic feature of certain photosynthetic prokaryotes and the non-negligible activity of LNA cells all over Mediterranean were reinforced.

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1 Introduction

The advent of flow cytometry (FCM) in the past 20 years allowed the differentiation and thorough exploration of 2 populations of heterotrophic prokaryotes (Hprok), according to their cellular nucleic content: the low nucleic acid (LNA) and the high nucleic acid (HNA) containing cells (Li et al., 1995; Troussellier et al., 1995; Jellett et al., 1996; Marie et al., 1997; Gasol et al., 1999; Lebaron et al., 2001). In many studies covering contrasted ocean areas and environmental conditions, abundances of HNA and LNA cells were compared to the bulk heterotrophic prokaryotic production (BP). Generally, a positive relationship was found between percentages of HNA abundances and BP (Lebaron et al., 2001; Moran et al., 2007). In other studies, radiolabeling coupled to cell sorting emerged as a powerful technique to assess specific activities of both populations. The content of nucleic acid was at first thought to be directly linked to the cellular activity. It was indeed shown that HNA cells were specifically responsible for more than 70% of the bulk ^{3}H-leucine incorporation activity, in various environments, due to their high cell-specific activity (Lebaron et al., 2002; Servais et al., 2003; Longnecker et al., 2005; Scharek and Latasa, 2007; Belzile et al., 2008; Stenuite et al., 2009).

However, not all studies reached similar conclusions. Some authors found that LNA must gather dormant, inactive, or dead cells (Jellett et al., 1996; Gasol et al., 1999; Gasol and del Giorgio, 2000; Lebaron et al., 2001; Seymour et al., 2004), while others did not support this hypothesis and validated a more dynamic role of LNA in the BP (Mary et al., 2006; Sherr et al., 2006; Bouvier et al., 2007; Hill et al., 2010). Part of the disparity in all results could be related to a high phylogenetic diversity of the populations sampled, and/or to varying biotic and abiotic factors driving the dynamics and the activity of the bacterioplankton. Indeed, various conclusions about the role of HNA and LNA in the environment were drawn after diversity studies. Molecular fingerprinting or FISH techniques applied on sorted fractions showed that HNA and LNA populations could reflect either comparable diversity picture, or very different patterns (Servais et

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al., 2003; Longnecker et al., 2005; Obernosterer et al., 2008). Roughly, most findings suggested that Hprok was rather homogenous in terms of diversity but heterogenous in terms of activity.

Among biotic and abiotic factors governing the dynamics and the activity of HNA and LNA, the most often examined are grazing and viral lysis rates of these 2 populations (Nishimura et al., 2005; Williams et al., 2008; Longnecker et al., 2010), the primary production and nature of neoproducts (Lebaron et al., 2001; Moran et al., 2007; Moran and Calvo-Díaz, 2009), temperature (Shiah and Ducklow, 1995; Longnecker et al., 2006b; Andrade et al., 2007), eutrophic gradients (coastal-open ocean gradient: Longnecker et al., 2005; Longnecker et al., 2006a; Sherr et al., 2006; Longnecker et al., 2010), vertical distribution along the water column (Scharek and Latasa, 2007; Stenuite et al., 2009), P depletion (in lakes: Nishimura et al., 2005).

Both populations were systematically found to contribute to the BP, and HNA was more responsive and fluctuating. The observed variability in the percentages of HNA and LNA in the total abundance or BP might be the reflection of the impossibility of categorizing cells as live-and-active or dead-or-inactive. Single-cell activity, as well as any ecological parameter, should be considered as a continuum from a minimum to a maximum (Servais et al., 1999; Smith and del Giorgio, 2003), with many intermediate states. Several potential interactions between the 2 populations were exposed by Bouvier and collaborators (Bouvier et al., 2007), with one-way cell migrations from one group to the other, exchanges of cells and looping (proper cell-cycle, isolated from input and output).

The oligotrophic to ultraoligotrophic status of the open Mediterranean Sea has important consequences on the shaping and functioning of the microbial food web, the picoplankton dynamics being largely influenced by the availability of N and P within the surface layer (Siokou-Frangou et al., 2010). Carbon fluxes through the microbial compartment are mostly channeled through Hprok populations (Scharek and Latasa, 2007). The food web is thus dominated by small-sized organisms involved in the microbial loop, including Hprok, whose carbon demand often exceeds

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phytoplankton primary production (Van Wambeke et al., 2002b). BP in the Mediterranean Sea was shown to remain lower than 200 ng C l^{-1} h^{-1} (Lemée et al., 2002; Van Wambeke et al., 2002a).The resulting metabolic balance is mostly net heterotrophic during the post bloom and stratification periods in the different basins (Regaudie-de-Gioux et al., 2009).

It is thus interesting to know which Hprok populations are highly involved in the C flux through the microbial loop (Yokokawa and Nagata, 2010), if it can change, and why it does change with varying nutrient limitations (e.g. along vertical profiles) and degrees of oligotrophy across the Mediterranean Sea. Our study aims at quantifying the flux of leucine incorporation through the bacterioplankton, at the population and single-cell levels, using cell sorting. The response of bacterioplanktonic populations after radiolabeling and cell sorting was studied in both in situ freshly collected samples and in mid-term amendment experiments (36 or 96 h incubations), in order to check potential control of biological processes by nutrients. For this purpose, bacterioplankton abundance and production were interrogated at selected stations during the stratification period across the main basins (Algero-Provencal, Ionian, Levantine). Not only Hprok populations were considered, but also, occasionally, *Synechococcus* (Syn) and *Prochlorococcus* (Proc) cyanobacteria, who are the main primary producers in the Mediterranean Sea (Hagström et al., 1988; Man-Aharonovich et al., 2010). Indeed, it was for long hypothesised that these 2 populations were occasionally mixotrophs (Shalapyonok et al., 1998; Gasol and del Giorgio, 2000; Bertilsson et al., 2005), which was confirmed by recent studies in diverse oceans (Zubkov et al., 2004; Eiler, 2006; Michelou et al., 2007; Mary et al., 2008a; Zubkov and Tarran, 2008), but so far not in the Mediterranean Sea.

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2 Material and Methods

2.1 Study area and sample collection

The work was carried out during the cruise "BOUM" on the r/v Atalante in June–July 2008. The cruise was planned as a transect of stations encompassing a large longitudinal gradient in the Mediterranean Sea (Fig. 1, see also Moutin et al., 2010). Water samples were collected using a rosette equipped with 24×12-l Niskin bottles mounted on a CTD system. At 5 stations (St. C, St. B, St. 21, St. A and St. 25, from East to West, Fig. 1), samples were collected within the euphotic zone (0–200 m) for the determination of bulk and cell-specific leucine incorporation in the prokaryotes, and for cell enumeration.

2.2 Enrichment experiments

Occasionally, onboard enrichment experiments were conducted. Notably, the effects of N, P, or N+P amendments on the whole microplanktonic food web were investigated through a multidisciplinary approach at stations A, B and C (Tanaka et al., 2010). For further cell sorting purpose, only the experiment at site B was investigated and thus we described here only this experiment. Surface seawater samples (8 m) were collected and dispensed in series of acid-washed polycarbonate bottles (60 l, Nalgene). Under simulated in situ conditions on the deck, with running surface seawater and neutral screens of 50% incident light, triplicate bottles of each combination (unamended control C, + ammonium N, + phosphate P, + both ammonium and phosphate NP) were followed during 4 days.

Subsamples were periodically withdrawn for, among many other parameters, Hprok abundance and BP. At St. B, conditions of enrichments were + N:1600 nM NH_4Cl, +P:100 nM KH_2PO_4, NP:1600 nM NH_4Cl + 100 nM KH_2PO_4. At the end of the experiment (day 4), one of the triplicates was sampled to estimate the specific activity using cell sorting technique.

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To estimate the limiting factors more frequently and deeper in the water column, amendments were also done using 60 ml-volumes of seawater at St. 21. Amendments of phosphate (P), nitrate+ammonium (N), glucose (G) and a combination of the 4 nutrients were processed in 60-ml flasks. Samples were incubated during 36 h under in situ simulated light and temperature conditions (using neutral screens) and after this delay and subsampling was done for bulk and cell-specific BP measurements (Van Wambeke et al., 2010), as well as cell enumeration.

2.3 Onboard sample treatment

For delayed cell enumeration, seawater samples (4 ml) were transferred into cryovials, and fixed onboard using formaldehyde (2% final concentration). After at least 10 min on the bench at room temperature in the dark, samples were frozen in liquid nitrogen during 24 h and, finally, stored at –80 °C until their processing on shore (Troussellier et al., 1995), 2 months after the cruise.

For measurement of bulk and cell-specific leucine incorporation rates, three 4-ml samples were incubated per depth or treatment with a 12 nM final concentration of pure ^{3}H-leucine (Perkin Elmer Life Science, specific activity 115.4 Ci mmol^{-1}), in the dark, at simulated in situ temperature. This concentration was previously shown to be saturating for most of the bulk measurements routinely done on board using the centrifuge technique (Van Wambeke et al., 2010). Incubation durations were enlarged (but still in the linear phase of incorporation) to ensure sufficient labeling before cell sorting. One of the 3 aliquots was used as a killed control, with addition of 20% filtered PFA (0.4 ml) 10 min before addition of the radiolabeled leucine. After incubation, samples were killed with 2% PFA (final concentration), put briefly in liquid nitrogen for 15 min and stored frozen at –80 °C until analysis on shore (6 months after the cruise).

2.4 Flow cytometric analyses on shore

2.4.1 Cell enumeration

Samples were thawed at room temperature, and then analyzed using a FACSCan or a FACSCalibur flow Cytometer (BD-Biosciences), both instruments being equipped with an air-cooled argon laser (488 nm, 15 mW), and presenting the same optical-set and performances.

Phototrophic cells were enumerated with the FACSCan, according to right-angle light scatter properties (SSC, roughly related to cell size), and the orange (585/42 nm) and red (>650 nm) fluorescence from phycoerythrin and chlorophyll pigments, respectively.

SyBR Green I (Molecular Probes) was used to stain heterotrophic prokaryotic populations, which were then discriminated and counted with the FACSCalibur by their right-angle light scatter and green fluorescence due to nucleic acid labeling (Marie et al., 2000).

Data acquisition was performed using the Cell-Quest software (BD-Biosciences) on both machines. Fluorescent 1.002 µm beads (Polysciences) were systematically added to each analysed sample to normalize cell fluorescence and light scatter emissions, thus allowing comparison of results. To estimate cell abundance, the exact volume of sample analysed was determined by measuring sample volumes before and after analysis.

2.4.2 Specific leucine incorporation rates of ^{3}H-radiolabeled sorts on preserved marine samples

After thawing, the duplicates were mixed so that a sufficient volume of sample could be dedicated to all measurements: (i) as a reference, bulk leucine incorporation rates (1.5 ml, see below, Sect. 2.5), (ii) cell sorting of Hprok and (iii) cell sorting of autotrophic cells. To achieve this latter objective, 3.5-ml aliquots were dedicated to the cell sorting of autofluorescent cells, and we attempted to measure leucine incoporation by

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autotrophs. The remaining volume (3 ml) was stained with a 1/10 diluted SyBR Green I solution (Molecular probes) 30 min before cell sorting, so as to determine the cell-specific activity of Hprok populations. Samples were kept in the dark at +4 °C between unfreezing and cell-sorting.

After collection of the target number of cells in 2-ml Eppendorf tubes, sorted fractions were treated based on the centrifuge protocol (Smith and Azam, 1992). We added a TCA solution to the sorted fractions adjusted to the volume of recovered sample, yielding a final volume of 1.5 ml, and 5% TCA final concentration. Tubes were vortexed before the first run of centrifugation (16 000 g, 10 min). Then the supernatant was discarded, 1.5 ml of TCA 5% added in the tube which was centrifuged. Then, the supernatant was discarded, 1.5 ml of 80% ethanol added in the tube which was centrifuged again. The supernatant was discarded, PCS scintillation liquid was added to the radioactive pellets, and the sample was counted on a Packard 1600 TR scintillation counter calibrated with a quenched curve of tritium and equipped with vented microtube holders.

Samples used as killed blanks were sorted in the same conditions as the incubated samples. This allowed obtaining a level of blank expressed in dpm per cell for each fraction. This blank value was subtracted from each corresponding incubated sample. We were able to obtain significant signals for most of the sorted groups down to 200 m depth at all stations, with signals being at least 2-fold higher than the corresponding blank value. Coefficient of variation of per cell activities between equal sorts ranged 2 to 18%, with an average of 7% ($n = 24$).

A FACSAria cell sorter (BD-Biosciences) was used to analyse and to sort ^{3}H-radiolabeled phototrophic and stained heterotrophic populations. The FACSDiva software (BD-Biosciences) was used to run the cell sorter and acquire data. For both photo- and hetero- trophic cells, excitation light was 488 nm from the argon laser. Acquisition settings were run in order to discriminate side scatter and fluorescence emissions, showing patterns comparable to data acquired with the FACSCalibur analyser. For phytoplankton, orange and red fluorescences were collected on photomultiplicators

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at 585/42 nm and 655/40 nm, respectively, while the green fluorescence of stained heterotrophic prokaryotes induced by the SyBR Green I was collected at 530/30 nm. Sterilized particle-free seawater passed through 0.2 µm (Stericap, Whatman) was used as the sheath fluid. Analysis and sorting were run using a 70 µm nozzle, with a sheath pressure of 85 PSI. When needed, the real sample flow rate was measured using BD Trucount^{TM} Tubes and evaluated at 87.53 ± 2.31,µl min^{-1} ($n = 5$). Sorting precision mode was 0/32/0, allowing 99% of recovery. The rates of total events per second were generally largely below 1000 for phytoplankton, and below 2500 for Hprok sorts. According to the population richness, 4, 3 or 2 sorting-ways were simultaneously used, allowing to isolate from 500 to 250 000 sorted cells in different Eppendorf centrifuge tubes. No difference was noticed in the cytograms originating from a radioactive and a non-radioactive sample.

The cell-specific activity of a population was determined by dividing the activity of the sorted fraction by the total number of cells sorted within this population. The volumetric activity of a population was determined by multiplying the cell-specific activity by the abundance of that population.

2.5 Bulk sea water leucine incorporation rates

After the thawing of a tube for further cell sorting, an aliquot of 1.5 ml was discarded for measuring total unsorted leucine incorporation rates so that the bulk activities were to be compared to the specific activities from the same incubated sample, and submitted to the same conditions of fixation and storage. Trichloracetic acid (TCA) was added, yielding 5% TCA final concentration, and then the centrifuge technique was used, as for the sorted fractions.

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2.6 Statistical analyses

Data are all presented as average ± standard deviation. Relationships between environmental parameters and leucine incorporation rates measured either in situ or in the nutrient enrichment experiments were tested by 1-way analysis of variance (ANOVA on Log_{10}-transformed data, followed by Fisher's LSD paired comparison), linear regression model I and II, Pearson's correlation matrices, but also non-/parametrical tests, all performed using the XLStats software.

3 Results

3.1 Environmental parameters and microbial community structure

Vertical distribution (0 down to 150 or 200 m, according to stations) of ancillary parameters followed a classical trend for this period of the year. Concentrations of both nitrate and phosphate were low in surface layers, often below the limit of detection (Pujo-Pay et al., 2010). The deep chlorophyll maximum deepened towards the East (Table 1), from 51 m at the most coastal site sampled for this study, i.e. St. 25, to 120 m at St. B. Among the samples collected in this study, the highest chlorophyll concentration was obtained at St. 25 (1.86 $\mu g\,l^{-1}$ at the DCM and 38 $mg\,m^{-2}$ throughout the 0–150 m layer). The mixed layer was restricted to about 9 to 26 m, with sharp gradients of temperature below. Sea surface temperature varied from 21.4 °C (St. 25) to 26.9 °C (St. B) in the surface and ranged 13.2 to 15.6 °C at 150 m depth.

The bacterial production integrated over the first 150 m increased westwards, emphasizing the longitudinal oligotrophic gradient (Table 1). Several populations were detected and enumerated by FCM: the phototrophic *Synechococcus* (Syn) and *Prochlorococcus* (Proc) cyanobacterial populations, few pico-/nano-phytoeukaryotes (Pic) on the one hand, and the Hprok population on the other hand (Fig. 2).

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Hprok was most of the time subdivided in 3 populations discriminated according to their SSC and fluorescence properties: the low nucleic acid content cells (LNA), the high nucleic acid content cells (HNA) characterized by a high or a low scatter signal (HNA-hs and HNA-ls, respectively, Fig. 2a). A fourth Hprok population, "HNA+", was found proliferating in "short-term" enrichment experiments (36 h) realized at St. 21. HNA+ cells exhibited very high SSC values (reaching beads' SSC, Fig. 2b). In in situ samples, Hprok ranged 1.8 to 11.2×10^5 Hprok cells ml^{-1}, with maximum values close to the DCM (St. 25), and percentages of LNA (LNA/(HNA-hs + HNA-ls + LNA)) ranged 45–63% (Table 2). The contribution of LNA cells to Hprok abundance was significantly higher in the subsurface layer ("surface", above DCM) compared to deeper layers (1-way ANOVA, $F = 6.318$, $p = 0.026$).

Syn ranged from undetectable values (especially at the bottom of the euphotic zone: 200 m St. C, 140 m St. B) to a maximum of 77×10^3 cells ml^{-1} at the most coastal station, i.e. St. 25 (40 m) where the greatest integrated abundances were found (2.7×10^{12} cells m^{-2}, Table 1). At this station, maximum integrated abundances of Pic were also reached (0.22×10^{12} cells m^{-2} at 50 m). Proc cells were generally undetectable by FCM above 25 m and their concentration peaked at less than 10 m above the DCM at all stations (maxima of 81.2×10^3 cells ml^{-1} at 75 m, St. A, and 82.4×10^3 cells ml^{-1} at 100 m depth, St. B). However, the highest Proc integrated abundances were found in the easternmost stations B and C (Table 1).

3.2 Cell-specific leucine incorporation

3.2.1 Heterotrophic prokaryotes

Cell-specific leucine incorporation rates of HNA-hs, HNA-ls and LNA cells, pooled for all sampled stations and all depths, showed values significantly decreasing with depth (regression coefficient r of 0.79, 0.61 and 0.78, respectively, $p < 0.0005$, see Table 2) and increased with the temperature (regression coefficient r of 0.77, 0.50 and 0.86, respectively, $p < 0.005$). However, no significant relationship was found between the

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Hprok cell-specific incorporation rates and the total chlorophyll *a*, except for the HNA-ls population ($r = 0.51$, $p < 0.005$), while a weak but significant and positive correlation linked the cell-specific activities to the dissolved organic carbon (DOC) concentration, with the exception of the HNA-ls population ($r = 0.53$ and 0.57 for HNA-hs and LNA populations, respectively, $p < 0.05$).

Correlation between cell-specific activities was significant within all Hprok populations (Pearson's $0.73 < r < 0.90$, $p < 0.0001$), particularly for HNA-hs versus LNA (r=0.90). These rates were systematically higher for HNA-hs than for LNA cells (unilateral paired t-test, $t = 5.73$, $p < 0.0001$). No such pattern was observed in the HNA-ls population, which exhibited the highest vertical variability, and had either lower or higher cell-specific activities than LNA cells (Fig. 3). As previously shown in Table 2, cell-specific activities decreased with depth, but the change of activity with depth is different from one population to another. HNA-hs exhibited higher cell-specific activities than both HNA-ls (HNA-hs/HNA-ls ranged 1.4–13.3, Fig. 3) and LNA (HNA-hs/LNA ranged 1.9–6.9) populations over the whole euphotic zone. Roughly, HNA-hs/HNA-ls ratio decreased from a range of 4–7 within the surface layer, to 1.5–4 within the DCM. HNA-ls/LNA ratio tended, on the contrary, to increase at the DCM and below (from 0.57–0.71 within the mixed layer to 0.7–2.4 below).

3.2.2 Autotrophic populations

Less data of cell-specific incorporation rates were acquired for autotrophic populations. Required volumes to harvest sufficient cells and signals were most of the time higher than the few ml that were dedicated to the sorting of autotrophic populations. Indeed, more than 20 sorts were processed for each one of the 3 populations, but only 7 out of 22, 22 out of 26 and 13 out of 21 attempts lead to measurable signals (data not shown) for Syn, Proc and Pic, respectively. We then examined the significant values, those superior to 2-fold the measured blank value, and did not take into account significant values that were obtained in sorts of less than 1000 cells, for a better representativity of the activity. Finally, the number of acceptable values was 3, 16 and 1 for Syn, Proc

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and Pic, respectively, which explains that we only produce in this study data obtained for *Prochlorococcus* (Table 3). Data from all depths were pooled. Proc represented between <1 up to 17% of the bacterioplanktonic abundance, with an average of 8% (Table 3). The average cell-specific leucine incorporation rate for this population was 5 20×10^{-21} mol cell^{-1} h^{-1} but could reach values as high as the surface HNA-hs cells (Table 3), above 50×10^{-21} mol cell^{-1} h^{-1}.

3.3 Volumetric leucine incorporation

3.3.1 Heterotrophic prokaryotes

Volumetric incorporation rates of leucine significantly decreased with depth for all Hprok 10 populations sorted ($p < 0.0001$). They also were significantly correlated to each other (Pearson correlation coefficient $0.636 < r < 0.927$, $p < 0.001$). We compared the activity of the total sorted Hprok population (as a single sort) and the summed activities of the 3 Hprok populations: HNA-hs, HNA-ls and LNA (Fig. 4a). The summed volumetric incorporation rates of the 3 populations were significantly correlated with the leucine in-15 corporation rates of the total sorted Hprok ($p < 0.0001$). The Hprok volumetric leucine incorporation rate (as a single sort) showed a strong positive correlation with the bulk leucine incorporation rate ($p < 0.0001$, Fig. 4b). However, the slope was higher than 1 and, on average, a fraction of 15 ± 31% was unrecovered. In samples from profiles, the sum of HNA-hs, HNA-ls and LNA volumetric incorporation rates represented 39 to 20 104% of the bulk leucine incorporation rates measured in the lab (Fig. 5).

Plotted against the contribution of each sorted population to the Bpk abundance, the contributing activity of each sorted Hprok population showed 3 clusters separating LNA, HNA-hs and HNA-ls (Fig. 6). Each cluster exhibited a narrow range of cell abundances (19% variation on average), and a 2-fold wider range of cell-specific ac-25 tivities (41% on average). LNA cells constituted a cluster lying below the 1:1 axis. It was characterized by the highest contribution to Hprok abundance (45–63%, see Table 2). Their contribution to the bulk activity (<1–58%) ranged close to the percentages

of contribution to the bulk activity by HNA-hs cells (2–54%), which, at the opposite, clustered above the 1:1 axis. Thus, HNA-hs cells contributed the less to the abundance with regard to their activity. HNA-hs cells contribution to leucine incorporation activity was depth-dependent, with a higher activity contribution found in the surface layer. HNA-ls cells formed an intermediate cluster with a lower range of contributions to activity than the 2 other populations (<1–11%) although most dots overlapped the 2 other clusters in terms of activity contribution between 20 and 45%. Two patterns were distinguished, one below the 1:1 axis gathering data from the surface layer, and the other very close and mostly above the axis. Contrary to HNA-hs cells, HNA-ls cells from the surface down to the DCM depth were less active, in regard to their abundance contribution, than the cells found deeper in the euphotic zone.

3.3.2 Autotrophic populations

We measured non-negligible signals in the autotrophic cells, thus not included in Hprok sorts, but which may significantly contribute to the bulk leucine incorporation. We explored the possibility that the missing fraction between total Hprok and whole seawater volumetric incorporation rates (Fig. 4) would be partly completed by the sorted autotrophic populations. Considering the few significant values of leucine incorporation rates obtained in autotrophic populations (see 3.2.2), only Proc was considered. Around the DCM, Proc contributed to the bulk leucine incorporation as much as the strictly heterotrophic populations or more (Fig. 5: St. A 75 m; St. B 100 m, St. C 110 m) with up to 2.6 pmol leu l^{-1} h^{-1}, yielding a contribution of up to 63% of the bulk leucine incorporation activity (Table 3). Compared to the average unsorted bulk volumetric leucine incorporation rates, the summed activities of the HNA-hs, HNA-ls, LNA and Proc sorted populations, showed good recovery at the several depths (Fig. 5). The contribution of Proc appeared as a potential major component of the bulk leucine incorporation activity (24 ± 20%, Table 3).

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3.4 Enrichment experiments

At stations B and 21, we assessed the potential nutrient limitation of the heterotrophic prokaryotic populations, both at the activity and abundance level (Fig. 7). Dealing with abundances, in the St. 21 surface experiment (left panel), HNA+ and LNA were the most abundant populations, ranging 1.9–2.9$\times10^5$ cells ml^{-1}, with the exception of a 2.5-fold increase of HNA+ cells after the NPG addition, reaching 5.5$\times10^5$ cells ml^{-1}. The abundance of HNA cells increased slightly after the P, G and NPG enrichments but, although irrespective of the nutrient addition, absolute HNA cell counts remained below 2.0$\times10^5$ cells ml^{-1}. In the experiments done at DCM depth from St. 21 (85 m, Fig. 7, middle panel), no control could be used as a reference. HNA+ cells numerically dominated the Hprok populations with abundances of 2.4 and 3.0$\times10^5$ cells ml^{-1} after P and N enrichments, i.e. almost 3 times as high as HNA and LNA abundances. The 3 populations responded to G and NPG additions. HNA and LNA cell numbers exhibited the same increase, abundances were multiplied by 5 after G amendment and by 2.5 after NPG amendment, respectively (compared to the N addition which lead to the lowest abundances). HNA+ cells increased mainly after NPG additions (by a factor of 2.7). Finally, in the St. B surface experiment, HNA-ls abundance did not vary compared to the control, remaining between 0.5 and 0.9$\times10^5$ cells ml^{-1}. HNA-hs cells remained as numerous as in the control after N and P additions but almost doubled after the NPG enrichment, to a concentration of 2.1$\times10^5$ cells ml^{-1}. LNA cells were very abundant in the control compared to the other samples (2.9$\times10^5$ cells ml^{-1}), but 3 times lower after N and NP enrichments.

As for the bulk leucine incorporation, drastic effects were observed after NPG addition at the surface of St. 21 and both G and NPG at 85 m. Indeed, at the DCM, the activity reached 214 pmol l^{-1} h^{-1} after G addition, i.e. more than 25 times as much as in the control. NPG amendments resulted in highest rates of 138 and 454 pmol l^{-1} h^{-1} at 5 and 85 m, respectively. In comparison, low or no increases of rates were measured after N or P enrichment (<20 pmol l^{-1} h^{-1}), except for the N amendment in the surface

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layer (2.3-fold higher than the control). At station B, the NP enrichment resulted in an incorporation rate of up to 49.2 pmol l^{-1} h^{-1} (4-fold higher than the control).

Cell-specific incorporation rates also varied depending on the nature of the amended nutrient. In the surface experiment from St. 21, after N addition, all 3 populations responded. The factor of increase was higher for HNA (x 2.7) than for the 2 other populations (x 1.4 and 2.0 for LNA and HNA+, respectively). After NPG addition, however, HNA+ was the most responsive population (13-fold increase compared to the control: 337×10^{-21} mol leu $cell^{-1}$ h^{-1}), while HNA rates were multiplied by 6, reaching 136×10^{-21} mol leu $cell^{-1}$ h^{-1}. In the 85 m experiment, low cell-specific rates were measured ($5–15\times10^{-21}$ mol $cell^{-1}$ h^{-1}), with values nevertheless 1.5 and 2 times as high after G and NPG enrichments, respectively, compared to the N addition. HNA+ was less active than the 2 other populations, with rates below 3.4×10^{-21} mol $cell^{-1}$ h^{-1} or even under the limit of detection, but reacted drastically (x 85) after G enrichments, reaching 286×10^{-21} mol $cell^{-1}$ h^{-1}. The absence of response in NPG remained unexplained, and we were unfortunately unable to process another sort of this population. HNA cells displayed the highest rates of leucine incorporation after N and P amendments with 16 and 11.3×10^{-21} mol $cell^{-1}$ h^{-1}, respectively. A 10-fold higher activity was measured after incubation with glucose, but there was only a 5-fold increase after NPG enrichment. In the experiment realised at St. B, HNA-hs systematically showed the highest cell-specific activities, 52.2×10^{-21} mol leucine $cell^{-1}$ h^{-1} in the control, slightly more after the P addition, and twice as much after N and NP amendments, suggesting a N limitation. The other 2 populations showed similar responses in terms of factor of increase, but with lower values in the control, 8.6 and 6.0×10^{-21} mol $cell^{-1}$ h^{-1} for HNA-ls and LNA, respectively.

Relative comparison of the populations based on their activity and abundance values showed stable proportions in the surface experiment of St. 21 for both parameters, with a numerical co-dominance of HNA+ and LNA cells (Fig. 7, lower panels). More HNA+ cells were found at 85 m, but they represented a lower proportion of the total Hprok activity than HNA cells. The contribution of LNA to the total activity generally decreased

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when the bulk leucine incorporation rates increased substantially (5 m NPG, 85 m G, St. 21). At St. B, LNA and HNA+ cells showed opposite trends to each other with a shift in dominance of abundance from LNA to HNA+, while the latter dominated the leucine incorporation in all samples, particularly after N and NP additions (>80%).

4 Discussion

4.1 Importance of LNA cells in the leucine incorporation fluxes

This is the first data set exploring cell-specific activities of HNA and LNA cells over different basins in the Mediterranean Sea. LNA incorporated significant amounts of leucine at all stations and depths sampled (3 to 20 times the blank values). Evenly distributed across the Mediterranean Sea, LNA cell-specific incorporation rates ranged $1.2–12.5 \times 10^{-21}$ mol leucine cell^{-1} h^{-1}, accounting for 19% to 34% of the Hprok incorporation activity at St. 21 and St. 25, and 28% to 61% at sites A, B and C. Lebaron et al. (2001; 2002) measured LNA cell-specific incorporation rates of $0.2–25 \times 10^{-21}$ mol leucine cell^{-1} h^{-1} at a coastal station of the NW Mediterranean Sea, using saturating concentrations of substrate (5 nM radioactive and 75 nM non-radioactive tritiated leucine). More recently, Longnecker et al. (2005) measured LNA-specific activities of $3.5–9 \times 10^{-21}$ mol leucine cell^{-1} h^{-1} in the subsurface of 3 stations sampled on a shore-basin transect on Oregon coast by using 20 nM pure radiolabeled leucine in waters presenting temperatures between 5 and 15°C, and 1.3 to 7.5 µg chl *a* l^{-1} at the DCM. During the BOUM cruise, higher temperatures were measured (15–26°C), but closer values of chl *a* at the DCM (0.27–1.86 µg chl *a* l^{-1}) and we also used saturating concentrations of leucine (12 nM). The difference between this latter study and ours resides in the width of the range, which is greater in our Mediterranean samples (25–38% versus <1–58%, see Table 2).

In Pacific samples (Longnecker et al., 2005), increased proportional abundances of LNA cells were observed towards the basin station, with vertically decreasing cell-specific activities along the euphotic zone. In the Mediterranean Sea, data showed

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a higher vertical than longitudinal variability, considering that the profiles we studied were sampled in the open sea with a deep water column and that the nutrient conditions did not significantly vary offshore. Only nutriclines and DCM depths varied longitudinally, and this was taken into account when comparing vertical patterns (Table 1).

From the results published to date, the participation of LNA in bulk incorporation rates increases in marine ecosystems compared to freshwater and brackish environments. Along the coast-open sea transect off the Oregon coast, the contribution of LNA to the total leucine assimilation activity increased. Furthermore, the vertical distribution of LNA cell-specific activities varied greatly over the first 100 m in the latter study, which is in agreement with our own observations. Indeed, we found significant correlations between LNA cell-specific activities and two environmental parameters: depth and temperature (Pearson's $r = -0.783$ and 0.849, $p < 0.0001$). This suggests that factors such as temperature, light, nutrient factors limiting BP, contribute to structuring prokaryotic activities within each Hprok population, as it was shown also for the diversity of Hprok (Moeseneder et al., 2001; Fuhrman and Steele, 2008; Ghiglione et al., 2008; Feingersch et al., 2009; Van Wambeke et al., 2009). LNA cells are thus a dynamic population whose role in the microbial communities is far from negligible. This is congruent with other data from the literature, based on dilution experiments or biomass measurements, showing that significant growth rates, sometimes higher than HNA, were also measured in LNA cells (Scharek and Latasa, 2007; Williams et al., 2008; Longnecker et al., 2010). LNA cells were proved to be active members of the Hprok community, not only in terms of protein synthesis, but also in cell division, as shown in fractions sorted after thymidine incorporation (Longnecker et al., 2006b). Finally, it was recently shown that LNA could also be quickly reactive (within 24 h) to environmental changes like dust addition (Hill et al., 2010), and that they could respond to P-deficiency in a lake by growing and dividing better than other cells, because their requirements for nucleic acid synthesis were by definition lower than that of HNA cells (Nishimura et al., 2005). During our experiments, LNA cells were also stimulated by nutrient amendments (specific activities increased up to 10 times after G enrichment

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compared to P addition, St. 129, 85 m), not as intensely as HNA cells, but still carrying another evidence of their plasticity.

4.2 Large variability of HNA leucine incorporation fluxes

Within the HNA population, cell-specific leucine incorporation rates have been shown to vary as much as 7-fold in a single sample (Lebaron et al., 2002), depending on the regions drawn on the cytograms before cell sorting. Dividing the HNA cluster in HNA-hs and HNA-ls populations based on their SSC was also done by Zubkov et al. on natural communities from Celtic Sea shelf waters (Zubkov, 2009). Using ^{35}S-labeled methionine as a tracer (<0.3 nM final concentration), they reported cell-specific methionine assimilation of the HNA-hs population twice as high in the surface layer as in the pycnocline or deeper layers. HNA-ls cell-specific activities were less variable between layers than those measured in HNA-hs, and sometimes lower than what was measured in LNA sorts. This is in agreement with our surface layer data from the Mediterranean Sea, using pure tritiated leucine concentration for protein synthesis rates measurement (Fig. 3, 6, Table 2). This feature was also observed in another environment (suboxic waters of Indian Ocean), although HNA-ls, due to their high relative abundance, were dominantly participating to methionine assimilation (30 to 60%, Zubkov et al., 2006).

However, we found that the range of specific activities within HNA group narrowed significantly in the vicinity of the DCM, as reflected by the decrease from 5.6 to 1.7 of the average ratio HNA-hs/HNA-ls of cell-specific activities (Fig. 3). The heterotrophic prokaryotic community would thus be less heterogeneous around the DCM, in terms of specific activity. According to Lebaron et al. (2001), the ratio HNA/LNA of cell-specific activities is lower when discrimination between the two populations is not as clear as they depicted in a coastal station (SOLA) compared to a marine harbour, lagoon or brackish system. The ratios of cell-specific activities we measured (HNA-hs/HNA-ls, HNA-hs/LNA and HNA-ls/LNA, Fig. 3), coming closer with shorter distance to the DCM, thus support the fact that the populations are less distinct around the DCM compared to the surface.

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Higher cell-specific activities within the HNA-hs population measured in most study areas suggest that larger cells can synthesize proteins at higher rates (compared for example to thymidine synthesis, see Longnecker et al., 2006b), which is a necessity due to their larger protein biomass (Zubkov et al., 2001). However, no significant correlation was found between SSC values of HNA-hs or HNA-ls and their cell-specific activity.

Distinction between HNA-hs and HNA-ls is not always easy. Examining Hprok dynamics and community structure during a phytoplankton bloom in the North Sea, Zubkov et al. (2002) established the existence of two HNA subgroups separated after a protein staining (SYPRO protein red fluorescence dye) and verified that no such clusters were visible with DNA stain.

Because few studies investigated specific activities within sub-populations of HNA, it is difficult to compare our results with data from the literature. Thus, abundances and specific activities of HNA-hs and HNA-ls were pooled into an equivalent HNA group as follows:

$$SA_{HNA} = [(SA_{HNA-ls} \times N_{HNA-ls}) + (SA_{HNA-hs} \times N_{HNA-hs})]/(N_{HNA-ls} + N_{HNA-hs}) \quad (1)$$

where SA is the specific activity (volumetric or cell-specific) and N the abundance of the considered population at a station. A summary of incorporation rates within HNA and LNA populations was compiled in a previous study (Table 5 in Longnecker et al., 2006b). Comparable HNA/LNA ratios of cell-specific activities were measured in our samples (2.1 ± 0.7, ranging 1.0–4.0) and in results reported by (2.3, ranging 1.9–2.6: Longnecker et al., 2006b)Scharek and Latasa (2007) explored growth and grazing rates by the dilution technique at a slope station in the NW Mediterranean Sea in May–June 2000. They found that growth rates of HNA were significantly higher and distinct from those of LNA within surface layers. They also found that growth rates of both categories decreased at the DCM, the difference between both populations lowering to become insignificant, and they attributed the specificity of the surface response to the influence of fluvial water. However, this trend existed also in open stations from

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our study, which were located far from any riverine influence. Thus, other factors were responsible for the surface properties we observed in the different populations. Indeed, differences between surface and DCM layers, in terms of habitat for Hprok, are noticeable under stratified conditions. Among varying limiting factors (switch from N-P limitation to C limitation, see Fig. 7: Sala et al., 2002; Van Wambeke et al., 2002a) and phytoplankton populations, Hprok populations must adapt by facing different resources and modifying their diversity composition (Ghiglione et al., 2008) and cytometric properties (Van Wambeke et al., 2010) along the water column.

4.3 Effect of nutrient addition on the Hprok leucine incorporation

These experiments showed that leucine incorporation was partly nutrient-controlled for all populations of interest. As previous studies showed, we observed enhanced activity in bottles amended with a single or a mixture of nutrients, compared to the control (Nishimura et al., 2005; Zohary et al., 2005; Pinhassi et al., 2006; Van Wambeke et al., 2007; Van Wambeke et al., 2009). This emphasized the bottom-up effect of either single or multiple nutrients. In our case, it was a co-limitation situation by N and P in surface waters (confirmed at St. B 8 m, probable at St. 21 5 m, because NP combination was not directly tested, Fig. 7) and glucose at 85 m at St. 21, confirming a switch from inorganic nutrients in surface to labile carbon limitation along the vertical gradient (Sala et al., 2002; Van Wambeke et al., 2002a).

We further investigated the hypothesis according to which limitations could differ in distinct functional groups (Zohary et al., 2005). Firstly, none of the tested combinations stimulated only one population. Then, we showed that nutrient additions could selectively affect the intensity of cell-specific activity. HNA was mostly responding (HNA+ and HNA at St. 21, HNA-ls at St. B).

In these experiments, abundances varied less than specific activities (Fig. 7), which may be explained by a top-down control by predation and viral lysis of the most active cells. Mortality rates of HNA cells in natural lake and estuary samples were found substantially higher than those of LNA (Nishimura et al., 2005; Williams et al., 2008).

We could assume that HNA cells initially present at the sampling time had been partly grazed, leaving ecological space for another opportunistic population. This population might be HNA+, having also high specific activities (Fig. 7), but this population could have an additional advantage on HNA by being protected from grazing due to their large size (Scharek and Latasa, 2007; Williams et al., 2008; Pfandl et al., 2009; Longnecker et al., 2010). Such a population could develop in 60 ml-flasks due to the relative scarcity of large-sized grazers (microzooplankton) and this dual capacity could explain their high abundance. The low-varying cell abundances in the St. B experiment may also be attributable to lower stimulation of cell-specific activities than with G and NPG additions, combined to a more top-down regulated community, by microplankton food web, considering the size of the microcosms (20 l) in the 96 h-incubated samples. Indeed, Tanaka et al. (2010) showed that NP addition resulted in a significant increase of HNF at St. B and also virus and ciliates, although for these 2 latter parameters the increase was not significant due to variability within triplicate mesocosms. The outcompeting number of cells developing in such conditions (high specific activity) are organisms with a "r-strategy", which would explain the classically reported loss or shift of the diversity in other amendment experiments (Massana et al., 2001; Nishimura et al., 2005; Zohary et al., 2005; Van Wambeke et al., 2007).

4.4 Importance of *Prochlorococcus* in amino acids assimilation

Within our natural samples, *Prochlorococcus* was able to assimilate leucine. In dark incubation conditions, Proc could be responsible for up to 60% of the total leucine incorporation into proteins (22 ± 17%, $n = 16$) when this molecule was added in the usual range of concentrations used to estimate bacterial production. Such high contributions of Proc to the bulk leucine incorporation were measured in the vicinity of the DCM where Proc cells were more abundant and allowed the detection of significant per cell activity. Proc total activity was in some cases sufficient to compensate the "unrecovered activity" observed when comparing bulk unsorted activity to the sum of activities reconstructed after cell sorting of strict Hprok cells (Fig. 5). Significant assimilation of

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leucine by natural *Prochlorococus* cells was shown in Atlantic Ocean using a concentration of radiolabeled leucine as low as 0.8 nM (Mary et al., 2008a). This mixotrophic capability could be enhanced by incubation in the dark, since visible light can enhance leucine assimilation by 50% (Paerl, 1991; Michelou et al., 2007; Mary et al., 2008b). However, in situ effects of simultaneous visible light + UV light exposure would require further investigation.

Mixotrophy, through amino acids uptake, gives cyanobacteria with an advantage in N-limited environments (Mary et al., 2008a). The balance between photosynthesis and heterotrophic assimilation of organic molecules by Proc cells may be influenced by the varying nature and/or quantity of pigmental units, also by the ecotypes present at a given depth in link with their metabolic ability to use nitrates and nitrites (Bouman et al., 2006; Garczarek et al., 2007; Martiny et al., 2009). Unfortunately, in the most N-depleted surface waters encountered along the transect, Proc was not detectable by FCM, due to their very low pigment content.

Being one of the top 3 contributors to the bulk leucine incorporation in some of our samples, this technical limitation concerning Proc has 2 major consequences on studies like ours. First, population-specific elemental fluxes (e.g., the volumetric incorporation) measured in surface waters for Proc are underestimated, and thus, Hprok contribution to the fluxes may be overestimated, by including Proc cells into Hprok sorts, whose red fluorescence signal is too weak to be discarded from the Hprok population, into Hprok sorts. Uncertainties linked to the "surface Proc bias" are not quantifiable, but it can be thought that Proc cells with low natural fluorescence would be counted or sorted within the HNA fraction (Fig. 2).

The fact that only few significant activities were measured for other phototrophic populations (Syn and Pic) addresses the problem of the limit of detection of the method. Indeed, this might be due to the limited number of cells that could be sorted due to small volumes of seawater incubated and stored. For the same reasons, we do not reject the possibility of measuring significant heterotrophic activity in other autotrophic populations such as Syn or Pic in oligotrophic environments, by using longer tracer-incubation

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time and/or by sorting higher number of cells.

The role of *Prochlorococcus* within surface layers could not be studied in the very transparent waters we sampled. A compromise must be found between the number of samples treated and the sorting time necessary to higher the number of sorted cells. The use of ^{35}S-methionine as an amino acid source allows enhancement of the radioactive signal (compared to ^{3}H) but restricts the use to sulfur-containing molecules.

Whether the capacity to incorporate leucine into protein by Proc and Syn could be transformed in terms of "heterotrophic prokaryotic production" or not remains unknown, due to the lack of knowledge on the capacity of these organisms to incorporate other organic molecules in the range of in situ concentrations (Mary et al., 2008b). Other substrates are commonly used as tracers of heterotrophic activity, such as the ^{35}S-methionine, ^{3}H-tyrosine, ^{3}H-glucose, ^{3}H-glucosamine, and ^{3}H-thymidine (Zubkov and Tarran, 2005; Mary et al., 2008a, b; Hill et al., 2010). Interpreting amino acids uptake by heterotrophic and mixotrophic organisms as the reflection of a single process appears slightly contentious though. Leucine and methionine are two amino acids representing sources of C, N and C, N, S, respectively. Cells may take up such substrates in case of N and/or P depletion: indeed, it was recently showed that one way to overcome N- or P-scarcity in picophytoplankton is to synthesize sulphur- or nitrogen-rich membrane lipids in place of the regular phospholipids (Van Mooy et al., 2009). Then, conclusions about the preferential uptake of a substrate towards another should be drawn with care. For instance, it is to be considered that another steric configuration of leucine, other amino acids, other dissolved organic molecules like amino sugars or nucleic acids (Jorgensen et al., 1993; Zubkov et al., 2007), could be taken up at higher rates.

5 Conclusions

In this study we investigated specific leucine incorporation activities in the bacterioplankton of the open Mediterranean Sea, at the cellular (cell-specific) and population (volumetric) level. We measured higher vertical than longitudinal variability in activities and abundances, with Hprok activities decreasing from surface to the bottom of the

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euphotic layer, thus positively correlated with temperature. All heterotrophic prokaryotic populations significantly contributed to the bulk leucine incorporation flux, with different ranges of variation. LNA cells were shown to be key-players in this flux in the Mediterranean Sea at that time of the year, as we would expect in all warm and stratified oligotrophic areas of the ocean. HNA cells exhibited simultaneously the highest contribution to the bulk activity and the lowest contribution to the Hprok abundance, all depths and sites considered. HNA and HNA+ were the most opportunistic populations after enrichments. Significant leucine incorporation activity was measured in *Prochlorococcus*, whose cell-specific activity could be as high as the highest HNA cell-specific activity. Biovolume estimations and a phylogenetic survey of sorted bacterioplanktonic populations would supply consistent additional information and allow linking the dynamics of Hprok populations, community structure, in situ and after nutrient additions.

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Table 1. Characterization of the environmental and biological parameters at the 5 stations. Chlorophyll *a*, abundances and bacterial production (BP) values were integrated over 0–150 m. Proc = *Prochlorococcus*, Syn = *Synechococcus*, Pic = pico + nano - phytoeukaryotes, Hprok=heterotrophic prokaryotes.

	DCM depth (m)	Mixed layer depth (m)	Mixed layer temperature (°C)	Chl *a* (mg m^{-2})	Proc (10^{12} cells m^{-2})	Syn (10^{12} cells m^{-2})	Pic (10^{12} cells m^{-2})	Hprok (10^{12} cells m^{-2})	BP (mg C m^{-2} d^{-1})
St. 25	51	26	21.4	55.0	0.82	2.73	0.22	80.71	0.05
St. A	88	11	24.3	16.3	2.60	0.41	0.13	59.48	0.04
St. 21	87	17	25	21.2	1.37	0.50	0.03	73.77	0.07
St. B	120	12	26.8	16.5	4.95	0.32	0.12	67.65	0.04
St. C	108	10	26.2	22.9	3.04	1.04	0.09	47.79	0.02

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Table 2. Characteristics of the in situ LNA population: abundance, cell specific leucine incorporation rates (V_c), and contribution to the total flux (bulk leucine incorporation rates). Values were computed in 3 layers: surface (above DCM), DCM (exact DCM depth) and deep (below DCM – bottom of the euphotic zone).

		Abundance (10^5 cells ml^{-1})	V_c (10^{-21} mol leu $cell^{-1}$ h^{-1})	Contribution to summed hprok leucine incorporation (%)	Contribution to the BP (%)
surface	LNA	1.3–5.7 (2.6)	2.5–12.5 (6.4)	26–58 (42)	13–43 (26)
($n=19$)	HNA-hs	0.3–1.6 (0.7)	7.2–54.3 (23.9)	11–65 (36)	10–55 (27)
	HNA-ls	0.6–2.8 (1.3)	0.9–11.1 (5.0)	3–21 (13)	5–25 (12)
DCM	LNA	1.7–5.7 (2.7)	1.9–3.9 (2.7)	19–55 (35)	12–30 (19)
($n=5$)	HNA-hs	0.3–1.3 (0.6)	5.4–21.8 (12.7)	17–43 (28)	12–37 (19)
	HNA-ls	0.9–3.1 (1.7)	1.7–7.7 (4.6)	16–38 (28)	12–34 (26)
deep	LNA	0.8–2.1 (1.4)	1.2–2.3 (1.6)	<1–45 (33)	9–32 (22)
($n=7$)	HNA-hs	0.3–0.5 (0.3)	2.3–6.8 (5.3)	14–32 (25)	12–37 (20)
	HNA-ls	0.6–1.0 (0.9)	1.7–3.9 (2.9)	28–45 (34)	31–53 (41)

Table 3. Description of the sorted Prochlorococcus (Proc) population: % of abundance in the total bacterioplankton (Bpk = Hprok + Proc + Syn), range of sorted cells number, cell specific leucine incorporation rates (V_c), and contribution to the total flux (bulk leucine incorporation rates). Out of 21 attempts, only 16 measurements resulted in significant values (>2-fold the blank), which were used to obtain the following results.

		% Bpk abundance	Number of sorted cells (x 10^3 cells)	V_c (10^{-21} leu cell^{-1} h^{-1})	Contribution to the bulk leucine incorporation (%)
25–200 m	Proc	<1–17 (8)	0.3–156 (35)	2–55 (20)	4–63 (24)

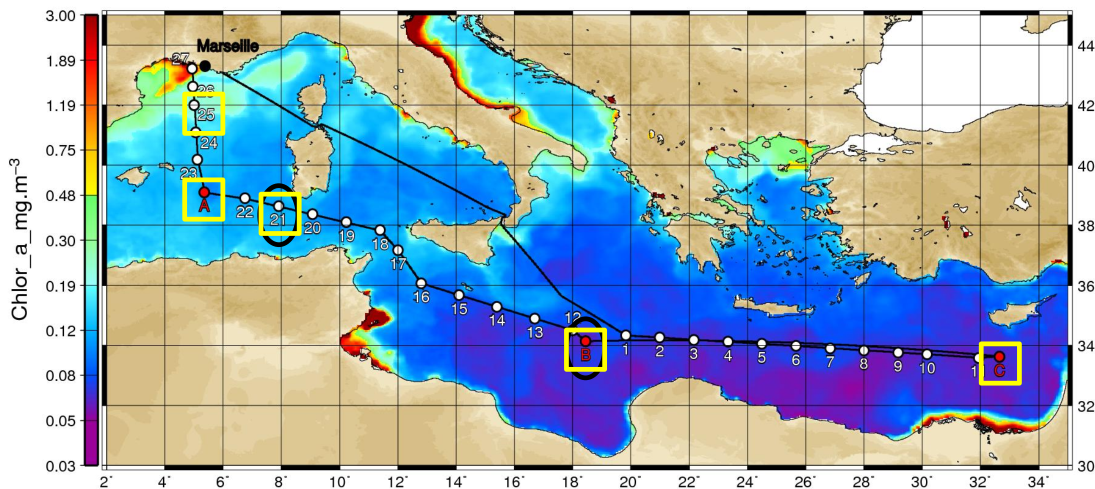

Fig. 1. Location of the BOUM transect and sampling sites (yellow squares): stations St. C, St. B, St. 21, St. A and St. 25 from East to West. Stations where enrichment experiments were conducted are encircled in black. Map of average sea surface chlorophyll *a* (15 June–15 July 2008), NASA, MODIS-Aqua.

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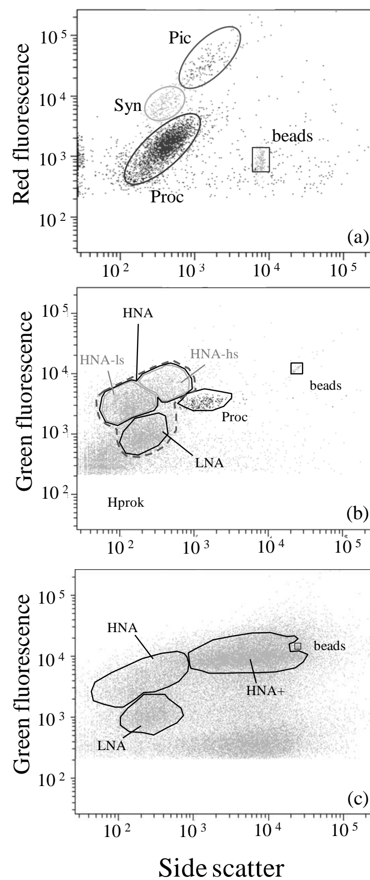

Fig. 2. Examples of FCM biparametric plots of the phototrophic populations discriminated by **(a)** their natural fluorescence and side scatter; b, c heterotrophic prokaryotic populations discriminated by their SyBR I-induced green fluorescence versus their side scatter. Cytogram b shows prokaryotes from an in situ sample, with Proc not excluded on the basis of their red versus green properties (St. 21, 85 m). Cytogram c shows a +NPG enriched sample (St. 21, 85 m), with the HNA+ population and Proc excluded.

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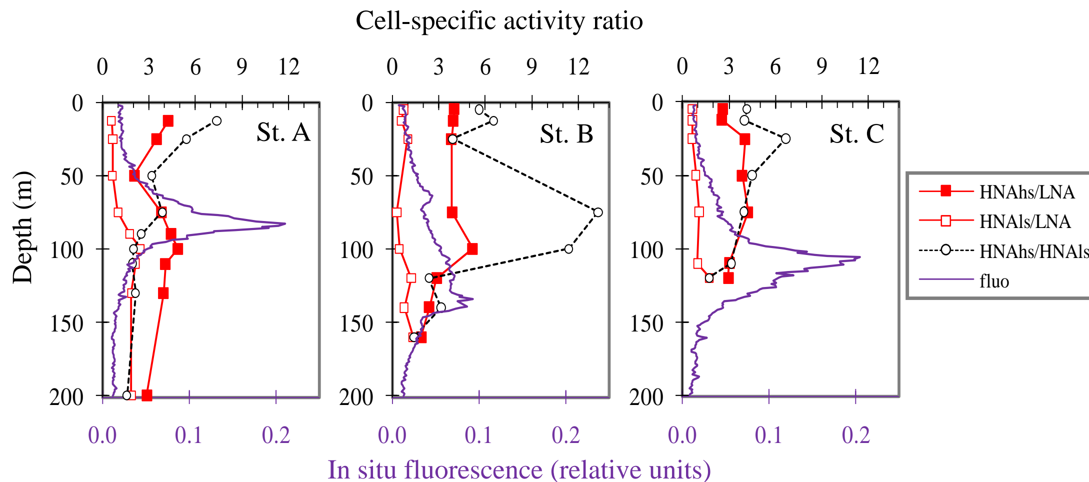

Fig. 3. Vertical distribution of the in situ fluorescence measured by the sensors mounted on the rosette and of of the HNA-hs/LNA, HNA-ls/LNA and HNA-hs/HNA-ls ratios of cell-specific activities (V_c)

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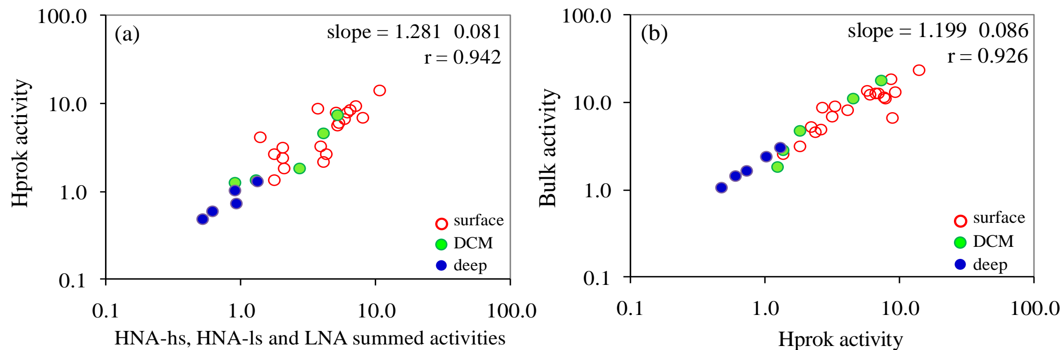

Fig. 4. Comparisons of **(a)** Volumetric leucine incorporation rates of Hprok (sorted as a single population) versus the sum of the volumetric rates measured in the sorted HNA-hs, HNA-ls and LNA populations (3 sorts) **(b)** Bulk leucine incorporation rates versus volumetric leucine incorporation rates of the Hprok. 3 layers were computed: surface = to DCM=DCM depth, deep=below DCM to the bottom of the euphotic zone. Slope = Tessier's slope ± se, r = coefficient of the Model II linear regression.

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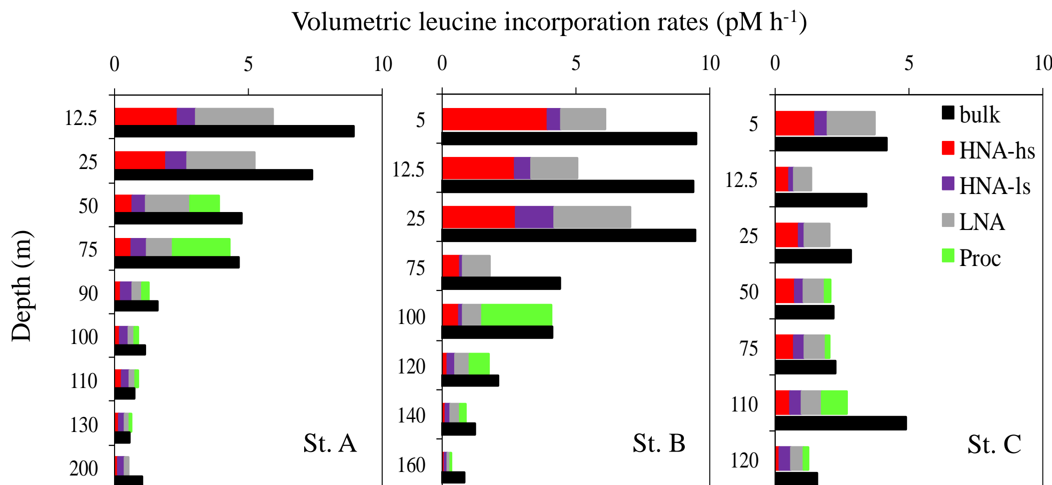

Fig. 5. Vertical distribution of summed activities of HNA-hs, HNA-ls, LNA and Proc compared to the bulk activity (black bars) at stations St. A, St. B and St. C. Note that the vertical scale varies from one profile to another.

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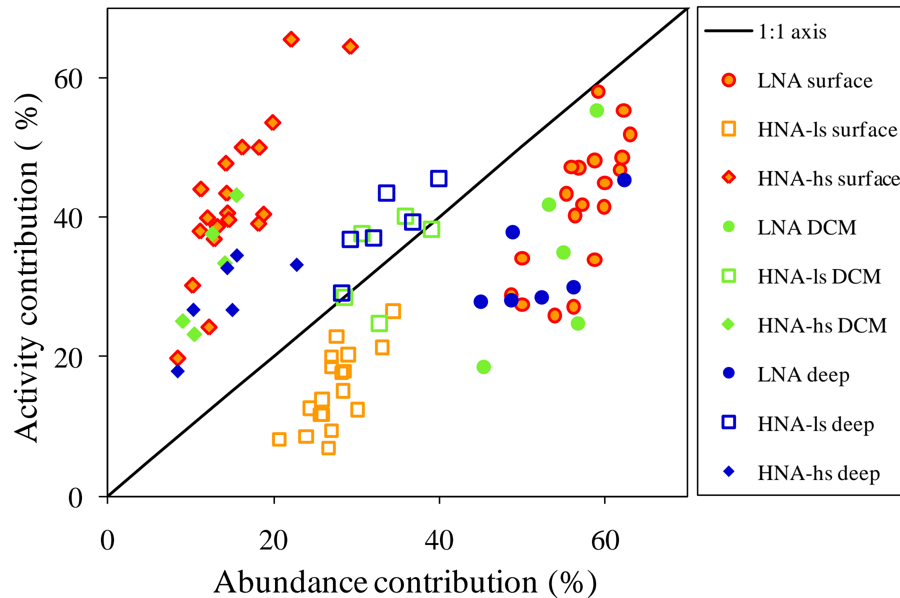

Fig. 6. Relationships between abundance and activity contributions of the sorted populations to the total Hprok abundance and summed activities of the 3 populations, LNA, HNA-hs and HNA-ls, respectively.

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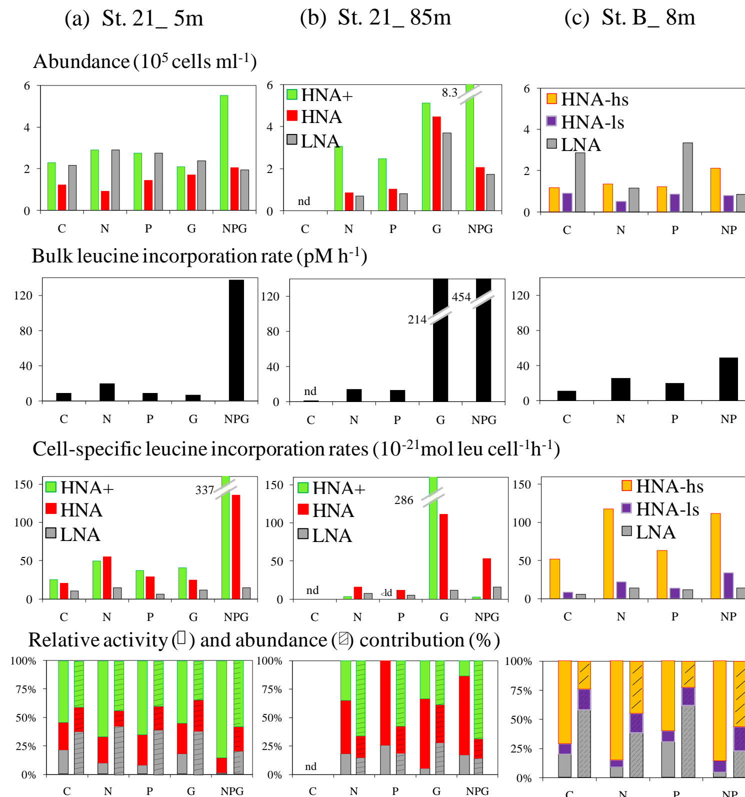

Fig. 7. Effects of enrichment experiments on the activities and abundances of the Hprok populations at two stations, St. 21, 5 m (left panels), St. 21, 85 m (middle panels) and St. B, 8 m (right panels). C = unamended control after the incubation period of 2 days (St. 21) and 3 days (St. B), P = +phosphate, N = + ammonium+nitrate (St. 21) or N = + ammonium (St. B), G = + glucose, NP = N + P. Note that HNA+ was only detected in the St. 21 experiments and that the C condition was not processed (no data, lost sample).

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