

# **Heterotrophic bacterial production and metabolic balance during the VAHINE mesocosm experiment in the New Caledonia lagoon**

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## Abstract

Studies investigating the fate of diazotrophs through the microbial food web is lacking although N<sub>2</sub> fixation can fuels up to 50% of new production in some oligotrophic oceans. In particular, the role played by heterotrophic prokaryotes in this transfer is largely unknown. In the frame of the VAHINE experiment, three replicate large-volume (~50 m<sup>3</sup>) mesocosms were deployed for 23 days in the new Caledonia lagoon and were intentionally fertilized on day 4 with dissolved inorganic phosphorus (DIP) to stimulate N<sub>2</sub> fixation. We specifically examined relationships between heterotrophic bacterial production (BP) and N<sub>2</sub> fixation or primary production, determined bacterial growth efficiency and established carbon budgets. . BP was statistically higher during the second phase of the experiment (P2: days 15-23), when chlorophyll biomass started to increase compared to the first phase (P1: days 5-14). Phosphatase alkaline activity increased drastically during the second phase of the experiment, showing adaptations of microbial populations after utilization of the DIP added. Notably, among autotrophs, *Synechococcus* abundances increased during P2, possibly related to its capacity to assimilate leucine and to produce alkaline phosphatase. Bacterial growth efficiency based on the carbon budget (27- 43 %), was notably higher than generally cited for oligotrophic environments and discussed in links with the presence of abundant species of Bacteria expressing proteorhodopsin. The main fate of gross primary production (particulate + dissolved) was respiration (67 %), and export through sedimentation (17 %). BP was highly correlated with particulate primary production and chlorophyll biomass during both phases of the experiment but slightly correlated, and only during P2 phase, with N<sub>2</sub> fixation rates. Heterotrophic bacterial production was strongly stimulated after mineral N enrichment experiments, suggesting N-limitation of heterotrophic bacteria all over the experiment. N<sub>2</sub> fixation rates corresponded to 17-37 % of the nitrogen demand of heterotrophic bacteria. Our results suggest that most of the diazotroph derived nitrogen fuelled the heterotrophic bacterial community through indirect processes generating dissolved organic matter and detritus, like mortality, lysis and grazing of both diazotrophs and non-diazotrophs.

## 1    **1    Introduction**

2    In the South West Pacific ocean, the natural occurrence of abundant and diverse plankton taxa  
3    capable of dinitrogen ( $N_2$ ) fixation ( $N_2$ -fixing or diazotrophic organisms) (e.g., Moisander et  
4    al., 2010) can fuel ~50 % of new primary production. (Garcia et al., 2007; Bonnet et al.,  
5    2015). However, little is known about the fate of the diazotroph-derived nitrogen (DDN) in  
6    this environment (Bonnet et al., 2016b). In particular, the role played by the microbial food  
7    web, and among them the heterotrophic bacteria in the transformation of DDN is largely  
8    unknown. In the central gyre of the South Pacific, where  $N_2$  fixation is lower than in the South  
9    West Pacific, nitrogen is the first element limiting growth of both phytoplankton and  
10    heterotrophic bacterioplankton as observed in short-term nutrient enrichment experiments  
11    (Bonnet et al., 2008; Van Wambeke et al., 2008a) or incubations with  $^{15}N$ - leucine or  $^{15}N$ -  
12     $NH_4^+$ , which significantly enhanced bicarbonate uptake (Halm et al., 2012). Such competition  
13    for nitrogen influences dissolved organic carbon accumulation in the surface layers and  
14    export. In the South West Pacific, however, the phytoplankton - heterotrophic bacterial  
15    coupling has mainly been investigated in the New Caledonia Lagoon. In this system,  
16    phytoplankton and bacterial production show seasonal patterns, with maxima in December-  
17    January and annual bacterial production representing 21 to 34 % of particulate primary  
18    production (Torreton et al, 2010), In the oligotrophic stations of the lagoon, based on a  
19    bacterial growth efficiency of 10% or less, dissolved phytoplankton release was not sufficient  
20    to sustain bacterial carbon demand (Rochelle-Newall et al., 2008). N-limitation of primary  
21    production is expected based on year-round dissolved inorganic nitrogen (DIN) to dissolved  
22    inorganic phosphorus (DIP) ratios and silicates to DIN ratios, respectively lower and higher  
23    than Redfield ratios (Torréton et al., 2010). However,  $N_2$  fixation is a recurrent feature in the  
24    lagoon (Garcia et al., 2007; Biegala and Raimbault, 2008), and no information is available on  
25    the potential role played by this process on the functioning of the microbial food web and how  
26    it could influence the factors limiting heterotrophic bacterial production. As blooms of  
27    diazotrophs are transient events, the production of varying sources and quality of organic  
28    matter is expected, which may influence biogeochemical fluxes, in particular heterotrophic  
29    bacterial production.

30    Through the VAHINE program (<http://mio.pytheas.univ-amu.fr/?VAHINE-Project>; Bonnet et  
31    al., 2016b), we experimentally investigated the fate of DDN in the planktonic food web and  
32    its potential impact on particle export. For this, we studied the development and the fate of a  
33    diazotroph bloom enhanced by intentional fertilization with DIP in large-volume (~50 m<sup>3</sup>)  
34    mesocosms deployed in the oligotrophic part of the New Caledonian lagoon, DIP being

considered to control the nitrogen input by dinitrogen fixation in the SW Pacific upper surface waters (Moutin et al., 2005, 2008). The VAHINE experiment provided a unique opportunity to study such phytoplankton-heterotrophic bacteria interactions by simultaneously using biogeochemical techniques assessing stocks and fluxes in the same body of water for a period of 3 weeks. In particular, our objectives were, i) to explore factors controlling heterotrophic bacterial growth, ii) to examine the links between heterotrophic bacterial production and the activity of N<sub>2</sub>-fixing organisms and primary producers and iii) to study the fate of carbon inside mesocosms and the balance of autotrophy *versus* heterotrophy. The factors controlling heterotrophic bacterioplankton were studied using short-term nutrient enrichment experiments and measurements of alkaline phosphatase activity. In oligotrophic systems, assimilation of organic nitrogen-containing molecules can also confer advantage for growth to some cyanobacteria (Zubkov et al., 2004, Mary et al., 2008a). Thus we quantified fluxes of leucine incorporation on a single cell basis, using flow sorting by cytometry (Talarmin et al., 2011).

## **2 Material and methods**

### **2.1 Mesocosm description and sampling strategy**

Three large mesocosms (~50 m<sup>3</sup>) were deployed as open tubes with unfiltered, nutrient-poor, waters of the Nouméa lagoon close to the Boulari passage (22°29.073 S - 166°26.205 E) located 28 km of the coast from January 13 to February 4, 2013 (Fig. 1). After 2 days for stabilizing mixing and verticality of mesocosms, they were closed at their bottom, which constituted the starting day of the experiment, and a sediment trap was screwed at the basis of the bottom cone of each mesocosm and changed every morning by SCUBA divers. The mesocosms design is based on Guieu et al. (2010) and the choice of the site in the lagoon, deployment and sampling strategy are described in details in Bonnet et al. (2016b). The three triplicate mesocosms were supplemented with 0.8 μM KH<sub>2</sub>PO<sub>4</sub> between day 4 and day 5 of the experiment to alleviate potential P limitation and induce a bloom of naturally present communities of N<sub>2</sub> fixing organisms. All samples for the parameters described below were collected every morning for 23 days using a clean Teflon pumping system from three selected depths (1 m, 6 m, 12 m) in each mesocosm (M1, M2 and M3) and in surrounding waters (i.e. outside the mesocosms, hereafter called Nouméa lagoon waters). Seawater from each mesocosm was first filled in a polypropylene 50 L tank for stocks measurements, 4.5 L polycarbonate bottles for rates measurements and 10 L carboys for diversity. All carboys were immediately transferred onto the R/V Alis anchored close to the mesocosms to serve as a lab

platform to ensure a quick processing of the samples. Subsampling procedure and analysis for inorganic nutrients, chlorophyll *a* (Chl) and their associated phaeopigments, DIP turnover time and N<sub>2</sub> fixation rates are detailed in a companion paper (Berthelot et al., 2015). Primary production (PP) is determined from short term (~ 4 h) incubations around noon using <sup>14</sup>C labeling technique (see details in Berthelot et al., 2015) and a model of photosynthesis is then applied which reconstitute daily fluxes (Moutin et al., 1999). Primary production (PP) was determined from short term (~ 4 h) incubations around noon with H<sup>14</sup>CO<sub>3</sub> (see details in Berthelot et al., 2015) and a model of photosynthesis applied to calculate daily fluxes (Moutin et al., 1999). This model allows estimation of 24h-fluxes (dawn to dawn) from hourly rates, independent of starting time or duration of incubations, or of the geographic origin of the samples or of the time of the year (i.e. systems with varying day-light periods). This model avoids the general biases introduced by the large variety of incubation conditions used in the Steemann-Nielsen (1952) <sup>14</sup>C methodology (Regaudie-de-Gioux et al., 2014 and ref therein). Another advantage of this model is that it allows estimation of both PP (24h dawn-to-dawn) and Gross Primary Production (GPP). For 24 h incubations, GPP is 1.72 x PP as determined from the model (Figure 5 in Moutin et al., 1999). This constant is applicable as long as 24h-fluxes (dawn-to-dawn) are calculated using the same model.

## **2.2 Heterotrophic bacterioplankton abundances**

Flow cytometry analyses were carried out at the PRECYM flow cytometry platform (<https://precym.mio.univ-amu.fr/>). Samples were analyzed using a FACSCalibur (BD Biosciences, San Jose, CA). For heterotrophic bacterial abundance (BA), 1.8 mL of seawater was fixed with formaldehyde (2 % final concentration, 15 minutes incubation at RT), frozen and stored in liquid N<sub>2</sub> until analysis in the laboratory. After thawing at room temperature, 0.3 mL of each sample was incubated with SYBR Green II (Molecular Probes, final conc. 0.05 % [v/v]) for 15 minutes at room temperature in the dark) to stain nucleic acids (Marie et al. 1997). Cells were characterized by 2 main optical signals collected from the 488 nm laser: side scatter (SSC, related to cell structure) and green fluorescence (530/40<sub>488 nm</sub>), related to nucleic acids staining. For the calculation of heterotrophic prokaryotes abundances, phytoplankton, in particular *Prochlorococcus* and *Synechococcus*, was gated out thanks to its red autofluorescence induced by the chlorophyll (Sieracki et al., 1995). We discriminated HNA (high nucleic acid) and LNA (low nucleic acid) cells and heterotrophic bacterial abundance (HBA) was calculated as the sum of both categories. TruCount beads (BD

Biosciences) and 2  $\mu\text{m}$  beads (Fluoresbrite YG, Polyscience) were added to the samples just before analysis. To determine the volume analyzed by the flow cytometer, the flow rate was estimated by weighing three tubes of samples before and after a 3 min run. The cell abundance was determined by dividing the number of cells by the volume analyzed determined both by the TruCount beads and flow rate. All data were collected in log scale and stored in list-mode using the CellQuest software (BD Biosciences). Data analysis was performed using the SUMMIT v4.3 software (Dako).

### 2.3 Heterotrophic bacterial production

Heterotrophic bacterial production (BP) was estimated daily using the  $^3\text{H}$ -leucine incorporation technique (Kirchman, 1993), adapted from the centrifuge method (Smith and Azam, 1992). For each sample, triplicate aliquots (1.5 mL) and one trichloroacetic acid (TCA) killed control were incubated with a mix of 6 nM hot leucine ( $\text{L-}[^3\text{H}]$  leucine, Perkin Elmer® specific activity ranging  $106 \text{ Ci mmol}^{-1}$ ) and 14 nM cold leucine, at *in situ* surface temperature (on-deck incubators equipped with 50 % light intensity screen and cooled with circulating surface seawater), for 1 h. Linearity of leucine incorporation was checked regularly by time series experiments. The live incubations were terminated with 5 % TCA (final concentration). After three runs of centrifugation/aspiration of the supernatant (once with the fixed sea water sample, once with a 5 % TCA rinse, once with an 80 % ethanol rinse), the pellet was resuspended in Packard Ultima Gold MW Scintillation liquid®. Radioactivity was counted using a Liquid Scintillation Analyzer Packard® 1600TR and the  $^3\text{H}$  counting efficiency was corrected for quenching. Concentration kinetic experiments showed that isotopic dilution factor ranged 1 to 1.56 and thus BP rates were calculated from leucine incorporation rates using conversion factor adjusted from 1.5 to  $2.4 \text{ kg C mol}^{-1}$  leucine. Daily rates were calculated assuming they are 24 times the hourly rate.

### 2.4 Nutrient addition experiments

The availability of phosphorus (P), nitrogen (N) and organic carbon (C) for heterotrophic bacteria was investigated by measuring changes in bacterial production following additions of DIP ( $0.25 \mu\text{M P}$ ),  $\text{NO}_3^-$  and  $\text{NH}_4^+$  ( $1 \mu\text{M}$  each) or glucose ( $10 \mu\text{M C}$ ) (final concentrations). Two bioassays were realised, one right before (day 4) and one two weeks after (day 20) the DIP fertilization in M1 (samples from 1 m depth). Eight combinations were tested (P, N, C, PN, PC, NC and PNC) including the non-enriched control T. Each bioassay condition was

tested in triplicate in 60 mL polycarbonate bottles incubated for 48 h under in situ-simulated conditions in the on-deck incubator (described in section 2.3). After incubation, each bottle was sub-sampled in order to measure BP using the leucine technique described in section 2.3.

## **2.5 Alkaline phosphatase activity**

Total alkaline phosphatase activity (APA) was measured at the three depths in M1, M2 and M3 and in Nouméa lagoon waters using the analog substrate methylumbelliferone phosphate (MUF-P, 1  $\mu$ M final concentration) (Hoppe, 1983). The linear increase in fluorescence of seawater with added MUF was measured over the incubation time (up to 8h)), in the dark with a TKO 100 Hoefer DNA fluorometer (single-wavelength with excitation/emission fixed at 365/460 nm but suitable for MUF). Concentration kinetics using a range from 25 to 2500 nM MUF-P were run on some occasions to check that the 1  $\mu$ M concentration used for routine measurements was sufficient to saturate enzyme activity. Blanks were run by adding the MUF-P to filtered boiled seawater and were shown to be insignificant. Calibration curves were made with MUF standards.

## **2.6 Statistical analyses**

Non parametric Mann-Whitney and Kruskal-Wallis tests were used to compare differences of each parameter studied between mesocosms, periods of time, or effect of various amendments on BP in the nutrient addition experiments. Model I linear regressions and Pearson correlation coefficient were used to study Log-log relationships between BP and Chl or PP; and evolution of DOC and POC with time.

## **3 Results**

Salinity and temperature measurements show that the water column was not stratified over the course of the experiment, except the first two days, which were characterized by a slight stratification both inside and outside of the mesocosms (Bonnet et al., 2016b). No vertical stratification was observed in the mesocosms for bacterial production or alkaline phosphatase activity (APA) (see exemplary data for M1 in Fig. S1) as for most of the parameters (Bonnet et al., 2016b; Turk-Kubo et al., 2015, Berthelot et al., 2015). For all description of biogeochemical stocks and fluxes, we thus used the average of the three depths to plot the temporal evolution within each mesocosm.

### **3.1 Chlorophyll stocks and net primary production**

Based on the Chl and PP dynamics, two periods P1 (days 5-14) and P2 (days 15-23) were identified after DIP fertilization, which were also identified by Berthelot et al. (2015) based on biogeochemical characteristics and by Turk-Kubo et al. (2015) based on changes in abundances of targeted diazotrophs. Diatom heterocyst-forming symbionts associated with diatoms were abundant during P1 while a bloom of the unicellular N<sub>2</sub>-fixing cyanobacteria from Group C (UCYN-C) occurred P2 (Leblanc et al., 2015; Turk-Kubo et al. 2015). Chl stocks significantly increased during P2 compared to P1 in the three mesocosms (statistics are presented in Table 1). Chl concentrations during P2 in all three mesocosms were significantly higher than those in the Nouméa lagoon for the same period. PP showed the same trend as for Chl, being higher during P2 in all three mesocosms (Fig. 3, Table 1). However, the PP rates and Chl concentrations reached during P2 were not identical between the three mesocosms: M3 exhibited higher Chl concentrations during P2 ( $0.71 \pm 0.30 \mu\text{g L}^{-1}$ ) than M2 ( $0.49 \pm 0.18 \mu\text{g L}^{-1}$ ) and M1 ( $0.42 \pm 0.14 \mu\text{g L}^{-1}$ ,  $p < 0.001$ ). This was equally true for PP rates ( $2.45 \pm 0.76 \mu\text{mol C L}^{-1} \text{d}^{-1}$  in M3 compared to  $1.47 \pm 0.35 \mu\text{mol C L}^{-1} \text{d}^{-1}$  in M2 and  $1.09 \pm 0.22 \mu\text{mol C L}^{-1} \text{d}^{-1}$  in M1,  $p < 0.001$ ). Significant increases of Chl and PP were also observed in lagoon waters (reaching in P2  $0.30 \pm 0.07 \mu\text{g Chl L}^{-1}$  and  $1.36 \pm 0.37 \mu\text{mol C L}^{-1} \text{d}^{-1}$ , respectively, Table 1).

### 3.2 Heterotrophic bacterioplankton abundance and production

Abundances of heterotrophic bacterioplankton (HBA) varied 10 fold, from 1.7 (day 9, M1, 1 m) to  $12.8 \times 10^5 \text{ cells mL}^{-1}$  (M2, day 11, 6 m). Peaks of HBA were sporadic, like on day 11 in M1,, but not repeated for the three depths sampled. They were possibly due to the presence of a patchy distribution of aggregates that could have biased some of the results. These peaks are occasional, and as they might reflect the reality of a patchy distribution, they were kept in the figures, statistics and estimates of means per day. Average HBA did not increase statistically between phase P1 and P2 in M1 and M2 and increased slightly ( $p < 0.05$ ) from ( $4.1 \pm 0.7$ ) to  $(5.0 \pm 1.4) \times 10^5 \text{ cells mL}^{-1}$  in M3 (Table 1, Fig. 3). Evolution of BP in the mesocosms was close to that in lagoon waters during P1, except for a peak only detected on day 4 (the morning before DIP fertilization) in M1 and M3, and at day 5 in M2. These peaks were not related to any HBA, Chl or PP increase but were related to a steep increase in *Rhodobacteraceae* 16S ribosomal RNA genes (Pfreundt et al., 2016b). Just like PP, BP significantly increased during P2 in all three mesocosms with higher values in M3 compared to M1 and M2 during P2 (Kruskal-Wallis test,  $p < 0.05$ ). This BP increase was also observed in lagoon waters but with lower amplitude (Table 1). In the three mesocosms, the log-log



relationship between BP and PP was significant only during P2 ( $r = 0.54$ ,  $p < 0.001$ ), whereas that between BP and Chl was significant during P1 and P2 ( $r = 0.4$ ,  $p < 0.001$  and  $r = 0.72$ ,  $p < 0.001$ , respectively, Fig. 4). In lagoon waters, the BP/PP ratio slightly increased (but significantly) between P1 and P2 (0.33 to 0.39,  $p < 0.05$ , Table 1). The BP/PP ratio significantly increased during P2 in M1 (0.48 to 0.65,  $p < 0.001$ ), significantly decreased in M3 (0.50 to 0.35,  $p < 0.05$ ) and remained stable in M2 (Table 1). Such differences probably depended on varying maximal values of PP in different mesocosms and the exact days when PP or BP started to increase. Thus we examined the trend of BP/PP ratio with PP, keeping in mind the risk of autocorrelation. BP/PP ratio decreased when PP increased, with higher intensities in the mesocosms compared to lagoon waters, and consistent for P1 or P2 (Table 2). The log-log relationship between  $N_2$  fixation rates ( $nM\ d^{-1}$ ) and BP ( $mg\ C\ m^{-3}\ d^{-1}$ ) was insignificant during P1 and significant during P2 ( $\log(BP) = 0.13 * \log(N_2\ fix\ rates) + 0.73$ ,  $r = 0.21$ ,  $p = 0.04$ , data not shown).

### 3.3 Alkaline phosphatase activity

APA was homogeneous between the three depths sampled from the mesocosms (example for M1 on Fig. S1), but this was not the case in the Nouméa lagoon, where activity was often higher at 1 m depth compared to the two other depths (data not shown). A slight but very reproducible decrease of APA occurred on days 5 and 6 in all three mesocosms where DIP fertilization took place, and in lagoon waters only on day 5 (Fig 3). DIP was consumed more rapidly in M1, mirrored by higher APA and lower TDIP between day 9 and 18 in this mesocosm (Fig. 3). APA then increased very rapidly in M1 and M2 after day 17, but only after day 21 in M3. Such delays were in agreement with the evolution of DIP, which was less rapidly consumed in M3 compared to M1 and M2 (Berthelot et al., 2015). Consequently, although mean APA increased significantly in all three mesocosms between P1 and P2 (Table 1), it was lower in M3 compared to M1 and M2 during P2 (3.1 vs 7.5-7.9  $nmol\ MUF-P\ hydrolyzed\ L^{-1}\ h^{-1}$ , respectively,  $p < 0.01$ ). Finally, APA also increased significantly between P1 and P2 in the lagoon waters, albeit to a lower extent as in the mesocosms (from 3 to 5  $nmol\ MUF-P\ hydrolyzed\ L^{-1}\ h^{-1}$ , Table 1). Noteworthy, APA in the lagoon waters exhibited the strongest increase between day 10 and 11 and stayed at this higher level until day 23, thus exhibiting different dynamics than in the mesocosms.

### 3.4 Enrichment experiments

In the two 48 h nutrient enrichment experiments performed on day 4 and on day 20, BP increased 3-fold after nitrogen addition ( $\text{NH}_4^+ + \text{NO}_3^-$ ) compared to the unamended controls. This significant increase was observed irrespectively of whether these N sources were added alone or in combination with DIP or glucose (N, NP, NC, NPC treatments, Fig. 5,  $p < 0.03$ ). Only PC and C addition on day 4 led to significant BP increase without N addition ( $p < 0.05$ ) however, to a much lower extent than with all N combinations (factor 1.5 and 1.1, respectively). On day 20, only N amendments led to significant BP increases after 48 h incubations, compared to the unamended control (Fig. 5,  $p < 0.03$ ).

### 3.5 Cell-specific leucine incorporation rates

Among the different groups sorted by flow cytometry, significant cell-specific leucine incorporation rates into macromolecules were obtained for heterotrophic bacterioplankton. LNA, HNA and hi-HNA cells had specific activities ranging from 4.6 to  $86 \times 10^{-21} \text{ mol cell}^{-1} \text{ h}^{-1}$ . Overall, cell specific leucine incorporation rates for LNA cells were lower than or equal to rates for HNA cells, and rates for Hi-HNA cells were 1.2 - 4.8 times greater than rates for HNA cells (Table 3). All cell-specific rates increased when bulk activities increased. Among autotrophic groups, significant leucine incorporation was detected for *Prochlorococcus* cells (PRO) only on day 21 and day 23, due to the low volume available for sorting and a significant decrease of PRO abundances in the samples to be sorted, when compared to the abundances determined on samples analysed only three months after the experiment. We checked on fresh Mediterranean samples that *Prochlorococcus* cells were clearly detectable with the flow cytometer setting chosen, and could discard any instrument problem. We assumed that the PRO cells lower detection was due to the long storage period of  $^3\text{H}$ -leucine labelled samples until cell sorting (two years at  $-80^\circ\text{C}$ ) that could induce a loss of fluorescence or cell damages. We obtained a maximum of 1200 PRO cells sorted. Thus, even when the signal was significant, it was associated with a high standard deviation (40 % on day 21, Table 3). On the opposite, *Synechococcus* cells (SYN) were well detected and their total abundance matched with the total counts determined on samples analysed three months after the experiment. Additional 651 nm and 355 nm laser excitations allowed us to distinguish two different sub-groups, not clearly distinguishable using only 488 nm laser excitation, separated mainly on the criterion of orange fluorescence (LO-SYN and HO-SYN) (Fig. 2), suggesting different relative amounts of accessory pigments (Neveux et al., 2010). Leucine incorporation was detected in both SYN groups for all samples analysed. For a given sampling date, cell

specific rates of both groups were almost equal, and increased on day 21 and 23 compared to days 15 and 19. They were lower than LNA cell-specific rates (from ~20 % of the LNA rates at day 15 to ~70 % at day 21). Cell-specific rates of LO-SYN and HO-SYN diverged only on day 23 (Table 3). At this date, cell specific rates for LO-SYN were twice as high as for LNA cells, reaching  $131 \times 10^{-21} \text{ mol cell}^{-1} \text{ h}^{-1}$ . Overall, the contribution of the two *Synechococcus* groups to the bulk activity was very low: it ranged 0.2 to 0.7 % for LO-SYN or HO-SYN (i.e. the contribution reached a max of 1.5 % for both SYN groups together), and 0.01-0.02 % for PRO (when detected), respectively. Contribution of LNA cells to the bulk activity was 4-12 %. Thus, the most important contribution to the bulk leucine activity was due to HNA and Hi-HNA cells.

### 3.6 Carbon budget

We used the advantage of a day to day sampling in an enclosed system to compute a carbon budget that will allow to estimate the fate of phytoplankton-derived organic carbon and the metabolic balance. This carbon budget was calculated using time-integrated data, and thus considered the whole data set. First, each time point was averaged for the three sampling depths, and then time integration was calculated separately for each mesocosm assuming a linear trend between 2 successive days. A mesocosm average was calculated based on the time-integrated data obtained in each of the three mesocosms, with error bars representing the standard deviation (sd) among the three mesocosms (Fig. 6a). Gross primary production (GPP) is derived from PP assuming  $\text{GPP} = \text{PP} \times 1.72$  (Moutin et al., 1999) and represents the whole photosynthetic source of organic matter, including both particulate and extracellular release forms. The cumulated GPP at day 23 was  $38 \pm 11 \mu\text{M C}$  (Fig. 6b). Carbon exported by sedimentation into the traps (Cexp) was corrected in  $\mu\text{M C}$  units based on a mean, constant water volume inside M1, M2, and M3 (see Berthelot et al., 2015 for details) and its cumulated value reached  $6.4 \pm 2.1 \mu\text{M C}$  on day 23. For POC and DOC, for which data were more irregular and showed outliers, we decided to calculate net variations of POC and DOC after a linear fit of the discrete data set between days 5 and 23 in each mesocosm (Table 4). POC increased linearly in M1 and M3 ( $0.12$  and  $0.48 \mu\text{mol C L}^{-1} \text{ d}^{-1}$ ,  $r = 0.32$   $p < 0.03$  and  $r = 0.70$   $p < 0.001$ , respectively) and showed no trend in M2. A significant increase of DOC was only observed in M2 (Table 4). Due to the high sd resulting from variability in net variation of POC and DOC versus time between the three mesocosms, the average accumulation of DOC and POC estimated for the carbon budget was negligible (Fig. 6a), and the most important measured fate of GPP was Cexp, representing 17 % of GPP (Fig. 6b).  $\text{GPP} - (\text{net DOC} + \text{net$

POC + C<sub>exp</sub>) can be considered as community respiration (CR). CR was calculated and reached  $27 \pm 11 \mu\text{M}$  cumulated from day 5 to 23, i.e. 71 % of GPP.

## **4 Discussion**

### **4.1 Variability within the triplicate mesocosms**

Overall, M3 exhibited maximum peaks of chlorophyll biomass reached, PP and BP rates and these different responses were particularly seen during P2. A time lag of a few days in the succession of the different planktonic populations was noticed, particularly for nitrogen fixers (Turk Kubo et al. xxx), and DIP was consumed more rapidly in M1. However, slight divergence in biological and chemical evolution among different replicated mesocosms is not uncommon, particularly after the first week of enclosure (Martinez-Martinez et al., 2006; Pulido-Villena et al., 2014). Here, the divergence was probably resulting from a combination of bottom up (availability of DIP and nitrogen), and top down controls (grazing pressure and viral lysis). The initial conditions prevailing before the DIP enrichment could be also at the origin of the divergence. Indeed mesocosms were closed 3 days before the DIP addition, and many species of diazotrophs exhibit a patchy distribution (Bombar et al., 2015). In addition, Hunt et al. (2016) noticed larger amounts of zooplankton individuals in M3 at the beginning of the experiment, some of which, stressed by the mesocosms, might have died (some larger amounts of 'swimmers' were recovered in the traps in M3), contributing to supplementary sources of N in M3. Nevertheless, overall the replicability among mesocosms was considered sufficiently correct for most of the biogeochemical stocks, fluxes and abundances of phytoplankton groups (Bonnet et al., 2016b) and thus our results are discussed based on averages.

### **4.2 N limitation and coupling between BP and N<sub>2</sub> fixation**

BP was significantly enhanced on a short-term scale (1 - 2 days) by  $\text{NO}_3^- + \text{NH}_4^+$  but not DIP or glucose amendments, indicating that BP was directly N-limited, and/or indirectly after stimulation of N-limited phytoplankton (Fig. 4). In the New Caledonia lagoon, N-limitation has previously been suggested based on a one-year survey of nutrient ratios (Torréton et al., 2010). N-limitation is a recurrent feature observed in the ultra-oligotrophic South Eastern Pacific Gyre (Van Wambeke et al., 2008a; Halm et al., 2012), as assessed from short-term (1-3 days) enrichment experiments or incubations. As N<sub>2</sub> fixation is assumed to be the only process providing a source of new nitrogen to the mesocosms in this experiment, we

examined the potential links between  $N_2$  fixation rates and BP. First, marine heterotrophic diazotrophs were detected at low abundances during the mesocosms experiment:  $\gamma$ -24774A11 with ca  $10^2$ - $10^3$  *nifH* gene copies  $L^{-1}$  (Turk-Kubo et al., 2015), and 16S tags corresponding to heterotrophic diazotrophs like *Bradyrhizobium* or *Mesorhizobium* were scarce (Pfreundt et al., 2016b). Therefore,  $N_2$  fixation directly performed by heterotrophic bacteria probably accounted for a minor fraction of bulk  $N_2$  fixation during the mesocosm experiment. Second, as the log-log relationship between  $N_2$  fixation rates and bacterial production was not significant during P1, and only slightly significant during P2 ( $r = 0.21$ ,  $p = 0.04$ ), the excretion of DON and  $NH_4^+$  by diazotrophs likely did not supply much nitrogen for heterotrophic prokaryotes directly, particularly during P1 when the main organisms responsible for diazotrophy were diatom-symbiotic (Turk-Kubo et al., 2015). Assuming a C/N molar ratio of around 6.8 for heterotrophic prokaryotic biomass (Fukuda et al., 1998),  $N_2$  fixation might have provided 17 to 30 % of the nitrogen demand of heterotrophic prokaryotes, depending on the phase and the mesocosm considered (Table 1). This proportion increases to 30 - 37 % if we consider a C/N ratio of 8.2, which seems more appropriate in the Pacific Ocean (Fukuda et al., 1998). Thus,  $N_2$  fixation contributed to but was not sufficient to sustain 100 % of the N requirements of heterotrophic bacteria during this study. Other potential sources were initial DON stocks, concentrations of which decreased slightly at the end of the experiment (Berthelot et al., 2015) and detritus. Indeed, there was a decay of larger phytoplankton cells after the closure of the mesocosms as discussed by Knapp et al. (2015) and Leblanc et al. (2015) following DIP availability (TDIP) as well as PP decreases (Berthelot et al., 2015) and *Synechococcus* 16S tags dropped substantially between day 2 and 4 (Pfreundt et al., 2016b). Such detritus probably also contributed to sustain BP. NanoSIMS analyses were performed during a parallel experiment done at the height of a bloom of diazotrophic *Cyanothece*-like cyanobacteria (UCYN-C) on days 17-20 in M2 (Bonnet et al., 2016a). After 24 h of  $^{15}N_2$ -incubations, these authors reported significant  $^{15}N$ -enrichment in picoplanktonic cells (0.2-2  $\mu m$  fraction). This confirmed a rapid (one day) transfer of DDN (also  $^{15}N$ -enriched) to picophytoplankton, and potentially heterotrophic bacteria. However, such transfer likely occurred indirectly through DON after mortality and grazing processes, as shown by model simulations run during the VAHINE project (Gimenez et al., 2015).

### 4.3 Alkaline phosphatase activity and P acquisition

A slight  $T_{DIP}$  decrease was noticed in the mesocosms before the DIP spike but not in the lagoon, suggesting a lower P availability inside and not outside the mesocosms. Therefore,  $N_2$

fixers might benefit from continuous and variable inputs of DIP sources in the lagoon waters during that period. This is also confirmed by the low values of alkaline phosphatase activity in the lagoon at the start of the experiment. Whether these sources were coming from the benthos (Torréton et al., 2002), the atmosphere (soot emission can influence lagoon waters inside and outside the barrier reef, Mari et al., 2014) and/or currents (Fichez et al., 2010) is beyond the scope of this study. Inside the mesocosms, when the DIP added was consumed, the increase of APA observed could be due to i) a population switch towards phosphatase producers, which can be heterotrophic bacteria and phytoplankton, and ii) increases in specific activities due to enzymatic induction; or both. We used POP as a proxy of living biomass (Duhamel et al., 2007) to estimate specific activities (nmole MUF-P hydrolyzed per unit POP per unit time) and found the same trend for specific activities and for bulk APA (i.e. specific activity increased up to 10-fold). APA was produced by different phylogenetic groups of heterotrophic bacteria, but also by cyanobacteria, as shown on a metatranscriptomic study in the special issue (Pfreundt et al., 2016a), with the highest levels of alkaline phosphatase transcripts originating from *Synechococcus* on days 14 and 20. Our results and those of Pfreundt et al. (2016a) suggested a switch towards a microbial population that produced phosphatase to escape P depletion after a transient P-replete period. Although  $T_{DIP}$  decreased and APA increased up to values analogous to those observed in P-limited areas (Moutin et al., 2002; Van Wambeke et al., 2002, respectively), heterotrophic bacteria stayed continuously N-limited but not P-limited. As discussed in Pfreundt et al. (2016a) and Pfreundt et al. (2016b), some acquisition mechanisms of large P-containing organic molecules and reduction of cellular P quota also helped microbial communities to resist P depletion during P2 phase.

#### 4.4. Cyanobacterial assimilation of leucine

BP was used in this study as a strict proxy of heterotrophic bacterial production. As we incubated  $^3H$  leucine under light conditions, photoheterotrophic activity and the possibility that some photosynthetic cyanobacteria incorporate leucine could biases BP estimates. Whether light stimulation of bacterial production can be explained by direct (assimilation or organic molecules by autotrophs), or indirect effects (stimulation of BP through release of organic molecules or photo-labilization of organic matter), or both, is difficult to determine (Béjã and Suzuki, 2008). Assimilation of methionine, leucine, and ATP was shown to be enhanced under light-incubation conditions in the North and South Atlantic Oceans and these increases are generally attributed to stimulation of *Prochlorococcus* and SAR11 (Evans et al., 2015), but the spectrum of organic molecules tested is low. In the New Caledonia lagoon,

incubation of samples under different light regimes influences estimates of BP determined by the thymidine technique (Rochelle-Newall et al., 2008), but so far there is no information available on the light effect on leucine uptake around New Caledonia. The capacity of both marine *Prochlorococcus* and *Synechococcus* to assimilate some organic molecules is evident from culture-studies, as well as flow cytometry cell sorting and gene studies (Béjà and Suzuki, 2008). Assimilation of leucine by cyanobacteria can also occur in the dark (Talarmin et al., 2011), but light clearly favours assimilation of leucine by cyanobacteria (Mary et al., 2008b). The polypropylene tubes used in this study to incubate BP attenuated the light intensity by 40 % without spectral distortion in the visible range (Richardson and Porter, 2005). Under such conditions which were intermediary between simulated *in situ* light conditions and dark conditions, significant incorporation of leucine into macromolecules was seen by flow cytometry sorting of *Synechococcus* cells. We could not unambiguously verify leucine incorporation into *Prochlorococcus* cells due to technical reasons (low volumes and long storage limitations). To conclude, although the relative contribution of cyanobacteria (PRO + SYN) to the bulk (community) leucine assimilation into proteins was less than 2 %, and could not be responsible of a bias in BP estimates, we estimate significant potential for leucine to be assimilated by cyanobacteria. Note that we used a 10 nM leucine concentration for cell sorting, but *in situ* natural concentrations could be much lower. More studies are needed, investigating the potential use of other organic molecules in lower, close to *in-situ* concentrations. Mixotrophy may be the rule rather the exception in these experimental systems (Moore, 2013; Evans et al., 2015).

#### **4.5 Phytoplankton-bacteria coupling and metabolic balance**

Torréton et al. (2010) report mean Chl concentration around  $0.3 \mu\text{g L}^{-1}$  over a seasonal cycle performed at an oligotrophic station in the New Caledonia lagoon. These concentrations are close to our reference conditions outside the mesocosms (lagoon waters), where means of Chl values were  $0.21$  and  $0.30 \mu\text{g L}^{-1}$  during P1 and P2 phases. Under these oligotrophic conditions, the seasonal variability of BP and PP in the Nouméa lagoon is much lower than in temperate waters, only about 3-fold with an average BP/PP ratio of 0.21, and some rare peaks reaching 0.6 (Torréton et al., 2010). This is in accordance with the range of BP/PP ratios encountered in the lagoon waters during our study, with average values ranging 0.33 to 0.39 during phases P1 and P2, respectively. On the opposite, inside the mesocosms, average BP/PP ratios were generally higher than in the lagoon waters whatever the phase or the mesocosm considered. A negative trend of BP/PP with increasing PP was obtained, suggesting that a

1 larger fraction of PP is channelled through the microbial food web when PP decreases, as  
 2 observed in the oligotrophic Mediterranean Sea (Conan et al., 1999). This is also the trend  
 3 when considering a large oceanic data set examined by Fouilland and Mostajir (2010). Indeed  
 4 in their study, regression of  $\log(\text{BP})$  as a function of  $\log(\text{PP})$  resulted in a slope of 0.57,  
 5 inferring that BP increases less rapidly than PP when PP increases. Contrarily to the weak  
 6 (during P2) or absence of (during P1) correlation between BP and  $\text{N}_2$  fixation rates described  
 7 above, strong relationships between BP and Chl, and between BP and PP were obtained  
 8 during both phases (Fig. 4). This suggests that  $\text{N}_2$  fixation stimulated autotrophic communities  
 9 during the VAHINE experiment, which may in turn have produced organic matter for  
 10 heterotrophic prokaryotes. BP and PP are determined routinely, but to estimate the flux of PP  
 11 channelled through heterotrophic bacteria, or to infer metabolic balance between autotrophy  
 12 and heterotrophy, bacterial carbon demand (BCD) and gross primary production (GPP) must  
 13 be also estimated. Such carbon fluxes are not directly estimated but are derived from PP and  
 14 BP using additional measurements of bacterial growth efficiency (or bacterial respiration) and  
 15 phytoplankton extracellular release of DOC. These parameters are less frequently acquired  
 16 due to time-consuming and difficult technologies (del Giorgio and Cole, 1998; Nagata, 2000),  
 17 which led to controversy on the metabolic balance in oligotrophic environments (Cole et al.,  
 18 1988; Ducklow et al., 2002; Van Wambeke et al., 2008b; Fouilland and Mostajir, 2010;  
 19 Moran and Alonso-Saez, 2010). BCD is derived from BP by the use of bacterial growth  
 20 efficiency (BGE) or respiration rates which are not often measured concomitantly with PP and  
 21 BP, and in many oligotrophic environments BCD/PP is higher than 1, or respiration exceeds  
 22 PP (del Giorgio et al., 1997). To explain this, different arguments are generally proposed.  
 23 First, other DOM sources than those deriving from phytoplankton (allochthonous sources) are  
 24 used to sustain BCD. In Pacific lagoons, excretion of mucus by coral has been proposed as a  
 25 supplementary source of DOM for heterotrophic bacterioplankton (Torr  ton et al., 2002, Wild  
 26 et al., 2004). Second, phasing between BP and PP peaks during seasonal blooms and  
 27 occasional presence of PP bursts (see for example Steinberg et al., 2001) are not always  
 28 detected due to inappropriate sampling frequency for BP and PP measurements. Daily and  
 29 parallel measurements of both BP and PP in the mesocosms avoided such problems in this  
 30 study.  
 31 Assuming negligible effect of a biofilm development on the mesocosms walls (Knapp et al.  
 32 (2015) on the plankton C budget, the main fate of photosynthetically fixed organic carbon  
 33 during the experiment was respiration (71 % of GPP) then sedimentation (17 % of GPP). The  
 34 different responses between the triplicate mesocosms led to a great propagation of errors and



1 thus on the variability on CR/GPP ratio ( $70 \pm 36 \%$ ). CR being lower than GPP, the biological  
2 system inside mesocosms was net autotrophic, with an upper error limit close to metabolic  
3 balance between autotrophy and heterotrophy.

4 In the lagoon, as close to Grande Rade Bay, long residence times favored local degradation,  
5 refractorisation of organic matter and not sedimentation (Mari et al., 2007). However, as these  
6 authors discussed, modification of phytoplankton community composition in Grande Rade  
7 Bay and the presence of metals could influence sticking properties of polymers. The  
8 confinement of the seawater inside the mesocosms probably favored to some extent the  
9 accumulation of UCYN-aggregates, as well as a possible reduction of grazing pressure (by a  
10 factor of 1.6) in the mesocosms compared to those in the lagoon waters (Turk-Kubo et al.,  
11 2015; Bonnet et al., 2016a; Hunt et al., 2016). However, UCYN-C formed large aggregates  
12 ( $100\text{--}500 \mu\text{m}$ ) embedded in an organic matrix that included TEP, which were largely  
13 responsible for enhanced export flux through sedimentation observed during P2 (Berthelot et  
14 al., 2015; Berman-Frank et al., 2016; Knapp et al., 2015). TEP evolution with time, however,  
15 and the TEP-C to TOC ratio were similar in the lagoon waters, where wave turbulence and  
16 tidal effects were present, and in the enclosed mesocosms, where these hydrodynamics were  
17 reduced, and concentrations were similar (Berman-Frank et al., 2016). In an unconstrained  
18 ordination analysis, Pfreundt et al. (2016b) described significant differences in bacterial  
19 communities between M1 and the lagoon, but similar temporal dynamics. Direct comparisons  
20 of our export results with findings from open ocean studies should be made cautiously as our  
21 mesocosms were both shallower (15 m) than in typical oceanic export studies ( $> 100 \text{ m}$ ) and  
22 exhibited reduced turbulence.

23 In order to calculate the fraction of GPP that channeled, directly or indirectly, through the  
24 microbial food web, the bacterial carbon demand (BCD) must be estimated through additional  
25 estimates of bacterial respiration (BR) or bacterial growth efficiency (BGE). In an  
26 oligotrophic site inside the Nouméa lagoon, BGE was estimated at 10 % using incubated  
27 samples where oxygen changes were followed with time in the dark (Briand et al., 2004).  
28 However, as suggested by Aranguren-Gassis et al. (2012), using consistently low BGE  
29 derived from size fractionation experiments and long-duration incubations leads to probable  
30 BGE underestimation. In the lagoon, the use of a 10 % BGE would lead to BCD values higher  
31 than GPP (Rochelle-Newall et al., 2008). If we assume such low BGE in the mesocosms (10  
32 %), cumulated BR from day 5 to 23 would rise to  $93 \pm 6 \mu\text{M C}$ , which is not realistic  
33 compared to CR estimated from the carbon budget. A more appropriate BGE of  $27 \pm 9\%$  to  
34  $43\% \pm 11\%$  could be calculated, based on minimum and maximum ranges admitted for

BR/CR ratio (from 100 to 50 %, Lemée et al., 2002), and based on propagation of errors due to the variability within triplicate mesocosms (Table S1). For sensitivity analysis of BR and BGE calculation from the carbon budget, we examined whether the errors of different methodological assumptions (conversion factors, analytical errors) were higher than those arising from variability between triplicate mesocosms. We considered different errors based on literature data for all the parameters used in computation of BR and BGE (i.e. GPP, C<sub>exp</sub>, DOC, POC, and BP). For GPP, we assumed  $GPP = 1.72 \times PP$ , i.e. PP/GPP ratio = 58 %. In the South Pacific Ocean, the mean average PP to GPP ratio (based on comparison between oxygen and <sup>14</sup>C technique) was 47% (Van Wambeke et al., 2008b). If we consider that the sum of dissolved and particulate PP in the lagoon (<sup>14</sup>C technique, Rochelle-Newall et al., 2008) is a good proxy of GPP, then an upper limit for this ratio is 65 % in the lagoon. We thus applied a 15 % variability to the PP/GPP ratio, leading to  $GPP = 1.36 \times PP$  to  $2.32 \times PP$ , i.e. approximately a 30 % variability on the conversion factor. For BP, we assumed a 25 % daily variability of BP (Church et al., 2006, Van Wambeke et al., 2008c, Torréton et al., 2010). For C<sub>exp</sub>, DOC and POC, we assumed analytical errors of 10 %. We then used propagation of errors to compute the error associated with BR and BGE (Table S1). For GPP, the errors resulting from triplicate mesocosms or resulting from conversion factor and analytical errors were the same. According to the propagation of errors, the error associated with GPP has the largest effect on estimates of BR. The uncertainty of DOC, POC, and C<sub>exp</sub> arising from variability within the triplicate mesocosms is higher than the methodological error, whereas it is the opposite for BP. Overall, the uncertainty of BGE estimates arising from variability within triplicate mesocosms or methodology is similar ( $27 \% \pm 9 \%$  or  $27 \% \pm 10 \%$  for BGE based on  $BR = CR$ ,  $43 \% \pm 11 \%$  or  $43 \% \pm 12 \%$  for BGE based on  $BR = CR/2$ )

The BGE values determined from C budget could be potentially related to a beneficial effect of photoheterotrophy. Indeed, in a companion metatranscriptomic study performed in M1 (Pfreundt et al., 2016a), accumulation of proteorhodopsin transcripts was recurrently detected among varying groups of bacteria notably Pelagibacteraceae and SAR86. These groups, belonging to the alpha- and gammaproteobacteria, respectively, were also abundant community members as observed through 16S sequencing (Pfreundt et al., 2016b). Aerobic anoxygenic phototrophic (AAP) bacterial abundances are reported to be particularly abundant in the South Pacific Ocean (Lami et al. 2007), but to date, AAP abundances are not available in the lagoon and they were not counted in this experiment. Nevertheless, Pfreundt et al. (2016a) detected expression of the *pufM* gene, encoding a photoreaction centre protein of AAP bacteria. Transcript abundances were an order of magnitude lower than for

1 proteorhodopsin, and only observed for a group of *Rhodocyclaceae* on day 14 and much  
2 weaker for *Rhodobacteraceae* on day 18. This suggests that AAP bacteria did not play a  
3 major role in the investigated system and did not influence the above calculation to a large  
4 extent. *Dokdonia* sp. strain MED134, proteorhodopsin containing flavobacteria, were shown  
5 to increase the maximum number of cells reached when growing in the light compared to  
6 darkness. However, if DOM was added initially, light vs dark responses changed depending  
7 on DOM concentrations (Gomez-Consarnau et al., 2007). Other laboratory experiment, at the  
8 opposite, showed no difference in growth rates or maximum cell yields of *Pelagibacter*  
9 *ubique* cultures grown in natural seawater (in in a diurnal light regime or in complete darkness  
10 (Giovannoni et al., 2005). The BGE of a bacteriochlorophyll-containing strain (*Erythrobacter*  
11 sp.) was shown to increase during light periods in a continuous culture (Hauruseau and  
12 Koblížek, 2012). Thus the energy benefits of photoheterotrophy remain controversial, and  
13 related to the difficulty to have true oligotrophic conditions in pure culture. Based on an  
14 energy budget, Kirchman and Hanson (2013) suggested that the net energy gained by light is  
15 mostly sufficient to meet maintenance cost of AAP but is not enough to meet that of  
16 proteorhodopsin-based phototoheterotrophic bacteria. Heterotrophic bacteria are limited by N  
17 but also by energy in the South Pacific (Van Wambeke et al. 2008a); this could give an  
18 advantage to photoheterotrophic prokaryotes for growth and their success in this area.  
19 Assuming BGE values ranging from 27 to 43%, the BCD/GPP ratio would range from 63 to  
20 99 %. A large part of the GPP is thus channelled through the microbial food web pathway  
21 within 20 days. To examine potential links between phytoplankton release and BP, we  
22 estimated a extracellular release of 35 %, as determined previously inside the Nouméa lagoon  
23 (Rochelle-Newall et al., 2008). Such values are in agreement with higher percent extracellular  
24 release that are generally obtained in nutrient limited environments (Nagata, 2000). According  
25 to Rochelle-Newall et al. (2008), contemporaneous DOC excreted by phytoplankton was  
26 sufficient to meet BCD only in the coastal part of the lagoon, but not in the offshore  
27 oligotrophic part of the lagoon where the VAHINE experiment was performed, but these  
28 authors used a 10 % BGE. In the mesocosms, still based on a extracellular release  
29 representing 35 % of GPP, DOC release was estimated at 13  $\mu\text{M}$  C produced between 5-23  
30 days. This is not sufficient to satisfy BCD cumulated for the same period (calculated as 24-38  
31  $\mu\text{M}$  C) although we used BGE varying from 27 to 43 % as discussed above. Thus,  
32 heterotrophic bacteria in the mesocosms used additional, not contemporaneous, sources of  
33 organic matter derived from phytoplankton after transformation through the food web like  
34 enzymatic hydrolysis of detritus, viral lysis, and/or sloppy feeding.

## Conclusions

This study confirms that in the Nouméa lagoon,  $N_2$  fixation is a relevant process to fuel the microbial food web and to sustain a biological system which is net autotrophic or close to metabolic balance. The relatively high BGE computed from the carbon budget (27-43 %) could be related to light-harvesting systems developed by abundant bacterial photoheterotrophs. The success of *Synechococcus* over *Prochlorococcus* described in companion papers (Leblanc et al., 2015; Pfreundt et al., 2016b) might be attributed to their ability to assimilate leucine, and possibly other amino acids, as well as reduction of their cellular P demand through sulfolipid synthesis. The relative importance of mixotrophy in these oligotrophic system implies that it is important to i) find alternative techniques to dark incubations to estimate bacterioplankton respiration, like with continuous measurements with oxygen microprobes during alternate light and dark periods (Pringault et al., 2007) and ii) to detect organisms responsible for the assimilation of a wide variety of organic molecules by cell sorting. From the carbon budget, a BCD to GPP ratio was estimated to range between 63 and 100 %, and thus a large part of the primary production is channelled through the microbial food web. Bacterial production was strongly coupled with Chl biomass and/or PP, rather than with  $N_2$  fixation rates, suggesting that indirect routes through lysis, grazing and mortality of phytoplankton were substantial for providing labile organic matter for heterotrophic bacteria.

**Author contributions** SB was the chief scientist responsible of the VAHINE program, she designed and executed the experiment in mesocosms. FV sampled for and analyzed BP and APA, TM sampled for and analyzed  $T_{DIP}$  and PP, AB performed the cell sorting, wrote the corresponding M&M section and made Fig. 2, HB contributed to the analyses of bacterial abundances by flow cytometry, MR sampled for and analyzed data, FV and UP equally wrote the manuscript and made the other figures. All the authors reviewed the manuscript.

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Table 1 Averages  $\pm$  standard deviations of some parameters during phases P1 (from day 5 to day 14) and P2 (from day 15 to day 23) in the three mesocosms M1, M2, M3 and in the lagoon waters.. HBA: heterotrophic prokaryotic abundances, BP: heterotrophic prokaryotic production, AOA: alkaline phosphatase activity, TDIP: turnover time of DIP. N<sub>2</sub> fixation contribution to BP (N<sub>2</sub>fix/BP ratio, in %) is based on a C/N of 6.8 for heterotrophic bacteria. Mann - Whiney tests were performed to test significant differences between P1 and P2: \* p < 0.05; \*\* 0.01 < p < 0.05;\*\*\* p < 0.001.

	M1 P1	M1 P2	Lagoon P1	lagoon P2
Chl ( $\mu\text{g L}^{-1}$ )	0.19 $\pm$ 0.05 ***	0.42 $\pm$ 0.14	0.21 $\pm$ 0.03 ***	0.30 $\pm$ 0.07
% pheopigments	24 $\pm$ 3 ***	28 $\pm$ 5	23 $\pm$ 6	26 $\pm$ 3
HBA ( $\times 10^5$ cells mL <sup>-1</sup> )	3.9 $\pm$ 1.9	4.5 $\pm$ 1.7	5.5 $\pm$ 0.95	6.2 $\pm$ 1.2
PP ( $\mu\text{mol C L}^{-1} \text{d}^{-1}$ )	0.71 $\pm$ 0.27 ***	1.09 $\pm$ 0.22	0.85 $\pm$ 0.17 ***	1.36 $\pm$ 0.37
BP ( $\text{ng C L}^{-1} \text{h}^{-1}$ )	157 $\pm$ 49 ***	348 $\pm$ 42	135 $\pm$ 24 ***	256 $\pm$ 60
DOC $\mu\text{M C}$	59 $\pm$ 3	60 $\pm$ 2	60 $\pm$ 3	60 $\pm$ 2
POC $\mu\text{M C}$	8 $\pm$ 3 *	9 $\pm$ 1	6.6 $\pm$ 1.1 **	7.6 $\pm$ 1.3
APA (nmole MUF-P hydr L <sup>-1</sup> h <sup>-1</sup> )	1.5 $\pm$ 0.9 ***	8.0 $\pm$ 5.4	3.0 $\pm$ 2.3 **	5.0 $\pm$ 3.1
TDIP (days)	16 $\pm$ 15 ***	0.5 $\pm$ 0.3	2.0 $\pm$ 0.9 ***	0.9 $\pm$ 0.4
BP/PP ratio	0.48 $\pm$ 0.18 ***	0.65 $\pm$ 0.20	0.33 $\pm$ 0.11 *	0.39 $\pm$ 0.10
N <sub>2</sub> fix/BP ratio (%)	21 $\pm$ 11*	29 $\pm$ 16	22 $\pm$ 13 *	15 $\pm$ 8

	M2 P1	M2 P2	M3 P1	M3 P2
Chl ( $\mu\text{g L}^{-1}$ )	0.22 $\pm$ 0.03 ***	0.49 $\pm$ 0.18	0.20 $\pm$ 0.04 ***	0.71 $\pm$ 0.30
% pheopigments	23 $\pm$ 2 ***	28 $\pm$ 6	23 2	26 $\pm$ 15
HBA ( $\times 10^5$ cells mL <sup>-1</sup> )	2.2 $\pm$ 2.2	4.9 $\pm$ 1.8	4.1 $\pm$ 0.7 *	5.0 $\pm$ 1.4
PP ( $\mu\text{mol C L}^{-1} \text{d}^{-1}$ )	0.75 $\pm$ 0.15 ***	1.47 $\pm$ 0.35	0.73 $\pm$ 0.15 ***	2.45 $\pm$ 0.76
BP ( $\text{ng C L}^{-1} \text{h}^{-1}$ )	227 $\pm$ 114 ***	338 $\pm$ 116	168 $\pm$ 52 ***	422 $\pm$ 132
DOC $\mu\text{M C}$	58 $\pm$ 3 **	61 $\pm$ 1	61 $\pm$ 3	60 $\pm$ 2
POC $\mu\text{M C}$	10 $\pm$ 3	9 $\pm$ 1	9 $\pm$ 2 ***	13 $\pm$ 3
APA (nmole MUF-P hydr L <sup>-1</sup> h <sup>-1</sup> )	1.0 $\pm$ 0.8 ***	7.6 $\pm$ 7.6	0.6 $\pm$ 0.5 ***	3.18 $\pm$ 2.61
TDIP (days)	27 $\pm$ 19 ***	1.8 $\pm$ 2.0	25 $\pm$ 12 ***	3.0 $\pm$ 3.1
BP/PP ratio	0.65 $\pm$ 0.41	0.47 $\pm$ 0.16	0.50 $\pm$ 0.24*	0.35 $\pm$ 0.08
N <sub>2</sub> fix/BP ratio (%)	17 $\pm$ 16 ***	30 $\pm$ 18	25 $\pm$ 15	22 $\pm$ 11

Table 2. Log - Log relationships between BP/PP ratio and PP (expressed in  $\text{mgC m}^{-3} \text{ d}^{-1}$ ). In mesocosms, phase P1 and P2 are separated for the regressions. r: Pearson correlation coefficient, p: probability.

	equation	r	probability
phase P1	$\log (\text{BP/PP}) = -0,87 \log (\text{PP}) + 0,49$	0,59	< 0.001
phase P2	$\log (\text{BP/PP}) = -0,53 \log (\text{PP}) + 0,33$	0,6	< 0.001
lagoon waters	$\log (\text{BP/PP}) = -0,24 \log (\text{PP}) -0,19$	0,28	< 0,01

Table 3 Specific leucine activities of main groups sorted. PRO : *Prochlorococcus*, LO-SYN (low orange fluorescence *Synechococcus*-like cells), HO-SYN (high orange fluorescence *Synechococcus*-like cells, PE (autotrophic pico-eukaryotes), LNA (low nucleic acid), HNA (high nucleic acid), Hi-HNA (high size and high nucleic acid) heterotrophic bacteria and bulk activities (total community leucine incorporation rates) corresponding to the same sample. When tests of reproducibility have been done, the corresponding data is indicated with its standard deviation. bdl: below detection limits, lag: lagoon waters

	PRO	LO SYN	HO SYN	PE	LNA	HNA	Hi-HNA	bulk
	$\times 10^{-21}$ mole leu cell <sup>-1</sup> h <sup>-1</sup>							pmole leu L <sup>-1</sup> h <sup>-1</sup>
d15 M1	bdl	4.6 ± 1.9	3.5 ± 0.3	19 ± 5	20.6 ± 0.2	67 ± 1	79	97 ± 3
d19 M1	bdl	5,4	3,5	17	27	16	80	126 ± 3
d21 lag	69 ± 28	30 ± 4	25 ± 4	79 ± 12	39	214	554	186 ± 6
d23 M1	22	131	42	108	56	113	356	242 ± 7

Table 4. Linear regression fits on temporal trends of POC and DOC in M1, M2 and M3 from days 5 to 23. DOC has been sampled only at 6 m depth in the 3 mesocosms. df: degree of freedom, r: Pearson correlation coefficient, p: probability, ns: not significant. For POC trend, some outliers have been suppressed from the regressions.

	Range $\mu\text{M}$	Outliers $\mu\text{M}$	slope	df	r	p
POC M1	4.7-12.4	19.3	0.12	35	0.32	0.02
POC M2	7.1-11.6	15.0, 15.0, 17.3	- 0.009	28	0.03	ns
POC M3	6.5-18.9	no	0,47	36	0.70	< 0.001
DOC M1	54-64	no	0.071	13	0.15	ns
DOC M2	53-62	no	0.25	13	0.48	0.04
DOC M3	54-66	no	- 0.12	14	0.22	ns



## Figure Legends

**Figure 1.** Position of mesocosms implemented in the southwest lagoon of New Caledonia.

**Figure 2.** Example (day 23 M3 1m) of flow cytometry cytogram dot plot of: a) naturally non-fluorescent bacterioplankton groups discriminated by their DNA content (SYBR green-induced fluorescence in arbitrary units (a.u.) versus cell size (side scatter), after 488 nm laser excitation); b) phototrophic groups discriminated by their chlorophyll *a* content (related to the red fluorescence intensity (a.u.) versus phycoerythrin related to the orange fluorescence intensity (a.u.) after 488 nm laser excitation); c) low-orange (LO-SYN) and high-orange (HO-SYN) *Synechococcus*-like sub-groups separated by their chlorophyll *a* content (after 661 nm laser excitation) versus their phycoerythrin content (after 488 nm laser excitation).

**Figure 3.** Evolution of: a) chlorophyll *a* (Chl), b) heterotrophic bacterial abundance (HBA), c) primary production (PP), d) heterotrophic bacterial production (BP), e) alkaline phosphatase activity (APA) and f) DIP turnover time (TDIP) in the three mesocosms M1, M2, M3 and in the lagoon waters (lagoon). Each point is the mean of the three depths sampled, error bars are standard deviations. For lagoon HBA, only data from 1 m and 12 m depth are available occasionally and discrete data are presented instead

**Figure 4.** Log-log relationships between heterotrophic bacterial production (BP) and a) primary production (PP) or b) chlorophyll *a* (Chl).

**Figure 5.** Response of heterotrophic bacterial production to the enrichment experiments conducted on days 2 and 20. Asterisks show significant responses in comparison to the unamended control (Co) after Mann Whitney test (\*:  $p < 0.05$ ).

**Figure 6.** Carbon budget of the mesocosms with time ( $\mu\text{M C}$ ). a) Evolution of time-integrated gross primary production (GPP). Cexp: C export in sediment traps (Cexp), time-integrated net POC and net DOC are calculated assuming linear fits of these variables between days 5 and 23 (see Table 3). b) Budget of time-integrated data on day 23. The difference  $\text{GPP} - (\text{Cexp} + \text{net DOC} + \text{net POC})$  was assumed to be community respiration (resp). The range of heterotrophic bacterial carbon demand (BCD) was calculated based on two hypotheses:  $\text{BR} =$

- 1 100 % CR (BCD 1) or BR = 50 % CR (BCD 2). Standard errors are plotted from the sum of
- 2 each category, using propagation of errors.

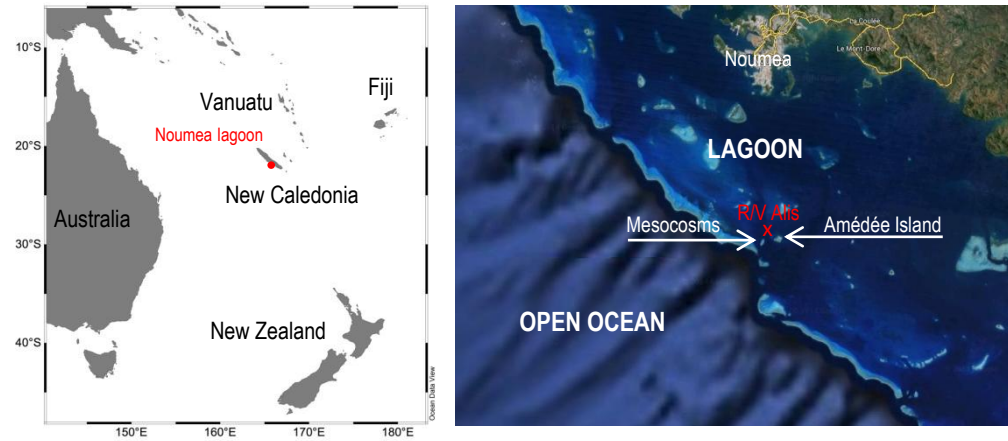


Fig. 1

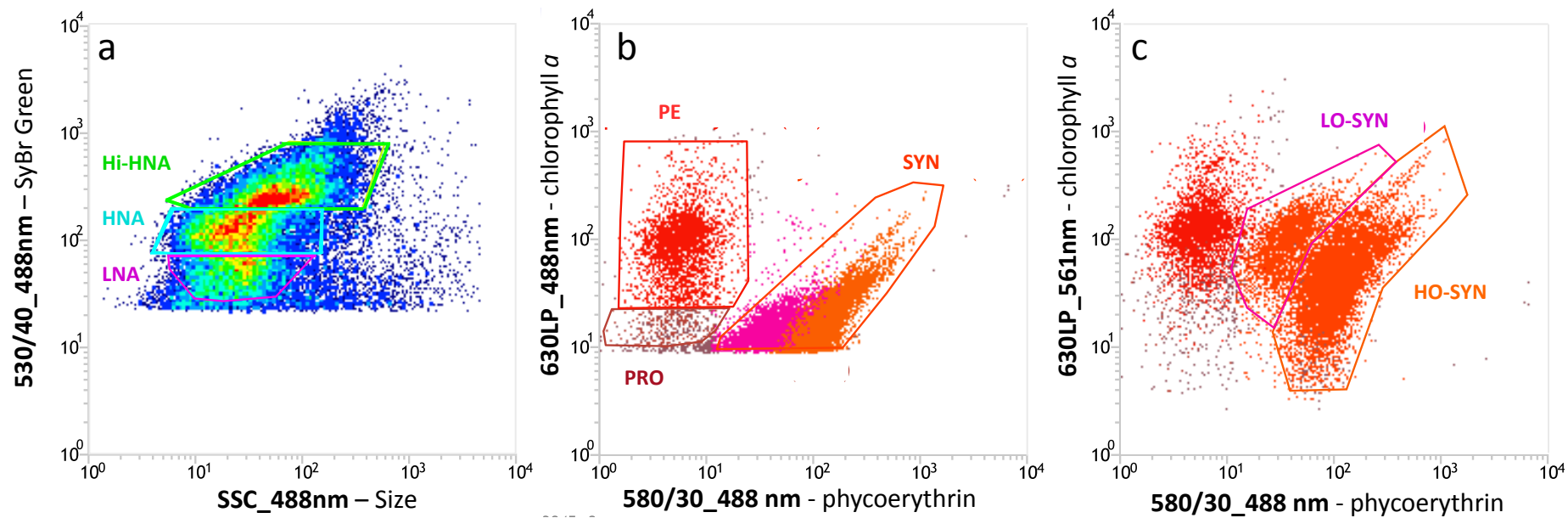


Fig. 2

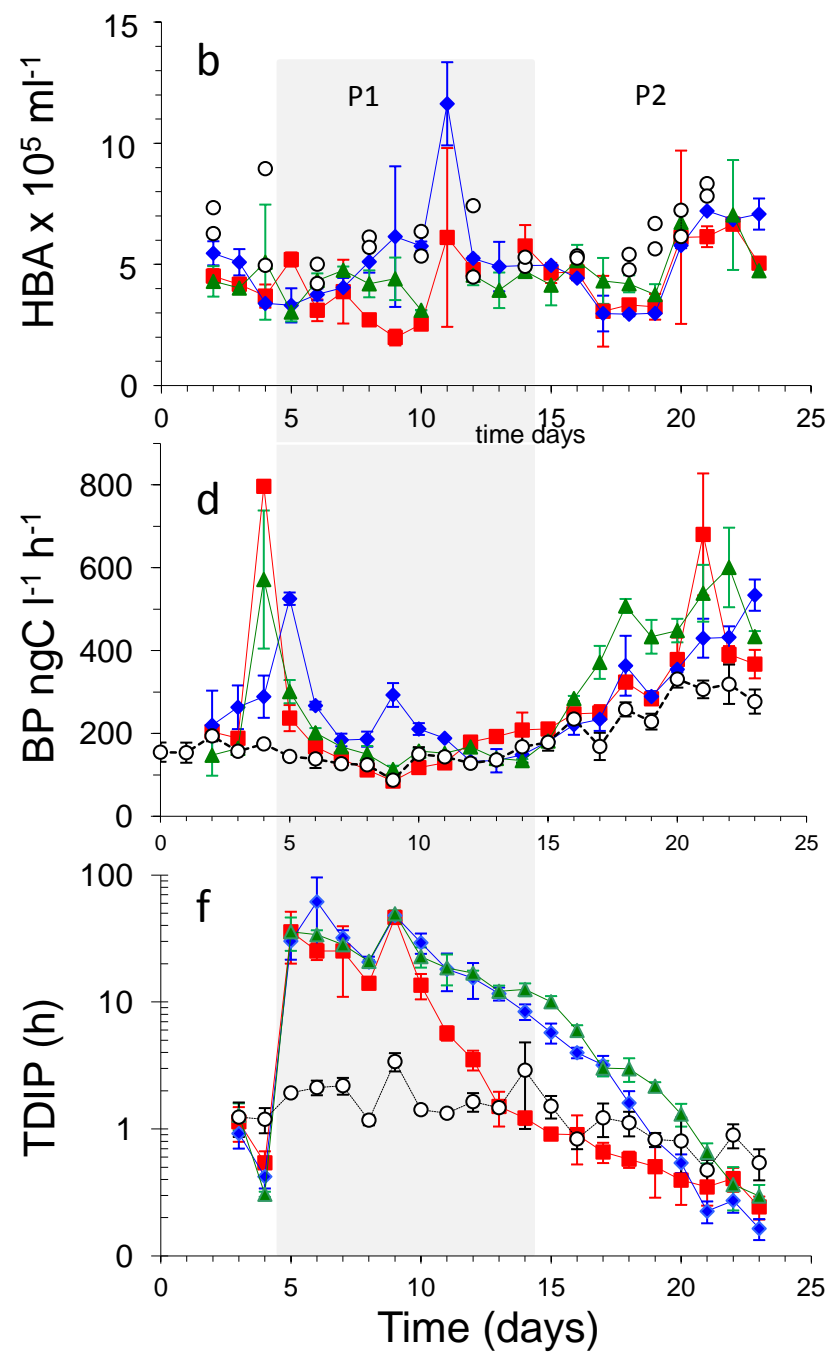
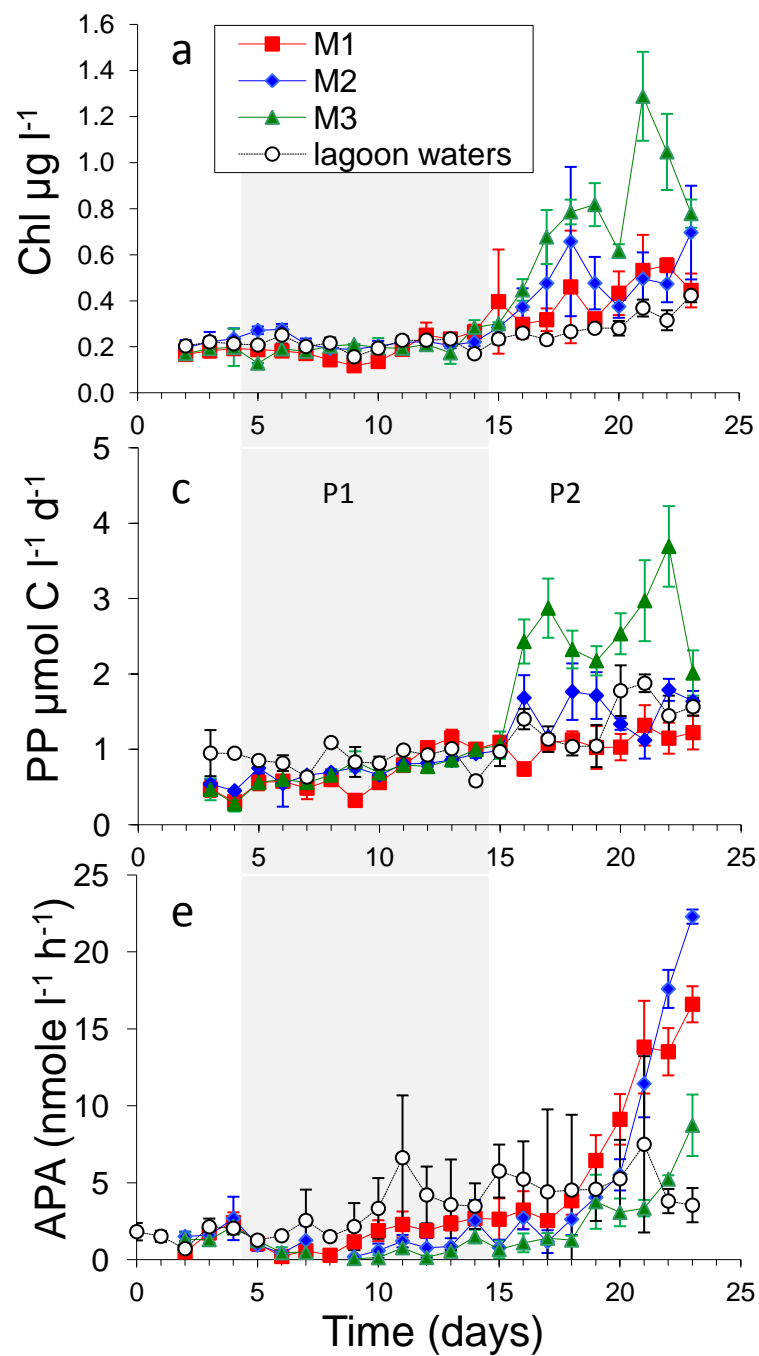


Fig. 3

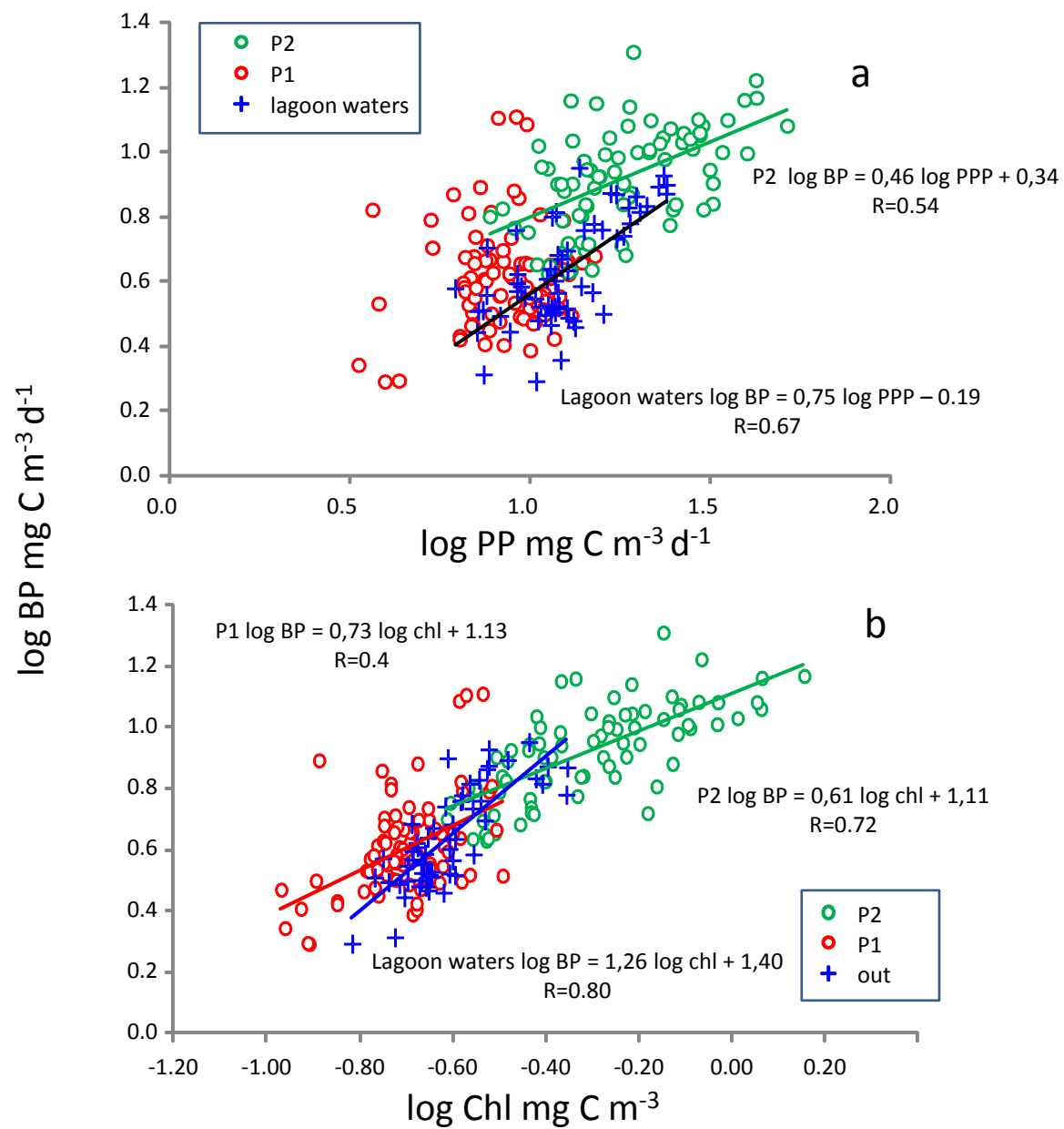


Fig. 4

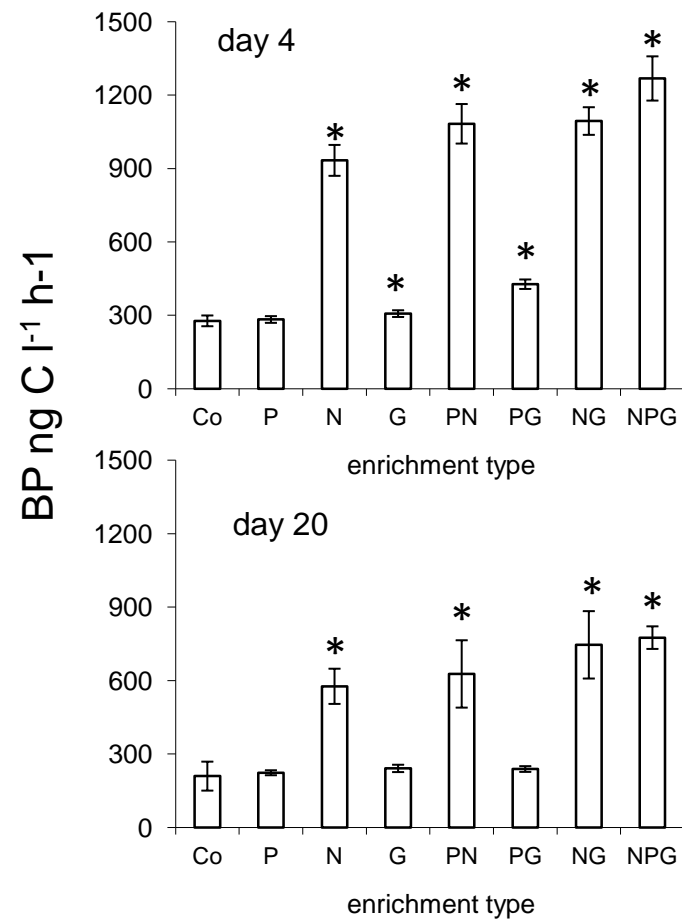


Fig. 5

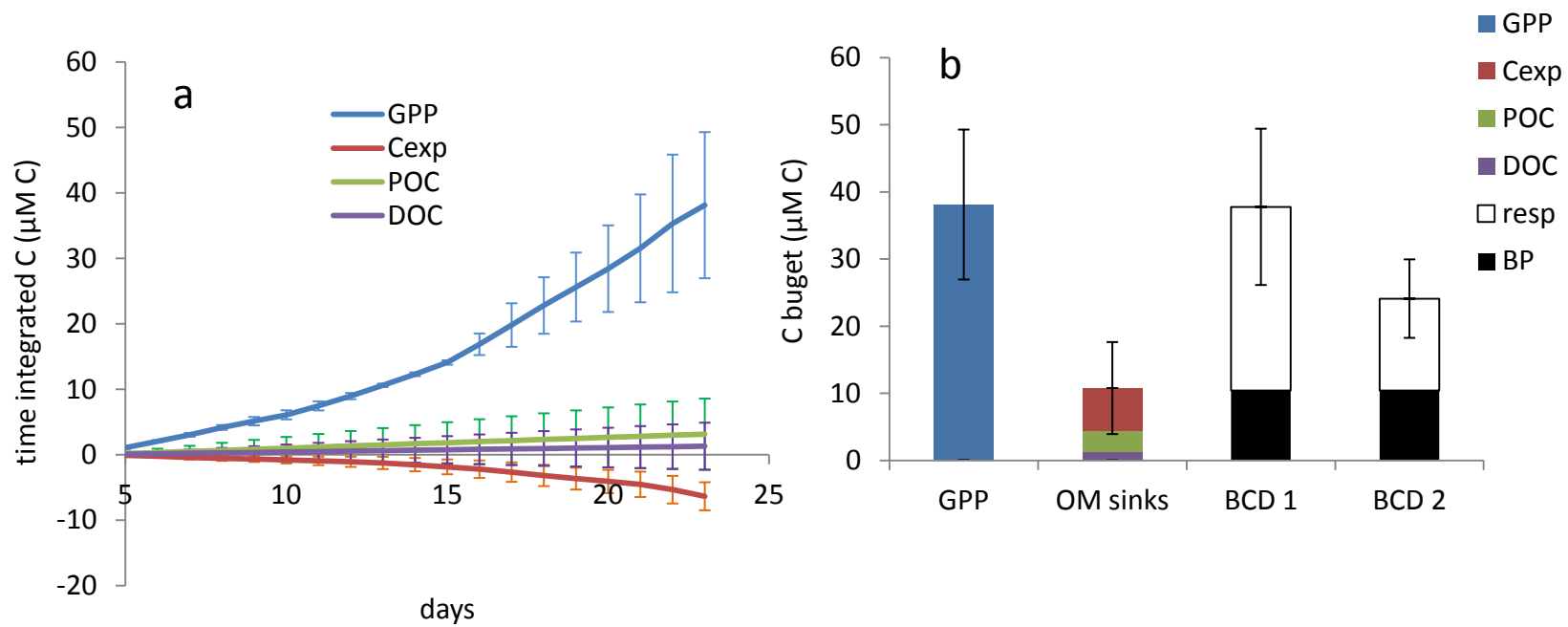


Fig. 6



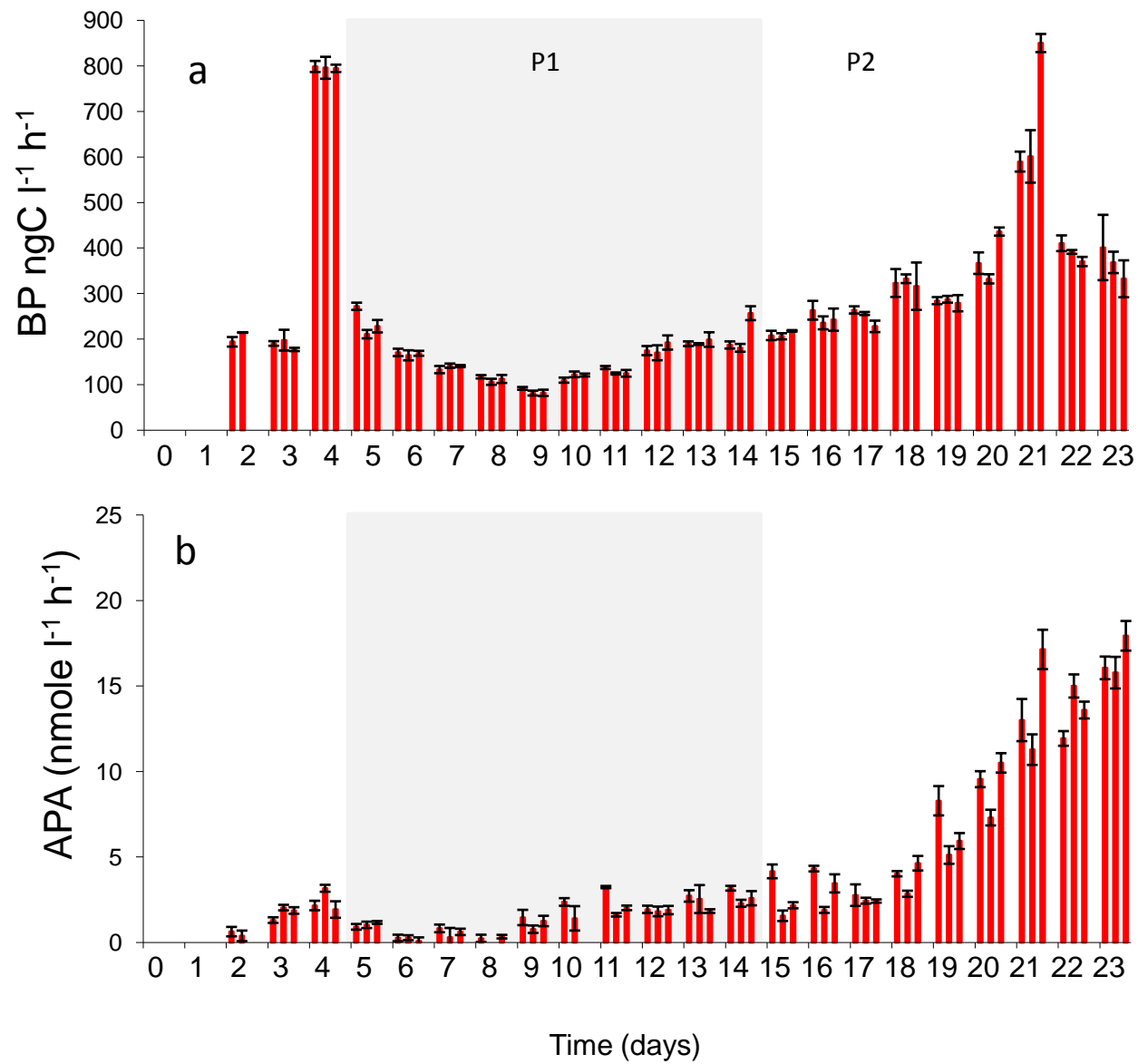


Fig. S1