Diel rhythmicity in amino acid uptake by *Prochlorococcus*

Isabelle Mary[1], Laurence Garczarek[2], Glen A. Tarran[3], Christian Kolowrat[2], Matthew J. Terry[4], David J. Scanlan[5], Peter H. Burkill[1,6,7], Mikhail V. Zubkov[1*]

[1] National Oceanography Centre, Southampton, SO14 3ZH, United Kingdom

[2] Université Pierre et Marie Curie et CNRS (UMR7144), Station Biologique, 29282 Roscoff, France

[3] Plymouth Marine Laboratory, Plymouth, PL1 3DH, United Kingdom

[4] School of Biological Sciences, University of Southampton, Southampton SO16 7PX, United Kingdom

[5] Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, United Kingdom

[6] Sir Alister Hardy Foundation for Ocean Science, Plymouth PL1 2PB, United Kingdom

[7] Marine Institute, University of Plymouth, Plymouth PL4 8AA, United Kingdom

* Corresponding author
Mailing address:
National Oceanography Centre
European Way
Southampton, SO17 3ZH
United Kingdom
Telephone: +44 (0)23 80596335
Fax: +44 (0)23 80596247
E-mail: mvz@noc.soton.ac.uk

Running title: **Diel rhythms in *Prochlorococcus* amino acid uptake**

Summary

The marine cyanobacterium *Prochlorococcus*, the most abundant phototrophic organism on Earth, numerically dominates the phytoplankton in nitrogen (N)-depleted oceanic gyres. Alongside inorganic N sources such as nitrite and ammonium, natural populations of this genus also acquire organic N, specifically amino acids. Here, we investigated using isotopic tracer and flow cytometric cell sorting techniques whether amino acid uptake by *Prochlorococcus* is subject to a diel rhythmicity, and if so, whether this was linked to a specific cell cycle stage. We observed, in contrast to diurnally similar methionine uptake rates by *Synechococcus* cells, obvious diurnal rhythms in methionine uptake by *Prochlorococcus* cells in the tropical Atlantic. These rhythms were confirmed using reproducible cyclostat experiments with a light synchronised axenic *Prochlorococcus* (PCC9511 strain) culture and ^{35}S-methionine and ^{3}H-leucine tracers. Cells acquired the tracers at lower rates around dawn and higher rates around dusk despite $>10^4$ times higher concentration of ammonium in the medium, presumably because amino acids can be directly incorporated into protein. Leucine uptake rates by cells in the $S+G_2$ cell cycle stage were consistently 2.2 times higher than those of cells at the G_1 stage. Furthermore, $S+G_2$ cells up-regulated amino acid uptake 3.5 times from dawn to dusk to boost protein synthesis prior to cell division. Because *Prochlorococcus* populations can account from 13% at midday, and up to 42% at dusk, of total microbial uptake of methionine and probably of other amino acids in N-depleted oceanic waters, this genus exerts diurnally variable, strong competitive pressure on other bacterioplankton populations.

Introduction

The marine cyanobacterium *Prochlorococcus* numerically dominates phytoplankton in the ocean's largest biomes – the nitrogen (N)-depleted oligotrophic gyres (Chisholm et al., 1988; Karl et al., 1997; Partensky et al., 1999). A high surface area to volume ratio gives *Prochlorococcus* a competitive advantage in nutrient uptake over eukaryotic algae (Chisholm, 1992) suggesting that the genus largely competes with heterotrophic bacteria for N. Experimental studies of *Prochlorococcus* strains (Moore et al., 2002) and bioinformatic analysis of their genomes (García-Fernández et al., 2004) has shown that *Prochlorococcus* predominantly uses reduced forms of inorganic N (ammonium) to meet its N demand. Whilst some strains can assimilate nitrite, nitrate seems unable to support growth although the latter is recently contested by field data (Casey et al., 2007). However, evidence for significant uptake of amino acids by *Prochlorococcus* has also recently been demonstrated, largely from studying oceanic populations (Zubkov et al., 2003; Zubkov et al., 2004). It seems ecologically rational for *Prochlorococcus* to use organic as well as inorganic sources of N, and amino acids could be preferred to ammonium, because the former can be directly incorporated into protein.

In oceanic surface waters sunlight provides energy for *Prochlorococcus* CO_2 fixation (Li, 1994) and also enhances uptake of amino acids at low ambient concentrations (Mary et al. 2008). Ecologically, the latter would be an efficient way of utilising an abundant light energy supply for amino acid acquisition in a N-depleted environment. It is already known that diel oscillations of sunlight synchronise the *Prochlorococcus* cell cycle (Vaulot et al., 1995) and hence it might be expected that amino acid uptake by *Prochlorococcus* cells could also vary diurnally, regulated either directly by light or indirectly by the cell cycle stage. A circadian

clock has been shown to regulate amino acid uptake in freshwater *Synechococcus* (Chen et al., 1991), and amino acid transport in *Prochlorococcus* cells could also be regulated by a similar mechanism. Despite the presence of some circadian clock genes in *Prochlorococcus* genomes (Rocap et al., 2003), and evidence for rudimentary clock function (Holtzendorff et al., in revision), the mechanism of regulation of this residual clock remains to be determined.

In order to test these ideas we conducted laboratory culture and field experiments using a combination of isotopic tracer and flow cytometric cell sorting techniques. Hence, the major objectives of this study were i) to assess diurnal changes in rates of amino acid uptake by *Prochlorococcus* and *Synechococcus* cells in oceanic surface waters; ii) to compare the specificity of amino acid transport in different *Prochlorococcus* strains; iii) to assess diurnal changes in amino acid uptake by a cultured *Prochlorococcus* strain; and iv) to determine the relationship between amino acid uptake and *Prochlorococcus* cell cycle stage.

Results and Discussion

Initial field experiments

Ambient methionine concentrations and its rate of uptake by bacterioplankton were bioassayed in the tropical Atlantic Ocean (Fig. 1a), using a ^{35}S-methionine precursor. Methionine uptake by natural cyanobacterial cells was determined using flow cytometric sorting of cells preloaded with the tracer. Compared to *Synechococcus* cells, a pronounced diel periodicity in amino acid uptake by *Prochlorococcus* cells was observed during the three days of this study (Fig. 1b). The *Prochlorococcus* population contributed 13±6% and 42±7% to the total bacterioplankton uptake of methionine at midday and after dusk, respectively (Fig. 1c).

The above field observations were conducted on a moving ship and the observed diel variability should have a spatial, potentially latitudinal, component. Even if the observations were made from a stationary ship, spatial variability could not be completely ruled out because of the ship's drift. Furthermore, competitive and trophic interactions with other microbial populations could also alter the physiological state of *Prochlorococcus* cells. Consequently, these field observations are insufficient to ascertain the diel rhythm of amino acid uptake by *Prochlorococcus*, or to explain the mechanism of its regulation. Therefore, we set out to reproduce the phenomenon in the laboratory using a *Prochlorococcus* culture.

Laboratory experiments with a light synchronised Prochlorococcus *culture*

The *Prochlorococcus* PCC 9511 strain was grown as an axenic culture in a cyclostat with a modulated light regime, which mimicked the natural light/dark cycle in equatorial surface waters (Fig. 2a). Because field studies have already shown differential uptake of amino acids

by dominant bacterioplankton groups (Mary et al. 2008), a mixture of ^{35}S-methionine and ^{3}H-leucine was used to examine any differences in the regulation of uptake of these two amino acids by *Prochlorococcus*. Uptake of both amino acids by cells in two independent experiments showed similar, reproducible diel rhythms with higher uptake before or at dusk and lower uptake around dawn (Fig. 2b,c), confirming the earlier field observations (Fig. 1b).

The concentration of leucine and methionine in the medium, bioassayed using an axenic *Prochlorococcus* culture, were 0.81±0.17 and 0.38 ± 0.05 nM, respectively. However, despite these very low concentrations of amino acids compared to the high concentration of ammonium (0.1 mM) in the medium, *Prochlorococcus* seemed to prefer to take up amino acids. The continuous cyclostat culture was constantly diluted with fresh medium in order to stabilise cell numbers, and, hence, amino acid concentrations in the culture were also kept constant. Thus, the observed similar rhythms of amino acid uptake reflected diurnally varying cell need in both amino acids for protein synthesis.

Flow cytometric analyses revealed that the cell growth cycle in cyclostat cultures was highly synchronised with reproducible daily alternations of cell cycle phases throughout the experiment (Fig. 3a). Approximately 95% of *Prochlorococcus* cells were at the G_1 stage, i.e. had a single chromosome copy according to SYBR Green I DNA staining (Marie et al., 1997), and from dawn to midday and this steadily decreased to 30% at dusk. At dusk approximately 70% of cells entered the S stage of synthesis of the second chromosome copy. Within three hours after the simulated sunset the S stage cells completed their chromosome synthesis and transited to the G_2 stage of the cell cycle, when two chromosome copies could be detected in cells, before cells divided during the night, returning to the G_1 stage and completing the cell growth cycle. The diel maximum of amino acid uptake preceded the

maximum percentage of cells in the $S+G_2$ stages by 2-3 hours, while being in anti-phase with the percentage of G_1 cells (Fig. 2b, 2c, 3a). Hence, a relationship between the cell cycle stage and cell amino acid uptake rate can be drawn.

In order to quantify cellular amino acid uptake at different stages of the growth cycle, the G_1 cells and $S+G_2$ cells were flow cytometrically sorted from subsamples, collected during one complete diel cycle and incubated with ^{3}H-leucine tracer for 3 min before being fixed and stained. $S+G_2$ cells took, on average, 2.2±0.7 times more leucine than G_1 stage cells (Fig. 3b). This observation is consistent with work performed on *Vibrio* cells, where 1.5 times higher methionine uptake by $S+G_2$ stage *Vibrio* cells was detected compared to G_1 cells (Zubkov and Sleigh, 2005).

$S+G_2$ cells showed clear diel variations in cellular leucine uptake with a maximum before dusk, when >95% of these cells were at the S stage, and a minimum after midnight, when only 10% of these cells were at the S stage and the remaining 90% of cells were at the G_2 stage (Fig. 3b). Therefore, the increase in uptake of amino acids by *Prochlorococcus* cells coincided with cells being at the S stage of their cell growth cycle. $S+G_2$ cells are bigger than G_1 cells because, before division, cell size and hence membrane surface area, increases up to 2 fold (Jacquet et al., 2001) and it might be excepted that these cells should possess more amino acid transporters. However, the diel increase in amino acid uptake by $S+G_2$ cells was up to 3.5 fold, which cannot be entirely explained by the increase in cell size. G_1 cells showed a similar diel 3.5 fold increase in amino acid transport, although it was more variable than that observed in $S+G_2$ cells (Fig. 3b). This variability in amino acid uptake by G_1 cells at 12:00 suggests that protein synthesis and DNA synthesis are not entirely synchronised in a growing

cell, consequently, the amount of cellular DNA is not a perfect indicator of the rate of cell protein synthesis (Fig. 3b).

Amino acid transporter specificity in different Prochlorococcus *strains*

Because previous bioinformatic analyses of genomic data revealed differences in the amino acid transporter gene complement of *Prochlorococcus* strains (Rocap et al., 2003, Kettler et al., 2007), we investigated leucine transport specificity using strains representing three main *Prochlorococcus* ecotypes, i.e. strain SS120 – a low-light (LL) II ecotype, strain PCC9511 – a high-light (HL) I ecotype and strain GP2 – a HLII ecotype (Rocap et al., 2002). In order to confirm that cells actually took up leucine rather than merely adsorbed it to the cell surface, leucine uptake was measured using a time series (up to nine sampling points between 1-60 min). Uptake of 0.5 nM leucine by the three strains was reproducibly linear in duplicated experiments (data not shown). Slopes of linear regressions were statistically significant at the 99.9% confidence level (the intercepts were statistically insignificant) and the corresponding regression coefficients were >0.985. Pulse-chase experiments showed that linear uptake of ^{3}H-leucine by the three strains was effectively stopped after an addition (chase) of non-labelled leucine at a 3 orders of magnitude higher concentration (data not shown). Thus, the ^{3}H-leucine pulse remained virtually unchanged up to 12 hours after the chase was added.

In order to examine whether even in potentially non-axenic batch cultures most of the ^{3}H-leucine was taken up by the *Prochlorococcus*, we flow-sorted *Prochlorococcus* PCC9511 cells and compared the uptake rates of sorted cells during a time series (1, 15, 30 and 60 min) with corresponding uptake rates of unsorted cells, calculated by dividing the amount of radioactivity in the filtered particulate material by the number of *Prochlorococcus* cells in the filtered sample. The correlation between the two sets of measurements was 99.2%; the

difference between the sets was statistically insignificant (data not shown). Therefore, in experimental batch cultures the bulk of ^{3}H-leucine in particulate material was that taken up by *Prochlorococcus* cells.

The specificity of leucine transport by the three *Prochlorococcus* strains was assessed using different non-labelled amino acids as ^{3}H-leucine competitors: leucine, methionine, tyrosine and leucine-methionine and leucine-tyrosine dipeptides, which are thought to block transporters more effectively than monomers (Kirchman and Hodson, 1984). Non-labelled leucine reduced ^{3}H-leucine uptake >99%, being the most effective competitor (Table I). Methionine inhibited ^{3}H-leucine uptake by the PCC9511 strain more effectively than ^{3}H-leucine uptake by the SS120 or GP2 strains. Tyrosine was a less effective inhibitor than methionine, while dipeptides were even less effective inhibitors than tyrosine (Table I). These results suggest that the selectivity of leucine transport in different *Prochlorococcus* strains is not absolute and that permeases which transport leucine may also transport other amino acids and dipeptides. Even so, some selectivity for branched chain amino acids, e.g. leucine and methionine, compared to aromatic amino acids e.g. tyrosine or a dipeptide which contained tyrosine, is apparent. Certainly, in freshwater cyanobacteria specific neutral, basic and acidic amino acid transporters have been described (Montesinos et al., 1995, 1997; Pernil et al., 2008). The neutral amino acid permease of *Anabaena* sp. strain PCC7120 is, however, capable of transporting several amino acid ‘types’ including hydrophobic (e.g. methionine), aliphatic (e.g. leucine), and aromatic (e.g. tyrosine; Picossi et al., 2005), perhaps hinting at differences in amino acid transport specificity between freshwater and marine cyanobacterial strains.

Concluding remarks

In this study, laboratory experiments (Fig. 2b,c) have confirmed the initial field 'evidence' (Fig. 1b) for diel rhythmicity in methionine uptake by *Prochlorococcus*. Cells at the S stage of the cell cycle showed the highest rates of amino acid uptake, presumably because at this stage proteins for a future daughter cell are synthesised. In order to maintain this synthesis, cells transport dissolved amino acids in preference to ammonium, likely due to the fact that amino acids can be directly used as protein building blocks, bypassing amino acid synthesis required for assimilation of ammonium. The rate of cellular amino acid transport is increased towards the end of the daytime before dusk, before or at the time when *Prochlorococcus* cells synthesise the second copy of their chromosome (Fig. 3).

The ecological significance of diel fluctuations in amino acid uptake observed in tropical surface waters and in culture is not trivial. The *Prochlorococcus* population consumed 13% and 42% of all methionine taken up by bacterioplankton at midday and after dusk, respectively (Fig. 1c). Because *Prochlorococcus* consumes a significant proportion of methionine, leucine and probably other amino acids in N-depleted oceanic waters, the *Prochlorococcus* population should exert a diurnally variable, strong competitive pressure on other bacterioplankton populations. Indeed, some of these heterotrophic populations 'respond' by enhancing their amino acid uptake using sunlight (Mary et al., 2008). Hence, our findings demonstrate a complexity in amino acid turnover in those oceanic regions where *Prochlorococcus* occurs, and suggest it is unsuitable to utilise microbial uptake rates of amino acids to estimate 'production' by heterotrophic bacterioplankton.

Experimental procedures

Sampling site

Field work was performed on board the Royal Research Ship James Clark Ross (Cruise No. JR91) in the Atlantic Ocean during September – October 2003, using a meridional transect of 7 stations from 17.1^{o}N and 19^{o}W to 2.9^{o}N and 24.1°W (Fig. 1a). At midday, seawater was collected with a rosette of 20 L Niskin bottles mounted on a conductivity-temperature-depth (CTD) profiler from 6 m and in the evening seawater was collected from the ship's glass lined, clean seawater supply system from 6 m. Cyanobacterial abundance, bioassayed methionine concentration, total microbial methionine uptake rates, and absolute rates of methionine uptake by *Prochlorococcus*, *Synechococcus* and flow sorted bacterioplankton cells were determined in each collected sample using previously published methods (Zubkov et al., 2004; Zubkov and Tarran, 2005; Mary et al. 2008). *Synechococcus* cells were only flow sorted from the first four samples, where their abundance was sufficiently high to flow sort without sample concentration.

Culture conditions

Non-axenic *Prochlorococcus* batch cultures of strains PCC9511, GP2 and SS120 strains were routinely maintained at 20°C and at 25 or 12 μmol m^{-2} s^{-1} white light in PCR-S11 medium (Rippka et al., 2000), using 0.1 mM ammonium as the sole source of N. *Prochlorococcus* were transferred into ammonium depleted medium and pre-incubated for one week before conducting leucine uptake kinetic, pulse-chase or amino acid competitor transport experiments.

Amino-acid uptake and uptake competition measurements

Prochlorococcus cultures (10^5 cells per ml), placed into crystal clear microcentrifuge tubes, were inoculated with L-[^{35}S]methionine (specific activity >37 TBq/mmol) at a final concentration of 0.5 nM or with [4,5-^{3}H]leucine (specific activity 6 TBq/mmol) at a final concentration of 0.8 nM. Uptake kinetics were followed using a time series of up to 9 time points during 60 min incubation. At each time point, duplicate tubes, containing 1.8 mL sample, were fixed with paraformaldehyde (PFA) at 1% (w/v) final concentration. Particulate material was collected onto 0.2 μm pore size polycarbonate filters by filtration and washed with deionised water. Radioactivity retained on filters was accurately measured using an ultra-low level liquid scintillation counter (1220 Quantulus, Wallac, Finland). In order to assess amino acid uptake specificity, non-labelled leucine, methionine, tyrosine, leucine-methionine dipeptide or leucine-tyrosine dipeptide were added at concentrations 1000 times higher than ^{3}H-leucine at the beginning of the experiment (Table I). ^{3}H-leucine retention by *Prochlorococcus* was assessed by adding 1000 times higher concentration of non-labelled leucine as a chase after 1 hour pulse incubation with the tracer.

Cyclostat experiments

Duplicate 3 L cyclostat cultures of the axenic *Prochlorococcus* sp. strain PCC 9511 were acclimated in PCR S11 medium (Rippka et al., 2000) in 8 L quartz flasks, placed in a thermo-regulated bath at 21 ± 1°C and under a cycle of 12 h of light and 12 h of dark (L/D) (light from 6:00 to 18:00), mimicking the light conditions of the ocean surface layer at the equator. Details about the cyclostat setup and light systems were reported previously (Holtzendorff et al. in revision). Briefly, during the light periods, cells were illuminated by two symmetrical computer controlled banks of light bulbs (OSRAM DuluxL 55 W daylight) providing a modulated irradiance varying in a sinusoidal way from 0 to 998 μmol m^{-2} s^{-1} (Fig. 2a).

Cyclostat cultures were sampled during three consecutive photocycles. The culture was maintained in exponential growth at an average density of $1.3\pm0.3\times10^8$ cells mL^{-1} by continuous dilution during the complete sampling period. The cyclostat culture was sampled every hour to determine the cell concentration and cell cycle stage using flow cytometric cell cycle analysis (Marie et al., 1997). In order to minimise physiological changes of cells during sample manipulations, short term amino acid uptake experiments were conducted every 3 hours during the light period and every 4 hours during the dark period. Amino acid uptake of *Prochlorococcus* cells was assessed as described above. However, because of the high cell concentration, ^{3}H-leucine uptake was measured after 1, 2 and 3 min incubations. In addition, *Prochlorococcus* cells, preloaded with ^{3}H-leucine, were preserved with 1% PFA, flash frozen in liquid nitrogen and stored at -80^{o}C for sorting cells at G_1 and $S+G_2$ cell cycle stages.

Flow cytometric cell enumeration and cell cycle analysis

Absolute concentrations of *Prochlorococcus* in culture were determined by flow cytometry (FACSort, BD Biosciences, Oxford, UK) after fixing samples with 1% PFA. Cells were stained with SYBR Green I DNA-specific dye as described previously (Marie et al., 1997). A yellow-green 0.5 μm bead standard (Fluoresbrite Microparticles, Polysciences,Warrington, USA) was used in all analyses to determine absolute cell concentrations. The CellQuest software (Becton Dickinson Biosciences, Oxford, UK) was used for operating the flow cytometer and for data analyses. Stained *Prochlorococcus* cells at different stages of their cell cycle could be differentiated and flow sorted to compare their rates of tracer uptake.

Flow cytometric sorting of radioactively labelled cells

For flow cytometric sorting, fixed samples were stored at 2°C for 1–2 days, or frozen at -80^{o}C for longer storage. Cells were stained with SYBR Green I, and target cells were flow sorted

using a FACSort instrument (BD Biosciences, Oxford, UK) in single-cell sort mode, sorting at a rate of 10–250 particles s^{-1}. Sorted cells were collected onto 0.2 μm pore size polycarbonate filters, washed with deionised water and radio-assayed. Three samples containing proportional numbers of cells were sorted, and the mean cellular tracer uptake was determined as the slope of the linear regression of radioactivity against the number of sorted cells. Sorting purity was assessed routinely by budgeting radioactivity of flow sorted cells from different clusters (Zubkov and Tarran, 2005) as well as by sorting one type of beads from a mixture of two 0.5 μm beads (Zubkov et al., 2007). The sorted material was 99% enriched with the target particles; the sorted particle recovery was >95%.

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Table I. Inhibition of ^{3}H-Leucine uptake by three strains of *Prochlorococcus* by saturating samples containing *Prochlorococcus* and 0.8 nM ^{3}H-Leucine with 1000 times higher concentration of either: unlabelled Leu, methionine (Met), tyrosine (Tyr), Leu-Met and Leu-Tyr dipeptides. Values are the percentage reduction in the ^{3}H-Leucine uptake compared to controls containing just *Prochlorococcus* and ^{3}H-Leucine.

	Prochlorococcus strain (ecotype)		
	PCC9511	GP2	SS120
Molecule	(HLI)	(HLII)	(LLII)
Leu	99.6	99.6	99.2
Met	91.9 ± 6.3	65.1 ± 5.5	88.2 ± 4.3
Tyr	76.7 ± 4.2	50.7 ± 3.8	68.7 ± 3.8
Leu-Met	48.1 ± 4.7	58.8 ± 4.5	57.4 ± 4.0
Leu-Tyr	28.6 ± 5.1	26.9 ± 4.9	26.1 ± 4.2

All percentages of inhibition were significantly ($P < 0.0001$) different from the control.

Figure legends

Fig. 1. Diel variability of methionine (Met) uptake by *Prochlorococcus* (*Pro*) and *Synechococcus* (*Syn*) cells in surface waters of the tropical Atlantic Ocean.

a. Schematic representation of the sampled area on the meridional transect cruise during October 2003. Solid line shows the cruise track. An arrow indicates the direction of the transect.

b. Diel variations in methionine uptake by flow sorted cells. Symbols show mean values and error bars indicate single standard errors of measurements.

c. Diel variations in the contribution of the *Prochlorococcus* population to total bacterioplankton (Bpl) uptake of methionine.

Fig. 2. Diel variations in leucine (Leu) and methionine (Met) uptake by *Prochlorococcus* sp. PCC9511 in a light synchronised axenic culture.

a. Simulation of light irradiance conditions typical for equatorial surface waters.

b. and c. Diel variation in cellular amino acid uptake in two independent continuous culture cyclostat experiments.

Fig. 3. Diel changes in leucine uptake at different cell cycle stages of a light synchronised axenic *Prochlorococcus* culture.

a. Percentages of cells at the G_1 and $S+G_2$ stages of the cell cycle. (See text for details).

b. Diel changes in leucine uptake by cells at the G_1 and $S+G_2$ stages and comparative changes in the percentage of cells at the S stage in the $S+G_2$ group of cells. Symbols show mean values and error bars indicate standard errors of measurements.

Fig. 1.

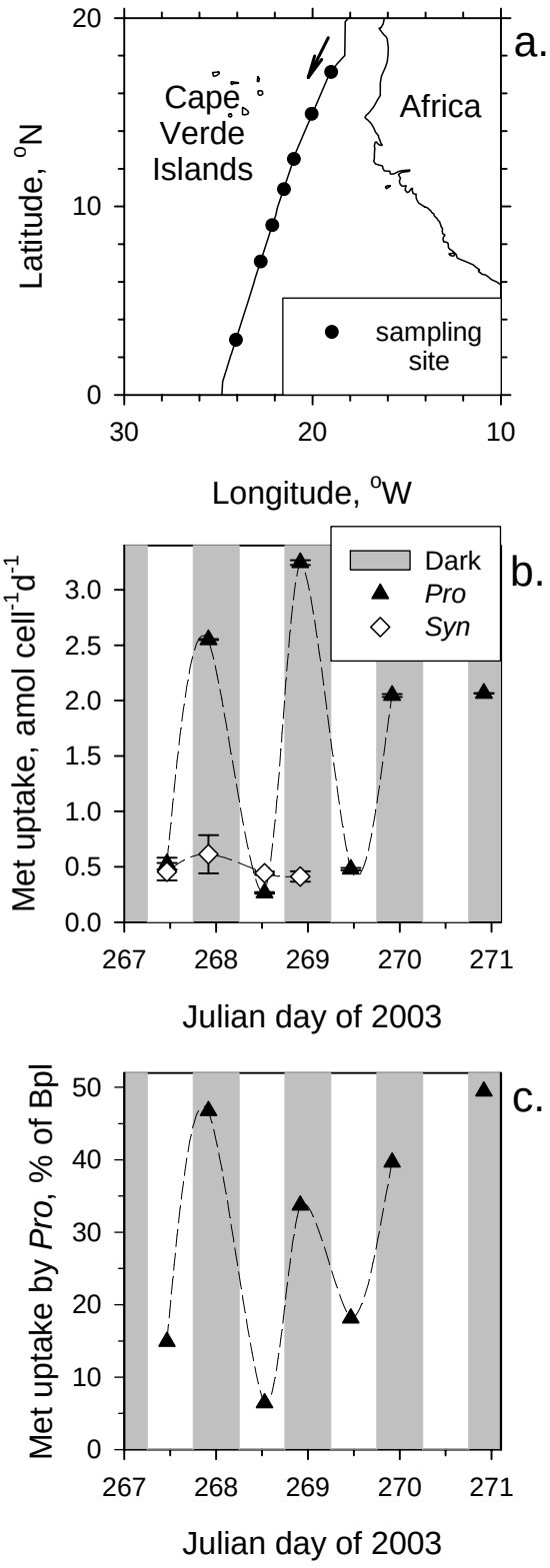

a.
Cape Verde Islands
Africa
sampling site
Latitude, °N
Longitude, °W
b.
Dark
Pro
Syn
Met uptake, amol cell-1d-1
Julian day of 2003
c.
Met uptake by Pro, % of Bpl
Julian day of 2003

Fig. 2.

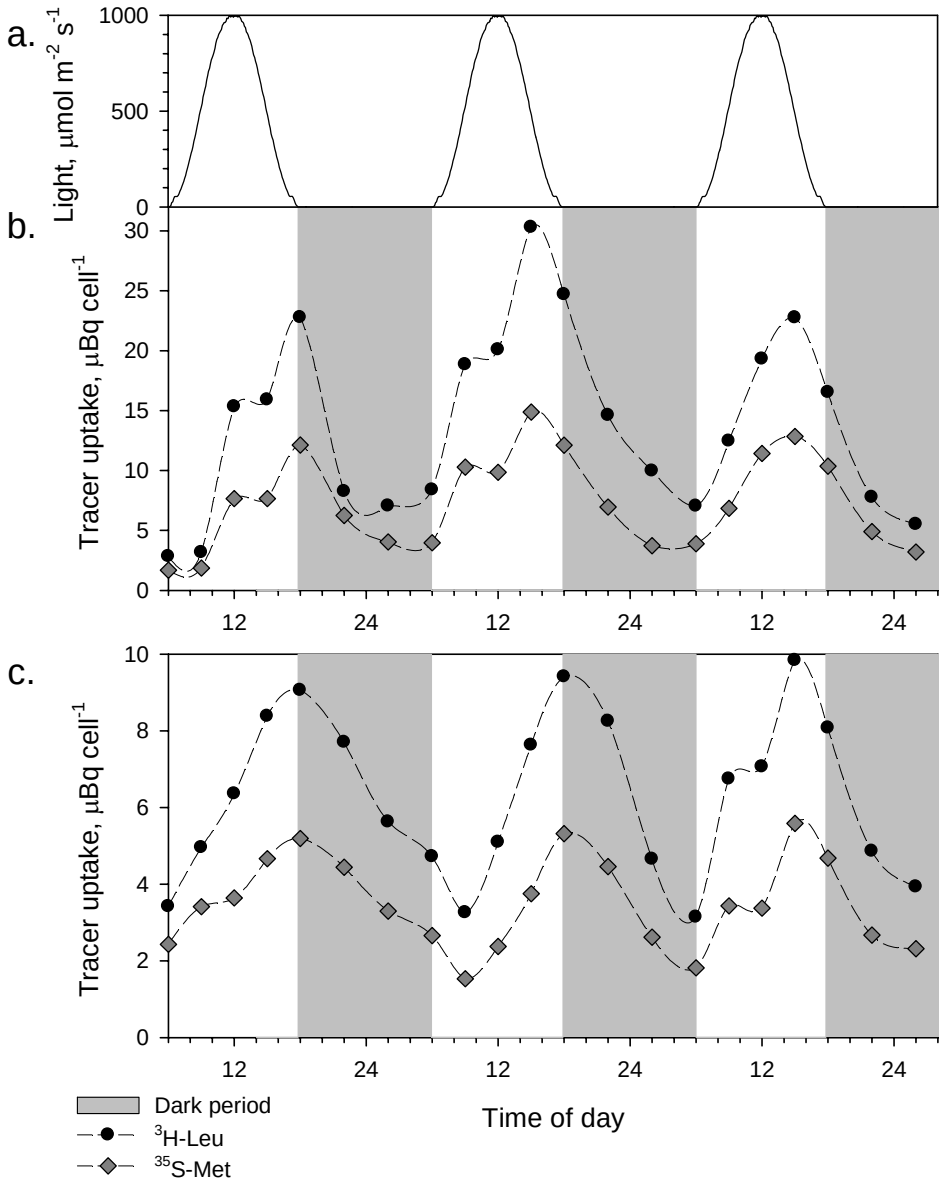
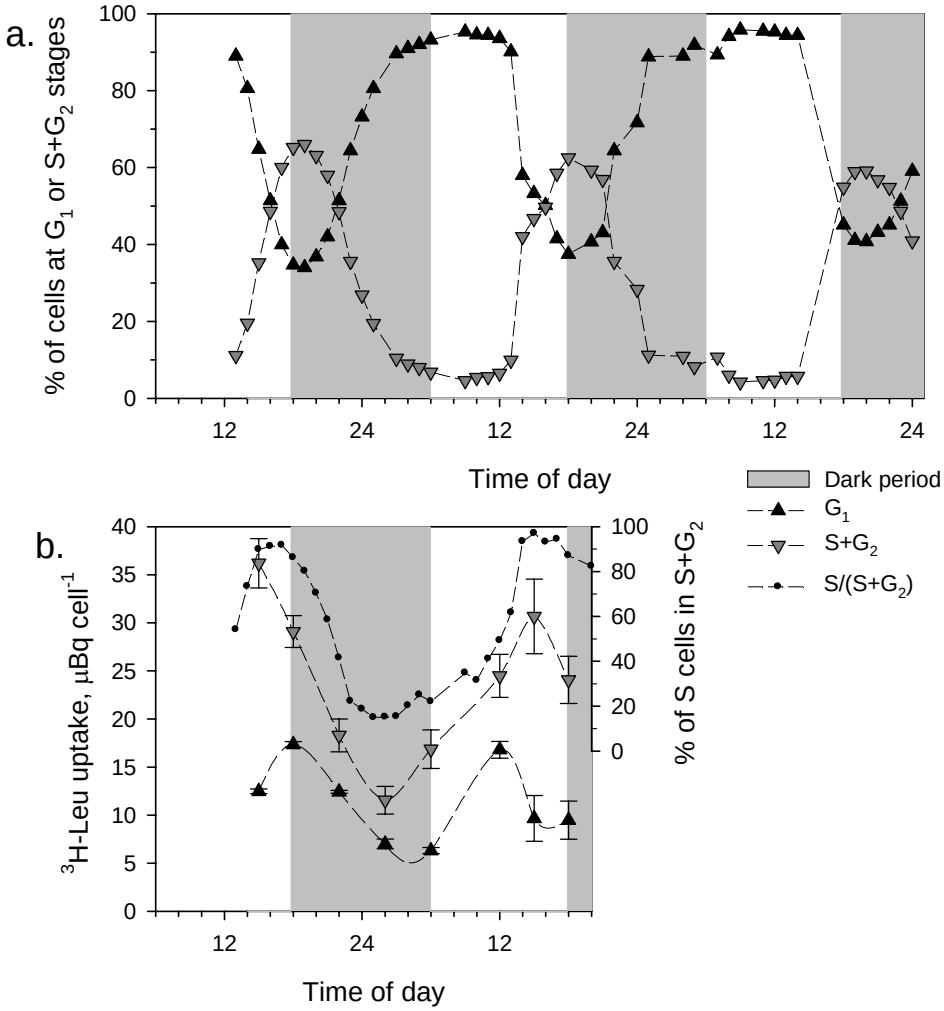

a.
Light, µmol m-2 s-1
1000
500
0
b.
Tracer uptake, µBq cell-1
30
25
20
15
10
5
0
12
24
12
24
12
24
c.
Tracer uptake, µBq cell-1
10
8
6
4
2
0
12
24
12
24
12
24
Dark period
3H-Leu
35S-Met
Time of day

Fig. 3.


a.
% of cells at G1 or S+G2 stages
Time of day
Dark period
G1
S+G2
S/(S+G2)
b.
3H-Leu uptake, μBq cell-1
% of S cells in S+G2
Time of day