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RELEASE OF DISSOLVED ORGANIC MATTER BY *PROCHLOROCOCCUS*

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PROCHLOROCOCCUS
DOC
EXCRETION
CARBOXYLIC ACIDS
NUTRIENT LIMITATION

ABSTRACT. – Phytoplankton release a variable fraction (0 to > 80%) of the photosynthetically fixed inorganic carbon as extracellular dissolved organic compounds. Despite this wide range and the potential effects on carbon fluxes and food webs, the knowledge on how the environment and phytoplankton species involved influence this process is incomplete. Notably, there are no estimates for release of dissolved organic carbon by *Prochlorococcus*, a marine cyanobacterium estimated to be the numerically dominant oxygenic phototroph in the oceans. Here we report extracellular release of dissolved organic compounds from two axenic *Prochlorococcus* strains representing different ecotypes (MED4 and MIT9312) cultured under nutrient replete and phosphorus limited conditions. Independent assays based on ¹⁴C-bicarbonate tracers and analyses of particulate and dissolved organic carbon suggest that the release of dissolved organic carbon ranged from 9 to 24% of the total assimilated inorganic carbon with slightly lower values for phosphorus limited cultures. Between 4 and 20% of the released organic matter consisted of low molecular weight carboxylic acids, compounds known to be highly labile substrates for heterotrophic bacteria. In oligotrophic oceans where *Prochlorococcus* is a dominant contributor to primary production and input of terrigenous organic matter is negligible, this process is likely a significant contributor to the pool of organic substrates available for microbial heterotrophs.

PROCHLOROCOCCUS
COD
EXCRETION
ACIDES CARBOXYLIQUES
OLIGOTROPHIE

RÉSUMÉ. – Les cellules phytoplanctoniques vivantes libèrent une fraction variable du carbone inorganique fixé lors de la photosynthèse sous forme de composés organiques dissous extracellulaires. Les pourcentages rapportés dans la littérature varient de 0 % à 80 %. Malgré cette grande étendue et des effets potentiellement importants sur les flux de carbone et les réseaux trophiques, l'état actuel des connaissances sur le rôle des variables environnementales et des espèces phytoplanctoniques de ce processus demeure incomplet. En particulier, il n'existe aucune estimation de la libération de carbone organique dissous par *Prochlorococcus*, une cyanobactérie marine considérée comme l'organisme oxygénique phototrophe dominant des océans. Nous rapportons des taux de libération extracellulaire de composés organiques dissous provenant de deux souches axéniques de *Prochlorococcus*, représentant des écotypes différents (MED4 et MIT9312), cultivées sous des conditions d'abondance en nutriments et limitées pour le phosphore. Des techniques indépendantes basées sur le traçage de ¹⁴C-bicarbonate et des analyses chimiques du carbone organique particulaire et dissous suggèrent que la libération de carbone organique dissous dans le milieu environnant varie entre 9 et 24 % du carbone inorganique total assimilé. Cette libération est légèrement inférieure pour les cultures limitées par le phosphore. Entre 4 et 20 % de la matière organique libérée est composée d'acides carboxyliques de faibles poids moléculaires, tous des composés connus comme étant hautement labiles pour les bactéries hétérotrophes. Dans les océans oligotrophes où *Prochlorococcus* domine la production primaire, ce processus pourrait contribuer de façon majeure au réservoir de substrats organiques disponibles pour les hétérotrophes microbiens.

INTRODUCTION

Prochlorococcus are small (0.4–0.8 μm diameter) unicellular marine cyanobacteria with a carbon content that ranges from 45 to 94 fg C cell⁻¹ (Partensky *et al.* 1999, Bertilsson *et al.* 2003). They are geographically restricted to tropical and subtropical oceans (e.g. 40°S to 40°N), but the high abundance of *Prochlorococcus* that extends throughout the euphotic zone in these regions makes them the most numerically abundant oxygenic phototroph in the oceans (Partensky *et al.* 1999). Hence, despite their small size, they can make up nearly half of the total microbial biomass (Campbell *et al.* 1994, Karl & Dobbs 1998). Growth rate estimates based on *in situ* cell cycle analyses suggest that depth-integrated growth rates of *Prochlorococcus* vary from 0.36 to 0.63 day⁻¹, with maximum rates at discrete depths of about 1 day⁻¹ in the upper part of the water column (Vaulot *et al.* 1995, Liu *et al.* 1999). Since growth appears to be balanced by loss processes, e.g. population abundances are rather stable over time and space (Partensky *et al.* 1999), the flux of carbon, nutrients and energy through *Prochlorococcus* can be expected to strongly influence oceanic aquatic food webs.

It has long been recognized that a significant portion of the inorganic carbon fixed in actively growing photosynthetic algae is released as dissolved organic carbon (DOC) (Bertilsson & Jones 2003). This release of extracellular DOC appears to be universal, though highly variable, among different phytoplankton species (Fogg 1983). The reported range of percent extracellular release of total fixed inorganic carbon in marine waters range from 0 to 80% whereas axenic cultures of marine phytoplankton appear to be somewhat more restricted (2.5–37%; reviewed in Bertilsson & Jones 2003). There is some evidence that smaller phytoplankton cells release a larger fraction of their total fixed inorganic carbon as extracellular DOC (Malinsky-Rushansky & Legrand 1996) and nutrient availability has also been identified as a factor that may regulate the release of extracellular DOC with higher DOC release under nutrient limitation (Fogg 1983, Lancelot 1983, Obernosterer & Herndl 1995). Several marine field surveys have reported strong negative correlations between percent extracellular release and nutrient concentrations (Lancelot 1983) as well as total productivity (Andersson & Zeutschel 1970, Mague *et al.* 1980, Morán & Estrada 2002). Hence, phytoplankton in oligotrophic waters should release a higher percentage of their assimilated carbon as DOC. However, there are surveys where no such correlations are observed (Baines & Pace 1991). Still, these combined tendencies suggest that *Prochlorococcus*, a small cyanobacterium that thrives in oligotrophic oceans, is likely to release a substantial fraction of

the assimilated inorganic carbon as DOC. Currently, no estimates are available for the magnitude of this process despite its potentially large impact on food webs. However, a study of its close relative *Synechococcus* suggests that the percent extracellular release from this group may in fact be equal to or lower than that of other groups of marine phytoplankton (Biddanda & Benner 1997).

Here we investigate the production of extracellular DOC by *Prochlorococcus* using two of the few existing axenic *Prochlorococcus* isolates (MED4 and MIT9312) and two independent methodological approaches. We demonstrate that extracellular DOC production by *Prochlorococcus* may be significant for both exponentially growing and nutrient limited cells and that part of the DOC is comprised of low molecular weight carboxylic acids.

MATERIALS AND METHODS

Isolates and culture conditions: Two axenic *Prochlorococcus* isolates were examined in this study; MED4 and MIT9312. Both are high-light adapted strains with a low chlorophyll *b/a* ratio (low-B/A) but represent separate ecotypes as they differ in both phosphorus acquisition mechanisms (e.g. phosphatase activity and the spectrum of phosphorus compounds available for utilization; Moore *et al.* 2005) and ecological distributions (West *et al.* 2001, Ahlgren *et al.* 2005, Zinser *et al.* 2005, Johnson *et al.* 2005). All batch incubations were at 22°C in constant light at 30–40 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ under cool-white fluorescent lamps. Borosilicate test tubes (25 mm inner diameter) filled with 25 ml Sargasso Sea-based media amended with 800 $\mu\text{mol L}^{-1}$ NH_4Cl and either 50 or 1 $\mu\text{mol L}^{-1}$ NaH_2PO_4 were used throughout the experiment (Bertilsson *et al.* 2003). All cultures were acclimated to the light conditions and the respective growth medium (P-replete, P-limited) for at least three transfers prior to the experiment. Cyanobacterial growth was monitored by non-invasive *in vivo* fluorescence using a Turner 10AU fluorometer whereas cell abundances were determined by flow cytometry (Bertilsson *et al.* 2003).

¹⁴C-tracer experiments: Triplicate batch cultures of each strain (MED4, MIT9312) were grown in P-replete media and monitored for growth as described above. Tracer incubations with $\text{NaH}_2^{14}\text{CO}_3$ (ICN Pharmaceuticals, 8.4 $\mu\text{Ci } \mu\text{mol}^{-1}$) were carried out in mid-exponential growth phase (Fig. 1). Subsamples of 5 ml were incubated in identical glass tubes for either 2 or 4 hours with tracer additions of ¹⁴C-bicarbonate to a final concentration of 0.12 mCi L⁻¹ (14 $\mu\text{mol L}^{-1}$). Cells were captured on 0.2 μm PVDF membrane filters (Millipore) under gentle vacuum (< 100 mBar). Filters were washed with 2 ml sterile growth medium, placed in 20 ml scintillation vials and acidified with 1 ml 0.1M HCl. Filtrates were collected directly in 20 ml scintillation vials, acidified with 1 ml 1M HCl and vigorously vortexed for 5 min. All vials were left open overnight to vent residual inorganic carbon. Scintisafe 50% (Fisher Scientific) was added in appropriate amounts (5 ml for filters, 15 ml for

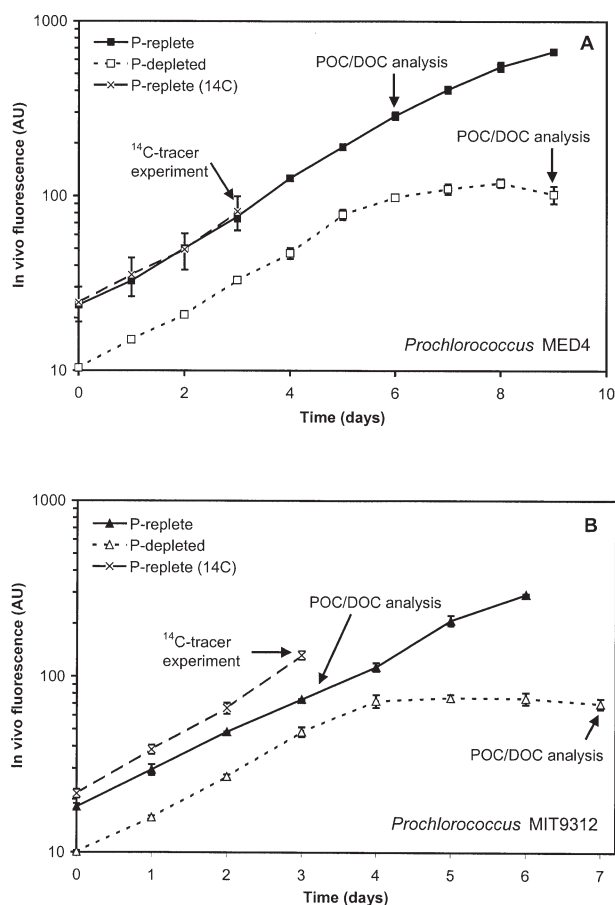


Fig. 1. – *In vivo* chlorophyll-*a* growth curves in arbitrary fluorescence units (AU) for (A) *Prochlorococcus* MED4 and (B) *Prochlorococcus* MIT9312. Parallel curves for P-replete (solid symbols), P-depleted (open symbols) and P-replete cultures for ^{14}C -tracer experiments (crosses) are shown. Growth rates in the exponential phase of MED4 were 0.39 day^{-1} for ^{14}C -tracer, 0.38 day^{-1} for P-limited and 0.39 day^{-1} for P-replete cultures. Corresponding rates for MIT9312 were 0.59 day^{-1} for ^{14}C -tracer, 0.50 day^{-1} for P-depleted and 0.47 day^{-1} for P-replete cultures. The incubation times used for the different analyses are indicated by arrows. Error bars are standard deviations ($n=3$).

liquid) and vortexed prior to liquid scintillation counting. Formaldehyde-killed controls (2% final concentration) were analyzed in parallel. The ^{14}C -signals for controls (filter and filtrates) were always less than 10% of corresponding live samples; e.g. $< 541\text{ dpm}$ and $< 76\text{ dpm}$, respectively. Analysis of variance was used to assess the significance of observed differences in percent extracellular release between treatments.

POC/DOC partitioning experiment: For each strain (MED4, MIT9312) and media (P-replete, P-limited), 12 replicate 25 ml batch cultures were inoculated and incubated as described above. For each treatment, 3 of these replicate tubes were used for continuous monitoring of growth. P-replete cultures were sampled in the mid-exponential growth phase whereas P-limited cultures were harvested in the early plateau phase (Fig. 1). The particulate and dissolved organic matter at the time

of sampling will thus reflect the combined processes from the point of inoculation to the time of harvesting. Analysis of variance was used to assess the significance of observed differences in percent extracellular release. Three POC samples were analyzed for each treatment. Each of these POC samples were based on three pooled batch cultures (75 ml total) harvested on a combusted Whatman GF/F filter (25 mm diameter, $0.7\ \mu\text{m}$ nominal pore size). All filtrations were carried out under gentle vacuum ($< 100\text{ mBar}$) in an acid-washed and thoroughly rinsed filtration funnel made of glass. All filters were dried at 60°C and stored dark until combustion and subsequent analysis of particulate carbon. Total abundances of the respective strain at the time of sampling ranged from $1.8\text{ to }3.2 \times 10^7\text{ cells ml}^{-1}$ for MIT9312 and from $5\text{ to }11 \times 10^7\text{ cells ml}^{-1}$ for MED4. The percentages of *Prochlorococcus* cells captured on filters were $> 95\%$ for both strains. Combined filtrates were further passed through pre-rinsed $0.2\ \mu\text{m}$ PVDF membrane filters (Millipore) to remove any remaining particles. Liquid samples were stored frozen in acid washed and teflon-lined glass vials until analysis of DOC and low molecular weight organic acids.

Chemical analyses: Particulate organic carbon was analyzed on a Fisons CN analyzer (NA1500 NC). Acetanilide (71.09% C and 10.36% N) was used as reference material. All samples were corrected for the background caused by adsorption of dissolved medium constituents (Bertilsson *et al.* 2003). DOC was analyzed on a Shimadzu TOC-5000 analyzer. Prior to analysis, potassium hydrogen phthalate standards and liquid samples were acidified by adding HCl to a final concentration of 0.02 mol L^{-1} , followed by 4 minutes sparging with TOC grade air. This procedure lowered the pH to < 2 and efficiently stripped the sample of inorganic carbon. The coefficient of variation between individually prepared standards at a concentration of 1 mg C l^{-1} was $< 10\%$ and the dose-response was linear between 0 and 10 mg C l^{-1} ($r^2 > 0.999$, $n=5$). Low molecular weight organic acids were measured by ion-pairing reversed-phase HPLC of their 2-nitrophenylhydrazide derivatives (Goldstone *et al.* 2002, Pullin *et al.* 2004). The method provides baseline resolution of glycolic, lactic, formic, acetic, levulinic, malonic and oxalic acid. The detection limit is 100 nmol L^{-1} for $100\ \mu\text{l}$ injections and the peak area response is linear to at least $100\ \mu\text{mol L}^{-1}$.

RESULTS

Observed partitioning of ^{14}C tracer between particulate and dissolved organic matter suggest that MED4 release from 9 to 11% of the total fixed inorganic carbon (particulate and dissolved) as DOC (Fig. 2). The DOC release was higher, though not significantly so ($p > 0.05$), for MIT9312 with a range of 15 to 21% of the total fixed inorganic carbon (Fig. 2). The release of ^{14}C -DOC relative to ^{14}C in biomass was always slightly lower but not significantly different ($p > 0.05$) for the 2 h incubations compared to the 4 h incubations (Fig. 2). This observed lag in DOC appearance is likely a kinetic effect caused by an initial isotopic imbalance

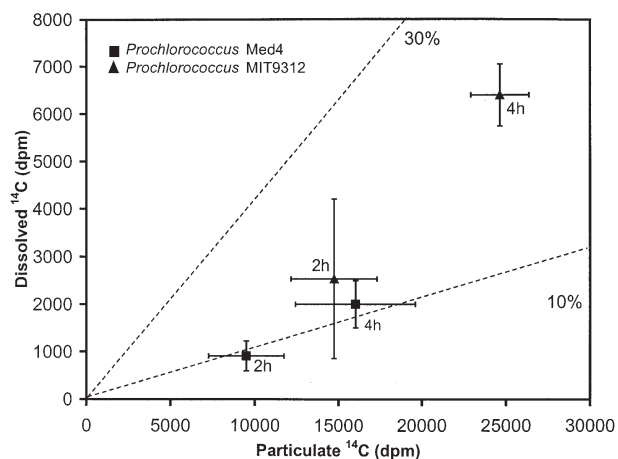


Fig. 2. – Distribution of photosynthetically fixed ^{14}C -bicarbonate into particulate and dissolved ($< 0.2 \mu\text{m}$) organic forms after 2 and 4 hours tracer incubations with MED4 and MIT9312 (see Fig. 1 for time in the growth curve when the cells were harvested). Each datapoint represents the mean of triplicates and error bars represent the standard deviation. Lines marking release of DOC corresponding to 10% and 30% of total fixed inorganic carbon (particulate + dissolved) are added for clarity.

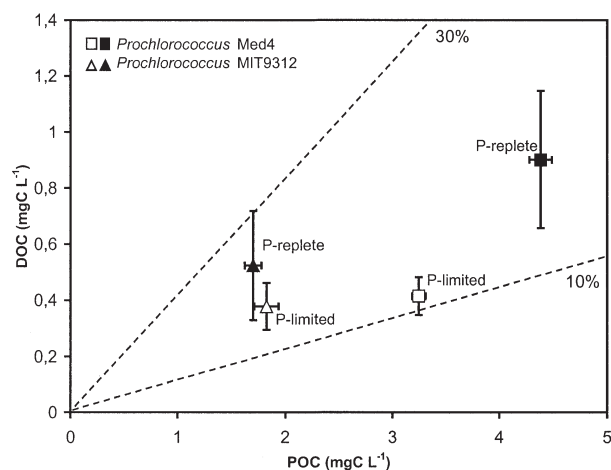


Fig. 3. – Partitioning of accumulating organic carbon between particulate and dissolved ($< 0.2 \mu\text{m}$) forms in MED4 and MIT9312 (see Fig. 1 for time in the growth curve when the cells were harvested). Both P-replete cultures harvested in the exponential growth phase and P-limited cultures harvested in the early plateau phase were analyzed (solid and open symbols respectively). Each datapoint represents the mean of triplicates and error bars represent the standard deviation. Lines marking release of DOC corresponding to 10% and 30% of total fixed inorganic carbon (particulate + dissolved) are added for clarity.

between the intracellular and extracellular pool of inorganic carbon (Morán & Estrada 2002).

Results from the ^{14}C tracer experiment were supported by results from the second experiment where the partitioning of organic matter between the particulate and dissolved phase in P-limited

and P-replete *Prochlorococcus* batch cultures was measured (Fig. 3). Between 11 and 24% of total produced particulate and dissolved organic carbon was released as DOC. Here the release from MED4 was significantly lower than for MIT9312 ($p < 0.05$). Both isolates released a significantly higher percentage of the total fixed inorganic carbon as DOC under P-replete growth conditions compared to P-limited cultures harvested in the early plateau phase ($p < 0.05$) (Fig. 3).

Glycolic and acetic acid accumulated in both P-limited and P-replete cultures of the two isolates (Table I, top). The accumulation of formic acid was more variable and could not be detected in the P-replete MIT9312 cultures. We did not observe any accumulation of levulinic, malonic or oxalic acid. Lactic acid was detected in all samples, but high analytical blanks of this particular compound prevented us from establishing whether or not this compound was produced during the incubations. The combined accumulation of glycolic, acetic and formic acid ranged between 19 and 139 $\mu\text{g C L}^{-1}$ (Table I, top) corresponding to between 4-12% of total DOC release for MIT9312 and 15-20% of total DOC release for MED4.

DISCUSSION

Our study shows that actively growing *Prochlorococcus* release a significant fraction (9-24%) of total fixed inorganic carbon as extracellular DOC. The observed values are within the range previously observed for axenic cultures of marine phytoplankton (Bertilsson & Jones 2003). The percent release is also close to, or slightly above, previous estimates for another marine unicellular cyanobacterium, *Synechococcus bacillaris*, which released 11 percent of total fixed inorganic carbon as DOC in non-axenic batch cultures (Biddanda & Benner 1997). This study also revealed that dissolved polymeric carbohydrates were important components of extracellular DOC produced by *Synechococcus* ($> 60\%$ of DOC). In the present study, we did not measure the production of carbohydrates, but described a substantial release of another group of organic compounds; low molecular weight carboxylic acids (e.g. glycolic, acetic, and formic acid), which contributed up to 20% of total released DOC. Production of the same suite of low molecular weight carboxylic acids has previously been reported for a *Synechococcus* strain isolated from a hot spring (Teiser 1993). The direct utilization of the extracellular DOC by heterotrophic bacteria, and hence the associated flux of energy and nutrients through the microbial loop and up the food web, is regulated both by the amount DOC released and its bioavailability. All organic acids detected in our study are highly la-

Table I. – Top, Concentrations of total dissolved organic carbons (DOC) and low molecular weight carboxylic acids in P-replete and P-limited cultures of *Prochlorococcus* MED4 and MIT9312; n.d. = not detected (± 1 standard deviation, $n=3$). Bottom, Comparison of bacterial production, *Prochlorococcus* production and estimated DOC release by *Prochlorococcus* integrated over 0–200 m depth in the equatorial Pacific Ocean.

Strain	Culture conditions	DOC $\mu\text{g C L}^{-1}$	glycolic	acetic	formic	total
			Carboxylic acid concentration ($\mu\text{g C L}^{-1}$)			
<i>Prochlorococcus</i> MED4	P-limited	410 \pm 70	45 \pm 9	14 \pm 5	21 \pm 4	80 \pm 6
	P-replete	900 \pm 240	68 \pm 11	45 \pm 4	26 \pm 2	139 \pm 13
<i>Prochlorococcus</i> MIT9312	P-limited	380 \pm 80	18 \pm 22	9 \pm 12	20 \pm 4	47 \pm 19
	P-replete	520 \pm 190	8 \pm 3	11 \pm 3	n.d.	19 \pm 4

Date	Conditions ^{1,2}	Production (0–200 m)		Estimated DOC release ³	
		(mg C m ⁻² day ⁻¹)		(mg C m ⁻² day ⁻¹)	
		Bacterial ¹	<i>Prochlorococcus</i> ²	9% release	24% release
April 1992	Warm El Niño	249	423	38	102
October 1992	non-El Niño	189	254	23	61

¹ Ducklow et al. (1995)

² Vaultot et al. (1995)

³ From depth-integrated *Prochlorococcus* production (Vaultot et al., 1995) and measured range of percent extracellular release in laboratory incubations (this study)

bile bacterial substrates and are most likely rapidly metabolized by co-localized heterotrophs (Bertilsson & Jones 2002, Bertilsson & Tranvik 1998). Polymeric carbohydrates, like the ones released from *Synechococcus bacillaris*, and likely also from our *Prochlorococcus* strains, are probably degraded much slower and may therefore accumulate over time (Aluwihare & Repeta 1999).

Short supply of mineral nutrients (e.g. nitrogen or phosphorus) may cause some phytoplankton to release a larger proportion of the total fixed inorganic carbon as extracellular DOC (Lancelot 1983, Obernosterer & Herndl 1995). Our study does not confirm such a general starvation-response for *Prochlorococcus*, since both MED4 and MIT9312 released a higher proportion of fixed inorganic carbon as DOC under nutrient replete vs. phosphorus limited conditions (Fig. 3). This feature could be an effect of changes in the biochemical composition and morphology of the cell as a result of the limited phosphorus supply (Bertilsson et al. 2003). An alternative explanation may be an increase in the potential of *Prochlorococcus* to take up and utilize dissolved organic compounds under phosphorus limiting conditions. The latter mechanism could be a strategy to assimilate dissolved and organic-bound nitrogen and phosphorus under nutrient-scarce conditions. In support of such a

mixotrophic lifestyle in *Prochlorococcus*, recent studies based on radiotracer incubations and flow cytometry have shown that *Prochlorococcus* effectively take up amino acids in oligotrophic regions such as the tropical Southern Atlantic and the Arabian Sea (Zubkov et al. 2003, 2004).

Both the ¹⁴C-tracer experiment and the DOC/POC partitioning experiment showed that the proportion of total fixed inorganic carbon released as DOC was higher for MIT9312 than for MED4 (Fig. 2–3). Furthermore, there were marked differences in the proportion of carboxylic acids in the released DOC between the two strains with much higher values for MED4. These differences may reflect physiological differences between the isolates and is not altogether surprising since they represent different ecotypes (Rocap et al. 2002). Both strains are high-light adapted but appear to be niche-separated with ecotype MIT9312 being dominant in warm surface oceans and MED4 being most abundant in colder waters (Johnson et al. 2005).

Release of organic compounds by phytoplankton may selectively promote specific subsets of the bacterial community that are able to utilize the released organic compounds as substrates for growth (Bell et al. 1974). *Prochlorococcus* can make up 21–43% of the total primary producer biomass

(Campbell *et al.* 1994, Vaultot *et al.* 1995, DuRand *et al.* 2001), reach growth rates of 1 day⁻¹ (Vaultot *et al.* 1995, Liu *et al.* 1999) and account for 13-57% of net primary production in tropical and subtropical oceans (Vaultot *et al.* 1995, DuRand *et al.* 2001, Li 1994). Hence the extracellular release of DOC from these cyanobacteria may constitute an important source of growth substrates for heterotrophic bacteria in these regions. The quantitative significance of the process can be illustrated by applying the range of percent extracellular release observed in our experimental incubations to previous estimates of depth-integrated *Prochlorococcus* production in the equatorial Pacific during spring and fall 1992 and compare the calculated release to bacterial production measured in parallel (Table I, bottom). Assuming 9% DOC release from *Prochlorococcus* (minimum release in the present study), this DOC input would still make up 12-15% of total bacterial production integrated over 0-200 m depth. With 24% DOC release (maximum release in the present study), DOC input would account for 32 to 41% of total bacterial production. Future studies should explore these emerging signs of strong food web linkages among the prokaryotic plankton of the ocean.

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