Biogeosciences, 11, 5115–5122, 2014
www.biogeosciences.net/11/5115/2014/
doi:10.5194/bg-11-5115-2014

Biogeosciences Open Access

An experimental study on the effects of nutrient enrichment on organic carbon persistence in the western Pacific oligotrophic gyre

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Received: 31 January 2014 – Published in Biogeosciences Discuss.: 21 February 2014
Revised: 18 August 2014 – Accepted: 20 August 2014 – Published: 23 September 2014

Abstract. Carbon sequestration in the ocean is of great concern with respect to the mitigation of global warming. How to hold the fixed organic carbon in the presence of tremendous numbers of heterotrophic microorganisms in marine environments is the central issue. We previously hypothesized that excessive nutrients would ultimately decrease the storage of organic carbon in marine environments. To test this, a series of in situ nutrient enrichment incubation experiments were conducted at a site (17.59° N, 127.00° E) within the western Pacific oligotrophic gyre. Five treatments were employed: glucose (Glu), algal exudation organic material (EOM), nitrate (N) and phosphate (P), N and P in combination with glucose and a control with no added nutrients. The results showed that the dissolved organic carbon consumption rates and bacterial community specific growth rates were enhanced by inorganic nutrient enrichment treatments during the initial 48 h incubation. At the end of 14 days of incubation, about one-third (average 3.3 µmol C kg^{-1}) more organic carbon was respired in the glucose-enriched incubation with the addition of inorganic nutrients compared to that without. In contrast, when nutrients were limiting, glucose could not be efficiently used by the bacteria and thus it remained in the environment. These results suggest that repletion of inorganic nutrients could facilitate microbial consumption of organic carbon and thus has a significant impact on carbon cycling in the environment.

1 Introduction

Dissolved organic carbon (DOC) in the ocean, as one of the largest carbon reservoirs on the earth, is comparable to the entire atmospheric CO_2 reservoir (~ 750 Gt; Hedges, 1992; Ogawa and Tanoue, 2003) and plays an important role in global carbon cycling and climate change. A great deal of research effort has been directed to the processes and mechanisms involved in DOC dynamics, including production, consumption, and long-term storage of DOC. Recalcitrant dissolved organic carbon (RDOC), which comprises the largest portion of the bulk ocean DOC reservoir, can persist for thousands of years in the water column (Blitz, 1992), constituting significant carbon sequestration in the ocean. Therefore, how RDOC is produced and stored is essential for understanding DOC dynamics and has been one of the hot topics among biogeochemists for more than a decade (Søndergaard et al., 2000; Kragh and Søndergaard, 2004; Eichinger et al., 2009; Kragh and Søndergaard, 2009). Recently, the role of microbes in formation of RDOC is proposed as the microbial carbon pump (MCP) (Jiao et al., 2010a). One of the MCP rationales lies in the constraints of microbial DOC consumption (Jiao et al., 2011).

Although great progress has been made in microbial growth and production under different environmental conditions in the past decades (e.g., Goldman, 1987; Zweifel et al., 1993; Carlson and Ducklow, 1996; Cherrier et al., 1996; Cotner et al., 1997; Kirchman and Rich, 1997; Rivkin and Anderson, 1997; Thingstad et al., 1998; Carlson et al., 2002; Caron et al., 2000; Sala et al., 2002; Pinhassi et al., 2006), controversy remains to be addressed regarding remaining DOC versus nutrient availability. It is generally considered that enrichment of inorganic nutrients can result in the enhancement of primary production (Falkowski et al., 1998), subsequently leading to enhanced DOC release into the environment (Carlson et al., 1994; Biddanda and Benner,

1997; Hansell and Carlson, 1998). However, such DOC is highly available to heterotrophic consumption, and thus cannot build up high concentrations (Roson et al., 1999; Chen et al., 2007). On the other hand, the so-called "malfunction of the microbial loop" effect could cause degradable DOC accumulation in productive surface water (Thingstad et al., 1997). Obviously carbon storage in the environment is not necessarily the result of nutrient enrichment. In fact, eutrophication waters such as estuaries are often sources rather than sinks of CO_2 (Laruelle et al., 2010; Yuan et al., 2010). Therefore, we proposed that excessive inorganic nutrients would play against organic carbon storage in the marine environment (Jiao et al., 2010b). To test this point, nutrient enrichment experiments should be carried out in oligotrophic waters rather than coastal waters where sources of nutrients and DOC were myriad (Coble et al., 1990; Cabaniss and Shuman, 1987). In the present study, we conducted a set of in situ microcosm experiments in the western Pacific gyre where the low background concentrations of nutrients (below detection limit) and DOC (81.5 µmol C kg^{-1}) allowed us to check the effects of nutrient enrichment on resistance of DOC to microbial consumption manifestly.

2 Materials and methods

2.1 Experimental design and sampling

Seawater was collected from a depth of 75 m in the western Pacific Ocean (17.59° N, 127.00° E), using a rosette sampler with a conductivity–temperature–depth instrument on 27 November 2012. The experiment was carried out with 10 microcosms (polycarbonate bottles, 20 L) which were pre-acid-washed and rinsed with sample water. The filter system was pre-cleaned with copious ultrapure water and seawater in turn to minimize carbon contamination. Each microcosm was filled with 20 L seawater pre-filtered through a 3 µm filter. Treatments were amended with organic carbon sources and inorganic nutrients as described in Table 1. Each treatment was conducted in replicate. The microcosms were incubated at 28 ± 0.5 °C in darkness. Cultures were sampled at hour 0, 3, 6, 12, 24, 36, 48, 96, 168 and 336, and water samples were stored at −20 °C until analysis.

2.2 Algal culture and dissolved organic material extraction

Axenic culture of *Phaeodactylum tricornutum* was incubated in f/2 medium with artificial seawater under a photon flux of 112 µE m^{-2} s^{-1} and a 10 h light/14 h dark cycle, with a temperature of 20 ± 0.5 °C. To separate the medium from any algal cells, the culture was filtered through a 3 µm filter when the stationary growth phase was reached. The algal exudation organic material (EOM) was extracted from the medium using solid-phase extraction cartridges (PPL, 1 g, Agilent, Bond Elut) following Dittmar et al. (2008). The algal filtrate was acidified with HCl (final pH = 2) prior to passing through the cartridges. Ultrapure water (also acidified to pH = 2 with HCl) was then used to remove excess salt from the cartridges, which secures the identical nutrient background between the EOM treatment and control. The EOM was eluted with methanol into pre-combusted glass vials (40 mL) after the sorbent was dried with nitrogen gas. The EOM was stored at −20 °C until the evaporation of methanol by nitrogen gas had been achieved. Before being transferred to the microcosms, the EOM was re-dissolved in 10 mL ultrapure water. Phytoplankton-derived organic matter plays a key role in carbon cycling due to its bioavailability. However the dissolved fraction was relatively low, accounting for only about 5 % of the total released organic carbon of *Phaeodactylum tricornutum* (Becker et al., 2014). Furthermore, it is noteworthy that solid-phase extraction does not concentrate all algae exudate molecule equally, and only about 3.5 % of total algae exudate organic carbon was recovered finally in this study.

2.3 Total DOC (TOC) analysis

Total dissolved organic matter was measured using the high-temperature combustion method with a Shimadzu TOC-V CPH TOC analyzer. To avoid potential organic carbon contamination, a filtration procedure was not applied in sampling. Consequently, bacterial biomass carbon was not ruled out, but it contributes less than 1 % of TOC (Carlson and Ducklow, 1996). The samples were collected in glass vials (40 mL) with glass pipettes. All the glass apparatuses employed in sampling were pre-acid cleaned and combusted (500 °C, 6 h). Water samples were then acidified to pH = 2 with H_3PO_4 and stored at −20 °C until analysis. TOC measurement was based on Callahan et al. (2004). Culture samples were taken at hour 0, 3, 6, 12, 24, 36, 48, 96, 168 and 336.

In this study, bacteria were considered as particulate organic carbon (POC) in the microcosm culture. POC concentration was estimated from bacterial abundance (BA) and the bacterial carbon conversion factor (CCF), which was assumed to be 20 fg C $cell^{-1}$ based on the natural planktonic assemblage (Lee and Fuhrman, 1987). The POC concentration could then be calculated using the following equation:

$$\text{POC concentration} = \text{BA} \times \text{CCF}. \quad (1)$$

DOC was defined as the TOC which did not include POC (the bacterial biomass carbon). Therefore, DOC concentration was calculated as

$$\text{DOC concentration} = \text{TOC concentration} - \text{POC concentration}. \quad (2)$$

In theory, the organic carbon decrease in the incubation system was the overall consequence of bacterial biomass carbon

Table 1. Initial organic carbon and inorganic nutrient enrichment treatments in the experiments.

Treatments	Carbon amendment		Inorganic nutrient amendment	
	Glucose (μmol C kg^{-1})	EOM (μmol C kg^{-1})	Inorganic nitrogen (μmol N kg^{-1})	Inorganic phosphate (μmol P kg^{-1})
Glu + N + P	7.9	non	1.53	0.11
Glu	7.0	non	nd	nd
EOM	non	7.0	nd	nd
N + P	non	non	1.54	0.11
Control	non	non	nd	nd

"non" means no organic carbon enrichment, and the background TOC concentration was 81.5 μmol C kg^{-1}.
"nd" means below the detection limit.

(POC) increase and bacterial respiration consumption which is comparable to the observed net TOC reduction (Carlson et al., 1999). Therefore, the bacterial respiration rate was estimated as

$$\text{bacterial respiration rate} = \Delta\text{TOC concentration}/\Delta t. \quad (3)$$

2.4 BA analysis and specific growth rate

The culture was sampled at hour 0, 3, 6, 12, 24, 36, 48, 96, 120, 144, 168 and 336. Samples were fixed with glutaraldehyde to a final concentration of 1 % (Vaulot et al., 1989) and frozen in liquid nitrogen prior to storage at −80 °C. Bacteria were stained with SYBR Green I (Marie et al., 1997) before using a flow cytometer (Becton Dickinson), and the autotrophs were run separately without being stained (Jiao et al., 2005).

Specific growth rate was measured from 0 to 48 h and from 120 to 168 h and calculated as follows:

$$\text{specific growth rate} = \ln(\Delta\text{BA})/\Delta t, \quad (4)$$

where ΔBA represents the observed net change in BA.

2.5 Analysis of dissolved inorganic nutrient concentration

The concentrations of dissolved inorganic nutrients in the samples were measured using a Technicon AA3 Auto-Analyzer (Bran-Lube, GmbH). The copper–cadmium reduction method was employed to determine the dissolved inorganic nitrogen (DIN, nitrate and nitrite) concentrations of each sample. The spectrophotometric method was employed to determine dissolved inorganic phosphorus (DIP) concentration (Knap et al., 1996). The detection limits for DIN and DIP were 0.1 μmol kg^{-1} and 0.08 μmol kg^{-1}.

3 Results and discussion

3.1 Dynamics of BA and growth rate

In general, BA in all treatments showed a similar growth pattern transitioning through lag, logarithmic and stationary phases but with different levels of maximum abundance (Fig. 1). Compared to the control, the most pronounced differences were observed in the Glu + N + P treatment, followed by the EOM and N + P treatments. In contrast, the glucose (Glu) treatment did not show much difference from the control. The specific growth rates during 0–48 h of all the treatments, except for the Glu treatment, were significantly higher than that of the control (analysis of variance (ANOVA) test, $p < 0.05$) (Table 2). In the Glu treatment the no/slight bacterial response to enrichment with Glu, a labile DOC source, seemed to be unreasonable, but such situations do exist in oligotrophic oceans as reported in the Sargasso Sea (Carlson et al., 1996). These results actually suggest a case of nutrient limiting rather than carbon limiting for the microbial community. In our study, the bacterial specific growth rate in N + P treatments was significantly higher than that in the control (ANOVA test, $p < 0.05$) (Table 2), and the same result was obtained for BA at 48 h (Fig. 1), indicating that inorganic nutrient addition could stimulate bacterial growth in the logarithmic phase. This was consistent with the results of Carlson et al. (2002) from the Sargasso Sea (2 days, 0.08 cell L^{-1} d^{-1} and 0.06 cell L^{-1} d^{-1}). When

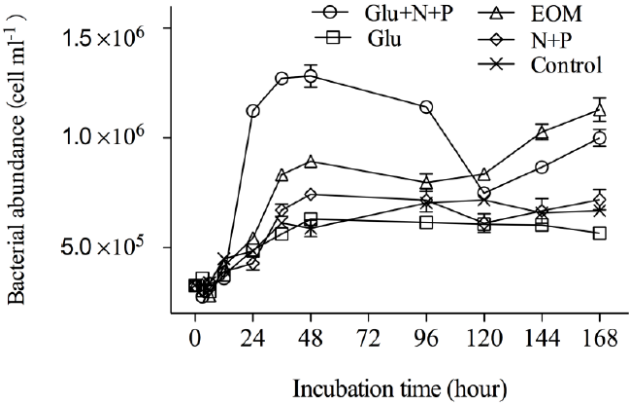

Figure 1. Variations of bacterial abundance during the incubation time course in various treatments. Error bars indicate the standard errors.

Table 2. The DOC consumption rate, bacterial community specific growth rate, bacterial abundance (BA) and bacterial respiration rate (BR) in the incubation experiments.

Treatments	DOC consumption rate (0–48 h)	Specific growth rate (h^{-1}) 0–48 h	120–168 h	BA (336 h) (10^5 cell mL^{-1})	BR (μmol C kg^{-1} d^{-1})
Glu + N + P	0.232 ± 0.038	0.022 ± 0.0008	0.005 ± 0.0005	12.82 ± 0.51	5.46 ± 0.19
Glu	0.141 ± 0.021	0.009 ± 0.0002	–	6.30 ± 0.06	3.45 ± 0.58
EOM	0.163 ± 0.028	0.015 ± 0.0003	0.006 ± 0.0008	8.94 ± 0.26	3.50 ± 0.88
N + P	0.170 ± 0.022	0.012 ± 0.0005	–	7.43 ± 0.17	4.03 ± 0.27
Control	0.134 ± 0.024	0.008 ± 0.0003	–	5.88 ± 0.37	3.46 ± 0.46

The DOC consumption rate was estimated from the absolute value of the slope of the linear regression on all collected data of DOC concentration during 0–48 h. The BR was estimated from the observed net TOC concentration reduction in the corresponding incubation time (0–48 h).
– Non-measurement during 120–168 h due to no obvious bacterial abundance enhancement.
Data were mean ± SE (standard error).

Glu was with N + P, it made the most robust difference, and the BA reached 1.25×10^6 cells mL^{-1} at 48 h, which was the highest value among all the treatments, with specific growth rates of 1.8- to 2.5-fold that of the N + P treatments during the 0–48 h incubation (Fig. 1 and Table 2). These results demonstrated that even labile DOC such as Glu could not be used by microbes and would be left over in the environment if no essential nutrients were available. On the one hand, this meant that bacterial growth not only required organic carbon but also required inorganic nutrients; it may be possible for a labile DOC molecule to become semi-labile or instantly refractory to bacteria if nutrients are not available. The argument could be raised here as to whether inorganic nutrients are essential for bacteria. The results of EOM treatment (without supplementary inorganic nutrients) showed that the BA and specific growth rate were significantly higher than in the control and even other treatments (except for the Glu + N + P treatment) during the 0–48 hour period. Since EOM must contain organic nitrogen, phosphorus and other elements, it is possible to say that elemental balance is a key for bacterial growth and abundance. In the case where the labile organic matter (which contains diverse elements) is not enough, inorganic nutrients could be substituted to meet the bacterial demand for growth elements in the oligotrophic ocean.

3.2 Dynamics of the inorganic nutrients

Dissolved inorganic nutrient (N and P) concentrations in non-nutrient enrichment treatments (Glu, EOM and control treatments) were all below the detection limits (Fig. 2a and b). For the nutrient (N and P)-enriched treatments, the variation of nutrient concentrations during the incubation time course was different between the Glu + N + P and N + P treatments. In the Glu + N + P treatment, after a lag phase (0–12 h), the DIN decreased rapidly from 1.45 to 0.77 μmol N kg^{-1} during the 12–48 h period, and then maintained a low level (around 0.66 μmol N kg^{-1}) during the 48–336 h period (Fig. 2a). In contrast, the DIN in the N+P treatment showed a prolonged gradual reduction over the 0–336 h incubation time course, decreasing from 1.54 to 1.13 μmol N kg^{-1} in total (Fig. 2a). Moreover, in the Glu + N + P treatment, the DIP was rapidly consumed during the first 6 h (0.11 to 0.09 μmol P kg^{-1}) and reduced to below the detection limit within 12 h (Fig. 2b). In the N + P treatment, however, DIP decreased slowly from 0.11 to 0.09 μmol P kg^{-1}, during 0–72 h, and then suddenly dropped to below the detection limit after 96 h (Fig. 2b). The difference in dynamics of the nutrients between the Glu + N + P and N + P treatments suggested that inorganic nutrient uptake could be significantly enhanced by labile organic carbon (e.g., Glu) enrichment.

3.3 Dynamics of TOC and DOC concentrations

TOC concentrations in all treatments, including the control, were monitored during the entire incubation time course (336 h) to check the differences in consumption of organic carbon between the treatments. Generally, TOC concentrations decreased rapidly in the initial 48 h and then became relatively stable during the later hours (48–336 h) at different levels in different treatments (Fig. 3). The fraction of DOC consumed in the first 48 h accounted for 8 to 13 % of the initial bulk DOC varying with different treatments (Table 2). The Glu + N + P treatment showed the highest DOC consumption rate (0.232 μmol C kg^{-1} h^{-1}) among all the treatments, most likely due to the combined effects of nutrient and organic carbon enrichment. DOC consumption rates in the N + P treatment ranked second highest (0.170 μmol C kg^{-1} h^{-1}), indicating that nutrient enrichment did stimulate the uptake of organic carbon as previously hypothesized (Jiao et al., 2010b). The DOC consumption rate in the EOM treatment (0.163 μmol C kg^{-1} h^{-1}) was similar to the N + P treatment. However, the mechanism behind DOC utilization could be quite different. EOM provided not only carbon but also the other elements including

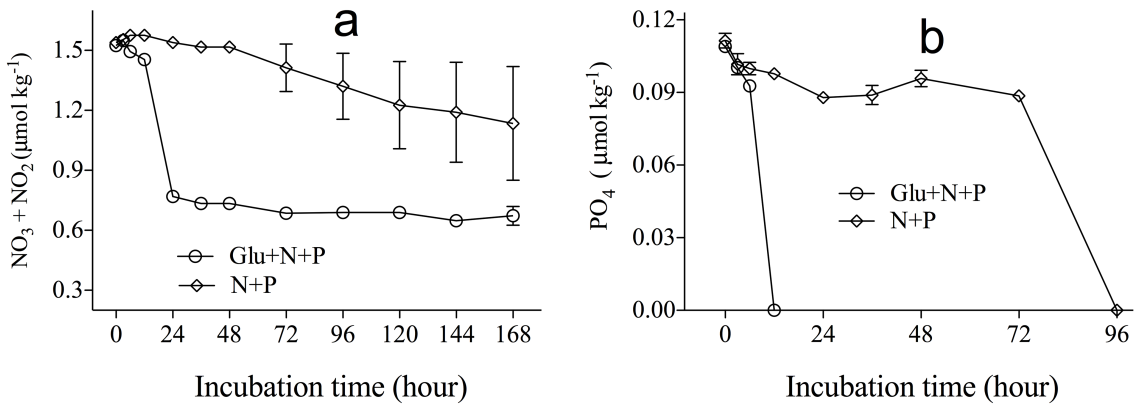

Figure 2. Variations of dissolved inorganic nitrogen and phosphate concentrations during the incubation time course of the nutrient-enriched treatments. (Non-inorganic-nutrient-enrichment treatments are not shown since they were below the detection limits.) Value "0" in **(b)** means below the detection limit. Error bars indicate standard errors.

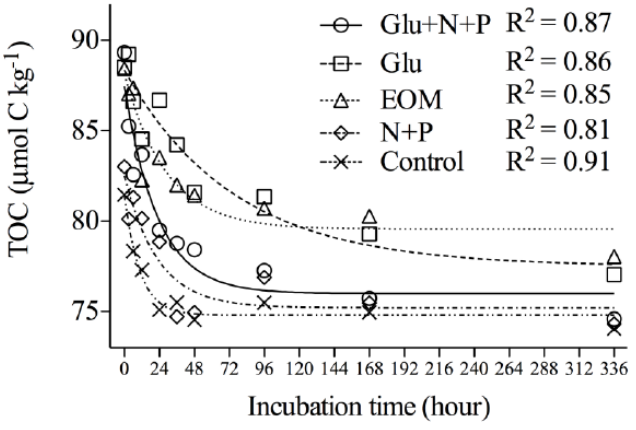

Figure 3. Variations of TOC concentrations during the incubation time course of various treatments. The non-linear regression lines were obtained from the corresponding TOC concentration observations during the entire incubation. Symbols shown in the figure are the mean values of TOC concentrations at each sampling point.

N and P necessary for the bacterial demand. In contrast if only organic carbon had been supplied, like the case of Glu treatment, bacteria may not have been able to use much carbon (0.141 μmol C kg^{-1} h^{-1}, similar to the control, 0.134 μmol C kg^{-1} h^{-1}) due to the elemental limitation of N and P (Table 2). A number of studies have shown that algal excretions are a complex mixture of organic material, comprised largely of polysaccharides, small nitrogenous compounds, lipids, vitamins, etc. (Goldman et al., 1992; Myklestad et al., 1989; Mague et al., 1980). Therefore EOM could have been more efficient than Glu alone for bacterial growth in oligotrophic waters. It is noteworthy that while the DOC consumption rates in the EOM and N + P treatments were similar, the bacterial respiration rates in the two treatments were quite different (higher in the latter; Table 2), which was likely due to the fact that more energy is needed for the synthesis of biomass (protein etc.) in the case of N + P treatment. Among all the treatments, the highest bacterial respiration rates were observed in the Glu + N + P treatment, contrasting to the lowest in the Glu treatment, which was actually the same as the control. Taking together the DOC consumption and bacterial respiration, it was clear that inorganic nutrients stimulated bacterial respiration and led to more carbon consumption in the same organic carbon availability scenario.

As the less organic carbon used by microbes, the more organic carbon would be left in the environment. Compared to the Glu treatment, more than 3.3 μmol C kg^{-1} TOC on average was consumed in the Glu + N + P treatment after 336 h incubation. This suggested that inorganic nutrient enrichment was not beneficial for organic carbon persistence, and that the bio-availability and lability of an organic carbon compound could be situation-specific. Therefore, the term RDOC could be refined as the deep ocean RDOC (which has generally been used by chemists), and situational RDOC (Jiao et al., 2014), which means that it can hold recalcitrance under certain conditions but may become bioavailable when conditions change (such as nutrient enrichment). Such situations actually exist in the oceans. For example, the highest DOC concentration among the oceanic waters of the world is located in the Southern Ocean gyre (Hansell et al., 2009), where nutrients are limiting, and stratification is strong.

Before the present study, there were many studies on the limiting factors of bacterial growth and/or bacterial production (Supplement Table S1), and the results differ among different experiments. In some cases enrichment of inorganic nutrient (N and/or P) stimulated bacterial growth and/or bacterial production (Rivkin and Anderson, 1997; Cotner et al., 1997; Thingstad et al., 1998; Caron et al., 2000; Sala et al., 2002; Pinhassi et al., 2006), while in other cases bacterial growth and/or production were proven to be limited by organic carbon (Kirchman and Rich, 1997; Rivkin and Anderson, 1997; Pinhassi et al., 2006). Meanwhile co-limitation

by organic carbon and inorganic nutrients was also reported (Pomeroy et al., 1995; Donachie et al., 2001; Hoikkala et al., 2009). These studies mainly focused on the microbial abundance/production/respiration rather than organic carbon left over in the environment, although some of them have discussed DOC dynamics. There were a few studies that followed DOC dynamics during the incubations, and found that organic carbon rather than nutrients is the limiting factor for bacterial activities (Cherrier et al., 1996; Carlson et al., 1996; Carlson et al., 2002).

In contrast, the direct coupling between DOC dynamics and bacterial activities in the present study showed that inorganic nutrients rather than organic carbon were the limiting factor in the Pacific oligotrophic gyre. Meanwhile our study showed the negative effects of nutrient enrichment on DOC persistence in the environment.

4 Conclusions and prospects

It is generally known that enrichment of inorganic nutrients would increase carbon fixation, but this is not necessarily true for carbon preservation in the environment. Our in situ incubation experiments in the western Pacific gyre showed that nitrate and phosphate addition stimulated organic carbon consumption and bacterial respiration and ultimately resulted in reduction of organic carbon remained in the environment. In contrast, if nitrogen and phosphorus are limiting (oligotrophic case), even if provided with labile carbon molecules such as glucose, bacteria may not take it up efficiently. On the other hand, natural labile organic matter such as EOM, containing multiple elements, would fuel bacteria efficiently. Taken together, these recognitions are useful for interpreting some paradoxes such as why eutrophic estuarine waters are often sources rather than sinks of CO_2. That is, although nutrients are rich in estuarine waters, autotrophs are limited by light availability, whereas heterotrophic microbes could be simulated by both labile DOC such as EOM and deplete nutrients. As a result, consumption of environmental organic carbon (including some of the river discharged) could exceed primary production resulting in outgassing rather than uptake of CO_2 in the system. These recognitions can be referred to for coastal water management regarding ecological health and carbon sequestration. Further studies are desired to explore the concentration ranges, optimum elemental ratios of nutrients which are most favorable for shifting the carbon cycle equilibrium towards organic carbon storage in a variety of marine environments.

The Supplement related to this article is available online at doi:10.5194/bg-11-5115-2014-supplement.

Acknowledgements. We thank the crew of the R/V *Kexue* – 1 for the smooth sampling. We also thank John Hodgkiss for the assistance with English. This work was supported by National Key Basic Research Program of China (grant no. 2013CB955700) and the SOA Project (GASI-03-01-02-05, 201105021).

Edited by: H. Thomas

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