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Abstract

The effects of grazing pressure and inorganic nutrient availability on the direct carbon transfer from freshly produced phytoplankton exudates to heterotrophic bacteria biomass production were studied in Mediterranean coastal waters. The short-term incorporation of ^{13}C ($H^{13}CO_3$) in phytoplankton and bacterial lipid biomarkers was measured as well as the total bacterial carbon production (BP), viral lysis and the microbial community structure under three experimental conditions: (1) High inorganic Nutrient and High Grazing (HN + HG), (2) High inorganic Nutrient and Low Grazing (HN + LG) and (3) under natural *in situ* conditions with Low inorganic Nutrient and High Grazing (LN + HG) during spring. Under phytoplankton bloom conditions (HN + LG), the bacterial use of freshly produced phytoplankton exudates as a source of carbon, estimated from ^{13}C enrichment of bacterial lipids, contributed more than half of the total bacterial production. However, under conditions of high grazing pressure on phytoplankton with or without the addition of inorganic nutrients (HN + HG and LN + HG), the ^{13}C enrichment of bacterial lipids was low compared with the high total bacterial production. BP therefore seems to depend mainly on freshly produced phytoplankton exudates during the early phase of phytoplankton bloom period. However, BP seems mainly relying on recycled carbon from viral lysis and predators under high grazing pressure.

Introduction

Phytoplankton, bacteria and micrograzers are major components of the microbial food web and primary sources of biogenic carbon production in aquatic systems. Phytoplankton in the oceans account for slightly < 50% of global primary production (Field *et al.*, 1998). Bacteria also play a crucial role in biogeochemical cycles, owing to their importance in regulating the decomposition of organic matter and nutrient cycling (Azam *et al.*, 1983; Cole *et al.*, 1988). Micrograzers (such as heterotrophic nanoflagellates and ciliates) are ubiquitous in marine environments and their importance in marine food webs is well recognised for transferring energy to the upper trophic levels and releasing dissolved matter (Azam *et al.*, 1983). Interactions between these microorganisms through competition, mutualism or grazing ultimately influence the carbon production of the ecosystem (Legendre & Rassoulzadegan, 1995). Competition for mineral nutrients between phytoplankton and bacteria, combined

with pressure from micrograzers, may drive the species composition of the phytoplankton and bacterial communities (Jacobsen *et al.*, 1995; Samuelsson *et al.*, 2002). It may also affect the functioning of the microbial ecosystem by changing the balance between phytoplankton and bacteria (Bratbak & Thingstad, 1985) as well as the bacterial degradation of organic matter (Pengerud *et al.*, 1987; Grossart *et al.*, 2006). The carbon dependence of bacteria on phytoplankton exudates (direct carbon coupling) depends on available organic substrates meeting the heterotrophic bacterial carbon (C) demand. Direct C coupling could be expected to be weak in coastal marine waters, including Mediterranean waters, where allochthonous organic C may satisfy the bacterial carbon demand (Morán *et al.*, 2002a,b). This was supported by the few studies performed in transitional ecosystems showing that the bacterial carbon demand may occasionally exceed the dissolved primary production (e.g. Pugnetti *et al.*, 2010). However, Morán *et al.* (2013) suggested recently that a larger than expected trophic dependence of bacteria on phytoplankton can occur in estuarine ecosystems. Nevertheless, carbon sources, other than dissolved organic carbon (DOC) freshly produced by phytoplankton or from terrestrial sources, may also be available for bacteria in coastal waters. The importance of the release of organic matter as a consequence of grazing (e.g. sloppy feeding) and viral lysis must be taken into account in the carbon budget, as it appears to be a major source of DOC for bacteria in all marine waters (Wilhelm & Suttle, 1999; Nagata, 2000). Moreover, the bacterial carbon demand to meet the bacterial C : N : P stoichiometry is directly affected by nitrogen and phosphorus availability. We therefore hypothesised that the direct C coupling between heterotrophic bacteria and freshly produced phytoplankton exudates in marine coastal productive waters is stronger with high inorganic nutrient availability and reduced grazing pressure on microorganisms. Under these conditions, the bacterial need for carbon provided by the freshly produced phytoplankton exudates would increase. However, heterotrophic bacteria may rely mainly on organic sources released by grazers in conditions with reduced inorganic nutrient availability and high grazing pressure on bacteria and phytoplankton. So far as we are aware, no experimental study has tried to focus specifically on the importance of both grazing and nutritional status on the direct carbon coupling between bacteria and phytoplankton in coastal waters.

Most of the studies on the direct carbon coupling between phytoplankton and bacteria compared the dissolved primary production rates with the bacterial production and respiration rates. This assumes that the entire bacterial community can easily take up and assimilate organic matter freshly released by phytoplankton. Such an assumption does not seem to be applicable to all marine bacterial groups (Sarmento & Gasol, 2012). The degree of direct carbon coupling between phytoplankton and bacteria can be assessed using a more direct approach involving the exposure of photosynthesising organisms to ^{13}C-labelled substrates. The isotope can then be traced using specific microbial biomarkers to link specific environmental microbial processes directly with the organisms involved (Boschker *et al.*, 1998). A number of recent studies have used this ^{13}C-labelling technique to label microbial fatty acids (FAs) to characterise functional groups of bacteria involved in specific processes (Knief *et al.*, 2003; Crossman *et al.*, 2005; Evershed *et al.*, 2006) and has also enabled bacteria to be included in food web studies (Boschker & Middelburg, 2002; Van den Meersche *et al.*, 2004, 2011). The tightness of the phytoplankton–bacteria carbon coupling can be assessed using this ^{13}C-labelling technique. Short-term (4–24 h) enrichment of bacterial biomarker in ^{13}C is generally attributed to the efficient growth of heterotrophic bacteria on freshly produced phytoplankton extracellular carbon exudates (Boschker & Middelburg, 2002). The cumulative ^{13}C enrichment in bacterial lipids over several days and weeks probably includes recycled phytoplankton carbon (algal cell death, viral lysis and sloppy feeding by mesozooplankton; Van den Meersche *et al.*, 2011).

In the present study, a short-term ^{13}C tracer experimental field study was carried out in the Mediterranean coastal lagoon of Thau (South of France) to track the transfer of carbon from phytoplankton exudates to bacterial communities under three different nutritional and trophic conditions (1) artificial High inorganic Nutrient and Low Grazing (HN + LG) and (2) artificial High inorganic Nutrient and High Grazing (HN + HG) and (3) natural Low inorganic Nutrient and High Grazing (LN + HG).

Materials and methods

Experimental set-up

The study was conducted in spring from 2 to 11 May 2006 in a Mediterranean lagoon, in the south of France (Thau lagoon, France: 43°24′N, 03°41′E) using MEDIMEER experimental facilities. Two transparent polyethylene bags of about 1000 L with transmission of 77% of photosynthetically active radiation (PAR) were used (Nouguier *et al.*, 2007) as *in situ* enclosures to simulate two trophic conditions with high inorganic nutrient concentrations. The experimental design is shown in Fig. 1. The first bag was filled on 2 May with *c.* 900 L of filtered (50, 10, 0.45 and 0.2 μm successively) seawater taken

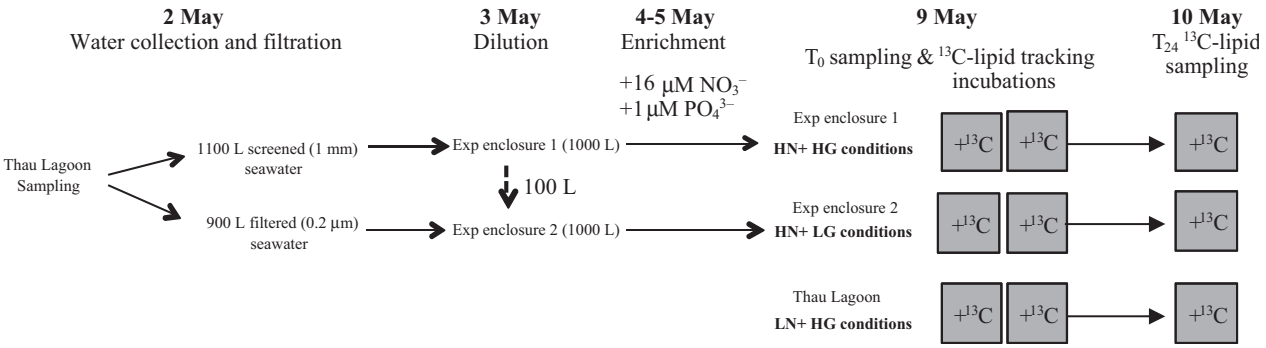

Fig. 1. Experimental design used for this study with three sets of trophic and nutritional conditions (High inorganic Nutrient and High Grazing: HN + HG, High inorganic Nutrient and Low Grazing: HN + LG, and Low inorganic Nutrient and High Grazing: LN + HG) and ^{13}C-lipid tracking incubations to assess the transfer of carbon from phytoplankton to bacterial communities. The full description of the experimental procedure is in the text.

from the lagoon surface waters adjacent to the MEDIMEER pontoon. The second bag was filled with *c.* 1100 L of screened (1 mm pore size) surface seawater from the same location. About 100 L from this second bag was transferred to the first bag on 3 May to dilute the seawater 10-fold to reduce the grazing pressure. The water in each bag was continuously mixed from 10 cm below the surface water to the bottom of the bag, using a small pump to ensure a turnover of the whole water mass within the enclosure every 2 h.

The two enclosures were enriched with nitrate and phosphate on 4 and 5 May with a final enrichment of about 16 µM NO_3 ($NaNO_3$) and about 1 µM PO_3 (H_3PO_4) to sustain significant phytoplankton growth in both enclosures for several days. The chlorophyll fluorescence and the concentrations of nitrate and phosphate in both enclosures were recorded every day. The experimental conditions expected were achieved when the chlorophyll concentration started to increase substantially (values > 1 µg Chl*a* L^{-1}), 4 days after adding the inorganic nutrients to give the two conditions: High inorganic Nutrient and High Grazing (HN + HG) and High inorganic Nutrient and Low Grazing (HN + LG). On 9 May, 80 L samples for ^{13}C enrichment and samples for chemical and biological characterisation were collected in four 20-L carboys from each enclosure. In addition to these two artificial conditions, *in situ* natural surface water samples (80 L) were also collected in Thau lagoon on 9 May and screened (1 mm pore size) to follow the short-term dynamics of a plankton community under natural conditions, Low inorganic Nutrient and High Grazing (LN + HG) (Fig. 1). On 9 May, seawater samples (20 L) for chemical and biological characterisation were taken once for the three sets of conditions as a snapshot of different plankton dynamics, while the remaining seawater samples (60 L) were used immediately for ^{13}C tracer experiments as described below.

Nutrient concentrations

Samples for nutrient concentration measurements were collected in triplicate from the two experimental enclosures before and 4 days after adding the inorganic nutrients. Concentrations of nitrate + nitrite ($NO_3 + NO_2$), nitrite, phosphate (PO_4), ammonium (NH_4) and dissolved organic carbon (DOC) were measured according to the protocols described in Fouilland *et al.* (2013).

Particulate primary production rates

The net carbon uptake rates were measured in seawater samples using ^{13}C stable isotope tracer according to the protocol described in Fouilland *et al.* (2013). When using this standard method (Slawyk *et al.*, 1977), unfixed ^{13}C-labelled DIC was usually not removed from the filters prior to POC analysis. The use of acid for removing unfixed ^{13}C is still controversial, with insignificant final effect on the ^{13}C : ^{12}C ratio of ^{13}C-enriched organisms (Bunn *et al.*, 1995).

Abundance of virioplankton, heterotrophic bacterioplankton and picophytoplankton

For microbial parameter measurements, samples were fixed with prefiltered (0.02 µm) buffered formaldehyde (2% final concentration) and stored at −80 °C until analysis.

For the enumeration of virioplankton, the number of virus-like particles (VLPs) contained in triplicates of 100-µL samples were determined after filtering on 0.02-µm-pore-size membranes (Anodisc) and stained with SYBR Gold, as described by Patel *et al.* (2007).

After thawing, samples for bacterioplankton and picophytoplankton abundance measurements were performed using a FACSCalibur flow cytometer (Becton Dickinson) as described by Pecqueur *et al.* (2011).

Diversity of heterotrophic bacteria and phytoplankton pigment concentrations

Changes in the bacterial community structure were assessed by catalysed reporter deposition fluorescent *in situ* hybridisation (CARD-FISH). Samples (5 mL) were fixed with formaldehyde (2% final concentration), filtered using 0.2-μm polycarbonate filters (Whatman) and kept at −80 °C until hybridisation according to Pernthaler *et al.* (2004). Oligonucleotide probes (Biomers) used were as follows: EUB 338 I + II + III, ALF968, BET42a, GAM42a, CF319a, targeting bacteria, *alpha*-, *beta*- and *gammaproteobacteria*, and the Bacteroidetes phylum, respectively. The NON338 probe was used as a negative control. The EUB338 detected on average 64% of the total number of cells and the NON338 detected < 2% of total cells.

For phytoplankton pigment analysis, 1–4 L of water was filtered onto 25-mm glass fibre filters (Whatman GF/F). Chlorophylls and carotenoids were extracted and analysed by high-performance liquid chromatography (HPLC; 600E, Waters) using a modified method of Zapata *et al.* (2000) as described by Vidussi *et al.* (2011). Pigments were used as chemotaxonomic markers of phytoplankton groups as described by Vidussi *et al.* (2000) and Pecqueur *et al.* (2011).

Plankton diversity and abundance

The main species dominating the phytoplanktonic community were identified by microscopy.

Heterotrophic flagellate (HF), naked ciliate and tintinnid abundances were measured using the protocols described by Pecqueur *et al.* (2011).

For mesozooplankton abundance measurement and identification, samples (60 L) were taken using three 20-L polycarbonate containers and screened onto a 60-μm sieve to collect and concentrate zooplankton in neutralised formalin (4% final concentration). Metazooplankton taxa were identified as described by Rose (1933) and Razouls *et al.* (2013), and enumerated using a Leica MZ6 dissecting microscope.

Heterotrophic bacterial production and viral mortality

The net bacterial production (BP) was estimated from the DNA synthesis rates measured by (^{3}H-methyl) thymidine (^{3}H-TdR) incorporation as described by Bouvy *et al.* (2011). BP was calculated using a conversion factor of 2×10^{18} cells mol^{-1} TdR (Bell, 1990) and 20 fgC cell^{-1} (Lee & Fuhrman, 1987). The lytic phage infection of heterotrophic bacterial cells was measured in formalin fixed water samples using the protocol described by Bouvy *et al.* (2011).

^{13}C tracing experiments

The water was collected in three 20-L carboys for each set of conditions (Fig. 1). After addition of ^{13}C-labelled sodium bicarbonate (99% of ^{13}C) at a final concentration of 60 μmol L^{-1} (^{13}C level was about 3% of total DIC concentration) to each carboy, the carboys were incubated for 24 h in the Thau Lagoon surface waters. Samples were taken from one carboy for each set of conditions twice (at the beginning and end of the 24-h incubation period), after which the carboy was removed. Sampled seawater was filtered (3–7 L max) onto 450 °C-precombusted GF/F filters, immediately (t0) and 24 h (t24) after adding the ^{13}C substrate. Only the particulate fraction collected on the GF/F filter was analysed for lipid biomarkers. A fraction of small bacterial cells may not have been retained on the precombusted GF/F filters resulting in potential underestimation of ^{13}C enrichment in bacterial biomass under all the three experimental conditions.

Lipid extraction and analysis

Filters containing the suspended particles were spiked with internal standards (5α–androstan–3β–ol and cholanic acid), and extracted by microwave oven with 40 mL of a mixture with CH_2Cl_2/MeOH (3 : 1) at 70 °C for 15 min (Tolosa *et al.*, 2008). The neutral and acid lipid fractions were isolated using the method described by Tolosa & de Mora (2004). Only data on fatty acids are shown and discussed in the present study.

Compound-specific isotope analysis

The lipid biomarkers were analysed for their stable carbon isotope composition using an HP 5890 GC equipped with a HP 7673 autoinjector and interfaced through a combustion furnace with a FINNIGAN MAT Delta C isotope ratio mass spectrometer (GC/C/IRMS). All δ^{13}C values are given as delta relative to the Pee Dee Belemnite (PDB) standard. Corrections for the isotopic change introduced in the derivatisation of fatty acids were determined by derivatisation of standards of known isotopic composition ($C_{18:0}$ fatty acid and $C_{18:0}$ FAME), applying the equation of Jones *et al.* (1991).

Calculating excess ^{13}C for the FA biomarkers and biomass production rates

The incorporation of ^{13}C in fatty acids (FA) was evaluated as the excess (above background) ^{13}C and was

expressed as specific uptake (i.e. $\Delta\delta^{13}C$ in‰ = $\delta^{13}C_{sample} - \delta^{13}C_{background}$).

The production rates or carbon uptake rates of each selected FA were calculated as follows:

$$\text{Production rate}\,(\mu g\,C\,L^{-1}\,h^{-1}) = (a_{is} - a_{ns})/(a_{ic} - a_{ns}) \times BCC/t, \quad (1)$$

where a_{is} is the atom% ^{13}C in the compound in the incubated sample, a_{ns} is the atom% ^{13}C percent of the compound in the natural sample, a_{ic} is the atom% ^{13}C in the ^{13}C-enriched inorganic carbon, BCC is the carbon concentration of the biochemical compound at the end of incubation (μg C L^{-1}), and t is the duration of incubation (hours) (Hama *et al.*, 1987). a_{ic} is derived from the natural abundance of ^{13}C in seawater and the amount of ^{13}C bicarbonate label added. When enriched seawater is used immediately after the addition of ^{13}C bicarbonate, then a_{ic} can be calculated from the mass balance such that:

$$a_{ic}C_{se} = a_{ns}C_{ns} + a_{13b}C_{13b} \quad (2)$$

where a_{ns} is atom% ^{13}C in natural seawater before enrichment, a_{13b} is atom% ^{13}C in $NaH^{13}CO_3$ added (=98%), C_{13b} is μmol ^{13}C bicarbonate added (1200 μmol), C_{ns} is μmol ΣCO_2 in natural seawater and C_{se} is total μmol of ΣCO_2 in enriched seawater at the beginning of the experiment ($C_{se} = C_{ns} + C_{13b}$).

C_{ns} was approximated from salinity using the equations described by Strickland & Parsons (1968), which gave C_{ns} values of 2218, 2206 and 2180 μmol C L^{-1} for the salinities of 35.6 (HN + HG), 35.4 (HN + LG) and 35.0 (LN + HG). The atom% ^{13}C in natural seawater (a_{ns}) was assumed to be equal to 1.1112% (=$^{13}\delta C$ of 0.0‰). The production rates of total bacterial and phytoplankton biomass were estimated from the sum of the production rate of the bacteria- and phytoplankton-specific FAs and using the conversion factors as described below.

A simple relationship between branched FAs and bacterial biomass was assumed because branched FAs are membrane components (largely phospholipids) from many groups of bacteria (Kaneda, 1991). Nevertheless, some bacteria do not produce branched FAs and Archaebacteria do not use FAs to build their membrane phospholipids. Therefore, the bacterial biomass estimates may be slightly underestimated. The first step was the conversion from bacteria-specific FAs-C (C14:0iso, C15:0iso, C15:0anteiso) into total bacterial FAs-C assuming these bacterial biomarkers account for 14% of total bacterial FAs (calculated from Boschker *et al.*, 1998). The second step was the conversion from total bacterial FAs-C to total bacterial C biomass. Bacteria in the particulate fraction were assumed to be mainly aerobic and conversion factors of 0.056 (De Kluijver *et al.*, 2010) and 0.073 (Van den Meersche *et al.*, 2011) gram of carbon FAs per gram of carbon biomass were both used.

Similarly, the phytoplankton-specific FAs-C (C20:5ω3 and C22:6ω3) were converted into total phytoplankton FAs-C assuming that these phytoplankton biomarkers account for about 15% of total FAs for all phytoplankton in general (Dalsgaard *et al.*, 2003). The label incorporated into algae was also obtained by subtracting the bacterial FAs from the total FAs. The phytoplankton-specific FAs were then converted into phytoplankton biomass using conversion factors of 0.035 (Middelburg *et al.*, 2000) and 0.046 (Van den Meersche *et al.*, 2004) gram of carbon FAs per gram of carbon biomass.

Statistical tests

All the measured variables, except ^{13}C-lipid data, were analysed by multidimensional scaling analysis (MDS) to characterise the three different conditions chemically and biologically. For this purpose, the chemical and biological variables measured in each condition were divided by the average of each variable for the three conditions and were then analysed using SYSTAT with a stress value of 0.01. The resulting graphical representation showed each variable as a plot where the relative importance between the three trophic conditions (LN + HG, HN + HG, HG + LG) can be visualised and interpreted as the distances between the points.

Results

Nutritional and trophic conditions

In the two experimental enclosures (HN + LG and HN + HG, respectively), the initial concentrations of dissolved organic carbon DOC (175 ± 12 and 181 ± 4 μmol L^{-1}), ammonium (0.19 ± 0.02 μmol L^{-1} for both enclosures), nitrate (0.18 ± 0.03 and 0.16 ± 0.04 μmol L^{-1}) and phosphate (undetectable) measured after the initial filtration and before inorganic nutrient additions were similar. The chemical and biological characteristics of the water sampled just before the ^{13}C tracking incubation under the three different conditions (HN + LG, HN + HG and LN + HG) are summarised in Table 1.

A comparison of chemical and biological characteristics by multidimensional scaling (MDS) analysis between the three conditions (Fig. 2) showed that the biomass and activity of primary producers with high inorganic nutrient concentrations and low grazing pressure (HN + LG) was much higher than in the two other conditions. The lowest abundance of microzooplankton communities (heterotrophic flagellates and ciliates) and mesozooplankton was under HN + LG conditions (Table 1). The

Table 1. Nutrient concentrations, plankton abundance and diversity, primary production, bacterial production and mortality (mean and standard deviation) measured under the three conditions: High inorganic Nutrient + Low Grazing (HN + LG), High inorganic Nutrient + High Grazing (HN + HG), Low inorganic Nutrient + High Grazing (LN + HG). n/a: not available, und: undetectable

	HN + LG		HN + HG		LN + HG	
	Mean	SD	Mean	SD	Mean	SD
Dissolved matter (μmol L^{-1})						
NH_4	0.06	0.00	0.18	0.02	0.05	0.01
NO_2	0.12	0.01	0.13	0.00	und	und
NO_3	14.97	0.11	9.62	0.04	0.04	0.020
PO_4	0.79	0.01	0.57	0.02	und	und
DOC	172	3	198	21	191	17
Planktonic abundance						
Virus (cell mL^{-1})	1.0E+08	n/a	1.5E+08	n/a	6.3E+07	n/a
Heterotrophic bacterioplankton (bac, in cell mL^{-1})	1.7E+06	1.7E+05	7.7E+06	4.1E+05	2.4E+06	4.3E+04
Cyanobacteria (cyano, in cell mL^{-1})	5.9E+02	5.5E+01	1.8E+04	1.7E+03	5.2E+03	1.4E+02
Picoeukaryote (pico, in cell mL^{-1})	5.2E+04	2.0E+03	2.2E+04	2.1E+03	4.9E+04	5.4E+04
Heterotrophic flagellates (Flag, in cell mL^{-1})	4.5E+02	1.3E+02	5.3E+02	3.5E+02	9.7E+02	4.0E+02
Naked ciliates (Cil, in cell mL^{-1})	0.19	0.09	0.63	0.24	1.32	0.14
Tintinnid (cell mL^{-1})	0.13	0.05	3.42	0.63	0.04	0.02
Mesozooplankton (Meso, in ind L^{-1})	6	n/a	42	n/a	87	n/a
Heterotrophic bacterial group (% of total EUB-bacteria)						
Alphaproteobacteria	4.10	0.60	2.30	0.30	7.10	1.10
Betaproteobacteria	0.90	0.10	0.10	0.01	0.80	0.10
Gammaaproteobacteria	11.30	1.70	17.40	2.70	63.30	9.70
Bacteroidetes	81.80	12.50	78.60	12.00	19.60	3.00
Phytoplanktonic pigments (μg L^{-1})						
Chlorophyll a (total phytoplankton biomass)	2.97	0.09	1.07	0.01	0.81	0.09
Chlorophyll b (green algae, pGreen Alg)	0.33	0.00	0.11	0.01	0.05	0.00
Alloxanthin (cryptophytes, pCryp)	0.04	0.01	0.03	0.01	0.01	0.01
19′-Butanoyloxyfucoxanthin (pelago-, prymnesio- and cryptophytes, pPelago)	0.31	0.01	und	und	und	und
Fucoxanthin (diatoms, pDiat)	3.46	0.16	1.29	0.05	1.15	0.15
19′-Hexanoyloxyfucoxanthin (prymnesiophytes, pPrym)	0.94	0.03	0.03	0.00	0.08	0.00
Peridinin (dinoflagellates, pDino)	0.06	0.01	0.10	0.01	0.03	0.01
Zeaxanthin (cyanobacteria, pCyano)	0.03	0.00	0.05	0.00	0.01	0.00
Microbial production and viral infection						
Particulate primary C production PP (μg L^{-1} h^{-1})	33.53	4.20	9.33	0.65	14.52	2.03
Heterotrophic bacterial C production BP (μg L^{-1} h^{-1})	1.21	0.06	3.85	0.09	1.61	0.01
Frequency of visibly infected bact cells (%)	0.9	n/a	3.7	n/a	1.8	n/a
Frequency of infected bact cell (%)	6	n/a	32	n/a	14	n/a
Viral-Induced Bacterial Mortality VIBM (%)	6	n/a	61	n/a	19	n/a
Mean burst size (virus. infected bact $cell^{-1}$)	30	n/a	41	n/a	30	n/a

phytoplankton community was dominated by diatoms (small 10 μm *Nitzschia*) and by flagellates (mainly prymnesiophytes). The abundance of picoeukaryote cells (5.2×10^4 cell mL^{-1}) measured was the highest under HN + LG conditions, confirming the great importance of autotrophic picoflagellates in the phytoplankton composition. The bacterial community was dominated by the Bacteroidetes group (81.8 ± 12.5%) in HN + LG conditions, the other groups being marginal. The highest particulate C primary production (PP) rate (33 μgC L^{-1} h^{-1}) and the lowest viral-induced bacterial mortality rate (6%) were also observed in HN + LG conditions. The concentration of DOC measured in HN + LG (172 μmol L^{-1}) was similar to the initial values measured before the addition of inorganic nutrient.

MDS analysis (Fig. 2) revealed that under high inorganic nutrient concentrations and grazing pressure conditions (HN + HG), there was a high abundance of tintinnid ciliates (3.42 cell mL^{-1}). Diatoms dominated the phytoplankton community (*Ceratoneis closterium* or *Nitzchia longissima*) under HN + HG with a significant proportion of various dinoflagellates (Fig. 2). Under these conditions, there was a high abundance of bacterioplankton (7.7×10^6 cell mL^{-1}), which was dominated by Bacteroidetes as in HN + LG conditions. The bacterial carbon production rate (BP) was three times greater

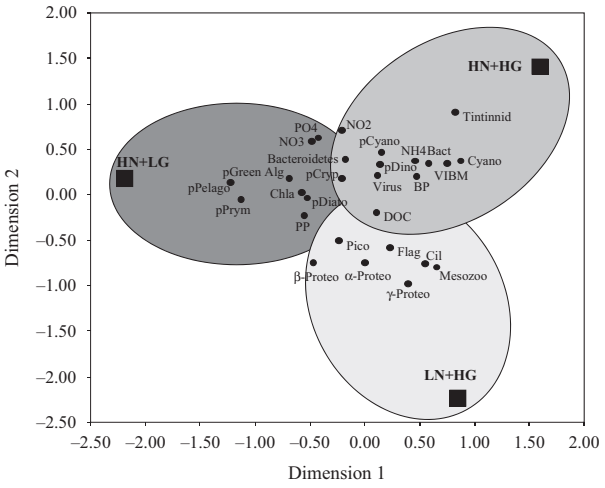

Fig. 2. Two-dimensional plots of MDS analysis of all the chemical and biological variables measured before ^{13}C trace addition under the three conditions. See Table 1 for abbreviations.

(3.85 μgC L^{-1} h^{-1}) than for the two other conditions, accounting for about 40% of PP (Table 1). The highest viral-induced bacterial mortality (61%) was observed under HN + HG conditions. The concentration of DOC was also the highest (198 μmol L^{-1}).

MDS analysis (Fig. 2) showed that under low inorganic nutrient concentration and high grazing pressure (LN + HG), there was a high abundance of mesozooplankton (especially appendicularians), naked ciliates and heterotrophic flagellates. Diatoms also dominated the phytoplankton community (similar dominant species as observed in HN + HG) with a barely significant proportion of other phytoplanktonic groups (Table 1). The bacterial community was dominated by the *Gammaproteobacteria* group (63.3 ± 9.7%) and the viral-induced bacterial mortality rate was low (19%) when compared to HN + HG conditions. The primary and bacterial production rates were in the range of values reported for the two other conditions (Table 1). The concentration of DOC (191 μmol L^{-1}) measured in LN + HG was close to that measured in HN + HG conditions.

Lipid biomarker composition and concentrations

The fatty acid (FA) concentrations (Table 2) measured just after the addition of ^{13}C (t0) under all the three conditions were mainly dominated by diatom biomarkers (C16, C16:1ω7, C14, C20:5ω3) and autotrophic flagellate biomarkers (C22:6ω3 and C18:5ω3). The highest concentrations of bacterial fatty acids (BAFAs) were found with high inorganic nutrient concentration and grazing pressure (HN + HG) with bacterial FA biomarkers, such as BrFAs and monounsaturated FAs (MUFAs) contributing up to 8% and 10% of the total FAs, respectively (Table 2). The initial concentrations (t0) of total FAs (Table 2), which is a measure of the algal and bacterial biomass together with detritus, were highest with low inorganic nutrient concentration and high grazing (LN + HG).

Specific ^{13}C uptake

The specific uptake ($\Delta\delta^{13}C$) of the lipid biomarkers for each experimental condition 24 h after introducing the ^{13}C-labelled substrate is shown in Table 2. Under all experimental conditions, $\Delta\delta^{13}C$ values for biomarker FAs for diatoms (C14, C16, C16:1ω7, C20:5ω3) and autotrophic flagellates (C18:5ω3, C18:2ω6, C18:3ω3, C18:1ω9, C22:6ω3) were generally much higher than those for heterotrophic bacteria (*i*-C14, *i*-C15, *a*-C15, C18:1ω7) after 24-h incubation.

The highest $\Delta\delta^{13}C$ values (Table 2) were measured for diatoms, autotrophic flagellates and heterotrophic bacteria with high inorganic nutrient concentrations and low grazing pressure (HN + LG). Generally, $\Delta\delta^{13}C$ values for diatom FA biomarkers were higher than for autotrophic flagellates with high inorganic nutrient concentrations and grazing pressure (HN + HG). However, similar ^{13}C enrichment was measured for the two phytoplankton groups with lower grazing pressure (HN + LG) and low inorganic nutrient concentrations (LN + HG).

The FA-derived C uptake by phytoplankton and bacteria

The total biomass production rates based on FA biomarkers ranged between 0.14 and 0.78 μgC L^{-1} h^{-1} and between 7.0 and 39.9 μgC L^{-1} h^{-1} for the heterotrophic bacterial and phytoplankton communities, respectively (Table 3). The lowest total biomass production rates were measured under high grazing conditions (HN + HG, LN + HG). The total phytoplankton biomass production rates based on ^{13}C-FAs (Table 3) were close to the particulate PP rates measured from ^{13}C-POC (Table 1) with a difference of 4% on average.

Discussion

Methodological and general considerations

The experimental procedures used in this study allowed us to investigate the effects of different inorganic nutrient availability and grazing pressure conditions on the direct carbon coupling between coastal bacterial and phytoplankton communities. It was assumed that the different initial filtration of the two experimental enclosures did

Table 2. Selected lipid biomarker concentrations (ng L^{-1}) and specific uptakes ($\Delta\delta$13C in ‰) measured during the first 24-h incubation period with the ^{13}C-labelled substrate under the three conditions: High inorganic Nutrient + Low Grazing (HN + LG), High inorganic Nutrient + High Grazing (HN + HG), Low inorganic Nutrient + High Grazing (LN + HG). Difference (expressed in%) between lipid concentrations measured between T24 and T0 is set in brackets. und: undetectable

	HN + LG			HN + HG			LN + HG		
	Lipid concentrations		Specific	Lipid concentrations			Lipid concentrations		Specific
	t0	t0+24 h	uptake	t0	t0+24 h	Specific uptake	t0	t0+24 h	uptake
Diatoms									
C14	3175	13286 (+318)	1657	2355	5608 (+138)	875	7109	13564 (+91)	1120
C16:4	1235	3218 (+161)	1313	533	1697 (+218)	880	692	970 (+40)	358
C16:1ω7	2504	11575 (+362)	1715	2968	6191 (+109)	798	7035	13235 (+88)	1047
C16	3067	12766 (+316)	1670	4656	6582 (+41)	654	7166	13513 (+89)	1228
C20:5ω3	2352	5736 (+144)	1428	1861	4834 (+161)	856	2085	4181 (+100)	581
C18:4ω3	1808	5450 (+201)	1674	693	1509 (+118)	922	1107	1895 (+71)	939
Autotrophic flagellates									
C18:5ω3	1104	3180 (+188)	1412	361	471 (+31)	577	589	1113 (+89)	878
C18:2ω6	722	1962 (+172)	1661	606	660 (+9	522	661	1331 (+101)	1202
C18:3ω3	863	2384 (+176)	1533	404	535 (+32)	825	796	1718 (+116)	1256
C18:1ω9	477	973 (+104)	1434	971	759 (−22)	381	556	897 (+61)	1002
C18	383	585 (+53)	903	957	632 (−34)	200	600	820 (+37)	983
C22:6ω3	1447	3662 (+153)	1234	1154	1692 (+47)	432	2811	5038 (+79)	418
Heterotrophic bacteria									
i -C14	281	486 (+73)	249	267	268 (+0)	84	129	193 (+50)	und
i -C15	253	449 (+78)	586	576	554 (−4)	104	345	472 (+37)	251
a -C15	123	225 (+82)	400	350	342 (−2)	91	166	255 (+54)	216
C15	159	451 (+184)	1334	260	326 (+26)	396	323	528 (+64)	641
i -C17	27	44 (+64)	und	66	64 (−3)	und	41	68 (+64)	und
a -C17	33	66 (+98)	und	69	66 (−5)	und	51	116 (+129)	und
C17:0cyclo	37	64 (+74)	und	57	77 (+35)	und	46	51 (+12)	und
C17	52	83 (+61)	und	100	90 (−9)	und	83	139 (+68)	und
C18:1ω7	1270	1748 (+38)	613	2352	1850 (−21)	131	1053	1647 (+56)	409
SAFAs*	7361	28293 (+284)		9055	14254 (+57)		15629	29144 (+86)	
BrFAs†	953	1790 (+88)		1864	1966 (+5)		1266	2111 (+67)	
MUFAs‡	4446	14868 (+234)		6579	9253 (+41)		9166	16684 (+82)	
PUFAs§	10759	28192 (+162)		6386	12904 (+102)		9843	18219 (+85)	
BAFAs¶	2481	4097 (+65)		4664	4329 (−7)		2800	4551 (+63)	
Total FAs	23520	73144 (+211)		23883	38378 (+61)		35905	66159 (+84)	

*Saturated FAs (*n*-C_{12}-C_{26}).

†Branched FAs (*i*-C_{13}, *a* -C_{13}, *i* -C_{14}, methyl$C_{14:1}$, *i* -C_{15}, *a* -C_{15}, methyl$C_{16:2}$, *i*-C_{17}, *a* -C_{17}, $C_{17:0}$cyclo).

‡Monounsaturated FAs ($C_{14:1}$, $C_{16:1\omega7}$, $C_{18:1\omega9}$, $C_{18:1\omega7}$, $C_{20:1}$, $C_{22:1}$, $C_{24:1}$).

§Polyunsaturated FAs ($C_{16:2}$, $C_{16:3}$, $C_{16:4}$, $C_{18:4\omega3}$, $C_{18:5}$, $C_{18:2\omega6}$, $C_{18:3\omega3}$, $C_{20:4}$, $C_{20:5}$, $C_{20:4}$, $C_{20:3}$, $C_{22:4}$, $C_{22:6}$, $C_{22:5}$).

¶Bacterial FAs (BrFAs, *n* -C_{13}, *n* -C_{15}, *n* -C_{17}, *n* -C_{19}, *n* -C_{21}, $C_{18:1\omega7}$).

not significantly affect the initial chemical conditions. This was confirmed by the similar nutrient concentrations measured just after the initial filtration and before adding the inorganic nutrients to the two enclosures. Samples for ^{13}C incubation (primary production and C tracking) collected from the three conditions were all incubated in the Thau Lagoon surface waters, and they may have experienced some light inhibition similar to that found in stratified hydrodynamic conditions. This may have affected the primary production and the release of dissolved organic matter. However, a positive or negative effect of photoinhibition on photosynthetic extracellular release has not been clearly demonstrated (Nagata, 2000). All the incubations for primary production rates and C tracking were performed under similar *in situ* light and temperature conditions. The effect of photoinhibition during the incubation period, if any, will have affected all the results similarly, with no consequence on the significant difference observed between the three nutritional and grazing conditions.

The addition of nitrate and phosphate combined with dilution by seawater (HN + LG) led to the growth of both diatoms and autotrophic flagellates helped by the low abundance of their potential predators (ciliates and mesozooplankton). With the same level of inorganic nutrient addition but without dilution by seawater

Table 3. Estimated biomass production rate (μgC L^{-1} h^{-1}) for heterotrophic bacteria and phytoplankton using a combination of different conversion factors, for the three conditions: High inorganic Nutrient + Low Grazing (HN + LG), High inorganic Nutrient + High Grazing (HN + HG), Low inorganic Nutrient + High Grazing (LN + HG)

Conversion factors used	HN + LG	HN + HG	LN + HG
Heterotrophic bacteria			
Σiso, anteiso = 14% total FAs			
0.056 g FAs-C/g C biomass	0.78	0.18	0.29
0.073 g FAs-C/g C biomass	0.60	0.14	0.23
Phytoplankton			
C20:5 + C22:6 = 15% total FAs			
0.035 g FAs-C/g C biomass	33.4	12.8	12.0
0.046 g FAs-C/g C biomass	25.4	9.8	9.2
Total FA-bacterial FAs uptake			
0.035 g FAs-C/g C biomass	39.9	9.2	22.3
0.046 g FAs-C/g C biomass	30.4	7.0	17.0

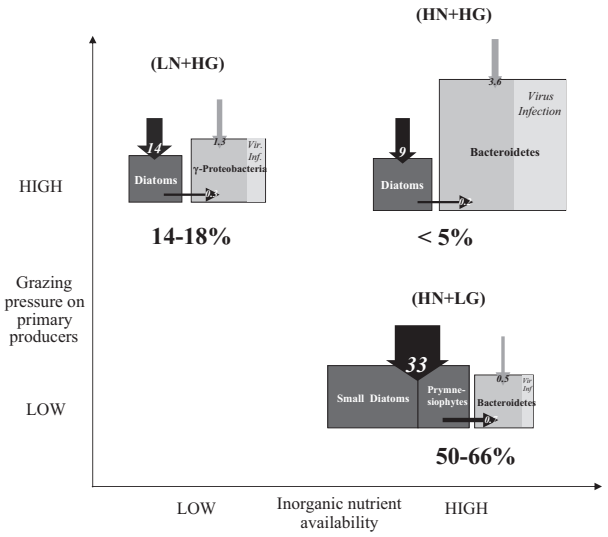

Fig. 3. Carbon fluxes estimated for phytoplankton and bacterial community under the three conditions (High inorganic Nutrient and High Grazing: HN + HG, High inorganic Nutrient and Low Grazing: HN + LG, and Low inorganic Nutrient and High Grazing: LN + HG). The contribution of freshly produced phytoplankton exudates to total bacterial production is expressed in%. The names of the dominant groups identified by pigment and CARD-FISH are shown for the two microbial communities. The phytoplankton and bacterial stocks are proportional to the phytoplankton pigment concentrations measured by HPLC and to the bacterioplankton abundance measured by flow cytometry under the three different conditions (see M&M section for method details). The fraction of the bacterial stock infected by viruses is represented within the bacteria boxes. The rates (μgC L^{-1} h^{-1}) of phytoplankton uptake of DIC, bacterial uptake of DOC and phytoplankton carbon exudates are shown within the corresponding proportional width arrows.

(HN + HG), the heterotrophic bacterial community grew, probably helped by the high availability of dissolved organic carbon (DOC) owing to the high grazing pressure on the phytoplankton community (high abundance of tintinnid ciliates). This is supported by the high ratio of total FAs to chlorophyll *a* reported under high grazing conditions (both HN + HG and LN + HG), strongly suggesting a high proportion of nonphytoplankton lipids, for example detritus and faunal material. The plankton community sampled in Thau Lagoon surface waters with low inorganic nutrient availability (LN + HG) also had a high abundance of predators on phytoplankton and bacteria (heterotrophic flagellates, naked ciliates and mesozooplankton such as appendicularians) relatively to the abundance of their prey, suggesting a strong grazing control on these two communities.

The effect of both inorganic nutrient availability and grazing pressure on the source of the carbon used for bacterial production was investigated for the first time by tracking the short-term transfer (24 h) of carbon from phytoplankton to the bacterial community using ^{13}C biomarkers. Incorporation of ^{13}C by bacteria over a short time scale (first 24 h after ^{13}C-labelled bicarbonate was added) is attributed to the bacterial growth using extracellular carbon exudates freshly produced by the phytoplankton community (Boschker & Middelburg, 2002). After several days, the ^{13}C enrichment in bacterial biomarkers may come from recycled phytoplankton biomass through cell death, viral lysis and sloppy feeding by mesozooplankton, as suggested by Van den Meersche *et al.* (2011). In the present study, a 24-h incubation with ^{13}C-labelled bicarbonate was used as a good compromise between ensuring that the levels of ^{13}C enrichment in lipids was high enough to provide significant quantitative detection and short enough to ensure that there was little ^{13}C recycling of phytoplankton biomass through lysis or grazing. We also assumed a constant bacterial incorporation rate of labelled ^{13}C over the whole 24-h incubation period. The lowest bacterial ^{13}C enrichments (Table 2) were found under High Nutrient and High Grazing (HN + HG) conditions where the highest bacterial production rate was measured (3.85 μgC L^{-1} h^{-1}). This supports the assumption that the transfer of labelled fixed carbon to bacteria through the action of grazers or lysis over the 24-h incubation was unlikely. All the results are shown in Fig. 3.

Strong direct C coupling between bacteria and phytoplankton under early phytoplankton bloom conditions

The addition of nitrate an phosphate with a reduced abundance of ciliates and mesozooplankton (HN + LG)

helped the growth of some phytoplankton species as shown by the high concentration of diatoms and autotrophic flagellate pigments (i.e. prymnesiophyte pigments). The high $\Delta\delta^{13}C$ values measured for HN + LG indicated high growth rates of the phytoplankton species. This was shown by the ^{13}C incorporation in phytoplankton biomarkers, both diatoms and autotrophic flagellates, being the highest under these conditions. The heterotrophic bacterial production (BP) and bacterioplankton abundance were the lowest for HN + LG, and BP accounted for < 4% of the primary production (PP). Such a low ratio is typical of highly productive marine waters (reviewed in Fouilland & Mostajir, 2010) as observed during the phytoplankton bloom development in coastal waters where phytoplankton grew exponentially under high nutrient availability (e.g. Ducklow & Kirchman, 1983). Under such conditions, a high level of phytoplankton exudates is expected if the exudation of freshly produced metabolites depends on the rate of total carbon fixation, as recently reported by López-Sandoval *et al.* (2013) for diverse marine phytoplankton species. A substantial use of phytoplankton exudates by heterotrophic bacteria was thus expected. The incorporation of ^{13}C into heterotrophic bacterial biomarker FAs showed the fastest uptake of newly produced phytoplankton-derived C by heterotrophic bacteria in HN + LG. The heterotrophic bacterial uptake of phytoplankton-derived carbon was estimated at about 0.6–0.8 μgC L^{-1} h^{-1} when estimated from the bacterial-specific FAs (Table 3) and accounted for more than half of BP (1.21 μgC L^{-1} h^{-1}, Table 1). Therefore, the bacterial use of freshly released phytoplankton carbon accounted for 50–66% of BP. The remaining fraction of BP (44–50%) may be explained by the use of other carbon sources (e.g. phytoplankton biomass recycling or background DOC from Thau Lagoon). No significant change in the bulk DOC concentrations was observed before and after 4 days of incubation with addition of inorganic nutrients under HN + LG conditions. This may be due to the low DOC bacterial uptake (about 1 μmol L^{-1} day^{-1}) measured in HN + LG, representing < 1% of the bulk DOC concentrations. Even though the reactive DOC pool may account for a large fraction of the total DOC during a phytoplankton bloom, the dynamics of this reactive pool may only partly explain the total DOC dynamics (Grossart *et al.*, 2007).

Our results suggest that bacterial C production may be strongly dependent on freshly produced phytoplankton exudates during the nutritional and grazing conditions leading to the phytoplankton bloom in Mediterranean coastal waters. This is in agreement with some previous field studies suggesting that heterotrophic bacteria are closely associated with phytoplankton blooming species in coastal marine environments (Rooney-Varga *et al.*, 2005).

Reduced direct carbon coupling between bacteria and freshly produced phytoplankton exudates under strong grazing control

Both the highest concentrations of BAFAs and the highest ratios of branched-C15:*n*-C15 FAs (a proxy for heterotrophic bacterial growth; Najdek *et al.*, 2002) were measured under high inorganic nutrient and high grazing pressure (HN + HG). These values were consistent with the high heterotrophic bacterial biomass and production under these conditions (BP accounted for 40% of PP). Despite the very high BP measured under HN + HG conditions, the heterotrophic bacterial uptake of freshly produced phytoplankton carbon was estimated at about 0.14–0.18 μgC L^{-1} h^{-1} using heterotrophic bacterial biomarkers over 24-h incubation, which accounted for < 5% of BP. This suggests that most of BP was based on other sources of carbon such as phytoplankton biomass recycled through grazing or viral lysis. The viral-induced bacteria mortality was estimated at 61% of BP under these conditions, which was much higher than for HN + LG and LN + HG conditions (6% and 19%, respectively). The high availability of DOC measured under HN + HG conditions supports the hypothesis that the DOC released from viral lysis and grazing on phytoplankton may have been the main contribution to BP, supporting the conclusions reached by Wilhelm & Suttle (1999) and Nagata (2000).

Similarly, the direct carbon coupling between bacteria and phytoplankton was also weak under low inorganic nutrient, high grazing conditions (LN + HG), when both bacteria and phytoplankton were under strong grazing pressure. The heterotrophic bacterial uptake of freshly produced phytoplankton exudates was estimated at about 0.23–0.29 μgC L^{-1} h^{-1} using lipid biomarkers and accounted for only 14–18% of BP under LN + HG conditions. Under low inorganic nutrient availability conditions, there should be a high release of organic carbon by phytoplankton owing to a self-induced release mechanism under stress (Nagata, 2000). Heterotrophic bacteria were therefore expected to assimilate the freshly produced DOC under limited inorganic nutrient conditions for phytoplankton, such as previously observed during an experimental postbloom phase (Norrman *et al.*, 1995). However, our results suggest that heterotrophic bacteria mainly used other sources of carbon for BP under LN + HG conditions. Under these conditions, bacterial production appeared to rely more on organic matter released from strong grazing pressure on microorganisms (both phytoplankton and

bacteria), indicating the important role of the microbial C recycling under such conditions.

Potential role of phytoplankton and bacterial community composition in determining the direct carbon coupling

There were some significant differences in phytoplankton and bacterial community composition between the three conditions. Although diatoms were the dominant phytoplanktonic group in all the three conditions, the strongest direct C coupling between bacteria and phytoplankton exudates was measured in HN + LG when small diatoms and autotrophic flagellates (i.e. prymnesiophytes) were the main components of the phytoplankton community. This suggests that heterotrophic bacteria may strongly depend on organic C released by small autotrophic cells.

With the bacterial phylum probes used in this study, no significant difference in bacterial community structure was observed between HN + LG and HN + HG conditions. The Bacteroidetes dominated even though there was a significant difference in the bacterial carbon dependence on phytoplankton exudates. Our results suggest that the level of phytoplankton-derived carbon used for bacterial production may not be sufficient to affect significantly the composition of the bacterial community. However, there are some doubts about the specificity of the probe used (Amann & Fuchs, 2008) and the phylum level oligonucleotide probes used may not have revealed the full diversity.

Conclusions

Our results illustrate the effect of both inorganic nutrient availability and grazing pressure on the direct carbon coupling between bacteria and freshly produced phytoplankton exudates in coastal waters. Strong direct carbon coupling appears to be a transient event occurring during phytoplankton blooms. Short-term heterotrophic bacteria production in coastal waters seems to rely mainly on organic matter provided by grazing and viral lysis on phytoplankton and bacteria. This apparently contradicts some recent observations indicating a larger than expected direct carbon coupling between bacteria and phytoplankton in a whole estuarine ecosystem (Morán *et al.*, 2013) based on measurements of dissolved primary production and bacterial carbon demand. Differences in the methodology used may explain this discrepancy. If some labelled phytoplankton biomass recycled through lysis or grazing was included in our measurements, this may have led to an overestimation of the bacterial uptake of freshly produced phytoplankton exudates. This would not change our main conclusions. Furthermore, our results are in agreement with previous studies performed in various geographical areas where the bacterial uptake of freshly produced phytoplankton exudates, estimated from size fractionation, represented on average < 40% of BP (Fouilland & Mostajir, 2010).

The present study highlights that the predation and viral lysis within the microbial food web plays a greater role in sustaining the bacterial production in coastal waters than direct use of carbon from freshly produced phytoplankton exudates, which is apparently restricted to the early bloom period.

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