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Factors Controlling the Year-Round Variability in Carbon Flux Through Bacteria in a Coastal Marine System

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Abstract

Data from several years of monthly samplings are combined with a 1-year detailed study of carbon flux through bacteria at a NW Mediterranean coastal site to delineate the bacterial role in carbon use and to assess whether environmental factors or bacterial assemblage composition affected the in situ rates of bacterial carbon processing. Leucine (Leu) uptake rates [as an estimate of bacterial heterotrophic production (BHP)] showed high interannual variability but, on average, lower values were found in winter (around 50 pM Leu^{-1} h^{-1}) as compared to summer (around 150 pM Leu^{-1} h^{-1}). Leu-to-carbon conversion factors ranged from 0.9 to 3.6 kgC mol Leu^{-1}, with generally higher values in winter. Leu uptake was only weakly correlated to temperature, and over a full-year cycle (in 2003), Leu uptake peaked concomitantly with winter chlorophyll a (Chl *a*) maxima, and in periods of high ectoenzyme activities in spring and summer. This suggests that both low molecular weight dissolved organic matter (DOM) released by phytoplankton, and high molecular weight DOM in periods of low Chl *a*, can enhance BHP. Bacterial respiration (BR, range 7–48 μg C l^{-1} d^{-1}) was not correlated to BHP or temperature, but was significantly correlated to DOC concentration. Total bacterial carbon demand (BHP plus BR) was only met by dissolved organic carbon produced by phytoplankton during the winter period. We measured bacterial growth efficiencies by the short-term and the long-term methods and they ranged from 3 to 42%, increasing during the phytoplankton blooms in winter (during the Chl *a* peaks), and in spring. Changes in bacterioplankton assemblage structure (as depicted by denaturing gradient gel electrophoresis fingerprinting) were not coupled to changes in ecosystem functioning, at least in bacterial carbon use.

Key words: bacterioplankton; production; respiration; carbon; marine; seasonality; growth efficiency; coastal.

Introduction

Marine bacterioplankton plays a central position in the flux of carbon in the ocean with two main ecological roles: (1) the incorporation of dissolved or-

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ganic matter (DOM) into biomass (that is, bacterial heterotrophic production, BHP) and (2) the remineralization of organic carbon (that is, bacterial respiration, BR). A large number of studies have focused on determining BHP and bacterial biomass in the ocean (Ducklow and Carlson 1992), resulting in a substantially larger dataset compared to BR measurements (Robinson and Williams 2005; Robinson in press). However, most (generally over 80%) of bacterial carbon demand (BCD) is accounted for by BR, and thus this parameter currently constitutes one of the biggest gaps in our understanding of carbon cycling by bacterioplankton (Janhke and Craven 1995; del Giorgio and Cole 2000).

Bacterial respiration measurements are needed to estimate not only BCD, but also bacterial growth efficiency (BGE), that is, the proportion of carbon consumed by bacteria that can be transferred to higher trophic levels (BGE = BHP/(BHP + BR)). A wide range of BGEs has been reported from marine waters (1 to >60%, del Giorgio and Cole 1998), and there is great interest in constraining these values across spatial and temporal scales for the purposes of large-scale modeling, especially in the oligotrophic areas, which cover most of the oceans. High uncertainties are not only associated with many current estimates of BGE because of methodological problems (Briand and others 2004; Alonso-Sáez and others 2007c), but also importantly the in situ controlling factors of BGE and BCD are still unclear, despite the great importance of these parameters for determining carbon biogeochemistry.

Temperature, nutrient concentrations, DOM lability, and bacterial taxonomic composition have been regarded among the main factors that can influence BGE and DOM consumption by bacteria. Significant correlations between temperature and BHP (White and others 1991) or BGE (Rivkin and Legendre 2001) have been found on large-scale comparative analyses. However, in particular field studies and with some exceptions (Apple and others 2006), the relative importance of temperature as a modulator of these parameters is less clear (del Giorgio and Cole 1998). The few previous seasonal studies carried out in marine waters that included BGE estimates suggest that this parameter is mainly determined by DOC lability (Lemée and others 2002; Reinthaler and Herndl 2005). Importantly, no attention has been paid to changes in bacterial assemblage structure, even if distinct phylogenetic groups of bacteria could carry out different patterns of organic carbon metabolism (for example, Cottrell and Kirchman 2000).

In this study we aimed to (1) characterize the flux of carbon through bacterioplankton in a coastal oligotrophic system, and (2) analyze the factors influencing the different elements of this flux. We used a comprehensive approach, including the measurement of parameters that are seldom assessed together in seasonal studies. These included BR and BGEs, empirical leucine-to-carbon conversion factors (eCFs) (to accurately constrain BHP), ectoenzyme activities (as indicators of the bacterial consumption of high molecular weight, HMW-DOM), and dissolved primary production (dPP) by phytoplankters, which is supposed to be one primary source of carbon for bacteria. Data from a full annual cycle were combined to longer datasets of some of the main variables. In analyzing the underlying controlling factors on BGE and BCD, we included not only environmental parameters [such as temperature, nutrients, chlorophyll *a* (Chl *a*)], but also the effect of changes in bacterial assemblage structure, which has been reported in detail elsewhere (Alonso-Sáez and others 2007b).

Materials and Methods

Sampling and Basic Parameters

We carried out a monthly study in a station located in the Northwestern Mediterranean, Blanes Bay (The Blanes Bay Microbial Observatory) from March 2001 to March 2006. From 4 March 2003 to 22 March 2004 a full study was conducted including a wider range of environmental and bacterial parameters (Table 1), some of which were measured until March 2006. During the two first years (2001 and 2002) only temperature, Chl *a*, and inorganic nutrients were measured (with the exception of phosphate, Table 1). Surface waters were sampled at about 1 km offshore (41°40′N, 2°48′E), filtered through a 200 μm mesh net and transported to the lab under dim light (within 1.5 h) in 25 l polycarbonate carboys. Surface water temperature was measured in situ with a mercury thermometer. For determination of Chl *a* concentration, 150 ml of seawater was filtered on GF/F filters (Whatman) and subsequently extracted in acetone (90% v/v) in the dark at 4°C for 24 h. Fluorescence was measured with a Turner Designs fluorometer.

Chemical Analyses

Samples were filtered through 0.2 μm pore size polycarbonate filters (47 mm diameter, Supor-200; Gelman sciences) using a polycarbonate filtration device (Millipore). Dissolved inorganic nutrient concentrations were determined spectrophotomet-

Table 1. Summary of the Variables Measured for this Study Including Sampling Period, Average (±SE), Maximum (max), and Minimum (min) Values

Variable	Period	Max	Min	Average (±SE)
Temperature (°C)	March 01–March 06	26	12	17 (±0.5)
Nitrate (μM)	March 01–March 06	7	0.03	1.49 (±0.17)
Silicate (μM)	March 01–March 06	7.6	0.26	2.02 (±0.17)
Phosphate (μM)	March 03–March 04	0.2	0.01	0.07 (±0.01)
Chl *a* (μg l^{-1})	March 01–March 06	3.93	0.02	0.73 (±0.07)
DOC (μM)	March 03–March 04	177	83	109 (±11)
Particulate PP (mgC m^{-3} d^{-1})	March 03–December 05	66	2	17 (±2)
PER (%)	March 03–March 04	73	21	45 (±4)
Bacterial abundance (10^6 cell ml^{-1})	January 03–March 06	1.98	0.34	0.94 (±0.43)
Leu uptake (BHP) (pmol Leu l^{-1} h^{-1})	January 03–March 06	493	2.8	73.1 (±12.5)
Empirical CF (kgC mol Leu^{-1})	January 03–January 04	3.62	0.98	1.73 (±0.24)
Bacterial respiration (μgC l^{-1} d^{-1})	March 03–March 04	48	4	22 (±4)
BGE short-term (%)	March 03–March 04	42	3	17 (±4)
BGE long-term (%)	March 03–March 04	20	1	8 (±2)
Beta-glucosidase act. (nmol l^{-1} h^{-1})	January 03–March 04	41	8	23 (±3)
Leu-aminopeptidase act. (nmol l^{-1} h^{-1})	January 03–March 04	1,150	133	451 (±72)
Beta-xylosidase act. (nmol l^{-1} h^{-1})	January 03–March 04	46	1	9.4 (±3.2)
Quitinase act. (nmol l^{-1} h^{-1})	January 03–March 04	36	1	7.8 (±2.6)

rically with an Alliance Evolution II autoanalyzer following standard procedures (Grasshof and others 1983), except for phosphate concentration, which was determined manually, using a 10-cm cuvette to increase the detection limit (Pinhassi and others 2006). For analysis of DOC concentration, filtered samples (20 ml) were acidified with 16 mM HCl (final conc.) in acid-clean polypropylene tubes and stored at 4°C until analysis. DOC was measured with a high temperature carbon analyzer (Shimadzu TOC 5000).

Bacterial Abundance and Biomass

Samples (1.6 ml) were preserved with 1% paraformaldehyde + 0.05% glutaraldehyde (final conc.). Bacterial abundance (BA) was analyzed by flow cytometry (FACSCalibur cytometer, Becton and Dickinson) after staining with Syto13 (Molecular probes). Bacteria were detected by their signature in a plot of side scatter versus FL1 (green fluorescence) as explained in Gasol and del Giorgio (2000). Picocyanobacteria were discriminated in a plot of FL1 versus FL3 (red fluorescence). Flow cytometric counts were calibrated with DAPI counts. Bacterial cell size (biovolume) was estimated using the relationship between average bacterial size (obtained by image analysis of DAPI preparations following common procedures) and average fluorescence of the SYTO-13 stained sample relative to beads as shown by Gasol and del Giorgio (2000). Bacterial biomass was calculated by using the volume-to-carbon relationship derived by Norland (1993): pgC $cell^{-1}$ = 0.12 pg (μm^3 $cell^{-1}$)$^{0.7}$.

Bacterial Heterotrophic Production

Bacterial heterotrophic production was estimated using the [^{3}H]-leucine (Leu) incorporation method (Kirchman and others 1985). For each sample, triplicate or quadruplicate aliquots (1.2 ml) and one or two TCA killed controls were incubated with 40 nmol l^{-1} Leu for about 2 h at in situ temperature in the dark. The incorporation was stopped with the addition of 120 μl of cold TCA 50% to the vials and samples were kept frozen at −20°C until processing, which was carried out by the centrifugation method of Smith and Azam (1992). Finally, samples were counted on a Beckman scintillation counter 24 h after the addition of 1 ml of scintillation cocktail (Optiphase Hisafe2, Perkin-Elmer). During 2003, eCFs were determined monthly in replicate dilution cultures, which were also used to determine one estimate of BGE (referred to as BGE long-term). Details on the preparation of the seawater dilution cultures are described below. Conversion factors were computed with the cumulative (Bjørnsen and Kuparinen 1991) and the integrative (Riemann and others 1987) methods.

Ectoenzymatic Activities

For the determination of the ectoenzyme activities, we used fluorogenic substrates (Hoppe 1983) fol-

lowing a modification of the methodology described in Sala and others (2001). The substrates 4-MUF-beta-D-glucoside for beta-glucosidase, L-leucine-7-amido-4-methyl-coumarin for Leu-aminopeptidase, 4-MUF-beta-D-xyloside for beta-xylosidase, and 4-MUF-*N*-acetyl-alpha-D-glucosaminide for chitinase were added at saturating concentrations (100 µM final concentration) to 0.9 ml replicate subsamples. Fluorescence was measured immediately after addition, and after an incubation of 1–3 h in the dark at room temperature. Fluorescence was read on a Shimadzu spectrofluorometer RF-540 at 365 nm excitation and 446 nm emission wavelengths. An increase in fluorescence units during the incubation time was converted into activity by preparing a standard curve with the end product of the reaction: 7-amino-4-methyl-coumarin for aminopeptidase and 4-methyllumbelliferone for the other enzymes. Samples for leu-aminopeptidase activity determination were previously filtered through 1 µm (Millipore) size pore filters to avoid phytoplanktonic activity.

Bacterial Respiration

Bacterial respiration was measured by following changes in dissolved oxygen during dark incubations of filtered water (0.8 µm, mixed esters of cellulose) to include only the bacterial fraction. We found that, after filtration, we recovered on average (±SE) 48 ± 4% of total bacteria and, on average (±SE), BR accounted for 71 ± 12% of total community respiration (CR). In October 2003, a strong increase in the respiration rate of the filtered fraction compared to the unfiltered water was found (almost threefold). Therefore, for this month, the measurement obtained from the unfiltered water, and not the filtered fraction, was used as the estimate of BR. Boro-silicate glass bottles were carefully filled and four to eight replicates were immediately fixed with Winkler reagents to determine the initial oxygen concentration. Eight to ten replicate bottles were incubated in the darkness at in situ temperature and fixed with Winkler reagents after 24 h. Dissolved oxygen measurements were made with an automatic titrator (DL50 Graphix, Mettler Toledo) based on potentiometric endpoint detection (Outdot and others 1988). The rate of respiration was determined by regressing the oxygen concentration against the time in which the samples were withdrawn. This estimation assumes that the disappearance of oxygen was linear (Model II regression) and the slope of the regression is equal to the respiration rate. We assumed a respiratory quotient of 0.88 (Williams and del Giorgio 2005).

Bacterial Growth Efficiency

Bacterial growth efficiency was estimated as the ratio of bacterial production to total carbon demand by two independent approaches. In the first approach (BGE short-term), BR was estimated as described in the previous section, and bacterial net production was estimated as the average between BHP at time zero of the incubation experiment (0.8 µm-filtered seawater, with eCFs) and as the change in bacterial biomass estimated from the increase in cell biomass during the 24 h incubation (Alonso-Sáez and others 2007c). In the second approach (BGE long-term), we followed the increase in bacterial biomass (the only POC produced in the experiment) and the decrease in the concentration of DOC in seawater cultures (Carlson and Ducklow 1996). For each dilution culture, approximately 1,900 ml of sample was filtered through a 0.2 µm pore size Sterivex filter capsule (Millipore) using a peristaltic pump. The inoculum (100 ml) was prepared by gravity filtration (0.8 µm pore-size polycarbonate filter, Nucleopore) and added to obtain a 20-fold dilution of BA. Seawater cultures were incubated at in situ temperatures in the dark for 2–4 days. Samples for BA and Leu uptake determination were taken every 8–24 h. Samples for DOC were taken in replicates at the beginning and at the end of the incubation, to determine DOC consumption (initial DOC–final DOC).

Primary Production

For the measurement of particulate primary production (pPP), thirteen 70 ml-bottles (Corning) and one dark control were filled with seawater and inoculated with (10 µCi) $NaH^{14}CO_3$. The incubation was carried out in a water bath at in situ temperature for 2 h in a gradient of light irradiance (ca 10–1,000 µmol photons m^{-2} h^{-1}). Light was measured with a small size spherical light meter (Illuminova AB, Sweden). After the incubation, the samples were filtered at low vacuum pressure through cellulose ester filters (Millipore 0.22 µm), and the filters were subsequently exposed overnight to concentrated HCl fumes. Scintillation cocktail (4 ml Optiphase Hisafe 2) was added to each filter, and the radioactivity was measured in a Beckman LS6000 scintillation counter. Total in situ pPP was determined from the P–E curve and the in situ irradiance obtained with a Li-Cor sensor (Li-193S). Parallel to these incubations, rates of dPP were estimated in a different set-up. Twenty-two

bottles (Corning) were filled with sample and ten of them were covered with aluminum foil. They were inoculated with the same amount of ^{14}C and incubated for 2.5 h at a fixed light intensity (ca. 500 µmol photons m^{-2} h^{-1}). Two clear bottles were taken at time zero, and after that, every half an hour two clear and two dark bottles were taken, the samples filtered through Millipore filters, and the radioactivity measured on both, filters (labeled POC, pPP) and filtrates (DOC, dPP). The filters were treated as described above and the filtrates were acidified with 1 ml of HCl 1 N and left open in an orbital shaker for 12 h to remove inorganic ^{14}C. Scintillation cocktail was then added to both filters and filtrates. Average percentages of labeled DOC over total labeled carbon (DOC + POC) over the time-course evolution were used to calculate the percentage of photosynthetically extra-cellular release (PER), manually checking, and removing data when DOC production had reached an asymptote or started to decrease due to bacterial consumption. Total primary production (tPP) was calculated as the sum of pPP and dPP. Rates of PP were converted from hourly to daily values using the average irradiance at the Blanes latitude and the Straskraba and Gnauck (1985) model. We assume that the in situ irradiance at 10:00 am (sampling time) was the average irradiance of the day (light period).

DGGE

Details of the analysis of seasonal samples by DGGE are given in Alonso-Sáez and others (2007b). In brief, genes encoding for 16S rRNA were amplified with primers 358f and 907rM, and DGGE analysis was performed as previously described (Schauer and others 2003). Each band was considered one OTU, and a similarity matrix was constructed for all DGGE lanes taking into account the relative contribution of each band (in percentage) to the total band intensity of the lane.

Statistical Treatment

Non-metric multidimensional scaling (MDS, Primer v5) and Ward's clustering method (Euclidean distances, Statistica 6.0) were used to analyze the similarities between the samples based on (1) bacterial assemblage structure (DGGE OTUs intensities), (2) environmental factors (temperature, Chl *a*, DOC concentration and nitrate), and (3) carbon processing including the following parameters: BHP (Leu uptake using eCF), BR, bacterial biomass, and ectoenzyme activities. Pearson *r* coefficients are given for correlation analyses with the exception of small data sets ($n < 20$), in which non-parametric correlation analyses were used, and Spearman *rho* coefficients are given.

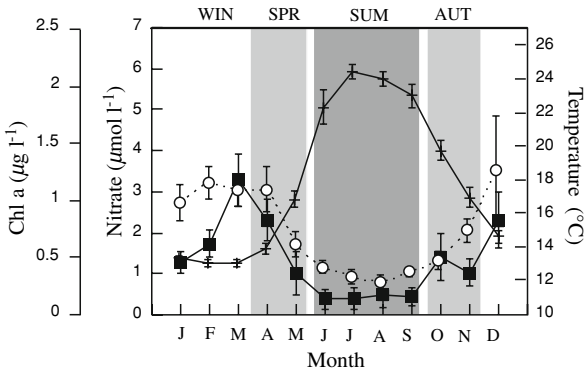

Figure 1. Monthly averaged measurements of temperature (*black line*), Chl *a* (*open circles*), and nitrate concentration (*squares*) during the period March 2001–March 2006, including a distinction of winter, spring, summer, and autumn periods based on the values of these parameters (see text).

Results

Environmental Factors

Average 6-year (March 2001–2006) series of monthly measurements of several environmental parameters such as temperature, nitrate, and Chl *a* concentration are shown in Figure 1. The summer period, from June to September, is characterized by high water temperature (over 22°C, Figure 1), and low concentrations of inorganic nutrients and Chl *a* (ca. 0.4 µg Chl *a* l^{-1}, Figure 1). In contrast, the winter period from December to March shows water temperatures around 13°C, and higher concentrations of nutrients and Chl *a* (over 1 µg Chl *a* l^{-1}, Figure 1). The concentrations of nitrate and silicate (details not shown) usually peak in March (Figure 1). Phosphate concentration, which was only analyzed during 2003, showed low values over the entire year (<0.06 µM, Pinhassi and others 2006), except for October (0.2 µM, coincident with a stormy period) and December (0.1 µM, coincident with a Chl *a* peak).

Phytoplanktonic Primary Production

Phytoplankton pPP was studied over nearly three entire seasonal cycles (March 2003–December 2005). Particulate PP showed a marked seasonality, with higher average values during the winter (over 1.5 mg C m^{-3} h^{-1}) compared to the summer (Figure 2A). A repeatable peak of pPP was found in February (2.6 mg C m^{-3} h^{-1}) and variable peaks in December and March (Figure 2A). From spring to

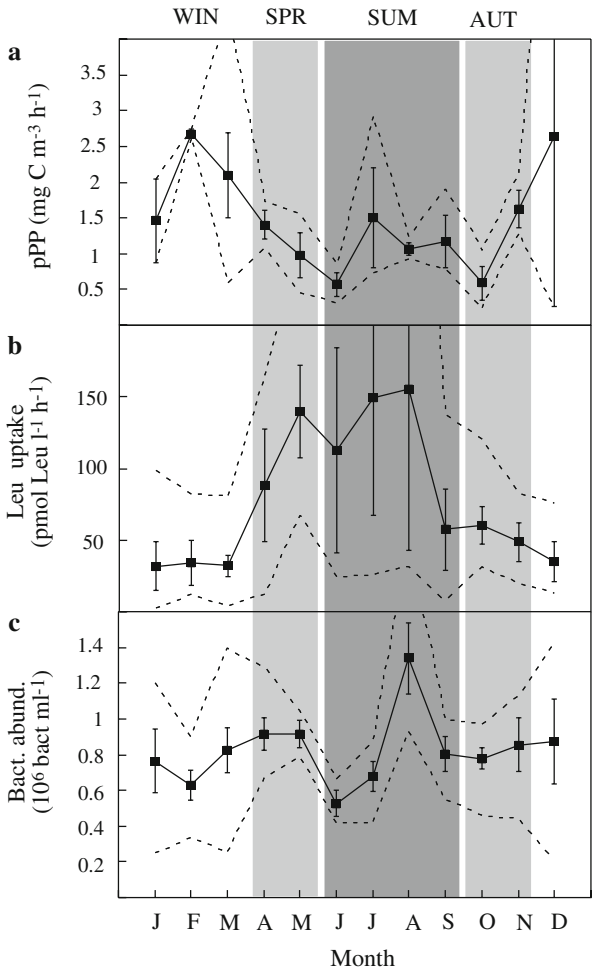

Figure 2. Monthly averaged (±SE) measurements of (**A**) pPP, (**B**) leucine incorporation rates, and (**C**) BA through the period March 2003–March 2006 in Blanes Bay. In the case of pPP, measurements were carried out only until December 2005. The *dashed lines* above and below the lines indicate the monthly maximum and minimum values observed for each measurement over the period studied.

autumn, the average pPP was around 1 mg C m^{-3} d^{-1}, with the exception of an unusually high value obtained during July 2003 (2.5 mg C m^{-3} d^{-1}, Figure 2A). The DOC released by phytoplankton was measured over an annual cycle (March 2003–March 2004). On average, the percentage of PER constituted 45% of total PP (range 22–73%), and the lowest values were found in winter (24 and 22% in December and January, respectively, details not shown).

Bacterial Carbon Flux

Bacterial abundance measurements showed a narrow range, and after a decline in June (average of 5.3×10^5 bact ml^{-1}), BA tended to increase during the summer period reaching maxima in August (1.3×10^6 bact ml^{-1}, Figure 2C). BA average values were quite stable (around 0.8×10^6 bact ml^{-1}) the rest of the year (Figure 2C). The average values of Leu uptake rates (as an estimate of BHP) tended to be higher in the spring and summer periods (Figure 2B), contrary to particulate PP. However, there was a substantial interannual variation in Leu uptake rates during summer, with values ranging about 14-fold. The high variability in Leu incorporation measurements was confirmed even at a much shorter time-scale in this site. Measurements carried out on consecutive days for several months during 2003 varied, on average, by a factor of 3 (details not shown).

During a full-year cycle (March 2003–March 2004, Figure 3), BR and BGE were determined monthly. eCFs were also measured during almost all this period (until January 2004, Table 2), to be able to calculate reliable estimates of BHP. Empirical CFs (range 0.97–3.62 kgC mol Leu^{-1}) showed generally higher values during the winter period (and May), compared to the summer–autumn period. Within this annual cycle and during the two winter periods (January–March 2003 and December 2003–March 2004), BHP followed the pattern of Chl *a*, with peaks in February and December 2003 (Figure 3A). However, maximal values of BHP were also found in May, when Chl *a* concentration was low (Figure 3A), and a secondary peak was found in August. BHP was low from the end of the summer through autumn. An unusually high BHP value was found in July 2003, which was not repeated in other years, and was concomitant with a drastic shift in bacterial assemblage structure as well as important changes in many other parameters (Gasol and others Unpublished). Therefore, the results of July 2003 have not been considered as a seasonal feature and are presented as outliers in Figure 3. Low activities of the bacterial ectoenzymes beta-glucosidase, Leu-aminopeptidase, beta-xylosidase, and chitinase were found during winter in 2003, although the activity of beta-glucosidase and Leu-aminopeptidase increased during the winter of 2004 (Figure 4). A common peak of activity of all the enzymes studied was found in May, and a second peak was found in August for all the enzymes except for Leu-aminopeptidase, which peaked in September and December. Leu uptake was significantly correlated with beta-glucosidase ($rho = 0.54$, $P < 0.05$, $n = 15$), chitinase ($rho = 0.65$, $P < 0.01$, $n = 15$), and marginally with beta-xylosidase ($rho = 0.46$, $P < 0.1$, $n = 15$), but not with Leu-aminopeptidase ($P > 0.1$, $n = 15$).

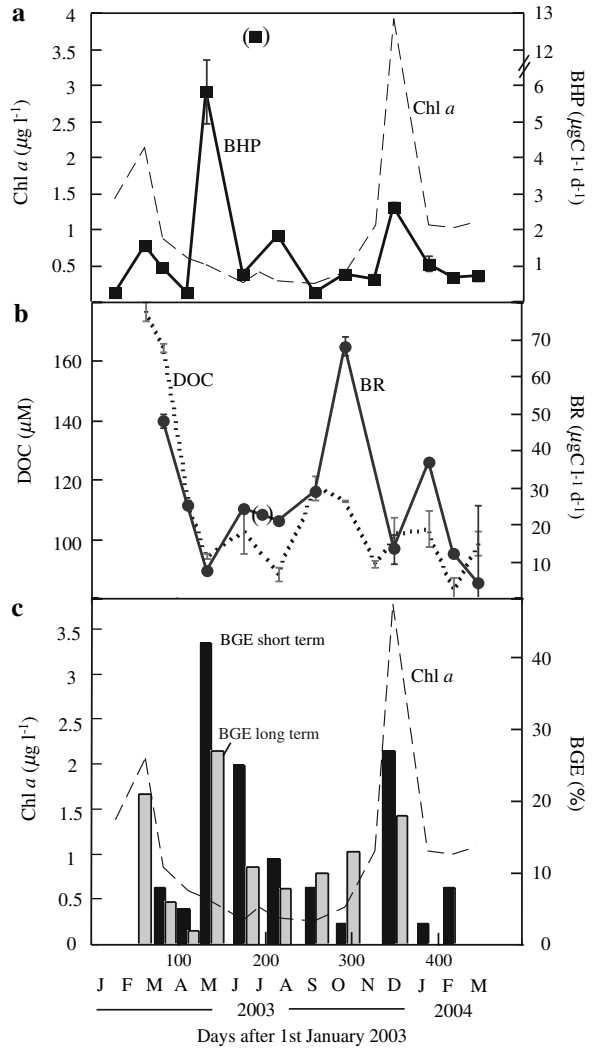

Figure 3. Determinations (±SE) of (**A**) bacterial heterotrophic production (BHP), (**B**) BR, and (**C**) growth efficiency over the period January 2003–March 2004 in Blanes Bay. *Dashed lines* indicate the concentrations of Chl *a* (**A**, **C** panels), or dissolved organic carbon (DOC, panel **B**) in the waters.

Changes in BR were not correlated to those of BHP ($P > 0.1$, $n = 11$) during the annual cycle. Remarkably, minimum values of BR were found at the time of the two BHP peaks (May and December 2003, Figure 3), leading to high BGEs in these months (Figure 3C). High values of BR were found in March 2003 and January 2004, during the decline of the Chl *a* bloom. Intermediate values of BR (around 25 μgC l^{-1} d^{-1}) were found during summer. During the spring and summer periods, peaks of BHP (May and August) were coincident with substantial decreases in the concentration of DOC (Figure 3B), which ranged between 83 and 176 μM with maximal values during the winter 2003 (Figure 3B). Values of total BCD (the sum of BHP and BR) were compared to measurements of dPP and tPP (Figure 5). Autochthonous dPP could cover BCD during some months of the winter period

Table 2. Empirical Leucine-to-Carbon Conversion Factors (±SD) Calculated by the Integrative (Bjørnsen and Kuparinen 1991) and Cumulative (Riemann and others 1987) Methods

Month	Emp. CF (integrative) (kgC mol Leu^{-1})	Emp. CF (cumulative) (kgC mol Leu^{-1})
28-Jan-03	3.40 ± 0.47	3.52 ± 0.50
4-Mar-03	1.66 ± 0.05	1.65 ± 0.08
25-Mar-03	1.04 ± 0.06	0.98 ± 0.02
22-Apr-03	1.07 ± 0.06	1.32 ± 0.43
13-May-03	2.46 ± 0.14	2.25 ± 0.04
25-Jun-03	1.36 ± 0.20	1.25 ± 0.18
14-Jul-03	1.29 ± 0.12	1.31 ± 0.26
4-Aug-03	1.37 ± 0.23	1.35 ± 0.11
16-Sep-03	1.38 ± 0.02	1.33 ± 0.06
21-Oct-03	0.97 ± 0.31	0.98 ± 0.25
25-Nov-03	1.36 ± 0.04	1.28 ± 0.11
16-Dec-03	3.62 ± 0.40	3.62 ± 0.53
26-Jan-04	1.67 ± 0.13	1.65 ± 0.15

SD refers to two replicate incubations.

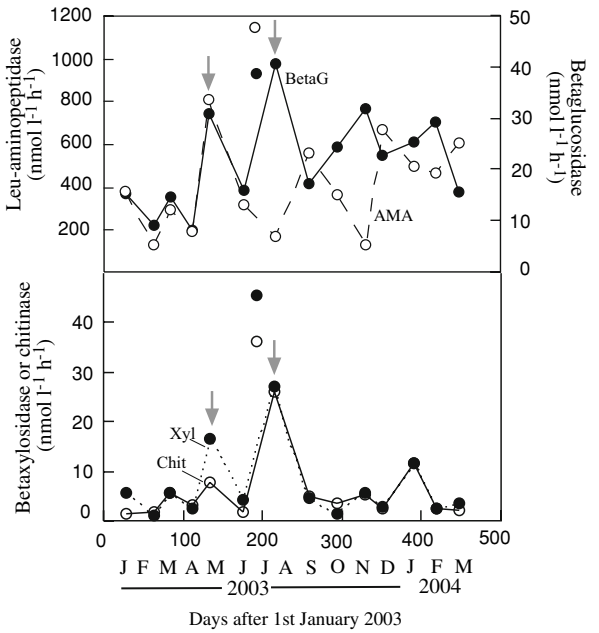

Figure 4. Activity of the bacterial ectoenzymes (**A**) beta-glucosidase (betaG) and Leu-aminopeptidase (AMA, in the fraction <1 μm), and (**B**) beta-xylosidase (Xyl) and chitinase (Chit) through January 2003–March 2004 in Blanes Bay. *Arrows* indicate peaks in ectoenzymatic activities.

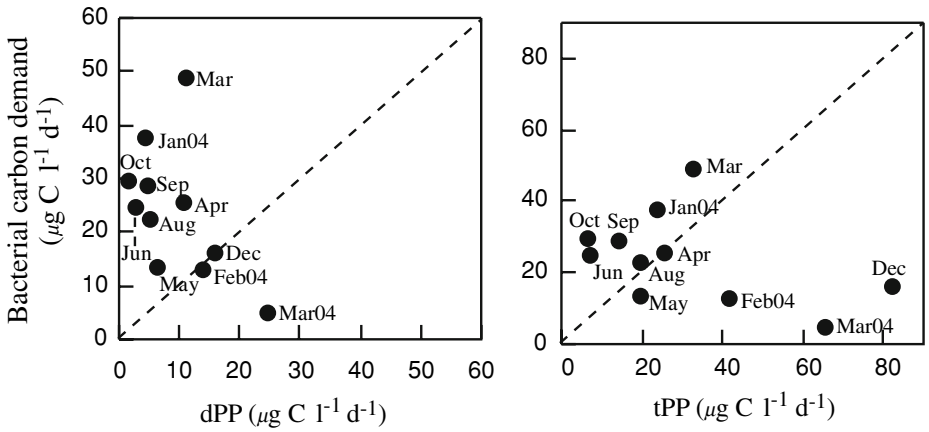

Figure 5. Dissolved (dPP) or tPP compared to bacterial carbon demand from March 2003 to March 2004. The *dotted line* indicates the 1:1 line.

(Figure 5). When total (dissolved plus particulate) PP was taken into account, BCD could also be balanced by total autochthonous PP during spring and August (Figure 5).

Bacterial growth efficiency was determined by two independent approaches: short-term and long-term incubations (see Sect. ''Materials and methods''). Although the range of BGE obtained with the two methods was somewhat different (3–42 and 2–27% for the short-term and long-term estimates, respectively), both approaches were correlated ($rho = 0.72$, $P < 0.05$, $n = 8$, excluding the data point from July 2003), and showed a similar trend throughout the year, with maximum values in May and December (Figure 3C). BGE decreased after the Chl *a* peaks, and from May to October (Figure 3C). The average BGE (using both estimates) was significantly correlated with BHP calculated with eCFs ($rho = 0.71$, $P < 0.05$, $n = 10$), inversely correlated with BR ($rho = -0.65$, $P < 0.05$, $n = 10$), and not correlated with pPP ($P > 0.1$, $n = 10$).

Effects of Environmental Factors and Bacterial Assemblage Structure on Carbon Metabolism

Leu uptake rates were significantly correlated to temperature ($r = 0.30$, $P < 0.05$, $n = 53$) and exoenzymatic activities ($rho \geq 0.5$, $P \leq 0.05$, $n = 15$), with the exception of Leu-aminopeptidase ($P > 0.5$, $n = 15$). Temperature was inversely correlated with pPP ($r = -0.32$, $P = 0.05$, $n = 37$), and no relationship was found with BR or BGE ($P > 0.1$, $n = 12$ and 11, respectively). Nitrate concentration was positively correlated with pPP ($r = 0.32$, $P = 0.05$, $n = 37$) and BA ($r = 0.32$, $P < 0.05$, $n = 45$). Phosphate concentration was inversely correlated with beta-xylosidase ($rho = -0.59$ $P < 0.05$, $n = 12$). Chl *a* was significantly correlated with BA ($r = 0.30$, $P < 0.05$, $n = 62$), but above the 0.05 probability threshold with the eCF ($rho = 0.49$, $P = 0.08$, $n = 13$). DOC concentration was positively correlated to BR ($rho = 0.78$, $P < 0.01$, $n = 12$), not correlated to Leu uptake ($P > 0.5$, $n = 17$), and inversely correlated to beta-glucosidase ($rho = -0.73$, $P < 0.01$, $n = 13$).

Non-metric MDS and the Ward's clustering method were used to explore the similarities between subsets of the samples (Figure 6) based on (1) environmental factors, (2) bacterial assemblage structure depicted by DGGE, as reported elsewhere (Alonso-Sáez and others 2007b), and (3) bacterial carbon metabolism. We found a reasonably similar grouping of seasonal samples based on environmental and assemblage structure parameters, suggesting that summer versus winter and spring conditions were differentiated. Opposite to environmental and assemblage structure parameters, carbon metabolism parameters did not produce a seasonal grouping of the samples, clustering together samples from different seasons.

Discussion

Bacteria's role in planktonic carbon flux is often poorly constrained, because not always are all the relevant fluxes measured, or they are not measured with empirically determined CFs and growth efficiencies. We have comprehensively studied the role of bacteria in the cycling of carbon in an oligotrophic area, Blanes Bay, which shows average environmental parameters (such as Chl *a* or nutrients) similar to those of open waters (Krom and others 1993). Indeed, the year-round measurements of BHP and BR were in the range found in previous studies of coastal oligotrophic sites (Ducklow and Carlson 1992; Robinson and Williams 2005), and the annual BR average (22 μgC l^{-1} d^{-1}) was closer to the mean respiration rate of open-ocean areas

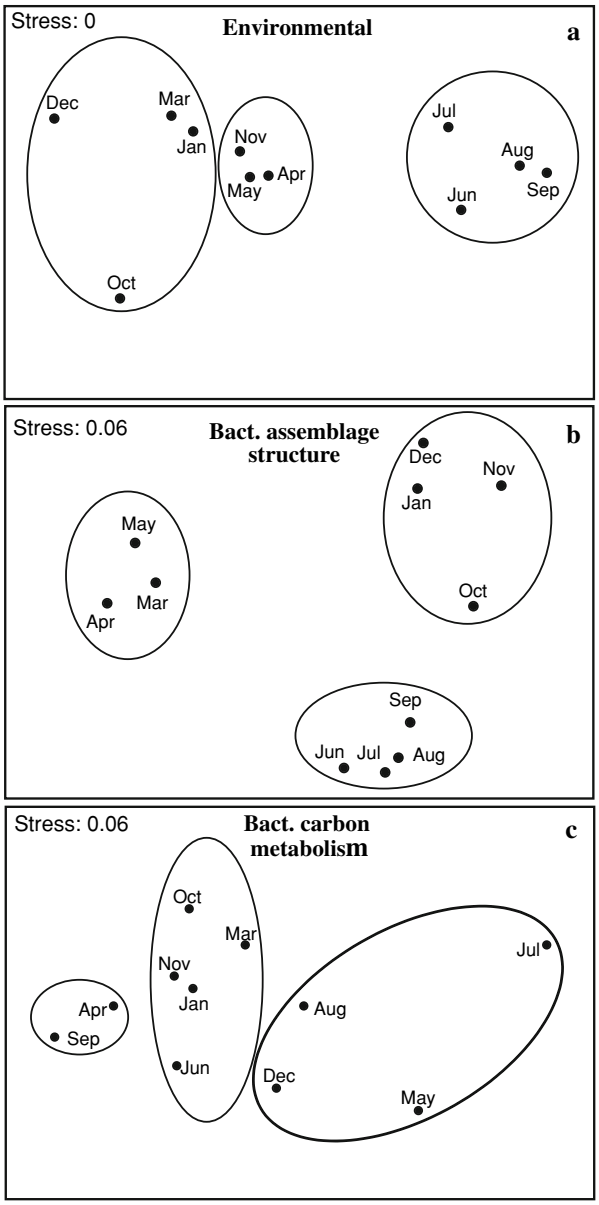

Figure 6. Nonmetric MDS of (**A**) environmental (temperature, Chl *a*, DOC concentration and nitrate), (**B**) bacterial assemblage structure (DGGE OTUs), and (**C**) carbon metabolism (bacterial biomass, production, respiration, and ectoenzyme activities) parameters. The stations have been grouped according to the results of a Ward's clustering method.

(35 μgC l^{-1} d^{-1}, Robinson and Williams 2005), than to those of coastal areas (78 μgC l^{-1} d^{-1}). Our goal was to explore the factors potentially regulating the role of bacteria in carbon use.

Empirical CFs and BGE measurements are crucial to constrain the BHP measurements obtained from Leu uptake rates (Ducklow and Carlson 1992; Alonso-Sáez and others 2007c) and the total C use by bacteria. However, their temporal variability is usually neglected in seasonal studies. To our knowledge, only Murrell (2003) has previously reported seasonal variability in eCFs in marine waters but he did not find any relationship between the eCFs and environmental parameters. Conversely, we found a positive tendency for eCFs to be related to Chl *a* suggesting a more efficient incorporation of Leu to biomass production in high-Chl *a* periods. Interestingly, the peaks of eCFs coincided with peaks in BGE (May and December). However, we could not find a significant positive relationship between eCF and BGE as found in oceanic North Atlantic waters (Alonso-Sáez and others 2007c), something that suggested that eCF and BGE were related, at least in that system.

Although scarce, there are some reports of seasonal measurements of BGE in the ocean. The range of BGE found in Blanes Bay (2–42%) agrees with previous estimates in an oceanic station of the NW Mediterranean (Lemée and others 2002), and other oligotrophic sites (Carlson and Ducklow 1996; Sherry and others 1999). We used the two most widely used approaches to measure BGE: short-term and long-term incubations (del Giorgio and Cole 1998) and, to the best of our knowledge, this is the first time both types of BGE estimates have been simultaneously estimated. We found a relatively good agreement ($rho = 0.72$, $P < 0.05$, $n = 8$) between them, although with higher values in the short-term incubations, something that could be due to an increasing use of refractory DOC fractions in long-term compared to short-term incubations. Also, the accumulation of toxic metabolic by-products could produce lower BGEs in long-term incubations (del Giorgio and Cole 1998).

Over a 3-year cycle, phytoplanktonic primary production showed a more marked seasonal trend (with peaks in winter and low values in summer) than bacterial production. Leu uptake (as an estimator of BHP) showed a much higher interannual variability, with an average trend opposite to that of PP, approximately threefold higher in summer than in winter. Because empirical CFs also increased threefold from summer to winter (Table 2), both factors would tend to smooth out seasonal variability in BHP.

Although positive correlations between phytoplankton and bacterial biomass or production have been observed in mesocosms (for example, Smith and Kemp 1995), and large-scale comparative studies (White and others 1991; Cole and others 1988; Gasol and Duarte 2000), this relationship is often not evident in field studies. This has been attributed to a decoupling of bacterial and phytoplanktonic production due to: (1) inefficient DOM

consumption due to inorganic nutrient limitation (Thingstad and others 1997), (2) a time lag between DOM production by phytoplankton and consumption by bacteria (Sherr and Sherr 1996) or, (3) consumption of allochthonous DOM.

Phosphate limits BHP in Blanes Bay throughout the year, especially during the summer (Pinhassi and others 2006). But the high Leu uptake rates found in summer suggest that nutrient limitation does not importantly account for the decoupling between the bacterial and phytoplankton productions. Furthermore, the high interannual variability of BHP in summer (Figure 3B), when nutrient and Chl *a* concentrations were consistently low, suggests that different factors can participate in the control of BHP. In support of this hypothesis, the analysis of ectoenzymatic activities indicated a shift in the type of DOM consumed at different times of the year. During winter 2003, BHP roughly followed Chl *a* concentration suggesting that bacteria were growing on the dissolved compounds exudated by phytoplankton, probably monomers easily transported and degraded. However, peaks of BHP during spring and summer were concomitant with high ectoenzymatic activities (Figures 3 and 4), suggesting that they could be associated with the HMW-DOM. The proteolysis:glicolysis ratio, that is, the ratio between leu-aminopeptidase and beta-glucosidase activities, depends on the functional features of the ecological system (Christian and Karl 1995) and was found to be higher during periods of high primary production and low during degradation events in the Ross Sea (Misic and others 2002). In the seasonal cycle at Blanes Bay, the ratio varied strongly, from 4 to 33. The lowest value was found in August coincident with a peak of BHP, suggesting high degradation of polysaccharides, something also indicated by the peaks of beta-xylosidase and chitinase activities. Similarly, Chróst (1992) showed low specific beta-glucosidase activity during the spring phytoplankton bloom in an eutrophic lake, and highest values at the collapse of the bloom. This author postulated that the excretion of photosynthetic products during active growth of phytoplankton repressed the bacterial synthesis of the enzyme, which was later induced by the high amount of polysaccharides released during algal lysis. The induction of the enzymatic systems responsible for the use of polymers in the nutrient-limited season (summer) could also be due to the increase in the release of HMW-DOM by phosphorous-limited phytoplankton (Obernosterer and Herndl 1995; Urbani and others 2005), or alternatively, to the increase in phytoplankton cell lysis during this season (Agustí and others 1998).

Finally, we assessed the need for allochtonous sources of carbon to meet the BCD by the simultaneous measurement of BHP, BR, and particulate and dPP, which is frequently neglected. The phytoplankton PER showed high variability, with lower values in the chlorophyll-rich season, in agreement with previous results (Morán and Estrada 2002; Teira and others 2001). The average PER value measured in our study (45%) is in the upper range of values reported (2–44%, Fernández and others 1994; Morán and Estrada 2001; Nagata 2000). BCD could only be met by autochthonous dPP in the winter period (with the exception of January) or winter, spring, and August, if pPP was also considered (Figure 5). These results agree with and support the previous reports of the net heterotrophy of this coastal system (Duarte and others 1999; Satta and others 1996; Lucea and others 2005). Other studies in marine systems, without considering dPP, found results compatible with ours (Sherry and others 1999) and, as a general rule, PP only covered BCD in some parts of the annual cycles (Reinthaler and Herndl 2005; Lemée and others 2002).

Even if we considered that total PP (including the dissolved and particulate forms) is entirely used to fuel BCD (which is clearly an overestimation), yearly integrated BCD would still slightly exceed total PP (factor of 1.058) suggesting that a source of allochtonous carbon is needed to meet the balance. The Bay of Blanes sporadically receives inputs of nutrients and terrestrial carbon from runoff during stormy periods (Satta and others 1996; Guadayol and others submitted), and this supply can be equivalent to about 50% of PP (Lucea and others 2005) over the year. These authors also noted that the excess net benthic community production could contribute to balance the pelagic carbon deficit. That allochtonous DOM is used as a source of carbon for bacterial growth in this system could also be deduced by the high activity of the enzyme beta-xylosidase, which degrades allochtonous plant-derived hemiceluloses.

Whatever the origin of the carbon (autochthonous or allochtonous), different environmental factors can influence its subsequent processing by bacterioplankton. We found a significant positive (although weak) relationship between temperature and BHP, suggesting that temperature could partly regulate BHP. Although the effect of temperature on BHP is contradictory (White and others 1991; del Giorgio and Cole 1998), our result is in agreement with previous seasonal studies in marine (Sherry and others 1999) and estuarine systems (Staroscik and Smith 2004; Shiah and Ducklow 1994). Bacterial biomass, conversely, was signifi-

cantly correlated with nitrate and Chl *a* concentration, suggesting a positive relationship with phytoplankton biomass.

The effect of environmental factors on BR and BGE has been less studied than those on BHP. Previous studies in Blanes Bay have shown a repeatable seasonality in total CR, with higher values during summer (Satta and others 1996; Lucea and others 2005). This pattern did not show up in our seasonal cycle, where BR also peaked after the collapse of the winter phytoplanton bloom (Figure 3B). BR measurements were uncoupled to BHP, contrary to expectations (del Giorgio and Cole 1998), and this lack of relationship suggests that BHP and BR should be controlled differently in Blanes Bay. In this sense, it is noteworthy that we found BR, and not BHP, to be positively correlated to DOC concentration ($rho = 0.78$, $P < 0.01$, $n = 12$) similarly to other reports (González and others 2003, but see Lemée and others 2002). BR is usually reported to be a more constant biogeochemical process compared to BHP, which can rapidly respond to environmental changes. The BHP measurement probably reflected the incorporation of the most labile fraction of DOC, whereas BR integrated the total use of DOC. Temperature, Chl *a* or nutrient concentration did not seem to influence BR during the annual cycle.

Few previous studies that have addressed the seasonality of BGE in marine waters found different trends (Sherry and others 1999), with maxima in spring and summer in the North Sea (Reinthaler and Herndl 2005), or during the spring bloom and fall in the NW Mediterranean (Lemée and others 2002). BGE was not correlated with temperature in Blanes Bay. Inorganic nutrient availability has been proposed as a controlling factor enhancing BGE in experimental (Goldman and others 1987) and field studies (Kroer 1993; Smith and Prairie 2004). Puddu and others (2003) found higher BGE in bacteria growing in P-balanced conditions compared to P-limited conditions, and we also found higher BGEs (over 20%) in nutrient-replete conditions (such as the winter bloom or May, when most exoenzymatic activity was taking place), versus nutrient limited assemblages, as those at the end of the summer, when BGE was as low as 5%.

DOC lability and its association to PP are a main factor that affects BGE in seasonal studies: Lemée and others (2002) found a positive relationship between BGE and Chl *a*, and Reinthaler and Herndl (2005) found higher BGE in months with higher PP. These authors concluded that BGE could be directly linked to the bioavailability of DOC, and indirectly to PP. We also found BGE peaks during Chl *a* maxima. However, a significant peak in BGE was also found in May, unrelated to Chl *a* concentration, which suggests that factors other than PP can also affect BGE. Interestingly, peaks of BHP and BGE in May and December were concomitant with abundance peaks of a very active bacterial group, Roseobacter (Alonso-Sáez and others 2007b; Alonso-Sáez and Gasol 2007). Although frequently neglected in this type of studies, the taxonomic composition of the bacterial assemblage can be a factor regulating carbon processing (Cottrell and Kirchman 2000), and we tested this hypothesis. We did not find, however, any relationship between bacterial assemblage structure and general carbon metabolism measurements in Blanes Bay, in contrast to what has been shown in other marine studies (Fuhrman and others 2006; Alonso-Sáez and others 2007a). Such lack of relationship suggests a high level of functional redundancy in the bacterial assemblage, at least at the phylogenetic resolution level analyzed (DGGE band pattern) and at the level of general functions studied, such as production or respiration. However, it is possible that carbon metabolism is mainly carried out by some active populations that represent a low percentage of the total bacterial community (such as the Roseobacter) and that, therefore, do not drive the variations in community structure.

In summary, we described the patterns of carbon metabolism in Banes Bay, which were not found to be related to changes in bacterial assemblage structure. Temperature was related to BHP but not to BGE or BR, and different factors seemed to control BHP and BR. Peaks of ectoenzymatic activities in spring and summer indicate the significance of allochtonous carbon fueling BCD in this system, resulting in BCD being higher than total PP for a large part of the year.

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