II

Paper II

Bacterial growth response to copepod grazing in aquatic ecosystems

Cristian A. Vargas*[∫§], L. Antonio Cuevas[†¶], Humberto E. González[†‡∫] and Giovanni Daneri[†∫]

*Aquatic System Unit, Environmental Sciences Center EULA, Universidad de Concepcion, PO Box 160-C, Concepcion, Chile.
†Centro de Investigaciones Oceanográficas en el Pacífico Sur-Oriental (COPAS), Concepción, Chile.
¶Present address: Department of Biology, University of Bergen, Jahnebakken 5, N-5020, Bergen, Norway.
‡Institute of Marine Biology "Dr. Jürgen Winter", Universidad Austral de Chile, PO Box 567, Valdivia, Chile.
∫Patagonian Ecosystems Research Center (CIEP), Bilbao 449, Coyhaique, Chile.
§Corresponding author, e-mail: crvargas@udec.cl

The growth rate response of bacterial communities to the potential increase of dissolved organic matter (DOM) produced by the copepod *Acartia tonsa* was assessed in experiments conducted in three stations representing three contrasting aquatic environments (coastal embayment, shelf and ocean). Bacterial assemblages were inoculated in filtered seawater where *A. tonsa* had previously grazed. Utilization of DOM over time was evaluated after the addition of bacterial inoculums as the biomass changes in both 'control' and 'copepod' treatments. In the embayment and ocean a high bacterial growth was observed in the treatments with seawater where copepod were feeding. Additional field measurements of bacterial, primary production and zooplankton biomass support the idea that bacterial communities living in oceanic environments can be efficient to utilize the newly available substrate. Copepods play a key role not only as conveyors of carbon up through the classical food-web, but also generated significant amounts of bacterial substrate in the microbial loop food-web.

INTRODUCTION

The significance of bacterioplankton in the cycling of organic matter in coastal and oceanic food webs has received considerable attention during the last decade (Azam, 1998). There is increasing evidence that the microbial food web is a fundamental and almost permanent feature in both oligotrophic and eutrophic marine systems (Vargas & González, 2004a,b). However, only half the high demands of dissolved organic matter (DOM) for heterotrophic bacteria in the microbial food web can be directly ascribed to the release of organic carbon from phytoplankton (Nagata, 2000).

Zooplankton act not only as consumers of an important fraction of the primary production (PP) and as nutrient regenerators, but also play an active role in the cycling of prey carbon into DOM (Peduzzi & Herndl, l992). Marine copepods may constitute up to 80% of the total zooplankton biomass (Verity & Smetacek, 1996), and they are a key group in the energy transfer through pelagic food webs. An important percentage of the carbon grazed by copepods (∼25 to 50%) may not be transferred directly to upper trophic levels, but may go directly to the microbial food web through the production of DOM (Møller, 2005). Copepods contribute to the pool of DOM through different mechanisms, such as, excretion, leakage from faecal pellets and sloppy feeding (Nagata, 2000). Therefore, it seems that copepods can play an important role influencing bacterial production (BP) in marine ecosystems through the production of alternative DOM sources, especially when copepods are feeding on long-chain forming cells (Møller, 2005), which typically occur in highly productive coastal regions (Vargas & González, 2004a).

Recent studies have shown differences in the ability of bacterial communities to utilize available substrate sources (e.g. Peduzzi & Herndl, 1992). In the present study we aimed to assess the effect of the grazing activity of *Acartia tonsa* on the biomass response of oceanic and coastal natural bacterial communities. Due to the potentially small changes in the DOM pool (against the large DOM background) produced by the addition of a small number of copepods, the potential utilization of DOM was evaluated as bacterial biomass changes over time in simple batch incubations. The added bacterial assemblages were treated as a total community and no effort to distinguish among neither species, nor functional groups were made.

MATERIALS AND METHODS

Study area

The study was carried out at a coastal area in the northern Humboldt Current System (HCS) off Mejillones Peninsula, Chile (23°S). Experiments were carried out at three stations representing an embayment (Station 1), shelf (Station 2) and ocean (Station 3) environment (Figure 1A). The first two sets of experiments took place during a research cruise between 5 and 20 April 2001 onboard the RV 'Abate Molina'. These experiments were conducted at two anchor stations, in an oceanic (80 n.m. from the coast) and shelf (5 n.m.) station (Figure 1A). The last experiment was conducted during 6 and 7 December 2002 during a field campaign onboard the RV 'Purihaalar' at a station located in Mejillones Bay (23°S 73°20′W; Figure 1A). The embayment and shelf stations were located close to
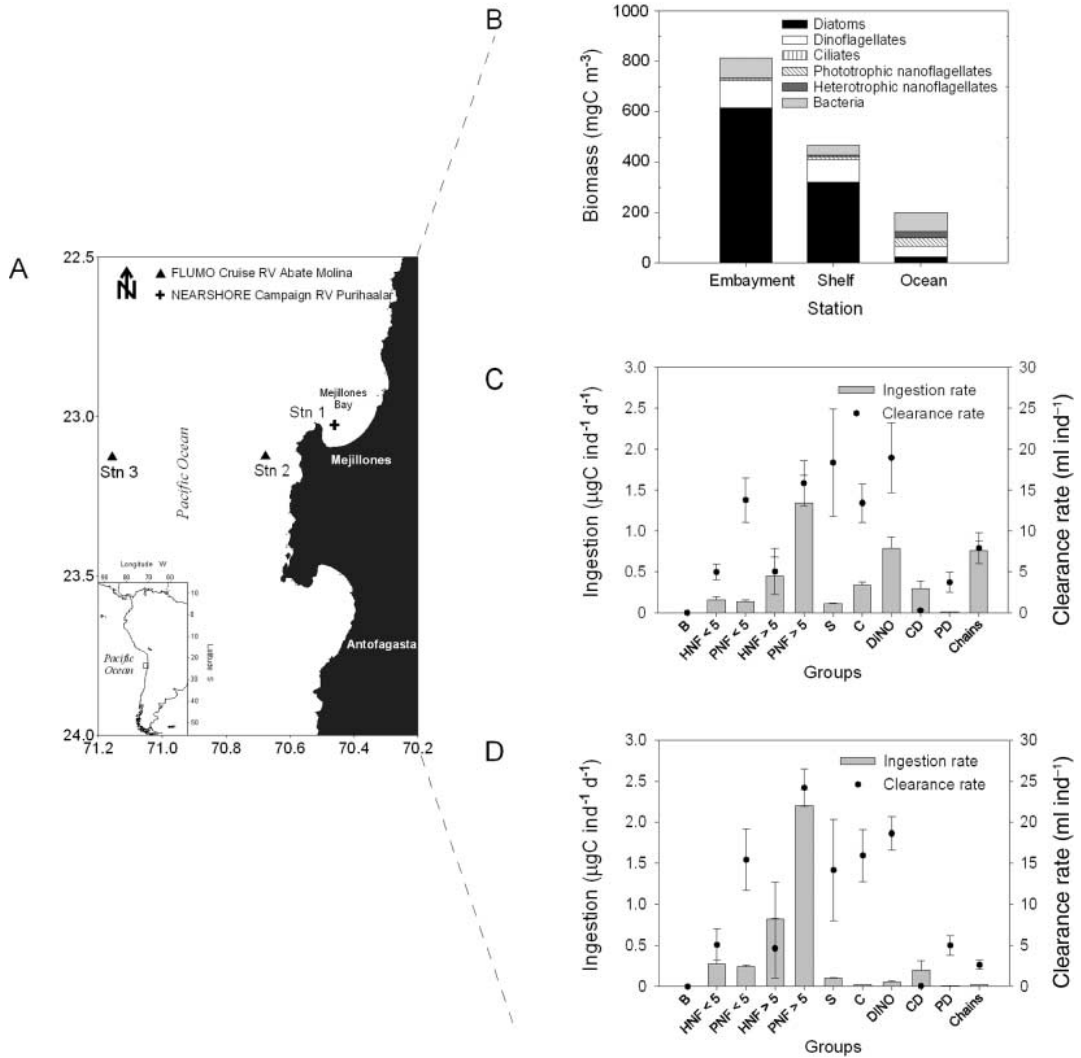
Figure 1. (A) Map showing the location of the study area and the position of the stations during two research campaigns; (B) biomass of autotrophic and heterotrophic cells at 5 m depth at each station where experiments were done; (C&D) clearance (–●–) and ingestion rate (bars) of major autotrophs and heterotrophs groups by *Acartia tonsa* at the (C) shelf and (D) oceanic station in April 2001. B, bacteria; HNF, heterotrophic nanoflagellates; PNF, phototrophic nanoflagellates; S, silicoflagellates; C, ciliates; DINO, dinoflagellates; CD, solitary centric diatoms; PD, pennate diatoms; Chains, chain forming diatoms. < or >5 = < or >5 μm cell size. Error bars are standard deviations from replicates (N=3).

Mejillones Peninsula; a nearshore area influenced by intermittent upwelling events and also one of the most biologically productive areas within the northern part of the HCS off Chile (Daneri et al., 2000).

Experiments

In the present experiments we estimated the grazing activity measured as clearance, ingestion, and specific egg production (SEP) of copepods feeding on natural food assemblages. After incubation for copepod grazing, the seawater with the food assemblages, with and without

copepods, was filtered and the subsequent growth response of natural bacterial inoculums was measured as an estimation of DOM availability. The experimental set-up was realized as follows: copepods for feeding and egg production experiments were collected by vertical hauls in the upper 30 m using a WP-2 net (mesh size 200-μm) with a large non-filtering cod end (40 l). Water for incubation was collected from 10 m with a clean Niskin bottle (30 l) and subsequently screened through a 200-μm net to remove other grazers. We also checked for potential small copepod nauplii that could have been included in the incubation bottles, which were removed by the screening.

Within 30 min after collection, five healthy *Acartia tonsa* were pipetted into 500-ml acid-washed incubation bottles (Duran Schott® borosilicate bottles) with mixed ambient water and filled to avoid air bubbles. Control bottles without copepods and experimental bottles with copepods were running in triplicate and placed on a plankton wheel (0.2 rpm) for approximately 20–24 h in darkness and at *in situ* temperature. An initial control sample was immediately preserved in glutaraldehyde (2% w/v in 0.2-μm-prefiltered seawater) for bacterial and nanoflagellates counts and a subsample was preserved with 2% acid Lugol for cell counts. At the end of the incubation, subsamples were taken for bacterial biomass, nanoflagellate counts, and preserved with glutaraldehyde. A subsample for cell concentration (50 ml) was preserved in 2% acid Lugol. Bacteria, microprotozoan and phytoplankton were counted and carbon content estimated according to Vargas & González (2004a). Copepods and eggs were collected, sized and counted. Corrections due to 'trophic effect' were done as suggested by Vargas & González (2004a). Potential ingestion of heterotrophic nanoflagellates, ciliates, and dinoflagellates was estimated by using the model proposed by Peters (1994). Growth rates of flagellates during incubations and values for potential ingestion rates by heterotrophic flagellates feeding on bacteria, ciliates and dinoflagellates feeding on small flagellates were used in these corrections. We applied a 3-component equation template that considers interactions among three grazers in differently structured food chains, which is described in detail in Tang et al. (2001). Corrections were done for the interactions bacteria−flagellates−zooplankton, flagellates−ciliates−zooplankton and diatoms−dinoflagellates−zooplankton. Clearance and ingestion rates were calculated according to Frost (1972). Ingestion was also estimated from SEP assuming a gross growth efficiency (GGE) of 33%, which is the GGE of *A. tonsa* females grazing on *Rhodomonas* sp., and for which there should not be sloppy-feeding (Møller, 2005).

Although we were not able to have a direct measurement of DOM production, we evaluated a potential C loss by sloppy-feeding of copepods during our grazing experiments. With this aim, we estimated a potential C loss as follow: first, by using the prey-size ingestion dependence in dissolved organic carbon (DOC) production as suggested by Møller (2005), and second, since ingestion estimated from SEP is actually what was ingested by the copepod (ingestion from clearance is only what was removed from suspension), we used the differences in ingestion estimated from clearance and SEP as a measurement of potential DOC production by sloppy feeding (C-loss=C removed from suspension−C ingestion estimated from SEP). During the experiment conducted in December 2002 at Mejillones Bay, only size-fractioned Chl-*a* (<5, 5 to 23 and >23 μm) depletion was estimated as a measured of grazing activity, and C loss was not possible to estimate by this second approximation.

Bacterial growth response

We assumed that although incubation water for clearance experiments already included natural DOM, bottles where copepods fed were enriched with labile DOM in relation to control bottles without copepods. Therefore,

differences in bacterial growth (BG) response were assumed to represent differences in substrate availability and lability (DOM) induced by grazing activity. Filtered seawater (<0.2-μm through polycarbonate Nucleopore® filters) from each treatment (with and without copepods) was distributed separately in four 200 ml glass bottles (100 ml per bottle for different times, t_1, t_2, t_3 and t_4). The rest of the content (100 ml) was refilled with 0.8-μm filtered seawater (i.e. as a bacteria inoculum and to reduce bacterial predators). Bottles were incubated in a plankton wheel, in darkness and *in situ* temperature. Over the experiment subsamples from each bottle were taken for biomass determination (10 ml). Bacterial counting was done using epifluoescence microscopy and by using acridine orange as fluorochrome. Cell concentrations were converted to carbon by assuming 20 fgC cell^{-1} (Lee & Fuhrman, 1987). Bacterial growth was estimated by measuring the change in bacterial carbon over time and fixed within a linear model. We estimated a potential bacterial carbon demand (BCD) needed to support the BG observed over the incubation, assuming a growth efficiency of 30% in the embayment and coastal station, which is representative for productive upwelling areas (Peruvian upwelling, Sorokin & Mameva, 1980), and 26% for the oceanic station (del Giorgio & Cole, 2000).

Field measurements

Additional field measurements were carried out during each campaign, including PP, BP, DOC, and zooplankton abundance at different stratum (Tucker net, 200 μm, depth-strata=0–50, 50–100 and 100–300). Water-column integrated PP was estimated from changes in the dissolved oxygen concentrations of light and dark bottle incubations. This procedure is fully described in Daneri et al. (2000). Ambient BP was estimated from incorporation rates of L-[^{14}C]-leucine and transformation of the rates of incorporation of leucine into bacterial carbon was done according to Simon & Azam (1989). For field DOC profiles 10 ml aliquots were drawn directly into clean, pre-combusted glass ampoules from the Niskin bottles. The samples were flame sealed and stored at −20°C until analysis in the laboratory. In the laboratory, the DOC samples were analysed using a TOC 5000 SHIMADZU DOC analyser. Water samples were acidified with 10 ml of concentrated phosphoric acid (pH 2) and decarbonated by purging with CO_2-free gas for 10 min. The carrier gas utilized was a high purity air (<1 ppm of CO_2 and CO). Prior to analysis, the furnace was given a series of injections of freshly prepared Milli-Q water. The signal of Milli-Q high purity water was used as an instrument blank. In order to minimize sample carryover, deep-water samples were run first. Calibration standards were prepared with potassium hydrogen phthalate.

RESULTS AND DISCUSSION

Natural food assemblages and grazing activity

In the embayment and shelf, phytoplankton was dominated by diatoms species characteristic of coastal blooms in northern Chile (Figure 1B). Diatoms were scarce in the oceanic station, in which most cells were dominated by

Table 1. *Clearance rate of* Acartia tonsa *based on size-fractioned Chl-*a *depletion during grazing experiments and Weight-specific Ingestion Rate estimated from (1) cell counts and (2) specific egg production.*

| Environment | Chl size-fractionated clearance | | Total specific carbon ingestion | |
	Chlorophyll fraction	Clearance rate $(ml\ ind^{-1}\ h^{-1})$	Ingestion (1) $(\mu gC\ \mu gC^{-1}\ d^{-1})$	Ingestion (2) $(\mu gC\ \mu gC^{-1}\ d^{-1})$
Embayment	$<5\ \mu m$	2.1 ± 1.8	$2.0 \pm 0.2*$	0.41 ± 0.02
	5–$20\ \mu m$	0 ± 0		
	$>20\ \mu m$	5.7 ± 1.7		
Shelf	$<5\ \mu m$	0.4 ± 0.1	1.1 ± 0.2	0.22 ± 0.03
	5–$20\ \mu m$	3.2 ± 0.9		
	$>20\ \mu m$	4.5 ± 1.2		
Ocean	$<5\ \mu m$	1.2 ± 0.3	1.0 ± 0.2	0.10 ± 0.01
	5–$20\ \mu m$	6.5 ± 2.2		
	$>20\ \mu m$	1.9 ± 1.1		

*For the embayment, ingestion rate was estimated only from Chl-*a* depletion.

Table 2. *Linear regression equation parameters of the increase in bacterial biomass over 24 h. r=correlation coefficient, initial bacterial biomass (BB = T$_0$) and bacterial growth (BG). Bacterial carbon demand (BCD) during each experiment was also included. BCD was estimated assuming 30% of bacterial growth efficiency in the embayment and coastal station (Sorokin & Mameva, 1980), and 26% for the oceanic station (del Giorgio & Cole, 2000).*

Survey	Area	Treatment	r	P-value	ANCOVA P	BB $(\mu gC\ l^{-1})$	BG $(\mu gC\ l^{-1}\ d^{-1})$	BCD $(\mu gC\ l^{-1}\ d^{-1})$
Mejillones	Embayment	Control	0.62	0.0481	**0.0041**	4.7	1.98	6.6
		Copepods	0.96	0.0001		4.7	6.75	22.5
Antofagasta	Shelf	Control	0.92	0.0015	0.11	1.3	1.14	3.8
		Copepods	0.94	0.0001		1.3	2.18	7.3
Antofagasta	Ocean	Control	0.98	0.0001	**0.0038**	1.3	2.29	8.8
		Copepods	0.96	0.0001		1.3	4.20	16.1

phototrophic (PNF) and heterotrophic nanoflagellates (HNF), and bacteria. Clearance based in Chl depletion estimated at the embayment, showed that *Acartia tonsa* was feeding mostly on Chl-a > 20 μm (Table 1). In this station, phytoplankton > 20 μm was mostly dominated by diatoms, such as *Leptocylindrus* and *Chaetoceros* sp. If we assumed a C:Chl-a ratio of ~50 (for a community in exponential growth phase, Booth et al., 1993), weight-specific carbon ingestion equalled ~2.0 μgC μgC^{-1} d^{-1} (Table 1). At the shelf station, *A. tonsa* was both clearing on Chl-a > 20 μm and on 5–20 μm, while at the oceanic station they were mostly feeding on nanoplankton (Table 1). Cell counts at shelf and oceanic station were in agreement with rates based on Chl depletion. Copepods were removing different prey in proportion to their abundance in field stations (Figure 1C,D). At the shelf, copepods ingested carbon from several sources, including small PNF, HNF, dinoflagellates (DINO), centric (CD) and chain-forming diatoms (Figure 1C). Conversely, most of the ingestion in the oceanic station was based on small prey, such as PNF and HNF, and few less-abundant CD and DINO (Figure 1D). Total ingestion in each station was similar between them and averaged ~4 μgC ind^{-1} d^{-1}. Our values are similar to those reported by Vargas & González (2004a) for *A. tonsa* (incubations: 3.6 to 7 μgC ind^{-1} d^{-1}) in the same area of the present research.

Weight-specific carbon ingestion based on cell counts was 1.1 and 1.0 μgC μgC^{-1} d^{-1} for shelf and ocean station, respectively. On the contrary, ingestion calculated from egg production was ~0.2 and 0.1 μgC μgC^{-1} d^{-1} (Table 1).

Potential DOM production and bacterial response

Once feeding experiments were finished, we inoculated seawater both from control and experimental bottles with natural bacterial communities. No copepod faecal pellets were included in the incubation water for bacterial inoculums. During the experiment at the shelf and oceanic station, the initial bacterial biomass in the 0.8-μm filtered seawater was 1.3 μgC l^{-1}. Conversely, during the campaign at the embayment, we observed a higher initial biomass of 4.7 μgC l^{-1} in the filtered seawater (Table 2). As the experiment was running we observed significant differences in the response of bacterial communities inoculated in 0.2-μm filtered seawater from grazing experiment (Figure 2). The increase in bacterial biomass over 24 h as found in our experiments can be described by linear regression equations (Table 2). Although incubations were too short to observe the maximum carrying capacity of bacterial biomass, the highest BG was always observed in the 'copepod' treatments. The slope of the 'copepod' treatment regressions was significantly different from 'controls'
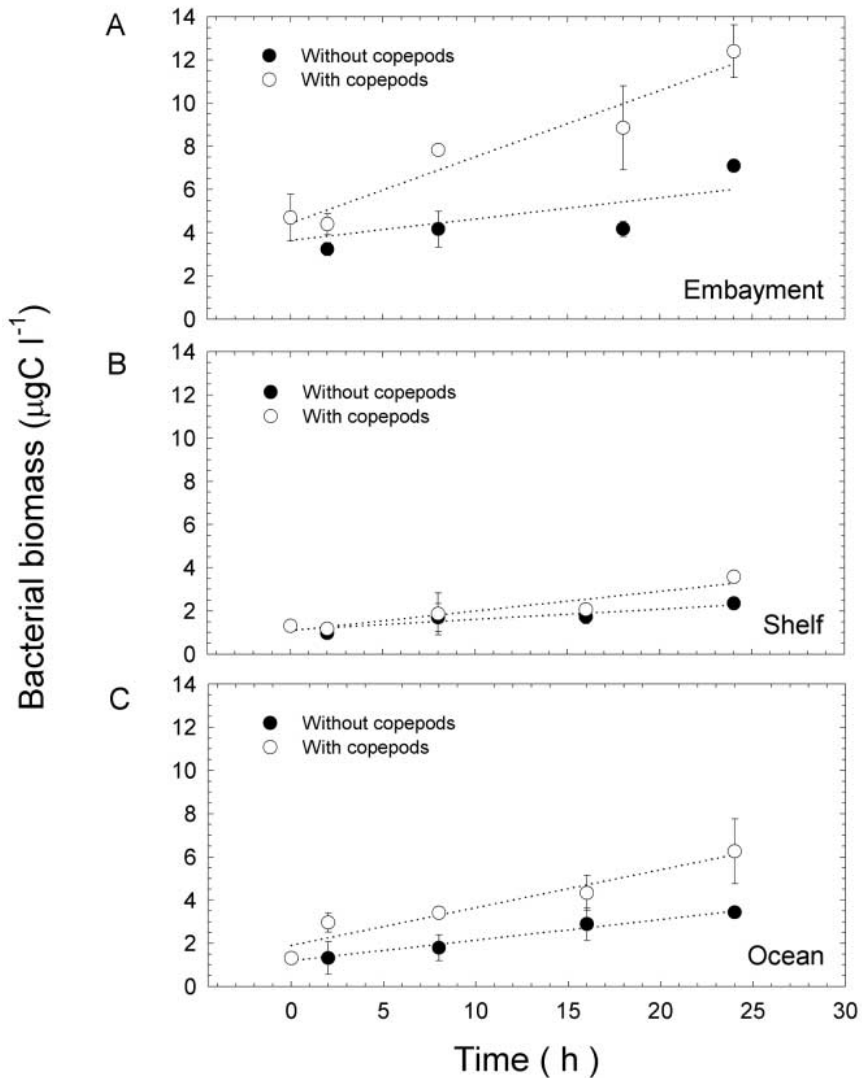
Figure 2. Development of bacterial biomass (μgC l^{-1}) in batch cultures with seawater from grazing experiments inoculated with natural bacterial assemblages from an (A) embayment, (B) shelf, and (C) oceanic station. Statistical analyses for linear regression are included in Table 2. Error bars means standard deviation.

in the embayment and oceanic stations (i.e. initial measurements T_0 were not included in the analysis; analysis of covariance (ANCOVA): $P < 0.0l$; Table 2) (Figure 2A&C). In general, in all stations BG response over the incubation was at least twice higher in 'copepod' than 'control' treatment. Since bacterial innoculum for each treatment were obtained from the same 0.8-μm filtered seawater stock, differences in bacterial substrate between treatments were most probably caused by the grazing activity in the 'copepod' treatment. Such production may have been mediated through mechanisms such as sloppy feeding, excretion or leaching from faeces (Nagata, 2000), however, our experimental set-up did not allow us to discriminate between these potential sources. Since BG

was higher in 'copepod' than 'control' treatments, the BCD need to support this BG was consequently higher in all the treatments (Table 2). In our experiments BG was observed both in treatments and controls implying that other DOM sources (e.g. phytoplankton exudates, refractory DOC, DOC from viral lysis) were supporting BG. We therefore estimated $BCD_{grazing}$ supported by copepod grazing activity as ($BCD_{grazing} = BCD_{copepod} - BCD_{control}$). These estimations resulted in a $BCD_{grazing}$ of 15.9, 3.5 and 7.3 μgC l^{-1} d^{-1}, for the embayment, shelf and oceanic station, respectively (Table 3).

Prey-size dependence production of DOM by copepods has been suggested and recently an equation predicting DOC production by sloppy feeding was developed by

Table 3. *Environmental conditions measured during each research cruise including euphotic zone depth (EZ), primary production (PP) and bacterial production (BP). Experimental results include temperature, carbon loss from grazing activity estimated both from (1) the prey-size dependent equation suggested by Møller 2005 and (2) the difference between C removed from suspension − C ingested (calculated from egg production). Bacterial carbon demand ($BCD_{grazing}$) to support bacterial growth mediated by copepod grazing ($BCD_{grazing} = BCD_{Copepods} - BCD_{control}$) is also included.*

| Area | Treatment | Environmental conditions | | | Experimental results | | | |
		EZ (m)	PP (mgC m^{-2} d^{-1})	BP (mgC m^{-2} d^{-1})	Temperature ($^{\circ}$C)	Carbon loss (μgC l^{-1} d^{-1}) (1)	Carbon loss (μgC l^{-1} d^{-1}) (2)	$BCD_{grazing}$ (μgC l^{-1} d^{-1})
Embayment	Control	–	–	–	20	–	–	–
	Copepods				20	11.0		15.9
Shelf	Control	25–27	800–3300	295–1264	20.5	–	–	–
	Copepods				20.5	9.0	8.2	3.5
Ocean	Control	76	270–300	53–69	14	–	–	–
	Copepods				14	2.5	9.7	7.3

Møller (2005). Because the equation can only be applied for an $ESD_{copepod}/ESD_{prey}$ ratio $< 55\,\mu$m (ESD: equivalent spherical diameter), which for a typical adult of *A. tonsa* of $\sim 850\,\mu$m involve cells or chains of at least 17 μm ESD (e.g. 4 to 5 chain-forming cells), potential DOC production could involve around 49% of carbon removed from suspension. If we assume a C:Chl-*a* ratio of 50 (Booth et al., 1993) in our Chl depletion experiment at the embayment, ingestion on Chl $> 20\,\mu$m was $\sim 2.2\,\mu$gC ind^{-1} d^{-1}, with a subsequent carbon loss of $\sim 11\,\mu$gC l^{-1} d^{-1}. Considering that during our study at the shelf, the ingestion by *A. tonsa* on prey $> 20\,\mu$m ESD was $\sim 1.8\,\mu$gC ind^{-1} d^{-1} (estimated from raw data in Figure 1C), a carbon loss of ~ 9.0 μgC l^{-1} d^{-1} could have been mediated by sloppy feeding during our incubations (Table 3). Similarly, at the oceanic station, ingestion on the few less-abundant centric diatoms and dinoflagellates accounted for a less significant ingestion on cells $> 20\,\mu$m ESD of $\sim 0.5\,\mu$gC ind^{-1} d^{-1} (estimated from raw data in Figure 1D), and a potential C loss of $\sim 2.5\,\mu$gC l^{-1} d^{-1} could have occurred. Under this approximation, these C losses were enough to support the differences in BCD over the incubation between 'control' and 'copepod' treatment only at the shelf station (Table 3). However, at the embayment station the potential DOM production could have been underestimated, since the breakage of large heterotrophic prey was not considered when clearance was only based in Chl depletion. Thus, the BGE used in our estimations of BCD could have also been different. These estimations of potential DOM production only include losses by sloppy-feeding, and do not include other mechanisms such as, excretion and leakage from faeces during the grazing incubation. Furthermore, excretion and leakage could have been high at the oceanic station if the contribution of DOM from sloppy feeding was small (copepods fed on small prey), because more prey carbon was actually ingested. For comparison, and considering that both at the shelf and oceanic stations whole cells were counted by epifluorescence and inverted microscope to obtain values for clearance, differences in carbon ingestion with those estimated from specific egg production can be also attributed to carbon losses by sloppy feeding (Møller, 2005). These C loss estimations resulted in values of 8.2 and 9.7 μgC l^{-1}

d^{-1} for shelf and ocean stations respectively, which are enough to support the potential BG and BCD observed (Table 3).

Implications for coastal and oceanic areas

Our results showed important differences in the level of response of bacterial assemblages from three contrasting marine environments. These differences may be related to the quantity and quality of the DOM produced by copepod activity and by the physiological condition of different bacterial assemblages found in each environment. Peduzzi & Herndl (1992) observed high monomeric carbohydrate concentration and bacterial activity in experiments where copepods were included. Furthermore, these authors observed that bacterial communities living in an oligotrophic environment were more efficient to utilize the newly available substrate source, perhaps as an initial response of starved bacteria to the new introduced substrate (Morita, 1984). Field measurements during the present research campaigns support this idea. Both PP and BP were lower at the oceanic station in comparison to the shelf (Table 3). At the shelf, BP represented $\sim 37\%$ of the PP. Since the high PP (Table 3) and high Chl and zooplankton abundance at the shelf, DOC concentration was almost twofold at the shelf than at the oceanic station (Figure 3). If we consider that the per cent of extracellular release from phytoplankton correspond to be 13% of the PP (Baines & Pace, 1991), 103–430 mgC m^{-2} d^{-1} from the PP may be allocated into the DOC pool and may support BP in the shelf. Therefore, there is a high concentration of bacterial substrate in this productive environment in comparison with the ocean station (Figure 3). In fact, no significant differences were observed in BG between 'control' and 'copepod' treatments at shelf, which suggest that potential C losses by copepod feeding activity were not important in fueling BG. In contrast, in the ocean only ~ 40 mgC m^{-2} d^{-1} from the PP may contribute to the DOC pool. For estimations, in our calculations in Table 3, we assumed that when copepods graze on diatoms and dinoflagellates they may contribute with $\sim 50\%$ of the carbon ingested to the DOC pool, and no loss when grazing on nanoflagellates (Møller & Nielsen,
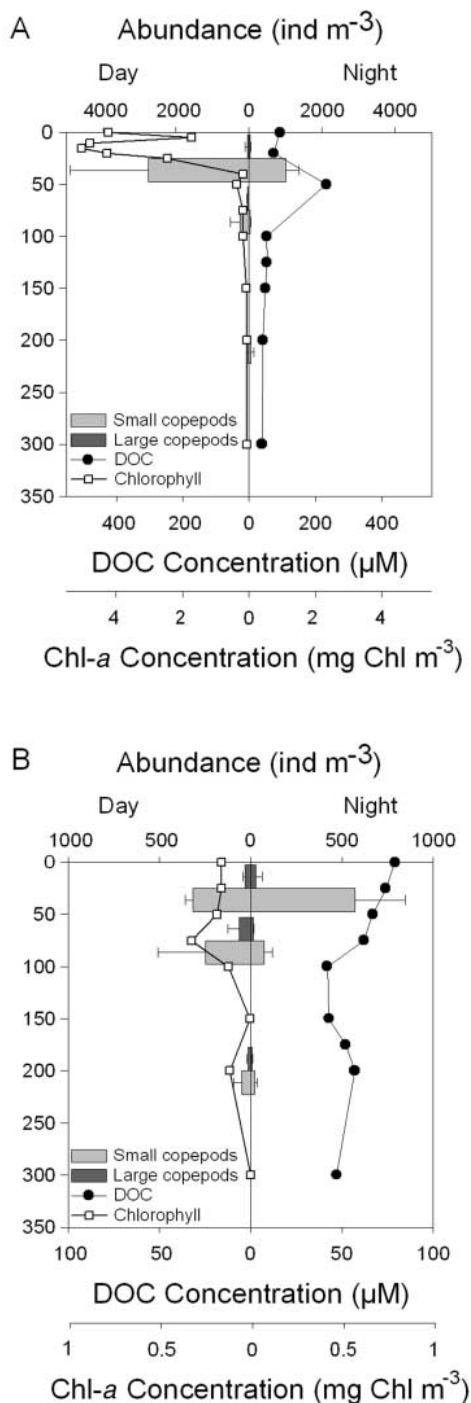
Figure 3. Mean day–night vertical distribution of small and large copepods in (A) shelf and (B) oceanic stations during the FLUMO Cruise. A mean profile of DOC and chlorophyll concentration is also included. Profiles were taken during the day, but they were separated in day and/or night cycle only to avoid plot overlap.

2001). If we consider the integrated abundance of small copepods at the oceanic station (i.e. including *A. tonsa* among others; Figure 3B), and although feeding of *A. tonsa* on large cells was relatively low at the oceanic station ($\sim$0.5 μgC ind^{-1} d^{-1}), $\sim$11 mgC m^{-2} d^{-1} from copepod activity may be allocated into the DOC pool, which is a significant contribution of substrate for bacterial communities in this oligotrophic environment ($\sim$4% PP). In fact, the extracellular release of DOC by phytoplankton both in the coast and ocean should have been an important source of DOC for bacterial communities, but not only vertical distribution of Chl but also the zooplankton abundance and distribution correlate well with distribution of DOC in the field (Figure 3). Although our study is based on some field information and specific laboratory experiments, evidence from previous studies and the present results suggest that the copepod activity in oligo-mesotrophic environments can affect the availability of substrate for BG. Consequently, small copepods not only may act as conveyors of carbon up through the classical food chain of upwelling areas, but also generated amounts of DOM readily available for bacterial communities and then to other grazers through the microbial loop food web.

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