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Annual changes of bacterial mortality due to viruses and protists in an oligotrophic coastal environment (NW Mediterranean)

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Summary

The impact of viruses and protists on bacterioplankton mortality was examined monthly during 2 years (May 2005–April 2007) in an oligotrophic coastal environment (NW Mediterranean Sea). We expected that in such type of system, (i) bacterial losses would be caused mainly by protists, and (ii) lysogeny would be an important type of virus–host interaction. During the study period, viruses and grazers together were responsible for 50.6 ± 40.1% day^{-1} of bacterial standing stock losses (BSS) and 59.7 ± 44.0% day^{-1} of bacterial production losses (BP). Over the first year (May 2005–April 2006), protists were the principal cause of bacterial mortality, removing 29.9 ± 20.4% day^{-1} of BSS and 33.9 ± 24.3% day^{-1} of BP, whereas viral lysis removed 13.5 ± 17.0% day^{-1} of BSS and 12.3 ± 12.3% day^{-1} of BP. During the second year (May 2006–April 2007), viruses caused comparable bacterial losses (29.2 ± 14.8% day^{-1} of BSS and 40.9 ± 20.7% day^{-1} of BP) to protists (28.6 ± 25.5% day^{-1} of BSS and 32.4 ± 20.0% day^{-1} of BP). In 37% of cases higher losses of BP due to viruses than due to protists were found. Lysogenic infection was detected in 11 of 24 samplings. Contrary to our expectations, lytic infections dominated over the two years, and viruses resulted to be a significant source of bacterial mortality in this oligotrophic site.

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Introduction

During the last decades, marine viruses have been recognized to be an important component of microbial food webs. It is known that viral lysis, along with grazing by protists, can be an important source of mortality in aquatic bacterial communities (Wommack and Colwell, 2000). Dominance of those two mortality processes varies among ecosystems. In marine habitats, bacteriophages were found to be the main factor of bacterial losses (Guixa-Boixereu *et al*., 1999a; Wells and Deming, 2006), to have similar significance (Fuhrman and Noble, 1995; Hwang and Cho, 2002) or to be less significant than protists (Guixa-Boixereu *et al*., 1996; Choi *et al*., 2003). Viruses transform particulate carbon and nutrients from prokaryotes to dissolved compounds, which can be assimilated again by bacterioplankton, allowing the retention of nutrients in the euphotic zone (Bratbak *et al*., 1990; Fuhrman, 1999). This process can be important especially in oligotrophic systems, where it could partially avoid nutrients' sinking to the deep sea (Fuhrman, 1999). Thus, in order to understand the functioning of nutrient-poor habitats, it is important to evaluate the carbon and nutrient fluxes at the very basic level – from prokaryotes to viruses and to grazers. Moreover, as the proportion of viral infections versus grazing can vary even within the hours (Winter *et al*., 2004), there is a need of simultaneously run experiments for relatively realistic prokaryotic loss processes evaluation. Yet, very few studies based on such comparisons have been performed (e.g. Guixa-Boixereu *et al*., 2002; Wells and Deming, 2006), and most of them were carried out sporadically or covering short time periods.

Mathematical models predict that contact rates between viruses and hosts increase at high bacterial abundances (Murray and Jackson, 1992; Thingstad, 2000), increasing the infection rate and prokaryotes losses due to viral lysis. According to that, many studies have found viruses as an important cause of prokaryotic mortality in eutrophic systems (Weinbauer and Peduzzi, 1995; Weinbauer and Suttle, 1999), and protists as a principal cause of bacterial losses in oligotrophic waters (Guixa-Boixereu *et al*., 1996; Bettarel *et al*., 2002). Other authors, however, reported higher proportion of lysed than

grazed bacteria in cold and nutrient-poor environments (Wells and Deming, 2006). Similarly, bacterial density can also affect the protists–bacteria interactions, and hence higher grazing is expected when the encounter probability increases (Peters, 1994; Vaqué *et al*., 1994). Moreover, the relative dominance of virus- or protists-mediated mortality of bacteria can also change in relation with predation on nanoflagellates, enhancing the mortality due to viruses.

In addition, system trophy apparently affects also the life strategy of viruses. Lysogeny seems to predominate over lytic infections in oligotrophic systems, characterized by low bacterial abundances and low productivity (e.g. Herndl, 1991; Sorokin and Mamaeva, 1991) as a survival strategy of phages at low host densities. In this type of systems, virus–host contact rates, and thus chances for successful infection and lytic replication, are low (Stewart and Levin, 1984). Moreover, lysogenic bacterial cells (lysogens) appear to be more competitive than non-lysogenic ones in low-nutrient conditions because once infected, they cannot be infected again by homologous phages (Lwoff, 1953) and can benefit from the products of the lysis of other cells. Higher percentage of lysogens was found in offshore than coastal environments (Jiang and Paul, 1998; Weinbauer *et al*., 2003) and during periods with low inorganic nitrogen and phosphate concentrations (Williamson *et al*., 2002). Nevertheless, other studies have not found a clear relationship between lysogeny and system trophy (Weinbauer and Suttle, 1999).

Here we present results of a 2-year study on the impact of grazers and viruses on the bacterioplankton community in a relatively oligotrophic coastal marine environment (Blanes Bay, NW Mediterranean), characterized by low nutrient concentration and plankton biomass (Duarte *et al*., 1999; Pinhassi *et al*., 2006; Alonso-Sáez *et al*., 2008). Due to the trophic status of this ecosystem we hypothesized that (i) grazing by predators would be the principal cause of prokaryotic mortality, and (ii) lysogeny would represent an important percentage of viral infection. We performed monthly, from May 2005 to April 2007, experiments to evaluate simultaneously losses of prokaryotes due to grazers and phages. To simplify, from now on, the period May 2005–April 2006 will be called 'the first year', and May 2006–April 2007 will be 'the second year'.

Results

Physicochemical parameters and chlorophyll a concentration

Over the sampling period the seawater temperature ranged from 12.0°C to 26.0°C (Fig. 1A), with similar mean values for both years (*c*. 18.5°C) (Table 1). Salinity ranged between 35.8 and 39.1, and light penetration was on average *c*. 15 m (Table 1).

Inorganic nutrient concentrations (PO_4^{-3}, NO_3^-) were relatively low during the whole sampling period (Table 1, Fig. 1B). Average NO_3^- concentration was higher during the first year than in the second (Table 1), especially during September 2005–April 2006 (Fig. 1B), although the difference between the two years was not statistically significant ($P = 0.085$). The lowest value of nitrate was reached in August 2005 (0.10 μM), and the highest in April 2006 (3.2 μM). PO_4^{-3} concentrations were highly variable between months. The lowest value was observed in July 2006 (0.02 μM), and the highest in July 2005 and March 2007 (0.2 μM) (Fig. 1B).

The average concentration of chlorophyll *a* (Chl *a*) was very similar between the two years, *c*. 0.6 μg l^{-1} (Table 1). Two peaks of Chl *a* concentration were detected in March and May in both years (Fig. 1A). The lowest value was recorded in August 2005 (0.02 μg l^{-1}), and the highest in May 2006 (2.5 μg l^{-1}). Chlorophyll *a* concentration was correlated with NO_3^- concentration (Table 2), but not with PO_4^{-3} concentrations.

Abundances of microorganisms

Abundances of microorganisms did not differ significantly between the two years of study (Table 1). However, within years viral and heterotrophic nanoflagellates' (HNF) abundances varied one order of magnitude, whereas bacterial abundance varied 2.5-fold only.

During the two years viral abundance showed the lowest value in February 2007 (8.9×10^6 viruses ml^{-1}), and the highest in May 2006 (6.0×10^7 viruses ml^{-1}) (Fig. 1C). No significant correlation between abundance of viruses and others parameters was found.

Bacterial abundance did not follow a clear seasonal pattern. The minimum value was detected in August 2005 (4.6×10^5 cells ml^{-1}), and two maxima in May 2005 (1.6×10^6 cells ml^{-1}) and May 2006 (1.5×10^6 cells ml^{-1}), coinciding with peaks of Chl *a* and viral abundance (Fig. 1A and C). Percentage of high-nucleic-acid-content bacteria (%HNA) reached the highest value in April 2006 (88.6%), coinciding with the peak of nitrates, while the lowest value of %HNA was detected in July 2006 (11.6%). Percentage of high-nucleic-acid-content bacteria presented a significant positive correlation with NO_3^-, PO_4^{-3} and Chl *a* concentrations (Table 2). However, no correlation was detected between total bacterial abundance and these variables. Average virus–bacteria ratios were similar over the two years (*c*. 25). The lowest value was recorded in August 2006 (9.7), and the highest in January 2007 (68.8) (Fig. 1C).

Abundance of HNF in both years showed a marked decrease in late autumn and winter, and two peaks of

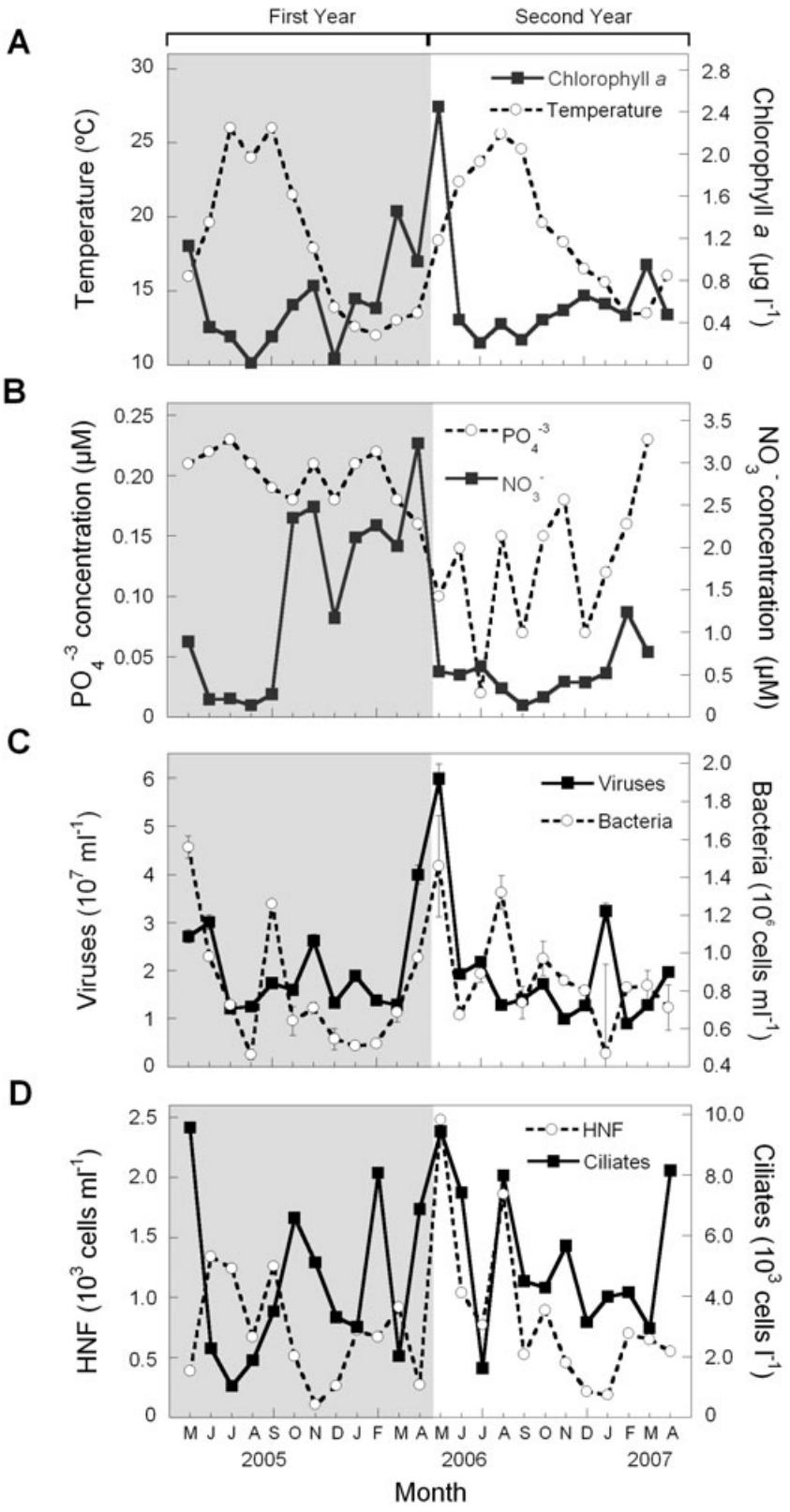

Fig. 1. Temporal variability of (A) temperature and chlorophyll *a* concentration, (B) nutrient concentrations, (C) abundances of viruses and bacteria and (D) abundances of heterotrophic nanoflagellates (HNF) and ciliates during a 2-year study in Blanes Bay, NW Mediterranean.

Table 1. Average, minimum and maximum values of the physicochemical and biological parameters in both study years.

Variable	First year Average (min.–max.)	Second year Average (min.–max.)
Temperature (°C)	18.0 (12.5–26.0)	19.0 (13.4–25.6)
Salinity (‰)	37.4 (35.8–39.0)	38.0 (36.3–39.1)
NO_3^- (μM)	1.4 (0.1–3.2)	0.5 (0.1–1.2)
PO_4^{-3} (μM)	0.2 (0.2–0.2)	0.1 (0.02–0.2)
Secchi disc (m)	16.1 (12.0–22.5)	15.3 (8.0–19.5)
Chlorophyll *a* (μg l^{-1})	0.6 (0.02–1.5)	0.6 (0.2–2.5)
Viruses ($\times 10^7$ viruses ml^{-1})	1.8 (0.5–4.0)	2.0 (0.9–6.0)
Bacteria ($\times 10^5$ cells ml^{-1})	8.0 (4.6–15.6)	8.8 (4.7–14.6)
HNF ($\times 10^2$ cells ml^{-1})	7.0 (1.1–13.4)	8.6 (1.9–24.8)
Ciliates ($\times 10^3$ cells l^{-1})	4.4 (1.0–9.6)	5.3 (1.6–9.4)
BP (10^5 cells ml^{-1} day^{-1})	8.7 (2.7–21.2)	7.8 (1.3–17.4)
VP_L ($\times 10^5$ viruses ml^{-1} day^{-1})	6.7 (0.8–16.4)	22.2 (1.9–131.0)
VP_{LG} ($\times 10^5$ viruses ml^{-1} day^{-1})	1.0 (nd–7.4)	1.6 (nd–6.3)
VMM_{BSS} (% day^{-1})	13.5 (2.5–64.7)**	29.2 (9.6–56.0)**
VMM_{BP} (% day^{-1})	12.2 (3.1–47.7)***	40.9 (9.6–84.1)***
PMM_{BSS} (% day^{-1})	19.9 (0.0–66.6)	28.6 (0.0–91.6)
PMM_{BP} (% day^{-1})	33.9 (0.0–77.6)	32.4 (0.0–77.0)

Significance of differences between the two years: $^{**}P < 0.005$, $^{***}P < 0.001$.
HNF, heterotrophic nanoflagellates; BP, bacterial production; VP_L, lytic viral production; VP_{LG}, lysogenic viral production; VMM, virus-mediated mortality of bacteria (BSS – % of bacterial standing stock; BP – % of bacterial production); PMM, protists-mediated mortality of bacteria; nd, no detectable.

abundance were recorded in spring and late summer (Fig. 1D). The lowest value was observed in November 2005 (1.1×10^2 cells ml^{-1}) and the highest in May 2006 (2.5×10^3 cells ml^{-1}). Peaks of HNF abundance coincided with peaks of bacterial abundance in spring and summer months (Fig. 1C and D). Heterotrophic nanoflagellates 2–5 μm size class, considered mainly bacterivores (Wikner and Hagström, 1988), represented 53.4 ± 13.7% of total HNF. Bacterial abundance showed a slightly higher correlation coefficient with HNF 2–5 μm than with total HNF abundance (Table 2).

Ciliate abundance during the first year showed a predator–prey pattern with HNF abundance, whereas this trend was less evident over the second year (Fig. 1D). The lowest ciliate abundance was reached in July 2005 (1.0×10^3 cells l^{-1}), and the highest in May 2005 (9.6×10^3 cells l^{-1}, Fig. 1D), as was observed for the other variables. The ciliate community was dominated by oligotrichs, mainly *Strombidium* sp. (54.5 ± 14.2% of total ciliates abundance in the first year and 67.8 ± 13.5% in the second, Table 1). Strictly bacterivorous ciliates, such as scuticociliates, represented only 3.0 ± 4.9% over the two years. Ciliates abundance showed significant correlation with HNF fractions of 5–10 and 10–20 μm (Table 2). No correlation was found between ciliates and total HNF abundance.

Table 2. Results of Pearson correlation analysis used to test for simple correspondence among variables.

Variable	*n*	*r*	*P*
Chl *a* – NO_3^-	23	0.583	< 0.01
Chl *a* – %HNA	20	0.579	< 0.02
%HNA – NO_3^-	19	0.572	< 0.05
%HNA – PO_4^{-3}	19	0.534	< 0.05
BA – HNF	24	0.403	< 0.05
BA – HNF 2–5 μm	24	0.428	< 0.05
Ciliates – HNF 5–10 μm	24	0.597	< 0.01
Ciliates – HNF 10–20 μm	24	0.524	< 0.01
BP – BA	24	0.501	< 0.02
BP – %HNA	20	0.677	< 0.01
VP_L – Chl *a*	24	0.604	< 0.01
VP_L – BA	24	0.515	< 0.01
VMM_{BSS} – BA	24	0.476	< 0.02
VMM_{BSS} – VP_L	24	0.551	< 0.01

Chl *a*, chlorophyll *a* concentration; BA, bacterial abundance; %HNA, percentage of high nucleic acid content bacteria. The rest of variable abbreviations are the same as in Table 1.

Bacterial production

Average bacterial production (BP) rates were similar between the two years (Table 1). The lowest value was observed in January 2007 (1.3×10^5 cells ml^{-1} day^{-1}), and the highest in May 2005 (2.1×10^6 cells ml^{-1} day^{-1}) (Fig. 2). For the whole study period, BP presented a higher correlation coefficient with %HNA than with total bacterial abundance (Table 2). Only for the second year BP was correlated with total HNF abundance ($r = 0.686$, $P < 0.02$).

Bacterial mortality

Lytic viral production (VP_L) was significantly higher in the second year than in the first ($F_{1,22} = 10.3$, $P < 0.005$) (Table 1). VP_L did not show a clear trend over the seasons. The lowest value was detected in August 2005

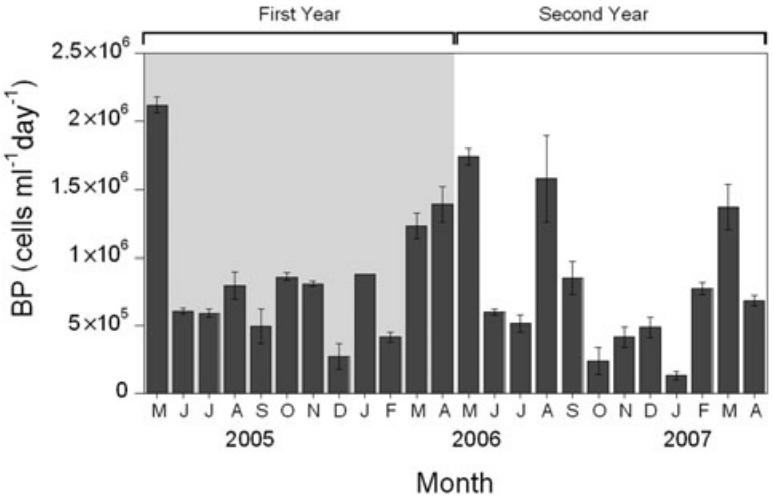

Fig. 2. Average bacterial production (BP) over the study period ($n = 3$). Bars indicate standard deviation.

(2.3×10^5 viruses ml^{-1} day^{-1}), and the highest in November 2006 (2.8×10^7 viruses ml^{-1} day^{-1}). VP_L was positively correlated with Chl *a* concentration and bacterial abundance (Table 2). Lysogeny was observed in four occasions along the first year, and seven times during the second. The lowest percentage of lysogeny to total viral production was detected in November 2006 (0.6%), and the highest in August 2005 (78.4%), coinciding with the highest and the lowest VP_L respectively. Lysogeny events occurred mainly in summer (four times) and winter (four times) months (Fig. 3).

Virus-mediated mortality of bacteria (VMM) was significantly different between the two years, either related to losses of bacterial standing stock (VMM_{BSS}, $F_{1,22} = 12.3$, $P < 0.005$) or to BP (VMM_{BP}, $F_{1,22} = 23.8$, $P < 0.001$) (Table 1). Average VMM_{BSS} and VMM_{BP} for the first year were 13.5 ± 17.0% day^{-1} and 12.2 ± 12.3% day^{-1}, respectively, while in the second, higher values were recorded for both variables (29.2 ± 14.8% day^{-1} and 40.9 ± 20.7% day^{-1}) (Table 1). For the whole period, VMM_{BSS} varied 26-fold between minimum and maximum value (2.5% day^{-1} and 64.7% day^{-1}, Fig. 4A), and 27-fold for VMM_{BP} (3.1% day^{-1} and 84.1% day^{-1}, Fig. 4B). VMM_{BSS} was significantly correlated with bacterial abundance and VP_L (Table 2). Only for the second year, VMM_{BSS} was correlated with %HNA ($r = 0.677$, $P < 0.05$) and BP ($r = 0.439$, $P < 0.05$), coinciding with higher values of VMM_{BSS} than in the first year.

Protists-mediated mortality of bacteria (PMM) did not differ between the two years (Table 1). PMM was not detectable in August 2005 and April 2007 (Fig. 4). When measurable, the lowest value of PMM related to the bacterial standing stock (PMM_{BSS}) was observed in October 2006 (6.3% day^{-1}, Fig. 4A), and to BP (PMM_{BP}) in March 2006 (3.9% day^{-1}, Fig. 4B). The highest value of PMM_{BSS} was observed in May 2006 (91.6% day^{-1}), and for PMM_{BP} in September 2005 (77.6% day^{-1}). In two occasions, viruses and grazers together removed *c.* 100% day^{-1} of BP (May and November 2006) (Fig. 4B). PMM_{BSS} in the second year was positively correlated with total bacterial

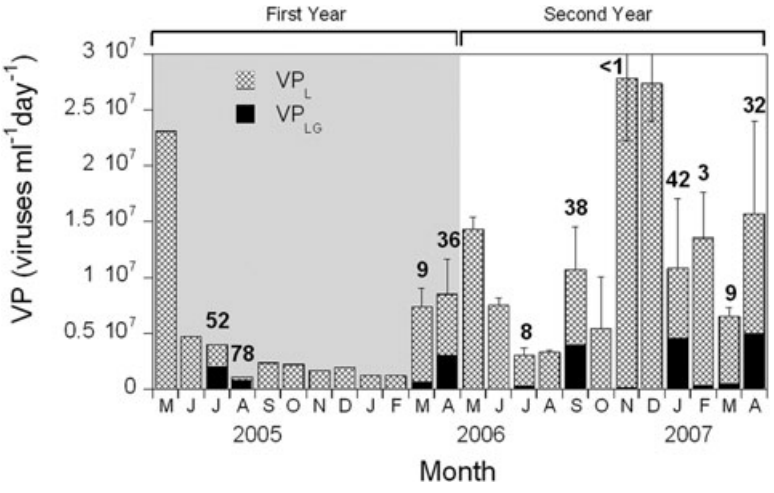

Fig. 3. Temporal variability of the average lytic (VP_L) and lysogenic (VP_{LG}) viral production. In bold: percentage of VP_{LG} in total viral production. Each value corresponds to experimental triplicates ± standard deviation.

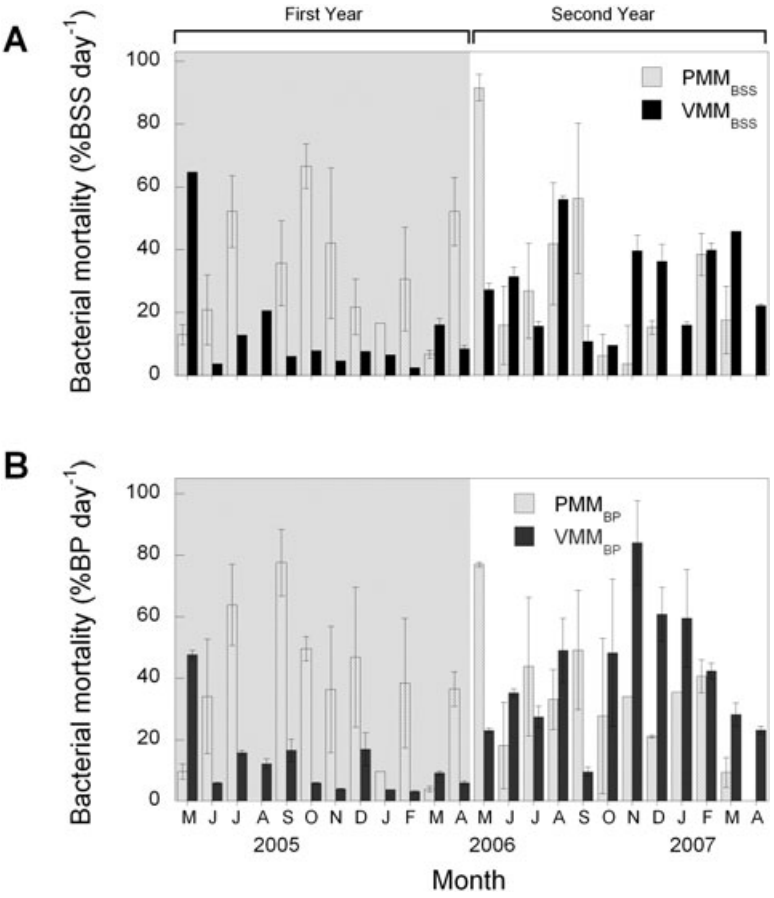

Fig. 4. Temporal variability of the average protists- (PMM) and virus-mediated mortality of bacteria (VMM), as a percentage of bacterial standing stock BSS (A) and bacterial production BP (B). Each value corresponds to experimental triplicates ± standard deviation.

abundance ($r = 0.620$, $P < 0.05$), BP ($r = 0.620$, $P < 0.05$) and HNF ($r = 0.585$, $P < 0.05$). The correlation with HNF was mainly due to 2–5 μm ($r = 0.596$, $P < 0.05$) and 5–10 μm ($r = 0.598$, $P < 0.05$) fractions.

Discussion

Evaluation of the used methodology

The methods to estimate complex ecosystem variables, such as PMM based on fluorescent labelled bacteria (FLB) disappearance and bacterial production, have their suite of assumptions and uncertainties. For grazing on bacteria one can refer to Strom (2000) and references therein, where the used method is widely discussed. The bulk disappearance of FLB as tracers of natural bacteria during 24 h incubation time is in our view the best option in studies where the dynamics of the whole bacterial assemblage is targeted, as it introduces the least water manipulation possible which is always a potential source of artefact. In order to evaluate bacterial growth enhancement owing to confinement, we compared BP rates measured as the sum of the net increase of bacterial abundance (BP_N), losses of bacteria due to protists (G) and viruses (RLC_{GR}) in the same incubation bottles as for grazing estimations, with *in situ* measurements of BP by ^{3}H-leucine incorporation method (BP_{Leu}; J.M. Gasol, unpublished), as described in Kirchman (1993). We obtained a significant relationship between both measurements ($\log BP_{Leu} = -1.65 + 1.17$ (± 0.28) $\log BP$, $n = 24$, $r = 0.670$, $P < 0.01$), with a slope not significantly different from 1. This indicates that our BP results are comparable to those obtained *in situ* with standard methods, although BP values in the experimental bottles were higher than the *in situ* values.

Viral mortality rates are subject to the calculation of burst size. We assumed that the only cause of bacterial decrease over short time periods (1 h) in the experimental falcon tubes was cell lysis, and we did not take into account the viral decay and bacterial production rate during this time interval. Burst size values reported in this study, between 6 and 375, are within the range obtained from different aquatic environments (Guixa-Boixereu *et al.*, 1996; Parada *et al.*, 2006), and are lower than values found in the anoxic part of an eutrophic lake (~500, Weinbauer and Höfle, 1998). Also, bacterial losses caused by lysis measured in this study are the potential losses, as we did not consider grazing on infected cells by

protists in our calculations. This process could reduce the percentage of bacteria that burst due to viral activity in natural communities.

The virus reduction approach (VRA) used to evaluate VP and VMM is based on the assumption that all viral production observed during the experiment is a result of infections previous to the incubation. It is also assumed that no new infections occur, and the filtration and incubation does not induce lysogenic bacteria. This method allows direct observation of changes in viral abundance over time, taking into account the bacterial losses during filtration, and allows the distinction between production of virulent and temperate phages. In addition, it is relatively easy and inexpensive to perform (Winget *et al*., 2005). Detection of lysogeny is based on lysis induction by mitomycin C. It is known that in some circumstances (i.e. nutrients availability, pH) this agent is not sufficient to induce the lytic cycle in all prophages (Cochran *et al*., 1998; Weinbauer and Suttle, 1999 and references therein). However, this method is widely used and mitomycin C is superior to other inducing agents, hence the obtained results are comparable to other studies. Finally, the main drawbacks of this technique are the considerable sample manipulation and the loss of a portion of bacterial community during filtration. Despite of these disadvantages, VRA is considered one of the best-suited incubation-based methods for VP and VMM estimations (Helton *et al*., 2005).

Abundances of microorganisms

Abundances of viruses and bacteria found in Blanes Bay were similar to others found in the Mediterranean Sea (Guixa-Boixereu *et al*., 1999b; Bettarel *et al*., 2002). Viral and bacterial abundances (total and %HNA) were not following the same pattern during the study period and there was no significant correlation between them, neither with Chl *a* concentration. This suggests that (i) the viruses detected could be pathogens of other organisms besides bacteria, and/or (ii) these phages could be in different phases of cell infection at the sampling moment. Other authors found a similar lack of correlation in temporal studies in the NW Mediterranean Sea (Bettarel *et al*., 2002; M.G. Weinbauer, unpublished) and in the North Sea (Winter *et al*., 2004). Nevertheless, Jiang and Paul (1994) have found a significant positive relationship between viral and bacterial abundances during an annual cycle in Tampa Bay (Florida). Abundance of HNF was similar to values found in previous years in Blanes Bay (Vaqué *et al*., 1997; Unrein *et al*., 2007). Heterotrophic nanoflagellates were weakly correlated with bacterial abundance, and this correlation did not improve much more when considering the bacterivore HNF 2–5 μm fraction (Wikner and Hagström, 1988), suggesting that trophic cascade effects (Calbet *et al*., 2001) could have blurred the real bacteria–HNF relationship. Thus, the lack of strong correlations observed between most variables in this study could be due to changes in metabolic processes and succession of communities at all trophic levels. We are also aware that the considered microbial processes could be highly variable within days, or even hours (e.g. Winter *et al*., 2004). Then, with the span of sampling we probably missed small oscillations within short periods of time, which would be reflected in weak correlations among variables. It is interesting that roughly constant bacterial abundances (variation about 2.5-fold) can be maintained in the environment in spite of highly oscillating abundances of predators within years.

Lysogeny

There are indications that lysogeny dominates in oligotrophic systems as a survival strategy of viruses at low host densities (Stewart and Levin, 1984). Weinbauer and colleagues (2003) have found that the frequency of lysogenic cells was inversely related to bacterial abundance and production in Mediterranean and Baltic Seas. Also, higher percentage of lysogens was observed in bacteria isolates from an offshore poor environment (Jiang and Paul, 1994), which could suggest that the trophic conditions, not only host density, determine the occurrence of lysogeny. During our study we did not find any correlation between bacterial abundance and lysogenic infection. However, we have detected higher number of lysogeny cases in the second year, when nutrient concentrations were lower than in the first year (Fig. 1B). Thus, our results seem to indicate that lysogeny might be influenced by the trophic status of the system. We are aware, however, that more data would be needed to prove this suggestion.

Bacterial losses due to protists and viruses

Average values of bacterial losses by protists per year were similar (Table 1), indicating constant PMM in this system over the two years. In the same study area, similar grazing rates were found in previous studies (Unrein *et al*., 2007). Within protists, HNF are considered the main bacterivores, while ciliates feed preferentially on larger cells as nanoflagellates (Stoecker and Capuzzo, 1990), and/or small phytoplanktonic cells (Sherr and Sherr, 2002). In agreement with this, HNF abundances, especially HNF 2–5 μm and HNF 5–10 μm size classes, were positively correlated with bacterial abundance, grazing rates and BP, while ciliates presented correlation with the HNF 5–10 μm and HNF 10–20 μm fractions.

Viruses were an important source of bacterial mortality in this oligotrophic environment, sometimes overweight-

Table 3. Bacterial production losses caused by protists and viruses detected simultaneously in different systems and methods used to determinate prokaryotic mortality caused by viruses.

System/geographical location	Method	Losses by protists	Losses by viruses	Reference
Extreme systems				
Arctic				
Bering and Chukchi Seas	TEM			Steward *et al*. (1996)
Integrated depths				
Bottom		23 (17–30)	23 (9–37)	
Bottom		5 (1–9)	23 (9–37)	
50 m		17 (6–27)	11 (3–20)	
Bottom		25 (9–40)	12 (4–21)	
50 m		3 (1–6)	11 (3–19)	
Bottom		4 (1–8)	9 (2–16)	
Franklin Bay, Canadian Arctic	VDA	nd to –0.004[a]	–0.006 to –0.015[a]	Wells and Deming (2006)
Antarctic				
Bellingshausen Sea	VDR	35.3	400	Guixa-Boixereu *et al*. (2002)
Bransfield Strait		37.2	180	
Gerlache Strait		0.9	44	
Eutrophic				
Santa Monica, California, USA	BDR	1.8–2.2[b]	1.2–1.4[b]	Fuhrman and Noble (1995)
		2.8–3.4[b]	2.7–2.8[b]	
Ria de Aveiro, Portugal	VDA			Almeida *et al*. (2001)
Marine waters		69	36	
Brackish waters		73	59	
Masan Bay, Korea	TEM	41	9.4	Choi *et al*. (2003)
Mediterranean Sea, Spain (Masnou harbour)	VDR	120	100	Guixa-Boixereu *et al*. (1999a)
Oligotrophic				
Mediterranean Sea, Spain	TEM	31.0	nd	Guixa-Boixereu *et al*. (1999b)
		36.5	nd	D. Vaqué (unpubl. data)
		74.0	nd	
Mediterranean Sea, Spain	TEM	19	17	Guixa-Boixereu *et al*. (1996)
East Sea, Korea	TEM	9.5 (1.6–32.9)	13.1 (6.9–26.9)	Hwang and Cho (2002)
Mediterranean Sea, France	TEM	26–80	nd–18	Bettarel *et al*. (2002)
Mediterranean Sea, Spain	VRA	33.9 (nd–77.6)	12.2 (3.1–47.7)	This study
		32.4 (nd–77.0)	40.9 (9.6–84.1)	

a. Bacterial mortality in h^{-1} (for details see reference).
b. Bacterial loss rate in % h^{-1} (for details see reference)
BDR, bacterial decay rate; VDR, viral decay rate; TEM, determination of frequency of visibly infected cells by transmission electron microscopy; VDA, virus dilution approach; VRA, virus reduction approach; nd, no detected

ing the impact of protists on the bacterial community. Comparison of bacterial losses due to protists and viruses in different marine systems shows that in eutrophic habitats those two predators cause similar losses of bacterial production (Fuhrman and Noble, 1995), in extreme systems (Arctic and Antarctic) viruses appear to be the main source of bacterial mortality (Guixa-Boixereu *et al*., 2002), and in oligotrophic waters impact of protists is often more important than viruses (Bettarel *et al*., 2002) (Table 3). However, in the second year of our study, viruses and protists had a similar impact on bacteria, and these results are in agreement with findings of Hwang and Cho (2002) in the oligotrophic East Sea, Korea. The type of bacterial mortality has a great importance for carbon and nutrient fluxes through the ecosystem. Grazing on bacteria by protists shifts the particular organic carbon (POC) to higher trophic levels, whereas viral infection causes lysis of bacterial cell and release of the dissolved and detrital organic matter. This dissolved organic matter (DOM) can be a source of nutrients, such as N, P or Fe, for other microorganisms (Fuhrman, 1999). We have calculated that during our study, the potential bacterial carbon flux to higher trophic levels mediated by HNF was on average 3.12 ± 3.35 μg C l^{-1} day^{-1} (range: no detectable to 16.1 μg C l^{-1} day^{-1}), assuming a cell-carbon factor of 12 fg C $cell^{-1}$ (Simon and Azam, 1989). In contrast, the input of bacterial carbon to the DOC pool due to VMM was in average 2.5 ± 2.9 μg C l^{-1} day^{-1} (range: 0.2–12.1 μg C l^{-1} day^{-1}). The dominance of one of these two processes will, thus, shape the carbon flow pattern through the food webs.

Mortality of bacteria due to phages during our study increased with the increase of bacterial abundance and production, suggesting that the metabolic status of the host is critical for viral infection and proliferation, as it was found in other studies (Steward *et al*., 1996; Weinbauer *et al*., 2003). The interesting fact is that the averages of VP_L and VMM over the first year were approximately threefold lower than during the second year, which means that bacterial mortality due to viruses was a more variable

process than mortality due to protists. On the other hand, the averages of viral and bacterial abundances remained similar between the two years, as well as other measured parameters, except nitrate, which had higher, yet not significantly, concentrations during the first year than during the second year (Fig. 1B). Thus, variation in VP_L and VMM could be related with the occurrence of structures named porins on the bacterial surface wall (Szmelcman and Hofnung, 1975; Delcour, 1997), which allow the continuous exchange of nutrients across the bacterial cell wall with the surrounding environment. These structures are also receptor sites for phages (Braun and Hantke, 1977). Porins' number on the cell wall can be modified in response to changing environmental conditions, e.g. can be lost under higher viral pressure (Lenski, 1988), or can appear under nutrients depletion (Korteland *et al*., 1982; Poole and Hancock, 1986) favouring new viral infections. It is also possible that changes in VMM during our study were caused by changes in bacterial community composition induced by nutrient conditions (Øvreås *et al*., 2003; Alonso-Sáez *et al*., 2007). The bacterial mortality due to viruses could be therefore enhanced or depleted due to distinct susceptibility on viral infection of newly dominating bacterial groups (Bouvier and del Giorgio, 2007).

In summary, preservation of bacterial population abundance at constant level, in spite of environmental changes and variation of predation pressure, is possible. Interestingly, in a microcosm experiment, Middelboe and colleagues (2001) also observed that bacterial abundance was not affected by phages during their experiments, since infection-resistant bacteria complemented the abundance decrease caused by viral lysis. It seems clear then that although the bacterial standing stocks are similar between years, different mechanisms can control bacterial growth and create different paths for the carbon flow in the system. However, further experimental studies are needed to determine factors that regulate the dominance of grazing or bacterial lysis through the year.

Conclusions

During our study, phages were an important source of bacterial mortality in an oligotrophic coastal area (NW Mediterranean), causing in 37% of cases higher losses of bacterial production than protists. Lytic infection dominated over lysogeny during the two years. We suggest that changes in nutrient concentrations in the environment play an important role in regulating viral infection of bacterial cells.

Experimental procedures

Study site and sampling strategy

Surface water samples (0.5 m) were collected from May 2005 to April 2007 in Blanes Bay, Spain (The Blanes Bay Microbial Observatory, NW Mediterranean, 41°40′N, 2°48′E, 20 m depth), at 0.5 miles from the shore. Samples were collected once a month, in 10 l polyethylene carboys, and kept in the dark and refrigerated until reaching the lab (~2 h). Water temperature and salinity were measured *in situ* with a CTD (conductivity, temperature, depth).

Chlorophyll a *and nutrient concentrations*

Chlorophyll *a* was extracted from 250–500 ml of water samples and determined fluorometrically (Yentsch and Menzel, 1963). Inorganic phosphorous and nitrates were analysed using standard methods (Grasshoff *et al*., 1983).

Abundances of microorganisms

Viral abundances were determined by flow cytometry. Subsamples (2 ml) were fixed with glutaraldehyde (0.5% final concentration), refrigerated, quick frozen in liquid nitrogen and stored at −80°C as described in Marie and colleagues (1999). Counts were made using FACSCalibur flow cytometer (Becton and Dickinson) with a blue laser emitting at 488 nm. Samples were stained with SYBR Green I, and run at a medium flow speed (Brussaard, 2004).

The term 'bacteria' used along this article refers to all prokaryotes, as in our study we did not distinguish between *Bacteria* and *Archaea*. Bacteria and HNF *in situ* abundances were obtained by epifluorescence microscopy (Olimpus BX40). Subsamples 100 ml were fixed with glutaraldehyde (1% final concentration). Aliquots of 20 ml were filtered through 0.2 μm (for bacteria), and 60 ml through 0.6 μm (for HNF) black polycarbonate filters, and stained with DAPI (4,6-diamidino-2-phenylindole; Porter and Feig, 1980) at a final concentration of 5 μg ml^{-1} (Sieracki *et al*., 1985). At least 200–300 bacteria and 20–100 HNF were counted per sample. Heterotrophic nanoflagellates were grouped into four size classes: ≤ 2, 2–5, 5–10 and 10–20 μm.

Ciliate abundances were determinate using the Utermöhl method. One litre of sample was fixed with acidic lugol (2% final concentration). After sedimentation of 100 ml aliquots, ciliates were counted in an inverted microscope (Zeiss) and identified to genera level when possible (Lynn and Small, 2000).

Bacterial mortality and production

Bacterial losses due to protists were evaluated following the FLB (Spanish Type Culture Collection, http://www.cect.org/index2.html, Burjassot, València) disappearance method (Sherr *et al*., 1987; Vázquez-Domínguez *et al*., 1999). Three polycarbonate (2 l) bottles were filled with 1 l of natural seawater, and other two (controls) with virus-free water. Fluorescent labelled bacteria were added to a final concentration of 15–20% of bacterial *in situ* concentration. Bottles were incubated in a thermostatic chamber during 24 h, simulating *in situ* temperature and light conditions. Samples were taken at time 0 and 24 h to evaluate abundances of bacteria, FLB and HNF by epifluorescence microscopy. Control bottles showed no decrease of FLB during the experiment.

Grazing rates of bacteria were obtained following the mathematical model #3 of Salat and Marrasé (1994), based on the specific grazing rate (g) and specific net growth rate (a). First, net bacterial production (BP_N) in the incubation bottles was obtained:

$$BP_N = BA_0 \times (e^{at} - 1)[\text{cells ml}^{-1}\ \text{day}^{-1}],$$

where BA_0 is bacterial abundance at the beginning of the experiment; t is time of experiment (1 day).

Then, grazing rate (G) was calculated

$$G = (g/a) \times BP_N[\text{cells ml}^{-1}\ \text{day}^{-1}].$$

Finally, protists-mediated mortality of bacteria (PMM) was calculated as the percentage of BSS and BP losses:

$$PMM_{BSS} = (G \times 100)/BA_0\,[\%\ \text{day}^{-1}],$$

$$PMM_{BP} = (G \times 100)/BP[\%\ \text{day}^{-1}].$$

The virus reduction approach (Weinbauer *et al*., 2002; Wilhelm *et al*., 2002) was followed to determine viral production and bacterial losses due to phages. Briefly, 1 l of seawater was pre-filtered by 0.8-μm-pore-size cellulose filter (Whatman), and next concentrated by a spiral-wound cartridge (0.22 μm pore size, VIVAFlow 200), obtaining 60 ml of bacterial concentrate. Virus-free water was collected filtering 1 l of seawater using a cartridge of 100 kDa molecular mass cut-off (VIVAFlow 200). A mixture of virus-free water (240 ml) and bacterial concentrate (60 ml) was prepared and distributed into six sterile falcon plastic tubes (50 ml in each one). Three of the tubes were maintained without any manipulations as controls, while in other three, mitomycin C (Sigma) was added (1 μg ml^{-1} final concentration) as inducing agent of the lytic cycle in lysogenic bacteria. All falcon tubes were incubated in a thermostatic chamber simulating *in situ* temperature and light conditions, during 12–19 h. Samples for bacterial and viral abundances were collected at time zero and each hour during the first 6 h of the experiment, and at the end of the experiment. The choice of the sampling period was made based on previous experiments that showed that almost 100% of the lysis occurs during the first 6 h. Samples for viral and bacterial counts were fixed with glutaraldehyde and stored as described before for viruses. Viruses were counted by flow cytometry (see above). Bacteria were stained with SYTO 13 and run in a flow cytometer at a low flow speed, using 50 μl of 0.92 μm yellow-green latex beads as an internal standard (del Giorgio *et al*., 1996; Gasol and del Giorgio, 2000). Burst size (BS) was estimated from viral production experiments, as in Middelboe and Lyck (2002) and Wells and Deming (2006). Briefly, an increase of viral abundance during an incubation period of viral production experiment was divided by a decrease of bacterial abundance at the same period of time. In all these experiments, growth of bacteria was observed and such calculations were possible only for very short time intervals (1 h). Burst size estimated ranged from 6 to 375 viruses per cell, with mean values of 93 ± 92 in the first year, and 91 ± 94 in the second year.

Estimation of virus-mediated mortality of bacteria (VMM) was performed following the model presented by Weinbauer and colleagues (2002) and Winter and colleagues (2004). Briefly, virus increase in the control tubes represents lytic viral production (VP_L), and an increase in mitomycin C treatments represents total (VP_T), i.e. lytic plus lysogenic, viral production. A difference between VP_T and VP_L represents lysogenic production (VP_{LG}). As during tangential flow filtration the loss of part of bacterial *in situ* standing stock occurred (from 1% to 78%, mean of 56.3 ± 20.3%), we multiplied VP_L and VP_{LG} by the bacterial loss factor (Winget *et al*., 2005) to compare the values between different months. The percentage of lysogeny in total viral production was calculated:

$$\%\ VP_{LG} = (VP_{LG} \times 100)/VP_T\,[\%].$$

Following the method used by Guixa-Boixereu (1997), the rate of lysed cells (RLC) was obtained dividing VP_L by BS:

$$RLC = VP_L/BS[\text{cells ml}^{-1}\ \text{day}^{-1}].$$

RLC was used to calculate VMM as a percentage of bacterial standing stock (VMM_{BSS}):

$$VMM_{BSS} = (RLC \times 100)/BA_0\,[\%\ \text{day}^{-1}],$$

where BA_0 is the initial bacterial abundance in the viral production experiment. Assuming that percentage of losses of bacterial standing stock due to viruses is the same in falcon tubes and in the grazing polycarbonate bottles, we used VMM_{BSS} to calculate the rate of lysed bacteria during the grazing experiment (RLC_{GR}):

$$RLC_{GR} = (VMM_{BSS} \times BA_{GR})/100[\text{cells ml}^{-1}\ \text{day}^{-1}],$$

where BA_{GR} is bacterial abundance in the grazing bottles at time zero. Finally, using RLC_{GR}, VMM as a percentage of bacterial production (VMM_{BP}) could be calculated:

$$VMM_{BP} = (RLC_{GR} \times 100)/BP[\%\ \text{day}^{-1}],$$

where BP is total bacterial production.

Bacterial production was calculated summing the BP_N, G and RLC_{GR}:

$$BP = BP_N + G + RLC_{GR}\,[\text{cells ml}^{-1}\ \text{day}^{-1}].$$

Statistical analyses

Normal distribution of data was checked using the Shapiro–Wilk *W*-test, and data were logarithmic transformed if necessary. Annual variations of different parameters were analysed by one-way ANOVA for normal distributions, and by Wilcoxon test for non-normal distributions. The Pearson correlation and regression analyses were used to determine the relationships between parameters. All statistical analyses were performed using the JMP program.

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