JPR Advance Access published July 29, 2011

Effect of viruses on marine stramenopile (MAST) communities in an oligotrophic coastal marine system

ANDREU SAURA[1], RAMON MASSANA[1], JULIA A. BORAS[1], IRENE FORN[1], GEMMA VILA-REIXACH[2] AND DOLORS VAQUÉ[1]*
[1]INSTITUT DE CIÈNCIES DEL MAR (CSIC), PASSEIG MARÍTIM DE LA BARCELONETA 37-49, 08003 BARCELONA, CATALONIA, SPAIN AND [2]DEPARTAMENT D'ECOLOGIA, UNIVERSITAT DE BARCELONA, AVINGUDA DIAGONAL 645, 08028 BARCELONA, CATALONIA, SPAIN

*CORRESPONDING AUTHOR: dolors@icm.csic.es

Received November 25, 2010; accepted in principle June 13, 2011; accepted for publication June 26, 2011

Corresponding editor: John Dolan

Viruses are the most abundant biological entities in aquatic systems, infecting from bacteria to mammals. However, there has been little study so far on their impact on marine heterotrophic flagellates (HFs). For this reason, four experiments were carried out between April 2006 and February 2007 in the Blanes Bay Microbial Observatory (NW Mediterranean coast). We investigated whether viruses could affect HF communities, and specifically two uncultured marine stramenopile groups (MAST-4 and MAST-1C). For each experiment, four microcosms containing 12 L of 5-μm filtered seawater were prepared, two received active viruses and the other two received heat-inactivated viruses. Microcosms were then incubated for 48 h in order to measure changes in the abundance of the target groups. In three of the four experiments, both the growth rates of HF and MAST-4 cells and the percentage of MAST-4 cells with respect to HF after 48 h were higher in the heat-inactivated treatment compared with the active viruses treatment. These results indicate that viruses can negatively affect the HF community either directly via lysis of protists or indirectly via lysis of bacteria, and highlight the interactions between, virus, bacteria and protists.

KEYWORDS: viruses; bacteria; heterotrophic flagellates; stramenopiles

INTRODUCTION

It was only two decades ago that viruses were first recognized as the most abundant and ubiquitous members of aquatic microbial communities (Bergh *et al.*, 1989). Research aimed at determining their ecological significance was initiated relatively recently and our knowledge on this topic is still limited. The hosts of aquatic viruses may be found among all marine organisms, including prokaryotes (*Bacteria* and *Archaea*), protists, invertebrates, fish and mammals. However, since bacteria are the most abundant cells in aquatic environments, it appears that the majority of natural viruses, in this case called bacteriophages, infect this group of microorganisms (Bergh *et al.*, 1989; Fuhrman, 1999). The direct effect of prokaryotic infections is the release of organic matter on which bacterioplankton feed in the so-called "viral loop" (Bratbak *et al.*, 1990). Besides bacteriophages, viruses infecting marine microbial eukaryotes can be important mortality agents, producing changes in the dynamics and structure of protist communities (Bratbak *et al.*, 1990; Brussaard *et al.*, 2004). The viral impact on important algal species has been observed in the field, with viruses preventing or terminating algal blooms. Reports of the viral control of populations of the DMS-producing *Emiliania huxleyi* (Bratbak *et al.*, 1996), of the harmful

doi:10.1093/plankt/fbr068, available online at www.plankt.oxfordjournals.org

bloom-forming *Heterosigma akashiwo* (Tarutani *et al*., 2000) and of the abundant coastal picoeukaryote *Micromonas pusilla* (Cottrell and Suttle, 1995; Brussaard *et al*., 1999) are examples of the effect of viruses on phytoplankton. Viruses that infect these and other algal taxa have been isolated and maintained in culture, where their lytic cycle, infectivity range and molecular features have been determined (Wilson *et al*., 2006; Zingone *et al*., 2006).

However, there are still very few studies on the viral infection of marine heterotrophic flagellates (HFs), and how this contributes to changing their performance within the microbial food web. For instance, Garza and Suttle (Garza and Suttle, 1995) isolated and maintained in culture a virus that infects *Bodo* sp., and Massana *et al*. (Massana *et al*., 2007) reported a crash of a population of the marine HF *Cafeteria roenbergensis* presumably due to viral infection. Recently, Fischer *et al*. (Fischer *et al*., 2010) used 454 pyrosequencing to sequence the genome of the *C. roenbergensis* virus.

In oligotrophic marine systems, most HFs are between 2 and 5 μm and reach abundances of 100–1000 cells mL^{-1} but their *in situ* diversity remains largely unknown (Arndt *et al*., 2000). Recently, in surface water samples, Massana *et al*. (Massana *et al*., 2004, 2006a, b) identified relatively abundant uncultured protist groups, called marine stramenopiles (MAST), by using environmental sequencing and fluorescent *in situ* hybridization (FISH) approaches. MAST protists were shown to be free-living bacterivorous HFs (Massana *et al*., 2009). They are widely distributed, occurring in the five world oceans and account for a significant fraction (up to 35%) of the HFs in diverse geographic regions. In the NW Mediterranean Sea, the numerically dominant MAST groups are MAST-4 and MAST-1C (Massana *et al*., 2006a). MAST-4 is also rather abundant in marine ecosystems worldwide and probably contributes to picoplankton grazing and nutrient remineralization (Rodríguez-Martínez *et al*., 2009). At this point, considering that MAST cells might play an important role in marine systems, it would be interesting to explore how viruses can interact with them in order to gain a wider picture of the functioning of microbial food webs.

The main goal of this study was to determine the impact of viruses on HFs in terms of abundance, growth rates and composition, and more specifically whether or not viruses affect two MAST groups. Therefore, we conducted four microcosm experiments with microbial communities from an oligotrophic coastal marine system (NW Mediterranean Sea). We added active or heat-inactivated viruses to the experimental microcosms and evaluated to what extent viruses modified the abundance and growth rate of the total community of HFs, and particularly of the MAST-4 and MAST-1C groups. Taxonomic shifts were observed with the FISH technique with oligonucleotide probes. It could be clearly observed that viruses had a negative effect on the total HFs and the MAST-4 group, although the actual mechanism of this effect could not be established.

METHOD

Study site

One hundred litres of surface water (0.5-m depth) were collected on four occasions (April, July and November 2006 and February 2007) in Blanes Bay, Catalonia, Spain (The Blanes Bay Microbial Observatory, NW Mediterranean Sea, 41° 40′ N, 2° 48′ E, 20-m depth bottom), at 0.5 miles from shore. Water was prefiltered *in situ* with a 50-μm mesh, collected in 50 L polyethylene carboys, and kept in the dark at a low temperature until reaching the laboratory (~2 h). The water temperature and salinity were measured *in situ* with a SeaBird.SBE25 CTD (conductivity, temperature, depth). Once in the laboratory subsamples for determining *in situ* chlorophyll *a* (Chl *a*), viral, bacterial and HF concentrations were taken immediately.

Experimental design

Fifty litres of the sampled seawater were filtered through 5-μm pore size polycarbonate Poretics filters (47 mm of diameter) to remove large nanoplankton and microzooplankton but retaining the natural communities of viruses, bacteria and small protists. This 5-μm filtered seawater was poured into two 25 L plastic carboys using a peristaltic pump. The remaining 50 L of seawater were filtered sequentially with 5, 0.8 and 0.2 μm Poretics filters (diameter, 47 mm) and concentrated to 1 L of viral concentrate using a cartridge of 100 kDa molecular mass cutoff (PREP/SCALE-TFF, 0.23 m^2). Half of the viral concentrate (0.5 L) was kept active (without further handling) and the other 0.5 L was heated in a water bath at 80°C for 30 min for virus inactivation. The time taken to complete all the manipulations for the experimental set-up was ~8 h. Before adding viral concentrates to the carboys, we took samples from the microcosms and from the active and inactivated viral concentrates in order to determine the viral abundances. Then, each treatment was prepared by adding 0.5 L of active or heat-inactivated viral concentrate to each 25 L carboy, which resulted in one virus-enhanced treatment (FBV, Flagellate, Bacteria and *in situ* viruses, + active Viruses) and one virus non-enhanced treatment (FB, Flagellate, Bacteria and *in*

situ viruses + inactivated viruses). From each treatment, 12 L duplicates were prepared in 20 L microcosms and incubated at *in situ* temperature and in the dark during 48 h. Samples for determining Chl *a*, viral, bacterial and total HF abundances were collected from all duplicates at the beginning ($t = 0$) and at the end of the incubation ($t = 48$ h). Samples for MAST-4 and MAST-1C FISH counts were collected from each treatment (25 L) at $t = 0$ and from each duplicate (12 L) at time 48 h.

Dissolved organic matter (DOC, DON and DOP)

Dissolved organic carbon (DOC), dissolved organic nitrogen (DON) and dissolved organic phosphorous (DOP) from samples collected *in situ* and from each microcosm at time zero were analysed. The seawater was filtered through precombusted (450°C, 4 h) 47-mm diameter Whatman GF/F filters in an acid-cleaned glass filtration system under low N_2-flow pressure. Samples for the analysis of DOC/DON were collected in 60 mL precombusted (450°C, 4 h) amber glass bottles. After $HgCl_2$ had been added the bottles were sealed with a polyethylene cap and stored in the dark at 4°C until analysis. DOC and DON concentrations were obtained following the technique developed by Cauwet (Cauwet, 1999), and were measured simultaneously with a nitrogen-specific TNM-1 unit with a nitrogen dioxide chemiluminescence detector coupled in series with a carbon-specific infrared gas analyzer of a Shimadzu TOC-5000 organic carbon system, using high-temperature catalytic oxidation. For DOP analysis, the filtrate was collected and kept frozen in 60 mL Nalgene bottles (polycarbonate) for a few months until analysis (Hansen and Koroleff, 1999) based on an acid persulphate digestion (at 120°C in an autoclave for 30 min).

Biological parameters

Chl *a* was extracted from 250 to 500 mL of seawater and determined fluorometrically (Yentsch and Menzel, 1963). Viral abundances were measured by flow cytometry. Subsamples (2 mL) were fixed with 4°C glutaraldehyde (0.5% final concentration), quick frozen in liquid nitrogen and stored at −80°C (Brussaard, 2004). Counts were made in a FACSCalibur flow cytometer (Becton & Dickinson) with a blue laser emitting at 488 nm. Samples were stained with SYBR Green I, and run at a flow rate ranging from 0.028 to 0.045 μL min^{-1}, which in our cytometer corresponds to the medium flow speed (Brussaard, 2004). Bacteria were fixed and stored as described for viruses, stained with SYTO 13 and processed at a flow rate from 0.010 to 0.016 μL min^{-1} (low flow speed), using 50 μL of a 0.92 μm yellow–green latex bead solution as an internal standard (del Giorgio *et al.*, 1996). The actual flow rate was measured every four samples by weighting the tubes before and after the sample was analysed with the flow cytometer. Furthermore, samples of viruses were diluted with TE-buffer and some samples of bacteria were prefiltered with Milli-Q water to adjust the concentration of particles to the flow speed and keep the counts <800 events s^{-1}. HFs were enumerated by epifluorescence microscopy (Olympus 20 BX40) under UV radiation. Subsamples (50 mL) were fixed with 4°C glutaraldehyde (1% final concentration), and 20 mL were filtered through 0.6-μm, 25-mm diameter, black polycarbonate filters after staining 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 50–100 HF cells were counted per sample. Ninety millilitre samples for FISH or CARD (Catalyzed Reported Deposition)-FISH were fixed with formaldehyde (3.7% final concentration), kept at 4°C for 24 h, filtered with 0.6-μm, 47-mm diameter, white polycarbonate filters and stored at −80°C until processed. Filter-immobilized protists were hybridized with CY3-probes (MAST-1C), and HPR-probes (MAST-4), counter-stained with DAPI and observed by epifluorescence microscopy under UV (DAPI staining), green light excitation (FISH signal) and blue light excitation (CARD-FISH signal). Hybridization conditions followed previously described protocols for FISH (Pernthaler *et al.*, 2001; Massana *et al.*, 2006b) and CARD-FISH (Urdea *et al.*, 1988; Not *et al.*, 2002; Pernthaler *et al.*, 2004). Four slice portions of the same filter and four to six transects (15–18 mm) in each portion (a total of 16–24 sub-replicates for each data point) were processed for each MAST count in order to reduce the effect of a heterogeneous cell distribution, and provide mean values.

The growth rates (μ; units in d^{-1}) of the total HF, MAST-1C and MAST-4 assemblages were estimated as follows:

$$\mu = (1/t) * \ln(F_t/F_o)$$

where t is the incubation time (48 h), F_t is flagellate (HF or MASTs) numbers at the final time, and F_o is the flagellate numbers at the initial time.

Statistical analysis

A Shapiro–Wilk W test was used to check that data were distributed normally, and data were logarithmically transformed if necessary. ANOVA tests were used to identify significant differences in MAST counts between subsamples (four portions of the same filter) and between

Table I: In situ *physico-chemical and biological parameters of seawater collected for the experiments in the Blanes Bay Microbial Observatory*

Samples	Temp (°C)	DOC	DON	DOP	Chl *a* (μg L^{-1})	Viruses (mL^{-1})	Bacteria (cells mL^{-1})	HF (cells mL^{-1})
Spring	13.5	69.39	3.96	0.06	1.23	3.03×10^7	1.74×10^6	148
Summer	23.7	87.11	2.08	0.05	0.09	1.88×10^7	3.94×10^6	772
Autumn	17.0	60.17	0.91	0.07	0.60	0.99×10^7	0.49×10^6	456
Winter	14.0	60.26	4.78	0.08	1.04	1.10×10^7	0.48×10^6	700

Temp., temperature; DOC, dissolved organic carbon (μmols L^{-1}); DON, dissolved organic nitrogen (μmols L^{-1}); DOP, dissolved organic phosphorous (μmols L^{-1}); Chl *a*, chlorophyll *a*; HF, heterotrophic flagellates (≤5 μm).

treatments, and also to detect differences in DOM between treatments at time 0. *T*-tests were also applied to determine differences in abundances and growth rates of the HF and MAST groups, as well as differences in the percentages of MAST groups respect to HF between treatments. These statistical analyses were performed using the JMP and Kaleidagraph programs.

RESULTS

Environmental and microbial parameters

Four experiments were prepared with seawater samples showing contrasting values of *in situ* temperature, (DOM) dissolved organic matter (DOC, DON, DOP), Chl *a* and microbial abundances (Table I). The temperature varied from 13.5°C in spring, when the viral abundance and Chl *a* values were highest, to 23.7°C in summer, when DOC, bacterial and HF abundance values were highest. The maximum DON and DOP values were detected in winter together with rather high values of Chl *a* and HF (Table I). Detailed information about the monthly and seasonal *in situ* variability of the physico-chemical and biological variables in the system studied can be found elsewhere (Boras *et al.*, 2009). Clearly, the initial conditions of each experiment were very different and represented contrasting situations at different times of the year in this ecosystem.

Dissolved organic matter (DOC, DON, DOP) in the experimental microcosms

The DOM concentrations in the experimental microcosms at $t = 0$ are shown in Table II. DOC values ranged between 63.5 and 75.7 μmol L^{-1} (spring and summer in the FBV treatment, respectively). These values are close to the *in situ* values, which were lowest in autumn (60.17 μmols L^{-1}) and highest in summer (87.11 μmols L^{-1}) (Table I). The maximum DON and DOP concentrations were detected in the spring and summer experiments, respectively (Table II), which contrasts with the highest *in situ* values in winter (Table I). The differences observed between *in situ* and experimental values were probably due to the manipulation of experimental samples and the time elapsed before sampling. Interestingly, the DOC, DON and DOP concentrations in the different viral treatments (FB versus FBV) were not significantly different ($n = 8$, $P > 0.05$).

Table II: Concentration (μmols L^{-1}) of dissolved organic matter (DOC, DON and DOP) in the experimental microcosms at t = *0 in the active viruses (FB) and inactivated viruses (FBV) treatments*

Experiment	Treatment	DOC	DON	DOP
Spring	FB	65.97	3.56	0.05
	FBV	63.48	3.08	0.03
Summer	FB	72.49	7.32	0.07
	FBV	75.68	5.55	0.07
Autumn	FB	64.62	3.91	0.04
	FBV	70.96	4.00	0.03
Winter	FB	68.36	5.39	0.05
	FBV	75.09	4.62	0.09

Changes in microbial abundances during the experiments

The abundances of viruses in the four experiments at $t = 0$ as well as the *in situ* samples were highest in spring and summer and lowest in autumn and winter (Table I and III). At $t = 0$, the concentrations of the viral communities in the four experimental microcosm duplicates before the viral concentrate was added were not significantly different (DF = 7, $t_{value} = -1.94$, $P > 0.05$). After the inoculation of active and inactivated viruses in each treatment, the viral abundance increased by 29 ± 14% in FB and by 19 ± 10% in FBV (Table III). Nevertheless, the concentration of the total active viruses in FBV (*in situ* viral communities plus active viruses) was higher than in the FB treatments (*in situ* viral communities plus inactivated viruses) (DF = 7, $t_{value} = -5.28$, $P = 0.0011$). Bacterial abundances reached minimum values in autumn and maximum values in the spring experiments, while in the *in situ* samples these minimum

Table III: Abundance of viruses (10^7 mL^{-1}), bacteria (10^6 mL^{-1}), heterotrophic flagellates (HF < 5 μm, cells mL^{-1}), MAST-4 (cells mL^{-1}), MAST-1C (cells mL^{-1}) after viruses were added at the beginning (t = 0) and the end (t = 48) of four experiments

Experiments	Time (h)	Treatment	Viruses	Bacteria	HF	MAST-4	% MAST-4	MAST-1C	% MAST-1C
Spring	0	FB	3.7 ± 0.1	1.9 ± 0.1	76 ± 19	2	2.7 ± 0.7	0.8	1.2 ± 0.3
	48	FB	3.2 ± 0.1	2.1 ± 0.0	162 ± 27	24 ± 4	14.4 ± 2.3	0.7 ± 0.4	0.5 ± 0.4
	0	FBV	3.4 ± 0.1	2.0 ± 0.2	105 ± 19	3	3.3 ± 0.6	0.5	0.5 ± 0.1
	48	FBV	3.6 ± 0.2	2.2 ± 0.1	164 ± 24	7 ± 2	4.3 ± 1.0	0.6 ± 0.2	0.4 ± 0.1
Summer	0	FB	1.9 ± 0.0	1.8 ± 0.0	1440 ± 31	86	6.0 ± 0.1	16	1.1 ± 0.0
	48	FB	1.6 ± 0.0	1.4 ± 0.8	5918 ± 1640	718 ± 127	12.2 ± 0.2	46 ± 10	0.8 ± 0.3
	0	FBV	1.9 ± 0.0	1.6 ± 0.0	2114 ± 220	128	6.1 ± 0.6	37	1.8 ± 0.2
	48	FBV	1.6 ± 0.0	0.9 ± 0.0	6383 ± 784	385 ± 69	6.0 ± 0.6	52 ± 25	0.8 ± 0.3
Autumn	0	FB	1.3 ± 0.0	0.6 ± 0.0	678 ± 30	72	10.7 ± 0.5	4	0.6 ± 0.0
	48	FB	1.5 ± 0.2	1.7 ± 0.1	424 ± 84	nd	Nd	5 ± 1	1.2 ± 0.2
	0	FBV	1.0 ± 0.0	0.8 ± 0.0	396 ± 35	17	4.4 ± 0.4	nd	nd
	48	FBV	1.5 ± 0.0	1.6 ± 0.1	282 ± 102	nd	nd	2 ± 1	0.8 ± 0.4
Winter	0	FB	1.0 ± 0.0	0.8 ± 0.0	381 ± 131	8	2.2 ± 0.8	nd	0.3 ± 0.1
	48	FB	0.8 ± 0.1	1.0 ± 0.1	2463 ± 102	40 ± 4	1.6 ± 0.1	9 ± 9	0.4 ± 0.4
	0	FBV	0.7 ± 0.0	1.4 ± 0.0	490 ± 137	8	1.8 ± 0.5	2	nd
	48	FBV	0.9 ± 0.0	1.4 ± 0.3	1742 ± 163	26 ± 4	1.5 ± 0.2	7 ± 7	0.4 ± 0.4

The standard deviation shows the maximum and minimum range of the duplicates (±SD); nd, not detected; FB, inactivated virus treatment; FBV, active virus treatment.

and maximum values were found in winter and summer, respectively (Tables I and III). The HF abundances were highest in summer and lowest in spring in both the *in situ* samples and the experimental carboys. MAST-4 abundances in the microcosms at $t = 0$ varied from 2 to 3 cells mL^{-1} in spring to 86–128 cells mL^{-1} in summer. These values did not correspond to the minimum and maximum HF percentages, which were detected in winter (1.8–2.2%) and autumn (4.4–10.7%), respectively (Table III). MAST-1C showed very low abundances in all experiments (≤1 cells mL^{-1}), except for summer when they reached 16–37 cells mL^{-1} (Table III). Percentages of MAST-1C with respect to HF varied from 0.3% in winter to 1.1–1.8% in summer. At $t =$ 48 h, the Chl *a* concentration decreased in all microcosms, but these changes were not statistically significant (data not shown). Viral abundances did not show important changes with respect to $t = 0$, and tended to decrease slightly in all experiments except in autumn, while bacterial abundances increased in autumn and decreased in summer and almost no variations were detected in the spring and winter experiments (Table III). The HF abundance increased with respect to $t = 0$ in all experiments except in autumn (Table III). Finally, the MAST groups also increased after 48 h except for MAST-4 in autumn, when no hybridized cells were detected, and MAST-1C in spring and summer.

Response of HF and MAST to viral treatments

First, we used a nested ANOVA to test whether differences in MAST abundances were due to variability in the counts between sub-replicates or treatments. This showed that abundances of MAST-4 cells were significantly different between treatments (FB and FBV, $P <$ 0.05) but not between sub-replicates ($P > 0.05$) in all experiments (except in autumn when no hybridized cells were found). For MAST-1C, significant differences between treatments ($P < 0.05$), but not between sub-replicates ($P > 0.05$), were only found in summer.

The HF abundances in the autumn and winter experiments reached higher values in treatments with heat-inactivated viruses added to them (FB), whereas in spring and summer the HF abundances were similar in the FB and FBV treatments (Table III). Therefore, differences between the two treatments in terms of HF abundance were not statistically significant (DF = 7, $t_{value} = 1.52$, $P = 0.172$). In contrast, the MAST-4 cells was significantly more abundant (DF = 5, $t_{value} = 3.02$, $P = 0.029$) and there was a higher percentage of HF (DF = 5, $t_{value} = 2.82$, $P = 0.037$) in the FB than in the FBV treatments, except for in autumn when no hybridized cells were detected (Table III, Fig. 1A). In addition, the highest percentage of MAST-4 cells was detected in spring in the FB treatment, and represented 14.4% of HF (Table III). Higher percentages of MAST-1C cells were only detected in autumn in the FB treatment. We could not estimate significant differences due to these low MAST-1C values (Fig. 1B).

Growth rates of heterotrophic flagellates

The observed increases in the HF abundance after 48 h incubation translated into positive growth rates in three out of the four experiments in both treatments (Fig. 2A,

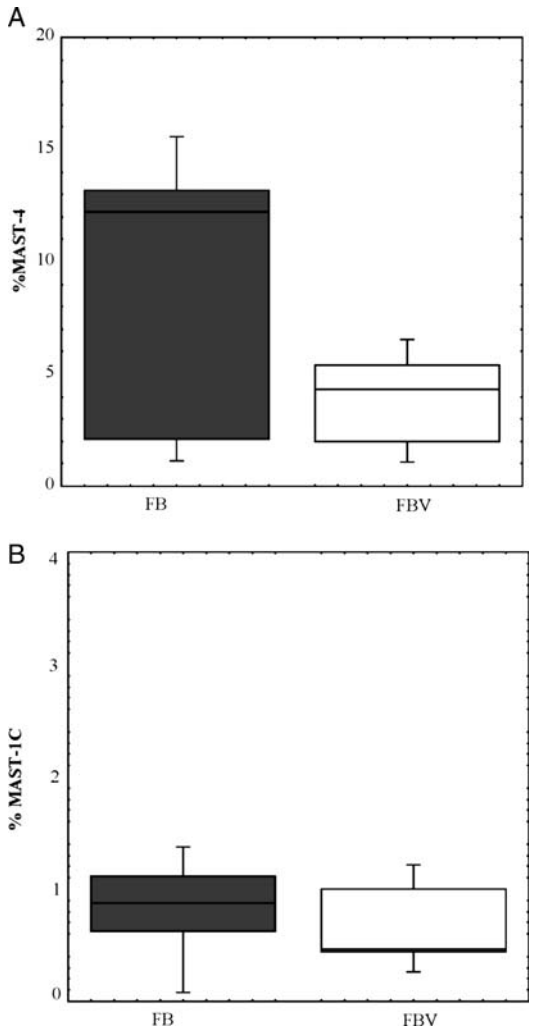

Fig. 1. Box and whiskers plots of the averaged percentage (pooling all data from all experiments for each treatment) of MAST-4 (**A**), and MAST-1C (**B**) with respect to the total HF at time 48 h of the experiment. The central horizontal line in each box represents the median of the distribution, while the other horizontal lines contain 50% of the values between them. The whiskers indicate the total range of values without outliers.

Table III). These growth rates were typically higher in heat-inactivated virus (FB) treatments than in active virus (FBV) treatments (Fig. 2A, Table III). These differences were significant when all experiments except for autumn were considered ($DF = 5$, $t_{value} = 4.25$, $P = 0.008$). Similarly, MAST-4 growth rates were always positive and significantly higher in the FB than in the FBV treatments considering all measurable experiments ($DF = 5$, $t_{value} = 3.08$, $P = 0.028$, Fig. 2B, Table III). As mentioned above, MAST-4 cells were not detected in autumn at 48 h, and therefore growth rates were not measurable (Fig. 2B, Table III). The MAST-1C growth rates did not show any

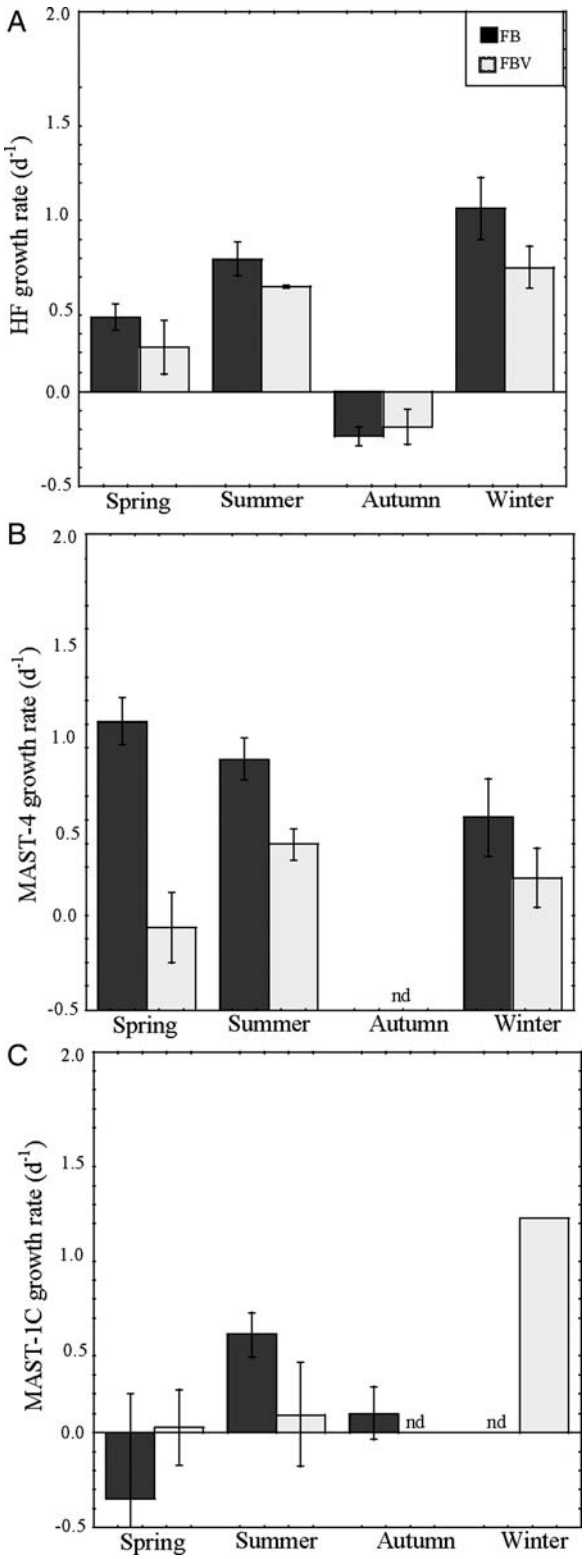

Fig. 2. Growth rates of total HF (**A**), MAST-4 (**B**) and MAST-1C (**C**) for both treatments (FB and FBV) in all experiments.

clear pattern, and there was large variability between duplicates (Fig. 2C, Table III). Finally, the growth rates of HF, MAST-4 and MAST-1C were only higher in FB than in FBV in the summer treatment (Fig. 2, Table III).

DISCUSSION

Methodological considerations

The experimental size fractionation set-up we used did not remove the natural viral community in either of the two treatments. We therefore assumed that in both treatments the effect of *in situ* viruses was the same, and the added inactivated viruses did not have any effect, while added active viruses were sufficient to enhance and provide evidence of the viral impact on flagellates (Figs 1 and 2). A similar set-up has been used in other studies examining the virus–bacteria–flagellate interactions (Šimek *et al.*, 2001; Sime-Ngando and Pradeep, 2005). Furthermore, Weinbauer *et al.* (Weinbauer *et al.*, 2007) also added active and heat-inactivated viruses to prepare the two treatments. A concern in this set-up is that adding heat-inactivated viruses could increase the DOM concentration in the microcosm due to viral material (proteins, nucleic acids) being destroyed by the heat, which would have an indirect effect on bacteria–HF interactions, and promote bacterial and HF growth. Our data showed that this was not the case because we did not find significant differences in the DOM concentration between treatments ($n = 8$, $P > 0.05$). We were also concerned that a part of viruses infecting eukaryotes may have been removed by the filtration through the 0.2-μm filter when the viral concentrate was prepared. It is well known that viruses of eukaryotes can be larger than bacteriophages. Thus, viruses infecting the 2–6-μm marine bicosoecid *Cafeteria roenbergensis* are between 230 and 300 nm in size (Garza and Suttle, 1995; Fischer *et al.*, 2010). In general, viruses belonging to the phycodnaviridae family have sizes between 100 and 220 nm in diameter (Wilson *et al.*, 2006), and viruses that infect eukaryotic photosynthetic and heterotrophic protists have a size range between *ca.* 20 and 220 nm (Nagasaki and Bratbak, 2010), as they are mimiviruses as examples of extremely large viruses that can be as large as 750 nm (La Scola *et al.*, 2003). Consequently, in order to cover the whole viral size range potentially capable of infecting HF and MAST cells, samples from the present study should have been filtered through 0.45 or 0.8 μm before the viral concentrate was prepared. However, this would have led to an extra amount of bacteria being added to the different treatments, which would have confused the results and interpretation. In addition, HF in our study always had sizes <5 μm and one of our target groups (MAST-4) is a picoeukaryote of 2–3 μm, so it is probable that their viruses would be in the lower size-range of phycodnaviridae and thus not excluded by our 0.2 μm prefiltration. We believe our approach is the best compromise in order to minimize manipulations of bacteria and nanoflagellates and enhance the viral effects on them.

Abundances and growth rates of the HF and MAST groups

In this study, the abundances of viruses, bacteria and HFs are in the range found in other studies in the Mediterranean Sea (Weinbauer *et al.*, 2003; Siokou *et al.*, 2010, and references therein) and in particular by the Blanes Bay Microbial Observatory (Alonso-Sáez *et al.*, 2007; Unrein *et al.*, 2007; Boras *et al.*, 2009). On a global scale, the MAST-4 group is the most abundant in temperate waters and represents ~10% of the total HF in surface waters (Massana *et al.*, 2006a, b; Rodríguez-Martínez *et al.*, 2009), while the MAST-1C group is generally less abundant but more abundant in polar systems (Massana *et al.* 2006a, b). In the present study, we found slightly lower abundances of MAST-4 cells compared with previously published values for the same site (Massana *et al.*, 2006a, b). Finally, the MAST-4 growth rates measured in the FB treatments were highest ($1.3\ d^{-1}$) in the spring experiment, and this increase in cells resulted in an HF percentage of 14.4% (Table III). However, the MAST-1C growth rates were always lower, reaching the highest value for the FB treatment in the summer experiment ($0.7\ d^{-1}$) (Fig. 2C). These growth rate values are in the range of the ones found by Massana *et al.* (Massana *et al.* 2006b) and Jürgens and Massana (Jürgens and Massana 2008). Hence, considering that there are still few data reported in the literature for these two MAST groups, our data seem to suggest that there are some differential functional roles between the two groups, in agreement with previous results (Massana *et al.*, 2009).

Complex interactions in the microbial food webs

Our general result is that the increase in viruses has an overall negative effect on HFs and MAST-4 cells, and these could be interpreted directly or through bacterial lysis. We already know that these two effects are very difficult to separate due to the multiple interactions among viruses, protists and bacteria (Miki and Jacquet, 2008). Current investigations into virus–bacteria–flagellate interactions can be grouped into three categories: (i)

experimental studies with natural communities that examine the direct lysis of HF by viruses, there are only two studies so far (Garza and Suttle, 1995; Massana *et al.*, 2007); (ii) studies that compare the effect of grazing on bacteria by HF with viral lysis as the main cause of bacterial mortality in natural systems (Bettarel *et al.*, 2002; Boras *et al.*, 2009, 2010), here the number of observations is starting to become relevant and (iii) experimental studies evaluating the interactions among viruses, bacteria and nanoflagellates, most of which are carried out in freshwater systems (reviewed in Miki and Jacquet, 2008). Regarding this last point, these investigations explore the synergistic or antagonistic interactions between viruses and HF in relation to bacterial mortality (Šimek *et al.*, 2001; Weinbauer *et al.*, 2007); however, there are no studies that assess the response of HF to the interaction between bacteria and viruses. In a previous study that used part of the same experimental set-up, Boras (Boras personal communication) found that bacterial mortality due to viral lysis was enhanced in treatments in which viruses and HF were present, and concluded that there are synergistic interactions between the two bacterial "predators". However, the effect of viruses on HF and on some of their components, such as MAST cells, has not yet been explored. In the present study, we observed a trend of decreasing growth rates for HF, MAST-4 and in some cases MAST-1C (not significant), and a decreasing percentage of MAST-4 cells, in treatments in which active viruses were added (FBV) (Figs 1A, 2A and B). Although, there is evidence of direct virus–HF interactions (Garza and Suttle, 1995; Massana *et al.*, 2007), we tend to support the alternative hypothesis of an indirect effect mediated by bacteria because HFs were never observed under the microscope to be clearly infected by viruses. Hence, the response of HF and MAST groups in the FBV treatments could be based on the competition of these two "predators" (viruses and HF) for the same prey (bacteria). Viral lysis of bacteria returns DOM to the water column, which can be taken up by other heterotrophic bacteria (Fuhrman, 1999). This promotes changes in the bacterial community structure that could have negative effects for the HF community. Consequently, bacterial lysis together with changes in bacterial diversity could favour a decline in HF growth rates in the FBV treatments, which supports the idea of competition for the same prey (Fig. 2). Nevertheless, this competition between HF and viruses for a certain prey does not exclude a synergistic effect on bacterial mortality due to viruses when both predators are present, as shown in previous studies (Weinbauer *et al.*, 2007; Miki and Jacquet, 2008). Nanoflagellates grazing on bacteria can also produce an increase in DOM due to the release of sloppy feeding material that enhances bacterial production, which in turn could increase viral infections and proliferation (Šimek *et al.*, 2001; Weinbauer *et al.*, 2007). Thus, bacteriophages benefit from the presence of protists by taking advantage of increased bacterial fitness.

In conclusion, our results show that viruses affected HF and MAST-4 communities in the Mediterranean Sea. Particularly relevant were the decreases in abundance and growth rates of HF and MAST-4 in incubations with active viruses added to them. Although, we cannot clearly relate the observed differences between treatments to a direct or indirect viral effect on HFs, our experimental approach illustrates a plausible virus–bacteria–protist interaction, which is complementary to what has been observed in recent investigations.

ACKNOWLEDGEMENTS

We wish to thank all our colleagues involved in the Blanes Bay Microbial Observatory for the sampling logistics and for providing the physico-chemical variables. We also thank C. Balestra for helping in the laboratory setting up the microcosms, and in the flow cytometer analysis.

FUNDING

This work was supported by the projects funded by the Spanish Ministry of Sciences and Innovation (CTM2004-04404-CO2-01 and CTM 2007-62140 to D.V., CGL2010-16304 to R.M. and CTM2008-06261-CO3-01 to M.L.)

REFERENCES

Alonso-Sáez, L., Balagué, V., Sà, E.L. *et al.* (2007) Seasonality in bacterial diversity in north-west Mediterranean coastal waters: assessment trough clone libraries, fingerprinting and FISH. *FEMS Microbiol. Ecol.*, **60**, 98–112.

Arndt, H., Dietrich, D., Auer, B. *et al.* (2000) Functional diversity of heterotrophic flagellates in aquatic ecosystems. In Leadbeater, B. S. C. and Green, J. C. (eds), *The Flagellates: Unity, Diversity and Evolution*. Taylor and Francis, London, pp. 240–268.

Bergh, O., Børsheim, K. Y., Bratbak, G. *et al.* (1989) High abundance of viruses found in aquatic environments. *Nature*, **340**, 467–468.

Bettarel, Y., Dolan, J. R., Hornak, K. *et al.* (2002) Strong, weak, and missing links in a microbial community of the NW Mediterranean Sea. *FEMS Microbiol. Ecol.*, **42**, 451–462.

Boras, J. A., Sala, M. M., Baltar, F. *et al.* (2010) Effect of viruses and protists on bacteria in eddies of the Canary Current region (subtropical northeast Atlantic). *Limnol. Oceanogr.*, **55**, 885–898.

Boras, J. A., Sala, M. M., Vázquez-Domínguez, E. *et al.* (2009) Annual changes of bacterial mortality due to viruses and protists in an oligotrophic coastal environment (NW Mediterranean). *Environ Microbiol.*, **11**, 1181–1193.

Bratbak, G., Heldal, M., Norland, S. *et al.* (1990) Viruses as partners in spring bloom microbial trophodynamics. *Appl. Environ. Microbiol.*, **56**, 1400–1405.

Bratbak, G., Wilson, W. and Heldal, M. (1996) Viral control of *Emiliania huxleyi* blooms? *J. Mar.* Sys., **9**, 75–81.

Brussaard, C. P. D. (2004) Optimisation of procedures for counting viruses by flow cytometry. *Appl. Environ. Microbiol.*, **70**, 1506–1513.

Brussaard, C. P. D., Noordeloos, A. A. M., Sandaa, R. A. *et al.* (2004) Discovery of a dsRNA virus infecting the marine photosynthetic protist *Micromonas pusilla*. *Virology*, **319**, 280–291.

Brussaard, C. P. D., Thyraug, R., Marie, D. *et al.* (1999) Flow cytometric analyses of viral infection in two marine phytoplankton species, *Micromonas pusilla* (Prasinophyceae) and *Phaeocystis pouchetii* (Prymnesiophyceae). *J. Phycol.*, **35**, 941–948.

Cauwet, G. (1999) Determination of dissolved organic carbon and nitrogen by high temperature combustion. In Grasshoff, K., Ehrhardt, M. and Kremling, K. (eds), *Methods of Seawater Analysis*. 3rd edn. Weinheim, Germany. Willey-VCH, pp. 407–421.

Cottrell, M. T. and Suttle, C. A. (1995) Dynamics of a lytic virus infecting the photosynthetic marine picoflagellate *Micromonas pusilla*. *Limnol. Oceanogr.*, **40**, 730–739.

del Giorgio, P. A., Bird, D. F., Prairie, Y. T. *et al.* (1996) Flow cytometric determination of bacterial abundance in lake plankton with the green nucleic acid stain SYTO 13. *Limnol. Oceanogr.*, **41**, 783–789.

Fischer, M. G., Allen, M. J., Wilson, W. H. *et al.* (2010) Giant virus with a remarkable complement of genes infects marine zooplankton. *PNAS*, **107**, 19508–19513.

Fuhrman, J. A. (1999) Marine viruses and their biogeochemical and ecological effects. *Nature*, **399**, 541–548.

Garza, D. R. and Suttle, C. A. (1995) Large double-stranded DNA viruses which cause the lysis of a marine heterotrophic nanoflagellate (*Bodo sp.)* occur in natural marine viral communities. *Aquat. Microb. Ecol.*, **9**, 203–210.

Hansen, H. P. and Koroleff, F. (1999) Determination of nutrients. In Grasshoff, K., Ehrhardt, M. and Kremling, K. (eds), *Methods of Seawater Analysis*. 3rd edn. Weinheim, Germany. Willey-VCH, pp. 200–201.

Jürgens, K. and Massana, R. (2008) Protistan grazing on marine bacterioplankton. In: Kirchman, D. L. (ed.), *Microbial Ecology of the Oceans*. 2nd edn. John Wiley and Sons, Inc., New York, USA, pp. 383–441.

La Scola, B., Audic, S., Robert, C. *et al.* (2003) A giant virus in amoebae. *Science*, **299**, 2033.

Massana, R., Balagué, V., Guillou, L. *et al.* (2004) Picoeukaryotic diversity in an oligotrophic coastal site studied by molecular and culturing approaches. *FEMS Microb. Ecol.*, **50**, 231–243.

Massana, R., Campo, J., Dinter, C. *et al.* (2007) Crash of a population of the marine heterotrophic flagellate *Cafeteria roenbergensis* by viral infection. *Environ. Microbiol.*, **9**, 2660–2669.

Massana, R., Guillou, L., Terrado, R. *et al.* (2006b) Growth of uncultured heterotrophic flagellates in unamended seawater incubations. *Aquat. Microb. Ecol.*, **45**, 171–180.

Massana, R., Terrado, R., Forn, I. *et al.* (2006a) Distribution and abundance of uncultured heterotrophic flagellates in the world oceans. *Environ Microbiol.*, **8**, 1515–1522.

Massana, R., Unrein, F., Rodríguez-Martínez, R. *et al.* (2009) Grazing rates and functional diversity of uncultured heterotrophic flagellates. *ISME J.*, **3**, 588–596.

Miki, T. and Jacquet, S. (2008) Complex interactions in the microbial world: underexplored key links between viruses, bacteria and protozoan grazes in aquatic environments. *Aquat. Microb. Ecol.*, **51**, 195–208.

Nagasaki, K. and Bratbak, G. (2010) Isolation of viruses infecting photosynthetic and nonphotosynthetic protists. In Wilhelm, S. W., Weinbauer, M. G. and Suttle, C. A. (eds), *Manual of Aquatic Viral Ecology*, pp. 92–101.

Not, F., Simon, N., Biegala, I. C. and Vaulot, D. (2002) Application of fluorescent *in situ* hybridization coupled with tyramide signal amplification (FISH-TSA) to assess eukaryotic picoplankton composition. *Aquat. Microb. Ecol.*, **28**, 157–166.

Pernthaler, J., Glöckner, F. O., Schönhuber, W. *et al.* (2001) Fluorescence *in situ* hybridization (FISH) rRNA-targeted oligonucleotide probes. *Methods Microbiol.*, **30**, 207–226.

Pernthaler, A., Pernthaler, J. and Amann, R. (2004) Sensitive multicolor fluorescence in situ hybridization for the identification of environmental microorganisms. In Akkermans, A. D. L., de Bruijn, F. J. and van Elsas, J. D. (eds), *Molecular Microbial Ecology Manual*. 2nd edn. Dordrecht, Netherlands: Kluwer Academic Publishers, pp. 711–726.

Porter, K. G. and Feig, Y. S. (1980) The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanog*, **25**, 943–948.

Rodríguez-Martínez, R., Labrenz, M., Campo, J. *et al.* (2009) Distribution of the uncultured protist MAST-4 in the Indian Ocean, Drake Passage and Mediterranean Sea assessed by real-time quantitative PCR. *Environ. Microbiol.*, **11**, 397–408.

Sieracki, M. E., Johnson, P. B. and Sieburth, J. M. (1985) Detection, enumeration and sizing of planktonic bacteria by image analyzed epifluorescence microscopy. *Appl. Environ. Microbiol.*, **49**, 799–810.

Šimek, K., Pernthaler, J., Weinbauer, M. G. *et al.* (2001) Changes in bacterial community composition and dynamics and viral mortality rates associated with enhanced flagellate grazing in a mesoeutrophic reservoir. *Appl. Environ. Microbiol.*, **67**, 2723–2733.

Sime-Ngando, T. and Pradeep Ram, A. S. (2005) Grazer effects on prokaryotes and viruses in a freshwater microcosm experiment. *Aquat. Microb. Ecol.*, **41**, 115–124.

Siokou-Frangou, I., Christaki, U., Mazzocchi, M. G. *et al.* (2010) Plankton in the open Mediterranean Sea: a review. *Biogeosciences*, **7**, 1543–1586.

Tarutani, K., Nagasaki, K. and Yamaguchi, M. (2000) Viral impact on total abundance and clonal composition of the harmful bloom-forming phytoplankton *Heterosigma akashiwo*. *Appl. Environ. Microbiol.*, **66**, 4916–4920.

Unrein, F., Massana, R., Alonso-Sáez, L. *et al.* (2007) Significant year-round effect of small mixotrophic flagellates on bacterioplankton in an oligotrophic coastal system. *Limnol. Oceanogr.*, **52**, 456–459.

Urdea, M. S., Warner, B. D., Running, J. A. *et al.* (1988) A comparison of non-radioisotopic hybridization assay methods using fluorescent, chemiluminescent, and enzyme labeled oligodesoxyribonucleotide probes. *Nucleic Acids Res.*, **16**, 4937–4956.

Weinbauer, M. G., Brettar, I. and Höfle, M. (2003) Lysogeny and virus-induced mortality of bacterioplankton in surface, deep, and anoxic marine waters. *Limnol. Oceanogr.*, **48**, 1457–1465.

Weinbauer, M. G., Hornák, K., Jezbera, J. *et al.* (2007) Synergistic and antagonistic effects of viral lysis and protistan grazing on bacterial biomass, production and diversity. *Environ. Microbiol.*, **9**, 777–788.

Wilson, W. H., Declan, O. S., Schroeder, C. *et al.* (2006) Phylogenetic analysis of PgV-102P, a new virus from the English Channel that infects *Phaeocystis globosa*. *J. Mar. Biol. Ass. U.K.*, **86**, 485–490.

Yentsch, C. S. and Menzel, D. W. (1963) A method for the determination of phytoplankton, chlorophyll and phaeophytin by fluorescence. *Deep-Sea Res.*, **10**, 221–231.

Zingone, A., Natale, F., Biffali, E. *et al.* (2006) Diversity in morphology, infectivity, molecular characteristics and induced host resistance between two viruses infecting *Micromonas pusilla*. *Aquat. Microb. Ecol.*, **45**, 1–14.