environmental microbiology

Environmental Microbiology (2011) **13**(7), 1842–1857 doi:10.1111/j.1462-2920.2011.02498.x

Trophic interactions between viruses, bacteria and nanoflagellates under various nutrient conditions and simulated climate change

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Summary

Population dynamics in the microbial food web are influenced by resource availability and predator/parasitism activities. Climatic changes, such as an increase in temperature and/or UV radiation, can also modify ecological systems in many ways. A series of enclosure experiments was conducted using natural microbial communities from a Mediterranean lagoon to assess the response of microbial communities to top-down control [grazing by heterotrophic nanoflagellates (HNF), viral lysis] and bottom-up control (nutrients) under various simulated climatic conditions (temperature and UV-B radiations). Different biological assemblages were obtained by separating bacteria and viruses from HNF by size fractionation which were then incubated in whirl-Pak bags exposed to an increase of 3°C and 20% UV-B above the control conditions for 96 h. The assemblages were also provided with an inorganic and organic nutrient supply. The data show (i) a clear nutrient limitation of bacterial growth under all simulated climatic conditions in the absence of HNF, (ii) a great impact of HNF grazing on bacteria irrespective of the nutrient conditions and the simulated climatic conditions, (iii) a significant decrease in burst size (BS) (number of intracellular lytic viruses per bacterium) and a significant increase of VBR (virus to bacterium ratio) in the presence of HNF, and (iv) a much larger temperature effect than UV-B radiation effect on the bacterial dynamics. These results show that top-down factors, essentially HNF grazing, control the dynamics of the lagoon bacterioplankton assemblage and that short-term simulated climate changes are only a secondary effect controlling microbial processes.

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Introduction

Three main regulators of bacterioplankton dynamics can be identified in pelagic systems: nutrient availability (bottom-up control), viral lysis and grazing (top-down control) especially by heterotrophic nanoflagellates (HNF) (Sanders *et al.*, 1992; Simek *et al.*, 1995; Fuhrman, 1999; Bouvy *et al.*, 2004). Traditionally, dissolved organic carbon has been considered to be the main factor limiting the growth of pelagic heterotrophic bacteria. However, numerous studies have shown that mineral limitation of growth rate is also widespread in various marine ecosystems (Thingstad *et al.*, 1998; Torréton *et al.*, 2000; Li *et al.*, 2004). The effects of grazing by HNF on bacterial abundance (BA) and metabolism have shown that freshwater and marine bacterial communities often react to escape predation (Hahn and Höfle, 2001; Gasol *et al.*, 2002a,b; Simek *et al.*, 2003; Karayanni *et al.*, 2008). Some studies revealed dominant types of grazers within nanoflagellate groups with clear seasonal changes in their grazing rates (Domaizon *et al.*, 2003). Since the discovery of high viral abundances 20 years ago (Bergh *et al.*, 1989), more attention has been paid to the major role played by the virioplanktonic community in the aquatic

food web (Wommack and Colwell, 2000; Weinbauer and Rassoulzadegan, 2004; Jacquet *et al*., 2010). Although some estimates indicate that viruses account for up to 90–100% of bacterial mortality in freshwater systems (Fischer and Velimirov, 2002; Colombet *et al*., 2006), most studies indicate that virus-induced bacterial mortality is around 10–50% (Weinbauer *et al*., 2002; Bettarel *et al*., 2005; Jacquet *et al*., 2005; Ory *et al*., 2010). This leads to the conclusion that viral lysis can be a major cause of mortality, sometimes comparable to grazing-induced mortality (Fuhrman and Noble, 1995; Jacquet *et al*., 2005). Weinbauer and colleagues (2003) found consumer-specific effects on bacterial activity and diversity, suggesting distinct vulnerability to the two sources of mortality (grazing and viral lysis). Despite the fact that bacteria are undoubtedly the principal constituent in the diet of HNF in most aquatic systems (Sanders *et al*., 1992; Boenigk and Arndt, 2002; Comte *et al*., 2008), some studies have reported that viruses are also a source of nutrition for HNF (Gonzalez and Suttle, 1993; Bettarel *et al*., 2005). However, this pathway only represents an alternative method of carbon transfer because the grazing rates are often very low (Bettarel *et al*., 2005). More recently, a number of studies conducted mainly in freshwaters have reported synergy between protozoan predation and viral lysis in removing bacteria (Simek *et al*., 2003; Sime-Ngando and Pradeep-Ram, 2005; Jacquet *et al*., 2007; Weinbauer *et al*., 2007; Pradeep Ram and Sime-Ngando, 2008). So far as we are aware, only two studies have reported antagonistic relationships with bacterial communities (Hornak *et al*., 2005; Weinbauer *et al*., 2007). Recently, antagonist top-down control was also reported for viruses and HNF against picophytoeukaryotes in a multivorous food web (Ory *et al*., 2010). Considerable research has been carried out on top versus bottom effects but few studies have considered how the two effects vary when the microbial communities are exposed to environmental forcing (Miki and Jacquet, 2008).

In addition to trophic interactions, the microbial food web is also controlled by a wide variety of climatic conditions, such as water temperature and incident UV-B radiation (280–320 nm), which can modify the dynamics of microbial communities. For instance, warming can increase microbial carbon respiration (Vázquez-Domínguez *et al*., 2007). Studying the effects of increased UV-B on whole communities, Mostajir and colleagues (1999) showed that the ecosystem shifts from a herbivorous to a microbial food web favouring bacteria and picophytoplankton communities. Microbial populations overcome the stress of solar UV (as well as predation and other adverse effects) by faster cell division and growth (Häder *et al*., 2003). According to Weinbauer (2004) and others, the UV-B portion of light can cause viral DNA damage and it is also suggested that UV-A (300–400 nm) has a harmful effect on capsid and tail proteins, reducing the adsorption ability of viruses. Furthermore, these two stressors (temperature and UV-B radiation) are expected to increase in the coming decades owing to simultaneous global warming and ozone depletion (Weatherhead and Andersen, 2006).

Trophic factors (defined here as bottom-up and top-down interactions) and/or climatic factors (defined here as temperature and UV-B modifications) on components of the microbial food web may directly affect the structure and interactions of biological assemblages. However, the relative importance of trophic versus climatic variables in determining environmental interactions within the planktonic food web has never been examined. Important ecological questions and concepts must be investigated: (i) Do the substrates play a major role compared with the biological control factors in the dynamics of microbial communities? (ii) What is the main control factor of bacterial mortality (HNF predation versus viral lysis)? (iii) What is the relative impact of temperature and UV-B versus top-down control factors on microbial dynamics?

To address these questions, an experiment was carried out based on a size fractionation approach of the microbial community (to separate planktonic compartments) and nutrient enrichments. This resulted in four trophic conditions with specific bottom-up and top-down controls, which were all incubated in four simulated climatic conditions (UV-B radiation and temperature) using floating enclosures located in a Mediterranean lagoon.

Results

A schematic diagram of the experiment set up is presented in Fig. 1. Briefly, four trophic conditions (TC) from the natural bulk lagoon water were obtained by filtration and nutrients addition. Every TC is characterized by specific top-down and bottom-up variables: TC1: viruses + bacteria + picophytoplankton (pico – defined here as cells < 1 μm), TC2: viruses + bacteria + pico- + nano-phytoplankton + heterotrophic nanoeukaryotes (defined here as cells < 6 μm), TC3: viruses + bacteria + picophytoplankton (< 1 μm) + nutrients, TC4: viruses + bacteria + pico- + nano-phytoplankton + heterotrophic nanoeukaryotes (< 6 μm) + nutrients (Fig. 1). See details in *Experimental procedures*. All have been incubated in triplicate for 4 days at four climatic simulations: at *in situ* temperature and UV conditions (C for control), and with an increase of +3°C (T), +20% of UV-B (UV) and +3°C and +20% UV-B (TUV) above the control conditions (Fig. 1).

BA, thymidine incorporation rates and activity index

At the start of the experiment the BA was twice higher in TC2 and TC4 than in TC1 and TC3, indicating that the

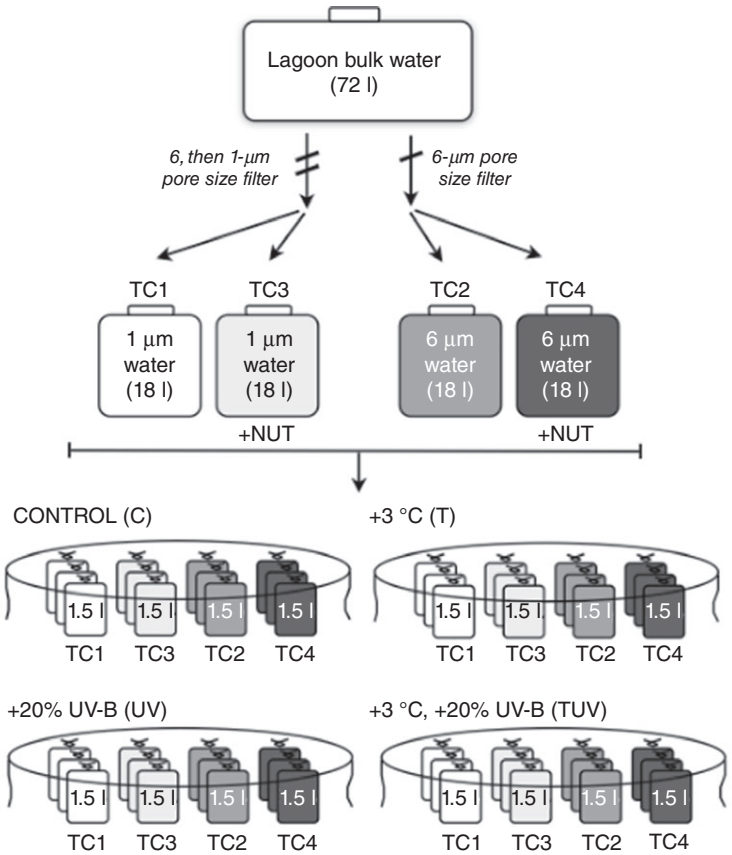

Fig. 1. Design of the protocol used in this study. Processing of the different trophic conditions (TC) and schematic representation of the incubations conducted in moored floating enclosures with specific temperature and UV-B radiations simulating four climatic conditions.

+NUT: mixture of leucine and phosphate. See Experimental procedures for details.
TC1: viruses + bacteria + picophytoplankton.
TC2: viruses + bacteria + pico- + nano-phytoplankton + heterotrophic nanoeukaryotes.
TC3: viruses + bacteria + picophytoplankton +NUT.
TC4 viruses +bacteria +pico- + nano-phytoplankton + heterotrophic nanoeukaryotes +NUT.

1 µm filtration procedure applied to TC1 and TC3 retained nearly 50% of the bacterial communities (Table 1). In TC1, BA increased during the first 48 h before flattening out, with no difference between the simulated climatic conditions. In all samples with added nutrients, BA was significantly higher ($P = 0.007$) than in those without added nutrients after 48 h. The highest abundance values were observed in T and TUV treatments, whereas abundance under UV alone was significantly lower than in the control ($P < 0.001$). In presence of flagellates, a significant

Table 1. Initial abundances of the biological components (bacteria, viruses, HNF and phytoplankton) composing the pelagic microbial food web.

Component Nutrient status Trophic conditions		Bact+virus No nutrient TC1	Bact+virus+HNF No nutrient TC2	Bact+virus Nutrient added TC3	Bact+virus+HNF Nutrient added TC4
Bacteria	Mean	4.10×10^6	7.56×10^6	3.85×10^6	7.92×10^6
(cells ml^{-1})	STD	2.17×10^4	2.48×10^5	5.34×10^3	1.33×10^5
^{3}H thymidine incorporation rate	Mean	10.5	51.1	11.2	46.0
(pM h^{-1})	STD	0.4	0.3	0.1	0.7
Phytoplankton (pico- or nano-)	Mean	4.61×10^3	7.60×10^4	5.91×10^3	7.55×10^4
(cells ml^{-1})	STD	1.58×10^2	5.78×10^2	7.70×10^1	6.70×10^2
Virus	Mean	1.82×10^8	1.98×10^8	1.90×10^8	1.95×10^8
(VLP ml^{-1})	STD	nd	nd	nd	nd
HNF	Mean	nd	7.24×10^2	nd	7.24×10^2
(cells ml^{-1})	STD	nd	6.10×10^1	nd	6.10×10^1

Bacterial production (^{3}H Tdr incorporation rates) was also reported. According to the size fractionation, phytoplankton is considered as picophytoplankton (< 1 µm) or pico-nanophytoplankton (< 6 µm). nd, not determined.

decrease in BA was observed for all climatic conditions, with and without nutrients (Fig. 2).

The results for thymidine incorporation were similar to those for BA. ^{3}H-TdR incorporation was higher at t_0 in TC2 and TC4 (42 pM h^{-1}) than in TC1 and TC3 (19 pM h^{-1}) (Table 1). In the absence of HNF and with nutrients (TC3), ^{3}H-TdR incorporation increased considerably, with values significantly higher ($P < 0.001$) for T and TUV treatments (close to 100 pM h^{-1}) than in C and UV treatments (Fig. 2).

Viral abundance and variables linked to viral infection

At the start of the experiment, no significant difference in viral concentration was observed for all treatments (close to 2×10^8 VLP ml^{-1}; Table 1). A slight reduction (%) was noted after 96 h for all treatments. However, with flagellates (TC2 and TC4), viral losses were slightly higher after 96 h (32% and 27% respectively) than without flagellates (30% and 22% respectively; $P < 0.001$; Fig. 2). The burst size (BS) linked to viral infection varied substantially during incubation (Table 2). With an average of 34 viral particles produced per bacterium at t_0 (STD = 12; $n = 16$), the lowest values were observed in TC4 (with HNF and nutrients) after 96 h (Table 2). The viral induced bacterial mortality (VIBM) determined from the frequency of infected cells (FIC, %) gave an indication of lysed bacterial production (BP). The frequency was low, ranging from 0% to 29% at t_0 in TC4 (with HNF and nutrients) under UV radiation (Table 2). VIBM values averaged 9.2% (STD = 7.0%) at t_0 and only 2.5% (STD = 2.9%) after 96 h incubation, irrespective of the climatic conditions (Table 2). The virus to bacterium ratio (VBR) varied significantly depending on the trophic conditions (with or without HNF) and climatic conditions (Table 2). The ratio decreased in both cases in the absence of HNF (TC1 and TC3), from 44 and 53 to a mean of 18 and 15 after 96 h respectively. However, in presence of HNF, this ratio increased significantly, especially in TC2 under C and UV treatments (from 26 to 141 and 146 after 96 h respectively). The increase in VBR was less marked in treatments with added nutrients (Table 2). There was a slight increase in VBR for T and TUV treatments in the presence of HNF (TC2 and TC4). Viral decay was noticed, with or without added nutrients, and was more marked in the presence of HNF (Fig. 3).

Phytoplankton and HNF abundance

The abundance of < 1 μm autotrophic cells was 90% lower in TC1 and TC3 (4.6 and 5.9×10^3 cells ml^{-1} respectively) than in the < 6 μm fraction TC2 and TC4 (7.6×10^4 cells ml^{-1}) (Table 1). Only 10% of phytoplankton was observed in the < 1 μm fraction, considered in this experiment as pico-phytoplankton. When nutrients were added to samples without HNF (TC3), the phytoplankton abundance increased for the first 48 h. Two types of response were then observed: there was a clear increase in C and UV treatments whereas the cell concentrations decreased to the initial values in T and TUV treatments (Fig. 2). In the presence of HNF, a slight decrease of < 6 μm autotrophic cells (considered here as pico-nanophytoplankton) was observed in all climatic conditions without nutrients during incubation. However when nutrients were added, the < 6 μm autotrophic cells increased in C and UV treatments for the first 48 h and then decreased significantly, suggesting a high predation pressure exerted by HNF on phytoplanktonic cells. At the start of the experiment, HNF abundance was 724 cells ml^{-1} (SD = 61) (Table 1). Without nutrients (TC2) a significant increase in their abundance was noted after 96 h in the TUV treatment compared with the C treatment (Fig. 4). With added nutrients (TC4), a slight decrease in HNF was observed after 96 h in TUV treatment compared with conditions without nutrients.

Bacterial and phytoplankton growth rates and grazing rates by HNF

In the absence of HNF, the growth rates of bacteria were significantly higher ($P < 0.001$) with nutrients (TC3, mean of 0.121 day^{-1}; STD = 0.014) compared with the rates observed without added nutrients (TC1, mean of 0.229 day^{-1}; STD = 0.023), for all climatic conditions (Table 3). In the presence of HNF, grazing rates were significantly higher ($P < 0.001$) in the control and UV treatments (5574 and 5819 bact HNF^{-1} day^{-1} respectively) compared with T and TUV treatments (2646 and 2912 bact HNF^{-1} day^{-1} respectively) (Table 3). Positive net growth rates of < 6 μm autotrophic cells were only observed in the presence of nutrients without HNF (TC3), with the highest values observed in the C and UV treatments (Table 3). Grazing rates of < 6 μm phytoplankton by HNF were similar irrespective of the treatments ($P > 0.05$), with or without nutrients (49.1 and 57.7 cells HNF^{-1} day^{-1} respectively).

Microbial responses to trophic versus simulated climatic conditions

Principal component analysis (PCA) of the environmental conditions (16 conditions: 4 trophic conditions × 4 climatic conditions) and the biological variables (seven variables) was performed excluding the unicellular eukaryote abundance (HNF) because these were absent from TC1 and TC3. The first three axes accounted for 81.3% of the total variance (Fig. 5). Projection of the environmental and biological variables in the reduced space formed by the first two axes (axis 1/axis 2; 67.3% of total variance) (Fig. 5A)

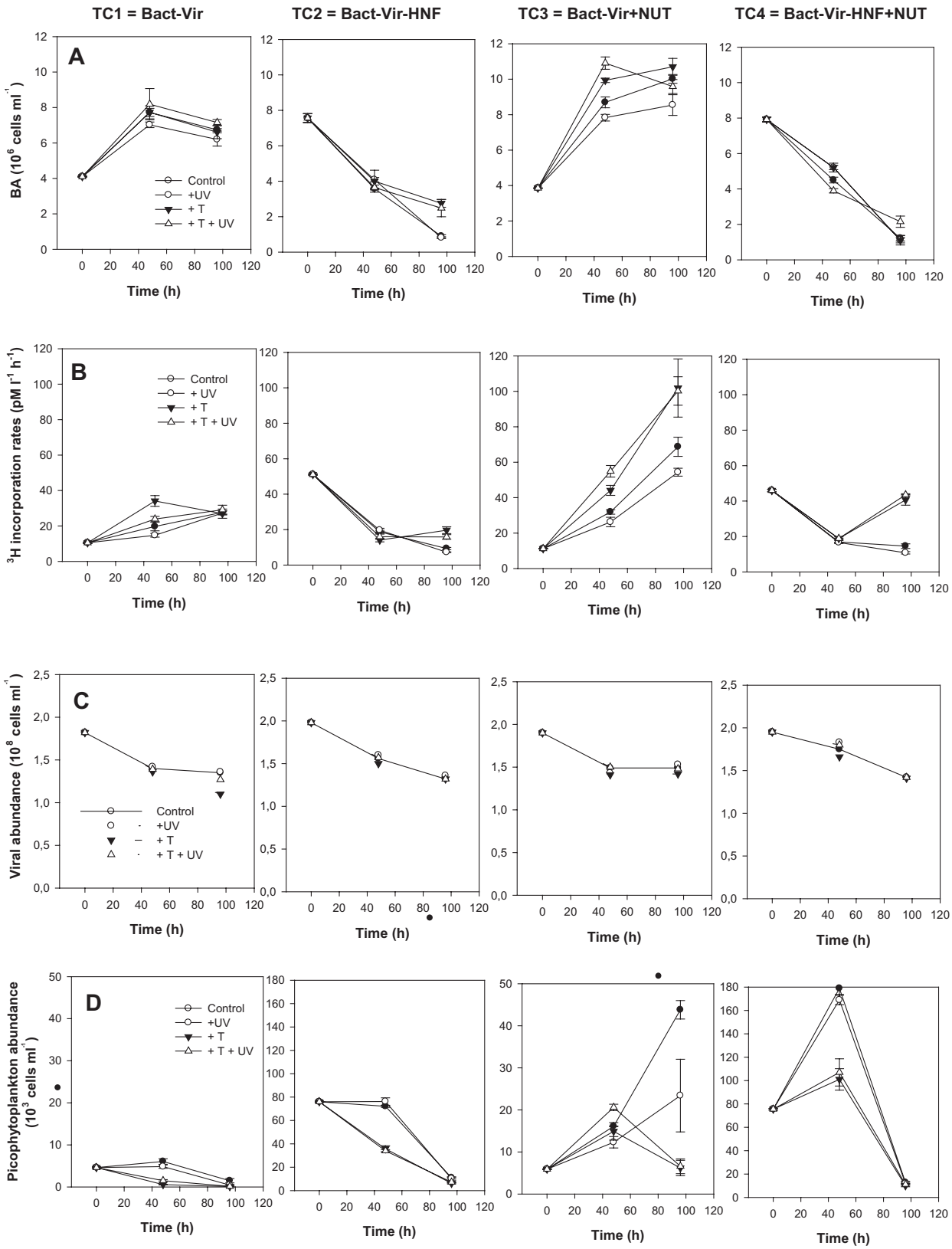

Fig. 2. Temporal variability of (A) BA, (B) ^{3}H incorporation rates, (C) viral abundance, (D) phytoplankton abundance during the experiment time (96 h). TC1 and TC3 were obtained from 1 μm filtrate. TC2 and TC4 were obtained from 6 μm filtrate. According to the size fractionation, phytoplankton is considered as picophytoplankton (< 1 μm) or pico-nanophytoplankton (< 6 μm).

Table 2. Values of BS, viral induced bacterial mortality (VIBM; %) and virus to bacterium ratio (VBR) in the different experimental trophic conditions (TC) under different climatic treatments (Control; UV: +20% of UV-B; T: +3°C of temperature; TUV: +3°C of temperature and 20% of UV-B); nd: not determined.

	BS				VIBM (%)				VBR			
	TC1	Bacteria+virus										
Time (h)	Control	UV	T	TUV	Control	UV	T	TUV	Control	UV	T	TUV
0	32	23	30	30	2.96	6.97	nd	5.67	44	44	44	44
48	24	40	18	14	8.64	13.73	nd	5.69	22	23	20	20
96	32	23	21	15	nd	2.27	nd	1.67	20	21	16	18
	TC2	Bacteria+virus+HNF										
Time (h)	Control	UV	T	TUV	Control	UV	T	TUV	Control	UV	T	TUV
0	35	24	53	29	4.27	19.39	7.40	9.92	26	26	26	26
48	17	19	14	56	17.51	11.97	10.36	0.63	54	46	47	56
96	15	nd	10	0	1.76	9.03	0.82	nd	141	146	46	49
	TC3	Bacteria+virus+nutrient										
Time (h)	Control	UV	T	TUV	Control	UV	T	TUV	Control	UV	T	TUV
0	41	42	46	53	8.04	7.83	nd	3.74	53	53	53	53
48	26	15	14	37	6.78	3.53	0.17	12.77	21	23	17	18
96	27	20	16	5	5.34	0	74.49	nd	15	16	14	16
	TC4	Bacteria+virus+HNF+ nutrient										
Time (h)	Control	UV	T	TUV	Control	UV	T	TUV	Control	UV	T	TUV
0	50	24	11	23	10.67	28.64	9.95	3.00	26	26	26	26
48	25	nd	24	35	11.84	nd	10.57	nd	50	42	46	61
96	0	0	0	6	1.84	nd	0	nd	101	111	46	63

showed a clear distinction on the first axis between TC2 and TC4 (with HNF, on the right of the axis) and TC1 and TC3 (without HNF, on the left of the axis). This suggests a clear impact exerted by HNF on the viral and bacterial parameters. Furthermore, there was a clear distinction on the second axis between TC1 (without nutrient; at the top of the axis) and TC3 (with nutrient; at the bottom of the axis) suggesting a significant impact of nutrients in the absence of HNF. The factorial plane defined by the first two axes discriminated experimental trophic relationships with two gradients based on the complexity of microbial composition (axis 1, with or without HNF) and nutrient status (axis 2, with or without nutrient). However, there was no clear discrimination by climatic condition, suggesting that the simulated climatic variables had less effect on the biological components. BP and abundance (BA) and BS were linked to specific trophic conditions (no HNF and nutrients added). On the other hand, TC2 and TC4 (with HNF) were only weakly discriminated, suggesting that nutrients had less effect on biological compartments in the presence of HNF. The highest VBR ratios were observed in the presence of HNF, irrespective of the simulated climatic treatment. The variable PHYTO (pico-nanophytoplankton abundances) appeared to be linked to the presence of nutrients rather than correlated with the bacterial variables (BA and BP).

Projection of the environmental and biological variables in the reduced space formed by the axes 1 and 3 (axis 1/axis 3; 57.4% of total variance) (Fig. 5B) confirmed the clear opposition between the two groups of trophic relationships (TC1 and TC3 against TC2 and TC4), and thus the role played by HNF community. This projection also confirmed the correlation between the highest values of bacterial variables (BA, BP) and the BS in the absence of HNF. The highest VBR values were observed in the presence of HNF. Axis 3 also confirmed the clear opposition between VIBM (virus-induced bacterial mortality) and VA (viral abundance).

Discussion

Microbial models are known to be useful for exploring food-web theory (Jessup *et al*., 2004), in particular as a means for determining the degree of complexity of the

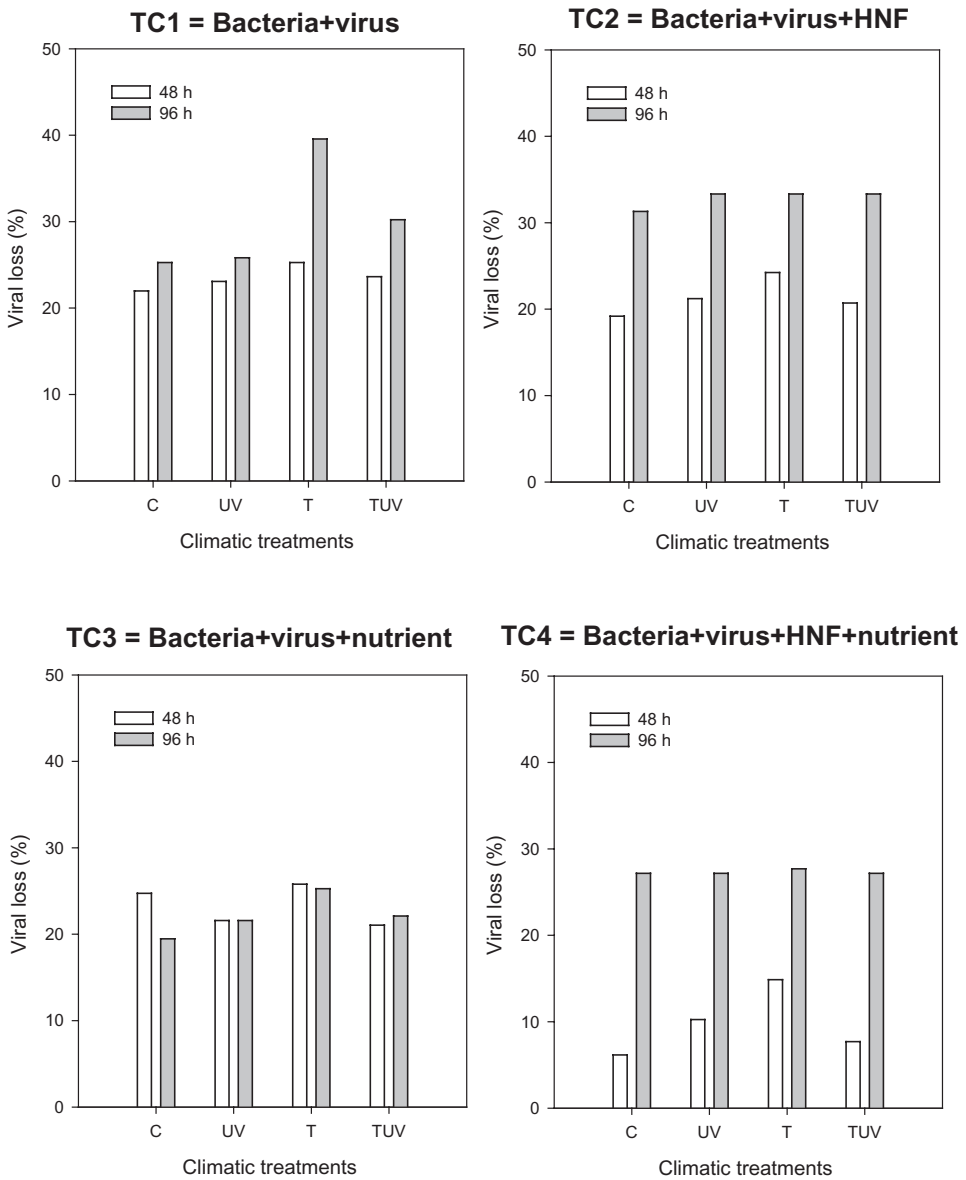

Fig. 3. Temporal variability of viral losses in the different experimental conditions (C: control; UV: +20%; T: +3°C; TUV: +3°C +20% UV) after 48 and 96 h.

microbial assemblage (Lawton, 1995). In aquatic systems, much attention is currently being paid to bacterioplankton and picophytoplankton which are among the major constituents in the production and recycling of energy and nutrients. Bacterioplankton account for 60% of the organic surface area in pelagic marine systems (Cho and Azam, 1988) and have been shown to process up to 90% of locally produced photosynthetic products by phytoplankton (Biddanda *et al*., 1994). Changing environmental conditions may disrupt the functioning and organization of these critical components, inducing far-reaching impacts on the system (Naeem and Li, 1997; Häder *et al*., 2003). Experimental approaches have been shown to be extremely useful tools for investigating the effects of environmental changes on the structure and dynamics on microbial communities (Jacquet *et al*., 2007; Zhang *et al*., 2007; Pradeep Ram and Sime-Ngando, 2008; Bonilla-Findji *et al*., 2009). In particular, the size fractionation method is considered to involve the least disturbance to the trophic webs (Chen *et al*., 2001). These miniature versions of field systems are not intended to reproduce nature in an experimental model system but to simplify nature so that it can be more easily understood.

The experiment reported here shows that individual components of the microbial food webs and their interactions have different responses under top-down (grazing

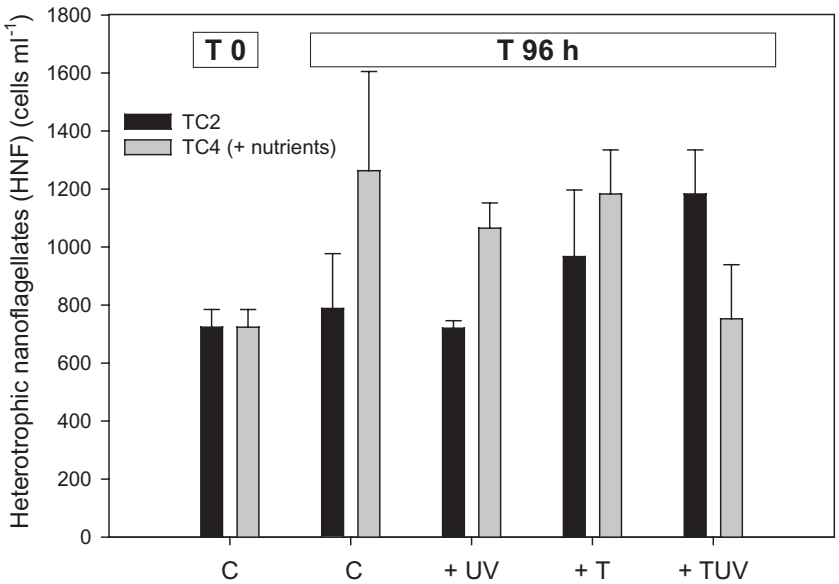

Fig. 4. Heterotrophic nanoflagellate abundances (HNF) in the different trophic conditions TC2 (no nutrient) and TC4 (nutrient added) in the different experimental conditions (C: control; UV: +20%; T: +3°C; TUV: +3°C +20% UV).

and viral lysis) and bottom-up (nutrient) controls. The results clearly show the dependence of bacterial growth rates upon nutrient availability, although flagellate grazing is the major factor controlling the biomass and thus the growth rates in a given nutrient context. The results also demonstrate the different responses of microbial components to simulated climatic variables, with a small negative effect of a 20% UV-B increase and a positive response to a 3°C increase in ambient water temperature. The conclusion is that the impact of simulated climatic factors (from short-term experiment) is largely secondary to the trophic biological mediated interactions, especially flagellate grazing on bacteria.

Top-down and bottom-up controls of bacterial and pico-nanophytoplankton communities

Heterotrophic bacteria obtain energy from the oxidation of dissolved organic matter, and the most common way of evaluating resource controls on bacterioplankton dynamics is to measure of potentially growth-limiting resources. Figure 6 depicts the bacterial responses to experimental treatments aimed at evaluating the relative influence of top-down and bottom-up processes changes in one or more proxies of bacterial biomass or production over time following the addition in controlling bacterial biomass. It is clear that the addition of nutrients stimulates bacterial

Table 3. Growth rates and grazing rates of bacteria and pico-nanophytoplankton with and without nutrients in presence or absence of HNF in the different experimental conditions (C: control; UV: +20%; T: +3°C; TUV: +3°C +20% UV) during the experiment time (96 h).

	Prey: bacteria; predator: HNF				Prey: picophytoplankton; predator: HNF	
	Without predators	With predators			Without predators	With predators
Units	Growth rate day^{-1}	Grazing rates bact HNF^{-1} day^{-1}	Clearance rates bact ml^{-1} day^{-1}	BP losses[a] %	Growth rate day^{-1}	Grazing rates cells HNF^{-1} day^{-1}
No nutrient						
C	0.124	5574	4.0×10^6	164	–0.280	50.79
UV	0.103	5819	3.6×10^6	148	–0.586	52.77
T	0.119	2646	1.6×10^6	67	–1.022	65.98
TUV	0.139	2912	1.8×10^6	74	–0.849	61.33
Nutrient added						
C	0.238	5123	3.2×10^6	145	0.500	46.33
UV	0.199	5396	3.4×10^6	152	0.344	48.63
T	0.255	2796	1.7×10^6	79	0.011	52.77
TUV	0.227	3567	2.2×10^6	101	0.027	48.81

a. BP computed from ^{3}H thymidine incorporation rates at t_0 using the conversion factor of 2×10^{18} cells mol^{-1} Tdr (Ducklow and Carlson, 1992). Clearance rates and losses by HNF as per cent of BP are reported for each climatic treatment.

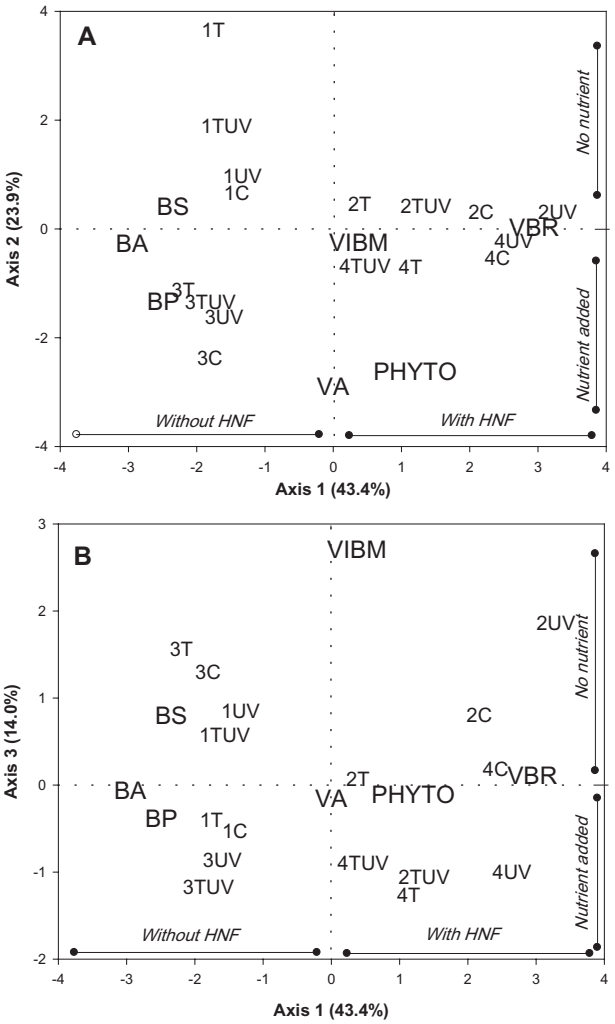

Fig. 5. Principal component analysis (PCA) on the two first axes (A) and on the axis 1 and 3 (B). Eigenvalues for each axis of the PCA are reported. Seven variables are identified in the PCA: viral abundance (VA, VLP ml^{-1}), virus-induced bacterial mortality (VIBM, %), burst size (BS, virus bacterium^{-1}), bacterial abundance (BA, cells ml^{-1}), bacterial production (BP, pM h^{-1}), viral abundance/BA (VBR, no unit), pico-nanophytoplanktonic cells (PHYTO, cells ml^{-1}).

growth (from 0.124 day^{-1} in absence of nutrients to 0.238 day^{-1} with nutrients added; Table 3) in the absence of grazers, confirming that the inorganic and organic substrate availability and flux of ready assimilated DOM limits bacterial growth in the Thau lagoon (Trottet *et al*., 2011). Furthermore, the abundance of autotrophic microorganisms only increased in samples with added nutrients and the growth rates of the pico- and nanophytoplankton were negative in the absence of nutrients, suggesting a clear nutritive limitation in the Thau ecosystem and/or great competition for nutrients between bacteria and picophytoplankton (Kirchman and Wheeler, 1998; Fouilland and Mostajir, 2010). Viral lysis products enhance the microbial loop by releasing dissolved organic matter available to bacteria and also to picophytoeukaryotes in microcosm experiments in the absence of predators (Ory *et al*., 2010).

The food-web organization revealed that the presence of HNF (see Fig. 6) had a significant negative effect on BA. Under natural climatic conditions (control), grazing rates varied between 5123 and 5574 bacteria per HNF per day, without and with added nutrients respectively (Table 3). A review of the published data on bacterivory in planktonic systems revealed that ingestion rates (using fluorescently labelled bacteria) were generally in the range of 48–480 bacteria per HNF per day (Vaqué *et al*., 1994). This study showed values well above the range of values previously reported in marine systems (24–2400 bacteria per HNF per day; Karayanni *et al*., 2008). With higher bacterial concentrations, these results support the theory that HNF bacterivory increases with prey concentration (Vaqué *et al*., 2004). Thus, in these conditions (control), the HNF community was the principal cause of bacterial mortality, removing 145–164% of daily BP (Table 3). Besides heterotrophic bacteria, cyanobacteria (e.g. *Synechococcus*) are also within the prey size range of nanoflagellates (Frias-Lopez *et al*., 2009). Worden and Binder (2003) revealed that clearance rates of HNF grazing on *Synechococcus* and on bacteria are similar. In these experimental conditions, grazing rates on cyanobacteria were 100-fold lower than grazing rates on heterotrophic bacteria (Table 3), suggesting that autotrophic species are of poor food quality and support only slow growth of HNF (Christaki *et al*., 2001).

Recent studies have shown that viral infections are another major source of bacterioplankton mortality (Wommack and Colwell, 2000; Weinbauer, 2004; Suttle, 2007). Virus-induced bacterial mortality (VIBM) is generally greater in nutrient-rich habitats where contact rates with potential hosts are high (Bettarel *et al*., 2005; Weinbauer, 2004; Lymer *et al*., 2008). However, other studies reported that BP was significantly reduced by flagellates and not by viruses (Pernthaler, 2005; Zhang *et al*., 2007). In fact, the effects of these two factors have been rarely investigated simultaneously (Boras *et al*., 2009; Personnic *et al*., 2009). VIBM is calculated using a standard formula from the BS measured by TEM and it is recognized that none of methods for estimating the virus-induced mortality of bacterioplankton is completely satisfactory (Weinbauer *et al*., 2003). These VIBM values averaged 9.2%, with no clear trend for any treatment (trophic and climatic conditions). However, in surface waters of the Mediterranean, it was estimated that viruses remove < 20% of the BP (Guixa-Boixereu *et al*., 1999), while Boras and colleagues (2009) reported, in the same region, values accounting for 48–84% of BP in oligotrophic areas. Our estimates suggest that viral infec-

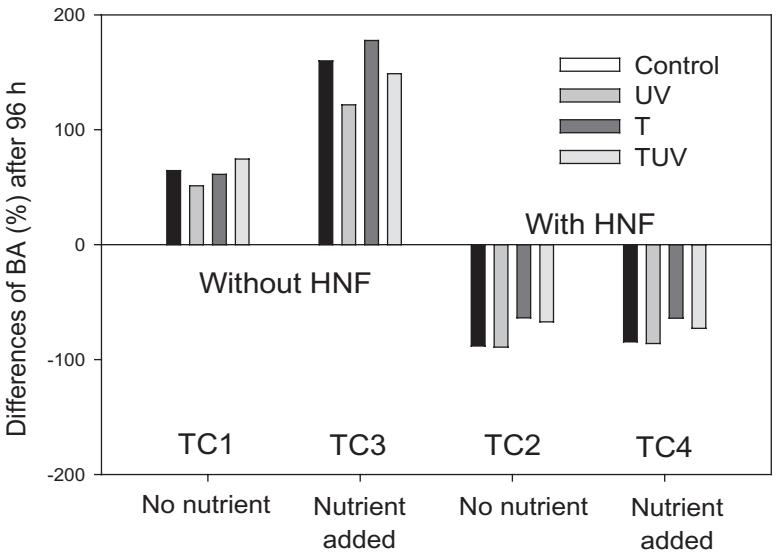

Fig. 6. Impact of heterotrophic nanoflagellates (HNF) on BA under different nutrient and climatic conditions after 96 h.

tion is not a significant factor for bacterial mortality in Thau lagoon, irrespective of the trophic and climatic conditions.

Nanoflagellates are eukaryotic organisms in the 1–10 μm range and, surprisingly, they have been reported to affect viral decay rates in both marine and freshwater environments (Gonzalez and Suttle, 1993; Maranger *et al*., 2002; Bettarel *et al*., 2005). To understand the observed viral decay (Fig. 3), it is necessary to describe the other viral variables, such as the BS and the VBR. The average BS calculated for natural marine communities is 24 viral particles per bacterium in eutrophic areas (Wommack and Colwell, 2000; Parada *et al*., 2006). In the control treatment, an average of 39 viral particles per bacterial cell was noted at the beginning of experiment and the lowest values of BS were observed after 96 h in the presence of HNF and added nutrients (BS below the limit of detection, Table 2) suggesting a preferential consumption of infected bacteria by HNF. Although the virus sizes are commonly between 20 and 200 nm and many studies have demonstrated an insignificant effect of flagellate grazing on viral decay, viruses can represent a minor food source for HNF, especially in oligotrophic systems (Bettarel *et al*., 2005). These results indicate that HNF may play a minor role in virus removal, either directly via ingestion or indirectly via ingestion of bacterial hosts, as suggested by Maranger and colleagues (2002). It was also observed that the VBR was greater in the presence of HNF, with higher values in TC2 and TC4 (141 and 101, respectively, Table 3), corresponding to a lower BA. Under grazing pressure, changes in the bacterial community structure may include the selection of virus-resistant bacteria species (Weinbauer *et al*., 2007; Pradeep Ram and Sime Ngando, 2008; Ory *et al*., 2010).

Effects of simulated climatic conditions on microbial components and their interactions

Among climatic factors, water temperature and incident UV radiation, in particular UV-B, are expected to increase in the next decades owing to simultaneous global warming and ozone depletion linked to anthropization and industrialization (Weatherhead and Andersen, 2006; IPCC, 2007). Temperature is a strong determinant of viral persistence and infectivity as has been demonstrated using phage isolates (Giladi *et al*., 1995). The susceptibility of natural virioplankton to temperature has rarely been investigated and temperature seems to have a significant effect on viral infectivity (Suttle and Cheng, 1992; Noble and Fuhrman, 1997). Temperature is known to influence the food-web structure (Sarmento *et al*., 2010) which has generally been attributed to the differential effects of temperature at a specific trophic level (consumer, producer or parasite) (Richardson and Schoeman, 2004; Muren *et al*., 2005; Vidussi *et al*., 2011). In this short-term experiment, temperature had a strong influence on bacterial growth rates (Fig. 2), as already demonstrated by White and colleagues (1991). However, pico-nanophytoplankton dynamics did not show a clear response to temperature, corroborating the results of Rae and Vincent (1998).

Few studies have addressed UV-induced changes in the dynamics of natural microbial communities, and their conclusions vary greatly (Mostajir *et al*., 1999; Davidson and Belbin, 2002; Joux *et al*., 2009; Vidussi *et al*., 2011). In this study, viral abundance consistently decreased along the incubation under UV-B treatment but they were not significant different from those measured in controls (Fig. 2; Table 2). It is important to mention that Whirl-Pak

bags only transmit 70% of the total UV-B, and thus it is obvious that our results only reported a 20% UV compared with the other treatments and the controls. However, our conclusion is surprising since UV radiation has been reported as the most important cause of viral decay in aquatic environments (e.g. Wommack *et al*., 1996; Noble and Fuhrman, 1997), accounting for 25–66% of the total viral loss. However, dark repair of damaged phages in infected cells (by expression of SOS genes) may be more active and may more than counterbalance the losses of viral infectivity Weinbauer (2004). Published results on the UV-B effects on bacteria and small planktonic organisms (nano and pico-plankton) are variable. In clear oceanic waters with high UV penetration, bacterial growth rates were reduced in the top layers of the water column (Maranger *et al*., 2002; Conan *et al*., 2008). However, little effect on the composition and dynamics of coastal marine bacterioplankton communities in the North Sea has been observed, probably owing to high turbidity and the presence of an efficient DNA repair system (Winter *et al*., 2001). Studies of picophytoplankton have demonstrated a great difference in UV sensitivity among species (Sommaruga *et al*., 2005). In this study, the increase of 20% of UV-B radiation had a low impact (compared with the control) on the abundance and thymidine incorporation rates of bacterial communities (TC1 and TC3, Fig. 2) suggesting an active dark repair system, perhaps with the contribution of viral genes (Weinbauer, 2004). Several studies reported that BP is often reduced in samples exposed to UV-B radiation (Maranger *et al*., 2002; Conan *et al*., 2008). However, positive effects of both combined factors (T + UV) were observed for each bacterial variable studied, suggesting that bacteria are likely to be more responsive to temperature than UV-B radiation. Thus, the results reported here show that the bacterial communities have various reactions to the climatic conditions tested, with an increased resistance to damage by UV-B, but a greater sensitivity to change in ambient temperature, corroborating the conclusions of Rae and Vincent (1998) and Vidussi and colleagues (2011) about the freshwater and marine microbial food-web structure.

Conclusions

Overall, based on these short-term experimental results (96 h), our results only reveal rapid physiological responses of organisms to the different treatments and do not pretend to predict the impact of long-term global change on the dynamic of marine microbial food webs. We suggest that the top-down factors, essentially HNF grazing, mainly shape the microbial assemblages in a coastal Mediterranean lagoon during spring, compared with other factors such as nutrients, temperature and UV solar radiation. Furthermore, short-term climatic change of +3°C increased the abundance of the microbial components, while the effect of UV-B was only marginal. Moreover, neither simulated climatic factor changed the significance of the HNF–bacterial interaction, taken individually or together. We suggest that short-term simulated climate changes are only a secondary effect controlling microbial processes.

Experimental procedures

Sampling strategy and treatments

The study was conducted in the coastal Mediterranean Thau lagoon (43°24′49″N, 3°41′19″E), and the experiments were performed using the Mediterranean platform for Marine Ecosystem Experimental Research (MEDIMEER, for details, see Nouguier *et al*., 2007; Vidussi *et al*., 2011). The spring period (18–24 April 2006) was characterized by stable environmental conditions owing to limited freshwater inputs.

The salinity of the area sampled was close to 36 and the mean water temperature was 16.4°C (range: 15.7–17.8°C). The detailed protocol used to set up the experiment is shown in Fig. 1. Different biological assemblages were obtained from bulk surface water by size fractionation. Seventy-two litres of bulk water was filtrated through 6-μm-pore-size polycarbonate membranes (47 mm in diameter). Half of this 6 μm filtrate (36 l) was then filtrated through 1-μm-pore-size polycarbonate membranes (47 mm in diameter). Each 6 μm and 1 μm 36 l filtrate was homogenized and equally distributed into two series of 12 × 1.5 l Whirl-Pak® polyethylene sterile bags which allow transmission of 70% UV radiations. One of the two Whirl-Pak® series of the 6 μm and 1 μm filtrates was amended with nutrients composed of a mixture of leucine (C and N) and phosphate at final concentrations of 1.14 μM of carbon, 0.50 μM of nitrogen and 0.05 μM of phosphorous. From these plankton size fractions and nutrient additions, the following four trophic conditions (TC) were obtained:

TC1: viruses + bacteria + picophytoplankton (pico – defined here as cells < 1 μm)
TC2: viruses + bacteria + pico- + nano-phytoplankton + heterotrophic nanoeukaryotes (defined here as cells < 6 μm)
TC3: viruses + bacteria + picophytoplankton (< 1 μm) + nutrients
TC4: viruses + bacteria + pico- + nano-phytoplankton + heterotrophic nanoeukaryotes (< 6 μm) + nutrients.

Each TC was incubated in triplicate in four simulated climatic conditions using four moored floating enclosures (polyethylene bags, 1.2 m diameter × 2 m depth, 2.3 m^3 volume), held at 1 m above the surface of the water by floating structures and filled with lagoon water. The enclosures and associated instruments in this experiment acted as incubators for the Whirl-Pak bags described above. The four simulated climatic conditions were: (i) *in situ* temperature and UV-B radiation (control: C), (ii) 3°C (T) above *in situ* conditions, (iii) 20% UV-B above *in situ* conditions (UV) and (iv) 3°C and 20% UV-B above *in situ* conditions (TUV). Temperature increase in the T and TUV enclosures was achieved using a submersible

heater (Galvatec, France), and UV-B radiation was increased in the UV and in the TUV enclosures using two UV fluorescent lamps (Philips TL20RS/01) as described in Nouguier and colleagues (2007) and Vidussi and colleagues (2011). Within each incubator, the 12 Whirl-Pak bags (TC1, TC2, TC3 and TC4, each in triplicate) were placed in the centre of the enclosure at 0.5 m depth and incubated for 96 h. At each sampling time (0, 48 and 96 h), subsamples were collected from each Whirl-Pak bag and preserved for analysis within 1 h after collection.

Viral parameters

To enumerate virus-like particles (VLPs), triplicate subsamples of 200–500 μl were taken and particles retained on 0.02-μm-pore-size membranes (Anodisc) and stained with SYBR Gold (Patel *et al*., 2007). Three hundred to 600 VLPs were counted in 1520 fields on each slide. Triplicate 8 ml aliquots of formalin-fixed samples were taken and bacterial cells were harvested by ultracentrifugation at 70 000 *g* for 20 min onto 400 mesh Cu grids, stained for 30 s with uranyl acetate (2% w/w) and examined at ×40 000 by TEM operated at 80 kV to distinguish between infected and uninfected bacteria (Weinbauer and Höfle, 1998). A bacterium was considered to be infected when at least five phages, identified by shape and size, were clearly visible inside the host cell and the BS was defined as the number of virus particles per bacterium. At least 600 bacterial cells were inspected per grid and the number of infected bacteria ranged from 15 to 20. To estimate virus-induced bacterial mortality (VIBM), the frequency of infected cells (FIC) was calculated from the frequency of visibly infected cells (FVIC) (as a per cent of bacteria) using the formula:

$$\mathrm{FIC} = 9.524\ \mathrm{FVIC} - 3.256 \text{ (Weinbauer } et\ al., 2002\text{)}.$$

The FIC was then converted to VIBM according to Binder (1999): $\mathrm{VIBM} = (\mathrm{FIC} + 0.6\mathrm{FIC}^2)/(1 - 1.2\mathrm{FIC})$. For each bag, the net viral decay was calculated using the formula:

$$\text{decay } (\text{day}^{-1}) = (\ln N_f - \ln N_i)/(t_f - t_i),$$

where N_f and N_i are the total free viruses at t_f and t_i (final and initial time) respectively.

Bacterial parameters

Bacterial cells were enumerated by flow cytometry using the method described by Marie and colleagues (1997). One millilitre of formaldehyde-fixed subsamples were incubated with SYBR Green I (Molecular Probes, Eugene, OR, USA) at a final concentration of 1/10 000 for 15 min at room temperature in the dark. For each subsample, three replicate counts were performed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an air-cooled argon laser (488 nm, 15 mW). Stained bacterial cells, excited at 488 nm, were enumerated according to their right-angle light scatter (RALS) and green fluorescence (FL1) measured using a 530/30 nm filter. These cell parameters were recorded on a four-decade logarithmic scale mapped onto 1024 channels. Fluorescent beads (0.94 μm, Polysciences, Warrington, PA, USA) were systematically added to each sample. Standardized RALS and FL1 values (cell RALS and FL1 divided by 0.94 μm beads RALS and FL1 respectively) were used as an estimation of the relative size and nucleic acid content of bacterial cells respectively (Troussellier *et al*., 1999). Listmode files were analysed using CYTOWIN software.

BP was estimated from the DNA synthesis rates measured by (^{3}H-methyl) thymidine (^{3}H-TdR) incorporation using the microcentrifuge method (Smith and Azam, 1992). A sample aliquot (1.4 ml) was added to a sterile polystyrene snap cap tube containing a final saturating concentration of 20 nM of ^{3}H-TdR (specific activity 53 Ci mmol^{-1}, Amersham). Triplicate live samples and a control were run for each assay. Killed controls were prepared by adding 70 μl of 100% of trichloroacetic acid (TCA) 15 min before the addition of ^{3}H-TdR. Bacterial growth was measured in the dark at *in situ* temperature for a short incubation time (no longer than 1 h) to minimize the repair of UV damage by bacteria (Kaiser and Herndl, 1997). Incorporation was terminated by adding 70 μl of 100% TCA. Samples were stored for at least 2 h at 4°C and then centrifuged for 14 000 *g* for 15 min. The precipitates were rinsed three times with 5% TCA and once with 70% ethanol and were resuspended in 1.5 ml of liquid scintillation cocktail (Ultima Gold LLT, Perkin Elmer) prior to determining the radioactivity by liquid scintillation counter (Beckman LS 6500).

Phytoplankton and unicellular eukaryotes parameters

Phytoplankton counts were performed with the same flow cytometer described above. Cells excited at 488 nm were detected and enumerated from rough, unfixed samples (within 1 h after sampling) according to their FALS and RALS properties and their orange fluorescence (585/42 nm) and red fluorescence (> 650 nm) from phycoerythrin and chlorophyll pigments respectively. Fluorescent beads (0.94 μm) were also systematically added to each sample. List mode files were analysed using CYTOWIN software. To enumerate heterotrophic and pigmented flagellates, water samples were fixed with glutaraldehyde (1% final concentration) and stored at 4°C for 24 h. Twenty-five millilitres of preserved water samples were then stained with DAPI (final concentration, 15 μg ml^{-1}) for 15 min, filtered onto black Nuclepore filter (0.8 μm pore size), stored at –20°C, and counted using an epifluorescence microscope (NikonEclipseTE200) with UV excitation (Boenigk *et al*., 2004, modified). In this study, the pigmented flagellates (revealed by blue and green fluorescence) were not considered in the biological assemblages.

Bacterial and phytoplankton growth rates and grazing rates by HNF

An exponential growth rate was assumed for bacteria and pico-phytoplankton in the absence of predators (< 1 μm fraction), and the net growth rate (μ expressed in day^{-1}) was calculated using the formula:

$$\mu = (\ln N_f - \ln N_i)/(t_f - t_i),$$

where N_f and N_i are the total abundance of cells at t_f and t_i (final and initial incubation time) respectively.

The grazing rate (g expressed in day^{-1}) was calculated using the same equation in the presence of predators (< 6 μm fraction). The ingestion (I, cell HNF^{-1} h^{-1}) rate of bacteria or pico-nanophytoplankton cells was calculated using the formula:

$$I = g \times N_{prey} / N_{HNF},$$

where g is the grazing rate (day^{-1}) and N_{prey} and N_{HNF} are the average concentrations of prey (bacterial or phytoplankton cells by cell size) and HNF respectively.

The clearance rates were obtained by multiplying the ingestion rate by the mean of HNF abundance during the experiment.

Principal component analysis

The relationships between viral parameters (total abundance, BS, viral-induced bacterial mortality), bacterial parameters (total abundance, ^{3}H-TdR incorporation as a production proxy), VBR and phytoplankton abundances (by cell size: < 1 μm and < 6 μm) were studied using multivariate analysis. Table 1 gives the abbreviations for the seven variables studied. As the distribution of several variables did not meet normality, a non-parametric Mann–Whitney *U*-test was applied in order to search for median differences (Sigma Stat version 3.5). Correlations between variables were tested to provide an overview of the major interactions between the microbial communities. Parametric tests (two variables Bravais-Pearson correlations) and multivariable centred PCA were conducted on $\log(x + 1)$ transformed data in order to meet normality requirements. Data analysis was based on a PCA using ADE-4 software (Thioulouse *et al*., 1997).

Acknowledgements

We gratefully acknowledge Jean Nouguier and Yvan Vergne for their technical help during the experiment. We also thank the Unit Research UMR 5235 and Sharon Wein for the access of the hall of radioactivity and for the use of the Beckman counter. This work was supported by the French program PNEC (10301705 to T.B.) and the ANR AQUAPHAGE (ANR07 BIODIV 015-02 to T.B.) This study was also supported by the Groupement De Recherche (GDR) 2476 Réseaux Trophiques Pélagiques. The experimental platform for Mediterranean Ecosystem Research (MEDIMEER) was founded by UMR 5119 ECOLAG, CNRS-INEE, Institut Fédératif de Recherche 129 A. Sabatier, GDR 2476 Réseaux Trophiques Aquatiques, Région Languedoc Roussillon. We also thank the two anonymous reviewers for their helpful comments that have contributed to improve the manuscript.

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