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Potential Effect of Freshwater Virus on the Structure and Activity of Bacterial Communities in the Marennes-Oléron Bay (France)

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Abstract:

Batch culture experiments using viral enrichment were conducted to test the response of a coastal bacterial community to autochthonous (i.e., co-existing) or allochthonous riverine viruses. The effects of viral infections on bacterial dynamics and activity were assessed by epifluorescence microscopy and thymidine incorporation, respectively, whereas the effect of viral infection on bacterial community composition was examined by polymerase chain reaction-single strand conformation polymorphism 16S ribosomal RNA fingerprinting. The percentages of high nucleic acid-containing cells, evaluated by flow cytometry, were significantly correlated (r^2 = 0.91, n = 12, p < 0.0001) to bacterial production, making this value a good predictor of active cell dynamics along the study. While confinement and temperature were the two principal experimental factors affecting bacterial community composition and dynamics, respectively, additions of freshwater viruses had significant effects on coastal bacterial communities. Thus, foreign viruses significantly reduced net bacterial population increase as compared to the enrichment treated with inactivated virus. Moreover, freshwater viruses recurrently and specifically affected bacterial community composition, as compared to addition of autochthonous viruses. In most cases, the combined treatment viruses and freshwater dissolved organic matter helped to maintain or even enhance species richness in coastal bacterial communities in agreement to the 'killing the winner' hypothesis. Thus, riverine virus input could potentially influence bacterial community composition of the coastal bay albeit with modest modification of bulk bacterial growth.

Introduction

Viruses are major players in the marine environment (see recent reviews in [41, 42]) and represent one of the most abundant biological entities in the water column (10^6-10^8 viruses mL^{-1}, [51]). While viruses can interact with all the components of the marine food web (primary producers, bacteria, grazers, carnivores), bacteria are assumed to be the principal host for viruses based on their abundance and the high frequency of bacterial cells containing recognizable viral particles in pelagic waters (up to 40%, [8]). Further investigations have shown significant correlations between viral and bacterial abundances, corroborating this trophic linkage (reviewed in [51]).

Interactions between bacteria and viruses have been conceptualized in terms of (i) nutrients and energy cycles under the "viral shunt" principle [7, 14, 48] and (ii) biodiversity and host specificity with the so-called "killing the winner" theory [44]. The net effect of the viral shunt on nutrients cycling increases community respiration and reduces the efficiency of carbon transfer to higher trophic levels [14] by catalysing transformation of host living communities to dissolved states of nutrients [41]. Grazing by protozoans and viral lysis affect the composition of planktonic bacterial assemblages by exerting size or host specific selection pressures, respectively [47]. Hence, the observed stability of bacterial richness in natural communities could be explained by infection on the bacterial "winner" (in the competition for nutrients) allowing non-dominant species to persist. In this case, bacterial diversity, in terms of activity or growth rates, would affect viral abundances, implying that a feedback exists between lytic viruses and bacterial species richness [43]. In a recent study it was suggested that only a small number of active bacterial populations are necessary to maintain high viral abundances [49]. Such population level interactions may explain the coupling between bacterial / viral diversity and biogeochemical cycles in terms of a transformation of living particulate matter into dissolved matter and non living forms [45]. Viral lysis potentially can influence the efficiency of the biological pump (sinking of particulate organic matter from surface to deep waters) whereas highly labile dissolved materials tend to be efficiently recycled in the photic zone [42].

Recent evidence shows that most aquatic viral genomes are rather widespread, and thus specific viral species may remain infectious in different aquatic environments and on a wide variety of bacterial hosts [1, 17]. River water contains an important number of viral particles (10^6 – 10^7 viruses mL^{-1}, [51]) that could maintain infectious capacity on marine bacterial hosts from coastal waters. However, the effects (in terms of dynamics and activity) of such riverine viral inputs on coastal planktonic bacterial populations have not been

considered. In the present work, we describe the results of a series of batch culture experiments designed to compare the response of coastal marine bacterial communities to both co-existing coastal viruses and allochtonous riverine virus in the Charente River (French mid-Atlantic coast). Viral production, bacterial activities and changes in bacterial diversity were compared in a large dataset of experimental samples. Changes in bacterial genetic diversity along the incubations were followed by PCR – single strand conformation polymorphism (PCR-SSCP) fingerprinting on the 16S rRNA gene, and compared to changes in viral diversity measured by pulsed field gel electrophoresis (PFGE).

Methods

Study site and water sampling**.** The Marennes-Oléron Basin (45° 59' 06" N, Fig. S1) is a shallow embayment (5 m average depth) characterised by short water residence time (4 to 10 days). The Charente River is the major source of freshwater and continental inputs to the basin and has an average yearly river discharge of 60 m^3 s^{-1}. The present study is part of a larger investigation on the transport and fate of freshwater viruses in a coastal environment; additional details about the study site are given in [4].
Surface water samples (first meter of the water column) were simultaneously collected at two stations located at each end of the Charente Estuary (Fig. 1): Station A in the Charente River, 50 km upstream from the mouth of the Charente Estuary (0.2 PSU, river water), and Station E located in the Marennes-Oléron Basin and still within the estuarine plume of the Charente River (> 30 PSU, coastal water). Sample collection was always done on the ebb cycle during spring tides. Water samples for the batch experiments were collected in February (in situ temperature 7.5°C), March (9.6°C), June (16.6°C) and July 2004 (20.2°C). Water was collected in opaque, acid-rinsed (10 % HCl) polypropylene bottles (15 - 40 l total sample volumes) and maintained at in situ water temperature during transport to the laboratory. Additional samples were collected (3 mL volume, in triplicate) at stations A and E, throughout the study period to monitor temporal in situ changes of both the viral and bacterial abundances.

Preparation of viral concentrates and grazer-free seawater**.** To prepare grazer-free seawater (defined here as a 0.8 µm filtrate), 15 to 40 L of water were prefiltered through a succession of nylon fibre sieves (Saratifil, see Fig. 1). Next, water was sequentially filtered through two membrane barriers (3 and 0.8 µm pore size cellulose acetate filters, 142 mm diameter,

Sartorius) mounted on stainless steel filter holders. Bacterial abundance in the 0.8 µm filtrate was 53 ± 27 % of total (unfiltered) bacterial abundance. Five liters of the 0.8 µm seawater filtrate (station E) the so-called "grazer-free seawater" was stored (approximately 2 h) at in situ temperature until the start of experiments.

Viral concentrates were made using the 0.8 µm filtrate after an additional filtration step on a 0.2 µm pore size membrane (cellulose acetate, 142 mm diameter, Sartorius) to remove most of the remaining bacteria (70 to 98% of unfiltered bacteria were removed). Next, viruses in the 0.2 µm filtrate were concentrated using tangential ultrafiltration (Ultraslice support, 30 KDa cut-off, polysulfon membrane, Sartorius) to a final volume of approximately 200 mL. Viral abundances were determined in the viral concentrates to calculate the concentration factor (CF = 7.0) as well as in the ultrafiltrates to check for the absence of virus.

Design of batch culture experiments. All the batch experiments used a "diluted grazer-free seawater" (see Fig. 1) from station E made after a ten-fold dilution of 0.8 µm grazer-free seawater and virus-free ultrafiltrate. Experiments were carried out at in situ temperature starting 5 - 7 hours after field sampling ended. Batch cultures were incubated for 90 h in the dark under gentle agitation and sub-samples for bacterial and viral counts were taken from each flask at regular intervals (every 6 h during the first 18 h, then every 12 h until the final sample at 90 h). Although all analyses were carried out in duplicate or triplicate, it was not possible to replicate batch cultures for each treatment due to the complexity of the experimental protocol.

In February and March 2004, three batch cultures experiments were carried out in parallel: one control (Ec) and two x2 viral enrichment treatments (Fig. 1). The added viruses were obtained either from station A (Ec + VA, river) or E (Ec + VE, coastal station). Acid-rinsed (10 % HCl) 5 L Erlenmeyer flasks each received 400 mL of grazer-free seawater from station E and were filled to a final volume of 4 liters with the station E ultrafiltrate and the selected viral concentrate from station A or E. Depending on the sample collection date and the type of treatment, 88 mL to 210 mL of viral concentrate were added (calculated according to a ×2 viral enrichment, based on diluted grazer free sea water). In June (16.6°C) and July 2004 (20.2°C), additional control experiments with aliquots of microwave-inactivated (two cycles of three min at 800 W) viral concentrates from stations A and E were also done.

A preliminary test of experimental conditions was done using water collected in November 2003 from station E. This test showed that: (i) the theoretical and the observed viral

enrichment factors were similar, and (ii) after addition of the viral concentrate, the difference among initial bacterial abundances in experimental flasks did not exceed 11 %.

Viral and bacterial counts. For enumeration of viruses and bacteria, water samples (1.5 mL, in duplicate) were collected with a sterile pipette, immediately fixed with 0.2 µm filtered formaldehyde (2 % v/v, final concentration) and stored at 4 °C until analysis (carried out within 24 hours after sampling). Virus and bacteria were counted simultaneously within two days of sampling, on the same membrane using nucleic acid staining (SYBR Green I, Molecular Probes) and epifluorescence microscopy [27].

Analysis of virioplankton communities**.** For all batch incubations, the structure of viral communities was analysed at the beginning (10 L of water from the field station) of each experiment using pulsed field gel electrophoresis (PFGE) as described in Auguet et al. [5]. This earlier study showed that the 0.2 µm filtration step did not modify virioplankton community structure. Thus, it was considered unlikely that the 1:10 dilution using ultrafiltrate fractions would change virioplankton structure in our experiments.
We attempted to look at the structure of virioplankton communities at the end of each experiment. However, the remaining volume in the batch cultures was a limiting factor to obtain reliable PFGE fingerprints.

Bacterial community analysis**.** Bacterial diversity in the field water and at the beginning and end of each experiment was assessed using PCR - Single Strand Conformation Polymorphism (PCR-SSCP) fingerprinting on the 16S rRNA gene. This technique uses single strands of amplified fragments (200 bp) which are separated electrophoretically based on their conformational polymorphism which depends on the precise sequence of bases [21]. The bacterial community assemblage is represented by a series of peaks. Each peak corresponds in the best case to a single DNA sequence and, by definition, to a bacterial OTU (operational taxonomical unit). The area below the peak roughly estimates the relative abundance of a phylotype within the targeted community. SSCP was chosen against other fingerprinting techniques such as DGGE because a fluorescently labeled internal size marker permits, after computing correction, a reliable comparison of SSCP banding patterns from a large set of samples. Co-migration, however, is a general limitation in fingerprinting methods and in some cases PCR products from different species may stop within the same peak or band (see more

details in Casamayor et al. 2000 and references therein). At any rate, most of the signals in the genetic fingerprints are single products [9].

For DNA extraction, water samples (300 mL) from each flask were filtered through a 0.2 µm polycarbonate filter (Sartorius). Filters were cut into small pieces and put in 2 mL microtubes containing 300 µl of lysis buffer (40 mM EDTA, 50 mM Tris-HCl at pH 8, 0.75 M sucrose). After extraction with phenol-chloroform-isoamyl alcohol (25:24:1, v:v:v), the DNA was precipitated in cold isopropanol (2 v) for 3 h at – 20 °C with 3 M sodium acetate (0.1 v). The air-dried pellets of DNA were then resuspended in 20 µL sterile distilled water [13]. Fragments of 16S rRNA gene (200 bp) were amplified using the bacterial primers W49 forward (E. coli position 331; 5'-ACG-GTC-CAG-ACT-CCT-ACG-GG-3') and W34 reverse (E. coli position 533; 5'-TTA-CCG-CGG-CTG-CTG-GCA-C-3') [12, 36]. These primers are universal and non-selective against any specific bacterial group. The PCR amplification program consisted of an initial denaturation step at 94 °C for 2 min, followed by 25 cycles: denaturation (30 s at 94 °C) then, annealing (30 s at 61 °C), and elongation (30 s at 72 °C). The final primer extension step was for 10 min at 72 °C.

All PCR products were purified using the QIAGEN PCR purification kit. For the SSCP fingerprint analysis, PCR products were heat denatured at 94 °C for 5 min and immediately placed on ice for at least 10 min. A fluorescently labelled internal size marker was added (Genescan-400 Rox, Applied Biosystems) that permitted, after correction, reliable comparison of patterns between runs. SSCP electrophoresis was carried out as described in [12] using an ABI 310 genetic analyser equipped with a capillary tube (Applied Biosystems).

Enumeration of high or low nucleic acid content cells by flow cytometry. Flow cytometry samples (3 mL each, in duplicate) were immediately fixed with formaldehyde, frozen in liquid nitrogen and stored at -80°C. A highly significant relationship between a subset of the microscopic bacterial counts (X) and flow cytometry counts (Y) ($Y = 0.75X$, $r = 0.94$, $n = 39$, $p << 0.001$) indicated a mean loss of 25% of total cells due to freezing. One mL of fixed sample was incubated with 0.5 µL of SYBR-Green I during 15 min at room temperature in the dark. Fluorescent beads (1µm diameter, Polysciences Inc, Warrington, Pa) were systematically added to each analysed sample to normalize cell fluorescence and light scattering values. Samples were counted with a Facscalibur flow cytometer (Becton Dickinson) equipped with an air-cooled argon laser (488 nm). Stained bacteria were discriminated and counted based on their 90° Side Scatter values (SSC, relates to bacterial cell volume, $V = 0.6636 \times SSC + 0.0166$) and their fluorescence emission at 530 ± 15 nm

(green fluorescence). Subgroups of cells with a high nucleic acid (HNA) content or a low nucleic acid (LNA) content were identified visually on the SSC versus green fluorescence plot by hand-selected windows. Cell abundances were determined in each window and then utilized to obtain the percent of HNA cells (%HNA) of total cytometry abundance (Y). HNA abundances were then calculated as % of epifluorescence (X) counts.

Bacterial activities and viral production. Bacterial production was estimated by the [^{3}H-methyl]-Thymidine (TdR) incorporation method [15]. Control and triplicate samples (20 mL each) were incubated for 30 to 60 min with 20 nM of [^{3}H] TdR (50 Ci mmole^{-1}, final concentration) at in situ temperature. Rates of ^{3}H thymidine incorporation were converted to cell production values assuming that 2.18 10^{18} cells were produced per mole of thymidine incorporated [15].

Bacterial growth rates (per day) were inferred from the exponential phase of bacterial growth in each flask. For the calculation of viral growth rates (per day), we averaged the slopes of all detected viral abundance peaks for each incubation. The number of viral particles produced during each abundance peak was assumed to be a measure of viral production. Total viral production (i.e. all the viruses produced during an incubation) corresponds to the sum of all viral productions over 90h. For each treatment, the viral production occurring during the bacterial exponential phase was expressed as a percentage of total viral production.

Viral production rates (virus mL^{-1} h^{-1}) were calculated by adding the net increase of viral abundance during each peaks divided by the time elapsed during the start of the first peak and the end of the ultimate peak [50].

Net Bacterial and Viral population increases (NBI and NVI, respectively) were calculated by comparison of final to initial abundances ratio for each experiment.

Nutrient concentrations. Water samples (70 mL) for dissolved inorganic nutrient concentrations (nitrate [NO_3], nitrite [NO_2], ammonium [NH_4] and phosphate [PO_4]) were collected at 0h, 18h, and then every 24 h. Samples were filtered immediately after sampling through Whatman GF/F filters (47 mm diam.) and stored in acid rinsed polypropylene bottles at –20°C until analysis with a Skalar auto-analyser as described by Strickland and Parsons [40]. Dissolved organic carbon (DOC) concentrations were estimated spectrophotometrically [29, 30].

Statistical analyses**.** Ordinary least square regressions were used to describe the experimental data. We used a standard software package for the statistical analyses (Excel Stat Pro with Microsoft Excel 97).

PFGE profiles were compared using a similarity analysis based on the presence / absence of each band within each sample [5]. The similarity matrix (Dice coefficient) permitted the construction of a dendrogram following the unweighted pair group aggregation method with arithmetic means (UPGMA).

The SSCP profiles of each sample were compared using a similarity analysis (Dice coefficient) based on the presence / absence of each phylotype. Only peaks >0.2% of the total community area were compared. A dendrogram was made using the similarity matrix, as for the case of virus. A second matrix that included the relative contribution of each phylotype to the total bacterial community (relative abundance) was used to calculate the Shannon diversity index (H'):

$$H' = -\sum_{i=1}^{k} pi \log pi$$

where k is the number of phylotypes in one sample and pi the relative abundance of each phylotype. As H' is susceptible to variations of k, we calculated heterogeneity or dominance (1-J)' as an index of diversity where the evenness or relative diversity index $J' = H'/H'_{max}$ and $H'_{max} = \log (k)$.

Results

Bacterio- and virio-plankton communities at the two stations. Bacterial and viral abundances were always higher at the river station (A) than at the estuary plume station (E), and higher in summer (June, July) than in winter (February, March) at both stations. Between the two seasons, bacterial and viral abundances at station E increased by a factor of 2.8. At this station, between February and June, bacterial cell production increased by a factor of 5.4 (Table 1).

In June and July bacterial diversity (i.e., SSCP peak richness) was slightly higher at the coastal station E (26 ± 6 bacterial phylotypes) than at riverine station A (19 ± 3 bacterial phylotypes). Samples clustered according to sampling station (Fig. 2), although the two communities shared some phylotypes. For example in June and July, 50 % of the riverine phylotypes were also detected at the coastal station.

Analysis of virus-like genomes (VLGs) identified 23 different genotypes with a mean of 6 ± 2 phylotypes per sample, and mean genome sizes of 34 kb (station E) and 46 kb

(station A). From February to July 2004, VLGs below 100 kb made up 97 % and 98% of total VLGs at stations A and E, respectively. The VLG fingerprints clustered by month, with higher similarity values for samples collected in June and July at both stations (Fig. 2). In particular, June samples from both stations had similar VLG community structures.

Microbial growth and activities in batch cultures. In the batch cultures, bacterial abundances followed a classic sigmoidal-type growth curve with a decreasing lag period from February (90 h) to July (6 h) (Fig. 3). The bacterial growth dynamics were similar for all viral treatments except in the inactivated treatments of June where bacteria reached significantly higher abundance than in the other treatments. Bacterial growth rates increased from March (mean ±SD: 0.84 ± 0.13 day^{-1}) to July (1.43 ± 0.33 day^{-1}). Total bacterial cell production was multiplied by a factor of 1000 over the same period (estimated from thymidine incorporation, data not shown). In February, no bacterial exponential phase appeared, and the Net Bacterial population Increase was close to one (mean ± SD for all treatments: 1.3 ± 0.5). In March, June and July, the NBI was 30.1 ± 6.1, 29.8 ± 23.9 and 29.5 ± 8.5, respectively. One of the most interesting results was the lower NBI in virus enriched treatments as compared to flasks enriched with inactivated concentrates for the June and July experiments (Fig. S2). Additions of freshwater viruses reduced NBI by a factor 3.3 and 2.0 in comparison to inactivated treatment, in June and July respectively.
HNA cells initially made up from 3 ± 1 % (February) to 39 ± 5 % (July) of the total bacterial assemblage. After 90 h, HNA cells were 88 ± 7 % of the population in March, June and July, and 38 ± 2% in February (controls and coastal viral enrichment). Independent of the season, the exponential phase started when HNA cells reached around 33-39% of the total assemblage. The HNA cell growth dynamics had the same pattern as the bacterial abundances and showed few differences as result of viral concentrate additions (Fig. 3). The %HNA was an excellent predictor of bacterial cell production (P): ($\log (P) = -2.277 + 3.701 \times \log (\%HNA)$; $r^2 = 0.91$, $n = 12$, $p < 0.0001$).

Viral dynamics and production. At the start of the batch cultures, viral abundances were significantly ($p < 0.05$) higher in treatments than in controls (enrichment factor x2.1 ± 0.3). In all the treatments, production - mortality cycles occurred as shown by the oscillations of viral abundance, even in February (Fig. 3). However, Net Viral population Increase, ranging from 0.8 ± 0.2 (February) to 1.8 ± 1.2 (July), was weak in all batch cultures as compared to

bacteria. As a result, the virus-to-bacteria ratio (VBR) declined during the incubations by 32 ± 17 % in February and 91 ± 5 % in March, June and July (Fig. S3).
Most of the viral production occurred during the exponential phase of bacterial growth (72 ± 20 %, Fig. 4) in March, June and July. In flasks containing inactivated viruses, this percentage declined to 56 ± 23 % (autochthonous co-existing viruses) and 28 ± 16 % (allochthonous riverine viruses), respectively (Fig. 4). Besides, mean viral production rates were significantly ($p<0.05$) lower in flasks enriched with heat-treated viral concentrates (Fig. 4), confirming the successful inactivation of viruses.

Relationships between microbial abundance and biotic/abiotic parameters. For each monthly experiment, correlations were estimated for mean data from all treatments and controls (Table S1). In all batch cultures, mean bacterial abundances correlated significantly and positively with %HNA values (Table S1). Viral abundances were significantly related to bacterial abundances in March, June and July, but also to %HNA in March and June. Whatever the month, bacterial abundances explained 45 to 87 % of the viral abundance variability in the control flask (Table S2), but addition of viral concentrate decreased the strength of this straight-line relationship. The addition of the microwave-inactivated viral concentrate eliminated the dependent relationship between bacterial and viral abundances ($0.094 \leq p \leq 0.75$, Table S2).
In the control flasks, a significant positive linear relation was found for viral production as a function of the active bacterial cell community (bacterial abundance corrected by %HNA) ($r^2 = 0.90$, $p = 0.013$, $n = 5$). Furthermore, using data from each identified viral production cycle in the controls, a significant linear relationship was obtained between viral production rate and initial abundance of active bacteria for each cycle (slope = 0.32; $r^2 = 0.49$, $p < 0.01$, $n = 13$). Conversely for treatment data, no significant relationships were found (co-existing virus, slope = 0.24; $r^2 = 0.217$, allochtonous riverine virus, slope = 0.12; $r^2 = 0.109$).

Effect of virus additions on bacterial community composition. Changes in bacterial assemblages between the beginning and the end of each experiment were assessed by SSCP fingerprinting. Dendrograms based on the similarity analysis of SSCP profiles showed that the final bacterial assemblages were relatively more similar to each other than the initial ones (Fig. 5). In all experiments, bacterial species richness (R_B) decreased (with a maximum loss of – 65 ± 15% in February) and dominance (1-J') increased (Table 2). We identified arbitrarily two bacterial phylotype groups on the SSCP profiles (Fig. 6). Group I dominated the initial

bacterial assemblage, accounting for 66 ± 11% of the total relative abundance (mean for all experiments). Group II dominated at the end of experiments (74 ± 11% of total relative abundance).

Despite the significant changes in bacterial community composition measured in the controls (due to confinement), the virus additions also had a measurable effect (positive or negative) on bacterioplankton assemblage richness or diversity. Overall, at 90 h, R_B was strongly and significantly related to viral abundance (V): ($\log (R_B) = - 0.962 + 1.436 \log (V)$; $r^2 = 0.66$, $p < 0.001$, $n = 16$). Addition of freshwater viruses had the same effect on R_B than co-occuring viruses. They both promoted an increase of the R_B in June and a decrease in July, as compared with inactivated concentrates (Table 2). In addition to the effect of viruses alone, DOM and nutrients contained in the active viral concentrates seemed also to increase R_B when compared to the controls (Table 2). Bacterial species richness decreased in two cases due to viral enrichment (by -11.5 and -18.2%, respectively), remained unchanged in two, but increased in four cases (by +12.5 to +87.5%). The effects of viral concentrates on bacterial community composition are illustrated by the SSCP profiles of the February incubations in Fig. 6 (profile Ec 90 versus profiles Ec + VE90, Ec + VA90). First, relative abundances of some phylotypes from group II consistently rose after viral additions. Second, we observed new dominant phylotypes that were not detected in the controls (profile Ec 90). In three of the four experiments, the addition of viral concentrate from station A (freshwater viruses) led to the development of significantly different bacterial communities as compared to flasks enriched with co-occuring viruses (station E) and to control flasks (Fig. 5, February, March, July). However, active and inactivated concentrates did not show a contrasted effect.

Discussion

Our results show that the addition of viral concentrates prepared with water from a freshwater station either were able to affect the dynamics of a coastal bacterial community, or supported the development of bacterial assemblages different from the ones obtained with addition of native viral concentrates. This suggests a potential effect of freshwater viruses on the composition of the neighboring coastal bacterial communities.

We carefully used the term “potential” because experimental data obtained herein must be translated to natural conditions with care since batch cultures introduce known biases (confinement and handling) as compared to in situ studies. In the present work some limitations should be considered, (i) 47 % of natural bacterial abundance were lost due to sequential filtration steps implying a potential loss of dominant microorganism species [16,

35, 50], (ii) the ten-fold dilution of the remaining 53 % bacteria in the ultrafiltrate may have created an ecological niche that could favor proliferation of opportunistic bacterial species, and (iii) elimination of grazers in the initial inocula may have modified the virus - bacteria interactions since it has been recently established that grazers and viruses act in synergy (positive or negative) on bacterial dynamics and diversity [38, 39, 47]. Finally, confinement generally results in a considerable decrease in species richness [24, 35, 50]. Without any virus addition, we found in the incubations a loss of 65 % of the species richness in February and 22.5 % in June and July (average for all treatments at 90h)). Viruses present in the original bacterial inoculate could hardly contributed to these important losses because of the known weak effect of viruses on bacterial community composition [18, 19, 35, 50]. In addition, loss in richness was inversely related to bacterial growth (higher in June and July). Thus, 14 phylotypes disappeared from group I during February incubations, but in the summer treatments these phylotypes either did not disappear or new phylotypes arose.
Given all these biases, our comparative approach based on an identical initial (prefiltered) community appears justified.

Transplantation of riverine viruses to coastal water. The Charente river contains an important number of viral particles (10^7 – 10^8 viruses mL^{-1}, [3]). Several lines of evidence obtained here and in former works suggest the introduction of riverine virus – host systems to the Marennes- Oleron Basin including: (i) the conservative abundances of bacteria and viruses in winter along the entire Charente River estuary [4], (ii) the presence of one half riverine phylotypes in the basin in June and July, and (iii) the very high similarity of genomic or morphologic structures between riverine and estuarine virioplankton [5]. Recent reports have shown that non-marine viral communities can actively infect marine bacteria [34] and that cyanophage g20 genes, with >99% identities, occur widely in marine and freshwater environments [37]. These investigators have suggested either that close phage-host systems are distributed across a wide range of environments or that some polyvalent phages may have broad host ranges allowing them to propagate onto distantly related or even unrelated hosts. Direct evidences of the propagation of freshwater viruses were not obtained in these experiments because it was impossible to distinguish between allochthonous viruses and the autochthonous (co-existing) viruses included in the bacterial inoculate. However, indirect evidence indicated that freshwater viruses were able to develop on coastal bacterial communities. First, viral growth rates estimated in flasks enriched with freshwater viral concentrates were not significantly different from the control and flasks enriched with

autochthonous viruses (Fig. 4). As a consequence, viral abundance in flasks enriched with freshwater viruses always remained higher than those in flasks enriched with inactivated viruses (Fig. 3). If freshwater viruses had not replicated, viral growth rates and viral abundance would have decreased significantly compared to flasks enriched with inactivated viruses. Second, NVI and active bacteria (in division) were linked in flasks enriched with freshwater viruses, contrary to the uncoupling observed in treatments with inactivated virus (Fig. 4). The same was true for the relationship between viral and bacterial abundance (Table S2).

Effect of viral additions on bacterial community composition. Viruses are believed to influence clonal composition rather than community composition, and consequently no changes in bacterial community composition should be detected by 16S rRNA fingerprinting [50]. In our study, incubations with allochthonous viruses promoted the development of bacterial assemblages that were significantly different from the controls and from the incubations with autochthonous viruses (except in June, Fig. 5). In June, the absence of differences may be explained by the similarity between estuarine and riverine viral communities (Fig. 2).

According to previous studies, the expected effects of viral enrichment on bacterial species richness are (i) a stimulation of some bacterial phylotypes because of nutrient input (from lysis products) and dissolved organic matter (DOM) associated with viral concentrates [25] and, (ii) a decrease of interspecies competition due to viral lysis [47]. In our study, virus enrichment increased (6 of 8 experiments) or at least maintained the bacterial diversity (R_B, Table 2), independent of virus origin. Concerning the second point, autochthonous viruses increased bacterial dominance compared to the allochthonous ones.

Selective grazing on infected and uninfected bacterial cells as well as DOM and nutrient release through grazing influence bacterial community composition [2, 20, 26, 33]. Here, grazers were removed, viral additions did not enhance mineral nutrients or DOC and any production of inorganic or organic nutrients produced by viral lysis was not detectable (Fig. S3). However, we consistently measured significantly higher carbohydrate (monomeric and polymeric) and lower dissolved free amino acid concentrations in the river (Delmas, unpublished data). Thus, changes in DOM quality between freshwater and coastal viral concentrates could have stimulated the growth of different bacterial phylotypes after enrichment [11, 28, 31] An accurate evaluation of the bias due to addition of DOM present in viral concentrates is difficult to establish. We consider that our negative controls (inactivated

viral concentrates) yielded good information on potential bias effect from DOM addition. The reduction of viral growth rates (Fig. 4) together with decrease in the bacteria to virus ratio (VBR) in flasks enriched with heat-treated viruses (Table S2, Fig. S3) confirmed good inactivation of viruses. Active as well as inactivated virus led to the same bacterial community composition in July (Fig. 5), suggesting a higher and / or faster impact of DOM and nutrients than that of viruses. Since viruses need 1 to 2 generations of infection to lyse their hosts [32], the five-day experiment may have been too short to see a strong lytic impact with relatively slow growing viruses (0.25 day^{-1} , Fig. 4).

Nonetheless, in three of four experiments we obtained a specific bacterial assemblage shift after the addition of freshwater virus. To our knowledge, such a recurrent bacterial community response to allochthonous viral concentrate has not yet been reported. Overall, our results emphasize the hypothesis of a combined action of freshwater viruses and associated DOM quality on bacterial assemblage structure.

In contrast to a previous report [49], we found a strongly positive relationship between bacterial diversity and viral abundance at the end of all experiments, in agreement with the "killing the winner" theory [43, 44]: Dominant bacterial populations are controlled by viral infection, allowing less competitive populations to co-exist and maintain a consistently higher bacterial diversity (species richness). The second key mechanism suggests a control of viral abundance by bacterial activity. Our observation (72 ± 20 % of viral biomass produced during the log phase of bacterial growth) is consistent with this view.

Bacterial resistance versus susceptibility to viral replication. As suggested by the reduction of NBI, another expected effect of viral enrichment was the control of virus-sensitive bacterial phylotypes. However, few phylotypes (22 ± 8 %, average for June and July experiments) were negatively affected by viral additions, albeit different for autochthones and allochthones. One explanation is that most bacterial cells are resistant to viral infections, as reported by chemostat and pure culture experiments [22, 46]. Another is that most of the phylotypes, or at least the dominant ones, acquired some resistance to viral infections during the incubation [22]. The strong decrease of VBR observed in all treatments suggests prevalence of the second mechanism [23].

Schwalbach et al. [35] have suggested co-existence of virus-sensitive and virus-resistant clones for each phylotype . In this case, the physiological cost of resistance allows the maintenance of the virus-sensitive clones (i.e. through conservation of a growth advantage [10]. Moreover, exponential development of one or two opportunist bacterial species (in the

group II of phylotypes) may have supported the major part of viral production. Thus, the most abundant taxa would be slow growing and resistant to viral infection cells whereas the most susceptible taxa would be scarce opportunist phylotypes with bloom and burst cycles [6, 42].

In conclusion, we show that freshwater viral concentrates have a significant and recurrent effect on coastal bacterial community composition as compared to autochtonous viruses. There is substantial evidence that virus themselves were key players, but our experimental time and size scales limited a clear separation of virus and DOM quality effects. Nonetheless, at the scale the Charente river which imports $\approx 5 \times 10^{19}$ viral particles per day to the Marennes - Oleron basin, the effects of freshwater viruses upon coastal bacterial assemblages may be more important than the influence of freshwater DOM.

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Table 1: Temperature and microbial parameters for the river (A) and coastal (E) stations. The values represent *in situ* conditions for the *in vitro* experiments. Due to technical problems, some data are absent. nd = non determined. Error in microbial counts and bacterial production measurement never exceed 10 % of the given value.

Month	Temperature *in situ*, °C		Virus, $10^7 ml^{-1}$		Bacteria, $10^6 ml^{-1}$		Bact. Production, 10^6 cells $ml^{-1} h^{-1}$
	A	E	A	E	A	E	E
February	nd	7.5	nd	1.23	nd	1.46	8.44
March	11	9.6	2.02	1.33	3.44	2.41	24.19
June	17	16.6	4.64	3.10	6.39	5.16	45.88
July	21	20.2	9.20	4.00	8.16	5.56	nd

Table 2: Dominance (D) and specific richness (R_B) values for bacteria in each treatment. Numbers in bold highlight the cases where richness in enriched treatments was equal to or higher than the control (Ec 90).

	E 0.8 µm		Ec 90		Ec + VE90		Ec + VA90		Ec + VE90inact		Ec + VA90inact	
	D	R_B	D	R_B	D	R_B	D	R_B	D	R_B	D	R_B
February	0.14	29	0.22	8	0.45	**15**	0.20	7	/	/	/	/
March	/	/	0.31	11	0.36	9	0.33	**11**	/	/	/	/
June	0.06	20	0.29	16	0.30	**16**	0.25	**18**	0.28	14	0.33	13
July	0.15	30	0.18	18	0.18	**23**	0.18	**22**	0.19	**27**	0.17	**27**

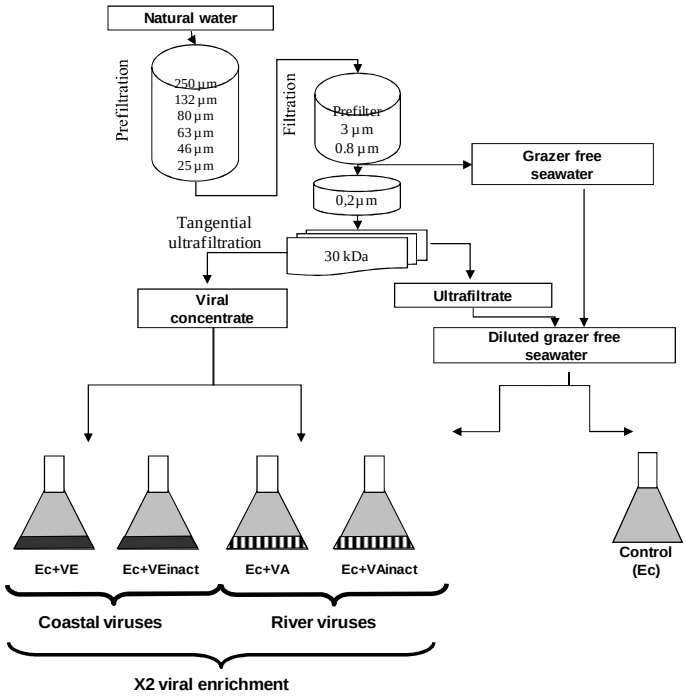

Figure 1: Preparation of samples for the batch culture experiments. Controls (Ec) contain diluted grazer-free seawater. Treatments were enriched with autochthonous viruses (Ec+VE), allochthonous viruses (Ec+VA), inactivated autochthonous viruses (Ec+VEinact) and with inactivated allochthonous viruses (Ec+VAinact), respectively.

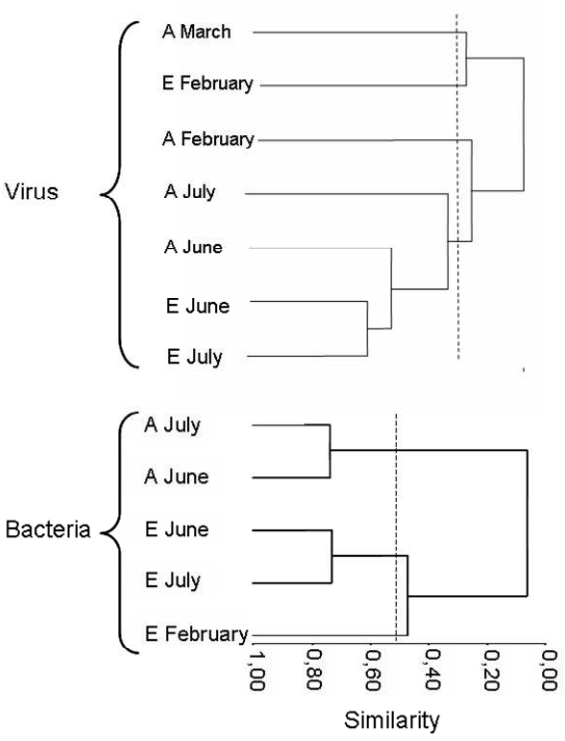

Figure 2: Dendrogram based on the similarity matrix generated from the PFGE (for virus) and the SSCP fingerprints (for bacteria) at stations A and E for all four experiments. The dotted line represents the threshold similarity below which SSCP or PFGE profiles were considered significantly different. Bacterial PCR amplifications failed for samples from station A in February and stations A and E in March. No PFGE fingerprint was obtained for samples from station E in March 2004.

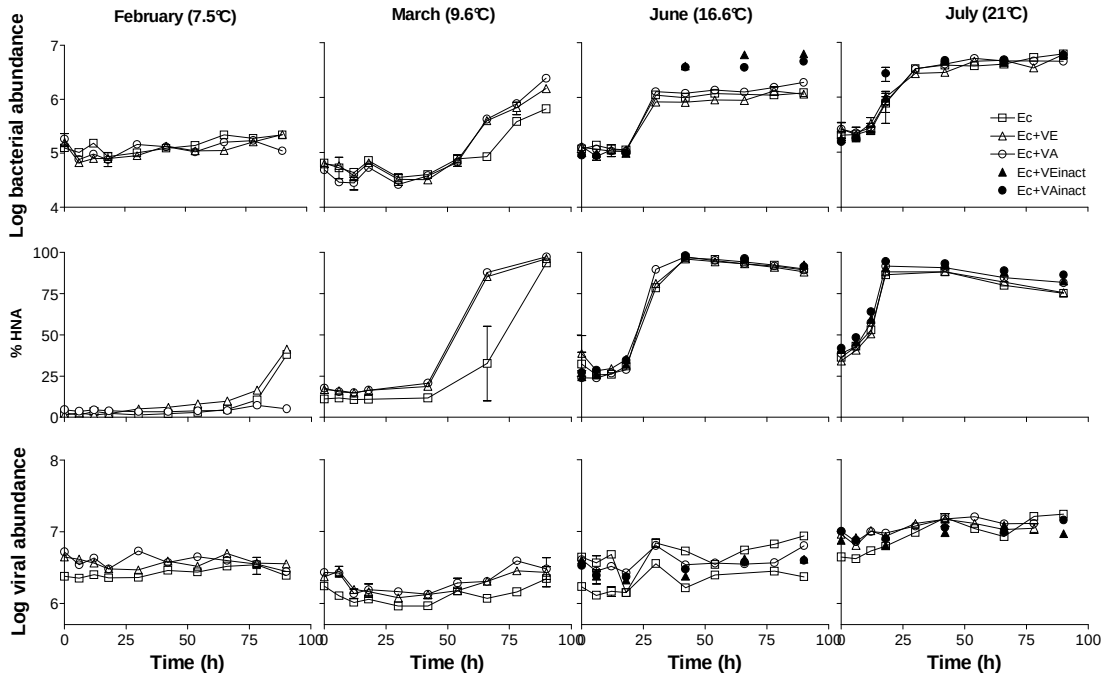

Figure 3: Variations in measured parameters of batch-cultures during 90 h in February, March, June and July 2004. Bacterial and viral abundances were estimated by epifluorescence counts. Percentage of active cells (%HNA) was estimated by flow cytometry. Ec: control flask, VE or VA: viral concentrate from station E or A, VEinact or VAinact: inactivated concentrate.

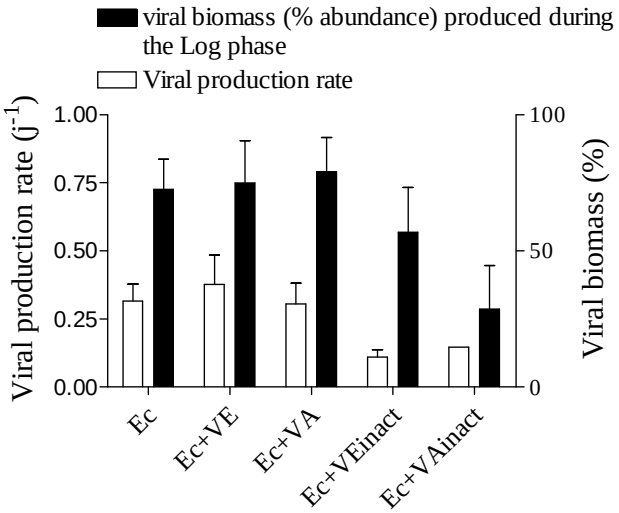

Figure 4 Viral production rate and percentage of the viral production occurring during the period of exponential bacterial growth. For each treatment (Ec: control flask, VE or VA: viral concentrate from station E or A, VEinact or VAinact: inactivated concentrate) average values from experiments in March, June and July were calculated with standard deviations.

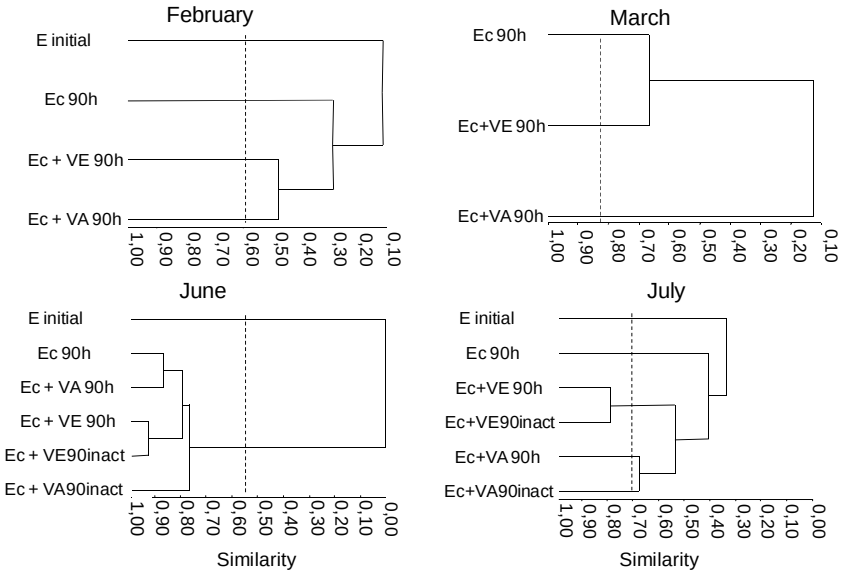

Figure 5: Dendrograms comparing bacterial assemblages from initial samples and samples after 90h incubation, based on the similarity matrix generated from SSCP chromatograms. E 0.8 µm are the initial samples, final samples are coded with '90' and inactivated viral concentrates with 'inact'. Ec is to the control, Ec+VE and Ec+VA are enrichments with station E (marine) and station A (river) viruses, respectively. Inactivation of viral concentrates was not tested for February and March. The dotted line represents the threshold beyond which SSCP profiles were considered significantly different

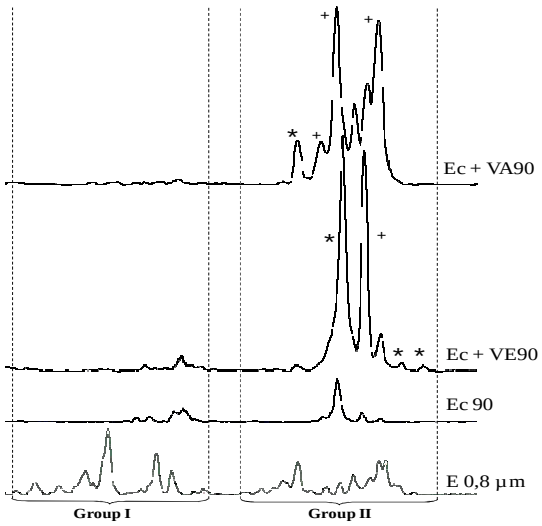

Figure 6: Example of SSCP scans obtained with treatments E 0.8 µm (initial state), Ec 90 (control after 90 h of incubation) and Ec + VE90 and Ec + VA90 (enriched treatments) in February experiments. The symbol (+) indicates main peaks which increased in the enriched treatment as compared to the control, and the symbol (*) represents the peaks absent in the control and / or in the 0.8 µm filtered water