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Natural and anthropogenic forcing on the dynamics of virioplankton in the Yangtze river estuary

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Seasonal investigation of virus dynamics by flow cytometry was conducted in the Yangtze river estuarine area in April, August, November 2002 and February 2003, and a supplemental investigation in the inner estuary and downstream of the river was conducted in October 2005. The majority of the total viral abundance was bacteriophage and only 5.4% of the total was algal virus. Total viral abundance varied with season and location, ranging from 6.75×10^5–1.68×10^7 particles/ml, and the virus:bacterium ratio (VBR) ranged from 1.52 to 72.02 with a mean of 8.7. In the present study, viral abundance peaked in both the summer and the winter, unlike the typical seasonal pattern reported in the literature, in which viral abundance peaks in the summer when bacterial hosts are also at their most abundant. However, the driving forces for the two peaks reported here were totally different, the summer viral abundance peak coupled with the development of bacterial hosts which were controlled largely by temperature year-round and by trophic state occasionally, while the winter one seemed to be multi-factor controlled. The host-phage interaction was no longer predominant in control of the winter viral abundance as bacterial abundance was lowest in this season. The winter low temperature would help maintain a high viral abundance as high temperatures might increase viral inactivation and viral decay; the VBR peak values actually occurred in the winter. More importantly, the high virus-containing freshwater discharge in winter due to a higher proportion of anthropogenic sewage relative to low natural flooding in winter run-off, turned out to be the first factor contributing to the high winter viral abundance and VBR values. In addition, the variation of intrusion of warm and relatively oligotrophic water from oceanic currents played a role alternating the distribution patterns of temperature, salinity and trophic conditions and consequently the distribution patterns of virus and bacteria seasonally and spatially. Dynamics of virus in the Yangtze river estuarine area is thus characterized by distinct seasonal and spatial variations due to natural forcing and by pronounced alternation of the regular patterns due to anthropogenic impacts.

INTRODUCTION

Virioplankton, being 10^5 to 10^8 particles/ml in seawater, is the most abundant and dynamic member of the microcommunity in the marine environment, responsible for a great portion of bacterial and phytoplanktonic mortality. Viruses play important biogeochemical and ecological roles in the marine ecosystem (Fuhrman, 1999). Such roles may include nutrient cycling, system respiration, particle size-distribution and sinking, bacterial and algal biodiversity and species distributions, algal bloom control, dimethyl sulphide formation and genetic transfer, etc. The virioplankton has been thus drawing great attention from microbial ecologists for the last decade. The dynamics of viral abundance has been studied in a variety of marine environments, thus a great deal of data has been acquired on geographical variation trends, seasonal patterns and the major controlling factors of viral abundance. The general understandings conclude that most of the viruses in the marine environments are bacteriophages (Bergh et al., 1989; Cochlan et al., 1993) that viral abundance significantly depends on the host bacterial abundance (Cochlan et al., 1993; Hewson et al., 2001; Corinaldesi et al., 2003; Auguet et al., 2005) and that seasonal patterns are recognized as highest viral abundance occurring in summer and lowest in winter (Jiang & Paul, 1994; Williamson et al., 2002; Auguet et al., 2005). However, conflicting conclusions about controlling mechanisms are often seen; for example, trophic/nutrient level is a controversial issue for virus regulation. There are evidences showing enhancement of viral abundance with increased nutrient availability (Maranger & Bird, 1995; Hewson et al., 2001; Danovaro et al., 2003) while some studies showed no causal relationship between viral abundance and nutrients in large scale investigations (Corinaldesi et al., 2003). Salinity is often reported to be related to viral abundance (Jiang & Paul, 1994; Auguet et al., 2005) but it can be an apparent parameter as suggested by the observation in an oligotrophic estuary without trophic gradients where no correlation between salinity and viral abundance is found (Hewson et al., 2001). Conclusions on controlling mechanisms of virus dynamics in marine ecosystems by different authors may be case by case, but one thing is without argument that the controlling mechnisms are complex and multi-factor regulated. Therefore, field investigations in different environments and over time are still the basic aim of current studies, and explorations of the controlling mechanisms of

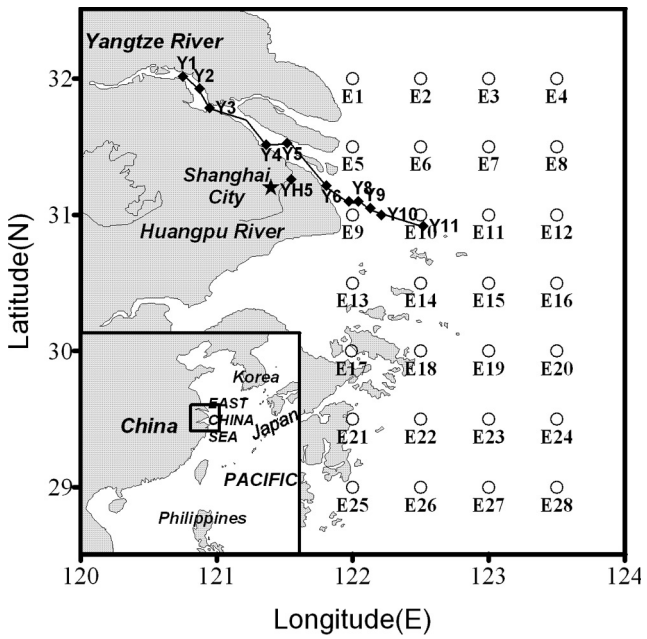

Figure 1. Locations of the Yangtze river estuary and the sampling stations. Stations E1–E28 in the estuarine area were sampled seasonally from April 2002 to February 2003; Stations Y1–Y11 in the inner estuary and downstream of the river were sampled in October 2005. The Station YH5 located in the Huangpu river, a branch of the Yangtze river, which passes through Shanghai City, was also sampled in October 2005.

virioplankton populations are especially desired. Coastal waters being most productive and most diverse in terms of environmental conditions have been the focus in this regard. However, little is known about the effects of anthropogenic impacts on the dynamics of virioplankton in coastal ecosystems. In the present study, we chose the Yangtze river estuarine area in the East China Sea as the investigation field, for the Yangtze river is the third largest river in the world, and the estuary receives great amount of input of water and materials from the river due to natural occurrences like flooding, and the estuary is highly disturbed by human activities as well. We performed seasonal investigations on the abundance of virioplankton, bacterioplankton, eukaryotic picoplankton and physico-chemical variables as well, aiming at a better understanding of the controlling mechanisms of dynamics of virioplankton under natural and anthropogenic stresses.

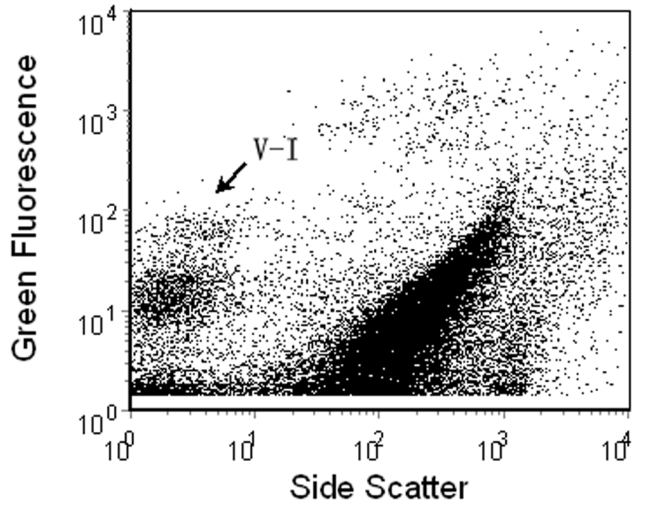

Figure 2. Flow cytogram of a field sample from the Yangtze river estuary showing the discrimination of the viral populations. V-I with high fluorescence are considered as algal viruses, the majority below V-I as bacteriophages.

MATERIALS AND METHODS

Description of the study area

The Yangtze river estuary, located in the East China Sea (Figure 1), is an area influenced strongly by the freshwater input of ~1000 billion cubic metres each year. The Yangtze river going from the west to the east across China is basically located in the temperate zone, thus the discharge from the river to the East China Sea is featured by a distinct seasonal pattern with 70% of the total water and 86% of the total sediment occurring in summer (Wang & Shen, 2001). The Yangtze river delta area is heavily inhabited and industrialized, great amounts of sewage are dumped in the river year-round, altering the natural structure of nutrients in the estuary (Shi et al., 2003). Thus the Yangtze river estuary is a very complex ecosystem that receives not only natural forcing but also anthropogenic impacts.

Sampling

An area between 122–123.5°E and 29–32°N off the Yangtze river mouth with water depth between 10 and 50 m was investigated seasonally. Water samples were collected from the 28 stations (E1–E28) on four cruises in April, 2002 (spring), August, 2002 (summer), November, 2002 (autumn) and February, 2003 (winter). In order to clarify the trend in viral abundance and its influencing factors, a supplemental cruise with 12 stations (Y1–Y11) along the salinity gradient from the river mouth to 160 km upstream was conducted in October 2005 (Figure 1). Samples were collected with 10-l Niskin bottles from the 1 m surface water and were fixed with glutaraldehyde (final concentration: 0.5%, Marie et al., 1999) for 15 min and then stored in liquid nitrogen for later analysis.

Flow cytometric analysis

There are a number of techniques that can be used for virus enumeration, such as transmission electron microscopy (TEM), epifluorescence microscopy (EFM) and flow cytometry (FCM). Among these, FCM is the most convenient one (Marie et al., 1999), and was employed in the present study.

Pico-eukaryotes, *Synechococcus*, heterotrophic bacteria and virioplankton were analysed separately by using an Epics Altra II flow cytometer (Beckman Coulter, USA) equipped with a 15-mW 488-nm air-cooled argon-ion laser and a standard filter set-up. Procedures were as described by Jiao et al. (2002). Pico-eukaryotes and *Synechococcus* were distinguished according to their positions in the plots of chlorophyll (FL3) vs 90°—angle light scatter (SSC), and phycoerythrin (FL2) vs SSC.

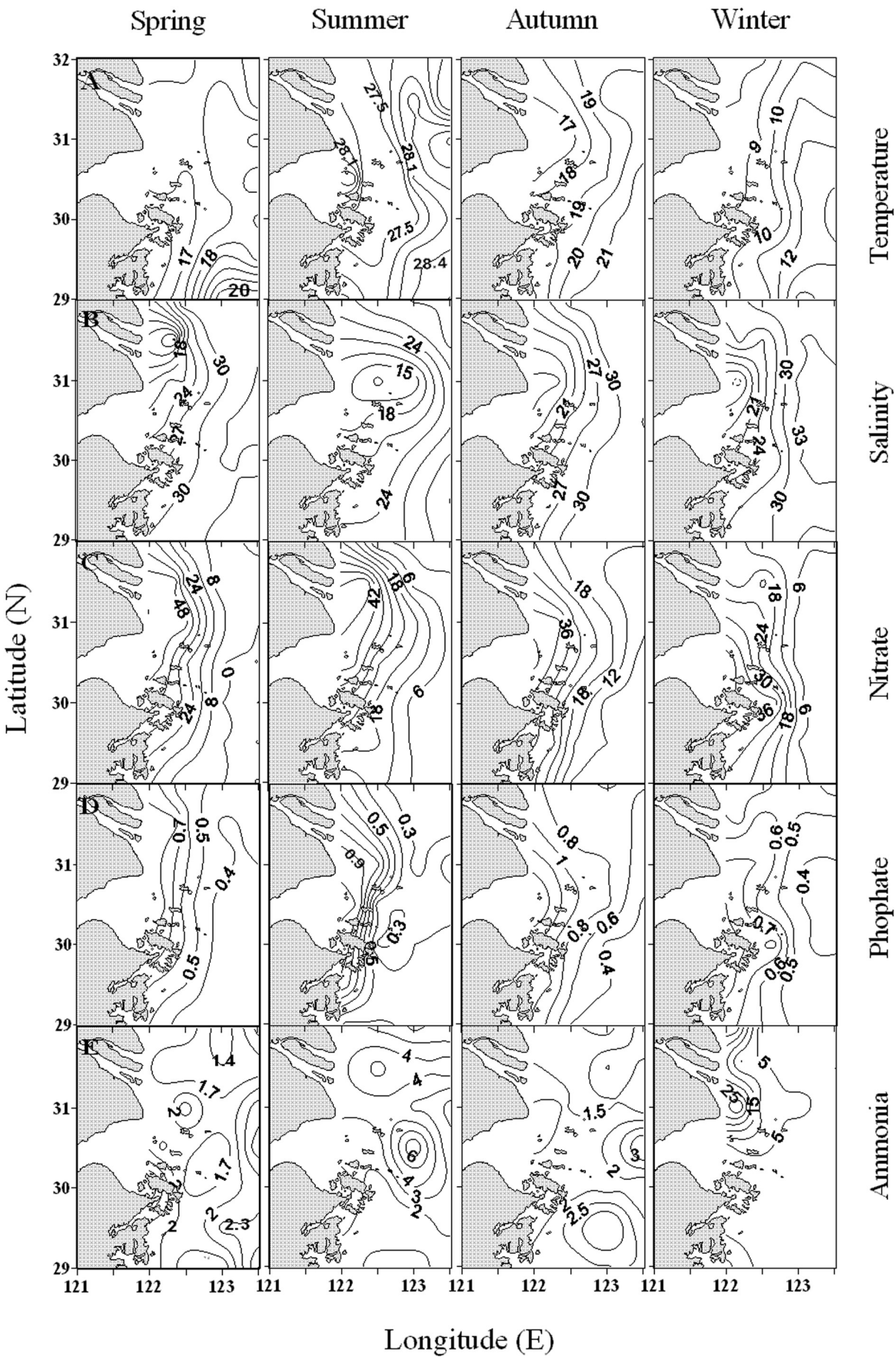

Figure 3. Distribution of (A) water temperature; (B) salinity (psu); (C) nitrate (μmol/l); (D) phosphate (μmol/l); and (E) ammonia (μmol/l) in the Yangtze River estuarine area in the four seasons.

Table 1. *Seasonal means of viral and bacterial abundance and virus-to-bacterium ratio.*

Season	Virus Group I (V–I) (particles/ml)	Total viral abundance (particles/ml)	Bacterial abundance (cells/ml)	Virus-to-bacterium ratio (VBR)
Spring	$7.19 \pm 1.80 \times 10^4$	$1.65 \pm 0.50 \times 10^6$	$3.34 \pm 1.80 \times 10^5$	7.23 ± 6.96
Summer	$1.60 \pm 0.59 \times 10^5$	$2.68 \pm 1.20 \times 10^6$	$6.71 \pm 4.05 \times 10^5$	4.53 ± 1.91
Autumn	$9.70 \pm 5.17 \times 10^4$	$1.80 \pm 0.87 \times 10^6$	$2.95 \pm 1.57 \times 10^5$	8.50 ± 7.97
Winter	$1.64 \pm 0.68 \times 10^5$	$2.85 \pm 1.63 \times 10^6$	$2.50 \pm 0.58 \times 10^5$	14.19 ± 14.63

The values are means ±SDs.

Pico-eukaryotes were identified by their large size and red fluorescence. SYBR Green I (Molecular Probes) was employed as the nucleic acid stain (Marie et al., 1997) for bacterium identification in plots of FL3 vs green fluorescence (FL1).

Virus enumeration was performed according to the method by Marie et al. (1999). Once thawed at 37°C, samples were diluted in 0.02-μm filtered TE (Tris-EDTA, pH=8) buffer 10 to 100 times as needed and heated for 10 min in the dark at 80°C after staining with the DNA dye SYBR Green I (1/20 000 final concentration, Molecular Probes), and then cooled for 5 min prior to analysis. Samples were run at a flow rate of 3–25 μl/min. The discriminant was set on green fluorescence. Viruses were discriminated on the basis of their green DNA-dye fluorescence vs SSC (Figure 2). Data were analysed with EXPOTM[32] MultiCOMP software (Beckman Coulter, USA). A subgroup of virus with higher DNA fluorescence was seen in the cytograms (Figure 2), which is called the V-I group (Marie et al., 1999).

Nutrients, temperature, salinity, and chlorophyll-*a* were analysed according to the standard procedures described in the JGOFS protocol (SCOR, 1996).

Statistical analysis

Analysis of variance (ANOVA) and *t*-test were employed to compare the differences in parameters among seasons and stations using the statistical software package on spss13.0. Spearman rank correlation analysis

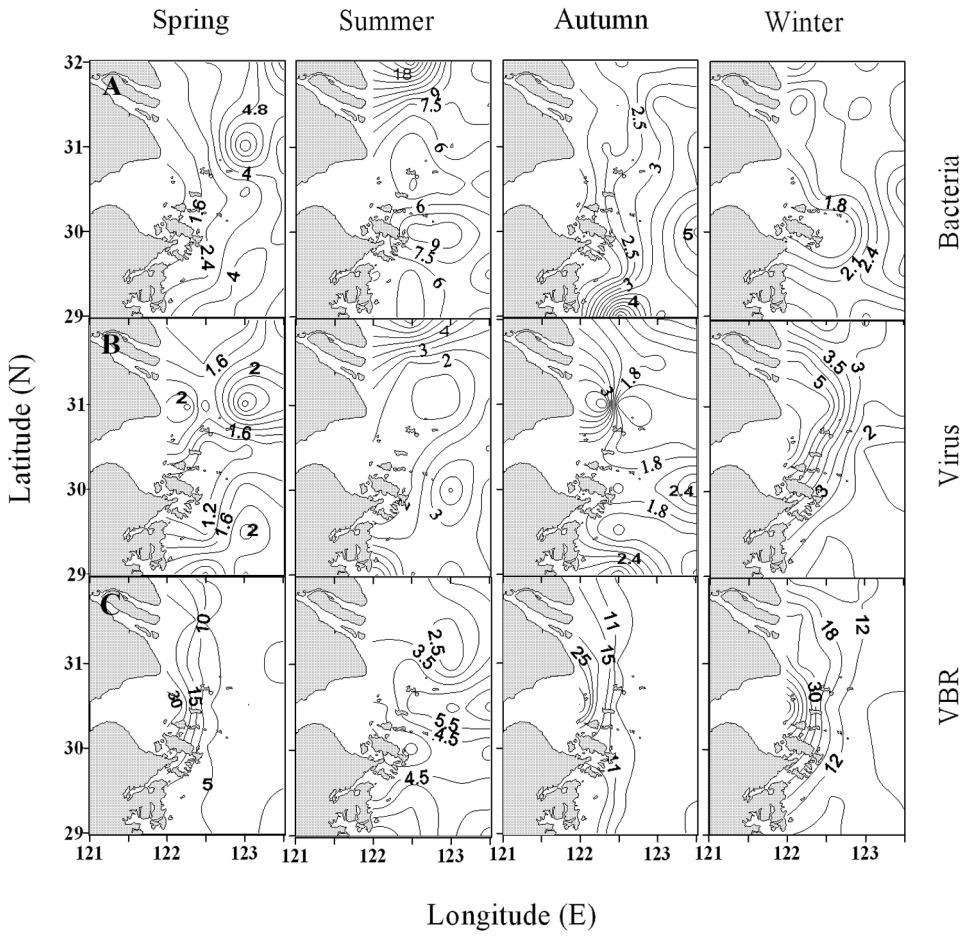

Figure 4. Distribution of (A) bacterial abundance ($\times 10^5$ cells/ml); (B) viral abundance ($\times 10^6$ particles/ml); and (C) virus-to-bacterium abundance ratio (VBR) in the Yangtze river estuarine area in the four seasons.

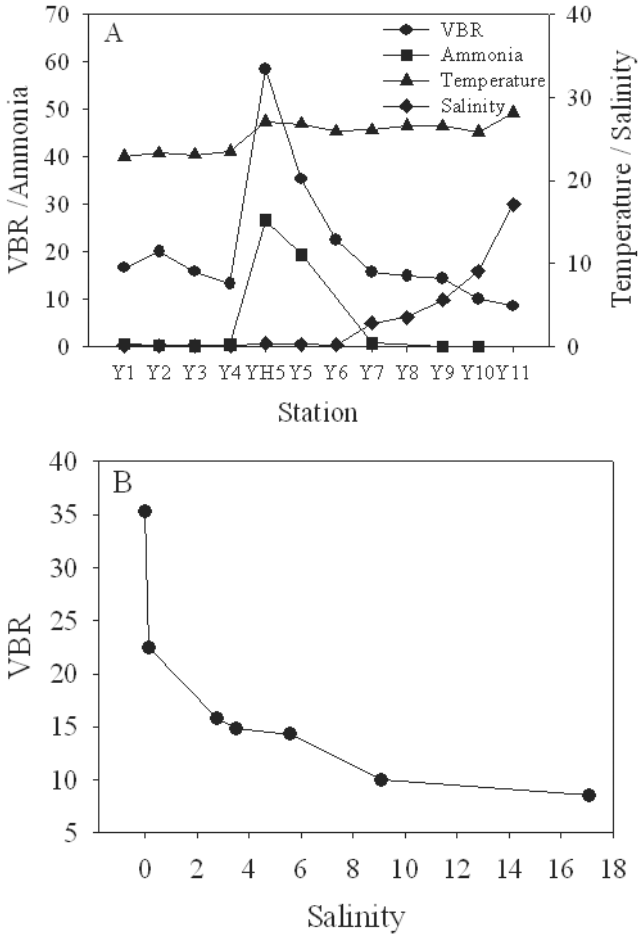

Figure 5. (A) Variation of salinity (psu), temperature (°C), ammonia (μmol/l), and VBR from downstream of the Yangtze river to the estuarine area; and (B) VBR as a function of salinity. Refer to Figure 1 for the locations of the Stations Y1–Y11. The extremes at Station YH5 was not in the Yangtze river but in the Huangpu river, a branch passing through Shanghai City

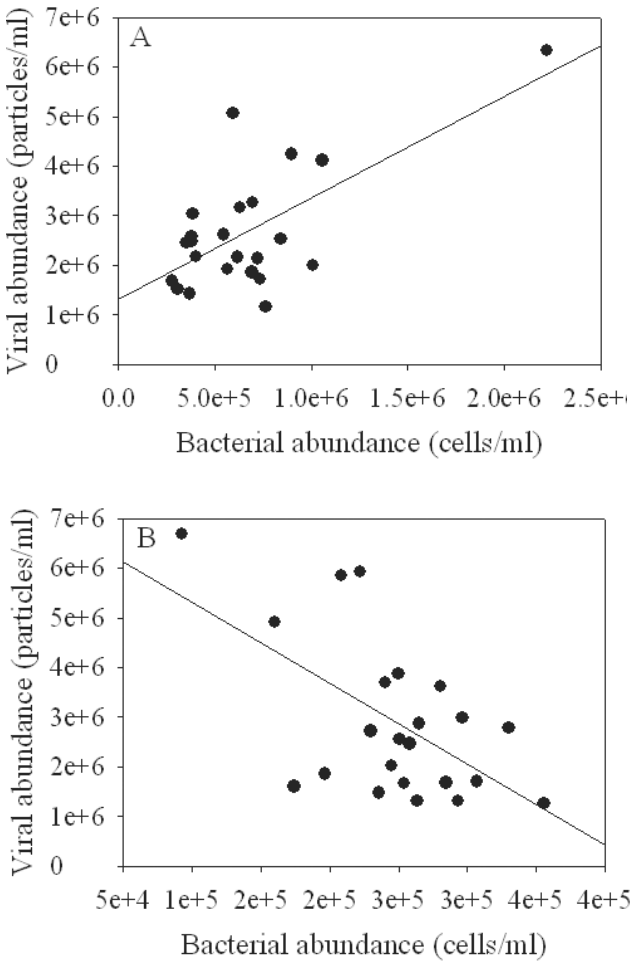

Figure 6. (A) Relationship between viral abundance and bacterial abundance in August, 2002; and (B) February, 2003.

was applied to the assessment of the degree of correlation among the investigated parameters.

RESULTS

Variation of environmental variables temperature, salinity, and nutrient concentrations in the study field were followed seasonally (Figure 3). Temperature displayed a significant seasonal variability with 17.46 ±1.03°C in the spring, 27.81 ±0.74°C in the summer, 19.42 ±1.68°C in the autumn and 10.60 ±1.88°C in the winter. Spatially, the temperature distribution patterns were controlled by the interaction between the cold water masses including freshwater from the Yangtze river and the coastal current from the north-west of the study area and the warm water mass intruded from the Kuroshio Current in the south-east of the study area. Thus there were existing temperature gradients increasing from north-west to south-east throughout the year (Figure 3A).

Influenced by seasonal variation in flooding, salinity in the Yangtze river estuary showed a seasonal variability with lowest values occurring in the summer and highest values in the winter. A clear increasing salinity from the river mouth to the outer margin of the study area was observed in all the seasons (Figure 3B).

Seasonal changes in nitrate and phosphate concentrations were not significant ($P > 0.05$). The overall averaged concentrations of nitrate and phosphate were 16.11 ±15.51 and 0.55 ±0.24 (μmol/l) respectively. Spatially, there were pronounced gradients decreasing in offshore directions (Figure 3C,D). In contrast, ammonia concentrations showed no consistent spatial gradients among the four seasons, but there were extremely high concentrations in the winter around the river mouth (Figure 3E). The supplemental investigation in October 2005 showed similar high ammonia concentrations in the inner river Stations Y5 (19.3 μmol/l) and YH5 (26.6 μmol/l).

Variation of bacterioplankton and virioplankton

Abundance of total bacteria showed a dramatic fluctuation ranging from 3.87×10^4–2.22×10^6 cells/ml with a clear seasonal pattern as high abundance occurring in the summer (mean, 6.71×10^5 cells/ml) and low abundance in the winter (mean, 2.50×10^5 cells/ml) (Table 1). Geographically, there were increasing gradients in offshore direction in all the seasons except the summer

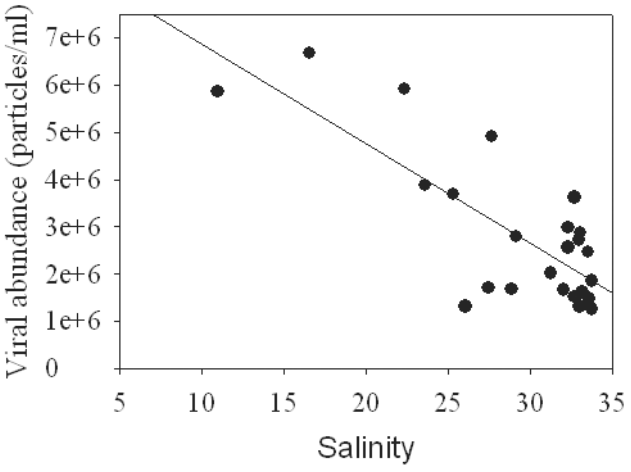

Figure 7. Correlation between viral abundance and salinity in February 2003.

(Figure 4A). Abundance of virus Group I (V−I) accounted only for 5.4 ±3.1% of the total averaged over the four seasons (Table 1). Total viral abundance over the investigation period fluctuated between 6.75×10^5 and 6.38×10^6 particles/ml in the estuarine area. Seasonally, there were no such regular patterns as observed in bacteria. The abundance peak values were recorded both in the summer (mean, 2.68×10^6 particles/ml) and in the winter (mean, 2.85×10^6 particles/ml) (Table 1). Geographically, there were similarities between the distribution patterns of viral abundance and bacterial abundance except in the winter when the variation trend in viral abundance was actually opposite to that in bacterial abundance. It is notable that there were always high values of viral abundance occurring near the river mouth area (Figure 4B). The virus-to-bacterium ratios varied dramatically with season and station ranging from 1.52 to 72.02, with an overall mean of 8.7. Seasonally, the highest mean value (14.19 ±14.63) occurred in the winter and the lowest in the summer (4.53 ±1.91). Spatially, there were distinct decreasing gradients in offshore directions except for the summer. The supplemental investigation in October 2005 showed high viral abundance and VBR values in the inner river areas (Figure 5). Particularly at the Y5 station where a branch, the Huangpu river, joins the Yangtze river, viral abundance reached 1.61×10^7 particles/ml, with a VBR of 35.29. Moreover, at Station YH5 where the Huangpu river passes through Shanghai City, the viral abundance was even 3.36×10^7 particles/ml making the VBR extremely high (58.38) (Figure 5A). A sharp drop down in VBR along the increasing salinity gradient from the sewage polluted area as indicated by the high ammonia concentrations in the Yangtze river to the centre of the estuarine area was clearly observed (Figure 5B).

DISCUSSION

Total viral abundance observed in the study area is overall comparable with values reported in the literature (Cochlan et al., 1993; Jiang & Paul, 1994; Hewson et al., 2001; Auguet et al., 2005). Although it is hard to discriminate viral sub-populations by flow cytometric analysis, a subgroup of virus with higher DNA fluorescence was seen in the cytograms in the present study (Figure 2). Close correlation existed between V−I and picoeukaryotes ($P<0.001$, N=80) especially in the summer (data not shown) suggesting that the V−I group are algal viruses (Marie et al., 1999). Since the V−I group accounted for only 5.4 ±3.1% of the total averaged over the four seasons, it will not be discussed separately hereafter. There were close correlations between total viral abundance and total bacterial abundance ($P<0.05$, N=95) but not chlorophyll-*a* ($P>0.5$, N=95; data not shown), which is consistent with most of the conclusions in the literature (Cochlan et al., 1993; Hewson et al., 2001; Corinaldesi et al., 2003; Auguet et al., 2005), suggesting that the majority of the viruses we observed in this study were bacteriophages. This agrees with the conclusion by TEM that the majority of hosts of viruses in marine waters are bacteria (Bergh et al., 1989; Cochlan et al., 1993).

Seasonal variability in viral abundance, with highest values in summer and lowest values in winter, is often reported in the literature (Jiang & Paul, 1994; Williamson et al., 2002; Auguet et al., 2005). However, the seasonal pattern we observed in the present study is quite different, in that high viral abundance was also recorded in the winter. The summer peak values are easily understood as the host bacteria were most abundant in the summer and thus more viruses would be released into the water. This is confirmed by the correlation between viruses and bacteria in the summer ($P<0.01$, N=26) (Figure 6A). Such correlations are also reported in other marine environments (Cochlan et al., 1993; Hewson et al., 2001; Corinaldesi et al., 2003; Auguet et al., 2005). However, the winter peak values can not be interpreted by host−phage interactions, as viral abundance is actually inversely correlated with bacterial abundance ($P<0.01$, N=23) (Figure 6B). The VBR, an important parameter indicating the numerical predominance of viruses over bacteria (Wommack & Colwell, 2000), also showed a wide range from 1.52 to 72.02 suggesting the uncertainties of virus−host interactions. The VBR values were actually lowest in the summer (mean, 4.53) and highest in the winter (mean, 14.19). That is, there were factors other than host influencing the dynamics of viral abundance in the study area in winter.

Although high temperatures favour bacteria, for viruses in the summer the effect may be more negative than positive because of enhanced bacterial enzymatic activity (such as protease and nuclease) with temperature, which would increase viral inactivation and viral decay (Noble & Fuhrman, 1997), and the greater summer influences of UV-induced virus decay (Noble & Fuhrman, 1997) and grazing (Gonzalez & Suttle, 1993). Therefore low summer VBR values are often observed.

Trophic status is reported to be related to virus production (Maranger & Bird, 1995; Hewson et al., 2001; Danovaro et al., 2003). Nutrients may play an indirect role in viral abundance through their hosts, as eutrophic environments allow a higher standing stock of bacteria (and consequently higher number of hosts for bacteriophages) than oligotrophic systems (Danovaro et al., 2003). Particularly, phosphate can even directly stimulate viral development by modulating the lysogenic response of natural populations and favouring viral replication and/or

prophage induction (Williamson et al., 2002). We did observe close correlations between VBR and nitrate and phosphate concentrations (both $P<0.001$, N=95), but the year-round high concentrations of both nitrate and phosphate in the study area indicated that trophic state is unlikely to be the key driving force controlling the seasonal variation of viral abundance in this study.

Looking at the viral and bacterial spatial distribution patterns, one would find that, although there are some significant similarities between the two, there is an important difference in that viral abundance often peaked near the river mouth area where bacterial abundance was quite low, especially in the winter. Meanwhile we recognized a reverse correlation between viral abundance and salinity especially in the winter ($r=-0.83$, $P<0.001$, N=23) (Figure 7). Such reverse correlations have been reported before (Jiang & Paul, 1994; Auguet et al., 2005) and can be interpreted as effects of ion strength on the steady status and replication ability and even absorption to particles (Maranger & Bird, 1995; Wommack & Colwell, 2000) but also could be apparent phenomena, and more likely to be a result of high virus-containing freshwater input (Jiang & Paul, 1994; Auguet et al., 2005). In the case of the present study, when the whole study area is divided into 'heavily diluted' and 'slightly diluted' regions based on a salinity value of 31 psu (Gong et al., 1996), the mean VBR values are 10.92 ± 12.33 in the low salinity area and 5.59 ± 2.54 in the high salinity area providing evidence that the high VBR values originated from freshwater input. However, this raises another question: since freshwater input is much greater in summer than in winter, why were the VBRs much higher in the winter than in the summer?

It turns out that the seasonal difference in the composition of the river discharge is responsible for the paradox. As the Yangtze river delta area is heavily inhabited and industrialized, a great amount of sewage is dumped into the river throughout the year. These polluted waters contain many more viral particles than the flooding water from upstream; this is shown in Figure 5 in which high viral abundance and VBR were observed in the Huangpu river which passes through Shanghai City. In summer, the majority of the Yangtze river discharge is upstream flood which carries a great amount of suspended particulates (mud and sand) which may adsorb virus particles, and take them out of the surface water. This relatively low virus-containing upstream water would dilute the viral abundance in the downstream water. In the winter, reduced upstream flooding and relatively greater downstream sewage led to a high viral abundance and VBR values in the estuary. The high winter ammonia concentration is solid evidence for the reduced dilution of the downstream water (sewage) by the upstream water in the winter (Figure 3E). There are likely to be two mechanisms contributing to the winter high VBRs. Sewage water contains more viruses as shown by our supplemental investigations at the Huangpu river (Station YH5) and Wusongkou sewage outlet (Station Y5), contributing directly to the high winter viral abundance. Meanwhile, pollutants including inorganic nitrogen, phosphate, hydrocarbon, oil and heavy metals into seawater would stimulate virus release from their hosts and induce the viral development by synergistic effects (Danovaro et al., 2003) and thus increase the VBR indirectly. In addition, freshwater bacterial cells would lyse more easily under ion strengthening in seawater and consequently release more viral particles into the water, whereas many viruses can move between different ecosystems and propagate (Sano et al., 2004). Therefore, high VBR values were observed around the river mouth area in all the seasons (Figure 4C).

In summary, the majority of the virus in the Yangtze river estuary was bacteriophages. Viral abundance peaked in both summer and winter, differing from the literature reported single peak in summer. The summer peak was basically host dependent while the winter one is multi-factor mediated, among which sewage input seemed to be the most important factor contributing to the high winter viral abundance and VBR value. Spatially the intrusion of warm water from the oceanic currents played a role in alternating the distribution patterns of temperature, salinity and trophic conditions and consequently the distribution patterns of virus and bacteria. Dynamics of virus in the Yangtze river estuarine area is thus characterized by distinct seasonal and spatial variations due to natural forcing and by pronounced alternation of the regular patterns due to anthropogenic impacts.

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