Viral Attachment to Biotic and Abiotic Surfaces in Seawater

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ABSTRACT Viruses influence microbial community structure and biogeochemical cycles in marine environments. Viral attachment to nonhost surfaces could influence host viral infection rates; however, the prevalence of such viral attachment is not investigated quantitatively. We used coastal seawater viral assemblages and, as models, marine vibriophage (SIO-2) and enterobacteriophages (T2 and T4) to investigate their attachment to probable nonhost marine bacteria. We also studied viral attachment to colloids and other abiotic surfaces in seawater. Centrifugation experiments with bacterium-virus mixtures showed substantial viral loss in the supernatant presumably due to the viral attachment to bacteria. This attachment (0.04 to 24 viruses μm^{-2} [bacterial surface area]) varied with bacterium-virus combinations. Surprisingly, filtering seawater on 0.2- μm Anodisc or polycarbonate filters retained ~12 to 84% of viruses presumably attached to $\geq 0.2\text{-}\mu\text{m}$ -sized particles and/or the filter surface. Enzymatic digestion followed by epifluorescence and atomic force microscopy suggested that 7 to 25% of the total viruses were attached via β -glycosidic linkages. Furthermore, a substantial proportion (7 to 48%) of viruses became attached to model abiotic surfaces (polycarbonate, polypropylene, and glass), and this has significance for laboratory protocols as well as studies of virus ecology in particle-rich marine environments. Substantial attachment of viruses to nonhost surfaces could influence virus-driven biogeochemical cycles and microbial community structure.

IMPORTANCE Viruses play important roles in altering microbial community structure and biogeochemical cycles in marine environments. Viral attachment to nonhost surfaces can influence host viral infection rates; however, the prevalence of viral attachment to nonhost surfaces and the ratio of attached viruses to total viruses are little known. We used coastal seawater viral assemblages and used marine vibriophage (SIO-2) and enterobacteriophages (T2 and T4) as models to investigate their attachment to abiotic and biotic surfaces in seawater. Viral attachment was observed on several surfaces, such as nonhost bacteria, polymers, filters, cover glasses, and tube surfaces. This study cautions against commonly used protocols that require viral incubation and seawater fractionation. More importantly, these results could influence virus-driven biogeochemical cycles and microbial community structure in the ocean.

KEYWORDS viral attachment, biotic and abiotic surfaces, marine ecology, nonhost bacteria, seawater

Viruses influence microbial community structure and biogeochemical cycles in marine environments (1). Diffusive transport enables viruses to perform their initial step of infection, i.e., attachment to the host (2). This process may also result in the attachment of viruses to nonhost surfaces, both biotic and abiotic, including nonhost bacteria, inorganic particles and clay minerals, and several classes of organic particles ranging in size from fine colloids to marine snow aggregates (3–7). Viral attachment to nonhost surfaces can influence infection rates both positively and negatively due to a loss of infectivity, protection from solar radiation, and alteration of collision frequency

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(6, 8–10). Murray and Jackson (2) predicted from the results of enteric viral studies conducted in marine environments that viral attachment to nonhost surfaces might be frequent in seawater. Metagenomic analysis in oxygen minimum zones off the coast of Chile detected viral genomes in 0.22- to 1.6- μm -size-fractionated seawater (11), which was consistent with viral attachment to nonhost surfaces (particles retained by $\geq 0.22\text{-}\mu\text{m}$ filtering). However, because quantitative data on viral attachment to nonhost surfaces are limited to marine aggregates (6, 12), transparent exopolymer particles (TEP) (13), clay minerals (8, 14), and black carbon (15), the prevalence and significance of viral attachments to nonhost biotic and abiotic surfaces remain largely unknown. This paucity of data hampers our understanding of the role of viruses in microbial community structures, food webs, and biogeochemical cycles in marine ecosystems.

We used viral assemblages collected from coastal seawater and viral isolates (e.g., myovirus enterobacteriophages T2 and T4 and marine siphovirus vibriophage SIO-2 [16]) in order to observe and quantify the viral attachment to nonhost surfaces and investigate the effects of viral species. In microcosm experiments, we tested viral attachment to nonhost bacterial cells, $\geq 0.2\text{-}\mu\text{m}$ -sized polymers and particles, as well as two types of abiotic surfaces, culture tubes and cover glass. We also made some preliminary observations on the composition of polymers responsible for virus attachment using enzymatic digestion experiments.

RESULTS

Viral attachment to natural bacterial assemblages. Incubating 0.6- μm -filtered seawater containing natural bacterial and viral assemblages did not show much viral attachment to bacteria (Fig. 1A and B). There were no large differences in sizes and shapes between attached and unattached viruses under epifluorescence microscopy (EFM). Also, we confirmed that these unattached viral assemblages could attach to the bacterial isolates used in this study (data not shown). In contrast, with added SIO-2 treatment, viral attachment was observed on the surfaces of most bacteria (Fig. 1C and D), suggesting that many marine bacterial surfaces had the potential for nonspecific viral attachment. To enumerate viral attachment to bacteria present in seawater assemblages (NBA), we incubated T2 (1 h) and SIO-2 (10 min) with NBA (0.6- μm -filterable fraction concentrated on a 0.22- μm filter), followed by centrifugation to sediment bacteria and any attached viruses. We found a significant decrease in viral abundances in the supernatant, presumably due to the viral attachment to bacteria. This apparent attachment increased by increasing bacterial concentration (Fig. 1E). Incubation of enterobacteriophage T2 (1.0×10^7 viruses ml^{-1}) with bacterial fractions (compared with a virus-only control) resulted in significant drawdowns of T2. The drawdown increased with increasing bacterial concentrations. At the bacterial concentration of 10^6 cells ml^{-1} , which is typical of coastal surface seawater, T2 drawdown was 10.8%, increasing to 61.7% at 10^7 cells ml^{-1} (Fig. 1E). Consistent with virus attachment by bacteria that we inferred from the decrease in the supernatant (Fig. 1E), our qualitative microscopic observations showed the attachment of T2 to bacterial cells (see Fig. S1A and B in the supplemental material).

A similar viral attachment was also found when we incubated marine phage SIO-2 (1.0×10^7 viruses ml^{-1}) with NBA (Fig. 1E and S1C and D). Phage drawdown was significant even at the lower bacterial concentration used in this experiment, and it was comparable (10.5% at 1.4×10^6 cells ml^{-1}) to the value for T2 at 1×10^6 cells ml^{-1} (Fig. 1E). Attachment of SIO-2 to bacteria was also qualitatively detectable by microscopy (Fig. S1C and D).

In the above-described viral enumeration experiment, the average surface area of bacteria in the natural assemblages was determined by EFM of 4',6-diamidino-2-phenylindole (DAPI)-stained cells to be $1.7 \pm 0.7 \mu\text{m}^2 \text{ cell}^{-1}$ ($n = 82$) and $2.0 \pm 1.1 \mu\text{m}^2 \text{ cell}^{-1}$ ($n = 238$) for incubation experiments with T2 and SIO-2, respectively. Virus attachment normalized to the bacterial surface area ($V_{\text{att}}B$) was 0.37 to 24 viruses μm^{-2} (mean $\pm$ standard deviation [SD] for T2, 2.6 ± 3.0 viruses μm^{-2} [$n = 9$]; and for SIO-2, 8.9 ± 9.9 viruses μm^{-2} [$n = 9$]). Virus attachment to the container tube

FIG 1 Viral attachments to probable nonhost bacteria and decrease in viral abundances by the addition of natural bacterial assemblages (NBA). (A to D) Epifluorescence microscopic images of NBA in 0.6-μm-filtered seawater in control (A and B) and with vibriophage (SIO-2) treatment (stained by SYBR green I) (C and D) with or without applying a homogenization filter (NIS Elements AR image software; Nikon) after 18 h of incubation (see Materials and Methods). SIO-2 and natural viral assemblages are shown as small green dots. Bacteria are shown as large green ellipsoid and rod shapes. Black arrows show representative unattached virus-like particles retained on a 0.2-μm-pore filter. White arrows show several virus attachments to a single bacterial cell. (E) Washed and concentrated viruses (T2 and SIO-2) (see Materials and Methods) were incubated with or without NBA (0.6-μm-filterable fraction concentrated on a 0.22-μm-pore filter) for either 1 h (T2) or 10 min (SIO-2) in artificial seawater. The percentages of either T2 or SIO-2 abundance in the supernatant after centrifugation with or without NBA are shown. Values were the mean $\pm$ SD ($n = 3$). Initial viral (T2 and SIO-2) abundances were 1.0×10^7 viruses mL⁻¹ and NBA abundances were 1.0×10^5 to 1.0×10^7 and 7.0×10^4 to 1.4×10^6 cells mL⁻¹ for T2 and SIO-2 incubations, respectively. Different letters indicate that the mean values are significantly ($P < 0.05$) different from each other in each viral addition experiment (1-way ANOVA followed by Tukey *post hoc* tests).

FIG 2 Viral attachments to nonhost bacterial isolates. T4 (open bars) and SIO-2 (closed bars) attachments per bacterial cell surface area (V_{attB}) after viral incubation with each bacterial isolate for 24 h. Values are the mean $\pm$ SD ($n = 3$), which were calculated by using the differences of viral abundances in the supernatant between the virus-only control and bacterial isolate addition treatments after centrifugation (see Equations 1 and 2 in Materials and Methods). The initial bacterial abundance was 0.6×10^6 cells ml^{-1} for each isolate and the initial viral abundances for T4 and SIO-2 were 5.5×10^6 and 5.7×10^6 viruses ml^{-1} , respectively. After incubation, the abundance of each bacterial isolate increased to ca. 10^7 cells ml^{-1} . The bacterial surface area was measured by microscopy. Different letters indicate that the mean values are significantly different ($P < 0.05$) from each other in each viral addition experiment (1-way ANOVA followed by Tukey *post hoc* tests).

was high and rapid (see below), but when normalized to attachment per surface area (V_{attT}), it was 2 to 4 orders of magnitude lower than V_{attB} .

Virus attachment to nonhost bacterial isolates. We studied the following four bacterial isolates that had previously been obtained from seawater off the Scripps Institution of Oceanography (SIO) Pier: *Alteromonas* spp. (TW7 [17] and AltSIO [18]), a *Bacteroidetes* sp. (BBFL7 [17]), and a *Vibrio* sp. (SWAT3 [19]). Enterobacteriophage T4 and marine vibriophage SIO-2 incubated individually with each bacterial isolate for 24 h, followed by centrifugation, showed V_{attB} values ranging broadly over 0.04 to 7.31 viruses μm^{-2} (Fig. 2). It is noteworthy that SIO-2 attachment per bacterial surface area was ~ 30 -fold greater than T4 attachment; coinoculation of SIO-2 and BBFL7 particularly stood out (7.31 viruses μm^{-2}). Further, the V_{attB} values for all virus-bacterium coinoculations in this study were 1 to 3 orders of magnitude higher than for virus-tube attachment (0.001 to 0.005 viruses μm^{-2} ; mean $\pm$ SD of V_{attT} , 0.002 ± 0.001 viruses μm^{-2} ; $n = 6$) (data not shown).

Viral attachment to polymers and particles in seawater. We observed that filtering seawater through 0.2- μm aluminum oxide (Anodisc) filters resulted in the retention of an unexpectedly large number of virus-like particles (VLP) (0.7×10^7 to 1.0×10^7 particles ml^{-1}) on the filter (Table 1, and see, e.g., Fig. 1B and D and S1B and D). This retention equaled 29 to 84% of the total VLP count on a 0.02- μm Anodisc filter (1.0×10^7 to 2.3×10^7 particles ml^{-1}). While we could not determine their size, the retained VLP did not appear to be larger than those captured on a 0.2- μm Anodisc

TABLE 1 VLP retention on 0.02- and 0.2- μm -pore Anodisc filters and the effect of Triton X-100 wash^a

Treatment	VLP abundance (mean $\pm$ SD) ($n = 3$) by sampling date (day/mo/yr)							
	03/01/18		09/01/18		11/01/18		12/02/18	
	Concn ($\times 10^7$ ml^{-1})	% total	Concn ($\times 10^7$ ml^{-1})	% total	Concn ($\times 10^7$ ml^{-1})	% total	Concn ($\times 10^7$ ml^{-1})	% total
0.02- μm filter (total VLP)	1.0 ± 0.2	100 ± 16	2.3 ± 0.4	100 ± 15	1.9 ± 0.4	100 ± 23	1.8 ± 0.1	100 ± 6
0.2- μm filter	0.8 ± 0.1	84 ± 8	0.7 ± 0.0	29 ± 2	0.8 ± 0.0	43 ± 2	1.0 ± 0.0	58 ± 1
0.2- μm filter, Triton X-100 ^b	—	—	—	—	—	—	0.3 ± 0.1	14 ± 3

^aWhole-seawater samples were filtered. —, no data.

^bFilters were washed thrice with 0.02- μm -filtered 0.1% Triton X-100.

TABLE 2 VLP retention on 0.2- and 0.02 μm -pore Anodisc filters and the effects of enzymatic treatments^a

Treatment	VLP abundance (mean $\pm$ SD) ($n = 3$) by sampling date (day/mo/yr) ^b							
	22/02/18				26/02/18			
	0.2 μm		0.02 μm		0.2 μm		0.02 μm	
	Concn ($\times 10^7 \text{ ml}^{-1}$)	% total	Concn ($\times 10^7 \text{ ml}^{-1}$)	% total	Concn ($\times 10^7 \text{ ml}^{-1}$)	% total	$\times 10^7 \text{ ml}^{-1}$	% total
MQ control	0.4 $\pm$ 0.1	31 $\pm$ 7	1.1 $\pm$ 0.1	100 $\pm$ 9	0.4 $\pm$ 0.1	30 $\pm$ 6	1.3 $\pm$ 0.1	100 $\pm$ 10
Proteinase K	0.4 $\pm$ 0.1	33 $\pm$ 5	1.4 $\pm$ 0.1	121 $\pm$ 9	—	—	—	—
β -Glucosidase	0.1 $\pm$ 0.0**	6 $\pm$ 3**	1.5 $\pm$ 0.1*	132 $\pm$ 5*	0.2 $\pm$ 0.0*	14 $\pm$ 1*	1.6 $\pm$ 0.0*	124 $\pm$ 3*

^aWhole-seawater samples were incubated with MQ or enzymes (activity units, $\geq 2 \text{ units mg}^{-1}$; final conc., 1 mg ml^{-1}) for 1 h at room temperature in the dark under static conditions before filtration. After sample filtration, filters were washed by 0.02- μm filtered and autoclaved ASW and 0.02- μm filtered 0.1% Triton X-100 thrice each.

^bA comparison of mean VLP abundances among control and enzymatic treatments were conducted using Student's *t* test with asterisks indicating $P < 0.05$ (*) and $P < 0.01$ (**). —, no data.

filter. We surmised that viral retention could have been due to their association with transparent particles, e.g., TEP (20) and Coomassie-stainable particles (CSP) (21). Washing the filter with a nonionic detergent such as 0.1% Triton X-100 might solubilize some of the transparent particles, thus releasing the associated VLP. It might also remove VLP attached to the filter surface. Indeed, these treatments, postfiltration, reduced the VLP count on 0.2- μm filters by 70% (Table 1; this treatment was done only on 12 February 2018).

In order to further explore the possibility that the retained VLP were attached to transparent organic matter, we considered that hydrolysis of proteinaceous particles (e.g., CSP) with proteinase K or polysaccharides (e.g., TEP) with β -glucosidase might render the retained VLP filterable through 0.2- μm filters. Proteinase K treatment of seawater samples prior to filtration on a 0.2- μm Anodisc filter did not change the fraction of total VLP trapped (i.e., the VLP count on a 0.2- μm Anodisc filter in Milli-Q [MQ] control) ($P > 0.05$; Table 2). However, β -glucosidase treatment strongly decreased VLP retention by 16 to 25% of the total VLP ($P < 0.05$). This equaled 53 to 81% of VLP abundances retained on 0.2- μm filters in the MQ control. We also note that in both enzymatic treatments, the VLP count on 0.02- μm Anodisc filters increased, with proteinase K at 21% ($P = 0.08$) and β -glucosidase at 24 to 32% ($P < 0.05$) of the total VLP.

It was desirable to further support our observation of VLP retention on a 0.2- μm polycarbonate filter by direct count of the retained VLP (in addition to the filtration loss described above). Direct enumeration of VLP retained on 0.2- μm polycarbonate filters (using SYBR green I) was not feasible due to high background fluorescence. In order to resolve this method limitation, we employed the filter-transfer-freeze (FTF) technique (22) that enabled us to transfer the filter-retained VLP onto a glass slide, followed by SYBR green I staining. We compared these counts to the total counts (i.e., with whole seawater on a 0.02- μm Anodisc filter) as well as to the VLP counts of seawater passing the 0.2- μm filter. We were thus able to confirm VLP retention on 0.2- μm polycarbonate filters (Fig. S2). The FTF-transferred VLP accounted for ~ 12 to 29% of the total VLP (Table 3). This is likely an underestimate since the transfer efficiency was $< 100\%$. In the 20 March 2019 sample, 27% of the total VLP in the seawater sample were “missing” (retained) in the filtrate; however, FTF accounted for 12% of the total count or transfer efficiency of $\sim 44\%$.

We also tried to observe the VLP associated with TEP in the above-mentioned FTF-treated sample by alcian blue and SYBR green I double staining (23), but little TEP were detectable by EFM (data not shown).

Atomic force microscopy observation of retained virus-sized particles. The significant retention of VLP by 0.2- μm Anodisc and polycarbonate filters prompted us to test whether these VLP were attached to transparent particles that might be detectable by atomic force microscopy (AFM). AFM observation revealed networks of abundant “string-like” materials with attached ~ 20 - to 200-nm particles on 0.2- μm

TABLE 3 Effect of seawater fractionation by a 0.2- μ m-pore polycarbonate filter on VLP abundances

Treatment	VLP abundance (mean $\pm$ SD) ($n = 3$) by sampling date (day/mo/yr) ^a					
	27/11/18		19/03/19		20/03/19 ^b	
	Concn ($\times 10^7$ ml ⁻¹)	% total	Concn ($\times 10^7$ ml ⁻¹)	% total	Concn ($\times 10^7$ ml ⁻¹)	% total
Whole seawater (total VLP)	3.0 $\pm$ 0.2	100 $\pm$ 16	2.2 $\pm$ 0.2	100 $\pm$ 7	3.3 $\pm$ 0.5 A	100 $\pm$ 14 A
Retained on 0.2- μ m-pore polycarbonate filter (after filter-transfer-freeze)	—	—	0.3 $\pm$ 0.1***	29 $\pm$ 6***	0.4 $\pm$ 0.1 B	12 $\pm$ 3 B
0.2- μ m filtrate	2.2 $\pm$ 0.1**	75 $\pm$ 7**	—	—	2.4 $\pm$ 0.2 C	73 $\pm$ 11 C

^aA comparison of mean VLP abundances among control and enzymatic treatments were conducted using Student's *t* test with asterisks indicating $P < 0.01$ (**) and $P < 0.001$ (***). —, no data.

^bDifferent letters indicate that the mean values are significantly ($P < 0.05$) different from each other (1-way ANOVA followed by Tukey *post hoc* tests).

polycarbonate filters (Fig. 3A and B). The abundance of virus-sized particles (VSP; diameter, 20 to 200 nm [24]) was 3.6×10^7 to 58×10^7 particles ml⁻¹. EFM of the same samples that we used for AFM showed a VLP abundance of 0.5×10^7 to 12×10^7 particles ml⁻¹, accounting for 13 to 21% of the VSP detected by AFM (Table 4). The AFM we used did not have an epifluorescence attachment; therefore, we could not distinguish between VLP and other VSP. We note that Triton X-100 treatment did not largely affect total VLP abundance and the structure of string-like materials (data not shown).

We considered that β -glucosidase treatment might reduce the retention of AFM-detectable VSP on 0.2- μ m polycarbonate filters (as was the case for EFM-detectable VLP). This treatment did indeed reduce the retained VSP abundance by $53\% \pm 7\%$ ($n = 3$) (Fig. 4A and B). The VSP abundance following β -glucosidase treatment ($27 \times 10^7 \pm 0.9 \times 10^7$ particles ml⁻¹, $n = 3$) was lower ($P < 0.01$) than in the MQ control ($58 \times 10^7 \pm 8.0 \times 10^7$ particles ml⁻¹, $n = 3$). Further, the VSP appeared to be associated with a network of string-like materials (Fig. 4A). As additional controls, we

FIG 3 A network of virus-sized particles (VSP; 20 to 200 nm), string-like materials, and a bacterium retained on 0.2- μ m polycarbonate filters. The sample was prepared by filtration of coastal seawater and formaldehyde fixation (final conc., 2%) with a 0.02- μ m-filtered and autoclaved artificial seawater wash, followed by 0.1% Triton X-100. (A and B) AFM images of a network of VSP and string-like materials (A) and VSP associated with string-like materials and a bacterium (shown in the dashed frame) (B) presented as height data. Color bars indicate the height difference (0 [black] to 20 [white] nm). Green and blue dots, VSP; red lines, string-like materials; large white ellipsoid, bacterium; black circles, 0.2- μ m filter pores.

TABLE 4 Abundances of VSP and VLP retained on 0.2 μm -pore polycarbonate and Anodisc filters enumerated by AFM and EFM^a

Category (enumeration method)	VSP and VLP abundance (mean $\pm$ SD) by sampling date (day/mo/yr)								
	15/02/18			16/02/18			23/02/18		
	Concn ($\times 10^7 \text{ ml}^{-1}$)	% total	<i>n</i>	Concn ($\times 10^7 \text{ ml}^{-1}$)	% total	<i>n</i>	Concn ($\times 10^7 \text{ ml}^{-1}$)	% total	<i>n</i>
VSP (AFM)	3.6 ± 1.1	100 ± 31	3	8.8 ± 3.1	100 ± 37	6	58 ± 8.0	100 ± 14	3
VLP (EFM)	0.5 ± 0.0	13 ± 4	3	1.2 ± 0.1	13 ± 5	6	12 ± 0.1	21 ± 3	3

^aAfter sample filtration, filters were washed with 0.02- μm FASW and 0.02- μm -filtered 0.1% Triton X-100 thrice each. Only VSP (20 to 200 nm) having a height of ≥ 5 nm were enumerated (see Materials and Methods).

counted VSP after (i) formaldehyde fixation or (ii) washing with 0.02- μm filtered autoclaved artificial seawater ($<0.02 \mu\text{m}$ FASW). These treatments did not significantly affect the abundance of VSP or the appearance of the fibrillar network detectable by AFM (data not shown).

Since the EFM counts were a small fraction (13 to 21%) of the AFM-based VSP counts, we sought to distinguish between VLP and other VSP using nanomechanical properties (e.g., adhesion). Using this AFM analysis, we could roughly classify most of the VSP into a high-adhesion (11 to 12 nN) category and a low-adhesion (9 to 10 nN) category. About 50% of the VSP belonged to the high-adhesion group and about 30% to the low-adhesion group (Fig. 5A to C). Further, the high-adhesion VSP group overlapped the peak of aggregates and string-like materials (at 11.5 nN) (Fig. 5C).

In the above-described experiment, the initial VLP abundance in seawater was $4.0 \times 10^7 \pm 0.4 \times 10^7$ particles ml^{-1} (EFM counts; $n = 3$), and the VSP abundance after the filtration and FTF was $3.6 \times 10^7 \pm 1.2 \times 10^7$ particles ml^{-1} (AFM counts; $n = 3$).

FIG 4 Effect of the enzymatic treatment on the retained virus-sized particles (VSP; 20 to 200 nm) and string-like materials on 0.2- μm -pore polycarbonate filters. The samples were prepared by filtration of coastal seawater after 1 h of incubation with the addition of 0.02- μm -filtered either MQ or β -glucosidase. These samples were formaldehyde fixed (final conc., 2%) and washed by 0.02- μm -filtered and autoclaved artificial seawater, followed by 0.1% Triton X-100. AFM images of VSP and string-like materials in MQ control (A) and β -glucosidase treatment (B) (activity units, ≥ 2 units mg^{-1} ; final conc., 1 mg ml^{-1}). Images are presented as height data, and color bars show the height difference (0 [black] to 33 [white] nm). The dashed frame indicates the representative remaining string-like materials. Green, blue, and red dots, VSP; red lines, string-like materials; large yellow-green ellipsoid, bacterium; black circles, 0.2- μm -pore filter pores.

FIG 5 Adhesion of virus-sized particles (VSP; 20 to 200 nm) and the distributions of VSP, aggregates, and string-like materials. Coastal seawater sample was retained on a 0.2-μm-pore polycarbonate filter and transferred to a glass slide by using the filter-transfer-freeze technique without fixation. AFM images of the same area presented as height data with a color bar (0 [black] to 10.5 [white] nm) (A) and adhesion data with a color bar (4 [yellow] to 15 [black] nN) (B). Arrows indicate the representative VSP, and the dashed frames indicate the aggregates and string-like materials. (C) Adhesion distributions of VSP and of aggregates and string-like materials. Data from 50 different VSP and the points of aggregates and string-like materials each were used (see Materials and Methods).

Assuming the range of VLP transfer efficiency from the filter to a cover glass of 12 to 29% (Table 3), the transferred VLP abundance would be 0.48×10^7 to 1.16×10^7 particles ml^{-1} , which accounted for 13 to 32% of the VSP. This value was roughly equal to the percentage of the lower adhesion peak of VSP (ca. 30%), which suggested that the lower adhesion peak of VSP was made by VLP.

Viral attachment by abiotic surfaces. Viral loss due to the container surface was large, and this required us to examine its significance for our experimental design as well as its possible importance in virus attachment to inorganic surfaces in the ocean. Using polypropylene tubes as incubation containers for viral assemblages and virus isolates, we measured virus loss from the water phase, presumably due to the attachment to the tube walls. The tube inner surface area-to-sample volume ratio (S/V), at 5.5 cm^{-1} , was constant for all samples. After 24 h, there was a large decrease in viral abundance in the seawater (Fig. 6A). The loss was greatest (43%) for the natural seawater assemblages. The mean viral loss for marine viruses (natural viral assemblages and SIO-2) was $36\% \pm 7.9\% \text{ day}^{-1}$ ($n = 6$), which was 1.7 times higher ($P < 0.01$) than that for T2 and T4 ($21\% \pm 4.7\% \text{ day}^{-1}$, $n = 6$). However, when normalized to the tube surface area, the attachment was 0.004 to 0.009 viruses μm^{-2} (which was 1 to 3 orders of magnitude less than for bacterial surfaces, as noted above).

FIG 6 Viral losses in polypropylene tubes. Viral loss (ratio) was calculated by using Equation 3 described in Materials and Methods. (A) Viral species (natural assemblages and isolates) effect on their losses. Values are the mean $\pm$ SD ($n = 3$). Different letters indicate that the mean values are significantly ($P < 0.05$) different from each other (1-way ANOVA followed by Tukey *post hoc* tests). Incubation time, 24 h; tube surface area-to-sample volume ratio (S/V), 5.5 cm^{-1} ; initial viral concentration, 1.1×10^7 viruses ml^{-1} . (B) Relationship between the viral loss rate and incubation time for T2 isolate. Each plot is the mean of 3 to 9 measurements, and errors are SDs. The line indicates the power regression obtained by nonlinear curve fitting: Viral loss rate = $6.78 (\pm 0.22) \times (\text{incubation time})^{-0.48 (\pm 0.02)}$ ($r^2 = 0.99$, $P < 0.001$, $n = 8$; error is the standard error). Incubation time, 1 to 120 h; S/V, 3.3 cm^{-1} ; initial viral concentration, 1.0×10^7

(Continued on next page)

T2 incubation in polypropylene tubes (S/V, 3.3 cm⁻¹) for 1 to 120 h showed that the viral loss rate decreased with increasing incubation time (Fig. 6B). The range of mean viral loss rates was 0.42% to 6.73% h⁻¹. An empirical equation that relates the viral loss rate to incubation time was obtained using a power regression model after averaging the data at each incubation time. A power rather than linear regression model was used because the *r*² value of the power regression exceeded that of the linear regression. The relationship between the viral loss rate (% per hour) and incubation time (hours) was described by the following equation: viral loss rate = 6.78 (± 0.22) × (incubation time)^{-0.48 (± 0.02)} (*r*² = 0.99, *P* < 0.001, *n* = 8; the error is the standard error). The *V*_{att}*T* was 0.002 to 0.015 viruses μm⁻².

Viral isolates (SIO-2 and T2) incubated in polypropylene tubes of different S/V ratios (1.7 to 6.3 cm⁻¹) for 24 h showed that viral loss increased with increasing S/V (Fig. 6C). The range of mean values of viral loss was 16 to 48% day⁻¹. An empirical equation that relates the viral loss to S/V was obtained using a power regression model after averaging the data at each S/V. A power rather than linear regression model was used because the *r*² value of the power regression exceeded that of the linear regression. The relationship between viral loss (percent per day) and the S/V (per centimeter) was described by the following equation: viral loss = 12 (± 2.3) × (S/V)^{0.74 (± 0.12)} (*r*² = 0.93, *P* < 0.001, *n* = 5; the error is the standard error). The *V*_{att}*T* was 0.011 to 0.013 viruses μm⁻².

Using a microscope cover glass, we were able to confirm that the loss of virus was due to the attachment to that abiotic surface. We incubated T2 suspension in <0.02-μm FASW on a cover glass (S/V, 4.2 cm⁻¹) for 1 min to 60 min and counted viruses by EFM (Fig. S3A to C). The mean abundances of 20 images were 510, 1,200, and 2,300 viruses mm⁻² for 1, 30, and 60 min of incubation, respectively. In a control experiment, we filtered T2 through a 0.02-μm syringe filter and then incubated the filtrate on cover glass for 60 min, followed by microscopy. The cover glass did not show detectable viruses (Fig. S3D). Similar to the attachment on the polypropylene tube surface, viral attachment by the cover glass was 0.001 to 0.002 viruses μm⁻².

Virus attachment and detachment. The effect of different tube materials on viral attachments was investigated by incubating SIO-2 in both polypropylene and glass tubes (S/V, 7.0 cm⁻¹) for 24 h. After centrifugation, viral abundances in the suspension phase in both tube types decreased (*P* < 0.05) to 89.3% ± 2.5% and 91.0% ± 2.5% (*n* = 3) for the polypropylene and glass tubes, respectively, which were not significantly different (*P* > 0.05) (Fig. 7). The *V*_{att}*T* was 0.007 to 0.008 viruses μm⁻².

To confirm viral attachment to tube surfaces during the 24 h of incubation, we tested whether the “missing” virus could be recovered. The liquid phase was removed from the tubes, and virus detachment was measured after 24 h of incubation of SIO-2 in polypropylene tubes for several detergent treatments or 30-kDa filtered autoclaved artificial seawater (<30-kDa FASW) as a control (S/V, 7.0 cm⁻¹) (Fig. 7). The least release was observed with the <30-kDa FASW addition, while 0.1% Triton X-100 released most of the attached viruses.

DISCUSSION

Viral attachment to nonhost bacteria and potential controlling factors. Viral incubation with nonhost bacteria showed that the tested viruses had high affinity for the bacterial cell surface (Fig. 1 and 2). Although the possibility of viral attachment by nonhost surfaces in seawater had previously been predicted (2, 11), we are not aware of any study quantitating the attachment of marine viruses to nonhost marine bacteria. Our data also indicated that the affinity and capacity for binding of viruses to nonhost

FIG 6 Legend (Continued)

viruses ml⁻¹. (C) Relationship between the viral loss and S/V for the SIO-2 and T2 isolates. Each plot is the mean of 3 to 7 measurements, and errors are SDs. The line indicates the power regression obtained by nonlinear curve fitting: viral loss = 12 (± 2.3) × (S/V)^{0.74 (± 0.12)} (*r*² = 0.93, *P* < 0.001, *n* = 5). Incubation time, 24 h; S/V, 1.7 to 6.3 cm⁻¹; initial viral concentration, 1.4 × 10⁷ viruses ml⁻¹.

FIG 7 The percentages of SIO-2 abundances before (open bar) and after 24 h of incubation in a polypropylene tube (hatched bar) and a glass tube (closed bar), and in four detachment treatments (10 min) followed by incubation in a polypropylene tube (hatched and shaded stacked bars) (see Materials and Methods). The upper and lower horizontal dashed lines indicate the percentages of SIO-2 abundance before incubation (100%) and after incubation (89.3%) in a polypropylene tube, respectively. Values are the mean $\pm$ SD ($n = 3$). Different letters indicate that the mean values are significantly ($P < 0.05$) different from each other (1-way ANOVA followed by Tukey *post hoc* tests). S/V, 7.0 cm^{-1} ; initial viral concentration, 5.4×10^7 viruses ml^{-1} ; FASW, $<30\text{-kDa}$ filtered and autoclaved artificial seawater.

bacteria (0.04 to 24 viruses μm^{-2}) were ~ 1 to 4 orders of magnitude higher than for the abiotic surfaces (polypropylene and glass tubes and a cover glass; 0.001 to 0.015 viruses μm^{-2}). Bacterium-sized particles, including organic and inorganic particles (10^8 to 10^9 particles ml^{-1} [4, 5, 25]), are 2 to 4 orders of magnitude more abundant in coastal surface seawater than are bacteria ($\sim 10^5$ to 10^6 cells ml^{-1} [26]). Viruses may thus compete with colloids in seawater, and their attachment may require high affinity and capacity of the bacterial attachment surface. We noted that our measurements of viral attachment to nonhost bacterial isolates by centrifugation experiments may be underestimates because of the production of a variety of bacteria produced DNA- and/or RNA-containing vesicles under some conditions (27).

The capacity of viruses to attach to the bacterial surface varied widely (ca. 600-fold; 0.04 to 24 viruses μm^{-2} ; Fig. 1 and 2), depending on the combination of bacterial and viral species. For instance, SIO-2 had 30 times greater capacity than did T4 (Fig. 2). (Whether marine viruses have a higher affinity and capacity for binding to marine bacteria than enterobacteriophages would be interesting to test.) Below, we discuss potential factors controlling the affinity and capacity of nonhost bacteria for virus attachment.

(i) Size, surface composition, and structure. Viral and bacterial sizes and surface structure and composition likely affect their binding affinity. The sizes of viruses and bacteria, and hence the surface area for interaction, are quite varied among species in natural marine assemblages. Bacterial cell size and surface area may also vary with the growth phase and the environmental nutrient conditions (28, 29). This could alter virus-bacterium collision frequency and attachment (30). Also, both viral and bacterial surface compositions (e.g., proteins, lipids, and polysaccharides) and structures (e.g., for viruses, capsid, tail, and envelope, and for bacteria, flagella, pili, fimbriae, and curli fibers) could alter the binding affinity and capacity (31, 32).

In our study, both the viruses (T2, T4, and SIO-2) and bacteria (TW7, Alt-SIO, BBFL7, and SWAT3) probably offered a range of different surface structures and surface areas involved in phage-bacterium interactions. For example, the viruses had different tail lengths (ca. 130 nm for T2 and T4 and ca. 210 nm for SIO-2) as well as capsid size (ca. 120 nm for T2 and T4 and ca. 90 nm for SIO-2), structure, and composition (e.g., with or without decoration protein) (33–35). The bacterial species that we used had various

mean diameters ranging from 0.8 to 1.7 μm (data not shown). These and other factors likely influenced the variability in viral affinity and capacity of nonhost bacteria.

(ii) Potential role of exopolymeric substances. Many bacteria produce extracellular polymeric substances (EPS) (e.g., TEP [36] and CSP [21]; reviewed by Decho and Gutierrez [37]). Bacteria are often covered with EPS-comprising capsules and slime layers. The chemical nature and the extent (area and volume) and permeability of the EPS to viruses may alter the affinity of viruses for the bacterial surface. In the ocean, both biotic and abiotic particles (e.g., viruses, bacteria, and clay mineral) are usually negatively charged (38). The EPS might increase viral attachments to bacterial surfaces (39). The variability in the affinities among bacterial isolates for viruses might be due in part to the differences in the nature and amounts of EPS displayed on the bacterial surface (37). The high surface area and perhaps also the volume of EPS might then explain why the bacterial surface capacity and affinity (calculated by assuming a smooth surface) are much higher than for abiotic tube surfaces. This is consistent with our AFM observations of VSP attached to string-like materials emanating from a bacterium in a seawater sample (Fig. 3B) (see below).

Other considerations are as follows: (i) bacterial motility and virus diffusion would enhance their collision frequency (40, 41). Bacterial isolates used in this study (except BBFL7) were motile. (ii) The nonhost bacteria might not only attach to but also degrade the viruses using exoenzymes (9, 42, 43). (iii) The high affinity of viruses for nonhost bacteria is consistent with previous reports that organic polymers, particles, and aggregates may be significant adsorbents in the sea (6, 9). Our observation of attachment of viruses to polymers suggests a possible mechanism for virus enrichment of marine aggregates, wherein attachment of viruses to polymer is followed by polymer flocculation to form large aggregates (marine snow) that are highly enriched with viruses (44). Marine snow is also enriched in bacteria (1 to 3 orders of magnitude higher on marine snow than in equal volumes of surrounding seawater [3]), possibly by a similar mechanism as viruses. This may contribute to enhancing the efficiency of viral infection to host cells. Thus, polymer attachment could bring together various viruses (and bacteria) to high concentrations and thus may have major implications for virus-bacterium interactions in marine aggregates.

VLP retention by 0.2- μm filter. Surprisingly, ~12 to 84% of the VLP in whole seawater were retained on the 0.2- μm filters (Tables 1 and 3), possibly because these VLP (i) became attached directly to the filter upon impact and/or (ii) were bound to larger particles. As 51 to 92% of marine viruses were nontailed and their capsid sizes ranged from 0.02 to 0.2 μm (24), the effect of large viruses (e.g., >0.2 μm) on the whole-virus retention on the filters might be minor in this study. Triton X-100 treatment reduced the retention to 14 to 31% of the total VLP (Tables 1 and 2). We think that these remaining VLP were attached to particles. β -Glucosidase treatment of seawater, followed by filtration on a 0.2- μm filter, resulted in a large decrease (53 to 81%) in VLP retention (Table 2). This suggests that 7 to 25% of the total VLP may have been attached to polysaccharides containing β -glycosidic linkages (e.g., TEP [36]) in our experiment. We could not directly detect TEP binding of VLP by EFM, probably because TEP was too diffuse, and hence not stained strongly enough, to detect a VLP-TEP association. Also, an EFM and AFM comparison showed that 13 to 32% of VSP trapped on a 0.2- μm filter could be VLP (Table 4 and adhesion experiment), and 53% of them were removed from the filter by β -glucosidase treatment (Fig. 4A and B). Although β -glucosidase treatment appeared to release most VSP from the polymer "strings" (AFM images, Fig. 4B), some still remained. The remaining VSP might be attached to polymers and particles with various compositions that resisted β -glucosidase treatments.

A previous study suggested that bacterial protease activity contributed to virus decay in seawater (9, 42). In the short-term (1-h) incubations with proteinase K, we did not observe net virus decay; in fact, there was a measurable increase in VLP count (Table 2). Interestingly, the β -glucosidase treatment (described above) also showed a comparable increase in the total VLP count. One interpretation is that protein enrich-

ment (in the form of the enzymes) caused a burst of phage production (prophage induction [45, 46]), resulting in a net increase in VLP count surpassing the expected phage decay, which might also enhance the virus-bacterium attachment. Another possibility is that a pool of VLP attached to bacteria (and not resolved by EFM) were released by hydrolytic action of the enzymes and thus became detectable. The effect of the two enzymes on 0.2- μ m-filter-retained VLP was dramatically different; proteinase K did not significantly affect the VLP count on a 0.2- μ m filter, but β -glucosidase greatly reduced the retained VLP count. Cumulatively, our results suggest that VLP attachment to ≥ 0.2 - μ m-sized particles was not mediated by proteinase-sensitive polymers (e.g., CSP), but polysaccharides containing β -glycosidic linkages (e.g., TEP) may have played a major role. This interpretation is consistent with a previous study showing that significant fractions of the viral community in coastal seawater are associated with TEP (47).

Our results also serve as a cautionary note that seawater fractionation using polycarbonate filters commonly used in experimental protocols in marine environmental studies might trap abundant <0.2 - μ m-sized particles, including a substantial fraction of viruses and other VSP. Our AFM-based individual particle adhesion measurements of VSP retained on 0.2- μ m polycarbonate filters suggested that ca. 30% of the VSP might be VLP and ca. 50% of the VSP might be small organic particles and colloids (e.g., precursors of larger aggregates and string-like materials) (5, 44). While not further investigated here, an additional explanation for VSP attachment to a 0.2- μ m filter is that seawater dissolved organic matter (DOM) conditioned the filter surface that resulted in the adsorption of VSP. Again, in the case of seawater fractionation and VSP counting by using such filters, attention should be paid to the effects of these viral attachments on the results.

Viral attachments to abiotic surfaces and possible control mechanisms. There was a remarkably high virus attachment at the polypropylene tube surface (Fig. 6A). The attachment was the highest (43%) for natural seawater assemblages. The attachment of viral isolates was somewhat lower (17 to 30%). Our measurements in Fig. 6A were done after 24 h of incubation. Time course analysis showed that the rate of virus attachment to the tube surface was highest during the first hour and then declined to reach a low but still measurable rate at 24 h (Fig. 6B). Perhaps not surprisingly, the virus attachment was positively correlated with the S/V of the tube (Fig. 6C). It is also noteworthy that the attachment varied with the virus species, so the attachment cannot be treated as a single correction, e.g., in experimental manipulations of viral assemblages. These results raise important questions on the potential for virus attachment by abiotic surfaces in the ocean and its influence on virus interactions with bacterial surfaces. Further, such high and rapid attachment is also relevant to the design (e.g., incubation time and particle concentration) and interpretation of laboratory experiments on viruses.

The difference in tube compositions (polypropylene versus glass) did not affect the attachment significantly (Fig. 7). However, the combination of viral species and abiotic surface compositions and structures might affect the affinities. Previous studies indicated that some abiotic particles (e.g., clay minerals, Saharan dust, and black carbon) played important roles as viral absorbents in seawater (6, 7, 14, 15). Further, polymers could mediate viral attachment to abiotic surfaces (39, 47).

Although the maximum area occupied by viruses (i.e., viral area $\times$ abundance) was >4 orders of magnitude smaller than the tube surface area, the relationship between virus (SIO-2 and T2) binding and tube S/V was not linear (Fig. 6C). The T2 loss rate was high during 1 to 10 h and decreased thereafter (Fig. 6B). An explanation is that some polymers in seawater (see above) attach to the viruses and are subsequently adsorbed to the tube surface along with the viral cargo. The time-dependent decline of the hypothesized dissolved polymer concentration would then explain the observed decline in virus loss rate from seawater. As seawaters contain abundant polymers (36) and abiotic particles (6, 7), polymer-mediated viral attachment to abiotic particles might be

common and affect the marine ecology and biogeochemical cycles. Viral attachment is also regulated by salinity, water temperature, pH, and composition and nature of the adsorbent (7, 48, 49).

Implications for marine ecology. Our data on viral attachments to biotic and abiotic surfaces suggested that the abundant biotic and abiotic nanometer- to micrometer-size particles in seawater are potential viral adsorbents and consequently affect virus-mediated processes in marine ecosystems. Extrapolating from our findings, such potential adsorbents might include polymers, colloidal particles, clay minerals, microplastics, microbes, and cell debris. Nonhost bacterial surfaces had ~ 1 to 4 orders of magnitude higher viral affinity than did polypropylene and glass surfaces, which suggested that biotic particles such as living organisms (e.g., prokaryotes, flagellates, and algae) and cell debris provide large viral attachment sites. These biotic particles might act as adsorbents or as vectors of viral infections to specific hosts in seawater (6).

In the nearshore seawater samples, we observed that 7 to 25% of the VLP were apparently attached to $\geq 0.2\text{-}\mu\text{m}$ -sized particles containing β -glycosidic linkages such as TEP. Such an association may affect virus fate and infectivity. While the attachment mechanism was not investigated, in the simplest scenario, the diffusive encounter of viruses with TEP and polymer networks would result in virus attachment within the polymer matrix. Intriguingly, polymer adsorption of viruses is likely intensive in the microenvironment of a lysing cell presumably containing polymers, including polysaccharides, at very high microspatial concentration. Viral attachment and gel association may also occur during spontaneous polymer gel assembly in seawater (44), as well as viral attachment to polysaccharides at the surfaces of various marine organisms (e.g., phytoplankton [50], bacteria [37], corals [51], and seaweed [52]). Polysaccharide excretion by organisms is thought to aid in the protection against viral infections (52).

To understand the influence of viruses attached to biotic and abiotic particles on marine ecology, it is necessary to investigate the extent to which these attached viruses are infective to their hosts. While the infectivity of attached viruses to particles in the sea is still controversial (6, 7, 12, 14), some viruses may enhance their infection ratio to their host cells by attachment to chemically specific nonhost surfaces (10). Further studies about the effects of viral species and attachment surfaces on their attachment are also needed in order to understand the influence on marine ecology and community composition. Nevertheless, our findings contribute to the emerging thinking that viral attachments to varied nanometer- and micrometer-sized polymers and particles influence marine virus ecology. These interactions could influence virus-driven biogeochemical cycles and microbial community structure.

MATERIALS AND METHODS

Viral isolates and natural viral assemblages. Viral isolates (T2 and T4) and their host bacterium (*Escherichia coli*) were purchased from the Carolina Biological Supply Company (NC, USA). SIO-2 was previously isolated from coastal Pacific surface waters at the pier of the Scripps Institution of Oceanography (SIO Pier), La Jolla, CA (16). SIO-2 infects one of the hosts used in this study, the marine bacterium *Vibrio* sp. strain SWAT3 (16, 19). To obtain T2, T4, and SIO-2, we followed the supplier's protocol for T2 and T4 and the protocol in a study by Baudoux et al. (16) for SIO-2. Briefly, freshly produced viruses were obtained by infecting host bacteria and filtering through a $0.22\text{-}\mu\text{m}$ -pore syringe filter (Merck Millipore, Ireland) to remove cell debris. This viral suspension of SIO-2 was used for the test of the potential viral attachments to NBA (see below). For the enumeration of viral attachments to bacteria, the virus suspension was concentrated using a centrifugal cartridge filter (Amicon Ultra-15 centrifugal filter, membrane nominal molecular weight limit [NMWL], 30 kDa; Merck Millipore) (centrifugation at $5,000 \times g$, 18°C , 20 min). Then, virus concentrates (ca. $140\text{ }\mu\text{l}$) were washed in a new centrifugal cartridge filter filled with 12 ml of $<30\text{-kDa}$ FASW and centrifuged ($5,000 \times g$, 18°C , 20 min). This wash treatment was repeated twice, yielding concentrated (ca. 10^9 viruses ml^{-1}) and washed viruses. The washed and concentrated viral suspension was stored at 4°C for up to 2 months, and it was filtered through a $0.22\text{-}\mu\text{m}$ syringe filter before use (to minimize the effects of resistant bacteria, aggregated viruses, and viruses attached to polymers and particles on the following experiments).

Natural viral assemblages were obtained by filtering coastal seawater collected off the SIO Pier through a $0.2\text{-}\mu\text{m}$ polycarbonate filter (47-mm diameter; Merck Millipore, Ireland) with a vacuum of $<150\text{ mm Hg}$. The filtrate containing viral assemblages was used for viral attachment experiments within 2 h after sampling.

Nonhost bacterial isolates and NBA. The overnight-cultured bacterial isolates (TW7, AltSIO, BBFL7, and SWAT3) in Marine broth 2216 medium (Becton, Dickinson and Company, MD, USA) at RT in the dark

were centrifuged ($5,000 \times g$, 18°C , 10 min), and the supernatant was discarded. The pellets were suspended in 10 ml of $<30\text{-kDa}$ FASW and centrifuged again to wash the bacteria. The pellets were then suspended in 1 ml of $<30\text{-kDa}$ FASW. The isolates were used for the following incubation experiments within 1 h.

To test the potential viral attachments to NBA, NBA were obtained by passing seawater collected at the SIO Pier through a $0.6\text{-}\mu\text{m}$ polycarbonate filter (Merck Millipore) (e.g., NBA in $0.6\text{-}\mu\text{m}$ -filtered seawater). For enumeration of the viral attachments to NBA, to reduce the influence of natural viral assemblages, NBA were obtained by filtering seawater collected at the SIO Pier as in a study by Yamada et al. (23). Briefly, seawater was filtered through a $0.6\text{-}\mu\text{m}$ polycarbonate filter, and 100 ml of the filtrate was gently filtrated onto a $0.22\text{-}\mu\text{m}$ syringe filter (25-mm diameter; Merck Millipore) with a $<150\text{-mm}$ Hg pressure differential. We assumed that most of bacteria were collected on the $0.22\text{-}\mu\text{m}$ filter. To remove natural viruses, the bacteria collected onto the filter were rinsed twice with 50 ml of $<0.02\text{-}\mu\text{m}$ FASW. Finally, 5 ml of $<0.02\text{-}\mu\text{m}$ FASW was used to flush back the syringe filter, and the containing NBA (ca. 10^7 cells ml^{-1}) were collected inside the syringe (e.g., NBA in the $0.6\text{-}\mu\text{m}$ -filterable fraction on a $0.22\text{-}\mu\text{m}$ filter). Note that the sample of NBA still contained some portion of viruses (less than 8.7×10^5 viruses ml^{-1}) and also might contain polymers and particles ($\geq 0.22\text{-}\mu\text{m}$) that could attach to viruses. The NBA were used for viral attachment experiments within 2 h of sampling.

Observation and enumeration of viruses and bacteria by epifluorescence microscopy. Viruses and bacteria were counted by EFM (53). Briefly, samples were fixed in the filtration funnel with formaldehyde (2% final concentration [conc.]) and immediately filtered to minimize the viral attachments to other particles and/or each other (54). The 0.2- or $0.02\text{-}\mu\text{m}$ (depending on experiments) Anodisc filters (Whatman) were used for viral enumeration which were stained with SYBR green I (Molecular Probes; final conc., $50\times$ in antifade mounting medium [Vectashield; Vector Laboratories, CA, USA]) for 10 min in the dark and mounted on a glass slide. Slides were observed and analyzed using a fluorescence microscope (Eclipse TE2000-U and Ti; Nikon, Tokyo, Japan). Digital images were captured using a charge-coupled-device camera (CoolSNAP_{HQ}; Roper Scientific, NJ, USA) and processed using the NIS Elements AR image software (Nikon). To enumerate viruses, virus-like particles (VLP), and bacteria, 20 areas for each sample were processed by image analysis or manually. For measurement of the bacterial surface area, bacteria were stained by DAPI (in Vectashield, H-1200; Vector Laboratories) instead of SYBR green I and observed by EFM. Then, the bacterial surface area was calculated assuming the cell to be a sphere after estimating the cell diameter by using the NIS Elements AR image software (Nikon) (55).

Potential viral attachments to NBA. To investigate whether virus attached to probable nonhost bacteria in seawater, NBA in $0.6\text{-}\mu\text{m}$ -filtered seawater (1.0×10^6 cells ml^{-1}) with or without the addition of SIO-2 (1.0×10^8 viruses ml^{-1}) was incubated for 18 h at room temperature (RT) in the dark, under static conditions. After the incubation, the sample was filtered through a $0.2\text{-}\mu\text{m}$ Anodisc filter (Whatman) and observed by EFM.

Enumeration of viral attachments to NBA and bacterial isolates. A $500\text{-}\mu\text{l}$ (for the attachments to natural assemblages) or 1-ml (for the attachments to isolates) aliquot of the viral (T2, T4, or SIO-2) suspension (0.6×10^7 to 1.0×10^7 viruses ml^{-1}) was incubated separately with either NBA or bacterial isolates (7.0×10^4 to 1.0×10^7 cells ml^{-1}) in 1.5-ml polypropylene tubes (bacterial addition treatment). Also, either T2, T4, or SIO-2 without bacteria was incubated as a control (virus-only control). For viral attachment to natural assemblages, T2 incubation was for 1 h and SIO-2 for 10 min (to minimize the possible SIO-2 infection to potential host bacteria in the natural assemblage). For viral attachment to nonhost isolates, T4 and SIO-2 incubation were for 24 h. Incubations were at RT in the dark under static conditions. After the incubation, samples were centrifuged ($4,000 \times g$, 18°C , 5 min) to settle the bacteria. We assumed that the attached viruses on bacterial cell surfaces would also settle to the bottom of the tube. Then, $300\text{-}\mu\text{l}$ of the supernatant was filtered through a $0.02\text{-}\mu\text{m}$ Anodisc filter (Whatman) for viral enumeration by EFM. To confirm the viral attachment to bacteria, samples before centrifugation were filtered by $0.2\text{-}\mu\text{m}$ Anodisc filters, and the filters were observed by EFM. The abundance of viruses attached to the tube (V_{attT}) was determined by the Equation 1:

$$V_{\text{attT}} \text{ (viruses per square micrometer)} \\ = \text{difference of viral abundance in a sample before and after incubation} \\ \left(D_{VA} \right) \text{ in virus-only control (viruses per milliliter) / tube surface} \\ \text{area-to-sample volume ratio (square micrometers per milliliter)} \quad (1)$$

Bacterial surface area (in square micrometers per cell) was determined by microscopy and multiplied by the bacterial abundance (cells per milliliter) to determine the total bacterial surface area per milliliter of sample (in square micrometers per milliliter). The value was used to calculate the abundance of viruses attached to the bacterial cell surface per unit area (V_{attB}) by Equation 2:

$$V_{\text{attB}} \text{ (viruses per square micrometer)} = \left(D_{VA} \text{ in bacterial addition treatment [viruses per milliliter]} \right. \\ \left. - D_{VA} \text{ in viral only control [viruses per milliliter]} \right) / \text{total bacterial} \\ \text{surface area (square micrometers per milliliter)} \quad (2)$$

VLP retention on 0.2- or $0.02\text{-}\mu\text{m}$ Anodisc filters. Seawater samples collected at the SIO Pier, fixed by 2% (final conc.) formaldehyde in the filtration funnel, were immediately filtered through either a 0.2- or $0.02\text{-}\mu\text{m}$ Anodisc filter (Whatman). Each filter was then rinsed 3 times with $300\text{-}\mu\text{l}$ of $<0.02\text{-}\mu\text{m}$ FASW. For only the experiment conducted on 12 February 2018, after a $<0.02\text{-}\mu\text{m}$ FASW wash, filters were subsequently washed 3 times with $300\text{-}\mu\text{l}$ of 0.1% Triton X-100 (dissolved in MQ [Millipore]) and filtered with a $0.02\text{-}\mu\text{m}$ syringe filter [Anotop, Whatman]; $<0.02\text{-}\mu\text{m}$ 0.1% Triton X-100). VLP abundances were enumerated by EFM.

Seawater fractionation by 0.2- μ m polycarbonate filter. One-milliliter aliquots of seawater were filtered through 0.2- μ m polycarbonate filters (Whatman) emplaced in an autoclaved filter holder (Swinnex; Merck Millipore) and using a sterile syringe. For TEP observation, the filters were stained by 1 ml of 0.2% alcian blue (8GX; Sigma-Aldrich) in 0.6% acetic acid and washed twice with 1 ml of <0.02- μ m filtered and autoclaved MQ (20). VLP retained on the filter were transferred to a glass slide and subjected to a FTF procedure as in a study by Hewes and Holm-Hansen (22). After transfer, 2 μ l SYBR green I (final conc., 50 $\times$) in antifade solution (Vectashield) with 2% (final conc.) formaldehyde was added to the slide and covered with a cover glass. Controls excluded the seawater filtration step. Also, whole seawater and the 0.2- μ m filtrate, fixed with 2% (final conc.) formaldehyde in the filtration funnel, were separately and immediately filtered through a 0.02- μ m Anodisc filter. VLP abundance was enumerated by EFM.

Effect of enzymatic digestion on VLP retention on 0.2- μ m Anodisc filter. Seawater samples from the SIO Pier were incubated with 1 mg ml⁻¹ (final conc.) proteinase K (from *Tritirachium album*; catalog no. P2308; Sigma-Aldrich) or β -glucosidase (from almonds; catalog no. G0395; Sigma-Aldrich) for 1 h at RT in the dark under static conditions. The controls omitted the enzyme. Seawater aliquots (300 μ l) fixed with 2% (final conc.) formaldehyde in the filtration funnel were immediately passed through either a 0.2- or 0.02- μ m Anodisc filter and washed thrice with 300 μ l of <0.02- μ m FASW and 300 μ l of <0.02- μ m 0.1% Triton X-100. The whole process was conducted within 3 h of sampling. Finally, VLP abundance was enumerated by EFM.

Sample preparation for AFM. Seawater aliquots (1 to 5 ml) collected off the SIO Pier, fixed with 2% (final conc.) formaldehyde in the filtration funnel, were immediately filtered onto 0.2- μ m polycarbonate filters (25 mm diameter, GTTP; Merck Millipore), washed thrice with 300 μ l of <0.02- μ m FASW and 300 μ l of <0.02- μ m 0.1% Triton X-100, transferred to the glass slide, and secured to the slide with tape. In order to test that β -glucosidase treatment of seawater would release TEP-associated VSP, the seawater samples were incubated with β -glucosidase (1 mg ml⁻¹; Sigma-Aldrich) for 1 h. Controls received <0.02- μ m filtered and autoclaved MQ instead of the enzyme (MQ control). These samples were filtered, washed, and transferred to the glass slide in the same manner as described above. Samples were observed by AFM within 1 month.

In order to measure adhesive properties, retained VSP, aggregates, and string-like materials on a 0.2- μ m polycarbonate filter without fixation were FTF transferred (as described above) from the filter to a cover glass (which enhanced the differences between measurement objects [e.g., VSP] and the background [i.e., glass] and facilitated the adhesion measurement). Then, the cover glass was secured to a glass slide with tape. All observations were completed within 4 h after sampling in order to minimize the time variation of the sample.

AFM observation and analysis. AFM images were acquired in dynamic mode (i.e., oscillating the AFM tip to reduce friction with the samples), and the scan rate was 1.69 Hz by using an atomic force microscope (Dimension Icon; Bruker, Santa Barbara, CA). The image resolution was 512 by 512 pixels. We used a silicon nitride cantilever (ScanAsyst-Fluid for the VSP, aggregates, string-like materials, and bacterial observation and ScanAsyst-Air for the adhesion measurement; Bruker) with a spring constant of 0.7 N m⁻¹. The ranges of peak force amplitude and peak force frequency were set as 150 to 300 nm and 2 to 8 kHz, respectively. AFM images (10- by 10- μ m size) were analyzed by using the software NanoScope Analysis (ver.1.8; Bruker). For enumeration of the VSP on the filter, particles $\geq$ 5 nm in height were counted in 10 discrete fields (1- by 1- μ m size) for each image because the background filter roughness of 300 nm length was 2.2 ± 0.8 nm (mean $\pm$ SD, $n = 90$). The number of images taken was 3 to 6 for each sample. VLP abundances for the same sample were enumerated by EFM, as described above, after filtration by a 0.2- μ m Anodisc filter with both 300 μ l of <0.02- μ m FASW and 300 μ l of <0.02- μ m 0.1% Triton X-100 washes (thrice each) to compare the abundances between VSP (counted by AFM) and VLP (counted by EFM).

For the adhesion of individual VSP and of aggregates and string-like materials, a constant area (60- by 60-nm size) of the center of individual VSP (diameter, $\geq$ 80 to 200 nm) and the random point of aggregates and string-like materials were measured. Then mean adhesion values were calculated for the 60- by 60-nm area. Also, the adhesion distributions were calculated by using the data from 50 different VSP and the points of aggregates and string-like materials each.

Viral attachments to polypropylene tube surfaces. First, to compare the attachment of different viral species to abiotic surfaces such as polypropylene, 1 ml of each viral suspension (natural assemblages, SIO-2, T2, and T4) in <30-kDa FASW at 1.1×10^7 viruses ml⁻¹ was incubated in 1.5-ml polypropylene tube at RT in the dark under static conditions for 24 h. Second, the effect of incubation time on viral loss rate (due to the surface attachment) was measured by T2 incubation for 1 to 120 h. An 11-ml T2 suspension in <30-kDa FASW (initial viral concentration, 1.0×10^7 viruses ml⁻¹) was incubated in a 15-ml polypropylene tube (Corning) for 1, 5, 10, 24, 48, 72, 96, and 120 h at RT in the dark under static conditions. Third, to determine the effects of collision frequency on viral attachments, the effect of the inner surface area of containers to sample-to-volume ratio (S/V , cm⁻¹) on viral loss was investigated. Half to 50 ml of either SIO-2 or T2 suspension in <30-kDa FASW (initial viral concentration, 1.4×10^7 viruses ml⁻¹) was incubated in 1.5- to 50-ml polypropylene tubes (S/V , 1.7 to 6.3 cm⁻¹, Table S1) for 24 h at RT in the dark under static conditions.

In the above-described experiments, viral abundances in the water phase were enumerated before and after incubation by EFM. Viral loss (ratio) was calculated using Equation 3:

$$\text{viral loss (\% per day) or viral loss ratio (\% per hour)} = \left(100 - 100 \times \left[\frac{\text{viral abundance after incubation}}{\text{viral abundance before incubation}}\right] [\%]\right) / \text{incubation time (days or hours)} \quad (3)$$

Viral attachments to a cover glass. A 1.5-ml T2 suspension in <30-kDa FASW (initial viral concentration, 4.0×10^7 viruses ml⁻¹) was incubated on a 25- by 25-mm cover glass (Fisher Scientific)

that was placed in a 10-cm petri dish (Corning, NY, USA) to prevent evaporation and contamination from the air for 1, 30, and 60 min at RT in the dark under static conditions. The T2 filtrate from a 0.02- μ m syringe filter (Whatman) was also incubated for 60 min in parallel as a control. After incubation, the suspension on the cover glass was discarded by tilting the dish, and the cover glass was rinsed twice with 1.5 ml of <30-kDa FASW. After depositing 10 μ l of SYBR green I solution (concentration of staining solution, 20 $\times$ dilution of stock [e.g., 50 $\times$] in Vectashield) with formaldehyde (final concentration, 2%) on the cover glass, the cover glass was put on (drop-side down) a glass slide and left in the dark for 10 min to stain the viruses. Finally, the attached viruses were observed and enumerated by EFM.

Viral attachment to different tube materials and detachment from a polypropylene tube. For equalization of the S/V (7.0 cm⁻¹; Table S1), either 300 μ l (in a 1.5-ml polypropylene tube [Fisher Scientific]) or 1.34 ml (in a 40-ml glass vial [Fisher Scientific]) of SIO-2 suspension in <30-kDa FASW (initial viral concentration, 5.4×10^7 viruses ml⁻¹) was incubated for 24 h at RT in the dark under static conditions. The SIO-2 remaining in suspension ("free fraction") fixed by 2% (final conc.) formaldehyde in the filtration funnel was immediately filtered on a 0.02- μ m Anodisc and enumerated by EFM. To enumerate the number of SIO-2 attached to the polypropylene tube, the SIO-2 suspension was pipetted out and the release of the surface-bound SIO-2 determined due to the addition, separately, of 300 μ l 0.1% Triton X-100, 1% Triton X-100, 0.1% Tween 20 (in 0.02- μ m-filtered autoclaved MQ) or <30-kDa FASW. After 10 min of incubation at RT in the dark under static conditions, the liquid phases fixed by 2% (final conc.) formaldehyde in the filtration funnel were immediately filtered through a 0.02- μ m Anodisc filter, and the abundances of detached SIO-2 in each treatment were enumerated by EFM.

Statistical analyses. Statistical comparisons of mean values were performed using SigmaPlot 13 (Systat Software, IL, USA). We used 1-way analysis of variance (ANOVA) followed by Tukey *post hoc* tests to compare the values of virus and VLP abundances and losses among the control and treatment groups. We also used Student's *t* test to compare the two mean values.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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