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Carreira, C.; Staal, M.; Middelboe, M.; Brussaard, C.P.D.

DOI
10.1128/AEM.02863-14
Publication date
2015

Published in
Applied and Environmental Microbiology

Link to publication

Citation for published version (APA):
Carreira, C., Staal, M., Middelboe, M., & Brussaard, C. P. D. (2015). Counting viruses and bacteria in photosynthetic microbial mats. *Applied and Environmental Microbiology*, *81*(6), 2149-2155. https://doi.org/10.1128/AEM.02863-14

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Counting Viruses and Bacteria in Photosynthetic Microbial Mats

Cátia Carreira,[a,b] Marc Staal,[b] Mathias Middelboe,[b] Corina P. D. Brussaard[a,c]

Department of Biological Oceanography, Royal Netherlands Institute for Sea Research (NIOZ), Den Burg, The Netherlands[a]; Section for Marine Biology, University of Copenhagen, Helsingør, Denmark[b]; Aquatic Microbiology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, The Netherlands[c]

Viral abundances in benthic environments are the highest found in aquatic systems. Photosynthetic microbial mats represent benthic environments with high microbial activity and possibly high viral densities, yet viral abundances have not been examined in such systems. Existing extraction procedures typically used in benthic viral ecology were applied to the complex matrix of microbial mats but were found to inefficiently extract viruses. Here, we present a method for extraction and quantification of viruses from photosynthetic microbial mats using epifluorescence microscopy (EFM) and flow cytometry (FCM). A combination of EDTA addition, probe sonication, and enzyme treatment applied to a glutaraldehyde-fixed sample resulted in a substantially higher viral (5- to 33-fold) extraction efficiency and reduced background noise compared to previously published methods. Using this method, it was found that in general, intertidal photosynthetic microbial mats harbor very high viral abundances ($2.8 \times 10^{10} \pm 0.3 \times 10^{10}$ g^{-1}) compared with benthic habitats (10^7 to 10^9 g^{-1}). This procedure also showed 4.5- and 4-fold-increased efficacies of extraction of viruses and bacteria, respectively, from intertidal sediments, allowing a single method to be used for the microbial mat and underlying sediment.

Photosynthetic microbial mats are vertically stratified benthic microbial communities that are found worldwide in environments ranging from hot springs to sea ice (e.g., see reference 1). The top layer of these mats is mostly composed of photoautotrophs (filamentous cyanobacteria and eukaryotic phytobenthos) that produce organic carbon, which is decomposed in a succession of layers of different heterotrophic prokaryotes reflecting concentration gradients in oxygen and other electron acceptors (e.g., see references 1 to 4). The intertwined filamentous cyanobacteria in the top layer and the excretion of exopolymric substances (EPS) make the microbial mats very stable and resistant to wind and wave erosion (5). Viruses are diverse, abundant, and ecologically important components of microbial communities, acting as major drivers of biodiversity and organic matter flux (e.g., see references 6 to 8). In sediments, viruses have been shown to affect prokaryote host mortality (9), spatial distribution (10), and biogeochemical cycling (11). However, while microbial mats have been intensively studied with regard to their biogeochemistry and biodiversity (e.g., see references 12 and 13), studies on the ecological role of viruses in these mats are, to our knowledge, lacking.

One of the challenges of assessing the role of viruses in sediments and other surface-associated environments, such as photosynthetic mats, is the need for reliable quantitative measures to determine their abundance. Depending on the type of sediment (intertidal, coastal, or deep sediments) (14–16), different methods have been used to extract viruses and bacteria. In microbial mats, EPS bind microorganisms, viruses, and particles together in a complex matrix (17), making it more challenging to extract viruses and bacteria than from bulk sediments. To allow detailed studies of viruses in microbial mats, modifications to protocols currently used for quantitative assessment of benthic viruses are necessary (14, 18, 19). Here, we report an improved assay allowing efficient extraction and enumeration by epifluorescence microscopy (EFM) or flow cytometry (FCM) of viruses from photosynthetic microbial mats, as well as intertidal sediments.

MATERIALS AND METHODS

Sample collection. Microbial mat samples were collected in Schiermonnikoog Island (The Netherlands; 53°29′24.29″N, 6°8′18.02″E) during March 2011 and July 2012. A detailed description of the coastal microbial mats in this area is provided in the work of Bauersachs and colleagues (20).

Ten samples of 15 by 8 by 4 cm (length by width by height) were individually collected and placed in clean plastic boxes at *in situ* temperature and taken to the laboratory within 3 to 4 h. In the laboratory, samples were kept at 8°C in a 16-h:8-h light/dark cycle with a low light intensity (15 μmol quanta m^{-2} s^{-1}) until sampled for viral and bacterial enumeration.

Subsamples were collected with a core (0.7-cm inner diameter). The top 1 mm (~100 mg), containing the photosynthetic microorganisms, was sliced with a knife, placed in a sterile 2-ml Eppendorf tube, and fixed with 800 μl of 25% glutaraldehyde (electron microscopy grade; Merck) (final concentration, 2%) diluted in sterile seawater. Samples were kept for 15 min at 4°C in the dark. Tests were performed with four replicate samples, each obtained from an individual core. As the various tests were not always performed with the same natural microbial mat samples, the obtained viral and bacterial abundances in the individual tests may show some variation.

Extraction of viruses and bacteria from photosynthetic microbial mats. The efficiency of extraction of viruses (and bacteria) from the top layer of the photosynthetic microbial mats was tested using a combination of chemical and physical treatments (Table 1). Solutions used for extraction were made with Milli-Q water (18.2 MΩ) and added only after fixation of the sample, therefore avoiding osmotic shock. To promote the

Received 3 September 2014 Accepted 7 January 2015
Accepted manuscript posted online 16 January 2015
Citation Carreira C, Staal M, Middelboe M, Brussaard CPD. 2015. Counting viruses and bacteria in photosynthetic microbial mats. Appl Environ Microbiol 81:2149–2155. doi:10.1128/AEM.02863-14.
Editor: K. E. Wommack
Address correspondence to Cátia Carreira, ccd.carreira@gmail.com.
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doi:10.1128/AEM.02863-14

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TABLE 1 Chemical and physical treatment parameters of the five methods (four previously published and the present study) used to extract and count viruses and bacteria from photoautotrophic microbial mat samples

Investigators and reference	Chemical treatment	Physical treatment
Lunau et al. (16)	MeOH (10–30%) at 35°C	Ultrasonic bath (15 min)
Kallmeyer et al. (15)	Acetate buffer (pH 4.6 in NaCl) for 2 h MeOH (10%) + EDTA (10 mM) + Tween 80 (0.1 %, vol/vol) + $Na_4O_7P_2$ (10 mM) 50 % Nycodenz	Vortexing for 30–60 min; ultrasonic probe (outside sample) (10 s, 3 times)
Danovaro and Middelboe (14)	$Na_4O_7P_2$ (5–10 mM) for 15 min on ice DNase (1 μl) + RNase (1 μl) for 15 min at room temp	Ultrasonic bath (1 min, 3 times)
Garren and Azam (21)	EDTA (0.01 mM) for 30 min on ice Trypsin (0.4 %) for 15 min at 37°C	NA[a]
Present study	EDTA (0.1 mM) for 15 min on ice Benzonase (1 μl) for 30 min at 37°C	Ultrasonic probe (10 s, 3 times)

[a] NA, not applicable.

release of particle-associated viruses (and bacteria) in the microbial mat samples, chemical treatment was first tested with tetrasodium pyrophosphate (TSPP) (14) and EDTA (18, 21) in combination with water bath sonication (14). TSPP is commonly used to extract viruses and bacteria from sediment particles (14). EDTA was chosen because it destroys cation links between EPS polymers and sediment particles, thus releasing EPS-bound viruses, and because it is known to permeabilize outer membranes, thereby facilitating dye uptake (22). Both tests were performed with water bath sonication as described by Danovaro and Middelboe (14).

The most efficient release of viruses from the microbial mat was achieved by addition of 0.1 mM EDTA, and this addition was then applied in the following comparison of the efficiency of water bath sonication versus probe sonication. Probe sonication resulted in a visual destruction of the microbial mat and showed improved extraction efficiency compared with the sonication bath treatment. From this comparison, probe sonication was then applied in a series of 10-s sonication cycles (0, 2, 3, 4, 6, or 8 cycles) with 10-s intervals while keeping the sample tubes on ice-water between cycles, using an ultrasonic probe (Soniprep 150; 50 Hz, 4-μm amplitude, exponential probe). Finally, different EDTA concentrations (no addition and 0.01, 0.1, and 1 mM final concentrations) were tested specifically in combination with probe sonication. Viruses and bacteria in the treated samples were enumerated using epifluorescence microscopy (EFM), as is standard for benthic microbial ecology (14).

One of the challenges of quantification of fluorescently stained viruses in sediment samples is the large background fluorescence due to the staining of free nucleic acids. To reduce this background fluorescence in the sample, three nucleases were tested: DNase I from bovine pancreas (~4,000 Kunitz units mg^{-1}; final concentration, 5 μg ml^{-1}; Sigma-Aldrich), RNase A from bovine pancreas (≥70 Kunitz units mg^{-1}; final concentration, 10 μg ml^{-1}; Sigma-Aldrich) and Benzonase endonuclease from *Serratia marcescens* (final concentration, >250 U μl^{-1}; Sigma-Aldrich). Benzonase degrades both free DNA and RNA in several forms (single stranded, double stranded, linear, circular, and supercoiled) and has been found to leave adenoviruses intact (23). A subsample of 1 μl from the extracted samples was diluted in 1 ml of sterile Milli-Q water, after which the enzyme was added and the sample incubated for 30 min at 37°C (optimal conditions provided by the manufacturer). Three enzyme combinations were tested: 1 μl of DNase I, a mixture of 1 μl of DNase I and 1 μl of RNase A, and 1 μl of Benzonase. EDTA concentrations above 1 mM can partly inhibit Benzonase activity (conditions provided by the manufacturer); however, the final concentration of EDTA after the addition of Benzonase was much lower (0.1 μM) and did not appear to inhibit nuclease activity in our test.

As the viral abundances in the microbial mats were high, small sample volumes were used. To test if the small sample size affected the results, subsamples of 1 μl and 10 μl were compared. Also, the effects of sample storage conditions and time on viral and bacterial abundances were examined. Fixed subsamples were directly snap-frozen with liquid nitrogen and stored at −80°C either before or after extraction and subsequently stored for 2 h, 1 to 2 weeks, 4 to 5 weeks, and 10 to 14 weeks before analysis. Lastly, we tested counting variability by analyzing four replicate subsamples of the same original sediment sample. A schematic overview of the procedure is given in Fig. 1.

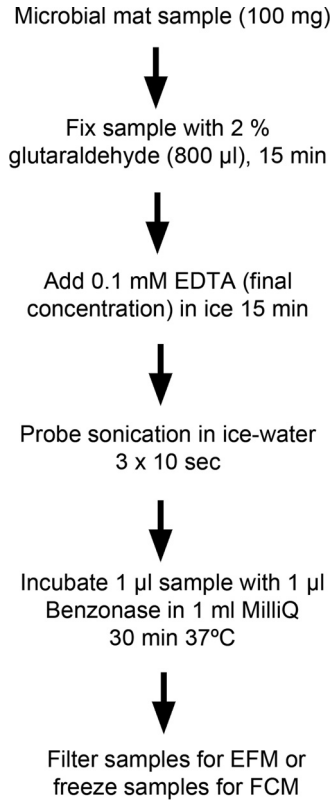

FIG 1 Flow diagram of the method established in the present study to extract viruses and bacteria from microbial mat samples and sediment.

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Epifluorescence microscopy. Filtration and staining procedures were performed according to the methods of Noble and Fuhrman (24). Samples were filtered onto 0.02-μm-pore-size filters (Anodisc 25; Whatman), stained with a green fluorescent nucleic acid-specific dye (400× dilution of commercial stock in Milli-Q water), and washed with sterile Milli-Q water (3 times). After staining, the filters were placed in glass slides with an antifade solution consisting of 50%:50% (vol/vol) glycerol-PBS (0.05 M Na_2HPO_4, 0.85% NaCl [pH 7.5]) with 1% *p*-phenylenediamine (Sigma-Aldrich, The Netherlands). Two different nucleic acid-specific fluorescent dyes, SYBR gold and SYBR green I (25) (Life Technologies, NY), were tested. Slides were stored at −20°C, and viruses and bacteria were counted within a 1- to 3-week period using a Zeiss Axiophot EFM (magnification, ×1,150). At least 10 fields and 400 viruses and bacteria were counted per sample and quantified per gram (wet weight).

Comparison to other methods. To assess the validity of our methodology, we compared the results of our optimized protocol with results obtained using previously published protocols: those of Lunau et al. (16), Kallmeyer et al. (15), Danovaro and Middelboe (14) (extraction from sediments), and Garren and Azam (21) (extraction from coral mucus). Further to testing existing methods, we also tested if the combination of each method with probe sonication yielded a better extraction of viruses (and bacteria). The details of each method and the physical treatment used are presented in Table 1.

Viral and bacterial abundance in sediment. As intertidal photosynthetic microbial mats are also closely associated with the underlying sediments beneath the layer of photosynthetic microorganisms, we examined the suitability of our method to extract viruses and bacteria from sediments and compared these results with those obtained by the method of Danovaro and Middelboe (14), using intertidal sediment (Mokbaai, Texel, The Netherlands).

Sediment samples were collected using a sediment core (5-cm internal diameter) and kept for about 1 h under *in situ* conditions, prior to processing in the laboratory. The top 1 cm was sliced and homogenized, and eight subsamples of 100 mg of sediment were used for viral and bacterial extraction. All samples were fixed with 2% glutaraldehyde (final concentration) for 15 min at 4°C. After fixation, four samples were treated according to our method (see schematic overview in Fig. 1), and the remaining four samples were treated according to the method of Danovaro and Middelboe (14). Briefly, the second set of samples received TSPP (10 mM final concentration) for 15 min in the dark after which they were sonicated (water bath sonicator [Pleuger, 50 to 60 Hz; Sonicor]) in three cycles of 1 min with 30 s of manual shacking in an ice bath. One microliter of DNase I from bovine pancreas (~4,000 Kunitz units mg^{-1}) and 1 μl of RNase A from bovine pancreas (≥70 Kunitz units mg^{-1}) were added, and the samples were incubated for 15 min in the dark. Filtration and staining was conducted as described above for all samples.

Flow cytometry counting of viruses. To examine if our extraction method could be used to count viruses by FCM, the sample extracts from the microbial mats and sediment beneath were either filtered, stained, and frozen for EFM analysis or flash frozen in liquid nitrogen and stained for FCM according to the method of Brussaard et al. (26). Flow cytometric enumeration of viruses was carried out using a standard benchtop Becton-Dickinson FACSCalibur flow cytometer equipped with an air-cooled argon laser (excitation wavelength, 488 nm; power, 15 mW). Samples were diluted (10 to 50 times) in TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]), stained with SYBR green I (Molecular Probes, Invitrogen Inc., Life Technologies, NY) to a final concentration of 10^{-4} of the commercial stock solution, and incubated for 10 min in the dark at 80°C. The trigger was set for green fluorescence, and the data were analyzed using CYTOWIN 4.31 freeware (27).

Statistical analysis. Prior to statistical analysis, normality was checked. All statistical analyses were performed in SigmaPlot 12.0 (SYSTAT Software) with a confidence level set at 95%. To determine differences between the different extraction methods, a one-way analysis of variance (ANOVA) with a *post hoc* Tukey honestly significant difference (HSD) test was performed. Linear regression analyses were performed to obtain the best-fitting coefficients between pairs of variables of regression model II (28) when comparing the EFM versus FCM viral counts.

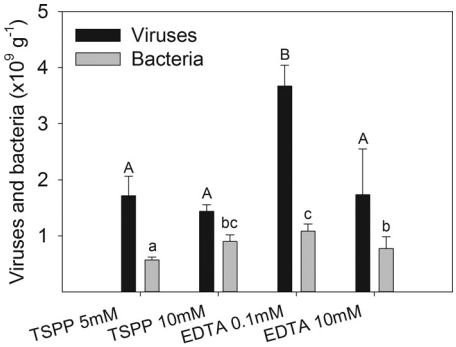

FIG 2 Viral and bacterial abundances in the top 1 mm of photosynthetic microbial mat samples using water bath sonication combined with the method of Danovaro and Middelboe (14) (5 and 10 mM TSPP) and the present method (0.1 and 10 mM EDTA). Standard deviations are shown ($n = 4$). Significant differences ($P < 0.05$) are noted by uppercase letters for viral abundance and lowercase letters for bacterial abundance.

RESULTS

Chemical and physical dispersion. The extraction of viruses from the photosynthetic layer of microbial mats was initially tested using a water bath sonication treatment in combination with the addition of EDTA (0.1 and 10 mM) or TSPP (5 and 10 mM), as used by Danovaro and Middelboe (14). Results showed a statistically significant ($P < 0.05$) increase after addition of 0.1 mM EDTA compared to TSPP or 10 mM EDTA, with a 2- to 2.5-fold increase in viral abundance compared to the other treatments (Fig. 2).

Comparison of water bath sonication versus probe sonication showed a 4.5-fold increase in the viral abundances ($P < 0.001$) and a 7.7-fold increase in the bacterial abundances ($P < 0.01$) when using probe sonication (data not shown). Moreover, probe sonication was less dependent on addition of EDTA for optimal extraction of the viruses from the photosynthetic, mat as there were no statistical differences between the concentrations of EDTA tested (0.01, 0.1, and 1 mM final concentrations). However, the addition of 0.1 mM EDTA improved microscope images (ease of counting) and the EDTA treatment was, therefore, maintained in subsequent tests. The ultrasonic probe disrupted the microbial mat (visible by eye) and significantly ($P < 0.001$) increased the extraction efficiency, up to 15- and 34-fold for viruses and bacteria after three cycles of 10 s, compared to no sonication (Fig. 3). Although the statistical analysis showed that the number of probe sonication cycles did not significantly affect the viral and bacterial abundances, we observed by light microscopy that 20 s of probe sonication did not completely disrupt the mat and that 60 s induced cell disruption. Therefore, three cycles of 10 s were chosen.

The addition of different combinations of enzymes (DNase I, DNase I plus RNase A, and Benzonase) resulted in comparable counts of viruses and bacteria without significant differences (data not shown). Nonetheless, the addition of Benzonase helped to produce substantially clearer images (lower background noise) (Fig. 4). Moreover, as Benzonase is able to digest both DNA and

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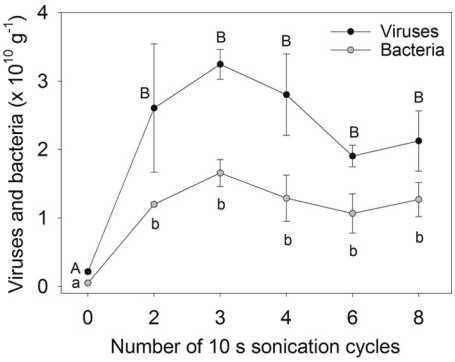

FIG 3 Effect of sonication cycles (10 s) on viral and bacterial abundance after the addition of 0.1 mM EDTA. Standard deviations are shown ($n = 4$). Significant differences ($P < 0.01$) are noted by uppercase letters for viral abundance and lowercase letters for bacterial abundance.

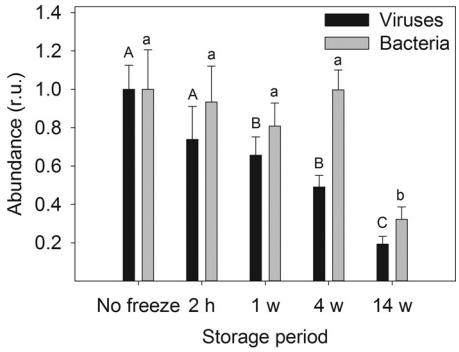

FIG 5 Effect of storage period on viral and bacterial abundances before extraction from top 1 mm of photosynthetic microbial mat samples (relative units [r.u.]). Samples were snap-frozen in liquid nitrogen (−80°C) before storage. Standard deviations are shown ($n = 4$). Significant differences ($P < 0.05$) are noted by uppercase letters for viral abundance and lowercase letters for bacterial abundance.

RNA, the addition of only Benzonase is more practical than using a combination of different enzymes.

Subsampling a volume of 1 or 10 μl from the extracted sample to count showed comparable viral and bacterial abundances without any significant statistical differences. The reproducibility of EFM counts of viruses and bacteria in the extracts was also tested by counting four subsamples of the same original sample. The coefficients of variation for viral and bacterial counts were 1.5 and 10%, respectively. This means that the standard deviation observed for viral and bacterial abundances in the various tests was the result of spatial heterogeneity in the distribution of viruses and bacteria among the collected subsamples rather than variablity in the actual counting analysis. Counting viruses and bacteria by EFM using SYBR gold showed 1.3-fold-higher counts for viruses ($P < 0.05$), but no differences for bacteria, than with SYBR green I-stained samples (data not shown).

Freezing of the fixed microbial mat sample before extraction resulted in a rapid statistically significant loss of viruses and bacteria (Fig. 5), i.e., the abundance of viruses after 1 week of storage was reduced ($P < 0.05$). However, when samples were stored frozen after the chemical and physical extraction, there was no significant loss, even after several months of storage.

In summary, the optimal protocol for extraction of viruses and bacteria from photosynthetic microbial mats (Fig. 1) comprises fixation with 2% glutaraldehyde (final concentration) for 15 min at 4°C, followed by incubation with 0.1 mM EDTA (final concentration) on ice and in the dark for another 15 min. Thereafter, probe sonication is applied in three cycles of 10 s with 10-s intervals, while keeping the samples in ice-water. A subsample of 1 μl is diluted in 1 ml of sterile Milli-Q water and incubated with 1 μl of Benzonase in the dark for 30 min at 37°C. Finally, the sample is placed on ice until filtration for EFM analysis or frozen in liquid nitrogen and kept at −80°C for EFM or FCM analysis.

Comparison to other methods. The selected existing procedures (Table 1) showed a significantly lower efficiencies of extraction of viruses and bacteria from photosynthetic mat samples than for the current protocol (5- to 33-fold-lower and 14- to 21-fold-lower abundances for viruses and bacteria, respectively) (Fig. 6). Addition of a probe sonication step to the published protocols

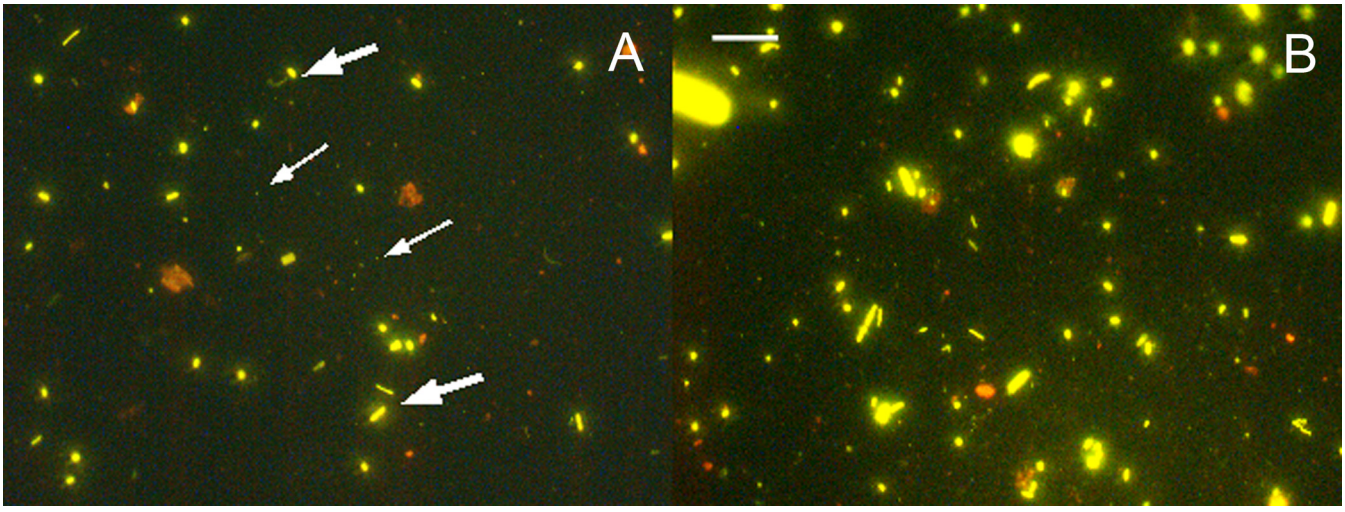

FIG 4 Epifluorescence microscopy images of viruses and bacteria from the top 1 mm of photosynthetic microbial mat samples with (A) and without (B) Benzonase. Scale bar indicates 5 μm. Small and big arrows indicate viruses and bacteria, respectively.

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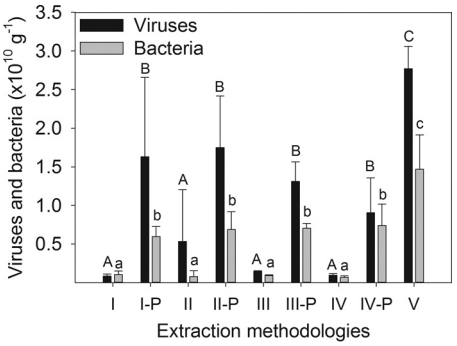

FIG 6 Viral and bacterial abundances in the top 1 mm of photosynthetic microbial mat samples, using the extraction methods of Lunau et al. (I) (16), Kallmeyer et al. (II) (15), Danovaro and Middelboe (III) (14), and Garren and Azam (IV) (21) and the method from the present study (V). P, probe sonication step added. Standard deviations are shown ($n = 4$). Statistical analysis showed a significant difference between the original and the combined method with probe sonication ($P < 0.001$ for viral and bacterial abundances) and significant differences between the present method and the other four methods ($P < 0.001$ and $P < 0.05$ for viral and bacterial abundances, respectively). Significant differences are noted by uppercase letters for viral abundance and lowercase letters for bacterial abundance.

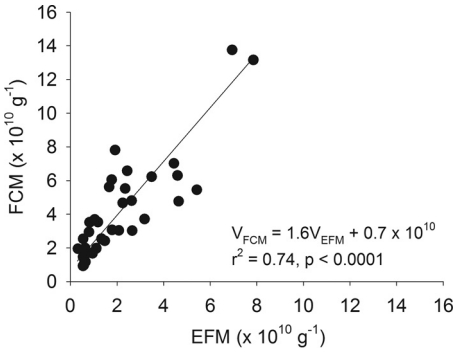

FIG 8 Comparison of viral counts ($n = 40$) using flow cytometry (FCM) and epifluorescence microscopy (EFM) after extraction with the present method.

resulted in a statistically significant increase ($P < 0.001$) in viral and bacterial abundances compared to those obtained in the original protocols. Still, our method produced an additional improvement, as illustrated by the significant increase in viral ($P < 0.001$) and bacterial ($P < 0.05$) abundances compared to those obtained by the published protocols even with the additional probe sonication step (Fig. 6). On average, our method gave 2.5- and 2.2-fold-higher viral and bacterial abundances, respectively, than did the other methods performed with probe sonication.

Sediment counts of viruses and bacteria. When applying our microbial mat extraction protocol to intertidal sediment samples, 4.5- and 4-fold increases ($P < 0.001$) in viral and bacterial abundances, respectively, were obtained compared to those obtained by the Danovaro and Middelboe (14) method, i.e., $(3.08 \pm 0.63) \times 10^9$ versus $(0.75 \pm 0.12) \times 10^9$ viruses g^{-1} and $(3.36 \pm 0.68) \times 10^9$ g^{-1} versus $(0.75 \pm 0.28) \times 10^9$ bacteria g^{-1}, respectively. Consequently, no change in the average ratio of viruses to bacteria was found for the two methods.

Flow cytometry. The present method allowed an easy analysis of viruses using FCM (Fig. 7). Two virus clusters with different green fluorescence intensities (V1 with the lowest intensity and V2 with the highest intensity) could be distinguished. Comparing virus quantification from microbial mats using EFM and FCM (Fig. 8) showed a good correlation ($r^2 = 0.74$; $P < 0.0001$), with FCM giving higher counts. As also observed for pelagic samples (29), bacterial abundances obtained by EFM and FCM matched well ($r^2 = 0.88$; $P < 0.0001$; $y = 1.02x$).

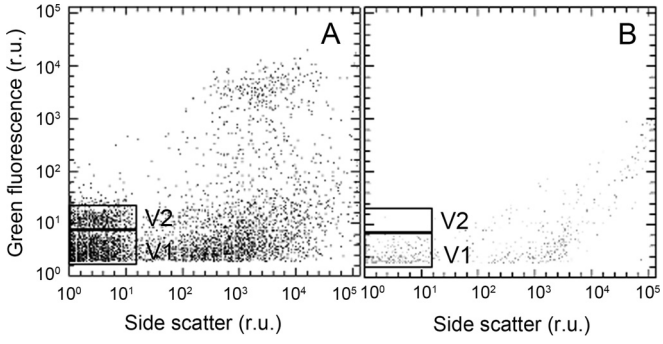

FIG 7 Cytogram of viruses from photosynthetic microbial mat samples using flow cytometry after staining with nucleic acid-specific dye SYBR green I (A) and from control sample without viruses (B). Green fluorescence (V1 and V2) allows the distinction of two virus clusters.

DISCUSSION

Intertidal photosynthetic microbial mats are mainly composed of intertwined filamentous cyanobacteria and microalgae, glued together in a biofilm composed of EPS, sediment particles, bacteria, and viruses (3, 17). To extract viruses and bacteria from such mats, a combination of chemical and physical treatments is necessary. This is most likely related with the need to disrupt the strong links between cyanobacterial filaments and EPS structures. The combination of probe sonication with a low EDTA concentration (0.1 mM) and a nuclease treatment provided an efficient method for the extraction of viruses and bacteria from microbial mat samples, as well as optimized conditions for subsequent counting by EFM or FCM.

EDTA has been widely used to extract EPS from both intertidal sediments (30) and microbial mats (31) because it chelates bivalent ions (Ca^{2+} and Mg^{2+}), destroying the links between the EPS polymers and between EPS and sediment particles and thereby releasing attached viruses and bacteria. EDTA has also been used previously for the extraction of bacteria from coral mucus (21) and in combination with other chemicals for bacterial and viral extraction from sediments and biofilms (15, 18, 19, 21). In these studies, EDTA was used in concentrations ranging from 0.01 to 10 mM and showed good results for the extraction of viruses and/or bacteria.

Our study is the first comprehensive study comparing water bath sonication with the effects of a probe sonication directly on microbial mat samples. Probe sonication had been previously used in a few studies for the extraction of viruses in marine sediments (e.g., see reference 32). In the current study, the application of probe sonication visually disrupted the microbial mats, significantly increasing the viral and bacterial abundances and strongly improving the counting yield of viruses and bacteria. Probe sonication proved more effective in viral extraction from microbial mat samples than water bath sonication, the methodology routinely used in sediments. Moreover, we did not observe cell disruption with the sonication times proposed in the current proto-

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col, contrary to previous studies in which probe sonication had disrupted bacterial cells during extended treatment (1 to 22 min) at high energy levels (33). Nonetheless, we recommend initial visual inspection of the material when utilizing our protocol.

The effect of nuclease addition on the extraction methodology has shown contradictory results in previous studies. Danovaro et al. (34) claimed that the increase in viral counts after nuclease addition was due to the disruption of bulks of matter where viruses could be found, and thus a release of attached viruses. Maruyama et al. (35) ascribed the decrease in the viral fraction to the degradation of uncoated DNA (or extracellular DNA [eDNA]) by DNase. Finally, Fischer et al. (36) showed no differences in viral counts after nuclease addition because of insignificant amounts of eDNA in the analyzed samples. In our study, the endonuclease Benzonase helped to optimize the counting efficiency by reducing the background fluorescence likely derived from staining free nucleic acids (eDNA). This is supported by previous measurements of high eDNA concentrations in marine sediments (3.5 to 55.2 $\mu g\ g^{-1}$) (37) and in activated wastewater biofilms (4 to 52 $mg\ g^{-1}$ of volatile suspended solids) (38), where it has been suggested to have an important structural role in bacterial microcolonies by binding bacterial cells together (38). Microbial mats are highly active biofilms (17) and most probably also contain high concentrations of eDNA. Nucleases have been shown not to degrade viral particles (39); therefore, the addition of nucleases does not have negative implications for viral abundance.

Clearly, probe sonication contributed most to the method improvement; however, the addition of EDTA (viruses and cells are shown brighter) and nuclease (cleaner samples) allowed easier counting. The extraction protocol presented in this study is an effective extraction method for recovery of viruses and bacteria from photosynthetic microbial mats. Using this method, viral and bacterial abundances obtained from intertidal microbial mats were 1.7- to 2.8-fold and 2- to 2.5-fold, respectively, higher than those found using other published methods for extraction of viruses and bacteria from sediments (14–16) and coral mucus (21), even after adding probe sonication to these protocols.

The microbial mat extraction protocol was shown to also improve the efficiency of extraction of viruses and bacteria from bulk intertidal sediment underlying the photosynthetic microbial mat compared to previous published methods (4.5- and 4-fold-higher abundances of viruses and bacteria, respectively). With this method, it is thus possible to count viruses and bacteria in both microbial mats and sediments, allowing a direct comparison of viral and bacterial abundances without biases derived from the use of different extraction methods.

Application of the assay to sediment samples in combination with FCM analysis showed two clear virus clusters, as has been observed also for pelagic samples (26). The higher virus counts with FCM than with EFM were most likely due to the reduced quenching of the green fluorescent signal using FCM in combination with sensitive detection of the green fluorescent signal (thus overall improved FCM counts of low-fluorescence viruses). Our method resulted in less background noise and an improved correlation between EFM and FCM virus counts ($r^2 = 0.74$) compared to what is published thus far (freshwater sediment; $r^2 = 0.55$) (40). FCM has the advantage of being faster and more accurate than EFM counting of viruses.

The application of our method to natural photosynthetic microbial mats showed that viral abundances in these environments are among the highest recorded in natural aquatic systems ($2.8 \times 10^{10} \pm 0.3 \times 10^{10}\ g^{-1}$). Higher viral numbers have been reported only for eutrophic sediments with large anthropogenic influence, e.g., the Chesapeake Bay ($1.5 \times 10^{11}\ ml^{-1}$) (41) and Brisbane River ($2.2 \times 10^{11}\ ml^{-1}$) (42). Furthermore, the presented extraction procedure may also be beneficial for capturing genetic information (e.g., next-generation sequencing) from the recovered microbes, thereby coupling quantitative abundance analysis to biodiversity information. However, for DNA extraction, we advise testing the protocol without the use of a fixative, as this might inhibit good DNA extraction. Alternatively, heat treatment has been suggested to reverse the cross-linking of DNA or RNA to proteins caused by fixatives (43). We anticipate that the methodology presented here will stimulate a systematic and quantitative exploration of viral ecology in benthic microbial mat systems.

ACKNOWLEDGMENTS

The study received financial support from Fundação para a Ciência e a Tecnologia (FCT) (SFRH/BD/43308/2008), the Royal Netherlands Institute for Sea Research (NIOZ), and The Danish Research Council for Independent Research (FNU). We are grateful to Christian Lønborg, Tim Piel, and Robin van de Ven for field and laboratory assistance.

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