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), 1790 AB 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]

Running head: Viral abundances in photosynthetic microbial mats

#Address correspondence to Cátia Carreira, ccd.carreira@gmail.com

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Abstract

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 to a glutaraldehyde fixed sample resulted in substantially higher viral (5 to 33-fold) extraction efficiency and reduced background noise as compared to previously published methods. Using this method it was found that in general, intertidal photosynthetic microbial mats harbour very high viral abundances ($2.8 \pm 0.3 \times 10^{10}$ g^{-1}) compared with benthic habitats (10^{7} - 10^{9} g^{-1}). This procedure also showed a 4.5 and 4-fold increased extraction efficacy of viruses and bacteria, respectively, from intertidal sediments, allowing a single method to be used for the microbial mat and underlying sediment.

Introduction

Photosynthetic microbial mats are vertically stratified benthic microbial communities that are found worldwide from hot springs to sea ice (e.g. 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. 1-4). 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. 6-8). 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 in regard to their biogeochemistry and biodiversity (e.g.(12, 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, as phtosynthetic 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.

Material 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 Bauersachs and colleagues (20).

Ten samples of 15 x 8 x 4 cm (L x W x H) were individually collected and placed in clean plastic boxes at *in situ* temperature and taken to the laboratory within 3-4h. In the laboratory, samples were kept at 8ºC in a 16:8h L:D 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 and placed in a sterile 2 ml Eppendorf tube and fixed with 800 µl of 2 % glutaraldehyde final concentration (25 %, EM-grade, Merck) diluted in sterile seawater. Samples were kept for 15 min at 4ºC 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 extraction efficiency 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 MilliQ water (18.2 MΩ) and only added

after fixation of the sample, therefore avoiding osmotic shock. To promote the release of particle-associated viruses (and bacteria) in the microbial mat samples, chemical treatment was first tested with tetrasodiumpyrophosphate (TSPP; 14) and Ethylenediaminetetraacetic acid (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 permeabilise 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 relase of viruses was obtained 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 and 8) using an ultrasonic probe (Soniprep 150; 50 Hz, 4 µm amplitude, exponential probe) with 10 s intervals while keeping the sample tubes on ice-water. Finally, different EDTA concentrations (no addition, 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 epifluoscence 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 (~4000 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 MilliQ water, after which the enzyme was added and the sample incubated for 30 min at 37ºC (optimal conditions provided by the manufacturer). Three enzyme combinations were tested: 1 µL of DNase I, a mixture of 1 µL DNase I and 1 µL 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 this small sample size affected the results, subsamples of 1 µl and 10 µl were compared. Also, the effect 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 -80ºC either before or after extraction, and subsequently stored for 2h, 1-2 weeks, 4-5 weeks and 10-14 weeks before analysis. Lastly we tested counting variability by analysing four replicate subsamples of the same original sediment sample. A schematic overview of the procedure is given in Figure 1.

Epifluorescence microscopy. Filtration and staining procedures were performed according to 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 (400x dilution of commercial stock in MilliQ water) and washed with sterile MilliQ water (3 times). After staining, the filters were placed in glass slides with an anti-fade solution consisting of 50:50 % (v/v) 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, USA), were tested. Slides were stored at -20ºC and viruses and bacteria were counted within a 1-3 week period using a Zeiss Axiophot EFM (x1150 magnification). 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: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 yield 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 of the Danovaro and Middelboe (14) method, using intertidal sediment (Mokbaai, Texel, The Netherlands).

Sediment samples were collected using a sediment core (5 cm, internal diameter) and kept for about 1h at *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 4ºC. 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 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, Sonicor, 50-60 Hz) in three cycles of 1 min with 30 s of manual shacking in an ice bath. One microliter of DNase I from bovine pancreas (~4000 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 (FCM) 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 Brussaard et al. (26). Flow cytometric enumeration of viruses was carried out using a standard bench top Becton-Dickinson FACSCalibur flow cytometer, equipped with an air-cooled argon laser (excitation 488 nm, 15 mW power). Samples were diluted (10-50 x) in TE buffer (Tris 10 mM, EDTA 1 mM, pH 8.0), stained with SYBR Green I (Molecular Probes®, Invitrogen Inc., Life Technologies™, NY, USA) to a final concentration of 10^{-4} of the commercial stock solution, and incubated for 10 min in the dark at 80ºC. The trigger was set for green fluorescence and the data was analysed 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 ANOVA with a post hoc Tukey HSD test was performed. Linear regression analyses were performed to obtain the best-fitting coefficients between pairs of variables of the regression model II (28), when comparing the EFM versus FCM viral counts.

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 2-2.5 fold increase in viral abundance compared to the other treatments (Fig. 2).

Comparision of water bath sonication versus probe sonication showed a 4.5-fold increase in the viral abundances ($p < 0.001$) and 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 concentration). 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 cyles of 10 s were chosen.

The addition of different combinations of enzymes (DNAse I, DNAse I + 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

214 substantially clearer images (lower background noise; Fig. 4). Moreover, as benzonase is
215 able to digest both DNA and RNA, the addition of only benzonase is more practical than
216 using a combination of different enzymes.

217 Subsampling a volume of 1 or 10 µl from the extracted sample to count showed
218 comparable viral and bacterial abundances without any significant statistical differences.
219 The reproducibility of EFM counts of viruses and bacteria in the extracts was also tested
220 by counting four subsamples of the same original sample. The coefficient of variation for
221 viral and bacterial counts was 1.5 and 10 %, respectively. This means that the standard
222 deviation observed for viral and bacterial abundances in the various tests was the result of
223 spatial heterogeneity in the distribution of viruses and bacteria among the collected
224 subsamples rather than variablity in the actual counting analysis. Counting viruses and
225 bacteria by EFM using SYBR Gold showed 1.3-fold higher counts for viruses ($p < 0.05$),
226 but no differences for bacteria as compared to SYBR Green I stained samples (data not
227 shown).

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

233 In summary, the optimal protocol for extraction of viruses and bacteria from
234 photosynthetic microbial mats (Fig. 1) comprised the fixation with 2 % glutaraldehyde (final
235 concentration) for 15 min at 4ºC, followed by incubation with 0.1 mM EDTA (final
236 concentration) on ice and in the dark for another 15 min. Thereafter, probe sonication is
237 applied in three cycles of 10 s with 10 s intervals, while keeping the samples in ice-water.
238 A subsample of 1 µL is diluted in 1 ml sterile MilliQ water and incubated with 1 µl of

benzonase in the dark for 30 min at 37ºC. Finally, the sample is placed on ice until filtration for EFM analysis, or frozen in liquid nitrogen and kept at -80ºC for EFM or FCM analysis.

Comparison to other methods. The selected existing procedures (Table 1) showed a significantly lower extraction efficiencies of viruses and bacteria from photosynthetic mat samples when compared to the currrent protocol (5-33 and 14-21-fold lower abundances for viruses and bacteria, respectively; Fig. 6). Addition of a probe sonication step to the published protocols resulted in a statistically significant increase ($p < 0.001$) in viral and bacterial abundances compared to the original protocols. Still, our method showed an additional improvement as illustrated by the significant increase in viral ($p < 0.001$) and bacterial ($p < 0.05$) abundances compared to 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, compared to the other methods performed with probe sonication.

Sediment counts of viruses and bacteria. When applying our microbial mat extraction protocol to intertidal sediment samples, a 4.5 and 4-fold increase ($p < 0.001$) in both viral and bacterial abundance, respectively, was obtained as compared to the Danovaro and Middelboe (14) method, *i.e.* $3.08 \pm 0.63 \times 10^9$ *vs.* $0.75 \pm 0.12 \times 10^9$ viruses g^{-1}, and $3.36 \pm 0.68 \times 10^9$ g^{-1} *vs.* $0.75 \pm 0.28 \times 10^9$ bacteria g^{-1}, respectively. Consequently, no change in average viruses to bacteria ratio 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 lowest and V2 with 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).

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, thereby releasing attached viruses and bacteria. EDTA has also been used earlier 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 in 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. 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 protocol, contrary to previous studies, where probe sonication had disrupted bacterial cells during extended treatment (1-22 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 analysed 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 (3.5-55.2 μg g^{-1}; 37) and in activated waste water biofilms (4-52 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 on viral abundance.

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

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

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

341 The application of our method to natural photosynthetic microbial mats showed that
342 viral abundances in these environments are among the highest recorded in natural aquatic

343 systems (2.8 ± 0.3 x 10^{10} g^{-1}). Higher viral numbers have been reported only in eutrophic
344 sediments with large anthropogenic influence, e.g. Chesapeake Bay (1.5 x10^{11} ml^{-1}; 41)
345 and Brisbane River (2.2 x 10^{11} ml^{-1}; 42). Furthermore, the presented extraction procedure
346 may also be beneficial for capturing genetic information (e.g. next-generation sequencing)
347 from the recovered microbes, thereby coupling quantitative abundance analysis to
348 biodiversity information. However, for DNA extraction, we advice on testing the protocol
349 without the use of a fixative, as this might inhibit good DNA extraction. Alternatively, heat
350 treatment has been suggested to reverse the cross linking of DNA/RNA to proteins caused
351 by fixatives (43). We anticipate that the methodology here presented will stimulate a
352 systematic and quantitative exploration of viral ecology in benthic microbial mat systems.
353
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464 **Table 1.** Chemical and physical treatment parameters of the five extraction methods (four
465 previously published and the present study), used to extract and count viruses and
466 bacteria from photoautotrophic microbial mat samples. n.a. – not applicable.

Reference	Chemical treatment	Physical Treatment
Lunau et al. 2005	MeOH (10 - 30 %) at 35ºC	ultrasonic bath (15 min)
Kallmeyer et al 2008	Acetate buffer (pH 4.6 in NaCl) 2 h MeOH (10 %)+EDTA (10 mM) + Tween 80 (0.1 % v/v)+$Na_4O_7P_2$ (10 mM) 50 % Nycodenz	vortex 30 - 60 min; ultrasonic probe (outside sample) (10 sec x 3)
Danovaro & Middelboe 2010	$Na_4O_7P_2$ (5 - 10 mM) 15 min on ice DNase (1 µL) + RNase (1 µL) 15 min at room temperature	ultrasonic bath (1 min x 3)
Garren & Azam 2010	EDTA (0.01 mM) 30 min on ice Trypsin (0.4 %) 15 min at 37 ºC	n.a.
Present study	EDTA (0.1 mM) 15 min on ice Benzonase (1 µL) 30 min at 37 ºC	ultrasonic probe (10 sec x 3)

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Figure Legends

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

Figure 2 Viral and bacterial abundance ($x10^9$ g^{-1}) in the top 1 mm of photosynthetic microbial mat samples using water bath sonication combined with the Danovaro and Middelboe 2010 extraction method (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 upper case letters for viral abundance and lower case for bacterial abundance.

Figure 3 Effect of sonication cycles (10 s) on viral and bacterial abundance ($x10^{10}$ g^{-1}), after the addition of 0.1 mM EDTA. Standard deviations are shown (n = 4). Significant differences ($p < 0.01$) are noted by upper case letters for viral abundance and lower case for bacterial abundance.

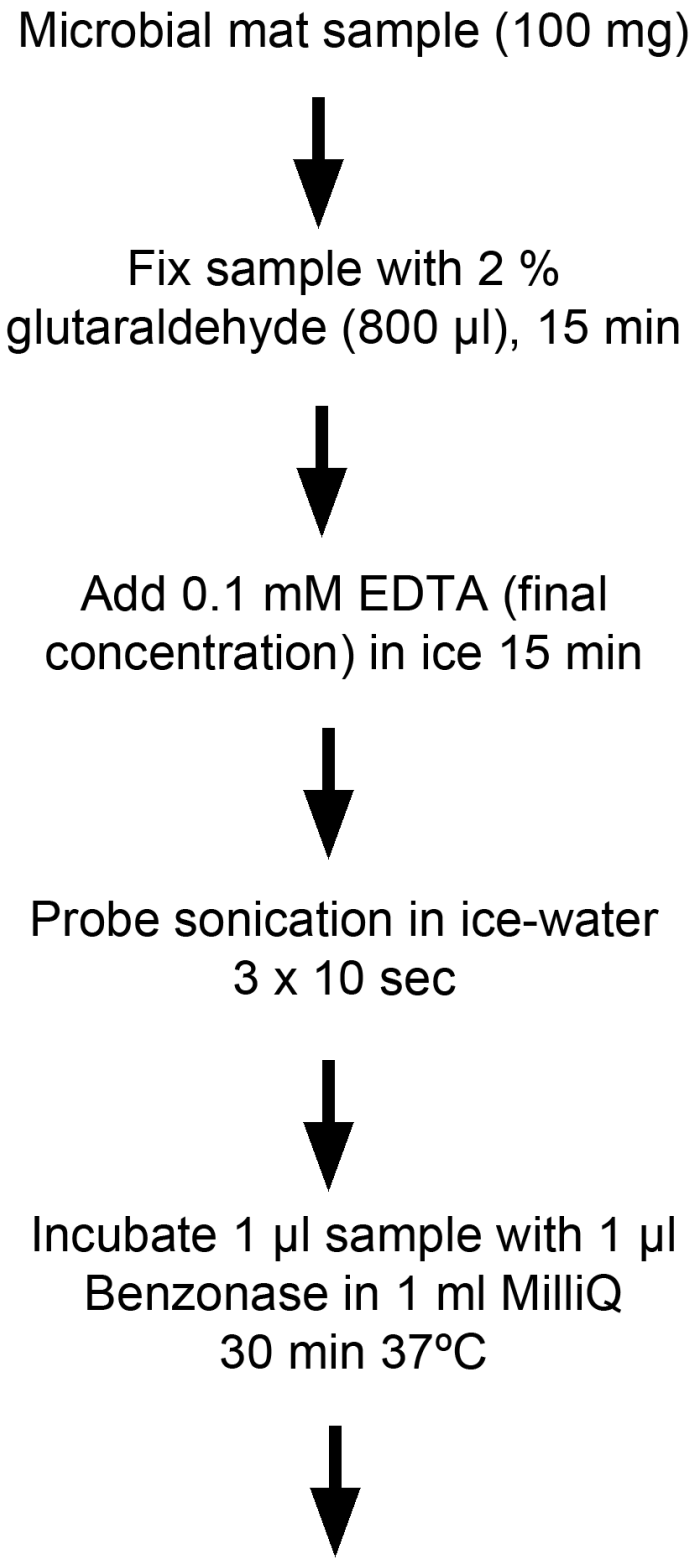
Figure 4 Epifluorescence microscopy images of viruses and bacteria from the top 1 mm of photosynthetic microbial mat samples (A) with and (B) without benzonase. Scale bar indicates 5 µm. Small and big arows indicate viruses and bacteria, respectively.

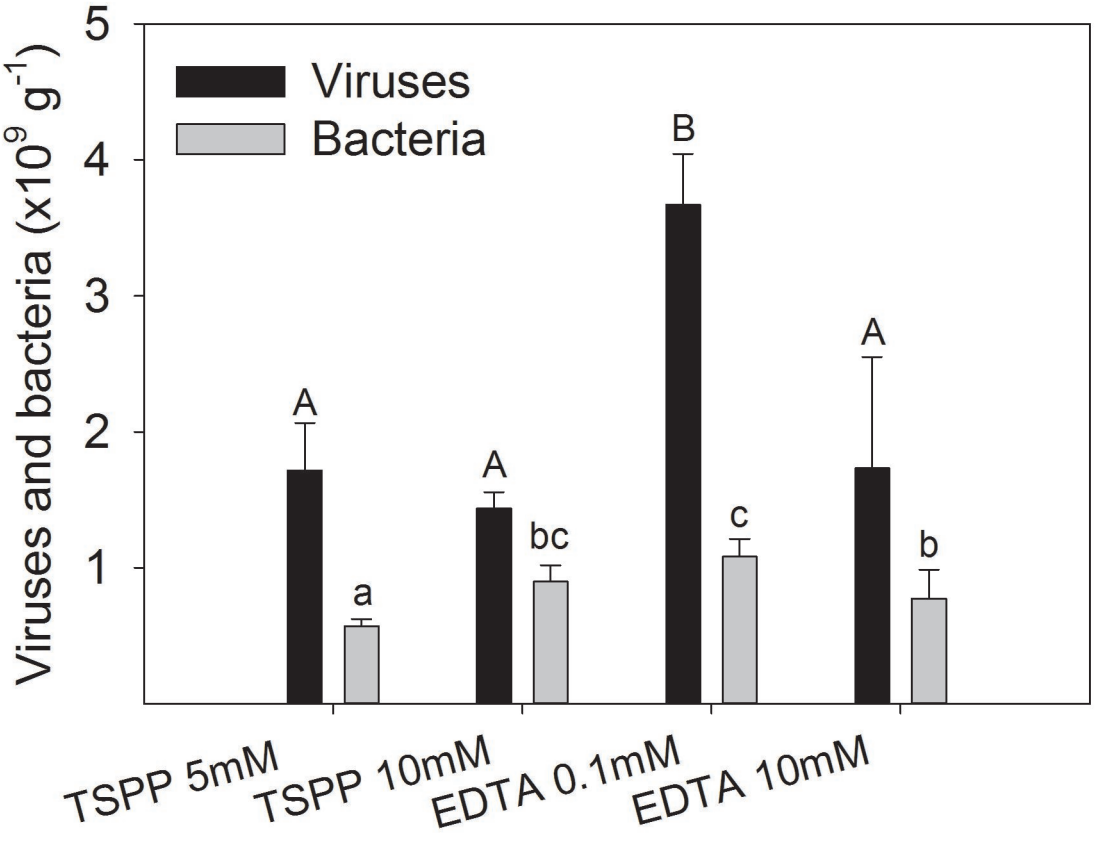
Figure 5 Effect of storage period on viral and bacterial abundance before extraction from top 1 mm of photosynthetic microbial mat samples (relative units). Samples were snap frozen in liquid nitrogen (-80 ºC) before storage. Standard deviations are shown (n = 4). Significant differences ($p < 0.05$) are noted by upper case letters for viral abundance and lower case for bacterial abundance.

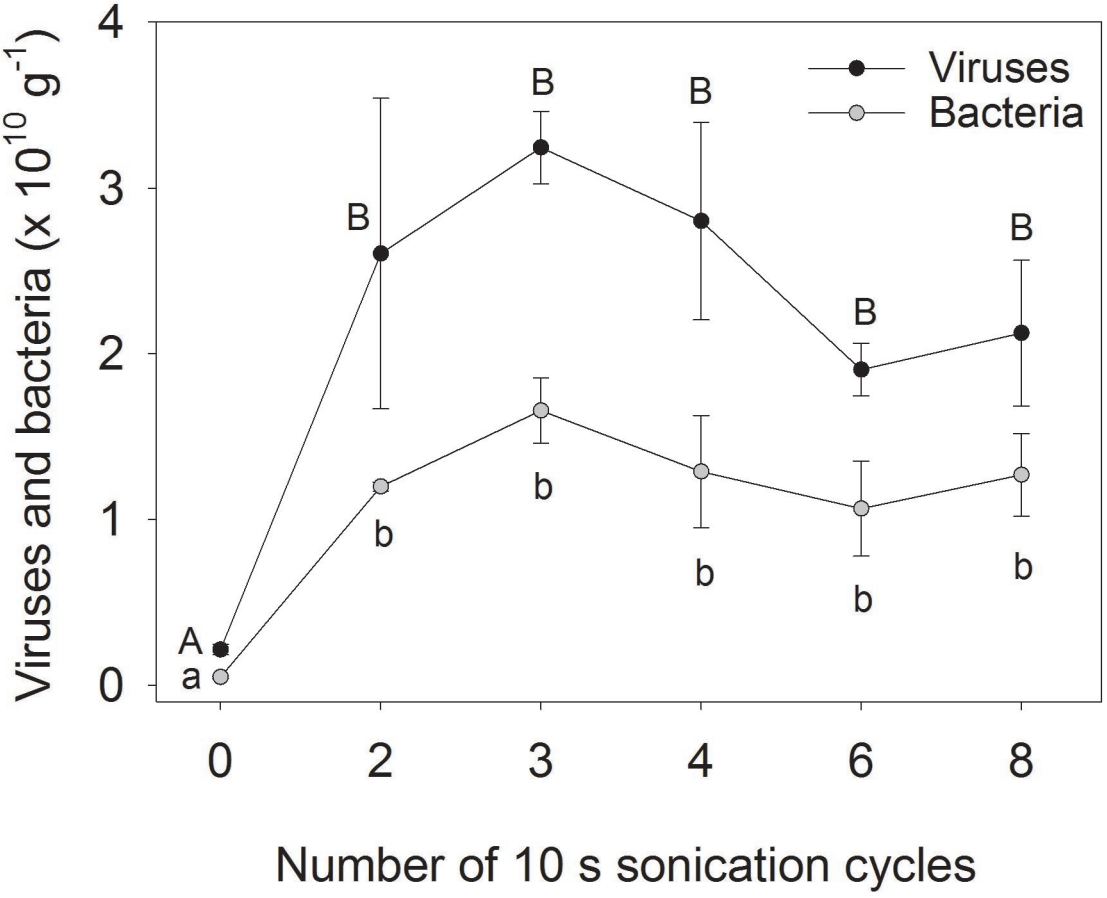
Figure 6 Viral and bacterial abundances ($x10^{10}$ g^{-1}) in the top 1 mm of photosynthetic microbial mat samples, using the extraction methods of Lunau et al. 2005 (I), Kallmeyer et al. 2008 (II), Danovaro & Middelboe 2010 (III), Garren & Azam 2010 (IV), and the method from the present study (V). P indicates 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 sonifcation ($p < 0.001$ for viral and bacteria abundance), and significant differences between the present method and the other four methods ($p < 0.001$ and $p < 0.05$ for viral and bacteria abundance, respectively). Significant differences are noted by upper case letters for viral abundance and lower case for bacterial abundance.

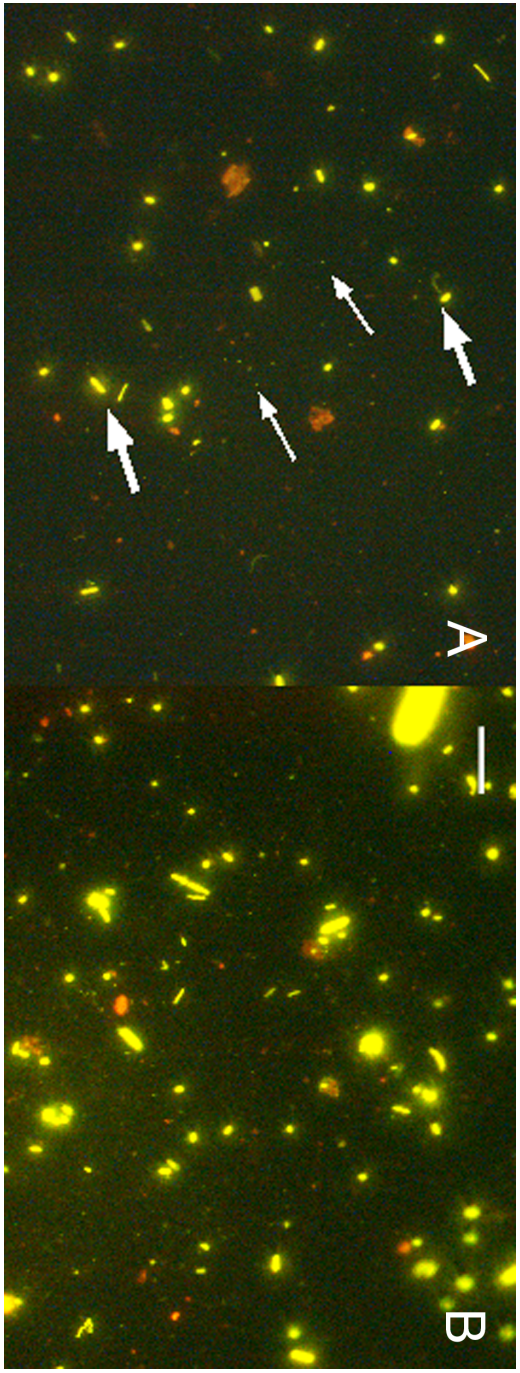
Figure 7 Cytogram (A) of viruses from photosynthetic microbial mat samples using flow cytometry after staining with nucleic acid-specific dye SYBR Green I, and (B) from control sample without viruses. Green fluorescence (V1 and V2) allows the distinction of two virus clusters. R.u. stands for relative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

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





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