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# Separation of free virus particles from sediments in aquatic systems

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## Abstract

The number of benthic viruses per unit of volume, at all depths (from shallow down to abyssal sediments), exceeds water column abundances by orders of magnitude. The need of methods for the determination of viral counting in aquatic sediment is becoming increasingly urgent along with the increasing evidence of the relevance of viruses in the benthic domain. The procedures used for determining viral abundances in sediments require specific modifications to release the viruses from sediment particles and to minimize the physical and chemical interferences of sedimentary matrix with the analysis. Dislodging viruses from sediment samples is the first crucial step for the analyses of viral abundance in benthic samples. Here we present the results of several tests aimed at optimizing the protocol for viral counts based on (i) the chemical treatment (type and modality of the use of surfactants), (ii) mechanical treatment (ultrasounds), (iii) cleaning of the samples (by enzymatic digestion of the extracellular DNA by means of DNases), and (iv) the limitations associated with viral recovery from the sediment (by serial washing steps). Sediment texture and composition vary considerably along horizontal and vertical gradients, and here we compare shallow sandy sediments with more silty deep-sea sediments. We found that the use of the surfactant tetrasodium pyrophosphate (final concentration 5 mM for 15 min), followed by ultrasound treatments (3 times for 1 min with 30 s intervals) and by the addition of an enzymatic cocktail composed of DNase I, nuclease P1, nuclease S1, and esonuclease 3, increased the detectability by staining with fluorochrome, thus resulting in significantly higher and more accurate viral counting, determined by epifluorescence microscopy. Our results also indicate that sediment samples processed using this optimized protocols displayed a significantly lower coefficient of variation, thus making sufficient the counting of a lower number of optical fields. Centrifugation of sediment samples after extraction procedures could underestimate viral counting, and we recommend here an accurate check of the potential loss or an alternative procedure based on sediment dilution prior to quantification by epifluorescence microscopy.

## Introduction

Viruses are the most abundant and diverse biological component of aquatic environments (Bergh et al. 1989; Proctor and Fuhrman 1990). The estimated overall abundance in the world's oceans is on the order of $10^{30}$ (Suttle 2005), a value that exceeds the abundance of prokaryotes by one order of magnitude (Whitman et al. 1998; Suttle 2005). Moreover, it has become increasingly evident that viruses play critical roles in shaping aquatic communities and determining ecosystem dynamics (Fuhrman 1999; Danovaro et al. 2008). More recently, the interest for the determination of viral enumeration has extended to the benthic compartment, as it has become increasingly evident that viral abundances in surface sediments at all depths down to abyssal sediments exceed those in the water column by orders of magnitude (typically reaching values of $10^8$–$10^9$ viral particles $mL^{-1}$; e.g., Danovaro and Serresi 2000; Danovaro et al. 2002; Corinaldesi and Danovaro 2003; Maranger and Bird 1996; Middelboe et al. 2006; Middelboe and Glud 2006; Siem-Jørgensen et al. 2008). Moreover, high viral abundances have been reported also in subsurface sediment (Bird et al. 2001.) There is a clear need to develop accurate protocols for viral enumeration in marine sediments for detecting potential changes in viral abundance

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and because of viral production's being typically assessed by use of total counts (Danovaro et al. 2008).

Approaches and procedures used for determining viral abundances in sediments are derived from those applied to water samples. However, due to the specific characteristics of the sediments and of the benthic environment, significant modifications of the protocols are needed to minimize the physical and chemical interferences of sedimentary matrix with the analysis. Efficient dislodging of viruses from sediment samples is the first crucial step for the analyses of viral abundance in benthic samples. This step is currently required for all of the available techniques of viral enumeration in sediments, including the technique based on the largely used epifluorescence microscopy (EFM; Danovaro et al. 2001; Patel et al. 2007), transmission electron microscopy (TEM; Middelboe et al. 2003), or the use of flow cytometry (FCM; Duhamel and Jacquet 2006).

Quantifying their abundance is a fundamental step in any attempt to understand the role of viruses in sediments, and their spatial and temporal dynamics. However, quantification of viral abundance in benthic environments is a much more complex task than is the case in the water column because the benthic viral particles have to be dislodged from the matrix in which they are embedded prior to quantification. Numerous approaches for extracting viruses from sediments have been proposed with quite variable results, and there is, therefore, a strong need for an evaluation of previous experiences and, subsequently, to move toward a consensus about which general extraction principle that provides the most accurate determination of viral abundance in sediments. The separation of viruses from sediment particles requires the breaking of the links between viruses and sediment particles. To do this, one of the most common approaches is based on a chemical treatment with surfactants, which create hydrophylic links among particles, thus enhancing their interdispersion. Among the available surfactants, tetrasodium pyrophosphate and polyoxyethylene-sorbitanmonooleate are the most widely used in the last decade (Maranger and Bird 1996; Hewson and Fuhrman 2003; Corinaldesi et al. 2007). The dislodgement of viruses from the sediment particles is typically accompanied by a mechanical shaking (by means of manual shaking, ultrasonication, and/or vortexing; Danovaro et al. 2001).

Here we present a detailed description of what we find is the most efficient protocol for dislodging viruses from sediment particles for subsequent analysis by EFM, flow cytometry, or TEM. The treatments compared in this study were performed with different types of sediments covering a wide range of depths (from shallow sandy sediments to silty deep-sea systems). To test the extraction efficiency, we focused our attention on EFM counting using SYBR Green I (or SYBR Gold) as a stain. The following methodological aspects were investigated: (i) virus dislodgment from sediment particles (using surfactant and ultrasound treatments), (ii) efficiency of virus extraction from bulk sediment (by the number of postsonication washings), (iii) stain-counting accuracy and efficiency (by removing possible interferences due to extracellular DNA in virus counting and by comparison with TEM counts), and (iv) effects of centrifugation versus dilution of the sediment prior to counting viral particles.

## Materials and procedures

*Extraction of viruses for the analysis on porewater samples*—Studies dealing with the determination of viral abundance or viral production in sediments have been carried out so far on both viruses dispersed in the porewater (nonattached to sediment particles) and on viruses attached to sediment grains. The counting of viruses in the porewater does not require a specific modification of the protocol used for aquatic samples, except the squeezing of the sediment for the extraction of the porewater (e.g., by centrifugation). Alternatively, wet sediment samples are filtered on glass fiber (GF/F) filters and the porewater recovered in a sterile flask for subsequent analysis (Hewson and Fuhrman 2003). Conversely, the analysis of viral abundance in sediments (i.e., including viruses attached to sedimentary matrix) requires a specific treatment.

*Extraction of viruses for the analysis on sediment samples*—Extracting viruses from sediments is a relatively recent and unexplored discipline in aquatic viral methodology, and there exist no established consensus on how this is done most efficiently. We present here studies for examining the efficiency of various treatments in extracting viruses for subsequent counting by epifluorescence microscopy.

Sediment samples can be collected by means of manual corers, multiple-corer, or other sampling devices allowing to recover a perfectly undisturbed sediment surface. Here we compared two sediment types: (a) sandy coastal sediments, collected in the Adriatic Sea (Mediterranean) and in Øresund, Denmark, and (b) muddy deep-sea sediments collected in the Northeastern Atlantic Ocean (at 4800-m depth).

Immediately after the recovery of samples, sediment slurries were made with about 0.5 mL of the top 1 cm of both sediment types (taken from independent cores and independent deployments), and 4.5 mL sterile and 0.02 μm prefiltered seawater, to avoid the burst of potentially infected cells (and the consequential release of viruses and DNA) due to changes of osmotic pressure. This protocol is also applicable for the estimate of viral abundance in freshwater sediments; in this case, the dilution steps should be performed with freshwater. Samples were immediately brought in the laboratory for their analysis without the use of preservatives, to avoid virus loss due to formalin fixation (Danovaro et al. 2001). Alternatively, the slurry is immediately frozen at –20°C or frozen in liquid nitrogen and stored at –80°C until further processing (see below). In our experience, no changes in viral counts can be detected within 6 months of storage. If sampling of the sediment is not possible at sea, the intact cores can be closed with rubber stoppers and transported to the laboratory in a water bath at in situ tem-

perature, where they are then processed. The spatial distribution of viruses in sediments is very heterogeneous even on micrometers to centimeter scales (Middelboe et al. 2006). It is therefore important to consider whether the given sediment should be homogenized before sampling to obtain an average viral density in a given sediment layer or whether one is interested in including the small scale variability in the analysis.

Materials used for the assessment and detailed information on buffers and solutions for the optimized protocol are given below. The optimized protocol for the separation of free virus particles from sediments is shown in Fig. 1.

*Chemical treatment*—The extraction of the porewater can be accomplished by centrifugation or filtration. Conversely, the first step for the detachment of the viruses from the sediment is a chemical treatment. Among the surfactants currently used by different authors to detach the viruses from sediment particles, the most widely used are (i) tetrasodium pyrophosphate (SIGMA; Danovaro et al. 2001), (ii) a mix of 10 mM pyrophosphate and 5 mM EDTA (Hewson and Fuhrman 2003), (iii) PBS (1:2 vol: vol, sediment and PBS, respectively; Hewson et al. 2001), (iv) polyoxyethylene-sorbitan monooleate (Tween 80, SIGMA). All of these surfactants are suitable for viral counting under epifluorescence microscopy, with the exception of Tween 80 that displays lower performance for interference with the visualization of the viruses on EFM but has been reported to be highly efficient for analysis by flow cytometry (Duhamel and Jacquet 2006).

In this study, we optimized the protocol for the counting of benthic viruses under epifluorescence microscopy using tetrasodium pyrophosphate (5 and 10 mM; i.e., 250 or 500 µL of a 100 mM solution in a 5 mL slurry). Samples were then incubated for 15 min in the dark at room temperature. Additional sediment samples ($n$ = 3; 0.5 mL) without pyrophosphate were added to 4.5 mL of MilliQ water and served as controls. After incubation, all samples were shaken manually for 1 min and then centrifuged (800$g$; 1 min) to reduce interference due to suspended particles.

Aliquots of the supernatant were diluted 500 to 1000 times before filtration onto 0.02-µm-pore-size Anodisc 25 membrane filters (Whatman) under void-pressure <100 mmHg. The final dilution of sediments depends on the actual viral abundance on the filter. Typically, the final dilution of the sediments is 100 times for sandy sediments, 500 times for sandy-muddy sediments, whereas in virus-rich sediment samples the optimal final dilution could be up to 1000 times. The choice of the correct dilution, which must be checked for all sediment types, is of vital importance for the final result: an excessive or an insufficient number of viruses on the filter could invalidate the final calculations. A low filtration pressure is needed to avoid the damages or burst of prokaryotic cells, and the consequential release of nucleic acids that, binding the fluorochrome, could interfere with virus counting.

For sediment samples, the filters are typically stained with 20 µL of SYBR Green I (Lot no. 4967-30; diluted to 500× in sterile MilliQ water; optical density at 495 nm = 1.357) and incubated for 15 min in the dark, rinsed twice with 1 mL MilliQ water (to eliminate fluorescence background noise), and analyzed by EFM using a Zeiss Axioplan microscope equipped with a 100-W lamp. Ten to 50 fields were viewed at 1000× magnification, and a minimum of 400 viruses was counted. Virus-like-particles (VLP) were discriminated from bacteria (0.2- to 2-mm diameter) by their dimensions (0.015- to 0.2-mm diameter; Noble and Fuhrman 1998). As sodium pyrophosphate–enhanced virus extraction efficiency, all subsequent steps were carried out using this surfactant. Details on the procedure for staining the samples before counting under epifluorescence microscopy are reported in Patel et al. (2007) and can be found also in Suttle and Fuhrman (2010, this volume).

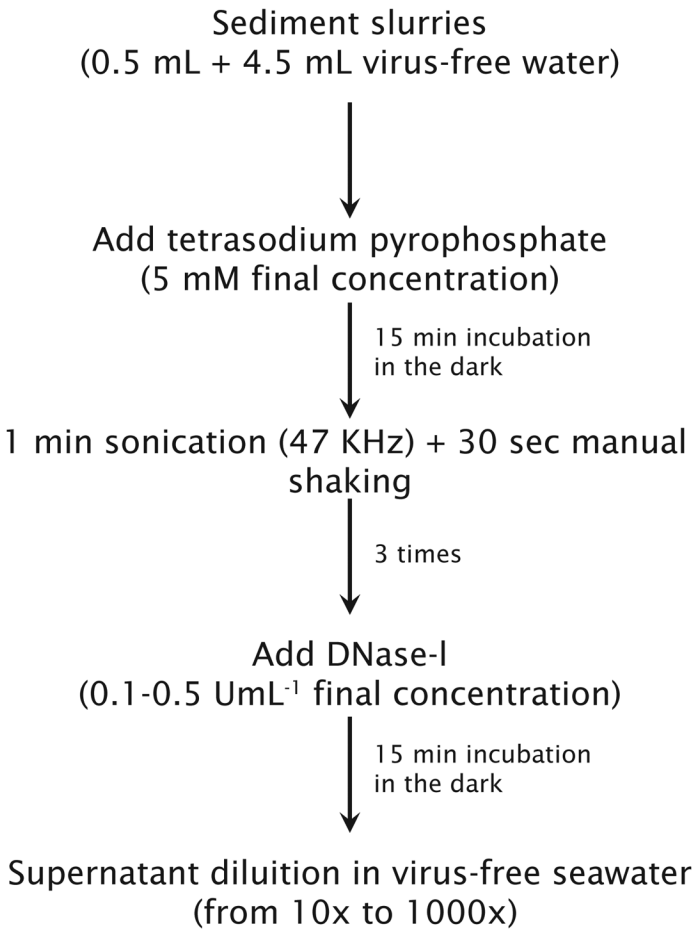


**Fig. 1.** Protocol illustrating the steps required for the separation of viruses from the sediment particle and subsequent counting.

*Physical treatment*—To test the combined effects of pyrophosphate and ultrasound treatments on virus extraction, muddy and sandy sediments ($n$ = 3; 0.5 mL for both sediment types) were added to pyrophosphate (5 mM final concentration) and treated by ultrasounds (Branson 2200 sonifier; 100 W; 47 kHz) for 0, 1, 3, 8, and 15 min. To prevent overheating,

ultrasonication was performed in ice bath. If the same sample is used for counting prokaryotes, it must be noted that the addition of ice to the sonication bath could result in a significant decrease of free prokaryotes in the sample (Duhamel and Jacquet 2006). The ultrasonication (with 30 s intervals every min) interval was alternated with gentle shaking. Centrifugation have been shown to remove a fraction of the viruses and prokaryotes in the sample, which are still associated with particles (see below), thus potentially underestimating their total abundance. In this case, however, it was used to analyze the efficiency of sonication treatment for releasing viruses from particles. After centrifugation, aliquots of the supernatant were processed as described above.

*Postsonication extraction efficiency*—Many of the initial studies of benthic viruses applied a centrifugation step and a series of washing steps following the sonication of the sediment slurry (e.g., Fischer et al. 2005; Danovaro et al. 2001; Middelboe et al. 2003). The purpose of centrifugation is to reduce the number of particles, which interfere with the subsequent analysis of viral particles by epifluorescence microscopy (e.g., by covering the viral particles and by autofluorescence of colloidal sized sediment particles). In the present study, the efficiency of virus detachment from sediment particles was checked by estimating the ratio of virus abundance after the first extraction with ultrasound and pyrophosphate treatment versus the cumulative virus abundance obtained by this procedure plus three further washing steps. The added steps were (i) an aliquot of supernatant obtained from deep-sea sediment samples (0.5 mL sediment plus 4.5 mL MilliQ water and sodium pyrophosphate) after sonication (3 min) and centrifugation was withdrawn and treated for counting as described above; (ii) the remaining supernatant was carefully discharged, the pellet was resuspended with 5 mL MilliQ water, shaken for 1 min, and centrifuged again, an aliquot of the supernatant was withdrawn, and viruses were counted as describe d above; and (iii) this procedure was repeated three times (since after the third washing, less than 5% of the total virus abundance was encountered).

Alternatively, instead of washing and centrifugation, the sample can be diluted to a point where the sediment particles do not interfere significantly with the analysis of viruses in the epifluorescence microscope. In that case the last sonication step should be followed by dilution of the sample with 0.02 µm filtered bottom water up to 40 mL (instead of centrifugation). It is convenient to perform the whole extraction procedure (slurry, incubation with surfactant, sonication, and dilution) in a 50 mL centrifuge tube. The diluted sample is then gently mixed, and one can either prepare a slide with 10-200 µL sample and/or take a 1 mL subsample, snap freeze it in liquid nitrogen, and store at –80°C.for later slide preparation.

One of the implications of this is that it is slightly more difficult and time consuming to count viruses in the microscope, especially with samples collected deeper sediment layers (e.g., >10 cm) as the ratio between background noise (from sediment particles) to actual viral abundance increases with sediment depth. The advantage, on the other hand is that the extraction procedure is faster when compared with a series of centrifugation and washing steps, hence also reducing the handling time of viruses and therefore the decay of viruses that goes on in the period between sampling and slide preparation.

*Interference with virus enumeration due to extracellular DNA*—To eliminate uncertainties in virus counting that we found sometimes associated with the presence of a matrix of extracellular DNA (Danovaro unpubl. data), we tested the effect of nuclease treatment on sediment samples. Twenty-five microliters of DNase I from bovine pancreas (1.9 U mL $mL^{-1}$), 10 mL nuclease P1 from *Penicillium citrinum* (4 U mL $mL^{-1}$), 10 mL nuclease S1 from *Aspergillus orizae* (2.3 U $mL^{-1}$), and 10 mL esonuclease 3 from *Escherichia coli* (1.9 U mL $mL^{-1}$) were added to 1.0 mL aliquots of the supernatant obtained from fresh sandy sediments and incubated for 15 min at room temperature. Additional aliquots of the supernatant (1.0 mL) without enzymes were incubated under the same conditions and served as controls.

## Assessment

The extraction optimization was divided into 3 phases, which were optimized to obtain the highest virus recovery from sediments as described here below.

*Chemical treatment*—The pyrophosphate extraction efficiency was tested comparing treated sediments (final concentrations 5 and 10 mM) with untreated sediments (Fig. 2). Results presented here indicate that the use of pyrophosphate is highly effective for the dislodgement of viruses from sediment particles, but the choice of an optimal concentration is crucial for the subsequent viral counting. Deep-sea sediment samples incubated with sodium pyrophosphate (5 mM final concentration) displayed higher viral counts than untreated samples ($14.6 \pm 2.79 \times 10^9$ versus $9.94 \pm 6.12 \times 10^9$ viruses $g^{-1}$, respectively). Pyrophosphate concentrations >5 mM in sediment samples did not increase significantly the extraction efficiency. On the contrary, too high pyrophosphate concentrations were found to decrease the stain contrast thus interfering with viral enumeration. Shorter incubation times (3-10 min) were less effective in detaching viral particles from the sediments, whereas longer incubation times did not increase the recovery efficiency (data not shown).

*Physical treatment*—The effects of sonication on virus counts was tested for 0, 1, 3, 8, and 15 min on coastal and deep-sea sediments. Results are reported in Fig. 3. The highest virus counts for both sediment types (1.1 and $11.1 \times 10^9$ viruses $g^{-1}$ for coastal and deep-sea sediments, respectively) were obtained after 3 min of sonication and were significantly higher (two- to fourfold; *t*-test; $P < 0.01$) than values obtained without sonication (i.e., with simple shaking). Treatments longer than 3 min resulted in a progressively lower viral count, and a sonication lasting 15 min reduced virus counts by about 1 order of magnitude (*t*-test; $P < 0.01$ for both sediment types). It must be underlined that our results were

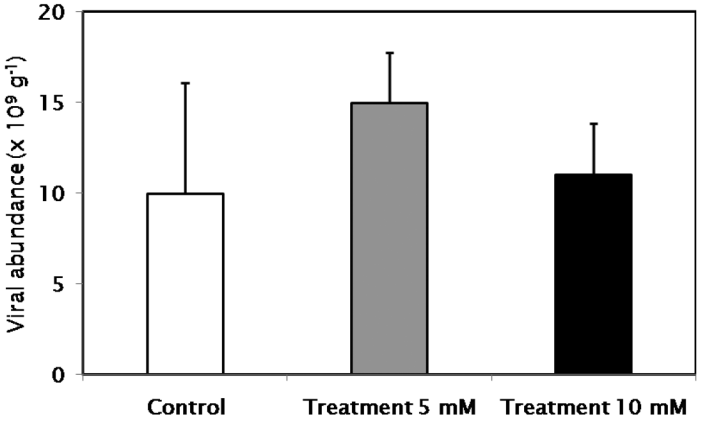


**Fig. 2.** Virus abundance in deep-sea sediments treated and untreated with tetrasodium pyrophosphate 5 mM final concentration. Standard deviations ($n$ = 10) are shown.

obtained in both coastal and deep-sea samples. However, the maximum extraction efficiency at the third minute of sonication varied significantly among the two sediment types, ranging from 55% in deep sea muds to 70% in coastal sands.

When we used the pyrophosphate-ultrasound treatment to dislodge viruses from sediment particles, we paid special attention to avoid the disruption of virus-infected prokaryotic cells, which might release virus particles, and thus falsifying the number of free viruses originally present in the sample. This risk was minimized by performing the ultrasound treatment in an ice bath and interrupting the treatment every minute for 30 s, to prevent overheating and further alterations of samples before counting. Finally, it should be taken into account that, as observed for prokaryotes, the optimal sonication time may strongly depend on the sonicator model and settings (Epstein and Rossel 1995), and may therefore vary considerably among laboratories.

*Postsonication extraction efficiency*—The efficiency of virus extraction by pyrophosphate-ultrasound treatment can vary significantly among different sediment types. Typically ca. 60% total extractable viruses are obtained after the first step, but the abundances of viruses extracted by this procedure were significantly lower than the total cumulative virus abundance ($t$-test; $P < 0.01$). The subsequent first and second washings recovered 27.5% and 9.0% of the total virus abundance, and after the third wash step, <5% was recovered (Fig. 4). A quick centrifugation (at 800$g$ per 1 min) can be conducted after the chemical and physical treatments to reduce the presence of sediment particles and pyrophosphate in the processed supernatant, but it has to be checked carefully the potential loss of viruses associated with this step. In muddy deep-sea sediment samples, a reduction of sediment particles by centrifugation can be required to count viruses on microscope slides without severe interference from nonviral particles. However, a recent study (Siem-Jørgensen et al. 2008) examining extraction of viruses and bacteria from a silty, shallow (35 m water depth) sediment, found that the number of viruses and bacteria kept increasing for 9 subsequent washings. In that case, the extracted fraction after three washes only represented ~60% and ~45% of viruses and bacteria, respectively, of the numbers that would have been extracted after 8 washes (Fig. 4b). Tests have been performed to compare the centrifugation/washing procedure with the more simple dilution procedure, where the sonicated sediment is diluted rather than going through repeated steps of centrifugation and washing. The tests showed that even very low centrifugation removes bacteria and viruses entrapped by settling particles, and underestimated viral and bacterial abundance by an average factor of 2.2 ± 0.17 and 7.7 ± 0.27 ($n$ = 15), respectively, relative to the dilution method (Fig. 4c). Centrifugation and washing of sediment samples during extraction can thus represent a significant source of error in the quantification of viruses in sediment compared with the more simple dilution procedure, which probably vary between different types of sediment, depending on the efficiency of viral attachment to the particles. Furthermore, the washing and centrifugation

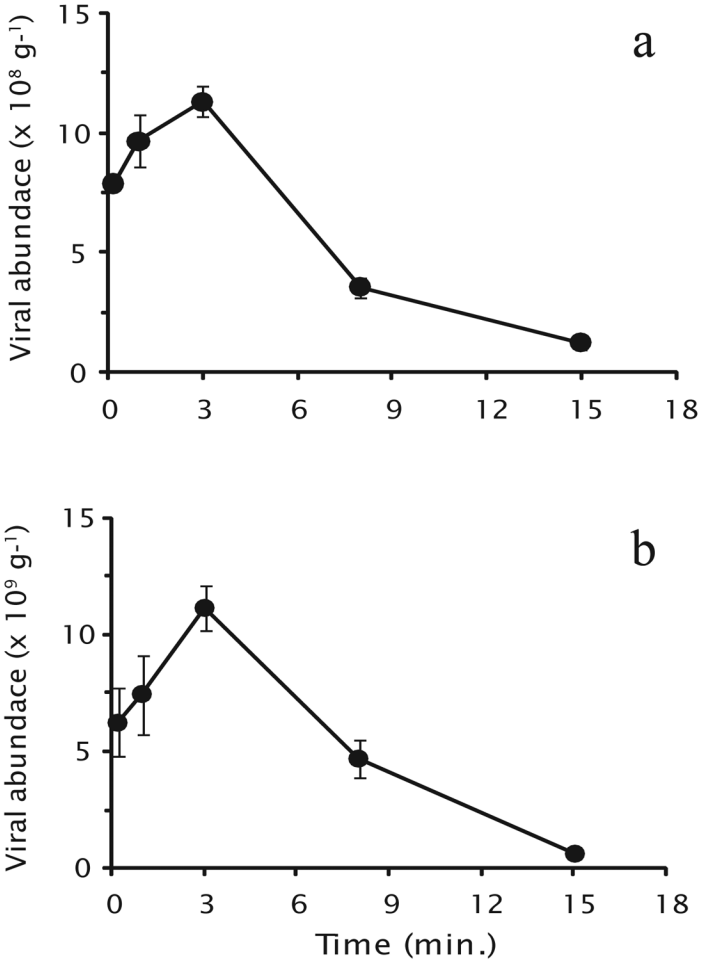


**Fig. 3.** Effect of sonication on virus abundance in surface (a) and deep-sea (b) sediments. Standard deviations ($n$ = 3) are shown.

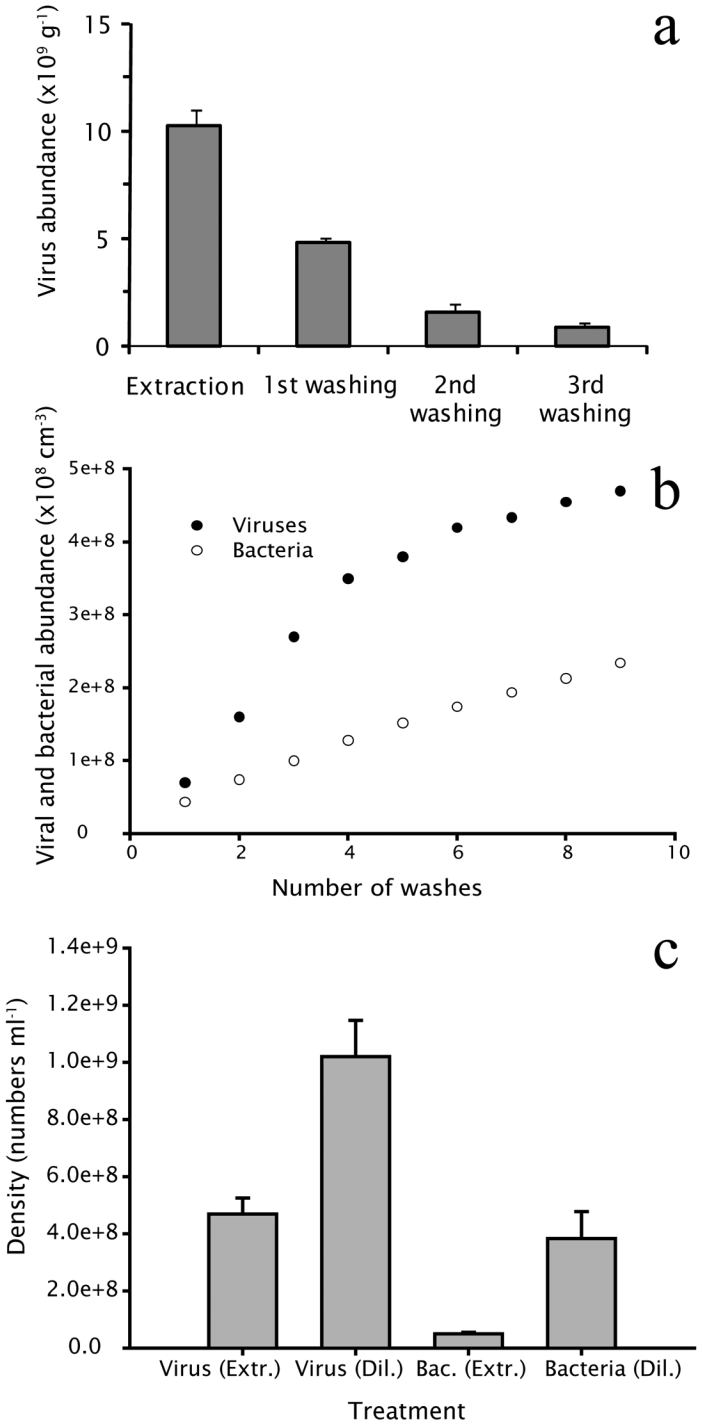


**Fig. 4.** Postsonication extraction efficiency of virus recovery from (a) deep-sea sediments and (b) from shallow coastal silty sediments (panel b redrawn from a supplemental image from Siem-Jørgensen et al. 2008 and used with permission). (c) Effects of centrifugation and dilution, respectively, of sediment slurries prior to preparation of EFM slides for the recovery of viruses and bacteria in samples from a shallow coastal sediment.

procedures are quite time consuming, especially if such tests need to be performed for each type of sediment, or perhaps even each sediment depth.

*Efficiency of DNase treatment*—Viral abundances obtained after nuclease treatment were significantly higher than those observed in untreated samples ($5.11 \pm 0.15 \times 10^8$ and $4.62 \pm 0.19 \times 10^8$ viruses $g^{-1}$ of sediment dry weight, respectively; *t*-test; $P < 0.05$).

## Discussion

In the present work, we have optimized the original protocol for viral extraction from aquatic sediments using as a model the extraction process of prokaryotes from sediment samples optimized by Ellery and Scheyer (1984). All of the extraction steps of the protocol were tested for two common sediment types of aquatic environments: shallow sands and deep-sea muds. The extraction efficiency of each step was determined as viral counts determined by epifluorescence microscopy.

The use of surfactants as first step of chemical treatment is widely used in the study of benthic microbial ecology, viruses included (Danovaro et al. 2001; Middelboe et al. 2003; Fischer et al. 2005). Surfactants are able to weaken hydrophilic links among sedimentary and biological particles thus allowing the subsequent separation between viruses and sediment grains. Among the different surfactants commercially available, the most widely used in studies of benthic viral abundance is tetrasodium pyrophosphate. The literature provides contrasting results about the use of this surfactant. Middelboe et al. (2003) reported that the use of tetrasodium pyrophosphate significantly increases the extraction efficiency of viruses from estuarine sands, in a percentage ranging from ~60 to ~70%, whereas Duhamel and Jacquet (2006) found that the use of the only pyrophosphate before FCM did not increase significantly viral counts in freshwater sands, and suggested to use a mix of pyrophosphate and Tween 80 (a nonionic detergent and an emulsifier) to obtain extraction efficiencies ranging from 25 to 40%. The analyses conducted on deep-sea muds revealed that the simple addition of pyrophosphate, although augmenting the average counts (Fig. 2), did not increase the extraction efficiency significantly (*t*-test; $P = 0.296$). The discrepancy observed comparing different sediment types was not due to differences in the final concentration of pyrophosphate solution (5 mM in all cases) or in the incubation time (15 min room temperature in all cases), and is likely dependent on the different mineralogical compositions of the substrates. Deep-sea muds, when compared with surface sands, displayed a finer sediment texture, and it is known that the silt fraction is able to create stronger electrostatic links with biological particles, including both benthic prokaryotes and viruses. This result suggests a lower extractability of viruses in silty sediments and requires the ultrasound treatment to enable the extraction of viruses (Fig. 3). Moreover, since viral sorption to sediment particles usually increases with increasing cation concentration in solution, particularly in the presence of divalent cations (Schijven and Hassanizadeh 2000), the observed differences in the extractability of viruses between marine and freshwater sediments might be due to differences in cation concentrations. Finally, because viral counts

are strongly influenced by the kind of analytical approach used, results obtained by epifluorescence microscopy (Middelboe et al. 2003) are difficult to compare with results obtained by flow cytometry (Duhamel and Jacquet 2006), and protocols developed for one approach could give different results if applied to the other.

Our results also indicate that pyrophosphate-treated samples were characterized by significantly lower CVs than untreated samples (19.1 versus 61.6%; *t*-test; $P < 0.05$). Similar results were reported for benthic prokaryotes (Epstein and Rossel 1995), suggesting that the use of pyrophosphate increases counting accuracy, making sufficient the counting of a lower number of optical fields. Minimizing the variability among replicates (i.e., obtaining low CVs) is also particularly important in viral counting, especially when differences among samples collected in different stations or in different incubation times are very low.

The extraction efficiency of viruses from sediment samples is significantly increased in all our samples with a further physical detachment by ultrasonication. In this work, we tested the optimal sonication time comparing virus abundance obtained by 6 different treatments, from 0 to 15 min (Fig. 3). By our results, the highest virus recovery is obtained after 3 min of sonication in both coastal sands and deep-sea muds. We obtained a higher extraction efficiency in sands (70%), whereas in deep-sea sediments viral abundance increased by 55% after 3 min of sonication. These results suggest that the optimal sonication time could vary among marine and freshwater sediments and could depend on the sediment grain size. Fischer et al. (2005) reported an optimal sonication time of 1 min in silty freshwater sediments, with an increase in virus counts of 11% to 27% compared with not-sonicated samples, whereas Middelboe et al. (2003) found that a 3-min sonication increased the extraction efficiency of 65% to 78% in estuarine sands.

The presented protocol allows extraction of most extractable viruses without postsonication washing. The postsonication extraction efficiency, tested in the deep-sea mud, revealed that extraction alone only released 60% of the extractable viruses. In these sediments, however, 90% of total extractable viruses were dislodged after a single subsequent washing. The effect of the washing procedure can vary among samples, but generally postsonication washings increase virus counts significantly from ca. 2% in coastal sands (Hewson and Fuhrman 2003) and ca. 11% to 40% in estuarine sediments (Middelboe et al. 2003; Siem-Jørgensen et al. 2008). Tests on the efficiency of the approach based on repeated washings versus the test based on sediment dilution have provided conflicting results. The first approach has the advantage of making clear slides easily read under epifluorescence microscopy, but requires a careful examination a priori to determine the extraction efficiency, the potential loss of viruses during centrifugation, and the subsequent coefficient for the calculation of the total viral abundance of the sediments. The approach based on sediment dilution has the advantage of avoiding the problems created by the centrifugation and the need for determining the coefficient of extraction efficiency, but depending on the sediment type, is possibly complicated by the difficulty of counting viruses in an optical field rich in sediment particles and the potential masking effects.

## *Comments and recommendations*

Our results also indicate that the use of DNase treatment is extremely useful, especially in muddy sediments (Fig. 5). It is conceivable that dissolved DNA within sediment samples could bind to fluorochromes and thereby inflate viral estimates. To eliminate uncertainties in viral counting due to extracellular DNA interference, we tested the effect of DNase treatment. Free, extracellular DNA could be extremely abundant in sediments (Danovaro et al. 1999; Dell'Anno et al. 1998; Dell'Anno et al. 1999; Dell'Anno and Danovaro 2005) and a nuclease treatment significantly reduce the fluorescence noise (due to the SYBR staining of extracellular DNA), which can have an important masking effect and determine the underestimation of the actual viral abundance in the sediment. We found that the sediment samples treated DNases displayed viral counts significantly higher than untreated samples (on average ~10% higher values). At the same time, it has been reported that in seawater samples DNases are able to degrade viral particles, thus influencing viral counts (Maruyama et al. 1993). To what extent this applies to the sediment is unknown yet, but data accumulated so far indicate that a final concentration of 1 U $mL^{-1}$ increases viral counts through the effect of increased visibility of the optical fields during counting.

Accurate quantification of benthic viral abundance is fundamental prerequisite for understanding spatial and temporal dynamics of benthic viruses. However, the study of benthic viruses is still a relatively new discipline in aquatic viral ecology and obviously more work is needed to optimize the extraction of viruses from sediments and clarify the efficiency of various procedures in dislodging viruses from particles in different types of sediments.

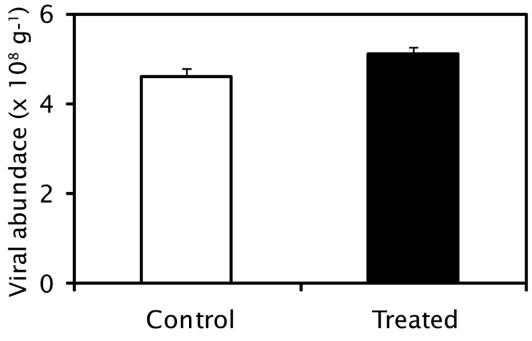


**Fig. 5.** Effect of DNase treatment extraction efficiency on muddy sediments. The standard deviations ($n = 3$) are shown.

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