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Flow cytometric enumeration of marine viral populations at low abundances

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ABSTRACT: Flow cytometric enumeration has advanced our ability to analyze aquatic virus samples and thereby our understanding of the ecological role viruses play in the oceans. However, low virus abundances are underestimated using the current flow cytometry (FCM) protocol. Our results revealed that low dilutions (<30-fold) not only decreased the total virus count but also limited the ability to differentiate between virus clusters. Here we report a simple and efficient method optimization for improving virus counts and optical resolution at low abundances. Raising the pH of the Tris-EDTA (TE) buffer to 8.2 successfully countered the effect of insufficient buffering capacity at low dilutions, which is caused by the higher proportion of acidic glutaraldehyde fixative in the final sample. The higher buffer pH did not interfere with virus counts at higher dilutions. We therefore recommend amendment of the standard FCM aquatic virus enumeration protocol using a TE buffer with pH 8.2 as a simple and efficient improvement.

KEY WORDS: Flow cytometry · Low abundance · Virus enumeration

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INTRODUCTION

Viruses are abundant, ubiquitous, and essential components of aquatic systems, playing key roles in the mortality of microbes, biogeochemical cycling, structuring host community composition, and genetic exchange between microbes (Sullivan et al. 2006, Suttle 2007, Brussaard et al. 2008b). Natural virus populations have been found to be highly dynamic, changing rapidly in abundance and diversity over a broad range of environments (Suttle & Chan 1994, Brussaard et al. 2008a, Winget & Wommack 2009). Therefore, aquatic viral ecology necessitates rapid and reliable methods for enumerating viruses.

The advent of flow cytometry (FCM) has vastly advanced our understanding of aquatic viral ecology (Brussaard 2004). Traditionally, viruses have been counted using culture-based methods (Cottrell & Suttle 1995, Suttle & Chan 1995), transmission electron microscopy (Bergh et al. 1989, Wommack et al. 1992, Bratbak et al. 1993), and epifluorescence microscopy (Hara et al. 1991, Hennes & Suttle 1995, Noble & Fuhrman 1998) in combination with nucleic acid-specific staining. However, these methods are limited by either the availability of a host system, cost, or time (Marie et al. 1999, Brussaard 2004, Duhamel & Jacquet 2006). FCM brought about immense improvements in the speed and accuracy of both the detection and enumeration of viruses in natural systems (Brussaard et al. 2000, Brussaard 2004). Rapid counting not only allows the analysis of more samples per time unit but also improves detection of stained viruses with low fluorescence compared to epifluorescence microscopy by reducing the poten-

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tial time for fading (Brussaard 2000). These improvements to the speed and accuracy of counting viruses using FCM, combined with the ability to discriminate different virus clusters (Brussaard et al. 2010), has increased the popularity of this method.

Low virus abundances (i.e. $\leq 10^6$ ml^{-1}), which can be found in the deep ocean or very oligotrophic waters or resulting from experimental design such as the viral production assay (Weinbauer et al. 2010), which reduces virus concentrations to permit the detection of the newly produced phage from infected bacteria, demand low dilutions in a buffer solution to obtain an optimal event rate (i.e. 200 to 800 events s^{-1}) (Brussaard et al. 2010). However, studies have shown that low dilution factors for virus samples can substantially underestimate the total virus concentration (Brussaard 2004). To overcome this limitation, the current study set out to (1) identify the factor responsible for the decline in viral abundance and the nucleic acid-specific staining signal at low dilution factors, and (2) improve the existing flow cytometric analysis method for the accurate enumeration of viruses present in natural samples at low abundances.

MATERIALS AND METHODS

Samples and FCM

FCM was used to measure the viral abundance in natural samples. Two geographical locations, i.e. the North Atlantic Ocean (STRATIPHYT summer 2009 cruise, samples obtained from 125 to 175 m, Stns 13 to 15, in the waters located between 42.3 to 44.3° N and 12.7° W) and the Southern Ocean (GEOTRACES austral spring 2008 cruise, samples obtained from 50 m in the central Weddell Sea at 66.2° S and 30.9° W) were used as model systems. These locations were chosen based on the abundance of viruses at these sites, which was around 10^6 ml^{-1} but sufficient to remain within the limits of measurement (i.e. 100 to 1100 events s^{-1}) at a 50-fold dilution (Marie et al. 1999, Brussaard 2004). As the viral abundance of these samples was not sufficient to test the influence of Tris-EDTA (TE) buffer pH on viral abundance at high dilution factors (i.e. 200- and 1000-fold), natural virus communities from the southern North Sea were used. Additionally, the effect of TE buffer pH 8.2 versus 8.0 was tested on natural virus communities originating from less saline locations, i.e. 2 estuaries (Wadden Sea and Baltic Sea) and 2 local freshwater ponds with very different pH values. To test these latter communities at a 10-fold dilution, natural samples were diluted (100×) using virus-free ultrafiltrate water from the same location prior to fixation (attained from tangential flow diafiltration using a 30 kDa VivaFlow 200 cartridge, Sartorius Stedim Biotech).

The method presented by Brussaard (2004) for counting viruses using flow cytometry was used as a standard. Briefly, samples were fixed with 25% glutaraldehyde (EM grade, Sigma-Aldrich) at a final concentration of 0.5% for 15 to 30 min at 4°C and subsequently flash frozen and stored at −80°C until analysis. In addition, formaldehyde (1 and 2% final concentration) fixation was also tested for a loss of virus counts at low dilution factors. Formaldehyde has been used for flow cytometric analysis and epifluorescence microscopy of natural virus samples and reported to show comparable counts to glutaraldehyde (Robinson et al. 1999, Patel et al. 2007)

In addition to diluting thawed samples in the standard TE buffer (pH 8.0, 10 mM Tris-HCl, 1 mM EDTA; Roche), Tris-borate-EDTA (TBE) (1×, 89 mM Tris, 89 mM boric acid, 2 mM EDTA) and Tris-acetate-EDTA (TAE) (1×, 40 mM Tris, 20 mM acetic acid, 1 mM EDTA) buffers were also tested. These buffers are recommended alongside TE buffer by the manufacturer of SYBR Green I (Life Technologies) and have not been tested previously. Other dilution solutions that have been tested (i.e. Tris, phosphate-buffered saline, dH_2O, and seawater) have shown that TE buffer provided the highest virus counts and green fluorescence signal (Brussaard 2004). After dilution in buffer solution, samples were stained with the nucleic acid-specific green fluorescence dye SYBR Green I at a final concentration of 5×10^{-5} the commercial stock concentration (Life Technologies) and heated at 80°C for 10 min in the dark. Cooled samples (5 min, room temperature) were analyzed using a 15 mW bench-top Becton-Dickinson FACSCalibur flow cytometer equipped with an air-cooled 488 nm argon laser and Milli-Q water (18 MΩ) as sheath fluid. The discriminator was set on green fluorescence, with the threshold at the lowest company-set level. The maximum voltage, at which no electronic or laser noise was detected, was used for the green fluorescence channel photo-multiplier according to the recommendations of Brussaard et al. (2010). Samples were analyzed for 1 min at a medium flow rate (~40 µl min^{-1}), after which the listmode files were analyzed using CYTOWIN (Vaulot 1989); www.sb-roscoff.fr/Phyto/index.php?option=com_content&task=view&id=72&Itemid=123). Virus counts were corrected for blanks consisting of TE buffer and

SYBR Green I prepared and analyzed in an identical manner to the samples. TE blanks were not found to be significantly different from blanks consisting of virus-free seawater samples generated by 30 kDa ultrafiltrate (2-sample *t*-test; df = 6; ns).

Treatments

Natural virus samples were subjected to different levels of sample dilution, salinity, and pH of TE buffer (Table 1), as well as type of fixative and type of buffer solution. The influence of salinity, which will vary according to the proportion of seawater present in the final sample because of the level of dilution with buffer, was tested. North Atlantic seawater ultrafiltrate (salinity 36.0) attained from tangential flow diafiltration (30 kDa VivaFlow 200, Sartorius Stedim Biotech) was subjected to slow evaporation using moderate heat until a maximum salinity of 41 was obtained. A range of salinities (Table 1) was then achieved by subsequent addition of increasing amounts of sterile ultrapure Milli-Q water (18 MΩ), while maintaining the ratios for constituent ions. Salinity was monitored using a digital conductivity meter (GMH 3430, Greisinger). The ultrafiltrate saline solutions were then used to dilute the North Atlantic viruses samples 5-fold, after which the samples were 10-fold diluted in the TE buffer at 3 different pH levels (7.8, 7.9, and 8.1). The result was a final dilution factor of 50, with the salinity impact of a factor 10 dilution.

The staining efficiency of SYBR Green I has been shown to be affected by pH, with significant drops in sensitivity occurring when pH is greater than 8.3 or less than 7.5 (Life Technologies, technical specifications). To test the effect of buffer pH on viral abundances measured at low dilutions, a range of pH was created by the addition of either 0.1 M NaOH (J. T. Baker) or 0.1 M HCl (J. T. Baker) to a working stock buffer solution. In addition, stepwise additions of 0.1 M HCl to glutaraldehyde-fixed North Atlantic samples at higher dilution (50× dilution in TE buffer at pH 8.0) were performed to verify the direct effect of pH, independent of the increase in glutaraldehyde. pH was monitored using a laboratory pH meter (827 pH lab with pH probe [6.0258.010], Metrohm Applikon). Total alkalinity was determined on a fixed-volume sample of unfiltered seawater poisoned with mercury chloride (Sigma-Aldrich) (0.05% final concentration of saturated mercury chloride). Potentiometric titration of seawater was performed using an open cell and computer-controlled titration instrument (Titrino DMP 785, Metrohm Applikon) and 0.1 M HCL + 0.6 M NaCl (Vstep of 0.05 ml). Total alkalinity was then calculated using the simple Gran and non-linear least-squares method (Dickson et al. 2003).

Table 1. Treatments investigated for effect on virus enumeration by flow cytometry

Treatment	Level
Dilution	10×, 15×, 20×, 30×, 50×
Salinity	26, 28, 30, 32, 34, 36, 38, 40
pH of TE buffer	7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5

Statistical analysis

Statistical analyses of different treatments were performed using R statistical software (R Development Core Team 2012). Assumptions for ANOVA were verified by the Shapiro-Wilks test for normality and the Bartlett's test for constancy of variance. If significant ($p < 0.01$) deviations were found, the Box-Cox transformation coefficient was used to find the optimal transformation. In the cases where lambda equaled 1, indicating that no transformations would improve data, non-parametric Kruskal-Wallis analysis was used. During ANOVA, all variables were initially included in the model with their interaction terms, and when necessary, the model was trimmed to remove any non-significant terms and interactions. When applicable, post-hoc analysis using Tukey's pairwise comparisons was performed. A probability of $\alpha < 0.01$ was used to conclude that the treatment levels differed significantly in the effect on the measured value. In the case where the effects of salinity (8 levels) and TE buffer pH (3 levels) were considered together, a factorial ANOVA model was fitted to data. Assumptions of equal variance for 2-sample Student's *t*-tests were verified by Fisher's *F*-test. When significant ($p < 0.01$) deviations were found, a nonparametric Wilcoxon rank-sum test was used.

RESULTS AND DISCUSSION

To optimize the staining of the viruses and avoid coincidence of particles during flow cytometric analysis, samples are diluted using TE buffer (Brussaard 2004). However, the enumeration of marine viruses in aquatic systems by FCM has been reported to be limited by a reduced efficiency in counts when the dilution in TE buffer is below 20-fold (Brussaard 2004).

Three virus groups (V1 to V3, Fig. 1) were differentiated based on green fluorescence and side scatter properties using bivariate scatter plots (Brussaard et al. 2010). We found that the dilution factor (i.e. 10 to 50×) of natural virus samples from the North Atlantic in TE buffer at the standard pH 8.0 had a significant effect on the measured abundance of total viruses (1-way ANOVA; $p < 0.0001$; Fig 2A) and V1 group viruses (1-way ANOVA; $p < 0.0001$; Fig 2B). Viral abundances dropped by 23, 13, and 5% for total virus counts and by 66, 49, and 19% for the V1 cluster when diluted 10-, 15-, and 20-fold, respectively.

Two constituents of a sample that could potentially account for the reduction in viral abundance at lower dilution are the increased proportion of the seawater and the fixative. Salinity, simulating the increasing proportion of seawater sample, did not significantly (factorial ANOVA) impact total virus or V1 counts. However, the pH of the TE buffer used for diluting samples was found to have a significant effect on the V1 group and total virus counts (both $p < 0.0001$). Adding glutaraldehyde (0.5% final concentration) to a 30 kDa ultrafiltrate seawater sample and diluting 10-fold in TE buffer at pH 8.0 demonstrated that the reduction in virus counts at low dilution was caused by the increased proportion of glutaraldehyde in the sample leading to a reduction in the pH, which was not sufficiently buffered against when using TE buffer at pH 8.0. The final sample pH was found to be 6.85, which fell out of the optimal range of 7.5 to 8.0 reported by Brussaard (2004) and the optimum pH of 8.0 recommended by manufacturer of SYBR Green I (Life Technologies, technical specifications). Stepwise additions of HCl to glutaraldehyde-fixed North Atlantic samples at a higher dilution (50× dilution in TE buffer at pH 8.0) showed that the general patterns for the decline of total and V1 virus abundance could be reproduced without increasing the proportion of glutaraldehyde, supporting the assumption that pH was the main cause for the underestimation of counts at low dilutions.

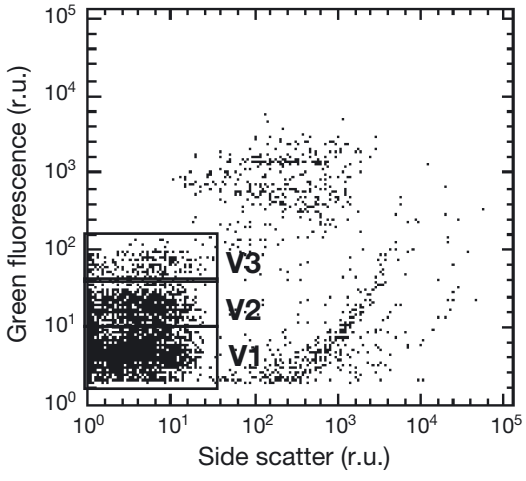

Fig. 1. Bivariate scatter plot of green fluorescent versus side scatter (relative units, r.u.) illustrating the V1, V2, and V3 virus groups, which together make up total viruses. Virus sample was obtained from the North Atlantic 2009 summer STRATIPHYT cruise (Stn 6, 60 m) and diluted 100× in TE buffer of pH 8.0

Adjusting the pH of TE buffer used for dilution led to improved total virus enumeration and differentiation between the different virus clusters for the North Atlantic Ocean and Southern Ocean virus communities (Fig. 3). At 10-fold dilutions, a pH of 8.2 showed the optimal balance between counts and staining signature. Raising the TE buffer pH from 8.0 to 8.2 at low dilutions resulted in an increase in the green fluorescence intensity of virus particles and subsequently increased the proportion of viruses that

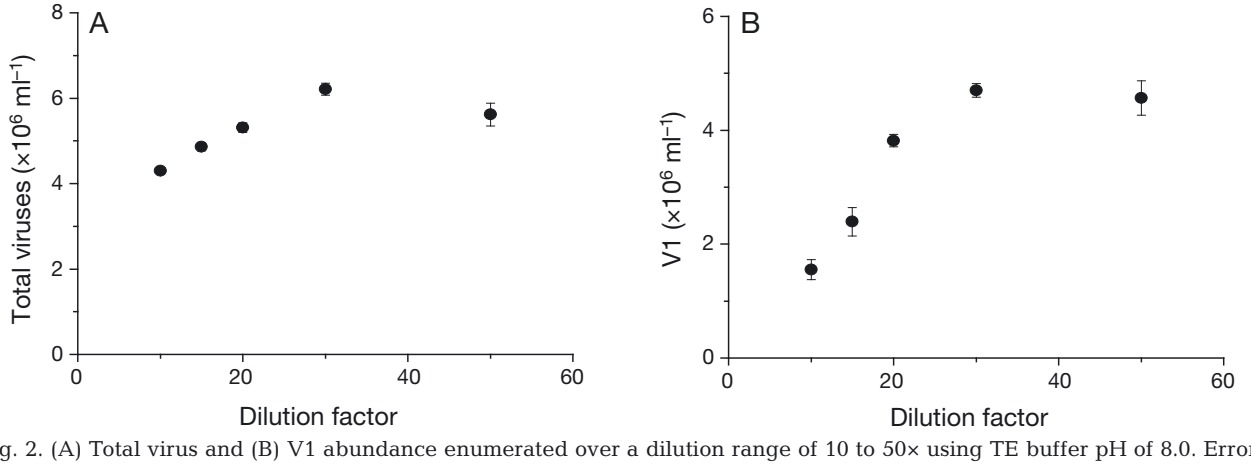

Fig. 2. (A) Total virus and (B) V1 abundance enumerated over a dilution range of 10 to 50× using TE buffer pH of 8.0. Error bars represent standard deviations (N = 3)

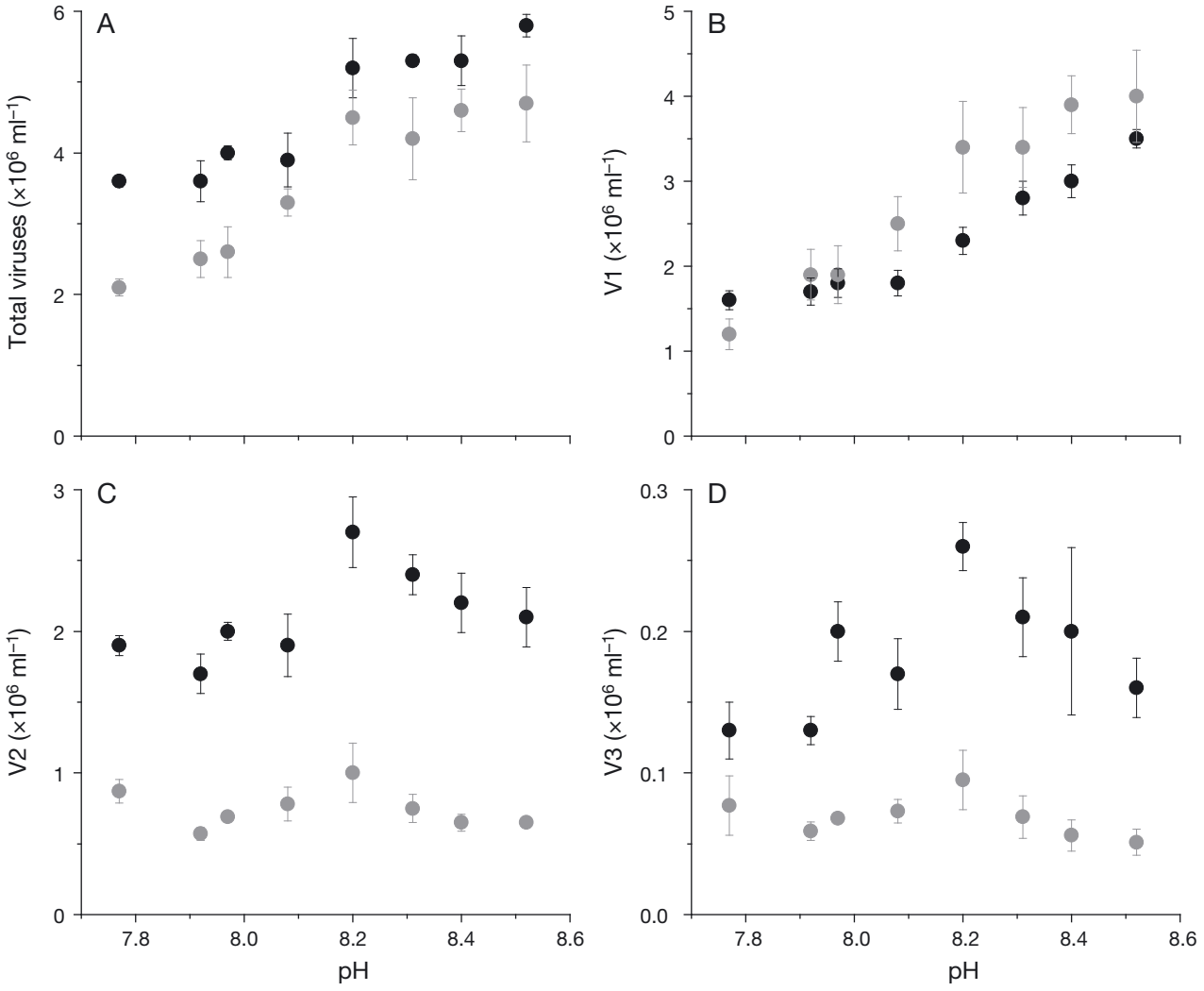

Fig. 3. Virus abundance enumerated at a 10-fold dilution using TE buffer at different pH levels. (A) Total virus abundance, (B) V1 virus abundance, (C) V2 virus abundance, and (D) V3 virus abundance in Southern Ocean (●) and North Atlantic (●) seawater. Error bars represent standard deviations (N = 4)

could be detected. The degree to which pH affected viral abundance, however, varied across the different sample locations, i.e. the 10-fold diluted North Atlantic virus samples had a proportionally higher reduction of total virus and V1 abundance at lower pH values compared to the Southern Ocean samples (Fig. 3A,B). Increasing the TE buffer pH to 8.2 when diluting 10-fold improved V1 and total virus counts by 28 and 31% in Southern Ocean samples and by 78 and 69% in North Atlantic Ocean samples, respectively, when compared to TE pH 8.0. The abundance of V2 and V3 groups of the North Atlantic samples remained relatively stable, with a small increase occurring in TE buffer pH 8.2 (Fig. 3C,D). However, the relative importance of V1 to V3 in Southern Ocean and North Atlantic samples remained comparable between TE pH 8.2 and 8.0, and as a consequence, there was little effect on the average green fluorescence. These results were not affected by the use of different FACSCalibur flow cytometers, as measurements for this experiment were performed simultaneously on 2 separate FACSCalibur cytometers and gave good correlations between counts (0.96 for the Southern Ocean and 0.93 for the North Atlantic samples).

When using TE buffer at pH 8.2, the dilution factor (i.e. 10 to 50×) no longer had a significant effect on the viral abundance (Kruskal-Wallis; df = 8; ns), demonstrating the effectiveness of pH 8.2 to alleviate the underestimation of virus counts at low dilutions. To verify that increased buffer pH did not affect viral counts at higher dilutions and would therefore be

applicable as a general method improvement, natural virus samples were diluted in TE buffer pH 8.0 and 8.2 at a 50-fold dilution for North Atlantic samples and at both a 200- and 1000-fold dilution for North Sea samples. At these high dilution factors, no significant differences (2 sample *t*-test; df = 8) were found in V1 or total viral abundance measured between samples diluted in TE buffer pH 8.0 and 8.2 for either location.

The effect of TE buffer pH 8.2 versus 8.0 was further tested on natural virus communities from different low salinity environments, i.e. 2 estuaries (Wadden Sea and Baltic Sea) and 2 freshwater ponds (Table 2). Increasing the pH of TE buffer to 8.2 had a relatively low (8%) but significant (2-sample *t*-test; $\alpha = 0.01$; N = 4) positive effect on the viral abundance measured in a 10-fold diluted sample from the Baltic Sea. However, no significant effect of TE buffer pH was found for the other locations tested (2-sample *t*-test; $\alpha = 0.01$; N = 4). The lack of effect for the Wadden Sea estuary was surprising considering that the alkalinity and pH of this water nearly matched that of the North Atlantic sample. The differences in the effect of pH 8.2 on viral abundance between samples are most likely dependent on variation in the staining sensitivity of the viral communities at these different locations.

The use of formaldehyde (1 and 2% final concentration) as a fixative was tested as an alternative to glutaraldehyde for both North Atlantic and Wadden Sea samples; however, no significant improvements were found (2-sample *t*-test; $\alpha = 0.01$; N = 3). Considering that commercially available formaldehyde (37%) has a pH range of 2.8 to 4.0, it presented the same methodological issues as glutaraldehyde (25%; pH range 3.0 to 4.0) when measuring at low dilutions in TE buffer pH 8.0. Moreover, the use of alternative buffer solutions (TAE and TBE, recommended in addition to TE by the manufacturer of SYBR Green I) did not lead to significant improvements in counts at low dilutions compared to dilution using TE buffer.

In summary, increasing the pH of TE buffer has the potential to significantly improve the efficiency of virus counts in aquatic systems when diluted to 10-fold dilution. TE buffer with a pH of 8.2 was found to be optimal, as it leads to significantly higher viral abundance (total and V1) compared to lower pH values, while providing the highest V2 and V3 counts. The beneficial effect of increased pH is not ubiquitous or consistent across all aquatic systems and does not appear to be dependent on sample pH or alkalinity, indicating that the magnitude of effect is dependent on the viral communities present in the sample. TE buffer at pH 8.2 did not have a significant negative effect on viral abundance in unaffected samples, was not found to affect the virus abundances at higher dilutions, and thus can be adopted for general use. While maintaining the best and most commonly used method for fixation and analysis, the modification in the pH of TE buffer is a simple and effective method to achieve vital improvement on viral enumeration at low dilutions. Increasing the accuracy and precision of virus counts in systems with low abundances has the potential to expand (or even open) the field of marine viral ecology, which is currently limited by the inability to measure viruses at low numerical abundances.

Table 2. Effect of TE buffer pH 8.2 (compared to 8.0) on total virus abundance in samples from various environments measured at a 10-fold dilution. TAlk: total alkalinity (Meq l^{-1}). Significant values in **bold**. Pond samples originate from Texel, Netherlands

Sample	Salinity	pH	TAlk	%Change
North Atlantic	35.7	7.87	2.37	**69**
Southern Ocean	34.3	8.09	2.41	**31**
Wadden Sea[a]	25.3	7.87	2.36	0
Baltic Sea[b]	5.7	8.15	1.59	**8**
NIOZ pond[a]	0.3	9.59	1.34	1
Den Hoorn pond[a]	0.4	7.94	2.17	0

[a]Samples were pre-diluted using virus-free sample to allow for 10-fold dilution. Salinity, pH, and alkalinity are reported for undiluted sample

[b]Viral production sample (Weinbauer et al. 2010) originating from a Baltic Sea mesocosm experiment

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