



Detection of Infectious Noroviruses from Wastewater and Seawater Using PEMAXTM Treatment Combined with RT-qPCR

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Abstract: Rapid detection of infectious noroviruses from environmental samples is essential to minimize the risk of norovirus outbreaks associated with environmental transmission. Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) methods are rapid and sensitive, but cannot differentiate between infectious and non-infectious noroviruses. In this study, a PEMAXTM treatment followed by RT-qPCR (PEMAXTM-RT-qPCR) method was developed for murine norovirus and norovirus GI/GII, and evaluated for the selective detection of infectious viruses following heat inactivation. The norovirus PEMAXTM-RT-qPCR method was then evaluated for the selective detection of infectious viruses from environmental samples. Following heat-treatment (90 °C for 3 min), the murine norovirus PEMAXTM-RT-qPCR showed at least a 2.04 log₁₀ reduction in detectable virus, compared to a $0.43 \log_{10}$ reduction for RT-qPCR alone. Under the same conditions, the norovirus PEMAXTM-RT-qPCR showed a 0.34 to 0.98 \log_{10} (GI.3) and 0.63 to 2.06 \log_{10} (GII.4) reduction in detectable viruses, compared to 0.05 to 0.18 \log_{10} (GI.3) and 0.06 to 0.25 \log_{10} (GI.4) for RT-qPCR alone. Evaluation of the norovirus PEMAXTM-RT-qPCR on norovirus-contaminated influent and effluent wastewater, and seawater indicated a high proportion of non-infectious norovirus GI and GII (i.e., 56 to 100% in seawater, 32 to 76% in effluent, and 11 to 79% in influent) was present in samples. While potentially overestimating the amount of infectious noroviruses, this approach has potential to provide better information on viral infectivity than RT-qPCR alone.

Keywords: norovirus; PEMAXTM-RT-qPCR; infectivity assessment; environmental water; health risks

1. Introduction

Understanding persistence and inactivation of norovirus in the environment has been limited due to the lack of a robust, reproducible, and quantitative detection method that is able to detect only infectious viruses. Currently, sensitive and specific reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) methods are widely used for the detection and quantification of noroviruses from a wide variety of samples including water and food [1,2]. A disadvantage of this approach is that a positive RT-qPCR result can be produced by non-infectious viruses present in the sample [3], and therefore RT-qPCR data may not be suitable for a health risk assessment.

A viral receptor binding approach utilizing histo-blood group antigens has been used with the aim of detecting infectious noroviruses, however, the process is expensive, labor intensive, and not suitable for all norovirus strains [4,5]. Recently an *in vitro* norovirus culture method was successfully developed [6,7] but it is not straightforward to implement, expensive, and currently limited to a few research laboratories worldwide. Therefore, other approaches for the detection of infectious noroviruses from environmental samples are needed.



Photoactivatable dyes such as propidium monoazide (PMA) and ethidium monoazide (EMA) (Biotium, Inc., Fremont, CA, USA) have been used with RT-qPCR with some success for the selective detection of infectious norovirus using RT-qPCR [8–13]. Theoretically, these dyes penetrate a damaged capsid and, in the presence of light, a covalent bond forms with the nucleic acid, resulting in a reduction in nucleic acid extraction efficiency and in the RT-qPCR signal [14]. In studies with bacteria, Nocker et al. [15] reported that PMA did not enter inactivated bacteria with an intact cell membrane, and consequently overestimated their infectivity. Conversely, EMA entered the intact cell membrane of viable bacteria and underestimated their infectivity. A study on the combined use of PMA and EMA for the detection of *Salmonella* Enteriditis found that this approach was more effective for discriminating non-viable bacteria from viable ones than PMA and EMA alone [16]. An alternative approach is the double photoactivatable dye PEMAXTM (GenIUL, Barcelona, Spain), which consists of both PMA and EMA. To our knowledge, one study has evaluated the performance of PEMAXTM for the detection of infectious noroviruses, but the study lacked optimization of the PEMAXTM concentration [9].

In our study, the concentration of PEMAXTM for the selective detection of infectious viruses was optimized, and the efficiency of PEMAXTM-RT-qPCR evaluated for the selective detection of infectious murine norovirus and norovirus GI.3 and GII.4 from heat-inactivated samples. The newly developed PEMAXTM-RT-qPCR method was further evaluated using norovirus-contaminated influent and effluent wastewater and seawater samples.

2. Materials and Methodology

2.1. Viral Stock Suspensions

Murine norovirus was propagated in RAW 264.7 mouse leukemic monocyte/macrophage cells and quantified using a monolayer plaque assay to determine the plaque forming units (PFU)/mL as described elsewhere [17,18] and stored at -80 °C until required. The murine norovirus was diluted to give a 10^5 PFU/mL stock suspension.

Clarified suspensions (10% (wt/vol) in viral transport media) of norovirus GI.3 and GII.4 positive human faecal samples, submitted to ESR for norovirus outbreak surveillance purposes, were prepared and the stock suspensions stored at 5 $^{\circ}$ C until required (~4 weeks).

2.2. Recovery of Noroviruses from Wastewater and Seawater

Archived virus concentrations from wastewater and seawater samples were used in this study. Briefly, viruses from influent wastewater (2 L) collected from six different wastewater treatment plants were concentrated using beef extract elution followed by polyethylene glycol 6000 precipitation (10% wt/vol) with the addition of sodium chloride (1.75% wt/vol) [19]. Viruses from effluent wastewater (10 L) collected from two treatment plants and seawater (10 L) collected from an estuarine harbor were concentrated using an ultrafiltration method, and further concentrated using polyethylene glycol 6000 and sodium chloride as above [1]. Final concentrates of between 5–10 mL were obtained and stored at -80 °C until required.

2.3. Heat Inactivation of Murine Norovirus and Norovirus

To achieve inactivation of murine norovirus and norovirus GI.3 and GII.4 for the method development and evaluation, 100 μ L aliquots of each virus stock suspension were transferred into 200 μ L PCR tubes and heated at 90 °C for 3 min using a BioRad CFX thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). Two 100 μ L aliquots were pooled to give 200 μ L volumes for subsequent use.

2.4. Optimisation of PEMAXTM Concentration

To optimize the PEMAXTM (GenIUL) concentration, 0.5 mg PEMAXTM was first dissolved in 500 μL dimethyl sulfoxide (20% vol/vol) (Sigma-Aldrich, Auckland, New Zealand) to obtain a 2 mM

working solution. Aliquots (200 μ L) of heat-inactivated and non-heat-inactivated murine norovirus was transferred into 1.5 mL transparent centrifuge tube (GenIUL), and either 0, 50, 100, or 200 μ M PEMAXTM solution added to give a total volume of up to 225 μ L. The tubes were then incubated in the dark for 30 min and treated with the "Photo Activation System for Tubes" PhAST blue light (GenIUL) for 15 min. Viral nucleic acid was then extracted from the entire sample (at least 200 μ L) and tested for murine norovirus using a one-step RT-qPCR method (Section 2.8). Triplicate samples were processed to ensure the reproducibility of the method.

2.5. Evaluation of PEMAXTM-RT-qPCR for the Selective Detection of Infectious Murine Norovirus

An initial experiment was conducted to evaluate the efficiency of the PEMAXTM-RT-qPCR method for the selective detection of infectious murine norovirus. Ten-fold serial dilutions $(10^{-1} \text{ to } 10^{-4})$ of murine norovirus stock suspension (10^5 PFU/mL) were prepared and divided into four $(200 \ \mu\text{L})$ aliquots. Two sets of aliquots of 10^0 to 10^{-4} dilutions were heat-inactivated (90 °C for 3 min). One set of aliquots was treated with the optimal concentration of PEMAXTM (Section 2.4), and another set used as a control. Two sets of aliquots of 10^0 to 10^{-4} dilutions were used to determine the percentage of infectious viruses in the stock suspension. This was achieved by treating one set of original (non-heat-inactivated) aliquots with PEMAXTM and the other remained untreated. Viral nucleic acid was extracted from the entire sample (at least 200 μ L) and tested for murine norovirus using a one-step RT-qPCR method (Section 2.8).

2.6. Evaluation of PEMAXTM-RT-qPCR for the Selective Detection of Infectious Norovirus

As above, experiments were conducted to determine the efficiency of the PEMAXTM-RT-qPCR method for the selective detection of infectious norovirus GI.3 and GII.4. Ten-fold serial dilutions $(10^{-1} \text{ to } 10^{-4})$ of each genotype were prepared from the stock suspension (Section 2.1) and treated with PEMAXTM (Section 2.4). Viral nucleic acid was extracted from the entire sample (at least 200 µL) and tested for norovirus GI and GII using a duplex one-step RT-qPCR method (Section 2.8).

2.7. Application of PEMAXTM-RT-qPCR for Detection of Infectious Norovirus from Environmental Samples

Two 200 μ L aliquots of each wastewater and seawater concentrate were prepared for each experiment. One aliquot was treated with PEMAXTM at the optimized concentration (Section 2.4) and the other remained untreated. Viral nucleic acid was then extracted from the entire sample (at least 200 μ L) and tested using a duplex one-step RT-qPCR method (Section 2.8).

2.8. Nucleic Acid Extraction and Virus Detection

Prior to analysis by RT-qPCR, viral nucleic acid was extracted from 200–225 μ L (depending on the volume) of each sample using the High Pure Viral Nucleic Acid Kit (Roche Life Science, Mannheim, Germany) according to the manufacturer's instructions. A reagent blank (water) was included during viral nucleic acid extraction. All nucleic acid samples were tested for potential PCR inhibition by using a sketa22 qPCR method as described elsewhere [20]. No PCR inhibition was detected in any of the samples and were used for downstream analysis.

Previously published primers and probes were used for the detection of norovirus GI/GII [21] and murine norovirus [18]. One-step RT-qPCR was performed using the SuperScriptTM III PlatinumTM One-Step RT-qPCR Kit (Invitrogen Corporation, Carlsbad, CA, USA). Each 25 μ L reaction mix contained 12.5 μ L 2× Reaction Mix, 0.5 μ L RNaseOUT, 0.5 μ L SuperScriptTM III PlatinumTM TaqMix, 2.5 μ L viral nucleic acid, and appropriate concentrations of primers and probes [18,21]. For each RT-qPCR run, corresponding positive controls (known viral RNA standards and DNA plasmids) and negative controls (DNase/RNase-free water) were included. One-step RT-qPCR assays were performed using a BioRad CFX 96 thermal cycler (Bio-Rad Laboratories). The qPCR C_T values were determined using the BioRad CFX ManagerTM 3.0 software (Bio-Rad Laboratories). All the RT-qPCR

runs were carried out in triplicate. To minimize cross-contamination, the sample preparation, nucleic acid extraction, RT-qPCR reagent preparation, and testing were performed in separate laboratories using dedicated equipment.

2.9. Statistical Analysis

The effect of PEMAXTM on original (non-heat-inactivated) and heat-inactivated viruses were determined by calculating log_{10} reduction of genome copies using the qPCR C_T values. Following equations were used to calculate the log_{10} and percentage reductions in detectable genome copies.

$$\Delta\Delta C_T = \Delta C_{TC} - \Delta C_{TS} \tag{1}$$

$$R_n = 100 - (1/POWER (2, (-\Delta \Delta C_T)) \times 100)$$
(2)

$$Log_{10} R_n = -(Log_{10}((-R_n/100) + 1))$$
(3)

where,

 $\Delta C_{TC} = qPCR C_T$ value for control samples $\Delta C_{TS} = qPCR C_T$ value for PEMAXTM-treated samples

 $\Delta\Delta C_T$ = Difference in qPCR C_T value

 R_n = Percent reduction

 $Log_{10} R_n = Log_{10}$ reduction

The statistical difference in qPCR C_T values and \log_{10} reductions in genome copies were assessed using analysis of variances (ANOVA) using Microsoft Excel.10. *p* values less than 0.05 were considered significant.

3. Results

3.1. Optimization of PEMAXTM Concentration

None of the PEMAXTM concentrations completely eliminated RT-qPCR amplification from heat-inactivated murine norovirus. The 200 μ M concentration produced the largest difference between the mean qPCR C_T value (4.4 \pm 0.04) of the PEMAXTM treated and untreated heat-inactivated murine norovirus. This was followed by 100 μ M (4.0 \pm 0.02 difference) and 50 μ M (3.3 \pm 0.03 difference) (Figure 1). However, the difference between the mean qPCR C_T values of 200 μ M and 100 μ M was not significant (p = 0.09). Therefore, a PEMAXTM concentration of 100 μ M was used for the further experiments.



Figure 1. Differences in qPCR C_T values (mean and standard deviation) for original (non-heat-inactivated) and heat-inactivated murine norovirus with and without PEMAXTM treatment.

3.2. Evaluation of PEMAXTM-RT-qPCR for the Selective Detection of Infectious Murine Norovirus

The original (non-heat-inactivated) murine norovirus with no PEMAXTM treatment was detected for all five concentrations (10⁰ to 10⁻⁴ dilutions) with mean qPCR C_T values ranging from 26.1 ± 0.1 (10⁰ dilution) to 41.5 ± 0.3 (10⁻⁴ dilution) (Figure 2). When the non-heated samples were treated with PEMAXTM, the virus was only detected in the 10⁰ to 10⁻² dilutions with mean qPCR C_T values ranging from 27.6 ± 0.1 (10⁰ dilution) to 37.7 ± 0.3 (10⁻² dilution).

For heat-inactivated murine norovirus with no PEMAXTM treatment, the last dilution (10^{-4}) was no longer detectable, with mean qPCR C_T values of the positive samples ranging from 28.1 \pm 0.1 (10⁰ dilution) to 39.5 \pm 0.2 (10⁻³ dilution). For these samples, the mean qPCR C_T values obtained after PEMAXTM treatment were significantly (p < 0.05) higher (34.7 \pm 0.2 (10⁰ dilution) to 40.4 \pm 0.4 (10⁻¹ dilution), respectively, than those which were not treated with PEMAXTM (Figure 2). The PEMAXTM treatment resulted in a significant reduction (1.98 to 2.14 log₁₀) of murine norovirus in heat-inactivated samples for the detected dilutions compared to the original (non-heat inactivated) samples (0.43 to 1.06 log₁₀ reduction) (Figure 3).



Figure 2. qPCR C_T values (mean and standard deviation) of serially-diluted original (non-heat-inactivated) and heat-inactivated murine norovirus with and without PEMAXTM treatment.



Figure 3. Log₁₀ reduction (mean and standard deviation) of original (non-heat-inactivated) and heat-inactivated murine norovirus after PEMAXTM treatment.

For original (non-heat-inactivated) norovirus GI.3, PEMAXTM treatment did not produce a significant difference in qPCR C_T values (Figure 4a). However, there was a significant (p < 0.05) difference in qPCR C_T values obtained for the heat-inactivated samples in all dilutions (10^0 to 10^{-4}) after PEMAXTM treatment. The difference in the mean qPCR C_T value was higher in the 10^{-4} (-3.3 ± 1.2) and 10^0 (-3.0 ± 1.0) dilutions. Similar results were observed for non-heat-inactivated norovirus GII.4 samples (Figure 4b). However, the difference in the mean qPCR C_T value for heat-inactivated norovirus GII.4 samples with and without PEMAXTM treatment were greater than for GI.3. The difference in the mean qPCR C_T value for heat-inactivated norovirus GII.4 samples with and without PEMAXTM treatment were greater than for GI.3. The difference in the mean qPCR C_T value for heat-inactivated norovirus GII.4 samples with and without PEMAXTM treatment were greater than for GI.3. The difference in the mean qPCR C_T value for heat-inactivated norovirus GII.4 samples with and without PEMAXTM treatment were greater than (GI.3. The difference in the mean qPCR C_T value for heat-inactivated norovirus GII.4 samples with and without PEMAXTM treatment ranged from -7.5 ± 1.2 (10^{-4} dilution) to -1.8 ± 0.6 (10^{-3} dilution) (Figure 4b).

The PEMAXTM treatment resulted in a 0.34 to 0.98 \log_{10} reduction of detectable norovirus GI.3 in heat-inactivated samples (Table 1). However, a significantly (p < 0.05) higher reduction (0.53 to 2.26 \log_{10}) was achieved for heat-inactivated norovirus GII.4 with the PEMAXTM treatment. The \log_{10} reduction was greater when lower virus concentrations (i.e., 10^{-4} dilution) were used for both norovirus genotypes.

Table 1. Relative \log_{10} reduction (mean and standard deviation) of norovirus GI.3 and GII.4 concentrations in original (non-heat-inactivated) and heat-inactivated suspensions after PEMAXTM treatment.

	Relative log ₁₀ Reduction in Norovirus Concentration						
Dilutions _	(GI.3	GII.4				
	Original	Heat-Inactivated	Original	Heat-Inactivated			
100	0.11 ± 0.02	0.91 ± 0.16	0.05 ± 0.08	1.12 ± 0.13			
10^{-1}	0.05 ± 0.25	0.47 ± 0.12	0.06 ± 0.01	2.03 ± 0.08			
10^{-2}	0.17 ± 0.11	0.34 ± 0.21	0.13 ± 0.03	0.63 ± 0.07			
10^{-3}	0.17 ± 0.28	0.43 ± 0.13	0.15 ± 0.01	0.53 ± 0.03			
10 ⁻⁴	0.05 ± 0.04	0.98 ± 0.35	0.25 ± 0.09	2.26 ± 0.02			

3.4. Application of PEMAXTM-RT-qPCR for Detection of Infectious Norovirus from Environmental Samples

A total of 10 samples [six influent wastewater (samples I1–I6), two effluent wastewater (samples E1 and E2) and two seawater (samples S1and S2)] were tested for norovirus GI and GII. With the exception of one influent wastewater sample (I4), that was negative for norovirus GI, all samples were positive for both norovirus GI and GII using RT-qPCR. For norovirus GI, PEMAXTM treatment resulted in its non-detection using RT-qPCR for both seawater samples (S1 and S2) compared to the PEMAXTM untreated samples (Table 2). The PEMAXTM treatment resulted in significantly higher qPCR C_T values for both effluent (E1 and E2) and 60% (3/5) of the influent samples. Similar results were observed for norovirus GII. The PEMAXTM treatment resulted in complete elimination of qPCR amplification for one of the seawater samples (S2) and resulted in a significant increase in mean qPCR C_T value for the other seawater sample (S1), both effluent samples and 50% (3/6) influent samples.

The analysis showed that although both seawater samples (S1 and S2) did not contain infectious norovirus GI, it did indicate that 44% of the norovirus GII detected in one seawater sample (S1) was infectious (Table 3). For the effluent samples E1 and E2, infectious norovirus GI (59% and 32%, respectively) and GII (24% and 52%, respectively) were detected. The analysis also indicated the presence of non-infectious norovirus (19 to 79% GI and 11 to 63% GII) in influent samples.

Sample Description	Sample ID	Norovirus GI			Norovirus GII		
		qPCR C_T Values (Mean \pm SD)		AA C _T	qPCR C_T Values (Mean \pm SD)		
		(-) PEMAX TM	(+) PEMAX TM		(-) PEMAX TM	(+) PEMAX TM	$\Delta\Delta C_T$
Influent wastewater	I1	34.9 ± 0.24	36.6 ± 0.73	-1.65	33.2 ± 0.61	33.8 ± 0.36	-0.53
	I2	36.5 ± 0.60	38.6 ± 0.15	-2.14	28.1 ± 0.04	29.5 ± 0.07	-1.42
	I3	34.9 ± 0.28	35.2 ± 0.23	-0.30	27.2 ± 0.07	27.4 ± 0.12	-0.17
	I4	ND	ND	NA	40.6 ± 1.01	40.9 ± 0.67	-0.22
	15	37.5 ± 0.10	39.8 ± 0.23	-2.23	31.5 ± 0.07	32.6 ± 0.14	-1.15
	I6	30.4 ± 0.36	31.9 ± 0.10	-1.42	31.4 ± 0.01	32.7 ± 0.23	-1.29
Effluent wastewater	E1	39.6 ± 0.32	40.4 ± 0.10	-0.76	37.2 ± 0.26	39.3 ± 0.98	-2.05
	E2	33.1 ± 0.26	34.7 ± 0.06	-1.63	28.3 ± 0.09	29.3 ± 0.12	-0.94
Seawater	S1	40.8 ± 0.59	ND	NA	37.8 ± 0.36	39.0 ± 0.04	-1.12
	S2	40.2 ± 1.05	ND	NA	37.4 ± 0.39	ND	NA

Table 2. Difference in qPCR C_T values (mean and standard deviation) for norovirus GI and GII in wastewater and seawater samples with and without PEMAXTM treatment.

 $\Delta\Delta C_T$ = Difference in qPCR C_T values. ND = not detected. NA = not applicable. Statistically significant (p < 0.05) results are shown in bold.

Table 3. Percentage of infectious norovirus GI and GII in wastewater and seawater samples as determined by PEMAXTM-RT-qPCR.

Commission Description	Sample ID –	Percentage of Infectious Norovirus Detected		
Sample Description		GI	GII	
Influent wastewater	I1	32	59	
	I2	23	37	
	I3	81	89	
	I4	NA	86	
	I5	21	45	
	I6	38	41	
Effluent wastewater	E1	59	24	
	E2	32	52	
Seawater	S1	0	44	
	S2	0	0	

NA = Not applicable (as norovirus GI not detected by RT-qPCR).



Figure 4. qPCR *C_T* values (mean and standard deviation) of serially-diluted original (non-heat-inactivated) and heat-inactivated (**a**) norovirus GI.3 and (**b**) norovirus GI.4 with and without PEMAXTM treatment.

4. Discussion

Municipal wastewater potentially contains high concentrations of enteric viruses, and physical (sedimentation, activated sludge and trickling filters, irreversible adsorption) and chemical (disinfectant) treatment processes can be inefficient for their inactivation and/or removal [22,23]. Irrespective of wastewater treatment type, enteric viruses including noroviruses are likely to be present at concentrations up to 5 log₁₀ genome copies in non-disinfected wastewater effluents. When virus-contaminated effluent is released into receiving environmental waters there are associated human health risks through environmental transmission [19,24]. Demonstration of virus infectivity in these waters is required to assess risk. To address the issue of RT-qPCR detecting non-infectious viruses, a norovirus PEMAXTM-RT-qPCR method was developed and evaluated for its applicability in the detection of infectious viruses from wastewater and seawater.

The photoactivatable dyes PMA and EMA have been used individually for the selective detection of infectious murine norovirus from heat-inactivated samples [8,13,25,26]. In those studies, PMA was less effective (0.14 to 1.38 log₁₀ reduction) in discriminating between non-infectious and infectious murine norovirus [13,25] than shown by our results using PEMAXTM (~2.0 log₁₀ reduction). This difference may be explained by the use of a 90 °C virus inactivation temperature in our study, much higher than the 65 °C and 72 °C temperatures used elsewhere [13,25]. As Millard and colleagues reported that complete inactivation of another enteric virus, hepatitis A virus, was only achieved when heated to 90 °C and maintained for 90 s [27], therefore, 90 °C for 3 min was chosen to ensure that complete capsid damage was achieved, with virus capsid would be likely permeable to the PEMAXTM dye [26].

Further studies should compare the effectiveness of PEMAXTM with other approaches, including methods that utilize an RNase pre-treatment to remove free viral RNA. In addition, different inactivation mechanisms (UV treatment, chlorination, etc.) should be explored because the effectiveness of photoactivable dye-based methods can provide different infectivity profiles [28].

Our results suggest that the effect of PEMAXTM was highest in samples containing a lower virus concentration. This finding is consistent with previous studies [25,29]. This could be that aggregation of inactivated virus particles would prevent entry of PEMAXTM through the damaged capsids [30]. In addition, more effective heat-inactivation may have been achieved in samples containing lower virus concentrations. Our study also indicated that complete discrimination of non-infectious norovirus using photoactivable dyes is difficult to achieve, as reported elsewhere [9,10,29]. This could be that the differential ability of dye-based RT-qPCR methods depend on the extent of capsid damage from heat-treatment. It has been reported that the extent and type of damage to the capsid of heat inactivated viruses is temperature dependent [13,30,31].

PEMAXTM was less effective on norovirus GI.3 (up to 1 log₁₀ reduction detected) than for GII.4 (up to 2 log₁₀). This may be associated with the finding that the structure of virus-like particles of GI is more heat resistant than GII [32]. However, our results contradict with the findings of a previous study where higher (0.41 log₁₀) reductions for norovirus GI compared to GII (0.23 log₁₀) were reported [9]. The minimal effect of PEMAXTM on that study could be associated with the lack of appropriate incubation step (i.e., 30 min in the dark) as recommended by the manufacturer. Therefore, direct comparisons between these two studies may not be feasible. Further inter-laboratory validation using a similar methodology and other norovirus genotypes would be beneficial to evaluate the efficiency of PEMAXTM for the selective detection of infectious viruses including noroviruses from environmental samples.

Finally, the efficiency of PEMAXTM for the selective detection of infectious norovirus in influent and effluent wastewater, and seawater was evaluated. Our results showed that we could not detect any infectious norovirus GI in seawater samples, possibly because of the low concentration of norovirus GI present in those samples. Similar results were reported in a previous study where 98% hepatitis A viruses present in river water were deemed non-infectious [33]. However, our results did indicate the low level of infectious norovirus GII present in one of the seawater samples.

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Despite the significant reduction in infectious norovirus (GI and GII) in effluent wastewater, the PEMAXTM-RT-qPCR method indicated the presence of infectious noroviruses. The detection of noroviruses by the PEMAXTM-RT-qPCR method in these samples may reflect that ineffective virus inactivation and/or that capsid damage may not be sufficient to allow entry of the PEMAXTM, both resulting in a minimal effect of this approach. Despite limitations, the PEMAXTM-RT-qPCR method may serve as a better approach than RT-qPCR alone for the detection of noroviruses from environmental samples and can be useful when associated public health risks are to be evaluated. However, improvements are needed, including comparison of the PEMAXTM-RT-qPCR results with a norovirus in vitro cultivation method when available.

5. Conclusions

Until a reliable culture method for the assessment of norovirus infectivity is widely available, we have to rely on RT-qPCR methods, preferably modified with photoactivable dyes including PEMAXTM, PMA and EMA. In this study, we developed and evaluated the efficiency of PEMAXTM using RT-qPCR for the selective detection of infectious norovirus from naturally contaminated environmental samples including effluent wastewater. The PEMAXTM-RT-qPCR provided better results on infectivity of norovirus than RT-qPCR alone; however, the method has limitations as it did not completely eliminate RT-qPCR amplification from heat-inactivated norovirus. Despite the limitations, this approach has the potential to provide information on viral infectivity to assess the potential human health risks associated with food and water. However, further improvements of the PEMAXTM-RT-qPCR method and the use of different norovirus genotypes inactivated with different inactivation strategies such as UV treatment and chlorination should be considered before adapting for the routine use.

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Conflicts of Interest: The authors declare that there is no conflict of interest.

Ethical Statement: This research does not contain clinical studies or patient data.

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