Simulated sunlight inactivation of norovirus and FRNA bacteriophage in seawater

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Keywords FRNA bacteriophage, norovirus, plaque assay, RT-qPCR, solar disinfection, virus inactivation.

Abstract

Aims: To investigate norovirus (NoV) and F-specific RNA (FRNA) bacteriophage inactivation in seawater under simulated sunlight and temperature conditions representative of summer (235 W m^{-2}; 1 7 °C) and winter (56 W m^{-2}; 1 0 °C) conditions in Ireland.

Methods and Results: Inactivation experiments were carried out using a collimated beam of simulated sunlight and 100 ml of filtered seawater seeded with virus under controlled temperature conditions. NoV concentrations were determined using RT-qPCR, and FRNA bacteriophage concentrations were determined using RT-qPCR and by plaque assay. For all virus types, the fluence required to achieve a 90% reduction in detectable viruses (S_{90} value) using RT-qPCR was not significantly different between summer and winter conditions. S_{90} values for FRNA bacteriophage determined by plaque assay were significantly less than those determined by RT-qPCR. Unlike S_{90} values determined by RT-qPCR, a significant difference existed between summer and winter S_{90} values for infectious FRNA bacteriophage.

Conclusions: This study demonstrated that RT-qPCR significantly overestimates the survival of infectious virus and is therefore unsuitable for determining the inactivation rates of viruses in seawater.

Significance and Impact of the Study: Results from this study provide initial data on the inactivation of NoV and FRNA bacteriophage in seawater under representative summer and winter conditions and will be of interest to shellfish and water management agencies alike.

Introduction

Norovirus (NoV) of the family *Caliciviridae* is the most significant cause of nonbacterial gastroenteritis in the developed world. NoV is shed in the faeces of infected persons at concentrations $>10^8$ g^{-1} (Lee *et al.* 2007) and thus can be commonly detected in wastewater (Nordgren *et al.* 2009; Flannery *et al.* 2012). Wastewater discharge to marine waters has been associated with the NoV contamination of shellfisheries and the subsequent illness amongst consumers (Lees 2000). Wastewater treatment is employed worldwide to reduce microbiological contaminants in wastewater prior to discharge to the environment. However, enteric viruses including NoV have been shown to persist in treated wastewater (Nordgren *et al.* 2009; Flannery *et al.* 2012). Knowledge of the viral inactivation kinetics for NoV in seawater would prove useful to shellfish and water management agencies to model the likely risk posed by wastewater discharges to shellfish harvest waters.

The sunlight that reaches the earth's surface is comprised of UV, visible and longer wavelengths of light and is the primary virucide in the environment (Lytle and Sagripanti 2005). Virucidal effects are elicited primarily through direct damage to the nucleic acids of viruses, where pyrimidine dimers or other photo-products form upon exposure of DNA or RNA to sunlight (Lytle and Sagripanti 2005). However, viral inactivation may also occur through indirect mechanisms caused by the excitation of endogenous or exogenous sensitizers (Kohn and

Nelson 2006; Romero *et al.* 2011). Endogenous sensitizers are located in the capsid, while exogenous sensitizers are present in the surrounding environment although the contribution to viral inaction has been difficult to determine (Davies-Colley *et al.* 1999; Kohn and Nelson 2006; Romero *et al.* 2011).

The extent to which NoV is inactivated through sunlight exposure in marine waters is poorly understood. Numerous studies have investigated the inactivation rates for enteric viruses and bacteriophage under UV-C at 254 nm (germicidal wavelengths) and NoV inactivation kinetics have been inferred from data involving FRNA bacteriophage (Simonet and Gantzer 2006), viruses within the *Caliciviridae* (De Roda Husman *et al.* 2004; Duizer *et al.* 2004) and other nonenveloped enteric RNA viruses (Meng and Gerba 1996; Crance *et al.* 1998). However, germicidal wavelengths are not found within terrestrial sunlight that is composed of wavelengths >293 nm (Kay *et al.* 2005). Notwithstanding, it has been shown that these nongermicidal wavelengths can directly damage enteric viruses and bacteriophages (Sinton *et al.* 1999, 2002; Love *et al.* 2010). One reason for the lack of inactivation kinetic data for NoV specifically is the absence of a reliable cell culture assay. The detection of NoV in environmental samples has relied on molecular methods such as RT-qPCR. However, it has been shown that these methods do not provide information on viral infectivity (Nuanualsuwan *et al.* 2002; Pecson *et al.* 2009).

Using solar simulated light, we determined the T_{90} and S_{90} values for NoV and FRNA bacteriophage GA in seawater under representative summer and winter temperature and sunlight conditions using RT-qPCR. FRNA bacteriophage GA is present in faecal waste originating from human sources (Wolf *et al.* 2008) and has been found to be a conservative indicator for virus persistence during water treatment (Boudaud *et al.* 2012). With the inclusion of a plaque assay for FRNA bacteriophage GA, we aimed to determine whether RT-qPCR is adequate for viral detection in marine waters and for use in modelling the risk posed by wastewater discharges to shellfish harvest areas.

Materials and methods

Solar simulator set-up

The solar disinfection experiments were performed using an LOT Oriel Low-Cost solar Simulator (LSO106; LOT Oriel, Leatherhead, UK) equipped with a 150W xenon arc lamp (LSB521; LOT Oriel). The solar simulator was equipped with an Oriel Air Mass 1.5 Global Filter (LSZ189; LOT Oriel) and provided a 35-mm-diameter beam of light with a constant intensity of 1000 W m^{-2} at a distance of 80 mm from the light source. A calibrated solar cell (15150-1; LOT Oriel) was used to measure the irradiance of the solar simulator.

Solar radiation data, consisting of the cumulative irradiance received each month in Ireland, were obtained from Met Éireann. From the monthly irradiance, the mean daily irradiance (per 24 h period) was calculated. Mean daily irradiances of 235 and 56 W m^{-2} were calculated as irradiances typical of summer and winter periods, respectively. To attenuate the irradiance required for summer and winter experiments, a collimated beam set-up was used (Qualls and Johnson 1983) at a distance of 45 and 155 cm from the solar simulator, respectively. Mean maximal summer (17°C) and mean minimum winter (10°C) seawater temperatures were obtained from Met Éireann.

Preparation of virus stocks

FRNA bacteriophage GA was propagated in *Salmonella enterica* serovar Typhimurium (*Salm*. Typhimurium) WG49 host by broth enrichment (International Organisation for Standardisation, 1995) and prepared as 10% glycerol/virus stocks. Briefly, following 6 h incubation, host cells were lysed with chloroform and stored overnight at 5°C. The aqueous layer was decanted and centrifuged at 2000 ***g*** for 20 min, and glycerol was added to make a 10% stock. Single-use aliquots were prepared and stored at −20°C. The concentrations of the FRNA bacteriophage aliquots were 10^9 plaque-forming unit (PFU) ml^{-1} and 10^9 genome copies ml^{-1} determined using the plaque assay and RT-qPCR assay, respectively. For NoV, two separate stool samples, each positive for NoV GI or NoV GII, were diluted 1 : 5 with PBS. Following centrifugation at 2000 ***g*** for 20 min, the supernatant was divided into single-use aliquots that were stored at −20°C. The concentrations of NoV in each aliquot were determined as 10^3 genome copies ml^{-1} for both NoV GI and NoV GII.

Experimental set-up for virus inactivation

All experiments were carried out in a darkened, temperature-controlled room at 17 ± 1°C for summer experiments and 10 ± 1°C for winter experiments. At each sampling point, the temperature of the irradiated and control samples was measured using a thermocouple to confirm that temperature deviation of >1°C did not occur. Two millilitres of each virus stock was inoculated into 200 ml of filtered seawater that was then mixed and divided into two 100 ml volumes. For the inactivation studies, 500-ml sterile Pyrex beakers containing 100 ml of inoculated filtered seawater at a depth of 18 mm were used for the irradiated samples. For control (dark)

conditions, 100 ml of inoculated filtered seawater was added to 500 ml bottles covered in aluminium foil. All inoculated seawater volumes in both the control and irradiated samples were magnetically stirred at 130 rev min^{-1} throughout the trial. For infectious FRNA bacteriophage GA, seawater samples were stored in the dark at 4°C and analysed within 24 h. Two sampling occasions were performed each day during the summer ($n = 3$) and winter experiments ($n = 3$) for total FRNA bacteriophage GA, NoV GI and NoV GII analysis by RT-qPCR. At each sampling occasion, 1 ml samples of seawater were taken and immediately stored at −20°C and were analysed within 1 week.

FRNA bacteriophage plaque assay

FRNA bacteriophage was enumerated using a double agar layer method (International Organisation for Standardisation, 1995) using *Salm.* Typhimurium WG49 as the host strain. Host cells were cultivated for 4–6 h at 37°C in tryptone yeast-extract glucose broth (to yield $>10^6$ CFU ml^{-1}) and then mixed with 2·5 ml of tryptone yeast-extract glucose 1% agar (soft agar) and 1 ml of appropriately diluted seawater. This was then poured onto the hard agar (tryptone yeast-extract glucose 2% agar). Following overnight incubation at 37°C, FRNA bacteriophage GA was identified as semi-transparent plaques that were then counted and multiplied by the dilution factor to obtain the titre in PFU ml^{-1}. The limit of detection (LOD) of the assay was determined as 10 PFU ml^{-1}.

Viral genomic extraction

Viral RNA was extracted from 1 ml seawater samples in duplicate using the NucliSENS® miniMAG® extraction platform and NucliSENS® magnetic extraction reagents (bioMérieux, Marcy-l'Étoile, France) following the manufacturer's instructions. Five hundred microlitres of seawater was used per extraction, and viral RNA was eluted into 100 μl of elution buffer (bioMérieux). The eluted RNA was stored at −80°C until RT-qPCR analysis was undertaken (<1 month). A negative RNA extraction control using molecular biology grade water was processed alongside seawater samples.

Preparation of double-stranded DNA plasmids for quantification of NoV and FRNA bacteriophage GA in wastewater and oysters

Double-stranded (ds) DNA plasmids for quantification of NoV GI and GII were prepared as previously described (Flannery *et al.* 2012). For quantification of bacteriophage GA, PCR products from the RT-qPCR were purified using the Wizard® SV PCR Purification kit (Promega, Southampton, UK) and cloned using the pGEM-T Easy vector system (Promega). Transformant clones were screened and purified using the PureYield™ plasmid miniprep kit (Promega). The purified plasmid was quantified using a spectrophotometer at 260 nm and subsequently diluted to 10^5 copies μl^{-1} for use as a quantitative standard in the RT-qPCR.

RT-qPCR assay for NoV and FRNA bacteriophage GA

An Applied Biosystems AB7500 instrument was used for RT-qPCR analysis. Twenty microlitres of the NoV or FRNA bacteriophage GA reaction mix was prepared using RNA Ultrasense one-step qRT-PCR system (Invitrogen, Carlsbad, CA, USA) containing 1× reaction mix, 500 nmol l^{-1} forward primer, 900 nmol l^{-1} reverse primer, 250 nmol l^{-1} probe, 1× μl Rox and 1·25 μl of enzyme. Previously described primers QNIF4 (Da Silva *et al.* 2007), NV1LCR and probe NVGG1p (Svraka *et al.* 2007) were used for NoV GI analysis, and primers QNIF2 (Loisy *et al.* 2005), COG2R (Kageyama *et al.* 2003) and probe QNIFS (Loisy *et al.* 2005) used for NoV GII analysis. Previously described Levivirus genogroup II forward and reverse primers and probe were used (Wolf *et al.* 2008). Duplicate 5-μl aliquots of sample RNA were added to adjacent wells of a 96-well optical reaction plate in addition to no template controls. RT-qPCR inhibitors were controlled for each virus as previously described (Flannery *et al.* 2012).

Norovirus GI and GII reaction conditions were as follows: initial incubation at 55°C for 60 min followed by 95°C for 5 min and then 45 cycles of 95°C for 15 s, 60°C for 1 min and finally 65°C for 1 min. For FRNA bacteriophage GA, reaction conditions involved an initial incubation at 55°C followed by 95°C for 5 min and then 45 cycles of 95°C for 15 s and 58°C for 1 min NoV and FRNA bacteriophage GA were quantified by comparing the C_q value to the standard curves in copies per microlitre and then adjusted to reflect the volume of RNA analysed (expressed as genome copies ml^{-1}). The LOD of the assay was determined as 20 detectable genome copies ml^{-1}.

Calculation of T_{90} and S_{90} values

To evaluate the log decrease in detectable virus, the virus concentrations in the initial inoculated seawater suspension at time 0 were expressed as N_0. The concentrations of detectable virus at all subsequent times were expressed as N_t. The linear relationship between log decrease in detectable virus and time is described by the inactivation rate constant (k_{obs}):

$$\log_{10}\left(\frac{N_t}{N_0}\right) = -k_{obs} \times \text{time}$$

All experiments were performed on three separate occasions. At each sampling occasion, the mean concentration of virus was determined from duplicate analysis. For comparison with other studies, the time taken to achieve a 1 log reduction in virus concentrations (T_{90} value) was calculated. The fluence (MJ m^{-2}) was determined as a product of the irradiance (W m^{-2}) and the exposure time (seconds). The fluence required for a 90% reduction in detectable virus (S_{90} value) was also determined. The mean data for N_t/N_0 from three independent summer and winter experiments were paired with the corresponding time or fluence and were plotted. Minitab statistical software version 15 (Minitab Inc., State College, PA, USA) and Sigma Plot software version 11 (Systat Software, Chicago, IL, USA) were used for the statistical analysis. A Student's *t*-test was carried out on log N_t/N_0 for each virus to determined whether a difference in S_{90} and T_{90} values existed between summer and winter conditions and between irradiated and control conditions.

Results

Decrease in total detectable viruses in seawater determined by RT-qPCR

S_{90} and T_{90} values were determined for all virus types under summer and winter conditions (Table 1). The S_{90} values for total NoV GI, NoV GII and FRNA bacteriophage GA, as measured by RT-qPCR, were not significantly different ($P > 0{\cdot}05$) between summer (GI; 18·18 MJ m^{-2}, GII; 17·30 MJ m^{-2}, GA; 13·96 MJ m^{-2}) and winter (GI; 18·09 MJ m^{-2}, GII; 16·92 MJ m^{-2}, GA; 15·73 MJ m^{-2}) experiments.

Under summer conditions, the reduction in all virus types as measured by RT-qPCR was significantly greater in the irradiated samples than in the control (dark) samples (Fig. 1). No significant difference ($P > 0{\cdot}05$) was found between the T_{90} values for NoV GI (21·5 h) and NoV GII (20·5 h) under summer conditions. However, the T_{90} value for FRNA bacteriophage GA (T_{90} 16·5 h) was significantly less than the T_{90} values for NoV GI and GII ($P < 0{\cdot}01$) under summer conditions.

Under winter conditions, no significant difference ($P > 0{\cdot}05$) was observed between the T_{90} values for NoV GI (89·3 h) and NoV GII (83·9 h) in irradiated samples. However, NoV GI and NoV GII T_{90} values were greater than the T_{90} value for total FRNA bacteriophage GA (78 h); however, this difference was not significant ($P > 0{\cdot}05$). Similarly in control samples, no significant difference ($P > 0{\cdot}05$) existed between T_{90} values for all virus types detected using RT-qPCR under winter conditions. Under winter conditions using RT-qPCR, the reduction of all viruses in irradiated samples was not significantly different ($P > 0{\cdot}05$) from the reduction in the control samples.

Detection of total and infectious FRNA bacteriophage GA in seawater

Figure 2 shows the reduction of infectious FRNA bacteriophage GA under summer and winter conditions. The S_{90} values for infectious FRNA bacteriophage GA determined by the plaque assay were significantly less ($P < 0{\cdot}001$) than those determined using the RT-qPCR at 0·20 and 0·86 MJ m^{-2} for summer and winter conditions, respectively. The difference between summer and winter S_{90} values for infectious FRNA bacteriophage GA was significant ($P < 0{\cdot}05$). Under summer and winter conditions, the T_{90} values for infectious FRNA bacteriophage GA in irradiated samples (0·25 and 4·0 h, respectively) were significantly less ($P < 0{\cdot}05$) than in the control samples (5·2 and 15 h, respectively). The T_{90} values for infectious FRNA bacteriophage GA under winter conditions were significantly greater than summer conditions ($P < 0{\cdot}05$). Under winter and summer conditions, the T_{90} of total FRNA bacteriophage GA as determined using the RT-qPCR was significantly different than that determined for infectious FRNA bacteriophage GA ($P < 0{\cdot}001$).

Table 1 T_{90} and S_{90} values for viruses in seawater

		S_{90} (MJ m^{-2})*		Irradiated T_{90} (h)		Control T_{90} (h)	
Virus	Detection method	Summer	Winter	Summer	Winter	Summer	Winter
Norovirus GI	RT-qPCR	18·18	18·09	21·5	89·3	59·8	85·9
Norovirus GII	RT-qPCR	17·30	16·92	20·5	83·9	41·0	101·5
FRNA phage GA	RT-qPCR	13·96	15·73	16·5	78	50·5	95·0
FRNA phage GA	Plaque assay	0·20	0·86	0·25	4·0	5·2	15·0

*The irradiance applied during the experiments were 20 MJ m^{-2} day^{-1} in summer and 5 MJ m^{-2} day^{-1} in winter.

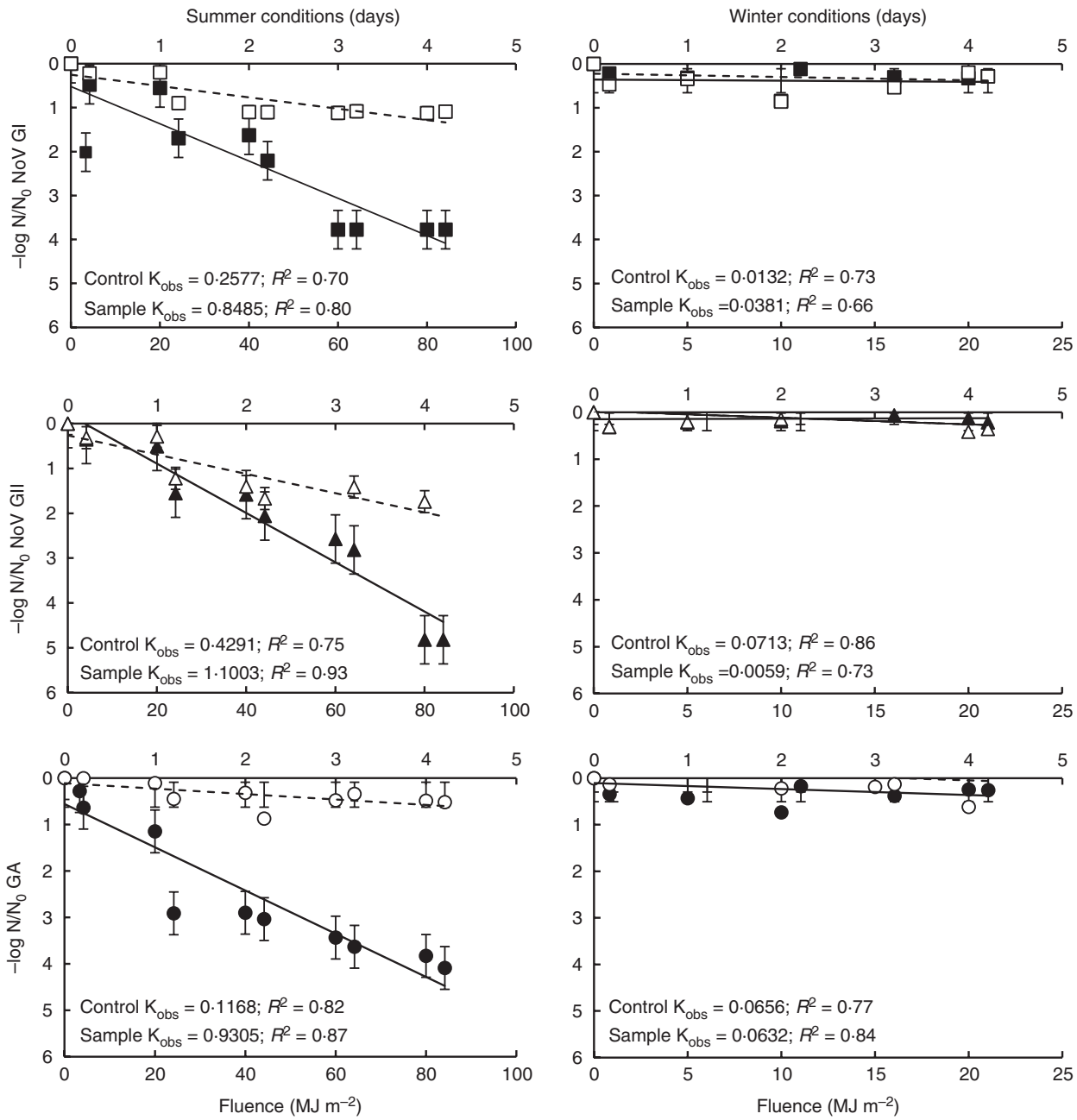

Figure 1 Simulated sunlight reduction of viruses in seawater using RT-qPCR. The reduction in NoV GI (squares), NoV GII (triangles) and FRNA bacteriophage GA (circles) are plotted against time (days) and fluence (MJ m^{-2}). Fittted regression lines for the irradiated (shaded markers) and dark (open markers) samples are shown. Data points are the mean value from three experiments. Summer and winter experiments were carried out at irradiances and temperatures of 235 Wm^{-2}; 17°C and 56 Wm^{-2}; 10°C, respectively. Error bars indicate SE of the mean, calculated from three experiments carried out on three separate occasions.

Discussion

We determined the S_{90} and T_{90} values for NoV and FRNA bacteriophage GA in seawater using RT-qPCR under simulated sunlight and temperature conditions representative of those found in Ireland during summer and winter periods. No significant difference was observed between S_{90} values under summer and winter conditions for any virus type when determined using the RT-qPCR. This suggests that temperature does not have a significant effect on viral inactivation. Given that the S_{90} values were constant between summer and winter, the T_{90} values for all virus

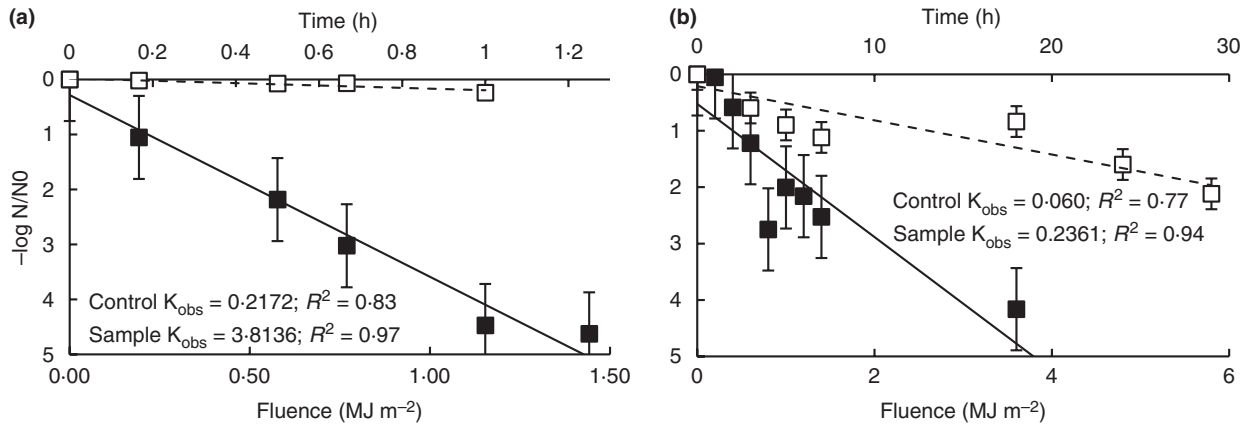

Figure 2 Solar simulation reduction of infectious FRNA bacteriophage. Summer (a) and winter (b) experiments were carried out for 75 min and 28 h respectively. The log N_t/N_0 of viral concentrations determined using the plaque assay in the irradiated samples (shaded squares) and control samples (open squares) are shown. Error bars indicate SE of the mean, calculated from three experiments carried out on three separate occasions.

types were greater under winter conditions than under summer conditions. The shorter T_{90} values in summer were a function of the greater irradiance that was applied in the summer experiments and therefore the shorter time required to reach the S_{90} value in these studies.

The S_{90} value for infectious FRNA bacteriophage GA determined by the plaque assay was significantly less than the S_{90} value determined using RT-qPCR in both winter and summer experiments. Clearly the RT-qPCR underestimates the extent of infectious FRNA bacteriophage inactivation under simulated environmental conditions. Primers used in the RT-qPCR target a short region (111-bp) of the ~3·5-kb FRNA bacteriophage GA genome, which is a normal feature of real-time PCR. However, provided this fragment is intact, the virus genome would still be detected and quantified even if there is damage to other regions of the virus genome which could prevent viral replication from occurring. Pecson *et al.* (2009), when studying UV inaction of bacteriophage MS2, did not detect a difference between the decrease in qPCR signals for primer sets directed towards different regions of the entire MS2 genome. Capsid-directed damage has been shown to occur in water impacted by sunlight due to interactions with reactive oxygen species and natural organic matter (Romero *et al.* 2011). RT-qPCR also detects virus particles that are not infectious because of damage to the capsid that would prevent virus attachment to host cell receptors (Nuanualsuwan *et al.* 2002). It is probable that a similar overestimation of infectious NoV concentrations is provided by RT-qPCR. Therefore, using RT-qPCR to determine T_{90} values for virus inactivation should be considered inappropriate. A significant difference existed between summer and winter S_{90} values for infectious FRNA bacteriophage GA, suggesting that temperature, possibly associated with capsid damage, as well as sunlight irradiation played a role in virus inactivation. This is in agreement with other studies that have reported a greater reduction of infectious virus in water under increased temperatures (Sinton *et al.* 1999; Duizer *et al.* 2004).

Under both summer and winter conditions, S_{90} values for NoV GI and NoV GII were greater than S_{90} values for total FRNA bacteriophage GA determined by RT-qPCR. FRNA bacteriophage and in particular FRNA bacteriophage GA have been proposed to be a conservative indicator of enteric viruses (Boudaud *et al.* 2012) in water treatment processes. The use of an RT-qPCR assay for FRNA bacteriophage alongside an RT-qPCR assay for NoV in inactivation studies has not been reported previously; therefore, FRNA bacteriophage GA may not be as conservative an indicator when used in this context. As the NoV genome is ~4 kb larger than the FRNA bacteriophage GA genome, it may mean that the NoV genome is more resistant to damage from sunlight.

Few data exist concerning the solar inactivation of viruses in seawater (Sinton *et al.* 1999, 2002; Love *et al.* 2010). Where such data are available, caution is required in comparing studies because of different experimental design and detection methods employed. To apply a standardized experimental regimen, we used a collimated beam set-up for the solar simulator, similar to other inactivation studies that used germicidal UV light (De Roda Husman *et al.* 2004; Duizer *et al.* 2004). We recognize that the results presented here represent an initial assessment of viral inactivation in seawater under simulated sunlight using ideal laboratory conditions. UV light is attenuated as it passes through the water column with UVA and longer wavelengths persisting at greater depths therefore shorter wavelengths of light predominate at shallow depths, such as those used in our study. These

shorter wavelengths have been shown to cause inactivation of viruses through direct mechanisms (Fisher *et al.* 2011). It has also been shown that the presence of organic material can provide an additive effect on virus inactivation through the generation of organic reactive species and that occur at greater depths (Romero *et al.* 2011). We carried out our experiments using filtered seawater and a shallow depth; therefore, the S_{90} values calculated in this study may represent the highest inactivation rate likely for FRNA bacteriophage under sunlight and temperature conditions representative of summer and winter periods in Ireland. There is therefore a clear requirement for further studies to determine more representative T_{90} values for NoV and viral surrogates under environmental conditions. Such studies should include consideration of the effect of seawater turbidity and depth. In addition, given the underestimation of infectious virus inactivation provided by RT-qPCR, such studies should consider the application of more appropriate approaches that may distinguish between infectious and noninfectious virus particles. Recently Pecson *et al.*, proposed a framework to assess UV inactivation of viruses and found that RT-qPCR can be used to provide an accurate estimation of MS2 inactivation under UV light (Pecson *et al.* 2011). Alternatively, the use of long range RT-qPCR has been shown to reflect the reduction in infectious murine NoV during UV disinfection (Wolf *et al.* 2009). Both approaches may provide additional relevant data in virus inactivation studies. In this study, FRNA bacteriophage inactivation in seawater determined using a plaque assay occurred at a greater rate than that determined using RT-qPCR. Therefore, RT-qPCR overestimated the survival of FRNA bacteriophage, and we consider this method unsuitable for use in determining inactivation rates of viruses in seawater.

Acknowledgements

This work was funded by the Environmental Protection Agency, Ireland as part of the Science, Technology, Research and Innovation for the Environment (STRIVE) Programme 2007–2013 (project code 2008-EH-MS-7-53).

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