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Estimating the distribution of norovirus in individual oysters

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ABSTRACT

Norovirus in oysters is a significant food safety risk. A recent ISO detection method allows for reliable and repeatable estimates of norovirus concentrations in pooled samples, but there is insufficient data to estimate a distribution of copies per animal from this. The spread of norovirus accumulated across individual oysters is useful for risk assessment models. Six sets of thirty individual *Crassostrea gigas* oysters were tested for norovirus concentration levels by reverse-transcription quantitative PCR (RT-qPCR): three from a commercial harvest site, and three post-depuration. Five sets had norovirus GII means above the limit of quantification (LOQ), and one below the LOQ, but above the limit of detection. No norovirus GI was detected in pooled tests, and individual oysters were not tested for norovirus GI. Depuration was shown to reduce the mean concentration of GII copies, but not to affect the shape of the distribution around the mean. Deconvoluting the uncertainty of the method, the coefficient of variation was stationary (0.45 ± 0.2). The best fit distribution was either a lognormal distribution or a gamma. Multiplying these distributions by the weight of oyster digestive tissues gave an estimate for the count mean. This was used as the parameter λ in three compound Poisson distributions: Poisson-lognormal, Poisson-gamma, and Poisson-K. No model was found to fit better than the others, with advantages for each. All three could be used in future risk assessments. Preliminary validation of sampling uncertainty using repeated testing data from a previous study suggests that these results have predictive power.

1. Introduction

Norovirus causes more than half of all foodborne microbial illness in the developed world (Kirk et al., 2015). More than nine million annual food-related illnesses occur in the US alone, with norovirus accounting for an estimated 58% of incidents (Scallan et al., 2011). Comparable rates of illness have been reported in Canada, the UK, and the Netherlands (Gormley et al., 2011; Havelaar et al., 2012; Thomas et al., 2013). Norovirus is a human enteric virus, not indigenous to marine waters: it enters the water system through sewage outflows (de Graaf et al., 2016). There are three principle transmission vectors for norovirus in food: first, the irrigation of crops with water contaminated with sewage effluent; second, the unhygienic preparation of food by infected handlers; and third, bioaccumulation in the shellfish that feed in contaminated waters (Mathijs et al., 2012; Moore et al., 2015). In the US, for all foodborne norovirus cases whose initial cause is known, this third, shellfish-borne, pathway accounts for up to 20% of incidents (13—19%) (Hall et al., 2012, 2013, 2014). Although data are sparse, similar estimates have been made for other countries: 16% in the Netherlands (Havelaar et al., 2008); 14% to 29% in the UK (ACMSF, 2015; Hassard et al., 2017); and 36% in Canada, for cases excluding where transmission by infected handlers (Davidson et al., 2011). Overall, the global proportion is significant.

Oysters that feed in waters contaminated with effluent will bioaccumulate norovirus copies in their digestive tissues (Maalouf et al., 2011). This internal accumulation means that the contaminating particles cannot simply be washed off or rinsed away. Norovirus levels that are insignificantly low when diluted in coastal waters can become highly concentrated within the oyster's digestive pathways (Ventrone et al., 2013). Norovirus is stable in the environment and infectious in low doses to the susceptible population (Atmar et al., 2014; Teunis et al., 2008). After an initial infection, secondary transmission among close contacts can rapidly follow (Teunis et al., 2015). Since oysters are often eaten raw or lightly cooked, there is no option for reducing or inactivating the virus by heat treatment. Depurating with sterile seawater, a method that can flush out bacteria within days or hours, is far less effective in removing much smaller, more stubborn, virus particles (Baggi et al., 2001). Standard faecal indicator bacteria like *Escherichia coli (E. coli)* show poor correlation with viral contamination, which makes them ineffective as a norovirus surrogate (Flannery et al., 2013). Different shellfish safety approaches are needed to tackle the specific hazard of norovirus in oysters.

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A recent international standard method for detecting and quantifying norovirus in shellfish is a significant milestone. ISO 15216-1:2017 (ISO, 2017) is a protocol that first extracts virus RNA from food matrices and then quantifies it with reverse transcription quantitative PCR (RT-qPCR). For oysters, the digestive glands of ten or more animals are combined and homogenised for a single pooled test. This is based on studies of how virus accumulates in oysters (McLeod et al., 2009; Wang et al., 2008) and has been validated extensively by national reference laboratories over the years of its development (Lees and CEN, 2010; Lowther et al., 2019a). The standard is recognised internationally as the most accurate available, given the challenges of quantifying norovirus in oysters (EFSA, 2019). In the EU, proposed regulations for oyster and shellfish safety are based on the results from this standard and the implications for public health and risk management (EFSA, 2016). Despite the comprehensive validation process, the standard presents some challenges for risk assessment. RT-qPCR detects genome copies and does not distinguish between infectious and non-infectious virus. The proportion may be under 2%, as proposed for Hepatitis A in shellfish (Pintó et al., 2009), or it may be high enough to cause outbreak events with relatively low concentrations (Thebault et al., 2013). Cell culturing of norovirus in stem-cell derived enteroids, first shown in Ettayebi et al. (2016), has made significant progress in recent years, but this is not a routine procedure, and has not been extended to food matrices (Cates et al., 2020; Estes et al., 2019; Green et al., 2020; Murakami et al., 2020). A more immediately approachable challenge is to extend the use of the ISO method as a tool for risk assessment.

Risk management means identifying uncertainties of measurement and using better data and modelling to reduce them where possible. A pooled sample is useful information when comparing overall contamination between oyster production sites, or across seasons. It is more limited when assessing the likelihood of illness following consumption. There is a high risk of infection posed by relatively low numbers of virus. This makes small variations in exposure more significant, which needs to be addressed in risk assessment (Haas et al., 2014). It is important to be able to estimate the expected distribution of norovirus concentrations (copies per gram) and counts (copies) across individual oysters from the same site. Oysters are individual animals, and therefore will accumulate virus copies at different rates, even in the same location. As virus is concentrated in the digestive glands, risk profiles recommend that exposure assessments be based on a serving unit of an individual oyster, rather than the total weight of flesh consumed (Greening et al., 2009). Stochastic estimates of per unit counts like this can be represented by lognormal, gamma, or compound Poisson distributions (Bassett, 2010; Gonzales-Barron and Butler, 2011).

The objective of this study is therefore to measure and compare the variability of norovirus concentrations and counts in individual oysters, and to find the distributions that fit best. The aim is a framework that takes as input an ISO 15216-1:2017 pooled estimate of norovirus concentration and produces a fully realised probabilistic estimate for copies per individual oyster, with associated uncertainties.

2. Materials and methods

2.1. Site selection and sampling

Six samples of live oysters (*Crassostrea gigas*) were taken, each from one of two field sites, at different times in the year. Three samples were taken from Site One, a Class A commercial shellfish production site in Ireland, with several years of monitoring data. Three samples were taken from Site Two, an unclassified site in a bay in Ireland, exposed to untreated sewage outflow. In production, oysters were grown in mesh bags of 100–200 oysters: one mesh bag was assumed to represent a single sampling point.

2.2. Sampling

In each of the six samples, forty oysters were taken from a single sampling point. Pooled samples of ten were tested by the standard method, for both norovirus GI and GII. Thirty were tested individually once contamination was confirmed. For depurated oysters, pooled samples were measured both before and after treatment.

2.2.1. Untreated production site oysters

The three samples tested directly after harvest, with no depuration treatment, came from Site One. Oyster bags were arranged on semi-submerged trestles in a production area of approximately 200,000m^2. Several years of monitoring data showed a predictable pattern of norovirus concentrations across the winter period of late October to early March, peaking in January/February. Norovirus GII is typically prevalent throughout the season, with concentration levels above the limit of quantification (LOQ) of the method. Norovirus GI, by comparison, shows less prevalence at this site, and when detected is often at sub-LOQ levels (Doré et al., 2010; Rupnik et al., 2018). During routine norovirus monitoring of the site, a sample of 40 oysters was taken on three separate occasions: November (Set 1: the beginning of the winter season); December (Set 2: mid-season, before the peak) and February (Set 3: close to peak concentration). Typical sea surface temperatures in this region during this time range from 12 °C to 14 °C in November/December and from 8 °C to 10 °C in February (Huang et al., 2017).

2.2.2. Depurated oysters

Shellfish at risk of faecal contamination from sewage are required to be treated by depuration in a tank with sterile seawater for periods of up to a week (EFSA, 2015). Samples of oysters to be later depurated were first relayed on an intertidal area of Site Two for a period of at least one month. After harvesting from the relay site, these oysters were placed in a temperature-controlled depuration tank for at least three days. One batch of oysters was depurated at a water temperature of 14 °C, where, after three days, 30 individuals were taken to be tested. A second batch of oysters was depurated at a water temperature of 18 °C. From this batch, 30 individuals were tested after three days, and another 30 tested after seven days.

2.2.3. Other oyster data sources

A stability study project from the same group provided useful archival data for validation purposes (Fahy, 2018). On two occasions, repeated testing for norovirus GI and GII was carried out on a single batch of oysters from Site Two, using samples of ten different pooled oysters for each test. One set had thirty pooled samples tested from one point, and the second had fifteen. The resulting distributions of mean concentrations was compared to the theoretical sampling uncertainty for a sample of that size, and the variance of GI results was compared with GII.

2.3. Virus extraction from oyster digestive tissues

Oysters were dissected and tested for the presence of norovirus genogroups I and II according to ISO 15216-1:2017 (ISO, 2017). In addition to the standard method of testing a pooled sample of 10 oyster digestive tissues (DT), individual DT were also tested using the standard method with modification. Oysters were cleaned by rinsing under running tap water and opened using a flame sterilised shucking knife.

2.3.1. Pooled samples

Each set of samples was tested for both norovirus GI and norovirus GII according to ISO 15216:2017–1. For pooled samples: DT of 10 oysters were transferred to a sterile petri dish, weighed, chopped, and homogenised. 100ul of Mengo virus strain MC_0 was added directly to a 2 g sample of the homogenised DT as an internal process control (IPC) virus, following the methodology of Costafreda et al. (2006). The

original stock of MC_0 was provided by the Community Reference Laboratory, CEFAS. Proteinase K solution (100 μg/ml; Sigma-Aldrich, Dorset, UK) was added in a ratio of 1 ml per 1 g. The sample was then incubated at 37 °C for 60 min, with shaking, followed by incubation at 60 °C for 15 min, centrifugation at 3000 x*g* for 5 min and extraction of the supernatant. Prior to RNA extraction, this proteinase K extract was stored for a period of no more than one month at −80 °C.

2.3.2. Individual oysters

When norovirus of either genogroup was confirmed at sufficient levels in the pooled samples, individual oysters from the same batch were tested for that genogroup. For individual oysters, the DT were similarly dissected, chopped finely and weighed before an equal volume (ml per g) of Proteinase K solution was added. As the method requires 500 μl of DT virus extract, oysters with DT weight in excess of 0.5 g were preferentially selected. DT weights below 0.5 g were had their shellfish proteinase K extract supernatant topped up to 500ul with molecular biology grade water. Concentration calculations were modified where necessary to reflect this lower DT mass.

2.3.3. RNA extraction

Viral RNA was extracted using NucliSENS® miniMAG® platform and NucliSENS® magnetic extraction reagents (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's protocol. Sample RNA was tested by RT-qPCR either immediately after extraction or stored at -80 °C until analysis was undertaken.

2.3.4. One-step real-time RT-qPCR quantification of norovirus in oysters

A previously described real-time RT-qPCR assay was used to detect and quantify norovirus GI and GII in the oyster samples (Flannery et al., 2013). Norovirus GI analysis used primers QNIF4 (Da Silva et al., 2007), and NV1LCR and probe.

NVGG1p (Svraka et al., 2007). For GII, primers QNIF2 (Loisy et al., 2005), COG2R (Kageyama et al., 2003) and probe QNIFS (Loisy et al., 2005) were used. Plasmids pGEM-3Zf(+) carrying the norovirus GI and GII target sequences containing restriction site (BamH1) to check for contamination (supplied by Dr. Francoise S. Le Guyader, Ifremer, Nantes, France) were used to enable quantification of norovirus RNA in copies per μl. A log dilution series of DNA plasmids (range 1×10^1 to 1×10^5 copies per μl) were included in duplicate on each RT-qPCR run to provide a standard curve for quantification. The limit of detection (LOD) for was 20 genome copies per gram (gc/g) of DT, and the published limit of quantification (LOQ) for the method was 100 gc/g.

2.3.5. Quality control

All the samples were assessed for RT-PCR inhibition using external control RNA (Flannery et al., 2013). Samples with a RT-PCR inhibition of greater than 75% were not accepted and in such cases the sample RNA was reanalysed at a 1:10 dilution. All the samples were also assessed for extraction efficiency using Mengo virus as internal process control. Primers Mengo209 and Mengo110, and probe Mengo147, were used as described in Pintó et al. (2009). Samples greater than 1% extraction efficiency were accepted for inclusion in this study.

2.4. Statistical analysis

RT-qPCR data was transferred from the Applied Biosystems® AB7500 software to Microsoft Excel (*Microsoft Excel Version* 2013, 2013) for data management and calculation of concentrations from Ct values and standard curves. All subsequent analyses were performed using R version 3.2.2 (R Core Team, 2017), with the packages described below. Code is available from the corresponding author.

2.4.1. Measurement error

The ISO 15216-1:2017 detection method has a published measurement uncertainty of ± 0.60 on a log base 10 scale, or a ratio of 3.98 on the linear. This is known to vary between laboratories (EFSA, 2019). To allow the greatest possible accuracy for future modelling, the exact measurement uncertainty for these tests was quantified by analysing previously carried out norovirus detection results, where 55 samples had been tested twice. The effect of measurement error on the distribution observations was modelled by the equation $log(Y) = log(X) + e$.

This form of equation is well-established in biological science (Wang and Wang, 2011). X is the distribution of the true, unobservable random variables, while e is a lognormally distributed error term with mean of zero. Y is the observed distribution, a convolution of the two, from which all measurements taken were generated. Two possible distributions for X were compared: lognormal and gamma. With lognormal X, log(Y) is the sum of two normal distributions, and its parameters are defined as $log(Y) \sim N(\mu_Y = \mu_X + \mu_e, \sigma_Y{}^2 = \sigma_X{}^2 + \sigma_e{}^2)$.

The distribution of X was therefore taken as $log(X) \sim N(\mu_X = \mu_Y - \mu_e, \sigma_X{}^2 = \sigma_Y{}^2 - \sigma_e{}^2)$

With gamma X, Y is the product of a gamma and lognormal distribution, which has been observed in both nature and engineering (Dogandzic and Jinghua Jin, 2003; Frank and Bascompte, 2019). It can be approximated by a lognormal (Stüber, 2017, p. 132). If Y and e are both approximately lognormal, then the moments of X can be calculated from their ratio as in equation x above, and a gamma distribution fit to them.

The distribution for 'e' was estimated by calculating the intraclass correlation coefficient (ICC) of the duplicate tests on the log scale, using the R package "icc" (Wolak et al., 2012). The ICC is a measure of reliability, and can be converted to an estimate of the proportion of total variance due to measurement error by the formula in Stratford and Goldsmith (1997): $VAR[Error] = VAR[Total] * (1 - ICC)$

Any uncertainty intervals were estimated by bootstrapping ($N = 5000$), followed by bias correction and acceleration (Efron and Tibshirani, 1994). Non-parametric deconvolution methods based on Fourier transformations (Stirnemann et al., 2012; Wang and Wang, 2011) were also considered and rejected, as simulations failed to predict with better accuracy or precision than the raw observed values. Parametric "empirical Bayes" methods (Efron, 2014) were also not used, due to unsuitability of the data sets.

2.4.2. Concentration distributions

The concentration of norovirus within each oyster of a set was modelled using either a gamma or lognormal distribution. The choice of these distributions follows standard microbiological concentration modelling practice (Bassett, 2010; Jongenburger et al., 2015). The coefficient of variation (CV) was used to assess the consistency of distribution between each set. (Wiens, 1999), and parameters were calculated using the first two moments, using bootstrapping (N = 5000) to include and propagate uncertainty. To assess measurements that fell below the LOQ or LOD, a probability plot method was used, following the recommendations of Helsel (2011). This ROS method assumes the lognormal approximation, then uses the un-censored upper values to fit a regression line which will estimate the values of the unknown points. Since each sample of oysters came from a site where contamination was known to be present, and concentrations can be infinitesimally small, the fitted distributions were not zero-inflated, even if some proportion of non-detects may have had a true count of zero.

To check results, the true concentration mean was then convoluted again with both aspects of measurement uncertainty, the overall error (lognormal), and the limit of detection based on the limits of the test. The quantiles of the resulting lognormal and gamma-lognormal distributions were estimated numerically. This was done by Monte Carlo simulation, where the sole input for each predicted distribution was the estimate of the sample mean. These quantiles were plotted against the quantiles of the observed data in QQ-plots, to assess the fit visually. If the predicted distributions are accurate, they should provide a reliable estimate of the variation in sample results due to sampling error,

$e \sim N\left(\mu, \frac{\sigma^2}{\sqrt{n}}\right)$. To test this prediction, a set of results were taken from archival data where the same batch of oysters had been tested thirty times (all pooled samples of $n = 10$ oysters).

2.4.3. Count distributions

The distribution of greatest interest for risk assessment is the number of NoV copies contained in each oyster. Count data was estimated by multiplying measured or estimated concentrations by the weight of the individual DT. Unlike concentrations, which can be assumed continuous and positive, the distribution for count data is ideally discrete and allows for zero values. Other recommended criteria are a potential reduction to the Poisson distribution when mixing is most homogenous, and an approximation to the lognormal distribution when values are high (Gonzales-Barron et al., 2010; Jongenburger et al., 2015). The number of norovirus copies expected in each oyster was also modelled. The form of the models compared were generalised Poisson distributions. λ, the mean of each of these, was set as the virus concentration per gram of oyster DT times the weight of the DT in grams.

The value for the weight of the DT was estimated using laboratory measurements. For the lognormal concentration, λ_L, a lognormal distribution was chosen. For the gamma distributed λ_G, two alternatives were compared: a point estimate for W, based on the average size of the oysters, and another gamma distribution. The first of these resulted in a Poisson-gamma, or negative binomial distribution, scaled by the mean weight of oyster DT. The second was modelled as a gamma distribution, parameterised in the form $\sim\Gamma(\mu, \theta)$, where μ is itself gamma distributed. This is equivalent to a K distribution (Redding, 1999).

The count frequencies for the observed datasets were approximated by multiplying the measured concentrations by the corresponding DT weight for each observation. All three count distributions–Poisson-lognormal (PLN), Poisson-gamma (PG), and Poisson-K (PK)–were combined with the measurement error and limit of detection to produce theoretical quantiles for six QQ plots. The set of data with LOQ values was represented by the actual observed values, including non-detects, not the ROS modelled values.

3. Results and discussion

3.1. Descriptive summary

Norovirus GI was not present at quantifiable levels in any of the sets. All six sets did have sufficient norovirus GII concentrations to justify testing individuals. All the results which follow therefore apply to norovirus GII in oysters only. All six sets of individual oysters showed a similar pattern of concentrations (units of genome copies per gram, or gc/g). The results display the basic shape of a common distribution, whose mean would be estimated by the standard pooling approach. Summary statistics are shown in Table 1. Each set had at least 29 oysters tested for norovirus gc/g. Table 1 also includes the lognormal-ROS model for set 1. This addresses the left-censorship observed, where 68.8% of values fell below the LOQ, and 25% below the LOD. Each distribution was unimodal and right-skewed. On the $\log_{10}$-scale (Fig. 1), the spread of values about the median was similar. The exception was the raw data from set 1, where non-detect values could not be transformed.

3.1.1. Measures of dispersion

The coefficient of variation (CV), a common measure of dispersion, was almost identical across the five sets above the LOQ. A CV that stays the same when the mean is higher or lower ("stationary" CV) is a feature of both lognormal and gamma distributions, and a strong indicator for either (Wiens, 1999). It would also mean that the variance of the distribution can be consistently estimated from the pooled detection method mean. Fig. 2 plots the mean of each set against its CV, with bootstrapped uncertainty intervals. Fig. 2 also shows the mass of the uncertainty intervals. The wider uncertainty interval for set 4 can be seen to come from the presence of one high outlier.

A test for equality between CVs, proposed in Feltz and Miller (1996) and implemented using the R package "cvequality" (Marwick and Krishnamoorthy, 2019), shows a high likelihood for equality between the CVs of sets 2 to 6 ($p = .30$). No difference in dispersion was observed between depurated and un-depurated samples. Adding the two versions of set 1 to the group rejects the raw, censored, values ($p = .025$) but does not reject lognormal model ($p = .27$). A robust alternative to the CV has been proposed in Arachchige et al. (2019): the absolute deviation from the median (MAD), divided by the median itself. It confirms the comparisons between the six sets (Fig. 3). Using this robust metric, the consistency between the sets is even clearer, and again the lognormal model is closer to the other sets than the censored set. The discrepancy between Set 1 and the other sets may partly be because of greater measurement uncertainty at lower, less reliable, levels of detection.

3.1.2. Measurement error

The duplicate measurements of samples are shown in Fig. 4, where they appear to be normally distributed on the log-scale (base 10). The estimate for the error distribution e (in base 10) is $e \sim Log_{10}(\mu = 0, \sigma^2 = (0.1789)\hat{}2)$.

The effect of this distribution for e on the parametric deconvolution of the CV estimates is shown in Fig. 5. The adjusted lognormal ROS CV estimates are relatively consistent with the five sets above the LOQ, suggesting that the concentration distribution will be the same at all levels. Assuming that the CV is stationary (that is, taking the same value in all cases), then the results for all six sets can be pooled for a single estimate. The results of this are also shown in Fig. 5: a reduction from 0.76 ± 0.21 to 0.45 ± 0.20. Fig. 7 plots the density curves of the implied distributions over the histograms of the data. The estimates for the true distribution keep the same mean as the sample, but a reduced variance. There is no discernible difference in shape between the gamma and lognormal fits.

Table 1
Summary statistics for Norovirus concentrations (copies/g) measured in individual oysters, including coefficient of variation (CV). Sets 1–3 were harvested from a commercial farm, where the seasonal trend of norovirus was known. Sets 4–6 were re-laid near a sewage outflow to obtain the high concentrations shown, then treated with depuration for the times and temperatures shown. The limit of detection is 20 copies/g, and the limit of quantification is 100 copies/g: values below the LOQ are included un-censored in set 1 and modelled using regression on order statistics (ROS) substitution.

Set	Depuration	N	< LOQ	< LOD	Mean	Median	StDev	CV
1	NA	32	22	10	80.2	51.4	94.5	1.18 (0.93—1.59)
1 (ROS)	NA	32	22	2	95.3	64.9	85.7	0.90 (0.72—1.13)
2	NA	30	0	0	1292.9	1161.4	816.9	0.63 (0.50—0.86)
3	NA	27	3	0	316.7	294.2	199.9	0.63 (0.49—0.84)
4	3 days at 14 deg. C	30	0	0	2756.5	2282.8	2549.3	0.92 (0.57—1.31)
5	3 days at 18 deg. C	30	0	0	3556.8	3392.1	2024.4	0.57 (0.45—0.75)
6	7 days at 18 deg. C	30	0	0	2417.7	1927.3	1608.5	0.67 (0.54—0.89)

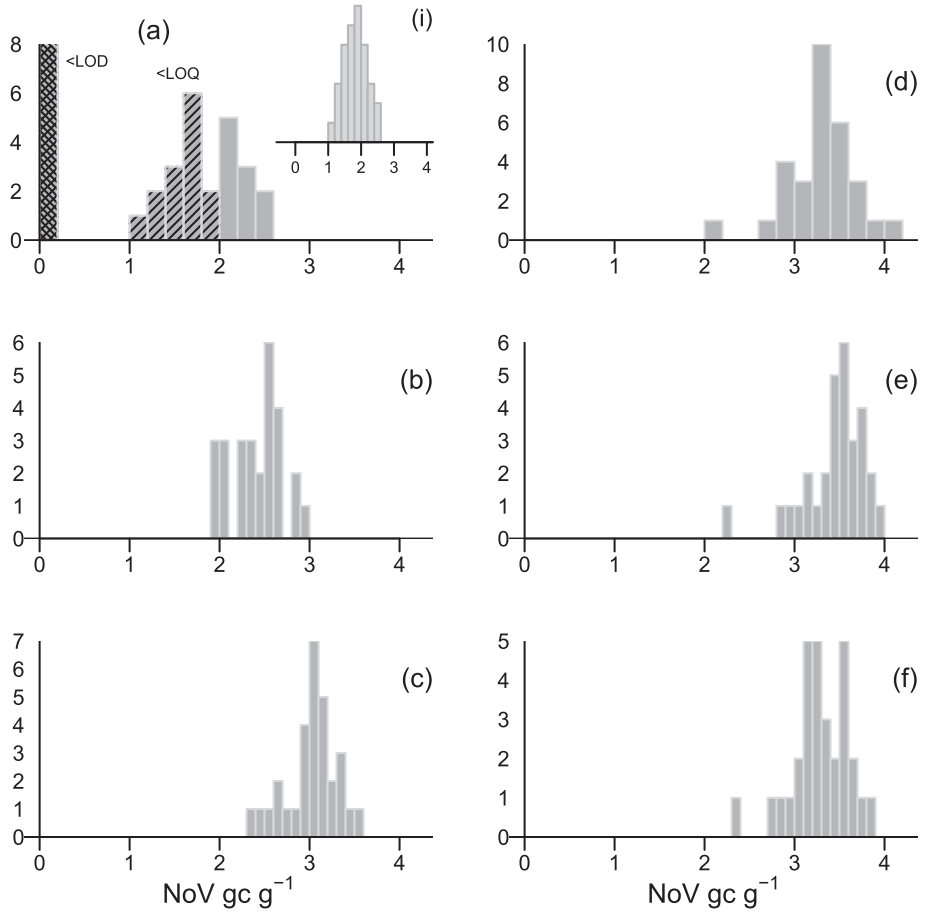

Fig. 1. Log-transformed histograms (base 10) of concentrations measured in all six sets. Censored values in Set 1 are modelled by ROS substitution methods, assuming a lognormal approximation, inset (i).

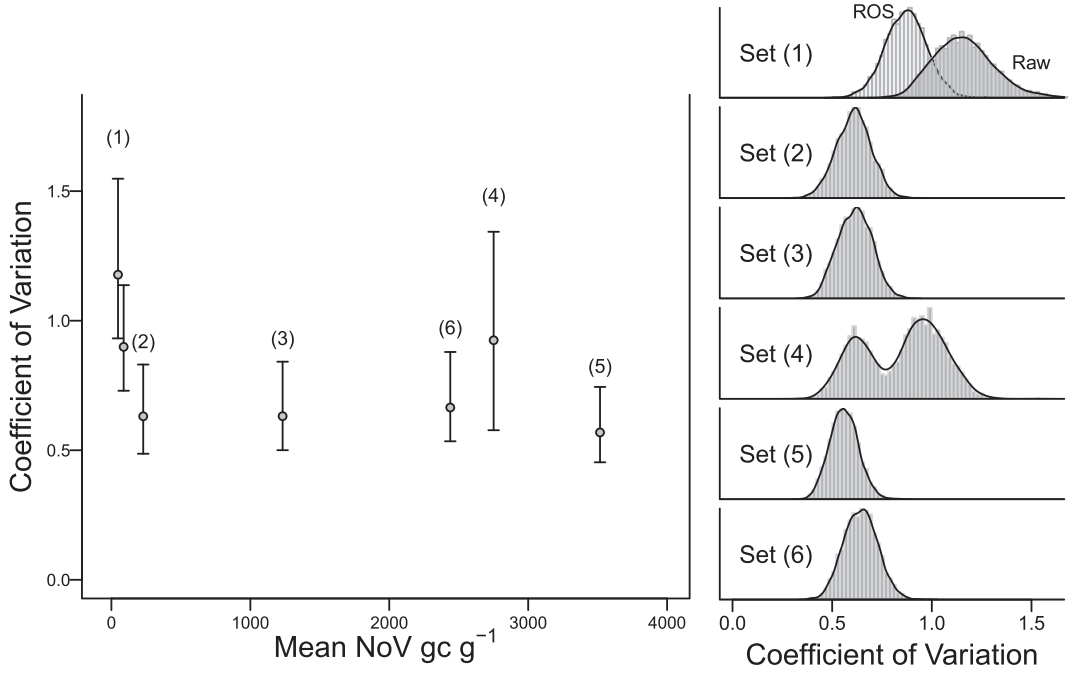

Fig. 2. Coefficients of variation (CV) for each set, with bootstrapped uncertainty densities shown on the right. The set with censored values, Set 1, is shown with both the raw data and ROS substitution, assuming a lognormal approximation.

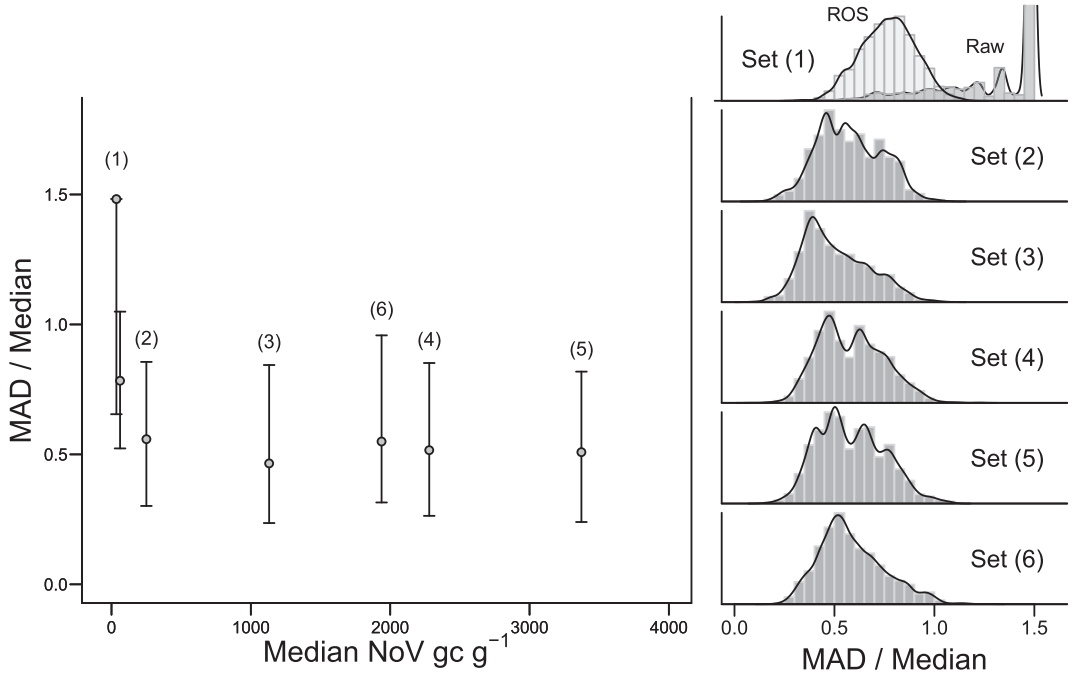

Fig. 3. A robust alternative to the coefficient of variation, median absolute deviation over median (MAD/Med), for each of six sets. The set with censored values, Set 1, is shown with both the raw data and ROS substitution, assuming a lognormal approximation.

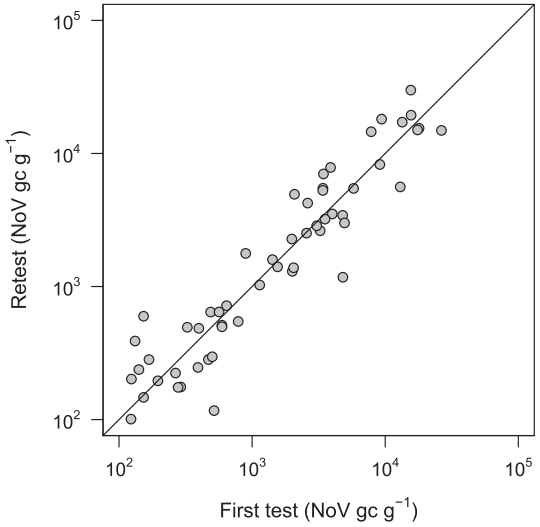

Fig. 4. Duplicate measurements of norovirus concentration in 55 different oyster DT samples.

3.2. Concentration distributions

The adjusted lognormal ROS CV estimates for set 1 in Fig. 5 are consistent with the five sets above the LOQ, as are the three depurated sets (4–6). This suggests that the concentration distribution will maintain the same shape at all contamination levels, if fractional concentrations are sufficient to predict counts of zero. Both the lognormal and gamma parameters for the concentration distribution can be estimated from the sample mean, by using the assumed CV ranges shown in Fig. 6 to predict the standard deviation and working out the relevant parameters. The sampling uncertainty of the mean will be the predicted standard deviation divided by the number of oysters pooled. All these parameters, and their uncertainties, can be calculated using only the estimate of the mean from the sample data. Visual assessment by QQ-plot for each predicted concentration distribution shows little to distinguish between them. The right tail of the lognormal is marginally heavier compared to the gamma.

The fit between what the results above predict and what was observed in an archival data set of repeated pooled samples is shown in Fig. 7a, for a set of GII results. Comparing the 95% uncertainty intervals predicted by each individual mean (Fig. 7b) shows that 28 out of 30 estimates overlap with the overall mean estimate of 2255.5 ± 227.1gc/g (95% level). Out of an expected 95% success rate, this is 78.1—98.2%, calculated by a Wilson score interval (Brown et al., 2001). Fig. 7c compares the results of repeated testing of pooled samples in a context where both GI and GII were quantifiable (n = 15). The difference between the log standard deviations for GI and GII is shown in Fig. 7d. The bias-corrected confidence interval for these data contains zero and does not show evidence of significant difference in dispersion between the two genogroups.

3.3. Count distributions

The count data for the six datasets was approximated by multiplying the weights of the digestive glands of each oyster by the associated concentration. The lognormal concentration distribution was multiplied by a lognormal weight distribution to produce another lognormal estimate. For the gamma concentration, the weights were represented by either a point estimate of the mean, or by another gamma distribution. The mean weight of the oyster DT was 1.04 g for the largest set and 0.39 g for the smallest. This likely represents a standard range for commercial oysters, from largest to smallest grade.

These three continuous estimates for the count were then each used as the mean in a compound Poisson distribution: Poisson-lognormal, Poisson-gamma (or negative binomial), and Poisson-K. Comparing the predicted outcomes to the measured values visually by QQ plotting shows a similar fit for all three, with a marginally heavier right tail observable in the lognormal. The only input taken by each estimate was the overall sample mean.

3.4. Main findings

For oysters from a single location, the distributions of norovirus GII

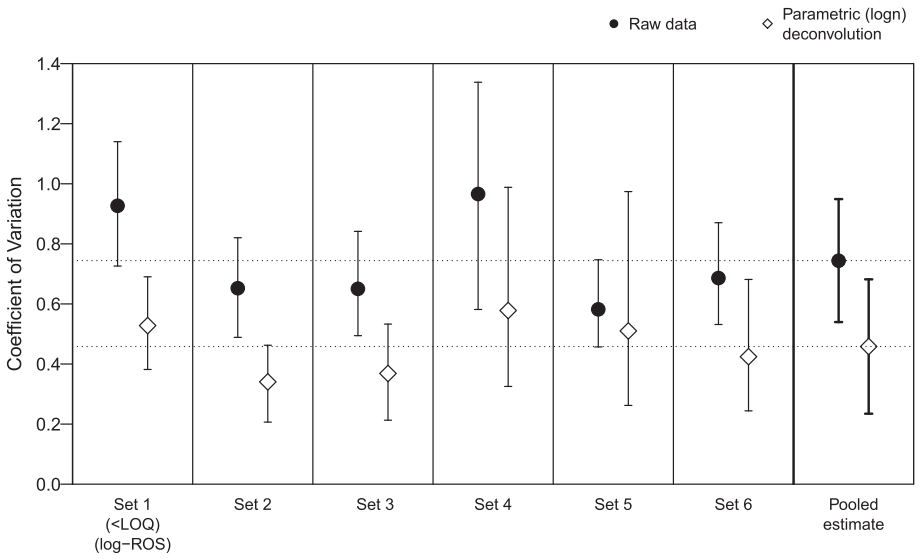

Fig. 5. Comparing the coefficients of variation (CV) for each set when measurement error is deconvoluted from each set. The raw data sets (black circles) are assumed to be approximately lognormal. Subtracting the effect of the lognormal error distribution 'e' by standard Gaussian arithmetic estimates the deconvoluted CV for each set (clear diamonds). Set 1, with many censored values, uses regression on order statistics (ROS) substitution, assuming a lognormal distribution.

counts and concentrations are consistently predictable, with several suitable models to fit. This consistency has been shown to hold for different site conditions, different sites, and different post-harvest treatments. The only measurement needed to estimate these distributions is the overall concentration mean, which can be done through a single test of pooled animals. The framework which connects this sample mean estimate with the final count distribution should account for measurement error, left censoring, sampling error, and oyster variation. All the data and distributions needed to construct this framework have been provided in this paper.

Oysters exposed to the same environmental conditions accumulate norovirus by a process with predictable results. The distribution is unimodal, right skewed, has a constant coefficient of variation, and can be modelled equally well by two-parameter lognormal or gamma models, taking only a sample mean as input. If the oysters have been depurated, the distribution of virus before and after treatment can be

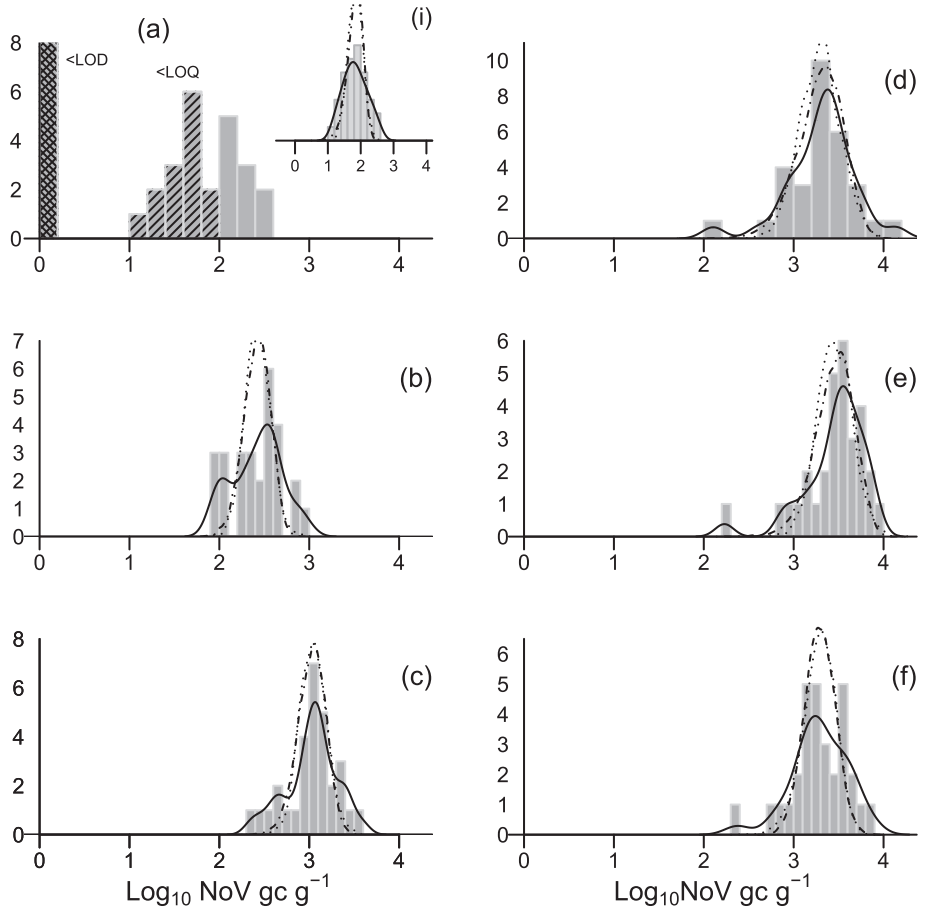

Fig. 6. Histograms of observed data with three overlaid density curves: the two deconvoluted distributions, lognormal and gamma (dotted and dashed lines) and the kernel density estimate for the data itself (solid line).

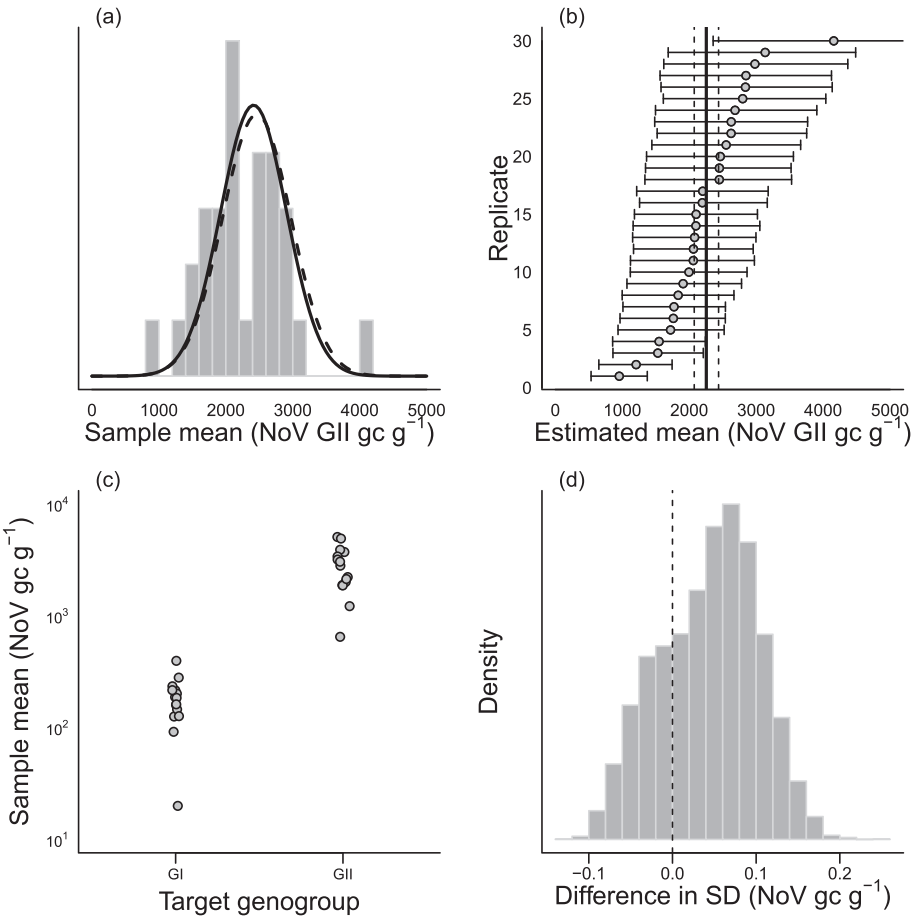

Fig. 7. (a) Predicted sampling error distribution overlaid on 30 real results. Each data point is a concentration value taken from a pooled sample of ten different oysters. (b) 95% confidence intervals for the mean predicted by each of 30 samples from the same site. The lines in the centre shows the combined estimate for the mean. (c) The results of fifteen repeated pooled samples from a large batch of oysters, comparing concentrations of GI and GII. (d) The difference between the $\log_{10}$ standard deviation of the two data sets shown in (c).

compared directly, using sample means. The sampling uncertainty of the mean can also be quantified reliably at the 95% confidence level. The results have revealed the shape of a distribution of virus counts which can be scaled to fit by a single estimate of a concentration mean.

3.5. Flexible framework

The results offer flexibility for future risk assessment models. Sources of error that lead to intractable composite distributions like the gamma-lognormal have been deconvoluted into their component parts. The measurement error assessed here only applies to the results from one lab; however, since the net effect on the mean should be zero, it is not critical in future results to know the reliability of the tester. The Poisson-gamma, Poisson-k, and Poisson-lognormal models off approximately similar estimates, with different utilities for modelling. Poisson-gamma results may be more useful when left-tail results are more relevant to risk assessment, or when the negative binomial is a more convenient choice of prior. Poisson-lognormal estimates have a heavier right tail and are easier to combine with other log-transformed variables. For Bayesian models, the parameters presented here can be taken as initial estimates for posterior fitting, as there may be more accurate fits available for individual circumstances.

3.6. Use for risk assessment

The Poisson count distributions allow relevant modelling for exposure assessments, particularly in monitoring risk over time. The only varying input is the sample mean, which can be tested routinely on production sites, before and after intervention treatments. It should be noted however that the lowest concentration set, which contained 25% zero counts, is predicted by each model to contain 6% zero counts or less, even when allowing for the error of the method. An additional distribution, to account for "zero inflation" (Gonzales-Barron et al., 2010, 2014) may be necessary at lower levels of contamination. Predictive modelling of site concentrations, based on historical or environmental data, can also be used as a framework input. This may be most suitable using a Bayesian modelling approach, particularly when the detection method itself has uncertain censoring limits. (Busschaert et al., 2011; Guo et al., 2019; Richardson and Gilks, 1993). After outbreak events associated with a site occur, monitoring data could be linked with infection rates and exposure assessment data to estimate the infectivity conditions of the virus consumed.

The ISO detection method is already a useful tool for assessing and comparing risk (Lowther et al., 2019b): these results show that it can be made more precise. The estimate for virus concentration across a population of oysters gives a robust prediction for the individual virus counts. This is relevant to any risk analysis that models variation across individual oysters, such as Thebault et al. (2013). The ISO method is an established tool for monitoring and investigating the risk posed by norovirus in oysters (Lowther et al., 2019b), and has been exhaustively validated for over a decade (Lees and CEN, 2010; Lowther et al., 2019a). Regulatory thresholds have been proposed in the EU based on the connection between a high estimate and a risk of outbreak (EFSA, 2016). There are challenges in managing the risks involved, given the limitations of the method and the economic interests of stakeholders. The proportion of detected virus which is infectious is an open question, as is the effect of the food matrix. In the absence of food-specific cell culturing techniques, methods proposed to address this include using FRNA bacteriophages as proxies for infectivity (Lowther et al., 2019b), and the use of viability PCR (Monteiro and Santos, 2018; Moreno et al., 2015). Whatever the proportion, variation in numbers between individual oysters will remain, with a central tendency defined by the

population estimate. The distributions seen in these data will stay relevant to the risk assessment of viruses in shellfish. Any such work that relates to the consumption of individual oysters will benefit from the greater precision in modelling offered by these results.

3.7. Future directions

Some questions of uncertainty do remain for these results. If an estimate for the ratio of infectious to non-infectious genome copies can be made, then this can be incorporated into the framework by multiplying the final count estimate by a scaling factor or distribution, taking a value between 0 and 1. The distributions provided can be scaled to different units if needed, while maintaining the same shape. The scaling parameters of the distributions observed may not apply equally to all genogroups, although the identical mechanism of accumulation suggests that the distributions themselves are as consistent, as does the comparison between pooled samples shown in Fig. 7. Depuration may be less effective on genogroup GI which has been suggested to bind to ligands inside the oyster flesh (Le Guyader et al., 2012, 2006; McLeod et al., 2017). It may not be the case that ten oyster DTs that have been homogenised, pooled, and tested as a unit will give the same result as the same ten oyster DTs tested individually. Some effects of inhibition or amplification may be observed, although the validation work of Fig. 7 suggests good predictability for this assumption. Finally, the work done here was carried out at single geographical points on sites. If there is significant variation across a site due to differing environmental conditions, then the mean parameter will also need to vary according to some, yet unknown, distribution, which would mean modelling a summation of individual distributions. This approach can also have application for samples from different sites mixed at retail locations, where Bayesian assessment of multiple tests would be most useful for estimating exposure to consumers.

4. Conclusion

The distribution of norovirus copies per oyster from a location can be inferred from a single measurement of concentration. The framework that has been presented here allows a flexible and reliable means of estimating the consumption of norovirus copies following a meal of oysters. The predicted distributions include uncertainty estimates, which have been validated by comparisons with real data. The effect of depuration, the most used method for risk management, has been confirmed not to change the shape off the distribution, but to lower the mean only.

The framework and data provided by this study allows better understanding of exposure to norovirus following oyster consumption, and risk assessment following. It can be used as a base for connecting environmental modelling to risk estimation, and a method of assessing the effectiveness of different intervention strategies. It can also be adapted for estimation of mixed distributions, for retail, or spatially diverse production areas. It provides better knowledge of the mechanics of norovirus accumulation, and better modelling of outbreak events in the future.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2020.108785.

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