A comparison of the MPN and pour plate methods for estimating shellfish contamination by Escherichia coli

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Abstract

Aims:

Shellfish production areas are classified for suitability for human consumption using counts of E.coli in shellfish samples. Two alternative laboratory methods are approved in the EU and UK for measuring E. coli in shellfish samples; the MPN and pour plate methods. These methods have inherently different statistical uncertainty and may give different counts for the same sample. Using two approaches: simulated data and spiking experiments, we investigate the theoretical properties of the two methods to determine their reliability for shellfish waters classification.

Methods and results:

Assuming a Poisson distribution of E. coli in shellfish samples, we simulate concentrations in 10,000 samples using the MPN and pour plate methods. We show that for higher concentrations of E. coli the pour plate method becomes increasingly more reliable than the MPN method. The MPN method has higher probabilities than pour plate of generating results exceeding shellfish classification thresholds, while conversely having higher probabilities of failing to detect counts that exceed regulatory thresholds. The theoretical analysis also demonstrates that the MPN method can produce genuine extreme outliers, even when E. coli are randomly distributed within the sampled material. A laboratory spiking experiment showed results consistent with the theoretical analysis, suggesting the Poisson assumption used in the theoretical analysis is reasonable.

Conclusion:

The large differences in statistical properties between the pour plate and MPN methods should be taken into consideration in classifying shellfish beds, with the pour plate method being more reliable over the crucial range of E. coli concentrations used to determine class boundaries.

Impact Statement:

Appreciation of the greater reliability of the pour plate method should lead to improved shellfish bed classification, improved management of the public health risk associated with shellfish consumption and greater confidence for shellfish producers that management decisions are based on sound evidence.

Keywords:

Shellfish; E. coli; most probable number (MPN); pour plate; theoretical comparison; spiking experiment

Introduction

Where shellfish are grown in areas impacted by anthropogenic pollutants, they can accumulate and concentrate a range of contaminants. These pollutants often include pathogenic bacteria and viruses associated with faecal matter that is introduced into the water column and originates from point and diffuse pollution such as from wastewater treatment facilities and agricultural runoff (Iwamoto et al, 2010; Malham et al 2014; Hassard et al 2017). As bivalve shellfish are often eaten raw or only lightly cooked, they can become foodborne vectors for these pathogens with a potential risk of human illness (Pouillot et al, 2021). To protect public health, regular monitoring of faecal indicator bacteria is undertaken with production areas classified according to the results of the monitoring. In the EU this framework is referred to as the Shellfish Official Control Regulations (OCR) and aims to ensure that the harvesting and selling of bivalve shellfish can only occur at sites that are deemed safe for consumption according to a standardised classification system (European Commission 2004; European Commission 2017; European Commission 2019/627); an aligned framework is also applied in the UK. Routine sampling is used to determine the appropriate classification of Class A, B or C depending on the concentrations of E. coli in shellfish flesh (Table 1). High levels of bacterial contamination can cause the closure of shellfish harvesting areas for periods of time to ensure the protection of public health or downgrading of classification which can mean that additional

preparation steps such as depuration are required before shellfish can be offered for human consumption. In other parts of the world, the use of E. coli counts in shellfish flesh as an indicator of faecal contamination is widely applied in national regulations for classification of shellfish areas. Some countries may utilise results from monitoring of water samples (eg. USA) or a combination of water and shellfish flesh samples (eg. Canada, New Zealand) rather than of shellfish flesh samples alone (Pinn and Le Vay 2023). Import controls may also require compliance with an upper limit for E. coli in shellfish flesh (eg. 230 E.coli $(100g)^{-1}$ from Class A waters for imports into the EU).

The most widely (almost universally) used method for measuring E. coli in shellfish flesh is the ISO-accredited Most Probable Number or MPN method (ISO 2016). This method uses dilutions across culture tubes and a probability calculation to estimate the concentration of viable organisms in a sample, based on the number of tubes that return a positive result (West and Coleman, 1986). This method specified in Codex Standard CXS 292-2008 (Codex Alimentarius, 2015) and European Commission Regulation No 2073/2005 (European Commission, 2005).. Significant advantages of the dilution tube MPN method are the simplicity of the laboratory process itself relative to molecular methods and the wide range of E. coli concentrations that can be measured, as well as comparability with long term historical data sets based on this method (Walker et al, 2018). Other methods are available for use to enumerate faecal indicator bacteria in shellfish, including pour plate/Tryptone Bile X-Glucuronide (TBX) testing, impedance testing and plate spreading (spread plate) methods. The pour plate method (EURL 2014; ISO 2001; Lowther 2024;) is a culture-based technique that relies on the counting of E. coli colonies on TBX or brilliance agar (Clements

et al, 2013; Walker et al, 2018), which has been validated and characterised against the MPN reference method (Walker et al, 2018; Pol-Hofstad and Jacobs-Reitsma 2021) and is approved for use in official bivalve shellfish control samples in the EU and UK, with an updated test protocol recently published by the UK National Reference Laboratory (Lowther 2024)

Both the MPN and the pour plate methods assume that the number of E. coli g^{-1} present in a sample follows a Poisson distribution. When analysing a sample of shellfish flesh, a laboratory assay and an accompanying statistical procedure generate an estimate of the sample E. coli concentration and its standard error. Official protocols for application of MPN in shellfish samples acknowledge the inherent variability in the method, with theoretical $\log_{10}$ standard deviations of around 0.24 and expanded uncertainty of 0.66 for $\log_{10}$ transformed results (Walker et al. 2018; Stockley 2023). However, the estimated parameter of that distribution is accepted as a point estimate of the number of E. coli present in a specified volume of shellfish and this estimate is used for classification. Clearly, the results of E. coli monitoring will have implications for shellfish business operators, who may be required to close areas or restrict harvesting when relatively high E.coli monitoring results are returned (Pinn and Le Vay 2023). In application of the OCR, the classification of a shellfish production area is typically informed by results from a series of MPN results for which the individual measurement uncertainty of each result is not directly considered. In-year E. coli results and classifications are reviewed on an ongoing basis, considering a rolling dataset; exceptionally high results may trigger a site-specific investigation that can result in their being disregarded. However, in some cases, a single unusually high E. coli result can potentially lead to imposition of harvest or export restrictions.

Several comparative and validation studies have been reported, assessing the pour plate method against the reference MPN method as applied to shellfish samples (e.g Mooijman et al, 2007; Pol-Hofstad and Jacobs-Reitsma 2021), which are the basis for acceptance of their equivalence in official control samples. However, such comparisons do not take account of the inherent measurement uncertainty in both methods. Given the differences in the statistical properties of the estimation methods, there is potential for different results from the same shellfish sample depending on which laboratory method is used. This may lead to different long-term shellfish area classifications or immediate regulatory actions such as harvest restrictions. In this paper, we investigate the theoretical statistical properties of the MPN and pour plate method, based on Monte Carlo simulation of data under the Poisson assumption. We also compare the two methods, in practical measurement of shellfish spiked with different concentrations of E. coli to simulate the range of values that may be experienced in shellfish production areas.

Materials and Methods

MPN

The theory and practice behind the MPN method, including detail of the statistical analysis, have been provided by Jarvis, Wilrich and Wilrich (2010), collating results from previous authors. E. coli are assumed to be randomly distributed in sample material at a concentration of μ colony-forming units (CFU) g^{-1}. Under this randomness assumption the number of counts in a sample approximates a Poisson distribution, and this distributional assumption is at the root of the MPN analysis. The laboratory procedure is to take the material to be analysed and make k dilutions $i = 1,...,k$, with d_i the dilution in each of n_i tubes, with w_i the volume of inoculum at dilution level i and x_i the number of positive tubes

at dilution i. The probability of obtaining a sequence of values $x_1, ?, x_k$ can be written following Jarvis, Wilrich and Wilrich (2010)

$$P = \prod_{i=1}^{k} \frac{n_i!}{x_i!(n_i - x_i)!} \left(1 - \exp\left(-d_i w_i \mu\right)\right)^{x_i} \left(\exp\left(-d_i w_i \mu\right)\right)^{(n_i - x_i)}$$

The maximum likelihood MPN estimate $\hat{\mu}$ is found by solving equation for each set of laboratory realisations.

$$\sum_{i=1}^{k} \left[\frac{x_i d_i w_i}{1 - \exp\left(-d_i w_i \hat{\mu}\right)} - n_i d_i w_i \right] = 0$$

Jarvis et al also provide estimates of the variance of μ and confidence intervals based on asymptotic theory. De Man (1975), De Man (1983) provide an alternative derivation, with further discussion by Garthright and Blodgett (2003). These authors derive confidence intervals for the true E. coli count given an MPN value. In contrast our analysis considers a range of values of the underlying parameter μ and estimates the distribution of MPN values for each. From that, misclassification probabilities can be estimated for samples with the underlying parameter value and selected classification threshold.

For a given set of x_i the MPN may be estimated using the **mpn** routine of the MPN library in the R statistical package (R Core Team 2021). This routine provides a maximum likelihood (unadjusted) estimate of MPN from equation , a bias-adjusted estimate and approximate confidence intervals using a choice of two methods. It also provides a rarity index (RI), the ratio of the likelihood of the sample being considered at the maximum likelihood estimate MPN_{sample} divided by the maximum likelihood over all possible samples, fixing MPN_{sample} as

the value of μ in equation . Samples with a low RI are suggestive of a deviation from the underlying Poisson distribution assumption, or other irregularity.

As an alternative to the R routine **mpn** the Excel routine (ISO Standards Maintenance Portal) may be used. The MPN values given by this routine correspond, within minor arithmetic differences such as degree of rounding, with the unadjusted MPN values given by the R function **mpn**. The Excel routine does not provide an adjusted MPN value but does give confidence intervals and an RI.

The MPN method is commonly used with four tenfold dilutions ($k = 4$) using 1g of shellfish sample in each of 5 ($n_i = 5$) tubes. However, four dilutions are not sufficient to estimate concentrations of the order of 46000 E. coli $(100g)^{-1}$, and in this theoretical analysis we use 5 dilutions to capture these high concentrations. For lower concentrations a fifth dilution will be superfluous in practice. Multiplication of the MPN from 1g samples by 100 then gives an estimate of E. coli $(100g)^{-1}$ and its confidence limits.

Pour plate

In the pour plate method, counts $x_i, i = 1,\ldots,n$ are made of E. coli CFUs in each of n samples. Commonly 5 samples each of 1g of shellfish are used, and we make this assumption in our simulations. Using the same Poisson assumption as the MPN method, an estimate of the mean μ is then

$$\hat{\mu} = \frac{\sum_{i=1}^{5} x_i}{5}; E(\hat{\mu}) = \mu$$

A property of the Poisson distribution is that the sum of independent Poisson distributed variables is also Poisson, with parameter the sum of the individual parameters, so if

$X \sim P(\lambda)$ and $Y \sim P(\mu)$ represent X and Y having Poison distributions with parameters λ and μ, then $X + Y \sim P(\lambda + \mu)$. Using this property, $\sum_1^5 x_i$ is Poisson distributed with parameter 5μ. From this, confidence limits for μ can be derived. Multiplication of $\hat{\mu}$ and the confidence limits by 100 will then give an estimate of E. coli $(100g)^{-1}$ and its 95% confidence limits.

Monte Carlo method

We investigate the sampling properties of MPN and pour plate analysis using simulated counts generated by repeated independent sampling for specified values of the parameter μ, according to the sampling and subsampling laboratory procedures of each method and under the Poisson assumption on the distribution of E. coli in shellfish samples.

For the MPN method we use the **`rpois`** function in R to generate Poisson realisations and assume a fivefold dilution sequence, that is a base sample, 0.1, 0.01, 0.001 and 0.0001 dilutions, with five tubes used at each dilution level. Once a set of realisations has been obtained, we use the R package MPN to derive estimates $\hat{\mu}$ of μ and their confidence interval. Note that the using the MPN method the full distribution of $\hat{\mu}$ across all realisations does not have a tractable algebraic form.

For the pour plate method, we assume five samples are taken, each generated as a Poisson realisation for the selected value of μ. An estimate $\hat{\mu}$ of the mean μ can be obtained from equation and the R routine **`qpois`** used to find confidence limits, given that the estimate $\hat{\mu}$ has a Poisson distribution for the pour plate method.

Simulations are made for the critical E. coli counts 230, 700, 4600 and 46000 E. coli $(100g)^{-1}$, which are used as classification boundaries between A/B and B/C. For 46000 E. coli $(100g)^{-1}$

we recognise that the pour plate method would in practice not be appropriate without dilution of the original sample to reduce concentrations. Results shown here are illustrative.

For each of the two analysis methods we generate 10000 simulated samples for values of μ of 230, 700, 4600 and 46000 E. coli $(100g)^{-1}$ and find the empirical distributions of the resulting MPN and pour plate counts. Because counts are discrete, only a limited number of combinations is possible, giving rise to discrete distributions for both methods, although individual estimates of MPN may not be integer values. The distribution of MPN values does not have a tractable form, but the theoretical distribution of the pour plate estimate is known to be Poisson, the Monte Carlo analysis for the pour plate method is simply confirmatory.

For MPN, at each dilution there are 6 possible presence or absence counts, the values 0 to 5. The total number of combinations is therefore 6^5 or 7776. Many of these combinations are highly unlikely, for example a count of 5 tubes with E. coli present in the most dilute sample and none at the remaining four dilutions. In our simulations of 10000 samples, fewer than 100 different outcomes were generated at least once. More samples would increase this number, but with little contribution to the probability distribution.

As an example, Figure 1 shows the distribution of MPN estimates for 10000 simulations, with the value of μ set to 230 E. coli $(100g)^{-1}$. The distribution is exceptionally ragged because combinations with greatly differing probability of occurring have similar associated MPN estimates, and the distribution never assumes a regular shape. For example, the combinations [4,0,0,0,1], [4,1,0,0,0,], [3,2,0,1,0] give unadjusted MPN estimates x 100 of 165.1, 168.5 and 169.1 E. coli $(100g)^{-1}$. Application of equation for μ set to 230 its respective estimate gives corresponding probabilities of [0.0002, 0.1205, 0.0002]. Very

similar discrete values of MPN may therefore have quite different associated probabilities of occurrence. Empirical estimates from different sets of 10000 simulations vary but results closely correspond to these values.

The ragged nature of the distribution means that exceedance probabilities do not vary smoothly with increasing counts. Note that despite the irregular shape of the distribution of the adjusted MPN values, its mean is very close to 230 E. coli $(100g)^{-1}$.

Spiking study

A laboratory-based experiment was conducted to compare the MPN and pour plate methods at known experimentally spiked E. coli concentrations. Thirty mussels were collected from the Menai Strait, North Wales before being placed in a small-scale depuration unit for 7 days to ensure that any E. coli present in the mussels had been removed. On removal from the depuration unit the mussels were opened using a sterile shucking knife and the flesh and liquor were decanted into a sterile beaker. One ml of 0.1 % peptone water was then added per gram of shellfish before homogenisation using a blender (EURL, 2014). The shellfish homogenate was then split equally into six batches, one for a negative control. The remaining five batches had varying amounts of E. coli K12 culture added, as described below. Each batch was then split equally into 3 sub-batches.

E. coli K12 (LZB 035), supplied by Blades Biological (Kent, UK), was cultured overnight in Luria-Bertani Miller's medium (LB) (Miller, 1972). The optical density of the culture at 600 nm was measured using a spectrophotometer to estimate the concentration of E. coli cells. The culture was then serially diluted in 0.1 % peptone water to reach concentrations that were appropriate for the spiking of the shellfish homogenate. Diluted K12 culture was added to the beakers at the targeted concentrations of E. coli per 100 g of shellfish flesh at

50, 150, 300, 1000, 2000 and 5000 E. coli per 100 g, with each concentration run in triplicate. To verify the concentration of E. coli in the culture, the culture was serially diluted and filtered through a 0.45 µm cellulose nitrate membrane filter and placed on Harlequin™ chromogenic coliform agar before incubation for 24 hours at 37°C.

Once the spike was added to each beaker, the homogenates were blended again before they were processed for subsequent enumeration using the MPN and pour plate methods. One set of control triplicates was spiked with 0.1 % peptone water only to ensure that the depuration had successfully reduced the E. coli in the mussels to an undetectable level.

Results

Monte Carlo simulations

Figures 2 and 3 show empirical distributions of simulated values for the parameter μ set to two of the four classification threshold values. Figures 2a and 3a show, for 230 and 4600 E. coli $(100g)^{-1}$, the unscaled empirical distribution of 10000 simulations for the adjusted MPN and pour plate methods as numbers of values of each count estimate. The sum of these numbers (the y axis) is therefore 10000. Maximum counts given by the unadjusted MPN (distribution not shown), bias-adjusted MPN and pour plate are shown bottom right. The precise numbers will vary according to the random samples selected by **`rpois`**, but their order of magnitude will be unchanged. Figures 2b and 3b show the empirical cumulative probabilities associated with Figures 2a and 3a.

Figures 2a and 2b show that for a true count of 230 E. coli $(100g)^{-1}$ the MPN method is capable of occasionally generating estimates up to an order of magnitude higher than the true value, while pour plate estimates are more stable. Nevertheless, most estimates are within a similar range for both methods. For a true count of 4600 E. coli $(100g)^{-1}$ (Figures 3a, 3b) the pour plate distribution is much more closely concentrated around the true mean than the adjusted MPN method, with much narrower confidence limits. Again, MPN estimated counts can be up to an order of magnitude higher than the true count.

Table 2 shows statistics from 10000 simulations at the four classification threshold E. coli $(100g)^{-1}$ counts for the three methods. Lower and upper CI's refer to a central 95% confidence interval for the empirical distributions. Note the large bias in the unadjusted MPN values, and the much lower upper CI's given by the pour plate method.

Table 3 gives the empirical probabilities of adjusted MPN and pour plate counts being at least two times and four times the true value set to the classification threshold values. While the probabilities of exceedance for pour plate become vanishing small at higher counts, these probabilities are much higher for MPN, and do not diminish at higher concentrations.

Inspection of the RI values for the very high counts does not indicate model inadequacy, as shown in Figure 4 for estimates of the true value 230 E. coli $(100g)^{-1}$. Note that the lower values of the RI, indicating model inadequacy correspond mainly to mid-range values of

MPN, and the highest adjusted estimate (1821), ten times the true value, has an RI value of 0.77 making it acceptable by this criterion. Nevertheless, the probability of this value occurring is 0.0004, making the associated combination [5,5,0,0,0] very rare.

We can also consider the event that a genuinely high E. coli count is underestimated by MPN and pour plate counts. Table 3 shows probabilities of counts being less than a half and less than a quarter of the true counts, using the two methods. The high probability of an MPN value less than 350 when the true count is 700 is largely attributable to the high point probability (0.21) of obtaining the combination [5,2,0,0,0], which gives an adjusted MPN value of 349. The probabilities shown in Table 4 demonstrate that in addition to providing over-estimates of true counts (Table 3), MPN has high probabilities compared to pour plate of failing to detect counts that exceed regulatory thresholds.

Figure 5 shows a barchart of summary results for true counts of 100, 230, 700, 4600 and 10000 E. coli $(100g)^{-1}$. Note from this figure the evident bias in the unadjusted MPN. The figure also emphasises at higher counts the very much smaller confidence intervals given by the pour plate method when compared with MPN. At lower counts, in terms of mean and confidence limits, the performance of the two methods is closer, though note that MPN can generate occasional very high estimates to which normal confidence limits are insensitive because of their infrequency.

Also of interest are probabilities of exceeding the classification thresholds, given a range of true values. Figures 6a and 6b show these probabilities for the thresholds of 230 and 4600 E. coli $(100g)^{-1}$. A perfect method would show a step from zero to one at the threshold value concerned. That is, if the true value were less than the threshold, the method would never (zero probability) generate an estimate above the threshold, with a similar argument if the true value were above the threshold. The closer the curve is to this zero-one function the better the method is at producing a correct decision. Visual inspection suggests the methods perform similarly for the 230 E. coli $(100g)^{-1}$, but that pour plate is markedly superior at 4600 E. coli $(100g)^{-1}$

Table 5 shows a selection of the values used to generate Figures 6a and 6b. The first four columns relate to values around a comparison threshold of 230 E. coli $(100g)^{-1}$, the final four columns to values around the threshold of 4600 E. coli $(100g)^{-1}$. Taking two examples, if the true concentration is 150 E. coli $(100g)^{-1}$, the probability that a shellfish sample will yield a count above 230 is [0.3,0.15,0.08] respectively for unadjusted MPN, adjusted MPN and pour plate. If the true concentration is 3700 E. coli $(100g)^{-1}$, the respective probabilities of a sample count being above 4600 E. coli $(100g)^{-1}$are [0.44,0.21,0.00].

Spiking study

Figure 7 shows the results of the spiking experiment. Note that this barplot is based on three samples for the two methods, with "error bars" ranging from the minimum to the maximum of the three values (i.e. not true confidence intervals). Nevertheless, the data

reflect the theoretical result, in that the precision of pour plate estimates on a log scale increases with increasing counts, although with some evidence of a slight underestimation of the true count. MPN estimates show consistent variability at a log scale with increasing counts, consistent with the theoretical results. The upward bias on the MPN results is more apparent in the spiked experiment than the theoretical bias.

All samples processed by the MPN gave results that were higher than the intended spiked concentration of E. coli whereas the same samples processed by the pour plate returned results lower than the intended spiked concentration.

Discussion

Estimation of bacterial abundance in food and environmental samples is inherently variable. Post sampling variability in the laboratory may be derived from uneven distribution of bacteria in homogenised samples (whichever test method is applied) and potential for differences in growth of cells inoculated into test cultures. As with any laboratory analysis, an additional level of variability may be introduced by human operator or process differences in sample handing, storage, processing, assay performance and record keeping. The guidance given in the official protocols is designed to minimise these sources of variability through standardisation of processes and methods, and quality assurance monitoring via within-lab and inter-lab comparability testing. However, the present results demonstrate that regardless of laboratory procedures there are substantial differences in the inherent statistical properties of the MPN and pour plate methods for measurement of E. coli in shellfish.

Our simulations demonstrate that for E coli concentrations of 230 E. coli $(100g)^{-1}$, the theoretical properties of the standard implementations of the pour plate method and the MPN method considered here are similar. At higher concentrations of E.coli, MPN becomes increasingly less reliable than the pour plate method, being capable of generating estimates an order of magnitude greater than the true value. For MPN, the probability of obtaining a count greater than twice the true count lies in the region of 0.05 and 0.1 regardless of the true count, while for higher counts the probability becomes vanishingly small for the pour plate method. The theoretical analysis also demonstrates that the MPN method can produce genuine extreme outliers, even when E. coli are randomly distributed within the sampled material.. In contrast, the pour plate method is not prone to generating extreme outliers. In addition to generating over-estimates of E. coli counts, MPN also gives greater probabilities than pour plate of under-estimating counts. Probabilities of counts of less than half the true value are of the order of 0.1 to 0.2. These are an indication of the risk of under recording of E.coli values that exceeed regulatory thresholds.

The differences in the statistical properties of the estimates are due to the methodology rather than assumptions about the sampled material; both the MPN and pour plate methods assume an underlying Poisson distribution of E. coli in the sampled material. While the accuracy and precision of estimates depends on, for example, the number of dilutions made in the case of MPN and the number of petri dishes used in the pour plate method, our results show that for the standard procedure at high concentrations of E. coli, there is a sound theoretical basis for greater accuracy and reliability of the pour plate method compared to MPN. This is consistent with modelling by Gronewold and Wolpert (2008) for measurement of faecal coliforms in water samples which indicated differences in intra-sample variability between MPN and plating methods were likely to arise from the intrinsic

uncertainty introduced by the statistical method of calculation of MPN results, rather than human or process error. Development of statistical approaches to account for this intrinsic measurement uncertainty has been integral to the evolution of the MPN method over the > 100 years since its first application (McCrady, 1915). Hence, the MPN assay generates estimates of E. coli abundance derived from statistical calculations, with 95% confidence intervals and a rarity ranking score that can help identify erroneous results (Jarvis et al, 2010). This limitation to the precision of the MPN method is acknowledged (Lee and Silk, 2013; Walker et al, 2018), and in practical terms by the provision of recommended methods for laboratory determination of measurement uncertainty by laboratories, even if these are not applied in regulatory interpretation of results for individual shellfish samples.

The results of the spiking experiment described here compare well with theory, suggesting that the Poisson assumption is a sound basis for analysis for both MPN and pour plate methods within the range of E. coli concentrations considered. This study compared results from individual samples split and analysed with two methods, focusing on measurement uncertainty rather than variability between different samples. The results are consistent across both the modelled and practical results, with relatively greater variability in MPN results at the higher end of the range of E. coli concentrations tested. There are relatively few examples of similar direct comparisons of spike-recovery of E. coli in shellfish by MPN and plate culture methods, especially for the two ISO methods compared here. Data for mussel samples spiked with E. coli, from Pol-Hofstad and Jacobs-Reitsma (2021, Annex 4), show similar results to those observed in the present study; measured MPN values were generally higher than the inoculated concentration, while pour plate results were closer to the inoculated values (though also higher in many cases). In other studies, the measured concentrations of E. coli have been found to be slightly lower or equivalent to the

introduced spiked concentrations. For example, Garcia et al (1995) compared spike recovery in soft shell clams (Mya arenaria) over a range of concentrations from 100 - 10,000 E. coli $(100g)^{-1}$, for which they found that 5-tube MPN yielded slightly but significantly lower results compared to the ETPC/mFC rosilic acid agar plating method, that also were below the intended 100 – 10,000 E. coli $(100g)^{-1}$ spike concentrations.

In practical monitoring of E. coli in shellfish, variability in results is compounded across a range of sources and factors before samples enter the laboratory. Spatial variation across individual shellfish beds has been reported, which in part may be due to proximity to sources of contamination such as sewage discharges and dispersal plumes (Beliaeff and Cochard 1995; Kay et al, 2008). However, some studies have also reported localised "hotspots" within a single shellfish bed that are not readily attributed to known sources of contamination and may in part be due to fine scale differences in tidal elevation and spatial differences in rates of bacterial uptake and elimination (Clements et al, 2015). Short-term temporal and longer-term seasonal variation in E. coli concentrations in shellfish may reflect a range of environmental variables, such as rainfall, river flow, temperature, water turbidity and sunlight (Malham et al 2023). This level of pre-sampling variation in E. coli concentrations in shellfish, responding to seasonal and environmental factors, is considered to be greater than variation between repeat samples within an area or measurement uncertainty for individual samples (Walker et al, 2018). However, the inherent statistical variability in MPN test results means that single unusually high results, that may not necessarily reflect actual food safety risk, can still impact both overall classifications and/or short-term downgrades. To some extent, there is mitigation for over-reporting of E. coli counts in the OCR regulations, in the 20% and 10% of results allowed above 230 and 4,600 thresholds for A and B classifications, respectively (see Table 1). However, this is not the

case for the absolute upper thresholds of 700 and 46,000 E. coli $(100g)^{-1}$ for these classifications. Conversely, the statistical variability of MPN results also carries the potential to miss samples where E. coli concentrations exceed classification thresholds (Table 3), with the consequence that shellfish water classifications may underestimate consumer health risk for production areas that are subject to relatively infrequent periods of high contamination of shellfish. Analysis of long term practical shellfish monitoring data sets has shown a trend of higher results with MPN compared to pour plate TBX, particularly for values >230 E. coli $(100g)^{-1}$, but also how applying the upper or lower limits of the confidence interval range for individual MPN results might substantially influence classification outcomes (Rubini et al, 2023). Similarly, Gronewold and Wolpert (2008) considered how differences in variability in MPN and CFU-based methods of measuring faecal coliforms in environmental water samples might result in differences in management decisions (e.g. closure of shellfish waters, which in the USA is based on bacterial concentrations in water rather than shellfish flesh).

In conclusion, based on the statistical properties of the approved MPN and pour plate tests, there is a strong case for adoption of the TBX pour plate method for measurement of E. coli in bivalve shellfish samples for official control monitoring. This would improve public health protection, and the more reliable results would help reassure the shellfish industry that any changes to classifications and/or area closures are well-evidenced. Currently the only country using the pour plate method in analysis of OCR shellfish samples is the Netherlands (Pinn and Le Vay, 2023), despite being approved for the enumeration of E. coli in shellfish samples in the EU and in the UK. The pour plate method has additional advantages, as a relatively simple and cost-efficient assay, providing results in 24 hours from the start of sample testing in the laboratory. This is in contrast to the MPN method, which is more

laborious and takes a minimum of 45 hours after sample arrival in the laboratory to yield results. The most recent official protocol, based on ISO 16649-2, has increased the upper detection limit for application of the pour plate method in bivalve shellfish samples to 150,000 E. coli $(100g)^{-1}$ (Lowther, 2024), enabling its use across all OCR classification thresholds.

Data availability

Data are available on request.

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Ethical approval

No ethical approval was required for this work.

Conflict of Interest

None declared.

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Author contribution

David Cooper contributed to conceptualization, data curation, formal analysis, methodology, visualisation and writing – original draft.

Finn Mannion contributed to conceptualization, investigation, methodology and writing – original draft.

Laurence Jones contributed to conceptualization, funding acquisition and writing – review and editing.

Eunice Pinn contributed to conceptualization, funding acquisition, methodology, project administration and writing – review and editing.

Rees Sorby contributed to investigation and methodology.

Shelagh Malham contributed to conceptualization, funding acquisition, methodology, supervision and writing – review and editing.

Lewis Le Vay contributed to conceptualization, funding acquisition, project administration, writing – original draft and writing – review and editing.

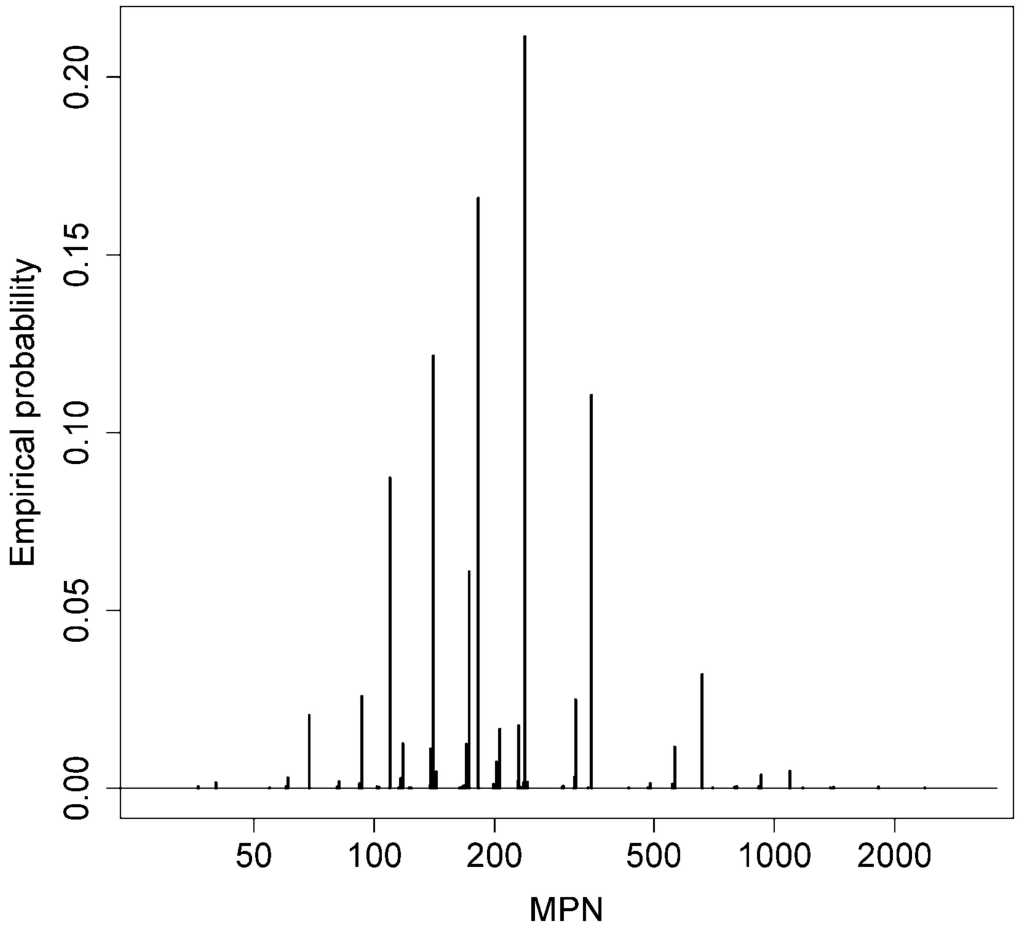

Figure 1. Empirical distribution of MPN from 10000 simulations with 230 E.coli $(100g)^{-1}$.

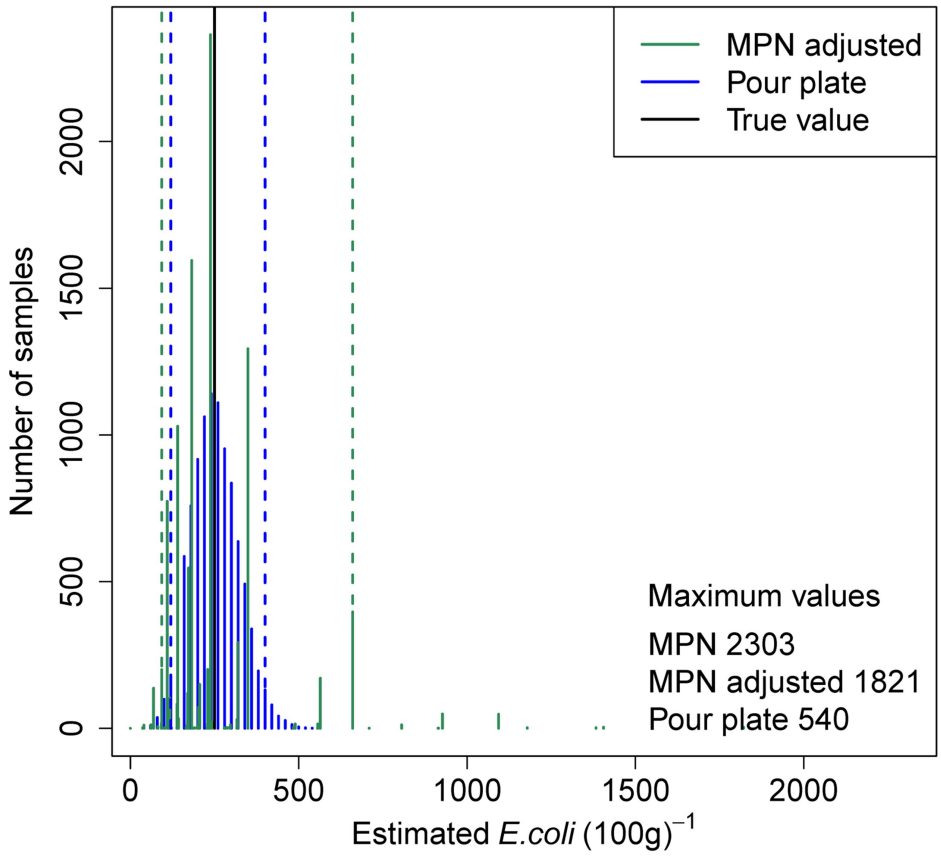

Figure 2a. Estimated MPN and pour plate unscaled empirical distribution and cumulative distribution for samples with 230 E.coli $(100g)^{-1}$. Vertical dashed lines indicate limits of an empirical 95% confidence interval (CI) for each method.

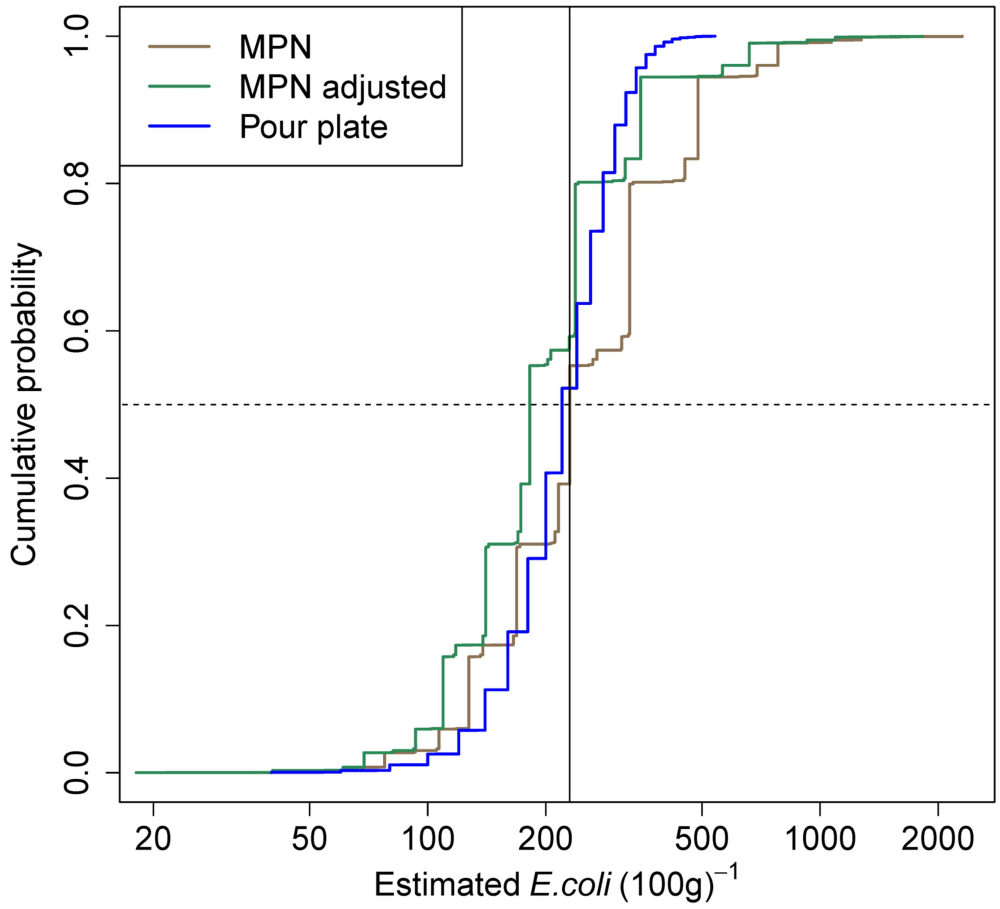

Figure 2b. Estimated MPN and pour plate cumulative distribution for samples with 230 E.coli $(100g)^{-1}$. The vertical line indicates the target E.coli $(100g)^{-1}$.

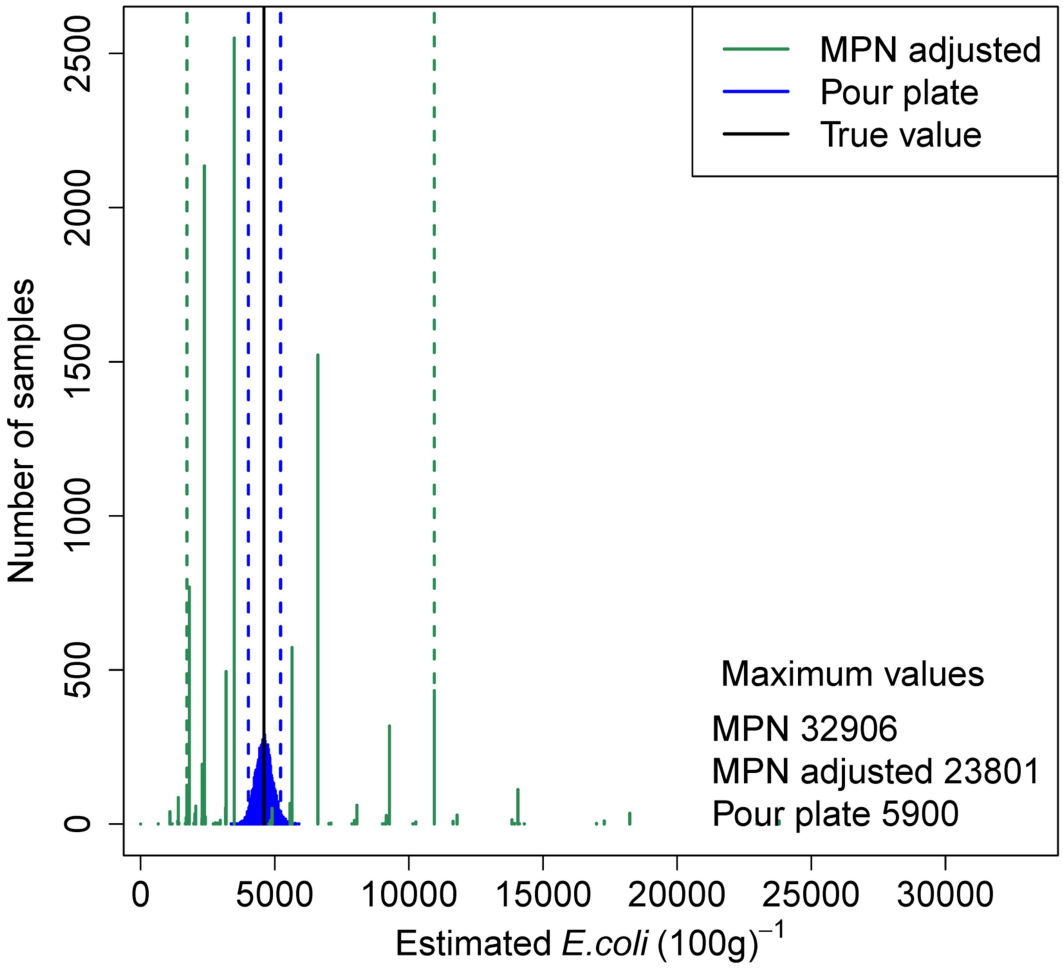

Figure 3a. Estimated MPN and pour plate unscaled empirical distribution for samples with 4600 E.coli $(100g)^{-1}$. Vertical dashed lines indicate limits of an empirical 95% confidence interval (CI) for each method.

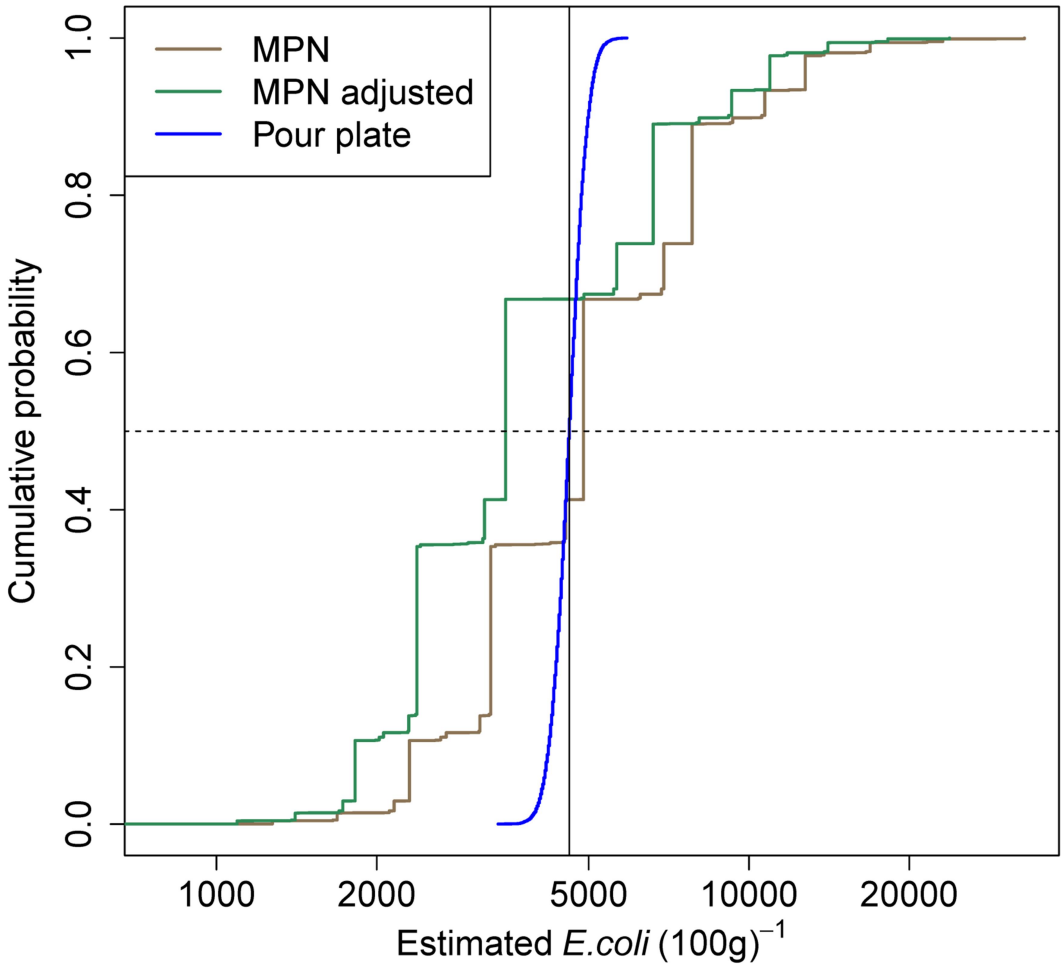

Figure 3b. Estimated MPN and pour plate cumulative distribution for samples with 4600 E.coli $(100g)^{-1}$. The vertical line indicates the target E.coli $(100g)^{-1}$.

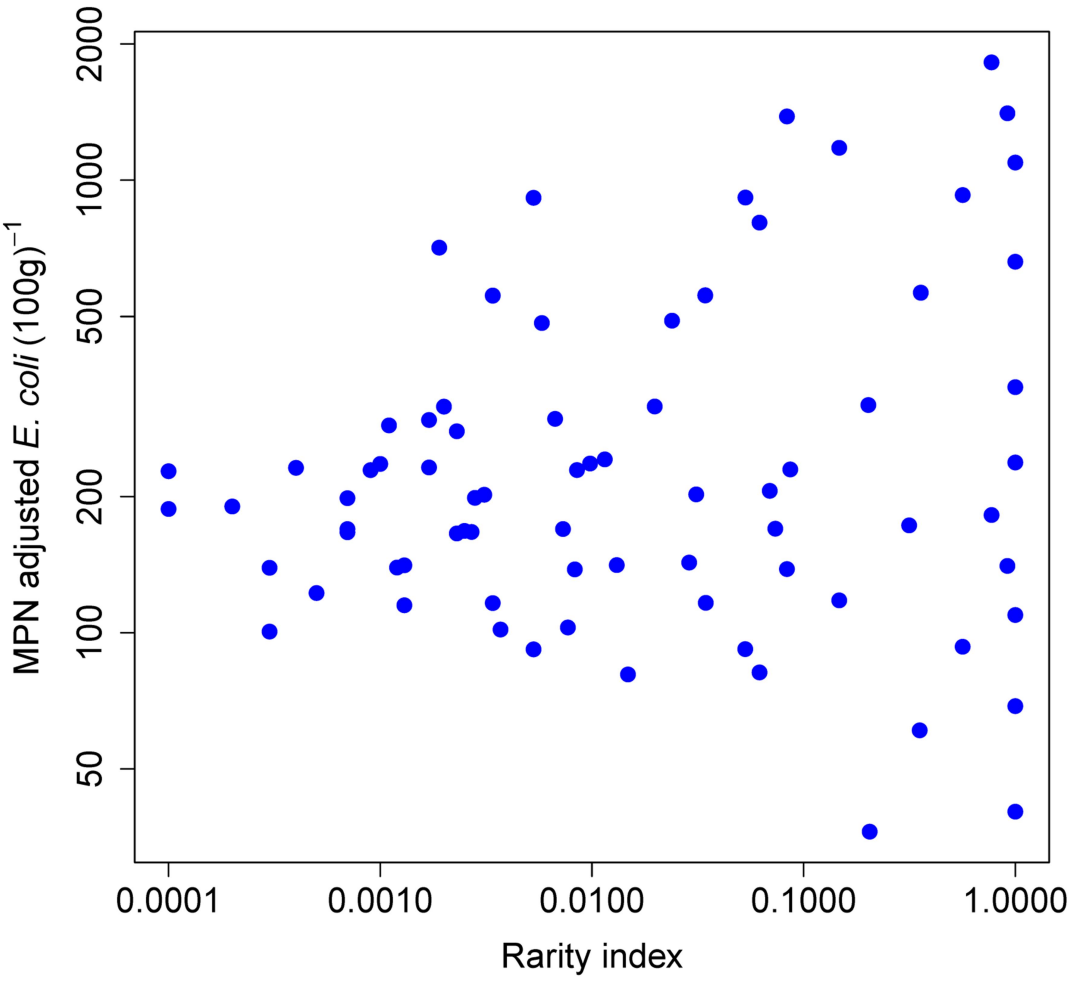

Figure 4. Rarity index (RI) for simulations of 230 E.coli $(100g)^{-1}$ (MPN adjusted values).

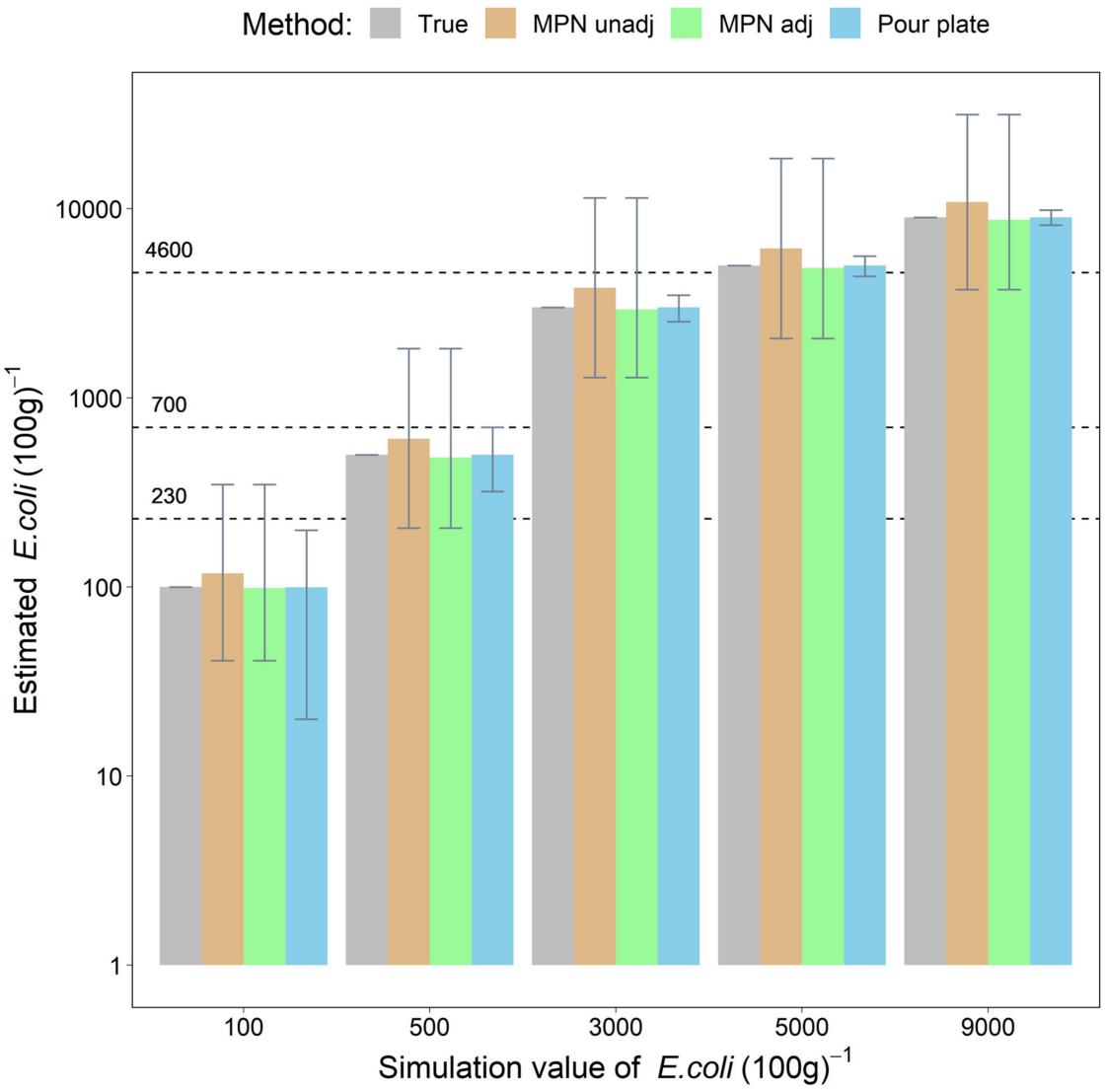

Figure 5. Simulated E.coli $(100g)^{-1}$ in shellfish samples, showing the true value and estimates using the unadjusted MPN, adjusted MPN and pour plate methods. Error bars indicate a 95% confidence interval.

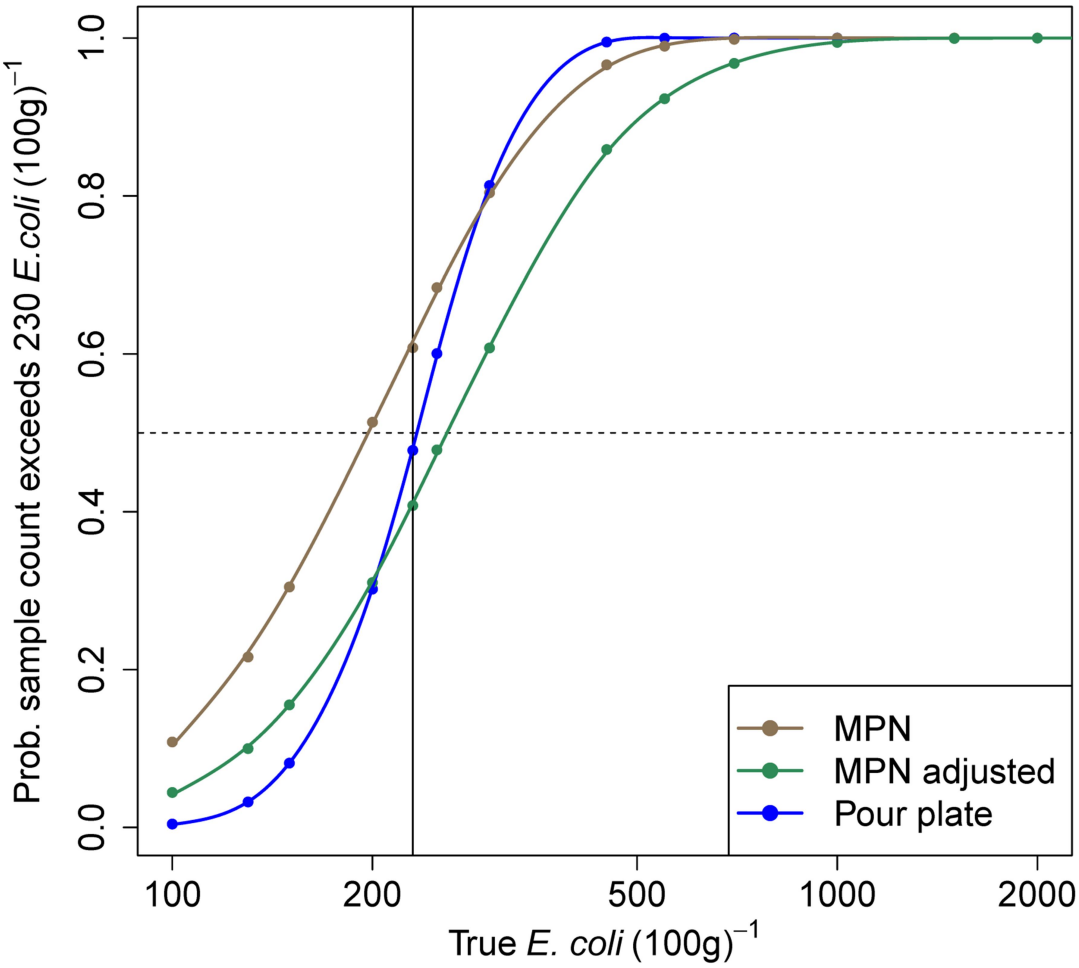

Figure 6a. Probability of estimating exceedances of the critical threshold value 230 E.coli (100g)$^{-1}$ for a range of true counts. The vertical line shows the location of the threshold value.

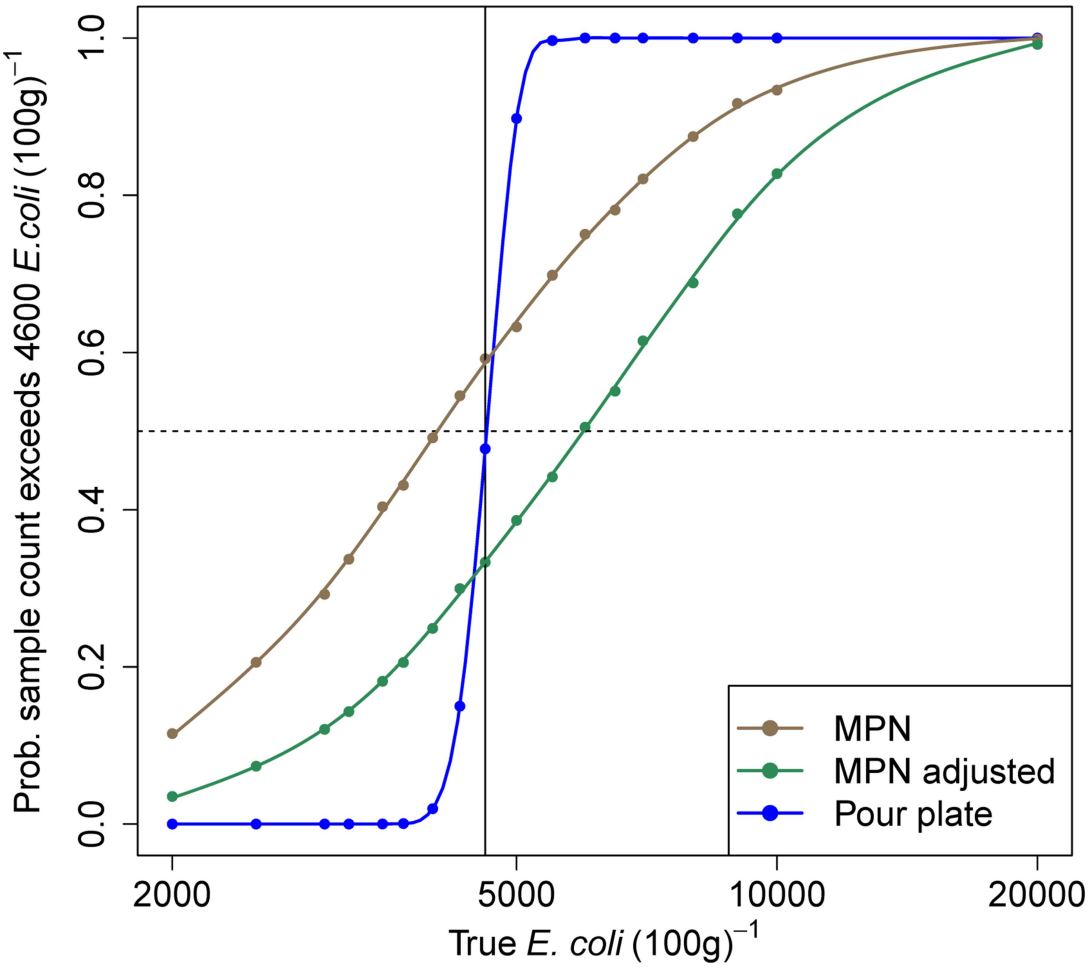

Figure 6b. Probability of estimating exceedances of the critical threshold value 4600 E.coli $(100g)^{-1}$ for a range of true counts. The vertical line shows the location of the threshold value.

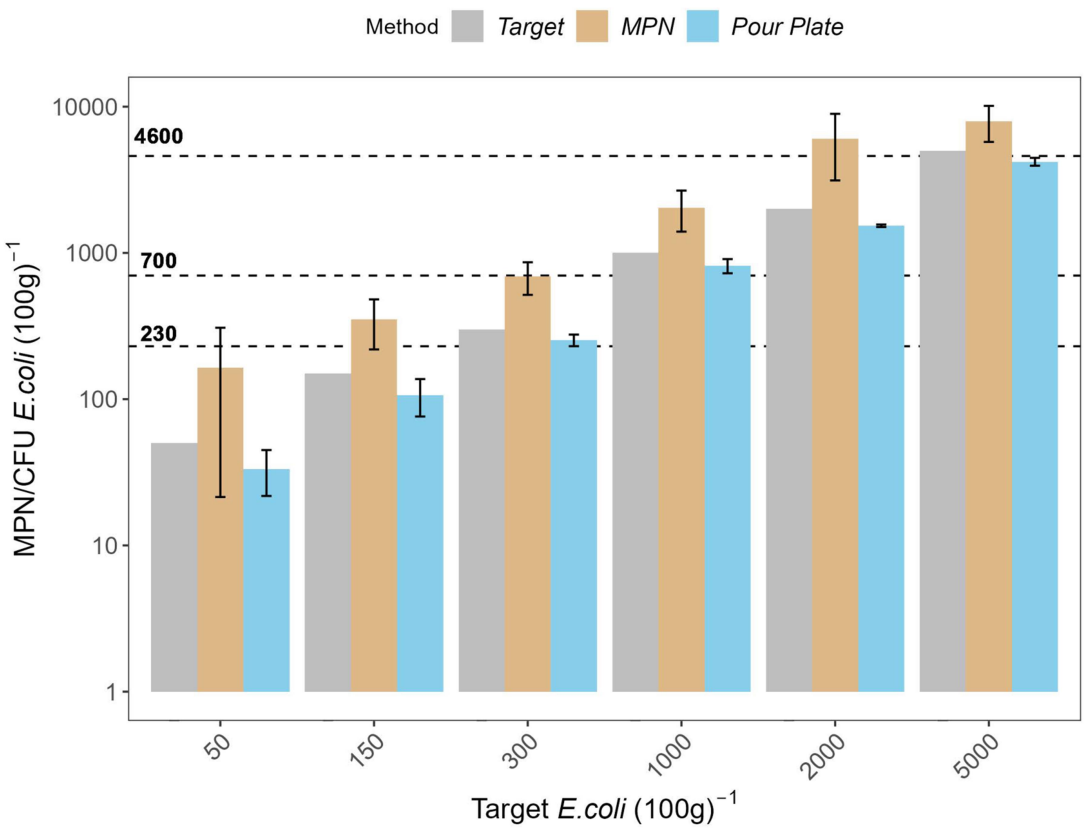

Figure 7. Comparisons of E.coli concentrations reported by the MPN and pour plate to the target E.coli concentration in a homogenised shellfish sample. Dashed horizontal lines indicate key classification boundaries.

Table 1. Outline of the classification system in place for EU and UK bivalve production based on the health standards set out in Annex III of European Community Regulation 853/2004 and Articles 53, 54 and 55 of European Commission Regulation 2019/627.

Classification	Samples Required	*E. coli* concentration Limits	Post-harvesting Treatment Options
A	Generally monthly (minimum of 10 samples per annum)	80 % of samples must be ≤ 230 *E. coli* $(100g)^{-1}$. No result > 700 *E. coli* $(100g)^{-1}$.	1. Shellfish can be harvested directly for human consumption.
B	Generally monthly (minimum of 8 samples per annum)	90 % of samples must be ≤ 4,600 *E. coli* $(100g)^{-1}$. No result > 46,000 *E. Coli* $(100g)^{-1}$.	1. Purification in an approved establishment. 2. Relaying for at least one month in a Class A relaying area. 3. An approved heat treatment process
C	Generally monthly (minimum of 8 samples per	All samples ≤ 46,000 *E. coli* $(100g)^{-1}$.	1. Relaying for at least 2 months in an approved Class B relaying area followed by treatment in an approved purification centre.

	annum)		2. Relaying for at least 2 months in an approved class B relaying area. 3. An approved heat treatment process.
Prohibited		Results > 46,000 *E. coli* $(100g)^{-1}$.	Shellfish from areas with consistently prohibited level results must not be subject to production or harvested.

Table 2. Summary statistics of empirical distributions from 10000 simulations.

E.coli (100g)$^{-1}$	Method	Lower 95% CL	Mean	Upper 95% CL
230	MPN unadj	77.7	289.9	781.0
	MPN adj	68.8	223.9	660.2
	Pour plate	100.0	230.5	380.0
700	MPN unadj	312.3	832.0	2303.5
	MPN adj	229.6	675.2	1821.1
	Pour plate	480.0	699.1	940.0
4600	MPN unadj	2156	5701.4	12756.1
	MPN adj	1727.4	4504.6	10948.0
	Pour plate	4020.0	4595.3	5200.0
46000	MPN unadj	21609.4	56765.8	129933.8
	MPN adj	17282.7	44503.2	110715.9
	Pour plate	44120.0	45993.0	47820.0

Table 3. 2x and 4x exceedance probabilities for MPN and pour plate methods for classification threshold *E. coli* $(100g)^{-1}$.

Target	MPNx2	MPNx4	PPx2	PPx4
230	0.054	0.008	0.001	0.000
700	0.080	0.002	0.000	0.000
4600	0.103	0.001	0.000	0.000
46000	0.103	0.007	0.000	0.000

Table 4. 0.5x and 0.25x under-estimation probabilities for MPN and pour plate methods for classification threshold *E. coli* $(100g)^{-1}$

Target	MPNx0.5	MPNx0.25	PPx0.5	PPx0.25
230	0.149	0.002	0.029	0.001
700	0.387	0.001	0.001	0.000
4600	0.140	0.004	0.000	0.000
46000	0.134	0.005	0.000	0.000

Table 5. Threshold exceedance probabilities around 230 and 4600 *E.coli* $(100g)^{-1}$.

True value	MPNunadj	MPNadj	PP	True value	MPNunadj	MPNadj	PP
100	0.11	0.04	0.01	**3000**	0.31	0.13	0.00
130	0.21	0.09	0.03	**3200**	0.35	0.15	0.00
150	0.30	0.15	0.08	**3500**	0.41	0.19	0.00
200	0.51	0.31	0.30	**3700**	0.44	0.21	0.00
230	0.62	0.40	0.48	**4000**	0.49	0.25	0.02
250	0.68	0.47	0.59	**4300**	0.55	0.29	0.14
300	0.80	0.61	0.82	**4600**	0.59	0.34	0.48
450	0.96	0.85	1.00	**5000**	0.64	0.39	0.89
550	0.99	0.93	1.00	**5500**	0.69	0.44	1.00
700	1.00	0.97	1.00	**6000**	0.75	0.51	1.00
1000	1.00	0.99	1.00	**6500**	0.78	0.57	1.00
1500	1.00	1.00	1.00	**7000**	0.82	0.61	1.00
2000	1.00	1.00	1.00	**8000**	0.87	0.69	1.00