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Determining the 95% limit of detection for waterborne pathogen analyses from primary concentration to qPCR

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ABSTRACT

The limit of detection (LOD) for qPCR-based analyses is not consistently defined or determined in studies on waterborne pathogens. Moreover, the LODs reported often reflect the qPCR assay alone rather than the entire sample process. Our objective was to develop an approach to determine the 95% LOD (lowest concentration at which 95% of positive samples are detected) for the entire process of waterborne pathogen detection. We began by spiking the lowest concentration that was consistently positive at the qPCR step (based on its standard curve) into each procedural step working backwards (i.e., extraction, secondary concentration, primary concentration), which established a concentration that was detectable following losses of the pathogen from processing. Using the fraction of positive replicates ($n = 10$) at this concentration, we selected and analyzed a second, and then third, concentration. If the fraction of positive replicates equaled 1 or 0 for two concentrations, we selected another. We calculated the LOD using probit analysis. To demonstrate our approach we determined the 95% LOD for *Salmonella enterica* serovar Typhimurium, adenovirus 41, and vaccine-derived poliovirus Sabin 3, which were 11, 12, and 6 genomic copies (gc) per reaction (rxn), respectively (equivalent to 1.3, 1.5, and 4.0 gc L^{-1} assuming the 1500 L tap-water sample volume prescribed in EPA Method 1615). This approach limited the number of analyses required and was amenable to testing multiple genetic targets simultaneously (i.e., spiking a single sample with multiple microorganisms). An LOD determined this way can facilitate study design, guide the number of required technical replicates, aid method evaluation, and inform data interpretation.
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1. Introduction

For analysis of waterborne pathogens by qPCR, the limit of detection (LOD) is used to validate and compare methods, qualify results (e.g., categorize as "below the LOD"), and impute values for non-detections. While LODs are often reported, the definitions, methods, and applications for the LOD vary and may not be explained clearly or precisely, making interpretation and comparison difficult. In environmental microbiology and elsewhere, the literature is full of formal and informal terms surrounding the LOD, including critical value, limit of blank, limit of quantification, determination limit, decision limit, detectable-not-quantifiable, sensitivity, lower limit of applicability, lower limit of linear range, distinguishable from zero, minimum detectable value, minimum reporting limit, minimum level, lowest concentration detected, method detection limit, and others (APHA, 2012; Armbruster and Pry, 2008; Burns and Valdivia, 2008; Bustin et al., 2009; Childress et al., 1999; CLSI, 2012; Currie, 1968, 1988; U.S. EPA 2004). In some cases, the LOD is not defined but simply stated with no evidence (e.g., Dunkel et al., 2016; Eichmiller et al., 2014; Harwood et al., 2013; McCall et al., 2015; Newton et al., 2011; Sauer et al., 2011; Villemur et al., 2015).

Following Bustin et al. (2009), we define the LOD as the concentration detected with reasonable certainty. As 95% is a common level of certainty, our definition may be more specifically given as the lowest concentration at which 95% of positive samples are detected. Defined this way, the LOD is a measure of analytical sensitivity (Bustin et al., 2009), and concentrations detected below

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the LOD are not false positives, rather, they have a lower probability of detection. An LOD determined based on this definition can be used to evaluate or validate an assay or method, interpret data, inform negative or censored results, provide information on false negatives, and aid in study design.

Like the definitions, methods used to determine the LOD vary. Methods based on measurement of blank samples or the variance of low-concentration replicate standards may be invalid for qPCR because blank samples do not produce a cycle of quantification (C_q) and qPCR data sets may violate parametric assumptions (i.e., normal distribution and homoscedasticity; Burns and Valdivia, 2008; CLSI, 2012; Kubista, 2014). Mathematical approaches to calculating an LOD (e.g., based on the 3 genomic copies (gc) per reaction (rxn) theoretical LOD for qPCR) should be validated to assure that they accurately represent the method for which they are being applied. An empirical LOD established by a single point (e.g., lowest concentration detected) is incomplete and has limited utility because the certainty aspect (e.g., 95%) is not precisely determined. Modeling approaches based on empirical data, such as probit analysis (Burd, 2010; Wolk and Marlowe, 2011), allow the calculation of an LOD at a desired level of certainty (e.g., 95%). However, empirical methods that establish an LOD for the qPCR reaction alone do not account for sample processing inefficiencies and do not necessarily represent the method as applied to actual sample analysis (Harwood et al., 2014; Staley et al., 2012), though some approaches account for sample processing mathematically, to varying degrees (e.g., Bae and Wuertz, 2012; Harwood et al., 2013; Rajal et al., 2007a).

The broad range of LOD definitions and methods has produced a lack of consistency in how LODs for qPCR-based analyses are determined, reported, and used in environmental microbiology. Some subdisciplines are making efforts toward consensus regarding assay development and validation, methodologies, and quality control (e.g., Harwood et al., 2013; Staley et al., 2012; Stoeckel and Harwood, 2007). However, there is currently not a standard definition or method for qPCR LOD determination in environmental microbiology, as there is in clinical settings (CLSI, 2012). Probit analysis is a standard, robust, empirical method for determining qPCR LODs that is commonly used in clinical microbiology (Burd, 2010; CLSI, 2012; Wolk and Marlowe, 2011), and it can be applied to environmental qPCR analyses. However, extending the probit analysis approach to environmental applications presents challenges due to multiple steps in sample processing and concentration, which add uncertainty in target recovery and increase cost of determining an LOD.

Our objective was to develop an efficient approach to empirically determine the 95% LOD for the entire process of waterborne pathogen detection. We selected the concentrations needed to determine the LOD using a process that limited the number of analyses required (compared to *a priori* selection). Replicate samples ($n = 10$) spiked with microbial targets were analyzed through the entire waterborne pathogen analysis process, and the LOD was calculated using probit analysis. We demonstrated our approach by using it to determine the 95% LOD for three pathogens in a tapwater sample matrix.

2. Materials and methods

2.1. Study design

Our approach to determining the 95% LOD included 1) producing an empirical data set by processing and analyzing water samples spiked with known concentrations of qPCR targets with each concentration replicated 10 times and 2) using regression to calculate the LOD. Each data point in the regression represented a concentration (independent variable) and a proportion of the 10 replicates detected (dependent variable), which was transformed to probits, a measure of detection probability. Regressing probits against concentration, the fitted model was solved for the concentration with a 0.95 probability of detection, which represents the 95% LOD. At least three microbial target concentrations that spanned the LOD were needed for regression. A concentration selection process was used to determine these concentrations while minimizing the number of samples that were analyzed (Fig. 1). To demonstrate our approach, we used it to determine the 95% LOD for three microbial targets.

2.2. Concentration selection process

Microbial target concentrations were selected and analyzed one at a time until three informative data points that spanned the LOD were generated (Fig. 1). Each concentration resulting in a unique combination of positive and negative results (which estimates the probability of detection) was informative. The concentration selection process is best illustrated using an example, so the process for adenovirus is described below (Table 1). Note that while one target is discussed all three were spiked into each sample (at different concentrations) and analyzed simultaneously. For consistency, spike concentrations were converted to the qPCR assay equivalent (i.e., gc rxn^{-1}) of the actual spike concentrations, which were calculated using the sample processing volumes (e.g., sample volume, nucleic acid extraction and elution volumes).

We began the selection process by referencing the adenovirus qPCR standard curve (Fig. 1 step 1). We selected the lowest concentration for which all replicates were detected (11 gc rxn^{-1}). This concentration was detected in 9 of 10 replicates when spiked at the extraction step (Fig. 1 step 2) and in 6 of 10 replicates when spiked at the secondary concentration step (Fig. 1 step 3). These results indicated that detection following full-process analysis was likely (we would have increased the concentration if no replicates had been detected). Moving forward, 11 gc rxn^{-1} was detected in 7 of 10 replicates (detection probability = 0.7) when spiked prior to primary concentration and analyzed through the entire process (Fig. 1 step 4). Based on this result we selected and analyzed 21 gc rxn^{-1} through the entire process to establish a concentration with detection probability = 1 as it is required to span the 0.95 probability level (Fig. 1 step 4). Specifically, we increased the concentration from the previous step but were conservative with the magnitude of increase in order to remain near the informative concentration range (subsequent concentration increases could be made if needed to achieve detection probability = 1). Next, we analyzed a low concentration (2 gc rxn^{-1}) through the entire process, which we selected based on the first two concentrations and by referencing the qPCR standard curve (Fig. 1 step 4). Specifically, we chose a concentration that was less than the first two (11 and 21 gc rxn^{-1}) while greater than standards that were not detected for the qPCR standard curve. At this point there were sufficient informative data points, three, for LOD calculation. However, we analyzed an additional concentration for adenovirus because another target required additional analysis (spiking with additional targets does not increase the processing load). For this final concentration we selected the midpoint of 2 and 11 gc rxn^{-1} (6 gc rxn^{-1}).

2.3. Probit analysis

Target concentrations analyzed through the entire sample process provided data points for calculating the 95% LOD using probit analysis (a regression technique; CLSI, 2012; Wolk and Marlowe, 2011). Experimental replicates were considered either a detection

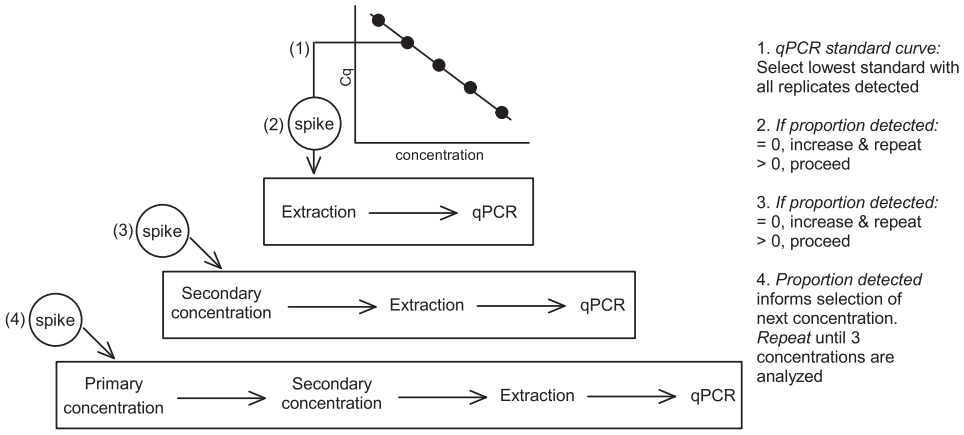

Fig. 1. Concentration selection and analysis process used to determine 3–4 concentrations that span the LOD. Step 1 involved selecting the lowest concentration on the qPCR standard curve with all replicates detected. The selected concentration was used in subsequent steps. Steps 2–4 involved spiking microbial targets prior to extraction (2), secondary concentration (3), and primary concentration (4) and analyzing through qPCR. Step 4 was repeated until 3 informative concentrations were analyzed.

Table 1
Summary of concentration selection process for adenovirus.

Concentration#	Process starting at	Concentration[b] (gc rxn^{-1})	Proportion detected
1	qPCR[a]	11	1.0
1	Extraction	11	0.9
1	Secondary concentration	11	0.6
1	Primary concentration	11	0.7
2	Primary concentration	21	1.0
3	Primary concentration	2	0.0
4	Primary concentration	6	0.5

[a] We referenced the qPCR standard curve to identify the first concentration for testing.
[b] Concentration of the spike, not the qPCR measurement. gc, genomic copies; rxn, reaction.

or non-detection. The fraction of positive replicates was calculated for each concentration by dividing the number of detections by 10 (the number of replicates per concentration) and interpreted as an estimate of the probability of detection. Probability of detection at each concentration was transformed to probits (short for "probability units") using the inverse of the normal cumulative distribution function available in Microsoft Excel® (Microsoft Corporation) and then plotted against $\log_{10}$-transformed concentration data. The 95% LOD was calculated by solving the regression equation for probability = 0.95 (probit = 1.64). Concentrations for other levels of certainty were also calculated.

Because the transformation to probits (inverse of the normal cumulative distribution function) results in values of infinity and -infinity for probabilities of 1 and 0, respectively, numerical values were imputed to allow ordinary least squares fitting of the model. Probit values of 3.72 and −3.72 (which translate to actual probabilities of 0.9999 and 0.0001, respectively) were imputed for probabilities of 1 and 0, respectively, following Bliss (1934). Imputation is unnecessary when a statistical platform that runs a single-step probit analysis with maximum likelihood is used (rather than transformation followed by regression), and while probit analysis is recommended by the Clinical and Laboratory Standards Institute for determining the 95% LOD (CLSI, 2012), other modeling approaches may be used to calculate the LOD.

2.4. Microbial stock cultures

Adenovirus stock was derived from A549 cell cultures of adenovirus 41, and poliovirus stock was derived from BGM cell cultures of vaccine-derived poliovirus Sabin 3. After cytopathic effect was observed, the viruses were released by freeze-thawing the infected cell monolayers three times followed by removal of cell debris by centrifuging at 900× *g* for 10 min. Virus stocks were stored at 4 °C in TE buffer before spiking. To quantify the virus stock preparations extraneous nucleic acids were removed by treatment with Benzonase® (Novagen, Madison, WI) for 30 min at 37 °C followed by incubation for 2 days at 4 °C. This method leaves behind only the nucleic acid protected by intact viral capsids so that when it is subsequently extracted and quantified the nucleic acid accurately reflects the actual number of virions. After Benzonase treatment, viral nucleic acid was extracted using the QIAamp® DNA blood mini kit, but without adding carrier RNA to the AVL buffer as this extra RNA would inflate the apparent virus copy number. Viral DNA or RNA mass was measured using a GloMax® Multi Jr fluorimeter (Promega, Madison, WI) and the DNA or RNA intercalating dyes PicoGreen® or RiboGreen®, respectively (Molecular Probes, Eugene, OR). Nucleic acid mass was converted to genomic copies using the nucleic acid molecular weight for each virus (Roche Molecular Biochemicals 2000).

Salmonella enterica serovar Typhimurium was grown on MacConkey plates overnight at 37 °C and suspended in TE buffer for spiking. *Salmonella* were quantified by diluting an aliquot from the stock in sterile phosphate buffer saline (PBS), fixing with 3% formalin, staining with 10 μg mL^{-1} of 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO), and filtering through a 0.22 μm pre-blackened polycarbonate membrane (Maine Manufacturing LLC, Sanford, ME). Membranes were rinsed with sterile PBS and mounted on glass slides. *Salmonella* spike densities were counted from 50 fields at 1000× magnification under UV light with an epifluorescent microscope (Nikon Eclipse 55i).

2.5. Experimental matrices

Dechlorinated tap-water was used when microbial targets were spiked for the full sample process beginning with primary filtration. All three targets were spiked into 10 L for each experimental replicate (n = 10). For samples analyzed starting at secondary concentration, targets were spiked into 5 L of dechlorinated tap-water with 1% desiccated beef extract, and 500-mL aliquots (n = 10) were processed as described below. For samples analyzed starting at extraction, the final concentrated sample volume resulting from secondary concentration was emulated by spiking targets into 50 mL of ultrafilter backflush solution (see below) containing also 8% polyethylene glycol 8000 (PEG), 0.2 M NaCl, and 1% desiccated beef extract. Aliquots of 280 μL (n = 10) were extracted and analyzed.

Previous evaluation of the tap-water used in this study showed no reverse transcription-qPCR inhibition. Inhibition was evaluated by spiking hepatitis G virus armored RNA (Asuragen Inc., Austin, TX) into the reverse transcription reaction mixture and performing reverse transcription-qPCR as described for poliovirus. The target hepatitis G virus concentration in the qPCR reaction was a quantification cycle (C_q) of 30. The C_q in tap-water for this study was less than 1 cycle higher than the expected C_q for the spiked hepatitis G virus, indicating no inhibition (Gibson et al., 2012).

2.6. Sample processing

Microbial targets spiked into 10 L dechlorinated tap-water samples were concentrated with dead-end ultrafiltration using Hemodialyzer Rexeed-25 s filters (Asahi Kasei Medical MT Corp., Oita, Japan) and backflushed from filters using 500 mL of a solution containing 0.5% Tween 80, 0.01% sodium polyphosphate (NaPP), and 0.001% antifoam Y-30 (Mull and Hill, 2012). Samples were further concentrated by PEG flocculation (Lambertini et al., 2008). Briefly, following addition of 8% PEG, 0.2 M NaCl, and 1% desiccated beef extract and overnight incubation at 4 °C, samples were centrifuged for 45 min at 4700× *g* at 4 °C, and the pellet was resuspended in TE buffer to a final concentrated sample volume of 3 mL. Nucleic acids were extracted from 280 μL of final concentrated sample volume with the QIAamp DNA blood mini kit and buffer AVL using a QIAcube® (Qiagen, Valencia, CA). Final volume of the nucleic acid suspension was 100 μL.

Poliovirus RNA was reverse-transcribed by adding 4.3 μL nuclease-free water and 0.35 μL random hexamers (ProMega, Madison, WI) to 4.3 μL of the extracted nucleic acids. This mixture was heated for 4 min at 99 °C and then mixed with 16.05 μL RT master mix consisting of the following components reported as final concentrations in the 25 μL total reaction volume: 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 0.8 mM dithiothreitol, 70 μM of each deoxynucleoside triphosphate (ProMega), 18 U RNAsin® (ProMega), 50 U SuperScript® III reverse transcriptase (Invitrogen Life Technologies, Rockville, MD). Reaction incubation was 42 °C for 60 min followed by 5 min at 95 °C and then held at 4 °C until PCR amplification.

2.7. qPCR

qPCR was performed with a LightCycler® 480 instrument (Roche Diagnostics, Mannheim, Germany) using the LightCycler 480 Probes Master kit. The 20 μL reaction volume consisted of 14 μL master mix and 6 μL extracted DNA (adenovirus and *Salmonella*) or cDNA from reverse transcription (poliovirus). Primers (Integrated DNA Technology, Coralville, IA) and hydrolysis probes (TIB Molbiol, Berlin, Germany) and their concentrations are reported in Table 2. Thermocycling began at 95 °C for 10 min followed by 45 cycles of 10 s at 95 °C and 1 min at 60 °C. Each qPCR batch included a qPCR negative (no template control), which was nuclease-free water in PCR master mix. qPCR was performed in duplicate, and the experimental replicate (n = 10) was considered positive if either technical replicate was positive (both technical replicates were positive for 73% of the detected experimental replicates).

Standard curves were generated by serially diluting gBlocks® (Integrated DNA Technology, Coralville, IA) of the target sequence in 0.02% bovine serum albumin. C_q values were calculated using the second derivative maximum method and regressed against the decimal logarithm of target concentration using the non-linear function provided by the LightCycler 480 software. Standard curve parameters are reported in Table 3.

3. Results and discussion

3.1. Concentration selection and analysis process

At least three microbial target concentrations that span the 0.95 probability level were needed to determine the 95% LOD. Wolk and Marlowe (2011) prescribed analyzing high, middle, and low concentrations to calculate the LOD using probit analysis, but for waterborne pathogen analyses relevant concentrations are difficult to determine *a priori* because the effect of target loss during sample processing on the probability of detection is unknown. As a result, it was a challenge to identify detectable concentrations that were also informative (i.e., concentrations that result in a mix of positive and negative replicates for calculating the probability of detection).

Using our selection process, we analyzed 3–5 concentrations for each pathogen that ranged from 2 to 100 gc rxn^{-1} (Table 4). In contrast, other methods to determine the LOD based on empirical data routinely involve analysis of all concentrations from a serial dilution (1:2 is common) over several orders of magnitude (e.g., Burns and Valdivia (2008) used 15 concentrations over 4 orders of magnitude). *A priori* selection of concentrations over a large range produces many data points and assures that the 0.95 probability level will fall within the range of samples analyzed. This approach is reasonable when considering the qPCR reaction alone, but such *a priori* selection of target concentrations may not be practical for determining an LOD that accounts for the entire sample process due to the time and cost of processing samples and the level of replication necessary. Moreover, selecting concentrations without considering prior knowledge produces many uninformative data points (i.e., multiple concentrations with 0 or 1 probability of detection; Fig. 2).

We point to three considerations that facilitated efficient selection of detectable, informative concentrations. First, we began analysis with the lowest pathogen concentration that was consistently positive at the qPCR step (based on its standard curve). We chose this concentration because the standard curve provided evidence that 1) it was detectable, and 2) it was near a level at which non-detections would occur in some replicates (non-detections occurred at the next lowest concentration standard). Second, rather than spiking this concentration at the filtration step, we initially spiked it at the extraction step, and then the secondary concentration step, to ensure that these processing steps did not cause target loss to the point of non-detection. When results indicated that detection following the entire sample process was likely, we spiked at the primary concentration step. Third, we selected the remaining concentrations one at a time using the probability of detection at each concentration to inform our selections. Using an iterative approach limited redundant data points and unnecessary analyses. Note that while we began our selection process by referencing the qPCR standard curve, any data that indicate the concentration breakpoint at which the probability of detections

Table 2
Primer and probe concentrations, target genes, and references for qPCR assays.

Microbe	Target gene	Primer/probe concentration (nM)	Primer and probe reference
Salmonella	invA	500/50	Hoorfar et al., 2000
Adenovirus	Hexon	500/50	Kuo et al., 2009
Poliovirus	5′ UTR	300/50	De Leon et al., 1990; Monpoeho et al., 2000

Table 3
qPCR standard curves quality assurance parameters.

Microbe	Efficiency	Mean square error	r^2[a]	Highest C_q measured
Salmonella	1.814	0.0297	0.9703	40.00
Adenovirus	1.924	0.0010	0.9990	39.26
Poliovirus	1.973	0.0094	0.9906	38.78

[a] The r^2 value was calculated as one minus the mean square error determined by the LightCycler software (Chuck Hardwick, Roche technical support, personal communication).

Table 4
Concentrations analyzed through the entire process (from primary concentration to qPCR) for LOD determination.

Concentration[a] in gc rxn^{-1} (proportion detected)		
Salmonella	Adenovirus	Poliovirus
100 (1.0)[b]	21 (1.0)	50 (1.0)[b]
40 (1.0)	11 (0.7)	15 (1.0)[b]
10 (0.9)	6 (0.5)	9 (1.0)
3 (0.5)	2 (0.0)	6 (0.8)
		3 (0.4)

[a] Concentration of the spike, not the qPCR measurement.
[b] Not included in regression (only the lowest concentration producing a detection proportion of 1.0 was used). gc, genomic copies; rxn, reaction.

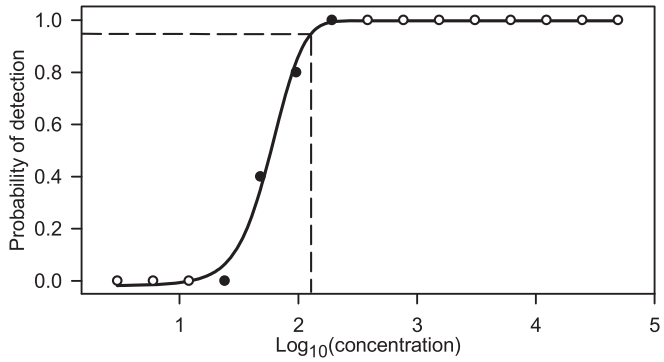

Fig. 2. Typical relationship between concentration and probability of detection. Data represent two-fold dilutions over four orders of magnitude. *A priori* selection of concentrations over this range yields many redundant/uninformative data points (open symbols). Dashed line indicates the 95% LOD.

becomes less than 1 may also be an effective starting point.

3.2. Probit analysis

Three or four concentrations that were analyzed through the entire process were used in the regression model to calculate the LOD for each microbial target (Table 4). The concentrations analyzed spanned the LOD (i.e., 0.95 fell within the range of the probabilities of detection for the concentrations analyzed). Multiple concentrations with detection probabilities of 0 or 1 were not included in the regression because they were uninformative (Fig. 2). Specifically, the following concentrations with detection probabilities of 1 were excluded from the regression because the next highest concentration also had a detection probability of 1: for *Salmonella*, 100 gc rxn^{-1}, and for poliovirus, 50 and 15 gc rxn^{-1} (Table 4). Probit transformation produced a linear relationship from one that is typically sigmoidal, allowing linear regression. Coefficients of determination (r^2) were 0.98, 0.97, and 0.85 for *Salmonella*, adenovirus, and poliovirus, respectively.

Because many arbitrarily high concentrations can produce an empirical detection probability of 1, the highest concentration in the regression may have undue influence on the slope and therefore the LOD. For example, if instead of using the 40 gc rxn^{-1} data point for *Salmonella* as the concentration yielding a detection probability of 1 we had settled on a concentration of 400 gc rxn^{-1} (still yielding a detection probability of 1), the calculated LOD would have doubled. It is important, therefore, to analyze low but detectable concentrations and to be cautious with concentrations that yield all positive or negative replicates.

3.3. LOD determination

We demonstrated our LOD approach by determining the 95% LOD for three pathogens. The 95% LOD for the entire process from dead-end ultrafiltration to qPCR was determined as 11, 12, and 6 gc rxn^{-1} for *Salmonella*, adenovirus, and poliovirus, respectively (Table 5). Based on the 1500-L sample volume prescribed in EPA Method 1615 (Fout et al., 2010) for tap-water and assuming no loss with increased volume, this is equivalent to 1.3, 1.5 and 4.0 gc L^{-1} for *Salmonella*, adenovirus, and poliovirus, respectively. The LOD at other levels of certainty can be calculated using the same probit regression model (Table 5).

The LOD is based on detection/non-detection criteria, not quantification. For example, while 11 gc rxn^{-1} of *Salmonella* would be detected 95% of the time, it may not be quantified as that concentration when detected. The disparity between actual concentration (in this case, the spike concentration) and measured concentration occurs because sample processing is not totally efficient and pipetted PCR template is Poisson-distributed. In effect, the process efficiency dictates how much target nucleic acid reaches the template vial, and the Poisson distribution describes the probability that target template will be pipetted from the template vial to the qPCR reaction. This fact has two important implications.

First, for an LOD encompassing the entire waterborne pathogen sampling and analytical process, changes to sample processing procedures (e.g., primary filtration elution volume, volume eluted following nucleic acid extraction, dilution to alleviate qPCR

Table 5
Limits of detection that account for the entire sample process at various levels of certainty for *Salmonella*, adenovirus, and poliovirus.

Certainty level (%)	*Salmonella*		Adenovirus		Poliovirus	
	gc rxn^{-1}	gc L^{-1}[a]	gc rxn^{-1}	gc L^{-1}[a]	gc rxn^{-1}	gc L^{-1}[a]
99	17	2.0	15	1.8	7.1	4.9
95	**11**	**1.3**	**12**	**1.5**	**5.8**	**4.0**
50	3.4	0.4	7.1	0.8	3.6	2.5
10	1.4	0.2	4.7	0.6	2.4	1.7

Bold numbers indicate the 95% limit of detection.
[a] gc L^{-1} based on 1500 L sample volume for tap-water (Fout et al., 2010). gc, genomic copies; rxn, reaction.

inhibition) or the efficiency of nucleic acid recovery (e.g., during nucleic acid extraction) can alter the LOD because the concentration or quantity of target nucleic acid is changed. As a result, the process used to determine the LOD must match the process that will be used for unknown sample analysis for the LOD to be relevant for those unknown samples. However, conveniently, after an LOD is determined for a microbial target, the effect of changing many processing parameters can be calculated, and, following validation, the LOD can be adjusted accordingly. For example, if the elution volume from the extraction step is increased (say doubled, diluting the nucleic acid template 1:2), the 95% LOD will increase proportionally (in this example the 95% LOD would double). In the same way, the increase in the 95% LOD from dilution to mitigate qPCR inhibition can be quantified.

Second, the effect of technical replication on the LOD can be calculated. The Poisson distribution describes the probability of a concentration being observed (i.e., pipetted) given an average concentration. The probability of drawing 0 gc rxn^{-1} from a vial that contains the target sequence is the probability of a false negative. For example, for a vial with an average concentration of 3 gc rxn^{-1}, there is a 5% chance of pipetting 0 gc rxn^{-1} (this is the basis for the often-cited qPCR theoretical LOD of 3; Appendix). When multiple draws are taken (i.e., technical replicates), the probability of pipetting the target sequence in *at least one* of the draws increases with the total number of draws, which decreases the LOD. Specifically, the relationship between the LOD and the number of technical replicates is inversely proportional (e.g., doubling the number of technical replicates halves the LOD) and is calculated as $\lambda = -\ln(1 - 0.95)/n$, where λ is the template concentration per reaction in the vial, 0.95 is the probability that one of the technical replicates contains the target sequence (i.e., positive result), and n is the number of technical replicates (Appendix). Furthermore, solving the equation for n allows calculation of the number of technical replicates needed to achieve an LOD of interest. Note that any probability level can be used with the above equation (e.g., 0.99 rather than 0.95).

Because the above equation is based only on the Poisson distribution, it applies to pipetting template for qPCR and does not account for the entire sample process. While the LOD (λ) calculated on a per reaction basis using the equation can be converted to a per L basis (thus reflecting the concentration and dilution steps in the sample process), it still does not account for target loss due to inefficiencies in sample processing. However, an LOD established for the entire sample process (that includes the effect of target loss) can also be easily adjusted. For example, the LOD we determined for adenovirus was 1.5 gc L^{-1} using two technical replicates. Based on the equation, using half the technical replicates (one) would theoretically result in an LOD that is double (3 gc L^{-1}). Calculations that determine how the LOD responds to changes in technical replication and sample processing parameters can be useful when considering study design. The accuracy of these calculations depends on the accuracy of the underlying LOD, which in turn depends on the LOD determination approach.

3.4. Key aspects of the approach

Our approach that accounts for the entire sample process reflects actual sample analysis, which contrasts with LODs determined for the qPCR reaction alone (e.g., Bae and Wuertz, 2012; Harwood et al., 2013; Jenkins et al., 2009; Kildare et al., 2007; Kirs and Smith, 2007; McQuaig et al., 2009; Oster et al., 2014; Rajal et al., 2007a; Sauer et al., 2011). In some cases assay-based LODs are extended to the entire sample process through unit conversions (e.g., gc rxn^{-1} to gc L^{-1}), which requires additional data or assumptions regarding target recovery (e.g., Bae and Wuertz, 2012; Harwood et al., 2013; Jenkins et al., 2009; Layton et al., 2013; Rajal et al., 2007a, 2007b; Sauer et al., 2011). Using data from samples analyzed through the entire process is more direct and accurate than extrapolating an assay-level LOD. This was made feasible by working the process backwards, beginning with spiking the lowest target concentration that gives all positive replicates at the qPCR step into the extraction step (then the secondary concentration and primary concentration steps) to find concentrations that are detectable after processing losses. Selecting concentrations *a priori* would likely produce many uninformative data points. The use of this concentration selection process reduces the number of unnecessary analyses and is a novel aspect of our approach.

Another key aspect of our approach is the use of several concentrations that are modeled by probit analysis to yield a level of detection certainty (e.g., 95%). In contrast, LODs in environmental microbiology are often defined by a single point, such as the lowest concentration detected or the lowest concentration with a given proportion (e.g., 2 of 3) of positive replicates (e.g., Ahmed et al., 2014; Gonzalez and Noble, 2014; Harwood et al., 2009; Kirs and Smith, 2007; Layton et al., 2006; McQuaig et al., 2009; Seurinck et al., 2005; Staley et al., 2012). While based on empirical data, relying on a single point to establish an LOD is less robust than modeling approaches that use multiple points, and a single-point LOD does not have a defined certainty (e.g., 95%) associated with it.

While many common LOD definitions and methods are not ideal, other LOD approaches incorporate the key aspects described here (modeling empirical data and accounting for the entire sample process) to varying degrees. In terms of modeling, probit analysis is often used in clinical applications when determining the LOD for a qPCR assay (Burd, 2010). Burns and Valdivia (2008) suggest a modeling and bootstrapping approach for qPCR assays used for trace detection that provides unique data analysis benefits, though the number of concentrations and technical replicates needed would not be practical if applied to the full sample process used in environmental applications. In terms of accounting for the entire sample process, Rajal et al. (2007a,b) used an internal recovery control to adjust an assay-level LOD for target loss due to processing inefficiencies, and doing so for each sample allowed determination of a sample-specific LOD. Also, new assays for environmental applications, like microbial source tracking, may be characterized in a sample matrix of interest and account for processing steps (e.g., Harwood et al., 2009; Layton et al., 2006; Seurinck et al., 2005). Notably, Staley et al. (2012) present a suite of three LOD methods, including for the qPCR assay alone, for extraction followed by qPCR, and for the full sample process, which demonstrates how the scope of a method can affect the LOD.

3.5. Limitations and application

There are several limitations for LODs determined by our approach. First, LODs were determined by analyzing 10-L water samples meant to represent field samples that may have volumes 100 times greater, which assumes that recovery is consistent across sample volumes. Similarly, LODs were determined in a single sample matrix, which may not account for differences in recovery or qPCR efficiency in other matrices (Borchardt et al., 2013; Harwood et al., 2009; Lambertini et al., 2008; Staley et al., 2012). While the impact of dilution to overcome RT-qPCR inhibition can be calculated, the matrix effect on other aspects of the sample process is more difficult to determine. Finally, LODs were determined based on the entire sample process, so they are specific to that process (e.g., ultrafiltration for primary concentration). However, the LOD may be adjusted for some changes in the sample process, like technical replication and processing volumes (e.g., volume of nucleic acid eluted from extraction).

The 95% LOD is defined as the concentration at which 95% of positive samples are detected (Bustin et al., 2009). By this definition, it is not appropriate to use the LOD as a detect/non-detect cut-off point to avoid false positives. Samples detected at concentrations below the LOD are positive, there is simply a lower probability of detecting them. Furthermore, the LOD does not assure diagnostic accuracy, nor does it establish a level at which quantification is accurate or precise. However, data obtained while determining the LOD may be suitable for determination of the limit of quantification (LOQ), but we have not attempted to extend our LOD approach to encompass the LOQ because of the open-ended nature of its definition. The LOQ is defined as the lowest concentration that can be measured with a stated level of accuracy and/or precision (CLSI, 2012; Kubista, 2014). The specific technical aspects of the LOQ (i.e., the stated level of accuracy and/or precision) vary with the application and purpose, so the data requirements (e.g., concentration range and interval, level of replication) vary as well. Specifically, data from this LOD approach may be unfit for LOQ determination because the concentrations analyzed may be below the LOQ. In addition, the LOQ determination method may not be compatible with the many non-detections in the LOD data set that are needed for LOD calculation, and low concentrations produce a binned terminal C_q on some qPCR platforms (e.g., our Roche LightCycler 480 assigns a C_q of 40 to all positive samples that amplify in cycles 40–45), which artificially reduces variation in quantification.

4. Conclusions

- Determining the 95% LOD for waterborne pathogen analyses by qPCR can be laborious and expensive because target losses during sample processing steps are unknown. Without knowledge of which target concentrations to choose, many of the concentrations tested produce unusable data because they do not yield the combination of positive and negative replicates necessary for LOD determination. We present a simple but efficient approach of spiking target concentrations iteratively and backwards through the analysis procedural steps as a means of quickly obtaining the most informative data for LOD determination.
- Probit analysis is well-established as a method for determining LODs of specified certainty, and it is applicable and easy-to-use for LOD determination of waterborne pathogen analyses.
- Based on the probability distribution for randomly pipetting target into a qPCR reaction, we present an equation for calculating the number of technical replicates (at the qPCR step) necessary to achieve the LOD desired.
- An empirically-determined qPCR LOD of specified certainty that accounts for the entire sample process can be used to compare and evaluate methods, interpret data, and inform negative or censored results. In addition, it can aid in study design, such as by determining an appropriate number of technical replicates, or determine if a pathogen concentration of interest (e.g., biologically significant or of concern for human health) is detectable.

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Appendix

The theoretical 95% limit of detection (LOD) for qPCR is often given as three copies per reaction. This value is derived from the probability mass function (PMF) of the Poisson distribution, which is:

$$P(X = k|\lambda) = \frac{\lambda^k}{k!}e^{-\lambda} \tag{A.1}$$

In this equation, X is a discrete random variable, k is an index representing possible values of X, and λ is the average rate at which discrete "events" occur in a fixed amount of time or space. For our purposes, we can define an individual "event" as an individual gene copy and the "space" in which this event occurs as the volume of sample pipetted into a PCR. Given these definitions, X represents the actual, randomly distributed number of genomic copies pipetted into a PCR.

We will assume that if even one gene copy is successfully pipetted into a PCR, then that gene copy will be amplified and result in a detection. With this assumption, we have defined the probability of a detection (P_d) as being the probability that X does not equal zero:

$$P_d = P(X \neq 0|\lambda) \tag{A.2}$$

Now we just need an expression to calculate the probability that X does not equal zero for a Poisson-distributed random variable. To start, we solve the Poisson distribution's PMF for the probability that X does equal zero:

$$P(X = 0|\lambda) = \frac{\lambda^0}{0!}e^{-\lambda} = \frac{1}{1}e^{-\lambda} = e^{-\lambda} \tag{A.3}$$

The probability of detection (i.e., of X not equaling zero) is simply the complement of Equation (A.3):

$$\begin{aligned} P_d &= 1 - P(X = 0|\lambda) \\ P_d &= 1 - e^{-\lambda} \end{aligned} \tag{A.4}$$

Now we can solve for λ, which is the average number of "events" per "space" (genomic copies per pipetted sample volume), as a function of the probability of detection:

$$\begin{aligned} e^{-\lambda} &= 1 - P_d \\ \lambda &= -\ln(1 - P_d) \end{aligned} \tag{A.5}$$

And now, we can substitute in any arbitrary probability of detection that we are interested in to find the value of λ that corresponds to that probability. For a 95% probability of detection, we get:

$$\lambda = -\ln(1 - 0.95) = 3.0$$

This is why the theoretical 95% LOD for qPCR is frequently cited as three copies per reaction. As an alternative example, we could calculate the theoretical 99% LOD:

$$\lambda = -\ln(1 - 0.99) = 4.6$$

The theoretical LOD for qPCR is also often cited as one copy per reaction. This definition is based on the physical limit of the analytical system rather than on a probability of detection. However, we can use Equation (A.4) to calculate the theoretical probability of detection for a sample in which the concentration is one copy per reaction:

$$P_d = 1 - e^{-1} = 0.63$$

Thus, the often cited theoretical LOD for qPCR of one copy per reaction is a 63% LOD. In other words, if a sample contains one gene copy per volume of template added to the reaction, then there is theoretically only a 63% chance of obtaining a positive result when that sample is analyzed once. Of course, based on intuition and experience, we know that analyzing a sample more than once should increase our probability of obtaining a detection for a truly positive sample.

This leads to another important point. Equations A.4 and A.5 can be generalized to consider any arbitrary number of technical replicates at the qPCR step. To do so, we return to Equation (A.3) and consider the probability of pipetting zero genomic copies from a sample when, in fact, some non-zero number of genomic copies are present.

If each act of pipetting is assumed to be independent of all the others, then the probability of pipetting an arbitrary number (n) of reaction volumes from a sample and obtaining zero genomic copies in each reaction volume can be calculated as the probability of multiple events in a series:

$$P(X = 0|\lambda, n) = \left(e^{-\lambda}\right)^n = e^{-\lambda n} \tag{A.6}$$

Now we can combine Equations A.4 and A.6 to obtain an expression for calculating the theoretical probability of detection for any arbitrary number of technical replicates:

$$\begin{aligned} P_{d|n} &= 1 - P(X = 0|\lambda, n) \\ P_{d|n} &= 1 - e^{-\lambda n} \end{aligned} \tag{A.7}$$

And Equation (A.7) can be solved for λ:

$$\begin{aligned} e^{-\lambda n} &= 1 - P_{d|n} \\ \lambda &= \frac{-\ln\left(1 - P_{d|n}\right)}{n} \end{aligned} \tag{A.8}$$

Note that λ and n are inversely proportional. This makes for an easy rule-of-thumb describing the relationship between the two. At a given probability of detection, doubling the number of technical replicates should theoretically reduce the LOD by half.

To derive Equation (A.7), we assumed that each pipette draw from a sample is independent of all other pipette draws. In other words, we assumed sampling with replacement. This assumption is convenient because it simplifies the derivation, but it is not technically correct. We are actually sampling without replacement, which means that λ for any particular draw is dependent on any prior draws that have occurred.

However, since the most likely value of X for a Poisson-distributed variable is something close to λ, the value of λ tends not to change on average. As a result, Equation (A.7) closely approximates sampling without replacement from a Poisson distribution, except when λ becomes very small. We confirmed this behavior with a relatively simple Monte Carlo simulation in R (R Core Team, 2015; code is available from us upon request).

Finally, several important points should be kept in mind related to how we derived Equation (A.4):

1. We have assumed that genomic copies in a sample tube are Poisson-distributed. This implies that each genomic copy is physically independent of all others in the sample tube. If this condition is not met, then genomic copies will tend not to be Poisson-distributed and other discrete distributions might be required to determine the theoretical LOD for qPCR.
2. We have also assumed that if even a single gene copy is successfully pipetted into a PCR, then that gene copy will amplify and result in a detection. Thus we have implicitly assumed that the PCR is well-optimized and that no confounding factors (e.g., the presence of PCR-inhibiting compounds) exist that would prevent amplification.
3. The theoretical LOD only considers the act of pipetting a subsample directly into a PCR. It does not consider all the steps that must occur to produce the sample as it exists immediately prior to qPCR analysis (e.g., primary concentration, secondary concentrations, nucleic acid extraction, etc.). Thus, when the theoretical LOD is extended to a theoretical full-process LOD through simple unit conversions, it is implicitly assumed that the combined efficiency of all prior sample-processing steps is 100%.

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