Level of spiked virus necessary to correctly assess enteric virus recovery in water matrices

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Abstract

Quantitative microbial risk assessment (QMRA) identifies human enteric viruses in municipal wastewaters as the pathogen group requiring highest log-reductions for various reuse applications. When estimating virus concentration, however, method performance is not well understood, which without inclusion actual risks are likely severely underestimated. To evaluate recovery efficiency for viruses in water, ‘known’ amounts of virus are often spiked into a water sample and then recovered after a series of analytical procedures. Yet for water matrices such as wastewaters, due to the unknown background concentrations of targeted viruses in the matrix and the variable recovery efficiency between individual processes, only an approximation of the recovery efficiency may be obtained from such spike-and-recovery experiments. Here, we theoretically demonstrated that for two widely-used approximations, the error in estimating virus recovery should be less than the ratio of the amount of target virus in the background sample to that in the spike. Furthermore, we developed an applicable method, based on this new understanding, to decide on the amount of virus for spiking before conducting a spike-and-recovery experiment, so that the approximation error is restricted to within an acceptable level for each individual process. Finally, we applied the method to a set of experimental data for viruses in wastewater, demonstrating its utility, and noting its general applicability to other pathogens or water matrices.

Importance Pathogen log-reduction performance is at the heart of new risk-based guidance/regulation globally, yet how to undertake an assessment of pathogen recovery is not standardized despite its fundamental impact on assessing log-reductions. Herein we describe what level of spiking agent(s) are necessary to correctly assess spiked pathogen/surrogate recovery with whatever method is deployed.
The significance of our research is in identifying the importance of the amount of spiking agents for reducing uncertainty in recovery estimates, which will allow the development of a recommendation for spiking experiments, proactively applying this understanding.

Keywords: QMRA, spiking, recovery, enteric viruses, water reuse

Introduction

National standards for microbial hazards in harvested rainwater/stormwater (1), direct potable reuse of municipal wastewater (2) and non-potable reuse guidelines (3) recently accepted in the State of California (4) require specified log-reductions for enteric viruses, bacteria and parasitic protozoa. There are significant uncertainties, however, when estimating microbial counts due to matrix effects and associated variable losses with the different processing and data analytical methods used (5-9). Enteric virus estimation often involves the most method steps of any microbial group (10) and the highest log-reductions for safe reuse (11). For example, analytical procedures for estimating infectious enteric virus concentrations may involve sample filtration, elution, concentration, culture and qPCR (10), each step known to cause inevitable loss of target viruses (9). The fraction of targeted virus in a water sample that is captured after the procedures is termed recovery efficiency, or more commonly, method recovery. Quantitative evaluation of method recovery is required to correct virus enumeration data for the fraction that is lost when using health-based, quantitative microbial risk assessment (QMRA) derived log-reduction targets (11-13).

When it is deemed necessary to use a spiked virus (14), known amounts of virions or viral genomic copies are added to a water sample and then recovery is estimated by the difference in
counts before and after processing the water. Selection of spike material for a target virus has
been discussed previously in the context of evaluating pathogen elimination in water treatment
systems (14). In summary, lab-grown pure virus cultures are the least preferable, while viruses
isolated from matrix water and propagated in vitro, or concentrated from matrix water are
preferred because they are more likely to provide genetically and physiologically diverse
populations that are more representative of indigenous environmental strains (14). These are
traits also preferred for the spike material to closely resemble the genetic and physiological
variability of the indigenous target(s) when evaluating method recovery.

To avoid matrix effect complications, a control, target-free water is often included for spiking
and measurement of method recovery. In these circumstances, the method recovery for the target
virus can be easily evaluated as the ratio of recovered to the spiked viruses. However, water
matrix effects are also important to address, since physiochemical (e.g. temperature, pH,
nutrients, and sorption to solids) and biological (predation by free-living protozoa, metazoa and
enzymatic decomposition) factors of matrix water impact on the remaining target microorganism
in the water (15-16). Thus, to be more relevant for performance-based log-reduction assessments,
target microbes are typically spiked into matrix water (14). Hence our aim is not to discuss the
choice of spiked microbe, but rather how to evaluate the recovery of the target group spiked into
the water matrix of interest.

The main concern here is that the matrix water may also bring with it the target group of interest,
which introduces an unknown and variable quantity, into the recovery calculation. Furthermore,
the variable recovery efficiency between individual water processes also makes it difficult to
assess the recovered background target viruses through processing duplicated samples. Together
these factors make the calculation of recovery uncertain.
While spiking is not a new concept for method recovery related to enteric viral, bacterial and protozoan pathogens and their surrogates (14), there is still no standard available on how much spike control is needed. Including high titers of the selected spike material is required to provide concentrations that are high enough to evaluate method recovery, however it is unclear whether elevated or reduced recovery may result from too high a spiking dose. Therefore, the aim of this paper was to describe an optimum spike dose that is enough for assessing microbial method recoveries without risking artifacts to recovery estimates, and to illustrate the approach with human enteric virus spiking of municipal wastewater.

Results

1. Theoretical Analysis

Regardless of the spike material, it is imperative that the target in the spike be quantified (virus count is quantified by an identical method, either of molecular, culture, or mixed method throughout the experiment) prior to inoculation. The target spike is then mixed with related microbes present in the matrix water sample, and the target is (partially) recovered with a certain recovery efficiency. A typical spike-and-recovery experiment for viruses is illustrated in Figure 1.

By definition, the overall recovery efficiency $r$ is expressed as:

$$ r = \frac{c}{a + b} \quad (0 \leq r \leq 1) $$

Eq. 1

where $a$ is the virus count in the spike, $b$ is the indigenous virus count in the matrix water, and $c$ is the virus count that is recovered and quantified.
Since the virus count in matrix water sample $b$ is unknown, it is challengeable to calculate the exact recovery efficiency from an individual sample, but approximations can be obtained using the following two widely-used approaches.

Approximation I:

When the virus count in a matrix water sample $b$ (e.g. wastewater, surface water etc.) is very low, as compared to the virus count in spike $a$, i.e., $\frac{b}{a} \approx 0$, it follows that

$$r \approx \frac{c}{a} \tag{Eq. 2}$$

Then $\frac{c}{a}$ in Eq. 2 is a good approximation of the recovery efficiency $r$ in Eq. 1. In practice, the virus count in the spike $a$ is controllable. In order to keep the condition for Eq. 2, i.e., $\frac{b}{a} \approx 0$ valid, we are able to adjust the spike dose $a$ based on the background virus count in the matrix water sample $b$. Furthermore, we need to understand how $\frac{b}{a}$ relates to the error of approximation of recovery efficiency $\frac{c}{a} - r$ so that we will be able to determine how close $\frac{b}{a}$ should be to zero to keep the error in the approximated recovery efficiency acceptable (for example $< 1\%$, $< 2.5\%$, or $< 5\%$), which is chosen subjectively.

To discuss the error of Approximation I, let:

$$k = \frac{a}{b} \quad (b > 0)$$

then the error of the approximated recovery efficiency can be expressed as:

$$\frac{c}{a} - r = \frac{cb}{a(a+b)} = \frac{b}{a} \frac{c}{a+b} = \frac{r}{k}.$$

Since $0 \leq r \leq 1$, it follows that
so recovery can be described (Eq. 3).

\[ 0 \leq \hat{r} - r \leq \frac{1}{k}. \]

Therefore, using \( \hat{r} \) to approximate recovery efficiency \( r \), the true recovery efficiency \( r \) will be overestimated, and the maximum error is less than \( \frac{1}{k} \).

**Approximation II:**

When there is no variation presented in recovery efficiencies across individual samples, a duplicate sample collected at the same time could be used jointly to estimate the mean recovery. Suppose that \( d \) virus particles are recovered from the duplicate sample without spiking, which has the same background virus count \( b \), it follows that the recovery efficiency for the duplicate sample without spiking is:

\[ r' = \frac{d}{b} \]  \quad \text{Eq. 4}

and its value should be the same as \( r \) defined for sample with spiking in Eq.1, i.e. \( r' = r \).

Combining Eq. 1 and Eq. 4, we could get Eq. 5.

\[ r = r' = \frac{c - d}{a} \]  \quad \text{Eq. 5}

However, it is known that virus recovery can vary considerable across samples (9), thus the condition for Eq. 5, i.e., \( r' = r \) is unlikely to be valid in most circumstances. For general circumstances, taking:

\[ \tilde{r}' = \frac{c - d}{a} \]
as an approximation of the recovery efficiency \( r \) in Eq. 1 will introduce an approximation error. We will discuss how \( \frac{b}{a} \) relates to the error of approximation of recovery efficiency \( \tilde{r}' - r \) as follows in Approximation II.

For Approximation II, following the above approach:

\[
\tilde{r}' - r = \frac{(a + b)(c - d) - ac}{a(a + b)}
\]

\[
= \frac{bc}{a(a + b)} - \frac{d}{a}
\]

\[
= \frac{b}{a} r - \frac{b}{a} r'
\]

\[
= \frac{r - r'}{k}.
\]

It follows that

\[
|\tilde{r}' - r| = \frac{|r' - r|}{k} \leq \frac{1}{k}.
\]

Since \( 0 \leq r \leq 1 \) and \( 0 \leq \tilde{r}' \leq 1 \), we derive Eq. 6, which demonstrates that the error of Approximation II is between \( -\frac{1}{k} \) and \( \frac{1}{k} \).

\[
\tilde{r}' - \frac{1}{k} \leq r \leq \tilde{r}' + \frac{1}{k}
\]

Eq. 6

The relationship between approximation error and the ratio of target (virus) count in the spike to that in the background matrix water sample is presented in Figure 2.

For a matrix water sample with unknown true recovery efficiency, using either Approximation I or II, the approximation error in the worst-case scenarios are illustrated as blue lines in Figure 2.

When the ratio of microbial target count in the spike to that in the background water sample \( k \) is
large, the approximation error can be neglected for either Approximation I or Approximation II. Based on this understanding, for a pre-determined error level $\sigma$ (for example, 5%), one can spike an appropriate microbial target (e.g. virus) count in the matrix water sample so that the maximum error term $\frac{k}{\sigma} < \sigma$, or $k > 1/\sigma$, for which, Approximation I and II both provide acceptable approximations of the true recovery efficiency.

To keep $k > 1/\sigma$ so that the error in the approximated recovery efficiency is less than the pre-determined error level $\sigma$, it is required that

$$a > \frac{b}{\sigma}$$

Eq. 7

In practice, however, the spike-in microbial target count $a$ cannot be determined directly based on the choice of $k$ because microbial target count in the background water sample $b$ is unknown. Some extra information needs to be collected prior to the spike-and-recovery experiment so that the background virus count $b$ can be evaluated approximately. Specifically, the following information is recommended to choose the spike-in microbial target count $a$:

1) Collect $N$ replicate samples (minimum of two replicates is recommended considering the variation in background virus count expected between replicates) and then process the water in the same way except without a spike step. It is assumed that the replicates have the same background target concentration. We denote the maximum recovered target amount as $d^0$, and denote its corresponding unknown background target count and unknown recovery efficiency as $b^0$ and $r^0$, respectively. When $N$ is large, it is most likely that $d^0$ is corresponding to the sample with both the largest background target count and the highest recovery efficiency considering the variation of replicate samples and the variation of recoveries.

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2) Obtain lowest recovery efficiency observed for cleaner water matrix as an approximation for the recovery efficiency, for example, virus recovery values obtained from spike-and-recovery experiments for pure water. It is likely that recovery for pure water is higher than that for other water matrices, so we treat the minimum recovery efficiency for pure water as an approximation of \( r \) in Eq. 1.

Since the replicate samples have the same background target concentration, \( b^0 = \frac{a^0}{r^0} \) is the most likely higher and closest approximation of \( b \), based on the available information.

It is worth noting that the aim here is to obtain the best spiking dose \( a \). Since \( \frac{b}{\sigma} \) is the lowest that \( a \) can go to ensure the estimated recovery estimation is valid (Eq. 7), choosing a larger approximation of \( b \) in calculation would result in a larger spiking dose than the lowest value of \( a \) but still fulfill the requirement. It is also the lowest value of \( a \) that is above its unknown lowest value and a value we are confident to work with. Therefore, the minimum dose of microbial target count required for spike-in is approximated as \( \frac{a^0}{\sigma r^0} \).

2. Application of spiking approaches to estimate recovery of human enteric viruses

The spike-and-recovery requirement of the minimum value of \( a/d^0 \) to deliver an approximation error of 5% for Approximation I and II (Eq. 3 & Eq. 5 respectively) is shown in Table 1. For the human enteric virus data presented in Table 1, all samples analysed were above the limit of detection (LOD) of 1 virus/sample volume, except for reovirus in unspiked samples (24/25 and 13/14 for WTP1 and WTP2 were below LOD, respectively, and half the LOD was substituted.)
Discussion

As expected, when comparing the results in Table 1 to that presented in a previous publication (18), the wastewater matrix had a lower virus recovery efficiency in general compared to the recovery efficiency with spiking in pure water (Norovirus GII: 32%-69%, Adenovirus 41: 20%-52%, and Enterovirus [Coxsackievirus-B]: 32% - 69%). Another important consideration is LOD for viruses, so as to interpret non-detects data. When virus concentration is low in the wastewater, i.e. the virus count in matrix water sample $d$ could be under the LOD, then the $a/d^0$ ratio could be calculated using the LOD as a conservative estimate of $d^0$.

Although Approximations I and II both gave acceptable approximations of recovery efficiency when the spike-in virus count fulfills the condition for Eq. 5, we could choose between Approximations I and II if we know more information about the distribution range of the recovery efficiency. For example, for viruses we could assume that the recovery efficiency itself is highly variable (9); hence Approximation I would be preferable over Approximation II. However, when the recovery efficiency itself is large but it spans only a small range in values, it is better to choose Approximation II over Approximation I.

In practice, determine recovery with each water sample is desirable, however this might be costly and laborious depending on the detection method. When it is impractical to have a replicate for every sample, for example, in monitoring programmes with time series of samples, collecting duplicate samples only at selected times with high virus loadings for the same water source would be acceptable, assuming some information is available on likely times of higher virus loadings. The highest recovered background virus count among all duplicate samples should then be used (as $d^0$) for calculating the preferred spiking dose. This simple approach will only result in a higher value of spiking dose and is not likely to alter the estimation for recovery efficiency.
We discussed the appropriate spiking dose for spike-and-recovery experiments, focusing on viruses. But the principles are generally true for method recovery any pathogen or microbial surrogate, and for all other water matrices, such as river water, storm water, etc.

### Materials and Methods

The methods described in this paper for determining target microbe count in the spike were applied to a set of human enteric virus recovery data for municipal wastewater treatment plant (WWTP) behaviour. In brief, secondary-treated wastewater samples were collected in duplicate at the pre-UV disinfection site from two WWTP in Calgary, Canada (17). A spiking mix consisting of four viruses (Norovirus GII, human adenovirus 2/4, Reovirus 2 and an Enterovirus [coxsackie virus-B] derived from human stool, human stool, wastewater and cell culture respectively), were used to assess virus recoveries. One millilitre of the virus mixture was spiked into one of the duplicate samples. Then all samples were processed similarly by concentration, viral nucleic acid extraction and qPCR to estimate total virus concentrations. Finally, the same aliquot of the virus mixture (1 mL) was added to 14 mL of deionized water as a baseline control and tested in parallel with the concentrated spiked water samples using qPCR. A detailed description of sample collection and processing was as previously described (17).

The spike-in virus count was determined based on estimating in pure water, therefore, we are not sure whether the spike-in virus count is enough for a reliable approximation of the recovery efficiency based on Eq. 5, which could be express as:

\[
\frac{a}{d_0} > \frac{1}{\sigma r_0}
\]

Eq. 5a

The minimum requirement for \(a/d_0\) to deliver an approximation error of 5% was calculated based on Eq. 5a. Then for each set of duplicate samples, we calculate the ratio \(a_0/d_0\), where \(a_0\)
is the qPCR results of the spike, and $d^0$ is the qPCR results of the duplicate sample without
spiking. As a result, duplicate samples that did not have an $a^0/d^0$ ratio above the threshold value
were excluded from the recovery efficiency calculation.

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Figure 1. Illustration of a typical spike-and-recovery experiment for evaluation of virus recovery in a water matrix.

Figure 2. Approximation error in relation to the ratio of microbial target count in the spike to that in the background sample. (A) Approximation I; (B) Approximation II.

Table 1. Spike-in requirement for the Calgary enteric virus wastewater study.
| Virus         | Minimum of $\frac{a}{d^0}$ for error < 5% | # of samples | Recovery results (%) | Mean (Standard Deviation) | Approximation I | Approximation II |
|--------------|-------------------------------------------|--------------|----------------------|--------------------------|----------------|----------------|
|              |                                           |              |                      |                          | WTP1 | WTP2            | WTP1 | WTP2            | WTP1 | WTP2            |
| Norovirus GII| 32%                                       | 62.6         | 22/32                | 21/21                    | 10.4 (6.0) | ND             | 10.2 (5.9) | ND             |
| Adenovirus   | 20%                                       | 100          | 4/32                 | 9/21                     | 13.7 (14.4) | 11.5 (5.4) | 13.5 (14.5) | 11.2 (5.4) |
| Enterovirus  | 32%                                       | 62.6         | 2/32                 | 13/21                    | 21.5 (6.6) | 31.9 (4.8) | 21.0 (6.6) | 31.1 (4.9) |
| Reovirus     | 1%                                       | 2000         | 0/25                 | 0/14                     | 18.9 (15.5) | 19.1 (11.8) | 18.9 (15.5) | 19.0 (11.8) |

*Minimum reference recovery (18). ND: no data. cAs no data available, 1% was assumed.