American Journal of Environmental Protection
2022; 11(2): 32-38
http://www.sciencepublishinggroup.com/j/ajep
doi: 10.11648/j.ajep.20221102.14
ISSN: 2328-5680 (Print); ISSN: 2328-5699 (Online)

Evaluation of Measurement Uncertainty from Pour Plate Method in Bacterial Enumeration in Water

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To cite this article:

Zhi Xuan Li, Wei Ma, Yi Jun Kong, Sin Neng Chio, Chang Li Zhang, Chi Tou Lei, She Xia Zhan. Evaluation of Measurement Uncertainty from Pour Plate Method in Bacterial Enumeration in Water. *American Journal of Environmental Protection.* Vol. 11, No. 2, 2022, pp. 32-38.
doi: 10.11648/j.ajep.20221102.14

Received: March 23, 2022; **Accepted**: April 8, 2022; **Published**: April 25, 2022

Abstract: The evaluation of measurement uncertainty (MU) is an important method for quantitative determinations derived by microbial cultivation, thus it could further improve the result accuracy. In this study, we aimed to evaluate the MU in total bacteria counts (TBC) in water. Experiments were carried out using Pour Plate Method. The bottom-up approach was used to access the MU of TBC and different mathematical models were set up based on microbial growth principles and test process. Two geometric and five non-geometric progression factors derived from fishbone diagram were selected in these models, especially considering the influence of the bacteria binary fission. The results showed that the calculated value of expanded uncertainty was between 82 and 120CFU/mL when the test result was 100CFU/mL with the 95% confidence level. The geometric progression factor had a greater impact on MU evaluation with almost 80% of contribution, whilst non-geometric progression factor dedicated 60%. Experimental procedures such as sample repeatability, reproducibility and inoculation time should be drawn more attention, thus the accuracy of results could be improved. These innovative models not only reasonable and reliable, but also easy to use. The achievements of this study illustrated that the MU evaluation method could also be applied for analyzing other microbial indicators in water.

Keywords: Measurement Uncertainty, Total Bacteria Count, Geometric Progression, Pour Plate Method

1. Introduction

Once water-borne pathogens enter water supply system, it may cause threats to public health and even lives. Therefore, water quality and consequent safety problems have always been our top concerns [1-2]. Bacteria enumeration allows to estimate the number of live, culturable bacteria in water and total bacteria count (TBC) and has been widely selected as one of the indicators to suggest the presence of bacteria and thus to assess the microbiological quality of water. The TBC analysis is highly affected by its intrinsic constraints, such as sampling conditions, cultivation parameters, growth process, analytical procedures, results counting methods, *etc*. Despite of all efforts to guarantee the reliability of enumeration, there will always be some uncertainties associated with the measured values. Therefore, this quantification would not be complete without evaluation of the measurement uncertainty (MU).

Formal concepts and terminology for MU were brought together with the publication of the Guide to the Expression of Uncertainty in Measurement (GUM) [3] (ILAC, 2001). Increasingly, both national and international accreditation organization/laboratory seeks to define the level of uncertainty which can be ascribed to a series of tests, such as China National Accreditation Service for Conformity Assessment (CNAS), International Standards Organization (ISO), International Laboratory Accreditation Cooperation (ILAC), International Diary Federation (IDF), the British Standards Institute, the Nordic Committee for Microbiological Standardization (NMKL) and AOAC (Association of Official Agricultural Chemists) International.

The MU has been widely accepted and applied in physical and chemical analysis for many years. Recently, the subject has been addressed by microbiologists [4-6]. Although primarily concerned with methods and techniques in food microbiology, the concepts and techniques for estimating MU

are applicable to other microbial areas since microbiological techniques are generic and often common to different matrices.

The MU normally can by calculated using two different approaches: "top-down" and "bottom-up" approaches. The "top-down" approach based on measurement of control samples where empirical data are typically precision estimates and bias data obtained from within-laboratory validation studies and quality control. The "bottom-up" approach, the typical method to estimating MU described in GUM, based on a comprehensive mathematical model of the measurement procedure where all possible sources of MU are identified and included in the mathematical model. The fishbone diagram normally helps to show the sources of MU. These two approaches are recognized and utilized as equally valid tools.

Evaluations of MU for colony count data which derived by examination of biological samples, including water, has been suggested to use "bottom-up" approach [6]. These data frequently conform to lognormal distribution and sometimes to a Poisson distribution. In the former case, the data should be transformed to logarithms before statistical analysis where in the latter case, a square root transformation should be used to conform to an approximately normal distribution.

2. Objectives

In this study, the MU of total bacteria enumeration derived by pour plate method in TBC analysis in water were discussed and evaluated. The main objectives are:

1) to select the MU evaluation approach,
2) to design the fishbone diagram and to identify the possible MU sources which caused variabilities,
3) to setup mathematical models of the analytical procedure and to calculate MU based on the above-mentioned sources,
4) to make recommendations concerning the important precautious to minimize MU associated with analytical procedure,
5) to work as a demonstration role in biological analysis.

3. Materials and Methods

3.1. Materials and Reagents

Difco™ Plate Count Agar was used for bacteria enumeration (BD, USA). Difco™ Nutrient Agar was used for the cultivation of bacteria in growth process study (BD, USA). Cell cultures used for repeatability analysis were purchased from Proficiency Testing Schemes (Fapas®, UK). The flasks, cylinders and graduated pipettes applied in this study were all glasswares (Duran, Germany). Sterilized disposable plastic (57cm^2) Petri dishes were used in pour plate method (Corning® Gosselin™, USA).

3.2. Instruments

The BD115 incubator was used to grow and maintain cell cultures (Binder, Germany). All aseptic experiments were performed under biological safety cabinet (B Science, Denmark). The plate count liquid medium was maintained melted in water bath (Techne, UK) before use.

3.3. Methods

3.3.1. Pour Plate Method

After thoroughly mixed, an aliquot of 1 mL of water samples was added into Petri dish followed by pouring 10~12 mL plate count liquid medium (maintained between 44 to 46°C) in each dish. Then the water sample and liquid medium was mixed by rotating the dish clockwise until well-mixed. After the medium solidified, all the plates were inverted and incubated at 37°C. The results were recorded as in colony forming unit (CFU)/mL after 48 hours incubation [7]. All the experiments were carried out in two replicates, unless otherwise stated.

3.3.2. Investigation of MU Calculation Approach

i. Selection of Calculation Approach

Recently, the "bottom-up" approach has been explored in the context of food and water microbiology [8-10]. In our study, this approach was selected based on an innovative mathematical model for total bacteria enumeration in water.

ii. Identification of Uncertainty Sources

Microbiological analysis involves in many processes and each stage could add variabilities into the results. All the corresponding sources in our study were identified and showed in the fishbone diagram (Figure 1). As shown below, the uncertainty components were mainly originated from 7 sources: repeatability, inoculation time (from sample arrival to inoculation), standard strain, sample volume, dilution, sampling and sample uniformity, and others (such as blank test).

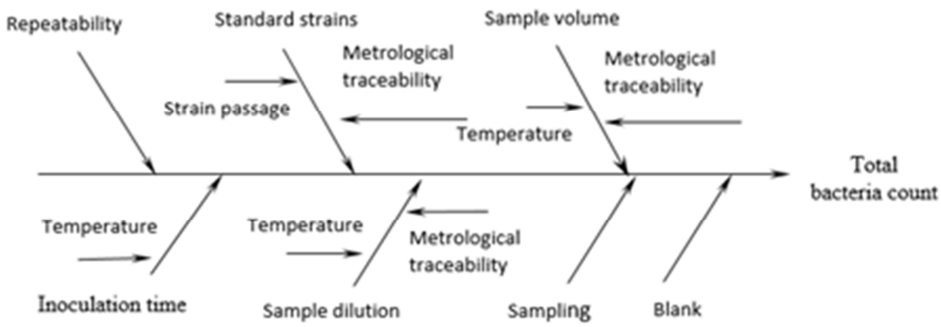

Figure 1. *The fishbone diagram of MU sources in bacteria enumeration.*

iii. Categorization of Uncertainty Sources

All the identified uncertainty sources were categorized into two groups: geometric and non-geometric sources (Table 1).

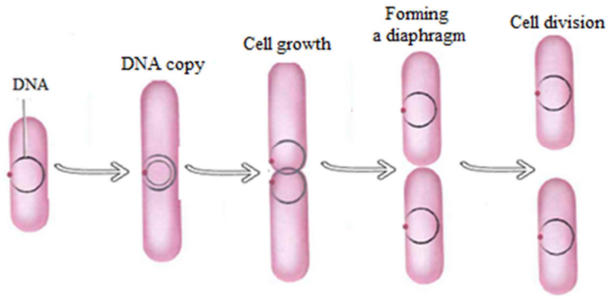

Figure 2. *The process of bacteria binary fission.*

The microbial growth process generally can be divided into five main phases: lag phase, logarithmic phase, stable phase, decline phase and death phase. In the logarithmic phase, total number of bacteria would multiply rapidly to the power of 2^n [11]. The process of bacteria binary fission was shown in Figure 2. The results (e.g. colony counting data) derived by analyzing biological specimens conform to the lognormal distribution. Therefore, in our study, the sources which related to bacteria growth, e.g. repeatability and inoculation time, were categorized into geometric sources.

For the rest sources, the results which mainly related to physical and chemical analysis conform to "normal" statistical distribution. Thus, these sources were categorized into non-geometric sources.

Table 1. *Categorization of uncertainty sources.*

No.	Geometric sources	No.	Non-Geometric sources
u_{rel1}	Sample Repeatability	u_{rel3}	Standard Strain Passage
u_{rel2}	Sample Inoculation time	u_{rel4}	Sample Volume
		u_{rel5}	Sample Dilution
		u_{rel6}	Sampling and Content Uniformity
		u_{rel7}	Blank

3.3.3. Setup of Mathematical Model for MU Calculation

i. Setup of mathematical model for geometric components

Based on the process of bacteria binary fission, the results of bacteria enumeration increased geometrically with time under certain conditions and could be expressed as below:

$$y = x^n \tag{1}$$

Where y was the bacteria enumeration result; n was the exponential index of bacteria binary fission process; x was the initial bacteria enumeration result.

To transform it to logarithms:

$$\lg y = \lg x^n = n \times \lg x \tag{2}$$

The geometric uncertainty component u_{relA} was evaluated according to (2), then u_{relA} was transformed by antilogarithm. The expanded uncertainty component U_A of all the geometric components was obtained by combination.

ii. Setup of mathematical model for non-geometric components

For the non-geometric components, the mathematical model for physical and chemical analysis was applied here.

$$y = a \times x \tag{3}$$

Where y was measurement result; x was the observed results; a was the dilution factor ($a = 5^b$, where b was the dilution times). The non-geometric uncertainty component u_{relB} was evaluated followed by calculation of the expanded uncertainty component U_B.

iii. Calculation of combined expanded uncertainty

A square root transformation was applied to calculate the combined expanded uncertainty (U) for both geometric (U_A) and non-geometric (U_B) uncertainty components as shown below.

$$U = \sqrt{{U_A}^2 + {U_B}^2} \tag{4}$$

4. Results and Discussion

4.1. MU Calculation for Geometric Components

4.1.1. Sample Repeatability, u_{rel1}

The result (x_i) of an aliquot of 1 mL cell cultures by pour plate method from Proficiency Testing Schemes was recorded as in CFU/mL. Then the results were transformed by logarithm ($\lg x_i$). The experiments were performed in 23 replicates. The colony counting and corresponding log-transformed results were listed in the Table 2.

Table 2. *Logarithm results of sample repeatability analysis.*

No.	Result x_i (CFU/mL)	$\lg x_i$	No.	Result x_i (CFU/mL)	$\lg x_i$
1	184	2.2648	13	201	2.3032
2	178	2.2504	14	178	2.2504
3	209	2.3201	15	212	2.3263
4	214	2.3304	16	217	2.3365
5	192	2.2833	17	161	2.2068
6	187	2.2718	18	180	2.2553
7	204	2.3096	19	180	2.2553
8	155	2.1903	20	192	2.2833
9	171	2.2330	21	192	2.2833
10	180	2.2553	22	187	2.2718
11	187	2.2718	23	192	2.2833
12	208	2.3181			

The logarithmic result of mean value ($lg\bar{x}$) for total samples was 2.2763. The standard deviation of the logarithmic value (S) was calculated as following:

$$s = \sqrt{\frac{1}{(n-1)}\sum_{i=1}^{n}(lgx_i - lg\bar{x})^2}$$=0.03808

Where *n* was the number of replicates.

For bacteria enumeration in water by pour plate methods, two replicates (*p*=2) were normally performed in our routing analysis. Therefore, the calculated MU value after taking sample repeatability into account was shown as below:

$$u_{rel1} = \frac{0.03808}{\sqrt{p}}$$=0.02693

4.1.2. Sample Inoculation Time, u_{rel2}

Even though water samples were analyzed at room temperature (normally 20±3°C) right after arrival, there was still a time period (marked as "inoculation time" in our study) between sample arrival and inoculation. The bacteria enumeration results might be affected during this time period [12]. Thus, the corresponding uncertainty should be considered and calculated.

$$u_{rel2} = \frac{0.002039}{\sqrt{3}} \times T$$=0.01766

Where *T* was the inoculation time, normally 15 minutes.

4.1.3. MU Calculation, u_{relA} and U_A

Considering the above-mentioned two geometric components, the MU of geometric components (u_{relA}) was calculated as below:

$$lg(u_{relA}) = \sqrt{(lgu_{rel1})^2 + (lgu_{rel2})^2} = \sqrt{0.02693^2 + 0.01766^2} = 0.03220$$

A 95% confidence interval was applied to indicate that the results would not exceed or less than a desired approximate statistical probability, thus the coverage factor (k=2) was chose in this study.

Take the measured TBC value of 100 CFU/mL (*x*) as an example:

$$lg(x_{A\,low}) = \lg(x) - 2\lg(u_{relA})$$=1.9356

$$lg(x_{A\,high}) = \lg(x) + 2\lg(u_{relA})$$=2.0644

Thus, the lowest and highest limit values were calculated by anti-log transformation:

$$10^{lg(x_{A\,low})} = 10^{1.9356}$$=86.2 CFU/mL

$$10^{lg(x_{A\,high})} = 10^{2.0644}$$=116.0 CFU/mL

The lowest and highest uncertainties were calculated as following:

$U_{A\,low}$=100-86.2=13.8 CFU/mL

$U_{A\,high}$=116.0-100=16.0 CFU/mL

The mean between $U_{A\,low}$ and $U_{A\,high}$ can be applied if the above results were close enough, as recorded in U_A:

$$U_A = \frac{U_{A\,low} + U_{A\,high}}{2} = \frac{13.8 + 16.0}{2} = 14.9\ \text{CFU/mL}$$

4.2. MU Calculation for Non-Geometric Components

4.2.1. Standard Strain Passage, u_{rel3}

The main purposes of using the standard strain after multiple passages in our study were to identify the bacteria as well as to validify both culture medium and test method. Therefore, it could hardly affect the MU result as shown below:

$$u_{\text{rel3}} = 0\%$$

4.2.2. Sample Volume, u_{rel4}

The glass graduated pipette (Grade B) with 10mL volume capacity was used to calculate the MU generated from sample volume. Since it's a glassware, two factors might affect the MU results: allowance error given by the manufacturer and temperature when performing the analysis.

i. Allowance Error of Pipette

An aliquot of 1.00 mL water sample was withdrawn to Petri dish by the glass graduated pipette (Grade B) with 10mL volume capacity. Given the allowance error of the pipette was 0.10mL, the result of corresponding calculated MU was shown below:

$$u_{1mL} = 0.10/\sqrt{3} = 0.0578mL$$

ii. Ambient Temperature

In case of ambient temperature could have effects on the results by using glass pipette. If the temperature change by an average of 3°C, the result of corresponding calculated MU was shown below:

$$u_{1mL-Tem} = 1.0 \times 2.1 \times 10^{-4} \times 3/\sqrt{3}$$=0.00037mL

iii. Combined MU

The combined MU generated by both pipette allowance error and ambient temperature was calculated as below:

$$u_{1mL} = \sqrt{0.0578^2 + 0.00037^2} = 0.0578\ \text{mL}$$

However, the result showed that the ambient temperature had little effect on MU result, thus this factor could be ignored.

u_{rel4}=0.0578÷1.00 ×100%=5.78%

4.2.3. Sample Dilution, u_{rel5}

Dilution is often applied in bacteria enumeration using pour plate method since the result of TBC should be lied within the range: 30 – 300 CFU/mL. The main factors that would affect the MU results were allowance error of glass wares and dilution times.

i. Allowance Error of Glasswares

In our study, the graduated glass pipette (Grade B) with the volume capacity of 10 mL was used to withdraw 10mL the water samples. Besides, the measuring cylinder with volume capacity of 50 mL was used to contain diluted solutions.

Given the allowance error of this pipette was 0.10 mL. The corresponding calculated MU was shown below:

$$u_{rel10mL} = \frac{0.10/\sqrt{3}}{10} \times 100\% = 0.58\%$$

Given the allowance error of this measuring cylinder was 1.0 mL. The corresponding calculated MU was shown below:

$$u_{rel50mL} = \frac{1.0/\sqrt{3}}{50} \times 100\% = 1.16\%$$

The combined MU generated from the above-mentioned glasswares was calculated as following:

$$u_{rel}=\sqrt{1.16\%^2 + 0.58\%^2}=1.30\%$$

ii. Dilution Times

Dilution times (*b*) would also affect the MU result. If taking dilution times into account, the calculated MU of sample dilutions would be shown as below:

$$u_{rel5}=b \times 1.30\%$$

4.2.4. Sampling and Content Uniformity, u_{rel6}

Since it is impossible to analyze the TBC of entire water bulk, the MU associated with the sampling process must inevitably contribute to the reported results. Thus, the uncertainty arising from the sampling process should be evaluated and calculated. The empirical approach usually uses repeated sampling and analysis to quantify the effects caused by heterogeneity of the analyte in the sampling. This approach has been applied crossing a range of areas (water, food, soil, *etc*).

In our study, we took total nitrogen (TN) as an example to evaluate and calculate the MU generated from sampling process. Water samples were taken in 30 seconds intervals and a total of 6 water samples were analyzed for TN concentrations. The analysis for each sample was performed in 6 replicates.

The mean values of TN concentrations for 6 samples were 1.739 mg/L, 1.744 mg/L, 1.751 mg/L, 1.753 mg/L, 1.740 mg/L and 1.753 mg/L, respectively. The standard deviation was 0.0062 mg/L. The relative standard deviation could also be applied in our study to calculate the MU introduced by sampling and its related content uniformity.

$$u_{rel6}=0.35\%$$

4.2.5. Blank, u_{rel7}

"Blank" was a sample which was identical to other water samples of interest except that the TBC to be measured was not present. Thus, the blank is considered as an integral part of testing procedure and its response must be metrologically considered.

In our study, blank experiments were performed to validate the culture medium and whole testing procedure, the same function as the passage of standard strains. Therefore, it had little effects on the results.

$$u_{rel7}=0\%$$

4.2.6. MU Calculation, u_{relB} and U_B

Considering the above mentioned non-geometric components, the MU of geometric components (u_{relA}) was calculated as below:

$$\mathrm{Lg}(u_{relB}) = \sqrt{(\lg u_{rel3})^2 + (\lg u_{rel4})^2 + \cdots + (\lg u_{rel7})^2}=\sqrt{0^2 + 5.78\%^2 + (0 \times 1.30\%)^2 + 0.35\%^2 + 0^2}= 5.79\%$$

Where Urel3 and Urel7 are zero, respectively. Besides, no dilution was applied in this study (dilution time b = 0).

A 95% confidence interval was applied to indicate that the results would not exceed or less than a desired approximate statistical probability, thus the coverage factor (k=2) was chose in this study.

Take the measured TBC value of 100 CFU/mL (*x*) as an example:

$$U_B = 2 \times x \times u_{relB} = 2 \times 100 \times 5.79\%=11.6 \text{ CFU/mL}$$

4.3. Calculation of Expanded Uncertainty, U

Both geo and non-geo components were considered into the calculation of expanded MU in bacteria enumeration. Take the measured TBC value of 100 CFU/mL (*x*) as an example:

Scenario 1: Given $U_{A\ low}$ and $U_{A\ high}$ were close enough, the expanded uncertainty (U) was calculated as below:

$$U = \sqrt{U_A^2 + U_B^2} =\sqrt{14.9^2 + 11.6^2}=19 \text{ CFU/mL}$$

Therefore, the confident value of expanded uncertainty was 100±19 CFU/mL.

Scenario 2: If the confidence level is 95%, the lowest and highest values of the MU would be calculated as followings:

$$x_{Low} = x - \sqrt{U_{A\ low}^2 + U_B^2}=100\text{-}18.0=82 \text{ CFU/mL}$$

$$x_{High} = x + \sqrt{U_{A\ high}^2 + U_B^2}=100+19.7=120 \text{ CFU/mL}$$

The confident values lied between 82-120 CFU/mL which was consistent with the result from scenario 1.

4.4. Contribution of Geo and Non-Geo components to MU

Taking the measured TBC value of 100 CFU/mL as an example, the contributions of geometric and non-geometric components to MU were calculated and the results were shown in Table 3.

Table 3. *Contributions of geo and non-geo components to MU.*

Category	MU of geometric component, U_A		MU of non-geometric components, U_B	
	Result (CFU/mL)	Contribution (%)	Result (CFU/mL)	Contribution (%)
Lowest	13.8	77	11.6	64
Highest	16.0	81	11.6	59

The results showed that the lowest and highest MU of geometric components were 13.8 CFU/mL and 16.0 CFU/mL, respectively. The corresponding contribution to the expanded MU was between 77%-81% where the contribution of non-geometric components to the expanded MU was between 59%-64%. It indicated that geometric components had greater effects on the expanded MU compared to non-geometric components in this study. Therefore, more precautious should be made to minimize the MU in procedures associated with bacteria growth, e.g. sample repeatability and sample inoculation time.

4.5. The Characteristic and Modularization of the Method

Derivation of MU provides a method to standardize the variabilities associated with any analytical procedures [13]. In the past, evaluation of MU was mainly explored in chemical and physical parameters in food and water [14-16]. There are some studies about the MU assessment in microbiology while the process of bacteria growth was rarely incorporated [17-20].

Although concerned primarily with variabilities associated with colony count procedure, we also discussed and evaluated the causes of variabilities based on binary fission theory of bacteria growth in this study. Seven components which caused MU including sample repeatability, sample inoculation time, standard strain passage, blank test, sample volume, sample dilution, sapling and content uniformity were discussed, evaluated and calculated. Therefore, these components were categorized into two groups: geometric components (associated with bacteria growth) and non-geometric components.

All these grouped components can be transferred to Excel. A formatted table was designed to calculate the MU of TBC. Thus, the computation of MU for other biological indicators will be implemented automatically if we input the raw data of difference testing parameter [21]. The modularization of this evaluation method will be developed through the smart computer tool. This requires further research and exploration.

5. Conclusion

In our study, MU associated with bacteria enumeration in water by pour plate method was evaluated and calculated. The MU results showed that geometric components had greater effects on the expanded MU compared to non-geometric components with almost 80% of contribution. Recommendations were made concerning the important precautious to minimize MU associated with procedures associated with bacteria growth. It conforms to the evaluation principles of Requirements for Measurement Uncertainty of CNAS. The newly setup calculation models are simple, accurate and reliable. They could be used routinely by microbiologists not only examining TBC in water, but also examining other indicators, (e.g. total coliforms and protozoa). Besides, this "robust" MU evaluation method could be applied in other areas such as clinics, pharmacy, environment, food, agriculture, *etc*.

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