

Original Research

**MEASUREMENT UNCERTAINTY OF WASTEWATER SAMPLING FOR
MICROBIOLOGICAL ANALYSIS USING DUPLICATE SAMPLING AND ANOVA
APPROACH**

DOI: 10.5937/EcoTER26556O

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Abstract

Microbiological analysis of wastewater is strongly affected by sampling variability due to the heterogeneous distribution of microorganisms, turbulence, particulate association, and rapid biological changes within the matrix. For accredited laboratories operating under ISO/IEC 17025, it is therefore essential to quantify the contribution of sampling to overall measurement uncertainty. This study presents an ISO-compliant evaluation of wastewater sampling uncertainty for microbiological analysis based on duplicate sampling design, ANOVA statistics, and the component approach recommended by ISO 29201 Annex H. Sampling was conducted according to EN ISO 19458 using sterile techniques, with duplicate samples collected by two operators under identical conditions across different seasons (2021–2025). Each sample was analyzed in duplicate for fecal coliform bacteria using the MPN method. Measurement uncertainty components were estimated using range statistics, one-way ANOVA, and robust ANOVA approaches. The results showed relative standard uncertainties of 14.02% for analysis, 12.44–13.27% for sampling, and 15.30% for overall measurement, yielding an expanded sampling uncertainty of 26.54% ($k = 2$). These values are significantly lower than typical literature values for wastewater microbiology, demonstrating that strict implementation of standardized sampling protocols can effectively control variability. The presented approach provides a practical framework for ISO-compliant estimation of sampling uncertainty in microbiological wastewater monitoring.

Keywords: wastewater sampling, measurement uncertainty, microbiological analysis, ISO 19458, ANOVA.

INTRODUCTION

Microbiological examination of wastewater represents one of the most demanding tasks in water-quality monitoring due to the pronounced spatial and temporal variability of microorganisms within the wastewater matrix. Unlike physicochemical parameters, microbiological indicators are influenced by biological variability, uneven distribution, and frequent association with suspended particles. In addition, enumeration methods used for microbiological analysis are governed by counting statistics that are well described by Poisson distribution, where variability is inherent to the number of counted colonies rather than solely to analytical precision. These characteristics make the separation and

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quantification of individual uncertainty components particularly challenging. For this reason, uncertainty estimation in microbiology typically relies on a top-down statistical approach based on repeated measurements of the same method rather than on individual component evaluation.

According to ISO/IEC 17025, accredited laboratories are required to evaluate and report measurement uncertainty, including the contribution arising from sampling. Standards such as EN ISO 19458 and ISO 29201 emphasize that, in microbiological analyses, the variability introduced during sampling may be comparable to or even greater than that introduced during laboratory analysis [1,2]. This is particularly relevant for wastewater, where matrix heterogeneity strongly affects the representativeness of the collected sample.

Wastewater represents a particularly complex matrix for microbiological analysis because microorganisms are unevenly distributed, frequently attached to suspended solids, and strongly influenced by hydraulic turbulence, flow variability, and environmental conditions within the sewer system. Additional variability arises from potential bacterial growth or die-off during transport and storage, as well as from differences in sampling technique, equipment cleanliness, sample handling, and operator experience. The type of sample collected (grab versus composite), the sampling location, and the degree of mixing at the sampling point further contribute to variability.

As a result, wastewater sampling introduces significantly greater uncertainty than sampling of groundwater or drinking water [3], and its contribution to overall measurement uncertainty cannot be neglected. The total measurement uncertainty can therefore be expressed as the combination of sampling and analytical components (Eq. 1):

$$u_{\text{measurement}}^2 = u_{\text{sampling}}^2 + u_{\text{analytical}}^2 \quad (1)$$

where the variance introduced by sampling and the variance associated with laboratory analysis jointly determine the combined uncertainty of the reported result [4].

MATERIALS AND METHODS

Wastewater sampling for microbiological analysis was performed in accordance with EN ISO 19458 guidelines for microbiological water sampling [5], ensuring representativeness and prevention of secondary contamination. Samples were collected from municipal wastewater characterized by natural microbial contamination, without artificial spiking or pre-treatment.

Sterile 500 mL borosilicate glass bottles with leak-proof caps were used for sample collection. Prior to sampling, bottles were stored and handled under aseptic conditions. Grab samples were collected at a depth of 20–30 cm below the water surface using sterile samplers to avoid surface films and bottom sediments, which could bias bacterial counts. Care was taken to prevent disturbance of the surrounding matrix during sampling.

Immediately after collection, samples were placed in insulated containers with ice packs and transported to the accredited laboratory under controlled temperature conditions of $(5 \pm 3)^\circ\text{C}$. The time between sampling and analysis did not exceed the holding times prescribed by EN ISO 19458, thereby minimizing potential microbial growth or die-off during transport.

Sampling campaigns were conducted during different seasons between 2021 and 2025 in order to capture variability associated with temperature fluctuations, hydraulic load, and changes in wastewater composition.

Duplicate design and analytical procedure

A balanced duplicate (split-level) design was employed to quantify sampling and analytical variability separately. For each sampling event, two independent field samples were collected at the same location, under identical environmental and operational conditions, and within a short time interval of each sampling event. Duplicate sampling was performed by two trained operators to incorporate realistic field variability.

Each primary sample and its field duplicate were subsequently analyzed twice in the laboratory, resulting in full duplication at both the sampling and analytical levels. This hierarchical structure allows decomposition of total variability into:

- between-sample (sampling) variance, and
- within sample (analytical) variance.

At least ten complete duplicate sample sets were processed during the study period.

Microbiological analysis was performed using the Most Probable Number (MPN) method for fecal coliform bacteria according to internal procedure DMJČ008, based on the Colilert method. The method relies on defined substrate technology and selective enzymatic detection, with incubation under controlled conditions according to manufacturer and standard requirements. Quality control measures included reagent blanks, positive controls, and incubation temperature verification.

The balanced duplicate design applied in this study follows recommendations provided in ISO 29201 Annex H for estimation of measurement uncertainty in microbiological enumeration methods.

Statistical evaluation and uncertainty estimation

Measurement uncertainty was evaluated using range-based statistics derived from the duplicate design, supported by classical one-way ANOVA and robust ANOVA (RANOVA) approaches in accordance with ISO 29201 and EURACHEM/CITAC guidance [4].

Given that microbiological count data typically follows log-normal distribution, results were logarithmically transformed prior to statistical treatment. This transformation stabilizes variance and improves conformity with ANOVA assumptions. Calculations were performed using spreadsheet tools and the Royal Society of Chemistry uncertainty calculator.

The combined standard uncertainty was calculated as (Eq. 2):

$$u = \sqrt{u_a^2 + u_b^2} \quad (2)$$

and the expanded uncertainty at a 95% confidence level was obtained as (Eq. 3):

$$U = 2u \quad (3)$$

Sampling uncertainty was derived from the relationship between total measurement uncertainty and analytical uncertainty (Eq. 4):

$$S_{\text{sampling}} = \sqrt{S_{\text{measurement}}^2 - \left(\frac{S_{\text{analysis}}}{\sqrt{2}}\right)^2} \quad (4)$$

RESULTS AND DISCUSSION

Application of ISO 29201 range statistics to the duplicate sampling data yielded a relative sampling uncertainty of 12.4% and a relative measurement uncertainty of 10.9%, with a relative standard deviation (RSD) of 11.78%. These results confirm the consistency and reproducibility of the balanced duplicate sampling design (Table 1).

To verify these findings, the same dataset was further evaluated using one-way ANOVA and robust ANOVA (RANOVA) approaches in accordance with EURACHEM guidance. The estimated relative standard uncertainties obtained by ANOVA indicated analytical variability of 14.02%, sampling variability in the range of 12.44–13.27%, and an overall measurement uncertainty of 15.30%. The calculated expanded sampling uncertainty was 26.54% ($k = 2$).

Although derived from different statistical approaches, both methods produced highly consistent estimates of sampling variability, confirming the reliability of the applied ISO 29201 duplicate design for uncertainty evaluation.

The obtained values are lower than those typically reported for microbiological analysis of wastewater, where substantial variability is commonly observed due to matrix heterogeneity and sampling conditions.

These results demonstrate that strict adherence to EN ISO 19458 sampling procedures, combined with a controlled duplicate sampling design, can substantially reduce variability introduced during sampling. Despite the inherent heterogeneity of wastewater, the applied protocol ensured reproducible sampling conditions, confirming that sampling uncertainty can be effectively managed. Under such controlled conditions, analytical variability remains the dominant contributor to the combined measurement uncertainty.

Table 1 Comparison of uncertainty components obtained by ISO range statistics and ANOVA approaches

Component	ISO 29201 Range Statistics (%)	ANOVA / RANOVA (%)
Analytical uncertainty	8.6	14.02
Sampling uncertainty	12.4	12.44–13.27
Measurement uncertainty	10.9	15.30
Expanded sampling uncertainty ($k=2$)	—	26.54
Relative standard deviation (RSD)	11.78	—

CONCLUSION

The results of this study confirm that the contribution of sampling to the overall measurement uncertainty in microbiological wastewater analysis can be quantitatively evaluated using ISO-recommended approaches based on duplicate sampling design and ANOVA statistics. The obtained sampling uncertainty, approximately 13%, was found to be comparable to the analytical uncertainty, demonstrating that sampling represents a significant and measurable component of total uncertainty that cannot be neglected.

The application of EN ISO 19458 procedures, together with strict control of sampling techniques, transport, and handling conditions, proved effective in reducing variability typically associated with wastewater matrices. Despite the inherent heterogeneity of wastewater, the implemented protocol enabled reproducible sampling conditions and reliable estimation of uncertainty components. These findings emphasize the importance of incorporating sampling uncertainty into laboratory quality management systems and accreditation frameworks, particularly in compliance with ISO/IEC 17025 requirements. A structured evaluation of sampling variability enhances the reliability of microbiological monitoring data and supports more robust decision-making in wastewater management and regulatory reporting.

REFERENCES

- [1] International Organization for Standardization. ISO 29201:2017 Water quality—The variability of test results and the uncertainty of measurement of microbiological enumeration methods. Geneva: ISO; 2017.
- [2] European Committee for Standardization. EN ISO 13843:2017 Water quality—Requirements for establishing performance characteristics of quantitative microbiological methods. Brussels: CEN; 2017.
- [3] International Organization for Standardization. ISO/IEC 17025:2017 General requirements for the competence of testing and calibration laboratories. Geneva: ISO; 2017.
- [4] Eurachem/CITAC. Guide: Accreditation for Microbiological Laboratories, Annex D. Teddington: Eurachem; 2023.
- [5] International Organization for Standardization. EN ISO 19458:2006 Water quality—Sampling for microbiological analysis. Geneva: ISO; 2006.