Simple enumeration of *Escherichia coli* concentrations in river water samples by measuring β-D-glucuronidase activities in a microplate reader

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**ABSTRACT**

Monitoring of *Escherichia coli* concentrations in river water (RW) is essential to identify fecal pollution of the river. The objective of this study was to assess the suitability of a novel, simple and high throughput method developed in our laboratory to enumerate *E. coli* concentrations in RW samples. The method is based on the use of the synthetic substrate specific for the β-D-glucuronidase (GUS) produced by *E. coli*. GUS activities and *E. coli* concentrations were monitored at eight selected sites in rivers running through Sapporo, Japan. Because the fluorescence intensities of the synthetic substrate in the RW samples increased linearly over a 4-h incubation period, we could estimate the GUS activities of the RW samples. The GUS activities were highly correlated with *E. coli* concentrations at >100 most probable numbers 100 mL⁻¹ with a correlation coefficient of 0.87. The GUS activities of the RW samples collected from all sampling sites fitted well to a single correlation equation, which indicates that it was applicable to the estimation of *E. coli* concentrations regardless of the sampling sites. This method is simple, rapid, reliable, inexpensive, and high throughput, and is therefore useful for monitoring *E. coli* in RW.

**Key words** | enzyme, fecal pollution, fluctuation, fluorescence, Sapporo, wastewater

**HIGHLIGHTS**

- A novel method was applied for enumeration of *Escherichia coli* in urban rivers.
- Response time was <3 h and detection limit was 430 most probable numbers 100 mL⁻¹.
- Specific enzyme activities of *E. coli* were similar in wastewater and river waters.
- Correlation between enzyme activity and *E. coli* concentration was site independent.
- *E. coli* concentrations were monitored at eight different sites over 9 months.

**GRAPHICAL ABSTRACT**

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INTRODUCTION

Rivers provide many ecosystem services, through the provision of drinking water, irrigation water, livestock watering, fisheries, and recreational activities, but also through supporting and maintaining aquatic ecosystem functions. Fecal pollution of an urban river negatively impacts those ecosystem services. Urban river water (RW) contaminated with the pathogenic bacteria, protozoa and viruses present in feces can pose a serious threat to human health. Combined sewer overflows, the treated wastewater discharged from WWTPs and septic tanks, and stormwater runoff contaminated with feces from livestock, wild animals and pets can be a source of pathogens in urban areas (Templar et al. 2016). Previous studies have reported that urbanization and rainfall events caused fecal pollution in Beiyun River, which flows through Beijing and Tianjin, China (Zhang et al. 2020); agricultural and urban drainage water and bird feces were the likely source of fecal contamination in the Yangtze lakes, the Columbia River and the Mississippi River, USA (Stadler et al. 2019). The microbiology of an urban river, especially the fate, transport and pathways of pathogens, is spatially and temporally complicated because of variability in their sources, survival, growth and complex transport processes (Jang et al. 2017).

Because of the high cost and complexity of direct pathogen monitoring, fecal pollution is usually identified by monitoring fecal indicator bacteria because they are present in the gastrointestinal tract of humans and most warm-blooded animals, and easily and effectively grow in or on common laboratory media (Jang et al. 2017). Escherichia coli has historically been used as a fecal indicator bacterium (Shrestha et al. 2016; Poopipattana et al. 2018). Currently, E. coli enumeration methods include multiple tube fermentation, membrane filtration, plate count methods (Rice et al. 2017), and quantitative polymerase chain reaction (qPCR; Zhang & Ishii 2018). However, growth-based methods can require laborious media preparation and long incubation periods for growth of E. coli (e.g., >18 h), while skilled technical staff are required to perform molecular biological techniques (e.g., qPCR) (Pala et al. 2020). Because of these drawbacks, current methods are not suitable for monitoring the dynamics of E. coli concentrations in an urban river, which requires extensive sampling and laboratory analysis efforts. This makes it difficult to facilitate effective and timely decision-making for mitigating fecal pollution of an urban river.

To overcome these problems, alternative rapid and simple methods have been developed based on the detection of β-D-glucuronidase (GUS), which is produced by most E. coli strains (Pala et al. 2020). Herein, a synthetic substrate, specific for GUS produced by E. coli, was added to a water sample. The synthetic enzyme substrate comprises β-D-glucuronic acid as the substrate for GUS, the fluorogenic molecule, which produces a detectable signal after the enzymatic reaction, and a labile spacer which connects the substrate to the fluorogenic molecule. Typically, the GUS activity of a water sample is detected using the synthetic substrate 4-methylumbelliferyl-β-D-glucuronide (MUG). When MUG is added to the sample with E. coli, its spacer is degraded via hydrolysis by GUS activity. Then, the fluorogenic substrate, such as methylumbelliferone (MU), is liberated and generates a fluorescence signal, the intensity of which is proportional to the GUS activity. Many previously developed methods using this approach have been applied to measure E. coli concentrations in drinking water (Hesari et al. 2016), wastewater (Satoh et al. 2020) and natural freshwater (George et al. 2000; Wildeboer et al. 2010; Heery et al. 2016; Stadler et al. 2019). The detection limit and response time of those methods were ca. 10 cells mL⁻¹ and <2 h, respectively. Specifically, the technology of automated GUS activity measurement allowed for simple and easy determination of E. coli concentrations in large freshwater bodies (Stadler et al. 2019), RW (Burnet et al. 2019) and streams (Stadler et al. 2016). However, they measure only one sample per test and are not suitable for high throughput determination. Briciu-Burghina et al. (2019) developed ColiSense for E. coli detection, which relies on the detection of GUS with 6-chloro-4-methylumbelliferyl-β-D-glucuronide as a fluorogenic substrate and measures a
direct kinetic response of extracted GUS. However, it requires lysis of *E. coli* cells and filtration for cell concentration, GUS extraction and purification (Briciu-Burghina et al. 2017). Analysis of fecal pollution in an urban river in which the *E. coli* distribution is spatially and temporally heterogeneous requires a high throughput monitoring system.

Previously, we developed a simple (no pretreatment), inexpensive (0.02 USD per sample), high throughput (up to 96 samples per test) method to enumerate *E. coli* concentrations in municipal wastewater samples based on GUS activity measurement (Satoh et al. 2020). In the previous study, *E. coli* concentrations in wastewater samples taken from different stages of the treatment process and different WWTPs were successfully determined within 3 h with a dynamic range of 10^1 to 10^4 most probable number (MPN) mL^-1. However, this technology has never been applied to RW samples that have *E. coli* at much lower concentrations. In this study, we applied our method to RW samples to assess the suitability of our method to enumerate *E. coli* concentrations in RW samples. The RW samples were taken at eight selected sites in rivers running through Sapporo, Japan from May 2017 to January 2018. Six of the sites were located upstream and downstream from discharge outlets of three municipal WWTPs to analyze the effect of treated wastewater on *E. coli* concentrations in urban rivers. The GUS activities determined by our method were compared with *E. coli* concentrations determined by the Colilert method to obtain the correlation equation used as a calibration curve and to examine the reliability of our method. Seasonal fluctuations in the *E. coli* concentrations of the RW samples were also analyzed. The results presented here show that our simple method allowed for high throughput determination of *E. coli* concentrations in RW samples.

### MATERIALS AND METHODS

#### Study site and river water sampling

The study was conducted in northern Sapporo, Japan (Figure 1). Sapporo is a city on the southwest part of the Ishikari Plain and the alluvial fan of the Toyohira River. The population of Sapporo is about 1,960,000. Major industries include information technology, retail, tourism, and manufacturing of various goods such as food and related products, fabricated metal products, steel, machinery, beverages, and pulp and paper. RW samples were collected weekly at eight sampling sites in Sapporo, Japan from May...
2017 to January 2018 (Figure 1). Six of them were at distances of several hundred meters upstream and downstream from discharge outlets of three municipal WWTPs. The scale, physicochemical characteristics and operating conditions of WWTPs-A, -B and -C were described in our previous report (Satoh et al. 2020). The WWTPs are connected to wholly or partly combined sewer. There are a few livestock farms and industries upstream from all the sampling sites. RW samples were collected in sterile one-liter polypropylene containers. The RW samples were processed within 60 min after collection.

**Quantification of *E. coli* by the Colilert method**

Conventional enumeration of *E. coli* was conducted using Colilert and Quanti-Tray/2,000 (IDEXX Laboratories) according to the manufacturer’s instructions. The RW samples were diluted with sterile physiological NaCl solution (0.9% NaCl) in 100-mL sterile bottles in our laboratory. Each analysis was carried out in duplicate for each ten-fold serial dilution tested. The coefficient of variation (CV, expressed in %) was calculated by dividing the standard deviation by the average and multiplying by 100.

**Microplate GUS assay**

A simple microplate assay was developed to measure *E. coli* GUS activity in our previous study (Satoh et al. 2020). Briefly, the phosphate buffer medium for incubation of *E. coli*, which contained organic and inorganic compounds, sodium dodecyl sulfate (SDS), 0.1 g of isopropyl β-D-thiogalactopyranoside (IPTG), and 0.1 g of MUG (Merck Millipore) was freshly prepared for each test. SDS was added as a selective agent for gram-negative bacteria, while IPTG was added to induce expression of GUS in *E. coli*. Chemicals and reagents, if not specified otherwise, were obtained from Sigma-Aldrich. An aliquot (20 μL) of the medium was added to one well of a 96-well microplate. Subsequently, 180 μL of RW samples or Milli-Q water as a blank control were added and thoroughly mixed with the medium by pipetting. Ten wells were used for each sample. The microplate was placed in a microplate reader (Tecan Infinite F200Pro) equipped with a 360-nm excitation filter and 460-nm fluorescence filter and set at 37 °C. Then, fluorescence intensity was measured every 10 min over 4 h.

The fluorescence intensity increased linearly between 1 and 3 h of the incubation period. The temporal change in the fluorescence intensity during this period was fitted by linear regression and the slope was taken as the substrate utilization rate. The obtained substrate utilization rate was converted to the enzyme activity of GUS and expressed in modified Fishman units per mL (MFU mL⁻¹), following the standard Sigma Quality Control Test Procedure (Sigma-Aldrich 1998). One MFU of GUS activity from *E. coli* liberates 1.0 μg of phenolphthalein from phenolphthalein β-D-glucuronide (P0501, Sigma-Aldrich) per hour at pH 6.8 at 37 °C. The GUS activity degrading one mole of phenolphthalein β-D-glucuronide is assumed to be same as that degrading same amount of MUG. For calibration, commercial enzyme standard (G7396-25KU, type IX-A β-D-glucuronidase from *E. coli*, Sigma-Aldrich) activities were determined in triplicate.

**RESULTS AND DISCUSSION**

The fluorescence intensity of MU in the medium-RW mixture decreased in the initial incubation period (about 0.5 h) and thereafter increased over the 4 h incubation period (Figure 2). The MU fluorescence intensities increased linearly between 1 and 3 h. Because the slopes of the MU fluorescence intensities of the samples could be related to the initial *E. coli* concentrations (Satoh et al. 2020), the

![Figure 2](http://iwaponline.com/wst/article-pdf/83/6/1399/865131/wst083061399.pdf)
E. coli concentrations in the RW samples at >100 MPN 100 mL\(^{-1}\), under which condition the GUS activities were highly correlated with E. coli concentrations with a correlation coefficient of 0.87 (\(y = 0.012 x^{0.51}\); \(p\)-value = 2.8 \times 10^{-32}). The average and the standard deviations (\(\sigma\)) of the GUS activities at <100 MPN 100 mL\(^{-1}\) of E. coli concentrations were 0.18 and 0.023, respectively. Based on these values, the limit of detection (LoD) of our method was calculated from the following equation: \(\text{LoD} = 5 \times \sigma / \text{slope of an equation, to be 430 MPN 100 mL}^{-1}\). This LoD was lower than that of our previous method for analysis of treated wastewater (2,200 MPN 100 mL\(^{-1}\); Satoh et al. 2020) and that of a hand-held fluorescence detector used for analysis of RW (700 colony-forming units 100 mL\(^{-1}\); Wildeboer et al. 2010).

The plots of the RW samples collected downstream of the discharge points of three WWTPs fitted well to the correlation equation, implying that the specific GUS activity of E. coli cells (i.e., the exponent value of the correlation equation) in the RW samples contaminated with treated sewage effluent (TSE) was comparable among the three WWTPs (Figure 1). The exponent value (0.51) for the RW samples was quite similar to those for TSEs (0.47 for
WWTP-A and 0.48 for WWTP-B) in Sapporo (Satoh et al. 2020), implying that once a correlation equation is established it could be applicable to various RW samples. Conversely, Cazals et al. (2020) reported that linear relationships and coefficients of correlation between GUS activity and E. coli concentrations in recreational freshwater samples were site-dependent. These results suggest that our method is useful for simply quantifying E. coli concentrations in TSE and RW samples. Because the exponent value of a correlation equation is identical to the specific GUS activity of E. coli, the result suggested that the GUS activities of E. coli strains present in the urban river system were similar to each other. Universality of specific GUS activities of E. coli in TSE and RW shown in this study will be confirmed in future studies.

Seasonal fluctuations in the E. coli concentrations and GUS activities of RW samples collected upstream and downstream of the discharge point of WWTP-A were analyzed (Figure 4). Similar seasonal fluctuations were observed for the Colilert method and our method. During the study period, the water level at monitoring site No. 1 and the flow rate at monitoring site No. 2 ranged from 0.65 to 1.43 m and from 1.04 to 14.62 m³ s⁻¹, respectively (Figure S1). The monthly average temperature in Sapporo ranged from -2.6 to 22.9 °C (Figure S1). The E. coli concentration upstream was usually <100 MPN 100 mL⁻¹. The TSE increased the E. coli concentration downstream by 1–2 log units. There were two peaks for the upstream RW samples in July and September and two peaks for the downstream RW samples in June and December; the downstream E. coli concentrations were relatively higher between July and October. Thus, our method allowed for identification of seasonal and temporal fluctuations in E. coli concentrations in RW.

Recently, several methods for enumeration of E. coli based on GUS measurement have been developed. (Briciu-Burghina et al. 2017) reported a novel protocol for the recovery and detection of E. coli using GUS activity, with LoDs of 26 MPN 100 mL⁻¹ for seawater and 110 MPN 100 mL⁻¹ for freshwater samples. Although the sample preparation (concentration, purification and cell lysis) reduced the LoD, it required a syringe filter and cell lysis reagent. They also designed and built a sensitive field-portable fluorimeter for E. coli enumeration (Heery et al. 2016), which also required pre-concentration of E. coli (Hesari et al. 2016). Conversely, our method does not require any pretreatment and the protocol (only mixing a sample with a liquid medium) is simpler and the running cost (0.02 USD per sample) is lower than the other fluorogenic substrate-based methods described above. Especially, our method can handle up to 96 samples simultaneously on a single microplate, which is advantageous for analysis of non-point source pollution in an aquatic system.

**CONCLUSIONS**

The novel, simple and rapid method for enumeration of E. coli concentration developed in our laboratory was applicable to RW samples with >100 MPN 100 mL⁻¹ of E. coli. Treated wastewaters discharged into the river increased E. coli concentration in RW by 1–2 orders of magnitude. Our method was suitable for monitoring temporal change...
in *E. coli* concentrations in RW. Future work will evaluate the method for enumeration of *E. coli* concentrations in groundwater as a drinking water source. Our method can be used as an alternative to conventional culture-dependent methods, providing a rapid and simple method to enumerate *E. coli* concentrations in water samples, which allows for effective and timely decision-making for action toward proper drinking water quality management.

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**DECLARATION OF INTERESTS**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**DATA AVAILABILITY STATEMENT**

All relevant data are included in the paper or its Supplementary Information.

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