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Simple Enumeration of *Escherichia coli* Concentrations in River Water Samples by  
Measuring  $\beta$ -D-glucuronidase Activities in a Microplate Reader

Short title: Simple *E. coli* enumeration by  $\beta$ -D-glucuronidase activity

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25

26 **Abbreviations**

27 GUS:  $\beta$ -D-glucuronidase

28 LoD: limit of detection

29 MFU: modified Fishman units

30 MPN: most probable number

31 MU: 4-methylumbelliferone

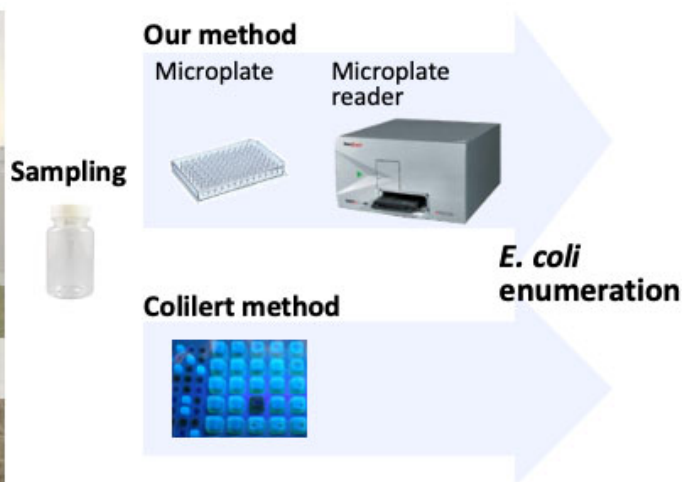
32 MUG: 4-methylumbelliferyl- $\beta$ -D-glucuronide

33 RW: river water

34 TSE: treated sewage effluent

35 WWTP: wastewater treatment plants

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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  $\beta$ -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  $100\text{ mL}^{-1}$  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.

## Keywords

Enzyme; Fecal pollution; Fluctuation; Fluorescence; Sapporo; Wastewater

## 1 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 wastewater treatment plants (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 2016). Previous studies have reported that urbanization and rainfall events caused fecal pollution in Beiyun River, which flows through Beijing and Tianjin, China (Zhang 2020); agricultural and urban drainage water and bird feces were the likely source of fecal contamination in the Yahara lakes, the Columbia River and the Mississippi River, USA (Stadler 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 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 2017). *Escherichia coli* has historically been used as a fecal indicator bacterium (Poopipattana 2018, Shrestha 2016). Currently, *E. coli* enumeration methods include multiple tube fermentation, membrane filtration, plate count methods (Rice 2017), and quantitative polymerase chain reaction (qPCR; Zhang and Ishii, 2018). However,

growth-based methods can require laborious media preparation and long incubation periods for growth of *E. coli* (e.g., >18h), while skilled technical staff are required to perform molecular biological techniques (e.g., qPCR) (Pala 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  $\beta$ -D-glucuronidase (GUS), which is produced by most *E. coli* strains (Pala 2020). Herein, a synthetic substrate, specific for GUS produced by *E. coli*, was added to a water sample. The synthetic enzyme substrate comprises  $\beta$ -D-glucuronic acid as the substrate for GUS, the fluorescence molecule, which produces a detectable signal after the enzymatic reaction, and a labile spacer which connects the substrate to the fluorescence molecule. Typically, the GUS activity of a water sample is detected using the synthetic substrate 4-methylumbelliferyl- $\beta$ -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 2016), wastewater (Satoh 2020) and natural freshwater (George 2000, Heery 2016, Stadler 2019, Wildeboer 2010). The detection limit and response time of those methods were ca. 10 cells mL<sup>-1</sup> 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 2019), RW (Burnet 2019) and streams (Stadler 2016). However, they measure only one sample per test and are not suitable for high throughput determination. Briciu-Burghina (2019) developed ColiSense for *E. coli* detection, which relies on the detection of GUS with 6-chloro-4-methyl-umbelliferyl- $\beta$ -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 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 (Sato 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)  $\text{mL}^{-1}$ . 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.

## **2 Materials and Methods**

### *2.1 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 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.

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

Conventional enumeration of *E. coli* was conducted using Colilert and Quanti-Tray/2000 (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.

### 2.3 Microplate GUS assay

A simple microplate assay was developed to measure *E. coli* GUS activity in our previous study (Sato 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  $\beta$ -D-1-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  $\mu$ L) of the medium was added to one well of a 96-well microplate. Subsequently, 180  $\mu$ L 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 h 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<sup>-1</sup>), following the standard Sigma Quality Control Test Procedure (Sigma Aldrich, 1998). One MFU of GUS activity from *E. coli* liberates 1.0  $\mu$ g of phenolphthalein from

phenolphthalein  $\beta$ -D-glucuronide (P0501, Sigma-Aldrich) per hour at pH 6.8 at 37°C. The GUS activity degrading one mole of phenolphthalein  $\beta$ -D-glucuronide is assumed to be same as that degrading same amount of MUG. For calibration, commercial enzyme standard (G7396-25KU, type IX-A  $\beta$ -D-glucuronidase from *E. coli*, Sigma-Aldrich) activities were determined in triplicate.

### 3 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 2020), the slopes were calculated. The slope decreased with decreasing *E. coli* concentrations; slope values of 1.2, 0.31 and 0.015 h<sup>-1</sup> were obtained for samples with *E. coli* concentrations of 3,500, 930 and 27 MPN 100 mL<sup>-1</sup>, respectively.

To calculate sample-specific GUS activity, we used the slope of the linear fit to the MU fluorescence intensities between 1 and 3 h of incubation. A linear increase in the MU fluorescence intensities over time (Figure 2) indicated that the degradation of MUG by GUS was a zero-order reaction within this period. The GUS activity was almost constant over the 4 h incubation period, which indicates poor or no growth of *E. coli* during the incubation and the presence of intrinsic GUS activity in the RW samples. These results agreed with those of previous studies on the analysis of GUS activity in treated sewage wastewaters (Satoh 2020), in recreational freshwaters (Cazals 2020) and in river water (Burnet 2019). GUS activities were

calculated by subtracting the slope of the blank sample determined for each test from that of the RW sample and converting the unit to milli-MFUs (mMFU) using the following equation obtained in the previous study (Sato 2020);  $GUS\ activities = 0.063 \times Slope + 0.16$ . The GUS activities were calculated to be 0.94, 0.35 and 0.16 h<sup>-1</sup> for *E. coli* concentrations of 3,500, 930 and 27 MPN 100 mL<sup>-1</sup>, respectively (Figure 2).

This result implied the presence of intrinsic GUS activity in the RW samples, which was high enough to catalyze MUG to emit a detectable level of the fluorescence signal within 3 h. These GUS enzymes might have been produced when the *E. coli* was present in the intestine of their hosts (e.g., humans), where glucuronidated compounds (i.e., substrates of GUS) are abundantly available (Little 2018). The amount and activity of GUS in *E. coli* cells might remain unchanged in a sewage system after being released from the host (Sato 2020, Wéry 2008) and even after discharge of the treated wastewater into a river as demonstrated in this study. Because both *E. coli* cells and GUS are diluted in a similar manner in a sewage system and a river into which treated wastewater is discharged, we hypothesized that it is possible to estimate the *E. coli* concentrations in RW samples only by measuring the GUS activity in the RW samples.

To confirm this hypothesis, we examined the correlation between GUS activities and the *E. coli* concentrations in RW samples (Figure 3). A total of 103 samples were collected from eight points in Sapporo (Figure 1) from May 11 2017 to January 16 2018. Although the GUS activities were unchanged (ca. 0.2 mMFU mL<sup>-1</sup>) at <100 MPN 100 mL<sup>-1</sup> of *E. coli*, they increased with the increase in *E. coli* concentrations in the RW samples at >100 MPN 100 mL<sup>-1</sup>, 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  $100 \text{ mL}^{-1}$  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:  $\text{LoD} = 3 \times \sigma / \text{slope of an equation}$ , to be 430 MPN  $100 \text{ mL}^{-1}$ . This LoD was lower than that of our previous method for analysis of treated wastewater (2,200 MPN  $100 \text{ mL}^{-1}$ ; Satoh 2020) and that of a hand-held fluorescence detector used for analysis of RW (700 colony-forming units  $100 \text{ mL}^{-1}$ ; Wildeboer 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 2020), implying that once a correlation equation is established it could be applicable to various RW samples. Conversely, Cazals (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 m<sup>3</sup> s<sup>-1</sup>, 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<sup>-1</sup>. 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 2017) reported a novel protocol for the recovery and detection of *E. coli* using GUS activity, with LoDs of 26 MPN 100 mL<sup>-1</sup> for seawater and 110 MPN 100 mL<sup>-1</sup> 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 2016), which also required pre-concentration of *E. coli* (Hesari 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.

#### 4 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<sup>-1</sup> 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.

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Figure legends

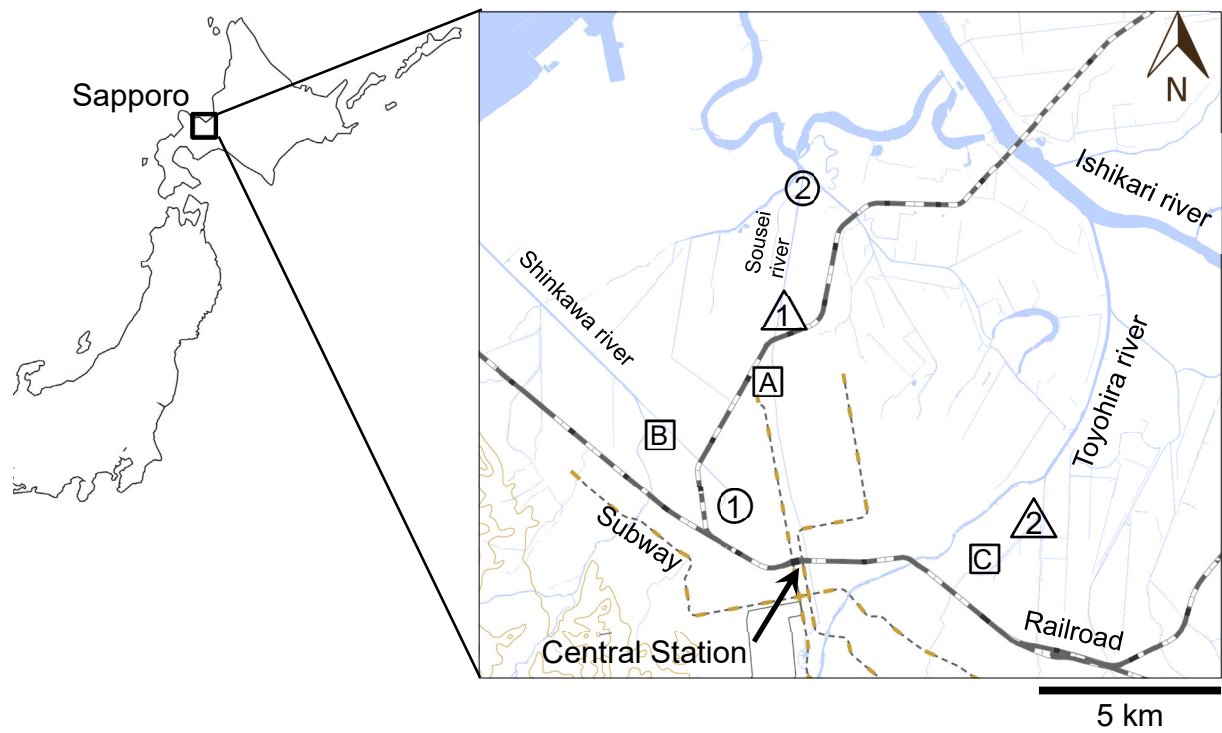
**Figure 1.** Map of the study area. Squares, circles, and triangles indicate wastewater treatment plants, sampling sites, and water level and flow rate monitoring sites, respectively. Square A: WWTP-A, Square B: WWTP-B, Square C: WWTP-C. Six samples were taken at distances of several hundred meters upstream and downstream from discharge outlets of three municipal WWTPs and other two samples were taken at circle 1 and circle 2.

**Figure 2.** Time-course changes in 4-methylumbelliferone fluorescence intensities in river samples with *Escherichia coli* concentrations of  $3,500 \pm 50$  most probable number (MPN)  $100 \text{ mL}^{-1}$  (●),  $930 \pm 21$  MPN  $100 \text{ mL}^{-1}$  (▲) and  $27 \pm 0.8$  MPN  $100 \text{ mL}^{-1}$  (■). All samples were incubated in the medium with 4-methylumbelliferyl- $\beta$ -D-glucuronide. Error bars indicate standard deviation ( $n=10$ ). The gray area indicates the period when  $\beta$ -D-glucuronidase activities were calculated.

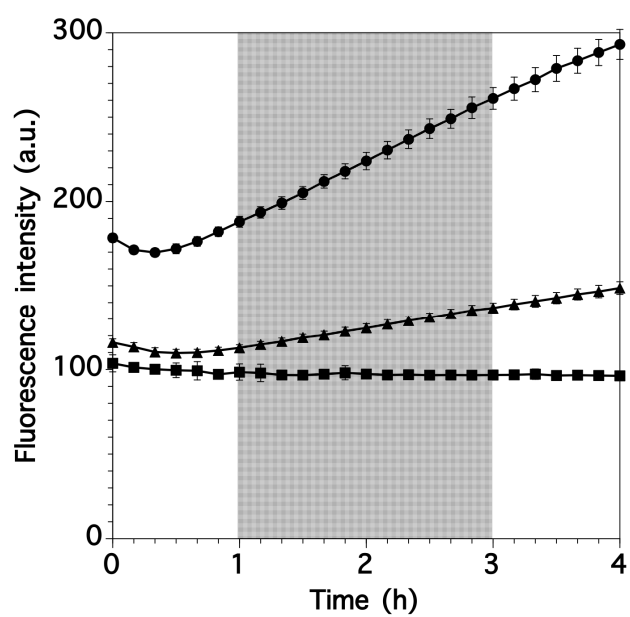
**Figure 3.** Correlation between  $\beta$ -D-glucuronidase (GUS) activities and *Escherichia coli* concentrations of 103 river water (RW) samples collected from eight points from 11 May 2017 to 16 January 2018. Symbols shown in black and gray indicate the RW samples upstream and downstream of the discharge point of the wastewater treatment plant, respectively. The horizontal and vertical error bars indicate standard deviations of the *E. coli* concentrations ( $n=2$ ) and GUS activities ( $n=10$ ), respectively. The solid, dashed, and gray lines indicate a regression line, 95%

confidence interval, and prediction interval, respectively. MPN: most probable number. MFU:  
modified Fishman unit.

**Figure 4.** Seasonal fluctuations in the *Escherichia coli* concentrations (gray triangle) and the  $\beta$ -  
D-glucuronidase (GUS) activities (black circle) of the river water samples collected upstream (a)  
and downstream (b) of the discharge point of wastewater treatment plant A from May to  
December 2017. Error bars indicate standard deviations in *E. coli* concentrations ( $n=2$ ) and GUS  
activities ( $n=10$ ), respectively. MPN: most probable number. MFU: modified Fishman unit.

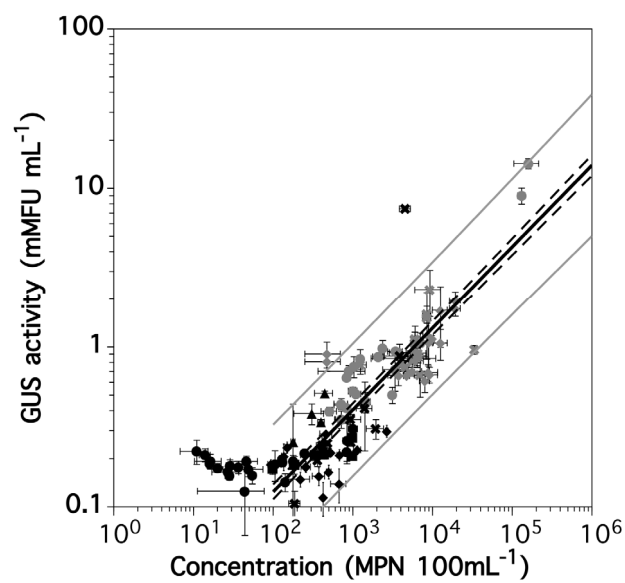


**Figure 1.**

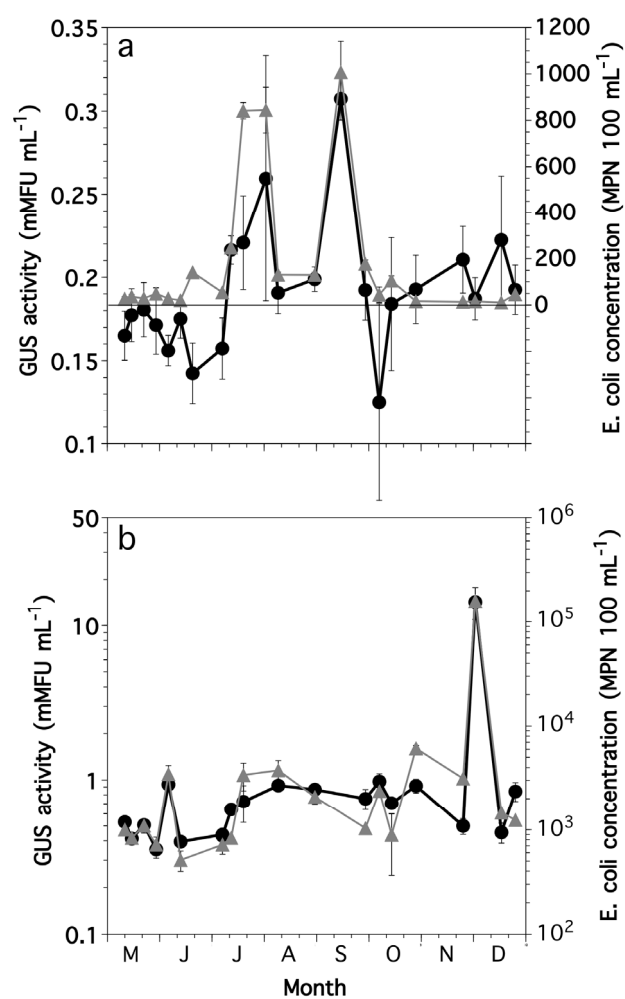


**Figure 2.**





**Figure 3.**



**Fig. 4**