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

Short title: Simple *E. coli* enumeration by β-D-glucuronidase activity

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Abbreviations

GUS: β-D-glucuronidase

LoD: limit of detection

MFU: modified Fishman units

MPN: most probable number

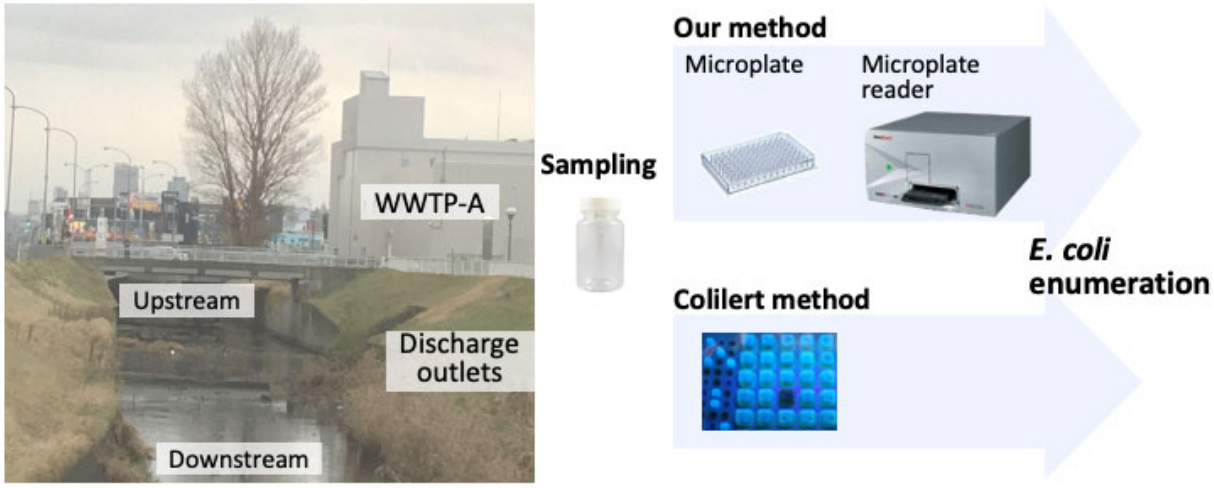
MU: 4-methylumbelliferone

MUG: 4-methylumbelliferyl-β-D-glucuronide

RW: river water

TSE: treated sewage effluent

WWTP: wastewater treatment plants


WWTP-A
Upstream
Discharge outlets
Downstream
Sampling
Our method
Microplate
Microplate reader
Colilert method
E. coli enumeration

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40

41 **ABSTRACT**

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

56 **Keywords**

57 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,

81 growth-based methods can require laborious media preparation and long incubation periods for
82 growth of *E. coli* (e.g., >18h), while skilled technical staff are required to perform molecular
83 biological techniques (e.g., qPCR) (Pala 2020). Because of these drawbacks, current methods are
84 not suitable for monitoring the dynamics of *E. coli* concentrations in an urban river, which
85 requires extensive sampling and laboratory analysis efforts. This makes it difficult to facilitate
86 effective and timely decision-making for mitigating fecal pollution of an urban river.
87
88 To overcome these problems, alternative rapid and simple methods have been developed
89 based on the detection of β-D-glucuronidase (GUS), which is produced by most *E. coli* strains
90 (Pala 2020). Herein, a synthetic substrate, specific for GUS produced by *E. coli*, was added to a
91 water sample. The synthetic enzyme substrate comprises β-D-glucuronic acid as the substrate for
92 GUS, the fluorescence molecule, which produces a detectable signal after the enzymatic
93 reaction, and a labile spacer which connects the substrate to the fluorescence molecule. Typically,
94 the GUS activity of a water sample is detected using the synthetic substrate 4-
95 methylumbelliferyl-β-D-glucuronide (MUG). When MUG is added to the sample with *E. coli*, its
96 spacer is degraded via hydrolysis by GUS activity. Then, the fluorogenic substrate, such as
97 methylumbelliferone (MU), is liberated and generates a fluorescence signal, the intensity of
98 which is proportional to the GUS activity. Many previously developed methods using this
99 approach have been applied to measure *E. coli* concentrations in drinking water (Hesari 2016),
100 wastewater (Satoh 2020) and natural freshwater (George 2000, Heery 2016, Stadler 2019,
101 Wildeboer 2010). The detection limit and response time of those methods were ca. 10 cells mL^{-1}
102 and <2 h, respectively. Specifically, the technology of automated GUS activity measurement
103 allowed for simple and easy determination of *E. coli* concentrations in large freshwater bodies

104 (Stadler 2019), RW (Burnet 2019) and streams (Stadler 2016). However, they measure only one
105 sample per test and are not suitable for high throughput determination. Briciu-Burghina (2019)
106 developed ColiSense for *E. coli* detection, which relies on the detection of GUS with 6-chloro-4-
107 methyl-umbelliferyl-β-D-glucuronide as a fluorogenic substrate and measures a direct kinetic
108 response of extracted GUS. However, it requires lysis of *E. coli* cells and filtration for cell
109 concentration, GUS extraction and purification (Briciu-Burghina 2017). Analysis of fecal
110 pollution in an urban river in which the *E. coli* distribution is spatially and temporally
111 heterogeneous requires a high throughput monitoring system.
112
113 Previously, we developed a simple (no pretreatment), inexpensive (0.02 USD per
114 sample), high throughput (up to 96 samples per test) method to enumerate *E. coli* concentrations
115 in municipal wastewater samples based on GUS activity measurement (Satoh 2020). In the
116 previous study, *E. coli* concentrations in wastewater samples taken from different stages of the
117 treatment process and different WWTPs were successfully determined within 3 h with a dynamic
118 range of 10^1 to 10^4 most probable number (MPN) mL^{-1}. However, this technology has never
119 been applied to RW samples that have *E. coli* at much lower concentrations. In this study, we
120 applied our method to RW samples to assess the suitability of our method to enumerate *E. coli*
121 concentrations in RW samples. The RW samples were taken at eight selected sites in rivers
122 running through Sapporo, Japan from May 2017 to January 2018. Six of the sites were located
123 upstream and downstream from discharge outlets of three municipal WWTPs to analyze the
124 effect of treated wastewater on *E. coli* concentrations in urban rivers. The GUS activities
125 determined by our method were compared with *E. coli* concentrations determined by the Colilert
126 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

150 laboratory. Each analysis was carried out in duplicate for each ten-fold serial dilution tested. The
151 coefficient of variation (CV, expressed in %) was calculated by dividing the standard deviation
152 by the average and multiplying by 100.
153
154 *2.3 Microplate GUS assay*
155 A simple microplate assay was developed to measure *E. coli* GUS activity in our previous study
156 (Satoh 2020). Briefly, the phosphate buffer medium for incubation of *E. coli*, which contained
157 organic and inorganic compounds, sodium dodecyl sulfate (SDS), 0.1 g of isopropyl β-D-1-
158 thiogalactopyranoside (IPTG), and 0.1 g of MUG (Merck Millipore) was freshly prepared for
159 each test. SDS was added as a selective agent for gram-negative bacteria, while IPTG was added
160 to induce expression of GUS in *E. coli*. Chemicals and reagents, if not specified otherwise, were
161 obtained from Sigma-Aldrich. An aliquot (20 µL) of the medium was added to one well of a 96-
162 well microplate. Subsequently, 180 µL of RW samples or Milli-Q water as a blank control were
163 added and thoroughly mixed with the medium by pipetting. Ten wells were used for each sample.
164 The microplate was placed in a microplate reader (Tecan Infinite F200Pro) equipped with a 360-
165 nm excitation filter and 460-nm fluorescence filter and set at 37°C. Then, fluorescence intensity
166 was measured every 10 min over 4 h.
167 The fluorescence intensity increased linearly between 1 h and 3 h of the incubation
168 period. The temporal change in the fluorescence intensity during this period was fitted by linear
169 regression and the slope was taken as the substrate utilization rate. The obtained substrate
170 utilization rate was converted to the enzyme activity of GUS and expressed in modified Fishman
171 units per mL (MFU mL^{-1}), following the standard Sigma Quality Control Test Procedure (Sigma
172 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.

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^{-1} were obtained for samples with *E. coli* concentrations of 3,500, 930 and 27 MPN 100 mL^{-1}, 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

196 calculated by subtracting the slope of the blank sample determined for each test from that of the
197 RW sample and converting the unit to milli-MFUs (mMFU) using the following equation
198 obtained in the previous study (Satoh 2020); GUS activities = 0.063 × Slope + 0.16. The GUS
199 activities were calculated to be 0.94, 0.35 and 0.16 h^{-1} for *E. coli* concentrations of 3,500, 930
200 and 27 MPN 100 mL^{-1}, respectively (Figure 2).
201
202 This result implied the presence of intrinsic GUS activity in the RW samples, which was
203 high enough to catalyze MUG to emit a detectable level of the fluorescence signal within 3 h.
204 These GUS enzymes might have been produced when the *E. coli* was present in the intestine of
205 their hosts (e.g., humans), where glucuronidated compounds (i.e., substrates of GUS) are
206 abundantly available (Little 2018). The amount and activity of GUS in *E. coli* cells might remain
207 unchanged in a sewage system after being released from the host (Satoh 2020, Wéry 2008) and
208 even after discharge of the treated wastewater into a river as demonstrated in this study. Because
209 both *E. coli* cells and GUS are diluted in a similar manner in a sewage system and a river into
210 which treated wastewater is discharged, we hypothesized that it is possible to estimate the *E. coli*
211 concentrations in RW samples only by measuring the GUS activity in the RW samples.
212
213 To confirm this hypothesis, we examined the correlation between GUS activities and the
214 *E. coli* concentrations in RW samples (Figure 3). A total of 103 samples were collected from
215 eight points in Sapporo (Figure 1) from May 11 2017 to January 16 2018. Although the GUS
216 activities were unchanged (ca. 0.2 mMFU mL^{-1}) at <100 MPN 100 mL^{-1} of *E. coli*, they
217 increased with the increase in *E. coli* concentrations in the RW samples at >100 MPN 100 mL^{-1},
218 under which condition the GUS activities were highly correlated with *E. coli* concentrations with

219 a correlation coefficient of 0.87 ($y = 0.012\ x^{0.51}$; *p*-value = 2.8×10^{-32}). The average and the
220 standard deviations (σ) of the GUS activities at <100 MPN 100 mL^{-1} of *E. coli* concentrations
221 were 0.18 and 0.023, respectively. Based on these values, the limit of detection (LoD) of our
222 method was calculated from the following equation: LoD = 3 × σ / slope of an equation, to be
223 430 MPN 100 mL^{-1}. This LoD was lower than that of our previous method for analysis of treated
224 wastewater (2,200 MPN 100 mL^{-1}; Satoh 2020) and that of a hand-held fluorescence detector
225 used for analysis of RW (700 colony-forming units 100 mL^{-1}; Wildeboer 2010).
226
227 The plots of the RW samples collected downstream of the discharge points of three
228 WWTPs fitted well to the correlation equation, implying that the specific GUS activity of *E. coli*
229 cells (i.e., the exponent value of the correlation equation) in the RW samples contaminated with
230 treated sewage effluent (TSE) was comparable among the three WWTPs (Figure 1). The
231 exponent value (0.51) for the RW samples was quite similar to those for TSEs (0.47 for WWTP-
232 A and 0.48 for WWTP-B) in Sapporo (Satoh 2020), implying that once a correlation equation is
233 established it could be applicable to various RW samples. Conversely, Cazals (2020) reported
234 that linear relationships and coefficients of correlation between GUS activity and *E. coli*
235 concentrations in recreational freshwater samples were site-dependent. These results suggest that
236 our method is useful for simply quantifying *E. coli* concentrations in TSE and RW samples.
237 Because the exponent value of a correlation equation is identical to the specific GUS activity of
238 *E. coli*, the result suggested that the GUS activities of *E. coli* strains present in the urban river
239 system were similar to each other. Universality of specific GUS activities of *E. coli* in TSE and
240 RW shown in this study will be confirmed in future studies.
241

242 Seasonal fluctuations in the *E. coli* concentrations and GUS activities of RW samples
243 collected upstream and downstream of the discharge point of WWTP-A were analyzed (Figure
244 4). Similar seasonal fluctuations were observed for the Colilert method and our method. During
245 the study period, the water level at monitoring site No. 1 and the flow rate at monitoring site No.
246 2 ranged from 0.65 to 1.43 m and from 1.04 to 14.62 $m^3 s^{-1}$, respectively (Figure S1). The
247 monthly average temperature in Sapporo ranged from −2.6 to 22.9°C (Figure S1). The *E. coli*
248 concentration upstream was usually <100 MPN 100 mL^{-1}. The TSE increased the *E. coli*
249 concentration downstream by 1–2 log units. There were two peaks for the upstream RW samples
250 in July and September and two peaks for the downstream RW samples in June and December;
251 the downstream *E. coli* concentrations were relatively higher between July and October. Thus,
252 our method allowed for identification of seasonal and temporal fluctuations in *E. coli*
253 concentrations in RW.
254
255 Recently, several methods for enumeration of *E. coli* based on GUS measurement have
256 been developed. (Briciu-Burghina 2017) reported a novel protocol for the recovery and detection
257 of *E. coli* using GUS activity, with LoDs of 26 MPN 100 mL^{-1} for seawater and 110 MPN 100
258 mL^{-1} for freshwater samples. Although the sample preparation (concentration, purification and
259 cell lysis) reduced the LoD, it required a syringe filter and cell lysis reagent. They also designed
260 and built a sensitive field-portable fluorimeter for *E. coli* enumeration (Heery 2016), which also
261 required pre-concentration of *E. coli* (Hesari 2016). Conversely, our method does not require any
262 pretreatment and the protocol (only mixing a sample with a liquid medium) is simpler and the
263 running cost (0.02 USD per sample) is lower than the other fluorogenic substrate-based methods
264 described above. Especially, our method can handle up to 96 samples simultaneously on a single

265 microplate, which is advantageous for analysis of non-point source pollution in an aquatic
266 system.
267
268 **4 Conclusions**
269 The novel simple and rapid method for enumeration of *E. coli* concentration developed in
270 our laboratory was applicable to RW samples with >100 MPN 100 mL^{-1} of *E. coli*. Treated
271 wastewaters discharged into the river increased *E. coli* concentration in RW by 1–2 orders of
272 magnitude. Our method was suitable for monitoring temporal change in *E. coli* concentrations in
273 RW. Future work will evaluate the method for enumeration of *E. coli* concentrations in
274 groundwater as a drinking water source. Our method can be used as an alternative to
275 conventional culture-dependent methods, providing a rapid and simple method to enumerate *E.*
276 *coli* concentrations in water samples, which allows for effective and timely decision-making for
277 action toward proper drinking water quality management.
278
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287

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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381 Figure legends

382

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

388

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

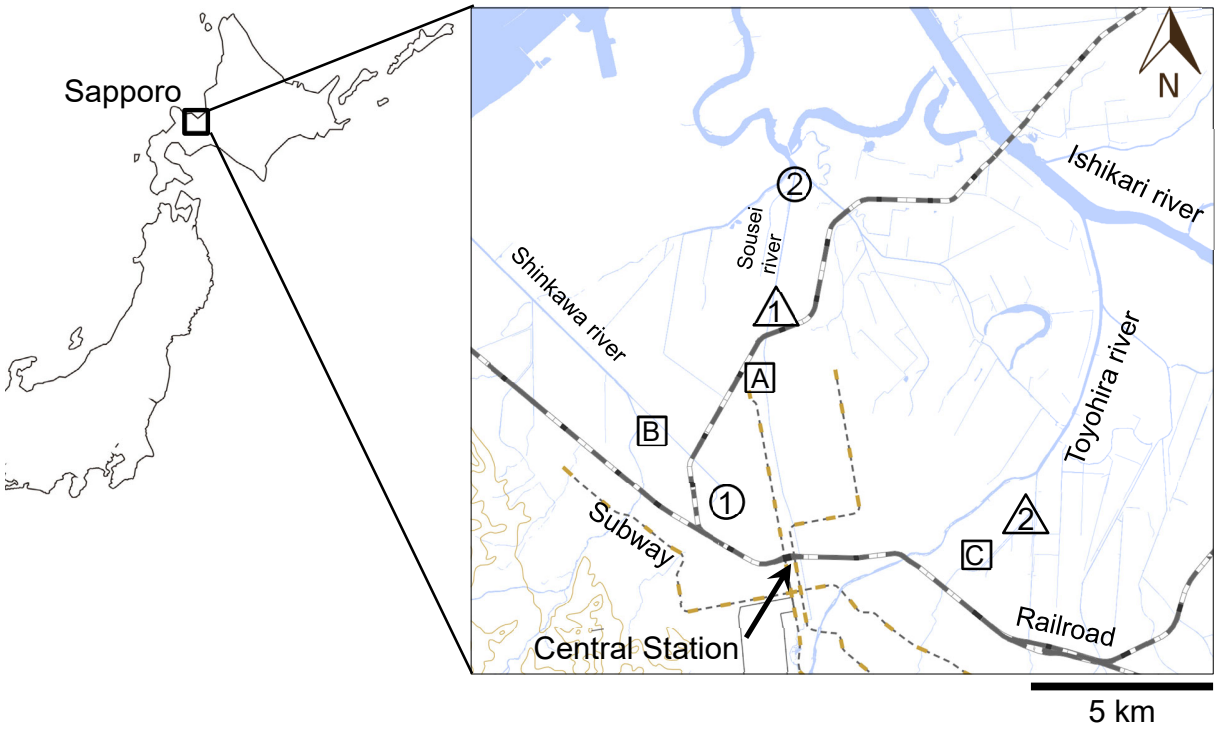
395

396 **Figure 3.** Correlation between β-D-glucuronidase (GUS) activities and *Escherichia coli*
397 concentrations of 103 river water (RW) samples collected from eight points from 11 May 2017 to
398 16 January 2018. Symbols shown in black and gray indicate the RW samples upstream and
399 downstream of the discharge point of the wastewater treatment plant, respectively. The horizontal
400 and vertical error bars indicate standard deviations of the *E. coli* concentrations (*n*=2) and GUS
401 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 β-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.

412



413

414

415 **Figure 1.**

416

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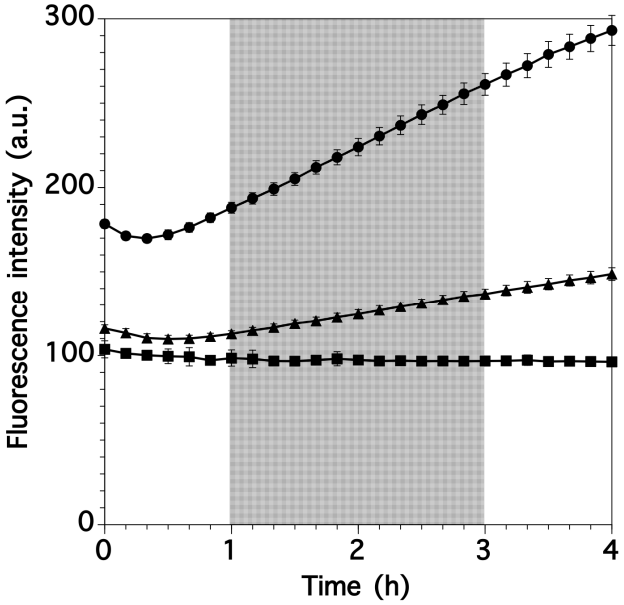
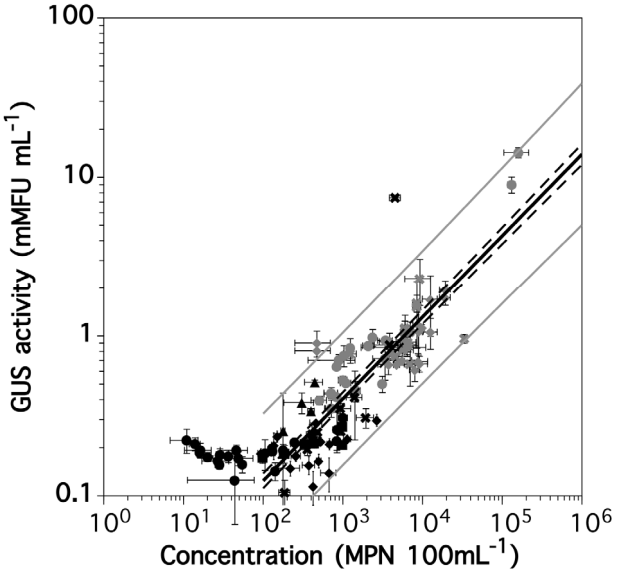


418

419 **Figure 2.**

420

421



422

423

424 **Figure 3.**

425

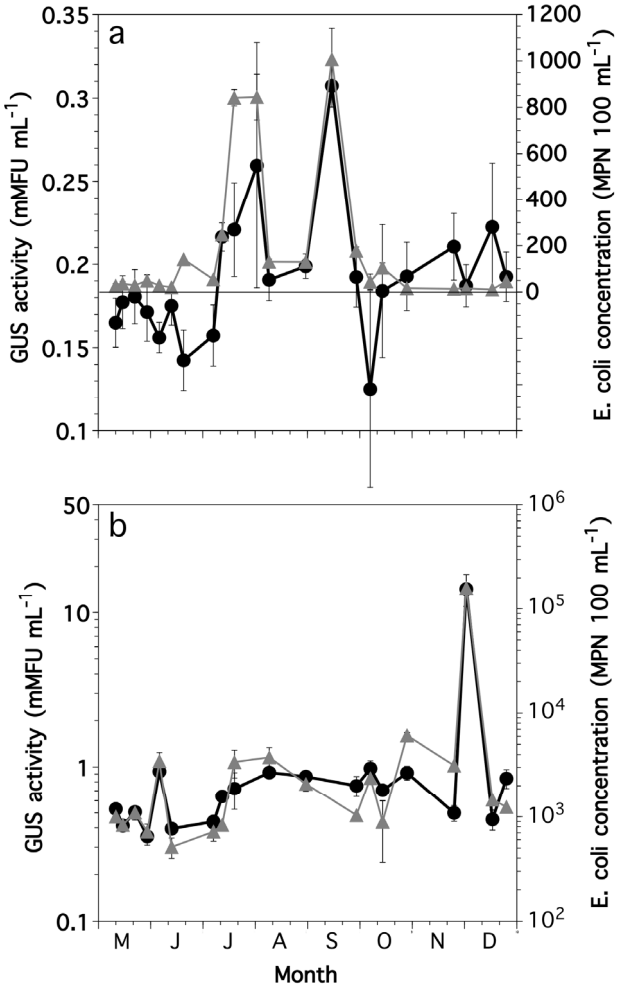

a
b
GUS activity (mMFU mL-1)
E. coli concentration (MPN 100 mL-1)
0.35
0.3
0.25
0.2
0.15
0.1
1200
1000
800
600
400
200
0
50
10
1
0.1
10^6
10^5
10^4
10^3
10^2
M
J
J
A
S
O
N
D
Month

Fig. 4