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A simple and rapid method for detecting fecal pollution in urban rivers by measuring the intrinsic β-D-glucuronidase activity of *Escherichia coli*

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ABSTRACT

As urban rivers are domestic, industrial, and agricultural water resources, fecal pollution poses human health and environmental risks. In this study, we developed a simple and rapid method to detect fecal pollution in urban rivers. Water samples were mixed with liquid medium, including a fluorescent substrate and fluorescence intensity (F.I.) was measured using a microplate reader to determine *Escherichia coli* (*E. coli*) β-D-glucuronidase (GUS) activity instead of *E. coli* concentration. GUS activities measurements in pure *E. coli* cultures revealed that *E. coli* incubated with a GUS substrate accumulated GUS enzymes in their cells, whereas those incubated without a GUS substrate did not. The increase in GUS activity corresponded to the proliferation of *E. coli* and the GUS activity increased linearly even during the lag growth phase of *E. coli*, indicating the presence of intrinsic GUS (iGUS) in *E. coli* cells before incubation. iGUS activity persisted at 81% in the chlorinated samples, even though the *E. coli* concentration was reduced by a factor of 10^6. The iGUS activity persisted for approximately three days. Therefore, we assumed that *E. coli* present in fecal contaminants, in which GUS substrates are present, could be distinguished from those surviving in the natural environment for three days or longer by measuring iGUS activity. River water samples were collected upstream and downstream of the discharge outlets of municipal wastewater treatment plants and a combined sewer outlet. The iGUS activities were <0.24 mMFU/mL for the upstream samples and >0.21 mMFU/mL for the downstream samples. Interestingly, *E. coli* concentrations were not necessarily associated with fecal pollution. This indicates that by setting a threshold for iGUS activity, our method can be used as a simple and rapid method for detecting fecal pollution in urban rivers. Because the limit of detection for our method is 20 CFU/mL, our method is applicable to detecting high fecal pollution in a small river.

Keywords

Optical analysis; intrinsic enzyme activity; enzyme persistence; chlorination; microplate reader; VBNC state

1 Introduction

Urban rivers are important resources for drinking, irrigation, and recreational water use. However, fecal pollution in rivers is greatly concerning because of the potential for waterborne disease outbreaks caused by pathogens and oxygen depletion (Cui et al., 2019). Potential sources of fecal pollution include wastewater treatment plant (WWTP) effluents (Satoh et al., 2021), combined sewer overflows (CSOs), sanitary sewer overflows (Templar et al., 2016) and fecal deposits from domestic animals, livestock, and wildlife (Derx et al., 2023). There is a positive relationship between enteric illness and exposure to sand-harboring fecal indicator organisms (Heaney et al., 2012) or male-specific coliphages in marine waters (Benjamin-Chung et al., 2017). Therefore, the detection of fecal pollution sources is essential to reduce serious risks to human health and environmental ecosystems.

Traditionally, fecal pollution has been identified by enumerating fecal indicator bacteria, mostly *Escherichia coli* (*E. coli*) and enterococci. However, because this is conducted using a culture-based method, it relies on the culturability of the bacteria in a sample, which is affected by the bacterial species investigated and environmental parameters (Rochelle-Newall et al., 2015). It cannot distinguish the bacteria in the polluted fecal sample from those present in the natural environment. Moreover, it is time consuming (>18 h) and laborious. Molecular techniques such as microbial source tracking (MST) have been used to determine fecal pollution sources (Hinojosa et al., 2020). In this method, the 16S rRNA genes of fecal anaerobes, such as *Bacteroidales*, are targeted as host-specific genetic markers and measured using polymerase chain reaction (PCR). This allows for the identification of the source (human, cow, dog, bird, etc.) of fecal pollution. Gene markers are more useful as conservative tracers of fecal pollution

than traditional fecal indicator bacteria because they persist in the environment longer than culturable bacteria (Calderon et al., 2022; Kim and Wuertz, 2015; Ren and Feng, 2023). However, PCR requires a laborious DNA extraction step and expensive enzymes (polymerase) and equipment (thermal cycler) and cannot distinguish live microorganisms from dead ones. The persistence of gene markers differs among them (Calderon et al., 2022; Kim and Wuertz, 2015; Ren and Feng, 2023). Moreover, inhibitors present in a sample impede DNA amplification. Chemical and biological markers present in fecal contaminants, such as sterols, stanols, caffeine, and the pain medicine acetaminophen, have been used to track fecal pollution sources (Rochelle-Newall et al., 2015). However, no single chemical indicator associated with the presence of fecal pathogens currently exists. Another problem is the difference in behavior (e.g., decay rate and growth of pathogens) between the chemical indicators and pathogens. Moreover, expensive equipment, such as gas and liquid chromatography-mass spectrometry (GC-MS and LC-MS, respectively) are necessary for the analysis of chemical indicators at low concentrations.

Instead of the methods described above, we focus on the enzyme β-D-glucuronidase (GUS) as a biomarker of fecal pollution in this study. Currently, GUS is used to enumerate *E. coli* concentrations (Cai et al., 2021; Demeter et al., 2020; Li et al., 2022; Satoh et al., 2021, 2020; Sylvestre et al., 2020) and is not used to detect fecal pollution. GUS hydrolyzes the glycosidic bonds between glucuronic acid and either small molecules or the terminal ends of polysaccharides (Pellock and Redinbo, 2017). Because most *E. coli* strains possess the *uidA* gene encoding the GUS enzyme (Martins et al., 1993), *E. coli* expresses the GUS enzyme in the gastrointestinal tract, which is rich in GUS substrates. Therefore, *E. coli* and other gastrointestinal microbes may play an important role in the health and disease of host mammals

by regenerating toxic drugs and carcinogens or processing hormones and neurotransmitters (Pellock and Redinbo, 2017). As GUS is an intracellular hydrolase produced within the cytoplasm of *E. coli* (Liang et al., 2005), *E. coli* accumulates GUS inside the cells. Consequently, *E. coli* concentrations can be estimated by monitoring the GUS activity in pure cultures, drinking water, drinking water sources, recreational water, and wastewater (WW) (Cai et al., 2021; Demeter et al., 2020; Li et al., 2022; Satoh et al., 2021, 2020; Sylvestre et al., 2020). For example, *E. coli* was monitored without pretreatment at a low cost (0.02 USD per sample) with high throughput with a microplate reader (MPR) in our previous study (Satoh et al., 2020). The method based on GUS activity measurement may detect *E. coli* in a viable but non-culturable (VBNC) state and even dead cells because *E. coli* growth is not necessary for analysis (Heery et al., 2016). In addition, George et al. demonstrated that the GUS enzyme is susceptible to environmental stresses, such as light irradiation, osmotic stress, nutritional stress, and light irradiation, causing a 50% loss in GUS activity after one week (George et al., 2000).

Based on the results described above, we hypothesized that the closer the analyzed site in a river is to the fecal pollution source, the higher the activity of the intrinsic GUS (iGUS) of *E. coli*, which is the GUS present in an *E. coli* cell. iGUS activity can be measured using a MPR, as described above. The detection time was reduced because incubating *E. coli* for iGUS to be determined was unnecessary. In this study, we developed a novel, simple, and rapid method for detecting fecal pollution sources in urban rivers. The iGUS activity of river water (RW) samples, instead of *E. coli* concentration, was used to estimate *E. coli*. The iGUS activity and growth of pure *E. coli* cultures were monitored using MPR and compared with the *uidA* gene and its mRNA concentrations. The effect of chlorination on iGUS activity in pure *E. coli* cultures was

also investigated. iGUS activity was compared with *E. coli* concentrations determined using a conventional culture-dependent method. Finally, fecal pollution sources were identified using the proposed method and the ability to discriminate fecal pollution was discussed.

2 Materials and Methods

2.1 Samples

Pure cultures of *E. coli* K-12 (ATCC 47076), *E. coli* (Migula) Castellani and Chalmers (ATCC25922), *E. coli* O157:H7 (RIMD 0509952), *E. coli* O111 (RIMD 05092017), *E. coli* O26:H11 (RIMD 05091992), *E. coli* O1:K1:H7 (JCM1649) and *Salmonella enterica* (JCM 1652) were used. All strains stored in 50% of glycerol solution at -80 °C were activated overnight using Luria-Bertani (LB) broth (BD Difco, # 244620, Franklin Lakes, NJ, USA) at 37 °C in a shaker incubator to reach the stationary phase. Some samples were further incubated for 1 h in LB medium with 4-methylumbelliferyl-β-D-glucuronide (MUG) or p-nitrophenyl β-D-glucuronide before the test, which was termed pre-incubation. Biologically treated WW samples were obtained from a secondary clarifier of WWTP-A in Sapporo City (Satoh et al., 2020) and used as *E. coli* sources.

The RW samples were collected between September and December 2022 from seven sampling sites in Sapporo, Japan (Figure 1). Six sampling sites were located: 620 m upstream and 180 m downstream, 500 m upstream and 120 m downstream, and 500 m upstream and 400 m downstream of the discharge outlets of municipal WWTP-A, WWTP-B, and WWTP-C, respectively. There were no WWTP upstream of WWTP-A, WWTP-B, or WWTP-C. Although there were combined sewer outlets (CSOut) upstream of WWTP-A, WWTP-B, and WWTP-C,

146 contamination by CSOs was negligible, because all samples were collected on fine days. At
147 average river flow rates, WWTP-A, WWTP-B, and WWTP-C effluents approximately contribute
148 to 65%, 40%, and 70% of the river flow rates, respectively. One sampling site was located 200 m
149 downstream of CSOut (Figure 1).
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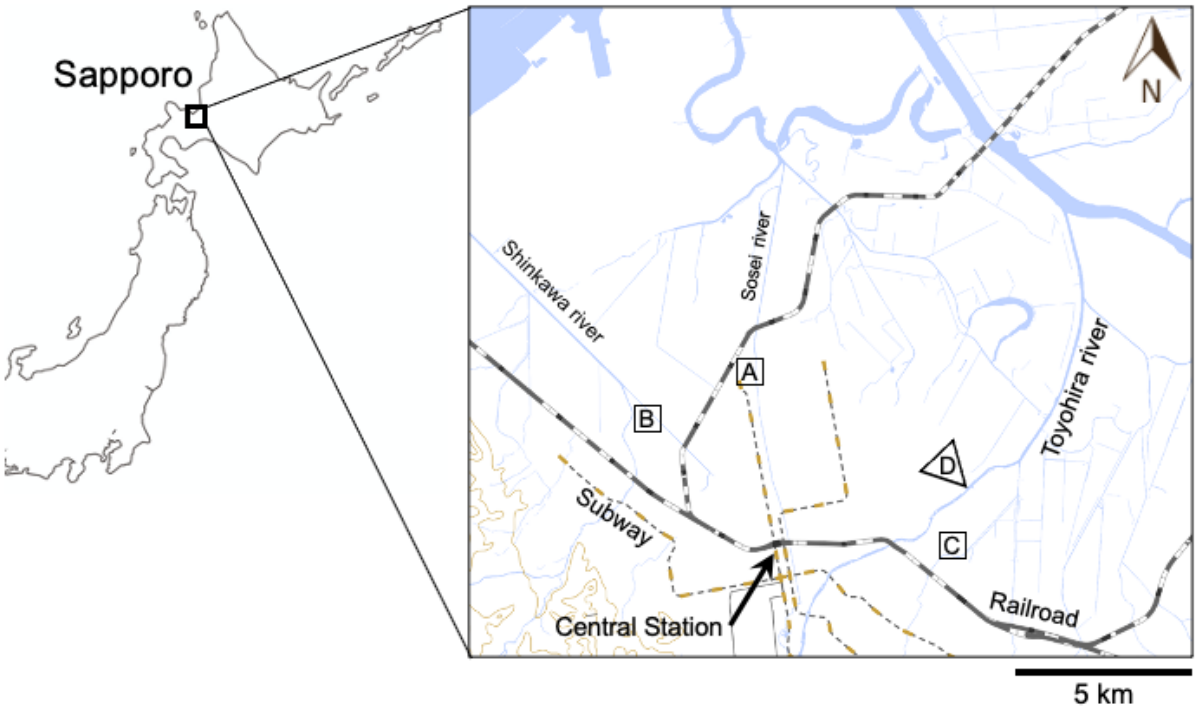

151
152 Figure 1. Map of the study area. Squares and the triangle indicate WWTPs and a CSOut,
153 respectively. Square A: WWTP-A, Square B: WWTP-B, Square C: WWTP-C. The samples were
154 collected at distances of several hundred meters upstream and downstream from discharge
155 outlets of three municipal WWTPs and a CSOut (Triangle D).
156
157 *2.2 Microplate iGUS activity assay*

The iGUS activity of *E. coli* was determined using a simple microplate assay developed in our previous studies (Satoh et al., 2021, 2020). Briefly, an aliquot (20 µL) of the medium including MUG for incubation of *E. coli* (MPR medium) was added to one well of a 96-well microplate (TPP Techno Plastic Products AG, 92696, Trasadingen, Switzerland). Subsequently, 180 µL of a sample was added and thoroughly mixed with the MPR medium by pipetting. For the measurement of the pure culture, the samples were prepared by diluting each culture to an optical density at 600 nm (OD600) of 0.3 and further diluting 10 times. Milli-Q water was used as blank control. Twelve wells were used for each sample. The microplate was placed in an MPR (Tecan Trading AG, Tecan Infinite F200Pro, Männedorf, Switzerland) equipped with a 360 nm excitation filter with 35 nm bandwidth and a 460 nm emission filter with 20 nm bandwidth for measuring fluorogenic 4-methylumbelliferone (MU) produced by degradation of MUG and set at 37 °C. The fluorescence intensity (F.I.) was measured every 10 min for 24 h. The results were reported as the mean of 12 measurements. F.I. increased approximately linearly between 1 and 3 h of incubation; therefore, the temporal change in F.I. during this period was fitted by linear regression, and the slope was considered as the substrate utilization rate. The substrate utilization rate was converted to iGUS activity and expressed in modified Fishman units per mL (MFU/mL), following the standard Sigma Quality Control Test Procedure (Sigma Aldrich, 1998). To investigate the effect of chlorination on the samples iGUS activity, the samples were disinfected with sodium hypochlorite for 1 h or 4 h. The hypochlorite concentrations were measured with a portable residual chlorine meter (DR300, HACH, Loveland, Colorado, USA) at arbitrary timing and the total Cl concentration was maintained between 0.8–1.2 mg-Cl_2/L. To investigate iGUS activity persistence, the pre-incubated sample was incubated in LB medium diluted 100 times or 0.1% NaCl solution. Pure *E. coli* culture and treated WW and RW samples

were counted by the conventional method using a Colilert® and Quanti-Tray/2000 system (IDEXX Laboratories, Westbrook, ME, USA) following the manufacturer's instructions. The *E. coli* concentration was indicated as the most probable number (MPN)/mL.

2.3 Extraction and determination of DNA and mRNA of uidA *gene*

For the microplate iGUS activity assay, DNA and mRNA were extracted from one set of the 200-μL samples collected from the microplate described above by using a Power Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA USA) and the RNeasy Mini Kit (Qiagen, Hilden, Germany), respectively, according to the manufacturer's instructions. Thereafter, cDNA was synthesized from the extracted RNA using a PrimeScript™ RT Reagent Kit (Perfect Real Time; Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. The extracted DNA and cDNA were quantified using the TaqMan assay (Frahm and Obst, 2003) according to a previous study (Satoh et al., 2020).

3 Results and Discussion

3.1 Induction of GUS activities in pure E. coli cultures

Figure 2 shows the changes in the F.I. of 360 nm excitation and 460 nm fluorescence to monitor MU over time generated by incubating pure *E. coli* K-12 cultures at different concentrations in the MPR medium. The original sample with 2.8×10^9 MPN/mL of *E. coli* (Sample 1) and the 10 times diluted sample (i.e., at 2.8×10^8 MPN/mL of *E. coli*, Sample 2) showed exponential increase in F.I. at 0 h. In contrast, the F.I. of the sample at 2.8×10^7 MPN/mL of *E. coli* (Sample 3) linearly increased until approximately 4 h of incubation and thereafter increased exponentially (Figure 2B). The F.I. of the samples at 2.8×10^6 MPN/mL (Sample 4) to

0.28 MPN/mL (Sample 11) of *E. coli* also showed a linear and subsequent increase in F.I., although the time at which the exponential F.I. increase started was delayed. The F.I. of the sample containing 0.028 MPN/mL (Sample 12) of *E. coli* did not exhibit an exponential increase of F.I. until 24 h.

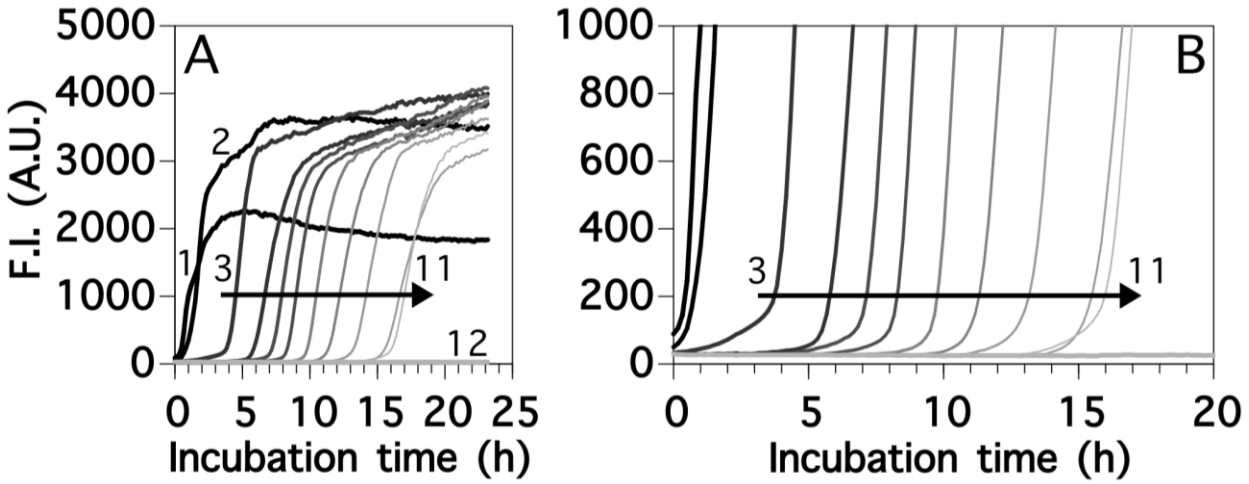

Figure 2. (A) Time-course changes in the F.I. of MU generated by pure *E. coli* K-12 cultures. (B) Magnified Figure 2A. The numbers on the graphs indicate the numbers of the samples. *E. coli* concentration in Sample 1 was 2.8×10^9 MPN/mL. Samples 2–12 were prepared by a 10-fold serial dilution of Sample 1.

Figure 3 shows time-course changes in the F.I. of MU and copy numbers of the *uidA* gene and its mRNA molecules in a treated WW sample incubated in the MPR medium. *E. coli* concentration of the sample was 3.2×10^3 MPN/mL. The F.I. of the sample increased from approximately 5 h and reached a plateau at approximately 8 h. The *uidA* gene concentration simultaneously increased exponentially (from 5–8 h). It demonstrated that the increase in the F.I. of the MPR medium directly corresponded to *E. coli* growth. The *uidA* mRNA concentration

increased at 6 h and decreased at 7 h. The result demonstrated that GUS was produced mainly at the early logarithmic growth phase because the levels of mRNA transcript of a gene (i.e., *uidA*) directly reflect its transcription rate. Therefore, we assumed a linear F.I. increase in the lag phase as the GUS activity of *E. coli* in an original sample before incubation in the MPR. It was defined as an iGUS activity of *E. coli* and the iGUS activity of the sample in Figure 3 was calculated to be 0.80 mMFU/mL from the slope (10 A.U./h). Moreover, the time of the exponential increase of the F.I. started was defined as the initiation time of logarithmic growth (Satoh et al., 2022, 2020). Based on the definition, the results shown in Figure 2 were translated as follows: Samples 1 and 2 had no lag phase of *E. coli* growth in the MPR tests due to high initial *E. coli* concentrations. Since the Samples 3–12 containing lower concentrations of *E. coli* underwent the lag phase, the iGUS activity of *E. coli* could be estimated from the slope of linear increase in F.I. The iGUS activities of *E. coli* in the samples shown in Figure 2 are listed in Table S1. The iGUS activity for Sample 3 was 2.5 mMFU/mL and decreased with decreasing *E. coli* concentrations in the sample.

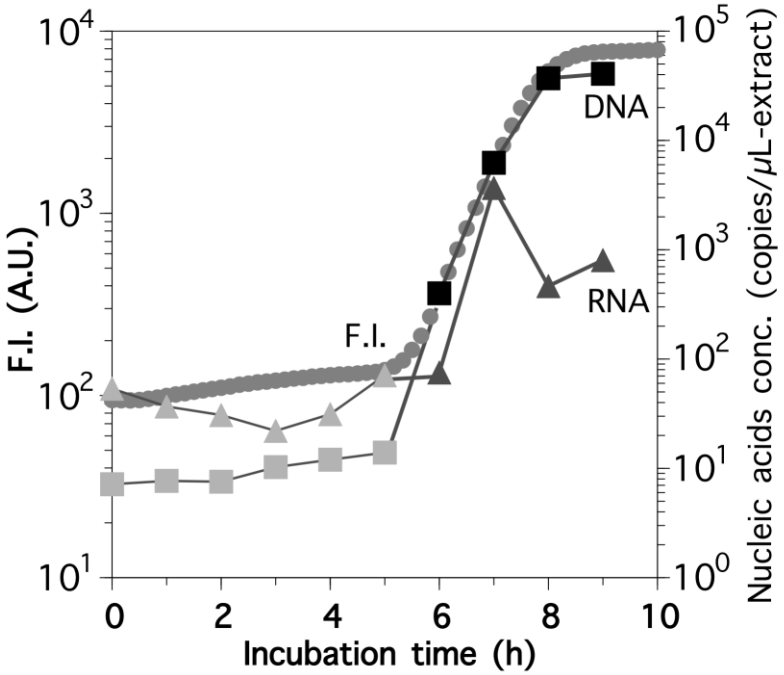

Figure 3. Time-course changes in the F.I. of MU and copy numbers of the *uidA* gene and its mRNA in a treated WW sample incubated in the MPR medium. Gray plots indicate the data below the detection limit (70 copies/μL-extract).

A pure *E. coli* K-12 culture was pre-incubated in LB medium with MUG for 1 h to accumulate GUS inside the *E. coli* cells, because iGUS activities of *E. coli* K-12 cultured in LB medium (Figure 2) were lower than those of the treated WW sample (Figure 3 and Table S1). Figure 4 shows the time-course changes in the F.I. of pure cultures of pre-incubated *E. coli* K-12. The pre-incubated sample contained 2.4×10^9 MPN/mL of *E. coli* (sample 1), which was serially diluted with Milli-Q water. Sample 1 F.I was very high (1350 ± 110 in average ± standard deviation) even at 0 h of incubation, indicating GUS was produced and MU was generated during preincubation. The pre-incubated and subsequently diluted sample at approximately 10^7 MPN/mL of *E. coli* (Sample 3 in Figure 4) exhibited higher iGUS activity than the sample without pre-incubation at approximately 10^7 MPN/mL of *E. coli* (Sample 3 in Figure 2) (Figure

S1 and Table S1 in the Supplementary Material). The iGUS activities of the pre-incubated samples at 10^6 MPN/mL (Sample 4) and lower concentrations of *E. coli* were also higher than those of the samples without pre-incubation, compared with the same orders of magnitude of *E. coli* concentrations (Figure S1 and Table S1 in the Supplementary Material). These results indicate that incubating a pure *E. coli* culture in a medium with a substrate for *uidA* gene expression resulted in the induction of GUS production. Little et al. explored the molecular basis of the GusR-mediated regulation of GUS expression in response to small-molecule glucuronides (Little et al., 2018). In the absence of a glucuronide, GusR may repress the downstream transcription of GUS operon proteins GusA, GusB, and GusC by binding to a specific DNA operator site. In the presence of a glucuronide molecule, GusR disassociates from the operator, allowing the expression of the GUS operon.

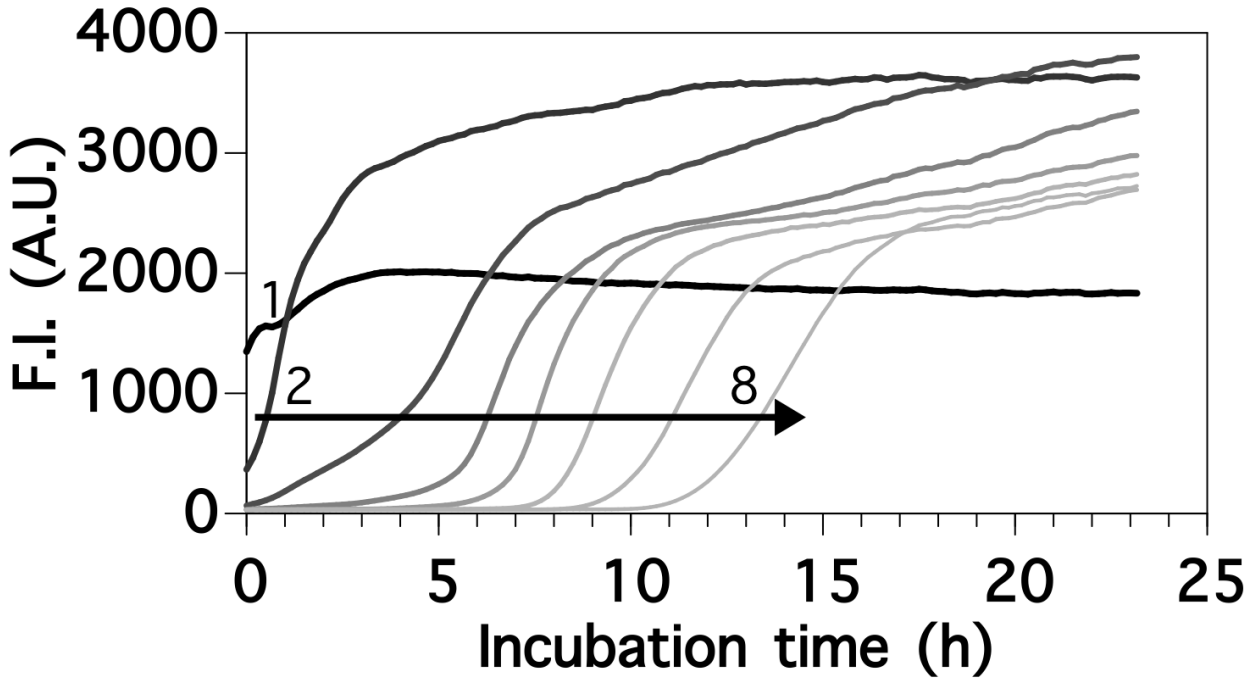

Figure 4. Time-course changes in the F.I. of MU generated by pure *E. coli* cultures pre-incubated in LB medium with MUG for 1 h. *E. coli* concentration in Sample 1 was 2.4×10^9 MPN/mL. Samples 2–8 were prepared by a 10-fold serial dilution of Sample 1.

Furthermore, iGUS activities of six *E. coli* strains and *Salmonella enterica* as a model bacterium affiliated with Enterobacteriaceae, were measured (Table S2 in Supplementary Material). The concentrations of the six *E. coli* strains were approximately 10^7 CFU/mL. The iGUS activities of four *E. coli* strains were similar (11.6–14.9 mMFU/mL) with a coefficient of variation of 0.12. These values are similar to those obtained at the same orders of magnitude as *E. coli* concentrations obtained in the test, as shown in Figure 4 (Table S1 in the Supplementary Material). Filtrates of the culture liquids from the four *E. coli* strains exhibited no iGUS activity, indicating that GUS was located inside the cells. Although both *E. coli* O157:H7 (RIMD 0509952) and *E. coli* O111 (RIMD 05092017) possess the *uidA* gene encoding GUS (Ishii et al., 2013), they exhibit no iGUS activity. This indicates that *uidA* expression is dependent on both genetic and environmental factors. Salmonella enterica (JCM 1652) did not exhibit iGUS activity because it does not possess the *uidA* gene (Ishii et al., 2013).

3.2 Effect of chlorination on iGUS activity and regrowth of pure E. coli cultures

A diluted pure *E. coli* culture at 2.7×10^6 MPN/mL was chlorinated at 1.0 mg-Cl_2/L for 1 h to investigate the effect of chlorination on the iGUS activity of *E. coli*. To accumulate GUS in *E. coli* cells, the cells were incubated with p-nitrophenyl-D-glucuronide as a GUS substrate for 1 h before chlorination. The F.I. of the samples before chlorination (i.e., intact sample) began exponential growth at 2.7 h with 2.1 mMFU/mL of the iGUS activity (Figure 5). As several *E. coli*

287 cells were killed by chlorination, the chlorinated sample showed a delay in the initiation time of
288 logarithmic growth to 9 h. In a previous study, we estimated the culturable *E. coli* concentration
289 by determining the incubation time at which the fluorescence intensity exceeded the threshold with
290 an MPR (Satoh et al., 2022). *E. coli* concentrations determined using our method were
291 approximately positively correlated with those determined using the Colilert® test. A delay in the
292 initiation time of logarithmic growth indicates a decrease in culturable *E. coli* concentrations in
293 the original sample. *E. coli* concentration in the chlorinated sample decreased to 2.9 MPN/mL.
294 The iGUS activity of the chlorinated sample was 1.7 mMFU/mL. The ratio of iGUS activity in
295 chlorinated (1.7 mMFU/mL) to intact samples (2.1 mMFU/mL) was 0.81. This indicates that,
296 although chlorination resulted in a 6-log reduction in *E. coli* counts in Colilert® test, iGUS activity
297 was reduced by only 19%. This may be because chlorination damaged only the *E. coli* cell wall
298 and most of the GUS present inside the chlorinated *E. coli* cells remained active. Wang et al. also
299 reported that chemically dosed chlorine damaged *E. coli* cell membrane integrity before disrupting
300 enzyme activity and the damaged *E. coli* entered the VBNC state (Wang et al., 2010).
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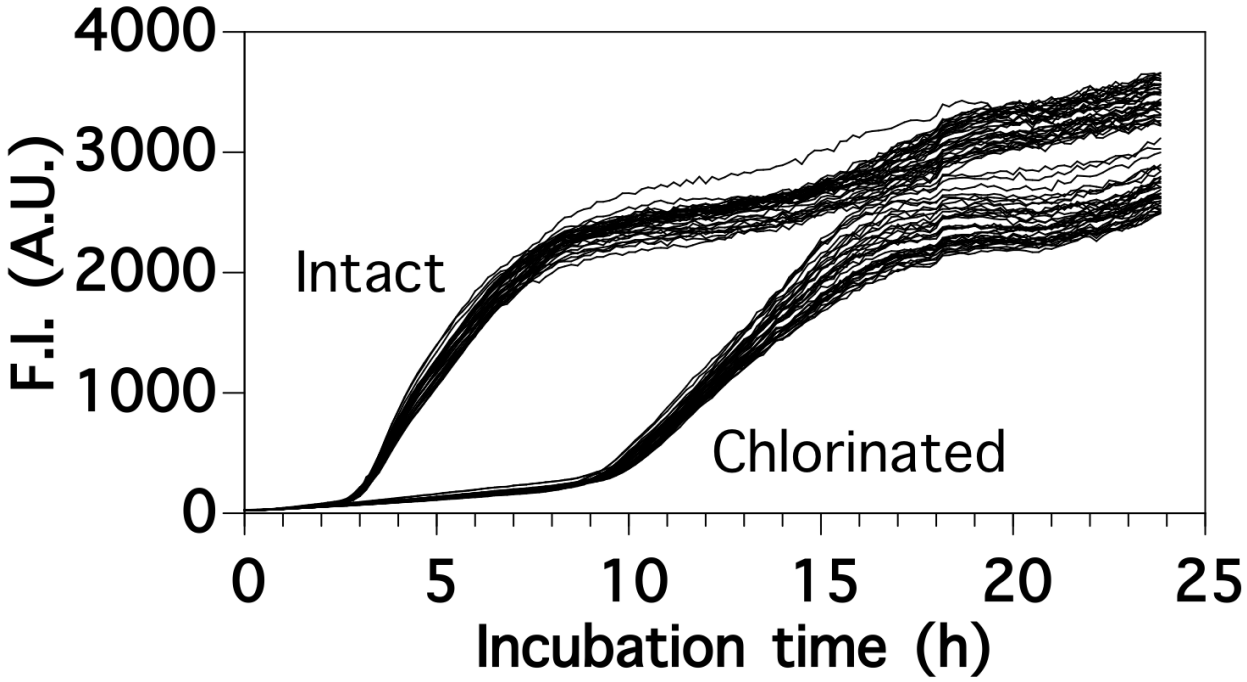

Figure 5. Time-course changes in the F.I. of MU generated by pure *E. coli* cultures pre-incubated in LB medium with p-nitrophenyl β-D-glucuronide for 1 h. After preincubation and dilution, half of the sample with 2.7×10^6 MPN/mL of *E. coli* was subjected to F.I. measurement using MPR, and the remaining was used for iGUS activity assessment after chlorination.

A pure *E. coli* culture pre-incubated in LB medium with p-nitrophenyl-D-glucuronide for 1 h was completely disinfected (0 MPN/mL of *E. coli*) by chlorination at 1.0 mg-Cl_2/L for 4 h. The F.I. of intact samples at 3.3×10^6 MPN/mL showed an exponential growth at 2.7 h with 1.5 mMFU/mL of iGUS activity (Figure 6). In contrast, among the 36 chlorinated samples, only one showed exponential growth in the MPR test, indicating that *E. coli* was inactivated in 35 of the 36 samples. Both the Colilert® test and our method showed that *E. coli* was almost completely inactivated (>9-log reduction). In contrast, the iGUS activity of the chlorinated sample was 0.28 mMFU/mL, accounting for 19% of the iGUS activity of the intact sample. These results indicate

316 that GUS was partly protected from chlorination and remained inside the chlorinated *E. coli*
317 cells.
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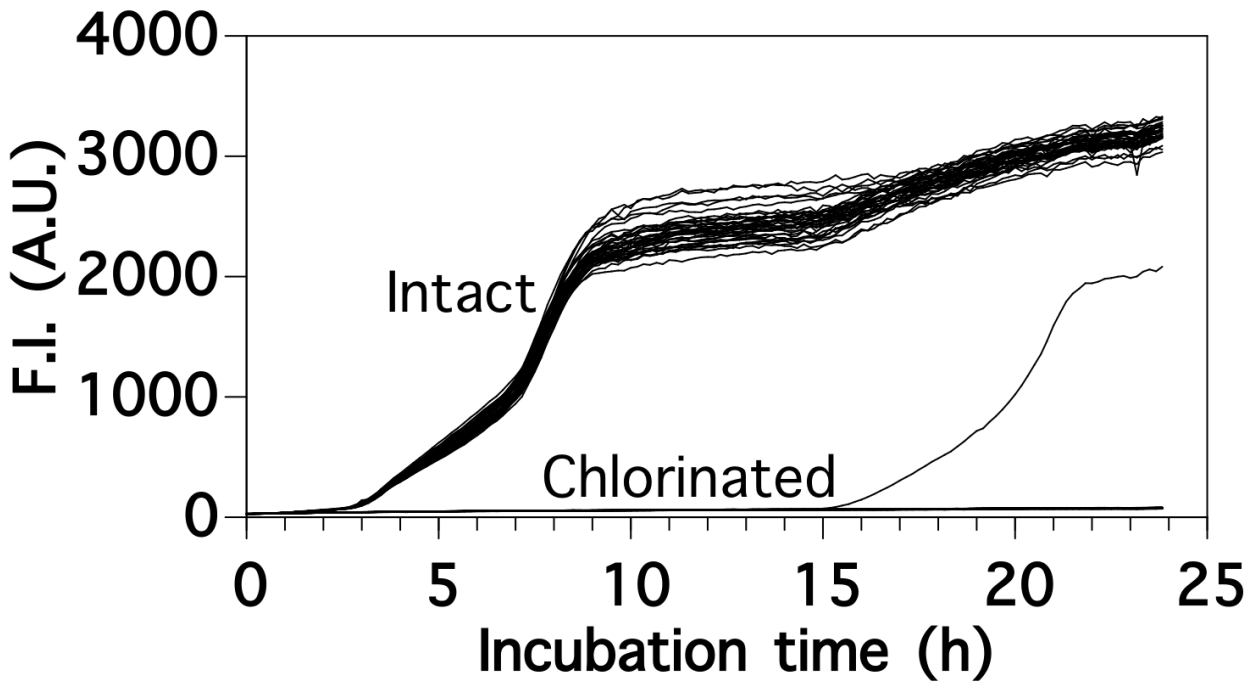


319
320 Figure 6. Time-course changes in the F.I. of MU generated by pure *E. coli* cultures pre-incubated
321 in LB medium with p-nitrophenyl β-D-glucuronide for 1 h. After preincubation and dilution, half
322 of the sample with 3.3×10^6 MPN/mL *E. coli* was subjected to F.I. measurement using MPR, and
323 the remaining was used for iGUS activity assessment after chlorination.
324
325 Figure 6 shows the presence of *E. coli* that could not be detected by the Colilert® test but
326 grew in the MPR medium. *E. coli* were defined as being in the VBNC state in the Colilert® test
327 (Wang et al., 2010). To further investigate the presence of VBNC *E. coli*, the subsamples were
328 collected from positive and negative wells in Colilert® and Quanti-Tray/2000 tests (IDEXX
329 Laboratories Inc., 2023). The Colilert-positive subsamples in the intact sample exhibited strong
330 fluorescence (500–1000 a.u.), even after 0 h of incubation (data not shown). Colilert-negative

subsamples in the intact sample showed neither iGUS activity nor exponential growth, indicating the absence of *E. coli* in the Colilert-negative wells in the intact sample (data not shown). In contrast, all the wells containing chlorinated samples were negative. Among the randomly selected 24 Colilert-negative subsamples in the chlorinated sample, nine (38%) showed exponential growth after 4–17 h of incubation in the MPR medium (Figure 7). This implied that there were VBNC *E. coli* in some Colilert-negative subsamples.

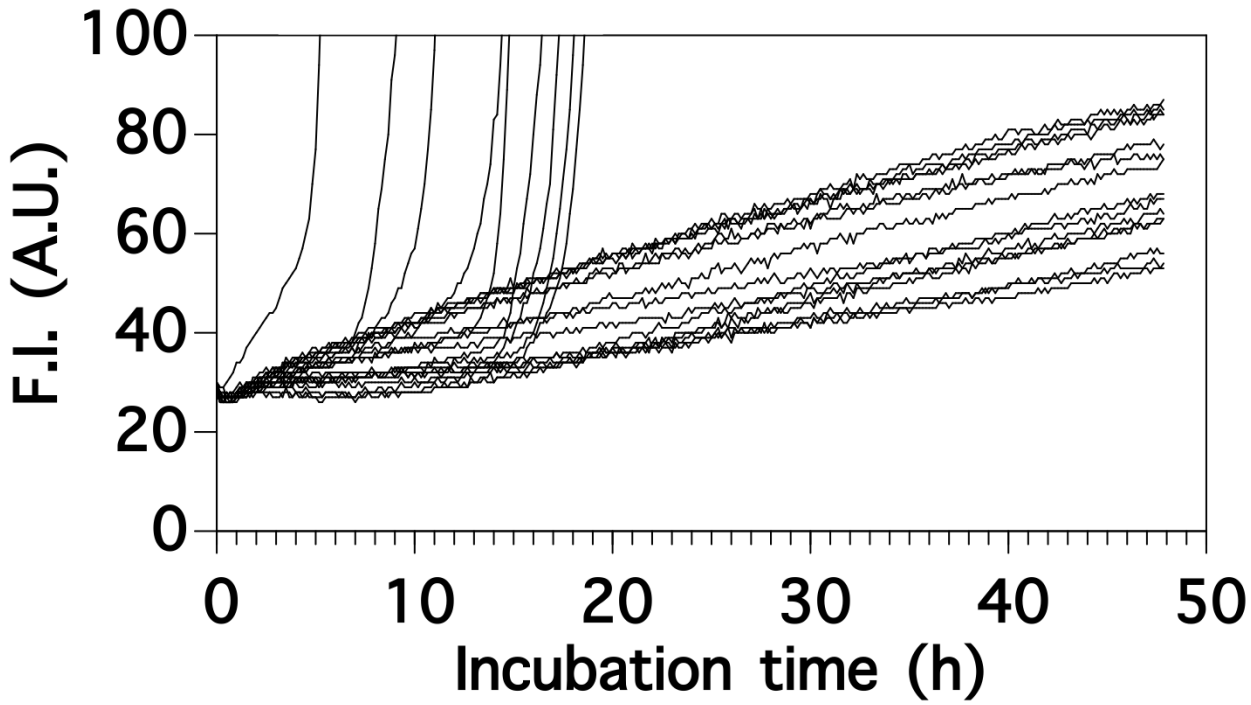


Figure 7. Time-course changes in the F.I. of MU generated by the subsamples collected from negative wells in Colilert® and Quanti-Tray/2000 tests.

3.3 iGUS activities of E. coli in river

Figure 8 shows the iGUS activity of the RW samples collected upstream and downstream of the WWTP discharge outlets and CSOut. The iGUS activity of the blank sample was

345 estimated to be 1.6 mMFU/mL by our method. iGUS activity in the upstream samples was <0.24
346 mMFU/mL, roughly showing a concentration-dependent increase. In contrast, the iGUS activity
347 in the downstream samples was >0.21 mMFU/mL. Interestingly, although some upstream
348 samples contained *E. coli* in the range of 10^4–10^5 MPN/mL, in which all downstream *E. coli*
349 were detected, the iGUS activities in most of these samples were lower than those in the
350 downstream samples. In contrast, although *E. coli* concentrations in some samples from
351 downstream CSOut were relatively low (<10^4 MPN/mL), probably because of dilution, their
352 iGUS activities were higher than those of the upstream samples with *E. coli* in the same
353 concentration range (<10^4 MPN/mL). This may be explained by the fact that GUS can survive
354 inside *E. coli* cells in untreated WW discharged from CSOut and in treated WW, even when
355 subjected to chlorination. These results demonstrate that fecal pollution sources can be easily
356 identified by setting a threshold (0.21 mMFU/mL) for iGUS activity for fecal pollution. A value
357 of 0.21 mMFU/mL corresponded to approximately 15 (Burnet et al., 2019) and 20 CFU/mL
358 (Satoh et al., 2021). Therefore, these values could be considered as the limit of detection for our
359 method.
360

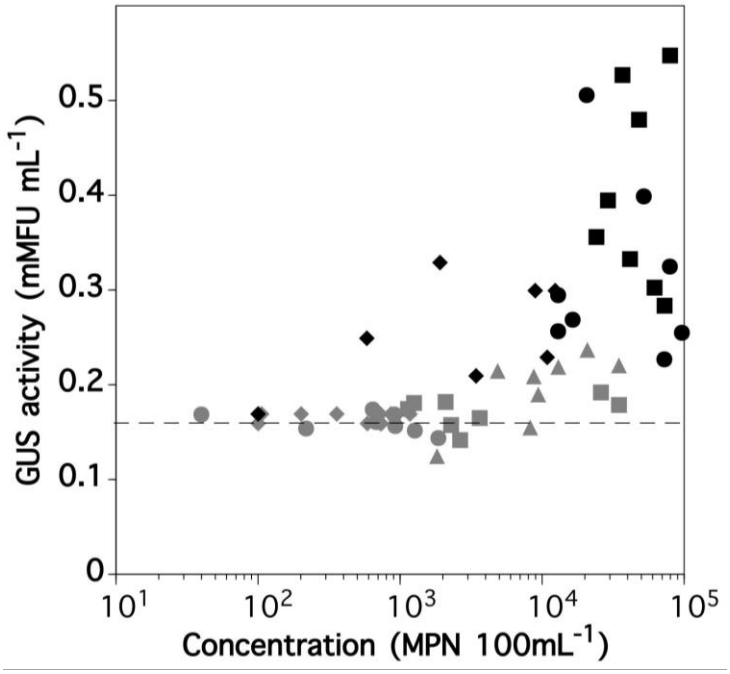


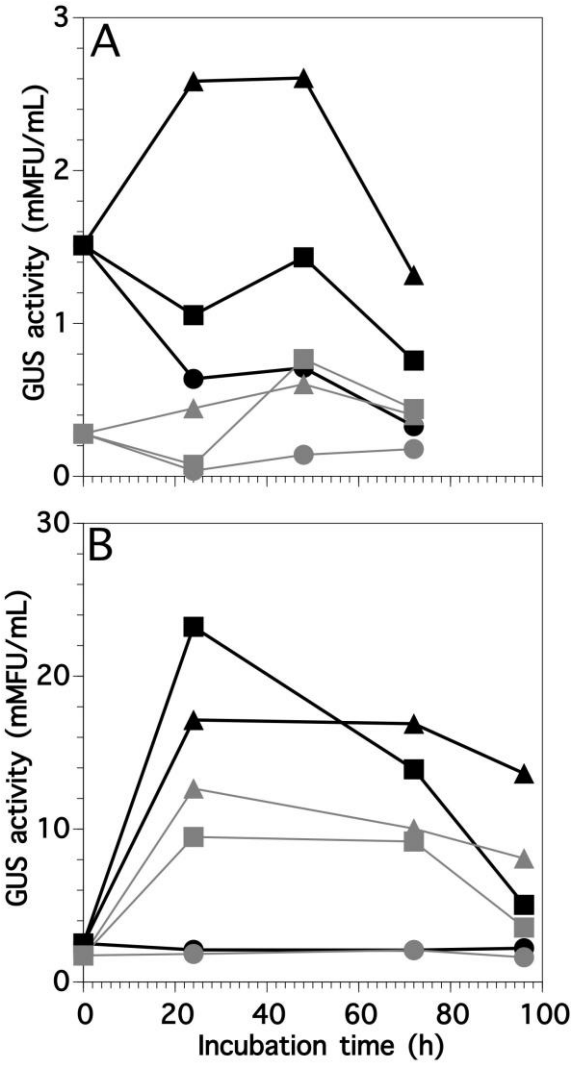
Figure 8. Correlation between iGUS activities and *E. coli* concentrations of the RW samples collected from WWTP-A (circles), WWTP-B (triangles), WWTP-C (squares), and CSOut (diamonds) from September to December 2020. Symbols shown in black and gray indicate the RW samples collected downstream and upstream, respectively, of each discharge point. The dashed line indicates the average GUS activity of the blank samples.

We assumed that *E. coli* actively produced GUS in the human gut, where GUS substrates (e.g., glucuronic acid-conjugated polysaccharides) are abundant (Little et al., 2018) and that GUS could remain inside *E. coli* cells in a sewer system. A comparison of the results in Figure 2 with those in Figure 4 (Figure S1 in the Supplementary Material) demonstrated that the presence of GUS substrates induced GUS production. In contrast, in the upstream parts of the WWTP and CSOut, GUS, which might have been previously produced in human or animal guts, must have already been degraded, deactivated, and/or not produced, whereas *E. coli* was present in the soil

and flowed down the river because of the absence of GUS substrates and/or the conditions unsuitable for *E. coli* growth.

3.4 Persistence of iGUS activities of E. coli

To verify this hypothesis, tests were conducted to determine the persistence of iGUS activity in the samples. Pure *E. coli* culture samples pre-incubated with p-nitrophenyl-d-glucuronide were chlorinated. Thereafter, the intact and chlorinated samples were kept in 0.1% NaCl solution at different temperatures, and iGUS activity was measured during a 72 h incubation (Figure 9A). The iGUS activities of the chlorinated samples did not significantly increase or decrease after 72 h. In contrast, the iGUS activities of the intact samples decreased at 0 °C and 37 °C, increased until 48 h at 25 °C, and then decreased quickly.



387

388 Figure 9. Time course changes in iGUS activities of the pre-incubated pure *E. coli* samples
389 incubated with (A) 0.1% NaCl solution and (B) LB medium diluted 100 times at 0 °C (circles),
390 25 °C (triangles), and 37 °C (squares). Symbols shown in gray and black indicate the samples
391 chlorinated for 1 h or not (intact), respectively, before the incubation.

392

393 Furthermore, the pre-incubated pure *E. coli* culture samples were maintained in LB
394 medium diluted 100 times at different temperatures for 96 h (Figure 9B). Compared with the
395 results shown in Figure 9A, iGUS activities significantly increased within 24 h despite

chlorination or not in the samples incubated at 25 °C and 37 °C. The increase in iGUS activity was greater in intact than in chlorinated samples. This indicates that the presence of organic matter and nutrients accelerated *E. coli* growth and GUS production without a GUS substrate, even in chlorinated samples, resulting in increased GUS activity. After 24 h, GUS activity decreased, significantly at 37 °C, indicating that the degradation of GUS was faster at higher temperatures. In contrast, at 0 °C GUS activity did not increase in the samples for 96 h. These results indicate that *E. coli* can grow in an environment where conditions (e.g., organic matter, nutrients, and temperature) are favorable, leading to an increase in GUS activity. However, GUS activity began to decrease after approximately two days, probably due to the limitation of organic matter and/or nutrients.

Therefore, our method based on the MPR assay could allow us to distinguish *E. coli* present in a recent fecal contamination sample from *E. coli* surviving approximately three days or longer after stool evacuation in the environment by calculating the ratio of iGUS activity to *E. coli* concentration; in other words, it could be used for the detection of recent fecal pollution in an aquatic system. However, our method cannot detect persistent fecal pollution (Calderon et al., 2022; Kim and Wuertz, 2015; Ren and Feng, 2023) or fecal pollution associated with viruses and protozoa. Furthermore, because the limit of detection for our method is 20 CFU/mL, our method is applicable to detecting high fecal pollution in a small river. One way to lower the limit of detection is to use a membrane filter to concentrate *E. coli* in a sample.

4 Conclusions

Currently, the water quality of urban rivers is deteriorating because of fecal pollution caused by the discharge of treated and untreated wastewater. Climate change, urbanization, and population growth in cities have severely affected fecal pollution. Therefore, we developed a simple and rapid method to detect fecal pollution in urban rivers We identified recent fecal pollution within 3 h without any pretreatment of a RW sample by monitoring the intrinsic GUS activity of *E. coli* present in the RW using an MPR along with *E. coli* concentration measurements. Our method might rely on the fact that GUS in *E. coli* cells survives for three days under nutrient- and/or substrate-limited conditions, which is called intrinsic GUS, whereas *E. coli* cells that persist under such conditions for three days or longer have lower GUS activity. Our method confirmed that the intrinsic GUS activity was higher downstream of WWTPs and at a combined sewer outlet than upstream. Therefore, fecal pollution sources can be easily identified by setting a threshold for intrinsic GUS activity for fecal pollution. Moreover, the *E. coli* concentrations were not necessarily associated with fecal pollution. A detailed study on the analyses of GUS activities for pure *E. coli* cultures supported the results of GUS activity measurements in RW samples. The presence of GUS substrates led to the accumulation of GUS inside *E. coli* cells and vice versa. The accumulated GUS remained active even after chlorination. Our method can be used to confirm the spatiotemporal dynamics of fecal pollution in urban rivers. However, at present our method is only applicable to detecting high fecal pollution in a small river. Our findings may be useful for decision makers and policymakers in the wastewater management sector.

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Declaration of interests

The authors declare that they have no competing financial interests or personal relationships that may have influenced the work reported in this study.

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Supplementary Material

A simple and rapid method for detecting fecal pollution in urban rivers by measuring the intrinsic β-D-glucuronidase activity of *Escherichia coli*

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Table S1. The iGUS activities of *E. coli* which were preincubated with MUG (Fig. 4) or not (Fig. 2). The iGUS activity of *E. coli* in the WW sample (Fig.3) was also shown.

Samples (MPN/mL)		GUS activities (mMFU/mL)	Samples (MPN/mL)	GUS activities (mMFU/mL)	Samples (MPN/mL)		GUS activities (mMFU/mL)
Fig. 2	10^{9}	N.D.	Fig. 3		Fig. 4	10^{9}	N.D.
	10^{8}	N.D.				10^{8}	N.D.
	10^{7}	2.5				10^{7}	11.4
	10^{6}	0.20				10^{6}	1.4
	10^{5}	LoD				10^{5}	0.27
	10^{4}	LoD				10^{4}	0.18
	10^{3}	LoD	10^{3}	0.80		10^{3}	LoD
	10^{2}	LoD				10^{2}	LoD
	10^{1}	LoD					
	10^{0}	LoD					
	10^{-1}	LoD					
	10^{-2}	LoD					

N.D.: Not determined.
LoD: Below the limit of detection. 0.16 mMFU/mL.

Table S2. The iGUS activities of six *E. coli* strains and *Salmonella enterica* and the filtrates of their culture liquids. OD600 of the samples was 0.03.

Samples	GUS activities (mMFU/mL)	
	Culture liquid	Filtrate
E. coli K-12	14.1	LoD
E. coli (Migula)	11.6	LoD
E. coli O157:H7	LoD	LoD
E. coli O111	LoD	LoD
E. coli O26:H11	12.2	LoD
E. coli O1:K1:H7	14.9	LoD
Salmonella enterica	LoD	LoD

LoD: Below the limit of detection. 0.16 mMFU/mL.

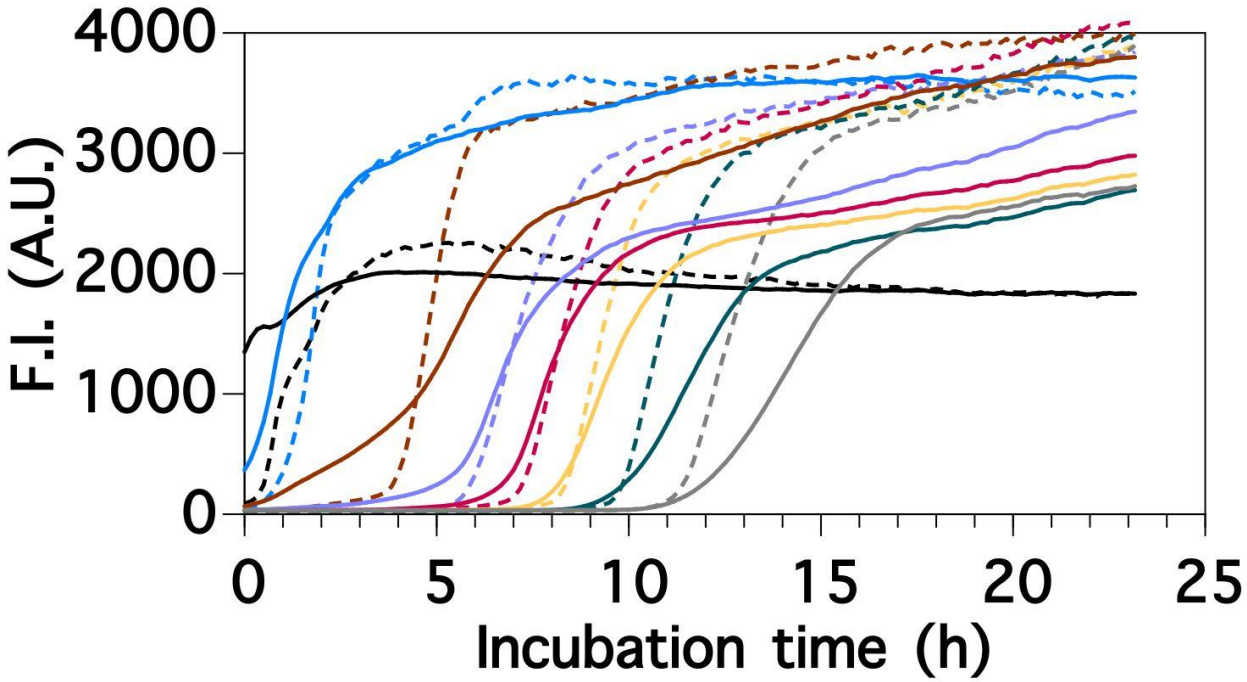

Figure S1. Time-course changes in the F.I. of MU generated by pure cultures of *E. coli*. The figure was created by a combination of Figure 2 (dashed line) and Figure 4 (solid line). The line in the same color indicates the sample in which the *E. coli* concentration was of the same order of magnitude.