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Simple and Reliable Enumeration of Escherichia coli Concentrations in Wastewater Samples by Measuring β-D-glucuronidase (GUS) Activities via a Microplate Reader

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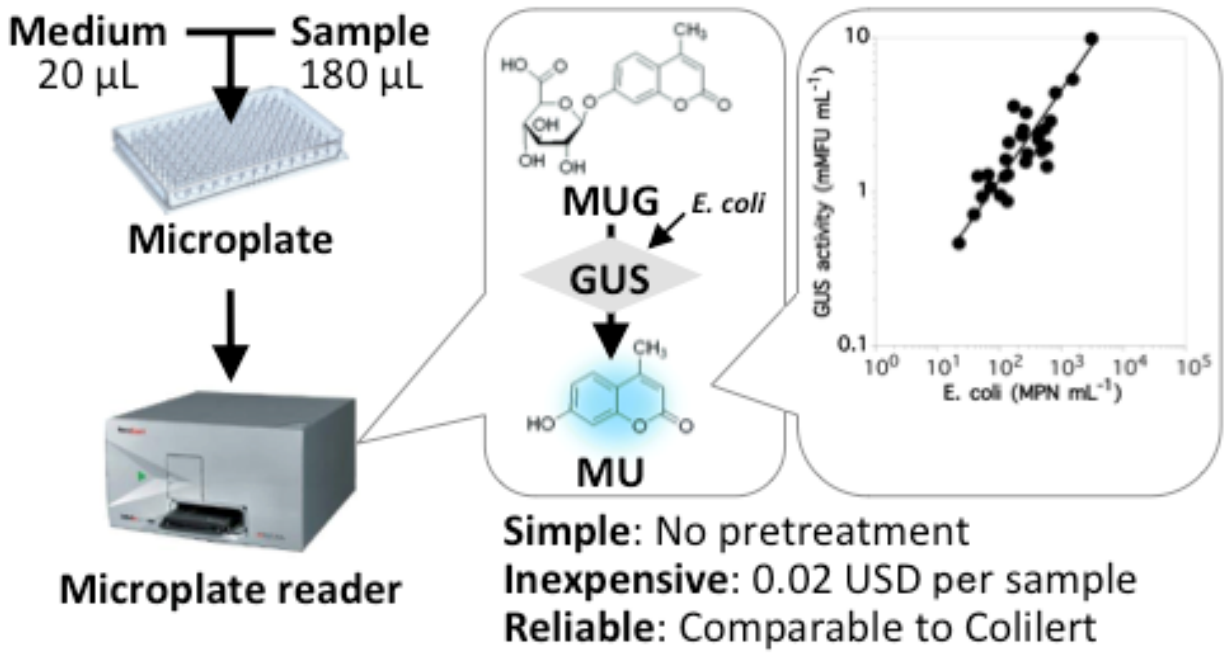

Medium
20 µL
Sample
180 µL
Microplate
Microplate reader
MUG
E. coli
GUS
MU
GUS activity (mMFU mL-1)
E. coli (MPN mL-1)
Simple: No pretreatment
Inexpensive: 0.02 USD per sample
Reliable: Comparable to Colilert

42

43

44

Highlights

- *E. coli* enumeration methods require high cost or skilled technique.
- *E. coli* could be enumerated only by measuring intrinsic GUS activity in wastewater.
- No pretreatment was required, and the cost was 0.02 USD per sample.
- *E. coli* could be monitored over ten months and at different treatment stages.
- The wastewater matrix did not inhibit the measurement.

ABSTRACT

Monitoring of *Escherichia coli* concentrations at wastewater treatment plants (WWTPs) is important to ensure process performance and protect public health. However, conventional *E. coli* enumeration methods are complicated and time- and labor-consuming. Here, we report a novel simple and reliable method based on β-D-glucuronidase (GUS) activity assay to enumerate *E. coli* concentrations in wastewater (WW) samples. An aliquot (20 µL) of the medium with fluorogenic enzyme substrate for *E. coli* and 180 µL of a WW sample were added to one well of a 96-well microplate. The microplate was placed in a microplate reader at 37°C. To this end, the fluorescence intensity of a fluorogenic enzyme substrate for *E. coli* was measured every 10 min over 3 h to determine GUS activity. The linear increase in the fluorescence intensity representing the GUS activities showed a positive correlation with *E. coli* concentrations in wastewater samples. However, the correlation equations were specific to WWTPs, which could be due to the difference in the *E. coli* population structures among WWTPs. We observed that the wastewater matrix is not a limitation to measure the GUS activity, and a WWTP-specific correlation equation can be used as a calibration curve to estimate the *E. coli* concentrations in the samples collected from that site. A comparison of the results with those of culture-dependent Colilert method proved that the current method is simple and useful for the enumeration of *E. coli* concentrations in wastewater samples reliably.

Keywords

Simple enumeration method; Fluorogenic enzyme substrate; *Escherichia coli*; Sewage wastewater; A microplate reader.

1. Introduction

Contamination of surface water by pathogenic microorganisms can cause water-related diseases. Municipal wastewater has been identified as one of the major sources of pathogenic microorganisms in urban areas (Baker et al., 2015; Perkins et al., 2016; Shrestha et al., 2016), thereby necessitating wastewater treatment plants (WWTPs) to monitor the pathogens before releasing the treated wastewater to the aquatic environment. However, direct pathogen monitoring is expensive and involves a complex process. Therefore, detection of fecal indicator bacteria, such as *Escherichia coli*, is the common route that is undertaken for this purpose (Rice et al., 2017; Poopipattana et al., 2018; Wéry et al., 2008). The current *E. coli* enumeration methods include multiple tube fermentation, membrane filtration, plate count methods (Rice et al., 2017), and quantitative PCR (qPCR) (Chern et al., 2009). However, these methods require time for *E. coli* to grow (e.g., >18h), the cost for a large volume of culture media, or skilled technicians to perform microbiology and molecular biology techniques (e.g., PCR), thus, hampering effective and timely decision-making toward proper monitoring of WWTPs and water quality.

To overcome these problems, alternative and simple methods have been developed based on the detection of β-D-glucuronidase (GUS), which is generally produced by most *E. coli* strains. The GUS activity is usually detected using fluorogenic substrates, such as methylumbelliferone (MU), which is liberated by the hydrolysis of 4-methylumbelliferyl-β-D-glucuronide (MUG) through GUS activity. Many of the previously developed methods have been applied to measure *E. coli* in natural freshwater (George et al., 2000; Heery et al., 2016; Wildeboer et al., 2010) and drinking water (Hesari et al., 2016). The detection limit and response time of those methods can be <10 cells mL^{-1} and <2 h, respectively. However all of these

methods required pre-treatments, such as removal of contaminants, concentration, fluorescence enhancer (e.g., NaOH) and/or lysis of *E. coli* cells. Recently, Burnet et al. (2019) developed an automated online fluorescence-based technology to measure GUS activity for rapid near-real-time monitoring of *E. coli* in river water that did not require any pretreatment (Burnet et al., 2019). However, these methods have only been used for river water or drinking water, which are relatively clean compared with wastewater. Wastewater contains complex matrix, which could interfere with GUS activity. In addition, production and degradation of GUS in wastewater samples could strongly affect the accuracy of these methods.

The objective of this study was to develop a simple and reliable method to measure *E. coli* concentrations in wastewater samples via GUS activity measurement. Here, we designed a protocol that does not require any sample pretreatment. We analyzed the correlation between GUS activity and *E. coli* abundance and examined the potential inhibitory effect of the wastewater matrix on GUS activity. The correlation equation was then used to estimate the *E. coli* concentrations in various wastewater samples. To examine the reliability of our method, resulting *E. coli* concentrations were compared with those measured using the conventional culture-dependent Colilert method. The results presented here show that our simple method may prove useful to enumerate *E. coli* concentrations in wastewater samples reliably.

2. Materials and Methods

2.1. Wastewater samples

Wastewater samples (WWs) were collected at three municipal wastewater treatment plants (WWTPs). WW characteristics and operating conditions of the WWTPs are described in Table S1. For WWTP-A, WWs were collected at the outlet of a primary clarifier (PC), a secondary

clarifier without chlorination (SC), a sand filtration process (SF), and a chlorination process (Chl); while WWs were collected only at an SC in WWTP-B and WWTP-C. The WWs were collected in sterile one-liter polypropylene containers and processed within 30 min after collection.

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

The conventional method for the enumeration of *E. coli* was conducted using Colilert and Quanti-Tray/2000 (IDEXX Laboratories) according to the manufacturer's instructions. The WWs were taken and diluted with sterile physiological NaCl solution (0.9% NaCl) in 100-mL sterile bottles. 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 β-D-glucuronidase (GUS) assay

A simple microplate assay was developed to measure the GUS activity of *E. coli*. The medium for incubation of *E. coli* was prepared by adding 50 mg of peptone (CAS. No. 73049-73-7), 50 mg of NaCl (CAS. No. 7647-14-5), 10 mg of $CH_3COCOONa$ (CAS. No. 113-24-6), 10 mg of KH_2PO_4 (CAS. No. 7778-77-0), 40 mg of K_2HPO_4 (CAS. No. 7758-11-4), and 10 mg of KNO_3 (CAS. No. 7757-79-1), 1.0 mg of $CH_3(CH_2)_{11}OSO_3Na$ (sodium dodecyl sulfate, SDS, CAS. No. 151-21-3), 1.0 mg of $C_9H_{18}O_5S$ (isopropyl β-D-1-thiogalactopyranoside, IPTG, CAS. No. 367-93-1), and 1.0 mg of 4-methylumbelliferyl-β-D-glucuronide (MUG, CAS. No. 6160-80-1) to 1 mL of Milli-Q water (Merck Millipore). SDS was added as a selection agent for gram-negative bacteria, while IPTG was added to induce expression of GUS of *E. coli*. The medium was based

146 on a medium of EC Blue (Nissui Pharmaceutical, Tokyo, Japan) for the growth of *E. coli*.
147 Chemicals and reagents, if not specified otherwise, were obtained from Sigma-Aldrich. All the
148 chemicals used were analytical grade. Ultrasonic treatment was applied to dissolve the substrates
149 in the medium. An aliquot (20 μL) of the medium was added to one well of a 96-well microplate
150 (TPP Techno Plastic Products AG, 92696). Subsequently, 180 μL of WWs or Milli-Q water as a
151 blank control were added and thoroughly mixed with the medium by pipetting. Ten wells were
152 used for each sample. The microplate was placed in a microplate reader (Tecan Trading AG,
153 Tecan Infinite F200Pro) equipped with a 360 nm excitation filter and 460 nm fluorescence filter
154 and set at 37°C. Then, fluorescence intensity was measured every 10 min over 10 h.
155 The temporal change of the fluorescence intensity during the incubation period was fitted
156 by linear regression, and its slope was defined as the "rate of increase in fluorescence intensity."
157 Since the fluorescence intensity increases when MUG is broken down by GUS, the rate should
158 reflect the substrate utilization rate, and therefore, the enzyme activity. The obtained substrate
159 utilization rate was converted to the enzyme activity of GUS and expressed in Modified Fishman
160 Units per one mL (MFU mL^{-1}), following the standard Sigma Quality Control Test Procedure
161 (Sigma Aldrich, 1998). One MFU of GUS activity from *E. coli* liberates 1.0 μg of
162 phenolphthalein from phenolphthalein β-D-glucuronide (P0501, Sigma-Aldrich) per hour at pH
163 6.8 at 37°C. For calibration, the commercial enzyme standard (G7396-25KU, type IX-A β-D-
164 glucuronidase from *E. coli*, Sigma-Aldrich) activities were used in triplicates.
165 The relationship between incubation time, GUS activity, and *E. coli* abundance was
166 investigated through the following experiment. Aliquots (1800 μL) of WWs were mixed with
167 200 μL of the medium in a deep-well 96 microplate (10 wells per sample) and incubated for 10 h

168 at 37°C. Aliquots (200 μL) of the mixture were taken from each well every hour for DNA
169 extraction.
170
171 *2.4. DNA extraction*
172 To quantify *E. coli* by qPCR, DNA was extracted from one set of the 200-μL WW-medium
173 mixtures collected from the microplate described above, by using a PowerSoil DNA Isolation Kit
174 (MO BIO Laboratories, Inc., Carlsbad, CA USA) according to the manufacturer's instructions.
175 DNA was also directly extracted from the WW samples collected from SC in WWTP-A, -B, and
176 -C once a week for three consecutive weeks (a total of nine WW samples) to analyze the
177 diversity of *E. coli* by *uidA* amplicon sequencing. One liter of the WWs was filtrated in triplicate
178 through a 0.2-μm-pore mixed-cellulose-ester membrane filter (47-mm dia) (A020A047A,
179 Advantec), which was sterilized before use. The membranes were cut by half using a clean
180 surgical scalpel. One half of the membrane was placed in a bead-beating tube supplied with a
181 PowerSoil DNA Isolation Kit, and DNA was extracted according to the manufacturer's
182 instructions. DNA samples were stored at -20°C until further use.
183
184 *2.5. qPCR targeting* uidA
185 qPCR was done to quantify *uidA* gene copies by using a TaqMan assay (Frahm and Obst, 2003).
186 The reaction mixture (20 μL) contained 10 μL of 2× Premix Ex Taq reagent (Takara Bio Inc.,
187 Kusatsu, Shiga, Japan), 20 pmol each of forward primer, 784F (5'-GTG TGA TAT CTA CCC
188 GCT TCG C-3') and reverse primer, 866R (5'-AGA ACG GTT TGT GGT TAA TCA GGA-3'),
189 0.8 pmol of TaqMan probe, EC807 (FAM-TCG GCA TCC GGT CAG TGG CAG T- BHQ1), 0.4
190 μL of ROX Reference Dye, and 1.0 μL of template. The oligonucleotides were synthesized by

191 Eurofins genomics K.K. (Tokyo, Japan). For no-template controls, sterile Milli-Q water was used
192 instead of a template. The qPCR was carried out in triplicate by using an ABI Prism 7500
193 sequence detection system (Applied Biosystems, Warrington, United Kingdom) under the
194 following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for
195 10 s and 60°C for 30 s. ROX was used as a passive reference dye. Amplification data were
196 collected and analyzed with Sequence Detection software version 2.0.4 (Applied Biosystems,
197 Warrington, United Kingdom).
198 For standard DNA, the nucleotide sequence corresponding to the qPCR-amplified region
199 of *uidA* was artificially synthesized and inserted into the pTAKN-2 vector by Eurofins Genomics
200 K.K. (Tokyo, Japan). The plasmid DNA was purified, and the concentration of the purified
201 plasmid DNA was determined fluorometrically using Quant-iT PicoGreen dsDNA Assay Kit
202 (Thermo Fisher Scientific, Waltham, MA USA), and serially diluted from 10 to 10^6 copies μL^{-1}
203 for the preparation of standard curves for qPCR assay.
204
205 *2.6.* E. coli uidA *amplicon sequencing analysis*
206 Structure and diversity of *E. coli* populations in the WWTP-A, -B and -C was analyzed and
207 compared by using *uidA* amplicon sequencing analysis. DNA samples (n=27) extracted as
208 described above, were used. Amplification of *uidA* was done using primers 298F (5'-
209 tcgtcggcagcgtcagatgtgtataagagacagAATAATCAGGAAGTGATGGAGCA-3') and 884R (5'-
210 gtctcgtgggctcggagatgtgtataagagacagCGACCAAAGCCAGTAAAGTAGAA-3'), which has been
211 used to examine diversity of *E. coli* strains (Ram et al., 2007). Bases shown in lower cases
212 indicate the Illumina adaptor sequences, while those shown in upper cases indicate *uidA*-specific
213 primer sequences. The size of the PCR amplicons excluding the Illumina adaptor sequences was

587 bp. The oligonucleotides were synthesized by Eurofins genomics K.K. (Tokyo, Japan). The PCR mixture (30 μL) contained 1× PCR buffer (Takara Bio Inc., Kusatsu, Shiga, Japan), 0.5 μM each primer, 0.2 mM dNTPs, 0.75 U of *Ex* Taq DNA polymerase (Takara Bio Inc., Kusatsu, Shiga, Japan), and 5 ng DNA template. The PCR thermal conditions were as follows: 35 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 60 s, followed by 72°C for 10 min. The PCR products were purified using the FastGene Gel/PCR Extraction Kit (Nippon Genetics Co, Ltd., Tokyo, Japan), and tagged with sample-specific index sequences at their 5'-end by PCR by using Nextera XT Index Kit v2 Set A (Illumina, San Diego, CA USA). The PCR mixture (20 μL) contained 1× KAPA HiFi HS ReadyMix (Nippon Genetics Co, Ltd., Tokyo, Japan), 1 μL each of forward and reverse index primers, 2 μL of the purified PCR products, and 6 μL of nuclease-free water. The PCR was done under the following cycling conditions: 95°C for 3 min; 10 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and 72°C for 5 min. After agarose gel electrophoresis, amplicons of 600–1,000 bp were excised from the gel and purified using the MagExtractor-PCR and Gel Clean-up Kit (Toyobo Co, Ltd., Osaka, Japan). Index-tagged amplicons were pooled and analyzed using a MiSeq paired-end sequencing reaction with the v3 reagent kit (300 cycles) (Illumina, San Diego, CA USA).

2.7. Bioinformatic analysis

The *uidA* sequence reads were analyzed using the MacQIIME 1.9.1 (Caporaso et al., 2010) and USEARCH 9.2 (Edgar et al., 2011) software packages. The generated *uidA* sequence reads were processed for removing adapter sequences using Cutadapt (Martin, 2013). The forward and reverse reads from the MiSeq paired-end sequencing were not assembled because the PCR amplicon size too large (>600 bp). Only forward sequence reads were used in the following

bioinformatics analyses because the number of high-quality sequences was greater in the forward reads (2,235,556 reads) than the reverse reads (873,717 reads). The sequence reads were demultiplexed and further filtered through the split_library_fastq.py script of QIIME with a minimum quality score parameter of 20 and a minimum sequence length of 75 bp. Chimeric sequences were detected and removed by script identify_chimeric_seqs.py and filter_fasta.py (Edgar et al., 2011). The quality-filtered reads were clustered into operational taxonomic units (OTUs) at 99.5% sequence identity by the USEARCH program. We selected the 99.5% identity of the *uidA* sequence as a threshold required for the OTU clustering to investigate community dissimilarity of *E. coli* species with high resolution. The sequence reads representing each OTU were subjected to a BLASTn search against the known 365 nucleotide sequences of *E. coli uidA*, and the reads with less than 50% of sequence identity were removed as non-*uidA* sequences. Alpha diversity (number of observed OTUs, Shannon index, Simpson index, Chao1, and Good's coverage) and beta diversity (principal coordinate analysis [PCoA] (Forsberg et al., 2014) based on Bray–Curtis dissimilarities) were analyzed using the MacQIIME. Alpha diversity indices were calculated at a sampling depth of 10,000 reads. The significance of community dissimilarity was tested by an analysis of similarity statistics (ANOSIM) using the vegan package of R version 3.4.3.

2.8. Nucleotide sequence accession numbers

Nucleotide sequence data obtained in the present study were deposited in the DDBJ/EMBL/ GenBank databases under the accession number DRA008169.

3. Results and Discussion

3.1. Effect of incubation on GUS activity

GUS activity measurement has been applied to enumerate *E. coli* in drinking and surface water samples without incubation to grow *E. coli* (Briciu-Burghina et al., 2017; Burnet et al., 2019; George et al., 2000; Gunda et al., 2016; Heery et al., 2016; Hesari et al., 2016; Wildeboer et al., 2010); however, it has not been applied to the analysis of wastewater. Because WWs could contain potential GUS inhibitors, incubation of samples may be necessary to increase *E. coli* GUS to reliably measure GUS activity. Therefore, we first analyzed the effect of incubation on GUS activity and how incubation influenced the quantitative performance of our GUS assay.

The fluorescence intensity of MU gradually increased until 5.5-h of incubation of the medium-WW mixture, and thereafter, exponentially increased (Fig. 1). The abundance of *E. coli*, as expressed as *uidA* copies μL^{-1}, exponentially increased after 4 h of incubation, while it was below the detection level (65 copies μL^{-1}) until 4 h. The increase in the GUS activity after 5.5 h of the incubation was likely associated with the increased number of *E. coli*. The time-course changes in the *uidA* copy numbers and fluorescence intensity of MU were reproducible (Supporting Information (SI) Fig. S1).

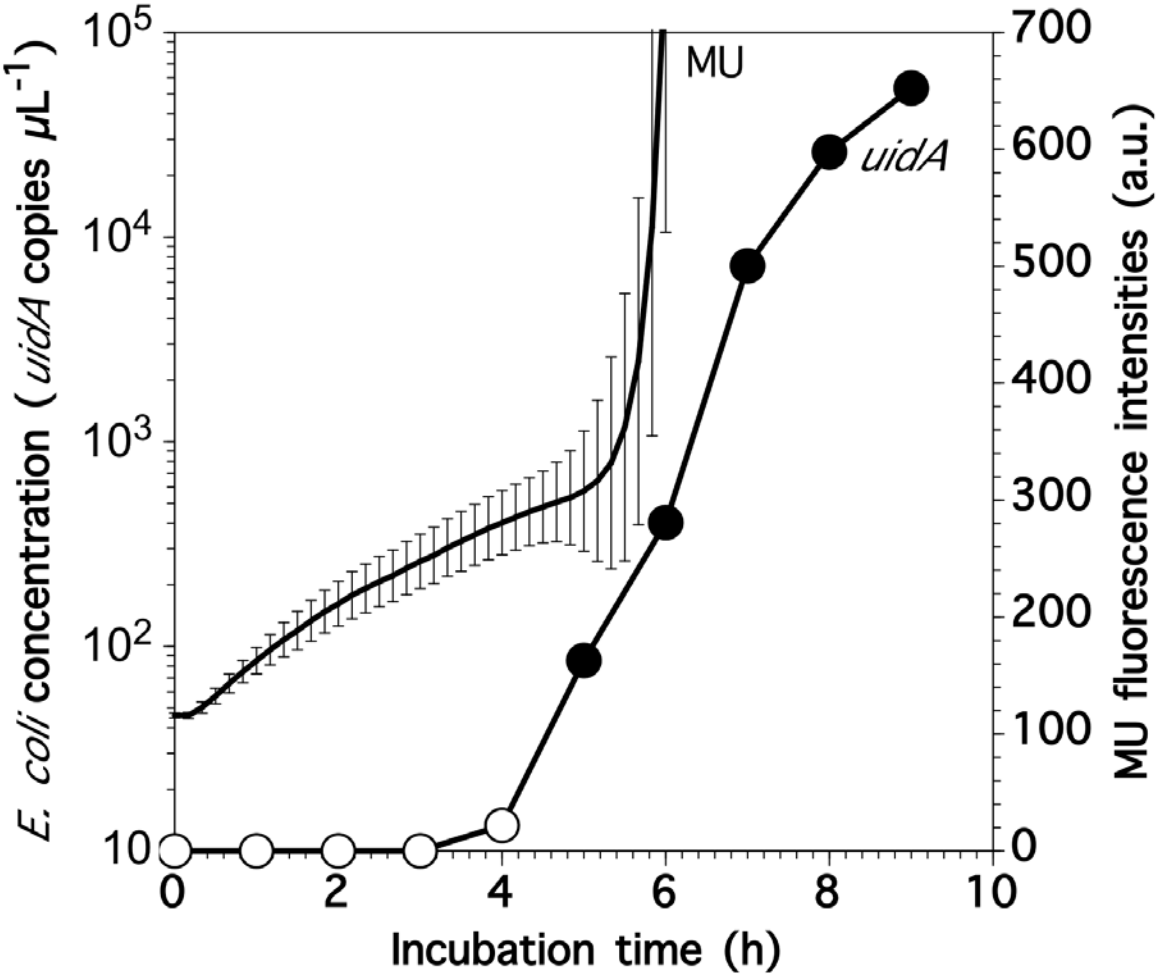


Figure 1. Changes in the *uidA* copy numbers and the MU fluorescence intensities by incubation of the WWs taken from SC in WWTP-A in the *E. coli* medium. Open symbols indicate the values below detection limits. Error bars indicate standard deviation (n=10). The reproducibility of the results is shown in Fig. S1.

It should be noted that the GUS activity was found even at the beginning of the incubation. This result implied the presence of intrinsic GUS activity in the WWs, which was high enough to catalyze MUG to emit the detectable level of the fluorescence signal. These GUS may have been produced when *E. coli* was present in the intestine of their hosts (e.g., humans),

in which glucuronidated compounds (i.e., substrates of GUS) is abundantly available (Little et al., 2018). The amount of GUS could remain unchanged in a sewage system after being released from the host (Wéry et al., 2008). Since both *E. coli* and GUS are diluted in a similar manner in a sewage system, we hypothesized that it is possible to estimate the *E. coli* concentrations in WWs by measuring the activity of GUS in the same sample.

To calculate sample-specific substrate utilization rates (i.e., GUS activities), we used the slope of the linear fit to the MU fluorescence intensities during the initial 5 h of the incubation. Linear increase of the MU fluorescence intensities by time (Fig. 1) indicated that the utilization of MUG by GUS was a zero-order reaction within 5 h of the incubation. Since incubation conditions, such as pH and temperature, were kept constant by using a pH-buffered medium and a temperature-controlled microplate reader, respectively, a substrate utilization rate should be equal to the GUS activity of the WWs. The GUS activity was almost constant within 5 h of the incubation, indicating poor or no growth of *E. coli* during this period.

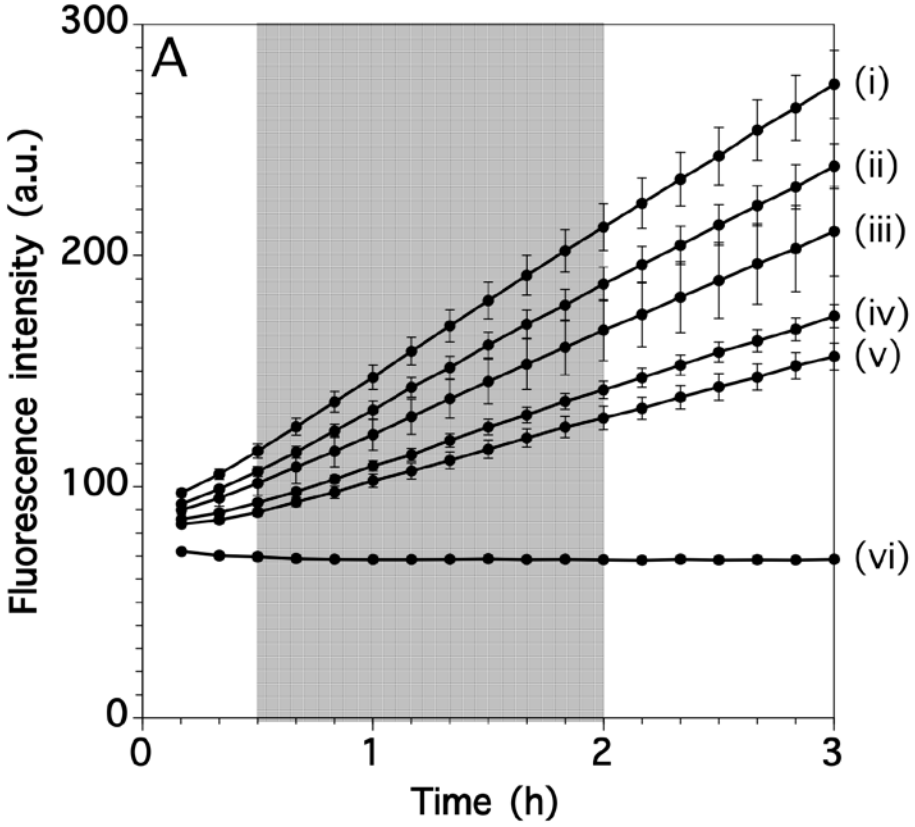
3.2. Potential inhibition assay by components in WWs

To examine potential inhibition of the GUS activity by components in WWs, we added commercial enzyme (GUS) standards (5.9 mg mL^{-1}) to a phosphate buffer solution (1.0 g-P L^{-1}, pH 6.8) and WWs collected from SC in WWTP-A. The GUS activities in the WW were identical and slightly higher than that in the phosphate buffer solution at 5 μg L^{-1} and >10 μg L^{-1}, respectively, of GUS concentration (Fig. S2), indicating that components in WWs do not inhibit GUS activity. Our results suggested that *E. coli* concentrations in WWs could be enumerated by measuring fluorescence intensity without any pre-treatments, similar to river water (Wildeboer et

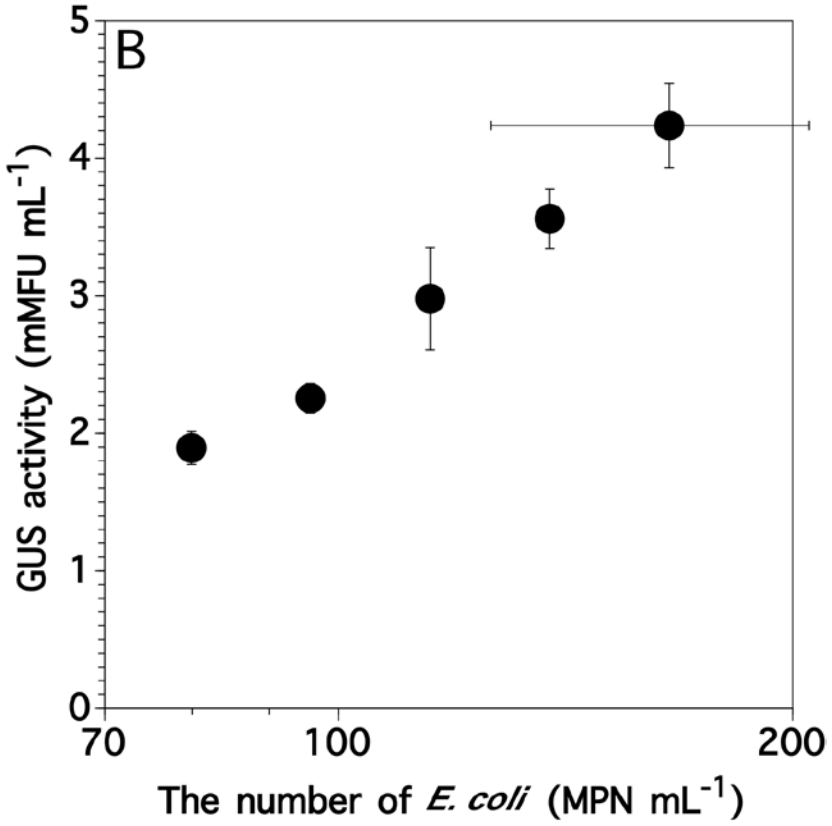
al., 2010), freshwater (George et al., 2000; Hesari et al., 2016) and drinking water (Hesari et al., 2016) samples.

3.3. Correlation between GUS activity and the number of E. coli

To analyze if there is a correlation between substrate utilization rates (i.e., GUS activities) and the initial *E. coli* concentrations in the sample, we serially diluted the WWs, with *E. coli* concentration of 166 ± 39 MPN mL^{-1}, four times by a factor of 1.2 with sterile physiological NaCl solution to obtain five samples. The MU fluorescence intensities of the five samples increased linearly from 0.5 h to 2.0 h after incubation (Fig. 2A). The slopes of serially diluted WWs decreased in the order of the dilution factor. The slope of the blank samples was -0.18 h^{-1}. Based on these results, substrate utilization rates were calculated by subtracting the slope of the blank sample from those of the WWs and converting the unit to GUS activities (mMFU mL^{-1}) (Fig. 2B). There was a strong positive correlation between the number of *E. coli* and the GUS activity of the WW with a correlation coefficient of 0.997. The standard deviation of our method was lower than that of Colilert because a large number of samples (n=10) were analyzed by our method than the Colilert method (n=2 in this study). In addition, the CV of the GUS activity assay was <12.5%, which was lower than that of Colilert (23.8%), implying that our method is more reliable than Colilert. Other studies also reported lower CVs of GUS activity assay than those of Colilert (Burnet et al., 2019). Due to low standard deviation, p-values from t-test for all of two neighboring data plots were <0.0005, indicating that our method can distinguish the difference between 80 MPN mL^{-1} and 96 MPN mL^{-1} of *E. coli* in the WWs with high accuracy and the sensitivity of our method was at least 16 MPN mL^{-1}.


A
Fluorescence intensity (a.u.)
Time (h)
(i)
(ii)
(iii)
(iv)
(v)
(vi)
0
100
200
300
0
1
2
3

332


B
GUS activity (mMFU mL-1)
The number of E. coli (MPN mL-1)
0
1
2
3
4
5
70
100
200

333

Figure 2. (A) Time-course changes in the MU fluorescence intensities in the WWs taken from SC in WWTP-A (i) and serially diluted WWs (ii to v). All samples were incubated in the medium. The samples (ii) to (v) were prepared by serial dilution of the sample (i) by a factor of 1.2 with physiological sodium chloride solution. The sample (vi) was the blank sample (Milli-Q water). Error bars indicate standard deviation (n=10). The gray area indicates the period when GUS activities were calculated. (B) GUS activities calculated based on the slopes of samples (i) to (vi) shown in Panel A. The horizontal and vertical error bars indicate standard deviations of the *E. coli* concentrations at 166 MPN mL^{-1} (n=2) and GUS activities of five samples (n=10), respectively.

To further examine the correlations between GUS activities and *E. coli* concentrations, we analyzed the WWs collected from SCs at three different WWTPs for 10 months (from May 2017 to February 2018). To obtain a wide range of *E. coli* concentrations, we also created spike-in samples by adding a small amount (<1%) of the PC-WWs to the SC-WWs. The GUS activities were highly correlated with the number of *E. coli* in the WWs taken from WWTP-A (Log y = 0.47 Log x -0.53; r = 0.86; p-value = 1.6×10^{-12}) and B (Log y = 0.48 Log x -0.87; r = 0.86; p-value = 4.8×10^{-9}), although correlation was not very strong in the WWs collected from WWTP-C (Log y = 0.24 Log x -0.45; r = 0.44; p-value = 0.04) (Fig. 3). The limit of detection (LoD) of our method was estimated from the following equation, LoD = (10 × σ - intercept) / slope of an equation, where σ is the standard deviations of 10 blank samples, to be 22 MPN mL^{-1} for WWTP-A. Spike-in samples fitted well to the correlation equations, suggesting that background matrix in the WWs such as non-*E. coli* bacteria and organic/inorganic compounds

had no or little interfering effects on the MU fluorescence measurement. This is also supported by the high selectivity of fluorogenic substrate for *E. coli* reported in the previous studies (Briciu-Burghina et al., 2017; Hesari et al., 2016; Wildeboer et al., 2010).

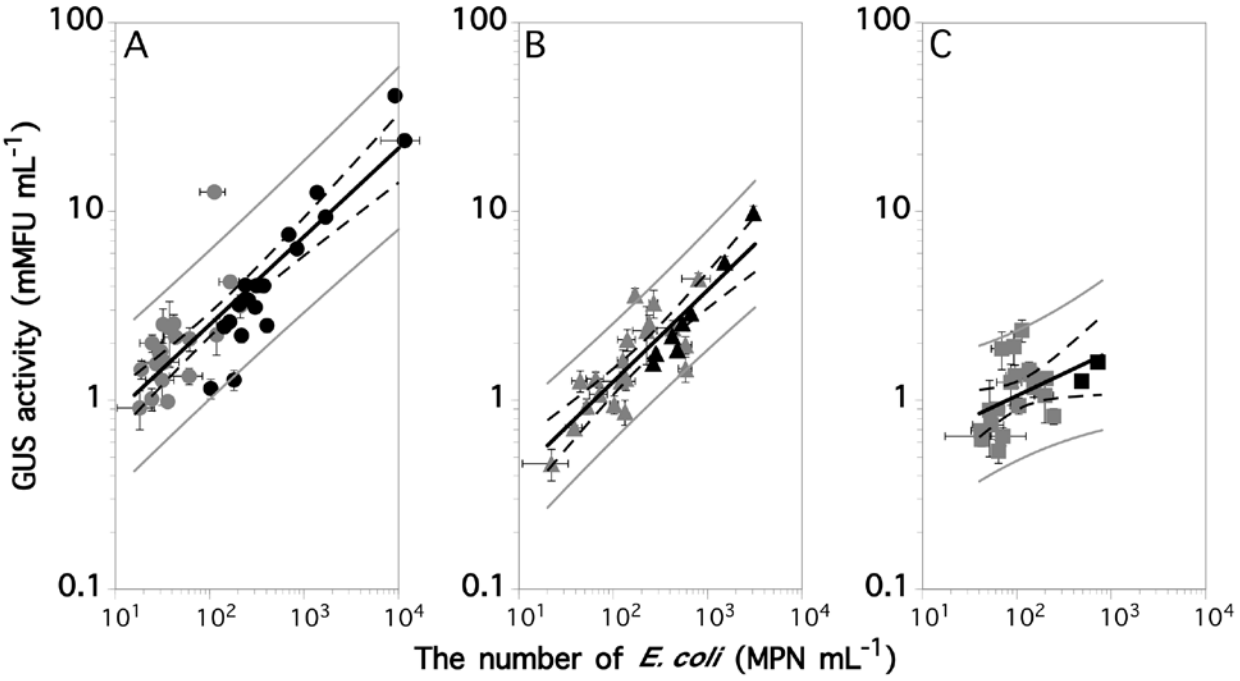


Figure 3. Correlation between GUS activities and the *E. coli* concentrations in the samples collected from SCs (light gray) at WWTP-A (A), WWTP-B (B) and WWTP-C (C) from May 2017 to February 2018. Symbols shown in black indicate the mixture of the WWs collected from SCs with those collected from PCs. Horizontal and vertical error bars indicate standard deviations in the number of *E. coli* (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.

In addition to the tolerance to background matrix, our GUS assay also detected seasonal fluctuations in the *E. coli* concentrations in the WWs. Similar seasonal fluctuations in *E. coli* concentrations were observed between the Colilert method and our GUS assay tested with WWTP-A and -B samples, except for several sampling dates indicated by dotted lines (Fig. S3). The *E. coli* concentrations ranged from 18 to 210 MPN mL^{-1} and from 22 to 800 MPN mL^{-1} in WWTP-A and -B, respectively, from May to February. These results suggested that our GUS assay is useful in quantifying *E. coli* concentrations in WWs. As far as we know, this is the first study showing the seasonal fluctuation in the *E. coli* concentrations by simple methods with enzyme substrates.

It is important to note that correlation equations were different by three WWTPs, and sometimes, not very strong (e.g., WWTP-C), suggesting that relationships were WWTP-specific. However, once a correlation equation is established at individual WWTPs, the same equation could be used over time at the same site.

Low correlation between GUS activities and *E. coli* concentrations in the WWTP-C samples might be due to the narrow range in the *E. coli* concentrations (40-730 MPN mL^{-1}) compared with wide *E. coli* concentration ranges seen in WWTP-A and -B samples (18-11,700 MPN mL^{-1} and 22-3,100 MPN mL^{-1}, respectively). Another possible reason was the weak GUS activity in the WWTP-C samples. The slope of the correlation equation, which is identical to the specific GUS activity of *E. coli*, was smaller (0.24) in the WWTP-C samples than those in the WWTP-A (0.47) and -B (0.48) samples. Therefore, it would be possible that *E. coli* populations in the WWTP-C samples were different from those in the WWTP-A and -B samples, and contained more *E. coli* strains that are negative or weak in GUS activity.

3.4. Analysis of E. coli *populations in the WWs*

To examine the *E. coli* population similarity among WWs collected from WWTP-A, -B, and -C, we analyzed diversity in the *E. coli*-specific *uidA* fragments. A total of 2,235,556 *uidA* sequence reads (17,542 to 147,641 reads per WWs) were obtained from 27 WWs (Table S2). The obtained sequence reads were grouped to 2,060 to 4,224 OTUs per sample by using 99.5% nucleic acid sequence identity as a threshold value. The results of the PCoA analysis showed that the *E. coli* populations obtained from the WWTP-A were clustered together with those from the WWTP-B, although they did not cluster with those from the WWTP-C (Fig. S4). Based on the ANOSIM analysis, *E. coli* populations in the WWTP-C were significantly different (p <0.001) from those in the WWTP-A and -B. Differences in the *E. coli* populations between the WWTP-B and -C samples and between the WWTP-A and -C samples (i.e., R values were 0.4904 and 0.3433, respectively) were greater than those between the WWTP-A and -B (0.1207) (Table S3). Different *E. coli* population structure between WWTPs, as assessed by *uidA* sequence dissimilarity, is interesting but cannot explain the reason for the difference in the GUS activities between WWTPs by itself. However, since proportion of *E. coli* strains that have negative or very weak GUS activity vary by human fecal samples (Chang et al., 1989), the WWTP-C samples likely contain more GUS-negative/weak *E. coli* strains than the WWTP-A and -B samples, which could explain the difference in the GUS activity between WWTPs (Fig. 3).

3.5. Enumeration of E. coli *concentrations in a WWTP at different treatment stages*

To examine the applicability of our GUS assay, we collected WWs from different treatment stages, PC, SC, SF, and Chl, from WWTP-A from June to August 2018. The GUS activities of these WWs were measured, and the *E. coli* concentrations were estimated by using the regression

equation (Log y = 0.47 Log x -0.53) determined based on the data shown in Fig. 3A. The number of *E. coli* in the PC was about 10^4 MPN mL^{-1} and reduced at 2-3 log units in the SC. Removal of *E. coli* in the SF was not significant as compared with a previous study (De Sanctis et al., 2017), probably due to the difference of filter media. The Chl reduced the number of *E. coli* to smaller than 1 MPN mL^{-1}. The results of the PC and SC correlated well to those determined with Colilert (Fig. 4) most likely because the regression equation was determined using the WWs taken from the same PC and SC (Fig 3A). In contrast, the results of the SF underestimated and the Chl overestimated those determined with Colilert. Overestimation might be associated with the survival of GUS present in chlorinated *E. coli* cells, and underestimation might be due to degradation and/or deactivation of GUS present in *E. coli* cells.

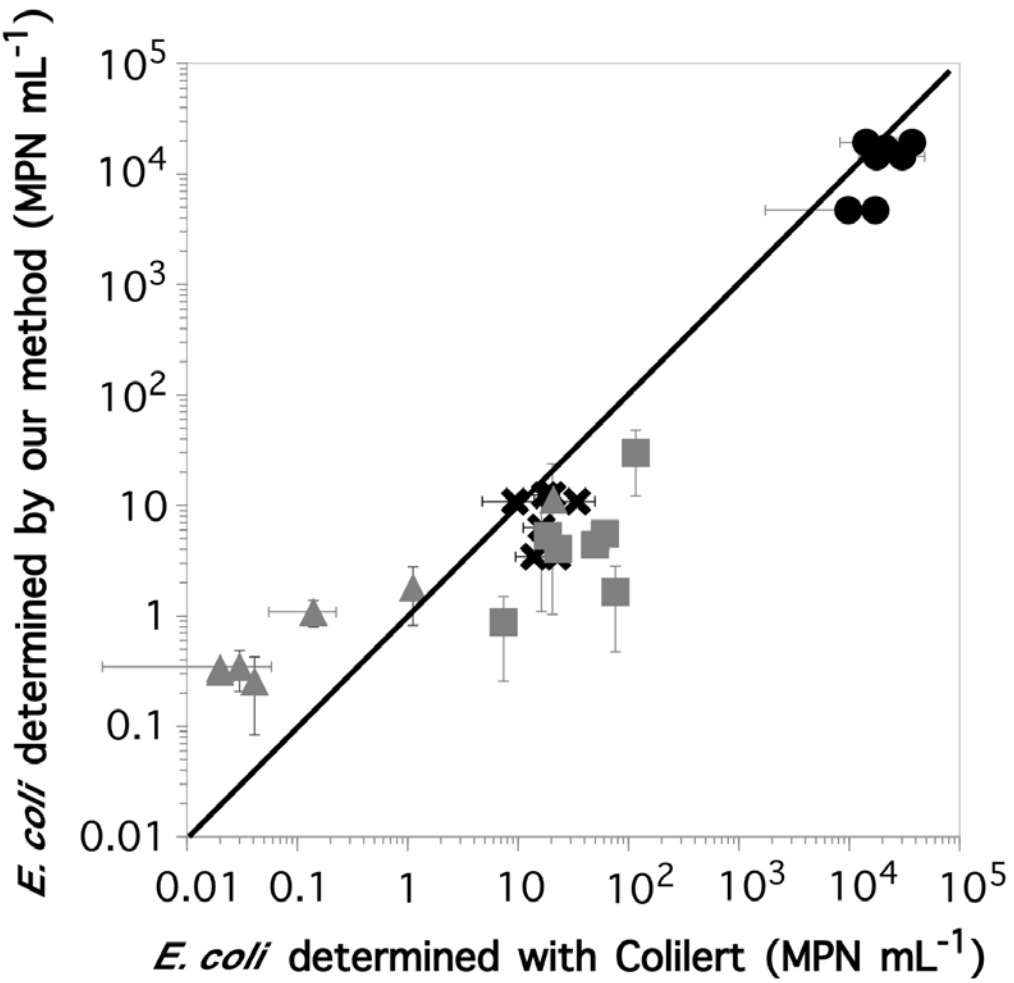


Figure 4. Correlation between the *E. coli* concentrations determined with Colilert (horizontal axis) and those estimated by our method using the regression equation shown in Fig. 3A as the calibration curve (vertical axis). The samples were taken from PC (circles), SC (crosses), SF (squares), and Chl (triangles) at WWTP-A from June to August 2018. Horizontal and vertical error bars indicate standard deviations in the *E. coli* concentrations determined by the Colilert method (n=2) and those estimated by our method (n=10), respectively.

3.6. Advantages and disadvantages of our E. coli *enumeration method*

Advantages of our method include (i) simple protocol, (ii) high throughput, (iii) low cost, (iv) wide detection range, and (v) ability to detect viable-but-non-culturable (VBNC) cells (Table S4).

A noteworthy advantage of our method is the simple procedure as compared with other enumeration methods. It involves only mixing a WW sample with a liquid medium in a microplate. No pretreatment, such as filtration and purification, nor the sequential addition of chemical reagents is required. Determination of fluorescent intensity with a microplate reader in our method effectively eliminated subjective bias and human errors. In contrast, the methods with chromogenic substrates cannot completely avoid the subjective views of assays because the signals are read directly with the naked eye (Gunda et al., 2016; Magro et al., 2014). Our method is simpler than other fluorogenic substrate-based methods, which require addition of NaOH (George et al., 2000; Wildeboer et al., 2010), lysis of *E. coli* cells (Heery et al., 2016), filtration (Briciu-Burghina et al., 2017; George et al., 2000), or cell concentration (Briciu-Burghina et al., 2017; Hesari et al., 2016). Recently, an automated online technology is reported (Burnet et al., 2019). Although we have not implemented automation with our assay protocol, it should not be too difficult to automate our protocol.

Our method can handle a large number of samples (up to 96 samples if no replicate is required) on a single microplate. We can increase the number of replicates to improve the accuracy of the analysis. Small-volume requirement (0.2 mL of WWs) and no need for reagents (e.g., cell lysis reagent and fluorescence enhancer) other than culture medium resulted in much lower running cost (0.02 USD per sample) of our method than the other methods. The running costs of consumable items per sample for the *E. coli* enumeration methods were 3 USD for an autonomous online fluorescence-based technology (Burnet et al., 2019) and <0.5 EUR (~0.5-0.6

USD) for a fluorescence measurement system based on a specific cellular biomarker (Ferrero et al., 2016), which were still cheaper than a growth-dependent method (e.g., 9 USD for IDEXX Colilert and Quanti-Tray/2000) (Burnet et al., 2019) but more expensive than our method. There is, however, a much cheaper method present (2-3 CAD [~1.5-2.3 USD] per 10,000 samples) to enumerate *E. coli* concentrations based on plunger-tube assembly with a hydrogel-based porous matrix (Gunda et al., 2016), although the applicability of this method to WWs is unknown.

The enumeration range of our method is relatively wide (at least 18-11,700 MPN mL^{-1}) as compared with conventional methods (e.g., 30 to 300 CFU per plate in membrane filter method) (Rice et al., 2017). In addition, our method can detect VBNC *E. coli* cells because it is incubation-dependent but culture-independent (Wildeboer et al., 2010).

Enumeration of *E. coli* by our method took up to 2 h, which is much shorter than conventional methods (e.g., 18 h when using IDEXX Colilert-18/Quanti-Tray), but longer than other methods previously reported (Table S4) (Briciu-Burghina et al., 2017; Burnet et al., 2019; Gunda et al., 2016; Heery et al., 2016; Hesari et al., 2016; Wildeboer et al., 2010). We believe that the longer detection time of our method is the offset of simplicity and low-running cost of our method.

We also notice some limitations of our method, including (i) necessity of obtaining calibration curve (i.e., correlation equation between GUS activities and *E. coli* concentration) for each WWTP, (ii) low correlation between GUS activities and *E. coli* concentration for some samples (as seen in WWTP-C in this study), and (iii) requirement of a microplate reader, which is not cheap. There is no universal calibration for our method. Correlation may become weak in WWTPs where *E. coli* concentrations are small, or the GUS activity of the *E. coli* population is weak. Nonetheless, once a suitable calibration curve is established, it can be applied to monitor *E. coli* concentrations over time (Fig 4). Although our protocol used a microplate reader, it can

be done using less expensive instruments. If the simultaneous high-throughput analysis is not necessary, the combined use of a handheld fluorometer and a 37°C heating block allows researchers to estimate *E. coli* concentrations by a simple two-point calibration method at 0 h and 2 h of incubation. This simple method enables on-site measurements of the number of *E. coli* of WWs.

4. Conclusions

A novel, simple (no pretreatment), reliable (as compared with the conventional culture-dependent method), inexpensive (0.02 USD per sample), and high throughput (up to 96 samples per test) method to enumerate *E. coli* concentrations in wastewater samples based on GUS activity measurement was developed herein. The procedure of our method involves only mixing a WW sample (180 µL) with the liquid medium (20 µL) in a microplate and incubating it at 37°C for 2 h. The GUS activity is estimated from a linear increment in fluorescence intensity measured every 10 min. The GUS activity was found even at the very beginning of the GUS activity assay and it was high enough to catalyze MUG to emit detectable levels of the fluorescence signal. Since there was a positive correlation between the number of *E. coli* and the GUS activity in the WW, *E. coli* concentrations could be estimated only by measuring the GUS activities in wastewater samples, although the relationships were WWTP-specific. The difference in the *E. coli* population structures among WWTPs may explain the difference in the correlation equations between GUS activities and *E. coli* concentration. *E. coli* concentrations in WWTPs were successfully monitored over ten months and at different treatment stages (i.e., a secondary clarifier, a sand filtration process, and chlorination process). Our method can distinguish the difference between 80 MPN mL^{-1} and 96 MPN mL^{-1} of *E. coli* in the WWs with high accuracy.

The enumeration range of our method was at least 18-11,700 MPN mL^{-1}. Our method detected seasonal fluctuations in the *E. coli* concentrations in the WWs. The wastewater matrix did not inhibit GUS activity measurement.

The notable advantages of the high throughput method developed here are the simple protocol involved, low cost, wide detection range, and the ability to detect viable-but-non-culturable (VBNC) cells. The procedure of our method involves simply mixing a WW sample with a liquid medium in a microplate. No pretreatment, such as filtration and purification, or the sequential addition of chemical reagents is required. Our method can handle up to 96 samples in a single test. The running cost of our method is very low (0.02 USD per sample) owing to a small sample volume and no need for expensive reagents. The enumeration range of our method is wider than that of conventional methods, enabling us to determine *E. coli* concentrations without dilution of samples. Nevertheless, there are some limitations to this method, including the necessity of obtaining a calibration curve for each WWTP, the low correlation between GUS activities and *E. coli* concentration for some WWTPs, and the requirement of a microplate reader. However, our simple method can serve as an alternative for enumeration of *E. coli* concentrations in wastewater samples to conventional culture-dependent methods, which allows for effective and timely decision-making for action toward proper control of WWTPs and water quality management.

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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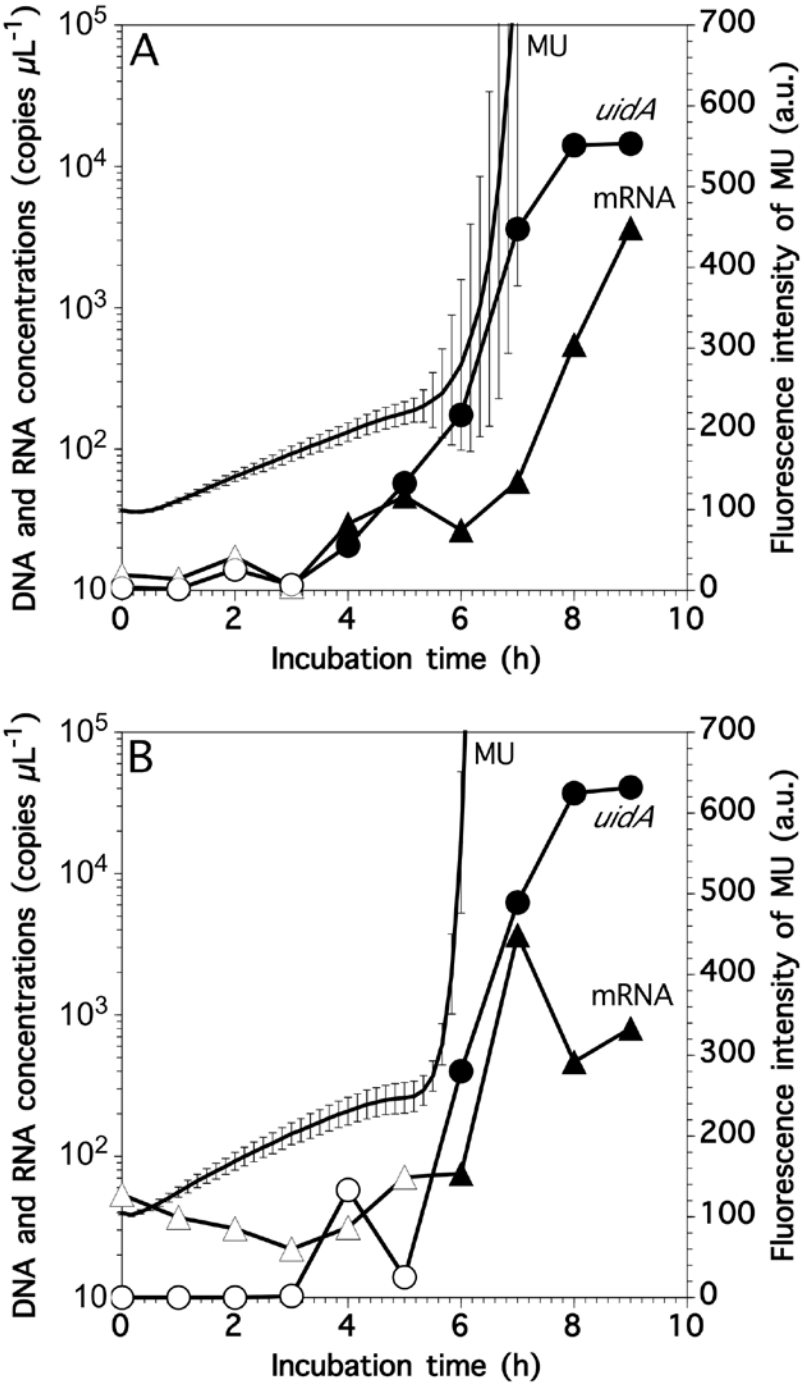

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636 Figure S1. Satoh *et al.*

637 The time-course changes in the copy numbers of *uidA* gene and its mRNA and fluorescence
638 intensity of MU from MUG in WWs taken from SC in WWTP-A in (A) October and (B)
639 November 2017. The samples were incubated in the medium for incubation of *E. coli*. The
640 unfilled plots indicate the values below the detection limit.

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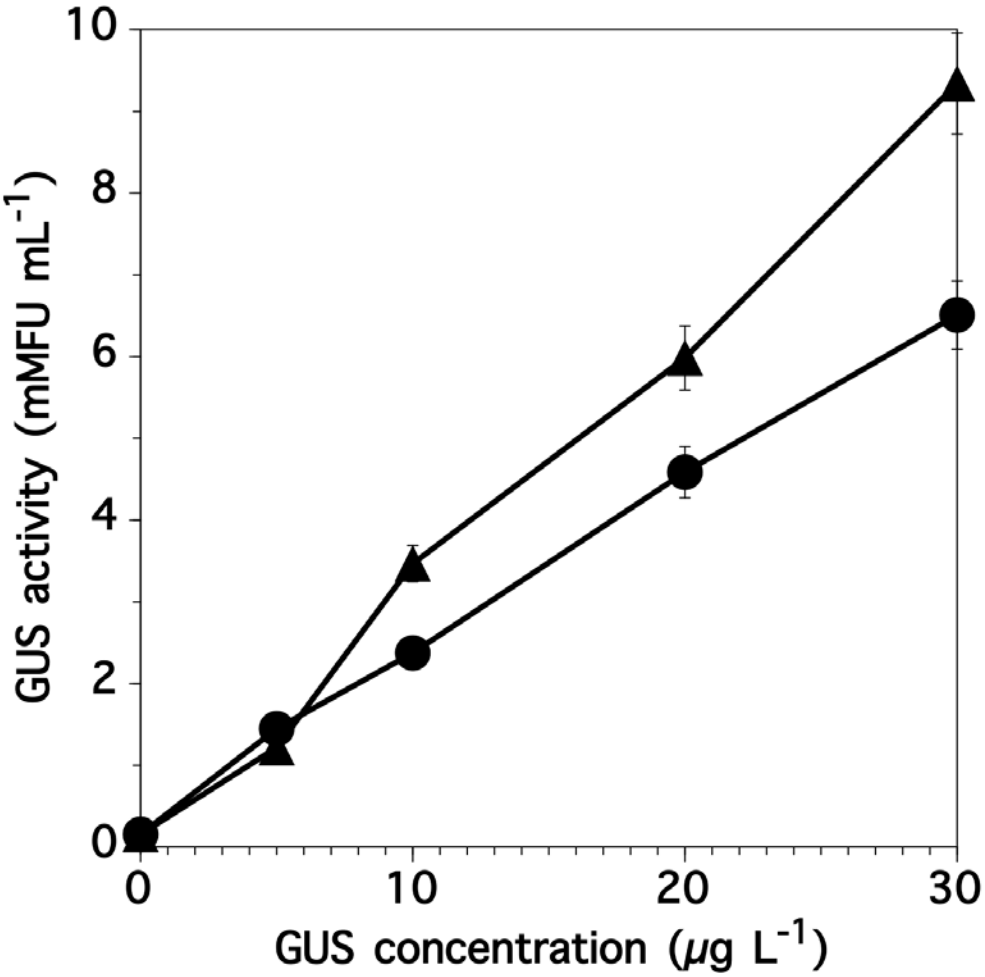

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644 Figure S2. Satoh *et al.*

645 The GUS activities in a phosphate buffer solution (circles) and wastewater (triangles).

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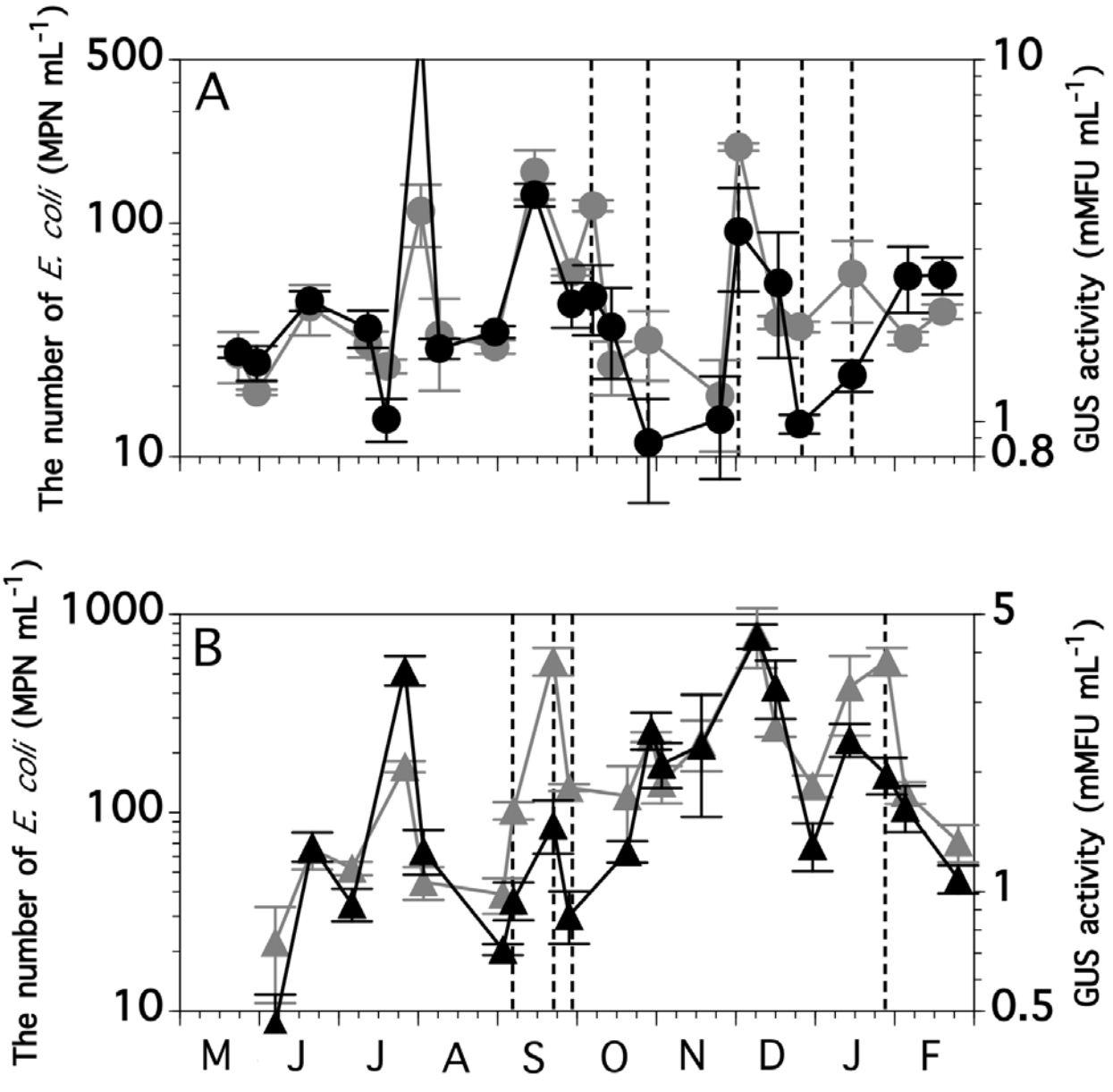

Figure S3. Satoh *et al.*

(A) Seasonal variation in the *E. coli* concentrations (gray plots) and GUS activities (black plots) in the samples collected from SCs at WWTP-A (A) and WWTP-B (B) from May 2017 to February 2018. Error bars indicate standard deviations in *E. coli* concentrations (n=2) and GUS activities (n=10), respectively.

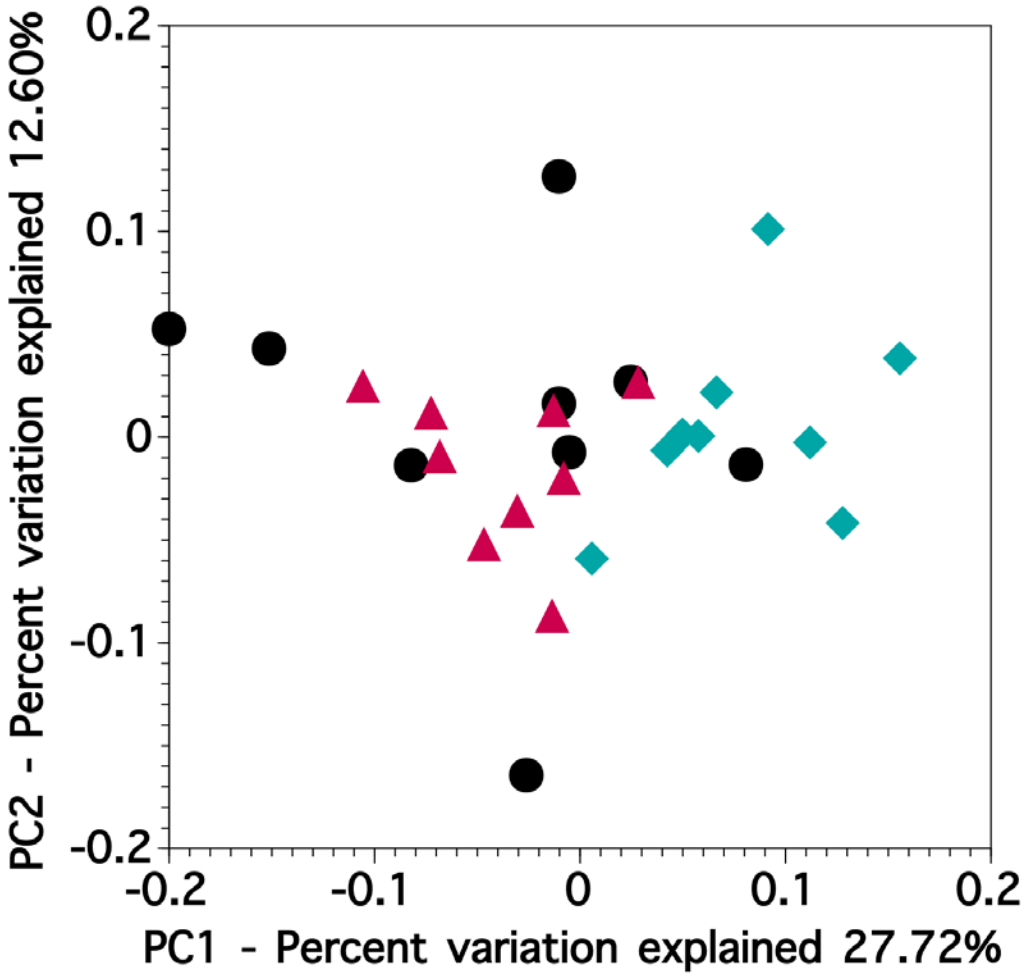

Figure S4. Satoh *et al.*

Principal coordinate analysis (PCoA) of the *E. coli* populations in the WWs collected from WWTP-A (circles), -B (triangles) and -C (diamonds), respectively.

Table S1 Wastewater characteristics (average ± standard deviation) and operating conditions of the wastewater treatment plant (WWTP) A, B and C. All the samples (n = 5) were collected in 2018.

Parameters			WWTP-A	WWTP-B	WWTP-C
Service area (ha)	Combined		2,066	2,905	2,773
	Separate	Wastewater	-	830	2,060
		Rainwater	-	807	2,022
Hydraulic load ($\times 10^3$ m^3/day)			144	238	186
Type of process			Standard activated sludge	Standard activated sludge	Standard activated sludge
Surface load on primary clarifier (m^3/m^2/day)			47	36	26
Activated sludge process					
Hydraulic retention time (with sludge return) (h)			5.9	4.7	4.4
BOD loading (kg/m^3/day)			0.35	0.33	0.23
Surface load on secondary clarifier (m^3/m^2/day)			18	29	25
Water quality					
Suspended solids in the influent (mg/L)			188±8	164±18	202±8
Suspended solids in secondary clarifier (mg/L)			4.2±0.8	8.8±2.3	7.6±2.5
Coliforms in secondary clarifier (CFU/mL)			506±195	298±151	310±115

WWTP: wastewater treatment plant, BOD: biochemical oxygen demand.

Table S2. Community richness, diversity, and evenness indices. All the samples were collected in 2018.

WWTP	Sampling campaign	replicate	read	OTU	Shannon	Simpson	Chao1	Good's coverage
WWTP-A	15-May	n=1	86155	2419	7.0	0.914	9545	81.3%
		n=2	17542	4224	9.0	0.968	26471	63.6%
		n=3	82953	2763	7.4	0.942	10606	78.4%
	22-May	n=1	71944	2361	6.8	0.903	8997	81.9%
		n=2	88515	2651	7.3	0.937	11248	79.3%
		n=3	76913	2383	7.0	0.926	9831	81.6%
	29-May	n=1	112517	2712	7.4	0.939	10848	78.7%
		n=2	118570	2861	7.7	0.949	11289	77.8%
		n=3	108944	2701	7.4	0.942	9845	79.3%
WWTP-B	17-May	n=1	73141	2437	7.0	0.934	9308	81.3%
		n=2	105853	2565	7.2	0.938	9376	80.4%
		n=3	86584	2694	7.3	0.937	11761	78.8%
	24-May	n=1	30002	2718	7.4	0.940	10960	78.6%
		n=2	37748	2487	7.1	0.939	8268	81.2%
		n=3	42615	2532	7.1	0.932	9259	80.5%
	31-May	n=1	34063	2628	7.3	0.937	10604	79.4%
		n=2	88768	2684	7.4	0.939	12001	78.8%
		n=3	48385	2679	7.3	0.937	10656	79.1%
WWTP-C	14-May	n=1	84227	2808	7.5	0.950	11333	77.8%
		n=2	87745	2494	7.1	0.942	9368	80.8%
		n=3	76785	2480	7.1	0.940	9032	81.1%
	21-May	n=1	122366	2060	6.6	0.929	7285	84.6%
		n=2	95264	2293	6.9	0.934	7864	82.6%
		n=3	110960	2449	7.0	0.932	9032	81.4%
	28-May	n=1	147641	2404	7.1	0.938	9261	81.6%
		n=2	100776	2359	6.9	0.934	9274	81.7%
		n=3	98580	2448	7.0	0.940	9492	81.0%

WWTP: wastewater treatment plant, OTU: operational taxonomic unit.

Table S3 Community dissimilarity among the wastewater treatment plant (WWTP) A, B and C as examined by an analysis of similarity statistics (ANOSIM). The values of *R* and *p* indicate community dissimilarity and statistical significance, respectively.

Pairwise comparison	*R* value	*p*-value
WWTP-A − WWTP-B	0.1207	0.026
WWTP-A − WWTP-C	0.3433	0.001
WWTP-B − WWTP-C	0.4904	0.001

Table S4 The comparison of analytical characteristics of our method with other ones in the previous studies.

Reference	Pretreatment	Chemical	Running cost	Detection time
This study	**No need**	**No need**	**0.02 USD**	120 min.
George et al., 2000	Filtration	NaOH	N.D.[a]	**30 min.**
Wildeboer et al., 2010	**No need**	NaOH	N.D.[a]	**30 min.**
Gunda et al., 2016	Filtration Cell concentration	**No need**	**0.0002 USD**	<60min
Heery et al., 2016	Filtration Cell lysis	Lysing agent	N.D.[a]	75 min.
Hesari et al., 2016	Cell concentration	**No need**	N.D.[a]	<120 min.
Briciu-Burghina et al., 2017	Filtration Cell lysis	Lysozyme	N.D.[a]	75 min.
Burnet et al., 2019	**No need**	A washing solution	3 USD	**30 min.**

[a] N.D.: not determined.