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3 Simple and Reliable Enumeration of Escherichia coli Concentrations in  
4 Wastewater Samples by Measuring  $\beta$ -D-glucuronidase (GUS) Activities via a  
5 Microplate Reader

6

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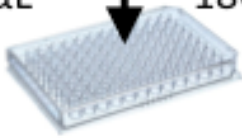
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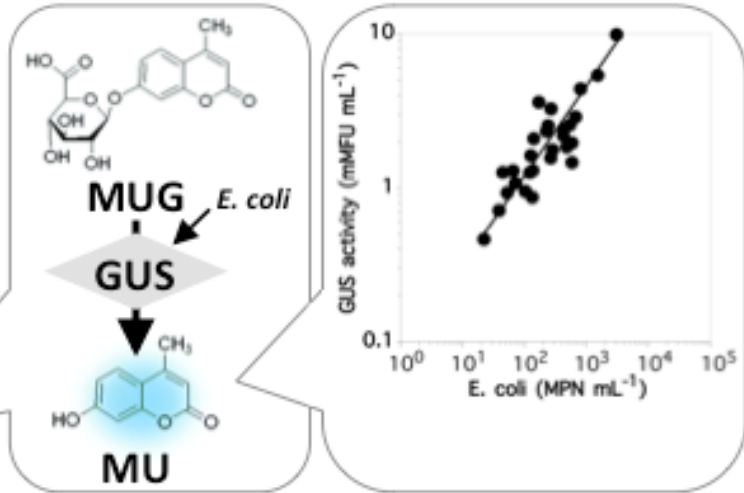
Medium 20  $\mu$ L    Sample 180  $\mu$ L



Microplate



Microplate reader



**Simple:** No pretreatment

**Inexpensive:** 0.02 USD per sample

**Reliable:** Comparable to Colilert

42

43

44

45 **Highlights**

46

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

52

53

54 **ABSTRACT**

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

72

73 **Keywords**

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

76

## 77 **1. Introduction**

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

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

100 methods required pre-treatments, such as removal of contaminants, concentration, fluorescence  
101 enhancer (e.g., NaOH) and/or lysis of *E. coli* cells. Recently, Burnet et al. (2019) developed an  
102 automated online fluorescence-based technology to measure GUS activity for rapid near-real-  
103 time monitoring of *E. coli* in river water that did not require any pretreatment (Burnet et al.,  
104 2019). However, these methods have only been used for river water or drinking water, which are  
105 relatively clean compared with wastewater. Wastewater contains complex matrix, which could  
106 interfere with GUS activity. In addition, production and degradation of GUS in wastewater  
107 samples could strongly affect the accuracy of these methods.

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

117

## 118 **2. Materials and Methods**

### 119 *2.1. Wastewater samples*

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



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

127

## 128 2.2. Quantification of *E. coli* by Colilert method

129 The conventional method for the enumeration of *E. coli* was conducted using Colilert and  
130 Quanti-Tray/2000 (IDEXX Laboratories) according to the manufacturer's instructions. The WWs  
131 were taken and diluted with sterile physiological NaCl solution (0.9% NaCl) in 100-mL sterile  
132 bottles. Each analysis was carried out in duplicate for each ten-fold serial dilution tested. The  
133 coefficient of variation (CV, expressed in %) was calculated by dividing the standard deviation  
134 by the average and multiplying by 100.

135

## 136 2.3. Microplate $\beta$ -D-glucuronidase (*GUS*) assay

137 A simple microplate assay was developed to measure the GUS activity of *E. coli*. The medium  
138 for incubation of *E. coli* was prepared by adding 50 mg of peptone (CAS. No. 73049-73-7), 50  
139 mg of NaCl (CAS. No. 7647-14-5), 10 mg of CH<sub>3</sub>COCOONa (CAS. No. 113-24-6), 10 mg of  
140 KH<sub>2</sub>PO<sub>4</sub> (CAS. No. 7778-77-0), 40 mg of K<sub>2</sub>HPO<sub>4</sub> (CAS. No. 7758-11-4), and 10 mg of KNO<sub>3</sub>  
141 (CAS. No. 7757-79-1), 1.0 mg of CH<sub>3</sub>(CH<sub>2</sub>)<sub>11</sub>OSO<sub>3</sub>Na (sodium dodecyl sulfate, SDS, CAS. No.  
142 151-21-3), 1.0 mg of C<sub>9</sub>H<sub>18</sub>O<sub>5</sub>S (isopropyl  $\beta$ -D-1-thiogalactopyranoside, IPTG, CAS. No. 367-  
143 93-1), and 1.0 mg of 4-methylumbelliferyl- $\beta$ -D-glucuronide (MUG, CAS. No. 6160-80-1) to 1  
144 mL of Milli-Q water (Merck Millipore). SDS was added as a selection agent for gram-negative  
145 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  $\mu\text{L}$ ) of the medium was added to one well of a 96-well microplate  
150 (TPP Techno Plastic Products AG, 92696). Subsequently, 180  $\mu\text{L}$  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  $\text{mL}^{-1}$ ), following the standard Sigma Quality Control Test Procedure  
161 (Sigma Aldrich, 1998). One MFU of GUS activity from *E. coli* liberates 1.0  $\mu\text{g}$  of  
162 phenolphthalein from phenolphthalein  $\beta$ -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  $\beta$ -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  $\mu\text{L}$ ) of WWs were mixed with  
167 200  $\mu\text{L}$  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<sup>6</sup> copies  $\mu\text{L}^{-1}$   
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

214 587 bp. The oligonucleotides were synthesized by Eurofins genomics K.K. (Tokyo, Japan). The  
215 PCR mixture (30  $\mu$ L) contained 1 $\times$  PCR buffer (Takara Bio Inc., Kusatsu, Shiga, Japan), 0.5  $\mu$ M  
216 each primer, 0.2 mM dNTPs, 0.75 U of *Ex* Taq DNA polymerase (Takara Bio Inc., Kusatsu,  
217 Shiga, Japan), and 5 ng DNA template. The PCR thermal conditions were as follows: 35 cycles  
218 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  
219 products were purified using the FastGene Gel/PCR Extraction Kit (Nippon Genetics Co, Ltd.,  
220 Tokyo, Japan), and tagged with sample-specific index sequences at their 5'-end by PCR by using  
221 Nextera XT Index Kit v2 Set A (Illumina, San Diego, CA USA). The PCR mixture (20  $\mu$ L)  
222 contained 1 $\times$  KAPA HiFi HS ReadyMix (Nippon Genetics Co, Ltd., Tokyo, Japan), 1  $\mu$ L each of  
223 forward and reverse index primers, 2  $\mu$ L of the purified PCR products, and 6  $\mu$ L of nuclease-free  
224 water. The PCR was done under the following cycling conditions: 95°C for 3 min; 10 cycles of  
225 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  
226 electrophoresis, amplicons of 600–1,000 bp were excised from the gel and purified using the  
227 MagExtractor-PCR and Gel Clean-up Kit (Toyobo Co, Ltd., Osaka, Japan). Index-tagged  
228 amplicons were pooled and analyzed using a MiSeq paired-end sequencing reaction with the v3  
229 reagent kit (300 cycles) (Illumina, San Diego, CA USA).

230

## 231 2.7. Bioinformatic analysis

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

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

254

#### 255 2.8. Nucleotide sequence accession numbers

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

258

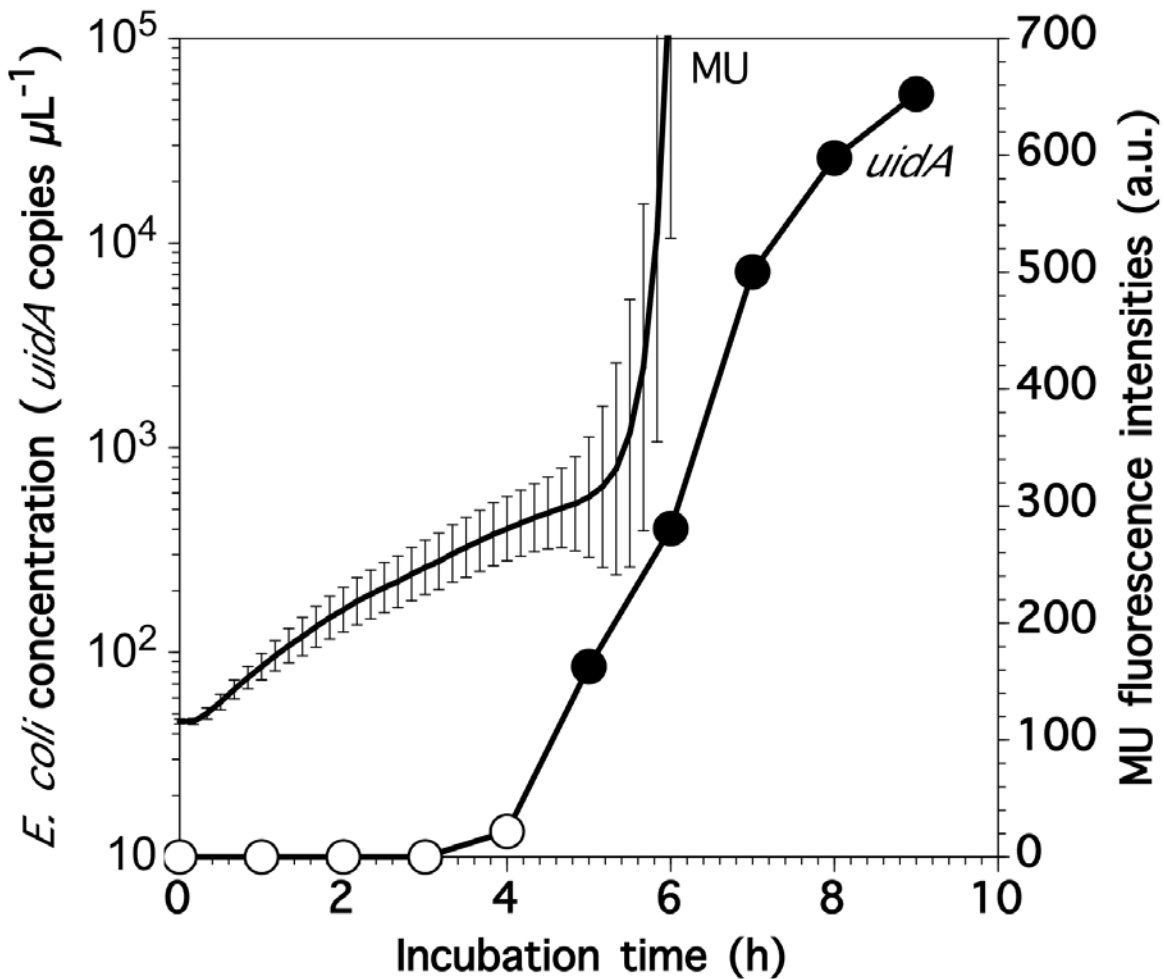
### 259 3. Results and Discussion

260 3.1. Effect of incubation on GUS activity

261 GUS activity measurement has been applied to enumerate *E. coli* in drinking and surface water  
262 samples without incubation to grow *E. coli* (Briciu-Burghina et al., 2017; Burnet et al., 2019;  
263 George et al., 2000; Gunda et al., 2016; Heery et al., 2016; Hesari et al., 2016; Wildeboer et al.,  
264 2010); however, it has not been applied to the analysis of wastewater. Because WWs could  
265 contain potential GUS inhibitors, incubation of samples may be necessary to increase *E. coli*  
266 GUS to reliably measure GUS activity. Therefore, we first analyzed the effect of incubation on  
267 GUS activity and how incubation influenced the quantitative performance of our GUS assay.

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

275



276

277

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

282

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



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

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

300

### 301 3.2. Potential inhibition assay by components in WWs

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

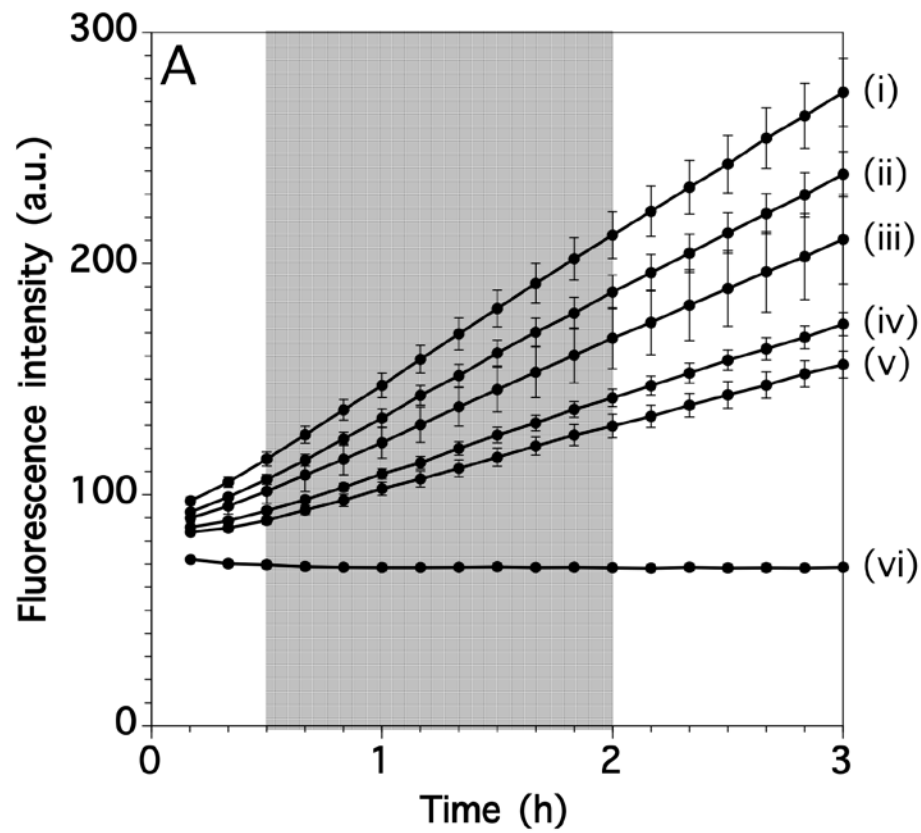
309 al., 2010), freshwater (George et al., 2000; Hesari et al., 2016) and drinking water (Hesari et al.,  
310 2016) samples.

311

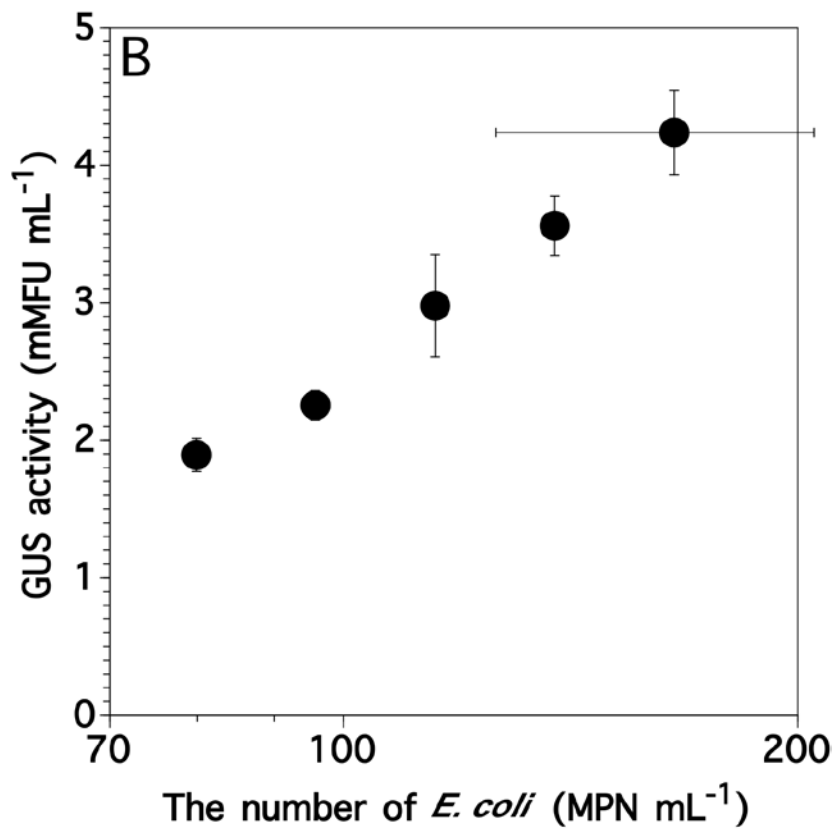
### 312 3.3. Correlation between GUS activity and the number of *E. coli*

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

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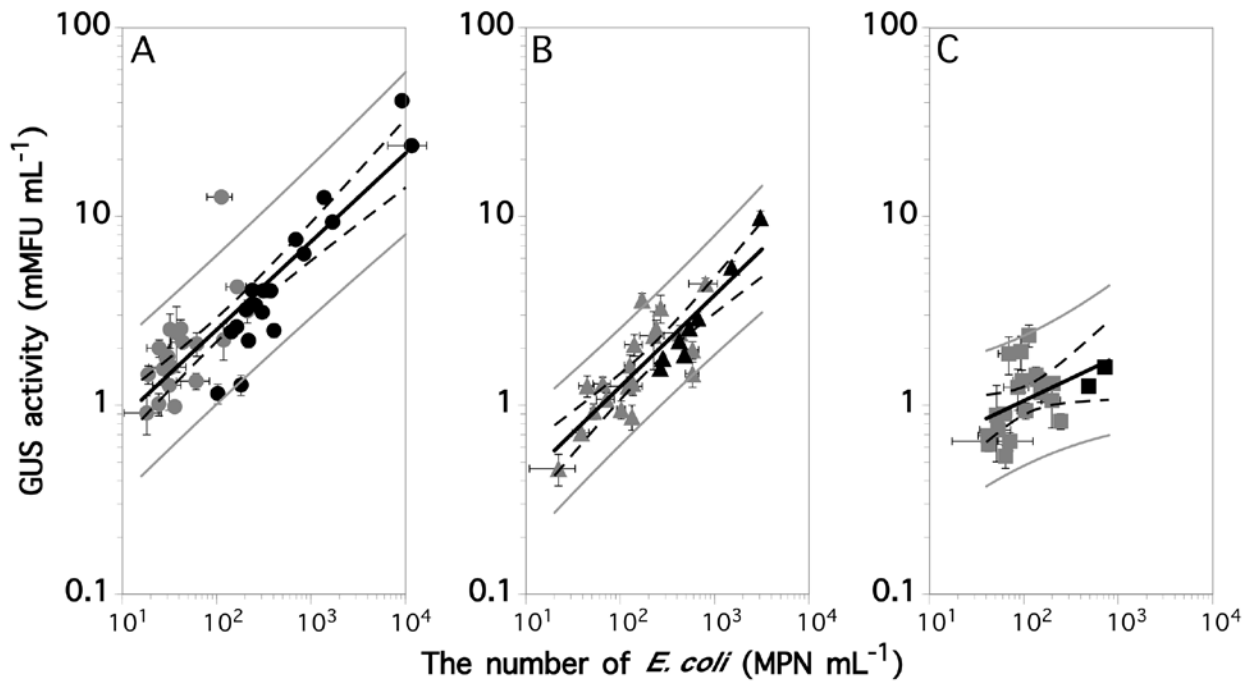
334

335 **Figure 2.** (A) Time-course changes in the MU fluorescence intensities in the WWs taken from  
336 SC in WWTP-A (i) and serially diluted WWs (ii to v). All samples were incubated in the  
337 medium. The samples (ii) to (v) were prepared by serial dilution of the sample (i) by a factor of  
338 1.2 with physiological sodium chloride solution. The sample (vi) was the blank sample (Milli-Q  
339 water). Error bars indicate standard deviation ( $n=10$ ). The gray area indicates the period when  
340 GUS activities were calculated. (B) GUS activities calculated based on the slopes of samples (i)  
341 to (vi) shown in Panel A. The horizontal and vertical error bars indicate standard deviations of  
342 the *E. coli* concentrations at 166 MPN mL<sup>-1</sup> ( $n=2$ ) and GUS activities of five samples ( $n=10$ ),  
343 respectively.

344

345 To further examine the correlations between GUS activities and *E. coli* concentrations,  
346 we analyzed the WWs collected from SCs at three different WWTPs for 10 months (from May  
347 2017 to February 2018). To obtain a wide range of *E. coli* concentrations, we also created spike-  
348 in samples by adding a small amount (<1%) of the PC-WWs to the SC-WWs. The GUS  
349 activities were highly correlated with the number of *E. coli* in the WWs taken from WWTP-A  
350 ( $\text{Log } y = 0.47 \text{ Log } x - 0.53$ ;  $r = 0.86$ ;  $p\text{-value} = 1.6 \times 10^{-12}$ ) and B ( $\text{Log } y = 0.48 \text{ Log } x - 0.87$ ;  $r =$   
351  $0.86$ ;  $p\text{-value} = 4.8 \times 10^{-9}$ ), although correlation was not very strong in the WWs collected from  
352 WWTP-C ( $\text{Log } y = 0.24 \text{ Log } x - 0.45$ ;  $r = 0.44$ ;  $p\text{-value} = 0.04$ ) (Fig. 3). The limit of detection  
353 (LoD) of our method was estimated from the following equation,  $\text{LoD} = (10 \times \sigma - \text{intercept}) /$   
354  $\text{slope of an equation}$ , where  $\sigma$  is the standard deviations of 10 blank samples, to be 22 MPN mL<sup>-1</sup>  
355 for WWTP-A. Spike-in samples fitted well to the correlation equations, suggesting that  
356 background matrix in the WWs such as non-*E. coli* bacteria and organic/inorganic compounds

357 had no or little interfering effects on the MU fluorescence measurement. This is also supported  
358 by the high selectivity of fluorogenic substrate for *E. coli* reported in the previous studies  
359 (Briciu-Burghina et al., 2017; Hesari et al., 2016; Wildeboer et al., 2010).  
360



361  
362  
363 **Figure 3.** Correlation between GUS activities and the *E. coli* concentrations in the samples  
364 collected from SCs (light gray) at WWTP-A (A), WWTP-B (B) and WWTP-C (C) from May  
365 2017 to February 2018. Symbols shown in black indicate the mixture of the WWs collected from  
366 SCs with those collected from PCs. Horizontal and vertical error bars indicate standard  
367 deviations in the number of *E. coli* ( $n=2$ ) and GUS activities ( $n=10$ ), respectively. The solid,  
368 dashed, and gray lines indicate a regression line, 95% confidence interval, and prediction  
369 interval, respectively.  
370

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

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

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

393

394 3.4. Analysis of *E. coli* populations in the WWs

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

412

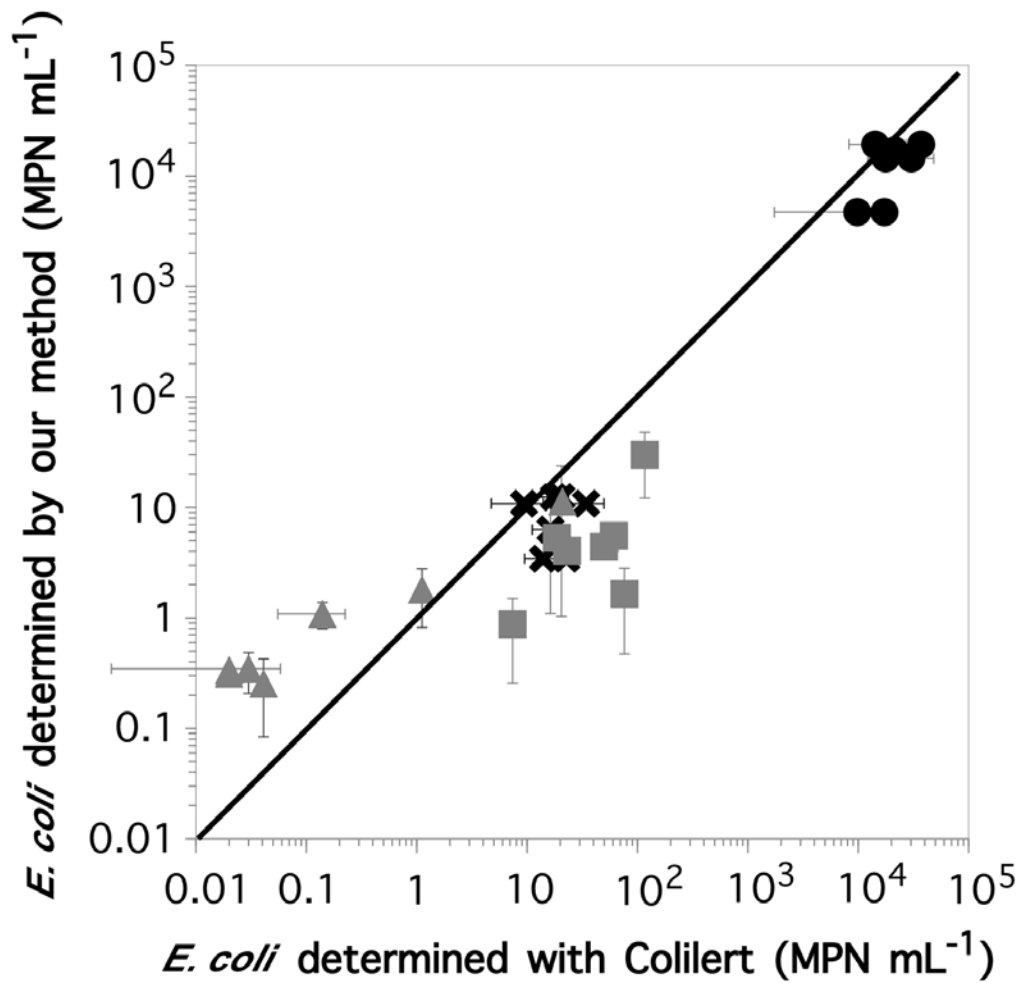
413 3.5. Enumeration of *E. coli* concentrations in a WWTP at different treatment stages

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

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

427





428

429

430 **Figure 4.** Correlation between the *E. coli* concentrations determined with Colilert (horizontal

431 axis) and those estimated by our method using the regression equation shown in Fig. 3A as the

432 calibration curve (vertical axis). The samples were taken from PC (circles), SC (crosses), SF

433 (squares), and Chl (triangles) at WWTP-A from June to August 2018. Horizontal and vertical

434 error bars indicate standard deviations in the *E. coli* concentrations determined by the Colilert

435 method ( $n=2$ ) and those estimated by our method ( $n=10$ ), respectively.

436

437 *3.6. Advantages and disadvantages of our E. coli enumeration method*

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

441 A noteworthy advantage of our method is the simple procedure as compared with other  
442 enumeration methods. It involves only mixing a WW sample with a liquid medium in a  
443 microplate. No pretreatment, such as filtration and purification, nor the sequential addition of  
444 chemical reagents is required. Determination of fluorescent intensity with a microplate reader in  
445 our method effectively eliminated subjective bias and human errors. In contrast, the methods  
446 with chromogenic substrates cannot completely avoid the subjective views of assays because the  
447 signals are read directly with the naked eye (Gunda et al., 2016; Magro et al., 2014). Our method  
448 is simpler than other fluorogenic substrate-based methods, which require addition of NaOH  
449 (George et al., 2000; Wildeboer et al., 2010), lysis of *E. coli* cells (Heery et al., 2016), filtration  
450 (Briciu-Burghina et al., 2017; George et al., 2000), or cell concentration (Briciu-Burghina et al.,  
451 2017; Hesari et al., 2016). Recently, an automated online technology is reported (Burnet et al.,  
452 2019). Although we have not implemented automation with our assay protocol, it should not be  
453 too difficult to automate our protocol.

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

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

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

471 Enumeration of *E. coli* by our method took up to 2 h, which is much shorter than conventional  
472 methods (e.g., 18 h when using IDEXX Colilert-18/Quanti-Tray), but longer than other methods  
473 previously reported (Table S4) (Briciu-Burghina et al., 2017; Burnet et al., 2019; Gunda et al.,  
474 2016; Heery et al., 2016; Hesari et al., 2016; Wildeboer et al., 2010). We believe that the longer  
475 detection time of our method is the offset of simplicity and low-running cost of our method.

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

484 be done using less expensive instruments. If the simultaneous high-throughput analysis is not  
485 necessary, the combined use of a handheld fluorometer and a 37°C heating block allows  
486 researchers to estimate *E. coli* concentrations by a simple two-point calibration method at 0 h and  
487 2 h of incubation. This simple method enables on-site measurements of the number of *E. coli* of  
488 WWs.

489

#### 490 **4. Conclusions**

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

507 The enumeration range of our method was at least 18-11,700 MPN mL<sup>-1</sup>. Our method detected  
508 seasonal fluctuations in the *E. coli* concentrations in the WWs. The wastewater matrix did not  
509 inhibit GUS activity measurement.

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

525

#### 526 **Declaration of interests**

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

529

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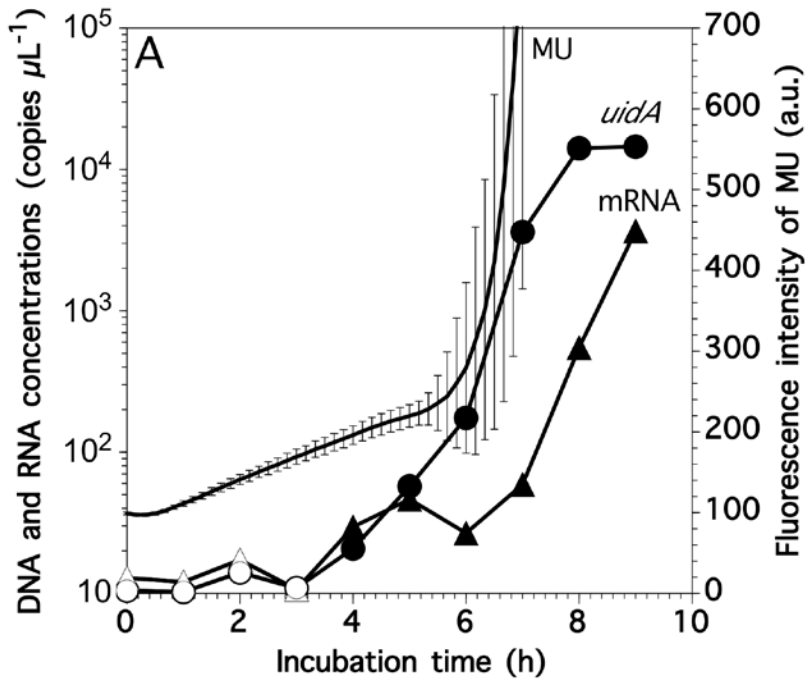
623 Wéry, N., Lhoutellier, C., Ducray, F., Delgenès, J.P., Godon, J.J., 2008. Behaviour of pathogenic  
624 and indicator bacteria during urban wastewater treatment and sludge composting, as  
625 revealed by quantitative PCR. *Water Research* 42, 53–62. doi:10.1016/j.watres.2007.06.048

626 Wildeboer, D., Amirat, L., Price, R.G., Abuknesha, R.A., 2010. Rapid detection of *Escherichia*  
627 *coli* in water using a hand-held fluorescence detector. *Water Research* 44, 2621–2628.  
628 doi:10.1016/j.watres.2010.01.020

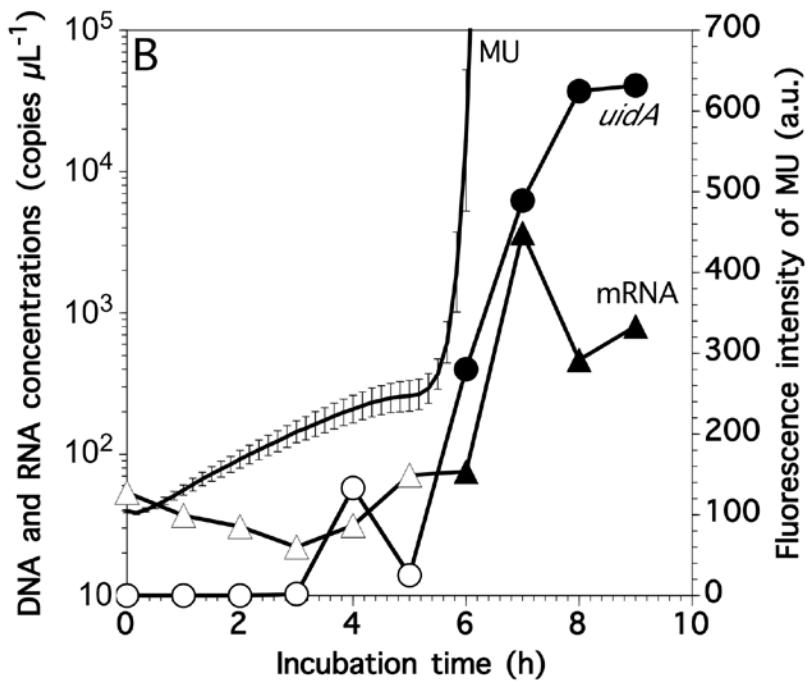
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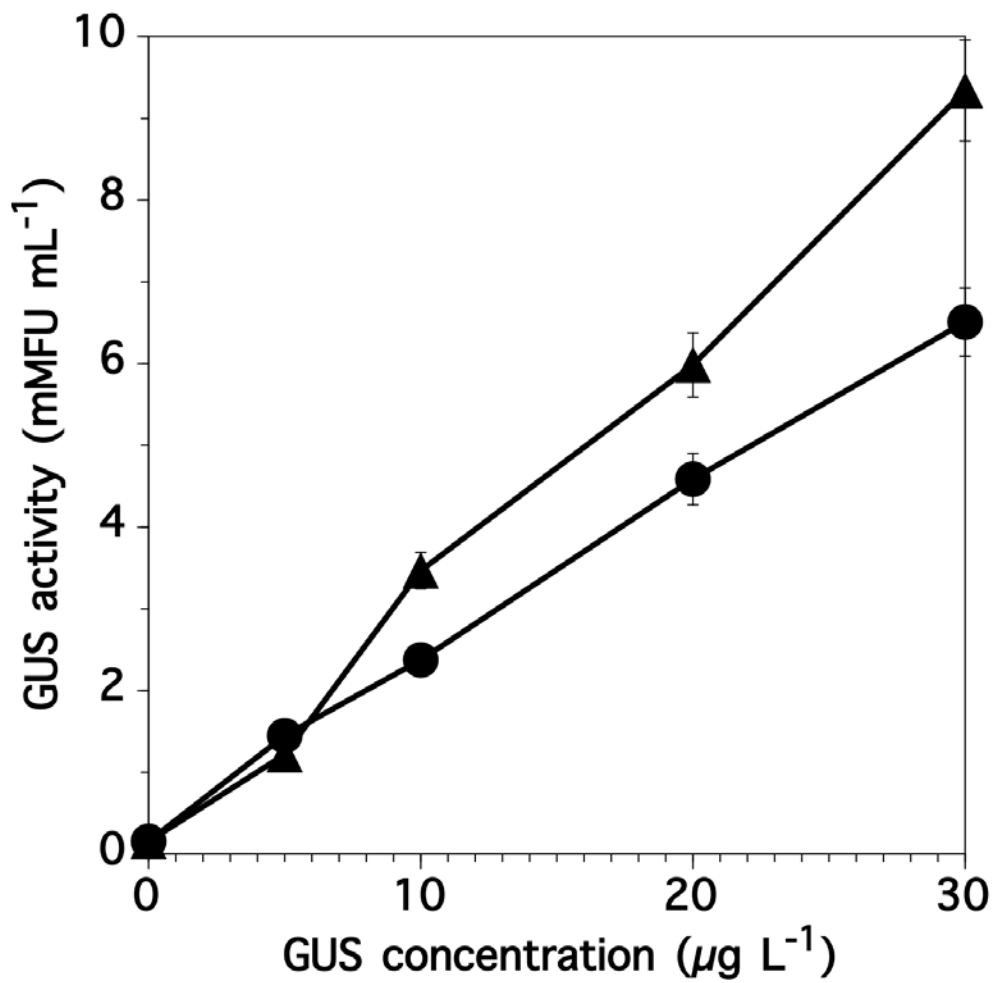
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636 Figure S1. Satoh *et al.*

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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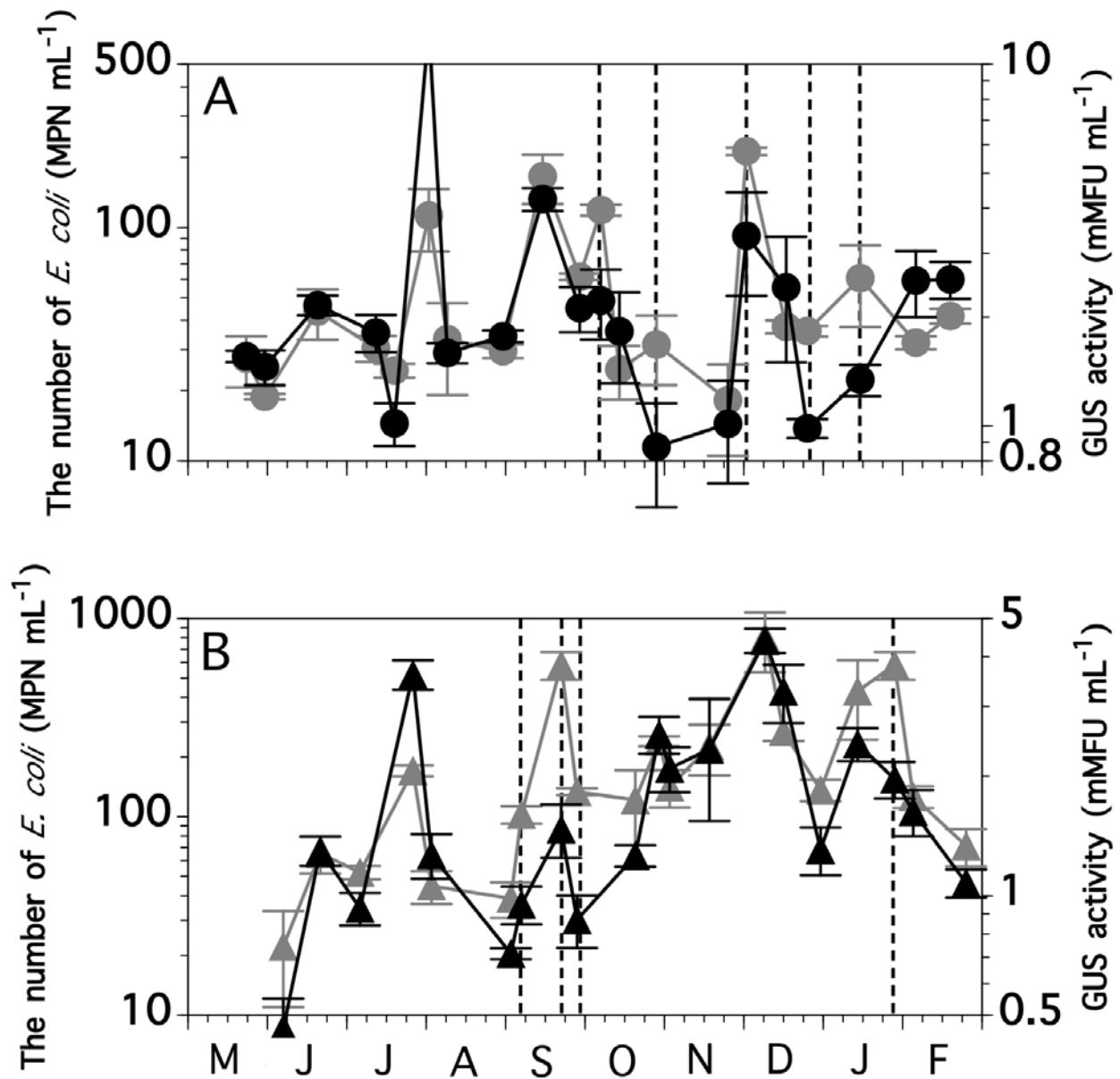
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644 Figure S2. Satoh *et al.*

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

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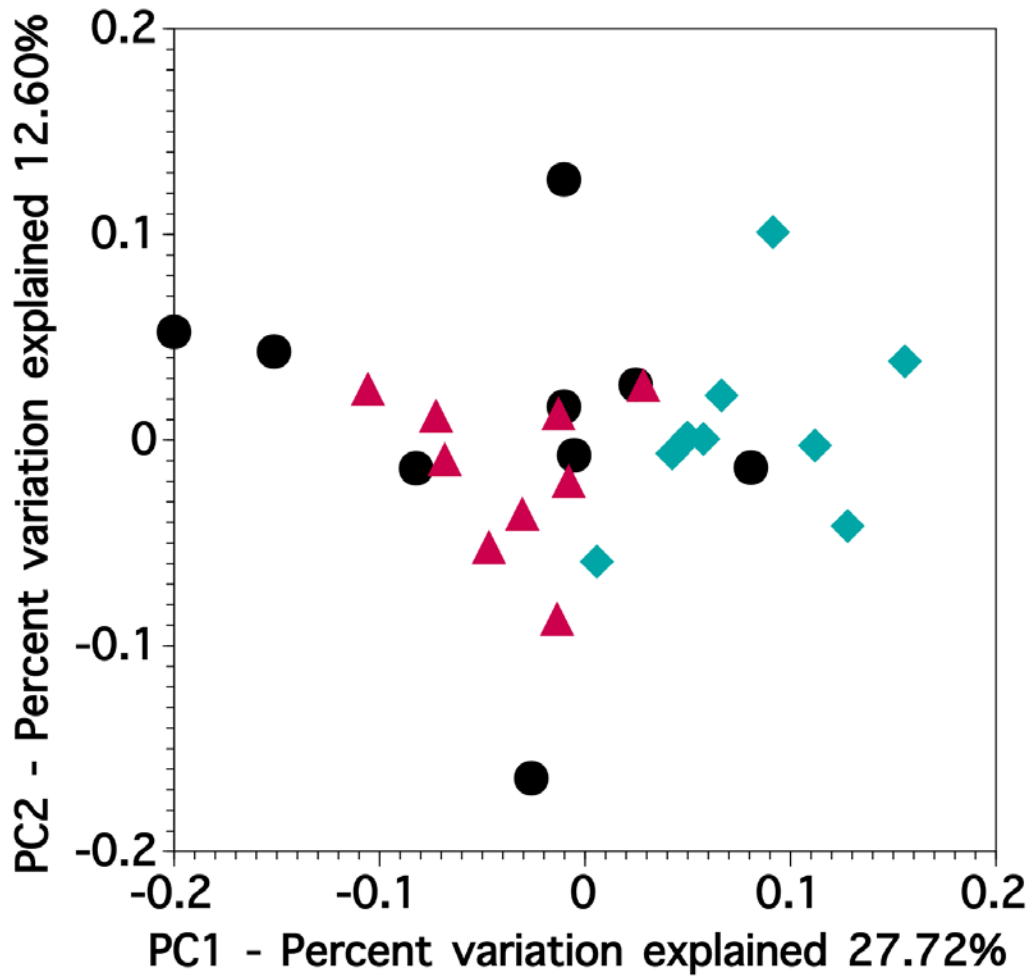
649 Figure S3. Satoh *et al.*

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

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658 Figure S4. Satoh *et al.*

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

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**Table S1** Wastewater characteristics (average  $\pm$  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 <sup>3</sup> /day)		144	238	186	
Type of process		Standard activated sludge	Standard activated sludge	Standard activated sludge	
Surface load on primary clarifier (m <sup>3</sup> /m <sup>2</sup> /day)		47	36	26	
Activated sludge process					
Hydraulic retention time (with sludge return) (h)		5.9	4.7	4.4	
BOD loading (kg/m <sup>3</sup> /day)		0.35	0.33	0.23	
Surface load on secondary clarifier (m <sup>3</sup> /m <sup>2</sup> /day)		18	29	25	
Water quality					
Suspended solids in the influent (mg/L)		188 $\pm$ 8	164 $\pm$ 18	202 $\pm$ 8	
Suspended solids in secondary clarifier (mg/L)		4.2 $\pm$ 0.8	8.8 $\pm$ 2.3	7.6 $\pm$ 2.5	
Coliforms in secondary clarifier (CFU/mL)		506 $\pm$ 195	298 $\pm$ 151	310 $\pm$ 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	<b>No need</b>	<b>No need</b>	<b>0.02 USD</b>	120 min.
George et al., 2000	Filtration	NaOH	N.D. <sup>a</sup>	<b>30 min.</b>
Wildeboer et al., 2010	<b>No need</b>	NaOH	N.D. <sup>a</sup>	<b>30 min.</b>
Gunda et al., 2016	Filtration Cell concentration	<b>No need</b>	<b>0.0002 USD</b>	<60min
Heery et al., 2016	Filtration Cell lysis	Lysing agent	N.D. <sup>a</sup>	75 min.
Hesari et al., 2016	Cell concentration	<b>No need</b>	N.D. <sup>a</sup>	<120 min.
Briciu-Burghina et al., 2017	Filtration Cell lysis	Lysozyme	N.D. <sup>a</sup>	75 min.
Burnet et al., 2019	<b>No need</b>	A washing solution	3 USD	<b>30 min.</b>

<sup>a</sup> N.D.: not determined.