**Changes of microbial substrate metabolic patterns through a
wastewater reuse process, including WWTP and SAT
concerning depth**

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19 **Abstract**

20 In this study, changes of microbial substrate metabolic patterns by BIOLOG assay were
21 discussed through a sequential wastewater reuse process, which includes activated
22 sludge and treated effluent in wastewater treatment plant and soil aquifer treatment
23 (SAT), especially focusing on the surface sand layer in conjunction with the vadose
24 zone, concerning sand depth. A SAT pilot-scale reactor, in which the height of packed
25 sand was 237 cm (vadose zone: 17 cm and saturated zone 220 cm), was operated and
26 fed continuously by discharged anaerobic-anoxic-oxic (A2O) treated water. Continuous
27 water quality measurements over a period of 10 months indicated that the treatment
28 performance of the reactor, such as 83.2% dissolved organic carbon removal, appeared
29 to be stable. Core sampling was conducted for the surface sand to a 30 cm depth, and
30 the sample was divided into six 5 cm sections. Microbial activities, as evaluated by
31 fluorescein diacetate, sharply decreased with increasing distance from the surface of the
32 30 cm core sample, which included significant decreases only 5 cm from the top surface.
33 A similar microbial metabolic pattern containing a high degree of carbohydrates was
34 obtained among the activated sludge, A2O treated water (influent to the SAT reactor)
35 and the 0–5 cm layer of sand. Meanwhile, the 10–30 cm sand core layers showed
36 dramatically different metabolic patterns containing a high degree of carboxylic acid
37 and esters, and it is possible that the metabolic pattern exhibited by the 5–10 cm layer is
38 at a midpoint of the changing pattern. This suggests that the removal of different organic

39 compounds by biodegradation would be expected to occur in the activated sludge and in
40 the SAT sand layers immediately below 5 cm from the top surface. It is possible that
41 changes in the composition of the organic matter and/or transit of the limiting factor for
42 microbial activities from carbon to phosphorus might have contributed to the observed
43 dramatic changes in SAT metabolic patterns.

44

45

46 **Keywords**

47 Wastewater reuse, Soil aquifer treatment, Surface sand layer, Activated sludge,
48 BIOLOG assay, Microbial substrate metabolic pattern

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59 **1. Introduction**

60 Inadequate water supply and deterioration of water quality are serious problems in
61 many parts of the world. These problems are attributed to population growth in urban
62 areas, contamination of surface water and groundwater, uneven distribution of water
63 sources and frequent droughts because of extreme global weather patterns (Asano and
64 Cotruvo, 2004). Wastewater reuse is one of the realistic solutions to the abovementioned
65 problems.

66
67 Soil aquifer treatment (SAT) offers advantages such as low cost, underground storage of
68 reclaimed water and the potential for water quality improvement through infiltration
69 (Asano and Cotruvo, 2004; Asano et al., 2007). Actual wastewater reuse systems using
70 SAT include the ‘Groundwater Replenishment System’ in California and the
71 ‘Sweetwater Recharge Facilities’ in Arizona (USA) and the ‘Dan Region Sewage
72 Reclamation Project’ in Israel (Chalmers and Patel, 2013; Oren et al., 2007; Quanrud et
73 al., 2003). Reclaimed water has been used for such applications as potable and irrigation
74 water.

75
76 It is widely known that water replenishment using the water reuse systems strongly
77 depends on microbial decomposition, including not only WWTP but also SAT (Drewes
78 and Fox, 1999; Xue et al., 2009; Zhang et al., 2012). A lot of past studies focused on

79 microbial transformation processes in WWTP (e.g. Xue et al. 2010; Al-Mutairi, 2009;
80 Guckert, et al., 1996) and SAT (Alfreider et al., 1997; Kolehmainen et al., 2009;
81 Långmark et al., 2004; Zhang et al., 2012), respectively. Moreover, the transformation
82 processes would change in WWTP and SAT, which consists of sequential water reuse
83 that replenishes the reused water qualities. However, there were few studies that focused
84 on the changes in the microbial processes in WWTP and SAT from the viewpoint of
85 WWTP and SAT being a sequential water reuse process.

86
87 Microbial transformation processes in SAT were characterized by the conversion rate of
88 certain chemicals by microbes (Alfreider et al., 1997; Långmark et al., 2004),
89 extracellular enzyme activities (Kolehmainen et al., 2009) and the range of used
90 substances using the BIOLOG assay (Zhang et al., 2012). These studies analysed the
91 changes in the transformation processes throughout the entire SAT system, and samples,
92 including water and soil, were collected at discrete distances. In addition, the removal of
93 organic matter in SAT occurred in the soil surface layer including the vadose zone
94 (Drewes and Fox, 1999; Quanrud et al., 1996; Quanrud et al., 2003; Zhang et al., 2012).
95 Therefore, dynamic changes in the microbial transformation processes are expected in
96 these layers. However, this is also not well understood (Schütz et al., 2010).

97
98 The BIOLOG assay is simple and yields a great deal of information for the

99 characterization of microbial transformation processes (Campbell et al., 1997; Salomo
100 et al., 2009). A single substrate, redox dye tetrazolium violet and nutrients are supplied
101 in well plates. The colour production from the reduction of tetrazolium violet is used as
102 an indicator of the metabolism of the substrates. Moreover, the microbial transformation
103 processes are characterized on the basis of the substrate metabolic pattern (Garland and
104 Mills, 1991; Salomo et al., 2009). The BIOLOG assay has been used in different media,
105 collected from different locations, including natural soil (Campbell et al., 1997; Garland
106 and Mills, 1991), soil from constructed wetlands and SAT (Salomo et al., 2009; Weber
107 and Legge, 2011; Zhang et al., 2012), freshwater (Garland and Mills, 1991) and
108 activated sludge (Al-Mutairi, 2009; Guckert, et al., 1996) to compare and discuss the
109 substrate metabolic patterns.

110

111 Based on the above-mentioned background, treated wastewater was continuously
112 discharged to a SAT pilot-scale reactor in an actual WWTP, and the following objectives
113 were considered using the BIOLOG assay: (1) to comprehend the changes in microbial
114 transformation processes through a sequential wastewater reuse process, including
115 activated sludge and treated effluent in WWTP and SAT, looking at the soil depth,
116 surface layer and the vadose zone, (2) to discuss relations between changes in the
117 processes and water quality parameters. In addition, Hazen et al. (1991) determined that
118 the number of bacteria in sediment (1.00×10^6 to 5.01×10^8 bacteria/g dry) was much

119 higher than that in the adjacent groundwater (1.00×10^3 to 6.31×10^4 bacteria/mL).

120 Therefore, microbes in SAT soil were evaluated in this study.

121

122

123 **2. Materials and methods**

124 **2.1. Pilot-scale reactor**

125 A stainless steel pilot-scale reactor was set in an actual wastewater treatment plant in

126 Kyoto Prefecture, and a diagram of the reactor is shown in Figure 1. The reactor was

127 cuboidal and its width, depth and height were 150, 150 and 300 cm, respectively

128 (volume: $6.75 \times 10^6 \text{ cm}^3$).

129

130 Sand, which was collected in Shiga Prefecture, was purchased and packed in the reactor,

131 and its characteristics are listed in Table A1. The reactor operations began on 20

132 October 2011. The height of the packed sand was initially 250 cm, but the surface sank

133 immediately after the initiation of operations. The height of the sand surface appeared to

134 be stable after approximately 1 month at 237 cm. A final effluent port was set at an

135 elevation of 220 cm from the bottom of the plant. Therefore, the thicknesses of the

136 vadose zone and saturated zone in the reactor were 17 and 220 cm, respectively. The

137 reactor also had two side ports at depths of 87 (Port 1) and 187 cm (Port 2), respectively,

138 from the sand surface to collect water and sand samples at the given depths.

139 The influent water to the reactor was effluent of an anaerobic-anoxic-oxic (A2O)
140 process, which is mainly used to treat domestic wastewater. The influent to the SAT
141 reactor was collected by a hose attached to a 0.05 cm mesh from a 100cm depth in the
142 final sedimentation tank before chlorination. The hydraulic retention time (HRT) of the
143 A2O process was 2.8 h in the anaerobic tank, 2.8 h in the anoxic tank and 5.6 h in the
144 aerobic tank, and the solid retention time (SRT) was 9.5 days.

145

146 First, the influent to the SAT reactor was piped into a storage tank made of polyethylene
147 and exposed to the atmosphere in the tank. And then, it was continuously pumped into
148 the reactor through a PTMG tube (Aoi, Japan). The Influent to the SAT reactor was
149 dripped using a final port, which was located at a height of 60 cm from the centre of the
150 sand surface. The influent to the SAT reactor was continuously discharged without
151 interruption.

152

153 The HRT was set at 30 days, and the inflow rate was 70 L/day.

154

155 **2.2. Continuous water quality measurement**

156 Water samples of the influent to the SAT reactor and effluents from Port 1, Port 2 and
157 the final effluent port were collected once every two weeks, in principal, from 4
158 November 2012 to 6 September 2013. The measured water quality parameters included

159 pH (n = 26), dissolved oxygen (DO) (n = 26), dissolved organic carbon (DOC) (n = 29),
160 UV₂₅₄ (n = 29), nitrogen species (dissolved total nitrogen [DTN], NH₄⁺, NO₂⁻, NO₃⁻
161 and organic N [as the difference between DTN and total inorganic N]) (n = 20),
162 dissolved total phosphorus (DTP) (n = 20), as well as water temperature (n = 126) of the
163 influent to the SAT reactor and the final effluent from the SAT reactor. With respect to
164 the influent to the SAT reactor, a sample for each parameter, except pH and DO, was
165 collected just after the final port, which was located at a height of 60 cm from the centre
166 of the sand surface, whereas the sample for pH and DO was collected in the storage tank
167 to prevent DO from increasing during sampling. Prior to the measurement of the water
168 qualities, except for pH and DO, the water samples had been filtered by GF/B with a
169 pore size of 1 µm (Whatman, USA).

170

171 The pH and DO were measured by D-52 and D-55 (Horiba, Japan) multi-parameter
172 metres, respectively. The DOC and UV₂₅₄ were measured by a TOC-L analyzer
173 (Shimadzu, Japan) and a Multi-Spec-1500S spectrophotometer (Shimadzu, Japan),
174 respectively. The specific ultraviolet absorbance (SUVA) was calculated by dividing the
175 UV₂₅₄ value by the DOC. DTN and DTP were measured by an AACS-II auto-analyzer
176 (Bran+Luebbe, Germany). The nitrogen component NH₄⁺ were measured by an AA-II
177 auto-analyzer (Bran+Luebbe, Germany), and NO₂⁻ and NO₃⁻ were measured by an
178 AA-III auto-analyzer (Bran+Luebbe, Germany). Water temperature was measured by a

179 HC-763 detector (TOA-DKK, Japan).

180

181 The effluents from Port 1, Port 2 and the final effluent port are referred to as effluent
182 (87 cm) from the SAT reactor, effluent (187 cm) from the SAT reactor and final effluent
183 (237 cm) from the SAT reactor, respectively, in this study.

184

185 **2.3. Evaluation of microbial substrate metabolic patterns**

186 **2.3.1. Sampling scheme**

187 Samples, including the water and sand samples described below, were collected four
188 times in the summer for reproducibility.

189

190 Activated sludge from the aerobic tank of the A2O process, influent to the SAT reactor
191 and sand samples from the SAT reactor were collected on 29 July and 5, 12 and 19
192 August 2013. The water temperatures at each sampling event were similar, ranging from
193 27.3 to 30.9°C.

194

195 The activated sludge was collected with a plastic ladle and preserved in a sterilized
196 polypropylene (PP) vial (Vioramo, AS ONE, Japan). The collected samples were used
197 for BIOLOG assay, fluorescein diacetate (FDA) assay and for the water quality
198 measurements, described below.

199 The influent to the SAT reactor was collected using the PP vial for BIOLOG assay and
200 FDA assay and glass bottles for the water quality measurements.

201

202 Core sampling was conducted to collect the surface sand down to a 30 cm depth using a
203 liner sampler (DIK-110C, Daiki Rika Kogyo, Japan) with a 5 cm diameter and 30 cm
204 height. The liner sampler and its sampling tube were sterilized at 250°C for 2 h and
205 using 10 mg-NaHOCl/L overnight, respectively. The 150 cm×150 cm area of the sand
206 surface was divided into 10 cm×10 cm sections, and the 30 cm depth sand core sample
207 was collected at a grid point. The four sample collections were conducted at different
208 grid points within 15 cm from the position of the final port.

209

210 The sand core sample was divided into six segments of 5 cm lengths (denoted as the
211 0–5 cm layer, 5–10 cm layer, 10–15 cm layer, 15–20 cm layer, 20–25cm layer and
212 25–30 cm layer) in the laboratory, and each layer was collected in a sterilized glass
213 beaker. The sand was well mixed with sterilized stainless spoons in the beaker and used
214 as sand samples. The beakers and spoons were sterilized at 250°C for 2 h in advance.

215

216 A 30-cm-deep hole was also created by the liner sampler at a point that was located 70
217 cm from the position of the final port, and a water sample taken from an approximately
218 25 cm depth from the top surface (denoted as effluent (25 cm) from the SAT reactor)

219 was collected with a PP syringe and tubes and stored in the PP vial. This sampling was
220 conducted five times from 12 August to 26 August 2013.

221

222 Sand and water samples were collected from Port 1 and Port 2 with PP vials. After the
223 sand mixed with the treated water, which flowed away from Port 1 and Port 2, was left
224 to rest for 30 sec, the supernatant was decanted to another PP vial, and the resulting
225 sand and supernatant were used as sand and water samples, respectively. The sand and
226 water samples from Port 1 were denoted as 87 cm layer and effluent (87 cm) from the
227 SAT reactor, respectively, and the sand and water samples from Port 2 were denoted as
228 187 cm layer and effluent (187 cm) from the SAT reactor, respectively.

229

230 **2.3.2. TOC measurement in sand**

231 The total organic carbon (TOC) of each collected sand sample was measured using a
232 SSM-5000A solid sample combustion unit (Shimadzu, Japan).

233

234 **2.3.3. FDA assay**

235 Several enzymes produced by microbes, such as protease, lipase and esterase, can
236 catalyse the transformation of fluorescein diacetate (3',6'-diacetylfluorescein (FDA)) to
237 fluorescein, and the FDA assay was used to assess the total microbiological activity
238 (Ichikawa et al., 2002; Schnürer and Rosswall, 1982; Weber and Legge, 2011). FDA

239 assay was applied to the activated sludge, influent to the SAT reactor and each sand
240 layer sample, in reference to Ichikawa et al., (2002) and Schnürer and Rosswall, (1982).
241
242 A 20 mL volume of 60 mM sterile sodium phosphate buffer (pH 7.6) was added to each
243 5 g-wet sand sample, 1 mL activated sludge and 5 mL influent to the SAT reactor. After
244 addition of 0.3 mL of FDA solution (2 mg FDA and 2 mL acetone), the samples were
245 shaken for 1 h. The amount of FDA to be added was determined based on the amount
246 sufficient for microbes in the 5 g-wet sample of the 0–5 cm sand layer, whose activity
247 was the highest. Immediately after shaking for 1 h, the sample was filtered using a No.1
248 filter (Advantec, Japan) followed by a polytetrafluoroethylene (PTFE) membrane filter
249 with a pore size of 0.2 μ m (Advantec, Japan). The amount of fluorescein was measured
250 as the absorbance at 490 nm by the Multi-Spec-1500S. The same process using samples
251 sterilized by autoclave at 120°C for 20 min was repeated for use as blank samples, and
252 the absorbance of the sterile sample was subtracted. A strong relationship was found
253 between the microbial amount and the absorbance ($y=0.0192$, $R^2=0.995$: x =g-wet of
254 sand, y =absorbance) using 1, 2, 3, 4 and 5 g-wet of the sand samples taken from a 1 cm
255 depth from the top surface.

256

257 **2.3.4. BIOLOG assay with EcoPlate**

258 EcoPlate (Biolog Inc. U.S.) was used to evaluate and compare microbial substrate

259 metabolic patterns among the samples.

260

261 The activated sludge and influent to the SAT reactor were diluted 100 and 10 times with
262 the 60 mM sterile sodium phosphate buffer, respectively, and 150 μ L of the diluted
263 samples were inoculated to the plates.

264

265 The suspension of microbes in the sand was conducted with a Waring blender (Ace
266 homogenizer AM-3, Nihonseiki, Japan). A mixture of 10 g-wet of each sand sample and
267 40 mL of the phosphate buffer were blended in the blender for 3 min. The homogenate
268 was centrifuged for 1 min at 1500 rpm (KUBOTA 5200, Kubota, Japan) to remove the
269 sand particles, and 150 μ L of the supernatant was inoculated to the plate.

270

271 EcoPlates were incubated at 25°C in an incubator (IS-41, Yamato, Japan) for 7 days,
272 and wet papers were set at the bottom of the incubator to prevent dryness.

273

274 Absorbance was measured using a Powerscan-PC (DS Pharma Biomedical, Japan) at
275 590 nm after the inoculation of the plates every 3 h during the first period of 5 days and
276 6 h during the last 2 days. The absorbance was also measured every 1.5 h during the
277 first period for samples, in which the absorbance rapidly increased.

278

279 The absorbance data were corrected by subtraction of the blank well data at each
280 sampling time. Average well colour development (AWCD) was calculated for each
281 sample at each time, and the absorbance data in each well was normalized by dividing
282 by the AWCD to compensate for the influences of inoculum density (Garland, 1996;
283 Rutgers et al., 2008).

284

285 Salomo et al., (2009) determined microbial substrate metabolic patterns from a
286 constructed wetland using the data at AWCD = 0.2 when the substrate utilization rates
287 were situated in the transition from lag phase to exponential phase. In this study, the
288 normalized absorbance data for each sample at each sampling date with AWCD = 0.2
289 was analysed by principal component analysis to evaluate and compare microbial
290 substrate metabolic patterns among the samples.

291

292 It is known that bacteria are one component of biomass in activated sludge and soil
293 (Thawornchaisit and Pakulanan, 2007; Wardle, 1992). Therefore, BIOLOG assay was
294 also applied to the activated sludge with different dilution levels to ensure that
295 normalization by the AWCD compensated for the influences of inoculum densities.
296 Nippon Steel & Sumikin Eco-Tech (Japan) measured 16S rRNA copies by real-time
297 PCR for the activated sludge, influent to the SAT reactor and the phosphate buffer after
298 the extraction of each sand layer on 19 August (DNA extraction with Extrap Soil DNA

299 Kit Plus ver. 2 [Nippon Steel & Sumikin Eco-Tech, Japan] and quantification with
300 PicoGreen® dsDNA assay kit [Invitrogen, US]). There were two digit differences in the
301 copy numbers among the samples (Table 1). Therefore, activated sludge samples diluted
302 10, 100 and 1000 times were assayed and analysed by principal component analysis
303 with the other samples on 19 August.

304

305 **2.3.5. Water quality measurements**

306 The pH and DO (except for effluent (25 cm) from the SAT reactor) and DOC, UV₂₅₄,
307 nitrogen species and DTP after filtration with GF/B were measured for each water
308 sample. The measurement methods were the same as described in Section 2.2. In
309 addition, fluorescence spectra were collected for the filtered samples on 12 August. The
310 samples were diluted to 0.7 mgC/L and adjusted to pH 7. To obtain excitation–emission
311 matrix (EEM) profiles, excitation wavelengths were incremented from 220 to 400 nm in
312 5 nm steps, and for each excitation wavelength, the emission was detected from 240 to
313 500 nm in 5 nm steps. Fluorescence of super purified water as a blank sample was
314 subtracted from each spectrum.

315

316 Suspended solid (SS): 105°C for 2 h and volatile suspended solid (VSS): 600°C for 30
317 min were measured for the activated sludge and treated wastewater (Japan Sewage
318 Works Association, 1997).

319 **2.4. Statistical analysis**

320 The Wilcoxon rank sum test was used to examine the statistical significance of the
321 measured sand and water quality indexes between two samples at subsequent points
322 along the flow path, such as the influent to the SAT reactor vs. effluent (25 cm) from the
323 SAT reactor and 0–5 cm layer vs. 5–10 cm layer. A significance level of 0.05 was used
324 for all tests.

325

326

327 **3. Results and discussion**

328 **3.1. Temporal changes in water qualities**

329 Temporal changes of the water qualities of each water sample are shown in Figure2-1
330 and 2-2. The distribution of each quality is arranged in order of the influent to the SAT
331 reactor, effluent (87 cm) from the SAT reactor, effluent (187 cm) from the SAT reactor
332 and final effluent (237 cm) from the SAT reactor unless otherwise noted, and median,
333 minimum and maximum values for each index are formatted as median
334 (minimum–maximum) in this section.

335

336 The pH varied 6.58 (6.16–7.67), 6.70 (5.88–7.03), 5.79 (5.31–6.52) and 5.64
337 (5.28–6.03), respectively. There were no significant differences between the influent to
338 the SAT reactor and effluent (87 cm) from the SAT reactor ($p>0.05$). Meanwhile, pH

339 significantly decreased from effluent (87 cm) from the SAT reactor to effluent (187 cm)
340 from the SAT reactor and from effluent (187 cm) from the SAT reactor to final effluent
341 (237 cm) from the SAT reactor ($p<0.05$). There were no noticeable seasonal changes in
342 each water sample.

343

344 When 10 g-wet sterilized sand was shaken with 40 mL of sterile influent to the SAT
345 reactor for 12 h, the pH in the influent to the SAT reactor was found to decrease from
346 7.34 to 6.16. Based on this result, the decrease of pH in the plant was partially caused
347 by the original sand characteristics, such as ion exchanges.

348

349 The DO ranged 3.36 (1.12–6.70), 7.87 (3.32–10.6), 4.38 (1.28–9.18) and 4.52
350 (1.30–7.56) mgO₂/L, respectively. The DO of the effluent (87 cm) from the SAT reactor
351 was significantly higher than that of the influent to the SAT reactor ($p<0.05$), and the
352 increase can be partially attributed to the contact of the influent to the SAT reactor with
353 air trapped in the sand voids of the vadose zone. DO significantly decreased thorough
354 percolation from the 87 to 187 cm depth ($p<0.05$). Meanwhile, there were no significant
355 differences in DO between the effluent (187 cm) from the SAT reactor and final effluent
356 (237 cm) from the SAT reactor ($p>0.05$).

357

358 Figure 2-1 also shows that DO in each effluent adversely increased and decreased

359 against the water temperature of the final effluent (237 cm) from the SAT reactor.
360 Therefore, changes in the saturated oxygen concentration of water because of the
361 temperature might influence the DO of each effluent.

362

363 Based on the results, DO in each effluent mostly exceeds 2 mgO₂/L, and it was
364 determined that the entire reactor operated under aerobic conditions.

365

366 The distribution of DOC was 3.91 (2.85–4.55), 1.02 (0.781–1.57), 0.734 (0.560–1.32)
367 and 0.658 (0.482–0.870) mgC/L, respectively, and DOC significantly decreased in each
368 effluent through the percolation path in the reactor ($p<0.05$). In comparison with the
369 median values of the influent to the SAT reactor, DOC removal percentages were 73.7%,
370 81.3% and 83.2%. Drewes and Fox (1999) obtained 76% DOC removal (from 11.5
371 mgC/L to 2.8 mgC/L) for the secondary effluent after 21 days percolation. In addition,
372 Zhang et al., (2012) obtained 79% DOC removal (from 4.31 mgC/L to 0.91 mgC/L) for
373 ozonated secondary effluent after 28.8 days retention. The DOC removal of the final
374 effluent (237 cm) from the SAT reactor in the present study is similar to these values
375 over a similar HRT. DOC was slightly low in the influent to the SAT reactor in the
376 summer season from June to September. Meanwhile, there were no noticeable seasonal
377 changes in each effluent through the sampling period.

378

379 UV₂₅₄ ranged 0.088 (0.046–0.128), 0.027 (0.008–0.062), 0.024 (0.008–0.048) and 0.023
380 (0.005–0.052) cm⁻¹, respectively. There was a significant decrease from the influent to
381 the SAT reactor to the effluent (87 cm) from the SAT reactor ($p<0.05$), which indicate
382 the removal of the aromatic components through 87 cm percolation from the top surface.
383 Meanwhile, there were no significant differences between effluent (87 cm) from the
384 SAT reactor and effluent (187 cm) from the SAT reactor and effluent (187 cm) from the
385 SAT reactor and final effluent (237 cm) from the SAT reactor ($p>0.05$). There were no
386 noticeable seasonal changes in each water sample through the sampling period.

387
388 The distribution of SUVA was 0.022 (0.013–0.035), 0.025 (0.0078–0.061), 0.030
389 (0.0099–0.065) and 0.033 (0.0077–0.079) L/(mgC·cm). There was a significant increase
390 from the influent to the SAT reactor to the effluent (87 cm) from the SAT reactor
391 ($p<0.05$). Therefore, it was found that non-aromatic components were preferentially
392 removed during organic matter removal through 87 cm percolation. Meanwhile, there
393 were no significant differences below the 87 cm depth ($p>0.05$). There were no
394 noticeable seasonal changes in each water sample.

395
396 DTN ranged 4.58 (3.36–7.50), 4.62 (3.56–8.14), 4.43 (2.90–6.83) and 4.69 (3.79–6.29)
397 mgN/L, respectively, and no significant differences were observed among the water
398 samples ($p>0.05$). In addition, using median values, NO₃⁻ accounted for 78.3%, 92.3%,

399 96.6% and 88.9% of DTN in each water sample. DTN was comparatively high in each
400 water sample during the winter season from December to March.

401

402 The distribution of DTP was 0.206 (0.090–1.13) mgP/L in the influent to the SAT
403 reactor. Meanwhile, it was not detected (<0.002 mgP/L) in each effluent. DTP was high
404 in the influent to the SAT reactor during the summer season from June to September. Ak
405 and Gunduz (2013b) showed that PO_4^{3-} precipitated in the soil surface. PO_4^{3-} accounted
406 for $56 \pm 21\%$ (average $\pm$ standard deviation, $n=34$) in DTP in the influent to the SAT
407 reactor (Kyoto City Waterworks Bureau); therefore, the PO_4^{3-} precipitation might have
408 contributed to the decrease in DTP in the SAT reactor.

409

410 The median mole ratio C:N:P of the influent to the SAT reactor during the period from
411 June to September, when DOC decreased and DTP increased, was 100:129:4.02, while
412 for all other periods it was 100:115:12.7. Therefore, it was determined that carbon was
413 the limiting factor for microbial activities in the influent to the SAT reactor, despite the
414 observed decrease and increase. Meanwhile, the median C:N:P ratio was
415 100:483:<0.196, 100:634:<0.273 and 100:734:<0.304 for effluent (87 cm) from the SAT
416 reactor, effluent (187 cm) from the SAT reactor and final effluent (237 cm) from the
417 SAT reactor, respectively, through the entire period, and was determined that
418 phosphorus was the limiting element.

419 Based on these results, it was determined that the treated effluents from the SAT reactor
420 exhibited no noticeable seasonal variations, except for slightly high DTN in the winter
421 season, and the treatment performance appeared to be stable under aerobic conditions,
422 including the sampling period for the analysis of the metabolic patterns.

423

424 **3.2. Changes of microbial substrate metabolic patterns**

425 **3.2.1. TOC in the sand along the flow path**

426 Ranges of TOC for all sand samples are listed in Table 1. Median TOC in the 0–25 cm
427 layers was high in comparison with a TOC of 0.0094% in the sand before reactor
428 packing (Table A1). In addition, TOC in the 0–5 cm layer was significantly higher than
429 that of the 5–10 cm layer ($p<0.05$). This indicates that microbial growth and organic
430 adsorption occurs in the layers through operation, especially in the 0–5 cm layer. The 87
431 cm and 187 cm layers exhibited lower TOC values than that before packing, which
432 indicates that organic matter and microbes originally adsorbed were washed out by
433 percolation of the treated water cleaned by the above layers.

434

435 **3.2.2. Microbial activities by FDA assay**

436 The results of FDA assay are listed in Table 1. The microbial activity of the activated
437 sludge and the 0–5 cm layer was significantly higher than that of the influent to the SAT
438 reactor ($p<0.05$). The maximum weight of water in the 0–5 cm layer sample was 0.16 g

439 in a 1 g-wet sand sample, and the absorbance was below 0.005 g^{-1} in the influent to the
440 SAT reactor, based on a water density of 1 g/mL . Therefore, under conditions where the
441 bulk water in the 0–5 cm layer sample was the influent to the SAT reactor, the microbial
442 activity of the influent to the SAT reactor could be calculated as less than 0.0008 cm^{-1}
443 in 1 g-wet of sand. Meanwhile, the minimum absorbance of the 0–5 cm layer was 0.044
444 $\text{cm}^{-1} \cdot \text{g-wet}^{-1}$. Therefore, the sample should contain some active microbes which were
445 directly discharged from the influent to the SAT reactor into the 0–5 cm layer. However,
446 the assay determined that this contribution to the overall activity in the 0–5 cm layer
447 was very small (less than $1/50$).

448
449 The SAT microbial activity sharply decreased over the flow path, and this corresponds
450 to the TOC trend. In comparison to the median absorbance of the 0–5 cm layer, the
451 absorbance of the 5–10 cm layer was 47% thereof and significantly low ($p < 0.05$). In
452 addition, low absorbance was obtained in the 10–15 cm layer (13%) and 15–20 cm layer
453 (5.6%).

454

455 **3.2.3. Microbial substrate metabolic pattern**

456 Figure A1 shows the AWCD of each sample at each sampling date. AWCD in activated
457 sludge, influent to the SAT reactor and 0–5 cm layer increased more rapidly than the
458 other samples.

459 Figure 3 shows the results of the principal component analysis. In addition, Table A2
460 shows the principal component loading rate of each substrate, and the 31 substrates
461 were classified based on their structure, in reference to previous reports (Salomo et al.,
462 2009; Zhang et al., 2012).

463

464 The contribution ratio of the first and second principal component ranged from 61.3%
465 to 71.6% and from 12.3% to 14.9%, respectively. The first principal component
466 exhibited much in common with substrates whose loading rate exceeded 0.5 (Sala et al.,
467 2005) among the sampling dates. Data plots of microbes with substantial metabolism
468 for the following substrates are located in the positive direction on the first principal
469 component axis: carboxylic acids (4-hydroxy benzoic acid, itaconic acid and
470 γ -hydroxybutyric acid), amino acids (L-asparagine, L-phenylalanine and L-threonine),
471 polymers (Tween 40 and Tween 80) and esters (pyruvic acid methyl ester). Meanwhile,
472 those with substantial metabolism for the following substrates are located in the
473 negative direction: carbohydrates (D-cellobiose, D-mannitol,
474 N-acetyl-D-glucosamine, α -D-lactose and β -methyl-D-glucoside), amino acids (L-serine),
475 polymers (glycogen) and phosphorylated chemicals (DL- α -glycerol phosphate). Roughly
476 speaking, microbes with substantial metabolism for carboxylic acid and esters are
477 plotted in the positive direction, while those with substantial metabolism for
478 carbohydrates are plotted in the negative direction. Meanwhile, the second principal

479 component exhibited little in common with substrates whose loading rate exceeded 0.5
480 among the sampling dates.

481

482 Activated sludge having different dilution levels are plotted with the data set taken on
483 19 August. The fact that the three data plots are located at approximately the same
484 position indicates that the two digit differences in the copy numbers among the samples
485 have little influence on the results of the principal component analysis.

486

487 The distribution of substrate metabolic patterns for the samples exhibited much in
488 common among the sampling date. It was determined that similar substrate metabolic
489 patterns, which metabolized carbohydrates to a high degree, were obtained for the
490 activated sludge, influent to the SAT reactor and 0–5 cm layer. In comparison to these,
491 quite a different metabolic pattern was demonstrated by the sand layers from 10 to 30
492 cm, which metabolized carboxylic acid and esters to a high degree, and it is possible
493 that the metabolic pattern exhibited by the 5–10 cm layer is at a midpoint of the
494 changing pattern from the 0–5 cm layer to the 10–30 cm layers. This result suggests that
495 the SAT sand layers immediately below 5 cm from the top surface removes different
496 organic compounds by biodegradation than the aerobic tank in WWTP. This also means
497 that several organic compounds, which are not easily removed by the microbial
498 activities in the activated sludge, which is operated with limited HRT and SRT, are

499 removed by microbial activities in the SAT sand layers below 5 cm from the top surface.
500 In addition, the boundary between the vadose zone and saturated zone was exhibited by
501 the 15–20 cm layer. Meanwhile, there were no changes observed in the metabolic
502 patterns of adjacent layers. Moreover, metabolic patterns demonstrated by the 87 cm
503 layer, especially the 187 cm are unique.

504

505 **3.2.4. Water qualities along the flow path**

506 SS and VSS for the activated sludge were (median (minimum–maximum)) 1140
507 (960–1360) and 975 (757–1200), respectively, while those for the influent to the SAT
508 reactor were 3.80 (2.90–4.75) mg/L and 3.60 (2.50–3.65) mg/L, respectively.

509

510 Table 2 shows water qualities along the flow path. In addition, Figure 4 shows the
511 profiles of the water quality parameters in the SAT reactor. The pH of the water samples,
512 except for the effluent (187 cm) from the SAT reactor, were similar. Meanwhile, the
513 effluent (187 cm) from the SAT reactor was significantly lower than that of the effluent
514 (87 cm) from the SAT reactor, as discussed previously. Median DO of the samples
515 varied from 1.47 to 4.34 mgO₂/L indicating that each water sample was under aerobic
516 conditions, as discussed previously.

517

518 Similar values of DOC were obtained for the activated sludge and influent to the SAT

519 reactor ($p>0.05$). Meanwhile, a significant decreasing trend along the percolation path
520 in the reactor was observed ($p<0.05$), as discussed previously. In comparison to the
521 median DOC of the influent to the SAT reactor, the median DOC removal ratio of
522 effluent (25 cm) from the SAT reactor was 59.0%. Therefore, it was determined that
523 over half the DOC of the influent to the SAT reactor was removed through 25 cm
524 percolation.

525

526 Similar values of UV_{254} and SUVA were obtained for the activated sludge and influent
527 to the SAT reactor ($p>0.05$). Median UV_{254} decreased from the influent to the SAT
528 reactor to the effluent (25 cm) from the SAT reactor; however, it was not significant
529 ($p>0.05$). Meanwhile, SUVA significantly increased from the influent to the SAT reactor
530 to the effluent (25 cm) from the SAT reactor, and this indicated the preferential removal
531 of non-aromatic components through 25 cm percolation. UV_{254} significantly decreased
532 from the effluent (25 cm) from the SAT reactor to effluent (87 cm) from the SAT reactor
533 ($p<0.05$); meanwhile, no significant difference was observed between the effluent (25
534 cm) from the SAT reactor and effluent (87 cm) from the SAT reactor ($p>0.05$). There
535 were no significant differences for UV_{254} and SUVA between effluent (87 cm) from the
536 SAT reactor and effluent (187 cm) from the SAT reactor ($p>0.05$).

537

538 Fluorescence EEM profiles of the activated sludge, influent to the SAT reactor, each

539 effluent from the SAT reactor samples are given in Figure 5. In general, peaks in the
540 short range of excitation wavelengths (Ex)/emission wavelength (Em) = <250 nm/<350
541 nm are related to simple aromatic proteins. Peaks in the range of Ex/Em = 250–280
542 nm/<380 nm are associated with soluble microbial by-product-like (SMP-like)
543 compounds. Peaks in the range of Ex/Em = 240–260nm/380–480 nm are related to
544 fulvic acid-like compounds. Peaks in the range of Ex/Em = 320–350 nm/400–480 nm
545 are associated with humic acid-like compounds (Chen et al., 2003; Xue et al., 2009).
546
547 Peaks representative of simple aromatic proteins, fulvic acid-like and humic acid-like
548 compounds were observed in the activated sludge and influent to the SAT reactor.
549 Meanwhile, effluent (25 cm) from the SAT reactor was dominated by SMP-like
550 compounds. In addition, peaks representative of simple aromatic proteins, fulvic
551 acid-like and humic acid-like compounds in effluent (25 cm) from the SAT reactor were
552 stronger than those in the influent to the SAT reactor. In comparison with the influent to
553 the SAT reactor, over half of the DOC was removed and the change of SUVA shows
554 preferential removal of non-aromatic components through 25 cm percolation. In
555 addition, Xue et al., (2009) determined that organic matter fractionated into hydrophilic
556 and transphilic neutral components were preferentially removed by SAT. Therefore, the
557 increase of the peaks indicated that the relative abundance of simple aromatic proteins,
558 fulvic acid-like and humic acid-like compounds increased because of the removal of

559 such type of organic substances. In addition, the appearance of the SMP-like substance
560 peak was possible because of the contribution of biodegradation to the DOC removal
561 through 25 cm percolation. This was supported by Ak and Gunduz (2013a) who
562 clarified the active removal of organic carbon in the top 10 cm soil surface owing to
563 biodegradation. Because the peak for SMP-like substance was not observed in the case
564 of the effluent (87 cm) from the SAT reactor, SMP-like fluorescing compounds
565 observed in the effluent (25 cm) from the SAT reactor would have been removed. The
566 fluorescence data also suggested a decrease of simple aromatic proteins; meanwhile,
567 little change in the peaks representative of fulvic acid-like and humic acid-like
568 compounds was observed. With respect to the effluent (87 cm) from the SAT reactor,
569 little change in the peak representative of simple aromatic compounds was observed;
570 meanwhile, decrease of fulvic acid-like and humic acid-like compounds was exhibited
571 in the case of effluent (187 cm) from the SAT reactor. This suggests that the relative
572 abundance of fulvic acid-like and humic acid-like organic compounds decreased
573 because of their removal through percolation.

574

575 DTN in the activated sludge was significantly lower than that of the influent to the SAT
576 reactor ($p<0.05$). In addition, DTN in the effluent (25 cm) from the SAT reactor was
577 significantly higher than that of the influent to the SAT reactor and the effluent (87 cm)
578 from the SAT reactor ($p<0.05$). Meanwhile, the relative abundance of NO_3^- was high

579 (median: 68.3%–85.2%) in each sample, as discussed previously.

580

581 DTP was detected in the activated sludge (median: 0.758 mgP/L) and influent to the
582 SAT reactor (median: 0.400 mgP/L); meanwhile, the concentration of 0.007 mgP/L in
583 the effluent (25 cm) from the SAT reactor was significantly lower than that of the
584 influent to the SAT reactor ($p<0.05$). In addition, DTP was not detected in the 87 cm
585 and effluent (187 cm) from the SAT reactor samples (<0.002 mgP/L), as discussed
586 previously.

587

588 The median mole ratios of C:N:P were 100:92:21, 100:120:11, 100:350:0.47,
589 100:530:<0.23 and 100:550:<0.30 for the activated sludge, influent to the SAT reactor,
590 effluent (25 cm) from the SAT reactor, effluent (87 cm) from the SAT reactor and
591 effluent (187 cm) from the SAT reactor, respectively, and it was determined that carbon
592 was the limiting factor for microbial activities in the activated sludge and influent to the
593 SAT reactor; meanwhile, phosphorus was the limiting factor in the effluent (25 cm)
594 from the SAT reactor, effluent (87 cm) from the SAT reactor and effluent (187 cm) from
595 the SAT reactor.

596

597 **3.2.5. Relationships between the changes in substrate metabolic**
598 **patterns and water qualities**

599 There were similar substrate metabolic patterns among the activated sludge, influent to
600 the SAT reactor and 0–5 cm layer; meanwhile, those in sand layers below 5 cm were
601 quite different from these. In this section, relationships between the changes in substrate
602 metabolic patterns and water qualities are discussed.

603

604 First, it is suggested that DO concentrations have little influence on the change in
605 metabolic patterns because the activated sludge, influent to the SAT reactor and the SAT
606 reactor operated under aerobic conditions.

607

608 Comparing the water qualities between the activated sludge and influent to the SAT
609 reactor, no significant differences, including similar organic components by SUVA and
610 EEM, except for the relative abundance of nitrogen species, were observed. Meanwhile,
611 carbon was the limiting factor for microbial activities in both samples. Therefore, the
612 observed significant differences of nitrogen species are expected to have little influence
613 on the microbial substrate metabolism in these cases. Based on the results, similar
614 metabolic patterns should be observed because of a similar water environment.

615

616 The metabolic patterns in the 0–5 cm layer were also similar to those of the activated
617 sludge and influent to the SAT reactor. The influent to the SAT reactor, whose water
618 qualities were similar to the activated sludge and which contained microbes that

619 originated from the activated sludge, was discharged onto the top SAT surface.
620 Therefore, it is possible that the similar water environment and the discharge of the
621 microbes would have an influence on the populations of microbes in the 0–5 cm layer,
622 resulting in metabolic patterns similar to that of the activated sludge and influent to the
623 SAT reactor.

624

625 Differences in the compositions of organic matter, as determined by SUVA and EEM,
626 and transit of the limiting factor from carbon to phosphorus were observed between the
627 influent to the SAT reactor and effluent (25 cm) from the SAT reactor. FDA assay of the
628 0–5 cm layer was significantly higher than that of the 5–10 cm layer ($p<0.05$). In
629 addition, Quanrud et al., (1996) showed that 8 cm percolation of the vadose zone
630 removed DOC from 8.6 to 6.7 mgC/L, and active removal was obtained by only 8 cm
631 percolation. Therefore, it is possible that the active DOC removal occurring in the 0–5
632 cm layer had the largest contribution to the change in the composition of organic matter
633 between the influent to the SAT reactor and effluent (25 cm) from the SAT reactor,
634 which is reflected by the different metabolic patterns observed between the 0–5 cm
635 layer and layers from 5 to 30 cm. In addition, it is also possible that microbial activity in
636 the 0–5 cm layer had the largest contribution to the change of the limiting factor from
637 carbon to phosphorus. Kunimoto and Nagaoka (2009) showed that BIOLOG assay
638 using ECO plate significantly differentiated metabolic patterns of microbes in forest soil,

639 which was cultivated with and without the addition of nitrogen and phosphorus.
640 Therefore, it is also possible that the observed changes in metabolic patterns can be
641 attributed to the changes in the limiting factor.

642

643 The large differences in the composition of organic matter can be partly contributed to
644 the change of metabolic patterns between the 87 cm layer and the layers above. In
645 addition, the change of the composition of organic matter and significantly low pH of
646 the effluent (187 cm) from the SAT reactor should contribute to the unique metabolic
647 patterns observed there.

648

649

650 **4. Conclusions**

651 In this study the changes in the microbial substrate metabolic patterns by using the
652 BIOLOG assay were discussed through a sequential wastewater reuse process, which
653 includes activated sludge and treated effluent in WWTP and SAT, especially focusing
654 on the surface sand layer in conjunction with the vadose zone. The substrate patterns in
655 SAT were analysed using a pilot-scale reactor, in which stable water quality parameters
656 were obtained. The major conclusions are as follows:

657 (1) FDA assay revealed that microbial activities in sand significantly decreased at a
658 depth greater than 5 cm from the top surface in SAT (0–5 cm layer: 0.090, 5–10 cm

659 layer: 0.042, 10–15 cm layer: 0.012 and 15–20 cm layer: 0.005 cm⁻¹·g-dry⁻¹).

660 (2) Similar microbial metabolic patterns with preferential carbohydrates were obtained

661 among the activated sludge, the influent to the SAT reactor and the 0–5 cm sand

662 layer. In addition, the 10–30 cm sand core layers showed dramatically different

663 metabolic patterns with high degree of carboxylic acid and esters. Thus, it is

664 possible that the metabolic pattern exhibited by the 5–10 cm layer is at the midpoint

665 of the changing pattern. This suggests that the removal of different organic

666 compounds by biodegradation is expected to occur in the activated sludge and in the

667 SAT sand layers immediately below 5 cm from the top surface.

668 (3) There were no significant differences, including similar organic components,

669 between the activated sludge and the influent to the SAT reactor, and similar

670 metabolic patterns between them should be observed because of similar water

671 environment. In addition, it is possible that the similar water environment and the

672 discharge of microbes would affect the populations of microbes in the 0–5 cm layer,

673 resulting in metabolic patterns similar to those of the activated sludge and influent to

674 the SAT reactor.

675 (4) DOC significantly decreased from the influent to the SAT reactor (3.68 mgC/L) to

676 the effluent (25 cm) from the SAT reactor (1.52 mgC/L). The composition of

677 organic matter also changed between them, represented as domination by SMP-like

678 compounds in the effluent (25 cm) from the SAT reactor, which suggests the

contribution of biodegradation to the DOC removal via 25 cm percolation. DTN significantly increased from the influent to the SAT reactor (4.43 mgN/L) and to the effluent (25 cm) from the SAT reactor (5.11 mgN/L); whereas DTP significantly decreased from 0.400 to 0.007 mgP/L. Consequently, limiting factor for microbial activities was carbon in the influent to the SAT reactor (C:N:P = 100:120:11); meanwhile it was phosphorus in the effluent (25 cm) from the SAT reactor (C:N:P = 100:350:0.47). Therefore, changes in the composition of organic matter and/or the transition of the limiting factor for microbial activities from carbon to phosphorus may contribute to the observed dramatic changes in the SAT metabolic patterns.

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Table 1 Ranges of TOC and FDA, 16S rRNA copy numbers and results of the Wilcoxon rank sum test, where ranges are given as median (minimum–maximum)

Sample	TOC (%)			FDA (cm ⁻¹ ·g-dry ⁻¹ or cm ⁻¹ ·mL ⁻¹)		16S rRNA copy numbers (copies/mL)
Activated sludge				0.160 (0.14–0.23)	$p < 0.05$	3.3×10^7
Influent to the SAT reactor				<0.005		8.2×10^5
0–5 cm layer	0.084 (0.032–0.094)	$p < 0.05$	$p > 0.05$	0.090 (0.051–0.186)	$p < 0.05$	2.4×10^7
5–10 cm layer	0.027 (0.012–0.038)			0.042 (0.010–0.057)		1.8×10^7
10–15 cm layer	0.015 (0.012–0.019)	$p > 0.05$	$p > 0.05$	0.012 (<0.005–0.038)	$p > 0.05$	2.1×10^7
15–20 cm layer	0.013 (0.0082–0.019)			0.005 (<0.005–0.011)		1.7×10^7
20–25 cm layer	0.010 (0.0078–0.011)	$p > 0.05$	$p > 0.05$	<0.005	-	2.0×10^7
25–30 cm layer	0.0072 (0.0063–0.011)			<0.005		1.1×10^7
87 cm layer	0.0017 (0.0011–0.0044)	$p > 0.05$	$p < 0.05$	<0.005	-	5.6×10^5
187 cm layer	0.0020 (0.00048–0.0057)			<0.005		8.2×10^5

Table 2 Ranges of water quality index and results of the Wilcoxon rank sum test, where ranges are given as median

(minimum–maximum)

Sample	pH (–)			DO (mgO ₂ /L)			DOC (mgC/L)			UV ₂₅₄ (cm ^{–1})		
Activated sludge	6.45 (6.32–6.50)	$p > 0.05$		2.56 (2.20–3.36)	$p > 0.05$		3.65 (3.41–4.13)	$p > 0.05$		0.104 (0.071–0.156)	$p > 0.05$	
Influent to the SAT reactor	6.43 (6.37–6.50)		$p > 0.05$	1.47 (1.13–1.75)		$p < 0.05$	3.68 (3.31–4.19)	$p > 0.05$		0.082 (0.070–0.128)		$p > 0.05$
Effluent (25 cm) from the SAT reactor	6.52 (6.29–6.90)	$p > 0.05$					1.52 (1.18–1.98)	$p < 0.05$		0.055 (0.031–0.094)	$p < 0.05$	
Effluent (87 cm) from the SAT reactor	6.55 (6.47–6.70)		$p < 0.05$	4.34 (3.54–4.77)	$p > 0.05$		0.848 (0.808–0.876)	$p < 0.05$		0.022 (0.020–0.053)		$p > 0.05$
Effluent (187 cm) from the SAT reactor	5.51 (5.34–5.65)			3.61 (3.27–3.93)			0.672 (0.608–0.740)	$p < 0.05$		0.024 (0.017–0.035)		
Sample	SUVA (L/(mgC·cm))			DTP (mgP/L)			DTN (mgN/L)			NH ₄ ⁺ (mgN/L)		
Activated sludge	0.029 (0.020–0.038)	$p > 0.05$		0.758 (0.570–0.865)	$p > 0.05$		3.25 (2.41–4.20)	$p < 0.05$		0.07 (0.05–0.12)	$p > 0.05$	
Influent to the SAT reactor	0.023 (0.021–0.031)		$p < 0.05$	0.400 (0.280–0.805)		$p < 0.05$	4.43 (3.87–4.89)	$p < 0.05$		<0.02 (<0.02–0.56)		$p > 0.05$
Effluent (25 cm) from the SAT reactor	0.032 (0.016–0.053)	$p > 0.05$		0.007 (0.004–0.012)	$p < 0.05$		5.11 (4.95–5.97)	$p < 0.05$		0.17 (0.05–0.18)	$p > 0.05$	
Effluent (87 cm) from the SAT reactor	0.026 (0.024–0.061)		$p > 0.05$	<0.002			4.54 (4.32–4.71)	$p > 0.05$		0.20 (0.16–0.26)		$p > 0.05$
Effluent (187 cm) from the SAT reactor	0.036 (0.024–0.056)			<0.002			4.30 (4.08–4.62)			0.39 (0.06–0.70)		
Sample	NO ₂ [–] (mgN/L)			NO ₃ [–] (mgN/L)			Organic-N (mgN/L)					
Activated sludge	0.006 (<0.003–0.022)	$p > 0.05$		2.31 (1.55–2.79)	$p < 0.05$		0.87 (0.78–1.28)	$p > 0.05$				
Influent to the SAT reactor	0.046 (0.014–0.080)		$p > 0.05$	3.41 (3.26–3.46)		$p < 0.05$	0.92 (0.53–0.97)		$p < 0.05$			
Effluent (25 cm) from the SAT reactor	0.005 (0.003–0.013)	$p < 0.05$		4.48 (4.27–5.38)	$p < 0.05$		0.50 (0.45–0.53)	$p > 0.05$				
Effluent (87 cm) from the SAT reactor	0.018 (0.006–0.023)		$p > 0.05$	3.80 (3.66–4.19)		$p > 0.05$	0.40 (0.33–0.64)		$p > 0.05$			
Effluent (187 cm) from the SAT reactor	<0.003 (<0.003–0.027)			3.64 (3.26–3.95)			0.30 (0.22–0.41)

Figure 1 Diagram of the SAT reactor and designations of various treated water and layer samples: (a) samples used for the determination of substrate metabolic patterns and (b) samples used for the determination of water qualities

◆ Influent to the SAT reactor ■ Effluent (87 cm) from the SAT reactor ▲ Effluent (187 cm) from the SAT reactor ● Final effluent (237 cm) from the SAT reactor ✕ Water temperature in the final effluent

Figure 2-1 Temporal changes in pH, DO, DOC, UV₂₅₄, SUVA and DTP for treated water sampled from various points of the SAT reactor

Figure 2-2 Temporal changes in DTN and its components for treated water sample from various points of the SAT reactor

Figure 3 Changes of microbial substrate metabolic patterns at each sampling event, where FPC refers to the first principal component, SPC to the second principal component, AS to activated sludge, Influent to influent to SAT reactor and DIL to dilution

Figure 4 Profiles of the water quality parameters in the SAT reactor. Plot and bars show median, and minimum and maximum values, respectively

Figure 5 Fluorescence emission–excitation matrices (EEM) profiles of treated water samples from various points of the SAT reactor

Change of microbial substrate metabolic patterns by BIOLOG assay through a wastewater reuse process, including WWTP and SAT