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Fate of *Escherichia coli* in Dialysis Device Exposed into Sewage Influent and Activated Sludge

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Abstract

Tracing the fate of pathogens in environmental water, particularly in wastewater, with a suitable methodology is a demanding task. We investigated the fate of *Escherichia coli* K12 in sewage influent and activated sludge using a novel approach that involves the application of a biologically stable dialysis device. The ion concentrations inside the device could reach that of surroundings solution when it was incubated in phosphate buffered saline for 2 h. Above 10^7 CFU·mL^{-1} of *E. coli* K12 (inoculated in distilled water, influent, activated sludge) were introduced into the device and incubated in influent and activated sludge for 10 days. Without indigenous microorganisms, *E. coli* K12 could survive even with the limited ions and nutrients concentrations in influent and activated sludge. *E. coli* K12 abundance in influent and activated sludge were reduced by 60% and 85%, respectively, after just one day. The establishment of microbial community in wastewater played an important role in reducing *E. coli* K12. Bacteriophage propagated in filtered influent or activated sludge when *E. coli* K12 was introduced, but not in raw influent or activated sludge. The methodology developed in this study can be applied in the actual environmental water to trace the fate of pathogens.

Keywords

Activated sludge, bacteriophage, dialysis device, influent, microbial community, pathogens

Introduction

There are many concerns about the proliferation of virulent enteric microorganisms in environmental water, especially in water sources that serve recreational activities in daily life (Abhirosh *et al*. 2010, Cai *et al*. 2014). The risks posed to human health and environmental vulnerability have captured much attention since water sources are threatened by large amounts of waste generated by domestic, industrial, and agricultural activities. In particular, domestic wastewater contains organic compounds, inorganic materials, and a variety of microorganisms (Sheng *et al*. 2010, Tan *et al*. 2012). Generated wastewater must be treated to mitigate its adverse effects on the environment and to sustain natural resources. There are several wastewater treatment technologies such as conventional activated sludge, membrane bioreactor, upflow anaerobic sludge blanket and so on that share a similar principle goal, namely to decontaminate wastewater. In fact, the design of the activated sludge process aims to reduce the high load of organic matter in sewage influent (influent) at low cost. Moreover, it is also known as a process for removing numerous pathogenic microorganisms from wastewater by biological processes (Li *et al*. 2015, Orruno *et al.* 2014, Wen *et al*. 2009a).

However, wastewater treatment plants (WWTPs) are known reservoirs for pathogenic microorganisms, antibiotic-resistant bacteria, and genes responsible for antibiotic resistance (Gao *et al*. 2012, Karkman *et al*. 2016). Many kinds of pathogenic bacteria derived from the human gut are transported to WWTPs. At least 113 species and 75 genera of pathogenic bacteria were found in wastewater examined by two different groups (Cai *et al*. 2014, Li *et al*. 2015). The reduction of pathogenic bacteria, which indicated by *E. coli* or coliforms, after aerobic and final settling process was observed (Abhirosh *et al*. 2010, Anderson *et al*. 2005, Costán-Longares *et al*. 2008). However, the reduction in pathogenic bacteria within the primary clarifier (influent) and aeration tank (activated sludge) is still not well-quantified, more information on their survivability in both compartments is needed. It is quite difficult to trace the fate of a certain type of bacteria in the mixed culture such as influent and activated sludge.

Regardless, experimental design using microcosm and mesocosms have been used in research to understand the pathogenic removal mechanism by predator or indigenous microbes. However, the maintaining of experimental condition was not accurately reflected to the actual environmental condition since the samples were withdrawn from originated source and incubated in laboratory or exposed to outdoor environment (Korajkic *et al*. 2013, Mantilla-Calderon & Hong 2017, Wanjugi *et al*. 2016). Mesocosms and microcosm bags have also been introduced in several studies but the selection and characterization of membranes were not clearly indicated (Brettar *et al*. 1994, Calero-Cáceres and Muniesa 2016, Gutiérrez-Cacciabue *et al*. 2016). Since ion and nutrient concentrations are key factors for the survival of microorganisms in the environment, membrane characteristics are critically important. Moreover, culture-based methods have been used to estimate the numbers of pathogenic bacteria in the environment. However, culture-based methods cannot reliably identify all bacteria found in wastewater. Recently, the relative abundance of the microbial community can be quantified by metagenomics analysis using next-generation sequencing (NGS) (Karkman *et al*. 2016, Ye & Zhang 2011).

Given that the development of appropriate experimental protocols coupled with molecular analysis to study the survival of pathogens in the real condition of environmental water is necessary compared to laboratory-scale or outdoor-incubated experiments. The main aim of this study is to focus on the fate of *E. coli* K12 in influent and activated sludge by using a biologically stable dialysis membrane as a support. Determining relative bacterial abundance before and after introducing the strain into the influent and activated sludge for extended incubation by gene-based analysis is necessary compared to culture-based methods. The osmolality induction and bacteriophage infection of *E. coli* in diluted phosphate buffered saline (PBS) and in filtered influent and activated sludge, respectively, were also examined.

Materials and Methods

Wastewater samples

In this study influent, activated sludge, effluent before chlorination (EBC), and effluent after chlorination (EAC) were taken from a domestic WWTP (Tokyo, Japan). The physicochemical parameters such as pH, conductivity, MLSS (mixed liquor suspended solids) and TOC (total organic carbon) were determined (Table 1). pH was measured with a pH meter (HORIBA, F-71). Conductivity was monitored with conductivity meter equipped with 3552-10D probe model (HORIBA, ES-71). After centrifugation at 6300 *g* for 10 min, pellet and supernatant of sample were used to determine the concentration of MLSS and TOC concentration, respectively (Tan et al., 2012). The TOC of supernatant was quantified using a TOC-V_{CPH} analyzer (SHIMADZU, Japan).

Dialysis membrane characterization

To trace the fate of *E. coli* in influent and activated sludge, we used a cellulose ester membrane dialysis device (Float-A-Lyzer® G2, Spectra/Por®) equipped with a floating ring (Fig. 1A). Cellulose ester membrane is biologically stable even when the device is incubated in influent or activated sludge for long periods. The dimensions of the device were: 10 mL volume, 16 cm length, 10 mm diameter and 100 kDa molecular weight cut-off (MWCO). Since ion and nutrient concentrations are important factors for the survival of microorganisms, we determined the penetration of ions and nutrients into the device. The dialysis devices were filled with DW (distilled water) and incubated separately in 1 L of PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 1 L DW), glucose solution (10 g·L^{-1}), influent, and activated sludge for 2 h. The bottles were incubated at 25ºC with rotation at 80 rpm by shaker, and 1 L·min^{-1} filtered air was introduced under each condition. Glucose concentration was measured by Anthrone reagent method (Pons et al., 1981). Ion and nutrient concentrations in influent and activated sludge were determined as the conductivity value and TOC concentration, respectively.

126 **Fate of *E. coli* K12 in influent and activated sludge**

127 *E. coli* K12 W3110 (*E. coli* K12) was used as a model strain of gastrointestinal bacteria. The
128 overnight culture in 2 mL Luria-Bertani (LB) broth was washed twice with PBS and
129 resuspended with DW. *E. coli* K12 was then inoculated into three different media of 10 mL
130 (DW, influent, and activated sludge) to obtain a final concentration of 10^7 CFU·mL^{-1}. The
131 inoculated *E. coli* K12 was transferred into the dialysis device and incubated in 1 L of influent
132 and activated sludge. Two control samples were made by using influent or activated sludge
133 without *E. coli* K12 inoculation. Experimental conditions were described as "inside the dialysis
134 device" and "device surroundings". For instance, (*E. coli* K12+DW)/Influent shows that the
135 inoculated *E. coli* K12 in DW was introduced into the interior of the device surrounded by the
136 influent. Filtered air at flow rate of 1 L·min^{-1} was supplied to each bottle. The bottles were
137 incubated on a rotating shaker at 80 rpm and at 25ºC for 10 days in the dark (Fig. 1B). 2 mL
138 of sample was withdrawn from the device and used it for culture-based and gene-based analysis
139 at each time course.

140 Activated sludge was homogenized using a glass homogenizer 10 times prior to determining
141 the concentrations of bacteria and bacteriophage. The concentrations of *E. coli*, coliforms, and
142 other bacteria in influent, activated sludge, EBC, and EAC were determined using
143 Chromocult® Coliform Agar (Merck KGaA, Germany).

144 Plaque assay was used to determine bacteriophage concentration. Sample was centrifuged at
145 11, 100 *g* for 5 min and the supernatant was collected. After addition of 0.1% chloroform, the
146 sample was shaken for 20 min before performing plaque assay on LB agar plate. *E. coli* K12
147 was used as a host strain for plaque assay. Bacteriophage propagation was examined by
148 introducing *E. coli* K12 into 100 mL filtered influent and activated sludge. Influent and
149 activated sludge were centrifuged at 6300 *g* for 10 min and the supernatants were collected.
150 The samples were filtered through 0.45 μm, then 0.22 μm membrane. The overnight culture of

E. coli K12, after washing, was introduced into the filtered influent and activated sludge at the final concentration above 10^5 CFU·mL^{-1} and incubated for 24 h following the above procedure. Samples were taken for counting bacteria on LB media and for plaque assays.

Additionally, the osmolality induction on the survival of *E. coli* K12 was examined by exposing them to different concentrations of ions in diluted PBS. The washed *E. coli* K12 was introduced into 100 mL of PBS buffer, 1/10 PBS, 1/100 PBS, 1/1000 PBS and DW with the conductivity of 1590, 177.1, 18.8, 1.9, and 0.08 mS·m^{-1}, respectively. The samples were incubated for one week under the same conditions as described before. Samples were plated on LB media and the colonies were count after incubation at 37ºC for 24 h.

Analysis of microbial community by next-generation sequencing (NGS)

Withdrawn samples from dialysis device were centrifuged at 6300 *g* for 10 min and the pellet was resuspended in PBS. The resuspended samples were used for DNA extraction. Glass beads (0.1 mm diameter) were used to break the cells for 45 s at 6 m·s^{-1}, twice, using a BeadBeater (FastPrep24, MP Biomedicals). The supernatant was collected after centrifugation at 1800 *g* for 5 min. The further DNA extraction process was described by Lee and Singh (Lee 2013, Singh *et al*. 2015).

The 16S rRNA gene sample was prepared by following the Illumina Miseq sequencing protocol (https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). DNA templates were subjected to amplification by two rounds of PCR. Miseq16S_341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and Miseq16S_805R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC C-3') were used to amplify the V3 to V4 region of the 16S rRNA gene. The program for the

first round of PCR amplification were as follows: 95°C for 5 min, 25 amplification cycles (95°C for 30 s, 55°C for 30 s, and 72°C for 60 s), and 72ºC for 5 min. DNA concentration in PCR products was estimated using a Nanodrop (Thermo Fisher Scientific) after purification, and subsequently diluted to 2.5 $ng \cdot \mu L^{-1}$ using TE buffer. The index primers (Nextera XT Index Kit v2 SetD, Illumina) were used in the second round of PCR. The amplification program was as follows: 95°C for 5 min, eight amplification cycles (95°C for 30 s, 55°C for 30 s, and 72°C for 60 s), and 72ºC for 5 min. PCR products were purified by QIAQuick PCR Purification Kit (Qiagen, Hilden, Germany) and DNA concentration was measured by NanoDrop and QuantiFluor® dsDNA System (Promega, Madison, WI, USA). All samples were gathered and sent to Hokkaido System Science Co., Ltd for sequencing. The data was analyzed by QIIME ver.1.9.1 pipeline (Caporaso *et al*. 2010). Chimeric sequences were checked using Usearch (version 6.1.544) coupled with GreenGenes Database (gg_13_8_otus.tar.gz). Less than 4% of the microbial community's relative abundance was classified into others.

DNA templates from influent and activated sludge were used to quantify the total number of 16S rRNA gene by real time quantitative PCR (qPCR). 341 F (5′-CCTACGGGAGGCAGCAG-3′) and 534 R (5'ATTACCGCGGCTGCTGG-3') primers were used to amplify a target DNA size of 194 bp. After primers were combined with all qPCR reagents including SYBR green, the mixture was monitored by using StepOne Real-Time PCR System (Applied Biosystems) with the template DNA standard (Tanji *et al.* 2014).

Results

Membrane penetration by ions and nutrients

The conductivity inside the dialysis device reached 96% of the conductivity of PBS (1590 ± 18.2 $mS \cdot m^{-2}$), 85% of that of influent (52.8 ± 5.9 $mS \cdot m^{-2}$) and 81% of that of activated sludge (41.0 ± 2.4 $mS \cdot m^{-2}$) after 2 h (Fig. 2A, 2B).

By incubating the membrane in a glucose solution (10 $g \cdot L^{-1}$), the concentration of glucose in the membrane increased and almost reached the value of the surrounding solution after 2 h (Fig. 2C). When the membrane was incubated in influent, the TOC within the membrane reached 34% after 10 min and increased to 42% of the surrounding TOC (44.3 ± 10.6 $mg \cdot L^{-1}$) after 2 h. However, when the membrane was incubated in activated sludge, the TOC in the membrane reached 96% after 10 min, and eventually reached 99.7% of surrounding (8.0 ± 1.1 $mg \cdot L^{-1}$) after 2 h (Fig. 2D).

Fate of indigenous *E. coli* and coliform bacteria in WWTP

Figure 3A showed that the concentration of indigenous *E. coli*, coliforms, and total bacteria decreased going from influent to EBC. The bacteria were undetected in EAC. The concentration of coliforms (1.2 x 10^6 $CFU \cdot mL^{-1}$) was about 4.6 higher than *E. coli* (2.6 x 10^5 $CFU \cdot mL^{-1}$) in influent. *E. coli* and coliforms bacteria were subsequently reduced in activated sludge and in EBC. However, the other bacteria, represented by white colonies, increased slightly in number going from influent to activated sludge. The numbers of other bacteria decreased greatly going from activated sludge to EBC and became undetectable after chlorination. The 16S rRNA gene, quantified by qPCR, demonstrated that the gene copy number of bacteria in activated sludge (6.97 x 10^9 $copies \cdot mL^{-1}$) was higher than that in influent (9.08 x 10^8 $copies \cdot mL^{-1}$) (Fig. 3B). Despite this, the total bacterial count determined in activated sludge was lower than that in influent on Chromocult® Coliform Agar.

Fate of *E. coli* K12 in influent and activated sludge

E. coli K12 (inoculated in DW and incubated in influent and activated sludge) maintained its high concentration (Fig. 4A). However, *E. coli* K12 (inoculated in influent and activated sludge and incubated in influent and activated sludge, respectively) was reduced during the incubation period. *E. coli* K12 inoculated in activated sludge were reduced compared with *E. coli* K12 inoculated in influent by about tenfold. Without the inoculation of *E. coli* K12, indigenous *E.*

coli was detected in influent and activated sludge. Based on the control samples, the concentration of indigenous *E. coli* contributed to *E. coli* K12 count from three days onwards. By introducing *E. coli* K12 into different ionic concentrations in 100 mL of diluted PBS, *E. coli* K12 was able to maintain a high concentration for one week (Fig. 4B). Hence, we determined that the ions in influent and activated sludge were not a main factor in the reduction of *E. coli* K12.

Consistent results from NGS at the genus level illustrate the relative abundance of the microbial community in influent and activated sludge before and after inoculation with *E. coli* K12 and when incubated in influent and activated sludge, respectively (Fig. 5A). A change in the relative abundance of bacteria was observed after the same amount of *E. coli* K12 was introduced into either influent or activated sludge, following an extended incubation period. *E. coli* K12 became the dominant strain in influent, making up about 40% of the total community. The relative abundance of *E. coli* K12 was reduced by about 60% while the relative abundance of dominant bacteria in influent, such as *Acinetobacter*, *Comamonadaceae*, *Cloacibacterium*, and *Chryseobacterium*, increased in just one day. The relative abundance of *E. coli* K12 decreased continuously and almost disappeared from the influent after five days. The same amount of *E. coli* K12 was introduced into the activated sludge, but the relative abundance of *E. coli* K12 was not the highest in the system, since the total copy number of 16S rRNA genes found in activated sludge was about tenfold higher than that in influent (Fig. 3B). *E. coli* K12 represented about 6% of the total microbial community in activated sludge. About 85% of *E. coli* K12 was decreased after one day while the relative abundances of indigenous bacteria such as *Saprospiraceae*, *Kouleothrix*, *Caldilinea*, and *Comamonadaceae* were changed slightly. *E. coli* K12 was almost undetected after five days. The microbial community in activated sludge revealed in this study was changed slightly compared to influent for 10 days under aerobic conditions. Based on the Shannon diversity index (Fig. 5B), the community diversity in

250 activated sludge before and after introducing *E. coli* K12 remained almost unchanged over 10
251 days. The community diversity in influent, however, was reduced after the introduction of *E.*
252 *coli* K12. Nevertheless, it was increased going from 1 to 10 days incubation.

253 **Bacteriophage infection of *E. coli* K12**

254 Bacteriophages, which are a biotic factor, play an important role in controlling the growth of
255 various bacteria in environmental water. We therefore quantified the concentration and
256 infectivity of bacteriophage on *E. coli* K12 inoculated and incubated in influent and activated
257 sludge (Fig. 6A). The concentration of bacteriophage in influent (2.9 x 10^3 PFU·mL^{-1}) was
258 about a hundredfold higher than that in activated sludge (60 PFU·mL^{-1}). Above 10^7 CFU·mL^{-1}
259 of *E. coli* K12 was inoculated in and incubated in influent and activated sludge. The
260 concentration of *E. coli* was decreased and was still detected after 10 days at low concentration
261 (Fig. 4A). However, there was no increase in bacteriophage concentration during the
262 incubation period. The concentration of bacteriophage in activated sludge decreased and
263 became undetectable after one day while the bacteriophage concentration in influent decreased
264 and was still detected at a lower concentration after 10 days. By introducing *E. coli* K12 into
265 influent and activated sludge, we determined that bacteriophage was not a potential factor that
266 affected the fate of *E. coli* K12.

267 *E. coli* K12 was introduced into the filtered influent and activated sludge. TOC (44.3 mg·L^{-1})
268 observed for influent was 5.5 times higher than that of activated sludge (8 mg·L^{-1}) (Table 1).
269 By eliminating indigenous microorganisms, the relatively high TOC in influent permitted the
270 growth of *E. coli* K12, which was followed by the propagation of bacteriophage and lysis of *E.*
271 *coli* K12 in less than 9 h (Fig. 6B). As a result, *E. coli* K12 abundance in filtered influent
272 decreased greatly between 6 h and 9 h of incubation while bacteriophage abundance in filtered
273 influent increased greatly between 3 h and 9 h of incubation. *E. coli* K12 was still detected in
274 filtered influent after 24 h incubation at low concentrations. However, *E. coli* K12 in filtered

activated sludge could maintain their number and there was no bacteriophage increase in filtered activated sludge after 24 h. The bacteriophage concentration in filtered activated sludge was observed to increase about one hundredfold over 3 days (data not shown). Hence, we observed bacteriophage propagation when the host was able to grow in number, even under limited nutrient conditions such as those found in influent and activated sludge.

Discussion

Understanding the fate of a certain type of bacteria in the environmental water such as influent and activated sludge is quite difficult. Device developed in this work enables to solve the problem. By enclosing the model of gastrointestinal bacterium (*E. coli* K12) in the biologically inactive dialysis membrane and exposing it to the different environments, we can trace the fate of *E. coli* K12 and identify factors influential.

The ions of PBS and glucose molecules were able to penetrate the membrane. However, the penetration of ions and nutrients from the influent or activated sludge into the dialysis device was limited (Fig. 2). The limitation of small molecules, less than 100 kDa, may have driven the numbers of ions and nutrients penetration into the membrane. Moreover, a portion of the ions and nutrients in influent or activated sludge may be bound to other organic compounds, and those organic compounds consist of a different fractions of aquatic humic substances, hydrophobic, and hydrophilic groups. The fate of nutrients and the partitioning of dissolved organic carbon with a number of hydrophobic pollutants has been demonstrated in the wastewater process (Gianico *et al*. 2013, Katsoyiannis & Samara 2007).

Without predators and nutrient competition, *E. coli* K12 can survive for extended periods. However, the concentration and relative abundance of *E. coli* K12 both decreased when *E. coli* K12 was inoculated in and incubated in influent and activated sludge (Fig. 4A, 5A). Several bacteria-removing mechanisms in WWTP and aquatic water have been reported. The aggregation and floc settling in the activated sludge process require at least 3–4 days of sludge

300 retention time coupled with a hydraulic retention time of around 10 h (Hreiz *et al*. 2015).
301 During an extended period, a portion of pathogenic bacteria may be adsorbed into the flocs and
302 settle down. At the same time, the biological factors may exert themselves on their survivability.
303 The highlight of predator action and bacterial competition on the removal of pathogenic
304 bacteria has been reported (Orruno *et al*. 2014, Sheng *et al*. 2010, Wen *et al*. 2009b). However,
305 osmolality induction was not a reason that drove the *E. coli* K12 reduction observed in this
306 study.
307 *E. coli* K12 is a foreign strain and may need time to adapt and compete for nutrients with
308 indigenous bacteria in influent and activated sludge. Since nutrients are limited in those
309 environments, competition for them must be high, particularly in activated sludge. As shown
310 in our study, the relative abundance of indigenous microbial community in wastewater, before
311 and after introducing *E. coli* K12 for extended incubation periods, was well-established (Fig.
312 5A). Even when large amounts of *E. coli* K12 was introduced into complex microbial structures,
313 they did not strongly affect indigenous bacteria. The establishment of the microbial community
314 and their diversity in wastewater are important factors contributing to the induction of foreign
315 microorganisms and the maintenance of efficiency of the wastewater treatment process.
316 Camper *et al.* showed that the growth rate of environmental strains of *Klebsiella pneumoniae*
317 was almost two times higher than the clinical strain in a low-nutrient environment. The
318 environmental strains were well-adapted to different environmental conditions (Camper *et al*.
319 1991). Consequently, the well-adapted strain coupled with the ability to use a variety of
320 nutrients enabled it to become a dominant species in the environment (Hibbing *et al*. 2010).
321 The presence of bacteriophage in wastewater is thought to be a biotic factor that controls the
322 numbers of pathogenic bacteria. However, there was no increase in bacteriophage numbers
323 when *E. coli* K12 was introduced into the influent or activated sludge and incubated for 10
324 days (Fig. 6A). The bacteriophage, introduced in filtered influent or activated sludge, showed

its ability to infect *E. coli* K12. Our results show that bacteriophage concentration increased while *E. coli* K12 concentration decreased (Fig. 6B). This result shows that the bacteriophage was able to infect the host strain when they were able to grow in either influent or activated sludge. Environmental bacteriophages may be able to infect a specific host strain, and the efficiency of infection is dependent not only on their biological function but also on host status. Particularly, infection by bacteriophage may increase greatly when the host is in the growing phase (Julia et *al.* 2014, You *et al.* 2002). However, bacteriophage was reported to not be a relevant factor that leads to a decrease of cells numbers in lake water mesocosms and wastewater. The reduction of cells was mostly affected by grazing and particulate adsorption (Brettar *et al.* 1994, Orruno *et al*. 2014). Wciłso and Chróst have showed that a concentration of 0–2 $PFU \cdot mL^{-1}$ bacteriophage was not able to infect *E. coli* that was inoculated into freshwater. They hypothesized that large numbers of bacteriophages and active host cells may be needed (Wciłso & Chróst 2000). Additionally, our study demonstrates that if bacteria are able to grow even in a low nutrient environment, infection by bacteriophage occurs; however, the bacteriophages are unable to eliminate all the *E. coli* K12. As reported, the co-evaluation of bacteriophage and host resistance normally occurs in either the natural environment or in co-culture experiments (Levin & Bull 2004, Tanji *et al*. 2005).

Conclusions

We have studied the fate of *E. coli* in WWTP using a novel approach that involves the use of a biologically stable dialysis membrane device. *E. coli* K12 concentration was considerably reduced when the bacterium was inoculated in and subsequently incubated in influent and activated sludge. In contrast, *E. coli* K12 inoculated in DW and subsequently incubated in influent or activated sludge were able to maintain their high concentration for 10 days. *E. coli* K12 was reduced about tenfold in activated sludge compared with influent. The native population of microbial communities in wastewater, particularly in activated sludge, were more

350 persistent than *E. coli* K12. The bacteriophage was proposed as one of the factors that control
351 the numbers of *E. coli* K12 if they were able to grow in wastewater. The ion and nutrient
352 concentrations in influent and activated sludge were sufficient to support the survival of *E. coli*.
353 The experimental protocol developed in this study can be widely used to trace the fate of
354 chemical and biological contaminants in the actual environmental water.

355 **Conflict of interest statement**

356 The authors declare that they have no conflicts of interest.

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Figure captions

Fig. 1 (A) Dimensions of the dialysis device used in the experiment. (B) Experimental conditions are described as “inside of the dialysis membrane” and “surrounding of the membrane”. (*E. coli* K12+DW)/Influent: inoculated *E. coli* K12 in DW and incubated in influent, (*E. coli* K12+DW)/Activated sludge: inoculated *E. coli* K12 in DW and incubated in activated sludge, (*E. coli* K12+Influent)/Influent: inoculated *E. coli* K12 in influent and incubated in influent, (*E. coli* K12+Activated sludge)/Activated sludge: inoculated *E. coli* K12 in activated sludge and incubated in activated sludge, Influent/Influent: without inoculated *E. coli* K12 in influent (control), Activated sludge/Activated sludge: without inoculated *E. coli* K12 in activated sludge (control).

475 **Fig. 2** Ion penetration into the dialysis membrane device from its surroundings: (A) PBS, (B)
476 influent and activated sludge and the nutrient penetration into the dialysis device from its
477 surroundings: (C) 10 $g \cdot L^{-1}$ glucose, (D) influent and activated sludge. The dotted lines
478 indicate the surrounding conductivity of PBS (1590 ± 18.2 $mS \cdot m^{-2}$), influent (52.8 ± 5.9
479 $mS \cdot m^{-2}$), activated sludge (41.0 ± 2.4 $mS \cdot m^{-2}$), and TOC of influent (44.3 ± 10.6 $mg \cdot L^{-1}$),
480 activated sludge (8.0 ± 1.1 $mg \cdot L^{-1}$).
481 **Fig. 3** (A) Concentrations of indigenous *E. coli*, coliform bacteria, other bacteria, and total
482 bacterial count in influent, activated sludge, effluent before chlorination (EBC), and
483 effluent after chlorination (EAC) on Chromocult® Coliform Agar. (B) Total 16S rRNA
484 gene in influent and activated sludge.
485 **Fig. 4** (A) The fate of *E. coli* K12 in influent and activated sludge over a 10-day period using
486 a dialysis membrane as a support. (B) Survival of *E. coli* K12 in 100 mL diluted PBS and
487 DW for 7 days. *E. coli* K12 incubated in PBS (PBS), *E. coli* K12 incubated in PBS diluted
488 10 times (1/10 PBS), E. coli K12 incubated in PBS diluted 100 times (1/100 PBS), *E. coli*
489 K12 incubated in PBS diluted 1000 times (1/1000 PBS), and *E. coli* K12 incubated in DW
490 (DW).
491 **Fig. 5** (A) The bacteria relative abundance in influent and activated sludge before and after
492 incubation with *E. coli* K12 for 10 days (genus with abundance > 4%). (B) Shannon
493 diversity index of influent and activated sludge before and after incubation with *E. coli*
494 K12 for 10 days. (*E. coli* K12+Influent)/Influent: inoculated *E. coli* K12 in influent and
495 incubation in influent, (*E. coli* K12+Activated sludge)/Activated sludge: inoculated *E. coli*
496 K12 in activated sludge and incubation in activated sludge.
497 **Fig. 6** (A) The evaluation of bacteriophage concentration in influent and activated sludge after
498 incubation with *E. coli* K12 for 10 days. (*E. coli* K12+Influent)/Influent: inoculated *E. coli*
499 K12 in influent and incubated in influent, (*E. coli* K12+Activated sludge)/ Activated

sludge: inoculated *E. coli* K12 in activated sludge and incubation in activated sludge. (B) The infection of bacteriophage on *E. coli* K12 in 100 mL filtered influent and activated sludge. *E. coli* K12 concentration in filtered influent (*E. coli* K12/Influent) and *E. coli* K12 concentration in filtered activated sludge (*E. coli* K12/Activated sludge), bacteriophage concentration in filtered influent (Phage/Influent) and bacteriophage concentration in filtered activated sludge (Phage/Activated sludge).

508

509 **Table 1** Physicochemical parameters of influent and activated sludge used in the experiment.

Parameters	**Influent**	**Activated sludge**
pH	7.2 ± 0.3	6.9 ± 0.3
Conductivity ($mS \cdot m^{-2}$)	52.8 ± 5.9	41.0 ± 2.4
TOC ($mg \cdot L^{-1}$)	44.3 ± 10.6	8.0 ± 1.1
MLSS ($mg \cdot L^{-1}$)	190.0 ± 54.8	5120 ± 1230

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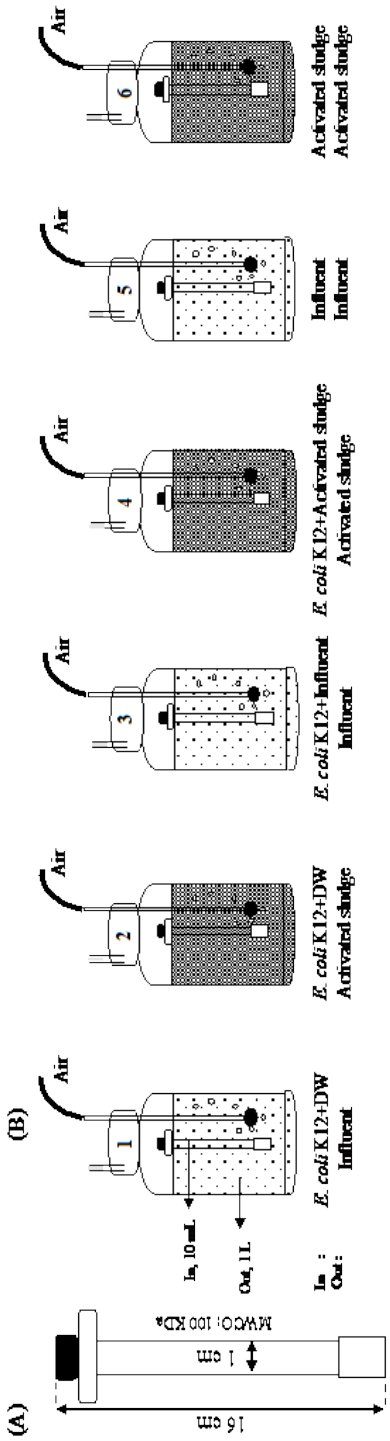

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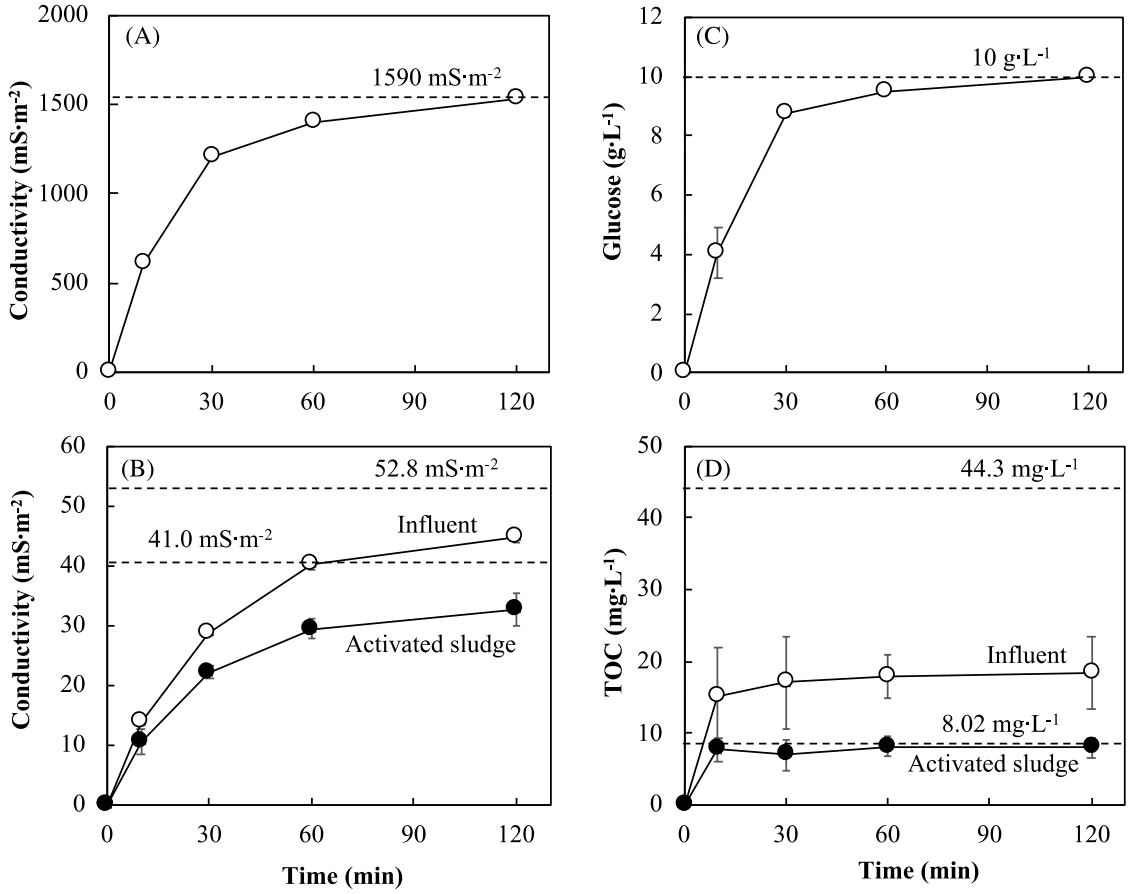
514 **Fig. 1 –** Ung *et al.*



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517 **Fig. 2 –** Ung *et al.*

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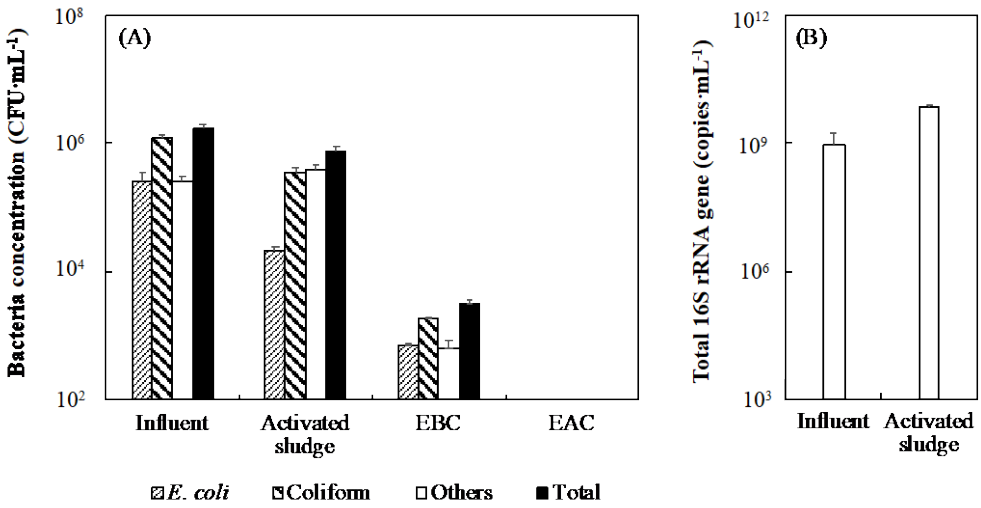

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522 **Fig. 3** – Ung *et al.*

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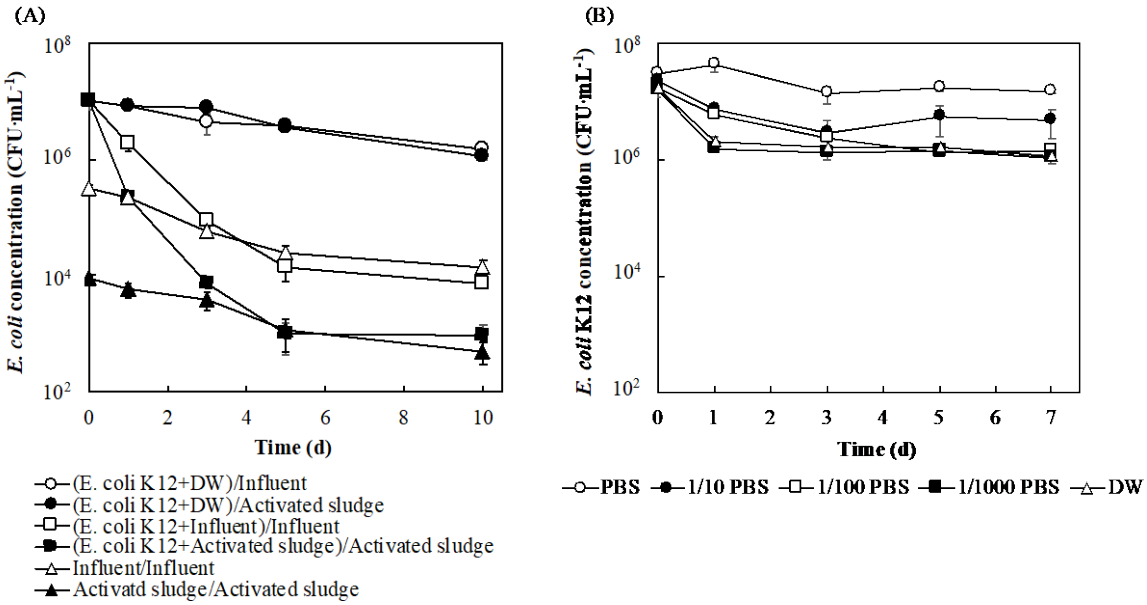

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527 **Fig. 4** – Ung *et al.*

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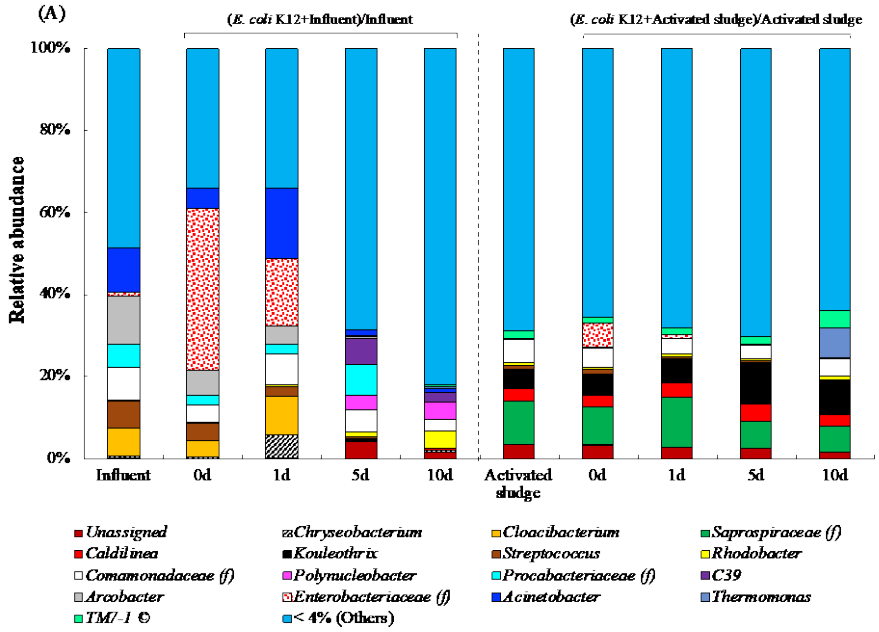

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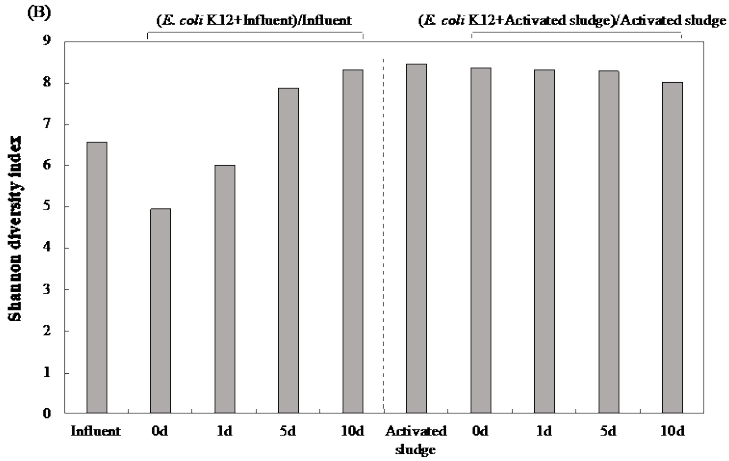

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532 **Fig. 5 –** Ung *et al.*

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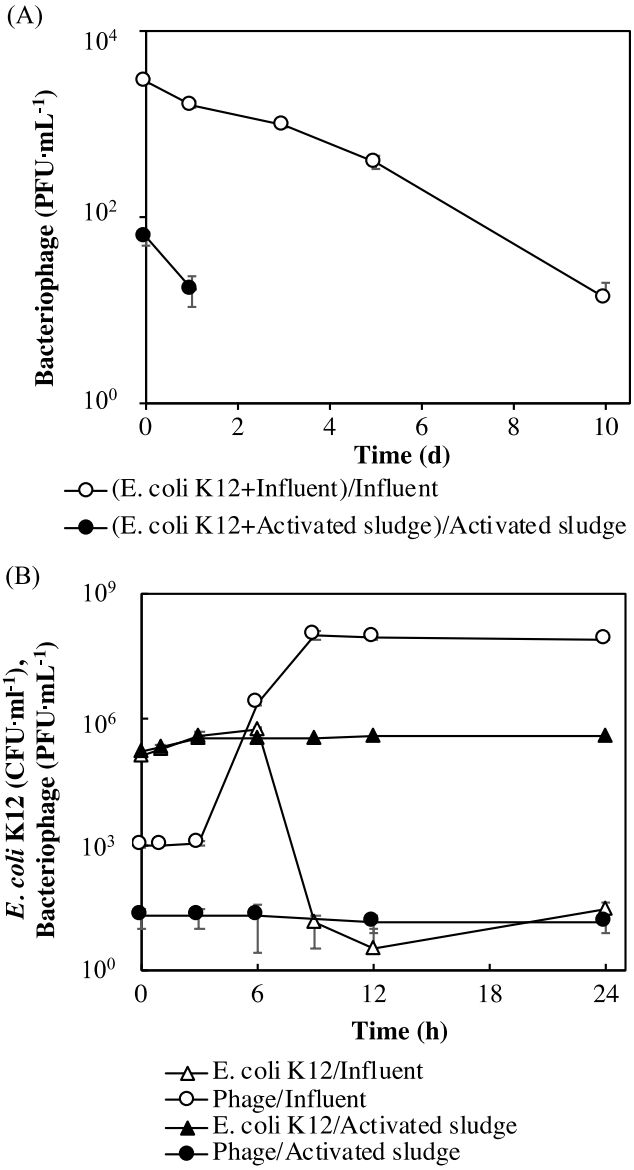

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537 **Fig. 6 –** Ung *et al.*

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