Fate of *Escherichia coli* in dialysis device exposed into sewage influent and activated sludge

Porsry Ung, Chanthol Peng, Sokunsreiroat Yuk, Vannak Ann, Hasika Mith, Reasmey Tan, Kazuhiko Miyanaga and Yasunori Tanji

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 surrounding solution when it was incubated in phosphate buffered saline for 2 h. *E. coli* K12 above 10^7 CFU mL^{-1} (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 1 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.

Key words | activated sludge, bacteriophage, dialysis device, influent, microbial community, pathogens

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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 principal 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 (Wen *et al.* 2009; Orruno *et al.* 2014; Li *et al.* 2015).

However, wastewater treatment plants (WWTPs) are known reservoirs for pathogenic microorganisms,

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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, indicated by *Escherichia coli* or coliforms, after aerobic and final settling processes was observed (Anderson *et al.* 2005; Costán-Longares *et al.* 2008; Abhirosh *et al.* 2010). 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 mixed cultures such as influent and activated sludge. Regardless, experimental design using microcosms and mesocosms have been used in research to understand the pathogenic removal mechanism by predator or indigenous microbes. However, the maintaining of experimental conditions was not accurately reflected to the actual environmental condition since the samples were withdrawn from the originating source and incubated in a laboratory or exposed to outdoor environments (Korajkic *et al.* 2013; Wanjugi *et al.* 2016; Mantilla-Calderon & Hong 2017). 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 & 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) (Ye & Zhang 2011; Karkman *et al.* 2016).

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 6,300 *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 (Figure 1(a)). Cellulose ester membrane is biologically

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	5,120 ± 1,230

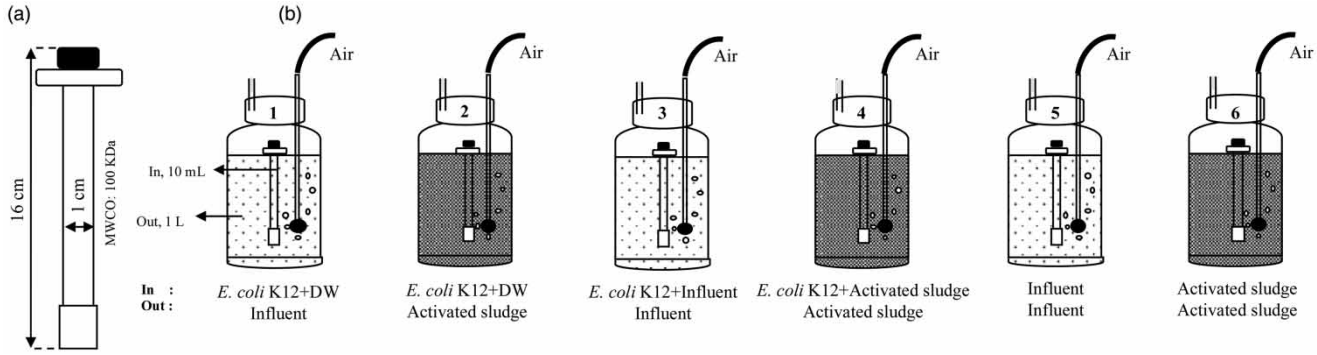

Figure 1 | (a) Dimensions of the dialysis device used in the experiment. (b) Experimental conditions are described as 'inside of the dialysis membrane' and 'surrounding of the membrane'. (*E. coli* K12 + DW)/Influent: inoculated *E. coli* K12 in DW and incubated in influent, (*E. coli* K12 + DW)/Activated 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).

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 25 °C 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.

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

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

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

Plaque assay was used to determine bacteriophage concentration. The sample was centrifuged at 11,100 *g* for 5 min and the supernatant was collected. After addition of 0.1% chloroform, the sample was shaken for 20 min before performing plaque assay on LB agar plate. *E. coli* K12 was used as a host strain for plaque assay. Bacteriophage propagation was examined by introducing *E. coli* K12 into 100 mL filtered influent and activated sludge. Influent and activated sludge were centrifuged at 6,300 *g* for 10 min and the supernatants were collected. The samples were filtered through a 0.45 and 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, 1/1,000 PBS and DW with the conductivity of 1,590, 177.1, 18.8, 1.9, and 0.08 mS m^{-1}, respectively. The samples were incubated for 1 week under the same conditions as described before. Samples were plated on LB media and the colonies were counted after incubation at 37 °C for 24 h.

Analysis of microbial community by next-generation sequencing

Withdrawn samples from the dialysis device were centrifuged at 6,300 *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 1,800 *g* for 5 min. The further DNA extraction process was described by Lee (2013) and 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 polymerase chain reaction (PCR). Miseq16S_341F (5′-TCG TCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGG GNGGCWGCAG-3′) and Miseq16S_805R (5′- GTCTCG TGGGCTCGGAGATGTGTATAAGAGACAGGACTACHV GGGTATCTAATCC-3′) were used to amplify the V3 to V4 region of the 16S rRNA gene. The program for the first round of PCR amplification was 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 72 °C 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 μL^{-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 72 °C 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 were analyzed by QIIME ver.1.9.1 pipeline (Caporaso *et al.* 2010). Chimeric sequences were checked using Usearch (version 6.1.544) coupled with the 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′-CCTACGGGAGG-CAGCAG-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 (1,590 ± 18.2 mS m^{-2}), 85% of that of influent (52.8 ± 5.9 mS m^{-2}) and 81% of that of activated sludge (41.0 ± 2.4 mS m^{-2}) after 2 h (Figure 2(a) and 2(b)).

By incubating the membrane in a glucose solution (10 g L^{-1}), the concentration of glucose in the membrane increased and almost reached the value of the surrounding solution after 2 h (Figure 2(c)). 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 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 the surrounding bulk solution (8.0 ± 1.1 mg L^{-1}) after 2 h (Figure 2(d)).

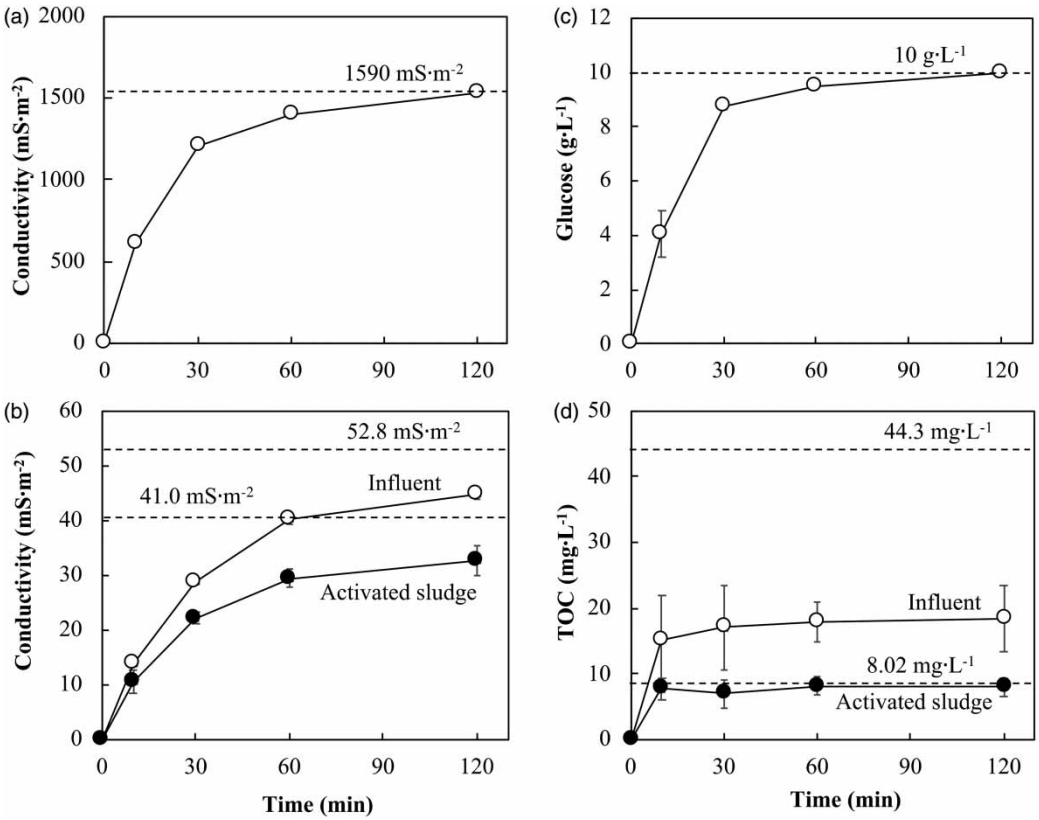

Figure 2 | Ion penetration into the dialysis membrane device from its surroundings: (a) PBS, (b) influent and activated sludge and the nutrient penetration into the dialysis device from its surroundings: (c) 10 g·L^{-1} glucose, (d) influent and activated sludge. The dotted lines indicate the surrounding conductivity of PBS (1,590 ± 18.2 mS·m^{-2}), influent (52.8 ± 5.9 mS·m^{-2}), activated sludge (41.0 ± 2.4 mS·m^{-2}), and TOC of influent (44.3 ± 10.6 mg·L^{-1}), activated sludge (8.0 ± 1.1 mg·L^{-1}).

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

Figure 3(a) shows 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 (1.2×10^6 CFU mL^{-1}) was about 4.6 higher than *E. coli* (2.6×10^5 CFU 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 (6.97×10^9 copies·mL^{-1}) was higher than that in influent (9.08×10^8 copies·mL^{-1}) (Figure 3(b)). 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 (Figure 4(a)). 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 10-fold. 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 the *E. coli* K12 count from 3 days onwards. By introducing *E. coli* K12 into

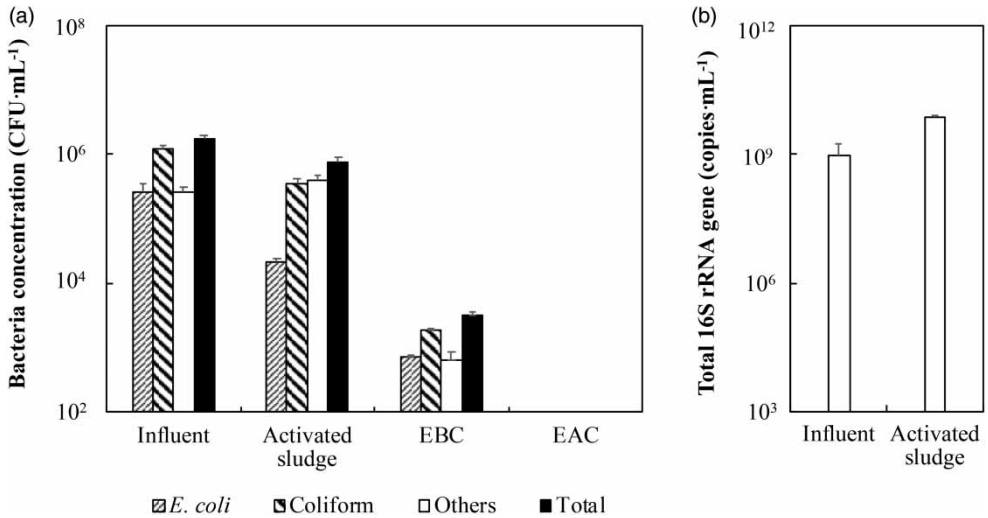

Figure 3 | (a) Concentrations of indigenous *E. coli*, coliform bacteria, other bacteria, and total bacterial count in influent, activated sludge, EBC, and EAC on Chromocult® Coliform Agar. (b) Total 16S rRNA gene in influent and activated sludge.

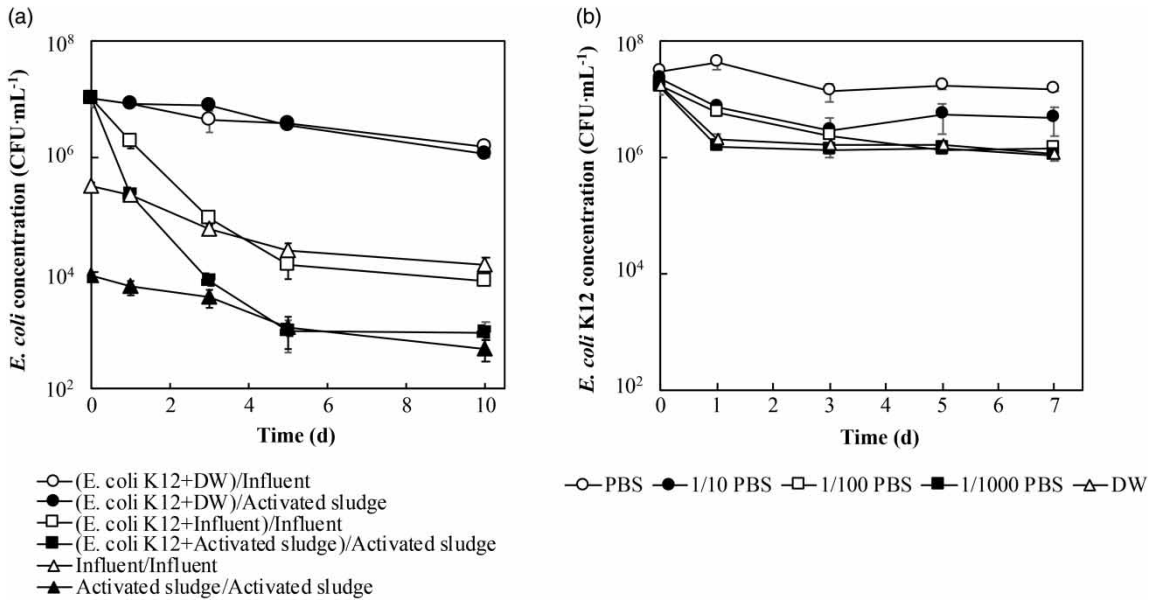

Figure 4 | (a) The fate of *E. coli* K12 in influent and activated sludge over a 10-day period using a dialysis membrane as a support. (b) Survival of *E. coli* K12 in 100 mL diluted PBS and DW for 7 days. *E. coli* K12 incubated in PBS (PBS), *E. coli* K12 incubated in PBS diluted 10 times (1/10 PBS), *E. coli* K12 incubated in PBS diluted 100 times (1/100 PBS), *E. coli* K12 incubated in PBS diluted 1,000 times (1/1,000 PBS), and *E. coli* K12 incubated in DW (DW).

different ionic concentrations in 100 mL of diluted PBS, *E. coli* K12 was able to maintain a high concentration for 1 week (Figure 4(b)). 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 (Figure 5(a)). 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,

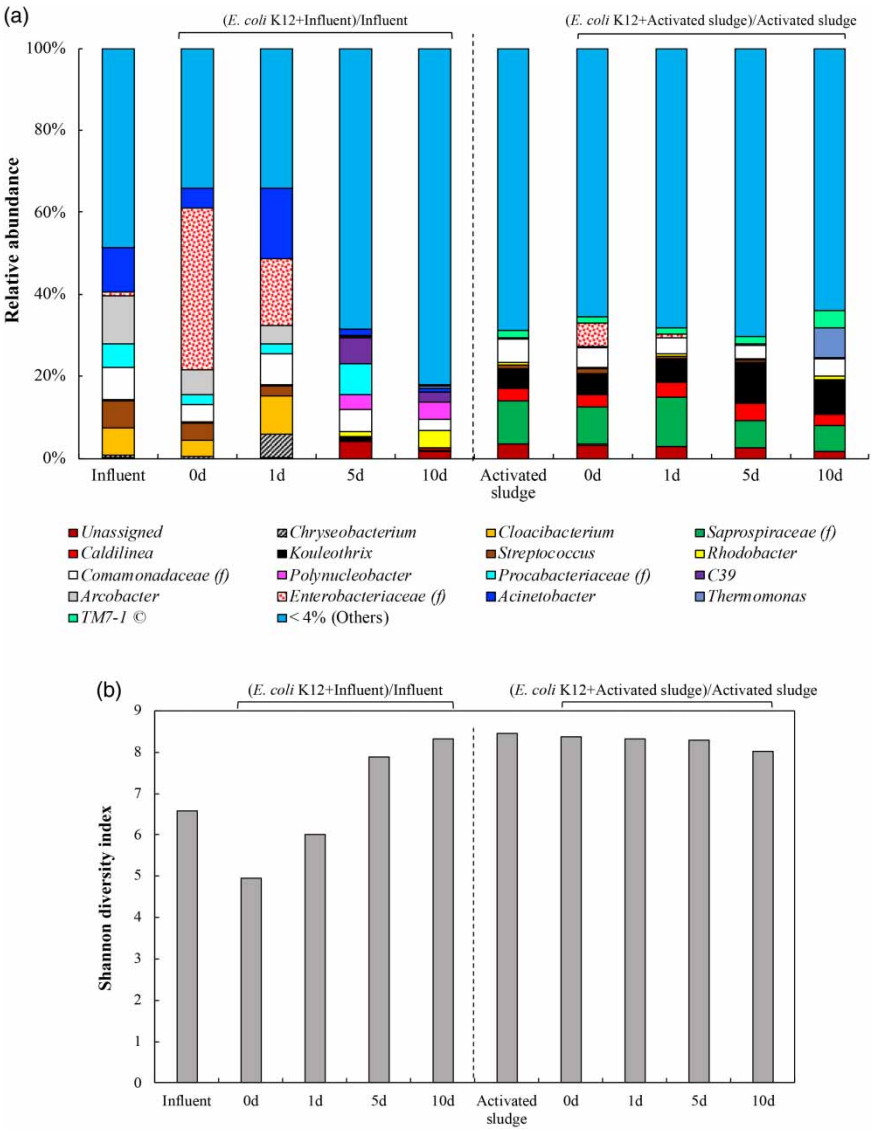

Figure 5 | (a) The bacteria relative abundance in influent and activated sludge before and after incubation with *E. coli* K12 for 10 days (genus with abundance >4%). (b) Shannon diversity index of influent and activated sludge before and after incubation with *E. coli* K12 for 10 days. (*E. coli* K12 + Influent)/Influent: inoculated *E. coli* K12 in influent and incubation in influent, (*E. coli* K12 + Activated sludge)/Activated sludge: inoculated *E. coli* K12 in activated sludge and incubation in activated sludge.

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 1 day. The relative abundance of *E. coli* K12 decreased continuously and almost disappeared from the influent after 5 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 10-fold higher than that in influent (Figure 3(b)). *E. coli* K12 represented about 6% of the total microbial community in activated sludge. About 85% of *E. coli* K12 was decreased after 1 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 5 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 (Figure 5(b)), the community diversity in activated sludge before and after introducing *E. coli* K12 remained almost unchanged over 10 days. The community diversity in influent, however, was reduced after the introduction of *E. coli* K12. Nevertheless, it was increased, going from 1 to 10 days' incubation.

Bacteriophage infection of *E. coli* K12

Bacteriophages, which are a biotic factor, play an important role in controlling the growth of various bacteria in environmental water. We therefore quantified the concentration and infectivity of bacteriophages on *E. coli* K12 inoculated and incubated in influent and activated sludge (Figure 6(a)). The concentration of bacteriophage in influent (2.9×10^3 PFU·mL^{-1}) was about 100-fold higher than that in activated sludge (60 PFU·mL^{-1}). Above 10^7 CFU·mL^{-1} of *E. coli* K12 was inoculated in and incubated in influent and activated sludge. The concentration of *E. coli* was decreased and was still detected after 10 days at low concentration (Figure 4(a)). However, there was no increase in bacteriophage concentration during the incubation period. The concentration of bacteriophage in activated sludge decreased and became undetectable after 1 day while the bacteriophage concentration in influent decreased and was still detected at a lower concentration after 10 days. By introducing *E. coli* K12 into influent and activated sludge, we determined that bacteriophage was not a potential factor that affected the fate of *E. coli* K12.

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

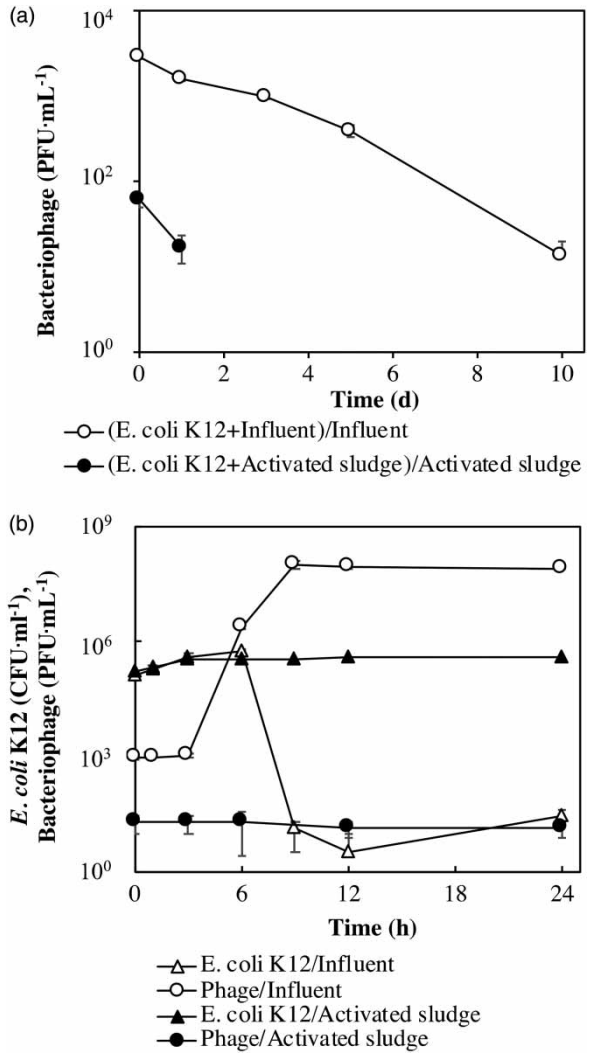

Figure 6 | (a) The evaluation of bacteriophage concentration in influent and activated sludge after incubation with *E. coli* K12 for 10 days. (*E. coli* K12 + Influent)/Influent: inoculated *E. coli* K12 in influent and incubated in influent, (*E. coli* K12 + Activated sludge)/ Activated sludge: inoculated *E. coli* K12 in activated sludge and 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).

no bacteriophage increase in filtered activated sludge after 24 h. The bacteriophage concentration in filtered activated sludge was observed to increase about 100-fold 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 environmental water such as influent and activated sludge is quite difficult. The device developed in this work enables the problem to be solved. By enclosing the model of gastrointestinal bacterium (*E. coli* K12) in the biologically inactive dialysis membrane and exposing it to different environments, we can trace the fate of *E. coli* K12 and identify the influential factors.

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 (Figure 2). The limitation of small molecules, less than 100 kDa, may have driven the numbers of ions and nutrients that penetrated 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 fraction 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 (Katsoyiannis & Samara 2007; Gianico *et al.* 2013).

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 (Figures 4(a) and 5(b)). 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 retention time coupled with a hydraulic retention time of around 10 h (Hreiz *et al.* 2015). During an extended period, a portion of pathogenic bacteria may be adsorbed into the flocs and settle down. At the same time, the biological factors may exert themselves on their survivability. The highlight of predator action and bacterial competition on the removal of pathogenic bacteria has been reported (Wen *et al.* 2009; Sheng *et al.* 2010; Orruno *et al.* 2014). As revealed in this study, the reduction of *E. coli* K12 in influent or activated sludge was not relevant to osmolality induction since ions concentration found in those environments was enough to maintain the cell concentration for 10 days.

E. coli K12 is a foreign strain and may need time to adapt and compete for nutrients with indigenous bacteria in influent and activated sludge. Since nutrients are limited in those environments, competition for them must be high, particularly in activated sludge. As shown in our study, the relative abundance of indigenous microbial community in wastewater, before and after introducing *E. coli* K12 for extended incubation periods, was well established (Figure 5(a)). Even when large amounts of *E. coli* K12 were introduced into complex microbial structures, they did not strongly affect indigenous bacteria. The establishment of the microbial community and their diversity in wastewater are important factors contributing to the induction of foreign microorganisms and the maintenance of efficiency of the wastewater treatment process. Camper *et al.* (1991) showed that the growth rate of environmental strains of *Klebsiella pneumoniae* was almost two times higher than the clinical strain in a low-nutrient environment. The environmental strains were well adapted to different environmental conditions (Camper *et al.* 1991). Consequently, the well-adapted strain coupled with the ability to use a variety of nutrients enabled it to become a dominant species in the environment (Hibbing *et al.* 2010).

The presence of bacteriophage in wastewater is thought to be a biotic factor that controls the numbers of pathogenic bacteria. However, there was no increase in bacteriophage numbers when *E. coli* K12 was introduced into the influent or activated sludge and incubated for 10 days (Figure 6(a)). 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 (Figure 6(b)). 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 (You *et al.* 2002; Julia *et al.* 2014). 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 & Chróst (2000) have shown that a concentration of 0–2 PFU 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 10-fold in activated sludge compared with influent. The native population of microbial communities in wastewater, particularly in activated sludge, were more persistent than *E. coli* K12. The bacteriophage was proposed as one of the factors that control the numbers of *E. coli* K12 if they were able to grow in wastewater. The ion and nutrient concentrations in influent and activated sludge were sufficient to support the survival of *E. coli*. The experimental protocol developed in this study can be widely used to trace the fate of chemical and biological contaminants in the actual environmental water.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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