Occurrence and Removal of Enterohemorrhagic *Escherichia coli* O157:H7 in Four Full-scale Sewage Treatment Systems in Beijing, China

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Enterohemorrhagic *Escherichia coli* O157:H7 (EHEC O157:H7) is zoonotic pathogen linked with severe human illnesses. In order to evaluate risks of the pathogen in wastewater treatment process, the occurrence and removal of them were studied in four municipal wastewater treatment systems every three months from November, 2011 to July, 2012. Real-time PCR for *E.coli* O157:H7 was performed on extracted DNA using primers targeting *eaeA* gene. The method was used to detect *E.coli* O157:H7 in raw wastewater, mixed liquor, secondary clarifier effluent, excess sludge and sludge liquor of the four systems mentioned above. The quantity of *E.coli* O157:H7 in raw wastewater varies with the season, ranging from 10^3 to 10^5 copies per milliliter(copies/ml) in summer, from 10^2 to 10^3 copies/ml in winter. However, effluent keeps relatively steady, 10 to 10^2 copies/ml. The removal of *E.coli* O157:H7 was about 1 to 3 orders of magnitude in copies per milliliter. The amount in sludge liquor is more close to influent than in effluent, which showed the sludge liquor should not be discharged into natural water bodies directly as it still threatens people's health. The result also indicated that EHEC might not be a kind of bacteria joining to make up zoogloea. In order to understand the mechanism of the removal of EHEC, the quantity of *E.coli* O157:H7 in effluent in copies per millimeter was compared with that of excess sludge, and the latter makes up a proportion ranged from 54.22% to 96.14%, which means the majority of *E.coli* O157:H7 came into returned sludge in secondary sedimentation tank. Thus, temporary transference from water phase to sludge is one reason of *E.coli* O157:H7 decreasing.

Key words: Enterohemorrhagic, *Escherichia coli* (EHEC), real-time PCR, wastewater treatment.

Enterohemorrhagic *Escherichia coli* (EHEC) (O157 and other serotypes) are zoonotic pathogens linked with severe human illnesses, such as diarrhea, abdominal pain, hemorrhagic colitis and hemolytic–uremic syndrome (HUS). EHEC O157:H7 is the serotype most commonly associated with large outbreaks. Water has been documented as a way of transmission of EHEC, which may contaminate waters (recreational, drinking or irrigation waters) through feces from humans and other animals(Muniesa *et al*., 2006). Wastewater is the source of many human enteric pathogens, such as *E.coli* O157:H7(Srinivasan *et al*., 2011). Many pathogens still exist even in influent of secondary sediment tank in municipal wastewater treatment plant(MWWTP)(Meric S, 2009). Thus, reclamation and reuse of wastewater, which are means for extending water supplies, and influent of MWWTP discharging into rivers, threatens people's health(Rajkowski and Rice, 1999).

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Conventional culture techniques for the detection of *E. coli* O157:H7 in water and environment are labor intensive and time consuming, and often fail to recover of the bacteria, when the concentration in the sample is low (below the threshold of sensitivity) or when cells enter a viable but non-cultivable state after stress or injury. Molecular methods detecting virulence genes, such as real-time PCR, have been developed for the direct identification of *E.coli* O157:H7 in food and environmental samples(Chassagne *et al.*, 2009; Gannon *et al.*, 1997; Sharma *et al.*, 1999; Spano *et al.*, 2005; Yoshitomi *et al.*, 2003).

The pathogenicity of EHEC O157: H7 is associated with a number of virulence factors, including Shiga toxins 1 and 2 (encoded by genes *stx1* and *stx2*), and intimin (encoded by the gene *eaeA*). Intimin facilitates the adherence to attachment and effacement(Moon *et al.*, 1983). PCR has become a useful diagnostic tool because it is quick, specific, sensitive and relatively inexpensive. Among the PCR-based methods, quantitative real-time PCR is capable of both identifying and quantifying single bacterial species in a complex background microbial population, as well as in a complex sample. Several authors have used a real-time PCR method to identify *E. coli* O157:H7 in Fresh-Cut Cabbage(Kim *et al.*, 2011), pure cultures (Yoshitomi *et al.*, 2003), meat products (Suo *et al.*, 2010), dairy products(Derzelle *et al.*, 2011), faeces(Sharma, 2002),surface water (Bonetta *et al.*, 2011) and wastewater (Srinivasan *et al.*, 2011).

In this research, The occurrence and removal of EHEC O157:H7 was studied in four municipal wastewater treatment systems (anaerobic-oxide, anaerobic-anoxic-oxide, sequencing batch reactor and oxidation ditch) from November, 2011 to July, 2012. Real-time PCR was used to detect EHEC O157:H7 in raw wastewater, mixed liquor, secondary clarifier effluent, returned sludge (or excess sludge) and sludge liquor of the four systems mentioned above, using primers targeting *eaeA* gene.

MATERIALS AND METHODS

Study sites and sample collection

Four municipal wastewater treatment plants investigated in this study all belong to typical activated-sludge systems. Physicochemical parameters of the wastewater, temperature, pH, conductivity, dissolved oxygen (DO) were measured on-site using a portable Hach (Sens-Ion) Multi meter(USA) (table 1). The occurrence and removal of EHEC was studied in these four wastewater treatment systems (anaerobic-oxide, anaerobic-anoxic-oxide, sequencing batch reactor and oxidation ditch) every three month over a period of one year (from November, 2011 to July, 2012). Raw wastewater (debris and grit removed by preliminary treatment), mixed liquor, final effluent, returned sludge and sludge liquor were obtained for microbiological analysis. Samples were preserved in an ice container at 4°C prior to the analysis and processed within 24 h of sample collection.

Enrichment of enterohemorrhagic *Escherichia coli*

As the quantity of enterohemorrhagic *Escherichia coli* in wastewater is low for conventional PCR to detect, enrichment of EHEC is necessary. Samples were enriched at 5ml mixed liquor of sequencing batch reactor to 100ml mEC broth supplemented with 20µg/ml Novobiocin, and incubated at 37°C overnight. After enrichment, broth was plated onto cefixine tellurite sorbitol MacConkey(CT-sMAC) supplemented with 0.01mg/L cefixime and 0.5mg/L potassium tellurite. Plates were incubated at 37°C overnight. The non-sorbitol-fermenting clones from the plates were picked and streak on CT-sMAC five times. Strains isolated were stored at -80°C in 30% sterile glycerol and ready for DNA extraction.

DNA extraction

Due to the varying amount of solid biomass contained in each type of wastewater sample, different volumes of the samples were used for the genomic DNA extraction. DNA was extracted from biomass collected from 100ml raw wastewater, 10ml mixed liquor, 10ml returned sludge, 500ml final effluents and 50ml sludge liquor. The samples above were filtered passing through 0.22-µm-pore-size filter membrane and biomass was remained on the membrane. Then the membrane was cut into pieces and collected into E-tube (included in FAST DNA exaction kit for soil, MP). DNA was extracted directly with the FAST DNA exaction kit for soil which is effective and efficient for DNA extraction of complicated wastewater and sludge samples, according to the manufacturer's

instruction. Total DNA of each sample were set as DNA template in real-time PCR. DNA of the isolated strain was extracted with the Genomic DNA purification kit (Fermentas, Canada).

Conventional PCR protocol

PCR for *E.coli* virulence gene amplification was performed on DNA of isolated strain using primers targeting gene associated with *eaeA* that mediates the intimate adherence of the organism to host cells *(Bonetta et al., 2011)*. Primers *eae*150 (Forward-GGC GGA TTA GAC TTC GGC TA, Reversed-CGT TTT GGC ACT ATT TGC CC)(Sharma *et al.*, 1999) were used for the PCR assay. The primers were synthesized by Invitrogen Life Technology. The PCR was performed with a thermal cycler (Eppendorf, Germany). The reaction was carried out as follows: initial denaturation at 94°C for 7 min followed by 35 cycles of denaturation at 94°C for 30 sec , annealing at 55°C for 30 sec and extension at 72°C for 30 sec, and a final elongation at 72°C for 10 min. PCR mixture contains 5µL Taq buffer, 4µL dNTP (1.5 mmol/L, final concentration), 1 µL of each primer (0.4mmol/L, final concentration), 0.2 µL BSA (10 mg/mL), 1µL template DNA, 0.4 µL Taq DNA polymerase (5 U/µL,Takara,China) and add RNase free dH$_2$O (Takara, China) to a final volume of 50 µL. The PCR product of 150 bp was checked using 1.0% agarose gel electrophoresis.

Cloning and sequencing

PCR products were purified with cycle pure kit (Fermentas, Canada) and then ligated into pGEM-T easy vector (Promega, USA). The recombinant plasmids were transformed into competent DH5α cells (Takara, China) via heat shock following the manufacture's protocol. Cells were incubated at 37°C overnight on agar plates containing 0.05% Ampicillin, IPTG and X-Gal. White clones were selected for Colony PCR, and then sent to be sequenced by Shanghai Majorbio Bio-pharm Biotechnology Company. The plasmid of positive one of sequencing was extracted and prepared for the standard curve of real-time PCR.

Real-time PCR protocol

The real-time PCR was carried out in ABI PRISM 7300 Real-Time PCR System (USA).The same primers for conventional PCR were still utilized and each 25µl reaction consisted of a 25µl total volume mixture with 12.5µl of SYBR Premix Ex Taq TM II(2×) (Takara, China), 0.2µM of each primer(10µM), 0.5µl of ROX Reference Dye(50×)

(Takara, China), 0.5µl of bovine serum albumin (BSA, 10 mg/mL), 2µl of DNA template, and water to volume. The PCR reaction was optimized to the conditions of 95°C for 30 sec, followed by 40 cycles of 95°C for 15 sec, 65°C for 20 sec, and 72°C for 15 sec, with fluorescence being measured during the extension phase. Melting curves were subsequently carried out and consisted of 95°C for 15 sec and 60°C for 20 min, increasing in 0.5°C increments to 95°C.

To create the standard curve for the PCR assay, serial dilutions of DNA were prepared from DNA of the plasmid containing *eae* gene as described in the previous section. The 10-fold serial dilutions of three independent experiments were used to determine the initial starting concentration of cells and template DNA copy numbers. The fluorescence along with the DNA template number results were used to construct a linear curve that correlated the first cycle number at which fluorescence was detected to the number of cells per milliliter. For each reaction, the threshold cycle number (C_t) was determined to be the cycle number at which fluorescence was >400 fluorescence units. Melting curves were created and analyzed using the 7300 System SDS software. Efficiency of amplification (E) was estimated from standard curve data by using the formula:

$$E = (10^{-1/slope}) - 1$$

A theoretical 100% efficiency reaction would have generated a slope of -3.32(Ibekwe and Ma, 2011). The unknown samples were set two parallel tests.

RESULTS

Isolation and Identification of enterohemorrhagic *Escherichia coli*

One strain of enterohemorrhagic *Escherichia coli* was isolated from the mixed liquor of wastewater treatment plant. DNA of the pure culture was extracted and amplified by PCR targeting V3 region of 16s rDNA using primers 27F, 1492R (Lane, 1991) and *eae* gene using *eaeA* primers mentioned above. The two PCR products were sent to be sequenced. Then the sequences were both compared in GeneBank using BLAST tool in NCBI. The former 1465bp PCR product was achieved 99% identity with other *E.coli* and confirmed to be a

Table 1. Physicochemical characteristics of municipal wastewater
samples from four wastewater treatment plants in Beijing*

Secondary treatment system	Source	Temp (°C)	pH	Conductivity (µS/cm)	Dissolved oxygen (mg/L)
Anaerobic-Oxide(AO)	Influent	18.1	7.69	1223	0.61
	Anaerobic	18.2	7.15	1044	1.03
	Oxide	17.9	7.01	985	3.55
	Effluent	17.9	7.00	1003	3.93
Anaerobic-Anoxic-Oxide(A²O)	Influent	16.3	7.65	1367	1.85
	Anaerobic	16.2	7.13	1285	0.22
	Anoxic	16.0	7.05	1094	0.41
	Oxide	16.7	6.98	1040	2.41
	Effluent	17.4	7.20	1030	4.01
Sequencing Batch Reactor(SBR)	Influent	17.5	7.88	2324	2.14
	Mixed liquor	18.4	7.23	1728	1.58
	Effluent	19.2	7.38	1736	6.46
Oxidation Ditch(OD)	Influent	16.9	7.43	1278	0.60
	Mixed liquor	16.7	7.28	1034	2.28
	Effluent	17.0	7.76	1040	4.40

*Values in the table are mean values of four sampling.

strain of *E.coli*. The 150bp PCR product of the later primers achieved 99% identity with *Escherichia coli eae* gene for intimin. Therefore, the isolate strain was confirmed to be a kind of enterohemorrhagic *Escherichia coli.*

PCR amplification of DNA extracted from the isolated EHEC

PCR for *eae* gene was first tested on pure DNA from isolated type to optimize the amplification conditions before real-time PCR (Fig.1). The PCR product was cloned, and the plasmid containing *eae* gene was set to make standard curve of real-time PCR.
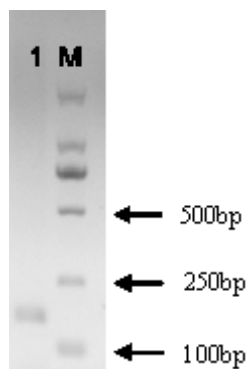
Real-time PCR

Real-time PCR with SYBR Green I was performed with DNA extracted from wastewater samples. Standard curve was generated from the plasmid containing *eae* gene ranging from 10^8 copies per millimeter to 10 copies per millimeter (Fig. 2). The slope of the curve was -3.1918, and the squared regression coefficient of the gene after the linear regression was 0.9942. The efficiency of amplification of total wastewater DNA extracted, calculated from standard curves by the formula given in supporting information of the Materials and methods, was all around 105%.
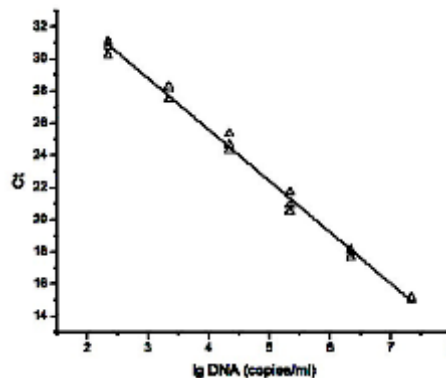

Fig. 1. Polymerase chain reaction (PCR) on DNA extracted from pure cultures of isolated strain EHEC- line 1, *eae* amplicon(150bp); M, DL 2000 DNA marker (Takara)

Fig. 2. Standard curves for the real-time polymerase chain reaction

The relative starting quantity of target DNA was calculated by comparing data from standard curves. Real-time PCR detection of *E. coli* O157:H7 in wastewater samples gave the results reported in Fig. 3. During all the four processes, the quantity of *E.coli* O157:H7 in influent was changed along with the season changing: the quantity ranged from 10^3 to 10^5 copies/ml in
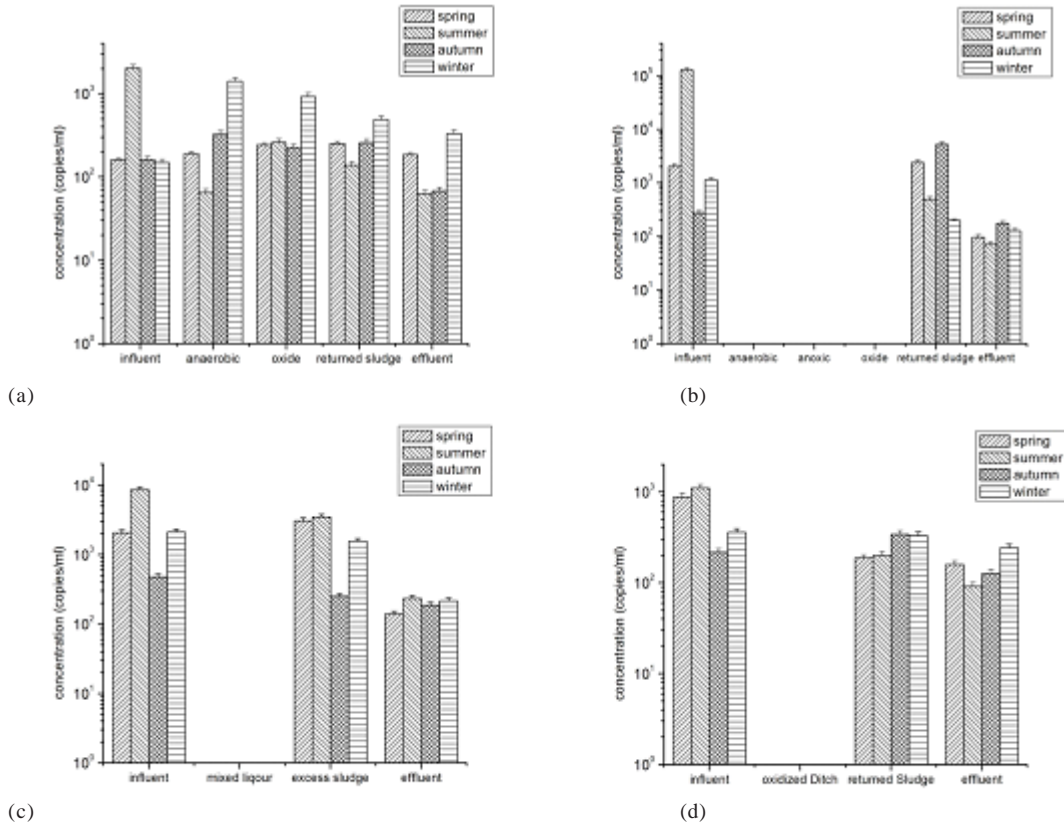

(a)

(b)

(c)

(d)

Fig. 3. (a) The quantity of *E.coli* O157:H7 during anaerobic-oxide system in four seasons;(b)The quantity of *E.coli* O157:H7 during anaerobic-anoxic-oxide system in four seasons;(c) The quantity of *E.coli* O157:H7 during sequencing batch reactor system in four seasons;(d) The quantity of *E.coli* O157:H7 during oxidation ditch system in four seasons
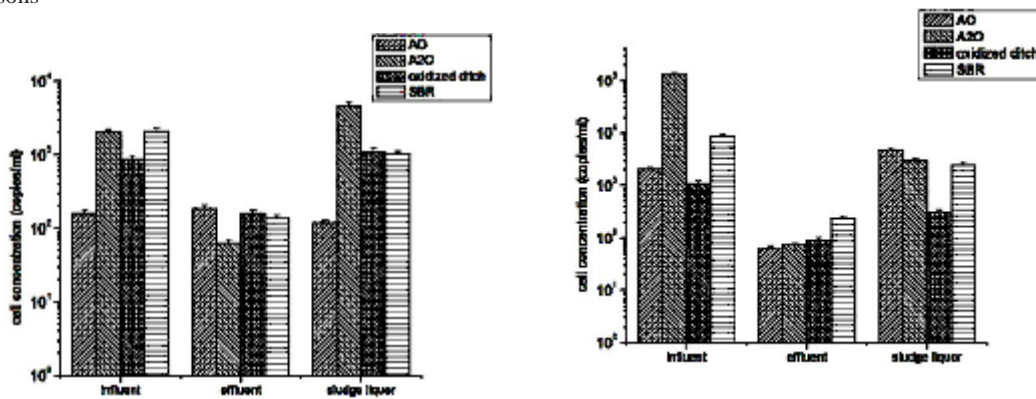

Fig. 4(a). The comparison of the amount of *E.coli* O157:H7 in influent, effluent and sludge liquor in the four systems in spring; (b) The comparison of the amount of *E.coli* O157:H7 in influent, effluent and sludge liquor in the four systems in spring

summer, and from 10^2 to 10^3 copies/ml in winter. On the contrary, effluent of each treatment system keeps relatively steady, 10 to 10^2 copies/ml. Therefore, the removal of *E.coli* O157:H7 was about 1 to 3 orders of magnitude in copies per milliliter. However, if the amount of *E.coli* O157:H7 was about 10^2 copies/ml or less in influent, it is difficult to make the amount of bacteria less after wastewater treatment systems based on certain data of the four systems in autumn and winter. In the mixed liquor, *E.coli* O157:H7 is few or below the detection limit, which might owe to dilution by activated sludge.

In secondary sedimentation tank, water and sludge was separated and the quantity measured in copies per millimeter of each was compared. The amount in returned sludge (or excess sludge) made up a proportion ranged from 54.22% to 96.14% (average value was 74.66%), which means the majority *E.coli* O157:H7 came into returned sludge in secondary sedimentation tank. Thus, the removal of EHEC is much more likely due to the temporary transference from water phase to sludge.

Returned sludge would be dewatered in the end, and be divided into dewatered sludge and sludge liquor. The quantity of *E.coli* O157:H7 in influent, effluent was compared with it in sludge liquor (Fig. 4). The data shows that the amount of *E.coli* O157:H7 in sludge liquor was more close to that in influent than in effluent, which showed that the sludge liquor should not be discharged into natural water bodies directly as it still threatens people's health.

DISCUSSION

Municipal WWTPs are not designed to completely remove human waterborne pathogens and therefore they are recognized as reservoirs of such microbiological contaminants occurring in surface waters (Cheng *et al*., 2009). *Escherichia coli* O157:H7 is a pathogenic type of *E. coli* and can lead to human diseases. This strain has emerged in recent years as important human pathogen associated with a wide spectrum of diseases ranging from mild nonbloody diarrhea to haemorrhagic colitis and haemolytic-uraemic syndrome(Karmali, 1989; Spano *et al*., 2005). *Escherichia coli* O157:H7 was detected, by real-

time PCR using SYBR Green I, in wastewater samples. The detection of EHEC by PCR has been achieved by amplification of the sequences located in the 5' two-thirds of the gene *eaeA* (Gannon *et al*., 1993; Sharma *et al*., 1999). This region of *eaeA* gene is highly conserved among EHEC serotypes. PCR assays that allow differentiation of EHEC O157:H7 from other EHEC use primers that have homology in the 3' one-third of the *eaeA* gene (RICHTER and BRUNTON, 1994). The 3' end of the *eaeA* gene is less conserved among EHEC serotypes. Although the use of the primers in PCR facilitated the detection of all EHEC O157:H7 strains, these primers also detected entero-pathogenic *E. coli*(EPEC) strains of serotype O55:H7 and an EHEC strain of serotype O145:NM, which is unavoidable as they contains highly homologous *eae* gene (Sharma *et al*.,1999). SYBR Green I real-time PCR or multiplex PCR has been used for the detection of *E.coli* O157:H7 in pure culture by others (Chassagne *et al*., 2009; Hu *et al*., 1999; Paton and Paton, 1998a). But our study proved poor specificity of the primers they used when employed for environmental samples which is consisted of various microbes (data not shown). SYBR Green I real-time PCR using primers *eaeA* to detect *E.coli* O157:H7 in wastewater samples was developed in this study; it is effective as the specificity of the primers. It is a low cost assay compared to Taqman probes methods. However, one key limitation of real-time PCR methods is the inability to differentiate between live and dead cells. It was reported significant difference between real-time PCR and cultivable levels of *E.coli* and enterococci following disinfection (chlorination). But it is not clear if this difference in number is solely due to the dead cells or if some of the cells had transformed into a viable but non-cultivable stressed state, which prevents their growth in culture media (Srinivasan *et al*., 2011). Therefore, to take real-time PCR methods or culture methods should be based on the objective of experiments. The present study demonstrated high effectiveness of reduction of *E.coli* O157:H7 in the four wastewater treatment plants from raw wastewater to the final wastewater effluent in certain conditions. The removal rate was ranged from 88.96% to 99.94% if the amount of *E.coli* O157:H7 was 10^3 copies/ml or more in raw wastewater; On the contrary, it did not show

obvious reduction if the amount was less than 10^2 copies/ml in raw wastewater. It might be caused by the limitation of the detection method or the background pathogen due to a period time of operation and recycle of activated sludge. Therefore, the removal by wastewater treatment system is limited and the lowest concentration that WWTPs can show effective reduction of *E.coli* O157:H7 in raw wastewater was about 10^2 copies/ml. The quantity in raw wastewater varies with the season, but the quantity in effluent keeps steady with season changing, which indicates the processing keeps relatively stable with season changing. The high quantity in raw wastewater in summer reflects the fitting conditions for *E.coli* O157:H7 in summer. As reported, *E.coli* O157:H7 infections are more common in warmer months than in colder months, with a peak incidence from June through September(Boyce *et al.*, 1995). Although the quantity is only 10-10^2 copies/ml in effluent of secondary sedimentation tank, it still threatens to people's health, as the enterohemorrhagic *E. coli* strains infect with a very low infectious dose (1-10^2 CFU)(Paton and Paton, 1998b). The result that the amount of *E.coli* O157:H7 in sludge liquor was more close to that in influent than in effluent, indicated that, *E.coli* O157:H7, as an external one, might not be a kind of bacteria joining to make up zoogloea.

During the four treatment systems, *E.coli* O157:H7 is little or below the detection limit in the mixed liquor, which might be caused by dilution of activated sludge mainly consisting of indigenous microorganisms. We proved one reason of *E.coli* O157:H7's reduction was the transference from water phase to sludge. It has been reported by others that reduction in numbers of pathogens is brought about by the combined effects of separation of solid material (most microorganisms are associated with solids), predation, competition from naturally occurring organisms, and inactivation due to changes in pH and temperature etc.(Godfree and Farrell, 2005).

In terms of public health, the management of sewage sludge both during and following wastewater treatment systems has epidemiological implications. On one hand, it might be dangerous to operatives as EHEC exists during the treatment process, especially in returned sludge and sludge liquor. Therefore, preventive measures are recommended for workmen. On the other hand, influent of secondary sedimentation tank is usually discharged into surface water, or for scenic environment use, agricultural use and industrial use in the place of conventional water resources, which brought risk to people although the quantity of EHEC O157:H7 is at a low level. Beijing has been integrating reclaimed wastewater as an important water resource since 2003 to solve the persistent problem of water shortage(Chang and Ma, 2012). Therefore, more effective treatment measures are needed for both excess sludge and effluent of secondary treatment.

Further studies might be focused on effective removal of EHEC or other pathogenic bacteria during wastewater treatment process. It was reported that there is potential for phage treatment to be used successfully in combination with biological sludge stabilization processes to reduce the abundance of specific pathogenic bacterial strains such as *E. coli* O157 (Withey *et al*., 2005). Therefore, phage treatment combined with activated-sludge process is a significant research area in the future.

CONCLUSION

In all the four systems, the concentration of *E.coli* O157:H7 in raw wastewater was different in different seasons, ranging from 10^3 to 10^5 copies/ml in summer, from 10^2 to 10^3 copies/ml in winter. However, effluent of each treatment system keeps relatively steady, 10 to 10^2 copies/ml. The removal of *E.coli* O157:H7 was about 1 to 3 orders of magnitude in copies per milliliter. Based on certain data of the four systems in autumn and winter, 10^2 copies/ml might be a treatment limit of these wastewater treatment systems in raw wastewater, or the difference between the quantity in raw wastewater and that in final effluent cannot be detected using real-time PCR.

The removal of EHEC is much more likely due to the temporary transference from water phase to sludge, as the majority of *E.coli* O157:H7 came into returned sludge in secondary sedimentation tank. Data shows that the amount of *E.coli* O157:H7 in sludge liquor was more close to that in influent than in effluent, which showed that the sludge liquor should not be discharged into natural water bodies directly as it still threatens people's health.

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