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Implication of environmental factors on the occurrence of pathogens in urban landscape ponds with reclaimed wastewater replenishment

Dong Ao[a,b], Yue Huang[b], Tao Xue[b], Nan Wang[b] and Rong Chen[b,*]

[a] School of Environmental and Chemical Engineering, Xi'an Polytechnic University, Xi'an 710048, China

[b] International S&T Cooperation Center for Urban Alternative Water Resources Development; Key Laboratory of Environmental Engineering, Shaanxi Province; Xi'an University of Architecture and Technology, No.13 Yanta Road, Xi'an 710055, China

*Corresponding author. E-mail: chenrong@xauat.edu.cn

Abstract

One year of continuous observation of bacterial and viral pathogen concentrations in overlying water and sediment of three urban landscape ponds replenished with reclaimed wastewater (RW) ponds was carried out to establish the distribution of pathogens and investigate the effects of environmental factors on that in RW ponds. The pathogens were represented by *Escherichia coli* and three common viral pathogens (enterovirus, norovirus, and rotavirus). Results indicated that the peak concentrations of pathogens occur from August to October. Pathogens present in sediment should be paid much more attention than those in overlying water, as they mainly contribute to the favorable conditions for survival and regrowth of pathogens in sediments. Cluster and redundancy analyses revealed that the environmental factors of chlorophyll a (Chl-a), organic matter, and water transparency have key impacts on the occurrence of pathogens. This infers that the practical way to reduce pathogenic risks in RW ponds is to control the algae bloom and improve the transparency of water bodies. Furthermore, based on breakpoint regression analyses, the appropriate ranges of Chl-a and transparency are suggested to be less than 57 mg/m^3 and greater than 68 cm, respectively, to reduce the concentration of pathogens in urban landscape ponds replenished with RW.

Key words: environmental factors, landscape pond, pathogen, reclaimed wastewater, replenishment

INTRODUCTION

The quality of urban ponds is often barely satisfactory because of inadequate water supply, and is a general problem in cities with water shortages (Yi *et al.* 2011). Due to its consistent availability and controllability, reclaimed wastewater (RW) has become a stable alternative source for urban landscape pond replenishment. Using RW can solve the water shortage problem of landscape pond replenishment, but the quality of RW is significantly different from that of surface water, and thus may have a great impact on the quality of the receiving water body. Current researchers are mostly concerned about algae growth in RW ponds, with the primary objective to improve its visual effects (Zhao *et al.* 2015). In fact, recreation is another important function of urban landscape ponds, particularly for those located in parks. Moreover, these ponds may also be the water source of garden irrigation (Yi *et al.* 2011). Therefore, people could be exposed directly to the water, which may have a negative impact on human health when the water quality in

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the ponds deteriorates. This is an important reason behind the public concern regarding the reuse of RW for landscape pond replenishment (Brookes *et al.* 2004; Teklehaimanot *et al.* 2015).

In recent years, health problems caused by the presence of pathogens in water bodies have drawn wide attention. These include acute gastroenteritis caused by rotavirus (RV), vomiting and abdominal pain caused by norovirus (NV), and enteric fever caused by bacterial pathogens (Brookes *et al.* 2004). RW is thoroughly processed in wastewater treatment plants (WWTPs), but residual pathogens in the RW may still exist (Tajima *et al.* 2007). In addition, the high amounts of nutrients in RW could promote the growth and prolong the survival of pathogens. Pathogens in ponds may also be the effect of non-point sources, such as direct rainfall, surface runoff, dust precipitation, animals (birds in particular), and human activities. Most studies found that the concentration of pathogens in the gulf area, beach sand, and urban landscape ponds was increased significantly after being polluted by the effluent of WWTPs, which could cause health risks for visitors to these facilities (Teklehaimanot *et al.* 2015). Our previous survey also indicated that the health risks of pathogens in ponds could be increased with RW replenishment (Zhou *et al.* 2015). Furthermore, as a result of the potentially favorable properties of sediment, the concentration of pathogens in sediment can reach up to hundreds or even thousands of times than that in overlying water (Millis 1989; Abia *et al.* 2016). In addition, urban landscape ponds are generally shallow, and the sediment and overlying water exchange easily during natural turbulence or human recreational activity. Thus, the health risk of ponds may be increased because of the resuspension of pathogenic micro-organisms from sediment. Previous research has confirmed that the main source of pathogens in overlying water is the releasing of pathogens from the sediment (Wu *et al.* 2009). Overall, it is necessary and worthwhile to analyze the effect of pathogens in the overlying water and sediment of RW ponds simultaneously.

The survival of pathogenic bacteria and viruses in water bodies is highly species and strain specific (Hassard *et al.* 2016). Related studies showed that the pathogen populations depend on various factors, such as pressure (Brookes *et al.* 2004), pH, salinity (Staggemeier *et al.* 2015), temperature (Pachepsky & Shelton 2011; Xagoraraki *et al.* 2014), turbidity (Hassard *et al.* 2016), light (McGuigan *et al.* 2012; Nguyen *et al.* 2015), predation (Bogosian *et al.* 1996), nutrients (Shelton *et al.* 2014), biological factors (Xagoraraki *et al.* 2014), sediment moisture content (Sologabriele *et al.* 2000) and organic matter (OM) content (Garzio-Hadzick *et al.* 2010). However, the focus of such studies was on rivers, the coastal and estuarine regions, and large lakes, with those being seldomly polluted directly by RW. As far as we could determine, no studies have been carried out on the influencing factors of the occurrence of pathogens in urban landscape ponds that are replenished with RW.

In this paper, three urban landscape ponds that are located in three cities and are all replenished with RW were selected to gain insight into the temporal effects of various pathogens in the overlying water and sediment of RW ponds, as well as to investigate their relationship with environmental factors. We examined the concentrations of *Escherichia coli* and three common viral pathogens (enterovirus, norovirus, and rotavirus) in the overlying water and sediment of the three RW ponds for a year. The physical and chemical indexes of RW ponds were analyzed simultaneously. Statistical analyses were conducted to analyze the relationships between environmental factors and pathogens. This study identified the key factors affecting the distribution of pathogens and attempted to find the threshold value for decreasing the pathogen concentration of those factors from a statistical perspective, which will be useful for landscape planners and managers to minimize the negative effects of urban ponds' replenishment with RW.

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MATERIALS AND METHODS

Selected ponds and water sources for replenishment

The landscape ponds of Cuihu in Kunming city (designated R1), Fengqing in Xi'an city (R2), and Lingang in Tianjin city (R3), in China, all of which are solely replenished by RW, were chosen for sampling and analysis. The three ponds are not vegetated and are mainly used for esthetic and recreational purposes. The basic information for the three ponds is listed in Table 1. All ponds have similar hydraulic and morphologic features, with hydraulic retention times ranging from 26 to 38 days, average depths from 1.5 to 2.0 m, and surface areas from 5×10^4 to 10×10^4 m^2. In addition, the replenishment frequency of the three ponds was once per day and the ponds did not receive input from storm water runoffs. RW is transferred from the WWTPs to the RW ponds by pipelines.

Table 1 | Physical information for the three selected ponds and their basic properties of reclaimed wastewater (RW)

Ponds		Cuihu	Fengqing	Lingang
Basic information	City	Kunming	Xi'an	Tianjin
	Replenishment source	RW[a]	RW[b]	RW[c]
	Abbreviation	R1	R2	R3
Hydrological properties	HRT(d)	28–34	27–38	26–38
	Mean depth (m)	2.0	1.5	1.9
	Surface area (m^2)	6×10^4	5×10^4	10×10^4
RW quality	COD (mg/L)	19.24 ± 3.32	23.12 ± 2.23	21.93 ± 4.21
	TN (mg/L)	6.39 ± 0.52	9.51 ± 0.40	10.18 ± 0.84
	NO_3^--N (mg/L)	4.63 ± 0.40	8.89 ± 0.42	8.16 ± 0.73
	NH_4^+-N (mg/L)	1.10 ± 0.10	0.35 ± 0.08	2.32 ± 0.82
	TP (mg/L)	0.31 ± 0.15	0.58 ± 0.20	0.42 ± 0.16
	PO_4^{3-}-P (mg/L)	0.25 ± 0.14	0.42 ± 0.21	0.38 ± 0.13

RW treatment processes: [a]anaeroxic-anoxic-oxic (A2O) + membrane bioreactor (MBR) + chlorination; [b]anaeroxic-oxic (A/O) + MBR + chlorination; [c]A2O + MBR + chlorination.

Sample collection and preparation

Field investigations were conducted from January to December 2015. Samples were collected once a month. Rainy days were excluded from sampling, and in the event of rainfall, there was no sampling for at least 5 days. Four sampling sites were arranged for each pond (the spatial distribution of the samplings in each ponds are described in Figure S1 in the supplementary material). The samples of overlying water were the mixture of those collected at a depth of 0, 0.5, and 1.0 m using a water sampler (JC-800A-3, Qingdao, China). Meanwhile, the sediment samples in the same positions as for the overlying water sampling were also sampled, and were collected using a Ponar Grab (HAD-XDB0201D, Beijing, China) at the bottom of each pond. Five liters of water samples were collected from each sampling site and kept in glass bottles. About 1 liter of sediment samples was collected and kept in polyethylene bags. Then, all the samples were transported to the laboratory on ice and stored at −4 °C. In particular, the samples of sediment (50 mL) for pathogen detection were kept separately, and LifeGuard™ Soil Preservation Solution (MoBio Inc., Carlsbad, CA, USA) was added to protect the viability of the pathogens, and those were kept dormant in storage at −20 °C. The temperature, pH value, dissolved oxygen (DO), total dissolved solids (TDS), and transparency (Secchi disk clarity, SD) were measured *in situ*.

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Sample analysis

Environment factors

The quality of overlying water and sediment samples from the three ponds was determined. For the overlying water, the samples were directly analyzed for total nitrogen (TN), total phosphorus (TP), chlorophyll a (Chl-a), and chemical oxygen demand (COD), whereas the filtered samples were analyzed for nitrate nitrogen (NO_3^--N), ammonium nitrogen (NH_4^+-N), and orthophosphate (PO_4^{3-}-P). For the sediment samples, the OM and water content (WC) were detected directly from fresh, wet samples. The sediment samples for total nitrogen (S-TN) and total phosphorus (S-TP) were first freeze dried and then homogenized by passing them through a 75 μm sieve after grinding. All of the analytical measurements were obtained in accordance with standard methods (Environmental Protection Agency of China 2002), and each measurement was carried out in triplicate. In addition, the total solar radiation in this study was calculated from the data of daily solar radiation, which was provided by the local city's meteorological bureau.

Pathogens

According to normal practices, the microbiological quality of water bodies is regulated using bacteria indicators such as fecal coliform. As *E. coli* is considered to be suitable to characterize fecal contamination, it was used as a representative indicator of the bacterial pathogens in RW ponds. In addition, due to the bacterial and viral pathogens having different abilities to resist varied environmental conditions, the level or concentration of bacteria indicators in water bodies may not represent or be proportional to the true level of viral pathogens (Brookes *et al.* 2004). Thus, three viral pathogens, which frequently cause illness in humans, were selected to represent the distribution of viral pathogens: enterovirus (EV), which causes a broad spectrum of diseases such as poliomyelitis and neonatal multi-organ failure, norovirus (NV), which causes nausea, forceful vomiting, and watery diarrhea, and rotavirus (RV), which causes acute gastroenteritis (Zhou *et al.* 2015). The concentrations of *E. coli* and the three viral pathogens in the source water, overlying water and sediment (represented by S-*E. coli*, S-EV, S-NV, and S-RV) of the three ponds were measured. For the sediment samples, the LifeGuard™ Soil Preservation Solution was removed by centrifugation (2,500 × g, 10 min), and the deposit was collected for extraction.

Bacterial DNA extraction

To detect *E. coli*, DNA was extracted from the deposit (5 g) using PowerMax Soil DNA Isolation Kits (MoBio Inc.) to obtain 100 μL of DNA, following the manufacturer's instructions. For water samples, 2,000 mL was first filtered through a mixed cellulose ester membrane with pore size 0.22 μm (diameter 50 mm, Beijing ShengHe Membrane Technology Development Center, Beijing, China) and then eluted with 30 mL of 3% beef extract solution (at pH 9.5) to make bacteria desorb from the membrane into the beef extract solution. The eluate was centrifuged at 14,000 rpm for 30 min at 4 °C. After centrifugation, the bacterial pellets deposited on the bottom of the centrifugal tubes were collected for DNA extraction. DNA was extracted from the deposits using a Genomic DNA Extraction Kit (Beijing Bioteke Biological Technology Co. Ltd, Beijing, China) to obtain 60 μL of DNA, following the manufacturer's instructions.

Virus RNA extraction and reverse transcription

For the detection of EV, RV, and NV, RNA was extracted from the deposit (10 g) using RNA PowerSoil Total RNA Isolation Kits (MoBio Inc.) to obtain 100 μL RNA, following the manufacturer's

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instructions. For the water samples, 2,000 mL was used, to which 25 mM $MgCl_2$ (pH 7.0–7.4) was added, passed through a mixed cellulose ester membrane with a pore size of 0.45 μm (diameter 150 mm, Beijing ShengHe Membrane Technology Development Center, Beijing, China). The membrane was rinsed with 1,820 mL of 0.5 mM H_2SO_4 (pH 3.0). Subsequently, 45 mL of 1 mM NaOH (pH 10.5–10.8) was added to elute viruses from the filter membranes. The elution was recovered in a tube containing 0.45 mL of 50 mM H_2SO_4 and 0.45 mL of 100× Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0) for neutralization. The obtained mixtures were further concentrated by PEG-6000 (Beijing Land Bridge Technology Co. Ltd, Beijing, China) precipitation. The mixture was stirred gently overnight at 100 rpm and 4 °C and then centrifuged at 9,000 × g for 30 min. The supernatant was discarded, and the deposit was re-suspended in 1,000 μL ultrapure water. The viral genomic RNA was extracted with 140 μL of the concentrate. This was achieved using a QIAamp Viral RNA Kit (Qiagen, Valencia, CA, USA) to obtain 60 μL RNA, following the manufacturer's instructions. Then, 5 μL of the extracted RNA of sediment and water samples was subjected to reverse transcription, which was carried out using a Prime Script RT Reagent Kit with gDNA Eraser (TaKaRa, Otsu, Japan) to acquire 20 μL cDNA, following the protocol described by the manufacturer. The total amount of sample extracted was recorded and used to determine the number of gene copies per gram of deposit weight after sediment centrifugation (copies/g CW).

Real-time quantitative polymerase chain reaction detection

Real-time quantitative polymerase chain reaction (PCR) was conducted for the quantification of the *E. coli* and NV using a SYBR Premix Dimer Eraser™ Kit (TaKaRa) in an iCycler iQ5 Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The primers and probes used are described in Table S1 (Zhou *et al.* 2015). The amplification of genomic cDNA was carried out in a reaction medium containing 12.5 μL of SYBR Premix Dimer Eraser™, 400 nmol/L of each primer and sterilized distilled water to a total volume of 25 μL.

The quantification of the EV and RV was performed using a Premix Ex Taq™ Kit (TaKaRa) in a 7500 Real-Time PCR system (Applied Biosystems, Framingham, MA, USA), using a 20 μL reaction volume containing 10 μL of Premix Ex Taq™, 0.4 μL of ROX Reference Dye II, 200 nmol/L of each primer, 400 nM of TaqMan probe, and 2 μL DNA or cDNA template. Detailed procedures are contained in this group's previous work (Zhou *et al.* 2015). A five-point standard curve was included on each qPCR plate. All samples and standards were run in triplicate. The amplification efficiency and correlation coefficient of the standard curve for all assays were larger than 0.94 and 0.992, respectively.

Calculation of pathogen concentration

The concentrations of the viruses and bacteria in the sediment, $C_{s\text{-}vir}$ and $C_{s\text{-}bac}$, respectively (copies/g CW), and that in the water samples, $C_{W\text{-}vir}$ and $C_{W\text{-}bac}$, respectively (copies/L), were determined as follows:

$$C_{s-vir} = \frac{(Vir/2\ \mu L) \times 20\ \mu L \times (1/5\ \mu L) \times 100\ \mu L}{ISW} \tag{1}$$

$$C_{W-vir} = \frac{(Vir/2\ \mu L) \times 20\ \mu L \times (1/5\ \mu L) \times 60\ \mu L \times (1/140\ \mu L) \times 1000\ \mu L}{ISV \times RE} \tag{2}$$

$$C_{s-bac} = \frac{(Bac/2\ \mu L) \times 100\ \mu L}{ISW} \tag{3}$$

$$C_{W-bac} = \frac{(Bac/2\ \ \mu L) \times 60\ \mu L}{ISV \times RE} \tag{4}$$

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where *Vir* is the number of viruses detected (copies), 2 μL is the amount of RNA per reaction tube, and 5 μL and 20 μL are the amounts of RNA and synthetic cDNA, respectively. The 100 μL and 60 μL terms are the volumes of the extracted RNA from the sediment and water samples, respectively. The 140 μL and 1,000 μL in Equation (2) are the volumes of the extracted water sample and the amount of concentrated elution after the last step of polyethylene glycol precipitation, respectively. In Equations (3) and (4), *Bac* is the number of bacteria detected (copies) and 2 μL is the amount of DNA per reaction tube, and 100 μL and 60 μL are the amounts of extracted DNA from the sediment and water samples, respectively. *ISW* is the initial weight of deposit after sediment centrifugation (g CW). *ISV* is the initial sample volume (mL) and *RE* is the recovery efficiency of pathogens, with the mean recovery efficiencies of bacteria and viruses being 71% and 65%, respectively. Estimates for these values were obtained for *E. coli* from ATCC 2592 and for poliovirus type 1 from Sabin, China, respectively. More details can be obtained from our group's previous work (Zhou *et al.* 2015).

Statistical analysis

The relationship between pathogens and environmental factors was analyzed via redundancy analysis (RDA) using CANOCO for Windows (version 4.5). Cluster analysis was performed to analyze the relationship between the pathogens in overlying water and sediment. Pearson correlations between *E. coli* and viral pathogens among environmental factors were determined using SPSS software (v. 19.0; SPSS IBM, Armonk, NY, USA). The thresholds of environment factors on pathogens were calculated using breakpoint regression analysis in SegReg software. To increase the normality of the data, all variables were logarithmically transformed, and the data for CANOCO software were standardized further by Z-score standardization.

RESULTS

Variations of environmental factors

Environment factors were monitored and provided, and the temporal variations of these factors are shown in Table 2. The temperature of R1, located in Kunming city, showed minimal seasonal variation. However, the temperatures of R2 and R3 had four distinct seasons. In addition, the annual average temperatures of R1, R2, and R3, were 18.1, 17.4, and 17.6 °C, respectively. For solar radiation, the temporal variation in RW ponds was almost the same as that of temperatures, as well as the comparisons between the three RW ponds. TDS were mainly dependent on geographical location, and which varied slightly with the seasons. TDS of R3, which is located at the coast, were higher than those of R1 and R2.

In the overlying water, the concentrations of nutrients and Chl-a in the three ponds varied significantly with the seasons, but the trends were different. The largest concentration of Chl-a appeared in summer, and the smallest during winter. For the nutrients, the largest concentrations were found during spring and the smallest during summer. From the comparison of nutrients in the three ponds, all fractions of nitrogen and phosphorus were the greatest in R2, followed by R1 and R3. In addition, the annual average concentration of Chl-a was 164.05, 117.42, and 92.75 mg/m^3 in R1, R2, and R3, respectively. For pH and COD, the temporal variations of the three ponds and the comparisons between the three ponds were similar to those of Chl-a. In contrast, those of the SD were the reverse of Chl-a. Due to photosynthesis of algae, there was oversaturation of DO in ponds, and which was also consistent with Chl-a.

The characteristics of sediment were more stable compared to those in overlying water (Table 2). The maximum monthly range of S-TN was no more than 20% in the three ponds, and for S-TP it

Table 2 | Environmental factors of the three RW ponds

		Spring: Mar.–May			Summer: Jun.–Aug.			Autumn: Sep.–Nov.			Winter: Dec.–Feb.		
Index	**Unit**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
T	°C	18.8 ± 1.2	19.2 ± 3.2	18.5 ± 7.9	23.0 ± 0.8	26.4 ± 2.0	28.9 ± 1.5	18.8 ± 1.1	18.8 ± 5.6	17.3 ± 6.2	11.8 ± 2.2	5.1 ± 0.9	5.7 ± 1.1
Solar	MJ/m^2	580 ± 61	434 ± 82	525 ± 108	471 ± 10	539 ± 15	548 ± 26	380 ± 26	348 ± 117	284 ± 63	408 ± 39	227 ± 27	246 ± 50
TDS	mg/L	530 ± 11	651 ± 71	1,162 ± 408	535 ± 20	569 ± 61	1,655 ± 802	601 ± 23	561 ± 21	2,334 ± 695	528 ± 11	633 ± 68	1,347 ± 168
TN	mg/L	2.75 ± 0.93	6.21 ± 0.93	2.45 ± 0.57	1.58 ± 0.71	3.71 ± 1.47	1.95 ± 0.24	1.27 ± 0.40	2.85 ± 0.51	1.89 ± 0.17	1.96 ± 0.48	4.67 ± 0.73	2.14 ± 0.21
NO_3^--N	mg/L	1.09 ± 0.35	3.90 ± 0.71	0.31 ± 0.14	0.89 ± 0.46	2.09 ± 1.03	0.28 ± 0.14	0.47 ± 0.18	1.42 ± 0.040	0.30 ± 0.07	0.95 ± 0.43	2.83 ± 0.38	0.34 ± 0.15
NH_4^+-N	mg/L	0.45 ± 0.40	0.43 ± 0.29	0.75 ± 0.25	0.22 ± 0.12	0.17 ± 0.09	0.80 ± 0.15	0.28 ± 0.16	0.14 ± 0.05	0.84 ± 0.13	0.14 ± 0.04	0.20 ± 0.20	0.90 ± 0.37
TP	mg/L	0.22 ± 0.09	0.78 ± 0.22	0.29 ± 0.20	0.16 ± 0.04	0.64 ± 0.19	0.20 ± 0.05	0.14 ± 0.03	0.47 ± 0.12	0.19 ± 0.04	0.16 ± 0.03	0.56 ± 0.17	0.21 ± 0.07
PO_4^{3-}-P	mg/L	0.07 ± 0.04	0.31 ± 0.13	0.16 ± 0.18	0.03 ± 0.02	0.28 ± 0.12	0.07 ± 0.05	0.05 ± 0.02	0.17 ± 0.05	0.08 ± 0.05	0.08 ± 0.01	0.41 ± 0.19	0.10 ± 0.05
Chl-a	mg/m^3	190.4 ± 18.2	114.4 ± 33.6	88.5 ± 18.4	232.6 ± 28.4	189.1 ± 31.8	151.5 ± 23.4	153.4 ± 57.6	105.8 ± 37.6	87.9 ± 19.2	81.4 ± 10.3	60.8 ± 19.3	43.4 ± 7.6
COD	mg/L	33.2 ± 10.2	30.6 ± 5.5	20.4 ± 5.7	72.5 ± 8.6	51.5 ± 11.6	40.0 ± 10.7	29.3 ± 6.2	25.1 ± 9.8	14.7 ± 1.9	21.9 ± 4.1	19.0 ± 3.2	13.6 ± 1.7
SD	m	0.46 ± 0.07	0.58 ± 0.04	0.70 ± 0.02	0.38 ± 0.02	0.52 ± 0.05	0.68 ± 0.04	0.47 ± 0.08	0.61 ± 0.09	0.70 ± 0.04	0.54 ± 0.04	0.72 ± 0.02	0.72 ± 0.04
pH	–	9.22 ± 0.32	8.72 ± 0.62	8.68 ± 0.32	9.40 ± 0.62	9.41 ± 0.31	8.84 ± 0.50	9.16 ± 0.45	9.15 ± 0.68	8.50 ± 0.36	8.91 ± 0.60	8.76 ± 0.55	8.40 ± 0.12
DO	mg/L	11.7 ± 2.0	14.2 ± 1.5	10.8 ± 2.6	9.4 ± 0.2	10.4 ± 1.7	10.2 ± 4.1	14.7 ± 2.2	12.8 ± 1.8	9.6 ± 2.4	15.6 ± 2.1	13.2 ± 4.4	14.4 ± 2.5
S-TN	g/Kg DW	2.88 ± 0.21	3.45 ± 0.14	3.14 ± 0.15	2.95 ± 0.22	3.51 ± 0.16	3.10 ± 0.12	2.91 ± 0.21	3.45 ± 0.18	3.13 ± 0.11	2.93 ± 0.26	3.45 ± 0.13	3.12 ± 0.11
S-TP	g/Kg DW	1.01 ± 0.06	1.18 ± 0.06	1.08 ± 0.05	1.01 ± 0.04	1.18 ± 0.06	1.09 ± 0.07	1.02 ± 0.03	1.17 ± 0.06	1.10 ± 0.06	1.01 ± 0.04	1.17 ± 0.05	1.09 ± 0.04
OM	g/Kg DW	160.1 ± 21.1	128.4 ± 20.3	110.2 ± 24.7	188.7 ± 29.3	158.5 ± 21.7	121.0 ± 21.2	173.1 ± 27.2	137.6 ± 38.3	112.5 ± 25.2	149.7 ± 20.1	103.2 ± 22.8	92.7 ± 12.7
WC	%	77.1 ± 4.1	76.7 ± 5.7	76.4 ± 4.1	84.3 ± 3.3	79.1 ± 4.6	71.6 ± 4.2	81.8 ± 4.9	76.1 ± 3.5	76.8 ± 6.4	78.9 ± 3.9	75.6 ± 5.0	73.6 ± 5.9

T means temperature.

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was lower than 10%. In addition, TN and TP in the sediment of the three RW ponds were all represented by R2 > R1 > R3. Furthermore, the temporal variation of OM and WC was also not obvious. The annual mean values of OM were 167.9, 131.9, and 109.1 g/kg DW in R1, R2, and R3, respectively. For WC, the mean values were 80.5%, 76.9%, and 74.6%, respectively.

Variations of *E. coli* and the three viral pathogens

The monthly concentrations of *E. coli* and the three viral pathogens in the three RW ponds are shown in Figure 1. Those concentrations in the overlying water and sediment presented different trends with temporal variation. The concentrations of pathogens in the overlying water were higher in summer and autumn (Figure 1(a)), while the concentrations of that in the sediment were higher during autumn and winter (Figure 1(b)). Moreover, the maximum concentrations of *E. coli* and the three viral pathogens in the overlying water appeared in September, and during October in the sediment. In summary, the peak values of *E. coli* and the three viral pathogens mainly occurred from August to October.

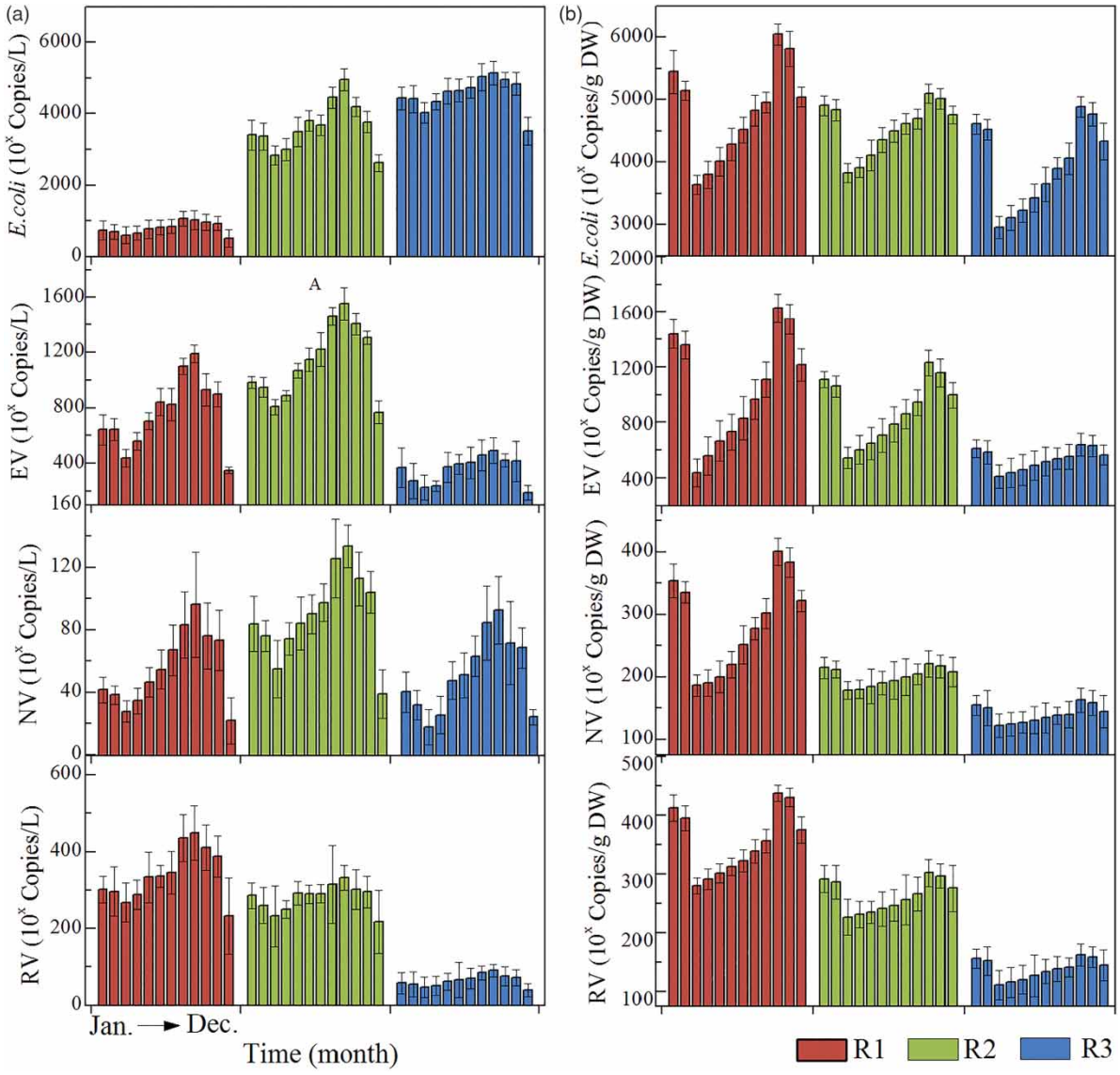

Figure 1 | Temporal variations of *E. coli* and three viral pathogens in the three RW ponds: (a) overlying water and (b) sediment. All data were transformed by $\log_{10}(x)$.

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By comparing the concentrations of pathogens in overlying water of the three RW ponds, *E. coli*, EV, NV, and RV in the sediment were on average about 4.2, 3.7, 4.3, and 3.8 log higher than those in the overlying water (the density of centrifuged sediment was assumed to be 5 g/mL). In addition, the distributions in the sediment were more stable. Specifically, the annual average concentration of *E. coli* in the overlying water was 801, 3,632, and 762 copies/L in R1, R2, and R3, respectively. For EV and NV, it was the highest in R2 (which was 1,129 and 89 copies/L, respectively), followed by R1 (760 and 55 copies/L) and R3 (354 and 31 copies/L). For RV, the concentration was 340, 280, and 64 copies/L, in R1, R2, and R3, respectively. However, in the sediment, the order of *E. coli* and the three viral pathogens in the three RW ponds was the same, which is indicated as $R1 > R2 > R3$.

Correlation of environmental factors with *E. coli* and the three viral pathogens

Based on irregular sample collections from RW sampling, the concentrations of *E. coli* and the three viral pathogens in source water were always close to or even below the detection line, and presented no significant differences in the three RW ponds. In addition, as Figure 1 shows, pathogen concentration in the overlying water presented a sharp decrease specifically in March, with a second minimum in December. However, precipitations were very small in March and December (Figure S2), and the dilution effect of rainfall on the pathogens' concentration in ponds was limited. Thus, the occurrence of these concentrations was mainly attributed to the environmental factors. RDA was performed to analyze the correlation of *E. coli* and the three viral pathogens with environmental factors, as well as to further understand the driving factors that affected the distribution of pathogens in RW ponds, in which the environmental factors involved temperature, solar radiation, TDS, TN, NO_3^--N, NH_4^+-N, TP, PO_4^{3-}-P, Chl-a, COD, SD, pH, DO, S-TN, S-TP, OM, and WC. Furthermore, cluster analysis was performed to understand the relationship of *E. coli* and the three viral pathogens in overlying water and sediment. The results of the RDA were visualized in the form of ordination diagrams based on axes 1 and 2, produced using CanoDraw for the Windows program. Quantitative environmental variables and species scores are presented as lines with arrows. The directions of arrows in the quadrant indicate the correlations between those and the axis, and the correlations of those depended on the length and directions of environment variables and species arrows. The results of RDA and cluster analysis (the details are shown in Figure S3) are shown in Figure 2, and the summary statistics for the first two axes of RDA are shown in Table 3.

The eigenvalues of axes 1 and 2 were 0.620 and 0.109, respectively. Together, these axes explained 72.9% of the total variance. Species–environment correlations for species axes 1 and 2 were 0.900 and 0.913, respectively. The first two species axes were approximately vertical, with a correlation of 0.007. Moreover, the correlation between the first two environmental axes was 0. These results showed that the ordination result was credible because the linear combinations of the ordination axes and the environmental factors provide a good representation of the relationship between the species and environmental factors.

As Figure 2 and Table S2 show, axis 1 correlated mostly with Chl-a, SD, OM, NH_4^+-N, and TDS, with the correlation coefficients of −0.643, 0.707, −0.676, 0.793, and 0.816, respectively. TP, S-TP, PO_4^{3-}-P, and temperature correlated mostly with axis 2, with the correlation coefficients of 0.653, 0.664, 0.501, and 0.593, respectively. It indicated that these nine environmental factors are closely related to the distribution of *E. coli* and the three viral pathogens in RW ponds. Furthermore, the results of cluster analysis showed that *E. coli* and the three viral pathogens were divided into three clusters. Cluster 1 (EV, NV, and RV) is mainly related to axis 1, and the three viral pathogens in the overlying water have a significant ($P < 0.01$) relationship with Chl-a ($r > 0.59$), SD ($r < -0.64$), OM ($r > 0.63$), and NH_4^+-N ($r < -0.71$). Cluster 2,

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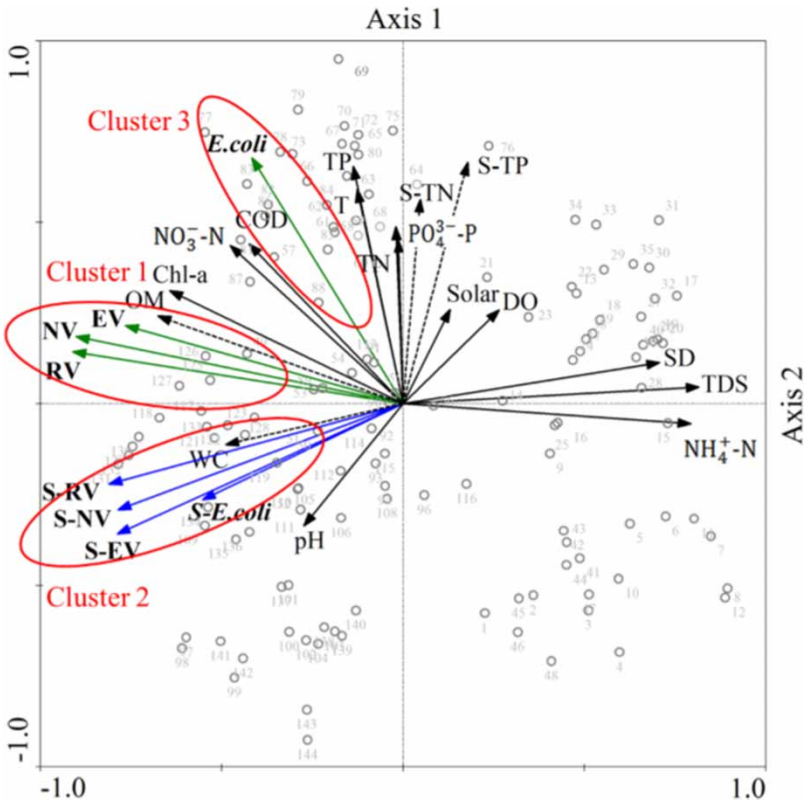

Figure 2 | Ordination diagram of RDA exhibits species and environmental variables in overlying water and sediment of the three RW ponds. S-TN and S-TP means TN and TP of sediment, respectively.

Table 3 | Summary statistics for the first two axes of RDA

	Axis 1	Axis 2
Eigenvalues	0.620	0.109
Species–environment correlations	0.900	0.913
Cumulative percentage variance		
of species data	62.0	72.9
of species–environment relation	79.1	93.0
Test of significance of first canonical axis	F = 3.570	$P = 0.037$
Test of significance of all canonical axes	F = 27.880	$P = 0.001$

which includes *E. coli* and the three viral pathogens in the sediment, also correlate significantly ($P < 0.01$) to Chl-a ($r > 0.28$), SD ($r < -0.36$), OM ($r > 0.34$), and NH_4^+-N ($r < -0.41$). For cluster 3 (*E. coli*), there is a significant ($P < 0.01$) relationship with NO_3-N ($r = 0.42$), TP ($r = 0.46$), Chl-a ($r = 0.45$), and SD ($r = -0.24$). In addition, *E. coli* and the three viral pathogens in the overlying water and sediment of the RW ponds displayed significantly negative relationships with TDS ($P < 0.05$). Furthermore, *E. coli* and the three viral pathogens in the overlying water of the three RW ponds were less affected by solar radiation ($P > 0.34$) and those in the sediment showed no correlation with temperature ($P > 0.06$). The details are presented in Table S3.

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DISCUSSION

Effects of RW replenishment on pathogens

Pathogens in the overlying water were divided into two clusters. Because bacterial pathogens can multiply by themselves, the relatively greater nutrient concentrations in RW ponds would promote the regeneration of bacterial pathogens (Garzio-Hadzick *et al.* 2010; Pachepsky & Shelton 2011); *E. coli* in the overlying water of RW ponds was significantly related to nutrients. As we know, viral pathogens are not directly involved in nutrient metabolism as they are obligate intracellular parasites and are not living entities, and nutrients will indirectly protect the virus by the promotion of algae and microbial growth. However, the nutrient concentration in RW ponds was much greater than the nutrient threshold of eutrophication (TN = 0.2 mg/L, TP = 0.02 mg/L) (Pu *et al.* 2001), which means that nutrients were not the limiting factors in algae growth, thus the viral pathogens were not significantly affected by nutrients. Furthermore, the bacterial and viral pathogens were all significantly positively related with Chl-a, and negatively with SD. First, the locations of bacterial and viral pathogens' adsorption with algae keep pathogens from being captured, and the survival of pathogens becomes longer (Brookes *et al.* 2004). Second, SD was mainly dependent on the amount of algae ($r = -0.81$, $P < 0.01$), thus lower transparency caused by the algae growth would reduce the damage effect of UV radiation on pathogens, which would be conducive to the survival of pathogens in the overlying water (McGuigan *et al.* 2012; Nguyen *et al.* 2015). Possibly due to the exchange of pathogens between sediment and overlying water, pathogens in the overlying water were significantly impacted indirectly by OM in the sediment.

Bacterial and viral pathogens in the sediment were directly affected by OM. In urban landscape ponds, OM comes from endogenous and exogenous sources (Ni *et al.* 2015), but OM content in the source water is lower (Gücker *et al.* 2006). The settlement of dead algae from overlying water was likely the main source of OM in sediment ($r = 0.84$, $P < 0.01$) (Ni *et al.* 2015). Therefore, the higher level of OM caused by algae growth could result in micro-aggregates that restrict predator access to pore spaces. Alternatively, higher levels of OM in sediments could contain slow-release, polymeric nutrients that retard pathogen die-off (Millis 1989; Abia *et al.* 2016). Furthermore, for viral pathogens in sediment, OM could be enhanced through virion attachment and could promote viral persistence in sediment by microbial biofilm formation and flocculation of extracellular polymeric substances *in situ* (Garzio-Hadzick *et al.* 2010). In addition, the pathogens in overlying water are easily adsorbed and accumulated in sediment, and the concentration of pathogens in sediment was also affected by those in overlying water; thus, these pathogens are related significantly to SD and Chl-a.

Usually, solar radiation is the main factor that affects the distribution of pathogens in the overlying water of natural water bodies (McGuigan *et al.* 2012; Nguyen *et al.* 2015), and temperature is an important factor for pathogens in sediment (Garzio-Hadzick *et al.* 2010; Pachepsky & Shelton 2011; Xagoraraki *et al.* 2014). However, the situation seems different in RW ponds. According to the results of RDA, pathogens in the overlying water were mainly affected by nutrients, Chl-a, and SD, rather than solar radiation, and in the sediment, the pathogens were largely affected by Chl-a and OM instead of by temperature. This may be due to the high nutrient content in RW and the rapid growth of algae in RW ponds. Lower SD caused by high algal biomass in RW ponds reduced the exposure effect on pathogens of UV radiation (McGuigan *et al.* 2012; Nguyen *et al.* 2015). Pathogens in sediment were little influenced by temperature, which can probably be attributed to the greater OM content, as that would result in the pathogens' lower sensitivity to temperature (Garzio-Hadzick *et al.* 2010).

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Key factors and their recommended values for controlling health risks

From the above analysis, the nutrients in RW might have a greater effect on the pathogens' concentration in ponds. On the one hand, this would inhibit the growth of bacterial pathogens. On the other hand, it would reduce the amount of algae, thereby decreasing the protection strength of pathogens by algae in the overlying water. Moreover, it would also reduce the OM content in sediment, thus decreasing the concentration of pathogens in sediment indirectly. However, it is difficult and uneconomical to decrease the level of nutrients in RW in order to restrict algae growth. Except for nutrients, and according to the results of the RDA, the pathogens in RW ponds were mainly affected by the environmental factors of Chl-a, OM, and SD. As analyzed in the section 'Effects of RW replenishment on pathogens', the settlement of dead algae was the main source of OM. Thus, the most important and practical way to reduce the occurrence of pathogens in RW ponds is to control the key factors of Chl-a and SD. As described above, it is difficult and uneconomical to reduce the nutrient concentrations in RW further to levels that limit the proliferation of algae growth. Therefore, the practical way to reduce the risk in RW ponds is to restrain algae growth by improving the flow conditions, or dredging. Moreover, bypass purification measures of strengthening the water purification capacity is also recommended, such as ozone oxidation, coagulation and constructed wetland. However, the specific control values of these two factors must be known.

Among the methods of determining control values, breakpoint value calculated by the pressure-response model is used extensively. In those models, SegReg is a free and user-friendly tool for linear segmented regression analysis to determine the breakpoint at which the relation between the dependent variable and the independent variable changes abruptly. Thus, breakpoint regression analysis of *E. coli* and the three viral pathogens against Chl-a and SD was carried out with SegReg, to attempt to identify any significant pressure thresholds in those relationships (Ye *et al.* 2014). That is, when the value of Chl-a is smaller or SD is larger than those thresholds, the concentration of *E. coli* and the three viral pathogens will be significantly decreased. Meanwhile, the control values for Chl-a and SD to reduce the concentration of pathogens in RW ponds could be determined.

The results of breakpoint regression analysis of *E. coli* and the three viral pathogens against Chl-a are shown in Figure 3. The concentrations of EV and RV in overlying water increased with Chl-a, and no breakpoint was found (Figure 3(a)). However, a breakpoint was found in the analysis of *E. coli* and NV, where the thresholds were 140 and 132 mg/m^3 for Chl-a, respectively. For the concentrations in sediment, as Figure 3(b) shows, there is no breakpoint found between Chl-a and *E. coli*, but the viral pathogens in sediment had thresholds with Chl-a. The threshold values of Chl-a with EV, NV, and RV were 57, 63, and 128 mg/m^3, respectively. Figure 4 presents the results of breakpoint regression analyses and indicates that *E. coli* and the three viral pathogens all had breakpoints in their relationships with SD. In overlying water, the breakpoint of SD against *E. coli*, EV, NV, and RV, were 54, 63, 68, and 63 cm, respectively. For those in sediment, the breakpoints were 49, 53, 47, and 46 cm, respectively. The abilities of different pathogens in RW ponds to be resistant to the external environment are not the same; thus, the control values of Chl-a and SD should be chosen appropriately. For Chl-a, the minimum threshold value was selected as the control value. In contrast, the maximum value was selected for SD. According to the results of breakpoint regression analysis, it is recommended that Chl-a should be controlled to less than 57 mg/m^3, and SD should be greater than 68 cm.

CONCLUSION

Reusing RW is a good way to solve the water shortage of urban landscape pond replenishment, but the opposition by the public is a major limitation to its implementation. The public opposition stems from

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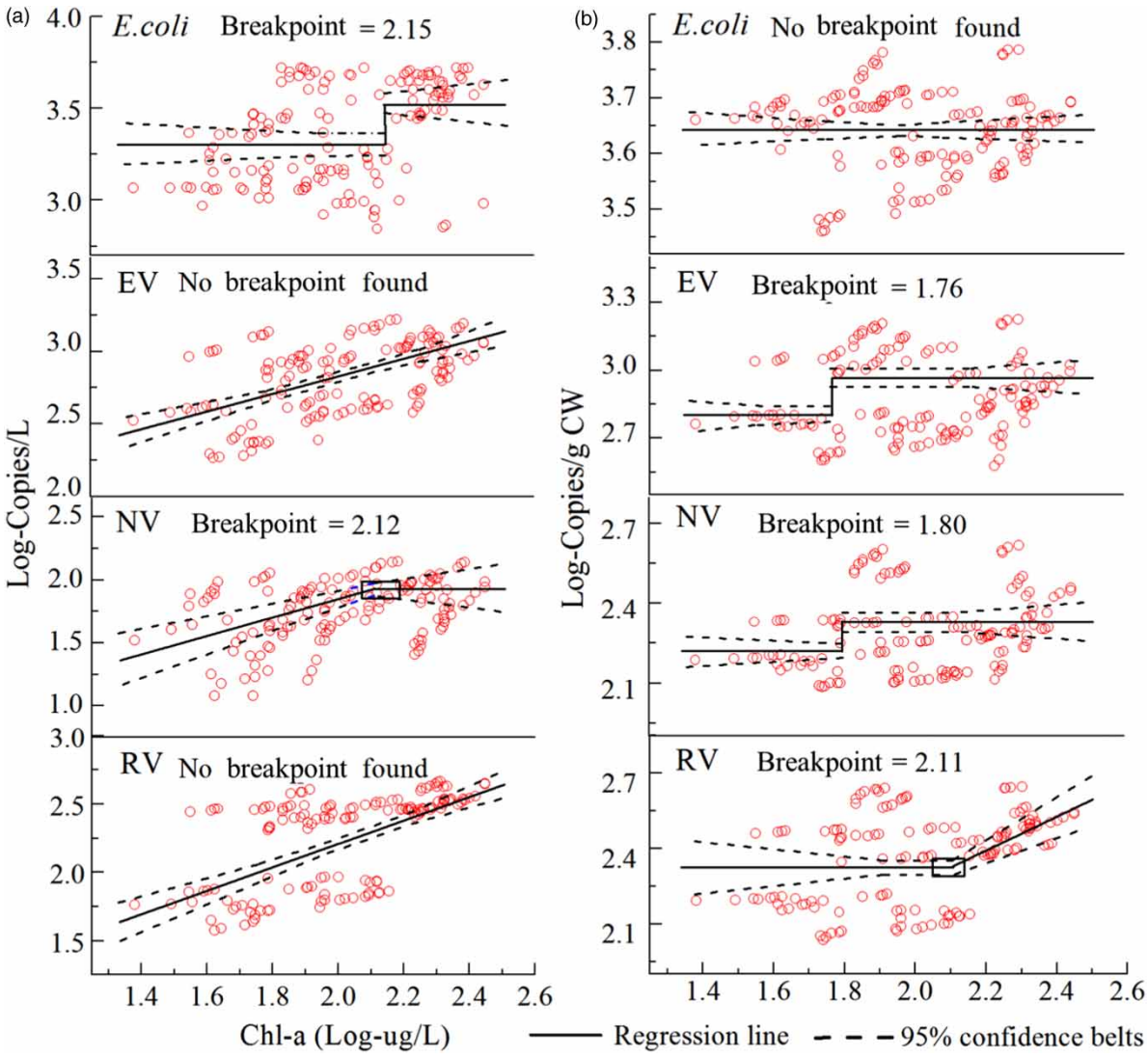

Figure 3 | Breakpoint regression analysis of *E. coli* and three viral pathogens against Chl-a of the three RW ponds. The data for regression analyses were logarithmically transformed. (a) Overlying water and (b) sediment.

concerns over the levels of pathogens in RW ponds. In order to analyze the distribution of pathogens and their influencing factors in RW ponds, the concentrations of *E. coli* and three common viral pathogens (EV, NV, and RV) in the overlying water and sediment of three RW ponds in three cities, as well as environmental factors, were observed for one year. The results of RDA and cluster analyses indicated that RW replenishment could make the concentrations of nutrient higher in those ponds, which would then promote the growth of algae, resulting in lower SD as well as greater OM content. Therefore, pathogens can easily accumulate in overlying water and sediment of RW ponds. Having analyzed the transport characteristics of the components, the practical way to control the occurrence of pathogens in RW ponds is to focus on decreasing the amount of algae and improving the SD, using methods such as reducing hydraulic retention time and strengthening the water purification capacity. Moreover, the results of breakpoint regression analyses showed that, with Chl-a less than 57 mg/m^3 and SD greater than 68 cm, the concentration of pathogens would abruptly reduce in RW ponds. These research results contributed to a better understanding of the relationships between pathogens and environmental factors of RW ponds, which can be used to improve the management of RW as a source for replenishing urban landscape ponds and to promote the appropriate utilization of RW.

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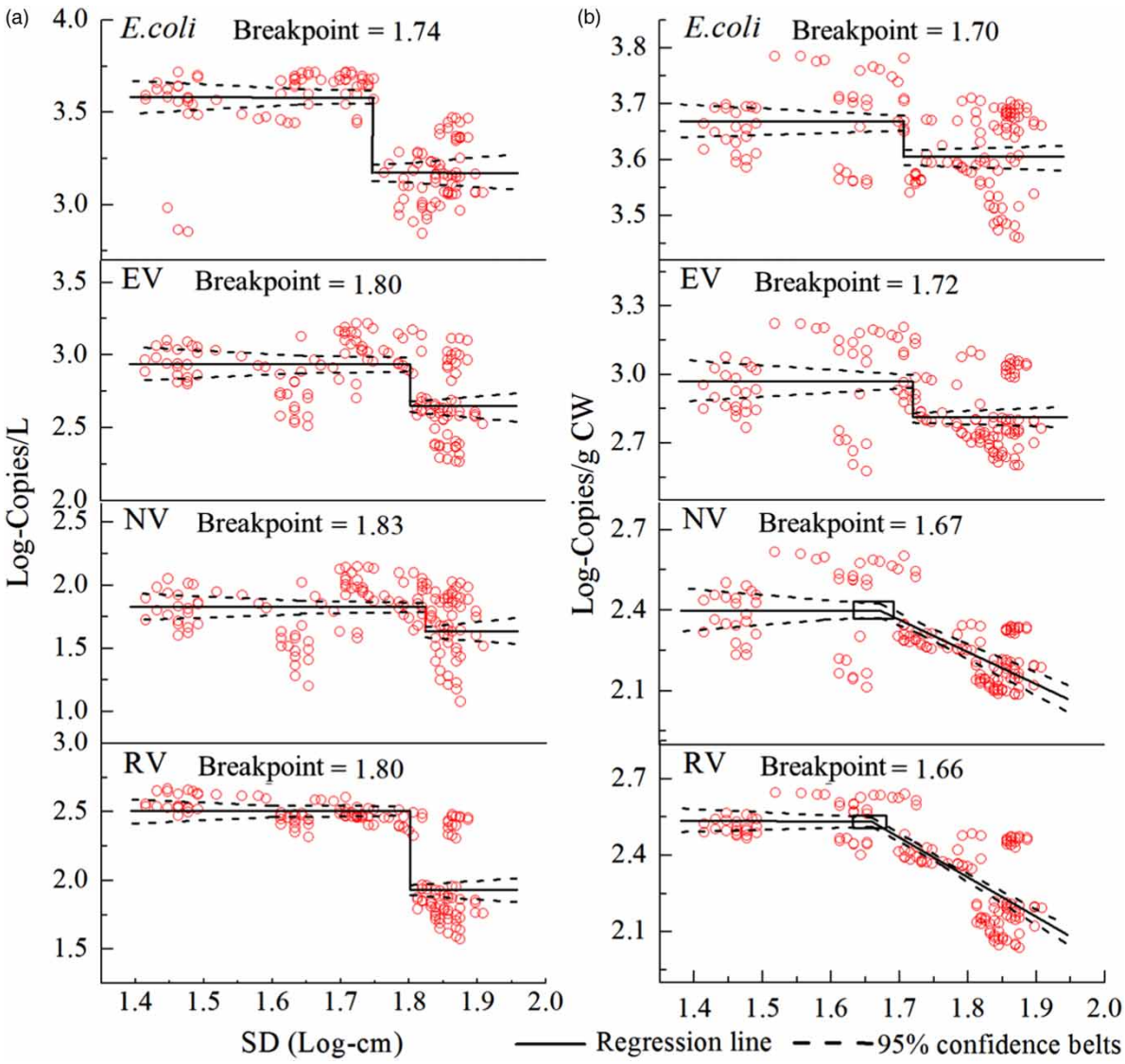

Figure 4 | Breakpoint regression analysis of *E. coli* and three viral pathogens against SD of the three RW ponds. The data for regression analyses were logarithmically transformed. (a) Overlying water and (b) sediment.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this paper is available at https://dx.doi.org/10.2166/bgs.2019.916.

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