water

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Supplementary Information

Stormwater biofilters as barriers against *Campylobacter jejuni*, *Cryptosporidium* oocysts and adenoviruses; results from a laboratory trial

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Tracer Test

The objective of the KCl tracer test was to estimate the submerged zone (SZ) pore volume. SZ pore volume was then used to differentiate old SZ water from the from the freshly treated stormwater in the biofilter outflow. Furthermore, the porosity of the SZ media was also derived using the tracer test results. It should be noted that the tracer test was conducted only on un-vegetated columns to avoid any unnecessary impacts on plants and plant-associated microbes due to the introduction of high salt concentration. Nevertheless, SZ of both vegetated and un-vegetated columns had the similar media layers, as such it was assumed that the results from un-vegetated columns apply to vegetated columns.

Dechlorinated tap water (using 0.83 mg/L $Na_2S_2O_3$) was used as the blank solution. Then 0.96 g/L KCL was added to the blank solution to prepare the salt solution with a target electrical conductivity of 2000 µS/cm. Each column was dosed with 26-L of salt solution, and the total outflow was collected into a series of 500-mL samples. Each outflow sample was analyzed for electrical conductivity. It should be noted that at the end of tracer test, each biofilter column was flushed with another 26-L blank solution, but this flushing caused a significant release of fine particles from the filter media (data not shown). As such, all three un-vegetated columns used for the tracer test were emptied and re-packed. These columns were subjected to accelerated dosing to receive the same amount of water as the rest of the columns to achieve the same level of hydraulic compaction.

The electrical conductivity of each outflow sample (C_{out}) was normalised to the measured electrical conductivity (C_{in}). The results of the tracer test are shown in Figure S1. It was assumed that the old SZ water was fully displaced when a sudden increase in the initial steady normalised outflow concentration (C_{out}/C_{in}) was observed. The sudden increase in C_{out}/C_{in} was observed when C_{out}/C_{in} reached approximately 0.1, and the cumulative outflow volume reached approximately 10-L. Therefore, the pore volume retained of the SZ was estimated to be 10-L, and the porosity was estimated to be 0.5.

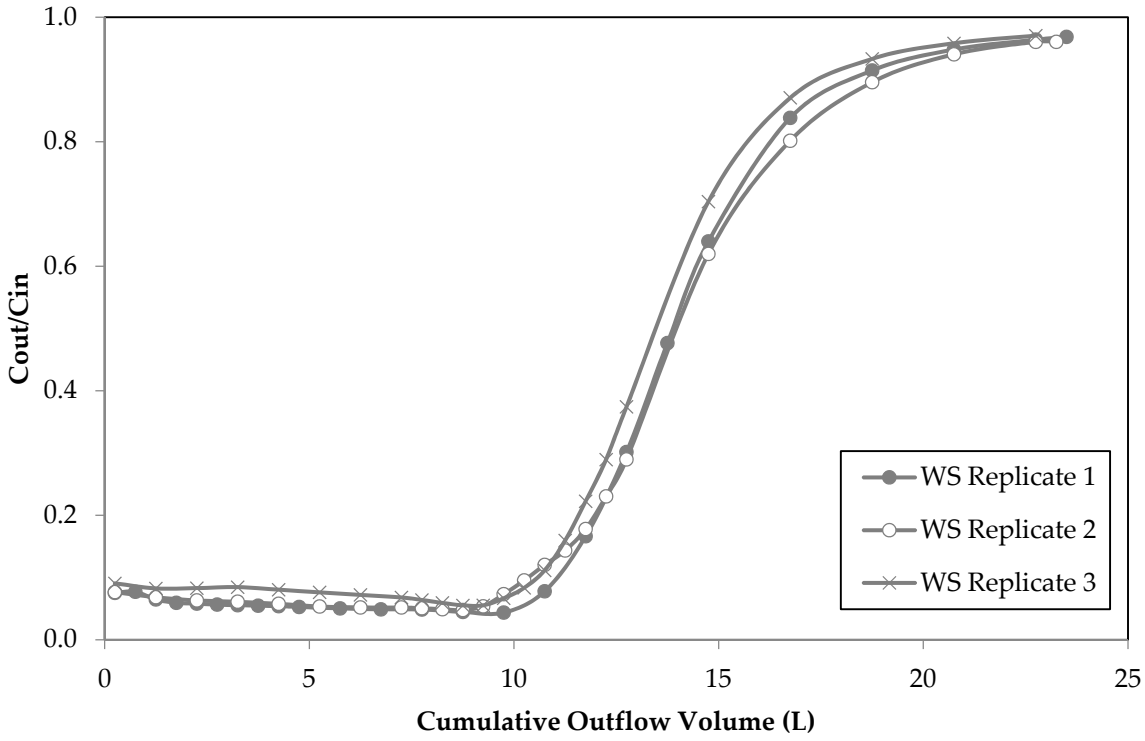

Figure S1. Change of outflow KCl concentration (normalised to the inflow concentration) with cumulative outflow during the tracer test trail in three replicates of WS un-vegetated columns.

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Table S1. Microbial assay description

Indicator/ Pathogen	Method of detection
E. coli	*E. coli* analyses were conducted following AS 4276.21 – 2005 – Method 21, using the Colilert™ procedure [1]. Samples were appropriately diluted and sealed in 97 well MPN plates, which were subsequently incubated at 37°C for 24 hours. *E. coli* were enumerated by placing the plates under ultraviolet light and counting the number of wells which fluoresce; indeed, *E. coli* will metabolise methyl-umbelliferone glucuronide using the enzyme β-glucuronidase to produce 4-methyl-umbelliferone which fluoresces under long wave (365 nm) ultraviolet light. The number of wells was converted to an estimate of Most Probable Number (MPN/100mL) using standard equations and/or charts. The detection limit for *E. coli* using the Colilert procedure is between <1 and 2,400 MPN/100mL, but serial dilution often ensures that all analysed samples are within this detection range.
FRNA coliphage	The method used for FRNA Phage analysis followed that presented in standards around the world, including: US EPA 1602, APHA 9224 and ISO 10705-1. In short, FRNA phages were detected and quantified through a single agar layer assay and using recombinant *E. coli* Famp or *Salmonella typhimurium* WG49 as the host bacteria. The sample was analysed with and without the addition of RNase A. RNase A inactivates FRNA bacteriophages and the difference in the number of plaques in the sample analysed with and without the addition of RNase A was used to calculate the number of FRNA bacteriophage plaque forming units (pfu).
Clostridium perfrignens	*C. perfringens* were quantified using the AS/NZS 4276.17.1:2000. After preliminary heat treatment to destroy vegetative bacteria, a sample of a known volume is passed through a membrane filter. Spores present in the sample were retained on one side of the membrane, being too large to pass through the pores. The membrane was then incubated anaerobically with a sulphite-containing medium. After incubation, black colonies that develop were counted and verified by subculture onto Nutrient Agar plates, which were incubated aerobically (as a control) and anaerobically. Further confirmation was carried out to demonstrate the presence of *C. perfringens*. The number of colony forming units (cfu) was calculated per 100mL or per volume analysed.
Campylobacter	*Campylobacter* were quantified by a method closely based on the standard MPN procedure and the AS 4276.19:2001. Five 0.45μm membrane filters were used to filter different volumes of the water samples: 1x500mL, 5x100mL, 5x10mL. These filters were then placed on selective agar and incubated at 42°C for 48hrs under microaerobic conditions (oxygen of around 5% and carbon dioxide of around 10%). The presence of thermophilic *Campylobacters* was confirmed by the recovery and development of colonies on selective agar media, cell morphology, Gram stain and oxidase test. Approximately 1L of sample was used in the analysis, thereby providing a detection limit of <1MPN/L.

Adenoviruses	Adenoviruses were detected and quantified using a cell culture technique. In brief, water samples were concentrated using ultrafiltration. Viruses were then cultured in A549 cells (American Type Culture Collection) (ATCC). The culture of adenovirus and/or enterovirus was confirmed by polymerase chain reaction (PCR). The PCR detection of adenovirus has been described in [2].
Giardia	For detection and quantification of *Cryptosporidium*, the USEPA 1623 method was used with some modifications. In brief, the samples were filtered (HV capsule) and the oocysts and other material was retained on the filter; the filter material was then eluted with Tween 8 buffer and the eluate was centrifuged (15g for 1min) to pellet oocysts and cysts. This process was undertaken within 72hrs of sample collection. The oocysts were magnetised by attachment of magnetic beads conjugated with anti-*Cryptosporidium* antibodies. The oocysts were then separated from other materials using a magnet. They were stained on well slides with fluorescently labelled antibodies and 4',6-diamidino-2-phenylindole (Waterborne AquaGlo and DAPI) and were counted for oocysts using fluorescence and DIP microscopy. In all samples, ColorSeed was added for quality assurance of the assay procedure. Approximately 5-10L of water was used for each analysis, providing a detection limit of <0.1-0.2oocysts/L.

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Table S2. A summary of inflow and outflow microbial concentrations of each sampling round.

Microorganism	Unit	Inflow concentration					Geometric mean outflow concentration (*Geometric standard deviation*)							
		Sampling Event[(a)]					Un-vegetated (WS)					Vegetated (LC)[(b)]		
							Sampling Event					Sampling Event		
		1 (Wet 40-L)	2 (Wet 40-L)	3 (Wet 20-L)	4 (Dry 20-L)	5 (Wet 40-L)	1 (Wet 40-L)	2 (Wet 40-L)	3 (Wet 20-L)	4 (Dry 20-L)	5 (Wet 40-L)	3 (Wet 20-L)	4 (Dry 20-L)	5 (Wet 40-L)
E. coli	MPN/L	2.3×10^{5}	2.2×10^{5}	6.3×10^{5}	2.8×10^{5}	1.8×10^{5}	4.6×10^{3} *(1.51)*	1.2×10^{4} *(1.12)*	1.5×10^{4} *(1.25)*	1.2×10^{4} *(1.57)*	1.5×10^{4} *(1.28)*	2.4×10^{4} *(1.18)*	1.5×10^{5} *(1.20)*	6.2×10^{3} *(1.04)*
FRNA coliphages	pfu/L	2.0×10^{1}	1.6×10^{4}	5.2×10^{2}	8.1×10^{2}	1.0×10^{1}	2.8×10^{1} *(2.65)*	2.9×10^{1} *(2.56)*	9.9×10^{1} *(7.38)*	1.3×10^{2} *(3.70)*	1.0×10^{1} *(1.00)*	2.3×10^{1} *(2.08)*	1.3×10^{1} *(1.49)*	5.5×10^{1} *(4.40)*
C. Perfringens	orgs/L	6.0×10^{3}	1.0×10^{4}	1.8×10^{4}	2.3×10^{4}	1.8×10^{4}	1.8×10^{2} *(1.74)*	0.0×10^{0} *(1.00)*	3.6×10^{2} *(1.18)*	1.8×10^{2} *(2.81)*	0.0×10^{0} *(4.35)*	1.8×10^{2} *(1.72)*	1.6×10^{2} *(2.23)*	2.0×10^{2} *(2.00)*
Campylobacter spp.	MPN/L			2.1×10^{0}	3.6×10^{2}	3.6×10^{2}						1.7×10^{0} *(1.00)*	1.9×10^{1} *(15.15)*	5.3×10^{1} *(2.87)*
Adenovirus	MPNIU/L			3.2×10^{0}	4.3×10^{0}	9.4×10^{1}						3.2×10^{-1} *(1.09)*	7.0×10^{-1} *(1.00)*	5.3×10^{0} *(1.00)*
Cryptosporidium oocysts	oocysts/L			3.3×10^{0}	9.7×10^{0}	2.1×10^{1}						1.4×10^{-1} [(c)] *(-)*	5.2×10^{-1} *(1.49)*	1.5×10^{-1} *(1.44)*

Notes: (**a**)Sampling Event Description: Events 1, 2 and 5 were wet-weather events (antecedent dry weather period of 2-3 days) with a high dosing volume (40-L); Event 4 was a wet-weather event with a regular dosing volume (20-L) and Event 4 was a dry-weather event (antecedent dry weather period of 4 weeks) event with a regular dosing volume. (**b**)Biofilter vegetated with a single Leptospermum continentale plan. (**c**)*Cryptosporidium* oocysts concentration was below detection limit in all three replicates. Therefore, outflow concentration was taken as the limit of reporting which was 1 oocyts/7 L

References

1. IDEXX-Laboratories *Colilert® Test Kit*; IDEXX-Laboratories: Maine, USA, 2007;
2. Allard, A.; Girones, R.; Juto, P.; Wadell, G. Polymerase chain reaction for detection of adenoviruses in stool samples. *J. Clin. Microbiol.* **1990**, *28*, 2659–2667.