Clostridium perfringens identifies source of pollution and reference streams in a tropical highland environment

Douglas Mushi

ABSTRACT

Clostridium perfringens was analysed in soils from a tropical highland catchment and corresponding headwater streams at baseflow condition in order to understand the contribution of soils to the microbiological quality of stream water and the feasibility of using these streams as surrogate for negative control (reference streams). The concentrations of *C. perfringens* depended on the sample matrix. *C. perfringens* concentrations were significantly higher in the catchment soil than in the stream water ($P < 0.05$, $n = 20$). In addition, *C. perfringens* concentrations in the catchment soil remarkably predicted *C. perfringens* concentrations in the stream water (i.e., 82% of variations in *C. perfringens* concentrations in water were predicted by *C. perfringens* concentrations in soil; $P < 0.05$, $n = 20$). This suggests that the catchment soils contributed *C. perfringens* to the stream water. Despite the observed contamination, the concentrations of *C. perfringens* (geometric mean of 32 cfu/100 cm^3) in the stream water was below the recommended safe level for tropical freshwater systems and extremely lower than that detected in anthropogenically influenced rivers. This concentration was defined as an acceptable level of disturbance, and a reference concentration that can serve as surrogate for negative control in the studied tropical environment.

Key words | catchment soil, *Clostridium perfringens*, headwater streams, reference concentration, reference streams, tropical environment

Douglas Mushi
Sokoine University of Agriculture, Solomon Mahlangu College of Science and Education, Department of Biosciences,
PO Box 3038, Morogoro,
Tanzania
E-mail: *douglas@sua.ac.tz*

INTRODUCTION

Water quality evaluation using *Clostridium perfringens* is performed around the globe (Bisson & Cabelli 1980; Fujioka & Shizumura 1985; Sartory 1988; Sorensen *et al.* 1989; Byamukama *et al.* 2005; Goto & Yan 2011) with the aim of understanding the microbiological safety associated with surface and drinking water systems. The occurrence of *C. perfringens* in the gastrointestinal tract of both humans and other animals (Rood & Cole 1991; Vierheilig *et al.* 2013), their correlation with human enteric pathogens in environmental waters (Ferguson *et al.* 1996) and their ability to predict gastrointestinal diseases (Kueh *et al.* 1995; Viau *et al.* 2011) support their use as a water safety determinant. While a significant number of previous studies shows a strong link between *C. perfringens* and anthropogenic influences (e.g., Skanavis & Yanko 2001; Byamukama *et al.* 2005; Farnleitner *et al.* 2010), little is known regarding the indication value of *C. perfringens* in water systems free from anthropogenic influences. Furthermore, water systems contaminated with *C. perfringens* in the absence of any known anthropogenic disturbance may not correlate with human enteric pathogens and/or gastrointestinal illness risks, limiting attempts to design and initiate sustainable water quality management.

On the other hand, understanding the source of *C. perfringens* in anthropogenically free water systems is critical because the management of water systems

doi: 10.2166/wh.2018.192

contaminated with *C. perfringens* depends on the known source(s) of *C. perfringens*. For example, surface water contaminated with *C. perfringens* from wildlife needs completely different management strategies compared to that from sewage. Consequently, there is a critical need to comprehensively investigate the source of *C. perfringens* in anthropogenically free water systems in order to improve microbiological quality and the overall ecological health of surface waters.

As in other highlands around the globe, Uluguru Mountain in Tanzania, which is representative of larger areas of the East African Arch Mountains (Kimaro *et al.* 2008), is the main source of drinking water for Morogoro municipality and its outskirts (Jiwa *et al.* 1991) due to the presence of headwater streams. Although these headwater streams are free from anthropogenic influences, they are contaminated by catchment soil as a result of the unstable slopes of Uluguru Mountain (Westerberg & Christiansson 1999). Given the critical health impacts associated with stream water contaminated with soil (Baumgardner 2012), understanding the magnitude of this environmental problem is critical so that appropriate plans for precluding the bacterial contamination of stream water are put in place.

Therefore, this paper has: (1) determined the contribution of soil from the steep slope of Uluguru Mountain to the microbiological quality of stream water during the baseflow condition; (2) conveyed the way *C. perfringens* may support the understanding of the link between microbiological quality of the stream water and the catchment soil; and (3) determined the feasibility of using these headwater streams as reference (i.e., negative control) in order to establish long-term water quality monitoring programmes and to assist management decisions. The hypothesis was that soil from the tropical steep slope highland is the dominant source of contamination in stream water resources at the baseflow condition and that *C. perfringens* is able to clearly demonstrate the existence of this influence and provide the information about the feasibility of using tropical highland streams as a reference. Given that catchment soil is slowly transported through soil erosion and that its impact on headwater streams is cumulative, evaluation of this kind of influence requires an appropriate bioparametric variable reflecting this situation. Consequently, the resistant nature (Davies *et al.* 1995; Vierheilig *et al.* 2013) and accumulation potential (Byamukama *et al.* 2005) of *C. perfringens* were the basis for its use as a suitable bioparametric variable for testing the present hypothesis.

MATERIALS AND METHODS

Study area and sampling strategy

The study was conducted on the northern slope of the Uluguru Mountain (coordinates 6.8°S, 37.6°E) in Tanzania with an anticipated area of approximately 137 km^2 at an altitude greater than 1,450 m above sea level. This area is characterised by mean annual rainfall ranging from 900 to 2,300 mm and air temperature ranging from 19 °C to 25 °C (Kimaro *et al.* 2008). The soil texture ranges from sandy-clay to sandy-clay-loam (Munishi *et al.* 2007). The area is composed of headwater streams that drain the steep slope of the mountain environment. These headwater streams are characterised by two pronounced discharge peaks per year as a result of long (March to May) and short (November to December) rainfall events. Generally, all these headwater streams and the associated environments have not been microbiologically investigated for their suitability as source of drinking water for Morogoro's urban population in Tanzania. Extensive survey of these headwater streams revealed that anthropogenic influences were non-existent and the surveyed headwater streams satisfied 20 presumptive criteria of reference streams (see Sánchez-Montoya *et al.* 2012). Although the surveyed environment was covered with vegetation (mainly shrubs and grasses), soil erosion and landslides were highly pronounced as a result of the unstable nature of the mountain slope. Additionally, an in-field visual counting survey revealed the presence of various types of faecal droplets ($>$5 faecal droplets/km^2) on the soil, signifying the potential influence from wildlife.

To understand the effect of the catchment soil on the microbiological quality of headwater streams, four independent plots (each with the size 20 m × 20 m) were randomly selected in the catchment with observable soil erosion and/or landslide. In order to obtain representative soil samples, each of the selected plots was further divided into 10 m × 10 m subplots resulting in four sub-plots per plot. Five grams of the first 10 cm top soil was sampled aseptically from each sub-plot of the selected plot to form a composite soil of 20 g per plot. Soil samples were immediately placed in sterile plastic bags before being carried to the laboratory and stored at 4 °C in the dark until analysis. Water samples from the streams corresponding to the selected plots was aseptically obtained under baseflow condition using sterile 500 mL glass bottles. Each sample matrix from the selected sites was separately sampled five times ($n = 5$) over the sampling period resulting in a total of 20 samples per sample matrix. Additionally, water samples from two independent river systems, one located in an agricultural environment (6.74° S, 37.77° E) and the other in an urban environment (6.8° S, 39.26° E) were sampled 25 times ($n = 25$) during the baseflow condition in order to evaluate the suitability of highland streams to serve as a negative control during the evaluation of tropical river systems located in anthropogenically influenced environments. All the water samples were taken to the laboratory and stored at 4 °C in the dark until analysis.

Sample processing

In the laboratory, soil samples were homogenised as described in Byamukama *et al.* (2005) before extraction of *C. perfringens* using a method that has proven to be efficient in recovering bacterial cells from soil matrix (Kingsley & Bohlool 1981). After extraction, the supernatant solution (10–100 mL) was analysed for *C. perfringens* concurrently with stream water (100–200 mL) samples using the standard technique as proposed in ISO 14189:2013 (ISO 2013). The membrane filtration method employing 47 mm diameter Whatman cellulose nitrate filters (Sartorius, Gottingen, Germany) with porosity of 0.45 μm was used for the enumeration of *C. perfringens* from soil extractants and stream water samples. The cellulose membrane filters that retained bacteria with greater than 0.45 μm size were placed on tryptose sulphite cycloserine (TSC) agar (Merck, Darmstadt, Germany) containing 0.4 g/L of *C. perfringens* supplement that was prepared according to the manufacturer's directions. It should be noted that TSC agar method was applied due to its higher specificity when compared to other available methods for detecting *C. perfringens* (Araujo *et al.* 2001). Additionally, the *C. perfringens* supplement test for acid phosphatase is a highly specific indicator for *C. perfringens* (Ryzinska-Paier *et al.* 2011). Triplicate TSC agar plates per sample were prepared before being incubated at 44 °C for 24 hours while in Anaerocult A anaerobic system containing anaerobic indicator (Merck). After incubation, presumptive black colonies surrounded by black precipitates that appeared light blue upon exposure to UV light were considered as *C. perfringens*. Enumerations were performed on Petri dishes (60 mm) containing between 20 and 200 positive colonies of *C. perfringens*. The counted colonies were expressed as colony forming unit per cubic centimetre (cfu/cm^3) assuming that $1\ \text{mL} \approx 1\ \text{g} \approx 1\ \text{cm}^3$.

Data analysis

Comparison of *C. perfringens* concentrations between sample matrices was performed using non-parametric Mann–Whitney *U*-test. Spearman rho test was used to measure the degree and statistical significance of the association between soil and water *C. perfringens* concentrations. The extent and significance differences among reference stream, agricultural and urban rivers, with respect to *C. perfringens* were determined using non-parametric Kruskal–Wallis test. Mean abundances of *C. perfringens* in soil and water samples were separately determined by calculating geometric means of abundances. Streams with a geometric mean below the recommended safe permissible value for tropical environment (geometric mean of 50 cfu/cm^3) were classified as reference streams. All statistics were executed on the Statistical Package for the Social Sciences version 16.0 (SPSS Inc., IL, USA) and probability (P) value below 0.05 was deemed significant.

RESULTS AND DISCUSSION

C. perfringens was frequently detected in the catchment soil (100%) and in the stream water (100%) (Figure 1) even though the sampling sites were free from anthropogenic influences and in different locations within the investigated mountain (>1 km from one site to another). The constant detection of *C. perfringens* in soil samples confirmed that this bacterium accumulates in the environment and thus can serve as a marker for persistent faecal contamination

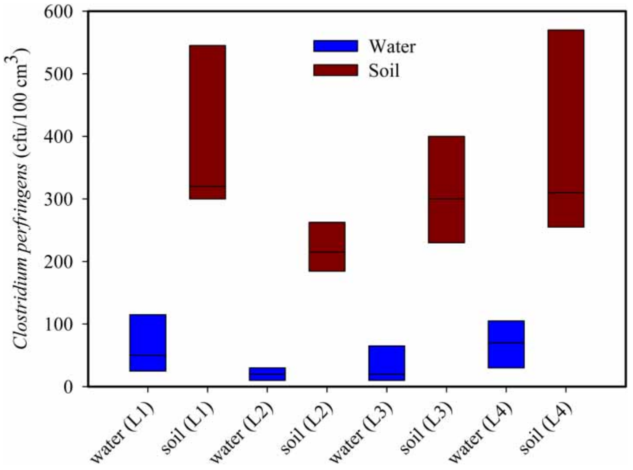

Figure 1 | Pairwise comparison of *C. perfringens* concentrations (in cfu/100 cm^3) between stream water and corresponding catchment soil as detected in various locations (L1 to L4) of the studied tropical highland. Boxes denote interquantile range (25 to 75 percentiles), the line within the box denotes the median. The concentrations were found to be significantly different between the sample matrices ($P < 0.05$, non-parametric Mann–Whitney *U*-test).

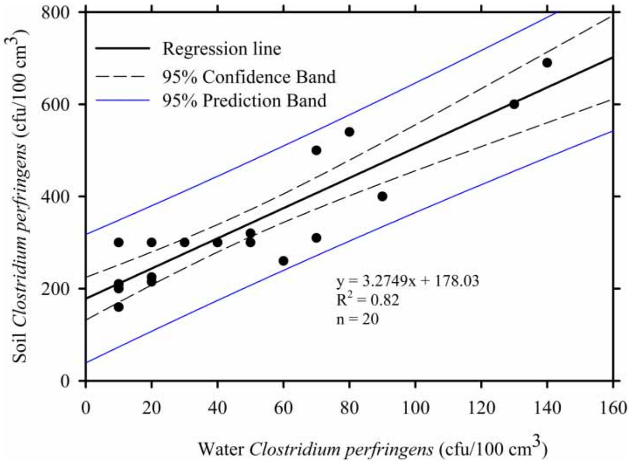

Figure 2 | Regression analysis of *C. perfringens* concentrations (in cfu/100 cm^3) between soil and stream water matrices.

(Farnleitner *et al.* 2010; Vierheilig *et al.* 2013). While the concentrations of *C. perfringens* in the soil matrix were within the range reported in the literature for tropical environments (Byamukama *et al.* 2005; Goto & Yan 2011), the water matrix showed significantly lower *C. perfringens* concentrations than previously reported in tropical river systems (Mushi *et al.* 2010). Unlike the headwater streams in this study, these tropical river systems were located in an urban environment characterised by anthropogenic influences, where higher concentrations are expected due to the strong link between *C. perfringens* and anthropogenic influences described in previous studies (e.g., Skanavis & Yanko 2001; Byamukama *et al.* 2005; Farnleitner *et al.* 2010). *C. perfringens* from soil had a geometric mean of 307 cfu/cm^3 (ranging from 160 to 690 cfu/cm^3) as compared to the geometric mean of 33 cfu/cm^3 (ranging from 10 to 140 cfu/cm^3) detected in water samples (Figure 1) suggesting that soil entrance into stream water through erosion resulted in 1 log reduction of *C. perfringens*. The low concentrations of *C. perfringens* detected in the stream water suggest that the catchment soil was the source of contamination in stream water rather than anthropogenic activities. This was supported by the fact that 82% of the variations of *C. perfringens* concentrations in the water were explained by *C. perfringens* concentrations from the catchment soil (Figure 2). Supposedly, wildlife present in the studied environment deposits *C. perfringens* to the catchment soil through faecal droplets, which are subsequently brought to the stream water by soil erosion and/or landslide enhanced by the poor stability of the studied steep slope catchment (Westerberg & Christiansson 1999). It should be noted that autochthonous *C. perfringens* are unlikely to occur in the investigated environment due to the fact that these bacteria require special growth conditions (Shimizu *et al.* 2002), which are not offered by environmental matrices such as water and soil.

The *C. perfringens* concentration in stream water was below the recommended concentration for safe water in tropical environments (Fujioka & Shizumura 1985). This concentration is associated with a negligible risk of gastroenteric illnesses (Kueh *et al.* 1995), suggesting an acceptable level of disturbance in the studied streams. Because the stream water samples were collected in an area free from anthropogenic influences during baseflow condition (dry season), the quantified geometric mean of *C. perfringens* concentration is suggested to denote the baseline concentration for tropical stream waters. This outcome is consistent with previous studies (Fujioka & Shizumura 1985; Byamukama *et al.* 2005), which reported that *C. perfringens* exists in the environment at low concentrations, but tends to increase to significant levels, when there is anthropogenic influence. Consequently, the value detected in this study is suggested as reference concentration for *C. perfringens* in tropical environments and the use of the investigated streams – from which this reference concentration was quantified – as reference streams is proposed.

Unlike the faecal indicator bacteria *Escherichia coli* (Verhougstraete *et al.* 2015) and *Enterococcus* sp. (Tiefenthaler *et al.* 2009), which were used to define acceptable levels of disturbance and reference concentrations in freshwater systems of moderate climate, *C. perfringens* is a more precise indicator of fecal contamination in tropical environments (Fujioka & Shizumura 1985; Byamukama *et al.* 2005). In contrast to *E. coli* and *Enterococcus* sp., *C. perfringens* has not been shown to be able to proliferate in the environment (Davies *et al.* 1995; Ishii & Sadowsky 2008; Byappanahalli *et al.* 2012), which makes them a suitable candidate for defining microbiological reference level. This is the first time the reference level of *C. perfringens* is defined in the tropical developing world despite it being used frequently for evaluating faecal pollution of their water systems (Byamukama *et al.* 2005; Mushi *et al.* 2010; Macharia *et al.* 2015). When the concentrations of *C. perfringens* from the headwater streams (denoted as reference) were compared with the river waters subjected to anthropogenic influences, concentrations of *C. perfringens* were significantly greater ($P < 0.05$) in agricultural and urban river systems than in reference streams (Figure 3). This suggests that the investigated headwater streams can be used as a surrogate for negative control during evaluation of anthropogenically influenced river systems in tropical environments.

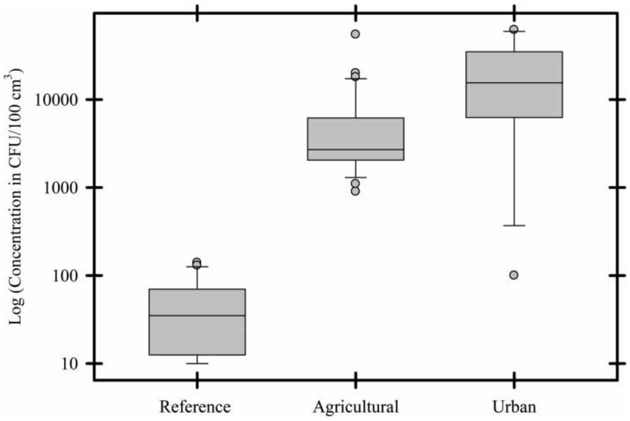

Figure 3 | Observed *C. perfringens* concentrations in different stream waters by degree of anthropogenic influence. Box plots show 10th, 25th, 50th, 75th and 90th percentiles, and individual data points outside the 10th and 90th percentiles denote outliers. Anthropogenic influences had a significant effect on *C. perfringens* concentrations ($P < 0.05$, Kruskal–Wallis test) when compared to the reference streams.

This study provides a useful basis for setting an acceptable contamination level that does not hamper critical services provided by tropical streams as there is no significant health risk associated with the low concentrations of *C. perfringens* detected in the investigated stream water. Despite the fact that the reference level of *C. perfringens* reported here can be directly applied to tropical environments similar to that of this study, the existing gaps regarding the concentration of *C. perfringens* contributed by each wild animal species, their accumulation and removal rates, the pathways in which these bacteria get into stream water systems, and their ecology in stream water have to be filled in order to validate their applicability in this type of environment.

CONCLUSION

This paper showed that *C. perfringens* was consistently detected in the catchment soil and in the stream water matrices of the investigated tropical highland with the catchment soil having significantly higher *C. perfringens* concentrations than the stream water matrix. The catchment soil was found to be the main source of *C. perfringens* in the stream water. Despite this contamination, the geometric mean of stream water was below the permissible safety level recommended for tropical environments. When the concentrations of *C. perfringens* in the stream water were compared with those of anthropogenically influenced rivers, significant differences were observed. Consequently, the geometric mean of *C. perfringens* concentration detected in the tropical highland streams was recommended as reference level and the streams in which this concentration was determined were suggested as reference streams.

ACKNOWLEDGEMENTS

I thank James Mwesongo for his assistance during sampling and processing of samples. This work was supported by funds from Ministry of Education and Vocational Training (MoEVT) of Republic of Tanzania awarded to DM.

REFERENCES

Araujo, M., Sueiro, R. A., Gómez, M. J. & Garrido, M. J. 2001 Evaluation of fluorogenic TSC agar for recovering *Clostridium perfringens* in groundwater samples. *Water Science and Technology* **43** (12), 201–204.

Baumgardner, D. S. 2012 Soil related bacterial and fungal infections. *Journal of American Board of Family Medicine* **25** (5), 734–744.

Bisson, J. W. & Cabelli, V. J. 1980 *Clostridium perfringens* as a water pollution indicator. *Journal of Water and Pollution Control Federation* **52**, 241–248.

Byamukama, D., Mach, R. L., Kansiime, F., Manafi, M. & Farnleitner, A. H. 2005 Discrimination efficacy of fecal pollution detection in different aquatic habitats of a high-altitude tropical country, using presumptive coliforms, *Escherichia coli*, and *Clostridium perfringens* spores. *Applied and Environmental Microbiology* **71** (1), 65–71.

Byappanahalli, M. N., Nevers, M. B., Korajkic, A., Staley, Z. R. & Harwood, V. J. 2012 Enterococci in the environment. *Microbiology and Molecular Biology Reviews* **76** (4), 685–706.

Davies, C. M., Long, J. A. H., Donald, M. & Ashbolt, N. J. 1995 Survival of fecal microorganisms in marine and freshwater sediments. *Applied and Environmental Microbiology* **61** (5), 1888–1896.

Farnleitner, A. H., Ryzinska-Paier, G., Reischer, G. H., Burtscher, M. M., Knetsch, S., Kirschner, A. K. T., Dirnböck, T., Kuschnig, G., Mach, R. L. & Sommer, R. 2010 *Escherichia coli* and enterococci are sensitive and reliable indicators for human, livestock and wildlife faecal pollution in alpine mountainous water resources. *Journal of Applied Microbiology* **109** (5), 1599–1608.

Ferguson, C. M., Coote, B. G., Ashbolt, N. J. & Stevenson, I. M. 1996 Relationships between indicators, pathogens and water quality in an estuarine system. *Water Research* **30** (9), 2045–2054.

Fujioka, R. S. & Shizumura, L. K. 1985 *Clostridium perfringens*, a reliable indicator of stream water quality. *Journal of Pollution Control Federation* **57**, 986–992.

Goto, D. K. & Yan, T. 2011 Effects of land uses on fecal indicator bacteria in the water and soil of a tropical watershed. *Microbes and Environments* **26** (3), 254–260.

Ishii, S. & Sadowsky, M. J. 2008 *Escherichia coli* in the environment: implications for water quality and human health. *Microbes and Environments* **23** (2), 101–108.

ISO 2013 *Water Quality–Enumeration of Clostridium perfringens–Method Using Membrane Filtration (ISO 14189:2013)*. International Organization for Standardization, Geneva, Switzerland.

Jiwa, S. F. H., Mugula, J. K. & Msangi, M. J. 1991 Bacteriological quality of potable water sources supplying Morogoro municipality and its outskirts: a case study in Tanzania. *Epidemiology Infection* **107**, 479–484.

Kimaro, D. N., Poesen, J., Msanya, B. M. & Deckers, J. A. 2008 Magnitude of soil erosion on the northern slope of the Uluguru Mountains, Tanzania: interill and rill erosion. *Catena* **75**, 38–44.

Kingsley, M. T. & Bohlool, B. B. 1981 Release of Rhizobium spp. from tropical soils and recovery for immunofluorescence enumeration. *Applied and Environmental Microbiology* **42**, 241–248.

Kueh, S. C. W., Tam, T. Y., Lee, T., Wong, S. L., Lloyd, O. L., Yu, I. T. S., Wong, T. W., Tam, J. S. & Basset, D. C. J. 1995 Epidemiological study of swimming-associated illnesses relating to bathing-beach water quality. *Water Science and Technology* **31** (5), 1–4.

Macharia, P. W., Wairimu, A. M., Yillia, P. T., Byamukama, D. & Kreuzinger, N. 2015 Microbial quality of domestic water: following the contamination chain in a rural township in Kenya. *Journal of Water, Sanitation and Hygiene for Development* **5** (1), 39–49.

Munishi, P. K. T., Shear, T. H., Wentworth, T. & Temu, R. A. P. C. 2007 Compositional gradient of plant communities in submontane rainforests of Eastern Tanzania. *Journal of Tropical Forest Science* **19** (1), 35–45.

Mushi, D., Byamukama, D., Kivaisi, A. K., Mach, R. L. & Farnleitner, A. H. 2010 Sorbitol-fermenting Bifidobacteria are indicators of very recent human faecal pollution in streams and groundwater habitats in urban tropical lowlands. *Journal of Water and Health* **8** (3), 466–478.

Rood, J. I. & Cole, S. T. 1991 Molecular genetics and pathogenesis of *Clostridium perfringens*. *Microbiological Reviews* **55** (4), 621–648.

Ryzinska-Paier, G., Sommer, R., Haider, J. M., Knetsch, S., Frick, C., Kirschner, A. K. T. & Farnleitner, A. H. 2011 Acid phosphatase test proves superior to standard phenotypic identification procedure for *Clostridium perfringens* strains isolated from water. *Journal of Microbiological Methods* **87**, 189–194.

Sánchez-Montoya, M., Arce, M. I., Vidal-Abarca, M. R., Suárez, M. L., Prat, N. & Gómez, R. 2012 Establishing physico-chemical reference conditions in Mediterranean streams according to the European water Framework Directive. *Water Research* **46** (7), 2257–2269.

Sartory, D. P. 1988 Faecal Clostridia and indicator bacteria levels in an eutrophic impoundment. *Water South Africa*, **14**, 115–117.

Shimizu, T., Ohtani, K., Hirakawa, H., Ohshima, K., Yamashita, A., Shiba, T., Ogasawara, N., Hattori, M., Kuhara, S. & Hayashi, H. 2002 Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. *Proceeding of the National Academy of Science USA* **99**, 996–1001.

Skanavis, C. & Yanko, W. A. 2001 *Clostridium perfringens* as the potential indicator for the presence of sewage solids in marine sediments. *Marine Pollution Bulletin* **42**, 31–35.

Sorensen, D. L., Eberl, S. G. & Dicksa, R. A. 1989 *Clostridium perfringens* as a point source indicator in non-point polluted streams. *Water Research* **23**, 191–197.

Tiefenthaler, L. L., Stein, E. D. & Lyon, G. S. 2009 Faecal indicator bacteria (FIB) during dry weather from Southern California reference streams. *Environmental Monitoring Assessment* **155** (1–4), 477–492.

Verhougstraete, M. P., Martin, S. L., Kendall, A. D., Hyndman, D. W. & Rose, J. B. 2015 Linking fecal bacteria in rivers to landscape, geochemical, and hydrologic factors and sources at the basin scale. *Proceeding of National Academy of Science USA* **112** (33), 10419–10424.

Viau, E. J., Lee, D. & Boehm, A. B. 2011 Swimmer risk of gastrointestinal illness from exposure to tropical coastal waters impacted by terrestrial dry-weather runoff. *Environmental Science and Technology* **45**, 7158–7165.

Vierheilig, J., Frick, C., Mayer, R. E., Kirschner, A. K. T., Reischer, G. H., Derx, J., Mach, R. L., Sommer, R. & Farnleitner, A. H. 2013 *Clostridium perfringens* is not suitable for the indication of fecal pollution from ruminant wildlife but is associated with excreta from nonherbivorous animals and human sewage. *Applied and Environmental Microbiology* **79** (16), 5089–5092.

Westerberg, L. & Christiansson, C. 1999 Highlands in East Africa: unstable slopes, unstable environments? *Ambio* **28** (5), 419–429.

First received 7 July 2016; accepted in revised form 24 May 2018. Available online 11 June 2018