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BACTEROIDALES: REVOLUTIONISING MICROBIAL SOURCE TRACKING

The age of using *Escherichia coli* and *Enterococci sp.* as standard faecal indicator bacteria is coming to an end.

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ABSTRACT

The age of using *Escherichia coli* and *Enterococci sp.* as standard faecal indicator bacteria is coming to end as new technological advancements enable us to identify which animals (i.e. human, birds, dogs, cattle etc.) are contributing faecal contamination in waterways and therefore provide a better understanding of the risks posed by this type of pollution.

Bacteroidales are increasingly being used to source human faecal pollution in urban streams and stormwater drains. Here, we clearly outline their favourable characteristics and provide a detailed discussion of analytical methods. Examples of their successful application in waterway assessments are also provided.

INTRODUCTION

Growing populations in urbanised, coastal areas are increasing pressure on water managers to provide safe recreational waters (Image 1). *Escherichia coli* and *Enterococci sp*. have long been preferred faecal indicators in water quality assessments because of their high abundance in the faeces of warm blooded mammals (McLellan & Eren, 2014).

Until the last few decades, it was widely accepted that quantification of *E. coli* was a satisfactory indicator of human health risk. Now, with advances in pathology, it is becoming evident that different sources of faecal contamination (i.e. human and animals), vary in the occurrence of zoonoses and consequently risk to human health. For example, dog faeces can transmit *Toxocariasis* infections in humans. This parasite can cause serious illness and blindness (Robertson & Thompson, 2002).

Similarly, cow faeces can transmit *Leptospirosis* in humans which can cause severe liver and kidney disease (Azizi *et al*., 2012). Correct sourcing of faecal contamination can not only inform on whether faecal pollution is present, but also likely health risk associated with contamination. Unfortunately, *Escherichia coli* and *Enterococci sp.* lack the host specificity required to discriminate human and animal faecal sources.

Another issue with standard faecal indicator bacteria is increasing evidence of environmental *Escherichia coli* and *Enterococci sp*. strains that have no faecal origin and which cannot be discriminated by current culture methods, reducing confidence that recorded levels are truly representative of faecal contamination (Power *et al.,* 2005, Byappanahalli *et al.,* 2012, Lleo *et al.,* 2005).

Many scientists and managers alike aver that current bacterial assessments are inappropriate, given the challenges and complexity of pollution sourcing; calling for more informative and rapid indicators of faecal contamination.

Image 1. Beachgoers enjoying a warm summers day on a beach in Melbourne, Victoria

BACTEROIDALES: A FAECAL INDICATOR OF THE FUTURE

New indicator bacteria that are increasing in popularity belong to the Archaean order *Bacteroidales* (Dick & Field, 2004, Stea *et al.,* 2015, Walker *et al*., 2015). *Bacteroidales*, unlike traditional indicator bacteria are obligate anaerobes and therefore are unlikely to propagate outside an animal's digestive system (Scott *et al*., 2002).

They are more abundant than either *Escherichia coli* or *Enterococci sp.* in faeces. Arguably, their greatest attribute is that they display high specificity, coevolving within their hosts' digestive system (Dick *et al.,* 2005, Witty *et al.,* 2009).

These assays reflect a change in protocol away from culture dependent assessments towards the application of genetic technologies, specifically polymerase chain reaction methods (PCR), to amplify bacteria to a quantifiable level i.e. quantitative-PCR or real-time PCR (qPCR and rt-PCR respectively). These methodologies have the potential to revolutionise microbial source tracking in waterways because the choice of indicator bacteria is no longer limited to those which can be easily cultured, but rather select bacteria that have desired traits such as high host-specificity and abundance (Roslev & Bukh, 2011, Witty *et al.,* 2009).

Another major advantage of genetic amplification methods is greater quantification of amplification efficiency. In culture-based approaches, it is assumed that bacterial levels successfully grown on culture medium directly correlate to environmental levels. Often, limited quality control measures are reported. Conversely, qPCR assays are specifically designed for each species marker. Each assays comprises a unique set of reagents and primers which are subjected to a pre-defined series of thermo-cycles i.e. hot / cold cycles to maximise amplification of target bacteria (Bernhard & Field, 2000).

During this process, target bacteria are fluorescently tagged and their associated fluorescence quantified in real-time during each thermo-cycle. The qPCR light cycler machine quantifies "crossing points" (Cp) values which are defined by the manufacturer as the cycle at which the fluorescence of a sample exceeds background levels i.e. the number of light cycles until the sample concentration is reliably detected. These Cp values are then compared against a standard curve and converted to concentrations (Dick & Field, 2004).

The standard curve is generated from replicate serial dilutions of known-concentration reference standards. Here, light cycler-generated Cp values are plotted against predetermined concentrations and a linear regression fit using the second derivative method.

The range of the standard curve should encompass the expected distribution of the samples to be tested to ensure accurate quantification. The fit of the data to the standard curve is then used to generate quality control indices. Broad assay performance indices report the accuracy of light-cycler quantification by assessing the deviation of light cycler-determined concentrations of standards from predefined concentrations and also amplification efficiency.

If the reaction is 100% efficient, we would expect the number of fluorescently tagged bacteria to exactly double each cycle representing a value of "2".

Generation of a standard curve for each species-marker also allows for distinct definitions of detection and quantification limits which provide the user with a complete understanding of the power and error within each species marker.

The limit of detection is commonly determined from the linear equation as the concentration when the Cp value is zero or a pre-set threshold i.e. five cycles. Another approach is to analyse serial dilutions until the light cycler can no longer detect any target DNA beyond background levels. The limit of quantification can be determined as the exact concentration at which the linear relationship between concentration and Cp value breaks down.

Another common approach uses ANOVA to determine the concentration which can no longer reliably be discriminated from previous levels.

Additional control measures are also added to assays. These commonly include: analysing replicate samples where the average Cp or concentration and standard deviations are reported; negative filtration controls (filtration of distilled water and extraction of DNA from associated membrane); negative PCR controls (replacement of environmental water sample with distilled water in certain PCR reactions); positive PCR controls (amplification of multiple known-concentration reference standards); internal amplification controls (all reactions are spiked with a reference material to determine if PCR inhibitors are present within each reaction); and recovery efficiency indicators/extraction control (each environmental water sample is spiked with a known concentration of non-target DNA, typically salmon sperm DNA, during the extraction process which is quantified in a separate qPCR assay).

Comprehensive research has investigated marker specificity (amplification in non-target species) and sensitivity (amplification in target species) to verify that the most accurate and reliable segment of *Bacteroides sp.* 16s rRNA or other gut fauna in the case of birds which have low levels of *Bacteroides sp.,* have been targeted (Ahmed *et al.,* 2012, Dick *et al.,* 2005, Fogarty & Voytek, 2005).

Results from one of these studies are provided in Table 1 (Kildare et al., 2007). If a marker is shown to cross-amplify in a non-target species, a major advantage of qPCR assays is that the limit of quantification for the target species can be altered accordingly to exclude these incorrect positive results.

Bayes' Theorem can also be applied combining assay sensitivity and specificity, with background levels (determined as the frequency of environmental samples tested with target concentrations above the limit of quantification) to determine the likelihood of results representing true positives.

Table 1. Accuracy of commonly applied host-specific genetic markers to discriminate target (sensitivity) and non-target (specificity) faeces. Percentage of positive identifications are reported within each listed assay (no. of samples positive/no. of samples tested) (from Kildare *et al.*, 2007).

	Non host specific / general faecal Bacteroidales (%)			Human-specific faecal Bacteroidales (%)			Cow- or bovine-specific faecal Bacteroidales (%)		Dog-specific Bacteroidales (%)
	BacUni-UCD	Total Bacteroidales	AllBac	BacHum-UCD	HuBac	HF183	BacCow-UCD	BoBac	BacCan-UCD
Faecal material									
Human	100 (18/18)	94.4 (17/18)	100 (18/18)	66.7 (12/18)	88.9 (16/18)	61.1 (11/18)	0.00 (0/18)	11.1 (2/18)	22.2 (4/18)
Cow	100 (8/8)	87.5 (7/8)	100 (8/8)	0.00 (0/8)	37.5 (3/8)	0.00 (0/8)	100 (8/8)	100 (8/8)	0.00 (0/8)
Horse	100 (8/8)	100 (8/8)	100 (8/8)	0.00 (0/8)	12.5 (1/8)	0.00 (0/8)	37.5 (3/8)	0.00 (0/8)	0.00 (0/8)
Dog	100 (8/8)	87.5 (7/8)	100 (8/8)	13.0 (1/8)	87.5 (7/8)	25.0 (2/8)	0.00 (0/8)	0.00 (0/8)	62.5 (5/8)
Cat	100 (7/7)	100 (7/7)	100 (7/7)	0.00 (0/7)	71.4 (5/7)	14.3 (1/7)	0.00 (0/7)	0.00 (0/7)	14.3 (1/7)
Seagull	100 (10/10)	100 (10/10)	100 (10/10)	0.00 (0/10)	0.00 (0/10)	0.00 (0/10)	0.00 (0/10)	0.00 (0/10)	0.00 (0/10)
WWTP influent	100 (14/14)	100 (14/14)	100 (14/14)	100 (14/14)	100 (14/14)	100 (14/14)	0.00 (0/14)	0.00 (0/14)	28.6 (4/14)

Distribution and abundance of markers within host populations across different geographic ranges and life history stages have also been tested (Ahmed *et al.*, 2008, Okabe *et al.,* 2007, Shanks *et al.*, 2014). Region-specific variation in marker sensitivity and specificity confirm it is paramount that the distribution and abundance of *Bacteroides sp.* markers in local populations be determined prior to any investigations.

At The University of Melbourne's Centre for Aquatic Pollution and Identification Management (CAPIM), we have tested the presence and cross amplification of human, dog, seagull and ruminant markers in a small subsample from Victoria (Table 2).

Our results support other research in the literature which confirmed the presence of high concentrations of human *Bacteroides sp.* in wastewater and stormwater across Australia (Ahmed *et al.,* 2016, Sidhu *et al.,* 2013), reiterating their applicability and value in Australian local water quality assessments. Age-related differences in *Bacteroides sp.* present in host populations are less clear but differences are suggested to be most prevalent in species with long weaning time for young, such as cattle (Shanks *et al.,* 2014). Long-term agricultural feeding strategies have also been shown to influence marker sensitivity in cattle (Shanks *et al.,* 2011). However, the presence of specific *Bacteroides sp.* is not only based on what food is digested, but also how food is digested. The strength of these qPCR assays is that these sources of error / variation can be quantified in pilot studies and the parameters of the assays adjusted accordingly to increase overall accuracy and confidence of results.

A standard protocol is currently being developed by the US EPA for sourcing human faecal contamination. Unique *Bacteroides sp.* have also been identified from ruminants, canine, seagull and duck digestive systems among others, although these have not yet been adapted into standard protocols.

Table 2. Comparison of sensitivity and specificity of Bacteroidales species markers from a subsample from Victoria, Australia. Percentage of positive stool samples are reported (no. of samples positive/no. of samples tested).

Faecal sources	BacHum-UCD (%)	BacCan-UCD (%)	qGull (%)	BacCow-UCD (%)
Human	80 (4/5)	0 (0/5)	0 (0/5)	20 (1/5)
Horses	0 (0/3)	100 (3/3)	0 (0/3)	100 (3/3)
Cows	33 (1/3)	66 (2/3)	0 (0/3)	100 (3/3)
Goats	0 (0/3)	100 (3/3)	0 (0/3)	66 (2/3)
Dog	0 (0/3)	100 (3/3)	0 (0/3)	0 (0/3)

Arguably one of the main challenges for the use of *Bacteroides sp.* markers to date is ensuring repeatability and reproducibility of results. Shanks *et al.,* (2016) have outlined data acceptance criteria for standardised human-associated faecal source identification based on a multi-lab investigation.

Modern sourcing of microbial pollution in waterways requires a complex suite of indicator organisms to equal the ever increasing number of mixed point and non-point sources of pollution. *Bacteroides sp.* are showing real promise in their successful sourcing of microbial pollution, particularly between human and non-human sources.

Their value is highest in combination with traditional faecal indicator bacteria such as *Escherichia coli* or *Enterococci sp.* and chemical markers in a multi-tiered approach to microbial source tracking.

SUCCESFUL APPLICATIONS OF *BACTEROIDES* TO SOURCE HUMAN AND NON-HUMAN FAECAL CONTRIBUTION

Staff from CAPIM in collaboration with local councils, Melbourne Water and EPA Victoria have successfully used *Bacteroides sp.* assays in studies to determine the presence of human faecal contamination in rivers and estuaries.

Bacteroides sp. assays in conjunction with traditional faecal indicator bacteria *Escherichia coli* and the chemical indicator ammonia have been used to source human faecal pollution in urban waterways within Melbourne city, including Dandenong Creek and Merri Creek and Frankston and Mornington shires on the Mornington Peninsular.

These assays have also been undertaken in the rural catchments of Wye River, The Otways Coast and Werribee, located 32 km south-west of Melbourne, Victoria.

The inclusion of a general *Bacteroides sp.* marker which detects all sources of faecal contamination, not just those of concern confirmed that faecal contamination was present in Dandenong Creek.

The subsequent use of species-specific *Bacteroides sp.* markers (dog, human and ruminant) showed that the major source of this contamination was non-human (dog and ruminant) although strong human-specific markers were present at some sites.

Measurements using *Escherichia coli* showed that faecal contamination was above recommended levels for recreational swimming. However *Bacteroides sp.* markers identified low levels of faecal contamination that was not detected by *E. coli* assessments (Kellar *et al.,* 2016).

A multi-tiered approach was implemented in the Frankston and Mornington surveys where we were able to use a combination of *Escherichia coli, Bacteroides sp.* and chemical ammonia indicators to identify human faecal contamination in streams (Figure 1).

These were then traced up the stormwater drains using real-time AMI Ammonia test kits® and *Bacteroides sp.* testing was used to confirm whether any faecal contamination was of human origin. We were able to identify three separate locations where cracked sewerage infrastructure was leaking untreated sewage directly into stormwater.

Once these leakages had been rectified, human faecal contamination was no longer detected. Such leakages can be identified by other approaches such as directly inserting high-tech cameras into stormwater drains and looking for visual evidence of cracks.

But such approaches are often extremely costly for local councils and often require timely surveys through complex networks before cracks are located.

The advantage of combining *Bacteroides sp.* assays with a targeted sampling approach is that large urbanised areas can be quickly monitored and key areas flagged for further inspection. On multiple occasions throughout this survey, we were able to source elevated *E. coli* levels as non-human derived, identifying them as from either seagull or dog faeces, confirming non-point source contamination (Sharley *et al*., 2014).

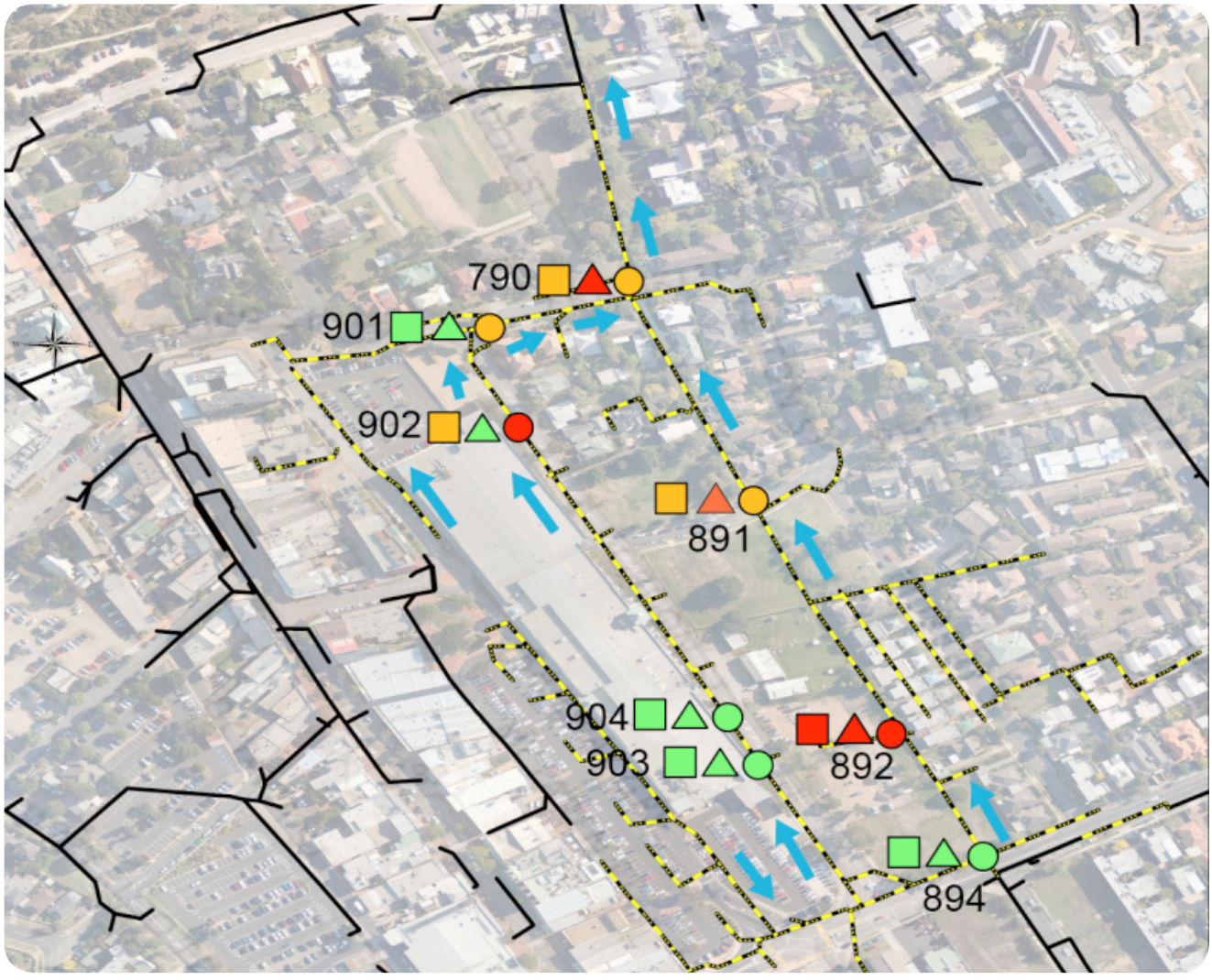

Figure 1. Microbial source tracking using multiple indices (chemical, *Bacteroidales sp.* & *Escherichia coli*) in Frankston, Victoria.

Non-human and human sources were identified. Cracked sewer pipes were found at sites 790, 891 and 892.

A second follow-up study was conducted again in Frankston. Similar faecal indicators were employed and again we were able to confirm that elevated *E. coli* levels were sourced from humans in the lower catchment and were able to identify an emergency spill and another damaged sewer discharging raw sewage into stormwater (Amos & Sharley, 2016).

The application of *Bacteroides sp.* markers in a rural catchment was able to confirm likely agricultural sources of contamination. In Wye River, sites were tested for human, ruminant, duck and universal *Bacteroides sp.* markers (Figure 2).

Results from the universal marker again confirmed faecal contamination at multiple sites. Both the duck and human markers identified levels of contamination that were below the limit of quantification indicating that faecal contributions from these sources are low. Conversely, the ruminant marker identified quantifiable levels of faecal contamination and, interestingly, the levels of contamination identified at each site was comparatively similar to those identified by the universal marker, albeit an order of magnitude less, suggesting that ruminant sources are likely to be a major contributor of faecal contamination at each site.

These results suggest a lower risk to human health than if the contamination was sourced as of human origin. In the Werribee surveys, the same markers were tested and again, *Bacteroides sp.* assays identified low-levels of dog and ruminant faecal contamination not detected by *Escherichia coli* and confirmed the high levels of *E. coli* recorded at one site was of human origin, suggesting a potential cracked sewer leaking into stormwater.

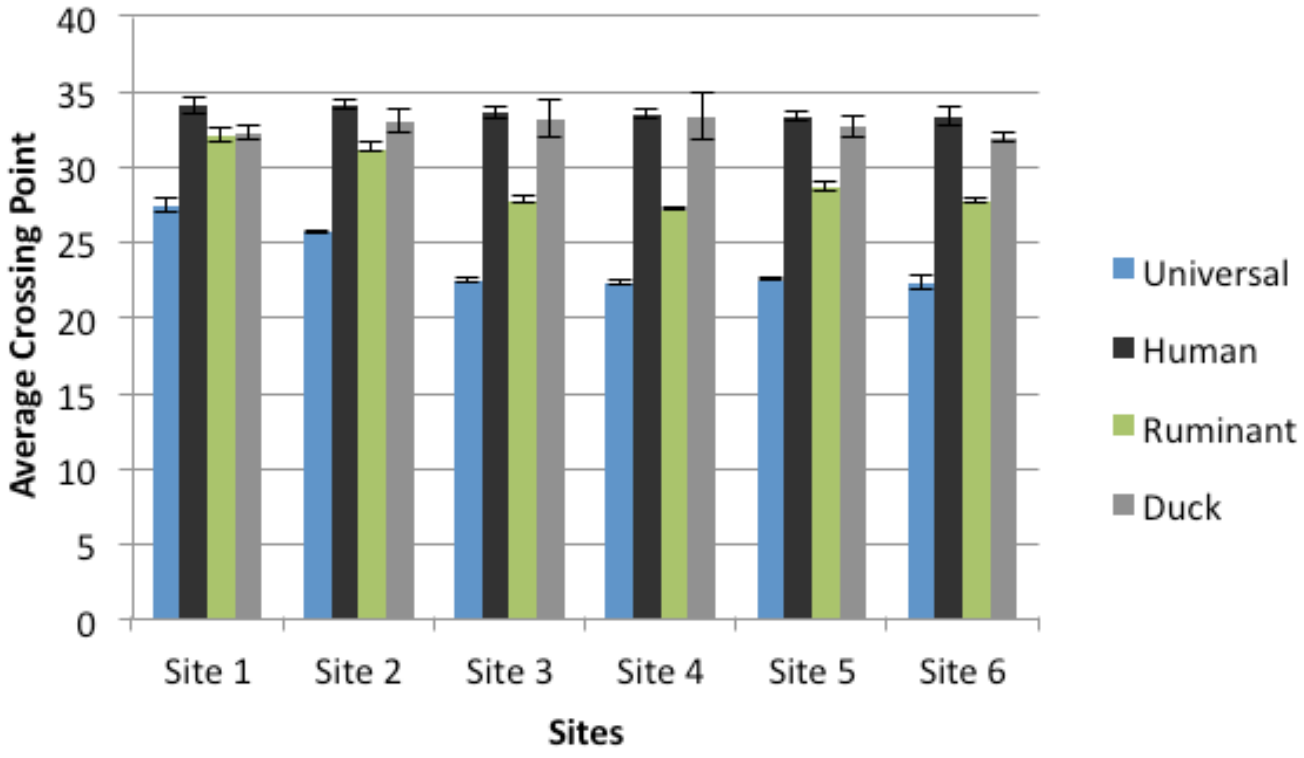

Figure 2. Wye River *Bacteroides sp.* assessment results. Average qPCR light cycler derived crossing point values (defined as the number of polymerase chain reaction (PCR) cycles until the sample fluorescence recorded by the PCR machine exceeds background levels) with associated standard deviations are reported.

Human and duck species markers were below the limit of quantification i.e. recorded levels are too low to be reliably quantified, and are in grey scale. Both the universal and ruminant markers recorded quantifiable levels of faecal contamination and displayed similar patterns across sampled sites.

The examples described above showcase the application of *Bacteroides sp.* assays in both snapshot and longer-term assessments of faecal contamination. In snapsot assessments, *Bacteroides sp.* assays provide a powerful, highly sensitive and specific approach to reliably source faecal contamination at a particular point in time.

In longer-term assessments, when combined with a targeted sampling strategy faecal signals can be traced within catchments to identify point sources and temporal changes in specific faecal sources of contamination, as opposed to general measures as currently provided by standard *E. coli* counts alone.

Exactly what environmental levels of *Bacteroides sp.* correlate to human health concerns is not yet defined for all pathogens. However, this approach does allow managers to prioritise risk based on whether the faecal contamination is human or animal and more specifically ruminant, bird or domestic. Each faecal source poses different and known risks to human health based on the presence of specific zoonotic pathogens.

CONCLUSION

The sole use of general indicators such as *E.coli* and *Enterococci sp.* is no longer sufficient to infer human faecal pollution in urban waterways. These standard faecal indicator bacteria are incapable of discriminating between the growing number of human and non-human sources of faecal contamination and consequently differing risks to human health. Furthermore, poor identification of contamination source restricts appropriate management to ensure the contamination event does not reoccur.

Bacteroides sp. can provide information on what animals are the sources of any observed faecal pollution and therefore provide managers with a better understanding of the risk posed by this contamination to human health and best management practices. Sourcing faecal contamination through complex stormwater systems requires an equally diverse toolbox of which *Bacteroides sp.* are proving to be a much valued, additional source information, than could be provided by *E. coli* counts alone.

Combined with a target sampling strategy, *Bacteroides sp.* assays provide a comparatively cheap and quick method to monitor both point and non-point sources in large, often urbanised catchments.

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