ALTERNATIVE INDICATORS FOR DETECTION AND QUANTIFICATION OF FAECAL POLLUTION

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Abstract

Traditional faecal indicator bacteria such as faecal coliforms, *Escherichia coli* and enterococci have long been used as indicators of faecal pollution in environmental waters. However, the reliability of these traditional indicators has been questioned in terms of their ability to predict the likely presence of pathogens. Another limitation of these indicators is that they cannot be used to distinguish the sources of faecal pollution which need to be known to ensure the improved management of water quality and the assessment of health risk. In recent years, the use of alternative microbial faecal indicators such as faecal anaerobes (i.e. *Bacteroides* spp., *Bifidobacterium* spp., *Clostridium perfringens)*, and viruses (phage), and chemical indicators (i.e. faecal sterols, caffeine, and optical brighteners) has become popular because these can provide sensitive and accurate measurement of faecal pollution in environmental waters. In this paper, the advantages and limitations of using alternative indicators for predicting the sources of faecal pollution are briefly evaluated. The correlations between alternative indicators and pathogens in environmental waters are discussed. A combination of traditional indicators along with alternative indicators and markers is suggested for monitoring faecal pollution, and future research directions for direct pathogen monitoring are also discussed.

Introduction

Coastal and inland waters are commonly polluted by pathogenic microorganisms, particularly following heavy rainfall. Non-point sources such as domestic and wild animals, poorly performing on-site wastewater treatment systems, urban stormwater runoff, and point sources such as industrial effluents and raw sewage are known to be potential sources of such pollution. Traditional faecal indicator bacteria such as faecal coliforms, *E. coli* and enterococci have long been used to assess the microbiological quality of environmental waters, but do not distinguish between sources. The public health risk from human-associated faecal pollution is well recognised and the risk is considered to be greater than from animal-derived faecal pollution (Field & Samadpour 2007). However, pathogens such as *Escherichia coli* O157:H7, *Salmonella, Campylobacter jejuni, Giardia* spp., *Cryptosporidium* spp., and hepatitis E viruses can also be spread via animal faecal pollution (Craun *et al.* 2004). Direct monitoring of pathogens in environmental waters is an attractive option as it can provide valuable information regarding public health risk. However, there are hundreds of different types of pathogens that can be found in water, and therefore it is not an economically, technologically or practically feasible option for the routine monitoring of water quality.

Epidemiological studies have established human health standards based on exposure to faecal indicator bacteria (Pruss *et al.* 1998). However, the ideal faecal indicator bacteria should satisfy a number of specific criteria. They should be universally native to the intestine of warm-blooded animals, should not be pathogenic, their concentration should be higher than pathogens, they should not multiply outside the host and they should be resistant to a variety of environmental stresses. Finally, ideal indicator bacteria should have a strong association with the presence of pathogens. The shortcomings of the traditional indicators in relation to these have been commonly reported in the literature. These include the following:

- may originate from non-faecal sources (Scott *et al.* 2002);
- ability to replicate in environmental waters in tropical regions (Desmarais *et al.* 2002);
- susceptible to the disinfection process (Hurst *et al.* 2002);
- cannot be used to differentiate the sources of faecal pollution (Field and Samadpour 2007); and
- weak association with the presence of pathogens (Hörman *et al.* 2004).

Distinguishing human from animal pollution.

In recent years, phenotypic and genotypic microbial source tracking (MST) methods have been developed to distinguish various sources of human and animal faecal pollution in surface waters (Scott *et al.* 2002). The most commonly used methods such as antibiotic resistance analysis (ARA), biochemical fingerprinting (BF), ribotyping, repetitive extragenic palindromic (rep) PCR require the development of a known source database of traditional indicators (i.e. *E. coli* and enterococci) from host groups, based on the hypothesis that phenotypic or genotypic characteristics of specific bacterial strains are associated with specific animals. The developed database is then used to compare fingerprints from these same indicator bacteria found in environmental waters (Field and Samadpour 2007).

Despite the successful application of these database-dependent methods, several questions have arisen regarding their utility. For instance, the size and the representativeness of the database need to be addressed prior to developing a database for optimal performance. It has further been reported that temporal and geographical variability exists in faecal indicators, which may restrict their use for a universal database. In response to these factors, it has been suggested that a specific database should be developed for each catchment of interest (Wiggins *et al.* 2003). This approach, however, is unlikely to be cost effective and for this reason may not become an accepted monitoring tool for regulatory authorities. Some of these limitations of MST using *E. coli* and enterococci could be partly overcome by using alternative faecal indicators such as faecal anaerobes (Bernhard & Field 2000), viruses (Borrego *et al.* 1987) and faecal organic compounds (Leeming & Nichols 1996). The most important feature with alternative indicators is that most of them could be used to distinguish the sources of faecal pollution without the need for developing a database. Therefore, better

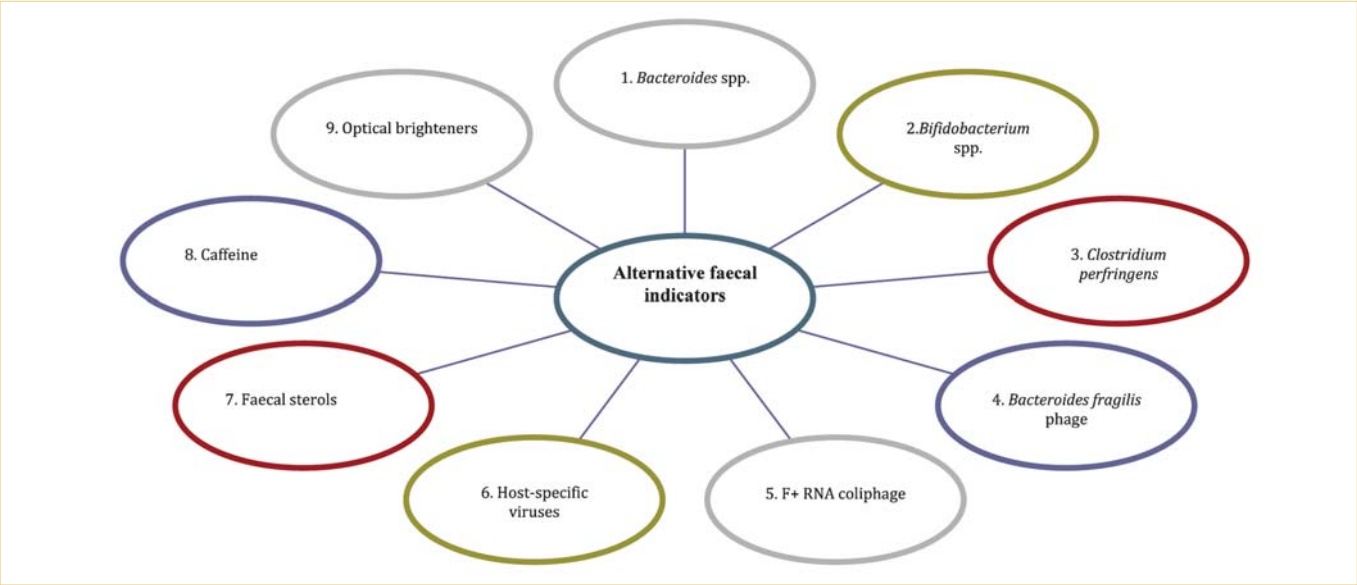

Figure 1. Commonly used alternative indicators of faecal pollution.

management practices can be implemented to minimise the potential health risk associated with faecal pollution. The use of some of these alternative indicators is gaining popularity as evidenced by a recent special issue of *Water Research* (volume 41, issue 16, 2007) which focused on faecal source tracking (FST) methods and their application. Of the 24 research papers, 13 reported the use of alternative indicators alone or in combination with traditional indicators.

The purpose of this review is to evaluate the advantages and limitations of alternative indicators for their ability to predict the sources of faecal pollution. The correlation between alternative faecal indicators with pathogens is also discussed. Furthermore, current methodologies for direct monitoring of pathogens in environmental waters and future research directions are discussed.

Alternative Indicators

The most commonly used alternative indicators are listed in Figure 1. The advantages and limitations of these indicators are discussed below.

1. *Bacteroides* spp.

The members of the *Bacteroides* genus hold promise as alternative indicators of faecal pollution due to a number of advantages including their short survival rates outside the hosts, their exclusivity to the gut of warm-blooded animals and the fact that they constitute a relatively larger portion (i.e. 1,000 fold) of faecal bacteria compared to traditional indicators (Sghir *et al.* 2000). However, the use of these anaerobes for water quality monitoring has been limited because of the difficulties in growing them using traditional culture methods. Nevertheless, recent advances in PCR technology can result in rapid detection and identification of these microorganisms (Field & Samadpour 2007). A recent study reported the identification of human and bovine specific *Bacteroides-Prevotella* 16S rRNA gene markers by using PCR, and concluded that these markers can be considered as potential faecal indicators to detect human or bovine origin faecal pollution (Bernhard & Field 2000). Due to these advantages, PCR detection of *Bacteroides* markers has emerged as a potential tool for MST, and field studies have been conducted in the USA (Bernhard & Field 2000), France (Gourmelon *et al.* 2007), UK, Portugal, Ireland (Gawler *et al.* 2007), Belgium (Seurinck *et al.* 2006), Japan (Okabe *et al.* 2006), Austria (Reischer *et al.* 2006) and Australia (Ahmed *et al.* 2007). Consequently, real-time PCR methods have also been developed to quantify the human-specific *Bacteroides* markers in environmental samples (Okabe *et al.* 2006; Seurinck *et al.* 2006). Such an assay would provide precise information regarding the extent of sewage pollution in environmental waters. A limitation of the *Bacteroides* markers is that geographical specificity must be assessed prior to application because horizontal transfer of faecal bacteria is possible amongst species in close contact such as humans and dogs (Dick *et al.* 2005).

2. *Bifidobacterium* spp.

Bifidobacterium spp. are an obligate anaerobic, non-spore-forming enteric bacteria, which are abundant in human faeces and rarely found in animals (Bonjoch *et al.* 2004). As such, this group of bacteria can be considered as a potential faecal indicator to identify human faecal pollution. The key advantage of *Bifidobacterium* spp. is that they do not replicate outside of the digestive tract due to strict growth requirements, and therefore provide evidence of recent faecal pollution. However, the use of these organisms for routine monitoring of water quality is also limited due to the difficulty in growing them using traditional culture methods. It has been reported that certain *Bifidobacterium* spp. are host-specific (Bonjoch *et al.* 2004). PCR and real-time PCR assays have been developed to detect and quantify these host-specific *Bifidobacterium* spp. for environmental samples (Bonjoch *et al.* 2004). An important characteristic of these bacteria is their limited persistency as the numbers can decrease by 3 to 4 orders of magnitude within 2 weeks in the environment. In addition, high background levels of predators and gram-positive bacteria can prevent growth and/or detection of *Bifidobacterium* spp. (Rhodes & Kator 1999). Little is known regarding the persistence and geographical distribution of *Bifidobacterium* markers.

3. *Clostridium perfringens*

C. perfringens are gram-positive spore-forming sulphite-reducing, anaerobic bacteria which are commonly found in the gut of warm-blooded animals. The advantage of using this bacterium is that unlike traditional indicators, they do not replicate in natural waters due to their strict growth requirements (Davies *et al.* 1995). *C. perfringens* are extremely

resistant to disinfection processes and environmental stresses as most of the populations form spores. As such, they persist longer in the environment than traditional faecal indicators and pathogens. Consequently, these microorganisms have been suggested as an indicator for the inactivation and removal of viruses in drinking water treatment (Payment & Franco 1993). It has been reported that the presence of *C. perfringens* significantly correlates with the presence of pathogens in environmental waters (Ferguson 1996). A limitation of *C. perfringens* is that they may not be suitable for identifying recent faecal pollution because their persistence results in detection long after the pollution event (Desmarais *et al.* 2002). Similar to many alternative faecal indicators, *C. perfringens* standards have not yet been evaluated based on epidemiological studies in relation to the acceptable risk associated with faecal pollution.

4. *Bacteroides fragilis* bacteriophage

B. fragilis is an anaerobic gram-negative rod-shaped bacterium present in high numbers in both humans and animals. The phages which infect *B. fragilis* have been proposed as an indicator for human faecal pollution. *B. fragilis* HSP 40 strain has been found in human samples but not detected in samples from animals (Tartera & Jofre 1987). For this reason *B. fragilis* bacteriophage is considered as a potential candidate for human faecal pollution tracking in surface waters. The key advantage of using *Bifidobacterium* spp. is that they do not replicate in the environment. In addition, their presence in the environment has been found to significantly correlate with the presence of human enteric viruses (Jofre *et al.* 1989). However, these phages do not occur commonly in some geographical areas including the USA and Canada (Scott *et al.* 2002). Additionally, the difficulty in recovering this phage from waters with low levels of faecal pollution limits the use of this organism as a faecal indicator.

5. F+ RNA coliphage

Coliphages are viruses that infect E. coli. It has been reported that animal and human faeces contain different serotypes of RNA coliphages, and therefore can be used to identify the sources of faecal pollution (Cole *et al.* 2003). The F+ RNA coliphages comprise of 4 sub groups namely I, II, III and IV. Members of group I are commonly found in both humans and animals, while group IV is associated only with animals. However, members from group II and III have been found to be associated with sewage. One important feature of phages is that their physical characteristics and genetic makeup are similar to human enteric viruses. As such, coliphages have been considered as an index of viral pollution. Another notable feature is that coliphages exhibit high resistance to the water purification process. Hence, they are valuable indicators for viral inactivation by both UV and chemical disinfectants (Tree *et al.* 2003). It has been reported that coliphages are relatively sensitive to high temperature and sunlight inactivation in seawater (Chung & Sobsey 1993). Nonetheless, coliphages exhibit much better resistance in freshwater systems where they could be considered as a potential indicator of enteric viruses (Sinton *et al.* 2002). Overall, further research into the differential survival characteristics and genetic characterisation of the various groups of coliphage is warranted.

6. Host-specific viruses

Over 100 different enteric viruses are found in the intestine of humans. Because of their high degree of host-specificity they could be regarded as excellent indicators of human faecal pollution. One major limitation is that many of them are not detectable using conventional cell culture techniques (Arraj *et al.* 2005). Furthermore, cell culture assays are laborious, time-consuming and lack sensitivity for unequivocal detection of viruses (Baggi *et al.* 2001). However, PCR-based methods have been developed to detect human-specific adenoviruses, polyomaviruses, and enteroviruses (Jiang 2002; McQuaig *et al.* 2006) in environmental waters. Bovine enteroviruses and porcine adenoviruses have also been proposed for the detection of animal faecal pollution. Additional viral targets could also be host-specific, but molecular assays are not available at this stage. One important feature of virus detection is that they are not only host-specific but also indicate public health risk. One limitation of host-specific viruses is that their concentration is low in receiving waters. Therefore a large volume of water need to be processed for detection.

7. Faecal sterols

Faecal sterols and stanols have also been used widely as alternative indicators of faecal pollution (Leeming *et al.* 1996; Shah *et al.* 2007a). Coprostanol is the major sterol (comprising about 40-60% of the total sterol content) in human faeces and is considered a biomarker of human faecal pollution (Leeming *et al.* 1994). However, the use of coprostanol alone as a biomarker can lead to a false indication of results as it is also present in the faeces of other animals such as pigs (Leeming *et al.* 1996). In addition, small amounts can be generated from cholesterol in anaerobic sediments (Mudge *et al.* 1999). As such, the ratio of coprostanol with other faecal sterols has been proposed as an improved method to identify the sources of human/animal faecal pollution (Leeming 1997). In recent studies, ratios across a range of C27:C29 sterols and 5β:5α stanols have given a more specific measure of pollution (Bull *et al.*, 2003; Leeming *et al.*, 1996). When C27:C29 and 5β:5α ratios are both greater than 1, the faecal source is likely to be of human origin. Ratios C27:C29 and 5β:5α) < 1, are indicative of mixed faecal pollution and C27:C29 <1 and 5β:5α > 1 ratios are indicative of herbivore faecal pollution. However, a recent study reported the poor performance of faecal sterols in determining the percentage contribution of sources in mixed faecal samples (Shah *et al.* 2007b). In addition, no direct relationship has been established between the presence of faecal sterols and pathogenic organisms or consequent health risks.

8. Caffeine

Caffeine is of anthropogenic origin, and is found in beverages and many pharmaceutical products. It is excreted in the urine of individuals who have consumed it. Because of this, it has been suggested that the presence of caffeine in the environment could indicate the presence of human sewage (Burkhardt 1999). Levels of caffeine in domestic wastewater have been reported to be between 20 to 300 µg/L (Roger *et al.* 1986). As such, dilution of more than 1:200 would make it difficult to detect in environmental waters. Little is known about the fate of the caffeine in the environment (Standley *et al.* 2000). Furthermore, similar to faecal sterols, no direct relationship has been established between the presence of caffeine and pathogenic microorganisms.

9. Optical brighteners

Optical brighteners have been suggested as potential indicators to detect the presence or absence of human faecal pollution in environmental waters. Optical brighteners (also known as fluorescent whitening agents) are white dyes, a common component of laundry detergents, which act to make light colours appear brighter (Kaschig 2003). Laundry effluent is a major component of human wastewater as plumbing systems collect wastewater from both toilets and washing machines. Because of this, optical brighteners serve as indicators of the presence of sewage in environmental waters. Optical brighteners

in environmental waters could be detected using several methods such as 1) leaving a cotton pad in environmental waters followed by detection using exposure to UV light, 2) high performance liquid chromatography (HPLC) detection, and 3) fluorometry detection. The combination of fluorometry detection and bacterial counts were successful in determining the sources of faecal pollution (Hagedorn *et al.* 2005). However, one major disadvantage of optical brighteners is the lack of specificity as background fluorescence can originate from various organic compounds (Gregor *et al.* 2002). A recent study reported high fluorometric value in areas with no faecal pollution (Hartel *et al.* 2008).

Correlation Between Alternative Indicators and Pathogens

The correlation between indicator bacteria (both traditional and alternative indicators) and pathogenic microorganisms is one of the most important issues in risk assessment. Little is known regarding the correlation between faecal anaerobes (i.e. *Bacteroides* spp. and *Biofidobacterium* spp.) and pathogens. A recent study found a positive correlation between general *Bacteroides* spp. and zoonotic pathogens (Walters *et al.* 2007). In the same study, ruminant-specific markers were also found to predict the presence of *E. coli* O157:H7 and Salmonella while the human specific markers predicted the presence of *Campylobacter* spp. Another recent study in California reported a moderate correlation between the presence of human-specific *Bacteroides* marker and human-specific polyomaviruses in surface waters (McQuaig *et al.* 2006). Human-specific *Bacteroides* markers were also found to show significant correlation with *E. coli* O157 and *Salmonella* spp. (Olga *et al.* 2007). Davies *et al.* (1995) reported a significant correlation between *C. perfringens* and the occurrence of pathogens in surface waters. Significant correlation was also observed between the presence of *C. perfringens* and *Salmonella* spp. in fresh and marine waters. Positive correlation between bacteriophage, enteric viruses and other pathogens have been demonstrated for marine waters by Rozen and Belkin (2001). However, to date there have been no published data on the correlation between chemical indicators/organic compounds and pathogens.

Molecular Methods for Direct Monitoring of Pathogens

The detection and enumeration of traditional indicators and pathogens using conventional culture and biochemical methods have some major limitations such as underestimation of the bacterial concentration due to injured or stressed cells. Furthermore, certain microorganisms in environmental waters could be viable but cannot be cultured using conventional culture techniques. In addition, some of the test methods are time consuming and labour intensive.

However, the application of PCR-based methods has generated interest in direct monitoring of pathogens in environmental waters. The advantages of PCR-based method are that they are rapid and can detect organisms that are difficult to grow using conventional techniques. PCR-based methods have been used to detect a wide array of pathogenic microorganisms in environmental waters (Olga & Okabe 2006). Multiplex PCR methods have been developed which are able to target multiple pathogens in a single tube; as have real-time PCR methods, which are able to quantify target genes in environmental waters. A major drawback of PCR-based methods is their inability to distinguish between viable and non-viable organisms since the DNA of both live and dead cells could potentially be amplified in a reaction. However, reverse transcriptase (RT) PCR can be used to detect viable cells only (Yaron 2002). RT is an enzyme able to synthesise single-stranded DNA from RNA which gives sensitive results without a pre-enrichment step (Deisingh 2004). To increase the sensitivity and specificity of the detection, PCR may also be used in conjunction with other techniques such as the most probable number counting method (MPN-PCR) (Rose *et al.* 1997), PCR enzyme-linked immunosorbent assay (PCR-ELISA) (Sails *et al.* 2002) or the fluorescence *in situ* hybridisation (FISH) technique (Moreno *et al.* 2003).

The immunology-based methods such as ELISA (Crowther 1995) and immunomagnetic separation (IMS) (Mine 1997) provide sensitive detection of a wide range of targets. ELISA combines the specificity of antibodies and the sensitivity of simple enzyme assays by using antibodies and antigens. IMS is a pre-concentration technique which can be used to capture and extract the targeted pathogen from bacterial suspension by using antibody coated magnetic beads. Microarrays are increasingly being used in pathogen detection. This method was developed for studies of gene expression and regulation in organisms for which the complete genome sequence is known (Kato-Maeda *et al.* 2001). Arrays exist for the detection of several virulence factors for *E. coli* O157:H7 and *Shigella* spp. and application of these suggests that virulence factors may be useful in the automated identification and characterisation of bacterial pathogens (Chizhikov *et al.* 2001).

Conclusions

The use of alternative faecal indicator bacteria appears to be promising for distinguishing between the sources of human and animal faecal pollution in environmental waters. A tabulation of advantages and limitations is given in Table 1. However, studies reporting the correlation between these alternative indicators and pathogens are limited and warrant further investigation. None of the traditional and/or alternative indicator bacteria can be seen as a 'gold standard' in terms of predicting the presence of pathogenic bacteria, viruses and protozoans. However, a combination of traditional indicators along with alternative indicators and markers could provide valuable information regarding the extent of faecal pollution, its origin and possible correlation with pathogens. This approach has been applied in only a few studies (for example Boehm *et al.* 2003; Hörman *et al.* 2004; Simpson *et al.* 2004). It has been reported that traditional indicators and alternative indicators showed significant cross-correlation with each other as well as significant correlations with enteropathogens in surface waters (Hörman *et al.* 2004). Since the correlation between indicators and the presence of pathogens in waters is controversial, the best approach could be direct monitoring of pathogens (where possible) so that public health risk could be assessed. The recent advances in PCR, immunology-based methods and microarray technologies will not only allow sensitive and specific detection of pathogens, but also will enable detection of multiple targets with a single assay and would provide important information on microbial water quality and consequent health risk.

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Craun, G.F., Calderon, R.L. & Craun, M.F.

Table 1. Advantages and limitations of alternative indicators of faecal pollution.

Alternative indicators	Advantages	Limitations
Bacteroides spp.	1. Certain species are host-specific. 2. Indicate recent faecal pollution. 3. Do not replicate in the environment. 4. PCR-based rapid detection.	1. Little is know about the relationship with pathogens. 2. Difficulty in growing using traditional methods.
Bifidobacterium spp.	1. Certain species are host-specific. 2. Do not replicate in the environment.	1. Little is know about the relationship with pathogens. 2. Limited persistency. 3. Low sensitivity of detection methods.
Clostridium perfringens	1. Do not replicate in the environment. 2. Highly resistant to environmental stress.	1. Do not exhibit host-specificity. 2. Indicate past faecal pollution. 3. Labour-intensive.
Bacteroides fragilis phage	1. Human-specific. 2. Do not replicate in the environment. 3. Good correlation with enteric viruses.	1. Present in low concentration. 2. Little is known about the relationship with pathogens. 3. Variable persistency.
F+ RNA coliphage	1. Human-specific. 2. Do not replicate in the environment. 3. Good correlation with enteric viruses. 4. Highly resistant to disinfection process.	1. Do not present in all geographical areas. 2. Variable persistency. 3. Warrants more genetic characterisation.
Host-specific viruses	1. Human and bovine specific. 2. Highly resistant to disinfection process 3. PCR-based rapid detection.	1. Present in low concentration 2. Can be absent in the presence of faecal pollution. 3. Concentration and purification of viral nucleic acid from environmental samples can be difficult.
Faecal sterols	1. Can be used to distinguish between human and animal sources.	1. Expensive analytical techniques. 2. Indicate past faecal pollution. 3. No direct relationship with pathogens. 4. Low sensitivity of detection methods. 5. Labour-intensive.
Caffeine	1. Indicate the presence of human faecal pollution.	1. Low sensitivity of detection methods. 2. No direct relationship with pathogens.
Optical brighteners	1. Indicate the presence of human sewage 2. Inexpensive	1. Low sensitivity of detection method 2. Lacks specificity. 3. Can yield false positive. 4. No direct relationship with pathogens.

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