The potential of lipopolysaccharide as a real-time biomarker of bacterial contamination in marine bathing water

Anas A. Sattar, Simon K. Jackson and Graham Bradley

ABSTRACT

The use of total lipopolysaccharide (LPS) as a rapid biomarker for bacterial pollution was investigated at a bathing and surfing beach during the UK bathing season. The levels of faecal indicator bacteria *Escherichia coli* (*E. coli*), the Gram-positive enterococci, and organisms commonly associated with faecal material, such as total coliforms and *Bacteroides*, were culturally monitored over four months to include a period of heavy rainfall and concomitant pollution. Endotoxin measurement was performed using a kinetic Limulus Amebocyte Lysate (LAL) assay and found to correlate well with all indicators. Levels of LPS in excess of 50 Endotoxin Units (EU) mL^{-1} were found to correlate with water that was unsuitable for bathing under the current European regulations. Increases in total LPS, mainly from Gram-negative indicator bacteria, are thus a potential real-time, qualitative method for testing bacterial quality of bathing waters.

Key words | faecal indicator bacteria, LPS, rapid method, sewage pollution, surfing/bathing water

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INTRODUCTION

The quality of beaches in the UK and Europe is governed by the European Bathing Water Directive (EU 2006) which sets legislation and defines standards that all beaches must comply with in order to be considered as a designated bathing beach. Faecal Indicator Bacteria (FIB), namely *Escherichia coli* and enterococci, are important indicators for determining the quality of marine bathing waters as they can eliminate the need for expensive and time consuming testing for pathogenic bacteria and viruses (Lucena *et al.* 1994). The FIB previously used were total coliforms, faecal coliforms, *E. coli* and faecal streptococci as these microorganisms can provide a general indication of faecal pollution; these were later revised and coliforms were substituted by enterococci. However, current quantitative culture-based FIB methods have drawbacks (Borrego *et al.* 1983; Anderson *et al.* 2005; Field & Samadpour 2007) as these indicators have a tendency to multiply in bathing waters and the results are retrospective, taking at least 24–48 h to inform regulatory bodies. Marine recreational water users themselves really need an instant, qualitative indication of possible bacterial pollution events in order to make an informed decision on whether to undertake an activity. The term lipopolysaccharide (LPS) is often used interchangeably with endotoxin and is the major component of the outer membrane of all Gram-negative bacteria (Raetz 1990; Rietschel *et al.* 1994). LPS has been targeted by previous studies for estimating total biomass and testing potable water (Jorgensen *et al.* 1976, 1979; Watson *et al.* 1977; Evans *et al.* 1978; Haas *et al.* 1983) but not as a potential biomarker for bacteria-polluted bathing water, possibly due to background levels from Gram-negative marine species.

Studies conducted by Fiksdal *et al.* (1985), Allsop & Stickler (1985) and Kreader (1995) have suggested the use of *Bacteroides* species as an excellent bacterial indicator candidate since it is shed in high numbers in faeces and is unlikely to reproduce in seawaters. However, because of its anaerobic nature and consequent difficult culture requirement, it has always been overlooked by legislators. *Bacteroides* species inhabit the intestines of human and warm-blooded animals where they comprise 30–50% of normal faecal matter in humans (Isar

doi: 10.2166/wh.2013.142

et al. 2006) and they are present in bathing waters when polluted with poorly treated sewage from urban discharge or runoff from farms, hence *Bacteroides* was included in this study.

Therefore, the aim of this study was to measure the *total* LPS levels in seawater and correlate them with the current culture-based methods for determining this water quality using bacterial indicators such as *E. coli* and *Bacteroides*. This was undertaken at a surfing/bathing beach during the UK bathing season, including a high rainfall pollution event.

MATERIALS AND METHODS

Seawater sampling regime

Challaborough beach was chosen as a popular bathing, body boarding and surfing beach located in the South Hams district of Devon, UK (Latitude: 50.287159, Longitude: −3.899052). It is a horseshoe-shaped bay divided by a small stream that runs from a valley down into the sea (Figure 1).

Challaborough village comprises two small fixed caravan sites and a few private houses which end just beside the beach. This beach was chosen because it is divided into two distinct areas: bathing and surfing.

Figure 1 | Topography of Challaborough beach at low tide and the surrounding caravan site. A, B, C and D represent sampling stations for shallow water and sediment at the bathing area. E, F, G and H represent sampling stations of approximately 1 m depth water sampling in the bathing area. I, J K and L represent sampling stations for shallow water and sediment at the surfing area. M, N, O and P represent sampling stations of approximately 1 m depth water sampling in the surfing area. Q represents the sampling site for stream water.

Composite samples, comprising the four stations from each area, were collected on 10 occasions over the summer of 2012 at Challaborough beach starting from early June to the beginning of September (UK bathing season), in addition to water samples from the stream. Samples were usually taken once a week with a random timing for low and high tide, except for one sample in which heavy rainfall and high level of runoff had been observed and which was considered as the 'polluted' sample (15/08/2012 sample in Figure 3). Samples were collected in 500 mL disposable, sterile, screw-capped wide-mouth pots for the bacteriological investigation whilst 50 mL endotoxin-free Falcon™ tubes (BD Biosciences, UK) were used to collect water samples for LPS detection. Shallow water samples were collected from the surface of the water whilst deeper water samples were taken from approximately 1 m depth. Sediment samples were also collected in similar pots from the shallow water area for both bathing and surfing areas. Samples were immediately taken to the laboratory and processed within 3 h. Samples for LPS detection were aliquoted into endotoxin-free glass tubes (Lonza, UK) and preserved at – 20 °C until assay.

Seawater sample filtration

Water samples were filtered aseptically through a 0.45 μm membrane (Whatman, UK) and placed on appropriate media; *Bacteroides* Bile Esculin (BBE) (Livingston *et al.* 1978), Membrane Lauryl Sulphate Broth, and Slanetz and Bartley agar (Oxoid, UK) to isolate *Bacteroides*, *total* coliforms/*E. coli* and enterococci, respectively. Appropriate volumes of the samples were aseptically filtered in duplicate using a vacuum pump attached to the water filtration system. A 1:10 dilution of the sediment samples (2 g) was prepared in sterile simulated sea water (Instant Ocean, Underworld, UK) (18 mL) and placed in stomacher bags that were placed in a stomacher (Seward Lab, UK) for 2 minutes, and left to settle for 10 minutes. Then, 10 mL of the supernatant was filtered in a manner similar to the method described for water sample filtration above. BBE agar cultures were incubated in an anaerobic incubator (Don Whitley, UK) at 37 °C for 48–72 h, Slanetz and Bartley

agar was incubated aerobically at 35 °C for 4 h then at 44 °C for 44 h, and Membrane Lauryl Sulphate total coliforms cultures were incubated aerobically at 35 °C for 24–48 h and for *E. coli* incubated aerobically at 35 °C for 4 h, then at 44 °C for 44 h.

Enumeration and isolation of bacterial colonies

The numbers of colony-forming units (CFU) were calculated and numbers were expressed as CFU 100 mL^{-1} of water or per g^{-1} of sediment.

Determination of total endotoxin concentration

In order to determine whether there was a correlation between the total LPS concentration in bathing water and the number of CFU of the bacterial indicators, a kinetic-QCL™ LAL assay (Lonza, UK) was conducted to determine the LPS concentration in the water samples. Briefly, the principle of the kinetic-QCL™ LAL assay is that the endotoxin sample from Gram-negative bacteria is mixed with Limulus Amebocyte Lysate (LAL, extracted from horseshoe crab *Limulus polyphemus*) substrate in appropriate condition and monitored over a period of time for the appearance of a colour change due to endotoxin presence. Reaction time is the time that a colour change (yellow) reaches an optical density absorbance of 0.2; this reaction time is inversely proportional to the concentration of endotoxin and the concentration of unknown samples is determined from standard curve.

A composite sample of the 10 water samples from the stream in addition to each area, i.e. shallow or deep (S or D), bathing or surfing (bathe or surf), was prepared. Four dilutions (undiluted sample, 1/10, 1/100 and 1/1000) from each composite sample were run for their total LPS activity using the kinetic-QCL™ method to investigate whether different sampling areas possess different LPS levels. In addition, 10 individual water samples from the bathing area were assayed for their endotoxin activity to assess whether the total LPS levels correlate to the number of *E. coli* and *Bacteroides* and followed a similar trend to these bacterial numbers. Water samples were diluted with endotoxin-free water and assayed for their endotoxin activity using a kinetic LAL assay kit (Kinetic-QCL™; Lonza, UK) according to the manufacturer's instructions. Endotoxin from *E. coli* O55:B5 (provided with the kit) was used as a standard to calibrate the assay as described by the manufacturer's instructions. Appropriate serial dilutions were prepared from the endotoxin standard and samples from 50 to 0.005 Endotoxin Units (EU)/mL. The US Food and Drug Administration (FDA) defined an EU as the endotoxin activity of 0.2 ng of Reference Endotoxin Standard depending on the source of endotoxin (Liebers et al. 2006). Presently, the conversion rate of the current reference standard used by the FDA (EC-6) is 10 EU/ng. Samples and standard endotoxins in duplicate were loaded into endotoxin-free 96-well plates (Costar, Corning, USA) and incubated for 10 minutes at 37 °C in a pre-warmed plate reader (BioWhittaker elx808, BioTek, UK). The default template parameters of WinKQCL Endotoxin detection and analysis software version 3.0.1 (Lonza) were set to 40 readings absorbance with a 405 nm measurement filter. Concentrations of unknown samples were calculated using the values of the standard curve to give a quantitative endotoxin EU value. All tips (Fisher Scientific, UK) and glass dilution tubes (Lonza, USA) used in each experiment were certified as endotoxin-free. As a quality assessment of the assay, a spike and recovery experiment using a known quantity of standard endotoxin was performed to investigate any inhibition/enhancement when assaying for endotoxin in seawater.

Statistical analysis

Statistical analysis was performed using Microsoft Office Excel 2010 and Minitab® version 16.1.1 (Kruskal-Wallis test and one-way analysis of variance). LAL assay concentrations were calculated using WinKQCL Endotoxin Detection and Analysis Software Version 3.0.1 (Lonza).

RESULTS

Bacterial enumeration and total LPS estimation

The mean CFU in the 10 composite samples of the four areas for *Bacteroides*, *E. coli*, enterococci and total coliforms ($n = 10$) over the sampling period are shown in Figure 2. The number of indicator bacteria generally

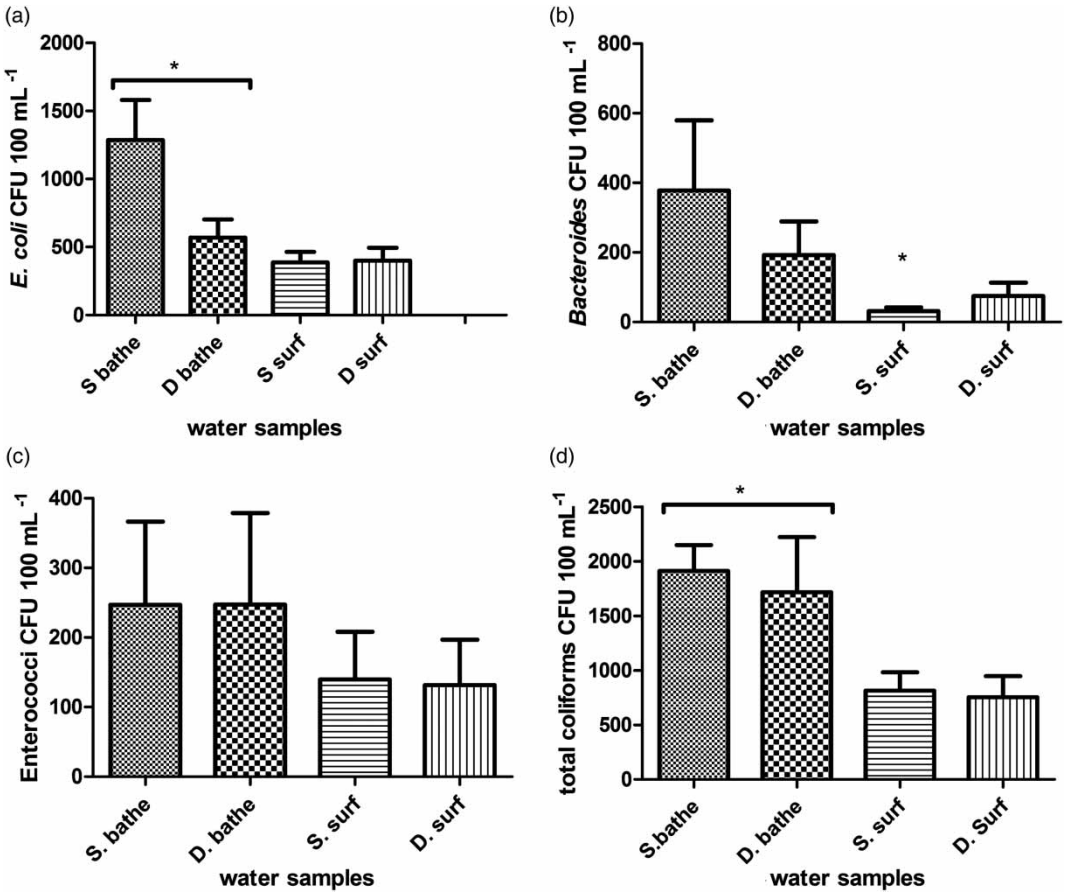

Figure 2 | Mean number of CFU 100 mL^{-1} bacteria in 10 composite bathing water samples for four areas. (a) *E. coli,* (b) *Bacteroides* species, (c) enterococci and (d) total coliforms. S. bathe represents shallow water samples collected from the bathing area, D. Bathe represents deep water samples collected from the bathing area, S. surf represents shallow water samples collected from the surfing area, and D. surf represents deep water samples collected from the surfing area. $n = 10$. Bars show standard error of means. * means *E. coli* $p <$ versus S. surf and D. surf; *Bacteroides* $p < 0.05$ versus S bathe; Total coliforms $p < 0.05$ versus S. surf and D. surf.

showed that the bathing area was more contaminated than the surfing area. There was a significant difference in the number of CFU of *E. coli* ($p = 0.03$) and total coliforms ($p = 0.01$) when comparing the surfing and the bathing areas.

Bacteroides results only showed a significant difference between S. surf and S. bathe areas. Enumerated enterococci showed no significant difference between the bathing and the surfing areas, however, the numbers of CFUs were still higher than in the bathing area. A 95th and 90th percentile was calculated according to the recommended formula by the 2006 European directive; results showed that all the water samples from both bathing and surfing areas were classified as 'poor' (data not shown). Sediment samples showed extremely low numbers of bacteria; the sediment consists of small rocks and sand grains which tend to have a poor surface for bacterial attachment and also therefore as a bacterial reservoir (Harrison 2012).

The mean LPS concentration over the sampling period for each area is shown in Figure 3 where LPS concentration varied between 34 and 135 EU mL^{-1}. There were highly significant differences among all the sampling sites.

When examined over the time period, a distinct increase in contamination was shown for all indicators after a period of heavy rainfall and concomitant runoff (data shown for *Bacteroides* and *E. coli* only, Figure 4). Endotoxin levels followed a similar trend to the number of FIB CFUs showing the highest increase at the polluted run-off event (15/08/12) and returning to threshold levels within the next sampling period (7 days).

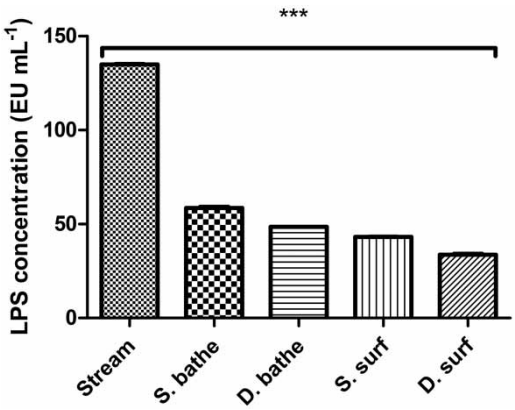

Figure 3 | | Mean LPS concentrations of 10 composite water samples from five areas. A one-way analysis of variance was conducted to evaluate the difference in LPS concentrations among different water sampling areas using Tukey's Multiple Comparison Test. *** means $p < 0.001$ among all sampling areas. Stream represents water samples collected directly from the stream area, S. bathe represents shallow water samples collected from the bathing area, D. bathe represents deep water samples collected from the bathing area, S. surf represents shallow water samples collected from the surfing area, D. surf represents deep water samples collected from the surfing area. $n = 4$. Error bars show standard error of means.

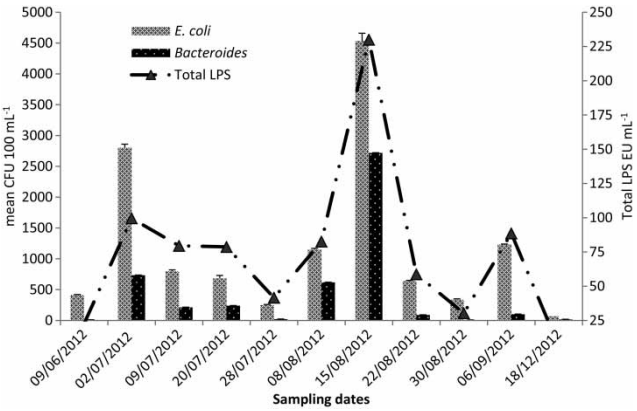

Figure 4 | Mean number of *Bacteroides* and *E. coli* CFU 100 mL^{-1} and total endotoxin concentration EU mL^{-1} in water samples ($n = 4$) of the shallow bathing area during the sampling periods. The dotted line represents the cut-off of LPS concentration above which readings are considered as unsuitable water for bathing.

Good correlations between the number of indicator bacteria and concentrations of total LPS were obtained (Figure 5). The European directive threshold value for *E. coli* is also shown in Figure 5(a) and is converted to EU of LPS.

The use of undiluted seawater samples indicated that there was inhibition of the LAL assay. To confirm the inhibition, a spike-and-recovery assessment was performed by spiking artificial seawater (Instant Ocean) to a final concentration of 0.5 EU mL^{-1} with appropriate controls and performing the LAL assay. The recovery of LPS was over 10-fold lower at undiluted levels but this inhibition was removed at dilutions of more than one in 10. Consequently, all seawater samples were diluted 1/100 for LPS measurements.

DISCUSSION AND CONCLUSIONS

Testing the quality of marine bathing waters is an essential procedure to ensure compliance with EU legislation and the health of bathers and watersports performers. However, a real-time cost effective method is needed as current culture-based methods are time consuming with retrospective results reflecting the water quality status at least 24–48 h previous. Results from this study have shown that total LPS can be used as a rapid, qualitative biomarker taking approximately 1.5 h and can be optimised to give qualitative results in 30 minutes to detect levels above a calculated threshold of 50 EU mL^{-1} of LPS in marine bacterial and faecal contamination. Studies conducted previously have explored the use of LPS as an indicator for testing contamination in drinking and other water. Jorgensen *et al.* (1976) ran a pilot study to explore the possibility of using a LAL assay to estimate the concentration of endotoxins in potable and reclaimed, advanced waste treatment waters and showed that chlorination of water interferes with the assay. Watson *et al.* (1977) used three techniques including the determination of LPS concentration to estimate the biomass and number of bacteria in marine water. That study showed that LPS can be related to the number of bacteria and suggested a factor to convert LPS to bacterial carbon. LPS concentration in water supplies from a stream water correlating with the culture-based bacterial count of coliforms, enteric, Gram-negative and heterotrophic bacteria was investigated by Evans *et al.* (1978) using the gel clot and a spectrophotometric LAL assay. This showed that the gel clot method was less sensitive and less reproducible than the spectrophotometric assay; Evans *et al.* (1978) study also suggested the continued refining of the LAL assay for implementation in water quality investigation.

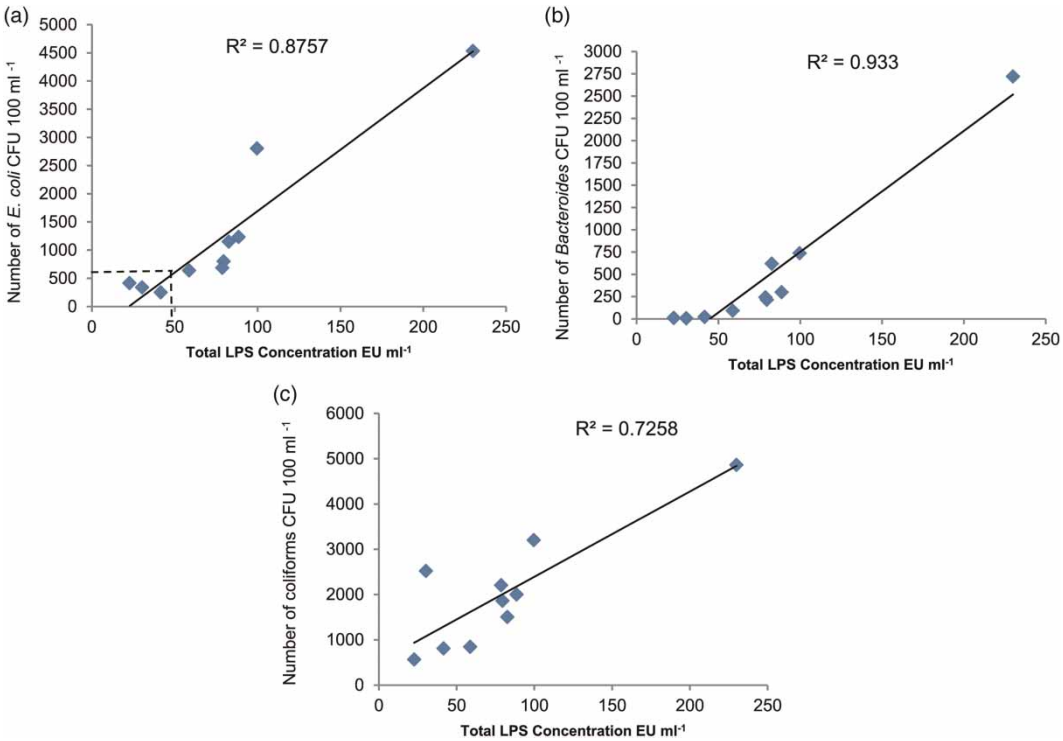

Figure 5 | Pearson correlation of 10 composite water sample means for CFU and total LPS at the shallow bathing area (S. bathe) (a) *E. coli*, Pearson correlation of LPS and *E. coli* = 0.936, $p < 0.001$ (b) *Bacteroides,* Pearson correlation of LPS and *Bacteroides* = 0.954, $p < 0.001$ and (c) total coliforms, Pearson correlation of LPS and total coliforms = 0.852, $p < 0.05$. A threshold as set by the European directive and the corresponding EU/mL of endotoxin is highlighted with the dotted line where appropriate.

In the current study, a more accurate and highly sensitive kit was used to investigate the bacteriological LPS quality of recreational bathing seawater. The kinetic-QCL™ is a highly sensitive method that can detect LPS down to 0.005 EU mL^{-1}. Results showed that total LPS concentration correlates very well with the current bacterial indicator, *E. coli* and with *Bacteroides*, and slightly less with total coliforms (Figure 5), all including a pollution event after heavy rainfall. Increases in LPS also appeared to dissipate from the bathing water within the time period of the next sample (7 days) and probably within the 70 h by the methods described by Shibata *et al.* (2009). LPS molecules are shed from the Gram-negative bacterial cell wall when the environmental factors are not suitable for these cells or when infected with bacteriophages (Fuhrman 2000; Nagata 2000). LPS is being constantly removed from seawater by flagellates (Shibata *et al.* 2009). The LPS threshold at which seawater can be considered as polluted or unsuitable for bathing was determined based on the current legislated number for a 'sufficient' level of bacterial indicators in the European directive 2006 guide for coastal bathing water quality. Using Figure 5, the authors estimate the corresponding LPS concentration at these limits to be equivalent to 50.3 EU mL^{-1}. This level could be set for a future real-time qualitative bathing water test kit development.

Marine bathing water represents a sanctuary for eukaryotes and prokaryotes, including indigenous Gram-negative bacteria. When estimating the *total* LPS from marine bathing waters, part of the total LPS measurement is due to the presence of 'background indigenous Gram-negative bacteria' which will affect the estimation of LPS using the LAL assay. However, many studies have shown that the major bacterial species present in seawater is characterised by low endotoxin virulence because low acylation and phosphorylation are encountered in marine Gram-negative bacteria (Ramos *et al.* 2001; Krasikova *et al.* 2004; Leone *et al.* 2007). The presence of low acylation – penta- and tetra-acyl species – and phosphorylation in the lipid A structure signifies low immunological

activity compared to endotoxically active molecules (Rietschel *et al.* 1994) and is also an indication of low activity with the LAL assay. Hence, even when there is an increase in the background levels it is likely to induce a low reactivity in the LAL assay. Collected water samples processed in this study have never shown a false positive in the LAL assay, and low CFU numbers of FIB (especially *E. coli*) were always accompanied by low LAL activity. The background level of LPS was measured in an excellent quality bathing water sample and results showed it was 6 EU mL^{-1}. However, there is a possibility of occasionally observing false positives, events above 50 EU mL^{-1}, in the absence of FIB. This may perhaps be true due to, for example, eutrophication (Nixon 1995) in seawater that allows marine Gram-negative bacteria to thrive. A less plausible source is from cyanobacterial blooms and an increase in vibrio densities. Although these increases in total LPS would be related to a non-faecal pollution event, this can still be useful as an indicator for the presence of these blooms which have been shown to cause serious human health problems such as gastrointestinal tract infections, cyanobacterial intoxications and even cholera (Morris & Acheson 2003; Dietrich *et al.* 2008). Our immunological studies (unpublished data) have shown that an increase in LPS levels in contaminated bathing water is directly linked to an increase in human cell line pro-inflammatory cytokines and the production of these cytokines was dose-dependent on LPS levels. Hence the LAL test has an advantage in detecting total LPS levels even in a non-faecal contamination event.

The quantitative kinetic-QCL™ assay was performed in approximately 1.5 h when using a 10-fold range of LPS standards 0.005–50 EU mL^{-1} and approximately 30 minutes to detect 0.5 EU and above, which is equivalent to 50 EU mL^{-1} after 100-fold dilution. This method could potentially be optimised as an endpoint chromogenic assay, manufactured and available for untrained bathers as a real-time, qualitative, single-use kit to examine the bacterial quality of bathing waters. The LAL method is not an attempt to replace the applicability of current culture or future PCR methods as these are a valuable method used in microbial source tracking but could be used as part of a 'tool box' approach to water quality management.

Whilst background levels of endogenous LPS appeared to remain low and stable during this study, a *specific* LPS assay for one indicator would be preferable. The authors are working on developing a more specific biomarker involving LPS of *Bacteroides* as real time biomarker using ELISA assay.

In conclusion, a rapid method has been developed to screen the bacterial quality of marine bathing water and has the potential to be developed and used as a qualitative, on-the-spot test by bathers and beach goers, based on an excellent correlation between the culture-based method and total LPS determination.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Wondwossen Abate for help in running the LAL assays.

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First received 3 August 2013; accepted in revised form 16 September 2013. Available online 26 October 2013