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Endotoxin, a novel biomarker for the rapid risk assessment of faecal contamination of coastal and transitional waters

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ABSTRACT

Current methods for testing water for faecal contamination rely on the culture of faecal indicator bacteria (FIB; *Escherichia coli* and *Enterococci*) that take 24–48 h, which leads to delays in taking proactive measures and poses a risk to public health. More rapid methods are therefore required. Here, we have tested a rapid, portable assay (Bacterisk) that detects the bacterial biomarker endotoxin in 30 min to quantify the bacterial biomass present, to evaluate 159 coastal water samples and to compare the results with the traditional culture of FIB. There was a significant correlation between the Bacterisk data given in endotoxin risk (ER) units and FIB culture that could accurately distinguish between poor and sufficient or good quality bathing water using the EU bathing directive values. Receiver operating characteristic analysis was used to determine the optimal ER threshold for coastal water samples, and the area under the curve was 0.9176 with a *p*-value of <0.0001. The optimal threshold was 7,300 ER units with a sensitivity of 95.45% and a specificity of 83.48%. In conclusion, we have shown that the Bacterisk assay provides a rapid and easy-to-use *in situ* method to assess bathing water quality.

Key words: Bacterisk, bathing water quality, endotoxin, faecal indicator bacteria, public health, rapid method

HIGHLIGHTS

- One hundred and forty-seven coastal water samples were analysed.
- New rapid water quality assessment method used (Bacterisk).
- Bacterisk results gave significant correlation with conventional culture method.
- Bacterisk provides a rapid method to assess bathing water quality.

GRAPHICAL ABSTRACT

INTRODUCTION

Water-based recreational activities are important for both mental and physical health (Overbury *et al.* 2023). Additionally, local economies benefit greatly from the tourism generated by such activities, with Europeans estimated to spend over €800 billion annually on recreational visits to open waters (Börger *et al.* 2021). Unfortunately, microbial pollution from various sources, such as sewage discharge and agricultural runoff, poses a substantial risk to public health. Bathers are 3.3 times more likely to show signs of gastrointestinal illness, respiratory infections, skin infections, ear and eye infections than non-bathers (Leonard *et al.* 2020). The risk of infection is made more serious by the increased prevalence of antimicrobial resistance associated with wastewater (Fouz *et al.* 2020). Furthermore, the perception of poor water quality costs an estimated €100 billion in lost annual revenue in Europe (Börger *et al.* 2021), as water quality has been identified as the primary reason for tourists' choice of destination (Dodds & Holmes 2018). Therefore, monitoring bathing water quality is vital to mitigate the health risks associated with the recreational use of bathing waters and to improve public perception and confidence in water quality.

Coastal water quality is monitored for faecal pollution using faecal indicator bacteria (FIB). The most widely used indicators are *Escherichia coli* and intestinal *Enterococci*. These bacteria are normally found in the intestinal tract of warm-blooded animals, including humans. When present in water, they indicate faecal contamination. The majority of FIB are not pathogenic but indicates the presence of faecal contamination that is a potential source of pathogenic organisms such as *Salmonella*, *Shigella*, *Clostridium*, *Legionella*, *Yersinia*, *Mycobacterium*, adenovirus, norovirus, rotavirus and coronavirus (Motlagh & Yang 2019), helminths and fungal species (Shah *et al.* 2011). Using FIB as a proxy for other potentially more harmful pathogens eliminates the need for multiple tests, saving resources.

In the EU, bathing water quality is currently regulated by monitoring *E. coli* and intestinal *Enterococci* (Bathing Water Directive, BWD, 2006/7/EC), and locations are given an annual classification of Excellent, Good, Sufficient or Poor based on the levels of these indicator bacteria present in the waters (Table 1) (Bathing Water Regulations 2013).

Culture-based methods to enumerate FIB are the current gold standard and have been used since the mid-20th century (Dufour 2021). Although these methods are well established, they have several major drawbacks. The most notable issue

Table 1 | Coastal and transitional water standards

Parameter (CFU/100 mL)	Excellent [a]	Good [a]	Sufficient [b]
Intestinal *Enterococci*	100	200	185
Escherichia coli	250	500	500

[a]Based upon a 95-percentile.
[b]Based upon a 90-percentile.

is that they require growth of the organism that takes at least 18–44 h, meaning that any result obtained is retrospective and delays proactive measures to address concerning polluted waters. In addition, the current methods require sample transportation to specialised laboratories and trained personnel to carry out these methods in a reproducible way. Moreover, by focussing solely on FIB, it is possible that other pathogens that are not necessarily of faecal nature may go undetected (Topić *et al.* 2021). These drawbacks to the current culture-based approaches highlight the need for not only rapid, but also more complete solutions to better indicate a broader set of pathogens.

Several investigators have suggested that endotoxin may be a useful biomarker for rapidly determining bacterial biomass and water quality (Jorgensen *et al.* 1973; Watson *et al.* 1977; Evans *et al.* 1978; Jorgensen *et al.* 1979; Haas *et al.* 1983). Endotoxin is the lipopolysaccharide present in the outer membrane of Gram-negative bacteria and some cyanobacteria. Previous work by our group has shown the applicability of using endotoxin as a marker of faecal contamination of seawater (Sattar *et al.* 2013) and surface water (Sattar *et al.* 2022) due to the high concentrations of Gram-negative bacteria, notably coliforms, in the natural gut microbiota and in sewage discharge (Holcomb & Stewart 2020). The team at Molendotech have developed a portable near real-time assay (Bacterisk) to detect endotoxin in water, which can be conducted by non-specialist staff *in situ*.

This study was undertaken to evaluate the performance of the Bacterisk assay as a rapid risk assessment tool to assess the quality of coastal and inland bathing waters in the southwest of England. This was achieved by testing samples in parallel using established culture-based methods for FIB, *E. coli* and intestinal *Enterococci*, as well as with Bacterisk.

MATERIALS AND METHODS

Water sampling

A total of 159 coastal and transitional water samples were collected from various locations in the southwest of England according to the Bathing Water Regulations (2013). Briefly, 500 mL of samples were taken using sterile bottles 30 cm below the water's surface in water at least 1 m deep. The samples were then transported in the dark and tested within 4 h or stored in a fridge (2–8 °C) and tested no later than 24 h after collection.

Membrane filtration

Appropriate volumes of each water sample (1, 10, and 100 mL) were aseptically filtered through a 0.45 μm membrane (Whatman, UK) using a 6-branch vacuum manifold (Sartorius, UK). Membranes were placed on membrane lactose glucuronide agar (MLGA) (Oxoid, UK) and incubated at 30 °C for 4 h, then at 37 °C for 14 h for the detection of presumptive *E. coli* or on Slanetz and Bartley medium (Oxoid, UK) and incubated at 36 °C for 44 h for the detection of presumptive intestinal *Enterococci*. The numbers of colony-forming units (CFUs) were then calculated and expressed as CFU/100 mL.

Bacterisk assay

The Bacterisk assay (Molendotech Ltd, Brixham, UK; www.bacterisk.com) was performed following the manufacturer's instructions. Briefly, the samples were diluted 1 in 100 in dilution buffer and then combined with the detection reagent in equal parts in endotoxin-free micro-cuvettes at a final volume of 200 μL. The samples were then incubated at 37 °C for 25 min using the integrated Bacterisk incubator and the reader. An endotoxin risk (ER) score was then calculated by the device based on the absorbance of the sample at 405 nm. Some samples were incubated and read using a Biotek ELx808 plate reader (Agilent) for increased throughput. Both a negative control (endotoxin-free water) and a positive control (endotoxin from *E. coli* 055:B5) were run with every assay.

Kinetic Limulus Amoebocyte Lysate assay

Endotoxin activity (EU/mL) was determined using a Kinetic Chromogenic Limulus Amoebocyte Lysate (LAL) assay according to the manufacturer's instructions.

Statistical analysis

Statistical analyses on the distribution of samples and the correlation between Bacterisk data and culture-derived water quality were performed with Fisher's exact test and the Chi-square test using GraphPad prism version 9 for Windows, GraphPad Software, Boston, Massachusetts, USA (www.graphpad.com).

RESULTS

The Bacterisk assay measures the concentration of endotoxin in a sample that is presented as endotoxin risk (ER) units, which is an arbitrary scale derived from the colour change of the reagents in an endpoint assay. As previously shown (Sattar *et al.* 2022), ER directly correlates with endotoxin activity (EU/mL), as determined using a kinetic LAL assay, up to 10,000 ER at which point the Bacterisk assay begins to saturate and lose linearity (Figure 1).

A total of 159 coastal and transitional water samples were analysed in parallel by the Bacterisk assay to calculate ER and by membrane filtration to enumerate the levels of *E. coli* and intestinal *Enterococci* (CFU/100 mL). The samples were further classified into two water quality groups, 'sufficient or better' or 'poor', based on the levels of *E. coli* (CFU/100 mL). The threshold chosen for 'poor' water quality was >500 *E. coli* CFU/100 mL based on the Bathing Water Directive 2006/7/EC. Using this threshold, there were a total of 107 'sufficient or better' samples with a median ER of 3,696, and 25th and 75th percentiles of 2,219 and 6,108, respectively, and 52 'poor' samples with a median ER of 12,475, and 25th and 75th percentiles of 8,827 and 20,316, respectively. Though intestinal *Enterococci* are also used as FIBs, they are Gram-positive bacteria. Therefore, they were not used to classify water samples but instead to understand if there was a correlation between intestinal *Enterococci* and ER.

While Bacterisk assay results could be used to obtain risk groups that differentiate different levels of water quality, we have used it here as a binary classification model to determine whether a water source is either polluted ('poor') or clean ('sufficient or better'). One of the most effective methods for checking the performance of such a model is plotting a receiver operating characteristic (ROC) curve, which can then be used to determine the optimal threshold ER value used to discriminate between the two water quality groups. The ROC curve uses 1 – specificity on the *x*-axis, as calculated:

$$\text{Specificity} = \frac{\text{True negatives}}{\text{True negatives} + \text{False positives}}$$

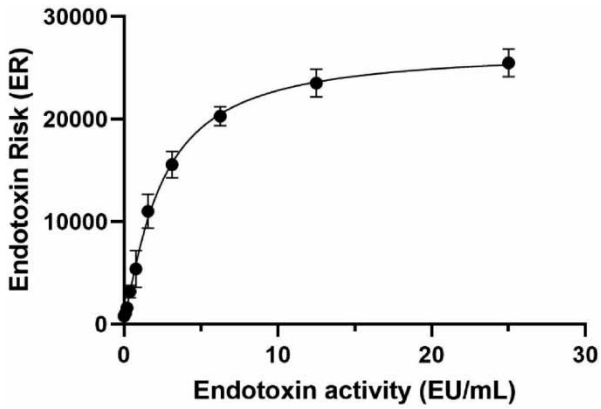

Figure 1 | A standard curve with a four-parameter fit showing the correlation between endotoxin activity (EU/mL) and ER. $R^2 = 0.9892$. Each point represents mean ± standard error of the mean for three independent experiments.

and sensitivity (true positive) on the *y*-axis, as calculated:

$$\text{Sensitivity} = \frac{\text{True positives}}{\text{True positives} + \text{False negatives}}$$

to plot points for each Bacterisk value. In doing so, the ROC curve provides a visual representation for discriminating between clean and poor samples over the entire range of Bacterisk results, by treating each result as a unique threshold with its sensitivity and specificity. The optimal threshold was then determined by choosing the threshold with the highest Youden index, a statistic that measures the overall diagnostic effectiveness (Youden 1950), as calculated:

$$\text{Youden index} = \text{Sensitivity} + \text{Specificity} - 1$$

The ROC curve also provides an area under the curve (AUC) value between 0 and 1. The closer the AUC value is to 1, the better the model is at predicting a correct classification, whereas a value of 0.5 represents a model with no ability to predict a correct classification. A model with an AUC of greater than 0.8 is considered acceptable (Nahm 2022). The ROC analysis determined an AUC of 0.9176 with a *p*-value of <0.0001 (Figure 2). The Youden index analysis determined a threshold of $\geq$7,300 (Youden index $= 0.7893$), which corresponds to a sensitivity of 95.45% and a specificity of 83.48%.

Having established the sensitivity and specificity of the Bacterisk assay, the ER results for the water samples were plotted against the *E. coli* and *Enterococci* culture results. Having used the EU directive threshold value for *E. coli* (>500 CFU/100 mL) to distinguish 'good or sufficient' bathing water quality from 'poor' quality, a correlation between the Bacterisk results and *E. coli* results was obtained, with the same also being done for *Enterococci* (Figure 3). When applying the ER threshold ($\geq$7,300 ER) and FIB thresholds (>200 CFU/100 mL for *Enterococci*) to the plots in Figure 3, a quadrant could be drawn, and the results split into true positive (TP) and true negative (TN) values. In addition, results that were 'positive' by Bacterisk and negative by culture are shown as 'off-target positive' (OP) because they are due to the presence of high levels of endotoxin produced by other Gram-negative organisms or potentially viable but non-culturable (VBNC) bacteria. Any samples that were negative by Bacterisk but positive by culture were labelled as 'false negative' (FN). Of the 44 culture positive samples, two were detected as negative by Bacterisk giving a FN rate of 4.5% (this compares with an FN rate of 7.4% found in Colilert-18® (Tiwari *et al.* 2016)), as calculated:

$$\text{False negative rate} = \frac{\text{False negatives}}{\text{True positives} + \text{False negatives}}$$

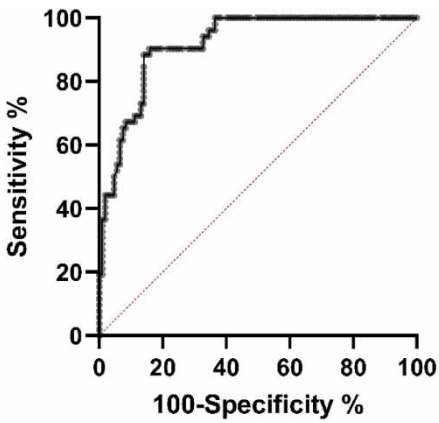

Figure 2 | ROCs for the ability of Bacterisk to discriminate the presence of contamination in coastal and transitional water samples (AUC = 0.9176, Youden index = 0.7893). AUC, area under the ROC curve.

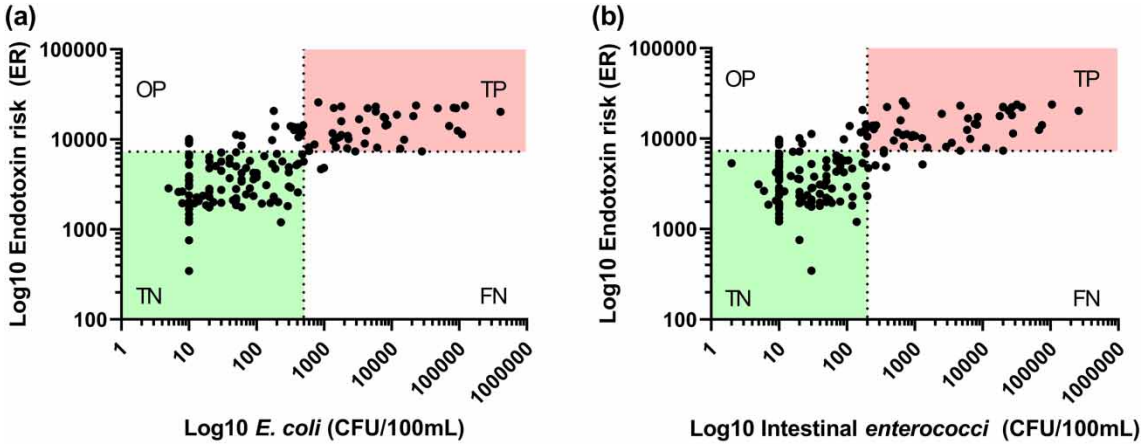

Figure 3 | Scatter plot displaying the Bacterisk ER against (a) *E. coli* and (b) intestinal *Enterococci* enumerated using the membrane filtration method. The chart is split into quadrants based on the ER threshold (*X*-axis 7,300) and (a) *E. coli* threshold (*Y*-axis >500 CFU/100 mL) and (b) *Enterococci* threshold (*Y*-axis >200 CFU/100 mL). $n = 159$. TN, true negative; TP, true positive; FN, false negative; OP, off-target positive.

Of the 159 water samples analysed, there was strong concordance between the Bacterisk ER results and both *E. coli* and *Enterococci* culture results (TP *E. coli* = 42; TP *Enterococci* = 46; TN *E. coli* = 94; TN *Enterococci* = 91; OP *E. coli* = 21; OP *Enterococci* = 17; FN *E. coli* = 2, FN *Enterococci* = 5).

Using the ER threshold value, a contingency table was produced and analysed using Fisher's exact test to examine the distribution of samples and the correlation between the chosen threshold and the two categories of water quality (Table 2), showing that ER is dependent on water quality ($p < 0.0001$).

To further improve the characterisation of water samples into each water quality group, three risk groups were created with different ER thresholds. These three risk groups are comprised of low, medium and high based on the likelihood of the sample being characterised as 'poor'. The low-risk group used the original threshold determined by the ROC analysis. These risk groups were put into a contingency table (Table 3) and analysed using a Chi-square test for trend ($X^2 = 88.93$, degrees of freedom = 1, *p*-value = <0.0001).

Table 2 | Contingency table for coastal and transitional water threshold

Thresholds (ER)	Poor	Sufficient or better	Total
<7,300	2 (1.26%)	96 (60.38%)	98
≥7,300	42 (26.42%)	19 (11.95%)	61
Total	44	115	159

Poor quality is >500 *E. coli* per 100 mL and sufficient or better is ≤500 *E. coli* per 100 mL. Fisher's exact *p*-value = <0.0001.

Table 3 | Contingency tables for coastal and transitional water risk groups

Risk group (ER)	Poor	Sufficient or better	Total
Low (<7,300)	2 (1.26%)	96 (60.38%)	98
Medium (7,300–14,000)	21 (13.21%)	16 (10.06%)	37
High (>14,000)	21 (13.21%)	3 (1.89%)	24
Total	44	115	159

Poor quality is >500 *E. coli* per 100 mL and sufficient or better is ≤500 *E. coli* per 100 mL. Chi-squared test for trend $X^2 = 88.93$, degrees of freedom = 1, *p*-value = <0.0001.

DISCUSSION

Faecal pollution of coastal waters impairs water quality and poses a serious health threat by promoting the spread of infectious diseases among humans and marine ecosystems. With the prevalence of illness in coastal bathers being 3.3 times higher than that of non-bathers (Leonard *et al.* 2020), it is unsurprising that the public's perception of poor water quality has a detrimental effect on the economy (Börger *et al.* 2021). Effective monitoring of faecal pollution is thus essential to protect human health and prevent or mitigate the spread of faecal pollution to the ocean and restore public confidence.

Current routine methods for testing water for faecal contamination detect the indicator bacteria *E. coli* and intestinal *Enterococci*. This requires the transportation of water samples to qualified laboratories, sample filtration, incubation, and biochemical tests and identification by specialist staff. This is a time-consuming process often taking 18–44 h to obtain results. The results are therefore retrospective, which inhibit proactive measures to be taken concerning polluted waters. While this approach can be used to develop a risk profile for a designated bathing location over time, it cannot give information on water quality at the present time, and this is a major limitation considering that the levels of FIB are known to vary throughout the day (Kim *et al.* 2009).

Previous work by our group has shown the applicability of using endotoxin as a marker of faecal contamination of seawater (Sattar *et al.* 2013) and surface water (Sattar *et al.* 2022). The Bacterisk assay, which detects the bacterial biomarker endotoxin, was developed to address the limitations of current culture-based testing. It is portable and produces results in under 30 min, allowing for near real-time monitoring of water quality. This makes it possible to gather information for daily beach management decisions. Additionally, it can notify the public on whether the water is suitable for bathing prior to a major social event (WHO 2018).

Bacterisk gives a measure of endotoxin as 'ER' units that correlate precisely with endotoxin quantity. Results presented here show that Bacterisk can effectively monitor coastal water quality and the results have significant correlation with levels of the FIB *E. coli* and *Enterococci*. The significant correlation with *Enterococci*, a Gram-positive bacterium that does not contain endotoxin, was surprising but most probably reflects the detection of Gram-negative bacteria that are also present in faecal contamination. ROC analysis determined the optimal ER threshold for coastal and transitional water samples of $\geq$7,300, which corresponds to a sensitivity of 95.45% and a specificity of 83.48%. Using this threshold, we found a significant correlation between Bacterisk ER values and the presence of *E. coli* or *Enterococci*. When looking at *E. coli,* 60.38% of the samples tested were classified as 'sufficient or better', with 26.42% being classified as 'poor'. Of the samples, 11.95% were detected as positive by Bacterisk but negative by culture ('OP'). This is due to Bacterisk detecting high levels of endotoxin produced by Gram-negative bacteria or Gram-negative bacteria in a VBNC state. The issue of VBNC bacteria can result in significant underestimation of viable cell counts when assessing bathing water quality. Enterotoxigenic *E. coli* has been shown to enter a VBNC state during stressful conditions yet remain infectious after long-term incubation in both sea and fresh water (Lothigius *et al.* 2010). This VBNC state would therefore negatively impact the accuracy of any assay that uses the enumeration of *E. coli* as an indicator of water quality. Bacterisk can therefore not only alert to the presence of VBNC bacteria but also other potential pathogens such as *Salmonella* spp. that have been found in bathing waters classified as 'good' by current indicator detection methods (Mansilha *et al.* 2010). Likewise, pathogens such a *Aeromonas* ssp. that are not necessarily of faecal nature and would not be detected by current methods either directly or as a proxy (Janda & Abbott 2010) but can be detected by Bacterisk due to their expression of endotoxin (Magaña *et al.* 2013).

Endotoxin may also be derived from non-pathogenic bacteria; however, our data show that for the 'sufficient or better' category, we see a median ER value of 3,696 compared to 12,475 for the 'poor' category, with 25th and 75th percentile values of 2,219 and 6,108, respectively, for the 'sufficient or better' category and 8,827 and 20,316, respectively, for the 'poor' category. This indicates that a background level of endotoxin from Gram-negative bacteria is detected in coastal and transitional waters, though it is during a pollution event that we observe a significant increase in endotoxin and not merely from fluctuations in the normal microbial community.

Of the 159 samples tested, two were detected as negative by Bacterisk but positive by culture giving an FN rate of 4.5%. These FN results could be due to a phenomenon known as low endotoxin recovery that can be due to the presence of surfactants, chelating agents or heavy metal ions in high concentrations leading to the disruption of endotoxin aggregates (Gorman & Golovanov 2022). The Bacterisk method incorporates a 100-fold sample dilution that is specifically optimised to circumvent this problem, except extreme cases (Milton *et al.* 1992). It is worth noting that the current standard method, Colilert-18®, was shown to have an FN rate of 7.3% when testing bathing waters in Finland (Tiwari *et al.* 2016), making the Bacterisk FN rate superior.

It is worth mentioning that many of the current reference methods for the selective isolation and enumeration of *E. coli* rely on the detection of the enzyme β-D-glucuronidase such as chromogenic agar (MLGA, MI, and Chromocult Coliform®) and different formulations of Colilert® (notably Colilert-18®). This reliance on detecting β-D-glucuronidase can result in high FN rates due to the absence or low levels of the enzyme in up to 34% of *E. coli* strains in human faeces (Chang *et al.* 1989) and 10–20% of *E. coli* strains isolated from environmental sources (Shadix & Rice 1991). Furthermore, as MLGA was used to determine the number of presumptive *E. coli* in this study, it may be that Bacterisk results that were classified as 'OP' may, in fact, be TP. Therefore, as all strains of *E. coli*, regardless of β-D-glucuronidase activity, express endotoxin, Bacterisk will be able to detect them.

CONCLUSIONS

In conclusion, we have shown that the Bacterisk assay provides a rapid and easy to use *in situ* method to assess bathing water quality. The strong statistical corelation between Bacterisk results and traditional culture results for FIB will give confidence for using Bacterisk as a rapid screening tool for coastal bathing water quality. With an estimated annual loss of €100 billion in European tourism resulting from the perception of poor water quality, the need for improved infrastructure and testing is vital to bolster public confidence in bathing water quality. The economic and health value of clean recreational waters combined with record fines for polluting companies has stimulated a desire for new, rapid testing solutions to monitor and risk assess bathing waters. By providing a near real-time assessment of water quality, Bacterisk will allow regulators and companies responsible for bathing water quality the ability to take immediate action to protect public health and the environment.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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