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Detection of β -D-glucuronidase activity in environmental samples using 4-fluorophenyl β -D-glucuronide and ^{19}F NMR†

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Common methods for establishing the presence of enteric bacteria polluting water supplies, or in other samples, rely on detecting the hydrolysis of model glucuronide substrates by glucuronidases to release a phenolic product quantifiable by absorbance or fluorescence. Substrates include the β -D-glucuronides of *p*-nitrophenol, and umbelliferyl or quercetin derivatives. One limitation is that it may be difficult or impossible to quantify the released phenolic moiety in samples that are strongly coloured or, that contain fluorescent compounds. Exploiting the sensitivity available from the ^{19}F nucleus to changes in chemical environment which can be detected by ^{19}F NMR spectroscopy, and the almost complete absence of ^{19}F from naturally-occurring samples containing organic matter, which provides background-free signals, we propose a model substrate; 4-fluorophenyl β -D-glucuronide (4FP-glucuronide). The ^{19}F NMR chemical shift position of 4FP-glucuronide changes from -121.0 ppm upon hydrolysis to release 4-fluorophenol, at -124.9 ppm (at pH 6.8), enabling detection of β -glucuronidase activity. We illustrate the use of this substrate with environmental samples from forest soil, standing water, and mud from cattle pasture. Each of these would challenge conventional methods, owing to their opacity or the presence of coloured organic material. The technique enables detection of glucuronidases, a widely-used proxy for enteric bacteria, extending the scope of testing beyond water to include environmental and other challenging samples.

1. Introduction

The provision of water free of significant levels of potentially pathogenic microorganisms, especially enteric bacteria, is a milestone in the history of Public Health that stands alongside vaccination in significance. Despite this apparent success, pressures on land use for agriculture combined with the challenges of sewage and water management, result in the pollution of rivers and water courses worldwide; a situation that also threatens the ecological balance.¹ Faecal coliform contamination of water courses can arise from a variety of sources which include sewage spills, agricultural or urban run-off, wild animals and storm over-flows. The bacteria that are most commonly cited include *Escherichia coli*, as well as *Streptococci*, *Clostridium* and *Klebsiella* species² although many others have been detected (see, for example, Table 2 in ref. 2). The bacterial load of water courses can vary widely; for example, the counts (quoted as most probable number: MPN) of *E. coli* in a variety of water sources in the Dangme West District of Ghana ranged from 0–50/100 mL MPN,³ that of drinking water in Hanoi, Vietnam, has been recorded as 2.4×10^3 /100 mL MPN,⁴ while that from the river Beiyun, Beijing, China, varied between 10^3 and 10^5 /L MPN (10^2 – 10^4 /100 mL MPN).⁵

Clearly, the ability to test the safety of water ideally includes an assessment of the level of any contamination. Also crucial is the capacity to assess the potential sources of that contamination, including detecting the presence of bacteria in environmental samples that may not necessarily be taken directly from water supplies, but which could originate from the surrounding catchment area or wider environment. The presence of enteric bacteria may be established by a number of means, requiring a variety of equipment and resources that include direct bacterial culture, MALDI-TOF analysis, a raft of immunological techniques and PCR-based approaches.^{6–9} One widely used proxy for detecting contamination with enteric gut bacteria is the presence of β -glucuronidase enzymes (GUS) capable of hydrolysing model β -D-glucuronides to their constituent D-glucuronate and phenolic moieties. Beta-D-glucuronides are D-

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glucuronic acid derivatives in which the β -linked aglycone moiety is typically a phenolic compound. Beta-D-glucuronides are formed in mammalian organs, particularly the liver, kidney and large intestine, to increase the solubility, and hence the rate of clearance of phenolic compounds, especially those of a dietary origin but, they serve a similar role with xenobiotics. Beta-glucuronidase enzymes (GUS) are produced by a wide range of gut bacterial classes; Firmicutes, Bacteroidetes, Verrucomicrobiota and Pro bacteria, and 279 GUS isoforms have been identified and grouped into six structural categories.¹⁰ These can include those capable of hydrolysing the glucuronides of naturally-occurring phenolic compounds, such as bilirubin, steroid hormones (estrone, estradiol, testosterone and androsterone diol) and neurotransmitters (dopamine and serotonin), as well as around one hundred xenobiotics¹¹ that include anti-cancer drugs and opioids, as well as dietary substances (reviewed¹⁰). It has also been established that there is considerable variation between individuals regarding GUS enzymes in animals and humans.^{12–14} Given these observations, it is unsurprising that there is little evidence of a direct correlation between simple bacterial numbers and disease type or severity and, indeed, while numerous enzymes capable of hydrolysing the model substrate, 4-methylumbelliferyl β -D-glucuronide (4MU) for example, have been identified,¹⁵ extensive diversity in the structure, function and cellular localization of GUS enzymes has also been noted.¹⁶

Despite these caveats, a method of screening that can identify the presence *per se* of such bacteria and thereby serve as an indicator of potential contamination, and that could complement the existing 4MU-based assay¹⁷ would have practical value. In particular, samples whose physical state, such as opacity resulting from extensive colouration arising from the presence of organic matter, that effectively precludes standard procedures could be more easily amenable to analysis.

Several tests have been devised for the detection of GUS enzymes. Often, these are based on the release of the phenolic product from a glucuronide resulting in a change of retention by HPLC.¹⁸ Common forms of such glucuronides are *p*-nitrophenyl β -D-glucuronide,¹⁹ 4-methylumbelliferyl β -D-glucuronide²⁰ and quercetin derivatives.²¹ For some of these, the excitation and emission maxima (*e.g.* 360 and 450 nm at pH 7 for 4-MU) change as a function of pH.²² Here, we address the challenge of assessing samples that are characterized by high optical opacity, hence display significant background absorbance, which limits the feasibility and sensitivity of

conventional testing. One route by which background signals can be eliminated is to exploit the properties of the ^{19}F nucleus in NMR spectroscopy in a suitable ^{19}F -containing glucuronide substrate. In addition to the simple spectra that it provides, the ^{19}F nucleus (spin 1/2 nucleus) is very sensitive to changes in chemical environment and, since there is almost no naturally-occurring fluorine in biological systems, it also provides background-free signals unaffected by whatever complex organic milieu is under observation.²³

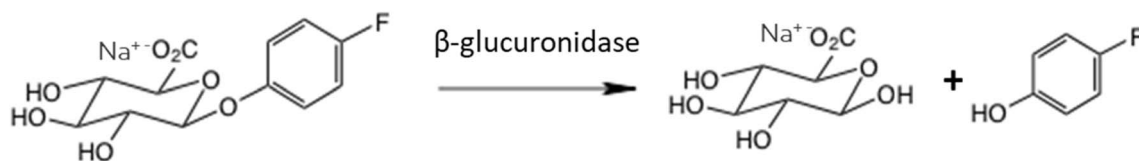
2. Results and discussion

During our recent programme focusing on the synthesis, properties and degradation of model glucuronides,^{24–26} we prepared the β -D-glucuronide of 4-fluorophenol *via* the β -anomer of tetraacetylated methyl ester of D-glucuronic acid as the glycosyl donor, based on a published method²⁴ (see ESI†). We reasoned that, owing to the sensitivity of the ^{19}F nucleus to its chemical environment, it may be possible to distinguish between the intact glucuronide and the hydrolysed form, in which free 4-fluorophenol would be the species detected (Scheme 1). We observed that, indeed, the ^{19}F NMR chemical shift of the intact glucuronide is distinct from that of the free phenol (−121.5 *vs.* −124.9 ppm under buffered (pH 6.8) conditions, Fig. 1A) released by hydrolysis. This provides a potential approach by which enzymatic hydrolysis by β -glucuronidases could be monitored using ^{19}F NMR in environmental samples, free of complicating background signals and irrespective of the optical opacity of the sample. This latter property precludes accurate conventional detection which uses, for example, the absorbance at 401 nm of the enolate (at pH 11.3) form of the released product when employing *p*-nitrophenyl β -D-glucuronide²⁷ or absorbance/emission at 350/450 nm of 4-methylumbelliferyl β -D-glucuronide.²⁰

The potential of the method to provide a means of detecting hydrolysis of the glucuronide in environmentally-derived samples was then explored using three samples that would, owing to their optical opacity, be intractable with standard glucuronide enzyme assays.

2.1 4-fluorophenyl β -D-glucuronide is a suitable substrate for the detection of β -glucuronidase activity in environmentally derived samples

The 4-fluorophenyl glucuronide is stable in aqueous buffered (1/4 strength phosphate buffered saline; NaCl 35 mM, pH 6.8) solution (Fig. 1A, upper), while the hydrolysed product in the



Scheme 1 Hydrolysis of 4-fluorophenyl β -D-glucuronide by β -glucuronidases releases 4-fluorophenol, whose ^{19}F NMR chemical shift (−124.9 ppm) is distinct from that of the intact glucuronide (−121.0 ppm) and provides a means of detecting β -glucuronidase activity in optically opaque samples.



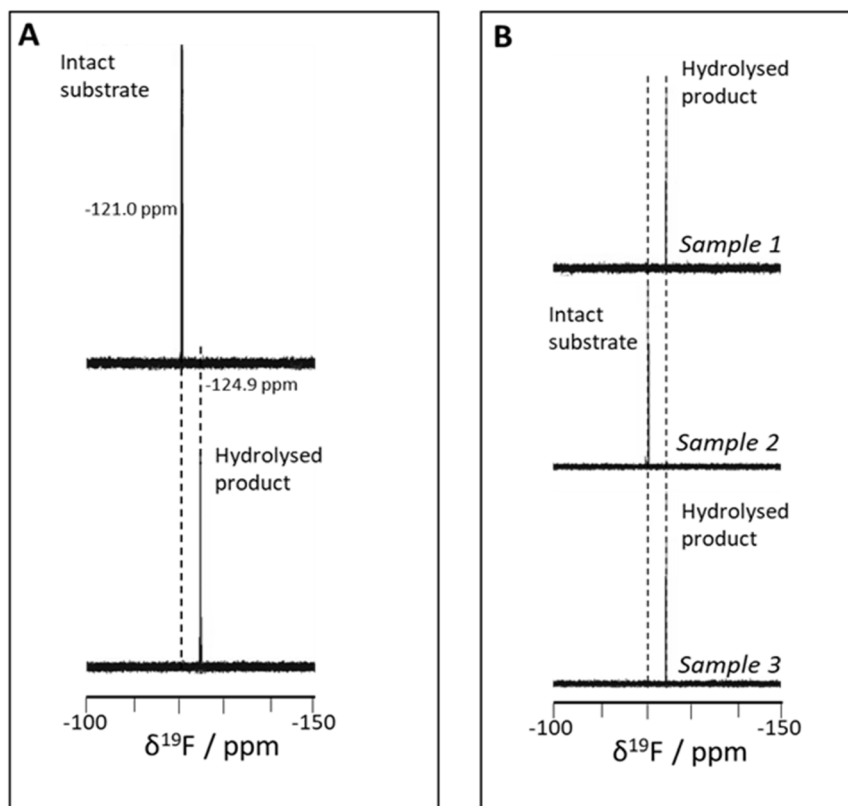


Fig. 1 ^{19}F NMR detection of hydrolysis of 4-fluorophenyl glucuronide by samples from the environment. (A) Control experiment; (upper) intact substrate, signal at -121.0 ppm. (lower) Hydrolysed product, 4-fluorophenol signal at -124.9 ppm. (B) Analysis of environment samples. (upper) Forest soil hydrolyses the substrate. (middle) Standing water from a static forest pond is unable to hydrolyse the substrate. (lower) Puddle water from cattle pasture is also able to hydrolyse the substrate.

same buffer (Fig. 1A, lower) exhibits a signal with clearly distinct ^{19}F NMR chemical shift value, shifted upfield by 3.9 ppm from -121.0 to -124.9 ppm.

The method was explored using three environmentally-derived samples: one of damp, deciduous forest soil (Sample 1), standing water from a forest pond (Sample 2), and water from a puddle on land in use as cattle pasture (Sample 3). The ^{19}F NMR spectra of these, following incubation with the substrate, are shown in Fig. 1B, in which samples 1 and 3 (Fig. 1B, upper and lower) clearly show complete conversion to product (-124.9 ppm) while the standing water (Sample 2) (Fig. 1B, middle) shows no product (intact glucuronide remains, at -121.0 ppm).

In principle, any ^{19}F -containing glucuronide could have been employed for this purpose but, the glucuronide of the simple phenol, 4-fluorophenol, the *para*-substituted analogue of phenol, is straightforward to prepare (see ESI, 1–8[†]). The reaction provides β -D-selectivity in good yield (69% for the coupling step) and, since the $\text{p}K_{\text{a}}$ of 4-fluorophenol is 9.95 ,²⁸ avoids the risk of low yield that can be associated with low $\text{p}K_{\text{a}}$ phenols. This route also avoids heavy metal salts employed in the traditional Koenigs–Knorr synthesis to achieve (1,2-*trans* configuration) β -D-glycosidic bond formation, although the 4-fluorophenyl β -D-glucuronide should also be accessible *via* several alternatives, including Schmidt imidate donors.

The ^{19}F nucleus, with 100% abundance and 87% the sensitivity of the ^1H nucleus, is extremely rare in biological systems, but highly sensitive to changes in chemical environment on account of its electronic configuration, $1s^2 2s^2 2p^5$. In the present system, this sensitivity provides an upfield shift of 3.9 ppm following hydrolysis, clearly separating the signals from glucuronide and released phenol (Fig. 1A). There have been attempts to form a comprehensive theoretical basis for predicting the chemical shifts in this class of molecules²⁹ which derive from earlier work in substituted aromatic systems,²⁸ and postulate the donation of p-electrons from fluorine into the π electrons of the aromatic system (p- π donation).

Practical subtleties regarding the method developed here are the need to add buffer to eliminate any pH dependent chemical shift changes, and to centrifuge the sample prior to addition of the glucuronide. Centrifugation precipitates solid material but, this does not necessarily render the sample transparent and amenable to conventional spectroscopic assays, owing to residual colouration from dissolved organic matter. Indeed, after centrifugation, the environmental samples retained significant absorbance values (>0.25 , 1 cm pathlength) in the range 350 – 450 nm, in which conventional spectroscopic measurements are made. The purpose of centrifuging the sample is two-fold; first, it was found that, if added without prior centrifugation, the glucuronide may bind any solid



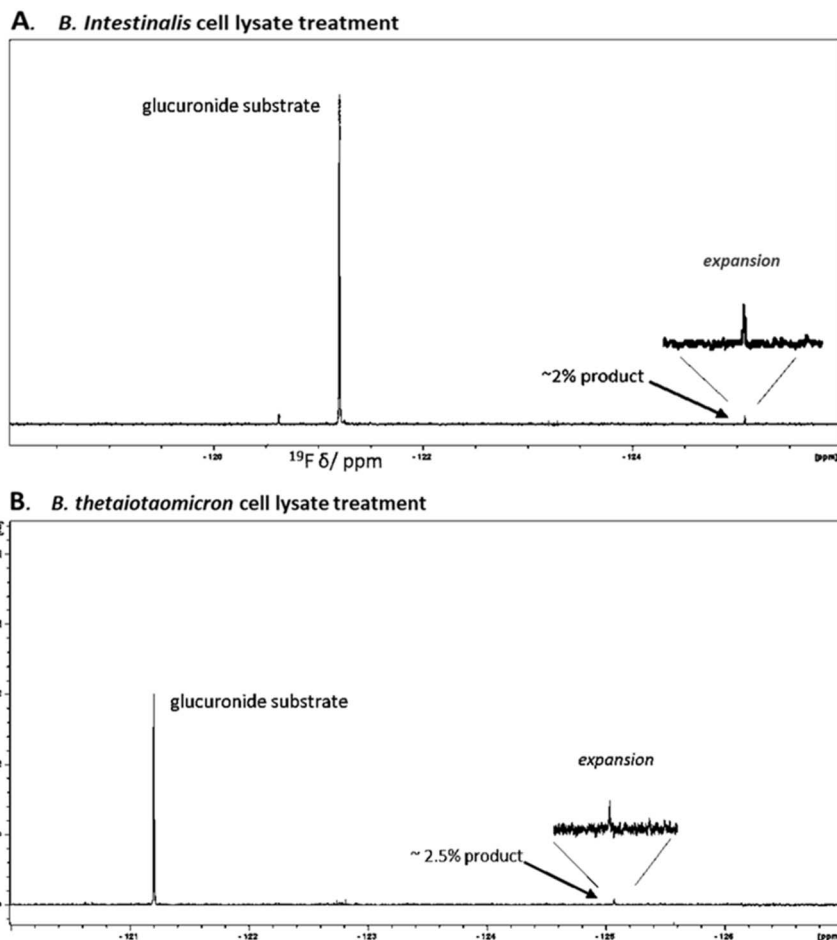


Fig. 2 Examples of limit of detection (proving signal to noise ratio of ~ 3 to 1) under standard recording condition (0.1 mg substrate, 64 scans on a 600 MHz NMR instrument) employing bacterial lysates of *Bacteroides* species. (A) Hydrolysis with *B. intestinalis* lysate detected product conversion of $\sim 2\%$ (inset). (B) Hydrolysis with *B. thetaiotaomicron* lysates detected product conversion of $\sim 2.5\%$ (inset).

matter, thereby causing the ^{19}F NMR signals to broaden or disappear (data not shown) and, second, removal of solid material renders the sample more physically homogeneous, making shimming the sample in the NMR magnet more straightforward. The samples were buffered to preclude chemical shift changes caused by pH differences, with phosphate buffered saline at 1/4 strength rather than full strength (137 mM NaCl), also to facilitate shimming. To confirm reproducibility, four independent measurements on one separate environmental sample provided an average value of 96.6% hydrolysis (3.4% intact glucuronide) with a standard deviation of 0.6% (see ESI, 9–18[†]).

2.2 Detection limit for the hydrolysed product under standard recording conditions (64 scans)

As with most direct spectroscopic methods, there is a trade-off between signal to noise ratio, the number of scans (effectively the time taken to record the spectrum) and the quantity (concentration) of sample. In the case of NMR, there are several other potential factors, including the magnetic field strength and the precise operating parameters such as the relaxation

delay. Under the conditions employed here (a standard 5 mm NMR tube, 600 MHz cryoprobe, 0.70 mL volume and 0.1 mg (~ 30 nmol) of sample, 64 scans) the signal of the product in cases where low conversion of substrate to product had occurred were investigated. To provide samples with appropriately small signals, we utilised the bacterial cell lysates of two *Bacteroides* species, *B. intestinalis* and *B. thetaiotaomicron* which generated only low levels of products ($\sim 2\%$) with which to explore the effective limit of detection under the standardized recording conditions employed here. The spectra of these samples provided clear signals of product, consistently at -124.9 ppm with signal to noise ratios (Fig. 2A and B, insets) of ~ 3 to 1 and correspond to detection of a 10's of μM solutions (10 s of nmol).

3. Conclusions

The samples, taken on the same day within a distance of 200 m of each other from temperate rain forest (British National Vegetation Classification W11) and neighbouring farmland in North Wales, UK, provided both β -D-glucuronidase positive and negative results. Unsurprisingly, the sample from cattle pasture



(Sample 3), exposed to regular contact with bovine faecal matter, was positive. Perhaps more surprisingly, given that GUS activity is often taken as a proxy for the presence of enteric bacteria, the forest soil sample (Sample 1) was also positive. The presence of bacterially-derived GUS cannot be excluded but, plants, fungi and bacteria that employ plant-derived materials as carbon sources could provide an alternative source of suitable enzymes, highlighting that caution needs to be exercised when employing simple proxy measurements to infer the presence of particular bacterial classes. Other enzymes that may be present include those from plants with specificities against, for example, xylans containing β -linked D-glucuronic acid³⁰ or, from fungi such as *Aspergillus niger*, that contain β -glucuronidases.³¹ It is important to note therefore that, although hydrolysis of the substrate constitutes β -glucuronidase activity by definition, it cannot be assumed that such activity arises from bacterial GUS enzymes alone. Hydrolysis of this substrate could also result from other carbohydrate hydrolysing enzymes in addition to those of faecal origin.¹⁴

The present paper provides an additional method for the detection of glucuronidase activity which is suitable in cases where conventional techniques would be hampered by opacity or the presence of coloured organic matter. This extends the scope of testing beyond water to include environmental and other challenging samples. It also provides a complementary approach to a range of other techniques by which bacteria can be detected which employ, for example, biosensors,³² optical chips³³ and chemiluminescence.³⁴ The method described here is suitable for use in an assay format, in which the ultimate signal to noise ratio will depend on many factors including the particular state of the samples under test but, since it is an NMR-based method, it is possible to optimise detection through increased scans and the use of higher magnetic field strength.

4 Materials and methods

4.1 Preparation of the substrate, 4-fluorophenyl β -D-glucuronide

The substrate, 4-fluorophenyl β -D-glucuronide, was prepared as part of our on-going programme in which a series of dietary glucuronides have been synthesized.^{24–26} Briefly, the synthesis of 4-fluorophenyl β -D-glucuronide, an unnatural analogue of the glucuronides of the simple phenols 4-methylphenol (*p*-cresol) and 4-methoxyphenol reported in ref. 24 and ref. 26 respectively, employed the β -anomeric tetraacetate intermediate of the methyl ester of D-glucuronic acid as donor. It utilized trimethylsilyl trifluoromethanesulfonate catalysis, with 4-fluorophenol as acceptor in dichloromethane, based on, ref. 24 to form the protected glucuronide intermediate (compound (1) in ESI†) which, following de-protection, provided the product (compound (2) in ESI†) in 69% yield in the coupling step (for full details and characterization, see ESI, 1–8†).

4.2 Hydrolysis of the substrate by environmental samples

Test samples (final PBS; NaCl 37 mM, pH 6.8) were prepared by mixing 0.5 mL of the test material with 0.5 mL 75 mM PBS, pH

6.8 containing 10% (v/v) D₂O, and spinning in a benchtop centrifuge for 2 min, at 13.5×10^3 rpm, in a 5 cm rotor. (Note that to employ the method as a detection system, as is the case in this instance, samples should be incubated overnight (18 h, as here) but, if it were to be used in an assay format for comparison of activities or rates, then incubation times appropriate for the enzymes level in the system under study would need to be employed, and an excess of substrate would need to be provided; all of which would have to be optimized for the particular system under investigation.) A calibration curve (ESI 9†, inset) shows that the peak area of the ¹⁹F NMR signal of 4-fluorophenol is linear in the range 0.01 to 0.1 mg. Following incubation, 0.7 mL of the supernatant was then taken for NMR analysis. Controls consisted of untreated 4-fluorophenyl β -D-glucuronide (0.3 μ moles, in 0.70 mL of 1/4 strength phosphate buffered saline (35 mM NaCl PBS, pH 6.8) containing 10% D₂O) – the no treatment control, and 0.3 μ moles 4-fluorophenol in the same buffer – the hydrolysed control.

4.3 Bacterial culture and lysate preparation

As a source available from our recent study with known lysate activity,²⁶ bacterial samples (*Bacteroides intestinalis*; DSM17393 and *Bacteroides thetaiotaomicron*; ATCC 29148) were grown anaerobically in BHI media supplemented with haematin (120 μ g mL^{−1}) to mid-late log phase (OD *ca.* 0.8). Five mL of culture medium were removed, spun and washed (2 \times) with PBS (5 mL) before resuspension in 1 mL of PBS. Cells were sonicated (Sonics Vibracell, with ultrasound probe for 2×15 s at 25% power). Twenty μ L of lysed bacterial supernatant was added to the glucuronide (5 μ L, 1 mg mL^{−1}) in PBS and incubated overnight (37 °C).

4.4 ¹⁹F NMR spectroscopy

One-dimensional ¹⁹F NMR spectra were recorded with proton decoupling (Bruker, zgpg30.2) on a Bruker Ascend 600 MHz instrument in 5 mm tubes (0.7 mL volume) at 300 K, recording 64 scans. Note that where the limit of detection is approached, improved signal to noise can be obtained by increasing the number of scans (*N*) providing signal to noise proportional to $N^{1/2}$. Spectra were processed using Bruker TopSpin software.

Data availability

The datasets supporting this article have been included or uploaded as part of the ESI.†

Author contributions

A. T.: investigation, formal analysis, experimental write-up. I. L. B.: investigation, formal analysis. A. C.: supervision, editing of MS, investigation, formal analysis. A. K. P.: investigation, formal analysis, editing of MS. A. V. S.: project administration, supervision, investigation, formal analysis, editing of MS. E. A. Y.: project conception and administration, supervision, investigation, formal analysis, experimental write-up, editing of MS.



Conflicts of interest

We have no conflicts of interest to declare.

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References

- 1 I. Stevenson, S. Thackeray and E. Ransome, *Delivering Biodiversity: Priority Actions for Fresh Water*, British Ecological Society, London, UK, 2024.
- 2 S. Some, R. Mondal, D. Mitra, D. Jain, D. Verma and S. Das, *Energy Nexus*, 2021, **1**, 100008.
- 3 S. T. Odonkor and T. Mahami, *Int. J. Microbiol.*, 2020, 2534130.
- 4 S. C. Crosby, N. C. Spiller, K. E. Tietz, J. R. Cooper and P. J. Fraboni, *Environ. Monit. Assess.*, 2019, **19**, 745.
- 5 L. Chen, L. F. Li, X. S. Zhi, P. Zhang, Y. Dai, Y. C. Xiao and Z. Y. Shen, *xuanjing kexue*, 2019, **40**, 633–639.
- 6 K. Gilbride, *Molecular Methods for the Detection of Waterborne Pathogens. Waterborne Pathogens*, Elsevier, Amsterdam, The Netherlands, 2014, pp. 231–290.
- 7 R. A. Deshmukh, K. Joshi, S. Bhand and U. Roy, *Microbiologyopen*, 2016, **6**, 901–922.
- 8 B. Pang, C. Zhao, X. Song, K. Xu, J. Wang, Y. Liu, *et al.*, *Anal. Biochem.*, 2018, **542**, 58–62.
- 9 A. F. Maheux, D. K. Boudreau, M. A. Bisson, V. Dion-Dupont, S. Bouchard, M. Nkuranga, *et al.*, *Appl. Environ. Microbiol.*, 2014, **80**, 4074–4084.
- 10 S. Gai, R. Sun, R. Singh, S. Y. So, C. T. Y. Chan, T. Savidge and M. Hu, *Drug Discov. Today*, 2022, **27**, 103316.
- 11 M. M. Elmassy, S. Kim and B. Busby, *PLoS One*, 2021, **16**, e0244876.
- 12 C. Ebuzoeme, I. Etim, A. Ikimi, J. Song, T. Du, M. Hu, *et al.*, *Pharmaceutics*, 2021, **13**, 1043.
- 13 Z. Xiang, H. Zhu, B. Yang, H. Fan, J. Guo, J. Liu, *et al.*, *Sci. Rep.*, 2020, **10**, 16628.
- 14 M. Mroczynska, M. Galecka, P. Szachta, D. Kamoda, Z. Libudzisz and D. Roszak, *Pol. J. Microbiol.*, 2013, **62**, 319–325.
- 15 Overview of structure-function relationships of glucuronidases. Glycoside Hydrolases, in *Foundations and Frontiers in Enzymology*, ed. S. B. Mohapatra and N. Manoj, in A. Goyal and K. Sharma, Academic Press, Elsevier, 2023, ch. 12, pp. 255–274.
- 16 R. M. Pollet, E. H. D'Agostino, W. G. Walton, Y. Xu, M. S. Little, K. A. Biernat, *et al.*, *Structure*, 2017, **25**, 967–977.
- 17 H. Nelis and S. van Pouke, *Water, Air, Soil Pollut.*, 2000, **123**, 43–52.
- 18 B. Sperker, M. Schick and H. K. Kroemer, *J. Chromatogr. B:Biomed. Sci. Appl.*, 1996, **685**, 181–184.
- 19 S. Uhlig, L. Ivanova and C. K. Faeste, *J. Agric. Food Chem.*, 2013, **61**, 2006–2012.
- 20 A. H. Farnleitner, L. Hocke, C. Beiwel, G. G. Kavka and R. L. Mach, *Water Res.*, 2002, **36**, 975–981.
- 21 C. Tong, G. Cai, Q. Wei, Y. Cao, Y. Chen and S. Shi, *Microchem. J.*, 2022, **174**, 107104.
- 22 D. W. Fink and W. R. Koehler, *Anal. Chem.*, 1970, **42**, 990–993.
- 23 H. Chen, S. Viel, F. Ziarelli and L. Peng, *Chem. Soc. Rev.*, 2013, **42**, 7971–7982.
- 24 J. A. London, E. C. S. Wang, I. L. Barsukov, E. A. Yates and A. V. Stachulski, *Carbohydr. Res.*, 2021, **499**, 108225.
- 25 M. K. Fraser, A. Gorecka, E. A. Yates, J. A. Iggo, K. Baj and A. V. Stachulski, *Org. Chem. Front.*, 2024, **11**, 2720.
- 26 A. Gorecka, H. Schacht, M. K. Fraser, A. Teriosina, J. A. London, I. L. Barsukov, *et al.*, *ACS Omega*, 2024, **10**, 1419–1428.
- 27 J.-J. Max, F. Meddle-Mouelhi, M. Beauregarde and C. Chapados, *Appl. Spectrosc.*, 2012, **66**, 1433–1441.
- 28 A. W. Shepard, *J. Am. Chem. Soc.*, 1965, **87**, 2410–2420.
- 29 C. Kasireddy, J. G. Bann and K. R. Mitchell-Koch, *Phys. Chem. Chem. Phys.*, 2015, **17**, 30606–30612.
- 30 M. Derba-Macleuch, M. Mitra, M. Hedenström, X. Liu, M. L. Gandla, F. R. Barbut, *et al.*, *New Phytol.*, 2023, **238**, 297–312.
- 31 T. E. Gottshalk, J. E. Nielsen and P. Rasmussen, *Appl. Microbiol. Biotechnol.*, 1996, **45**, 240–244.
- 32 L. Zhang, Y. Chen, N. Cheng, Y. Xu, K. Huang, Y. Luo, P. Wang, D. Duan and W. Xu, *Anal. Chem.*, 2017, **89**, 10194.
- 33 W. Wu, B. T. T. Nguyen, P. Y. Liu, G. Cai, S. Feng, Y. Shi, *et al.*, *Sens. Actuators, B*, 2022, **368**, 132198.
- 34 W. Wu, B. T. T. Nguyen, P. Y. Liu, G. Cai, S. Feng, Y. Shi, *et al.*, *Biosens. Bioelectron.*, 2022, **215**, 114594.

