

Automated online monitoring of fecal pollution in water by enzymatic methods

Katalin Demeter^{1,2,a}, Jean-Baptiste Burnet³, Philipp Stadler^{2,4}, Alexander Kirschner^{5,6,a}, Matthias Zessner⁴ and Andreas H. Farnleitner^{1,6,a}

Abstract

To facilitate the prompt management of public health risks from water resources, the fluorescence-based detection of the enzymatic activity of β -D-glucuronidase (GLUC) has been suggested as a rapid method to monitor fecal pollution. New technological adaptations enable now its automated, near-real-time measurement in a robust and analytically precise manner. Large data sets of high temporal or spatial resolution have been reported from a variety of freshwater resources, demonstrating the great potential of this automated method. However, the fecal indication capacity of GLUC activity and the potential link to health risk is still unclear, presenting considerable limitations. This review provides a critical evaluation of automated, online GLUC-based methods (and alternatives) and defines open questions to be solved before the method can fully support water management.

Addresses

¹ Institute of Chemical, Environmental and Bioscience Engineering, E166/5/3, TU Wien, Gumpendorferstraße 1a, A-1060 Vienna, Austria

² Center for Water Resource Systems E222, TU Wien, Karlsplatz 13, A-1040 Vienna, Austria

³ Department of Civil, Geological, and Mining Engineering, Polytechnique Montreal, Montreal, Quebec, H3C 3A7, Canada

⁴ Institute of Water Quality and Resource Management, E226/1, TU Wien, Karlsplatz 13, A-1040 Vienna, Austria

⁵ Institute for Hygiene and Applied Immunology, Unit Water Microbiology, Medical University of Vienna, Kinderspitalgasse 15, A-1090 Vienna, Austria

⁶ Department of Pharmacology, Physiology, and Microbiology, Division Water Quality and Health, Karl Landsteiner University of Health Sciences, Dr.-Karl-Dorrek-Straße 30, A-3500 Krems an der Donau, Austria

Corresponding author: Farnleitner, Andreas H (andreas.farnleitner@kl.ac.at)

^a Interuniversity Cooperation Centre Water & Health (www.waterandhealth.at).

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Keywords

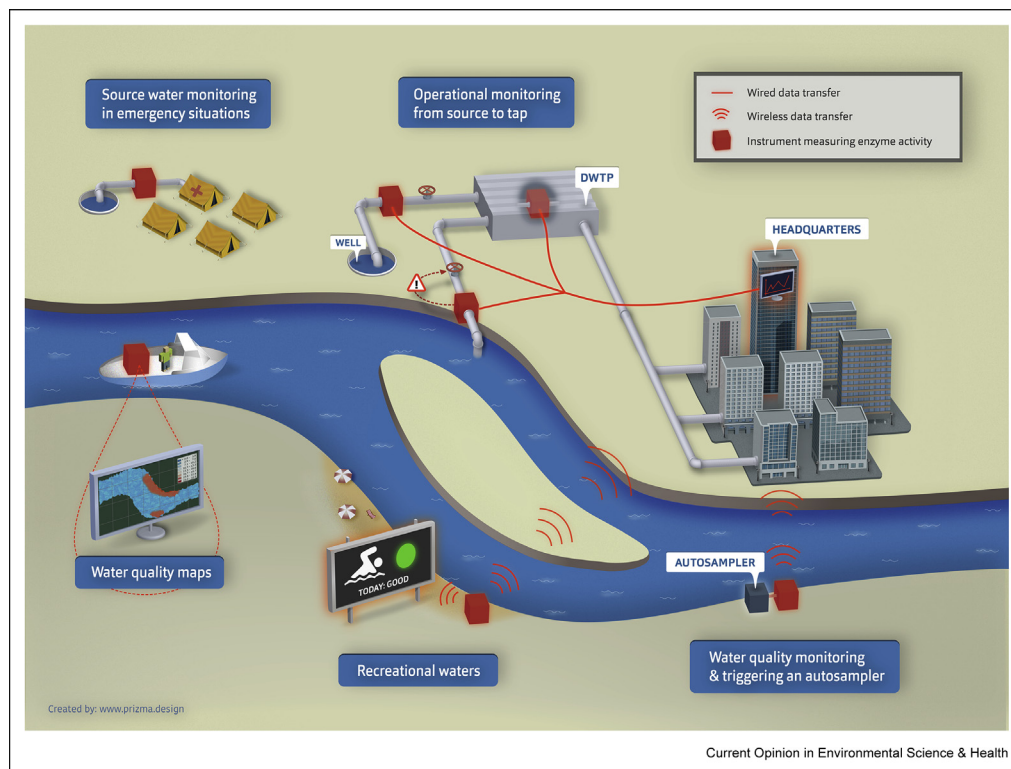
Fecal pollution, Rapid enzymatic methods, β -D-Glucuronidase, Fecal indicator bacteria, *E. coli*, Water safety.

Introduction

The prevention of waterborne diseases requires a systemic framework including water quality monitoring, pollution characterization, and health risk assessment [3]. The monitoring of fecal pollution is a key element in this approach. Fecal pollution patterns in water may vary greatly on short temporal and spatial scales [4,5]. However, culture-based monitoring standards using fecal indicator bacteria (FIB, such as *Escherichia coli*, intestinal enterococci) only provide a result after 18–24 h and grab samples are collected at large intervals (often $\gg 1$ day). Pollution peaks might be missed, or if caught, the result is only available retrospectively. Therefore, there is a need for continuous and (near-)real-time monitoring of fecal pollution in water. Such devices may be applied for monitoring and strategic management throughout the water sector, from drinking water supply to recreational waters (Figure 1). Wired or wireless data transmission enables remote control and thus the method may become an integral part of an increasingly digitalized water industry.

Methods based on the fluorometric measurement of the enzymatic activity of β -D-galactosidase (GAL) and β -D-glucuronidase (GLUC) in water were suggested over two decades ago as rapid surrogates for the culture-based determination of coliforms (GAL) and fecal coliforms or *E. coli* (GLUC) [8–11]. Fluorogenic and chromogenic enzymatic substrates had been well known for a long time as diagnostic supplements in bacterial media (e.g. Ref. [13] included now in ISO 9308-2:2012 for the detection of *E. coli* [14]). During the last decade, fluorogenic substrate technologies were incorporated into online instruments enabling the automated and

Figure 1



Potential applications of rapid online enzymatic methods for the detection of fecal pollution in water. The instruments may be placed at various monitoring points in natural waters or at critical control points along the drinking water supply chain. Connection with existing infrastructure allows the instrument triggering the action of another instrument, such as an autosampler to allow cross-comparison with laboratory-based standard microbiological assays. Connection to the headquarters and/or to cell phones allows data management and central monitoring. DWTP, drinking water treatment plant.

rapid determination of specific enzymatic hydrolysis rates in water [15–17].

Here, we provide an update and extension of the milestone review of Fiksdal and Tryland [9] by focusing on online, automated enzyme measurement platforms intended for fecal pollution monitoring in water resources. The emphasis lies on the direct determination of enzymatic hydrolysis rates in water (not involving a culture step) because the short time to result supports near-real-time monitoring applications. The focus is on GLUC activity rates, because the studies available to date in peer-reviewed literature cover almost exclusively this parameter.

Does the automation work? *The technical realization of rapid-automated GLUC measurement*

Device principles

The technical developments necessary for the enzymatic assay to be operated remotely and fully automated have been achieved and are well documented [15–18]

(Table 1, upper panel). The devices typically consist of a sample intake, reagent stocks, a temperature-controlled reaction chamber, a UV emitter and optical sensor as well as a control unit and a user interface (for references,

Definitions

Rapid detection: there is no widely accepted definition, Noble and Weisberg suggest 'methods that provide results in less than 4 h' [1].

Online measurement: continuous and automatic monitoring of a parameter. Intranet and/or Internet connection allows controlling the results remotely [2].

Proxy or surrogate parameter: a parameter that is used as an indicator of the presence of another parameter in the absence of a direct measure [6,7].

Automated: carried out by machines or computers without needing human control [12].

Table 1

Published methods for the laboratory-independent measurement of enzymatic activities intended for the monitoring of fecal pollution.

Enzyme	Method	Measurement principle	Substrate	Time to result	Automated or manual	Literature reference: method	Literature reference: field applications	Commercial realization
<i>Automated devices for the direct measurement of enzymatic activity</i>								
GLUC (GLU, GAL)	Fluorometric	Direct enzymatic	4-Methylumbelliferyl- β -D-glucuronide	15 min	Automated online	[16,19]	[19,21,22,25,27–29]	ColiMinder (VWMS, Austria)
GLUC (GAL)	Fluorometric	Direct enzymatic	4-Methylumbelliferyl- β -D-glucuronide	75 min ^a	Automated online	[15,17,20]	[17,19]	BACTcontrol (microLan, The Netherlands), previously ColiGuard (mbOnline, Austria)
<i>Alternative methods for laboratory-independent monitoring of fecal pollution based on enzymatic activities</i>								
GLUC	Fluorometric (ColiSense)	Enzymatic after lysis	6-Chloro-4-methylumbelliferyl-beta-D-glucuronide (6-CMUG)	75 min	Manual, field-portable	[26,46,47]	[48]	–
GAL	Fluorometric	Enzymatic after selective culture	GAL: 4-methylumbelliferyl-D-galactoside	15–120 min	Manual, field-portable	[42]	[42]	Colifast Field kit (Colifast AS, Norway)
GLUC, GAL	Fluorometric	Enzymatic after selective culture	GAL: 4-methylumbelliferyl-D-galactoside GLUC: not disclosed	2.5–15 h	Automated online	[42,43]	[42,43]	Colifast ALARM, Colifast CALM (Colifast AS, Norway)
GLUC, GAL	Fluorometric	Enzymatic after selective culture	Pyrene-glucuronide and anthracene-galactoside	2–18 h	Automated online	[45]	[21]	Tecta B16 (ENDETEC, Canada)
GLUC	Fluorometric	Enzymatic after selective culture	4-Methylumbelliferyl- β -D-glucuronide	2–12 h	Automated field-deployable and manual field-portable	[44]	–	ALERT System, ALERT Lab (Fluidion SAS, France)
GLUC	Voltammetric (EcoStat)	Enzymatic after selective culture	Methyl- β -D-glucuronide sodium salt	\leq 10 h	Automated	[49]	–	–

GLU, β -D-glucosidase.^a Including a sample concentration step.

Table 2

Applications of automated GLUC enzymatic activity measurement devices.

Enzyme	Intended application	Duration	Water resource type (mean discharge)	Location	Land use (major fecal pollution sources)	Meteorological conditions	Literature reference
GLUC	Automated near-real-time monitoring of source water quality	2 years	Karst aquifer spring (5 m ³ /s)	Northern Alps, Austria	Forested and summer pastures (domestic and wildlife ruminants)	Dry weather, rainfall	[17]
			Alluvial aquifer	Danube River, Vienna, Austria	Protected wetland and floodplain forest (wildlife ruminants)	Dry weather, rainfall	
GLUC	Comparison of two automated online technologies for investigation of catchment-based transport of <i>E. coli</i>	1 year	Stream (2.7 10 ⁻³ m ³ /s)	Hydrological Open-Air Laboratory (HOAL) catchment, Austria	Agricultural cropland (swine manure)	Dry weather, rainfall	[19]
GLUC	Automated near-real-time monitoring of source water quality in remote and resource-limited settings	10 days	Karst spring (0.6–0.9 m ³ /s)	Seo Ho River, Vietnam	Agricultural (livestock, manure, untreated domestic sewage)	Dry weather, rainfall	[22]
GLUC	Ship-borne automated surface water quality mapping at various spatial scales	3 h–1 day	Lake	Yahara lakes, Wisconsin, USA	Predominantly agricultural with urban areas (diffuse agricultural pollution, leaks from sanitary sewers, urban stormwater outfalls, birds)	Dry weather, rainfall	[28]
		5 days	River (5700 m ³ /s)	Lower Columbia River, Oregon/Washington, USA	Agricultural and urban	Dry weather	
		1 day	River (1300 m ³ /s)	Upper Mississippi River, Wisconsin, USA	Predominantly agricultural with urban areas (diffuse agricultural sources, wastewater treatment plant effluents)	Dry weather	
GLUC	Investigation of catchment microbial dynamics at seasonal to hourly time scales	2 years	Stream (2.7 10 ⁻³ m ³ /s)	Hydrological Open-Air Laboratory (HOAL) catchment, Austria	Agricultural cropland (swine manure)	Dry weather, rainfall	[25]
GLUC	Identification of dominant fecal pollution sources in an urban drinking water supply	1.5 years	River (300 m ³ /s)	Greater Montreal Area, QC, Canada	Predominantly urban, small agricultural tributaries (treated and untreated sewage discharges, diffuse agricultural sources)	Dry weather, rainfall, and snowmelt, spring flood	[27]
GLUC	Automated near-real-time monitoring of recreational water quality	2 months	River (7500 m ³ /s)	Greater Montreal Area, QC, Canada	Combined sewer overflows	Dry weather, rainfall	[29]
		4 months	River (300 m ³ /s)	Greater Montreal Area, QC, Canada	Predominantly urban, small agricultural tributaries	Dry weather, rainfall	

(continued on next page)

Table 2. (continued)

Enzyme	Intended application	Duration	Water resource type (mean discharge)	Location	Land use (major fecal pollution sources)	Meteorological conditions	Literature reference
		2 months	River (12,600 m ³ /s)	Quebec City, QC, Canada	(treated and untreated sewage discharges, diffuse agricultural sources) Mixed (Combined sewer overflow discharges, diffuse agriculture runoff, gulls)	Dry weather, rainfall	
		3 months	River (150–450 m ³ /s)	Waikato River, Hamilton, New Zealand	Agricultural (diffuse runoff from livestock grazing, effluent spreading and wildlife, stormwater outfalls)	Dry weather, rainfall	

see Table 1). Technical applications (casing, power supply, etc.) have been reported for operation in buildings, remotely as a stationary device or as a mobile outdoor device [17,19,20]. Reported sample volumes range from 6 to 5000 mL, with the possibility to concentrate large sample volumes [15–17]. Measurement intervals between 15 and 180 min have been described [17,19,20].

Analytical performance

The available evaluations have indicated high analytical precision for the automated GLUC activity measurements with coefficients of variation below 5% [21]. Widely used cultivation-based FIB standards achieved a lower analytical precision with coefficients of variation between 16% and 31% [21]. The general performance of GLUC activity measurements was reported to be comparable with manually performed analysis and the simultaneous determination of the limit of quantification can be integrated into the automated data analysis by the instrument [17]. It should be noted that the reported units differ among manufacturers and studies (hydrolysis rate *versus* Fishman units per volume), although conversions can be achieved.

The *robustness* of the automated GLUC activity measurements in freshwater types having a wide range of physicochemical and microbiological characteristics was demonstrated by recent studies in pristine waters [17], surface waters with elevated suspended solid loads [19,22], and waters impacted by treated and/or untreated municipal sewage [21,23]. However, marine waters were only tested so far using laboratory-based direct GLUC assays [10,24]. Reported environmental factors influencing measurement accuracy and error-free running time are ambient temperature and suspended organic matter [9,19]. Both factors are now managed well by specific adaptations of the devices, including the specific design of the reaction chamber, sample pre-filtration, adapted cleaning procedures, and data-correction algorithms [20]. Such devices were successfully operated outdoors *in situ* for up to 2 years (e.g. Ref. [17,25]).

Alternative laboratory-independent methods based on GLUC activity

A portable device has been developed based on the direct measurement of GLUC activity after cell lysis ([26]; Table 1, lower panel). In addition, automated devices based on enrichment in selective growth media before the measurement of enzymatic activity have been successfully realized, with several fluorometric and one voltammetric method based on this principle. Some instruments have been designed for online monitoring others as field-deployable devices (Table 1, lower panel).

Where has it been used? *Field studies using automated GLUC measurement*

Automated GLUC activity measurement devices have been deployed with the aim to characterize the temporal and spatial patterns of GLUC activity and describe the relationship to cultivation-based standard *E. coli* detection methods in various water resources (Table 2).

Vulnerability assessment of water resources

The first demonstration of the technical feasibility of near-real-time monitoring of GLUC and GAL activities was provided by Ryzinska-Paier *et al.* [17] at an alpine karst spring and an alluvial aquifer in Austria over a period of 2 years. The seasonal dynamics of GLUC activity at a karstic spring environment were described for the first time (>5000 successful automated measurements). In a freshwater resource for urban drinking water supply in Canada, Burnet *et al.* [27] used a 1.5 year long GLUC activity time series to identify the dominant fecal pollution source among multiple wastewater discharges and to uncover the hydraulic connection between an upstream wastewater treatment plant and the drinking water treatment plant. Ender *et al.* [22] demonstrated the feasibility of automated near-real-time monitoring of GLUC activity in a remote karst spring in Northern Vietnam using a portable instrument designed to operate under limited resources settings.

Catchment microbial/biochemical dynamics

The automated near-real-time monitoring of GLUC activity as biochemical indicator has a considerable potential. Stadler *et al.* [19] first demonstrated that two different commercially available instruments were able to detect rapid fluctuations in enzymatic activity caused by episodic changes in hydrological conditions. The authors reported seasonal variations in the transport of GLUC activity, which peaked more often and at higher amplitudes in summer, although several of these GLUC activity peaks occurred in absence of rainfall and suspended sediment peaks [19,25]. Through the screening of GLUC activity in stream water and sediments and using stable isotopes in stream water, the authors suggested that a large portion of the transported GLUC originated from the resuspension of streambed sediments and reflected the existence of a remnant reservoir of GLUC in the catchment [25]. In an urban catchment affected by multiple treated and untreated wastewater discharges, Burnet *et al.* [27] similarly illustrated the large temporal scale of variation in GLUC activity in water. GLUC activity peak episodes occurred exclusively between late fall and early spring and were caused by intense precipitation (24–48 h before GLUC activity peak) and/or snowmelt events, which triggered the local discharges of untreated sewage into the river.

Besides the seasonal and event-based fluctuations in GLUC activity, recurrent daily patterns have been

reported in various habitats, although the peak activities did not occur at the same time of the day [19,21,22,27]. The origin of these daily patterns was attributed to the likely temperature dependence of bacterial activity in a small agricultural stream [19], and in a karst spring [22], although the causal link requires further investigations. Another type of daily pattern of GLUC activity was described at an urban drinking water intake and was traced back to the discharge pattern of an upstream wastewater treatment plant [27].

Surface water quality mapping

Using a ship-borne instrument, Stadler *et al.* [28] recently demonstrated the feasibility of rapid GLUC activity assessment for surface water quality mapping. These first high-resolution spatial data on GLUC activity illustrated the effect of rainfall-induced runoff on surface water quality along urbanization gradients and indicated tributaries and confluences as main fecal pollution hotspots in these large waterbodies.

Recreational water quality assessment

Cazals *et al.* [29] illustrated the usefulness of online GLUC activity monitoring for rapid identification of impaired waters in recreational freshwater bodies. Threshold GLUC activity values were developed to match the regulatory ('gold standard') *E. coli* beach action values while minimizing the rates of failures to act and false alarms. Near-real-time monitoring of GLUC activity enabled to identify fecal pollution peaks and determine the exact timing of GLUC activity threshold exceedance.

What does it tell us? *Indicator capacity of GLUC*

Relationship to cultivation-based FIB

All field studies using automated GLUC determination (Table 2) performed cross-comparisons with cultivation-based *E. coli* standards [17,19,21,22,25,27–29] and one study reported data also for coliforms [17]. Reported correlations between GLUC activity and cultivation-based *E. coli* standards (expressed in linear or non-parametric correlation coefficients r) varied widely among the studied water resources. For freshwaters influenced by urban sewage, r ranged between 0.33 and 0.84 on non-transformed data [21,27,28] and between 0.10 and 0.79 on log-transformed data [29], with an apparently strong dependence of hydrometeorology and contamination characteristics [27,29]. Among the studied watersheds influenced by agriculture (manure spreading and/or cattle grazing), r ranged between 0.53 and 0.56 at karstic springs of remote mountains [17,22], whereas a small brook revealed $r = 0.72$ [19,25]. Stronger correlations were found at higher pollution levels [21,27] and during events (with the highest r reported being 0.89 [25]). Notably, GLUC activities often resulted in stronger correlations with

Table 3

Open research topics and future development goals regarding the automated, cultivation-independent determination of enzymatic activities intended for online fecal pollution monitoring at water resources.

Some open research topics and future development goals

Fecal and health-risk indication capacity of GLUC activity

- What are the limits to use GLUC as a biochemical fecal indicator (i.e. fecal pollution level, age, treatment)?
- Which aquatic habitats are most suitable for GLUC activity monitoring in respect to its fecal indication ability?
- Which habitats or situations are not suitable for GLUC activity monitoring and strong interference or bias from non-fecal sources is to be expected?
- In which situations may GLUC activity become indicative of the occurrence of intestinal pathogens?
- In which situations may GLUC activity become indicative of infection and health risks?
- Can GLUC activity be used as a conservative indicator for pathogen removal during treatment?

GLUC activity of fecal origin: persistence and fate in the (aquatic) environment

- How long does cell-associated enzyme activity of intestinal populations persist?
- How does GLUC activity compare to other cell-viability parameters?
- What are the relative abundances of culturable, VNBC, dead cells/cell debris, free and particle-attached enzymes under various environmental conditions? Do they have a differential persistence?
- Is there a difference in GLUC activities between human versus animal sources?
- Which intestinal microbiota contribute to GLUC activity in water?
- Do different microbiota show differential GLUC activity persistence?
- Could the ratio GLUC to cultivation-based fecal indicator standards indicate contamination age?
- Do free enzymes re-attach to abiotic particles, such as to silt-colloids? How does re-attachment influence the enzymatic persistence? Do catchments with high turbidity and GLUC adsorption rates limit the application?
- Which GLUC inhibiting substance may occur in water samples and under what conditions?

GLUC activity of fecal origin: resistance and fate during water treatment and disinfection

- What is the resistance of GLUC activity of fecal origin to the various steps of wastewater treatment, including ozonation, UV disinfection and chlorination? Do the various GLUC compartments (culturable, VNBC, free enzymes, etc.) have a differential resistance?
- What is the resistance of GLUC activity of fecal origin to the various steps of drinking water treatment, including chlorination, UV disinfection and ultrafiltration? Do the various GLUC compartments have a differential resistance?
- How does GLUC activity compare to other cell-viability parameters during the treatment steps?

GLUC activity of non-fecal origin

- Under which conditions does algae-associated GLUC activity become significant?
- Under which conditions does environmental bacteria-associated GLUC activity become significant?
- What are other potential non-fecal associated GLUC sources?
- Is it possible to differentiate or correct for non-fecal associated GLUC activity?
- Does significant GLUC activity occur from 'naturalized' (re-grown) intestinal populations in the environment?
- What is the exact nature and origin of daily GLUC fluctuations that are not related to the fecal pollution source dynamics?

Fecal pollution-associated enzymes other than GLUC (questions above are all relevant)

- What are the sources and fate of β -D-galactosidase? Is it a useful fecal indicator?
- What are the sources and fate of β -D-glucosidase? Is it a useful fecal indicator?
- Are there any other enzymes or combinations demonstrating enhanced fecal indicator capacity?
- How can enzymatic substrates be improved to increase their sensitivity and specificity for fecal pollution?

Technical realization of automated, online instruments

The field needs

- ... Validation guidelines (precision, robustness, specificity, sensitivity)
- ... Quality control and quality assurance protocols
- ... Uniform, standardized measurement units
- ... Strategies to trigger microbiological autosampling, based on online GLUC and/or physicochemical measurements

VNBC, viable but not culturable.

environmental parameters than cultivation-based *E. coli* data. For example, correlations up to $r = 0.87$ with turbidity (2–3 μm particle fraction, karst spring, rain event) [22] and $r = 0.93$ with chlorophyll *a* (lake, dry weather) were observed [28].

GLUC does not qualify as a general proxy parameter for cultivation-based *E. coli* enumeration

The above correlation analysis supports previous observations that GLUC activity is not a general proxy for cultivation-based *E. coli* enumeration [9]. Enzymatic activity was demonstrated to be a more persistent biochemical parameter against environmental and treatment (disinfection) stresses as compared to the culturable fraction of FIB in water resources [9]. Indeed, there is evidence that GLUC activity is able to detect culturable cells as well as viable but non-culturable cell populations [30]. Furthermore, persistent GLUC activity was also reported for damaged or dead *E. coli* cells [31] and from the fraction of free enzymes in river water with fecal pollution [32]. It can be argued that free or particle-associated GLUC activity may be relevant for the detection of low, remote, old, or treated (disinfected) fecal pollution.

GLUC activity can also be associated with biotic or abiotic compartments other than *E. coli*

Without a selective cultivation-based enrichment step for *E. coli*, a significant amount of GLUC activity in water samples may also originate from other microbiota and substances [9]. Recent investigations highlight that microbiome-encoded GLUC activities play an important role in the human gastrointestinal system [33]. By genomic and proteomic tools, hundreds of different β -glucuronidase enzymes, grouped into six distinct categories, could be identified in abundant microbial phyla of Bacteroidetes, Firmicutes, Verrucomicrobia, and Proteobacteria in human stool samples [34], confirming previous observations before the genomic era [35]. However, possible interference from non-intestinal microbiota in water resources was also reported, including environmental bacteria and algae [9,36,37]. As a result, GLUC activity is considered to be of fecal origin, especially under the situation of high fecal pollution (culturable cells, viable but non-culturable, cell debris, and free enzymes), but interfering GLUC activity of non-fecal origin (biotic and abiotic) can also occur [9].

Status quo and open questions

Without any doubt, the automated online GLUC activity determination in water resources has been successfully realized during the last decade, offering fascinating new possibilities to support water safety management in the future (Figure 1). This technology may not be restricted to GLUC and related enzymes,

but could support any type of enzymatic online monitoring (if technically feasible) that can inform about microbial and biochemical water quality issues [38–41]. As opposed to the original suggestion almost 20 years ago [11], it is now obvious—after the many cross-comparison efforts—that GLUC activity is not a general surrogate for the cultivation-based determination of *E. coli*. Depending on the habitat, fecal pollution characteristics and hydrometeorology, the relationship between culturable *E. coli* concentrations and GLUC activity rates can vary substantially. In cases where the direct comparison with cultivation-based *E. coli* standards is essential, online GLUC determination using automated pre-enrichment procedures by selective growth would be a more suitable approach (Table 1, [42–45], with reported correlation coefficients to standard *E. coli* methods ranging between 0.90 and 0.94 [21,42,44]). However, a trade-off between this stronger relationship and a significantly longer sample-to-result time has to be taken into account (Table 1). The rapid online prediction of culturable *E. coli* based on GLUC direct determination may only be possible in special cases: at certain sites and under certain pollution scenarios allowing a sufficiently high statistical relationship. This, however, requires further investigations.

In contrast to the achieved progress in the automated determination of enzymatic hydrolysis rates, the scientific evaluation of the GLUC indication capacity for fecal pollution monitoring has been almost neglected for more than a decade [9]. There is an urgent research need to understand more comprehensively the sources and sinks, the persistence and mobility, and the link of GLUC activity with the actual cellular states. Such investigations should cover all important water resource systems and should also include essential water treatment and disinfection processes (Table 3). Based on the information currently available, we propose *GLUC activity as a conservative biochemical proxy-parameter for bacterial fecal pollution* (not only associated with *E. coli* or fecal coliforms) in water resources. Furthermore, for specific system conditions and exposure scenarios, GLUC activity may also indicate pathogen occurrence and infection risk from fecal pollution and could therefore be part of the strategic management of the given water resource (Table 3). However, as highlighted a decade earlier [9], GLUC activities from non-fecal compartments may interfere with the intended indication capacity, especially in the case of low, old, or remote fecal pollution. The above-mentioned gaps of knowledge currently limit the application of automated online GLUC activity monitoring in the water management sector and warrant further detailed investigations.

Declaration of Competing Interest

Nothing declared.

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