Journal of Water & Health

IWA PUBLISHING

Journal of Water and Health Vol 22 No 2, 290 doi: 10.2166/wh.2024.207

Detection of endotoxins from selected drinking water microbiota using an LAL-based assay and its implications for human health

Harmen Hawer *, Rebecca Burmester, Nadine Sonnenberg and Katja Weiß

Panpharma GmbH, Bunsenstraße 4, 22946, Trittau, Germany
*Corresponding author. E-mail: hhawer@panpharma.de

HH, 0000-0003-0451-6840

ABSTRACT

Endotoxins are pyrogenic lipopolysaccharides from Gram-negative bacteria that are known to induce fever, septic shock, and multiple organ failure, posing a substantial risk to human health. Drinking water systems are especially prone to home microbiomes containing a large variety of Gram-negative bacteria. Consumption of water from these systems in developed countries is generally regarded as non-hazardous to humans due to the low number of non-pathogenic bacterial cells per milliliter and oral admission. To assess potential risks posed by endotoxins in drinking water systems, we conducted a conventional microbiological investigation on a local community water system in the north of Germany and mined the resulting data to investigate the endotoxin contents of some of the most abundant microbiota found during these analyses. Using a *Limulus* amoebocyte lysate (LAL) -based endotoxin detection method, average normalized endotoxin content was determined. Although the average culturable amounts of microbiota in the drinking water system were insufficient to exert endotoxin levels critical to human health, peaks and acute contaminations may pose substantial health risks.

Key words: *Brevundimonas*, drinking water, endotoxins, LAL, *Sphingomonas*, *Stenotrophomonas*

HIGHLIGHTS

- Endotoxin detection from cell suspensions of specific drinking water microbiota.
- Endotoxin release from different bacterial species is highly variable.
- Implications of species-specific endotoxin levels for human health.

INTRODUCTION

Endotoxins are lipopolysaccharides (LPS) that naturally occur in the outer cell membrane of Gram-negative bacteria. Their function is likely connected to the structural integrity of the outer membrane and their amphiphilic properties may protect cells against challenging environments and antimicrobial agents (Nikaido 2003). In humans, infections with Gram-negative bacteria can result in the accumulation of chemically stable LPS molecules in the bloodstream, which readily induces fevers, septic shocks, and even multiple organ failure (Su & Ding 2015). Despite enormous advances in modern medicine, sepsis and septic shocks remain medical conditions with an exceptionally high mortality rate (Cecconi *et al.* 2018), rendering endotoxemia a severe health risk.

Strikingly, common drinking water presents a major environment for Gram-negative microbiota (Brumfield *et al.* 2020; Thom *et al.* 2022). Even though drinking water is used mostly for oral consumption or dermal application, leaving little possibility of direct and efficient endotoxin uptake, there may still be a risk of LPS entering the bloodstream via injured intestinal mucosa (Bhattacharyya *et al.* 2004) or inhalation (Anderson *et al.* 2007). At high concentrations, such uptake could suffice to induce endotoxemia, posing a risk especially for immunosuppressed patients. Monitoring endotoxins therefore presents a major quality parameter for all water sources (Sattar *et al.* 2022). Large-scale conventional studies on the microbiome of water systems have become rare since the emergence of genomic approaches, which generate the benefit of readily depicting viable but non-culturable organisms (Liu *et al.* 2023) and creating large amounts of data via mostly automized systems (Bruno

et al. 2022). However, high-throughput methods mostly cease to generate data on numbers of culturable microorganisms, which remains subject to recent research (Knecht *et al.* 2023) and is an essential quality aspect in water monitoring.

Although urban water systems are generally well monitored and controlled in developed countries (Perrin *et al.* 2019; Hopland & Kvamsdal 2022), there is no comprehensive data available on the potential endotoxin-threats of these waters or the endotoxin-potency of the most abundant drinking water microbiota to distribute hazardous LPS molecules. We therefore comprehensively monitored a community drinking water system over a period of 5 years using conventional microbiological and modern endotoxin testing methodologies to identify potential endotoxin-threats among some of the most abundant species. Furthermore, we assessed endotoxin concentrations from normalized cell input to identify species-specific differences. Our findings present an important step in identifying details on the potential origins and roles of endotoxins from drinking water systems in human disease.

METHODS

Sampling

Sampling occurred weekly or monthly in randomized intervals over a period of 5 years and at four independent sampling points fed from the same urban water supply. Valves were disinfected with 70% Isopropanol and a minimum of 10 l of water was discarded before sampling to rinse valves of residual disinfectant. Samples were taken in polypropylene beakers sterilized via electron beam irradiation.

Bioburden analysis

For each microbiological analysis, 200 ml of sample was taken and sterile filtered through a 0.45 μm filter disk using a Milliflex Oasis pump system under a sterile work bench. Filter membranes were placed on R2A agar plates and incubated at 32.5°C for 7 days. Colonies were counted manually and verified by two independent analysts.

Gram-staining and identification

Gram-staining was performed using an automized Biomerieux PREVI-Color staining system and the outcome was visualized using standard light microscopy (Froböse *et al.* 2020). Species identification was performed using standardized procedures such as biochemical profiling via VITEK 2 as well as a mass-spectrometry-based MALDI-TOF VITEK MS system (Martins *et al.* 2018).

Detection of endotoxins via LAL-assay

Bacterial species were isolated from their respective original plates and used for further analysis. Colonies from fresh tryptic case soy agar (TSA) plates were diluted in endotoxin-free water. Cell numbers were normalized using McFarland standards and optical measurements. Further dilutions of the cell suspensions were prepared in endotoxin-free water. LAL assays were conducted using a Lonza kinetic-turbidimetric testing system. Analyses were conducted as biological replicates and each replicate consisted of technical duplicates on the LAL system with two additional positive controls to ensure detectability. All analyses were verified by simultaneously running standard curves using samples with 0.01, 0.1, 1 and 10 Endotoxin Units (EU)/ml of reconstituted endotoxin from *Escherichia coli*. Results were normalized to cell numbers and significance was determined using Mann–Whitney tests (Qu *et al.* 2008).

RESULTS AND DISCUSSION

Assessment of a local community water microbiome using conventional methods

We monitored a drinking water system in the north of Germany over a period of 5 years applying conventional microbiological methods and taking a total of 223 samples. During this time, a total of 492 individual isolates were identified to the genus and/or species level, revealing predominantly Gram-negative bacteria (Figure 1(left)) – which is in line with available high-throughput studies on drinking water microbiomes (Brumfield *et al.* 2020; Thom *et al.* 2022). Specific colony forming unit (CFU)/ml values were determined when permitted by visible plate growth. Total CFU/ml values for all analyses were documented with an average of 40.3 CFU/ml. As expected of a drinking water microbiome containing large varieties of biofilm-producing bacteria, CFU/ml values varied heavily between measurements with 69.5% under the average (<40.3 CFU/ml), 20.6% over average (>40.3 CFU/ml) and 4.5% uncountable due to indistinguishable growth. In three samples, particulate contamination was observed. These instances correlated with technical interventions as well as cleaning and rinsing

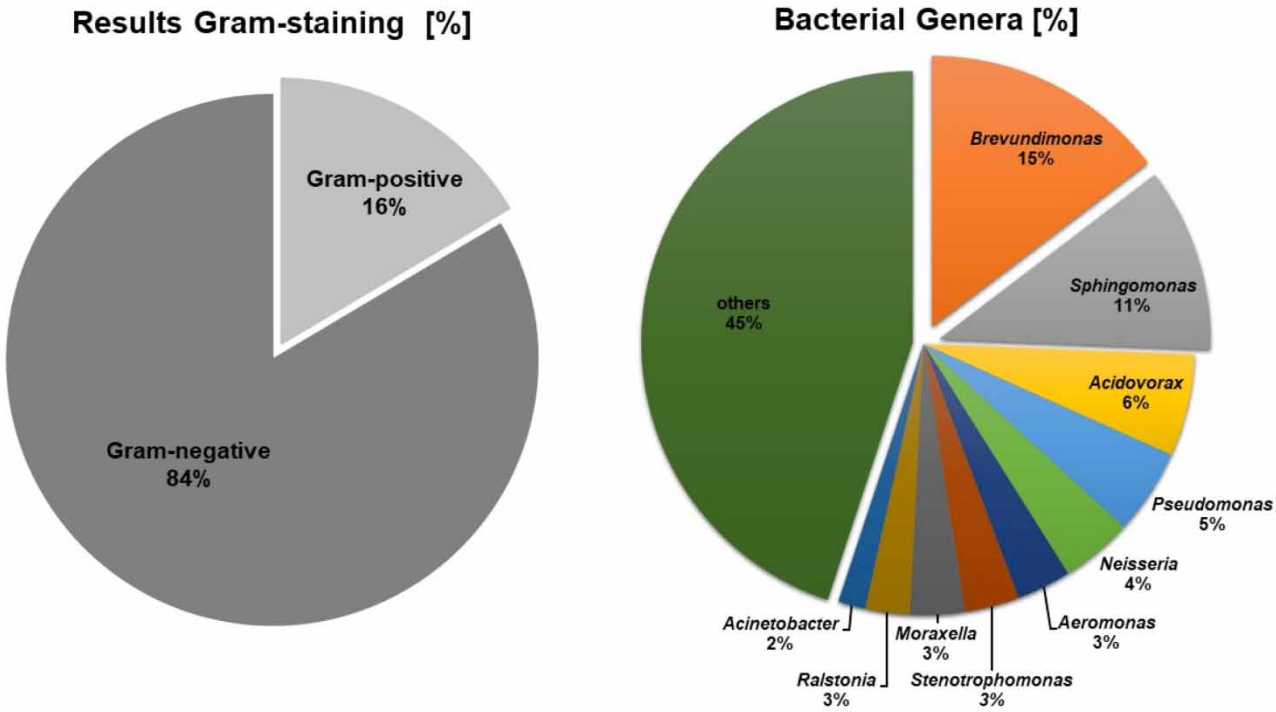

Figure 1 | Overview of 5 years of microbiological drinking water monitoring. Left: Percentage distribution of Gram-staining results. Right: Percentage abundance of the 10 most frequent bacterial genera. Data were calculated for 223 samples and a total of 492 individual identified bacterial isolates.

events conducted by the water supplier. However, there was no significant change in CFU numbers or bacterial species spectrum identified in the affected samples.

Human pathogenic bacteria of at least class 3 or higher were not identified during the entire study. Classical inhabitants of water systems that are traditionally considered public health threats, such as *E. coli*, *Pseudomonas aeruginosa* or *Legionella* species were not found. The 10 most abundant genera included exclusively Gram-negative bacteria that jointly comprised a total of 55.1% of all identifications (Figure 1(right)). The 10 most abundant species were *Sphingomonas paucimobilis* (10.8%), *Brevundimonas vesicularis* (8.7%), *Brevundimonas diminuta* (5.9%), *Acidovorax delafieldii* (4.9%), *Aeromonas salmonicida* (3.3%), *Stenotrophomonas maltophilia* (3.3%), *Ralstonia pickettii* (2.6%), *Neisseria animaloris* (2.2%), *Neisseria zoodegmatis* (2.0%) and *Acinetobacter lwoffii* (1.6%).

Detection of endotoxins from selected drinking water microbiota

To date, few studies exist that comprehensively compare detectable endotoxin levels between different Gram-negative species (Luchi & Morrison 2000) with a focus on water microbiota. We therefore attempted the detection of endotoxins from a selection of the most common drinking water microbiota using standard LAL-tests (Harm *et al.* 2021) and our results present a small starting point to foster differential studies on the risk of LPS from drinking water microbiomes. To closely resemble a potential endotoxin health risk scenario from respective water sources, we refrained from using additional cell lysis methods or buffers, to simulate endotoxin exposure to human consumers by the selected bacteria more accurately. Endotoxin tests were conducted from fresh and diluted cell suspensions and normalized to approximately 10^8 cells/ml. Considering detectable LPS molecules supposedly requires efficient release from the surrounding membrane (Su & Ding 2015), we were satisfied with the immediate detectability (Figure 2).

B. diminuta suspensions exhibited the highest endotoxin concentration (80.7 EU/ml $\pm$ 16.6 EU/ml) trailed by results detected from *S. paucimobilis* (47.4 EU/ml $\pm$ 9.4 EU/ml). Lower concentrations were detected from suspensions of *S. maltophilia* (16.5 EU/ml $\pm$ 4.9 EU/ml), *Pseudomonas fluorescens* (10.2 EU/ml $\pm$ 2.5 EU/ml) and *R. pickettii* (8.6 EU/ml $\pm$ 1.7 EU/ml). Indeed, our findings indicate that populations of Gram-negative bacteria release basal endotoxin

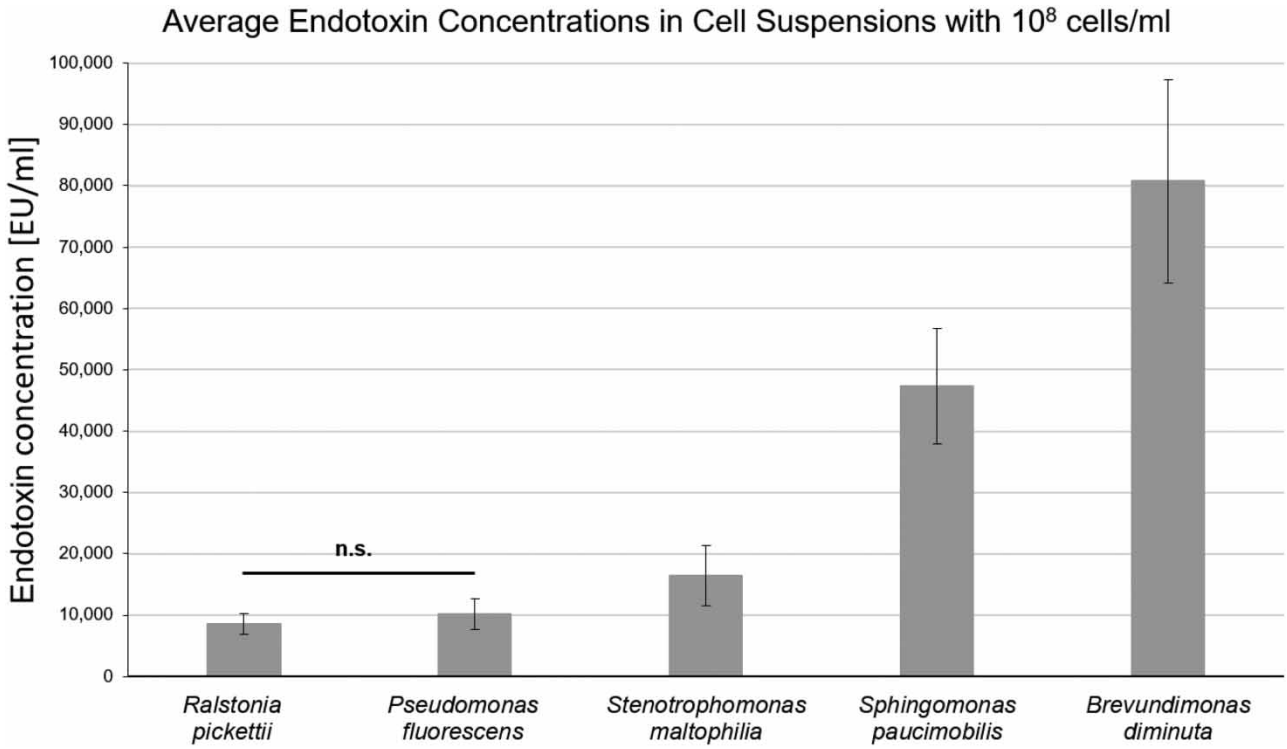

Figure 2 | Detection of endotoxins from selected Gram-negative bacteria. Average endotoxin concentration was determined from diluted cell suspensions normalized to 10^8 cells/ml. Biological replicates ($n \geq 6$) were measured in technical duplicates on the analytical system. Reconstituted endotoxin was used to generate positive controls for every sample. (n.s. = not significant; all other differences determined $P < 0.01$ using two-tailed Mann–Whitney tests).

levels, potentially from average amounts of old, damaged and/or dying cells or random release/errors during processes such as vesicle separation or cell divisions.

During the experiment, suspensions with *Brevundimonas vesicluaris* and *Methylobacterium fujisawaense* failed to produce significant amounts of detectable endotoxins within the utilized LAL system (data not shown). Strikingly, positive controls with spiked reconstituted endotoxin from *E. coli* were readily detected in these tests, indicating no interference of cellular components with the assay or the activation cascade in the lysate. Native LPS concentrations of these species may therefore either be under the detection limit, inhibited from being released from the membrane, or masked by structural interactions with other cell constituents (Cao *et al.* 2021). Similar effects were observed within clinical diagnostic trials indicating human serum proteins may inhibit proper endotoxin detection by LAL assays (Gnauck *et al.* 2015).

Including non-culturable cells, the typical range of bacterial concentrations in drinking water systems is highly variable and may reportedly fluctuate between 10^3 and 10^6 cells/ml (Prest *et al.* 2016) with mean endotoxin concentration of 7–8 EU/ml (Korsholm & Søgaard 1987). Considering our data (see Figure 2), we speculate that high *B. diminuta* or *S. paucimobilis* cell numbers may exhibit critical endotoxin concentrations during situations of active biofilm release or acute contamination, as these scenarios may increase endotoxin concentrations manifold. Previous reports of high endotoxin concentrations (750 EU/ml) in drinking water systems after technical shutdown events of biological filters (Anderson *et al.* 2007) provide striking evidence of such a risk. Another report of a fever outbreak with 100 people affected was attributed to contaminated bathwater containing an endotoxin concentration of up to 2,000 EU/ml (Anderson *et al.* 2007), which further underlines the resulting risk for public health. Our data therefore indicate peak concentrations of *B. diminuta* and *S. paucimobilis* provide a potential health threat in communal water systems. As drinking water systems are not commonly tested towards endotoxin concentrations, most cases of endotoxin-induced illnesses are likely never identified.

A meta study of water systems in different regions of Manitoba suggests Gram-negative bacteria from water sources present a potential trigger for inflammatory bowel disease (IBD) (Forbes *et al.* 2016). Since the connection between systematic endotoxemia and IBD is well described (Gardiner *et al.* 1995), our findings support the notion that high cell density of specific populations of Gram-negative bacteria may potentially facilitate the presence of harmful endotoxin levels in drinking water (Figure 2). In fact, both *B. diminuta* and *S. paucimobilis* have been described as opportunistic pathogens that induce classical symptoms of endotoxemia (Ryan & Adley 2010; Ryan & Pembroke 2018). Considering our data, the latter pathogenicity is likely connected to LPS release and subsequent activation of host recognition receptors (Gorman & Golovanov 2022).

In addition, severe endotoxemia has recently been associated with neurodegeneration and Parkinson's disease (Brown 2019; Brown *et al.* 2023). Health threats like the above emphasize the importance of research towards endotoxin removal therapies, which may provide a potent medical tool to fight the high mortality rates in patients between sepsis and septic shock (Śmiechowicz 2022). Conventional monitoring of drinking water microbiota therefore remains important, as monitoring CFU/ml numbers in combination with species spectra profiling and endotoxin testing effectively helps maintain water quality and protect the public from potential risks of endotoxin-related disease or infection outbreak.

CONCLUSIONS

Advances to foster a worldwide drinking water microbiome analysis project would undoubtably help optimize the protection of public health from potential drinking water system-related threats. Our analyses suggest endotoxins may play a role in drinking water-associated health risks and a global drinking water project may comprise collaborations to unlock more comprehensive and species-specific endotoxin data. Although the average culturable concentration of microbiota in the drinking water system investigated here was too low to express detectable or harmful amounts of endotoxins, we demonstrated that high cell numbers during peaks or acute contaminations may potently release critical amounts of endotoxins even without targeted cell lysis. Together with recent data that connects endotoxemia with thresholds of specific water microbiota, our data confirms the potential for endotoxin-driven health threats from drinking water systems, specifically for *B. diminuta* and *S. paucimobilis*, and affirms the necessity of consistent water quality monitoring.

ACKNOWLEDGEMENTS

We thank Maike Sasse, Olaf Heinz, Curley Hohmann and Lena Knaack for help with sampling, filtration and species identification. Furthermore, we thank Dr Arnd Karnatz for his support and guidance.

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.

REFERENCES

Anderson, W. B., George Dixon, D. & Mayfield, C. I. 2007 Estimation of endotoxin inhalation from shower and humidifier exposure reveals potential risk to human health. *J. Water Health* **5** (4), 553–572. doi: 10.2166/wh.2007.043.

Bhattacharyya, J., Biswas, S. & Datta, A. G. 2004 Mode of action of endotoxin: Role of free radicals and antioxidants. *Curr. Med. Chem.* **11** (3), 359–368. doi: 10.2174/0929867043456098.

Brown, G. C. 2019 The endotoxin hypothesis of neurodegeneration. *J. Neuroinflammation* **16** (1), 180. doi: 10.1186/s12974-019-1564-7.

Brown, G. C., Camacho, M. & Williams-Gray, C. H. 2023 The endotoxin hypothesis of Parkinson's disease. *Mov. Disord.* doi: 10.1002/mds.29432.

Brumfield, K. D., Hasan, N. A., Leddy, M. B., Cotruvo, J. A., Rashed, S. M., Colwell, R. R. & Huq, A. 2020 A comparative analysis of drinking water employing metagenomics. *PLoS One* **15** (4), e0231210. doi: 10.1371/journal.pone.0231210.

Bruno, A., Agostinetto, G., Fumagalli, S., Ghisleni, G. & Sandionigi, A. 2022 It's a long way to the tap: Microbiome and DNA-based omics at the core of drinking water quality. *Int. J. Environ. Res. Public Health* **19** (13), 7940. doi: 10.3390/ijerph19137940.

Cao, Y., Zhang, Y. & Qiu, F. 2021 Low endotoxin recovery and its impact on endotoxin detection. *Biopolymers* **112** (11), e23470. doi: 10.1002/bip.23470.

Cecconi, M., Evans, L., Levy, M. & Rhodes, A. 2018 Sepsis and septic shock. *Lancet* **392** (10141), 75–87. doi: 10.1016/S0140-6736(18)30696-2.

Forbes, J. D., Van Domselaar, G., Sargent, M., Green, C., Springthorpe, S., Krause, D. O. & Bernstein, C. N. 2016 Microbiome profiling of drinking water in relation to incidence of inflammatory bowel disease. *Can. J. Microbiol.* **62** (9), 781–793. doi: 10.1139/cjm-2016-0219.

Froböse, N. J., Bjedov, S., Schuler, F., Kahl, B. C., Kampmeier, S. & Schaumburg, F. 2020 Gram staining: A comparison of two automated systems and manual staining. *J. Clin. Microbiol.* **58** (12), e01914–e01920. doi: 10.1128/JCM.01914-20.

Gardiner, K. R., Halliday, M. I., Barclay, G. R., Milne, L., Brown, D., Stephens, S., Maxwell, R. J. & Rowlands, B. J. 1995 Significance of systemic endotoxaemia in inflammatory bowel disease. *Gut* **36** (6), 897–901.

Gnauck, A., Lentle, R. G. & Kruger, M. C. 2015 The Limulus Amebocyte Lysate assay may be unsuitable for detecting endotoxin in blood of healthy female subjects. *J. Immunol. Methods* **416**, 146–156. doi: 10.1016/j.jim.2014.11.010.

Gorman, A. & Golovanov, A. P. 2022 Lipopolysaccharide structure and the phenomenon of low endotoxin recovery. *Eur. J. Pharm. Biopharm.* **180**, 289–307. doi: 10.1016/j.ejpb.2022.10.006.

Harm, S., Schildböck, C., Strobl, K. & Hartmann, J. 2021 An in vitro study on factors affecting endotoxin neutralization in human plasma using the Limulus amebocyte lysate test. *Sci. Rep.* **11** (1), 4192. doi: 10.1038/s41598-021-83487-4.

Hopland, A. O. & Kvamsdal, S. F. 2022 Tap water quality: In the eye of the beholder. *J. Water Health* **20** (9), 1436–1444. doi: 10.2166/wh.2022.151.

Knecht, A. E., Ettenauer, J., Posnicek, T., Taschl, M., Helmecke, M., Haller, H., Gölß, S. & Brandl, M. 2023 Differences in microbial communities in drinking water from conventional electronic and manual taps in dependence on stagnation and flushing cycles. *Water* **15** (4), 784. doi: 10.3390/w15040784.

Korsholm, E. & Søgaard, H. 1987 Comparison of endotoxin (LPS) measurements and plate counts for bacteriological assessment of drinking water. *Zentralbl. Bakteriol. Mikrobiol. Hyg. B Umwelthyg. Krankenhaushyg. Arbeitshyg. Prav. Med.* **185** (1–2), 121–130.

Liu, J., Yang, L., Kjellerup, B. V. & Xu, Z. 2023 Viable but nonculturable (VBNC) state, an underestimated and controversial microbial survival strategy. *Trends Microbiol.* **31**, 1013–1023. doi: 10.1016/j.tim.2023.04.009.

Luchi, M. & Morrison, D. C. 2000 Comparable endotoxic properties of lipopolysaccharides are manifest in diverse clinical isolates of gram-negative bacteria. *Infect. Immun.* **68** (4), 1899–1904. doi: 10.1128/IAI.68.4.1899-1904.2000.

Martins, K. B., Ferreira, A. M., Mondelli, A. L., Rocchetti, T. T. & Lr de S da Cunha, M. 2018 Evaluation of MALDI-TOF VITEK®MS and VITEK® 2 system for the identification of *Staphylococcus saprophyticus*. *Future Microbiol.* **13**, 1603–1609. doi: 10.2217/fmb-2018-0195.

Nikaido, H. 2003 Molecular basis of bacterial outer membrane permeability revisited. *Microbiol. Mol. Biol. Rev.* **67** (4), 593–656. doi: 10.1128/MMBR.67.4.593-656.2003.

Perrin, Y., Bouchon, D., Delafont, V., Moulin, L. & Héchard, Y. 2019 Microbiome of drinking water: A full-scale spatio-temporal study to monitor water quality in the Paris distribution system. *Water Res.* **149**, 375–385. doi: 10.1016/j.watres.2018.11.013.

Prest, E. I., Hammes, F., van Loosdrecht, M. C. & Vrouwenvelder, J. S. 2016 Biological stability of drinking water: Controlling factors, methods, and challenges. *Front. Microbiol.* **7**, 45. doi: 10.3389/fmicb.2016.00045.

Qu, Y., Zhao, Y. D. & Rahardja, D. 2008 Wilcoxon-Mann-Whitney test: Stratify or not? *J. Biopharm. Stat.* **18** (6), 1103–1111. doi: 10.1080/10543400802369103.

Ryan, M. P. & Adley, C. C. 2010 *Sphingomonas paucimobilis*: A persistent Gram-negative nosocomial infectious organism. *J. Hosp. Infect.* **75** (3), 153–157. doi: 10.1016/j.jhin.2010.03.007.

Ryan, M. P. & Pembroke, J. T. 2018 *Brevundimonas* spp: Emerging global opportunistic pathogens. *Virulence* **9** (1), 480–493. doi: 10.1080/21505594.2017.1419116.

Sattar, A. A., Good, C. R., Saletes, M., Brandão, J. & Jackson, S. K. 2022 Endotoxin as a marker for water quality. *Int. J. Environ. Res. Public Health* **19** (24), 16528. doi: 10.3390/ijerph192416528.

Śmiechowicz, J. 2022 The rationale and current status of endotoxin adsorption in the treatment of septic shock. *J. Clin. Med.* **11** (3), 619. doi: 10.3390/jcm11030619.

Su, W. & Ding, X. 2015 Methods of endotoxin detection. *J. Lab. Autom.* **20** (4), 354–364. doi: 10.1177/2211068215572136.

Thom, C., Smith, C. J., Moore, G., Weir, P. & Ijaz, U. Z. 2022 Microbiomes in drinking water treatment and distribution: A meta-analysis from source to tap. *Water Res.* **212**, 118106. doi: 10.1016/j.watres.2022.118106.

First received 19 June 2023; accepted in revised form 25 January 2024. Available online 7 February 2024