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Verification and application of qPCR and viability-qPCR for *Legionella* monitoring in evaporative cooling systems complementing the conventional culture method

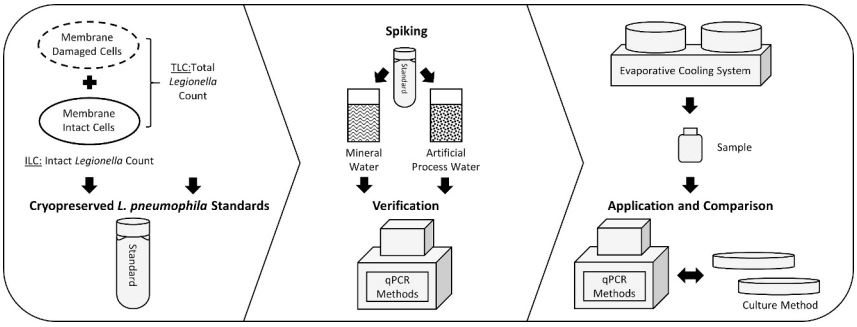
J. Redwitz [a,*], P. Streich [c], M. Zamfir [a], S.M. Walser-Reichenbach [a], M. Seidel [c], C.E.W. Herr [a,b], S. Heinze [a,b], C. Quartucci [a,b]

[a] Department of Occupational and Environmental Health, Epidemiology, Bavarian Health and Food Safety Authority, Munich, Germany
[b] Institute and Clinic for Occupational, Social and Environmental Medicine, University Hospital, LMU Munich, Munich, Germany
[c] Chair of Analytical Chemistry and Water Chemistry, TUM School of Natural Sciences, Technical University of Munich, Munich, Germany

HIGHLIGHTS

- Successful verification of viability-qPCR (v-qPCR) with cryopreserved *L. pneumophila*
- Cryopreserved standards enabled a comparison of qPCR, viability-qPCR, culture method.
- Detection of *Legionella* with v-qPCR in many samples that were culture-negative
- Better *Legionella* control through application of culture-independent methods

GRAPHICAL ABSTRACT



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ABSTRACT

To date, in many countries the only legally valid method for evaporative cooling system (ECS) monitoring is the culture method. However, a duration of up to 14 days and a risk of underestimation of *Legionella* concentrations are seen as limitations of cultivation methods. Rapid cultivation-independent methods are an important step towards a more practicable monitoring of ECS to quickly control interventions if elevated concentrations of *Legionella* are found.

Two commercial kits for quantitative polymerase chain reaction (qPCR) and viability-qPCR (v-qPCR) were studied, comprising sample filtration and DNA extraction. Cryopreserved *Legionella pneumophila* were established as calibration standard with intact (ILC) and total *Legionella* count (TLC) determined by flow cytometry before conducting spiking experiments in commercial mineral water and artificial process water. Final assessment was carried out using real ECS samples.

Recovery and robustness ranged from 86 to 108 % for qPCR with a drop to 40–60 % for v-qPCR when compared to direct extraction, possibly attributable to cell damage during sample concentration. All methods including culture did perform well regarding linearity with $R^2 \geq 0.95$ for most trials. Detected concentrations in

* Corresponding author at: Department of Occupational and Environmental Health, Epidemiology, Bavarian Health and Food Safety Authority, Pfarrstrasse 3, 80538 Munich, Germany.
E-mail address: johannes.redwitz@lgl.bayern.de (J. Redwitz).

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comparison to spiked *Legionella* counts differed with culture averaging 25 ± 7 % of spiked ILC and v-qPCR being closest to spiked concentrations with 65–144 %. In comparison, qPCR was several fold above spiked TLC concentrations. For real ECS samples *Legionella* spp. were detected in concentrations above 10^3 GU/100 mL by v-qPCR in 70–92 % of samples, depending on the kit used. Most of these samples were either culture-negative or not evaluable on agar plates.

This study showed that a cryopreserved bacterial standard based examination is applicable and can be used for future v-qPCR verification. For assessment of differences in results between culture and v-qPCR/qPCR in ECS samples expert knowledge about the operating mode and used analytical methods is required. Guidelines addressing this issue could be a solution.

1. Introduction

The term legionellosis comprises diseases caused by the genus *Legionella*; gram-negative bacteria found in the aquatic environment and wet soil (Bartram et al., 2007). The species most commonly detected in patients is *Legionella pneumophila* serogroup 1–15 (Sg 1–15). It is responsible for around 90 % of infections with Sg 1 having a share of approximately 80 % (Helbig et al., 2002; von Baum and Lück, 2011). The pneumonic form named Legionnaires' Disease (LD) is characterized by severe atypical pneumonia requiring treatment with antibiotics and often hospitalization (Fields et al., 2002). With case fatality rates in Europe of around 10 %, LD is one of the most relevant diseases in environmental medicine (ECDC, 2015; RKI, 2021). The main path of transmission is inhalation of *Legionella* containing bioaerosols, generated in most cases by engineered water systems and installations like evaporative cooling systems (ECS), fountains, whirlpools or car washes (Bartram et al., 2007). Especially ECS have been heavily associated with *Legionella* outbreaks in the past with 19 identified outbreaks occurring between the years 2000 and 2012 resulting in 1609 cases and 102 deaths (Walser et al., 2014). Some outbreaks that are more recent occurred in Germany, Belgium and Spain between 2013 and 2016 with 365 confirmed cases and 5 deaths (Cebrian et al., 2018; Hammami et al., 2019; Maisa et al., 2015).

Due to this, many countries introduced regulations for the operation of ECS addressing the prevention of *Legionella* outbreaks with coordinated approaches to control the growth of *Legionella* and to keep quantities below defined concentrations deemed safe (Van Kenhove et al., 2019).

Control of *Legionella* concentrations in water systems like ECS is traditionally done by plate culture method, which is still considered the "gold standard" (Walker and McDermott, 2021). In many regulations around the world reference units are given in colony forming units (CFU) per sample volume (Van Kenhove et al., 2019). Fractions of the sampled water are plated on buffered charcoal yeast extract (BCYE) containing substances like Cycloheximide, Vancomycin and Polimyxin B to suppress accompanying microorganisms often found in ECS (ISO, 2017). In addition, heat and acid washes are part of the ISO 11731 regimen to further suppress the accompanying flora (ISO, 2017; Lee et al., 1993). Other components and sample handling, including biocides, sample transport and storage can affect the culture method, so that measures to stabilize the sample are required (Flanders et al., 2014). For instance, the neutralization of oxidative biocide with sodium thiosulfate and sample transport and storage at 5 ± 3 °C limited to 24 to 48 h (UBA, 2020). Although, as listed in ISO 11731, several precautions are taken, the determined culture forming units (CFU) of *Legionella* can still underestimate the *Legionella* concentration (Donohue, 2021; Lee et al., 2011). This can be attributed to suppression resistant accompanying flora, *Legionella* in amoeba, some *Legionella* species being hard to cultivate, the heat and acid washes and the effect of biocides (Díaz-Flores et al., 2015; Lee et al., 1993; Nisar et al., 2023; Rowbotham, 1980). *Legionella* in a viable but non-culturable (VBNC) state that forms due to environmental stressors or stressors like heat and acid washes as part of the culture regimen have also been documented (Alleron et al., 2008; Hussong et al., 1987; Nisar et al., 2023). *Legionella* in a VBNC state can revert to an infectious state (Dietersdorfer et al., 2018), which poses a potential risk. Another drawback of the culture method is the extended duration of ten to fourteen days from sampling to result and up to four weeks if the isolate has to be sub- or serotyped (ISO, 2017).

Due to the long duration of the culture method, rapid cultivation-independent methods are desired in situations when there is a current risk of infection. Some guidelines already recommend rapid molecular detection methods in outbreak scenarios among others the quantitative polymerase chain reaction (qPCR) (Walker and McDermott, 2021). The qPCR can be performed in under 24 h from sampling to result, which is given in genomic units (GU) per sample volume. qPCR is inhibited less by accompanying flora, it can detect *Legionella* in Amoeba and VBNC *Legionella* in contrast to culture (Collins et al., 2017; Donohue, 2021; Whiley and Taylor, 2016). These properties could complement the culture method in regular monitoring of ECS. Especially under circumstances where ECS are not running as expected, system changes are performed, critical action levels are exceeded (e.g. ≥10,000 CFU/100 mL) or are recommissioned. In summary, situations in which the operation status of the system must be closely monitored, and quick action is required to prevent outbreaks.

Because of enumerated reasons, qPCR is seen as a beneficial addition to the culture method and has been assessed in parallel for the examination of environmental samples to gain further insight (Collins et al., 2017; Díaz-Flores et al., 2015; Krøjgaard et al., 2011; Toplitsch et al., 2021). Over time, multiple different primers and probes have been published and several companies have released single or multiplex qPCR kits for the detection of *Legionella* spp., *L. pneumophila* or *L. pneumophila* Sg 1 (Ahmed et al., 2019; Ginevra et al., 2020; Monteiro et al., 2021; Omiccioli et al., 2015). Since 2012 there exists the ISO Norm "Water quality - Detection and quantification of *Legionella* spp. and/or *Legionella pneumophila* by concentration and genic amplification by quantitative polymerase chain reaction (qPCR)" that was updated in 2019 (ISO, 2019). The norm defines requirements for the validation of qPCR kits and consequently offers the manufacturers and laboratories a way to evaluate and compare qPCR performance. The examination of a variety of samples from ECS with qPCR and culture method did show discrepancies between the methods regarding determined *Legionella* concentrations (Collins et al., 2017; Lee et al., 2011). Nevertheless, offering a high sensitivity, the qPCR was shown to have a very good negative predictive value (NPV) for culture with rates of 98.6 % for *Legionella* spp. and 99.6 % for *L. pneumophila* (Collins et al., 2017). Resulting GU per sample determined by qPCR attributed higher concentrations of *Legionella* on average in comparison to the culture determined CFU in various studies (Collins et al., 2017; Donohue, 2021; Young et al., 2021). Probable reasons are aforementioned VBNC, *Legionella* in Amoeba, accompanying flora and problems with the cultivation of some species on agar media (Collins et al., 2017; Donohue, 2021; Whiley and Taylor, 2016). Additionally, the traditional qPCR does not differentiate between DNA of living and dead cells and therefore potentially overestimates the concentration of infectious *Legionella* (Whiley and Taylor, 2016). Therefore, the development of v-qPCR based on photoactivated cross-linkers of DNA intercalating dyes like propidium monoazide (PMA) and ethidium monoazide (EMA) or enzyme-based approaches gained momentum in recent years (Kirschner, 2016; Reyneke et al., 2017). These

methods offer a way to make free DNA or DNA in membrane-damaged cells inaccessible or degrade it enzymatically. Only DNA of cells with intact membrane is available for amplification by qPCR after treatment. The efficiency of v-qPCR techniques has been shown to correlate positively with amplicon length, samples with low turbidity and low accompanying flora (Ditommaso et al., 2015a; Kontchou and Nocker, 2019; Taylor et al., 2014; Yanez et al., 2011). This can in turn lead to an over- or underestimation of viable *Legionella* by v-qPCR if applied incorrectly requiring thorough verification.

Despite existing studies regarding the performance of v-qPCR/qPCR kits and ISO norms for the validation of qPCR, challenges remain when it comes to selecting the method of choice for the investigation of different systems. One factor is the recovery method i.e. filtration and DNA extraction of the sample, that is often neglected in the validation since it heavily depends on the sample matrix. For ECS this holds especially true since the combination of biocides, corrosion inhibitors, freshwater source and microorganisms are unique for most systems. Additionally, for comparison to the culture method and the detection of living and therefore infectious *Legionella* sp. it is essential that v-qPCR is evaluated intensively because no standard or guideline exist. The aim of the present study was therefore to develop a quick and conclusive procedure to evaluate readily available v-qPCR kits as used in commercial and government laboratories by establishing cryopreserved standards containing living *L. pneumophila*. As a basis, the ISO/TS 12869 was utilized to verify the standards with traditional qPCR and in a first attempt application of the norm for v-qPCR was tested. Furthermore, linearity over relevant action levels commonly used for ECS was investigated and compared to culture method. Ultimately, all methods were applied to environmental samples from ECS and compared.

2. Material and methods

2.1. Artificial samples

Matrices for artificial samples were Evian mineral water (Danone) or Evian mineral water supplemented with chemicals (Coragard® OS 587, Aqua Concept) used to treat water of ECS according to the manufacturer's instructions, referred to as artificial process water (aPW). Biocides were not added to prevent a change in intact cell count. Respective matrices were spiked with defined concentrations of total or intact *L. pneumophila* Philadelphia-1 (DSMZ 7516) Sg 1, *L. pneumophila* Sg 4 (DSMZ 7514) or *L. pneumophila* Sg 6 (DSMZ 25182), using cryopreserved *Legionella* standards (Table 1) stored at −80 °C and thawed at 37 °C for 5 min. Cryopreserved standards were prepared by rqmicro AG according to following protocol and shipped within 24 h on dry ice. Inoculation of 50 mL liquid growth medium [homogenous solution of 500 mL ultrapure water, 5 g yeast extract, BCYE growth supplement (VWR) filtered using 0.2 μm PES filter discs] with one colony of a pure culture. Shaking incubation of the culture at 36 °C and 180 rpm for 16–18 h. After incubation, a dilution series up to 10^{-5} was prepared in 1× phosphate buffered saline (PBS). Two 250 μL aliquots of each dilution were treated using *L. pneumophila* Sg 1, Sg 4 or Sg 6 specific antibodies (rqmicro AG) and either 2.5 μL 0.25 μM propidium iodide (PI) or 2.5 μL 100× SYBR Green (Sigma-Aldrich) and incubated for 1 h in the dark at room temperature. SYBR Green stains all bacteria inside the sample and PI can only penetrate in damaged cell membranes. By combining both fluorescence stains, dead cells can be discerned from viable ones (Koetzsch et al., 2012). After incubation, the intact *Legionella* count (ILC) and the total *Legionella* count (TLC) were determined by flow cytometry using the rqmicro.COUNT system (rqmicro AG) according to manufacturer's instructions. The culture had to contain >60 % intact *Legionella* and a contamination of <10 % to pass quality control. In the next step, 250 mL of sterilized Evian water were spiked with the mother suspension to a concentration of 10^6 ILC/mL based on the counts determined by flow cytometry and incubated at 25 °C, 120 rpm for 48 h to condition the cells. After conditioning two 1:10 dilutions were prepared as described for flow cytometry and measured. Results were used to calculate the TLC/mL and ILC/mL of the cryopreserved standards by also taking into account the final 1:1 dilution with buffer for cryoconservation [9.55 g 10× PBS, 10 g Bovine serum albumin (BSA), 60 g Dextran 40, 400 mL double distilled water – filtered after mixing using 0.2 μm filters] that immediately followed the conditioning. Afterwards, cryopreserved standards were inverted a few times and stored at −20 °C for the night before being transferred into a −80 °C freezer. After eight months of storage Sg 1 batches were tested via flow cytometry detecting a change of 0.0 ± 1.5 % for TLC and 7.7 ± 1.2 % for ILC.

Table 1
Concentrations of cryopreserved *Legionella* standards determined by flow cytometry for total (TLC) and intact *Legionella* count (ILC).

Cryopreserved standard	TLC/mL	ILC/mL
L. pneumophila Sg1	9.60×10^5	6.4×10^5
L. pneumophila Sg4	1.35×10^6	1.21×10^6
L. pneumophila Sg6	1.07×10^6	8.50×10^5

2.2. Environmental samples

For the period from March to June 2022, 25 environmental samples were collected from 19 different ECS; the operator and exact location were anonymized. All ECS were operated with non-oxidizing biocides and subsequently all samples were collected in sterile containers without a neutralizing agent and protected from light. Part of the samples were collected by Aqua Concept GmbH and shipped using express delivery. Samples arriving on the following day could be processed within 24 h, if shipping delays occurred, they were noted and are indicated in Supplement Table 1 accordingly.

2.3. DNA extraction/purification and treatment for viability-qPCR

For DNA extraction with the Aquadien Kit (Bio-Rad Laboratories) 50 or 100 mL of sample, depending on sample consistency and filter clogging, were filtered with 0.4 μm pore size polycarbonate filters (Merck Millipore). Subsequent DNA extraction followed the manufacturer's protocol. The iQ-Check® Free DNA Removal Solution (FDRS) (Bio-Rad Laboratories), an enzyme -based approach to v-qPCR was used according to the manufacturer's instructions in combination with iQ-Check® Quanti *L. pneumophila* Real-Time PCR Quantification Kit (Bio-Rad Laboratories). For DNA extraction with the foodproof® StarPrep Two Kit (Hygiena, formerly Biotecon) 50 or 100 mL of sample, depending on filterable volume, were filtered with 0.4 μm pore size polyethersulfone (PES-Polyethersulfon, Hygiena) filter discs. DNA extraction with the foodproof® StarPrep Two Kit (Hygiena) followed the manufacturer's extraction procedure A and was performed using Reagent D (Hygiena) a crosslinking dye-based approach for determination of viable *Legionella* via v-qPCR. For photoactivation of the Reagent D the PAUL device (Photo Activation Universal Light, Geniul Company) was used exposing the sample to wavelengths between 464 and 476 nm for 5 min. The identical protocol was applied for DNA extraction without Reagent D treatment if total *Legionella* count was determined.

Direct DNA extraction without a prior filtration step was carried out using the foodproof® StarPrep Two Kit (Hygiena) following the manufacturer's extraction procedure B. If Reagent D was used in combination with procedure B, the photoactivation used the PAUL device.

2.4. Legionella qPCR and viability-qPCR

Every qPCR and v-qPCR were performed on a Real-Time PCR Detection System consisting of a CFX96 optical module (Bio-Rad Laboratories) in combination with a C1000 Touch™ Thermal Cycler base (Bio-Rad Laboratories). The software utilized was CFX Manager industrial diagnostic edition (Bio-Rad Laboratories). DNA extracted with

Aquadien Kit (Bio-Rad Laboratories) for qPCR or v-qPCR (usage of FDRS during the extraction process) was amplified with the associated iQ-Check® Quanti *L. pneumophila* and iQ-Check® Quanti *Legionella* spp. kit from Bio-Rad Laboratories according to manufacturer's instructions in quantities of 5 μL per reaction. The kits are designed for the detection of *L. pneumphila* and *Legionella* spp., respectively. After each Bio-Rad qPCR run, the Bio-Rad CFX Manager software automatically determined the threshold for the run and associated Cq values for each reaction. The software also calculated the concentrations in GU/100 mL for each sample based on sample volume and subsequent steps that occurred during the DNA extraction.

Both Bio-Rad qPCR kits have been validated by the Association française de normalisation (AFNOR, 2023) to meet the requirements of the ISO/TS 12869 (ISO, 2019). The limit of detection (LOD) for the Bio-Rad qPCR Kits was determined to be at 5 genomic units (GU) per reaction by meeting the 90 % confidence limit of 30 independent dilutions of the desired LOD. The upper quantification limit (UQL) and the limit of quantification (LOQ) are dependent on the lowest and highest concentration of the iQ-Check® *Legionella* quantification DNA standards (Bio-Rad Laboratories) used for quantification of the runs. In the case of this study 20 GU for iQ-Check® Quanti *L. pneumophila*, 15 GU for iQ-Check® Quanti *Legionella* spp. and 31,000 GU for both kits as the lowest and highest standard concentration, respectively. The amplification results were considered valid if the PCR efficiency was between 75 and 125 % and the correlation coefficient (R^2) was above 0.99 (in accordance with ISO/TS 12869) for the iQ-Check® standards. Inhibition was measured based on the internal controls (IC) of the samples. If the control was not detected or the quantification cycle (Cq) of the control was greater than the mean Cq value of all quantification standards internal controls plus three times their standard deviation the reaction was considered inhibited. For inhibited samples, the DNA extract was diluted 10-fold and the run repeated.

The DNA extracted with the foodproof® StarPrep Two Kit (Hygiena, formerly Biotecon) was amplified with the associated microproof® *Legionella* Quantification LyoKit (Hygiena, formerly Biotecon) according to manufacturer's instructions in quantities of 25 μL per reaction. The microproof® *Legionella* Quantification LyoKit is based on 8 well PCR Tube strips that contain a ready to use lyophilized master mix for multiplex qPCR and only needs the addition of 25 μL of DNA extract. The multiplexed qPCR enables detection of *Legionella* spp., *L. pneumophila* and *L. pneumophila* Sg 1. After the qPCR run, the threshold for each fluorophore was set at 3 % of the maximum fluorescence measured for the specific run. The results where then transferred to the calculation template (Version 2.1, based on ISO/TS 12869:2019) that was provided by Hygiena. The template calculated if PCR efficiency was between 75 and 125 % and R^2 was $\geq$0.98 for the standards provided. Inhibition was indicated if the IC of a sample had a deviation of >1.5 cycles from the IC of the negative control and no strong amplification (Cq <29) was present in the sample for one of the target reactions. Results (C_L) for each sample were calculated in GU/100 mL using formula (1). Calculations were based on determined GU per qPCR reaction (C_r), elution volume for washing the filter disc (V_s) and recovery factor (a) being the quotient of the used Rinse Buffer for washing the filter disc divided by the recovered volume. Also PCR reaction volume in μL (V_r) and sample volume in mL (V_s).

$$C_L = C_r \times V_e \times a/V_r \times V_s \quad (1)$$

The LOD for the Hygiena qPCR Kit was determined to be at 3 GU for *Legionella* spp. and 5 GU for *L. pneumophila* as well as *L. pneumophila* Sg1 per reaction by meeting the 90 % confidence limit of 20 independent dilutions of the target concentrations in accordance to validation data provided by Hygiena. The upper and lower LOQ is dependent on the lowest and highest concentration of the DNA standards (microproof® *Legionella* Quantification LyoKit, Hygiena) used for quantification of the runs. In the case of this study 25 and 25,000 GU per reaction, respectively.

2.5. Culture method

For the detection and quantification of *Legionella*, Evian mineral water and artificial process water samples were cultured on Buffered Charcoal Yeast Extract agar medium containing glycine, vancomycin, polymyxin, cycloheximide (GVPC, Xebios Diagnostics) in volumes of 0.5 mL and 0.1 mL as duplicates each. Additionally, 100 mL of sample were filtered using mixed cellulose esters (MCE) membranes with a pore size of 0.45 μm (Merck Millipore) that were placed on GVPC agar. GVPC agar plates for artificial samples were incubated at 36 ± 2 °C in a humidified environment and colonies were checked and counted subsequently starting on the second day. Environmental samples were plated in accordance with the suggestion of the German Federal Environment Agency for ECS (UBA, 2020) and DIN EN ISO 11731 (ISO, 2017). The environmental samples were plated as follows, 0.1 mL without treatment, 0.1 mL heat treated, 2× 0.5 mL heat treated, 2× 0.5 mL acid treated, membrane filtration of 20 mL heat treated sample and membrane filtration of 20 mL sample including acid treatment, all plating was done on GVPC agar (Xebios Diagnostics) and mixed cellulose esters (MCE) membranes with a pore size of 0.45 μm (Merck Millipore) were used for filtration. Plated environmental samples were incubated at 36 ± 2 °C in a humidified environment and colonies were checked subsequently starting on day 2–4, 3–5, 7–10. Suspicious colonies were checked by picking at least three colonies of the same phenotype, if possible. These were streaked on BCYE+AB agar (Xebioas Diagnostic) and trypticase soy agar (TSA) plates and incubated at 36 ± 2 °C in a humidified environment. TSA is a non-selective growth medium that lacks cysteine. Potential *Legionella* colonies grow on BCYE+AB plates, but not on TSA plates. A further confirmatory test was conducted after a positive culture result using the immunochromatographic test VIRAPID® LEGIONELLA CULTURE (Vircell Microbiologists) for *Legionella* spp. and *L. pneumophila* serogroups 1–15.

2.6. Data analysis for extraction and robustness verification

The whole method (filtration, DNA purification and qPCR) was verified for extraction performance and robustness by adapting the ISO/TS 12869 protocol (ISO, 2019) to be used with cryopreserved standards and v-qPCR. For verification of matrix influence, Evian water (Danone) and artificial PW free of *Legionella* DNA were spiked with cryopreserved standards of *Legionella pneumophila* Sg 1 to concentrations of 10^3 and 10^4 total or intact *Legionella*/100 mL. In total, 10 samples per concentration and matrix were produced for each of the tested v-qPCR/qPCR kits and processed following the recommended protocols for each kit provided by the manufacturer (see Section 2.3). Additionally, three samples of the stock suspension used for spiking were processed by applying the protocol for direct extraction (see Section 2.3). Results of the direct extraction were used to calculate the recovery of the whole method. Results were given as the percentage of the determined GU/100 mL of the whole method divided by the GU/100 mL of the direct extraction. Recovery calculation according to ISO/TS 12869:2019 was performed using formula (2) to get the decimal logarithm of recovery for sample x (ISO, 2019). Where A is the reference value for the concentration of the mother suspension, expressed as decimal logarithm of the number of genome units per millilitre, B is the measured value of GU per mL expressed as a decimal logarithm, D is the decimal logarithm of the dilution factor between mother suspension and spiked sample and V_{pe} being the volume of the spiking suspension in μL.

$$log_{10}\eta_x = \mathrm{B} - A + D + log_{10}{}^{1,000}/V_{pe} \quad (2)$$

Following statistical analysis including average recovery ($\overline{\eta_x}$), variance (s^2) and overall expanded uncertainty ($U_{overall}$) of the whole method was performed according to ISO/TS 12869:2019 protocols for samples x

= 1…n with the total number of samples for all matrices and spiked levels being n using formulas (3) to (5) (ISO, 2019).

$$\overline{\eta_x} = \sum_{x=1}^{n} \eta_x \Big/ n \tag{3}$$

$$s^2 = \frac{\sum_{x=1}^{\eta} \eta_x^2 - \left[\left(\sum_{x=1}^{\eta} \eta_x \right)^2 \Big/ n \right]}{n-1} \tag{4}$$

$$U_{overall} = 2 \times \sqrt{\eta_x^{-2} + s^2} \tag{5}$$

3. Results

For evaluating the applicability of qPCR and v-qPCR for process water samples of ECS, a prior verification of the full bioanalytical method was conducted. This included the reliability of the qPCR reaction itself as well as the filtration and elution for concentration of *L. pneumophila* followed by cell lysis, DNA extraction and purification as well as robustness of the method regarding different matrix compositions. The International Organization for Standardization provides an extensive guideline for development and validation of quantitative PCR with ISO/TS 12869 (ISO, 2019). While the norm provides valuable protocols for the verification of the whole bioanalytical method, v-qPCR is generally excluded from the norm. Therefore, this study aimed to test if verification of the whole bioanalytical method using cryopreserved standards containing living *L. pneumophila* is feasible. TLC and ILC of *L. pneumophila* were determined by flow cytometry. Verification was carried out in Evian water as a matrix with a defined content of bacteria and water chemistry. Evian water supplemented with chemicals used in ECS was defined as aPW to resemble the chemical composition of ECS process water. Lastly, methods were tested on process water samples from 19 different ECS.

3.1. Extraction and robustness performance (adapted after ISO/TS 12869)

Evaluated *L. pneumophila* concentrations were 10^3 and 10^4 using TLC/100 mL for qPCR and ILC/100 mL for v-qPCR as a reference. The ISO/TS 12869 defines that the average recovery for 10 replicates of two concentrations levels must be between −0.6 and 0.3 $\log_{10}$GU/100 mL. This corresponds to 25 to 200 % of the GU/100 mL determined with the direct extraction without concentration steps like filtration. For Evian and aPW Bio-Rad qPCR, Hygiena qPCR as well as Hygiena v-qPCR did meet the specifications (Table 2). Enzyme-based Bio-Rad v-qPCR gave results below −0.6 $\log_{10}$GU/100 mL in Evian (Table 2). Moreover, the gap between spiked and measured concentrations for enzyme-based v-qPCR was below the results for culture method that found 25 ± 7 % of the spiked ILC/100 mL (Fig. 4). This led to removal of the BioRad v-qPCR from verification since incompatibility with cryopreserved standards was suspected. The observations between Evian and aPW showed similar results and scattering of the single replicates was around ±20 % of the mean recovery (Fig. 1). Differences in recovery were observed between qPCR and v-qPCR with the latter having a reduced average recovery (40–60 %) compared to the direct extraction while the average loss for qPCR was minimal and recovery at 86–108 % (Table 2, Fig. 1).

3.2. Analysis of linearity

Evaluation of Bio-Rad qPCR was performed in Evian water and aPW spiked with cryopreserved *L. pneumophila* Sg 1, Sg 4 and Sg 6 resulting in concentrations of 10^2, 5×10^2, 10^3, 5×10^3, 10^4, 5×10^4, 10^5 TLC/100 mL. TLC/100 mL was used as reference unit for Bio-Rad kit since

Table 2
Results of recovery and robustness testing of qPCR and v-qPCR for *L. pneumophila* adapted after ISO/TS 12869 (ISO, 2019). Ten samples per concentration level were spiked in Evian water and/or artificial process water (aPW) using *L. pneumophila* Sg 1 cryopreserved standards. Additionally DNA of three aliquots of the stock suspension (10^5 *Legionella*/100 mL) were extracted using a direct extraction method without filtration steps and used as a reference value for the calculations of the recovery of *L. pneumophila*.

Matrix	Spiked concentration [qPCR = TLC/100 mL; v-qPCR = ILC/100 mL]	Recovery [$\log_{10}$GU/100 mL]							
		qPCR				Viability-qPCR			
		Bio-Rad	Hygiena			Bio-Rad	Hygiena		
		L. pneumophila	*Legionella* spp.	*L. pneumophila*	*L. pneumophila* Sg 1	*L. pneumophila*	*Legionella* spp.	*L. pneumophila*	*L. pneumophila* Sg 1
Evian water	1000	−0.01	0.09	0.00	−0.08	<−0.60	−0.31	−0.39	−0.33
	10,000	−0.07	0.08	0.00	−0.02	<−0.60	−0.26	−0.34	−0.31
aPW	1000	0.03	n. p.[a]	n. p.[a]	n. p.[a]	n. p.[b]	−0.07	−0.20	−0.22
	10,000	−0.05	n. p.[a]	n. p.[a]	n. p.[a]	n. p.[b]	−0.06	−0.19	−0.20
Average recovery [$\log_{10}$GU/100 mL]		−0.02	n. p.[a]	n. p.[a]	n. p.[a]	n. p.[b]	−0.17	−0.28	−0.27
Variance (s^2)		0.00	n. p.[a]	n. p.[a]	n. p.[a]	n. p.[b]	0.02	0.01	0.01
Global expanded uncertainty, $U_{overall}$		0.12	n. p.[a]	n. p.[a]	n. p.[a]	n. p.[b]	0.45	0.61	0.58

[a] n. p. not performed due to limited reaction volume.
[b] n. p. not performed due to lack of compatibility of Bio-Rad viability-qPCR with cryopreserved standards.

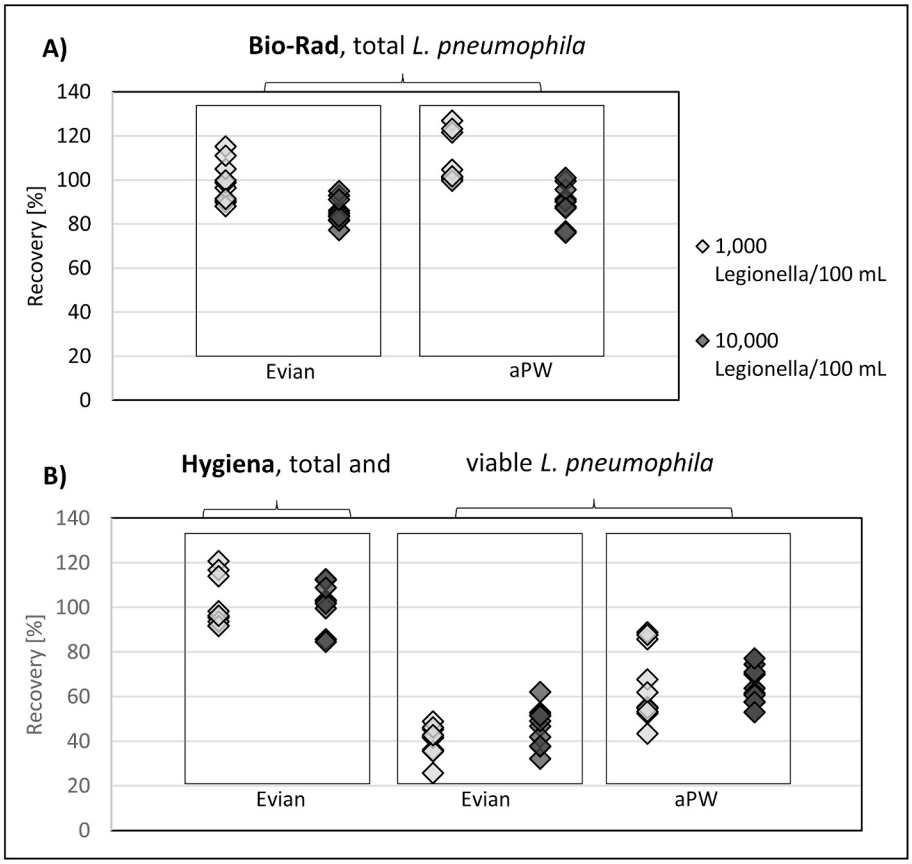

Fig. 1. Recovery of *L. pneumophila* in Evian water and artificial process water (aPW) using qPCR and viability-qPCR. Ten samples per concentration level and matrix were produced by spiking with cryopreserved *L. pneumophila* Sg 1 standards. Additionally, DNA of three aliquots of the stock suspension (10^5 *Legionella*/100 mL) were extracted using the direct method without filtration steps and used as a reference value for the calculation of the recovery of *L. pneumophila*.

emphasis was on qPCR performance due to poor recovery with the enzyme-based Bio-Rad v-qPCR (see chapter extraction and robustness). The coefficient of determination (R^2) predicted a good fit to the model for each of the three extraction replicates of the dilution series in spiked Evian water for Sg 1 ($R^2 = 0.99$; m = 6, n = 3), Sg 4 ($R^2 \geq 0.98$; m = 5, n = 3) and Sg 6 ($R^2 \geq 0.97$; m = 6, n = 3) (Fig. 2). Determined GU/100 mL were at 483, 567 and 587 % of spiked concentrations in TLC/100 mL for extraction replicates of Sg 1, indicating increased detection by qPCR in comparison to flow cytometry. For Sg 4 and Sg 6 determined GU/100 mL were at 104, 114, 117 % and 140, 148, 150 % of spiked TLC/100 mL, respectively, showing only a moderate increase compared to Sg 1. aPW results were comparable to Evian water with a good fit to the linear model for Sg1 ($R^2 \geq 0.97$; m = 6, n = 3), Sg 4 ($R^2 \geq 0.98$; m = 6, n = 3) and Sg 6 ($R^2 \geq 0.94$; m = 6, n = 3) (Fig. 2). Comparing determined GU/100 mL and spiked TLC/100 mL in aPW for Sg 1 a strong (377, 388, 400 %) and a moderate increase for Sg 4 (136, 138, 150 %) and Sg 6 (135, 147, 151 %) was observable similar to Evian. Nevertheless, for aPW a decrease in recovery was seen with an increase in spiked TLC concentration. This led to a flattening of the regression curve making it drop below the reference value in the case of Sg 4 and Sg 6 (Fig. 2). Determined concentrations were at 68–91 % compared to spiked TLC at the point of biggest difference.

For samples spiked with Sg 1 reproducible quantification with the Bio-Rad Kit was achieved at 500 TLC/100 mL, for Sg 4 and Sg 6 at 10^3 TLC/100 mL. At these concentrations ≥2 qPCR replicates for all three extraction replicates of a dilution series were quantitatively detected. This was the case for both Evian and aPW. Quantification was possible until the highest spiked concentration of 10^5 TLC/100 mL in both matrices.

Using the multiplexed Hygiena kit, the focus was on evaluating v-qPCR performance and consequently ILC/100 mL was used as reference. Evian water and aPW were spiked to concentrations of 10^2, 5×10^2, 10^3, 5×10^3, 10^4, 5×10^4, 10^5 ILC/100 mL.

For *L. pneumophila* detection the two extraction replicates of each dilution in Evian showed good approximation of the data points to the model for v-qPCR with an R^2 of 0.99 (m = 6, n = 2) (Fig. 3). Measured GU/100 mL showed a decrease compared to spiked ILC/100 mL detecting only 65 and 70 % of spiked concentrations for the extraction replicates. In aPW results were comparable to Evian water with a good fit to the linear model ($R^2 \geq 0.98$; m = 6, n = 2) (Fig. 3). Comparison of determined GU/100 mL for v-qPCR and spiked ILC/100 mL showed an increase of 135 and 144 % corresponding to a moderate rise in contrast to the results in Evian. Detection reactions for *L. pneumphila* Sg 1 and *Legionella* spp. were comparable with the latter showing a tendency to give increased concentrations in comparison to reactions for detection of *L. pneumophila* and *L. pneumophila* Sg 1 (Supplement Fig. 1).

For all three detection reactions reproducible quantification for v-qPCR was at 10^3 ILC/100 mL in Evian. At this concentration all duplicates were quantitatively detected. In aPW reproducible quantification was at 500 ILC/100 mL for *L. pneumophila* Sg 1 and *L. pneumophila* while for *Legionella* spp. it was below 100 ILC/100 mL. Quantification with v-qPCR was possible until the highest spiked concentration of 10^5 ILC/100 mL in both matrices. For qPCR reproducible quantification was possible for the lowest concentration of 100 ILC/100 mL and up to a concentration of 5×10^4 ILC/100 mL for all detection reactions in both matrices. Reason for this was the average difference of 280 % and 360 % for Evian and aPW between the spiked concentrations and the detected GU/100 mL, limiting the detection of higher spiked concentrations.

For comparison, culture method was performed using the same *L. pneumophila* Sg 1 cryopreserved standards and concentrations. The fit

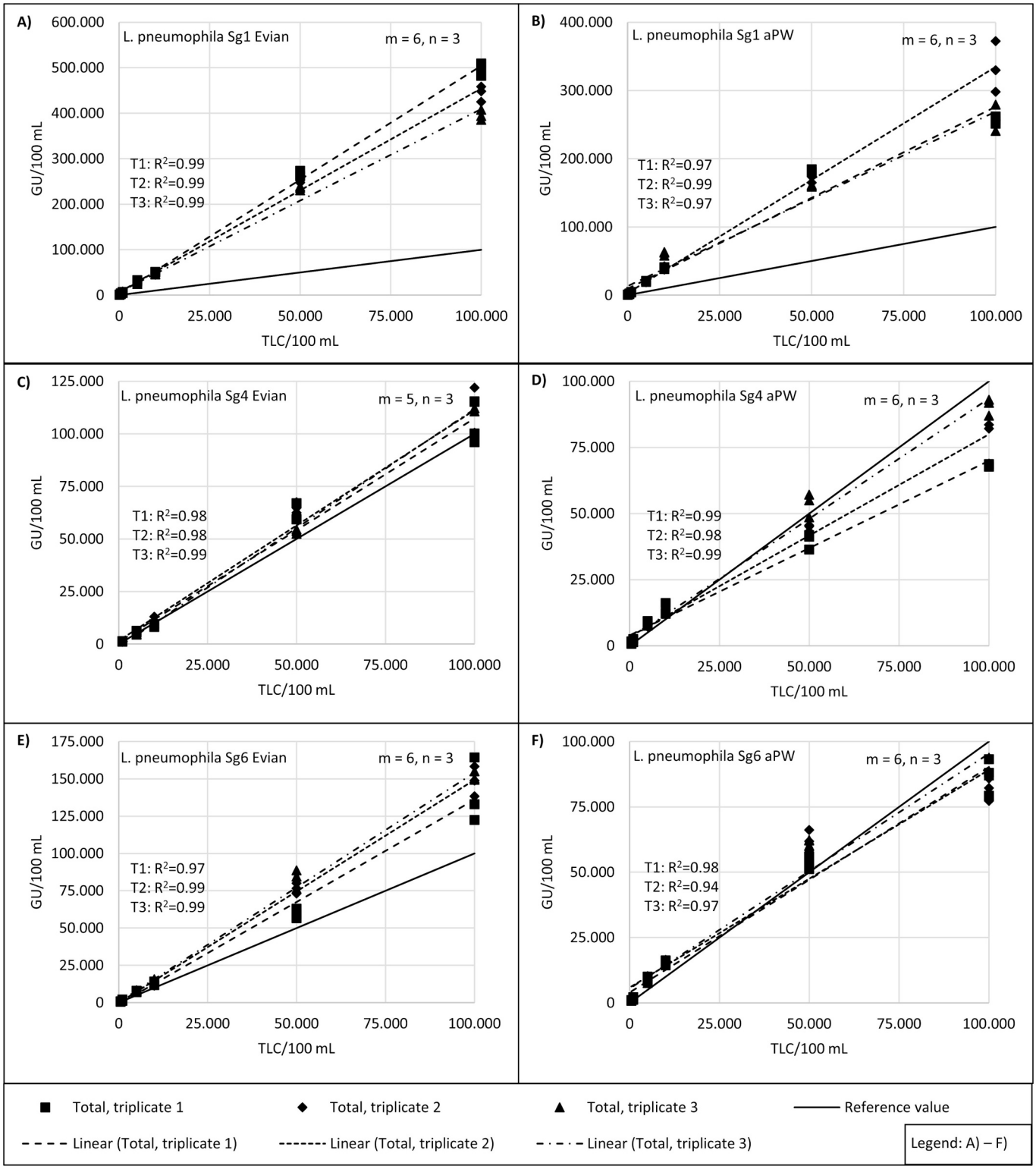

Fig. 2. Verification of linearity for the Bio-Rad *L. pneumophila* qPCR kit using artificial samples based on Evian water or artificial process water (aPW). Matrices were spiked with *L. pneumophila* Sg 1, Sg 4 or Sg 6 cryopreserved standards over a range of seven concentrations (10^2, 5×10^2, 10^3, 5×10^3, 10^4, 5×10^4, 10^5 TLC/100 mL). Each concentration was spiked in triplicates for qPCR (T1 to T3) using dilutions based on one stock suspension (10^5 TLC/mL) and the resulting DNA extracts were run as triplicates. Results of the qPCR in genomic units (GU) for each triplicate (n = 3) are plotted against spiked concentrations (m) based on total *Legionella* count (TLC). The reference value is indicating the curve if TLC and GU were identical. For linear regression, results below the limit of quantification were omitted.

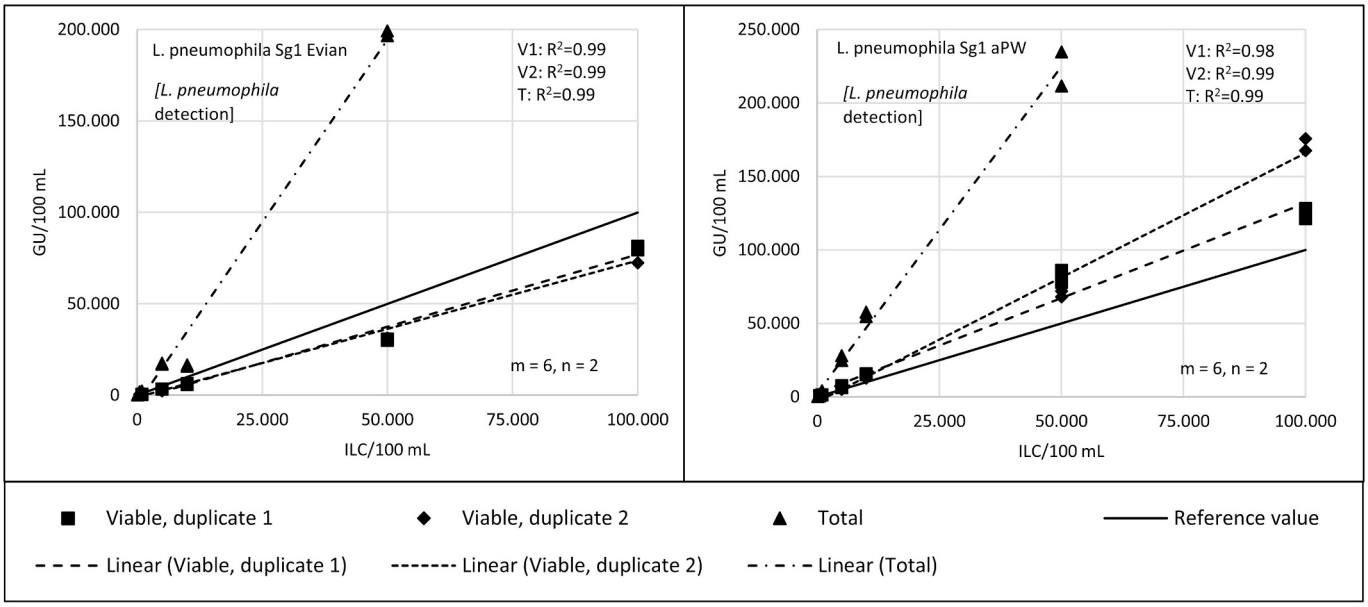

Fig. 3. Verification of linearity for the Hygiena qPCR kit using artificial samples based on Evian water or artificial process water (aPW). Matrices were spiked with *L. pneumophila* Sg 1 cryopreserved standards over a range of seven concentrations (10^2, 5×10^2, 10^3, 5×10^3, 10^4, 5×10^4, 10^5 ILC/100 mL). Each concentration was spiked in duplicates per matrix for viability-qPCR (V1, V2) and once for qPCR (T) and all resulting DNA extracts were run as duplicates. Results of the viability-qPCR or qPCR in genomic units (GU) for each reaction duplicate (n = 2) are plotted against spiked concentrations (m = 6) based on intact *Legionella* count (ILC). The reference value is indicating the curve if ILC and GU were identical. For linear regression results below the limit of quantification were omitted.

of the linear regression curve was very good with R^2 being ≥0.98 (m = 7) for three repetitions in Evian water. In aPW one repetition had an R^2 of 0.87 (m = 7) and the second one an R^2 of 0.98 (m = 7). For both matrices a sharp decrease was noticeable for determined CFU/100 mL in comparison to the spiked ILC/100 mL (Fig. 4). The amount of determined CFU/100 mL in relation to spiked ILC/100 mL for repetition one to three in Evian was 24, 25, 29 % and for repetition one and two in aPW 26 and 21 %, respectively.

3.3. Analysis of environmental samples

Environmental samples collected from 19 different cooling towers at 25 different dates were analyzed in duplicates using each of the qPCR and v-qPCR kits as well as culture. Shipping delays lead to some ECS samples not being analyzed within 24 h after sampling. Nevertheless, the focus was on comparing different analytical methods and not determining the *Legionella* concentrations at the point of sampling as accurately as possible.

Using the Bio-Rad kit, *L. pneumophila* was detected in 8 of 25 samples

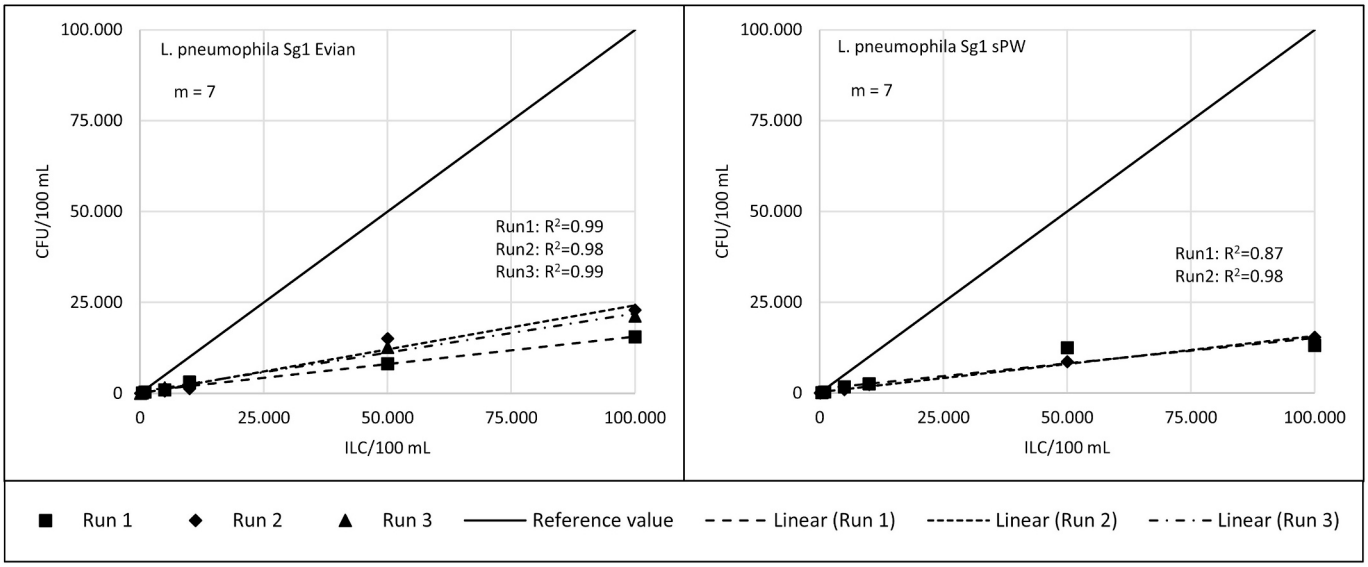

Fig. 4. Verification of linearity for the culture method using artificial samples based on Evian water or artificial process water (aPW) spiked with *L. pneumophila* Sg 1 cryopreserved standards over a range of seven concentrations (10^2, 5×10^2, 10^3, 5×10^3, 10^4, 5×10^4, 10^5 ILC/100 mL). For each concentration the weighted mean of evaluable plating (on GVPC agar) of a series of five (2×0.1 mL, 2×0.5 mL, membrane filtration of 20 mL) was used for linear regression. Results of the culture method (colony forming units, CFU) are plotted against spiked concentrations (m = 7) based on intact *Legionella* count (ILC). One additional run (Run3) was performed in Evian mineral water due to bigger deviations between the first two runs compared to aPW.

below the LOQ (<640 GU/100 mL) and 1 of 20 samples gave a positive result for viable *L. pneumophila* below the LOQ. Hygiena qPCR detected *L. pneumophila* in 14 of 25 samples, three of them contained concentrations above the LOQ (>180 GU/100 mL). Hygiena v-qPCR detected *L. pneumophila* in 9 of 25 samples, but none contained concentrations above the LOQ. All samples that gave a positive signal applying the Hygiena kit were also positive in the Bio-Rad qPCR except for one (Supplement Table 1). Viable *L. pneumophila* Sg 1 were detected in three samples using the multiplexed Hygiena kit, but below the LOQ.

Legionella spp. were detected in all but one sample by the corresponding Bio-Rad kit and all samples by the Hygiena multiplexed kit. Using v-qPCR 35 % and 48 % of samples showed concentrations of viable *Legionella* spp. above 10^4 GU/100 mL applying the Bio-Rad and Hygiena kit, respectively (Fig. 5). In contrast to spiked samples enzyme-based Bio-Rad v-qPCR did show a ratio of viable to total *Legionella* spp., which was comparable to the dye-based Hygiena v-qPCR (Supplement Table 1).

Applying the culture method *Legionella* spp. were detected only in three out of 25 samples in concentrations of 500 to 800 CFU/100 mL. However, colony counts were insufficient to obtain a reliable quantification (<10 CFU). *L. pneumophila* was not detected by culture.

Inhibition of qPCR and v-qPCR that did not resolve after a 1:10 dilution of DNA extract was observed for a portion of the samples, even though the starting samples did not show irregularities upon visual inspection. Both qPCR and v-qPCR did show inconsistencies for samples with suspected low concentrations of *Legionella* DNA, most likely at the border of the LOD, leading to one extraction or qPCR replicate being negative (Supplement Table 1).

3.4. Analysis of spiked environmental samples

Spiking with cryopreserved *L. pneumophila* Sg 1 to concentrations of 10^3 and 10^4 ILC/100 mL was performed for eight ECS samples. Reasons were low *L. pneumophila* concentrations in the environmental samples and to test the influence of highly complex matrices on the performance of cryopreserved standards. After spiking, qPCR could detect quantifiable concentrations of *L. pneumophila* and viable *L. pneumophila* in all samples. Although determined concentrations could differ up to threefold between samples (Fig. 6), for no sample the measured concentration was below the spiked concentration. Dilution steps spiked in the same sample matrix using identical cryopreserved *Legionella* standards showed good approximation to the reference value representing an exact ten-fold increase in concentration (Fig. 6). As also shown in robustness testing for enzyme-based Bio-Rad v-qPCR, the detection of the cryopreserved viable *Legionella* was drastically reduced with determined concentrations dropping below the LOQ for six out of eight spiked samples (Fig. 6).

4. Discussion

The current gold-standard for *Legionella* detection is culture. However, the method has its limitations which include failure to detect VBNC *Legionella*, inhibition by accompanying flora and long turnover time (Whiley and Taylor, 2016). Since prevention of infections is the main goal of ECS monitoring, underestimation of *Legionella* should be viewed critically as undetected *Legionella* can still cause infections (Dietersdorfer et al., 2018). This results in a need for complementing methods like v-qPCR. Prerequisite for future acceptance is a targeted verification of the whole method, including sample concentration by filtration and DNA extraction, which is considering the respective application and correct interpretation of the data.

4.1. Verification of qPCR and v-qPCR utilizing cryopreserved Legionella standards

For qPCR and v-qPCR a standardized approach for validation is imperative and exists to an extent in the form of the ISO/TS 12869 (ISO, 2019). However, this norm does not contain a guideline for the verification of v-qPCR, which attempts to limit detection to viable and therefore potentially infectious *Legionella* (Kirschner, 2016). In addition, validation is often based on DNA extracts, disregarding the impact of the whole method, including concentration, dilution and elution of the sample during filtrations steps. While extraction performance and robustness are assessed in different matrices following the ISO/TS 12869, the in the ISO predefined standards consist of freshly cultured *Legionella* making them difficult to reproduce and highly susceptible, especially if verification stretches over several days and/or is performed in different laboratories. Cryopreserved bacterial standards are an alternative and were also successfully applied for verification of immunomagnetic separation coupled with flow cytometry (Streich et al., 2024). Cryopreserved standards used for this study could be stored for several months showing only minor changes in composition and permitted a verification of extraction and robustness meeting the specifications given in the ISO/TS 12869. Both tested qPCRs had an excellent recovery for the whole method of 86–108 % compared to a direct extraction without filtration steps for the Evian matrix and also the more complex aPW, imitating the chemical composition of process water. In case of v-qPCR one of two kits was successfully verified using cryopreserved standards but had a lower recovery of 40 to 60 % compared to qPCR. It is probable that filtration steps prior to treatment for viability detection led to damaged cells explaining the decline in recovery compared to the similar direct extraction protocol without filtration steps. This could be an indication, that v-qPCR underreports viable cells if concentration of the sample is performed before treatment. For enzyme-based v-qPCR recovery was below −0.6 $\log_{10}$GU/100 mL and therefore not meeting the specifications of the ISO/TS 12869 (ISO, 2019). In comparison validation for AFNOR certification that used

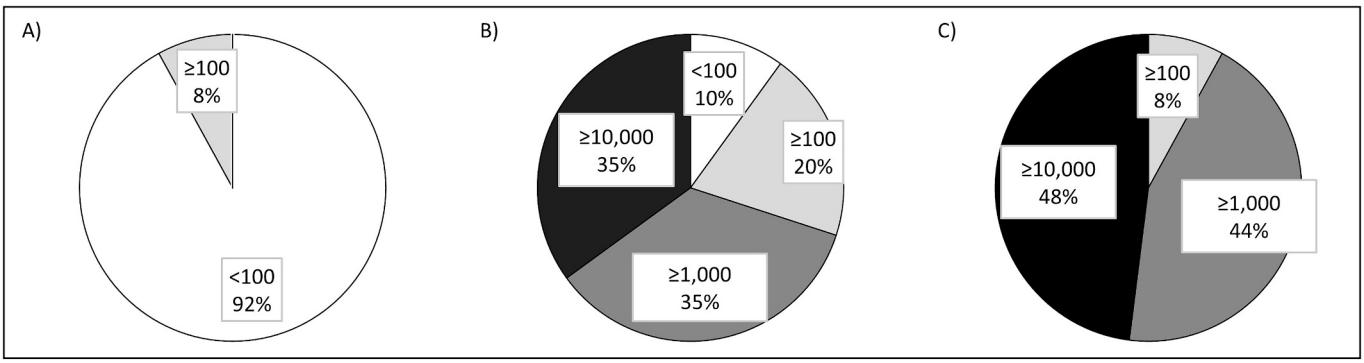

Fig. 5. Proportion of evaporative cooling tower sample results for A) culture method (n = 25), B) viability-qPCR Bio-Rad (n = 20) and C) viability-qPCR Hygiena (n = 25), that exceed *Legionella* concentrations of 10^2, 10^3 or 10^4 CFU/100 mL for culture or GU/100 mL in case of v-qPCR.

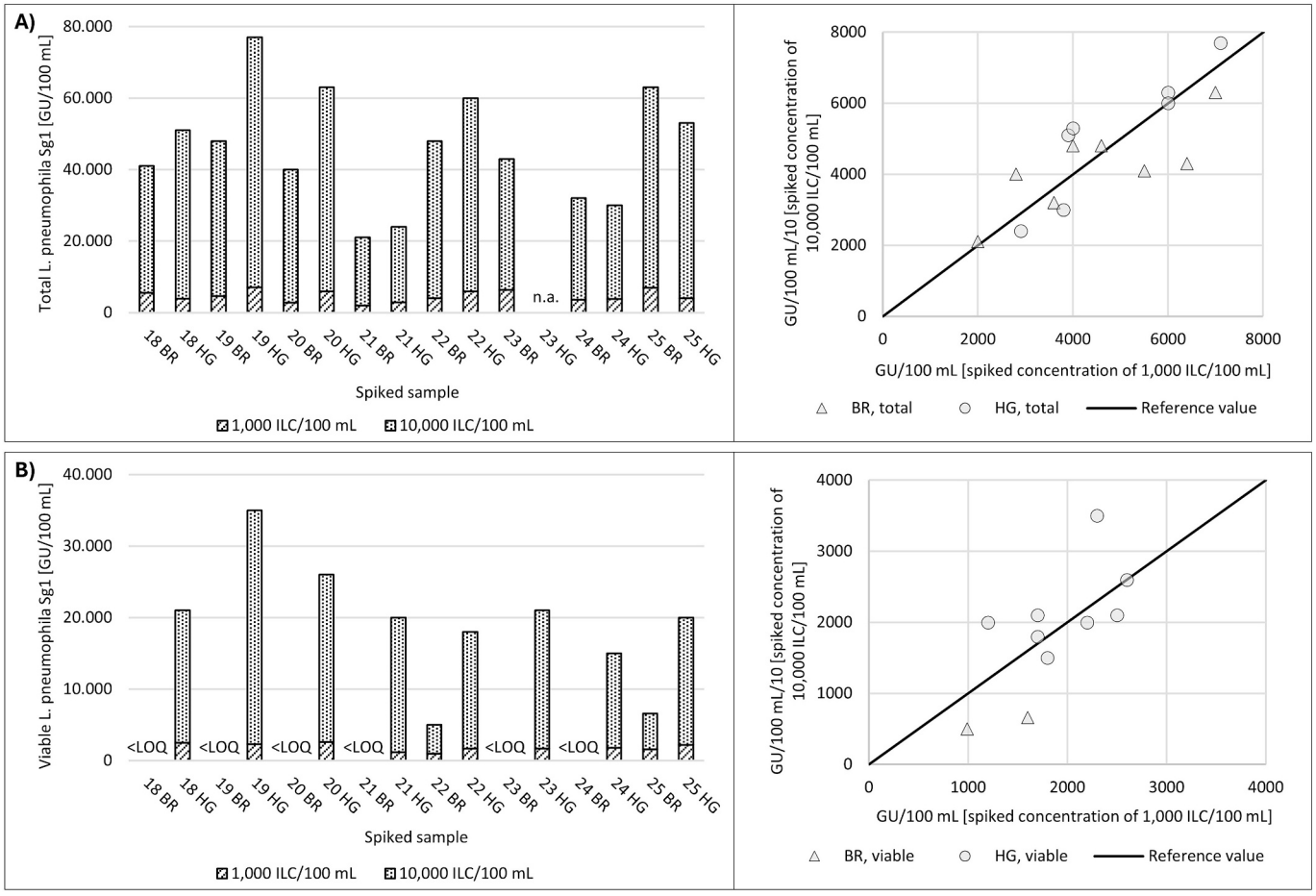

Fig. 6. qPCR (A) and viability-qPCR (B) results for environmental samples of evaporative cooling systems spiked with cryopreserved *L. pneumophila* Sg 1 to concentrations of 10^3 and 10^4 ILC/100 mL. Bar chart on the left is showing determined GU/100 mL for samples 18 to 25 for Bio-Rad (BR) and Hygiena (HG) kit. Results below Limit of quantification (<LOQ) as well as not analyzed (n.a.) samples are indicated. The scatter plot on the right shows the results for the samples spiked with 10^4 ILC/100 mL divided by the factor 10, plotted against the results of the same samples spiked to a concentration of 10^3 ILC/100 mL. The reference value is indicating the point where both spiked concentrations would meet if the difference in concentration was exactly 10-fold.

freshly cultured cells was successful (AFNOR, 2023), but comes with the disadvantages previously mentioned. Therefore, likely cause is the cryopreservation since environmental samples treated with an enzyme-based v-qPCR showed a higher percentage of viable *Legionella* spp. comparable to crosslinking dye-based v-qPCR. Spiked environmental samples on the other hand displayed again a great disparity between the two v-qPCR approaches.

Culture method was able to quantitatively detect *L. pneumophila* at concentrations of 100 ILC/100 mL demonstrating its high sensitivity under optimal conditions. Still, it detected only 21 to 29 % of spiked ILC/100 mL independent of the matrix. In comparison GU/100 mL determined by qCPR and v-qPCR were above or only slightly below spiked ILC or TLC/100 mL. CFU being below reported GU or cell counts determined by flow cytometry was also described in other studies citing several reasons including VBNC state (Díaz-Flores et al., 2015; Lizana et al., 2017). For examined artificial samples, the main difference should be attributable to VBNC *Legionella*, due to absence of inhibitors, Amoeba and accompanying flora, with cryopreservation-induced stress potentially increasing the proportion (Wolkers and Oldenhof, 2021). Linear regression analysis did attest good linearity for culture, qPCR and v-qPCR data with an R^2 that was $\geq$0.97 for a majority of runs demonstrating successful sample preparation using cryopreserved standards and good intra-assay precision. For Bio-Rad qPCR increasing *Legionella* concentrations led to a drop in recovery in aPW for all tested serogroups that might be missed following current norms. A drop in recovery was not detected during robustness testing that was adapted from ISO/TS 12869 for the aPW, highlighting the need for additional application-related verification.

Spiking of ECS samples was conducted in order to verify performance of qPCR and v-qPCR with cryopreserved *L. pneumophila* standards in highly complex matrices. Verification of qPCR and v-qPCR revealed adequate accuracy with traceable dilution steps for spiked concentrations of 10^3 and 10^4 *Legionella*/100 mL demonstrating reproducible extraction for common concentration ranges in routine monitoring (Van Kenhove et al., 2019). In addition, a drop below spiked levels was only observed for enzyme-based v-qPCR already observable in artificial samples. Despite reproducible dilution levels, the variation between matrices was in some cases 3-fold indicating possible influences of the matrix on the methods. These could be attributed to inhibiting compounds in the sample matrix (Díaz-Flores et al., 2015; Miyamoto et al., 1997) and in case of v-qPCR additional effects like turbidity (Fittipaldi et al., 2012; Taylor et al., 2014). Although no sign of inhibition was given by the included internal control for *L. pneumophila* detection. Another explanation could be differences within the cryopreserved standards. The tests were carried out on different days using newly thawed cryopreserved standards. Even though preparation was standardized, small deviations for example during thawing can cause a change in concentration, i.e. through residual water that damages the cell membrane (Pegg, 2015). A larger scale verification in different matrices on the same day with identical standards could provide more insight. Along with a more elaborate approach based on the addition method, as performed for biotrickling filters with cryopreserved

standards, where viable target *Legionella* are used not only to verify but calibrate the whole method in the target matrix (Schwaiger et al., 2024).

4.2. Analysis of environmental samples from evaporative cooling systems

Examination of environmental samples by v-qPCR revealed a high *Legionella* spp. burden over 10,000 GU/100 mL in 35–48 % of samples with ≥90 % of samples testing positive depending on the kit used. In contrast, only 12 % of samples tested positive for *Legionella* spp. by culture with colony counts ≤9 that did not allow reliable quantification. *Legionella* spp. have been found in numerous ECS (Llewellyn et al., 2017) and in high numbers when analyzed by qPCR in contrast to culture similar to this study (Collins et al., 2017; Lee et al., 2011; Lizana et al., 2017; Young et al., 2021). Comparable to previous studies, samples with higher concentrations of *Legionella* spp. in qPCR were also predictive of a positive culture result (Collins et al., 2017). The most frequently cited reasons for CFU being lower compared to v-qPCR results are VBNC state and inhibition because of accompanying flora (Whiley and Taylor, 2016). This was probably amplified for samples with shipping delays in this study, since the non-oxidative biocides used in sampled ECS cannot be inactivated and can lead to *Legionella* switching into the VBNC state (Díaz-Flores et al., 2015). Additionally, compared to *L. pneumophila* some *Legionella* spp. are hard to grow on the commonly used media, contributing further to the low detection rate (Lee et al., 1993). Also heat and acid treatment used to suppress the interfering accompanying flora have been shown to reduce colony counts by inducing VBNC status (Nisar et al., 2023). The burden of *L. pneumophila* was considerably lower with only one or nine samples testing positive below the LOQ by v-qPCR with the kits having a LOD of 640 or 180 GU/100 mL, respectively, and culture being negative for all samples. Interpretation of v-qPCR is difficult in particular in regard to the high concentrations for *Legionella* spp., since for example German legislation does not differentiate between *Legionella* species (42. BImSchV, 2017), even though there seems to be a bias towards *L. pneumophila* concerning LD cases (von Baum and Lück, 2011). To date, culture is the only legally valid method for routine monitoring according to many legislations and correlation with qPCR has not been demonstrated yet, although some conversion factors have been proposed ranging from four to five-fold and even 28-fold (Ditommaso et al., 2015b; Lee et al., 2011; Yaradou et al., 2007). Sample composition is a factor that can make v-qPCR results hard to evaluate and prevent a universal conversion factor. Currently v-qPCR performance is known to be influenced especially in environmental samples containing for example high organic matter or ferric ions (Fittipaldi et al., 2011; Fittipaldi et al., 2012). In general, a negative qPCR result is seen as a good negative predictive value (NPV) for culture (Collins et al., 2017; Guillemet et al., 2010; Lee et al., 2011; Toplitsch et al., 2021). In this study, this was the case for *L. pneumophila* as all samples that tested negative in qPCR and v-qPCR also tested negative using culture. Besides providing a high NPV other studies suggest using action and alert levels, which are specifically defined for qPCR and distinguish between *L. pneumophila* and *Legionella* spp. for advanced control of water systems (Collins et al., 2017; Lee et al., 2011). This is supported by the difference in determined concentrations between *Legionella* spp. and *L. pneumophila* of up to 5-fold in this study. Some sources suggest action and alert levels for *L. pneumophila* only (ANSES, 2011; PWGSC, 2016). With the addition of v-qPCR, result interpretation can moreover consider the proportion of viable cells if carried out in combination with standard qPCR. For this scenario Lizana et al. propose a 4-tier risk evaluation approach distinguishing between major threat to well managed at a cutting point of 10^3 GU/L in relation to the proportion of dead to viable cells (Lizana et al., 2017). Applying the model to this study, most ECS would fall either into the category for immediate action due to high levels of viable *Legionella* or into the category indicating a potential risk due to few but mostly viable cells. In contrast to this, an approach differentiating additionally between *L. pneumophila* and *Legionella* spp. as proposed by Nocker et al. could certify the sampled ECS as well maintained due to low concentrations of *L. pneumophila* and *Legionella* spp. $<10^6$ GU/100 mL (Nocker et al., 2020). Overall, with the low positive results for *L. pneumophila* applying culture and v-qPCR, the sampled ECS appear to be in good condition, which could be attributed to a successful implementation of the German 42nd Federal Emission Control Act (42. BImSchV, 2017). Nevertheless, the high results for *Legionella* spp. show a need for regular monitoring and the results should be considered when performing a qualitative risk assessment.

5. Conclusions

In conclusion, cryopreserved standard based verification of the full bioanalytical method, i.e. including filtration and DNA extraction did attest a performance of qPCR sufficient to meet current ISO/TS 12869 specifications. The procedure was also feasible for v-qPCR for which no official guideline exists to date. Nevertheless, for v-qPCR care must be taken in the choice of standards, as not every v-qPCR method seems compatible with cryopreserved standards. Overall, this approach could be a step towards data that is more comparable due to it not being centered on freshly cultured cells enabling validations extending over a longer time period or inter-laboratory trials. Analysis of environmental samples from ECS did show expected differences between culture and v-qPCR with the latter detecting high concentrations of viable *Legionella* spp. in most samples. Especially for v-qPCR data, the correct interpretation of the data is a crucial aspect for future application in routine monitoring to complement culture. A differentiation between *L. pneumophila* and *Legionella* spp. as well as simplified evaluation approaches for result interpretation as proposed in other studies (Lizana et al., 2017; Nocker et al., 2020) may be needed. This is particularly important for selection of adequate measures regarding the determined concentrations and responsible *Legionella* species to avoid costly and unnecessary action (Krøjgaard et al., 2011). This way, the benefits of a fast turnover time and detection of *Legionella* otherwise missed by culture, for example VBNC and growth inhibited *Legionella*, could help to reduce the risk of outbreaks while also avoiding unnecessary measures and associated costs. Moreover, parallel and regular application of new methods alongside culture in routine monitoring could not only help to better assess the status of ECS and comparable systems but also set differences between the results into context, since every system has its own dynamic. For this, however, the incentive to use culture-independent methods has to be promoted.

CRediT authorship contribution statement

J. Redwitz: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **P. Streich:** Writing – review & editing, Resources, Methodology. **M. Zamfir:** Writing – review & editing, Methodology. **S.M. Walser-Reichenbach:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization, Methodology. **M. Seidel:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **C.E.W. Herr:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **S. Heinze:** Writing – review & editing, Supervision. **C. Quartucci:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.176011.

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