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Chip-based duplex real-time PCR for water quality monitoring concerning *Legionella pneumophila* and *Legionella* spp.

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Keywords
bacteriological; drinking water; risk assessment; water quality.

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Abstract

Based on biomolecular methods, rapid and selective identification of human pathogenic water organisms becomes an important issue. *Legionella* spp., are pathogenic water bacteria with worldwide significance. Prevalent detection methods for these microorganisms are time and/or cost intensive. We describe a detection setup and relating DNA assay. A miniaturized real-time polymerase chain reaction (real-time PCR) for direct on-line discrimination of *Legionella pneumophila* and *Legionella* spp. was established and integrated into a real-time PCR-chip-system. The PCR-chip device combines a temperature controlling unit and a fluorescence intensity measurement. It was designed to achieve rapid amplification, using an approach of real-time fluorescence read out with the intercalating dye EvaGreen® and melting curve analysis, without requiring multiple probes. The presented results exhibit reproducibility and good sensitivity, showing that the setup is suitable for robust, rapid and cost-efficient detection and monitoring of a variety of *Legionella* spp.in urban water samples.

Introduction

Legionella bacteria constitute a public health concern in water systems. Inhalation or aspirations of contaminated aerosols represent the usual process of infection. The *Legionellae* may so enter the human respiratory tract and cause Legionnaires' disease or Pontiac fever. Outbreaks of both diseases are frequent. More than 61 *Legionella* species (*Legionella* spp.) have been identified (http://www.bacterio.net/legionella.html, 2019). Nearly one-half of *Legionella* spp. have been associated with human diseases (Fields *et al*., 2002). Recent reports have shown that the majority of human infections (more than 90%) have been caused by *Legionella pneumophila*, especially serogroups 1 and 6 (Riffard *et al*., 1998; Yu *et al*., 2002). In order to prevent infections, a rapid diagnostic assay is required that detects and monitors the presence of *Legionella pneumophila* and other *Legionella* species, and simultaneously differentiates from other waterborne pathogens in hot and cold water systems.

Bacterial culture remains the gold standard for the diagnosis of *Legionella* based upon the ISO 11731 standard, but it is time-consuming, requires relatively complex culture media and takes too long (several days) to obtain definitive and reliable results. There is a need for more rapid and reliable detection strategies. Several methods for an improved detection of waterborne pathogens have been proposed, especially for *L. pneumophila*. It was found that PCR-based methods are not only faster, but also have a higher rate of detection than culture methods, because of the presence of viable but noncultivable *Legionella* spp. (Whiley and Taylor, 2014). Identification methods which are already investigated are microarray setups. DNA-Microarrays for *Legionella* have been successfully used to determine *Legionella pneumophila* (Zhou *et al*., 2011; Ranjbar *et al*., 2017). However, the sensitivity was low and multiple steps are involved in this technology. Alternatively, numerous real-time PCR (Brandão *et al*., 2015) and digital droplet PCR (Baume *et al*., 2018) methods that avoid the need for post-PCR analysis have been established. Several assays use fluorescently labelled hybridization (Reischl *et al*., 2002; Wilson *et al*., 2003; Stolhaug and Bergh, 2006; Yang *et al*., 2010; Merault *et al*., 2011; Benitez and Winchell, 2013; Gruas *et al*., 2014; Collins *et al*., 2017). Newer studies investigate the more recent quantitative PCR (qPCR) method used in laboratories as standard method as described in ISO 12869:2012 (Toplitsch *et al*., 2018). However, these methods require a precise probe design, expensive FRET probes and, in addition, extensive optical equipment. To overcome those problems, there is the possibility using rather simple dsDNA-intercalating dyes and miniaturization. Miniaturized Lab-on-chip systems for other

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bacteria already exist. The concept of Lab-on-a-chip technologies is under intense research. Platforms with integrated solid phase PCR (Hung *et al*., 2017), or integrated isolation of pathogens (Sandetskaya *et al*., 2017), and highly sensitive PCR-chips have been developed using flow-through PCR-systems. Thereby the PCR fluid is guided through microchannels that are integrated into a chip with different temperature profiles (Schneegass *et al*., 2001; Reichert *et al*., 2008; Markey *et al*., 2010; Ahrberg *et al*., 2016; Liu *et al*., 2018). Other miniaturized technologies work in a stationary regime: As film-based PCR chip (Bae *et al*., 2018), or the reaction mixture is put as droplet onto the chip surface (Guttenberg *et al*., 2005) before heated to realize the needed temperature regime.

In this report, we describe the development of a beneficial real-time chip-PCR device for the simultaneous detection, identification and discrimination of *Legionella pneumophila* from *Legionella* spp. and other waterborne pathogens in a single droplet, using a dsDNA-intercalating dye and melting curve analysis. Compared to standard approaches, the presented detection setup offers time and cost savings (small volumes), can be implemented on a common fluorescence microscope and is also suitable as a portable device for point-of-care (POC) analysis.

Assay concept

Two primer sets were used in the PCR reaction mixtures to amplify more than one target sequence of the template DNA in parallel (Figure 1). Such a duplex PCR has the potential for considerable savings regarding time as well as reagents.

Materials and methods

Bacterial strains

Eleven *Legionella* strains and five non-*Legionella* strains (waterborne pathogens) were obtained from BioSolutions Halle GmbH (Halle, Germany) (Table 1). Isolation of total bacterial DNA was performed using the innuPREP DNA Micro Kit (Analytik Jena, Jena, Germany). For cell lysis overnight cultures (GVPC medium at 37°C) were centrifuged for 5 min at 16,000 ×*g*, the respective cell pellets were resuspended in 200 µl lysis buffer and 20 µl Proteinase K, and incubated for 15 min at 70°C and 1400 rpm in a thermoshaker (Eppendorf Thermomixer compact, Hamburg, Germany) followed by an incubation step at 95°C for 10 min and 1.400 rpm. Afterwards the cell lysates were mixed with binding buffer and applied on a silica column and washed following the manufacturers protocol. Total bacterial DNA was eluted with 50 µl elution buffer and stored at −20°C until further use.

PCR assay

PCR primer sequences targeting the 16S rDNA of *Legionella pneumophila* and of the complete genus *Legionella* were designed (Table 2). The primers were initially tested and optimized in singleplex format. Their final concentrations for duplex PCR were determined. A 3.0 µl real-time PCR reaction mixture was prepared using 0.3 µM of each forward primer and 0.3 µM of each reverse primer (biomers.net, Ulm, Germany), 0.1 U/µl Taq DNA polymerase (Genaxxon, Ulm, Germany), 0.2× SYBR® Green (Molecular Probes, Eugene, OR) or 1 × EvaGreen® (Biotium, Hayward,

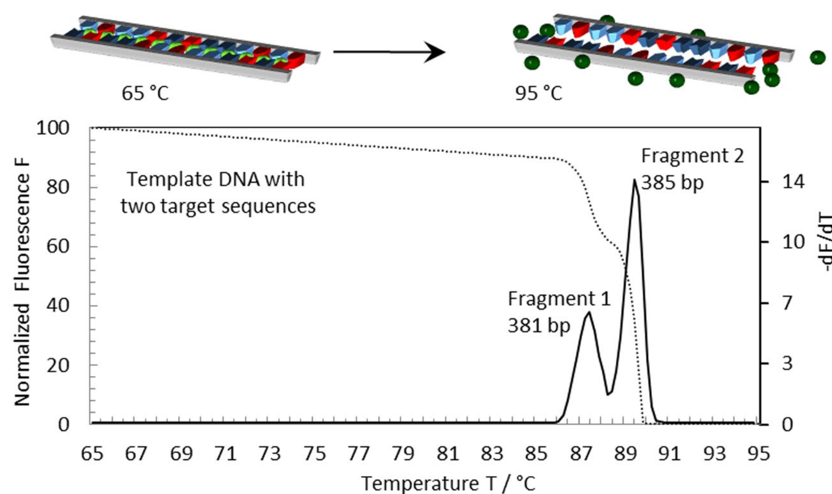

Fig. 1. General scheme of the duplex real-time PCR amplicons detection by melting curve analysis (MCA), showing the DNA amplification products of the two different fragments and the resulting melting curve with specific peaks for each fragment using intercalating dyes (spots in the scheme).

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Table 1 Bacterial strains

Name	Species and serogroups (SG)	ATCC strains no.
L. pneu_neu	*L. pneumophila*	ATCC 33152
L. dum	*L. dumoffi*	ATCC 33279
L. feel	*L. feeleii*	ATCC 35072
L. boz	*L. bozemanii*	ATCC 33217
L. mic	*L. micdadei*	ATCC 33218
L. pari	*L. parisiensis*	ATCC 35299
SG2	*L. pneumophila* SG2	ATCC 33154
SG6	*L. pneumophila* SG6	ATCC 33215
SG8	*L. pneumophila* SG8	ATCC 35096
SG9	*L. pneumophila* SG9	ATCC 35298
SG15	*L. pneumophila* SG15	ATCC 32251
E. fas	*Enterococcus faecalis*	ATCC 19433
E. fam	*Enterococcus faecium*	ATCC 19434
E. coli	*Escherichia coli*	ATCC 11775
E. clo	*Enterobacter cloacae*	ATCC 13047
P. aer	*Pseudomonas aeruginosa*	ATCC 10145

Note: Legionella strains and other waterborne human pathogens used in this study.

Table 2 Primers and amplicons

Primer	Sequence (5′-3′)	T_m (°C)	Amplicon (bp)	Species
Lspp1_f	gccttcgggaacactgatac	59.4	262	L. pneu
Lspp1_r	taaggatttgctccaggtcgc	59.8		
Lspp2_f	gatcggaaggaacaccag	59.4	297	L. spp
Lspp2_r	tgtatgtcaagggtaggtaagg	58.4		
Lspp3_f	aacctgggacggtcagat	56.0	381	L. pneu
Lspp2_r	tgtatgtcaagggtaggtaagg	58.4		
Lspp1_f	gccttcgggaacactgatac	59.4	385	L spp
Lspp4_r	gtgacgggcggtgtgtac	66.7		

Note: Primer pairs (f = forward, r = reverse) and their specific *Legionella pneumophila* and *Legionella* spp. 16S rDNA amplicons.

CA), 0.2 mM dNTPs mix (Genaxxon, Ulm, Germany), 2.0 mM $MgCl_2$ (Genaxxon, Ulm, Germany), 1× Puffer S (Genaxxon, Ulm, Germany) and nuclease-free water (DEPC H_2O) (Carl Roth, Karlsruhe, Germany). A volume of 0.3 µl (1.0 ng/µl final concentration) total genomic DNA was added. Different *Legionella* species and other waterborne human pathogens were chosen applying the PCR-chip system, listed below (Table 1). The negative template control (NTC) contained 0.3 µl DEPC H_2O instead of DNA.

The reactions were carried out with the real-time PCR-chip system implemented on an inverted fluorescence microscope. Furthermore the obtained results were compared and confirmed with the conventional Rotor-Gene™ 6000 real-time instrument (Qiagen, Hilden, Germany), where data acquisition was carried out on the green channel (excitation at 470 nm, detection at 510 nm). The final thermal profile includes 30 s at 94°C for DNA denaturation, 30 s at 57°C for primer annealing and 30 s at 72°C for DNA amplification using Taq DNA polymerase. This three step reaction is repeated 40× with an initial DNA denaturation step of 180 s and a final elongation step of 180 s. No template controls (NTCs), were included in all batches of PCR. To prevent evaporation of the 3.0 µl PCR solution in the PCR-chip-system it was covered with 10.0 µl mineral oil (Sigma-Aldrich, St. Louis, MO).

Data analysis

Considering the sequence information of 16S rDNA genes of different *Legionella* species, we selected special fragment combinations in duplex format, which were well suited with respect to melting curve analysis (MCA) including the discrimination between *Legionella pneumophila*, other *Legionella* species and some other important waterborne pathogens. These observations can be attributed to differences in primer binding and fragment structure.

The estimated melting temperature of the different fragments was calculated by the thermodynamic standard term, for determining an appropriate temperature differences in melting points (Howley *et al*., 1979; Rychlik *et al*., 1990). This term is only an approximation and was compared to the actual melting points. The PCR solution was heated up constantly from 65.0°C to 95.0°C for MCA, with a resolution of 0.2 K per 2 s. Double stranded DNA intercalating dyes show a sudden decrease of the fluorescence intensity that indicates the specific melting temperature of the amplified DNA fragment (Fig. 1, black curve). The fluorescence signal was monitored at each temperature step and the melting points were determined by plotting the first negative derivative of the fluorescence over the temperature (–dF/dT) (Fig. 1, blue curve).

To ensure the reproducibility and obtain accurate values all the melting points for the same protocol on the PCR-chip and the same stock solutions for both assays with respect to the conventional device were collected.

Gel electrophoresis

Additionally, 1.0 µl of the PCR products were analyzed with agarose gel electrophoresis (2% agarose GTQ) stained with 1× GelRed™ (Biotium, Hayward, CA), using standard methods (Russel, 2001) and showing the banding pattern obtained with DNA amplification products. Documentation took place under a UV-transilluminator (Herolab GmbH Laborgeräte, Wiesloch, Germany).

Setup

A real-time PCR-chip-system implemented on an inverted fluorescence microscope was used (Fig. 2a). The setup consists of an excitation laser (at 488 nm, Omicron Laserage,

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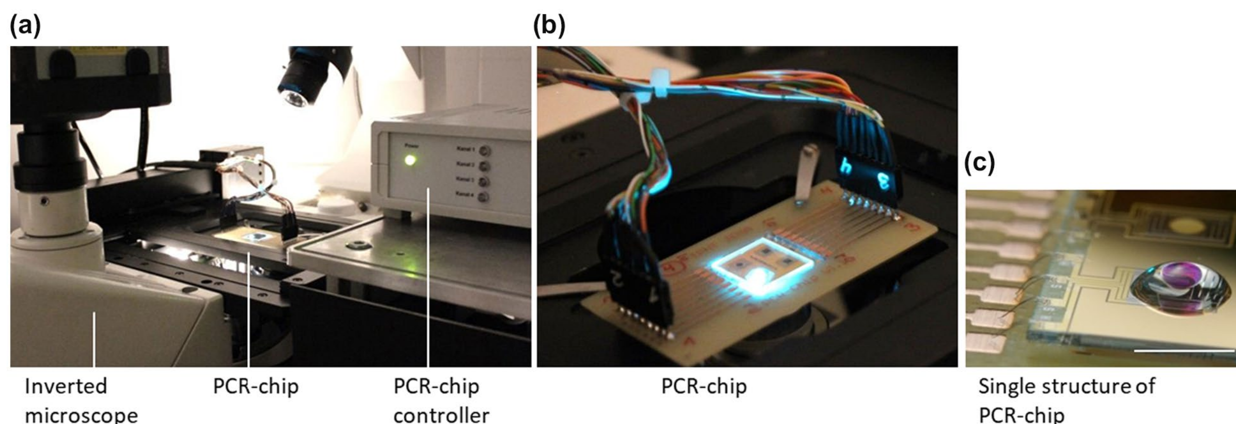

Fig. 2. Real-time detection setup. Fluorescence microscope setup (a), with integrated PCR-chip-device with four assay-windows (b). The PCR solution (purple coloured) is covered with transparent mineral oil to prevent evaporation, scale bar: 4 mm (c).

Rodgau-Dudenhofen, Germany) and an optical microscope providing a very high local resolution (Zeiss Axiovert 200M MAT, Jena, Germany), and the flexibility to work with a variety of fluorescence dyes during establishment. The signal of the emitted fluorescence was detected with an Avalanche Photodiode (APD) photo-receiver (Hamamatsu Photonics Deutschland GmbH, Herrsching am Ammersee, Germany). Furthermore, the setup includes fluorescence filters (FITC), a PCR-chip-controller (Leibniz-IPHT, Jena, Germany), an analogue-to-digital converter, an amplifier (Leibniz-IPHT, Jena Germany) and a separate netbook (ASUS Eee PC) for control and implementation of amplification protocols. The stationary PCR-chip-cycler, with a size of 20 × 20 mm, consists of specific micro structured thin platinum layers serving as heating and sensing structures, with heating- and cooling rates of up to 50 K/s and 5 K/s (Guttenberg *et al*., 2005). As a result of small volumes and the relatively large surface, a passive cooling system was used as a thermal control (Fritzsche *et al*., 2012).

Four controllers with a transparent window in the centre of each structure allow for a real-time detection of four different PCR assays in parallel including subsequent melting curve analysis. Until now, amplification curve can be acquired for only one assay. Nonetheless, by switching the objective to the next detection window (Fig. 2b) for the other assays, melting curve data are detectable. PCR reactions were performed onto a disposable, coated glass slide with hydrophobic ring structures. This glass surface was hydrophobized by precleaning steps, plasma etching (200-G Plasma System, Heidolph Instruments GmbH & Co KG, Schwabach, Germany) and silanization with a Teflon-AF-solution. The ring structure serves as boundary for a volume of 0.5 to 3 µl PCR reaction mixture, which is covered by 10 µl mineral oil (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), to prevent evaporation during heating/denaturation steps (Fig. 2c).

The PCR-chip can be implemented on several inverted microscopes. After set-up and optimization, the microscope could be replaced by dedicated fluorescence detection. We already developed a setup for the described PCR-chip in order to miniaturize the system and for further functional integration for practical use (Seise *et al*., 2011; Singh *et al*., 2017).

Results and discussion

Conventional real-time PCR

The PCR assay was initially established and optimized in a conventional real-time cycler Rotor-Gene^{TM}6000. The specificity of the real-time PCR assay was confirmed by MCA, yielding a characteristic melting temperature (T_m). In comparison, melting points determined by the melting curve analysis of *Legionella pneumophila* give a well-defined double peak pointing to the presence of two products, compared to those of *Legionella* spp. with a single peak/product (Fig. 3) and other relevant waterborne pathogens where no peak appeared (data not shown).

The intercalating dye SYBR® Green has gained prominence as the most widely used in real-time PCR applications. However, it suffers from several disadvantages, like PCR inhibition that prohibits the use of saturation dye concentration for maximal signal of PCR products, and by the need for implementation of a melting curve analysis because of dye redistribution (Eischeid, 2011). EvaGreen® dye was determined as more robust and performed better than SYBR® Green in general, and high reaction efficiencies could be achieved. The use of EvaGreen® instead of the

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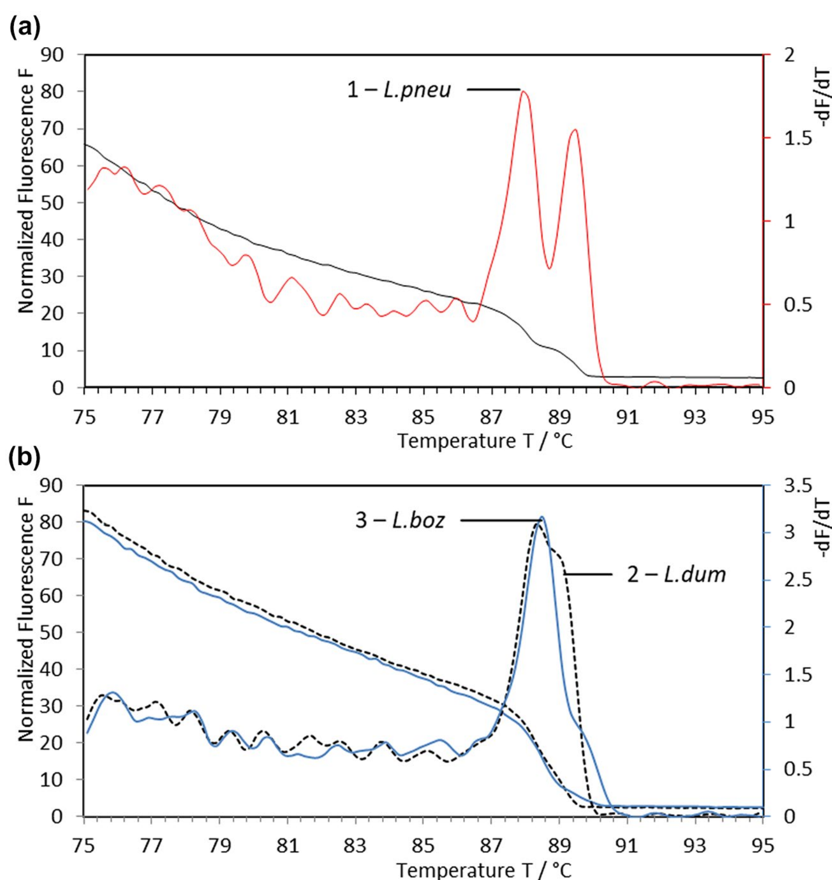

Fig. 3. Optimization of the duplex real-time PCR assay. MCA on a conventional device of the 262 bp and the 297 bp fragment of *L. pneumophila* T_m = 87.9°C and 89.5°C (A, 1), and some L*egionella* spp. such as *L. dumoffi* T_m = 88.3°C (B, 2) and *L. bozemanii* T_m = 88.5°C (B, 3). The peak difference is used for discrimination.

conventionally used SYBR® Green dye required minimal optimization. In the presented duplex real-time PCR, the double peak in MCA could be resolved, that indicates the presence of *Legionella pneumophila*.

The duplex reaction mix targeting fragments with 381 bp and 385 bp (Fig. 3) were first investigated; the primer pair Lspp3_f and Lspp2_r is specific for the template sequence of *L. pneumophila,* and the primer pair Lspp1_f and Lspp4_r specific for *Legionella* spp. This results in specific product amplification and peak formation as mentioned above. The T_m of both fragments is affected by a number of factors and the characteristics melting points of each fragment have a defined mean distance of 1.8°C.

Sensitivity

To determine the analytical sensitivity of the real-time PCR assay and lowest amplifiable concentration of DNA template, total genomic DNA of *L. pneumophila* SG6 was used at concentrations ranging from 0.02 pg/µl to 5.0 ng/µl. Fluorescence intensity curves and MCA were plotted. The detection limit for the developed assay was 2.0 pg/µl of total genomic DNA.

The standard curve for 0.0002 ng/µl to 5.0 ng/µl is shown in Fig. 4, and confirmed by agarose gel electrophoresis of the PCR amplicons of 381 bp and 385 bp fragments. Above 20.0 pg/µl total genomic DNA, an identification of *L. pneumophila* is reproducible and specific.

The number of copies of the *Legionella* genome in the initial DNA solution was calculated to an acceptable approximation by assuming a molecular mass of 660 Da for 1 bp of dsDNA and using the following equation: number of copies = quantity of DNA (fg)/mean mass of the total *L. pneumophila* genome. The mean mass of the total *L. pneumophila* genome was calculated to be 3.7 fg from the assumed mean size of 3.4 Mb of the genome. The value determined would be about 500 molecules for a quantity of 2.0 pg/µl of initial total DNA concentration. Quantitative real-time PCR gives the number of genome units (GU) per liter, but equivalence with the number of colony forming units (CFU) has not been established (Wellinghausen *et al*., 2001; Joly *et al*., 2006)

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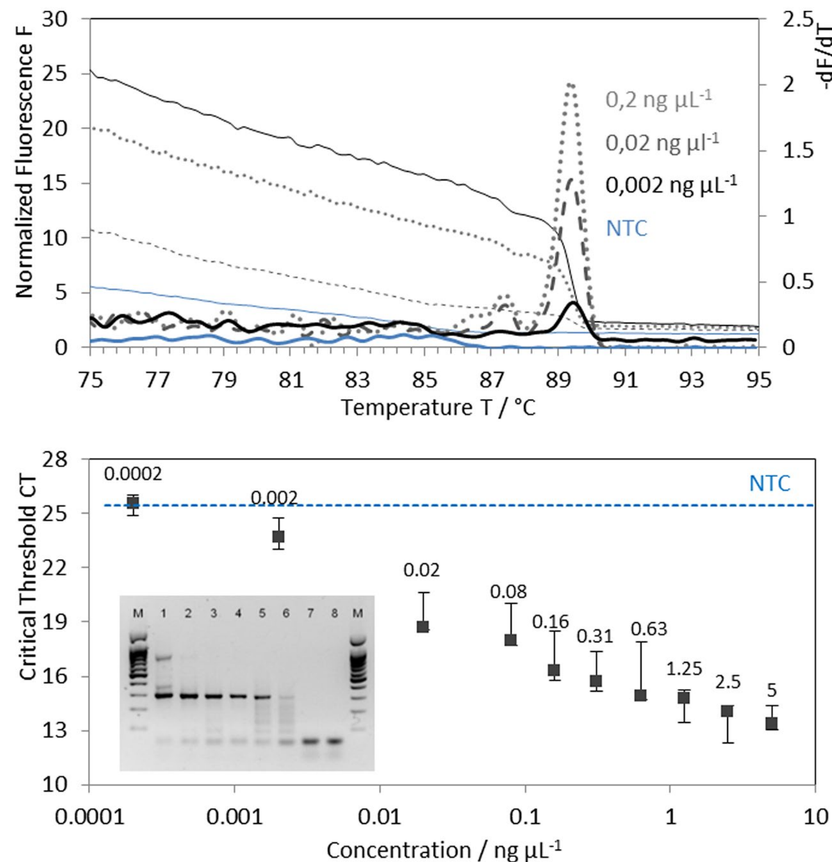

Fig. 4. Sensitivity of the real-time PCR assay. Limit of detection of the developed real-time PCR for the 381 bp and 385 bp fragment-pair, 16S rDNA fragment of total genomic DNA amplified with conventional thermocycler, Inset: Agarose gel electrophoretic analysis of PCR amplicons. M = 100 bp DNA ladder, 1 = 5.00 ng/µl, 2 = 1.25 ng/µl, 3 = 0.31 ng/µl, 4 = 0.08 ng µl, 5 = 0.02 ng/µl, 6 = 0.002 ng/µl, 7 = 0.0002 ng/µl, 8 = NTC (no template control)

(Whiley and Taylor, 2014; Toplitsch *et al*., 2018). As orientation an action level of 1000 CFU/L water has found wide acceptance as the maximally tolerable concentration of *Legionella* in hot and cold water systems (ISO_ 11731:2017; Lee, 2018), and quantitative PCR (qPCR) proposed action levels were 1000 GU/L (Lee *et al*., 2011; Diaz-Flores *et al*., 2015). Nevertheless, more *Legionella* bacteria are detectable with PCR assays than with culture methods (viable but noncultivable *Legionella*, VBNC) (Slimani *et al*., 2012).

Real-time PCR in PCR-chip-system

The initial evaluation of the real-time duplex PCR assay was performed in a conventional thermo-cycler (tube). Nonetheless, in on-chip (droplet) experiments the results were disappointing in the beginning when using exact the same PCR mixture. No PCR amplification curve and melting curve could be reproduced, apparently an optimization was required. For optimization, we tested different additives, such as BSA (bovine serum albumin) (Genaxxon, Ulm, Germany), Q-Solution (Qiagen, Hilden, Germany) and dimethyl sulphoxide (DMSO). BSA was not necessary for the conventional assay and had no significant influence on the PCR performance, but our results show that for the PCR-chip-device it was crucial for surface passivation (Fig. 5). On the chip, only reactions carried out with addition of 1.0 µM BSA performed successfully. Q-Solution is not optimal for the desired real-time purpose, especially for melting curve analysis, because it changes the melting behaviour of the dsDNA.

Comparison of PCR-chip and conventional thermocycler

The discrimination of *Legionella pneumophila* from other *Legionella* species was obtained by specific melting peaks, following the real-time PCR amplification. Only the *Legionella* species were amplified successfully. Furthermore, a positive PCR result could be interpreted with MCA. We could show that the results at a miniaturized scale with 3.0 µl PCR solution are comparable to

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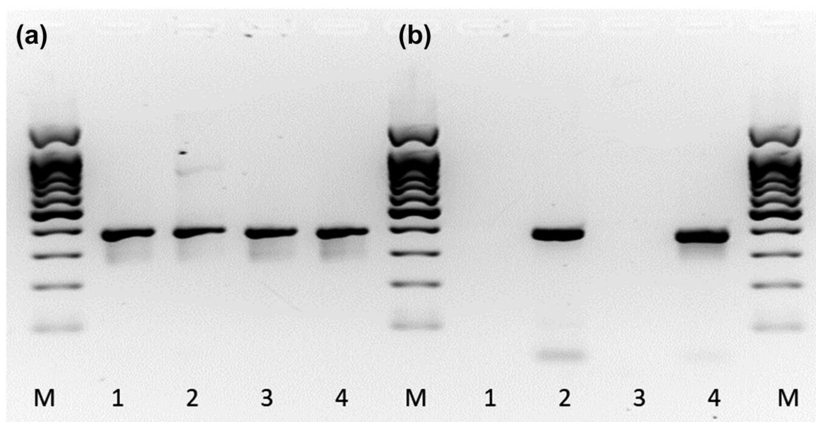

Fig. 5. Comparison of conventional thermocycler and PCR-chip. Agarose gel electrophoresis of real-time PCR amplicons, showing the banding pattern obtained with DNA amplification products for *L. pneumophila* SG6. 16S rDNA fragment amplified with conventional thermocycler (a) and a PCR-chip-cycler (b), respectively. M = 100 bp DNA ladder, 1 = without additive, 2 = BSA, 3 = Q-Solution, 4 = BSA and Q-Solution

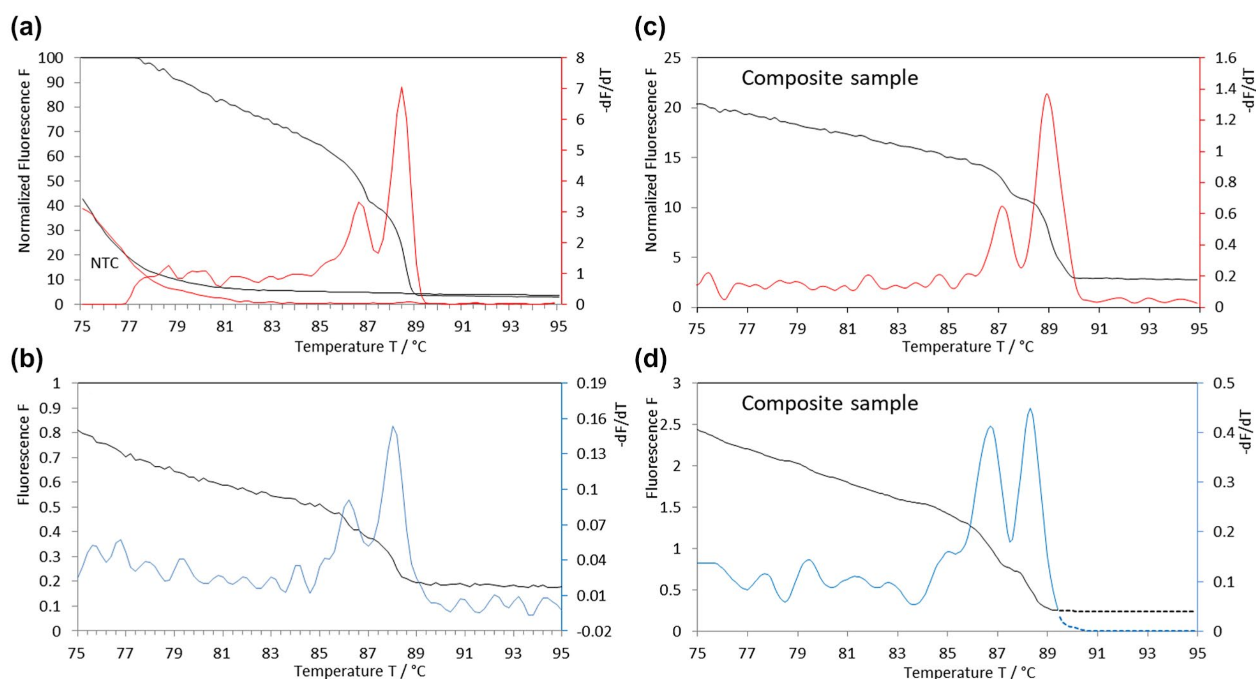

Fig. 6. Comparison of the conventional device to the PCR-chip. Selectivity. MCA, conventional device (a, 10.0 µl tube) in comparison to the PCR-chip (b, 3.0 µl droplet with 10.0 µl mineral oil), *L. pneumophila*: conventional: T_m = 86.7°C and 88.5°C, PCR-Chip: T_m = 86.2°C and 88.0°C. Composite samples. MCA, conventional device (c, 5.0 µl tube with 10.0 µl mineral oil) in comparison to the PCR chip (D, 3.0 µl droplet with 10.0 µl mineral oil), composite sample *L. pneumophila/L. dumoffi:* conventional: T_m = 87.1°C and 88.9°C, PCR-chip: T_m = 86.7°C and 88.3°C (T offset of chip device subtracted)

the conventional one, with 10.0 µl reaction volumes (Fig. 6a,b). Lower volumes reduce costs. Further significant advantages of this chip-setup are higher heating (15 K/s) and cooling (5 K/s) rates, resulting in a significant shortening of the PCR duration by a higher efficiency: $t_{PCRconventionally}$ > $t_{PCRchip}$: 132 min> 85 min – pure melting curve analysis time: 7.5 min in the PCR-chip-system – 28% related to the conventionally cycler.

The developed real-time chip-PCR assay was evaluated using composite samples, including all listed *Legionella*

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species, *Legionella pneumophila* and other waterborne pathogens (Fig. 6c,d). The experiments confirm the assay results, no secondary cross reactions with primers and templates were observed. All *Legionella* samples were amplified and detected by MCA. *Legionella pneumophila*-specific amplicons could be easily distinguished by their characteristic melting temperature (T_m). The specificity of the 16S rDNA assay was confirmed by producing a specific melting point (mean melting temperature value T_m) of 87.0 ± 0.5°C and 89.0 ± 0.5°C conventional, and 86.6 ± 0.5°C and 88.1 ± 0.5°C for the PCR-chip-cycler that corresponds to the detection of a 381 bp and 385 bp fragment with agarose gel electrophoresis.

All nontarget strains were not amplified and non-*pneumophilia* stains induced a single peak. For the *Legionella pneumophila* strain on the PCR-chip-cycler, a clearly resolved double peak was detected with a lower detection limit of 20.0 pg/µl.

The suggested device and duplex assay allows fast detection of the pathogens *Legionella pneumophila* and *Legionella* spp. for a minimal sample volume and cost-efficiency regarding consumables. For that reason, we plan to transfer the developed assay into a reverse transcriptase PCR protocol in order to detect viable *Legionella* and waterborne pathogens. This reaction mixture is leading towards further steps in increasing the amount of primer pairs for multiplexing if needed.

Besides multiplexing (addressing different target sequences in one reaction), also parallelization (how many reactions can be simultaneously detected) is an important aspect for application. In the case of the described prototype chip, four different assay windows are accessible, allowing for four reactions in parallel. Larger arrays with higher numbers of windows are possible, requiring respective technologies for positioning the target solution droplets (e.g. multichannel pipettes or even microarray spotters) as well as parallel optical readout (e.g. fibre or parallel LED-based).

Conclusions

(1) The presented PCR-chip system offers a rapid analysis of urban water samples and has been demonstrated to allow a reliable preliminary risk assessment of urban water samples regarding the detection and quantification of *Legionella*. It is capable of a simultaneous detection and differentiation of *Legionella* spp. and *Legionella pneumophila* with low requirements for material, time and initial sample volume. Fluorescence-based real-time detection as well as melting curve analysis can be implemented on chip avoiding cross contamination and the requirement for any post-PCR analysis. Intercalating dyes were chosen in this study for their simplicity and cost-efficiency; certainly probe-based assays are also possible and would be considered when, for example, needed during the adaptation to real matrix conditions. Complete analysis at miniaturized scale can be reduced to 2-5 h (versus 7-10 days) and is easier to interpret than culture. Moreover, the presence of noncultivable *Legionella* spp. (VBNC) can be detected.

(2) The sensitivity matches to the requirements as given by the European Guidelines where the action level lies at 1000 CFU/L (ISO_, 11731:2017; Lee, 2018). Quantitative PCR (qPCR) proposed action levels of 1000 GU/L (Lee *et al*., 2011; Diaz-Flores *et al*., 2015) and in France an action target value of 5000 GU/L is recommended (French Agence nationale de sécurité sanitaire de l'alimentation, 2011). Nevertheless, the distinction between live and dead cells was not taken into account (Omiccioli *et al*., 2015) and conversion equations from GU to CFU for Legionella are not yet established (Whiley and Taylor, 2014; Toplitsch *et al*., 2018). Other studies revealed a higher proportion of *Legionella* positive samples by qPCR (Collins *et al*., 2015) compared to culture methods.

(3) On-chip real-time PCR using an intercalating dye is particularly suited for quantitative analysis of *Legionella* DNA because fluorescence intensity is directly related to the amount of DNA contained in the PCR mixture. The number of sequences that can be analysed in parallel is restricted. The assay enables to expand the diagnostic possibility to detect species other than *L. pneumophila*. For future investigations the presence of vital bacteria can be tested using this system in combination with propidium monoazide (Yanez *et al*., 2011). The determination of the melting points was very stable run-to-run wise, but weak long-term variations require normalizations to a sample standard.

(4) As a result of the aspects of a rapid, sensitive and specific detection the PCR-chip system offers a great potential to be further developed (including the adaptation of the assay onto real matrix conditions) into an on-site enabled point-of-care diagnostic, to provide early and accurate information related to the presence of pathogens in urban water samples.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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