**Incorporation of a screening system for amoebal hosts into a routine screening programme for legionellae in cooling towers**

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**Abstract**

Free-living amoebae (FLA) are widely spread in the environment and known to cause rare but often serious infections. Besides this, FLA may serve as vehicles of dispersal for bacterial pathogens. In particular, *Legionella pneumophila* is known to replicate within FLA thereby triggering its infectivity and resistance against biocides. Cooling towers have been the source of periodical outbreaks of Legionnaires’ disease in the past and are thus usually screened for legionellae on a routine basis. However, standard screening programmes do not consider FLA and their vehicle function and may deliver biased results. The aim of this study was to incorporate a screening system for amoebal hosts into a routine screening programme for legionellae in cooling towers. Consecutive water samples from three cooling towers (n=66) and various tap water facilities (n=12) were screened over the period of one year for FLA, *Legionella* and *Pseudomonas aeruginosa* by culture and molecular methods in parallel. A new real-time PCR-based screening system for *Acanthamoeba*, Vahlkampfiidae and *Vermamoeba*, was established. Additionally, all amoebal isolates were screened for intracellular bacteria. Altogether, 71.2% of the cooling tower samples and 50% of the tap water samples were positive for *Acanthamoeba* spp. Moreover, 57.6% of the cooling tower samples were positive for Vahlkampfiidae and 7.6% for *Vermamoeba*. Plate culture revealed also other genera as e.g. *Cochliopodium* or *Stenamoeba*. Interestingly, 68.8% of the cooling tower samples were not suitable for standard screening methods for *Legionella* due to their high organic burden. In the remaining samples positivity for *Legionella* spp. was 25%, but positivity was 50% in a random set of samples screened with molecular methods. Moreover, several amoebal isolates revealed intracellular bacteria by fluorescence in situ hybridization. Our study highlights the need for a regular screening of and modified screening protocols for cooling towers.

1. Introduction

Free-living amoebae (FLA) have a worldwide distribution and are found in various natural habitats like soil, freshwater and seawater (Berk et al., 2006; Declerck et al., 2007; Geisen et al., 2014; Smirnov and Brown, 2004). Additionally they can colonize engineered water facilities, including water treatment plants, air conditioning, plumbing systems and drinking water networks or cooling towers (Canals et al., 2015; Delafont et al., 2013; Retana-Moreira et al., 2014). In any of these habitats FLA play an important role as vehicles of replication and dispersal for bacteria (Cirillo et al., 1997; Greub and Raoult, 2004; La Scola and Raoult, 2001; Siddiqui and Khan, 2012; Winiecka-Krusnell and Linder, 2001). The extremely resilient genus *Acanthamoeba* is a particularly suitable host for several bacteria, including *Legionella pneumophila,* which can cause Legionnaires´ disease, a severe pneumonia (Rowbotham, 1980). Intracellular legionellae are protected from changes in pH, temperature changes or biocides (Ohno et al., 2003; Wadowsky et al., 1985), enabling them to survive disinfection (Cervero-Aragó et al., 2014; Dupuy et al., 2011; Hwang et al., 2006). Moreover, under environmental stress, legionellae can enter a physiological dormant state, the viable but non-culturable (VBNC) state, in which they cannot be detected by standard cultivation techniques (Hussong et al., 1987; Robertson et al., 2014). Passage through FLA*,* seems to resuscitate VBNC legionellae and to enhance their infectivity (Cirillo et al., 1999; Steinert et al., 1997; Garcia et al., 2007).

Man-made habitats like open cooling towers can disseminate legionellae via aerosols (Walser et al., 2014). These aerosols can be distributed over long distances from 1 to 6 km (Addiss et al., 1989). Cooling towers of large public buildings pose a particular risk and have been reported as sources of community-acquired and nosocomial outbreaks of Legionnaires´ disease (Buse et al., 2012; Freudenmann et al., 2011; Hugosson et al., 2007; Nguyen et al., 2006; Sala Ferré et al., 2009). An example for such an incidence in Austria was reported in 2007 (Wewalka, 2013), when the cooling towers of a hospital were the source of a *Legionella* outbreak. 16 cases of legionellosis occurred not only in the hospital, but also in its surroundings and 3 of these cases were lethal. Until now, legislation in Austria does neither require the registration of wet cooling systems nor are regular microbiological monitoring or standard disinfection mandatory. Most public cooling towers are, however, screened by the respective operating company. In a previous study, we have shown that FLA and legionellae are abundant in Austrian industrial waters (Scheikl et al., 2014). Thus, a project was initiated to incorporate a screening system for amoebal hosts into a routine screening programme for legionellae in cooling towers. A particular aim of this study was to synchronously assess the diversity of FLA relevant as bacterial hosts and to investigate all amoebal isolates for intracellular bacteria. Thus, a rapid and reliable screening system for the detection and synchronous differentiation of the amoebal hosts was established.

1. Materials and methods

**2.1. Sample collection and processing**

The study included three open cooling towers that are under permanent observation for legionellae, the screening including also *Pseudomonas aeruginosa* and total bacteria, and under regular albeit not standardized disinfection. Two of these cooling towers were hospital cooling towers, located at two different hospitals, one in the centre of Vienna (CT-Hospital 1) and the other one in the periphery of Vienna (CT-Hospital 2). Cooling towers of Hospital 1 had been the source of the *Legionella* outbreak in 2007 and since then had been dismantled and renewed. The third cooling tower (CT-Company) was from a complex of company buildings, located in the same district as CT-Hospital 1 and comprising several large office buildings with bureaus, shops, restaurants, a kindergarten and also an outpatient clinic. Moreover, 12 tap waters (Tap) from various sites were sampled over the same period of time for comparison, including one sample from a *Legionella*-contaminated shower head in another hospital in Vienna. During the study period of one year, water samples were obtained every 14 days, corresponding to the regulations for routine *Legionella* screening. CT-Company was not sampled between November 2013 and March 2014 as the system was not in operation. There is no standardized disinfection protocol for cooling towers, cooling towers in this study were disinfected with heat and/ or oxidative biocides (chlorine- and bromine-based). As we have shown in a previous study that amoebae re-colonize waters rapidly after disinfection and to have most stringent conditions for screening the prevalence of amoebae, samples were always obtained one day after disinfection. Water temperatures from all sites were recorded throughout the study, on site-pH values were obtained from CT-Hospital 2 and were between 8.3 and 9.1.

The bacterial screening was performed according to the respective international regulations. In detail, the sampling and evaluation strictly followed the new requirements for evaporative re-cooling plants (ÖNORM B 5020:2013 Austrian Standards Institute, 2013). From each sampling site, 3 L of water were collected in sterile plastic bottles, stored at 4°C and processed within 48 hours. The water samples were analysed for *Legionella* spp. CFU/100 ml (colony forming units in 100 ml) after centrifugation and filtration of 100 ml untreated water or after acid treatment (ISO 11731-2:2004 International Organization for Standardization, 2004). *Legionella* species were identified by sequencing the mip-gene and *Legionella pneumophila* was serotyped according to the EWGLI- (European Working Group for Legionella Infections, 2011) criteria. *Pseudomonas aeruginosa* was evaluated in 100 ml of water (ISO 16266:2008 International Organization for Standardization, 2008) and total heterotrophic bacteria were counted as CFU in 1 ml at 36°C (ISO 6222:1999 International Organization for Standardization, 1999).

**2.2. Amoeba culture and evaluation of intracellular bacteria**

For isolation of FLA, 250 ml of well mixed water samples were vacuum-filtered through a cellulose nitrate filter with 0.45 µm pore size (area 12.5 cm2, Sartorius, Germany). After filtration, the filter was cut into 2 pieces and placed onto a NN (non-nutrient) agar plate covered with 100 µl of a 48-h-old culture of *Escherichia coli* in brain heart infusion broth (BHI). The NN plates were sealed with Parafilm® and stored at room temperature for up to four weeks. Every day, the plates were examined for amoebic migration from the filter pieces by inverted phase contrast microscopy (Nikon TMS). Detected FLA were transferred to a fresh *E. coli*-coated NN plate using a sterile inoculation loop. All amoebal isolates were cloned by sub-culturing to generate pure cultures for later DNA isolation. Morphological identification was accomplished by inverted phase contrast microscopy and phase contrast microscopy (Nikon Eclipse E800) using the identification keys of Page (Page, 1991) and Smirnov (Smirnov et al., 2011). All isolates were screened for intracellular bacteria. Endosymbionts were detected by FISH (fluorescence in situ hybridization) using the probe EUK516 (5´- ACCAGACTTGCCCTCC -3´), detecting most Eukaryotes, a mix of bacterial probes, namely EUB338 I-III (5´-GCTGCC TCCCGTAGGAGT-3´, 5´-GCAGCCACCCGTAGGTGT-3´, 5´-GCTGCCACCCGTAGGTGT-3´; Amann et al., 1990; Daims et al., 1999) and the negative control probe NONEUB (5´-ACTCCTAC GGGAGGCAGC-3´). Amplification and identification were performed by 16S rRNA gene sequencing using barcoded primers Bakt\_341F (5´-CCTACGGGNGGCWGCAG-3´) and Bakt\_805R (5´-GACT ACHVGGGTATCTAATCC-3´) as described (Herlemann et al., 2011).

**2.3. DNA extraction from cell culture and water samples**

Trophozoites from clonal cultures were harvested with cotton swabs and re-suspended in 15 ml centrifuge tubes filled with 5 ml 0.9% sodium chloride (NaCl). The samples were centrifuged for 10 min at 800 x g, the supernatant was discarded and the pellet was re-suspended in 200 µl 0.9% NaCl. Total genomic DNA was extracted from the cells using the QIAmp® DNA Mini Kit (QIAGEN, Hilden, Germany). For direct DNA isolation from water samples, 2 L were filtered and DNA was extracted from cellulose nitrate filters using the MO BIO PowerWater® DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA).

**2.4. Amplicon sequencing and analysis**

Amplicon sequencing was performed as described in Herbold et al. (2015) (Herbold et al., 2015). V3 and V4 regions of the bacterial 16S rRNA were amplified with barcoded versions of the primers Bakt\_341F (CCTACGGGNGGCWGCAG) and Bakt\_805R (GACTACHVGGGTATCTAATCC) (Herlemann et al., 2011). Each PCR reaction included 1x DreamTag Green Buffer (Fermentas, Thermo Fisher Scientific, Vienna, Austria), 2 mM MgCl2, 0.2 mM dNTP mix (Fermentas), 0.1 mg mL-1 bovine serum albumin, 1 µM of each of the forward and reverse primers, 0.025 U DreamTag polymerase (Fermentas) and 1 µL of template. The PCR was performed with a cycle ratio of 25:10. Clustering into operational taxonomic units (OTUs) was performed as described previously (Herbold et al., 2015). Taxonomic classification was carried out using the mothur classify.seqs function (Schloss et al., 2009) and the Silva 1.19 SSU database as reference (Quast et al., 2013). The bootstrap threshold for the taxonomic assignment was set to 80%.

**2.5. PCR and sequencing of amoeba cultures**

Genotyping of *Acanthamoeba* isolates was performed by amplifying and sequencing a 385-450 bp (depending on genotype) long fragment of the *Acanthamoeba*-specific amplimer ASA.S1 located in the 18S rRNA-gene using the newly designed primers AcF1 5´-TGCCACCGAATACATTAGCAT-3´ and AcR1 5´-ACAAGCTGCTAGGGGAGTCA-3´ modified from primer JDP2 from (Schroeder et al., 2001). PCRs were run with 1 µl, 3 µl and 6 µl whole cell DNA in a total reaction volume of 50 µl for each sample under the following conditions: 15 min pre-heating at 95 °C, followed by 35 cycles at 95 °C for 1 min, 60 °C for 2 min, 72 °C for 3 min and a final extension for 7 min at 72 °C. An ASA.S1 amplicon clone of a T4 genotype strain was used as a positive control. *Acanthamoeba* genotypes were assessed with the model assumption of a <5% sequence dissimilarity within one genotype (Gast et al., 1996). DNA extracted from other amoebae isolated by culture was amplified and sequenced using universal eukaryotic primers binding in the 18S rRNA gene, namely the modified primers SSU1 5´-CGACTGGTTGATCCTGCCAGTAG3´ and SSU2 5´-TCCTGATCCTTCTGCAGGTTCAC-3´ (Gast et al., 1994) and P1fw 5´-CAAGTCTGGTGCCAGCAGC-3´, P1rev 5´-GCTGCTGGCACCAGACTTG-3´, P2fw 5´-GATCAGATACCGTCGTAGTC-3´, P2rev 5´-GACTACGACGGTATCTGATC-3´, P3fw 5´-CAGGTCTGTGATGCCCTTAG-3´ and P3rev 5´-CTAAGGGCATCACAGACCTG-3´ (Walochnik et al., 2004). PCR was performed with 1 µl, 3 µl and 6 µl of whole cell DNA in 50 µl reaction volume running a standard amplification program (35 cycles; 95 °C for 1 min, 52 °C for 2 min, 72 °C for 3 min). Amplified DNA was detected by gel electrophoresis on a 2% agarose gel and visualized with GelRed™ (BIOTREND, Germany). Gel bands were extracted with the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK) and directly sequenced in both directions with the ABI PRISM® BigDye sequencing kit and an ABI PRISM 310® automated sequencer (PE Applied Biosystems, Germany). All sequences obtained were compared to sequences from GenBank with the NCBI Nucleotide BLAST search and aligned with sequences of highest similarity using ClustalX (Thompson et al., 1997) or CLC Main Workbench (CLC bio, QIAGEN). Multiple alignments were processed with the GeneDoc sequence editor (Nicholas et al., 1997).

**2.6. Real-time PCR**

For the detection of *Acanthamoeba* spp., a real-time PCR assay (Qvarnstrom et al., 2006) was adapted, using the primers AcantF900 5´-CCCAGATCGTTTACCGTGAA-3´, AcantR1100 5´-TAAATATTAATGCCCCCAACTATCC-3´ and the Cy5-labeled probe AcantP1000 5´-Cy5-CTGCCACCGAATACATTAGCATGG-BHQ3-3´ and amplifying fragments of 170 to 230 bp, depending on the genotype. For design of the primers and probe for the detection of Vahlkampfiidae, particularly *Naegleria* spp., we retrieved partial or full length 18S rDNA sequences from GenBank (NCBI, National Center for Biotechnology Information) and included them in multiple sequence alignments. Sequences of *N. jamiesoni, N. andersoni*, *N. clarki*, *N. andersoni*, *N. fultoni*, *N. pagei*, *N. australiensis*, *N. lovaniensis*, *N. fowleri,* some unidentified *Naegleria* spp. strains and additionally, 13 sequences from other vahlkampfiids including *Paravahlkampfia*, *Vahlkampfia*, *Singhamoeba*, *Willaertia* and *Tetramitus* were evaluated and compared for conserved and variable regions resulting in the new primers VahlNaegF 5´-GTATAGTCGCAAGACCGAAAC-3´, VahlNaegR 5´-CAAGACAGATCACTCCACGA-3´ and the Cy5-labeled probe VahlNaegP 5´-Cy5-GAAAGGCACCACCAGGAGTG-BHQ2-3´, amplifying 190-200 bp fragment. The same procedure was followed for the design of the primers and probe for the detection of *Vermamoeba vermiformis,* namely VermHartF 5´-TAACGATTGGAGGGCAAGTC-3´, VermHartR 5´-ACGCCTGCTTTGAACACTCT-3´ and the HEX-labeled probe VermHartP 5´-HEX- TGGGGAATCAACCGCTAGGA-BHQ1-3´. The amplicon is approximately 240 bp long. The specificity of all primers and probes were evaluated with Primer3Plus, BLAST Nucleotide search and multiple alignments with other amoebal genera. Moreover, PCR test runs were performed with several reference strains, to check specificity and sensitivity. The *Acanthamoeba*- and the Vahlkampfiidae-PCRs were duplexed with an Exogenous Internal Positive Control (IPC) containing a VIC-labelled probe, to distinguish true target negatives from PCR inhibition (Behets et al., 2007). Duplexing with the IPC was not possible for the *Vermamoeba-*assay, as the HEX- and the VIC-fluorescence dyes have the same excitation/emission range and other fluorochromes did not work equally well. Real-time PCRs were performed in a final reaction volume of 20 µl, containing 1x TaqMan® Fast Universal PCR Mastermix (Applied Biosystems, USA), forward primer (0.9 µM), reverse primer (0.9 µM), probe (0.25 µM), 1x Exo IPC Mix, 1x Exo IPC, 3 µl DNA and sterile H2O (for DNA analysis, Carl Roth, Germany). *Acanthamoeba*- and Vahlkampfiidae-PCRs with no IPC signal were repeated with tenfold diluted DNA. These diluted DNA samples that were suspected to contain inhibitors were also repeated with *Vermamoeba*-PCR. Real-time PCRs were performed in a Light Cycler® LC 480 Instrument (Roche, Germany) with an initial activation step at 95°C for 10 min followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Fluorescence was measured at the end of the 60°C anneal/extend step. Samples with a Ct (threshold cycle) value below 40 were considered to be positive. The cell detection limit for *Acanthamoeba* and Vahlkampfiidae was below one cell whereas the detection limit for *V. vermiformis* was about 3 cells. Data were analysed with the LightCycler® 480 Software (version 1.5) and calculated using the second-derivate maximum algorithm.

**2.7. Reference strains**

For the establishment of the real-time PCR assays and as controls, we used amoeba reference strains from our culture collection, namely *Acanthamoeba polyphaga* strain 4CL, genotype T4 (ATCC PRA-107TM (Walochnik et al., 2000), *A. castellanii* strain 1BU, genotype T4 (ATCC PRA-105TM), *Vermamoeba vermiformis* strain 1282-2 (isolated from a contact lens case, 2010), *Hartmannella cantabrigiensis* strain Hc (Walochnik, 1997), *Naegleria lovaniensis* strain 12N (veterinary stool sample, 2005) and *N. gruberi* strain 40N (GenBank accession no. AF114439). From each reference strain DNA was extracted from tenfold dilution series (105 cells/ml to 1 cell/ml), so that the highest diluted sample contained less than one amoebal cell per 20 µl reaction mix.

**2.8. Statistical Analysis**

The collected data were analysed with IBM SPSS Statistics, version 19 (SPSS Inc., Chicago, USA). Frequency distributions were compared using chi-square tests or Fisher’s exact probability test, as appropriate. Dependent frequencies were compared by McNemar tests. For all analyses p-values below 0.05 were considered significant.

1. Results

**3.1. Free-living amoebae relevant as bacterial hosts**

Altogether, 83.3% of all cooling tower samples (55/66) were positive for FLA. As shown in Table 1, *Acanthamoeba* was most prevalent, namely in 71.2% of all cooling tower samples, as well as in 50% of the tap water samples (Table 1). *Acanthamoeba* was the only detected amoebal genus in tap water, whereas cooling waters also showed a high prevalence of Vahlkampfiidae (57.6% positive samples). *Vermamoeba* always co-occurred with Vahlkampfiidae in cooling tower samples (7.6%) and in 4.5% all three FLA groups were detected simultaneously. *Acanthamoeba* and Vahlkampfiidae co-occurred in 45.5% of all cooling tower samples, with the highest numbers of samples being positive for both taxa in CT-Hospital 1 (65.4%). Compared to that, CT-Hospital 2 showed significantly higher frequency of *Acanthamoeba* than of Vahlkampfiidae (p=0.01) with Vahlkampfiidae always co-occurring with *Acanthamoeba*. Altogether, CT-Hospital 1 showed the highest frequency of FLA with 89% positive samples (Table 1). This cooling tower also showed the highest abundance of Vahlkampfiidae (84.6%) and *Vermamoeba* *vermiformis* (11.5%), as shown in Table 1. The shower head sample was negative with all real-time PCR tests (not shown in Table 1).

The two hospital cooling towers had temperatures varying between 20°C and 30°C throughout the entire year, mainly between 25°C and 30.5°. For the company’s cooling tower, temperatures below 20°C were measured at the beginning of the operating time, as this cooling tower was not in use during the winter (November to March).

**3.2. Screening for bacteria**

3.2.1. *Legionella* spp.

Due to the high organic burden resulting in bacterial and fungal overgrowth, 7/66 cooling water samples had to be totally excluded. Despite acid-treatment, for further 39 samples (39/59; 66.1%) smaller volumes, i.e. 1 ml–10 ml, had to be used for the routine screening as is standard procedure.Thus, the optimal volume of 100 ml could be analysed only from 33.9% (20/59) of the cooling tower samples. Of these, 25% (5/20) were positive for *Legionella* spp. (Figure 1). From the 5 positive samples, 4 samples from CT-Hospital 1 were positive for *L. rubrilucens* in increased (>100–1000 CFU/100 ml) to highly increased (>1000 CFU/100 ml) concentrations and one sample from CT-Company was positive for *L. pneumophila* (serogroup 2–14) in low concentration (≤100 CFU/100 ml). The sample taken from a shower head in a hospital also showed low concentrations (6 CFU/100 ml) of *L. pneumophila* serogroup 2-14 (Table 2), whereas all 10/12 examined tap water samples were negative. All smaller volumes investigated remained negative in routine screening. Thus, for comparison, a random set of twenty-eight cooling tower samples were also analysed by amplicon sequencing for the presence of OTUs classified as members of the genus *Legionella*. Of these 14 were positive for at least one *Legionella* OTU (50%). CT-Hospital 1 showed three samples positive for *Legionella* spp. (21.4%) in contrast to eleven positive samples from CT- Hospital 2 (78.6%). Each hospital cooling tower showed a unique set of *Legionella* OTUs, with no shared OTUs between the two. In total, six different OTUs were taxonomically assigned to the genus *Legionella*.

3.2.2. *P. aeruginosa*

Standard screening for *P. aeruginosa* was performed for 64/66 samples taken from cooling towers, 25% (16/64) being positive. Eleven of these 16 samples showed low bacterial counts (1–100 CFU/100 ml) and 5 samples showed increased concentrations (>100–1000 CFU/100 ml). Increased concentrations were measured in both hospital cooling towers, while the company cooling tower showed low concentrations. Altogether, 56.3% (9/16) of the *P.* *aeruginosa* positive samples also were positive for FLA. All tap water samples investigated (4/12) were negative.

3.2.3. Total bacteria

Total heterotrophic bacteria counts were calculated for 64/66 cooling water samples and they all were positive. 56.3% (36/64) of the samples showed only low bacterial counts (1–10,000 CFU/ml) while increased numbers (>10,000–100,000 CFU/ml) were detected in 34.4% (22/64) samples taken from cooling towers and 9.4% (6/64) showed highly increased concentrations (>100,000 CFU/ml), all of them from hospital cooling towers. With 82.8% (53/64), the majority of samples revealed FLA, detected by real-time PCR.

Total CFU counts were not done for the shower head sample, but for 9/12 tap water samples with 4/9 (44.4%) showing low concentrations of total bacteria.

**3.3. Isolation of FLA and identification of their endosymbionts**

We aimed to obtain clonal monoxenic subcultures from all isolates, however, several cultures were lost due to fungal overgrowth. Anti-mycotics/antibiotics were applied, but nevertheless amoebae often did not survive. Altogether, 31/66 cooling water samples were positive for FLA in the initial cultures and 26 of these could be identified. From these, 16 isolates were successfully brought into monoxenic cultures and subjected to further PCR and sequencing (Table 2). Filtration of the cooling water and culturing the amoebae from filters was time-consuming regarding the high organic burden and the high turbidity of the samples. Especially the water collected from CT-Hospital 1 usually contained a high number of mites, nematodes, nematode eggs and fungi.

Sequencing of the 18S rRNA gene was performed, with DNA obtained from monoxenic subcultures of amoebae. An overall of nine different taxa could be identified morphologically and by sequencing from 16 isolates. In contrast to the cooling towers, the Tap and the Shower samples were all negative for FLA in culture. CT-Hospital 1 showed the highest amoebal diversity with four different genera and two species (Table 2). Two isolates from CT-Hospital 2 were identified as *Naegleria* spp., with 100% sequence homology to one another and with equal sequence identities to N. clarki and *N. pagei*. Both were shown to grow at 37°C.

Altogether, three amoebal isolates, all from cooling towers, revealed endosymbionts (Table 2). In an *Acanthamoeba* isolate from CT-Hospital 1, the facultative intracellular bacterium *Paracaedibacter acanthamoebae* (order *Rickettsiales*) was detected. *Cochliopodium minus* contained bacteria belonging to a new genus distinct from *Legionella* within the order *Legionellales* (Tsao et al., in preparation), whereas another *Acanthamoeba* strain from CT-Hospital 2 contained a novel member of the order *Rickettsiales* (Tsao et al., unpublished).

1. Discussion

In the current study, 83.3% (55/66) of all water samples from cooling towers were positive for amoebae suitable as bacterial vehicles, despite regular disinfection and already one day after disinfection. With our new screening system based on the combination of three group-specific real-time PCRs we achieved a synchronous rapid and highly sensitive detection of those amoebae that are most important as bacterial hosts in treated waters. Moreover, it was shown, that the routine screening for *Legionella* spp. does not give reliable results for waters with high organic burden.

Interestingly, hospital cooling towers (CT-Hospital 1 and CT-Hospital 2) had higher frequencies of amoebae compared to the company cooling tower and, against expectations, the cooling tower from Hospital 1 that had been the source of an *Legionella* outbreak in 2007 and since then had been dismantled and renewed, showed the highest frequency of amoebae. FLA are a natural reservoir for legionellae and open wet cooling towers provide optimal growth- and dissemination conditions for both of these microorganisms. With the real-time PCR-based screening system for amoebae we achieved a significantly higher sensitivity compared to similar screenings using culture and conventional PCR (Scheikl et al., 2014). Also in the current study, culture had low sensitivity, only 52.7% (29/55) of the real-time PCR positive samples being positive however, the advantage of culture is that basically any viable species of FLA can be found and isolated amoebae can be screened for intracellular bacteria.

*Acanthamoeba* showed the highest overall prevalence followed by Vahlkampfiidae, a majority of samples being positive for both. Atlan et al. (2012) revealed either *Acanthamoeba* or *Vermamoeba* as dominating genera in cooling towers and a predominance of *Acanthamoeba* was reported from water treatment plants (Magnet et al., 2013), whereas *V. vermiformis* was found to be the most abundant FLA species in drinking water (Delafont et al., 2013; Pagnier et al., 2015; Thomas et al., 2008; Wang et al., 2012), and in hot water systems (Rohr et al., 1998). In the current study, *V. vermiformis* was absent in tap water and also rarely found in cooling towers (7.6%). This might be attributed to *Vermamoeba*´shigher sensitivity to biocides compared to *Acanthamoeba* (Coulon et al., 2010; Fouque et al., 2015). The higher prevalence of Vahlkampfiidae compared to a previous study on industrial waters (Scheikl et al., 2014) may be attributed to the higher sensitivity of the real-time PCR, but probably also to the constantly warm water temperatures. Generally, Vahlkampfiidae are known to occur in cooling towers (Canals et al., 2015; Rohr et al., 1998). In the current study, also two thermophilic *Naegleria* strains related to *N. clarki* and *N. pagei*, were isolated. Although both species are not considered pathogenic, they are from the same phylogenetic cluster as *N. australiensis* and *N. italica*, which were shown to be pathogenic in animal experiments (De Jonckheere, 2014).

Altogether, the investigated cooling towers showed a high positivity and diversity of FLA. The cooling tower from Hospital 1 had the highest organic burden and also the highest number of different amoebal taxa, although it had been disinfected with oxidative biocides, always one day before the sampling. At eight time points, disinfection had even been performed shortly before (1.5 – 3.5 hrs) the sampling. All those samples still showed a high amoebal diversity, with e.g. *Acanthamoeba*, *Stenamoeba* and *Cochliopodium* growing in culture, demonstrating that disinfection had no effect on amoebal viability. Surprisingly, also Vahlkampfiidae were detected in all cooling towers and *V. avara*, *Naegleria* spp. and other unidentified vahlkampfiids could be isolated from the hospital cooling towers. This shows that disinfection did not have a significant impact on even these sensitive amoebae and that cooling towers provide an optimal environment for thermophilic and potentially pathogenic amoebae (Canals et al., 2015; Pagnier et al., 2009). Besides, *Acanthamoeba* and also *Cochliopodium* are known to be stimulated by biocides recommended for cooling towers (Srikanth and Berk, 1993). This is even more interesting, as we demonstrated the co-occurrence of *C. minus* and *L. rubrilucens* in CT-Hospital 1 and additionally, we detected intracellular bacteria in *C. minus*, belonging to the order *Legionellales*. This once more corroborates the reservoir function of amoebae for potentially pathogenic bacteria (Greub and Raoult, 2004), and that these amoebal hosts are also resistant against conventional disinfection procedures for cooling tower waters.

Thus, the cooling towers provided ideal conditions for *Legionella* spp. Also the weak alkaline pH is within the range for the growth of *L. pneumophila* and other legionellae species (Wadowsky et al., 1985), as well as for FLA, including amoebae carrying bacteria (Berk et al., 2006; Buse et al., 2012; Huang et al., 2011). All *Legionella*-positive samples from CT-Company and CT-Hospital 1 showed elevated temperatures, i.e. between 26°C and 30°C. The tap waters with temperatures around 12°C were all negative, but the sample from the shower head was positive for *L. pneumophila* serogroup 2–14 although it had been cleaned and disinfected before. Three samples showed highly increased concentrations of *L. rubrilucens* (18,000–22,000 CFU/100 ml) and one sample increased numbers of *L. rubrilucens* (600 CFU/100 ml) although disinfected one day before sampling. *L. rubrilucens* always co-occurred with *Acanthamoeba* or Vahlkampfiidae or both amoebal groups. No legionellae were detected in CT-Hospital 2 by the standard method, despite the presence of amoebae. However, our study indicates that the standard protocol for *Legionella* screening on routine basis is not suitable for waters from open cooling towers with a high organic burden. For the majority of these samples, only reduced volumes can be investigated, resulting in unreliably low recovery rates for *Legionella*, in our study this was 7.6% positive samples. In comparison, the amplicon-based screening revealed the presence of *Legionella* spp. in nearly 80% of the samples investigated. In fact, the ISO 11731-2 (2004) standard protocol was established for waters with low bacterial counts (e.g. tap water), it is however, widely used for *Legionella* detection also from engineered waters. Non-standard molecular biological methods like CARD-FISH reveal higher numbers of *Legionella* positive samples and also higher concentrations compared to the standard method (Kirschner et al., 2012). Co-culture with amoebae (Magnet et al., 2015) is another sensitive tool to isolate *Legionella* and other potentially pathogenic bacteria like chlamydiae, *Pseudomonas* spp. or mycobacteria from various sampling sources (Collingro et al., 2005; Corsaro et al., 2010; Kebbi-Beghdadi and Greub, 2014; Thomas et al., 2008). However, both techniques have not yet been implicated in routine screenings.

*P. aeruginosa* was generally detected in low concentrations in the cooling towers, increased concentrations were measured at elevated temperatures (27°C). Increased counts of *P. aeruginosa*, respectively highly increased counts of total bacteria were only detected in the hospital cooling towers. The majority of *P. aeruginosa* positive samples also were positive for FLA, whereby *Acanthamoeba* always co-occurred with *P. aeruginosa*. All *Legionella*-positive samples were negative for *P. aeruginosa*. It has been shown, that *P. aeruginosa* inhibits the growth and biofilm formation of *L. pneumophila* (Kimura et al., 2009).

Altogether, our study highlights the need for a regular and regulated screening of cooling towers, possibly also including FLA, and for standardized modified screening protocols for these types of waters.

1. Conclusions

* There is a high prevalence of amoebae suited as bacterial vehicles in cooling towers.
* Regular disinfection does not affect amoebal survival.
* The newly established real-time PCR-based screening system for amoebae is well-suited for regular and synchronous screening for amoebae.
* Results obtained from *Legionella* routine screenings are not reliable for water samples with high organic burden.

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**Table legend**

**Table 1.** Positivity rates of FLA in cooling towers and tap waters, evaluated by real-time PCR.

**Table 2.** Diversity of microorganisms per sampling site. FLA and *Legionella* isolated by culture and identified by DNA sequencing. Endosymbionts detected by FISH in isolates of FLA and identified by sequencing.

**Figure legend**

**Figure 1.** Number of *Legionella*-positive samples in relation to the investigated sample volume.

Table 1. Positivity rates of FLA in cooling towers and tap waters, evaluated by real-time PCR.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| FLA |  | Cooling tower Hospital 1 | Cooling tower Hospital 2 | Cooling tower Company | Tap water | Total |
| *Acanthamoeba* | percentage[[1]](#footnote-1)  number[[2]](#footnote-2) | 69.2%  18 | 84.6%  22 | 52.0%  7 | 50.0%  6 | 67.9%  53 |
| Vahlkampfiidae | percentage  number | 84.6%  22 | 42.3%  11 | 35.7%  5 | -  - | 48.7%  38 |
| *Vermamoeba* | percentage  number | 11.5%  3 | 3.8%  1 | 7.1%  1 | -  - | 6.4%  5 |
| *Acanthamoeba* + Vahlkampfiidae | percentage  number | 65.4%  17 | 42.3%  11 | 14.3%  2 | -  - | 38.5%  30 |
| *Acanthamoeba* +Vahlkampfiidae + *Vermamoeba* | percentage  number | 7.7%  2 | 3.8%  1 | -  - | -  - | 3.8%  3 |
| TOTAL | number | 88.5%  (23/26) | 84.6%  (22/26) | 71.4%  (10/14) | 50.0%  (6/12) | 78.2%  (61/78) |

**Table 2.** Diversity of microorganisms per sampling site. FLA and *Legionella* isolated by culture and identified by DNA sequencing. Endosymbionts detected by FISH in isolates of FLA and identified by sequencing.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Cooling tower Hospital 1 | | | | |
| **FLA** | | | **Legionella** | |
| **Taxa** | **No. of isolates** | **Endosymbionts** | **Species** | **No. of isolates** |
| *Acanthamoeba* sp.  *Cochliopodium minus*  *Stenamoeba* sp.  *Thecamoeba*  *Protostelium*-like amoeba  *Vahlkampfia avara* | 3  3  2  5  1  1 | *Paracaedibacter acanthamoebae*  *Legionellales*  -  -  -  - | *L. rubrilucens* | 4 |
| **Cooling tower Hospital 2** | | | | |
| *Acanthamoeba* sp.  Vahlkampfiidae[[3]](#footnote-3)  *Naegleria* spp.[[4]](#footnote-4) | 5  1  2 | *Rickettsiales*  -  - |  |  |
| **Cooling tower Company** | | | | |
| *Acanthamoeba* sp.  *Leptomyxa reticulata* | 1  2 | -  - | *L. pneumophila* SG 2-14 | 1 |
| **Shower head** | | | | |
| - | - | - | *L. pneumophila* SG 2-14 | 1 |
| Total | 26 | 3 | 6 |  |

1. Relative frequencies of amoeba-poitive samples in different sampling sites [↑](#footnote-ref-1)
2. Absolute numbers of positive samples [↑](#footnote-ref-2)
3. Mixed culture of several genera grown at 30° [↑](#footnote-ref-3)
4. Thermophilic [↑](#footnote-ref-4)