**Title**

Free-living amoebae and their associated bacteria in Austrian cooling towers and tap water

**Running title**

FLA colonize Austrian cooling towers

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**Abstract**

Free-living amoebae (FLA) are widely spread in the environment and also known to cause rare but often serious infections. Besides this, FLA have an indirect public health significance as they may serve as vehicles of dispersal and replication for bacterial pathogens. In particular, *Legionella pneumophila*, the causative agent of Legionnaires´ disease, replicates within FLA, triggering *Legionella*´s infectivity and resistance against biocides. Additionally, intracellular legionellae in FLA are protected against disinfection. As cooling towers have caused periodical *Legionella* outbreaks in the past and there is no mandatory regular monitoring in Austria, we screened selected cooling towers for *Legionella*, *Pseudomonas aeruginosa* and FLA. Additionally, we screened all amoebal isolates for intracellular bacteria. A new screening system for FLA including real-time PCR assays specific for *Acanthamoeba*, Vahlkampfiidae and *Vermamoeba*, was established. Water samples were taken periodically from three cooling towers of public buildings and various tap water facilities from 2013 to 2014 and investigated by culture and molecular methods in parallel. Altogether 78 samples were investigated. With real-time PCR, 50% of the tap water samples and an overall of 71.2% cooling tower samples were positive for *Acanthamoeba* spp. More than half of the samples from cooling towers were positive for Vahlkampfiidae (57.6%), whereas *Vermamoeba* was detected in 7.6% of these samples. Less than half of the cooling water samples were also positive for FLA by culture, revealing however also other genera, as e.g. *Cochliopodium* or *Stenamoeba*. Interestingly, a high number of cooling tower samples 68.8% were not evaluable with *Legionella*-standard cultivation techniques or had to be re-examined, due to the composition of this special sample type (high organic burden). Of the 20 cooling water samples that could be screened by standard techniques, five were positive for *Legionella* spp. and 16 for *P. aeruginosa* and additionally,one sample taken from a hospital´s shower was also *Legionella* positive. Moreover, some amoeba isolates revealed intracellular bacteria by fluorescence in situ hybridization (FISH). As cooling towers are of public health concern it would be useful to adapt standard protocols and additionally use more sensitive methods like *Legionella*-PCR and particularly considerate the amoebal reservoir when screening cooling towers.

**Introduction**

Free-living amoebae (FLA) have a worldwide distribution and are found in various natural habitats like soil, freshwater and seawater (1–4). Additionally they can colonize engineered water facilities, including water treatment plants, air conditioning, plumbing systems and drinking water networks or cooling towers (5–7). In any of these habitats FLA play an important role as predators of bacteria, but amoebae are also known to serve as vehicles of replication and dispersal for bacteria (8–12). Especially the extremely resistant genus *Acanthamoeba* is a suitable host for several bacteria, including *Legionella pneumophila,* which can cause a severe pneumonia, the “Legionnaires´ disease” (13). Intracellular legionellae are protected and gain resistance to e.g. changes in pH, temperature changes or biocides (14, 15), enabling them to survive disinfection measures (16–18). 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. Passage through FLA*,* seems to resuscitate VBNCs (19–22) and to enhance the infectivity of *Legionella* spp. (23–25). Man-made habitats like open cooling towers can disseminate legionellae via aerosols if they are not regularly serviced and controlled (26). These aerosols can be distributed over long distances from 1 to 6 km (27). Particularly cooling towers of large public buildings pose a risk for public health and have been reported as sources of community-acquired and nosocomial outbreaks of Legionnaires´ disease (28–32). An example for such an incidence in Austria was investigated and reported 2007, 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 3/16 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. In a previous study, we have shown that FLA and legionellae are abundant in Austrian industrial waters and cooling towers (33). Thus, a project was initiated to screen waters from cooling towers for FLA, *Legionella* spp. and *Pseudomonas aeruginosa* in parallel. A particular aim of this study was to also assess the diversity of FLA relevant as bacterial hosts and to investigate all amoebal isolates for intracellular bacteria. A rapid and reliable screening system for the detection and synchronous differentiation of the amoebal hosts was established. The detection of *Legionella* spp. and *P. aeruginosa* was achieved by standard cultivation techniques.

**Materials and Methods**

Sample collection and processing

79 cooling water and tap water samples were taken from different Austrian sites. Three open cooling towers were sampled every 14 days during operation over the period of one year, resulting in 66 water samples. Two of these cooling towers were hospital cooling towers, located at two different hospitals (CT-Hos1 and CT-Hos2), the third cooling tower was located at a company (CT-Comp). CT-Comp was not sampled between November 2013 and March 2014 as the system was not in operation. 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 a hospital.

From each sampling site, 3 L of water were collected in sterile plastic bottles, stored at 4°C and processed within 48 hours. The bacterial screening was performed according to the new requirements for evaporative re-cooling plants (ÖNORM B 5020:2013 (34)). In brief, the water samples were analyzed 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 (ÖNORM EN ISO 11731-2 (35)). *Legionella* species were identified by sequencing the mip-gene and *Legionella pneumophila* was serotyped according to the EWGLI- (36) criteria. *Pseudomonas aeruginosa* was evaluated in 100 ml of water (ÖNORM EN ISO 16266 (37) and total heterotrophic bacteria were counted as CFU in 1 ml at 36°C (ÖNORM EN ISO 6222 (38).

PCR, amplicon-sequencing

The amplicon-sequencing as well as the purification, quantification and sequencing was performed as described in Herbold et al. (2015). V3 and V4 regions of the bacterial 16S rRNA was amplified with barcoded-version of the primers Bakt\_341F (CCTACGGGNGGCWGCAG) and Bakt\_805R (GACT ACHVGGGTATCTAATCC) (Herlemann et al., 2011). Each PCR reaction includes 1x DreamTag Green Buffer (Fermentas), 2mM MgCl2, 0.2mM dNTPmix (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.

Sequences processing analysis

The OTU-clustering was achieved by using the pipeline described in Herbold et al. (2015). Taxonomic classification was done with mothur classify.seqs function (Schloss et al., 2009) and the Silva 1.19 SSU database as the reference (Quast et al., 2013). The bootstrap threshold for the taxonomic assignment was set to 80. OTUs with a lower value has been assigned as unclassified.

Amoeba culture and evaluation of intracellular bacteria

For isolation of FLA, 250 ml of well mixed water samples were vacuum-filtrated 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 (BHI). The NN plates were sealed with Parafilm® and stored at room temperature for up to four weeks. Every day, the filters 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 receive 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 key of Page (39) and Smirnov (40). All isolates were screened for intracellular bacteria. Endosymbionts were detected using FISH (fluorescence in situ hybridization) and identified by 16S rRNA gene sequencing as described (ref).

DNA extraction from cell culture and water samples

Trophozoites from clonal cultures were harvested with cotton swabs and resuspended 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 resuspended in 200 µl 0.9% NaCl. Total genomic DNA was extracted from the cells according to the “DNA purification from tissues” protocol” of the QIAmp® DNA Mini Kit (QIAGEN, Hilden, Germany).

For direct DNA isolation from water samples, 2 L were filtrated and DNA was extracted from cellulose nitrate filters using the MO BIO PowerWater® DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA).

PCR and sequencing of amoeba cultures

Genotyping of *Acanthamoeba* isolates was performed by amplifying and sequencing a 385-450 bp 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 (41). 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 determined with the model assumption of a <5% sequence dissimilarity within one genotype (42).

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´ (43) 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´ (44). 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 obtained sequences were compared to sequences from GenBank with the NCBI Nucleotide BLAST search and aligned with sequences of highest similarity using ClustalX (45) or CLC Main Workbench (CLC bio, QIAGEN). Multiple alignments were processed with the GeneDoc sequence editor (46).

Real-time PCR

For the detection of *Acanthamoeba* spp., a real-time PCR assay from Qvarnstrom et al. (47) 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 the design of primers and probe specific for the Vahlkampfiidae and particularly for *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 primers and a probe for the detection of the *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-labeled probe, to distinguish true target negatives from PCR inhibition. 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. 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 analyzed with the LightCycler® 480 Software (version 1.5) and calculated using the second-derivate maximum algorithm.

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, (48), *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 (49), *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.

Statistical Analysis

The collected data were analyzed with IBM SPSS Statistics, version 19 (SPSS Inc., Chicago, USA), using crossing tables and chi-square test (asymptotic significance, 2-tailed) or Fisher´s exact test and McNemar test. Significance was set at p < 0.05.

**Results**

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 and occurred 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 FLA in CT-Hos1 (65.4%). Compared to that, CT-Hos2 showed significantly higher *Acanthamoeba* rates than Vahlkampfiidae rates (p=0.01) whereas Vahlkampfiidae always co-occurred with *Acanthamoeba*. Altogether, CT-Hos1 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).**

Screening for bacteria by standard techniques

***Legionella* spp.**

Due to the high organic burden, 7/66 cooling water samples had to be excluded. Thus, 59 cooling waters were screened for *Legionella*, however, the majority of the samples (39/59; 66.1%) had to be diluted to volumes of 1 ml–10 ml despite acid-treatment.Only from 33.9% (20/59) of the cooling tower samples the standard volume of 100 ml could be analysed and out of these 25% (5/20) were positive for *Legionella* spp. (Figure 1). From these, 4 samples from CT-Hos1 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-Comp 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, whereas the 10/12 examined tap water samples were all negative (Table 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 companies cooling tower only showed low concentrations. Altogether, 56.3% (9/16) of the *P.* *aeruginosa* positive samples also were positive for FLA. The shower head sample was not checked for *P. aeruginosa* and from the tap water samples 4/12 were examined and they were all negative.

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 all cooling towers and 9.4% (6/64) showed highly increased concentrations (>100,000 CFU/ml), detected in both hospital cooling towers. With 82.8% (53/64), the vast majority of these 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.

In addition to the routine methods, 28/66 cooling tower samples were analyzed for the presence of OTUs classified as member of the genus *Legionella* by amplicon sequencing. In total, six OTUs have been taxonomically assigned to *Legionella.* 14 out of the 28 samples are positive for at least one of the OTUs (50%). Each hospital cooling tower has its unique set of *Legionella*-OTUs, with no shared OTUs between the them. In total, CT-Hos1 has three *Legionella*-OTU-positive samples (3 of 14 = 21,4%) in contrast to the eleven positive samples taken from C**T-Hos2** (11/14 = 78,6%).

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 culture and 26 could be specified. 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-Hos1 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 of 16 isolates. CT-Hos1 showed the highest amoebal diversity with four different genera and two species (Table 1). Especially interesting was the detection of *Cochliopodium minus*, as to our knowledge, this is the first isolation of a *Cochliopodium* species in Austria. Two isolates from CT-Hos2 grown at 37°C and room temperature, respectively, showed highest similarity to *N. clarki* and *N. pagei*. In contrast to the cooling towers, the Tap and the Show samples were all negative for FLA in culture.**

**As shown in** Table 2**, three amoeba isolates from cooling towers revealed intracellular bacteria. In an *Acanthamoeba* isolate from CT-Hos1, the facultative intracellular** *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 C**T-Hos2 contained a novel member of the order Rickettsiales** (Tsao et al., in preparation)**.**

**Discussion**

**FLA are a natural reservoir for legionellae, and both groups of microorganisms are regularly found in engineered water systems. Especially open wet cooling towers provide optimal growth- and dissemination conditions for these organisms and therefore cooling towers pose a potential threat to public health.** Altogether, in the current study we found a high positivity of the cooling towers for FLA (83.3%; **55/66)** and a particularly high abundance of amoebae in hospital cooling towers (CT-Hos1 and CT-Hos2) compared to the cooling tower of the company and to tap water. With our screening system based on the combination of three group specific real-time PCRs we achieved a synchronous rapid and highly sensitive detection of the genera of FLA most important as bacterial hosts under disinfection, particularly well-suited for this type of water samples. Moreover, our study indicates that the standard protocol for *Legionella* detection is not suitable for cooling water samples.

Generally, *Acanthamoeba* showed the highest overall prevalence with 71.2%, respectively 75.8% including culture, followed by Vahlkampfiidae with 57.6%. 63.8% (30/47) of the *Acanthamoeba*-real time PCR positive cooling water samples were also positive for Vahlkampfiidae. *Acanthamoeba* was the predominant species in CT-Comp (52%) and CT-Hos2 (84.6%), whereas Vahlkampfiidae were predominant in CT-Hos1, also with 84.6%. In contrast, other studies like from Atlan et al. (50) revealed either *Acanthamoeba* or *Vermamoeba* as dominating genera in cooling towers.

By using real-time PCR, the number of *Acanthamoeba*-positive samples was about three times higher compared to a previous study (33) based on culture and conventional PCR (75.8% versus 24.8%). Other studies also revealed a predominance of *Acanthamoeba* in other man-made habitats like water treatment plants (51), whereas *V. vermiformis* was found to be the most abundant FLA species in drinking water (7, 52–54), and in hot water systems (55). In the current study, *V. vermiformis* was absent in tap water and also rarely found in cooling towers by real-time PCR (7.6%). This might be attributed to *Vermamoeba*´shigher sensitivity to biocides, e.g. free chlorine, compared to *Acanthamoeba* (56, 57).

Interestingly, in our previous study, Vahlkampfiidae, were only detected in 12.4% (16/129) of the cooling tower samples, whereas our recent data show a high prevalence of this amoeba group in cooling towers, especially in CT-Hos1 (84.6%). This fact is due to the newly established real-time PCR method, but probably also because of the constantly warm water temperatures mainly between 25°C and 30.5°. From CT-Hos2 *N. clarki* and *N. pagei* were isolated and grown at 37°C. Though these species are thermophilic, they not pathogenic. Nevertheless, they occur in the same phylogenetic cluster as *N. australiensis* and *N. italica*, which were shown to be pathogenic in animal experiments (58). Anyway, Vahlkampfiidae are regularly isolated from cooling towers (5, 55). In contrast, the only FLA we could detect in tap water was *Acanthamoeba* in 6/12 samples and only by real-time PCR.

The culture method, as shown before, has relatively low sensitivity, only 52.7% (29/55) of the real-time PCR positive samples also were positive in the initial culture, however. The advantage of culture is, that basically any species of FLA can be found that feeds on bacteria.

The cooling tower samples showed a high organic burden and massive growth of accompanying microbial flora. Thus, for the majority of the samples, only smaller volumes of the original samples (10 ml or 1 ml respectively) could be subjected to the standard protocol, which resulted in low recovery rates of *Legionella* (7.6%; 5/66). Compared to that, the amplicon-based screening revealed that some investigated cooling tower samples could almost be up to 80% positive for *Legionella* ssp.. Thus, results obtained from waters with high organic burden are not reliable. In fact, the ISO 11731-2 standard protocol was actually established for waters with low bacterial counts (e.g. tap water), it is however, widely used for *Legionella* detection also from engineered waters. Other, molecular biological methods like the newly established CARD-FISH have been demonstrated to reveal higher numbers of *Legionella* positive samples and also higher concentrations compared to the standard method (59). Moreover, Kirschner et al. showed that after disinfection *L. pneumophila* still remained detectable by CARD-FISH and that after a short drop, the concentration re-increased quickly. Hence, *L. pneumophila* cells that enter the VBNC state after disinfection are still detectable by this method. In other studies, aiming to detect and enumerate legionellae in waters with high turbidity and massive bacterial growth, similar problems with the culture method arose and standard protocols had to be modified (60). Magnet et al. (61) detected *Legionella* by *Acanthamoeba* co-culture in 75.7% of the investigated wastewater- and drinking water treatment plants. As other studies have shown, co-culture is another sensitive tool to isolate *Legionella* and other potentially pathogenic bacteria like chlamydiae, *Pseudomonas* spp. or mycobacteria from various sampling sources (53, 62, 63).

Independently from seasons, temperatures in the two hospital cooling towers varied between 20°C and 30°C and therefore provided ideal temperatures for *Legionella* growth. For the companies 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). All *Legionella*-positive CT-Comp and CT-Hos1 samples showed higher temperatures between 26°C and 30°C.

The tap waters were all negative, but the sample from the shower head was positive for *L. pneumophila* serogroup 2–14) in low concentration (6 CFU/100 ml), even though cleaning and disinfection measures were carried out before. Increased numbers of *L. rubrilucens* (600 CFU/100 ml) were detected in a sample that was taken from CT-Hos1 shortly after disinfection, whereas all other samples (3/4) were disinfected one day before sampling and showed highly increased concentrations of *L. rubrilucens* (18000–22000 CFU/100 ml). As shown by real-time PCR, *L. rubrilucens* always co-occurred with *Acanthamoeba* or Vahlkampfiidae or both amoebal groups. Compared to that, no legionellae could be detected in CT-Hos2 by the standard method, whereas *Acanthamoeba,* Vahlkampfiidae and even *Vermamoeba* were detected.

*P. aeruginosa* was generally detected in low concentrations in cooling towers, whereas 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. However, 56.3% (9/16) of *P. aeruginosa* positive samples also were positive for FLA with real-time PCR, whereby *Acanthamoeba* always co-occurred with *P. aeruginosa*. It has been shown, that *P. aeruginosa* inhibits the growth and biofilm formation of *L. pneumophila* (64).

Data for the pH were only available for CT-Hos2, with values between 8.3 and 9.1. This weak alkaline pH range is usual in cooling towers and is within the range for the growth of *L. pneumophila* and other legionellae species (14), as well as for all detected FLA, including infected FLA (3, 65, 66).

Altogether, the investigated cooling towers showed a high positivity and diversity of FLA. CT-Hos1 had the highest organic burden and also the highest number of different amoebal taxa, including the species *Cochliopodium minus*. To our knowledge, this is the first isolation of a representative of the genus *Cochliopodium* in Austria. CT-Hos1 was treated with oxidative biocides (Chlorine- and Bromine-based) and that disinfection was regularly performed a day before the sampling. Additionally, at eight different time points, disinfection was performed shortly before (1.5 – 3.5 hrs) the sampling. All those samples were still positive for amoeba by culture, with e.g. *Acanthamoeba*, *Stenamoeba* and *Cochliopodium* growing, demonstrating that disinfection had no effect on FLA´s viability. Surprisingly, Vahlkampfiidae were detected with real-time PCR in all cooling towers and additionally we cultivated *V. avara*, *Naegleria* spp. and other unidentified vahlkampfiids 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 (5, 67). Besides, *Acanthamoeba* and also *Cochliopodium* are known to be stimulated by biocides recommended for cooling towers (68). This is even more interesting, as we demonstrated the co-occurrence of *C. minus* and *L. rubrilucens* in CT-Hos1 and additionally, we detected intracellular bacteria in *C. minus*, belonging to the order Legionellales. These findings support the assumption, that FLA are an important reservoir for potentially pathogenic bacteria, especially legionellae (8), and that these amoebal hosts are also very resistant against conventional disinfecting agents used for cooling towers. In addition to a regular *Legionella*-screening, we propose FLA-screenings on a random basis, to monitor the microbial burden of open cooling towers and thereby also assess the potential risk of a community acquired or nosocomial pneumonia.

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**Table legend**

**Table 1. Observed frequencies of occurrence of FLA in cooling towers an tap water (CT-Hos1=cooling tower of hospital 1 and CT-Hos2= cooling tower of hospital 2, CT-Comp=company´s cooling tower, Tap=tap water), evaluated by real-time PCR.**

**Table 2. Diversity of microorganisms per sampling site. FLA and Legionella isolated by culture and identified by DNA sequencing. Endocytobionts 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. Observed frequencies of occurrence of FLA in cooling towers an tap water (CT-Hos1=cooling tower of hospital 1 and CT-Hos2= cooling tower of hospital 2, CT-Comp=company´s cooling tower, Tap=tap water), evaluated by real-time PCR.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| FLA |  | CT-Hos1 | CT-Hos2 | CT-Comp | Tap | Total |
| *Acanthamoeba* | percentage  number | 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.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| CT-Hos1 | | | | |
| **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 |
| **CT-Hos2** | | | | |
| *Acanthamoeba sp.*  *Vahlkampfiidae[[1]](#footnote-1)*  *Naegleria* spp.[[2]](#footnote-2) | *5*  *1*  *2* | Rickettsiales  *-*  *-* |  |  |
| **CT-Comp** | | | | |
| *Acanthamoeba sp.*  *Leptomyxa reticulata* | *1*  *2* | *-*  *-* | *L. pneumophila SG 2-14* | *1* |
| **Show** | | | | |
| *-* | *-* | *-* | *L. pneumophila SG 2-14* | *1* |
| Total | 26 | 3 | 6 |  |



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

1. Mixed culture of several genera grown at 30° [↑](#footnote-ref-1)
2. *N. clarki* or *N. pagei* grown at room temperature and 37°C [↑](#footnote-ref-2)