Strategies for the reduction of *Legionella* in biological treatment systems

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ABSTRACT

A community-wide outbreak of Legionnaire's disease occurred in Warstein, Germany, in August 2013. The epidemic strain, *Legionella pneumophila* Serogruppe 1, was isolated from an industrial wastewater stream entering the municipal wastewater treatment plant (WWTP) in Wartein, the WWTP itself, the river Wäster and air/water samples from an industrial cooling system 3 km downstream of the WWTP. The present study investigated the effect of physical–chemical disinfection methods on the reduction of the concentration of *Legionella* in the biological treatment and in the treated effluent entering the river Wäster. Additionally, to gain insight into the factors that promote the growth of *Legionella* in biological systems, growth experiments were made with different substrates and temperatures. The dosage rates of silver micro-particles, hydrogen peroxide, chlorine dioxide and ozone and pH stress to the activated sludge were not able to decrease the number of culturable *Legionella* spp. in the effluent. Nevertheless, the UV treatment of secondary treated effluent reduced *Legionella* spp. on average by 1.6–3.4 log units. Laboratory-scale experiments and full-scale measurements suggested that the aerobic treatment of warm wastewater (30–35 °C) rich in organic nitrogen (protein) is a possible source of *Legionella* infection.

Key words | activated sludge, industrial wastewater, *Legionella pneumophila*, *Legionella* spp.

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INTRODUCTION

Legionella pneumophila is the main causative agent of Legionnaire's disease or legionellosis acquired by inhalation of aerosolized droplets produced in water installations (shower, tap), air conditioning and cooling towers (Percival & Williams 2014). This pathogen grows preferentially at temperatures above 25 °C and uses amino acids as a source of carbon and energy (Price *et al.* 2014). In the environment *L. pneumophila* can multiply intracellularly in protozoa (several species of amoeba and ciliates), which confers to this pathogen special virulent traits (Scheikl *et al.* 2014).

In Warstein a community-wide outbreak of Legionnaire's disease occurred in August 2013 linked to an industrial cooling system, extracting water from the river Wäster. The epidemic strain was identified as *L. pneumophila*, Serogruppe 1, Mab Subtyp Knoxville, ST 345. In an exhaustive investigation of the etiology of the problem the epidemic strain was detected in the river Wäster, in the Warstein municipal wastewater treatment plant (WWTP) 3 km upstream of the cooling system and in an industrial sewer discharging to the WWTP. A probable source for the pathogen was then identified as the industrial sewer transporting a warm wastewater rich in organic nitrogen. At the WWTP a UV disinfection unit was immediately installed to reduce the concentration of *Legionella* entering the river Wäster. In parallel a research project was initiated to investigate strategies to reduce *Legionella* in wastewater treatment systems.

The present study is focused on the occurrence and fate of *Legionella* in the municipal WWTP in Warstein between August 2013 and July 2015. The original layout of the plant and the two successive upgrades are shown in Figure 1(a)–1(c). Briefly, until July 2014, aerobically pre-treated industrial wastewater was discharged to the municipal WWTP. Afterwards, this pre-treatment was shut down and a high-load activated sludge system was constructed at the municipal WWTP and has been in operation since then to cope with the increase in the average organic load from 3,000 kg/d to 10,000 kg/d of chemical oxygen demand (COD). In the near future, an anaerobic pre-treatment

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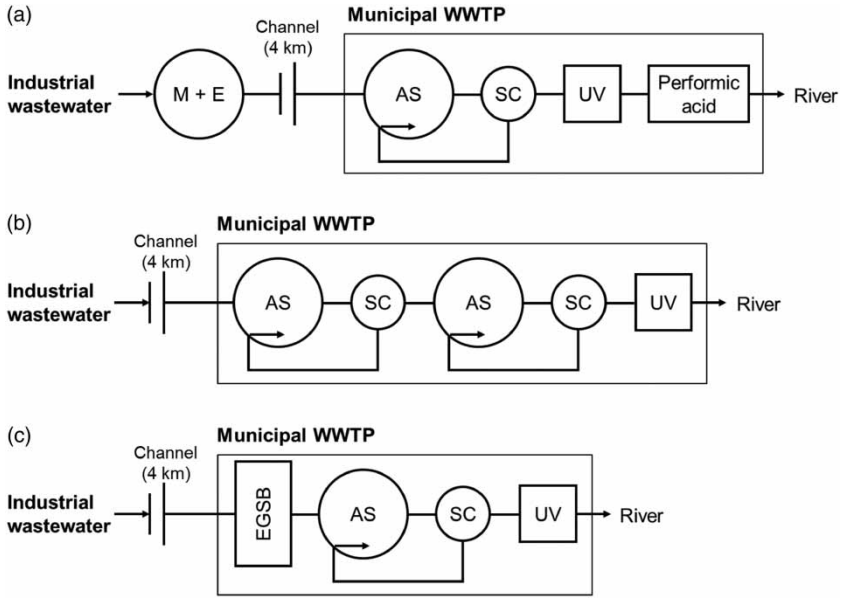

Figure 1 | Layout of the WWTP in Warstein: (a) until July 2014, (b) at present (shut-down of the aerobic pre-treatment of the industrial wastewater), (c) near future (construction of an anaerobic pre-treatment for the industrial wastewater). AS: activated sludge, EGSB: expanded granular sludge bed reactor, M + E: mixing and equalization tank, SC: secondary clarifier, UV: ultraviolet disinfection.

process for the industrial wastewater, already under construction, will replace the high-load activated sludge process, converting organic matter to methane.

The aims of the present study were: (i) to assess the effect of several chemical and physical methods on the reduction of *Legionella* both in the activated sludge and in the effluent discharged to the river Wäster; and (ii) to understand how wastewater characteristics (temperature, carbon source and aerobic pre-treatment of industrial influent) affect the growth of *Legionella*.

MATERIALS AND METHODS

The influent entering the Warstein municipal WWTP comprises two-thirds domestic wastewater and one-third industrial wastewater flowing through a separate channel (Figure 1). The industrial wastewater has a temperature around 30–35 °C and is rich in organic nitrogen (protein concentration ca. 400 mg/l).

Experimental design

The concept for the present study is depicted in Figure 2 and comprises two main research activities, briefly explained as follows.

Investigating the wastewater characteristics influencing the growth of *Legionella*

Effect of the aerobic pre-treatment

The municipal WWTP was sampled before (until July 2014) and after (from August 2014 to July 2015) the shutdown of the aerobic pre-treatment of the industrial wastewater. Samples were collected from the industrial wastewater entering the municipal WWTP and at the effluent discharged to the river Wäster for quantification of *Legionella* spp. by the culture method.

Effect of temperature and substrate

To study the effect of temperature on the growth of *Legionella* in activated sludge a defined substrate was selected. Preliminary experiments showed that *L. pneumophila* grew well at 35 °C in a synthetic wastewater containing meat extract and peptone (DIN 38412-26 1994) and in a suspension of yeast cells (spent yeast). In the present study three laboratory-scale activated sludge reactors (1 l) were fed with spent yeast (0.65 kg COD/m^3·d) and operated at a sludge retention time of 10 d for 8 weeks. Three different temperatures were tested: 15 °C, room temperature (22–26 °C) and 35 °C. Samples were taken weekly for the quantification of *L. pneumophila*

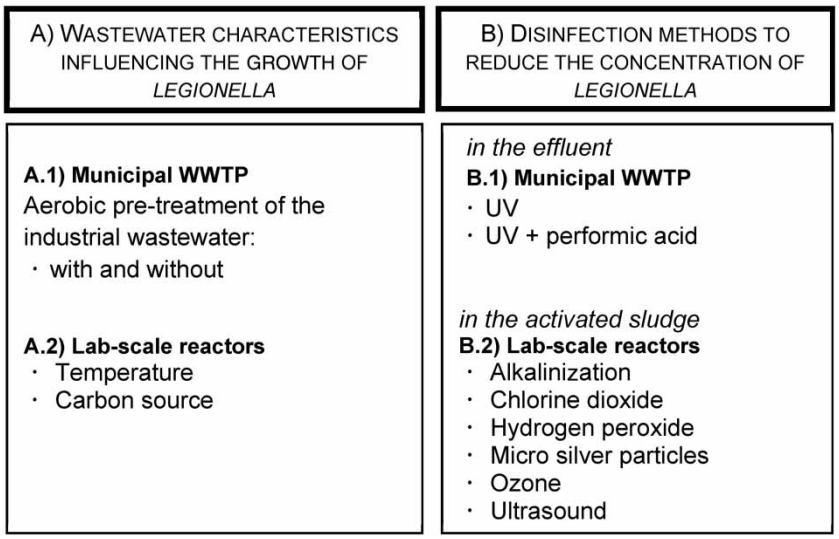

Figure 2 | Reduction measures (chemical–physical methods) and wastewater characteristics investigated in this study.

concentrations by the real-time quantitative polymerase chain reaction (qPCR) method.

Testing disinfection methods to reduce the concentration of *Legionella*

In the effluent from the secondary treatment

A UV (254 nm) disinfection facility was installed at the municipal WWTP after the secondary treatment to reduce the concentration of *Legionella* discharged to the river Wäster. An additional chemical disinfection with performic acid (a mixture of hydrogen peroxide and formic acid) was implemented from October 2013 to February 2014. Samples were taken before and after chemical disinfection for the quantification of *Legionella* spp. concentration by the culture method in the period between August 2013 and July 2015.

In the activated sludge

Six parallel laboratory-scale activated sludge sequencing batch reactors (SBRs, 0.8 l each) were fed with industrial wastewater (230 mg COD/l) and operated at a hydraulic retention time of 10 h for 12 weeks at room temperature (24 °C). The SBRs were operated in cycles of 2 h (12 cycles/d). Each cycle started with 90 min of aeration with influent addition during the first 10 min. Subsequently, there was a sludge settling phase of 25 min, after which the effluent was withdrawn for 5 min.

In five of the six reactors, a defined volume of activated sludge (10% and 25% of the reactor's volume) was removed from the reactors and treated three times a week with the following chemical agents: Microsil (silver micro-particles), hydrogen peroxide (H_2O_2), chlorine dioxide (ClO_2), ozone (O_3), and alkalization (pH 12). After treatment the activated sludge was returned to the reactors. One control reactor was operated without dosing of chemicals at room temperature (24 °C). Four different testing periods can be distinguished in which different dosages, reaction times and volumes of activated sludge were applied for each treatment (Table 1). The effect of each treatment on the concentration of *Legionella* spp. in the reactor's effluent was evaluated. In testing periods 2 and 3, the instantaneous effect was also evaluated by measuring the concentration of *Legionella* spp. in the supernatant of the treated activated sludge volume before returning it to the reactors. In a separate experiment ultrasonic treatment of the activated sludge was also tested (2 W·h/l and 50 W·h/l, 20 kHz). The concentration of *Legionella* spp. was measured by the culture method and the concentration of *Legionella pneumophila* was measured by the qPCR method.

Methods

The concentration of *Legionella* spp. was quantified by the culture method (ISO 11731-2 2004) and expressed as CFU/100 ml (CFU: colony forming units). Briefly 1 l samples were collected in sterile polyethylene containers and transported at room temperature to the laboratory.

Table 1 | Overview of the applied treatments on the activated sludge in the laboratory-scale SBR (dosages are expressed per unit of total suspended solids (TSS))

Treatments	Microsil	Hydrogen peroxide	Chlorine dioxide	Ozone	Alkalization (pH 12)
Period 1 (0–21 d)					
Daily dosage	6.4 μg Ag/g TSS.d	122 mg/g TSS.d	212 μg/g TSS.d	9.8 mg/g TSS.d	1.7 meq/g TSS.d
Treated volume	10%	10%	10%	10%	10%
Contact time	0.5 h	0.5 h	0.5 h	0.5 h	0.5 h
Period 2 (22–48 d)					
Daily dosage	4.5 μg Ag/g TSS.d	99 mg/g TSS.d	163 μg/g TSS.d	24 mg/g TSS.d	1.7 meq/g TSS.d
Treated volume	10%	10%	10%	10%	10%
Contact time	1 h	1 h	1 h	1 h	1 h
Period 3 (49–63 d)					
Daily dosage	11 μg Ag/g TSS.d	372 mg/g TSS.d	0.68 mg/g TSS.d	111 mg/g TSS.d	3.2 meq/g TSS.d
Treated volume	10%	10%	10%	10%	10%
Contact time	1 h	1 h	1 h	2 h	2 h
Period 4 (64–84 d)					
Daily dosage	73 μg Ag/g TSS.d	2,504 mg/g TSS.d	2.4 mg/g TSS.d	237 mg/g TSS.d	6.8 meq/g TSS.d
Treated volume	25%	25%	25%	25%	25%
Contact time	2 h	2 h	2 h	2 h	2 h

The samples were homogenized and pre-treated with HCL (pH 2.2 for 5 min) or heat (50 °C for 30 min) to suppress the growth of background flora. Subsequently the samples were serially diluted and plated on GVPC agar (the medium contains the antibiotics cycloheximide, polymyxin and vancomycin). The plates were incubated at 37 °C for 7 to 10 d.

The concentration of *L. pneumophila* was quantified by the qPCR method and expressed as GU/ml (GU: genomic units). The samples for DNA extraction were processed using the Qiagen QIAamp Fast DNA Stool Mini Kit DNA extraction kit (Quiagen, Germany). The fluorescence-based qPCR was performed on a Rotor-Gene Q duplex (Quiagen) with the mericon Quant *L. pneumophila* Kit (Qiagen) for the detection and quantification of *L. pneumophila* in water. The mericon PCR assays include an internal control to monitor potential inhibition that was co-amplified in each qPCR reaction with the target DNA. Quantification of the target *Legionella* species was done using a standard curve comprising a concentration range of 25,000–25 copies per reaction. The assay can detect six copies of *Legionella* DNA in a reaction (limit of detection). The qPCR amplification curves were processed and concentrations were determined using the Rotor-Gene Q-series system software, version 2.3.1 (Qiagen).

RESULTS AND DISCUSSION

Wastewater characteristics influencing the growth of *Legionella*

The concentration of *Legionella* spp. in the industrial wastewater entering the municipal WWTP was high in the period from August 2013 to 2014 (Figure 3) and reached maximum values around 10^9 CFU/100 ml. A possible explanation for this result is that in the aerobic pre-treatment of the industrial wastewater (at the source) the growth of *Legionella* was favored by the presence of dissolved oxygen, a temperature above 30 °C and the availability of organic nitrogen (protein). Similar results have been reported in the literature for industrial wastewater from the paper mill industry, petrochemical industry and dairies (Allestam *et al.* 2006; Fykse *et al.* 2013; Lund *et al.* 2014).

The subsequent flow of the pre-treated wastewater through the channel and the contact with the biofilm on the walls might have had a multiplicative effect on the concentration of *Legionella*. As expected, following the shutdown of the aerobic pre-treatment in August 2014, the concentration of *Legionella* in the wastewater entering the WWTP decreased considerably (Figure 3). This observation suggests that *Legionella* had been growing in the aerobic pre-treatment under the prevailing optimal conditions and

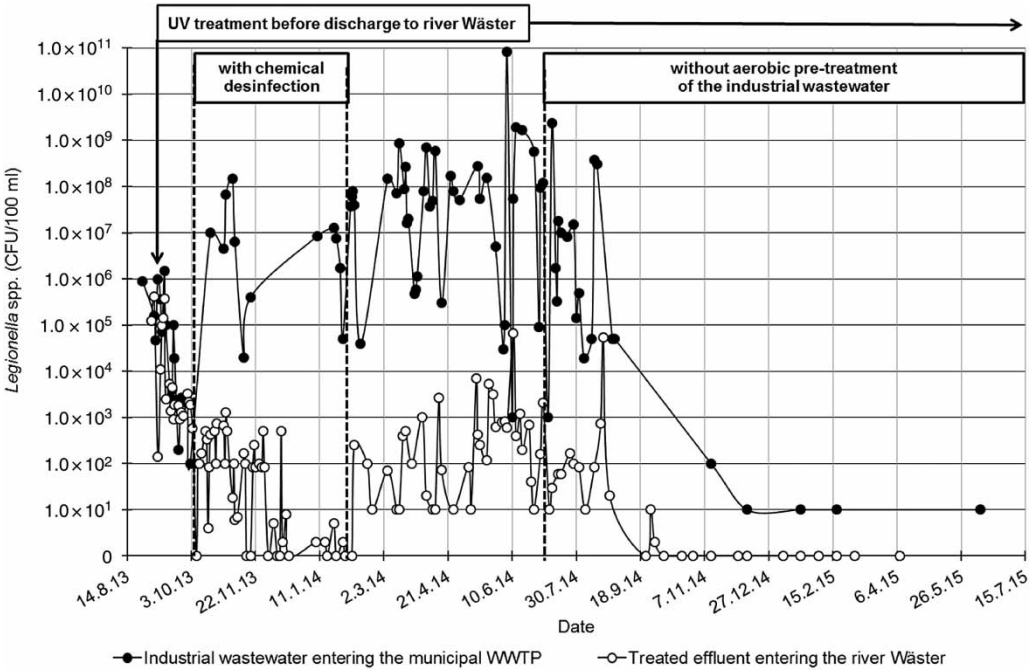

Figure 3 | *Legionella* spp. concentrations at the industrial wastewater entering the municipal WWTP and at the effluent discharged to the river Wäster. The vertical arrow indicates the beginning of the operation of the UV unit installed after the secondary treatment.

that the industrial wastewater was continuously inoculating the WWTP with *Legionella*. The shutdown of the pre-treatment had a positive effect on the microbiological quality of the treated effluent discharged to the river Wäster, the concentration of *Legionella* being below the limit of detection of the cultivation method after October 2014.

Laboratory-scale experiments (Figure 1) were done to assess the specific growth rate of *Legionella* under optimal conditions and the effect of temperature on the growth. Spent yeast was used as substrate because yeast is a known source of amino acids. The results showed that the growth of *L. pneumophila* was favored at 35 °C compared to room temperature, whereas at 15 °C *L. pneumophila* did not grow. The specific growth rate obtained for *L. pneumophila* at 35 °C is 3.8 d^{-1}. A similar growth rate was obtained with a synthetic wastewater containing pepton and meat extract, two protein-rich substrates.

Interestingly, it was observed that 15 °C selected for the growth of non-*L. pneumophila* species in the activated sludge. Our findings support those of Wullings and co-workers (Wullings & van der Kooij 2006; Wullings *et al.* 2011), who reported the presence of putative psychrophilic *Legionella* species in water at temperatures below 15 °C. In the present study the affiliation of the non-*L. pneumophila* species is being investigated and will allow an evaluation of the hygienic relevance of these bacteria to human health.

Disinfection methods to reduce the concentration of *Legionella*

To reduce the concentration of *Legionella* in the treated effluent entering the river Wäster, a UV disinfection unit was installed after the secondary treatment in August 2013 and has been in operation since then. Figure 4 compares the concentration of *Legionella* in the wastewater before and after the UV treatment and a reduction of about 1.6–3.4 log units can be observed. For a short period of time, between October 2013 and February 2014, a combination of UV and performic acid disinfection was tried. After an initial period of optimization of the chemical dosage, it can be observed that the performic acid potentiated the effect of the UV treatment, decreasing the concentration of *Legionella* to values close to the limit of detection of the culture method.

Chemical disinfection agents to reduce the concentration of *Legionella* in the activated sludge were tried at laboratory scale. Table 2 summarizes the concentrations of *Legionella* spp. in the effluents from the reactors treated with different chemicals. In general the treatment of activated sludge with Microsil (6.4–73 μg Ag/g TSS·d; TSS: total suspended solids), hydrogen peroxide (122–2,504 mg/g TSS·d), chlorine dioxide (0.2–2.4 mg/g TSS·d), and ozone (9.8–237 mg/g TSS·d) as well as an increase in pH to 12 (alkalization) did

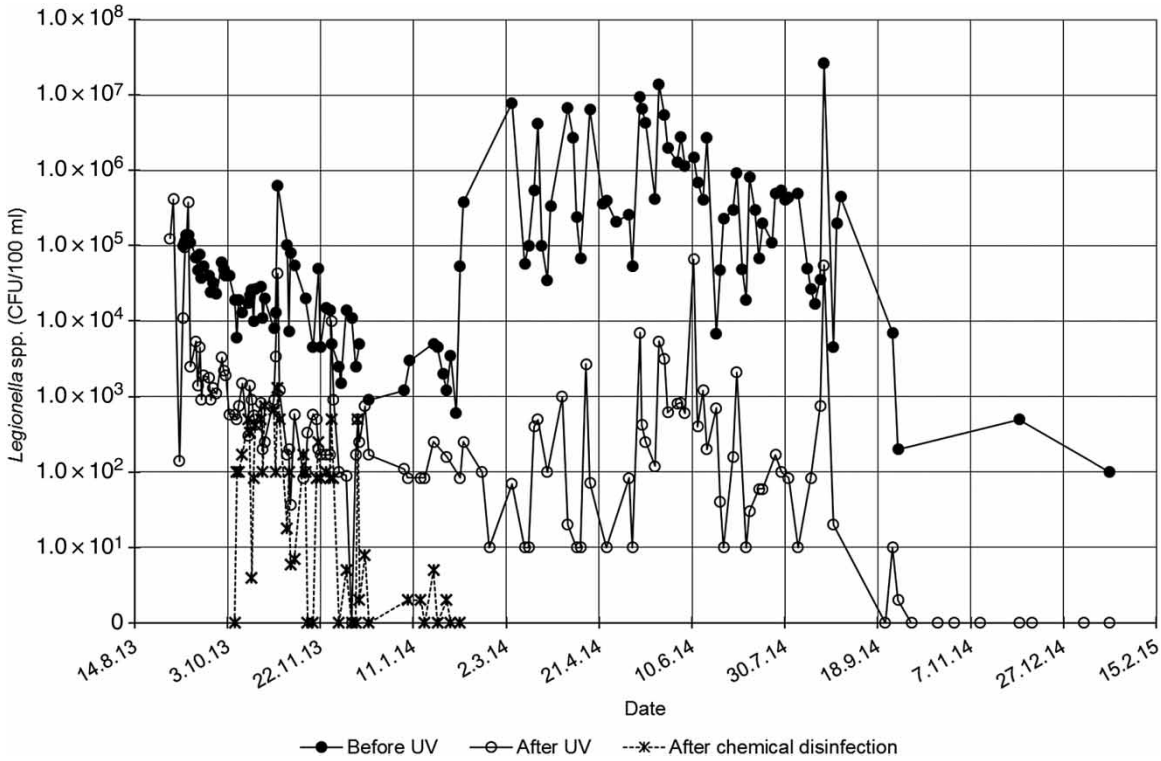

Figure 4 | *Legionella* spp. concentrations before and after the UV treatment and after chemical disinfection.

Table 2 | Concentration of *Legionella* spp. in the different effluent samples of the six activated sludge SBRs (measurements done in duplicate)

	***Legionella* spp. (CFU/100 ml)**			
Treatments	Period 1 (0–21 d)	Period 2 (22–48 d)	Period 3 (49–63 d)	Period 4 (64–84 d)
Influent	8.05×10^7	6.45×10^6	7.00×10^6	5.05×10^6
Effluent				
Control (24 °C)	9.20×10^7	4.35×10^4	7.40×10^4	1.15×10^5
Microsil	$>1.00 \times 10^8$	2.55×10^4	4.60×10^5	7.40×10^4
Hydrogen peroxide	5.15×10^7	4.10×10^4	3.50×10^6	5.20×10^5
Chlorine dioxide	1.28×10^8	6.60×10^4	4.05×10^5	1.27×10^5
Ozone	$>1.00 \times 10^8$	4.85×10^6	4.80×10^4	1.53×10^5
Alkalization (pH 12)	7.15×10^5	6.05×10^4	2.35×10^5	5.35×10^5

not result in the reduction of the concentration of *Legionella* spp. in the effluents in comparison to the situation without dosing of chemical agents (control reactor). A significant instantaneous reduction in the concentration of *Legionella* spp. in the activated sludge was observed for the treatment with pH (pH 12 for 1 h) and chlorine dioxide at 16 mg/g TSS. However, the instantaneous effect of these two treatments on the activated sludge did not result in a decrease of the concentration of *Legionella* spp. in the effluent of the reactors. The application of higher chemical dosages is not recommended because they decrease the nitrifying capacity of the activated sludge.

The ultrasonic treatment of the activated sludge with 2 W·h/L destroyed completely naked amoeboid cells, protozoa known to serve as hosts for the intracellular multiplication of *Legionella*, but had no significant effect on

the concentration of *L. pneumophila* measured with the qPCR method. An increase in the energy input to 50 W·h/L decreased slightly the concentration of *L. pneumophila* (below 1 log unit) but disrupted considerably the structure of the activated sludge flocs, hindering their fast sedimentation. The reduction in *L. pneumophila* obtained with the qPCR method is an underestimation because this method, besides viable cells, also quantifies viable-but-not-cultivable and dead cells. Supplementary studies showed that amoeboid cells were able to recolonize the activated sludge previously treated with ultrasound (2 W·h/L) during an incubation step at 35 °C with the addition of substrate (yeast cells). This result suggests that, under optimal growth conditions, new amoeboid cells developed from existing amoeba cysts (a very resistant cell structure) in the activated sludge. Consequently, under favorable environmental conditions, amoeboid cells may serve as a host for the intracellular multiplication of *Legionella*, making the ultrasonic treatment of activated sludge unsuitable for a practical application.

Strategies to minimize the growth of *Legionella* in activated sludge

The present study showed that industrial wastewater with a temperature of 30–35 °C and containing organic nitrogen (protein) has the potential to stimulate the growth of *Legionella* under aerobic conditions. Lowering the temperature of the industrial wastewater (<20 °C) can certainly minimize the growth of *L. pneumophila*, but the associated high-energy costs for cooling (without heat recovery) may make this measure unfeasible. An alternative strategy is to remove the organic nitrogen from the industrial wastewater in an anaerobic pre-treatment before mixing with the domestic wastewater. Under anaerobic conditions, unsuitable for the growth of *Legionella*, organic nitrogen in the form of proteins is converted to methane and ammonium. In the subsequent aerobic treatment planned for the removal of the remaining carbon and ammonium, a significant growth of *Legionella* is not to be expected due to substrate limitation and a lower temperature resulting from the mixing with domestic wastewater. An anaerobic reactor is under construction at the Warstein WWTP (Figure 2(c)) and in the near future the efficacy of this strategy on the reduction of *Legionella* growth in activated sludge systems can be assessed.

Recommendations for reducing aerosols emission

Reduction of aerosols emission from sewers and WWTPs where positive results were obtained for *L. pneumophila* is an integral part of a prevention strategy to minimize the risk of infection. It is recommended to substitute surface aerators with diffusers placed at the bottom of the activated sludge tanks and to cover the tanks. Knowledge about the aerosolization efficiency and travelling distance and survival of *Legionella* in the aerosols is needed to avoid over dimensioned strategies for managing risks (Nguyen *et al.* 2006).

In case the treated wastewater contains *L. pneumophila* in concentrations considered to be not acceptable for a direct discharge in surface waters, a disinfection step needs to be included. At the Warstein WWTP a UV disinfection of the effluent discharged to the river Wäster was successfully implemented.

CONCLUSIONS

WWTPs receiving warm wastewater rich in proteins have an increased risk of contamination with *L. pneumophila*, especially in the aerobic treatment. Chemical and physical methods to reduce the concentration of *Legionella* in activated sludge are not feasible due to their non-specificity, thus decreasing the activity of other key microorganisms, i.e. nitrifiers. It is also not possible to wash out *Legionella* by reducing the sludge retention time (<10 d) without compromising the nitrification performance. Future research is needed to determine precisely the temperature and substrate combination that trigger the growth of *L. pneumophila* in activated sludge, the influence of an anaerobic pre-treatment of the protein-rich wastewater on the subsequent growth of *Legionella* under aerobic conditions and the protozoan hosts of *Legionella* in the activated sludge.

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