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Temperature-driven growth of *Legionella* in lab-scale activated sludge systems and interaction with protozoa

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ABSTRACT

The occurrence of *Legionella pneumophila* in activated sludge systems has been reported in the literature. However, the factors triggering its growth are not yet well understood. This knowledge is needed to develop strategies to minimize the risk of the spread of Legionnaires' disease that originates in these systems. In the present study, *L. pneumophila* multiplied exponentially at 35 °C in activated sludge, but lower temperatures (24 °C and 15 °C) did not favour the growth of the pathogen despite the presence of its protozoan hosts (free-living amoeba and ciliates). L. non-*pneumophila* species, which are adapted to low temperature, prevailed at 15 °C. *Legionella* was found dispersed in the activated sludge, forming clusters of different sizes and also inside protozoa. This study shows that temperature is a key parameter triggering the growth of *L. pneumophila* in activated sludge. The detection of infected protozoa suggests that these are important vehicles for the multiplication of *Legionella*. However, in this study it was not possible to exclude the growth of *Legionella* directly on the substrate or on the activated sludge matrix.

1. Introduction

L. pneumophila is the main causative agent of Legionnaires' disease, a lung infection that occurs through the inhalation of contaminated aerosols. This bacterium is an ubiquitous inhabitant of various natural and human-made habitats, including wastewater treatment plants (WWTP) (Allestam et al., 2006; Kusnetsov et al., 2010; Nogueira et al., 2016). In these treatment systems, activated sludge tanks are the main sources of aerosols generation (Medema et al., 2004). Until 2015, WWTP were reported as the infection source of 169 cases of Legionnaires' diseases, with *L. pneumophila* being the isolated strain (Van Heijnsbergen et al., 2015).

L. pneumophila is strictly aerobic (Wullings and Van Der Kooij, 2006) and requires amino acids as its main source of carbon and energy (George et al., 1980; Tesh and Miller, 1981). However, *L. pneumophila* is auxotrophic for several amino acids, namely arginine, cysteine, isoleucine, leucine, methionine, threonine and valine. To overcome this limitation, *L. pneumophila* parasitizes protozoan hosts and highjacks their amino acids synthesis machinery for self-profit (Price et al., 2014). In nutrient poor water systems, the interaction with protozoa is considered to be the main strategy to overcome the amino acids limitation (Caicedo et al., 2016; Taylor et al., 2009). However, the multiplication of *Legionella* independently from protozoa cannot be ruled out. *Legionella* can uptake essential nutrients from dead microorganisms (Temmerman et al., 2006), from nutrients excreted by other bacteria or blue-green algae (Tison et al., 1980) and from sediments or decaying organic matter (Devos et al., 2005).

Several studies have showed that *L. pneumophila* can multiply in free-living amoeba (FLA), namely *Hartmannella*, *Acanthamoeba.,* and *Naegleria* (Anand et al., 1983; Rowbotham, 1980). FLA are not only a nutrient source, but also a shelter when environmental conditions are unfavourable. Both amoeba and *Legionella* are extremely resistant to disinfection agents, particularly during the cyst stage, where *Legionella* increases its virulence after amoeba passage (Scheikl et al., 2014). Interestingly, temperature plays a key role not only in the multiplication of *L. pneumophila,* but also in the bacteria-host interaction. Ohno et al. (2008) reported that *L. pneumophila* infects and multiplies in *Acanthamoeba castellani* at temperatures higher than 20 °C, but at lower temperatures *L. pneumophila* is eliminated by its host.

In the recent years, high concentrations of *L. pneumophila* have been reported in biological treatment systems receiving effluents from the forest and paper mill industries, 10^9 CFU/mL (Allestam et al., 2006), and from the brewery industry, 10^4–10^7 CFU/mL (Nogueira et al., 2016). A temperature around 35 °C is the common factor in both industrial effluents, having a completely different wastewater composition. Until now, the influence of operational and environmental factors on the proliferation of *L. pneumophila* in activated sludge systems is still poorly understood. Further research to elucidate the growth strategies

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of *L. pneumophila* in activated sludge systems can lead to the development of feasible long-term strategies to prevent the proliferation of L. pneumophila in WWTP. Therefore, the aims of this study were: i) to investigate the effect of temperature (15 ± 1 °C, 24 ± 2 °C and 35 ± 1 °C) on the multiplication of *L. pneumophila* and ii) to assess the *Legionella*-protozoa interaction in activated sludge systems.

2. Material and methods

2.1. Lab-scale sequencing batch reactors

Experiments were conducted in sequencing batch reactors (SBR) of 2 L (1.5 L liquid volume) operated at a constant sludge retention time of 10 days, aerated with fine bubble diffusers, and fed daily with 18 mL of 1:10 diluted spent yeast (liquid waste from the food and beverage industry) to reach a volumetric loading rate of 0.65 kg COD/m^3 d (being COD the chemical oxygen demand).

2.2. Physicochemical analyses

The organic content of the spent yeast was characterized by its COD concentration, measured with the standardized photometric cuvette test from Hach-Lange (LCK 514). The nitrogen content was characterized based on the total nitrogen, protein, and amino acids concentrations. The total nitrogen was calculated as the sum of the Kjeldahl nitrogen plus nitrite and nitrate. The Kjeldahl nitrogen was determined according to DIN (DIN EN 25663:1993-11) and nitrite and nitrate were determined by HACH Lange cuvettes (LCK 341, LCK 340). The protein was determined with the standardized Lowry Assay Kit (SERVA Cat. No.39236). Amino acids were determined by high performance liquid chromatography described in detail in Caicedo et al. (2016). The total phosphorus concentration was determined with the HACH Lange cuvettes (LCK 350). The inoculum was characterized by the total suspended solids (TSS) concentration (DIN 38414-2,3).

The COD, total nitrogen, total phosphorous concentrations in the SBR immediately after the addition of the spent yeast were: 393 mg/L, 17 mg/L and 2,3 mg/L. The protein and amino acid concentrations were: 1,8 mg N/L and 1,6 mg N/L respectively. To determine the performance of the reactors, two samples were taken weekly from the three SBR for the analysis of the dissolved COD concentration (Results shown in Fig. S1 Appendix A in Supplementary material). The performance of the SBR was on average between 88% within the experimental period.

2.3. Selection of the inoculum

In order to select an inoculum that contained *L. pneumophila,* and its growth could be observed at 35 °C, two different inocula were tested: activated sludge from a WWTP receiving only domestic wastewater (500,000 P.E., 65,000 m^3/day), and activated sludge from a WWTP receiving 1/3 of its influent from the food and beverage industry (45,200 P.E., 10,767 m^3/d), later referred to as domestic sludge and municipal sludge, respectively. Two SBR were fed with spent yeast and run in parallel at 35 ° C for 6 days under the operating conditions previously described. Mixed liquor activated sludge samples for the quantification of *L. pneumophila*, and FLA were taken at the beginning and at the end of the experimental period.

2.4. Experiments at different temperatures

Three SBR were inoculated with municipal sludge, fed with spent yeast and operated at 15 ± 1 °C, 24 ± 2 °C and 35 ± 1 °C for 26 days. Mixed liquor activated sludge samples were taken twice a week for the quantification of *L. pneumophila* and *Legionella* spp.

2.5. Legionella-amoeba interaction in activated sludge

To clarify the role of protozoa in the multiplication of *Legionella*, experiments were performed with untreated activated sludge and activated sludge that was previously treated with ultrasound to eliminate FLA. Municipal sludge (75 mL) was treated at a frequency of 20 kHz with an ultrasonic energy of 2 W h/L in a closed double-glass Rosett cell (100 mL). An ultrasonic disruptor Sonifier II model W-250 coupled to a 6.5 mm tapered microtip with amplitude of 59.5–247 μm was used in the experiments (Branson, Germany). Mixing was provided by circulation of the sludge driven by the pressure of the ultrasonic energy through the 3 arms of the Rosetta cell and the main chamber. Cooling water was circulated in the water jacket, keeping the temperature of the samples below 20 °C. Two SBR were subsequently inoculated with untreated and ultrasonic treated sludge and operated in parallel at 35 °C for 6 days, as previously described, to assess the effect of the presence/absence of amoebic trophozoites on the growth of *Legionella*. Mixed liquor activated sludge samples for the quantification of *L. pneumophila*, *Legionella* spp. and FLA were taken at the beginning and at the end of the experimental period.

2.6. Microbiological analyses

2.6.1. Sampling and sample preservation

Mixed liquid activated sludge samples collected from the lab-scale reactors were frozen at −20 °C for qPCR analysis or immediately processed for the microscopic quantification of FLA. For fluorescence *in situ* hybridization (FISH), mixed liquor activated sludge samples were collected from the SBR, fixed with 4% paraformaldehyde and stored at −4 °C (Manz et al., 1995).

2.6.2. qPCR for the detection and quantification of Legionella

Quantification of *L. pneumophila* and *Legionella* spp. concentrations was done by using the qPCR method as described in detail in Caicedo et al. (2016). Briefly, the samples were processed for DNA extraction using the extraction kit QIAamp Fast DNA Stool Mini Kit DNA (Qiagen, Germany). The extracted DNA was measured with a Qubit 2.0 Fluorometer and stored at −20 °C prior to PCR amplification. The fluorescence-based qPCR was performed on a Rotor-Gene Q duplex (Qiagen) with the mericon Quant *Legionella* spp. Kit (Qiagen) for the detection and quantification of *Legionella* spp. in water, and the mericon Quant *Legionella pneumophila* Kit (Qiagen) for the detection and quantification of *Legionella pneumophil*a 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 6 GU (Genomic Units) of *Legionella* DNA in a reaction (limit of detection of the qPCR) and 60 GU in 1 mL sample (limit of detection of the method including the DNA extraction). The limit of quantification of the qPCR is 12 GU in a reaction and the limit of quantification of the method is 120 GU in a 1 mL sample. A Rotor-Gene Q-series system software version 2.3.1 (Qiagen) was used to process the qPCR amplification curves and to determine the target *Legionella* concentrations. The qPCR results are expressed as GU/mL.

2.6.3. Microscopic quantification of FLA

The microscopic cell counts of FLA were made with a Fuchs-Rosenthal chamber as described in Madoni (1994). Untreated activated sludge samples freshly collected from the SBR were immediately analysed.

2.6.4. Fluorescence in situ hybridization (FISH)

L. pneumophila, Legionella spp. and eukaryotes were detected using the rRNA-targeted oligonucleotide probes LEGPNE (Grimm et al., 1998), LEG705 (Manz et al., 1995) and EUK516 (Amann et al., 1990),

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