Detection of *Legionella pneumophila* in a Biological Treatment Plant by Co-Cultivation with *Acanthamoeba castellanii*

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Abstract: *Legionella pneumophila* was identified in the aeration ponds of a biological wastewater treatment plant at the pulp and paper industry Borregaard, Sarpsborg, Norway. After 3 outbreaks of Legionaires' disease reported in this area in 2005 and 2008, the aeration ponds were shut down by the Norwegian authorities in September 2008. During the shutdown of these ponds, September to December 2008, the viable counts of *L. pneumophila* decreased from 10^7 to < 10 CFU/mL measured using the International Standard growth (ISO11731) method. The aim of this work was to use amoebal co-culture with *Achantamoebae castellanii* to recover and detect *L. pneumphilia* from the complex microbial community in the pond during the shutdown period. This work shows that the viable counts of the environmental *L. pneumophila* ST 462 outbreak strain present in the pond samples during shutdown, was increased from 0-10 CFU/mL (no amoebae added) to 10^7 -10^8 CFU/mL in co-culture with *A. castellanii*. This indicates that pathogenic *L. pneumophila* isolates present in the environment may not be detected using standard culture methods. As a consequence, methodological improvements are needed to ensure more reliable detection and isolation of *Legionella*. By using amoebal co-culture, the concentration of *L. pneumophila* increased by 5-7 log units, allowing low concentrations and bacteria not detected using standard growth methods (according to the ISO11731), to be detected. Cells in the viable but non-culturable (VBNC) form will not be detected using the ISO 11731 standard culture method, and growth on agar media may be inhibited by other organisms and inhibitors present in complex environmental samples. The methodological procedure described in this paper may assist in providing a general more robust and sensitive approach to detect *L. pneumophila* in more complex environmental samples and may assist in providing improved hazard assessments.

Keywords: *Achantamoebae castellanii*, aeration pond, amoebal co-culture, *Legionella pneumophila*, wastewater treatment plant.

INTRODUCTION

Legionella pneumophila is the etiological agent of Legionaires' disease (LD) and the non-pneumonic legionellosis Pontiac fever. More than 50 *Legionella* species with more than 70 distinct serogroups have been classified. An infection is usually caused by inhalation of aerosols containing this bacterium. *L. pneumophila* is responsible for 90 % of the clinical cases of LD, of which 92 % are caused by *L. pneumophila* serogroup 1(SG1) [1]. In 2005 and 2008, three outbreaks of LD were reported in the Fredrikstad/Sarpsborg community, Norway. In 2005, 56 people were infected and 10 died [2] and later, an additional 50 people were diagnosed with LD. The outbreak strain in 2005 was *L. pneumophila* ST15. In 2008, five cases of LD were caused by another strain *L. pneumophila* ST462, SG1. *L. pneumophila* ST462 was identified at concentrations up to 10^7 CFU/mL in the aeration ponds of the biological treatment plant at the wood and pulp factory Borregaard Ind. Ltd., Sarpsborg, Norway which is a world leading supplier of lignin-based chemicals [3]. *L. pneumophila* has also been measured at concentration levels up to 3300 CFU/L (*L. pneumophila* BLA3 SG4) in air samples taken above the aeration ponds and up to 200 m downwind from the ponds [4, 5]. Based on these findings the Norwegian Climate and Pollution Agency decided in 2008 to shut down the aeration ponds of Borregaard's biological treatment plant. Prior to the shutdown in September 2008, the biological treatment plant consisted of two large aeration ponds kept at 37 °C for optimal growth of microorganisms including *Legionella* species to obtain degradation of organic compounds, e.g. lignin [3-6]. Using the ISO 11731 standard cultivation method demonstrated that the concentration level of *L. pneumophila* in the aeration ponds decreased by 6-7 log units during the shutdown process from September to December 2008. Quantitative real-time PCR analysis of the *L. pneumophila mip* gene complemented the findings and showed a similar decrease of legionellae DNA [3, 6]. At 17 November, biocide (hypochlorite) was first added to the ponds and after 15 December no growth of legionellae was identified using the ISO11731 standard method. The ISO11731 standard culture method may underestimate the legionellae concentration in environmental samples containing inorganic and organic compounds in addition to a diverse bacterial community [7, 8]. The non-legionellae bacteria can overgrow the agar plates and prevent detection of legionellae. Therefore, if *Legionella* is not detected by the ISO 11731 standard method in a complex sample, it cannot be ruled out that viable *Legionella* is present.

The presence of legionellae in wastewater treatment plants is well documented [2, 3, 9-12]. In Norway, legionellae have been identified in 27 % of the biological treatment plants analyzed, and in pulp and paper industry 75 % of the plants contained legionellae [10, 13]. Two cases of LD's have also

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been reported among employees at two industrial wastewater treatment plants in Finland [11]. It is assumed that protozoa such as amoebae play a role for growth and survival of *Legionella* in wastewater treatment plants, and protozoa has been identified in pond samples from Borregaard by 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) staining at 10^5cells/mL, and at the same time the legionellae count was 10^7 CFU/mL [9, 10]. However, the complete understanding of the environmental and other, yet unidentified factors, that contributes to survival, growth and dissemination of *Legionella* as aerosols in ambient air or in complex aqueous environment (e.g. wastewater plants) along with other microorganisms and protozoa is limited [6].

The survival of *Legionella* in the environment is enhanced by their symbiotic relationships with protozoa, algae, and other bacteria, providing them with advantages (protection, source of nutrients) in the natural environment and in potable water distribution systems [14]. Multiplication of *L. pneumophila* in *Achanthamoeba* has previously been shown to enrich pathogenic strains present in the environment [14, 15], and legionellae proliferated within amoebic hosts tend to infect macrophages at a higher rate than cells replicated extracellularly [16]. A mouse model of co-inhalation of *L. pneumophila* and the amoebae *Hartmannella vermiformis* showed that co-inhalation with *H. vermiformis* significantly enhanced the intrapulmonary growth of *L. pneumophila*, resulting in greater mortality than that from inhalation of legionellae [17]. It is assumed that the presence of disinfectants in potable water systems promote selection of *Legionella* strains that have been protected within amoebae and thus have the potential to become pathogenic and cause illness if disseminated to humans [18]. *L.pneumophila* can persist for long periods in natural and artificial aquatic environments and eradication by disinfection of the bacterium from plumbing systems is often difficult [19, 20]. *Legionella* may be present as viable, viable-but-not-culturable (VBNC) or non-viable cells in the environment, and VBNC *Legionella* is able to survive in the environment and to be resuscitated *via* co-culture with the *A. castellanii* [21, 22]. It is shown that *L. pneumophila* could persist for long time in biofilm in a VBNC state after treatment of the system with monchloramine, and the VBNC bacteria could be resuscitated by co-culture in the amoeba *A. castellanii* [23]. Such cells may constitute potential sources of contamination and should be taken into account in monitoring water systems.

The most commonly used technique for environmental surveillance of *Legionella* spp. is the standard ISO11731 culture method [7, 24], which fails to detect low levels of legionellae in complex samples containing inorganic and organic compounds and high microbial diversity. The aim of the present study was to use the amoebal co-culture method to recover and detect pathogenic *L. pneumophila* cells present in a complex microbial community in pond samples collected during the shutdown of the aeration pond at the biological treatment plant at Borregaard in 2008. The results showed that the outbreak *L. pneumophila* ST462 strain was recovered and replicated in co-culture with *A. castellanii* in aeration pond samples harvested throughout the shutdown period.

MATERIAL AND METHODS

Sample Collection

Based on previous findings of *Legionella* in a biological wastewater treatment plant at Borregaard Ind. Ltd., Sarpsborg, Norway [3, 4] the Climate and Pollution Agency (under the Ministry of the Environment) initiated a four month long shutdown process of the aeration ponds (activated sludge) (September to December 2008), and a biocide (hypochlorite) was injected first into the system the 17 November. The activated sludge facility consisted of two large aeration ponds (each 2500 m^2) each containing 30,000 m^3 of liquid kept at 36-38 °C, and 30 000 m^3 of air was pumped through it every hour for optimal growth of bacteria. From 4 September the influx of waste material (substrate for the bacteria) and the airflow into the ponds (3503 and 3504) were stopped. Samples (500 ml) were collected from the two aeration ponds at the same position and they were immediately transported to the laboratory (1.5 hours) and stored at 4 °C on arrival [3, 6]. In this work samples (500 mL) collected 25 September, 3 November and 1 December from the aeration pond (3503) were used in all experiments. These samples were stored at 4 °C for 36 months before the co-culture experiments.

Bacterial Strains and Cultivation

The following *L. pneumophila* strains from Borregaard were used: ST462 SG1[1], ST15 SG1[2], ST458 SG1[3], and BLA3 SG4[4]. The Colitax SG2-14 strain was obtained from the European Working Group for *Legionella* Infections (EWGLI). The following *L. pneumophila* strains were obtained from the American Type Culture Collection (ATCC); Bloomington SG3 ATCC 33155, Philadelphia SG1 ATCC 33152, SG2 ATCC 103856, SG3 ATCC 103857, SG4 ATCC 103858, SG5 ATCC 103859, SG6 ATCC 103860, SG7 ATCC 103861, SG8 ATCC 103862, SG9 ATCC 103863, SG10 ATCC 103864, SG11 ATCC 103865, SG12 ATCC 103866, SG13 ATCC 103867, SG14 ATCC 103869.

The *L. pneumophila* strains used in this study were grown for 48 -72 hrs at 37 °C at buffered charcoal yeast extract (BCYE, Oxoid, Cambridge, UK) agar. The bacterial cells were suspended to approximately 10^9 CFU/mL of legionellae in Page's amoebae saline buffer (PAS) (4 mM $MgSO_4$, 0.4 mM $CaCl_2$, 0.1 % sodium citrate dehydrate, 0.05 mM $Fe(NH_4)_2(SO_4)$ x $6H_2O$, 2.5 mM Na_2HPO_4 x $7H_2O$, 2.5 mM KH_2PO_4 (Sigma Chem. Co. St. Louise, Mo, USA) and dilutions were used in the amoebal co-culture experiments. Enrichment of *L. pneumophila* from the indicated aeration pond samples was performed according to the ISO11731 standard.

Amoebal Co-Culture

Amoebal co-culture experiments were performed basically as described [21, 25]. *Achantamoeba castellanii* ATCC 30234 obtained from ATCC was grown in a 25 cm^2

[1]Outbreak strain Borregaard, 2008. The strain present in the aeration pond during shutdown [3].
[2]Outbreak strain Borregaard, 2005 [2].
[3]Strain isolated at Borregaard, detected in a drying machine of the cellulose plant
[4]Strain isolated from air samples harvested above the aeration pond at Borregaard in 2006 [4].

cell culture flask (Sarstedt, Newton, NC, USA) containing PYG medium (PAS buffer containing 2 % proteose peptone (Oxoid), 0.1 % yeast extract (Sigma) and 0.1 M glucose (Merck, Darmstadt, Germany)) until the cells had formed a confluent layer in the flask ($1.0x10^6$ cells/mL). The day before the experiments, the amoebae were refreshed with new PYG medium. The number of cells was determined in a Bürker haemcytometer and amoebae (1.0×10^5 cells/mL) were added to a 24-well cell culture microplate (Costar, Corning, NY, USA) and incubated for 18 hrs at 20°C. The amoebae were washed three times with 1 ml of PAS buffer and incubated at 20°C for 30-60 min before they were used in co-culture experiments. Aliquots of 1 mL of *L. pneumophila* (different strains or concentrations diluted in PAS buffer) or aeration pond sample were added to the wells, and were present throughout the whole experiments in order to mimic the situation in a real biological treatment plant. The aeration pond samples were heated to 50 °C for 30 min before the co-culture experiments to inactivate non-legionellae bacteria. The co-culture samples were incubated at 37 °C for 1 to 6 days. At day 1, 2 and 3 the samples were screened under phase contrast microscope for the presence of legionellae inside the amoebae and for bacteria released into the medium. To determine the number of CFU/mL of *Legionella* cells at the different time points, the co-culture samples were lysed using a 27-gauge needle before applied to BCYE (legionellae strains) or GVPC (BCYE agar containing a glycine, vancomycin, polymixin B, and cyclohexamide supplement, Oxoid) agar (aeration pond sample). These agar plates were inspected for *Legionella* colonies after incubation at 37 °C for 1, 3 and 10 days. In general, in all co-culture experiments (strains and aeration pond samples) the viable counts of legionellae were 10^6 – $5x10^8$ CFU/mL after 3-6 days of incubation in co-culture. The counts never exceeded $5x10^8$ CFU/mL due to lack of nutrient and lysis of the amoebae in the PAS buffer. Negative control samples without *A. castellanii*, otherwise treated similarly, were always run in parallel. In general, legionellae was not replicated in the negative controls.

In follow up experiments, an infection time of 4 hrs for amoeba and aeration pond samples from 25 September, 4 November and 1 December was used. This was done to visualize the bacteria recovered from the aeration pond samples since the complexity of these samples prevents visualization of bacteria under the microscope. In those experiments the aeration pond sample was removed after 4 hrs and the amoebae were washed with PAS buffer 3 times and 1 mL of PAS was added to the wells. This time point was denoted as 0 hr (T0). The samples were incubated at 37 °C for 6 days in co-culture and viable counts were measured using GVPC agar. The samples were regularly inspected under the microscope during the time period.

In order to optimize the ratio of aeration pond sample and *A. castellanni* in the co-culture experiments, 3 different concentrations of *A. castellanii* were tested (10^4, 10^5, 10^6 cells/mL. In general, 10^5 cells/mL was optimal for proliferation of legionellae from aeration pond samples.

In each experiment, amoebal co-culture of *L. pneumophila* SG3 Bloomington was used to control intra-amoebal replication. The legionellae recovered from the aeration pond samples and replicated in co-culture experiments were distinguished from the controls (Bloomington SG3) by positive real-time PCR of primers specific for *L. pneumophila* SG1. In control co-culture experiments *L. Pneumophila* (Bloomington) (inoculums concentrations 10^3 or 10^4 CFU/mL) was proliferated to 10^7-10^8 CFU/mL.

In general, the *Legionella* replicated in the amoebal co-culture experiments with aeration pond samples was identified as *L. pneumophila* SG1 using real-time PCR of the *L. pneumophila* specific *mip* gene and the *L. pneumophila* SG1 specific primers.

The results were plotted using the Origin Software (Origin Lab Corporation, Northampton, MA, USA).

Real-Time PCR

Colonies from the GVPC agar plates after co-culture with *A. castellanii* were confirmed to be *L. pneumophila* SG1 by real-time PCR. The following primers were used: *L. pneumophila* SG1; mip-f */mip*-r, which amplified a 186 bp fragment of the *L. pneumophila mip* gene [26] and *L. pneumophila* SG1 specific primers (P1-f/P2-r), which amplified a 294 bp fragment specific for *L. pneumophila* SG1 [27]. Representative colonies from the GVPC agar plates from all co-culture experiments were picked and the colonies were solubilzed in water and heat inactivated at 95 °C for 10 min. PCR were run directly on 2 µl of the supernatant. The reaction mixture contained in 20µl; 10µl LightCycler 480 SYBRGreen I Master mix (Roche Diagnostics, Indianapolis, USA), primers (1 µM) and 2µl of template DNA. PCR was run on a Light Cycler 480 instrument (Roche Diagnostics) with the following PCR profiles; *mip-f/mip-r*: 95°C for 5 min, followed by 35 cycles of 95°C for 5 sec, 62°C for 10 sec and 72°C for 15 sec; P1-f/P2-r: 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. A minimum of 20 morphological similar colonies were tested from each plate. DNA extract from a colony of *L. pneumophila* ST462 was used as positive control. The *L. pneumophila* Bloomington control (co-culture) was confirmed by positive PCR amplification of the *mip* gene and negative for the SG 1 specific primers.

The P-FLA-F/ P-FLA-R primers targeting free living amoebae (FLA) were used for real-time PCR amplification of DNA isolated from the aeration pond samples as described [28]. DNA was isolated using the DNeasy Blood and Tissue kit. The reaction mixture contained in 20µl; 10µl LightCycler 480 SYBRGreen I Master mix (Roche), primers (1 µM) and 2µl of isolated DNA. PCR was run on a Light Cycler 480 instrument (Roche Diagnostics) with the following PCR profile: 95°C for 5 min, followed by 45 cycles of 95°C for 20 sec, 58°C for 20 sec and 72°C for 40 sec. DNA isolated from a culture of *A. castellanii,* using the DNeasy Blood and Tissue kit, was used as positive control. In general, deionized water was used as negative controls. The specificity of the PCR products was confirmed by melting point analysis. The size of the PCR products was verified in selected samples by gel electrophoresis (Bio-Analyzer, Angilent Technology, USA). The isolated DNA contained no PCR inhibitors shown by Real-Time PCR of the Lambda phage DNA [6].

Sequenced Based Typing

Selected samples of the *Legionella* proliferated from the aeration pond sample in co-culture with *A. castellanii* was genotyped using sequence based typing (SBT) as described [3].

Microscopy

A phase-contrast fluorescence microscope (Zeiss, Germany) was used for microscopic investigations of samples for the presence of legionellae in the amoebae and for bacteria released to the medium. The infected amoebae in the 24-well cell culture microplate were carefully resuspended using a pipette and 10-15 µl were transferred to sterile microscopic glass slide overlaid with sterile cover slips before investigation under the microscope (100 x). Photomicrographs were taken using a digital microscope camera (AxioCam, Zeiss, Germany). The number of amoebae cells was counted in a Bürker haemcytometer.

Statistical Analysis

Results in Figs. (**1-3**) were expressed as mean ± standard deviation (SD). Statistical significance was determined using the unpaired, two-tailed Student t-test.

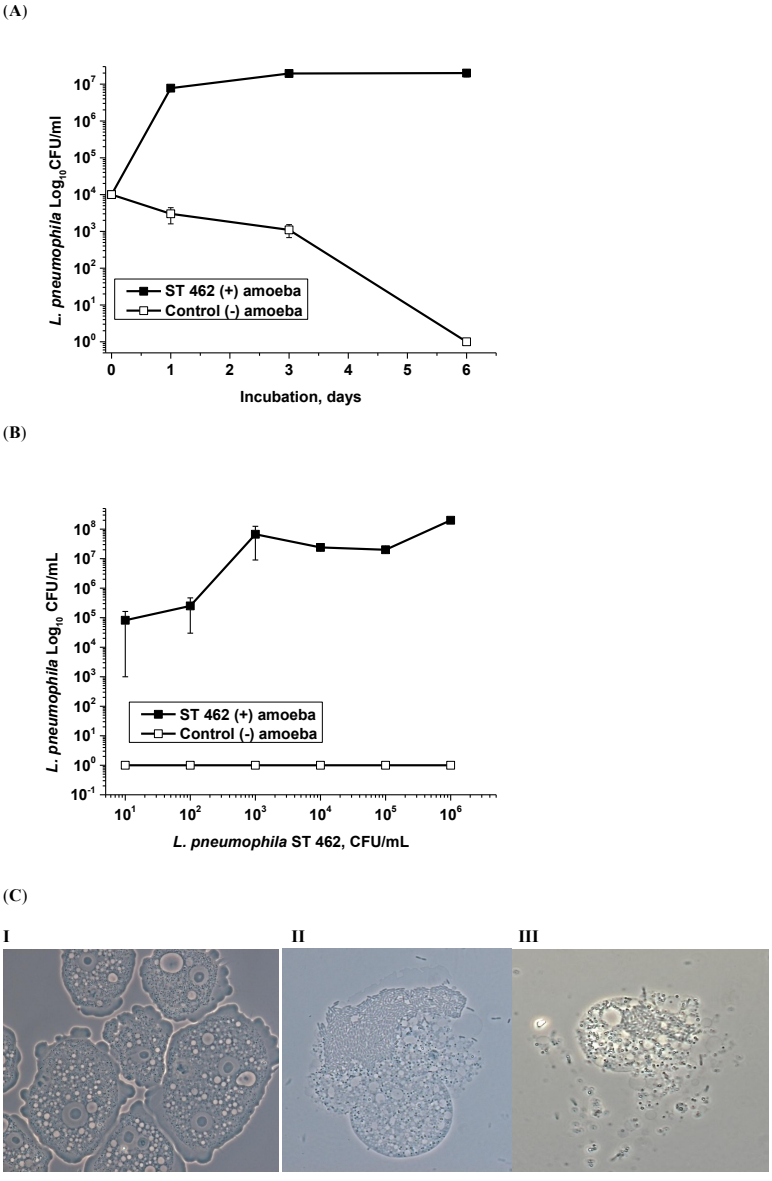

Fig. (1). Infection and replication of the *L. pneumophila* in co-culture with *A. castellanii* (10^5 cells/mL). Samples were incubated for 6 days and plated on BCYE agar in duplicates for identification of viable counts. *L. pneumophila* were identified with real-time PCR (*mip*, SG1 specific primers). Negative controls were performed similarly at the same time, but without *A. castellanii*. The figures are based on the results of one experiment and the data is expressed as mean±SD. Similar experiments are repeated 2-3 times. **A**) Time course of the replication of the *L. pneumophila* strain ST462 (outbreak strain from 2008). **B**) A 10-fold serial dilution of the *L. pneumophila* ST462 (10 to 10^6 CFU/mL) was replicated in *A. castellanii* for 6 days. *A*ll concentrations of legionellae replicated to 10^5-10^8 CFU/mL. **C**) Microscopic investigation of the replication of *L. pneumophila* (ST462) in *A. castellanii* (100 X). **I**; *A. castellanii* before infection; **II**; *A. castellanii* 1 day post infection; **III**; *A. castellanii* 2days post infection.

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RESULTS

Replication of Different *L. pneumophila* Strains in Co-Culture with *A. castellanii*

The ability of *L. pneumophila* to survive and replicate in amoebae has been linked to human pathogenicity. Therefore, the ability of the *L. pneumophila* outbreak strain ST462 from 2008 (the strain present in the aeration ponds during the shutdown [3]) to replicate in *A. castellanii* was investigated and compared to replication of other *L. pneumophila* strains (Table **1**). The ST462 outbreak strain showed an increase in viable counts of 3-4 log units during three days of incubation in co-culture compared to controls incubated without amoebae which showed no replication (Fig. **1A**). After 3 days of incubation in amoebal co-culture, a *Legionella* plateau phase was reached. *A. castellanii* infected with 10-fold serial diluted *L. pneumophila* ST462 (10 to 10^6 CFU/mL) resulted in viable counts of 10^5-10^8 CFU/mL after 6 days of incubation (Fig. **1B**). Microscopic investigations showed *L. pneumophila* inside the amoebae one day post infection. At 2-3 days post infection, the amoeba cells were lysed and *Legionella* cells were released to the PAS medium (Fig. **1C**). Screening of *L. pneumophila* strains belonging to different serogroups (1 to 14) for their ability to infect and replicate inside *A. castellanii* demonstrated that all strains except for the *L. pneumophila* strain Colitax SG2-14, the *L. pneumophila* strains belonging to SG4, 5, 6, and 9 and *L. pneumophila* Philadelphia SG1 replicated in amoebae (Table **1**). The *L. pneumophila* SG4 strain isolated from air at Borregaard BLA3 [4] replicated; however, the other *L. pneumophila* SG4 strain dis not replicate in *A. castellanii* co-culture.

Table 1, Co-culture of different *L. pneumophila* strains of different serogroups with *A. castellanii* (10^5 cells/mL). In all co-culture experiments *L. pneumophila* (Bloomington ATCC 33155) at 10^3 CFU/mL was used as control.

L. pneumophila Strain (10^3 CFU/mL)	Amoebal Co-Culture with *A. castellanii* (10^5 Cells/mL), Day 7
BloomingtonSG3 ATCC 33155	Yes
Philadelphia SG1 ATCC 33152	No
Colitax SG 2-14	No
ST462 SG1	Yes
ST15 SG1	Yes
ST458 SG1	Yes
BLA3 SG4	Yes
SG2 ATCC 103856	Yes
SG3 ATCC 103857	Yes
SG4 ATCC 103858	No
SG5 ATCC 103859	No
SG6 ATCC 103860	No
SG7 ATCC 103861	Yes
SG8 ATCC 103862	Yes
SG9 ATCC 103863	No
SG10 ATCC 103864	Yes
SG11 ATCC 103865	Yes
SG12 ATCC 103866	Yes
SG13 ATCC 103867	Yes
SG14 ATCC 103869	Yes

All strains used are *L. pneumophila*. The start concentration of all *L. pneumophila* strains was $1x10^3$CFU/mL. Average replication of the strains of the different serogroups was $2x10^8$ $\pm 4x10^7$ (SEM).

Replication of *L. pneumophila* in the Aeration Pond Samples in Co-Culture with *A. castellanii*

During the shutdown from September to December 2008, the concentration level of *L. pneumophila* ST462 decreased gradually and after 1 December 2008, no growth of *L. pneumophila* was detected using the ISO11731 standard method after 15 December [3, 6]. At 17 November, 2008 hypochlorite was first added to the aeration ponds in order to inactivate the remaining bacterial species. The growth of *L. pneumophila* according to the ISO11731 standard method was estimated to 10^2- 10^3, 10^2 and 0-10 CFU/mL (below detection limit) in the samples harvested at 25 September, 3 November and 1 December in 2008, respectively. However, in amoebal co-culture experiments, viable counts of *L. pneumophila* from these samples increased in general by 5-7 log units ($p<0.05$). There were no viable counts of *L. pneumophila* in the aeration pond sample from 1 December diluted 10 times using the ISO11731 standard, but in amoebal co-culture, the viable counts were in general always 10^7 -10^8 CFU/mL (Fig. **2**). ~~In the diluted aeration pond~~

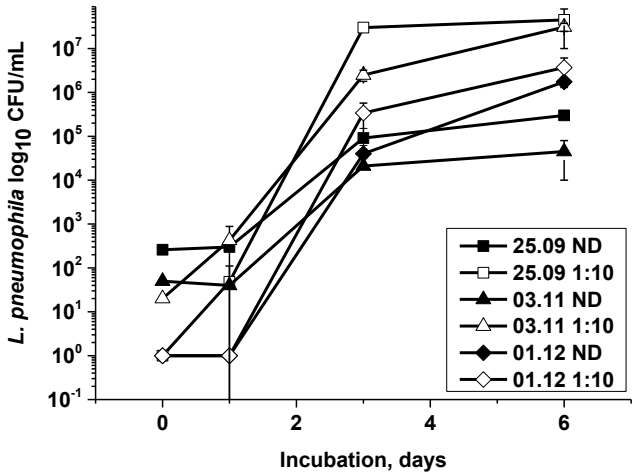

Fig. (2). Co-culture of aeration pond samples from 25 September, 3 November and 1 December with *A. castellanii* (10^5 cells/mL). The aeration pond samples were present throughout the experiment and after 1, 3 and 6 days of incubation with amoebae, the samples were plated on GVPC agar in duplicates for identification of viable counts of recovered *L. pneumophila*. *L. pneumophila* were confirmed by real-time PCR of the *L. pneumophila* specific *mip* and SG1 specific primers. The *L. pneumophila* ST462 strain was confirmed by genotyping in selected samples using SBT. ND: Non-diluted aeration pond sample; 10xD: 10 x dilution of aeration pond sample. The figure is based on one experiment performed in duplicate, which is repeated at least three times with similar results. Data are mean±SD. Significant growth ($P<0.05$) was observed for all samples except for the 25.09 ND sample. No growth was detected in the control (no amoebae) samples.

In the diluted aeration pond samples (1:10) more efficient replication of *L. pneumophila* samples were observed compared to replication in the non-diluted samples, indicating that inhibitors of *L. pneumophila* a replication might be present in the pond samples. These results, showing replication of *L. pneumophila* in the aeration pond samples in co-culture with *A. castellanii*, were confirmed by the absence of replication of legionellae in heat inactivated aeration pond samples (September 25) (100 °C 20 min) and the absence of replication when using heat inactivated amoebae. No growth of legionellae was detected in the controls in which no amoebae were added, and after 3-6 days of incubation the legionellae cells was not cultivable. In all co-culture experiments, *L. pneumophila* was confirmed by specific PCR using the *L. pneumophila* SG1 specific primers [27] and the *L. pneumophila mip* primers [26]. Genotyping (SBT) of legionellae [3] confirmed that the *L. pneumophila* ST462 strain was recovered from the aeration pond samples and replicated in the co-culture experiments.

The presence of amoebae in the aeration pond sample was investigated using real-time PCR amplifying the FLA-primers. These results indicated a decreasing amount of amoebae DNA in the pond samples from 25 September, 3 November and 1 December (results not shown). These findings were supported by the results [10] showing high concentrations of protozoa (approximately 10^5cells/mL) in the aeration pond samples.

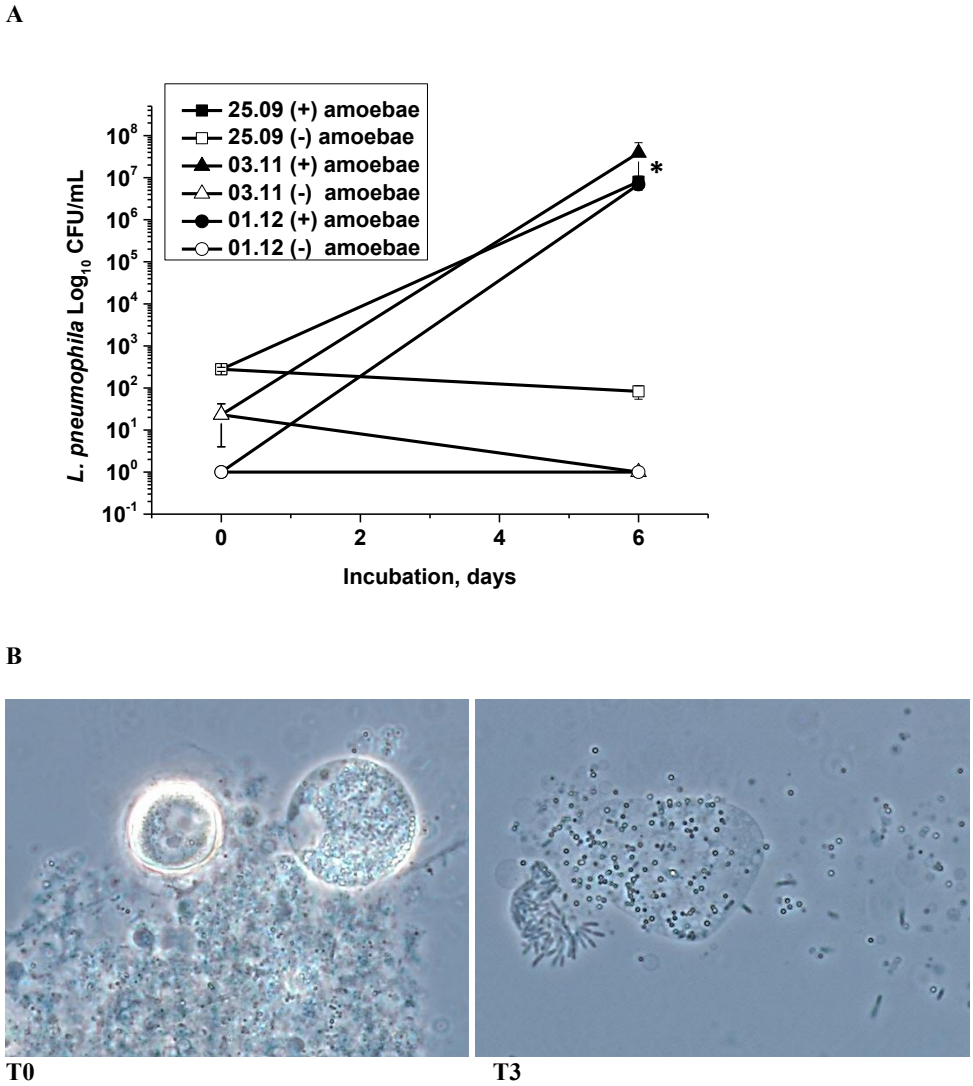

Fig. (3). (**A**) Co-culture of aeration pond samples from 25 September, 3 November and 1 December with *A. castellanii* (10^5 cells/mL). After 4 hrs incubation of amoeba and aeration pond samples, the aeration pond sample was removed and PAS buffer added. The growth of legionellae was inspected under the microscope daily, and at day 6 the samples were plated on GVPC agar in duplicates for identification of viable counts of recovered *L. pneumophila*. *L. pneumophila* were confirmed by real-time PCR of the *L. pneumophila* specific *mip* and SG1 specific primers. The *L. pneumophila* ST462 strain was confirmed by genotyping in selected samples using SBT. The figure is based on one typical experiment performed in duplicate, which was repeated 2 times and $p<0.05$ for all 3 aeration pond samples. Data are mean±SD. No growth was detected in the control (no amoebae) samples. (**B**) Microscopic investigation of the replication of bacteria recovered from the aeration pond sample (1 December) in *A. castellanii* (100x). At T0 *A. castellanii* is added to the aeration pond sample. At day 3 (T3) bacteria has been replicated in *A. castellanii* and released to the PAS medium and identified as *L. pneumophila* by culture and real-time PCR.

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Intracellular Replication of the Outbreak Strain *L. pneumophila* ST462 in Aeration Pond Samples

In co-culture experiments performed in this study, aeration pond samples were present throughout the experiments to mimic the real situation in a biological wastewater treatment plant. Due to the microbial and chemical complexity and the turbidity of the aeration pond samples [6], it was not possible to visualize bacteria recovered from the aeration pond sample in the amoebae under the microscope. Therefore, in follow up experiments the pond samples were incubated with *A. castellanii* for 4 hrs before the aeration pond samples were removed and replaced by PAS buffer. After 2-3 days, microscopic investigation revealed *A. castellanii* cells containing bacteria and *L. pneumophila* ST462 was identified by growth on GVPC agar, by real-time PCR (*mip* and SG 1 primers) and genotyping as described in material and methods (Fig. **3**). These experiments showed that *L. pneumophila* was recovered from the aeration pond samples and replicated in *A. castellanii*.

In general, a concentration of *A. castellanii* of 10^5 cells/mL was used for co-culture experiments. However, in samples containing low levels of legionellae (samples from 3 November and 1 December diluted 10 fold), a 10^6 cells/mL of *A. castellanii* resulted in higher viable counts of legionellae, indicating that a higher ratio of amoebic cells and legionellae was required. Another observation was that heat inactivated (100 °C for 20 min) aeration pond sample inhibited the replication of the *L. pneumophila* strains Bloomington, ST462 and ST15 in *A. castellanii*. However, dilutions (10 and100 folds) of the heat inactivated aeration pond sample from September 25 were less inhibitory (10-100 folds) (results not shown).

DISCUSSION

The health impact associated with pathogenic *Legionella* cells in wastewater treatment plant is well documented [2, 4, 10, 11, 29]. Replication of *Legionella* inside amoebae (ubiquitous in nature) may lead to unexpected accumulation of potential pathogenic bacteria in nature or in artificial water systems [8, 30-32]. Increased knowledge of resistance of amoebae and legionellae associated with amoebae to disinfection and the impact of amoebae on selecting potential pathogenic legionellae strains from complex environmental microbial flora raises important questions for wastewater treatment plants [33]. In this study, the level of viable pathogenic *L. pneumophila* cells in samples harvested from the aeration ponds of the biological treatment plant at Borregaard Ind. Ltd., Sarpsborg, Norway during the shutdown of the aeration ponds in 2008 has been studied. All strains of legionellae isolated at Borregaard including the outbreak SG1 strains ST15 and ST462, the BLA3 SG4 strain isolated from air and the ST458 SG1 strain isolated in a drying machine of the cellulose plant, were able to infect and replicate in *A. castellanii*. The BLA3 and ST458 isolates were not associated with outbreaks. However, the results of the amoebal co-culture experiments show that they have the potential to become pathogenic and they may have an impact on human health if disseminated to air. The SG4 ATCC 103858 strain tested in this study did not replicate in the amoebae. This may indicate differences in the sensitivity for co-cultivation with *A. castellanii* reflecting the potential to become pathogenic. However, it may also imply that other species of amoebae is required for replication [34].

In general, it is difficult to detect low levels of legionellae in complex environmental samples by culture methods due to overgrowth of the plates by other bacteria present [8] and the potential of legionellae cells to become VBNC [23]. The aim of the present study was to use amoebal co-culture with *A. castellanii* to detect and recover *L. pneumphilia* from the complex microbial community in the pond during the shutdown period in 2008. During the shutdown period several measures (as described in the methods; influx of waste material and injection of air into the ponds were stopped) including injection of hypochlorite, which first was injected into the ponds at 17 November 2008, were used to reduce the concentration of legionellae. The recovery of the pathogenic *L. pneumophila* ST462, using the ISO11731 standard growth method on BCYE and GVPC agar, decreased during the shutdown [3] and in samples from 1 December 2008, the CFU/mL varied between 0 and 10. However, in most cases it was not detectable and when this sample was diluted 10 fold, no viable counts were ever detected. However, using the amoebal co-culture method with *A. castellanii*, *L. pneumophila* was replicated to 10^7 -10^8 CFU/mL. Previous findings, during the shutdown of the aeration pond samples, have shown a rapid decrease of legionellae *mip* DNA from 10^6 GU/mL to 10^3GU/mL and at 1 December the DNA level was stabilized at the detection limit (10^3 GU/mL) [6], which correlated with the decrease of cultivable *L. pneumophila* cells [3]. It is difficult to speculate on if it was viable cells of legionellae present in the pond samples or VBNC cells present that was recovered and replicated in the present co-culture experiments. However, the present results indicate that care should be taken regarding disinfection of wastewater treatment systems, cool towers and drinking water systems. If the growth conditions become optimal and protozoa are present in the environment *L. pneumophila* may start to replicate and become a risk to the population. Previously, it has also been revealed that disinfection of human water systems does not lead to total eradication of the legionellae bacteria [29, 35-37]. Intracellular growth of legionellae within amoebae can causes resistance to chemical disinfectants and biocides since the bacteria can be protected inside amoebae and biofilms [23, 29, 38].

The co-culture experiments can be divided into two different phases, an infection phase when legionellae is added and enters the amoebae, and a replication phase [25]. To mimic a real situation of a biological wastewater treatment plant in the laboratory the aeration pond samples were incubated with the amoebae throughout the experiments and not removed after the initial infection of the amoebae as performed by others [25]. However, due to the turbidity of the aeration pond samples containing filaments and a high background of other bacteria [6], it was not possible to visualize *L. pneumophila* inside the amoebae under the microscope. Therefore, in follow up experiment the aeration pond sample was removed from the amoebae after 4 hrs incubation and *L. pneumophila* recovered from the aeration pond samples was replicated and detected. The aeration pond samples were in general turbid and contained filaments and filamentous bacteria, in particular the sample from September 2008. The November and December

samples were less turbid [6] and these samples showed in general, higher viable counts in the co-culture experiments compared to the samples from September. Indicating that compounds present in the aeration pond including dead bacteria can inhibit replication of legionellae in *A. castellanii*. The observation that samples from September showed higher viable count compared to samples from November and December using the ISO 11731 standard culture method support this theory [3, 6]. These findings also supports the general observation that the viable counts of *L. pneumophila* in co-culture experiments with the aeration pond samples 10 fold diluted were higher compared to non-diluted aeration pond samples. This may also explain that the growth in the co-culture of *L. pneumophila* ST462 recovered from the non-diluted samples from September did not turn out to be significant ($p>0.05$).

Microscopic investigation indicated the presence of amoebal cysts in the aeration pond samples (not shown). However, fragments and filaments in the samples made it difficult to confirm these results [6]. The previous findings of protozoa (approximately 10^5 cells/mL) in the aeration ponds at Borregaard [10] indicate that protozoa could be present in the aeration pond at the time of legionellae proliferation in the ponds [3, 4]. A concentration of 10^5 cells/mL of amoebae in the ponds is optimal for replication of legionellae [21, 25]. Therefore, it is possible that such levels of protozoa may have contributed to the high concentration of the *L.pneumopila* outbreak strain ST462 in the aeration ponds in 2008 [3]. *L. pneumophila* has been detected within the cyst wall of amoebae [39], which indicates that the cyst may protect legionellae during unfavorable condition, e.g. treating of wastewater with hypochlorite. In the present work it was difficult to confirm the presence of amoebae or cysts in the aeration pond samples. However, PCR amplification indicated the presence of DNA of free living amoebae (FLA) in the pond samples and the PCR indicated a decreasing amount of amoebae DNA in the samples from 25 September, 3 November and 1 December. A disadvantage using FLA-PCR primers is that amplification also occurs for other protozoans [40].

The presence of amoebal vacuoles filled with legionellae bacteria show that free-living amoebae in nature may act as a reservoir for the internalized, bacteria [41, 42]. During replication of *Legionella* strains in protozoa, virulent bacteria are selected, and which is also an adaption of the internalized bacteria to life within human macrophages [16, 43]. Not surprisingly, preliminary experiments at our institute have shown that *L. pneumophila* ST462 was able to proliferate within the THP-1 human cell line. It is shown that amoeba grown l*egionellae* have higher ability to colonize or to develop biofilm, which protects bacteria from environmental stress factors and increases the survival in e.g. water systems [44].

The results of this study showed that the ISO 11731 standard cultivation method was not able to detect the pathogenic *L. pneumophila* outbreak strain ST462 present at low concentration in the aeration pond samples. However, in amoebal co-culture experiments this strain was replicated to 10^7 -10^8 CFU/mL in the pond samples, including samples treated with hypochlorite. This observation shows that the ISO 11731 standard method may not be sensitive enough to detect all pathogenic legionellae bacteria in complex biological treatment plant samples containing high background of microbial flora. As a consequence, methodological improvement is needed to ensure more reliable detection of *Legionella*, and it is recommended that the amoebal co-culture method is used in addition to the ISO11731 standard method. Another precaution to prevent legionellae proliferation could be to monitor the amount of protozoa able to support growth of legionellae and other potential pathogenic bacteria also known to proliferate in amoebae in biological treatment plants [42].

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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