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Prevalence of *Legionella* spp. in Bioscrubbers at 36 Livestock Farms

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Abstract

At many livestock farms bioscrubbers are used for emission abatement. The pH and temperature of the recirculating water might be favourable for growth of *Legionella* spp. bioscrubbers recirculate water over a packing, force air through the packing with fans, and use spraying nozzles to create water droplets that moisten the packing. If *Legionella* is present in the water, spread to the environment is probable. The aim of this study was to assess whether *Legionella* spp. were present in bioscrubbers at animal farm houses. This was done by determining the presence of *Legionella* spp. in the recirculating water of 36 farm site bioscrubbers by using culture and amoebal coculture methods. Additionally, on-site environmental conditions that could favour *Legionella* growth were assessed. Neither *Legionella* spp. nor *L. pneumophila* was detected in any of the investigated bioscrubbers. The bacterium may however have been present in the recirculation water, but at concentrations below the detection limit of the used methods. Based on the results of this study, the risk of legionellosis from bioscrubbers at farms seems limited for people that live near those farms.

Keywords

Animal House; Biofilm; Bioscrubber; Biotrickling Filter; *Legionella* spp.

Introduction

In European countries like the Netherlands, Belgium, Germany and Denmark, air scrubbers are often used at animal houses to comply with emission standards for removal of ammonia (NH_3), odour, and fine dust (PM_{10}). Air scrubbers normally use tap water or ground water as washing fluid. Depending on the type of air scrubber, either chemicals are added to the water ('chemical scrubber') or the removal is driven by bacterial conversion ('bioscrubber' or 'biotrickling filter') [1]. In a bioscrubber, nitrifying bacteria convert ammonia to nitrite and nitrate resulting in accumulation of ammonium nitrite and ammonium nitrate in the recirculating water, which is partly discharged and replenished by addition of fresh water. It is estimated that about 2,500 bioscrubbers are used at animal houses in the Netherlands [2,3].

Recently, Walser et al. [4] detected *Legionella* spp. and *L. pneumophila* in aerosols near a bioscrubber at a breeding sow facility in Germany. *Legionella* spp. cause mild (Pontiac fever) to severe (Legionnaires' disease) pneumonia in humans. Infections commonly originate from inhalation of aerosols that may be produced by e.g. whirlpools showers, fountains, and cooling towers [5]. The majority of outbreaks of legionellosis are caused by *Legionella pneumophila* [6]. *Legionella* spp. inhabits biofilms and growth occurs in protozoa grazing these biofilms [7]. Growth of *L. pneumophila* is possible between 22°C and 45°C with an optimum growth temperature of 37°C, and at a pH within the range of 5.5 - 9.2 [8-10]. Other *Legionella* spp. have a minimum growth temperature of 17°C [9].

Bioscrubbers resemble wet cooling towers. Wet Cooling Towers (WCTs) are part of the climate control system of large buildings, or used for cooling of industrial processes. Air scrubbers and WCTs recirculate water over a porous packing (usually made of plastic), force air through the packing with fans, and use spraying nozzles to create water droplets that moisten the packing. The high specific surface area of the packing ensures a high air-water contact area, thus promoting evaporation (in WCTs) and mass transfer of pollutants from air to water (in bioscrubbers).WCTs are held responsible for most of the recorded outbreaks of legionellosis [11], as *Legionella* present in the recirculating water of WCTs can be emitted into the air. Therefore, water treatment is often in place in WCTs to minimize biofilm formation and microbiological fouling. In contrast to WTCs, the presence of a biofilm on the packing and (detached) biomass flocks suspended in the recirculation water is an essential characteristic of bioscrubbers. Typical operating conditions in bioscrubbers are a pH of 6.5 - 7.5 and a temperature of 15 – 30°C [12]. Since evaporative cooling cools the air, the air outlet temperature is usually equal to the water temperature, and the air inlet

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temperature is 4-5°C higher [13]. These conditions may be favourable for growth of *Legionella*.

Legionella was detected in an industrial air scrubber which was suspected to be responsible for an outbreak of Legionnaires' disease in Norway [14]. However, in a later study, a wastewater treatment plant and a near by river were suggested as more likely sources of the aforementioned outbreak [15].

The aim of this study was to assess whether bioscrubbers at animal houses could pose a risk of legionellosis to people working at or living in the vicinity of these farms, by determining the presence of *Legionella* spp. in the recirculating water of 36 farm site bioscrubbers and investigating on-site environmental conditions that could favour *Legionella* growth.

Materials and methods

Sampling

This study was performed in the period August-September 2014, because the highest number of notified cases of legionellosis is reported in the late summer in the Netherlands [16,17]. All 36 livestock farms that were included in the study had one bioscrubber, and were visited once. All but two bioscrubbers were located at pig farms; the other two were located at poultry farms. The farms were located in the Southern and Eastern part of the Netherlands.

From each bioscrubber, three samples with a volume of 200 or 500 ml of the recirculation water were taken. When possible, samples were collected at three places: directly from the spray nozzles, from the recirculation water basin at a point where the water flow was minimal, and from the tap of the recirculation water tubing system. When the spray nozzles or recirculation water tubes were difficult to access due to the construction of the bioscrubber, an extra sample was taken from the recirculation water basin.

The pH (WTW-pH340/SenTix41-SensoLytSE-TFK325, WTW, Germany), electrical conductivity (EC) (WTW-Cond340i/TetraCon325, WTW, Germany), and temperature (Testo 950/Pt100, Testo, Germany) of the recirculation water samples were measured on-site, immediately after sampling.

Additionally, air temperatures (Testo 950/Pt100, Testo, Germany) were measured at the inlet of the bioscrubber (this is the exhaust air of the animal house), in the technical room where the water piping and pumps were located, and very close to the pumps where the air temperature might be highest.

Water samples were cooled on melting ice within half an hour after sampling and during transport to the laboratory, where they were stored at 4°C in a refrigerator until processing, which was initiated within 24 hrs after sampling.

Sample analysis

The water samples were analysed for the presence of *Legionella* by culture on GVPC (Glycin Vancomycin Polymyxin Cycloheximide) agar plates (n=108) and by amoebal coculture (n=100). Of each sample, a 100 µl volume was spread onto each of two GVPC agar plates (Oxoid Ltd., Hampshire, UK). Plates were incubated for 7 days at 36 ± 2°C, and inspected for the presence of *Legionella* colonies, based on morphological characteristics. The detection limit for the plate method was 5,000 cfu/L [18]. The amoebal coculture method was performed as described previously [19,18]. Briefly, *Acanthamoeba castellanii* ATCC #30234 (American Type Culture Collection, Rockville, Md., USA) were grown in 75 cm^2 culture flasks (Corning Inc., New York, USA) with 15 ml of Peptone-Yeast extract-Glucose (PYG) broth at 25°C. Prior to the infection, the PYG broth was removed and the amoebae were resuspended in 15 ml Page's Amoebal Saline (PAS). The amoebae suspension was centrifuged at 850 g for 10 minutes and the pellet was subsequently resuspended in 15 ml PAS. This washing step was repeated twice. Cells were then seeded in a 12-well micro plate (Corning) at a density of 5×10^5 cells/ml of PAS. Per well, 1 ml of PAS with amoebae was inoculated with 100 µl of sample. Each sample was tested in triplicate by inoculating three wells, yielding a total analysed sample volume of 300 µl. The amoeba plates were incubated at 32°C for 3 days. Subsequently, the amoeba was re suspended, and 100 µl from each well was sub cultured on a freshly seeded plate with amoebae. After another 3 days of incubation at 32°C, the amoeba was again resuspended and 100 µl from each well was 10-fold serially diluted in PAS. Of the 10^5- 10^9 fold dilutions, 100 µl was plated on GVPC plates (Oxoid Ltd., Hampshire, UK). After 3-7 days of incubation at 36 ± 2°C, the GVPC plates were inspected for the presence of *Legionella* colonies based on morphological characteristics. A maximum of 5 suspected colonies were confirmed by culturing on BCYE (Buffered Charcoal Yeast Extract) agar plates with and without cysteine (both Oxoid Ltd., Hampshire, UK). *Legionella* colonies do not grow on BCYE without cysteine.

For two bioscrubbers, the pH of the water samples was below pH 5, and therefore analysis by amoebal coculture was not done, since amoebal growth is very unlikely at such low pH values. The theoretical detection limit for the amoebal coculture method was 3,300 cfu/L [18].

Results

All water samples had a yellow-brownish color, which indicates the presence of organic material and biomass. In some bioscrubbers, foam was visible on top of the recirculation water basin, showing brown biomass and/or dust. Furthermore, for some bioscrubbers it was possible to access the supporting structure and packing at the bottom of the bioscrubber, where a brownish biofilm was observed.

In most bioscrubbers, the pH values of the spraying water, the recirculation basin water and the water in the recirculation piping and tubing differed minimally (differences ≤ 1.2), which indicates that the systems were mixed well. In one bioscrubber, that had two separate water recirculation systems, a larger difference occurred (pH of 3.0 in biotrickling step and pH of 6.4 in prior dust removal step).The mean pH for the individual bioscrubbers ranged between 2.8 and 8.6, with an overall mean of 6.7 and an overall median of 7.2. About half of the bioscrubbers (n=17) operated at a pH between 6.5 and 7.5, which is considered a normal operational pH. Ten bioscrubbers operated at a lower pH (2.8-6.3), whereas eight bioscrubbers operated at higher pH (7.6-8.6). In 30 of the investigated bioscrubbers (83% of total) the pH was in the range that generally allows growth of *Legionella* (5.5 -9.2).

Mean EC values in the water samples from the bioscrubbers ranged between 2.0 and 49 mS/cm, and the differences in conductivity between samples from the spraying water, the recirculation basin and the tubing were mostly minimal (<2.5 mS/cm). In 13 bioscrubbers, the average EC was higher than 18 mS/cm, which is considered the maximum value for normal operation.

In all bioscrubbers, the temperature of the spraying water, the recirculation basin water and the water in the tubing was almost equal, with differences of less than 2°C. The mean water temperature for the individual bioscrubbers ranged between 17 and 23°C, with an overall mean and median of 20°C. In all bioscrubbers, water temperatures were in the range that generally allows growth of *Legionella* spp. In eight bioscrubbers water temperatures of over 22°C were detected.

The air temperature at the inlet of the investigated bioscrubbers ranged from 20 to 32°C, with a mean and median of 24°C. The air temperature in the technical rooms was slightly lower, ranging from 17 to 30°C, with a mean and medium of 22°C. The air temperature close to the surface of the pumps ranged from 20 to 29°C (mean and median 22°C). There was no indication that the pumps caused a significant local increase of the air or water temperature. No other equipment was observed that might lead to warming up of air or water.

In the samples from 31 of the 36 bioscrubbers, the presence of *Legionella* spp. could not be determined by culture on GVPC agar plates, since the cultures were overgrown by other bacteria. On the culture plates from samples from three bioscrubbers, no colonies were observed at all, most probably because of the low pH of the samples (2.7 – 4.0). On the culture plates from samples from two bioscrubbers, with sample pH of 4.7 and 6.0, respectively, separate and countable colonies were observed but these were confirmed not to be *Legionella* bacteria. The amoebal coculture method resulted in separate colonies for all 34 tested samples; however, suspected colonies were also confirmed not to be *Legionella*. A positive control (addition of *Legionella* to one well in the 12-wells plates), demonstrated that *Legionella* growth in amoebal coculture was not hampered by the presence of the recirculation water of the bioscrubbers.

Discussion and conclusion

The presence of biofilm, as was observed on the packing of the bioscrubbers, indicates possible growth opportunities for *Legionella* bacteria [7]. However, in none of the investigated bioscrubbers, *Legionella* spp. were detected in the recirculating water with the culture methods used, although temperature and pH conditions were in the ranges that allow *Legionella* growth in most (n=30) of the bioscrubbers.

Growth of nitrifying bacteria can be inhibited by accumulated nitrogen salts when the EC is above 18 mS/cm [20], an EC value that was observed in 13 bioscrubbers. The EC is mainly determined by the sum of NH_4^+, NO_2^-, and NO_3^-, where 1g N/l equals an EC of about 5 mS/cm [13]. However, there is no indication that nitrogen salts at the observed EC values inhibit growth of *Legionella* bacteria. Furthermore, the total salt concentration does not seem to be inhibitive either since it was shown that *Legionella pneumophila* can even survive in water with 1.5 – 3.0% sodium chloride [21], which equals EC values of about 23 - 46 mS/cm. Only at one bioscrubber the EC was (slightly) higher, viz. 49 mS/cm.

In the study published by [4], which investigated the presence of *Legionella* spp. at one farm with a bioscrubber, no *Legionella* was detected using culture methods. However, by using qPCR and micro array methods, DNA and antigens of *Legionella* spp. were detected in both the recirculation water and aerosols that were emitted from the bioscrubber. These results indicate a possible health risk in case viable *Legionella* bacteria would become airborne. Van der Heyden et al. [22] demonstrated that members of the family *Legionella*ceae were present at low abundancy (<0.5%) in water samples from two bioscubbers at pig farms, in which they studied the microbial community by 16S rRNA sequencing. These results match those obtained by [23], also using16S rRNA sequencing, who concluded that a strong selective pressure in one bioscrubber that they studied at a pig farm resulted in limited establishment and survival of species of the genera *Legionella*, *Mycobacterium* and *Clostridium*.

Although *Legionella* in the current study was not detected in any of the 36 investigated bioscrubbers with culture and amoebal coculture methods, the bacterium may nevertheless have been present in the recirculation water, but at concentrations below the detection limit. In future research, the updated culture method for analysing heavily contaminated water can be used [24]. In addition, to confirm the presence of *Legionella* DNA, molecular techniques such as qPCR can be used. However, caution with interpretation of qPCR results is warranted, because false positive results are possible with heavily contaminated water, such as water from bioscrubbers. Moreover, it is currently not possible to determine which percentage of the detected DNA originates from dead bacteria [25]. Alternatively, the presence of *Legionella* could be investigated in the air surrounding bioscrubbers and farms with bioscrubbers, because air samples contain less background flora that may have hampered the culture methods in this study.

To date no patients with Legionnaires' disease have been linked to bioscrubbers used at livestock farms. Positive environmental samples are rarely linked to Legionnaires' disease patients and it is not known if bioscrubbers used at livestock farms can spread *Legionella* bacteria to the environment.

Based on this study and the published results of other studies [4,21,22], the risk of legionellosis from bioscrubbers at farms for people that live near those farms seems limited

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