About:	Author:	Version:	date:
Legionella in waste water	Kevin Kanters	12	May 24th, 2021

Changes from version:

3 Life cycle Legionella and protozoa in wastewater described;
4 Deepening aerosol emissions and updating legal framework
5 Deepening aerosols, detection of methods, concentrations per purification, nutrient flows
6 Differences per type of aeration, reliability detection method, elaboration of the dose-effect relationship
7 Deepening nutrients and purification processes
8 Feedback processed from Petra Brandsema (RIVM), Jan Rullens (Infomil) and Imke Leenen (STOWA)
9 Analytical methods deepened, dose-effect relationship rewritten, aeration systems rewritten, information seed sludge processed, risk assessment approach added, feedback Frits Hollebekkers, Royal Haskoning DHV processed
10 Deepening analysis techniques, description Legionella growth after anaerobic reactor, processing case Legionella longbeachae, nutrient iron expanded
11 Legionella in communal influent after rainfall, description of Gammaproteobacteria, analysis technique sequencing added, new source-oriented measures described

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1. Introduction

In 2017 and 2018, two Dutch industrial waste water treatment plants were identified as a likely source for Legionella patients in the area. Since then, Hydroscope has been active in carrying out risk assessments, sampling, drawing up management plans and testing measures for effectiveness.

This document is a collection of relevant literature sources, practical experiences and input from interested parties. The document is updated regularly.

2. Cases Legionella in wastewater

In 2002, research was conducted into Legionella risks on Dutch WWTPs [1]. Legionella was present in wastewater and in the ambient air of the WWTPs. The levels of Legionella in the air are low compared to Legionella cases in cooling towers and air conditioners. Due to the low concentrations of Legionella in wastewater, activated sludge and sludge, exposure to Legionella on a WWTPs is low. Contrary to the cases described below, the temperature of the investigated waste water was low (<20°C).

In relation to cooling towers and drinking water installations, few Legionella cases with wastewater have been described:

- In 1999, five employees in Denmark got infected with Legionella while working on a wastewater treatment plant at a food company. The employees worked in a confined space and wore the wrong respiratory protection [9].
- In 2003 and 2004, 86 people in France fell ill with Legionella. Legionella was found in the wastewater treatment plant and cooling tower of a petrochemical company.
- In 2004, an employee in Sweden fell ill after working at a wastewater treatment plant at a paper mill [19].
- In 2005 and 2008, 64 people in Norway fell ill with Legionella. Legionella bacteria are found in a wastewater treatment plant at a wood processing company [3, 5, 6, 7].
- In 2005, an employee in Finland falls ill and works 200 meters away from a wastewater treatment plant. Legionella is found in both the wastewater treatment and the nearby cooling tower [2].
- In 2006, an employee in Finland falls ill after working on an aeration basin [2].
- In 2013, 78 people fall ill in Germany. Legionella is found in the wastewater treatment plant of a beer brewery. Most likely, legionella spread through a nearby cooling tower [11].
- In 2017, two employees fall ill in Finland after cleaning an air scrubber of a wastewater treatment plant [21].
- In 2016 and 2017, 15 people in Boxtel fall ill with Legionella. After source detection, Legionella is found at a wastewater treatment plant of a meat processing company. The sequence type ST1646 corresponds to five patients. The patients live up to two kilometers from the wastewater purification.
- At the beginning of 2018, Legionella was found at a wastewater treatment plant in Son. Since 2013, there have been more reports of Legionella than expected in that area. In the period from 2013 to March 2018, there were 56 patients, compared to only 11 patients in the previous 5 years (2008-2012). This increase is greater than the national increase. In the period 2013-2017, an identical ST type was detected in 7 out of 17 patients (ST1646). In 2018, there was also a patient seen where the typing indicated an ST1646 [18].
- In 2018, the water boards recommend not to spray water from ‘the Roer’, after high concentrations of Legionella were found in ‘the Roer’. A paper factory in Düren (Germany) has discharged high concentrations of Legionella into the sewer. The water ended up in the Roer via the sewage treatment plant.
- In 2020, a person in Amerika got infected with Legionella Longbeachae after using dry waste from a water treatment plant to fertilize his garden [104].

In various studies, Legionella concentrations up to 10^9 cfu/l have been found in wastewater treatment plants. Legionella in air is difficult to measure. In Norway, up to 3,300 cfu/m³ of air was measured directly above the aeration basin. The concentration of Legionella bacteria measured in this study in the air, directly

above the basin, was a factor of 10^2-10^7 lower than in the water. Bacteria have been found in the air up to a distance of 200 meters from the treatment plant. At the WWTP in Norway, two basins were aerated at 500 m^3/min normally[7]. For comparison, the WWTP in Boxtel is aerated at 140 m^3/min normally. The air throughput in a cooling tower is normally 10^3 to 10^4 m^3/min.

Legionella is regularly detected in wastewater treatment plants. In most wastewater treatment plants, aerosol formation also takes place. In 2019, the environmental services and STOWA made an inventory of the wastewater treatment plants [38]. Through a literature study, RIVM has drawn up four risk criteria to estimate the legionella risk at these WWTP's. Based on these criteria and the inventory, the risk of Legionella multiplication and spread was estimated as plausible in 69 of the 382 industrial wastewater treatment plants (18%) and 12 of the 327 sewage treatment plants (4%) [38].

Using a calculation model, RIVM has estimated how many aerosols containing Legionella bacteria spread through the air from wastewater treatment plants. Compared to people without pneumonia due to Legionella, the "Legionellosis" patients appeared to have been exposed to more aerosols from wastewater treatment plants. This is an indication that these aerosols have caused this form of pneumonia in recent years [61].

3. Dutch Legal framework and guidelines

3.1 Health and safety guidelines

The Occupational Health and Safety Legislation serves to ensure that employees work safely and healthily. The Working Conditions Decree Articles 4.84 to 4.102 pay attention to biological agents and Legionella. In accordance with the occupational hygiene strategy, the Working Conditions Decree prescribes the following measures:

- Remove the source where possible;
- Where possible, taking technical measures to prevent or reduce workers' exposure to biological agents;
- Where it appears that technical measures have insufficient effect, organizational measures must be taken;
- Personal protective equipment should be used as a last resort.

Occupational health and safety information sheet nine aims to minimize risks from biological agents [12]. According to the Health Council, it is not possible to prescribe a health-based recommended exposure limit for biological agents, due to lack of knowledge. Rules have been drawn up by various bodies. However, these should not be considered as a safe limit. NVvA/NVAB rule from 1989 sets 10,000 cfu/m³ of room air as the limit for the total of bacteria and 1,000 cfu/m³ of room air as a limit for each specific type of gram-negative bacteria.

VLA (Association of Suppliers of Air Technical Devices) applies stricter practical limits for the indoor environment. Legionella is considered "Group 3: high harmfulness". A value <10 cfu/m³ is considered good, a value between 10 and 20 cfu/m³ is considered moderate and a value >20 cfu/m³ is considered poor.

Stowa published a report in 2004: 'Exposure to endotoxins and the prevention of complaints among employees of sewage treatment plants'. The research shows that employees are exposed to endotoxins during cleaning work and in areas (such as sludge dewatering and screening). The following recommendations were made in this report:

- Limit the release of endotoxins at the source. If this fails, shield the source. Provide PPE as a last resort;
- Forced ventilation of rooms where there is aerosolization of sludge, influent and/or effluent takes place;
- Ensure cover or canopy of the sources;
- Avoid high-pressure cleaning;
- Do not use effluent indoors for cleaning work;
- Reduce the length of stay of employees near less-favoured areas;
- Provide information;
- Ensure sufficient availability of personal protective equipment.

Occupational health and safety information sheet 32 focuses specifically on Legionella prevention in process water systems to protect personnel [13]. Chapter 6.8 describes the risks and measures in WWTP's and AWZI's. For grate- and auger installations, rooms with covered oxidation beds and sometimes the aeration basins, a relatively large amount of aerosol formation takes place. These locations pose an increased risk,

but in general the stay of staff here is only short. In rooms where the sludge is processed (belt filter press), the staff will be present longer and the risk will be slightly higher. In this room, aerosols are mainly created by carrying out cleaning work with a high-pressure cleaner.

The A&O Fund Water Boards has released an Occupational Health and Safety Catalogue. Part 5 Chapter 4 describes the risks of biological agents in waste water [36]. In the 2011 version, the VLA guideline was used for Legionella in room air. The guideline is no longer included in the 2016 version. The following measures are recommended to prevent aerosol formation:

- Applying closed systems instead of open systems to limit aerosol formation;
- Take into account the accumulation of aerosols in indoor processes. In the context of the occupational hygiene strategy, source extraction should first be applied. If this is not sufficient, the room extraction must be increased. A number of process components in the purification process are already equipped with source extraction as standard. Examples include belt filter presses, filter presses, screen removal installations and centrifuges;
- Preventing/minimizing sludge in aerosols;
- Prevention of heating of pipes to prevent further growth of biological agents. At a temperature of more than 20°C, the growth of micro-organisms should already be taken into account;
- High exposure installations: performing easy-to-clean floors and walls;
- The application of forced ventilation in indoor processes;
- Belt filter building: prevent belt filter presses from being cleaned manually;
- Screenings treatment building: if it is necessary to prevent foaming in the screenings building, technical measures must be taken to minimize aerosol formation;
- Automate sampling, create sampling taps instead of hatches, and realize sampling points in the safest possible place;
- When designing and renovating a treatment plant, objects (installations and facilities) that may be a possible source of contamination of biological agents must be mapped. The walkways and (logical) walking routes should be designed and constructed in such a way that the possible exposure is minimal.

Within the Health and Safety Catalogue, cleaning activities, inspection work/sampling and work on sludge dewatering equipment are considered to be high-risk.

For cleaning work, it is important that:

- Spilled sludge is removed dry as much as possible;
- If it is not possible to remove sludge dry, clean water may be used. Try to avoid the use of effluent when removing sludge;
- High pressure cleaning is used as little as possible (both with effluent and clean water);
- Respiratory protection (FFP3 mask) is worn during indoor work and when aerosols are formed.

For taking water samples and carrying out of checks, it is important that:

- Spilled sludge is removed dry as much as possible;
- If it is not possible to remove sludge dry, clean water may be used. Try to avoid the use of effluent when removing sludge;
- High-pressure cleaning is used as little as possible (both with effluent and clean water);
- Respiratory protection (FFP3 mask) is worn during indoor work and when aerosols are formed.

3.2 Environmental Management Act

The Environmental Management Act does not set specific requirements for Legionella prevention with regard to wastewater treatment. This applies to wet cooling towers. The Activities Decree (Articles 3.16a and 3.16b) describes specific regulations regarding risk assessment, prevention and management.

Article 1.1a describes the general duty of care. The general duty of care assumes that everyone takes sufficient care of the environment. In any case, it means that any person who knows- or can reasonably suspect- that his actions or omissions may cause adverse effects on the environment is obliged to refrain from such actions, or to take measures.

Pursuant to Chapter 17 of the Environmental Management Act, a municipality may designate a Legionella contamination that has occurred as an "unusual occurrence". This allows the municipality to impose additional permit requirements and/or enforce a plan of action.

The competent authority may impose a periodic penalty payment if the measures are not followed.

In the case of new permits to be issued, the municipality can include additional customized regulations for Legionella prevention.

3.3 Environmental Law General Provisions Act (Wabo)

Environmental permits are issued from the Wabo. The environmental permit can be adjusted on the basis of Article 2.31 of the Wabo. This can be done, for example, on the basis of:

- Technical developments
- New environmental insights
- To prevent serious adverse effects on the physical living environment
- Announced new administrative order (AMvB)

The permit can be changed ex officio. A draft decision shall be made available for inspection for this purpose.

The Ministry of Infrastructure and Water Management is working on a guide for 'Legionella management in WWTP's'. This is expected in July 2021. After adoption of the guideline, the Environment Activities Decree will be amended by means of an Order in Council, which will provide for national regulation of Legionella in WWTP's. The aim is for the amendment to come into effect on January 1st 2022.

3.4 Approach to high-risk locations

The RIVM and the environmental services have mapped out industrial biological treatment plants. 69 purifications have been designated as high-risk on the basis of the following criteria [38]:

- Industry: Food industry, wood and paper industry, and rendering- and petrochemical companies;
- Water temperature between 25°C and 45°C;
- Distribution via aerosols and/or effluent plausible.

In July 2019, the Ministry of Infrastructure and Water Management urged the Environment Services to supervise the high-risk treatment plants. It is being called for to cover the aeration tanks and to make an inventory of the (re)use of effluent.

The Environment Services have defined an approach in carrying out their supervisory task. This approach is based on three scenarios [39]:

1. An industrial water treatment plant, but no Legionella was found:
    - Carry out risk analysis, draw up a management plan and take samples;
    - Urgent advice to cover the aeration tanks and not to use effluent for atomization.

2. An industrial water treatment plant and Legionella has been found:
    - Same as scenario 1;
    - Conduct research into system customization.

3. An industrial water treatment plant, Legionella has been found and there may be patients:
    - Same as scenarios 1 and 2;
    - Report as unusual occurrence;
    - Adjusting the environmental permit;
    - Take measures for Legionella reduction in effluent;
    - Proceed to air sampling.

3.5 German guidelines 'Legionella Expert Commission'

In Warstein (Germany) there was a Legionella outbreak in 2013. Legionella was found in a wastewater treatment plant. The treated wastewater was discharged into a river. A nearby cooling tower was fed From the river water. Commissioned by the Ministry for Climate Protection, Environment, Agriculture, Nature and Consumer Protection of the State of North Rhine-Westphalia, an expert committee made recommendations for Legionella prevention [26].

The following recommendations have been made for the discharge of treated waste water into a river.

Legionella result	recommendation
< 1,000 cfu/100 ml	No measures required.
≥ 1,000 < 10,000 cfu/100 ml	Inform the environment and carry out further research.
≥ 10,000 cfu/100 ml	Take measures to reduce the numbers. Possibly enact a ban on the abstraction of river water.

As far as is known, the recommendations are not incorporated into standards or regulations, but are used by industry.

4. Growth factors

This chapter describes the different species, the habitat, and growth factors of Legionella bacteria.

4.1 Type of Legionella

Not all Legionella species are equally dangerous. Legionella pneumophila serotype 1 causes 70% of cases. Legionella pneumophila serotype 2-14 cause 20-30% of cases. Legionella species/Legionella non-pneumophila, a collective name of all Legionella species other than Legionella pneumophila, cause 5-10% of cases [54].

The Ministerial Regulation on legionella prevention in drinking water and hot tap water describes 20 Legionella non-pneumophila species that have been linked to cases of disease in the WHO context. The following species lead to the most cases of disease [55]:

- L. micdadei (60%);
- L. bozemanii (15%);
- L. dumoffii (10%);
- L. longbeachae (5%);
- other species (10%).

Since 2015, Of the various Legionella non-pneumophila species, L. Longbeachae has mainly been found in patients in the Netherlands [96].

Hydroscope has encountered many different types of legionella in wastewater treatment plants, including L. pneumophila (ST 1, 2, 3, 5, 6, 7-14), L. oakridgensis, L. dumoffi, L. bozemanii, L. gormanii and L. feeleii.

From the point of view of occupational health and safety legislation, Legionella is considered biological agents. In accordance with the class classification according to European Directive 2000/54/EC Annex 2, Legionella pneumophila belongs to pathogenicity class 2. Pathogenicity class 2 applies to a micro-organism that may cause a disease in humans, which is unlikely to spread to the population, while there is an effective prophylaxis, treatment or control, as well as a micro-organism that may cause disease in plants or animals [12].

4. 2 Temperature

The temperature determines the growth (multiplication) and the death of Legionella bacteria. Legionella pneumophila belongs to the group of mesophilic bacteria.

temperature	state
Below 20°C	Almost no growth
20 to 51°C	Able to grow
32 to 42°C	Optimal temperature to multiply

In the past, the death of the Legionella bacterium was assumed at temperatures above 50°C. However, more recent studies have shown growth at 51°C. In addition, some of the bacteria do not appear to die, but survive for a long time in a kind of dormant form (VBNC), in which it is no longer growable in the laboratory with the usual cultivation techniques [98].

In 2019, research showed long survival time at high temperatures of this dormant form. Legionella can no longer be cultivated with the usual laboratory techniques in:

- 3-8 hours at 55°C,
- 60 minutes at 60°C,
- 2 minutes at 70°C.

However, some of the bacteria survive high temperatures in a kind of dormant form for a longer time. A log 2 reduction of live bacteria is achieved at:

- 9 days at 55°C,
- 8 hours at 60°C,
- 20 minutes at 70°C.

Although legionella in VBNC form is less infectious, true loss of infective capacity was only seen after:

- 85 days at 55°C and 60°C,
- 8 days at 70°C.

These values were found for two investigated Legionella pneumophila strains [98]. However, the optimum growth temperature and heat tolerance differs per Legionella pneumophila strain and Legionella species.

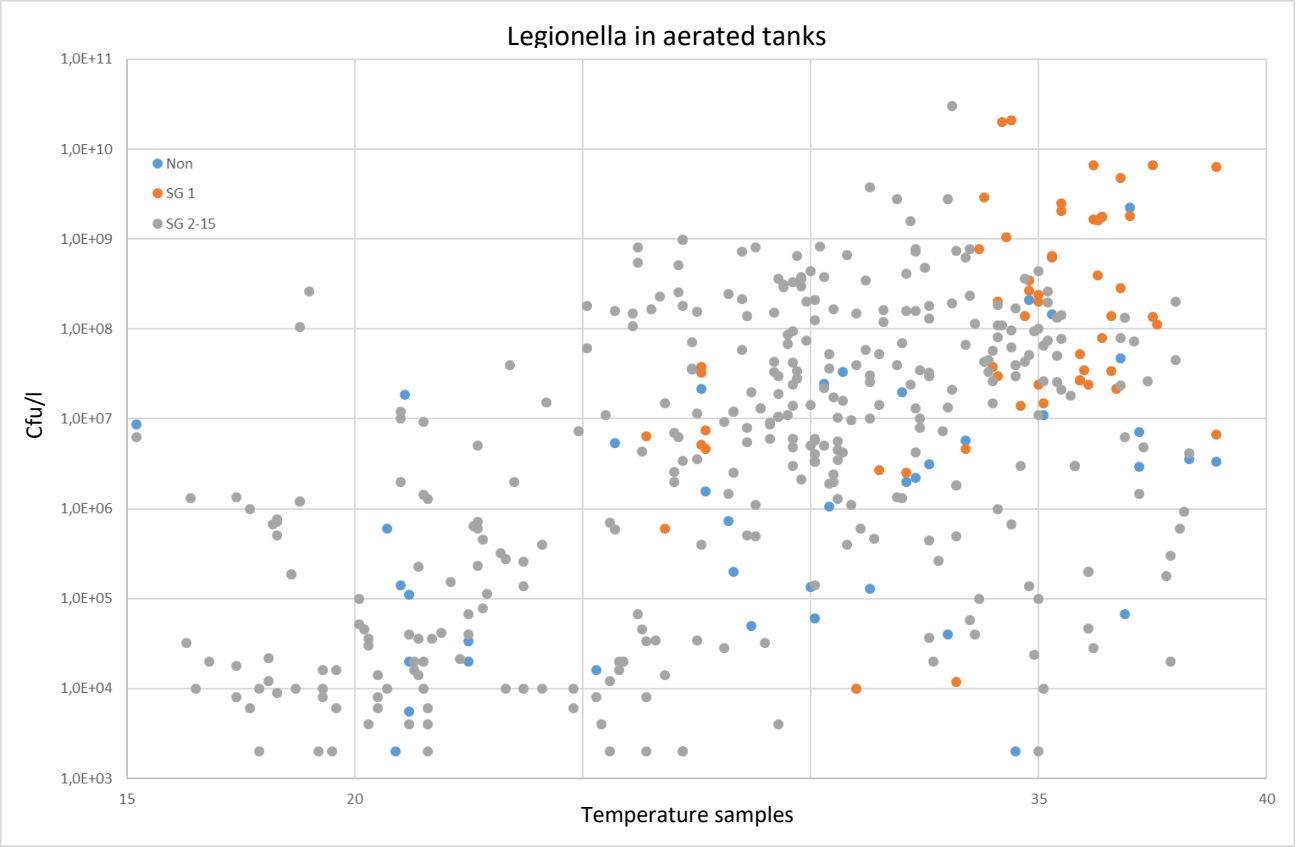
The residence in biofilm and protozoa also gives the legionella bacteria some protection against higher temperatures and also against disinfectants.

On a laboratory scale, research has been carried out into the growth of Legionella in activated sludge at temperatures of 15, 24 and 35°C. At a temperature of 35°C, Legionella pneumophila grows exponentially. At a temperature of 15°C, Legionella species prevail. The research shows that temperature is the most important parameter in the growth of Legionella in activated sludge [17].

For biological wastewater treatment plants, RIVM uses the following [38]:

temperature	Condition
< 25°C > 45°C	Propagation to high concentrations of Legionella pneumophila not to be expected. Some growth is not excluded.
25-29°C 39-45°C	Propagation to high concentrations of Legionella pneumophila is likely.
30-38°C	Propagation to high concentrations of Legionella pneumophila is very likely.

Hydroscope has taken 1,400 Legionella samples at 20 hot reactors, with a water temperature between 15° C and 39°C. In these measurements there appears to be a log factor increase in legionella concentration per 5°C temperature increase.



4. 3 Nutrients

Legionella is a heterotrophic bacterium, so it uses organic compounds as a carbon source.

Carbon source	name	energy source	name	Aerobic/anaerobic	Examples
Organic compounds	Heterotrophic	light	Phototrophic		
		Light & redox reaction	Mixotroph		
		Redox reaction	Chemotrophic	Aerobic	Legionella
				Anaerobic	
				optional	E-coli
Inorganic compounds	Autotrophic	light	Phototrophic		
		Light & redox reaction	Mixotroph		
		Redox reaction	Chemotrophic	Aerobic	
				Anaerobic	Anammox
				optional	

Legionella belongs to the strain Proteobacteria and class Gammaproteobacteria. Proteobacteria are gram negative.

Amino acids are the main carbon source for Legionella. Legionella pneumophila needs the following amino acids [47]:

Code	name	Autotroph for	In vitro	In vivo	Extracted from amoeba
area	Alanine				X
Arg	Arginine	X	X	X	
Asn	Asparagine				
Asp	Aspartic acid				X
Cys	Cysteine	X	X	X	
Gln	Glutamine			X	
Glu	Glutamic acid				X
Gly	Glycine				
His	Histidine				
with	Isoleucine	X	X		
Leu	Leucine	X	X		
light	Lysine				
with	Methionine	X	X		
Phe	Phenylalanine				X
for	Proline				X
be	Serine	X		X	X
Thr	Threonine	X	X		
Trp	Tryptophan				X
Tyr	Tyrosine				
Val	Valine	X	X		

High concentrations of Legionella are detected especially in wastewater from industries with a high protein and amino acid content [27]. Research at three wastewater treatment plants has shown that there is a positive correlation between Legionella species and COD, Kjeldahl nitrogen and protein concentration [47].

Legionella is able to build up a fuel reserve. This reserve is used to survive in the event of a lack of nutrients. The fuel reserve is built up in the form of poly-beta-hydroxybutyric acid or poly-3-hydroxybutyrate (PHB). Legionella pneumophila SG1 is able to convert 16% of its dry weight to PHB [91].

Under optimal conditions, Legionella does not store PHB. When Legionella is under stress, it starts building PHB. This in case there is a food shortage. PHB is made up of serine and later on by glucose [93].

Legionella needs iron to grow. Legionella can obtain iron in various ways, such as through dissolved metals or directly from its host. The construction and consumption of PHB is controlled by iron. In case of iron deficiency, PHB is produced. The iron deficiency can arise because the host of Legionella becomes exhausted and dies. If there is sufficient presence of iron, the PHB reserve is also reduced again [91]. Legionella is limited in water with iron concentrations below 75 mg/l. At iron concentrations above 300 mg/l, Legionella is more common [105]. High concentrations of iron (FE^{3+}) in wastewater appear to be growth-inhibiting [47].

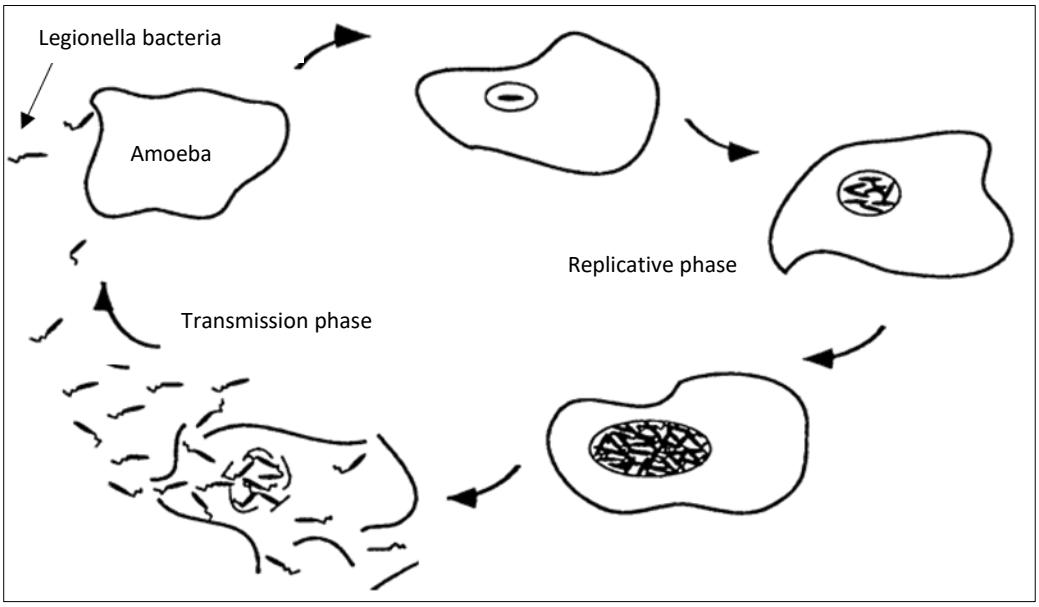
Legionella can survive between a pH of 5 and 9. Legionella grows optimally at pH between 6.5 and 7.5 [28]. Legionella is usually located in biofilm. The pH within the biofilm may differ from the pH in the water system.

Legionella needs oxygen to multiply. Legionella grows well, with dissolved oxygen saturated (6-6.7 mg/l) water. Under anaerobic conditions (1.7-2.2 mg/l), less Legionella is manifested after the application of the culture method. However, Legionella has been isolated from water with dissolved oxygen of 0.3 to 9.6 mg/l. Survival under anaerobic conditions is therefore (temporarily) possible [28]. No information is yet known about the VBNC status of Legionella at low oxygen levels.

4. 4 Life cycle

Legionella grows and multiplies preferably in protozoa, such as amoebae. The bacterium has a large number of different life stages. At least 14 different stages of life have been described and the life cycle of the Legionella bacterium can vary depending on the environment and the protozoa present [99].

A common life cycle of Legionella in drinking water consists of the transferable phase (transmission phase) and the growth phase (replicative phase). During the growth phase, the Legionella bacteria multiply in the protozoa. Under ideal conditions, Legionella bacteria can multiply in 99 minutes [70]. The bacteria are immobile, long and filamentous (wire-shaped) at this growth stage. The bacteria are 2 to 6 µm long and can form wires up to 20 µm. The Legionella bacteria extract nutrients from their host. Amino acids are an important source of energy. Oxygen consumption increases with growth [93]. When the nutrients run out, Legionella starts to build up PHB. The offspring also change and move into the transferable phase. The offspring escape from their host. They can survive outside the host and look for a new host. Legionella uses the accumulated PHB as a fuel source at this stage [92]. The bacteria in this transferable phase are short and very mobile rods [62]. They are approximately 1.3 µm long at this stage [70].



In drinking water, it is assumed that Legionella can only multiply within protozoa. However, in wastewater, Legionella bacteria have been shown to multiply outside protozoa [48].

With long-term food deprivation, Legionella goes into a sleep state. Legionella still uses PHB as fuel, but it is used up less quickly. Legionella is capable of surviving for up to 600 days [91]. In the dormant form, the Legionella bacteria are not very infectious, although VBNC's may still be able to infect other cells [99]. Via an extra step with protozoa, such as with Acanthamoeba castellanii, the VBNC form can be reactivated to the growable form.

Practical measurements show that legionella concentration in wastewater can fluctuate greatly. Within a period of several weeks, the concentration can increase or decrease many log factors. When there are many

amoebae in the water, Legionella bacteria can multiply quickly. Because of the Legionella growth, the amoebae eventually die. As a result, amoeba concentration and subsequently Legionella concentration decreases again [110].

4. 5 Protozoa in wastewater

Legionella develops mainly within protozoa. Protozoa are mainly located in the biofilm of pipe material, but also in wastewater. Protozoa can swim freely in wastewater, but are also part of sludge flakes. In addition to protozoa, Legionella bacteria can also multiply within rotifers. Unlike protozoa, they are multicellular. Rotifers also occur in wastewater.

Protozoa

Protozoa can be divided into four groups according to the way they move:

1. Flagellates or dinoflagellates;
2. Ciliophora, Ciliates or ciliated protozoan;
3. Amoebozoa, Amoebae or Sarcodina;
4. Apicomplexa or spore-forming animals.

Legionella does not develop in all protozoa. Amoeba in particular is known to host them. About 20 species of amoeba have been described as hosts [59]. Some Ciliates are also known to serve as host, including Paramecium caudatum [64].

Amoebae are often divided into two types: scale amoebae and naked amoebae. Naked amoebae in particular are known to host Legionella [66]. In any case, the following amoeba are known to occur in waste water [65, 68]:

- Scale amoebae
    1. Arcella: Can host Legionella [66];
    2. Centropyxis: Can host Legionella [67];
    3. Rhogostoma: Can host Legionella [109];
    4. Fisculla: Can host Legionella [109];
    5. Thecofilosea: Can host Legionella [109];
    6. Euglypha: Legionella growth is limited [67];
    7. Trinema [68]: Unable to find information related to Legionella;
    8. Bullinularia [68]: Unable to find information related to Legionella;
    9. Difflugia [68]: Unable to find information related to Legionella.
- Naked amoebae
    1. Hartmannella vermiformis: Can host Legionella;
    2. Naegleria: Can serve as host for Legionella;
    3. Acanthamoeba: Can host Legionella.

Other amoebae may also be present.

Activated sludge

Activated sludge is mainly intended for the removal of the organic fraction from wastewater. Nitrogen and phosphate are also removed to a limited extent. Activated sludge is a mixture of microorganisms (bacteria, protozoa and rotifers). In the presence of sufficient oxygen, these organisms are able to oxidize organic

components from the water to CO_2 and oxygen (dissimilation). However, part of the organic matter is used for making biomass, which causes the sludge to grow. The excess sludge is drained. Part of the sludge is returned to the beginning of the process (returned sludge) to promote the production of sludge.

Activated sludge flakes are conglomerates of living and dead bacterial cells, often including wire-forming species, precipitated salts and captured inorganic particles (sand) and organic fibers. The whole is held together by a mucus matrix of polymeric compounds around the cells and chemical bonding forces [69]. In activated sludge plants, protozoa can grow to 3-20 $x^{10\,6}$ cells/l. It is estimated that the protozoa biomass can reach values of 250 mg/l (dry weight), which makes up more than 9% of volatile solids [58].

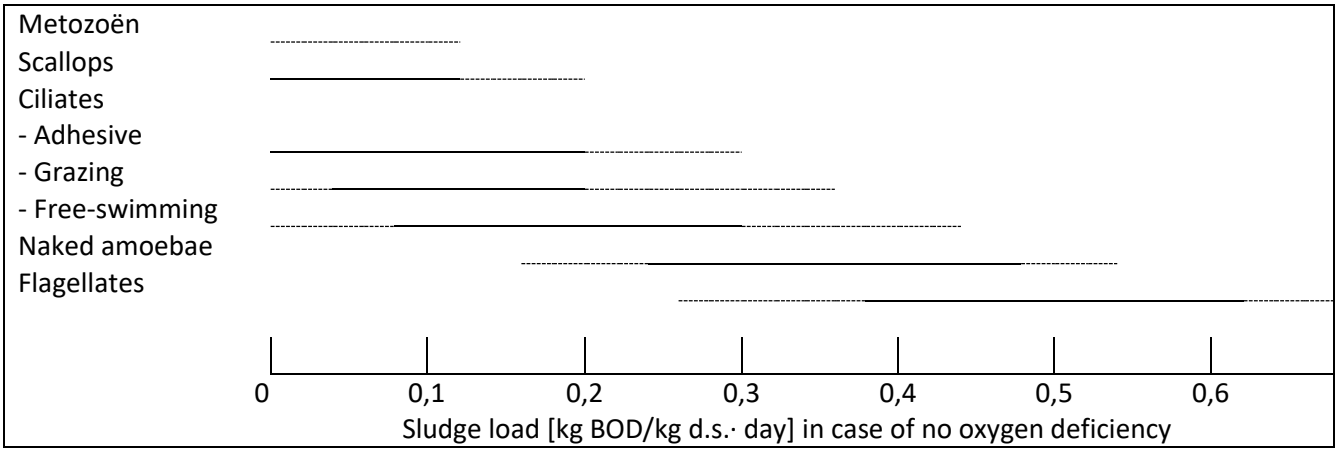
The form of activated sludge flakes varies from rounded to irregular. A purification works optimally if rounded flakes are present. That way, the sludge settles best. Bacteria form flakes to maintain themselves in nutrient-poor environments. The need to form flakes decreases as more food becomes available and fast-growing species start to dominate the population. The firmness of the flakes is therefore mainly determined by the sludge load. Sludge load is the ratio between nutrients and formed biomass. At a low sludge load, firm flakes are formed and gram-negative bacteria prevail (Legionella is gram negative). When there is a high sludge load, the sludge becomes more irregular, the number of fast-growing gram-positive bacteria increases [69].

Three phases can be distinguished at the start-up of an activated sludge installation. In the beginning, few nutrients are present. Flagellates predominate, because they need few nutrients. In the second stage, free-swimming Ciliates grow. Ciliates then attach to the sludge flakes. The third phase, the stabilization phase, creates a balance between sludge growth and sludge discharge [58].

Loose cells are not part of the sludge flake and are freely present in the water. The cells do not settle and cause a deterioration in effluent quality. In case of a short sludge stay time or high sludge load (above 0.3 to 0,4 kg BOD/kg d.s.· day) many separate cells are present.

Composition of activated sludge in relation to Legionella

Legionella can use different protozoa as host. Microscopic analysis of activated sludge may give an indication of which Legionella growth-promoting protozoa are present.



Ciliate Paramecium may contain Legionella. These free-swimming Ciliate (paramecium) are mainly present at a sludge load of 0.1 to 0.3 kg BZV/kg d.s. · day [69].

Naked amoebae can grow to 50 to 400 µmin in size. Naked amoebae mainly occur at a higher sludge load of 0.1 to 0.4 kg BZV/kg d.s. · day and/or oxygen deficiency. Therefore, they are only rarely observed in low-load systems with extensive nutrient removal [69]. Free-swimming amoebae can occur in all process steps of a purification. Free-swimming amoeba have been detected in the influent, anaerobic reactor and aerobic reactor at a purification plant in South Africa [63].

Scale amoebae occur in large numbers in low-load sludge. Arcella can host Legionella and is the most common species. Arcella mainly occurs under nitrifying conditions [69]. Euglypha are also regularly found in aeration tanks [58]. Legionella growth in Euglypha is limited [67]. Scale amoebae of the Rhizaria group have been found in sequencing research as the dominant amoebae in wastewater treatment plants. This includes the scale amoebae Rhogostoma, Fisculla and Thecofolosea sp. [109].

Scale amoebae usually occur in young sludge, but can also grow during sudden high biochemical oxygen demand (BOD). Even after disturbances, the growth of scale amoebae can suddenly be high [66]. Scale and naked amoebae can survive well with a low amount of dissolved oxygen [65].

In the case of old sludge, in the presence of rotifers and nematodes, large concentrations of scale amoebae can suddenly arise [66].

Theoretically, bacteria can only develop in activated sludge if their multiplication time is shorter than the sludge age. Under ideal conditions, legionella bacteria can multiply in 99 minutes [70].

Sludge retention time can make a difference in the life form and growth rate of Legionella. Legionella prefers string-shaped growth with a short sludge retention time (2 to 2.5 days) and a clustered form with a long sludge retention time (10 days) [48].

In conclusion, Legionella can survive within protozoa under different conditions. Based on the amount of literature available, free-swimming amoeba in particular are the main host for the growth, or spread, of pathogens. Further research is needed to establish the relationship between protozoa and optimal Legionella growth. A number of industrial treatment plants are characterized by large fluctuations in Legionella concentrations. Sludge research may provide an explanation for this.

4.6 Impeding growth factors

Low concentrations of salt (0.1-0.5 percent NaCl) promote the growth of Legionella in water. A high salt concentration can hinder Legionella growth. Seawater research has shown that Legionella bacteria can survive up to salt concentrations of 3% NaCl at a temperature of 4°C and 20°C. Amoebae can also survive in salt water [45]. At temperatures of 30°C and 37°C, the Legionella concentration decreases at a salinity above 1.5% NaCl [44].

Heterotrophic bacteria, such as Pseudomonas spp. and Aeromonas spp. may inhibit the growth of Legionella [56]. These bacteria use the same nutrients as Legionella. They compete with each other, or excrete substances that kill Legionella bacteria (Declerck, 2010).

Acanthamoeba castellanii is inhibited in growth by peptide. The growth of Legionella within this host is also inhibited [94].

The presence of high concentrations of organic acids and ammonium in anaerobically pretreated waste water caused growth inhibition [48].

Excessive concentrations of amino acids can also hinder in the growth of Legionella bacteria. Despite high concentrations of amino acids, activated sludge samples have shown that Legionella growth is possible. This could mean that Legionella bacteria multiply within protozoa [47].

Research into Legionella control in wastewater has shown that UVc treatment, copper/silver treatment, hydrogen peroxide, chlorine dioxide and ozone are ineffective. The amoebae provide adequate protection for legionella bacteria [16].

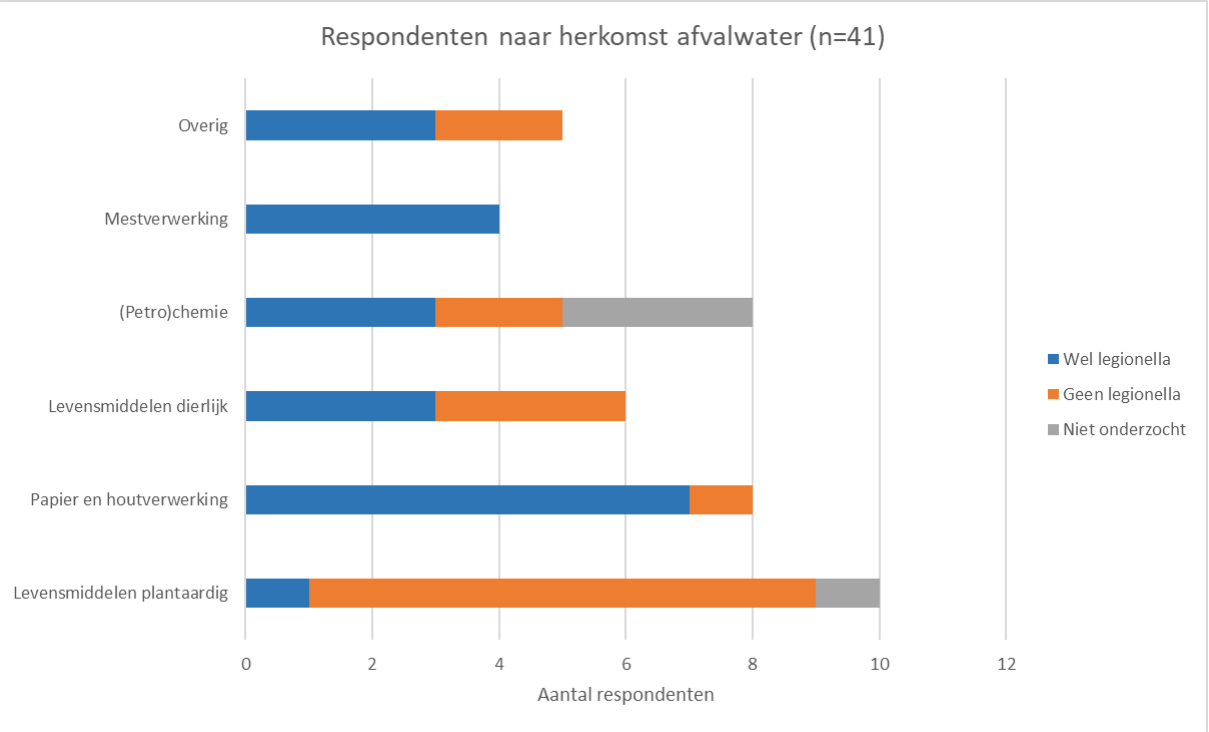
4. 7 Type of purification process

Type of industry

Case studies show that Legionella has been found in biological treatment plants of the food industry, paper industry, wood processing, petrochemicals, rendering plants, sewage treatment plants and textile industry [38].

VEMW conducted a survey among its members. The survey was sent to the operators of 69 industrial biological wastewater treatment plants. For 41 treatments (59%) the survey has been completed [88].

The questionnaire asked for the origin of the waste water. There was a big spread in answers. As a result, the number of categories has been reduced and the food industry has been redistributed to 'animal' and 'plant-based'. Generically was asked if Legionella was found on the treatment. In the following graph, the origin of wastewater is plotted against the detection of Legionella.



Wastewater from food companies that work with animal ingredients appears to be more sensitive to Legionella growth than the wastewater from companies that work with plant-based ingredients. The food industry with animal ingredients consists of one purification at a meat processor and five purifications at dairy related farm companies. Manure processing appears to be sensitive to Legionella growth. The 4 purifications with manure processing all contain Legionella. More than average Legionella has been found in the paper industry. It has been noted that the presence of Legionella fluctuates greatly per purification and over time [88].

Legionella in communal influent

During heavy rainfall, after a previous warm period, relatively many people become infected with Legionella. In addition to water, Legionella also grows in the soil. During heavy rainfall, the bacteria wash out to the water. Research into heavy rainfall during a warm summer period has shown that 2 out of 77 rain puddles contain Legionella. Six samples were taken from water on the street. Legionella was found in 3 samples. Legionella was also found in 38% of samples at wastewater treatment plants (9 out of 24 samples) [106, 107].

Process parameters and addition of nutrients

The process parameters were queried in the VEMW survey. This has only been stated by a limited number of respondents. Legionella seems to grow to higher concentrations at high concentrations of Kjeldahl nitrogen and COD. The eight treatments with the highest concentrations (above 10^7 cfu/l) have accordingly a water temperature above 33 degrees Celsius, a COD supply above 2,000 mg/l, a BOD supply above 1,000 mg/l and a pH between 6.5 and 7.7 [88].

Nutrients are added to promote the purification process. Nutrients are mainly added to treatment plants with non-animal-related influent, such as at paper companies, chemical companies and in companies in the category 'other'. Most nutrients are used to make up for a shortage of nitrogen or phosphorus. Within the category of non-animal-related influent, Legionella appears to occur more frequently in the treatment plant when nutrients are used [88].

It is not clear whether Legionella also plays a functional role in the purification process. Urea is often used as a nutrient for nitrogen supply. This is added before the anaerobic process step. Urea may be converted into valuable nutrients for Legionella via the nitrogen cycle.

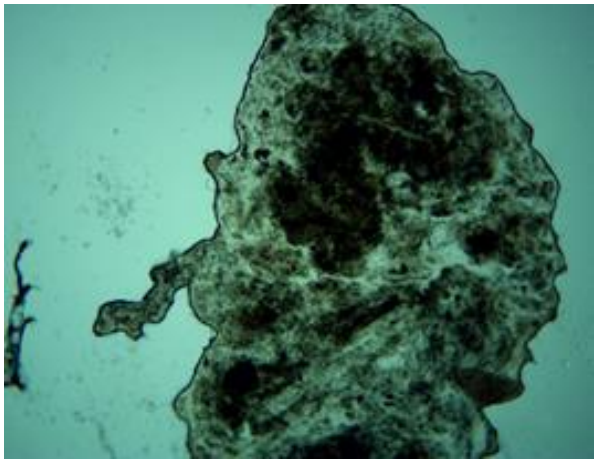
Legionella may have a role in the forfor-removal process. Phosphate-accumulating organisms (PAO) absorb acetate under anaerobic conditions and convert it to PHB via glycogen. The organisms release phosphate to the water, causing the phosphate content to increase. In the aerobic phase, it works the other way around. PHB is oxidized, phosphate is absorbed and converted to polyphosphate. Legionella is also able to build up PHB. With Legionella this seems to happen mainly in the final stage within the amoeba, when the extracted nutrients from the host run out.

Life forms in different purifications

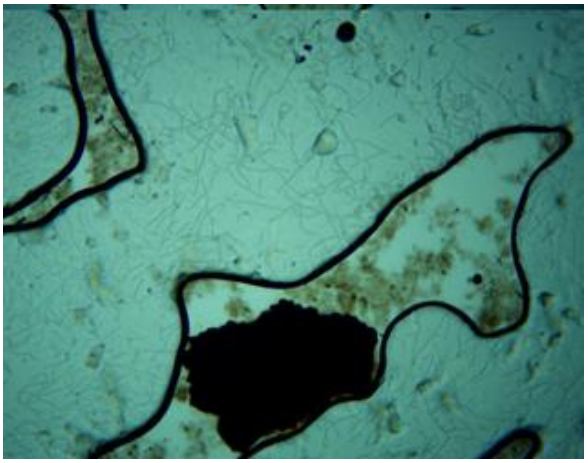
In German research, two reactors have been compared. A fully mixed aerobic reactor with 2.5 days of residence time and a membrane bioreactor with 10 days of residence time. The Legionella bacteria grew to different concentrations at 35°C. In the case of the mixed reactor, the legionella concentration was 3×10^3 to 4.8×10^6 CFU/l. In the membrane bioreactor, the concentration was 3×10^5 to 4.7×10^6 CFU/l [48].

The morphology of Legionella bacteria is different in both reactors. In the membrane bioreactor, the bacteria grew in clusters, while in the mixed reactor filaments predominate and show a higher growth rate [48].

Hydroscope conducted sludge testing on two types of treatments. Although Legionella is not marked as such in the photos, there is a clear difference between the types of purifications.



Aerated basin after anaerobic step 100x enlarged



MBR 100x enlarged

Legionella after an anaerobic reactor

In some cases, Legionella appears to grow rapidly after an anaerobic purification step. Three aspects play a possible role in this:

- Amino acids: In an anaerobic reactor, organic matter is converted into biogas in four steps [103]:
    1. Hydrolysis: amino acids are released from proteins, among other things
    2. Fermentation: further conversion to simple compounds, such as ammonia and volatile fatty acids
    3. Acetogenesis: further conversion to acetic acid and acetate, for example
    4. Methanogenesis: further conversion to methane and carbon dioxide

  The suboptimal course of the anaerobic process can lead to the effluent of the reactor containing many amino acids. Amino acids are an important building block for protozoa and Legionella.
- Free-swimming organisms: sludge granules are formed in an anaerobic reactor. At the end of the reactor step, the sludge granules and water fraction are separated from each other. The effluent from the reactor contains few sludge particles. Certain free-swimming amoebae, such as Acanthamoebae, can survive under anaerobic conditions and are contained in the effluent of the anaerobic reactor. Oxygen is used to create an environment that promotes the growth of free-swimming organisms, such as amoebae. This is also beneficial for Legionella growth. Sometimes an aerator is placed between the anaerobic reactor and activated sludge plant. This gives free-swimming amoebae longer time to grow, before they settle on sludge particles. The use of such an intermediate/pre-aerator appears to promote Legionella growth.
- Temperature: A mesophilic anaerobic purification works optimally at temperatures between 30°C and 40°C. The water from the anaerobic reactor has an ideal temperature for Legionella growth.

Type of purification and maximum concentrations found

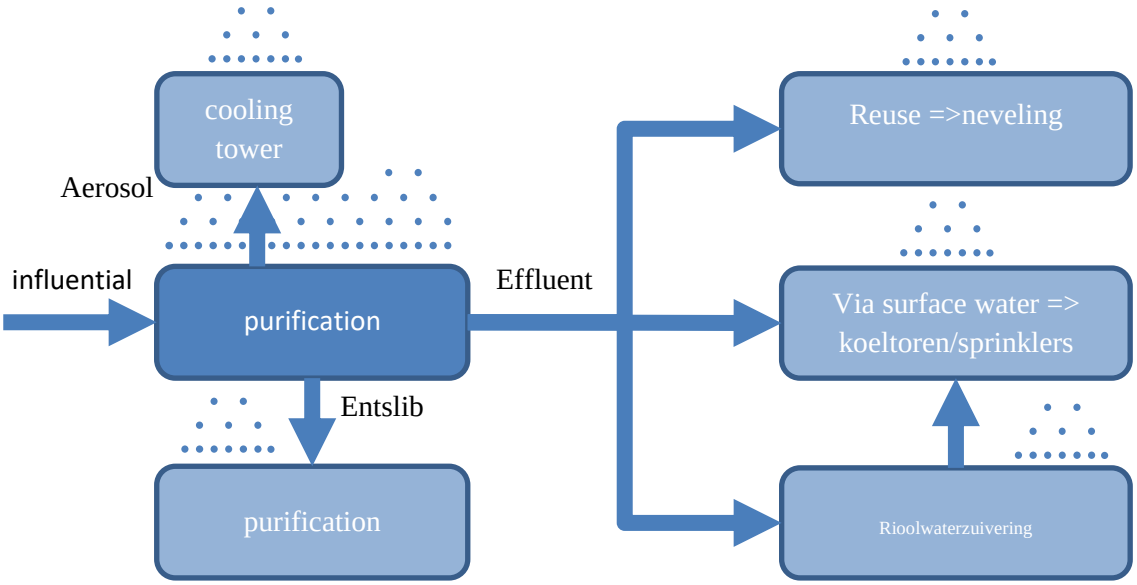
Hydroscope sample results show differences in maximum Legionella concentrations:

- Membrane bioreactors all 10^{10} cfu/l;
- Sub-process in sewage treatment for nitrogen removal through combination anaerobic/aerobic up to 10^{10} cfu/l;
- Paper industry with additional nitrogen source through combination anaerobic/aerobic up to 10^{9} cfu/l;
- Manure processing through combination anaerobic/aerobic up to 10^{8} cfu/l;
- Aerobic treatment only up to 10^{7} cfu/l.

Since the Legionella bacteria remain within the installation in MBR systems (with well-functioning membranes), this can also lead to higher concentration of Legionella in these systems [48].

5. Distribution routes

Legionella bacteria can spread directly from the purification or via an indirect route and be inhaled by a patient [31].



Direct diffusion takes place via aerosols released from the treatment plant. These can be inhaled by employees or bystanders.

The effluent from wastewater treatment plants is reused in some cases, for example as water for industrial use. Misting this water can pose a risk.

Some treatment plants discharge the effluent into surface water. There are known cases [6, 11] where high legionella concentrations have been detected in surface water. Ingestion and nebulization, for example by irrigation, of this water can pose a risk.

As described in the cases, cooling towers pose a danger when spreading Legionella bacteria over long distances through the air. Legionella bacteria can enter a cooling tower by air or after ingesting discharged wastewater. Poor management or technical defects in the cooling tower pose a danger to the environment.

The sludge from a wastewater treatment plant is normally drained and incinerated. When starting a purification, the sludge of another purification is often used to seed.

5. 1 Aerosol formation per type of aeration system

Legionella infection occurs through the lungs by inhaling the bacteria in very small droplets of water (aerosols). Aerosols are created, for example, by spraying with water or aerating water. Many biological treatment plants are aerated. Aerosols are released to a greater or lesser extent. This section describes aerosol formation by type of aeration system. The next paragraph discusses the other aerosol-forming process steps.

Aeration is mainly used to add oxygen to the wastewater. In addition, the aim may be to mix the wastewater, to move the wastewater, to cool water or to keep membranes clean.

Aeration systems can be divided into types [8]:

- Bubble aeration
    1. Aerate with pure oxygen
    2. Fine bubble aeration
    3. Coarse bubble aeration
- Surface aeration
    1. Rotors
    2. Point aeration
    3. Water jet aeration
- Atomization of wastewater itself
    1. Spray arms
    2. Rotors
    3. Cascade

The intensity of the mist during aeration may vary due to the amount of air throughput and the type of aeration. Depending on the droplet size, osmolarity and humidity, the level of the emission and meteorological conditions, the nebula can move further or less far through the air.

Condensation of water vapor also produces aerosols. Because water vapor consists of separate molecules, it cannot contain Legionella.

Here are some examples of aeration:

Example light form of fine bubble aeration, with foaming

Example intensive form of fine bubble aeration

Example coarse bubble aeration, partly to keep membranes clean

Example point aeration

Example spray arm

Example aeration with pure oxygen

An aerosol particle must be large enough to carry a Legionella bacteria. A Legionella bacterium is elongated and approximately 2 µm by 0.3 - 0.9 µm in size [61]. Breathing through the nose or mouth makes big difference in reaching an aerosol droplet to the lungs. Aerosols smaller than 3 µm can easily reach the deep lungs (alveoli). Particles larger than 8 µm settle in the nose or upper respiratory tract [78]. Other research describes that particles can be up to 5 µm in size to reach the lungs [79].

The ciliated epithelium in the upper part of the lungs can remove the inhaled bacteria in a large number of cases without disease occurring. Amoebae, or vesicles secreted by these amoebae, may also be present in aerosols, which may contain hundreds or even thousands of Legionella bacteria [62].

Surface aeration produces fewer aerosols than bubble aeration [102]. Fine bubble aeration produces less aerosols than coarse bubble aeration [72]. The intensity of aeration determines the amount of aerosol formation.

In the Legionella case, Norway has established that the concentration of Legionella bacteria in the air directly above the basin is a factor of 10^2 - 10^7 lower than in the water [3].

A study carried out at a WWTP in 2007 found that the concentration of bacteria in the water was at least 10^8 times higher than in the air above the tank [35]. However, the results depend on the sensitivity of the measurement method. The emission of germs increased to a bacterial concentration of approximately 10^7/ml of wastewater. At higher concentrations, emissions remained almost constant [72].

Bubble aeration sometimes releases fewer aerosols than surface aeration, but the aerosols from bubble aeration are smaller and can therefore reach the lungs more easily [102].

The micro-organisms experience a high degree of stress from the sampling, which is likely to cause some of the bacteria in the air sample to die. Most likely, the amount of bacteria shown in an air sample is therefore an underestimate of the actual amount of bacteria present in the air.

Hydroscope conducted an experiment in Boxtel at a basin with intensive fine bubble aeration. At the time of the investigation, the basin was covered. The air wasn't extracted. Within the canopy, the Merck Mas 100 has measured Legionella in the air. This method is less sensitive than some other methods.

Legionella concentration [cfu/l water]	Number of air samples	Number of times Legionella in air demonstrated	Legionella concentration [cfu/m³ air]
10^6	3	0	
10^7	4	3	740
10^8	3	2	3.600
10^9	4	4	7.200

In addition, Hydroscope has carried out aerosol measurements with an OPS meter TSI 3330 at various locations. The measurements were carried out directly above or on the edge of the basins. At a particle size of 2.0 to 10.0 µm, the number of particles per cm^3 is determined. Particles larger than 10 µm cannot be determined by this measurement. Below are measurements of three typical purifications.

system	Aerosol particles [#/cm^3]							
	2,0 – 2,5 µm	2,5 – 3,0 µm	3,0 – 4,0 µm	4,0 – 5,0 µm	5,0 – 6,0 µm	6,0 – 8,0 µm	8,0 – 10,0 µm	total
Coarse bubble aeration inside canopy	328	392	479	384	435	522	238	2.778
Point aeration	0,61	0,37	0,64	0,34	0,15	0,20	0,14	2,46
Fine bubble aeration	0,09	0,06	0,05	0,04	0,01	0,01	0,01	0,27

Although this is a limited experiment, with a range between 2 µm and 10 µm, it is clear that coarse bubble aeration gives significantly more aerosol formation than fine bubble aeration. In addition, with coarse bubble aeration, more large aerosol droplets appear to be formed.

5. 2 Aerosol dispersion to the environment

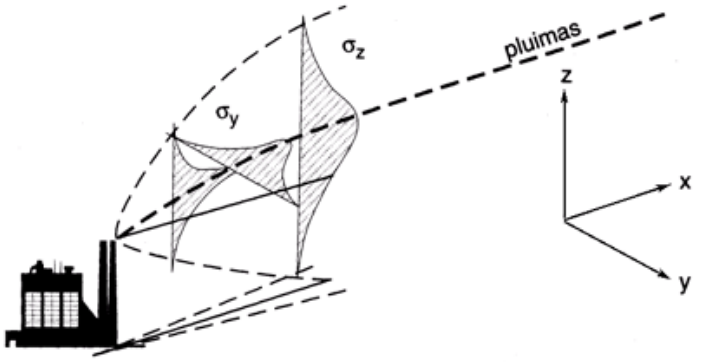
Due to an interplay of temperature and humidity, aerosols can either evaporate (and therefore become smaller) or grow [35]. Aerosols with a higher salinity are hygroscopic and can initially grow at high humidity and later become smaller again due to evaporation. Meteorological conditions also determine how the aerosols spread. In certain weather conditions (inversion), horizontal dispersion occurs mainly in the low air layers, while in other weather types vertical displacement to higher air layers occurs.

In conditions where there is virtually no direct sunlight (e.g. at night and in severe cloudy weather), an increased survival rate has been observed [50].

It is estimated that the large aerosols (> 100 µm) can spread up to about ten meters, and the smaller ones over a distance of several hundred meters [35]. Studies show that there is a considerable dilution at such a distance.

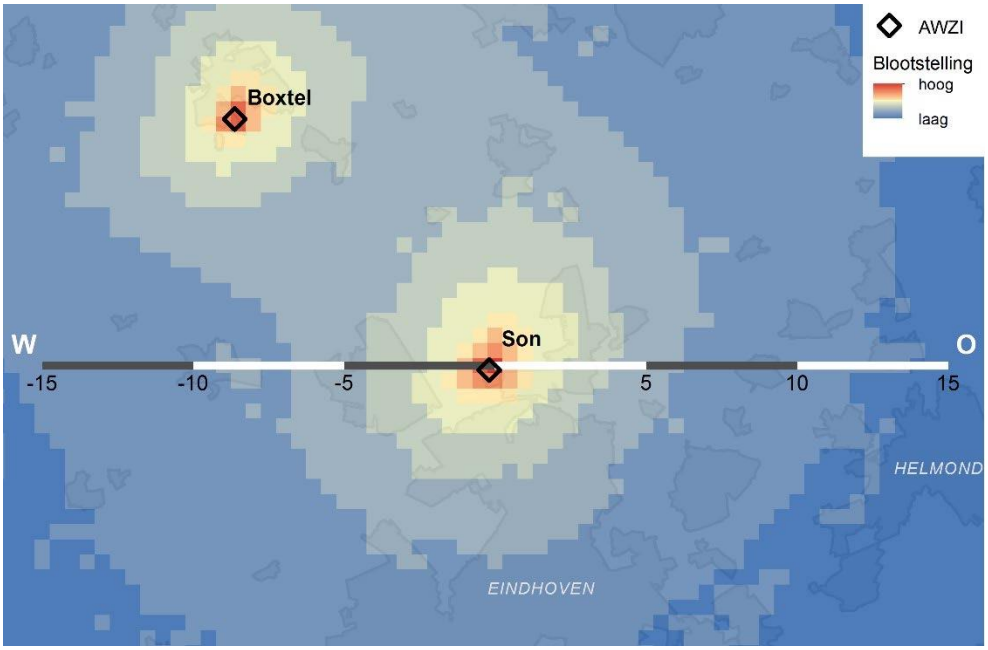
During a windless period, aerosols can accumulate above the aeration basin, which are blown into the environment when the wind picks up again. At night there is often a period with little wind. Aerosols then accumulate above aeration basin. When the wind picks up in the morning, these aerosols are dispersed.

In order to determine the aerosol emission to the environment, the Gaussian plume model is used [72].



Gaussiah plume model, InfoMil [73]

RIVM has developed an Operational Priority Substances Model (OPS model) to simulate the dispersion of aerosols [61]. This model has been made available for free.



Calculated concentrations of aerosols to which patients are exposed (OPS exposure, mean 2013 – 2018) [61].

In addition to the vulnerability of the people and the distance to the source, the duration of exposure determines the risk. Short-term exposure to nebulization is less risky than continuous exposure to nebulization.

5. 3 Other aerosol-forming processes

In addition to aeration, there can also be other aerosol-forming processes on treatment plants. The risk of Legionella infection depends on the extent to which people are exposed to Legionella and the frequency with which this occurs. Commissioned by the Ministry of Social Affairs and Employment, the "Blueprint RI&E biological agents" has been developed [14]. The method is aimed at staff and not local residents. The risk of exposure is determined per task and function. The frequency of exposure, exposure duration and distance between the breathing zone and emission are also decisive for the risk qualification.

Stowa issued a report in 2002 on Legionella risks at wastewater treatment plants [1]. A qualification has been made of the amount of aerosol formation per process step. In 2002 Stowa subsequently issued a report on the occurrence of endotoxins on WWTP's [37]. The amount of endotoxin in the air was measured per process step.

source	Aerosol formation	Leg*	Endo-toxin**	source	Aerosol formation	Leg	Endo-toxin
Cleaning installation	+++++	++	++++	Overflow settler, not covered	++	+	0
Screen removal	++++	+	+++	Sludge centrifuge space	++	+	++++
Point aerator without oxicap.	+++	+	0	In sludge digester	++	+	++++
Point aerator with oxicap.	+++	+	0	Above sludge containers	+	+	0
Bubble aeration	+++	+	+	Divider	+	+	+++
Brush aeration	+++	+	0	Sludge pre- and post-thickener	+	+	0
Production of chemicals	+++	+	0	Floating layer of suction dredgers	+	+	++
Compost filter/Lava filter	+++	+++	0	Pre-settlement basins, covered	0	+	+
Belt screen press room	++	+	++	Overflow of sludge return, covered	0	+	++
Overflow auger, not covered (return sludge)	++	+	++++	Overflow fermentation/sludge buffer	0	+	+

** Possible presence of Legionella in the water, estimated on the properties of Legionella*

*** Endotoxin concentration: > 400 EU/m3 ++++, 200-400 EU/m3 +++, 100-200 EU/m3 ++, 50-100 EU/m3 +, < 50 EU/m3 0*

Hydroscope has taken air samples in several places. Especially on treatments where high Legionella concentrations have been found, dewatering sludge is risky. Legionella bacteria have been observed in the air when tire thickeners are used without a good covering.

The Dutch Knowledge Centre for Labour and Lung Diseases has created a blueprint for biological agents. The blueprint assumes that from 4 minutes of exposure, within one meter of the source, respiratory protection is necessary. Respiratory protection must be worn from 39 minutes outside the range of one meter [14]. RIVM advises employees to wear effective respiratory protection while working at an WWTP that does not mention duration or distance [38].

5. 4 Distribution via effluent

The effluent of a wastewater treatment plant can contain Legionella bacteria. As long as this water is not atomized, it does not have to pose a danger. A case study shows that effluent from a wastewater treatment can lead to Legionellosis patients.

During a purge in Warstein (DE), 78 people became ill. Legionella was observed in wastewater treatment, but also downstream in sewers, surface water and cooling tower. Presumably the cooling tower in particular allowed the bacteria to spread through the air over a long distance [11]. Legionella was also found in the air above the drains of the gravity sewer.

In Norway, 64 people have become ill with Legionella. Legionella was found in high concentrations in wastewater treatment. It is not entirely clear how the patients got infected, but most people live near a river downstream from the treatment. The river contains some waterfalls [3, 5, 6, 7].

In some cases, effluent is also reused. This usually happens with small quantities, for example for cleaning work or feeding an air scrubber. Sometimes large quantities are also reused, for example within the paper industry, for irrigation purposes or the precipitating of dust during dry periods.

Many industrial treatment plants discharge the waste water into the sewage system. The sewer leads to the municipal sewage treatment plant, from where the wastewater flows into the surface water. At the Legionella case in Boxtel, Legionella was found in the industrial treatment plant, sewage treatment plant and the River “de Dommel”.

In 2018, water boards recommend not to spray water from the Roer after high concentrations of Legionella were found in the Roer. A paper mill in Düren (DE) has discharged high concentrations of Legionella onto the sewer. The water ended up in the Roer via the sewage treatment plant.

Ingestion of surface water by misting systems, such as irrigation systems and cooling towers, may pose a risk of further dispersion.

5. 5 Spread via seed sludge

Wastewater is a mixture of water and sludge particles. Too little is known about the development of Legionella in wastewater, but there are indications that Legionella may be present in high concentrations in aerobic sludge. The Legionella concentration in the purification process decreases as more sludge has settled and removed. In addition, Legionella is also found in the centrate water during sludge dewatering processes. Due to the high dry matter content and large amount of disturbing flora, sludge can hardly be analyzed, so the Legionella concentration in sludge can hardly be determined.

Anaerobic sludge is less likely to contain high Legionella concentrations. Due to the anoxic/poor conditions, Legionella can survive for some time, but probably not multiply.

Aerosol formation can take place during sludge dewatering and thickening processes. Modern processes are often covered. Sludge dewatering processes usually have a limited scope. They can pose a risk to employees, but are often too small in size to pose a risk to the environment as well. (Dewatered) sludge is usually removed by truck and incinerated at sludge processing plants.

Wet sludge or concentrate of sludge is sometimes also used for fertilization in agriculture. Driving out over land, where aerosol formation is released, is rare. Manure injection is mainly used in agriculture.

During cleaning activities, the splashing of sludge can pose a risk to employees. Hydroscope found Legionella in the air at various locations during cleaning work.

In America, a man became infected with Legionella Longbeachae after using dry waste (presumably sludge) from a treatment plant to fertilize his garden. The waste was scooped into bags and unloaded in his garden [104].

At the start of a purification or poorly performing purification, sludge from another purification is used for inoculation. Other water systems also use seeding material, such as air scrubbers. Legionella pneumophila serogroup 1 ST 1646 was found in both the purification in Boxtel and Son. For a while it was thought that the bacteria had been transmitted via inoculation material, but no sludge inoculation took place.

Hydroscope has experienced at various treatment plants that the Legionella concentration can increase or decrease significantly after inoculation of a treatment plant. The mechanism behind this is not clear. In addition to Legionella, inoculum may also contain growth-promoting protozoa or nutrients.

5.6 Risk qualification RIVM

In 2019, RIVM made a preliminary risk qualification for biological wastewater treatment plants [38].

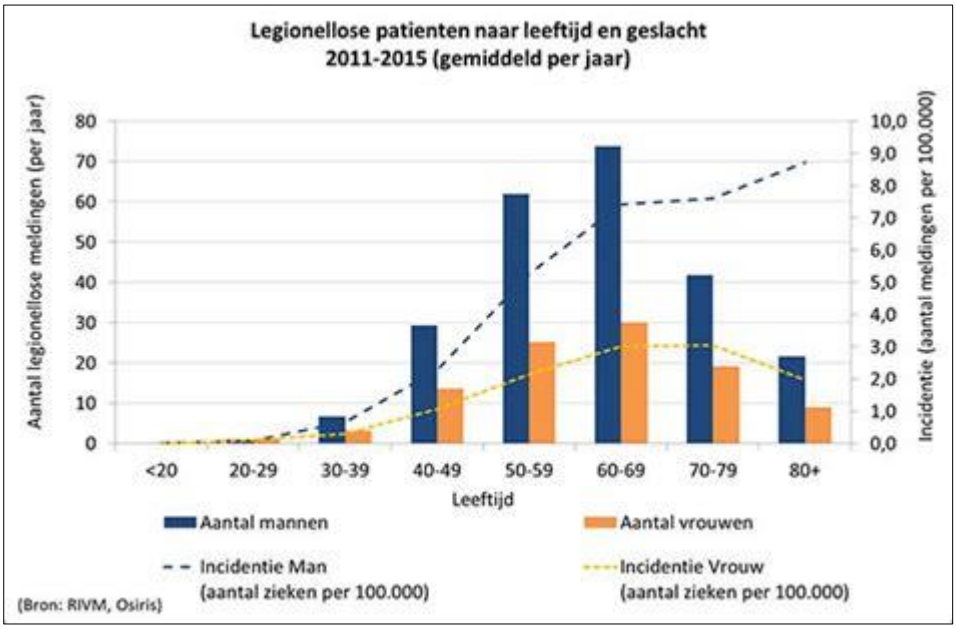
Industry type	Temperature process	aeration	Airborne dispersion	Distribution via water
foodstuffs Paper and wood petrochemistry Rendering companies Sewage treatment	30-38°C	yes	Very plausible	Very plausible
		no	plausible	Very plausible
	25-29°C of 39-45°C	yes	plausible	plausible
		no	possible	plausible
	< 25°C of > 45°C	yes	possible	possible
		no	Not plausible	possible

6. Vulnerability of the environment

6.1 User vulnerability

Most people who get infected with Legionella don't get sick. Only a small part of the people who become infected will get Legionnaires' disease or Legionella flu. The following people are at greater risk of getting sick from Legionella:

- People over the age of 65;
- Smokers;
- People with poorer health;
- People with reduced resistance.



Graph age Legionellosis patients

6.2 Vulnerable locations in the environment

In general, it can be said that that the greater the distance to the source of contamination source, the risk of contamination decreases. Legionella infections with cooling towers have made people up to more than 10 kilometers away ill. At a wastewater treatment plant in the Netherlands, contaminations up to a distance of 6 kilometers were described. In the case of cooling towers, which is not fully comparable, the following risk classification has been used [13]:

Cat.	Location purification
1	In the vicinity (< 200 m) of a hospital, nursing home or other (medically oriented) care institution where people with reduced immune systems reside.
2	In the vicinity (< 200 m) of retirement homes, hotels or other buildings in which many people are located.
3	In the vicinity (< 600 m) of a residential environment.
4	At a distance of (> 600 m) a residential environment.

6. 3 Dose-effect relationship

There is a lot of scientific research into the dose-effect relationship of Legionella. However, there are many factors that have an influence, such as:

- Virility of legionella species, type and sequence type
- Life form/phase of the bacterium
- Demographic characteristics of the population
- Health aspects
- De exposure time
- Number of aerosols in the air
- Aerosol size and number of bacteria in an aerosol

The many factors mean that a lot of additional research is needed. The RIVM LCI guidelines therefore assume that the dose-effect relationship is not yet sufficiently known [30].

However, various studies and calculation methods give direction to a dose-effect relationship. However, the results cannot be directly applied to the Dutch situation and wastewater treatment plants.

Risk assessments often distinguish between the probability of:

- getting infected,
- getting so ill that hospitalization is required,
- dying

Various animal experiments have been carried out to find out which concentrations can be harmful. The animal experiments with Guinea Pigs provide the best comparison with humans. From these animal studies, the following was concluded [41]:

- The probability is 1:25 that an individual inhaled Legionella bacterium leads to infection;
- The probability is 1:11.111 than an individual inhaled Legionella bacterium leads to hospitalization;
- Exposure to 2,400 to 100,000 bacteria can be fatal [78].

Other studies describe:

- Inhaling 1,000 Legionella cells can be fatal for a vulnerable person [31].;
- Infection is possible with 15 minutes of showers under a Legionella air concentration of 35 to 3,500 CFU/m³ [42];

In 2012, the RIVM made a risk assessment for Legionella pneumophila in showers and hot tubs [86]. At a concentration of 100 CFU/l, there is a 2% chance that someone will become infected with Legionella pneumophila during 15 minutes of showering. When using a hot tub, this probability is about 50%. A person breathes about 0.41 m³/hour of air.

Recently, calculations have been made expressed in DALY (years of life adjusted with limitations). The calculations are based on a conversion factor between water and air concentration, distribution of aerosols by size and the infection numbers based on animal studies [82]. The calculations have been performed for a sink, shower and toilet. For shower use, the following results have been presented.

Target value risk	Critical concentration (CFU/l Legionella pneumophila)
10^{-4} infections per person per year	1.410
10^{-6} DALY per person per year	14,4

People are frequently exposed to Legionella bacteria. Based on the probability calculations, more people should get sick than is actually the case. Researchers expect that not all infection factors are known. For example, it is thought that inhaling an aerosol with an amoeba, which can contain hundreds of Legionella cells, is more harmful than inhaling some loose cells [78].

7. Sampling and analysis

Wastewater is a very difficult medium to analyze. Inhomogeneity of the sample, snapshot, the amount of weaving particles and disturbance by other microorganisms make the measurement uncertainty great. Select the right technique based on the application scope and research goal. Do not judge directly on the basis of one analysis result. Build up a series of measurement results and consider the measurement uncertainty.

7.1 Water sampling

Sampling waste water is particularly difficult due to its inhomogeneous nature. Bacteriological sampling, including Legionella, is described in NEN-EN-ISO 19458. It is essential to periodically take the sample in the same place and under the same conditions.

The NEN 6600-1 focuses more specifically on the sampling of waste water. The standard describes how a sample plan is drawn up and by which method the sample can best be taken. Only sampling the total waste water flow of well-mixed waste water provides a representative picture. When determining the sampling point, the following preference is used in order:

1. Take a sample directly through a drain valve;
2. Take a sample directly by placing it diagonally in the wastewater stream;
3. Direct sample using a sample holder;
4. Indirect sample using a sample scoop.

To estimate the legionella risk, sampling at aerosol-forming process components is essential. This is often at the aeration tank. It is very difficult to take a representative sample. There can be major differences in the mixture of sludge and water per sampling round.

Since large sludge particles, depending on the type of aerosol formation, cannot get into the air, in some cases it is preferable to let the sample settle first. A sample is then taken from the water fraction.

Samples should preferably be transported refrigerated and used in the laboratory within 48 hours. Because bacteriological regrowth is still possible within the sample, it is preferable to use the sample as soon as possible.

7. 2 Grow

Rivm has identified the cultivation method as the most suitable for Legionella detection in wastewater [38]. The water analyses are carried out in accordance with NEN-EN-ISO 11731.

Matrix

The standard describes three matrices:

- Matrix A: little disturbing flora to be expected, e.g. drinking water;
- Matrix B: much expected disturbing flora, for example process water;
- Matrix C: very much expected disturbing flora, for example wastewater.

In matrix A and B, the water is first passed over a filter. In case of wastewater, the filter becomes clogged too quickly. Characteristic of matrix C is therefore that a small amount of water is applied directly, without filtering, to the petri dishes. Wastewater with relatively little disturbing flora and suspended particles can sometimes also be used as matrix B. This includes highly purified effluents.

Analysis process

With matrix C, in accordance with NEN-EN-ISO 11731 Annex J, heat and acid treatment is applied. 0.1 to 0.5 ml of the sample is plated directly on the culture medium. A selective medium (GVPC or MWY) is used. With this medium, disturbing flora is better suppressed than with BCYE medium, but it is also more likely that Legionella is (partly) killed [80]. Optionally, one petri dish with BCYE-cys medium is also used as a control. A sample is normally plated on 10 petri dishes (9x GVPC or MWY, 1x BCYE-cys). The laboratory reports the results with the highest number of bacteria counted.

Petri dish with Legionella bacteria (left) and overgrown petri dish (right)

By suppressing the disturbing flora, there is also a chance that some of the Legionella bacteria will be suppressed. A number of laboratories, such as Streeklab Haarlem and Cyprio, therefore also offer their own method of analysis. For example, the heat or acid treatments are skipped.

The petri dishes are placed in an incubator of 36°C for seven to ten days. After three days, the petri dishes are checked for overgrowth by other micro-organisms.

Dilution

In wastewater, the petri dishes quickly get overgrown with other micro-orgasms present. As a result, Legionella is indistinguishable and countable. To prevent this, the water is used diluted. The lab technician determines the necessary dilution at his discretion. It is better that the laboratory uses a dilution series, but this also makes the analysis more expensive. Experiences of previous sampling rounds are therefore used to limit the dilution series.

When using a diluting series, 10 petri dishes are used per dilution. For example, a sample is diluted 1:10, 1:100, and 1:1,000. The lower detection limit increases with the dilution. In the case of drinking water samples, the lower limit is 100 cfu/l. For matrix C, the lower limit is 2,000 cfu/l. By applying a dilution series, the lower limit is increased to as much as 10.000.000 cfu/l. The lower limit shall be calculated in accordance with NEN-EN-ISO 11731 Annex J.

Lower limit for dilution	Start 0,5 ml	Start 0,1 ml
undiluted	2,000 cfu/l	10.000
1:10	20,000 cfu /l	100.000
1:100	200,000 cfu /l	1.000.000
1:1.000	2,000,000 cfu /l	10.000.000

The dilution with the first result to be determined is reported. The analyzed volume and/or lower detection limit is indicated on the analysis report. Optionally, the laboratory issues a declaration of conformity. The result is tested against a standard. The analysis report should then clearly state whether or not the measurement uncertainty has been considered. Discounting the measurement uncertainty is not common in Legionella samples.

Measurement uncertainty

A measurement uncertainty of around 30-40% is common for matrix A (little disturbing flora to be expected), the measurement uncertainty for matrix C is expected to be a multiple of this.

The Nen-EN-ISO 11731 describes the results of intra-laboratory comparison (n=8). For direct plating after dilution and with an extremely high amount of disturbing flora, repeatability is 18.0% and reproducibility is 46.0%. This means that there can be big differences in results.

In 2017 a renewed accreditation standard NEN-EN-ISO 17025 was released. Laboratories must disclose measurement uncertainty of the sampling and analysis. This can usually be found on the website of the laboratories.

Confirmation and serotyping

To ensure that the bacteria found are actually Legionella bacteria, a confirmation technique is used. This technique often also allows you to find out the type of Legionella bacteria. Confirmation is not mandatory in accordance with the NEN-EN-ISO 11731 standard, but it is common in the Netherlands. To confirm whether it is indeed Legionella, three common confirmation techniques are used:

- Subculture: the bacterium is cultured 'clean' for up to five extra days;
- Maldi-cool: a mass spectrometer determines the protein profile of the bacterium;
- PCR: A DNA profile is used to determine whether it is a Legionella bacterium;
- UV: new method under development at Vitens to confirm Legionella bacteria by means of UV [81].

When many colonies are found on a petri dish, a sample is taken for confirmation. It is therefore possible that not all bacterial species are confirmed in a sample.

The NEN-EN-ISO 11731 describes the performance of the confirmation of an intralaboratory comparison:

- Culture: false positive 3.3% false negative 1.4%
- PCR: false positive 1.8% false negative 2.1%

Optionally, the sequence type of the Legionella bacterium can also be determined. This is only common when conducting source research. Only a limited number of laboratories can carry this out, including Streeklab Haarlem.

Viable but not culturable

A well-known problem with the cultivation method is that Legionella bacteria enter a viable but not cultivatable stage in a nutrient-poor environment (viable-but-not-culturable, VBNC) [74]. Bacteria can also reach the VBNC level due to lack of oxygen or temperature changes [75]. This makes the cultivation method less suitable for analysis of anaerobic water or water immediately after anaerobic treatment.

Accreditation

The Accreditation Council verifies whether laboratories operate according to the accreditation standard NEN-EN-ISO 17025. STERLAB was the predecessor of the Dutch Accreditation Council. On the website of the Accreditation Council you can find which laboratories are accredited in accordance with NEN-EN-ISO 11731 matrix C. Matrix C was rarely used until recently. Only since the Legionella problem in wastewater have laboratories started offering this analysis. Preparing, applying for and accrediting an analysis takes about 1,5 years. That is why only a few laboratories have been accredited.

7. 3 qPCR

In PCR, small amounts of DNA material from the Legionella bacterium are multiplied until there is enough material to be able to analyze them. Often the technique stops at the determination of the Legionella species. Further sequencing ultimately allows the sequence type of the Legionella bacterium to be determined, which is essential in source detection. A match between the patient's sequence type and an environmental sample makes it plausible that the source has been found.

qPCR makes it possible to obtain a quantitative result of the sample. Internationally, it is common to analyze wastewater samples using qPCR for Legionella pneumophila. Detection of Legionella is described in NEN 6254. The turnaround time of qPCR is significantly shorter than a culture method at 24-48 hours.

As with the cultivation method, water is preferably filtered first. Therefore, 1 to 2 ml of wastewater is added directly to lysis buffer. However, this increases the measurement uncertainty.

In qPCR, the number of DNA copies found (mip gene) is reported. It concerns DNA from both living and dead/broken Legionella cells. Most qPCR techniques available on the market only detect Legionella pneumophila serotype 1 or Legionella pneumophila.

A mip value of three or more is reported to have been associated with Legionella. With a mip value of 5 or more, the result is also quantified.

qPCR generally shows higher Legionella rates. The disadvantage of qPCR is that both living and dead Legionella bacteria(virulent/non virulent) are shown.

7. 4 Other methods

There are also alternative methods of Legionella detection in water [33], such as:

- Viability-PCR: By adding a chemical that binds only to dead bacteria, a distinction can be made in living and dead bacteria.
- FISH: The DNA is made fluorescent, making it countable under a microscope. This method gives less good results than the qPCR method.
- MPN method: In the 'most likely number' method, a water sample is briefly cultured in test tubes in different dilution series. By using a reaction liquid, the test tube turns cloudy in the presence of at least one Legionella bacterium. From the applied dilution series, the concentration of Legionella is calculated [84]is taken back. Test kit Legioalert® indicates that it gives more reliable results in samples with a lot of disturbing flora than the traditional cultivation method [85].
- Use antibodies: These methods detect Legionella proteins using antibodies. The antibodies are then stained or made magnetic to be able to quantify them.
- Flow cytometry: Legionella bacteria are magnetized using antibodies and isolated from the water sample. The particles are counted by flow cytometry. Flow cytometry uses a laser. The reflection of the laser light and its fluorescent character make a Legionella bacterium recognizable and countable [83].

Hydroscope has varying experiences with detection methods. Wastewater is a very difficult matrix to analyze. Many methods require a filtration step or a lot of dilution for the analysis process to run smoothly. Legionella bacteria can be found in protozoa, making them difficult to detect. Analysis results are expressed in different units and are difficult to compare.

7. 5 Air sampling

Air samples can be taken in different ways. Air sampling must provide insight into the risks for bystanders, the degree of aerosol formation and the relationship between the concentration of Legionella in water and air.

Aerosol measurement

Legionella spreads to the environment via aerosols. Aerosols must be 1 to 10 µm to be able to contain legionella bacteria and be inhaled. However, larger aerosols can become smaller due to evaporation and reach inhalable size. By performing aerosol measurements, an image is formed of how many aerosols spread to the environment. The volume of aerosols is also determined, which is used to estimate the risk of inhalation of Legionella. There are several optical particle meters on the market. Hydroscope uses a portable OPS meter TSI 3330 to perform aerosol measurements.

The results from the aerosol measurement can be used as input for the OPS model of RIVM.

TSI 3330

Air sampling using a filtration method

The air is sucked in by the sampler and passed over a filter. The bacteria remain on the filter. The bacteria can be detected with qPCR. The method is not suitable for culture research [60].

Air sampling by impaction method:

Air over a nutrient medium 100 to 500 liters of air are sucked in per sample and passed over a petri dish. The method is described in VLA measurement protocol 3A [15]. Hydroscope uses the Merck MAS 100 for aerial research. The method is less accurate than the other impaction method [29].

Merck MAS 100 from Hydroscope

Air sampling by impaction method: Air through a liquid

A better way of air research is to suck in air, after which the bacteria are brought into a liquid [5].
Hydroscope has three brands/types of devices:

- SAS2000/2300;
- SKC Biosampler;
- Coriolis µ.

Coriolis µ from Hydroscope

The air can be sucked in over a longer period of time, ranging by type of device from a few minutes to two days.

Based on the Coriolis µ Hydroscope uses the following method:

- Weather: The weather is a very determining factor. For a representative measurement, the weather pattern should be as close as possible to the most unfavourable situation for Legionella spread. The most representative weather picture is inversion (foggy weather in the morning). Measurements in case of precipitation or strong winds do not give a good picture.
- Position: Perform a measurement directly above/near the emission source. If necessary, use tools. Running downwind samples quickly gives a distorted picture. When determining a correct downwind position, keep drawing with the wind direction and air currents. If necessary, use an airflow model to determine the correct position. Determine the correct downwind distance based on the purpose of the study. Experience shows that measurements beyond 50 meters downwind hardly produce any useful results.
- Hygiene: Avoid contamination by previous samples. Autoclave the clones and neck before using them. When sampling, start with the sample in which the least Legionella bacteria can be expected (order: upwind sample, furthest downwind sample, above the emission source).
- Medium: Hydroscope uses BPW (in accordance with NEN-EN-ISO 8199) as an impaction fluid. Make sure that foaming does not occur. By sucking in the air, liquid can evaporate (dry weather) or vapor can be collected (humid conditions). According to experience and insight, choose the starting volume in such a way that 10 µm of liquid remains after taking the air sample.
- Settings: Excessive suction power can cause fewer bacteria to be caught in the liquid and/or too much liquid to evaporate. Hydroscope sucks in up to 150 liters/minute of air. A total of 3 m^3 of air is sucked in. To do this, two cycles per sample must be started. Set sufficient delay time at the start of the cycle to be able to distance yourself in time and thus prevent contamination.
- Transport: Work hygienically. Seal the sample immediately with the cap. Start the analysis as soon as possible (maximum within 24 hours). Transport the sample conditioned in accordance with NEN-EN-ISO 19458.

The liquid can then be analysed by various methods:

- Culture: The samples are cultured in accordance with NEN-EN-ISO 11731. Only growable live Legionella bacteria are detected. The result is quantitative;
- qPCR: In accordance with NEN 6254. Both live and dead Legionella bacteria are detected. The result is quantitative;
- Amoeba culture + qPCR: With amoeba culture it is also possible to detect bacteria that are not culturable (viable but nonculturable, VBNC). After amoeba culture, only live Legionella bacteria that are detected by qPCR remain. This method indicates only the result present/absent[33].

According to Italian research [29], an analysis using qPCR reliably gives better results than a culture. With amoeba culture, the RIVM has demonstrated Legionella bacteria in air samples that were not detectable with qPCR and culture. Nevertheless, analyzing Legionella pneumophila in air remains very difficult.

The microorganisms experience a high degree of stress from sampling, which is likely to cause some of the bacteria in the air sample to die. Most likely, the amount of bacteria shown in an air sample is therefore an underestimate of the actual amount of bacteria present in the air. It is still unclear what the exact rate of dieback is. [60].

Static method

A final method of air research is to perform a static measurement. A sticky plate is set up over a period of several days. Because of the air blowing along the plate, bacteria remain on the plate. The bacteria are being analyzed. This method is used less frequently. In the Italian study [29], this method did not produce as good results.

7. 6 Sludge research

Microscopic

Sludge Research can provide insight into the presence and lifestyles of Legionella and its hosts. Sludge is magnified under a microscope. By adding markers, various parts light up. For example, a marker that responds to PHB can be used to demonstrate protozoa. Sludge research is also combined with FISH technology.

Sequencing

Based on DNA and RNA, it can be investigated which bacterial groups are actively present and what percentage this represents of the total population of bacteria in the sludge [108]. It can be analyzed for Gammaproteobacteria and for Legionella.

8. Approach to risk assessment

Performing a Legionella risk analysis for wastewater requires a lot of expertise and is tailor-made. Compared to other water systems, a lot is still unclear about the growth and spread of Legionella. Only a few wastewater treatment plants have been associated with contamination, while 54% of industrial warm biological wastewater treatment plants have been found to contain Legionella [88] and often in high concentrations. There are large differences in Legionella concentrations in the air over purifications. In the vast majority of treatments, no Legionella is detected in the air. It should be noted that air research is often an underestimate of reality. The norm values used for drinking or process water cannot be applied to waste water, due to limitations in analysis technology.

Where legionella growth factors can be easily influenced in drinking, bathing or process water, this is not yet possible or hardly possible with wastewater. Wastewater treatment plants are very different in configuration. Solutions are often customized and not generically applicable.

Where possible, Legionella risks must be reasonably minimized. However, there are also cases where Legionella growth and/or spread cannot be prevented. Not in all cases a technical or economic solution is available. In these cases, it is necessary to assess whether the (residual) risk is acceptable or whether the purification should be shut down.

Making a risk assessment is a careful process that can be different for each purification. Sometimes one location visit is enough. In other cases, an extensive sampling plan should be drawn up and intensive coordination is required. The following paragraphs describe the process steps.

8.1 Process assessment

A risk assessment starts with a document study and location visit. The process is mapped out and the growth and spread risks are assessed per process component. Attention is paid to:

- the nature and quality of the water and seed sludge used/treated;
- the temperature of the water in the various process steps;
- the type of aeration and atomization of the water;
- the operational management of the biological (waste) water purification (can aerosol formation/misting take place).

In addition, attention is paid to clarity of process responsibilities, administration and embedding.

8.2 Analysis of history and vulnerability of the environment

Check the location of the purification in relation to the environment, taking into account:

- the distance to the built environment;
- the vulnerability of employees and local residents;
- presence of other aerosol-forming water systems, such as cooling tower and air scrubbers;
- the downstream route of the effluent;
- making seed sludge available
- Drain route of the surplus sludge;
- history of Legionella patients in the area.

8.3 Further analysis by sampling

If an initial risk assessment shows that there is insufficient measurement data available to properly assess the risk, it may be recommended to carry out additional measurements. It is conceivable that:

- Sampling additional water flows to map out growth;
- Carry out sludge research;
- Aerosol measurement and air sampling to determine the spread to the environment.

8.4 Matching action levels

In the absence of standard values, it is important to agree on action levels in accordance with the Environment Service. For example, legionella may have been found in wastewater treatment, but no Legionella in the air was measured at the relevant concentration and no cases of contamination are known. An action level may involve measures being taken above the agreed concentration, additional research being carried out or the installation being shut down.

8.5 Step-by-step plan, recalibration and management plan

If legionella is found in the treatment plant, a step-by-step plan is set up to carry out further research and/or to take additional measures. Taking measures, such as placing a canopy, can be stressful and take time. Therefore, it is important to continue to monitor and manage during the implementation of the step-by-step plan.

After the measures have been implemented, the risk assessment will be recalibrated and the final management plan drawn up.

9. Source-oriented measures

This chapter describes the possible source-oriented measures to prevent Legionella growth.

9. 1 Seed sludge

Often the sludge of one biological purification is used to inoculate another purification. When inoculating a purification plant, it is important to use inoculum sludge that does not contain Legionella bacteria. In practice, this is hardly feasible. Sludge is difficult to analyze. Due to the high risk of overgrowth, due to dilution, the detection limit is very high (rising above 10^6 CFU/l). qPCR is a more suitable method of analyzing seed sludge. In addition, small amounts of Legionella bacteria will grow into large quantities under favorable growing conditions in the inoculated purification. When inoculation occurs, it is especially important to know where the inoculum sludge comes from and whether specific sero- and sequence types have been present that may lead to the risk of infection, such as Legionella pneumophila serotype 1.

Hydroscope is familiar with some systems that have been (re)grafted. The experiences are very varied and cannot be fully explained. There are known systems in which the entire installation has been disinfected and re-grafted, after which Legionella problems have not occurred. There are also systems where this effect was only temporary. Unfortunately, there are also known systems in which, after inoculation, the Legionella concentration temporarily increased.

9.2 Legionella reduction in influent

In Boxtel, research was carried out into Legionella concentrations in the influent in relation to the concentrations in the warm reactor. In most cases, no Legionella was found in the influent. In cases where Legionella was found in the influent, this had no significant effect on the concentration in the reactor.

Other practical examples show the same picture. Hydroscope has reviewed several reactors where the centrate from the fermentation process is treated in an aerobic reactor. The centrate has been without oxygen for more than 30 days. No Legionella was found in the analyzed samples of the centrate. Nevertheless, high Legionella concentrations can be observed in the downstream warm aerobic reactor.

The VEMW survey shows that Legionella is common in industrial influent. In 23 treatments, the influent was studied for Legionella. Legionella was found in 9 purifications (39%) [88].

9. 3 Legionella reduction in nutrient additives

In some purifications, it is necessary to adjust the nutrient ratio in order for the purification process to run smoothly. Additional nitrogen, organic matter, phosphorus or micronutrients are added to the waste water. For example, to solve a nitrogen deficiency, residual flows from other processes are used, such as the leachate from waste processing. It is important to know the origin of nutrient flows and, if necessary, to investigate them for the presence of Legionella. Adjusting nutrient flows may help with Legionella reduction.

Process automation can help improve and stable nutrient dosing, potentially preventing spikes in legionella growth.

9. 4 From mesophilic to thermophilic fermentation

In the Netherlands, mostly mesophilic digesters are used. The temperature range from 20°C to 45°C is also an ideal temperature for Legionella growth [101]. Thermophilic digesters operate at a temperature of at least 52°C [100]. This type of digesters is more commonly used in the Scandinavian countries. The use of thermophilic digesters may be applicable in manure processing. The effluent from this type of digesters may be less susceptible to Legionella growth. Further investigation is needed.

9. 5 Eliminate aerobic process step

In some cases, it is possible to apply membrane filtration immediately after the anaerobic process step. Legionella therefore does not get a chance to multiply. Two companies with such a treatment were included in vemw's investigation. No Legionella was found in both purifications [88].

9. 6 Eliminate biological purification step

The use of an air stripper can serve as an alternative for the removal of ammonia from wastewater. By intensively mixing water and air at a high pH, volatile components, such as ammonia, are blown out. An air stripper can act as an alternative purification step for biological purification [110].

9. 7 Legionella reduction within reactors

The temperature inside the reactors is ideal for Legionella development. To reduce the growth of Legionella pneumophila, the temperature should be reduced to below 25°C. This has a negative effect on the effectiveness of bacteria present in the reactors. When the temperature is raised to 40°C, amoebae die, which inhibits the growth of the Legionella bacteria.

It has been investigated whether the Legionella bacteria can be specifically killed in the water, without affecting the effectiveness of the other beneficial bacteria. The following options have been reviewed:

- Biological biocide BioMeba: cannot survive in wastewater;
- Bacteriophages: no supplier found;
- Clorious2: this chlorine solution was found to have a negative effect on anammox® bacteria.

There are currently (advanced) studies on the development of biocides based on substances that other bacteria secrete to specifically inhibit Legionella growth. The interim results were presented at the ESGLI congress in 2019. This may provide a way to selectively prevent legionella growth with a biological biocidal health in the future.

After the Legionella outbreak in Warstein Germany, research was carried out into the reduction of Legionella in biological water treatment plants. The administration of silver, hydrogen peroxide, chlorine dioxide, ozone and pH change had no effect on the killing of Legionella [16].

Acanthamoeba castellanii is growth inhibited by peptide. The growth of Legionella within this host is also inhibited [94]. It is not known what the effect of peptide is in a wastewater treatment plant.

An incomplete conversion of proteins into methane in an anaerobic purification step can lead to more amino acids leaching to a downstream aerobic purification step. This can promote Legionella growth. Thus increasing the purification efficiency of the anaerobic purification can inhibit Legionella growth. There are

several possibilities to increase efficiency, such as process automation, applying a thermal pre-treatment (heat treatment) or pre-acidification [111].

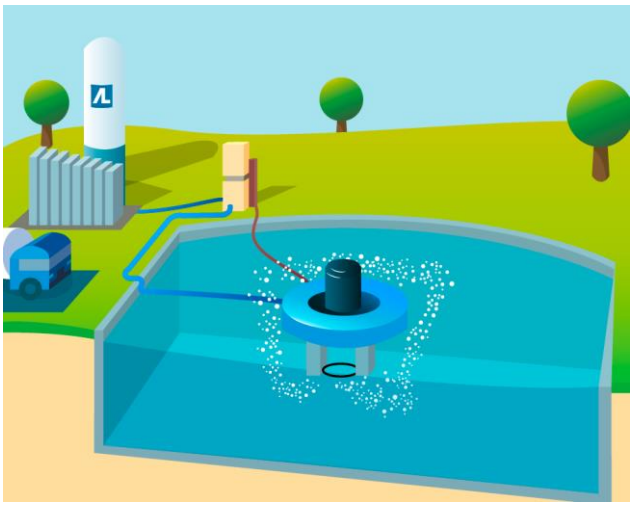
For the time being, there do not appear to be any possibilities to completely combat Legionella growth in the reactor.

10. Preventing airborne spread

The following measures are conceivable to reduce aerosol formation or prevent aerosol spread.

10. 1 Type of aeration

Surface and point aeration are known to form more aerosols than fine bubble aeration. By aerating with liquid oxygen, air throughput can be reduced by 80% to achieve the same amount of oxygen dosing. Oxygen aeration has been used in France, among other things, to reduce Legionella spread. An oxygen buffer tank is required. A mixing system must be used to properly mix the oxygen. Aeration with pure oxygen is expensive, but can (partly) pay for itself through a better purification efficiency.



Example oxygen aeration Air Liquide Turboxal™

10. 2 Floating covering

In the Netherlands, various pilots are carried out with floating covers. None of the covers have yet been sufficiently tested for effectiveness.

Cover/manufacturer	explanation
 *Hexacovers*	Octagonal discs that need to slide into each other. Applied in Boxtel and proved ineffective. The pilot has stopped. The parts float too much to the sides.
 *AWTT Hexoshield*	Angular balls that connect better than round balls. The parts float less and lie on top of each other less. At the most powerful points of air entry, openings remain visible.
 *Floating balls*	The floating balls are superimposed in three layers. In China, a study was carried out on the effectiveness of the cover in preventing E-coli spread. 50% or more of the bacterial spread is prevented [40]. Small balls and multiple layers increase effectiveness.

 *Candock*	A floating pontoon is being installed. Aerosol spreading is significantly reduced. Limited aerosol formation can be observed along the edges of the cover.

Air measurements by Hydroscope with the Coriolis µ show that floating covers reduce Legionella spreading to a limited extent. The measured concentration in the air is usually reduced by 1 to 2 log factors.

Floating covers have an effect on the temperature of the wastewater. From experience, the wastewater temperature seems to be about 2°C to 4°C higher. The cover can also have an effect on the aeration intensity. Adjustment of purification may be necessary.

10. 3 Covering

Covering or covering a treatment plant can be done by sail or other construction. The aerosols largely settle within the canopy. The air, including some of the aerosols, is discharged through the openings and cracks. When performing air measurements, low Legionella concentrations are still visible in the air.

Within the cover, the aerosols and, for example, sulfur gases accumulate. The risk for employees who have to work within the canopy increases. Due to the presence of large quantities of aerosols, visibility is poor.

This can be taken into account during the design of new treatment plants. It is not always possible to place a cover at existing treatment plants. The surface to be spanned can be too large or the concrete structure to which the sail needs to be attached is insufficiently designed for it.

As with a floating cover, a cover with sail or a construction has a temperature-increasing effect.

10. 4 Dehumidification

To prevent emissions to the environment, it is more effective to purify the air and then dehumidify and/or treat it. From experience in Boxtel, air treatment techniques are much less effective at high humidity. Filters silt up faster and UV lamps are attacked faster by algae. The service life of the air handling units is also significantly shortened. It is strongly recommended to (partly) dehumidify the air first.

Knowledge Centre InfoMil has drawn up factsheets for air emission abatement techniques [71]. These are not primarily intended to combat aerosols from wastewater treatment plants. A number of techniques are used to remove wet dust, which do show parallels.

Cyclone

A cyclone operates on the basis of centrifugal force. The air is guided into a cylindrical chamber. Due to the rotating movement of the air, the heavier particles are thrown towards the wall and discharged. This technique is especially effective for particles > 5µm. The efficiency for particles between 6 and 10 µm is only 50%. Above 10 µm, the efficiency is considerably greater.

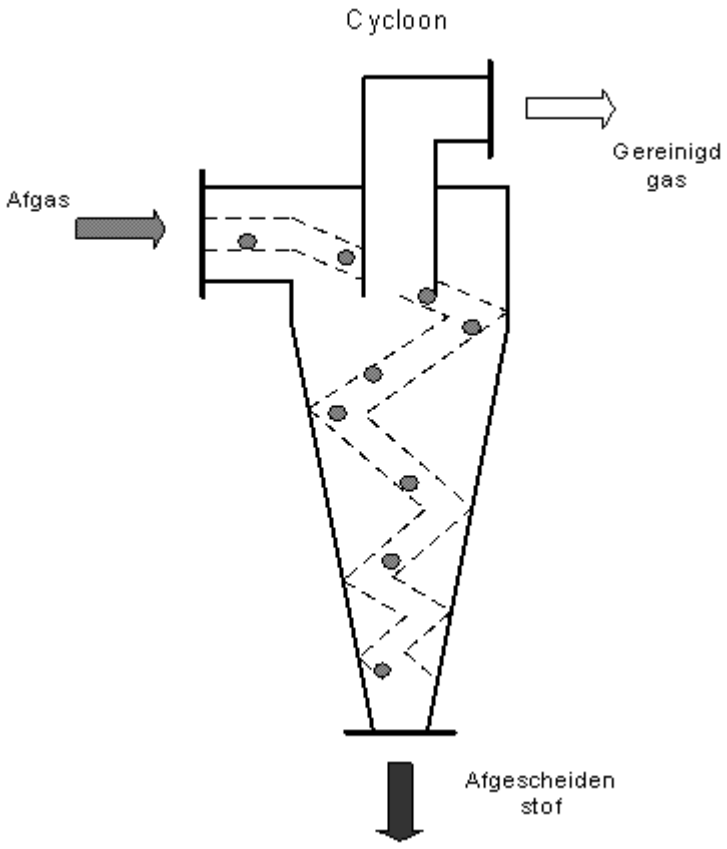


Image rotation washer of InfoMil

Spray tower/Rotary washer

Due to centrifugal forces and the rotating atomization of washing water, particles are dragged to the wall of the scrubber. A droplet separator provides further dehumidification. For particles < 10 µm, the efficiency is 70 to 99%.

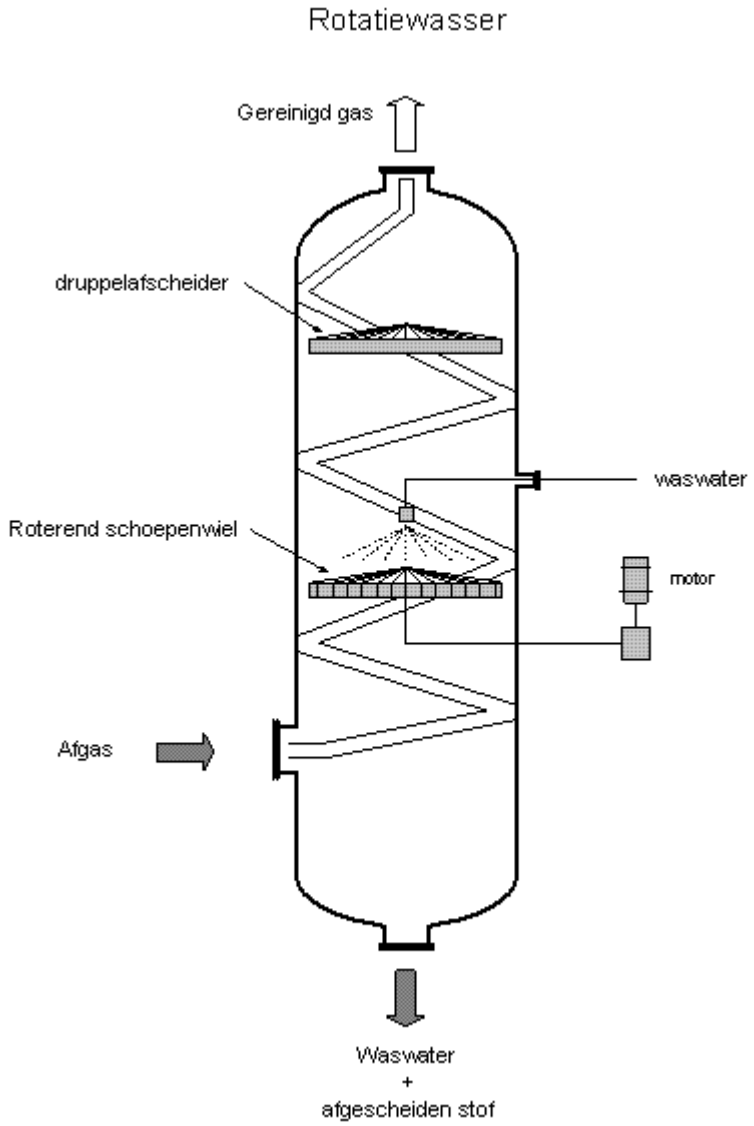


Image rotation washer of InfoMil

Fog filter

Most fog filters are woven elements of metal or synthetic material. The filters work on the principle of mechanical capture and depend on the speed at which the particles or drops pass through the filter. The efficiency of fog filters can be up to 99%. The smaller the mesh size, the more efficient it is for small particles (1 - 3 µm). However, the risk of clogging increases.

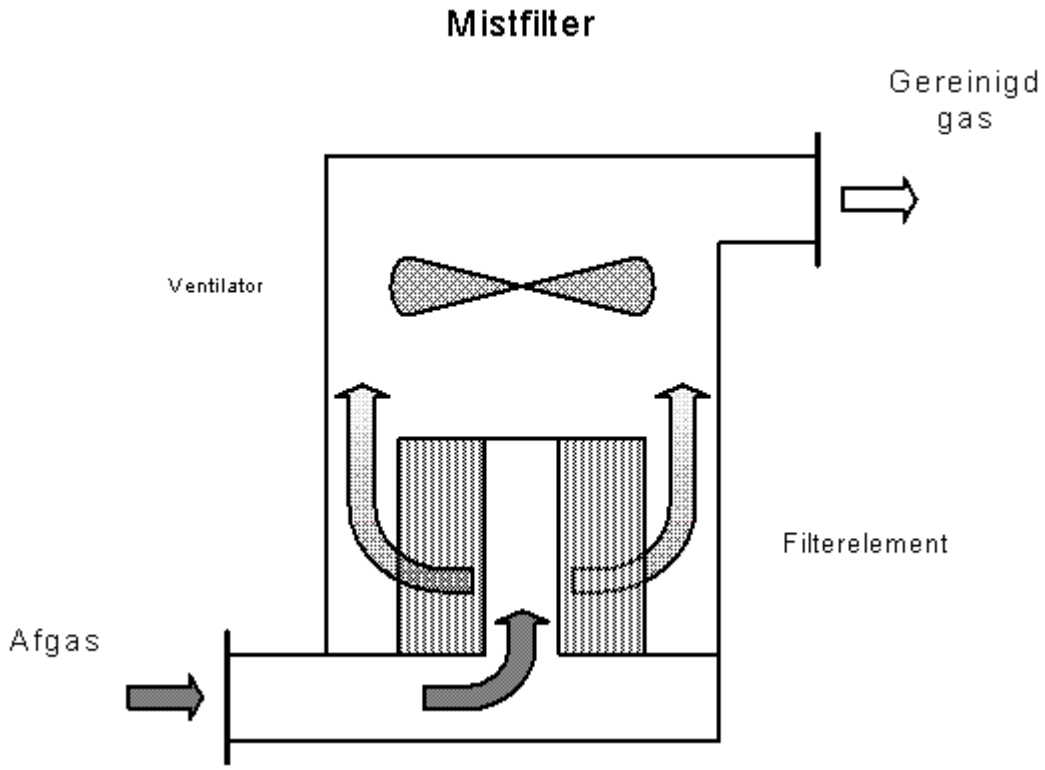


Image fog filter from InfoMil

Droplet collectors or fog filters are also used in cooling towers to prevent aerosol dispersion. Up to 98% of aerosols are captured. When applying this type of droplet eliminators, a high air velocity is essential. The air must be forcibly extracted.

In Boxtel, a dropper has been used to capture aerosols before the air is treated by Uvc.. The droplet eliminators only stopped a limited amount of aerosols. The technique does not appear to be suitable as a single barrier.

Condensation

By letting the air to flow on a cold surface for a long time, condensation occurs. The condensation is collected and discharged. Nitrogen cooling can be applied to cool the surface and control the amount of condensation.

10. 5 Air treatment

Conceivable additional measures on top of covering is the treatment of the air. The simplest shape is to make openings in the cover and provide them with filter mats. Legionella bacteria are rod-shaped. They have a diameter of 0.3 to 0.9 µm and are 2.0 to 3.0 µm long. Filter mats in accordance with ISO 16890 class ePM1 stop a large part of the bacteria. Activated carbon filters can also be used to capture Legionella bacteria.

A more effective solution is to extract and additionally treat the air. A slight negative pressure can be created to prevent escape of aerosols. The extracted air can be treated by:

- UV disinfection;
- Air filters;
- Of pasteurization

Example WWTP Boxtel: test set-up air failure with air filters *and Uvc treatment.*

Initial experience shows that droplet eliminators can be used at high air velocities. Only part of the aerosols is captured. Due to the high humidity, air filters have only a limited service life and silt quickly. UVc treatment seems to have the best results. However, its effectiveness decreases according to the age of the lamps. A combination of techniques often gives a better result.

Another idea for air treatment is the use of air scrubbers. No experiences of this are known in relation to Legionella prevention. Misting takes place within a number of types of air scrubbers, which can lead to Legionella risks if insufficient control is provided [28].

10.6 Minor exposure sources

Chapter 5.2 discusses sources of exposure at the treatment plant. These are generally small sources of exposure with little air throughput. This can pose a particular risk to employees. Especially in sludge dewatering, high Legionella concentrations may be present. For example, Hydroscope has detected Legionella in the air near a tire thickener$^{10\,3}$ CFU/l. Especially with older installations, the process components are still open. Cover the processes. Modern process components are often covered.

10. 7 Cleaning work

No dose-effect relationship has been demonstrated between the concentration of Legionella bacteria in a water source and the risk of getting sick after exposure [30]. The following calculation is debatable, but is the best approach that can currently be given. Norwegian research [31] assumes that 1,000 inhaled Legionella bacteria can be deadly. With 'aerated grit removal', the concentration of bacteria in the water is at least 10^8 times higher than in the air above the tank [35]. As the residence time in the vicinity of the reactor increases, the risk to the employee increases. At a Legionella concentration of 10^9 cfu/l of water, a vulnerable employee who works on top of the reactor can get a lethal dose of inhaled Legionella bacteria within 36 minutes.

As long as no high Legionella concentrations have been found in the wastewater, the regular health and safety measures are sufficient. Employees are at risk in the vicinity of nebulous processes. Respiratory protection shall be worn within one meter of a nebulizer source for exposures exceeding 4 minutes. Respiratory protection shall be worn outside one meter from the neve source at an exposure of more than 39 minutes [14].

Places symbols for dust masks at the places where aerosol formation takes place.

Symbol dust mask is sufficient, but to indicate that it concerns FFP3.

If you have long-term maintenance work, turn off the aeration 15 minutes in advance.

In accordance with the AI-09 health and safety information sheet, it is not recommended to use effluent for cleaning purposes. In addition, it is recommended to dry remove as much pollution as possible. When carrying out cleaning work, misting is released from the nozzle of the cleaning hose and the splashing water from the surface to be cleaned. Avoiding high pressure reduces the amount of atomization. At least a face mask with filter class FF P3 [32] must be worn for cleaning with effluent, surface water or drinking water. Wearing a half-face mask offers much better protection than a snout. A half-face mask is made more suitable for long-term use and therefore is more comfortable to wear.

Instruct employees on how to work with biological agents and wear respiratory protection. Run an RI&E on biological agents. Prepare job risk analyses and offer employees a periodic medical examination.

11. Preventing spread via effluent

The following measures are conceivable to reduce effluent spread.

11.1 Settlement

Assuming a water temperature of <25°C, the Legionella concentration decreases after a secondary settlement step. Sludge and water are separated. Some of the Legionella bacteria are removed via the sludge line. From experience with some purifications, the legionella concentration decreases by one to two log factors.

11.2 Membrane filtration

Membrane filtration is used in Boxtel. The membranes very effective at stopping Legionella bacteria. At Legionella concentration of 10^{10} cfu/l, the membranes manage to keep the concentration in the effluent below 10,000 cfu/l. The membranes are in the reactor. Unlike a reactor without membranes, the Legionella concentration in the reactor remains unabated. In a reactor without membranes, the Legionella concentration fluctuates more.

In case of membrane breakage, the effectiveness of the membranes decreases. It is therefore important to measure integrity and to block and replace leaking membranes in a timely manner.

11.3 UVc treatment

A pilot has been carried out with UVc on the effluent of a partial current treatment. Due to shadow formation, UVc is not sufficiently effective.

11.4 Other disinfection

Possible other alternatives to disinfection is the application of disinfectants.

Techniques developed for the fourth purification step may also be effective for Legionella control. Think of ozone and hydrogen peroxide in combination with UV.

Appendix 1 Literature list

	title	Year	author	Hyperlink
1	Risks of Exposure Legionella at WWTPs	2002	Dr. G.J. Medema, ir. D. Koot, A. Brouwer	Link
2	Two Legionnaires' disease cases associated with industrial waste water treatment plants: a case report	2010	Jaana Kusnetsov, Liisa-Kaarina Neuvonen, Timo Korpio, Søren A Uldum, Silja Mentula, Tuula Putus, Nhu Nguyen Tran Minh and Kari-Pekka Martimo	Link
3	Tracking Airborne Legionella and Legionella pneumophila at a Biological Treatment Plant	2008	Janet Martha Blatny, Gunnar Skogan, Bjørn Anders Pettersson Reif, Øyvind Andreassen, Gunn Merethe Bjørge Thomassen, Tone Aarskaug, Else Marie Fykse and Jaran Strand Olsen	Link
4	Microorganisms in bioaerosol emissions from wastewater treatment plants during summer at a Mediterranean site	2007	Eleftheria Katsivela, Styliani Karra	Link
5	Sampling and identification of Legionella spp. that Borregaard Ind. Ltd.	2007	Janet Martha Blatny, Gunnar Skogan, Bjørn Anders Pettersson Reif, Øyvind Andreassen, Gunn Merethe Bjørge Thomassen, Tone Aarskaug, Else Marie Fykse and Jaran Strand Olsen	Link
6	Alternative routes for dissemination of Legionella pneumophila causing three outbreaks in Norway	2010	Olsen JS, Aarskaug T, Thrane I, Pourcel C, Ask E, Johansen G, Waagen V, Blatny JM.	Link
7	Tracking Legionella in air generated from a biological treatment plant - A case study of the outbreak of Legionellosis in Norway	2011	Janet Martha Blatny, Jaran Strand Olsen, Øyvind Andreassen, Viggo Waagen and Bjørn Anders Pettersson Reif	Link
8	Procedure for the choice of aeration system	1999	and. Mr Kruit, and. E.G. Wypkema en dr. and. A. Vissa	Link
9	Pontiac fever at a sewage treatment plant in the food industry	1999	Gregersen P, Grunnet K, Uldum SA, Andersen BH, Madsen H.	Link
10	Strategies for the reduction of Legionella in biological treatment systems	2016	Nogueira R, Utecht KU, Exner M, Verstraete W, Rosenwinkel KH	Link
11	Epidemiological investigation and case–control study: a Legionnaires' disease outbreak associated with cooling towers in Warstein, Germany, August–September 2013	2015	Anna Maisa, Ansgar Brockmann, Frank Renken, Christian Lück, Stefan Pleischl, Martin Exner, Inka Daniels-Haardt, Annette Jurke	Link
12	Occupational health and safety information 09 Biological agents, safe working with microorganisms	2014	Dr. W.J.T. van Alphen, Dr. Ir. R. Houba	

	title	Year	author	Hyperlink
13	Occupational health and safety information 32 Legionella, risk management in process water systems	2013	Ir. F.I.H.M Oesterholt, Ing. A.J. van Pelt	
14	Blueprint RI&E biological agents	2007	Remko Houba	Link
15	Measurement protocol Air measurements microorganisms industry Part 3A	2011	VLA Indoor Environment Advice	Link
16	Strategies for the reduction of Legionella in biological treatment systems	2016	R. Nogueira, K-U Utecht, M. Exner, Willy Verstraete, K.H. Rosenwinkel	Link
17	Temperature-driven growth of Legionella in lab-scale activated sludge systems and interaction with protozoa	2018	Caicedo C., Rosenwinkel K.H., Nogueira R.	Link
18	Legionella pneumophila in second biological wastewater treatment plant	2018	T. Leenstra	Link
19	A community-wide outbreak of legionnaires disease linked to industrial cooling towers--how far can contaminated aerosols spread?	2006	Nguyen TM, Ilef D, Jarraud S, Rouil L, Campese C, Che D, Haeghebaert S, Ganiayre F, Marcel F, Etienne J, Desenclos JC	Link
20	Exposure to endotoxins and the prevention of complaints in workers of sewage treatment plants	2004	Drs. S. Spaan, Ir. L. Smit, M.J. Visser, Ir. H.J.J.M. Arts, Dr. Ir. I.M. Wouters, Prof. Dr. Ir. D.J.J. Heederik	Link
21	Legionnaires' disease in Finland, 2014-2017	2018	Sari Jaakola, Jaana Kusnetsov, Silja Mentula, Pia S. Räsänen, Piia Airaksinen, and Outi Lyytikäinen	Link
22	Two community clusters of Legionnaires' disease directly linked to a biological wastewater treatment plant in the Netherlands, 2016-2017	2018	Anna Loenenbach, Christian Beulens, Sjoerd M. Euser, et al.	
23	Biological nutrient removal	2018	Energy and environmental information system for the Flemish Region	Link
24	ESGLI-presentation: Monitoring of Legionella pneumophila in an industrial wastewater treatment plant	2018	Regina Nogueira	Link
25	Outbreak management of the Legionella outbreak in Warstein	2013	Martin Exner	Link
26	Report of the Expert Commission Legionella	2015	Mrs. Dericks and Mr. Oesterbeck	Link

	title	Year	author	Hyperlink
27	Legionella occurrence in municipal and industrial wastewater treatment plants and risks of reclaimed wastewater reuse	2018	C. Caicedo, K.-H. Rosenwinkel, M. Exner, W. Verstraete, R. Suchenwirth, P. Hartemann, R. Nogueira	Link
28	Can air scrubbers spread legionella bacteria to the environment?	2013	Bartels AA, Schalk JAC, Melse RW	Link
29	Evaluation of Legionella Air Contamination in Healthcare Facilities by Different Sampling Methods: An Italian Multicenter Study	2017	Maria Teresa Montagna, et al	Link
30	LCI Directive Legionellosis	2018	National Centre for Infectious Diseases	Link
31	Airborne Legionella bacteria from pulp waste treatment plant	2011	Janet Martha Blatny, et al	Link
32	Health and Safety Catalogue P3 Mask	2018	Arbocatalogus	Link
33	Detection methods for legionella in water	2010	J.A.C. Schalk, A.M. by Roda Husman	Link
34	Isolation of Legionella pneumophila from Pluvial Floods by Amoebal Coculture	2014	J.A.C. Schalk, A.E. Docters van Leeuwen, W.J. Lodder, H. de Man, S. Euser, J.W. den Boer, A.M. de Roda Husman	Link
35	Health complaints at RWZI Harnaschpolder	2007	A. Dusseldorp, P. Morgenstern, IMD	Link
36	Health and safety catalogue sector Water boards; Part 5 biological agents	2016	A&O Fund Water Boards	Link
37	The occurrence of endotoxins on rwzis	2002	And. H.J.J.M Arts, Prof. Dr. and. D.J.J. Heederik	Link
38	Inventory of legionella risks at wastewater treatment plants	2019	A.A. Bartels et al.	Link
39	Symposium "Prevent Legionella in wastewater" lecture ODBN	2019	Diana Stoel	
40	Use of floating balls for reducing bacterial aerosol emissions from aeration in wastewater treatment processes.	2010	H.F. Hung, et al.	Link
41	A quantitative microbial risk assessment model for Legionnaires' Disease: animal model selection and dose-response modeling	2007	T. Armstrong, C.N. Haas	Link
42	An in-premise model for Legionella exposure during showering events	2011	M.E. Schoen, N.J. Ashbolt	Link

	title	Year	author	Hyperlink
43	Ten Questions Concerning the Aerosolization and Transmission of Legionella in the Built Environment.	2017	A.J. Prussin, et al.	Link
44	Effect of salt concentration and temperature on survival of Legionella pneumophila	1998	R. Heller, et al	Link
45	Amoebae and Legionella pneumophila in saline environments	2011	R.J. Guest, et al	Link
46	Evaluation of the environmental impact of microbial aerosols generated by wastewater treatment plants utilizing different aeration system	2000	Brandi G., Sisti M., Amagliani G.	Link
47	Occurrence of Legionella in wastewater treatment plants linked to wastewater characteristics	2016	C. CaicedoS. Beutelt. Scheperk. H. RosenwinkelR. Walnut	Link
48	Growth kinetics of environmental Legionella pneumophila isolated from industrial wastewater	2019	C. CaicedoW. VerstraeteK--H. RosenwinkelR. Walnut	Link
49	Bacterial-aerosol emission from wastewater treatment plant	2013	Mohammad Malakootian, et al	Link
50	Stench and aerosols	1980	Ir. W.G. Werumeus	Link
51	Effect of the aeration system on the levels of airborne microorganisms generated at wastewater treatment plants.	2008	Sánchez-Monedero MA1, Aguilar MI, Fenoll R, Roig A.	Link
52	Effect of aeration mode on aerosol characteristics from the same wastewater treatment plant	2020	Yunping Hanab, Tang Yangab, et al	Link
53	Characterization of aerosols containing Legionella generated upon nebulization	2016	Séverine Allegra, Lara Leclerc, Pierre André Massard, Françoise Girardot, Serge Riffard & Jérémie Pourchez	Link
54	Surveillance of Legionnaires disease in Europe	2002	Joseph C.	
55	Legionella and the prevention of legionellosis	2007	WHO	Link
56	Inhibition of growth of Legionella species by heterotrophic pate-count bacteria isolated from chlorinated drinking water	1990	Toze S, Sly LI, Macrae IC, Fuerst JA.	

	title	Year	author	Hyperlink
57	Biofilms: the environmental playground of Legionella Pneumophila	2010	Declerck P.	Link
58	Protozoa in wastewater treatment processes	2009	P. Madoni	Link
59	Free-living protozoa in drinking water supplies: community composition and role as hosts for Legionella pneumophila	2011	Rinske Marleen Valster	Link
60	Potential measures against the spread of Legionella from wastewater treatment plants	2019	WJ Lodder, HHJL van den Berg, RC van Leerdam, AM de Roda Husman	Link
61	Possible air dispersion of Legionella by wastewater treatment plants: a patient-control study	2019	LC Vermeulen, PS Brandsema, J van de Kassteele, BCJ Bom, HHJL van den Berg, AM de Roda Husman	Link
62	Legionella in water; Development of an amoebe-culture PCR method for detection of legionella	2009	J.A.C. Schalk, W.J. Lodder, S.A. Rutjes, F.M. Sketch, A.M. de Roda Husman	Link
63	Detection of Free-Living Amoebae Using Amoebal Enrichment in a Wastewater Treatment Plant of Gauteng Province, South Africa	2014	P. Muchesa, O. Rock, T. G. Barnard, C. Bartie	Link
64	Ciliate Paramecium is a natural reservoir of Legionella pneumophila	2016	Kenta Watanabe, Ryo Nakao, Masahiro Fujishima, Masato Tachibana, Takashi Shimizu, Masahisa Wataraia	Link
65	Amoeba	2020		Link
66	Diverse Legionella-Like Bacteria Associated with Testate Amoebae of the Genus Arcella	2018	Fatma Gomaa, Maxim Gersh, Colleen Cavanaugh	Link
67	From Many Hosts, One Accidental Pathogen: The Diverse Protozoan Hosts of Legionella	2017	David K. Boamah, Guangqi Zhou, Alexander W. Ensminger, Tamara J. O'Connor	Link
68	The Testate lobose amoebae in the wastewater treatment	2009	A.C. Tomasini Ortiz, G. E. Moeller Chávez, M. Garzón-Zuñiga and Y. Hornelas Orozco	Link
69	Manual process monitoring by microscopic process examination	1999	STOWA	Link
70	Legionella		WHO	Link
71	Overview factsheets air emission mitigation techniques	2020	InfoMil Knowledge Centre	Link
72	Stench and aerosols	1980	Ir. W. G. Werumeus Buning	Link
73	Gaussisch pluimmodel	2020	InfoMil Knowledge Centre	Link

	title	Year	author	Hyperlink
74	Detection of legionella with a amoebekweekmethode	2011	J.A.C. Schalk et al.	Link
75	Starved viable but non-culturable (VBNC) Legionella strains can infect and replicate in amoebae and human macrophages	2018	Dietersdorfer, Elisabeth et al.	Link
76	Hazard prevention and control in the work environment: Airborne dust	1999	WHO	Link
77	Concepts in Inhalation Toxicology	1989	Schlesinger, R. B. In R. O. McClellan, R. F. Henderson	
78	Bioaerosols emission and exposure risk of a wastewater treatment plant with A2O treatment process	2019	Yunping Han, Kaixiong Yang, Tang Yang, Mengzhu Zhang, Lin Li	Link
79	The Variability of the Concentration of Bioaerosols Above the Chambers of Biological Wastewater Treatment	2018	Michał Michałkiewicz, Izabela Kruszelnicka, Małgorzata Widomska	Link
80	Comparisonof insulation media for Legionella: H2O edition 13-2005	2005	Harm Veenendaal, Simon in't Veld	Link
81	Fixing legionella colonies with UV exposure; a sustainable alternative	2020	Adrie Atsma, Ananda van der Spek, Sharda van Willigen (Vitens)	Link
82	Risk-Based Critical Concentrations of Legionella pneumophila for Indoor Residential Water Uses	2019	Kerry A. Hamilton, Mark T. Hamilton, William Johnson, Patrick Jjemba, Zia Bukhari, Mark LeChevallier, Charles N. Haas, and P. L. Gurian	Link
83	Management of Legionella in Water Systems	2020	National Academies of Sciences, Engineering, and Medicine	Link
84	MPN-method	2020	In his Wiersema	Link
85	Evaluation of Legiolert for Quantification of Legionella pneumophila from Non-potable Water	2017	Melanie M. Rech, et al.	Link
86	Interpretation of risk estimates for Legionella pneumophila	2012	M Bouwknegt, JAC Schalk, AM de Roda-Husman	Link
87	Legionella		WHO	Link
88	Legionella in wastewater; survey industrial biological wastewater treatment plants	2020	K. Kanters commissioned by VEMW	
89	Manual nitrogen and phosphorus removal from community waste water on RWZI's	2017	Annete Buunen, et al.	Link

	title	Year	author	Hyperlink
90	Industrial Production of Poly-β-hydroxybutyrate from CO2	2020	Roberta Carpine, Giuseppe Olivieri, Klaas J. Hellingwerf, Antonino Pollio, Antonio Marzocchella	Link
91	Poly-3-Hydroxybutyrate in Legionella pneumophila, an Energy Source for Survival in Low-Nutrient Environments	1999	W. Stuart Mauchline, P. Julian Dennis, Charles William Keevil	Link
92	Growth-related Metabolism of the Carbon Storage Poly-3-hydroxybutyrate in Legionella pneumophila	2016	Nadine Gillmaier, Eva Schunder, Erika Kutzner, Hana Tlapák, Kerstin Rydzewski, Vroni Herrmann, Maren Stämmler, Peter Lasch, Wolfgang Eisenreich, Klaus Heuner	Link
93	Legionella pneumophila CsrA regulates a metabolic switch from amino acid to glycerolipid metabolism	2017	Ina Häuslein, Tobias Sahr, Pedro Escoll, Nadine Klausner, Wolfgang Eisenreich, Carmen Buchrieser	Link
94	Potent antimicrobial peptides against Legionella pneumophila and its environmental host, Acanthamoeba castellanii	2015	Margot Schlusselhuber, Vincent Humblot, Sandra Casale, Christophe Méthivier, Julien Verdon, Matthias Leippe, Jean-Marc Berjeaud	Link
95	Manual biological phosphate removal	2001	STOWA	Link
96	Annual report Surveillance of influenza and other respiratory infections in the Netherlands	2019	RIVM Reukers et al.	Link
97	Viability and infectivity of viable but nonculturable Legionella pneumophila strains induced at high temperatures.	2019	Sílvia Cervero-Aragó, Barbara Schrammel, Elisabeth Dietersdorfer, Regina Sommer, Christian Lück, Julia Walochnik, Alexander Kirschner	Link
98	Impact of water heater temperature setting and water use frequency on the building plumbing microbiome.	2017	Ji P, Rhoads WJ, Edwards MA, Pruden A	Link
99	The many forms of a pleomorphic bacterial pathogen - the developmental network of Legionella pneumophila	2014	Robertson et al 2014	Link
100	Higher efficiency due to thermophilic fermented manure	2016	Mestportaal.nl	Link
101	The fermentation process	2020	Infomil	Link
102	Characteristics and formation mechanism of intestinal bacteria particles emitted from aerated wastewater treatment tanks	2019	Yanjie Wang, Lin Li,SongXue, Yunping Han, Kaixiong Yang	Link

	title	Year	author	Hyperlink
103	ECP technology description: Wet Anaerobe fermentation	2012	Energy Conversion Parks	Link
104	Legionella longbeachae pneumonia: Case report and review of reported cases in non-endemic countries	2021	Harrison Bella, Sai Chintalapati, Preet Patel, Ameer Halim, Andrew Kithas, Sarah A. Schmalzle	Link
105	Iron Content as an Indicator for Legionella Species in Artificial Water Systems	2021	Rajeshwari Vittal, Juliet Roshini Mohan Raj, Ballamoole Krishna Kumar, Indrani Karunasagar	Link
106	Thesis defence: There are also live legionella bacteria in garden soil and rainwater lakes	2017	H.H.L. van Heijnsbergen	Link
107	Water in public space has risks for health	2014	Heleen de Man	Link
108	Profiling Bacterial Diversity and Potential Pathogens in Wastewater Treatment Plants Using High-Throughput Sequencing Analysis	2019	Cecilia Olusey, Osunmakinde, Ramganesh Selvarajan, Bhekie B. Mamba and Titus A.M. Msagati	Link
109	Novel Endosymbionts in Rhizarian Amoebae Imply Universal Infection of Unrelated Free-Living Amoebae by Legionellales	2021	Marcel Dominik Solbach, Michael Bonkowski, Kenneth Dumack	Link
110	Control of legionella on rwzis	2021	Wim Wiegant as Martijn van Leusden (Royal HaskoningDHV)	Link
111	Methane production of thick manure fractions: with and without pretreatment	1995	Mr. Timmerman, F.E. de Buisonjé	Link

Appendix 2 Example completed blueprint biological agents

Werkblad 5b: Proces en blootstelling
Voor de procesanalyse uit (aard, mate en duur) en beheersmaatregelen

EU-klasse: 2 — Risico beoordeling voor agens: *Legionella*

				Blootstellingsroute		*Duur*			*Inhalatoire maatregelen*			*Dermale maatregelen*	*Risicoscore per route zonder maatregelen*			*Risicoscore per route met maatregelen*		
Stapnr	Processtap	Procesmateriaal	Werkzaamheden	inhalatoir	dermaal	frequentie	# per freq	duur in min	bronisolatie en ventilatie	afscheiding van de werknemer	adembescherming	handschoenen gebruik	Risico inhalatoir	Risico dermaal	Risico oraal	Risico inhalatoir na maat-regelen	Risico dermaal na maat-regelen	Risico oraal na maat-regelen
1	**Reguliere proces**																	
R1	Innameput	Afvalwater	Visuele controle	afwezig	afwezig	dagelijks	1	5										
R2	Grofilter	Afvalwater	Visuele controle	afwezig	afwezig	dagelijks	1	5										
R3	Selector	Afvalwater	Visuele controle	afwezig	afwezig	dagelijks	1	5										
R4	Zuurstofloze ruimte	Afvalwater	Visuele controle	afwezig	afwezig	dagelijks	1	5										
R5	Zuurstofarme ruimte	Afvalwater	Visuele controle	afwezig	afwezig	dagelijks	1	5										
R6	Beluchtingsbassins	Afvalwater	Visuele controle	< 1 meter van ademzone	afwezig	dagelijks	1	5	geen bronmaatregelen	niet cabine	Filtermasker (snuitje) P3 (FFP3)							
R7	Nabezinktanks	Afvalwater	Visuele controle	afwezig	afwezig	dagelijks	1	5										
R8	Effluentput	Effluent	Visuele controle	afwezig	afwezig	dagelijks	1	5										
R9	Aanmaak polymeren	Afvalwater	Visuele controle	afwezig	afwezig	dagelijks	1	5										
R10	Slibindikking	Slib	Visuele controle	< 1 meter van ademzone	afwezig	dagelijks	1	5	geen bronmaatregelen	niet cabine	Filtermasker (snuitje) P3 (FFP3)							
R11	Slibgisting	Slib	Visuele controle	afwezig	afwezig	dagelijks	1	5										
R12	Slibontwatering	Slib	Visuele controle	afwezig	afwezig	dagelijks	1	5										
R13	DEMON	Reactorinhoud	Visuele controle	afwezig	afwezig	dagelijks	1	5										
R14	Biobedden	Effluent	Visuele controle	afwezig	afwezig	dagelijks	1	5										
R15																		
R16																		
R17																		
R18																		
R19																		
R20																		
R21																		
2	**Reguliere onderhoud en schoonmaak**																	
O1	Grofilter	Effluent	Schoonspuiten	< 1 meter van ademzone	afwezig	wekelijks	1	5	gebruik van product dat de emissie vermindert	niet cabine	Filtermasker (snuitje) P3 (FFP3)							
O2	Slibindikking	Slib	Losschudden slib	< 1 meter van ademzone	afwezig	dagelijks	1	5	geen bronmaatregelen	niet cabine	Filtermasker (snuitje) P3 (FFP3)							
O3																		
O4																		
O5																		
O6																		
O7																		
O8																		
O9																		
O10																		
O11																		
3	**Storingen**																	
S1	Beluchtingsbassins	Afvalwater	Bijv pomp vervangen	< 1 meter van ademzone	afwezig	jaarlijks	1	240	bronafscherming	niet cabine	Filtermasker (snuitje) P3 (FFP3)							
S2																		
S3																		
S4																		
S5																		
S6																		
4	**Lab en Kwaliteitscontrole**																	
LK1	Innameput	Afvalwater	Ophalen 24-uursmonster	afwezig	afwezig													
LK2	Effluentput	Effluent	Ophalen 24-uursmonster	afwezig	afwezig													
LK3																		
LK4																		
LK5																		