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Legionella detection in wastewater treatment plants with increased risk for *Legionella* growth and emission

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ABSTRACT

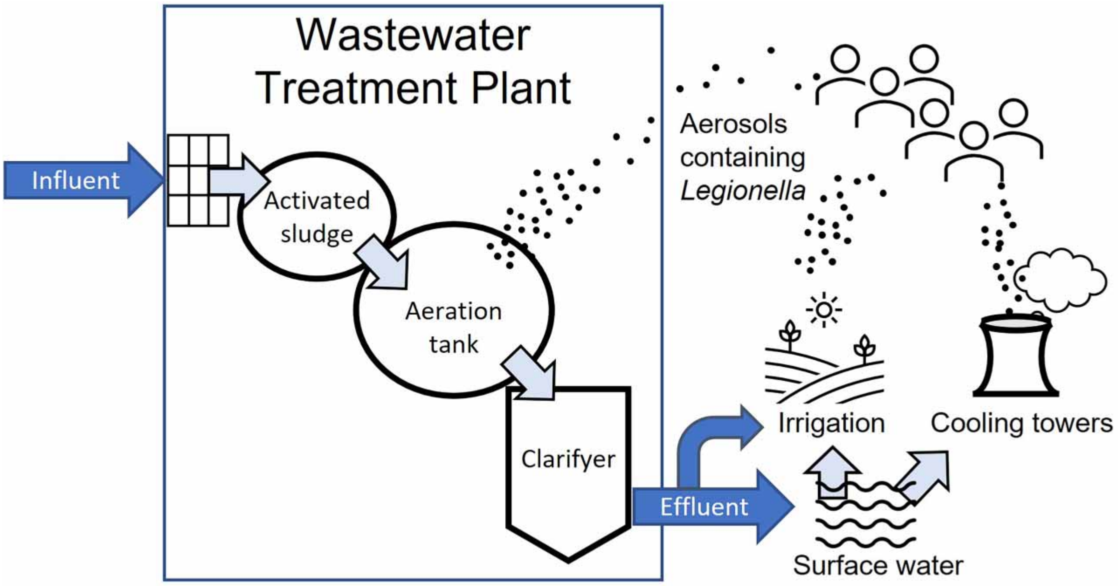
Legionnaires' disease (LD) is a severe pneumonia mainly caused by the bacterium *Legionella pneumophila*. Although many environmental sources of LD have been described, the sources of the majority of non-outbreak LD cases have not been identified. In several outbreaks in the Netherlands, wastewater treatment plants (WWTPs) were identified as the most likely source of infection. In this study, four criteria for *Legionella* growth and emission to air and surface waters were selected based on the literature and a risk matrix was drafted. An inventory was made of all WWTPs and their characteristics in the Netherlands. The risk matrix was applied to identify WWTPs at risk for *Legionella* growth and emission. Wastewater was collected at WWTPs with moderate to high risk for *Legionella* growth and emission. In 18% of the sampled WWTPs, *Legionella* spp. was detected using culture methods. The presented risk matrix can be used to assess the risks of *Legionella* growth and emission for WWTPs and support surveillance by prioritizing WWTPs. When *Legionella* is detected in the wastewater, it is recommended to take action to prevent emission to air or discharge on surface waters and, if possible, reduce the *Legionella* concentration.

Key words: aerosol, airborne, emission, industrial wastewater, Legionnaires' disease, sewage

HIGHLIGHTS

- The developed risk matrix to assess the risks of *Legionella* growth and emission from WWTPs supports prioritizing WWTP for *Legionella* surveillance.
- *Legionella* spp. was detected in 18% of the WWTP with moderate to high risk.
- This work contributed to the development of a guidance document to support owners of WWTPs to identify and control *Legionella* risks.

GRAPHICAL ABSTRACT



INTRODUCTION

Legionnaires' disease (LD) is a severe pneumonia mainly caused by *Legionella pneumophila*. In the Netherlands, the LD incidence increased in recent years (Reukers *et al.* 2019). *Legionella* bacteria can occur naturally in water and soil, often in low concentrations (Steinert *et al.* 2002). Higher concentrations are sometimes observed in water installations, such as building water systems, wet cooling towers and whirlpools, because the temperature is more favorable for growth and competition from other bacteria is lower (Steinert *et al.* 2002). Although many possible and confirmed environmental sources of LD have been described (van Heijnsbergen *et al.* 2015), a source of infections cannot be identified for the majority of sporadic (non-outbreak) cases. In the Netherlands, potential sources were systematically sampled as part of source-finding investigations, of which the majority of sampled sources were drinking water systems. Between 2002 and 2012, all potential sources of exposures were sampled for 392 non-outbreak patients from whom a clinical isolate was available, and the clinical and environmental isolates were compared using sequence-based typing. Only for 11% of these patients a genotypic match between the clinical isolate and the environmental isolate was found, showing that the source of infection remained unknown for the majority of patients, despite systematic source investigations. Moreover, there appears to be a mismatch in the Netherlands between the sequence types (STs) found in isolates from patients and environmental isolates from drinking water systems (Euser *et al.* 2013; Den Boer *et al.* 2015). The *L. pneumophila* serogroup 1 strain detected most frequently in sampled water systems was the strain ST1, but this strain was found in only a small portion (4–5%) of all clinical isolates. On the other hand, the ST-type of the *L. pneumophila* isolates found most frequently in patients (ST47) was not found in the sampled tap water systems at all (Euser *et al.* 2013; Den Boer *et al.* 2015). These data indicate that important sources of infections were not yet included in the source investigations and suggest that other sources of *Legionella* are present in the environment, that are causing LD.

In 2017, a wastewater treatment plant (WWTP) at a food processing company was identified as the likely source of contamination for a local increase of pneumonia caused by *L. pneumophila* observed (Loenenbach *et al.* 2018). In this installation, high *Legionella* concentrations were detected in the wastewater of an identical ST as found in five patients: *L. pneumophila* serogroup 1 ST1646. Another LD cluster with 54 patients could be linked to an industrial WWTP located at a rendering company (Loenenbach *et al.* 2018; Reukers *et al.* 2018). Both WWTPs had a biological process which treated nutrient-rich process water at temperatures between 30 and 35 °C (Loenenbach *et al.* 2018). Other studies also reported LD in employees or local residents in which WWTPs may have played a role (Nguyen *et al.* 2006; Nygård *et al.* 2008; Kusnetsov *et al.* 2010; Olsen *et al.* 2010; Maisa *et al.* 2015; Nogueira *et al.* 2016).

In several studies, *Legionella* was detected in wastewater samples using culture and/or molecular methods (Allestam *et al.* 2006; Blatny *et al.* 2008; Schalk *et al.* 2012; Lund *et al.* 2014; Loenenbach *et al.* 2018). High concentrations (up to 10^9 colony forming units (CFUs)/L) of *L. pneumophila* have been detected in the aeration tanks of industrial WWTPs (iWWTPs; Olsen *et al.* 2010; Loenenbach *et al.* 2018). During the wastewater treatment process, aerosols are formed, which may disperse *Legionella* from WWTPs to the environment. In air samples at WWTPs *Legionella* bacteria were found, with concentrations of up to 3,300 CFU/m^3 directly above aeration tanks (Medema *et al.* 2004; Blatny *et al.* 2008; Loenenbach *et al.* 2018). Air measurements showed that aerosols contaminated with *Legionella* emitted from the aeration tanks can spread through the air over a distance of at least 3 km (Reukers *et al.* 2018). Vermeulen *et al.* (2021) showed that exposure to aerosols from WWTPs likely caused LD in residents living near WWTPs. *Legionella* may also be discharged to surface waters through contaminated WWTP effluent (Olsen *et al.* 2010; Nogueira *et al.* 2016; Loenenbach *et al.* 2018). In the Netherlands, an overview of all WWTPs is missing and no legal requirements are in place for monitoring *Legionella* at WWTPs. To better understand the contribution of WWTPs to LD more information on the amount, location and characteristics of WWTPs as well as the presence of *Legionella* in the installations is needed.

In this study, an inventory was made of WWTPs. Furthermore, we developed a risk matrix to identify and prioritize WWTPs in the Netherlands at risk for *Legionella* growth and emission. *Legionella* spp. and *L. pneumophila* were quantified in process water of moderate to high risk WWTPs employing culture methods.

MATERIAL AND METHODS

Risk matrix

In 2019, a literature study was performed to identify risk criteria associated with *Legionella* growth and emission from WWTPs (Bartels *et al.* 2019). Peer-reviewed English-language articles describing WWTPs that were recognized as the direct or indirect source of *Legionella* infections were selected. In eight case studies, four similar characteristics were described that may have led to *Legionella* growth and emission to air and surface waters from WWTPs (Gregersen *et al.* 1999; Isozumi *et al.* 2005; Allestam *et al.* 2006; Nguyen *et al.* 2006; Blatny *et al.* 2008; Kusnetsov *et al.* 2010; Olsen *et al.* 2010; Maisa *et al.* 2015; Nogueira *et al.* 2016; Loenenbach *et al.* 2018). These characteristics were considered criteria for increased risk, as listed below.

Type of WWTP (biological or non-biological)

All WWTPs described in the case studies were biological WWTPs using aerobic bacteria for wastewater treatment. These bacteria have similar requirements for optimal growth as *Legionella* bacteria, including oxygen demand (via aeration) and temperature. *Legionella* growth is not inhibited or outcompeted by these bacteria (Caicedo *et al.* 2019). Non-biological WWTPs were not described in the literature as a source of LD.

Type of industry

Relevant industries for *Legionella* growth are industries where many organic compounds such as proteins, and nutrients such as phosphorus and ammonia are discharged into the wastewater. This organic/nutrient-rich wastewater is beneficial for *Legionella* growth, and high *Legionella* concentrations have been detected in this water (Caicedo *et al.* 2019). The following industries with organic/nutrient-rich wastewater were linked to patients with legionellosis:

- Food industry, including meat processing and a brewery (Nogueira *et al.* 2016; Loenenbach *et al.* 2018);
- Paper and wood industry (8, Allestam *et al.* 2006; Olsen *et al.* 2010);
- Rendering companies (processing cadavers) (Loenenbach *et al.* 2018; Reukers *et al.* 2018); and
- Petrochemical companies (Nguyen *et al.* 2006).

These types of industries were included in the risk matrix (Table 1). The occurrence or growth of *Legionella* in treatment plants from other industrial sectors cannot be excluded, including WWTPs receiving wastewater from other industries. However, no case studies for these industries were described at the time of this study, and therefore, the risks for *Legionella* growth and emission in these other industries are classified as 'unknown'.

Temperature of the process water

L. pneumophila grows within a temperature range from approximately 25 to 45 °C with an optimum between 35 and 37 °C (Wadowsky *et al.* 1985; Falkinham *et al.* 2015). WWTPs that were directly or indirectly identified as the source

Table 1 | Risk matrix for *Legionella* growth and emission from WWTPs in the Netherlands

Type of WWTP	Type of industry from which wastewater is treated	Temperature process water	Aeration	Risk category for emission to air	Risk category for emission to surface waters
Biological	• Food industry	30–38 °C	Yes	High	High
	• Paper and wood		No	Moderate	
	• Rendering companies	25–29 °C or	Yes	Moderate	Moderate
	• Petrochemical	39–45 °C	No	Low	
	• Communal WWTP[a]	< 25 °C or >45 °C	Yes	Low	Low
			No	Very low	
	Other industries[b]	25–45 °C	Yes	Unknown	Unknown
			No		
		< 25 °C or >45 °C	Yes	Unknown	Unknown
			No		
Non-biological	Not relevant	Not relevant	Not relevant	Very low	Very low

[a]In WWTPs without an elevated water temperature (<25 °C), very high concentrations of *Legionella* may present when wastewater is received from industries with high concentration of *Legionella* (influent).

[b]No case studies were available for other industries and therefore the risk is unknown. However, it cannot be ruled out that other industries with nutrient-rich influent might have an increased risk.

of LD all had a wastewater temperature between 30 and 37 °C, mainly between 35 and 37 °C (Allestam *et al.* 2006; Kusnetsov *et al.* 2010; Olsen *et al.* 2010; Loenenbach *et al.* 2018). Based on these case studies, water temperatures between 30 and 38 °C were considered as high risk for *Legionella* growth. For water temperatures between 25 and 29 °C and between 39 and 45 °C, the risk was categorized as moderate. When the water temperature at a WWTP is always below 25 °C, *L. pneumophila* growth to high concentrations is unlikely (low risk). However, WWTPs with water temperatures below 25 °C may receive wastewater with high *Legionella* concentrations (influent). Furthermore, some other *Legionella* spp. besides *L. pneumophila* may be able to multiply at these low temperatures. For water temperatures higher than 45 °C, *Legionella* growth was categorized as possible. These temperature ranges were included in Table 1 as well as their indicative risk level.

Aeration

Aeration of wastewater in aeration basins plays a role in spreading *Legionella* as described in multiple articles (Olsen *et al.* 2010; Loenenbach *et al.* 2018; Caicedo *et al.* 2019) and therefore included as a risk criterium. Aerosols formed through aeration allow *Legionella* bacteria to spread over a distance of more than 3 km (Reukers *et al.* 2018). Unfortunately, available literature did not yield sufficient information to differentiate the degree of risk of *Legionella* emission through the use of different types of aeration such as surface aeration or bubble diffusers.

Based on the four identified risk criteria, a risk matrix was developed for assessing the risk of *Legionella* growth and emission from WWTPs (Table 1). In this matrix, four risk categories were distinguished:

- *High risk*: High to very high *Legionella* concentrations in aeration tanks ($\geq 10^6$ CFU/L) and effluent ($\geq 10^4$ CFU/L) can be expected, as shown in the case studies. This may result in a high risk of exposure to *Legionella* bacteria if aerosols are emitted from the wastewater or (discharged) effluent.
- *Moderate risk*: (Temporary) *Legionella* growth to high concentrations is possible depending on the conditions. There is a risk of exposure to *Legionella* bacteria if aerosols are formed and emitted from wastewater or effluent.
- *Low risk*: *Legionella* may be present, but a high concentration ($\geq 10^6$ CFU/L) is not expected under these conditions. Incidentally, the concentration of *Legionella* in the process water might be increased, due to influent water with high concentrations of *Legionella*. Possible risk of exposure if aerosols are emitted from wastewater or effluent.
- *Very low risk: Legionella* is not likely to be present or is present at a very low concentration. Very low risk of exposure if aerosols are emitted from the wastewater or effluent.

Risk assessment for *Legionella* growth and emission to air and surface waters from WWTPs

To assess the number of WWTPs that potentially pose a risk for *Legionella* growth and emission to air and surface waters, an overview of existing WWTPs and their characteristics was needed. In November 2018, the Association of environmental agencies in the Netherlands (Omgevingsdienst NL (ODNL)) asked all 29 regional environmental agencies (ODs) to provide an overview of all iWWTPs in their region. Based on a questionnaire, additional information was gathered about the WWTPs. The Foundation for Applied Water Research (STOWA) made an inventory of all communal WWTP (cWWTPs) managed by the Dutch Water boards.

Based on the information received, the risk matrix was used to classify the WWTPs into four risk categories for *Legionella* growth and emission to air and discharge on surface waters from WWTPs: high, moderate, low, and very low risk. These risk levels do not provide information on the risk of infection or illness caused by *Legionella*.

Detection of *Legionella*

Sampling locations and sampling procedure

To verify the presence of *Legionella* in WWTPs with moderate to high risk of *Legionella* growth and emission, these WWTPs were sampled. Water samples were taken from the selected WWTPs in June and July 2019. Samples were taken at one time, so-called grab samples, according to the procedures described in ISO 19458:2006, if possible directly from the aeration tank or using a tap (ISO 2006). Water temperature (PT100 thermometer, Hanna Instruments) was measured on-site, in all samples. After sampling, samples were directly placed on melting ice or cooling-elements, and transported to the laboratory for microbiological analysis within 24 h after sampling.

Microbiological analysis

The water samples (500 mL) were concentrated using membrane filtration. Filtration was done by vacuum filtration (550 bar) with the aid of a vacuum controller (Innotech Europe BV; Moergestel; NL). The scraping technique, as described in ISO 11731: 2017 annex E, was used for the removal of the organisms from the membrane (ISO 2017). Residues were resuspended in 1 mL of sterile water. Due to the composition, not all samples could be concentrated. When this was the case, direct material was used. Of the suspension or direct material, 100 μL was inoculated without dilution and after a 10-fold and 100-fold dilution on three different agar plates at 35 °C, with increased humidity. The three agar plates used were (i) buffered charcoal yeast extract (BCYE) supplemented with α-ketoglutarate and L-cysteine (BCYE-α L-cysteine), (ii) the antibiotics polymyxin B, cefazolin, and pimaricin (BCYE AB); and (iii) the antibiotics polymyxin B, anisomysin, and vanomycin (MWY) (Thermo Fisher Scientific, Cheshire, UK). To reduce the growth of other microorganisms than *Legionella*, which can interfere with the recovery, portions of the water samples were also subjected to heat treatment and acid treatment. Heat treatment was done by adding the sample (concentrated or not concentrated) to a sterile container and placing it in a water bath at (50 ± 1) °C for (30 ± 2) min. Acid treatment was done by diluting one volume of the sample (concentrated or not concentrated) with nine volumes of the acid solution as described in ISO 11731: 2017 annex D, mixing well and leaving it for (5.0 ± 0.5) min (ISO 2017). The first examination was performed on day three of the total incubation period of 7 days using a dissection microscope. Suspected colonies were isolated and identified using MALDI-TOF. *L. pneumophila* strains were serotyped using commercially available kits containing antisera against *L. pneumophila* serogroups 1–14 according to manufacturer's protocol (*Legionella* latex test, Oxoid Limited, Hampshire, UK; *Legionella* antisera 'Seiken', Denka Seiken Co. Ltd, Tokyo, Japan).

RESULTS AND DISCUSSION

Risk assessment of WWTPs

In total, 451 iWWTPs were identified by the Dutch environmental agencies (OD) as competent authority. The number of iWWTPs listed per environmental agency varied between 0 and 74 implying that not all iWWTPs were identified in some regions. Some environmental agencies only reported iWWTPs with biological treatment, which indicates there might be underreporting of the number of non-biological treatment iWWTPs. In addition to the iWWTPs, a total of 327 cWWTPs owned and managed by the Dutch Water Authorities were reported by the Foundation for Applied Water Research (STOWA). In contrast to iWWTPs, for cWWTPs, an up-to-date database was available. In total, 778 WWTPs were identified as shown in Figure 1.

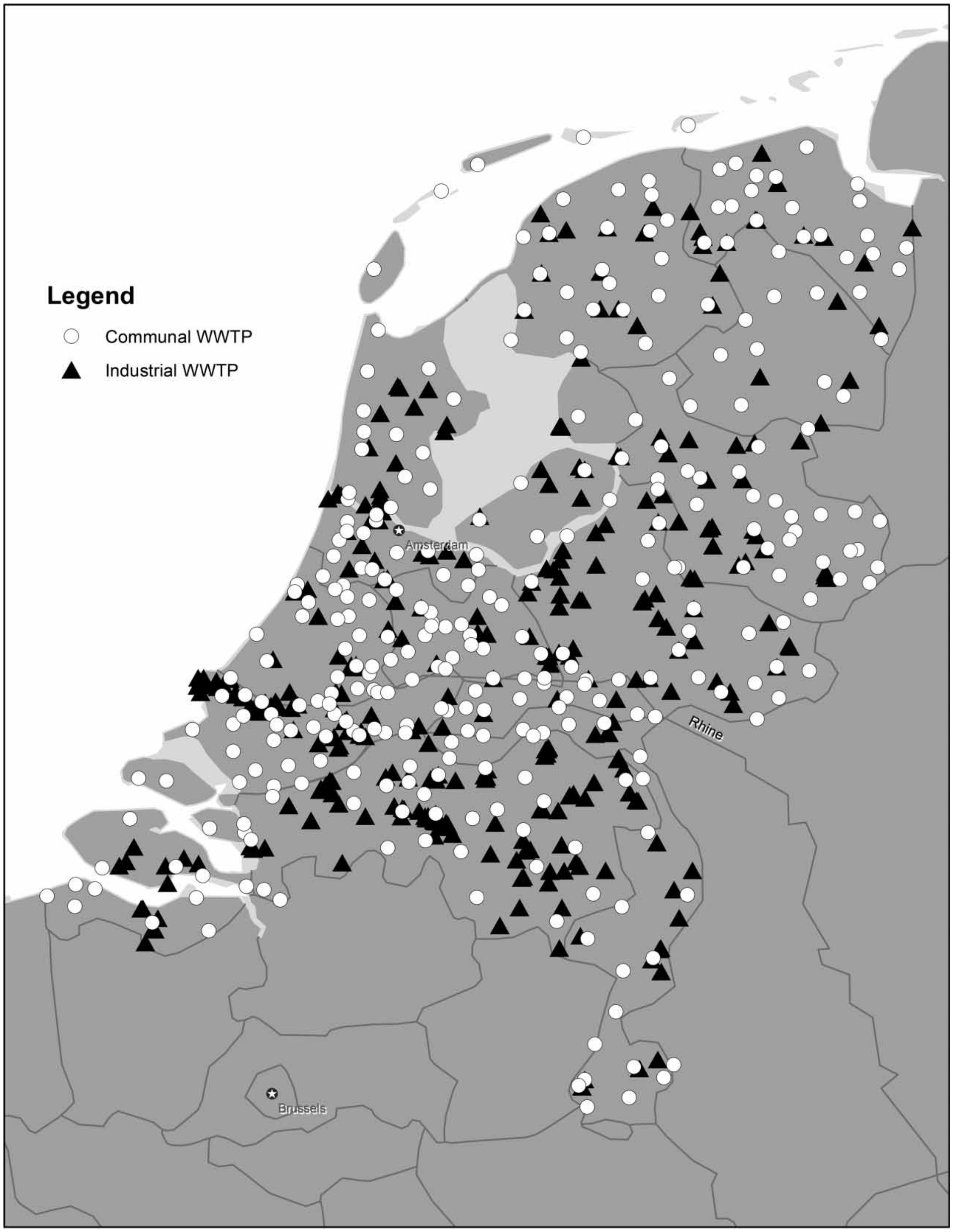


Figure 1 | Overview of communal (white dots) and industrial (black triangles) WWTPs in the Netherlands, based on the inventory.

A risk assessment was done for all 778 WWTPs using our newly developed risk matrix (Table 1), based on the four risk criteria for *Legionella* growth and emission to air or effluent. A biological treatment process was part of all 327 cWWTPs and they belonged to the type of industries with organic/nutrient-rich wastewater. Aeration was used in all 327 cWWTPs during biological (aerobic) treatment. The temperature of the process water of 315 cWWTPs (96%) depended on the ambient temperature and varied between 8 and 20 °C and these were identified as 'low' risk. Twelve cWWTPs (4%) (partly) operated between 30 and 38 °C for optimal operation in the conversion of ammonium and nitrite into nitrogen gas using Anammox bacteria. These 12 cWWTPs were classified as 'high' risk (Table 2).

For iWWTPs, 219 out of 451 (49%) had a biological treatment process. The risk of the 232 non-biological iWWTPs was categorized as 'very low'. There were 90 (41%) biological iWWTPs that received wastewater from other industries. Insufficient information about the composition of the influent was available and no case study for these industries was described. Therefore, no risk could be estimated and these 90 iWWTPs were classified as 'unknown' risk. More than half of the biological iWWTPs (59%, $n = 129$) belonged to the selected industries with organic/nutrient-rich wastewater: food industry ($n = 101$), petrochemical companies ($n = 10$), wood and paper industry ($n = 9$), rendering companies ($n = 1$), and (partly) communal wastewater ($n = 8$). For three out of these 129 iWWTPs, the risk for *Legionella* growth and emission could not be assessed (unknown) as the temperature of the process water was not reported. The risk for *Legionella* growth and emission was categorized as 'low' for 45 out of these 129 iWWTPs with a temperature of the process water not in the range of 25–45 °C. For 81 out of these 129 iWWTPs, the temperature of the process water was listed between 25 and 45 °C. For 18 iWWTPs, the temperature of the process water range was reported to be completely within the range of 30–38 °C, and therefore, the risk for *Legionella* growth and emission was categorized as 'high'. For the remaining 63 iWWTPs with a biological treatment process, the water temperature was reported as between 25 and 45 °C or only indicated as elevated temperature. Therefore, the risk was estimated as 'moderate to high' (Table 2).

For some locations, the requested information was not specified, incomplete or not correct. At some locations, for example, the reported temperature of the process from the inventory differed from the measured water temperature during sampling. If data is not correctly reported, this might have a negative effect on the risk assessment and WWTPs with a 'moderate' to 'high' risk might be assessed lower. Compared to cWWTPs for iWWTPs, it was more complicated to collect this information as a central registration of iWWTPs does not exist. To provide more information on the role of *Legionella* growth and emission from WWTPs and to assess the risk, an accurate overview of all WWTPs and their characteristics should be made available and kept up-to-date.

Detection of *Legionella*

Together with STOWA, the Dutch Water Authorities responsible for all cWWTPs developed voluntarily their own sampling strategy for high risk cWWTPs and were not included in this study. The *Legionella* detection focused on iWWTP classified as 'moderate to high' or 'high' risk (Table 2). In total, 81 iWWTPs were selected for sampling and analysis of which 63 iWWTPs with 'moderate to high' risk and 18 iWWTPs with 'high' risk. At the request of the environmental agencies, 11 iWWTPs were added to the final selection ending up with 92 iWWTPs. Finally, 85 of the 92 iWWTPs were sampled and analyzed for the detection of *Legionella* (Table 3). At seven iWWTPs, no samples could be taken due to maintenance, miscommunication, or problems during sampling.

During sampling, the water temperature sometimes differed from the reported information at the inventory. The water temperature of eight iWWTPs during sampling was below 25 °C compared with the reported temperature range of 25–40 °C, resulting in a lower risk than expected. One iWWTP that was added at the request of the environmental agency, had a temperature of 33 °C, but had been reported as below 25 °C in the inventory and was therefore originally not included in the

Table 2 | Overview of the number of WWTPs in the Netherlands with the risk of *Legionella* growth and emission to air or surface waters based on the reported characteristics in the inventory

	Number of WWTPs	
Risk of *Legionella* growth and emission to air and/or surface waters	**Communal**	**Industrial**
High	12	18
Moderate to high	0	63
Low	315	45
Very low	0	232
Unknown: missing information or no case studies available	0	93

Preventive measures to prevent the emission of *Legionella* were not taken into account.

Table 3 | Overview of the results of *Legionella* detection in iWWTPs categorized on the risk level

Risk level	Measured temperature of process water (°C)	Number iWWTPs sampled (*Legionella* positive iWWTP) per type of industry: Food industry	Petrochemical company	Wood and paper industry	Rendering company	Non-specific WWTP	Sludge and manure	Total
Low	<25	9 (0)	1 (0)					10 (0)
Moderate	25–29	13 (1) 1: L.p sg5	3 (1) 1: L.spp	2 (2) 1: L.p 7–14 2: L.p 7–14		3 (1) 1: L.p sg1		21 (5)
High	30–38	32 (3) 1: L.p sg1 and L.p sg2 2: L.p sg3 and L.p sg5 3: L.p sg2	5 (2) 1: L.p sg1 and L.p sg5 2: L.p sg5	6 (2) 1: L.p sg1 2: L.p sg6	1 (1) 1: L.p sg1	3 (0)		47 (8)
Moderate	39–45	3 (0)						3 (0)
Unknown	30–38					1 (0)	2 (2) 1:*.L. bozemanii* 2: L.p sg3 and L.p sg6	3 (2)
Total		57 (4)	9 (3)	8 (4)	1 (1)	8 (1)	2 (2)	85 (15)

The number of sampled iWWTPs is given per industry and the number of *Legionella* positive iWWTPs is shown between brackets. For the *Legionella* positive iWWTPs, the species including serogroup is given per positive iWWTP. L.p = *Legionella pneumophila* and sg = serogroup.

selection of high risk iWWTPs. iWWTPs were categorized based on the registered temperature of the water during sampling and was used for assessing the risk level (Table 3).

In this study, we found *Legionella* spp. 15 iWWTPs (18%). At 13 locations (15%), *L. pneumophila* was detected and at two locations *Legionella* non-*pneumophila* was found of which at one location it was determined as *L. bozemanii* (Table 3). Lund *et al.* (2014) found comparable results: 21 out of 130 analyses (16%) from iWWTPs samples were positive for *Legionella* spp. and 12 (9%) were positive for *L. pneumophila* (Lund *et al.* 2014). The concentrations for *Legionella* varied between 1×10^5 and 3×10^8 CFU/L which was similar to concentrations found in other studies (Olsen *et al.* 2010; Loenenbach *et al.* 2018).

Serotyping of *L. pneumophila* showed a diversity of serogroups and in five iWWTPs serogroup 1 was found (Table 3). *L pneumophila* serogroup 1 is the causative agent for most of the patients with diagnosed LD in the Netherlands (Reukers *et al.* 2019). Sequence typing of *L. pneumophila* serogroup 1 gave the following STs: ST47, ST474, ST1095, and ST1646. One iWWTP sample contained both ST1095 and ST1646. Notable, one *L. pneumophila* serogroup 1 isolate was ST-type 47. This virulent strain is found in 41% of the clinical isolates of Dutch non-travel-associated patients (Den Boer *et al.* 2015). Although this ST-type has also been detected in spa pools and soil (Schalk *et al.* 2014), it was not found in other water samples during 10 years of systematic sampling of potential sources (Den Boer *et al.* 2015).

The positive *Legionella* results were directly communicated to the responsible OD for follow-up with the owner of the iWWTP and investigated if additional control measures are required to protect public health. When *Legionella* is detected in the wastewater, it is recommended to take action to prevent emission to air or discharge on surface waters and, if possible, reduce the *Legionella* concentration. The first step is treating biological systems for selectively reducing *Legionella* without interfering with the biological process in the system, such as adding an 'Expanded Granular Sludge Blanket' reactor (Nogueira *et al.* 2016). If reduction of *Legionella* is not possible, then minimize the emission of *Legionella* from the WWTP. To reduce emission from WWTPs to air, the aeration basins could be covered with a tarpaulin or floating balls although the effect depends on how well the surface is covered (Lodder *et al.* 2019). When the air was extracted from closed aeration basins and subsequently filtered and disinfected with UV radiation the number of *Legionella* bacteria in the air decreased dramatically (Lodder *et al.* 2019). An additional treatment of effluent water, such as (membrane) filtration or UV treatment, could be applied to improve the water quality of the receiving surface waters (Collivignarelli *et al.* 2018).

ISO 11731: 2017 is often used for determining the number of *Legionella* bacteria in water, but when applied to wastewater, this method can give a wide variation of results (ISO 2017). Due to high concentration of other (interfering) microorganisms and the high detection limit for *Legionella* (approximately 10,000 CFU/L), it is rather difficult to detect *Legionella* bacteria in highly polluted wastewater. The sample pre-treatment conditions for wastewater are harsh and can affect *Legionella*'s cultivability (Whiley & Taylor 2016). Furthermore, culture-based methods such as ISO 11731 are not able to detect *Legionella* in the viable but non-culturable (VBNC) state (Whiley & Taylor 2016, Caicedo *et al.* 2019). This leads to an underestimation of the real *Legionella* concentration in the sample (Whiley & Taylor 2016). *Legionella* bacteria in the VBNC state can grow under the correct conditions. If *Legionella* cannot be detected in a wastewater sample with the used method, this means that no viable *Legionella* or less than 10,000 CFU/L *Legionella* bacteria are present in the sample. The iWWTPs in our study were sampled only once by taking a grab sample in the months of June and July. It is unknown how many WWTPs false negative results were obtained because of the high detection limit or because of other factors like the time period when samples were taken. The consequence of false negative results can be that no control measures are taken or control measures might appear more effective than they really are (Caicedo *et al.* 2019). Using molecular techniques, *L. pneumophila* and *Legionella* spp. are more frequently detected as compared with culture methods (Medema *et al.* 2004; Lund *et al.* 2014). Lund *et al.* (2014) investigated various industrial and communal WWTPs for the presence of *Legionella* and found that most samples (99%) were *Legionella* spp. positive by PCR, whereas *Legionella* could be detected less frequently (16%) using culture methods (Lund *et al.* 2014). We found similar results in our study (data not shown). The benefits of PCR are rapidly available results and the detection of *Legionella* bacteria in the VBNC state. The PCR method does not differentiate between alive or dead *Legionella* bacteria, and therefore, no information is gathered on the viable *Legionella* bacteria present (Caicedo *et al.* 2019). However, based on the limitations of the culture method for wastewater and the high detection rates with molecular techniques, the number of *Legionella* positive iWWTPs in this study is expected to be underestimated.

The majority of sampled iWWTPs (67%) processed wastewater from food industries whereas only one iWWTP from a rendering company was sampled. In all different types of industry, at least one iWWTP tested positive for *Legionella* varying between 7 and 100% *Legionella* positive iWWTPs per industry, which is in line with other published studies (Blatny *et al.* 2008; Kusnetsov *et al.* 2010; Loenenbach *et al.* 2018; Caicedo *et al.* 2019). In addition to industries with organic/nutrient-rich process water as described in the literature, other industries may also produce nutrient-rich wastewater, which promotes *Legionella* growth (Caicedo *et al.* 2019). In this study, two industries treating manure and sludge were *Legionella* positive but were not included as high risk industries. Furthermore, industries without nutrient-rich wastewater may sometimes receive nutrient-rich wastewater from other companies for processing in their WWTP. This may be the case for both cWWTPs and iWWTPs. Therefore, it is recommended to assess the risk for other industries, especially if nutrient-rich water is expected. If other industries should be included the risk matrix needs to be adapted.

The process water of the 15 *Legionella* positive samples had a temperature between 25 and 29 °C ($n = 5$) or 30 and 38 °C ($n = 10$). Similar to other studies, all *Legionella* positive samples were derived from iWWTP with process water temperatures between 25 and 38 °C (Blatny *et al.* 2008; Kusnetsov *et al.* 2010; Maisa *et al.* 2015; Loenenbach *et al.* 2018). This indicates that the selected range is an important criterion with increased risk. The percentages of *Legionella* positive iWWTPs with water between 25–29 °C and 30–38 °C were respectively 24 and 17%. Ten iWWTPs with temperatures of the process water below 25 °C and three iWWTPs with a temperature of the process water above 39 °C tested negative for the detection of *Legionella* spp. (Table 3). Based on the findings, we would suggest to divide the process water temperature below and above 25 °C. Furthermore, if WWTPs with a water temperature below 25 °C receive wastewater with high *Legionella* concentrations this may also result in high concentrations in the aeration tanks, although growth will be limited. *Legionella* growth does not only depend on the water temperature. There may also be circumstances where the temperature is stratified in the basin or changes temporarily, so the concentration could increase or decrease over time (Caicedo *et al.* 2016).

The risk matrix for assessing the risk of *Legionella* growth and emission from WWTPs was based on available information in the literature. New data might lead to an addition to or change in risk criteria.

For WWTPs with 'moderate' to 'high' risk of *Legionella* growth and emission, it is needed to check the existing measures in place. Existing control measures might contribute to a lower risk of *Legionella* growth or reduce the concentration of *Legionella* are a prolonged anaerobic step or treating effluent with an extra purification step, e.g. UV or additive biocides (Nogueira *et al.* 2016; Collivignarelli *et al.* 2018). Control measures to reduce the emission of *Legionella* include covering the aeration tank with concrete, tarpaulin or balls, and use of pure oxygen instead of normal aeration (Caicedo *et al.* 2019). These control measures aim to limit or prevent the emission of *Legionella*. However, when the high concentration of *Legionella* in the WWTP remains and in case of a hazardous event, it may still spread to the environment. When treated effluent or surface waters receiving treated effluent containing *Legionella* is used in cooling towers or for other activities, aerosols with *Legionella* can be spread (Nogueira *et al.* 2016). Due to water scarcity re-use of treated wastewater becomes an important alternative water source. For aerosol-forming applications such as irrigation and cooling towers, the presence of *Legionella* in treated wastewater might pose a health risk (Caicedo *et al.* 2019). Regulation of *Legionella* in effluent water used for application with a risk of aerosolization is needed to protect human health, such as the requirement of a maximum of 1,000 *Legionella* CFU/l in the EU regulation (EU 2022).

For high risk WWTPs, it is recommended that both operational monitoring of the WWTP is initiated and a management plan is developed. More research on control measures is needed to identify how *Legionella* growth and emission could be controlled. For existing WWTPs, it is recommended to adjust the treatment process so that *Legionella* growth is prevented or at least controlled as much as possible similar to *Legionella* prevention in wet cooling towers. If *Legionella* is present in high numbers it is recommended to reduce the concentration and avoid emission of *Legionella*, especially via air. A sustainable solution for new WWTPs is to design a process that limits the growth and/or emission of *Legionella*. To support owners of WWTPs to identify, interpret, and control *Legionella* risks, a guidance document was drafted using the findings of this study (Oesterholt & Hollebekkers 2022).

To improve the risk assessment for the emission of *Legionella* to air or surface water from WWTPs, existing measures and their local application to prevent the emission of aerosols should be taken into account.

CONCLUSION

Biological WWTPs are possible sources of LD and need to be considered during source investigation. In this paper, a risk assessment methodology was drafted to identify WWTPs at risk of *Legionella* growth and emission. The risk matrix is based on information about the WWTPs that is relatively easy to identify, and therefore, this approach is likely applicable for many countries to identify possible sources for *Legionella* emission from WWTPs. To provide more information on the role of *Legionella* growth and emission from WWTPs and to assess the risk, an accurate overview of all WWTPs and their characteristics should be made available and kept up-to-date.

In this study, 18% of the iWWTPs with moderate to high risk for growth and emission of *Legionella* were *Legionella* spp. positive, and two iWWTPs with unknown risks were positive. The true proportion of *Legionella* positive WWTPs may be even higher, as the culture method does not detect low concentrations of *Legionella* in wastewater. Based on the risk assessment of WWTPs and *Legionella* spp. detected with culture methods revising the risk matrix based on new scientific evidence and information on *Legionella* outbreaks is needed. Additional research is needed to further improve the risk matrix. Nevertheless, the current risk matrix can be used to develop a risk-based monitoring program starting at WWTPs with the highest risk of *Legionella* emission.

For WWTPs with a high risk, it is recommended that both operational monitoring of the WWTP is initiated and a sampling plan is drawn up and implemented, a so-called management plan. For assessing the risks for WWTPs, it is crucial that the location of such installations is known, therefore registration of WWTPs is recommended. For WWTPs still to be built or when new treatment technologies are installed, it is recommended to guarantee that *Legionella* growth is prevented or at least controlled as much as possible.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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