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Sea water whirlpool spa as a source of *Legionella* infection

Dijana Tomic Linsak, Darja Kese, Dalibor Broznic, Darija Vukic Lusic, Arijana Cenov, Milan Moric and Ivana Gobin

ABSTRACT

Bacterial pneumonia caused by the inhalation of aerosols contaminated with *Legionella* spp. is also known as Legionnaires' disease. In this study, we report a case of pneumonia caused by *Legionella pneumophila* sg.1 in a 58-year-old man who visited a sea water-filled whirlpool within a hotel and spa complex. The patient's *Legionella* urine antigen test was positive for *L. pneumophila* sg.1. During the field study, samples were taken from both the outdoor and indoor sea water-filled pools. Samples from the whirlpool were culture positive for *L. pneumophila* sg.1. Typing results indicated sea water isolate belonged to Sequence type ST82 and Allentown/France MAb subgroup. *In vitro* experiments showed that *L. pneumophila* strains are able to survive within sea water up to 7 days, and survival time is prolonged with sea water dilution. Also, our results indicate that *L. pneumophila* Allentown strain was the most resistant to adverse conditions in sea water with the highest values of DT50 (420 min) and DT90 (1,396 min). The possible source of infection was adding potable water for filling up the whirlpool. The survival of the *L. pneumophila* in additionally conditioned sea water should be considered in a further study.

Key words | genotypic investigation, LD90, *Legionella pneumophila*, legionellosis, sea water

HIGHLIGHTS

- Sea water in spa resorts may act as an environmental reservoir for *Legionella* spp.
- Sea and brackish water are suitable media for the survival of the *Legionella pneumophila.*
- *In vitro* experiments show that most *Legionella* spp. can survive in sea water up to 7 days.
- *L. pneumophila* Allentown strain shows better survival ability in seawater compared to other strains.
- The possible source of infection was filling up the whirlpool with potable water.

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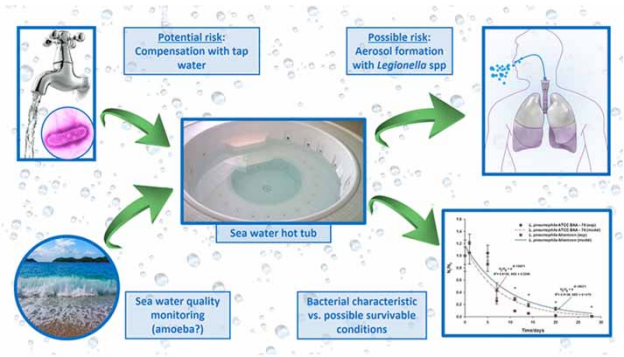
GRAPHICAL ABSTRACT



INTRODUCTION

Legionnaires' disease (LD) is a pneumonia-like infection caused by *Legionella pneumophila*, and it was first identified in an outbreak of LD in 1976 (McDade *et al.* 1977). *Legionellae* are gram-negative bacteria known as inhabitants of both natural and man-made aquatic environments such as cooling towers, ships, factories, homes, hotels and spas, fountains, and respiratory therapy equipment (Bartram *et al.* 2007; WHO 2007). However, only a few studies reported that sea water may also act as a potential reservoir for *Legionella* spp. (Ortiz-Roque & Hazen 1987; Palmer *et al.* 1993). Moreover, *Legionella*, especially *L. pneumophila*, was considered unable to survive or persist in saline waters. Furthermore, *in vitro* studies showed that higher concentration of NaCl was critical for the *Legionella* survival (Catrenich & Johnson 1989). However, a study by Heller *et al.* (1998) showed that the sensitivity of *Legionella* to NaCl depends on the temperature of the medium, namely the higher the temperature, the higher the sensitivity of bacteria. Also, *L. pneumophila* could be recovered from natural Baltic and North Sea sea water with 15 and 30‰ salinity, even after 7 days of inoculations (Fields *et al.* 2002).

Another study also suggests that *Legionella* in saline environments, such as coastal marine sites and estuaries, could persist in viable forms (Gast *et al.* 2011). The presence of estuarine and coastal water may be variable due to terrestrial runoff. The microbial content of these environments may also be greatly influenced by freshwater or soil impact. Marine environment positive for *Legionella* has most likely been contaminated by wastewater. The presence of *Legionella* in these environments may be a transient result of such freshwater or soil impact rather than multiplication within the marine environment.

These results strongly suggest that *Legionella* spp. could survive in saline environments, such as coastal marine sites and estuaries. Another factor that could facilitate the survival of *Legionella* in sea water are amoebae (Gast *et al.* 2011). Zbikowska *et al.* (2013) showed coexistence and interactions between free-living amoebae (Naegleria and Hartmannella) and *L. pneumophila* in thermal saline baths used in balneotherapy. *Legionella* are known to be amoebae parasites and their relationship has been studied extensively in freshwater environments (Abu Kwaik 1998; Fields 1998). Although contradictory, research shows the possible presence of amoebae in sea water, so there is a risk of their presence in recreational sea water environments (Tsvetkova *et al.* 2004).

Bacterial survival in a particular environment usually involves numerous complex reactions whose mechanism can be determined by mathematical modelling. It requires the input of experimentally obtained data that are processed by non-linear estimation. The model most commonly used to describe bacterial survival kinetic is the Single First-Order Kinetic Model whose inhibition rate constant is a function of environmental variables (Xiong *et al.* 1999). To give a more detailed account of environmental impact on the bacterial survival process, a more complex model such as

Biphasic First-Order Non-linear Model can be used (Hellweger *et al.* 2009; Roth *et al.* 2010; Phaiboun *et al.* 2015; Brouwer *et al.* 2017). This model assumes that the bacterial population consists of two subpopulations which can react by different inhibition rates during the exposure to environmental stressors.

The first aim of this study is to present the epidemiological and environmental investigation of a single case of LD in the northern Adriatic region after using a whirlpool filled with sea water. The second aim is to evaluate the kinetics and mechanism of bacterial inactivation in different environmental media using mathematical modelling.

MATERIALS AND METHODS

Patient characteristics

A 58-year-old man, who was a smoker, stayed in an apartment complex within a hotel and spa in the northern Adriatic region from 9–23 August 2016. He had no history of respiratory illness in the preceding weeks. During his stay at the hotel, he used the apartment shower as well as the whirlpool bathtub several times. The whirlpool is situated in the spa area. On 29 August 2016, he was admitted to the hospital with flu-like symptoms and a fever. At the time of admission, a radiological investigation showed bilateral infiltration of multiple lobes. A urine sample was obtained from the patient for the investigation of *Legionella* infection.

Hotel characteristics

The hotel was completely renovated in 2006 when the hotel's spa facilities were converted to a sea water system. At first, the whirlpool tub was not considered to be the point source of infection since it was filled with sea water. The apartment has an electric hot water heater with a tank capacity of 80 L and is also equipped with a newly installed air-conditioner without a water-cooling system. The spa zone with a pool, the whirlpool is situated close to the apartment building. All of them have sea water additionally conditioned with pH and chlorine chemicals. On-site investigation conducted by the public health service showed that the whirlpool was filled with sea water from a nearby pool. A pump fills the whirlpool tub with sea water and afterwards that water is conditioned with chlorine and pH chemicals. Regular sampling has been done every month for testing microbiological and chemical parameters. In the case of evaporation, the whirlpool water is compensated by adding water from the nearby tap. The schematic diagram of the whole installation is shown in Figure 1. Volumetric data of the pool and whirlpool located in the spa zone are as follows: Pool (width: 12 m; length: 16.8 m; height: 1.4 m; volume: 282 m^3; pool sea water temperature: 25 °C), Whirlpool (accommodates 10 persons; diameter: 2.93 m; volume: 2 m^3; whirlpool sea water temperature: 28 °C).

After the official authority reported a single LD case, the epidemiological team conducted an on-site investigation by collecting samples from the apartment where the guest stayed (water samples from the shower), as well as from the pool, whirlpool tub in the spa zone.

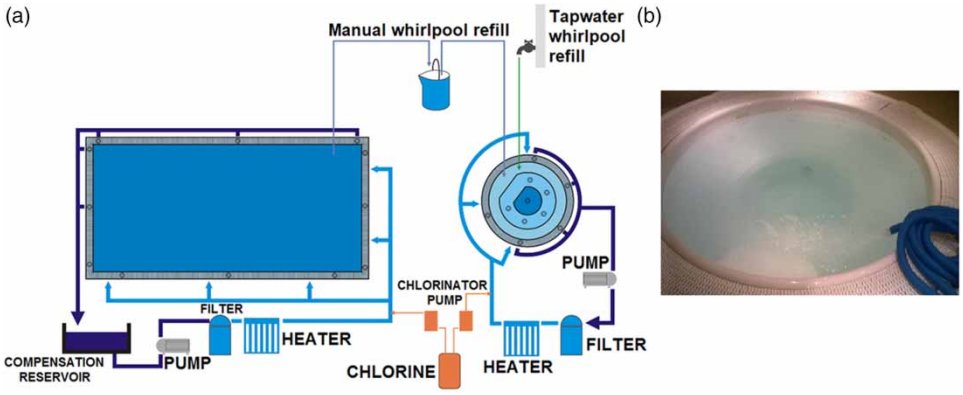


Figure 1 | A schematic diagram of a swimming pool and whirlpool (a); Manual filling of the whirlpool with sea water (b).

Microbiological investigations

Patient urine was tested for *Legionella* antigen using BinaxNOW and Binax Legionella Urinary Antigen EIA (Alere-Abbott, Scarborough, USA). *L. pneumophila* sg.1 was determined by a DFA reagent (Pro-Lab, Richmond Hill, Canada), following the manufacturer's instructions.

Isolation of *L. pneumophila* from environmental samples was performed in accordance with the standard HRN EN ISO 11731: 2000. The grown colonies were identified by using a latex agglutination test (Pro-Lab Richmond Hill, Canada).

In our study, we also wanted to check the microbiological and chemical parameters of tap water in the hotel and sea water in the spa zone. Examined parameters were tested in accordance with ISO methods, which are presented in Table 1.

Pseudomonas aeruginosa and *Escherichia coli* were tested in accordance with ISO methods EN ISO 16266:2008 and EN ISO 9308-1:2014 (Water quality), respectively. *Staphylococcus aureus* was determined using APHA Standard Methods for the Examination of Water and Wastewater (Standard Methods).

Phenotypic and genotypic investigation of *Legionella pneumophila*

Since *Legionella* was not isolated from the patient, only environmental *L. pneumophila* isolate was sent, under special transport conditions, to the Institute of Microbiology and Immunology, MF Ljubljana, Slovenia, for phenotype and genotype identification. Isolate was tested by PCR using the r-gene *L. pneumophila* primers/probe premix (ARGENE, France) and further determined *L. pneumophila* sg.1 by 3plex qPCR (Mentasti *et al.* 2015).

Isolated *Legionella* strain was typed using Dresden Panel of Monoclonal antibodies (MAb) to determine the phenotype subgroup of *L. pneumophila* sg.1 (Lück *et al.* 2013). The environmental isolate was further genotyped using the standard sequence-based typing (SBT) scheme according to the protocol of European Working Group for Legionella Infections (EWGLI database) using seven gene targets, namely flaA, pilE, asd, mip, mompS, proA and neuA (Gaia *et al.* 2005; Ratzow *et al.* 2007). Sequence type (ST) was determined with the sequence quality tool.

Table 1 | Parameters and methods used in tap and sea water examination

Parameter	Method
Air temperature	Alcohol thermometer with graduation intervals of 0.1 °C
Sample temperature	Alcohol thermometer with graduation intervals of 0.1 °C
Free chlorine	EN ISO 7393-2:2018 (Water quality – Determination of free and total chlorine – Part 2: Colorimetric method using *N,N*-diethyl-1,4-phenylenediamine for routine control purposes; quantification limit 0.02 mg Cl_2/L)
Colour	Standard Methods 23rd Ed. 2017. 2120 C. APHA, AWWA, WEF (2017) (Determination of colour; quantification limit 5 units Pt/Co scale)
Turbidity	EN ISO 7027-1:2016 (Water quality – Determination of turbidity; quantification limit 0.10 NTU)
pH	EN ISO 10523:2012 (Water quality – Determination of pH)
Conductivity	EN 27888:1993 (Water quality – Determination of electrical conductivity; quantification limit 9 μS/cm)
$KMnO_4$ consumption	EN ISO 8467:1995 (Water quality – Determination of permanganate index; quantification limit 0.25 mg O_2/L)
Trihalomethanes	EN ISO 10301:1997 (Water quality – Determination of highly volatile halogenated hydrocarbons – Gas-chromatographic methods; quantification limit Chloroform: 0.75 μg/L, Bromodichloromethane: 0.75 μg/L, Dibromochloromethane: 0.75 μg/L, Bromoform: 0.75 μg/L)
Salinity	Standard Methods 23rd Ed. 2017. 2520 B. APHA, AWWA, WEF (2017) (Determination of salinity – conductometry method; range 0–40‰)

In vitro studies

The ability of *Legionella* to survive in sea water was examined *in vitro*.

Water samples

Each of the water samples was collected in aliquot of 1 L and kept in a separately labelled bottle. The raw sea water

sample originated from the coast of the island of Lošinj, chlorinated sea water from the whirlpool and tap water from the public water supply of the island of Lošinj. Samples were tested for biochemical parameters and analysed for *Legionella* survival. Sea water samples were filter sterilised through a 0.45 μm CA Sterile Syringe Filter. For de-chlorination and sterilisation, the sampled tap water was sterilised by autoclaving (121 °C /15 min) and stored at 4 °C until use.

Bacterial strains

To determine the survival of *Legionella* in sea water, different strains of *L. pneumophila* and three other species of *Legionella* (*L. anisa*, *L. bozemanii* and *L. longbeachae*) were collected. The clinical isolate of *L. pneumophila* ATCC BAA-74 and *L. longbeachae* sg.1 strains were obtained by courtesy of Prof E. Hartland, PhD from the University of Melbourne, Australia. Other *Legionella* strains were obtained from the collection of the Institute of Microbiology and Immunology, University of Ljubljana, Slovenia (Table 2). The bacteria were stored at −80 °C in 10% glycerol broth. After culturing for 3–5 days on BCYE medium at 35 ± 2 °C, the bacteria were used in the experiments.

Table 2 | *Legionella* strains used in *in vitro* studies

Legionella species	Strain	ST	Source of isolation
L. pneumophila sg.1	Philadelphia	1	CS
	Philadelphia	37	CS
	Benidorm	1,299	CS
	Benidorm	1,299	WS
	Bellingham	728	CS
	Bellingham	334	CS
	Bellingham	728	WS
	Oxford	1	CS
	Oxford	1	WS
	Oxford	1	WS
	Allentown	82	SWS
L. pneumophila sg.1 (ATCC BAA-74)	Wadsworth		CS
L. pneumophila sg.8	Concord3	1,324	CS
L. pneumophila sg.9	Chicago2	421	CS
L. anisa	–	–	WS
L. bozemanii	–	–	WS
L. longbeachae sg.1	NSW150	–	CS
L. longbeachae sg.1	L6C9	–	SS

CS, clinical sample; WS, water sample; SWS, sea water sample; SS, soil sample.

Survival studies

Legionella strains grown on BCYE agar were collected, bacterial suspensions in sterile sea water were made. The OD_{600} (optical density) was determined and was about 0.1, which equalled 10^8 bacterial per mL. The number of bacteria was confirmed by inoculation of ten-fold dilutions on BCYE agar. Survival of different *Legionella* strains (Table 1) in sterile sea water was monitored on the 1st, 3rd and 7th days. The number of *Legionella* was determined after plating a series of ten-fold dilution on non-selective BCYE agar and incubating for up to 10 days at 35 ± 2 °C.

Furthermore, the survival dynamics of the two selected *Legionella* strains, *L. pneumophila* ATCC BAA-74 and *L. pneumophila* Allentown strain, in sterile sea water (SW), sterile tap water (TW) and sterile chlorinated sea water samples (cSW) was monitored for 28 days (1st, 5th, 7th, 10th, 14th, 20th and 28th day) at a temperature of 33 ± 2 °C. The initial bacterial count in the samples was 10^6 CFU/mL. In cSW samples, chlorine levels were maintained *in vitro* by the addition of Na-hypochlorite. The number of *Legionella* was determined at different points in time (0, 1, 5, 7, 14, 20 and 28 days after inoculation) on a non-selective BCYE agar. In addition, bacterial survival in sterile cSW samples diluted with sterile non-chlorinated tap water (1:1) was examined.

Kinetic analysis of bacterial activity inhibition

Bacterial activity inhibition of *Legionella* strains was estimated and analysed using the Single First-Order (SFM) and Double First-Order Exponential Biphasic (DFBM) kinetics models expressed by the following equations:

$$\frac{N_t}{N_0} = e^{-k \cdot t} \tag{1}$$

$$\frac{N_t}{N_0} = f \cdot e^{-k_1 \cdot t} + (1 - f) \cdot e^{-k_2 \cdot t} \tag{2}$$

where N_t, N_0, f and $(1–f)$ are bacterial concentrations at time t or at $t = 0$, the number of bacteria in the first and second phase of inactivation, respectively, while k, k_1 and

k_2 are inactivation rate constants. Time for 50 (DT50) and 90% (DT90) bacterial inhibition are also determined according to the following equations:

$$DT50 = \frac{\ln 2}{k} \quad (3)$$

$$DT90 = \frac{\ln 10}{k} \quad (4)$$

$$DT90_1 = \frac{\ln 10}{k_1} \quad (5)$$

$$DT90_2 = \frac{\ln 10}{k_2} \quad (6)$$

Statistical analysis

All experiments were performed in a minimum of five replications. The number of bacteria at different time intervals was presented by the mean ± standard deviations. Statistical analyses were performed using commercial software Statistica® v. 13.0 (StatSoft, Inc, Tulsa, OK, USA) at a significance level of $p < 0.05$, while modelling of bacterial inhibition was performed using the software package Wolfram Research Mathematica® Version 9.0 (Champaign, IL, USA). The normality distribution of inactivation rate constant for *Legionella* strains in sterile raw water, as well as the amount of *L. pneumophila* ATCC BAA-74 and Allentown strains in tap water, sea water, chlorinated sea water/tap water and chlorinated sea water at different time intervals, were tested using the Kolmogorov–Smirnov test. The normal distribution was not achieved, so nonparametric Mann–Whitney *U*-test was used. The agreement of the proposed mathematical models with the experimental data was estimated using the Pearson correlation coefficient (R^2) and the Standard Error of Estimate (SEE).

RESULTS

Table 3 shows the detection of *L. pneumophila* sg.1 confirmed in the sea water-filled whirlpool at a concentration of 6×10^2 (CFU/L). A potential source of infection was examined and a total of four samples were collected from the apartment shower, spa pool and whirlpool tub.

The environmental isolate of *L. pneumophila* sg.1 was typed as Allentown/France MAb subgroup. *L. pneumophila* sg.1 isolate was then successfully genotyped by SBT and complete allelic profiles for ST82 were obtained. Table 4 shows *L. pneumophila* sg.1 positive sample which was further processed for chemical analysis.

Based on the survival curves of three groups of *Legionella*, *L. pneumophila* water strains, *L. pneumophila* clinical strains and groups of other *Legionella* species, the reduction parameters *k*, DT50 and DT90 were determined (Table 5). Statistical analysis did not show a difference between water and clinical isolates of *L. pneumophila*. In the group of water isolates, *L. pneumophila* strain Allentown seems to be the most resistant to survival in sea water, with the highest values of DT50 (420 min) and DT90 (1,396 min). Among the clinical isolates of *L. pneumophila*, the *L. pneumophila* ATCC BAA-74 strain showed upward values of DT50 (235 min) and DT90 (781 min). Other types of *Legionella* are extremely sensitive and do not survive in sea water, so their DT50 and DT90 values are low.

Figure 2 shows the *L. pneumophila* inactivation *in vitro*, where NCFU/mL (*t*)/NCFU/mL (0) at different time intervals are presented together with the SFM or DFBM models. Statistically significant differences of bacterial inactivation were found only in TW (at 7th, 11th, 14th and 20th day) and SW (at 11th, 14th, 20th and 28th day) samples. Correspondence between experimental and data obtained through a model was evaluated by statistical indices

Table 3 | Microbiological parameters in samples from the site

Sample No.	Source	Sample specification	CFU/mL (22 °C)	*P. aeruginosa*	*E. coli*	*S. aureus*	*Legionella* spp.
1	Apartment	Tap water	12	Negative	Negative	Negative	Negative
2	Spa Pool	Sea water	5	Negative	Negative	Negative	Negative
3	Whirlpool	Sea water	25	Negative	Negative	Negative	*L. pneumophila* sg.1 Positive (600 CFU/L)
4	Jacuzzi	Sea water	8	Negative	Negative	Negative	Negative

Table 4 | Chemical analysis of collected samples (tap water, sea water and whirlpool sea water)

Parameter	Measuring unit	Whirlpool sea water sample[a]	Tap water sample	Raw sea water sample	Whirlpool sea water sample[b]
Air temperature	°C	28.0	26.0	26.5	24.0
Sample temperature	°C	34.7	18.0	24.7	32.0
Free chlorine	mg/L	0.90	0.05	ND	1.05
Colour	°Pt/Co	5	<5	ND	<5
Turbidity	NTU	0.28	2.6	ND	0.20
pH	pH unit	7.6	8.2	ND	7.1
Conductivity	μS/cm	10,780	398	52,400	52,900
$KMnO_4$ consumption	mg/L O_2	1.0	0.58	ND	0.7
Trihalomethanes	μg/L	94	ND	ND	95
Salinity	psu	ND	ND	36	ND

ND, not done.
[a]Sample was taken at the time of incident.
[b]Sample in which survival experiments were performed.
All chemical parameters were in accordance with regulations.

Table 5 | Reduction parameters (*k*, DT50 and DT90) and goodness of fit (R^2 and SEE) for the Single First-Order Kinetic Non-linear Model describing inactivation of *Legionella* strains in sterile raw sea water

Bacterial strain	k (day^{-1})	DT50 (min)	DT90 (min)	R^2	SEE
L. p. sg.1 Oxford WS	8.38	119	395	1.0000	2.07×10^{-5}
L. p. sg.1 Benidorm ST1299 WS	7.69	130	431	1.0000	4.12×10^{-5}
L. p. sg.8 Concord 3 ST1324 WS	5.35	187	620	1.0000	1.30×10^{-2}
L. p. sg.1 Bellingham ST334 WS	7.90	126	420	1.0000	3.81×10^{-5}
L. p. sg.1 Allentown ST82 SWS	2.37	420	1,396	0.9998	7.40×10^{-3}
L. p. sg.1 Philadelphia CS	8.20	122	405	1.0000	2.29×10^{-5}
L. p. sg.1 Oxford CS	8.46	118	392	1.0000	1.26×10^{-5}
L. p. sg.1 Philadephia CS	7.31	137	454	1.0000	9.75×10^{-5}
L. p. sg.6 Chicago 2 CS	7.36	136	451	1.0000	3.18×10^{-5}
L. p. sg.1 Bellingham ST334 CS	6.65	150	498	1.0000	3.00×10^{-4}
L. p. sg.1 Bellingham ST728 CS	8.23	121	403	1.0000	2.68×10^{-5}
L. p. sg.1 Benidorm ST1299 CS	6.50	154	510	1.0000	4.90×10^{-5}
L. p. sg.1 ATCC BAA-74 CS	4.25	235	781	0.9999	3.60×10^{-3}
L. bozemanii WS	477.69	2	7	1.0000	1.04×10^{-6}
L. anisa WS	156.63	6	21	1.0000	1.50×10^{-5}
L. longbeachae sg.1 NSW150 CS	211.67	5	16	1.0000	1.42×10^{-6}
L. longbeachae A5H5 SS	448.10	2	7	1.0000	1.20×10^{-6}

k, inactivation rate constant; DT50, time for 50% of bacterial inactivation; DT90, time for 90% of bacterial inactivation; R^2, Pearson correlation coefficient; SEE, Standard Error of Estimate. Experiments were performed in a minimum of five replications.

(R^2, SEE) and is presented in Figure 2 (and Supplementary Tables S6 and S7). Statistical indices showed that the SFM model was applicable to describe bacteria inactivation except for Allentown strain in TW media ($R^2 = 0.9521$ and SEE = 0.0716) and for both strains in cSW media ($R^2 = 1.00$ and SEE > 1.26×10^{-6}). According to the SFM model, the fastest inactivation for both *L. pneumophila* strains was observed in cSW/TW media, where *L. pneumophila*

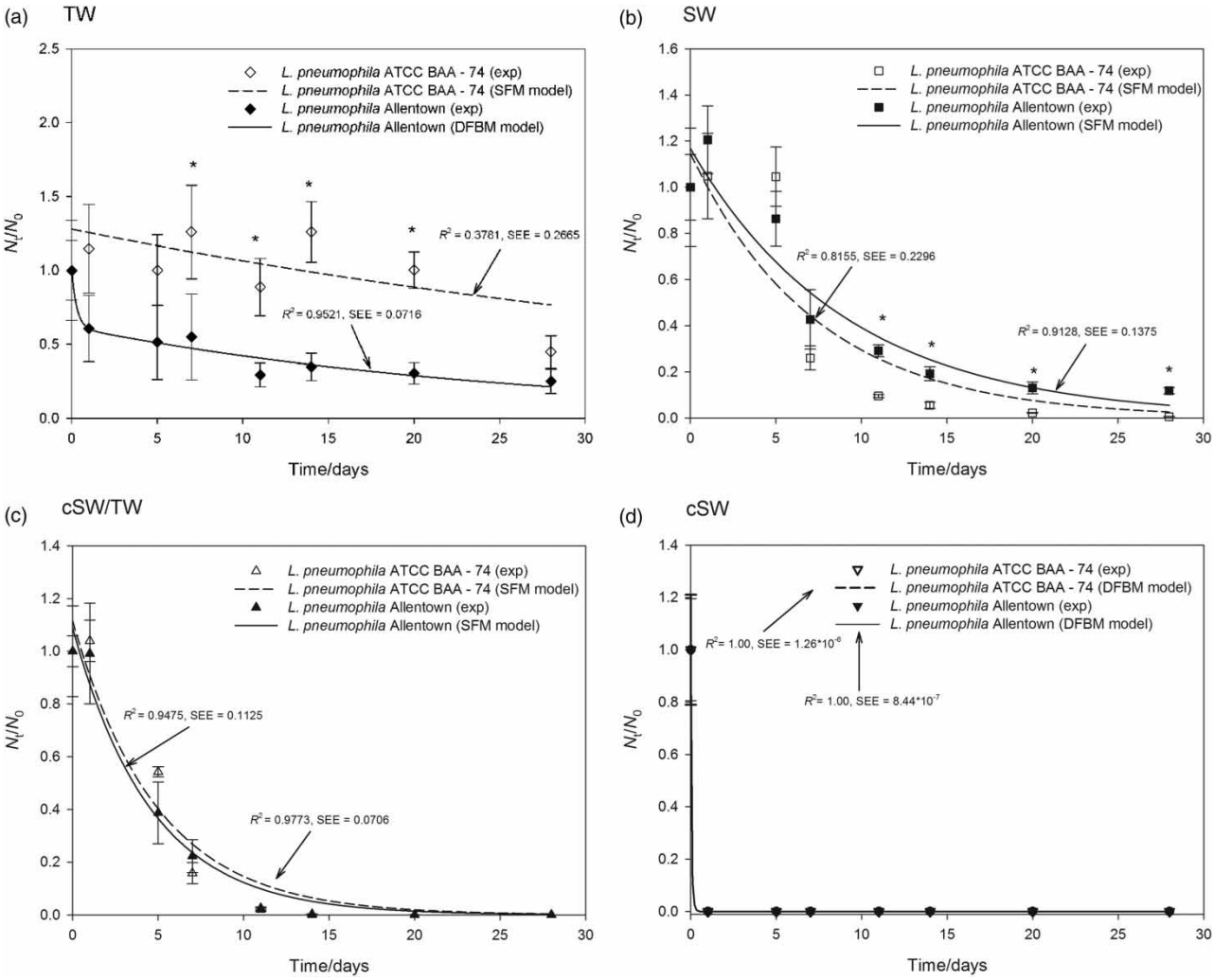


Figure 2 | Reduction of *L. pneumophila* ATCC BAA-74 and Allentown strains in (a) tap water (TW), (b) sea water (SW), (c) chlorinated sea water/tap water (cSW/TW, 50:50 ratio) and (d) chlorinated sea water (cSW). Symbols represent the experimental data, while lines represent the theoretical curves described by the Single First-Order (SFM) and Double First-Order Exponential Biphasic (DFBM) kinetics mathematical models. Data are expressed as mean ± standard deviation (at least five replicates).

Allentown strain was inactivated with a higher reaction rate constant ($k = 0.2182\ \text{day}^{-1}$) compared with *L. pneumophila* ATCC BAA-74 strain ($k = 0.2037\ \text{day}^{-1}$). The analysis of sensitivity of bacterial strains to TW and SW media showed that both strains were inactivated with a higher rate constant in SW ($k = 0.1360$ and $0.1092\ \text{day}^{-1}$ for ATCC BAA-74 and Allentown, respectively) than in TW media ($k = 0.0183$ and $0.075\ \text{day}^{-1}$ for ATCC BAA-74 and Allentown, respectively). Furthermore, the time for the 50% (T50) and 90% bacterial inhibition (T90), respectively, was also analysed (Supplementary Table S6). The shortest time for both bacteria was achieved in the reactions of both bacteria with cSW/TW media (T50: 3.4 and 3.2 days; T90: 11.3 and 10.6 days for ATCC BAA-74 and Allentown, respectively). In chlorinated samples, cTW and cSW, bacteria were not cultivable within 24 h (data not shown for the cTW sample).

DISCUSSION

The risk of illness or infection associated with recreational water is primarily associated with faecal contamination of the water. Environmental waterborne bacteria such as *Pseudomonas* and *Legionella* increasingly attract our attention. Most cases of legionellosis have been associated with exposure to recreational waters, such as in hot tubs and natural spas.

Most of the recreational water-associated legionellosis has been associated with hot tubs and natural spas. Such facilities create an ideal habitat (warm, nutrient-containing aerobic water) for the proliferation of these bacteria. Most recreational water guidelines focus on freshwater, while there are no precise recommendations for recreational sea water. Although it is already known that *Legionella* can survive in brackish and salt water, we did not find data on sea water whirlpool bathtubs as a source of *Legionella* infection (WHO 2006).

This study describes a case of LD in a person who was exposed to sea water in an indoor whirlpool spa. A male patient aged 58, with no pre-existing health conditions, was admitted to hospital on 29 August 2016 with radiologically confirmed pneumonia. His urine tested positive for *L. pneumophila* antigen. Namely, after the outbreak, the patient returned to his place of residence where he was diagnosed with legionellosis. We were unable to obtain *Legionella* isolates from the patient because the patient already received treatment and was recovering. The county epidemiological service was notified on 1 September 2016.

The patient was discharged from the hospital a week later, and the case was reported to the Croatian Institute of Public Health as an LD case. No other cases of the disease were reported at that time.

The use of whirlpools and hot tubs in spa facilities has been recognised as a particular risk factor for infection (Leoni *et al.* 2018). Bouwknegt *et al.* (2013) estimated that the risk of infection after a 15-min stay in an active whirlpool ranged from 3% up to >95% for *L. pneumophila* (bacterial count of 10 and ≥1,000 CFU/L, respectively). These findings suggest that the risk of infection is possible even at very low concentrations. *L. pneumophila* ST82 is not such a common strain in Europe or elsewhere either according to the ESGLI *L. pneumophila* SBT database. Interestingly, a small, suspected outbreak of LD caused by *L. pneumophila* ST82, subgroup Allentown/France was reported in Denmark, in the North Zealand region, between July and November 2014 (Schjørring *et al.* 2017). It has been shown that the Allentown strain is more frequently associated with ST47, which is a common cause of infection in Belgium, United Kingdom, France and the Netherlands (Harrison *et al.* 2009) but not in Germany or in Israel (Moran-Gilad *et al.* 2014).

In addition to microbiological analysis, at the time of the incident, various water samples listed in Table 3 underwent further chemical analysis. According to the results for the conductivity parameter of whirlpool sea water sample, we drew the conclusion that a lower value shows that sea water was diluted with added potable water. The usual conductivity of sea water is a far higher value as it was measured at the time of an incident, and we assume that this is a crucial finding. Additionally, the raw sea and whirlpool sea water samples that we used in the survival experiments were chemically characterised.

Recent studies have explored sea water as a potential environmental reservoir of *Legionella* spp. which strongly suggests that *Legionella* could persist in saline water environments, like coastal marine sites and estuaries (Mezrioui *et al.* 1995; Heller *et al.* 1998; Sinton *et al.* 2007; Gast *et al.* 2011). One of the most useful and commonly measured water quality parameters of sea water is conductivity. Furthermore, conductivity in a water system is an early indicator of chemical changes (Fondriest 2014). Findings from this investigation indicated that on the whole spa pool facilities should be expected to have pure sea water with the usual conductivity of over 45,000 µS/cm. Conductivity parameter from whirlpool sea water contaminated with *Legionella* is rather low and amounts to 10,780 µS/cm which indicates that freshwater has been added to this particular sea water bathtub. At the beginning of the epidemiological investigation, this information was not presented to the team experts, which is why an additional sample of added freshwater was not taken for analysis at the most important period of time (before the measurements were implemented). Further to the foregoing, we could assume that the source of *Legionella* was tap water used to fill up sea water within the whirlpool. Additionally, we should state that guidelines for levels of chlorine in tap water and those for recreational water differ. Therefore, it was expected that chlorine levels in tap water are significantly lower, so additional chlorine adjustment should have been performed after adding tap water to the whirlpool. Knowing that, as well as the fact that this tap is not used often, it is possible that after the pipe was flushed, *Legionella* ended up in the pool, and we could not subsequently prove it in tap water by way of the classical method.

After the confirmed environmental and laboratory research, appropriate environmental disinfection measures were implemented. The measures were implemented by technical experts and supervised by environmental health

professionals to prevent new cases of LD. Complete disinfection of the bathtub and all other equipment used during the filling of the whirlpool with sea water was carried out. Before being put back into service, the whirlpool tub was filled again with fresh sea water, additionally conditioned with higher levels of chlorine and balanced pH chemicals.

After conducting anti-epidemic measures, samples of sea water and tap water were taken from different sources. *Legionella* were not detected and chemical indicators were in accordance with the legislation.

Although earlier studies suggested the possibility of *Legionella* survival at different salt concentrations and in sea water, we wanted to examine *in vitro* survival studies of different *Legionella* species and different *L. pneumophila* strains in the raw sea water samples used to fill whirlpools.

To test whether *Legionella* survival in sea water is a strain or species-dependent characteristic of *Legionella*, we tested 14 strains of *L. pneumophila*, of which 4 isolates were from water, 9 clinical isolates and one isolate from sea water. Comparing the results of clinical and environmental isolates of *L. pneumophila,* there was no statistically significant difference in the reduction parameters (k, DT50 and DT90). If we look at different species of *Legionella*, *L. pneumophila* proved to be more resistant to survival in sea water than other tested species (*L. bozemanni*, *L. anisa* and *L. longbeachae*). Furthermore, the kinetics of reduction of two strains of *Legionella* in sea water, tap water and diluted sea water were examined. Our results showed that both *L. pneumophila* strains survive equally well in sterile TW, while *L. pneumophila* Allentown strain survives better in SW. In diluted cSW (cSW/TW), *L. pneumophila* Allentown strain survives for more than 20 days, while the ATCC BAA-74 strain loses its cultivability within 20 days. In addition, we also examined chlorinated samples of sea and tap water where there was a 100% reduction after 24 h. The reduction certainly happened earlier, but we did not follow it in the earlier stages.

Bacterial inactivation of Allentown strain in TW media and both strains in cSW media took place by a different reaction mechanism. As can be seen in Figure 2, inactivation was a two-step process; first rapid exponential decay and second slower step with extended tailing. For that reason, a DFBM model was used to describe bacterial inactivation in these media. This model was used since the two bacterial subpopulations reacted at different rates (k_1 and k_2) with environmental media and caused fast and slow bacterial inactivation. We assumed that the two subpopulations of bacteria existed in media representing two different domains of microorganisms, more susceptible in the first domain and resistant in the second one. Inactivation of resistant subpopulation was very slow with k_2 values lower 87 and 53 times than k_1 in TW and cSW media, respectively, causing prolonged tailing on the inactivation curve. The DT90 of rapid phase was shorter than DT90 of extended phase in both analysed media (Supplementary Table S7). Sommer *et al.* (1998) and Rincon & Pulgarin (2004) analysed the prolonged tailing process of bacteria caused by slow inactivation of the resistant subpopulation. They hypothesised that this phenomenon is induced by the occurrence of cell-repair enzymes, which are synthesised when the cell is exposed to stress conditions. Phaiboun *et al.* (2015) have studied the survival kinetics of *E. coli* wild-type K12 strain exposed to various environmental stresses. They demonstrated that the bacterial response to stress is the expression of the RpoS gene representing the master regulator in the expression of stress genes. Then, the RpoS gene is activated and, consequently, promotes the expression of new stress genes protecting the bacterial cell. Furthermore, Nair & Finkel (2004) found that during exposure to stress, bacteria aggregate in large clusters interconnected by hydrophobic bonds, protecting the cells from adverse effects.

The results of our study are in concordance with previous studies indicating the possibility of *Legionella* survival in sea water. However, the surprising fact is that *L. pneumophila* Allentown sea water isolate survives longer in marine conditions and it remains to be investigated whether this strain possesses properties that enable it to survive in sea water or whether adaptation of this strain to sea water survival may have occurred.

CONCLUSIONS

Information from this LD case investigation suggests that even if we take precautions by making spa resorts (sea water) with low potential risk, it is possible to become an environmental reservoir for *Legionella* spp. In order to understand more and prevent other possible pathways of LD, we should endeavour to develop appropriate control measures in artificial water systems to prevent possible new LD cases.

This single case of LD disease is a confirmation that even in the saline environment in certain chemical conditions for a short time, *L. pneumophila* can survive conditions that can result in an outbreak. It seems that contamination of a tap water system with *Legionella* may be inevitable. But it is critical to have well-planned maintenance and monitoring systems to avoid *Legionella* outbreaks.

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CONFLICT OF INTEREST

All authors agree to disclose any financial conflict of interest that might be construed to influence the results or interpretation of this manuscript.

ETHICAL STANDARDS

This work was undertaken as part of an urgent public health investigation; therefore, it did not require institutional ethical review or approval.

DATA AVAILABILITY STATEMENT

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

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