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Occurrence of Fecal Bacteria and Zoonotic Pathogens in Different Water Bodies: Supporting Water Quality Management

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Abstract: Water contaminated with microbiological and chemical constituents can cause a variety of diseases. Water bodies may become contaminated by wild and domestic animal feces, agricultural runoff or sewage, and are often overlooked as a reservoir and source of human infection by pathogenic microorganisms. The objectives of this study were to evaluate the presence of the zoonotic pathogens, *Salmonella* spp. and *Listeria monocytogenes*, in various water bodies located in urban and rural areas in the north of Portugal. Water samples were collected from six sites, including natural and artificial ponds, in two different time periods. Several water quality physicochemical parameters, as well as fecal indicator bacteria, were evaluated. High levels of total coliforms (>1.78 log CFU/100 mL) were detected in all samples, and substantial numbers of *Enterococcus* (>2.32 log CFU/100 mL) were detected in two ponds located in a city park and in an urban garden. *Escherichia coli* counts ranged from undetectable to 2.76 log CFU/100 mL. *Salmonella* spp. was isolated from two sites, the city park and the natural pond, while *L. monocytogenes* was isolated from three sites: the city garden, the natural pond and the artificial pond, both in the rural area. These data show that artificial and natural ponds are a reservoir of fecal indicator bacteria and enteric and zoonotic pathogens. This may impact the potential risks of human infections by potential contaminants during recreational activities, being important for assessing the water quality for strategic management of these areas.

Keywords: *Listeria*; *Salmonella*; water; pathogens; *Escherichia coli*; zoonoses; biological swimming pool; ponds; rural; urban

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1. Introduction

Zoonoses, i.e., infections caused by pathogenic organisms (bacteria, viruses or parasites) that jump from an animal reservoir to humans, either through direct contact or through contaminated food, water or environment, are a continuous threat to human health [1]. It is estimated that more than 60% of human infections have an animal source [2] and the frequency of illness caused by microorganisms originating from animal reservoirs is increasing due to unsustainable human activities [3].

Listeriosis and salmonellosis, caused by the bacteria *Listeria monocytogenes* and *Salmonella* spp., respectively, are within the major zoonotic foodborne diseases of relevance to public health in the European Union [4]. Salmonellosis was the second most reported zoonosis, and listeriosis presented the highest case fatality in 2020 [4]. The severity of the diseases caused by these pathogens varies from mild symptoms to life-threatening conditions and presents a high individual, societal and economic burden [5].

Listeriosis and salmonellosis are more frequently associated with the consumption of contaminated animal and animal-derived food products (e.g., fish, meat, poultry, eggs, dairy

products, etc.), or by non-animal products (e.g., fresh produce) contaminated by agricultural environments, via soil, irrigation water or manure used as fertilizer [6]. Although rare, some cases of disease caused by direct contact with infected animals or indirect contact with a contaminated environment have been reported [7,8]. A diverse range of reservoirs have been associated with these pathogens, including the gut of healthy farm animals (cattle, swine or poultry) and also wild animals, pets, fish or rodents [9], and, therefore, they are expected to be present in the natural environment.

Several studies have reported the survival of *Salmonella* spp. and *L. monocytogenes* in soil and different water sources, which serve as key reservoirs, contributing to the transfer of these pathogens through different environments [10–15]. Farm environments and agricultural waters are commonly associated with the presence of *Salmonella* spp. and *L. monocytogenes* [16,17], but these have also been isolated from other water sources, such as rivers and other fresh water sources [17–19]. Their presence is also more frequently associated with urban settings when compared to rural or pristine environments [20,21].

While there are extensive studies on the distribution of *L. monocytogenes* and *Salmonella* spp. in humans, animals, foods and food-processing environments [21–23], limited data is available regarding the distribution of these species in the general environment. A better understanding of the pathogen dynamics associated with different water ecosystems is indispensable for the development of effective strategies to mitigate the risk of human disease.

The intensive urban growth has been long linked to environmental pollution and ecological imbalance. Particularly, the contamination of water reservoirs by microbial pathogens is currently a major water quality issue worldwide [24]. Protection of water reservoirs, either to be used as a source of drinking water, agriculture irrigation or recreational use, is essential to ensure human health. Developing protective and preventive measures against microbial contamination of water requires a deep understanding of the relative risks associated with the frequency of pathogen occurrence in different water systems. The quantification of indicator organisms, such as *Escherichia coli*, total and fecal coliforms, has been traditionally used for water quality monitoring and to estimate the levels of potentially pathogenic bacteria. There is, however, much discussion concerning the ability of these organisms to truly represent potential contamination by pathogenic bacteria, as contradictory results have been reported among studies [25–28].

The aim of this study was to investigate the prevalence of *Salmonella* spp. and *L. monocytogenes* in various water ecosystems located in urban and rural areas in the north of Portugal. Simultaneously, several microbiological and physicochemical water quality parameters were evaluated.

2. Materials and Methods

2.1. Study Sites and Water Sampling

Surface water samples were collected from six water bodies in urban (#3) and rural (#3) landscapes in Portugal's northern region, based on their geographic situation (rural and urban) and use, in order to include artificial and natural ponds (Table 1). Climate conditions are considered temperate with rainy winters and dry summers at mild temperatures, classified as Csb according to the Köppen classification [29].

Briefly, two artificial ponds were located in a city garden (P1, Figure 1a) and a city park (P2, Figure 1b), respectively; one small pond was located on a roof terrace in the city, at the level of the seventh floor (P3, Figure 1c); an artificial pond was set up as a biological swimming pool, i.e., a swimming pool where the traditional chemical disinfection is replaced by natural biological processes for water purification, located in a private house in a rural area, serving three persons (P4, Figure 1d); a natural pond located in a rural protected area (P5, Figure 1e); and, an artificial pond (P6, Figure 1f), receiving treated wastewater by a constructed wetland in a tourism house (details described in [30]).

Table 1. Location and description of sampling sites.

Site Code	Location	Site Description	Area (m^2)	Coordinates
P1	Urban	Artificial pond at city garden	954	(41.145622, −8.616537)
P2	Urban	Artificial pond at city park	8600	(41.167868, −8.678431)
P3	Urban	Artificial pond at roof terrace, 7th floor in a city building	3	(41.176330, −8.605600)
P4	Rural	Artificial pond set up as biological swimming pool	210	(41.213734, −8.632291)
P5	Rural	Natural pond at a protected area	3.46×10^6	(41.766471, −8.642062)
P6	Rural	Artificial pond receiving wastewater treated by a constructed wetland in a tourism house	5	(41.805819, −8.567038)

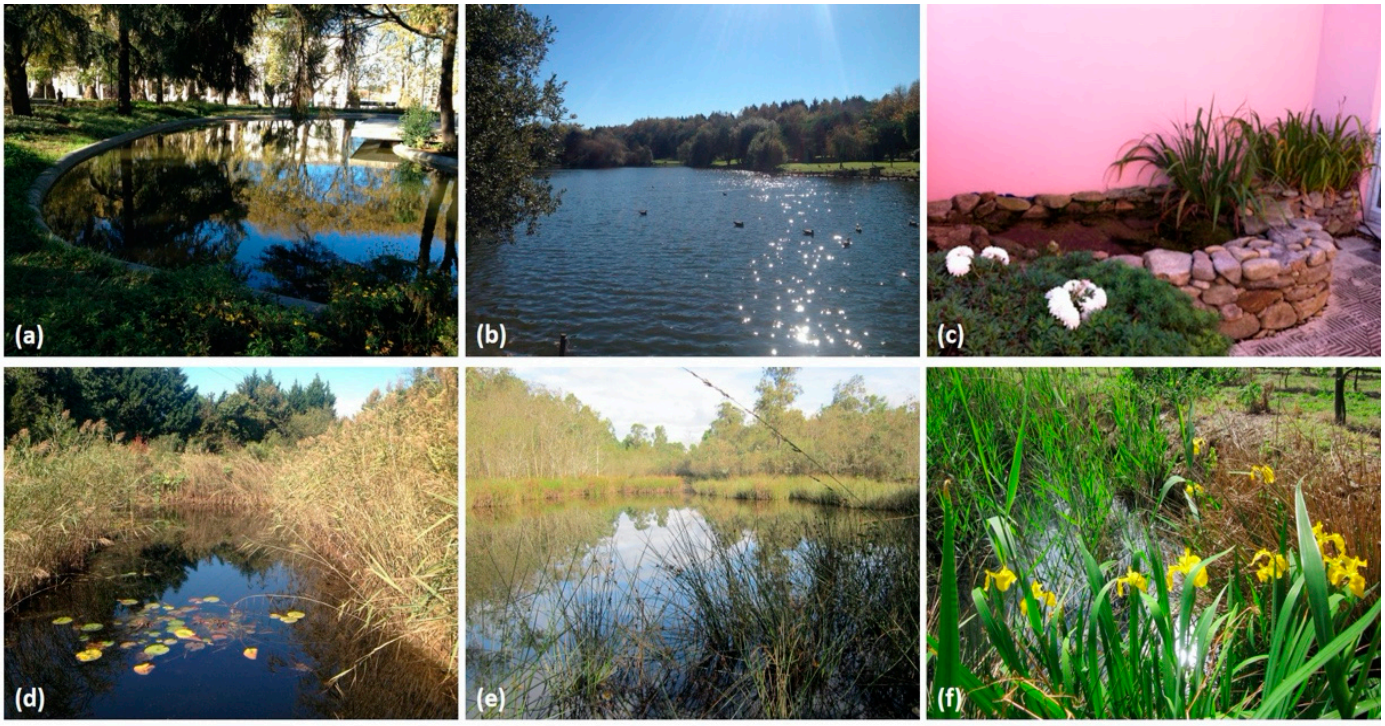

Figure 1. Water bodies sampling locations: (**a**) P1—artificial pond located in a city garden (**b**) P2—artificial pond located in a city park, (**c**) P3—artificial pond located on a seventh-floor roof terrace in the city, (**d**) P4—artificial pond set up as biological swimming pool in a rural area, (**e**) P5—natural pond located in a rural protected area, and, (**f**) P6—artificial pond receiving treated wastewater by a constructed wetland in a tourism house in a rural area.

Samples were collected from each site in two different time periods of the same year (September and October) in sterilized glass bottles of 1 L, directly dispatched to the laboratory and immediately analyzed for physicochemical and microbiological analysis (Sections 2.2 and 2.3).

2.2. Physicochemical Analysis

Samples were analyzed based on standard methods [31]: chemical oxygen demand (COD; closed reflux, titrimetric method) and total suspended solids (TSS). Phosphorous, ammonium, nitrite and nitrate (PO_4^{3-}, NH_4^+, NO_2^- and NO_3^-) concentrations were determined with photometric test kits (Spectroquant®, Darmstadt, Germany). On-site measurements, at the time of water sampling, were taken for water temperature, pH and conductivity, with a multi-parametric system HANNA HI-98130, and for relative humidity and air temperature with a thermometer/hygrometer OH HAUS OH 513, Greutor.

2.3. Microbiological Analysis

Microbial water analysis was performed by using the membrane filtration technique according to International Standards Organization (ISO) protocols for the detection of *E. coli* and total coliforms [32] and *Enterococcus* spp. [33]. Briefly, water samples of 1, 10 and 100 mL were filtered through a 0.45 µm pore size nitrocellulose filter of 47 mm in diameter (Millipore Corporation; Bedford, MA, USA). After filtration, filters were immediately placed onto sterile Petri dishes containing selective agar media, Tergitol 7 agar (Biokar Diagnostics) for *E. coli* and total coliforms enumeration (incubated at 37 °C for 24 h), and in Slanetz and Bartley agar (Biokar Diagnostics) for *Enterococcus* spp. enumeration (incubated at 37 °C for 48 h). Colonies having a yellow or orange color inside a yellow hallo were counted as coliforms, and yellow colonies inside a yellow hallo as *E. coli*. At least five typical colonies per sample, on each media type, were further isolated by the streak plate method in Tryptose Soy agar (Biokar) and confirmed by standard procedures. Briefly, typical colonies were tested for oxidase activity and production of indole from tryptophane broth at 44 °C. Oxidase-negative colonies were considered total coliforms, and those being oxidase-negative and indole-positive, *E. coli*. All red or maroon colonies were scoured as presumptive enterococci and further confirmed onto Bile Esculin Azide agar (Biokar) incubated for 24 h at 44 °C. After confirmation of suspected colonies, results were recorded as colony-forming units per 100 mL (CFU/100 mL).

To test the presence of *L. monocytogenes* and *Salmonella* spp. in water samples, the VIDAS® assay—a specific enzyme-linked fluorescent immunoassay (ELFA) rapid method performed in the automated VIDAS® instrument (bioMérieux, Marcy-l'Étoile, Lyon, France)—was used. For that, water samples of 1 L each were filtered, as previously described, and the filters were placed into sterile stomacher bags with 225 mL of appropriate pre-enrichment medium and analyzed according to VIDAS® SLM [34] and VIDAS® LMO2 [35] protocols. For *Salmonella* spp. detection, membranes were placed in 225 mL of buffered peptone water (BPW, Merck), homogenized and incubated at 37 °C for 24 h. Subsequently, two samples of 1 mL of pre-enriched buffered peptone water were subcultured into 10 mL of Rappaport–Vassiliadis (bioMérieux) and into 10 mL of Muller–Kauffmann tetrathionate (bioMérieux) and incubated for 8 h at 42 °C and 37 °C, respectively; 1 mL of each selective enrichment broth was diluted separately in 10 mL of M-broth (Merck, Darmstadt, Germany) and incubated at 42 °C for 18 h. One milliliter of each M-broth tube was mixed in another tube and boiled for 15 min and loaded into a VIDAS® SLM strip (bioMérieux) according to manufacturer's instructions. For *L. monocytogenes* detection, membranes were placed into 225 mL of 1/2 Fraser selective broth (bioMérieux), homogenized and incubated at 30 °C for 24 h. Subsequently, 0.1 mL of this broth was transferred to 10 mL of Fraser selective broth (bioMérieux). After incubation at 37 °C for 24 h, 0.5 mL of the secondary enrichment broth was loaded into a VIDAS® LMO2 strip (bioMérieux) according to manufacturer's instructions. For all positive samples, one loop of the Fraser selective broth was streaked onto ALOA selective chromogenic medium (bioMérieux) and incubated at 37 °C for 48 h. Presumptive *L. monocytogenes* colonies were subcultured on tryptone soy agar plates supplemented with 0.6% yeast extract (TSAYE) and confirmed by standard procedures according to the standard techniques described in the International Standard ISO 6579 (ISO, 2002), including selected sugar (rhamnose, xylose, mannitol) fermentation tests and the Christie—Atkins—Munch—Petersen (CAMP) test [36] with *Staphylococcus aureus* ATCC (American Type Culture Collection) 25923 and *Rhodococcus equi* ATCC 6939 on sheep blood agar plates (bioMérieux). The results were recorded as the presence or absence of *Salmonella* spp. or *L. monocytogenes* in 1 L.

2.4. Determination of Listeria Monocytogenes Isolates Serotype

Confirmed isolates of *L. monocytogenes* were stored in tryptic soy broth with 30% (*v*/*v*) glycerol at −80 °C. Isolates' serotypes were determined by multiplex PCR as described by Doumith et al. [37] using primers targeting fragments of genes lmo0737, ORF2819, ORF 2110, lmo1118, and prs (MWG-Biotech, Muenchenstein, Switzerland). This assay differentiates

five major subtypes, each representing more than one serotype: geno-serogroup IVb (serotypes 4b, 4d, and 4e), geno-serogroup IIa (serotypes 1/2a and 3a), geno-serogroup IIb (serotypes 1/2b, 3b, and 7), geno-serogroup IIc (serotypes 1/2c and 3c) and geno-serogroup IV (serotypes 4a and 4c).

3. Results and Discussion

3.1. Water Bodies Physicochemical Parameters

Values of physicochemical levels measured during the sample collection for each water body are displayed in Table 2. The lower pH values were verified for the natural pond (P5) (5.570), while in the artificial ponds, pH varied between 6.138–7.860, whereas the highest value (8.993) was detected in the city park pond (P2). In general, nitrogen and phosphorus were detected at low concentrations, with higher values recorded for P6, that was the pond receiving treated domestic wastewater coming from a constructed wetland. Concerning COD, it was below the detection limit for P3, P4 and P6, although for P1, P2 and P5, concentrations between 16 and 60 mg/L were detected. This can be partially attributed to the presence of animals that were observed in the area, such as ducks, birds and amphibians. In the case of P2, fish were also observed. Flores et al. [38] mentioned that the lack of physical barriers in water bodies, such as lakes, allows for free contact of animals, such as pigeons, seagulls and/or ducks, dogs and cats, making these sites more exposed to fecal contamination. Moreso, the animal droppings near water spots (trees and bushes) can be leached through wind or stormwater, and thus, influence the water quality of the water bodies. Additionally, the water of lakes has a tendency to be more turbid and stagnated, decreasing the bactericidal effects of UV radiation.

Table 2. Physicochemical analyses in each pond, on two sampling times (September (TI) and October (TII)).

Site Code/Sampling Time		Air			Water						
		Temperature (°C)	Relative Humidity (%)	pH	Temperature (°C)	TSS (mg/L)	COD (mg/L)	PO_4^{3-} (mg/L)	NO_2^- (mg/L)	NH_4^+ (mg/L)	NO_3^- (mg/L)
P1	TI	24.5	44	7.647	19.7	<dl	81	0.13	0.12	0.09	2.2
	TII	15.0	43	6.919	15.9	<dl	94	0.11	0.06	<dl	2.0
P2	TI	25.8	41	8.993	23.6	21	25	0.26	0.18	0.04	2.3
	TII	19.0	42	8.897	18.5	23	16	0.24	0.19	0.05	2.0
P3	TI	21.0	60	7.161	18.0	<dl	<dl	0.25	0.09	<dl	1.6
	TII	15.0	48	7.165	13.0	<dl	<dl	0.24	0.06	<dl	1.9
P4	TI	29.3	42	7.860	23.4	16	<dl	0.10	0.07	0.06	1.9
	TII	18.0	49	6.710	17.5	<dl	<dl	0.07	0.07	<dl	1.7
P5	TI	19.5	51	5.570	17.5	<dl	42	0.11	0.18	0.23	1.8
	TII	19.5	51	5.979	16.5	<dl	60	0.25	0.18	0.17	1.5
P6	TI	20.3	58	6.138	19.2	<dl	<dl	0.26	0.15	0.11	3.0
	TII	18.5	61	6.517	17.5	<dl	<dl	0.38	0.18	0.08	2.1

Note: (P1) artificial pond located in a city garden, (P2) artificial pond located in a city park, (P3) artificial pond located on a seventh-floor roof terrace in the city, (P4) artificial pond set up as biological swimming pool in a rural area, (P5) natural pond located in a rural protected area, and (P6) artificial pond receiving treated wastewater by a constructed wetland in a tourism house in a rural area. dl: detection limit.

3.2. Occurrence of Fecal Indicator Bacteria and Zoonotic Pathogens in Surface Water Bodies

The 6 sites were sampled on two occasions, resulting in 12 water samples. The contamination levels of the indicator organisms varied considerably among sites and sampling events. Total coliforms and *Enterococcus* spp. were detected in all samples while *E. coli* was detected in five samples, i.e., at least once in the P1–4 and P6 sites (artificial ponds) and was absent in both P5 (natural pond) samples (Figure 2). Overall, levels of total coliforms ranged from 1.78 to 2.85 log CFU/100 mL, *Enterococcus* spp. ranged from 0.95 to 2.79 log CFU/100 mL, and *E. coli* ranged from undetectable to 2.76 log CFU/100 mL.

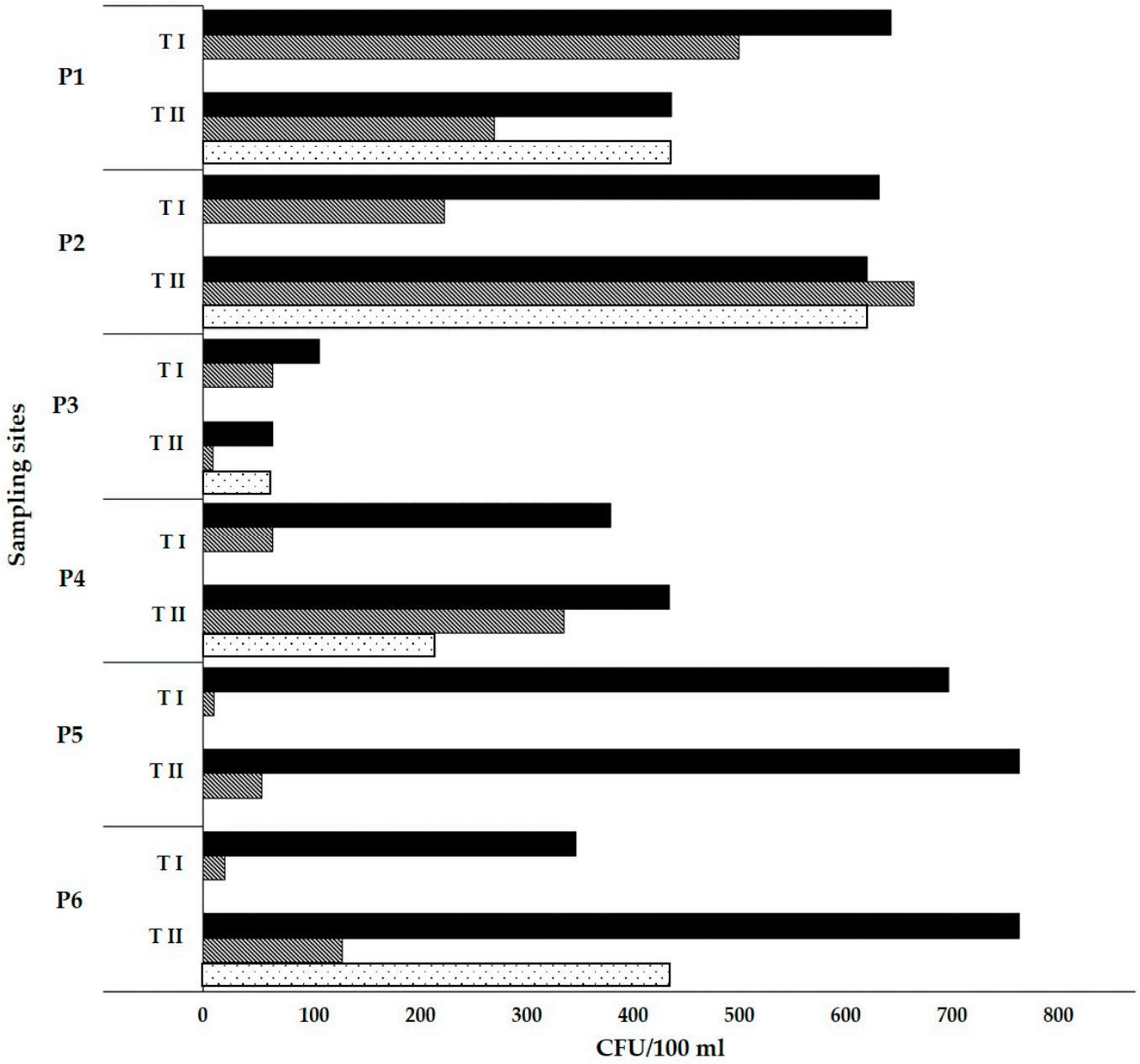

Figure 2. Comparison of total coliforms (■), *Enterococcus* (▨), and *E. coli* (⊡) levels (CFU/100 mL) in each pond at two sampling times (TI and TII). (P1) artificial pond located in a city garden, (P2) artificial pond located in a city park, (P3) artificial pond located on a seventh-floor roof terrace in the city, (P4) artificial pond set up as biological swimming pool in a rural area, (P5) natural pond located in a rural protected area, and (P6) artificial pond receiving treated wastewater by a constructed wetland in a tourism house in a rural area.

Salmonella spp. and *L. monocytogenes* were present in seven samples (Table 3). These included samples from the ponds of the city's garden and park that are frequently used for leisure and recreational activities, which highlight the importance of these aquatic environments as reservoirs of zoonotic pathogens and a potential risk for public health and safety.

The presence of pathogens tended to vary within site and sampling events. Two sites always tested negative for the presence of Salmonella and *L. monocytogenes*, the pond located in the terrace (P3) and the biological swimming pond (P4). The former is a small pond that retains rainwater in a roof terrace with a minor circulation of people, and that

serves mainly as a source of drinking water for the city's small birds; this might explain the low numbers of indicator organisms found. In addition, environmental factors, such as the wind-protected position and high exposure to solar radiation, may help to reduce the levels of bacterial contamination. It has been demonstrated that sunlight and higher environmental temperatures increase inactivation rates of indicator bacteria [39].

Table 3. Pathogens detection in each water body at two sampling times (September (TI) and October (TII)).

Site Code	Sampling Time	*Salmonella* spp. (per Liter)	*Listeria monocytogenes* (per Liter)
P1	TI	Negative	Negative
	TII	Negative	Positive
P2	TI	Positive	Negative
	TII	Positive	Negative
P3	TI	Negative	Negative
	TII	Negative	Negative
P4	TI	Negative	Negative
	TII	Negative	Negative
P5	TI	Positive	Negative
	TII	Negative	Positive
P6	TI	Negative	Positive
	TII	Negative	Positive

Note: (P1) artificial pond located in a city garden, (P2) artificial pond located in a city park, (P3) artificial pond located on a seventh-floor roof terrace in the city, (P4) artificial pond set up as biological swimming pool in a rural area, (P5) natural pond located in a rural protected area, and (P6) artificial pond receiving treated wastewater by a constructed wetland in a tourism house in a rural area.

Concerning the natural swimming pool, it is located in a private household in a rural area and is used mainly in the summer by the family. Currently, there is a lack of microbiological quality data concerning these specific environments, thus it is not possible to establish comparisons [40,41]. *Salmonella* spp. was isolated in both sampling visits from the urban city park (P2) and once in the natural wetland, located in a rural area (P5). Both sites are characterized by the presence of a high number of animal species, particularly birds and amphibians. Feces from these animals are known to contain fecal indicator bacteria, which may contribute to the low water quality observed in the samples collected in both ponds and are known to play an important role as reservoirs of this pathogen [42–45], however, other sources cannot be discarded. Correlations have also been established between the presence of fecal coliform concentrations in a river and environmental factors, including water turbidity and concentrations of nitrate, phosphate, chloride, and BOD_5 (biochemical oxygen demand) [46].

Listeria is widely distributed in the natural environment [47] and has been widely recovered from surface waters with a variable prevalence. In this study, *L. monocytogenes* was detected in two sites located in rural areas, in the natural pond and artificial pond receiving treated wastewater, and in the garden pond located in an urban area (overall prevalence of 33%). For comparison, an overall *L. monocytogenes* prevalence of 10% was detected in surface waters in Ontario, Canada [48], 12.8% of spring and river samples in Switzerland [49], 16% in water sources intended for irrigation in farm environments [50], 30% in rivers, lakes and stream samples along the central California coast [51], and 43% in five Californian watersheds [52]. A two-year survey of natural and urban regions located in New York yielded a higher prevalence of *L. monocytogenes* in surface water from urban regions (33%) than from natural regions (16%; [20]). Oppositely, Stea et al. [53] reported *L. monocytogenes* being more prevalent in the rural watershed than in the urban watershed. Constructed wetlands have been widely used as ecological treatment systems for different types of wastewaters [54]. The presence of *L. monocytogenes* in the pond receiving treated wastewater by a constructed wetland might indicate that the pathogen survived the treatment processes; however, the contamination post-treatment through

contact with wild animals cannot be excluded. Calheiros et al. [55] has previously assessed the fate of potentially pathogenic bacteria within a constructed wetland and the pond that is presently being studied, finding an Enterobacteriaceae level of 3.2 ± 0.7 Log_{10}CFU/g and *E. coli* of 1.9 ± 0.4 Log_{10}CFU/g. Concerning *Salmonella* spp., it was never detected. Although all pond samples were positive for *L. monocytogenes*, it was only by the detection technique using culture-based methods, since its levels were below the detection limit of the enumeration technique.

The number of samples analyzed in the present study is not sufficient to make general assumptions, however, the samples with the highest loads of total coliforms were also contaminated with *L. monocytogenes* or *Salmonella*, i.e., samples collected from P1, P2, P5 and P6, in comparison with those collected from P3 and P4. The same link could not be established for *Enterococcus* spp. and *E. coli* as samples positive for *Salmonella* spp. or *L. monocytogenes* presented both high and low contamination levels of these indicator organisms (e.g., P5 samples and second sampling of P1), thus these fecal indicators may not be adequate for the evaluation of surface water contamination by these specific pathogens. Stea et al. [53] found that *E. coli* levels ≥ 100 CFU/100 mL were associated with a higher likelihood of *Listeria* spp. presence but could not be correlated with contamination by *L. monocytogenes*. Results from prior studies also show no correlations between fecal indicators and the prevalence of *Salmonella* spp. or *L. monocytogenes* in diverse water systems [26,56–58].

Of the twelve samples analyzed, seven presented contaminations with the zoonotic pathogens, *Salmonella* (*n* = 3) and *L. monocytogenes* (*n* = 4); the two pathogens were never isolated from the same sample simultaneously. *Salmonella* spp. was isolated from two sites: the city park artificial pond (P2) was positive in both sampling visits, and the natural pond in the rural area (P5) was positive in one sampling visit. *Listeria monocytogenes* was present in one sample collected from the urban artificial pond of the city garden (P1), one sample collected from the P5, and both samples collected from the artificial pond receiving wastewater treated by a constructed wetland (P6). One isolate from each sample was further characterized in terms of serotype. All four *L. monocytogenes* isolates belonged to the PCR serogrouping profile IVb (4b, 4d, and 4e). In spite of the limited number of isolates, our results indicate that *L. monocytogenes* populations present in water environments belong to the major serotype associated with human listeriosis (4b). This serotype was also found to be prevalent in isolates from Canada [52]. Other studies reported serotype 4b, as well as 1/2a as the prevalent serotypes in *L. monocytogenes* populations collected from surface waters [14,48].

4. Conclusions

The occurrence of *Salmonella* spp. and *L. monocytogenes*, as well as high levels of fecal indicators in the collected samples, highlight the potential of artificial and natural ponds as reservoirs of important zoonotic pathogens. Particularly, the presence of these pathogenic agents in recreational public spaces (ponds of the city's garden and park) is worrisome. In addition, results demonstrate that *L. monocytogenes* circulating in these water ecosystems belong to serotypes that are frequently isolated from human clinical cases. Our data contributes to a better understanding of the prevalence and distribution of *Salmonella* spp. and *L. monocytogenes* in rural and urban water bodies, which is critical for managing disease risk. Limitations of our study include a limited number of sampling events. Following the methodology carried out in the present study, it may be useful to consider indicators' analysis to address pathogens' presence in water bodies. Future longitudinal studies that investigate how climate influences pathogen occurrence, as well as molecular typing of isolates will be useful to better elucidate the prevalence and diversity of these zoonotic pathogens in aquatic environments from urban and rural areas.

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C.S.C.C.; writing—review and editing, V.F., R.M., P.T., P.M.L.C. and C.S.C.C.; funding acquisition, P.T. and P.M.L.C. All authors have read and agreed to the published version of the manuscript.

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