Quantitative survival of *Leptospira interrogans* in soil and water microcosms

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Running title: Survival of *Leptospira* in soil and water

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ABSTRACT

Leptospira interrogans is the etiological agent of leptospirosis, a globally distributed zoonotic disease. Human infection usually occurs through skin exposure with water and soil contaminated with the urine of chronically infected animals. In this study, we aimed to quantitatively characterize the survival of *Leptospira interrogans* serovar Copenhageni in environmental matrices. We constructed laboratory microcosms to simulate natural conditions and determined the persistence of DNA markers in soil, mud, spring water and sewage using a qPCR and a PMA-qPCR assay. We found that *L. interrogans* does not survive at high concentrations in the tested matrices. No net growth was detected in any of the experimental conditions and in all cases the concentration of the DNA markers targeted decreased from the beginning of the experiment following an exponential decay with a decreasing decay rate over time. After 12 and 21 days of incubation the spiked concentration of 10^6 *L. interrogans* cells/mL or g decreased to approximately 100 cells/mL or g in soil and spring water microcosms, respectively. Furthermore, culturable *L. interrogans* persisted at concentrations under the limit of detection by PMA-qPCR or qPCR for at least 16 days in soil and 28 days in spring water. Altogether our findings suggest that the environment is not a multiplication reservoir, but a temporary carrier of the *L. interrogans* Copenhageni, although the observed prolonged persistence at low concentrations may still enable the transmission of the disease.

IMPORTANCE

Leptospirosis is a zoonotic disease caused by spirochetes of the genus *Leptospira* that primarily affects impoverished populations worldwide. Although leptospirosis is transmitted by contact with water and soil, little is known about the ability of the pathogen to survive in the

47 environment. In this study, we quantitatively characterized the survival of *L. interrogans* in
48 environmental microcosms and found that although it cannot multiply in water, soil or sewage, it
49 survives for extended time (days to weeks depending on the matrix). The survival parameters
50 obtained here may help to better understand the distribution of pathogenic *Leptospira* in the
51 environment and improve the predictions of human infection risks in endemic areas.
52

53 **INTRODUCTION**

54 Leptospirosis is a globally distributed life-threatening zoonotic disease that affects
55 humans and other mammals. The current estimates put the number of cases over 1,000,000
56 annually with almost 60,000 deaths making leptospirosis one of the most prevalent zoonotic
57 diseases worldwide (1). Leptospirosis is caused by motile spirochetes from the genus *Leptospira*.
58 Pathogenic leptospires colonize the kidneys of animal hosts and are chronically excreted with the
59 urine. Humans and other animals get infected through abrasions or cuts in the skin or mucous
60 membranes by contact with water or soil previously contaminated with infected urine (2).
61 Leptospirosis outbreaks are reported seasonally in endemic areas following rainfall events which
62 lead to an increased human exposure to flood water, mud and run-off (3–7). Therefore, the
63 environment plays a central role in the spillover infections to humans and the circulation of the
64 bacteria within the animal reservoir.

65 Currently, there is a very limited knowledge about the persistence of pathogenic
66 leptospires in environmental matrices and the factors affecting their fate (8). Persistence ranging
67 from few hours to several months have been reported for different species and serovars in
68 aquatic matrices such as tap, river, sea and distilled water (9–13). Similarly, in soil the reported
69 survival ranges span from few hours to 193 days (14–18). A number of factors have been

identified as affecting the persistence including pH, salinity, soil moisture, temperature and the presence of accompanying microorganisms (9, 10, 19–23). However, these studies were based on the isolation of leptospires by culture techniques or direct animal inoculation. These approaches are time-consuming, insensitive and prone to errors such as the overgrowth by the autochthonous microbiota. Furthermore, their results were qualitative and left a knowledge gap regarding the quantitative survival dynamics of pathogenic leptospires in environmental matrices.

The ability of pathogenic leptospires to survive or even multiply in environmental matrices is particularly critical to determine the extent to which they serve as a reservoir of the disease. In this study, we aimed to quantify the survival of pathogenic leptospires in spring water, sewage and soil under controlled laboratory conditions using qPCR. For this purpose, we selected two species: *Leptospira interrogans* serovar Copenhageni, a highly virulent serovar that has been associated with large seasonal outbreaks in urban slums in Brazil (5, 24); and *Leptospira biflexa* serovar Patoc, a saprophytic species. We constructed laboratory microcosms to simulate natural conditions and spiked them with known concentrations of leptospires. DNA was extracted from each microcosm over a period of 28 days and quantified by qPCR and/or PMA-qPCR. Finally, we developed a statistical model to describe the fate of *Leptospira* DNA markers in the microcosms.

RESULTS

Decay model

We developed a statistical model based on Weibull distributions to model the survival of *Leptospira* DNA markers in the microcosms. Starting with a full model including the covariates species (*L. interrogans* and *L. biflexa*), medium (spring water, soil, mud and sewage), treatment

(sterile and non-sterile), and quantification method (qPCR and PMA-qPCR)), the final model included species, medium, and quantification method (Table S1). Treatment (sterile/non-sterile microcosm) did not contribute significantly to the model fit ($p = 0.19$), and was therefore not selected as a covariate in the final model. The modeled shape of the decay curves was lower than 1 ($k = 0.715 \pm 0.03$), which indicated that the death hazard was not constant during the experimental time, but instead decreased gradually after spiking. The modeled initial marker concentration (μ_0) was 5.673 ± 0.041 $\log_{10}$ units, which reflected the loss of DNA due to the extraction procedure (see Supplementary Methods). Modeled decay parameters (ϕ and α) for *Leptospira* DNA markers in each of the experimental condition are presented in Table 1. All comparisons between markers below were based on this model.

Differential persistence of *Leptospira* DNA markers in spring water and soil

The concentration of markers for both *L. interrogans* and *L. biflexa* decreased in all the microcosms after spiking (Fig. 1). No differences were observed between decay rates of *L. interrogans* and *L. biflexa* markers in spring water or soil. In spring water, *Leptospira* markers presented an almost flat decay curve ($\phi = 51.5$ and 42.2 for *L. interrogans* and *L. biflexa*, respectively) in which the DNA concentration had decreased by approximately 0.5 $\log_{10}$ units at the end of the experimental time. By contrast, the decay in soil microcosms was significantly faster ($\phi = 16.3$ and 13.4, for *L. interrogans* and *L. biflexa*, respectively) with a rapid decrease during the first 8 days followed by stabilization at concentrations around 2.50×10^2 GE/g, marginally over the limit of detection. Leptospires cultured in EMJH media did not show any time lag before entering the exponential phase confirming that they were in good physiological

115 conditions at the beginning of the experiments. Taken together these results indicate that there
116 was no net growth of *Leptospira* in spring water or soil.
117
118 **Effect of moisture and soil characteristics on persistence**
119 To evaluate the effect of soil moisture on the persistence of *L. interrogans* and *L. biflexa*
120 markers, we compared their decay in two soils with different physicochemical characteristics
121 adjusted to different moistures. We observed that the increase in moisture from field capacity to
122 muddy conditions did not have any effect on the persistence of *L. interrogans* or *L. biflexa* in
123 Brazilian and US soils as the decay parameters ϕ and α were not statistically different (Fig. 2;
124 Table 1). The decay rates (ϕ) in Brazilian soil and mud were significantly smaller for *L.*
125 *interrogans* and *L. biflexa* in comparison to the ones in US soil. Conversely, the proportion of
126 persistent markers (α) was significantly higher for both species in US soil and mud than in
127 Brazilian soil and mud, except for *L. interrogans* in Brazilian soil that showed no difference (Fig
128 2; Table 1). These observations indicated that moisture and intrinsic physicochemical
129 characteristics of the soil such as pH, organic content, and texture affected the persistence of
130 *Leptospira*.
131
132 **Persistence of *Leptospira* DNA markers in sewage**
133 In sewage microcosms, *Leptospira* markers presented a rapid decay (ϕ = 2.23 and 1.83
134 for *L. interrogans* and *L. biflexa*, respectively), significantly faster than the decays observed in
135 other media (Fig 2E and 2F; Table 1). In addition, we observed that *L. interrogans* markers could
136 only be consistently quantified above the limit of detection for eight days (Fig. 2E) as opposed to
137 *L. biflexa*, which was detected until the end of the experiment (Fig. 2F). This result is consistent

with the estimated decay parameter (α) that indicated that a larger proportion of *L. biflexa* markers than *L. interrogans* persisted beyond the experimental time (Table 1). Thus, the experimental data suggest that *L. biflexa* survives better than *L. interrogans* in sewage.

Persistence of *L. interrogans* cells measured by PMA-qPCR in soil and spring water

To determine whether the leptospiral DNA markers were suitable surrogates for live cells, we monitored the decay curves of heat-killed *L. interrogans* and *L. biflexa* in spring water and Brazil soil. In spring water, both *L. interrogans* and *L. biflexa* markers showed an almost flat decay curve indicating that the DNA from dead cells was being degraded at a very slow pace (Fig. S1). The long persistence of DNA in spring water evidenced that the markers were not suitable surrogates for live cells. In contrast, in soil the persistence of DNA from heat-killed cells was shorter with a 3 $\log_{10}$ unit reduction in the first 4 to 6 days (Fig. S1), which indicated that DNA from dead cells was being quickly degraded.

To discriminate between live and dead *L. interrogans* cells in the microcosms, we optimized a PMA-based qPCR (Supplementary Methods). Briefly, PMA-qPCR is a viability qPCR in which propidium monoazide (PMA), a DNA-binding dye, is added to the sample before DNA extraction. PMA penetrates cells whose membrane is compromised and binds covalently to DNA upon photoactivation interfering with its amplification. Therefore, the PMA treatment allows for the selective detection of DNA from membrane-intact "live" cells (25). After optimization of the PMA-qPCR procedure, we compared the persistence of markers in spring water and Brazilian soil using qPCR and PMA-qPCR. In addition, we tested sterile and non-sterile microcosms to explore the role of the autochthonous microbial communities on the survival. As anticipated by the previous experiment, the behavior of the markers in spring water was completely different when measured by qPCR or PMA-qPCR. In the first case, an almost

flat decay was observed, indicating a long persistence of the markers in the system. Conversely, when using PMA-qPCR the decay rates of *L. interrogans* markers were faster (ϕ = 25.8) and there were no long-term persisting markers (α not statistically different from 0) (Fig. 3A and 3B; Table 1). These results indicate that *L. interrogans* cells were dying in the microcosm, but the extracellular DNA persisted for a long time in spring water without being degraded. Consequently, the qPCR measurement did not represent appropriately the fate of live *L. interrogans* cells in spring water. In addition, we did not observe major differences in decay parameters between sterile and non-sterile microcosms, which suggested that the spring water microbiota was not a major factor involved in the persistence of *L. interrogans*. Regarding the isolation of cells by culture, positive results were obtained in all sterile and non-sterile microcosms up to day 21. At day 28 only two replicates each showed still positive results, in agreement with the results obtained with PMA-qPCR (Table 2).

In Brazilian soil, the decay of markers measured by PMA-qPCR was also faster than that measured by qPCR (ϕ = 8.2 and 16.3, respectively). At days 16 and 21 we detected markers by qPCR in all the experiments in both sterile and non-sterile microcosms, but when using PMA-qPCR most replicates were negative (Fig. 3C and 3D), in agreement with the prediction of the model that no cells were long-term persistent (α = 0). Overall, these results showed that DNA markers persisted better than live *L. interrogans* cells in soil. However, as opposed to spring water, the decay shape was similar. Indeed, the average difference between the concentrations quantified by qPCR and PMA-qPCR before reaching the detection limit is 0.69±0.34 $\log_{10}$ units, with a maximum of 1.15 $\log_{10}$ units at day 4 (Fig 3C). These relatively small differences indicated that qPCR could be used as a reasonable surrogate for live cells in soil, although it may overestimate the concentration of live cells. Furthermore, *L. interrogans* cells were consistently

185 isolated by culture up to day 12 in all sterile microcosms and two of three non-sterile ones. At
186 day 16, two sterile and two non-sterile microcosms were still positive (Table 2). Altogether,
187 these data indicate that despite the decay of live *L. interrogans* in soil, culturable cells were still
188 present in Brazilian soil after 16 days at concentrations under the limit of detection by PMA-
189 qPCR.
190
191 **DISCUSSION**
192 In this study, we aimed to characterize the survival of the pathogenic spirochete *L.*
193 *interrogans* Copenhageni in the environment. As with other environmentally dispersed bacteria,
194 the transmission from host to host depends largely on the pathogen's ability to survive and
195 remain infectious for a certain time outside of the host. Our findings indicate that this species
196 cannot survive at high concentrations in soil, spring water or sewage. Yet, it exhibits a prolonged
197 persistence in the environment that extends for over 3 weeks in soil and spring water.
198 *L. interrogans* did not show any net growth in the microcosms after spiking. The
199 concentration of DNA markers decayed in all the environmental matrices. We observed that after
200 approximately 14 and 5 days of incubation in spring water and soil microcosms, respectively, the
201 initial concentration of 10^6 *L. interrogans* serovar Copenhageni cells/mL or g decreased by 3
202 $\log_{10}$ units (Table 1 and Fig. 3). This leads us to hypothesize that *L. interrogans* cannot multiply
203 in the environment after excretion from its animal reservoirs and thus, the environment is not a
204 reservoir from an epidemiological point of view, but rather a temporary carrier of the pathogen.
205 Consequently, although the environment is essential for the dispersion of the pathogen (4, 6, 26–
206 29), it might not be sufficient to solely sustain the transmission cycle of the pathogen from
207 animal to animal and the spillover infections to humans.

208 The experimental data collected in the microcosms fitted an exponential model with a
209 decreasing decay rate over time. Various explanations have been proposed to explain this
210 behavior that has been reported for *Salmonella enterica*, *E. coli*, *Enterococcus spp*.,
211 *Campylobacter jejuni* and *Bacteroidales*, among other microorganisms (30–32) such as the
212 regulation of the population though quorum-sensing (33). Alternatively, initial populations may
213 be rapidly reduced due to predation or nutrient limitation until the carrying capacity of the
214 ecosystem is reached (34). The mechanisms of survival of *Leptospira* in the environment are still
215 poorly understood (8, 35), but the formation of biofilms and the interaction with other
216 microorganisms (20, 23) could explain the decreasing decay rates observed. Unfortunately, after
217 12 and 21 days the concentrations in soil and spring water reached the limit of detection of the
218 molecular methods (≈ 100 cells/g or mL) and the fate of *L. interrogans* serovar Copenhageni
219 could not be followed quantitatively thereafter. We succeeded, however, in culturing *L.*
220 *interrogans* serovar Copenhageni from non-sterile field-capacity soils and spring water in all
221 microcosms for at least 16 and 28 days, respectively (Table 2), even after the molecular approach
222 yielded negative results. Furthermore, the decay model predicted that a small proportion of the
223 initial population persisted in soil microcosms beyond the time at which the limit of detection
224 was reached (Table 1; Fig 3C). These low concentrations are consistent with those reported in
225 waters and soils in surveys of the pathogen in endemic areas (27, 36, 37). Overall, this suggests
226 that prolonged persistence at low concentrations may be sufficient to enable the transmission of
227 the disease.

228 Our culture-based results for soil microcosms fall within the ranges reported previously
229 for other *L. interrogans* serovars. For instance, *L. interrogans* serovar Australis survived for 15
230 days in moist silt loams from Australia (15), and *L. interrogans* serovar Hardjo was successfully

231 cultured for up to six days from Malaysian moist loam and clay soils under natural shaded
232 conditions (11). *L. interrogans* serovar Pomona survived for 42 days in saturated sterile soils
233 under field conditions in New Zealand (16). Conversely, previous studies have found longer
234 survival times in water than the ones reported in this study. *L. interrogans* serovar
235 Icterohaemorragiae remained culturable for 316 days when incubated in spring water at 30°C
236 (13). However, the addition of 1% of culture medium in their tested water clouds the
237 interpretation of the results. In distilled water at lower temperature (20°C), *L. interrogans*
238 serovar Canicola showed longer persistence (up to 110 days) (22). Despite the methodological
239 differences with these studies, our results suggest that *L. interrogans* may have a shorter
240 persistence in water at higher temperatures. This finding may be relevant to understand the role
241 of freshwater and other aquatic matrices in the transmission dynamics of *L. interrogans* in
242 tropical countries.

243 Sewage was not a suitable carrier for *L. interrogans* serovar Copenhageni. Although in
244 this case our data were based exclusively on qPCR results, the decays of *L. interrogans* in
245 sewage was faster than in soil and spring water (Fig. 2E). This decay is in agreement with Chang
246 et al. (1948), who reported that *L. interrogans* serovar Icterohaemorrhagiae was viable for no
247 more than 2-3 days after spiking in undiluted sewage. Despite this relatively short persistence,
248 exposure to sewage and flooding water after seasonal rainfall are widely recognized risk factors
249 for leptospirosis infection (3, 38–40). Thus, the role that sewage plays in the pathogen
250 mobilization, transportation and distribution, especially during heavy rainfall and flooding events
251 and, consequently, in the transmission of the disease, should not be disregarded.

252 Unexpectedly, *L. biflexa* serovar Patoc did not survive at high concentrations in any of
253 the conditions tested (Fig. 2). Nevertheless, the decay rate of *L. biflexa* markers was slower than

254 that of *L. interrogans* in soil and sewage (Fig 2 and 3). Specifically, the proportion of markers
255 that persisted beyond the experimental time (α) was significantly higher for *L. biflexa* than for *L.*
256 *interrogans* (Table 1). This suggests that a small proportion of the inoculated *L. biflexa* persisted
257 in soil and sewage 4 weeks post-inoculation at low concentrations ($<10^3$ cells/g or mL). The
258 concentration of naturally occurring *L. biflexa* or other saprophytic *Leptospira* in the
259 environment has not been determined, but it is likely lower than the starting concentration of 10^7
260 cells/mL or g used in the microcosms to simulate the presumed excretion of *L. interrogans* by
261 animal reservoirs. As indicated by the α parameters, *L. biflexa* may be decaying until the
262 carrying capacity of the ecosystem is reached at concentrations close to the limit of detection by
263 qPCR and surviving at low concentrations thereafter. Since we did not attempt to culture *L.*
264 *biflexa* in the microcosms beyond the experimental time of 4 weeks, this hypothesis remains to
265 be verified. Alternatively, *L. biflexa* may require a specific ecological niche to thrive different
266 from the conditions tested here to simulate the environmental phase of *L. interrogans* in a
267 tropical urban slum. Finally, future studies should preferably use recent isolates as the *L. biflexa*
268 strain has been preserved in laboratory conditions for decades after isolation (41), which might
269 have reduced its ability to thrive in the environment.

270 Microcosms are a convenient tool to study the persistence of microorganisms under
271 controlled conditions, although the decay rates estimated using these systems might not perfectly
272 predict the ones found in a variety of real settings (42, 43). For instance, we kept the microcosms
273 at a constant incubation temperature of 29 °C, which is a common temperature in standing water,
274 small open sewers and sun-exposed soil surfaces in tropical areas (44). In a real situation,
275 however, this temperature may oscillate throughout the day and across different areas. Further
276 studies should validate the results obtained here in more realistic settings that account for the

277 variability of natural conditions. Another limitation of this study is that the long-term persistence
278 of *L. interrogans* serovar Copenhageni seems to occur at concentrations close or below to the
279 limit of detection by qPCR. Other alternative techniques should be developed to better explore
280 the concentrations occurring in this phase of the decay and the mechanisms behind this survival.
281 Moreover, future research should also explore the potential loss or reduction in infectivity of *L.*
282 *interrogans* during its environmental phase using animal models of infection.

283 Despite these limitations, we succeeded in characterizing quantitatively the survival of *L.*
284 *interrogans* in environmental matrices. Our results showed that *L. interrogans* exhibits a
285 prolonged survival in the environment for periods ranging from a few days in sewage to at least
286 4 weeks in spring water. Although it does not survive at high concentrations in the environment,
287 small subpopulations might persist in concentrations below 100 cells/g or mL for a prolonged
288 time. Since the infectious dose in humans and animal reservoirs is unknown, the role that these
289 small populations play in the spillover infections to humans and the maintenance of the pathogen
290 within the animal reservoir should not be underestimated. Altogether our results provide novel
291 information that may have important ramifications regarding the life cycle of pathogenic
292 *Leptospira*. The decay parameters reported here need to be integrated into models of the
293 distribution of pathogenic *Leptospira* in the environment to improve the predictions of human
294 infection risks and inform public health interventions to reduce the transmission of leptospirosis.
295

296 **MATERIAL AND METHODS**

297 **Bacterial strains and culture**

298 *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 (45) and *Leptospira*
299 *biflexa* serovar Patoc strain Patoc1 (41) were cultured in liquid Ellinghausen-McCullough-

Johnson-Harris (EMJH) (46, 47) in agitation (100rpm) at 29°C for 3 to 5 days. A late-exponential culture was used in all the assays. After the incubation, 5 mL of the culture were centrifuged at 4,000 *g* for 5 min and the pellet was washed twice with the same volume of sterile spring water. The number of cells was determined using a Petroff-Hausser counting chamber (Hausser Scientific, PA) under dark-field microscopy and the culture was adjusted to a concentration of 10^8 cells/mL with sterile spring water. For experiments requiring heat-killed cells, cultures were placed at 80°C for 15 min in a water bath and immediately cooled at room temperature for 20 min.

Soil and water samples

The persistence of *Leptospira spp.* was investigated in two soils: a sandy loam soil (60% sand, 35% silt, 5% clay and 3.17% organic matter) collected in an urban slum in Salvador (Bahia, Brazil) and a loam soil (40% sand, 35% silt, 25% clay and 12.3% of organic matter) collected in New Haven (Connecticut, US). In addition, two water matrices were evaluated: bottled spring water obtained from a local retailer, and sewage collected from the New Haven wastewater facility after the bar screen and grit removal. For the sterile controls, spring water was autoclaved once at 121°C for 20 minutes and soil was autoclaved three times with 24h of incubation at 29°C between cycles.

Microcosms

Microcosms were prepared by distributing either 40 g of soil or 40 mL of water or sewage in sterile Pyrex glass beakers. The surface of the microcosm was spiked by dispersing droplets of *Leptospira spp.* suspensions to achieve a concentration of 10^6 cells/g or mL and

thoroughly mixed. The volume of spiking suspension varied to adjust the moisture of the soils to 25% and 35% for the Brazilian and US soil respectively, which corresponded approximately to their field capacity. To create mud conditions, soil moisture was increased to 35% and 45%, respectively. After spiking, microcosms were thoroughly homogenized, sealed with plastic paraffin film to protect them from external inputs and prevent evaporation, and placed in a humid thermostatic chamber at 29°C under dark conditions. Samples of 1 g or 1 mL were withdrawn from each microcosm at 0, 1, 2, 4, 6, 7, 12, 16, 21 and 28 days, for a total of 10 sampling time points. A growth control was carried out using EMJH medium instead of the environmental matrix. All microcosms were conducted in three independent biological replicates for *L. interrogans* serovar Copenhageni and in two for *L. biflexa* serovar Patoc.

DNA extraction methods

Three DNA extraction methods for both spring water and soil samples were evaluated and compared (Supplementary Methods). Based on those results, soil samples and sewage were subsequently extracted using the Power Soil™ DNA Isolation Kit (Mobio), with minor modifications. Spring water and EMJH samples were extracted using a bead beating method with CTAB and phenol/chloroform/isoamyl alcohol. For the PMA assays, spring water was extracted with the automated Maxwell® 16 Cell DNA Purification Kit (Promega).

qPCR assays

lipL32 gene was selected as a marker for *L. interrogans* and quantified using a TaqMan® assay described elsewhere (48) with minor modifications (27). *rpoB* gene was selected as a marker for *L. biflexa* and was quantified using a newly designed SYBR-Green® reaction

(Supplementary Methods). Calibration curves were constructed using genomic DNA obtained from strains Fiocruz L1-130 or Patoc1 with concentrations ranging from 10^7 to 10^0 genomic equivalents (GE)/5µL, based on its respective genome size (49, 45). A standard curve was run on each plate and used to transform quantification cycles (Cq) to concentrations (GE/reaction). Non-template controls were randomly included in all rows of each plate to discard the presence of contaminating DNA. All negative controls were negative in all cases. qPCR inhibition was monitored using a previously described Internal Amplification Control (IAC) plasmid tested in singleplex reactions (27). There was no evidence of inhibition of the molecular assays. See Supplementary Material for further details on the qPCR assay, calibrators and inhibition assay.

Isolation of *Leptospira spp.* cells by culture

From soil microcosms, 1 g sample was mixed for 1h with 4 mL of PBS in a horizontal mixer followed by sedimentation of the big particles for 30 min. Then, 3 mL of the supernatant were recovered and inoculated into 3 mL of 2X concentrated EMJH supplemented with 500 µL of a 10X concentrated antimicrobial combination (sulfamethoxazole, 400µg/mL; trimethoprim, 200 µg/mL; amphotericin B, 50 µg/mL; fosfomycin, 4 mg/mL; 5-fluoroacil, 1 mg/mL) (50). From spring water microcosm, 1 mL sample was inoculated into 5 mL of EMJH liquid medium. When a culture showed contamination, 1mL sample was filtered through a 0.45 µm filter and the filtrate inoculated into 5mL of EMJH containing the antibiotic cocktail. All cultures were incubated at 29°C with agitation and checked twice a week for *Leptospira* growth by dark-field microscopy. Samples were considered negative when no growth was observed after 30 days.

Detection of intact *L. interrogans* serovar Copenhageni cells

369 The ability of propidium monoazide (PMA) to selectively amplify DNA from membrane-
370 intact *L. interrogans* cells in spring water and Brazilian soil was investigated. After optimization,
371 a 60-min treatment with 5 µM PMA was selected for spring water and a 15-min treatment with
372 100 µM PMA was selected for Brazilian soil (Supplementary Methods).
373
374 **Statistical modeling**
375 To model the survival curves of *Leptospira* markers and determine decay differences
376 between species (*L. interrogans* and *L. biflexa*), medium (spring water, soil, mud and sewage),
377 treatment (sterile and non-sterile), and quantification method (qPCR and PMA-qPCR), we
378 assumed that cell death and marker disappearance from the microcosms were probabilistic events
379 (51, 52). Thus, to describe the survival curves, a probabilistic Weibull distribution function was
380 applied to the experimental data:

$$S(t;\phi,k) = P(T > t) = \exp\left(-\left({}^{t}/_{\phi}\right)^{k}\right) : t \geq 0$$

381 where $k = 1$ is a special case of the exponential function with a scale parameter ϕ. k defines the
382 shape of the survival curve and ϕ defines how stretched the shape is. Now, considering a set of
383 experiments *i=1,..,r*, which *ith* is defined by the values of a set of covariates x_i and the
384 concentration of the bacteria were measured at each time $t_j : j = 1,2,\ldots,m$. Therefore, the
385 concentration expected in a given time *jth* is based on the initial concentration (μ_0), the
386 proportion of cells that survive beyond the time of the experiments (α), and the family of survival
387 functions, in this case Weibull distributions:

$$\mu_{ij} = \mu_0 * (\alpha_i + (1 - \alpha_i) * \left(S(t_j;\phi_i,\kappa)\right)$$

The effects of the covariates on the two parameters, ϕ and α, were explored to determine if there were any difference between species, treatment, method of quantification and substrates. Maximum likelihood methods were used to estimate the parameters, assuming normality of the residuals. The log-likelihood function was optimized using *optim* function in the R software package (53). See Supplementary Methods for a full description of the survival model and the incorporation of samples below detection limits in the analysis.

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572 **TABLE LEGENDS**

573 **Table 1.** Modelled decay parameters (ϕ and α) and 95% confidence intervals of *L. interrogans*
574 and *L. biflexa* markers in spring water, soil, mud and sewage microcosms. Estimates with
575 intervals that overlap are not significantly different at the 95% significance level.

576

577 **Table 2**. Proportion of positive culture of *L. interrogans* Copenhageni from spring water and
578 Brazilian soil microcosms after 12 days. All culture attempts before day 16 were successful.

579

580 **FIGURE LEGENDS**

581 **Figure 1.** Persistence of *L. interrogans* (A) and *L. biflexa* (B) markers measured by qPCR in
582 microcosms of spring water (squares), soil (circles) and EMJH media (triangles). The solid line
583 represents the modeled decay curve in spring water and the dashed line in soil. Open symbols
584 represent data points for which at least one observation was below the limit of detection. Error
585 bars indicate standard deviations. The horizontal dashed line indicates limit of detection in soil
586 samples.

587 **Figure 2.** Persistence of *L. interrogans* and *L. biflexa* measured by qPCR in microcosms of
588 Brazilian soil (A and B), US soil (D and E) and sewage (F and G). In soil microcosms, circles
589 denote soil adjusted to field capacity and squares denote mud soils. Sewage samples are
590 represented by triangles. The solid line represents the modeled decay curve in field capacity soil
591 and the dashed line in mud soils. Open symbols represent data points for which at least one
592 observation was below the limit of detection. Error bars indicate standard deviations. The
593 horizontal dashed line indicates the limit of detection.

Figure 3. Persistence of *L. interrogans* measured by qPCR and PMA-qPCR in sterile and non-sterile microcosms. (A and B) Spring water. (C and D) Brazilian soil. Squares denote measurements by qPCR and circles by PMA-qPCR. The solid line represents the modeled curve for qPCR measurements and the dashed line for PMA-qPCR ones. Open symbols represent data points for which at least one observation was below the limit of detection. Error bars indicate standard deviations. The horizontal dashed line indicates the limit of detection.

Table 1.

			ϕ	LCI	UCI	α	LCI	UCI
L. interrogans	qPCR	Spring Water	51.5	38.4	68.9	0.90	0.80	0.95
		Brazilian Soil	16.3	13.2	20.3	0.08	0.03	0.17
		Brazilian Mud	14.1	11.1	18.0	0.10	0.05	0.18
		US Soil	4.3	3.1	6.1	0.21	0.14	0.29
		US Mud	5.7	4.1	7.8	0.28	0.21	0.35
		Sewage	2.2	1.7	3.0	0.18	0.13	0.23
	PMA-qPCR	Brazilian Soil	8.2	7.4	9.1	0.00*	0.00	1.00
		Spring Water	25.8	22.5	29.7	0.00*	0.00	1.00
L. biflexa	qPCR	Spring Water	42.2	27.4	64.8	0.96	0.92	0.98
		Brazilian Soil	13.4	9.2	19.5	0.21	0.11	0.37
		Brazilian Mud	11.6	7.9	16.9	0.25	0.15	0.39
		US Soil	3.6	2.4	5.2	0.45	0.39	0.51
		US Mud	4.7	3.2	6.9	0.54	0.48	0.60
		Sewage	1.8	1.3	2.5	0.40	0.36	0.44

* Not significantly different from 0.

Table 2.

		16 days	21 days	28 days
Spring water	Sterile	3/3	3/3	2/3
	Non-sterile	3/3	3/3	2/3
Brazilian Soil	Sterile	2/3	1/3	0/3
	Non-sterile	2/3	0/3	0/3