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Pathogenic Leptospira and water quality in African cities: a case study of

Cotonou, Benin

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1 1. INTRODUCTION

2 Leptospirosis is a bacterial disease caused by spirochetes of the genus Leptospira. Leptospires 3 infect and develop inside renal tubules of a variety of Vertebrates, essentially mammals, notably rodents, 4 which excrete the pathogen into the external environment. Humans are infected following contact with 5 contaminated water or humid soils. Symptoms range from null to very severe and death (reviewed in 6 Haake & Levett 2015). It is considered that one million cases occur annually in the world, among which 7 60,000 have fatal issues (Costa et al. 2015). Leptospirosis has been mainly associated with rice 8 agriculture and breeding as well as water recreational activities (Mwachui et al. 2015). Flooding 9 episodes may also trigger local leptospiral epidemics (e.g., Thaipadungpanit et al. 2013), with one out 10 of eight flood event-associated epidemics being due to leptospirosis (Cann et al. 2013). In addition, more and more evidence suggest that the disease may occur in urban settings, especially socio-economically 11 12 disadvantaged, informal and poorly sanitated ones, such as slums (e.g., Ko et al. 1999; Cornwall et al. 2016), sometimes with higher waterborne leptospires concentrations compared to surrounding rural 13 areas (e.g. in Peru, Ganoza et al. 2006). As a consequence, ongoing urbanization together with 14 multiplying extreme climatic events are expected to increase the risk of human leptospirosis (Lau et al. 15 2010). This may be particularly true in Africa where the disease is present but yet remains poorly 16 documented (reviews in de Vries et al. 2014; Allan et al. 2015). In particular, we are aware of no study 17 focusing on soil- or waterborne leptospires in Africa (see Tab. 2 in the review by Bierque et al. 2020). 18

19 The so-called « Abidjan-Lagos corridor » (ALC) is a 700km-long conurbation that sprawls 20 along the West African Atlantic coast. It currently houses >25 million people who live within and in the 21 surroundings of large adjacent cities such as Lagos (11.8 million inhabitants), Porto-Novo (570,000 22 inhabitants), Cotonou (1.5 million inhabitants), Lomé (1.7 million inhabitants), Accra (4.4 million 23 inhabitants) and Abidjan (4.7 million inhabitant) (2015 data; OECD/Africapolis project, 24 www.africapolis.org, 2020). It is expected to reach ca. 34 million city dwellers by 2025 (UN Habitat, 25 2014). This very rapid and mostly uncontrolled urbanization translates into the creation and expansion 26 of vast socially disadvantaged areas where pollution, access to basic services (e.g., health care, 27 education, transport, sanitation, waste management) and acceptable housing conditions are rare, thus

raising important environmental and health issues. In addition, the ALC comprises many lakes, 28 mangroves and swamps which form a dense hydrographic network. Together with a subequatorial 29 30 climate, low altitude and flatness, this makes this West African coastal region extremely susceptible to flooding events. For instance, 43% of Cotonou, Benin, is flooded one to two months a year either 31 following Lake Nokoué overflows or rain accumulation in shallows. Such episodes directly affect 32 200,000 inhabitants and indirectly impact many others through service interruption, resource 33 unavailability or degraded water quality (PCUG3C, 2010; Houéménou et al. 2019b). In addition, they 34 35 are often enhanced by poor drainage due to defective or crowded sanitation network as well as anarchic land use (PCUG3C, 2010). 36

37 These conditions as well as the omnipresence of anthropophilous rodents (Houéménou et al. 2019a) elevate the risk of leptospiral contamination in the ALC (Dobigny et al. 2018). Accordingly, up 38 39 to 18.9% of commensal rodents were found pathogenic Leptospira-positive in cities from south Benin (Houéménou et al. 2014, 2019a), suggesting that leptospires may massively circulate in the ALC urban 40 environment, though with strong prevalence variations in both space and time (Houéménou et al., 41 2019a). Mean annual temperatures (ca. 27°C) and rainfalls (ca. 800-1,600mm) are highly favorable to 42 Leptospira survival. However, although waters and humid soils appear as the corner stone of human 43 contamination (Barragan et al. 2017; Bierque et al. 2020), little is known about the precise environmental 44 conditions that are compatible with pathogenic Leptospira outside of its mammalian host (see for 45 instance Chang et al. 1948; Gordon-Smith & Turner 1961; Khairani-Bejo et al. 2004; Wojcik-Fatla et 46 47 al. 2014; André-Fontaine et al. 2015; reviewed in Barragan et al. 2017 and Bierque et al. 2020), especially in urban habitats where pollution may be very important (Lapworth et al. 2017). The present 48 49 study aims are: (i) to confirm the presence of pathogenic leptospires out of their hosts in Cotonou waters, (ii) to determine the physico-characteristics of waters where it was found, and (iii) to compare these 50 51 physico-characteristics with the chemical spectrum of all waters within the city. Altogether, our preliminary data allow us to explore for the first time the ability of leptospires to evolve in water within 52 53 an urban polluted habitat in Africa.

55 2. MATERIAL AND METHODS

- 56
- 57 2.1 Study area and sampling
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Cotonou town is located in the coastal sandy plain of Benin between the Atlantic Ocean at the south and Lake Nokoué at the north (Fig. 1). The littoral zone in the south of Benin is characterized by a subequatorial climate. The average annual rainfall for Cotonou is 1,300 mm (Yabi and Afouda, 2012). Seasonal variations during the year are marked by a large rainy season from mid-March to mid-July followed by a small rainy season from mid-September to mid-November alternated, respectively, by a small dry season from mid-July to mid-September and a large dry season from mid-November to mid-March.

66 The study area involves 3 districts located in the core city of Cotonou: Ladji, Agla and Saint 67 Jean (Fig. 1a) that are distinct in the sources and duration of inundation and pond waters. Ladji is located at the edge as well as upon the Lake Nokoué, thus displaying houses built both on hard ground and on 68 stilt pegs (Fig. 1b). Lacustrine waters are permanent, but flooding of zones adjacent to the lake only 69 70 occurs at the end of the rainy season (i.e., September and October) following the rise in lake levels. 71 Therefore, the area has both long-standing lake water and temporary pond waters. Permanent ponds are also present due to dry season groundwater discharge (Houéménou et al. 2019b). Ladji is densely 72 populated, has essentially informal housing development and is a very poor area where even the most 73 basic public services are usually missing. In particular, waste management is almost inexistent, garbage 74 are omnipresent and are even often used as embankment material. Such an environment favors 75 proliferation of rodents, which are abundant and infest 60-100% of households depending on the season 76 (Dobigny et al. 2019). 77

Agla is a recent but rapidly expanding district within a vast lowland that is extensively flooded early in the rainy season (i.e., starting from June) following rainfall accumulation. Similar to Ladji, permanent ponds are also present in Agla due to the extensive low-lying areas. The permanent ponds are formed by groundwater discharge in the dry months, whereas during the winter months the rainfall
inputs to the ponds reverse the hydraulic gradients, resulting in groundwater recharge ponds
(Houéménou et al. 2019b). The habitat in Agla includes hard-built houses as well as very precarious
cabins, with the poorest inhabitants usually gathering around or within these floodable low-lying areas
(Fig. 1b). These low-lying areas are also widely used as dumping sites for household waste (Fig. 1b).
Rodents are abundant in households, with infestation rates always ≥ 90% (Dobigny et al. 2019).

87 Saint-Jean is an old and formal district that has large open sewers and many houses that are
88 solidly constructed. This district is not floodable *per se*, but large ponds may stand for several days after
89 heavy rains. Rodents are also abundant in ca. 90% of households (Dobigny et al. 2019).

90

In 2017 and 2018, the three districts of Cotonou (Agla, Ladji and Saint Jean; Fig.1b) were 91 92 monitored for both water quality parameters (physical parameters, major ions and trace elements) and leptospire presence. Water sampling was organized concomitantly (i.e. within the same week) in the 93 three districts during the dry season (March 2017 and February 2018) as well as at the beginning (June 94 2017 and June 2018) and at the end (October 2017) of the rainy season. In total, 193 water samples were 95 collected: 85 in Agla, 53 in Ladji and 55 in Saint Jean. Eighty-three samples were tap waters and 61, 29, 96 97 17 and 3 samples were from groundwater wells, temporary ponds, permanent ponds and Lake Nokoué, 98 respectively. This corresponds to 51, 64, 55, 5 and 18 samples collected in March 2017, June 2017, 99 October 2017, February 2018 and June 2018, respectively. The detailed distribution of samples among 100 districts, periods and water types are provided in Table 1. The temporary ponds were differentiated from 101 the permanent ponds by noting locations where the waters were present in the wet season but not in the dry season. Both temporary and permanent ponds were sampled in Ladji (8 and 6, respectively) and 102 103 Agla (21 and 11, respectively), but not in St Jean where ponds only last a few days.

104

105 Cartography of each studied area was performed within the same month immediately after each106 of the five seasonal sampling campaigns. The land use categories and the extent of surface waters

107 (diameter > 3m) were systematically delineated using Open Street Map Tracker v.0.6.11 (Tools Open
108 Street Map) on an Android smartphone. A Geographic Information System was produced under QGIS
109 v.2.14 (QGIS Development team 2016). Surface water superficies were computed using the
110 LandscapeMetric package (option level class) in RStudio v.3.6.1 (RStudio Team 2016).

111

112 2.2 Screening of waterborne pathogenic Leptospira

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114 2.2.1 Test of a filtration-based method of water-borne Leptospira screening

115 Published studies on water-borne leptospires usually rely on high-speed centrifugation methods 116 and freezing preservation of field samples (e.g., Ganoza et al. 2006; Munoz-Zanzi et al. 2014; Lall et al. 117 2016; Riedeger et al. 2016; Thibeaux et al. 2017; Sato et al. 2019). These approaches are poorly adapted to field surveys, especially in developing countries where lab facilities are usually hardly available 118 and/or distantly located. In order to adapt protocols of Leptospira screening of waters to the latter 119 120 conditions, we developed a simple filtration-based method that can be used in the field in absence of 121 costly and high-tech materials. To do so, we first experimentally compared the percentage of bacteria 122 recovery from mineral water (EC=180µS/cm) using centrifugation on the one hand, and filtration on the other hand. For centrifugation tests, lab water samples were inoculated with various amounts (from 123 124 36,800 to 3,680,000 cells per sample, each sample in sextuplets) of living culture-originating *Leptospira* 125 interrogans serovar Icterohaemorragiae strain Verdun (Spirochete Laboratory, Pasteur Institute of Paris, 126 National Reference Center for Leptospirosis and WHO Collaborative Center). Centrifugation consisted 127 in spinning 15mL of inoculated lab water samples at 4,000 rpm during 30 minutes. Water-borne DNA 128 was extracted immediately after centrifugation. Filtration was performed on 500mL of inoculated lab water samples (representing 2,000 to 2,000,000 cells per sample, each sample in triplicates) that were 129 130 sequentially filtered on 0.45µm and 0.1µm nitrocellulose membranes. Filters were then stored in ethanol 131 96° for subsequent DNA extraction.

Total DNA was extracted from both centrifuged and filtered samples using the Biobasics EZ10 Spin column Genomic DNA Minipreps kit, and eluted in 50µL of elution buffer. The same amounts
of expected bacteria were extracted directly from 20µL of culture medium as a positive control.

135 Leptospires detection and quantification were performed following a probe-based qPCR method 136 that targets a 199bp-long fragment of the *LipL32* gene using a LightCycler® 480 (Roche Diagnostics) in 96-well micro-titre plates with a 10µL final volume for each reaction (see Dobigny et al. 2015). All 137 138 qPCR investigations were conducted in duplicates. Negative and positive controls were systematically 139 included in each qPCR detection plates. Extraction negative controls consisted in lab water that was 140 filtered and extracted in the same conditions than the inoculated water samples, while negative qPCR 141 controls consisted in molecular grade water used for qPCR mix preparation steps. Positive controls consisted in the qPCR reactions that were used to construct the standard curve (i.e. DNA directly 142 143 extracted from Leptospira culture).

144

145 2.2.2 Leptospires screening in field water samples

For each of the 193 samples, one liter was collected during the morning. The 1 L bottles were 146 rinsed at least twice with the sample water before collection. Up to 500ml of water aliquots were filtered 147 148 for Leptospira screening within the same day, once in the lab. All field water samples were first filtered using 5μ m filter papers to retain the large suspended particles. Waters were then filtered using 0.45 μ m 149 filter papers. Clogging sometimes occurred, especially when filtering turbid waters. In such cases, 150 successive filters were used until 500mL in total had been filtered (e.g. filter 1 used to filter, say, 250mL; 151 152 filter 2 to filter 150mL; filter 3 to filter the last 100mL). Each filter was preserved in an independent 153 tube, thus allowing us to perform independent lysis. Digestion products were then pooled to go through the same single DNA extraction Qiagen© column. DNA extraction and leptospirosis quantification 154 155 proceeded as described above, except that for each water sample both 5µm and 0.45µm filter papers were pooled for DNA extraction. 156

158 **2.3 Physico-chemical analysis of waters**

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A wide spectrum of physico-chemical parameters were measured for each water sample investigated for *Leptospira*. These included the field parameters temperature, electrical conductivity (EC), dissolved oxygen (DO) and pH, and laboratory analysed parameters stable isotopes, major ions and trace elements. The methods and results of these hydrochemical data are detailed in Houéménou et al. (2019b, 2020).

165 The comparison of physico-parameters of leptospire-positive and leptospire-negative waters was 166 performed using univariate plots. Principal Component Analysis (PCA) was used under XLSTAT 167 (Addinsoft 2019) to explore in a multivariate manner the chemical characteristics of water samples 168 where pathogenic leptospires were found compared to those with no leptospires. In addition, using the 169 same parameters as for the PCA, a hierarchical cluster analysis (CA) was performed to identify 170 homogeneous groups of leptospire-negative pools that have similar chemical characteristics to leptospire-positive pool samples. The degree of association between pond sampling sites is represented 171 by the euclidian distance between groups. 172

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174 **3. RESULTS**

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176 **3.1** Centrifugation *vs*. filtration-based approaches

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Our experimental comparison of centrifugation- vs. filtration-based approaches showed that spinning allowed us to recover only $24.45\% \pm 10.14$ of the initial inoculated number of bacteria, while $98.30\% \pm 23.78\%$ was recovered by filtration (see Suppl. Fig. 1), thus demonstrating that the latter method provides an excellent opportunity to survey environmental leptospires. Importantly, the recovered bacteria were unevenly distributed among the 0.45μ m and 0.1μ m filter membranes, with $87.50\% \pm 23.78\%$ 183 22.26% and $10.80\% \pm 5.36\%$, respectively. Importantly, there was a high retention of leptospires on the 184 0.45µm filter papers, which was also observed by Kaboosi et al. (2010). We did not detect any major 185 differences in recovery rates between the different amounts of inoculated bacteria, except for very low 186 initial concentrations that resulted in higher variations between experimental replicates as well as lower 187 recovery rates by filtration (65% of recovery of the inoculum of 2,000 bacteria in 500mL) – which still 188 remained much better than with centrifugation (<10%; Suppl. Fig. 1).

189

190 **3.2** *Leptospira*-positive waters

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192 In total, six water samples were found pathogenic Leptospira-positive, with Ct ranging from 193 37.3 to 43.6 (i.e., 9 to 282 Leptospira genome equivalents per litre; gEq/L). Such Ct values may appear 194 high. However, we considered them as unambiguous tru positive signals that clearly differ from all other 187 negative samples that displayed perfectly flat fluorescence curves despite up to 60 amplification 195 196 cycles (data not shown). Though close to (and even sometimes beyond) theoretical detection limit, such 197 high Ct values are regularly observed in rodent samples in which 16S rDNA Leptospira sequence can yet be obtained (P. Gauthier & G. Dobigny, unpublished data). However, they probably correspond to 198 199 quite low bacterial load, thus precluding quantification that would make sense.

200 Five positive samples (9, 18, 44, 270 and 282 gEq/L, respectively) originated from pond water 201 in Agla, whilst a sixth positive sample (109 gEq/L) was from groundwater collected inside a well at 202 Ladji (Table 1). In Agla, *Leptospira*-positive waters were from both temporary and permanent ponds. 203 For temporary ponds the total prevalence was 9.5% (2/21). In comparison, for the permanent ponds the 204 prevalence was 27.3 % (3/11) (Table 1). All positive samples were obtained in June (2 temporary ponds 205 in Agla and one groundwater well in Ladji in June 2017; 3 permanent ponds in Agla in June 2018; 206 Fig.1a, 1d and 1e). No positive samples were found in Saint Jean, and no leptospires were detected in 207 the 83 tap waters analyzed.

210

211 The results of the physicochemical analysis of the early wet season (June) Leptospira-positive 212 pond waters in Agla, Ladji and Saint-Jean indicated there was little distinction with early wet season 213 pond waters that were Leptospira-negative (data presented in Suppl. Figure 1). Leptospira-positive ponds in Agla had water temperatures ranging between 28.2 and 31.9 °C, which is within the range for 214 215 all pond waters analysed (24.9-35.9°C). The pH of the Leptospira-positive pond waters (6.72-8.67) were 216 also within the range of pH for all other ponds sampled (6.69-9.85). This only partly overlaps the pH range for waters usually recognized for pathogenic Leptospira survival (i.e. pH 5.5 - 7.6; reviewed in 217 Barragan et al., 2017). The DO and EC values of the Agla Leptospira-positive pond waters ranged from 218 219 0-7.5 mg/L and 62-796 µS/cm, respectively, which are within the same range for all of the pond waters 220 sampled (0-12.4 mg/L and 28-4090 µS/cm respectively). Major ion and trace element concentrations of 221 positive ponds are also within the same ranges as ponds that were *Leptospira*-negative. For example, 222 the concentrations of the cations Na and Ca in Leptospira-positive ponds ranged between 6.8-78.9 and 0.4-62.7 mg/L, respectively, and between 1.1-8415.5 and 0.2-276.9 mg/L, respectively, for negative 223 ponds. The concentrations of the anions in the positive ponds include HCO₃ and Cl that range between 224 225 26.0-284.3 and 4.5-74.6 mg/L, respectively, and in the negative ponds these range from 11.7-1115.1 and 226 0.7-16114.9 mg/L. The results of selected trace elements for Leptospira-positive ponds includes 37.6-65.9 µg/L for Al, 3.1-194.2 µg/L for Mn, 7.9-3099 µg/L for Fe, 13.8-51.1 µg/L for B and 3.0-24.6 µg/L 227 228 for Zn.

The univariate plots also highlight that the *Leptospira*-containing waters have T°C, pH, EC, DO, major ions and trace elements that overlap with the range of values observed for all urban pond water samples. Similarly, the multivariate analyses shows overlaps of the four first principal components (that together represent 62% of the total variance) between *Leptospira*-positive waters and other urban pond waters (Fig. 2). The results of the hierarchical cluster analysis (AC; illustrated in the dendrogram on Fig. 3) also show that the positive pond samples (N38, N21, N18 and N25) are grouped within the same class as several other negative pond samples (e.g. A2, A1, A7, A3, A17, A16, A10, A12, A14).

The only groundwater sample to be Leptospira-positive also had values of chemical parameters 236 237 that were similar to ranges observed in groundwater sampled from the shallow aquifer that were 238 Leptospira-negative. In Ladji, the Leptospira-positive groundwater sample had a temperature of 29.4°C 239 and a pH of 7.69, which is within the range of values for other groundwater samples (26.2-31.1°C and 240 5.85-7.84 respectively). In addition, the DO and EC of the Leptospira-positive groundwater (4.4 mg/L and 1357 µS/cm respectively) was also within the range for all other Leptospira-negative groundwater 241 242 values (0.1-8.5 mg/L and 286-2480 µS/cm respectively).

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3.4 Seasonality of Leptospira-positive waters 245

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247 Leptospira-positive ponds (both temporary and permanent) were found only in the early wet season month of June (2/10 in June 2017, and 3/5 in June 2018), whilst those from the dry season month 248 249 of October 2017 (N=6) were all Leptospira-negative. In addition, the results of the stable isotope data 250 from Agla waters (Fig. 4) show that for four of the Leptospira-positive ponds (stable isotope data for 251 the fifth pond is not available) the pond water was recharged by light to moderate rainfall (≤ 250 252 mm/month). Two of the Leptospira-positive permanent ponds have stable isotope values close to rainfall 253 values when rainfall levels are between 50 and 250 mm/month. For the other two Leptospira-positive ponds (one permanent and one temporary) the stable isotope values are close to rainfall values when the 254 volumes are < 50 mm/month (Fig. 4). The sable isotope values of these 4 ponds (-3.6 to -1.3 % for δ^{18} O 255 and -0.3 to -16.6 % for δ^2 H) also show that the pond waters are neither evaporated nor affected by 256 257 mixing with the saline lake water.

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259 In addition, the hydrochemical results indicate low or insignificant groundwater inflows to these Leptospira-positive ponds during the wet season in June. For example, based on the (Na+Ca)/Cl ratios 260

and Cl concentrations (data from Houéménou et al. 2019b), the four Leptospira-positive ponds (the fifth 261 pond does not have major ion data) from Agla have waters with relatively higher (Na+Ca)/Cl molar 262 263 ratios and lower Cl concentrations compared with most groundwater samples (Fig. 5). Therefore, despite the permanent ponds being sustained by groundwater inflows during the dry season, during the wet 264 season the Leptospira-positive permanent ponds have received greater inflows from recent rainfall and 265 show a greater resemblance to the Leptospira-positive temporary ponds in terms of major ion 266 267 composition. This is also illustrated by the time series data of one of the Leptospira-positive permanent ponds. For example, when this pond is a groundwater discharge site and Leptospira-negative during the 268 dry season, the EC is 2,900 µS/cm (February 2018). In comparison, when the pond is Leptospira-269 270 positive and has higher rainfall contributions in the wet season, the EC has decreased by over 80% (June 271 2018, EC = 537 μ S/cm) and is closer to the EC values of temporary *Leptospira*-positive ponds (62-862) 272 μ S/cm).

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274 **3.5 Cartography of pond waters**

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In Agla, GIS-based evaluation of land use at the beginning of the rainy reason shows that ponds represent quite wide surfaces with 14.7% and 14.1% of the whole surface of the study area in June 2017 and June 2018, respectively. Ratios were similar (16% in June 2017) or lower (4% in June 2018), and close to null (1.6% in June 2017 and 0.17% in June 2018) in Saint-Jean. The other values range between 0.01% (in Agla in October 2016) to 2.99% (in Ladji in March 2018), with one exception in Agla in October 2017 (10.4%).

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284

286 4. DISCUSSION

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Leptospirosis is considered as one of the most widely distributed zoonosis, but its impact is 288 289 probably greatest the tropics, especially in Africa where it remains poorly documented (Pappas et al., 290 2008; Costa et al., 2015). Therefore, studies of leptospirosis in Africa are urgent to improving public health. The filtration-based protocol developed in this study may be particularly useful in remote areas 291 292 and/or in areas suffering from the lack of well-equipped technical facilities. First, it shows that, when 293 investigating waterborne leptospires in samples that have been filtered, the leptospire screening should include both the water sample and the filter paper (e.g. Wojcik-Fatla et al., 2014). Second, although our 294 method may potentially be less sensitive than those involving specific DNA extraction kits, high-speed 295 centrifugation, bacterial culture and/or high-throughput genetics (e.g., Lall et al. 2016; Riediger et al. 296 297 2016; Sato et al. 2019), it appears simple, cheap and easily implementable in the field. However, it may 298 further be improved by being coupled with higher efficiency DNA extraction kits (Riediger et al. 2016), 299 something that we did not test here. In addition, we did not investigate possible negative impacts of 300 suspended matter in field water samples on our filtration approach as well as on our qPCR-based 301 detection method (Riedeger et al. 2016). In some instances, we noted that after filtration, filter papers 302 appeared quite coloured, thus suggesting the presence of undetermined suspended particles. The latter 303 may have inhibited to some extent subsequent DNA extractions and qPCRs. However, Leptospira-304 positive samples were among the most turbid water samples (not shown), thus providing confidence on 305 our ability to indeed detect leptospires in environmental samples. To highlight the possible presence of 306 inhibitory compounds in these coloured filter paper samples, a known amount of leptospire DNA 307 obtained from a bacterial culture was added after extraction. These Leptospira-enriched samples 308 remained qPCR-negative, suggesting the presence of qPCR inhibitors co-extracted with the DNA (data 309 not shown). Consequently, we cannot definitely exclude the possibility of false negatives where the waters were very turbid, and our results from the field should be cautiously interpreted as potentially 310 311 underestimated. Additional in vitro tests would be useful to address this particular issue and to validate 312 and optimize our centrifugation-based approach for further studies of environmental samples. In a more

general manner, our experience confirms previous results that highlight the importance of testing for the
possible existence of PCR inhibitors when genetically investigating for the presence or quantity of
leptospires in environmental samples (Casanovas-Massana et al., 2018).

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The results retrieved from Cotonou waters demonstrate for the first time that water-borne leptospires circulate in the heart of this large African city (3.1% of total prevalence; 5.5% when tap waters are excluded; see Table 1).

320 It is noteworthy that leptospire-infected waters were found in the two most insalubrious areas, 321 namely Agla and Ladji, whilst none were observed in the popular but non-floodable Saint Jean district. 322 A survey of 780 rodents was conducted in the exact same areas of these three districts, and during the 323 exact same periods (Dossou et al. unpublished data). Interestingly, data in rodents fit well to patterns 324 retrieved in waters since 11.8% and 14% of rodent-borne leptospire prevalence were observed in Agla 325 and Ladji, respectively, while only 0.4% were positive in Saint Jean. This expectedly points towards a higher environmental Leptospira contamination in poor, lowland and lake-edge districts where both 326 327 commensal rodents and surface water are omnipresent (Houéménou et al. 2014; Dobigny et al. 2018).

328 However, different patterns are observed between the high-risk districts of Agla and Ladji. The 329 positive water sample found in Ladji was collected in a groundwater well, thus suggesting that either 330 groundwater in the aquifer was contaminated from infiltration of Leptospira-positive water from the 331 surface, or by accident (e.g., use of an infected bucket or rope, or an infected rodent falling down the well). At this stage, we cannot decipher between the two hypotheses, and since the sample size is low 332 333 we cannot draw robust conclusions on the potential of groundwater contamination by leptospires. 334 Further investigation of leptospires in groundwater in Cotonou deserves special attention, whatever its origin, since one well out of two (50%) was affected in Ladji in June 2017. It is also worth noting that 335 336 although the groundwater in Agla is shallow, and in contact with permanent ponds water via recharge 337 in the wet season and discharge in the dry season, no leptospires were detected in the Agla groundwater. 338 In comparison, other studies have found groundwater well to be contaminated by leptospires (i.e. in rural Chile; Munoz-Zanzi et al. 2014). In Cotonou, human activities in the immediate proximity of groundwater wells are plentiful, with many children and women searching water daily with their bare feet in close contact with humid soils and the water surrounding the well. Therefore, on-going investigations of leptospires in groundwater is of critical importance.

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In Agla, all Leptospira-positive waters originated from ponds, both temporary and permanent, 344 345 and all were collected in June. Although more intensive sampling is required during different rainfall 346 events, such a pattern strongly suggests that the water-mediated risk of leptospirosis infection may be higher at the beginning of the rainy season through ponds. Our results agree with the conclusions drawn 347 348 from other studies where leptospiral risk is variable in both time and space, and is strongly dependent 349 on local environmental conditions (reviewed in Barragan et al. 2017). Similar to the results in Cotonou, 350 seasonality patterns were also observed in several regions, such as the Pau da Lima slum (Brazil), where 351 sewage and puddle samples were detected positive more often during the rainy season (Casanovas-352 Massana et al. 2018). Pathogenic leptospires were also detected in several other urban surface waters 353 (see Tab. 1 in Barragan et al. 2017) where spatial heterogeneity is observed. For example, on the south 354 Andaman Island, the water-borne leptospires were only found in household drainage (18.7%) and 355 sewage (9.8%) waters and, unlike in Cotonou, there were no leptospire found in the pond waters (Lall et al. 2016). The variable results from each study once again highlights the importance of understanding 356 the local socio-environmental context in determining leptospire transmission pathways to humans (e.g., 357 358 Maciel et al. 2008; Himsworth et al. 2013; Mwachui et al. 2015; Houéménou et al. 2019a; reviewed in 359 Barragan et al. 2017).

The hydrochemical analysis of contaminated waters strongly suggest there are key hydrological processes that may favor the presence of leptospires in the surface waters of Cotonou. Indeed, stable isotopes and major ion data (from Houéménou et al., 2019b) indicate that leptospires are found in temporary and permanent ponds at the beginning of the rainy season that have recently formed as a result of low to moderate precipitation, and have relatively high runoff inputs compared to groundwater or lake water inputs. This agrees with the expectation that there will be larger concentrations of the pathogen in small water bodies (Barragan et al. 2017). It also corresponds well with the proposed model
of soil-to-water dispersion of leptospires recently recapitulated by Bierque and colleagues (2020).
Finally, and although the causal relationship remains untested, these results are strikingly consistent
with previous data on south Benin urban rodents in which *Leptospira* prevalence was found higher
during (or soon after) periods of moderate rains (100-200 mm; Houéménou et al. 2019b).

371

372 Though data are quite scarce, some physico-chemical parameters are known to have an influence 373 on Leptospira presence, survival and virulence (e.g., Chang et al. 1948; Khairani-Bejo et al. 2014; André-Fontaine et al. 2015; Lall et al. 2018). Our observations strongly suggest that most negative pond 374 samples have similar physico-chemical characteristics to those positive for pathogenic Leptospira. In 375 376 particular, it was recently suggested that iron, manganese and copper could influence the survival of 377 leptospires in Andamanese soils (Lall et al. 2018). However, our data do not confirm such a trend since leptospire-positive and negative water samples did not show any noticeable differences in these ions 378 379 concentrations (see Suppl. 2). Overlaps observed with both univariate and multivariate approaches mean that no association between physicochemical parameters and presence (Agla, Ladji) or absence (Saint 380 Jean) of water-borne leptospires can be identified (Suppl. Fig. 3). This means also that the possible 381 382 ecological spectrum of water-borne leptospires extensively covers the physico-chemical spectrum of Cotonou waters as a whole. For instance, pH and temperature of Cotonou waters, which are suspected 383 384 to be of primary importance for leptospires biology (Chang et al. 1948; Gordon-Smith & Turner, 1961; 385 Khairani-Bejo et al. 2004; André-Fontaine et al. 2015), are perfectly compatible with pH and 386 temperature ranges that allow for the long-term survival of pathogenic leptospires (André-Fontaine et al. 2015). In other words, the potential distribution of waters compatible with Leptospira survival, hence 387 388 human contamination risk, is quite wide in the city, meaning that, though quite polluted (Lapworth et 389 al. 2017; Houéménou et al. 2019b), urban waters in Cotonou are widely compatible with the leptospire 390 life cycle. In the city and, beyond, along the West African Abidjan-Lagos urban corridor, surface waters 391 inundate many zones of the public space, including areas that are used daily by the inhabitants: walking pathways and streets, courtyards and even homes may be flooded for several days if not weeks. 392

Consequently, the population has extensive and on-going contact with potentially contaminated water.
Altogether, this indicates that millions of people living along the extensively urbanizing Guinea Gulf
are at risk for falling sick of leptospirosis.

396

397 **5. CONCLUSION**

398

399 Future studies should include two major aspects of leptospires ecology, hence leptospiral 400 contamination risk, that were not taken into consideration here: water microbiota and biofilms (e.g., 401 Trueba et al. 2004; Kumar et al. 2015) on the one hand, and soil-associated leptospires on the other hand 402 (e.g., Thibeaux et al. 2017; Bierque et al. 2020). Nevertheless, our filtration-based results, though 403 potentially underestimated, clearly show that the Cotonou inhabitants live in an urban environment that 404 exposes them to leptospirosis infection risk, potentially at a wide spatial scale. Everyday uses and habits 405 as well as a lack of street and sanitation infrastructures results in many people in close contact with standing waters on a regular basis, especially during the rainy season and associated flooding events. 406 Spatio-temporal investigations of water-borne leptospires point towards a higher risk at the beginning 407 of the rainy season, especially via ponds that are recharged by rainfall. We believe that awareness 408 409 increase about leptospirosis should be a public health priority since the disease is very poorly documented and obviously overlooked in Benin and, further, along the rapidly extending Abidjan-Lagos 410 411 urban corridor.

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Figure 1. Sampling sites. Map of Cotonou showing the three neighborhoods Agla, Ladji and Saint Jean (a). Pictures of precarious and stilt habitats in Ladji (b), Saint-Jean (c) and Agla (e and f). A few of the

sampling sites ((c)Google Earth) to illustrate the sampling protocol in Agla (d) with blue, yellow and red dots corresponding to groundwater, Leptospira-negative and Leptospira-positive ponds, respectively. A19 indicates a Leptospira-positive pond presented in the photo (f).



Figure 2. Principal Component Analysis showing the physico-chemical characteristics of the *Leptospira*-positive samples (red dots) among all urban water samples collected in Agla and Ladji.



Figure 3. Hierarchical cluster analysis dendrogram showing the relationship between the *Leptospira*-positive pond samples (*) and *Leptospira*-negative samples in Agla.



Figure 4. Stable isotopes of rainwater, pond water and shallow groundwater samples in Agla during the wet and dry 2017 and 2018 seasons (modified from Houéménou et al. 2019).



Figure 5. (Na+Ca)/Cl molar ratios and Cl concentrations of pond water and shallow groundwater samples in Agla during the wet and dry 2017 and 2018 seasons (modified from Houéménou et al. 2019).

Table 1. *Leptospira* screening results by sampling sites and dates. "ok": physico-chemical data are available; "NA": not assessed; "t.ponds": temporary ponds; "p.ponds": permanent ponds.

AGLA		Mar-17	Jun-17	Oct-17	Feb-18	Jun-18
N=85	Groundwater	7	7	8		1
	Temporary ponds		10	6		5
	Permanent ponds				3	8
	Tap water	10	10	10		
	Total	17	27	24	3	14
	Positive leptospires		2+(t.ponds.)			3+(p.ponds.)
	Total prevalence	0.00	0.07	0.00	0.00	0.20
	Ponds prevalence	NA	0.20	0.00	0.00	0,21
	Measures		ok	ok	ok	1/2
LADJI		Mar-17	Jun-17	Oct-17	Feb-18	Jun-18
N=53	Groundwater	3	2	3		
	Temporary ponds		5	3		
	Permanent ponds	1			2	3
	Tap water	10	10	8		
	Lake					
	Total	14	18	15	2	4
	Positive leptospires		1+(well)			
	Total prevalence	0.00	0.06	0.00	0.00	0.00
	Groundwater prevalence	0.00	0.50	0.00		
	Measures	NA	ok	ok	ok	ok
St JEAN		Mar-17	Jun-17	Oct-17	Feb-18	Jun-18
N=55	Groundwater	10	10	10		
	Tap water	10	9	6		
	Total	20	19	16		
	Positive leptospires	0	0	0		
	Total prevalence	0.00	0	0.00		
	Measures	NA	ok	ok		

