Pathogenic Leptospira and water quality in African cities: A case study of Cotonou, Benin

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Pathogenic *Leptospira* and water quality in African cities: a case study of Cotonou, Benin

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1. INTRODUCTION

Leptospirosis is a bacterial disease caused by spirochetes of the genus *Leptospira*. Leptospires infect and develop inside renal tubules of a variety of Vertebrates, essentially mammals, notably rodents, which excrete the pathogen into the external environment. Humans are infected following contact with contaminated water or humid soils. Symptoms range from null to very severe and death (reviewed in Haake & Levett 2015). It is considered that one million cases occur annually in the world, among which 60,000 have fatal issues (Costa et al. 2015). Leptospirosis has been mainly associated with rice agriculture and breeding as well as water recreational activities (Mwachui et al. 2015). Flooding episodes may also trigger local leptospiral epidemics (e.g., Thaipadungpanit et al. 2013), with one out 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 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 areas (e.g. in Peru, Ganoza et al. 2006). As a consequence, ongoing urbanization together with multiplying extreme climatic events are expected to increase the risk of human leptospirosis (Lau et al. 2010). This may be particularly true in Africa where the disease is present but yet remains poorly documented (reviews in de Vries et al. 2014; Allan et al. 2015). In particular, we are aware of no study focusing on soil- or waterborne leptospires in Africa (see Tab. 2 in the review by Bierque et al. 2020).

The so-called « Abidjan-Lagos corridor » (ALC) is a 700km-long conurbation that sprawls along the West African Atlantic coast. It currently houses >25 million people who live within and in the surroundings of large adjacent cities such as Lagos (11.8 million inhabitants), Porto-Novo (570,000 inhabitants), Cotonou (1.5 million inhabitants), Lomé (1.7 million inhabitants), Accra (4.4 million inhabitants) and Abidjan (4.7 million inhabitant) (2015 data; OECD/Africapolis project, www.africapolis.org, 2020). It is expected to reach ca. 34 million city dwellers by 2025 (UN Habitat, 2014). This very rapid and mostly uncontrolled urbanization translates into the creation and expansion of vast socially disadvantaged areas where pollution, access to basic services (e.g., health care, 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, mangroves and swamps which form a dense hydrographic network. Together with a subequatorial 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 following Lake Nokoué overflows or rain accumulation in shallows. Such episodes directly affect 200,000 inhabitants and indirectly impact many others through service interruption, resource unavailability or degraded water quality (PCUG3C, 2010; Houéménou et al. 2019b). In addition, they are often enhanced by poor drainage due to defective or crowded sanitation network as well as anarchic land use (PCUG3C, 2010).

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 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 environment, though with strong prevalence variations in both space and time (Houéménou et al., 2019a). Mean annual temperatures (ca. 27°C) and rainfalls (ca. 800-1,600mm) are highly favorable to *Leptospira* survival. However, although waters and humid soils appear as the corner stone of human contamination (Barragan et al. 2017; Bierque et al. 2020), little is known about the precise environmental conditions that are compatible with pathogenic *Leptospira* outside of its mammalian host (see for instance Chang et al. 1948; Gordon-Smith & Turner 1961; Khairani-Bejo et al. 2004; Wojcik-Fatla et 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 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 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 an urban polluted habitat in Africa.

2. MATERIAL AND METHODS

2.1 Study area and sampling

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.

The study area involves 3 districts located in the core city of Cotonou: Ladji, Agla and Saint 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 stilt pegs (Fig. 1b). Lacustrine waters are permanent, but flooding of zones adjacent to the lake only occurs at the end of the rainy season (i.e., September and October) following the rise in lake levels. 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 populated, has essentially informal housing development and is a very poor area where even the most basic public services are usually missing. In particular, waste management is almost inexistent, garbage are omnipresent and are even often used as embankment material. Such an environment favors proliferation of rodents, which are abundant and infest 60-100% of households depending on the season (Dobigny et al. 2019).

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

81 are formed by groundwater discharge in the dry months, whereas during the winter months the rainfall
82 inputs to the ponds reverse the hydraulic gradients, resulting in groundwater recharge ponds
83 (Houéménou et al. 2019b). The habitat in Agla includes hard-built houses as well as very precarious
84 cabins, with the poorest inhabitants usually gathering around or within these floodable low-lying areas
85 (Fig. 1b). These low-lying areas are also widely used as dumping sites for household waste (Fig. 1b).
86 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

91 In 2017 and 2018, the three districts of Cotonou (Agla, Ladji and Saint Jean; Fig.1b) were
92 monitored for both water quality parameters (physical parameters, major ions and trace elements) and
93 leptospire presence. Water sampling was organized concomitantly (i.e. within the same week) in the
94 three districts during the dry season (March 2017 and February 2018) as well as at the beginning (June
95 2017 and June 2018) and at the end (October 2017) of the rainy season. In total, 193 water samples were
96 collected: 85 in Agla, 53 in Ladji and 55 in Saint Jean. Eighty-three samples were tap waters and 61, 29,
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
102 dry season. Both temporary and permanent ponds were sampled in Ladji (8 and 6, respectively) and
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 each
106 of the five seasonal sampling campaigns. The land use categories and the extent of surface waters

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

2.2 Screening of waterborne pathogenic *Leptospira*

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

Published studies on water-borne leptospires usually rely on high-speed centrifugation methods and freezing preservation of field samples (e.g., Ganoza et al. 2006; Munoz-Zanzi et al. 2014; Lall et al. 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 and/or distantly located. In order to adapt protocols of *Leptospira* screening of waters to the latter conditions, we developed a simple filtration-based method that can be used in the field in absence of costly and high-tech materials. To do so, we first experimentally compared the percentage of bacteria 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 36,800 to 3,680,000 cells per sample, each sample in sextuplets) of living culture-originating *Leptospira interrogans* serovar Icterohaemorragiae strain Verdun (Spirochete Laboratory, Pasteur Institute of Paris, National Reference Center for Leptospirosis and WHO Collaborative Center). Centrifugation consisted in spinning 15mL of inoculated lab water samples at 4,000 rpm during 30 minutes. Water-borne DNA 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 sequentially filtered on 0.45µm and 0.1µm nitrocellulose membranes. Filters were then stored in ethanol 96° for subsequent DNA extraction.

Total DNA was extracted from both centrifuged and filtered samples using the Biobasics EZ-10 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.

Leptospires detection and quantification were performed following a probe-based qPCR method 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 qPCR investigations were conducted in duplicates. Negative and positive controls were systematically included in each qPCR detection plates. Extraction negative controls consisted in lab water that was filtered and extracted in the same conditions than the inoculated water samples, while negative qPCR 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 extracted from *Leptospira* culture).

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 rinsed at least twice with the sample water before collection. Up to 500ml of water aliquots were filtered for *Leptospira* screening within the same day, once in the lab. All field water samples were first filtered using 5µm filter papers to retain the large suspended particles. Waters were then filtered using 0.45µm filter papers. Clogging sometimes occurred, especially when filtering turbid waters. In such cases, successive filters were used until 500mL in total had been filtered (e.g. filter 1 used to filter, say, 250mL; filter 2 to filter 150mL; filter 3 to filter the last 100mL). Each filter was preserved in an independent 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 proceeded as described above, except that for each water sample both 5µm and 0.45µm filter papers were pooled for DNA extraction.

2.3 Physico-chemical analysis of waters

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).

The comparison of physico-parameters of leptospire-positive and leptospire-negative waters was performed using univariate plots. Principal Component Analysis (PCA) was used under XLSTAT (Addinsoft 2019) to explore in a multivariate manner the chemical characteristics of water samples where pathogenic leptospires were found compared to those with no leptospires. In addition, using the same parameters as for the PCA, a hierarchical cluster analysis (CA) was performed to identify 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 by the euclidian distance between groups.

3. RESULTS

3.1 Centrifugation *vs*. filtration-based approaches

Our experimental comparison of centrifugation- *vs*. filtration-based approaches showed that spinning allowed us to recover only 24.45% ± 10.14 of the initial inoculated number of bacteria, while 98.30% ± 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 0.45µm and 0.1µm filter membranes, with 87.50% ±

22.26% and 10.80% ± 5.36%, respectively. Importantly, there was a high retention of leptospires on the 0.45µm filter papers, which was also observed by Kaboosi et al. (2010). We did not detect any major differences in recovery rates between the different amounts of inoculated bacteria, except for very low initial concentrations that resulted in higher variations between experimental replicates as well as lower recovery rates by filtration (65% of recovery of the inoculum of 2,000 bacteria in 500mL) – which still remained much better than with centrifugation (<10%; Suppl. Fig. 1).

3.2 *Leptospira*-positive waters

In total, six water samples were found pathogenic *Leptospira*-positive, with Ct ranging from 37.3 to 43.6 (i.e., 9 to 282 *Leptospira* genome equivalents per litre; gEq/L). Such Ct values may appear 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 cycles (data not shown). Though close to (and even sometimes beyond) theoretical detection limit, such 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 quite low bacterial load, thus precluding quantification that would make sense.

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

3.3 Chemical characteristics of *Leptospira*-positive and -negative waters

The results of the physicochemical analysis of the early wet season (June) *Leptospira*-positive pond waters in Agla, Ladji and Saint-Jean indicated there was little distinction with early wet season 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 all pond waters analysed (24.9-35.9°C). The pH of the *Leptospira*-positive pond waters (6.72-8.67) were 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 Barragan et al., 2017). The DO and EC values of the Agla *Leptospira*-positive pond waters ranged from 0-7.5 mg/L and 62-796 µS/cm, respectively, which are within the same range for all of the pond waters sampled (0-12.4 mg/L and 28-4090 µS/cm respectively). Major ion and trace element concentrations of positive ponds are also within the same ranges as ponds that were *Leptospira*-negative. For example, 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 ponds. The concentrations of the anions in the positive ponds include HCO_3 and Cl that range between 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 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 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 that were similar to ranges observed in groundwater sampled from the shallow aquifer that were *Leptospira*-negative. In Ladji, the *Leptospira*-positive groundwater sample had a temperature of 29.4°C and a pH of 7.69, which is within the range of values for other groundwater samples (26.2-31.1°C and 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 values (0.1-8.5 mg/L and 286-2480 µS/cm respectively).

3.4 Seasonality of *Leptospira*-positive waters

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 of October 2017 (N=6) were all *Leptospira*-negative. In addition, the results of the stable isotope data from Agla waters (Fig. 4) show that for four of the *Leptospira*-positive ponds (stable isotope data for the fifth pond is not available) the pond water was recharged by light to moderate rainfall (≤ 250 mm/month). Two of the *Leptospira*-positive permanent ponds have stable isotope values close to rainfall 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 volumes are < 50 mm/month (Fig. 4). The sable isotope values of these 4 ponds (-3.6 to -1.3 ‰ for $\delta^{18}O$ and -0.3 to -16.6 ‰ for $\delta^{2}H$) also show that the pond waters are neither evaporated nor affected by mixing with the saline lake water.

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

and Cl concentrations (data from Houéménou et al. 2019b), the four *Leptospira*-positive ponds (the fifth pond does not have major ion data) from Agla have waters with relatively higher (Na+Ca)/Cl molar 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 season the *Leptospira*-positive permanent ponds have received greater inflows from recent rainfall and show a greater resemblance to the *Leptospira*-positive temporary ponds in terms of major ion 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 dry season, the EC is 2,900 µS/cm (February 2018). In comparison, when the pond is *Leptospira*-positive and has higher rainfall contributions in the wet season, the EC has decreased by over 80% (June 2018, EC = 537 µS/cm) and is closer to the EC values of temporary *Leptospira*-positive ponds (62-862 µS/cm).

3.5 Cartography of pond waters

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%).

4. DISCUSSION

Leptospirosis is considered as one of the most widely distributed zoonosis, but its impact is probably greatest the tropics, especially in Africa where it remains poorly documented (Pappas et al., 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 and/or in areas suffering from the lack of well-equipped technical facilities. First, it shows that, when 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 method may potentially be less sensitive than those involving specific DNA extraction kits, high-speed centrifugation, bacterial culture and/or high-throughput genetics (e.g., Lall et al. 2016; Riediger et al. 2016; Sato et al. 2019), it appears simple, cheap and easily implementable in the field. However, it may further be improved by being coupled with higher efficiency DNA extraction kits (Riediger et al. 2016), something that we did not test here. In addition, we did not investigate possible negative impacts of suspended matter in field water samples on our filtration approach as well as on our qPCR-based detection method (Riedeger et al. 2016). In some instances, we noted that after filtration, filter papers appeared quite coloured, thus suggesting the presence of undetermined suspended particles. The latter may have inhibited to some extent subsequent DNA extractions and qPCRs. However, *Leptospira*-positive samples were among the most turbid water samples (not shown), thus providing confidence on our ability to indeed detect leptospires in environmental samples. To highlight the possible presence of inhibitory compounds in these coloured filter paper samples, a known amount of leptospire DNA obtained from a bacterial culture was added after extraction. These *Leptospira*-enriched samples remained qPCR-negative, suggesting the presence of qPCR inhibitors co-extracted with the DNA (data 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 underestimated. Additional *in vitro* tests would be useful to address this particular issue and to validate 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).

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).

It is noteworthy that leptospire-infected waters were found in the two most insalubrious areas, namely Agla and Ladji, whilst none were observed in the popular but non-floodable Saint Jean district. A survey of 780 rodents was conducted in the exact same areas of these three districts, and during the exact same periods (Dossou et al. unpublished data). Interestingly, data in rodents fit well to patterns retrieved in waters since 11.8% and 14% of rodent-borne leptospire prevalence were observed in Agla 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 commensal rodents and surface water are omnipresent (Houéménou et al. 2014; Dobigny et al. 2018).

However, different patterns are observed between the high-risk districts of Agla and Ladji. The positive water sample found in Ladji was collected in a groundwater well, thus suggesting that either groundwater in the aquifer was contaminated from infiltration of *Leptospira*-positive water from the 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 we cannot draw robust conclusions on the potential of groundwater contamination by leptospires. 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 although the groundwater in Agla is shallow, and in contact with permanent ponds water via recharge in the wet season and discharge in the dry season, no leptospires were detected in the Agla groundwater. 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.

In Agla, all *Leptospira*-positive waters originated from ponds, both temporary and permanent, and all were collected in June. Although more intensive sampling is required during different rainfall 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 from other studies where leptospiral risk is variable in both time and space, and is strongly dependent on local environmental conditions (reviewed in Barragan et al. 2017). Similar to the results in Cotonou, seasonality patterns were also observed in several regions, such as the Pau da Lima slum (Brazil), where sewage and puddle samples were detected positive more often during the rainy season (Casanovas-Massana et al. 2018). Pathogenic leptospires were also detected in several other urban surface waters (see Tab. 1 in Barragan et al. 2017) where spatial heterogeneity is observed. For example, on the south Andaman Island, the water-borne leptospires were only found in household drainage (18.7%) and 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 the local socio-environmental context in determining leptospire transmission pathways to humans (e.g., Maciel et al. 2008; Himsworth et al. 2013; Mwachui et al. 2015; Houéménou et al. 2019a; reviewed in 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

366 pathogen in small water bodies (Barragan et al. 2017). It also corresponds well with the proposed model
367 of soil-to-water dispersion of leptospires recently recapitulated by Bierque and colleagues (2020).
368 Finally, and although the causal relationship remains untested, these results are strikingly consistent
369 with previous data on south Benin urban rodents in which *Leptospira* prevalence was found higher
370 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;
374 André-Fontaine et al. 2015; Lall et al. 2018). Our observations strongly suggest that most negative pond
375 samples have similar physico-chemical characteristics to those positive for pathogenic *Leptospira*. In
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
378 leptospire-positive and negative water samples did not show any noticeable differences in these ions
379 concentrations (see Suppl. 2). Overlaps observed with both univariate and multivariate approaches mean
380 that no association between physicochemical parameters and presence (Agla, Ladji) or absence (Saint
381 Jean) of water-borne leptospires can be identified (Suppl. Fig. 3). This means also that the possible
382 ecological spectrum of water-borne leptospires extensively covers the physico-chemical spectrum of
383 Cotonou waters as a whole. For instance, pH and temperature of Cotonou waters, which are suspected
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
387 al. 2015). In other words, the potential distribution of waters compatible with *Leptospira* survival, hence
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
392 pathways and streets, courtyards and even homes may be flooded for several days if not weeks.

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.

5. CONCLUSION

Future studies should include two major aspects of leptospires ecology, hence leptospiral contamination risk, that were not taken into consideration here: water microbiota and biofilms (e.g., Trueba et al. 2004; Kumar et al. 2015) on the one hand, and soil-associated leptospires on the other hand (e.g., Thibeaux et al. 2017; Bierque et al. 2020). Nevertheless, our filtration-based results, though potentially underestimated, clearly show that the Cotonou inhabitants live in an urban environment that exposes them to leptospirosis infection risk, potentially at a wide spatial scale. Everyday uses and habits 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. Spatio-temporal investigations of water-borne leptospires point towards a higher risk at the beginning of the rainy season, especially via ponds that are recharged by rainfall. We believe that awareness 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 urban corridor.

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421 **7. REFERENCES**

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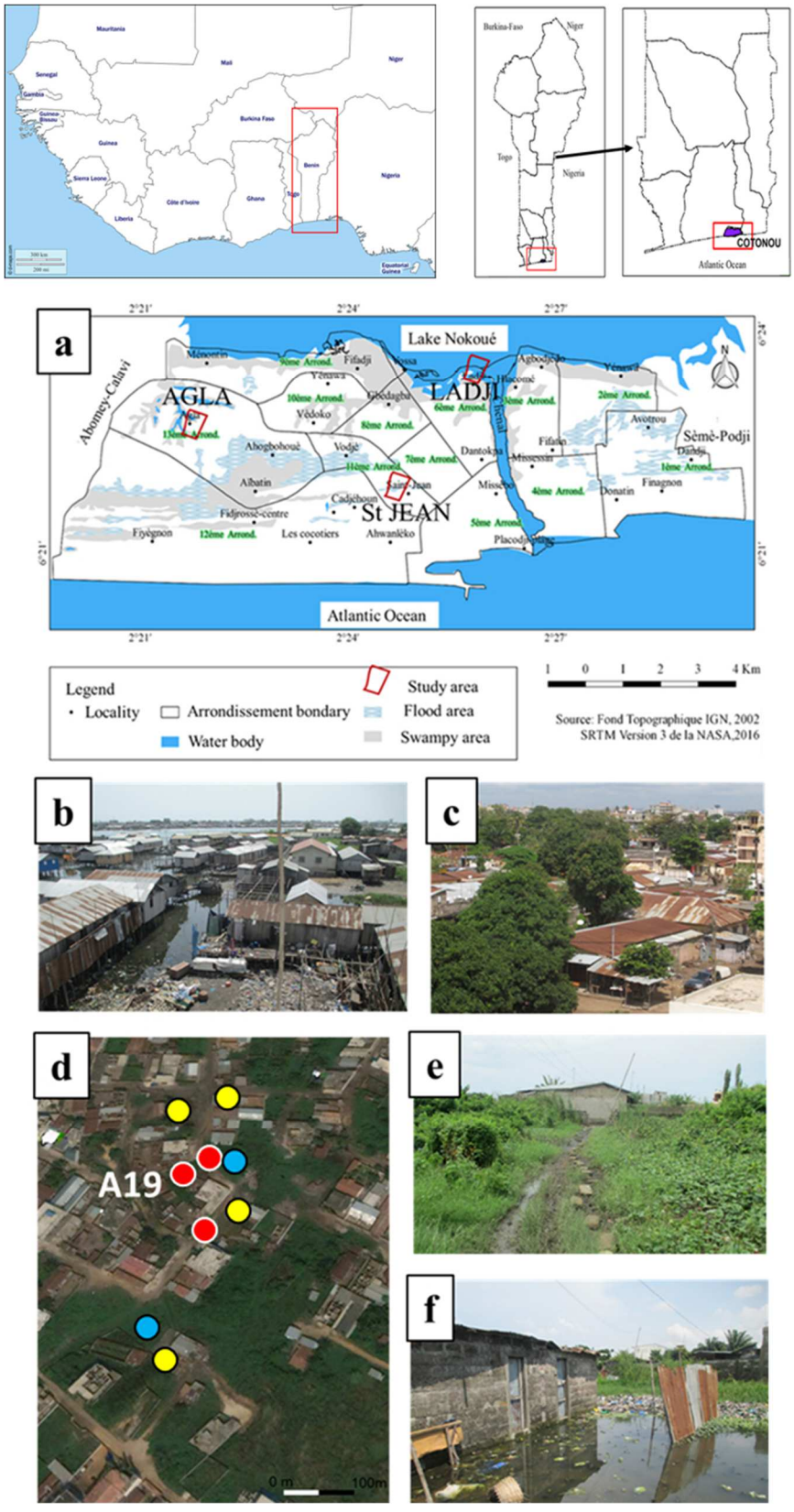


Figure 1. Sampling sites. Map of Cotonou showing the three neighborhoods Agla, Ladji and Saint Jean (a). Pictures of precarious and stilt habitats in Ladji (b), Saint-Jean (c) and Agla (e and f). A few of the

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).

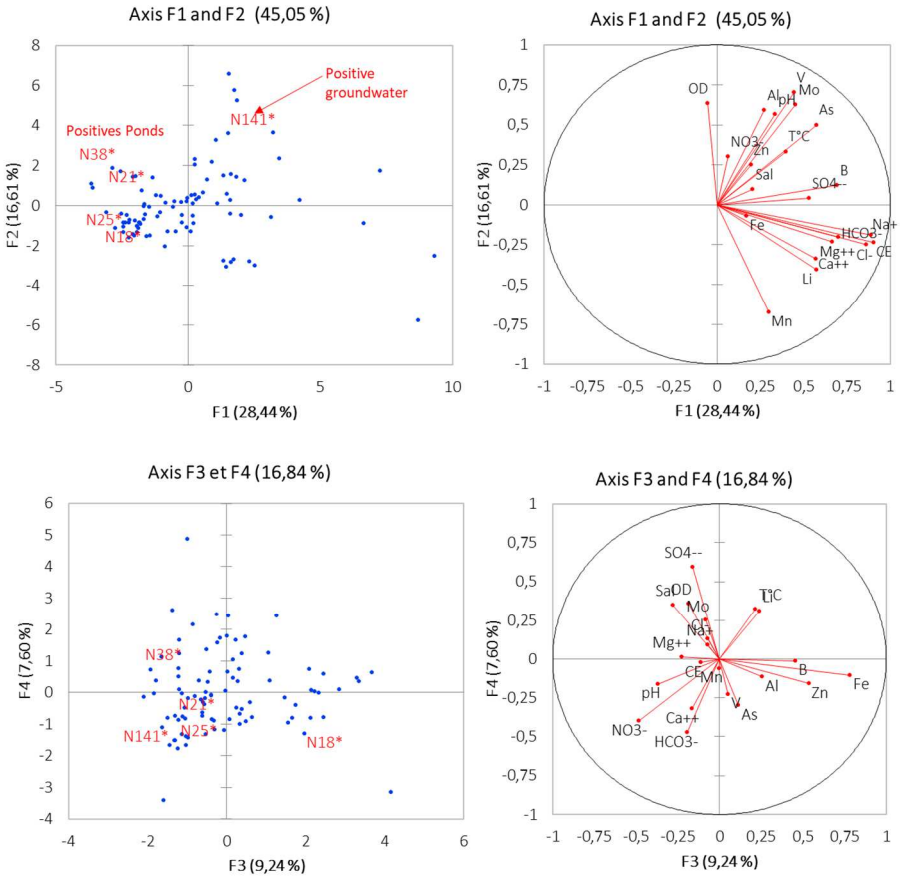


Figure 2. Principal Component Analysis showing the physico-chemical characteristics of the *Leptospira*-positive samples (red dots) among all urban water samples collected in Agla and Ladji.

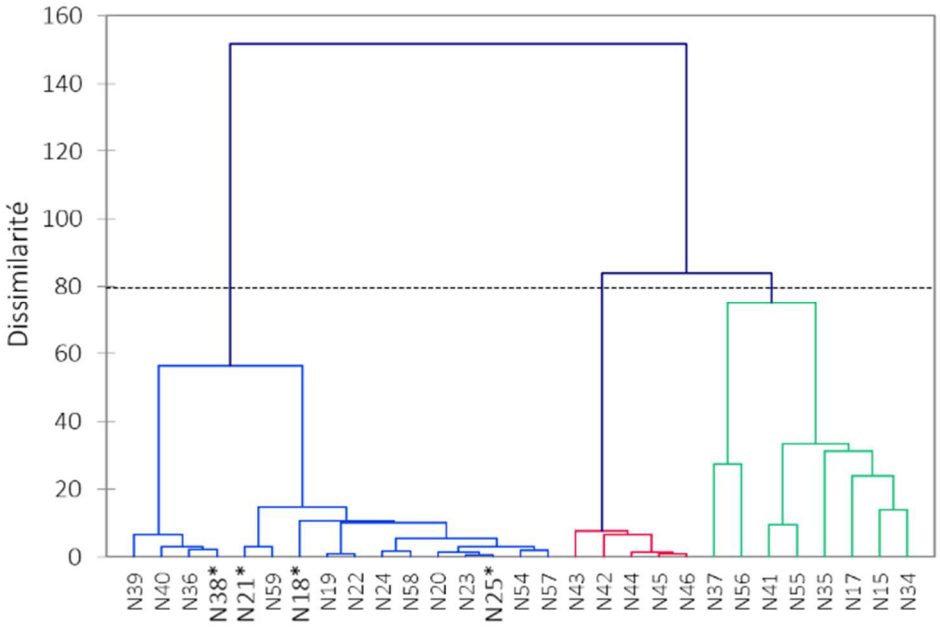


Figure 3. Hierarchical cluster analysis dendrogram showing the relationship between the *Leptospira*-positive pond samples (*) and *Leptospira*-negative samples in Agla.

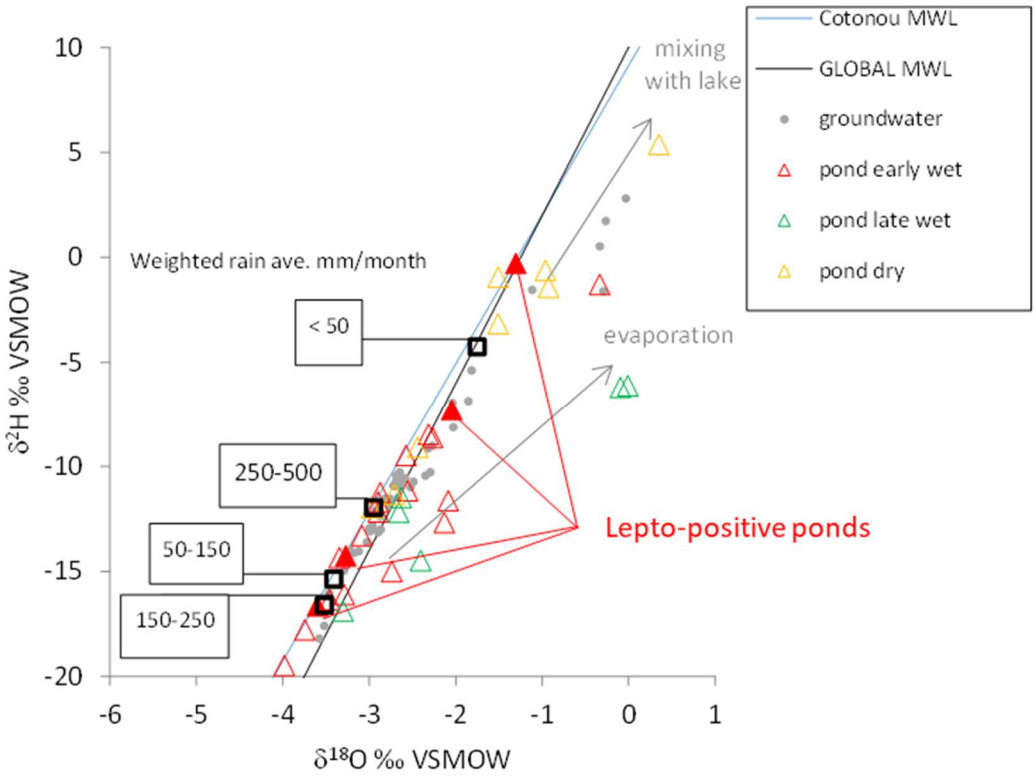


Figure 4. Stable isotopes of rainwater, pond water and shallow groundwater samples in Agla during the wet and dry 2017 and 2018 seasons (modified from Houéménou et al. 2019).

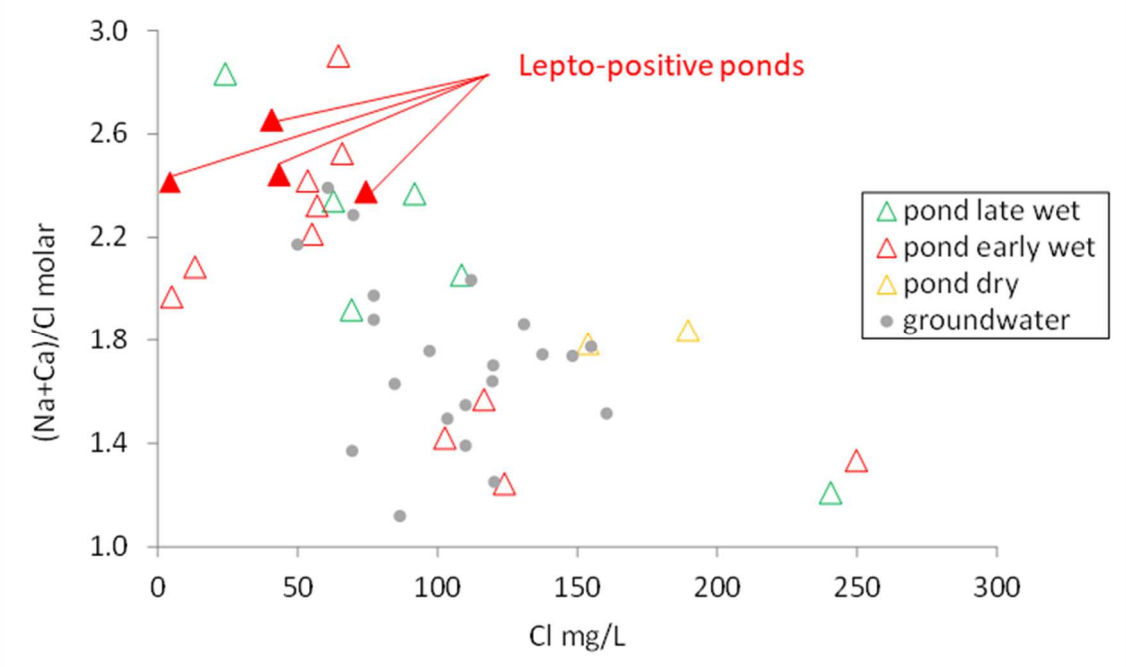
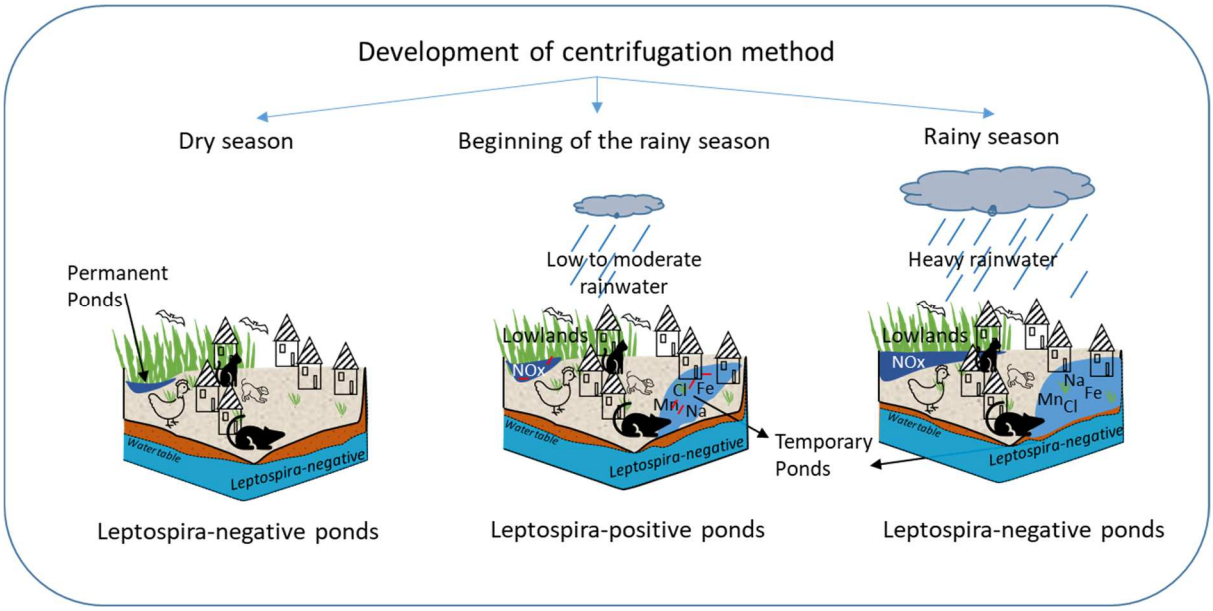


Figure 5. (Na+Ca)/Cl molar ratios and Cl concentrations of pond water and shallow groundwater samples in Agla during the wet and dry 2017 and 2018 seasons (modified from Houéménou et al. 2019).

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		


Development of centrifugation method
Dry season
Beginning of the rainy season
Rainy season
Permanent Ponds
Low to moderate rainwater
Heavy rainwater
Lowlands
NOx
Cl
Fe
Mn
Na
Water table
Leptospira-negative
Temporary Ponds
Leptospira-negative ponds
Leptospira-positive ponds
Leptospira-negative ponds