ANALYSIS OF ENVIRONMENTAL PARAMETERS CORRELATING WITH THE PRESENCE OF LEPTOSPIRACEAE IN HE‘EIA FISHPOND

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I certify that I have read this thesis and that, in my opinion, it is satisfactory in scope and quality as a thesis for the degree of Bachelor of Science in Global Environmental Science.

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To my mom, dad and siblings, whose life-long support and sacrifices have granted me the privilege to study at the University of Hawai‘i and obtain a college degree.

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ABSTRACT

Leptospirosis is a zoonotic disease caused by the bacteria *Leptospira interrogans* and is disseminated in the urine of mammalian hosts (rats, swine). Mild cases involve flu-like symptoms, vomiting, and jaundice while severe cases may involve renal failure and pulmonary hemorrhage. Hawai‘i has the highest incidence of leptospirosis in the United States due to its warm and humid climate, yet cases are likely under-diagnosed due to the non-specific nature of clinical symptoms. Taro farmers are at highest risk as freshwater habitats are near mammalian carriers. In the He‘eia watershed in windward O‘ahu, Indigenous agriculture (*lo‘i)* and mariculture (*loko i‘a)*, channelized streams, and established populations of feral pigs may facilitate transmission of leptospirosis. We hypothesized that precipitation and storms increase dispersal of *Leptospira*. We carried out water quality sampling at the He‘eia *loko i‘a* (HFP) between 2014-2015 and 2017-2019. Precipitation data were retrieved from the Hawai‘i Climate Data Portal. Metabarcoding of the 16S rDNA gene was performed on water samples. Leptospiraceae relative abundance co-varied with the seasonal precipitation and were highest in the wet season. Leptospiraceae presence is strongly correlated with storm events ($R^2 = 0.68$, $p=0.086$). Relative abundance was inversely correlated with salinity within HFP ($R^2 = 0.69$, $p < 0.05$) and correlated to the freshwater input ($R^2 = 0.27$, $p<0.05$) suggesting a riverine origin. Quantification of pathogenic *Leptospira* by qPCR, targeting the *lipL32,* which is conserved across pathogenic strains of *Leptospira,* showed no amplification of the target gene. Results of this project provide a better understanding of the presence of Leptospiraceae in HFP and indicate relatively risks of infection for communities working in the fishpond. **Keywords**: Leptospirosis, Zoonotic disease, Microbial Source Tracking

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1.0 - INTRODUCTION

1.1 - LEPTOSPIROSIS: AN EMERGING ZOONOTIC DISEASE

Leptospirosis is a zoonotic disease caused by bacteria in the *Leptospira* genus, transmitted by mammals (Bharti et al., 2003; Haake et al., 2000). *Leptospira* is a spirochete bacterium that belongs in the family of Leptospiraceae along with other species of spirochetes such as *Leptonema spp.* and *Turneriella spp.* (Levett, 2015). Leptospires are free living organisms and can survive outside of a host for up to 16 days in soils and 28 days in spring water (Casanovas-Massana et al., 2018). *Leptospira* can infect humans through direct contact with urine and feces of an infected mammal carrier, or through indirect contact via contaminated soil, water, or food (Figure 1). Symptoms develop within 7 to 14 days after the infection and clinical onsets of leptospirosis are variable. Mild cases of leptospirosis may involve febrile illness, myalgia, headache, nausea/vomiting, and jaundice while severe cases of leptospirosis may lead to renal failure, dysrhythmia, and pulmonary hemorrhage. Symptomless illnesses are also possible and common, especially in areas where leptospirosis is endemic (Bharti et al., 2003). Diagnosis of leptospirosis can be challenging as the early symptoms of the disease are nonspecific and common in other diseases, such as influenza, the common cold, or dengue fever (Ellis et al., 2008). The severity of the disease can vary greatly and can result in acute and chronic manifestations (Bharti et al., 2003).

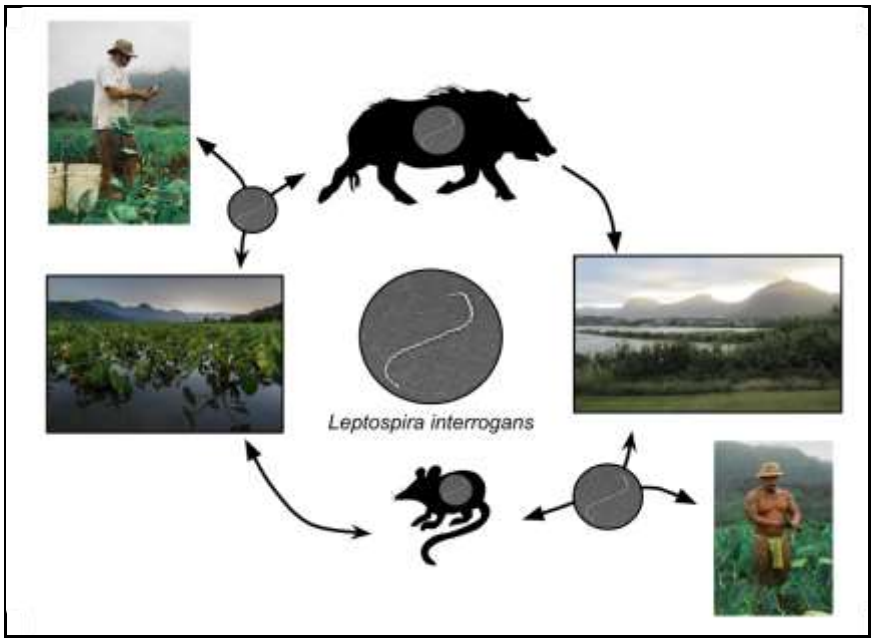

Figure 1 : Transmission of the *Leptospira* from environmental reservoirs to humans. *Leptospira* is maintained in the environment by mammal reservoirs such as rats (*Rattus spp.*) and feral swine (*Sus scrofa*). Humans are accidental hosts to *Leptospira*, and infections occur through environmental transmission.

1.2- GLOBAL INCIDENCE OF LEPTOSPIROSIS

Worldwide, the morbidity attributed to leptospirosis is estimated to be 14.77 cases per 100,000 population and the mortality is 0.84 deaths per 100,000 population (Costa et al., 2015). The distribution of leptospirosis globally is influenced by the geography, climate, and socioeconomic factors. Tropical regions account for 73% of the estimated cases in the world due the warm and humid climate that occurs at such latitudes. Tropical climate allows the establishment of reservoir populations, the survival of *Leptospira spp.* in soil and water, and favors the exposure to humans due to social and economic considerations (lifestyle, recreational activities, subsistence and commercial farming)

(Bharti et al., 2003; Costa et al., 2015; Felzemburgh et al., 2014). Transmission of leptospirosis in tropical climates has been noticed to be seasonal and increases during periods of heavy rainfall (Lau et al., 2010). The highest incidences of the disease occurred in Oceania (150.68 cases per 100,000), South-East Asia (55.54 cases per 100,000) and the Caribbean (50.68 cases per 100,000) (Costa et al., 2015). Clinical and animal studies in tropical islands estimated a high incidence of leptospirosis in the Western Pacific region and the circulation of pathogenic *Leptospira* in mammal carriers (Costa et al., 2015; Guernier et al., 2018). As such, Pacific Islands are especially susceptible to the disease due to environmental factors such as climatic events (rainy season, storms, floods) and individual factors such as farming, hunting, and recreational activities (Guernier et al., 2018).

Leptospirosis is considered an emerging disease by health authorities world-wide as countries with active leptospirosis surveillance have reported an increase in the incidence of the disease (WHO, 2003). In Malaysia, the state of Sarawak has experienced a four-fold spike in the number of cases. Epidemiologic surveys showed that in 2011, the case fatality rate was 6.9% which is above the six-year average of 6.42% between 2004 and 2009. The number of cases (186) reported in 2011 alone was already equivalent to 60% of the combined total from 2004 to 2009 (Thayaparan et al., 2013). Following the implementation of an active surveillance program, the 2012 report showed a record high of 271 documented cases (Thayaparan et al., 2013). In Thailand, the annual incidence of leptospirosis has increased ten-fold in a decade, from 0.3 per 100,000 persons (1982-1995) to 3.3 per100,000 persons (1997-1998) (S & Faine, 1999; Vijayachari et al., 2008). Additionally, in India, public health investigations indicated that leptospirosis accounts

for about 12.7% of cases of acute febrile illness (Sehgal et al., 2003; Vijayachari et al., 2008). In Europe, the incidence of leptospirosis was proved to be stable (0.13 per 100,000 inhabitants in 2010). However, epidemiological reports may be underestimating the incidence of disease as data available may only represent the most easily identifiable cases. Outbreaks of leptospirosis are closely associated with environmental risk factors that were identified in epidemiological studies, i.e., rainfall and flooding, temperature, exposure to animals, and poor sanitation and inadequate waste disposal (Lau et al., 2010; Vijayachari et al., 2008). As a result, climatic changes associated with global warming are hypothesized to impact the rate of infection of leptospirosis as rainfall, hurricanes and flood events may become more ubiquitous (Lau et al., 2010). Higher global temperature may also increase the survival rate of pathogenic *Leptospira* outside a mammalian host (Lau et al., 2010) .

1.3 - LEPTOSPIROSIS IN THE UNITED STATES

In the United States, incidence of leptospirosis was 100–200 cases reported annually before the disease was taken down from the list of notifiable diseases in 1994 (Center for Disease Control and Prevention, 1994; Katz et al., 2002). Epidemiological reports indicated that between 1998–2009, the average annual rate of hospitalization associated to leptospirosis was stable (0.6 hospitalizations per 1,000,000 population), with a higher rate in the Western region compared to the Eastern region of the country (0.7 hospitalizations per 1,000,000 population and 0.4 hospitalizations per 1,000,000 population respectively (Traxler et al., 2014). Cases of leptospirosis are predominantly reported during the summer and fall with a high proportion of hospitalizations occurring between June and September (41.2%) (Traxler et al., 2014). However, these national

statistics did not include the US territories where the disease is endemic (Traxler et al., 2014). Previous reports have mentioned that the states with the highest incidence of leptospirosis were Hawai‘i, Texas, California, and Puerto Rico (Guerra, 2013). Cases of leptospirosis have been associated with recreational activities involving freshwater (adventure races and triathlons), natural disasters (heavy rainfall, hurricanes, floods), and densely populated urban centers where *Leptospira* is maintained in mammalian hosts such as dogs and rats (Guerra, 2013; Moore et al., 2006; Smith et al., 2022). Active surveillance of the disease in the United States is currently nonexistent. Due to the range of clinical manifestation and the non-specific symptoms of leptospirosis, reports of leptospirosis are likely under-diagnosed by healthcare professionals and under-reported to health authorities (Ellis et al., 2008; Katz et al., 2011).

1.4 - LEPTOSPIROSIS IN HAWAI‘I

Epidemiological reports have indicated that Hawai‘i has the highest incidence of leptospirosis in the United States (2.5 per 100,000 pers), at about a hundred-fold the national incidence (0.02 per 100,000 pers) (Katz et al., 1991). In Hawai‘i, leptospirosis has been associated with varying risk factors over the years. Historically, the disease was associated with sugarcane farmers and was considered an occupationally acquired disease. However, with the rise of the tourism industry and the decline of the sugarcane industry in the 1970s, exposure to *Leptospira* became associated with recreational exposures due to activities such as hiking, hunting, and freshwater swimming. In recent years, exposures to leptospirosis have been associated with habitational risks which are suspected to be linked to the renewed interest in traditional taro farming state-wide (Anderson & Minette, 1986; Katz et al., 2002). Indeed, the cultivation of taro is

comparable to rice cultivation which involves the use of flooded wetlands and leptospirosis is a well-recognized occupational hazard to rice farmers (Faine, 1994; Vijayachari et al., 2008). Leptospirosis displays a seasonal pattern of occurrence with differences between the dry season (May - September) and the wet season (October - April) (Katz et al., 2011). *Leptospira* is maintained in the environment due to established populations of mammal reservoirs, mainly rats (*Rattus rattus*, *R. norvegicus* and *R. exulans*), mongooses (*Herpestes auropunctatus*), and feral swine (*Sus scrofa*) (Desvars et al., 2011). Each maintenance reservoir has been tested for antibodies targeting leptospiral antigens and serological tests have shown the association of specific serogroups of *Leptospira* with a particular reservoir. As such, serovar Icterohaemorrhagiae is most associated with rats, serovar Sejroe with mongooses, and serovar Australis with feral swine (Desvars et al., 2011). An increased prevalence of serovar Australis in human leptospirosis suggests an increased risk associated with the transmission of leptospirosis from feral swine (Anderson & Minette, 1986).

Microbial source tracking (MST) is a technique that traces the origin of a contamination, usually fecal pollution, using microbiological, genotypic, and/or chemical methods (Scott et al., 2002). MST plays an important role in monitoring the water quality in places used for water storage, aquaculture, and recreational activities (Scott et al., 2002; Simpson et al., 2002). Contaminated water, specifically with human feces, constitute a public health hazard as enteric pathogens, such as *Salmonella* spp., *Escherichia coli*, and *Cryptosporidium* spp., may be present in the water (Scott et al., 2002). As such, MST allows a direct monitoring of human pathogens and can help to determine the origin and the presence of biological contamination. For an effective

detection, the targeted pathogen must be adapted to the environment of interest and the genetic assays must employ markers that are conserved within a group pathogen (Scott et al., 2002; Simpson et al., 2002). Using this method, MST can be applied for the detection of *Leptospira* as the bacteria can survive outside of the host for extended periods, and genetic markers have been developed for the detection of the pathogenic strains of *Leptospira* (Rawlins et al., 2014; Riediger et al., 2016; Stoddard et al., 2009; Vadde et al., 2019). More specifically, the pathogenesis of leptospirosis is partially attributed to the gene *lipL32* present in certain species of *Leptospira*, which is responsible for the transcription of major outer membrane lipoproteins. *LipL32* is prominent and conservative across pathogenic strains of *Leptospira* and can therefore be used for the selective detection of pathogenic *Leptospira* in the environment (Haake et al., 2000).

1.5 - *AHUPUA'A* : TRADITIONAL AGRO-ECOSYSTEMS

An *ahupua'a* is a traditional Hawaiian land division (social-ecological community) and was an important unit of resource management and governance (Gonschor & Beamer, 2014; Winter et al., 2020) (Figure 2). An *ahupua'a* extends from the mountain ridge to the end of the back reef, therefore encompassing a diversity of ecological habitats. These diverse and complex agro-ecosystems were maintained and managed by the residents to optimize a broad range of ecosystem services such as food production, medicine, freshwater, timber and non-timber products, and biodiversity (Aoude, 1999). The physical management of natural resources and ecosystems was central to social and political strategies. Proper management of ecosystems, population dynamics, and species connectivity, have promoted resilience in the food system and improved food security (Winter et al., 2018). Two specific ecotones are the focal point of

food production within an *ahupua‘a*: the riparian/wetland ecotone and estuarine ecosystems (Winter et al., 2020) .

The riparian/wetland boundary is characterized by frequent flood events. These extensive wetlands are zones of active sediment deposition and nutrient runoff that Native Hawaiians have utilized for the production of taro (*kalo, Colocasia esculenta*), referred to as *lo‘i kalo*. Estuarine systems exist at the boundary between fresh and saltwater. *Loko i‘a* are constructed estuaries consisting of a rock wall with openings (*makahā*), built for the purpose of mariculture. Nutrient rich water circulating through the taro patches is channeled to the *loko i‘a* allowing the mixing of freshwater and saltwater in the fishpond. Estuaries are known to be highly productive as their ecological function is to serve as a nursery area for juvenile fish and invertebrates. *Loko i‘a* are an example of trophic engineering in which ecosystem processes are mimicked to create environments that favor the growth of microalgae (phytoplankton), macroalgae, and invertebrates that can support the production of fish (Hiatt, 1947).

Altogether, *ahupua‘a* are highly productive systems and are estimated to have been able to produce more than 1.02 million metric tons (mt) of food per year and support a maximum estimate of over 1.2 million people per year (Kurashima et al., 2019). Restoration of ancient aquaculture and mariculture practices may be able to address current issues pertaining to food security and sustainable agricultural practices.

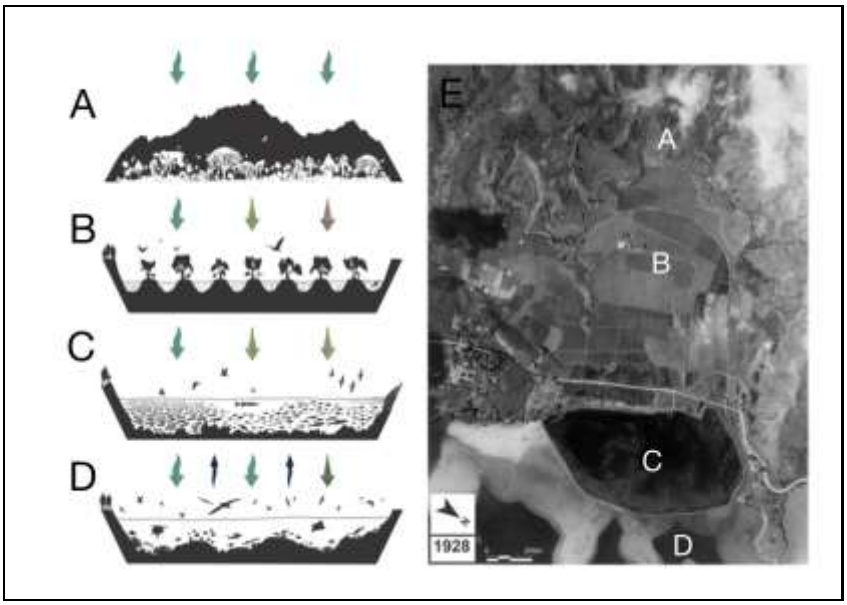

Figure 2 : Path of water flow in an *ahupua‘a*. *Ahupua‘a* land divisions closely follow those of a watershed. They are highly interconnected systems in which water flows from mountain to ocean. In He‘eia, rain water is collected throughout the watershed (E). The mountains (A) channel the precipitation in streams that flow throughout the taro patches (B) before discharging into the He‘eia fishpond (C) where it mixes with marine water (D) (Winter et al., 2020).

Figure 3 : *Loʻi kalo* in an *ahupuaʻa*. Example of *loʻi kalo* on land managed by Papahana Kuaola (left) with a close-up of taro plants (*kalo*, Colocasia esculenta) (right) (photo: Keanu Rochette-Yu Tsuen).

1.6 - HEʻEIA *AHUPUAʻA*: CONCERNS ASSOCIATED WITH LEPTOSPIROSIS IN A TRADITIONAL FOOD PRODUCTION SYSTEM

The Heʻeia *ahupuaʻa* is located on the windward side of the island of Oʻahu in the State of Hawaiʻi and is an example of an *ahupuaʻa* system undergoing biocultural restoration (Figure 2). Two wetland systems exist in Heʻeia, managed by three community-driven organizations: Kākoʻo ʻŌiwi and Papahana Kuaola oversee the upper watershed consisting of agricultural areas which produce taro in *loʻi* (taro patches) (Figure 3), and Paepae o Heʻeia in the coastal area which manages *loʻi* and *loko iʻa*, or fishpond (figure 4), traditionally used for aquaculture. Established populations of feral swine have been reported by local hunters and community partners, which represent a potential reservoir for pathogenic *Leptospira* to be maintained in the environment (Buchholz et al., 2016).

This agricultural system utilizes a network of streams and water channels which requires human management and may give a route for the dispersal of *Leptospira* in the watershed with increased proximity between human and potentially shedded bacteria in water. As a terminal point in the He‘eia watershed, He‘eia Fishpond receives water that underwent nutrient transformations and collected sediment throughout the watershed. As a result, microbial data from the fishpond could be used to estimate the presence and abundance of a pathogenic *Leptospira* maintained in the watershed and to draw relationships with environmental factors that correlate with the presence of the bacterium in the estuary.

Figure 4 : *Loko i;a o He‘eia*. He‘eia Fishpond was traditionally used for aquaculture. As fresh stream water and marine water mix within the fishpond, He‘eia Fishpond displays the similar characteristics as an estuary and makes it a very productive ecosystem (Photo credit: Paepae o He‘eia and Keli‘i Kotubetey).

1.7 - HYPOTHESES

Based on epidemiological reports, rainfall and flooding, temperature, exposure to animals, and poor sanitation and inadequate waste disposal are the most likely environmental factors associated with the transmission of leptospirosis (Lau et al., 2010; Vijayachari et al., 2008). However, epidemiological studies have not been able to establish robust statistical analyses between the potential risk factors leading to human leptospirosis due to a lack of surveillance of the disease in the region (Guernier et al., 2018). For this project, we will be focusing on correlating rainfall events with the presence and abundance of Leptospiraceae. Salinity will also be investigated as our study site is a constructed estuary and the pattern of occurrence of Leptospiraceae can be investigated in brackish water.

1.7.1 - HYPOTHESIS 1: LEPTOSPIRACEAE IS MORE PRESENT DURING RAINFALL EVENTS

H1.1 - Leptospiraceae is more likely to be detected during the wet season.

Null - There will be no differences in presence and abundance of Leptospiraceae between the wet and dry season.

H1.2 - Leptospiraceae are more likely to be detected during storm events.

Null - Leptospiraceae will have the same abundance during storm and non-storm conditions.

Rationale: *Leptospira* has been shown to have a positive association with rainfall and flood events as bacteria display the ability to aggregate on particles, form colonies and produce biofilm to increase their survivability in the environment (Trueba et al., 2004). As a result, we hypothesize that high flow events caused by storms, i.e., daily precipitation greater than 50 mm (Groisman et al., 1999; Karl et al., 1996), will result in the presence of Leptospiraceae in He‘eia Fishpond. Additionally, Leptospiraceae should be more detectable during storm events due to higher rainfall.

1.7.2 - HYPOTHESIS 2: LEPTOSPIRACEAE ABUNDANCE DECREASES IN SEAWATER

H2.1 - Leptospiraceae is more abundant in fresh water than seawater.

Null - There is no difference in abundance between fresh water and seawater.

H2.2 - Leptospiraceae is more abundant at sites in He‘eia Fishpond that are closer to the stream input.

Null – Proximity to the stream shows no difference in the relative abundance.

Rationale: *Leptospira* was shown to survive in brackish water (900mg/L NaCl or 0.9 PSU at 30°C) in controlled conditions (Faine, 1959). As a result, the distribution of Leptospiraceae in the fishpond is hypothesized to be more densely present in zones with more freshwater and to decrease in areas of more saline water, thus suggesting a riverine origin.

2.0 – METHODS

2.1 - SITE DESCRIPTION

He‘eia Fishpond (*Loko i‘a o He‘eia*) is located on the island of O‘ahu, in the *moku* of Ko‘olaupoko, in the *ahupua‘a* of He‘eia. The fishpond is estimated to have been built over 600-800 years ago as part of a traditional mariculture technique, nested in the *ahupua‘a* system (Paepae o He‘eia, n.d.). This traditional mariculture practice consists of a 2.5 km rock wall (*kuapā*) made of basaltic rocks and fossilized corals, enclosing a coastal body of water off of the coast of the He‘eia watershed. Covering about 0.356 km^2, He‘eia Fishpond is estimated to be one of the largest fishpond structures in the Hawaiian islands (Kikuchi, 1973; Lopera, 2020). The fishpond is bordered by fringing reef of Kāne‘ohe Bay to the East, He‘eia Stream to the North, and man-made water channels (*‘auwai*) that are adjacent to the He‘eia wetlands on the western and southern parts of its wall (Lopera, 2020). The *kuapā* is interspersed throughout the fishpond by sluice gates (*mākāhā*), allowing for inward and outward motions of water caused by tidal changes (Kikuchi, 1973). As a result of the mixing of marine water from Kāne‘ohe bay and riverine water from He‘eia Stream within the fishpond, He‘eia *loko i‘a* can be considered a constructed estuary.

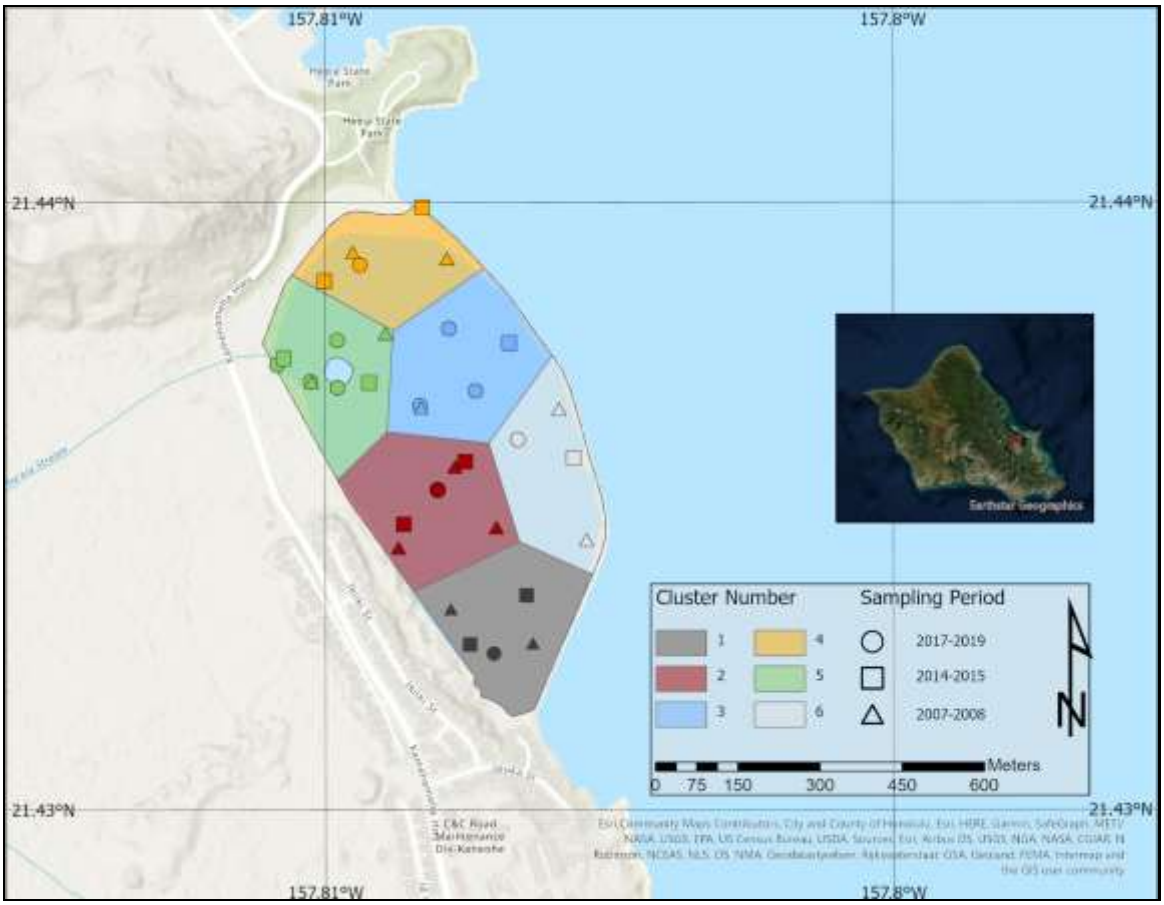

Figure 5 : Map of the sampling sites for three sampling campaigns in He‘eia Fishpond (Beebe, 2021).

2.2 - SAMPLE COLLECTION AND BIOGEOCHEMICAL ANALYSIS

This project utilizes data collected during the 2014-2015 and 2017-2019 sampling campaigns conducted through a collaboration between Paepae o He‘eia and the Alegado Lab (Beebe, 2021). Water samples were collected from He‘eia Fishpond from 2014 to 2015 (2014-campaign), and 2017 to 2019 (2017-campaign). Sampling events occurred every two weeks during the 2014-campaign and every quarter during the 2017-campaign. Approximately 1.5 L of seawater was collected at each site (Figure 5) using a Teflon-lined Niskin bottle (General Oceanics Inc., Miami, FL) 30 cm below the water surface.

During the water sampling, environmental parameters measurements were taken using a YSI multiparameter sonde (YSI Pro Plus for 2017-19, YSI 6600 v2 for 2014-15; YSI Incorporated, Yellow Springs, OH) for turbidity, temperature, and salinity. Dissolved inorganic macronutrient data was collected in both campaigns. During the 2014-campaign, nutrient data from unfiltered water was measured using a DR900 Multiparameter Portable Colorimetric kit (Hach® Company, Loveland, CO) (Beebe, 2021). During the 2017-campaign, water samples were filtered through 47-mm diameter Pall membranes (0.45-µm pore size) (GH Polypro, Pall Gelman Inc., Ann Arbor, MI) and stored at -20°C until analysis. Water samples were processed at the SOEST Laboratory for Analytical Biogeochemistry (Honolulu, HI) (Beebe, 2021). Additionally, daily precipitation data were retrieved from the Hawai‘i Climate Data Portal (HCDP) from January 1, 2014, to December 31, 2015, for the 2014-campaign, and January 1, 2017, to December 31, 2019, for the 2017-campaign. Precipitation data were collected via a rain gauge in the watershed of He‘eia at station KANEOHE 838.1 (21.4235°N, 157.8039°W) (Figure 6).

Figure 6 : Environmental data collection sites. Physico-chemical parameters originated from He‘eia Fishpond (yellow pin), precipitation data were collected from the rain gauge KANEOHE 838.1 (rain cloud) and the location of the stream mouth (blue pin) was used to measure the distance between sampling sites and the stream (see below).

2.3 - DNA EXTRACTION AND SEQUENCING

Seawater was filtered through a 47-mm diameter, 0.45-µm pore-sized hydrophilic polypropylene membrane (GH Polypro, Pall Gelman Inc., Ann Arbor, MI). The filters were used to extract genomic DNA using the PowerWater DNA Isolation Kit (MoBio Labs, Carlsbad CA) according to manufacturer's instructions.

Amplification of the 16S rRNA gene on DNA extracts was verified using polymerase chain reaction (PCR). PCR reaction mix consisted of 10 µL of SYBR Green Master Mix (2X), forward (BACT1369F) and reverse (Prok1541R) primers (10 µM) (Table 1), and 2µL of template DNA for a total volume of 20 µL (Suzuki et al., 2000). Each sample was tested in triplicates. PCR reactions took place in a Realplex

Mastercycler (Eppendorf® Mastercycler®, Eppendorf, Hamburg, Germany) and each plate run consisted of an initial denaturation at 94°C for 10 min, 40 cycles with denaturation at 94°C for 1 min, an annealing at 59°C for 1 min, an extension at 72°C for 1 min and a reading step at 80°C for 10s (adapted from Amend et al., 2022).

Samples were then prepared for sequencing using the SequalPrep™ kit. 25µL of PCR products were mixed with 25µL of SequalPrep™ Normalization Binding Buffer and incubated for 1h at room temperature. DNA samples were washed with 50µL of SequalPrep™ Normalization Wash Buffer and then eluted with 20µL of SequalPrep™ Normalization Elution Buffer. 200µL of the pooled DNA sequences (1-2ng/µL) were mixed with 20µL of sequencing primers (R1.read1.515F, R2.read2.806F, and R2.p7_index.806Rc) (Table 1) (Kozich et al., 2013; Naqib et al., 2018). The prepared amplified DNA sample was then sent to the Hawai‘i Institute of Marine Biology (HIMB) for sequencing with 250 bp paired-end MiSeq sequencing with V2 chemistry (Beebe, 2016).

2.4 - BIOINFORMATICS

MetaFlow|mics is a novel framework developed by the collaborative work of the Center for MICROBIOME Analysis through Island Knowledge and Investigations (C-MAIKI), the Hawaii EPSCoR Ike Wai project and the Hawaii Data Science Institute which uses a collection of pipelines to analyze microbiome marker data (Arisdakessian et al., 2020; Cleveland et al., 2022). The 16S data analysis pipeline can be divided into four distinct stages: 1) sequencing reads are trimmed, filtered and denoised, 2) sequencing reads are merged into longer sequences, and outlier sequences are filtered out, 3) DNA

sequences are clustered into taxonomically related sequences, 4) figures and tables are produced to summarize the results and facilitate potential troubleshooting (Arisdakessian et al., 2020). For this project, the DNA sequences were clustered by amplicon sequence variants (ASV) through the MetaFlow|mics pipeline. ASV clusters were annotated at each taxonomic level, from kingdom to genus *spp*.

Leptospira belongs in the family of Leptospiraceae, and an initial screening was performed through which the dataset was filtered to only select samples containing ASVs for the Leptospiraceae family, as well as identifying the number of ASV corresponding to Leptospiraceae in each sample. The relative abundance of Leptospiraceae was calculated by dividing the number of Leptospiraceae ASV by the total number of ASV in a sample. A comprehensive data set was created by merging environmental data to the data on relative abundance, which included temperature and salinity at the corresponding dates and sites in which Leptospiraceae was detected. Precipitation data were added by their corresponding date to the positively screened samples.

2.5 - QUANTIFICATION OF PATHOGENIC *LEPTOSPIRA* IN WATER SAMPLES

The detection and quantification of pathogenic Leptospira was performed using quantitative PCR. Amplification of the *Leptospira* genome was performed using protocols from Viau & Boehm, 2011, targeting the lipL32 gene. qPCR reaction mix consisted of 10 µL of TaqMan qPCR Master Mix (product reference), forward and reverse primers (400 nM), FAM BHQ1 probe (200nM) (Table 1) and 5 µL for DNA (< 250 ng) for a total volume of 20 µL. Each sample was tested in triplicate. A standard curve was generated by using a dilution series of a targeted synthetic oligonucleotide

gBlocks© Fragment (Integrated DNA Technologies, IA, USA) replicating *lipL32* genomic sequence. Standards were prepared at concentrations 50 000, 10 000, 5000, 1000, 500, 100, 50 and 10 genomes/reactions. A "pooled" calibration curve was then calculated by plotting the Cq values of the standards of known concentrations. Each dilution series was done in triplicates. Additionally, positive, and negative control, as well as a no-template control were added in triplicates to each plate run. qPCR reactions took place in a QuantStudio5 (Thermo Fisher Scientific Inc., USA) and each plate run consisted of 40 cycles with denaturation at 95°C for 15s and annealing-extension: 60°C for 60s.

2.6 - GEOSPATIAL DATA ANALYSIS

The open-source Quantum Geospatial Information System (QGIS) software (version 3.24.2 - Tisler) was used to analyze the spatial distribution of different parameters associated with the Leptospiraceae family and *Leptospira* (*QGIS*, 2019). A polygon was created from the outline of He'eia Fishpond using Google Earth Pro (version 7.3.6.9345 (64-bit)) and the resulting file was exported in a KMZ format. GIS data was consolidated using RStudio such that the file contained data on the sample names, the sites at which the samples were taken, the geographic coordinates (latitude and longitude), and the parameters of interests (salinity, relative abundance). The resulting file was exported as a CSV file for future analyses.

In QGIS, the polygon from the KMZ file was added to the layers panel and the CSV file containing geospatial data was imported using the Add Delimited Text Layer. An Inverse Distance Weighted (IDW) raster was calculated to interpolate the spatial

distribution of Leptospiraceae in the fishpond. The symbology render type was changed from the default single band gray to single band pseudo color. The interpolated surface generated was then clipped to the fishpond outline to fit the shape of the study site. This method was repeated to represent the salinity profile of the fishpond at the time Leptospiraceae can be detected in the water samples.

To further understand the relationship between sample site location and the abundance of Leptospiraceae, distance between the site of freshwater input and the sampling sites were measured using Google Earth Pro (Google, version 7.3.6.9345 (64-bit)). The He‘eia stream boarders the North side of the fishpond and the area is referred to as the *muliwai*. The *mākāhā* constitute points of freshwater entry in the fishpond. The *mākāhā* closest to the stream (marked “Freshwater input” on the map) was taken as the point of freshwater input for this project (Figure 6). The sampling site locations were placed on the map using a csv file containing information on latitude and longitude for each site. The measuring tool integrated in Google Earth Pro was used to measure the distance separating a sampling site from the site of freshwater input in meters. Each measurement was recorded and associated with the appropriate site for future analyses.

Table 1 : Summary of primers used for PCR amplification and DNA sequencing.

Target Primer	Sequences	Reference
BACT1369F	5'-CGGTGAATACGTTCYCGG -3'	(Suzuki et al.,
PROK1541R	5'-AAGGAGGTGATCC RGCCGCA -3'	2000)
R1.read1.515F	5'-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3'	(Kozich et al.,
R2.read2.806F	5'-AGTCAGTCAGCCGGACTACHVGGGTATCTAAT-3'	2013)
R2.p7_index.806Rc	5'-ATTAGATACCCBDGTAGTCCGGCTGACTGACT-3'	
gBlocks© Fragment	5' -	
sequence	ATGAAAAAACTTTCGATTTTGGCTATCTCCGTTGCACTCTTTGCAAGCATTACCGCTTGTG	
	GTGCTTTCGGTGGTCTGCCAAGCCTAAAAAGCTCTTTTGTTCTGAGCGAGGACACAATCCC	
	AGGGACAAACGAAACCGTAAAAACGTTACTTCCCTACGGATCTGTGATCAACTATTACGG	
	ATACGTAAAGCCAGGACAAGCGCCGGACGGTTTAGTCGATGGAAACAAAAAAGCATACT	
	ATCTCTATGTTTGGATTCCTGCCGTAATCGCTGAAATGGGAGTTCGTATGATTTCCCCAAC	
	AGGCGAAATCGGTGAACCAGGCGATGGAGACTTAGTAAGCGACGCTTT - 3'	
lipL32-45F	5'- AAG CAT TAC CGC TTG TGG TG -3'	(Rawlins et al.,
lipL32-286R	5'- GAA CTC CCA TTT CAG CGA TT -3'	2014 ; Stoddard et
lipL32-189P	FAM-5'-AA AGC CAG GAC AAG CGC CG-3'-BHQ1	al., 2009)

2.7 - DATA ANALYSIS

Statistical software RStudio (Posit Software, Boston, MA) was used for data analysis throughout the project (RStudio Team, 2022). Taxonomic data, relative abundance, and water sampling metadata, providing information on the sample name, genetic barcode, sampling site, and date, were loaded in RStudio and processed using the Phyloseq and Tidyverse packages.

A one-way ANOVA test and Tukey's test were used to evaluate the variations in the Leptospiraceae relative abundance found in the dry season and the wet season and assess the significance of the variations observed in the dataset. The relative abundance was tested against seasons as an independent predictor.

Relationship between Leptospiraceae ASV and environmental parameters was tested through linear regression. A linear regression was performed using the lm()

function and visualized using ggplot2 to examine the nature of the association between bacterial data and environmental data. The strength of the association was determined using the lm() function which calculated R^2 values and p-values which were used to determine the significance of the results.

The R package ggplot2 was used to perform the data visualization in RStudio and plot the linear regressions, histograms, and boxplots in this project.

3.0 - RESULTS

3.1 - CORRELATION BETWEEN PRECIPITATION PATTERNS AND LEPTOSPIRACEAE DISTRIBUTION

3.1.1 - SEASONAL VARIATIONS IN LEPTOSPIRACEAE DISTRIBUTION

Due to the association of leptospirosis outbreaks with rainfall and flooding events, we hypothesized that Leptospiraceae would be more abundant during the wet season. The overall seasonal precipitation pattern suggests that the wet season (October to April) (max = 5702.3 mm, mean = 107.6 mm ± 288.9, n = 1087) has greater precipitation than the dry season (May to September) (max = 4935.2 mm, mean = 102.1 mm ± 266.0, n = 1104) (Figure 7).

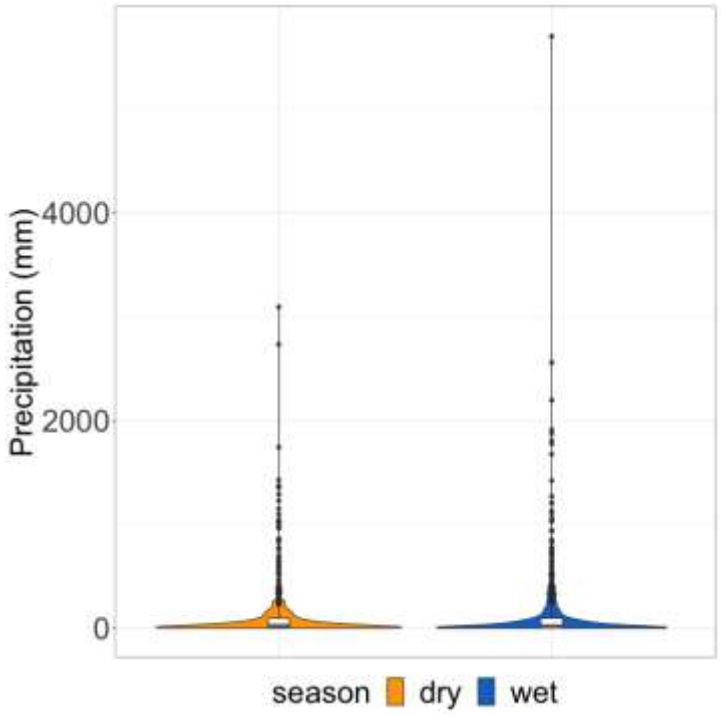

Figure 7: Distribution of precipitation data between the dry season (orange) and the wet season (blue).

Comparison between sampling years show great variations in the precipitation pattern (Figure 8). Between 2014 and 2015, precipitation seems higher in the dry the season compared to the wet season. This may be due to greater numbers of storm events during that sampling campaign. In 2017 and 2018, precipitations are higher in the wet season, with a very significant storm event in the wet season of 2018. In 2019, the precipitation pattern is comparable between seasons with slightly higher precipitation in the dry season.

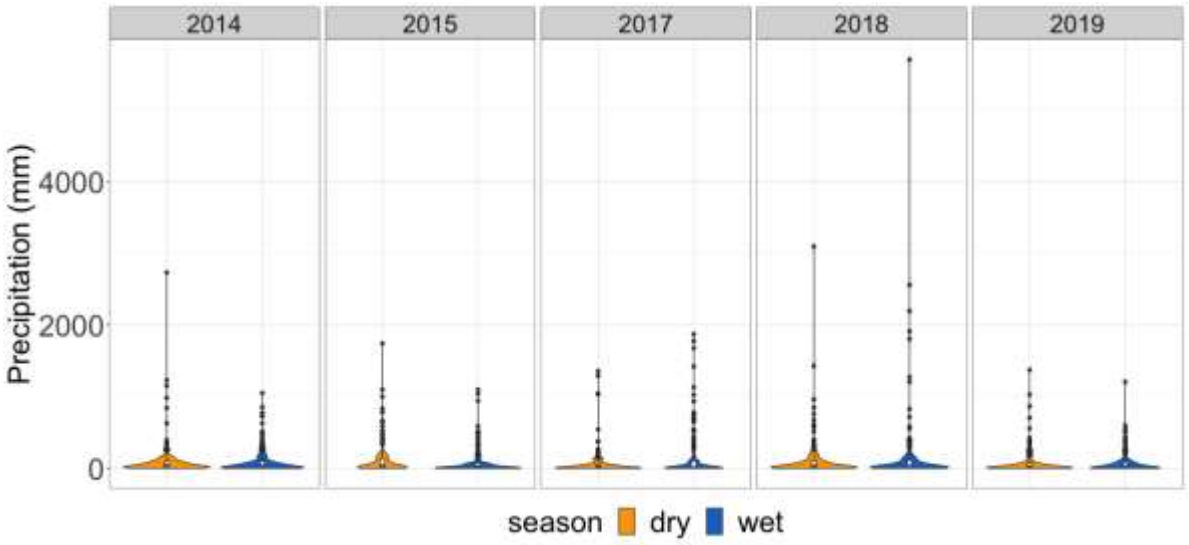

Figure 8: Distribution of precipitation data by year and by season (dry season in orange and wet season in blue).

To test the association between Leptospiraceae and the wet season, the relative abundance of Leptospiraceae was recorded by season and by year. Results have shown that the relative abundance in He‘eia Fishpond changes and varies by seasons, regardless of the year. During the wet season, relative abundance ranged from 7.79×10^{-5} to 9.48×10^{-4} with an average of $3.27 \times 10^{-4} \pm 2.55 \times 10^{-4}$, while during dry seasons, relative abundance varied between 6.85×10^{-5} to 5.29×10^{-4} with an average of $1.95 \times 10^{-4} \pm 1.60 \times 10^{-4}$ (Figure 9). One-way ANOVA test showed that there was no difference in the relative

abundance found between seasons each year (Table 2). The difference in the mean relative abundance between seasons, all years combined, was determined using a one-way ANOVA and Tukey's test which showed no significant differences between seasons. Though the difference was not significant, there is to be more Leptospiraceae detected in the wet season (Figure 10).

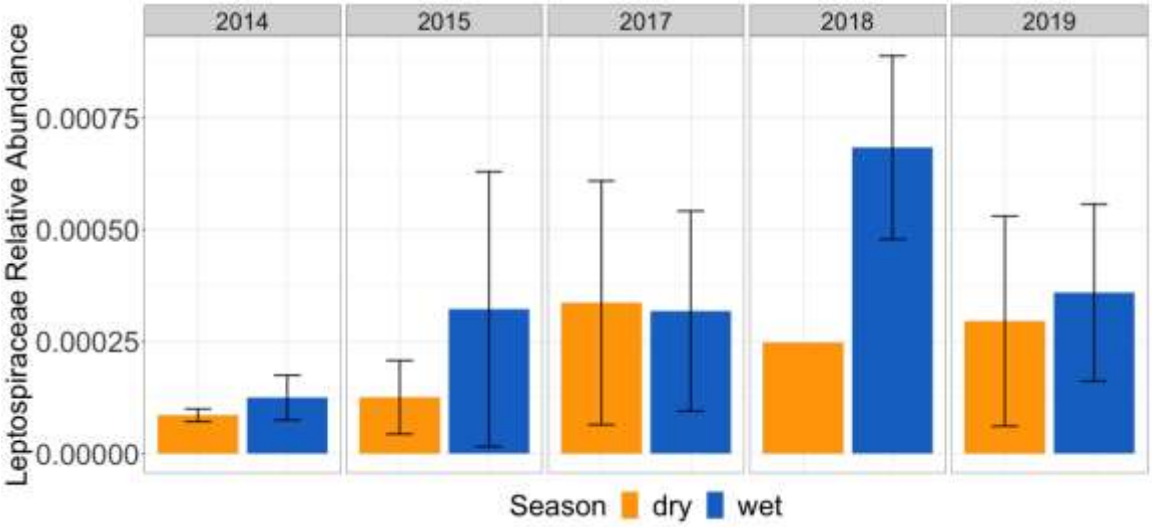

Figure 9 : Difference in relative abundance of Leptospiraceae between the dry and wet seasons (2014-2015 and 2017-2019).

Table 2 : Summary statistics of the Leptospiraceae mean relative abundance and standard deviation.

Year	Season	Mean	Standard Deviation
2014	Dry	8.52×10^{-5}	1.40×10^{-5}
2014	Wet	1.25×10^{-4}	5.01×10^{-5}
2015	Dry	1.25×10^{-4}	8.22×10^{-5}
2015	Wet	3.22×10^{-4}	3.07×10^{-4}
2017	Dry	3.37×10^{-4}	2.72×10^{-4}

2017	Wet	3.18×10^{-4}	2.24×10^{-4}
2018	Dry	2.47×10^{-4}	N/A
2018	Wet	6.83×10^{-4}	2.05×10^{-4}
2019	Dry	2.95×10^{-4}	2.35×10^{-4}
2019	Wet	3.59×10^{-4}	1.98×10^{-4}

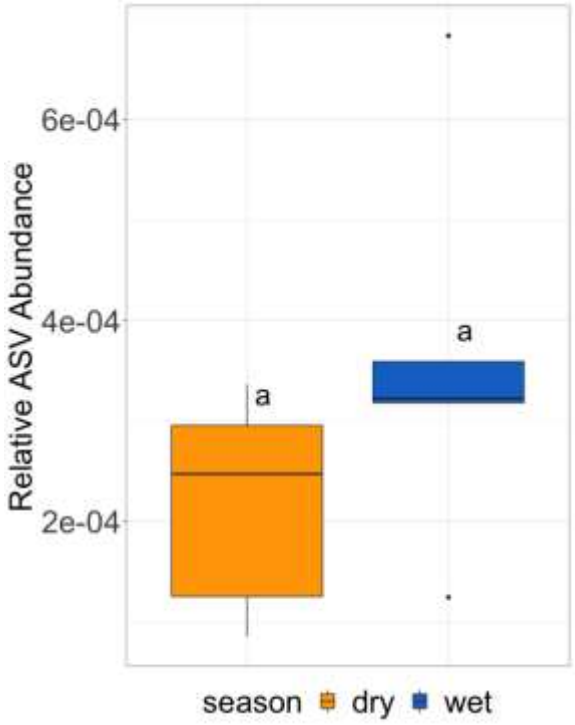

Figure 10 : Variation of Leptospiraceae relative abundance between seasons (2014-2015 and 2017-2019 combined). The boxplots show an apparent higher relative abundance in the wet season. Results from ANOVA indicate no significant difference between the relative abundance between seasons. Tukey's test indicating no significant difference between groups is represented by the same letter label “a”.

3.1.2 - VARIATIONS IN LEPTOSPIRACEAE DISTRIBUTION WITH PRECIPITATION AND STORM EVENTS

Next, to understand the relationship between precipitation and the relative abundance of Leptospiraceae, we first represented the data as a time series showing the average monthly precipitation and the monthly relative abundance found in both sampling campaigns. To test the strength of the association, we performed a linear regression between relative abundance and precipitation to obtain a correlation coefficient (R^2).

The time series of the mean monthly precipitation shows that precipitation varied in He‘eia over the years and changes corresponded to the seasonal pattern of precipitation of Hawai‘i. The 2014-campaign experienced abundant precipitation in the wet season which dwindled as the dry season approached and vice versa (Figure 11, top). The 2017-campaign did not exhibit the expected patterns, possibly due to a sparser sampling frequency in 2017 (Figure 11, bottom). Additionally, precipitation data was also only representative of precipitation in He‘eia associated with the positive detection of Leptospiraceae. Nevertheless, a co-variation between the average monthly precipitation and relative abundance was observed in both campaigns; therefore, qualitative analysis showed an association between precipitation and relative abundance of Leptospiraceae.

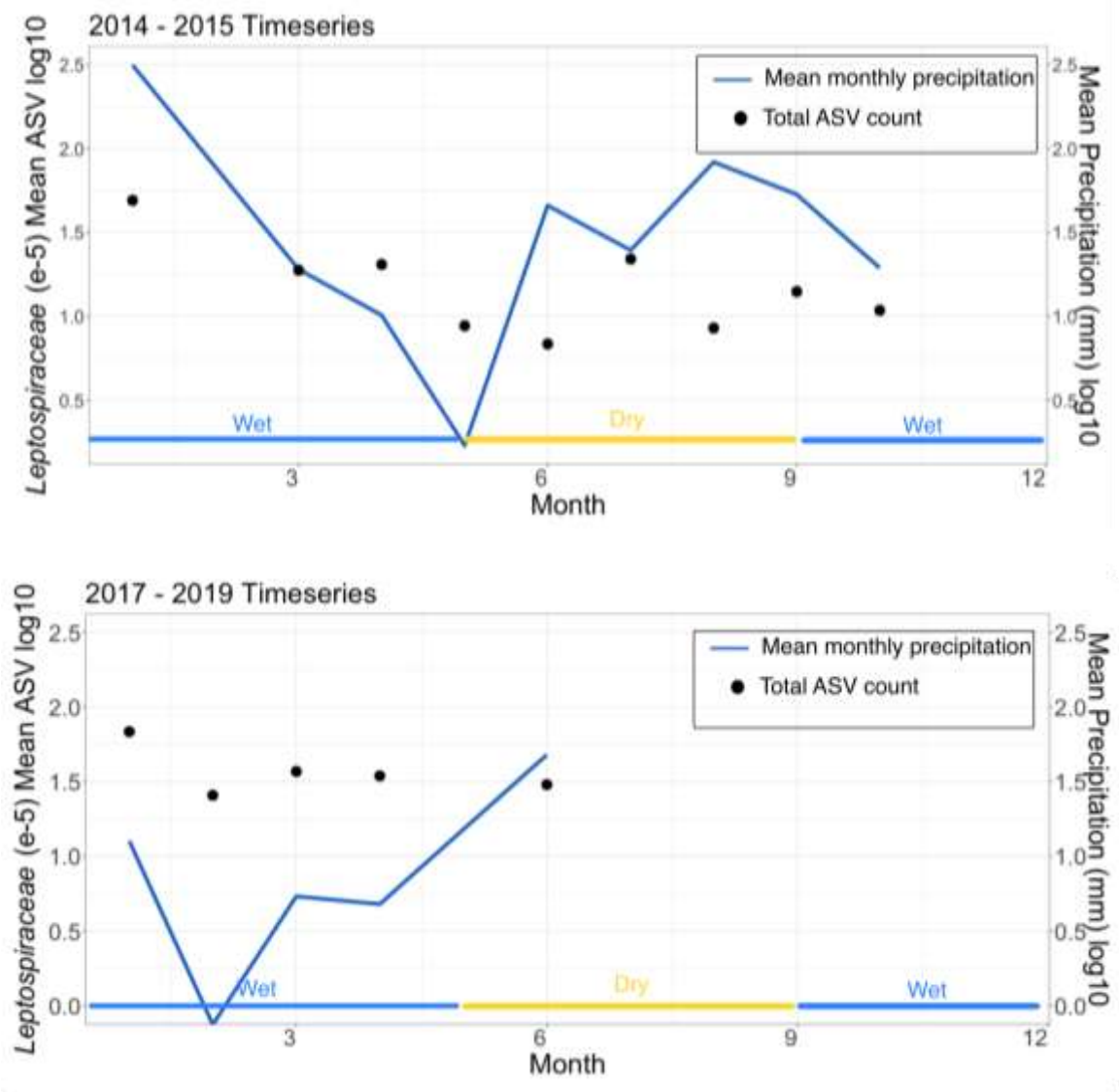

Figure 11 : Time series of mean precipitation and mean relative abundance. Covariation between precipitation and relative abundance can be observed. Seasons are colored at the bottom of the time series (yellow for the dry season and blue for the wet season). The blue line represents the mean monthly and the black dots represent average relative abundance. Precipitation data were retrieved from the Hawaii Climate Data Portal (HCDP).

Linear regressions were performed to measure the strength of association between precipitation events and relative abundance. In the 2014-campaign, a positive trend with a strong and significant correlation ($R^2 = 0.64$, $p < 0.05$) was observed between the precipitation and relative abundance (Figure 12, top). Outliers corresponding to significant storm events (>50 mm) had a visible impact on the association between the variables of interest. The 2017 campaign had much lower recorded precipitation as compared to 2014 - 2015. These data suggest no correlation between the precipitation and relative abundance ($R^2 = 0.01$, $p = 0.682$) (Figure 12, bottom). The 2014 - 2015 regression can be further analyzed by comparing non-storm events (< 50 mm) and storm events (≥ 50 mm). During non-storm events, the linear regression shows no correlation between precipitation and relative abundance ($R^2=0.02$, $p = 0.637$) (Figure 13, top), similar to the trend observed in 2017-2019. During storm events, a strong positive trend between the data also shows a strong but not significant correlation between precipitation and relative abundance ($R^2= 0.68$, $p = 0.086$) (Figure 13, bottom). The lack of significance in the results may be attributed to the low number of samples available for analysis (n=5).

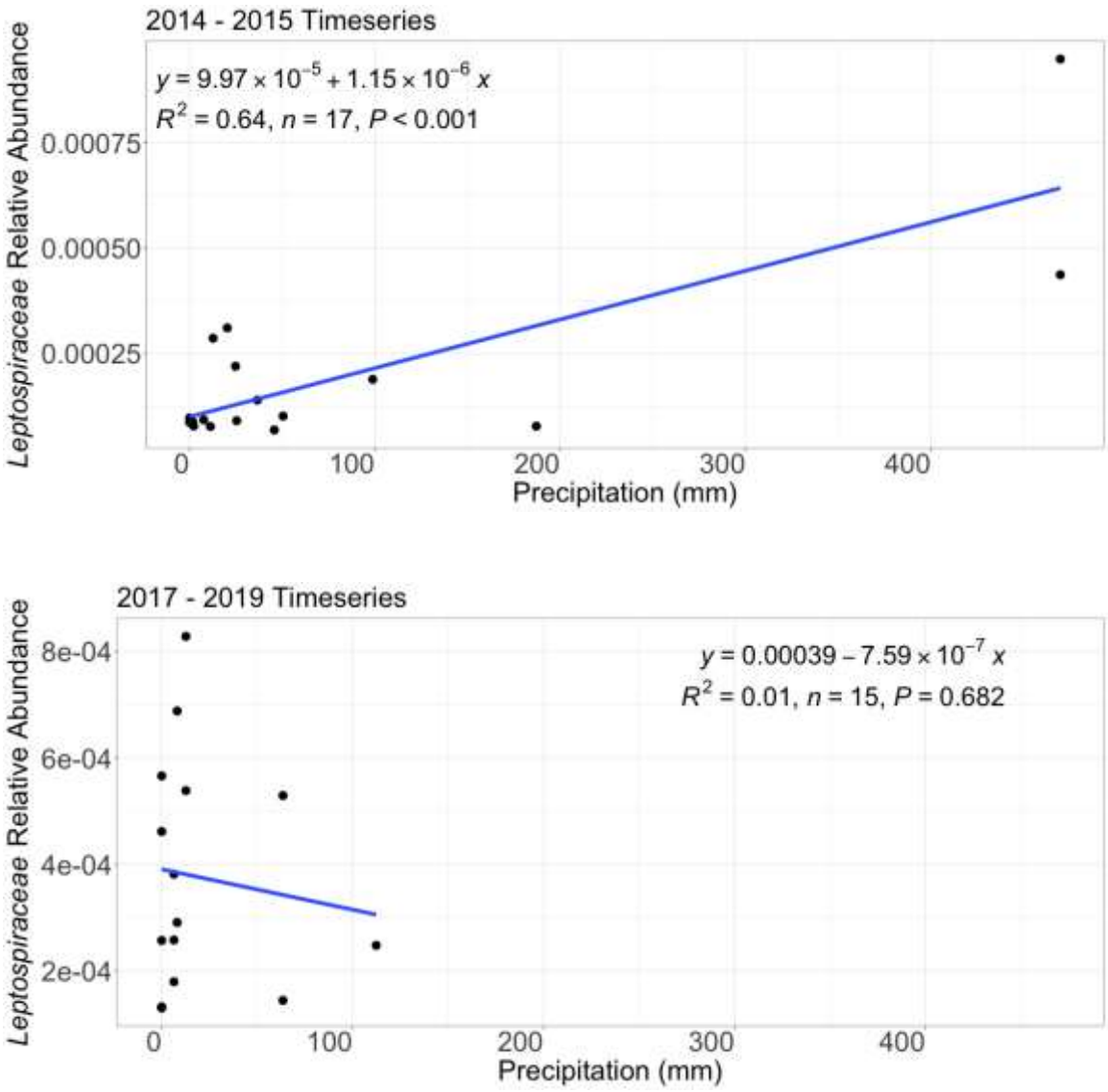

Figure 12 : Linear regression (blue line) between precipitation and Relative abundance in 2014 (top) and 2017 (bottom).

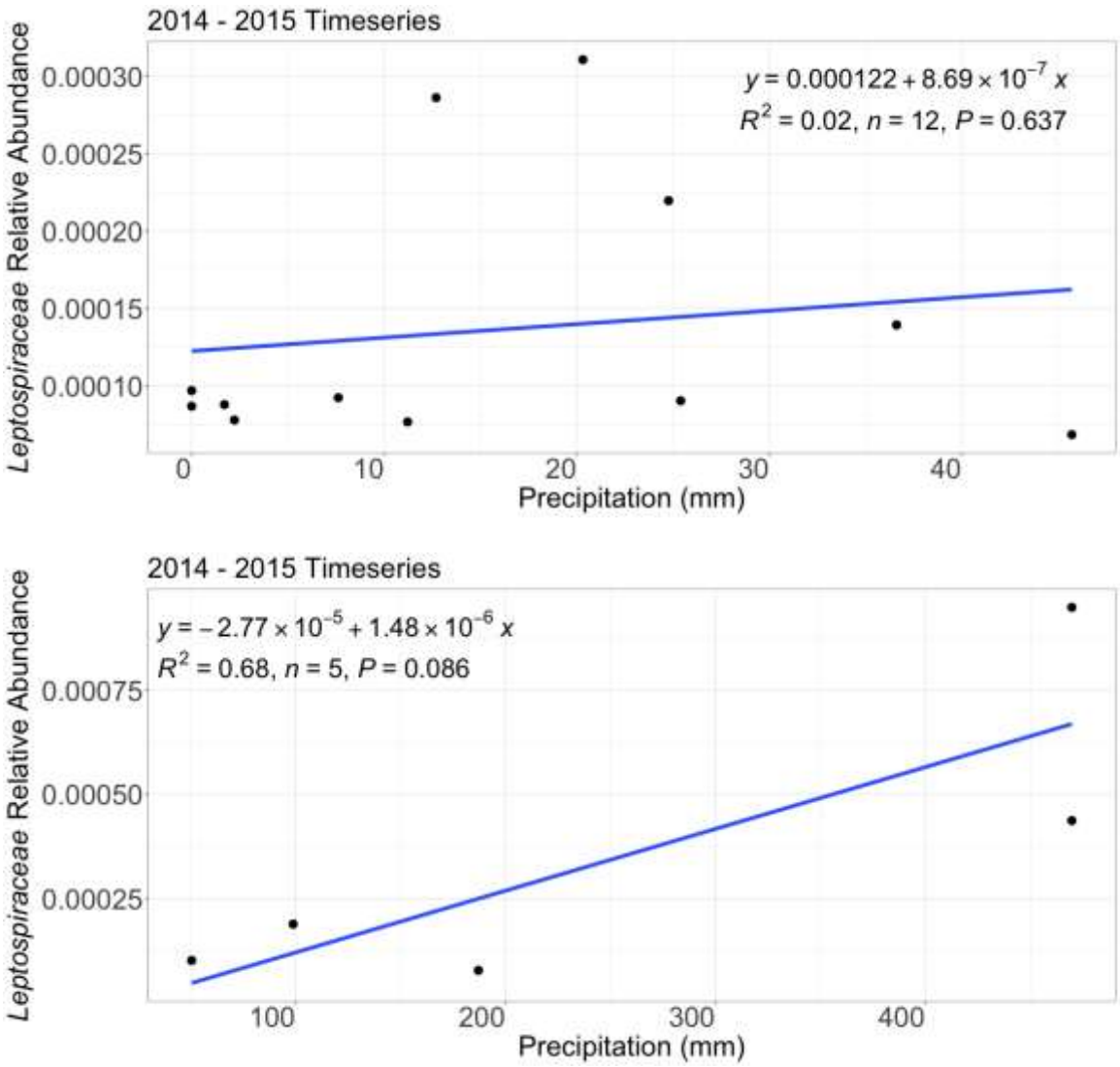

Figure 13 : Difference between non-storm (top) and storm (bottom) precipitation events. The linear regression is represented by the blue line.

3.2 - CORRELATION BETWEEN SALINITY AND LEPTOSPIRACEAE DISTRIBUTION

Given that Leptospiraceae ASVs are associated with significant rain events, bacteria must be entering the fishpond via stream input, thus being associated with inflow of freshwater. Maps of the salinity profile in the fishpond were made to visualize the

salinity profile and the distribution of the average relative abundance of Leptospiraceae found per site between years and by season. Between 2014 - 2015, maps indicate that more Leptospiraceae can be found during the wet season, as indicated by earlier analyses. Sites with lower salinity have an apparent association with higher relative abundance detected, which can be observed in sites P2 and P3 in the wet season and site P3 in the dry season (Figure 14).

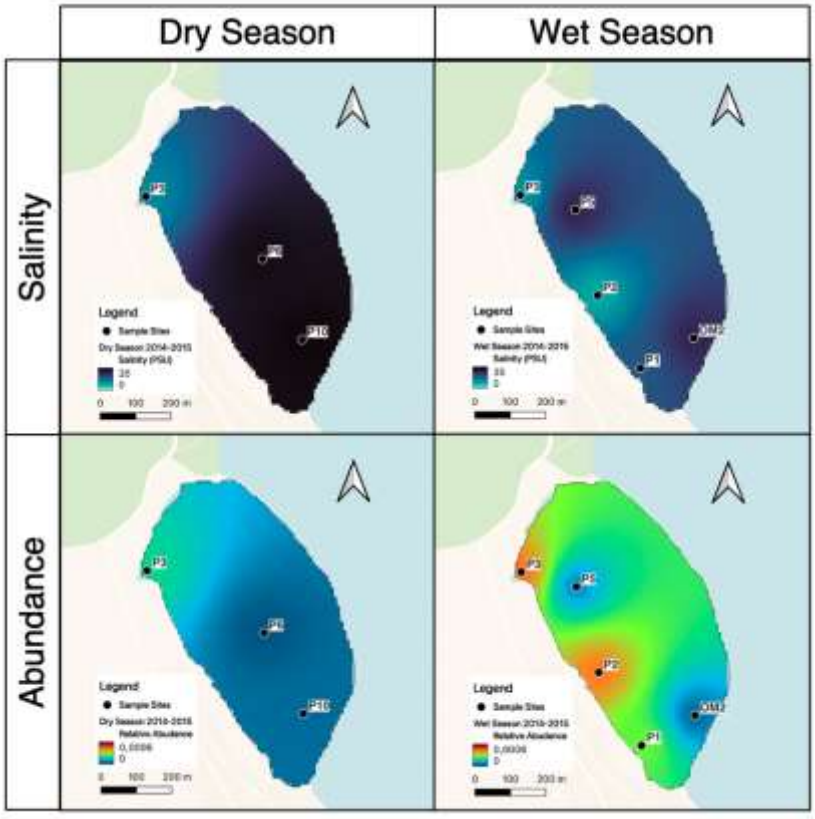

Figure 14 : Average salinity profile and distribution of average Relative abundance in 2014-campaign between seasons.

Generally, the patterns of Leptospiraceae distribution are similar in 2017-2019; e.g., more Leptospiraceae can be detected in the wet season than the dry season and low salinity sites are associated with higher relative abundance (Figure 15). This pattern is

especially visible for site E02 in the dry season and M01 in the wet season. However, Leptospiraceae were also detected at sites with higher salinity such as sites M02 in the wet season. However, M02 is located on the *muliwai*, thus in close proximity with He‘eia stream which may explain a higher relative abundance. M02 is also strongly tidally influenced due to its proximity with the ocean, which explains its higher salinity.

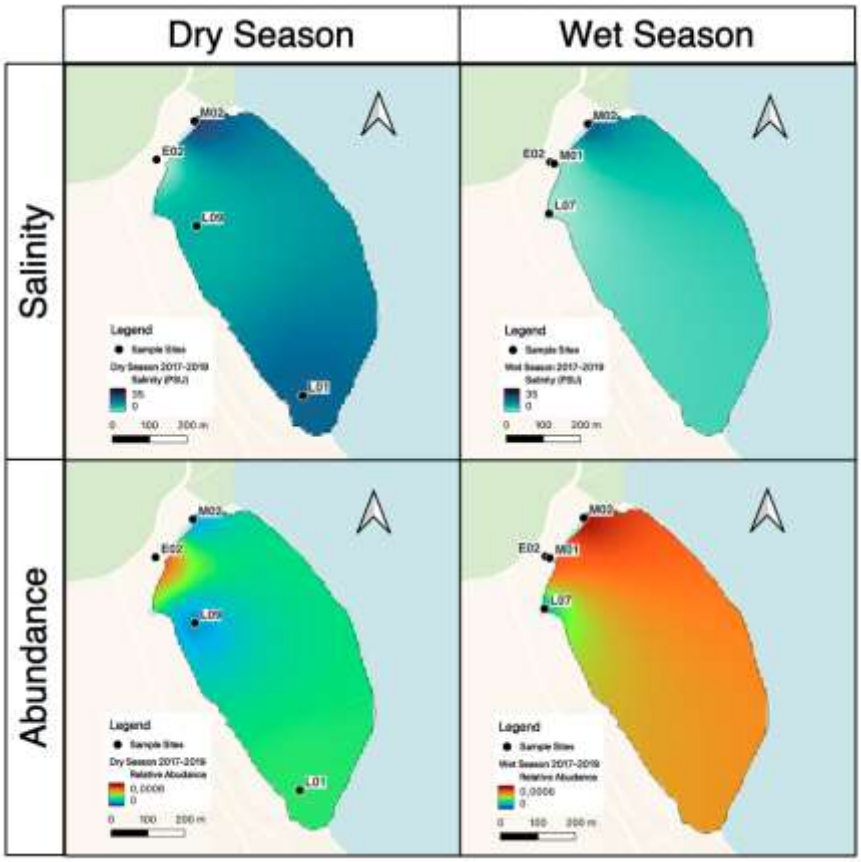

Figure 15 : Average salinity profile and distribution of average Relative abundance in 2017-campaign between seasons.

Linear regressions between salinity and relative abundance show distinct patterns between the 2014 campaign and the 2017 campaign. Between 2014-2015, there was a negative trend with a strong and significant correlation between the variables of interest

(R^2 = 0.69, p < 0.05) (Figure 16, top). Between 2017-2019, there was little to no correlation between the two variables (R^2 = 0.00, p = 0.992) (Figure 16, bottom). The difference of trends between the two campaigns may be explained by grouping data points by site. Sites with the highest relative abundance, such as sites P3, E02, M01 and M02, are in close proximity with the site of freshwater input, suggesting that freshwater input plays a role in the regression pattern observed.

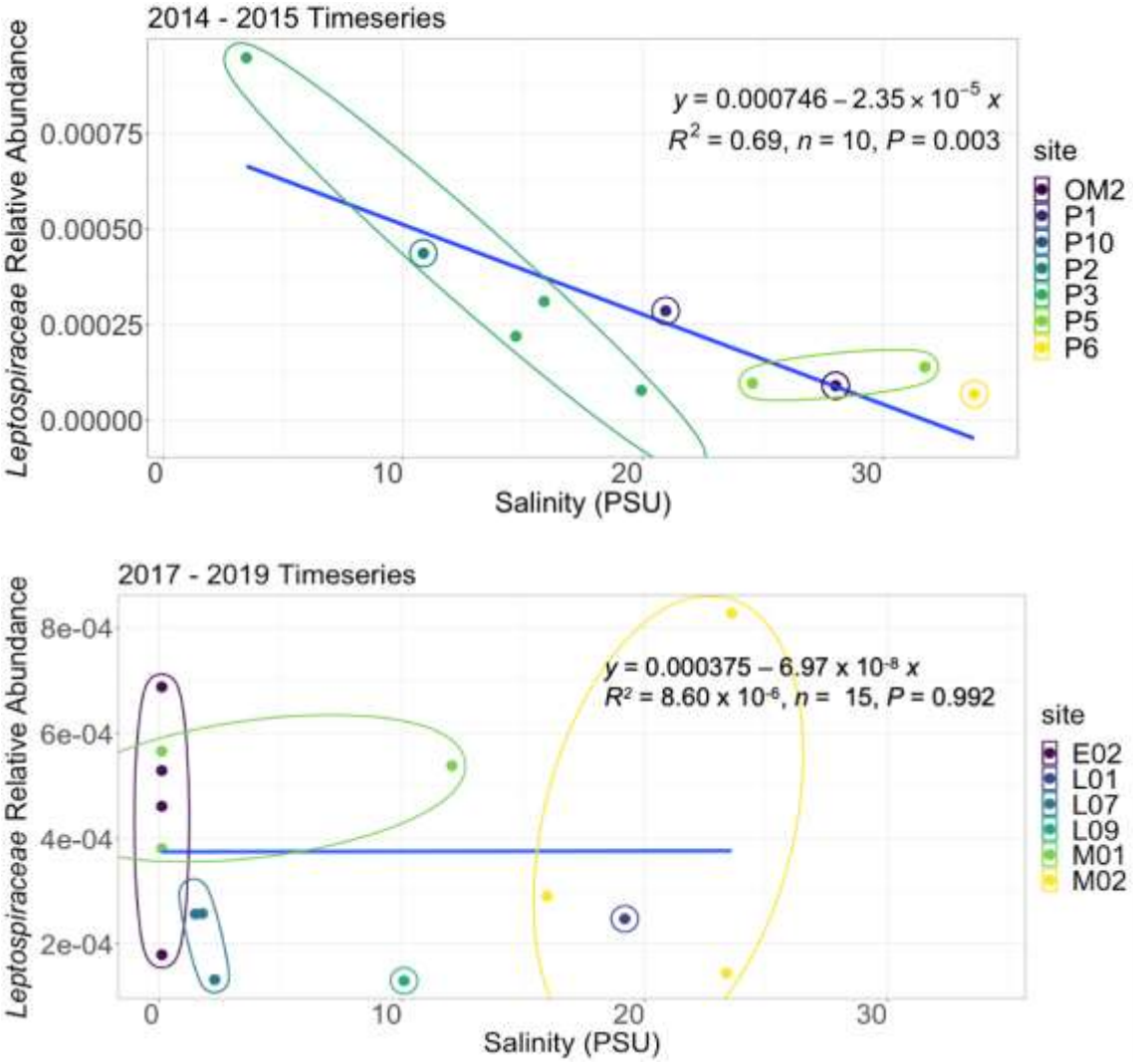

Figure 16 : Linear regressions (blue lines) between salinity (PSU) and relative abundance per site. The top plot shows the regression for the 2014-campaign and the bottom plot the

2017-campaign. Data points taken at the same sample site were circles in the same color as the data points.

Lastly, to understand the relationship between sample location and the presence of Leptospiraceae, the association between the distance between sites and the "freshwater input" site and Leptospiraceae relative abundance per site was studied. The linear regression shows a negative trend with a marginal correlation between variables that is statistically significant ($R^2 = 0.27$, $p < 0.05$) (Figure 17), confirming that proximity with the stream results in a higher probably to detect Leptospiraceae ASV. Leptospiraceae is therefore likely to have a riverine origin.

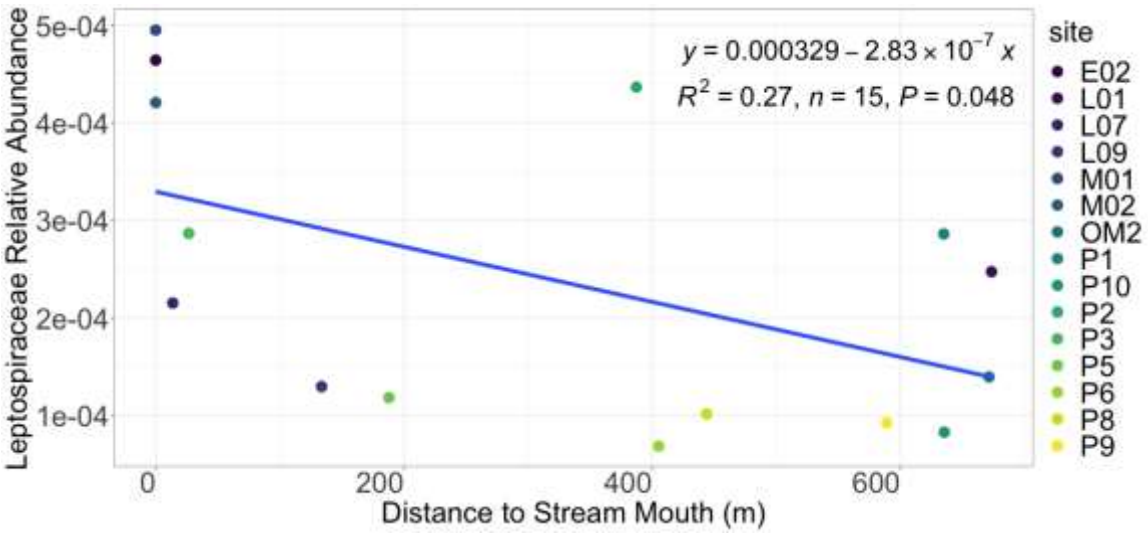

Figure 17 : Linear regression between distance to stream mouth (m) and relative abundance per site. The linear regression is represented by the blue line.

3.3 - DETECTION OF PATHOGENIC *LEPTOSPIRA* USING qPCR

Samples were tested for pathogenic *Leptospira* in two batches due to space limitation on 96-well plates. qPCR results consist of amplification curves (see Appendix) showing that only the gBlock© standards amplified passed the detection limit set by the thermocycler. As such, the samples tested did not amplify past the threshold of detection and were therefore considered negative to pathogenic *Leptospira*. Pooled standard curves were obtained for each plate by averaging the Cq values of the replicates of a given concentration. Pooled standard curves had a correlation coefficient $R^2 = 0.977$ and $R^2 = 0.953$ respectively indicating that the amplification of the *lipL32* gene was successful and the negative results obtained in the tested samples were correct (Figure 18). As a result, no pathogenic *Leptospira* was detected in the samples tested, indicating a low risk of leptospirosis infection in He‘eia Fishpond.

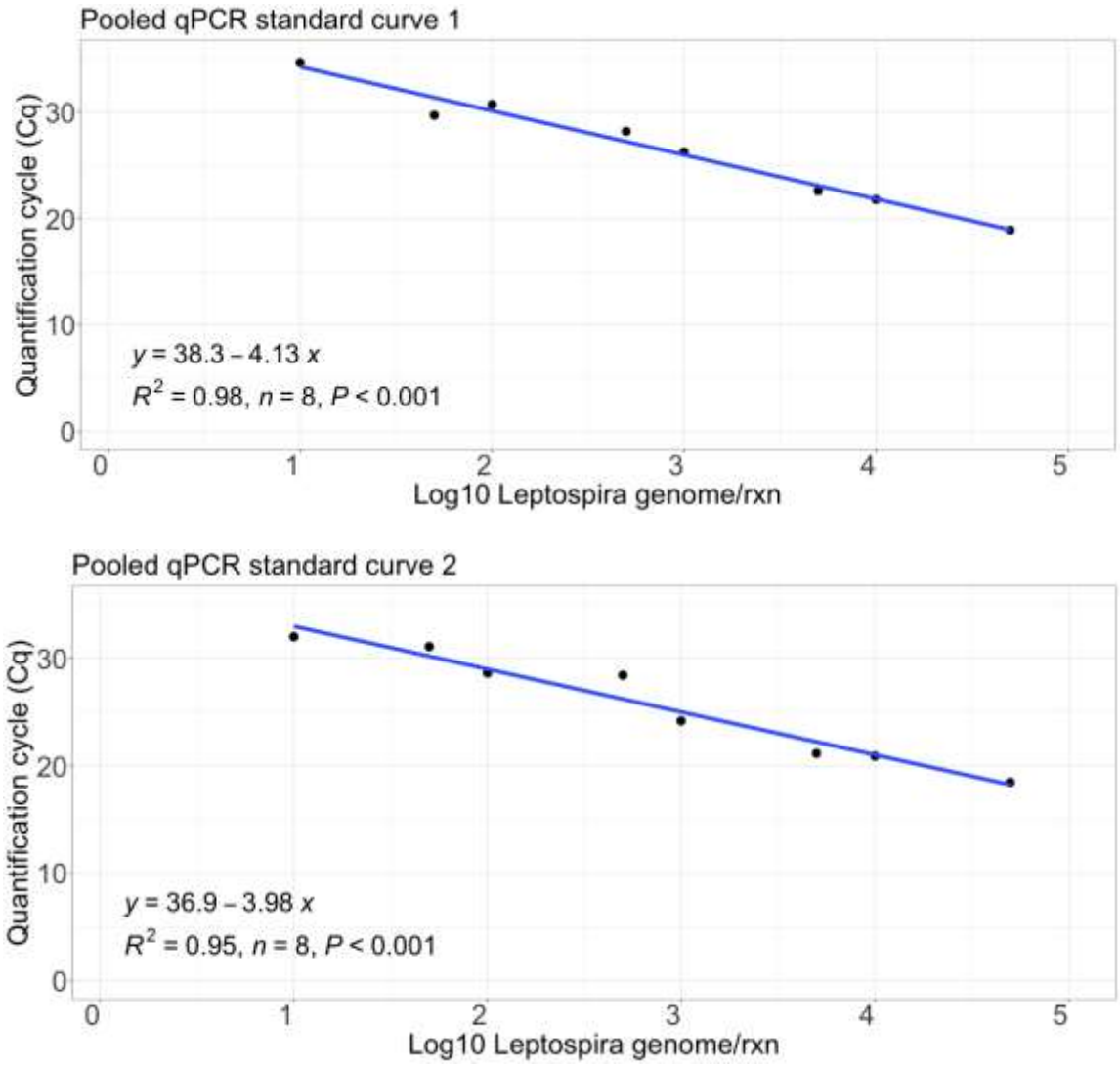

Figure 18 : Pooled amplification standard curve. The linear regression is represented by the blue line.

4.0 - DISCUSSION

4.1 - CORRELATION BETWEEN LEPTOSPIRACEAE AND PRECIPITATION

This project demonstrated that among environmental factors that correlate with outbreaks of leptospirosis, Leptospiraceae is associated with significant precipitation typically associated with the wet season and significant storm events, which is consistent with previous work on *Leptospira* (Lau et al., 2010). Previous studies have demonstrated a strong association of *Leptospira* (genus) with turbidity, which can be explained by the ability of bacteria to aggregate to suspended particles and form biofilms, which enables the bacteria to persist in the environment, independent from a host (Trueba et al., 2004; Viau & Boehm, 2011). As such, high velocity and turbulent water flow occurring during precipitation contribute to the suspension of bacteria in the water column. As a result, large amounts of precipitation during storms likely led to greater numbers of Leptospiraceae found in our water samples. This phenomenon can also explain the association of leptospirosis outbreaks with flood events (Gaynor et al., 2007; Monahan et al., 2009). However, the association of Leptospiraceae with turbidity has not yet been tested in He‘eia Fishpond.

Differences in sampling frequency between research campaigns represent a challenge in analyzing and comparing results obtained during this project. Indeed, sampling was more frequent during the 2014-sampling campaign (every two weeks) compared to the 2017-campaign (every quarter). Additionally, there is a time gap where no sampling was performed in 2016. As a result, the datasets had to be treated separately and data analysis could not benefit from a cumulative dataset. Future sampling

campaigns should keep consistent sampling intervals so that sampling campaigns can be more accurately compared between one another.

Precipitation data were collected from a rain gauge that was in close proximity to He‘eia Fishpond (1.4 km) because no data was available *in situ*. Precipitation data are a challenge to analyze as there may be a time delay before changes are reflected in the environment (hysteresis). Current data may not have the necessary resolution to account for hysteresis in order to provide an accurate representation of the association between precipitation and Leptospiraceae presence. The establishment of rain gauges on-site would provide more accurate precipitation data. Additionally, this study has been focusing on He‘eia Fishpond, which was used as a proxy site due to its terminal location in the He‘eia watershed. However, in order to have a better overview of the circulation of Leptospiraceae in the water and perform a risk assessment on human infection, water samples should be taken throughout the watershed, especially in taro fields where humans are most at-risk of getting infected by *Leptospira* (Tharmaphornpil et al., 2000).

4. 2 - CORRELATION BETWEEN LEPTOSPIRACEAE, SALINITY AND STREAM INPUT

This study indicated that Leptospiraceae presence inversely correlate with salinity in the 2014-campaign while a non-correlation could be observed in the 2017-campaign. This difference in the result between sampling campaigns can be explained by the inclusion of endmember sites in the 2017-campaign which are located outside and on the outer edge of the fishpond wall. The physico-chemical parameters outside of the fishpond are greatly influenced by the He‘eia Stream on the northern and western sides of the

fishpond while the ocean influences the eastern and southern sides. Correlations between the presence of *Leptospira* and salinity in previous projects have shown positive associations, potentially indicating tolerance of *Leptospira* to brackish and saline waters (Faine, 1959; Viau & Boehm, 2011). As a result, there seems to be inconsistent associations between leptospires and salinity. However, when sample locations were accounted for in data analyses, sites closer to the "freshwater input" site seem to show higher detection of Leptospiraceae. Correlations between distance to the stream and the relative abundance of Leptospiraceae show a marginal and statistically significant association between the two variables, thus supporting our hypothesis. This result is consistent with previous studies which indicated that *Leptospira* are widespread in Oahu coastal streams (Viau & Boehm, 2011). As a result, this confirms the riverine origin of Leptospiraceae in He‘eia Fishpond, reinforcing the need for a watershed-centered sampling effort to understand the origin of the bacteria, their dispersion within the watershed and the wetlands and their residence time at different locations, before flowing into the fishpond.

4.3 - DETECTION OF PATHOGENIC *LEPTOSPIRA*

qPCR experiments have shown no amplification of the *lipL32* gene, suggesting the absence of pathogenic *Leptospira*, potentially indicating low risks of infection in He‘eia fishpond. However, those results are only applicable to the sampling campaigns presented in this project. Previous studies have detected the presence of pathogenic *Leptospira* in O‘ahu streams and coastal water, indicating a sporadic presence of the pathogen in nature (Viau & Boehm, 2011). As such, consistent environmental detection

should be conducted to provide up-to-date status on the presence of pathogenic *Leptospira* in streams and coastal waters.

Many factors are involved in the shedding of the bacteria in the environment. As such, *Leptospira* was correlated with fecal indicators of feral swine, which are the suspected maintenance reservoir of *Leptospira* in He‘eia (Chapman, 2021; Poudel et al., 2020). Additional molecular assay detecting the presence of *Bacteroidales,* feral swine fecal indicator (Pig-2-back), should be used in conjunction with the detection of *lipL32* to inform the presence of both the host in the watershed and the pathogen in coastal water (Mieszkin et al., 2009). Indeed, though *Leptospira* was not detected, the host reservoir remains and may shed bacteria in periods outside of the sample collection.

5.0 - CONCLUSION

5.1 - IMPLICATIONS OF LEPTOSPIRACEAE DETECTION IN THE ENVIRONMENT

This project aimed to link environmental phenomena to the presence of Leptospiraceae and use molecular assays that would facilitate the environmental detection of pathogenic *Leptospira*. Current detection methods of leptospirosis are more reactive as they involve serological tests (microscopic agglutination test and enzyme-linked immunosorbent assay) following a potential infection in a patient (Plank & Dean, 2000). Rapid molecular detection techniques of pathogenic *Leptospira* have been developed using PCR techniques, however, protocols are developed for clinical applications (Merien et al., 2005; Picardeau, 2013). Environmental detection of pathogenic *Leptospira* aims to be a preventive intervention to reduce the risks of infection in healthy individuals. As a result, developing methodologies specific to environmental samples could be used for preventive screening of streams and coastal waters, especially because clinical protocols cannot be applied to environmental samples as the concentration of bacteria in a patient is much higher than in environmental samples ($8.0x10^1$ - $3.9x10^4$ leptospires/mL of blood *vs.* 0.05 - 100 genomes/mL of water) (Merien et al., 2005; Viau & Boehm, 2011).

5.2 - MANAGEMENT IMPLICATION OF THE PROJECT

Though results indicated the negative detection of pathogenic *Leptospira*, data on the presence of Leptospiraceae family provides information on the origin and the dispersal of the bacteria in the fishpond. Correlating Leptospiraceae presence and

abundance to environmental factors provide noticeable environmental cues that can be recognized by communities. Environmental factors associated with Leptospiraceae can then be incorporated in management plans to avoid outside activities that may be associated with risks of infection (during the rainy season, significant rain events, flooding events and/or turbid streams) without the need for constant screening of waterways.

APPENDIX

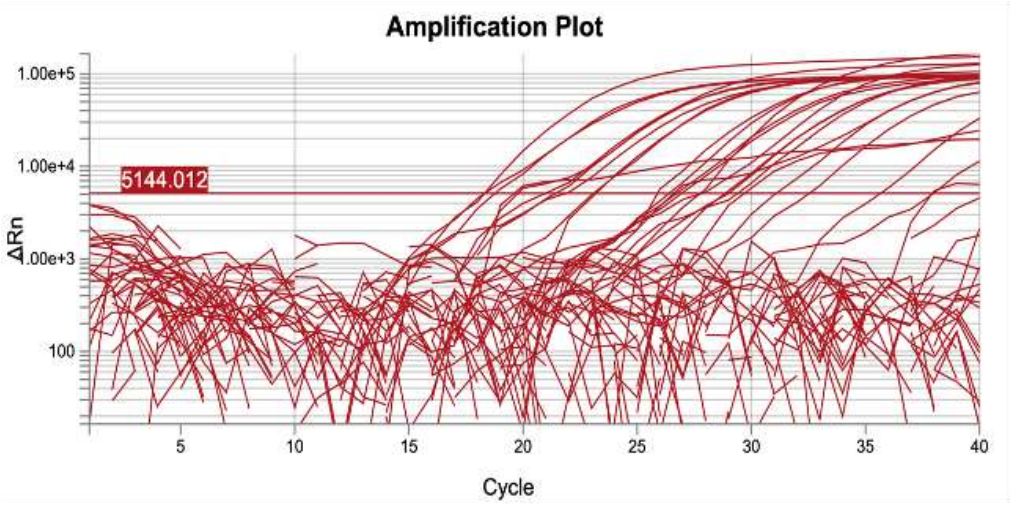

Figure 19 : Amplification plot for the first PCR plate. The horizontal line at 5144.012 represents the threshold of detection which is set by the qPCR machine. The standards are represented by the curves that have passed the threshold of detection. The samples tested did not amplify past the threshold of detection, indicating the absence of pathogenic *Leptospira*.

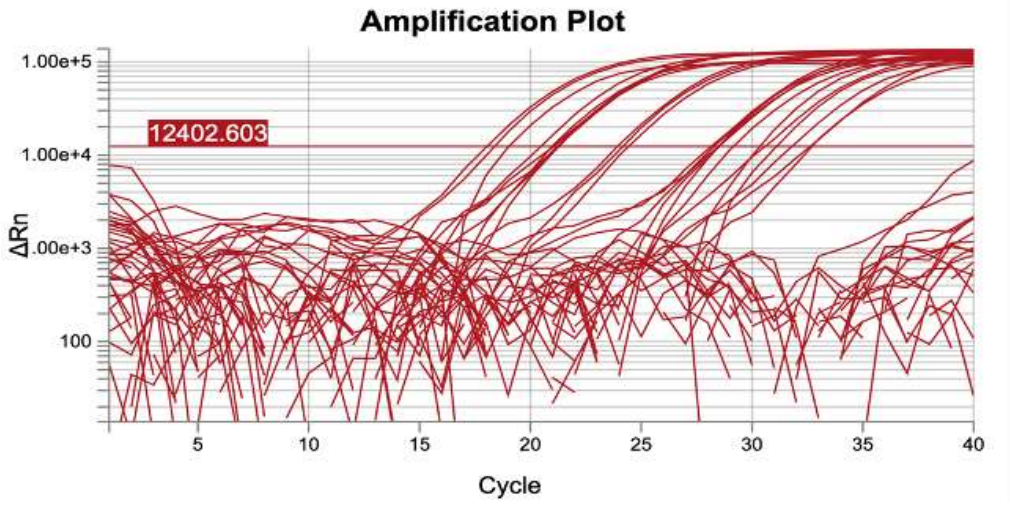

Figure 20 : Amplification plot for the second qPCR plate. The horizontal line at 12402.603 represents the threshold of detection which is set by the qPCR machine. The

standards are represented by the curves that have passed the threshold of detection. The samples tested did not amplify past the threshold of detection, indicating the absence of pathogenic *Leptospira*.

LITERATURE CITED

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