Science of the Total Environment 760 (2021) 143340

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Detection and assessment of the antibiotic resistance of *Enterobacteriaceae* recovered from bioaerosols in the Choqueyapu River area, La Paz – Bolivia

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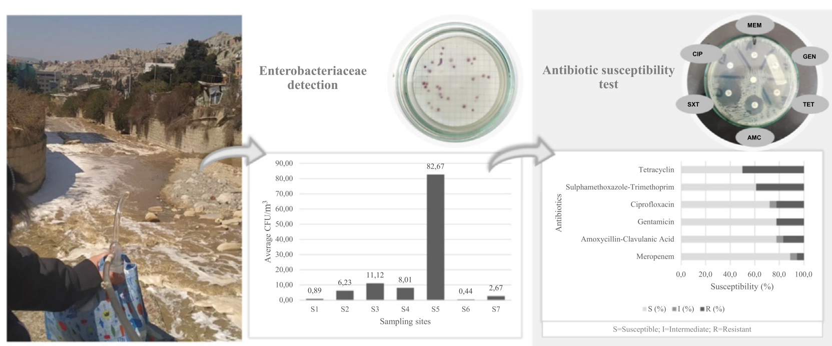
HIGHLIGHTS

- Antibiotic-resistant *Enterobacteriaceae* were detected on air samples from a high-polluted river on an urban area.
- Impingement devices were used to collect air samples.
- The membrane filter method and the Kirby-Bauer Disk Diffusion Susceptibility Test were performed in laboratory procedures.
- The detection of airborne bacteria is linked to the environmental conditions of the season and sampling sites location.
- Susceptibility tests for six types of clinical-use antibiotics were performed.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 24 August 2020
Received in revised form 20 October 2020
Accepted 21 October 2020
Available online 29 October 2020

Editor: Frederic Coulon

Keywords:
Antibiotic resistance
Bioaerosols
Enterobacteriaceae
Sanitation
Urban

ABSTRACT

As a highly contaminated waterway flowing through a densely populated urban area, microbiological pollution associated with the Choqueyapu River and the absence of a wastewater treatment plant in La Paz city threatens public health. We collected air samples adjacent to this river using impingement. Laboratory analyses identified the presence of *Enterobacteriaceae*, reporting a maximum concentration of 86,11 CFU/m^3 of sampled air. Positive samples were tested for antibiotic susceptibility against the antibiotics amoxicillin-clavulanic acid, ciprofloxacin, gentamicin, meropenem, sulfamethoxazole-trimethoprim and tetracycline via disk diffusion. The highest percentages of antibiotic resistance were registered for tetracycline (50% of isolates) and sulfamethoxazole-trimethoprim (38,9%), while the lowest resistance profile was reported for meropenem (5,6%). A comparison of results obtained on the pilot studies [elaborated during the wet season of 2018 by Chavez, 2019 and Salazar et al., 2020] and the present study has been done, highlighting seasonal effects over airborne *Enterobacteriaceae* concentration. Also, it was determined an increase of antibiotic resistance for tetracycline, gentamicin and ciprofloxacin; and a reduction for sulfamethoxazole-trimethoprim, meropenem and amoxicillin-clavulanic acid.

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https://doi.org/10.1016/j.scitotenv.2020.143340

1. Introduction

Waterborne or water-related diseases are caused by infectious and often enteric pathogenic agents including bacteria, virus, protozoa and helminths (Marcheggiani et al., 2015; Ramírez-Castillo et al., 2015). These agents can be transmitted to people by inhalation, in-direct or direct contact, or ingestion of contaminated water (Marcheggiani et al., 2015). The spread of infections mainly depends on the pathogen survival in the environment and the minimal infectious dose (MID) required to infect a susceptible host, which is influenced by the bacteria latency and ability for multiplication (Leclerc et al., 2002). To counteract these infections, antibiotic treatments are usually prescribed. However, microorganisms are likely to develop antibiotic resistance, which would result in prolonged illness and greater risk of death, among other consequences, becoming a public health concern (Cosgrove, cited in Marti et al., 2014).

Increasing cases of waterborne infections, including diarrhea, gastrointestinal infections and systematic illnesses have been registered worldwide, leading to an estimated of 2.2 million deaths per year, children being the most affected group (World Health Organization (WHO) cited in Ramírez-Castillo et al., 2015). The vulnerability of a population before the occurrence of waterborne diseases is conditioned by the political will to address water-related issues, which usually is deficient among developing countries, implying a slow development of public policies and inadequate investments in water and sanitation infrastructures (Moe and Rheingans, 2006). The municipality of La Paz (which territorially includes La Paz city, one of the largest cities of Bolivia) presented 38,873 cases of acute diarrheal diseases in the year 2019, 1 to 4 year- old children being the most affected population (Sistema Nacional de Información en Salud SNIS, 2019). Therefore, generating and improving the knowledge about the ways of spread and transmission of waterborne infections is a necessity to enhance the life quality of people, especially children, reducing their potential exposure to diseases.

Sanitation issues are latent and present among Bolivian cities. La Paz city is located on a sector of the La Paz River basin geographical area, which originates in the Bolivian Andes and it is part of the Amazon macro basin, thus it contributes to the pollution of this watershed (Poma et al., 2016). The most important problem in this basin is the contamination that its main contributor, the Choqueyapu River, receives as it flows through La Paz city, polluting as well irrigation fields located downstream. The Choqueyapu River and its tributaries receive domestic, hospital and industrial untreated sewage; moreover, the city does not have a wastewater treatment plant, which causes deeper sanitation problems (Contraloría General del Estado, 2013a). One of the most important tributaries of the Choqueyapu River is the Orkojahuira River, which is strongly affected by industrial and domestic wastewater, acquiring a very bad water quality by the point where both rivers intersect (Contraloría General del Estado, 2013a). Among the polluting substances discharged into the rivers, the presence of pathogenic bacteria and antibiotic compounds has been reported.

Domestic and hospital discharges contain *Enterobacteriaceae* with potential pathogenicity. The presence of *Escherichia coli*, *Salmonella*, *S. enterica*, *Shigella* and *Klebsiella pneumoniae* has been registered along the Choqueyapu River (Poma et al., 2016; Guzman-Otazo et al., 2019; Chavez, 2019; Rocha-Melogno et al., 2020; Salazar et al., 2020). Multiple-antibiotic resistant enteropathogens have been also identified, implying the presence of antibiotic compounds in the river, which generally occurs in low concentrations and originates from the overuse and misuse of antimicrobial agents in human and veterinary medicine, animal farming, or industrial settings (Marti et al., 2014; Poma et al., 2016; Guzman-Otazo et al., 2019; Salazar et al., 2020). Furthermore, some sections along the river and its tributaries have been channelized and covered, while others have been left uncovered with natural and man-made aeration mechanisms potentially leading to aerosolization of the water. Certain uncovered sections are located nearby public places throughout the urban area, where the presence of people and the possible contact and inhalation of aerosolized microbes, or bioaerosols, from the river may represent a public health issue.

During a pilot study performed in the year 2018, Chavez (2019) and Salazar et al. (2020) reported the spread of coliform bacteria from the Choqueyapu River through bioaerosols. Furthermore, Salazar et al. (2020) assessed their antibiotic resistance. This study, carried out during the wet season, registered the highest resistant profiles (i.e. percentage of resistant total coliform samples) for sulfamethoxazole-trimethoprim (73%) and amoxicillin-clavulanic acid (60%). Coliform bacteria include several genera of *Enterobacteriaceae*, which have a critical position on the priority list of antibiotic-resistant bacteria, developed by the World Health Organization (WHO, 2017). Moreover, *Enterobacteriaceae* presence can indicate that circumstances are suitable for the occurrence of enteric pathogens (Halkman and Halkman, 2014).

The impact of pathogens on bioaerosols is related to their viability (i.e. capacity to survive and grow in a medium), which in turn is influenced by environmental conditions, seasonal factors and microbial mechanisms of protection against extreme conditions (Löndahl, 2014; Kim et al., 2018). Moreover, the transfer of antibiotic resistance genes among bacteria and the formation and dispersion of bioaerosols colonized by pathogenic bacteria with potential contact with humans, depend on different factors which may change according to the season (Bengtsson-Palme et al., 2017; Kim et al., 2018). Thus, it is necessary to monitor and generate periodic information about the presence of pathogens and their resistance to antibiotics in different environmental compartments (i.e. air, water and soil) across all seasons.

This study aimed to detect *Enterobacteriaceae* in bioaerosol samples from the Choqueyapu River area and to assess the occurrence of antibiotic resistance among the *Enterobacteriaceae* positive samples. We also sought to analyze the evolution of this phenomenon through the comparison between data from the wet and dry season of 2018 and 2019 respectively.

2. Materials and methods

2.1. Study area and sampling sites

La Paz city is located in a geographical depression where a change of biomes between plateau and valley is evident. The Choqueyapu River, originating at Pampalarama lagoon, receives domestic, hospital, industrial and commercial untreated wastewater (Guzman-Otazo et al., 2019). After its pass through La Paz city, it changes the name to La Paz River, which is an important tributary of the Amazon basin and it is also used for agricultural purposes downstream (Poma et al., 2016).

We considered the following main aspects to define the sampling sites along the Choqueyapu and Orkojahuira rivers: i) public spaces with the concurrence of people; ii) proximity to aeration cascades; and iii) open-air conditions for direct sampling feasibility. We considered the inclusion of two sampling sites along the Orkojahuira River which were not considered in the pilot study, in order to verify its possible impact on the Choqueyapu River in terms of effect of increase of the concentration of aerosolized pathogenic bacteria. Thus, we accomplished sampling at seven sites: three were located before the intersection between Choqueyapu and Orkojahuira rivers; one at the intersection of the rivers; and three after the intersection.

As shown in Table 1, sites S1, S2, S3, S6 and S7 were sampled during June and July of 2019. However, later we decided to sample two additional sites (S4 and S5) in August of the same year, since we analyzed their location and considered potential results of high concentration of bioaerosols with presence of *Enterobacteriaceae*. The geographical location of sampling sites from the studies of 2018, according to Salazar et al. (2020), and 2019 can be visualized in Fig. 1.

2.2. Sample collection

We collected samples during the dry season (winter), between June, 6th and August, 31st of 2019, achieving a total of 34 samples. Sampling

Table 1
Location and description of sampling sites - dry season.

Site	Number of samples per site	Notation	Sampling period (2019)	Location	River	L-intersection[a]	Location description[b]
1	6	S1	June 6th–July 11th	−16,50°; −68,12°	Orkojahuira	Upstream	On a footbridge for cars and pedestrians
2	6	S2	June 6th–July 11th	−16,51°; −68,12°	Choqueyapu	Upstream	Close to an output of a covered section of the river
3	6	S3	June 6th–July 11th	−16,52°; −68,11°	Orkojahuira	Upstream	Close to an output of a covered section of the river
4	2	S4	August 20th	−16,52°; −68,12°	Intersection	Intersection	Intersection between Choqueyapu and Orkojahuira rivers
5	2	S5	August 22nd–August 31st	−16,52°; −68,11°	Choqueyapu	Downstream	Nearby a municipal public building
6	6	S6	June 6th–July 11th	−16,53°; −68,10°	Choqueyapu	Downstream	Nearby a community market
7	6	S7	June 6th–July 11th	−16,54°; −68,09°	Choqueyapu	Downstream	On a footbridge between two aeration cascades

[a] Location in reference to the intersection between Choqueyapu and Orkojahuira rivers.
[b] Remarkable characteristic of the sampling site location.

sites were located nearby an aeration cascade, at an approximate height of 2 to 5 m above the cascade, due to the height of the retaining walls, and a horizontal distance of 2 to 10 m. The methodology applied for the sampling and laboratory procedures was established in the first pilot study, performed in the year 2018 by Chavez (2019) and Salazar et al. (2020).

For bioaerosol collection, we used the All-Glass Impinger (AGI-30, Ace Glass Inc., NJ, USA), which collects microorganisms by inducing airborne particles to collide with the agitated surface of a collection fluid, directing the airstream downward through a single jet forming a vigorous rolling of the fluid (Duchaine et al., 2001). On site, impingers were connected to a vacuum which maintained a nominal air flow rate of 12.5 ± 0.5 L/min. We set the sampling time to 30 min, due to the high efficiency (100%) of *E. coli* recovery demonstrated using peptone dissolved on DI water as collection fluid (Dungan and Leytem, 2015).

As described by Salazar et al. (2020), we used Tween mixture as the collection fluid, which consisted of a distilled water-based solution containing 1% peptone (Thermo Scientific TM, USA), 0,01% Tween 80 (Thermo Scientific TM, USA) and 0,005% Antifoam Y-30 (Sigma-Aldrich, Inc., Darmstadt, Germany). Peptone maintains the viability of microbial cells by preventing osmotic shock (Dungan and Leytem, 2015). Tween 80 is a non-ionic surfactant with hydrophilic polyoxyethylene and hydrophobic fatty acid, which we employed to improve the suspension of bacteria in liquid (Chang and Chou, 2011 cited in Salazar et al.,

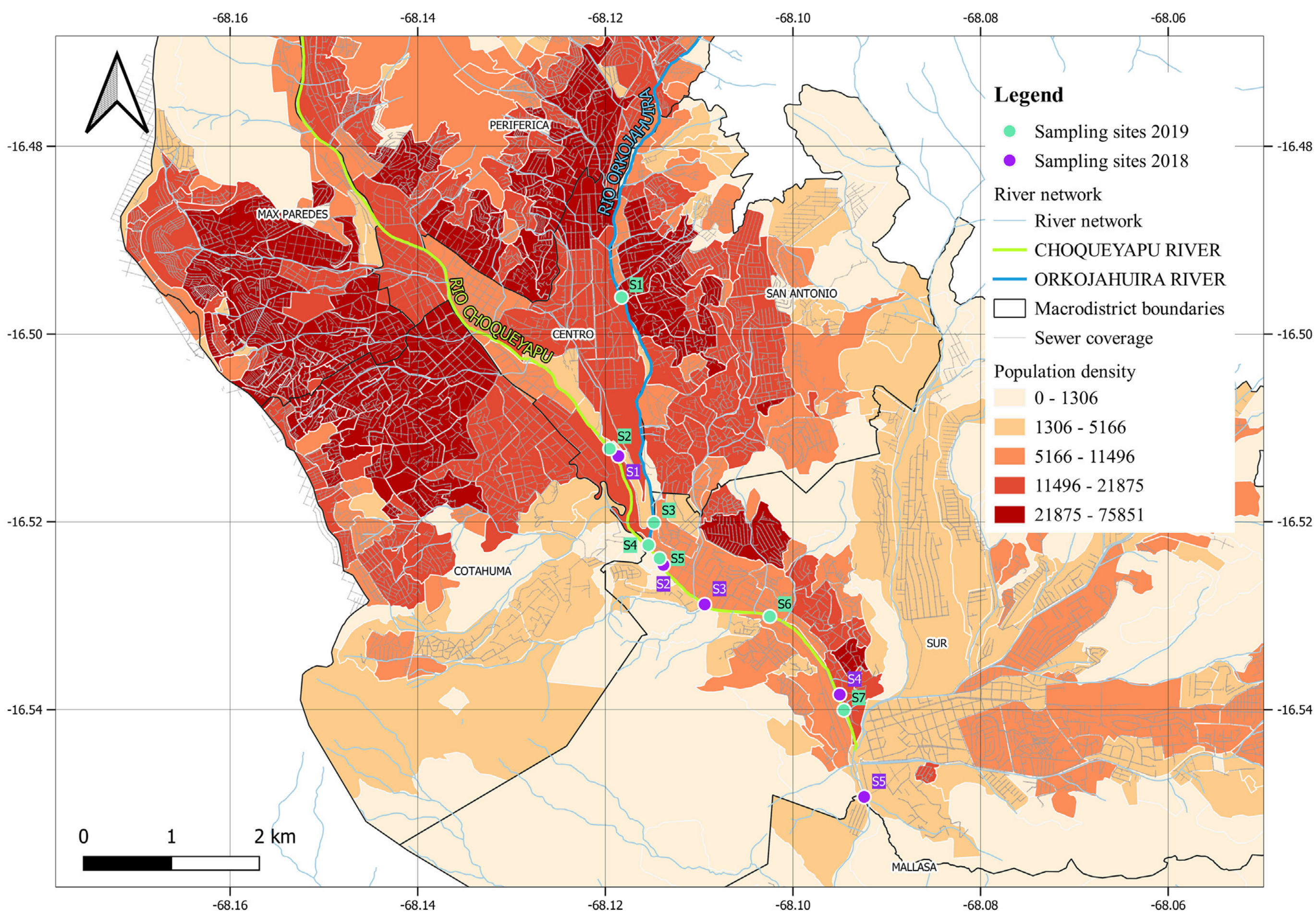

Fig. 1. Sampling area, contrast between population density (classified in quantiles), sewer coverage and sampling sites. Reference system of coordinates: WGS 84 (EPSG:4326). Data sources: i) Map of the La Paz municipality macro district boundaries (Gobierno Autónomo Municipal de La Paz, 2013c), ii) map of the La Paz municipality hydrographic network (Gobierno Autónomo Municipal de La Paz, 2013d), iii) map of the La Paz municipality population density (Gobierno Autónomo Municipal de La Paz, 2013b), iv) map of the La Paz city Sanitary Sewer Coverage (Gobierno Autónomo Municipal de La Paz, 2013a).

2020). We added Antifoam Y-30 to avoid re-aerosolization losses due to the foaming of peptone solutions during impinger operation (Springorum et al. 2011 cited in Dungan and Leytem, 2015). For sampling campaigns preparation, we distributed an amount of 20 mL in each impinger, then autoclaved at 121 °C for 20 min to achieve sterilization. During the sampling process, we covered the impingers from sunlight to avoid evaporation. After each sampling procedure we transported AGIs on ice to the laboratory, typically a duration between 15 min and 3 h depending on the sampling schedule.

2.3. Enterobacteriaceae detection and antimicrobial susceptibility test

We processed the samples contained in the impingers using the membrane filter method (American Public Health Association APHA, 2017). However, in order to report the bacteria colonies as total *Enterobacteriaceae*, we did not perform the urea confirmation procedure indicated by the Standard Methods (APHA, 2017) and we set the incubation temperature to 37 °C. We performed sample filtration using 0.45 μm pore sized membranes (DelAgua, Marlborough, UK), which we then placed on CRITERION™ m-TEC agar (Hardy Diagnostics, CA, USA) prepared plates, which according to Kaplan et al., 2013 selects for *Enterobacteriaceae*, and incubated at 37 °C for 22 ± 2 h.

We calculated the concentration of total *Enterobacteriaceae* for each sampling site using an expression derived from the Method 1603 for water samples stated by the Environmental Protection Agency EPA (2014). This equation divides the Colonies Forming Units (CFUs) with the sampling volume, obtaining the number of CFU (of *E. coli*) present on each m^3 of the sample. In our case, the sampling air flowrate and sampling time are related to the sampled quantity of CFU (i.e. sampled number of colonies of *Enterobacteriaceae*). Thus, we calculated the *Enterobacteriaceae* CFU/m^3 of sampled air according to Eq. (1) assuming a 100% efficiency due to the 30-minute sampling time and the fact that we did not use heads for size differentiation of aerosols.

$$Enterobacteriaceae concentration\left[\frac{\mathrm{CFU}}{\mathrm{m}^3}\right] = \frac{CFU}{vacuum\ air\ flowrate\left[\frac{L}{\min}\right]*sampling time[\min]*\frac{\mathrm{m}^3}{10^3 L}} \tag{1}$$

We performed the antibiotic resistance procedure through the Kirby-Bauer Disk Diffusion Susceptibility Test (Hudzicki, 2009). The plates that presented *Enterobacteriaceae* growth within 24 h were individually analyzed using this procedure. Using a sterile loop, two colonies were taken from the m-TEC agar and suspended on 2 mL of sterile saline PBS solution with an adjusted standard turbidity of 0.5 McFarland (Hardy Diagnostics, CA). Within the next 15 min, the inoculum was distributed covering the entire surface of a Mueller-Hilton agar (Hardy Diagnostics, CA, USA) plate. After 3 to 5 min, we placed the antimicrobial discs on the agar and incubated it at 37 °C for 18 h.

On the procedure, we tested six types of clinical-use antibiotics: 20/10 μg amoxicillin-clavulanic acid (AMC), 5 μg ciprofloxacin (CIP), 10 μg gentamicin (GEN), 10 μg meropenem (MEM), 1.25/23.75 μg sulfamethoxazole-trimethoprim (SXT) and 30 μg tetracycline (TET) (Thermo Scientific TM, USA); which are employed on treatments against *E. coli*. We determined the antibiotic susceptibility of the *Enterobacteriaceae* by measuring the diameters of the inhibition zone around each antibiotic disk after the incubation time. Then, our results were compared to the Performance Standards for Antimicrobial Susceptibility Testing from the Clinical and Laboratory Standards Institute (2018), which according to the inhibition zone diameters, provides the classification of the bacteria under the categories: Resistant, intermediate and susceptible.

2.4. Statistical analyses

We performed the analysis of variance (ANOVA) method using the software Microsoft Excel, based on a confidence level of 95% ($\alpha = 0,05$), which allows to identify the sources of variability on the first (*Enterobacteriaceae* detection) and second (antibiotic susceptibility test) parts of the study. This statistical technique, applying a completely random design, separates the treatments (i.e. the sampling sites and the antibiotics, respectively) variability from the error variability, which allows to accept or reject a hypothesis of equality among treatments (Gutiérrez and De La Vara, 2008).

The Least Significant Difference (LSD) method was performed after rejecting the equality hypothesis from the ANOVA analyses carried out for the first and second parts of the study. This statistic test identifies significant differences between treatments, which are compared in pairs, by calculating the minimum difference which must exist between their respective means (Dodge, 2008; Gutiérrez and De La Vara, 2008).

2.5. Meteorological data collection

We considered temperature, humidity, wind speed and solar radiation as atmospheric factors that may have affected the dispersion, survival and viability of *Enterobacteriaceae* in the sampling environment. Since the Bolivian Meteorology and Hydrology National Service (SENAMHI) database does not have an available register of the required parameters for the sampling area during the sampling period, we based in available data from the Reprocessed GLDAS-2.1: Global Land Data Assimilation System dataset provided by NASA (NASA GES DISC at NASA Goddard Space Flight Center). Using the Google Earth Engine software, we gathered the monthly mean temperature, humidity, wind speed and solar radiation for the sampling area during the sampling periods of both studies, 2018 and 2019. Nevertheless, the global horizontal solar radiation for the central area of La Paz city was obtained from Birhuett (2009), since this parameter is available in this database only. This information is contained in Table 2.

3. Results

3.1. Total Enterobacteriaceae detected on bioaerosols

We tested a total of 34 samples for *Enterobacteriaceae* detection. Among them, 58,8% showed positive results (i.e. presence of *Enterobacteriaceae*). Samples of site 5 presented a maximum average concentration of 82,67 CFU/m^3, being the highest average concentration detected on the study. All results are shown in Table 3.

We considered only the five sites sampled during the period June 6th–July 11th (2019) for the statistical analyses, since they present the same number of samples. Results from the single-factor analysis of variance show a statistically significant difference among them: the critical value for F (2,76) is lower than the obtained F value (6,30) and the obtained p-value was 0,0012 which is lower than the alpha value (5%). Thus, the null hypothesis of equality between the five sampling sites is rejected. Moreover, the Least Significant Difference method was applied using the mean values of CFU/m^3 from the sampling sites. Significant differences were obtained for the comparison of site 3 with sites 1, 6 and 7.

3.2. Detection of antibiotic resistance

A total number of 18 samples with *Enterobacteriaceae* presence were tested for antibiotic susceptibility in the present study. Numeric results of these tests are shown in Fig. 2. Moreover, in order to obtain resistance profiles for each antibiotic, we used resistance percentages as shown in Fig. 3. The highest profiles of resistance correspond to tetracycline (50%) and sulfamethoxazole-trimethoprim (38,9%), while the lowest profile was registered for meropenem (5,6%). Therefore, the bioaerosols of the Choqueyapu and Orkojahuira rivers contain antibiotic resistant *Enterobacteriaceae*.

Antibiotics and sampling sites are both significant variability sources in a two-way analysis of variance, which was done using percentages of

Table 2
Meteorological data for wet and dry season sampling periods.

Wet season – Year 2018					Year 2009
No.	Month	Mean temperature (°C)	Mean wind speed (m/s)	Mean specific humidity (kg/kg)	Global horizontal solar radiation (kWh/m^2 $*$ day)
1	November	11,79	3,31	0,007	5,9
2	December	11,15	3,24	0,007	4,7
Dry season – Year 2019					Year 2009
No.	Month	Mean temperature (°C)	Mean wind speed (m/s)	Mean specific humidity (kg/kg)	Global horizontal solar radiation (kWh/m^2 $*$ day)
1	June	7,43	2,06	0,005	4,6
2	July	7,12	2,23	0,005	4,8
3	August	7,75	2,37	0,005	5,5

resistance to each antibiotic per sampling site, since each site presented a different number of positive samples with presence of *Enterobacteriaceae*. Both of the F values result higher than the critical values for F (2,6 > 2,5 for antibiotics and 6,2 > 2,4 for sampling sites) and both of the *p*-values (0,047 for antibiotics and 0,00026 for sampling sites) are lower than the alpha value (5%). Thus, the analysis allows the rejection of both null hypothesis: no differences among the percentages of resistance to the different antibiotics, and no differences between the sampling sites.

The Least Significant Difference procedure was also applied in this part to identify the significant differences between the average values of the resistance percentages for each antibiotic. Therefore, three statistically significant differences were detected, two involve tetracycline differences with gentamicin and meropenem, being the last one between sulfamethoxazole-trimethoprim and meropenem.

3.3. Comparison between antibiotic resistance results from the wet season of 2018 (Salazar et al., 2020) and the dry season of 2019

We considered comparing the data reported by Salazar et al. (2020) and the present results since the location of sampling sites follows the same logic of before and after the intersection of the Choqueyapu River with the Orkojahuira River, also the same laboratory procedures were performed in both studies. Thus, a site by site comparison was done together with a comparison between antibiotic susceptibility profiles in order to observe and analyze the evolution of the antibiotic susceptibility of *Enterobacteriaceae* over time and the contrast between wet and dry seasons data.

The differences between resistance percentages are notable. There has been a reduction in resistance for amoxicillin-clavulanic acid (from 60% to 16,7%), meropenem (from 7,5% to 5,6%) and sulfamethoxazole-trimethoprim (from 62,5% to 38,9%). Meanwhile, antibiotic resistance has increased for ciprofloxacin (from 15% to 22,2%), gentamicin (from 10% to 22,2%) and tetracycline (from 30% to 50%). Thus, the antibiotic susceptibility variates between the wet season of 2018 and the dry season of 2019. These differences are represented in Fig. 4.

4. Discussion

4.1. Total Enterobacteriaceae detected on bioaerosols from the Choqueyapu River

It is important to mention that both the pilot and the present studies were limited to perform air sampling only. Nevertheless, different water quality studies along the Choqueyapu River area have been performed by different institutions through time and relevant data for this study purposes are reported throughout the introduction and discussion sections. Our samples were taken during the dry season, which also corresponds to the Bolivian winter, meanwhile the sampling period of the pilot study corresponded to the wet season and Bolivian summer. The detection of *Enterobacteriaceae* on bioaerosols may have been influenced by the degree of pollution of its source, the sampling sites location and/or environmental factors.

According to the audit report about the environmental impacts on the La Paz River basin elaborated by the Contraloría General del Estado, 2013b, the Choqueyapu and Orkojahuira rivers present water quality indexes of 20,24/100 and 20,70/100 respectively, which means a very bad water quality status. The mentioned study considered ten measured parameters, from which the Choqueyapu River (at the location of site S7) and the Orkojahuira River (at the location of site S3), reported weighted values of: 0,45/15 and 1,65/15 for chemical oxygen demand, 0,75/15 and 0,00/15 for thermotolerant coliforms, 1,10/10 for dissolved oxygen, 0,40/10, and 0,40/10 for electrical conductivity, respectively. This means that both rivers are highly polluted due to domestic and industrial wastewater.

As shown in Fig. 1, the Choqueyapu River receives domestic wastewater from more densely populated areas than the Orkojahuira River. However, the difference between the concentration of airborne *Enterobacteriaceae* from the sites S1 and S2 is not statistically significant. Also,

Table 3
Positive samples and concentration of total *Enterobacteriaceae* (CFU/m^3).

Sampling site	n[a]	Positive samples[b]	Concentration			River	L-intersection[d]
			Minimum CFU/m^3	Maximum CFU/m^3	Average[c] CFU/m^3		
1	6	1	0,00	5,34	0,89	Orkojahuira	Upstream
2	6	5	2,67	10,68	6,23	Choqueyapu	Upstream
3	6	4	0,00	18,69	11,12	Orkojahuira	Upstream
4	2	2	2,67	13,35	8,01	Intersection	Intersection
5	2	2	79,49	86,11	82,67	Choqueyapu	Downstream
6	6	1	0,00	2,67	0,44	Choqueyapu	Downstream
7	6	3	0,00	10,68	2,67	Choqueyapu	Downstream
Total	**34**	**18**	**0,00**	**86,11**	**16**	–	–

[a] n = Number of samples taken at each site.
[b] Positive samples are the petri plates with *Enterobacteriaceae* colonies detected.
[c] Including both positive and negative samples (registered as 0 CFU/m^3).
[d] Location in reference to the intersection between Choqueyapu and Orkojahuira rivers.

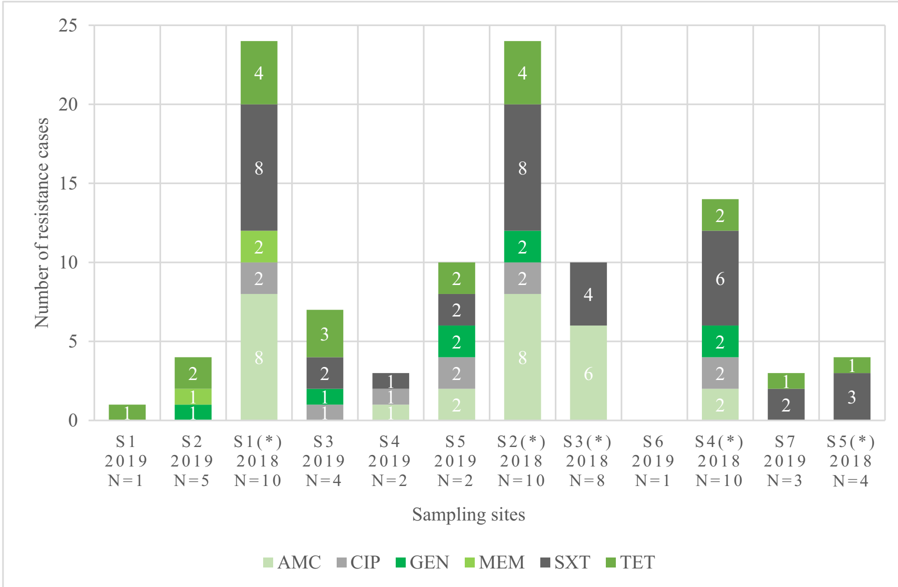

Fig. 2. Number of resistance cases per sampling site. Data from the pilot study of 2018 were recovered from Salazar et al. (2020). N = number of samples tested for antibiotic susceptibility (i.e. positive samples with presence of *Enterobacteriaceae*).

the concentrations on sites S6 and S7 after the rivers intersection are not significantly higher than the concentrations on sites before the intersection. Furthermore, the site S4, located on the intersection of the rivers, registered a lower concentration than its precedent site S3. The site S5, which registered the overall highest concentration, was located after the intersection; however, it was also the closest one to an aeration cascade. These facts suggest that the spatial variation of the concentration of *Enterobacteriaceae* on air may be conditioned by the environmental factors and the sampling sites location more than the degree of the fecal pollution of its source. However, the bacterial concentration existing on its source is still an important factor to consider; thus, further investigation is required.

Regarding the role of environmental factors, the impact of atmospheric conditions on the obtained results can be analyzed through a site by site comparison between data from the present study and the pilot study of 2018, taking into account the meteorological data reported in Table 2, the sites location illustrated in Fig. 1 and the average concentrations of *Enterobacteriaceae* on samples shown in Fig. 5. Three sites present a similar location: sites S1(*), S2(*) and S4(*) from 2018, and correspondently sites S2, S5 and S7 from 2019. Chavez (2019) reported the highest average concentrations of Enterobacteriaceae of the pilot study for S1(*) and S2(*), being 72,22 CFU/m^3 and 32,22 CFU/m^3 respectively; and S4(*) registered an average concentration of 2,78 CFU/m^3. On the other hand, the results of this study show interesting differences for the correspondent sampling sites: Site S2 presented an average bacteria concentration of 6,23 CFU/m^3, site S5 registered the highest average concentration being 82,67 CFU/m^3 and site S7 registered 2,67 CFU/m^3. Sites S4 (*) and S7 presented similar results, while the opposite situation was registered for the other two pairs of sites. Two initial factors may have had a strong influence over the differences between the 2018 and 2019 studies: the flowrate of the rivers and the relative humidity.

According to the National Institute of Statistics for the period 2006–2008, the Choqueyapu River flowrate during the dry season reaches a maximum value of 3 m^3/s, meanwhile during the wet season it increases reaching values of 4 m^3/s in November and 9 m^3/s in December (Instituto Nacional de Estadística INE, 2018). The increase of flowrate causes a dilution of the contaminants, therefore a decrease of bacteria concentration. This could explain the drastic difference between 72,22 CFU/m^3 (2018) and 6,23 CFU/m^3 (2019) for sites S1(*)

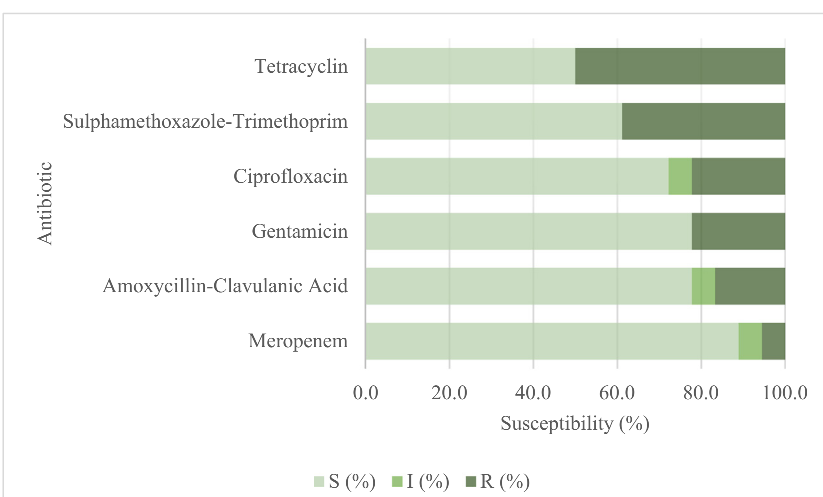

Fig. 3. Antibiotic susceptibility profile for n = 18 tested samples. S (%) = SUSCEPTIBLE. I (%) = INTERMEDIATE; R (%) = RESISTANT.

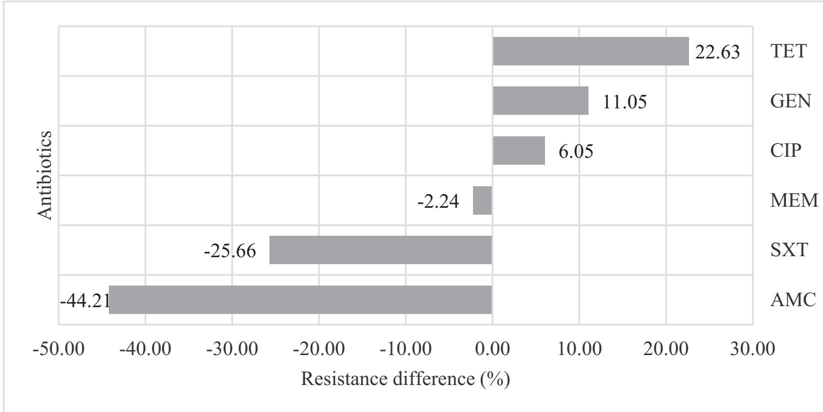

Fig. 4. Comparison between 2018 (Salazar et al., 2020) and 2019 results for antibiotic resistance. AMC = amoxicillin-clavulanic acid, CIP = ciprofloxacin, GEN = gentamicin, MEM = meropenem, SXT = sulfamethoxazole-trimethoprim, TET = tetracycline.

and S2. Moreover, relative humidity is an important factor for the survival of airborne microorganisms, if it ranges between 20% and 95%, bacterial cells on bioaerosols can absorb water from the atmosphere and, reaching >50% increased water sorption, protect themselves from UV-induced inactivation (Karra and Katsivela, 2007). As shown in Table 2, the specific humidity for the dry season is 0,005 kg/kg, while for the wet season it reaches 0,007 kg/kg. Thus, during the wet season, humidity increases and elevates the possibility of survival and viability of bacteria. This may be another factor which causes the difference between sites S1(*) and S2.

Temperature and solar radiation are environmental factors that also influence the survival and viability of airborne bacteria. Similar studies on wastewater treatment plants reported the major presence of total coliforms and enteric bacteria at cold seasons, such as autumn and winter. Szyłak-Szydłowski et al. (2016) reported the higher airborne total coliforms concentration at 10,64 °C and Korzeniewska et al. (2013) reported a higher number of *E. coli* on water and air during autumn months. Meanwhile, Gandolfi et al. (2011) registered the major presence of gram-negative bacteria, including *Enterobacteriaceae*, from urban samples of particulate matter PM10 in winter. In our case, the temperatures reported for the sampling area, which are shown in Table 2, reach 11,79 °C for the wet season and 7,43 °C for the dry season. This factor may explain the difference between 32,22 CFU/m^3 (2018) and 82,67 CFU/m^3 (2019) for sites S2(*) and S5 respectively, since the higher value was obtained during the dry season, when temperature is lower than during the wet season. However, the global horizontal solar radiation present values above 4 kWh/m^2 $*$ day for June, July and December, and values above 5 kWh/m^2 $*$ day for August and November; this suggest that the highest value registered during our study, 82,67 CFU/m^3 (2019), was obtained during a month with high solar radiation. Therefore, seasonal changes affect the airborne *Enterobacteriaceae* concentration, the wet season has an appropriate relative humidity for airborne bacteria survival, meanwhile winter presents suitable temperature. Nevertheless, further research is needed to determine a relation between concentration of airborne bacteria and global horizontal solar radiation.

On the other hand, according to Chavez (2019), wind speed is a factor that influences the dispersion of bioaerosols in the environment. According to Table 2, values above 3 m/s were registered for the 2018 sampling period, while the dry season of 2019 presented values above 2 m/s. This means that the dispersion of bioaerosols is more likely to reach higher distances during the wet season than during the dry one. However, we did not perform an analysis of wind direction in order to find a relation between the obtained bacteria concentrations and the wind as an influent meteorological factor.

Another important factor for the detected quantity of bacteria colonies on positive samples is the proximity of the sampling device to a waterfall. Studies from wastewater treatment plants reported that usually the highest emission of microorganisms occurs during pretreatment, where aeration and separation processes take place and therefore, the movement of water facilitates the aerosolization of particles (Szyłak-Szydłowski et al., 2016, Heinonen-Tanski et al., 2009, Karra and Katsivela, 2007). Therefore, it is worth to mention that sites S2 and S3 were located on outputs from long covered sections of Choqueyapu

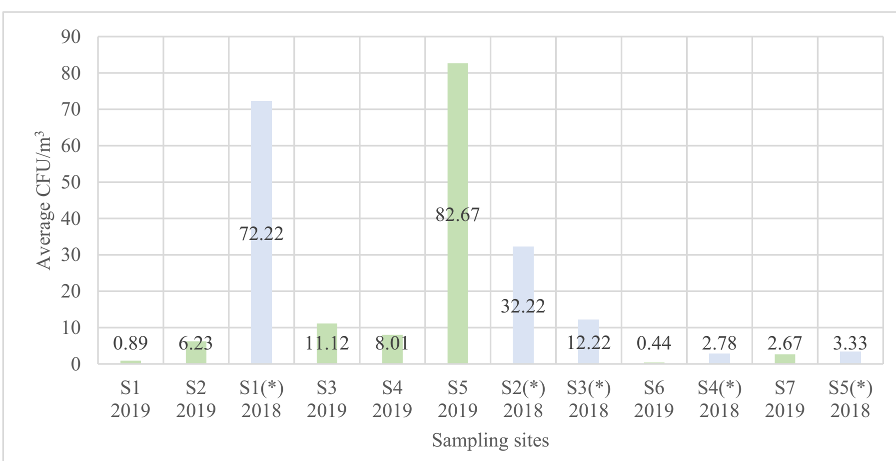

Fig. 5. Average concentration of *Enterobacteriaceae* per sampling site. Data from the pilot study of 2018 were recovered from Salazar et al. (2020).

and Orkojahuira rivers, respectively, and reported high CFU concentrations. It may be attributed to the lack of oxygen that the rivers experience on the covered sections, which together with the absence of UV radiation, provides appropriate environmental conditions for the survival of these anaerobic facultative bacteria.

A final factor that may have affected the *Enterobacteriaceae* detection process in samples is the actual lack of cultivable bacteria in samples. This phenomenon does not exclude the presence of viable non-cultivable bacteria and non-viable bioaerosols, causing an underestimation of the actual bioaerosol concentrations (Alvarez et al., 1995; Stewart et al., 1995; Bunger et al., 2000 cited in Szyłak-Szydłowski et al., 2016).

4.2. Detection of antibiotic resistance

After the processing of 18 samples, we detected antibiotic resistance in six sampling sites, from which the highest number of resistance cases were reported for sites S5, S3 and S2. Site S5 presented resistance to AMC, CIP, GEN, SXT and TET, while S3 reported resistant samples to CIP, GEN, SXT and TET and S2 to GEN, MEM and TET. The sites location represents an interesting factor in the analysis, since S2 was located upstream in the Choqueyapu River, S3 upstream in the Orkojahuira River and S5 downstream in the intersection between both rivers. The potential exchange of antibiotic resistance genes among strains in the environment can be associated to the enteric pathogens distribution in the environment, which is related to the human fecal contamination (Poma et al., 2016). Therefore, the difference among the number of resistance cases per sampling site may be related with the density of urban discharges in both rivers, since the Choqueyapu River receives domestic wastewater from more densely populated areas than the Orkojahuira River, as shown in Fig. 1. Finally, Guzman-Otazo et al. (2019) reported a positive association between water electrical conductivity and DNA concentration (i.e. antibiotic resistance genes), and both the Choqueyapu and Orkojahuira rivers present elevated values of this parameter (Contraloría General del Estado, 2013b).

Furthermore, we found that tetracycline and sulfamethoxazole-trimethoprim were the antibiotics for which *Enterobacteriaceae* samples presented the highest resistance, with resistance percentages of 50% and 38,9% respectively. Similar studies evaluating tetracycline and sulfamethoxazole-trimethoprim resistance of airborne bacteria reported similar or lower results. Gandolfi et al. (2011) reported a tetracycline resistance of 20% and 10% on winter and summer bacteria isolates, respectively, noticing the presence of tet genes in almost all resistant isolates. For sulfamethoxazole-trimethoprim, Zhang et al. (2017) studied this phenomenon on a pharmaceutical wastewater treatment plant, ranging resistance from 12,7% to 50%; and Korzeniewska et al. (2013) reported airborne *Escherichia coli* strains from a wastewater treatment plant ranged resistance from 18,4% to 20%.

The principal mechanisms of resistance to tetracycline on gram-negative enteric bacteria are the active efflux of the antimicrobial agent across the cell membrane and the ribosomal target protection (Opal and Pop-Vicas, 2015; Rossolini et al., 2017). According to Chopra and Roberts (2001), resistance to tetracycline emerges due to the acquisition of tet genes, which may be found on plasmids and transposable genetic elements (Opal and Pop-Vicas, 2015). Thus, tet genes can move from species to species and into a wide range of genera by conjugation, being found on bacteria from humans, animals and the environment; the transfer of these genes and the ability of the bacterial host to spread them is increased by low levels of tetracycline (Chopra and Roberts, 2001).

On the other hand, sulfamethoxazole-trimethoprim is a combination of trimethoprim and sulfonamide that has a synergistic effect and allows a broader spectrum of activity and bactericidal action (Rossolini et al., 2017). The intrinsic resistance mechanism presented by some bacteria is the reduced drug uptake (Rossolini et al., 2017). Meanwhile, acquired resistance to trimethoprim reported for *E. coli* is due to an overproduction of the chromosomal enzyme *DHFR* (Huovinen, 2001). Moreover, acquired resistance to sulfonamide is due to an increased production of para-aminobenzoic acid and alterations of *DHPS* that low the enzyme affinity for sulfonamides; on gram-negative bacteria, the main genes involved are *sul1* and *sul2* (Rossolini et al., 2017).

A rapid spread of resistance to sulfamethoxazole-trimethoprim has been reported, which together with serious side effects, declined its clinical importance; however, since it is a low-cost available antibiotic, it has been widely used for clinical therapy on developing countries (Huovinen, 2001). Chopra and Roberts (2001) mentioned that tetracyclines administration can be intravenous or oral, penetrating into body fluids and tissues and being excreted in the urine. Tetracyclines are used on clinical therapies for humans, veterinary medicine, animal growth promotion and aquaculture, generating direct linkages between its use on food animals and the development of resistant infections in humans, showing a transfer of resistant bacteria (Chopra and Roberts, 2001).

Previous studies of water and soil samples from La Paz river basin recorded resistance to these antibiotics and the presence of important antibiotic resistance genes. Poma et al. (2016) registered that most of the enteropathogenic isolates, including enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC) and enteroaggregative *E. coli* (EAEC), were commonly resistant to ampicillin, nalidixic acid, trimethoprim–sulfamethoxazole and tetracycline. Amoss et al. (2018) reported resistance to amoxicillin, ciprofloxacin, and tetracycline among *E. coli* detected on water samples. Moreover, Guzman-Otazo et al. (2019), identified resistance genes for tetracycline (*tetA*), sulfamethoxazole (*sul1* and *sul2*), quinolones (*qnrS1* and *qnrB1*) and beta-lactams, including cephalosporins (*blaTEM*, *blaOXA-1* and *blaCTX-M-3*), on *E. coli* isolates. Finally, the pilot study of 2018 and our study of 2019 registered resistance cases from airborne *Enterobacteriaceae* samples to TET, SXT, CIP, GEN, AMC and MEM.

As described, different studies reported the wide spread antibiotic resistance along the La Paz River basin, which is exacerbated by the urban and hospital untreated wastewater, which provide a constant discharge of contaminants and pathogenic bacteria across the year (Guzman-Otazo et al., 2019). Unfortunately, there is a lack of information regarding the concentration of antibiotics on water. However, it is known that sub-lethal antibiotic concentrations allow the increase of resistance mutations and the genetic rearrangements that are involved in mobilization and spread of resistance (Sandegren, 2014).

4.3. Comparison between antibiotic resistance results from the wet season of 2018 (Salazar et al., 2020) and the dry season of 2019

The pilot study was done during the wet season of 2018 and this one was performed during the dry season of 2019, being differences in the rivers flowrate, specific humidity, solar radiation and temperature between the respective sampling campaigns. A site by site comparison based on the nearby location of the sites can be carried out for the following pairs: S1(*)–S2, S2(*)–S5 and S4(*)–S7. Site S1(*) from 2018 registered resistance to AMC, CIP, MEM, SXT and TET, while site S2 from 2019 presented resistance to GEN, MEM and TET only; therefore, the bacteria detected from the Choqueyapu River upstream its intersection with the Orkojahuira River decreased their resistance to AMC, CIP and SXT and increased their resistance to GEN. On the other hand, sites S2(*) and S5 registered resistance to the same antibiotics, AMC, CIP, GEN, SXT and TET; thus, bacteria detected downstream the intersection between both rivers maintained their resistance to the same antibiotics. Finally, site S4(*) presented resistance to AMC, CIP, GEN, SXT and TET, while site S7 registered resistance to SXT and TET only; therefore, bacteria at this site reduced their resistance to AMC, CIP and GEN. The reduction of antibiotics for which sampled bacteria presented resistance may be attributed to the number of positive samples which were tested for antibiotic susceptibility in both studies. However, for site S2

(*) ten positive samples were obtained and site S5 had only two positive samples, but in both studies the sampling sites presented resistance cases to the same antibiotics. Thus, the differences in the other two pairs of sites may be due to the variation in the widespread use of the tested antibiotics in human clinical therapy, their concentration on water bodies and the dissemination of antibiotic resistance genes to different bacteria species through integrons, transposons and plasmids, which are determinative factors for antibiotic resistance (Nikaido, 2009 cited in Poma et al., 2016).

Regarding the antibiotic resistance profiles, we found a considerable increase of antibiotic resistance to tetracycline (22,63%), and important reductions of resistance to sulfamethoxazole-trimethoprim (22,66%) and amoxicillin-clavulanic acid (44,21%), according to Fig. 4. These differences may be related to the mobilization of tet genes, sul genes and TEM-derived enzymes which encode resistance to tetracycline, sulfamethoxazole-trimethoprim and amoxicillin-clavulanic acid respectively (Stapleton et al., 1995). Moreover, since the Contraloría General del Estado, 2013b reported the presence of some potentially toxic elements, such as arsenic, cadmium, copper, chrome, mercury, plumb and zinc, in different sites along the rivers, it is worth to mention that some of the antibiotic resistance mechanisms are also used by bacteria to protect themselves against toxic compounds (Munita and Arias, 2016). Thus, bacteria survival and the spread of antibiotic resistance genes over both Choqueyapu and Orkojahuira rivers may be affected by these factors, since these water bodies receive not only domestic and hospital sewage, but also wastewater from industries, which contain toxic elements that might generate pressure over bacteria mechanisms of survival and its natural selection.

Finally, a remarkable result is that resistance to meropenem has maintained low profiles, registering a value of 5,6% in 2019 and a decrease of 2,24% in relation to the results of 2018. Previous studies performed in the La Paz River basin did not present meropenem as a tested antibiotic. This antibiotic, as a member of the carbapenems beta-lactams antibiotics, is one of the most effective against Gram-positive and Gram-negative bacteria and less vulnerable to most beta-lactam resistance determinants, thus the spread of carbapenems resistance constitutes a public health problem of major importance (Meletis, 2016). However, within the pilot study and this one, there has been registered only three cases of resistance to meropenem, two on site S1(*) from 2018 and one on site S1 from 2019, which does not constitute a significant difference.

5. Conclusions

- Seasonal changes can affect the concentration of bacteria on bioaerosols, due to differences on environmental factors such as humidity, precipitation, temperature and solar radiation. The highest concentration of airborne *Enterobacteriaceae* from the Choqueyapu River was detected during the dry season (Bolivian winter), being 82,67 CFU/m^3.
- The detection of airborne *Enterobacteriaceae* suggests suitable conditions for enteric pathogens to be spread as bioaerosols from the Choqueyapu and Orkojahuira rivers.
- Tetracycline and Sulfamethoxazole-Trimethoprim registered the highest resistance profiles: 52,6% and 36,8% respectively.
- Meropenem, from the carbapenems family, is the antibiotic with the lowest resistance profile: 5,3%. This antibiotic maintained low resistant percentages between 2018 and 2019, which suggests that *Enterobacteriaceae* are not developing resistance at a considerable rate.
- In order to control the evolution of antibiotic resistance among enteric pathogenic bacteria recovered from bioaerosol and water samples from the La Paz River basin, it is important to periodically perform standardized susceptibility tests with clinically used antibiotics. Also, an evaluation of the actual health risk implied by the aerosolization of *Enterobacteriaceae* is also necessary for a better understanding of this public health issue.
- Appropriate wastewater disposal and treatments are necessary to improve the water quality and sanitation conditions over La Paz River basin.

CRediT authorship contribution statement

Claudia Medina: Conceptualization, Data curation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Olivia Ginn:** Methodology, Resources, Writing - review & editing. **Joe Brown:** Methodology, Resources, Writing - review & editing. **Freddy Soria:** Project administration, Resources, Supervision, Writing - review & editing, Funding acquisition. **Carolina Garvizu:** Resources, Supervision, Writing - review & editing. **Daniela Salazar:** Methodology. **Alejandra Tancara:** Investigation. **Jhoana Herrera:** Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The research has been carried out with the support of Georgia Institute of Technology (USA) funded by the National Science Foundation under the grant no. 1653226 and the Centro de Investigación en Agua, Energía y Sostenibilidad (Environmental Engineering Department - Universidad Católica Boliviana "San Pablo", Bolivia). We acknowledge Alejandra Tancara and Jhoana Herrera for their collaboration in field sampling and laboratory analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.143340.

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