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Vulnerability of Groundwater to Hospital Wastewater Driving Antimicrobial-resistant *Pseudomonas aeruginosa*, in Cameroon Central Africa

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Abstract In Cameroon, the dissemination of antibiotic-resistant bacteria in groundwater via hospital wastewater has not been sufficiently explored, despite the growing volume of lesstreated wastewater generated. This study aimed at assessing the impact of hospital wastewaters on the prevalence of antimicrobial-resistant *P. aeruginosa* in the groundwater, in two urban areas, Douala and Yaounde (Cameroon, Central Africa). In each urban area, 12 wells water and 2 hospitals effluents were sampled for water analysis. The wells water were then divided into two groups. Those close to hospitals (WCH) and those far from hospitals (WFH). The level of resistance among *P. aeruginosa* strains was assessed against 16 antimicrobial agents belonging to the β-Lactam, Aminoglycosid, Quinolone, and Polymyxin groups. *P. aeruginosa* resistance rate was significantly higher in WCH than WFH (p<0.05). This result was observed in Douala with seven antibiotics: ticarcillin/clavulanic acid (31.3% vs 9.86%), ticarcillin (47.3% vs 4.93%), piperacillin (35.9% vs 4.32%), ceftazidime (44.3% vs 7.04%), gentamicin (37.4% vs 9.15%), ofloxacin (24.4% vs 4.22%), and ciprofloxacin (14.5% vs 0%); and in Yaounde with six drugs: ticarcillin/clavulanic acid, ticarcillin, piperacillin, cefepime, gentamicin, and ofloxacin, (30% vs 8.63%; 44.4% vs 8.63%; 21.8% vs 10.79%; 39.1% vs 7.91%; 21.1% vs 2.88%; 56.4% vs 15.11%, respectively). The WCH are vulnerable to the hospital effluents.

Keywords: *Groundwater, vulnerability, hospital wastewater, antimicrobial-resistance, P. aeruginosa*

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1. Introduction

Microbiological pollution of groundwater in developing countries is a serious public health concern because waters harbor multidrug-resistant microorganisms [1,2,3]. In Cameroon, groundwater is contaminated with diverse bacterial microflora, primarily consisting of commensal or pathogenic fecal bacteria [4,5]. The opportunist bacterium *Pseudomonas aeruginosa* isolated from wells of Douala and Yaounde expressed higher acquired resistance rate (45%-70%) against several antibiotics to which it is normally sensitive: ticarcillin, piperacillin, gentamicin, cefepime, 3rd cephalosporin generation, ciprofloxacin, doxycycline, cotrimoxazole, tetracycline and ampicillin [1,2].

The origin of resistant bacteria in groundwater environments remains unclear, particularly because the selection pressure of antibiotics, which is recognized as the main cause of resistance, remains weak in groundwater [6]. To address this concern in our context, we previously conducted the first study in 2019 [2] to evaluate the impact of environmental stresses related to groundwater (acid stress, nutrient starvation, and oxygen limitation) on the occurrence of antibiotic-resistant bacterium *Pseudomonas aeruginosa*, as microorganisms frequently exposed to environmental stresses express molecular adaptation mechanisms that intersect with antibiotic resistance mechanisms [7,8,9]. This study showed that the groundwater of Douala and Yaounde are less stressful environments and have less influence on antibiotic resistance [2]. Regarding this previous work, it was necessary to carry out further investigations in order to find the origin of drug-resistant *P. aeruginosa* strains in groundwater of Douala and Yaounde (Cameroon).

Following this objective, this article attempts to analyze the impact of hospital effluents on the prevalence of resistant *P. aeruginosa* strains in the groundwater of Douala and Yaounde. Indeed, the intensive use of antibiotics in the referenced hospital of Douala and Yaounde has led to the selection of multidrug-resistant *P. aeruginosa* [10,11,12]. As a result, their wastewater would contain pollutants such as resistant bacteria [13,14]. Therefore, it is necessary to assess the prevalence of resistant bacteria in these wastewaters, which will make it possible to appreciate their potential for disseminating antibiotic resistance into groundwater [15,14,16]. This investigation was the primary objective of this study.

Several studies conducted in Cameroon showed that the vulnerability of groundwater to microbiological pollution sources can be influenced by soil properties [17,18,19] and hydrogeological characteristics, such as aquifer depth and depth to water level [20,21]. Although these findings prove to be relevant when comparing groundwater vulnerability in two areas with different pedological and hydrogeological properties, they do not provide sufficient explanations for the variations in vulnerability often observed in the same area with uniform soil and hydrogeological characteristics [19]. Based on this observation, we considered as the second objective of this article the analysis of an environmental parameter likely to fill this information gap. This parameter is distance between the source of pollution (hospital) and receiving aquifer. Indeed, the authors reported that hospital wastewater containing resistant bacteria can self-purify during flow. Therefore, their potential for groundwater contamination decreases with distance between the hospital (source) and wells (receptors) [6,22]. This means that groundwater close to the hospital would be vulnerable to hospital wastewater pollution than groundwater located far away. However, no study has so far verified this hypothesis in Cameroon.

The aims of this study were to: (i) assess the prevalence of drug-resistant *P. aeruginosa* in hospital wastewater in Douala and Yaounde cities; (ii) evaluate the impact of hospital wastewater on the dissemination of resistant pathogens in groundwater; and (iii) assess the influence of the hospital-aquifer distance on the vulnerability of groundwater to hospital wastewater pollution.

2. Materials and Methods

2.1. Site Description

This study was carried out in the two main urban areas in Cameroon, Douala and Yaounde.

2.1.1. Geography and Climate

2.1.1.1. Douala

Douala, city and chief port of Cameroon is situated on the southeastern shore of the Wouri River estuary, on the Atlantic Ocean (Gulf of Guinea) between latitudes 3° 5' and 4° 15' North and longitudes 9° 37' and 9° 50' East. The relief is relatively flatted with mean altitude of 25 m above the sea level.

The climate of Douala is humid equatorial type characterized by strong marine and monsoon influences [23]. The annual rainfall regime is unimodal with a long rainy season from March to November and a very short "dry season" in December–February. Thirty years (1980−2011) of meteorological data from the national archive in Douala show that the average annual rainfall in the study area is 3 854.3 mm/year, the maximum and minimum monthly values are respectively obtained in August (742.4 mm) and December (34 mm).

The peculiar climate of Douala is also linked to the relative uniformity of air temperature and very high atmospheric humidity conditions. The mean annual temperature is 27°C; lowest and highest temperatures are observed respectively in August (25.4°C) and February (28.6°C), giving low annual thermal amplitude of 3.2 °C. The relative humidity ranges between 79% and 90% during the rainy season (March to November) and between 77% and 81% during the dry season (December to February), with an annual mean of 85% [23].

2.1.1.2. Yaounde

Yaounde is located is located at about 250 km of the Atlantic Ocean. It is bounded by the 3° and 5° parallels of the north latitude and the 11° and 12° meridians of the east longitude. The climate is equatorial with four seasons: a long dry season (from mid-November to mid-March), a short rainy season (from mid-March to mid-June) a short dry season (from mid-June to mid-September) and a long rainy season (from mid-September to mid-November) [24]. According to the climatological data of Yaoundé during the period from 1951 to 2017, the annual average rainfall is about 1 600 mm, interannual monthly precipitations ranged from 18.8 mm recorded in January to 284.2 mm recorded in October. The temperature of the ambient air of Yaounde varies slowly during the year. The highest, 25.4 C, is generally observed in February and the weakest, 22.8 C, is observed in July. The relative humidity values of the ambient air of the studied space ranged from 71% (in February) to 82.5% (in July and August) for an average monthly interannual value of 78.4%.

The topography is undulating, and dominated by hills alternating with valleys thus, shaping the area into domes and basin's structure, with an average altitude of 800 m above sea level [25].

2.1.2. Hydrogeology

2.1.2.1. Douala Area

[26] reports that the Douala basin is made up of four principal aquifers: the Pleistocene and Pliocene alluvia of the Wouri Formation, the Palaeocene sand of the Nkappa Formation, and the Cretaceous basal sandstone of the Moundeck Formation. The study area (Douala) reposes directly on the Mio-Pliocene to recent alluvial sediments of the basin, which constitutes the Wouri Formation. Generally, it consists of unconsolidated fine to coarse-grained sand and gravel with intercalation of silt and clay in varied proportions. The alluvium is composed predominantly of quartz and kaolinite, with a general thickness that ranges between 50 - 60 m [27].

The aquifer system in the study area (Douala sedimentary basin), can be classified into two major classes, the shallow aquifers (<50 m depth) and the deep aquifers (>50 m depth) [26,28,29]. All groundwater samples considered in this study was shallow groundwater from wells with the total depth ranging from 4.7 m to 8.7 m, and depth to water level oscillating between 1.43±0.16 m and 6.07±0.15 m. Hydraulic conductivity changes from 1.1 × 10^{-4} to 0.2 m/s, and specific yield from 2.2 to 4.9 [30]. The groundwater flow regime is multidirectional from SE to NW, NW to W and from NE to SW [31].

The shallow aquifers of the Mio-Pliocene and Pleistocene alluvia are the most exploited (about 13 000 m^3/day) for domestic uses through wells and boreholes [31]. These aquifers are unconfined and characterized by a porous vadose zone. They are mainly recharged by precipitation. Additionally, the topography of the area is such that flooding is a common occurrence following heavy rainfall. As a result of low altitude, the aquifer in the area is very vulnerable to contamination. The quick response to rainfall implies rapid recharge through a highly permeable vadose zone.

2.1.2.2. Yaounde Area

Yaounde is occupied by weathered and cracked precambrian basement rocks constituted by plutonic and metamorphic rocks (gneiss, migmatite and schist) [32,33]. These hard rocks were transformed in the humid tropical zone into a thick alteration mantle (10-20 m) due to the infiltration of rain on the ground surface [32]. The alteration mantle is composed of the top toward the base of sandy clay-sandy layer, iron duricrust blocks, alloterite later, and isalterite layer [34].

Due to the variability of the topography made of various hills and valleys, the hydrodynamic characteristics of shallow aquifer in this region are heterogeneous. The yield of groundwater ranges from 0.06 to 0.5 m^3/h. The total depth of well considered in this study ranges from 2 m to 11.4 m, while the depth to water level oscillates between 1.5 ±0.01 m and 6.8±0.1 m depending on the season and the relief. Besides, the hydraulic conductivity varies between 10^{-4} and 10^{-6} m/s [35]. The general groundwater flow direction follows the topography and the major geological features (faults, fractures). The most representative groundwater flow directions are NE-SW, NNE- SSW, E-W, ESE-WNW, and NW- SE [25,36].

The shallow aquifers constitute for many households of Yaounde one of the main sources of water supply. Theses aquifers are exploited through wells, boreholes and springs for the supply of drinking water. The proximity of these waters to the surface makes them vulnerable to pollution. They are recharged directly by infiltration of precipitation without any notable change due to evaporation [34]. The relationship between groundwater behavior and rainfall has a lag of about 1 month that is attributed to morphopedological processes [34].

2.1.3. Soil

2.1.3.1. Douala

Douala has two types of soils: ferralitic and hydromorphic, their pH is acidic 5.5 [37,38,39] [27]. In both cases, the soil is made up of a large proportion of fine and coarse sands (45 to 80%), and clays (10 to 50%). The proportions of kaolinite (50-60%) are the most important while goethite (35-42%) is significant and gibbsite (2-10%) is negligible [40]. Ferralitic and hydromorphic soils occupy respectively 80% and 20% of the study region. The latter is mainly observed in alluvial plains and valley bottoms.

2.1.3.2. Yaounde

In Yaounde, red ferritic soils are more abundant. They are very thick (sometimes more than 20 m), clayey, and acidic (pH ≈ 5.5) and consist mainly of kaolinite, hematite, goethite, gibbsite, and quartz. Their profile shows from base to top an isalterite or saprolite level, an alloteritic level, a level of accumulation of iron oxyhydroxides and kaolinitic clay, and a thin level of topsoil [41].

2.1.4. Socio-Demographic Factors and Access to Drinking Water

The population of Yaoundé in 2010 was estimated to be 2,200,000 inhabitants [42]. Less than 50% of households have direct access to drinking water networks [43]. Groundwater plays a fundamental role in supplying water for bathing, drinking, and crop irrigation. It has been established that approximately 36% of households in Yaoundé have a well near the concession to solve the problem of water deficit [43]. However, 53% of households using well water have been exposed to waterborne diseases [43].

The population of Douala in 2010 was estimated to be more than 2,300,000 inhabitants [42]. About 2/3 of the population use boreholes, wells, and natural springs, regardless of the health risks incurred [43]. Approximately 3/4 of boreholes and wells are doubtful [43].

2.1.5. Antibiotic Consumption

The average total in-country antimicrobial consumption (AMC) in the hospital sector is about 5.1 DDD per 1 000 inhabitants per day [44].

Considering the class of antibiotics, the most consumed antimicrobial class in Cameroon is a combination of sulfonamides and trimethoprim, including derivatives, and combinations of penicillins, including beta-lactamase inhibitors and tetracyclines. The five most commonly consumed antimicrobials in the hospital sector were sulfamethoxazole/trimethoprim, amoxicillin/clavulanic acid, doxycycline, amoxicillin, and fluconazole. Together these accounts for 68% of total consumption [44].

2.2. Characterization of the Hospitals Producing Wastewater

2.2.1. Volume of Wastewater Discharged

The hospitals chosen for this study were referenced health structures housing several departments, including medicine, surgery, intensive care, gynecology/obstetrics, pediatrics, and emergency. The hospitals selected in Douala were Laquintinie Hospital, with 732 active beds (150,000 patients/year), and General Hospital, with 210 active beds. In Yaoundé, Teaching Hospital (250 active beds) and General Hospital (254 active beds) were selected.

According to the American Hospital Association [45], the number of active beds is an indicator that enables qualitative and quantitative evaluation of the volume of wastewater discharged into the environment. Based on this assumption, the AHA classifies hospitals into eight groups. For class 1 hospitals, the volume of effluent discharged into environment is approximately 1700 m^3/year. For classes 2, 3, 4, 5, 6, 7, and 8 hospitals, the volumes of effluent discharged were approximately 9,000, 17,000, 35,000, 40,000, 70,000, 90,000, and 130,000 m^3/year, respectively [45,46]. According to the above criteria, Laquintinie Hospital belongs to Class 8. The General Hospital of Douala, the Teaching Hospital and the General Hospital of Yaoundé belong to Class 5.

2.2.2. Hospital Wastewater Treatment

Wastewater management at health facilities in Cameroon is alarming. At the time of their construction, the four reference hospitals chosen for this study (Laquintinie Hospital, Douala General Hospital, Yaounde General Hospital, and Teaching Hospital) were equipped with wastewater treatment plant [47]. The general treatment process in all the wastewater treatment plants was an activated sludge system. But due to maintenance failures, these treatment plants are currently nonfunctional. Wastewater from these hospitals is discharged into the environment without appropriate treatment [47].

2.3. Sampling

This study was conducted in the two main metropolises of Cameroon, Douala, and Yaounde, from April 2018 to April 2019. The sampling duration was 13 months. In each town, 12 well waters distributed in 12 neighborhoods and two hospital wastewaters were selected as sampling sites for water analysis. Their locations are listed in Table 1 and Table 2. These wells are of public health interest, particularly when they are contaminated with microorganisms. The total well depth and water table were measured using a sterile graduated line with an attached weight.

In assess the impact of hospital sewage on groundwater, the 12 wells in each town was divided into two groups. The first group included six wells close to hospitals (WCH) receiving hospital wastewater. The distances between WCH and the hospital were 450-1600 m and 700-1900 m in Douala and Yaounde, respectively (Table 1 and Table 2). In Douala town, six WCH were selected around the General Hospital (3 WCH) and Laquintinie Hospital (3 WCH). In Yaounde, six WCH were selected near the General Hospital (3 WCH) and the Teaching Hospital (3 WCH). The spatial representation of the wells and selected hospitals is presented in Figure 1.

The second group involved six wells far from hospitals (WFH) in each town that did not receive hospital wastewater. Their distance from the hospital ranged from 11 300 m to 18 600 m in Douala and from 9 200 m and 13 000 m in Yaounde. For this study, WFH was considered as a control for assessing the potential impact of hospital effluents on the emergence of resistant *P. aeruginosa* in groundwater. The principle underlying this distribution is that hospital effluents contain resistant bacteria and genes [14]. They have the capacity to self-purify during flow. Therefore, their potential for groundwater contamination decreases with distance between the hospital (source) and well (receptor) [1,6] [22].

Well water and hospital sewage samples were aseptically collected in a 500 ml sterile glass bottle for bacteriological analysis according to standard methods [48]. Water samples from the wells were collected in the first layer of the water table, as this zone is sufficiently oxygenated and constitutes the preferential microhabitat of *P. aeruginosa*, whose metabolism is strictly respiratory. Hospital wastewater was collected from the wastewater treatment plant outlet before contact with soil. The collected samples were stored inside an ice box, refrigerated at 4°C, and analyzed within 8 h of collection. All analyses were performed at the Hydrobiology and Environment Laboratory of the University of Yaounde 1, Cameroon.

2.4. Bacterial Isolation and Identification

In each well and hospital wastewater, *P. aeruginosa* cells were isolated and counted by filtration of 100 ml of raw or diluted water through a cellulose acetate filter membrane with a porosity 0.45 µm [49,50]. The membranes were then placed on Cetrimide and Nalidixic acid agar (Difco Laboratories, Detroit, MI, USA) in Petri dishes. The inoculated dishes were incubated at 37°C. Bacterial count (CFU/100 mL) was determined after 24h of incubation. The bacteria were identified according to [51]. The blue-green colonies (production of pyocyanin) were considered *P. aeruginosa* and were not subjected to confirmatory tests. Non-blue-green fluorescent colonies were considered to be *P. aeruginosa* and were subjected to tests for ammonia production from acetamide and casein hydrolysis for confirmation [51]. To test for ammonia production, a tube containing the acetamide broth was inoculated with the isolated culture and incubated at 37°C for 24 h. After incubation, two drops of Nessler's reagent were added to the broth, and when the test result was positive, a yellow color appeared. The red-brown colonies were also considered presumptive *P. aeruginosa*; they were confirmed by oxidase test, fluorescence on King B medium, production of ammonia from acetamide, and casein hydrolysis. The search for oxidases was performed using the Kovac technique. Fluorescence in King B medium was observed using a 365 nm UV lamp (Spectroline, Q-22/F) [51].

2.5. Antimicrobial Susceptibility Testing

The Kirby-Bauer disk-diffusion method (on Mueller Hinton agar) was used for performing antimicrobial susceptibility patterns according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [52]. Base on it and also common locally antibiotic used, 16 antimicrobials (Difco Laboratories, Detroit, MI, USA.) were tested against all *P. aeruginosa* strains including: Ticarcillin (TIC, 75µg), Ticarcillin/clavulanate (TICC, 75/10µg), Piperacillin (PIP, 75µg), Piperacillin/tazobactam (PTZ, 75/10µg), Ceftazidime (CAZ, 30µg), Cefotaxime (CTX, 30µg), Cefepime (FEP, 30µg), Cefsulodine (CFD, 30µg), Aztreonam (AZT, 30µg), Imipenem (IMP, 5µg), Tobramycin (TOB, 10µg), Gentamicin (GM, 15µg),

Amikacin (AMK, 30µg), Ofloxacin (OFX, 5µg), Ciprofloxacin (CIP, 5µg), and colistin (CT, 50µg).

Table 1. Caracteristics of sampled wells in Douala and distances to the hospital settings

Wells	Neighborhood	GPS coordinates (altitude)	Depth (m)	Depth to water level ±SD (m)	Distance from hospital (m)
PD1	Ndogbong	N 3°54.1’ E 9°41.1’ (26 m)	5.9	2.93 ± 0.11	980 Receiving General Hospital wastewater
PD2	Ndogbong	N 4°03.9’ E 9°45.3’ (22 m)	6.4	4.2 ± .12	650 Receiving General Hospital wastewater
PD3	Ndogbong	N 4°03.8’ E 9°45.4’ (20 m)	7.6	6.07 ± 0.15	1600 Receiving General Hospital wastewater
PD4	Deido	N 4°02’ E 9°44.8’ (35 m)	8.7	4.77 ± 0.1	12300 No receiving hospital wastewater
PD5	Sic cacao	N 4°05.1’ E 9°44.7’ (23 m)	4.7	1.62 ± 0.08	13800 No receiving hospital wastewater
PD6	Sic cacao	N 4°02.7’ E 9°42.7’ (25 m)	4.8	2.38 ± 0.17	12900 No receiving hospital wastewater
PD7	New-Bell	N 4°02 E 9°42.7 (30 m)	6.2	1.43 ± 0.16	11300 No receiving hospital wastewater
PD8	Mboppi (Ecobank)	N 4°00 ‘ E 9°44.9’ (22 m)	5	3.71 ± 0.1	15700 No receiving hospital wastewater
PD9	Akwa	N 4°02.8’ E 9°42.8’ (24 m)	5.4	3.48 ± 0.06	450 Receiving Laquintinie Hospital wastewater
PD10	Akwa	N 4°03.4 ‘ E 9°43.7’ (24 m)	5.8	4.19 ± 0.09	500 Receiving Laquintinie Hospital wastewater
PD11	Akwa	N 4°03.7’ E 9°43.3’ (23 m)	4.7	3.73 ± 0.07	750 Receiving Laquintinie Hospital wastewater
PD12	Ndockpassi	N 4°03.5’ E 9°43.3’ (20 m)	6.3	4.72 ± 0.19	18600 No receiving hospital wastewater

Table 2. Caracteristics of sampled wells in Yaounde and distances to the hospital setting

Wells	Neighborhood	GPS coordinates (Altitude)	Depth (m)	Depth to water level ±SD (m)	Distance from hospital (m)
PY1	Nkolmbong	N 3°54’ E 11°31’ (751 m)	8,8	4.7 ± 0.06	13000 No receiving hospital wastewater
PY2	Miboman	N 3°52.4’ E 11°33’ (737)	2	3.8 ± 0.12	10300 No receiving hospital wastewater
PY3	Ngousso	N 3°54.2’ E 11°32.2’ (727 m)	10,3	3.45 ± 0.13	850 Receiving General Hospital Wastewater
PY4	Ngousso	N 4°54.3’ E 11°32.3’ (733 m)	7	5.18 ± 0.09	700 Receiving General Hospital Wastewater
PY5	Ngousso	N 3°51’ E 11°29.8’ (738 m)	7,1	4.4 ± 0.06	1200 Receiving General Hospital Wastewater
PY6	Obili	N 3°52.7’ E 11°30.2’ (738 m)	7,2	3.6 ± 0.04	1300 Receiving Teaching Hospital Wastewater
PY7	Obili	N 3°51.2’ E 11°29.7’ (722 m)	4,6	2.9 ± 0.07	2100 Receiving Teaching Hospital Wastewater
PY8	Obili	N 3°54.2’ E 11°32.2’ (717 m)	6,8	2.2 ± 0.06	1900 Receiving Teaching Hospital Wastewater
PY9	TKC Mendong	N 3°50.6’ E 11°28.9’ (729 m)	11,1	6.8 ± 0.1	9200 No receiving hospital wastewater
PY10	Odza	N 3°53.1’ E 11°31.7’ (740 m)	6,3	1.5 ± 0.01	11700 No receiving hospital wastewater
PY11	Minkan	N 3°50.3’ E 11°28.4’ (724 m)	11,4	3.5 ± 0.05	10200 No receiving hospital wastewater
PY12	Ekounou	N 3°50.7’ E 11°31.3’ (727 m)	7,2	3.2 ± 0.09	11900 No receiving hospital wastewater

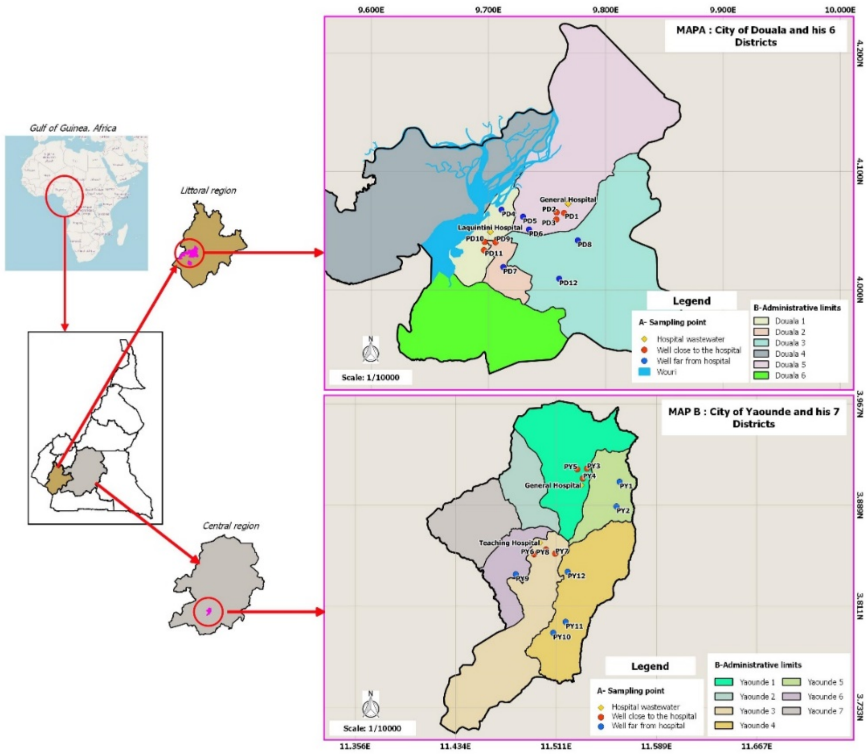

Figure 1. Localizations of sampling sites in Douala (A) and Yaounde (B) towns

The result of each antibiotic testing was determined in the cases where inhibition zone diameters of strains was within performance ranges according to those recommended by manufacturer's and EUCAST guidelines. Clinical interpretation [resistant (R), and sensitive (S)] was determined by the diameter from the zone of inhibition (Table 3).

Table 3. Standard zone chart of different antibiotics for P. aeruginosa [52]

Antibiotic	Code	Resistance (mm)	Sensitive (mm)
Ticarcillin 75µg	TIC	< 18	≥ 50
Ticarcillin/clavulanate 75/10µg	TICC	< 18	≥ 50
Piperacillin 75µg	PIP	< 18	≥ 50
Piperacillin/tazobactam 75/10µg	PTZ	< 18	≥ 50
Ceftazidime 30µg	CAZ	< 17	≥ 50
Cefotaxime 30µg	CTX	Natural resistance	Natural resistance
Cefepime 30µg	FEP	< 21	≥ 50
Cefsulodine 30µg	CFD	< 15	≥ 22
Aztreonam 30µg	AZT	< 22	≥ 25
Imipenem 5µg	IMP	< 17	≥ 20
Tobramycin 10µg	TOB	< 16	≥ 16
Gentamicin 15µg	GM	< 15	≥ 15
Amikacin 30µg	AMK	< 15	≥ 18
Ofloxacin 5µg	OFX	< 22	≥ 25
Ciprofloxacin 5µg	CIP	< 26	≥ 26
Colistin 50µg	CT	< 15	≥ 15

2.6. Quality Control/ Quality Assurance

Sterility of media was done by incubating one plate from each autoclaved of medium overnight at 35°–37°C and examine it for contaminants. Control strains *P. aeruginosa* ATCC 27853, were used to ensure both: ability to support growth, ability to produce appropriate biochemical reactions and adequate inhibition zone diameters.

2.7. Multiple Antibiotic Resistance Index (MAR)

MRA index is an indicator of antibiotic resistance. When evaluated at the strain level, the MRA Index simply corresponds to the ratio of the number of antibiotics to which the strain resists to the number of antibiotics tested. In this study, the MRA index was calculated at the sampling sites (wells and hospital wastewater). This calculation considered the number of *P. aeruginosa* strains isolated at each site, as recommended by [53] using the following equation:

$$MAR\ index = \frac{a}{b \times c} \qquad 0 \leq \text{MAR Index} \leq 1$$

Where **a** is the aggregate antibiotic resistance score of all isolates from the site, **b** is the number of antibiotics used (n = 16 in this study) and **c** is the number of isolates originating from the site.

Calculation of the MRA index at the sampling sites made it possible to assess the level of global resistance at each site. It also makes it possible to compare different sites and to appreciate the spatial distribution of resistance in the two cities

2.8. Resistance Phenotypes

The β-lactam resistance phenotypes were determined using antimicrobial susceptibility tests [54] (Livermore, 1995). Three resistant phenotypes were considered in this study. A penicillinase phenotype was detected in strains that were resistant or moderately resistant to piperacillin, cefotaxime, and aztreonam. The high-level cephalosporinase (AmpC) phenotype corresponds to strains resistant to piperacillin, cefotaxime, aztreonam, and ceftazidime. The low-level cephalosporinase phenotype corresponds to cefotaxime resistance.

As with the data from the susceptibility tests, the data from the β-lactam resistance phenotype were stratified into hospital wastewater, well close to the hospital (WCH), and well far from the hospital (WFH).

2.9. Statistical analysis

Data were stratified into hospital wastewater, wells close to the hospital (WCH) and wells far from the hospital (WFH). The prevalence of antibiotic resistance in *P. aeruginosa* was compared between WCH and WFH using the Mantel-Haenszel Chi-squared test and 2-tailed Fisher's exact test. The software used was the Statistical Package for Social Sciences (version 20.0; SPSS, Inc., Chicago, IL, USA). The aim of this comparison was to verify the hypothesis that a higher prevalence of resistance in WCH is linked to their physical proximity (distance) and vulnerability to hospital wastewater.

To quantify the impact of environmental factors on the vulnerability of groundwater to hospital effluents, correlation tests using Spearman's correlation coefficient «r» were performed between the MAR index values of well water and the distances between well water and the hospital (producing wastewater). The MAR index represented the level of global resistance at each site. In addition to distance, hydrogeological parameters such as well depth and depth-to-water level were also correlated with the MAR index. Statistical significance of the above tests was set at p-value <0.05.

3. Results

3.1. Distribution of *P. aeruginosa* Isolates among Sampling Sites

The population densities of *P. aeruginosa* at the study sites are presented in Table 4 and Table 5.

Table 4. P. aeruginosa frequencies in sampling sites in Douala

Sampling sites	Codes	Frequency
General Hospital wastewater	GHWD	34
Laquintinie Hospital wastewater	LHW	49
	Total	83
Wells receiving General Hospital wastewater	PD1	28
	PD2	24
	PD3	23
Wells receiving Lanquintinie hospital wastewater	PD9	25
	PD10	28
	PD11	20
Wells not receiving hospital wastewaters	PD4	25
	PD5	11
	PD6	25
	PD7	27
	PD8	22
	PD12	15
	Total	273

In Douala (Table 4), the highest concentration of *P. aeruginosa* was observed in Laquintinie Hospital wastewater (49 CFU/100 ml). This Hospital also produced the largest volume of effluent. The distribution of *P. aeruginosa* in the wells showed two trends. In WCHs, the distribution of germ is almost equitable, and the cellular abundances (not cumulative) remain lower than those observed in the hospital effluents they receive. In the WFH group, the distribution of bacterial cells was uneven. WFH PD5 had the lowest microbial density (11 CFU/100 ml). WFH PD7 had the highest bacterial density (27 CFU/100 ml). It is also interesting to note that the bacterial density in WFH PD7 was greater than that of several WCHs (PD2, PD3, and PD4), which nevertheless received hospital effluent.

Table 5. *P. aeruginosa* frequencies in sampling sites in Yaounde

Sampling sites	Codes	Frequency
General hospital wastewater	GHWY	40
Teaching Hospital wastewater	THW	29
	Total	70
Wells receiving General Hospital wastewaters	PY3	18
	PY4	30
	PY5	23
Wells receiving Teaching Hospital wastewaters	PY6	16
	PY7	31
	PY8	45
Wells not receiving hospital wastewaters	PY1	25
	PY2	16
	PY9	23
	PY10	17
	PY11	14
	PY12	14
	Total	272

In Yaounde, the distribution of *P. aeruginosa* was uneven in hospital effluents (Table 5). The effluent from the General Hospital was more loaded with microorganisms (40 CFU/100 ml) than that from the Teaching Hospital (29 CFU/100 ml). On the other hand, in the wells, the distribution of *P. aeruginosa* seems to be slightly uniform. However, three wells (PY8, PY10, and PY11) stand out from this distribution. The well with the highest bacterial density was WCH PY8 (45 CFU/100 ml). However, its bacterial load was higher than that of the hospital effluent it received (29 CFU/100 ml). The lowest bacterial concentrations were observed in WFH PY10 and PY11 (10 FCU/100 ml).

3.2. Prevalence of Resistant *P. aeruginosa* Strains

The level of resistance among *P. aeruginosa* strains was assessed using 16 antimicrobial agents. The results are shown in Figure 2. According to the results obtained in Douala, resistance to CT was not observed among *P. aeruginosa* strains at any sampling sites. However, susceptibility to IMP was observed only in WFH. All isolates were susceptible to PTZ in both WCH and WFH, except in hospital wastewater where the sensitivity to these drugs was below 100%. *P. aeruginosa* strains expressed intrinsic resistance to CTX at both sampling sites in Douala (Figure 3a). In hospital wastewater, the prevalence of acquired resistance greater than 50% was expressed against seven antibiotics: GM (81.9%), TIC (75.2%), PIP (63.2%), CAZ (62.4%), AMK (68.6%), OFX (66.8%), and TICC (58.3%). Among WCH isolates, 47.3 and 44.3% were resistant to TIC and CAZ, respectively, which was lower than the prevalence obtained in hospital wastewater. In addition, 9.86 and 9.15% of the isolates from WFH were resistant to TICC and GM, respectively, which were lower than those from the hospital wastewater and WCH.

In Yaounde, all *P. aeruginosa* strains were susceptible to CT and PTZ at both the sampling sites (Figure 2b). They showed natural resistance to CTX (Figure 2b).

All strains were susceptible to CFD at the WCH and WFH sites. When examining acquired resistance, the prevalence of resistant isolates greater than 50% was obtained with six antibiotics in hospital wastewater: TIC (88.2%), OFX (78.2%), FEP (67.6%), PIP (63.2%), TICC (54.3%), and AMK (52.3%). However, in the WCH sites the proportion of resistant isolates over 50% was observed only with one antibiotic, OFX (56.4%). Whereas 10.79 and 15.11% of the isolates from WFH were resistant to PIP and OFX, respectively, which were lower than those noted in WCH (Figure 2b).

The prevalence of the resistance phenotypes is shown in Figure 3. The high-level AmpC phenotype was highest in hospital wastewater (37.2%), followed by WCH (12.33%) and WFH (3.1%), in Douala town (Figure 3a). Similarly, the prevalence of the penicillinase phenotype was highest in hospital wastewater (23.1%), followed by WCH (10.2%), and WFH (2.5%). The low-level AmpC phenotype was particularly high in WFH compared to the other two sampling sites (Figure 3a).

In Yaounde (Figure 3b), the penicillinase and high-level AmpC phenotypes were highest in hospital wastewater (29.7 and 21.9%, respectively), followed by WCH (17.2 and 10.62%, respectively) and WFH (6.1 and 4.4%, respectively).

The general trend of resistance in both Douala and Yaounde towns is as follows: the prevalence seems to be higher in hospital effluents, then they decrease in WCH and become weaker in WFH.

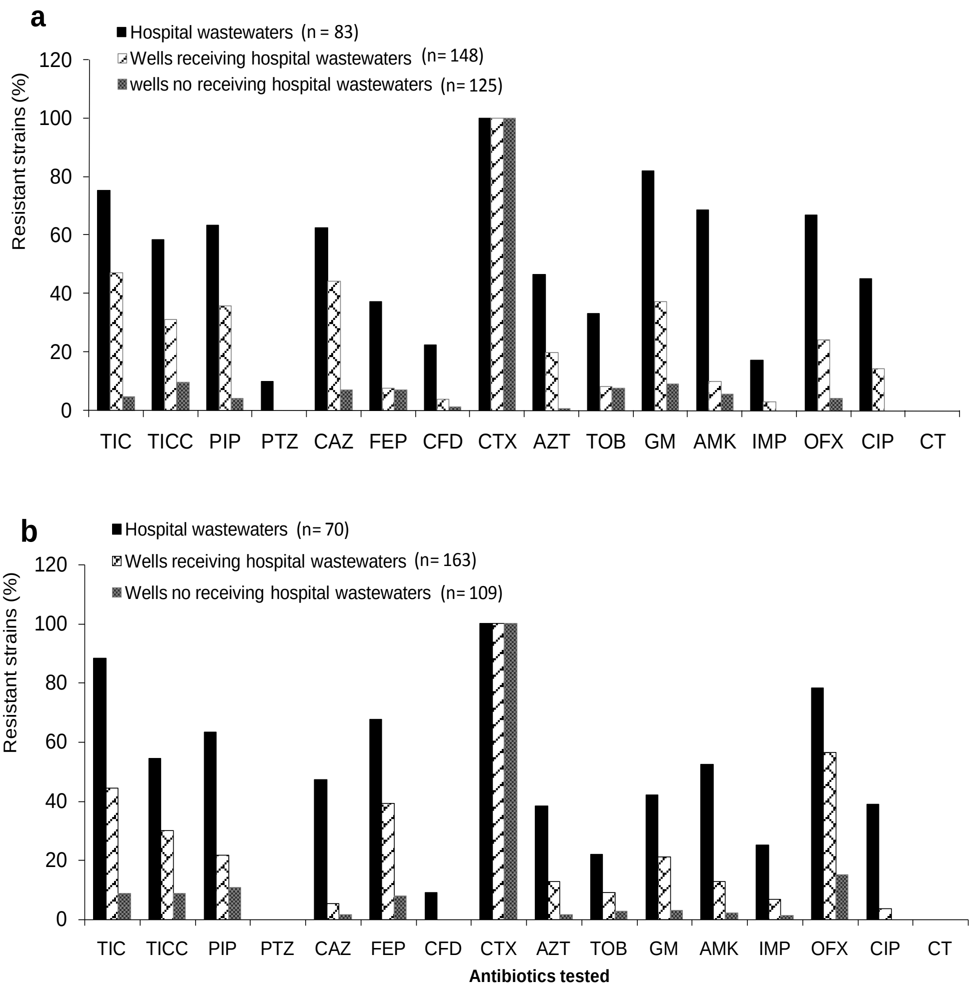

Figure 2. Antimicrobial resistance rate in *Peudomonas aeruginosa* isolates. **a** in sampling sites of Douala, **b** in sampling sites of Yaounde. TIC ticarcillin, TICC ticarcillin/clavulanate, PIP piperacillin, PTZ piperacillin/tazobactam, CAZ ceftazidime, FEP cefepime, CFD cefsulodin, CTX cefotaxime, AZT aztreonam, TOB tobramycin, GM gentamicin, AMK amikacin, IMP imipenem, OFX ofoxacin, CIP ciprofoxacin, CT colistin

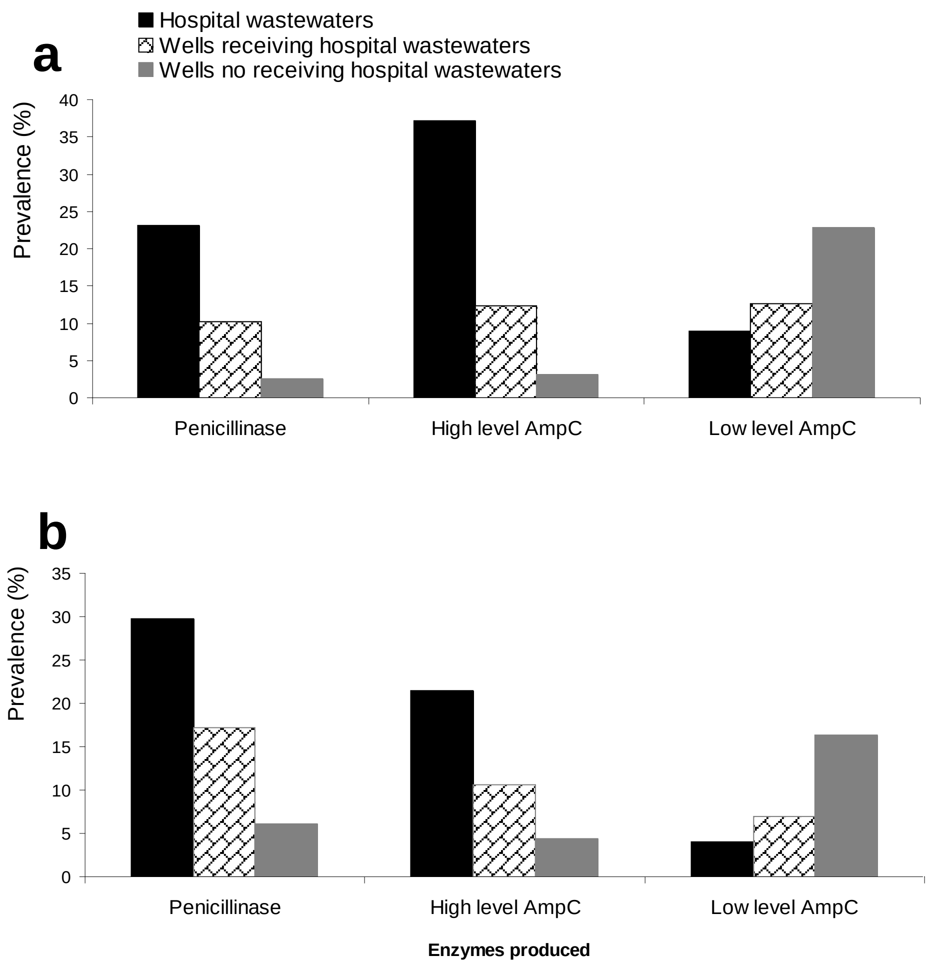

Figure 3. Resistance phenotype rate in *P. aeruginosa* strains. **a** in sampling sites of Douala, **b** in sampling sites of Yaounde. AmpC: Cephalosporinase enzyme

3.3. Multiple Antibiotic Resistance (MAR) Index in Sampling Sites

In Douala, the MAR index varied according to the sampling site (Table 6 and Table 7). Overall, its value is greater in hospital effluents, particularly in Laquitinie Hospital wastewater, where the index reaches a maximum value of 0.83 (Table 6). The MAR index decreased in General hospital effluent and reached a value of 0.74. In WCH, the MAR index oscillates between 0.48 and 0.79. The lowest index was obtained in WCH PD11 (0.48) and the highest index in WCH PD1 (0.79). It is remarkable to note that PD11 well, which has the lowest index among the WCHs, receives Laquintinie hospital wastewater, which has the highest MAR index among hospital wastewater. While the PD1 well, which has the highest index among the WCHs, receives the effluent from the General Hospital which has le lowest index MAR. In WFH, the MAR index values were lower than those at the other sampling sites. The MAR index varies from 0.2 (PD4) to 0.44 (PD12). Overall, the MAR index in Douala was highest for hospital wastewater (0.83), followed by WCH (0.66) and WFH (0.2).

In Yaounde, the MAR index varied in hospital effluents from 0.57 to 0.74 (Table 7).

Table 6. Multiple antibiotic resistance (MAR) index in sampling sites in Douala

Sampling sites	Codes	MAR index
General Hospital wastewater	GHW	0.74
Laquintinie Hospital wastewater	LHW	0.83
Wells receiving General Hospital wastewater	PD1	0.79
	PD2	0.51
	PD3	0.67
Wells receiving Lanquintinie Hospital wastewater	PD9	0.61
	PD10	0.66
	PD11	0.48
Wells not receiving hospital wastewaters	PD4	0.2
	PD5	0.21
	PD6	0.28
	PD7	0.4
	PD8	0.32
	PD12	0.44

The difference between the MAR indexes of the two hospital effluents was greater than that observed in Douala. In WCH, the MAR index oscillates between 0.47 (PY8) and 0.68 (PY3). These values were lower than those obtained for the hospital sewage. Contrary to the trend obtained in the city of Douala, WCH PY8 with the lowest MAR index was the one that received Teaching hospital effluent, which had the lowest index. The WCH PY3 with the highest index is the one that receives the General Hospital effluent, which has the highest index. In WFH, the MAR index values were lower than those of the previous sampling sites. Overall, the MAR index trend at all sampling sites was similar to that observed in Douala. The MAR index values obtained in Yaounde were highest for hospital wastewater (0.74), followed by WCH (0.68), and WFH (0.1).

Table 7. Multiple antibiotic resistance (MAR) index in sampling sites in Yaounde

Sampling sites	Codes	MAR index
General hospital wastewater	GHW Y	0.74
Teaching Hospital wastewater	THW	0.57
Wells receiving General Hospital wastewaters	PY3	0,68
	PY4	0.53
	PY5	0.65
Wells receiving Teaching Hospital wastewaters	PY6	0.62
	PY7	0.56
	PY8	0.47
Wells not receiving hospital wastewaters	PY1	0.1
	PY2	0.23
	PY9	0.25
	PY10	0.12
	PY11	0.35
	PY12	0.42

3.4. Comparison of the Prevalence of Resistance Between WCH and WFH

To verify the hypothesis that a higher prevalence of resistance in WCH is linked to their physical proximity to hospital wastewater, comparison of prevalence of resistance between WCH and WFH was performed. Results are presented in Table 8 and Table 9.

In Douala town, the prevalence of resistant isolates was significantly higher in WCH than in WFH (Table 8). This result was obtained with 7 antibiotics including TICC (31.3% vs. 9.86%, respectively, *p*-value <0.01), TIC (47.3% vs. 4.93%, respectively, *p*-value <0.001), PIP (35.9% vs. 4.32%, respectively, *p*-value <0.01), CAZ (44.3% vs. 7.04%, respectively, *p*-value <0.01), GM (37.4% vs. 9.15%, respectively, *p*-value <0.01), OFX (24.4% vs. 4.22%, respectively, *p*-value <0.01), and CIP (14.5% vs. 0%, respectively, *p*-value <0.01). However, when comparison was made using antibiotics FEP, CFD, AZT, TOB, AMK, and IMP, no significant difference in resistance rate was observed between WCH and WFH (*p*-value >0.05) (Table 8).

In Yaounde, comparative analysis showed a significant difference in resistance rate between WCH and WFH, with the following drugs (Table 9): TICC (30% vs. 8.63%, respectively, *p*-value <0.01), TIC (44.4% vs. 8.63%, respectively, *p*-value <0.001); PIP (21.8% vs. 10.79%, respectively, *p*-value <0.01), FEP (39.1% vs. 7.91%, respectively, *p*-value <0.01), GM (21.1% vs. 2.88%, respectively, *p*-value <0.01) and OFX (56.4% vs. 15.11%, respectively, *p*-value <0.01). However, the difference in the proportion of resistant strain between WCH and WFH was not significant (*p*-value >0.05) for the following antibiotics: CAZ, AZT, TOB, AMK, IMP, and CIP (Table 9).

Table 8. Comparison of Antimicrobial resistance rate in *P. aeruginosa* isolates between WCH and WFH in Douala

Antibiotics and enzyme produced	Number of resistant isolates / totals of isolates tested (%)		*p*-value
	WCH	WFH	
Ticarcillin/clavulanate	41/131 (31.3)	14/142 (9.86)	<0.01[a]
Ticarcillin	62/131 (47.3)	7/142 (4.93)	<0.001[a]
Piperacillin	47/131 (35.9)	6/142 (4.32)	<0.01[a]
Piperacillin/Tazobactam	0/131(0)	0/142 (0)	—
Ceftazidime	58/131 (44.3)	10/142 (7.04)	<0.01[a]
Cefepime	10/131 (7.63)	10/142 (7.04)	0.91
Cefsulodine	5/131 (3.82)	2/142 (1.41)	0.74
Cefotaxime	131/131 (100)	142/142 (100)	-
Aztreonam	26/131 (19,8)	1/142 (0.7)	0.91
Tobramycin	11/131 (8.4)	11/142 (7.75)	0.93
Gentamicin	49/131 (37.4)	13/142 (9.15)	<0.01[a]
Amikacin	13/131 (9.92)	8/142 (5.63)	0.67
Imipenem	4/131 (3.1)	0/142 (0)	0.89
Ofloxacin	32/131 (24.4)	6/142 (4.22)	<0.01[a]
Ciprofloxacin	19/131 (14.5)	0/142 (0)	<0.01[a]
Colistin	0/131 (0)	0/142 (0)	—
Penicillinase	13/131 (10)	4/142 (2.8)	<0.05[a]
High level AmpC	16 /131 (12.2)	5/142 (3.5)	<0.05[a]
Low level AmpC	17/131 (12.9)	32/142 (22.5)	<0.05[a]

[a] significant

Table 9. Comparison of Antimicrobial resistance rate in *P. aeruginosa* isolates between WCH and WFH in Yaounde

Antibiotics and enzyme produced	Number of resistant isolates / totals of number of isolates tested (%)		*P* value
	WCH	WFH	
Ticarcillin/clavulanate	40/133 (30)	12/139 (8.63)	<0.01[a]
Ticarcillin	59/133 (44.4)	12/139 (8.63)	<0.001[a]
Piperacillin	29/133 (21.8)	15/139 (10.79)	<0.01[a]
Piperacillin/Tazobactam	0/133 (0)	0/139 (0)	—
Ceftazidime	7/133 (5.26)	2/139 (1.44)	0.88
Cefepime	52/133 (39.1)	11/139 (7.91)	<0.01[a]
Cefsulodine	0/133 (0)	0/139 (0)	—
Cefotaxime	133/133 (100)	139/139 (100)	-
Aztreonam	17/133 (12,7)	2/139 (1.44)	0.96
Tobramycin	12/133 (9)	4/139 (2.8)	0.98
Gentamicin	28/133 (21.1)	4/139 (2.88)	<0.01[a]
Amikacin	17/133 (12.8)	3/139 (2.1)	0.77
Imipenem	9/133 (6.8)	2/139 (1.4)	0.86
Ofloxacin	75/133 (56.4)	21/139 (15.11)	<0.001[a]
Ciprofloxacin	5/133 (3.7)	0/139 (0)	0.92
Colistin	0/133 (0)	0/139 (0)	—
Penicillinase	23/133 (17.2)	9/139 (6.4)	<0.05[a]
High level AmpC	14/133 (10.6)	6/139 (4.4)	<0.45
Low level AmpC	9/133 (6.8)	23/139 (16.5)	<0.05[a]

[a] significant

3.5. Relationship Between MAR Index and Environmental Factors

To quantify the relation between the groundwater vulnerability and environmental factors, correlation test was performed between MAR index in well and the distance well-hospital, well depth, and depth to water level, using the Spearman's correlation coefficient. The results are presented in Table 100 and Table 111.

In both towns, the relationship between the MAR index of wells and well-hospital distance was strong overall. The correlation between these parameters was significant and negative in both cases (r = -0.77, p-value = 0.003 in Douala; r = -0.89, p-value =0.001 in Yaounde) (Table 100).

Table 10. Spearman correlation "r" coefficients (and *p*-values) between MAR index of well and distance

Distance	MAR index Douala wells	MAR index Yaounde wells
Distance well-hospital	-0.77 ** (0.003)	-0.89 ** (0.001)

*: *p*-value <0.05; * * *p*-value <0.01; df = 11

Table 11. Spearman correlation "r" coefficients (and p-values) between MAR index of well and hydrogeological factors

	Douala wells		Yaounde wells	
Hydrogeological factors considered	WCH	WFH	WCH	WFH
Well depth	0.35 (0.16)	-0.11 (0.83)	0.57* (0.03)	-0.3 (0.22)
Depth to water level	-0.05 (0.9)	0.07 (0.89)	0.3 (0.14)	0.03 (0.9)

*: *p*-value <0.05; df = 5

In Douala, the relationship between the MAR index in wells and hydrogeological factors was weak in both WCH and WFH. No significant correlation was obtained between these different parameters (-0.05 ≥ r ≥ 0.36, 0.11 ≥ p-value ≥ 0.89) (Table 111). In Yaounde, correlation between MAR index in wells and hydrogeological factors was different from those obtained in Douala. In WCH of Yaounde, well depth significantly impacted the MAR index and the correlation between these parameters was positive (r= 0.57; p-value = 0.03). However, relationship with the depth to water level was not significant (r= 0.3; p-value = 0.14). In WFH of Yaounde, there were no significative correlation between MAR index and well depth (r= -0.3; p-value = 0.22). Similar result was obtained between MAR index and depth to water level (r = 0.03; p-value = 0.9).

4. Discussion

This study affords useful information about the vulnerability of groundwater to hospital wastewater as well as the dissemination of antimicrobial resistance in the environment, and it relevant for population health. By analysing the health aspect of this work, it appears that *P. aeruginosa* strains have critical acquired resistance rates to commonly used antibiotics. In the prospected wells water, a high incidence of resistance was expressed by *P. aeruginosa* to the penicillins, 3^{rd} and 4^{th} generation cephalosporins, aminoglycosids and to the ofloxacin. These drugs are commonly prescribed against *P. aeruginosa* infections in the country. In shanty towns such as Douala and Yaounde where access to potable water and sanitation is limited, the presence of resistant *P. aeruginosa* strains in groundwater widely consumed by population, is a serious public health threat. When bacterial infection occurs during consumption of these contaminated groundwater, therapeutic possibilities are reduced and may result in severe morbidity and mortality [55,56,57]. The occurrence of antibiotic-resistant bacteria in groundwater reported in this study has been already evidenced by previous studies [1,2]. However, the main limitation of these studies was the absence of data stating on the origin of resistant bacteria in groundwater.

The present work was aimed to provide firsthand information about the impact of hospital wastewater on the prevalence of drug-resistant bacteria in groundwater, and by this impact, to assess the vulnerability of groundwater to hospital sewage as well as the environmental factors that can influence groundwater vulnerability. Firstly, we evaluated the prevalence of resistant bacteria in hospital wastewaters in order to assess the potential of hospital sewage for disseminating resistance into groundwater. Then we divided the wells into two groups according to their distance from the hospital, the wells close to the hospital (WCH) and the well far from the hospital. We also chose 3 indicators of bacterial resistance (resistance rate, MAR index and enzyme produced) which were used to compare WCH and WFH.

The hospital effluents analyzed in this study presented a very high prevalence of resistance reaching sometimes 88.2% with several antibiotics, as well as a high MRA index close to 0.85. According to [58], hospital wastewater presenting these high levels of resistance acts as reservoirs of resistance and can permanently transfer resistance to natural waters. The results of resistance rate comparison between WCH and WFH showed that in Douala, MAR index values and resistance rate (%), were significantly higher in WCH than in WFH (p-value <0.05). A similar result was obtained in Yaounde with 7 antibiotics. The fact that resistance rate and MRA indexes were higher in WCH could be explained by the proximity of WCHs to the hospitals facilities and their vulnerability to hospital wastewater. Hospital wastewater infiltration would further impact the WCH and would be responsible for the high resistance rate observed in these wells [14,16,22].

In this study, correlation test showed that the MAR index and well-hospital distance was strongly et negatively correlated. This negative correlation would mean that reducing the well-hospital distance would significantly increase the vulnerability of groundwater to the hospital wastewater pollution.

Besides, transfers of resistant bacteria in natural waters have been previously demonstrated by [59]. They examined the antibiotic resistance of *P. aeruginosa* in hospital effluents and in the surrounding natural urban waters. They showed that the two ecosystems were genetically linked. This suggests that multi-resistant strains of *P. aeruginosa* isolated from natural waters originate from hospital wastewater [59]. Even if this genetic link was not sought in the present study, the highest resistance rate and MRA indexes observed in hospital effluents and WCH when compared to WFH, constitutes an important indicator of the impact of hospital effluents on emergence of resistance in wells.

It is important to note that the highest resistance rates in WCH was not observed with all the antibiotic tested. These highest resistance rates were only obtained with TICC, TIC, PIP, CAZ, GM, OFX, and CIP. The reason is that, these antibiotics are widely used in hospitals against *P. aeruginosa* infections [60,61]. The intensive use of these drugs could have induced the selection of resistant strains, which would have contaminated the groundwater via hospital wastewater infiltration.

5. Conclusion

This study showed that hospital wastewater of Douala and Yaounde act as reservoir of drug-resistant *P. aeruginosa*. A significant difference in the resistance rate between WCH and WFH was observed in both towns. The resistance rates with the following antibiotics TICC, TIC, PIP, CAZ, GM, OFX, and CIP were significantly higher in WCH than in WFH. Similar results were obtained with the penicillinase, high-level cephalosporinase (AmpC) phenotype rate, and MAR index values. Hospital wastewater of Douala and Yaounde plays an important role in the spread of antibiotic resistance in the surrounding aquatic environments. They must be taken into account when developing strategies to combat the spread of antibiotic resistance.

Authors Contributions

This manuscrit was written through contributions of all authors. All authors gave approval to the final version of the manuscrit.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

This study does not involve human participants and then does not require any ethical consideration.

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