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Raw wastewater irrigation for urban agriculture in three African cities increases the abundance of transferable antibiotic resistance genes in soil, including those encoding Extended Spectrum β-Lactamases (ESBLs)

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Abstract

A study was conducted to investigate the impact of raw wastewater use for irrigation on dissemination of bacterial resistance in urban agriculture in African cities. The pollution of agricultural fields by selected antibiotic residues was assessed. The structure and functions of the soil microbial communities, presence of antibiotic resistance genes of human clinical importance and *Enterobacteriaceae* plasmid replicons were analysed using high throughput metagenomic sequencing. In irrigated fields, the richness of *Bacteroidetes* and *Firmicutes* phyla increased by 65% and 15.7%, respectively; functions allocated to microbial communities' adaptation and development increased by 3%. Abundance of antibiotic resistance genes of medical interest was 27% greater in irrigated fields. Extended spectrum β-lactamase genes identified in irrigated fields included $bla_{\text{CARB-3}}$, $bla_{\text{OXA-347}}$, $bla_{\text{OXA-5}}$ and bla_{Rm3}. The presence of ARGs encoding resistance to amphenicols, β-lactams, and tetracyclines were associated with the higher concentrations of ciprofloxacin, enrofloxacin and sulfamethoxazole in irrigated fields. Ten *Enterobacteriaceae* plasmid amplicon groups involved in the wide distribution of ARGs were identified in the fields. IncQ2, ColE, IncFIC, IncQ1, and IncFII were found in both farming systems; IncW and IncP1 in irrigated fields; and IncY, IncFIB and IncFIA in non-irrigated fields. In conclusion, raw wastewater irrigated soils in African cities could represent a vector for the spread of antibiotic resistance, thus threatening human and animal health. Consumers of products from these farms and farmers could be at risk of acquiring infections due to drug-resistant bacteria.

Key words: Wastewater irrigation; agricultural fields; bacterial resistance; metagenomics; ESBLs; Africa.

1. Introduction

Antibiotics are known for their properties to either stop bacteria from growing or to kill them. Bacterial drug resistance emergence and dissemination has compromised healthcare systems and public health policy in many countries. This is a source of concern in low- and middle-income countries (LMICs) where antibiotics of last resort are not available to most people (WHO, 2014). Furthermore, in LMICs, a high infectious disease burden commonly co-exists so antibiotic resistance compromises the treatment of many infections that were, until recently, treatable (Laxminarayan et al. 2016). Drug-resistant bacteria are a critical public health concern even in developed countries (Piddock, 2012). In 2011, an epidemic of *Escherichia coli* infections caused by contaminated bean sprouts affected up to 5,000 people in Europe, with over 48 deaths (Buchholz et al. 2011). To efficiently tackle the increasing bacterial resistance, environmental, agricultural, and medical aspects need to be handled at a global scale (Wellington et al. 2013).

Arable lands reported to be irrigated with wastewaters worldwide cover approximately 20 million hectares; which equates to 10% of the total global irrigated land (Mateo-Sagasta et al. 2011). Wastewaters originating from slaughterhouses, private use and applications in hospitals can contain high concentrations of antibiotics along with many drug-resistant pathogenic bacteria. Their application on agricultural fields can influence the structure of the soil microbiota (Dickin et al. 2016). Whereas in the past several research teams have addressed questions related to the application of manure, which is contaminated with antibiotics used in animal husbandry, much less is known about the effect of raw wastewater irrigation on the development of antibiotic resistant bacteria (ARB) in irrigated fields. This is despite the fact that raw wastewater irrigation is often applied in LMICs as a cheap alternative during water scarcity and to avoid expensive commercial fertilizers. In many cities in LMICs, wastewater irrigation has been a common practice for decades (Adegoke et al. 2018). Wastewaters can contain high concentrations of antibiotics from private use and applications in hospitals, along with pathogenic or drug-resistant bacteria or both (Igbinosa et al. 2011).

The release of pharmaceuticals in the environment selects for drug-resistant bacteria (Andersson and Hughes, 2010). To combat antibiotics in ecosystems, bacteria have evolved a plethora of different antibiotic resistance genes (ARGs) of which many are mobile and can easily spread between species including human and animal pathogens. Environmental drug-resistant bacteria can transfer ARGs to pathogenic bacteria by horizontal gene transfer (Forsberg et al. 2012). Owing to its complex intrinsic and acquired antibiotic resistome, some studies have highlighted the importance of soil as the potentially largest reservoir of genes coding for antibiotic resistance (Wang et al. 2014; Nesme and Simonet, 2015). There is a crucial need to identify the principal reservoirs of ARGs

for humans, animals and the environment, since there is insufficient information about the conditions and factors that lead to the mobilization, selection and movement of resistant drug bacteria into and between environment, human and animal populations (Wellington et al. 2013).

Next generation sequencing has been successfully used to track drug-resistant bacteria and ARGs which may spread quickly across the soil microbial community following selection pressure due to antibiotic application (Fahrenfeld et al. 2014; Guo et al. 2018). Metagenomics provides an understanding of the factors driving transfer of ARGs and the prevalence of different antibiotic resistance mechanisms (Amos et al. 2014).

In this study, we assessed the influence of raw wastewater commonly used in urban agriculture in LMICs on the structure and functions of soil microorganisms, and presence of drug-resistant bacteria and genes. It is postulated that in agricultural fields irrigated with raw wastewater, bacteria have adapted to survive antibiotic exposure by vertical and horizontal gene transfer producing high numbers of bacteria containing clinically relevant ARGs.

2. Material and methods

2.1. Experimental design and soil sampling

The experiment was conducted in three cities, in two African countries, namely Ouagadougou (12°23′ N, 1°29′) in Burkina Faso, Ngaoundere (7°19′ N, 13°35′) and Yaounde (3°52′ N, 11°31′) in Cameroon. Their respective annual mean of temperature and precipitations are for: Ouagadougou (30ºC; 867 mm); Ngaoundere (22ºC; 1497mm) and Yaounde (24ºC; 1628 mm), respectively. At each city two blocks were investigated, comprising three agricultural fields that were irrigated (IRI) with raw wastewater, and as control soils, 500 m away, three non-irrigated agricultural fields (NIR), with comparable soil properties. Wastewater was coming from dwellings, hospitals, agriculture, markets and slaughterhouses (Bougnom et al. (2019a; 2019b). Tomato and salad were regularly cultivated in the fields. We had Ouagadougou (IRI1 and NIR1); Ngaoundere (IRI2 and NIR2), and Yaounde (IRI3 and NIR3). The agricultural fields were approximately 0.2 ha each and watered manually twice per day with watering cans. In each field, 100 g of soil was randomly sampled at 10 different places from 0-20 cm depth, using soil cores. Replicate samples were pooled together, receiving 1 kg-composite samples. The collected soil samples were kept on ice during transport and stored at -80ºC before analysis.

2.2. Soil physical and chemical analysis

Soil pH was measured in a 1:2.5 (soil: demineralised water) ratio using a glass electrode. Total C and N were analysed using a TOC-V_{CPN}-analyzer (Shimadzu, Duisburg, Germany).

Soil antibiotic residues were extracted using solid phase extraction (SPE) according to Blackwell et al. (2004). The detailed description is reported in supplementary text S1. SPE was conducted from 4 g of air-dried soil, using SAX and HLB SPE cartridges (Thermofisher, Massachusetts, USA) set up in tandem. Prior to SPE, the cartridges were conditioned with 5mL methanol then conditioned with 5mL buffer. After SPE, the SAX cartridges were removed and the HLB cartridges were washed with 5ml conditioning buffer. Thereafter, the HLB cartridges were air dried for 10 min and antibiotic residues were eluted with 2 × 1 mL of methanol. Liquid chromatography-mass spectrometry (LC-MS) was used to quantify antibiotic residue concentrations (Michelini et al. (2012). Ciprofloxacin, enrofloxacin, chlortetracycline, sulfadimidine, sulfadiazine, sulfamethoxazole, oxytetracycline, tetracycline, trimethoprim and tylosin were the analyzed pharmaceuticals. These pharmaceuticals were analysed because of their molecular structure and physicochemical properties which allow them to resist transformation or degradation in soils (Cycon et al. 2019; Kumar et al. 2019).

2.3. Microbiological analysis

2.3.1. Soil Biomass Purification

Soil biomass purification was conducted to collect mainly the bacterial cells from the different soils (Sentchilo et al. 2013). Briefly, 15g soil samples were homogenized by magnetic stirring for 15min, in ice-cold poly (beta-amino) esters (PBAE) buffer (PBAE buffer is 10mM Na-phosphate, 10mM ascorbate, 5mM EDTA, pH 7.0), at 10 mL g^{-1} of soil. Low speed centrifugation in 50-mL conical tubes at 160 g for 6 min was used to remove bigger particles (>10 μm) such as coarse particles, large eukaryotic cells and bacterial flocs. Microbial biomass was collected after centrifugation of the supernatants at 10,000 g/5 min.

2.3.2. DNA extraction and quantification, and sequencing

DNeasy PowerSoil Kit (Qiagen, Germany) was used to extract soil DNA according to the manufacturer's instructions. Fluorometry was used to determine DNA concentration, using the Qubit™ 3.0 Fluorometer (Qubit, Life Technologies, USA). The three DNA samples extracted from each block were pooled together in equal nanogram quantities. Six DNA samples representative of the three cities were sent to Edinburgh Genomics for high-throughput sequencing. Sequencing was conducted using Illumina Hiseq4000 (Illumina, Inc, USA), TruSeq DNA. Nano gel free library (350 bp insert) was used to prepare the libraries. Raw data consisted of 190.5 Gb sequences. The sequenced reads raw data have been deposited in MG-RAST server (project IDs: mgm4815682.3 ; mgm4815683.3; mgm4815684.3; mgm4815685.3; mgm4815686.3; and mgm4815687.3).

2.4. Bioinformatic analyses

2.4.1. Taxonomic and functional annotations

The raw sequence reads were uploaded to the metagenomics analysis server (MG- RAST) version 4.0.3 (Glass et al. 2010). Soil microbial communities' profile and metabolic functions was determined with the use of both SEED database and SEED subsystems database, respectively. The SEED subsystems is a collection of protein families sharing the same functions. Both microbial and metabolic profiles were generated using a minimum identity ≥ 80% and minimum alignment length ≥ 20 amino acids, at E-value 10^{-5} (Glass et al. 2010).

2.4.2. Identification and quantification of antibiotic resistance genes.

Short Better Representative Extract Dataset (ShortBRED) was used to identify and quantify antibiotic resistance genes (ARGs) from the metagenome (Kaminski et al. 2015). ShortBRED profiles protein family abundance in metagenomes in two-steps: (i) *ShortBRED-Identify* isolates representative peptide sequences (markers) for the protein families, and (ii) *ShortBRED-Quantify* maps metagenomic reads against these markers to determine the relative abundance of their corresponding families based on reads per kilobase million (RPKM). Minimum identity of 95% and minimum fragment length of 30 amino acids were considered positive. ARG markers were generated from the Comprehensive Antibiotic Resistance Database (CARD) (McArthur et al. 2013) was used to generate, using UniRef50 as a reference protein database. Antibiotic resistance ontology (ARO) numbers in CARD was used to aggregate, annotate and associate the ARGs to the corresponding resistance family.

2.4.3. Identification of plasmid amplicons of clinical relevance

Enterobacteriaceae plasmid replicon sequences were downloaded from the PlasmidFinder database 1.3 (https://cge.cbs.dtu.dk/services/PlasmidFinder). The nucleotide sequences were aligned against the metagenomic reads using BLAST-like alignment tool (BLAT). The parameters were BLAT hit with a sequence identity ≥ 80% and E-value cut-off of 10^{-5} (Carattoli et al., 2014).

2.5. Data Analysis

The differences in relative abundance of the different bacterial phyla and families of interest, functional categories present in the metagenomic reads, ARGs, and *Enterobacteriaceae* plasmid replicon groups detected in the agricultural fields were analysed using the Student's *t*-test, at $P < 0.05$. Heatmaps were generated to visualize tabular abundance of ARGs in the fields, using hclust2 (Asnicar et al. 2015).

3. Results and Discussion

3.1. Antibiotic residues in soil

149 Enrofloxacin oxytetracycline and sulfamethoxazole concentrations were greater in irrigated fields. The
150 concentration of enrofloxacin was greater (1.10 ng. g^{-1}) in irrigated fields while that of sulfadimidine was greater
151 (0.81 ng. g^{-1}) in non-irrigated fields. In irrigated fields, antibiotic concentrations ranged from 0.09 to 0.92; in non-
152 irrigated fields, they ranged from 0.04 to 0.44 ng g-1 (Table 1). Sulfadiazine, chlortetracycline, tetracycline and
153 tylosin residues were not detected in any soil from either farming systems. In irrigated fields, the concentrations
154 of sulfadimidine and oxytetracycline was greater than in the non-irrigated fields by 10 %, ciprofloxacin by 52%,
155 enrofloxacin by 63.6%, and sulfamethoxazole by 77.8%.

156 The 20 years irrigation of soils with raw wastewater (Cisse et al. 2002; Kengne et al. 2002) containing
157 substantial amounts of organic matter led to higher pH, organic carbon and nitrogen. This could be consequent to
158 the increase in the soil organic matter (SOM) pool (Bougnom et al. 2009). The levels of antibiotic residues
159 concomitantly increased in irrigated soils. This confirms the studies reporting the accumulation of antibiotic
160 compounds in agricultural fields following irrigation with treated or raw wastewater (Calderon-Preciado et al.
161 2011; Grossberger et al. 2014; Wang et al. 2014). Soil pH, organic matter content and soil texture are reported to
162 be among the factors impacting the fate of pharmaceuticals in soil (Thiele-Bruhn et al. 2004; Du and Liu, 2012).
163 Antibiotic residues are less bioavailable, and thus less biodegradable in soils with high SOM and clay content,
164 owing to stronger sorption to SOM and the formation of non-extractable residues (Luo et al. 2011; Müller et al.
165 2013; Cheng et al. 2016). Both higher SOM content and higher contents of enrofloxacin, oxytetracycline and
166 sulfamethoxazole residues were found in irrigated fields. The prevalence of fluoroquinolones and
167 sulfamethoxazole may be due to the input or related to the stronger retardation of these antibiotics in acidic and
168 iron oxide-rich tropical soils (Essington et al. 2010). Antibiotic residues were also determined in non-irrigated
169 farms but at lower contents. This contamination could be due to previous (not reported to us) use of animal manure
170 as fertilizer, deposition of wastewater aerosol and soil dust, respectively, derived from the nearest irrigated sites
171 by wind erosion, and human transport of agricultural materials between fields (Dalkmann et al. 2012).

172 **3.2. Effects of urban agriculture and wastewater irrigation on soil microorganisms**

173 **3.2.1. Microbial diversity and functionality**

174 The taxonomic analysis of the microbial communities at kingdom level in irrigated and non-irrigated fields
175 showed that the highest proportion of metagenomic reads mapped to Bacteria (99.1% and 99.2%), followed by
176 Archaea (0.48% and 0.42%), Eukaryota (0.26% and 0.34%), and unassigned (0.05% and 0.06%). Metagenomic
177 reads allocated to viruses represented 0.01% in samples from both farming systems. Ten and nine bacterial phyla
178 with a relative prevalence ≥ 0.5% of the reads were identified in irrigated and non-irrigated fields, respectively

(Figure 1a). The dominant bacteria phyla in both farming systems were *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* (≥ 89.6% of all bacterial phyla). In irrigated fields, the relative abundance of *Bacteroidetes* and *Firmicutes* were 65% and 15.7% greater, respectively. The phylum *Gemmatinodetes* was not among the most prevalent phyla in irrigated fields. Figure 1b shows the top 22 bacterial families found in the different farming systems. The relative abundance and order of bacterial families differed in the farming systems. In irrigated agricultural fields, the 10 most prevalent bacterial families were 1. *Xanthomonadaceae*, 2. *Caulobacteraceae*, 3. *Comamonadaceae*, 4. *Sphingomonadaceae*, 5. *Flavobacteriaceae*, 6. *Mycobacteriaceae*, 7. *Pseudomonadaceae*, 8. *Planctomycetaceae*, 9. *Bradyrhizobiaceae*, and 10. *Nocardioidaceae*. The top 10 in non-irrigated fields were (deviating numbers from irrigated fields are added in brackets) 1. (2) *Caulobacteraceae*, 2. (1) *Xanthomonadaceae*, 3. *Comamonadaceae*, 4. (7) *Pseudomonadaceae*, 5. *Flavobacteriaceae*, 6. (-) *Conexibacteraceae*, 7. (6) *Mycobacteriaceae*, 8. (9) *Bradyrhizobiaceae*, 9. (8) *Planctomycetaceae and* 10. *Nocardioidaceae.*

Soil pH, texture, nutrients, carbon content, pollutants, and agricultural management influence soil microbial structure and functions in soil (Jangid et al. 2011; Kuramae et al. 2011). In this context, it is noted that irrigation wastewater contains nutrient elements, chemical, physical and biological pollutants, as well as degradable organic matter from anthropogenic activities (Deblonde et al. 2011). Since many pollutants can have a stimulating or inhibitory effect on microbial cells, changes in both soil microbial structure and functions following irrigation with wastewater were as expected. The identified bacterial phyla in both irrigated and non-irrigated fields are generally encountered in soil (Fierer et al. 2012; Nacke et al. 2014). Previous field and mesocosm-scale studies have indicated the transfer of *Bacteroidetes* phyla members into the soil following wastewater irrigation (Broszat et al. 2014; Frenk et al. 2018). The increased number of this phyla is explained by its copiotrophic activity and high grow rate in the presence of nutrients, moisture and labile organic C found in wastewater (Broszat et al. 2014). This elucidates their greater prevalence in irrigated fields. *Firmicutes* can form spores in unfavourable environmental conditions and will then sporulate and give rise to bacterial growth in response to the additional nutrients (Onyenwoke et al. 2004). This could explain their increase in irrigated fields, which contain nutritious organic matter. Tropical soils are depleted of nutrients because of the high mineralisation rate. *Acidobacteria*, *Chloroflexi*, *Verrucomicrobia* and *Planctomycetes* have been reported to follow oligotrophic strategies with limited growth rates and thriving capacity in nutrient-poor ecosystems (Lauber et al. 2013; Kielak et al. 2016). *Caulobacteraceae* family members are primarily known as oligotrophic microorganisms (Poindexter et al. 1981). This could explain the prevalence of these bacterial phyla and families in non-irrigated fields. The

greater abundance of *Xanthomonadaceae* family members following wastewater irrigation could present a health issue. The family *Xanthomonadaceae* contains plant-pathogenic genera including *Xanthomonas* and *Xylella* (Hajri et al. 2009). Therefore, plants grown in irrigated fields are more exposed to bacterial infections affecting crop productivity. Outbreaks of Xanthomonas wilt disease of banana are frequently reported in the region (Ocimati et al. 2019).

Functional metabolic diversity analysis of the six metagenome reads from the soil samples using the SEED database revealed that 14 subsystems were most frequent in the soil microbial communities (Table 2). The most prevalent functional categories were in both farming systems "Carbohydrates", "Clustering-based subsystems" and "Amino acids and derivatives". Comparative analysis using the Student's *t*-test showed that sequence reads coding for functional subsystems ''Clustering-based subsystems'', ''DNA metabolism'', ''Nucleosides and nucleotides'' and ''Stress response'' were significantly higher in irrigated fields ($P < 0.05$).

The analysis of metagenome reads using SEED provided insights into the functional metagenomic profiling of microorganisms living in the investigated fields. Considering changes in the microbial diversity structure observed between the two farming systems, some differences in the metabolic potential of the soil microbiota were expected. The functions 'clustering-based subsystems' (functional coupling evidence but unknown function), 'DNA metabolism' (DNA repair, bacterial), 'nucleosides and nucleotides and stress response' translate a higher bacterial and enzymatic activity in irrigated fields consequent to the introduction of nutrients, organic matter and several pollutants. The soil microbiota must develop functional redundancy and adopt mechanisms to adapt, survive and grow. Therefore, wastewater irrigation affects microbial community structure and functions.

3.2.2. Antibiotic resistance genes and *Enterobacteriaceae* plasmid replicons

Transferable ARGs abundance was 27% greater in irrigated soils. ARGs commonly associated with mobile genetic elements accounted for 33 and 26 out of the 45 and 39 detected ARGs in irrigated and non-irrigated fields sequence reads, respectively (Figure 2). The transferable ARGs confer resistance to trimethoprim (2) and nine major classes of antibiotics that encode resistance to aminoglycosides (10), β-lactams (7), amphenicols (6), tetracyclines (5), sulphonamides (3), macrolides (2), quinolones (1), phosphonic antibiotics (1) and nucleoside antibiotics (1). Twenty-one types were common to both farming systems, 12 (*aac(6')-Ib7*, *ant(9)-Ia*, *catIII*, *catQ*, $bla_{\text{CARB-3}}$, $bla_{\text{OXA-347}}$, $bla_{\text{OXA-5}}$, *bla*Rm3, *fosB*, *sul3*, *tetC* and *tetX*), and five (*mphG*, $bla_{\text{LCR-1}}$, *ereA2*, *qnrVC1*, and *tetB(P)*) were found in irrigated and non-irrigated fields, respectively. The relative abundance of ARGs common to both farming systems did not show any significant difference ($P<0.05$). The heat map reporting the relative

abundance (RPKM) of ARGs showed that agricultural fields clustered per origin. The only exception was in block 2, where the ARG imprint following wastewater irrigation was more pronounced. Bivariate correlation analysis between prevalence of both ARGs and antibiotic residues showed positive correlations between the concentration of sulfamethoxazole, ciprofloxacin, enrofloxacin, trimethoprim and some ARGs (Table 3). The concentration of sulfamethoxazole and ciprofloxacin had the greatest number of positive relationships (nine), followed by enrofloxacin (eight), and trimethoprim (one). Trimethoprim was positively correlated to *dfrA1*. There was a positive correlation between sulfamethoxazole, ciprofloxacin and enrofloxacin, and the presence of *catIII*, *floR*, $bla_{\text{OXA-347}}$, $bla_{\text{OXA-5}}$, $bla_{\text{CARB-3}}$, bla_{rm3}, *sul3*, *tetC*, and *tetX*; except *floR*.

Because of the presence of an abundant and diverse community of antibiotic producers, soil is the potentially largest reservoir of drug-resistant bacteria and genes (Nesme and Simonet, 2015). The presence and abundance of transferable ARGs in agricultural fields in three African cities is in accordance with previous studies from China, the US and Lithuania (Zhu et al. 2013; McKinney et al. 2018; Armalytė et al. 2019). Furthermore, an additional effect of wastewater irrigation on the soil resistome was found. Studies of Chen et al. (2016) ; Broszat et al. (2014) ; Dungan et al. (2018) in China, Mexico, and US, have reported an increase of ARGs in soils following wastewater irrigation. Some pathways likely to select for bacterial resistance in soil include, influx of antibiotic residues which can induce a selective pressure; transfer and survival of ARB; and influx of transferable plasmids harbouring ARGs (Von Wintersdorff et al. 2016).

In previous studies, the wastewaters used to irrigate the investigated fields were reported as strong vectors for bacterial resistance dissemination (Bougnom et al. 2019a; 2019b). Thus, a more diverse ARB community and ARGs in irrigated fields was anticipated. All the ARGs present in the fields had been found in the wastewaters. Nevertheless, the abundance of ARGs reported in the soil was lesser. This is most likely a consequence of the death of many ARB when transferred from wastewater to soil where the bacteria must develop mechanisms to adapt, survive and grow. Among the transferable ARGs found solely in irrigated fields, four among the 12 were genes encoding ESBLs. Gram-negative bacteria are primarily carriers of ESBL genes, and application of animal manure to soil sustains the survival and growth of pathogens, ARB and ARGs (Rawat and Nair, 2010; Sharma and Reynnells, 2016). *Enterobacteriaceae* producing ESBLs can survive for a long time in irrigated fields and contaminate humans and animals via direct contact or the food chain. ESBL producers pose critical issues in clinical settings since they are able to inactivate β-lactams, thus requiring the administration of more expensive antibiotics. Direct contact with urban farm workers and consumption of crops from these fields could pose a serious health risk. Changes in the distribution of the mechanisms of antibiotic resistance in the irrigated field

was consequent to the modification of the soil resistome following wastewater irrigation. Fertilisation or irrigation of soil with material rich in organic material could introduce ARB and ARGs (Binh et al. 2008; Negreanu et al. 2012; Nesme and Simonet, 2015). This could explain the greater abundance of transferable ARGs in soils from irrigated fields. Two non-irrigated farms from Yaounde and Ngaoundere used to be fertilised with cow manure, possibly explaining the greater prevalence of enteric bacteria in these fields.

There was a positive correlation between the concentrations of sulfamethoxazole and trimethoprim and relative prevalence of *sul3* and *dfrA1*, respectively, suggesting that pollution by these antibiotics influences the selection of these genes. Our data add to growing evidence that antibiotic residues in the environment exert a selective pressure on the acquisition of ARGs coding resistance against them (Cheng et al. 2016; Pan and Chu 2018). We found that sulfamethoxazole, ciprofloxacin and enrofloxacin were associated with multiple and cross resistance. Thus, presence of these antibiotics could foster the maintenance of drug-resistance genes for antibiotics of other classes and hence multidrug resistant plasmids (Levy, 2002; Blanco et al. 2016). Botts et al. (2017) in the US have reported multidrug resistant plasmids encoding resistance genes for amphenicols, aminoglycosides, β-lactams, fluoroquinolones, tetracyclines, and sulfonamides, in coastal wetland impacted by wastewater. Therefore, abundance of multidrug resistant plasmids could be observed in the case of selective pressure exerted by one of these antibiotics. This shows the complex nature of factors selecting ARB and ARGs in the environment.

Enterobacteriaceae plasmids are well known to be the main carriers of clinically relevant ARGs (Carattoli, 2009). Ten *Enterobacteriaceae* plasmid amplicon incompatibility (Inc) groups were identified in the agricultural fields (Figure 3). Five (IncQ2, ColE, IncFIC, IncQ1, and IncFII) were common to both, while two (IncW and IncP1) and three (IncY, IncFIB, and IncFIA) were found in irrigated and non-irrigated fields, respectively.

Considering the wide and diverse ARGs in the wastewaters used to irrigate these fields (Bougnom et al. 2019a; 2019b), the presence of *Enterobacteriaceae* plasmid replicons was not surprising. However, the different amplicon groups found in the fields were less diverse that those found in the wastewater. This suggests that following transfer of some ARBs from wastewater to soil, there is loss of some ARGs. IncFIB has been reported to be predominant in cattle faeces in Nigeria (Inwezerua et al. 2014); thus, fertilisation with animal manure could explain the presence of plasmid replicons in non-irrigated fields. Considering the epidemiological aspects, *Enterobacteriaceae* plasmid replicons found in irrigated farms represent greater public health issues. All Gram-negative bacteria are potential hosts of IncP and IncW plasmids (Dröge et al., 2000), while IncF and incY have a "narrow" host range (Hawkey, P. 2008; Mshana et al. 2009).

4. Conclusions

In conclusion, LC-MS and high throughput sequencing allowed the assessment of bacterial resistance dissemination in urban agriculture in African cities. Following wastewater irrigation, distribution and metabolic functions of soil microbial community were influenced. Sulfamethoxazole, ciprofloxacin and enrofloxacin antibiotic residues may select for ARGs not oriented against their action. Wastewater irrigation increases the abundance of ARGs of clinical importance in soil, including *Enterobacteriaceae* plasmid replicons. The collected information suggests that raw wastewater irrigated soils in African cities could represent a vector for the spread of antibiotic resistance, thus threatening human and animal health. Consumers of products from these farms and farmers could be at risk of acquiring infections due to drug-resistant bacteria.

Conflict of Interest

The authors declare no conflict of interest.

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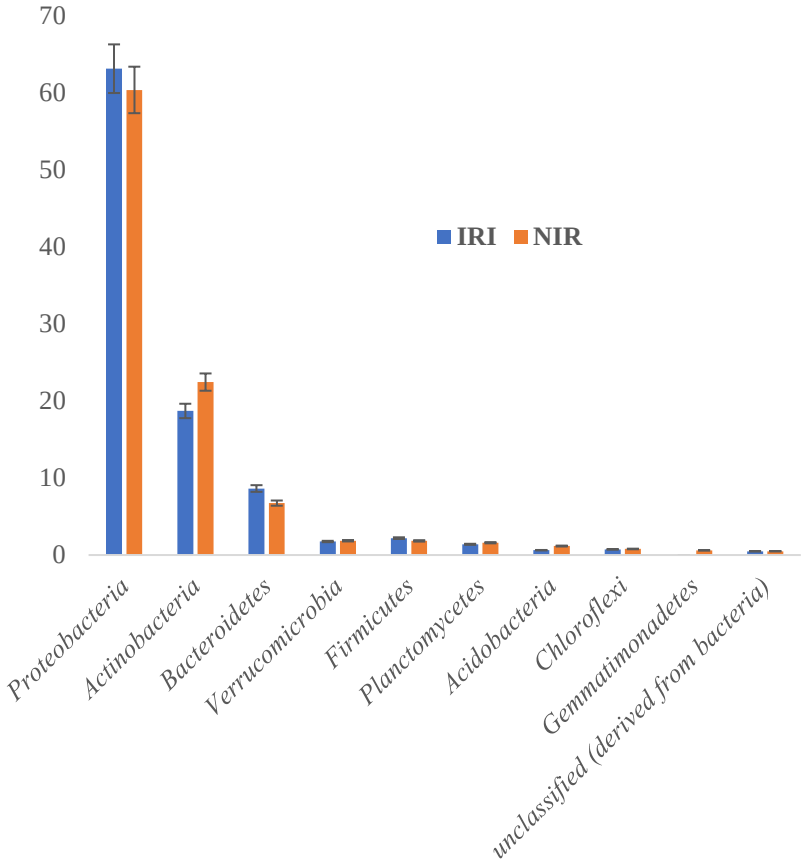
a)



b)

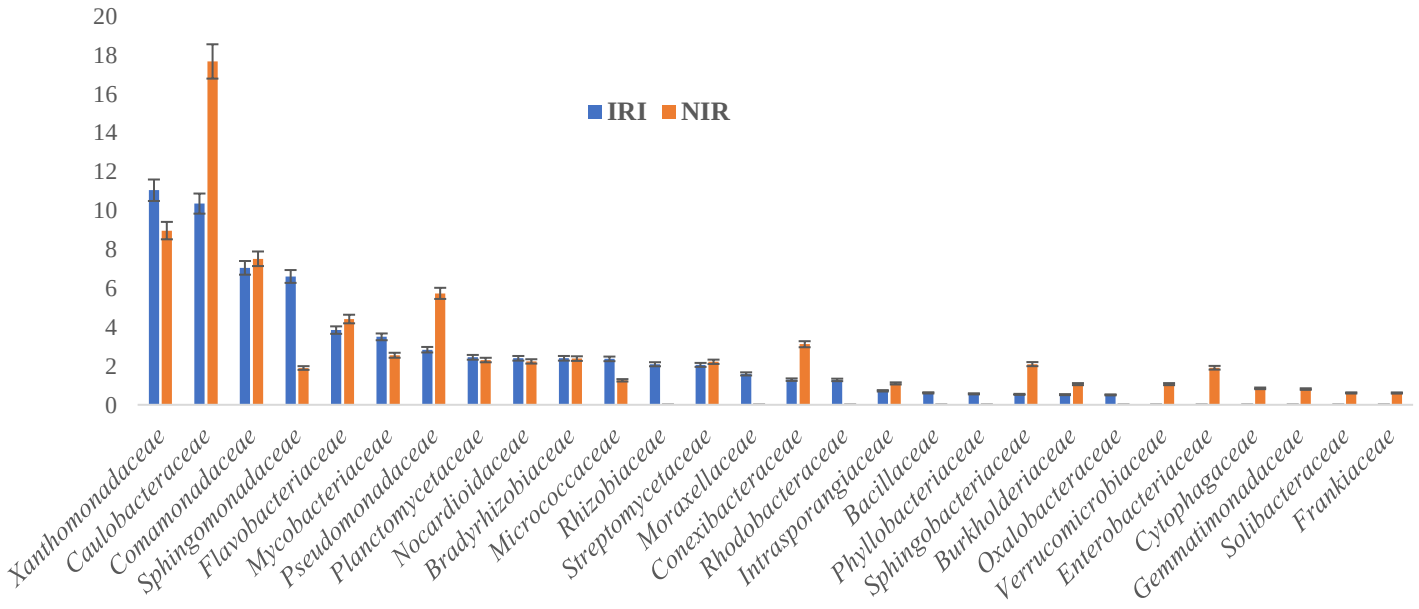


Figure 1. Relative abundances (%) of soil a) bacterial phyla and b) families derived from the metagenomic reads in irrigated (IRI) and non-irrigated (NIR) fields. Bacterial phyla and families with average relative abundance > 0.5% are visualized. (n=3)

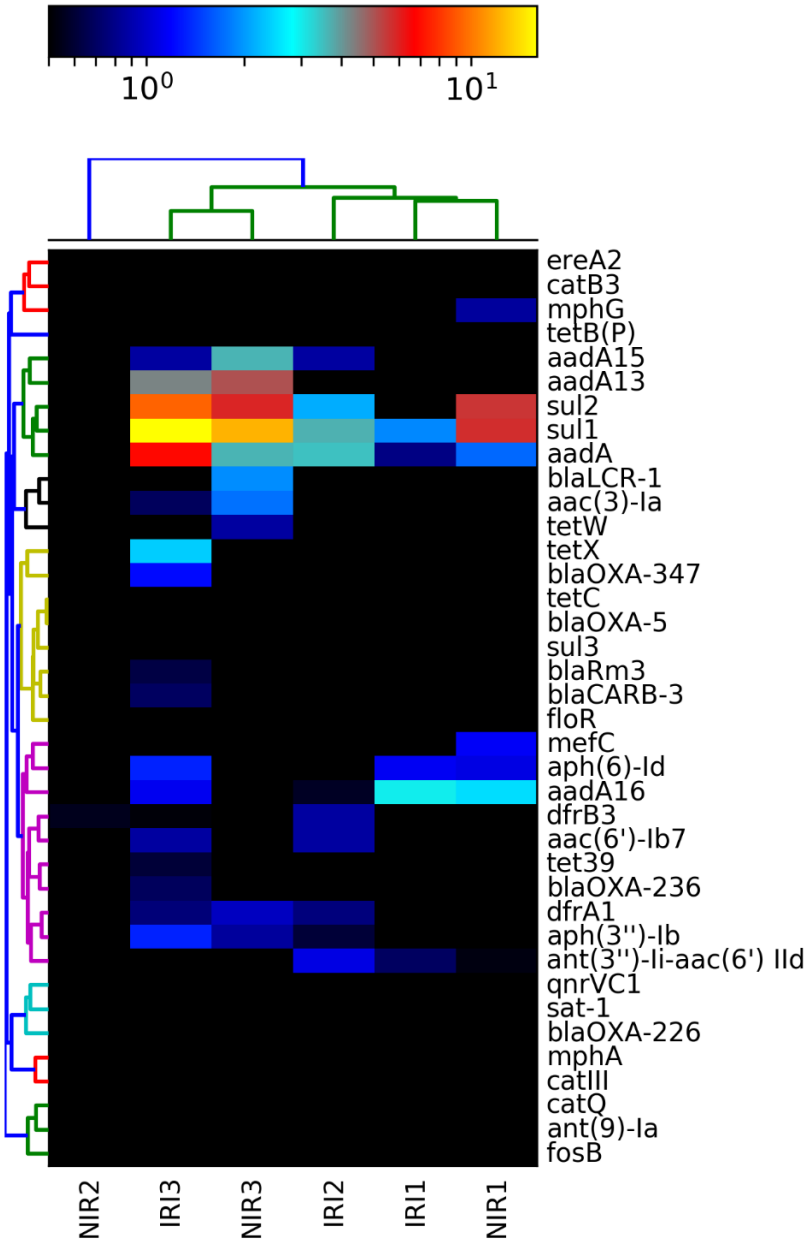


Figure 2. Heatmap representing the relative percentage (RPKM unit) of the different antibiotic resistance genes abundance in the agricultural soil samples. *RPKM* Reads Per Kilobase Million; *IRI* Irrigated; *NIR* Non-irrigated.

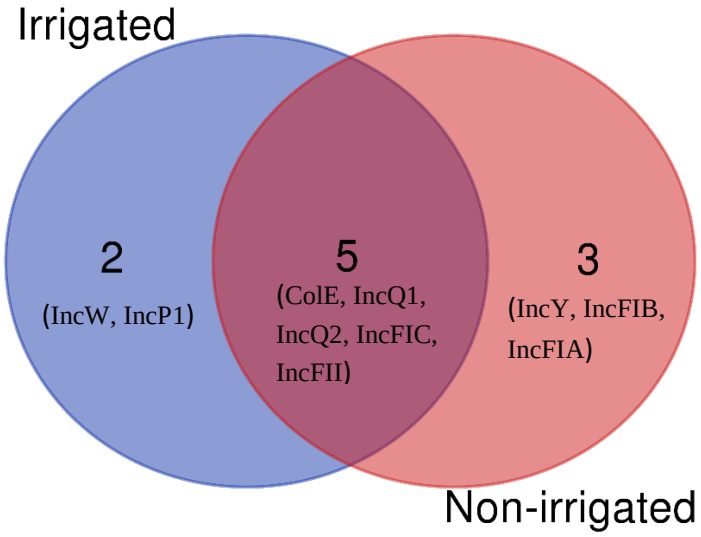


Figure 3: Venn diagram showing the identified *Enterobacteriaceae* plasmid replicons in the irrigated and non-irrigated agricultural fields. (n=3)

Table 1. pH, carbon, nitrogen content and concentration of antibiotic residues (ng. g^{-1}) in the samples collected in irrigated and non-irrigated fields

	Irrigated fields				**Non-irrigated fields**			
Parameters	**IRI1**	**IRI2**	**IRI3**	**Mean (±SD)**	**NIR1**	**NIR2**	**NIR3**	**Mean (±SD)**
pH	7.26	6.49	7.00	6.92 (±0.39)	7.00	5.29	5.78	6.02 (±0.88)
C (% dw)	2.16	4.54	2.38	3.03 (±1.31)	2.14	2.77	1.22	2.04 (±0.78)
N (% dw)	0.15	0.33	0.19	0.22 (±0.09)	0.17	0.22	0.11	0.17 (±0.05)
Sulfadimidine	n.d.	2.64	0.07	0.90 (±1.50)	n.d.	2.42	n.d.	0.81 (±1.40)
Sulfadiazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sulfamethoxazole*	n.d.	n.d.	0.54	0.18 (±0.31)	n.d.	0.11	n.d.	0.04 (±0.06)
Trimethoprim	0.04	0.33	0.38	0.25 (±0.18)	0.25	0.25	0.44	0.31 (±0.11)
Ciprofloxacin	0.44	n.d.	2.33	0.92 (±1.24)	0.19	0.95	0.18	0.44 (±0.44)
Enrofloxacin*	0.76	0.57	1.98	1.10 (±0.77)	0.47	0.31	0.43	0.40 (±0.08)
Oxytetracycline*	n.d.	0.26	n.d.	0.09 (±0.15)	n.d.	n.d.	n.d.	n.d.
Chlortetracycline	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tetracycline	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tylosin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

* The distribution is significantly different between the two agricultural systems (Student's *t*-test, $P < 0.05$).
dw dry weight; *SD* Standard deviation; *n.d.* not detected; *IRI* irrigated; *NIR* non-irrigated. Detection limits (5 to 20 μg L^{-1}); Quantification limits (2 to 10 μg L^{-1}).

Table 2. Relative distribution of sequencing reads (RPKM units) in major level 1 subsystems in irrigated and non-irrigated fields. Metagenomic data were annotated against SEED subsystems in MG-RAST at a cut off of E-value < 10^{-5}.

	Agricultural fields							
	Irrigated				Non-irrigated			
Functional subsystems	**IRI1**	**IRI2**	**IRI3**	**Mean (±SD)**	**NIR1**	**NIR2**	**NIR3**	**Mean (±SD)**
Carbohydrates	2.62x 10^6	2.35x 10^6	2.35x 10^6	2.44x 10^6 (±1.56x 10^5)	2.51x 10^6	2.11x 10^6	2.59x 10^6	2.40x 10^6 (±2.57x10^5)
Clustering-based subsystems*	2.55x 10^6	2.46x 10^6	2.48x 10^6	2.50x 10^6 (±4.59x 10^4)	2.63x 10^6	1.90x 10^6	2.82x 10^6	2.45x 10^6 (±4.88x 10^5)
Amino acids and derivatives	2.12x 10^6	2.08x 10^6	2.08x 10^6	2.09x 10^6 (±2.49x 10^4)	2.14x 10^6	1.65x 10^6	2.38x 10^6	2.06x 10^6 (±3.74x 10^5)
Miscellaneous	1.34x 10^6	1.33x 10^6	1.38x 10^6	1.35x 10^6 (±2.58x 10^4)	1.41x 10^6	9.85x 10^5	1.61x 10^6	1.33x 10^6 (±3.19x 10^5)
Protein metabolism	1.31x 10^6	1.21x 10^6	1.23x 10^6	1.25x 10^6 (±5.10x 10^4)	1.32x 10^6	9.68x 10^5	1.40x 10^6	1.23x 10^6 (±2.27x 10^5)
Cofactors, vitamins, prosthetic groups, pigments	1.24x 10^6	1.19x 10^6	1.20x 10^6	1.21x 10^6 (±2.33x 10^4)	1.25x 10^6	9.46x 10^5	1.39x 10^6	1.20x 10^6 (±2.27x 10^5)
RNA metabolism	8.85x 10^5	9.30x 10^5	9.76x 10^5	9.30x 10^5 (±4.58x 10^4)	9.84x 10^5	6.15x 10^5	1.14x 10^6	9.13x 10^5 (±2.70x 10^5)
Fatty acids, lipids and isoprenoids	8.01x 10^5	8.19x 10^5	7.60x 10^5	7.93x 10^5 (±3.01x 10^4)	8.26x 10^5	6.38x 10^5	8.54x 10^5	7.73x 10^5 (±1.17x 10^5)
DNA metabolism*	7.20x 10^5	7.23x 10^5	7.39x 10^5	7.27x 10^5 (±1x 10^4)	7.60x 10^5	5.06x 10^5	8.48x 10^5	7.05x 10^5 (±1.78x 10^5)
Cell wall and capsule	6.69x 10^5	7.23x 10^5	7.61x 10^5	7.18x 10^5 (±4.62x 10^4)	7.38x 10^5	4.86x 10^5	8.76x 10^5	7.00x 10^5 (±1.98x 10^5)
Respiration	4.84x 10^5	4.42x 10^5	4.34x 10^5	4.53x 10^5 (±2.72x 10^4)	4.70x 10^5	3.81x 10^5	4.94x 10^5	4.48x 10^5 (±5.93x 10^4)
Nucleosides and nucleotides*	4.54x 10^5	4.37x 10^5	4.46x 10^5	4.45x 10^5 (±8.73x 10^3)	4.63x 10^5	3.45x 10^5	5.04x 10^5	4.37x 10^5 (8.25x 10^4)
Virulence, disease and defense	4.36x 10^5	5.13x 10^5	5.85x 10^5	5.11x 10^5 (±7.48x 10^4)	5.24x 10^5	3.12x 10^5	7.46x 10^5	5.27x 10^5 (±2.17x 10^5)
Stress response*	4.06x 10^5	4.20x 10^5	4.23x 10^5	4.16x 10^5 (±9.04x 10^3)	4.44x 10^5	2.93x 10^5	4.99x 10^5	4.12x 10^5 (±1.07x 10^5)

* The distribution is significantly different between the two agricultural systems (Student's *t*-test, $P < 0.05$). *SD* Standard Deviation. *IRI* Irrigated; *NIR* Non-irrigated.

Table 3. Positive correlations between relative abundances of antibiotic resistant genes and antibiotics concentrations. In each cell, the value represents Spearman's coefficient (*r*).

	$^{bla}_{\text{CARB-3}}$	*catIII*	*dfrA1*	*floR*	$^{bla}_{\text{OXA-347}}$	$^{bla}_{\text{OXA-5}}$	^{bla}rm3	*sul3*	*tetC*	*tetX*
Sulfamethoxazole	0.979**	0.979**	-	0.864*	0.979**	0.979**	0.979**	0.979**	0.979**	0.946**
Ciprofloxacin	0.926**	0.926**	-	0.899*	0.926**	0.926**	0.926**	0.926**	0.926**	0.881*
Enrofloxacin	0.970**	0.970**	-	-	0.970**	0.970**	0.970**	0.970**	0.970**	0.936**
Trimethoprim	-	-	0.869*	-	-	-	-	-	-	-

*Asterisk denoted significant correlation at $p < 0.05$ level (2- tailed).
**Double asterisk denoted significant correlation at $p < 0.01$ level (2- tailed).