

RESEARCH ARTICLE

High abundances of class 1 integrase and sulfonamide resistance genes, and characterisation of class 1 integron gene cassettes in four urban wetlands in Nigeria

Olawale Olufemi Adelowo^{1,2*}, Therese Helbig^{1,3}, Camila Knecht^{1,4}, Franziska Reincke¹, Ines Mäusezahl¹, Jochen A. Müller¹

1 Department of Environmental Biotechnology, Helmholtz Centre for Environmental Research GmbH - UFZ, Leipzig, Germany, **2** Environmental Microbiology and Biotechnology Laboratory, Department of Microbiology, University of Ibadan, Ibadan, Oyo State, Nigeria, **3** Institute of Biology/Microbiology Martin-Luther-University Halle-Wittenberg, Halle, Germany, **4** Institute of Instrumental & Environmental Technology, Otto-von-Guericke-University Magdeburg, Magdeburg, Germany

* onomewaleadelowo@yahoo.co.uk



OPEN ACCESS

Citation: Adelowo OO, Helbig T, Knecht C, Reincke F, Mäusezahl I, Müller JA (2018) High abundances of class 1 integrase and sulfonamide resistance genes, and characterisation of class 1 integron gene cassettes in four urban wetlands in Nigeria. PLoS ONE 13(11): e0208269. <https://doi.org/10.1371/journal.pone.0208269>

Editor: Andrew C Singer, Natural Environment Research Council, UNITED KINGDOM

Received: August 5, 2018

Accepted: November 14, 2018

Published: November 29, 2018

Copyright: © 2018 Adelowo et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: The sequence of the 16S DNA and Class 1 integron gene cassettes are available in the GenBank under accession numbers MG859672 - MG859730 and MK093863 - MK093896. All qPCR data are deposited on Figshare at <https://doi.org/10.6084/m9.figshare.7334066.v1>.

Funding: This work was supported by a Georg Forster (HERMES) Postdoctoral Fellowship of the Alexander von Humboldt-Foundation (<https://www.ahf-hamburg.org/>).

Abstract

There is little information about environmental contamination with antibiotic resistance genes (ARG) in Sub-Saharan Africa, home to about 1 billion people. In this study we measured the abundance of three genes (*sul1*, *sul2*, and *int11*) used as indicators of environmental contamination with ARGs in the sediments of four urban wetlands in southwestern Nigeria by qPCR. In addition, we characterised the variable regions of class 1 integrons in sulfamethoxazole/trimethoprim (SMX/TRI)-resistant bacteria isolated from the wetlands by PCR and DNA sequencing. The indicator ARGs were present in all wetlands with mean absolute copy numbers/gram of sediment ranging between 4.7×10^6 and 1.2×10^8 for *sul1*, 1.1×10^7 and 1×10^8 for *sul2*, and 5.3×10^5 and 1.9×10^7 for *int11*. The relative abundances (ARG/16S rRNA copy number) ranged from about 10^{-3} to 10^{-1} . These levels of ARG contamination were similar to those previously reported for polluted environments in other parts of the world. The integrase genes *int11* and *int2* were detected in 72% and 11.4% SMX/TRI-resistant isolates, respectively. Five different cassette array types (*dfrA7*; *aadA2*; *aadA1|dfrA1*; *acc(6')Ib-cr|arr3|dfrA27*; *arr3|acc(6')Ib-cr|dfrA27*) were detected among 34 (59.6%) *int11*-positive isolates. No gene cassettes were found in the nine *int2*-positive isolates. These results show that African urban ecosystems impacted by anthropogenic activities are reservoirs of bacteria harbouring transferable ARG.

Introduction

There are increasing concerns about the presence of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) in the environment [1,2] where they pose special challenges as environmental contaminants. ARB and ARGs can persist and multiply, and ARGs can

humboldt-foundation.de/) to OOA. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

spread by horizontal gene transfer (HGT) mediated by mobile genetic elements (MGE) within and beyond the original point of occurrence [2], making possible the exchange of resistance traits between environmental bacteria and human pathogens [3]. ARGs and MGE can be enriched in natural ecosystems impacted by human activities such as wastewater discharge [4], solid waste disposal [5,6], metal contamination [7,8], agriculture [9,10,11], aquaculture [12], and drug manufacture [13]. However, very little is known about environmental contamination with ARB and ARGs in developing countries, especially in sub-Saharan Africa. These are critical knowledge gaps since various risk factors favoring the development and spread of antibiotic resistance exist in these environments [14,15].

Aquatic ecosystems are considered important matrices for the release, mixing, persistence and spread of ARB and ARGs associated with horizontally transferable genetic elements [16,17]. In Nigeria, Africa's most populous nation, wetlands (both coastal and inland) are a key aquatic ecosystem covering about 2.6% of the country's total land surface [18] with the most extensive being the coastal wetlands found in the southern region including the Lagos and Lekki lagoons and the wetlands of the Niger Delta and Cross Rivers [19]. In addition to the coastal wetlands, several riverine wetlands which are extensively used for livestock grazing, farming and fishing activities are scattered across the country [20]. Presently, existing regulations give little attention to the protection and management of Nigerian wetlands [20], hence, they are constantly exposed to human excreta, raw sewage, untreated wastewater and other pollutants from diverse sources. These forms of anthropogenic impact make the urban wetland ecosystems of Nigeria potential reservoirs of ARB carrying ARGs that might spread to other bacteria through HGT mediated by MGE. However, few studies have investigated pollution of natural wetlands with ARGs and none of those studies emanated from Nigeria.

Integrans are important MGE involved in the capture, mobilization and spread of antibiotic resistance genes in bacterial species [21,22]. The integron platform consists of an integrase gene that can recombine discrete units of circularised DNA known as gene cassettes, primary recombination site *attI*, and a PC promoter that directs transcription of the captured genes [23,24]. Using the sequences of integrase proteins, several classes of integrans have been recognized out of which only a few are important for spreading multidrug resistance in bacteria [25]. Integrans also serve as platform of bacterial evolution [23] and vehicles of gene exchange between the environmental resistome and commensal and pathogenic bacterial species [26] through HGT. Class 1 integrase and sulfonamide resistance genes on has recently been proposed as indicator of pollution by ARB, ARGs and other anthropogenic pollutants [2,27]. In view of this and their association with HGT, an important process for the spread of resistance in environmental reservoirs, it is important to examine genes associated with integrans when investigating antibiotic resistance in the environment.

A few previous studies have reported the detection of clinically relevant ARG in cultivable bacteria isolated from environmental sources in Nigeria [28,29,30,31], but none that we know of used culture-independent quantification to assess the contamination of the Nigerian wetland ecosystems with ARGs. The purpose of this study was to quantify the copy numbers of *sul1*, *sul2*, and class 1 integrase gene (*intI1*) as markers for ARG contamination in the sediments of four polluted wetlands located in Lagos and Ibadan, two of Africa's most populous cities. Additionally, because integrans are commonly reported in members of the *Enterobacteriaceae* [32] and are found in human impacted ecosystems [25], we investigated the occurrence of class 1 and 2 integrans and their gene cassette contents in 79 sulfamethoxazole/trimethoprim (SMX/TRI)-resistant bacteria of the family *Enterobacteriaceae* isolated from the wetlands.

Materials and methods

Sample sites and sample collection

Sediment samples (n = 16) were collected monthly from four wetlands located in Lagos and Ibadan, southwestern Nigeria between October 2014 and January 2015 (Fig 1). The sampling period represented the two major Nigerian seasons “rainy” (sampling in October and November) and “dry” (sampling in December and January). The two wetlands sampled in Ibadan are Awba (AW) (07.4468°N, 03.8763°E) and Apete (AP) wetlands (07.4577°N, 03.8828°E) while in Lagos the two wetlands sampled are Abule-Agege (AA) (06.5145°N, 03.4002°E) and Ogbe Creek (OC) (06.5135°N, 03.3937°E). AW, AP and AA receive untreated wastewater and raw sewage from student hostel facilities of two universities. In addition, the wetlands receive waste streams from fish farms (AW and AA), a Zoological Garden (AW), and seepages from upland solid waste dumpsites (AP and OC). Triplicate sediment samples collected from the upper 1 cm portion of each wetland were pooled to form a composite and stored at -80 °C until processed for analysis.

DNA extractions

Total sediment community DNA was extracted using FastDNA Spin Kit for soil (MP Biomedicals, Ohio, USA) according to the manufacturer’s instructions. DNA concentrations were estimated with a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE). Genomic DNA extraction from isolated SMX/TRI-resistant bacteria (see below) was performed by the microwave boiling method [33]. Extracted DNA was stored at -20 °C until used.

Quantitative PCR analysis

The abundances of *sul1*, *sul2*, and *intI1* in the total sediment community DNA of the four wetlands were determined by SYBR-green based real-time PCR with 4 technical replicates per

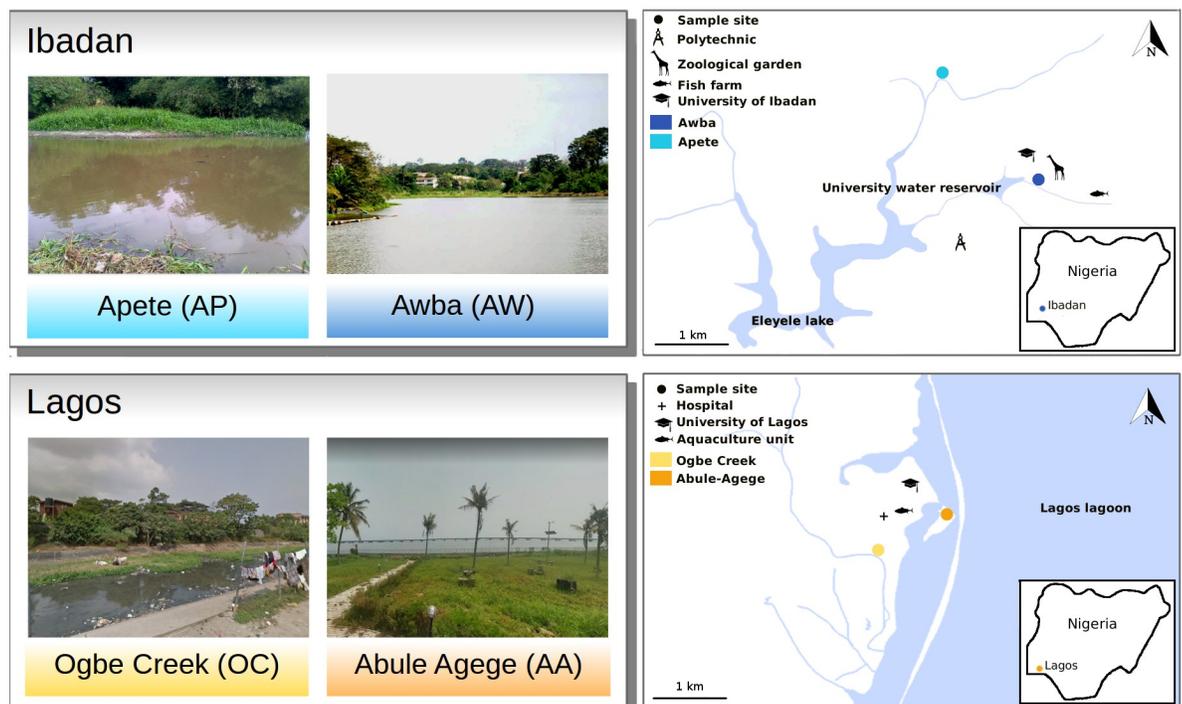


Fig 1. The study sites showing Awba wetland (AW), Apete wetland (AP), Abule-Agege wetland (AA) and Ogbe Creek (OC).

<https://doi.org/10.1371/journal.pone.0208269.g001>

sample. The assay was run on a StepOne Plus Cyclor (Applied Biosystems) in a 20 μ l reaction mixture. The PCR conditions were 95°C for 2 min, followed by 40 cycles of 95°C for 20 s, 20 s at the respective melting temperatures and 72°C for 20 s. Primers for *sul* genes were those reported by Wang et al. [34] and *intI1* primers those by Mazel et al. [35]. Dilutions of template DNA were used for each sample to compensate for the effect of PCR inhibitors in the samples. The copy number of the 16S rRNA gene in the samples was determined by using Primers 519F and 909R [36] as a measure of total bacterial abundance. Standards were PCR-amplified fragments of the 16S rRNA gene from *Escherichia coli* as well as the *sul* genes and *intI1* from environmental isolates obtained in this study. DNA concentrations of the standards (*c*) were measured by Nanodrop spectrophotometry and the copy number (CN) per gram of wetland sediment was calculated using the relation:

$$\text{CN} = (c \times 6.022 \times 10^{23}) / 660 \times N$$

where *c* is the measured DNA concentration ($\mu\text{g}/\mu\text{l}$) and *N* is the DNA fragment length in bp.

Efficiency values were 84.5% for *sul1*, 94.1% for *sul2* and 91.3% for *intI1*.

All qPCR data can be found on figshare.com under the title of this report (<https://doi.org/10.6084/m9.figshare.7334066.v1>).

Statistical analysis

Measured copy numbers of the genes were log-transformed to normalise the distribution before the statistical significance of the differences in the copy numbers of the genes at each sampling site was determined by analysis of variance (ANOVA) at 5% level of significance ($P < 0.05$). Pearson's Correlation analysis was used to test the association between measured copy numbers of the 16S rRNA gene, *sul1*, *sul2*, and *intI1*.

Isolation of SMX/TRI-resistant bacteria

Sediment samples (1 g) were suspended in saline and 100 μ l of appropriate dilutions spread on Eosine Methylene blue (EMB) agar plates supplemented with SMX/TRI (56 and 8 $\mu\text{g}/\text{ml}$, respectively). The SMX/TRI combination (co-trimoxazole, Septrin) is commonly used in Nigeria. Bacterial colonies growing on the plates following incubation for 48 h at 30°C were randomly selected, re-streaked on Mueller-Hinton Agar (MHA) plates with SMX/TRI until apparent purity, and the isolates stored in Müller-Hinton broth with 15% (v/v) glycerol at -80°C until further molecular analysis.

The 16S rRNA gene of SMX/TRI-resistant isolates was PCR amplified with universal primers 27F and 1439R [37], amplicons were sequenced (GATC Biotech, Cologne, Germany), and the strains identified via BLASTn queries in the GenBank. The 16S rDNA sequences were deposited in the GenBank database under accession numbers MG859672—MG859730.

Detection of SMX/TRI resistance genes and integron analysis in SMX/TRI-resistant bacteria

The presence of sulfonamide resistance genes *sul1*, *sul2* and *sul3* and the most commonly found trimethoprim resistance genes *dfrA1*, *dfrA5*, *dfrA7*, *dfrA12* and *dfrA17* was investigated in the SMX/TRI-resistant bacteria by PCR as described [34,38] using either QIAGEN *HotStar-Taq* Mastermix (QIAGEN GmbH, Hilden, Germany) (*sul1* and *sul2*), KAPA HiFi *HotStart* PCR Kit (KAPA Biosystems, Boston, USA) (*sul3*), *Dream Taq Green* PCR Mastermix (2x) (ThermoFisher Scientific, Waltham, USA) (*dfrA1*), or Kappa 2G Robust PCR kit (KAPA Biosystems, Boston Massachusetts, USA) (*dfrA5*, *dfrA7*, *dfrA12* and *dfrA17*). The presence of

integrase genes *intI1*, *intI2* and *intI3* was queried for by PCR and the variable region of class 1 integron characterised by PCR and Sanger sequencing as described elsewhere [39]. The diversity of promoters associated with detected class 1 integrons were characterised by Sanger sequencing as already described [40,41]. The sequenced sections of the class I integrons were deposited at GenBank under accession numbers MK093863 –MK093896.

Literature survey for qPCR data on *sul* and *intI1* genes in aquatic sediments

In order to compare abundances of *sul* and *intI1* genes in the Nigerian wetlands with values reported in the literature we searched the ScienceDirect and Scopus databases using the keywords “antibiotic resistance genes” and “wetlands”. We chose studies that reported the genes’ relative abundances for sediments in natural aquatic environments. Eleven studies from China, Pakistan, Poland, USA, Sweden and Switzerland but none from Africa met our criteria [42–53]. In cases where more than one season was examined in a study, we extracted the values for summer in order to be closer to the climatic conditions in Nigeria. When data from different sample sites was provided, we took the highest values.

Ethical statement

There are no specific permits required for sample collection in the field studies. The wetlands not protected and not privately owned, hence there are no regulations that restrict collection of sediment samples for research purposes from the four wetlands. The field study does not involve any endangered or protected species, only sediment samples were taken from the sites.

Results and discussion

Abundances of *intI1* and *sul* genes show medium to heavy pollution with antibiotic resistance genes in four urban Nigerian wetlands

The *intI1*, *sul1*, and *sul2* genes were detected in all the samples analysed. The mean absolute copy numbers/gram of wetland sediment of the genes and the 16S rRNA gene are shown in Fig 2. The mean copy numbers of 16S rRNA gene ranged from 1.6×10^8 (December 2014, AA) to 5.7×10^9 (December 2014, AP) copies per g of sediment. The *intI1* gene was detected in the wetlands at values ranging from 5.3×10^5 (January 2015, OC) to 1.9×10^7 (January 2015, AW) copies/gram sediment. These abundances were in the range reported for aquatic sediments in other regions of the world [25]. It should be mentioned that the respective studies cited in [25] used various primers for the quantification of the *intI1* gene, which hampers the comparative assessment of the levels of *intI1* contamination in the respective contaminated sites.

The copy number/gram sediment of *sul1* in the four wetlands ranged from 4.7×10^6 (November 2014, OC) to 1.2×10^8 (November 2014, AW). The absolute abundances of *sul2* was higher than that of *sul1* at 1.1×10^7 (November 2014, OC) to 1×10^8 (December 2014, AP) copy number/gram sediment. There was no universal trend regarding the ratio of *sul1* to *sul2* in sediments. Greater abundance of *sul2* over *sul1* was found by Luo et al. [43] similar to what we found in our study sites, similar abundance of both genes were measured by Jiang et al. [54] and Lu et al. [55], while a higher abundance of *sul1* was reported by Gao et al. [56] and Koczura et al. [48].

To the best of our knowledge, there are no similar studies of ARG contamination of the Nigerian environment available for the comparative analysis of our results. Overall, the absolute levels of *sul* contamination in the wetlands of this study are in the upper range of values reported for wetlands and river sediments in other parts of the world [42,46,48,51,55–59]. The abundance of the *sul* genes as well as those of *intI1* were significantly different ($p < 0.01$) across

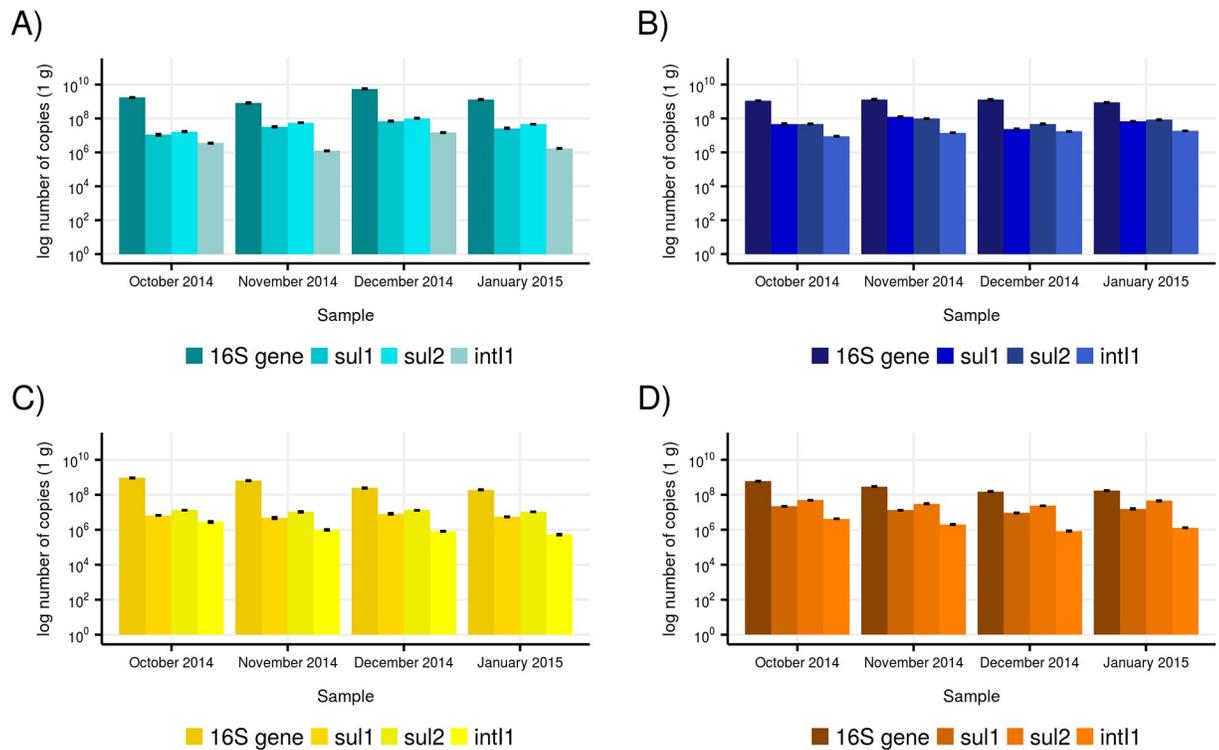


Fig 2. Mean absolute abundances of 16S rRNA, class 1 integrase (*intI1*) and sulfonamide resistance (*sul1* and *sul2*) genes in Awba (A), Apete (B), Abule-Agege (C) and Ogb Creek (D) for October 2014, November 2014, December 2014 and January 2015.

<https://doi.org/10.1371/journal.pone.0208269.g002>

the four wetlands. In particular the AW and AA sites had *sul* abundances mirroring those of heavily contaminated sites elsewhere [42,60]. These differences in ARG abundance in the four wetlands correlates with the different anthropogenic activities associated with the wetlands. AW receives direct input of untreated domestic wastewater, discharges from a fish farm and a Zoological Garden. The only known point source of anthropogenic input identified for OC, the wetland with the lowest ARG abundance, is an upland solid waste dumpsite which appears to be relatively new at the time of sample collection. There was no significant difference in the abundance of the ARGs measured for the samples collected in the rainy season (October and November) and those collected in the dry season (December and January).

The absolute abundance of the *sul* genes showed positive correlation with the copy number of *intI1* ($P < 0.01$ or $P < 0.05$). The correlation coefficient of *sul1* (0.794) is slightly higher than that of *sul2* (0.729). This is expected as *sul1* is typically located in the 3' conserved segments of class 1 integrons [32]. However, the mean absolute copy number of *intI1* in all the samples was generally lower than the mean absolute copy number of *sul1*, which is similar to what has been reported in previous studies [47,48].

It should be mentioned that the *intI1*-directed primers [35] used in our study did not exclusively target clinical *intI1* and thus may have resulted in an overestimation of anthropogenic pollution with the clinical class I integron. Yet that overestimation might not be substantial. Antelo et al. [61] used the same primers as in our study in a PCR-based survey on integron diversity with samples from King George Island, Antarctica, and found a prevalence rate of 42% of clinical class I integrons. On the other hand, the *sul*-targeting primers used here were designed based on the sequence of a single allele of the respective *sul* gene. This could have resulted in an underestimation of the *sul* gene abundances in our study sites as a recent study

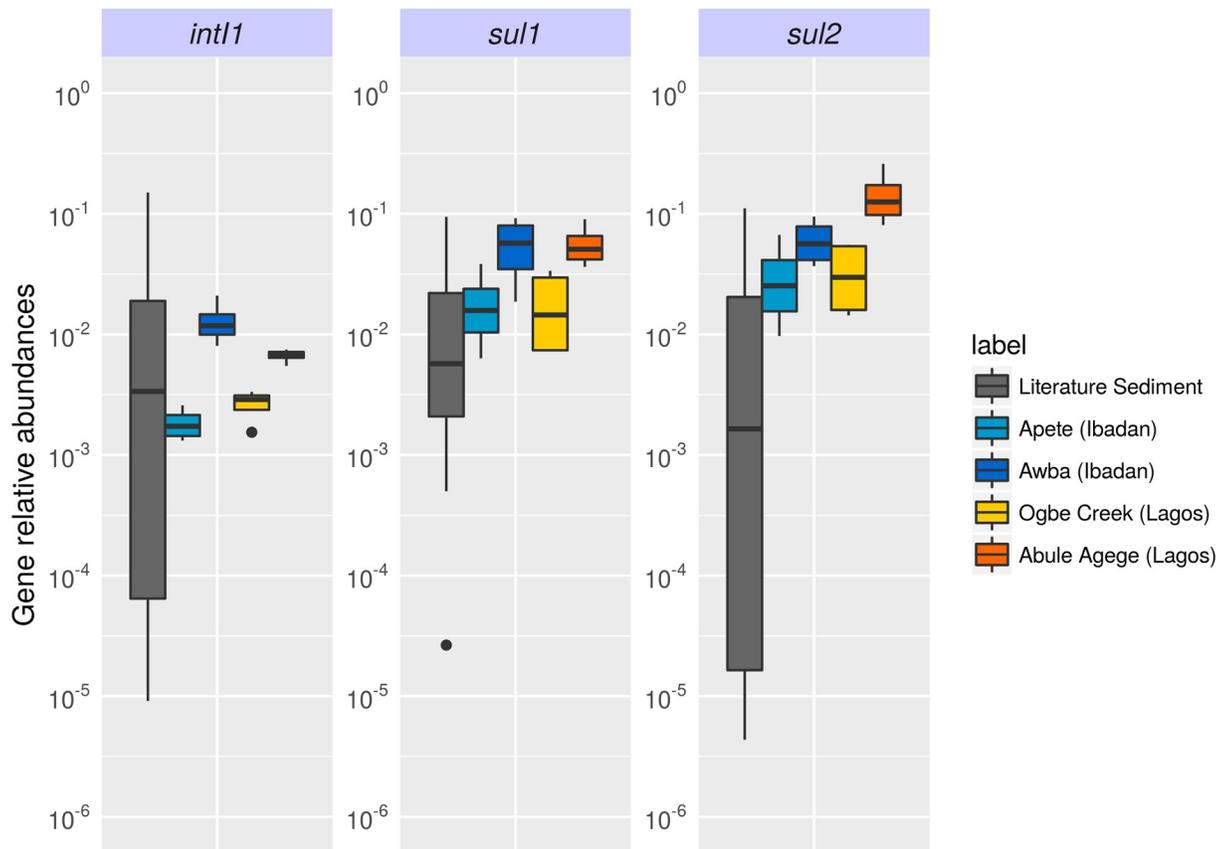


Fig 3. Relative abundance of indicator ARGs from our study sites (coloured bars) compared to similar sites in China [43,46,47,49–51], Pakistan [52], Poland [48], Sweden [45], Switzerland [44] and USA [42] (grey bars).

<https://doi.org/10.1371/journal.pone.0208269.g003>

has shown that a wide variety of both clinical and non-clinical *sul* alleles are present in polluted natural ecosystems [62].

Essentially the same picture of the level of contamination of the wetlands investigated here is apparent from the relative abundance of *intI1*, *sul1*, and *sul2* over 16S rRNA gene copy numbers (Fig 3). The relative abundance of *intI1* was similar to the values reported for aquatic sediments elsewhere [25], while the relative abundance of *sul* genes indicate heavy contamination, especially of the AW and AA sites. These relative abundances are similar to values found at a pig farm where SMX was used [63], mariculture sites in China [64], and water bodies around a drug formulation facility in Pakistan [52].

SMX/TRI genes and integrons are prevalent in bacterial isolates from the wetlands

To understand the genetic context of the SMX/TRI resistance genes and identify other transferable genes associated with detected ARG markers, SMX/TRI-resistant *Enterobacteriaceae* were isolated from the four wetlands and screened by PCR for sulfonamide resistance genes *sul1*, *sul2*, *sul3*, the trimethoprim resistance genes *dfrA1*, *dfrA5*, *dfrA7*, *dfrA12*, *dfrA17*, and integrons class 1, 2 and 3. Despite its obvious limitations, data from cultivable bacteria are still important in the study of antibiotic resistance. They provide insight into the phenotypic and genotypic characteristics of bacteria isolates and are therefore integral to national and

international antibiotic resistance surveillance and tracking efforts. Further, they also provide full insight into the resistome of natural ecosystems and the link between antibiotic resistances detected in the environment and clinical settings when combined with data generated from high throughput culture-independent methods [65].

Out of a total of 79 strains (AW = 23, AP = 19, AA = 17, OC = 20), 50 strains (63%) carried at least one of the SMX/TRI resistance genes tested for. 16S rRNA gene sequencing identified these 50 isolates as *Citrobacter* sp. (27), *Enterobacter* sp. (16), *Escherichia* sp. (3), *Pseudomonas* sp. (3), and *Achromobacter* sp. (1). Some of the isolates shared identical 16S rRNA sequences, however, the pattern of genes detected in the isolates were either different or where the isolates shared identical pattern of genes, they were isolated at different time periods. *sul1* was the most frequently detected gene, found in 40/79 (51%) strains (AW = 18, AP = 16 and AA = 6), followed by *sul2* found in 32/79 (41%) strains (AW = 10, AP = 10, AA = 10, OC = 2), and *sul3* found in only 1 strain from AW also carrying *sul1* and *sul2*. *dfrA1*, *dfrA12* and *dfrA7* were detected in 1, 2 and 10 isolates, respectively, *dfrA5* and *dfrA17* were not detected in any of the isolates. The gene *dfrA7* occurred with *sul1* in 4 isolates and with *sul1* and *sul2* in five isolates.

intI1 and *intI2* were detected in 57 (72%) and 9 (11.4%) SMX/TRI-resistant isolates respectively, including eight bacteria (*intI1* = 7, *intI2* = 1) where none of the SMX/TRI genes were found. *intI3* was not detected in any of the bacterial strains investigated. This is consistent with previous reports that class 1 and 2 integrons are the most frequently detected integrons in Gram negative bacteria [66] with class 2 integrons detected less frequently than class 1 integrons.

Characterisation of integrons gene cassettes

Because of the important role of integrons in the spread of resistance in natural ecosystems, we carried out PCR analysis together with DNA sequencing of the variable regions of detected *intI1* and *intI2* among isolated SMX/TRI-resistant bacteria to identify additional ARGs that may potentially spread by HGT. In all *intI2*-positive isolates, the primer pair attI2 and orfX failed to yield any useful amplification product in all cases indicating that they are likely to be novel integrons or integrons without gene cassettes. In contrast, variable regions containing gene cassettes were amplified in 34 of 57 (59.6%) *intI1*-positive isolates including three isolates where none of the tested SMX/TRI genes was detected. The remaining *intI1*-positive isolates either lack gene cassettes or might be derivatives of *Tn5090*-like class 1 integrons that lack the 3' conserved end [67,68]. Seven different types of genes arranged in five different arrays were found within the variable regions of the class 1 integrons in the 34 isolates (Table 1). The genes encode resistance to trimethoprim (*dfrA1*, *dfrA7*, *dfrA27*), aminoglycosides (*aadA1*, *aadA2*), rifampicin (*arr-3*) and fluoroquinolones (*acc(6')-Ib-cr*). The *dfrA* genes were the most frequently detected gene cassettes occurring in (82%) of the gene cassette-bearing isolates. This is similar to previous reports by Lin et al. [69] and corroborated previous reports that class 1 integrons are important for the dissemination of SMX/TRI resistance genes [70].

Type 1 (769 bp) gene cassettes shared 99% nucleotide sequence identity with class 1 integron from *Acinetobacter baumannii* 607460 (EU340417.1) containing *dfrA7* and was found in 10 isolates (AW = 1, AP = 8 and AA = 1). Type 2 (2.2 kb) cassette array shared 99% sequence identity with class 1 integron of *Citrobacter freundii* strain S12 (KR259319.1) containing the plasmid mediated quinolone resistance gene *acc(6')Ib-cr*, the rifampicin resistance gene *arr3* and the trimethoprim resistance gene *dfrA27* and was found in 16 isolates (AW = 4, AP = 9 and AA = 3). The order of arrangement of the Type 2 gene cassettes varied. In the isolates from Ibadan (AW and AP) it was *acc(6')Ib-cr|arr3|dfrA27* (Type 2a) while in the isolates from Lagos (AA) it was *arr3|acc(6')Ib-cr|dfrA27* (Type 2b). Type 3 (1.5kb) array, found in two

Table 1. Resistance genes, class 1 integron gene cassettes and promoter types in SMX/TRI-resistant bacteria from the four wetlands in Ibadan and Lagos, Nigeria.

Isolates	Phylogenetic placement	Date of isolation	Resistance genes/Integrans	Integron gene cassettes	Promoter Types
Awba Wetland					
PAW1-6	<i>Citrobacter</i> sp.	October 2014	<i>sul1, sul2, sul3, intI1, intI2</i>	<i>aadA1 dfrA1</i>	PcH1-P2
PAW1-4	<i>Citrobacter</i> sp.	October 2014	<i>sul1, intI1, intI2</i>	<i>acc(6')lb-cr arr3 dfrA27</i>	PcW-P2
PAW2-4	<i>Enterobacter</i> sp.	November 2014	<i>sul1, intI1</i>	<i>aadA2</i>	PcW-P2
EAW2-1	<i>Citrobacter</i> sp.	November 2014	<i>sul1, sul2, intI1</i>	<i>aadA2</i>	PcW-P2
EAW2-3	<i>Enterobacter</i> sp.	November 2014	<i>sul1, intI1</i>	<i>aadA2</i>	PcW-P2
PAW2-6	<i>Enterobacter</i> sp.	November 2014	<i>sul1, sul2, intI1</i>	<i>aadA2</i>	PcW-P2
PAW3-1	<i>Enterobacter</i> sp.	December 2014	<i>sul1, sul2, intI1</i>	<i>acc(6')lb-cr arr3 dfrA27</i>	PcW-P2
PAW3-3	<i>Citrobacter</i> sp.	December 2014	<i>sul1, sul2, intI1, intI2</i>	<i>acc(6')lb-cr arr3 dfrA27</i>	PcW-P2
PAW4-5	<i>Enterobacter</i> sp.	January 2015	<i>sul1, dfrA7, intI1</i>	<i>dfrA7</i>	PcW-P2
EAW4-6	<i>Citrobacter</i> sp.	January 2015	<i>sul1, dfrA1, intI1</i>	<i>aadA1 dfrA1</i>	PcH1-P2
PAW4-4	<i>Citrobacter</i> sp.	January 2015	<i>sul1, intI1, intI2</i>	<i>acc(6')lb-cr arr3 dfrA27</i>	PcW-P2
Apete Wetland					
EAP1-2	<i>Enterobacter</i> sp.	October 2014	<i>sul1, sul2, intI1</i>	<i>acc(6')lb-cr arr3 dfrA27</i>	PcW-P2
EAP1-3	<i>Citrobacter</i> sp.	October 2014	<i>intI1</i>	<i>acc(6')lb-cr arr3 dfrA27</i>	PcW-P2
EAP1-5	<i>Pseudomonas</i> sp.	October 2014	<i>sul1, sul2, intI1</i>	<i>acc(6')lb-cr arr3 dfrA27</i>	PcW-P2
PAP2-5	<i>Citrobacter</i> sp.	November 2014	<i>intI1</i>	<i>acc(6')lb-cr arr3 dfrA27</i>	PcW-P2
PAP2-6	<i>Citrobacter</i> sp.	November 2014	<i>sul1, sul2, dfrA7, intI1</i>	<i>dfrA7</i>	PcW-P2
EAP2-1	<i>Citrobacter</i> sp.	November 2014	<i>sul1, dfrA7, intI1</i>	<i>dfrA7</i>	PcH1-P2
EAP2-3	<i>Citrobacter</i> sp.	November 2014	<i>sul1, sul2, dfrA7, intI1</i>	<i>dfrA7</i>	PcW-P2
PAP2-2	<i>Citrobacter</i> sp.	November 2014	<i>sul1, sul2, dfrA7, intI1</i>	<i>dfrA7</i>	PcW-P2
PAP2-3	<i>Citrobacter</i> sp.	November 2014	<i>sul1, sul2, dfrA7, intI1</i>	<i>dfrA7</i>	PcW-P2
PAP4-16	<i>Citrobacter</i> sp.	January 2015	<i>sul1, dfrA7, intI1</i>	<i>dfrA7</i>	PcW-P2
PAP4-3	<i>Citrobacter</i> sp.	January 2015	<i>sul1, sul2, dfrA7, intI1</i>	<i>dfrA7</i>	PcW-P2
EAP4-6	<i>Achromobacter</i> sp.	January 2015	<i>dfrA7, intI1</i>	<i>dfrA7</i>	PcH1-P2
EAP1-1	<i>Citrobacter</i> sp.	January 2015	<i>sul1, sul2, intI1</i>	<i>acc(6')lb-cr arr3 dfrA27</i>	PcW-P2
PAP4-5	<i>Citrobacter</i> sp.	January 2015	<i>sul1, intI1</i>	<i>acc(6')lb-cr arr3 dfrA27</i>	PcW-P2
EAP4-1	<i>Citrobacter</i> sp.	January 2015	<i>sul1, intI1</i>	<i>acc(6')lb-cr arr3 dfrA27</i>	PcH1-P2
PAP4-1	<i>Citrobacter</i> sp.	January 2015	<i>sul1, sul2, intI1</i>	<i>acc(6')lb-cr arr3 dfrA27</i>	PcW-P2
EAP4-5	<i>Pseudomonas</i> sp.	January 2015	<i>sul1, sul2, intI1</i>	<i>acc(6')lb-cr arr3 dfrA27</i>	PcH1-P2
Abule-Agege					
PAA1-2	<i>Escherichia</i> sp.	October 2014	<i>sul1, sul2, intI1</i>	<i>arr3 acc(6')lb-cr dfrA27</i>	PcW-P2
PAA2-7	<i>Citrobacter</i> sp.	November 2014	<i>sul1, intI1</i>	<i>aadA2</i>	PcW-P2
PAA2-8	<i>Citrobacter</i> sp.	November 2014	<i>sul1, sul2, intI1</i>	<i>aadA2</i>	PcW-P2
EAA3-5	<i>Citrobacter</i> sp.	December 2014	<i>drfA12, intI1</i>	<i>arr3 acc(6')lb-cr dfrA27</i>	PcH1-P2
PAA4-1	<i>Citrobacter</i> sp.	January 2015	<i>sul1, sul2, intI1</i>	<i>arr3 acc(6')lb-cr dfrA27</i>	PcW-P2
EAA4-2	<i>Enterobacter</i> sp.	January 2015	<i>intI1</i>	<i>dfrA7</i>	PcH1-P2

ND: Not Detected, PcW: Weak Promoter, PcH1: Hybrid promoter 1, P2: Inactive promoter 2

<https://doi.org/10.1371/journal.pone.0208269.t001>

isolates from AW, shared 95% identity with class 1 integron of *E. coli* strain Ec171 (GU590934.1) containing the streptomycin/streptogamin resistance gene *aadA1* and trimethoprim resistance gene *drfA1*, while Type 4 (730 bp) cassette array shared 99% sequence identity with the streptomycin/streptogamin resistance gene *aadA2* on the class 1 integron of *Proteus mirabilis* strain NF991579 (HQ880254.1). Type 4 was found in six isolates from AW (n = 4) and AA (n = 2). Four cassette array types (1, 2a, 3 and 4) were represented in bacteria from AW, while isolates from AP carried Type 1 and 2a, and those from AA harbored Types 1,

2b and 4 arrays respectively, supporting previous reports that gene cassette populations can vary markedly even within small physical distances [71] with type of selection pressure playing important roles in shaping cassette diversity [68].

All the array types reported in this study have been previously reported in the literature. Array types 1 and 3 in particular have been reported widely in bacteria from human clinical sources, aquaculture, farm animals, rivers and hospital wastewater in Nigeria and Ghana [29,72,73], Central African Republic [74], Egypt [75], Europe [76–83], China [84–86], Brazil [87], Canada [54] and US [88]. The Type 1 cassette (*dfra7*) was found associated with a widely disseminated Tn21-type transposon in Nigeria and Ghana [72]. Cassette array Type 4 has also been reported in bacteria from wastewater in Mozambique [89], rivers in Nigeria [90] and China [86], *E. coli* isolates from Nigeria [72] and an on-farm bio-purification plant [9]. Only recently, type 2a cassettes was reported in six bacteria species (*Klebsiella pneumoniae*, *Acinetobacter nosocomialis*, *C. freundii*, *Pantoea agglomerans*, *Stenotrophomonas maltophilia* and *Staphylococcus xylosus*) isolated from American eel (*Anguilla rostrata*) and pond water in Fujian Province, China [69]. We additionally detected this array type in *Pseudomonas* and *Enterobacter* spp. confirming its potential for wide dissemination among bacterial species.

Diversity of promoters of class 1 integrons

We analysed the promoter types in the *intI1*-positive isolates. Two different Pc promoter types, the weak promoter PcW and the Hybrid Type 1 promoter PcH1 were detected in 26 and 8 isolates, respectively, of 34 *intI1*-positive isolates. In all cases, the detected promoters were associated with an inactive second promoter type P2 where the -35 and -10 hexamers of the respective promoters were separated by 14 bp instead of the 17 bp expected with an active P2 [91]. Jové et al. [40], Vinue et al. [41] and Moura et al. [92] similarly reported PcW as the most frequently detected promoter type in bacteria from clinical and environmental sources. Since promoter strength and integrase excision activity are inversely correlated, thus affecting the propensity for the dissemination of resistance genes [40]; the prevalence of weak PC variants in this study suggests that there is a high propensity for further dissemination of the antibiotic resistance genes carried on the class 1 integrons among bacteria in the wetlands.

In summary, indicator ARGs *sul1*, *sul2*, and *intI1* were detected in all four Nigerian wetlands at high absolute and relative (vs. the 16S rRNA gene) gene frequencies. Class 1 and 2 integrons were found in 72% and 11.4% of 79 culturable bacteria from the wetlands with five gene cassette arrays containing 7 different resistance genes found in 59.6% of detected class 1 integrons. No gene cassette was detected in the class 2 integrons. Our results demonstrate that the Nigerian wetlands are contaminated with ARGs at levels similar to those usually reported for highly contaminated sites in other parts of the world. Although only a limited number of ARGs and wetlands were selected, this study provides the first data on the status of ARG contamination in any Nigeria's aquatic ecosystem and suggested a role for anthropogenic activities in environmental contamination with ARGs. Larger studies are needed for an overall evaluation of the magnitude of ARG contamination in the Nigerian aquatic ecosystem and its associated risks.

Acknowledgments

The authors are grateful to Miss Nancy Rümmelein of the Department of Environmental Biotechnology at the Helmholtz Center for Environmental Research (UFZ) for laboratory assistance and to Mr Bassey Okon Bassey and Mr S. T. Lawal of the Department of Marine Biology, University of Lagos, Nigeria for assistance with sample collection in Lagos.

Author Contributions

Conceptualization: Olawale Olufemi Adelowo, Jochen A. Müller.

Data curation: Camila Knecht, Ines Mäusezahl, Jochen A. Müller.

Formal analysis: Olawale Olufemi Adelowo, Camila Knecht.

Funding acquisition: Olawale Olufemi Adelowo.

Investigation: Olawale Olufemi Adelowo, Therese Helbig, Franziska Reincke, Ines Mäusezahl.

Methodology: Olawale Olufemi Adelowo, Jochen A. Müller.

Supervision: Olawale Olufemi Adelowo, Jochen A. Müller.

Writing – original draft: Olawale Olufemi Adelowo, Camila Knecht, Jochen A. Müller.

Writing – review & editing: Olawale Olufemi Adelowo, Therese Helbig, Camila Knecht, Franziska Reincke, Ines Mäusezahl, Jochen A. Müller.

References

1. Pruden A, Joakim-Larsson DG, Amézquita A, Collignon P, Brandt KK, Graham DW, et al. Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. *Environ Health Perspect*. 2013; 121:878–885. <https://doi.org/10.1289/ehp.1206446> PMID: 23735422
2. Berendonk TU, Manaia CM, Merlin C, Fatta-Kassinos D, Cytryn E, Walsh F, et al. Tackling antibiotic resistance: the environmental framework. *Nat Rev Microbiol*. 2015; 13: 310–317. <https://doi.org/10.1038/nrmicro3439> PMID: 25817583
3. Ribeiro AF, Bodilis J, Alonso L, Buquet S, Feuilloley M, Dupont J-P, et al. Occurrence of multiantibiotic resistant *Pseudomonas* spp. in drinking water produced from karstic hydrosystems. *Sci Total Environ*. 2014; 490:370–378. <https://doi.org/10.1016/j.scitotenv.2014.05.012> PMID: 24875257
4. Lamba M, Graham DW, Ahammad SZ. Hospital wastewater releases of carbapenem-resistance pathogens and genes in urban India. *Environ Sci Technol*. 2017; 51:13906–13912. <https://doi.org/10.1021/acs.est.7b03380> PMID: 28949542
5. Wu D, Huang X-H, Sun J-Z, Graham DW, Xie B. antibiotic resistance genes and associated microbial community conditions in aging landfill systems. *Environ Sci Technol*. 2017; 51:12859–12867. <https://doi.org/10.1021/acs.est.7b03797> PMID: 28990771
6. You X, Wu D, Wei H, Xie B, Lu J. Fluoroquinolones and β -lactam antibiotics and antibiotic resistance genes in autumn leachates of seven major municipal solid waste landfills in China. *Environ Int*. 2018; 113:162–169. <https://doi.org/10.1016/j.envint.2018.02.002> PMID: 29425900
7. Xu Y, Xu J, Luo Y. Effect of the selective pressure of sub-lethal level of heavy metals on the fate and distribution of ARGs in the catchment scale. *Environ Pollut*. 2017; 220:900–908. <https://doi.org/10.1016/j.envpol.2016.10.074> PMID: 27876226
8. Garbisu C, Garaiyurrebaso O, Lanzén A, Álvarez-Rodríguez I, Arana L, Blanco F, et al. mobile genetic elements and antibiotic resistance in mine soil amended with organic wastes. *Sci To Environ*. 2018; 621:725–733.
9. Martini MC, Quiroga MP, Pistorio M, Lagares A., Centron D, Del Papa MF. Novel environmental class 1 integrons and cassette arrays recovered from an on-farm bio-purification plant. *FEMS Microbiol Ecol*. 2018; 93:fix190. Manyi
10. Nõlvak H, Truu M, Kanger K, Tampere M, Espenberg M, Loit E, et al. Inorganic and organic fertilizers impact the abundance and proportion of antibiotic resistance and integron-integrase genes in agricultural grassland soil. *Sci Tot Environ*. 2016; 562:678–689.
11. Peng S, Feng Y, Wang Y, Guo X, Chu H, Lin X. Prevalence of antibiotic resistance genes in soils after continually applied with different manure for 30 years. *J Hazard Mater*. 2017; 340:16–25 <https://doi.org/10.1016/j.jhazmat.2017.06.059> PMID: 28711829
12. Muziasari WI, Pitkänen LK, Sørnum H, Stedtfeld RD, Tiedje JM, Virta M. The resistome of farmed fish feces contributes to the enrichment of antibiotic resistance genes in sediments below Baltic Sea fish farms. *Front Microbiol*. 2017; 7:2137. <https://doi.org/10.3389/fmicb.2016.02137> PMID: 28111573
13. Marathe NP, Janzon A, Kotsakis SD, Flach CF, Razavi M, Berglund F, et al. Functional metagenomics reveals a novel carbapenem-hydrolyzing mobile beta-lactamase from indian river sediments

- contaminated with antibiotic production waste. *Environment International*. 2018; 112:279–286. <https://doi.org/10.1016/j.envint.2017.12.036> PMID: 29316517
14. Devarajan N, Laffite A, Mulaji CK, Otamonga J-P, Mpiana TP, Mubedi JL, et al. Occurrence of antibiotic resistance genes and bacterial markers in a tropical river receiving hospital and urban wastewaters. *PLoS ONE*. 2016; 11(2): e0149211. <https://doi.org/10.1371/journal.pone.0149211> PMID: 26910062
 15. Laffite A, Kilunga PI, Kayembe JM, Devarajan N, Mulaji CK, Giuliani G, et al. Hospital Effluents are one of several sources of metal, antibiotic resistance genes, and bacterial markers disseminated in Sub-Saharan Urban Rivers. *Front Microbiol*. 2016; 7:1128. <https://doi.org/10.3389/fmicb.2016.01128> PMID: 27499749
 16. Taylor NGH, Verner-Jeffreys DW, Baker-Austin C. Aquatic systems: maintaining, mixing and mobilising antimicrobial resistance? *Trends Ecol Evol*. 2011; 26:278–284. <https://doi.org/10.1016/j.tree.2011.03.004> PMID: 21458879
 17. Yang Y, Song W, Lin H, Wang W, Du L, Xing W. Antibiotics and antibiotic resistance genes in global lakes: A review and meta-analysis. *Environ Int*. 2018; 116:60–73. <https://doi.org/10.1016/j.envint.2018.04.011> PMID: 29653401
 18. Uluocha NO, Okeke IC. Implications of wetlands degradation for water resources management: Lessons from Nigeria. *GeoJournal*. 2004; 61:151–154
 19. Hughes RH, Hughes JS. A directory of African wetlands. World Conservation Union, United Nations Environment Programme and World Conservation Monitoring Centre. Gland, Switzerland. 1992. <https://www.iucn.org/content/directory-african-wetlands>
 20. Adekola O, Whanda S, Ogwu F. Assessment of policies and legislations that affect management of wetlands in Nigeria. *Wetlands*. 2012; 32:665–677
 21. Stokes HW, Gillings M. Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into gram-negative pathogens. *FEMS Microbiol Rev*. 2011; 35:790–819 <https://doi.org/10.1111/j.1574-6976.2011.00273.x> PMID: 21517914
 22. Stalder T, Barraud O, Jové T, Casellas M, Gaschet M, Dagot C, et al. Quantitative and qualitative impact of hospital effluent on dissemination of the integron pool. *ISME J*. 2014; 8:768–777. <https://doi.org/10.1038/ismej.2013.189> PMID: 24152716
 23. Mazel D. Integrons: agents of bacterial evolution. *Nat Rev Microbiol*. 2006; 4:608–620 <https://doi.org/10.1038/nrmicro1462> PMID: 16845431
 24. Gillings MR. Integrons: past, present and future. *Microbiol Mol Biol Rev*. 2014; 78:257–277. <https://doi.org/10.1128/MMBR.00056-13> PMID: 24847022
 25. Gillings MR. DNA as a pollutant: the clinical class 1 integron. *Current Pollution Reports*. 2018; 4:49–55.
 26. Stalder T, Barraud O, Casellas M, Dagot C, Ploy MC. Integron involvement in environmental spread of antibiotic resistance. *Front Microbiol*. 2012; 3:119. <https://doi.org/10.3389/fmicb.2012.00119> PMID: 22509175
 27. Gillings MR, Gaze WH, Pruden A, Smalla K, Tiedje JM, Zhu Y-G. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *ISME J*. 2015; 9:1269–1279. <https://doi.org/10.1038/ismej.2014.226> PMID: 25500508
 28. Adelowo OO, Fagade OE. The tetracycline resistance gene *tet39* is present in both Gram negative and Gram positive bacteria from a polluted river, southwestern Nigeria. *Lett Appl Microbiol*. 2009; 48:167–172. <https://doi.org/10.1111/j.1472-765X.2008.02523.x> PMID: 19196439
 29. Adelowo OO, Caucci S, Banjo OA, Nnanna OC, Awotipe EO, Peters FB, et al. ESBL-producing bacteria isolated from hospital wastewater, rivers and aquaculture sources in Nigeria. *Environ Sci Poll Res*. 2018; 25:2744–2755.
 30. Obasi A, Nwachukwu SC, Ugoji E, Kholer C, Ghöler A, Balau V, et al. Extended-spectrum β -lactamase-producing *Klebsiella pneumoniae* from pharmaceutical wastewaters in southwestern Nigeria. *Microb Drug Resist*. 2017; <https://doi.org/10.1089/mdr.2016.0269>
 31. Adelowo OO, Vollmers J, Mäusezahl I, Kaster A-K, Müller JA. Detection of the carbapenemase gene blaVIM-5 in members of the *Pseudomonas putida* group isolated from polluted Nigerian wetlands. *Sci Rep*. 2018; 8:15116. <https://doi.org/10.1038/s41598-018-33535-3> PMID: 30310126
 32. Fluit AC, Schmitz FJ. Class 1 integrons gene cassettes mobility and epidemiology. *Eur J Clin Microbiol Infect Dis*. 1999; 18:761–770. PMID: 10614949
 33. Orsini M, Romano-Spica V. A microwave-based method for nucleic acid isolation from environmental samples. *Appl Microbiol*. 2001; 33:17–20.
 34. Wang N, Yang X, Jiao S, Zhang J, Ye B, Gao S. Sulfonamide-resistant bacteria and their resistance genes in soils fertilized with manures from Jiangsu Province, Southeastern China, *PLoS ONE*. 2014; 9(11): e112626. <https://doi.org/10.1371/journal.pone.0112626> PMID: 25405870

35. Mazel D, Dychinco B, Webb VA, Davies J. Antibiotic resistance in the ECOR collection: integrons and identification of a novel *aad* gene. *Antimicrob Agents Chemother.* 2000; 44:1568–1574. PMID: [10817710](https://pubmed.ncbi.nlm.nih.gov/10817710/)
36. Wang Y, Qian PY. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. *PLoS ONE.* 2009; 4, e7401. <https://doi.org/10.1371/journal.pone.0007401> PMID: [19816594](https://pubmed.ncbi.nlm.nih.gov/19816594/)
37. Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, et al. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl Environ Microbiol.* 1998; 64: 795–799. PMID: [9464425](https://pubmed.ncbi.nlm.nih.gov/9464425/)
38. Grape M, Motakefi A, Pavuluri S, Kahlmeter G. Standard and real-time multiplex PCR methods for detection of trimethoprim resistance *df*r genes in large collections of bacteria. *Clin Microbiol Infect.* 2007; 13:1112–1118. <https://doi.org/10.1111/j.1469-0691.2007.01807.x> PMID: [17725650](https://pubmed.ncbi.nlm.nih.gov/17725650/)
39. Machado E, Canton R, Baquero F, Galan JC, Rollan A, Peixe L, et al. Integron content of extended-spectrum- β -lactamase-producing *Escherichia coli* strains over 12 years in a single hospital in Madrid, Spain. *Antimicrob Agents Chemother.* 2005; 49(5):1823–1829. <https://doi.org/10.1128/AAC.49.5.1823-1829.2005> PMID: [15855502](https://pubmed.ncbi.nlm.nih.gov/15855502/)
40. Jové T, Da Re S, Denis F, Mazel D, Ploy MC. Inverse correlation between promoter strength and excision activity in class 1 Integrons. *PLOS Genet.* 2010; 6(1), e1000793. <https://doi.org/10.1371/journal.pgen.1000793> PMID: [20066027](https://pubmed.ncbi.nlm.nih.gov/20066027/)
41. Vinué L, Jové T, Torres C, Ploy MC. Diversity of class 1 integron gene cassette P_c promoter variants in clinical *Escherichia coli* strains and description of a new P₂ promoter Variant. *Int J Antimicrob Agents.* 2011; 38:526–529. <https://doi.org/10.1016/j.ijantimicag.2011.07.007> PMID: [21917427](https://pubmed.ncbi.nlm.nih.gov/21917427/)
42. Pruden A, Pei R, Storteboom H, Carlson KH. Antibiotic resistance genes as emerging contaminants: Studies in Northern Colorado. *Environ Sci Technol.* 2006; 40:7445–7450. PMID: [17181002](https://pubmed.ncbi.nlm.nih.gov/17181002/)
43. Luo Y, Mao D, Rysz M, Zhou Q, Zhang H, Xu L, et al. Trends in antibiotic resistance genes occurrence in the Haihe River, China. *Environ Sci Technol.* 2010; 44:7220–7225. <https://doi.org/10.1021/es100233w> PMID: [20509603](https://pubmed.ncbi.nlm.nih.gov/20509603/)
44. Czekalski N, Berthold T, Caucci S, Egli A, Bürgmann H. Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their dissemination into Lake Geneva, Switzerland. *Front Microbiol.* 2012; 3:106. <https://doi.org/10.3389/fmicb.2012.00106> PMID: [22461783](https://pubmed.ncbi.nlm.nih.gov/22461783/)
45. Berglund B, Fick J, Lindgren P-E. Urban wastewater effluent increases antibiotic resistance gene concentrations in a receiving northern European river *Environ Toxicol Chem.* 2015; 34:192–196. <https://doi.org/10.1002/etc.2784> PMID: [25331227](https://pubmed.ncbi.nlm.nih.gov/25331227/)
46. Xiong W, Sun Y, Ding X, Zhang Y, Zeng Z. Occurrence of antibiotic resistance genes and genera associated with potentially opportunistic pathogens in the Liuxi River. *Front Environ Sci.* 2014; 2:61.
47. Chen B, Liang X, Nie X, Huang X, Zoum S, Li X. The role of class 1 integrons in the dissemination of sulfonamide resistance genes in the Pearl River and Pearl River Estuary. *J Hazard Mater.* 2015; 282:61–67. <https://doi.org/10.1016/j.jhazmat.2014.06.010> PMID: [24994022](https://pubmed.ncbi.nlm.nih.gov/24994022/)
48. Koczura R, Mokracka J, Taraszewska A, Łopacinska N. Abundance of class 1 and 2 integrase and sulfonamide resistance genes in Warta river water and sediments is affected by anthropogenic pressure and environmental factors. *Microb Ecol.* 2016; 72:909–916. <https://doi.org/10.1007/s00248-016-0843-4> PMID: [27599709](https://pubmed.ncbi.nlm.nih.gov/27599709/)
49. Fang H, Zhang Q, Nie X, Chen B, Xiao Y, Zhou Q, et al. Occurrence and elimination of antibiotic resistance genes in a long-term operation integrated surface flow constructed wetland. *Chemosphere.* 2017; 173:99–106. <https://doi.org/10.1016/j.chemosphere.2017.01.027> PMID: [28107719](https://pubmed.ncbi.nlm.nih.gov/28107719/)
50. Ma L, Li A-D, Yin X-L, Zhang T. The prevalence of integrons as the carrier of antibiotic resistance genes in natural and man-made environments. *Environ Sci Technol.* 2017; 51: 5721–5728. <https://doi.org/10.1021/acs.est.6b05887> PMID: [28426231](https://pubmed.ncbi.nlm.nih.gov/28426231/)
51. Zhu Y-G, Zhao Y, Li B, Huang C-L, Zhang S-Y, et al. Continental-scale pollution of estuaries with antibiotic resistance genes. *Nat Microbiol.* 2017;16270. <https://doi.org/10.1038/nmicrobiol.2016.270> PMID: [28134918](https://pubmed.ncbi.nlm.nih.gov/28134918/)
52. Khan GA, Berglund B, Khan KM, Lindgren PE, Fick J. Occurrence and abundance of antibiotics and resistance genes in rivers, canal and near drug formulation facilities—a study in Pakistan. *PLoS ONE.* 2013; 8:e62712.
53. Lévesque C, Piché L, Larose C, Roy PH. PCR mapping of integrons reveals several novel combinations of resistance genes. *Antimicrob Agents Chemother.* 1995; 39:185–191. PMID: [7695304](https://pubmed.ncbi.nlm.nih.gov/7695304/)
54. Jiang L, Hu X, Xu T, Zhang H, Sheng D, Yin D. Prevalence of antibiotic resistance genes and their relationship with antibiotics in the Huangpu River and the drinking resources Shanghai, China. *Sci Total Environ.* 2013; 458–460:267–282.

55. Lu H, Na G, Gao H, Wang L, Bao C, Yao Z. Fate of sulfonamide resistance genes in estuary environment and effect of anthropogenic activities. *Sci Tot Environ*. 2015; 527–528:429–438.
56. Gao P, Mao D, Luo Y, Wang L, Xu B, Xu L. Occurrence of sulfonamide and tetracycline-resistant bacteria and resistance genes in aquaculture environment. *Water Res*. 2012; 46:2355–2364. <https://doi.org/10.1016/j.watres.2012.02.004> PMID: 22377146
57. Liu L, Su J-Q, Guo Y, Wilkinson DM, Liu Z, Zhu Y-G. Large-scale biogeographical patterns of bacterial antibiotic resistance in the water bodies of China. *Environ Int*. 2018; 117:292–299. <https://doi.org/10.1016/j.envint.2018.05.023> PMID: 29891393
58. Yang Y, Liu G, Ye C, Liu W. Bacterial community and climate change implication affected the diversity and abundance of antibiotic resistance genes in wetlands on the Qinghai-Tibetan plateau. *J Hazard Mater*. 2019; 361:283–293. <https://doi.org/10.1016/j.jhazmat.2018.09.002> PMID: 30212791
59. Makowska N, Koczura R, Mokracka J. Class 1 integrase, sulfonamide and tetracycline resistance genes in wastewater treatment plant and surface water. *Chemosphere*. 2016; 144:1665–1673. <https://doi.org/10.1016/j.chemosphere.2015.10.044> PMID: 26519797
60. Pei R, Kim SC, Carlson KH, Pruden A. Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res*. 2006; 40:2427–2435. <https://doi.org/10.1016/j.watres.2006.04.017> PMID: 16753197
61. Antelo A, Romero H, Batista S. Detection of integron integrase genes on King George Island, Antarctica. *Advances in Polar Science*. 2015; 26:30–37.
62. Nardelli M, Scalzo PM, Ramírez MS, Quiroga MP, Cassini MH, Centrón D. Class 1 integrons in environments with different degrees of urbanization. *PLOS ONE* 7(6): e39223. <https://doi.org/10.1371/journal.pone.0039223> PMID: 22761743
63. Zhang S, Gu J, Wang C, Wang P, Jiao S, He Z, et al. Characterisation of antibiotics and antibiotic resistance genes on an ecological farm system. *Journal of Chemistry*. 2015; Article ID 526143. <https://doi.org/10.1155/2015/526143>
64. Chen CQ, Zheng L, Zhou JL, Zhao H. Persistence and risk of antibiotic residues and antibiotic resistance genes in major mariculture sites in southeast China. *Sci Tot Environ*. 2017; 580:1175–1184.
65. McLain JE, Cytryn E, Durso LM, Young S. Culture-based methods for detection of antibiotic resistance in agricultural ecosystems: advantages, challenges, and gaps in knowledge. *J Environ Qual*. 2016; 45:432–440. <https://doi.org/10.2134/jeq2015.06.0317> PMID: 27065389
66. Goldstein C, Lee MD, Sanchez S, Hudson C, Phillips B, Register B, et al. Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics. *Antimicrob Agents Chemother*. 2001; 45:723–726. <https://doi.org/10.1128/AAC.45.3.723-726.2001> PMID: 11181350
67. Saenz Y, Brinas L, Dominguez E, Ruiz J, Zarazaga M, Vila J, et al. Mechanisms of resistance in multiple-antibiotic-resistant *Escherichia coli* strains of human, animal, and food origins. *Antimicrob Agents Chemother*. 2004; 48:3996–4001. <https://doi.org/10.1128/AAC.48.10.3996-4001.2004> PMID: 15388464
68. Partridge SR, Tsafnat G, Coiera E, Iredell JR. Gene cassettes and cassette arrays in mobile resistance integrons. *FEMS Microbiol Rev*. 2009; 33:757–784. <https://doi.org/10.1111/j.1574-6976.2009.00175.x> PMID: 19416365
69. Lin M, Wu X, Yan Q, Ma Y, Huang L, Qin Y, et al. Incidence of antimicrobial-resistance genes and integrons in antibiotic-resistant bacteria isolated from eels and aquaculture ponds. *Dis Aquat Org*. 2016; 120:115–123. <https://doi.org/10.3354/dao03013> PMID: 27409235
70. Sukumaran DP, Durairaj S, Abdulla MH. Antibiotic resistance of *Escherichia coli* serotypes from Cochin estuary. *Interdiscip Perspect Infect Dis*. 2012;124879. <https://doi.org/10.1155/2012/124879>
71. Michael CA, Gillings MR, Holmes AJ, Hughes L, Andrew NR, Holley MP, et al. Mobile gene cassettes: a fundamental resource for bacterial evolution. *Am Nat*. 2004; 164:1–12. <https://doi.org/10.1086/421733> PMID: 15266366
72. Labar AS, Millman JS, Ruebush E, Opintan JA, Bishar RA, Aboderin AO, et al. Regional dissemination of a Trimethoprim resistance gene cassette via a successful transposable element. *PLoS ONE*. 2012; 7(5): e38142. <https://doi.org/10.1371/journal.pone.0038142> PMID: 22666464
73. Odetoyin BW, Labar AS, Lamikanra A, Aboderin AO, Okeke IN. Classes 1 and 2 integrons in faecal *Escherichia coli* strains isolated from mother-child pairs in Nigeria. *PLoS ONE*. 2017; 12(8): e0183383. <https://doi.org/10.1371/journal.pone.0183383> PMID: 28829804
74. Frank T, Gautier V, Talarmin A, Bercion R, Arlet G. Characterization of sulphonamide resistance genes and class 1 integron cassettes in Enterobacteriaceae, Central African Republic (CAR). *J Antimicrob Chemother*. 2007; 59:742–745. <https://doi.org/10.1093/jac/dkl538> PMID: 17350987

75. Ishida Y, Ahmed AM, Mahfouz NB, Kimura T, El-Khodery SA, Moawad AA, et al. Molecular analysis of antimicrobial resistance in Gram-Negative bacteria isolated from fish farms in Egypt. *J Vet Med Sci*. 2010; 72:727–736. PMID: [20145377](#)
76. Vinué L, Sáenz Y, Rojo-Bezares B, Olarte I, Undabeitia E, Somalo S, et al. Genetic environment of genes and characterisation of integrons in *Escherichia coli* isolates of blood origin in a Spanish hospital. *Int J Antimicrob Agents*. 2010; 35(5): 492–496. <https://doi.org/10.1016/j.ijantimicag.2010.01.012> PMID: [20188519](#)
77. Ozgumus OB, Sandalli C, Sevim A, Celik-Sevim E, Sivri N. Class 1 and class 2 integrons and plasmid mediated antibiotic resistance in coliforms isolated from ten rivers in northern Turkey. *The Journal of Microbiology*. 2009; 47:19–21. <https://doi.org/10.1007/s12275-008-0206-z> PMID: [19229487](#)
78. Moura A, Jové T, Ploy MC, Henriques I, Correia A. Diversity of gene cassette promoters in class 1 integrons from wastewater environments. *Appl Environ Microbiol*. 2012; 78:5413–5416. <https://doi.org/10.1128/AEM.00042-12> PMID: [22582073](#)
79. Peters ED, Leverstein-van Hall MA, Box ATA, Verhoef J, Fluit AC. Novel gene cassettes and integrons. *Antimicrob Agents Chemother*. 2001; 45:2961–2964. <https://doi.org/10.1128/AAC.45.10.2961-2964.2001> PMID: [11557503](#)
80. Leverstein-van Hall MA, Paauw A, Box ATA, Blok HEM, Verhoef J, Fluit AC. Presence of integron-associated resistance in the community is widespread and contributes to multidrug resistance in the hospital. *J Clin Microbiol*. 2002; 40:3038–3040. <https://doi.org/10.1128/JCM.40.8.3038-3040.2002> PMID: [12149373](#)
81. Baggesen DL, Sandvang D, Aarestrup FM. Characterization of *Salmonella enterica* serovar Typhimurium DT104 isolated from Denmark and comparison with isolates from Europe and the United States. *J Clin Microbiol*. 2000; 38:1581–1586. PMID: [10747147](#)
82. Skurnik D, Ruimy R, Andrement A, Amorin C, Rouquet P, Picard B, et al. Effect of human vicinity on antimicrobial resistance and integrons in animal faecal *Escherichia coli*. *J Antimicrob Chemother*. 2006; 57:1215–1219. <https://doi.org/10.1093/jac/dkl122> PMID: [16581916](#)
83. Da Silva MF, Vaz-Moreira I, Pajuelo MG, Nunes OC, Manaia CM. Antimicrobial resistance patterns in *Enterobacteriaceae* isolated from an urban wastewater treatment plant. *FEMS Microbiol Ecol*. 2007; 60:166–176. <https://doi.org/10.1111/j.1574-6941.2006.00268.x> PMID: [17250754](#)
84. Su HC, Ying GG, Tao R, Zhang RQ, Zhao JL, Liu YS. Class 1 and 2 integrons, *sul* resistance genes and antibiotic resistance in *Escherichia coli* isolated from Dongjiang River, South China. *Environmental Pollution*. 2012; 169:42–49. <https://doi.org/10.1016/j.envpol.2012.05.007> PMID: [22683479](#)
85. Chen B, Zheng W, Yu Y, Huang W, Zheng S, Zhang Y, et al. Class 1 integrons, selected virulence genes, and antibiotic resistance in *Escherichia coli* isolates from Minjiang River, Fujian Province, China. *Appl Environ Microbiol*. 2011; 77:148–155. <https://doi.org/10.1128/AEM.01676-10> PMID: [21057021](#)
86. Guo X, Xia R, Han N, Xu H. Genetic diversity analysis of class 1 integrons and their associated antimicrobial resistance genes in *Enterobacteriaceae* strains recovered from aquatic habitats in China. *Lett Appl Microbiol*. 2011; 52: 667–675. <https://doi.org/10.1111/j.1472-765X.2011.03059.x> PMID: [21496063](#)
87. Canal N, Meneghetti KL, de Almeida CP, Bastos MR, Otton LM, Corção G. Characterisation of the variable region in the class 1 integron of antimicrobial-resistant *Escherichia coli* isolated from surface water. *Brazilian Journal of Microbiology*. 2016; 47: 337–344. <https://doi.org/10.1016/j.bjm.2016.01.015> PMID: [26991286](#)
88. Bass L, Liebert CA, Lee MD, Summers AO, White DG, Thayer SG, et al. Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian *Escherichia coli*. *Antimicrob Agents Chemother*. 1999; 43:2925–2929. PMID: [10582884](#)
89. Taviani E, Ceccarelli D, Lazaro N, Bani S, Cappuccinelli P, Colwell RR, et al. Environmental *Vibrio* spp., isolated in Mozambique, contain a polymorphic group of integrative conjugative elements and class 1 integrons. *FEMS Microbiol Ecol*. 2008; 64:45–54. <https://doi.org/10.1111/j.1574-6941.2008.00455.x> PMID: [18318712](#)
90. Adelowo OO, Fagade OE. Phylogenetic characterization of antibiotic resistant bacteria isolated from a poultry waste polluted river, southwestern Nigeria. *Turkish Journal of Biology*. 2012; 36:37–45.
91. Lèvesque C, Brassard S, Lapointe J, Roy PH. Diversity and relative strength of tandem promoters for the antibiotic resistance genes of several integrons. *Gene*. 1994; 42: 49–54.
92. Moura A, Pereira C, Henriques I, Correia A. Novel gene cassettes and integrons in antibiotic-resistant bacteria isolated from wastewaters. *Appl Environ Microbiol*. 2012; 78:5413–5416