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**Site specific bacterial load, enterobacterial occurrence and antibiotic susceptibility patterns in Nile Tilapia (*Oreochromis niloticus*), water and sediment from earthen aquaculture ponds**

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## Abstract

***Introduction:*** *aquaculture is the most rapidly growing food production sector. The intensive nature of freshwater fish aquaculture is associated with practices which predispose aquaculture produce to bacterial contamination. To prevent losses due to bacterial diseases, there has been an increase in the uncontrolled use of antibiotics, a good prerequisite to the development of antibiotic resistance - a major global problem. Hence, we aimed at investigating the enterobacterial diversity and occurrence of antimicrobial resistance in Nile Tilapia and its earth pond environment.* ***Methods:*** *fish samples, pond water and sediments were collected from four earthen ponds located at Yato, Cameroon, in three different collection trips for Total Heterotrophic Aerobic Plate Count (THAPC) and enterobacterial isolation. Bacterial characterisation and antibiotic sensitivity were assessed using the API 20E kit and the standard Kirby-Bauer disc diffusion method, respectively.* ***Results:*** *THAPC ranged from 1.0 x $10^2$to 3.0x$10^9$CFU/mL across samples and within each pond, bacterial loads were highest in fish parts and lowest in the surrounding environment. Sixteen enteric genera and 24 species were identified, with Escherichia coli(16.49%) and Enterobacter cloacae(12.37%) being most abundant, especially in the gills and intestines. The highest degree of resistance was observed with Amoxicillin and Ampicillin, with more than 60% of tested isolates being resistant. Multiple drug resistance was also observed in all sample types, ponds and collection time points, and highest in fish parts.* ***Conclusion:*** *this study re-emphasizes the potential of freshwater fish aquaculture as carrier of human pathogenic and multiple drug resistant bacteria.*


## Introduction

Aquaculture, which presently accounts for about 50% of the global fish production for food is the most rapidly growing animal production sector [1], and because of increased marine pollution, overfishing and global climate change, the push has been towards a rapid transition from species capture to aquaculture production model [2]. Fish is one of the preferred sources of cheap and healthy proteins in developing countries [3], such as Cameroon - where fish farming has been reported to be a profitable business [4]. Freshwater aquaculture is still an emerging practice in Cameroon with great potential since the gap between demand and production is huge. The effect of this huge demand for fish has led to shift towards intensive freshwater fish aquaculture, characterized by several risks factors that lead to microbial contamination of fish [5]. These include manipulation of breeding cycles, high stocking density of fishes in pond and the feeding or fertilisation of ponds with agricultural by-products including animal droppings, imposing a certain amount of stress on the fish and increasing the predisposition of fish to bacterial contamination.

Several studies have reported on pathogenic bacteria associated with fish in aquaculture systems [6-9], and some of which are human pathogens [10,11]. In an attempt to promote growth, prevent or treat bacterial infections in intensive aquaculture setups, antimicrobials have been massively administered to fish. This increases the risk of emergence and spread of resistant bacteria capable of causing infection in both animals and humans as there is no discrimination between the classes of therapeutic antimicrobials used in human and food producing animal [12]. Moreover, fish do not effectively metabolize these antibiotics hence 70 - 80% of drugs fed to the fish are excreted in the aquaculture environment [13]. These unmetabolized drugs subsequently contaminate products meant for human consumption as well as the human handlers [14]. These drugs are also environmentally very persistent, thus exert selective pressure on the microbiota of aquaculture ecosystem [15]. Due to the rise in antibiotic resistance, there is increasing recognition that urgent action is needed to avoid inappropriate use of antibiotics in animal husbandry and aquaculture, as well as humans to maintain low resistance levels and prevent the

proliferation of bacteria resistant to certain drugs [16].

Additionally, antimicrobial resistance patterns observed in inland animal husbandry have also been observed in aquaculture [17]. Tilapia is one of the most grown freshwater fish species in the world and the most cultivated species in Cameroon. This species is environmentally resilient, capable of resisting harsh temperatures, low dissolved oxygen and heavy stocking density, which exposes tilapia to bacterial contamination and encourage antimicrobial abuse. In light of the fact that there is a drive by the government to increase aquaculture production nationwide towards addressing national demand, this study was aimed at investigating the enterobacterial diversity of Nile Tilapia, from earthen ponds and to assess the occurrence of antibiotic resistant bacteria of food safety importance in earth ponds that might greatly impact public health and economic sectors.

## Methods

**Pond conditions:**four earthen ponds located at Yato, Littoral Region of Cameroon, along the banks of the River Mungo (9° 33' 30" N, 4° 03' 02" E) were chosen for the study. Around the Mungo bridge, the river is about 150-200 m wide and has a depth of 5-15 m [18] and the soil type consists mainly of sandstone, intercalated with limestone and shale [19]. The site belongs to the Equatorial Monsoon climatic zone, characterized by annual rainfall ranging from 2000 to 10000 mm with an mean annual temperature of 21 ± 2.2°C [20]. The main activities in this region are artisanal in-channel sand mining, fishing, water supply, oil palm and banana farming [18]. These ponds are opened-air rectangular earth ponds 8-10 metres apart, surrounded by vegetation, palm trees, banana plants and fruit trees. The ponds are fed with waste from poultry, piggery, and palm oil production. These ponds established at the banks of River Mungo are not operated under flow-through systems and must be refilled about 3 times every 2 weeks with water from River Mungo to compensate for evaporation and seepage.

**Sample collection:** samples of tilapia, pond water and sediments were purposively collected from four earthen ponds. Nile Tilapia was fished out of the pond using a fishing line and a bait (earthworms or chicken intestine). Harvested fish samples were placed into transparent containers with pond water. A total of 52 tilapia were sampled from 4 ponds (Pond 1 to 4) within three collection trips; April (C1), December 2016 (C2) and May 2017 (C3). Twelve fish samples were collected at first trip and 20 each during the second and third trips, respectively. Fish samples from each pond were collected into separate containers. Six water samples were collected at different positions around each pond ~20 cm below the water surface into 60 mL sterile plastic collection cups. Using a handmade sediment sampler [21], six sediment samples were also collected at the same areas of the pond where water samples were collected. The containers were appropriately labelled and then placed in between ice packs for transportation to the laboratory within an hour of collection.

**Sample processing and analysis:** upon arrival at the laboratory, live fish were humanely killed through a sharp blow to the head. The skin of each fish was passively washed by pouring sterile water over the skin surface of the fish to wash off any transient bacteria before processing. A labelled sterile cotton swab was used to swab both sides of the fish, avoiding the opercular and anal regions, to collect the mucosal bacteria population on the surface of the fish's skin. Each fish sample was then aseptically weighed, its length measured and recorded. The fish skin was then disinfected with 70% alcohol and dissected to expose the gills and intestine of the fish. Aseptically, one gram each of gill and intestine was weighed and ground separately from each fish sample. The skin swab, ground gill and intestine were homogenised into 9 mL of 0.1% sterile peptone water and serially diluted (10-fold) to the $10^{-8}$dilution. The six water and sediment samples collected per pond were divided into two, and

three water/sediment samples were mixed to generate a composite sample, giving two composite water and sediment samples per pond. Therefore, each pond had 2 composite sediment samples and 2 composite water samples. One gram of each composite sediment sample was weighed and homogenised into 9 mL of sterile 0.1% peptone water. One mL of each composite water sample was also homogenised with 9 mL of sterile 0.1% peptone water. The homogenised water and sediment samples were serially diluted (10-fold).

**Bacterial culture and microbial loads:** total Heterotrophic Aerobic Plate Count (THAPC) was used to assess the total microbial loads in each sample by spreading 0.1 mL from each dilution on nutrient agar. To isolate enteric bacteria, each sample was also streaked on Eosine Methylene Blue (EMB) agar (Liofilchem, srl, Roseto, Italy) and *Salmonella Shigella*(SS) agar (Liofilchem). These plates were incubated for 24 hours at 30°C. For THAPC plates, the number of colonies were counted and recorded. The isolated bacteria from EMB and SS agar plates were sub-cultured to obtain pure colonies that were then stored in nutrient agar stabs at 4°C for 2-4 weeks prior to characterisation. All media were prepared according to manufacturers' instructions and cultured using standard methods.

**Phenotypic characterization of isolates:** gram staining was done on enteric bacterial pure isolates from nutrient agar and oxidase tests were performed using oxidase test strips (Liofilchem). Catalase tests were performed using 3% hydrogen peroxide (Gilbert Laboratories, Caen, France). Cells from pure cultures were grown on nutrient agar (Liofilchem) at 30°C, re-suspended in 0.85% NaCl API medium (BioMérieux, Inc., Marcy l'Etoile, France), and later inoculated into API 20 E strips (BioMérieux, Inc.) for additional phenotypic testing and characterisation. The test procedure was performed following the manufacturer´s instruction. The strips were incubated at 35°C, and results were recorded at 24h. Characterised isolates were then stored in 20% glycerol.

**Antibiotic susceptibility testing:** the phenotypically characterised isolates were then subjected to antibiotic susceptibility testing using the standard Kirby-Bauer disc diffusion method [22] as recommended by the Clinical and Laboratory Standards Institute [23]. Susceptibility to 11 antibiotics which belong to the major antibiotic classes (the penicillins, cephalosporincs, macrolide, aminoglycosides, carbapenams, fluroquinolone, sulphonamides, tetracyclines and nitrofurantoin) were tested. The antibiotics investigated were sulfamethoxazole and trimethoprim (23.75 µg/1.25 µg), gentamicin (10 µg), cefuroxime (30 µg), ceftriaxone (30 µg), imipenem (10 µg), tetracycline (30 µg), ampicillin (10 µg) and ofloxacin (5 µg) all obtained from BD BBL™, USA. Erythromycin (15 µg), nitrofurantoin (100 µg) and amoxicillin (10 µg) were obtained from Abtek, UK. Each plate was incubated at 30°C for 24 hours, followed by the measurement of the zones of inhibition using a transparent ruler.

**Statistical analysis:** data collected was entered into Microsoft Excel 2013 datasheets and analysed using both Microsoft Excel and GraphPad Prism 8.0.2 (GraphPad Software, San Diego, USA). Microsoft Excel was used to generate $Log_{10}$of bacterial loads initially calculated and expressed as CFU/mL or CFU/g. Hypothesis investigated was the difference between mean microbial loads across collection trips, ponds and sample units (skin, gills, intestines water and sediment). To test for normality of the data, the Shapiro-Wilk test was employed. For normally distributed data, the one-way ANOVA was used to test this hypothesis and the Turkeys multiple comparison test was employed to test which means differed significantly from the others when the one-way ANOVA was significant. Wherever the number of groups were less than 3, the unpaired t-test was used to test the difference among means. For data that did not pass the Shapiro-Wilk test, the Kruskal-Wallis test was used to test the difference between groups and when significant, the Dunn's multiple comparisons test was used to determined which means differed significantly from the others. In the event where

the Dunn's multiple comparisons test showed no difference despite a significant Kruskal-Wallis test, a Mann Whitney test was used to compare pairs of datasets. Where the number of groups for comparison equalled 2, the Mann Whitney test was used to test for differences. Statistical significance was set at P<0.05.

## Results

**Experimental fish:** a total of 52 fish samples were collected from the four ponds with a total of 13 tilapia per pond. Their weights ranged from 29.3g to 278.7g, with the lengths ranging from 11.2cm to 25.9cm (Table 1). Since none of the sampled fish presented with any skin abnormality and no fish mortality was observed in any of the ponds during sampling, the fish samples were considered as healthy.

**Bacterial loads:** in general, the THAPC recorded ranged from 1.0 x $10^2$to 3.0 x$10^9$CFU/mL. When the bacterial loads of each sample type were considered across ponds (Figure 1A,B,C,D,E), THAPC did not differ significantly between the sample units across the ponds except for the gills (p=0.04) (Figure 1B). When considered across sample collection rounds (Figure 1F,G,H,I,J), bacterial loads differed significantly between collection time points for skin (p=0.028) (Figure 1F), gills (p=0.0012) (Figure 1G), intestines (p=0.0004) (Figure 1H), pond sediment (p<0.0001;) (Figure 1I), and pond water (p=0.0001) (Figure 1J). Considering the pooled THAPC picture irrespective of collection trip or pond, the bacterial loads differed significantly among sample units and was significantly highest in fish intestines and lowest in water and sediment (p<0.0001;) (Figure 1K). When pooled per pond (Figure 1A,B,C,D) or per collection trip (Figure 1E,F,G), the bacterial loads differed significantly among sample units in all ponds, highest in fish parts and lowest in the surrounding environment.

**Biochemical characterization:** biochemical and API characterisation of the presumptive bacteria isolates revealed the presence of 22 enteric genera and 43 species, with *Escherichia coli*being the most abundant (16.49%), followed by Enterobacter cloacae (12.37%). The bacteria species identified included: *Acinetobacter spp, Aeromonas hydrophila, Aeromonas salmonicida, Escherichia coli, Enterobacter cloacae, Plesiomonas shigelloides, Citrobacter freundii, Edwardsiella tarda, Erwinia spp, Klebsellia pneumonia, Pseudomonas fluorescens, Raoultella ornithinolytica*and *Serratia liquefaciens* (Table 1). *The API database did not identify 3/194 of the isolates*. All the ponds were contaminated and among the samples, the intestines, followed by the skin and gills were the most contaminated. The most abundant characterised isolate from the intestinal samples was *Escherichia coli*(20%) and *Enterobacter cloacae*was most abundant in the gills (23.4%) and skin (15.1%) respectively (Table 1).

**Antimicrobial susceptibility:** the antibiotics used in this study belong to the following classes of antibiotics; aminoglycosides, macrolides, penicillins/cephalosporins, quinolones, sulfonamide and tetracycline, which are the most commonly used classes of antibiotics in animal production and human medicine. The highest degree of resistance was observed with the penicillins amoxicillin and ampicillin, with 72% and 65.5% of tested isolates respectively, being resistant to these two antibiotics. Resistance was also high for Erythromycin as 58.5% of isolates tested were resistant. Gentamicin and ofloxacin inhibited most of the isolates (Figure 2). This general picture of resistance profile was also presented as per ponds and therein a similar trend was observed across the four ponds for each antibiotic (Figure 2Aa). When presented across collection time points, resistance increased in the third phase for the drugs with highest resistance (AMX, AM and E) but reduced for the drugs with least resistance (IPM, CN and OFX) (Figure 2Bb). Multiple drug resistant (MDR) species were also identified and presented as per their site of occurrence (Figure 3A), per pond (Figure 3B) and

per collection time point ( Figure 3C). Two of the most prevalent resistant profiles (E and AMX-AM-IPM) were present in all five sample types, 2 (AMX-AM-E and AMX-AM-E-SXT-TE) in four samples, while 2 were found only in fish samples (AMX-AM-CRO and AMX-AM-E-F) (Figure 3A), highlighting that all combinations of obtained MDR isolates can be found in fish and thus, can affect humans through the consumption of fish. The three most prevalent resistant profiles (AMX-AM-E, E and AMX-AM-IPM) were present in all ponds while 8 of the most prevalent profiles (AMX-AM-CRO, AMX-AM-E-F, AMX-AM, AMX-AM, E-SXT-TE and AMX-AM-CXM-F) where found in 3 of four ponds (Figure 3B). For the distribution of MDR across collection time points, the 3 most prevalent MDR profiles were found in all 3 collection while the next two most prevalent were recorded only in the 3rd collection trip. Seven of most frequent MDR profiles were found in 2 of 3 collection trips (Figure 3C).

## Discussion

The quantitative and qualitative assessment of the bacterial loads, enterobacterial microbiota and their antibiotic resistance profile of the Nile Tilapia and its freshwater earth pond environment from Yato is revealed to be quite diversified. From our observation, this is the first study which seeks to investigate the presence and antimicrobial resistance profile of enterobacterial species found in tilapia fish and its freshwater aquaculture earth pond environment in Cameroon. The fish gills, skin and intestines, and the pond water and sediment constituted the samples, and the results demonstrate the diversity of enterobacteria across the different sample categories investigated. The variation in loads and species observed in this study is quite similar to results obtained by Pakingking [9] and Al-harbi and Uddin [24], when investigating bacterial microbiota in pond water and sediment, fish gills and intestines of tilapia that was cultured in earthen ponds in the tropical country of the Philippines and brackish waters in Saudi Arabia, respectively. Generally, loads were higher in the intestines compared to the other tested samples, and lowest in the pond environment. The observed difference could be because most of the organisms from the environment accumulate in the intestines followed by gills. It was also observed that the loads are higher in the warmer season and this was probably because of higher temperatures which increase microbial metabolism.

Looking at the pooled microbial loads by pond, it was generally discovered that the loads between sample types (fish skin, gills and intestines, and pond water and sediment) differed significantly ($P<0.5$) (Figure 1A,B,C,D). When each sample type was pooled per collection (Figure 1E,F,G), the loads between samples were still statistical different ($P<0.5$). Bacterial loads in the fish were generally significantly higher than the loads in the environment and this is in accordance with other authors. In all, when all the loads were pooled irrespective of collection trip or pond, the loads were highest in intestines, followed by skin, gills and lowest in the environment ($P<0.0001$) (Figure 1K). This is similar to trends reported by Kaktcham *et al.*[10] and Al-harbi and Uddin [24], who performed similar studies in earth ponds and brackish water but slightly different from that reported by Pakingking *et al.*[9] in that the loads were higher in gills.

The bacterial count of the skin samples ranged from $10^5$- $10^8$CFU/g and is slightly lower compared to mean count of $10^9$CFU/g reported by Shinkafi and Ukwaja [25] in skin of tilapia sold in the market. The higher count in the study by Shinkafi and Ukwaja [25] could be as a result of post-harvest handling and storage since these tilapia samples were collected from the market. The load in the skin is slightly higher than that in the surrounding water and this is similar to what is reported by [26] in common carp ponds in India where similarity was reported between the loads in the pond water and skin of the common carp fish samples.

A closer look at each sample type across collection trips and across ponds revealed a better picture of the variation in bacterial loads. Looking at the loads

when pooled across ponds (Figure 1A), there was no statistical difference among skin loads across the four ponds. This clearly shows that there is no variation in bacterial loads across ponds at each collection time point, indicating that population of bacteria on the skin are more the same constant at same temperature. The pooled bacterial load of the skin differed between the three collection phases (P=0.028) (Figure 1F). These loads where significantly highest in the warmer months of December and is most likely due to the increase microbial activity due to higher temperatures.

Taking a look at the tilapia gill samples, the THAPC of the gill samples ranged from $10^4$- $10^8$log CFU/g and was higher than that reported by Parkingking *et al.* [9] and Al-harbi and Uddin [24] who reported counts of $10^5$- $10^7$CFU/g and $10^6$CFU/g respectively. The counts across ponds and collection phases of the intestines ranged from $10^5$- $10^9$CFU/g. This was slightly less than the total heterotrophic bacteria count range of $10^3$- $10^8$CFU/g, $10^7$- $10^8$CFU/g and $10^9$- $10^{10}$CFU/g reported by Pakingking *et al.*[9] and by Kaktcham *et al.*[10] respectively. The higher loads might be as a result of the accumulation of microbes from the environment as water filters through the gills and during feeding of the fish with waste from agriculture and animal husbandry. The pooled THAPC count of the gills was statistically different across ponds, being highest in pond 2 (P=0.04) (Figure 1B). When pooled per collection round, there was also a significant difference among counts on gills across the three collection trips (P=0.0012) (Figure 1G), highest during the warmer month of collection 2. The loads being different across ponds and collection trip, imply that factors other than temperature contribute to the variation and the loads in the gills vary much more than that observed in the skin which is influenced largely by temperature of surrounding pond water.

The bacterial load of the intestines when pooled did not differ across ponds (Figure 1C) but were significantly different across collection round and significantly highest in both second and third collection phases (P<0.0004;) ( Figure 1H). This difference in pattern where the highest loads were not only higher in collection trip 2 is indicative of the fact that temperature might be less influential on the intestinal loads as compared to that of the skin. It is important to note that the similarity in loads in intestines across ponds but the difference across collection time points might be indicative of the implication of temperature or practices in increasing or decreasing loads.

The THAPC of the sediment samples ranged from $10^2$- $10^8$CFU/g and as expected, were higher than that obtained for the pond water. The THAPC count in sediments reported in this study is similar to the range of $10^5$- $10^6$and $10^6$- $10^7$CFU/g reported by Thanh *et al.*[27] and Al-harbi and Uddin [24] in sediments of earth and brakish water ponds, but higher than those reported by Pakingking *et al.*[9] who reported counts ranging from $10^3$- $10^5$CFU/g in sediments of earth ponds. Kaktcham *et al.*[10] working in freshwater earthen ponds in the Western Highlands of Cameroon reported even higher counts of $10^9$- $10^{10}$CFU/g in sediments, higher than what we obtained in the coastal region around the Mungo River. In addition to pond fertilization and manipulation practices, the soil type can account for the differences between both studies.

When pooled, there was no difference among bacterial loads of sediments across the four ponds (Figure 1D) and this indicates that the sediment population is similar across ponds at each collection time point. Considering the collection phases, the pooled bacterial load of the pond sediment was different between the three collection phases (p<0.0001;) (Figure 1I). Strikingly, the loads were highest in the third collection trip, implying that the difference in loads were not mainly influenced by temperature, but surely other factors which favour the accumulation of bacteria in the sediments.

Irrespective of collection phase or pond, the THAPC count of the water samples ranged from $10^3$- $10^7$CFU/mL. Our findings are similar to the THAPC

range of $10^3$- $10^4$CFU/mL obtained by both Pakingking *et al.*[9] and Al-harbi and Uddin [24] who investigated loads in water of earthen ponds in the Philippines and brackish water in Saudi Arabia respectively, but less than that of $10^9$-$10^{10}$CFU/ml reported in Cameroon by Kaktcham *et al*. [10] in the Western Highlands of Cameroon. Since these are all tropical regions with generally similar climatic conditions, the differences between our result and that of Kaktcham *et al*. [10] can therefore be as a result of differences in the quality of source water, pond fertilization and manipulation practices across study sites. The bacterial load of the surrounding pond water was similar across ponds and did not differ between ponds when pooled (Figure 1E). When considered as per collection round, the loads were significantly different across collection trips and lowest in the first strip (P=0.0001) (Figure 1J). It is important to note that the water samples during second collection were lost as the containers leaked out.

Generally, it was expected that bacterial loads will be higher during the second collection which was in the warmer month of December compared to those of the first or third sample collection. This was observed for skin and gills samples and is in line with results obtained by Karimi [**28**] who worked with tilapia from freshwater aquaculture farms and the Mosinga Dam in Kenya. The author attributed the higher bacteria loads in the dry season compared to the wet season to increased bacteria growth at higher temperatures. Markosova and Jezek [**29**], in a 6-year long study, equally reported a similar trend with an increase in the load of indicator bacteria in carp fish ponds with increasing water temperature as they reported highest loads in the summer months.

It is also important to note that the variation in bacteria load across ponds for skin and pond water was negligible compared to that among gills, intestines and sediment samples. This may be because of the inability of these two sample categories to accumulate organisms, thus maintaining stable bacterial loads across the ponds. When looking at the variation at each collection period and across collection periods, it was realized that only the loads of the gills differed significantly at each collection and across collection trips. The reason for this is not very clear but may probable be evidence of more influence of environmental parameters on the gills more than any other sample type. Considering the bacterial loads across sample types per pond, it was observed that the loads were different across sample types, being significantly higher in the fish and lower in the environmental pond water and sediment. Some researchers have postulated that the microbiota of the fish is a reflection of the environment and according to Pakingking *et al.*[**9**], the bacterial quantity and quality of the rearing environment of tilapia is a mirror image of the bacterial quantity and quality of the tilapia gills and intestines. The findings do not give enough evidence to corroborate such suggestion since this study reported significantly lower loads and genera in the pond environment than in the fish. There were 10 genera present only in fish tissues, while 6 genera were found only in pond water or sediment. This could suggest that the fish microenvironment supports the propagation of some bacteria that cannot flourish in the environmental pond water or sediment. Given that no study is without potential limitations, it is important to highlight that a further description of the biological, physical or chemical characteristics of the sample units (fish, water and sediment), could have given other opportunities for analysis and discussion of the measured outcomes (loads and isolates).

Phenotypic characterization of the isolates revealed that bacteria isolated during this study are similar to enteric bacteria reported by other authors in fish or aquaculture environment. The bacteria identified using the API 20E kit belonged to 22 gram negative bacteria genera and 41 species. The genera identified included *Acinetobacter, Aeromonas, Citrobacter, Enterobacter, Erwinia, Escherichia, Morganella, Plesiomonas, Pseudomonas, Edwardsiella, Raoultella,*

*Salmonella, Serratia, Pasteurella*and *Vibrio*(**Table 1**). These findings are in partial accordance to the study on the bacteria flora of Nile Tilapia cultured in brackish water by Pakingking *et al.*[**9**]. Even though [**9**] documented a similar number of bacteria genera, the absence of *Escherichia coli*from their work is a major difference with our study which strikingly was the most abundant bacteria isolated from the Nile Tilapia in earth pond aquaculture farms at Yato, Mungo. This high prevalence of *E. coli*in our study can have its source from handlers during pond fertilization practices using waste from chicken and pig farming, and palm oil extraction or from the source water which is collected from River Mungo. Given that the river is highly used for economic activities of in-channel sand mining and fishing which exposes the river to contamination by the human pathogen *E. coli.*

Karimi [28], working with tilapia harvested from freshwater fish ponds and the Masinga Dam also reported on bacteria genera similar to those we reported in our study, notably; *Acinetobacter, Aeromonas, Citrobacter, Escherichia, Edwardsiella, Enterobacter, Klebsiella, Plesiomonas, Pseudomonas, Salmonella*and *Vibrio*. There are also some similarities with the study on Nile Tilapia from four lakes in Northern Cameroon by Donkeng *et al.* [30] and another study carried out by Musefiu *et al.* [31] on Catfish and Nile Tilapia in Nigeria. Akoachere *et al.* [32] in their investigation of bacterial species associated with various species of fish from the coastal regions of Cameroon, also reported the presence of *Escherichia, Citrobacter, Enterobacter Serratia, and Klebsiella*which was observed in this study. It is important to point out that in their study, *Escherichia coli*was also the most abundant bacteria they reported.

Amongst the bacteria identified were species pathogenic to human especially members of the coliform group with *Escherichia coli*being the most abundant. In addition to being the most abundant species, only *Escherichia*was present in all sample types. It is important to mention that this is an important human pathogenic species which is of significant public health concern. Other important pathogenic species that have been reported include *Pseudomonas aeruginosa*and *Klebsiellia pneumonia*. Kaktcham *et al.* [10] also reported on presence of human pathogenic genera of public health interest in earth ponds including *Escherichia, Salmonella*and *Vibrio*which was also observed in this study. The findings from our study, similar to that from [10] and Akoachere *et al.* [32] clearly underline the fact that these fish samples are a clear potential health hazard to humans, be it handlers or consumers. In this light, there has been evidence of transmission of fishborne resistant bacteria to humans [33] even though most often, genetic evidence fails to link many of these suspected piscine zoonoses to human infections [34]. Besides being a potential source of human infection, these species can also contribute in the transfer of antibiotic resistance from aquaculture bacteria to human pathogens, even those from non-aquatic environments which widen the public health hazard that can result directly or indirectly from the aquaculture environment. Many studies have raised this call for concern concerning antibiotic resistance in the environment and food systems that can affect humans [35,36] and it is thus clear that it is of prime importance to stop the use of antibiotics in food animals [16]. The antibiotic susceptibility assessment of the bacteria microbiota of the Nile Tilapia from Yato, along the River Mungo revealed that they were mostly resistant to the penicillin class of drugs i.e. Amoxicillin and Ampicillin, followed by the macrolide Erythromycin, while highest susceptibility was to the fluroquinolone, ofloxacin and the aminoglycoside Gentamycin (Figure 2). The high resistance to penicillins is in line with the report by Donkeng *et al.*[30], and Dib *et al.*, [37] who reported the increasing environmental incidence of resistance to beta-lactam antibiotics. It is worth pointing out that these macrolide and penicillin drugs form part of the critically important antimicrobials for human medicine due to their high frequency of use in human medicine and also form part of the drug classes with limited available therapies to treat serious bacterial infections in people [38].

It is important to underline that our study focuses on earthen pond aquaculture which is practiced by approximately 83.3% of the fish farmers in the country, with 39.2% of which use animal manure (chicken droppings (20.5%) and pig dung (18.7%) to fertilize the ponds [39]. Looking at the most prevalent resistant profiles overall, resistance to the penicillin-macrolides profile was highest and this is similar to reports by Akinbowale *et al.*[40] and Kathleen *et al.*[41], who arrived at similar conclusions while working with bacteria isolated from aquaculture sources in Australia and Borneo. This resistance profile was followed by macrolides only, penicillins-carbapenams and penicillins-cephalosporins-nitrofurantoins in frequency of occurrence. In all, this trend was spread across all four ponds (Figure 2A) and collection time points (Figure 2B). It was observed that the more prevalent multiple drug resistance profiles were spread across fish and pond environment samples (Figure 3A), across ponds (Figure 3B) and across collection time points (Figure 3C), except for the AMX-AM-CRO and AMX-AM-E-F resistance profiles which were reported only in fish samples but not in pond environment (Figure 3A). This indicates a circulation in the pond system of MDR species underlining the importance of this for veterinary and public health because of the risk this poses to both fish and humans.

Taking a closer look at the most prevalent genera, the *E. coli*strains were not of a prevalent resistance phenotype, as opposed to the Aeromonas, Enterobacter, Klebsiella and Pleisomonas genera. The AMX-AM-E resistance profile occurred most in the Enterobacter and Klebsiella genera, the AMX-AM-IPM phenotype was more present in the genus Aeromonas, and the AMX-AM occurred most in the genera Pleisomonas. *Aeromonas hydrophilia*was resistant to both ampicillin and tetracycline and this is in line with findings by [42]. Lee and Wendy [42] also reported on the high resistance of *Edwardsiella tarda*to ampicillin and tetracycline which differs from our findings as we report no resistance in our study. It is worth highlighting that in contrast to reports by Martins *et al.* [6], who reported Pleisomonas as a high carrier of tetracycline resistance, we did not observe resistance to tetracycline by Pleisomonas species. This may suggest that the plesiomonas species in our study sites may have acquired resistance genes from other organisms. This demonstrates further the health risk that is posed by these organisms found in earth pond aquaculture systems.

## Conclusion

Our results clearly show the resident bacteria and their antimicrobial resistance patterns in the skin, intestines and gills of tilapia, and the rearing environment (pond water and sediment). Moreover, bacteria residing in the tilapia and its rearing environment consist of genera of opportunistic and/or fish pathogens and human pathogenic species. Therefore, the need for good aquaculture practices of tilapia and its pond environment cannot be overemphasized. Our findings and reports from other authors [14,43,44] further emphasize the importance of this call for concern about antimicrobial use and antimicrobial resistance organisms in the environment including the aquaculture environment. Follow-up studies will be needed to clarify the mechanisms of resistance, how they are transferred, their relationship with fish feed and animal droppings added to the ponds, and investigate alternatives to antibiotics that can be suitable in the tilapia earth pond setting.

***What is known about this topic***

- *Specific enteric bacteria have been reported in aquacultured fish or environment;*
- *The excessive use of antibiotics in food production systems has worsened the threat of antimicrobial resistance in both animal and human health.*

***What this study adds***

- *The occurrence of enterobacterial speciesin earth pond aquaculture setting of importance to human and animal health;*

- *The resistance to antibiotics of isolated strains is high with occurrence of multiple drug resistance profiles detected in all ponds, collection time points and pond environment.*

## Competing interests

The authors declare no competing interests.

## Authors' contributions

Gordon Takop Nchanji: field activities (equal); methodology (equal); formal analysis (lead); original draft (lead); review and editing (equal). Andrielle L Kemajou Tchamba: field activities (equal); methodology (equal); formal analysis (supporting); original draft (supporting); review and editing (supporting). Nancielle T Mbiatong: methodology (equal); field activities (equal); review and editing (supporting). Manuel Ritter: conceptualization (supporting), review and editing (equal). Bertrand Tatsinkou Fossi: conceptualization (lead), supervision (lead), review and editing (equal). Samuel Wanji: conceptualization (lead), supervision, review and editing (equal). All the authors have read and agreed to the final manuscript.

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## Table and figures

**Table 1**: distribution of isolated enterobacterial species

**Figure 1**: pooled total heterotrophic aerobic bacterial load by sample unit across ponds and collection time points. *Data represent the $Log_{10}$(± SD) CFU per gram or ml of sample. Data columns with different superscripts are significantly different from each other at p<0.05*

**Figure 2**: antibiotic resistance profile of isolates (A) across ponds and (B) across collection time points. (*AMX (Amoxicillin), AM (Ampicillin), CRO (Ceftriaxone), CXM (Cefuroxime), IPM (Imipenem), SXT (Sulfamethoxazole and Trimethoprim), E (Erythromycin), CN (Gentamicin), TE (Tetracycline), NIT (Nitrofurantoin), OFX (Ofloxacin*))

**Figure 3**: most frequent multiple drug resistant profile distributed across (A) fish parts and pond environment; (B) ponds and (C) collection time points. (*AMX (Amoxicillin), AM (Ampicillin), SXT (Sulfamethoxazole and Trimethoprim), CXM (Cefuroxime), CRO (Ceftriaxone), IPM (Imipenem), TE (Tetracycline), E (Erythromycin), NIT (Nitrofurantoin*))

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| **Table 1:** distribution of isolated enterobacterial species | | | | | | |
|---|---|---|---|---|---|---|
| **I.D. Organism** | **Gills (%)** | **Intestine (%)** | **Sediment (%)** | **Skin (%)** | **Water (%)** | **Total (%)** |
| Acinetobacter ssp | - | - | - | -1(1.89) | 1(5) | 2(1.03) |
| Acinetobacter baumannii/calcoaceticus | - | - | - | 2(3.77) | 3(15) | 5(2.58) |
| Aeromonas hydrophilia | 6(12.77) | 2(3.64) | 1(5.26) | - | 2(10) | 11(5.67) |
| Aeromonas hydrophilia/caviae/sobria 2 | - | - | 1(5.26) | - | - | 1(0.52) |
| Aeromonas salmonicida | - | - | 1(5.26) | - | - | 1(0.52) |
| Bordetella/Alcaligenes/Moxarella spp | - | - | - | - | 1(5) | 1(0.52) |
| Cedecea davisae | 1(2.13) | - | - | - | - | 1(0.52) |
| Citrobacter freundii | - | 2(3.64) | 1(5.26) | 1(1.89) | - | 4(2.06) |
| Citrobacter koseri | 1(2.13) | 1(1.82) | - | - | - | 2(1.03) |
| Edwardsella tarda | - | 4(7.27) | 1(5.26) | - | - | 5(2.58) |
| Enterobacter aerogenes | 4(8.51) | 5(9.09) | 1(5.26) | 4(7.55) | - | 14(7.21) |
| Enterobacter amnigenus/cloacae | - | 1(1.82) | - | - | - | 1(0.52) |
| Enterobacter cloacae | 11(23.40) | 4(7.27) | - | 8(15.09) | 1(5) | 24(12.37) |
| Enterobacter sakazakii | 2(4.26) | 1(1.82) | - | 4(7.55) | 1(5) | 8(4.12) |
| Enterobacter, Citrobacter | 1(2.13) | - | - | - | - | 1(0.52) |
| Erwinia spp | - | 3(5.45) | - | - | - | 3(1.55) |
| Escherichia coli 1 | 8(17.02) | 11(20) | 4(21.05) | 6(11.32) | 3(15) | 32(16.49) |
| E. fergusonii | - | - | - | 2(3.77) | - | 2(1.03) |
| Ewingella americana | - | - | - | - | 1(5) | 1(0.52) |
| Flavi oryzihabitans | - | - | - | - | 1(5) | 1(0.52) |
| Klebsellia pneumoniae | 1(2.13) | 4(7.27) | - | 11(20.76) | - | 16(8.25) |
| Klebsiella oxytoca | 2(4.26) | - | - | - | - | 2(1.03) |
| Kluyvera spp/ E. coli 1 | - | 1(1.82) | - | - | - | 1(0.52) |
| Morganella morganii | - | 1(1.82) | - | - | - | 1(0.52) |
| Pasteurella pneumotropica/ Mannheimia sp | 1(2.13) | - | - | - | - | 1(0.52) |
| Pleisomonas shigelloides | - | 3(5.45) | 1(5.26) | - | 4(20) | 8(4.12) |
| Pseudomonas aerogenosa | - | - | - | 1(1.89) | - | 1(0.52) |
| Pseudomonas fluorescens | 1(2.13) | 1(1.82) | 5(26.32) | 1(1.89) | - | 8(4.12) |
| Pseudomonas luteola | 1(2.13) | - | - | - | 1(5) | 2(1.03) |
| Pseudomonas oryzihabitans | - | - | - | - | 1(5) | 1(0.52) |
| Rahnella aquatilis | - | - | 1(5.26) | - | - | 1(0.52) |
| Raoultella ornithinolytica | 1(2.13) | 3(5.45) | - | 3(5.66) | - | 7(3.61) |
| Salmonella arizonae | - | - | - | 1(1.89) | - | 1(0.52) |
| Salmonella choleraesuis ssparizona | - | - | - | 1(1.89) | - | 1(0.52) |
| Salmonella enterica ssp arizonae | 1(2.13) | 1(1.82) | - | 2(3.77) | - | 4(2.06) |
| Salmonella ssp | - | - | 1(5.26) | - | - | 1(0.52) |
| Serratia fonticola | - | - | - | 1(1.89) | - | 1(0.52) |
| Serratia liquefaciens | 3(6.38) | - | - | 1(1.89) | - | 4(2.06) |
| Serratia odorifera | - | 2(3.64) | - | 2(3.77) | - | 4(2.06) |
| Shigella sp | - | - | 1(5.26) | - | - | 1(0.52) |
| unacceptable | 2(4.26) | - | - | 1(1.89) | - | 3(1.55) |
| Vibrio fluvialis | - | 4(7.27) | - | - | - | 4(2.06) |
| Vibrio parahaemolyticus | - | 1(1.82) | - | - | - | 1(0.52) |
| Total | **47** | **55** | **19** | **53** | **20** | **194** |

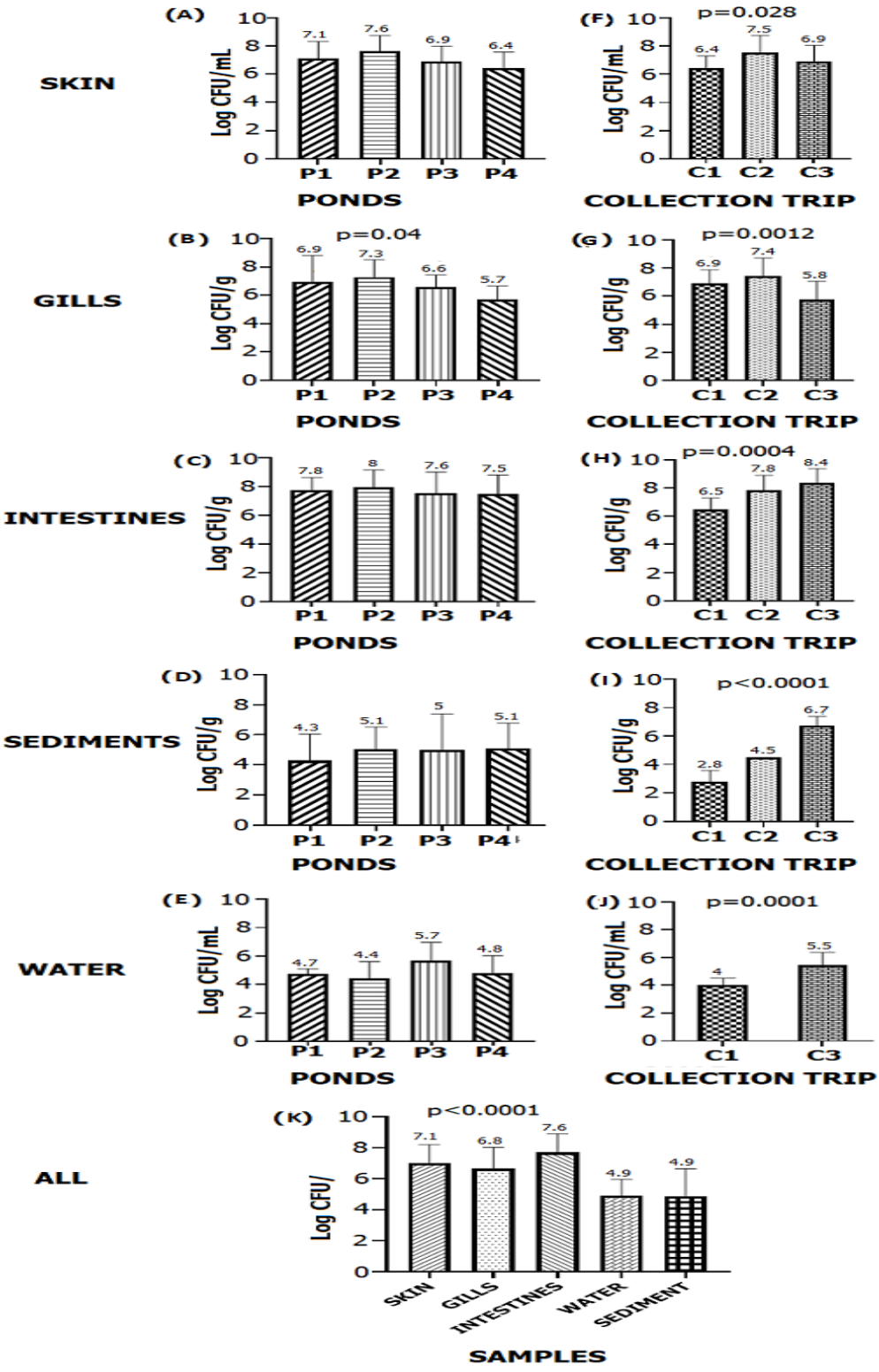


**Figure 1**: pooled total heterotrophic aerobic bacterial load by sample unit across ponds and collection time points. *Data represent the $Log_{10}$ (± SD) CFU per gram or ml of sample. Data columns with different superscripts are significantly different from each other at p<0.05*

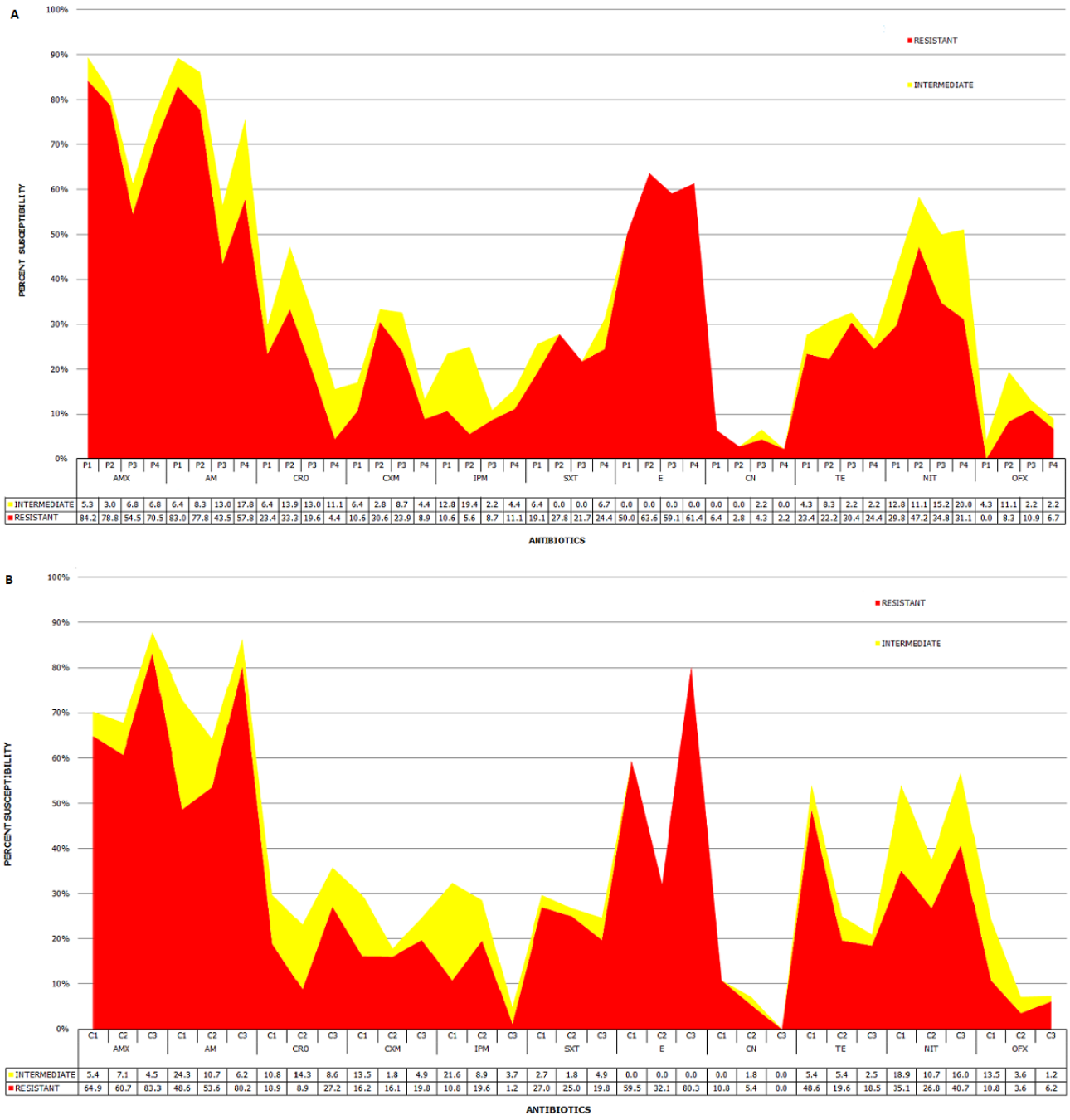


**Figure 2**: antibiotic resistance profile of isolates (A) across ponds and (B) across collection time points. (*AMX (Amoxicillin), AM (Ampicillin), CRO (Ceftriaxone), CXM (Cefuroxime), IPM (Imipenem), SXT (Sulfamethoxazole and Trimethoprim), E (Erythromycin), CN (Gentamicin), TE (Tetracycline), NIT (Nitrofurantoin), OFX (Ofloxacin*))

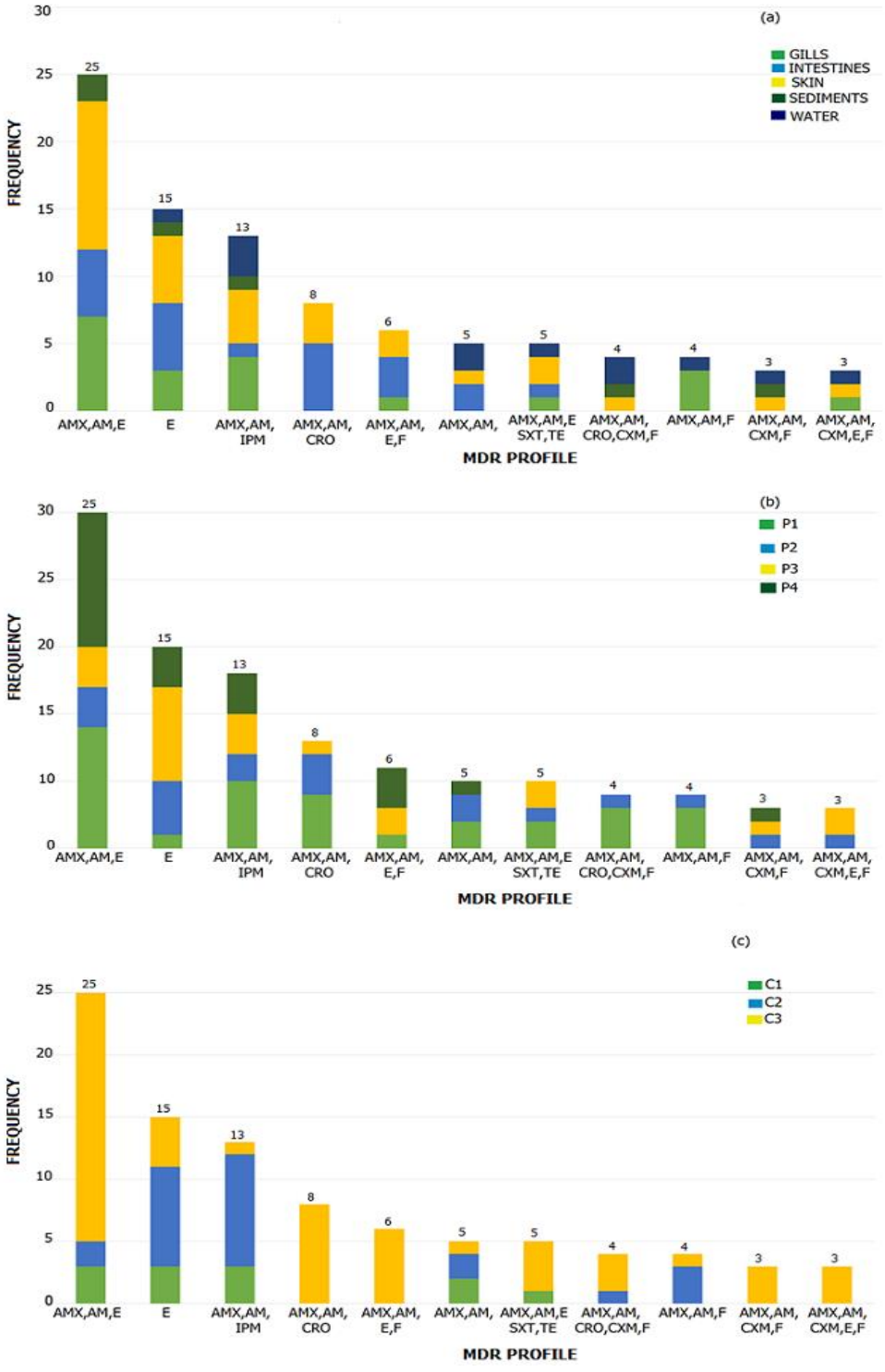


**Figure 3**: most frequent multiple drug resistant profile distributed across (A) fish parts and pond environment; (B) ponds and (C) collection time points. (*AMX (Amoxicillin), AM (Ampicillin), SXT (Sulfamethoxazole and Trimethoprim), CXM (Cefuroxime), CRO (Ceftriaxone), IPM (Imipenem), TE (Tetracycline), E (Erythromycin), NIT (Nitrofurantoin*))