World Applied Sciences Journal 35 (2): 168-176, 2017
ISSN 1818-4952

DOI: 10.5829/idosi.wasj.2017.168.176

Assessment of Metal Pollution and Antimicrobial Resistance in Bacterial Species Isolated from Aquaculture Sources South Eastern Nigeria

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Abstract: This study assessed the bacteriological and metal qualities of fish ponds in Afikpo, Southeast, Nigeria between June and October, 2015. Water samples was collected from four fish ponds and the isolated bacteria were biochemically identified and antimicrobial susceptibility patterns and metal pollution was determined. Bacteria count ranged from 1.52 - 3.29×10^7 CFU/mL. The highest number of colonies was seen in the water obtained from Mavchi fish farm (3.29×10^7 CFU/mL) followed by Akunna fish farm (2.81×10^7 CFU/mL) and Ahaobi (1.92×10^7 CFU/mL), while the least was from Cincom fish farm (1.52×10^7 CFU/mL). In general, bacteria isolated from fish ponds showed higher multiple antibiotic resistances. Resistance was widespread for nitrofurantoin, clarithomycin and ampicillin, while there was general resistance among members of the *E. coli* and *Salmonella* spp All the metals tested were found to be present in the water samples although at a level below the WHO permissible limit. The presence of these multi-drug resistant strains in the fish ponds could act as a vehicle to disseminate antibiotic resistant bacteria to humans.

Key words: Antimicrobial Agent・Bacterial Counts・Fish Pond・Prophylactic Agent・Sampling Area

INTRODUCTION

Aquaculture is extremely valuable in developing countries. It serves as a chief source of protein to population, income generation, employment and collateral for obtaining loans.

Aquaculture production is increasing at about 9.25% per year [1] and the FAO had previously estimated that half of the world's seafood demand will be met by aquaculture in 2020 [2].

Aquaculture in Nigeria hangs essentially on catfish culture and its development which will enhance fish supply. Although aquaculture is growing rapidly in Nigeria disease prevention and treatment practices are far from standardization or regulation. However, the use of antimicrobial agent in aquaculture has resulted in increased prevalence of resistant bacteria.

Over the past few years, aquaculture has intensified and diversified leading to movement of animal and animal products such as fingerlings (seeds) and feeds which are largely responsible for the introduction and spread of pathogen and disease into aquaculture system. The use of antimicrobials in aquaculture as a profitable commercial activity has been supported which may result in potential risks for the output of fish farming [3]. Thus, antibiotic therapy remains important as a prophylactic agent and/or a therapeutic method, especially considering the cost-effective aquaculture production chain [4].

However because of the need to control diseases and also to enhance productivity of the aquaculture products, antimicrobial agents have been introduced as food components or directly into water used for culture [5]. As a consequence, there is a deposition of antimicrobial residues in sediments, resulting in continuous selective pressure, thereby stimulating the sediment microbiota towards the selection of resistant bacteria [7,8]. In addition, resistance genes are highly persistent and do not disappear from aquaculture sites, even after several years without antibiotic use [9].

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Along with the concerns associated with using antimicrobial drugs, toxic metals, often introduced into the environment via anthropogenic or natural sources, may also interfere with different bacterial communities. It is well established that environmental metal contamination has an important role in the maintenance and proliferation of antibiotic-resistant bacteria due to co-selection mechanisms [10]. Unlike organic pollutants, chemical and biological agents, toxic metals are not subject to degradation and may remain as agents of selective pressure over long periods [10, 11].

This work aimed to identify microorganisms and their susceptibility patterns to antimicrobial drugs and toxic metals in fish farms inAfikpo area in Ebonyi State, Nigeria.

MATERIALS AND METHODS

Description of Study Area: Afikpo, also known as ‘Ehugbo’ is a town and the second largest urban area in EbonyiState, Nigeria. It is the headquarter of Afikpo North Local Government Area. It is situated in the Southern part of Ebonyi State and is bordered to the North by the town of Akpoha, to the South by Unwana, to the South west by Edda in Afikpo South Local Government Area, to the East by the Cross River and to the West by Amasiri. Afikpo spans an area approximately 164 square kilometers in size. Afikpo is a hilly area despite occupying a region low in altitude, which rises 350 feet above sea level. It is a transitional area between open grassland and tropical forest and has an average annual rainfall of seventy-seven inches (198cm).(Fig. 1). There are many streams in and around the town and as a result many inhabitants are known to engage in fish farming in addition to agricultural farming. There are also several fish ponds within the town which supplement the fish need of the growing population.

Sampling: Water samples was collected from the four different fish ponds in Afikpo. Five samples was collected from each of the ponds (n= 5) between June – October, 2015. The samples were collected aseptically in sterile 500mL glass bottles from different sampling points by directly dipping the bottles into the surface of the water. The samples were labeled properly and transported on ice to the laboratory for analysis. Aliquots of the samples was used for selective isolation of isolation of targeted bacteria based on standard microbiological procedures [14].

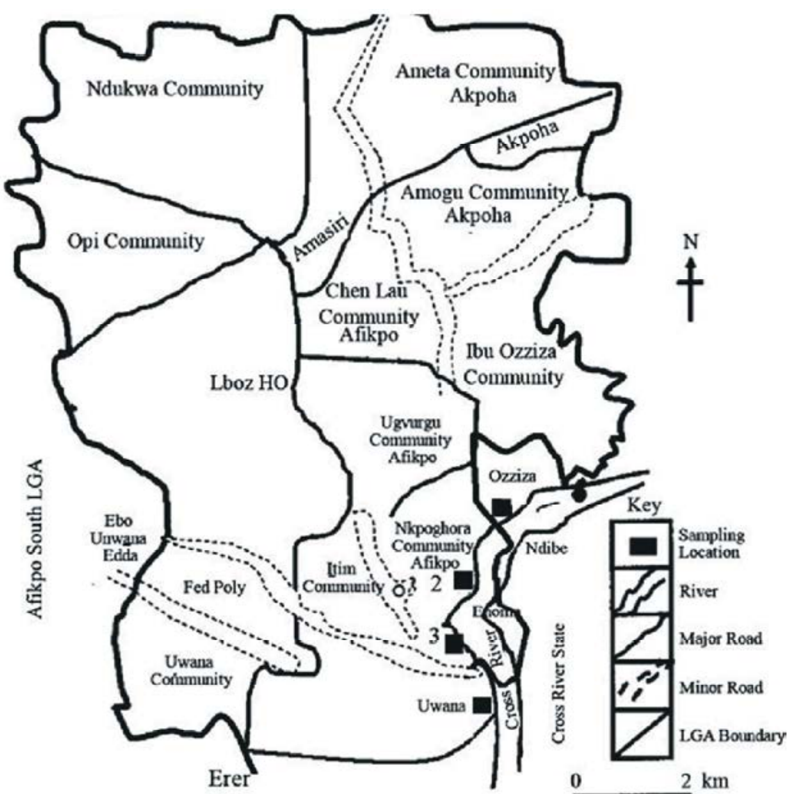

Fig. 1: Map of Afikpo showing the sampling location

Microbiological Analysis

Isolation of Bacteria: Isolation of bacteria from the water samples was aseptically carried out using standard microbiological techniques as described by Cheesbrough [14]. Samples were serially diluted using sterile water after which they were pour- plated on nutrient agar in Petri dishes and incubated at 37°C for 24 hours. After incubation period, colonies, which developed on the plates, were counted and recorded as colony forming units per milliliter (CFU/mL) of the sample. Distinct colonies from the agar stock culture and inocula from the nutrient broth were sub-cultured (using streak plate method) individually on freshly prepared selective and differential media to obtain pure isolates.

The selective and differential culture media used were Salmonella-Shigella agar (for the isolation of *Salmonella* and *Shigella* sp.),Cetrimide selective agar (for the isolation of *Pseudomonas aeruginosa*), Eosin methylene blue agar of *Escherichia coli*. The pure isolates were maintained on agar slants for further characterization and identification.

Identification and Characterization of Isolates: The bacteria isolates were identified by comparing their morphology, microscopy, Gram's reaction and biochemical characteristics with those of known taxa using method prescribed in Cheesbrough [14].

Disc Diffusion Susceptibility Test: Bacteria isolates were subjected to in-vitro susceptibility test against commonly used antimicrobial agents using disk diffusion method following guidelines established by the Clinical and Laboratory Standards Institute [15]. In brief, by taking pure isolated colony, bacterial suspension was adjusted to 0.5 McFarland turbidity standards. The diluted bacterial suspension was then transferred to Mueller-Hinton agar plate using a sterile cotton swab and the plate was seeded uniformly by rubbing the swab against the entire agar surface followed by 24 h incubation. After the inoculums were dried, antibiotic impregnated disks were applied to the surface of the inoculated plates using sterile forceps. The plates were then incubated aerobically at 37 °C for 24 h. Finally, the zone of inhibition was measured including the disk diameter. The susceptible and resistant categories were assigned on the basis of the critical points recommended by the CLSI and according to the manufacturer's leaflet attached to them. The standard antibiotic discs (Oxoid, England) and their concentrations used against the isolates were Nitrofurantoin (NIT) – 10 µg, cetriaxone (CET) – 30 µg, ciproloxacin (CIP) – 10 µg, gentamycin (GEN) – 30 µg, augmentin (AUG) – 10 µg, Perfloxacin (PEF) – 30 µg, ampicillin (AMP) – 30 µg, clarithomycin(CLA) – 10 µg, chloramphenicol (CHL) – 30 µg and ofloxacin (OFL) – 30 µg. These antibiotics were chosen because they are either used in both human medicine and animal veterinary practice or because previous studies have reported microbial resistance to them.

Determination of Metals in Fish Pond Water: Water sample was taken below the water surface from the fish pond using one (1) liter acid-leached polythene bottles. About 0.5L of the water samples were taken at each sampling site. Samples were acidified with 10% HNO_3, placed in an ice bath and brought to the laboratory. The samples were filtered through a 0.45ìm micropore membrane filter and kept at 4°C until analysis. Metals (Mg, Na, Cu, Fe, K) was determined in the water samples using an Atomic Absorption Spectrophotometer, Perkin Elmer Model 306.

Statistical Analysis: Data obtained was presented as mean ± SE (standard error). Significance of difference between different treatment groups was tested using one-way analysis of variance (ANOVA) and significant results were compared with Duncan's multiple range tests using SPSS window 7 version 1.6 software. For all the tests, the significance was determined at the level of $P<0.05$.

RESULTS

Total Bacterial Count: The result of the mean heterotrophic bacteria count from the ponds were computed and are presented in table 1. From the results, the number of colonies per ml for the four (4) sample sites ranged from 1.52 - 3.29×10^7CFU/mL. The highest number of colonies were seen in the water obtained from Mavchi fish farm (3.29×10^7CFU/mL) followed by Akunna fish farm (2.81×10^7 CFU/mL) and Ahaobi (1.92×10^7CFU/mL), while the least was from Cincom fish farm (1.52×10^7 CFU/mL) (Table 1).

Table 1: Total mean count (CFU/mL) of bacterial isolates

S/N	Sample point/location	Colony count (CFU/mL) ($\times 10^7$)
1	Mavchi	3.29
2	Cincom	1.52
3	Akunna	2.81
4	Ahaobi	1.92
	Mean	2.39

Antimicrobial Resistance: The result of the antibiotics susceptibility studies of the isolates showed that all the bacterial isolates exhibited varied degree of resistance to the antibiotics. Generally, resistance was widespread for nitrofurantoin, clarithomycin and ampicillin, almost all the isolated organisms was completely resistant to them. While, there was general resistance among members of the *E. coli* and *Salmonella* species (Fig. 2,3,4,5).

Analyzing the resistant profile of each of the isolates from four different fish ponds it was observed that, all the isolates had multiple resistance with at least four antibiotics out of the ten antibiotics used against them,

From Mavich fish pond it observed that, *Salmonella* spp was resistant to four antibiotics: nitrofurantoin, gentamicin, clarithromycin and ampicillin, while *E. coli* was resistant to five of the antibiotics: ciprofloxacin, gentamicin, augmentin, perfloxacin and ampicillin, *Shigella* spp was resistant to, nitrofurantoin, clarithromycin, chloramphenicol and ampicillin, while *P.aeruginosa* was resistant to ofloxacin, nitrofurantoin, ceftriaxone, augmentin and ampicillin (Fig. 2).

Results from Cincom fish farm showed that all the isolates were resistant to four to five antibiotics out of the ten antibiotics used against them, while among the antibiotics there was complete resistance of all the isolates against clarithromycin and nitrofurantoin (Fig. 3).

Similar trend was equally observed in the remaining two fish ponds, Akunna and Ahaobi farms, were there was equally multi-drug resistance of the isolates to more than 3 antibiotics, with almost all the isolates completely resistant to clarithromycin and ampicillin (Fig. 4, 5).

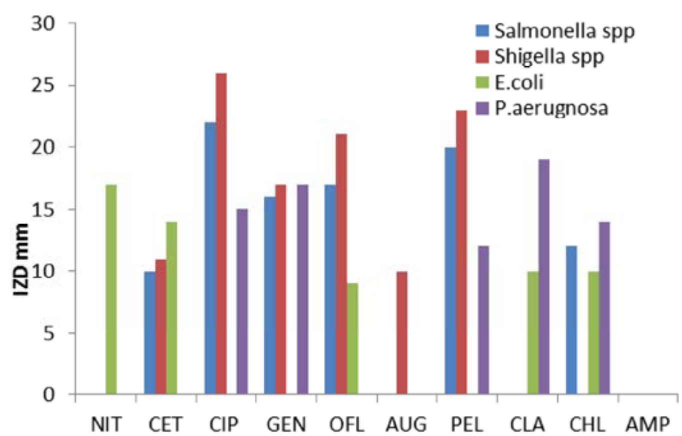

Fig. 2: Antibiotic resistance pattern of bacterial isolates from Mavchi Pond
KEY: IZD = Inhibition Zone diameter(mm); NIT = Nitrofurantion, CET= Cetriaxone, CIP= Ciprofloxacin, GEN=Gentamincin, OFL = Ofloxacin, AUG = Augmentine, PEL = Pefloxacine, CLA = Clarithomycin, CHL= Chloramphenicol, AMP = Ampicilin.

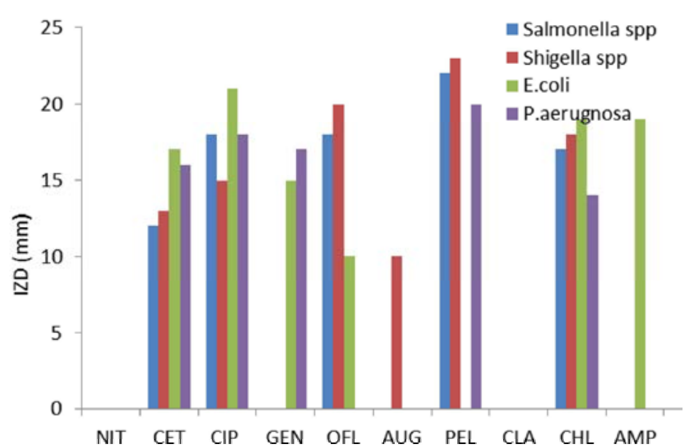

Fig. 3: Antibiotic resistance Pattern of Bacterial isolates from Cincom Fish Pond

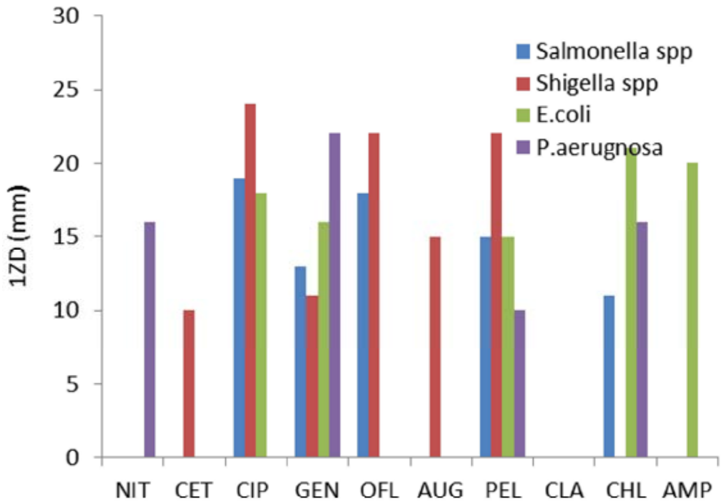

Fig. 4: Antibiotics resistance pattern of bacterial isolates from Akunna Fish Pond

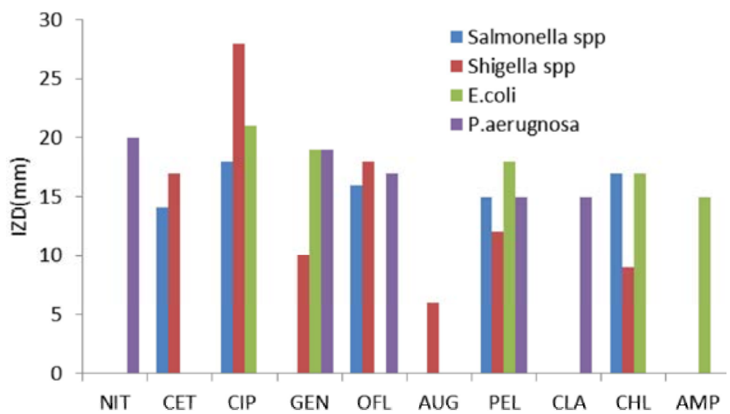

Fig. 5: Antibiotic resistance pattern of bacterial isolates from Ahaobi fish Pond

Metal Quality of Water Samples from the Ponds: The result of metal concentration in water samples from the different fish pond are presented in Fig. 6-10. Analysis of the different metals from different sample collection points showed some slight variation in the level of the concentration of the metals found in them. The result obtained for iron showed that Akunna had the highest concentration (0.78±0.01), it was followed by Mavchi fish pond (0.58±0.07), then Ahaobi(0.37±0.06), while the least concentration (0.23±0.09), was recorded from Cincom water samples, statistically there was no significant difference at $P < 0.05$ for the values obtained from the ponds (Fig. 6).

Also, the result obtained for copper showed that Ahaobi had the highest concentration(0.305±0.01), it was followed by Akunna fish pond (0.205±0.01), then Cincom (0.2±0.04), while the least concentration (0.12±0.03), was recorded from Mavchi water samples, statistically there was no significant difference at $P < 0.05$ among the different values obtained from the ponds (Fig. 7).

The concentration of magnesium in the four water samples had similar trend to that of copper, the highest concentration of magnesium was as well highest in Ahaobi (1.16±0.12),it was followed by Akunna (1.±0.15), then Cincom (0.52±0.07) and the least concentration was recorded in Mavich(0.44±0.56), there was no statistical difference between the values obtained from the four samples sites (Fig. 8).

Potassium was highest in Cincom fish pond (0.59±0.09), followed by Mavich with (0.30±0.04), then Ahaobi had (0.16±0.04), while the least concentration was obtained from Akunna with (0.06±0.06)(Fig.9) While, in sodium concentration, the reverse was the case, Ahaobi recorded the highest concentration of sodium(0.21±0.01), followed by Mavchi(0.14±0.02), then Akunna(0.13±0.02), while Cincom had the least value of (0.12±0.07) (Fig.10).

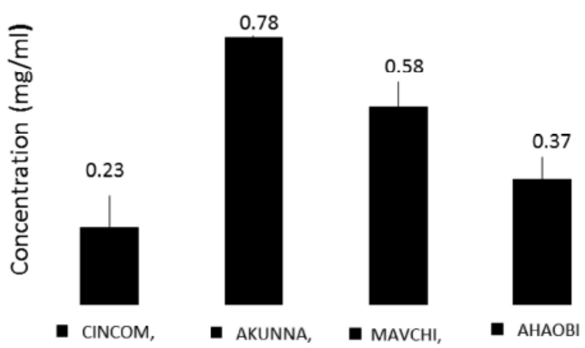

Fig. 6: Concentration of iron in different fish Ponds in Afikpo

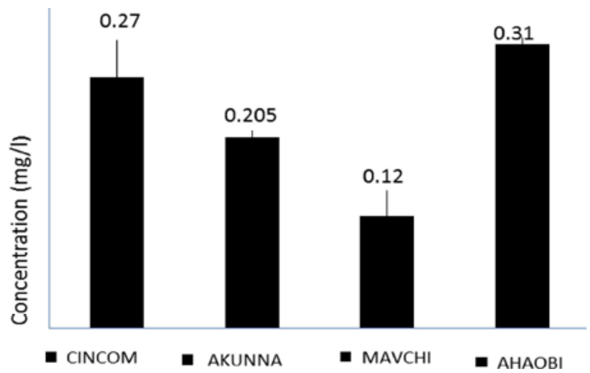

Fig. 7: Concentration of copper in different fish Ponds in Afikpo

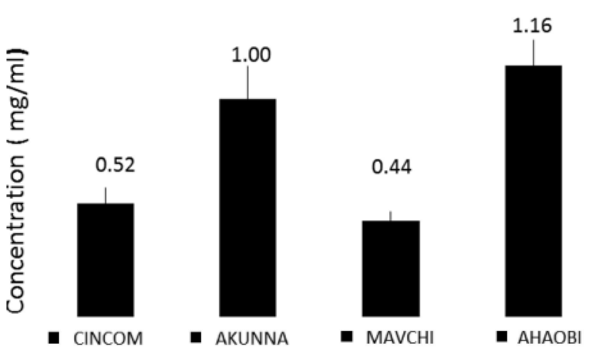

Fig. 8: Concentration of magnesium in different fish Ponds in Afikpo

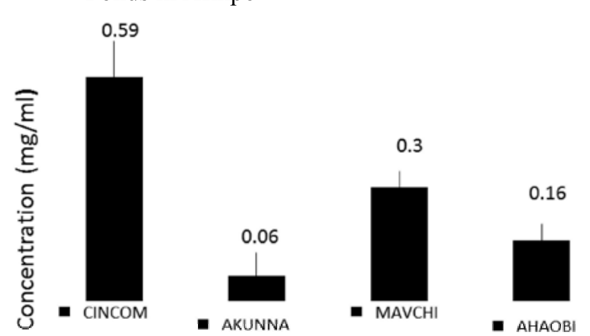

Fig. 9: Concentration of pottasium in different fish Ponds in Afikpo

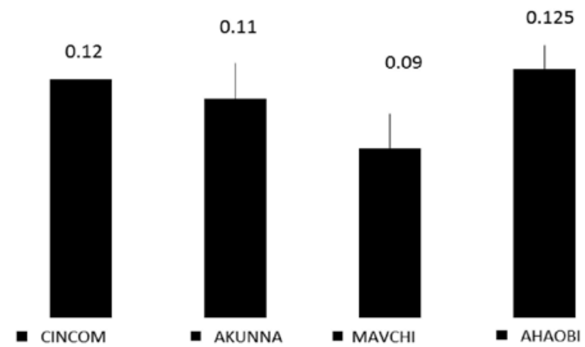

Fig. 10: Concentration of Sodium in different fish Ponds in Afikpo

DISCUSSIONS

In view of the rapid growth of aquaculture fish business in Nigeria and also because of the weak regulatory controls of the use of antibiotics and toxic metals often introduced into the environment. Isolation of antibiotic resistant bacteria from aquaculture products and aquaculture environment indicates the health risk associated with the aquaculture. There had been reports on detection of antibiotic resistance genes in bacteria isolated from aquaculture products that can be transferred to human microbiota. [16].

The result of the mean heterotrophic bacteria count from four fish ponds sampled were computed and are presented in table 1. From the results, it indicated very high microbial loads. The high number of bacteria load in the fish pond suggested that the bacteria have a consistent source of entry into the pond and not by chance inoculation alone. Chicken excreta and intestines, cow and pig extract are used generally by many of the farmers to enrich the pond in place of the very expensive feeds and since they contain high bacteria loads, it is likely they may be among the principal source of these organisms because of the fact that they were not in any way treated before they were dump into the pond [17&18].

Five species of bacteria were targeted and isolated. The bacteria isolated from the waters were *Escherichia coli, Pseudomonas aeruginosa, Salmonella* and *Shigella* species. The presence of faecal coliform like *E. coli* in some samples is an indication of recent pollution by sewage from untreated domestic and poultry wastes normally used as sources of feeds.

In antibiotic resistance analysis of the isolated bacteria, it was observed that varieties of resistance patterns was observed in the study, the result could not be correlated with antibiotic use as none of the antibiotics used are actually registered to use in aquaculture in

Nigeria. However, the result of this study is not different from those seen in countries where antibiotics are known to be used in aquaculture. There was resistance to majority of the antibiotics used in this study, with much resistance widespread in nitrofurantoin and ampicillin among all the isolated bacteria. Also, high percentage of nitrofurantoin and ampicillin resistance as observed in this study is similar to earlier study by Zhang *et al* [21], in their study on antibiotic resistance detection in *E. coli* strains isolated from two different aquaculture systems in South China. Hatha *et al.*[22] also recorded high resistance of ampicillin. In another study, identified isolates from mangrove soil in Malaysia was 100% resistant towards ampicillin and penicillin, while 77.8% of the isolates were resistant towards streptomycin Jalal *et al*. [23]. Son *et al*. [24] in their study on *Aeromonas hydrophila* isolated from *Tilapia mossambica* recorded 100% ampicillin resistance and 57% streptomycin resistance. Abdullahi *et al.*[25] observed 100% ampicillin resistance in *Pseudomonas* spp. isolated from Sarawak aquaculture environment. A study carried out by Kathleen *et al.* [26] and Akinbowale *et al.* [27] recorded 56.8% and 54.8% ampicillin resistance on aquaculture bacteria respectively.

However, in this study moderate susceptibility was observed towards gentamicin, fluoroquinolones and chloramphenicol when compared to other antibiotics used in the study. Similarly, Lim and Kasing [28] and Hatha *et al.*[22] in their respective studies observed that almost none of the bacteria tested were resistant to gentamicin and chloramphenicol. Low frequency of antibiotic resistant bacteria may indicate the less activity associated with the contamination of antibiotics in the area. The use of chloramphenicol in aquaculture has been banned in certain countries including Malaysia, Korea and Japan since 1983 [29]

In general, the results obtained from this study showed resistance to more than one class of antibiotic. Multiple drug resistance has been reported in a number of studies of fish pathogens and aquaculture environments [22].

The result of the assessment of the level of metal pollution in the fish ponds showed that all the metals tested were present in the pond. The result of iron concentration as obtained from the study showed variation from the different pond waters, but such variations were not statistically significant at $P = 0.05$. The iron content of the samples were found to be higher than the WHO (0.3 mg/L) standard in three of the four fish ponds sampled, the highest was in Akunna pond with concentration of (0.775±0.01, followed by Mavchi (0.575±0.07) and Ahaobi (0.365±0.0), while Cincom had the lowest concentration 0.225±0.09 which is below the WHO Standard value (Fig. 6). Fe is involved in the haemoglobin synthesis in the red blood corpuscles of the blood, Fe also help with red blood cell production. It is a necessary element in human diet and plays a significant role in metabolic processes, though an essential element, it has the tendency to become toxic to living organisms, even when exposure is low.

The result of the copper concentration from the ponds showed that the values in the four ponds varied but were not statistically significant and also the values were below WHO World Health Organization (1.00 - 2.00 mg/L) limits (Fig.7). Copper is used in aquaculture for a variety of purposes, including control of the blue-green algae responsible for off-flavors in culture animals, treating certain diseases and parasites, eliminating mollusks from ponds and avoiding fouling of fish cage netting [30]. Although copper is an essential nutrient for plants and animals, an excess can negatively affect the environment and human health. The amount of copper in aquaculture products does not appear to be harmful to humans. However, excessive exposure to copper in humans can cause nose, mouth and eye irritations, as well as headaches. Chronic exposure can lead to Wilson's disease and liver, brain, nervous system, kidney and eye damage. Also, excessive copper concentrations in the environment can be toxic to plants, contaminate forage and harm livestock and other animals and damage the soil biota. Elevated copper concentrations in water can harm fish and other aquatic life. [30]. From the result recorded in this study, the concentrations of copper from the four ponds were all below the WHO (1.00- 2.00 mg/L) permissible limit.

Magnesium was present in the ponds and the concentration ranged from 0.44±0.56 mg/L - 1.16±0.12 mg/L(Fig. 8), the value from our study is lower than those obtained by other authors. Ehiagbonare and Ogurinde [31] and Trivery and Khatavker [32] reported magnesium concentration which ranged from 1.21 – 5.46 mg/l and 7.32 – 18.00 mg/l respectively and these are higher values than the result of this study. Also Desia [33] reported higher value of 70 mg/l. Magnesium element contributes to both carbonate and non-carbonate hardness in water usually at a high concentration much lower than that of the calcium component. Magnesium is essential for bone formation, for reproduction and for normal functioning of the nervous system and it is also known to be a part of the enzyme system [34].

The values obtained for sodium and potassium fall within the WHO/USEPA guidelines [35]. Sodium and potassium are needed in small amount in fish pond. Optimum concentration of this element is unknown, however it is readily available and needed in stimulating the growth of aquatic flora. Sodium controls body water balance and plays a role in muscle contraction. Potassium spares sodium in the human body and its concentration in food when higher than sodium has a nutritional advantage.

CONCLUSION

The results of this finding present a valuable baseline data on the bacteria and metal contamination of fish ponds in Afikpo, area of Ebonyi State. It was observed that the pond water was contaminated with multi- drug resistant bacteria and also that some of metals analysed were found in the pond water. However the concentrations of majority of the metals recorded in the water samples were below the WHO & FEPA recommended limits. Also, the results confirm that aquaculture as currently practiced may have important public health consequences due to the occurrence of antimicrobial resistance pathogenic bacteria. Further prospective studies are needed to better assess microbial diversity and sanitary risks in the aquaculture environment, since aquaculture fish farming is increasingly becoming one of the most profitable commercial activities in Nigeria

ACKNOWLEDGEMENT

The author is grateful to the owners of the fish ponds who allowed the use of their facilities for the research. Funding for this study was from personal contributions from the author, no external funding.

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