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Comparative Study of the Microbiota of Fish Ponds in Awka, Anambra, Nigeria

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ABSTRACT

A pond is a man-made or natural water body that is between 1 m^2 and 20,000 m^2 in area and holds water for at least four months of the year or all year around, depending on geographic location. Since the health issues associated with fish have become a threat to human life, A comparative analysis was carried out among fish ponds in Awka metropolis. The locations chosen were: Aroma fish pond, Kamu fish pond, Unizik fish pond, and Agu-Awka fish pond. The samples were diluted serially and cultured in the laboratory using Sabouraud Dextrose Agar and Nutrient Agar for the isolation of fungi and bacterial pathogens, respectively. The fungi isolated from the water samples include *Aspergillus spp., Penicillum spp.,* and *Rhizopus spp. Aspergillus spp.* was the most prevalent in the four sample sites. The bacterial plate count ranges from 9.8 x 10^3 to 1.62 x 10^4 cfu/ml. The bacteria isolates were identified on the basis of their colonial and morphological features as the species Enterobacter spp., *Streptococcus spp., Klebsiella spp., Bacillus spp.,Escherichia coli, and Staphylococcus aureus. Escherichia coli* was isolated from the four different samples. This analysis was done to determine the prevalence of fish diseases in fish farming. Fish diseases are one of the most important problems that severely affect the economic balance of aquaculture farmers as well as the consumption of fish by humans. The study suggests the need for periodic water quality control in fish ponds in order to ensure fish safety and prevent the transmission of potential pathogens to humans.

Keywords: Fish Farming, *Aspergillus spp*, *Penicillum spp* and *Rhizopus spp*.

INTRODUCTION

Much of the earth's surface is occupied by water and about 71% of the Earth's surface is covered by water, with continents and islands making up the remaining 29% (Agu *et al.,* 2014; Agu *et al*., 2017; Agu *et al*., 2021). A pond is a man-made or natural water body which is betweenlm^2 and 20,000m^2 in area in area, which holds water for at least four months of the year or all year around depending on geographic locations (Gogoi and Sharma, 2013). It is also a body of standing water, either natural or manmade that is usually smaller than a lake. They may arise naturally in floodplains as part of a river system. The type of life in pond is generally determined by a combination of factors including water level regime(particularly depth and duration of flooding) and nutrient levels, but other factors may also be important, including presence or absence of shading by trees, presence or absence of streams, effects of grazing animals, and salinity. Pond waters are also facing pollution just like other water bodies are getting polluted due to discharge of effluents from various industries, domestic waste, land and agricultural drainage resulting in the degradation of water quality of these water resources (Rajiv *et al.,* 2012).

In human health, water plays a very important role and quality of the water supplied is important in determining the health of individuals and the whole communities. Safe water quality is a major concern with reference to public health importance as health and well-being of the human race is closely tied up with the quality of water used (Rajiv *et al.,* 2012). Recently, epidemics of cholera have been reported from different parts of the World, including India, Nigeria and Zimbabwe. The outbreak was caused by *Vibrio cholera* isolated from municipal taps and wells (Uzoigwe and Agwa, 2012). Outbreaks of typhoid fevers and dysentery were linked to unsanitary

mixing of some water supplies and sewage. The World Health Organization (WHO, 2003) has also reported that, 80% of sicknesses and deaths among children in the world are caused by unsafe drinking water.

Estuaries are unique in many respects, their calm, nutrient rich water, serves as nurseries for juvenile forms of many commercially important fish and invertebrates. Studies have revealed that the microbial flora of caught fish and other aquatic specimens is largely a reflection of the microbial quality of the water where they have harvested (Pelczar*etal*., 2008). Fungi were originally found as part of the plant kingdom and grouped to the kingdom protista, but in 1969, Whittaker argues that there multicellular nature meant they should be in their own kingdom. Fungi cells are different from the cells of plant not only by absence of chloroplast but forming a cell wall that possees chitin instead of cellulose (Sylvia, 2001).

Fungi are a group of organisms called heterotrophs that require living or death matter for growth and reproduction. Unlike plants they are incapable of manufacturing their own nutrient by photosynthesis. The fungal spore is like seed which is resistant to heat, drying disinfectants and the natural defense system of aquatic organisms and fishes in water due to this they are capable of causing diseasesin fish (Ruth *et al*., 2006).

Bacteriological monitoring is based on knowledge of the sanitary condition of the water supply, which is based on the detection of coliform bacteria and the specific indicator of humanfeacalcontamination, *Escherichia coli* (Idakwo and Abu, 2004). The term "indicator organisms" are used in water analysis, which refers to microorganisms whose presence in water shows that the water is polluted with feacal material from humans or other warm-blooded animals (Pelczer*etal.,* 2008). This kind of pollution means that the opportunity exists for the various pathogenic organisms, which periodically occur in intestinal tract, to enter the water, such water is described as non-potable water, and it is not safe for drinking. The use of intestinal organisms as indicators offaecal contamination is a universally acceptable process for monitoring and assessing the microbiological safety of water supply before

distribution. Coliform bacteria are a group of intestinal bacteria used as indicators to determine if treated water is acceptable for human consumption. Coliforms will not likely cause illnesses. However, the presence of coliforms in drinking indicates the presence of disease-causing organisms (Nwachukwu and Otokunefor, 2006). The Coliform includes the members of the family Enterobacteriaceae, e.g. *Escherichia coli, Enterobacteraerogenes, Salmonella* and *Klebsiella.*

This study is aimed to isolate and characterize microorganism associated with fish ponds.

MATERIALS AND METHODS

3.1.1 Study area

Awka town, Anambra State, South Eastern Nigeria is situated along the highway between Enugu and Onitsha. The four fish pond effluents where located in this area. They were constructed with blocks and tarpaulins.

3.2.2 Collection of Samples

The fish ponds samples were aseptically collected from four different fish ponds in Awka town, in Awka South Local Government Area of Anambra State, Nigeria. Samples were collected in 250ml sterile sample containers and appropriately labeled. It was taken to the Microbiology Laboratory of NnnamdiAzikwe University for analysis.

3.2.3.Isolation of Microorganisms

The pour plate method was used to isolate microorganisms from the fish ponds under aseptic conditions. The sample was diluted serially in 3 test tubes each containing 9ml of sterile distilled water each. 0.1ml each of the 10^{-2} and 10^{-3} was transferred using pipette into steriliepetric dishes (in duplicate) then, about 15ml of cool liquefied Sabouraud Dextrose Agar (SDA) and Nutrient Agar (NA) media and antibacterial and antifungal agents (choramphenicol and Nystatin) were added into the plates and gently swirled to mix the aliquot and the media. These plates were allowed to incubate for 24-72hours at room temperature. Developing microorganism were

purified by repeat subculture techniques. Pure cultures were maintained on agar slope at 4^0C in refrigerator.

3.3 Characterization by Identification of the isolates

This was done based on cultural, morphological and biochemical characteristics of the various isolates obtained. Isolates were picked from the stock culture and subcultured on a nutrient agar and incubated at 37^0C for 24 hours.

The bacterial strain was identified by microscopic examination (Gram Staining and Motility test) and Biochemical tests such as Citrate utilization test, Methyl red test, Indole production, motility, sugar fermentation test, spore forming test and catalases test.

3.3.1 Gram staining

This method was first described by Christian gram. This is the most widely use method for characterizing bacteria as it divides it into two groups: gram negative which is red or pink in colour and gram positive which stains purple

Procedure

• A smear of an isolated was made on a slide with normal saline, allowed to dry and heat fixed by passing over a bursen burner.

• The smear was flooded with crystal violet and left for 1minute before washing with water.

• Lugol’s iodine was added and allowed to stand for 1minute before washing off with water.

• The slide was then decolorized with acetone and allowed to stand for few 5 seconds then washed off with water immediately.

• The slide was finally stained with safranineand allowed to stand for 30seconds and washed off with water.

It was allowed to air dry and viewed under oil immersion lens of the microscope.

3.3.2 Catalase test

Catalase enzyme protects bacteria from hydrogen peroxide accumulation. In this test, small amount of test organism was smeared on a clean grease free slide. Then a drop of hydrogen pereoxide was added to the smear using a pipette. The presence of bubbles indicated a positive result while the absence indicated a negative result.

3.3.3 Motility test

The test was used to check if the bacteria a way from a line of immigration using physical features like flagella. Here, the organism was inoculated into a semi-solid media in a test tube. The slanted media was stabbed in a straight line as possible and it was drawn very carefully to avoid destroying the straight line. After incubation for 24hours the observation were made. Migration away from the line of inoculation showed a positive result. Lack of migration showed a negative result.

3.3.4 Indole test

This test was carried out to differentiate graqm negative rods, especially E. coli. Here, tryptophan is broken down with the release of indole which react with dimethlyllaminobenzqaldehyde. A pure culture was was grown in sterile peptone broth and allowed to grow for 24 hours at 37^0C. Five drops of Kovac reagent (isoamyl alcohol, P- dimethylaminobenzaldehyde, concentrated hydrochloric acid) was added to the culture broth. Positive result were was shown by the presence of a red nor red-voilet color in the surface layer of the broth, while negative result appeared yellow.

3.3.5 Citrate utilization test

Simmon citrate agear was used as the medium. The tetwqas carried out using 5ml of simmon citrate agar medium distributed into various test tubes. It was sterilized by autoclavingat121^0C for 15 minutes and cooled while slanted. Isolates were then innoculatedd by streaking and

incubated for 24hours at 37^0C. Utilization of citrate was indictaed by a color change from green to blue.

3.3.6 Methyl red test

This test is used to determine if glucose can be converted into acidic products like lactate, acetate and formate. The test was carried was carried using methyl red media which was distributed into test tubes and autoclaves at 121^0C for 15minutes and cooled. The text organism was inoculated in the test tube for 37^0C for 48 hours. Five drops of methyl red reagent was added and mixed properly. Red color change indicated positive results while yellow indicated negative test results.

3.3.7 Coagulase Test

Coagulases are enzymes that clot blood plasma and they produced by *Staphylococcus aureus.* The enzyme protease converts fibrinogen to fibrin resulting to blood clotting. The Slide method was used. Here, clean slide was divided into two sections, to one section the test organism was smeared on it using a sterile wire loop while a drop of distilled water was added to the other section serving as control. Then human plasma was added to both sections and the slide was rocked gently for some minutes. A clumping/agglutination of the plasma indicate the presence of coagulase.

3.3.8 Spore formation test

This test is used to determine if the organism sporulates. Smears of organism to be tested for presence of endospores was taken on a clean microscopic slide and air dried. It was heat fixed and a small piece of blotting paper was placed over the smear and slide was placed on a wire guaze on a ring stand. The slides were heated gently till evaporation was noticed and then placed over a boiling water bath. It was heated again and as the paper began to dry 2-3 drops of malachite green was added and left for 5minutes. The blotted paper was then removed and rinsed off then stained with safranin for 2minutes. It was rinsed and air dry then viwed under the microscope.

3.3.9 Sugar fermentation test

The test is used to detect which organism has the ability to utilize different sugars as source of carbon and energy with the production of either acid alone or acid and gas. The sugars used were glucose, lactose and maltose. Peptone water broth was prepared and bromothymol blue indicator solution was added including the test sugars. The preparation was dispensed into test tubes containing inverted test tubes. It was sterilized in the autoclave for 15 minutes at 121^0C and cooled. The test organism was inoculated into each test tube and incubated for 24hours. Acid production was indicated by color change of the medium while gas production was indicated by avoid in the durham tubes.

3.4Characterization and identification of the Fungal Isolates

3.4.1 Macroscopy

This was carried out by observing the morphological characteristics like underside, pigmentation and growth rate of the fungi on the plate. The identification of fungi and fungus-like organisms involve the observation of morphological features such as shape, size of hyphae, shape of sporangia, conidia, conidiophore and spores

Microscopy

The improved slide culture technique of Agu and Chidozie (2021) was employed in this study. A sterile glass slide was placed on the bottom of a sterile petri dish. With the aid of a sterile 2 ml syringe, 0.5 ml of the molten Saboraud Dextrose Agar (SDA) maintained at 45 ^{O}C in a water bath was dispensed on the sterile glass slide. The cover of the petri dish was replaced and the molten agar allowed to gel. Upon gelling, a sterile inoculation needle was used to inoculate the agar bump with a small amount of fungus at the centre of the bump. Therefter, a heat-sterilized coverslip was laid just over the agar bump without pressure. The plates were incubated at room temperature for 3 to 5 days depending on the growth rate of the fungus. When desired growth was observed, few drops of Lactophenol cotton blue stain was dropped at the

interface of the developing cultures on the slide and the coverslip so as to preserve the integrity of the culture and allowed to permeate the entire culture before viewing under the microscope. Referencing was done using Fungal Atlases (Barnett and Hunter, 2000; Watanabe, 2002; Ellis *et al*., 2007).

RESULTS

Table 4.0.1 microbial count of the water sample from fish pond.

Site	Sample	Bacteria plate count (cfu/ml)	Total coliform count (cfu/ml)	Feacal coliform count (cfu/ml)	Mold
Site A	Aroma fish pond	8.8×10^3	4.2×10^3	3.4×10^3	7
Site B	Kamu fish pond	5.1×10^3	3.1×10^3	18×10^3	5
Site C	Unizik fish pond	8.6×10^3	4.6×10^3	3.2×10^3	9
Site D	Agu-awka fish pond	1.12×10^4	7.7×10^3	4.4×10^3	11

Table 4.0.2: Biochemical characteristics of the isolates

Colony morphology	Gram reaction	Citrate utilization	Motility test	Coagulase test	Catalase test	Indole test	Methyl red test	Oxidase test	Spore test	Glucose fermentation	Sucrose fermentation	Lactose fermentation	Organism
Whitish medium sized mucoid colonies	+ (R)	+	+	-	+	-	-	-	+	A	AG	-	*Bacilliusspp*
Large colonies with golden-yellow color	+ (C)	-	-	+	+	-	+	-	-	A	AG	A	*Staphylococcus aureus*

Large mucoid colonies with grayish-white	- (R)	-	+	-	+	+	+	-	-	AG	-	AG	*Escherichia Coli*
Circularsmoth colonies white	+ (C)	-	-	-	-	-	-	-	-	A	AG	A	*Streptococcus spp*
CircularYellowconvex colonies	- (R)	+	+	-	+	-	-	-	-	AG	AG	AG	*Enterobactersp p*
Large mucoid colonies, shinny and dark-pink color	- (R)	+	-	-	+	-	-	-	-	A	A	AG	*Klebsiellaspp*

+ = Positive R= rod

- = negative C= cocci

A = Acid Production

AG = Acid and Gas Production

Table 4.0.3: occurrence of the isolates in different sites sample

Isolates	Site A	Site B	Site C	Site D
Bacilliusspp	+	-	+	-
Serratiaspp	-	+	-	+
Staphylococcus spp	+	-	+	+
Escherichia coli	+	+	+	+
Streptococcus spp	-	+	-	+
Enterobacterspp	+	-	+	+
Klebsiellaspp	-	+	+	+

Salmonella spp	+	+	+	+

+ = presence

- = absence

Table 4.0.4: Percentage frequency of occurrence of bacteria isolates in the different samples

Isolates	Site A		Site B		Site C		Site D	
	Number of colonies isolated	Frequency of isolation (%)	Number of colonies isolated	Frequency of isolation (%)	Number of colonies isolated	Frequency of isolation (%)	Number of colonies isolated	Frequency of isolation (%)
Escherichia Coli	34	38.64	18	35.29	32	37.21	44	39.29
Staphylococcus aureus	18	20.45	0	0	17	19.77	18	16.07
Bacilliusspp	8	9.09	0	0	7	8.14	0	0
Enterobacters pp	28	31.82	0	0	15	17.44	23	20.54
Klebsiellaspp	0	0	22	43.14	15	17.44	21	18.75
Streptococcus spp	0	0	11	21.57	0	0	6	5.36
Total	88	100	51	100	86	100	112	100

Table 4.0.5: Characteristics of the mold Isolates

Isolates	Colony colour on the surface	Microscope features	Texture	Organism
1	White to blue-green or grey green ate the centre with white patches surrounding it	Hyphae are septate while conidiophores are branched which are terminated by metullae carrying flask-shaped phialides which form brush-like	Velvety	*Penicilliumspp*
2	Whitish, becoming brown-black with age	Non-septate mycelia with sporagiosphores which were directly opposite to branched rhizoids. Sporangia and the columella were subglobose while the sporangiophores were smooth-walled.	Wolly	*Rhizopusspp*
3	White colony which turns black	Hyaline septate hyphae with noticeable condiospore and spines. Conidial heads are large, globoses, dark brown. Condiophores are short smooth-walled	Smooth	*Aspergillusspp*

Table :4.0.6: Occurrence of the isolates in different site samples

Isolates	Site A	Site B	Site C	Site D
Aspergillusspp	+	+	+	+
Penicillumspp	+	-	+	+
Rhizopusspp	+	+	-	+

Table 4.0.7:Percentage Frequency of occurrence of fungi isolates in the different site samples

Isolates	Number of colonies isolated	Frequency of isolation (%)	Number of colonies isolated	Frequency of isolation (%)	Number of colonies isolated	Frequency of isolation (%)	Number of colonies isolated	Frequency of isolation (%)
Aspergillusspp	3	42.86	2	40	4	44.44	3	27.27
Penicilliumspp	2	28.57	0	0	5	55.56	2	18.18
Rhizopusspp	2	28.57	3	60	0	0	6	54.55
Total	7	100	5	100	9	100	11	100

DISCUSSION

A detailed comparative study was made using microbiological examination on selected fish ponds water in Awka, Anambra for the detection of various bacteria and fungi and their population. Though microbes can serve as food source to fishes, some nutrients can also be obtained through the sediment sources; hence, high microbial load can be inimical to health.

In the present study, total bacteria count of 8.8 x 10^3, 5.1x 10^3, 8.6x 10^3 and 1.12 x 10^4cfu/ml was obtain from site A, site B, site C and site D respectively as shown table 4.0.1. As coliforms are facultative anaerobes, so it may be assumed that their number should be less at bottom of water body than its surface or sub surface part. But the reason for the highest number of

coliforms of the samples in this experiment may be depth of pond.Kroger and Noll (2009) found *E. coli* and Gram's negative rod shaped bacteria in tube well water due to the low depth of water source.This is in line with the findings of Ogbondeminu and Olayemi (2013) who reported that 50% of the microorganisms recovered from both fish and water of earthen pond fertilized with animal faecal waste were members of the family *Enterobacteriaceae*.

Another reason for the high count of coliform may be that fecal coliforms, after settling down might had encountered a favorable environment for reproduction (Davies *et al.*, 2005). The sediment's surface area and nutrient content promote the growth of bacteria (Sherer*et al.*, 2008). Fecal coliforms can survive for up to 60 days in freshwater sediments (Davies *et al.*, 2005) and can persist for a long time under hot and dry summer range conditions (Okafo*et al.*, 2003).

Differentbacteria isolates obtained in site Aincludes*Escherichia coli, staphylococcus spp,Bacillusspp,* and *Enterobacterspp.*withthe frequency of occurrence as 34, 18, 8 and 28 respectively as shown in table 4.0.4.

Bacteria isolates from site Bincludes*Escherichia coli,Klebsiellaspp*and*streptococcusspp*with the frequency of occurrence as 18, 22 and 11 respectively as shown in table 4.0.4.

Bacteria isolates obtained in site C includes *Escherichia coli, Klebsiellaspp, Staphylococcus spp, Bacillus spp*and*Enterobacterspp*with the frequency of occurrence as 32, 15, 17, 7 and15 respectively as shown in table 4.0.4.

Also different bacteria isolates obtained in site Dincludes*Escherichiacoli,Klebsiellaspp,Staphylococcusspp,Enterobacterspp*and*streptococcussp p*with the frequency of occurrence as 44, 21, 18, 23 and 6 respectively as shown in table 4.0.4.

Daboor (2008) found *E.coli* as the most dominant bacterial species in most samples of fish farm. In another study *Salmonella* has been isolated from tannery polluted fish pond with higher number of total coliforms as compared to fresh water (Begum *et al.*, 2007).

Different fungi isolates obtained in site Aincludes*Aspergillusspp*, *Penicillumspp*and*Rhizopusspp*with the frequency of occurrence as 3, 2 and 2respectively as shown in table 4.0.7

Fungi isolates from site Bincludes*Aspergillusspp and Rhizopusspp*with the frequency of occurrence as 2 and 3respectively as shown in table 4.0.7.*Penicillumspp*is absent in site B

Fungi isolates obtained in site Cinclude*Aspergillusspp*and*Penicillumspp*with the frequency of occurrence as 4 and 5 respectively as shown in table 4.0.7.*Rhizopusspp* is absent in site C

Also different fungi isolates obtained in site Dincludes*Aspergillusspp*, *Penicillumspp*and*Rhizopusspp*with the frequency of occurrence as 3, 2 and 6respectively as shown in table 4.0.7.

Birds visiting the ponds may be a source of fecal contamination. In fact magnitude and diversity of microbial load of a water bodies from many factors (Jones, 2002). Generally, it is believed that fecal coliforms are present only in feces of warm-blooded animals but it may be speculated that the two genera isolated in the present study may be present in feces of cold-blooded fish inhabiting the fish pond; as several coliforms including fecal *Citrobater*, *Enterobacter*and *Klebsiella*havebeen isolated by Harwood *et al.* (2009) from the fecal matter of fresh water turtle which is also a cold blooded animal.

Another source of contamination of pond may be the water entering the pond through water pipeline. A study done by Kirmeyer*et al.*(2009) showed that even post-treatment contamination may be detected in surrounding distribution system of pipelines. *Enterobacter*and *Klebsiella*have been found to multiply in the water mains and storage tanks under favourable environment (Edberg *et al.*, 2000).

CONCLUSION

This study shows the presence of some imminent contaminants in fish pond water sources that are of ecological threat and inimical to health. This includes the knowledge and observation of the following factors that could affect the microbial quality of fish pond water. Therefore, it is recommended that proper construction of fish pond should be ensured. The environment where the fish ponds are located should be protected from pollutants and weeds which can harbor microorganisms that find ways into fish pond by themselves or by passive process through wind, rainfall, etc. thereby affecting the fishes negatively. Similarly, water supply to the fish pond should be clean and free of contamination. Sample of the fish pond should be taken and examined in the laboratory for its microbiological quality before stocking. This will also give an insight to the possible presence of certain types of microorganisms, hence provide enabling environment for aquaculture purposes. Such practices must be considered compulsory in order to increase fish productivity and avoid post-harvest infections to the consumers.

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