**DETERMINATION OF OCCURENCE OF NITRIFYING BACTERIA IN A FRESHWATER FISHPOND OF CIRCULATORY AQUAPONICS SYSTEM**

**PRESENTED**

**BY**

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# APPROVAL

**THIS PROJECT WAS SUBMITTED AND APPROVED BY THE DEPARTMENT OF BIOTECHNOLOGY BIOLOGICAL SCIENCES, GODFREY OKOYE UNIVERSITY, ENUGU.**

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# DEDICATION

This project is dedicated to God Almighty whose grace, love and compassion has kept and sustained me till date.

# ACKNOWLEDGEMENT

I wish to acknowledge the Almighty God, who made it possible for me to be alive this day, and has given me the privilege to complete this research work successfully.

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# ABSTRACT

Nitrification is the biological oxidation of ammonia into nitrite, followed by the oxidation of nitrite into nitrate by small groups of autotrophic bacteria and Achaea. NH3 removal is beneficial to the plant system as build up is dangerous. Presence and activities of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) in freshwater fishpond of a circulating aquaponics system was examined in respect to random points in the aquaponics system (the fish tank, the bio-filter, and the line supplying water to the plants). The samples FT4, FT6, BF5, BF6, BF1, LN1, LN2, and LN3 were all rod-like in shape, when viewed with 40 objective light compound microscope.While samples BF7 and FT7 were both circular in shape. samples FT4, FT6, FT7, BF5, BF6, BF1, LN1, LN2 and LN3 retained their primary dye ( blueblack colouration). Sample BF7 retained its secondary colour (pink colouration). , samples FT4, FT5, LN2, LN3, LN1 were all in chains. Samples FT6, BF6, and BF1 were spaced. Samples BF7 FT7 were clustered together. From the statistical analysis, it was deduced that nitrifying bacteria can be isolated from any point in the aquaponics unit. Most nitrifying bacteria were discovered to have good yield of plasmid DNA. The potential

nitrification activities and oxidation rates were shown to be linear and activity of ammonia-oxidizing and nitrite oxidizing bacteria was highest in samples from the bio-filter.

**CHAPTER ONE**

**1.1 INTRODUCTION**

Most of the nitrogen available to the biosphere exists as N2 in the atmosphere, and is not useful to most organisms until it is "fixed" either biologically or abiotically (by lightning or aurorae, or industrially). Once it is fixed into NH3, usually it is either assimilated and transformed into organic N or nitrified into NO3-. (NASA-Amens 1996). Nitrification is the process by which ammonia is converted to nitrites (NO2-) and then nitrate (NO3-). This process naturally occurs in the environment, where it is carried out by specialized bacteria (Remay, 2000). The bacteria that carry out nitrification are called “Nitrifying bacteria” (AOBs and NOBs).

In the environments with high inputs of a nutrient such as freshwater fish ponds, mineralization of organic substances as a result of over-feeding and excretion increases the ammonia concentration which is harmful to fish and shrimp (Goldman *et al.,* 1985). Since microbial processes affect water quality parameters such as dissolved oxygen (DO), ammonium, nitrite, nitrate etc. (Moriarty, 1996), hence bacteria in ponds play important role in maintaining the water column chemistry (Vibha, 2011).

Aquaponics is the integration of a hydroponic plant production system with a recirculating aquaculture system. In a simple aquaponic system, nutrient-rich effluent from the fish tank flows through filters (for solids removal and biofiltration) and then into the plant production unit before returning to the fish tank (Christopher, 2015).

Ammonia becomes toxic to plants at certain concentration. This toxicity ranges from causing stunted growth in the plant to inhibiting germination of the seedling (Brain, 2014).

The present study was undertaken to determine the occurrence of nitrifying bacteria (Ammonia oxidizing bacteria [AOB] and Nitrite oxidizing bacteria [NOB]), in relation to the plants ability to utilize the ammonia produced from fresh-water fishpond in an aquaponic system in National Biotechnology Development Agency (NABDA) Abuja.

**1.2 STATEMENT OF PROBLEM**

In large urban areas, conventional agriculture is almost impossible, and this is as a result of lack of space for establishment of agricultural field. And it has consequently resulted to unsustainable supply of fresh, local, organic produce.

Aquaponics system which should have provided a reliable answer to the problem also has a little challenge on its own. Ammonia is produced by the fish’s respiratory system and is discharged through their gills. Buildup of ammonia in the fish tank eventually kill them (dead fishes will also produce ammonia). Also buildup of ammonia concentration in the system becomes toxic to the plant, such as causing stunted growth (Brain, 2014). Introduction of Nitrifying bacteria can help shorten the lag phase in starting up aquaponics system, which usually pose loss of resources and frustration on beginners.

**1.2 AIMS AND OBJECTIVES**

The general objective of the study is to determine if there are ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria NOB in Fresh water pond of Aquaponics system in NABDA Abuja. The specific objectives include;

1. Isolation of bacteria from the fresh water fishpond of aquaponics system in NABDA.
2. To identify ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) associated with aquaponics system .
3. To identify specific locations of these bacteria in the culture system.
4. To identify and characterize these bacteria.

**1.3 SCOPE OF STUDIES**

Under the auspices of this study, microbial work was carried out in the aspect of isolation of the organisms from the fishpond and gram-identification of the isolates.

Also a biochemical test will be carried out to evaluate the nitrifying potential of the isolates.

Molecular work will be also done by isolating plasmids from the bacteria.

Then a Nano technique will be used to quantify the concentration of the plasmids per isolate, using a Nano-drop spectrophotometer.

**1.4 SIGNIFICANCE OF STUDY**

This research work will provide information on the presence and types of bacteria or a bacteria culture in the Aquaponics or hydroponics system. Also the work will explore the plasmids available in the bacteria for future use in recombinant DNA technology for cloning and possibly in bio-remediation.

**CHAPTER TWO**

**LITERATURE REVIEW**

Nitrification is the bio3logical oxidation of ammonia or ammonium to nitrite followed by the oxidation of the nitrite to nitrate. (Nitrification Network 2014). The transformation of ammonia to nitrite is usually the rate limiting step of nitrification. Nitrification is an important step in the nitrogen cycle in soil. Nitrification is an aerobic process performed by small groups of autotrophic bacteria and achaea. This process was discovered by the Russian microbiologist Sergei Winogradsky.

2.1 **MICROBIOLOGY AND ECOLOGY OF NITRIFICATION**

The oxidation of ammonia into nitrite is performed by two groups of organisms, ammonia-oxidizing bacteria (**AOB**) and ammonia-oxidizing archaea (**AOA) (**Hatzenpichler, 2012). (Treusch., *et al* 2005) AOB can be found among the β-proteobacteria and gammaproteobacteria. (Purkhold, *et al* 2000) Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys.

The second step (oxidation of nitrite into nitrate) is done (mainly) by bacteria of the genus *Nitrobacter* and *Nitrospira*. Both steps are producing energy to be coupled to ATP synthesis. Nitrifying organisms are chemoautotrophs, and use carbon dioxide as their carbon source for growth. Some AOB possess the enzyme, urease, which catalyzes the conversion of the urea molecule to two ammonia molecules and one carbon dioxide molecule. *Nitrosomonas europaea*, as well as populations of soil-dwelling AOB, have been shown to assimilate the carbon dioxide released by the reaction to make biomass via the Calvin Cycle, and harvest energy by oxidizing ammonia (the other product of urease) to nitrite. This feature may explain enhanced growth of AOB in the presence of urea in acidic environments (Marsh, *et al*. 2005).

**2.2 TYPES OF NITRIFYING BACTERIA**

Nitrifying bacterium**,** plural Nitrifying Bacteria, any of a small group of aerobic bacteria (family Nitrobacteraceae) that use inorganic chemicals as an energy source. They are microorganisms that are important in the nitrogen cycle as converters of soil ammonia to nitrates, compounds usable by plants. The nitrification process requires the mediation of two distinct groups: bacteria that convert ammonia to nitrites(Ammonia Oxidizing Bacteria) [*Nitrosomonas*, Nitrosospira, Nitrosococcus, and Nitrosolobus] and bacteria that convert nitrites (toxic to plants) to nitrates (Nitrite Oxidizing bacteria) [*Nitrobacter*, Nitrospina, and Nitrococcus]. In agriculture, irrigation with dilute solutions of ammonia results in an increase in soil nitrates through the action of nitrifying bacteria. (Encyclopædia Britannica 1998)

**IDENTIFICATION OF NITRIFYING BACTERIA**

Table 1: NITRIFYING BACTERIA THAT OXIDIZE AMMONIA

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Genus** | **Phylogenetic group** | **DNA (mol% GC)** | **Habitats** | **Characteristics** |
| *Nitrosomonas* | Beta | 45-53 | Soil, Sewage, freshwater, Marine | Gram-negative short to long rods, motile (polar flagella)or non-motile; peripheral membrane systems |
| *Nitrosococcus* | Gamma | 49-50 | Freshwater, Marine | Large cocci, motile, vesicular or peripheral membranes |
| *Nitrosospira* | Beta | 54 | Soil | Spirals, motile (peritrichous flagella); no obvious membrane system |

(Schaechter, 2009)

Table 2: Nitrifying bacteria that oxidize nitrite

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Genus** | **Phylogenetic group** | **DNA (mol% GC)** | **Habitats** | **Characteristics** |
| *Nitrobacter* | Alpha | 69-62 | Soil, Freshwater, Marine | Short rods, reproduce by budding, occasionally motile (single subterminal flagella) or non-motile; membrane system arranged as a polar cap |
| *Nitrospina* | Delta | 58 | Marine | Long, slender rods, nonmotile, no obvious membrane system |
| *Nitrococcus* | Gamma | 61 | Marine | Large Cocci, motile (one or two subterminal flagellum) membrane system randomly arranged in tubes |
| *Nitrospira* | Nitrospirae | 50 | Marine, Soil | Helical to vibroid-shaped cells; nonmotile; no internal membranes |

(Schaechter, 2009).

**2.3 NITRIFYING POTENTIAL**

The net nitrification rates were determined by linear regression of NO2-NO3 accumulation vs. time, while the substrate concentration was calculated as the time weighted average of the measured NH4 concentration

**2.4 POND**

A **pond** is a body of standing water, either natural or artificial, that is usually smaller than a lake.

They may arise naturally in floodplains as part of a river system, or they may be somewhat isolated depressions (examples include vernal pools and prairie potholes). Usually they contain shallow water with marsh and aquatic plants and animals. *(*John, 1986).

Ponds are frequently human-constructed. In the countryside farmers and villagers dig a pond in their backyard or increase the depth of an existing pond by removing layers of mud during summer season.

Ponds can result from a wide range of natural processes. Any depression in the ground which collects and retains a sufficient amount of precipitation can be considered a pond, and such depressions can be formed by a variety of geological and ecological events. Rivers often leave behind ponds in natural flood plains after spring flooding, and these can be very important to breeding fish, particularly in large river systems like the Amazon.( McConnell, 1975).

Retreating glaciers can leave behind landscapes filled with small depressions, each developing its own pond; an example is the Prairie Pothole Region of North America.(Arnoud, 1989).

Many areas of landscape contain small depressions which form temporary ponds after spring snow melt, or during rainy seasons; these are called vernal ponds, and may be important sites for amphibian breeding. *(*Aram and Phillip, 2008).

Some ponds are created by animals. Beaver ponds are the best known example, but alligators also excavate ponds as well. *(*Paul, 2010). In landscapes with organic soils, fires can also create depressions during periods of drought; these become open water when normal water levels return. *(*Steven, 1994).

Ponds are used for the provision of fish and other wildlife including waterfowl which a source of food for humans. Pollutants entering ponds are often substantially mitigated by the natural sedimentation and biological activities within the water body. Ponds are also a major contributor to local ecosystem richness and diversity for both plants and animals.

A pond can be defined as a body of water (normally fresh water, but occasionally brackish), which can vary in size between 1 square meter and 2 hectares (this is equivalent in size to about 2.5 football pitches), and which holds water for four months of the year or more.

**2.5 FRESHWATER**

Freshwater is defined as having a low salt concentration — usually less than 1%. Plants and animals in freshwater regions are adjusted to the low salt content and would not be able to survive in areas of high salt concentration (i.e., ocean). There are different types of freshwater regions:

1. **Ponds and lakes:**  These regions range in size from just a few square meters to thousands of square kilometers. Many ponds are seasonal, lasting just a couple of months (such as sessile pools) while lakes may exist for hundreds of years or more. Ponds and lakes may have limited species diversity since they are often isolated from one another and from other water sources like rivers and oceans.
2. **Streams and rivers:**  These are bodies of flowing water moving in one direction. They get their starts at headwaters, which may be springs, snowmelt or even lakes, and then travel all the way to their mouths, usually another water channel or the ocean.
3. **Wetlands:** Wetlands are areas of standing water that support aquatic plants. Marshes, swamps, and bogs are all considered wetlands. Plant species adapted to the very moist and humid conditions are called hydrophytes. These include pond lilies, cattails, sedges, tamarack, and black spruce.

## Freshwater ecosystems–lakes, rivers, and the smaller ponds and streams–make up only two percent of Earth's water resources, and only one percent remains drinkable

Fish breathe by drawing water in their mouths and forcing it out of the body via a number of gill slits. The gill slits are rich in blood vessels, enabling the direct absorption of oxygen. While all fish have gills, some fish are also able to extract oxygen from the air via gulping.

Most fish have swim bladders that are permanently sealed, but other fish have swim bladders that can hold varying amounts of air. Salmon have open swim bladders, and they must gulp air at the surface to keep them full. Some fish, such as sharks, lack swim bladders entirely. This causes them to sink if they do not maintain forward motion.

Fish have fins that are adapted to their lifestyle. Some have evolved into long, barbed structures, suitable for defense. Other fish have fins suited for achieving great speed or for walking along the bottom of the ocean.

**2.6 CHEMISTRY OF NITRIFICATION**

Nitrification is a process of nitrogen compound oxidation (effectively, loss of electrons from the nitrogen atom to the oxygen atoms):

2 NH4+ + 3 O2 → 2 NO2− + 2 H2O + 4 H+ (Nitrosomonas, Comammox)

2 NO2− + O2 → 2 NO3− (Nitrobacter, [Nitrospira](https://en.wikipedia.org/wiki/Nitrospira), [Comammox](https://en.wikipedia.org/wiki/Comammox))

-OR-

NH3 + O2 → NO2− + 3H+ + 2e−

NO2− + H2O → NO3− + 2H+ + 2e−

## 2.7 Nitrification in the marine environment

In the marine environment, nitrogen is often the limiting nutrient, so the nitrogen cycle in the ocean is of particular interest (Zehr, *et al*,. 2011).

The nitrification step of the cycle is of particular interest in the ocean because it creates nitrate, the primary form of nitrogen responsible for "new" production. Furthermore, as the ocean becomes enriched in anthropogenic CO2, the resulting decrease in pH could lead to decreasing rates of nitrification. Nitrification could potentially become a "bottleneck" in the nitrogen cycle (Hutchins, *et al,.* 2009).

Nitrification, as stated above, is formally a two-step process; in the first step ammonia is oxidized to nitrite, and in the second step nitrite is oxidized to nitrate. Different microbes are responsible for each step in the marine environment. Several groups of ammonia-oxidizing bacteria (AOB) are known in the marine environment, including *Nitrosomonas*, *Nitrospira*, and *Nitrosococcus*. All contain the functional gene ammonia monooxygenase (**AMO**) which, as its name implies, is responsible for the oxidation of ammonia. (Hatzenpichler, 2012) (Nitrification and Denitrification, 1996).

More recent metagenomic studies have revealed that some Thaumarchaeota (formerly Crenarchaeota) possess AMO. Thaumarchaeotes are abundant in the ocean and some species have a 200 times greater affinity for ammonia than AOB, leading researchers to challenge the previous belief that AOB are primarily responsible for nitrification in the ocean. ( Zehr and Kudela, 2011).

In the second step, nitrite is oxidized to nitrate. In the oceans, this step is not as well understood as the first, but the bacteria *Nitrospina* and *Nitrobacter* are known to carry out this step in the sea. (Zehr and Kudela, 2011).

**2.7.1 Nitrogen Cycle**

The nitrogen cycle is the biogeochemical cycle by which nitrogen is converted into various chemical forms as it circulates among the atmosphere and terrestrial and marine ecosystems. The conversion of nitrogen can be carried out through both biological and physical processes. Important processes in the nitrogen cycle include fixation, ammonification, nitrification, and denitrification. The majority of Earth's atmosphere (78%) is nitrogen, (Steven, et al,. 2004) making it the largest source of nitrogen.

However, atmospheric nitrogen has limited availability for biological use, leading to a scarcity of usable nitrogen in many types of ecosystems. The nitrogen cycle is of particular interest to ecologists because nitrogen availability can affect the rate of key ecosystem processes, including primary production and decomposition. Human activities such as fossil fuel combustion, use of artificial nitrogen fertilizers, and release of nitrogen in wastewater have dramatically altered the global nitrogen cycle. (Galloway, et al. (2004).

## 2.7.2 Marine nitrogen cycle

The nitrogen cycle is an important process in the ocean as well. While the overall cycle is similar, there are different players and modes of transfer for nitrogen in the ocean. Nitrogen enters the water through precipitation, runoff, or as N2 from the atmosphere. Nitrogen cannot be utilized by phytoplankton as N2 so it must undergo nitrogen fixation which is performed predominately by cyanobacteria (Miller, 2008).

Nitrogen sources are removed from the euphotic zone by the downward movement of the organic matter. This can occur from sinking of phytoplankton, vertical mixing, or sinking of waste of vertical migrators. The sinking results in ammonia being introduced at lower depths below the euphotic zone. Bacteria are able to convert ammonia to nitrite and nitrate but they are inhibited by light so this must occur below the euphotic zone (Miller, 2008).

Ammonification or Mineralization is performed by bacteria to convert organic nitrogen to ammonia. Nitrification can then occur to convert the ammonium to nitrite and nitrate (Boyes, *2011).*

Nitrate can be returned to the euphotic zone by vertical mixing and upwelling where it can be taken up by phytoplankton to continue the cycle. N2 can be returned to the atmosphere through denitrification.

### 2.7.3 Environmental impacts of Marine Nitrogen Cycle

Additional risks posed by increased availability of inorganic nitrogen in aquatic ecosystems include water acidification; eutrophication of fresh and saltwater systems; and toxicity issues for animals, including humans (Camargo and Alonso, 2006).

Eutrophication often leads to lower dissolved oxygen levels in the water column, including hypoxic and anoxic conditions, which can cause death of aquatic fauna. Relatively sessile benthos, or bottom-dwelling creatures, are particularly vulnerable because of their lack of mobility, though large fish kills are not uncommon. Oceanic dead zones near the mouth of the Mississippi in the Gulf of Mexico are a well-known example of algal bloom-induced hypoxia (*Rabalais*, *et al* 2002)

**2.8 PLASMID EXTRACTION**

### Minipreparation

Minipreparation of plasmid DNA is a rapid, small-scale isolation of plasmid DNA from bacteria. It is based on the alkaline lysis method. The extracted plasmid DNA resulting from performing a miniprep is itself often called a "miniprep". Minipreps are used in the process of molecular cloning to analyze bacterial clones. A typical plasmid DNA yield of a miniprep is 50 to 100 µg depending on the cell strain. Miniprep of large number of plasmids can also be done conveniently by on filter paper lysing, the elution of the filter paper that contains plasmid can be directly sequenced to produce more than 700 bp high quality sequencing data with CE sequencing (Birnboim and Doly J 1979).

**2.9 The plasmid extraction by GeneJET™ Plasmid Miniprep Kit.**

**2.9.1 DESCRIPTION**

The GeneJET™ Plasmid Miniprep Kit is designed for rapid and cost-effective small-scale preparation of high quality plasmid DNA from recombinant E.coli cultures. The kit utilizes an exclusive silicabased membrane technology in the form of a convenient spin column. Each GeneJET™ spin column can recover up to 20 μg of plasmid DNA. The kit can be successfully used for efficient purification of any size plasmids and cosmids. The actual plasmid yield and optimal culture volume depend on the plasmid copy number and medium used for cultivation.

**2.9.2 PRINCIPLE**

Pelleted bacterial cells are resuspended and subjected to SDS/alkaline lysis (1) to liberate the plasmid DNA. The resulting lysate is neutralized to create appropriate conditions for binding of plasmid DNA on the silica membrane in the spin column (2). Cell debris and SDS precipitate are pelleted by centrifugation, and the supernatant containing the plasmid DNA is loaded onto the spin column membrane. The adsorbed DNA is washed to remove contaminants, and is then eluted with a small volume of the Elution Buffer (10 mM Tris-HCl, pH 8.5). The purified plasmid DNA is ready for immediate use in all molecular biology procedures such as conventional digestion with restriction enzymes, fast digestion with FastDigest® restriction enzymes, PCR, transformation and automated sequencing. [GeneJET™ Plasmid Miniprep Kit(3) Revised 05.2008].

**2.10 NANODROP SPECTROPHOTOMETER**

The NanoDrop Spectrophotometer from NanoDrop Technologies is designed for measuring nucleic acid concentrations in sample volumes of one microliter. The key to this advanced spectrophotometer is its unique sample retention technology that overcomes the need for cuvettes when taking measurements. This is accomplished by placing the sample directly on top of the detection surface and using the surface tension to create a column between the ends of optical fibers. Thus the measurement optical path is formed. The sensitivity range for DNA detection is between 2 and 3700 ng/ul. The spectral range of the device is 220 to 750nm and it is possible to scan all of the wavelengths. A single measurement cycle takes only 10 sec. The instrument is driven by a PC, which allows you to archive a large number of measurements (Michal, 2007)

**CHAPTER THREE**

**3.1 MATERIALS AND METHOD**

The study was carried out in the aquaponics section of Agricultural Biotechnology, in National Biotechnology Development Agency located at (Umar Musa Yar’adua express way, Lugbe, Abuja), to determine the presence and activities of nitrifying bacteria in a cycling fishpond used in an aquaponics system.

This chapter discusses research design, sources of data collection, tools for data collection, the population of the study, sample and sampling technique, instrumentation, reliability and validity of data and test instruments and data analysis techniques

3.2 RESEARCH DESIGN

The study design adopted for this study is completely randomized design (CRD). To gather primary data directly from the observation.

3.3 DATA COLLECTION

The sources of data for this research work was from primary sources

Primary source: The data was collected from direct observation through experiment.

3.4 MATERIALS AND REAGENTS

* TSA (Tryptic Soy Agar) media.
* Falcon tubes
* Petri dishes
* Incubator
* McCartney Bottles
* LB media
* Wire-loop
* Measuring cylinder
* Conical flask
* Weighing balance
* Spatula
* Ethanol
* Distilled water
* Aluminum foil
* Shaker incubator
* Methylene blue/ crystal violet
* Safranin red
* Iodine
* Glass slides
* Cover slides
* Staining rack
* Bunsen burner
* Microscope
* Micro pipette (1000µl, 200µ, 10µ)
* Pipette tips (1000µl, 200µ, 10µ)
* Micro centrifuge spin
* Vortex
* Water bath
* Micro centrifuge tubes
* 100% ethanol
* Gloves
* Plasmid extraction kit
* Nuclease-Free water
* Nano-drop Spectrophotometer
* Statistical analysis

3.5 SAMPLE AND SAMPLING TECHNIQUES

The researcher took samples from different points in the aquaponics system, the Fish tank, the line supplying water to the plants from the fish tank, and from the Bio-filter.

**3.6 PROCEDURES**

* Water samples from 3 different points in the fishpond (the fish tank, the line supplying water to plants, and the Bio-filter), was collected using sterile capped container
* Serial dilutions were run into 8 folds for the 3 samples.
* An aliquot of 1ml of dilutions (101, 102, 107) were cultured in Tryptic Soy Agar using pour plate method, by taking 1ml of each of the aliquot of the chosen dilutions and placing them into a sterile petri dish for each sample, and 5 ml of TSA was add into it.
* The plates were swirled 3 times clock-wise and 3 times anti-clock-wise directions.
* The samples were incubated for at least 21 hours at 370c.
* LB agar was prepared into petri dishes.
* Distinct colonies were aseptically sub-cultured into freshly prepared LB agar plates, one for each plate. Using streaking method.
* Samples were incubated for 24 hours at 370c.

**3.7 STOCK CULTURE**

* A stock culture was made by preparing LB agar into McCatheny bottles.
* The bottles were kept in a slant position to gel.
* The slants were inoculated with the pure cultures.

**3.8 GRAM STAINING**

* A fresh subculture was prepared was made from the stock culture into test tubes containing LB broth.
* The culture broth was incubated for 24 hours at 37 0C.
* Smear was made on grease-free clean slides.
* The slides were both dried and heat fixed in flame
* The fixed smear were flooded with crystal violet for 1 min
* They were washed with clean water by allowing water run on then gently
* They were flooded iodine for 1 minute
* They were then washed with clean water
* The slides were then flooded Acetone for about 15-20 seconds and were wash quickly.
* They were then flooded with safranin red for 1 minute and were washed with clean water.
* The slide were allowed to dry
* The slides were viewed under a light microscope with 40 objective and 100 objective lenses.
* The photograph of the cells under oil immersion were taken with an inverted microscope.

**3.10 NITRIFICATION POTENTIALS**

* Nitrification potential was measured using shaken slurry method. (S.C. Hart, J.M., Stark, E.A. Davidson and M.K., Firestone. Nitrogen Mineralization, and immobilization and nitrification. In: methods of soil analysis. 1994).
* An inorganic medium of (NH4) So4 was prepared into falcon tubes.
* The media in tubes were inoculated with the isolates
* The tubes were capped with parafilm to allow gas exchange during the potential nitrification assay.
* All tubes were shaken at 160 rpm for 24 hours.
* Aliquots of 10ml were removed from each flask after 2, 8 and 24 hours and centrifuged at 8000 rpm for 5 minutes
* The supernatant was decanted and filtrate was analyzed for the NO3 – N content by spectrophotometer (543nm)

**. . 3.11 PLASMID EXTRACTION PROCEDUE**

* The provided RNase was added into A solution to Re-suspension solution and mix.
* Absolute (100%) ethanol was added to the wash solution at the rate; 35ml ethanol with 20ml wash solution.
* From the culture in the Falcon tube, 1ml was transferred into a micro-centrifuge tube and spin at 8000rpm for 2 minutes.
* The supernatant was decanted and all remaining medium were removed.
* A 250µl of Re-suspension solution was added to the pellet and vortexed until no cell clumps remained.
* A 250µl of lysis solution was added and mixed thoroughly by inverting the tube 4-6 times until the solution became vicious and slightly clear.

NB: do not vortex. And do not incubate for more than 5 minutes.

* A 350µl of the Neutralization solution was added and mixed immediately and thoroughly by inverting the tube 4-6 times.
* The solution was centrifuged for 5 minutes at 1200rpm.
* The supernatant transferred to the supplied GeneJet spin column.

NB: avoid transferring the white precipitate.

* The supernatant was centrifuged for 1 minute, and the flow-through was discarded and then placed back on the column into the same collection tube.
* A 500µl of wash solution was added to the GeneJet spin column and centrifuged for 1 minute and the flow-through was discarded.
* The samples were placed back on the column in the same collection tube.
* Repeat the wash procedure was repeated using 500µl of the wash solution.
* The samples were transferred to the GeneJet spin column into a fresh 1.5ml micro-centrifuge tube.
* The elution buffer was pre warmed at 70⁰c for 10 minutes.
* A 50µl of the Elution Buffer was added to the center of the GeneJet spin column membrane to elute the plasmid DNA.
* The samples were incubated for 2 minutes at room temperature and centrifuged for 2 minutes.
* The column was discarded and stored the purified Plasmid DNA at -20⁰c.

**3.12 NANODROP SPECTROPHOTOMETER**

* The Nano-drop machine was set up properly and all the necessary connections where well done.
* A 5µl of Nuclease free water was placed on the pedestal and calibrated so as to clean the surface of the pedestal.
* The surface was gently cleaned with a dry cotton wool.
* A 1µl of the elution buffer used during the plasmid was used to blank the Nano-drop spectrophotometer.
* The surface was gently cleaned with a dry cotton wool.
* A 1µl each sample where carefully placed on the pedestal and measured with the arm closed.
* The pedestal was cleaned each time before a fresh measurement.

**3.13 STATISTICAL ANALYSIS OF NITRIFYING POTENTIAL**

ƴij = µ+zj + Ʃij j=1,2,…k, į=1,2,…,n.

ƴĳ is the observation from the ith experimental unit using jth treatment.

µ is general effect.

Zij is the effect of the jth treatment.

Ʃij is random experimental error with mean 0 and variance ơ

The appropriate test statistics to be used for comparing the completely randomized design of the two treatments is

ȶ = ȳ1-ȳ2

Sp

Where ȳ1 and ȳ2 are the sample means, n1 and n2 are the sample sizes, Sp2 is an estimate of common variance.

Ơ12 = ơ22 = ơ32 conjugated from

Sp2 = (n1 - 1) 521 + (n2 - 1) 522

n1 + n2 -2

and S12 and S22 are the individual sample variance ơ. To determine whether to reject Ho = µ1 = µ2, to would be compared to the t distributed with n1 + n2 – 2 degree of freedom.

If ̸to/ ˃ t α/2, n1 + n2 – 2 is the α/2 upper percentage point of the t distribution with n1 + n2 – 2 degree of freedom, Ho would be rejected and be concluded that the mean treatment differ.

FT BF

0.200 0.212

0.200 0.215

0.217 0.200

0.206 0.215

0.211

Hypothesis

Ho: FT = BF

H1: FT ≠ BF

**Level of Signifiance**

α = 0.05

Test statistics = to = ȳFT - ȳBF

Sp

Where ȳA and ȳB are the means of each treatment.

**CRITICAL VALUE**

t tab = t (1 – α/2 ), n1 + n2 – 2, t(1 – 0.05/2 )5

**DECISSION RULE**

Reject H0 if /t cal/ ˃ t tab otherwise accept calculation

n1 = n2 = 4

ȳ FT = 0.200 + 0.200 + 0.217 = 0.206

3

ȳ BF = 0.212 + 0.215 + 0.200 + 0.217 = 0.211

4

Variance of FT = Ʃ (y FT - ȳ FT)

n1 -1

= (0.200 – 0.206)2 + (0.200 – 0.206)2 + (0.217 – 0.206)2 = 0.0000956

3-1

= (0.212 – 0.211)2 + (0.215 – 0.211)2 + (0.200 – 0.211)2 + (0.215 – 0.211)2 = 0.0000513333

4-1

SP =

3 + 4 – 2

=

=

= 0.0083

T cal = 0.206 – 0.211

0.0083

= 0.206 – 0.211

0.0083 (0.7937)

= 0.206 – 0.211

0.0063

= -0.7937

T tab = 2.571

**DECISION**

Ho = Accept

FOR (BF and LN)

BF LN

0.212 0.211

0.215 0.204

0.200 0.219

0.215 ȳ=0.211

ȳ=0.211

**HYPOTHESIS**

H0: BF = LN

H1: BF ≠ LN

**LEVEL OF SIGNIFICANCE**

α= 0.05

Test statistics = t0 = ȳBF - ȳLN

Sp

Where ȳA and ȳB are the mean of each treatment

**CRITICAL VALUE**

T tab = t (1- α/2), n1 + n2 – 2, t (1 – 0.005/2)5

= 0.975,5

**DECISION RULE**

Reject H0 if /t cal/ ˃ t tab otherwise accept.

**CALCULATION**

n2 – 4, n3 = 3

ȳBF = 0.212 + 0.215 + 0.200 + 0.215 = 0.211

4

ȳLN = 0.211 + 0.204 + 0.219 = 0.211

3

Variance of BF

(0.212 – 0.211)2 + (0.215 - 0.211)2 + (0.200 – 0.211)2 + (0.215 – 0.211)2 = 0.000051

4-1

Variance of LN

(0.211 – 0.211)2 + (0.204 – 0.211)2 – (0.219 – 0.211)2 = 0.000057

3 -1

Sp =

4 + 3 – 2

=

5

=

Sp = 0.0073

T cal = 0.211 - 0.211

0.0073

= 0.211 – 0.211

0.0073 (0.7616)

T cal = 0

T tab = 2.571

**DECISION**

H0 = Accept

Since Ho says FT=BF=LN in regards to the presence of nitrifying bacteria, this means that nitrifying bacteria can be isolated from any point in the circulating aquaponic system.

**CHAPTER FOUR**

**4.0 RESULT**

**KEY**: The alphabets represent the location where the samples were collected. The suffix number represents serial dilution plate of where it was sub-cultured from.

**FT= SAMPLE FFROM FISHTANK, BF= SAMPLE FROM THE BIO-FILTER, LN= SAMPLE FROM WATER LINE TO THE PLANTS.**

Table 3: Result from gram staining and microscopic view.

|  |  |  |  |
| --- | --- | --- | --- |
| **SAMPLES** | **SHAPE** | **AGGREGATION** | **GRAM STAIN** |
| FT4 | ROD-LIKE | IN CHAINS | BLUE BLACK |
| FT6 | ROD-LIKE | SPARCE | BLUE BLACK |
| BF5 | ROD-LIKE | IN CHAINS | BLUE BLACK |
| BF6 | ROD-LIKE | SPARCE | BLUE BLACK |
| BF7 | COCCI | CLUSTERED | PINK |
| BF1 | ROD-LIKE | SPARCE | BLUE BLACK |
| LN2 | ROD-LIKE | IN CHAINS | BLUE BLACK |
| LN3 | ROD-LIKE | IN CHAINS | BLUE BLACK |
| LN1 | ROD-LIKE | IN CHAINS | BLUE BLACK |
| FT7 | COCCI | CLUSTERD | BLUE BLACK |

Table 4. Nitrifying Potential of the bacteria

|  |  |  |  |
| --- | --- | --- | --- |
| SAMPLE | 2 HOURS (nM) | 8 HOURS (nM) | 24 HOURS (nM) |
| FT4 | 0.200 | 0.323 | 0.208 |
| FT6 | 0.200 | 0.425 | 0.389 |
| FT7 | 0.217 | 0.357 | 0.322 |
| BF1 | 0.212 | 0.451 | 0.408 |
| BF5 | 0.215 | 0.220 | 0.260 |
| BF6 | 0.200 | 0.282 | 0.270 |
| BF7 | 0.215 | 0.448 | 0.360 |
| LN1 | 0.211 | 0.320 | 0.370 |
| LN2 | 0.204 | 0.435 | 0.245 |
| LN3 | 0.219 | 0.302 | 0.328 |

Figure 1: A CHAT REPRESENTING THE NITRIFICATION ACTIVITIES

The observed changes in the nitrification activities during the nitrifying potential test.

Table 5. The Nano-drop spectrophotometer.

|  |  |  |  |
| --- | --- | --- | --- |
| SAMPLES | CONC. (ng/µl) | A260 | A280 |
| FT4 | 8.5 | 0.170 | 0.148 |
| FT6 | 14.4 | 0.288 | 0.137 |
| FT7 | 21.6 | 0.432 | 0.124 |
| BF1 | 28.8 | 0.576 | 0.111 |
| BF5 | 58.2 | 1.165 | 0.058 |
| BF6 | 25.2 | 0.504 | 0.118 |
| BF7 | 61.2 | 1.224 | 0.052 |
| LN1 | 45.9 | 0.918 | 0.080 |
| LN2 | 37.8 | 0.756 | 0.095 |
| LN3 | 58.1 | 1.162 | 0.058 |

FIG 2: Chart of the plasmid DNA (Concentrations at A260 and A280)

The observed differences in the absorbance of plasmid DNA at A260 and protein at A280.

**CHAPTER FIVE**

**5.1 DISCUSSION**

This study shows that the aquaponics contained some nitrifying bacteria which are responsible for the aerobic conversion of ammonia into nitrates. This stands to be one of the most important functions in an aquaponics system as it reduces toxicity of the water for fish, and allows the resulting nitrate compounds to be removed by the plant according to a study by (Rakocy, *et al*. 2006). Variations were observed in abundance of nitrifiers in different points in the aquaponics. Although the sample mean of the sample sizes from the bio-filter and the line of water to the plants are the same, recording at ȳ = **0.211.** whereas the sample mean of the sample sizes from the fish tank is insignificantly lower, recording at ȳ = **0.206**. this agrees with the observations of vibha, et al. 2010 on the seasonal variations in the abundance of nitrifying bacteria in fish pond ecosystem. In a study carried out on “Nitrification and denitrification in the activated sludge process” by Micheal, 2002. And according to an encyclopedia on microbiology Schaecterdam, 2009. It confirms that sample FT7 is of the family of *Nitrosococcus*, samples FT4, FT6, BF5, BF6, BF1, LN1, LN2 and LN3 are of the family of *Nitrosobacter*, and sample BF7 is of the family of *Nitrosomonas*. Most nitrifying bacteria have good yield of plasmid DNA, which could be explored and or biotechnological applications.

**CONCLUSION**

Nitrifying bacteria (AOB and NOB) are present and play major roles in the conversion and maintenance of ammonia and dissolved oxygen in an aquaponics system.

Most nitrifying bacteria have good yield of plasmid DNA, and I recommend further research is carried out on them to explore all their potentials.

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