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Antimicrobial resistance (AMR) - prevalence in isolated bacteria in tilapia-farms in Zambia

Forekomst av antibiotikaresistens i isolerte bakterier fra tilapia-oppdrett i Zambia.

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Preface

I was very fortunate to be able to travel to Zambia and study such an important and interesting topic as AMR. Its prevalence in Africa is not well documented and this study was an exciting opportunity to contribute to the literature available. I was lucky to be accompanied with the brilliant professors and scientists from the University of Zambia, and the Norwegian University of Life Sciences, which are experts in their field and provided me with much knowledge and wisdom. This study has given me an insight into the important work being done by scientists all over the world every day. I was happy to be able to participate with this small contribution. This study was an interesting and fulfilling ending for my six years as a veterinary student at the Norwegian University of Life sciences.

Summary

Title: Antimicrobial resistance (AMR) -prevalence in isolated bacteria in tilapia-farms in Zambia

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Antimicrobial resistance is an emerging threat to human and animal health. It is driven by increased use and poor practices. The use of antimicrobials in aquaculture has been shown to increase the prevalence of antimicrobial resistance. This study examined the prevalence of antimicrobial resistance against commonly used antimicrobials in tilapia farms in Zambia. Samples were collected at eight different farms in the Copperbelt region, and around Siavonga and lake Kariba. Bacterial isolates were tested for antimicrobial resistance through disk diffusion on Mueller-Hinton agar. The bacteria were sequenced through 16S-sequencing for identification. A questionnaire given to the fish farms reported a low usage of antimicrobials in the farms visited. The antimicrobial resistance observed was generally low among most antimicrobials tested.

Terms and abbreviations

AMR	Antimicrobial Resistance
BLAST	Basic Local Alignment Search Tool
BTB agar	Bromo Thymol Blue Lactose Agar
CLSI	Clinical and Laboratory Standards Institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ECOFF's	Epidemiological Cut-Off Value
MALDI-TOF	Matrix-assisted laser desorption/ionization-Time of flight
MAR Index	Multi-antibiotic Resistance Index
NMBU	Norwegian University of Life Sciences
UNZA	University of Zambia
WHO	World Health Organization
WOAH	World Organization for Animal Health

Introduction

Tilapia

In Africa and the Middle East, Tilapiines and haplochromines are the two major native lineages (tribes) of cichlids (Nagl et al., 2001; Salzburger & Meyer, 2004) belonging to the family *Cichlidae* of the order Cichliformes. Tilapia is the general name given to all fish in the cichlid group consisting of three genera; *Oreochromis*, *Sarotherodon* and *tilapia* (Trewavas, 1983). They were introduced to the Americas and Southeast Asia in the 20th century (Munguti et al., 2022). It naturally inhabits freshwater sources such as ponds, streams, lakes and rivers (Elangovan et al., 2019). Globally, close to 70 species of tilapia have been identified. Genetic improvement projects have been underway for more than 10 years focusing on improved growth of farmed tilapia (Bentsen et al., 2012).

Tilapia Farming

Illustrations found in tombs in Egypt, dated 2500 B.C., depict early tilapia culture (El-Sayed, 2013), and over 140 countries are currently involved in tilapia-culturing (Fitzsimmons, 2015).

Tilapia-farming employs several species of tilapia, some of these include the Nile tilapia (*Oreochromis niloticus*), Blue tilapia (*Oreochromis aureus*) and the three spotted tilapia (*Oreochromis andersonii)* (El-Sayed & Fitzsimmons, 2023). *O. niloticus* is by far the most popular tilapia-species to use in aquaculture, accounting for 62% of all aquaculture production across all tilapia species (Munguti et al., 2022). Tilapia is the second most cultured freshwater fish globally, second only to carp (FAO, 2020). Aquaculture in sub-Saharan Africa has grown at a rate of 11% per year since the year 2000-2019, a 16% annual growth is observed in tilapia-farming in Zambia during the same time frame (Ragasa et al., 2022).

Production practices

While practices and methods vary, tilapia is usually cultivated in artificially built ponds in close proximity to a freshwater source. Members of the *Oreochromis* genus are uniparental (female) mouth brooders (Iq & Shu-Chien, 2011). *Sarotherodon galilaeus* are biparenteral mouth brooders while *Sarotherodon melanotheron* is a uniparental mouth brooder where the males carry the eggs and the fry in their mouth (Lopes et al., 2015). On the other hand, members of the *tilapia* genus are substrate spawners and both species protect their nest (Lopes et al., 2015). Tilapia can be kept in ponds and eggs extracted frequently through non-

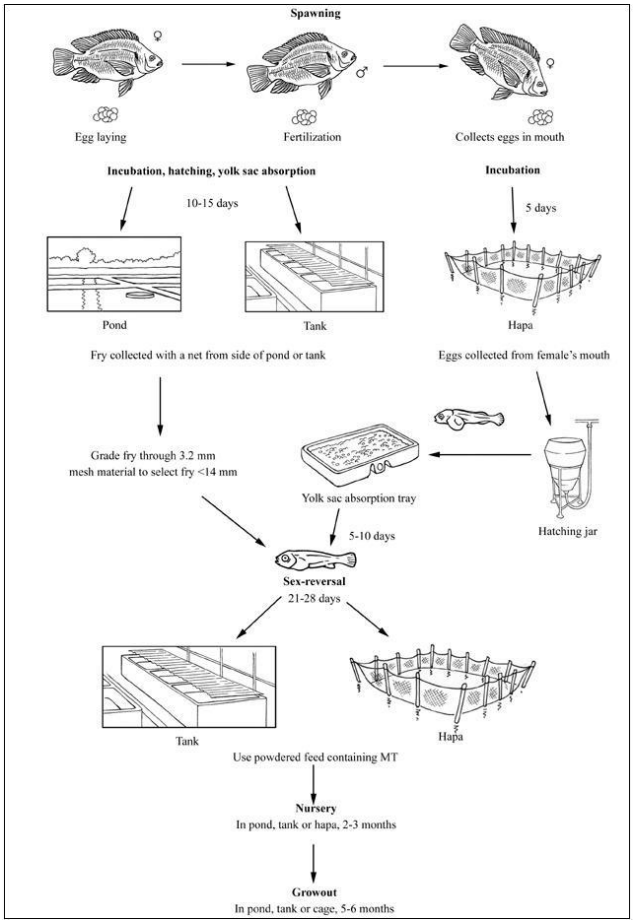

Figure 1. Nile tilapia production cycle, Credit of the Food and Agriculture Organization of the United Nations.

invasive means. Tilapia usually breed freely in ponds. Different ratios between male and female is used, usually at a 1:3/1:4 male to female ratio. One can collect fry directly from the breeding ponds or collect the eggs and hatch them in a hatchery. Once the fry grows to become fingerlings, they are transferred to ponds or hapas, where they will grow to size before being harvested. The process is illustrated in Figure 1.

All male tilapia culture is preferable, due to the faster growth of the males. Mixed culture would create more variation in fish size and fish would start to breed before they are slaughtered. Tilapia usually undergo sex reversal at start feeding using methyl testosterone to achieve an all-male culture (Mojekwu, 2014). This results in a 100% masculinization of the tilapia, from the suppression of key steroidogenic enzymes (Bhandari et al., 2006). These principles were also observed during our field work during the fish farm visits.

Rising AMR Prevalence

Antimicrobial resistance lists among the top global threats to human health. The increasing occurrence of resistance against commonly used antibiotics endangers the efficacy of treatment against bacterial infections in animals and man. Coinciding virulence and antimicrobial resistance have been observed in bacteria found in fish culture, predicting an emerging threat to public health (Sreedharan et al., 2012). Antibiotic usage in both human and in livestock production drives the increasing prevalence of resistance further. Antibiotic use in animal husbandry is a massive issue, that still contribute markedly to resistance against antibiotics. Restrictions and stronger regulations are important steps to reduce antibiotic use, and to slow down the increasing AMR problem (Manyi-Loh et al., 2018). Underdeveloped countries with lax regulations may use low doses of antimicrobials in feed, to promote faster growth (Cogliani et al., 2011). Antimicrobial use in animals can leave residues through

manure and consequently affect the soil and nearby water. It has been established that soil can be a vital reservoir for AMR genes (Riesenfeld et al., 2004).

Several bacterial diseases that impact tilapia may be treated using antimicrobials, such as streptococcosis, francisellosis, vibriosis and septicaemia caused by motile *Aeromonas* spp. The growing fish farming industry is increasingly troubled by bacterial infections due to the intensification of their production driven by increased demand (Haenen et al., 2023). 80 % of antimicrobials used in aquaculture enters the environment while still active. While in the soil and water, they select native bacterial population for resistance, thus increasing their presence (Cabello et al., 2013). The resistant bacteria can through horizontal gene transfers, spread their resistance genes to other bacteria (Watts et al., 2017). The nature of their use in aquaculture results in sub-therapeutic concentrations for prolonged periods, creating ideal condition for resistance selection (Santos & Ramos, 2018). Studies have shown that bacteria of the same species, from different environments share similar genetic resistance profiles, pointing to interspecies transmission as the possible cause (Del Castillo et al., 2013; Dubey et al., 2022). Whole-genome sequencing has previously shown that individual genes can be solely responsible for high prevalence of resistance, and that mobile genetic elements related to resistance may be disseminated to bacteria of different species or genera (Ying et al., 2019). Steps need to be taken to avoid the increasingly detrimental effects seen globally caused by antimicrobial resistance. This includes aquaculture (Cabello et al., 2016).The shortage of data regarding antimicrobial resistance in aquaculture, and lack of unified and validated methods to perform them has made risk analysis difficult, further complicating the work needed to predict the consequences or establish plans to prevent them (Smith, 2008).

Aims of the study

The aim of this study was to survey the prevalence of antimicrobial resistance from isolated bacteria from farmed tilapia, from soil and from the water bodies in fish farms in Zambia.

Materials and Methods

Sampling Protocol

Samples were collected in November of 2022, in various fish farms across Zambia. 40 different samples were collected, and from these samples, 135 bacterial isolates were examined for antimicrobial resistance. The locations were mainly in the Copperbelt region of Zambia, as well as the south-eastern region of Zambia, around Siavonga and Lake Kariba. Details regarding the sites and what type of samples were collected at each site is presented in Table 1.

Names	Production type	No. of samples collected	Avg. fish weight	Types of samples gathered	Area
Site 1	Fingerlings	5	33.3 g	Full set	Copperbelt area
Site 2	Full production cycle	6	Fingerling	Full set	
Site 3	Breeding program	5	109.4 g	2 fish samples, 1 water sample, 2 sediment samples	
Site 4	Full production cycle	6	66.0 g	Full set	
Site 5	Full production cycle	6	140.0 g	Full set	

Site 6	Cage-farming, buys fingerlings for stocking	4	292.5 g	2 fish samples, 2 water samples	Siavonga area
Site 7	Cage-farming, buys fingerlings for stocking	4	220.6 g	2 fish samples, 2 water samples	
Site 8	Fingerling production	4	Fingerling	2 water samples, 2 sediment samples	

Table 1. Sampled Sites, full set meaning 2 fish samples, 2 sediment samples and 2 water samples.

The sampling was performed to collect bacteria from two environments the fish were in contact with, as well as one sample was taken from the fish itself (Table 1). The three different sample types were:

1. Sediment
2. Water
3. Fish vent

Two samples of each category (1-3) were collected, and each sample was inoculated on two agar-dishes, one blood agar (0.5% NaCl) and one bromothymol blue lactose agar (BTB agar). Water and sediment samples were collected for bacteriological examination and used for study of water contamination (environmental contaminants). Samples for bacteriological and chemical examination were of the same origin, with few exceptions. Samples for chemical examination were not included as part of this study.

Sediment Samples

Samples (50 ml) were collected from locations at each fish farm that were considered most likely to contain contamination and medicinal residue, *i.e.*, sediment in runoff ponds where

the water was exiting the facility, and in the ponds where sex reversion was carried out using methyl-testosterone that were fed to the fry over a period of 28 days. A plastic inoculation loop was submerged in collected sediments, rotated and further inoculated onto the agar plates.

Water Samples

Water samples (500 ml) were collected using the same principles as for the sediment samples, *i.e.*, ponds/waterways with the highest probability of finding contamination were included. 100 µL of water was applied onto the blood and BTB plates from each water sample and spread evenly onto the entire surface of the plates, using a sterile plastic L-rod.

Fish Samples

Fish samples were collected from the vents of euthanized tilapia from the different sites as shown in Table 1. The fish were first sedated using clove powder to a point where no reflexes were observed when taken out of the water. The inoculation loop was introduced into the vent and pushed 3-6 mm cranially, and then rotated along the walls of the hind guts. For the smallest fish, a small incision was necessary to ease the entry of the loop into the fish vent. The incision was made using a sterile scalpel blade. For the smallest fish sampled, faeces was mechanically forced out of the vent by pressing the abdomen of the fish using the index finger and thumb on each side of the abdomen, and obtained faeces was collected by use of the inoculation loop.

Samples from 3 fish were pooled, and the pooled samples were transferred to two agar plates, using a sterile plastic inoculation loop.

Storage during field work

The samples were stored in sealed plastic bags in a plastic box. During the transport from the sampling sites to the hotel, the samples were stored at ambient temperature, but while in the hotel they were refrigerated (to avoid overgrowing of the agar plates). The samples from the

first week of field work (In the Copperbelt area) were stored cooled (4 °C) at the University of Zambia (UNZA) for 7 days until examined.

The sampling during the second week was done in the Siavonga area and the same type of samples (Table 1) and the same methods were used during sampling and further processing locally.

Laboratory Analysis

The laboratory analysis was initiated after the completion of sampling the 2nd week. The samples collected during the 1st week were examined first. Some plates with overgrowth were sub-cultured on agar corresponding to the original plates used (Blood or BTB agar).

Colonies of morphological similarity to *E. coli, Aeromonas sp. or pseudomonas sp.* was chosen for sub-cultivation, if present. Otherwise, other colonies were chosen for sub-cultivation. Single colonies with minimal chance of contamination were prioritized.

If multiple good candidates for testing were available, multiple were chosen. The colonies that were sub-cultured were noted. The initial sub-cultivation was conducted on a Friday, and the agar dishes were refrigerated over the weekend at 4 °C. The dishes used for subculturing were of the same make and batch as the previous ones. These extra dishes were stored refrigerated up to this point.

Kirby-Bauer Test (Disk Diffusion Test)

The standardized method for antimicrobial resistance testing, Kirby-Bauer, was used for. The method is based on applying liquified colonies evenly to Mueller-Hinton agar and applying tablets of different antimicrobial substances to evaluate the susceptibility of the bacteria to these substances. Evaluation is done after 24 hours of incubation and usually is measured using a millimeter ruler that measures the inhibition zone diameter across the tablets. Seven antimicrobial drugs were included (Table 2).

Name	Antimicrobial class	Concentration
Neo-Sensitabs Tetracyclines (oxytetracycline)	Tetracyclines	30 µg
Neo-Sensitabs Neomycin	Aminoglycosides	120 µg
Neo-Sensitabs Trimethoprim	Diaminopyrimidines	5 µg
Neo-Sensitabs Amoxicillin	Beta-lactam	30 µg
Neo-Sensitabs Florfenicol	Amphenicols	30 µg
Neo-Sensitabs Sulfonamides	Sulfonamides	240 µg
Neo-Sensitabs Flumequin	Fluoroquinolones	30 µg

Table 2. Antimicrobials used for susceptibility testing

Bacteria were dissolved in sterile 0.9% NaCl and to mixed match a 0.5 Mcfarland standard of opacity when dissolved. 2ml Eppendorf tubes were used but their opacity made the reading difficult, and to adjust for the opacity of the wall of the tube, solutions of 0.5 Mcfarland standard were prepared in the transparent glass tubes, which was then transferred to the Eppendorf-tubes to be used as reference. This method of visual adjustment was practiced before traveling to Zambia.

Colonies for sensitivity testing were chosen to avoid contamination, *i.e.*, that multiple bacteria were included on the same MH-plate. The previously sub-cultured plates were usually monocultures. If several good colonies were present on a plate, more than one would be chosen for sensitivity testing.

The solution was made using 1 mL sterile saline and colonies of bacteria were collected and mixed with saline using a sterile plastic inoculation loop. The solution was mixed using a vortex mixer for 8-10 seconds, to obtain an opacity of 0.5 Mcfarland standard. During this process the Mueller-Hinton plates was dried at 37 °C to avoid condensation and to allow quicker drying.

The bacterial solution was applied to the Mueller-Hinton agar using a sterile cotton swab. Excess water in the swab was removed by pressing the swab against the tube wall. The swab was rotated around its axis while applying the bacteria evenly using a back-and-forth motion to cover the entire plate. The plate was then rotated 60 degrees, and the process repeated. Two rotations were performed to ensure even coverage of the bacteria. Subsequently the plates were dried for a few minutes before the antimicrobial tablets were applied. They were applied using a proprietary NEO-SENSITABS applicator with a capacity of 7 different tablets. Evaluation was performed the following day after incubation at room temperature, with some exceptions because of slow growth that were evaluated at 48 hours after inoculation. Evaluation was done using a proprietary NEO-SENSITABS ruler and determined to the closest millimeter. Some plates showed no growth and were excluded.

Shipping of samples to Norway

The bacterial colonies used for AMR (Antimicrobial resistance testing) were simultaneously inoculated on blood agar ordered after their sampling location and labelled. Up to 8 samples were inoculated on each plate. Bacteria from the same sites were placed onto the same plates. The samples were to be analyzed for identification of selected bacterial strains using 16S-sequencing. The separate blood agar plates were shipped to the Norwegian University of Life Sciences (NMBU) using an international medical courier. The inoculated agar plates were stored (at 4 °C) until shipment from UNZA, and local staff was informed of the details of the shipment. The package was to remain upright, if possible, to avoid condensation resulting in mixing of the bacterial isolates inoculated onto the plates. The samples were also to be shipped with cooling elements to keep the bacteria from overgrowing. The shipment was delayed by almost 8 weeks and arrived in early January. Upon inspection of the plates, most plates were overgrown and filled with various amounts of liquid, presumably from condensation. 101 of 135 colonies were lost due to this event.

16S-Sequencing

DNA extraction

DNA-extraction began with regrowing the remaining 34 colonies on blood agar at 30 degrees °C for 24 hours. Single colonies were transferred to 15 ml centrifuge tubes containing 5 mL LB broth. The tubes were incubated for 24 hours at 30 degrees °C at 150 rpm. The extraction was done using a Qiagen "DNeasy Blood & tissue kit".

1. The LB-Broth was spun at 300 x *g* using a centrifuge for five minutes.
2. The remaining liquid was discarded.
3. 200 µl PBS was used to resuspend the pellets left at the bottom of the tubes after spinning. 20 µl Proteinase K was also added.
4. 200 µl Buffer AL was added before being mixed with a vortex mixer.
5. 200 µl ethanol (96%) was added and the mixture was further mixed with a vortexer.
6. The solution was placed in a DNeasy Mini spin column with a 2 ml collection tube and centrifuged at 6000 x *g* for 1 minute.
7. Flow-through discarded before replacing the collection tubes.
8. 500 µl Buffer AW1 was added before centrifuging at 6000 x g for 1 minute. Flow-through discarded and collection tubes replaced.
9. 500 µl Buffer AW2 was added to the spin column before centrifuging again at 20,000 x *g* for 3 minutes. Flow-through discarded and the spin columns were placed in 1,5 ml Eppendorf tubes.
10. The DNA was eluted using nuclease free water (50 µl) and centrifuging at 8000 x *g* for 1 minute.

Concentrations of DNA was measured using absorbance spectrophotometry. This task was performed using a VWR mySPEC micro-volume spectrophotometer. 2 µl sample was used to measure the concentration. A blank sample of nuclease free water is used to calibrate the

machine before testing. During the measurement, the optical density of the sample at 260 and 280 nm is measured to quantify the concentration of nucleic acid present in the sample.

Polymerase Chain Reaction trial run

A trial run of 8 samples were conducted to verify the proper functioning of the protocol. The PCR mix was comprised of these components per sample:

- 10 µl Phusion High-fidelity Master Mix (Thermo Fisher)
- 11,5 µl Nuclease free water
- 0,5 µl 27F Forward Primer (Eurofins Genomics)
- 0,5 µl 1492R Reverse Primer (Eurofins Genomics)
- 2,5 µl Template
- Total Volume: 25 µl

The trial run samples were run under these following conditions:

1. 98 °C for 10 seconds
2. 98 °C for 30 seconds
3. 55 °C for 30 seconds
4. 72 °C for 120 seconds
5. 72 °C for 5 minutes.

Point 2-4 were repeated for 34 cycles.

Gel electrophoresis trial run

To verify the presence and purity of the samples, a gel electrophoresis was run. 1% agarose gel was made using 0,5 g agarose mixed in 50 ml 1xTAE-buffer with 5 µl Thermo Fisher "SYBR Safe DNA gel stain". 10 µl sample was mixed with 2 µl Thermo scientific DNA Gel Loading Dye (6x) in a 0.2 ml PCR tubes using a pipette before being applied into the gel

wells. 0,5 µl to 1 µl Thermo Scientific 1 kb Generuler DNA Ladder was added to the first and last well.

The electrophoresis was performed using the following settings:

- 90V
- 400 mA
- 60 minutes.

Final run

A final run was performed after dilution and concentrating the samples to a concentration of around 100 µg/ml. Samples of low concentrations were concentrated by applying more template and reducing the amount of nuclease free water accordingly. The adjusted samples were ran using the same adjusted PCR conditions as the previous run. 5 µl sample was mixed with 1 µl of the same loading dye. The PCR product was run through a gel of the same recipe, using the previous settings and conditions. This time using one of the previously positive samples from the former trial run as a positive control.

DNA-Elution

The DNA was eluted from the PCR amplicon using a Qiagen "QIAquick Gel Extraction Kit". Gel cutting was therefore not performed, since the visible bands showed no contamination from other sources. The kit can also be used to purify DNA from enzymatic reactions such as PCR product.

1. Added 5 volumes of Buffer PB to the PCR sample, in this case 80 µl buffer to 20 µl PCR product. Mixed.
2. Check to see if the mixtures color is yellow, since it was, no steps were performed to correct this.
3. Mixture placed in spin columns in 2 ml collection tubes and spun at 13,000 RPM for 1 minute.

4. Flow-through was discarded and collection tubes reused.
5. 0.75 ml Buffer PE was added to the spin column and centrifuged for 1 min at 13,000 RPM.
6. Flow-through was discarded and the collection tubes reused.
7. The columns were spun for an additional minute to ensure complete removal of residual ethanol.
8. The spin columns were placed in clean 1.5 ml Eppendorf tubes.
9. 40 µl of nuclease free water was added to center of the spin column and left standing for 1 minute to increase DNA concentration before the final centrifuging for 1 minute at 13,000 RPM.
10. Spin columns were discarded.

The eluted DNA was subsequently measured for concentration using the same VWR mySPEC absorbance spectrophotometry machine as previously mentioned. Concentrations were noted and the samples were sent for sequencing. Results were analyzed using the National Center for Biotechnology Information (NCBI) BLAST Database, using the standard nucleotide BLAST. The longest chains (Forward or Reverse) were analyzed.

Data management

All data was entered into Microsoft Excel. All graphs were also made with Excel.

Results

Some samples collected during week 1 were overgrown and needed sub-cultivation to be tested. The overgrowth made selection of preferred bacteria more difficult.

Resistance results

Resistance against tetracyclines

The Clinical and Laboratory Standards Institute (CLSI) has guidelines available for *Aeromonas hydrophila, caviae and veronii* Complex and tetracyclines available in M45 (CLSI, 2015b). The zone diameters for this specific match are defined as follows:

- Susceptible=≥15 mm
- Intermediate=12-14 mm
- Resistant=≤11 mm

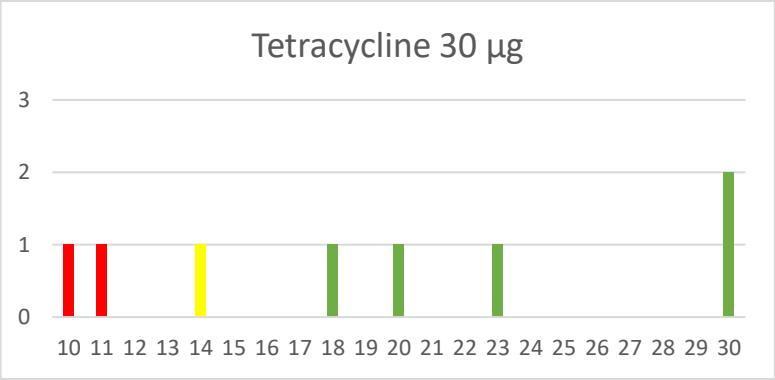

Figure 2. Known Aeromonas spp. *(8 isolates) Tetracycline resistance. Y-axis: No. of isolates, X-axis: Millimeter value, Colonies in red are considered resistant, yellow intermediate and green susceptible.*

Applying these values to the eight known *Aeromonas* Isolates, two isolates are resistant (Red), one intermediate (Yellow) and five susceptible (Green), resulting in a percentage of resistance at 25% (Figure 2). Similar color coding was used for all graphs where guidelines are available.

If we apply these same values to the entire dataset (Figure 3), 11% of the bacteria tested are scored as resistant, *i.e.*, the majority of isolates are susceptible under these criteria. To note, these parameters are not meant to be applied directly to an unknown group of bacteria. Adjusting the criteria to, *e.g.*, ≤17 mm as a threshold for resistant score, there is no significant increase in percentage of isolates that falls into this category since the majority of isolates showed a large inhibition zone, *i.e.*, 71% of isolates are ≥26 mm. Few strains are resistant towards tetracyclines.

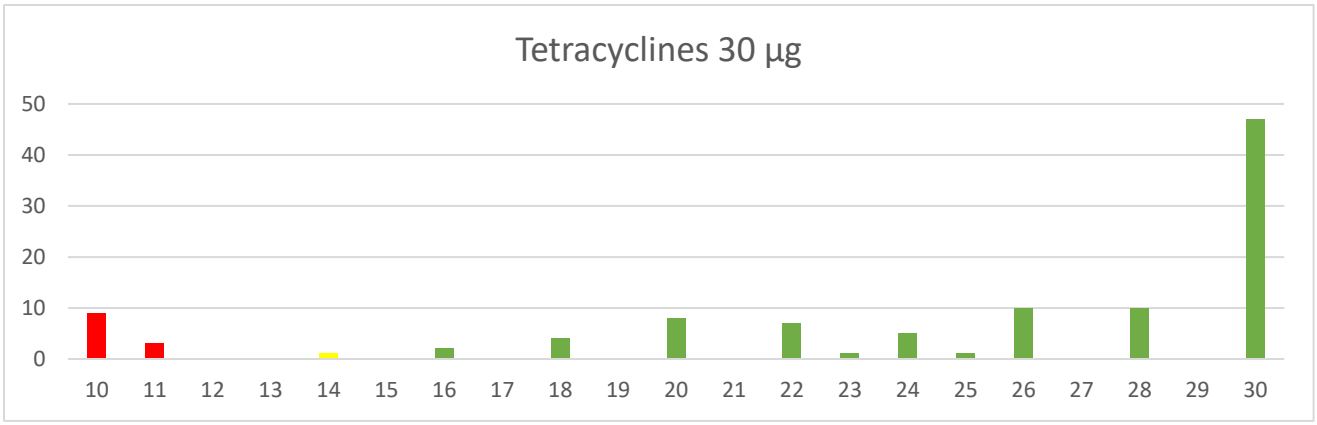

Figure 3. Tetracycline resistance, using Aeromonas spp. *values, whole dataset, (108 isolates) Y-axis: No. of isolates, X-axis: Millimeter value, Colonies in red are considered resistant, yellow intermediate and green susceptible.*

Variations did occur between sites, as shown in Figure 4. A simple chart showing the percentage of isolates with a zone diameter of 10 mm (resistant) for each given sites. The site with a high prevalence of resistance (site 4) reported no use of tetracyclines or other antimicrobials. Site 6 did however report use of oxytetracyclines, yet they did not exhibit higher prevalence of resistance.

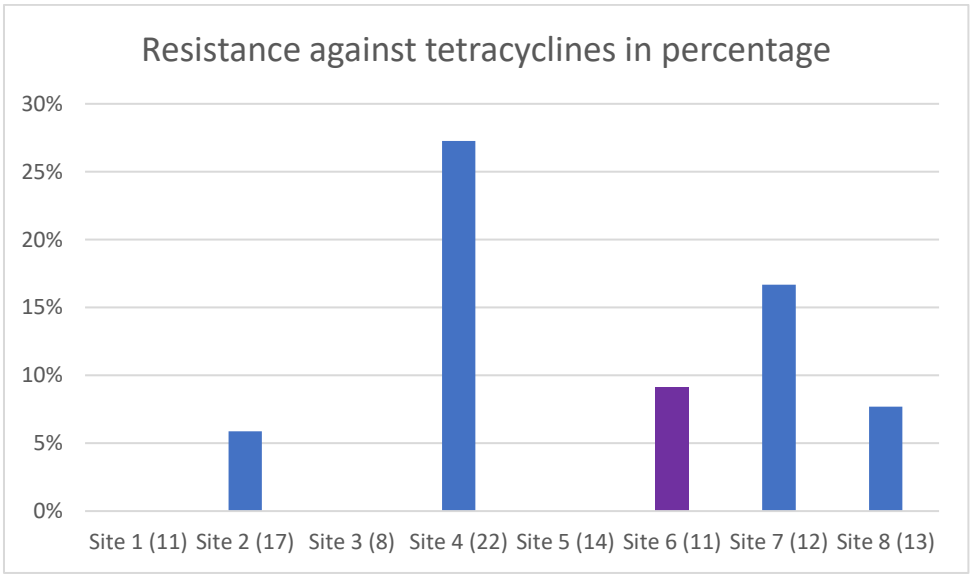

Figure 4. Variations of percentages resistant (10mm) between sites, sites with reported use of tetracyclines highlighted in purple. Number of isolates from each site in parenthesis.

Resistance against trimethoprim

CLSI or EUCAST guidelines are not available for *Aeromonas* and trimethoprim. However, values for the selected disk (trimethoprim 5 µg) are available for *Enterobacteriaceae (Rosco-Diagnostica, 2011).* The manufacturers user guide (based on CLSI and EUCAST's work) defines these values for the selected antimicrobial:

- Susceptible: ≥20 mm
- Intermediate: 19-17 mm
- Resistant: ≤16 mm

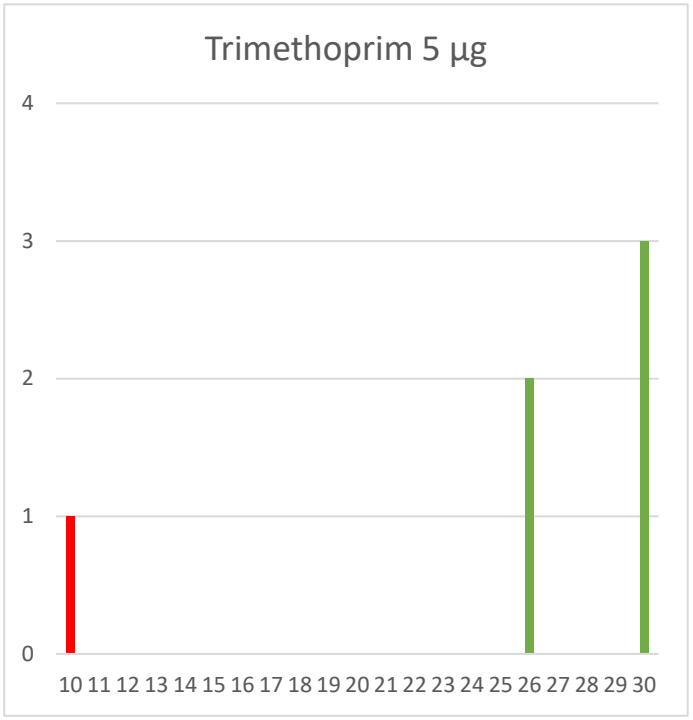

Figure 5. Resistance against Trimethoprim in the six known isolates that belong to Enterobacteriaceae Y-axis: No. of isolates, X-axis: Millimeter value, Colonies in red are considered resistant, yellow intermediate and green susceptible.

Applying these parameters to the six known isolates belonging to *Enterobacteriaceae* yields the following results shown in Figure 5. Only one of these isolates are by default counted as resistant, since no growth inhibition was present (10 mm is the diameter of the disk). The one resistant being the *Enterobacter asburiae* isolate.

Distribution of zone diameters applied to the whole dataset is shown in Figure 6. 23.3% of the isolates present are by default counted as resistant since they showed no zone of inhibition.

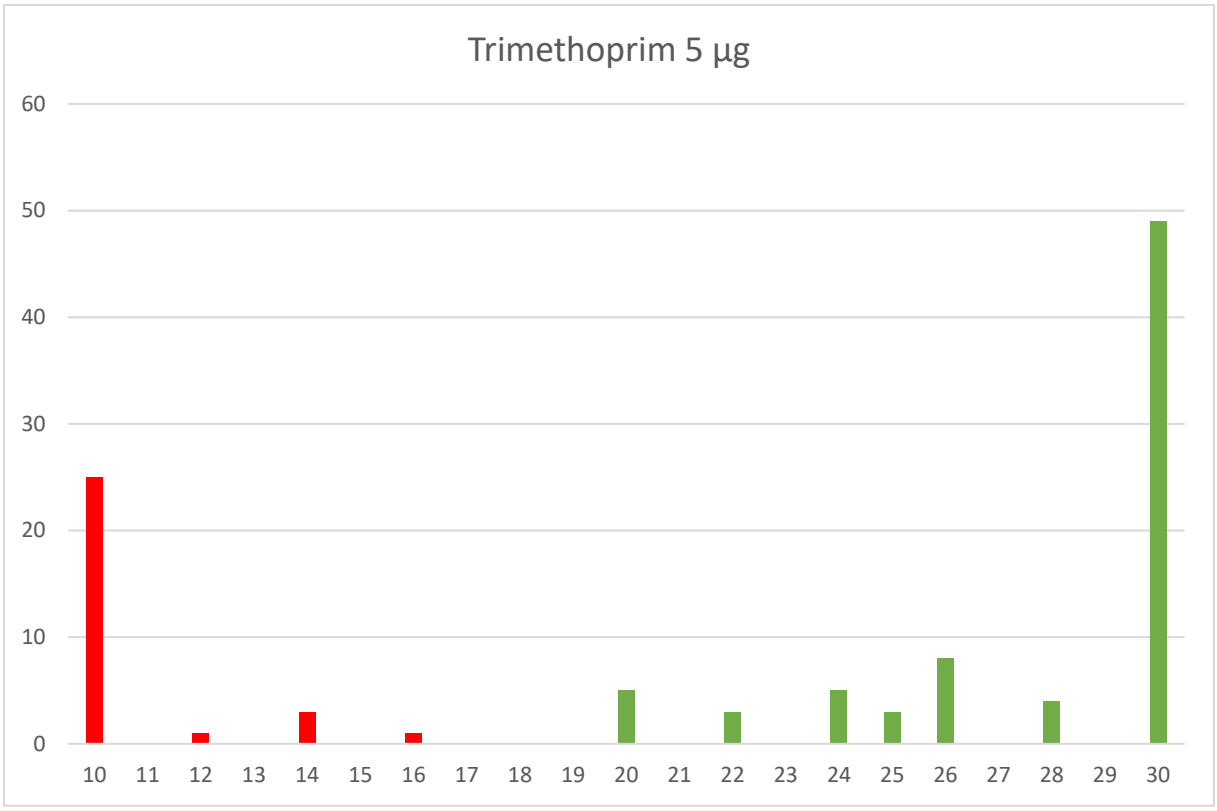

Figure 6. Distribution of zone diameters for trimethoprim (107 isolates) Y-axis: No. of isolates, X-axis: Millimeter value, Colonies in red are considered resistant and green susceptible.

Variations between the different sites was observed, shown in Figure 7 as a percentage of isolates resistant to trimethoprim. Total number of isolates from the corresponding site in parenthesis. Two sites (1 & 2) showed a low degree of resistance compared to the other sites.

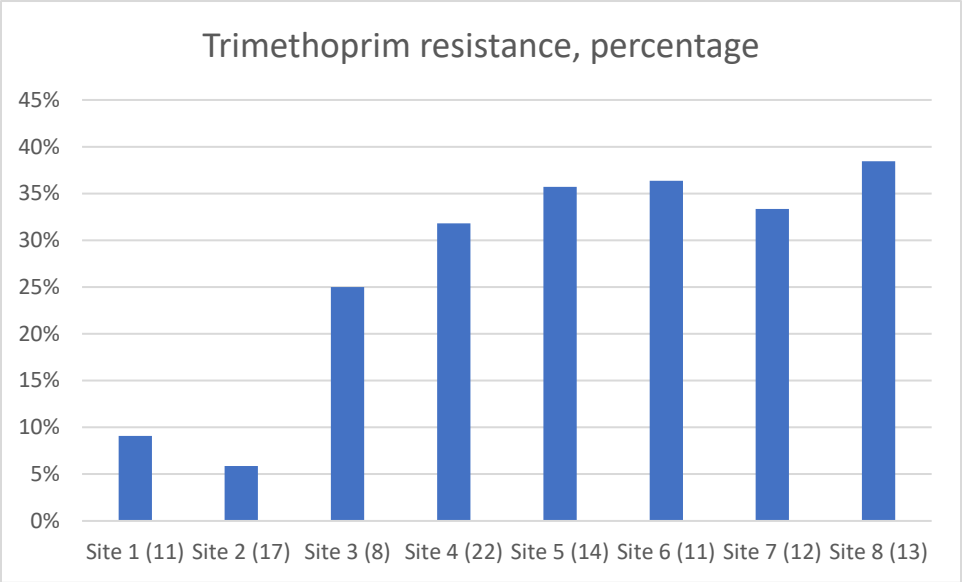

Figure 7. Variation in resistance of different sites, percentage of isolates deemed resistant. Amount of isolates from each site in parenthesis.

Resistance against amoxicillin

Amoxicillin resistance is the one of most common innate resistances, since amoxicillin and other penicillin-like antibiotics bactericidal effects are generally limited to gram-positive bacteria. As expected, a high degree of resistance was present in the dataset. Distribution of zone diameters is shown in Figure 8. Forty-seven isolates were by default resistant to amoxicillin (44%). The percentage of true amoxicillin resistance is by all accounts higher, since a cutoff has not been chosen in this figure. The user guide for NEO-SENSITABS presents these values for amoxicillin zone diameters for *Enterobacteriaceae* (colors in the chart reflect these values):

- Susceptible: ≥20 mm
- Intermediate: 19-17 mm
- Resistant: ≤16 mm

Half of the known isolates in the gram negative *Enterobacteriaceae*-family showed no zone inhibition for amoxicillin, the other half falling into the susceptible category (≥20 mm). 75%

of the known *Aeromonas* spp.-isolates (gram-negative) Is also resistant to amoxicillin, with only two of eight isolates being susceptible.

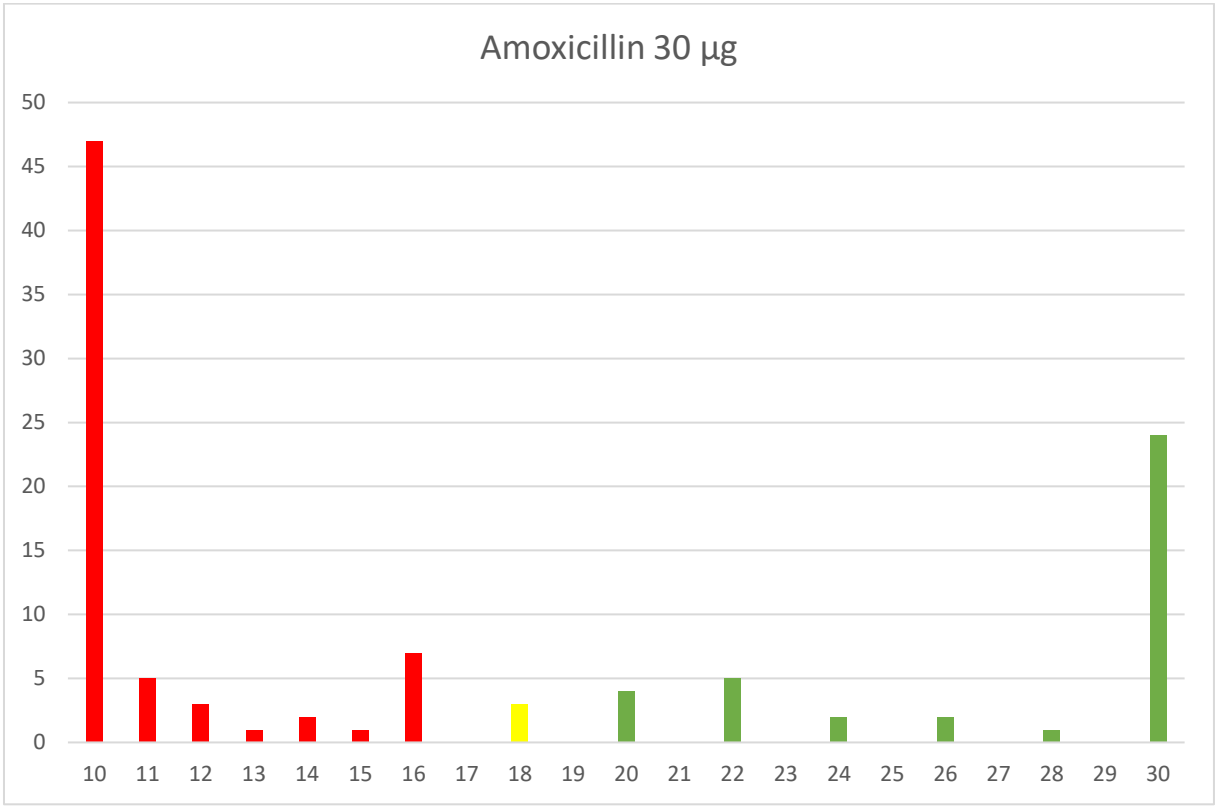

Figure 8. Distribution of zone diameters for amoxicillin (107 isolates) Y-axis: No. of isolates, X-axis: Millimeter value, Colonies in red are considered resistant, yellow intermediate and green susceptible.

Reported usage

Examination of the dataset revealed that the site with reported use of penicillin-like antimicrobials regularly (Site 5), had the highest observed prevalence of resistance (79%). This is shown in Figure 9. Site 8 is also close with 77% resistance, but with no reported use of antimicrobials. The values presented in the previous figure is used here as well (R=≤16 mm).

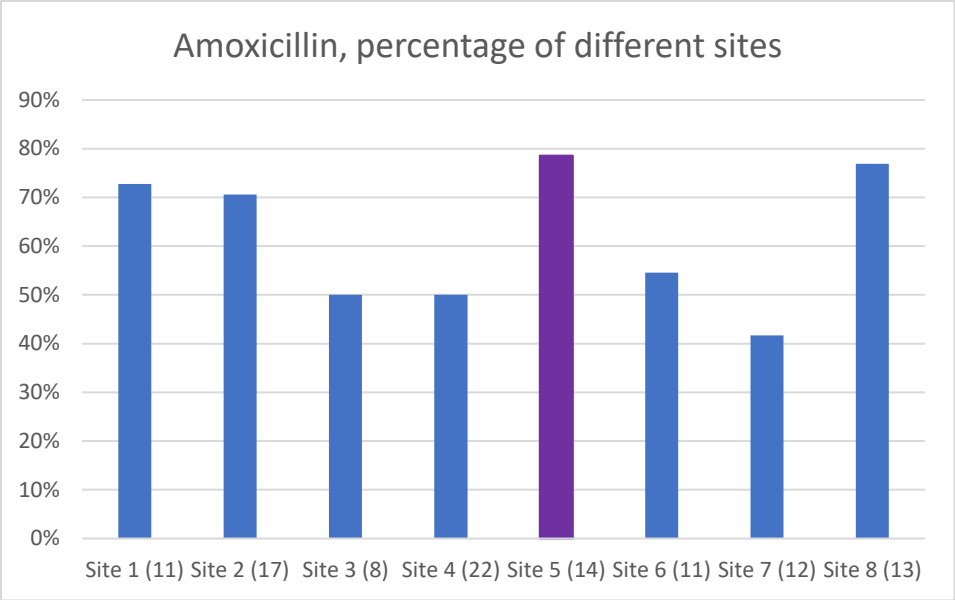

Figure 9. Variation in observed resistance between the sampled sites. Sites with reported use of penicillin-like antimicrobials highlighted in purple.

Resistance against florfenicol and flumequine

Florfenicol and flumequine are antimicrobials of different classes and mechanisms of action, yet they showed very similar distributions of zone diameters. Generally, the zone diameters are large, hence the probable prevalence of resistance against these antimicrobials are low in this dataset, shown in Figure 10. No guidelines are available from either EUCAST or CLSI for the pairing of these two antimicrobials with *Aeromonas* spp. We can still conclude that the general susceptibility and hence the resistance present is low, since the vast majority of isolates' zones were measured to over 30 mm. For florfenicol 73 of 107 isolates (68%) tested are considered susceptible by default, with only 9 isolates showing no inhibition of growth (8%). For flumequine, an even lower percentage of default resistance is observed, with only 2 isolates showing no inhibition of growth, with 75% of the isolates measuring ≥ 30 mm. No defined millimeter values shown in the figure, reflect susceptibility. Guidelines are available through CLSI M45 for *Aeromonas caviae, hydrophila* and *veronii* for chloramphenicol, a closely related antimicrobial of florfenicol, sharing the same mechanism of action (CLSI, 2015a). The breakpoint for chloramphenicol of similar concentration (30 µg) is defined as R=≤12 mm. This value would classify 10% of the isolates as resistant.

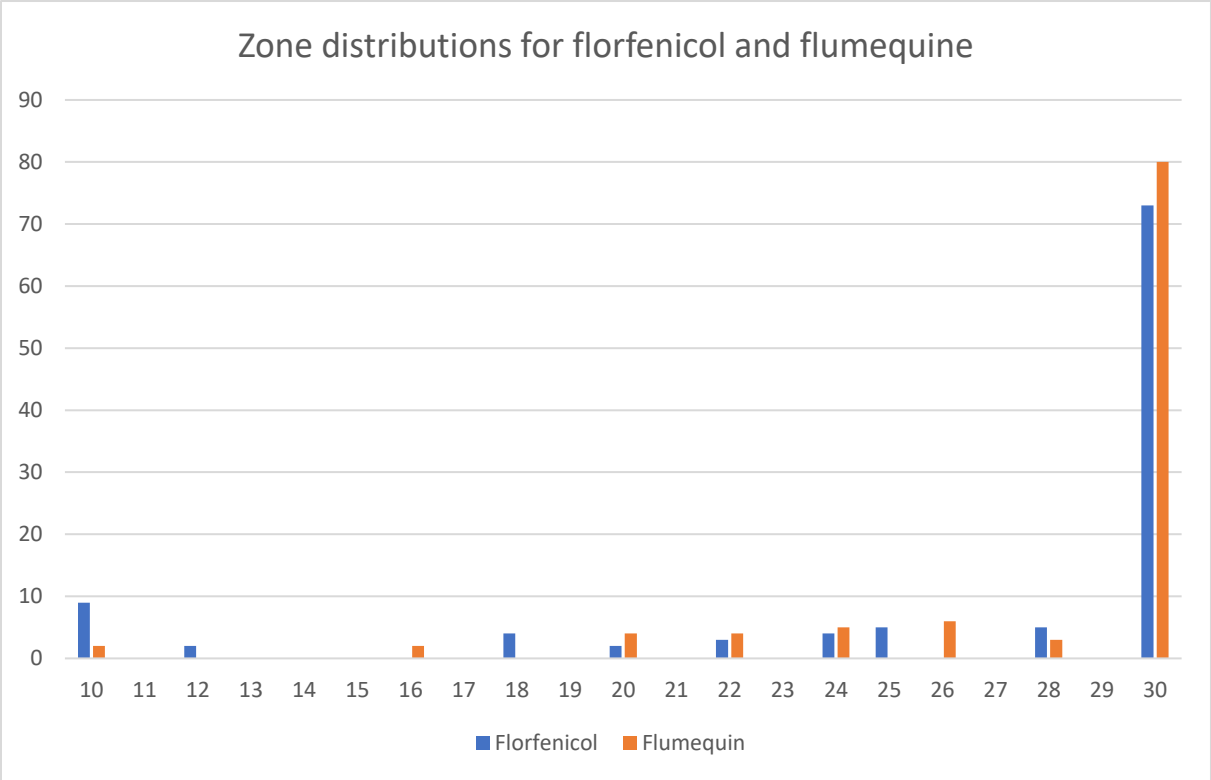

Figure 10. Zone distributions for florfenicol and flumequine (107 isolates) Y-axis: No. Of isolates, X-axis: Millimeter value

Resistance against neomycin

No guidelines are available for *Aeromonas* spp. and neomycin from CLSI or EUCAST. The user guide for NEO-SENSITABS has guidelines available for *Enterobacteriaceae* and neomycin. Zone distribution is shown in Figure 11. Those values are:

- Susceptible: ≥25 mm
- Intermediate: 24-21 mm
- Resistant: ≤ 20 mm

Only one isolate, stemming from a water sample from site 6 exhibited no inhibition of growth. If applying the breakpoints valid for Enterobacteriaceae, 93% of isolates would be counted as intermediate or susceptible (55% susceptible).

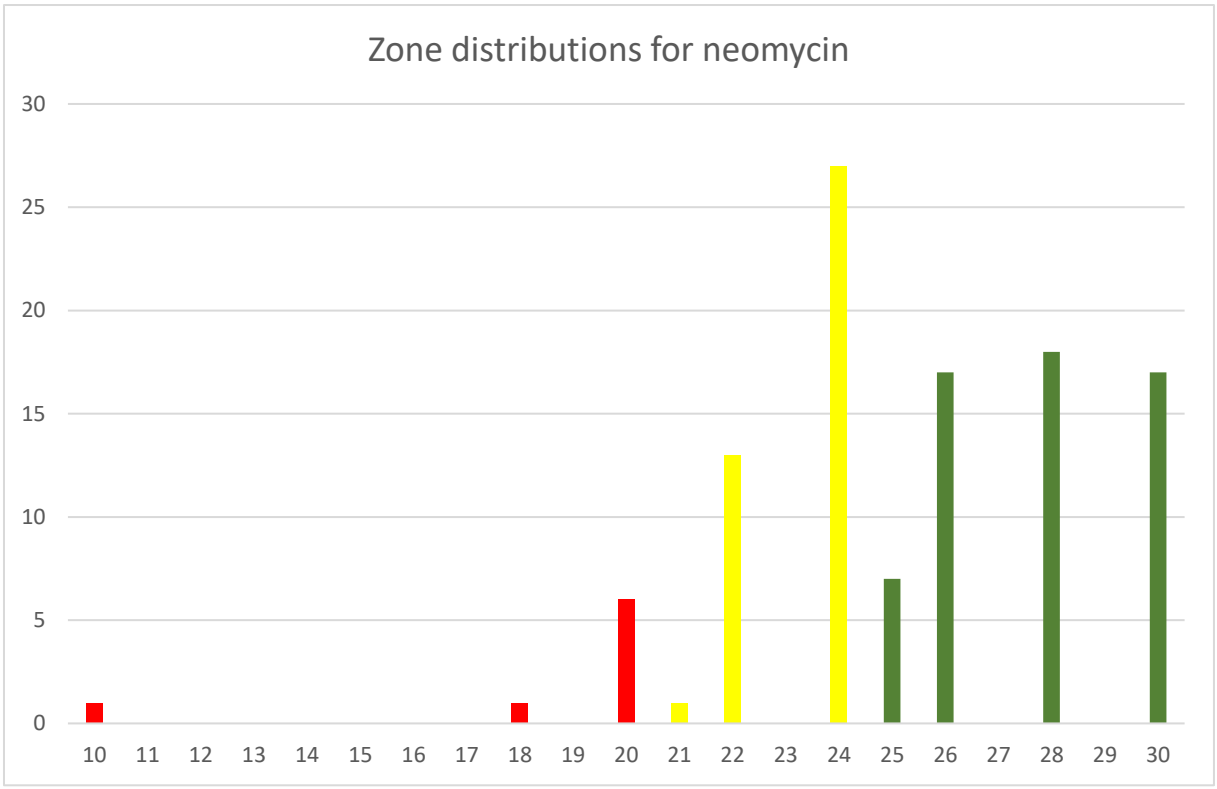

Figure 11. Zone distributions for whole dataset (108 isolates), neomycin Y-axis: No. of isolates, X-axis: Millimeter value, Colonies in red are considered resistant, yellow intermediate and green susceptible.

Resistance against sulfonamides

Established guidelines are not available from CLSI or EUCAST for sulfonamides and *Aeromonas* spp. guidelines are however available for *Enterobacteriaceae* through the manufacturer's user guide. The values they define are as follows:

- Susceptible: ≥ 17 mm
- Intermediate: 16-13 mm
- Resistant: ≤12

These values are applied in Figure 12. Under these definitions, 21 isolates are considered resistant (20% of isolates). The majority of isolates are considered susceptible for sulfonamides.

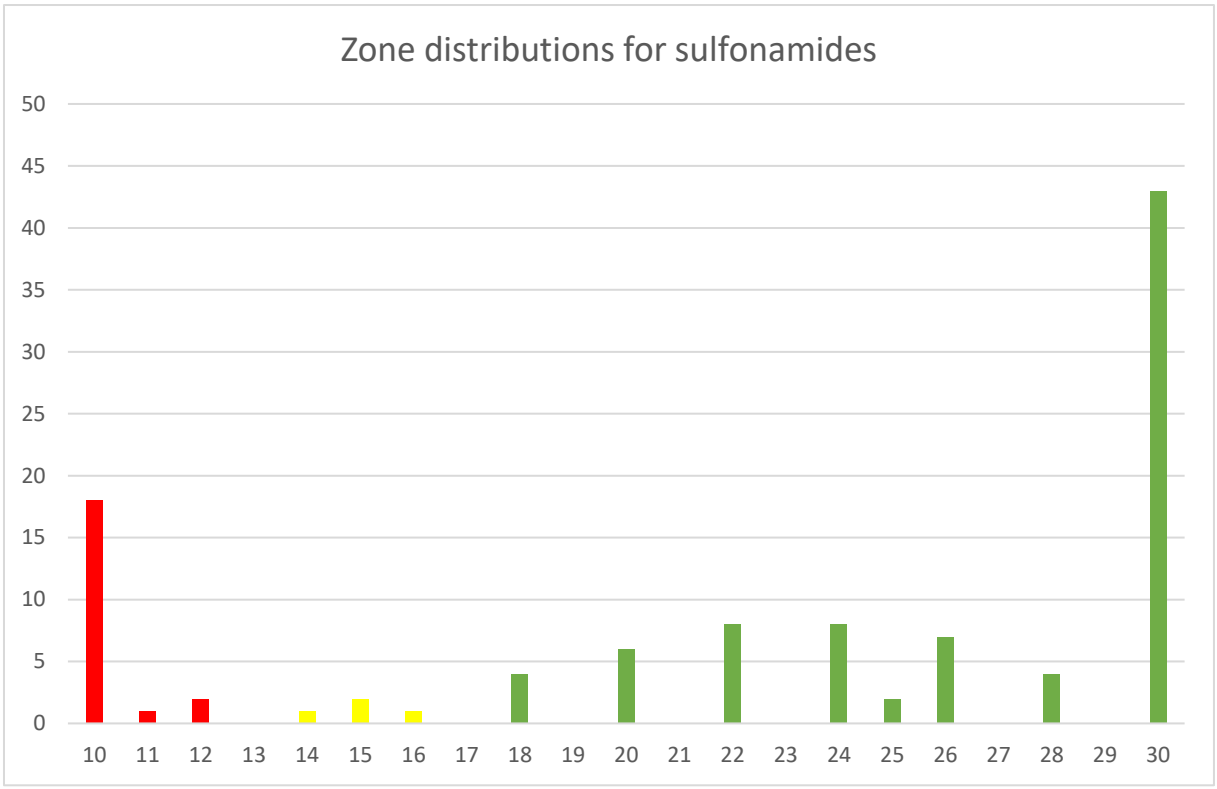

Figure 12. Resistance against sulfonamides, Y-axis: No. of isolates, X-axis: Millimeter value, Colonies in red are considered resistant, yellow intermediate and green susceptible.

PCR and Gel electrophoresis

No bands from samples were observed during the first trial run. Through a calculation of annealing temperatures using Thermo Fisher's Annealing Temperature Calculator, a new temperature of 59,5 °C was chosen (Thermo-Fisher, 2023). A second run was performed, every other factor being equal except the annealing temperature. This trial run was successful, with visible bands on 1500 kb (the approximate length of the 16S rRNA gene). The bands were however widely ranging in intensity and color; hence dilution was needed of the samples with higher concentrations of DNA. The third and final gel electrophoresis yielded abnormal results, with most bands visible, yet some not visible with no apparent reason. The positive and negative controls were both negative. Due to time constraints, it was decided that all the samples were to be sent for 16S-Sequencing regardless.

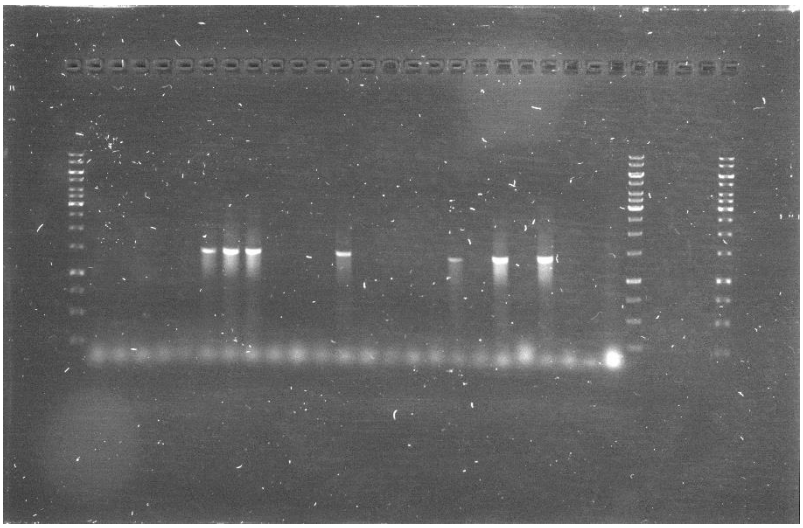

Figure 13. One of two gel electrophoresis results final run.

Sequencing results

Since many of the samples were lost during the transportation to Norway all remaining bacteria were sequenced. In total, 34 samples were sent for sequencing, of which 16 were identified at genus or species level (Table 3). Eight sample came out with sequences that matched with piscine orthoreovirus (PRV), to varying percentages, and another 8 samples were inconclusive. Site 5 unfortunately had no conclusive matches.

Location	Sample ID	Suspected Bacteria	Perc. Match
Site 1	2	*Aeromonas veronii*	97.73%
Site 1	4	*Aeromonas veronii*	98.34%
Site 1	9	*Aeromonas veronii*	97.82%
Site 1	10	*Enterobacter asburiae/Klebsiella pneumoniae*	97.77%/97.77%
Site 1	12	*Citrobacter freundii*	97.10%
Site 4	49	*Serratia ureilytica*	97.67%

Site 4	50	*Enterobacter asburiae*	96.47%
Site 6	97	*Aeromonas veronii*	97.44%
Site 6	98	*Aeromonas veronii*	97.91%
Site 6	100	*Leclercia adecarboxylata*	97.84%
Site 7	114	*Enterobacter hormaechei*	97.74%
Site 7	118	*Aeromonas (Low match perc.)*	86.37%
Site 7	119	*Aeromonas veronii (Low match perc.)*	76.16%
Site 8	121	*Pseudomonas putida*	98.17%
Site 8	125	*Pseudomonas plecoglossicida*	96.41%
Site 8	132	*Aeromonas taiwanensis*	98.37%

Table 3. Bacteria identified using BLAST, color coded for the different sites.

The bacteria belong to the genera *Aeromonas*, *Enterobacter* and *Pseudomonas*. Some other bacteria were present, such as *Serratia ureilytica*, *Leclercia adecarboxylata* and *Citrobacter freundii*, all belonging to the *Enterobacteriaceae* family. Sample 10 had two similarly matching percentages for bacteria of different genera. All these bacteria can be found in soil or water.

Several samples were identified with varying percentages of matching identity to Piscine Orthoreovirus (PRV). This suggests mix-up of samples at the sequencing facility or that that reagents used for sequencing were contaminated and the results were omitted. This will be further discussed later.

Reported use of antimicrobials

Each site where samples were collected was given a questionnaire with questions regarding their operations and practices, questions about antimicrobial use were also included. Six of the eight sites reported no previous use of any antimicrobials. Two sites reported use, shown in Table 4. The reason and extent of use of tetracyclines at site 6 is unknown.

Sites that reported use of AM	Reported AM used
Site 5	Penicillin*
Site 6	Oxytetracycline

*Table 4. Sites who reported use of antimicrobials (*This specific site feeds swine manure into the ponds to create edible algae for the fish, and reported use of penicillin for treatment of the swine.)*

Data interpretation

Of the 135 bacterial colonies tested, 25 plates were unreadable, usually due to a lack of bacterial growth on the plates. Plates that were unreadable were left for another 24 hours for a total of 48 hours before being measured. Those still exhibiting no growth were registered as blank. Some of these bacteria grew on the transport medium.

Zone diameters and their interpretation are generally exclusive to each bacterium and antimicrobial used. Therefore, a universal millimeter value defining susceptibility (or resistance) for different unknown bacteria cannot be used. The millimeter values contained in this thesis' dataset ranges from 10mm (the diameter of the tablet) to 30 mm (maximum of the ruler used). Ranges over 30 mm were not recorded, but simply noted as "S", meaning susceptible. There are several other ways to interpret zone diameters. One method would be using the clinical breakpoints/interpretive criteria determined by the European Committee On Antimicrobial Susceptibility Testing (EUCAST) or from the Clinical & Laboratory Standards Institute (CLSI), however this would require identification of all the bacteria present in the dataset, unfortunately not possible due to the loss of the samples (or negative results from 16S rRNA sequencing). Another approach is using Epidemiological Cut-off Values (ECOFFs). ECOFFs are a type of biological breakpoint used in susceptibility testing. The ECOFF is determined to separate a "wild-type" (Susceptible) bacteria population from a subpopulation of bacteria with acquired resistance (Turnidge et al., 2006). These values are based on statistical analysis of extensive data (Kahlmeter & Turnidge, 2022). With enough data, two

groups of bacteria will emerge, which allows a separation of the two. A cut-off value will be chosen based on these curves to separate the two groups, defining one as a "Wild-type population" and the other one as a resistant population (Giske et al., 2022).

Loss of samples during shipping

The result of this unfortunate event was the complete loss of samples from two locations (2 & 3), and the loss of 101 colonies. The contaminated dishes were discarded, and the laboratory work after receiving the samples at NMBU focused on those not contaminated. The dishes were stored after arrival at 4 °C. Some of the uncontaminated bacterial isolates would not grow on new plates, which could stem from the length of time they were stored. Identification of the bacteria trough means of 16S-gene sequencing was chosen. Originally, identification of the bacteria was to be performed using Maldi-Tof, but since this method relies heavily on previously established databases it was decided to move on with 16S-sequencing (Rychert, 2019). It was uncertain whether the database would be sufficient to identify bacteria from fish and environmental samples from rural Zambia. 16S-sequencing is a useful method of determining genus and species of bacteria that might not be as well researched and documented, or easily identifiable (Janda & Abbott, 2007).

Figure 14. Ruined plates received from Zambia, note the fluid in the bottom of the plastic bag.

Discussion

The results provided insights into what bacteria were present in the samples collected. Bacteria from primarily three genera were found, those being *Aeromonas* spp., *Pseudomonas* spp. *and Enterobacter* spp. The remainder of isolates belonged to the *Enterobacteriaceae*

family. Originally, bacteria of the genera *Escherichia, Aeromonas and Pseudomonas* were preferred for selection. BTB lactose agar plates were included to aid in the specific selection of lactose fermenting bacteria such as *E. coli*. However, *E. coli* was not identified among the isolates obtained from the samples which would have indicated fecal contamination from mammals in the fish farms since *E. coli* is generally found in warm-blooded organisms. The presence of *E. coli* in water is widely used as an indicator of fecal contamination by humans or mammals (Ferdous et al., 2021). Very few bacterial colonies were observed with yellow pigmentation during selection for testing, reinforcing the notion of low presence of fecal contamination. Without the loss of many isolates, this claim would be more certain.

The high prevalence of *Aeromonas* spp. (50% of successful sequences) in the sequenced samples, and the preferential selection of which might indicate a high number of its genera being present in the complete dataset. The loss of samples, however unfortunate, was almost entirely random in its selection, hence the isolates remaining are also random. The elimination of two sites is very unfortunate and may possibly skew the results. Graphs of zone distributions for trimethoprim and tetracyclines are included to compare the distribution of the small known dataset to the complete dataset. The graphs resemble each other, which could indicate a similar resistance profile present in the complete dataset, and therefore support the notion that the dataset contains a significant portion of *Aeromonas* spp. Therefore, making slight analogues and extrapolation between the datasets, and the application of breakpoints available for *Aeromonas* spp. not entirely unhelpful. Admittedly, this is a suboptimal way of interpreting data, but the best available in this scenario. Where possible, applicable values for *Aeromonas* spp. have been used to illustrate the graphs and calculate resistances. Situations where guidelines for *Aeromonas* spp. were unavailable, guidelines for the *Enterobacteriaceae* family, the second most prevalent in the dataset (38% of known isolates), was used instead. The reasoning behind is first that it constitutes the second largest group in of the known

isolates, and secondly, that several of the available guidelines are already adapted from *Enterobacteriaceae* guidelines (CLSI, 2015b, p. 18). Studies have been carried out exploring the level of agreement between the breakpoints for the two, finding a significant agreement (>95 % agreement) for many antimicrobials, tetracycline being one of them (Lamy et al., 2012). Unfortunately, the referred study did not include trimethoprim. Because of *Aeromonas* spp. ability to acquire resistance mechanisms, and its abundant presence in different water sources, makes it a good candidate for becoming a recognized indicator-bacteria for aquatic environments. The lack of literature to date regarding its use as an indicator bacterium, and its lack of guidelines is however unfortunate (Baron et al., 2017).

The observed resistance profiles varied widely, also between different sites. The results have been focused on the antimicrobials with highest likelihood to produce relevant and usable data, and those with reported use, mainly tetracyclines, amoxicillin and trimethoprim. Only two sites reported previous use of antimicrobials, with only one of those exhibiting a higher rate of resistance than the other sites when compared across sites, this being the site which reported regular use of long-acting penicillin-like antimicrobials for treatment of swine disease. The manure of which was used to promote algae growth in the ponds, hence exposing the fish and its environment to any remnants of the drug present in the manure. One other site (site 8) also exhibited an almost equally high prevalence of resistance to amoxicillin yet reported no use of any antimicrobial. This might indicate that the use of penicillin-like antimicrobials has caused an increase of resistance dissemination in the bacterial environment of this particular farm. The number of isolates from each site is generally low, and therefore statistical analysis of differences between sites is not relevant. Generally, the prevalence of resistance against amoxicillin observed across the sites are high, as is to be expected from a class of antimicrobial mainly limited to gram-positive bacteria.

The resistances against the individual antimicrobials and their zone distributions vary, as previously mentioned, and resistance against amoxicillin is high, found in about 70% of strains examined. Similar values have been obtained in other countries in Africa, like Tanzania (Shah et al., 2012). The observed resistance against tetracyclines is 25%, much lower than observed in Tanzania by Shah et al (49.2%), and the observed resistance for trimethoprim is 28%, much lower than observed in Tanzania (68.3%)(Shah et al., 2012). It is worth mentioning that Shah et al. did not specify any preferred phenotypic selection of bacteria, and the results might therefore not be fully comparable. Breakpoints were not easily available for florfenicol and flumequine, but based on the zone distributions presented, a low degree of resistance was present for both antimicrobials. Resistance against neomycin was also considered low, at 20%, and the same goes for sulfonamides at 20%. A south-African study examining antimicrobial resistance present in drinking water sources found an overall resistance at 51.7% against trimethoprim (Ateba et al., 2020), significantly higher than in this study. The referred found somewhat similar levels of resistance against tetracyclines (20%), but lower levels for neomycin resistance (7.4%) (Ateba et al., 2020). A similar study conducted in Uganda, exploring the prevalence of AMR in fish farms, suggested a low level of acquired antibiotic resistance. The study also focused on *Aeromonas* spp. due to its prevalence in the samples collected (Wamala et al., 2018). A higher prevalence of resistance phenotypes in farmed fish was also observed.

Limitations

Sampling limitations

Firstly, the voluntary basis of our sampling. The locations we sampled from gave their voluntary consent to sample their fish and ponds. We also visited sites that did not approve any sampling from their ponds or fish and the reason for this was not given. If this would reflect any underlying worry of use of results or possibly findings cannot be speculated upon. Businesses might want to protect their reputation and therefore not risk anything that would

tarnish their reputation. Overall, the limited number of sites from which bacterial isolates were obtained would raise questions as to the general applicability of the findings reported herein.

Bacterial overgrowth

Overgrowth on the agar plates was also an issue that hindered optimal selection of bacterial isolates, thankfully, assistance was provided in this regard to choose preferred isolates from the plates. Plates that were overgrown could not as easily provide several colonies for susceptibility testing. To rectify this issue, refrigeration during travel would have been necessary. The warm temperatures present in sub-Saharan Africa places limitations for the time bacteria can be stored at ambient temperature on plates. It is likely that this may have impacted the selection of isolates, and the results might be minimally skewed as a result.

Loss of samples during transport

The transportation of bacteria to Norway was catastrophic for this study, since it had a severe impact on how many bacteria could be isolated by 16S sequencing. As previously mentioned, zone diameter values are usually defined by a specific bacterial species to antimicrobial-combination, by extent, making it difficult to reach conclusions. The complete loss of isolates from two sites also removed all ability to compare the bacteria found between these sites and the others. This is certainly the most influential problem for this study, since the impacts of this directly hinders the results that were supposed to be presented, requiring a different and non-ideal approach to the complete dataset.

Challenges during sequencing

The first PCR-run was unsuccessful, as previously mentioned the annealing temperature was changed and the amplicon yielded no visible bands on gel. The second run however, was successful. The two first runs were only run with 8 of the 34 samples with no controls. The

third and final run was performed with all 34 isolates, however the results were unsatisfactory. Many of the samples did not provide visible bands, neither did the positive control. Due to time constraints, it was not possible to redo this step. The true nature of the missing bands is unknown, it may stem from human errors that occurred during the final dilution/concentration of the samples, or during the pipetting and mixing of the samples for the gel electrophoresis. The gel recipe and the setting were the same during all gel runs. The samples were sent for sequencing regardless. This is admittedly suboptimal but was necessary considering for the time constraints.

Concentrations of the final samples sent were measured, and multiple samples were below the ideal concentration of 10 µg/ml. Most of the samples that provided inconclusive BLAST results were those of low concentrations. It was expected that these samples may not provide good sequences for analyzing before shipping of the samples.

Bizarre sequencing results

The eight sequences matching with Norwegian isolates of PRV was very surprising, and the true nature of this is unknown. A similar situation has happened previously when samples were sent to this laboratory before. The existence of viral genome in the samples sent for sequencing is highly improbable. The bacterial colonies were sub-cultured several times, and primers for the 16S-gene, which only exists in bacteria, was used for the polymerase chain reaction. The 16S gene is approximately 1550 bp long (Clarridge, 2004). No DNA chains of other lengths was observed on the results from the gel electrophoresis during this whole process. Since the likelihood of viral genome being present in the samples sent for sequencing is insignificant, it is reasonable to assume that this is an error that has occurred at the laboratory used for sequencing. The previous and similar situation supports this assessment. It is possible that samples may have been swapped upon delivery, or that some carry-over of genome may have occurred in the sequencing equipment. This would call into question the

validity of all the samples; however the remaining results seem plausible considering the bacteria found and were considered valid.

Difficulty of interpretation of the results

As previously discussed, the loss of samples complicated the process of interpreting the results. The original plan was to base the analysis of zone diameters on the specific genera of bacteria isolated, to the specific antimicrobial combinations, since this was not possible, a more creative approach was necessary. This hinders this study's ability to make claims of significant strength.

Lack of literature and guidelines available

Connected to the difficulty with interpreting the results are the choice of antimicrobials. The consideration of what antimicrobials were to be tested should have included more thought regarding the availability of literature and supporting guidelines. This would have eased the analysis of the zone diameters. This was not sufficiently explored before travelling to Zambia. Few similar studies exist, making comparison difficult.

Application and validity

This thesis has given some indication into the level of prevalence of AMR against commonly used antimicrobials, in tilapia farms in Zambia, where little literature exists. The results suggest low levels of acquired resistance. Similar studies conducted in other countries in Africa has revealed both higher and lower prevalence of AMR to different antimicrobials than observed in this study.

This study is limited by several factors, some akin to human errors, and the results should be viewed with this in mind. Still, some level of application is possible. Further work and a more exhaustive study should be conducted to assess the true level of antimicrobial resistance present in fish farms in Zambia. Surveilling resistance dissemination and its consequences is

an important step to address the future problems that may arise from indiscriminate use of antimicrobials in man and animal.

Conclusion

Zambia has been ranked at the top on a global MAR (multi-antibiotic resistance) index out of 40 countries, in a study analyzing antimicrobial resistance in aquaculture (Reverter et al., 2020). Meaning, it has a very high prevalence of multi-resistant bacteria related to aquaculture compared internationally. While this thesis has not included calculations on multi-resistance, the level of resistance is low against most antimicrobials tested.

While this thesis is limited due to multiple factors, it can still suggest that the level of acquired AMR present in tilapia fish farms in Zambia is low. Only two of the eight sites sampled reported use of antimicrobials, only one using them regularly. The site with regular use of penicillin-like antimicrobials did also exhibit the highest percentage of resistance against amoxicillin, as can be expected. AMR is a global challenge to animal and man, and addressing it is of utmost importance. Increased usage of antimicrobials in African aquaculture contributes to increases in antimicrobial resistance (Limbu, 2020). Antibiotic use in Africa is plagued with poor prescription practices, and lax regulation. (Kimang'a, 2012). A change in sentiments in doctors, governments and the general populace regarding treatment strategies and antimicrobial use is much needed. Introducing strict regulations regarding the use of antimicrobials in aquaculture and disease control is important steps to reduce the use of antimicrobials. Success has been observed in Norway, where the use of antibiotics in aquaculture has been significantly reduced due to the introduction of effective vaccines against the most common bacterial infections in salmonids (Bondad-Reantaso et al., 2023).

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Oppsummering

Tittel: Forekomst av antibiotikaresistens i isolerte bakterier fra tilapia-oppdrett i Zambia.
Forfatter: Vebjørn Nygaard Woll
Veileder: Professor Øystein Evensen
Antibiotikaresistens er et økende problem i verden, og er en trussel mot folke og dyrehelsen. Økt forbruk og dårlige holdninger knyttet til bruk er et problem. Økt forbruk av antibiotika i akvakultur er knyttet til økt forekomst av antibiotikaresistens. Denne studien undersøkte forekomsten av antibiotikaresistens mot vanlige antibiotika i tilapia-oppdrett i Zambia. Prøver ble tatt fra 8 forskjellige tilapia-farmer i Copperbelt-området, Siavonga og Kariba-innsjøen. Bakterieisolater ble testet for resistens med disk-diffusjonsmetoden på Mueller-Hinton agar. Bakterieisolatene ble identifisert med 16S-sekvensering. Et spørreskjema ble gitt til oppdrettslokalitetene, der det ble rapportert lite bruk av antibiotika. Den observerte antibiotikaresistensen var lav mot de fleste antibiotikaene testet.

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Appendices

Sample ID	Concentration	Unit	A (260 nm)	A (280 nm)	260/280	260/230	Sample Type	Factor	Baseline corr.	Factor of dilution	Final concentration	Parts added
2	937.103	µg/ml	18.742	9.213	2.034	2.155	DNA	50	340	10	93.71	9
3	77.714	µg/ml	1.554	0.854	1.820	0.913	DNA	50	340	0.5	155.43	-0.5
4	880.796	µg/ml	17.616	8.617	2.044	2.188	DNA	50	340	9	97.87	8
5	243.367	µg/ml	4.867	2.356	2.066	1.905	DNA	50	340	2	121.68	1
6	123.994	µg/ml	2.48	1.288	1.925	1.125	DNA	50	340	1	123.99	0
8	88.151	µg/ml	1.763	0.861	2.048	1.32	DNA	50	340	0.5	176.30	-0.5
9	425.582	µg/ml	8.512	4.139	2.057	2.092	DNA	50	340	4	106.40	3
10	483.074	µg/ml	9.661	4.681	2.064	2.145	DNA	50	340	4	120.77	3
12	500.589	µg/ml	10.012	4.924	2.033	1.762	DNA	50	340	5	100.12	4
49	414.072	µg/ml	8.281	4.146	1.997	1.54	DNA	50	340	4	103.52	3
50	170.373	µg/ml	3.415	1.685	2.027	1.486	DNA	50	340	1.5	113.58	0.5
51	15.831	µg/ml	0.317	0.177	1.791	0.623	DNA	50	340	0.1	158.31	-0.9
52	59.745	µg/ml	1.195	0.599	1.995	1.372	DNA	50	340	0.5	119.49	-0.5
53	19.021	µg/ml	0.380	0.189	2.011	0.484	DNA	50	340	0.1	190.21	-0.9
55	53.299	µg/ml	1.066	0.548	1.945	0.970	DNA	50	340	0.5	106.60	-0.5
75	201.164	µg/ml	4.023	2.092	1.923	1.362	DNA	50	340	2	100.58	1
79	54.451	µg/ml	1.089	0.584	1.865	1.200	DNA	50	340	0.5	108.90	-0.5
97	386.185	µg/ml	7.724	3.785	2.041	1.725	DNA	50	340	3	128.73	2
98	532.503	µg/ml	10.650	5.247	2.030	2.001	DNA	50	340	5	106.50	4
100	484.938	µg/ml	9.699	4.775	2.031	1.945	DNA	50	340	4	121.23	3
101	57.007	µg/ml	1.14	0.575	1.983	1.088	DNA	50	340	0.5	114.01	-0.5
102	74.354	µg/ml	1.487	0.759	1.959	1.527	DNA	50	340	0.5	148.71	-0.5
108	73.842	µg/ml	1.477	0.751	1.967	0.932	DNA	50	340	0.5	147.68	-0.5
114	227.914	µg/ml	4.558	2.251	2.025	1.588	DNA	50	340	2	113.96	1
115	171.184	µg/ml	3.424	1.784	1.919	1.421	DNA	50	340	1.5	114.12	0.5
116	68.001	µg/ml	1.360	0.685	1.985	1.033	DNA	50	340	0.5	136.00	-0.5
118	511.613	µg/ml	10.232	4.948	2.068	2.158	DNA	50	340	4	127.90	3
119	169.027	µg/ml	3.381	1.685	2.007	1.678	DNA	50	340	1	169.03	0
121	929.595	µg/ml	18.592	9.045	2.056	2.275	DNA	50	340	9	103.29	8
122	141.753	µg/ml	2.835	1.696	1.672	0.655	DNA	50	340	1	141.75	0
125	1219.28	µg/ml	24.386	11.848	2.058	2.173	DNA	50	340	11	110.84	10
127	87.803	µg/ml	1.756	0.891	1.971	1.152	DNA	50	340	0.5	175.61	-0.5
132	335.112	µg/ml	6.702	3.285	2.040	1.738	DNA	50	340	3	111.70	2
134	271.498	µg/ml	5.430	2.653	2.047	1.914	DNA	50	340	2	135.75	1

Appendix 1. Concentrations and dilutions performed of eluted DNA from bacterial samples received in Norway.

Colony	Amoxicillin	Florfenicol	Flumequine	Neomycin	Sulphonamide	Tetracycline	Trimethoprim
1	13	30	30	25	30	30	30
2	10	30	30	24	26	11	28
3	30	30	30	28	30	28	20
4	10	30	28	24	12	14	30
5	10	30	30	24	22	30	24
6	30	24	30	30	30	28	14
7	10	30	30	28	30	30	30
8	10	30	30	24	20	28	30
9	10	30	30	22	20	30	30
10	20	30	30	22	20	30	30
11							
12	10	22	30	20	22	24	30
13							
14	10	30	30	24	22	30	26
15	10	30	30	22	26	10	30
16	10	30	30	24	18	28	26
17	11	30	30	28	30	30	30
18	18	30	30	30	30	30	30
19	10	30	30	24	26	30	30
21	10	12	26	28	26	22	10
20	30	30	30	24	26	30	30
22	10	12	30	18	10	18	28
23	10	30	30	26	28	30	30
24	30	30	30	30	26	30	30
25	30	30	22	30	30	30	16
26	10	30	30	24	30	30	30
27	10	30	30	24	30	30	30
28	10	30	30	24	26	30	30
29	10	30	30	26	30	30	30
30	30	30	30	30	30	30	30
31							
32	24	18	30	28	30	22	14
33	10	30	30	25	24	30	30
34							
35	10	30	30	22	22	30	30
36	16	30	30	24		30	30
37							
38							
39	30	30	30	30	30	30	30
40	10	22	30	25	28	26	30
41							
42							
43							
44							
45	22	18	30	30	30	30	30
46	22	18	28	24	30	22	12
47							
48							
49	30	24	30	26	30	10	30
50	10	24		24	10	10	10
51							
52	30	30	10	30	10	30	20
53	30	30	30	30	10	10	10
54	30	30	30	26	30	30	30
55	26	30	22	26	24	10	10
56	11	30	30	26	28	30	30
57							
58	10	30	S	21	20	30	25
59	30	30	22	30	30	10	10
60	30	30	26	30	30	10	10
61	10	28	30	26	30	28	30
62	15	10	24	28	22	20	10
63	10	25	30	24	30	24	24
64	10	30	26	30	30	26	30
65							
66	30	30	30	28	30	30	30
67							
68	10	25	30	26	28	28	30
69	10	30	30	25	30	30	30
70	30	30	30	30	30	30	30
71	10	30	30	28	30	30	26
72	10	28	30	28	30	24	25
73	18	30	30	30	30	30	10
74	30	30	24	30	30	30	30

Appendix 2. Site 1 to 4 Zone diameters in millimeters. Sites are color coded in the leftmost column in numerical order. Site 1 being the topmost section.

75	22	22	30	24	24	24	30
76	10	10	20	24	14	18	10
77	11	30	28	24	22	22	10
78	16	30	30	24	10	26	30
79							
80	10	30	30	26	30	30	28
81	10	30	30	24	24	30	30
82	10	30	30	26	10	26	26
83	10	10	24	20	18	16	10
84	10	30	30	20	22	28	24
85							
86	10	30	30	20	10	26	20
87	10	30	30	20	10	26	22
88	28	30	16	30	10	28	10
89							
90	26	30	30	26	18	22	30
91	16	30	30	22	24	22	10
92	10	30	30	28	22	30	22
93	30	30	30	30	30	30	30
94							
95	10	30	30	26	25	30	25
96	24	30	30	28	12	20	10
97							
98	20	28	30	22	30	20	14
99	12	10	22	22	16	20	10
100	30	28	30	22	25	25	26
101							
102							
103	10	25	30	10	10	10	10
104	16	10	20	25	15	16	10
105	10	30	30	22	15	30	28
106	30	30	30	26	30	28	30
107	20	30	30	22	30	30	30
108	30	30	30	26	10	30	R (30)
109	16	30	30	24	30	30	30
110	20	20	24	24	10	10	10
111	R (20)	R (24)	30	28	10	11	10
112	14	24	30	24	10	24	26
113	30	30	30	28	30	30	30
114	10	18	30	22	24	26	26
115	30	30	30	28	30	30	30
116							
117							
118	12	10	24	26	18	18	10
119	30	30	26	26	30	30	30
120	18	30	30	24	30	30	30
121	12	10	16	24	10	26	10
122	16	10	30	28	30	20	20
123	30	30	26	26	30	28	30
124	22	30	30	24	10	20	30
125	11	10	20	25	11	18	10
126	10	25	30	28	30	22	24
127	22	20	26	24	24	20	24
128	14	30	30	28	30	26	10
129	10	30	10	25	20	11	20
130	16	30	30	28	24	26	10
131	11	25	20	20	10	20	10
132	10	28	30	22	10	23	22
133							
134	10	30	30	22	20	30	26
135							

Appendix 3. Site 5 to 8, zone diameters in millimeters. Sites are color coded in the leftmost column in numerical order. Site 5 being the topmost section.

BLAST- Sequencing						
Sample id	Site	F - Length	R - Length	Genus	Likely subsp.	Perc. Identity
2	Site 1	968	1007	Aeromonas	Veronii	
3	Site 1	478	424	PRV		
4	Site 1	1243	1211	Aeromonas	Veronii	
5	Site 1	677	467	Aeromonas		73.84
6	Site 1	672	921	?		
8	Site 1	1231	5	PRV		83.44
9	Site 1	1107	1187	Aeromonas	Veronii	
10	Site 1	1208	1211	Enterobacter/Klebsiella		
12	Site 1	1267	1245	Citrobacter	Freundii	
49	Site 4	1062	1250	Serratia/enterobacter/leclercia/lelliottia		
50	Site 4	1300	1242	Enterobacter	asburiae	
51	Site 4	555	5	No result		
52	Site 4	793	840	Enterobacteriaceae/gamma proteobacterium		79
53	Site 4	666	503	No result		
55	Site 4	754	778	No result		
75	Site 5	866	584	No result		
79	Site 5	913	991	PRV/klebsiella		77.49
97	Site 6	1203	1248	Aeromonas	Veronii/hydrophila	
98	Site 6	1229	1242	Aeromonas	Veronii/sobria	
100	Site 6	956	987	Leclercia	adecarboxylata	97.84
101	Site 6	922	833	PRV		
102	Site 6	786	729	No result		
108	Site 7	671	378	No result		
114	Site 7	1276	1252	Enterobacter	Hormaechei	
115	Site 7	683	324	PRV		
116	Site 7	1036	380	No result		
118	Site 7	629	845	Aeromonas	Veronii/hydrophila	86.34
119	Site 7	872	391	Aeromonas	Veronii/caviae	77.92
121	Site 8	1199	1269	Pseudomonas	Putida/plecoglossida	
122	Site 8	1011	702	PRV		90.88
125	Site 8	1263	965	Pseudomonas	Plecoglossicida/asiatica	98.13
127	Site 8	740	904	PRV		
132	Site 8	1168	1139	Aeromonas	Caviae/taiwanensis/hydrophila	98.52
134	Site 8	556	677	PRV		93.29

Appendix 4. Results from the sequencing, including lengths of both forward and reverse sequences.

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