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Dual resistance to heavy metals and antibiotics of *Aeromonas hydrophila* isolated from *Carassius carassius* (Linnaeus, 1758) in Lake Tonga, Algeria

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Abstract. *Aeromonas hydrophila*, a bacterium with significant virulence potential, is the predominant pathogenic bacteria naturally infecting fish. This study aims to identify the antibiogram and heavy metal resistance pattern of *Aeromonas hydrophila* obtained from both *Carassius carassius* fish and their surrounding water environment in Lake Tonga, Algeria. A total of 59 strains of *Aeromonas hydrophila* were isolated from 168 *Carassius carassius* samples and 144 waters samples of Lake Tonga. All the strains were tested for resistance to 13 antibiotics and three types of heavy metals (Cobalt, copper and cadmium) using disk diffusion and two-fold agar dilution method, respectively. Clinical macroscopic examination of the fish was also carried out. More than 14% of the examined fishes showed the characteristic clinical signs. Drug screening showed high levels of resistance to β-lactam antibiotics, 100% of the strains were resistant to ampicillin followed by cefalotin (91.53%) and ticarcillin (88.14%). More than 40% of the strains exhibited resistance against gentamicin, amikacin and chloramphenicol. The multiple antibiotic resistance (MAR) indexing of *A. hydrophila* strains showed that all of them originated from high-risk sources. Among tested heavy metals, bacterial isolates exhibited resistant pattern of Co>Cu>Cd. A positive correlation was observed between antimicrobial resistance and metal tolerance (Odds Ratio>0.1). These resistant profiles could be useful information to avoid unnecessary use of chemical and antimicrobial products in the aquatic environment and to provide a novel approach to manage bacterial infection in fish.

Keywords: *Aeromonas hydrophila*, antimicrobial resistance, *Carassius carassius*, heavy metal, MAR index.

Introduction

The crucian carp (*Carassius carassius*) is a North European freshwater fish that often inhabits small ponds (Sollid *et al*., 2003). Currently, *Carassius carassius* (*C. carassius*) is a widespread fish in Lake Tonga (northeast Algeria). It is a non-cultivated wild fish that is commonly consumed in the region. Several studies have demonstrated *Carassius carassius*'s ability to withstand exposure to high concentrations of contaminants. However, this species remains vulnerable to bacterial infections (Shuvho *et al*., 2016).

Aeromonas have been identified as significant pathogens responsible for numerous disease outbreaks in finfish farming worldwide (Lee and Wendy, 2017). The genus *Aeromonas* comprises species that are commonly isolated from the environment, especially from aquatic samples, but also from a variety of foods, such as fish, mussels, meat products, milk and vegetables (Stratev and Odeyemib, 2016). *Aeromonas hydrophila* (*A. hydrophila*) has garnered attention due to its recurrent involvement in infections affecting both humans and fish, and its growing resistance to antimicrobial agents. This bacterium is responsible for causing hemorrhagic septicemia, skin ulcerations, and gastrointestinal tract infections in various fish hosts, including crucian carp (Jiang *et al*., 2020; Lü *et al.*, 2016).

The presence of drug and heavy metal pollution poses a significant and widespread environmental issue, disrupting microbial ecology. Cobalt, cadmium, and copper are among the major contaminants frequently found in the environment, and at high concentrations, they prove to be extremely toxic to microbes (Benhalima *et al.*, 2020). Nevertheless, bacteria strains possess various mechanisms to cope with elevated levels of these pollutants. The increasing resistance of *A. hydrophila* to antibiotics and heavy metals is a source of concern for public health (Yu *et al*., 2017). Hence, it is imperative to evaluate the resistance potential of this opportunistic pathogen worldwide in order to help farmers and veterinarians to establish more efficient and suitable chemical management practices for farm operations.

In this study, the main hypothesis posits that a considerable proportion of fish aeromonads in non-aquaculture freshwater environments are likely to exhibit resistance to multiple antibiotics and metals. Furthermore, it is suggested that these resistance traits can be transferred between different genera, thereby posing a significant risk to both consumers and the ecosystem. For this reason, the purpose of the present study was, for the first time, to (i) search for the presence of *A. hydrophila* in water and crucian carp of a non-aquaculture environmental (Lake Tonga), (ii) determine the antimicrobial and heavy metals sensitivity of the isolates, and identify the high-risk source.

Materials and methods

Water and fish samples

Water samples were collected from Lake Tonga (brackish water, 2400 ha; 36°53'N; 08°31'E), Northeastern Algeria (Fig.1). It is designated as a Ramsar site since 1983 and forms an integral part of the El Kala National Park, which is renowned for being one of the primary reservoirs of biodiversity in the Mediterranean Basin (Djamai *et al.,* 2019). All water samples (n = 144) were collected between January and July 2022 at four different sampling points in the system: El Bir (S1; 36.51231 N; 08.31432 E), Chalet (S2; 36.55017 N; 08.26483 E), Maizila (S3; 36.57485 N; 08.35331 E), and Oum jedour (S4; S3; 36.58615 N; 08.35847 E) (Fig.1).

A total of 168 adult crusian carp (*Carassius carassius*) weighing 15-45g were sampled. The fish was trapped and wiped down with a sterile drag-swab to collect samples of the skin's mucus layer. Subsequently, the fish samples were then gathered in sterile plastic bags and sent to the laboratory in a sterile container containing ice blocks, where the analyses were done. The collected fish (n > 30 at each sample site) were clinically examined in situ to identify any potential alterations or lesions. The overall prevalence of affected fish (P %) was then determined using the "pathological code" methodology (Girard and Elie, 2007). Mucus was scraped from the dorsal body of 3 or 4 fish 10-14 cm in length for bacteriological examination; ventral skin mucus was not collected to avoid intestinal tract and sperm contamination. The samples were collected in sterile tubes containing 5 ml of sterile alkaline peptonic water with 1% NaCl (w/v) (Oxoid, UK). The samples were incubated at 37°C for 24h (Harnisz and Tucholski, 2010). Gills and fish flesh were harvested aseptically using normal aseptic technique. Ten grams of each sample were suspended in 90 mL of sterile alkaline peptonic water (Oxoid, UK) containing 1% NaCl (w/v), homogenized for 2 minutes at high speed, and incubated at 37°C for 24 h (González-Fandos and Herrera, 2013).

Isolation and growth conditions of Aeromonas

Water samples and the fluid cultures of mucus, flesh, and gills were inoculated on thiosulfate citrate bile sucrose (TCBS) agar (Oxoid, UK). Bacteria colonies subjected to the Gram stain, oxidase and catalase tests, to identify potential *Aeromonas* spp. Several phenotypic tests (API ID 20E, BioMerieux, France) were also used in order to differentiate members of the aeromonads (Abott *et al.,* 2003).

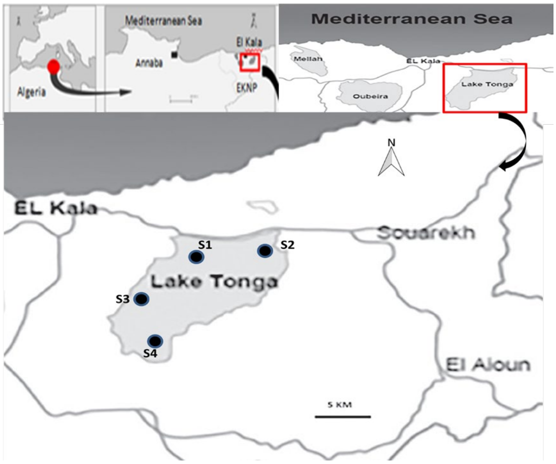

Figure 1. Study area with location of sampling sites.

Antimicrobial susceptibility testing

Antibiotic susceptibility was determined by the standard disk-diffusion method (CLSI, 2015) on Mueller-Hinton (MH) agar plates (Oxoid, UK) using 13 antibiotic disks (Lab. Pvt. Mumbai, India): Ampicillin (AM, 10 µg), ticarcillin (TI, 75 µg), imipenem (IPM, 10 µg), cephalotin (CF, 30 µg), cefotaxim (CTX, 30 µg), tetracycline (TE, 30 UI), gentamycin (GEN, 15 µg), amikacin (AKN, 30 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), nalidixic acid (NA 30 µg), nitrofurantoin (NIT, 300 µg), and fosfomycin (FOS, 15 µg + 50 µg G6P).

The *A. hydrophila* strain was grown overnight in MH broth, the turbidity of the cell suspensions was adjusted to that equivalent of a 0.5 McFarland standard and used to inoculate MH agar plates, which were incubated at 30˚C for 24 h. The results were interpreted as susceptible, intermediate, or resistant according to Clinical and Laboratory Standard Institute (CLSI, 2015). Reference strain, *E. coli* ATCC 25922 (Institut Pasteur CIP 7624), was used as antimicrobial susceptibility testing control, according to CA-SFM recommendations.

The MAR index, when applied to a single isolate, is defined as a/b, where "a" represents the number of antibiotics to which the isolate was resistant and "b" represents the number of antibiotics to which the isolate was exposed. MAR index values greater than 0.2 are considered to originate from high risk sources of contamination such as humans, commercial poultry, swine and dairy cattle, where antibiotics are widely used. MAR index value less than or equal to 0.2 is considered to be the origin of the strain from animals in which antibiotics are rarely or never used (Sarter *et al*., 2007).

Heavy metal tolerance assay

Three metals (cobalt, copper, and cadmium) were selected for the metal resistance assay based on their projected toxicities and presence in water samples. Adjusted bacterial suspensions (10^9 CFU) were subsequently disseminated onto trypticase soy agar medium TSA (Oxoid, UK), which contained various concentrations ranging from 12.5 to 3200 g mL^{-1} of one of the three metal salts, $Co(NO_3)_2$, $CuSO_4{\cdot}5H_2O$ and $CdCl_2{\cdot}2H_2O$ (Sigma-Aldrich, Germany). The inoculated media was incubated for 24 h at 37°C. The Minimal Inhibitory Concentration (MIC) for each strain for the three toxic metals was determined. The bacteria were considered resistant to the tested heavy metals if they are grown at a higher concentration than the reference strain *Escherichia coli* K12. In this study, the operational definition of tolerance was determined by observing a favorable increase in bacterial growth when the concentration of heavy metals exceeded the specified resistance threshold (Lee and Wendy, 2017).

Statistical analysis

Two way analysis of variance (ANOVA) was performed to determine the significant difference among the proportions of resistant *A.hydrophila* from different sources. The relationship between bacterial strains possessing a MAR index of ≥0.2 and their high tolerance to toxic metals was assessed by calculating the odds ratio (OR).The confidence interval used was 95%. An OR value of ≤1.0 suggested a negative correlation, indicating a lower probability of the condition in the first group compared to the second or an equal likelihood in both groups. Conversely, an OR value >1.0 indicated a positive correlation (Resend *et al*., 2012). All analyses were performed using the software R Development Core Team, 2014 Version 3.1.2. Critical p-value was set at 0.05.

Results

Clinical investigation of fish and bacteriological examination

A clinical macroscopic analysis of *C. carassius* revealed various clinical indications, including hemorrhages, erosions, and necrosis. The findings from the examination of the captured fish are presented in Table 1. The total prevalence (P) of fish with external abnormalities amounts to 14.04%. The highest prevalence values were observed at station S3 in February, April, June, and July. Meanwhile, station S1 exhibited elevated prevalence values in March and July. It is important to note that water and habitat quality were found to be subpar during these specific months and at the mentioned stations.

Table 1: Prevalence of external lesions of *Carassius carassius.*

Sampling period	Station	Number of fish examined	Prevalence of fish affected (%)	Lesions	Disturbance	Water and habitat quality
January	S1	35	14.29	E	Medium	Mediocre
	S2	30	10	N	Medium	Mediocre
	S3	30	13.33	H, E	Medium	Mediocre
	S4	37	8.11	N	Medium	Mediocre
February	S1	40	10	E	Medium	Mediocre
	S2	37	8.11	E	Medium	Mediocre
	S3	45	24.44	H, E, N	High	Poor
	S4	50	12	H, E	Medium	Mediocre
March	S1	34	23.53	H, E	High	Poor
	S2	30	13.33	E	Medium	Mediocre
	S3	42	19.05	N, E	Medium	Mediocre
	S4	53	9.43	N, H	Medium	Mediocre
April	S1	33	12.12	E	Medium	Mediocre
	S2	32	6.25	H, E, N	Medium	Mediocre
	S3	42	21.43	N	High	Poor
	S4	40	12.5	E	Medium	Mediocre
May	S1	38	18.42	E	Medium	Mediocre
	S2	34	8.82	H	Medium	Mediocre
	S3	45	11.11	H, E	Medium	Mediocre
	S4	43	6.98	E	Medium	Mediocre
June	S1	33	9.1	E	Medium	Mediocre
	S2	31	6.45	N	Medium	Mediocre
	S3	40	22.5	H, N	High	Poor
	S4	44	13.64	N	Medium	Mediocre
July	S1	40	22.5	E, H, N	High	Poor
	S2	42	11.9	E, N	Medium	Mediocre
	S3	40	22.5	H, E	High	Poor
	S4	45	13.33	H, E	Medium	Mediocre

H: hemorrhage, E: erosion, N: necrosis.

A total of 28 *A. hydrophila* bacteria were identified in water samples collected from four distinct stations. Among the *A. hydrophila* isolates, 31 were recovered from *C. carassius* fish, with 23 originating from the gills, 5 from the mucus, and 3 from the flesh. However, a significant difference (p<0.05) was observed in the prevalence of *A. hydrophila* among the various fish specimens. All the *A. hydrophila* strains were identified as Gram-negative and non-swarming. They showed positive results in oxidase and glucose-fermentative tests and were found to utilize L-arabinose, D-mannose, D-mannitol, D-lactose, D-sorbitol, and sucrose.

Antibiotic susceptibility

The percentage of *A. hydrophila* strains showing resistance against each antibiotic is given in Figure 2A. All the strains showed the highest resistance rate for beta-lactam and ticarcillin. More than 40 % of the strains were resistant to aminoside and chloramphenicol. All isolates were found to be susceptible to imipinem, ciprofloxacin, nalidixic acid, nitrofurantoin, and fosfomycin. No significant differences in resistance frequencies were found among the strains of both fish and water (ANOVA, F= 0.26, p = 0.532). The resistance of the *A. hydrophila* pattern isolate toward 13 antimicrobial agents tested is shown in Table 2. Among the 59 selected isolates, 37 % were multiresistant. *A. hydrophila* strains from fish exhibited resistance from 3 to 7 antibiotics, while 75% of the strains from water showed resistance to 3 antibiotics (Fig. 2B). The MAR index ranged from 0.23 to 0.54. Among the isolates 20.34% exhibited MAR indices ≥ 0.4, while 13.59% had MAR indices > 0.3. All of isolates indicate high-risk contamination originating from humans or animals where antibiotics are often used.

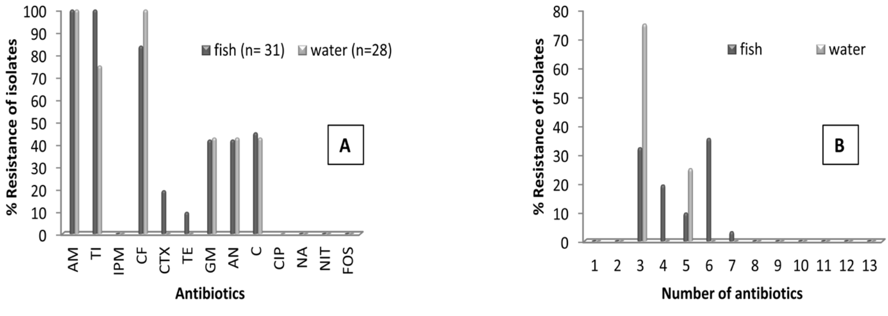

Figure 2. (A) Percentage frequency of antibiotic resistant and (B) multiple antibiotic resistance in *Aeromonas hydrophila* strains from fish (*Carassius carassius*) and water of Lake Tonga. AM: ampicillin; TI: ticarcillin; IPM: imipinem; CF: cephalotin; CTX: cefotaxim; TE: tetracyclin; GEN: gentamycin; AN: amikacin; C: chloramphenicol; CIP: ciprofloxacin; NA: nalidixic acid; NIT: nitrofurantoin, FOS: fosfomycin.

Table 2. Resistance patterns of the *Aeromonas hydrophila* isolates from fish (*Carassius carassius*) and water of Lake Tonga.

	Origin			
	Fish			**Water**
Resistance patterns	**Gill**	**Mucus**	**Flesh**	
AM/TI/CF	6	2	2	21
AM/TI/CF/CTX	5		1	
AM/CF/GM/AN/C				7
AM/TI/CF/GM/AN	1			
AM/TI/CF/TE/C		2		
AM/TI/CF/GM/AN/C	11			
AM/TI/CF/TE/GM/AN/C		1		

AM: ampicillin; TI: ticarcillin; CF: cephalotin; CTX: cefotaxim; TE: tetracyclin; GEN: gentamycin; AN: amikacin; C: chloramphenicol.

Heavy metal tolerance essay

The bacterial isolates displayed different levels of tolerance to the three metals used in this study. All of the isolates displayed higher tolerance towards Co, while 99.3% of the bacterial isolates in the current study showed resistance to Cu. The MBC showed a similar pattern, indicating that cadmium exhibited the highest bactericidal activity (Tab. 3). Fifty six (94.91%) *A. hydrophila* isolates showed Co/Cu/Cd combination pattern. Positive correlations were observed between multidrug-resistant *Aeromonas hydrophila* (MAR >0.2) recovered from water and fish in Lake Tonga and high tolerance to all toxic metals (OR>1.0; 95% confidence interval).

Table 3. Heavy metal resistance of *Aeromonas hydrophila* strains isolated from water and *Carassius carassius.*

Metal salts	**Source**	**MIC ($\mu g\ mL^{-1}$)**	**MBC ($\mu g\ mL^{-1}$)**	**Resistant strains (%)***
$Co(NO_3)_2$	Water	1200±1.38	2400±0.75	100
	Mucus	1600±0	3200±0	100
	Gills	1200±1.38	2400±2.75	100
	Flesh	800±0	2400±2.75	100
$CuSO_4 \cdot 5H_2O$	Water	1200±1.04	2400±1.35	100
	Mucus	1200±0.68	2400±2.75	98.4
	Gills	1200±0.25	2400±1.35	98.8
	Flesh	800±0	2400±1.35	100
$CdCl_2 \cdot 2H_2O$	Water	400±0	1600±0	100
	Mucus	400±0	800±0	100
	Gills	200±0	800±0	100
	Flesh	150±1.56	200±0	66.66

*Resistance Concentration: Co and Cu (200 $\mu g\ mL^{-1}$); Cd (100 $\mu g\ mL^{-1}$), values obtained with *E. coli* K-12 standard strain.

Discussion

Fish, with their heightened sensitivity to environmental factors, serve as valuable bioindicators in monitoring programs, aiming to assess fish food quality, monitor water and aquatic environment quality, and evaluate the health status of fish populations (Outa *et al*., 2020). In the present study, approximately a thousand common carp were examined in Lake Tonga, revealing 153 pathological alterations, with 24.18% corresponding to hemorrhages and 75.82% to erosions and necroses of fins. These alterations appear to be more common in fish inhabiting polluted environments, likely caused by a decline in bacteriological quality and chemical contamination of the surroundings. Various bacteria, including *Aeromonas* sp., are involved in causing lesions in freshwater fish, as shown by other studies (Lee and Wendy, 2017; Zhang *et al*., 2016). According to the 'Pathological code' methodology and integrated diagnoses, the health of fish in the Lake Tonga is considered precarious, with an overall prevalence of lesions well over 5%, indicating impaired habitat quality. Stations S3 and S1 exhibit the highest prevalence of affected fish, possibly due to the increased microbiological contamination of water in these sites (Liu *et al*., 2020).

In the last two decades, there has been a growing interest in bacteria of the genus *Aeromonas*, mainly because of their pathogenicity to both aquatic organisms and humans. In this study, *A. hydrophila,* which causing many disease outbreaks, were found in water and crucian carp captured from Lake Tonga. According to Atef *et al.* (2016), the bacterial community associated with fish is generally related to the characteristics of the aquatic habitat, such as the bacterial load in the water. Interestingly, *A. hydrophila* were more frequently present in some of the healthy fishes. Zhang *et al*. (2016) suggested that these pathogenic strains are usually present in the healthy fishes and they may act not only as opportunistic pathogens, but also as important players of other functions. However, Cao *et al.* (2022) have reported that *A. hydrophila* is recognized for its high pathogenicity to aquatic animals and is prevalent in diseased aquatic organisms worldwide, affecting a variety of fish species and the primary indications of *A. hydrophila* infection in fish consist of necrosis, ascetic fluid accumulation, darkening of the spleen and kidney, and liver pallor.

The isolation of *A. hydrophila* resistant to beta-lactams in various freshwater environments and from fish was previously reported by several authors. Olumide and Asmat (2015) as well as Shuvho *et al*. (2016) documented complete resistance to ampicillin among all *Aeromonas* species isolated from various aquatic sources. Additionally, the substantial resistance of *A. hydrophila* to cephalotin has been documented in earlier studies (Wickramanayake *et al.,* 2020; Pfeifer *et al*., 2010). However, the incidence of resistance to chloramphenicol in our study was less than what was observed in the research conducted by Michel *et al.* (2003), wherein 80% of fish bacteria from brown trout, Atlantic salmon, brook trout, and their hybrids exhibited resistance to chloramphenicol. Conversely, it was higher than the findings of Zdanowicz *et al.* (2020), who reported a lower percentage (5–6%) of *Aeromonas* strains resistant to chloramphenicol. The lower occurrence of tetracycline-resistant strains of *A. hydrophila* was consistent with the findings of Shao-wu *et al.* (2013). Imipenem belongs to the carbapenem class of b-lactams, has a very broad spectrum of activity, and acts mostly on gram-negative and gram-positive bacteria (Cheng *et al*., 2019). Also, quinolones are synthetic antibiotics used as drugs of choice for the treatment of *Aeromonas* infections in humans (Alcaide *et al*., 2010). Fortunately, our study showed that *A. hydrophila* was 100% susceptible to imipenem, ciprofloxacin and acid nalidixic, which aligns with the findings of Asadpour *et al.* (2016), and Stratev and Odeyemi (2016). In the case of nitrofuran, Shamsun *et al.* (2016) demonstrated that 20% of selected *Aeromonas hydrophila* isolates from fish were resistant to this antibiotic, a result that contrasts with our own findings.

All isolates had MAR indices > 0.2, which is unexpected, considering that most of the isolates had been previously exposed to antimicrobial agents. This finding is in agreement with the results reported by Vivekanandhan *et al.* (2002). Numerous factors contribute to the presence of multi drug resistance *A. hydrophila* isolates. According to Fang *et al*. (2019), the extensive utilization of antibiotics across medical, agricultural, and livestock sectors leads to the introduction of antibiotic remnants into the ecosystem. Consequently, the interplay of genetic resistance traits between diverse settings becomes possible through direct or indirect interaction, facilitated by mobile genetic components. The rapid surge in the prevalence of antibiotic-resistant and highly resistant aquatic *Aeromonas* species can be traced back to these organisms' ability to propagate antibiotic resistance via mobile genetic components (including plasmids, transposons, IS elements, gene cassettes, and class 1 integrons) within bacterial populations through direct cell-to-cell contact as highlited by Zdanowicz *et al*. (2020).

Aeromonas hydrophila isolates exhibited high resistance for Co, Cu and Cd. The detected tolerance to toxic metals in *A. hydrophila* strains might be attributed to the presence of these metals in our site, given its location in agricultural and livestock farming areas. Cobalt's resistance is higher than that of other metals, which could be explained by its role as an essential micronutrient for bacteria at low concentrations. Nevertheless, at high intracellular concentration the redox active metal ion Co is highly toxic and when the content of this metallic element surpasses the established limits and persists over extended periods, it has an unfavorable impact on the operational dynamics and assortment of microbial populations. Unfortunately, this phenomenon can lead to the emergence of bacterial resistance to heavy metals (Jiang *et al.*, 2020). Consistent with our findings, Yi *et al*. (2014) identified a prevalence of 74.4% Cd-resistant *Aeromonas* spp. among farmed fish and imported pet fish in Korea. Moreover, Wei *et al.* (2015) demonstrated that 25% of *Aeromonas* spp. isolated from red hybrid tilapia exhibited resistance to Cu. The variances in bacterial resistance to distinct metals could be elucidated through two interconnected mechanisms: co-resistance, in which genetically linked factors are concurrently expressed, and cross-resistance, where a single factor or gene confers resistance to multiple antimicrobials (Chettri and Joshi, 2022). Similar to many previous studies, resistance of *A. hydrophila* to antibiotics and heavy metals were found to be positively correlated (Lee and Wendy, 2017; Resend *et al.*, 2012). According to Pathak and Gopal (2009), water pollution caused by heavy metals increases the likelihood of bacterial resistance and infection in aquatic macrofauna, particularly fish populations and their consumers, representing a risk to public health. Jiang *et al*. (2020) indicate that the coselection or the coexistence of certain antibiotic resistance genes and heavy metal resistance genes may be beneficial to bacteria for increasing fitness in various environments. In the studies conducted by Wickramanayake *et al.* (2020) and Benhalima *et al.,* (2019), Copper and cadmium in agricultural fields and hospitals select for resistance to these substances and co-select for bacterial resistance to antimicrobials, including beta-lactams, cephalosporin and chloramphenicol.

Conclusion

This study demonstrates the presence of dual antibiotic and metal-resistant *Aeromonas hydrophila* strains in fish and their aquatic environment, which have not been subjected to any aquaculture practices thus far. *Carassius carassius*, serving as a bioindicator, can effectively monitor pollution levels. The MAR index values indicate a significant antibiotic exposure risk source in Lake Tonga. The evident phenotypic resistance to both antibiotics and heavy metals highlights the critical need to manage human activities that may contribute to resistance proliferation within Lake Tonga. The study clearly indicates that the consumption of fish infected with *A. hydrophila* could potentially pose a public health concern.

Ethics Statement

Animal studies involved in this manuscript adhere to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and maintained according to the standard protocols. All the experiments were reviewed and approved by the department of Ecology and Environmental Engineering of Guelma University.

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