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ASSESSING THE IMPACT OF ENVIRONMENTAL FACTORS ON GROWTH TRAITS OF *Oncorhynchus mykiss* (Walbaum, 1792) IN A RECIRCULATING AQUACULTURE SYSTEM: A CASE STUDY

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Abstract

Considering the growing demand for food to support the human population, along with the depletion of natural resources and global climate change, this case study presents a potential solution for fish meat production by utilizing a system that mimics the natural growing conditions of rainbow trout - Oncorhynchus mykiss (Walbaum, 1792), and also the associated problems related to microbial infections. Therefore, trout fry was used to populate circular tanks (T1-T4), where they were fed four types of commercial feed, one of the feeds being supplemented with 10% Nannochloropsis sp. flakes. After eight weeks, the impact of the feed on the length-weight relationship, growth type, morphology, and body indices of the rainbow trout was assessed. The results showed that the feed formulas do not significantly influence growth parameters. In tandem, due to massive infections, a histological characterization was performed to evaluate the fish's adaptability to feeding and the impact of environmental conditions on the gastrointestinal tract. Our study confirmed that Aeromonas veronii species was connected to septicemia and skin lesions in rainbow trout.

Key words*: biometry, body indices, feed, fish morphology, rainbow trout, RAS.*

INTRODUCTION

In recent years, the production of salmonid fish through aquaculture has expanded significantly due to the nutritional potential and economic opportunities (Pepe-Victoriano et al., 2024). *Oncorhynchus mykiss* (Walbaum, 1792) is one of the most relevant species in aquaculture production, being reared in a variety of systems, in many areas around the world (Nenciu et al., 2022; Cheng et al., 2023; de Araujo Jr et al., 2023). Compared to other salmonids, *O. mykiss* is suitable for intensive rearing, due to its rapid growth rate and high assimilation degree of the commercial feed (Shah et al., 2009; Docan et al., 2011).

The nutrition factor can influence the growth performances and health status of cultured fish (Gabor et al., 2012). Various research highlighted the relevance of supplementing fish meal nutrition in order to improve the final weight and fat metabolism and to decrease the mortality rate in fish farms (Nastova et al., 2014; Welker et al., 2017; Mahato et al., 2023).

For optimal growth of *O. mykiss*, high quality and quantities of protein and energy in diets are recommended (Janampa-Sarmiento et al., 2020). However, fishmeal-based diets supplemented with alternative protein sources did not affect the growth performance of rainbow trout reared in recirculating aquaculture systems (Fanizza et al., 2023). Instead of the traditional fishmeal, feeding rainbow trout with microalgae meal benefits both the aquaculture business (sector) and the environment (Velichkova et al., 2024). Several algae

including *Nannochloropsis* sp. have been well-accepted as ingredients in aquaculture feeds (Estévez et al., 2022). Also, an effective alternative to fish meal in rainbow trout is the plant-based diet supplemented with *Schizochytrium* sp. microalgae (Cardona et al., 2022). Velichkova et al., (2024) have found that a 50% algae meal-fed diet based on *Chlorella* and *Spirulina* is sufficient to improve the values of the studied histological structures in the intestines of the rainbow trout. Other research (Aulia et al., 2024) has shown that microalgae feed additives improve the growth and immunity of juvenile *O. mykiss*.

In order to match the diet formulation of any farmed animal, a good understanding of the digestive mechanism is essential (Kamalam et al., 2020).

Various studies investigated the gut physiology and digestive function of rainbow trout influenced by feeding (Calo et al., 2024; Frohn et al., 2024).

Regarding the optimal environment for the healthy growth and development of rainbow trout, it was postulated before the importance of ensuring the water quality (Mocanu et al., 2011; Uiuiu et al., 2020; Pepe-Victoriano et al., 2024). Among limiting factors in the aquaculture production system in trout farming are oxygen and ammonia, a product associated with the catabolism of protein (Fornshell, 2002).

Therefore, this research aimed to assess the adaptation of rainbow trout *Oncorhynchus mykiss* reared in a recirculatory aquaculture system, identifying the optimal living parameters, the growth type, and the impact of feeding upon the studied specimens, in the context of worldwide demand for increasing safety and quality in aquaculture production.

MATERIALS AND METHODS

Biological material consisted of rainbow trout fingerlings provided by the National Directorate of Forests, Ploiesti Forestry, Romania. The supplier farm of rainbow trout is located in Pietroșani, 4 km from the DN1 (Bucharest-Ploiești) highway. The farm operates over an area of 12.300 square meters, of which 2.600 square meters consist of water spread across 12 large basins. The site sits at an average elevation of 150 meters and is supplied by two water sources: the Recea River (101 l/s) and the Soava River (90 l/s). The trout farm is one of the main suppliers to wholesale chains nationwide.

The fishes were reared in a recirculating aquaculture system (RAS). The system consisted of four circular tanks (T1-T4), and filters, UV lamps, submersible pumps, and aerators for each one of the tanks. Also, the system was provided with oxygen, pH, and ammonia sensors for each tank.

Stocking of tanks

Thus, the stocking of basins with trout was preceded by a series of preparatory operations, including:

- Cleaning the basins, testing temperature and oxygen sensors, testing pH and ammonia (NH_3) sensors, filling the tanks with tap water, and allowing sedimentation for two weeks to eliminate chlorine. Additionally, the functionality of the air conditioning was checked to ensure a relatively constant temperature that does not exceed the critical threshold of 20°C.
- Inoculating bacteria (commercial name Biobactur) at concentrations of 10 g/1000 l of water in each filter of the four tanks.

Biometric measurements

The measurements were carried out in four stages, according to the Table 1.

Table 1. Biometric measurements over time and abbreviations

Measurement code	Tank code	Measurements interval
Measurement 1 (I)	Tank 1 (T1)	2 weeks
Measurement 2 (II)	Tank 2 (T2)	4 weeks
Measurement 3 (III)	Tank 3 (T3)	6 weeks
Measurement 4 (IV)	Tank 4 (T4)	8 weeks

During the study period, the length-weight relationship, growth type, biometric measurements, and body indices were calculated according to Lustun (1985), Turliu (2010), Pricope et al. (2013), and monitored, as it follows:

- *Total body length* (TL) measured from the tip of the snout to the tip of the tail;
- *Standard body length* (SL) measured from the snout to the base of the caudal fin;

- *Maximum body height* (H) measured in the highest area of the body;
- *Maximum body thickness* (T) measured in the area where the body has the greatest thickness;
- *Total body weight* (TW) established through weighting;
- *Profile index* (PI) highlights the body shape of the fish, being calculated as the ratio of total body length to maximum body height;
- *Thickness index* (TI) calculated as ratio between maximum body thickness and total body length, following the equation TI = (T/TL) x 100;
- *Fulton* condition factor (K) calculated as TW/TL^3 x 100;
- *Length-weight relationship* (LWR), computed as $TW=aTL^b$, where coefficient *a* (intercept) describes the rate of change of weight with length and parameter *b* (slope) gives data on the growth pattern of the fish or allometry. The relationship between the length and weight of rainbow trout was assessed through linear regression, as Log TW= Log a+ b Log TL;
- *The coefficient of determination* (r^2) of the length-weight relationship is a measure of the quality of the prediction of a linear regression (a value close to 1 meaning a better model).

Microbiological Examination

In order to identify the causes that led to some mortalities of the fish, samples were taken from the liver and gills. Thus, the samples collected from the liver were inoculated on Columbia blood culture media, respectively Cled and incubated at 37°C for 24 hours.

Gill samples were plated on Columbia blood and Chocolate culture media in the same conditions

The identification of the colonies was carried out by using MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization, Time-of-Flight, Mass Spectrometry). To identify a bacterium using Maldi-Tof, spread the sample on a plate and add Matrix after drying. The matrix is a solution made of an organic compound that absorbs energy. The sample is automatically ionized with a laser beam that produces protonated ions. The ions are accelerated to a constant potential and analyzed individually based on the mass-to-charge ratio. During the analysis, the mass-to-charge ratio of an ion is measured by determining the time it takes to travel the flight tube.

After the identification of the bacteria, the diffusimetric antibiogram was performed.

Carrying out antibiogram

A 0.5 dilution was made on the MacFarland scale, then dispersed on Mueller-Hinton medium in three different directions. It was necessary to completely cover the culture medium. Using the dispensers, the antibiotic discs were placed on the culture media, and then incubated at 37°C for 24 hours.

Antibiograms were read using the Adagio device that measures the diameter formed by the bacterial inhibition zone (Figure 1). The reference ranges used for each antibiotic are recorded in Adagio and based on these the bacterial sensitivity was determined.

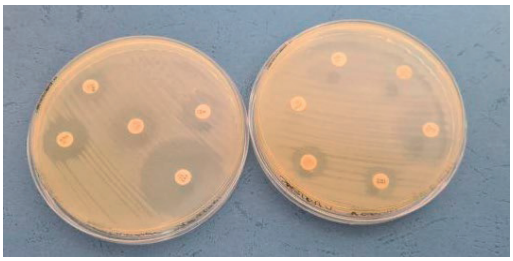

Figure 1. Carrying out an antibiogram - culture media with antibiotic discs after incubation at 37°C for 24 hours

Feed fish formulas

In this experiment, commercial feed formulas were supplemented with *Nannochloropsis* sp. biomass. The feed was manually distributed. Feeding was carried out according to the Table 2.

Table 2. Types of fish feed used

Tank	Feed code	Significance
T1	AlgaeBrew 3	Aller silver
T2	AlgaeBrew 2	AquaGarant
T3	AlgaeBrew 1	AquaGarant + 10% *Nannochloropsis* sp.
T4	AlgaeBrew 4	Aller gold

Calculation of feed ration

The amount of feed was calculated by multiplying the total weight of the fish by the percentage recommended by the feed manufacturer for AlgaeBrew 2 (2.6%), for AlgaeBrew 3 (1.68%), for AlgaeBrew 4 (1.53%), depending on the weight, size, age of

the fish, as well as the water temperature (16-18 °C). The calculation of the amount of food was updated every two weeks, after the measurements.

IR spectroscopy

Near-infrared reflection spectroscopy (NIRS), based on the absorption of light at near-infrared wavelengths by the molecules constituting the sample, is a non-destructive, rapid, and predictive technique. Furthermore, the NIRS technique is often presented as one of the most suitable approaches for product quality control. This technique has extensive applications for the analysis of feed, crop, and food constituents. NIR spectroscopy, although much simpler and faster than traditional analytical methods, typically requires grinding samples to a fine particle size to provide a smooth and homogeneous surface for reflection and increased accuracy. Once the calibrations are done, it takes only a minute to get the result of one or more constituents, whereas with conventional analyses it can take hours or days for this analysis (Khaleduzzaman & Salim, 2020).

The grounded samples were placed in the integrating macrosphere in a layer of 5 cm in the quartz cup which has a diameter of 97 mm. The ground samples were scanned in triplicate (32 scans at 16 cm^{-1} resolution, for each repetition) with FT-NIR (Bruker, MPA, Germany) in the range of 3600 and 12500 cm^{-1}, using OPUS 7.5 software and AQUA calibration software - FEED Bruker, for acquiring the spectra and obtaining the results. Results are presented ± standard deviation.

Statistical analysis

The statistical analysis was conducted based on the following hypothesis (H) at a significance level of 0.05: (H1) biometric measurements are influenced by the feed formulas. A univariate general linear model was applied, with the morphology characteristic as the dependent variable and the feed formula as fixed factors. Duncan's post hoc test was used to evaluate pairwise differences, while Bonferroni correction was applied for estimating marginal means. Statistical analysis was performed using SPSS software (version 26.0). The results are presented as the mean ± standard deviation (SD).

RESULTS AND DISCUSSIONS

Results on FT-NIR analysis of fish feed

Results are the mean values of 12 measurements, with error bars in the graph representing the standard deviation (Figure 2).

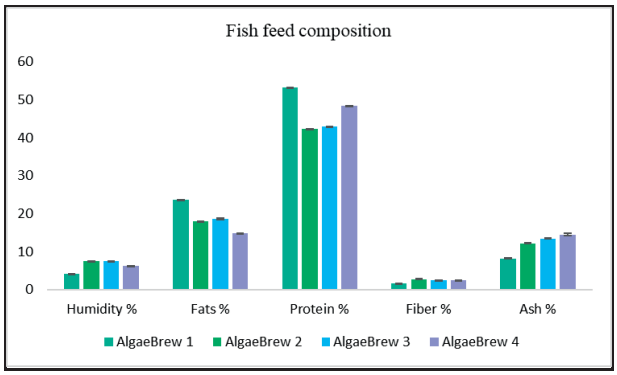

Figure 2. Samples of feed fish analysed by IR spectroscopy

Fish muscle is primarily composed of proteins (16-21%), lipids (0.5-2.3%), ash (1.2-1.5%), and water (52-82%). The proteins found in fish are biologically complete, containing all essential amino acids, making them comparable to those in terrestrial meats. Most fish species are carnivorous and have evolved to utilize protein as their primary energy source instead of carbohydrates, necessitating high dietary protein levels (30-60%) (Kim, 1997). All feed formulations used in this experiment fell within this range.

Regarding the lipid level, according to Liu et al. (2021) rainbow trout feted with different diets containing varying lipid levels (10.03%, 12.97%, 17.22%, 20.16%, 23.19%, and 26.06%), showed an increase of some parameters like weight gain (WG), specific growth rate (SGR), and feed intake (FI). Therefore, the fish were grown with higher dietary lipid levels. Also, in this case study, the lipid content varies between 14-23%.

Total ash refers to the inorganic residue left after water and organic matter are removed through heating with oxidizing agents. It serves as an indicator of the total mineral content in the feed (Khaleduzzaman & Salim, 2020). Therefore, in this case, AlgaeBrew 4 had the highest content of minerals.

Results on Total body length (TL)

Following the first measurement (M1), the total body length values for the analysed rainbow

trout individuals ranged between 16.81 cm in T4 and 17.42 cm for those in T1 (according to Figure 3). An increase in total length was observed so that at the last measurement the values ranged between 20.95 cm for the fish in T3 and 22.14 cm for those in T1. Thus, the fish in T1 had an average growth of 4.72 cm greater than the initial one, those in T2 by 4.02 cm, those in T3 by 3.57 cm, and those in T4 by 4.96 cm (Figure 3).

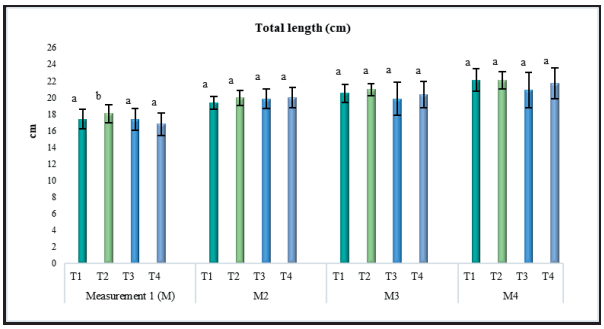

Figure 3. Variation in total body length of trout in the four tanks

According to the obtained results, it can be observed that during the measurements (M1-M4) there were no significant differences due to different feed formulas. An exception was identified, in the case of T2 at the first measurement, but this was not maintained until the end of the experiment.

Results on standard body length **(SL)**

The mean values for the first measurements ranged from 14.82 cm for T3 to 15.41 cm for T1. As for total body length, for standard body length, there was a progressive increase in length measurements from the first to the last measurement, as follows: for T1 there was an increase of 4.24 cm, for T2 a value of 4.18 cm, for T3 a value of 3.42 cm, and for T4 the fish grew by 5.01 cm (Figure 4).

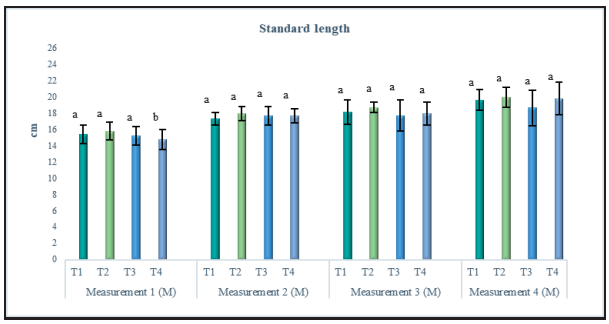

Figure 4. Variation in standard body length of trout in the four tanks

For both TL and SL, the greatest increase in rainbow trout length was observed in T4.

Statistically, no significant differences were recorded during each measurement. However, an increasing trend was recorded during the experiment (from M1 to M4).

Results on total body weight **(TW)**

The body weight of the rainbow trout varied from 51.62 g in T4, to 62.07 g in T2, for the first measurement. As in the case of TL and SL, an exponential increase in the weight of the trout was observed, so that at the last stage of the biometric measurements, the weight increase was: for the fishe in T1 with 74.65 g, for T2 with 75.61 g, for T3 with 45.73 g, and for T4 with 79.01 g compared to the initial moment of the population (Figure 5).

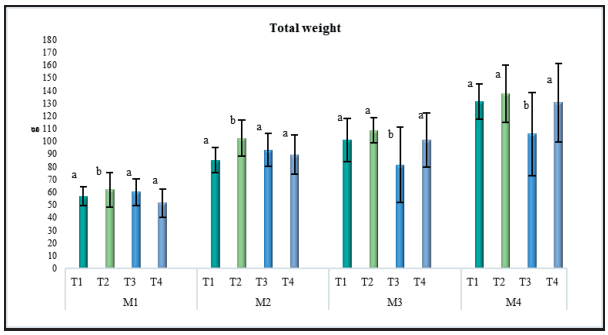

Figure 5. Variation in body weight of trout in the four tanks

The results regarding weight and length are consistent with those obtained by Ma et al. (2023) for juvenile rainbow trout raised in a recirculating system, thus for length, the authors obtained values between 16.45 cm and 18.25 cm, and for weight, values between 86.23 g and 96.91 g, corresponding to measurements M3 in our experimental variants.

According to the international database FishBase, for the species *Oncorhynchus mykiss*, the maximum body length ever recorded was 122 cm, the usual length being 60 cm, while the maximum body weight can reach up to 25.4 kg for an adult specimen.

There was also a proportional increase in weight versus length, ranging from 51.62 g for 16.81 cm long fish to 131.56 g for 22.14 cm long fish during the study period. The same proportional increase between the mentioned parameters was also found by Sharma & Bhat (2015) and Gallego-Alarcon & Fonseca (2019).

***Results on maximum body height* (H)**

Regarding the maximum body height, from the observations made in the present study, it can be noted a similar evolution to the other parameters previously analysed, so in the first stage of the experiment, a variation between 3.24 cm (T4) and 3.41 cm (T1) was registered. At the last measurement, there was an increase of 1.29 cm for T1, 1.17 cm for T2, 0.7 cm for T3, and 1.37 cm for the fishes in T4 compared to the first measurements. For T3 it can be noticed that there is an important increase in height (by 0.75 cm) until M2 period, then the value was insignificant (by 0.05 cm until M3 and by 0.12 cm in the last measurement period compared to the M2) (Figure 6).

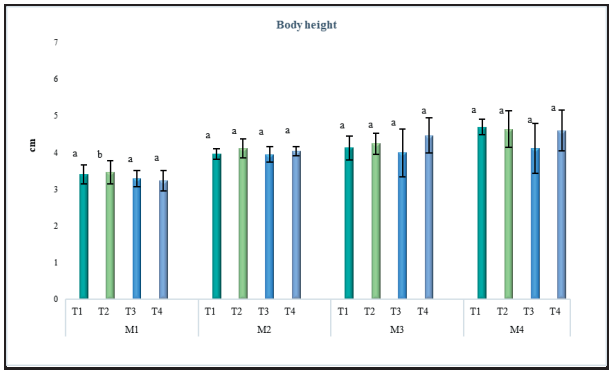

Figure 6. Variation in body height of trout in the four tanks

Also, in the case of the analysis of this parameter, the statistical analysis showed that there are no significant differences between the four tanks within each of the four measurements.

***Results on maximum body thickness* (T)**

The rainbow trout body thickness ranged from 1.85 cm to 2.57 cm in Tank 1, from 1.96 cm to 2.72 cm for Tank 2, from 1.85 cm to 2.42 cm for Tank 3, and from 1.84 cm to 2.67 cm for Tank 4, respectively. In both Tank 3 and Tank 4 a slight decrease in maximum body thickness can be observed at measurement M3. The largest increase was recorded for the fish in Tank 4 with 0.83 cm (Figure 7).

From a statistical point of view, it can be observed that in the case of the last measurement, the body thickness values from T1 and T3 are significantly different compared to those from T2 and T4, showing that the feed formulas may influence this parameter.

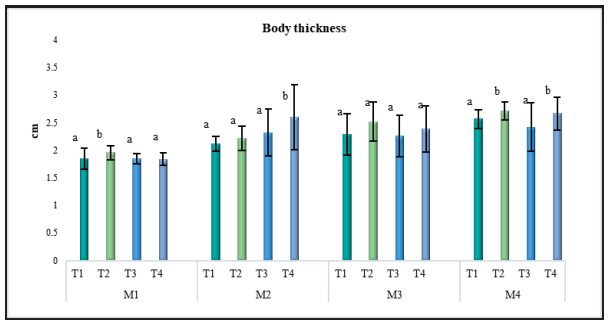

Figure 7. Variation in body thickness of trout in the four tanks

***Results on the profile index* (IP)**

The profile index (Figure 8) ranged from 4.71 cm to 5.11 for T1, from 4.85 to 5.22 for T2, from 5.03 to 5.25 for T3, and from 4.56 to 5.20 for T4. The values obtained here are higher than those presented by Nistor et al. (2012) and Pagu et al. (2012) for the same fish species.

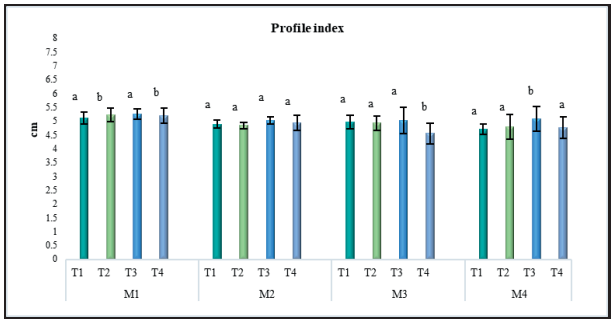

Figure 8. Variation in body profile index of trout in the four tanks

Statistically, there are significant differences at the end of the experiment between T3 and T1, T2, and T4, which means that there are some differences between the fish in terms of profile index.

***Results on thickness index* (TI)**

The thickness index (Figure 9) varied from 10.67% to 11.59% for trout in T1, from 10.87% to 12.29% for T2, from 10.73% to 11.47% for T3, and from 10.98% to 12.93% for T4. For T1 and T2, thickness index values increased exponentially, instead for T3 and T4, the values had their maximum value at the time of measurement 2 (M2).

There are no significant differences between the experimental variants, which means that there are no differences between the fish in terms of development.

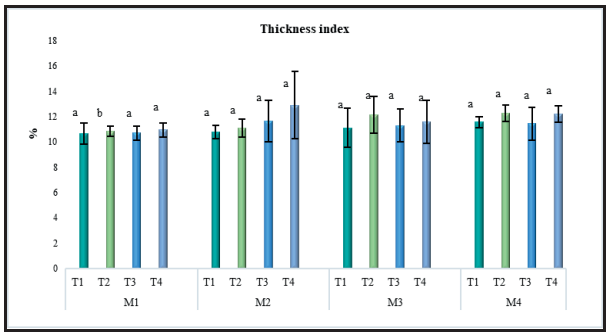

Figure 9. Variation in body thickness index of trout in the four tanks

***Results on the Fulton's condition factor* (K)**

For the analysed rainbow trout specimens during the study, K factor ranged between 1.01 and 1.28 (Figure 10), this coefficient indicating the health status of the fish.

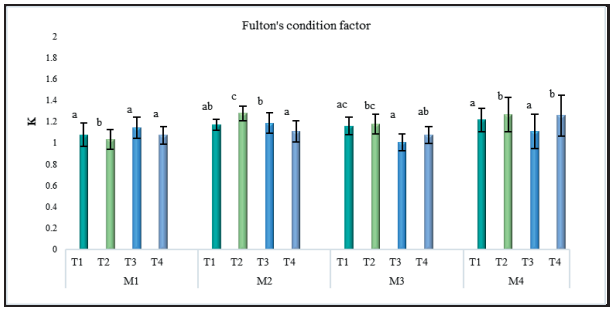

Figure 10. Variation of the Fulton coefficient in trout in the four tanks

For salmonids such as rainbow trout, the value of the condition factor K is considered acceptable if it reaches the threshold of 1.20. A K value of 1.00 indicates that the fish are in poor condition (long, thin body). However, it must be considered that the value of this coefficient is closely dependent on many factors, such as gender, season, development stage of the reproductive organs, age, the degree of muscle development, or the type of food consumed (Barnham & Baxter, 1998; Dekic et al., 2016).

Results on the b coefficient (slope)

With b values less than three, the growth pattern for trout was allometrically negative for most records in all four tanks, with the exception of trout in T2 and T3, for which positive allometric growth was observed (at M1 in T2, respectively to M3 and M4 in T3). These results (Figure 11) highlight the fact that most specimens of *O. mykiss* kept under observation tend, over time, to elongate their bodies, increasing in length more than the value related to weight gain. When the value of b>3, a positive allometric growth (A+) is suggested, which means that the fish grow faster in weight than in length, i.e. as they grow, they become less elongated or wider (Karachle & Stergiou, 2012; Sharma & Bhat, 2015).

The b slope values should be in the expected range of 2.5-3.5 (Froese et al., 2011). When b slope < 3, the growth pattern is negative allometric/hypo allometric (A-), which means that the fish grow faster in length than the estimated value related to weight gain (Karachle & Stergiou, 2012).

During the experiment, the water quality in the recirculating aquaculture system in all four tanks underwent several changes.

Maintaining good water quality is of utmost importance for recirculating systems. Poor water quality does not necessarily lead to the death of the cultured species, but it can reduce their growth rate, cause stress, and increase the incidence of diseases. Therefore, the following factors must be monitored and maintained within optimal parameters for the cultured species through the system installations: dissolved oxygen, ammonia, nitrites, nitrates, carbon dioxide, pH, suspended solids, etc.

Fish release carbon dioxide, ammonia, and waste into the culture environment. The components of the system must remove these substances and prevent their harmful effects. Thus, to maintain appropriate water quality, it must be continuously drained from the rearing tank and undergo filtration, biofiltration, oxygenation, and sterilization processes before being pumped back into the tank.

Based on the recorded values of oxygen and temperature, they exhibited a normal trend (at a temperature of 16-18°C, with dissolved oxygen concentrations of ≥ 9 mg/l). However, fluctuations were observed when the temperature or NH_3 levels increased. During the water renewal process, it was noted that all parameters became optimal.

Monitoring pH is also very important, especially in recirculating aquaculture systems, because a sudden change in this parameter can inhibit the growth of beneficial bacteria in the filter. An increase in pH value leads to a rise in ammonia, as these two parameters are correlated.

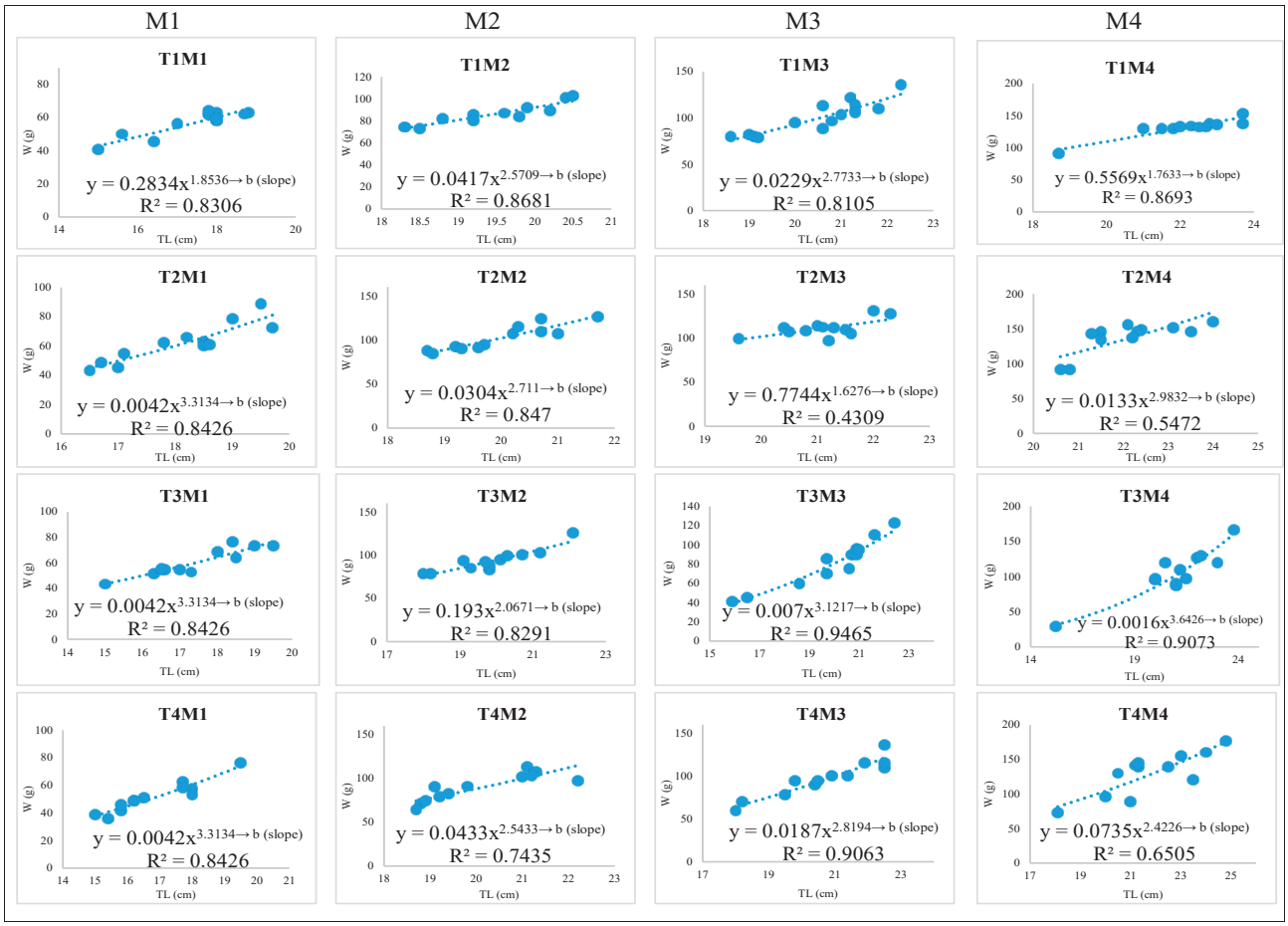

Figure 11. Variation of the coefficient b (slope) of the length-weight relationship in trout in the four tanks

In this case, it can be seen (Figure 12) that the pH value remained within normal limits (6.4-8.3) for trout throughout the analysed period. Throughout the experiment, it can be observed that there are fluctuations in ammonia levels (Figure 13) that far exceed the maximum allowable limit for trout. This parameter is influenced by temperature and pH (NH_3 increases if pH rises and temperature decreases) (Hewa Kinyage & Pedersen, 2016).

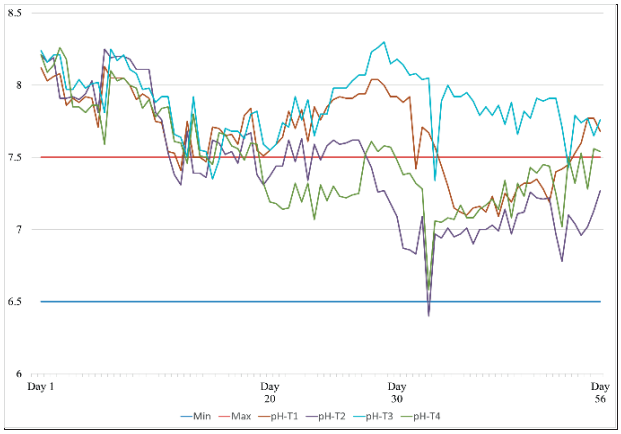

Figure 12. Variation of the water pH in the experimental tanks

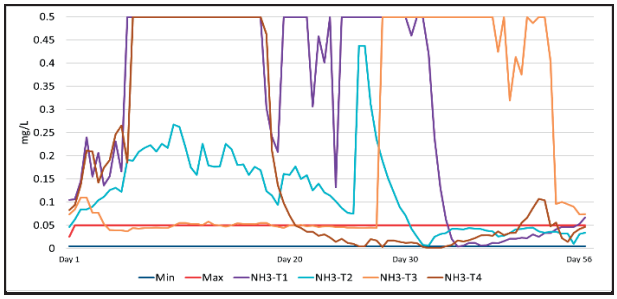

Figure 13. Variation of ammonia concentration of the water

Additionally, the stress caused by handling during the morphometric measurements led to a weakening of their immune system. According to the study realized by Pickering (1992), there are several physiological and endocrinological changes in rainbow trout under aquaculture stress. It highlights that elevated plasma cortisol, triggered by activation of the hypothalamic-pituitary-interrenal axis, is a critical factor in the negative impact of stress on the fish's survival, growth, and reproduction.

Thus, in our experiment an opportunistic bacteria developed, resulting in a decrease in fish numbers as follows: T4-80%, T2-60%, T3-

70%, T1-20%. According to the percentages, the bacteria first appeared in T4, followed by T3 and then in T2 and T1. During this period, measures were taken to determine the exact cause of mortality. In this regard, a detailed description of the histopathological analyses was necessary, along with the antibiogram that formed the basis for the applied medication. The latter consisted of administering oxytetracycline in the water of each tank at various concentrations calculated in g/kg of fish/volume of water. At this time, the total number of fish remained the same in all four tanks.

Necropsy examination - macroscopic description

Rainbow trout juveniles (T1-T4) were received at 7 months old. The length of received corpses varies between individuals. During the external examination, the inconstant presence of hyperemia at the level of the operculi was observed, at the level of the gills, ischemic aspects (Figure 14A) or marked hyperemia, occasionally with the presence of petechiae (Figure 14B), accompanied by sero-mucous catarrh, were identified; inconstant coelomic distension (Figure 14C) with evidence of discrete anal prolapse.

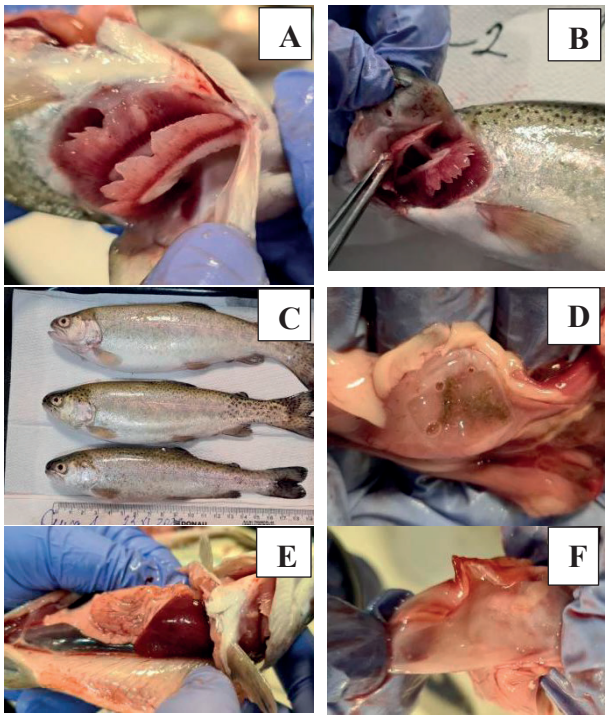

Figure 14. Macroscopic examination of gills - images A and B; general appearance of carcasses, Basin 1 - image C; examination of gastric contents - images D and F; examination of viscera in the coelomic cavity - image E

After opening the coalomic cavity, internal organs were sampled together, in a single piece (Figure 14E), and included in separate blocks for each litter. Parallel transverse sections in the cranio-caudal direction on the longitudinal axis were then performed for each fish, and all tissue fragments were included in separate paraffin blocks, through which serial sections were performed with the help of a microtome. Staining used: hematoxylin - eosin.

Results on microscopic examination (histopathological examination)

Multiple organs were sampled from all fish of the 4 tanks. Examined sections showed similar microscopic features.

<u>Gills</u> - the gill epithelium was occasionally necrotic, with most cells containing intracytoplasmic optically-empty vacuoles, consistent with granular and/or granulo-vacuolar degeneration; karyopyknosis, karyorrhexis and karyolysis were often observed. Necrotic cells were surrounded by low to moderate numbers of inflammatory cells, consisting of mononuclear and rare heterophilic cells. Within the central cartilage tissue in the primary lamina, connective fibers were dilacerated and edematous; congested blood vessels and ectatic lymphatics were also observed, with scarce inflammatory infiltrates. Occasional epithelial hyperplasia with fusion of the secondary lamellae was seen.

<u>Small intestine</u> - marked loss of the normal histological architecture, with replacement of the mucosa by necrotic debris, abundant sero-cellular exudate and hemorrhage, and admixed with bacillary structures, leukocyte debris composed mainly of heterophils, lesser macrophages and lymphocytes embedded in abundant fibrin deposition. Multifocally within the lamina propria or extending transmurally through the intestinal wall, there were infiltrates of numerous heterophils, macrophages, lymphocytes and plasma admixed with erythrocytes; highly congested blood vessels edema were observed.

<u>Cecums/pyloric appendages</u> - *lamina propria* was moderately infiltrated by polymorphous inflammatory cells (Figure 15 G); focally necrotic enterocytes were observed, occasionally with McKnight cell (MCK) aspects. Lamina contained cell debris, small

amounts of food debris and abundant sero-mucous exsudate.

Liver - the histological architecture was disrupted by focal areas of coagulative necrosis and marked degenerative changes. Multiple areas showed loss of hepatocytes cords; hepatocytes were frequently swollen, with hyperchromatic nucleus with a constant tendency to assume a pyknotic appearance. At the cytoplasmic level, hepatocytes show elements of granular and/or granulovacuolar degeneration or, very rarely, of lipid (microvacuolar) degeneration. Variable degrees of hyperemia (moderate to severe) of sinusoids were evident.

Pancreas - disruption of normal histological architecture by multifocal necrotic changes. At the level of exocrine acinar cells, cytoplasmic vacuolization was evident, with rare elements of karyopyknosis, karyorrhexis and karyolysis. The endocrine pancreas is focally characterized by granulo-vacuolar degenerative changes. Interstitial, focal, inflammatory infiltrates of mononuclear cells (lymphocytes and macrophages/monocytes) was observed. Occasionally, tissue necrosis extends into the adjacent adipose tissue surrounding the digestive tube (steatonecrosis).

Kidneys - swollen nephrocytes, with granulo-vacuolar degeneration, frequent pyknotic or absent nuclei and nephrocyte detachment from the basement membrane (tubulonecrosis). Lymphoid tissue showed moderate to severe lymphocytolysis with apoptotic bodies. The renal tubules were affected by hydropic degeneration. At the stromal/interstitial level, hyperemia and edema were observed, with rare erythrocyte extravasations. Occasionally, areas with inflammatory infiltrate composed of mononuclear and heterophil cells are observed.

Spleen - splenic parenchyma was characterized by lymphocyte depletion and infiltration by heterophils. Multiple areas of cellular necrosis were observed, accompanied by frequent leukocytes with karyopyknosis and karyorrhexis and rare erythrocyte extravasations (hemorrhages).

Heart - histological architecture was moderately preserved, with separation or rupture of myocardiocytes by a slightly fibrillar content mixed with a variable number of inflammatory cells consisting of heterophils, lymphocytes and histiocytes. Thickening of the epicardium was also observed, as a result of the accumulation of sero-cellular exsudate, admixed with frequent degenerate heterophils.

Muscular tissue - within areas adjacent to the renal tissue, a discrete inflammatory infiltrate was identified, extending deep into the dorsal muscle, with subsequent atrophy and hyaline degeneration at the level of the muscle fibers.

Histopathological examination - interpretation

Gills: proliferative, lymphocytic and histiocytic bronchitis, with epithelial hyperplasia, edema, hemorrhage, and necrotic-erosive foci (Figure 15 A, B, C).

Intestine: severe, diffuse, acute catarrhal enteritis, with intralesional bacteria (Figure 15 D, E and F).

Liver: diffuse microvesicular lipidosis; acute, multifocal necrotizibg hepatitis (Figure 15 I).

Pancreas: moderate, multifocal, acute necrosis.

Spleen: multifocal, acute necrotizing splenitis.

Heart: acute transmural myocarditis (Figure 15 H).

Kidneys: hydropic degeneration and multifocal tubular necrosis; multifocal lymphocytic, histiocytic and neutrophilic interstitial nephritis (Figure 15 J).

Striated muscle tissue: moderate, multifocal necrotizing and neutrophilic myositis.

The histological aspects are suggestive of a bacterial disease with septicemic evolution. In trouts, the most common species of bacteria associated with such lesions are represented by: *Aeromonas* spp., and *Flavobacterium* spp. Bacterial infections can be favored by high temperatures, low oxygen level, low water level. They can also be secondary to viral infections.

During the necropsy examination, samples were collected for the microbiological examination, from the gills and liver. The histopathological findings were similar with those found by Zepeda-Velázquez et al. (2015).

Results on microbiological assays

After 24 hours of incubation, smooth, convex, glossy, round-oval colonies showing hemolysis were observed, and *Aeromonas veronii* was identified (Figure 16).

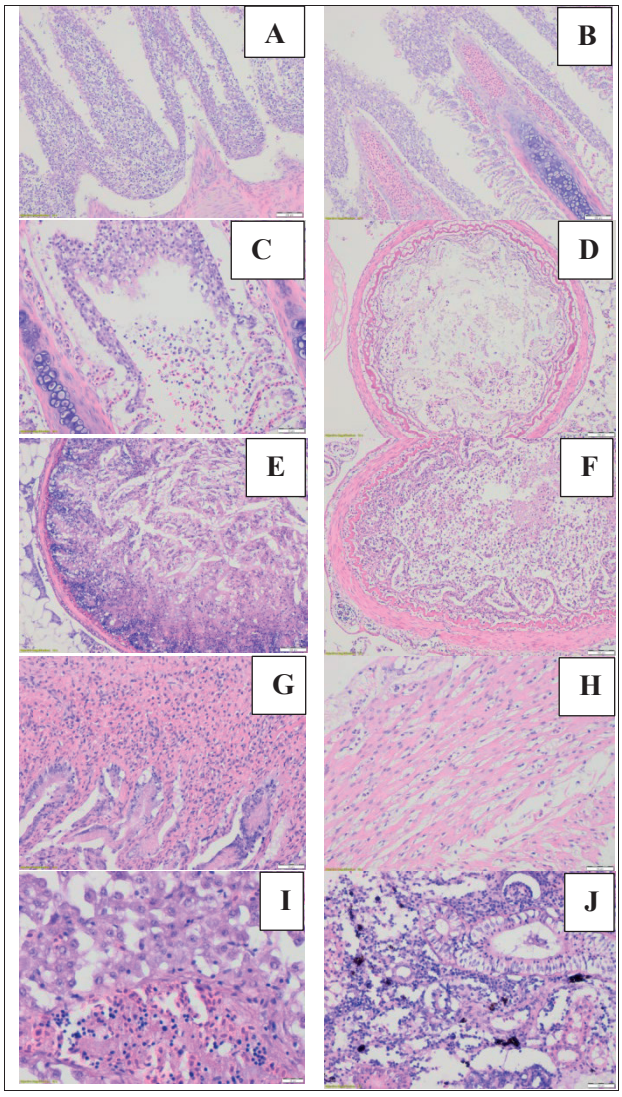

Figure 15. Microscopic (histopathologic) examination: gills showing necrotic elements and severe inflammatory infiltrate - images A and B, obj. 10x, image C, obj. 20x; small intestine - images D, E, and F, all with obj. 10x; pyloric cecum - image G, obj. 20x; cardiac wall - image H, obj. 20x; hepatic parenchyma - image I, obj. 20x; renal parenchyma - image J, obj. 20x

Aeromonas species are commonly found in aquatic environments and are primary pathogens in farmed aquatic animals. In recent years, *A. veronii* has been increasingly associated with infections in aquatic animals, exhibiting symptoms and histological lesions similar to those caused by *Aeromonas hydrophila*. Additionally, *A. veronii* has been linked to mass mortalities in various aquatic species (Liu et al., 2022).

Aeromonas veronii is a Gram-negative, rod-shaped, facultative anaerobic bacteria that is widely distributed in nature and exhibits strong environmental adaptability. The bacteria are known to infect a variety of species, including freshwater fish, amphibians, birds, and red meat animals, leading to significant losses in the aquaculture industry and posing a threat to food safety.

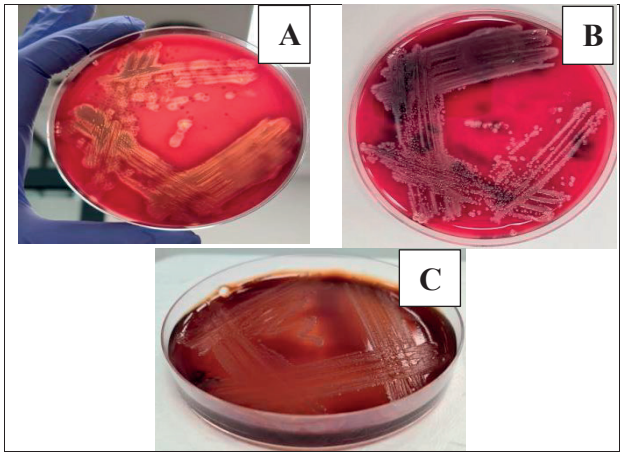

Figure 16. Identification of *Aeromonas veronii* Colonies on Columbia Blood (A and B) and Chocolate (C) agar media after 24 hours of incubation

Additionally, can infect humans, particularly the elderly and children with weakened immune systems, causing conditions such as sepsis, gastroenteritis, and other illnesses. Recent studies have also indicated that even individuals with healthy immune systems may be susceptible to infection. The growth of the aquaculture industry has been accompanied by an increase in bacterial diseases, and the overuse of antibiotics has contributed to the rise of antibiotic-resistant strains of *Aeromonas*, with antibiotic residues in aquatic products posing risks to human health. Reports of infectious diarrhea and food poisoning caused by pathogenic bacteria have also been on the rise (Li et al., 2020).

After the identification of the bacteria, the diffusimetric antibiogram was performed. Following the antibiogram assessment, the results showed that *A. veronii* is resistant to: amoxicillin, erythromycin, and penicillin, susceptible to chloramfenicol, doxycycline, trimethoprim with sulfamethoxazole, enrofloxacin, florfenicol, and intermediate susceptible to neomycin.

Study limitations

This research had several limitations due to environmental factors. Firstly, pH values did not correlate with ammonia levels during the experiment, and no explanation was found for this situation. Secondly, nitrite levels were not

monitored, and are toxic at concentrations >0.2-0.4 mg/L (Davidson et al., 2014). Thirdly, during the experiment, feces were collected only once every 3-4 days, which led to an increase in ammonia levels. Lastly, water renewal in the tanks and cleaning filters were realized at extended intervals (seven to ten days).

CONCLUSIONS

In conclusion, our findings confirmed that *A. veronii* is linked to septicemia and skin lesions in rainbow trout. These results may be valuable for future research on rainbow trout in cases where an *Aeromonas* species could be implicated. Additionally, the assessment of the recirculating system showed that after eight weeks, alterations in the physicochemical parameters of the water impacted the fish's well-being, highlighting the necessity for enhanced filtration mechanisms to optimize conditions.

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