Pol. J. Environ. Stud. Vol. 33, No. 1 (2024), 341-349
DOI: *10.15244/pjoes/170768*

Original Research

Study of Bottom Substrate Variation in Zero Water Discharge Aquaculture for Mahseer Fish *Tor soro* Nursery

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Received: 12 June 2023
Accepted: 7 August 2023

Abstract

Mahseer fish *Tor soro*, an endemic species in Indonesia, is currently declining its population due to the overreliance on wild captures to meet consumption demands. Furthermore, the survival rate of fry is alarmingly low, necessitating the implementation of sustainable fry cultivation initiatives to bolster the demand for Mahseer fish. Environmentally friendly aquaculture practices, particularly those employing the zero-water discharge principle, offer a promising solution for establishing Mahseer fish nurseries. In cultivation methods, adding bottom substrates to resemble the natural habitat of Mahseer fish can enhance their physical performance and improve fish welfare. This study aimed to evaluate the effects of different substrates on the nursery of Mahseer fish, specifically utilizing (A) coral rock, (B) limestone, (C) crushed stone, and (D) no substrate (control) as treatments. The results demonstrated that the use of substrate media in the cultivation tanks positively influenced water quality, stress levels, and production performance of Mahseer fish. Among the treatments, the utilization of coral rock (A) as a substrate medium yielded the best results in terms of water quality, stress response, and production performance throughout the study compared to the other treatments.

Keywords: bottom substrate, *Tor soro*, stress, water quality, production performance

Introduction

Mahseer fish *Tor soro* was classified as one of Indonesia's endemic species distributed in Sumatra, Kalimantan, and Java islands [1]. Currently, among the 18 assessed Mahseer fish species by the International Union for Conservation of Nature (IUCN), 50% are classified as threatened to critically endangered. However, the *T. soro* and *T. douronensis* species, originating from Indonesia are not included in the IUCN Red List [2]. The Mahseer fish population is declining primarily due to the ongoing dependence on wild captures to fulfill consumer demands. Moreover, anthropogenic activities on deforestation, land conversion, and pollution of river or lake can disrupt crucial habitat conditions and environmental quality for Mahseer fish [1]. Addressing this issue necessitates the implementation of cultivation activities

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to decrease reliance on natural captures and ensure that the demand for Tor fish is not solely dependent on wild catches.

The escalation of organic effluents from aquaculture activities [3] has prompted the adoption of environmentally friendly zero water discharge aquaculture methods such as recirculation aquaculture system (RAS), bio floc, and other cultivation technologies [4-6]. The bio floc technology relies on heterotrophic processes, wherein excess nutrients in the pond, uneaten feed, and fish excreta are converted into floc of single-cell protein, which can be utilized as a food source by the fish [7].

On the other hand, the nitrification process in RAS facilitates the conversion of toxic ammonia into nitrate, effectively regulating nitrogen levels in water [8]. Nitrifying microbial biofilm, hosted by biofilter media such as rocks, plastic, and ceramics, is a carrier for this process [9]. Nitrogen-degrading microorganisms experience enhanced biomass with an increased specific surface area of the biofilter media [10]. Filter media with higher values of a specific surface area generally demonstrate improved capabilities in rejecting total ammonia nitrogen (TAN), nitrite, and acetate [10-11].

Studies have consistently demonstrated the positive effects of habitat enrichment on the well-being of organisms, including improved growth performance and reduced stress levels in aquaculture species [12]. The incorporation of substrate materials such as gravel, plastic plants, and PVC pipes during the rearing of *Tor putitora* has been found to enhance their predatory abilities [13] and alleviate stress by significantly reducing cortisol and glucose levels [14]. Recent research has focused on utilizing biochemical parameters such as hematocrit, cortisol, red blood cell count, and haemoglobin as stress indicators in organisms [15-19].

Ongoing research in the field of Mahseer fish cultivation investigates various aspects of the environment, including the utilization of various filter media in RAS for fish nursery [20], temperature effects on fry [21-22], and the impact of low temperature on spermatozoa motility [23-24]. However, the study of controlled habitat enrichment for Mahseer fish cultivation still needs to be explored.

This study implemented controlled habitat enrichment by utilizing the attachment growth biofilm function on the bottom substrate to regulate water nitrogen levels. Mahseer fish prefer shallow water habitats characterized by gravel and rocky substrates and clear water conditions [25]. To replicate the natural habitat of the fish, the carrier biofilm in this research comprised natural solid materials, including coral rock, crushed stone, and limestone. The primary objective of this study was to assess the efficacy of different natural rocky bottom substrates in cultivating Mahseer fish fry, focusing on their impact on water quality improvement, and fish's physiological and production responses.

Material and Methods

Experimental Design

This research employed an experimental approach, utilizing a completely randomized design (CRD) with four treatments and three replications. The treatments implemented in this study encompassed the utilization of distinct substrates (Fig. 1), specifically: (A) coral rock, (B) limestone, (C) crushed stone, and (D) no substrate (control). The dimensions of the stones used (length x width x diagonal) in this study were: 11.0 x 2.0 x 11.0 cm (A), 7.5 x 4.5 x 6.5 cm (B), and 3.1 x 2.0 x 3.5 cm (C).

The cultivation containers used in this study were 12 units of 60 x 40 x 50 cm (length x width x height) fibre tanks. The fibre tanks were filled with 60 litres of water for each treatment, and aeration was installed in each tank. Additionally, a substrate with a height of 10 cm was placed in each fibre tank of the respective treatment. Mahseer fish fry, measuring 3.15 to 3.86 cm in length, were employed as the test animals, and stocked at 20 individuals per fibre tank density. The Mahseer fish were nourished with commercial feed with approximately 30% protein content. The feed was offered ad libitum, with a feeding frequency of twice daily at 6 AM and 4 PM. During the study period (40 days), the fish were kept in a closed system with zero water discharge without water replacement. Water was added to the system to compensate for the water lost through evaporation.

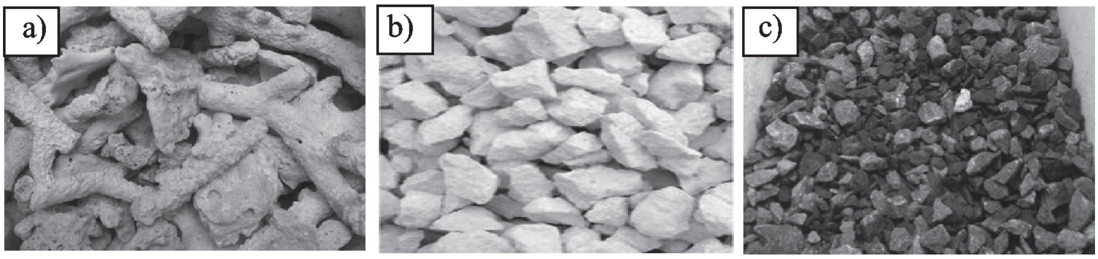

Fig. 1. The substrate media used in this study: a) coral rock, b) limestone, and c) crushed stone.

Sampling

The YSI Multi-parameter Model 556 was utilized for daily measurements of water quality parameters, such as dissolved oxygen (DO), temperature, and pH. The NH_3, NO_2, and NO_3 parameters were collected every ten days and analyzed using spectrophotometric [26]. Fish biometry and blood biochemical data were recorded at the same interval. Blood samples were collected during each replication from 5 fish, with a volume of 0.1 ml per fry. Subsequently, the gathered blood samples were thoroughly mixed, ensuring homogeneity before commencing the biochemical analysis. Glucose analysis was performed using spectrophotometry [27], while red blood cell and haemoglobin levels were analyzed using a hemocytometer and Sahli's method [27-28]. The abundance of nitrifying and denitrifying bacteria in the water was measured at the beginning and end of the study using the MPN (Most Probable Number) method [29-30]. The formulas used to calculate Fish biometry and blood biochemical are as follows:

$$\mathrm{SGR_L} = \frac{Ln\ Lt - LnLi}{T} \times 100 \tag{1}$$

Ln is the logarithmic natural, Lt is the fry final length, Li is the initial length, and T is the experiment period in a day.

$$\text{Survival Rate / SR (\%)} = \frac{Nf}{Ni} \times 100 \tag{2}$$

Nf is the number of live fry at the end of the experiment, and Ni is the initial number of fry.

$$\text{Blood glucose concentration (mg dL}^{-1}\text{)} = \frac{SpAbs}{StdAbs} \times \text{Standard glucose concentration (mg dL}^{-1}\text{)} \tag{3}$$

SpAbs is the sample solution's absorbance, and StdAbs is the absorbance of the standard solution.

$$\text{Number of Red Blood Cell (RBC)} = \text{count of erythrocytes x } 10^4 \text{ cell mm}^{-3} \tag{4}$$

Statistical Analysis

This study's biometric, water quality and blood biochemical data underwent statistical analysis using Minitab Statistical Software, specifically version 16.1.1. The statistical analysis encompassed the application of ANOVA with an F test at a 95% confidence level. The Fisher method was employed to ascertain significant differences between treatments.

Results and Discussion

Water Quality

Water quality, considered crucial in aquaculture, significantly influences the health, survival, and growth of aquaculture species. The overall well-being and development of fish are substantially impacted by water's physical, chemical, and biological characteristics [31-32]. The water quality test results during the study are presented in Fig. 2. The dissolved oxygen (DO) levels observed throughout the study remained within the acceptable range for aquaculture activities, ranging from 3.16 to 7.81 mgL^{-1}. The minimum DO requirement for aquaculture activities is 3 mgL^{-1} [33], while the recommended optimal DO level is above 5 mgL^{-1} [32, 34]. This study's concentrations of ammonia, nitrite, and nitrate ranged from $0.32\text{-}4.30 \times 10^{-3}$, $1.10\text{-}50.50 \times 10^{-2}$, and $4.29\text{-}75.19 \times 10^{-2}$ mgL^{-1}, respectively. The recommended standards for ammonia, nitrite, and nitrate are below 0.10, 50.00, and 100.00 mgL^{-1}, respectively [16]. The pH and temperature levels during the study ranged from 6.50-7.50 and 26.20-28.20ºC, respectively. The recommended pH range is 6.50-8.50 [20], while the recommended temperature range is 26.00-30.00ºC [21]. Overall, the water quality conditions in the cultivation pond meet the requirements for aquaculture activities.

Oxygen is considered the foremost critical factor for the survival and well-being of aquatic organisms [35]. It plays a vital role in various physiological processes and is essential for maintaining aerobic metabolism in aquatic animals. During the study, treatment A tended to have higher dissolved oxygen (DO) levels than the other treatments. The oxygen requirement for fish metabolism and the biological processes of bacterial degradation of organic matter from leftover feed and faeces during aquaculture activities influences the DO level. A proposed method for eliminating nitrogen compounds, such as ammonium and nitrates, from water is simultaneous autotrophic nitrification and anoxic denitrification [36-37]. In this process, nitrifying bacteria convert ammonia to nitrite, which is subsequently transformed into nitrate. During nitrogen removal, denitrifiers convert the nitrate into N_2 gas [37-38]. If the nitrification mechanism can effectively occur in water, it plays a crucial role in minimizing the levels of toxic pollutants in aquaculture ponds, such as ammonia and nitrite.

The presence of organic matter significantly influences the consumption of dissolved oxygen. As the quantity of undegraded organic waste or pollutants in the water increases, the DO level decreases accordingly. In this process, the porosity of the rock substrate employed in this study emerges as an additional factor that affects the quantifiable quantities of nitrifying and denitrifying bacteria. Porosity correlates with the surface area of the rock substrate, whereby a higher porosity leads to a greater contact surface area.

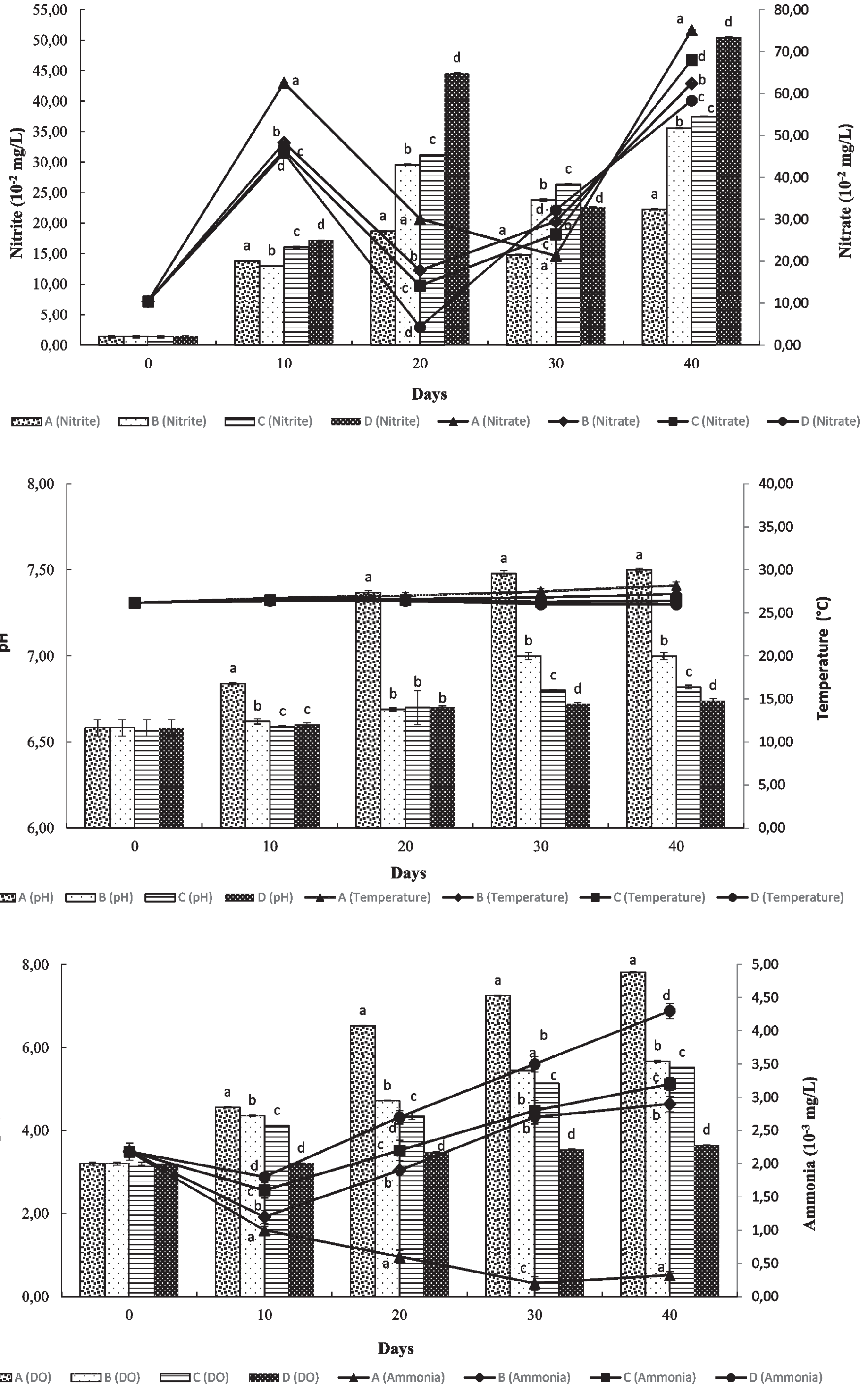

Fig. 2. Physical and chemical parameters of water during the study at (A) coral rock, (B) limestone, (C) crushed stone and control (D) treatments.. The use of distinct lowercase letters in the graph indicates significant difference ($p<0.05$).

A large surface area in the substrate media is crucial as it facilitates biofilm growth [39]. A greater surface area allows for a higher density of bacteria per unit of substrate media volume, resulting in enhanced ammonia removal in aquaculture ponds. The coral rock substrate exhibits superior porosity compared to limestone (treatment B) and crushed stone (treatment C). The coral rock, characterized by a brittle texture and an extensively developed pore structure, demonstrates a high degree of interconnectivity and can possess a porosity of 50% or more [40].

Table 1 shows that the highest numbers of nitrifying bacteria are found in the coral rock substrate treatment (treatment A). In contrast, the lowest numbers are observed in the limestone substrate treatment (treatment B). This finding indicates that treatment A pond's

Table 1. The abundance of nitrifying and denitrifying bacteria in water for each of the bottom substrate treatments at the beginning and day 40th.

Bacterial abundance	Bottom substrate media			
	Coral rock	Limestone	Crushed stone	Control
The quantity of nitrifying bacteria at the beginning of the study (cell/ml)	1.10±0.00 x 10^5	1.10±0.00 x 10^5	1.10±0.00 x 10^5	1.10±0.00 x 10^5
Number of Nitrifying bacteria on the 40th day (cell/ml)	1.10±0.00 x10^{11b}	2.97±1.15 x 10^{8a}	7.34±6.35 x 10^{10ab}	7.70±5.72 x 10^{10ab}
The quantity of nitrifying bacteria at the beginning of the study (cell/ml)	7.70±5.70 x 10^7	7.70±5.70 x 10^7	7.70±5.70 x 10^7	7.70±5.70 x 10^7
Number of Denitrifying bacteria on the 40th day (cell/ml)	7.70±5.70 x 10^7	7.70±5.70 x 10^7	7.70±5.70 x 10^7	2.86±0.46 x 10^7

Different lowercase letters in the table show a significant difference ($p<0.05$) between treatments

nitrification and denitrification processes are more efficient than the other treatments. Furthermore, treatment A showed the lowest levels of ammonia, nitrite, and nitrate throughout the study. The lower concentration of pollutants in the aquaculture water leads to a reduced oxygen requirement for bacterial degradation of organic matter. Consequently, this results in higher DO levels in treatment A compared to the other treatments.

Blood Biochemical

Blood consists of an intricate blend of red blood cells (erythrocytes), white blood cells (leukocytes), platelet analogues (thrombocytes), electrolytes, and nutrient components. [15, 41-42]. Modifications in informative blood-derived markers, encompassing metabolic and enzymatic constituents, hormonal profiles, and transcript abundances, manifest the systemic responses to perturbations or disruptions in homeostasis.

Hence, these markers can serve as monitoring tools for assessing the physiological well-being of an individual fish, a cohort, or an entire population [15, 43]. The physiological homeostasis of fish is influenced by changes in blood osmoregulation, levels of various ions, and glucose due to their adaptation to different environmental conditions [15, 44].

Based on Fig. 3, the blood glucose levels in treatment A were lower and more stable throughout the study than in other treatments. Under stress conditions, the blood glucose levels in fish exhibited an increase. As a response to stress conditions, the elevation in glucose levels can be interpreted as an indicator that the fish undergo metabolic adjustments to cope with the increased energy demands for homeostatic processes [15, 45-47].

The lower stress levels observed in treatment A are likely attributed to the superior water quality conditions observed during the study. According to the data presented in Fig. 2, treatment A demonstrated higher concentrations of DO, lower levels of ammonia, and increased abundances of nitrifying and denitrifying bacteria compared to the other treatments. These favorable environmental conditions significantly influenced the metabolic processes in treatment A, facilitating more efficient metabolic functioning and consequently reducing stress levels.

In the presence of stress, fish exhibit an augmentation in oxygen uptake facilitated by a multifaceted integration of physiological, behavioural, biochemical, and molecular mechanisms [15]. In stressful conditions marked by reduced oxygen levels, fish exert efforts to uphold oxygen delivery by increasing red blood cells (RBC) and haemoglobin (Hb) [48-49, 15].

Based on Fig. 3, the concentrations of RBC and Hb in treatment A tend to be lower compared to the other treatments. The lower Hb and RBC concentrations signify a lower stress level in fish subjected to treatment A. Lower glucose levels accompany this downward trend in RBC and Hb values than the other treatments. This observation can be attributed to the superior water quality, particularly in terms of dissolved oxygen (DO), in treatment A compared to the other treatments. Under conditions of reduced DO, fish will increase hematocrit (HCT) levels to restore the normal viscosity and functionality of RBC.

The elevation in Hb concentration facilitates improved oxygen transfer during fish stress [15]. Overall, the utilization of coral rock substrate significantly influences lower stress levels in fish during the nursery phase. However, the implementation of limestone substrate, crushed stone, and the control group did not yield any notable effect on reducing stress levels throughout the study.

Production Performance

Fish production performance can be analyzed by quantifying the growth and survival of fish throughout the study period. The growth and survival of fish are contingent upon a myriad of internal and external factors. Internal factors, such as physiological conditions and genetic characteristics, can significantly affect growth dynamics. Meanwhile, external factors,

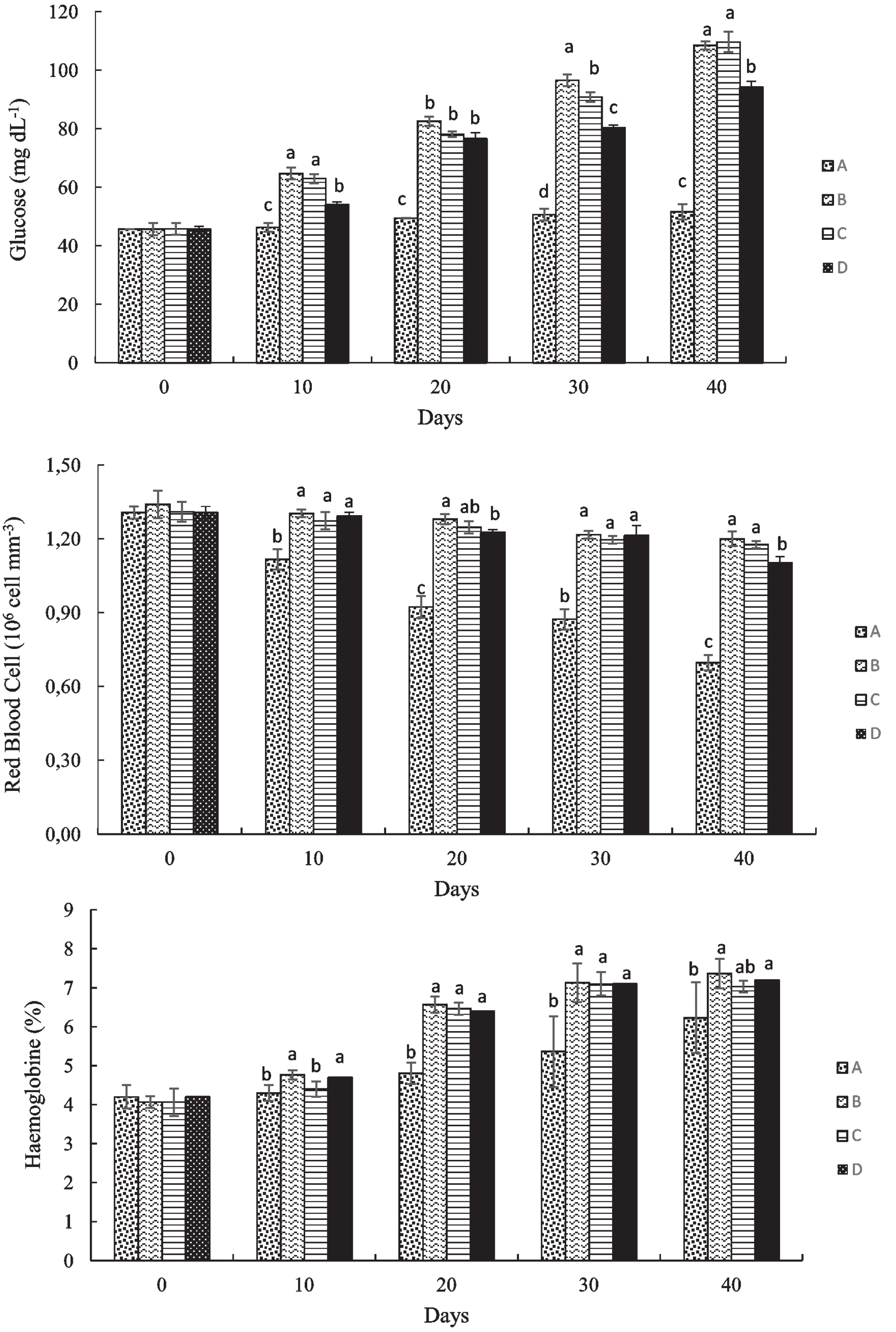

Fig. 3. Blood biochemical parameter at (A) coral rock, (B) limestone, (C) crushed stone and control (D) treatments. The use of distinct lowercase letters in the graph indicates significant difference ($p<0.05$).

such as the aquatic environment and feeding practices, exemplify the extrinsic influences impacting fish growth and survival [16].

Based on Table 2, treatment A achieved the highest Specific Growth Rate of Fish Length (SGR_L) at 1.95±0.05% day^{-1} and a survival rate of 91.00±4.18%. The utilization of coral rock substrate significantly and positively influenced the production performance of the cultivated fish. This effect can be attributed to the optimal environmental conditions and blood biochemical parameters observed in treatment A throughout the study. Treatment A exhibited the best water quality conditions, with superior levels of DO, ammonia, nitrite, nitrate, and abundances of nitrifying and denitrifying bacteria compared to the other treatments. The optimal environmental conditions during the study resulted in lower stress levels, leading to improved growth and survival rates compared to the other treatments.

Table 2. The production performance of Mahseer fish fry after being reared for 40 days on various bottom substrates media.

Production performance	Bottom substrate media			
	Coral rock	Limestone	Crushed stone	Control
Initial length (cm)	3.57±0.18	3.50±0.18	3.46±0.24	3.39±0.21
Final length (cm)	11.53±0.41^a	9.14±0.53^b	8.70±0.58^{bc}	8.40±0.44^c
Survival Rate (%)	91.00±4.18^a	87.00±2.74^a	82.00±2.74^b	79.00±2.24^b
SGR_L (% day^{-1})	1.95±0.05^a	1.60±0.03^b	1.54±0.02^b	1.52±0.18^b

Different lowercase letters in the table show a significant difference ($p<0.05$) between treatments

Conclusions

Based on this study's findings, substrate media utilization in the cultivation tanks positively impacts water quality, stress level, and production performance of Mahseer fish. Treatment with coral rock (A) as a substrate media resulted in the best water quality, stress response, and production performance throughout the study compared to other treatments.

Acknowledgments

We want to express our gratitude for the collaboration between the Ministry of Maritime Affairs & Research Center for Fisheries, and the National Research and Innovation Agency (BRIN), which made this study possible. We would also like to thank Mr Supendi, Mr Samsul and Mr Irwan for their valuable technical support.

Conflict of Interest

The authors hereby state that there are no conflicts of interest.

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