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Investigation Effects of Methylene Blue Exposure on Behavior and Adsorption in Baramundi (*Lates calcarifer*)

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Abstract
This study aims to investigate the effect of Methylene Blue (MB) exposure on the behavior and adsorption in the body of Baramundi (*Lates calcarifer*). The fish were kept for 28 days in aquariums with five levels of MB concentration (1-5 ppm) and one control group without exposure. The parameters observed included behavior, morphology, water quality, and MB concentration in the tissue through spectrophotometric analysis. The results showed a decrease in absorbance values in the 500–800 nm wavelength range in the treated fish compared to the control, indicating degradation or a reduction in MB concentration in the tissue. Water quality remained stable across all treatments, as indicated by relatively constant salinity, conductivity, salt content, pH, and ammonia values. Variations were only seen in lower Total Dissolved Solids (TDS)in some treatment aquariums and a decrease in phosphate compared to the control. Behaviorally, fish in the control group exhibited normal swimming activity, rapid response to feed, and stable social interactions. Meanwhile, fish exposed to MB, especially at higher concentrations, showed slower movements, decreased feeding responses, a tendency to remain at the bottom, and signs of mild stress. The treatment aquarium environment also appeared cleaner with minimal algae or other biota, unlike the control. Overall, the use of MB did not disrupt the stability of environmental quality but affected behavioral responses.

Keywords: Aquaculture, Behavior, Bioaccumulation, *Lates calcarifer*, Methylene Blue,

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1. Introduction

Seed health is a crucial factor that determines the success of fish farming, including leading commodities such as Baramundi (*Lates calcarifer*). During the seed phase, susceptibility to disease is relatively high due to attacks by parasites, bacteria, and fungi, which can reduce survival rates and productivity. To address this issue, one common approach is to soak the seeds in chemical compounds with antimicrobial activity, one of which is Methylene Blue (MB) (Mphuthi *et al*., 2023).

MB is a cationic dye with antiseptic, antimicrobial, and antiparasitic properties, which has long been used in aquaculture to prevent and control diseases in fish eggs and fry (Mitrowska *et al*., 2023). This compound works by disrupting the cell membranes of pathogens, inhibiting the replication of microorganisms, and interfering with energy metabolism (Khan *et al*., 2022). MB has a broad spectrum of activity against bacteria, fungi, and parasites, and is stable in solution, making it effective for use in the fry quarantine stage (Park *et al*., 2019; Wang,*et al*., 2020). This effectiveness has made MB popular among Baramundi farmers as an early preventive measure against disease. However, the use of MB is not without problems. A number of studies have reported that MB can affect fish metabolism and behavior and leave residues in tissues (Xu *et al*., 2021). Its ability to interact with negatively charged biomolecules causes accumulation in metabolic organs such as the liver and muscle tissue, which are the main products of consumption (Mitrowska *et al*., 2023). This raises concerns about food safety and export quality standards. The case of Australia's rejection of Indonesian Baramundi exports due to the discovery of MB residues shows that this issue has a direct impact on economic losses, product reputation, and the sustainability of international trade.

Based on these conditions, this study aims to investigate MB residues in the gills of Baramundi and changes in fish behavior as a physiological response to MB exposure. UV-Vis spectrophotometry analysis is an effective approach for measuring MB absorption levels in tissue, as this method enables the detection of residue concentrations through absorbance values at specific wavelengths. The results of this study are expected to provide a scientific basis regarding the risk of MB residues in Baramundi fish while supporting efforts to find safer, environmentally friendly disease control alternatives that meet international export standards

2. Methods

2.1 Study area

This study used an experimental design with six treatment groups, consisting of one control group and five test groups with different concentrations of Methylene Blue-(MB). The control group consisted of Baramundi (*Lates calcarifer*) fry that were not exposed to MB. Meanwhile, the treatment groups were differentiated based on the concentration of MB in the aquarium medium, namely:

a Aquarium A: fry was exposed to a concentration of 1 ppm MB.
b Aquarium B: fry was exposed to a concentration of 2 ppm MB.
c Aquarium C: fry exposed to a concentration of 3 ppm MB.
d Aquarium D: fry exposed to a concentration of 4 ppm MB.
e Aquarium E: fry exposed to a concentration of 5 ppm MB.

These concentrations were selected based on commonly applied therapeutic ranges of MB in aquaculture as reported in previous studies, and to evaluate the progressive physiological and behavioral responses of Baramundi fry across increasing exposure levels.

Each treatment group was maintained in controlled aquarium conditions with 5 fish per tank, adequate aeration, and uniform stocking density. Feeding was conducted twiceas based on the nutritional requirements and feeding behavior of the fry, while water quality (temperature, pH, salinity, and dissolved oxygen) was routinely monitored to remain within the optimal range for Baramundi fry.

The immersion treatment was carried out for 48 hours to evaluate behavioral responses, survival rates, and MB residue absorption in fish tissue. Subsequently, MB residue measurements were performed using UV-Vis spectrophotometry at specific wavelengths to obtain quantitative data on MB concentrations in the tissue.

2.2 Dilution of Methylene Blue (MB) Solution

A stock solution of MB with a parent solution concentration of 1000 ppm was prepared in advance by dissolving 1 g of MB powder in distilled water. The stock solution was then diluted to 100 ppm to obtain working concentrations suitable for the treatment, namely concentrations of 1, 2, 3, 4, and 5 ppm. The dilution process was carried out using the formula M1.V1 = M2.V2, so that the volume of stock solution added to the maintenance container could be accurately adjusted to achieve the desired final concentration. The diluted solution was then thoroughly mixed into the fish maintenance medium, with gentle stirring to ensure the solution was homogeneous.

2.3 Presentation of Methylene Blue Solution

The MB solution was administered by adding a mixture of solutions with treatment concentrations (1, 2, 3, 4, and 5 ppm) to the fish maintenance medium. The experimental fish were kept in aquariums with a specific capacity that had a recirculation system, so that the MB concentration could be maintained at a stable level throughout the study period. The culture medium was only displayed during each water change with the same MB solution at the initial concentration to avoid degradation of the active substance and maintain water quality. The exposure lasted for 28 days, accompanied by routine monitoring of temperature, pH, and dissolved oxygen levels to remain within the ideal range for fish life. Each MB aquarium received 1 drop in aquarium A, 2 drops in aquarium B, 3 drops in aquarium C, 4 drops in aquarium D, and 5 drops in aquarium E.

2.4 Analysis of Methylene Blue Detection in Fish

Detection of MB in fish tissue was carried out by taking fish samples at the end of the exposure period. Additionally, baseline measurement was conducted by analyzing MB levels in fish tissue prior to exposure to obtain the initial (zero) MB value, ensuring that any detected MB residue originated from the experimental treatment. Tissue samples were weighed according to the analysis requirements, then extracted using 1 mL of Phosphate Buffered Saline (PBS) solvent and homogenized until well mixed. The extraction results were then purified using a fortex process and centrifugation for 30 minutes at 25°C to separate the supernatant from the solid particles. The liquid produced from the extraction was analyzed using a high-performance liquid chromatography instrument combined with a spectrophotometer to measure the MB content.

2.4 Water Quality Analysis

Water quality monitoring in aquariums must be performed regularly to ensure that the environment remains conducive to the life and growth of fish. The parameters analyzed include temperature, pH, salinity, dissolved oxygen levels, and ammonia and phosphate concentrations. Temperature is controlled within the optimal range for Baramundi fry (28–32°C) to prevent fish from experiencing stress due to temperature changes, while pH is regulated so that it is not too acidic or alkaline, which can affect the physiological functions of fish. Salinity is closely monitored in fish that inhabit brackish and marine waters, as sudden changes can affect the fluid balance in the body. Dissolved oxygen is checked to ensure oxygen availability for respiration, while ammonia, nitrite, and nitrate levels are assessed as indicators of water quality, which are usually affected by food residues and fish metabolic products. By maintaining the stability of these parameters, water quality is maintained so that fish can live well, grow optimally, and remain disease-free (Can *et al*., 2023).

2.5 Behavioral Observation

Behavioral parameters observed in Baramundi include swimming patterns, responses to external stimuli, feeding behavior, interactions between individuals, and signs of stress such as irregular swimming, loss of balance, or remaining at the bottom of the water. Morphological parameters observed in Baramundi include body shape, body color, fin condition, eye condition, and the presence of physical abnormalities such as wounds, spots, or deformities on certain parts of the body. Spectrophotometer analysis parameters observed in Baramundi include maximum absorption wavelength, absorbance intensity, and target compound concentration in the analyzed tissue sample, which are analytical chemical measurements and not part of the behavioral observations.

3. Results and Discussion

3.1 Analysis of MB Detection in Tested Fish

The results of analysis using a spectrophotometer show the effect of each concentration on MBexposure and control samples in the organs of Baramundi (*Lates calcarifer*) in Table 1.

Table 1. MB exposure at each concentration and control samples in the organs of *Lates calcarifer* based on spectrophotometer analysis.

Treatment	Spectrum	Absorbance
Methylene Blue	400.000 nm	2.109 abs
	500.000 nm	1.817 abs
	600.000 nm	1.626 abs
	700.000 nm	1.450 abs
	800.000 nm	1.296 abs
Control	400.000 nm	2.109 abs
	500.000 nm	2.118 abs
	600.000 nm	1.880 abs
	700.000 nm	1.630 abs
	800.000 nm	1.370 abs

Description: Absorbance Phosphate Buffered Saline (PBS) 0,0073

Based on the MB absorbance spectrum data in the wavelength range of 400–800 nm, it can be seen that in both the control and treatment samples, the absorbance value decreases as the wavelength increases. At a wavelength of 400 nm, the absorbance values between the control and treatment were the same, namely 2.109, indicating identical initial conditions. However, from 500 nm to 800 nm, the absorbance values of the treatment samples were lower than those of the control. At 500 nm, the absorbance of the treatment was 1.817, while the control value was 2.118; at 600 nm, the treatment was 1.626, while the control was 1.880; at 700 nm, the absorbance of the treatment was 1.450 compared to 1.630 in the control; and at 800 nm, the treatment was 1.296 compared to 1.370 in the control. The variation in control absorbance values across wavelengths occurs because absorbance naturally depends on wavelength and represents the spectral characteristics of the non-exposed sample These differences indicate that the treatment caused a decrease in the light absorption intensity of Methylene Blue, which indicates degradation or changes in the molecular structure, resulting in a decrease in concentration. Rather than directly concluding that the treatment was "effective," the reduced absorbance may suggest a lower MB residue level in the treated samples compared to the control baseline, consistent with previous reports that MB may undergo metabolic transformation or degradation in aquatic organisms.

3.2 Performance of Interpolation Method Maps

The results showed clear differences in behavioral parameters, morphology, and aquarium environmental conditions between Baramundi exposed to MB and those not exposed to (Table 2).

From a behavioral perspective, fish in the control group (without MB exposure) exhibited active and regular swimming activity, rapid responses to external stimuli, and agile movements when snatching food. In contrast, fish exposed to MB exhibited irregular swimming activity, often remained motionless at the bottom of the aquarium, and responded slowly to stimuli and feeding. This indicates a disturbance in the nervous system or metabolism of fish due to the accumulation of Methylene Blue in line with previous findings that chemical exposure can reduce motor activity and sensitivity of fish to external stimuli (Soltanian *et al*., 2021).

Morphologically, the control group exhibited a normal and proportional body shape with a natural body color and growth. In contrast, the group exposed to MB slower growth, a smaller body size, and a paler body color. This change in body color is caused by physiological disturbances in pigment production or metabolic processes, which may be influenced by the interaction of Methylene Blue with important biomolecules in the tissue (Mitrowska *et al.,* 2023).

Growth inhibition also supports the hypothesis that MB, despite its antimicrobial effects, may disrupt the metabolic balance of target organisms.

Table 2. Analysis of behavior in Baramundi (*Lates calcarifer*).

Parameters	Without MB Exposure	With MB Exposure
Behavior (Swimming activities)	Active and regular swimming	Irregular swimming activity, often staying at the bottom
Behavior (Response to external stimuli)	Quick response to stimuli	Slow and less sensitive response
Behavior (Response when fed)	Fast and active movement when fed	Slow response, less enthusiastic about food
Morphology (Body shape)	Normal and proportional body shape	Slow growth, relatively smaller body size
Morphology (Body color)	Fish color according to natural conditions	Color appears cleaner or paler
Growth Morphology	Rapid growth	Stunted growth
Aquarium environment	Algae grows quickly, many other organisms attach themselves to the glass	Clean aquarium, algae is difficult to grow

Differences were also observed in the conditions of the aquarium environment. In the control group, the aquarium was quickly overgrown with algae and other biota attached to the glass surface, whereas in the aquarium exposed to MB , the water conditions were cleaner and algae growth was inhibited. This is consistent with the antimicrobial properties of MB, which not only suppresses the growth of pathogenic microorganisms but also non-target organisms such as algae and biofilm, making the aquarium environment appear more sterile (Khatoon *et al*., 2023).

Overall, these results show that although MB is effective in maintaining aquarium cleanliness and preventing the growth of nuisance organisms, direct exposure to Baramundi can have negative effects on behavior, physiological responses, morphology, and growth. These conditions emphasize that the use of MB in fish farming must be carefully considered, especially given the potential for residues that can interfere with fish health and reduce the quality of fishery products (Zhang *et al*., 2021).

3.3. Water Quality Analysis

The results of water quality parameter tests in Baramundi fish farming media show that the addition of MB causes variations in several chemical indicators compared to the control group (Table 3).

Table 3. Analysis of water stability in Baramundi fish aquariums (*Lates calcarifer*)

Water stability test	Control	MB A	MB B	MB C	MB D	MB E
Salinity (ppt)	40	40	41	40	40	40
Electrical Conductivity (µs/cm)	16,5	16,5	16,8	16,7	16,5	16,5
TDS (ppm)	8250	7722	8188	5896	8281	8219
Salt Level Test (%)	0,89	0,89	0,90	0,90	0,89	0,89
pH	7,71	7,71	7,79	7,66	7,72	7,63
Phosphate level test (mg/L)	0,5	0,1	0,1	0,25	0,1	0,25
Ammonia level test (mg/L)	< 0,15	0,25	0,25	< 0,15	0,15	0,15

Salinity parameters salt level (‰) were stable across all treatments, ranging from 40 to 41 ‰ with a salinity of 0.89 to 0.90% measured using a digital refractometer. This stability indicates that the presence of MB does not significantly affect the balance of major ions in seawater. According to Boyd (2019), salinity stability is an important factor for the survival of marine fish, including white snapper, which have limited osmotic tolerance to sudden fluctuations.

The Electrical Conductivity (EC) value was relatively constant with a slight increase in MB B (16.8 µS/cm) and MB C (16.7 µS/cm) compared to the control (16.5 µS/cm). This small increase can be attributed to the presence of additional ions from MB, although it is measured using a digital conductivity meter and considered statistically insignificant. Research by Khan et al. (2022) also reported that the use of redox dye-based compounds in aquaculture can affect water conductivity through the release of charged ions.

The Total Dissolved Solids (TDS) parameter shows a more noticeable difference. The control had a TDS of 8250 ppm, while the MB treatment ranged from 5896 to 8281 ppm, with the lowest value in MB C. The decrease in TDS may indicate an interaction between MB molecules and dissolved particles that form complexes, thereby reducing the number of measurable free particles. This is in line with the findings of Mitrowska, et al. (2023), who stated that MB has a high affinity for negatively charged biomolecules, which can also occur in dissolved ions in water media.

The pH value remained in the range of 7.63–7.79, which is still considered optimal for marine fish maintenance (Boyd & Tucker, 2012). However, small fluctuations, especially in MB B (7.79), which is slightly more alkaline, may indicate the effect of MB on the redox process in the water medium.

The phosphate level test showed a striking difference. The control recorded a value of 0.5 mg/L, while all MB treatments experienced a decrease to 0.1-0.25 mg/L. This is consistent with visual observations that aquariums treated with MB tended to be cleaner and free of algae, as phosphate is one of the main nutri ents that supports algae growth (Wetzel, 2001). Thus, MB not only controls pathogens but also inhibits non-target organisms such as phytoplankton and biofilm.

Conversely, for the ammonia parameter, there was a tendency for an increase in several MB treatments. The control showed an ammonia level of <0.15 mg/L, while MB A and MB B increased to 0.25 mg/L. This increase was likely due to the effect of MB on decomposer microorganisms that play a role in the nitrogen cycle. As reported by Xu et al. (2021), the use of antimicrobial chemicals in fish culture media can disrupt the population of nitrifying bacteria, thereby inhibiting the conversion of ammonia to nitrite and nitrate. This condition has the potential to pose a toxicity risk if MB is used in high concentrations or for long periods.

Overall, the results of this study indicate that MB has a dual effect on water quality: on the one hand, it reduces phosphate and keeps the medium more sterile, on the other hand, it increases ammonia, which can be harmful to fish health. Therefore, the use of MB in Baramundia quaculture needs to be done carefully, paying attention to the dosage and duration of application, and supported by regular monitoring of water quality.

4. Conclusion

The use of Methylene Blue has been proven effective in controlling disease in Baramundi (*Lates calcarifer*) fry, but it leaves residues in the tissue that have serious implications for food safety and lead to export rejection. UV-Vis spectrophotometry analysis results show a characteristic Methylene Blue absorption pattern that can be used as an accurate method for detecting residues. This study emphasizes the importance of monitoring Methylene Blue residues while opening opportunities for the development of alternative disease control methods that are safer, environmentally friendly, and comply with international standards. Based on this study, the recommended concentration for safe use without leaving significant residues is the lowest tested dose (MB A), which demonstrated effective disease control while minimizing tissue accumulation. However, further validation under commercial farming conditions is required before determining a definitive safe dosage.

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