RESEARCH ARTICLE

REVISED Effect of environmental factors on blood counts of *Gambusia affinis* caught at Brantas River watershed, Indonesia [version 2; peer review: 2 approved]

Previous Title: Effect of environmental factors on hematology profile of *Gambusia affinis* caught at Brantas River watershed, Indonesia

Asus Maizar Suryanto Hertika[1], Diana Arfiati[1], Evellin Dewi Lusiana[1], Renanda B.D.S. Putra[2]

[1]Water Resource Management, Universitas Brawijaya, Malang, East Java, 65144, Indonesia
[2]Aquacultue PSDKU, universitas Brawijaya, Kediri, East Java, 64111, Indonesia

v2 **First published:** 17 Nov 2021, **10**:1169
https://doi.org/10.12688/f1000research.74117.1
Latest published: 23 Mar 2022, **10**:1169
https://doi.org/10.12688/f1000research.74117.2

Abstract

Background: Contamination of freshwater ecosystems has become a major issue as it threatens public water sources as well as aquatic life. It is important to predict changes in organism health, given a known number of environmental factors and pollutant concentrations, in order to better manage contaminants through biomarker analysis. This study aims to examine the ecosystem health of the Brantas River based on its environmental condition and the hematology profile of *Gambusia affinis* fish present in the river. This species was chosen because of its wide distribution along the Brantas River, and because it is very tolerant, adaptable, highly abundant, and easy to catch.
Methods: The study area included 10 sampling sites along the Brantas River watershed. In total, six water quality parameters were observed (temperature, pH, dissolved oxygen (DO), biological oxygen demand (BOD), ammonia concentration, and phenol concentration) and hematology measurements consisted of erythrocyte, leucocyte, and micronuclei analyses.
Results: The results showed that the upstream area of Brantas River, located in Batu, was the least polluted region, while Mojokerto was the most polluted. The erythrocyte level of *Gambusia affinis* caught in most sampling sites was quite low. Furthermore, research revealed that the status of *Gambusia affinis*' hematological profile was significantly correlated ($p<0.05$) with water quality parameters, particularly DO, BOD, ammonia, and phenol.
Conclusions: It can be concluded from these results that the hematological profile of the fish is poor due to high levels of organic waste and harmful substances.

Open Peer Review

Approval Status ✓ ✓

	1	2
version 2 (revision) 23 Mar 2022	✓ view	✓ view
version 1 17 Nov 2021	? view	? view

1. **Akhmad Mukti**, Universitas Airlangga, East Java, Indonesia

2. **Mohammad Tamrin Mohamad Lal**, Universiti Malaysia Sabah, Kota Kinabalu, Malaysia

Any reports and responses or comments on the article can be found at the end of the article.

Keywords
biomarker, blood parameter, ecosystem health, water quality, river pollution

Corresponding author: Asus Maizar Suryanto Hertika (asusmaizar@ub.ac.id)

Author roles: Hertika AMS: Conceptualization, Data Curation, Methodology, Project Administration, Validation, Writing – Original Draft Preparation; **Arfiati D**: Data Curation, Formal Analysis, Supervision; **Lusiana ED**: Data Curation, Formal Analysis, Project Administration, Software, Writing – Review & Editing; **Putra RBDS**: Data Curation, Project Administration, Writing – Original Draft Preparation

Competing interests: No competing interests were disclosed.

Grant information: This work was supported by the Ministry of Research and Technology of Higher Education Indonesia through Penelitian Terapan Unggulan Perguruan Tinggi (PTUPT) number 267/SP2H/LT/DRPM/2021.
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

How to cite this article: Hertika AMS, Arfiati D, Lusiana ED and Putra RBDS. **Effect of environmental factors on blood counts of *Gambusia affinis* caught at Brantas River watershed, Indonesia [version 2; peer review: 2 approved]** F1000Research 2022, **10**:1169 https://doi.org/10.12688/f1000research.74117.2

First published: 17 Nov 2021, **10**:1169 https://doi.org/10.12688/f1000research.74117.1

REVISED **Amendments from Version 1**

The following manuscript have been revised according to reviewer suggestions. The title was changed for better comprehension to the content. In addition, some results and discussion as well as references were modified.

Any further responses from the reviewers can be found at the end of the article

Introduction

The contamination of freshwater ecosystems with a variety of pollutants has become a major concern in recent decades.[1,2] This is not only because of hazards to public water sources, but also because of the harm done to aquatic life.[3] Pollution from both point and non-point sources can have an impact on water quality in freshwater ecosystems.[4] Contamination from a single identifiable source, such as industrial effluents and wastewater treatment plants, is known as point source pollution.[5] Non-point sources include runoff linked to certain land use patterns, for example storm water and sewage outflows in urban areas, or fertilisers, pesticides and animal manure in agricultural or forested areas.[6] Changes in abiotic elements such as precipitation and temperature levels resulting from climate change can also impact the regular functions of aquatic ecosystems, including reproduction and feeding.[7]

Environmental contaminants can impair aquatic organisms' survival in a range of ways, including direct toxicity (both short- and long-term).[8] Usually, the effects are insidious, eventually affecting organism health.[9] Common laboratory metrics of growth, reproduction, and survival can be poor predictors of the myriad indirect stressor effects which exist in the field.[10] It is therefore necessary, given a known number of environmental factors and pollutant concentrations, to be able to forecast changes in ecosystem structure and function, as well as organism health in order to better manage contaminants.[8] Biomarker analysis of organisms gathered in the field is appropriate for this purpose because it can offer information on the state of the environment without the requirement for extrapolating laboratory results, which is associated with uncertainty.[11]

The use of particular biomarkers for assessing environmental quality and the health of fish in degraded environments has proven to be useful and widely-used.[12,13] Fish are commonly employed to assess the quality of the aquatic environment and are recognized as bio-indicators of pollution.[14,15] Fish live in close proximity to their environment and are thus vulnerable to physical and chemical alterations to it, which may manifest in components of their blood.[16] Hematological characteristics are an excellent diagnostic for physiological dysfunction, since their values exhibit genetic and physiological variations.[17–19] Genetic variation can be caused by both interspecific and intraspecific influences within species.[20] Moreover, differences between hematological values can be used to assess the links between these variables, and correlate them with the state of an organism's health in response to environmental conditions.[21,22] The species utilized in biomonitoring programs must be sensitive, and the biomarkers evaluated should provide a consistent response.[23]

The Brantas River, located in the East Java province of Indonesia, is the second largest river on Java Island.[24] It is prone to various types of contamination because of the extensive human activities along the course of the river: Java Island is the most populated region and largest industrial center in Indonesia.[25] The Brantas River is rich in aquatic biota, including *Gambusia affinis* which has frequently been used in biomonitoring activities in many previous studies.[11] This species was used in this study because it has a wide distribution along the course of the Brantas River,[26] and is also very tolerant, adaptable, abundant, and easy to catch.[11,27] Assessment of water quality in the Brantas River using common methods such as pollution indices and the Storet method have been performed in several preceding studies.[25,28,29] However, understanding of the ecosystem health of Brantas River, which is associated with the biological systems of aquatic biota, is still limited. Therefore, the purpose of this study is to evaluate the effect of physico-chemical water parameters on the blood counts of mosquito fish, Gambusia affinis in Brantas River.

Methods

Ethical considerations

Ethical approval was not needed for this research as the fish species used is highly abundant in the research area and not classified as an endangered species according to International Union for Conservation of Nature (IUCN) list. Moreover, according to the Regulation of the Indonesia Minister of Marine Affairs and Fisheries Number PER.17/MEN/2009 the fish species is not protected.

Study area

This research was carried out in the Brantas River. In total, ten sampling sites were chosen based on the geomorphological characteristics of the Brantas River watershed, as depicted in Figure 1. Each sampling site was divided into three carefully chosen sub-sites. Sampling was performed monthly between February and April of 2019.

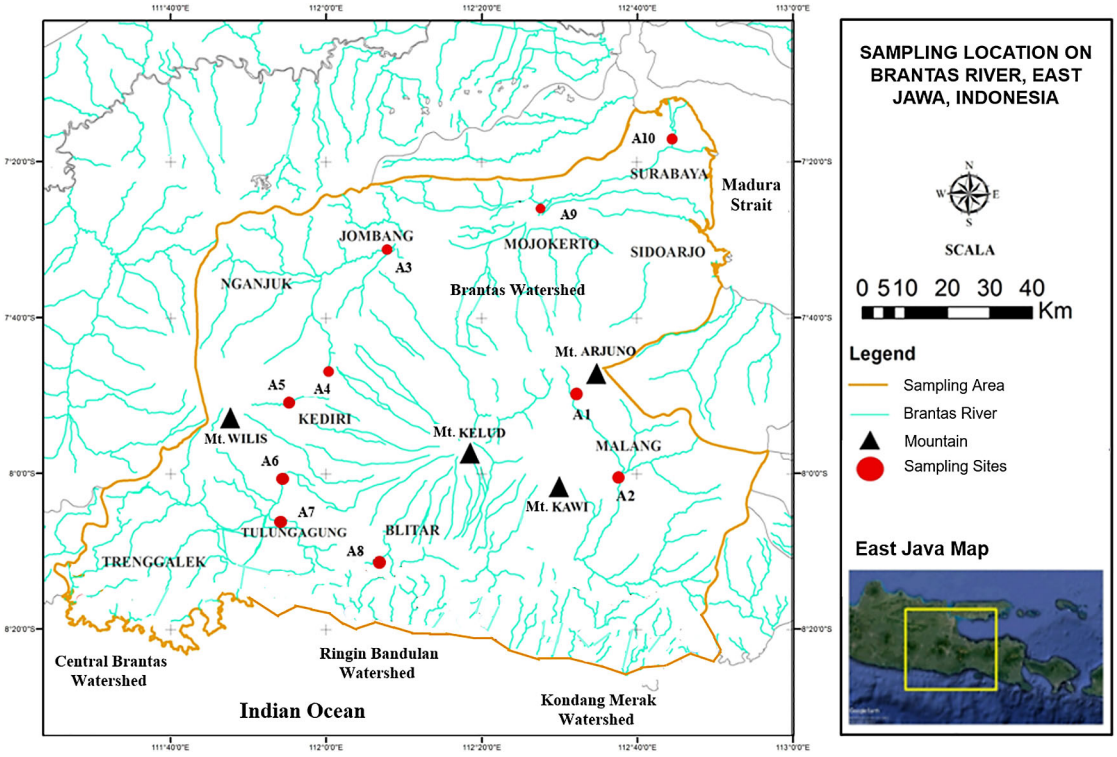

Figure 1. Sampling locations in the Brantas River watershed.

Water quality measurement

Several physico-chemical water quality parameters were measured in this research in order to assess the environmental condition of the study area. The parameters were temperature, pH, dissolved oxygen (DO), biological oxygen demand (BOD), ammonia (NH_3) concentration, and phenol concentration. The methods and instruments utilized for these measurements are presented in Table 1.

For BOD calculation, we measured the initial dissolved oxygen level at the beginning of sampling period, then re-measured it after incubation with a temperature of 20 ^{o}C for 5 days by using the Winkler method.[30,31] In the Winkler method or iodometric technique, divalent manganese solution is added to the solution in a glass-stopper bottle, followed by the addition of strong alkali. DO quickly oxidizes an equal quantity of distributed divalent manganese hydroxide precipitates to higher valence state hydroxides. When oxidized manganese is exposed to iodide ions in an acidic solution, it reverts to the divalent state, releasing the iodine equivalent of the initial DO concentration. The iodine is then titrated with a stranded thiosulfate solution. A starch indicator can be used to visually determine the titration end point.[30] The BOD value was the difference between the initial and final dissolved oxygen level. Moreover, phenol level was estimated using 4-aminoantipyrine method.[32] The phenolic compound was mixed with 4-aminoantipyrine along with alkaline oxidant at high solution pH, which formed a red quinone dye.[33]

Table 1. Methods and instruments used for water quality evaluation.

Parameter	Unit	Method/Instrument
Temperature	°C	Lutron PDO-520
pH	-	pH meter
Dissolved Oxygen (DO)	mg/L	Lutron PDO-520
Biological Oxygen Demand (BOD)	mg/L	Winkler method[30]
Ammonia (NH_3)	mg/L	Spectrophotometer Genesys 10S UV-Vis
Phenol	mg/L	4-Aminoantipyrine method[32]

Fish blood sample collection and hematology study

We took mosquito fish (*Gambusia affinis*) directly from the Brantas River from ten sampling sites. The fish were caught using nets on the banks of the river. The fish samples were in good health. The fish species includes the endemic fish of the Brantas River which are cosmopolitan (available at every station) and in large numbers. Each sampling site consisted of 3 sub-sites, and we took 3 fish at every sub-sites. Hence, we used a total of 90 fish in this study. The fish we took were adult fish with sizes ranging from 5 to 6 cm with a weight of 4-5 grams. The samples taken were female adult fish which have a larger body size than male fish. Therefore, the female adult fish had sufficient blood volume for hematology analysis.

Blood samples of *Gambusia affinis* were collected straight from the lateral line of the caudal fin. These samples consisted of roughly 90 µL of blood collected using a 1 mL syringe with 10 µL sodium citrate anticoagulant. This method of extracting blood from the fish was lethal. The samples were moved to a 1.5 mL Eppendorf flask and homogenized.[34] The homogenization was done by shaking the Eppendorf flask manually. After that, the samples were placed in a cool box with ice gels and promptly delivered to the Laboratory of Parasites and Fish Diseases, Faculty of Fisheries and Marine Sciences, Brawijaya University, Malang, Indonesia and immediately observed.

The hematological parameters measured in this study were the total values of erythrocytes, leucocytes, and micronuclei. A hemocytometer was used to quantify erythrocytes and leucocytes.[35,36] Micronuclei were measured in accordance with micronuclei test procedures.[37]

Data analysis

Data produced in this study were statistically analyzed, both descriptively and inferentially. For descriptive analysis, numerical measures (mean and standard deviation) and graphical technique (boxplot and scatter plot) were employed. For inferential analysis, one-way analysis of variance (ANOVA) and the Tukey test[38] were utilized to compare variables across sites, and Pearson correlation analysis[39] was used to analyze relationships between environmental conditions and hematological characteristics. For ANOVA, we also conducted assumption test such as normality (Shapiro-Wilk test) and homoscedasticity (Bartlett test). All of these analyses were performed using the statistical analysis software R (version 3.6.1).

Results and discussion

Water quality factors in Brantas River

Table 2 shows that the Batu and Malang sites had the lowest water temperatures, while water temperature in the other regions was higher. Batu is located at a high elevation in the upstream area of the Brantas River.[28,40] Malang is situated next to Batu, but has a high population density related to intensive residential development and industrial and agricultural activities.[41] Acidity levels at all sampling sites were considered normal, since the pH values recorded all lay within the recommended range of 6.5-8.5.[42] Fluctuations in pH values are influenced by the discharge of organic and inorganic waste into river bodies.[43] Very low pH conditions can result in the death of aquatic biota.[44]

DO values measured in Batu, Kediri, Tulungagung, and Blitar were higher than those measured in other regions. The lowest DO value was found in Mojokerto which significantly differed compared to other sites. Nevertheless, the results of DO measurement were all above 6 mg/L. The optimum DO for fish is generally greater than 6 mg/L.[45] If DO levels are not favourable, this will cause stress to fish because the brain will not be supplied with enough oxygen. This condition can cause death due to lack of oxygen (anoxia), as the fish body tissue will not be able to bind oxygen dissolved in the blood.[46] BOD measurement results contrasted with DO results. The lowest BOD level was found in Batu, and the highest in Mojokerto. Some BOD values (Tulungagung, Blitar, Mojokerto, Surabaya) were beyond the regulatory standard level (14 mg/L) set by the Indonesian Ministry of Environment.[47] An increase in BOD can disrupt biota, because it is accompanied by a decrease in DO.[48] This happens because of the disruption of the activity of bacteria that break down waste and foreign substances in the water.[49] As a result, polluted waters can be indicated by high BOD.[48]

Ammonia and phenol levels in the Brantas River watershed ranged from 0.45 to 1.11 mg/L and 0.65 to 1.14, respectively. The variation in concentration of these parameters was similar to that of BOD, in that Mojokerto had the highest value. According to the Indonesian Ministry of Environment, the maximum permittable value of ammonia is 0.5 mg/L and that of phenol is 0.001 mg/L. Therefore, the majority of ammonia measurements in the study area surpassed the standard, while all of the phenol values measured in this study were far beyond the standard. The high level of ammonia at this study sites affected by the land use along the Brantas river which primarily functioning as agricultural land, industry, and residential area. It is because ammonia could reach the aquatic ecosystems through direct sources such as municipal effluent discharges and animal excretion of nitrogenous wastes, as well as indirect sources such as nitrogen fixation, air deposition, and runoff from agricultural fields. Ammonia can diffuse through fish body tissues if the concentration is high, and it can potentially be toxic.[50] The level of ammonia toxicity is influenced by several factors, including fish

Table 2. Physicochemical water quality factor in Brantas River.

Site	Temperature (°C)	pH	DO (mg/L)	BOD (mg/L)	Ammonia (mg/L)	Phenol (mg/L)
Batu	23.52 ± 0.34^{g}	7.48 ± 0.08^{ab}	7.91 ± 0.25^{a}	8.17 ± 0.90^{g}	0.45 ± 0.07^{f}	0.65 ± 0.05^{c}
Malang	27.10 ± 1.01^{f}	7.38 ± 0.10^{abc}	6.46 ± 0.27^{cd}	10.34 ± 0.60^{f}	0.61 ± 0.04^{e}	0.77 ± 0.04^{bc}
Jombang	29.37 ± 1.10^{abc}	7.29 ± 0.07^{bcd}	6.38 ± 0.05^{cd}	11.94 ± 0.74^{e}	0.66 ± 0.04^{de}	0.71 ± 0.06^{bc}
Kediri1	28.44 ± 0.59^{cde}	6.87 ± 0.15^{f}	6.59 ± 0.22^{c}	12.41 ± 0.96^{de}	0.73 ± 0.03^{bcd}	0.80 ± 0.04^{b}
Kediri 2	30.22 ± 0.13^{a}	7.42 ± 0.10^{abc}	7.46 ± 0.07^{b}	13.33 ± 0.09^{cd}	0.78 ± 0.04^{bc}	0.77 ± 0.05^{bc}
Tulungagung 1	30.16 ± 0.34^{ab}	7.49 ± 0.05^{a}	7.32 ± 0.07^{b}	13.96 ± 0.32^{bc}	0.79 ± 0.03^{b}	0.76 ± 0.04^{bc}
Tulungagung 2	27.87 ± 0.15^{ef}	7.26 ± 0.16^{cd}	6.50 ± 0.34^{cd}	14.22 ± 0.32^{bc}	0.68 ± 0.07^{cde}	0.72 ± 0.07^{bc}
Blitar	28.19 ± 0.31^{def}	7.42 ± 0.13^{abc}	7.10 ± 0.29^{b}	14.23 ± 0.13^{bc}	0.49 ± 0.03^{f}	0.70 ± 0.04^{bc}
Mojokerto	29.08 ± 0.74^{bcd}	7.17 ± 0.10^{de}	6.12 ± 0.28^{d}	18.40 ± 0.85^{a}	1.11 ± 0.20^{a}	1.14 ± 0.24^{a}
Surabaya	29.73 ± 0.29^{ab}	6.98 ± 0.12^{ef}	6.68 ± 0.41^{c}	14.77 ± 0.27^{b}	0.66 ± 0.05^{de}	0.77 ± 0.04^{bc}

F1000Research 2022, 10:1169 Last updated: 04 APR 2022

species, level of exposure to ammonia, duration of exposure, and the effect of acclimatization. Certain fish species can accumulate high levels of ammonia in the brain or defense against ammonia toxicity by enhancing the effectiveness of ammonia excretion through active NH+4transport, manipulation of ambient pH, or reduction in ammonia permeability through the branchial and cutaneous epithelia.[51] Fish exposed to high levels of ammonia will experience damage to their gills and blood cells.[52] Erythrocyte levels will decrease as ammonia levels in the water increase.[53] Sources of phenol include waste discharged from various industries involving the production and disposal of coal, phenols, pharmaceuticals, resins, paints, textiles, leather, and petrochemicals.[54] Exposure of animals to phenol through inhalation causes liver damage, kidney damage, neurological effects, developmental effects, skin effects and even death.[55,56] The presence of phenol in excess of the water threshold can be a chemical stressor for aquatic organisms.[57]

The physico-chemical conditions of the Brantas River watershed indicate that the Batu region was the least polluted site, since the values of its water quality factors were better than the other sites. On the other hand, Mojokerto is the most polluted region, as half of its water quality parameters surpassed the standard values. One-way ANOVA and Tukey test results also suggested that the environmental states of the sampling sites, specifically Batu and Mojokerto, were significantly different, as none of the water variables in these regions shared the same letter notation.

Hematology profile of *Gambusia affinis* caught in the Brantas River

The hematology profile of fish is one of the early warning parameters that can be used to assess the level of fish health in relation to the aquatic environment.[18] If the environment is favorable, the blood parameters will also show normal conditions. Otherwise, the hematology profile of fish will show abnormal conditions when the environment is polluted.[58] Figure 2(a) is a box plot of the erythrocyte count of *Gambusia affinis* samples caught at each of the sampling sites. It shows that the highest erythrocyte level was found in Batu, with a median of around 1,300,000 cells/mm^3. Meanwhile, the erythrocyte levels of *Gambusia affinis* samples from Jombang, Kediri, Malang, and Tulungagung were recorded as slightly more than 1,000,000 cells/mm^3. Samples from both Blitar and Surabaya had erythrocyte levels slightly below 800,000 cells/mm^3. Lastly, the erythrocyte levels in samples obtained in Mojokerto were measured as around 400,000 cells/mm^3.

It is clearly visible in Figure 2(b) that a large number of sampling sites resulted in leucocyte values which ranged between 1,000,000 and 1,400,000 cells/mm^3. Leucocyte levels in *Gambusia affinis* caught in Mojokerto were significantly higher, at more than 1,800,000 cells/mm^3. Similarly, micronuclei levels in fish from this sampling site were around 25 cells/1000: this was the highest level found in this study (Figure 2(c)). Recorded micronuclei levels in *Gambusia affinis* from the remaining sites were a little above 10 cells/1000.

Erythrocytes are blood components which play a role in the process of transporting O_2 and CO_2 for respiration and in nutrient metabolism in fish.[59] When oxygen and iron levels in the cytoplasm increase, reactive oxygen in the form of O_2^- (superoxideanion), H_2O_2 (hydrogen perioxide) and OH (hydroxyl radical) are produced.[60] Erythrocytes are very sensitive to the oxidative processes of unsaturated fatty acids present in the membrane.[61] In fish and other aquatic animals, erythrocytes can be damaged by pollutants dissolved in the water.[62]

Leukocytes play an important part in the immune system. These cells help rid the body of foreign substances, including invading pathogens, through the immune response system.[63] Unhealthy fish will produce high levels of leukocytes in order to phagocytize bacteria and synthesize antibodies.[64] As fish try to defend themselves from poor environmental conditions by producing antibodies due to exposure to bacteria, leukocyte levels increase.[65]

Micronuclei are small extranuclear chromatins surrounded by a nuclear envelope containing DNA.[66] Micronuclei are formed during cell division at the anaphase stage when a chromosome fragment or part of a chromosome is not incorporated into a nucleus.[67] Micronucleus analysis performed on fish can be used to detect the clastogenic and aneugenic effects of genotoxic materials present in the waters.[68] Micronuclei analysis using blood cells is preferred. This is because blood cells are very sensitive, and because the kidneys, which are the main hematopoietic organs in fish, are easy to sample.[69] Micronuclei analysis is a method used to monitor aquatic pollutants with mutagenic properties. Fish can respond to mutagens present in aquatic habitats even at very low concentrations. These mutagens can lead to the formation of micronuclei in cells, with high levels of micronuclei formed in fish living in polluted waters[70]

In normal fish, the number of erythrocyte cells ranges from 1,050,000 to 3,000,000 cells/mm^3,[13] while the number of leucocyte cells ranges from 20,000-150,000 cells/mm^3.[71] Therefore, erythrocyte levels in *Gambusia affinis* caught in Blitar, Surabaya, and Mojokerto can be considered low. On the other hand, all sampling sites indicate an extremely high number of leucocytes in the *Gambusia affinis* samples. This may suggest the presence of health problems in fish due to pollution.[72] According to the field survey and,[26] these regions are dominated by industrial, agricultural and dense

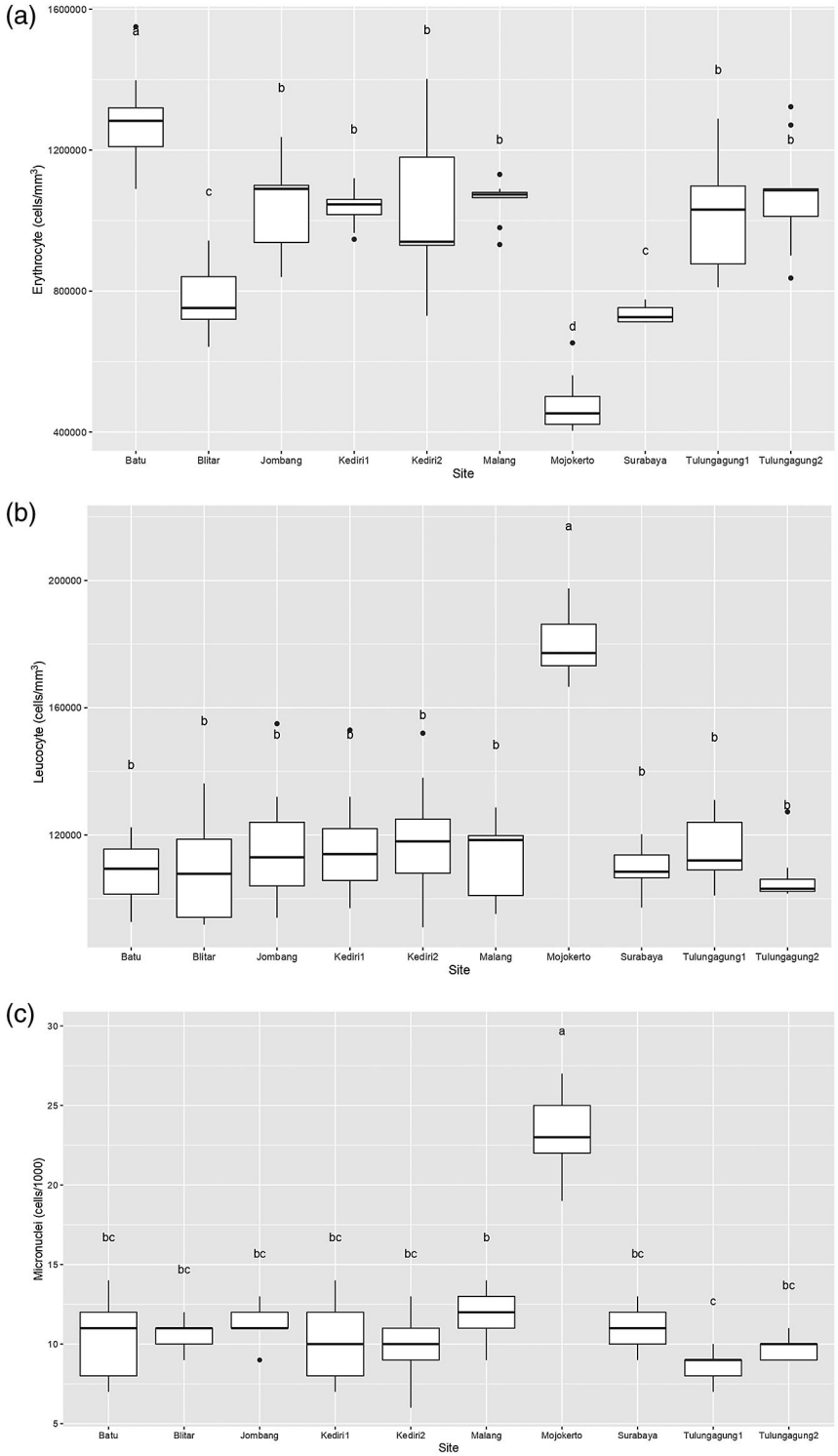

Figure 2. Boxplot of *Gambusia affinis*' hematology profile which caught in study area (a) Erythrocyte; (b) Leucocyte; (c) Micronuclei.

residential areas where domestic wastes from various anthropogenic activities are channeled into river bodies, resulting in low erythrocyte counts. Physical or chemical environmental changes will affect the blood components of fish. Exposure of fish to certain chemical compounds can reduce erythrocyte levels.[52] Furthermore, the fish sampled at these sites experienced health problems such as gill damage, and injuries around the body and tail. Decrease in erythrocyte levels can

be caused by deterioration of the gills disrupting their function, which has an impact on the ability of hemoglobin (Hb) to bind oxygen.[73] Reduced erythrocyte levels are also thought to be caused by anemia in fish. Anemia is a disorder which occurs in the blood tissue of fish. This disorder occurs as a result of the exposure of fish to chemical pollutants or heavy metals, resulting in dysfunction of the osmoregulatory organ. The supply of food to cells, tissues and organs will also be reduced, so that the metabolic processes of fish are hampered.[74,75] In addition, leukocyte and erythrocyte counts in fish are negatively correlated: the higher the number of erythrocytes, the lower the number of leukocytes.[76] Leukocytes help rid the body of foreign substances, including invading pathogens, through the immune response system. Unhealthy fish will produce more leukocytes in order to phagocytize bacteria and synthesize antibodies.[64]

Relationship analysis of hematology properties and water quality

All water quality parameters in this study were significantly correlated ($p < 0.05$) with erythrocyte levels in *Gambusia affinis* caught in the Brantas River watershed (Figure 3). Temperature, BOD, and ammonia and phenol concentrations were negatively correlated with erythrocyte levels, while pH and DO were positively correlated with erythrocyte levels. On the other hand, temperature and pH were not significantly correlated ($p > 0.05$) with leucocyte levels (Figure 4). DO was negatively correlated with leucocyte levels while ammonia and phenol concentrations along with BOD were positively correlated with leucocyte levels ($p < 0.05$). As shown in Figure 5, there was no significant correlation between micronuclei and water quality factors.

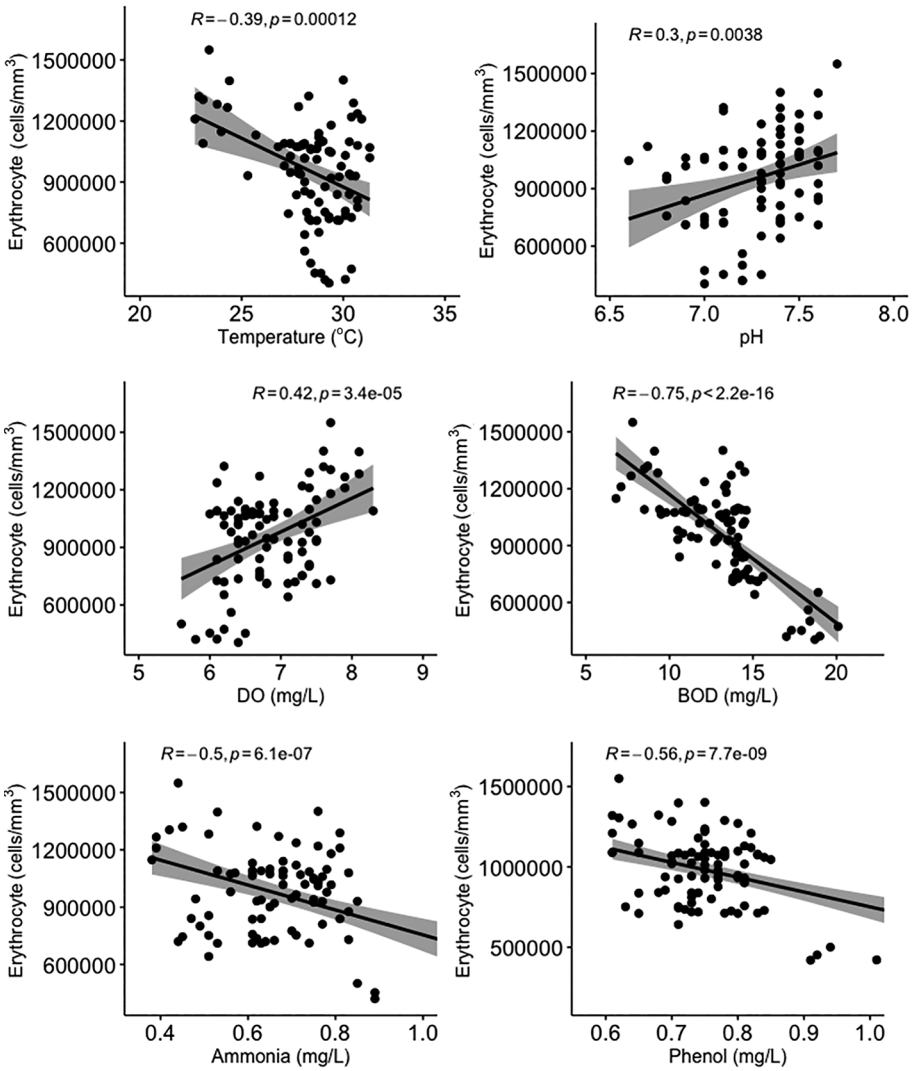

Figure 3. Scatter plot and Pearson correlation analysis between erythrocyte and water quality parameters. DO = dissolved oxygen; BOD = biological oxygen demand.

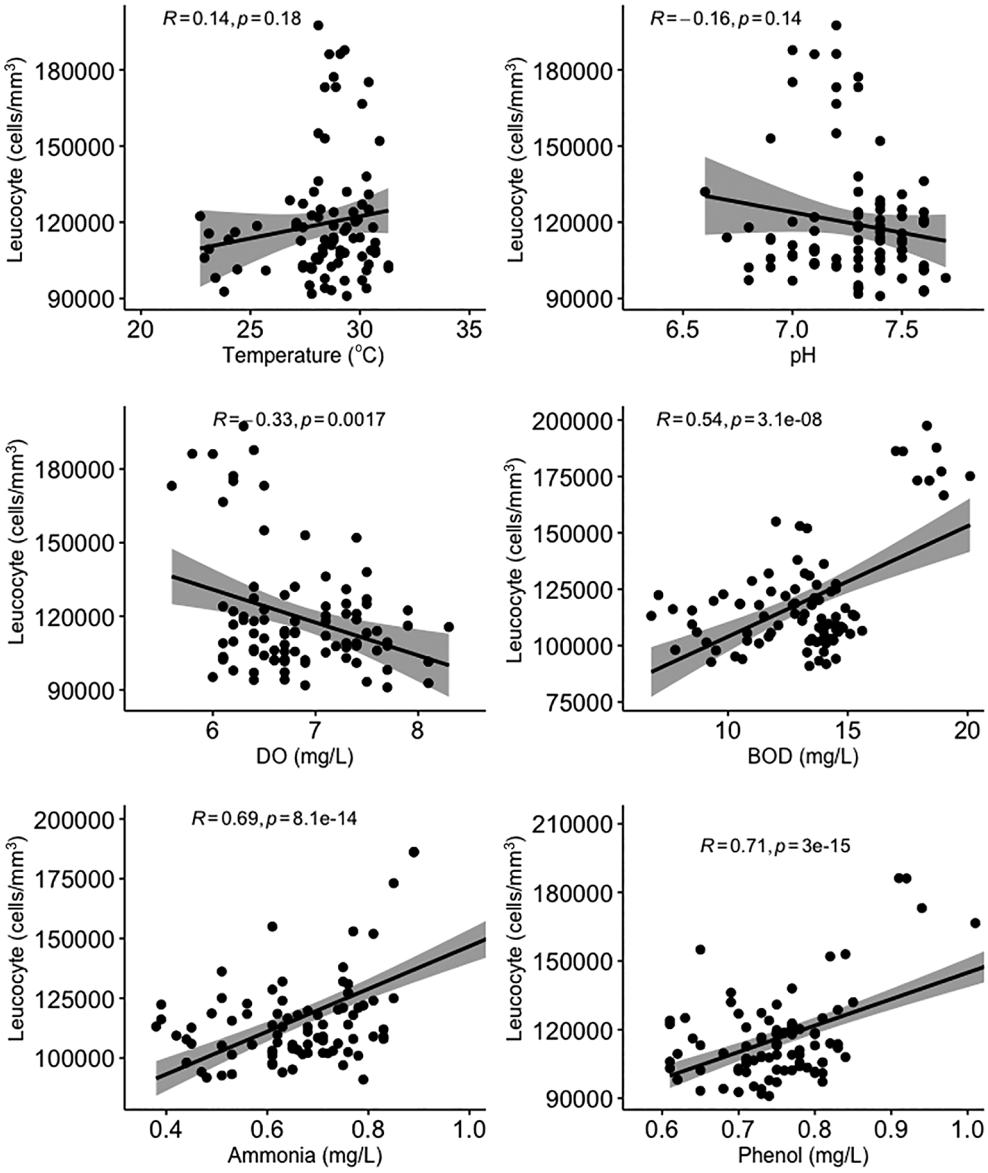

Figure 4. Scatter plot and Pearson correlation analysis between leucocyte and water quality parameters. DO = dissolved oxygen; BOD = biological oxygen demand.

Fish life is closely related to their habitat. Physical or chemical environmental changes will affect the blood components of fish.[77] Erythrocytes are very sensitive to the oxidative processes of unsaturated fatty acids present in the membrane.[61] Fish in poor health and fish in polluted environments have lower erythrocyte levels than healthy fish.[78] Stressed fish have increased leukocyte levels, as a reaction to variations in environmental circumstances or the presence of foreign substances.[19] However, the hematology profile of fish is not only caused by the environment but also by disease. Therefore, when taking fish blood, it is ensured that the sampled fish are in good health by showing normal morphological conditions and actively swimming in the water column.

Ammonia is the end product of organic and inorganic nitrogen metabolism. It can be toxic, and this can affect biota living in water.[79] Ammonia has a sub-lethal effect on fish, causing narrowing of the gill surface which results in a decrease in the speed of the gas exchange process in the gills.[80] Sub-lethal effects of ammonia can also include a decrease in the number of blood cells, a decrease in oxygen levels in the blood, reduced physical resistance and resistance to disease, and structural damage to various types of organs, including the liver parenchyma.[81] As a result, high levels of ammonia cause an increase in leucocyte levels and a decrease in erythrocyte levels.[53]

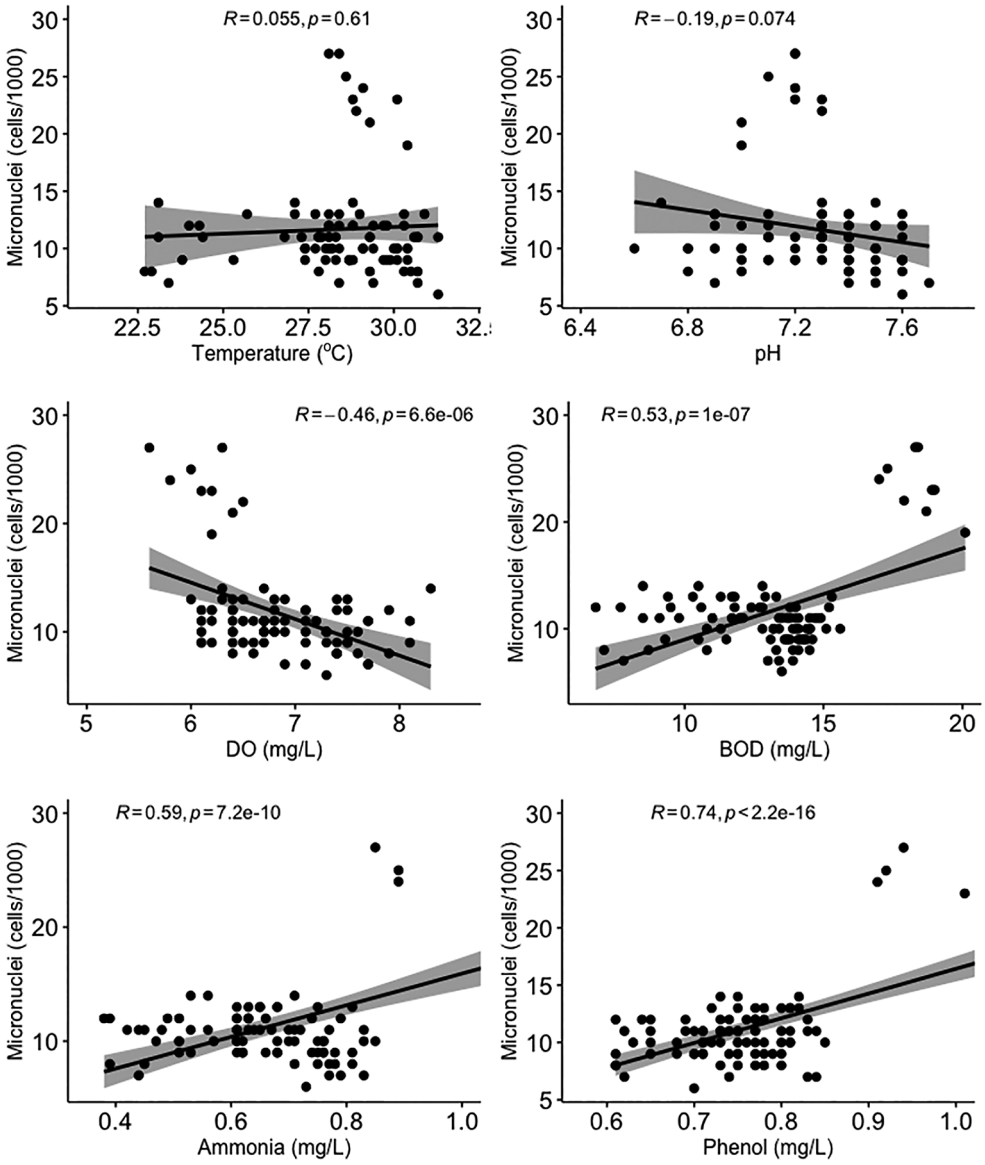

Figure 5. Scatter plot and Pearson correlation analysis between micronuclei and water quality parameters. DO = dissolved oxygen; BOD = biological oxygen demand.

BOD is commonly used to determine the level of water pollution and to trace the flow of pollution from upstream to estuary.[82] BOD is closely related to DO. As pollution increases in a body of water, BOD increases while DO decreases.[48] High BOD can cause problems in aquatic animals, for example paralysis in fish, because sufficient oxygen cannot be supplied to the brain.[83] This condition can also result in the death of fish due to lack of oxygen (anoxia), which is caused by the inability of fish body tissues to bind oxygen dissolved in the blood, which causes damage to blood cells.[84] High BOD can also affect micronuclei levels in fish.[85] This implies the existence of a toxic impact in which fish have been directly exposed to wastewater containing toxic substances where aneugenic and/or clastogenic activities are taking place. High micronuclei levels may indicate aneugenic and/or clastogenic activity associated with micronuclei formation due to poor spindle function of the chromosomes. Increases in micronuclei counts in fish can indicate an increase in wastewater levels in the river and this can cause environmental damage resulting in cytogenic damage to fish erythrocytes.[86]

Phenol has high toxicity in water and can cause damage to both aquatic ecosystems and human health. Phenol can damage blood cells, the central nervous system and organs such as the kidney and liver, cause low blood pressure, and potentially be lethal.[55] A decrease in the number of erythrocytes can indicate the occurrence of anemia, which can be triggered by the

entry of phenol into blood cells.[87] Phenol compounds have pathological effects which include gill necrosis, increased production of gill mucus, histological changes in the heart, liver, spleen and skin, and destruction of erythrocytes. This condition can also increase leucocytes in fish, which indicates poor health. In addition, high levels of phenol in the water can cause damage to the endocrine system, disrupt liver performance and have an impact on genotoxicity.[88] Genotoxic agents in fish are related to the frequency of pollution in the waters. Genotoxicity testing of the aquatic environment can be carried out using the micronuclei test. Micronuclei are formed from chromosomal fragments or entire chromosomes that are left behind during cell division due to lack of centromeres, defects in the centromere or defects during cytokinesis. In tissue division, the micronuclei test can determine the presence of clastogenic or aneugenic compounds.[89]

Conclusion

The Brantas River is one of the longest rivers on Java Island and is highly susceptible to pollution, especially as a result of anthropogenic activities. This study aimed to evaluate the ecosystem health of the Brantas River by linking physico-chemical conditions and the hematological profile of *Gambusia affinis* caught in the river. The results indicate that the upstream area of the Brantas River located in Batu is the least polluted stretch of the river and is in good condition in terms of water quality, while all other areas of the river were in poor condition, especially in Mojokerto. With regard to the hematological profile of the sampled fish species, the erythrocyte levels of *Gambusia affinis* caught in most of the sample sites were quite low, while the leucocyte and micronuclei levels were high. This indicates the presence of contaminants in the water which have triggered the immune systems of the fish. Further investigation showed that the hematological profile of the *Gambusia affinis* samples significantly correlated with the water quality parameters measured, especially DO, BOD, ammonia, and phenol. It can therefore be concluded that the unsatisfactory hematology profile of the fish may primarily be due to the presence of high levels of organic waste and toxic substances in the river.

Data availability

Figshare: Water quality parameters and hematology profile of Gambusia affinis caught at Brantas River watershed, Indonesia. https://doi.org/10.6084/m9.figshare.16895227.v1.[90]

Acknowledgements

We would like to show our gratitude to our colleagues for their comments on an earlier version of the manuscript.

References

1. Ali H, Khan E, Ilahi I: **Environmental Chemistry and Ecotoxicology of Hazardous Heavy Metals: Environmental Persistence, Toxicity, and Bioaccumulation.** Yang Y, editor. *J. Chem.* 2019; **2019**: 1–14.
Publisher Full Text

2. Bashir I, Lone FA, Bhat RA, *et al.*: **Concerns and Threats of Contamination on Aquatic Ecosystems.** Hakeem KR, Bhat RA, Qadri H, editors. *Bioremediation Biotechnol Sustain Approaches to Pollut Degrad.* 2020 Jan 27; 1–26.
Publisher Full Text | Reference Source

3. Campanale C, Massarelli C, Savino I, *et al.*: **A detailed review study on potential effects of microplastics and additives of concern on human health.** *Int. J. Environ. Res. Public Health.* 2020; **17**(4):
PubMed Abstract | Publisher Full Text | Free Full Text

4. Wu Y, Chen J: **Investigating the effects of point source and nonpoint source pollution on the water quality of the East River (Dongjiang) in South China.** *Ecol. Indic.* 2013; **32**: 294–304.
Publisher Full Text | Reference Source

5. Lapworth DJ, Baran N, Stuart ME, *et al.*: **Emerging organic contaminants in groundwater: A review of sources, fate and occurrence.** *Environ. Pollut.* 2012; **163**: 287–303.
PubMed Abstract | Publisher Full Text | Reference Source

6. Vadde KK, Wang J, Cao L, *et al.*: **Assessment of Water Quality and Identification of Pollution Risk Locations in Tiaoxi River (Taihu Watershed), China.** *Water.* 2018; **10**.
Publisher Full Text

7. Weiskopf SR, Rubenstein MA, Crozier LG, *et al.*: **Climate change effects on biodiversity, ecosystems, ecosystem services, and natural resource management in the United States.** *Sci. Total Environ.* 2020; **733**: 137782.
Publisher Full Text | Reference Source

8. Hook SE, Gallagher EP, Batley GE: **The role of biomarkers in the assessment of aquatic ecosystem health.** *Integr. Environ. Assess. Manag.* 2014/05/12. 2014 Jul; **10**(3): 327–341.
PubMed Abstract | Publisher Full Text | Free Full Text | Reference Source

9. Saaristo M, Brodin T, Balshine S, *et al.*: **Direct and indirect effects of chemical contaminants on the behaviour, ecology and evolution of wildlife.** *Proc. Biol. Sci.* 2018 Aug 22; **285**(1885): 20181297.
Reference Source

10. Connon RE, Beggel S, D'Abronzo LS, *et al.*: **Linking molecular biomarkers with higher level condition indicators to identify effects of copper exposures on the endangered delta smelt (Hypomesus transpacificus).** *Environ. Toxicol. Chem.* 2011 Feb 1; **30**(2): 290–300.
PubMed Abstract | Publisher Full Text

11. Huang G-Y, Liu Y-S, Liang Y-Q, *et al.*: **Multi-biomarker responses as indication of contaminant effects in Gambusia affinis from impacted rivers by municipal effluents.** *Sci. Total Environ.* 2016; **563-564**: 273–281.
Publisher Full Text | Reference Source

12. Dalzochio T, Rodrigues GZP, Petry IE, *et al.*: **The use of biomarkers to assess the health of aquatic ecosystems in Brazil: a review.** *Int Aquat Res.* 2016; **8**(4): 283–298.
Publisher Full Text

13. Hertika AMS, Supriatna DA, Nugroho BA, *et al.*: **The hematological profile of Barbonymus altus to evaluate water quality in the Badher bank conservation area, Blitar, East Java, Indonesia.** *Biodiversitas.* 2021; **22**(5): 2532–2540.
Publisher Full Text

14. van der Oost R, Beyer J, Vermeulen NPE: **Fish bioaccumulation and biomarkers in environmental risk assessment: a review.** *Environ. Toxicol. Pharmacol.* 2003; **13**(2): 57–149.
Publisher Full Text | PubMed Abstract | Reference Source

15. Linde-Arias AR, Inácio AF, Novo LA, *et al.*: **Multibiomarker approach in fish to assess the impact of pollution in a large Brazilian river, Paraiba do Sul.** *Environ. Pollut.* 2008; **156**(3): 974–979.
PubMed Abstract | Publisher Full Text

16. Olusanya HO, van Zyll de Jong M: **Assessing the vulnerability of freshwater fishes to climate change in Newfoundland and Labrador.** *PLoS One.* 2018 Dec 3; **13**(12): e0208182.
Publisher Full Text

17. Witeska M, Kondera E, Ługowska K, *et al.*: **Hematological methods in fish – Not only for beginners.** *Aquaculture.* 2022; **547**: 737498.
Reference Source

18. Grant KR: **Fish Hematology and Associated Disorders.** *Clin. Lab. Med.* 2015; **35**(3): 681–701.
PubMed Abstract | Publisher Full Text | Reference Source

19. Fazio F, Marafioti S, Arfuso F, *et al.*: **Comparative study of the biochemical and haematological parameters of four wild Tyrrhenian fish species.** *Vet Med (Praha).* 2013; **58**(11): 576–581.
Publisher Full Text

20. Moya-Laraño J: **Genetic variation, predator-prey interactions and food web structure.** *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* 2011 May 12; **366**(1569): 1425–1437.
PubMed Abstract | Publisher Full Text | Free Full Text | Reference Source

21. Ahmed I, Reshi QM, Fazio F: **The influence of the endogenous and exogenous factors on hematological parameters in different fish species: a review.** *Aquac. Int.* 2020; **28**(3): 869–899.
Publisher Full Text

22. Fazio F: **Fish hematology analysis as an important tool of aquaculture: A review.** *Aquaculture.* 2019; **500**: 237–242.
Publisher Full Text | Reference Source

23. Viarengo A, Lowe D, Bolognesi C, *et al.*: **The use of biomarkers in biomonitoring: A 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms.** *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* 2007; **146**(3): 281–300.
PubMed Abstract | Publisher Full Text | Reference Source

24. Hayati A, Tiantono N, Mirza MF, *et al.*: **Water quality and fish diversity in the Brantas River, East Java, Indonesia.** *Berk Penelit Hayati.* 2017 May 13; **22**(2 SE-Articles): 43–49.
Publisher Full Text | Reference Source

25. Roosmini D, Septiono MA, Putri NE, *et al.*: **River water pollution condition in upper part of Brantas River and Bengawan Solo River. IOP Conf Ser Earth.** *Environ. Sci.* 2018; **106**(1): 012059.
Publisher Full Text

26. Buwono NR, Risjani Y, Soegianto A: **The concentration of microplastic in water and fish (Gambusia affinis) collected from Brantas River.** *AIP Conf Proc.* 2021 May 25; **2353**(1): 30048.
Publisher Full Text

27. Adam MA, Maftuch M, Kilawati Y, *et al.*: **The effect of cadmium exposure on the cytoskeleton and morphology of the gill chloride cells in juvenile mosquito fish (Gambusia affinis).** *Egypt. J. Aquat. Res.* 2019; **45**(4): 337–343.
Publisher Full Text | Reference Source

28. Kustamar WLK: **the Pollution Index and Carrying Capacity.** *Int. J. Geomate.* 2020; **19**(73): 26–32.
Publisher Full Text

29. Sholichin M, Othman F, Limantara LM: **Use of Pi and Storet Methods To Evalute Water Quality Status of Brantas River.** *J. Math. Technol.* 2010; **1**(3): 116–124.

30. Bryan JR, Rlley JP, Williams PJL: **A winkler procedure for making precise measurements of oxygen concentration for productivity and related studies.** *J. Exp. Mar. Biol. Ecol.* 1976; **21**(3): 191–197.
Publisher Full Text | Reference Source

31. Daroni TA, Arisandi A: **Analisis BOD (Biological Oxygen Demand) Di Perairan Desa Prancak Kecamatan Sepulu, Bangkalan.** *J. Juv.* 2020; **1**(4): 558–566.

32. Ettinger M, Ruchhoft C, Lishka R: **Sensitive 4-Aminoantipyrine Method for Phenolic Compounds.** *Anal. Chem.* 1951 Dec 1; **23**(12): 1783–1788.
Publisher Full Text

33. Selvakumar PM: **Phenol Sensing Studies by 4-Aminoantipyrine Method-A Review.** *Org. Med. Chem. Int. J.* 2018; **5**(2).
Publisher Full Text

34. Arfiati D, Hertika AMS, Lukito DA, *et al.*: **Haematology Profile in Silver Barb (Barbonymus Gonionotus) Caught from Jagir River, Surabaya City, East Java, Indonesia. IOP Conf Ser Earth.** *Environ. Sci.* 2020; **416**(1).
Publisher Full Text

35. Priya KK, Ramesh M, Saravanan M, *et al.*: **Ecological risk assessment of silicon dioxide nanoparticles in a freshwater fish Labeo rohita: Hematology, ionoregulation and gill Na+/K+ ATPase activity.** *Ecotoxicol. Environ. Saf.* 2015; **120**: 295–302.
PubMed Abstract | Publisher Full Text | Reference Source

36. Nwani CD, Nwadinigwe AO, Joshua PE, *et al.*: **Hepatic antioxidant status and hematological parameters in african catfish, clarias gariepinus juvenile exposed to sublethal concentration of psychotria microphylla.** *J Anim Plant Sci.* 2016; **26**(1): 275–281.

37. Naqvi GEZ, Shoaib N, Ali AM: **Genotoxic potential of pesticides in the peripheral blood erythrocytes of fish (Oreochromis mossambicus).** *Pak. J. Zool.* 2016; **48**(6): 1643–1648.

38. Musa M, Lusiana ED, Buwono NR, *et al.*: **The effectiveness of silvofishery system in water treatment in intensive whiteleg shrimp (Litopenaeus vannamei) ponds, probolinggo district, East Java, Indonesia.** *Biodiversitas.* 2020; **21**(10): 4695–4701.
Publisher Full Text

39. Lusiana ED, Mahmudi M: *Teori dan Praktik Analisis Data Univariat dengan PAST.* Malang: UB Press; 2020; 216.

40. Widiatmono BR, Anugroho F, Nurlaelih EE, *et al.*: **Assessment of the Impacts of Land Use on Water Quality of Brantas Upstream, Batu City, Indonesia.** *J Enviromental Eng Sustain Technol.* 2017; **4**(1): 18–25.
Publisher Full Text

41. Buchori I, Sugiri A, Maryono M, *et al.*: **Theorizing spatial dynamics of metropolitan regions: A preliminary study in Java and Madura Islands, Indonesia.** *Sustain. Cities Soc.* 2017; **35**(August): 468–482.
Publisher Full Text

42. Rahmanian N, Ali SHB, Homayoonfard M, *et al.*: **Analysis of Physiochemical Parameters to Evaluate the Drinking Water Quality in the State of Perak, Malaysia.** Katsoyiannis A, editor. *J. Chem.* 2015; **2015**: 1–10.
Publisher Full Text

43. Feng Z, Su B, Xiao D-D, *et al.*: **Study on pH value and its variation characteristics of the main rivers into Dianchi lake under the anthropogenic and natural processes, Yunnan, China.** *J. Inf. Optim. Sci.* 2017 Oct 3; **38**(7): 1197–1210.
Publisher Full Text

44. Moiseenko TI: **Effects of acidification on aquatic ecosystems.** *Russ. J. Ecol.* 2005; **36**(2): 93–102.
Publisher Full Text

45. Franklin PA: **Dissolved oxygen criteria for freshwater fish in New Zealand: a revised approach.** *New Zeal J Mar Freshw Res.* 2014 Jan 2; **48**(1): 112–126.
Publisher Full Text

46. Hayes DS, Branco P, Santos JM, *et al.*: **Oxygen Depletion Affects Kinematics and Shoaling Cohesion of Cyprinid Fish.** *Water.* 2019; **11**.
Publisher Full Text

47. Ministry of Environment: *Peraturan Pemerintah Republik Indonesia Tentang Pengelolaan Kualitas Air Dan Pengendalian Pencemaran Air.* Indonesia: 2001.

48. Rizki N, Maslukah L, Sugianto DN, *et al.*: **Distribution of DO (Dissolved Oxygen) and BOD (Biological Oxygen Demand) in the Waters of Karimunjawa National Park using Two-Dimensional Model Approach. IOP Conf Ser Earth.** *Environ. Sci.* 2021; **750**(1): 012014.
Publisher Full Text

49. Susilowati S, Sutrisno J, Masykuri M, *et al.*: **Dynamics and factors that affects DO-BOD concentrations of Madiun River.** *AIP Conf Proc.* 2018 Dec 14; **2049**(1): 20052.
Publisher Full Text

50. Ip YK, Chew SF: **Ammonia production, excretion, toxicity, and defense in fish: a review.** *Front. Physiol.* 2010 Oct 4; **1**: 134.
Publisher Full Text | Reference Source

51. Randall DJ, Tsui TKN: **Ammonia toxicity in fish.** *Mar. Pollut. Bull.* 2002; **45**(1): 17–23.
PubMed Abstract | Publisher Full Text | Reference Source

52. Ishikawa NM, Ranzani-Paiva MJT, Lombardi JV, *et al.*: **Hematological parameters in nile tilápia, oreochromis niloticus exposed to sub-letal concentrations of mercury.** *Braz. Arch. Biol. Technol.* 2007; **50**(4): 619–626.
Publisher Full Text

53. Shin KW, Kim S-H, Kim J-H, *et al.*: **Toxic effects of ammonia exposure on growth performance, hematological parameters, and plasma components in rockfish, Sebastes schlegelii, during thermal stress.** *Fish. Aquat. Sci.* 2016; **19**(1): 44.
Publisher Full Text

54. Naguib DM, Badawy NM: **Phenol removal from wastewater using waste products.** *J. Environ. Chem. Eng.* 2020; **8**(1): 103592.
Publisher Full Text | Reference Source

55. Saha NC, Bhunia F, Kaviraj A: **Toxicity of Phenol to Fish and Aquatic Ecosystems.** *Bull. Environ. Contam. Toxicol.* 1999; **63**(2): 195–202.
PubMed Abstract | Publisher Full Text

56. Waskita Sari AH, Risjani Y, Mahendra APW: **Efek Konsentrasi Sublethal Fenol Terhadap Total Haemocyte Count (THC) dan Histologi Insang Kepiting Bakau (Scylla serata).** *J. Exp. Life Sci.* 2012; **2**(2): 82–88.
Publisher Full Text

57. Capolupo M, Sørensen L, Jayasena KDR, *et al.*: **Chemical composition and ecotoxicity of plastic and car tire rubber leachates to aquatic organisms.** *Water Res.* 2020; **169**: 115270.
PubMed Abstract | Publisher Full Text | Reference Source

58. Fazio F, Ferrantelli V, Fortino G, *et al.*: **The Influence of Acute Handling Stress on Some Blood Parameters in Cultured Sea Bream (Sparus Aurata Linnaeus, 1758).** *Ital J. Food Saf.* 2015 Mar 11; **4**(1): 4174.
PubMed Abstract | Publisher Full Text | Free Full Text | Reference Source

59. Nikinmaa M, Berenbrink M, Brauner CJ: **Regulation of erythrocyte function: Multiple evolutionary solutions for respiratory gas transport and its regulation in fish.** *Acta Physiol.* 2019 Oct 1; **227**(2): e13299.
PubMed Abstract | Publisher Full Text

60. Sharma P, Jha AB, Dubey RS, *et al.*: **Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions.** Polle A, editor. *J. Bot.* 2012; **2012**: 1–26.
Publisher Full Text

61. Maurya PK, Kumar P, Chandra P: **Biomarkers of oxidative stress in erythrocytes as a function of human age.** *World J. Methodol.* 2015 Dec 26; **5**(4): 216–222.
PubMed Abstract | Publisher Full Text | Free Full Text | Reference Source

62. Santos CA, Lenz D, Brandão GP, *et al.*: **Acute toxicity of the water-soluble fraction of diesel in Prochilodus vimboides Kner (characiformes: Prochilodontidae).** *Neotrop. Ichthyol.* 2013; **11**(1): 193–198.
Publisher Full Text

63. Rodrigues MV, Zanuzzo FS, Koch JFA, *et al.*: **Development of Fish Immunity and the Role of β-Glucan in Immune Responses.** *Molecules.* 2020 Nov 17; **25**(22): 5378.
PubMed Abstract | Publisher Full Text | Free Full Text | Reference Source

64. Rahman MM, Bae Kim H, Ja Baek H: **Changes in Blood Cell Morphology and Number of Red Spotted Grouper, Epinephelus akaara in Response to Thermal Stress.** *Dev. Reprod.* 2019/06/30. 2019 Jun; **23**(2): 139–148.
PubMed Abstract | Publisher Full Text | Free Full Text | Reference Source

65. Ángeles EM: **An Overview of the Immunological Defenses in Fish Skin.** Bensussan A, Puccetti P, Flaño E, Hayball JD, editors. *ISRN Immunol.* 2012; **2012**: 1–29.
Publisher Full Text

66. Maass KK, Rosing F, Ronchi P, *et al.*: **Altered nuclear envelope structure and proteasome function of micronuclei.** *Exp. Cell Res.* 2018; **371**(2): 353–363.
PubMed Abstract | Publisher Full Text | Reference Source

67. Beedanagari S: *4.11 - Genetic Toxicology.* Chackalamannil S, Rotella DWard SEBT-CMCIII, editors. Oxford: Elsevier; 2017; pp. 195–203.
Reference Source

68. Osman AGM: **Genotoxicity Tests and Their Contributions in Aquatic Environmental Research.** *J. Environ. Prot (Irvine, Calif).* 2014; **05**(14): 1391–1399.
Publisher Full Text

69. Luzhna L, Kathiria P, Kovalchuk O: **Micronuclei in genotoxicity assessment: from genetics to epigenetics and beyond.** *Front. Genet.* 2013 Jul; **4**(4): 131.
Publisher Full Text | Reference Source

70. Kousar S, Javed M: **Studies on Induction of Nuclear Abnormalities in Peripheral Blood Erythrocytes of Fish Exposed to Copper.** *Turk. J. Fish. Aquat. Sci.* 2015; **15**: 879–886.

71. Fauzan M: **The Influence Of The Level Of Lead (Pb) Exposure On The Blood Profiles Of Tilapia Fish (Oreochromis nilloticus).** *Jimvet.* 2017; **01**(4): 702–708.

72. Arkoosh MR, Casillas E, Clemons E, *et al.*: **Effect of Pollution on Fish Diseases: Potential Impacts on Salmonid Populations.** *J. Aquat. Anim. Health.* 1998 Jun 1; **10**(2): 182–190.
Publisher Full Text

73. Mohanty J, Nagababu E, Rifkind J: **Red blood cell oxidative stress impairs oxygen delivery and induces red blood cell aging.** *Front. Physiol.* 2014; **5**: 84.
Publisher Full Text

74. Seibel H, Baßmann B, Rebl A: **Blood Will Tell: What Hematological Analyses Can Reveal About Fish Welfare.** *Front. Vet. Sci.* 2021; **8**: 194.
Publisher Full Text

75. Galbraith ED, Le Mézo P, Solanes Hernandez G, *et al.*: **Growth Limitation of Marine Fish by Low Iron Availability in the Open Ocean.** *Front. Mar. Sci.* 2019; **6**: 509.
Publisher Full Text

76. Shen Y, Wang D, Zhao J, *et al.*: **Fish red blood cells express immune genes and responses.** *Aquac. Fish.* 2018; **3**(1): 14–21.
Publisher Full Text | Reference Source

77. Onyia LU, Ochokwu IJ, Edison ES: **Comparison of haematological indices, blood group and genotype of *Chrysicthys nigrodigtatus and Synodontis batensoda* from Geriyo Lake.** *J. Aquat. Sci.* 2020; **34**(1): 49–56.
Publisher Full Text

78. Corrêa SA d S, Abessa DM d S, dos Santos LG, *et al.*: **Differential blood counting in fish as a non-destructive biomarker of water contamination exposure.** *Toxicol. Environ. Chem.* 2017 Mar 16; **99**(3): 482–491.
Publisher Full Text

79. Robinson C, Tomek S, Schumaker R: **Test of Moderation Effects: Difference in Simple Slopes versus the Interaction Term.** *Mult Linear Regres Viewpoints.* 2013; **39**(16–24).

80. Beaumont MW, Butler PJ, Taylor EW: **Exposure of brown trout Salmo trutta to a sublethal concentration of copper in soft acidic water: effects upon gas exchange and ammonia accumulation.** *J. Exp. Biol.* 2003 Jan 1; **206**(1): 153–162.
Publisher Full Text

81. Imamuddin H: **Profil Perubahan Amonium, Nitrit Dan Nitrat Pada Percobaan Curah Dari Pdam Bogor Dan Surabaya.** *J. Teknol. Lingkung.* 2016; **11**(3): 443.
Publisher Full Text

82. Mocuba JJ: *Dissolved oxygen and biochemical oxygen demand in the waters close to the Quelimane sewage discharge.* Bergen, Norway: University of Bergen; 2010.

83. Jaishankar M, Tseten T, Anbalagan N, *et al.*: **Toxicity, mechanism and health effects of some heavy metals.** *Interdiscip. Toxicol.* 2014/11/15. 2014 Jun; **7**(2): 60–72.
PubMed Abstract | Publisher Full Text | Free Full Text | Reference Source

84. Abdel-Tawwab M, Monier MN, Hoseinifar SH, *et al.*: **Fish response to hypoxia stress: growth, physiological, and immunological biomarkers.** *Fish Physiol. Biochem.* 2019; **45**(3): 997–1013.
PubMed Abstract | Publisher Full Text

85. Serrano-García L, Montero-Montoya R: **Micronuclei and chromatid buds are the result of related genotoxic events.** *Environ. Mol. Mutagen.* 2001 Jan 1; **38**(1): 38–45.
PubMed Abstract | Publisher Full Text

86. Hoshina T, Takizawa K, Li J, *et al.*: **Domain Size Effect on Dielectric Properties of Barium Titanate Ceramics.** *Jpn. J. Appl. Phys.* 2008; **47**(9): 7607–7611.
Publisher Full Text

87. Tilak KS, Veeraiah K, Butchiram MS: **Effect of phenol on haematological components of Indian major carps Catla catla, Labeo rohita and Cirrhinus mrigala.** *J. Environ. Biol.* 2007; **28**(2): 177–179.
PubMed Abstract

88. Gad NS, Saad AS: **Effect of Environmental Pollution by Phenol on Some Physiological Parameters of Oreochromis niloticus.** *Glob Vet.* 2008; **2**(6): 312–319.

89. Baršienė J, Dedonytė V, Rybakovas A, *et al.*: **Investigation of micronuclei and other nuclear abnormalities in peripheral blood and kidney of marine fish treated with crude oil.** *Aquat. Toxicol.* 2006; **78**: S99–S104.
PubMed Abstract | Publisher Full Text | Reference Source

90. Suryanto AMH, Lusiana ED, Arfiati D, *et al.*: **Water quality parameters and hematology profile of Gambusia affinis caught at Brantas River watershed, Indonesia.** *figshare. Dataset.* 2021.
Publisher Full Text

Open Peer Review

Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 30 March 2022

https://doi.org/10.5256/f1000research.122571.r128615

✔ **Akhmad Mukti**
Department of Aquaculture, Faculty of Fisheries and Marine, Universitas Airlangga, East Java, Indonesia

I have reviewed and approved the revisions that have been made by the author in the manuscript

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Fish genetics, fish reproduction, an endocrine disorder, genotoxic, karyology, aquaculture biotechnology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 30 March 2022

https://doi.org/10.5256/f1000research.122571.r128616

✔ **Mohammad Tamrin Mohamad Lal**
Borneo Marine Research Institute, Universiti Malaysia Sabah, Kota Kinabalu, Malaysia

The manuscript was well revised. I have no further comment on this manuscript.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: aquaculture

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 16 February 2022

https://doi.org/10.5256/f1000research.77833.r121423

?

Mohammad Tamrin Mohamad Lal
Borneo Marine Research Institute, Universiti Malaysia Sabah, Kota Kinabalu, Malaysia

Overall, this manuscript is good. This paper can serve as a guideline for monitoring pollution in selected areas and can be used as a basis in other places. I recommend this paper for approval with further correction and clarification.

Specific Comments

The title: I would suggest the title to be change to "Effect of environmental factors on blood counts of mosquito fish, *Gambusia affinis* caught at Brantas River watershed, Indonesia" because your result only focused on the blood counts and no other haemtological parameters (SOD, hemoglobin counts, respiratory burst, phagocytic index, etc.)

Abstract: Well written abstract. However, please remove "because it is" the sentence "This species was chosen because of its wide distribution along the Brantas River, and because it is very tolerant, adaptable, highly abundant, and easy to catch", as it is a repetition.

Introduction

The aim/objective of this study is not in line with the title and the conclusion. I would like to suggest the objective of this paper to be changed to "To evaluate the effect of physico-chemical water parameters on the blood counts of mosquito fish, *Gambusia affinis* in Brantas River".

Methods

Some parts are not clear:

1. Put more details on the specific sampling time (morning, noon or evening).
2. What was the weather condition during the samplings?
3. How do you collect the water samples?
4. Is there any reason for not including the other water factors such as heavy metal concentration in this study?

5. Why don't you add male fish in this study? Is it that you won't be able to get the amount of blood needed? Since you did mention the fish is abundant in the area, therefore, the number of fish samples should not be a problem. Is there any reason for using small number of fish from each sampling location?
6. How do you do the blood sampling? Please put more detail on this (size of the needle, do you anaesthetize it? What method do you use to anaesthetize the fish? Do you refer to any specific method? Please cite it.
7. As for the blood analysis, is it a pooled samples from each sub-site? You should mention this.

Result and Discussion

What is the meaning of (90) in this sentence .... the other region was higher (90)...?

Delete "...discussed previously..."

In the paragraph 6 of Hematology profile of *Gambusia affinis*, authors mentioned the fish sampled in this site experiencing health problem. However, in the earlier method for fish sampling, only healthy fish were sampled and in paragraph 8. This is contradicting sentences I found in this paper.

Some references I found quite old. Probably the author can replace it with the newest reference.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: aquaculture

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have

significant reservations, as outlined above.

Author Response 17 Mar 2022

Asus Hertika, Lecturer at brawijaya university, Indonesia

Thank you for the comments from the reviewer of our article. In the following, we present clarifications as well as revisions to the manuscript that we have corrected

- Title: In this study, the haematology parameters were only measured on female fish, not all sexes. This must be clear.

At first, we have tried to take blood sample from male fish. However, due to its small size, we had difficulties to take the blood samples. Therefore, in this research we took blood sample from the female fish in which its size was 3-4 times bigger than the male and the blood sample was easier to be taken. Furthermore, we using small number of fish from each sampling site because of several reasons. First, at each sampling site was consisted of 3 sub-sites and we consider it was representative. Second, it was due to research ethics code.

- Abstract: Delete the words "and because it is"

Done

Methods - Things are not entirely clear.

- What is the sampling time? Is it the rainy or dry seasons and in the morning, afternoon, evening, and/or night for water quality and/or specimens?

The sampling was done during noon and rainy season.

- How many times to take? For each sampling, how many times is sampling done as a replication for data validity, for both water quality and specimens.

Sampling was replicated 3 times, every two weeks. AS each sampling site, we put samples from 3 sub-site.

- In the current study, these months include the rainy season, how about during the dry season? Data is needed here too.

We did not performed research during dry season. We plan for having sampling during dry season in the future.

- What about COD and other water qualities, including hormones (EDC)? These should be included too.

We did not measure these parameters. Thank you for the suggestion and we will consider these parameters for our nest research.

- How about nitrate/nitrite and heavy metals? Do these have no effect?

Jawab: *Nitrite and nitrate were not measured in this research, instead we measured ammonia. As for heavy metal, actually we also measured Hg, Pb and Cd. However, these variables were not presented in this manuscript, because it is being a part of another article which submitted in different journal.*

- What parameters are used as good health indicators? It would be better if the phenotypic or morphology performance of the fish was observed too.

To make sure the fish sampled were healthy, we observed their morphological features.

- The only fish observed were female: was it not possible to describe the entire population of fish (in terms of sex)?

At first, we have tried to take blood sample from male fish. However, due to its small size, we had difficulties to take the blood samples. Therefore, in this research we took blood sample from the female fish in which its size was 3-4 times bigger than the male and the blood sample was easier to be taken. Furthermore, we using small number of fish from each sampling site because of several reasons. First, at each sampling site was consisted of 3 sub-sites and we consider it was representative. Second, it was due to research ethics code.

- Is anesthesia done and what method was used when taking blood samples? This must be clear.

We did not anaesthetize the fish

- What about the other blood components? There should be measured data too.

We did not measure other blood components such Hb because the blood sample volume was not sufficient to perform this measurement.

- What about haemoglobin? This parameter is very important due to change of environmental conditions.

We did not measure other blood components such Hb because the blood sample volume was not sufficient to perform this measurement.

- What about haematocytes or monocyte and thrombocytes?

Thank you for the suggestion to measure those parameters. We will consider them for our future research.

Results and Discussion:

Water quality factors in Brantas River:

Table 2:

- 'While Malang is joint lowest with Batu, the gap between them is significant, and it is in fact closer in temperature to the other regions: explain why it is included with Batu.

We have tried to explain it in the manuscript.

- '...(90)...' what is this? A unit? à *Thank you for your correction.*

We believe that it is a writing error. We have deleted it from our manuscript

- 'DO values'....Check again the data as in Table 2, out of sync!

We have made correction, and add Tulungagung too as the region with high DO.

- While Mojokerto has the lowest DO levels, they are not significantly different from the other areas, please explain the significance further.

According to our data analysis, DO values in Mojokerto was the lowest and significantly different compared to other sites. It is because the notation letter of Tukey test for Mojokerto was not shared to other regions. In addition, the water quality condition of Mojokerto was explained altogether with other variables at the end of this section, which suggesting the poor condition of water quality in this site.

- 'Thus, the higher...' Correct the sentences.

We have made the correction.

- 'Therefore, the majority...' Why? What caused it? This should be explained.

W*e have added explanation for this in the manuscript.*

'The level of ammonia toxicity is influenced by several factors, including fish species...' How do the processes or mechanisms of fish species affect ammonia toxicity? This is not made clear.

W*e have added explanation for this in the manuscript.*

'Fish exposed to high levels ...' The sentences should be mentioned in the haematology results section.

We briefly described the effect of ammonia on fish which related to water quality discussion.

'The physico-chemical conditions of the Brantas River ...' This statement is not in accordance with the data obtained (Table 2) because it is based on data that ammonia and phenol in all locations relatively exceed the standard limits.

We explained that Batu as the least polluted region, since the values of its water quality factors were better than the other sites. Meanwhile, Mojokerto as the most polluted because the water quality was the poorest. We did not imply that other region were not polluted, since we emphasized on the pollution comparison between sites.

Hematology profile of Gambusia affinis caught in the Brantas River

- '... 25 cells/1000 or 10 cells/1000...' What is this (1000) unit?

It is indicate cells unit

- 'Erythrocytes are blood components which...' What about haemoglobin? This component plays a role very important too.

We did not measure other blood components such Hb because the blood sample volume was not sufficient to perform this measurement.

Relationship analysis of hematology properties and water quality
Relate and contrast the findings here ('Temperature, BOD, ...') with existing data, e.g. that found in Table 2 and Figure 2 (a and b).

The relationship between these table and figure has been explicitly represented by Figure 3-5.

'As a result, high levels of ammonia cause ...' Check again as the data in the current study, they appear out of sync.

We have checked the data, and as shown by Figure 3 and Figure 4, the statement is supported by our observed data.

- It would be better if the histopathological results were added in the study, including to know anoxia, as described.

Thank you for the suggestion. We will try to apply it for our next research.
'white blood cells ...' change to "leucocytes"

Done

- 'Genotoxicity testing of the aquatic ...' The sentences are more accurately mentioned in the Introduction section.

Thank you. We have added it in the manuscript.

Competing Interests: We have no competing interests

Reviewer Report 11 January 2022

https://doi.org/10.5256/f1000research.77833.r100540

?

Akhmad Mukti
Department of Aquaculture, Faculty of Fisheries and Marine, Universitas Airlangga, East Java, Indonesia

Title: In this study, the haematology parameters were only measured on female fish, not all sexes. This must be clear.

Abstract: Delete the words "and because it is"

Methods - Things are not entirely clear.

1. What is the sampling time? Is it the rainy or dry seasons and in the morning, afternoon, evening, and/or night for water quality and/or specimens?
2. How many times to take? For each sampling, how many times is sampling done as a replication for data validity, for both water quality and specimens.
3. In the current study, these months include the rainy season, how about during the dry season? Data is needed here too.
4. What about COD and other water qualities, including hormones (EDC)? These should be included too.
5. How about nitrate/nitrite and heavy metals? Do these have no effect?
6. What parameters are used as good health indicators? It would be better if the phenotypic or morphology performance of the fish was observed too.
7. The only fish observed were female: was it not possible to describe the entire population of fish (in terms of sex)?
8. Is anesthesia done and what method was used when taking blood samples? This must be clear.
9. What about the other blood components? There should be measured data too.
10. What about haemoglobin? This parameter is very important due to change of

environmental conditions.

11. What about haematocytes or monocyte and thrombocytes?

Results and Discussion:

Water quality factors in Brantas River:

Table 2:

- 'While Malang is joint lowest with Batu, the gap between them is significant, and it is in fact closer in temperature to the other regions: explain why it is included with Batu.
- '...(90)...' what is this? A unit?
- 'DO values'....Check again the data as in Table 2, out of sync!
- While Mojokerto has the lowest DO levels, they are not significantly different from the other areas, please explain the significance further.

Furthermore:

1. 'Thus, the higher...' Correct the sentences.
2. 'Therefore, the majority...' Why? What caused it? This should be explained.
3. 'The level of ammonia toxicity is influenced by several factors, including fish species...' How do the processes or mechanisms of fish species affect ammonia toxicity? This is not made clear.
4. 'Fish exposed to high levels ...' The sentences should be mentioned in the haematology results section.
5. 'The physico-chemical conditions of the Brantas River ...' This statement is not in accordance with the data obtained (Table 2) because it is based on data that ammonia and phenol in all locations relatively exceed the standard limits.

Hematology profile of Gambusia affinis *caught in the Brantas River*

1. '... 25 cells/1000 or 10 cells/1000...' What is this (1000) unit?
2. 'Erythrocytes are blood components which...' What about haemoglobin? This component plays a role very important too.

Relationship analysis of hematology properties and water quality

1. Relate and contrast the findings here ('Temperature, BOD, ...') with existing data, e.g. that found in Table 2 and Figure 2 (a and b).
2. 'As a result, high levels of ammonia cause ...' Check again as the data in the current study, they appear out of sync.
3. It would be better if the histopathological results were added in the study, including to know anoxia, as described.
4. 'white blood cells ...' change to "leucocytes".
5. 'Genotoxicity testing of the aquatic ...' The sentences are more accurately mentioned in the Introduction section.

Please also see annotated manuscript for further comments, as well as further context for points raised here.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Fish genetics, fish reproduction, an endocrine disorder, genotoxic, karyology, aquaculture biotechnology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 17 Mar 2022

Asus Hertika, Lecturer at brawijaya university, Indonesia

Thank you for the comments from the reviewer of our article. In the following, we present clarifications as well as revisions to the manuscript that we have corrected

Specific Comments

- The title: I would suggest the title to be change to "Effect of environmental factors on blood counts of mosquito fish, Gambusia affinis caught at Brantas River watershed, Indonesia" because your result only focused on the blood counts and no other haemtological parameters (SOD, hemoglobin counts, respiratory burst, phagocytic index, etc.)

The title has changed accordingly

- Abstract: Well written abstract. However, please remove "because it is" the sentence "This species was chosen because of its wide distribution along the Brantas River, and because it is very tolerant, adaptable, highly abundant, and easy to catch", as it is a repetition.

W*e have made change to the abstract. Thank you for the correction.*

Introduction

- The aim/objective of this study is not in line with the title and the conclusion. I would like to suggest the objective of this paper to be changed to "To evaluate the effect of physico-chemical water parameters on the blood counts of mosquito fish, Gambusia affinis in Brantas River".

Thank you for the suggestion. We have changed the study purpose as suggested

Methods

Some parts are not clear:

- Put more details on the specific sampling time (morning, noon or evening).

The sampling was conducted at noon

- What was the weather condition during the samplings?

The weather was rainy

- How do you collect the water samples?

Temperature, pH, and DO were measured in-situ. Meanwhile, water samples to measure the rest parameters were taken as much as 1 liter, then put to the cold box. Afterward, the samples were analyzed at laboratory.

- Is there any reason for not including the other water factors such as heavy metal concentration in this study?

In this research, we also measured heavy metals such as Hg, Cd, and Pb. However, the results were not presented in this manuscript. It is because they are part of another article which is being submitted to other journal.

- Why don't you add male fish in this study? Is it that you won't be able to get the amount of blood needed? Since you did mention the fish is abundant in the area, therefore, the number of fish samples should not be a problem. Is there any reason for using small number of fish from each sampling location? à *we chose only female fish because it has larger body size than male.*

At first, we have tried to take blood sample from male fish. However, due to its small size, we had difficulties to take the blood samples. Therefore, in this research we took blood sample from the female fish in which its size was 3-4 times bigger than the male and the blood sample was easier to be taken. Furthermore, we using small number of fish from each sampling site because of several reasons. First, at each sampling site was consisted of 3 sub-sites and we consider it was representative. Second, it was due to research ethics code.

- How do you do the blood sampling? Please put more detail on this (size of the needle, do you anaesthetize it? What method do you use to anaesthetize the fish? Do you refer to any specific method? Please cite it.

The blood sampling method is explained in Fish Blood Sample Collection and Hematology Study. In addition, we did not anaesthetize the fish.

- As for the blood analysis, is it a pooled samples from each sub-site? You should mention this.

Yes, it is true that the samples were pooled from each sub-site. We have added this to the

manuscript

Result and Discussion

- What is the meaning of (90) in this sentence .... the other region was higher (90)...?

Thank you for your correction. We believe that it is a writing error. We have deleted it from our manuscript
Delete "...discussed previously..."

D*one*

- In the paragraph 6 of Hematology profile of Gambusia affinis, authors mentioned the fish sampled in this site experiencing health problem. However, in the earlier method for fish sampling, only healthy fish were sampled and in paragraph 8. This is contradicting sentences I found in this paper.

As mentioned in our methodology, we chose health fish in accordance to its appearance. However, since the leucocyte and erythrocyte count of the sampled fish were quite low, then it might indicate the presence of fish' health problem internally that probably did not visible appearance wise.

- Some references I found quite old. Probably the author can replace it with the newest reference.

W*e have replaced the reference before 2000 with the updated ones.*

Competing Interests: We Have no Competing interests