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Gonadal microplastic-like particle occurrence and gamete quality of *Cyprinus carpio* along the Brantas River Basin gradient, East Java, Indonesia

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Abstract. *Residiwati G, Sabila A, Wiguna IA, Bello U, Budiono, Aulanni'am A, Raharjo DKWP, Sidi S, Lesmana MA, Hartono D, Hendrawan VF, Agustina GC, Tuska HSA. 2026. Gonadal microplastic-like particle occurrence and gamete quality of* Cyprinus carpio *along the Brantas River Basin gradient, East Java, Indonesia. Biodiversitas 27 (5): d270529. https://doi.org/10.13057/biodiv/d270529.* Microplastic contamination is an emerging concern in freshwater ecosystems because of its potential to impair fish reproductive performance. This field-based comparative study evaluated site-level patterns of environmental particle occurrence, gonadal occurrence of microplastic-like particles, and gamete quality in *Cyprinus carpio* collected from four locations along the Brantas River Basin, East Java, Indonesia, during May-July 2025. A total of 32 males and 32 females were examined, with 8 males and 8 females obtained from each site through four sampling replicates, in which two fish of each sex were collected per replicate. Water particle occurrence was derived from one processed sample per site and was therefore interpreted descriptively, whereas gonadal tissues were examined for morphologically identified microplastic-like particles. Reproductive quality was assessed through sperm motility, viability, morphological abnormalities, and DNA integrity, as well as oocyte diameter, survival rate, and germinal vesicle breakdown (GVBD). Microplastic-like particles were detected at all sites in both water and gonadal tissues, with higher occurrence at downstream locations than at the uppermost site. This pattern was accompanied by poorer reproductive parameters. In males, downstream fish showed lower sperm motility and viability together with higher morphological abnormalities and greater DNA damage. In females, downstream fish exhibited smaller oocyte diameter, lower survival rate, and reduced GVBD. One-way ANOVA indicated significant differences among sampling sites for all reproductive endpoints ($p<0.001$). Overall, higher site-level particle occurrence co-occurred with poorer gamete quality under field conditions. These results provide comparative field-based evidence that reproductive condition in *C. carpio* varied systematically along a site gradient in the Brantas River Basin.

Keywords: Brantas River Basin, *Cyprinus carpio*, gamete quality, microplastic-like particles, reproductive endpoints

INTRODUCTION

Global plastic production has increased rapidly since the early 20th century and reached more than 414 million tons in 2023 (de Lena et al. 2025). Although plastic has economic value due to its lightweight, low cost, and durability, its low recycling rate has led to the accumulation of plastic waste in the environment (Li et al. 2025a). In Indonesia, plastic waste is the second-largest contributor after food waste, accounting for 19.71% of total waste generation in 2024 (Soegianto et al. 2025). As larger plastic debris fragments into particles measuring ≤5 mm, microplastics become persistent contaminants that may adsorb hazardous chemicals and remain in aquatic systems for prolonged periods (Jaiswal et al. 2025; Mutlu et al. 2025). Since rivers function as major transport pathways for land-based waste and plastic degradation products, freshwater systems are increasingly recognized as important compartments of microplastic contamination.

The Brantas River is one of the major freshwater systems in East Java and passes through several reservoirs, including Karangkates, Sengguruh, and Selorejo (Sujono 2019). Domestic, industrial, and mining activities along the river contribute to the plastic pollution load entering the watershed (Maulidah et al. 2023). In addition, pollution pressure in the Brantas region has also been reflected in public health concerns in Malang, where ISPA cases remained high over four consecutive years, reaching 1,826 cases in 2019, 1,761 cases in 2020, 1,138 cases in 2021, and 862 cases in 2022, indicating a persistent burden of environmentally associated exposure in the broader watershed context (ECOTON 2023). Previous studies have reported microplastic abundance of up to 8,570 particles/m³ in the Brantas River, and contamination has also been documented in fish inhabiting this system (Faqih et al. 2021). These findings indicate that the Brantas River Basin provides an ecologically relevant setting for examining how site-level particle occurrence may co-occur with biological responses in freshwater organisms.

Cyprinus carpio is one of freshwater fish commonly found and consumed by communities surrounding the Brantas watershed (Andriyono and Fitrani 2021). Its omnivorous feeding habit further supports its relevance for this study, as it may increase contact with suspended particles and contaminated food items in the aquatic environment. Its ecological relevance, local availability, and food importance make this species suitable for evaluating reproductive condition under field settings. Male reproductive condition is particularly important since gamete quality plays a central role in determining fertilization success and, ultimately, recruitment potential in natural populations (Valdebenito et al. 2015; Zadmajid et al. 2019). In externally fertilizing fish, a single male may release sperm capable of fertilizing many oocytes during spawning; therefore, impaired sperm quality may influence reproductive output beyond the individual level (Browne et al. 2015). Poor gamete quality, indicated by reduced motility or viability, increased morphological abnormalities, or compromised DNA integrity, may reduce fertilization success and, over time, species persistence when reproductive performance is consistently impaired (Tuska et al. 2025). More broadly, microplastic-associated reproductive toxicity in fish has been linked to oxidative stress, altered steroidogenesis, gonadal cell apoptosis, and disruption of the hypothalamus-pituitary-gonadal axis, ultimately affecting fertility (Bhat et al. 2024; Hasan et al. 2024; Ghosh 2025). However, no study has specifically examined male and female gamete quality of *C. carpio* in the Brantas River.

The novelty of the present study therefore lies not merely in the study area, but in the integration of three levels of observation within a single freshwater field gradient: environmental particle occurrence in water, occurrence of microplastic-like particles in gonadal tissues, and sex-specific gamete-quality endpoints in both males and females. Accordingly, this study was designed as a field-based comparative study across a site gradient in the Brantas River Basin. The objective was to examine whether sites with higher environmental particle occurrence also showed greater gonadal occurrence of microplastic-like particles and poorer reproductive quality in male and female *C. carpio*. Specifically, the study evaluated site-related variation in sperm morphology abnormalities, motility, viability, and DNA integrity, as well as oocyte diameter, GVBD, and oocyte survival rate. By focusing on reproductive endpoints under field conditions, this study aimed to provide comparative evidence of site-gradient co-occurrence patterns between particle occurrence and reproductive condition in an ecologically and locally important freshwater fish species.

MATERIALS AND METHODS

Study area

This study was conducted in the Brantas River watershed, East Java, Indonesia, a major freshwater system subjected to anthropogenic pressures from domestic, agricultural, and industrial activities. Fish sampling was carried out in the morning from May to July 2025 to maintain a consistent sampling time across sites and sampling events. Four sampling locations were selected along an upstream-downstream gradient: the upper Brantas Sector, Selorejo Reservoir, Sengguruh Reservoir, and Karangkates Reservoir. The purpose of this selection was to capture a comparative field gradient of sites differing in upstream-downstream position and in the visible intensity of surrounding human activity.

Within the present study design, the upper Brantas Sector was treated as the comparatively less disturbed upstream site rather than as a fully validated reference site, because supporting environmental variables such as water quality, hydrology, and sediment characteristics were not measured directly. Fish representing this upstream category were not collected directly from Brantas Headwater Site (Titik Nol Sungai Brantas), since immediate headwater area is not a suitable habitat for *Cyprinus carpio*. Instead, samples were obtained from the nearest floating-cage site in the upper Brantas area, which was used to represent the upstream sector within the study gradient.

The upper Brantas sector is located in the uppermost section of the watershed and was considered the least impacted site in the present study area based on its upstream position and lower observed human activity. Selorejo Reservoir is situated downstream of mixed agricultural land and rural settlements. Sengguruh Reservoir lies further downstream in an area with higher population density and broader anthropogenic activity. Karangkates Reservoir, the most downstream site, represents the furthest point in the present sampling gradient, where cumulative upstream inputs may converge and where hydrological conditions may favor particle retention. Taken together, these four locations provided an ecologically meaningful spatial gradient for comparing site-level particle occurrence and reproductive parameters in *C. carpio*. Geographic coordinates at each site were recorded using a handheld GPS device to ensure spatial accuracy and reproducibility of sampling locations (Figure 1, Table 1).

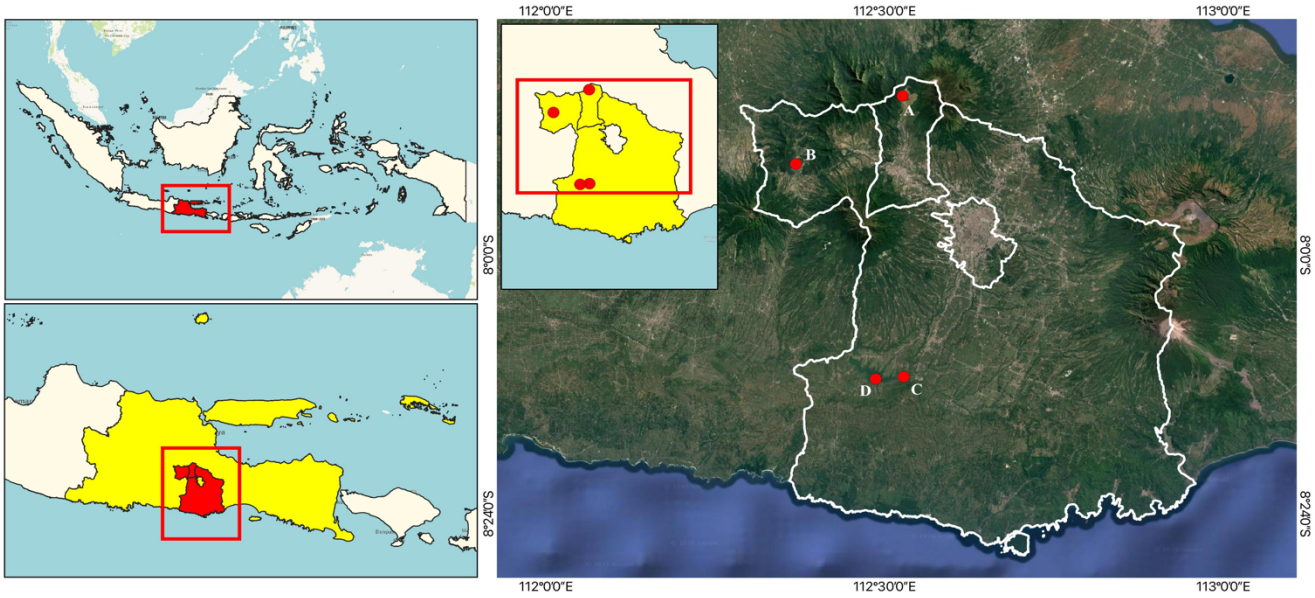

Figure 1. Map of sampling sites in the Brantas River watershed, East Java, Indonesia. A. Upper Brantas Sector, B. Selorejo Reservoir, C. Sengguruh Reservoir, D. Karangkates Reservoir

Table 1. Geographic coordinates and hydrological position of sampling sites in the Brantas River watershed, Indonesia

Sampling site	Hydrological position	Latitude (S)	Longitude (E)
Upper Brantas Sector	Uppermost upstream site	7.7540°S	112.5265°E
Selorejo Reservoir	Upstream	7.8593°S	112.3689°E
Sengguruh Reservoir	Midstream	8.1881°S	112.5281°E
Karangkates Reservoir	Downstream	8.1915°S	112.4867°E

Note: Geographic coordinates are presented in decimal degrees (WGS84 coordinate system). Hydrological positions indicate the relative position of each sampling site along the watershed gradient

Sampling design

Fish were collected by purposive sampling, with selection restricted to externally and anatomically healthy male and female *C. carpio* weighing 80-300 g. A total of 64 fish were examined, consisting of 32 males and 32 females. At each sampling site, 8 males and 8 females were collected across four sampling occasions during the May-July 2025 study period, with two males and two females obtained during each occasion. These repeated sampling occasions were used to distribute field collection over time during the spawning season and to improve consistency of sample acquisition under field conditions; however, they were not treated as separate statistical units. For subsequent reproductive analyses, the individual fish remained the analytical unit, whereas sperm cells and oocytes were treated as subsamples nested within each fish. The selected period coincided with the spawning season and was intended to increase the likelihood of obtaining reproductively active fish with macroscopically developed gonads. Seasonal rainfall may have influenced suspended particle dynamics within the watershed; however, rainfall and hydrological variables were not measured directly and were therefore not included as explanatory variables in the present analysis. Fish were captured using a 0.5 cm mesh net and transported in 15-L aerated containers. Euthanasia was performed by immersion in 2-phenoxyethanol (0.5 mL/L). After loss of reflexes, including cessation of opercular movement, pithing was carried out to confirm death. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) under ethical approval number 41-KEP-FKHUB-2025.

Microplastic assessment procedures in gonadal and water samples

Microplastic analysis in gonadal samples

Gonadal organs from each individual fish were placed into glass beakers and submerged completely in 10% potassium hydroxide (KOH) solution (Geppner et al. 2023). The whole gonadal organ from each fish was used for digestion. Although gonads were processed individually, the particle data presented in this manuscript were summarized as site-level occurrence data rather than retained and analyzed as individual fish-level burden values. Accordingly, these data are suitable for describing comparative occurrence patterns among sites, but they do not support direct fish-level association testing between gonadal particle counts and reproductive endpoints. Samples were incubated in an oven at 40°C for approximately 24 h to facilitate tissue degradation. The digested solution was then filtered using Whatman No. 42 filter paper with a particle retention of approximately 2.5 µm. The filter paper was allowed to dry at room temperature for several days until fully dehydrated. After filtration, the surface of the filter was gently rinsed with 0.9% sodium chloride (NaCl) solution to help disperse particles retained within the filter matrix and to remove residual digestion solution. The filters were subsequently dried again before microscopic examination. Particles were

examined directly on the filter surface using light microscopy (Hove et al. 2023). This approach was chosen to minimize particle loss during transfer and to allow visual inspection of all retained material on the filter. Suspected particles were evaluated using commonly applied visual criteria based on morphology, including shape, color, and surface texture, and were categorized as fibers, fragments, or films (Yasmin et al. 2024). No Fourier-transform infrared (FTIR) spectroscopy or Raman spectroscopy was performed. Therefore, all particles reported in this study are interpreted as morphologically identified microplastic-like particles rather than polymer-confirmed microplastics. This distinction is important because visual identification alone has recognized limitations, and the absence of QA/QC controls may increase the risk of artefactual results in biological tissue analysis (Lusher et al. 2020; di Fiore et al. 2024). The present study did not include field blanks, procedural blanks, filtered solutions, or airborne-fiber contamination controls during laboratory processing. This is an important methodological limitation, particularly because gonadal filters were dried under room conditions for several days and because morphology-based identification alone cannot exclude all non-plastic or exogenous particles. Consequently, absolute particle counts should be interpreted with substantial caution.

Microplastic analysis in water samples

Water samples were collected at four locations using a grab sampling method, with one sample representing each site. A total volume of 300 mL of water was collected from each sampling point and processed individually. These water samples were obtained as secondary data; therefore, only one processed sample was available per site and no true within-site analytical replication was available for inferential statistical analysis. Each water sample (300 mL) was first poured through nylon mesh filter No. 80 to remove large debris and suspended material. The filtrate was then subjected to oxidation to digest organic matter. For this purpose, 3 mL of 0.05 M Fe(II) solution and 3 mL of H_2O_2 were added, and the mixture was heated on a hotplate at 75°C for 30 min to facilitate the Fenton oxidation reaction (Tagg et al. 2017; Lestari et al. 2025). After heating, 1.8 g NaCl was added and stirred until dissolved completely. The solution was allowed to cool for approximately 1 h before filtration. Filtration was carried out using Whatman No. 42 filter paper (particle retention approximately 2.5 μm) arranged in a funnel to avoid filtration gaps. After filtration, the used filter paper containing retained particles was transferred to a Petri dish and dried in an oven at 90°C for 15 min, following Satiyarti et al. (2022). The dried filters were then examined under a microscope for particle presence. Water particle abundance was expressed as particles/L. As in the gonadal samples, suspected particles were identified visually according to shape, color, and surface texture and were categorized as fibers, fragments, or films (Koelmans et al. 2019; Lusher et al. 2020). Because the water dataset consisted of one processed sample per site and was derived from secondary data, it was used descriptively to characterize site-level environmental particle occurrence rather than as a replicated dataset for inferential testing. In addition, the processed water volume was relatively small for environmental characterization, and the resulting values should therefore be interpreted as descriptive occurrence estimates rather than as robust representations of within-site variability. No field blanks, procedural blanks, airborne contamination controls, or polymer-confirmation analyses were available for the water dataset. As with the gonadal particle data, this limitation should be considered central to the interpretation of the results.

Reproductive assessment procedures in male fish

Sperm motility assessment

Semen samples were first diluted with 0.9% NaCl to maintain spermatozoa in an immotile state and to prevent clumping. Motility was then activated by adding distilled water at a ratio of 1:10. Distilled water or other hypotonic media are commonly used to activate freshwater fish sperm during motility assessment (França et al. 2020). A semen aliquot was placed on a glass slide, covered with a coverslip, and examined under a microscope at 1000× magnification. All procedures were performed at room temperature (20-25°C). Motility evaluation was conducted independently by three observers who were blinded to sample identity. At least five microscopic fields were assessed, corresponding to approximately 200 spermatozoa per fish (Gallego et al. 2018; Residiwati et al. 2020). All samples were scored within the same post-activation interval, from the third to the tenth second after activation or until movement ceased, to maintain procedural consistency. Sperm were classified as progressively motile or non-progressively motile. Progressive motility was defined as linear movement toward a specific direction, whereas non-progressive sperm showed movement without forward displacement or remained stationary. Motility was expressed as the percentage of sperm exhibiting active forward movement; sperm vibrating in place were not counted as motile. The percentage was calculated as the number of progressively motile spermatozoa divided by the total number of spermatozoa observed, multiplied by 100% (Residiwati et al. 2020; Tuska et al. 2025a).

Sperm morphology abnormality assessment

Semen was mixed with 0.1% Rose Bengal solution, which stains sperm structures, particularly the head and tail, and facilitates the identification of morphological abnormalities. Abnormalities were classified as primary or secondary. Primary abnormalities were those mainly affecting the head and presumed to arise during spermatogenesis, including macrohead, microhead, and other head defects. Secondary abnormalities involved the midpiece and tail, including bent and curly tails.

The sample was placed on a glass slide in a 1:1 ratio with Rose Bengal solution, mixed, left for 30 s, smeared, and allowed to dry. Smears were examined under an Optilab microscope with oil immersion at 1000× magnification. Approximately 200 spermatozoa were evaluated across ten randomly selected fields per smear. Observations were made independently by three observers. The percentage of abnormal spermatozoa was calculated as the number of

abnormal sperm divided by the total number of sperm observed, multiplied by 100% (Gallego et al. 2018; Tuska et al. 2025a).

Sperm viability assessment

One volume of sperm suspension (10 µL) was mixed with an equal volume of 1% eosin solution and left for 30 s to allow eosin to penetrate damaged sperm membranes. An equal volume of 10% nigrosine solution was then added to create a dark background. The mixture was thinly smeared onto a glass slide, air-dried, and examined under a light microscope at 1000× magnification. Viability was assessed by evaluating approximately 200 spermatozoa across ten microscopic fields. Live spermatozoa did not absorb eosin and therefore appeared unstained or transparent, whereas dead spermatozoa took up the dye. Viability was calculated as the number of live spermatozoa divided by the total number of spermatozoa observed, multiplied by 100% (Gallego et al. 2018). Counts of live and dead sperm were further analyzed using ImageJ software, followed by manual confirmation by the observer (Guo et al. 2021; Tuska et al. 2025a).

Sperm DNA integrity assessment

Sperm smears were air-dried and dehydrated in a 1:1 mixture of acetone and 70% ethanol for 15 min at 4°C, then dried at room temperature. Slides were immersed in 0.1 M HCl for 5 min to denature DNA, rinsed three times with distilled water, and stained with 0.05% toluidine blue for 15 min. Dark-blue spermatozoa were classified as having abnormal chromatin packaging, whereas light-blue or unstained sperm were interpreted as showing better chromatin integrity (Pourentezari et al. 2016). Because toluidine blue had not previously been applied to fish sperm in this exact context, a preliminary internal procedural check was performed by inducing DNA damage using liquid-nitrogen cold shock. Approximately 200 spermatozoa across ten microscopic fields were examined. Counts of intact and damaged DNA were analyzed using ImageJ with observer verification (Guo et al. 2021). DNA integrity (%) was calculated as the number of intact-DNA sperm divided by the total number of sperm observed, multiplied by 100% (Gallego et al. 2018). To support the staining procedure, sperm samples with presumed intact DNA and experimentally damaged DNA were compared after liquid-nitrogen exposure and warming treatment. The analyzed samples included 200 spermatozoa per tube, with DNA damage levels categorized into five levels: 100%, 75%, 50%, 25%, and 0% (Tuska et al. 2025a). However, the quantitative outcomes of this preliminary procedural check were not included in the present manuscript. Therefore, this step should not be interpreted as a formal validation of the assay, but only as internal procedural support for the staining approach used. Accordingly, this endpoint is interpreted as a provisional chromatin-integrity indicator rather than as a fully validated species-specific DNA-damage assay.

Reproductive assessment procedures in female fish

Oocyte diameter assessment

Oocyte diameter was assessed using 50 oocytes from each female fish. The individual female fish was treated as the experimental unit, and the 50 oocytes were treated as subsamples used to generate one mean value per fish for statistical analysis. Oocytes were transferred from a 6-well plate to a concave object glass using a plastic pipette. Because plastic consumables were used during oocyte handling, the possibility of exogenous particle contamination cannot be excluded completely and should be considered an additional limitation when interpreting gonadal particle occurrence. Each oocyte was examined under a binocular microscope (Olympus CKX53, Japan) equipped with an OptiLab Advance Plus camera (Indonesia). Prior to measurement, the imaging system was calibrated using a micrometer. Captured images were analyzed with ImageJ software to determine oocyte diameter, and the resulting data were processed in Microsoft Excel. Final values were expressed in millimeters (mm) and used for statistical analysis (Residiwati et al. 2024; Tuska et al. 2025b).

Oocyte survival rate assessment

Oocyte survival rate was assessed using 50 oocytes from each female fish. As above, the female fish was the biological replicate and the 50 oocytes served as nested subsamples. A total of 1 mL of 0.1% Trypan Blue solution was added into a 6-well plate. Oocytes were transferred into the dye solution using a plastic pipette, and contact time was recorded for 40 s. Samples were then rinsed three times with distilled water (Santos et al. 2017). After the final rinse, oocytes were placed on a concave object glass and examined under a binocular microscope (Olympus CKX53, Japan) equipped with an OptiLab Advance Plus camera (Indonesia). Because the cited reference reports limitations of Trypan Blue for some fish oocyte applications, this assay was interpreted cautiously as an operational membrane-integrity indicator rather than as a definitive standalone measure of oocyte viability (Tuska et al. 2025b).

Oocyte GVBD assessment

Germinal vesicle breakdown (GVBD) was evaluated using 50 oocytes from each female fish. Again, the female fish served as the replicate and the oocytes served as nested subsamples. Oocytes were first transferred into a 6-well plate containing phosphate-buffered saline (PBS), then placed onto a concave object glass using a plastic pipette. Each oocyte was examined under a binocular microscope (Olympus CKX53, Japan) equipped with an OptiLab Advance Plus camera (Indonesia). Oocyte maturation stage was determined according to the presence or absence of the germinal vesicle (GV). Oocytes with a clearly visible GV were classified as being at the GV stage, whereas oocytes in which the GV was no longer visible were classified as having undergone GVBD. No maturation-inducing treatment was applied. Therefore, GVBD was used as an observational endpoint of oocyte maturation status at the time of collection (Tuska et al. 2025b).

Data analysis

The quantitative variables analyzed in this study included sperm quality parameters (morphological abnormalities, motility, viability, and DNA integrity) and oocyte parameters (oocyte diameter, survival rate, and germinal vesicle breakdown/GVBD). All statistical analyses were performed using SPSS version 29. Data were first tested for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test. Datasets meeting both assumptions were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Datasets not meeting parametric assumptions were planned for analysis using the Kruskal-Wallis test followed by Mann-Whitney pairwise comparisons. In the final dataset presented in this manuscript, all reported reproductive endpoints were analyzed using the parametric pathway after assumption checking. For sperm endpoints, the individual male fish was the analytical unit (n: 8 males per site). For oocyte endpoints, the individual female fish was the analytical unit (n: 8 females per site), and measurements obtained from 50 oocytes per female were first averaged at the fish level before statistical analysis to avoid pseudo-replication. Fish were collected across four sampling occasions at each site, with two males and two females obtained during each occasion; however, these sampling occasions were used to distribute field collection over time and were not treated as separate statistical units. Accordingly, comparisons among sites were performed using individual fish values rather than sampling occasions, individual sperm cells, or individual oocytes as independent observations. Water particle abundance was analyzed descriptively and used only to describe spatial variation in site-level environmental particle occurrence. Because the water dataset consisted of one processed sample per site, no replicated statistical comparison was performed for that endpoint. Similarly, gonadal particle data were interpreted primarily as tissue-level occurrence data across the site gradient. The manuscript was therefore framed as a site-gradient comparative study rather than as a direct exposure-response analysis linking individual environmental particle measurements with individual reproductive outcomes.

RESULTS AND DISCUSSION

Accumulation of microplastic-like particles in water and gonadal tissues

Morphologically identified microplastic-like particles were observed in both water samples and gonadal tissues, with visible variation in form and apparent size (Figure 2). In water samples, fragments and fibers were the most frequently observed particle categories, whereas similar categories were also detected in testicular and ovarian tissues. Particles observed in gonadal tissues generally appeared smaller than those observed in water samples. This visual difference may reflect several non-exclusive possibilities, including selective retention of smaller particles in biological tissues, reduced detectability of larger irregular particles within tissue-derived matrices, or differential persistence during digestion and filtration. Because particle classification was based solely on morphology, these observations should be interpreted as the occurrence of microplastic-like particles rather than as polymer-confirmed plastic composition.

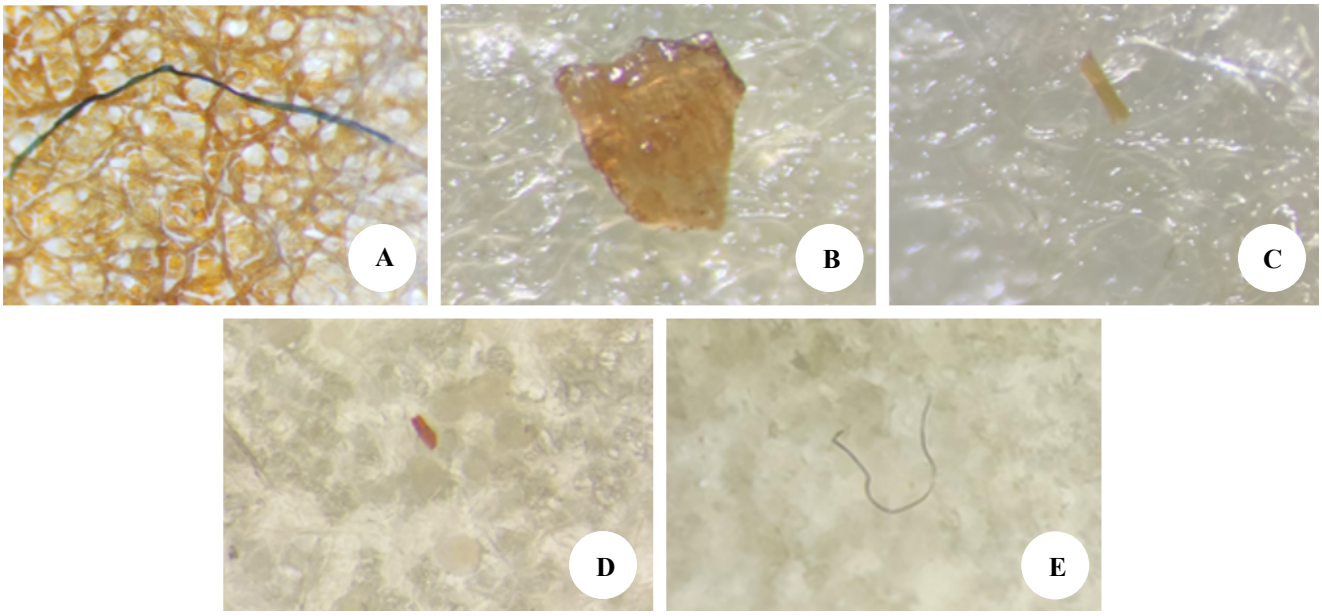

Figure 2. The microplastics observed in the water and gonadal tissues were estimated to range in size from 50 µm to 1 mm based on visual observation under microscopy. A. fiber in water sample, B. fragment in water sample, C. film in water sample, D. fiber in gonadal tissue, E. fragment in gonadal tissue

The predominance of fragments and fibers in the water samples is ecologically plausible in a river-reservoir system influenced by mixed domestic, agricultural, and industrial activities. Fragments are commonly associated with secondary breakdown of larger plastic debris, whereas fibers may originate from household wastewater, textile residues, fishing materials, or degraded synthetic ropes and nets. In reservoir environments, slower water movement relative to free-flowing river sections may favor the retention of suspended particles, especially where cumulative upstream inputs converge. Under such conditions, downstream reservoirs may function not only as transport corridors but also as depositional or accumulation zones for microplastic-like particles. This interpretation is consistent with studies showing that downstream or semi-lentic freshwater sections often accumulate higher particle loads than less disturbed upstream areas (Li et al. 2025b). Microplastic-like particles were detected at all sampling locations in both water samples and gonadal tissues of *C. carpio* (Table 2). Water particle occurrence, expressed as particles/L, followed an upstream-downstream pattern, with the highest value recorded at Karangkates (170.0 particles/L), followed by Sengguruh (140.0 particles/L), Selorejo (103.3 particles/L), and the lowest at the Upper Brantas Sector (26.7 particles/L). Because the water dataset was based on one processed sample per site (n: 1 per site), these values are interpreted descriptively and do not represent replicated estimates of within-site environmental variability.

A comparable gradient was also observed in gonadal tissues. Testicular particle occurrence increased from 3 particles/site at the Upper Brantas Sector to 8 at Selorejo, 12 at Sengguruh, and 19 at Karangkates, whereas ovarian particle occurrence increased from 3 to 8, 18, and 23 particles/site, respectively. Although the gonadal dataset was summarized at site level and therefore does not support individual-level association testing, the parallel increase in water and gonadal particle occurrence suggests that sites characterized by higher environmental particle presence also tended to show higher tissue-level particle occurrence. This pattern is biologically compatible with the possibility that fish inhabiting more particle-rich environments experience greater contact with suspended particles and greater opportunity for internal particle occurrence. Even so, the present data do not allow direct inference about uptake pathways, translocation efficiency, or retention dynamics at the level of individual fish. Taken together, the particle data identify a clear site gradient rather than a causal chain. The strongest conclusion supported by these observations is that environmental particle occurrence and gonadal tissue occurrence co-varied across the Brantas River Basin gradient, with the more downstream reservoirs showing consistently higher values than the uppermost site.

Male reproductive endpoints

Sperm morphology abnormalities

Representative sperm abnormalities observed across sites are shown in Figure 3. Rose Bengal staining revealed structural alterations involving the head, midpiece, and tail. Qualitatively, sperm from the Upper Brantas Sector appeared to show the least variation in abnormal forms, whereas preparations from Sengguruh and Karangkates displayed more frequent primary and secondary abnormalities. Samples from Selorejo appeared to be dominated mainly by secondary abnormalities, particularly in the tail region. These observations are presented only as qualitative morphological context for the quantitative comparison.

Quantitatively, sperm morphology abnormalities differed significantly among sampling sites (Figure 4). The highest abnormality percentage was recorded at Karangkates (15.50±1.00%), followed by Sengguruh (12.00±1.10%), Selorejo (9.50±1.10%), and the Upper Brantas Sector (7.00±0.67%). One-way ANOVA showed a significant difference among sites ($F(3, 28)$: 423.733, $p<0.001$), and Tukey's HSD indicated that all four sites differed significantly from one another.

Biologically, this pattern suggests that sperm structural integrity was poorest at the more downstream sites. Abnormalities affecting the head, midpiece, and tail may compromise fertilization capacity because they can interfere with directional movement, cellular energy supply, or the physical integrity of sperm cells. The fact that the difference among sites was not merely descriptive but statistically significant across the full gradient strengthens the interpretation that male reproductive condition varied meaningfully among locations. Even so, because this was a field-based observational study, these abnormalities cannot be attributed solely to microplastic-like particles. Other unmeasured environmental stressors may also have contributed to the same pattern. Pollutant-related disruption of spermatogenesis has been discussed in relation to oxidative imbalance and altered reproductive physiology, but such mechanisms were not measured directly here (Rochman et al. 2015).

Sperm motility

Microscopic observation showed that *Cyprinus carpio* spermatozoa displayed a range of movement patterns, including progressive forward motility, non-progressive movement, circular movement, backward movement, and complete immobility. These visual observations formed the basis for the quantitative motility analysis shown in Figure 5.

Table 2. Microplastic accumulation in water and gonadal tissues of *Cyprinus carpio* across sampling sites in the Brantas River watershed

Location	Water (particles/L)	Testis tissue (particles/site)	Ovary tissue (particles/site)
Upper Brantas Sector	26.7	3	3
Selorejo	103.3	8	8
Sengguruh	140.0	12	18
Karangkates	170.0	19	23

Note: Water microplastic abundance is expressed as particles/L. Water data were based on one processed sample per site (n: 1 per site) and are therefore interpreted descriptively. Gonadal tissue values are presented as site-level occurrence summaries of morphologically identified microplastic-like particles and not as replicated inferential exposure data

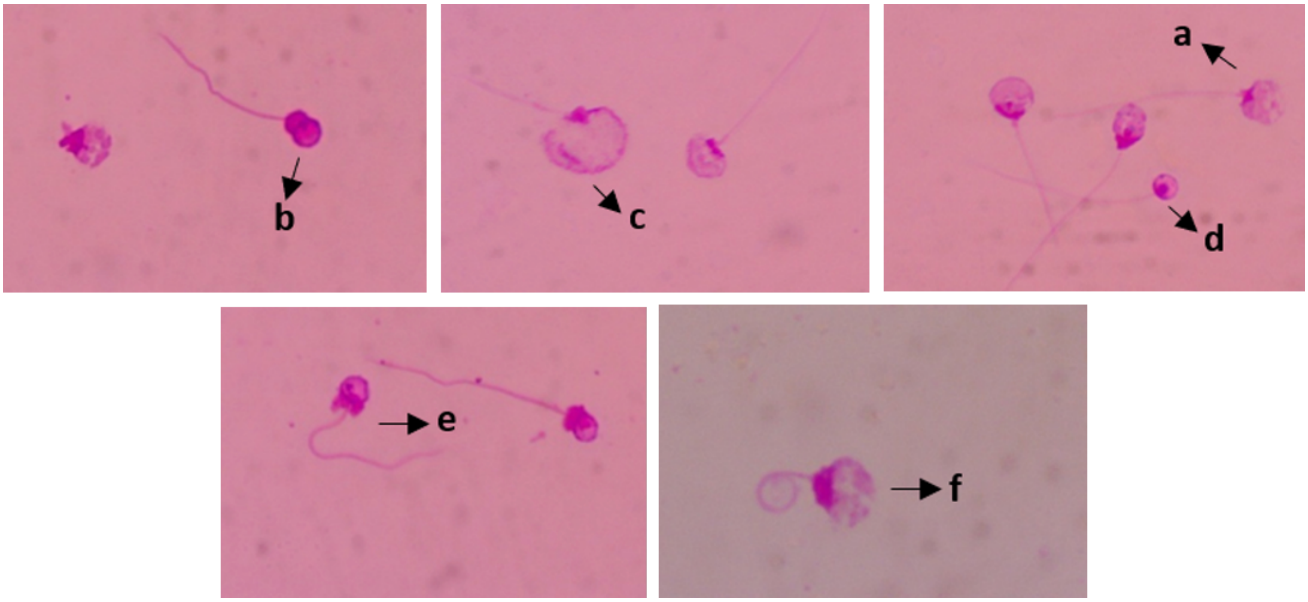

Figure 3. Examination of morphological abnormalities at four locations in the Brantas watershed with Rose Bengal staining at 1000× magnification with an Optilab microscope revealed several variations, including: a: defective head, b: swallowed midpiece, c: macrocephaly, d: microcephaly, e: bent tail, f: curled tail

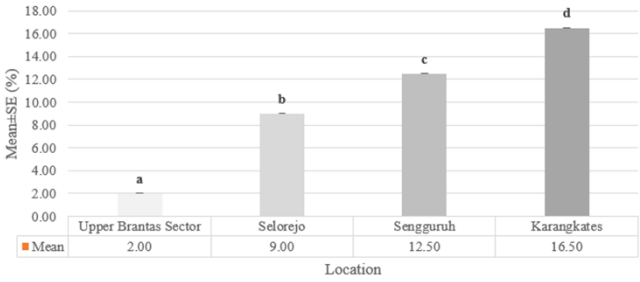

Figure 4. Percentage of sperm abnormalities at each sampling site. Data are presented as mean±SD (n: 8 per group). One-way ANOVA showed a significant difference among groups ($F(3, 28)$: 423.733, $p<0.001$). Different letters (a-d) indicate significant differences among groups based on Tukey's HSD post-hoc test ($p<0.05$)

Mean sperm motility declined significantly from upstream to downstream sites, with values of 88.56±1.02% at the Upper Brantas Sector, 82.75±1.22% at Selorejo, 76.94±1.45% at Sengguruh, and 69.19±1.33% at Karangkates. One-way ANOVA showed significant differences among sites ($F(3, 28)$: 341.733, $p<0.001$), and Tukey's HSD confirmed that all groups differed significantly from one another. This result is biologically important since motility is one of the most immediate determinants of fertilization capacity in externally fertilizing fish. A reduction in motility implies reduced ability of spermatozoa to reach and fertilize oocytes within the limited functional time window after activation. The consistent decline from the uppermost site toward the downstream reservoirs therefore suggests a progressive reduction in sperm functional competence along the site gradient. Reduced sperm motility has been associated in previous work with membrane instability, mitochondrial dysfunction, and oxidative disturbance under pollutant stress (Rochman et al. 2015). In the present manuscript, however, those mechanisms remain contextual explanations rather than directly demonstrated pathways.

Sperm viability

Representative eosin-nigrosine staining results are shown in Figure 6, in which unstained spermatozoa indicate viable cells and stained spermatozoa indicate non-viable cells. The quantitative distribution of sperm viability is shown in Figure 7. The highest viability was observed at the Upper Brantas Sector (87.81±1.33%), followed by Selorejo (81.25±1.22%), Sengguruh (76.37±1.45%), and Karangkates (70.44±1.02%). One-way ANOVA indicated a significant difference among sites ($F(3, 28)$: 386.583, $p<0.001$), and Tukey's HSD showed significant differences among all groups.

The viability results reinforce the same biological pattern seen in the motility data. Viability reflects membrane integrity and survival potential of sperm cells; therefore, lower viability at downstream sites indicates that a smaller proportion of spermatozoa remained functionally intact. In practical reproductive terms, reduced viability can limit the proportion of cells capable of successful fertilization even before motility or chromatin integrity is considered. As with the other male endpoints, this pattern is compatible with environmentally associated reproductive stress, but the study design does not allow identification of a single causal factor.

Sperm DNA integrity

Representative toluidine blue staining patterns are shown in Figure 8. Sperm with better chromatin integrity appeared light blue, whereas sperm with poorer chromatin integrity appeared dark blue.

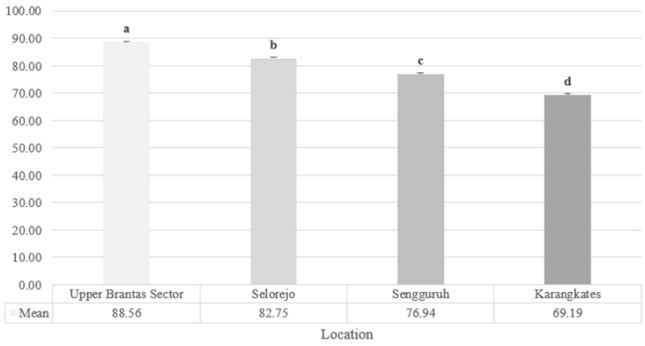

Figure 5. Percentage of sperm motility at each sampling site. Data are presented as mean±SD (n: 8 per group). One-way ANOVA showed a significant difference among groups ($F(3, 28)$: 341.733, $p<0.001$). Different letters (a, b, c, d) indicate significant differences among groups based on Tukey's HSD post-hoc test ($p<0.05$)

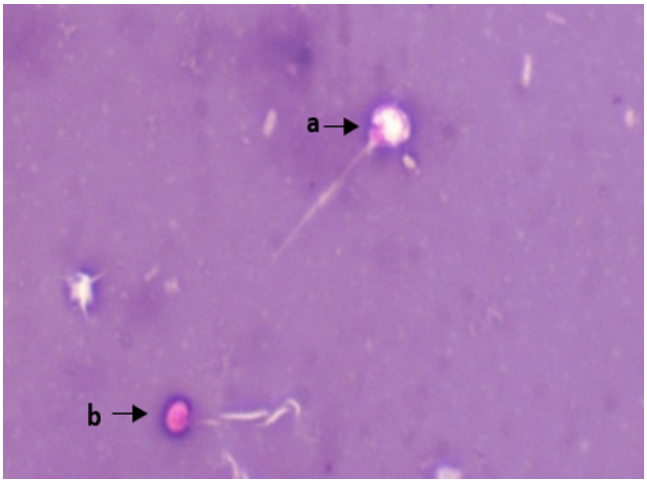

Figure 6. Examination of the viability of *Cyprinus carpio* spermatozoa using eosin-nigrosin staining at 1000× magnification with an Optilab microscope: A. live sperm, B. dead sperm

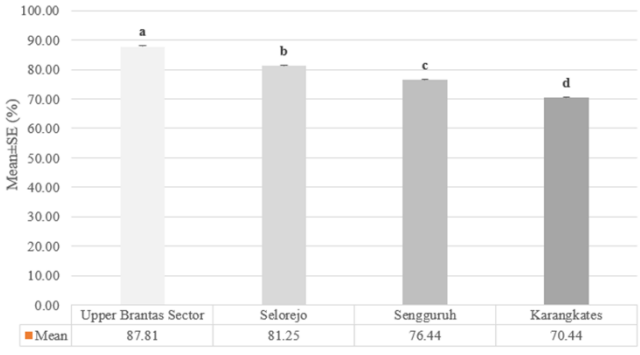

Figure 7. Percentage of sperm viability at each sampling site. Data are presented as mean±SD (n: 8 per group). One-way ANOVA showed a significant difference among groups ($F(3, 28)$: 386.583, $p<0.001$). Different letters (a, b, c, d) above bars indicate significant differences between groups based on Tukey's HSD post-hoc test ($p<0.05$)

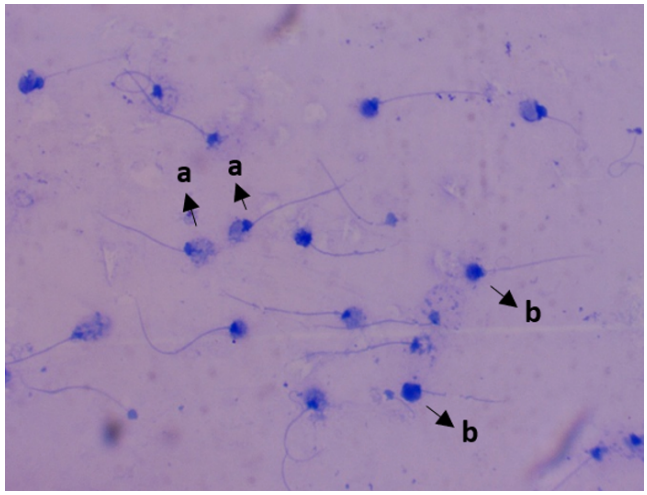

Figure 8. Examination of DNA integrity in *Cyprinus carpio* spermatozoa using toluidine blue staining at 1000× magnification under a microscope: A. sperm with good integrity appear light blue, B. sperm with poor integrity appear dark blue

The quantitative gradient in sperm DNA damage is presented in Figure 9. DNA damage was the highest at Karangkates (28.44±1.02%), followed by Sengguruh (21.75±1.22%), Selorejo (13.50±1.00%), and the Upper Brantas Sector (5.00±0.65%). One-way ANOVA showed that these differences were highly significant ($F(3, 28)$: 833.737, $p<0.001$), and Tukey's HSD indicated that all four sites differed significantly from one another.

Among the male reproductive endpoints, sperm DNA damage showed the steepest gradient. This suggests that chromatin integrity was especially sensitive to site-related differences in environmental condition. From a reproductive perspective, elevated sperm DNA damage is important because successful fertilization does not depend only on cell motility and viability, but also on the integrity of paternal genetic material. Mature sperm cells possess limited DNA repair capacity, making chromatin damage particularly relevant under toxicant-associated stress (Liu et al. 2024). In the present study, the strong statistical separation among sites indicates that this endpoint contributed substantially to the overall pattern of reduced male reproductive quality at the downstream locations. When considered together, the male reproductive endpoints showed a coherent and biologically meaningful gradient.

Sperm abnormalities and DNA damage increased downstream, whereas motility and viability declined in the same direction. This convergence across structural, functional, and chromatin-related measures strengthens the interpretation that male reproductive quality was poorer at sites with higher site-level particle occurrence. However, the interpretation remains comparative and associative rather than causal.

Female reproductive endpoints

Oocyte diameter

Representative oocyte morphology and the diameter measurement approach are shown in Figure 10. Observation

at 40× magnification enabled measurement along two perpendicular axes and provided the basis for comparison among sites.

The quantitative distribution of oocyte diameter is shown in Figure 11. The highest mean value was recorded at the Upper Brantas Sector (1.395±0.005 mm), followed by Selorejo (0.700±0.004 mm), Sengguruh (0.646±0.004 mm), and Karangkates (0.594±0.005 mm). One-way ANOVA indicated a significant difference among sampling locations (F: 7271.452, p<0.001). Post-hoc analysis showed that the Upper Brantas Sector differed significantly from all other locations, whereas Sengguruh and Karangkates did not differ significantly from one another but were both significantly lower than Selorejo.

These results indicate a strong decline in oocyte diameter from the uppermost site toward the downstream reservoirs. Biologically, smaller oocyte diameter may reflect less favorable conditions for oocyte growth and maturation, because oocyte size is closely related to the accumulation of reserves and developmental readiness. The statistical separation among sites therefore suggests that female gamete growth condition differed meaningfully along the watershed gradient.

Oocyte survival rate

Representative trypan blue staining results used to distinguish intact and damaged oocytes are shown in Figure 12. The quantitative distribution of oocyte survival rate is shown in Figure 13. The highest survival rate was observed at the Upper Brantas Sector (92.00±0.54%), followed by Selorejo (71.00±1.13%), Sengguruh (65.00±1.07%), and Karangkates (51.75±1.75%). One-way ANOVA showed a significant difference among sites (F: 194.583, p<0.001), and the Upper Brantas Sector differed significantly from the other three locations.

The progressive decline in oocyte survival toward downstream sites indicates poorer membrane integrity and reduced cellular stability of female gametes at the more downstream locations. This result is biologically relevant because membrane damage may reduce the likelihood that oocytes remain competent for normal reproductive processes. Because trypan blue was used here as an operational indicator rather than a species-specific fully validated viability assay, the survival-rate findings should be interpreted cautiously; however, the endpoint remains informative when considered together with oocyte diameter and GVBD.

Germinal vesicle breakdown (GVBD)

Representative GV and GVBD stages are shown in Figure 14. At the GV stage, the nucleus remained visible, whereas in GVBD the nucleus was no longer visible and the cytoplasm appeared more translucent, indicating progression of oocyte maturation.

The quantitative distribution of GVBD is shown in Figure 15. The highest GVBD value was observed at the Upper Brantas Sector (86.50±1.18%), followed by Selorejo (64.00±1.31%), Sengguruh (57.50±1.05%), and Karangkates (43.00±1.73%). One-way ANOVA indicated a significant difference among sampling locations (F: 181.723, p<0.001).

The decline in GVBD toward downstream sites suggests reduced oocyte maturation potential at sites with higher particle occurrence. Because GVBD reflects progression from an immature state toward maturation, lower values indicate that a smaller proportion of oocytes had reached a more advanced developmental condition at the time of collection. Nevertheless, no endocrine markers were measured and no maturation-inducing treatment was applied. GVBD should therefore be interpreted as an observational indicator of maturation status rather than as a direct mechanistic marker of endocrine disruption. Taken together, the female endpoints also showed a coherent site gradient. Oocyte diameter, survival rate, and GVBD were highest at the Upper Brantas Sector and declined toward Selorejo, Sengguruh, and Karangkates. This consistency suggests that female gamete quality, like male gamete quality, was poorest at the more downstream sites. As with the male data, the pattern is best interpreted as a spatial association within a comparative field design rather than as direct proof of microplastic toxicity.

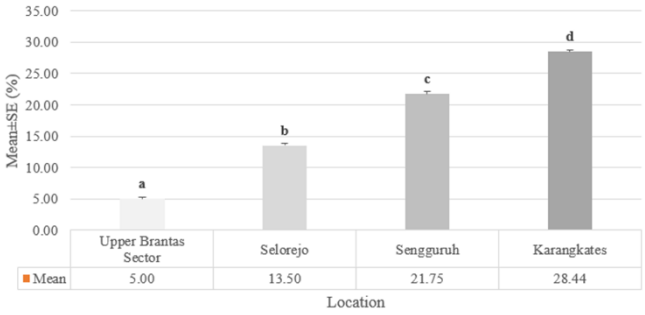

Figure 9. Percentage of sperm DNA damage at each sampling site. Data are presented as mean±SD (n: 8 per group). One-way ANOVA showed a significant difference among groups (F(3, 28): 833.737, p<0.001). Different letters (a, b, c, d) above bars indicate significant differences between groups based on Tukey's HSD post-hoc test (p<0.05)

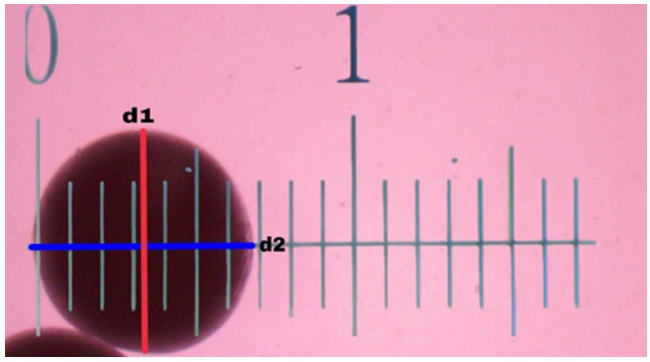

Figure 10. Examination of oocyte diameter in *Cyprinus carpio* at 40× magnification. Oocyte diameter 1 (d1) (red line) and oocyte diameter 2 (d2)

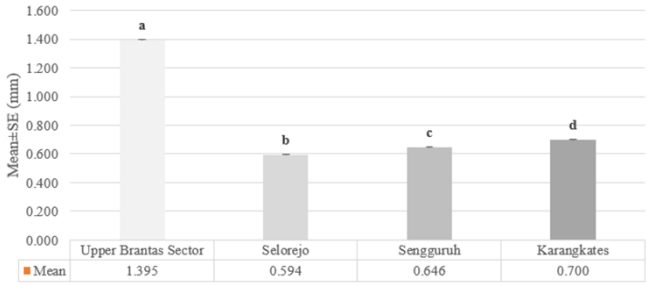

Figure 11. Mean oocyte diameter at each sampling site. Data are presented as mean±SE (n: 8 per group). One-way ANOVA showed a significant difference among groups (F: 7271.452, df: 31, $p<0.001$). Different letters above bars indicate significant differences among groups based on Tukey's HSD post-hoc test ($p<0.05$)

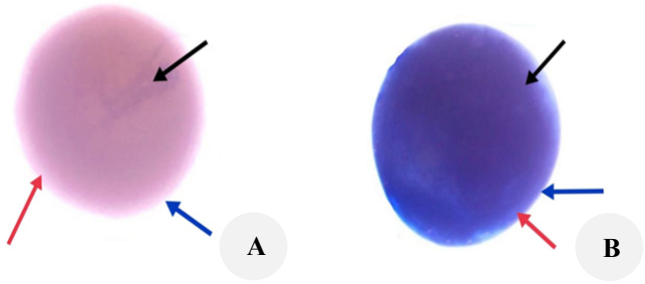

Figure 12. Examination of oocyte survival in *Cyprinus carpio* using trypan blue staining at 40× magnification under a microscope. A. Intact oocyte membrane, B. Damaged oocyte membrane. Blue arrow: zona radiata, red arrow: oolemma, black arrow: cytoplasm

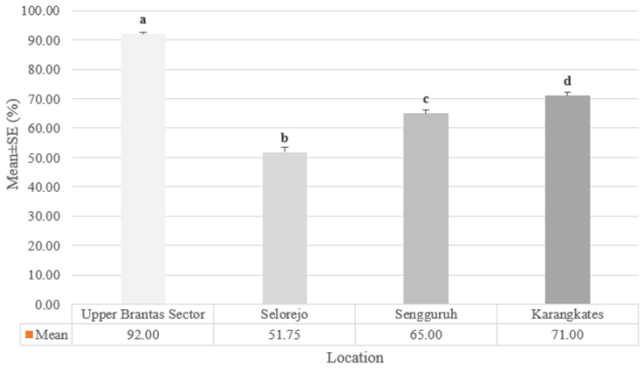

Figure 13. Percentage of oocyte survival rate at each sampling site. Data are presented as mean±SE (n: 8 per group). One-way ANOVA showed a significant difference among groups (F: 194.583, df: 32, $p<0.001$). Different letters above bars indicate significant differences among groups based on Tukey's HSD post-hoc test ($p<0.05$

Integrated interpretation of reproductive responses along the site gradient

When the male and female reproductive endpoints are considered together, a consistent spatial pattern emerges across the Brantas River Basin gradient. Sites with higher environmental particle occurrence and higher gonadal particle occurrence, particularly Sengguruh and Karangkates, were also characterized by poorer reproductive parameters. In males, this pattern was reflected by increased sperm abnormalities and DNA damage together with reduced motility and viability. In females, it was reflected by reduced oocyte diameter, survival rate, and GVBD. The concordance of these endpoints across sexes strengthens the interpretation that reproductive quality declined from the comparatively less disturbed upstream site toward the more downstream reservoirs. At the same time, the present findings must be interpreted strictly within the limits of the study design. The dataset does not support direct exposure-response inference linking individual environmental particle measurements, individual gonadal particle burden, and individual reproductive outcome. Several important methodological limitations further constrain interpretation, including the lack of polymer confirmation, the absence of field and procedural blanks, the lack of airborne contamination controls, the small processed water volume, the use of single processed water samples per site, and the possibility of contamination during laboratory handling. In addition, biomarker data were not collected, and potentially important environmental covariates were not measured directly.

Within these limits, the observed patterns remain compatible with mechanisms discussed in the literature. In males, pollutant-associated reproductive impairment has been linked to disrupted spermatogenesis, oxidative stress, chromatin damage, and endocrine interference (Rochman et al. 2015; Liu et al. 2024). In females, compromised oocyte development and maturation have been discussed in relation to reduced membrane integrity, oxidative disturbance, and impaired follicular support (Zitouni et al. 2020; Huang et al. 2022). These mechanisms were not tested directly in the present study and are therefore presented only as biologically plausible context rather than demonstrated processes. A particularly important interpretive issue is the role of co-varying environmental stressors. Microplastic-like particle occurrence in the present dataset may co-vary with other site-related pressures, including heavy metals, nutrient imbalance, hydrological differences, temperature variation, suspended matter, and other pollutants. Any of these factors, alone or in combination, may influence reproductive endpoints similar to those measured here. For that reason, co-varying stressors should be considered a central limitation rather than a minor caveat. The present study therefore does not isolate microplastic-like particles as the single driver of reproductive decline; instead, it shows that poorer reproductive condition occurred at the same sites where particle occurrence was also higher. Despite these limitations, the study contributes meaningful field-based evidence. Previous reports have indicated that microplastic exposure may produce sublethal yet biologically relevant reproductive effects in fish, including reduced oocyte size, impaired follicular development, decreased sperm motility, and increased sperm abnormalities (Siddique et al. 2026).

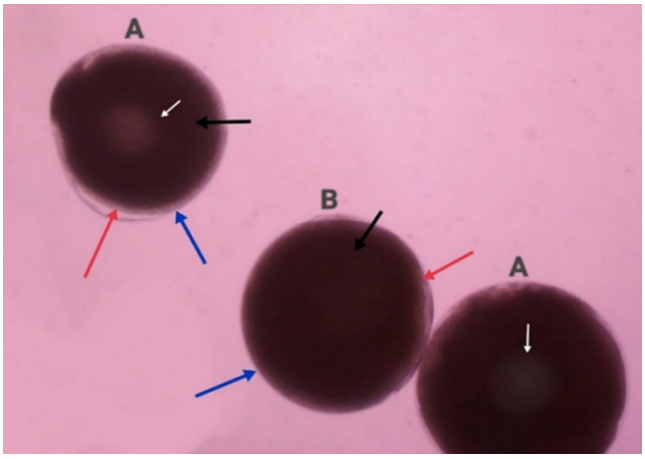

Figure 14. Examination of germinal vesicle breakdown (GVBD) at 40× magnification under a microscope. A: Germinal vesicle (GV), B: Germinal vesicle breakdown (GVBD). Blue arrow: zona radiata, red arrow: oolemma, black arrow: cytoplasm, white arrow: nucleus

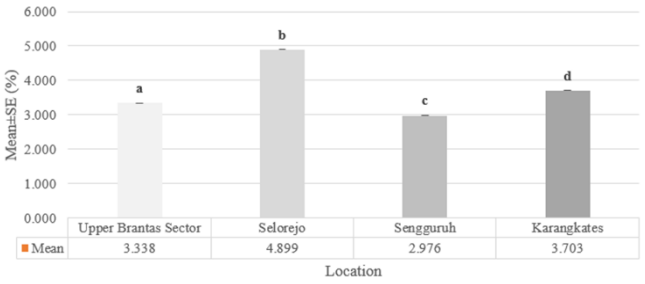

Figure 15. Percentage of oocyte GVBD at each sampling site. Data are presented as mean±SE (n: 8 per group). One-way ANOVA showed a significant difference among groups (F: 181.723, df: 31, $p<0.001$). Different letters above bars indicate significant differences among groups based on Tukey's HSD post-hoc test ($p<0.05$)

The present study extends that discussion by showing that, in *C. carpio* multiple male and female reproductive endpoints varied systematically along a freshwater site gradient characterized by increasing environmental and gonadal particle occurrence. This comparative pattern, observed simultaneously in both sexes, represents the strongest and most defensible contribution of the current work. Overall, microplastic-like particles were detected in both the aquatic environment and gonadal tissues of *C. carpio* across the Brantas River Basin gradient, and higher site-level particle occurrence coincided with poorer reproductive parameters in both sexes. These findings are compatible with the interpretation that site-level particle occurrence may be associated with reduced reproductive condition under field conditions, but they do not demonstrate direct causality. Future studies should incorporate polymer confirmation, replicated environmental sampling, stronger contamination control, biomarker analysis, and better characterization of co-varying environmental stressors to clarify more precisely the contribution of particle occurrence within the broader environmental context of freshwater fish reproduction.

In conclusion, this field-based comparative study showed that morphologically identified microplastic-like particles were present in both water samples and gonadal tissues of *C. carpio* along the Brantas River Basin gradient. Sites with higher site-level particle occurrence, particularly the more downstream reservoirs, were consistently associated with poorer reproductive condition in both sexes. In males, this pattern was characterized by increased sperm abnormalities and DNA damage together with reduced motility and viability, whereas in females it was reflected by reduced oocyte diameter, lower survival rate, and decreased GVBD. These findings indicate that site-level particle occurrence and reduced gamete quality co-occurred along the watershed gradient. However, the evidence should be interpreted cautiously. Because the study was observational and constrained by the absence of polymer confirmation, contamination-control procedures, replicated environmental sampling, biomarker analysis, and direct assessment of co-varying environmental stressors, the observed patterns remain associative rather than causal. The present study therefore does not demonstrate that microplastic-like particles alone caused the reproductive decline, but it does provide comparative field-based evidence that reproductive condition varied systematically along a site gradient where environmental and gonadal particle occurrence also increased. Overall, the main contribution of this study lies in integrating environmental particle occurrence, gonadal occurrence of microplastic-like particles, and sex-specific gamete-quality endpoints within a single freshwater field gradient in *C. carpio*. Future studies should incorporate stronger contamination control, polymer confirmation, replicated environmental measurements, individual-level gonadal particle analysis, biomarker assessment, and broader environmental characterization to better clarify the contribution of particle occurrence within the wider environmental context affecting freshwater fish reproduction.

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