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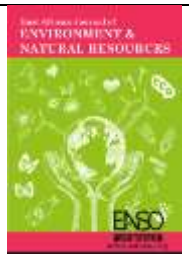

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Original Article

A Microcosm Analysis of Species-Specific Responses of Chironomidae on Heavy Metal Pollution in The Nyanza Gulf of Lake Victoria

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ABSTRACT

The Chironomidae family, known as "non-biting midges" in their adult stage and "bloodworms" in their larval stage, consists of diverse dipteran insects inhabiting various global aquatic environments. Despite extensive global research, data on Chironomidae in the polluted Nyanza Gulf of Lake Victoria, Kenya, is scarce, and molecular identification methods have not been explored. This study aimed to quantify heavy metal concentrations in water, sediment, and insect samples and assess their impact on Chironomid species identified using mitochondrial DNA barcoding of the cytochrome oxidase subunit 1 (COI) gene. Analysis of Variance was used to determine if there were any statistically significant differences in heavy metal concentrations across different sample types or locations along the pollution gradient. Chironomids were collected from Nyanza Gulf, focusing on a pollution gradient. Results showed that concentrations of arsenic (As), lead (Pb), and cadmium (Cd) in insect, water, and sediment samples exceeded standard limits, while mercury (Hg) concentrations were within limits. Significant variations ($p \leq 0.05$) in Pb levels were observed in water samples, and heavy metal concentrations in sediment samples varied significantly ($p \leq 0.05$), with Pb showing the highest variation ($p \leq 0.0001$). Insect samples exhibited significant differences ($p \leq 0.0001$) in As and Hg contents. Genetic analysis identified two known species: Chironomus transvaalensis at the heavily polluted Kisumu station and Chironomus pseudothummi at the moderately polluted Kendu Bay and Homa Bay stations. Additionally, a unique Chironomus species was found on Ndere Island, a relatively clean site with restricted human activities. Sequence comparisons indicated proximity to global data but also highlighted the evolutionary significance and uniqueness of the identified species. This study demonstrated the potential use of genetic methods in determining Chironomid species diversity, community structure, and abundance in relation to heavy metal concentration. It suggests that heavy metal pollution may act as a selective pressure, driving the evolution of Chironomid species. The study recommends combining genetic approaches with other pollution sources for a comprehensive understanding of using this species in monitoring pollution.

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INTRODUCTION

Chironomus, Meigen, 1804 commonly known as the non-biting midge/lake flies belonging to the Family Chironomidae-Diptera has been extensively studied due to its ecological significance and potential use as a bioindicator in environmental monitoring. The family has approximately 6359 species identified globally, (Ashe and O'Connor, 2009), highlighting its extensive diversity and ecological importance. Research on the non-biting midge has been conducted in Europe, Asia, Siberia, North America, Mexico, Canada, and Alaska (Karima, 2021). However, little research has focused on the Mediterranean region and Sub-Saharan Africa. Additionally, the existing studies have primarily focused on the description of adult non-biting midges and their distribution (Otieno et al., 2023); their use as food (FAO, 2013; Van Huis et al., 2013; Ayieko et al., 2016; Macadam & Stockan, 2017; Akhtar & Isman, 2018; Oganyo et al., 2022); and biomonitoring (Nyakeya et al., 2017). This indicates a need to broaden the scope by studying the immature stages and exploring other areas to discover unknown species. Lake Victoria, a biodiversity hub, is no exception.

The genus *Chironomus*, within the subfamily Chironominae, is among the largest in the class Insecta and includes many macro-invertebrates. However, morphological analysis of the larvae has been hindered by the concealed features of the organism, making their description difficult. Additionally, the high similarity in species phylogeny presents a significant limitation (Castello et al., 2017). Therefore, further studies are required to provide more reliable, accurate, and rapid data on the benthic larval forms with obscured features that limit identification (Fayram et al., 2022; Hutchings, 2017). To address these limitations, there is a growing need to utilize genomic structures or barcodes for identification (Hebert et al., 2003; Hübner et al., 2017). Hebert et al. (2003) recommended the use of the Cytochrome c oxidase subunit I (COI) gene, which is associated with a high range of phylogenetic signals, conserved sequences, and well-established primer utility in the taxonomic identification of benthic organisms.

The Cytochrome c oxidase subunit I (COI) gene is a highly conserved molecular marker that is ideal for distinguishing between immature and morphologically similar organisms (Fayram et al., 2022; Lobo et al., 2017). This gene is strictly inherited and remains stable across generations, making it reliable for genetic identification. Karima (2021) observed that previous studies on *Chironomus* focused primarily on the adult stage, utilizing morphological features that are only applicable to adults. There is a critical need for research dedicated to the larval stage of the non-

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biting midge, which plays a vital role in aquatic food chains and food webs as environmental cleaners and bioindicators of environmental and climatic conditions (Karima, 2021).

Furthermore, *Chironomus* larvae are essential for assessing and classifying aquatic systems based on eutrophication levels and testing for heavy metal pollution toxicity (Di Veroli et al., 2014; Zerguine et al., 2018; Youbi et al., 2020), yet this area has received minimal attention. Additional features of *Chironomus* larvae include their physiological adaptability to harsh environmental conditions, such as resistance to desiccation (Jindal & Singh, 2020), low oxygen levels (Sharma & Rawat, 2009; Klaus et al., 2008), and their ability to survive in various water types—fresh, brackish, or marine—whether polluted or clean (Sharma & Chowdhary, 2011; Sharma et al., 2015).

The genus *Chironomus* is known for its distinctive red coloration, attributed to the hemoglobin protein, which allows for survival in low oxygen habitats (Matthews-Bird et al., 2016; Sharma & Rawat, 2009; Klaus et al., 2008; Karima, 2021). *Chironomus* species exhibit varying tolerance to pollution and sensitivity to environmental factors such as temperature, pH, salinity, and dissolved oxygen concentrations (Grover et al., 2022; Matthews-Bird et al., 2016). These differences affect the community structure, abundance, and distribution of *Chironomus* populations (Sharma et al., 2015).

Given their adaptability to dynamic conditions, *Chironomus* species are excellent indicators for environmental biomonitoring (Karima, 2021; Li et al., 2010; Morse et al., 2007; Belle et al., 2017). The genus *Chironomus* serves as a valuable bioindicator for detecting changes due to anthropogenic effects such as urbanization, industrialization, and climate change. However, the impact of chemical pollutants, including heavy metals, persistent organic pollutants, and radioactive substances, on *Chironomus* species has not been extensively studied, particularly regarding their physiological adaptations.

The environment, especially the substrate, influences the physiology of *Chironomus* species. It is essential to determine if varying concentrations of heavy metals exhibit different toxicity levels, affecting the community structure. The specific identification of conspecific or heterospecific *Chironomus* species remains challenging due to the subtle morphological features. Furthermore, it is unclear how the divergences and convergences resulting from changing aquatic habitats impact these species.

This study aims to elucidate the occurrence of *Chironomus* species using the Cytochrome c oxidase subunit I (COI) gene and assess the influence of heavy metal concentrations on *Chironomus* populations in the Kenyan Nyanza Gulf of Lake Victoria.

Materials and methods

Study Area

The study took place in the Nyanza Gulf on the Kenyan side of Lake Victoria, which contributes about 30% of the lake's riverine inflow. This inflow comes from five major rivers—Nzoia, Yala, Kuja, Nyando, and Sondu—all originating in highlands and passing through agricultural areas (Gikuma-Njiru, 2005). Smaller rivers like Kisat, Kisian, and the highly polluted Nyamasaria also flow through agricultural and suburban zones. The Sondu and Miriu rivers are used for hydropower and traverse regions with sugarcane farming, industrial activities, and rice paddies integrated with small-scale aquaculture

The region faces considerable environmental challenges due to industrial activities, including breweries, tanneries, fish processing, agro-processing, and abattoirs, which discharge significant pollutants into the lake. Furthermore, the area is characterized by livestock farming, gold mining, sand harvesting, and oil depots, all contributing to environmental degradation. The study's focus on assessing heavy metal concentrations in relation to Chironomus populations is crucial, as these metals are likely present due to the diverse and significant pollution sources in the catchment area. Understanding how

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these pollutants affect Chironomus species will provide insight into the ecological health of the gulf and the impact of land use practices on aquatic life.

The Nyanza Gulf has a high population density, approximately 440 people per km² (KPHC, 2009), exerting pressure on available land and leading to deforestation and elevated pollution levels in the gulf. Major towns along the lake shores, including Kisumu City, Homa Bay, and Kendu Bay, face significant pressure in terms of sewage facilities and waste disposal, contributing to non-point source pollution that affects lake water quality. Stormwater runoff carries these pollutants into the lake, compromising water quality and disrupting the ecosystem. By examining the influence of these metals on Chironomus species, the study addresses a key challenge: determining the extent to which industrial and agricultural effluents impact the aquatic ecosystem, particularly the diversity and health of benthic organisms like Chironomus.

The influx of pollutants has detrimental effects on the gulf's ecosystem, destroying habitats for aquatic flora and fauna. The lake ecosystem is zoned into areas supporting the growth of emergent and submergent macrophytes in the littoral zone and the water column, creating habitats for insects that are integral to aquatic food chains and webs, facilitating energy flow through trophic levels. By focusing on Chironomus species, the study seeks to elucidate how these disruptions manifest in the aquatic fauna, particularly through the lens of genetic identification and the relationship between species occurrence and heavy metal concentrations. This approach underscores the relevance of the study in addressing the broader environmental challenges that threaten the ecological balance of the gulf

Sampling and Sampling Design

Sampling took place from September 8th to 11th, 2020, at three urban stations—Kisumu Bay, Homa Bay, and Kendu Bay—to assess the impact of urban pollution. These shallow stations (max depth ~3.5 meters) are heavily polluted by domestic effluents, raw sewage, industrial waste, and stormwater runoff. Kisumu Bay, in particular, is affected by the polluted Kisat River, which primarily receives sewage effluent.

Ndere and Maboko Islands, with minimal human activity and the protected Ndere Island National Reserve, were chosen to represent clean inshore stations. Fish landing beaches at Osieko, Usenge, and Goye were included to assess the impact of fishing activities.

A systematic random sampling design was used, with a 50-meter belt along the lake shores representing littoral and sub-littoral zones, habitats for macroinvertebrates like chironomids. The first sampling point was randomly chosen within this belt, with triplicate sampling at six sites, yielding 18 water and sediment samples. The first site of collection was Kisumu Bay located at the heart of Kisumu City, where triplicate samples were obtained from Coca Cola point (00º05.686.097’S,034º44.086’E,1134m); Kisat River mouth (00º 05.212’S ,034º44.969’E,1134m; and Hippo point (00º 07.474’S,034º44.630’E,1136 m). Kendu bay the second collection site located within the Kendubay town environment known for beaching activities and fishing landing. Sampling was done at Kendubay 1(00º 20.971’S,034º39.321’E,1139 m); Kendubay 2(00º 02.968’S,034º44.095’E,113 8m) and Kendubay3(00º 20.968’S,034º44.264’E, 1135m). The third collection was Homabay located in Homabay Town associated with beach activities, fishing and sewage disposal treatment plant entry point. The three sampling sites included Homabay 1(00º 31.334’S,034º26.735’E, 1134m); Homabay2(00º 30.784’S,034º26.735’E, 1129m) and; Homabay 3(00º 30.337’S,034º26.20 3’E,1134m). The fourth sampling site uniquely located within the Ndere National Park where human activities are prohibited. The sampling points denoted as Ndere 1 (00º 10.999’S,034º31.142’E,1134m; Ndere 2(00º 11.662’S,034º31.187’E,1138m) and; Ndere 3(00º 11.450’S,034º31.168’E,1134m). The fifth sampling site was an offshore station -Maboko island located furthest end of Kisumu Bay and

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12km away from Ndere National Park. The station is associated with hotel activities and Fishing. The sampling points were three: Maboko 1(00º 09.569'S,034º37.003'E);Maboko 2(00º 10.238'S,034º36.358'E);and Maboko 3(00º 10.097'S,034º36.537'E).The sixth sampling site was the Fish Landing Beaches, FLB whose mainly known for fishing activities: Osieko-FLB1(00º08.751'S,034º44.152'E),Usenge-FLB2(00º 01.506'S,034º00.809'E) and Goye-FLB3(00º 03.651'S,034º02.571'E).

Water and sediment samples were collected in triplicate at each point and pooled into a composite sample for heavy metal analysis. For insect larvae, triplicate grabs were pooled into a composite sample, from which three larvae were randomly selected for heavy metal and molecular analyses.

Sample collection and handling

Water samples for heavy metal analyses were collected using a polycarbonate sampling bar with a polytetrafluoroethylene container, thoroughly pre-washed with acid and deionized water. The samples were preserved in liquid HNO3 at a pH of <2, placed in cooler boxes at 4ºC, and transported to the laboratory for analysis.

Sediment samples were collected according to the U.S. EPA (2017) standard operating procedure (ID: LSASDROC-200-R4) and Ohio EPA (2001) guidelines. A 400 cm² (20 x 20 cm) AISI 316 stainless steel Eckman bottom corer grab was used. The spring-tensioned, scoop jaw-like part was mounted on pivot points and set with a trigger assembly activated from the surface by a messenger. Three grabs per sampling point were made, dumped on a tray, and pooled to form a composite sample. The samples were placed in 1000 ml polyvinyl chloride (PVC) bottles, covered with aluminum foil, placed in a cooler box at 4ºC, and transported to the laboratory for heavy metal analysis.

Insect samples were collected using a profundal lake sampling method as per SFS 3536 (1835). Sampling was conducted 50 meters offshore using a boat, with an Eckman grab–Birge dredge sampler used for random triplicate grabs of submerged insect larvae. The larvae were placed in a plastic bucket with a fine mesh sieve and pooled into a composite sample. The contents were sorted, washed with alcohol (to prevent decay and maintain structural integrity of the samples over time), and placed in labeled paper slips detailing location, date, time, collector, sampling method, habitat, weather, and sample number. The samples were filled with 80% alcohol as per ISO-EN 4243-3 (1840), closed, and packed in readiness for transportation in cooler boxes at 20ºC for further identification. Sampling was carried out in the morning hours between 7:00 AM and 11:30 AM following methodologies outlined by Moller (2013), Mehmet & Sertakaya (2015), and McMurtrie et al. (2011).

Sample Analyses (water, sediment and insect)

Heavy metal analyses in water samples were conducted using vacuum filtration to handle high particulate matter, turbidity, and suspended solids. Pre-treatment involved adding a mixture of HNO_3:H_2O_2, in a ratio 1:3 for acid digestion. The contents were placed in a hollow solid fiber micro-extractant coated with graphene oxide silica, then raised to a 100 ml mark for analysis using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) following EPA Methods (2008). Mercury analysis was performed using Cold Vapor Atomic Fluorescence Spectroscopy (CV-AFS) as per EPA Methods 245.1 (U.S. EPA, 1840).

For sediment samples, they were dried at room temperature, ground, and sieved using a 2 mm sieve. Approximately 2.00 g of the dried sample was placed in a 100 ml beaker with 15 ml HNO3, then heated at 130ºC to boiling for 5 hours until the volume reduced to 3 ml. The heated content was filtered, washed with 0.1M HNO3, and diluted to 100 ml with distilled water for analysis using Graphite Furnace Atomic Absorption Spectrophotometry (GF-AAS), as described in EPA Methods 200.9 (U.S. EPA, 1840) for metals excluding Mercury and Arsenic, which were analyzed using the Cold Vapor Absorption

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technique (CV-AFS) as per EPA Methods 245.1 (U.S. EPA, 1840).

Heavy metal analyses on preserved insect samples involved oven drying at 120ºC and weighing on a Mettler Toledo microbalance, providing six decimal place readability (1 µg). Triplicate samples were digested in a flask with a 1:1 mixture of HNO3 and H2O2, heated at 130ºC until dissolution. The samples were then diluted to 100 ml with distilled water for metal analysis using GF-AAS, as outlined in EPA Methods 200.9 (U.S. EPA, 1840) for metals except Mercury and Arsenic, which were analyzed using CV-AFS according to EPA Methods 245.1 (U.S. EPA, 1840).

Molecular analysis of Chironomids

Genomic DNA was extracted from insects' isolates using the DNeasy Blood and Tissue Kit Qiagen™ kit according to the manufacturer's specifications. The concentration and purity of DNA was estimated using a DNA Nanodrop™ Lite Spectrophotometer (Thermo Scientific Inc., USA) at 260-280 nm and by horizontal gel electrophoresis (Thistle Scientific Ltd, USA) on a 0.8% (w/v) agarose gel at 100 V for 30 min and visualized under UV after staining with GelRed™ (Thermo Scientific, USA).

Amplification was performed in a programmable master cycler thermocycler (C1000-BioRad, USA) using established specific primers that target *COI* gene of mitochondrial DNA, LCO1490 and HC02198. The thermal cycler conditions were as follows: initial denaturation at 98°C for 2 minutes followed by 35 cycles at 98 °C for 30 seconds, annealing at 47.3 °C for30 seconds, elongation at 75 °C for 30 seconds and final elongation at 75 °C for 10 minutes. 50µL PCR mix constituted DNA polymerase enzyme 1U/50µL reaction, 5X Buffer 10µL, 10pm dNTP, 50Mm Mg Cl_2 1µL,10pm primers 1µL each and distilled water.

PCR products were separated by horizontal gel electrophoresis on 1.5 % (w/v) agarose gel at 100 V for 45 mins and visualized under UV after staining with 2 μl Gel Red™ (Thermo Scientific). PCR amplicons purified using the Thermo Scientific® Gene JET Purification Kit (EU Lithuania). A ratio of 1:1 volume of binding buffer added to the completed PCR mixture and vortexed to mix properly. When the color of the mixture remained orange or- violet, 10 μl of 3M Sodium acetate (pH 5.2) was added to alter the color to yellow. Eight hundred microliters of the solution transferred to the Gene-JET purification column and centrifuged at 10,000rpm for 30 sec. and the flow-through was discarded. Seven hundred microliters of the buffer (diluted with ethanol) added and centrifuged at 10,000 rpm on a rotor for 30 sec. and the flow-through discarded. Additional centrifugation done to completely remove any residual buffer. The purification column was transferred to clean 1.5 *ml* microcentrifuge tubes and 50 μl of elution buffer added followed by centrifugation at 10,000 rpm for 1 min to obtain pure DNA amplicons.

Purified PCR products were sequenced by capillary sequencing on an ABI 3730xl DNA Analyzer (Applied Biosystems) using the same forward and reverse primers and the ABI BigDye® Terminator v3.1 Cycle Sequencing reaction kit (Applied Biosystems, USA). Forward and reverse sequences were obtained and edited using Chromas Pro v3.1 to produce consensus sequences. Further editing and alignment were performed using BioEdit Sequence Alignment Editor ver. 7.0. The sequences were then converted into FASTA file format for analysis. Sequences obtained in this study were submitted to GenBank under accession numbers ON455096-ON455103. Additional COI sequences for Chironomus from other parts of the world were obtained from GenBank (www.ncbi.nlm.nih.gov) for phylogenetic analyses.

Statistical Analyses

Descriptive statistics were employed to evaluate data on heavy metals across stations. The data was analyzed by ANOVA to determine if there were any statistically significant differences in heavy metal concentrations across different sample types or locations along the pollution gradient. Chironomids were collected from Nyanza Gulf,

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focusing on a pollution gradientand a Tukey's pairwise post hoc test was used to confirm significant variations. The composition of aquatic insects was analyzed independently based on morphological and molecular approaches. The non-biting midge, Chironomids a species representative sample was identified across stations for genetic analysis. Molecular analysis, assembled sequences were transferred to MEGA v.11 software and pairwise sequence alignment of the nucleotides done using CLUSTAL W according to Tamura *et al*. (2011). Sequences were submitted to the NCBI BLAST portal (www.ncbi.blast.nlm.nih.gov) for a sequence homology search, and sequences with greater than 97% similarity retrieved for phylogenetic analysis. Evolutionary history was inferred using the Neighbor-Joining algorithm and distances computed using the Maximum Composite Likelihood (Tamura & Kumar, 2004; Tamura et al., 2011). Bootstrap tests (1000 replicates) were used to cluster associated taxa, with replicate trees showing likelihoods above 50% indicated on the branches.

Results

Chironomids, benthic macro invertebrates are known to have a wide range in habitat preferences at different developmental stages. The organisms are low in mobility, are pollution sensitive and tolerant to changes in environmental stressors. The stressors include physical and chemical parameters in water quality though not very accurate (Sumudumali & Jayawardana, 2021). Some pollutants are not easily delectable at low levels though very detrimental for example heavy metals and pesticides. Their bioaccumulation and bio magnification properties up the food chains and food webs during the energy transfer in aquatic systems. Therefore, there's need to quantify the pollutants in water, sediment and the organism and for monitoring their effects on their molecular, cellular and entire body and the population. The present study assessed the heavy metals as environmental stressors on chironomids habitat.

Heavy metal concentration in water, sediment and insect samples

Heavy metal concentration in water, sediment and insect samples were as outlined in **Table 1.** Significant variations ($p \leq 0.05$) were detected in the level of Pb from the water samples. However, no variations were observed in levels of Ar, Hg, and Cd between the sampling stations. All sediment samples significantly varied ($p \leq 0.05$) in heavy metal concentrations, with Pb having highest ($p \leq 0.0001$) variation. Insect samples differed significantly ($p \leq 0.0001$) in Ar and Hg contents, although Pb and Cd displayed insignificant variations.

Overall mean concentration of 0.411±0.15(Ar), 0.019±0.01(Hg), 1.340±0.52(Pb), and 0.717±0.08(Cd) was observed in insect samples, which was greater that KEBS recommended standards. The concentration of Ar, Pb, and Cd in insect, water and sediment were greater than KEBS standards while Hg concentration was within the standard limits.

The levels of arsenic (Ar) in water samples were exceedingly high in Kisumu bay (1.62±0.04 mg/l) and Ndere island (<0.34±0.03 mg/l) while that of cadmium (Cd) was highest at Kisumu (0.731±0.002 mg/l) followed by Ndere island (0.587±0.1 mg/l). Mercury (Hg) recorded the lowest concentrations in water, which was below the KEBS standards.

The concentration of all heavy metals assessed in sediment samples were exceedingly elevated beyond the KEBS standards except Hg (<0.05) in all stations. The highest level in Ar was in sediment samples from Kisumu bay (2.377 mg/l) while the lowest level was from Kendu bay (0.011±0.0 mg/l). Similarly, highest levels of Pb were from Kisumu bay (2.78±0.08 mg/l) samples, and the lowest concentrations were from Ndere Island (0.137±0.0 mg/l) samples. Sediment samples from Ndere island, an offshore station, recorded the highest levels of Cd (0.763±0.4 mg/l) while Kendu bay samples had the least concentrations (0.481±0.0 mg/l).

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Observations on heavy metal concentration in insect samples showed Ar, Pb and Cd were exceedingly high beyond acceptable KEBS limits in all stations. Insects from Kisumu bay had outstandingly the highest concentration in all heavy metals: Ar (1.337±0.06 mg/l), Hg (0.087±0.01 mg/l), Pb (4.387±0.03 mg/l) and Cd (1.127±0.04 mg/l), while Kendu bay displayed the lowest concentration of Hg (0.003±0.0 mg/l) and Cd (0.497±0.0 mg/l). Samples of insects from the two islands studied recorded the lowest concentration of Ar (0.035±0.0 mg/l) at Maboko and Pb (0.087±0.01 mg/l) at Ndere Island. Levels of Ar exceeded KEBS acceptable limits in all stations except in Kendu-bay stations (0.018±0.0 mg/l).

Table 1. Heavy metal concentrations in water, sediment and insect samples expressed as mean (±SE) in milligrams/liter

Heavy metal (mg/l)	Sample type	Maboko Island	Kisumu Bay	Fish LB	Ndere Island	Kendu Bay	Homa Bay	KEBS STDS	F Stat	*p* value	*Sig.*
Arsenic (Ar)	Insect	0.035±0.00^{c}	1.33±0.06^{a}	0.387±.03^{b}	0.350 ±0.03^{b}	0.018±0.00^{c}	0.343 ±0.01^{b}	0.02	2016.70	<0.001	HS
	Water	0.021±0.00	1.62±0.04	0.22±0.01	0.34±0.03	0.013±0.00	0.147±0.01	0.02	1.55369	0.245913	NS
	Sediment	0.031±0.00^{c}	2.37±0.06^{a}	0.307±0.02^{b}	0.58±0.03^{b}	0.011±0.00^{c}	0.253±0.03^{b}	0.02	3.78681	0.0273018	S
Mercury (Hg)	Insect	0.035±0.00^{a}	0.08 ±0.01^{a}	0.007±0.00^{b}	0.087 ±0.00^{a}	0.003 ±0.00^{b}	0.006 ±0.00^{b}	0.05	543.452	<0.001	HS
	Water	0.001±0.00	0.001±0.00	0.00±0.00	0.003±0.00	0.001±0.00	0.002±0.00	0.05	1.57884	0.239117	NS
	Sediment	0.002±0.00^{b}	0.005±0.0^{a}	0.002±0.00^{b}	0.001±0.00^{b}	0.003±0.00^{b}	0.0017±0.0^{b}	0.05	4.72680	0.012847	S
Lead (Pb)	Insect	0.257 ±0.00	4.38 ±0.03	0.740±0.03	0.087 ±0.01	0.314±.020	2.253 ±0.01	0.01	0.00019	0.99981	NS
	Water	0.192±0.00^{c}	2.43±0.01^{a}	0.443±0.01^{c}	0.0867±0.01^{d}	0.254±0.00^{c}	1.877±0.01^{b}	0.01	5.70992	0.006356	S
	Sediment	0.250±0.00^{b}	2.78±0.08^{a}	0.503±0.04^{b}	0.137±0.00^{b}	0.336±0.02^{b}	2.177±0.01^{a}	0.01	896.344	<0.001	HS
Cadmium, (Cd)	Insect	0.512±0.02	1.12±0.04	0.573±0.01	0.639 ±0.01	0.497 ±0.00	0.953 ±0.01	0.01	0.00019	0.99981	NS
	Water	0.01±0.01	0.73±0.002	0.320±0.00	0.587±0.01	0.520±0.00	0.573±0.00	0.01	1.07426	0.4216749	NS
	Sediment	0.507±0.02	0.701±.025	0.397±.007	0.763±0.04	0.481±0.00	0.763±0.04	0.01	3.21848	0.0450694	S

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Characterization of Chironomids-Non-biting midge

Table 2: Chironomidae (Diptera) collected from sampling sites

Order: Diptera							
Family: Chironomidae							
Genus	Maboko island	Kisumu bay	Fish landing beache-FLB	Ndere island	Kendu bay	Homa Bay	Total
Ablebesmyia	01	17	02	23	00	01	44
Chironomus	01	01	01	00	09	12	14
Total	02	18	03	23	09	13	68

A total number of sixty-eight (68) Chironomids were collected from six sampling stations in Nyanza gulf of Lake Victoria Kenya based on morphological approach. Forty-four (44) belonging to the genus Ablebesmyia while 24 to genus Chironomus respectively as outlined in **Table 2.** Notably, the absence of genus Ablebesyia from Ndere island and Genus Chironomus from Kendubay was a striking feature. The striking and distinctive bright red color due to haemoglobin in the blood was used for the identification of Chironomidae. Hence the name blood fluke. In addition, the presence or absence of setae from the non-biting midge was used to define the family; subfamily and even the tribe. The segmented and elongated body form without the thoracic legs was also a key feature. Besides, the non-retractile head capsule with a long pair of antennae's; presence and absence of teeth, their location and number distinguished one family from the other. The location of teeth whether medial or lateral or both also offered a separating line. The description of the mentum and ventral plates, shape, length with or without lateral plates or both also offered distinctive features. The details were analyzed in reference to guides and works outlined in (Olafsson, 1992; Cranston, 1995a; Armitage et al., 1995; Coffman, 1996; Coffman & Ferrington, 1996; Brooks et al., 2008; Andersen et al., 2013) but not limited reference material. The study revealed the presence of non-biting midge larvae (*Chironomids*), across all the study sites (Plate 1). However, similarity and differences in *Chironomids* species was unclear. Hence, the need for molecular techniques to establish whether the species were similar or dissimilar across the stations.

Plate 1: Samples of Chironomid lavea collected from Winam gulf, Lake Victoria

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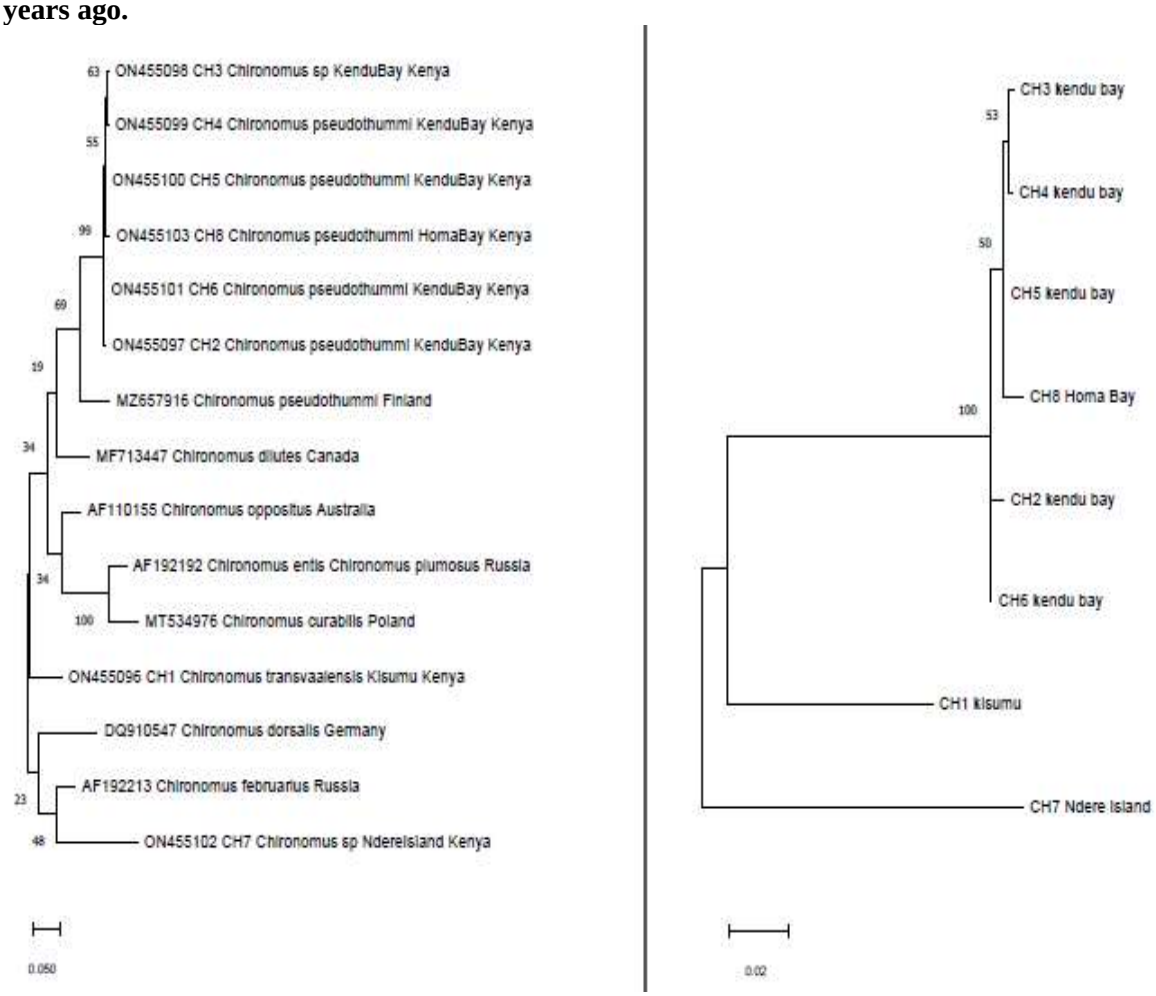
DNA Barcode Analysis

Eight sequences (CH1 – CH8) of the cytochrome oxidase 1 (*COx*1) gene were obtained from *Chironomus* insects which were distributed Winam Gulf (Figure 1). Comparison of the sequences with those from NCBI database through BLAST analysis resulted in above 97% similarity with *Chironomus pseudothummi* except isolate CH1 and CH7 which had 100% similarities with *Chironomus transvaalensis* strain and *Chironomus februarius* respectively (Fig. 2).

A neighbor-joining phylogenetic tree built from eight (8) sequences of the *COx*1 gene showed *Chironomus* insect species coalesced into three clades (Fig. 2). Insect species identified as CH1 from Kisumu bay and CH7 from Ndere Island separated into their own clades with NCBI species identified as *Chironomus februarius* strain AF1922314 from Russia and *Chironomus transvaalensis* strain EM5 from insect species CH2, CH3, CH4, CH5, CH6 and CH8 appeared to have had similarity in ancestry and clustered within the same clade.

Figure 1. Evolutionary relationships among the taxonomic groups inferred using the NJ method. Bootstrap probabilities are indicated on nodes as percentages while the scale represents millions of years ago.



A phylogenetic tree was constructed from sequences, revealing distinct clades and clusters with independent operational taxonomic units (OTUs) among insects. Reconstruction employed Neighbor Joining, Maximum Likelihood, and Maximum Parsimony algorithms, with the latter chosen for its in formativeness. A bootstrapping test, setting a threshold of >50% for significance, supported the analysis. Figure 1 results indicated that CH7 was an outlier from an offshore station—Ndere Island, located within Ndere National Park, known for minimal human disturbances and presumed cleanliness. CH1, CH2, CH3, CH4, CH5, CH6, and CH8 shared

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similar ancestry, evolving over 0.02 million years from CH7. CH7's evolution led to CH1 and CH2, followed by CH6, CH3, and finally CH5 and CH8. CH4 evolved into CH5 and CH8. CH1's mutation from a highly polluted station, possibly due to urbanization and industrialization in Kisumu, highlighted human-induced impact. Stations CH2, CH3, CH4, CH5, and CH6 exhibited close ancestry, linked to similar environmental and bio geographical characteristics in Homa Bay and Kendu Bay. Mutations occurred, indicating the influence of environmental closeness on clades. Additional CO1 sequences from NCBI Gen Bank showed spatial variations in Chironomus sp., identifying nine species—three from the study and six from Gen Bank. *Chironomus pseudothummi* predominated in Kendu Bay and Homa Bay (CH2; CH4; CH5; CH6), aligning with the accession numbers from Finland. Probability >55% in Chironomus spp. in Kendu Bay and Homa Bay suggested similar bio-geophysical characteristics, confirming intraspecific divergences and evolutionary histories in the Chironomus genus.

Estimation of average evolutionary divergences over sequence pairs within groups was carried out. The base substitution per station from the average of all sequence pairs within each group was approximated as in **Table 3** below with maximum value and minimum values of base substitution rate in a range of 0.002-0. 175. The species were closely related with a divergence value of 0.05 among the clades.

Table. 3: Estimates of Evolutionary Divergence between Sequences

	CH1_kisumu	**CH2_kendu_Bay**	**CH3_kendu_bay**	**CH4_kendu_Bay**	**CH5_kendu_Bay**	**CH6_kendu_Bay**	**CH7_Ndere_Island**	**CH8_Homa_Bay**
CH1_kisumu								
CH2_kendu_bay	0.144							
CH3_kendu_bay	0.142	0.011						
CH4_kendu_bay	0.140	0.011	**0.002**					
CH5_kendu_bay	0.142	0.008	**0.002**					
CH6_kendu_bay	0.140	**0.004**	0.006	0.006	0.004			
CH7_Ndere_Island	0.156	0.178	0.173	0.175	0.175	0.173		
CH8_Homa_Bay	0.142	0.015	0.008	0.008	0.006	0.011	0.173	

The numbers of base differences per site from between sequences are shown. This analysis involved 8 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There was a total of 473 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

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Table 4. Estimates of nucleotide substitution between sequences

From\To	A	T	C	G
A	-	7.7826	4.4320	**9.3727**
T	11.2474	-	**4.8275**	5.3534
C	11.2474	**8.4772**	-	5.3534
G	**19.6919**	7.7826	4.4320	-

The numbers of base substitutions per site from between sequences are shown in **Table 4** above. The substitution pattern and rates were estimated under the Tamura-Nei model, (Tamura & Nei, 1993). Analysis was conducted using the Maximum Composite Likelihood model [Tamura et al., 2004]. This analysis involved 8 nucleotides sequences. The nucleotide frequencies were: A = 39.03%, T/U = 27.01%, C = 15.38 %, and G = 18.58%. A tree topology was automatically computed. The maximum Log likelihood for this computation was -1179.657. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 473 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

Discussion

Freshwater ecosystems face significant threats from anthropogenic pollutants, particularly chemical hazards such as heavy metals. Heavy metal pollution leads to toxicity and the degradation of water's ecological integrity, altering ecological paradigms. These pollutants particularly affect vulnerable, sensitive, and pollution-intolerant aquatic species, thereby changing the structure of biological communities. Moreover, toxic elements within the ecosystem have a profound impact on the biodiversity of aquatic systems.

In this study, heavy metals were detected in both water and sediment samples, as well as in insect samples. This presence is linked to the physiology of the insects and the environment in which their larvae developed. The heavy metals identified included lead (Pb), arsenic (As), cadmium (Cd), zinc (Zn), and mercury (Hg), all known for their toxicity due to bioaccumulation and biomagnification within food chains and food webs. These metals were hypothesized to influence the development of Chironomid larvae in both water and sediment substrates. The study found that concentrations of these metals varied across different substrates, with relatively higher concentrations of Pb in water samples and lower levels of Hg within acceptable limits.

Cd levels were notably higher in urban areas such as Kisumu, Homa Bay, and Kendu Bay, which are associated with sewage, urbanization, and industrial effluents. Overall, toxic metal concentrations were significantly higher in sediment samples across all sampling stations, except for Hg, which remained below WHO standard limits. These results align with previously observed patterns of seasonal and spatial heterogeneity in metallic pollution (Su et al., 2022; Batool et al., 2020; Yuan et al., 2022; Olando et al., 2020).

For instance, studies on freshwater lakes have documented the presence of Pb, Zn, Ni, and Hg, generally within acceptable limits (Hashima et al., 2020; Olando et al., 2020; Showqi et al., 2018). Rehman et al. (2018), Gutiérrez-Ravelo et al. (2020), and Balalai-Mood (2021) also identified Pb and Cd as toxic metals and categorized metallic components into essential micronutrients (Cu, Zn, Fe, Mn, Co, Mo, Cr, Se) and macronutrients (Ca, Na, Mg, P, S), non-essential metals (Pb, Cd, Ni, As, Hg), and toxic metals (Pb, Cd). Additionally, Hare (1992) observed mercury concentrations exceeding WHO standards, while Tchounwou et al. (2012) and Shuhaimi-Othman (2012) confirmed the toxicity of metallic components in freshwater environments.

The present study identified a significant positive correlation between the concentrations of metals in water and sediment, particularly arsenic (As)

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and lead (Pb). A similar positive association was observed between the metallic components in water and those in insects, with arsenic showing a particularly strong correlation. Furthermore, a robust and significant correlation was also detected between the metal concentrations in insect samples and those in sediment.

Investigation into the macronutrients in insect samples revealed a negative correlation with the metal concentrations in water and sediment samples. However, a stronger correlation was found between the metallic components in sediment and those in insect samples compared to the correlation between water and insect samples. This can be attributed to the larval stage of the non-biting midge, which occurs and develops within the sediment, a substrate known to contain high concentrations of metallic elements. This developmental stage explains the strong positive correlation observed.

The non-biting midge undergoes four developmental stages, starting with the laying of eggs on the water surface. These eggs are influenced by the dynamics of water properties before sinking to the bottom, where they hatch into larvae. The larvae feed on suspended organic matter in the sediment, which forms the substrate for their development. As a result, the sediment, serving as the external environment for larval development, significantly impacts the insects. This explains the strong correlation between the metallic components in the insects and those in the sediment.

Any effluent into the water, particularly insoluble matter, can cause physiological variability in the larval forms of these insects (Jindal & Singh, 2020; Mohamed et al., 2020). Additionally, pollutants may lead to genetic variability in the organisms (Pedrosa et al., 2017a, 2017b). The presence of insoluble matter negatively impacts the life of the non-biting midge larvae, emphasizing the importance of understanding these correlations in environmental monitoring and management.

The present study on aquatic insects revealed the widespread presence of non-biting midge larvae, known as chironomids, across all sampling stations in the Winam Gulf. The abundance of non-biting midges, a species tolerant to pollution, is attributed to their ability to withstand harsh conditions such as low oxygen levels, nutrient-rich environments, and decomposing organic matter (Popovic et al., 2022; Prat & Castro-Lopez, 2023; Kranzfelder & Ferrington, 2015). These attributes make non-biting midges valuable bioindicators of environmental quality and useful for assessing ecosystem integrity.

Our findings are consistent with previous studies (Nicacio & Juen, 2015; Floss et al., 2012; Rossaro et al., 2022; Armstrong et al., 2021; Kranzfelder & Ferrington, 2015; Sumudumali & Jayawardana, 2021). For example, the hemoglobin in non-biting midge larvae, which gives them their red color, allows them to respire in anaerobic environments. They feed through filter feeding on suspended organic matter and mud. As they develop into pupae, they migrate through the water column to the surface, where they reach adulthood and fly off, increasing their survival chances (Frouz et al., 2003; Kranzfelder et al., 2015).

Beyond being indicators of water quality, non-biting midges play a crucial role in environmental cleanup by recycling organic matter, making them key components in aquatic food chains and webs. Predatory fish, particularly bottom feeders in benthic and profound zones, depend on midge larvae, as do other predatory insects, birds, and decomposers (Armitage, 1995; Cerba et al., 2023; Grzybkowska et al., 2020). This ecological role facilitates energy flow up the food chain, a critical function that has received minimal attention despite the fact that adult midges are also consumed by humans.

This research further explores the potential impacts of pollution on non-biting midges, highlighting their role as pollution-tolerant indicators and economically valuable species. The study underscores the importance of these insects in maintaining environmental health and their

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potential use as food and feed.Phylogenetic analyses of Chironomids

Distinguishing species using morphological similarities or dissimilarities among the Dipterans, remains a big challenge to Biologists and entomologists. Advancement in molecular biology has greatly resolved the problem of misidentification, (Hubner et al., 2017). Use of DNA sequences and development in bioinformatics has increased the speed, simplicity and accuracy. Moreover, species can be identified from partial remains and where species numbers have declined and facing the threat of extinction. Non-invasive methods such as eDNA can be employed for identification using species-specific primers, (Ruppert et al., 2019).In the present study, morphological characteristics were used for identification of Chironomids. The species being tolerant were detected in all stations presumably influenced by human activities. This inclination probably occurs due to adaptability and tolerance of the species. Pollution could also be a factor driving evolution acting as a selective pressure. This is supported by the rate of nucleotide substitution between the sequences (Table 4).

Sequencing of approximately 500 base pairs of the mt *CO1* region revealed three species-specific differences. The study was able to delineate the genus *Chironomus* into three species *Chironomus transvaalensus; Chironomus pseudothummi* and *Chironomus* species using cytochrome I oxidase subunit 1 as a vital barcode region. The study corroborates with a study by (Herbert et al., 2003; and Rodrigues et al., 2017) which was able to confirm that COX1 is a barcode for animal life for the ability to discriminate closely related species. This also agrees with the study of (Ekrem et al., 2007; Lin et al., 2015; Mrozińska & Obolewski, 2023) who demonstrated that mtDNA*CO1* sequences (along with *COII* and tRNA leu) could be used to identify the immature larvae of other forensically important *Diptera* (such as Lucilia *illustris*, *Phormia regina*, and *Phaenicia sericata*).

The results also prove that morphological and physiological characteristics are not conclusive in the study of diversity; rather a more reliable technique is to be used to distinguish the specimen in question that is *Chironomus.* This agreed with writings of Wink, 2007 which further indicated that genetic barcodes ids were more reliable (Rodrigues et al., 2017). COI gene is a perfect biomarker for the ability of detecting species with higher precision often misidentified manually, similar looking groups and with immature specimens (Nell et al., 2024; Ekrem et al., 2010; Cranston et al., 2013; Lehmann et al., 1998; Liu et al., 2023)

The present study showed interspecific variation between the three distinct *Chironomid species as* supported by calculations of pairwise differences between individuals providing validity of a grouping in the analysis. Each species formed distinct conspecific and monophyletic clusters. In the phylogenetic tree, the monophyletic separation of *C. transvalensis* (Kisumu), *C. psedothummi* (Kendu Bay) *and Chironomus sp.* (Ndere Island) supported by over 50% bootstrapping values confirmed the power of resolution of the genetic marker used in the present study to distinguish between closely related species. The observed nucleotide divergence as confirmed by evolutionary divergence between sequences or groups is consistent with the findings of (Nell et al.,2024). However, separation by COI was not supported significantly when considering sequences deposited in NCBI from other continents e.g., *C. psedothummi* from Kenya were separated from those of Canada and Russia with weak bootstrap values below 50%. The lack of support in this scenario could be due to geographical separation or allopatric speciation, each group evolving separately as a unique taxonomic unit. Moreover, species from regions with the similar climatic conditions e.g. Russia and Poland were closely related supported at 100% bootstrap values.

The present study therefore, delineated the genus *Chironomus* into three species *Chironomus transvaalensis, Chironomus pseudothummi* and *Chironomus* species using cytochrome I oxidase subunit 1 as a vital barcode region. The research in line with a study by Herbert *et al.*, (2003)

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confirmed COX1 barcode for animal life for the ability to discriminate closely related species. The results also proved that morphological and physiological characteristics were not conclusive in the study of diversity. Furthermore, a reliable technique was employed to distinguish the specimen in question - C*hironomids*. The results from the study agreed with writings of Wink, (2007) which further indicated that genetic barcodes ids were more reliable (Han et al., 2023; Gadawski et al.,2021; Nell et al.,2024).

Conclusion

In conclusion, the water analysis indicated significantly elevated levels of lead (Pb) beyond WHO/KEBS standards, while mercury (Hg) levels remained within limits. Cadmium (Cd) exhibited notable variations in Kisumu, Homa Bay, and Kendu Bay. Sediment analysis revealed excessive levels of metallic components, except for Hg, which was within limits. Insect analysis demonstrated exceedingly high Cd and Pb levels across all stations, with elevated arsenic (Ar) levels in water and sediment, in contrast to Hg staying below limits. Comparisons between inshore and offshore stations revealed significant variations in Pb and Zn. Overall; the results indicated a trend towards homogeneity in water quality parameters with minimal differences between inshore and offshore stations. The presence of heavy metals, including Ar, Hg, Cd, and Pb, suggested escalating toxicity levels in the Nyanza Gulf environment, signaling a deteriorating health state.

The study employed COX 1 to identify three Chironomidae species, namely *Chironomus transvaalensis*, *Chironomus pseudothummi*, and *Chironomus sp*., in Nyanza Gulf. DNA sequencing indicated distinct species from different locations, revealing mutations in CH3 Kendubay. The findings affirmed the significance of COX 1 in identifying larval Chironomidae, offering a more precise solution than traditional morphological identification. The evolutionary history results showed divergences likely linked to environmental stressors, particularly pollutants. Chironomus emerged as effective indicators of environmental changes, supporting bio-monitoring. Early warnings from these organisms can inform conservation and mitigation efforts in aquatic systems, preventing potential health hazards through food chains and webs. This study underscores the importance of monitoring and intervention for the sustainability of aquatic ecosystems and human health.

Statements and declarations

Ethics approval and consent to participate

National Commission for Science, Technology and Innovation, NACOSTI in partnership with Jaramogi Oginga University of Science and Technology, JOOUST provided ethical approval and consent to participate in current research.

Availability of Data and Material

All data will be availed upon request. In addition, all generated sequences are available to the public on the NCBI- website: https://www.ncbi.nim.nih.gov/genbank Accession number ON455096-ON455103.

Competing interests

Authors declare no competing interest.

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Author's contribution

MFM data collection, data analysis and drafting the manuscript. BO, TB conceptualization, designing, acquisition and analysis of data, DA and TB general supervision analysis of data, interpretation and review. POA and PO analysis, interpretation, uploading of molecular data to NCBI, review and editing of the manuscript. All authors discussed the results and approved the final manuscript.

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