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## Molecular Profiling of Malaria Vectors Adapted to Toxic Petroleum Contaminated Ecosystems in Akwa Ibom, Nigeria

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### ABSTRACT

Petroleum pollution imposes ecological stress capable of altering malaria vector distribution and composition. *Anopheles gambiae* sensu stricto (s.s.), a principal malaria vector in Africa, belongs to the *Anopheles gambiae* complex, which comprises morphologically indistinguishable sibling species. Fixed X-chromosome differences separate *An. gambiae* s.s. into the Mopti (M) and Savanna (S) molecular forms, identifiable only using genetic markers. These forms exhibit differential adaptation to toxicologically stressed environments, potentially influencing local vector dynamics. Consequently, molecular tools are essential for accurate species and form identification. This study investigated *Anopheles* species adapted to petroleum-contaminated habitats in Akwa Ibom State, Nigeria, between March and November 2024. Larvae and pupae were collected from breeding sites in Eastern Obolo and Ibeno LGAs, reared to adults, and morphologically identified. Genomic DNA was extracted from individual mosquitoes and analyzed using Polymerase Chain Reaction (PCR) targeting the ribosomal DNA Internal Transcribed Spacer 2 (ITS2) region to confirm species complex membership and identify sibling species. Molecular forms of *An. gambiae* s.s. were determined using PCR–Restriction Fragment Length Polymorphism (PCR-RFLP). Physicochemical and hydrocarbon analyses of breeding-site water were also conducted. Hydrocarbon contamination was confirmed at all sites. All specimens belonged to the *An. gambiae* complex, with only *An. gambiae* s.s. and *An. arabiensis* detected. *An. gambiae* s.s. predominated (94.7%), and both M and S forms occurred sympatrically, with the M form dominant (80.3%). The dominance of *An. gambiae* s.s. and the Mopti form showed strong positive correlations with hydrocarbon concentrations, indicating hydrocarbon-driven selection. Persistent petroleum pollution appears to exert selective pressure favoring ecotoxicologically resilient *Anopheles* lineages, underscoring the importance of integrating molecular surveillance with environmental monitoring for effective malaria risk prediction.

**Keywords:** *Anopheles* mosquitoes; Molecular taxonomy; rDNA; Petroleum pollution; Akwa Ibom state; Nigeria

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## INTRODUCTION

Female *Anopheles* mosquitoes are the primary transmitters of malaria, a disease that remains one of the most significant vector-borne parasitic diseases globally (Aribodor et al., 2016; WHO, 2024; Miriam et al., 2025). To date, over 500 *Anopheles* species have been documented (Charles & Godfray, 2013; Aschale, 2023). Among the approximately 70 species identified as malaria vectors, the most efficient transmitters worldwide are members of the *An. gambiae* complex and *An. funestus* complex (Charles & Godfray, 2013; Pimenta et al., 2015; Takken et al., 2024). In Africa, the most effective malaria vectors belong to the *An. gambiae* complex (Awolola et al., 2007; Tovi & Abdoulaye, 2008; Awolola, 2017; Ogola et al., 2018), which comprises seven sibling species that masquerade as a single indistinguishable species (Jan, 2016); exhibiting remarkable genetic heterogeneity in their rDNA internal transcribed spacer (ITS) and intergenic spacer (IGS) regions (Favia et al., 1997; della Torre et al., 2001; Favia et al., 2001). The principal African malaria vector, *Anopheles gambiae sensu stricto*, is one of these seven morphologically indistinguishable species (Awolola et al., 2007; Tovi & Abdoulaye, 2008; Awolola, 2017). Others are *An. arabiensis*, *An. bwambae*, *An. merus*, *An. melas*, *An. quadrinalutus A* and *An. quadrinalutus B*. Fixed differences in the rDNA intergenic spacer on the X chromosome of *An. gambiae s.s.* result in the Mopti (M) and Savanna (S) chromosomal forms (Favia et al., 1997). These genetic variants are cryptic isomorphic species that cannot be differentiated using classical morphological features but can be distinguished based on genetic variability. It becomes necessary to deploy both morphological and molecular protocols for accurate identification so as to detect diversity at local and regional levels (Jan, 2016).

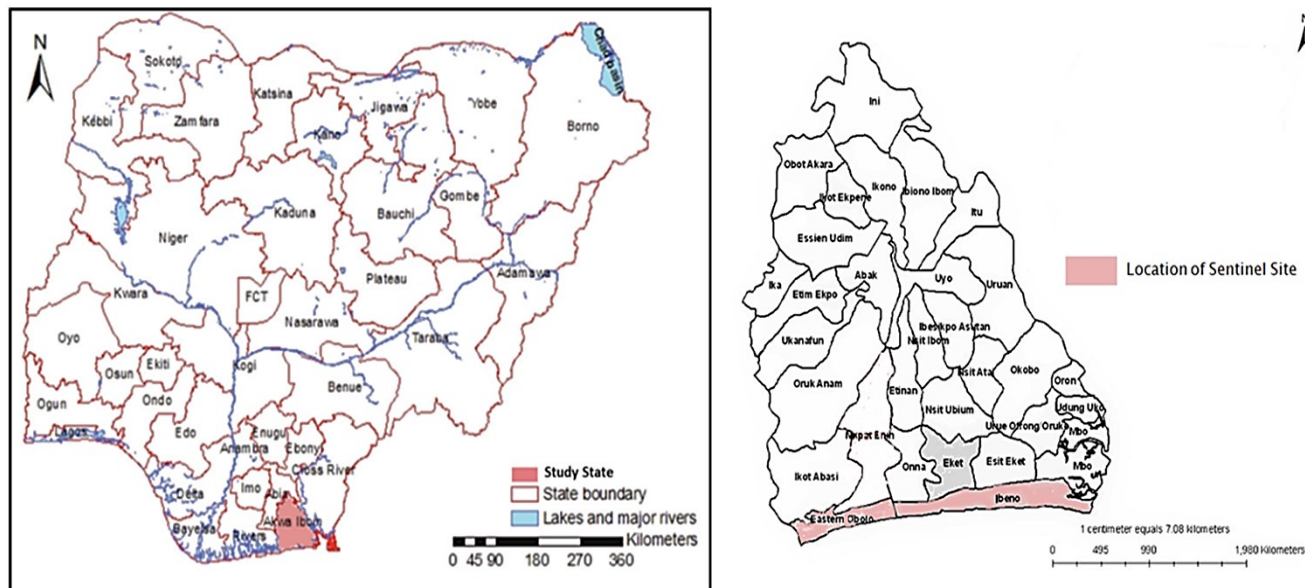
*Anopheles* mosquitoes generally breed in clear water with suitable pH, temperature, and nutrient composition (Service, 2012; Awolola, 2017). Pollution at breeding sites is known to influence vector distribution (Bilali et al., 2011; Ekpo et al., 2018). Physicochemical characteristics of larval habitats, including pH, temperature and concentrations of ammonia, nitrate and sulfate, affect larval development and survival. For instance, water with near-neutral pH (6.8–7.2) has been found optimal for weakening egg shells, allowing first-instar larvae to emerge (Okogun et al., 2003). However, some species have adapted to polluted habitats - sites previously considered unsuitable (Service, 2012). Successful breeding in oil-impacted waters, resulting in chronic exposure to petroleum-derived pollutants, has been reported (Imam & Yusuf, 2015; Miriam et al., 2025). Eastern Obolo and Ibeno Local Government Areas of Akwa Ibom State, Nigeria, experience frequent crude oil contamination of water bodies. Presence of petroleum contamination at breeding habitat of anopheles larvae is toxic to these immature aquatic stages and can kill them by suffocation or any specific toxic mechanism. Interestingly, while this can potentially reduce mosquito

populations, high mosquito densities persist in these areas, suggesting successful adaptation. According to WHO (2013), despite the oil-spillage events, the Niger Delta remains malaria-endemic. There is a significant variation amongst mosquito species on choice of breeding sites, distribution, vulnerability to insecticides, host preference, resting site preference and vectorial status (Awolola et al., 2002; WHO, 2015; Imam & Yusuf, 2015; Awolola, 2017). Effective, site-specific vector control interventions require comprehensive knowledge of local malaria vector species (Awolola, 2017). There remains a knowledge gap regarding the species of malaria vectors in these areas. This present study was aimed at addressing the knowledge gap.

## Materials and Methods

### Study site

The study areas, Eastern Obolo and Ibeno Local Government Areas of Akwa Ibom State, Nigeria (Figure 1), are crude oil rich environments constantly faced with crude oil pollution of water bodies. Eastern Obolo LGA is located in the Niger Delta fringe between Imo and Qua Iboe Rivers estuaries and lies between latitudes 4° 28' and 4° 53' and longitudes 7° 50' and 7° 55' East. It is bounded in the North by Mkpat Enin Local Government Area, North East by Onna and West by Ikot Abasi, South East by Ibeno Local Government Areas and in the South by the Atlantic Ocean. It has a total landmass of 117,008 square kilometers with an estimated shoreline of about 184 km long. Ibeno LGA lies in the Mangrove Forest Belt of the Niger Delta region of Nigeria. It is bounded in the West by Eastern Obolo local Government area, to the North by Onna, Esit Eket and Eket, to the South by the Atlantic Ocean. Ibeno Local Government Area is located at the South end of Akwa Ibom State, occupying a vast coastal area of over 1,200 km<sup>2</sup>. It stretches from Okposo I at the Eastern flank, bordering Mbo Local Government Area and Bakassi Peninsula to Atabrikang village on its Western flank. A total of thirty two (32) and thirty one (31) villages are gazette for Eastern Obolo and Ibeno LGAs respectively (AKS Ministry of Economic Development, 2016). Both study areas are located in the mangrove swamp ecozones where malaria transmission by the vectors is more prevalent between March and November (Ayanlade et al., 2010). With the aid of local guards, a pre-survey of the study areas was conducted. Villages notorious for high population density of mosquitoes were identified and considered as study sites. In Eastern Obolo LGA, these included Emereoke I, Emereoke II, Emeremen and Agansa. In Ibeno LGA, the study sites were Okuritip, Iwo-opom, Upenekang and Iwuonchang. All the study sites were characterized by thick bushes, swamps, forest, clustered residential buildings, water logs as well as shallowly dug wells vastly distributed. All these present good breeding ground (s) for mosquitoes; and such breeding sites were identified.



**Figure 1:** Map showing location of study sites

### Larvae collections and laboratory rearing of mosquitoes

This was done following the procedures of [Kabula et al. \(2011\)](#) with slight modifications. The study sites were surveyed on foot to locate the breeding sites such as muddy water, edges of rivers, swamps, shallowly dug wells, ponds, small pools of stagnant water, and irrigated vegetable farms in the study areas. *Anopheles* larvae were identified from their horizontal position on the surface of water and other species identified by their angular position. They were carefully collected with a 350 ml dipper and transferred into 10 litres plastic jerrycan which were loosely capped to allow aeration. The water samples containing larvae and other aquatic stages of *Anopheles* were then transported to the USAID/PMI/VECTORLINK Malaria Research Laboratory/Insectary in the Department of Animal and Environmental Biology, Faculty of Science, University of Uyo for rearing. In the laboratory, they were transferred into a rectangular plastic container (with a capacity of 20 liters) and covered with a net of tiny meshes to for allow aeration as well as prevent the adult that will emerge from escaping. The larvae were fed with a blend of cabin biscuit and yeast prepared by grinding two pieces of cabin biscuits and 10 tablets of yeast together. Following emergence of the adults, they were aspirated using aspirators into adults' mosquito cages. The adult populations were maintained by feeding them with ten percent (10 %) sugar solution.

### Analysis of physico-chemical parameters of water samples

Physico-Chemical parameters were measured from water samples collected from breeding sites of *Anopheles* using standard methods as described by The [American Public Health Association \(2005\)](#). Parameters that were measured included turbidity, temperature, pH, electrical conductivity,

total dissolved solids (TDS), total hardness, total alkalinity, Biological oxygen demand (BOD), Dissolved oxygen (DO), calcium, magnesium, sulphate, nitrite, nitrate, phosphate and total hydrocarbon content (THC). Some of physico-chemical parameters were measured *in situ*. Water samples were collected from the mosquito breeding sites into 1.5 liters plastic bottles for physicochemical analyses. The water samples were taken to the USAID/PMI/VECTORLINK Malaria Research Laboratory/Insectary and preserved for *ex situ* physicochemical analysis.

### Morphological identification

The adult mosquitoes were first knocked down at freezing temperature ( $-4^{\circ}\text{C}$ ) using a freezer (Haier THERMOCOOL). They were morphologically identified following standard protocol and using the morphological keys ([Kent, 2006](#)) and dissecting microscope (Olympus, USA). Female *Anopheles* mosquitoes were identified and isolated from male *Anophelines* by recognizing the males as having feathery antennae while the females lacked this morphological feature. Morphological identification was based on diagnostic characteristics such as wing scale patterns, palpal morphology and tarsal banding. The females (a total of 3,600) were then preserved individually in Eppendorf tubes over desiccated silica gel and labeled with unique identification number for molecular identification.

### Standard polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) assays for authentication and species composition profiling

Genomic DNA was extracted from individual mosquitoes following standard protocols. Species within the *Anopheles*

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*gambiae* complex were identified by a ribosomal DNA-based PCR assay using species-specific primers (UN universal forward; GA, ME, AR, QD reverse primers targeting *An. gambiae* s.s., *An. melas*, *An. arabiensis*, and *An. quadriannulatus*, respectively) as previously described (Scott et al., 1993; Koekemoer et al., 2002). Briefly, 50 µL PCR reactions contained 14 µL OneTaq 2× Master Mix (New England Biolabs, USA), 2 µL each primer, 21 µL nuclease-free water, and 5 µL DNA template. Amplification was performed in a thermal cycler with an initial denaturation at 95 °C for 5 min, 35 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 60 s, and a final extension at 72 °C for 5 min (Scott et al., 1993). PCR products were resolved on 2% agarose gels stained with ethidium bromide and visualized under UV illumination; fragment sizes were estimated against a 100 bp ladder to assign species identity (Scott et al., 1993; Kabula et al., 2011).

Specimens identified as *An. gambiae* s.s. were further differentiated into M and S molecular forms using a PCR-RFLP protocol that exploits single nucleotide polymorphisms in the ribosomal DNA intergenic spacer region (Fanello et al., 2002). Aliquots of PCR product were digested with HhaI restriction enzyme at 37 °C for 3 h and separated on 2% agarose gels. Digestion patterns corresponding to M (315 bp) and S (290 bp) molecular forms were determined by comparison with positive controls run alongside samples (Scott et al., 1993; Fanello et al., 2002).

All reactions included negative controls (no DNA template) to monitor contamination. Species and molecular form assignments were based on diagnostic band sizes established in the literature (Scott et al., 1993; Fanello et al., 2002).

### Statistical analysis

Elementary techniques of descriptive statistics (mean, standard deviation, range and percentages) were employed to analyze the physico-chemical parameters' data and specie profile data. Analysis of variance and Turkey krammer post-

hoc test were used in establishing the significant variations in physico-chemical parameters. The relationship between physicochemical properties of water in breeding sites and distribution of genetic variants of *An. gambiae* s.l were evaluated using Pearson product moment correlation (PPMC) analysis. The level of significance in testing the hypothesis was 0.05 i.e, 95 % confidence level.

## Results

### Physicochemical parameters of breeding sites

Physicochemical parameters of mosquito breeding waters exhibited marked spatial variability across study sites (Tables 1 and 2). pH values remained within EPA guideline limits, whereas water temperatures at all sites exceeded recommended thresholds for freshwater ecosystems. Electrical conductivity and total dissolved solids were consistently below regulatory limits. Dissolved oxygen concentrations were uniformly low, indicating hypoxic conditions, while biological oxygen demand remained within acceptable limits. Total alkalinity and hardness were generally compliant with EPA standards, although localized exceedances were observed, particularly at Agansa. Turbidity levels were low across all sites. In contrast, nitrite nitrogen concentrations exceeded EPA limits at all sites, indicating nutrient enrichment. Total hydrocarbon concentrations exceeded regulatory thresholds at all sites except Iwuonchang, reflecting widespread petroleum contamination of mosquito breeding habitats. Multivariate analysis revealed significant site-specific differences ( $p < 0.05$ ). In Eastern Obolo LGA, breeding waters at Agansa showed significantly lower temperatures, while Emereoke 2 exhibited significantly elevated conductivity and hydrocarbon concentrations. In Ibeno LGA, most physicochemical parameters at Iwuonchang differed significantly from other sites, except pH, temperature, and magnesium.

**Table 1:** Physico-chemical parameters of larval breeding Sites from Ibeno LGA

Parameters	Upenekang	Iwo Okpom	Iwuonchang	Okoroutip	EPA MPL
pH	7.54 ± 0.52	7.85 ± 0.23	7.30 ± 0.09	7.82 ± 0.19	6.5-9.0
Temperature (°C)	34.16 ± 0.69	34.20 ± 0.93	34.07 ± 0.85	33.88 ± 0.69	< 3°C
Conductivity (µS/ cm)	383.52 ± 16.42	358.02 ± 21.49	277.98 ± 59.41 <sup>ab</sup>	257.18 ± 21.16 <sup>ab</sup>	2500 µS/ cm
TDS (ppm)	114.60 ± 4.83	122.80 ± 7.98	84.82 ± 9.05 <sup>ab</sup>	122.36 ± 7.58 <sup>c</sup>	50 mg/l
DO (mg/l)	3.06 ± 0.21	3.09 ± 0.22	2.07 ± 0.19 <sup>ab</sup>	3.03 ± 0.28 <sup>c</sup>	50 mg/l
BOD (mg/l)	1.09 ± 0.11	1.11 ± 0.14	0.69 ± 0.15 <sup>ab</sup>	1.08 ± 0.12 <sup>c</sup>	5 mg/l
Total alkalinity (mg/l)	169.60 ± 11.57	199.20 ± 18.98 <sup>a</sup>	121.80 ± 12.91 <sup>ab</sup>	196.70 ± 18.71 <sup>c</sup>	200 mg/l
Total hardness (mg/l)	181.20 ± 8.70	212.60 ± 19.20 <sup>a</sup>	133.60 ± 12.95 <sup>ab</sup>	212.32 ± 18.74 <sup>ac</sup>	<200 mg/l
Calcium (mg/l)	23.38 ± 2.48	28.78 ± 1.98 <sup>a</sup>	13.76 ± 1.61 <sup>ab</sup>	28.58 ± 2.11 <sup>ac</sup>	-
Magnesium	20.46 ± 2.65	25.58 ± 1.98	25.07 ± 2.13	20.46 ± 2.65	30 mg/l
Turbidity (NTU)	0.40 ± 0.04	0.44 ± 0.11	0.37 ± 0.042	0.42 ± 0.08	5 NTU
Phosphate (mg/l)	1.65 ± 0.17	1.56 ± 0.21	0.73 ± 0.13 <sup>ab</sup>	1.50 ± 0.19 <sup>c</sup>	-
Nitrite (mg/l)	1.32 ± 0.21	1.05 ± 0.33	0.38 ± 0.14 <sup>ab</sup>	0.87 ± 0.11 <sup>ac</sup>	0.5 mg/l
Nitrate (mg/l)	1.98 ± 0.12	1.81 ± 0.13	1.00 ± 0.23 <sup>ab</sup>	1.71 ± 0.11 <sup>c</sup>	50 mg/l
Sulphate (mg/l)	2.80 ± 0.52	2.48 ± 0.49	1.59 ± 0.44 <sup>ab</sup>	2.40 ± 0.38	100 mg/l
Total hydrocarbon (mg/l)	8.34 ± 1.31	8.90 ± 1.33	4.22 ± 0.26 <sup>ab</sup>	8.77 ± 1.29 <sup>c</sup>	5 mg/l

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*a* =  $p \leq 0.05$  (comparing values in other study sites to what was observed in Upenekang); *b* =  $p \leq 0.05$  (comparing values in other study sites to what was observed in Iwo Okpom). *c* =  $p \leq 0.05$  (comparing values in other study sites to what was observed in Iwuonchang).

**Table 2:** Physico-chemical parameters of larval breeding Sites in Eastern Obolo LGA

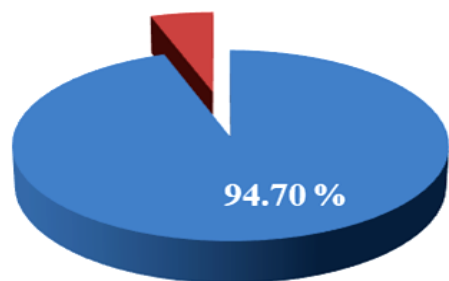
Parameters	Emereoke 1	Emereoke 2	Emeremen	Agansa	EPA MPL
pH	7.65 ± 0.16	7.99 ± 0.09 <sup>a</sup>	7.88 ± 0.04	7.76 ± 0.14 <sup>b</sup>	6.5-9.0
Temperature (°C)	34.01 ± 1.25	34.72 ± 0.76	34.43 ± 0.51	29.93 ± 1.09 <sup>a,b,c</sup>	< 3°C
Conductivity (µS/ cm)	373.20 ± 43.21	480.00 ± 26.96 <sup>a</sup>	367.35 ± 32.78 <sup>b</sup>	382.16 ± 14.68 <sup>b</sup>	2500 µS/ cm
TDS (ppm)	126.00 ± 9.81	129.90 ± 10.45	128.30 ± 11.81	123.00 ± 9.20	50 mg/l
DO (mg/l)	3.18 ± 0.36	3.18 ± 0.55	3.14 ± 0.46	3.06 ± 0.25	50 mg/l
BOD (mg/l)	1.17 ± 0.22	1.23 ± 0.41	1.16 ± 0.46	1.09 ± 0.17	5 mg/l
Total alkalinity (mg/l)	196.50 ± 21.17	188.20 ± 22.28	187.00 ± 15.34	201.25 ± 21.27	200 mg/l
Total hardness (mg/l)	208.25 ± 20.35	198.80 ± 18.51	195.75 ± 16.36	215.50 ± 20.87	<200 mg/l
Calcium (mg/l)	28.10 ± 2.21	26.38 ± 4.11	27.40 ± 0.67	29.13 ± 2.10	
Magnesium	25.08 ± 2.13	23.92 ± 3.78	23.55 ± 0.82	25.78 ± 2.23	30 mg/l
Turbidity (NTU)	0.39 ± 0.03	0.33 ± 0.11	0.39 ± 0.04	0.43 ± 0.12	5 NTU
Phosphate (mg/l)	1.79 ± 0.12	1.87 ± 0.12	1.90 ± 0.17	1.60 ± 0.17	
Nitrite (mg/l)	1.39 ± 0.41	1.52 ± 0.13	1.59 ± 0.23	1.14 ± 0.30	0.5 mg/l
Nitrate (mg/l)	2.23 ± 0.51	2.27 ± 0.14	2.31 ± 0.46	1.85 ± 0.11	50 mg/l
Sulphate (mg/l)	2.16 ± 0.25	2.48 ± 0.47	2.16 ± 0.25	2.48 ± 0.57	100 mg/l
Total hydrocarbon (mg/l)	9.80 ± 1.54	12.58 ± 1.17 <sup>a</sup>	8.75 ± 0.66 <sup>b</sup>	8.83 ± 1.52 <sup>b</sup>	5 mg/l

*a* =  $p \leq 0.05$  (comparing values in other study sites to that of Emereoke 1); *b* =  $p \leq 0.05$  (comparing values in other study sites to what was observed in Emereoke

**Morphological and Molecular Identification of Anopheles Species**

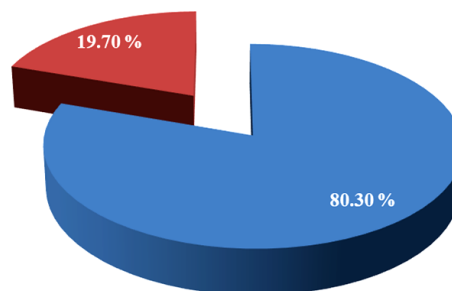
Morphological identification confirmed that all female mosquitoes belonged to the *Anopheles gambiae* complex. Molecular characterization using PCR amplification of the ITS2 region and agarose gel electrophoresis (Figures 4 and 5) showed that *Anopheles gambiae* sensu stricto (s.s.) was the predominant species across all study sites, accounting for 94.7% of specimens and producing the diagnostic 390 bp fragment, while *Anopheles arabiensis* represented 5.3% and exhibited a 315 bp fragment. Both species

occurred sympatrically at all eight sampling locations, with *An. gambiae* s.s. consistently dominating the populations (Figure 2; Table 3); and no additional members of the *An. gambiae* complex were detected. PCR-RFLP analysis using *HhaI* revealed the presence of both the Mopti (M) and Savanna (S) molecular forms of *An. gambiae* s.s. throughout the study area (Figure 6). The M form was predominant, comprising 80.3% of specimens, while the S form accounted for 19.7% (Table 3; Figure 4), with both forms occurring in sympatry across all site.



■ *An. gambiae* sl  
 ■ *An. arabiensis*

**Figure 2:** Percentage proportion of *Anopheles gambiae* sibling species in the *Anopheles gambiae* complex recorded in the study.



■ M form  
 ■ S form

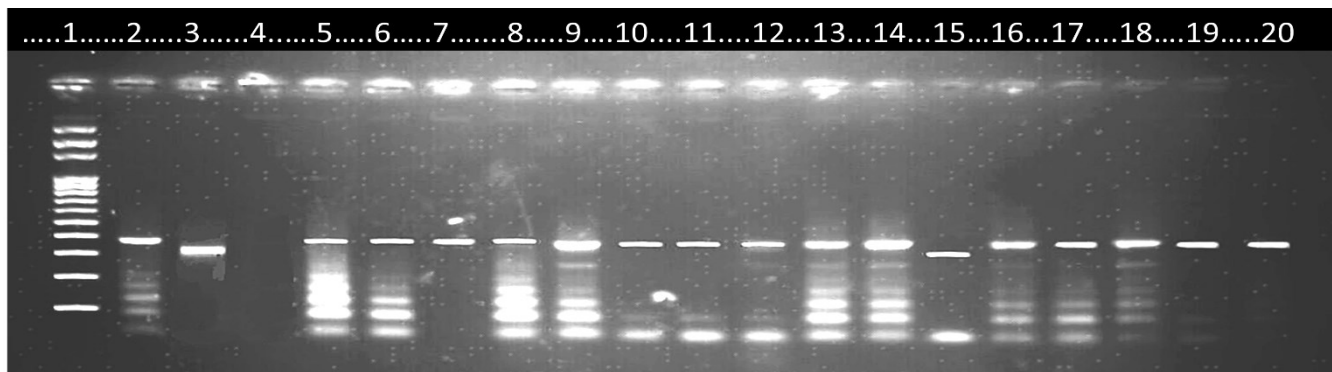
**Figure 3:** Percentage proportion of *Anopheles gambiae* s.s. molecular forms recorded in the Study

**Table 3:** Summary of AS-PCR specie identity of the *Anopheles* mosquitoes across the eight study sites

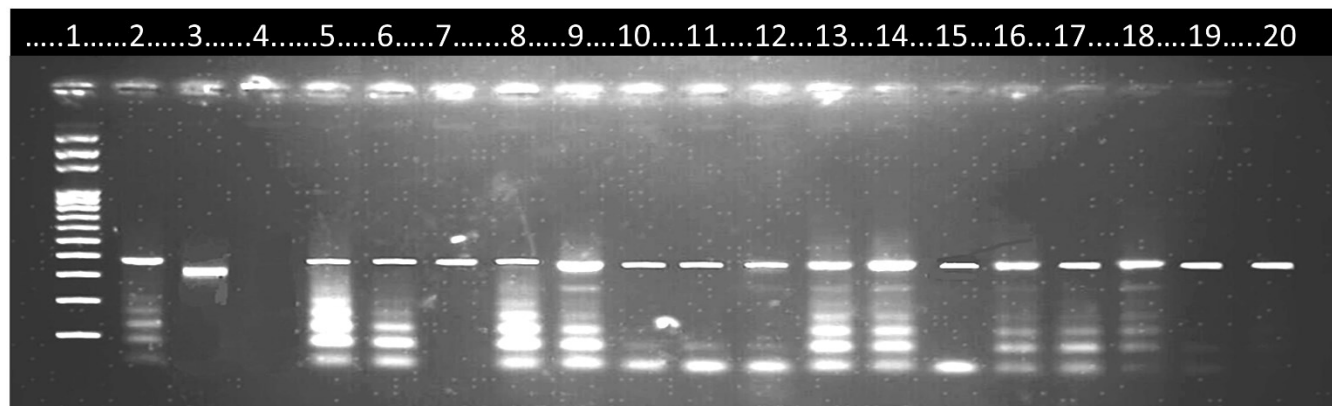
Study Sites	No Sampled	<i>An. gambiae</i> s.s (%)	<i>An. Arabiensis</i> (%)
Emeroke 1	45	42 (93.3)	3 (6.7)
Emereoke 2	45	44 (97.8)	1 (2.2)
Emeremen	45	42 (93.3)	3 (6.7)
Agansa	45	43 (95.6)	2 (4.4)
Upenekang	45	43 (95.6)	2 (4.4)
Iwo Okpom	45	43 (95.6)	2 (4.4)
Iwuonchang	45	41 (91.1)	4 (8.9)
Okoroutip	45	43 (95.6)	2 (4.4)
Total	360	341 (94.7)	19 (5.3)

**Table 4:** Summary of PCR-RFLP molecular forms identity of the *Anopheles gambiae* s.s across the eight study sites

Study Sites	Number sampled	M form	S form
Emeroke 1	40	35(87.5)	5(12.5)
Emereoke 2	40	38(95)	2(5)
Emeremen	40	33(82.5)	7(17.5)
Agansa	40	32(80)	8(20)
Upenekang	40	31(77.5)	9(22.5)
Iwo Okpom	40	30(75)	10(25)
Iwuonchang	40	25 (62.5)	15 (37.5)
Okoroutip	40	33 (82.5)	7 (17.5)
Total	320	257(80.3)	63(19.7)

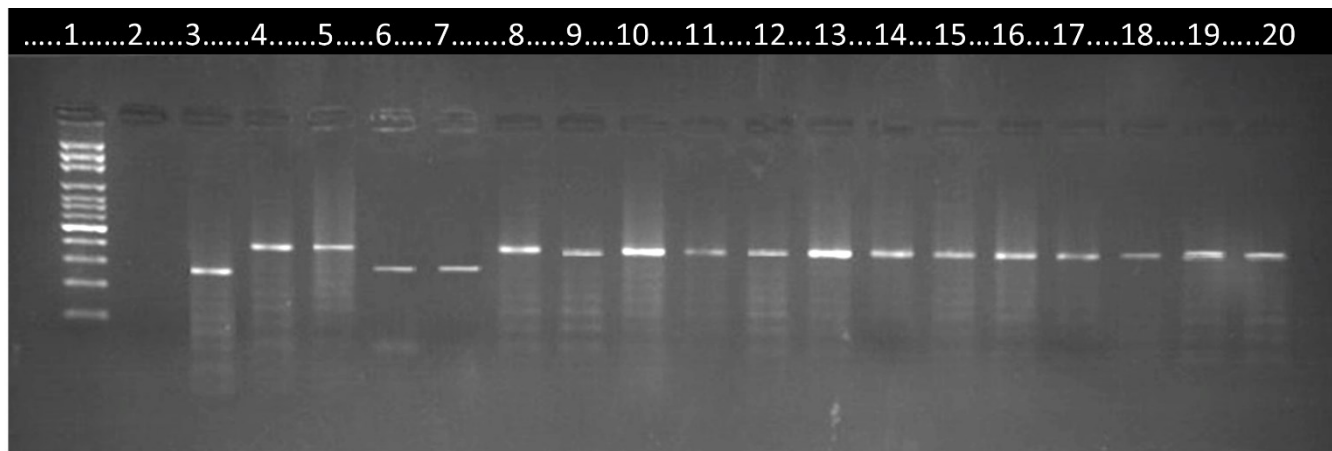


**Figure 4:** UV illuminated Agarose gel showing PCR amplified DNA fragments bands for identification of members of *An. gambiae* complex from study sites. Lane 1: 100-basepair, DNA size marker ladder, Lane 2: *An. gambiae* s.s. positive control (390 bp), Lanes 3: *An. Arabiensis* positive control (315 bp), Lane 4: Negative control, , Lane 5-14; 16-20: *An. gambiae* s.s (wild samples), Lane 15: *A. Arabiensis* (wild samples).



<https://dx.doi.org/10.4314/br.v24i1.17>

**Figure 5:** UV illuminated Agarose gel showing PCR amplified DNA fragments bands for identification of members of *An. gambiae* complex from study sites. Lane 1: 100-basepair, DNA size marker ladder, Lane 2: *An. gambiae* s.s. positive control (390 bp), Lanes 3: *An. Arabiensis* positive control (315 bp), Lane 4: Negative control, Lane 5-20: *An. gambiae* s.s (wild samples)



**Figure 6:** UV illuminated Agarose gel showing DNA fragments bands of PCR amplification for identification of the molecular M and S forms of *An. gambiae sensu strictu* from study sites. Lane 1: 100-basepair DNA size marker ladder, Lane 2: Negative control, Lanes 3: S form positive control (290 bp) Lane 4: M form positive control (315 bp), Lanes 5 - 20: M forms (wild samples). Lane 6 and 7: S forms (wild samples).

**Pearson Product–Moment Correlation Between the Distribution of Sibling Species/Molecular Forms of Female *Anopheles* Mosquitoes and Physico-chemical Characteristics of Breeding Habitats**

The bivariate correlation analysis using Pearson’s correlation coefficient (Table 5) demonstrated that the relative abundance of *Anopheles gambiae* s.s. was significantly ( $p < 0.05$ ) and positively associated with pH ( $r = 0.737, p = 0.037$ ), total hydrocarbons ( $r = 0.813, p = 0.014$ ), biochemical oxygen demand (BOD;  $r = 0.715, p = 0.046$ ), and sulphate concentrations ( $r = 0.844, p = 0.008$ ). Conversely, these same parameters exhibited significant negative correlations (identical r and p values)

with the abundance of *An. Arabiensis*. Similarly, Table 5 shows that the molecular M form exhibited significant ( $p < 0.05$ ) positive correlations with alkalinity ( $r = 0.708, p = 0.049$ ), pH ( $r = 0.777, p = 0.023$ ), total hydrocarbons ( $r = 0.955, p < 0.001$ ), dissolved oxygen ( $r = 0.820, p = 0.032$ ), phosphate ( $r = 0.866, p = 0.005$ ), nitrite ( $r = 0.809, p = 0.015$ ), nitrate ( $r = 0.866, p = 0.005$ ), and magnesium concentrations ( $r = 0.720, p = 0.044$ ). In contrast, all these physicochemical parameters were significantly ( $p < 0.05$ ) and negatively correlated with the S molecular form, with correlation coefficients and p-values identical to those reported for the M form

**Table 5:** Pearson product moment correlation on the association between the distribution of sibling species/molecular forms of malaria vectors and the physicochemical parameters of water

	<i>An. gambiae</i> s.s	<i>An. Arabiensis</i>	M form	S forms
Alkalinity	0.639	-0.639	0.708*	-0.708*
Temperature	-0.085	0.085	0.096	-0.096
pH	0.737*	-0.737*	0.777*	-0.777*
THC (mgL <sup>-1</sup> )	0.813*	-0.813*	0.955**	-0.955**
EC (µs/cm)	0.376	-0.376	0.346	-0.346
DO	0.697	-0.697	0.820*	-0.820*
BOD	0.715**	-0.715**	0.894**	-0.894**
PO <sub>4</sub> <sup>2-</sup> (MgL <sup>-1</sup> )	0.605	-0.605	0.866**	-0.866**
Nitrite (MgL <sup>-1</sup> )	0.495	-0.495	0.809*	-0.809*
Nitrate (MgL <sup>-1</sup> )	0.517	-0.517	0.866**	-0.866**
SO <sub>4</sub> <sup>2-</sup> (MgL <sup>-1</sup> )	0.844**	-0.844**	0.494	-0.494
Magnesium (MgL <sup>-1</sup> )	0.649	-0.649	0.720*	-0.720*
Calcium (MgL <sup>-1</sup> )	0.617	-0.617	0.697	-0.697

Values represent the correlation coefficients (r-values) \*\*. Correlation is significant at 0.01 levels (2-tailed). \*. Correlation is significant at 0.05 levels (2-tailed).

## Discussion

Understanding vector diversity in ecologically stressed environments is critical for predicting malaria transmission risk. In this study, morphological identification showed that all female anopheline mosquitoes collected from larval habitats in Ibeno and Eastern Obolo LGAs belonged to the *Anopheles gambiae* complex. This agrees with reports that the *An. gambiae* complex dominates malaria vector populations across sub-Saharan Africa, including Nigeria (Samdi et al., 2006; Sinka et al., 2010; Adeogun et al., 2023). Although *Anopheles funestus* has historically been a major vector in Nigeria (Molineaux & Gramiccia, 1980), only *An. gambiae* sensu lato was detected in the petroleum-polluted habitats studied, suggesting habitat filtering under chemical stress. Because sibling species in a complex are morphologically indistinguishable and they differ greatly in vectorial capacity and environmental tolerance (Jan, 2016), molecular identification was essential. Molecular assays revealed only *Anopheles gambiae* sensu stricto ( $\approx 95\%$ ) and *Anopheles arabiensis* in the *An. gambiae* complex populations across the study sites. This is consistent with recent distributional and molecular studies in Nigeria (AIRS, 2015; Adeogun et al., 2023). The dominance of *An. gambiae* s.s. across all sites likely reflects its ecological resilience to chemically degraded environments. Although direct evidence linking petroleum pollution to sibling-species composition is limited, *An. gambiae* s.s. has been shown to breed successfully in polluted waters with high conductivity, turbidity, hydrocarbons, and heavy metals (Awolola, 2002; Imam & Yusuf, 2015; Awolola et al., 2017), supporting earlier assertions of its adaptive advantage in chemically stressed habitats (Awolola et al., 2002; WHO, 2015; Imam & Yusuf, 2015; Awolola, 2017). Both M and S molecular forms of *An. gambiae* s.s. were present, but the M form predominated (83%), consistent with its reported adaptation to permanent, predator-rich, or polluted larval habitats (Diabaté et al., 2008). The absence of M/S hybrids aligns with observations from Cameroon, Ghana, and Angola (Yawson et al., 2004; Bilali et al., 2011), supporting partial reproductive isolation. The predominance of the M form in petroleum-contaminated sites suggests strong selective pressures driving molecular-level adaptation, indicative of ecotoxicogenomic responses in polluted ecosystems.

Physicochemical analyses revealed elevated total hydrocarbon content (THC), biochemical oxygen demand (BOD), sulphates, altered pH, and surface oil films, with several parameters exceeding regulatory limits, confirming petroleum contamination of breeding sites. Such conditions are established indicators of polluted aquatic habitats and are associated with shifts in invertebrate community structure (Omoigberale et al., 2020). Significant positive correlations ( $p < 0.05$ ) were observed between *An. gambiae* s.s. abundance and elevated THC,

BOD, sulphates, and pH. These associations reflect ecotoxicogenomic signatures, suggesting genetic or physiological traits that confer tolerance to hydrocarbon pollution, potentially involving altered development (Kats & Dill, 1998; Juliano & Gravel, 2001). The dominance of pollution-tolerant *An. gambiae* s.s., particularly the M form, in petroleum-impacted habitats presents important epidemiological implications. Such environments may act as ecological refugia for resilient vector genotypes capable of sustaining malaria transmission under degraded conditions.

Overall, this study demonstrates that petroleum pollution in Akwa Ibom State imposes strong ecological and genomic selection on malaria vector populations, shaping their composition and adaptive structure. These insights are essential for informing targeted vector control strategies in Nigeria's oil-producing regions.

## Conclusion

This study demonstrates that petroleum-polluted larval habitats in Ibeno and Eastern Obolo LGAs of Akwa Ibom State are dominated by *Anopheles gambiae* sensu stricto, particularly the M molecular form, reflecting its ecological resilience and tolerance to chemically degraded environments. Molecular analyses confirmed the near absence of other sibling species, highlighting selective pressures imposed by hydrocarbon contamination. Significant correlations between vector abundance and physicochemical parameters - including total hydrocarbon content, BOD, sulfates, and altered pH - indicate the presence of ecotoxicogenomic signatures in these mosquito populations. These findings suggest that petroleum-impacted ecosystems may serve as ecological refugia for robust, pollution-tolerant vectors capable of sustaining malaria transmission. Understanding such adaptive responses is critical for guiding targeted vector control strategies in Nigeria's oil-producing regions and for predicting malaria risk under environmental stress. Previous works have shown that these genetic variants can successfully resist pyrethroids; and as such, it can also be concluded that the use of pyrethroid based materials in these areas for their control may likely be compromised.

### Conflicts of Interest

The authors declare no conflicts of interest.

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