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Monitoring mosquito richness in understudied area: Can environmental DNA metabarcoding be a complementary approach to adult trapping?

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Abstract

Mosquito surveillance programs are essential to assess the risks of local vector-borne disease outbreaks as well as for early detection of mosquito invasion events. Surveys are usually performed with traditional sampling tools (i.e., ovitraps and dipping method for immature stages or light or decoy traps for adults). Over the past decade, numerous studies have highlighted that environmental DNA (eDNA) sampling can enhance invertebrate species detection and provide community composition metrics. However, the usefulness of eDNA for detection of mosquito species has, to date, been largely neglected. Here, we sampled water from potential larval breeding sites along a gradient of anthropogenic perturbations, from the core of an oil palm plantation to the rainforest on São Tomé Island (Gulf of Guinea, Africa). We showed that (i) species of mosquitoes could be detected via metabarcoding mostly when larvae were visible, (ii) larvae species richness was greater using eDNA than visual identification, (iii) new mosquito species were also detected by eDNA approach. We provide a critical discussion of the pros and cons of eDNA metabarcoding for monitoring mosquito species diversity and recommendations for future research directions that could facilitate the adoption of eDNA as a tool for assessing insect vector communities.

Keywords: invasive species, metabarcoding, oil palm plantation, rainforest, vectors.

43 **Introduction**

44 Factors associated with global change, such as temperature increase, land-use change
45 and the increasing spread of invasive species, are leading to a considerable loss and
46 reorganization of biodiversity (Hobbs, 2000; Segan et al., 2016; Eriksson et al., 2019),
47 with important consequences for the emergence of infectious diseases that affect
48 wildlife, livestock and human populations (Caminade et al., 2019; Smith et al., 2019).
49 Among global emerging infectious disease events, vector-borne diseases are
50 disproportionately over-represented (Swei et al., 2020) and constant efforts for
51 monitoring insect vector populations should be carried out in locations at risk (Pedersen
52 et al., 2009). Among insect-vectors, mosquitoes (Culicidae), with more than 3,500
53 described species widely distributed around the world (Harbach, 2013), are considered
54 among the main insect-vectors involved in the transmission of pathogens including
55 viruses, protozoans and nematodes. Three main genera, *Anopheles*, *Aedes* and *Culex*
56 are considered of medical importance for humans and transmit pathogens causing
57 diseases to more than 700 million people annually, resulting in over one million deaths
58 (WHO, 2020). In the last decades, the rapid worldwide spread of the invasive yellow
59 fever mosquito *Aedes aegypti* and the Asian tiger mosquito *Aedes albopictus* is
60 producing novel epidemiological scenarios (Bonizzoni et al., 2013; Iwamura et al.,
61 2020). Early detection of mosquito invasion events, as well as continued surveillance
62 of such invasions, is becoming essential to assess the risks of local mosquito-borne
63 disease outbreaks. In addition, it seems essential to understand the ecological
64 interactions between mosquito species at breeding sites to evaluate the competitiveness
65 of indigenous species (Juliano et al., 2009).

66

67 To date, surveys of mosquito species have been performed with traditional
68 sampling using ovitraps and dipping method for immature stages or with light/decoy
69 traps and human landing catches for adults (Focks, 2004). Skilled entomologists are
70 able to identify specimens using morphological traits (Besansky et al., 2003; Hajibabaei
71 et al., 2007), however some species are indistinguishable morphologically (e.g., cryptic
72 species of *Anopheles*) (Coetzee and Koekemoer, 2013). In addition, the identification
73 of different mosquito stages (i.e., eggs, larvae and adult mosquito specimens) needs
74 solid knowledge from experts in entomology. The identification can be time-
75 consuming, especially if the specimens are too damaged, and in particularly in the
76 tropics where the diversity is often high (Foley et al., 2007). Developments in molecular
77 techniques over the past decade, coupled with reduced sequencing costs, have made the
78 use of environmental DNA (eDNA) as an approach with a huge potential to survey
79 micro-biodiversity in the field. Environmental DNA is DNA that is shed by organisms
80 (e.g., through faecal waste, dead skin, gastrointestinal tract cells, gametes or via post-
81 mortem degradation), and it has formed the basis of numerous studies focussed on
82 vertebrate detection (Ficetola et al., 2008; Goldberg et al., 2011; Jerde et al., 2011;
83 Minamoto et al., 2012; Thomsen et al. 2012; Spear et al., 2015; Egeter et al., 2018), and
84 more recently for invasive invertebrates (Clusa et al., 2017; Klymus et al., 2017;
85 Mychek-Londer et al. 2019). In natural habitats, eDNA is affected by a variety of
86 factors, such as temperature, microbial activity, pH (Seymour et al. 2019), conductivity
87 (Collins et al., 2018), water chemistry or ultraviolet radiations. It is degraded over time,
88 but can remain at detectable levels weeks after an organism's removal (Dejean et al.,
89 2011; Barnes et al., 2014; Pilliod et al, 2014). Hence, most eDNA detection is expected
90 to indicate a current or recent colonization of the habitat (Piaggio et al., 2014), making

91 it a potentially suitable method for contemporary surveillance of aquatic populations,
92 such as mosquito aquatic stages.

93 Although studies have shown the usefulness of eDNA metabarcoding for the
94 monitoring of numerous invertebrate species, to the best of our knowledge, only few
95 studies have demonstrated the usefulness of this technique for detection of mosquito
96 species in particular. Schneider et al. (2016) analysed the potential of eDNA for the
97 detection of invasive *Aedes* mosquitoes in Europe. They collected water samples in the
98 field and used both quantitative real-time PCR (qPCR) and eDNA metabarcoding of a
99 short fragment of the 16S rRNA gene of the Culicidae family. Both molecular methods
100 gave comparable results and performed better than the traditional survey methods,
101 however, the detection capacity decreased by half 10 days after the removal of the
102 larvae. Those authors recommended for the eDNA approach to be used as a complement
103 to traditional captures. Two other studies compared the effectiveness of eDNA
104 approaches with traditional sampling techniques to detect mosquito larvae diversity in
105 the field (Boerlijst et al., 2019; Krol et al., 2019). These both studies used eDNA
106 primers targeting the COI gene. Boerlijst et al. (2019) found that 98% of the Culicidae
107 species were correctly identified using eDNA, suggesting that eDNA-based approaches
108 are reliable and can be even more reliable than traditional dipping methods for certain
109 species. However, both studies yielded only a subset of the adult community known in
110 their field sites. Species that were detected with eDNA were generally the most
111 abundant species in the traps indicating that the eDNA metabarcoding method was more
112 likely to pick up more abundant species than rare mosquito species (Krol et al, 2019).
113 Although eDNA metabarcoding can increase the accuracy of identification, while
114 reducing the cost and time, compared to classical barcoding, it must be integrated with
115 classical taxonomy and molecular methods for comprehensive ecological studies

116 (Ruppert et al., 2019). The use of environmental DNA is a booming technique, but also
117 has many limitations, including the degradation of eDNA in the environment, especially
118 in tropical regions, as well as the methods of conservation of the samples. In addition,
119 one of the important considerations in eDNA metabarcoding studies is the primer
120 design (Ruppert et al., 2019). Primers for different genes vary in coverage, resolution,
121 and inter-taxon bias. Cytochrome oxidase I gene (COI) is the standard gene for the
122 barcode of life for animals, but other regions such as 12s or 16s ribosomal RNA may
123 be more appropriate for different taxa (Epp et al., 2012; Taberlet et al., 2012; Deiner et
124 al., 2017; Hering et al., 2018). Primers for eDNA metabarcoding must be short enough
125 to amplify degraded samples, identical for the same species, but variable between
126 species, allowing amplification of a variety of species (Epp et al., 2012).

127 In our study on São Tomé Island, Gulf of Guinea (Africa), we wanted to evaluate
128 the richness of mosquito species along a gradient of anthropogenic disturbances in order
129 to confront the assemblage of species between human habitation areas (i.e., village with
130 domestic animals), intensive agricultural areas (i.e., oil palm plantations), and natural
131 neighbouring forested areas. To assess the mosquito richness at these three habitat
132 types, we collected i) water from larval breeding sites to perform eDNA metabarcoding
133 using COI and ii) adult specimens using CDC light traps set up in trees. The aims of
134 this study were (i) to compare our metabarcoding results with the visual identification
135 of larvae and the light traps captures, taking into account the samples characteristics
136 (i.e., water turbidity, containers), ii) to identify the assemblage of mosquito species
137 along a gradient of anthropogenic disturbance, (iii) to detect the presence of the invasive
138 tiger mosquito *Ae. albopictus* which recently colonized the island (Reis et al., 2017)
139 and finally, (iv) to perform a short review of the pros and cons of the eDNA
140 metabarcoding as a complementary methodological approach to traditional ones.

141

142 Materiel and Methods

143 *Study sites and sampling*

144 Water sampling took place in three different types of habitats in October 2019 on São
145 Tomé Island (Gulf of Guinea, Africa): (i) a small village located in the middle of the
146 oil palm plantation (0º6'57.308" N; 6º35'33.414" E), (ii) the oil palm plantation that
147 surrounds the village, and (iii) the secondary rainforest adjacent to the plantation at 1
148 km from the village (Figure 1).

149 We collected 37 water samples (30 mL each, with 10 mL of Longmire solution
150 added for preservation) (Williams et al., 2016), from a variety of containers, either
151 natural or artificial, that presented variation in water turbidity (defined as either clean
152 or dirty; Figure 2, Table 1). Eighteen (48.65 %) of the water samples were taken in
153 larval development sites where larvae were present, while 19 samples (51.35%) came
154 from sites with no larvae detected. When larvae were visually detected, they were
155 identified at least at the genus level (Table 1), except for three samples for which a
156 correct *de visu* identification was not possible.

157 A total of 47 CDC light traps were set up to collect adult mosquitoes three
158 consecutive nights in each habitat in parallel of the water sampling (Figure 2). Eleven
159 traps were in the village, 18 in the oil palm plantation and 18 in the forested areas. Every
160 morning, traps were gathered and placed in a freezer for 15 minutes. Then all arthropods
161 were sorted and dipterans of interest were identified morphologically using a Leica S9E
162 stereomicroscope (Leica Microsystems GmbH, Germany). Adults and larvae mosquito
163 were identified to species or species group using different morphological keys and
164 detailed descriptions provided in Edwards (1941), Hopkins (1952), Gillies and Coetzee
165 (1987), Service (1990) and Ribeiro et al., (1998). Our sources of data on species naming

166 were based on that recorded in the Walter Reed Biosystematics Unit Mosquito
167 Catalogue (http://www.mosquitocatalog.org).
168
169 *Molecular methods*
170 DNA extractions were performed in a low-copy DNA laboratory (in CIBIO, Portugal)
171 equipped with UV radiation where strict protocols are followed for the prevention of
172 contaminations (disposable laboratory clothing, UV sterilization of all equipment
173 before entering the laboratory and laboratory cleaning with a 60% dilution of bleach
174 between extraction batches). Prior to filtration, the water samples were manually shaken
175 for five minutes (Civade et al., 2016; Lopes et al., 2017) to homogenize the water
176 column within the 50 mL Falcons. To concentrate material to a suitable volume for
177 subsequent extraction, we filtered each sample (40 mL; water + Longmire) by pouring
178 it into a sterile container (100-mL filtering cup; Nalgene Polysulfone Filter Holder with
179 Funnel, Thermo Scientific, USA) through sterile 47 mm nitrocellulose disc filters, 0.45
180 µm pore size (Whatman, UK), using a vacuum pump. The disc filters were cut into
181 small pieces and placed in a 50 ml Falcon tube with 1.5 ml 3M sodium acetate and 33
182 ml absolute ethanol for the water samples. These samples were placed in a rotor for 2
183 hours to homogenize the samples. Subsequently, the water samples were stored for 24
184 hours at -20 ºC. Filter manipulation was performed with sterilized forceps between
185 samples. Subsequently, the samples were centrifuged at 3184 g for 45 min, at 10 °C to
186 recover the precipitated DNA and/or cell debris (Peixoto et al. 2021). The supernatant
187 was discarded (Valiere & Taberlet 2000) and we performed DNA extraction on the
188 pellet using the Dneasy Blood and Tissue Kit following the manufacturer's instructions
189 (Qiagen, Hilden, Germany) (Gutiérrez-López et al. 2015). The pellet was exposed to
190 enzymatic lysis using proteinase in a rotor for 1 hour at 56 ºC and the supernatant was

191 spun through the column purification of DNA. We include a negative control in each
192 set of extractions to monitor potential contaminations. The DNA was eluted in 80 μL
193 of ultrapure sterilized MilliQ water. After extraction, DNA was quantified using the
194 Qubit High Sensitivity dsDNA Assay (Thermo Fisher Scientific). DNA metabarcoding
195 libraries were prepared by amplifying a 200 bp fragment of the COI genomic region
196 using the following primers: eCul-F (5′ GGRKCHGGDACWGGDTGAAC 3′) and
197 eCul-R (5′ GATCAWACAAATAAAGGTAWTCGATC 3′) (Krol et al., 2019).
198 Illumina sequencing primer sequences were attached to the 5' ends of PCR primers
199 with i7 and i5 as indexes (*Index 1 (i7) Adapter: P7-P5'*
200 *CAAGCAGAAGACGGCATACGAGAT[i7]GTGACTGGAGTTCAGACGTGTGCTCTT*
201 *CCGATC; Index 2 (i5) Adapter: P5-P7'*
202 *AATGATACGGCGACCACCGAGATCTACAC[i5]ACACTCTTTCCCTACACGACGC*
203 *TCTTCCGATCT)*. PCRs were carried out in a final volume of 25 μL, containing 2.5 μL
204 of template DNA, 0.5 μM of each primer, 12.5 μL of Supreme NZYTaq 2x Green
205 Master Mix (NZYTech), and ultrapure water up to 25 μL. The thermocycler program
206 for DNA amplification started with an initial denaturation step at 95 ºC for 5 min,
207 followed by 40 cycles of 95 ºC for 30 s, 58 ºC for 45 s, 72 ºC for 30 s, and a final
208 extension step at 72 ºC for 10 min.

209 The oligonucleotide indices, which are required for multiplexing different
210 libraries in the same sequencing pool, were attached in a second PCR round with
211 identical conditions but for only 10 cycles and 60 ºC as the annealing temperature. We
212 used in-house designed indexes, which are a combinatorial set of 24 i5 and 24 i7
213 indexes, which we have pre-mixed and randomized. They are 8-bp long and the
214 Levenshtein distance between any two indexes is at least 3. A negative control
215 containing no DNA was included in every PCR round to check for contamination

216 during library preparation. The libraries were run on 2 % agarose gels stained with
217 GreenSafe (NZYTech), and imaged under UV light to verify the library size. Libraries
218 were purified using the Mag-Bind RXNPure Plus magnetic beads (Omega Biotek). We
219 pooled the samples and purified the resulting pool following the same method (1X of
220 magnetic beads). The purified pool was run through a Size-select eGel to precisely
221 select the band of interest. Libraries were quantified using the Qubit High Sensitivity
222 dsDNA Assay (Thermo Fisher Scientific).

223 Very low library quantification was detected in 18 samples that were removed
224 for sequencing. These samples corresponded to water samples in which only one larva
225 (n=2) or none were detected visually (n=16; Table 1). Therefore, 19 samples were
226 selected for sequencing and were pooled in equimolar amounts and re-purified for
227 double size selection in an e-gel system (Life Technologies) for primer dimer
228 elimination. The pool was sequenced in a fraction (1/16) of a MiSeq PE300 run
229 (Illumina). Library preparation and sequencing were carried out by AllGenetics &
230 Biology SL (www.allgenetics.eu).

231

232 *Bioinformatic analyses and taxonomic assignment*

233 Illumina paired-end raw files consist of forward (R1) and reverse (R2) reads sorted by
234 library and their quality scores. The indices and sequencing primers were trimmed from
235 the samples using the software CUTADAPT (Martin, 2011) and the quality of the
236 FASTQ files was checked using the software FastQC (Andrews, 2010). Plots
237 summarizing the quality across bases of R1 and R2 reads were generated by using
238 MultiQC (Ewels et al., 2016) (see Supplementary file). The merging of the R1 and R2
239 reads was performed with FLASH2 (Magoč & Salzberg, 2011). The mismatch
240 resolution in the overlapping region (minimum overlap of 30 base pairs) was

241 accomplished by keeping the base with the higher quality score. We used the
242 CUTADAPT software 1.3 (Martin, 2011) to remove sequences that did not contain the
243 PCR primers (allowing up to 2 mismatches) and sequences that ended up being shorter
244 than 145 nucleotides and larger than 210 nucleotides. The sequences were quality-
245 filtered (minimum Phred quality score of 20), then were dereplicated (-derep fulllength)
246 and clustered at a similarity threshold of 97 % (-cluster fast, -centroids option) and
247 sorted (-sortbysize) using VSEARCH (Rognes et al., 2016). *De novo* chimera detection
248 was carried out using the UCHIME algorithm (Edgar et al., 2011) implemented in
249 VSEARCH.
250
251 We conducted the taxonomic assignment of each Operational Taxonomic Unit (OTU)
252 using a customized taxonomic COI reference database. The database including (i)
253 newly generated mosquito sequences of four species sampled during the fieldwork
254 using light traps (*Aedes nigricephalus, Culex cambournaci, Uranotaenia micromelas*
255 *and Ur. connali;* Genbank accession number ON504276-ON504279), and (ii)
256 sequences downloaded from the National Center for Biotechnology Information and
257 the BOLD databases (Ratnasingham & Hebert, 2007) (accessed on March 2022). These
258 mosquito sequences (from mosquito species known to be present on São Tomé; Table
259 S1) were added to the database build using RESCRIPt (Robeson et al., 2021) (last
260 version on July 2020) based on the BOLD reference database (Ratnasingham & Hebert,
261 2007).
262
263 We employed the script *feature-classifier classify-consensus-vsearch* implemented in
264 Qiime2 (Bokulich et al., 2018) and the VSEARCH algorithm (Rognes et al., 2016) with
265 a sequence similarity threshold of 95 %. In addition, we used the *top-hits-only* option

266 in the VSEARCH command to recover only the hit with the highest percentage of
267 identity. In spite of the multiple top hits used in the consensus taxonomic assignment
268 carried out by VSEARCH, this option allows the assignment of the query to the closest
269 reference sequence. The table resulting from this step lists the number of sequences
270 from each OTU found in each sample and their corresponding taxonomic information
271 (Table S2 - Before OTUs filtering). Subsequently, based on the results of this table, we
272 applied several different filters. We removed singletons (i.e., OTUs containing only
273 one-member sequence in the whole data set). In DNA metabarcoding studies, it has
274 been observed that a low percentage of the reads of a library can be assigned to another
275 library. This phenomenon, referred to as mistagging, tag jumping, index hopping, index
276 jumping, etc. is the result of the misassignment of the indices during library preparation,
277 sequencing, and/or demultiplexing steps (Esling et al., 2015; Bartram et al., 2016;
278 Guardiola et al., 2016; Illumina, 2018). In order to correct for this phenomenon, OTUs
279 occurring at a frequency below 0.01 % in each sample were removed. Finally, only the
280 OTUs that matched any reference sequence in the database at a minimum similarity
281 threshold of 85 % were kept in the OTU table. Therefore, the unidentified OTUs
282 ('Unassigned') were removed from the OTU table for downstream analysis (Table S3
283 - After OTUs filtering). Six samples (V16, V17, P4, F1, F7, F9) had no OTUs assigned
284 to the family Culicidae.
285
286 The alpha rarefaction plots show the number of OTUs obtained with a rarefied number
287 of sequences in each sample. These plots were generated using the OTU table before
288 (Table S2) and after (Table S3) the OTU filtering (Figure S1). The vertical axis displays
289 the number of OTUs observed at different subsampling depths. When the rarefaction
290 curves tend towards saturation, the sequencing depth is considered to be sufficient to

291 retrieve most of the taxa diversity. We have to note that curve from sample V8 did not
292 reach the plateau in the number of OTUs observed (see Sup. file Rarefaction plot after
293 the OTU filtering).
294
295 In order to easily visualize the breakdown of taxonomic classification, stacked bar plots
296 showing the relative abundance of each OTU in each sample were generated at the
297 order, family and species level (Figure 3). In DNA metabarcoding studies, OTU relative
298 abundance is defined as the number of reads assigned to that OTU divided by the total
299 number of reads. Please note that the PCR may cause biases due to differences in primer
300 specificity. These biases can cause taxa with low representation in the original DNA
301 sample to become more abundant in the final results. As a result, this bias prevents from
302 correctly inferring the abundance of species in the original DNA sample. For example,
303 if SPECIES A is represented by the 35 % of the sequences in SAMPLE 1, and SPECIES
304 B is represented by the 50 % of the sequences in the same sample, we cannot reliably
305 conclude that there was more SPECIES B DNA in the original sample. That being said,
306 it is expected that, within the same study, the PCR bias always go in the same direction.
307 Therefore, it is possible to compare how the abundance of a given taxon varies across
308 different samples with a similar composition. For example, if SPECIES A is represented
309 by the 35 % of the sequences in SAMPLE 1 and by the 10 % in SAMPLE 2, we can
310 conclude that there was less SPECIES A DNA in SAMPLE 2 (Geisen et al., 2015;
311 Thomas et al., 2016; Matesanz et al., 2019).
312 Finally, we extracted the representative sequences for each of the picked OTUs before
313 and after the OTU filtering process. For the particular case of the taxonomic assignment
314 of OTUs to *Eretmapodites intermedius*, we performed a blast in NCBI and the results
315 are shown in Figure S2.

316 DNA metabarcoding analyses were carried out by AllGenetics & Biology SL
317 (www.allgenetics.eu).
318
319 **Results**
320 *Visual and genetic detection*
321 Of the 19 water samples collected from sites where no larvae were detected visually,
322 one was positive for *Aedes albopictus* (5%; Table 1), two others were found with
323 chironomids (Diptera) or coleopterans (10%), and 16 could not be sequenced because
324 of the low library DNA quantities (84%). Of the 18 water samples in which larvae were
325 seen, eDNA metabarcoding detected Culicidae in 13 (72%), three of which had
326 detections of other dipterans and branchiopodans (16%), and two could not be
327 sequenced because of the low library DNA quantities (11%; Table 1). When larvae
328 were present at the collection site, one or two Culicidae genera were identified visually
329 in each sample, whereas eDNA metabarcoding detected up to four genera per sample
330 (Table 1).
331 We recovered DNA sequences in 14 water samples out of the 26 considered as
332 clean (53,8%), and in 4 out of 7 considered as dirty (57%). Although our sample sizes
333 remain small, we found that the turbidity of the water did not appear to be a limitation
334 for eDNA metabarcoding (Chi-square test χ^2=0,33).
335 Overall, the taxonomic assignments revealed 4 orders of arthropods that
336 comprised 13 families. Within Culicidae, taxonomic assignments at the species level
337 for the genus *Anopheles* returned *Anopheles coluzzii,* the main human malaria vector
338 on the island (Chen et al., 2019). For the genus *Aedes*, the taxonomic assignments at
339 the species level returned the invasive tiger mosquito *Aedes albopictus* and *Aedes*
340 *aegypti*. All OTUs that matched the genus *Eretmapodites*, an endemic genus of the

341 Afrotropical region and vector of various viruses (Bamou et al., 2021), were assigned
342 to *Eretmapodites intermedius* (Supplementary files Figure S2). As for the *Culex* genus,
343 OTUs were assigned to *Culex cambournaci, Cx. decens* and *Cx. sasai*.
344 In summary, 12 species of Culicidae were detected, 7 with eDNA
345 metabarcoding, and 9 with CDC light traps. Four species were common to both
346 approaches: *Aedes albopictus*, *Anopheles coluzzii/gambiae*, *Culex cambournaci* and
347 *Culex decens*, all collected in the village (Table 2; Figure 4).
348
349 *Habitat effects on species detection*
350 In the village, five orders and eight families of arthropods were found. The Culicidae
351 was the dominant family found in the village, with 78% of the total reads from the
352 village attributed to the genera *Aedes*, *Anopheles*, *Culex* and *Eretmapodites*. The
353 invasive mosquito *Ae. albopictus* and the malaria vectors *An. coluzzii* were present
354 respectively in 57% (N=8) and 50% (N=7) of the samples collected in the village that
355 led to amplification. *Aedes albopictus* was found in both artificial and natural breeding
356 sites, while *Aedes aegypti* was totally absent from the village, a pattern that had already
357 been noted in previous surveys (Reis et al, 2017). *Culex* spp. were present in half of the
358 village samples that could be sequenced (7 out of 14; Figure 3).
359 In the plantation, in the 8 potential breeding sites that were sampled, we did not
360 detect any larvae visually. The only sample whose amplification worked gave 2 OTUs
361 affiliated to the Chironomidae family (order Diptera; see Supplementary file Tables S2
362 and S3).
363 In the forest, 4 orders and 4 families of arthropods were found, with the
364 Chironomidae being the dominant family with 73% of the reads (Figure 3). In the forest,
365 *Culex sasai* and *Ae. aegypti* were detected in the same sample (Figure 3).

366

367 **Discussion**

368

369 Our study showed that eDNA metabarcoding could be a complementary method to the
370 light or decoy traps to recover mosquito diversity, and help to evaluate the assemblage
371 of species using the same breeding sites. In particular, eDNA metabarcoding was able
372 to detect species that were not capture with light traps and picked up different
373 assemblage of mosquito species associated with the degree of anthropogenic
374 disturbance.

375 In the oil palm plantation, we found larvae of mosquitoes by *visu* at one sampling
376 location. Environmental DNA metabarcoding detected only one family of diptera
377 (Chironomidae) with very few reads, but no mosquito species. This result is not
378 surprising and is consistent with the view that the core of oil palm plantations is overall
379 poor in terms of arthropod diversity (Koh & Wilvoce, 2008; Turner & foster 2009;
380 Fayle et al., 2010; Ghazali et al., 2016). Recently, Young et al. (2021) also found that
381 mosquito abundance in oil palm plantations in Borneo was lower than in the forest. On
382 the contrary, in the village, the arthropod diversity was much higher than in the
383 surrounding plantations with eight families of Diptera recorded. Culicidae was the
384 predominant family: *Ae. albopictus* accounted for 36% of the reads, followed by *Culex*
385 *species* (33.5%), while *Anopheles* genus was the least abundant, with 3.3% of the reads.
386 Although more surveys are needed, *Ae. albopictus*, which recently colonized the island
387 (Reis et al., 2017), shared breeding sites with *Culex, Eretmapodites* and *Anopheles*
388 species. Co-occurrence with the latter was less expected since these species do not
389 usually use the same niche. Finally, in the forest, among the four families of Diptera
390 detected, Chironomids were the predominant one, with 73% of the reads, while

391 mosquito species were found in lower abundance (17%). Interestingly, the yellow fever
392 mosquito *Aedes aegypti* was detected in only one sample, inside a bamboo stalk. It used
393 to be very common and widespread on the island, and found equally in both natural and
394 artificial breeding sites (Ribeiro et al., 1998). However, recent on-going mosquito
395 projects and, surveys on the island revealed that *Ae. aegypti* became quite rare and
396 seems to have been replaced by *Ae. albopictus* in lowland and disturbed habitats (Reis
397 et al., 2017; Loiseau et al., 2022). This replacement pattern has been largely
398 documented in Florida, USA (Yang et al., 2021) but is less evident in mainland Central
399 Africa (Simard et al., 2005; Paupy et al., 2010; Kamgang et al., 2013; Tediou et al.,
400 2019). Nonetheless, our eDNA metabarcoding approach corroborates the actual known
401 distribution of these two *Aedes* species on the island (Loiseau et al., 2022). Finally, the
402 other Culicidae species found in the forest was *Culex sasai*. It is highly unlikely that
403 this mosquito is present on the island, since to date it has been detected only in Asia
404 (Phanitchakun et al., 2017), and is not known to be present on São Tomé Island (Loiseau
405 et al., 2022). Because *Culex sasai* belongs to the *Culiciomyia* subgenus, we probably
406 detected here a mosquito species belonging to this same subgenus. There are actually
407 four species of this subgenus on São Tomé Island: *Culex cambournaci*, *Culex*
408 *nebulosus, Culex cinerellus* and *Culex macfiei* (Loiseau et al., 2022), with only two
409 having barcoding sequences on online databases (*Cx. cambournaci* and *Cx. nebulosus*).
410 One could speculate that the species found in this forest sample could be either *Culex*
411 *cinerellus* or *Culex macfiei* and not *Culex sasai*. This error highlights one of the
412 limitations of the eDNA metabarcoding approach which is discussed below, i.e.,
413 incomplete reference databases.
414

415 ***Challenges of eDNA metabarcoding: sample quality and taxonomic assignment***
416 ***issues***

417 As with any new methods, some weaknesses and concerns need to be addressed. Some
418 critical factors for the application of eDNA methods to detect aquatic species have
419 already been reviewed (Goldberg et al., 2016), including contamination in the field and
420 in the laboratory, choosing appropriate sample analysis methods, validating assays or
421 testing for sample inhibition. Here, we highlight concerns that are specific for insect
422 vector monitoring using eDNA approaches.

423 First, mosquito larvae are mostly found in small and turbid breeding sites or in
424 stagnant water bodies. While water from some larval breeding sites (e.g., rock pools,
425 puddles, artificial containers) is easy to sample, it can be difficult to collect from other
426 sites (e.g., tree holes, plant axils). Traps and sampling procedures, such as aspiration of
427 resting mosquitoes, collection on human or animal bait, allow collecting a greater
428 diversity of species. For inventory purposes, eDNA techniques may need a great water
429 sampling effort in order to be comparable to other techniques (Krol et al., 2019). In
430 addition, sampling small volumes of water can lead to false negative detection when
431 the density of targeted organisms is low (Ulibarri et al., 2017). Another potential
432 sampling issue is the large amount of soil and humic substances found in breeding sites
433 that may act as PCR inhibitors, increasing the chance to obtain false negative results
434 (Buxton et al., 2017). In our case, we managed to amplify COI even from dirty samples,
435 although these samples contained many larvae. One study experimentally tested the
436 success of PCR detection of eDNA samples from containers with two different water
437 volumes (50mL and 1 L) (Odero et al., 2018). They found that the volume of water
438 required in relation to the density of larvae has an effect on the mosquito detection by
439 eDNA analysis. The detection was better when the samples had many larvae at low

440 densities than few larvae at higher densities (Odero et al., 2018). In addition, the effect
441 of different substrates in the eDNA analysis as well as the preservation methods are
442 parameters that should not be overlooked since metabarcoding analyses require good
443 DNA quality (Ball et al., 2014).
444 Secondly, it seems appealing to evaluate and compare mosquito diversity from different
445 type of samples (water vs. bulk samples) using the metabarcoding approach because
446 traditional dipping methods to survey larvae in breeding sites may not always reflect
447 the adult diversity that can be found with CDC traps (and inversely). In fact, in our
448 survey, only four species were shared between the two techniques (eDNA vs. CDC
449 traps). It is worth noting that some species may be very difficult to detect with
450 traditional trapping because not all insect vector species are equally attracted to dry ice
451 or light (Reisen & Lothrop, 1999). It is especially true for daytime biting mosquitoes.
452 On the other hand, it might be difficult to sample water in breeding sites, such as plant
453 axils or tree holes, which can be high up. More investigations in controlled conditions
454 are needed to compare the efficacy of metabarcoding water samples with trapped adults
455 to characterise insect-vector communities.
456 Thirdly, in the Barcode of Life Data System (BOLD), of about 3,500 species of
457 Culicidae known globally, barcodes are only available for 1,329 species (38%; accessed
458 on 2021-05-25) and, among the 41 known mosquito genera, three genera alone (*Aedes*,
459 *Anopheles* and *Culex*) account for 78% of the occurrences. Similar patterns are found
460 when gathering data on different genes in NCBI (COI, 18S rRNA and 28S rRNA).
461 While *Aedes*, *Culex* and *Anopheles* species account for only 60% of the total mosquito
462 species, 90% of the sequences on average correspond to these three genera (see Figure
463 S3 for illustration of these data). Sequences belonging to unknown taxa are still a
464 common problem in eDNA barcoding and therefore, when starting a new monitoring

465 program to assess the mosquito diversity in a region or locality, creating a good quality
466 reference database is an indispensable first step. This means that a considerable amount
467 of essential taxonomic work is required to setup eDNA-based monitoring protocols. In
468 this study, we managed to get DNA sequences of four mosquito species that were not
469 deposited in online databases yet. Eleven species out of the 34 known on the island
470 (Loiseau et al. 2022) still have to be captured and sequenced to have a full reference
471 database for future research work. Taking all this into account, and considering that
472 certain limitations can be surpassed, then eDNA metabarcoding can have significant
473 advantages for mosquito surveys.
474

475 ***Advantages of eDNA metabarcoding: easy sampling and less entomological expertise***
476 ***required***

477 Sampling for eDNA can be as simple as collecting freshwater samples in tubes and
478 adding preservation buffers (Williams et al. 2016), which drastically reduces the cost
479 and time allocated to fieldwork, as well as equipment and resources required for
480 sampling. This is particularly relevant for research projects carried out in remote
481 regions. The effort required for the traditional trapping methods is substantial.
482 Logistically it requires the transport of traps and batteries (which are voluminous and
483 heavy), the availability of freezers (to kill mosquitoes before identification) and of high-
484 quality stereomicroscopes. Once this material is in the field, traps must be set up for
485 several hours, with light that attracts mosquitoes together with a wide range of flying
486 insects, or with traps containing odour products to attract more specifically females
487 (BG-Sentinel or Gravid Mosquito traps). Since light traps are not selective, a great
488 amount of time is spent on sorting all the flying insects from the mosquitoes, separating
489 engorged individuals and labelling individual tubes. Once back in the laboratory,

490 experts may spend a great amount of time at the microscope identifying and dissecting
491 individuals. Identification of mosquito eggs and larvae implies mounting, which is time
492 consuming, and require a specific training. Although an alternative solution could be
493 rearing larvae into adults for unambiguous identification, this is logistically challenging
494 when doing fieldwork in remote places. In addition, for the identification of many adult
495 insect vectors, dissecting male genitalia is required, which is the case for example for
496 most of the species of the African genus *Eretmapodites* (Service 1990). Molecular
497 identification of eDNA is able to circumvent time-consuming morphological
498 investigation and to detect the presence of species without requiring a strong
499 entomology expertise. The efficacy of eDNA-based surveys will increase as reference
500 databases become more complete. Interestingly, in our study, we detected the species
501 *Eretmapodites intermedius* for the first time on the island, as until now *Eretmapodites*
502 *chrysogater* was the only known representative of this genus on the island (Ribeiro et
503 al., 1998). This detection would have been almost impossible using traditional light
504 traps since *Eretmapodites* species are day-biting mosquitoes and males are generally
505 less attracted to them. Finally, the ease of water sampling procedures for eDNA
506 protocols will allow developing large-scale citizen science monitoring programs and
507 integrating non-specialists in research projects (Biggs et al. 2015).
508
509 **Concluding remarks**
510 To date, numerous studies have demonstrated that eDNA sampling generally provides
511 greater detection probabilities than traditional techniques (Thomsen et al., 2012;
512 McKelvey et al., 2016; Valentini et al., 2016), but it still remains to be formally
513 demonstrated for mosquito communities. In fact, eDNA methods could surely help in
514 applied medical and veterinary entomology and significantly improve i) the detection

of invasive species and ii) the evaluation of the composition of mosquito communities in understudied regions. In our study, we showed that CDC light traps and adult identification methods recovered more species than the eDNA metabarcoding per habitat. However, eDNA metabarcoding was able to detect i) more species at a mosquito breeding site than *de visu* larval identification, and ii) different species than traditional methods. Therefore, our results highlight the fact that it is best to use in conjunction traditional survey methods and eDNA metabarcoding to enhance detection rates and increase confidence in the monitoring results.

Like any ecological survey tool, eDNA metabarcoding will always suffer biases and uncertainties which have to be taken into account at each step of the study (i.e., fieldwork, labwork, bioinformatic analyses). The building up of the BOLD is required to expand the potential of eDNA metabarcoding, a task where taxonomic expertise will be essential. However, the relative simplicity of field sampling protocols can create opportunities to collect samples using volunteers and even to develop citizen science programs such as (i) for monitoring and surveillance of invasive species such as *Ae. albopictus*, and (ii) for improving our understanding of ecological systems (competition and predation at breeding sites) that could definitely help in vector control management (Dambach 2020).

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552

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Table 1. Characteristics of water samples (n=37) collected on São Tomé Island (village n=17; oil palm plantation n=8; forest n=12; with A or N for anthropogenic or natural containers respectively) and the species identification, either visually or by metabarcoding (COI marker). In five sequenced samples, we did not detect Culicidae species but other arthropod families (see Figure 3).

	Field			***Visual***		***eDNA metabarcoding***	
ID	Location	Container	Water	Larvae (quantity)	Species	Seq	Species assignment
V1	Village	N: puddle	Clean	Yes (one)	Unknown	Yes	*Aedes albopictus*
V2	Village	A: tire	Dirty	Yes (>20)	*Ae. albopictus, Eretmapodites sp.*	Yes	*Ae. albopictus, Anopheles coluzzii, Er. intermedius, Culex cambournaci*
V3	Village	A: pot	Clean	Yes (>20)	*Ae. albopictus*	Yes	*Ae. albopictus, An. coluzzii, Er. intermedius, Cx. cambournaci*
V4	Village	A: tire	Clean	Yes (>20)	*Ae. albopictus, Culex sp., Eretmapodites sp.*	Yes	*An. coluzzii, Cx. decens*
V5	Village	A: tire	Dirty	Yes (>20)	*Eretmapodites sp.*	Yes	*Ae. albopictus, An. coluzzii, Er. intermedius*
V6	Village	A: tire	Clean	Yes (>20)	*Ae. albopictus, Culex sp.*	Yes	*An. coluzzii, Cx. decens*
V7	Village	A: tire	Clean	Yes (>20)	*Ae. albopictus, Eretmapodites sp.*	Yes	*Ae. albopictus, Cx. decens*
V8	Village	N: bamboo	Clean	Yes (<10)	*Ae. albopictus*	Yes	*Ae. albopictus*
V9	Village	N: fruit shell	Dirty	Yes (<10)	*Ae. albopictus, Eretmapodites sp.*	Yes	*Ae. albopictus, Cx. cambournaci, Er. intermedius*
V10	Village	A: bottle	Clean	Yes (one)	Unknown	No	
V11	Village	N: crab hole	Dirty	No		No	
V12	Village	A: can	Clean	No		No	
V13	Village	N: puddle	Clean	Yes (<10)	*Anopheles sp.*	Yes	*An. coluzzii*
V14	Village	N: puddle	Clean	Yes (<10)	*Anopheles sp.*	Yes	*An. coluzzii*
V15	Village	A: tire	Clean	Yes (>20)	*Ae. albopictus*	Yes	*Ae. albopictus, Cx. cambournaci*
V16	Village	N: puddle	Clean	Yes (<10)	*Anopheles sp.*	Yes	Other family
V17	Village	A: bottle	Clean	Yes (one)	Unknown	Yes	Other family
P1	Plantation	N: puddle	Clean	Yes (one)	*Anopheles sp.*	No	
P2	Plantation	N: puddle	Clean	No		No	
P3	Plantation	N: rocks	Dirty	No		No	

P4	Plantation	N: puddle	Clean	No		Yes	Other family
P5	Plantation	N: puddle	Clean	No		No	
P6	Plantation	N: leaf	Clean	No		No	
P7	Plantation	N: rocks	Dirty	No		No	
P8	Plantation	N: rocks	Clean	No		No	
F1	Forest	N: leaf	Clean	No		Yes	Other family
F2	Forest	N: rocks	Clean	No		No	
F3	Forest	N: tree hole	Dirty	No		No	
F4	Forest	N: river	Clean	No		No	
F5	Forest	N: tree hole	Dirty	No		No	
F6	Forest	N: rocks	Clean	No		No	
F7	Forest	N: rocks	Clean	No		No	
F8	Forest	N: rocks	Clean	No		No	
F9	Forest	N: tree hole	Clean	Yes (one)	*Culex sp.*	Yes	Other family
F10	Forest	N: tree hole	Dirty	No		No	
F11	Forest	N: tree hole	Dirty	No		No	
F12	Forest	N: bamboo	Dirty	Yes (<10)	*Ae. albopictus + Culex sp.*	Yes	*Ae. aegypti; Culex sasai*

828

829 **Table 2.** List of mosquito species detected in the water samples, visually and with eDNA metabarcoding, and with CDC traps along the gradient

830 of anthropogenic disturbance in São Tomé Island.

831

	Water sample		**CDC light trap**
	Visual identification (larvae)	eDNA	Visual identification (adults)
Village	*Aedes albopictus*	*Aedes albopictus*	*Aedes albopictus*
	Anopheles sp.	*Anopheles coluzzii*	*Anopheles coluzzii/gambiae*
	Culex sp.	*Culex cambournaci*	*Culex cambournaci*
	Eretmapodites sp.	*Culex decens*	*Culex decens*
		Eretmapodites intermedius	*Culex micolo*
			Uranotaenia connali
			Uranotaenia micromelas
Oil palm plantation	*Anopheles sp.*	none	*Anopheles coustani*
			Culex cambournaci
			Lutzia tigripes
			Uranotaenia micromelas
Forest	*Ae. albopictus*	*Aedes aegypti*	*Culex cambournaci*
	Culex sp.	*Culex sasai*[1]	*Uranotaenia micromelas*

832 [1] Incorrect taxonomic assignment likely due to incomplete molecular reference database.

833

834 **Figure legends**

835 **Figure 1.**

836 On the left: map of São Tomé Island (Gulf of Guinea, Africa), with the black frame
837 representing the sampling area in the southeast of the island. On the right, a satellite
838 picture of that area, with the village (circled in red), surrounding by the oil palm
839 plantation; the green line being the border between the oil palm plantation and
840 secondary forest.

841

842 **Figure 2.**

843 Photography representing the sampling methods used in our study: A) sampling water
844 in an artificial container, B) sampling in a natural rock hole, C) a CDC light trap in the
845 oil palm plantation.

846

847 **Figure 3.**

848 Stacked bar plots of the various arthropods detected along the anthropogenic gradient
849 using eDNA metabarcoding (COI marker): (a) order level, (b) family level for the
850 Diptera order, (c) species level for the family Culicidae. (V = village; P = plantation; F
851 = forest).

852

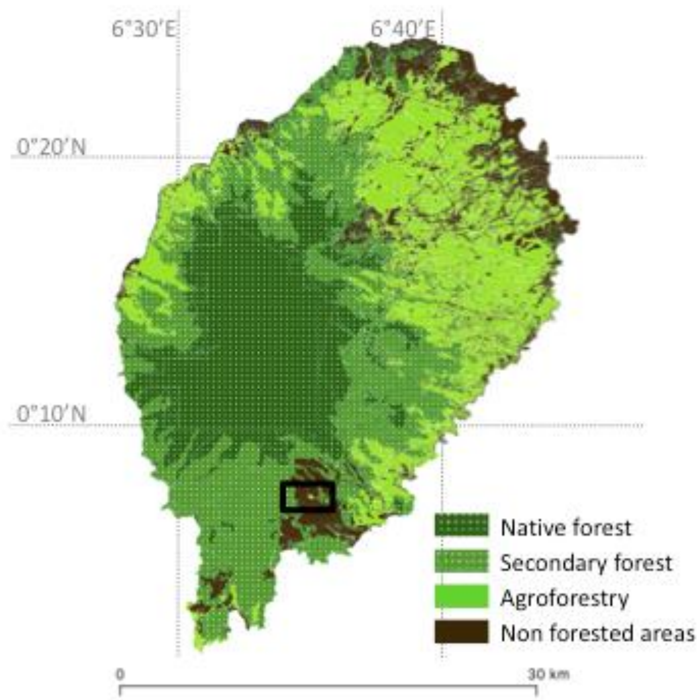
853 **Figure 4.**

854 List of mosquito species by habitat recovered using both methods: CDC traps (depicted
855 by the adult mosquito) and eDNA metabarcoding (depicted by the water bottle). Species
856 detected uniquely with eDNA metabarcoding are in bold.

857 *Icons from Freepik.*

858

859 **Figure 1**



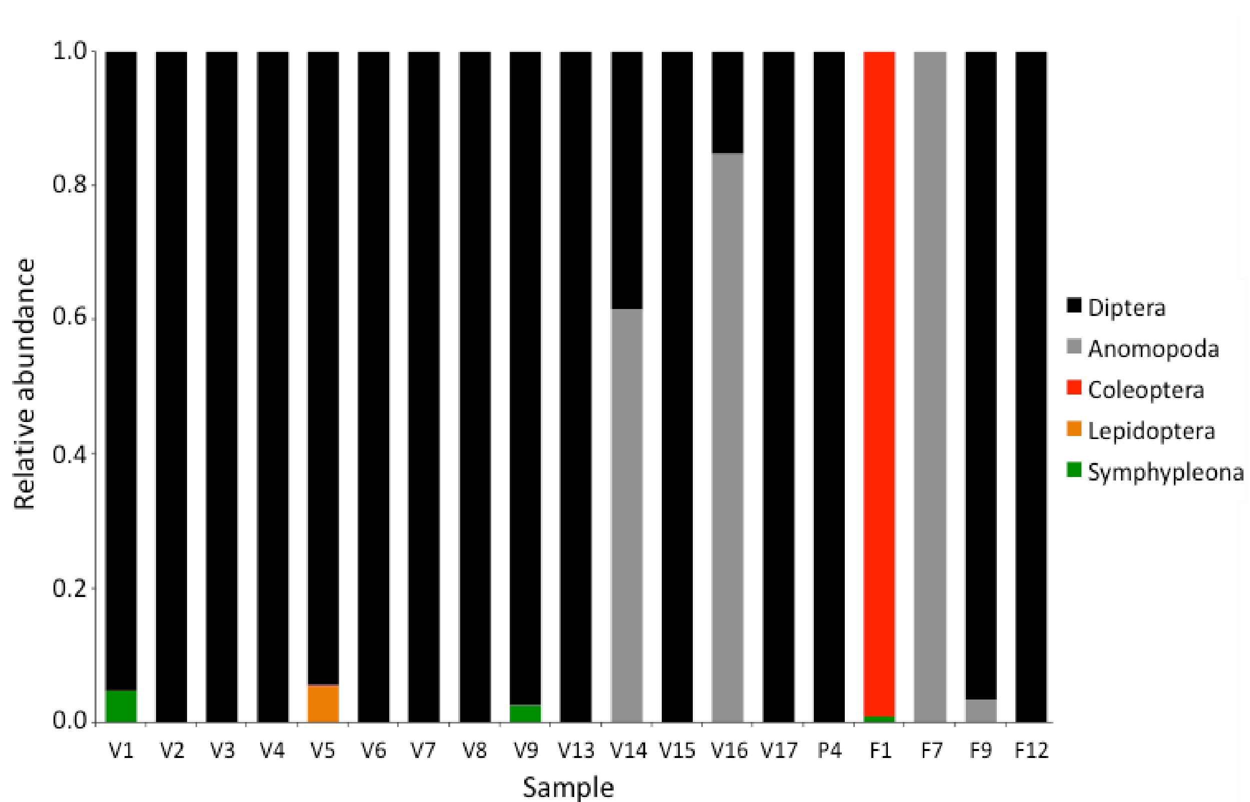
860

861 **Figure 2**

862

863 **Figure 3**

864 **a)**



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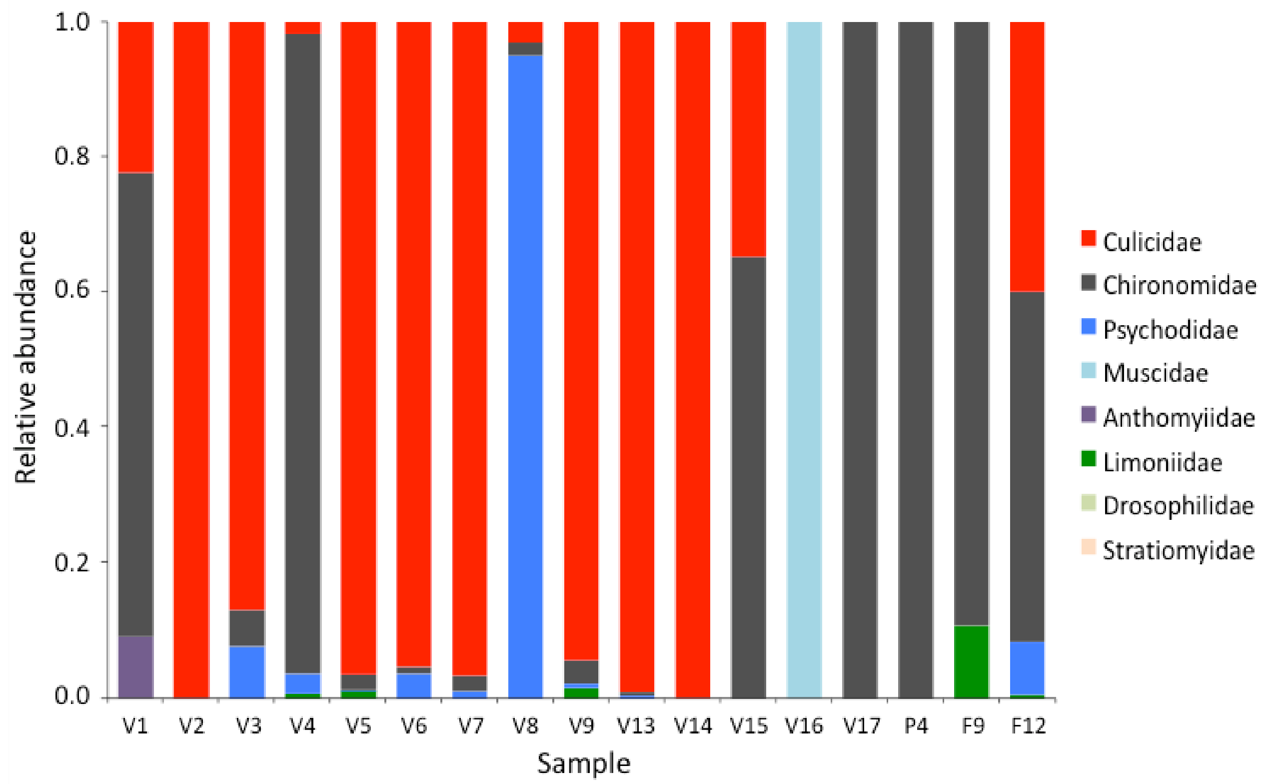
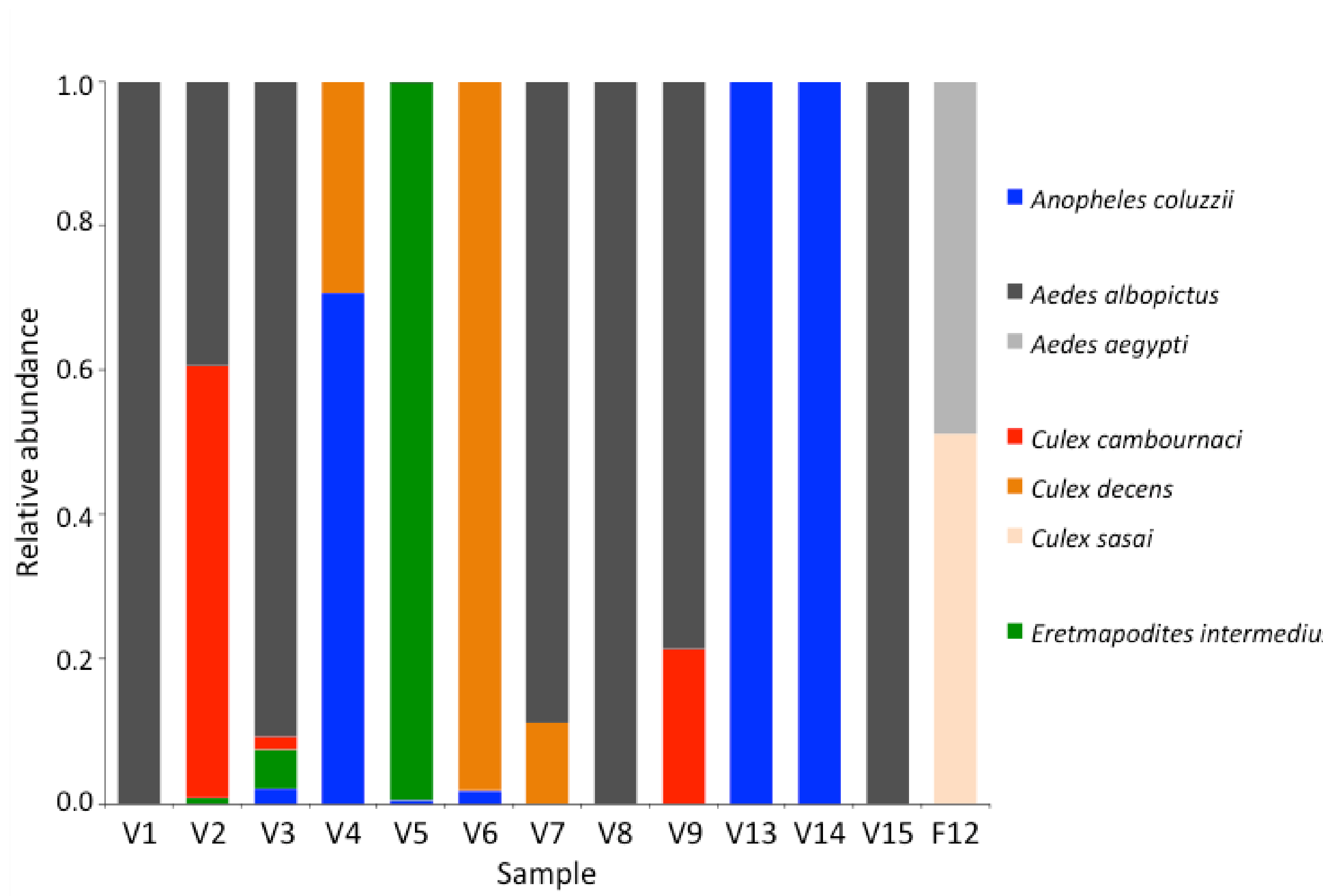
866 **b)**



867

868 **c)**



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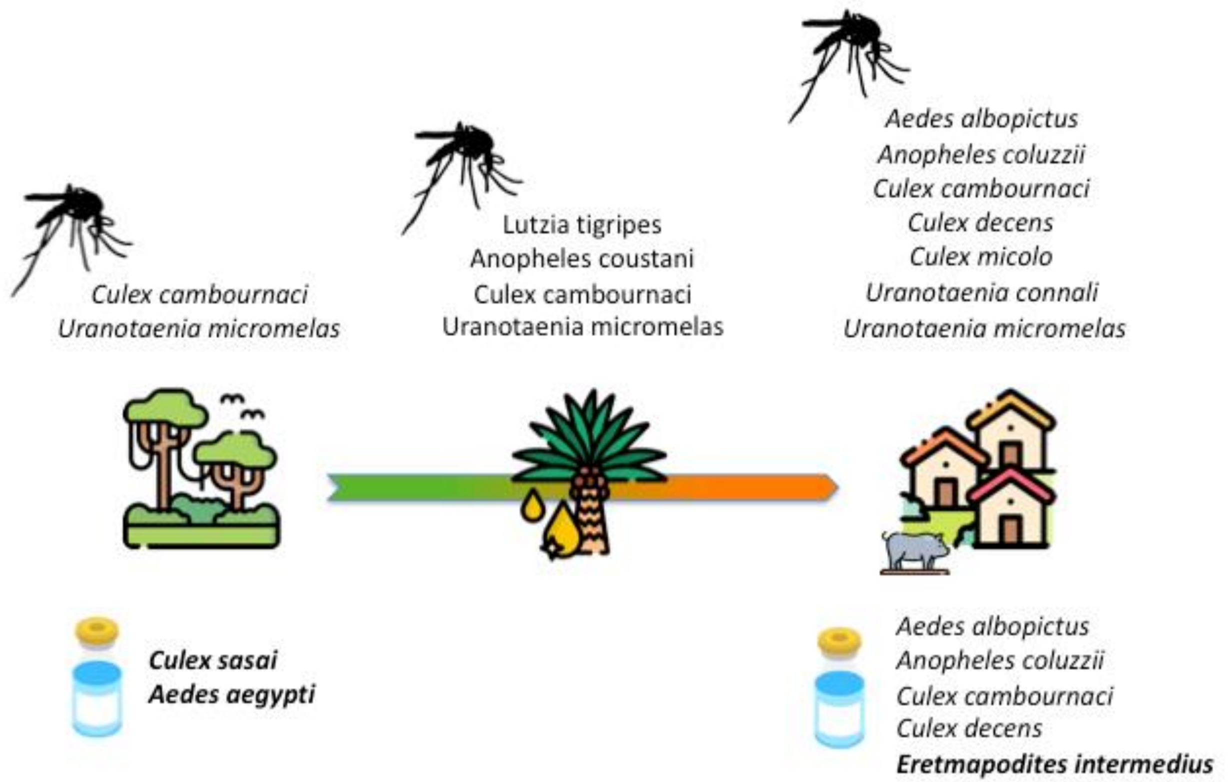
870

871 **Figure 4.**



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